# Muscle Growth and Development in Intrauterine Growth Restricted Pigs

Haibo Zhu

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Samer W. El-Kadi, Chair

Dave E. Gerrard

Sally E. Johnson

Robert P. Rhoads

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## ABSTRACT

Intrauterine growth restriction causes impaired growth and development of mammalian fetus, and leads to long-term negative effect on postnatal growth. Among domestic animals, pigs exhibit the most severe naturally occurring IUGR and reduced postnatal muscle growth. The objectives of this research project were to: 1) determine muscle stem cell characteristics in IUGR pigs; 2) determine how intrauterine growth restriction alters protein deposition in skeletal muscle; 3) investigate whether branched-chain amino acids (BCAA) are able to enhance protein synthesis in intrauterine growth restricted (IUGR) pig muscle. Newborn piglets were considered normal body weight (NBWT) or IUGR when birth weight was within $\pm 0.5$ SD and -2 SD of litter average respectively. Muscle satellite cell numbers, believed to be the major nuclei source for postnatal muscle growth, were lower in newborn IUGR pigs which could result in reduced muscle hypertrophy potential. In addition, cultures derived from IUGR muscle satellite cells had a lower fusion percentage. Fewer satellite cells and impaired differentiation ability may contributor to impaired muscle growth in these pigs. Protein synthesis rate was significantly lower in IUGR pig hindquarter in the first hour after feeding, but BCAA supplementation had no effect on protein synthesis in IUGR pigs. Further, eukaryotic translation initiation factor 4E (eIF4E) expression is down regulated in IUGR pig muscle. These results suggest that impaired translation initiation may provide a plausible explanation for the lower protein synthesis rates observed in IUGR pigs. Overall, reduced muscle stem cell number and changes in their activity, as well as impaired translation initiation may be important explanations for compromised postnatal muscle growth in intrauterine growth restricted pigs.

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# Chapter 1.

# Introduction and Literature Review

## Introduction

Animal fetal growth and development are influenced by genetic, epigenetic, nutritional and hormonal factors (Gootwine, 2004; Redmer et al., 2004; Wu et al., 2004a). Among these, maternal nutrition plays a key role in regulating embryo and fetal growth throughout gestation (Ferguson, 2005; Rehfeldt et al., 2004). The birth weight of an animal is determined not only by its genetic potential, but also by the maternal ability to deliver nutrients and oxygen to the fetus (Cox and Marton, 2009). When maternal nutrients supplies fail to satisfy the requirements of growing fetus, intrauterine growth restriction (IUGR) occurs. This is defined as impaired growth and development of the mammalian fetus, and it has a critical effect on postnatal growth (Wu et al., 2006).

Naturally occurring (e.g., placental insufficiency) and environmentally induced (e.g., heat stress, disease and toxins) IUGR are well documented in domestic animals (Baker et al., 1969; McEvoy et al., 2001; Pond et al., 1969; Redmer et al., 2004). Among domestic animals, pigs exhibit the most severe naturally occurring IUGR (Wu et al., 2006). Naturally occurring IUGR in pigs are mainly caused by placental insufficiency; that means the nutrient needs of multiple conceived fetuses surpass functional uterine capacity of the sows resulting in nutrient deficiency to the fetus (Wu et al., 2004a; Wu et al., 2006). Before d 35 of gestation, pig embryos are evenly

distributed inside the uterine horn, however the capacity of the uterus becomes limited later in gestation (Anderson and Parker, 1976; Knight et al., 1977). Therefore, some fetuses that are located in the middle of uterus have smaller placenta which results in limited nutrient transport (Foxcroft et al., 2006; Godfrey, 2002; Wu et al., 2004a). At birth, the weights of IUGR pigs are significantly lower (50% or even lower) than their normal body weight (NBWT) littermates (Widdowson, 1971).

Human neonates born full term with birth weight lower than 10 percentile are considered as IUGR infants (WHO, 1995). Every year, approximately 10 – 15 % of the infant born in the US and up to 24 % of those born in developing countries are affected by IUGR (Berghella, 2007; Saleem et al., 2011). Many external and internal factors induce IUGR in human pregnancy. In developed countries, maternal smoking, drugs and alcohol use, as well as placental insufficiency are the main causes of IUGR; whereas in developing countries, poor maternal nutrition and/or nutrient imbalance intake are the major causes for IUGR pregnancy (Briana and Malamitsi-Puchner, 2009; Klaric et al., 2013; McMillen and Robinson, 2005; Stocker and Cawthorne, 2008).

Intrauterine growth restriction not only increases the risk of still born and preterm birth, but also leads to long-term negative outcomes such as: chronic hypertension, heart and lung disease and type 2 diabetes (Alisi et al., 2011; Hales and Barker, 1992; Jones et al., 2012; Pallotto and Kilbride, 2006; Wienerroither et al., 2001). Thanks to improved medical care, the survival rate of IUGR babies has increased; however, they still suffer from these metabolic syndromes later in life (Karimi et al., 2011).

In the past decade, many animal models has been used in human IUGR research,

but the majority of these models are experimentally induced such as maternal protein restriction (rat and mouse) (Bhasin et al., 2009; Fernandez-Twinn et al., 2003), passive smoking (rat and mouse) (Esposito et al., 2008; Jauniaux and Burton, 2007), and partial ligation of uteroplacental vessels (sheep and rabbit) (Duncan et al., 2004; Eixarch et al., 2012). Using the pig as a clinical IUGR model has great advantages including the similarity of pre- and postnatal growth and development to human, as well as the mechanisms of growth restriction and natural occurrence of IUGR (Eiby et al., 2013; Ferenc et al., 2014; Guilloteau et al., 2010; Wu et al., 2004a). Although the reproductive systems are different in pigs and women (two bicornate horns with multiple conceived fetuses *vs* simplex uterus with one pregnancy normally), the primary cause of natural occurrence IUGR in pigs is quiet similar to that of human, namely poor nutrient delivery or placental insufficiency (Blomberg et al., 2010). Intrauterine growth restriction in both species results in significantly low birth weight neonates and causes long-term postnatal complications (Ferenc et al., 2014; Wu et al., 2006).

To date, the mechanisms underlying impaired growth and metabolic syndromes of IUGR animals have not been well explored; impeding researchers from finding approaches to prenatal IUGR prevention and postnatal growth rescue. Therefore, the objective of our studies is to use pig as a model to investigate the regulatory signaling of impaired muscle growth and development in IUGR neonates.

**Maternal Nutrition**

Sufficient maternal nutrition is critical for fetal growth and development. Accordingly, maternal protein and energy intake or dietary amino acid profile may affect fetal growth and birth weight. These essential substrates drive the fetal growth and thus any alteration in supply impact fetal growth. When sows are fed with 28% of energy requirement throughout gestation piglets birth weights are decreased (Buitrago et al., 1974). In other studies, feeding low energy diets to sows during gestation did not significantly affect litter average birth weight (Bee, 2004; Lawlor, 2007; Martineau, 2009). This suggests that sows are capable of utilizing maternal reserves to support fetal growth when dietary energy is insufficient. In addition, feeding sows with extra energy (5% to 10% more than requirement) has no effect on litter birth weight (Azain, 1993; Gatlin et al., 2002; Laws et al., 2009). These studies suggest that low dietary energy may not be the cause of IUGR pigs.

*Protein*. The effect of maternal low energy diet on fetal birth weight is debatable; however low dietary protein or amino acid supply to sows has been proven that reduce fetal birth weight. When low protein (from 0.5% to 8.5%) diets were given to gestating sows, piglets born to these sows had reduced birth weights and impaired postnatal growth (Atinmo et al., 1974; Kusina et al., 1999; Rehfeldt et al., 2011; Schoknecht et al., 1993). Furthermore, feed gestating sows led excessive dietary protein also negatively affected piglet birth weights (Mickiewicz et al., 2012; Rehfeldt et al., 2011; Rehfeldt et al., 2012). Rehfeldt and his colleagues (Rehfeldt et al., 2012) have conducted an isoenergetic feeding experiment with low (6.5%), adequate (12%) and high (30%) levels of crude protein. In that study, feeding gilts a low protein diet

resulted in reduced average birth weight, decreased total muscle fiber number and total content of muscle DNA in the neonates (Rehfeldt et al., 2012).

*Amino acids.* In monogastric animals (i.e., human and pig), balanced amino acid profile is more important than the absolute quantity of protein (Kim et al., 2009). Feeding gestating sows a balanced amino acid diet can increase litter uniformity by 4% compared to an amino acid restricted diet (Kim et al., 2009). Over the past decade, great efforts were made to increase piglets' birth weights via maternal dietary interventions. Some interventions have demonstrated that increased average birth weights and/or reduced IUGR piglets could be achieved by supplementation of some amino acid or their derivatives (L-arginine, L-glutamine and carnitine) supplementation (Mateo et al., 2007; Ramanau, 2008; Wu et al., 2010; Wu et al., 2011). To date, however, no method has been reported that can prevents IUGR in pigs completely.

**Intrauterine Growth Restriction**

In recent years, swine breeders selected for prolific sows in order to improve farm efficiency which led to dramatic increase in litter size (8.87 pigs per litter *vs* 10.2 pigs per litter from June to November farrowing) (USDA, 2000). Unfortunately, the increase in litter size reduced uteroplacental blood flow and thus nutrient delivery to each fetus, which eventually resulted in increased number of IUGR piglets (Beaulieu et al., 2010; Milligan et al., 2002; Quiniou, 2002). It has been reported that 15 to 20% of new born piglets are affected by intrauterine growth restriction within the same litter (Quiniou, 2002; Wu et al., 2006).

*Mortality*. A common outcome of IUGR is increased mortality and morbidity rates in domestic animals (Mellor, 1983; van Rens et al., 2005). The death rate of piglets is as high as 35 % when their birth weight is below 0.8 kg (IUGR), by comparison with 4 % for birth weights between 1.2 to 1.4 kg (NBWT) (Quiniou, 2002). At wean, the survival rate declines from 95% to 15% as piglet birth weights drop from 1.8 to 0.61 kg (Quiniou, 2002). Similar observations were also reported in other livestock animals (i.e., horse, sheep and cattle) (Azzam et al., 1993; USDA, 1998, 2003). Further, many studies have shown that IUGR animals have increased risk of suffering from intestinal disorder, respiratory problems and reduced immune function (D'Inca et al., 2010; Joss-Moore et al., 2011; Zhong et al., 2012).

*Feed efficiency*. In addition to increased mortality and morbidity, IUGR affects daily gain and feed conversion. IUGR pigs have reduced feed efficiency (Gondret et al., 2006; Nissen and Oksbjerg, 2011; Powell and Aberle, 1980), as well as lower average daily body weight gain (ADG) during postnatal growth compared to their normal birth weight littermates (Gondret et al., 2005; Quiniou, 2002). In a more recent study, Nissen et al. (Nissen and Oksbjerg, 2011) reported that ADG was reduced by 30 g/d in IUGR piglets before weaning, and this reduction persisted until slaughter where the lower ADG increased to 87 g/d compared to high birth weight pigs. In the same study, feed conversion ratio of IUGR pigs increased by 80 g/kg gain which indicates they have lower feed efficiency compared to high birth weight pigs (Nissen and Oksbjerg, 2011). Similarly, IUGR lambs and low birth weight calves also exhibit lower efficiency of energy or feed utilization for protein and fat deposition (Greenwood

et al., 1998; Guerra-Martinez et al., 1990).

*Muscle growth.* Skeletal muscle of IUGR animals are not only smaller in size, but also contain fewer myofibers. A lower number of myofibers is apparent in IUGR pig muscle as early as 64 d of gestation and the difference remains until birth compared to their NBWT siblings (Dwyer et al., 1993; Gondret et al., 2006; Losel et al., 2009; Wigmore and Stickland, 1983). In addition, IUGR animals have lower intramuscular concentration of DNA which suggests that these animals also have fewer myonuclei (Greenwood et al., 2000; Rehfeldt and Kuhn, 2006), and this differences persist between low and high carcass weight pigs until slaughter (Nissen et al., 2004). Reduced myofiber number and DNA concentration in muscles implies compromised muscle growth potential in IUGR animals. In fact, evidence suggest that reduced muscle growth in IUGR animals is a general phenomenon across many species of farm animals including lambs, pigs, cattle and horses (Allen et al., 2004; Cundiff et al., 1986; Greenwood et al., 2000; Hegarty and Allen, 1978).

*Asymmetric growth*. In IUGR pigs, muscle growth is disproportional to internal organs and brain throughout postnatal life (Bauer et al., 2003; Rehfeldt and Kuhn, 2006). In the fetuses, skeletal muscle is more sensitive to maternal nutrient restriction because it has a lower priority for nutrient repartitioning compared to the brain and internal organs (Zhu et al., 2006). IUGR is associated with impaired muscle growth and altered body composition with less carcass lean compared to NBWT pigs (Gondret et al., 2006; Rehfeldt and Kuhn, 2006). As a result of asymmetric growth, IUGR pigs produce meat with more fat compared to their NBWT littermates (Gondret et al., 2006;

Poore and Fowden, 2004).

**Muscle Growth and Development**

Skeletal muscle is composed of bundles of muscle fibers or myofibers, and the formation of muscle fiber is termed myogenesis (Gerrard and Grant, 2003; Sciote and Morris, 2000). Myogenesis is a complex process includes somitic cell commitment to the myogenic lineage, myoblast proliferation, fusion and differentiation to myotubes (Le Grand and Rudnicki, 2007). All skeletal muscles of the trunk and limbs derive from the somitic dermomyotome (Buckingham et al., 2003). Myogenesis is initiated when a wave of myogenic progenitor cells originating from the epithelial borders of the dermomyotome form the primary myotome where muscle differentiation first occurs (Brent et al., 2003; Christ et al., 2007). The sequential growth of skeletal muscle during embryonic, fetal and postnatal period is attributed to a population of muscle progenitor cells derived from the center region of the dermomyotome that co-express Paired box protein 3 and 7 (Pax3/7). These cells can differentiate to embryonic myoblasts (form primary fibers during embryonic myogenesis), fetal myoblasts (form secondary fibers during fetal myogenesis), satellite cells (contribute to postnatal muscle growth and repair) or remain as a reserve cell pool within the growing muscle during the pre- and postnatal stages (Biressi et al., 2007; Buckingham and Vincent, 2009; Le Grand and Rudnicki, 2007).

*Prenatal myogenesis regulation*. Myogenesis is tightly regulated by a group of muscle specific transcriptional factors. In the embryonic stage, the first genetic

markers that label myogenic precursors are Pax3 and Pax7 (Biressi et al., 2007; Otto et al., 2006). The expression of Pax3 and Pax7 in somitic cells induces the subsequent expression of myogenic regulatory factors (MRFs) and therefore initiates the myogenic program (Bailey et al., 2001; Grifone et al., 2007; Stewart and Rittweger, 2006). Pax3 and Pax7 play an important role in prenatal myogenesis and postnatal muscle growth because they control the expression of MRFs (Bajard et al., 2006; Sato et al., 2010) and support proliferation and survival of myoblasts before differentiation in somites, limb muscle and satellite cells (Buckingham and Relaix, 2007; Collins et al., 2009). Downstream of Pax3 and Pax7, are four identified MRFs: myogenic factor 5 (Myf5), myoblast determination protein (MyoD), muscle-specific regulatory factor 4 (MRF4) and myogenin (Stewart and Rittweger, 2006). These factors have distinct functions and a certain level of redundant function as well (Roth et al., 2003; Rudnicki et al., 1992). MRF4 is mainly expressed in the early stage of undifferentiated proliferating cells and act as a determination gene (Kassar-Duchossoy et al., 2004). MRF4 also is expressed later and act as a dominant MRF in postnatal muscle (Stewart and Rittweger, 2006). Myf5 is the earliest marker of committed muscle cells and closely followed by MyoD (Lepper and Fan, 2010). The expression of MyoD leads to exit from cell cycle of the cells and enter terminal differentiation process (Manceau et al., 2008). MyoD and Myf5 can compensate with each other to a certain extent, and both induce multipotent precursor cells to the muscle lineage (Roth et al., 2003). Myogenin is essential for terminal differentiation and fusion of committed myoblasts (Barnoy and Kosower, 2007).

*Primary and secondary myogenesis*. In pigs, primary fibers formation occurs from 35 to 55 d gestation and secondary myogenesis occurs from 55 to 90 d gestation (Wigmore and Stickland, 1983). During embryonic myogenesis, only a small number of primary fibers are formed which then serve as a template for secondary fiber mygenesis in the fetal stage (Bailey et al., 2001). Due to the limited number of primary fibers formed during embryonic myogenesis, secondary myogenesis has major impact on muscle size and total fiber number (Du et al., 2010). In pigs, the primary and secondary fibers ratios can be as high as 20:1 to 24:1 (Oksbjerg et al., 2013). Therefore, secondary myogenesis is critical for muscle growth and development since myogenesis is completed at 80 to 90 d of gestation in pigs and fiber numbers within each muscle are fixed prior to birth (Brameld, 2008; Wigmore and Stickland, 1983). The formation of secondary fibers is largely determined by the fetal myoblasts number as well as their activity (Biressi et al., 2007). However，these cells are highly sensitive to nutrients which makes maternal nutrient supply a critical factor for muscle development at fetal stage (Dwyer et al., 1994; Rehfeldt and Kuhn, 2006; Wu et al., 2010). In fact, many studies have shown that IUGR piglets have reduced muscle size and total fiber number (mainly secondary fibers) which significantly affect postnatal muscle growth (Gondret et al., 2006; Losel et al., 2009; Wigmore and Stickland, 1983).

*Postnatal muscle growth*. Since muscle fiber number remains constant after birth, postnatal muscle growth mainly depends on an increase in muscle fiber size (hypertrophy) rather than new myofiber formation (Brameld, 2008; Wigmore and Stickland, 1983). The main contribution of nuclei for postnatal muscle growth is from

muscle satellite cells (Moss and Leblond, 1971). Satellite cells were first discovered by Alexander Mauro in electron micrographs of frog skeletal muscle in 1961 (Mauro, 1961). These cells were found closely attached to muscle fiber, between fiber membrane and basal lamina, which were then named "satellite cell" (Mauro, 1961). Lineage tracing studies have shown that satellite cells are derived from embryonic somite, same compartment as the embryonic myogenic cells as mentioned earlier (Gros et al., 2005; Kanisicak et al., 2009). The function of satellite cells are regulated by Pax3/7, MRFs as well as other environmental stimuli (Buckingham and Relaix, 2007). These cells are mitotically quiescent under normal condition, and in response to internal or external signals, are activated (express Pax7 but not MyoD), undergo proliferation (express Pax7 and MyoD) and differentiation (lose Pax7 and express myogenin) (Collins et al., 2005; Cornelison and Wold, 1997). Once activated, satellite cells undergo rapid proliferation, with a small portion of daughter cells renewing the original satellite cell pool, while the majority of these cells differentiate to myoblasts (Kuang et al., 2007; Yin et al., 2013). These myoblasts fuse with existing muscle fibers and provide external nuclei, thereby increasing DNA content and protein synthetic capacity in each fiber (Le Grand and Rudnicki, 2007). The majority of adult muscle nuclei originate from the muscle satellite cell which suggests postnatal muscle growth potential is highly related to satellite cell number per muscle fiber, as well as their proliferation and differentiation (Allen and Rodman, 1979; Orcel et al., 1979).

*Muscle fiber types*. Skeletal muscle is composed of heterogeneous populations of muscle fibers that differ in their ability to contract and metabolize energy (Bassel-Duby

and Olson, 2006; Schiaffino and Reggiani, 1996). On the basis of myosin heavy chain (MyHC) isoforms, muscle fibers can be classified as type I, type IIA, type IIX and type IIB fibers (Lefaucheur et al., 1998). Type I muscle fibers are the slowest contracting fibers, whereas type IIB are fastest contracting fibers, and type IIA and IIX are intermedial (Berchtold et al., 2000). Muscle containing more type I and type IIA fibers utilize more oxidative phosphorylation as energy source, which allows muscle for sustained activity (Berchtold et al., 2000; Lefaucheur et al., 1998). Type IIX and IIB fibers rely more on glycolytic metabolism for ATP production (Berchtold et al., 2000; Lefaucheur et al., 1998). During fetal myogenesis, primary myotubes in slow muscle mature to become type I fibers, whereas majority secondary myotubes in slow muscle mature to become type II fibers. In fast muscle, primary and secondary myotubes mature to become type II with only 3% of the secondary myotubes become type I fibers (Lefaucheur et al., 1995). As mentioned above, IUGR pigs muscle have fewer total muscle fiber at birth which is probably due to reduced amount of secondary fibers (Powell and Aberle, 1981). Therefore, one can predict that muscle from IUGR animals has higher proportion of type I fibers.

*Nutritional effect on satellite cells.* Muscle satellite cell activation, proliferation and differentiation are significantly influenced by nutrient supply. In malnourished children, satellite cell nuclei density (satellite cell relative nuclei to myonuclei ) is up to 45% lower than well-nourished children implying impaired proliferation of satellite cells due to insufficient nutrient supply (Hansen-Smith et al., 1979). In turkeys, satellite cell mitotic activity is higher in amino acid supplemented compared to feed

deprived group (Nierobisz et al., 2007). Further, in C2C12 myoblasts, differentiation is inhibited by glucose restriction (Fulco et al., 2008). Although the underling mechanism of muscle satellite cell response to nutrient restriction is unclear, it is obvious that nutrition level has a significant impact on their development.

Nutrition could affect satellite cell number and their activity throughout life. For example, prenatal undernutrition results in reduced muscle satellite cell number in mice at 6 weeks (Woo et al., 2011). Cultured satellite cells derived from IUGR sheep proliferate slower in media containing 10% fetal sheep serum (FSS) from normal and IUGR neonates, indicating that intrinsic factors impair satellite cell proliferation (Yates et al., 2014). Likewise, in pigs there was a tendency for slower proliferation and a significant lower differentiation of satellite cells in cultures derived from low weight compared to high weight pigs (Nissen and Oksbjerg, 2009). In another study, satellite cell numbers per fiber were lower in smaller pig compared to NBWT littermates (Brown and Stickland, 1993). Thus these studies provide evidence to show the impact of maternal undernutrition or low birth weight on muscle satellite cells number and their activity. However, none of these reports used a naturally occurring IUGR model. The impact of IUGR caused by placental insufficiency on muscle satellite cell activity needs further investigation.

*Muscle side population*. Although satellite cells are widely recognized as the primary cells responsible for postnatal muscle growth and regeneration, other type of cells are able to contribute to postnatal muscle growth including myogenic- endothelial progenitors, pericytes, bone marrow derived stem cells and muscle side population (SP)

cells (Dellavalle et al., 2007; Mitchell et al., 2010; Sampaolesi et al., 2006; Tamaki et al., 2002). Muscle SP cells reside in skeletal muscle interstitium juxtaposed to blood vessels, and can be isolated by Fluorescence Activated Cell Sorter (FACS) according to their unique exclusion of Hoechst 33342 DNA dye (Gussoni et al., 1999; Jackson et al., 1999). Muscle SP cells are a heterogeneous population, and while some lineage tracing experiments suggested that the main population of SP cells is derived from hypaxial somite and share a common ancestor with the satellite cell, others suggested they may have distinct origins such as bone marrow (McKinney-Freeman et al., 2003; Schienda et al., 2006). Nevertheless, adult muscle SP cells are thought to be multipotent and distinct from satellite cells since they are present in Pax7-/- mice which entirely lack muscle satellite cell (Asakura et al., 2002; Peault et al., 2007). In spite of the unclear origin, side population cells are able to differentiate into myogenic lineage by co-culture with myoblasts or forced expression of Pax7 or MyoD (Asakura et al., 2002; Seale et al., 2004). More importantly, SP cells could engraft into skeletal muscle, and repopulate the myogenic stem cell pool following intravascular delivery, which suggests they could have long term effect on postnatal muscle growth (Bachrach et al., 2006; Gussoni et al., 1999; Tanaka et al., 2009). In addition, studies revealed that these cells are capable of secreting growth factors that promote the proliferation of adjacent myogenic cells which further highlight their importance in postnatal muscle growth and repair (Frank et al., 2006; Motohashi et al., 2008).

**Regulation of Postnatal Muscle Hypertrophy**

Muscle growth is a process that includes an increase in myonuclei and protein deposition (Allen et al., 1979; Koohmaraie et al., 2002). As mentioned earlier, the majority of muscle DNA content is contributed by satellite cell activity during postnatal muscle growth (Allen and Rodman, 1979; Moss and Leblond, 1971). The process of protein deposition is determined by the balance between protein synthesis and degradation. Any net protein accretion (amount of protein synthesized minus amount of protein degraded) will result in muscle hypertrophy (Koohmaraie et al., 2002). The mechanisms regulate protein synthesis and degradation involve both anabolic and catabolic pathways includes the insulin Like Growth Factor 1 - Protein Kinase B - Mammalian Target of Rapamycin (IGF1-PKB-mTOR) pathway, Mitogen Activated Protein Kinase - Extracellular Signal Regulated Kinase (MAPK-ERK) pathway, translation initiation, ubiquitin-proteasome pathway, autophagy-lysosome pathway, calpain pathway and caspase proteolysis pathway (Fuentes et al., 2011; Kandarian and Jackman, 2006).

*IGF-1 pathway.* IGF-1 is 70 amino acids long, evolutionary conserved peptide that is synthesized in liver and local tissues including skeletal muscle, kidney and brain (Isgaard et al., 1989; Le Roith et al., 2001). Locally produced IGF-1 in skeletal muscle plays an important role in regulating postnatal muscle growth and development by stimulating myogenic cell proliferation, differentiation and enhancing myofiber protein synthesis (Coleman et al., 1995; Rommel et al., 2001; Velloso, 2008). The addition of exogenous IGF-1 into either myogenic cell culture or muscle fiber via direct infusion results in a significant increase in myotube or myofiber size (Adams and

McCue, 1998; Ge et al., 2013; Rommel et al., 2001; Shavlakadze et al., 2010; Vyas et al., 2002). Likewise, overexpression of muscle specific IGF-1 gene leads to enlarged muscle fibers (Musaro et al., 2001; Shavlakadze et al., 2005), and upregulates muscle IGF-1 mRNA and protein in regenerating damaged muscles (Matheny et al., 2010; Pelosi et al., 2007). Collectively, these studies suggest that local IGF-1 is able to stimulate muscle hypertrophy and regeneration effectively. The two major IGF-1 dependent mechanisms are IGF1-PKB-mTOR pathway and IGF1-MAPK-ERK pathway.

*mTOR signaling pathway.* The mTOR (mammalian target of rapamycin) is a serine/threonine protein kinase that acts as a key regulator of protein synthesis in skeletal muscle, and can be stimulated by insulin, growth factors (IGF-1 and IGF-2) and amino acids (Hay and Sonenberg, 2004). In eukaryotes, two independently regulated and functionally distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) exist (Loewith et al., 2002; Oh and Jacinto, 2011; Sengupta et al., 2010). mTORC1 contains regulatory associated protein (RAPTOR) (Kim et al., 2002), and mTORC2 contains rapamycin insensitive component (RICTOR) (Guertin et al., 2006; Zoncu et al., 2011b). The crucial role of mTORC1 has been intensively studied and found to regulate protein synthesis, myogenic cell proliferation and differentiation in response to hormones, growth factors, and nutrients (Foster et al., 2010; Hay and Sonenberg, 2004; Schiaffino et al., 2013). Binding of IGF-1 to its receptor activates its intrinsic tyrosine kinase and leads to autophosphorylation which then provides a docking site for insulin receptor substrate (IRS). Phosphorylated IRS

by IGF-1 receptor recruits and activates phosphatidylinostitol-3-kinase (PI3K), which converts phosphoinositide-4, 5-biphosphate ($PIP_2$) to phosphatidylinositol-3, 4, 5-triphosphate ($PIP_3$). $PIP_3$ provides docking sites for two kinases: phosphoinositide-dependent kinase 1 (PDK1) and PKB. Phosphorylation of PKB at serine 308 by PDK1 leads to its activation. PKB in turn activates mTORC1 that mediated by tuberous sclerosis 1/2 complexes (TSC1/2) (Atherton and Smith, 2012; Egerman and Glass, 2014; Schiaffino and Mammucari, 2011).

*Regulation of mTOR pathway.* mTOR is a key regulatory protein in this pathway that stimulates several downstream targets including p70 ribosomal protein S6 kinase 1 (p70S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (Avruch et al., 2009). The phosphorylation and activation of S6K1 by mTORC1 promotes mRNA translation initiation and elongation through several substrates, such as eukaryotic elongation factor 2 kinase (eEF2K), eukaryotic initiation factor 4B (eIF4B) and ribosomal protein S6 (Ma and Blenis, 2009). Dephosphorylated 4EBP1 binds to eukaryotic translation initiation factor 4E (eIF4E) and interferes the formation of translation initiation factor 4F (eIF4F) complex, thus blocking the cap-dependent mRNA translation initiation (Pause et al., 1994). mTOR phosphorylates 4EBP1 and thus releases eIF4E, therefore stimulates translation initiation (Beretta et al., 1996; Gingras et al., 1999).

*Translation initiation*. In eukaryotes, mRNA translation includes three steps: initiation, elongation and termination. The translational rate is regulated primarily at the initiation step and is a process of delivery of methyonyl-tRNA (Met-tRNA) to the

start codon and elongation-competent 80 S ribosome assembly (Jackson et al., 2010; Lopez-Lastra et al., 2005). The first step is the binding of 40S ribosome onto mRNA leads to the formation of a ternary complex (TC) includes GTP-bound form of eukaryotic initiation factor 2 (eIF2) and tRNA initiator. The 40S ribosomal subunit recruits the TC to form 43S initiation complex (Lopez-Lastra et al., 2005; Sonenberg and Hinnebusch, 2009). The 43S ribosomal complex is recruited to 5' end of the mRNA and scans the 5' untranslated region until it reaches the AUG initiation codon which requires a group of eIFs (eIF4E, 4A, 4G, 4B) (Richter and Sonenberg, 2005; Scheper and Proud, 2002).

The eIF4F complex is a crucial factor in translation initiation that is composed of eIF4E, eIF4A and eIF4G and can promotes binding of 43S to mRNA via a cap- eIF4E-eIF4G-eIF3 interaction to form 48S complex. The eIF4G is a scaffold protein that harbors binding domains for poly (A)-binding-protein (PABP), eIF4E, eIF4A and eIF3. The interaction of eIF4G with PABP connects the 5' and 3' ends of mRNA. Interaction of eIF4G and eIF3 establishes a protein bridge between 43S complex and the 5' end of mRNA (Hinnebusch, 2011; Korostelev, 2014; Lorsch and Dever, 2010). Once bound to the mRNA, 43S scans the mRNA in the 5' to 3' direction for a start codon followed by Met-tRNA base pairs with AUG codon in the peptidyl-tRNA site, forming 48S initiation complex (Hinnebusch, 2011; Kolitz et al., 2009). The 48S complex joins the 60S subunit to produce 80S initiation complex containing Met-tRNA base-paired to start codon and ready for the elongation step of protein synthesis (Hinnebusch, 2011; Kolitz et al., 2009; Korostelev, 2014).

*Peptide elongation*. The second step of translation is elongation, during which a ribosome moves one codon forward and peptidyl tRNA moves from the A to P site, forming a peptide bond (Wang and Proud, 2006). This process is considered a cycle and in each cycle the complex forms one peptide bond and moves 3 ribonucleotides along the mRNA until it reaches the stop codon (UAA, UAG or UGA). The peptide elongation is mainly mediated by eukaryotic elongation factor 2 (eEF2) which facilitates the translocation of ribosome (Christophersen et al., 2002). The phosphorylation of eEF2 catalyzed by eEF2 kinase (eEF2K) inhibits its binding to ribosomes and thus results in down-regulation of elongation (Christophersen et al., 2002; Connolly et al., 2006). In response to increased cellular energy demand or reduced ATP production, eEF2 is phosphorylated by AMP-activated protein kinase (AMPK) (Hardie, 2007).

*Energy sensitivity of protein synthesis*. Protein synthesis in mammalian cells is a costly process that not only uses amino acids as substrates but also consumes a large proportion of cellular energy. More specifically, protein synthesis costs the cells 5 ATPs for each peptide bond formation and 2300 ATP for a typical protein (Princiotta et al., 2003). Therefore, protein synthesis is sensitive to cellular energy status and is down-regulated when ATP production is insufficient (Bolster et al., 2002; Winder, 2001). The AMPK is a cellular energy sensor that coordinates many signaling pathways to maintain energy homeostasis (Hardie, 2007; Lim et al., 2010). The protein senses the change of AMP:ATP ratio caused by energy deprivation and inhibits cellular protein synthesis (Bolster et al., 2002; Hardie, 2007). Under low energy

conditions, activated AMPK inhibits the activity of mTORC1 and eEF2 which in turn down regulate translation initiation and elongation resulting in diminished protein synthesis (Inoki et al., 2012; Thomson et al., 2008).

*Variation of protein synthesis*. Protein synthesis rate is not constant across different tissues and organs. The overall protein synthesis rate in adult humans is much lower in skeletal muscle than other tissues such as liver (Arfania et al., 1981). In rats, the fractional protein synthesis rate is highest in liver and lowest in skeletal muscle; while in juvenile rats it is highest in the spleen and lowest in muscle (Shahbazian et al., 1987). In several other vertebrates (goldfish, bullfrog, lizard, chicken and mouse), protein synthesis rates in muscle are approximately 8 to 10 fold lower than in liver (Sayegh and Lajtha, 1989). Similar results are found in pigs, which is probably because muscle fibers are long lived multinucleated cells with relatively constant protein turnover rate, and do not serve as a major endocrine tissue (Bregendahl et al., 2004; Burrin et al., 1992). Protein synthesis rate also differs at diverse ages. Rates of growth and protein synthesis are greatest during the neonatal period (Davis et al., 1989; Denne et al., 1991), and decline with age (Short et al., 2004).

*IGF-1 pathway in IUGR.* Due to the important role in mediating protein synthesis and muscle growth, numerous scientists have investigated the IGF1-PKB-mTOR pathway. Many studies have shown plasma IGF1 level in human IUGR infants or induced IUGR animal models was reduced at birth and in the neonatal period (Ohkawa et al., 2010a; Ostlund et al., 2002; Setia and Sridhar, 2009). There are some indications that a significant association exists between reduced plasma IGF1 level and

metabolic syndrome as well as type 2 diabetes in IUGR infants (Buckingham and Vincent, 2009). The longissimus muscle of the IUGR pig fetus has greater mRNA expression of IGF-1 receptor at d 65 and d 100 of gestation compared to normal fetuses (Tilley et al., 2007). IGF-1 mRNA expression is significantly less in IUGR neonatal pig muscle (Rongjun Chen, 2010a). More importantly, IUGR modifies the histone code of the hepatic IGF-1 gene in rat which suggests permanent genetic alteration in IUGR neonates (Fu et al., 2009). Therefore low plasma IGF-1 levels may be a consequence rather than a cause of IUGR in prenatal period. In addition, amniotic IGF-1 administration significantly enhances fetal growth rate of IUGR sheep which provides a potential approach for treatment of the IUGR infant (Eremia et al., 2007).

According to the current literature, two key proteins are altered downstream of IGF-1 and its receptor in IUGR human and animal models. First, PKB expression is 40% lower in skeletal muscle while eIF4E was reduced by 45% in liver suggesting that translation initiation signaling limiting protein synthesis rate in muscle and liver (Thorn et al., 2009). Second, eIF3 subunit 2 β (also called eIF3i) expression is significantly diminished in neonatal IUGR pig muscle which also implies a possible negative regulation of translation initiation in IUGR (Wang et al., 2008). Although, little information is available about PKB-mTOR and/or translation initiation in IUGR animal muscle, one can expect inherent flaws in these pathways due to the fact that IUGR animals have impaired muscle growth, higher feed conversion ratio and more fat deposition on carcass.

*Branched-chain amino acids.* Branched-chain amino acids (BCAA) are

essential amino acids that have a critical role in stimulating protein synthesis (particularly leucine) (Stipanuk, 2007). Leucine not only serves as a substrate for protein synthesis, but also directly stimulates mTOR signaling to enhance translation initiation (Cota et al., 2006; Yin et al., 2010). When compared to other essential amino acids, leucine supplementation to C2C12 myoblasts showed the greatest stimulation of anabolic response and was the only amino acid that activated mTOR and phosphorylated 4EBP1 (Atherton et al., 2010). Earlier in vivo studies revealed that oral leucine administration stimulated protein synthesis rate in muscle through mTOR dependent mechanism (Anthony et al., 2000a; Lynch et al., 2002). Additionally, intravenous leucine infusion in fasted neonatal pigs increased protein synthesis in skeletal muscle by activating two key proteins downstream of mTOR: S6K1 and 4EBP1 (Escobar et al., 2005). Further, the effect of BCAA on protein synthesis is due exclusively to leucine, since valine and isoleucine failed to enhance protein synthesis and activate mTOR pathway (Escobar et al., 2006). Although the stimulatory effect of leucine on protein synthesis is rapid, it is also transient, protein synthesis rates return to normal level within 2 h despite the continued infusion of leucine and activation of mTOR pathway (Escobar et al., 2005, 2006). This is probably because some substrates (amino acids) reach an insufficient level that limits protein synthesis.

The anabolic effect of leucine is believed to occur through an mTOR dependent mechanism (Crozier et al., 2005; Vary and Lynch, 2006), and is mediated through eIF4G phosphorylation and eIF4E and eIF4G association (Anthony et al., 2000a; Crozier et al., 2005; Lang, 2006). For instance, coinfusion of leucine and rapamycin

(mTORC1 inhibitor) prevents any increase in fractional protein synthesis rates and phosphorylation of S6K1 and 4EBP1 were blocked which suggestes that the effect of leucine is mTORC1 dependent (Suryawan et al., 2008). As the most effective single amino acid stimulator for protein synthesis, leucine may be an ideal dietary supplementation to mitigate the effect of IUGR in humans and animals. To date, however, no study has investigated the effect of leucine supplementation on muscle growth in IUGR neonates.

*MAPK signaling pathway.* The MAPK signaling pathway is another mechanism by which skeletal muscle growth and development are regulated. MAPKs are a group of Ser/Thr protein kinases that mediate gene expression, mitosis, metabolism, translation, apoptosis and differentiation in response to extracellular stimuli such as growth factors and stress (Cargnello and Roux, 2011; Lawan et al., 2013; Meloche and Pouyssegur, 2007; Roberts and Der, 2007). The MAPK pathway consists of several members that are sequentially activated: MAPK kinase kinase (MAPKKK or MEKK), which phosphorylates MAPK kinase (MAPKK or MEK), which then phosphorylates MAPK (Lawan et al., 2013). MAPK phosphorylates a set of downstream proteins, among which ERK1 and ERK2 are known to be important for mRNA translation. Upon the activation of MEK, ERK1/2 phosphorylates MAPK interacting kinase 1(MNK1) which regulates translation initiation via eIF4E phosphorylation (Ellederova et al., 2008; Fukunaga and Hunter, 1997). ERK1/2 phosphorylates several ribosomal S6 kinases (RSK1-4), which have a set of target proteins such as ribosomal protein S6 (spS6), eIF4B, and eukaryotic elongation factor 2 kinase (eEF2K) (Romeo et al., 2012).

The rpS6 is a component of 40S ribosomal subunit that is phosphorylated by S6K1/2 when mTOR is activated (Ruvinsky and Meyuhas, 2006). Recent studies suggest that rpS6 phosphorylation can also occur in an mTOR independent manner (Pende et al., 2004; Roux et al., 2007). MEK/ERK pathway also stimulates mTORC1 complex by direct phosphorylation of tuberous sclerosis complex 2 (TSC2) which provides a connection between two pathways (Ma et al., 2005; Rolfe et al., 2005; Roux et al., 2007).

As an important target downstream of ERK1/2, MNK1/2 plays a critical role in translation initiation. As mentioned above, during translation initiation, eIF4G serves as a scaffold and binds to 40S ribosomal subunits to the 5' end of mRNA through association with eIF3 (Gingras et al., 1999; Mahoney et al., 2009). The interaction of eIF4E with eIF4G are part of the eIF4F complex is considered as the rate limiting step in translation initiation (Jackson et al., 2010). This step is extensively studied and is reported to be under the regulation of both mTOR and MAPK pathways (Ellederova et al., 2008; Rajasekhar et al., 2003; Sonenberg and Hinnebusch, 2009; Walsh and Mohr, 2014). In a non-phosphorylated form, 4EBP1 binds to eIF4E preventing it from forming a complex with eIF4G. Conversely when 4EBP1 is phosphorylated by mTOR, it releases eIF4E and allows eIF4E • eIF4F complex formation (Gingras et al., 1999; Sengupta et al., 2010). Once released, eIF4E is phosphorylated at Ser209 upon activation of ERK1/2 or p38 MAPKs a process mediated by MNK1/2 (Ueda et al., 2004; Waskiewicz et al., 1997). MNK2 accounts for most of the basal eIF4E phosphorylation under basal condition, while MNK1 phosphorylates eIF4E in response

to p38MAPKs and/or ERK1/2 activation (Kohn et al., 2003; Scheper et al., 2001). Further, eIF4E phosphorylation is completely blocked in MNK1/2 knockout animals, suggesting that MNK1/2 are the dominant kinases for eIF4E phosphorylation (Ueda et al., 2004). MNK1 is recruited to the eIGF4F complex through association with the C terminus of eIF4G (Pyronnet et al., 1999; Quan et al., 1995; Ueda et al., 2004). The recruitment of MNK1 to the eIF4F complex depends on eIF3 subunit e (eIF3e), and eIF3e can effectively promote the binding of MNK1 to eIF4G (Walsh and Mohr, 2014). Although the mechanism of translation initiation has been extensively studied for over two decades in normal animals, it is still unclear so far that whether this step is altered in IUGR animals.

**Protein degradation**

Muscle hypertrophy depends on the dynamic relationship of protein synthesis and degradation. As the largest amino acid pool, skeletal muscle undergoes proteolysis to release amino acids when dietary protein supplies fail to provide sufficient amino acids and/or energy for the vital organs and the brain (Ventadour and Attaix, 2006). There are several mechanisms that control protein degradation in animals: ubiquitin-proteasome, autophagy-lysosomal, calpain and caspase proteolysis systems.

*Ubiquitin proteasome pathway*. One of the major protein degradation pathways in mammalian cells is ubiquitin-proteasome. In skeletal muscle, degradation of sarcomeric proteins is controlled by the ubiquitin-proteasome (Bodine et al., 2001; Sandri, 2008; Ventadour and Attaix, 2006). Once a protein is tagged by ubiquitin for

proteolysis, it is broken down first by 26S proteasome to short peptides, and followed by quick degradation to amino acids (Ventadour and Attaix, 2006). The proteasome is able to degrade sarcoplasmic protein, however it cannot degrade myofibrillar proteins until they have been removed from myofibril (Bonaldo and Sandri, 2013; Ventadour and Attaix, 2006). Proteins are "tagged" for degradation by attaching multiple ubiquitin proteins to lysine residues on the target, and this process requires ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin protein ligase (E3) (Komander, 2009). Ubiquitin ligases E3 are thought to be the rate limiting step in proteasome dependent degradation. Two of the most important ligases in skeletal muscle are: muscle atrophy F-box (MAFbx or atrogin-1) and muscle RING fiber 1 (MuRF1) (Bodine et al., 2001; Lecker et al., 2006; Yang et al., 2009). Mice lacking of MAFbx and MuRF1 genes have reduced muscle degradation, indicating the critical roles of these two proteins in proteolysis (Bodine et al., 2001). So far, the specific mechanisms of MAFbx and MuRF1 in muscle are still not clear. MAFbx is implicated in mediating growth pathways in muscle such as MyoD, myogenin and eukaryotic translation initiation factor 3 subunit F (eIF3f) (Cong et al., 2011). In contrast, MuRF1 interacts with many muscle structural proteins such as myosin heavy chain, myosin light chain, titin and troponin suggesting its important role in facilitating muscle structural protein degradation (Baehr et al., 2011; Cohen et al., 2009; Fielitz et al., 2007; Polge et al., 2011).

*Autophagy-lysosomal pathway*. Autophagy-lysosomal is another important proteolysis pathway which plays a crucial role in protein turnover in response to

extrinsic stimulation, including cellular stress, nutrient deprivation and amino acid insufficiency (Mizushima et al., 2008; Sandri, 2010a). Autophagy delivers the damaged proteins and organelles to the lysosome via autophagosomes, and then fuse with lysosomes (Bechet et al., 2005; Schneider and Zhang, 2010). Lysosome are membrane bound vesicles containing various enzymes includes proteases, glycosidases, lipases, nucleases and phosphatases which are responsible for degradation of macromolecules (Bonaldo and Sandri, 2013; Sandri, 2010b). There are three autophagic mechanisms in mammalian cells: microautophagy, chaperone-mediated autophagy and macroautophagy. Macroautophagy is a major degradation mechanism in skeletal muscle. Macroautophagy sequesters cytoplasm or organelle proteins, and expand to form an autophagosome which is mediated by microtubule associated protein 1 light chain 3 (LC3) (Hanna et al., 2012). Addition of phosphatidylethanolamine to LC3-I (cytosolic form) produces LC3-II, which binds to the membrane of autophagosome until it fuses with the lysosome (Mizushima et al., 2010).

*Calpain*. The calpain system is a family of 16 intracellular calcium dependent non-lysosomal cysteine proteases, which mediates the cleavage of specific tertiary structure substrates (Cuerrier et al., 2005; Huang and Wang, 2001). m-calpain and μ-calpain, are encoded by CAPN1 and CAPN2 genes respectively, and are dependent on millimolar and micormolar of $Ca^{2+}$ concentration for their half- maximal proteolytic activation (Goll et al., 2003; Wu and Lynch, 2006). There are many targets of calpain in skeletal muscle, including nebulin, titin, desmin and talin which are important cytoskeletal proteins connecting myofilaments and Z-disk (Bonaldo and Sandri, 2013;

Lim et al., 2004). Unlike other proteolytic system, calpain do not degrade polypeptides completely but rather cleaves protein substrates from skeletal muscle myofibrils, thereby generating polypeptide fragments for further degradation by other systems, including ubiquitin proteasome or autophagy lysosome (Bonaldo and Sandri, 2013).

*Caspase*. Caspases are cysteine proteases that play critical roles in protein degradation during apoptosis (Fan et al., 2005). Activation of caspases can be stimulated by receptor mediated mechanisms or by the release of proapoptotic factors from mitochondrial permeability transition pore (Dupont-Versteegden, 2005). Normally, muscle fibers are long lived cells that are resistant to apoptosis (Dupont-Versteegden, 2006). Therefore, caspases are more active in atrophying fibers than healthy muscle fibers.

*Regulation of protein degradation*. Many studies have shown that IGF1/PKB signaling controls both protein synthesis through mTOR, and protein degradation via forkhead box proteins （FOX0） family which includes three isoforms: FOX01, FOX03 and FOX04 (Raben et al., 2008; Sandri et al., 2004; Stitt et al., 2004). PKB phosphorylates FOX0 proteins and promotes their translocation from the nucleus to the cytoplasm (Cadwell et al., 2008; Stitt et al., 2004). Inhibition of PKB in various muscle atrophy models caused reduced phosphorylation of FOX0 in the cytoplasm with an increase in the nucleus (Calnan and Brunet, 2008), and results in enhanced MAFbx, MuRF1 expression and autophagy activity (Southgate et al., 2007; Stitt et al., 2004). In FOX01 overexpressing transgenic mice, muscle mass was significantly reduced

(Southgate et al., 2007). In addition to PKB/FOX0 signaling, MAFbx and MuRF1 can also be inhibited, at least partially, by activation of mTORC1 (Herningtyas et al., 2008; Shimizu et al., 2011). Nutrients, especially amino acids also regulate protein degradation via stimulation of mTORC1 signaling pathway and thus promote lysosomal dependent protein degradation (Zoncu et al., 2011a). To date, protein degradation in IUGR animals muscle remains unclear and needs further investigation.

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integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol 12: 21-35.

# Chapter 2.

# Characterization of muscle satellite cell and side population in neonatal IUGR pig

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**Abstract**

Satellite cells are myogenic precursors that support muscle fiber hypertrophy. A decline in the pool size or propensity of these cells to proliferate and differentiate may hinder growth. The aim of this study was to define the characteristics of satellite cell and other muscle specific stem cell in intrauterine growth restricted (IUGR) pigs. Satellite cells were isolated from semitendinosus (ST) muscle of normal (NBWT) and low birth weight neonatal pigs. Immunohistochemical staining was applied to ST muscle sections to determine fiber number, size and satellite cell number. Flow cytometry was used to assess the percentage of muscle side population (SP) cells. Newborn IUGR pigs had 30% fewer fibers ($P \leq 0.05$) and a 38% smaller fiber size ($P \leq 0.05$) compared to NBWT littermates. In IUGR pigs, ST muscle contained 60% fewer ($P \leq 0.01$) satellite cells per hundred fibers than their NBWT siblings. Ultimate satellite cell fusion percentage of IUGR pigs was 8% lower ($P \leq 0.05$) than that of NBWT; however no differences in proliferation was observed between the two groups. Compared to their NBWT littermates, IUGR pigs have a lower percentage of muscle SP cells ($P \leq 0.05$). These data suggest that impaired postnatal muscle may be a result of reduced muscle stem cell number and their ability to fuse.

**Keywords:** IUGR, satellite cell, muscle fiber, pig

## Introduction

In domestic animals, maternal nutrition plays an important role in fetal growth and postnatal performance. Both prenatal nutrient deficiency or imbalance may lead to intrauterine growth restriction (IUGR) which is defined as impaired fetal growth and development, and has critical effects on postnatal growth (Wu et al., 2006). In pigs, IUGR develops from d 30 to 45 until term, and is mainly caused by multiple conceived fetuses that surpass functional uterine capacity of the sow (Kim et al., 2009). Consequently, IUGR pigs have increased morbidity and mortality, reduced growth performance, as well as unfavorable meat quality that causes large economic losses (Gondret et al., 2006; Nissen and Oksbjerg, 2011; Wu et al., 2006).

Skeletal muscles largely represent the profit in swine industry. In prenatal myogenesis, primary myofiber hyperplasia is completed around 35 to 55 days of gestation, while secondary fibers is completed in late gestation between 85 to 95 days in pigs, resulting in muscle fiber number being pre-determined prenatally (Wigmore and Stickland, 1983). Myofiber number are fewer in IUGR pigs as early as 64 days gestation compared to their normal body weight (NBWT) siblings (Dwyer et al., 1993; Wigmore and Stickland, 1983). With fewer muscle fiber at birth, IUGR pigs undoubtedly have lower postnatal growth potential compare to their heavier littermates.

Muscle fiber growth is associated with increment of nuclei number and protein amount (Dayton and White, 2008). The main contribution of nuclei to postnatal skeletal muscle growth and development is from muscle stem cells termed satellite cells (SC). Satellite cells reside between fiber membrane and basal lamina, and represent the primary source of myogenic cells for muscle growth and regeneration (Mauro, 1961). Postnatal muscle hypertrophy potential is highly related to SC number per fiber and their propensity to proliferate and differentiate (Allen and Rodman, 1979). Previous studies have investigated SCs in different low birth weight animal models. There is an agreement that satellite cell number per fiber is lower in smaller compared to heavier littermates in rodents (Brown and Stickland, 1993), and that proliferation of SC is affected by birth weight in pigs and sheep (Nissen and Oksbjerg, 2009; Yates et

al., 2014). Similarly, differentiation of SC is also decreased in lowest birth weight of 6-week old pigs (Nissen and Oksbjerg, 2009).

Another population of multi-potent muscle stem cell referred to as side population (SP), could differentiate into myogenic cells (Asakura et al., 2002; Bachrach et al., 2006; Gussoni et al., 1999; Tanaka et al., 2009). Muscle SP cells are distinct stem cell population that can be identified by Fluorescence Activated Cell Sorter (FACS) according to their unique exclusion of Hoechst 33342 DNA dye (Gussoni et al., 1999). Importantly, SP cells can migrate through blood vessel walls and repopulate the SC pool which could have long term effect on postnatal muscle growth (Tanaka et al., 2009).

To date, it is still unclear whether impaired postnatal muscle growth of IUGR pigs is associated with a decline in SC pool size or propensity of these cells to proliferate and differentiate. Therefore, the aim of this study was to characterize muscle satellite cells and SP in IUGR neonatal pigs.

## Material and Methods

*Animals and satellite cell isolation.* New-born piglets from Virginia Tech Swine Center were defined as normal body weight (NBWT) when birth weight was between 1.4 to 1.6 kg, and IUGR when weight was below 0.9 kg. Four IUGR and four NBWT neonatal pigs were euthanized for muscle collection and satellite cell isolation. Left semitendinosus (ST) muscle was isolated for muscle histology. Right ST muscle was dissected for satellite cell isolation. About 10 mg muscle was minced and digested for 50 min at 37 $^{o}$C in an enzyme solution containing 0.8 mg • $mL^{-1}$ protease (Sigma-Aldrich, St Louis, MO) in PBS. After protease digestion, cells were differentially centrifuged at 1200 × g for 5 min and followed by 300 × g for 10 min. The cell suspension was then filtered through 250-micron nylon mesh and cells were harvested by centrifugation at 1200 × g for 15 min. All experimental procedures were approved by Virginia Tech Institutional Animal Care and Use Committee.

*Immunohistochemical analysis.* Approximately 1 cm long ST muscle sample was first embedded in Tissue-Tek medium (VWR International, Radnor, PA), followed by

freezing in super-cold isopentane, and 10 μm muscle sections were collected onto glass microscope slides. After air drying, sections were fixed in 4% paraformaldehyde (PFA) for 10 min, washed in Phosphate Buffered Saline Tween-20 buffer (PBST) and incubated in PBST buffer containing 3% BSA and 5% goat serum for 1 h at room temperature (RT). Primary anti-Pax7 antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa city, IA) was applied at 4 $^{o}$C overnight. Anti-wheat germ agglutinin (WAG) and DAPI (Abcam, Cambridge, MA) were added with secondary antibody (DyLight 488 green goat anti-mouse IgG, Thermo Fisher Scientific) for 1 h at RT for visualization of membranes and nuclei. Photomicrographs were captured with Nikon TI-U inverted microscope (Nikon, Lewisville, TX) at a magnification of 20×. Cross-sectional area (CSA) of the fibers was determined by the region within the WAG staining boundary. Fiber number was determined by dividing total section area by average muscle fiber area. Pax7 positive cells: fibers ratio was calculated as the number of Pax7 positive cells divide by total fiber number. Primary and secondary fibers were determined by their physiological position, and their mean CSA was measured from at least 120 fibers of each type per animal.

*DNA, RNA and protein content.* Muscle RNA, DNA and protein were quantified using the Schmidt-Thannhauser's method ((Munro and Fleck, 1966). Briefly, 250mg of ST muscle was ground in liquid nitrogen, mixed with 4 mL of 2% (wt/vol) perchloric acid (PCA), and centrifuged at 2,800 g for 15 min at 4 ℃. Pellets were re-suspended with 4mL of 0.3N NaOH and incubated at 37 ℃ for 1 h. The remaining solution was mixed with 2 mL of 12% PCA, incubated on ice for 10 min and centrifuged for 10 min at 4 ℃ at 12,100 × g. After washing with 2% PCA, the acid-soluble supernatant was analyzed by spectrophotometer for measuring RNA concentration (Ushida et al., 1985). The acid-insoluble pellet was re-suspended by incubation with 10 % PCA for 1 h at 70 ℃ and DNA was quantified using the diphenylamine method (Myers, 1965). Protein was quantified using bicinchoninic acid (BCA) assay following manufacturer's recommendations (Pierce, Rockford, IL).

*Cell proliferation and myogenic differentiation*. Satellite cells were seeded on 2% growth factor reduced Matrigel (BD Bioscience, San Jose, CA) coated 96-well flat-

bottom plates at a density of $2 \times 10^3$ cells per well in growth medium containing Minimum Essential Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) and 1% antibiotics (Sigma-Aldrich, St Louis, MO) and allowed to attach for 24 hours. Cell proliferation rate was evaluated every 24 h over 5 d culture period using the Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI). To measure myogenic differentiation, satellite cells were seeded on 2% Matrigel coated 6-well plates at $2 \times 10^5$ cells per $cm^2$. Cells were induced to differentiate when they reach 80% confluence by switching to differentiation medium (2% horse serum in Minimum Essential Medium with antibiotics). When fully differentiated, cells were fixed and stained with anti-myosin antibody and DAPI to visualize myotube and nuclei. Terminal fusion percentage was calculated by dividing the number of fused nuclei in myotubes by the number of total nuclei within a given region of interest. Fusion density was determined by dividing the number of fused nuclei by the area of selected myotubes.

*Flow cytometry.* Muscle cell isolates ($5 \times 10^6 \cdot mL^{-1}$) were pre-warmed to 37 ℃, and Hoechest 33342 (Sigma-Aldrich, St Louis, MO) was added to reach a final concentration of $5\ \mu g \cdot mL^{-1}$. Cells were incubated for 90 min at 37 ℃ for 10 min and centrifuged at 1500 × g. Cell pellets were re-suspended in Hanks's balanced saline solution containing 2% FBS and 2mM HEPES buffer (Sigma-Aldrich, St Louis, MO). Flow cytometry analyses were performed on Coulter ALTRA Cell Sorter (Beckman Coulter Inc. Miami, FL).

*Statistics*. Data was analyzed using the PROC MIXED procedure (SAS Inst. Inc., Cary, NC). Pairwise comparisons between the least square means of the factor levels were determined using the PDIFF option of the LSMEANS statement. Data are represented as least squares means ± SEM. Statistical significance was established at $P < 0.05$.

**Results**

*Muscle histology.* Intact left side ST muscle was used for muscle weight and size comparison. The muscle from IUGR pigs was lighter ($P \leq 0.05$) and smaller ($P \leq 0.05$)

compare to those from NBWT pigs (Table 2-1). IUGR pigs had 30% fewer ($P \leq 0.05$) fibers than their NBWT siblings in ST muscle at birth (Fig.1A). Average fiber CSA in ST muscle of newborn IUGR pigs was 61% of NBWT (150.2 $\mu m^2$ *vs* 92.5 $\mu m^2$, $P \leq 0.05$) (Fig.2-1B). Primary and secondary fibers were identified and analyzed according to their physiological location. In IUGR pigs, secondary fibers were significantly smaller ($P < 0.001$) than primary fibers (Fig.2-2B). However, the sizes of primary and secondary fibers in NBWT pig were too close so that we were not able to distinguish them. Nevertheless, the mean CSA of all fibers in NBWT was not differ from the CSA of primary fibers in IUGR pigs (Fig.2-2C).

*Protein, RNA and DNA content.* Muscle protein and RNA contents ($mg \bullet g^{-1}$) were not different between IUGR and NBWT pigs, however, total amounts of protein and RNA were greater in NBWT pigs given that their muscle weight was significantly greater. Muscle DNA content was less ($P \leq 0.05$) in IUGR pigs which implies fewer nuclei per gram muscle (Table 2-1).

*Satellite cell and side population content.* Immunostaining of Pax7 was used to identify satellite cells in muscle sections. NBWT pigs had 60% more ($P \leq 0.01$) satellite cells per hundred muscle fibers (Fig.2-3). Flow cytometry was used for muscle side population identification and quantification. Compared to their NBWT littermates, the proportion of side population from the total number of cells was lower in IUGR pig ($P \leq 0.01$) (Fig.2-4).

*Proliferation and fusion.* Satellite cell proliferation was assessed for 5 days, during which no difference was observed between IUGR and NBWT (Fig.2-5A). Final satellite cell fusion percentage in IUGR pigs was 8% lower ($P \leq 0.05$) than those form NBWT pigs (Fig.2-5B). Furthermore, in myotubes derived from IUGR satellite cells, nuclei density ($nuclei \bullet \mu m^{-2}$) was lower than in NBWT pigs ($P < 0.001$) (Fig.2-5C).

**Discussion**

In modern swine industry, the genetic selection for high prolific sows resulted in an increase in litter size, however this improvement led to a decrease in piglets'

average birth weight. Intrauterine growth restriction causes permanent consequences for muscle development and growth (Berard et al., 2010; Gondret et al., 2006; Town et al., 2004). During the biphasic prenatal myogenesis, primary myofibers are formed between day 35-55 of gestation (Wigmore and Stickland, 1983). From day 55-90 of pregnancy, a population of myogenic precursor cells use the primary fibers as templates to fuse and form secondary fibers (Picard et al., 2002; Wigmore and Stickland, 1983). Consequently, total muscle fiber number is fixed prior to birth which is of critical importance for postnatal muscle growth (Greenwood et al., 2000; Nissen et al., 2003). In this study, total muscle fiber number of IUGR pig was 30% fewer than that of NBWT at birth which shows strong concordance of previous studies (Berard et al., 2010; Pardo et al., 2013b; Town et al., 2004; Zhu et al., 2004). Reduced muscle mass at birth in IUGR pigs has long-lasting negative effect on postnatal growth performance (Gondret et al., 2006; Nissen and Oksbjerg, 2011). In pigs, insufficient uterine capacity starts around d 35 gestation which coincident with the myogenesis (Anderson and Parker, 1976; Knight et al., 1977). To date, how intrauterine growth restriction affects the process of myogenesis is still not well understood. Some reported that primary fiber numbers was not different between IUGR and NBWT pigs at d 80 of gestation, yet total fiber number was 17% less at birth in semitendinosus muscle (Wigmore and Stickland, 1983). Therefore it strongly suggested that the reduction in fiber number in IUGR pigs was exclusively due to a fewer secondary fibers. In fact, others reported that secondary fiber formation and growth are affected by intrauterine crowding (Pardo et al., 2013a). This is further supported by the fact that IUGR pigs had a higher proportion of type I and fewer type II fibers in semimembranosus muscle at birth than that of NBWT littermates (Powell and Aberle, 1981), since primary fibers tend to have high probability of becoming type I while secondary fibers have high probability of becoming type II fibers (Beermann et al., 1978; Lefaucheur et al., 1995). Despite these findings, we and others (Dwyer et al., 1993) only reported the total fiber number due to the difficulty of distinguishing primary and secondary fibers after d 80 of gestation (Wigmore and Stickland, 1983).

Uterine nutrient insufficiency causes fetal growth restriction which not only reduces muscle hyperplasia but also affected myofiber hypertrophy (Pardo et al., 2013b; Wang et al., 2013; Wu et al., 2006). In our study, the secondary fiber size of IUGR pigs was smaller than the primary fibers. We could not distinguish primary and secondary fiber in NBWT pigs because fiber sizes were too close. However, no differences were observed when primary fiber size of IUGR pigs was compared with average fiber size of NBWT pigs. These data suggest that the impaired prenatal muscle hypertrophy of IUGR pigs mainly occurs in secondary fibers. IUGR pigs have smaller primary fibers at d 60 of gestation (Wang et al., 2013). Smaller size of primary fibers provides less surface area for secondary fiber myogenesis, which could explain the reduced number of secondary fibers in IUGR pigs (Wang et al., 2013). Postnatal muscle growth is mainly caused by muscle fiber hypertrophy and elongation (Dayton and White, 2008). During postnatal growth, IUGR pigs have larger fibers at market weight, which implies that IUGR pig muscle may have altered fiber type, metabolism as well as meat quality (Gondret et al., 2006). Hence, reduced muscle growth performance in IUGR pigs is influenced by both fiber size and number at birth (Wang et al., 2013).

Muscle fiber is a multinucleated cell that requires extrinsic nuclei to support its growth (Le Grand and Rudnicki, 2007). Satellite cells undergo proliferation, differentiation and fusion into their host fibers. Extra DNA would enhance protein synthesis and therefore support muscle growth (Allen and Rankin, 1990). Our immunohistology indicated that IUGR pig muscles have significantly less SCs per fibers. First, less SCs per fiber at birth may probably be a result of lower myogenic precursor cells (MPC) prenatally. When intrauterine growth restriction occurs around d 30 of gestation, nutrient restriction may have negative impact on MPCs proliferation, thus leads to reduced prenatal myogenesis (less fibers) and decreased number of SCs at birth. Second, less SCs per fiber means compromised ability to provide external DNA for fiber growth which may lead to declined DNA content, lower protein synthesis and impaired muscle growth (Nissen et al., 2004; Wang et al., 2008). Furthermore, our flow cytometry analysis showed lower percentage SP cells of total isolates. As a type

of multi-potential progenitors, SP cells can give rise to muscle SCs and fuse to myofibers when injected into injured muscle or co-culture with primary myoblasts or C2C12 cells (Asakura and Rudnicki, 2002; Bachrach et al., 2004; Gussoni et al., 1999). Muscle SP has been extensively studied because of its unique ability in muscle fiber repair and regeneration. Although the role of SP cells in normal postnatal muscle hypertrophy has not been well understood, their contribution to myogenesis as a minor external nuclei source is a possibility. Taken together, reduced number of SCs and SP cells would decrease the muscle stem cell pool, and therefore affect postnatal muscle growth potential.

In this study, the DNA content of muscle was lower in IUGR pigs implying a decrease in muscle nuclei. In fact, lower fusion percentage was observed in SC cultures derived from our IUGR pig muscle. Such impaired differentiation in vitro indicates that muscle SCs may have reduced ability to fuse into myofibers. Further, in vivo study showed that IUGR pigs had more fibers with centrally located nuclei than NBWT pigs, suggesting intrauterine growth restriction delays myofiber differentiation during myogenesis (Aberle, 1984). Therefore, according to our data, the delayed myofiber differentiation may result from impaired muscle satellite cell fusion ability.

**Conclusion**

In conclusion, intrauterine growth restriction reduced muscle fiber number and size in pigs. Moreover, in IUGR pigs the proportion of SP cells to total myogenic cells is lower which may be a factor contributing to reduced muscle growth observed in these pigs. Muscle SC amount per fiber and its differentiation ability are two important indexes for muscle growth potential which were lower in IUGR pigs. Consequently, the poor postnatal growth performance of IUGR pigs may be collectively caused by pre-determined conditions: impaired muscle fiber number, stem cell pool and SCs differentiation.

Table 2-1. Body and semitendinosus (ST) muscle weight, ST cross section area, DNA, RNA and Protein content of newborn normal body weight (NBWT) and intrauterine growth restricted (IUGR) pigs.

| | NBWT | IUGR | SE | P value |
|---|---|---|---|---|
| Birth Wt, Kg | 1.52 | 0.79 | 0.03 | < 0.001 |
| ST Wt, g | 4.0 | 1.78 | 0.57 | 0.042 |
| ST area, $mm^2$ | 87.4 | 36.6 | 8.6 | 0.018 |
| DNA Content, mg/g | 0.79 | 0.72 | 0.05 | 0.035 |
| RNA Content, mg/g | 0.12 | 0.14 | 0.03 | NS |
| Protein Content, mg/g | 68.5 | 65.1 | 1.4 | NS |

Values are expressed as means ± SEM; $n = 4$. NS= not significant

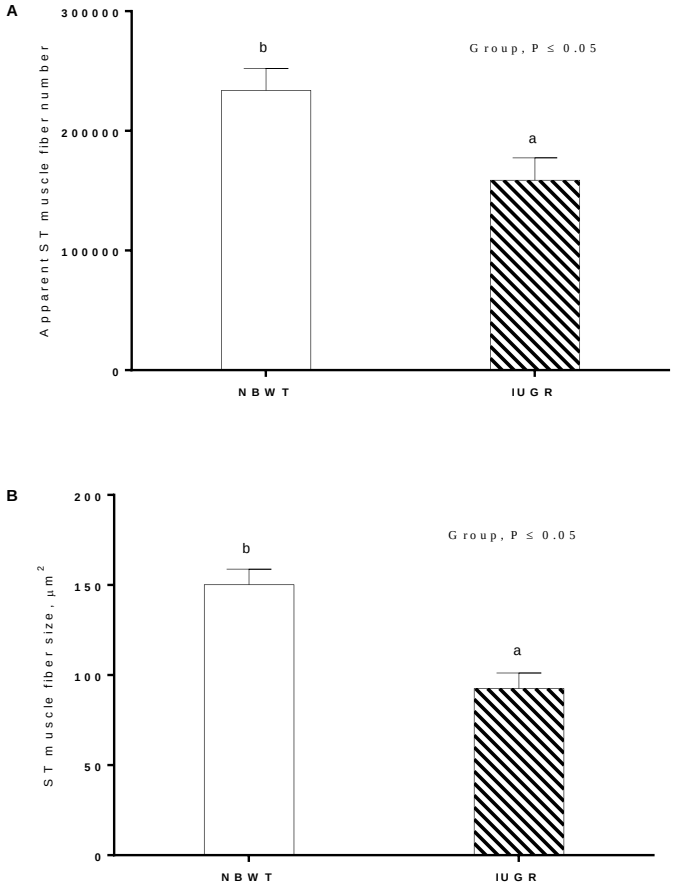



Fig 2-1. Apparent semitendinosus muscle fiber number (*A*) and average fiber size (*B*) of newborn normal body weight (NBWT) and intrauterine growth restricted (IUGR) pigs. Values are expressed as means ± SEM; $n = 4$; different letter indicate significantly difference of means.

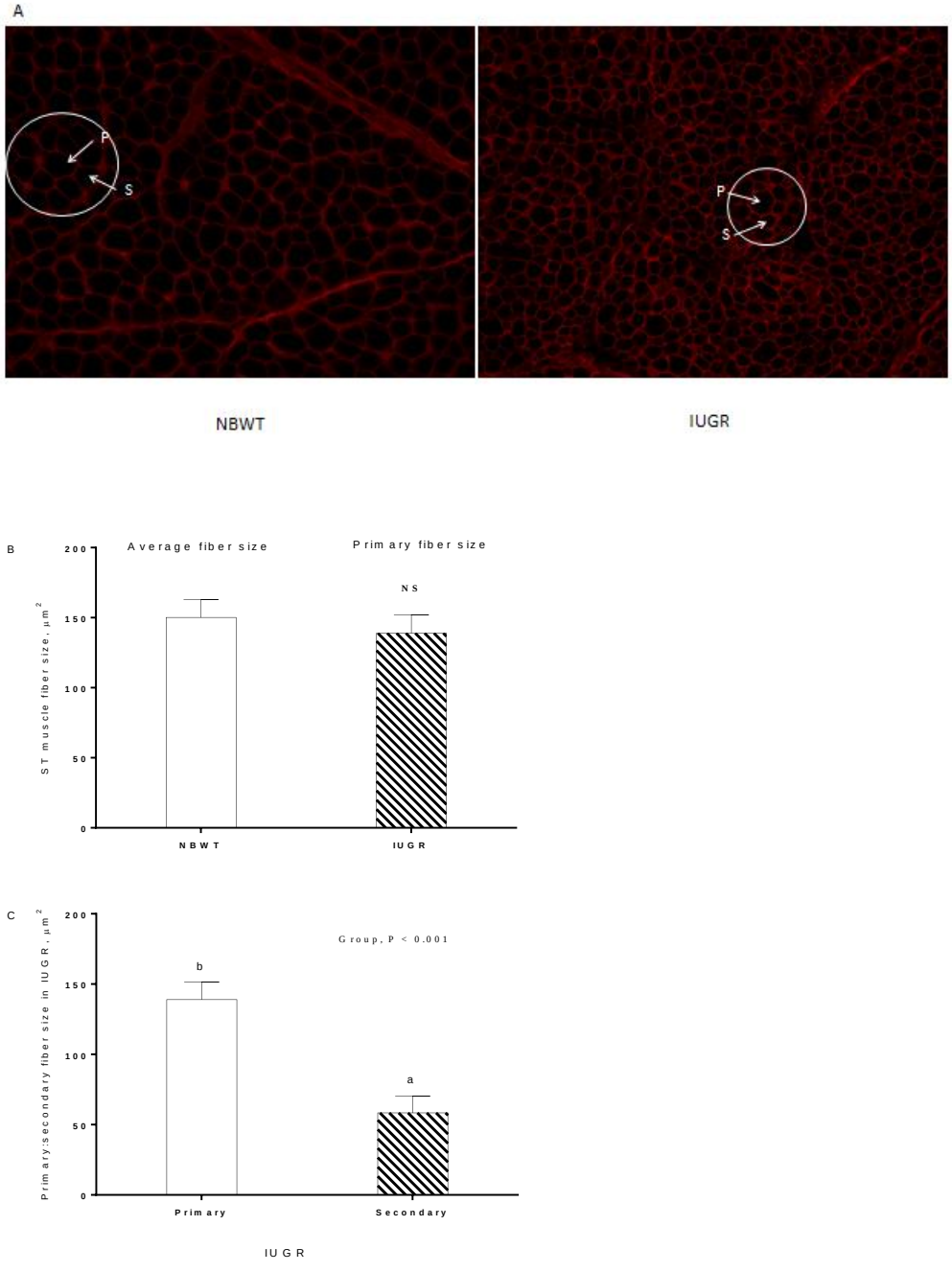
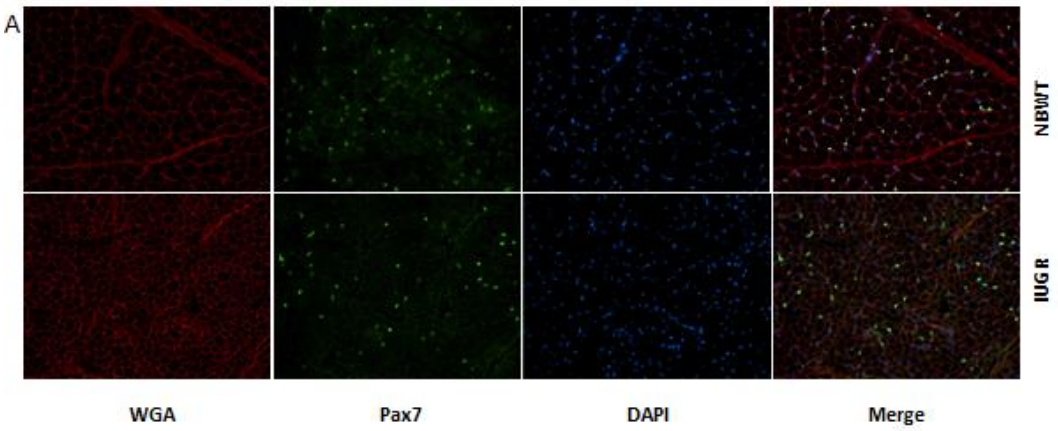



Fig 2-2. Primary (P) and secondary (S) muscle fibers of semitendinosus (ST) muscle section under 20 × magnification of normal body weight (NBWT) and intrauterine growth restricted (IUGR) pigs (*A*); primary fiber size of ST muscle in IUGR compare to average fiber size of ST muscle in NBWT newborn pigs (*B*); primary and secondary ST muscle fiber size of newborn IUGR pigs (*C*). Values are expressed as means ± SEM; $n = 4$; different letter indicate significantly difference of means.



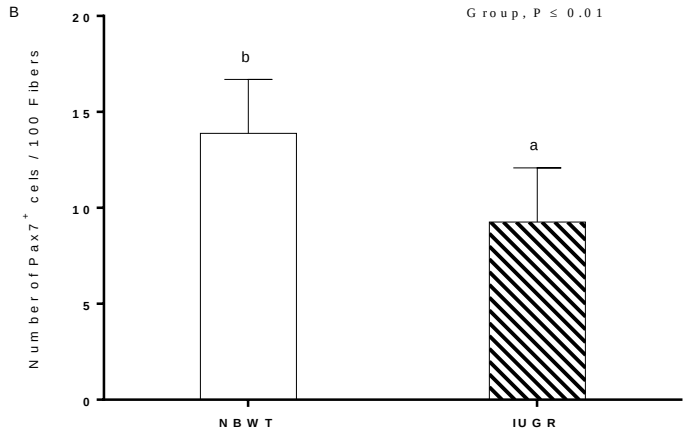




Fig 2-3. Semitendinosus (ST) muscle section staining of Anti-wheat germ agglutinin (WAG), paired box protein 7 (Pax7) and 4',6-diamidino-2-phenylindole (DAPI) (*A*) and the number of pax7 positive cells per 100 muscle fibers (*B*) of newborn normal body weight (NBWT) and intrauterine growth restricted (IUGR) pigs. Values are expressed as means ± SEM; $n = 4$; different letter indicate significantly difference of means.

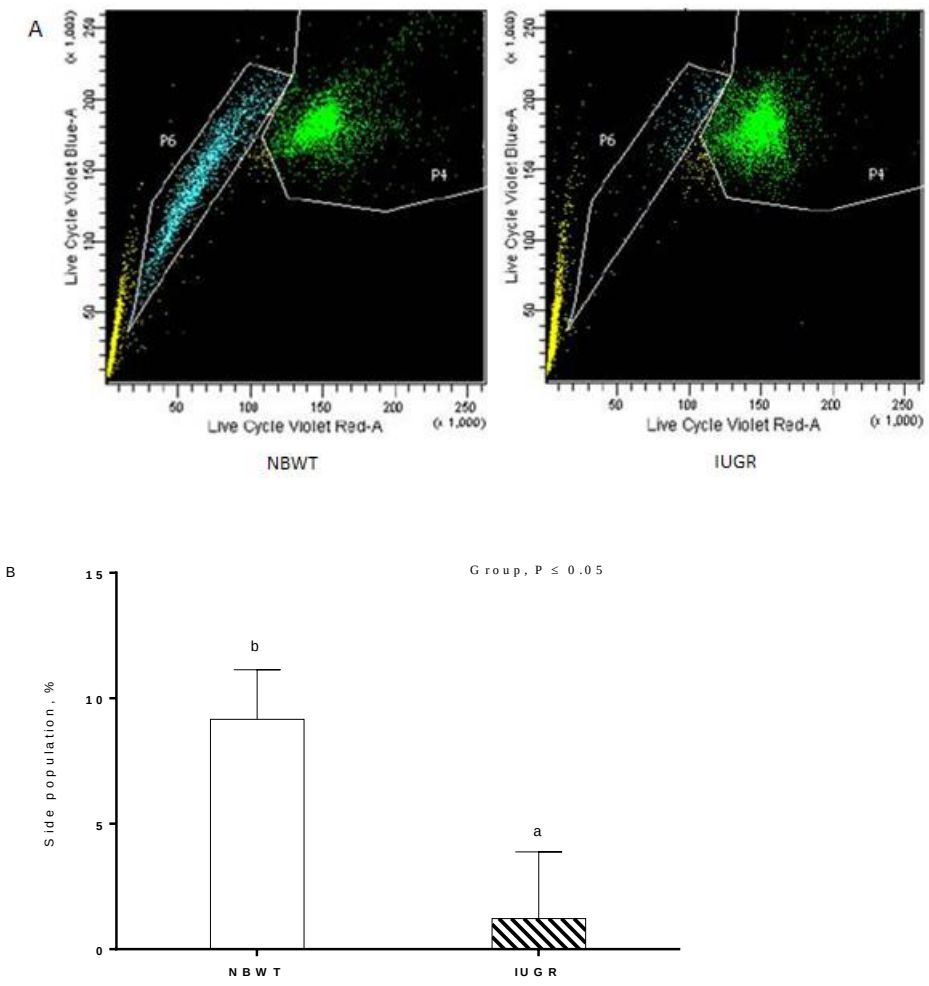



Fig 2-4. Flow cytometry of muscle side population (blue dots) (*A*) and it's percentage over total isolated cells (*B*) of newborn normal body weight (NBWT) and intrauterine growth restricted (IUGR) pigs. Values are expressed as means ± SEM; $n = 4$; different letter indicate significantly difference of means.

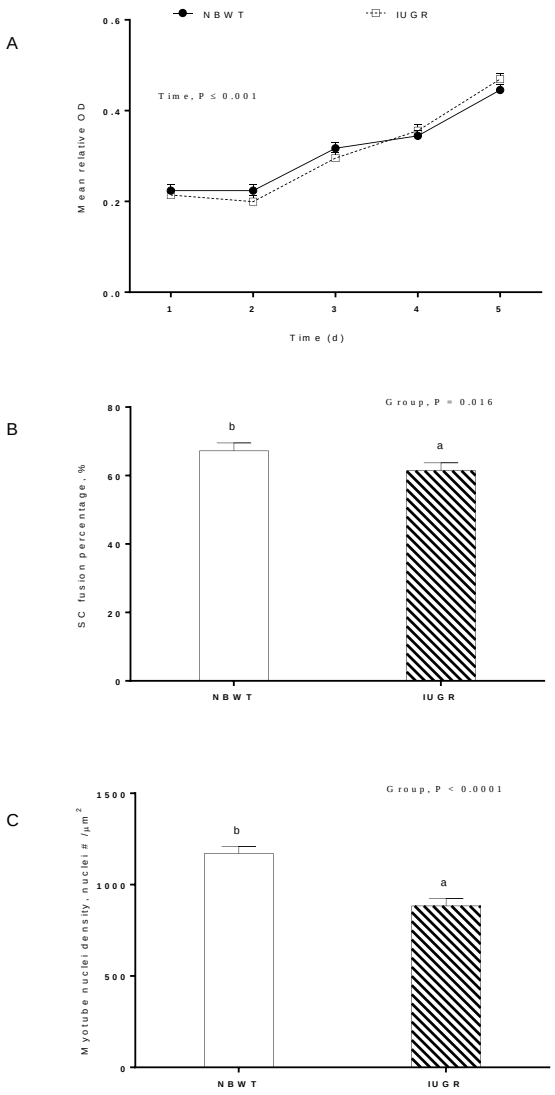



Fig 2-5. Semitendinosus muscle satellite cell proliferation (*A*), fusion percentage (*B*) and myotube nuclei number per area (*C*) of newborn normal body weight (NBWT) and intrauterine growth restricted (IUGR) pigs. Values are expressed as means ± SEM; *n* = 4; different letter indicate significantly difference of means.

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# Chapter 3.

## Intrauterine growth restriction alters insulin and IGF-I signaling in pig skeletal muscle

**Abstract**

Intrauterine growth restriction impairs muscle growth and development in mammalian fetus. Insulin and IGF-I play critical roles in muscle growth and development, however little is known of insulin and IGF-I signaling in intrauterine growth restricted (IUGR) animals. The aim of this study was to investigate changes in plasma insulin and IGF-I levels and the expression of their receptors in IUGR pigs. Pigs were considered IUGR when body weight was 90% below litter average at 85-d gestation (prenatal group), or birth weight below 2 SD of litter average in birth and wean groups, and NBWT when birth weight was within 0.5 SD of litter average. Plasma insulin and IGF-I concentrations, muscle IGF-I, IGF-I receptor and insulin receptor mRNA expression were measured. Plasma insulin and IGF-I concentrations of IUGR pigs in birth group were significantly lower ($P \leq 0.01$) than their NBWT littermates. Muscle IGF-I and insulin receptors mRNA expression were higher in IUGR compared to those of NBWT pigs ($P \leq 0.01$). These data suggest altered insulin and IGF-I signaling in IUGR pig muscle, and these changes may lead to compromised muscle growth and development.

**Keywords:** pig, IUGR, insulin, IGF-I

## Introduction

Insufficient maternal nutrient supply to fetus causes intrauterine growth restriction (IUGR), which negatively affects fetal growth and development. In pigs, IUGR develops from 30 to 45 d until the end of gestation (Anderson and Parker, 1976; Kim et al., 2009; Wu et al., 2006). After d 30 of gestation, fetuses in the center of the uterine horns have smaller placentae that could lead to reduced blood flow and therefore insufficient nutrient delivery to the fetuses (Foxcroft et al., 2006; Wu et al., 2004b). Coincidentally, sequential myogenesis during fetal stages occurs from d 35 to 90 of gestation in pigs (Wigmore and Stickland, 1983). With insufficient nutrient supply, muscle fiber growth and development is markedly affected in IUGR pigs, and these pigs have reduced muscle size and total fiber number at birth which in turn significantly affect postnatal muscle growth (Gondret et al., 2006; Losel et al., 2009). In humans, it is reported that IUGR affects up to 5% of all births in the US (Romo et al., 2009), and babies suffering from IUGR show increased death rates, higher risk of metabolic diseases including insulin resistance, type II diabetes and obesity in later life (Jones et al., 2012; Pallotto and Kilbride, 2006).

Insulin and insulin like growth factor-I (IGF-I) are two important activators in stimulating muscle growth and development. The stimulatory effect of insulin and IGF-I on muscle growth is mainly via binding to their receptors and activating the protein kinase B (PKB)/mammalian target of rapamycin (mTOR) signaling pathway (Huang and Manning, 2009; Sonenberg and Hinnebusch, 2009; Weigl, 2012). Briefly, binding of insulin or IGF-I to their receptors leads to auto-phosphorylation of the tyrosine kinase domain, and sequentially phosphorylates/activates PKB and mTOR complex 1 (mTORC1), which ultimately stimulates muscle protein synthesis by up-regulation of translation initiation and

peptide elongation (Egerman and Glass, 2014; Foster and Toschi, 2009; Schiaffino and Mammucari, 2011). The stimulatory effect of insulin on muscle protein synthesis has been shown by numerous studies under normal growth conditions (Davis et al., 1996; Kitamura et al., 1998; Stump et al., 2003; Wilson et al., 2009). In IUGR pigs, insulin level is not altered (Davis et al., 1997; Mostyn et al., 2005), but it is still questionable whether the insulin receptor and its downstream targets are not impaired.

Plasma IGF-1 level in IUGR human or animals is reduced in the neonatal period (Ohkawa et al., 2010b; Ostlund et al., 2002; Setia and Sridhar, 2009). In pigs, muscle IGF-I receptor mRNA expression is increased in the prenatal period (Tilley et al., 2007), however IGF-I mRNA expression in muscle is reduced during that same period (Rongjun Chen, 2010b). Whether a difference in plasma IGF-I between IUGR and NBWT pigs exists is less clear. For instance, plasma IGF-I concentration was shown to be lower in IUGR pigs compared to their NBWT littermates (Davis et al., 1997; Schoknecht et al., 1997), while others found no differences (Morise et al., 2008; Mostyn et al., 2005). Nevertheless, these studies may suggest a possible altered IGF profile in IUGR animals.

Postnatal muscle growth, or more specifically hypertrophy, largely depends on protein accretion in muscle fiber (Harbison et al., 1976; Koohmaraie et al., 2002). Protein accretion occurs when protein synthesis exceeds degradation. In IUGR pigs, whether insulin and IGF-I signaling is altered in skeletal muscle, and how altered signaling further affects protein turnover remains largely unknown. The aim of this study was to explore the plasma insulin and IGF-I concentrations and their receptors expression in IUGR pigs, and provide preliminary evidence for future protein synthesis study.

**Material and Methods**

*Experiment design.* Pregnant sows had free access to water and fed a diet to meet NRC (2012) requirements. At 85 days gestation, one group of sows was delivered by Caesarean section. Fetuses were considered as normal body weight (NBWT) or IUGR when fetal body weight was within 0.5 SD or 90% below litter average. Fetuses (Fetal group; *n*=8) were euthanized and muscle sample were collected into cryonic tubes filled with RNAlater solution (Life Technologies, Gaithersburg, MD) immediately. Mixed blood samples from the umbilical cord were collected for hormone and metabolite analysis into heparinized tubes. Plasma was separated and stored in -80 ºC. Another group of sows were allowed to reach parturition. Pigs born to these sows were defined as NBWT when the birth weight was within 0.5 SD or IUGR when weight was 2 SD below the litter average. A subgroup of these pigs were euthanized on day-1 (Birth group; *n*=8), and samples collected as described in fetal pigs, whereas a second subgroup was allowed to nurse with their sows for 3 weeks until weaning (Wean group; *n*=8) before euthanasia and sample collection. Blood and longissimus dorsi muscle samples were frozen at -80ºC for further analysis.

*Plasma Insulin and IGF-I.* Plasma samples were isolated by centrifuging blood at 10,000g for 20 min at 4 ℃. A commercial porcine insulin ELISA kit (R&D System, Minneapolis, MN) and IGF-I ELISA kit (Biotang, Lexington, MA) were used to measure plasma insulin and IGF-I concentrations. The measurements were performed accordingly to manufacturers' instructions.

*Quantitative real-time PCR.* Muscle mRNA expression of IGF-I, IGF-I and insulin receptors were assayed by real-time qPCR. Briefly, 20-30 mg gastrocnemius muscle

RNA was extracted using Qiagen RNeasy Plus Universal Kit (Qiagen, Valencia, CA) following the manufacture's guide. First strand cDNA was synthesized from 1 μg RNA using random hexanucleotide-primed cDNA synthesis (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed on ABI 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA). Primer sequences used in qPCR are listed in Table 1. All data were normalized to β-actin and expressed in arbitrary units.

*Statistics*. Data was analyzed using the SAS version 9.3 (SAS Inst. Inc., Cary, NC). All data were considered in repeated measure analyses where appropriate using the PROC MIXED procedure. When a significant treatment effect was detected, means were compared using Tukey-Kramer Multiple Comparison Test. Data are expressed as the least squares mean ± SEM and differences considered significance at $P < 0.05$, unless otherwise noted.

**Results**

*Plasma Insulin and IGF-I*. Plasma insulin concentration was highest in both IUGR and NBWT pigs at birth compared to prenatal and weaning age groups. Significantly lower ($P \leq 0.01$) plasma insulin concentration was observed in new born IUGR pigs compared to their NBWT siblings (Fig.3-1). No differences in plasma insulin concentration were found between IUGR and NBWT pigs at 85 d gestation and weaning ages. Similarly, plasma IGF-I concentration was also lower ($P \leq 0.01$) in birth group IUGR pigs than their NBWT littermates, but was not different between IUGR and NBWT pigs of both prenatal and wean groups (Fig.3-1).

*qPCR*. Muscle IGF-I expression does not differ between IUGR and NBWT pigs in

birth group. The expressions of muscle IGF-I receptor and insulin receptor were significantly higher (2.8 folds and 1.8 fold, respectively, $P \leq 0.01$) in IUGR pigs compared to their NBWT littermates in birth group (Fig.3-2).

**Discussion**

It is well established that IGF-I and insulin play critical roles in animal growth (Bark et al., 1998; Davis et al., 1996; Oksbjerg et al., 2004). The objective of this study was to investigate IGF-I and insulin signaling in IUGR pigs at different developmental stages. To date, data of circulating IGF-I concentration is inconclusive, where some showed lower plasma IGF-I concentration in IUGR pigs compared to their NBWT littermates (Davis et al., 1997; Schoknecht et al., 1997), while others did not observe any differences (Morise et al., 2008; Mostyn et al., 2005). In this study, plasma IGF-I concentration was lower in new born IUGR pigs, which may contribute to the poor postnatal growth performance. Muscle mRNA expression of IGF-I receptors was higher in IUGR pigs compared to their NBWT siblings in the birth group. This is consistent with a previous IUGR study which showed similar increase in prenatal pig muscle at d 65 and 100 of gestation (Tilley et al., 2007). In this study, we did not measure the IGF-I receptor mRNA expression in prenatal and wean groups, but data from others studies (from literature or our lab) have confirmed similar changes in prenatal (Tilley et al., 2007) and weaning (data shown in chapter 4) IUGR pigs. A previous study has shown reduced IGF-I mRNA expression in weaning IUGR pig muscle, liver and kidney (Rongjun Chen, 2010b). However, no differences of muscle IGF-I mRNA expression were observed between IUGR and NBWT pigs at birth and weanling age (data shown in chapter 4) in our studies.

IUGR pigs in the birth group have lower plasma insulin concentration but higher muscle insulin receptor mRNA expression compared to the NBWT littermates. Pigs of the birth and wean groups used for this study were kept with the sows before euthanasia and sample collection. Therefore, food intake was not properly controlled which is a weakness of this measurement. Further, earlier studies showed no differences in plasma insulin level between IUGR and NBWT pigs (Davis et al., 1997; Mostyn et al., 2005; Schoknecht et al., 1997). Even so, the mRNA expression of insulin receptor in IUGR pigs suggest altered insulin signaling in IUGR neonates. In fact, studies in IUGR sheep have shown increased insulin sensitivity (Limesand et al., 2007), attenuated insulin release and storage (Limesand et al., 2006) compared to NBWT controls. In human, IUGR babies have higher risk of metabolic diseases including insulin resistance and type II diabetes (Jones et al., 2012; Pallotto and Kilbride, 2006), which is possibly due to defects in their insulin signaling. The changes of plasma insulin and IGF-I concentrations, as well as their receptors expression in IUGR pigs muscle warrant further investigation in their downstream signaling pathways.

**Conclusion**

Attenuated plasma insulin and IGF-I concentration and increased mRNA expression of their receptors strongly suggest that IUGR pigs have altered insulin and IGF-I signaling which may be potential reasons for impaired postnatal muscle growth. Future studies is needed to further explore the downstream signaling of insulin and IGF-I receptors.

Table 3-1. Nucleotide sequences of primers used for qPCR

| Gene | Direction | Primer sequence | Accession No. |
|---|---|---|---|
| β-actin | Forward | 5’-CCA TCC AAT CGG TAG TAG CG-3’ | U_07786 |
| | Reverse | 5’-CTA ACC CGT TGA ACC CCA TT-3’ | |
| IGF-I | Forward | 5’-GCACATCACATCCTCTTCGC-3’ | XM_214256.1 |
| | Reverse | 5’-ACCCTGTGGGCTTGTTGAAA-3’ | |
| IGF-I receptor | Forward | 5’-GTCTTCACCACGCACTCTGA-3’ | NM_214172.1 |
| | Reverse | 5’-TACTGTTTGCACGGGATCGG-3’ | |
| Insulin receptor | Forward | 5’-GAAAGGGGGCAAGGGTCTAC-3’ | XM_005654749.1 |
| | Reverse | 5’-CTCGGGTGCTTTGTTCTCCT-3’ | |

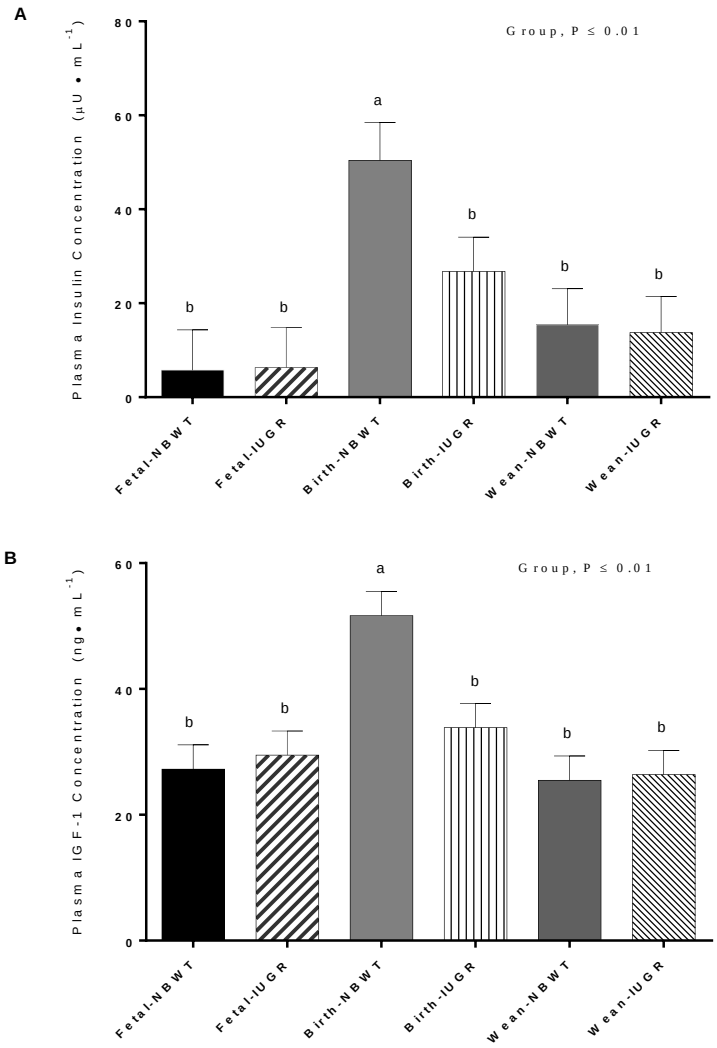


Fig.3-1. Plasma insulin concentration (*A*), plasma insulin like growth factor-I (IGF-I) concentration (*B*) in normal body weight (NBWT) and intrauterine growth restricted (IUGR) pigs at 85 d gestation (fetal), birth and wean ages. Values are means ± SEM; *n* = 8; different letter indicate significantly difference of means.

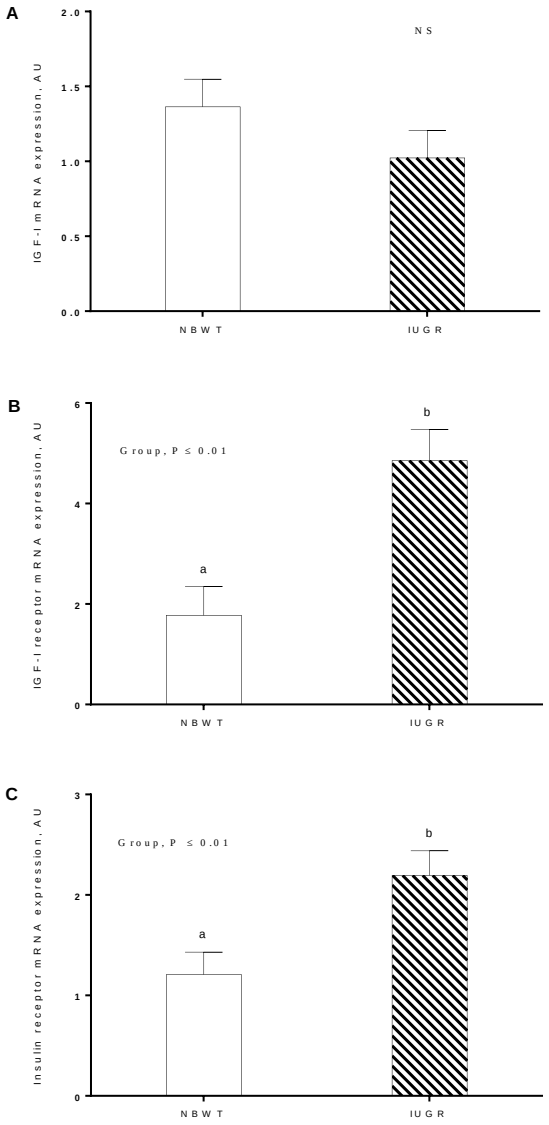


Fig. 3-2. Semitendinosus muscle mRNA expression of insulin like growth factor-I (IGF-I) (*A*), IGF-I receptor (*B*) and insulin receptor (*C*) in normal body weight (NBWT) and intrauterine growth restricted (IUGR) pigs at birth. Values are means ± SEM; $n$ = 8; different letter indicate significantly difference of means.

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# Chapter 4.

## Protein synthesis is impaired in low compared to normal birth weight neonatal pigs

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**Abstract**

Low birth weight neonates have impaired postnatal muscle growth. The aim of this study was to determine whether intrauterine growth restriction affects muscle protein synthesis and degradation in neonatal pigs, and investigate the efficacy of supplementing intrauterine growth restricted pigs with branched-chain amino acids to enhance the rate of protein accretion. NBWT pigs were fed control diet, while IUGR pigs were fed either the control diet or the same diet supplemented with 0.68% branched-chain amino acids (BCAA). On day 21, pigs were infused with [$^2H_5$] phenylanaline and [$^2H_2$] tyrosine for 8 h, and amino acids net balance in hindquarters were measured during the last 4 h of tracer infusion. Protein synthesis rate was significantly higher at 30 and 60 min after feeding in NBWT pigs compared to their IUGR siblings ($P \leq 0.01$). Supplementing with BCAA had no effect on protein synthesis and degradation, phenylalanine oxidation, feed conversion ratio, the efficiency of energy and protein deposition. No differences were observed in total and phosphorylated PKB, S6K1 and 4EBP1 among all groups. However, total and phosphorylated eIF4E were lower in IUGR compared to NBWT pigs ($P \leq 0.05$). In addition, mRNA expression of IGF-I receptor and MNK1 were higher ($P \leq 0.01$ and $P = 0.07$, respectively) in IUGR pig muscle. These results suggest that the slower growth of IUGR pig muscle may be due to reduced protein synthesis rates in IUGR pig muscle caused by the defect in translation initiation that may be due to down-regulation eIF4E.

**Keywords:** pig, IUGR, protein turnover, translation initiation

## Introduction

Fetal growth depends on proper maternal nutrient supply. Intrauterine growth restriction (IUGR) occurs when supply do not meet the demands of growing fetus. In humans, neonates whose birth weight for gestational age is lower than $10^{th}$ percentile are considered intrauterine growth restricted. It is estimated that IUGR affects 5% of all births in the US (Romo et al., 2009). IUGR infants have increased mortality and morbidity rates, higher risk of metabolic diseases including insulin resistance, type II diabetes and obesity later life (Jones et al., 2012; Pallotto and Kilbride, 2006). In the past decade, pigs have been used as a clinical IUGR model due to the similarity of pre- and postnatal growth and development to humans as well as natural occurrence of IUGR (Zhang, 2012). The frequency of IUGR in pigs accounts for up to 15% of the litter at birth which is more severe than any other farm animals (N Quinioua, 2002). IUGR develops in pigs between d 30 and 45 of gestation (Kim et al., 2009) and is mainly caused by placental insufficiency and/or maternal undernutrition (Wu et al., 2006).

In addition to higher mortality and morbidity rates, IUGR pig have compromised growth performance indicated by a reduction in feed intake and daily body weight gain, lower feed conversion ratio, and decreased carcass lean percentage when compare with their normal body weight (NBWT) littermates (Gondret et al., 2006; Nissen and Oksbjerg, 2011). A recent study done in juvenile pigs demonstrated that higher fat deposition in IUGR is associated with lower fat but higher carbohydrates oxidation (Krueger et al., 2014).

Postnatal muscle growth namely muscle hypertrophy largely depends on protein accretion in muscle fiber (Harbison et al., 1976; Koohmaraie et al., 2002). Protein accretion occurs when protein synthesis exceeds degradation. In IUGR pigs, impaired

postnatal lean growth implies a potential imbalance in muscle protein synthesis and/or degradation. Protein synthesis includes three processes: translation initiation, peptide elongation and termination. Translation initiation is the rate-limiting step and major regulatory mechanism for translational control (Gebauer and Hentze, 2004; Pestova et al., 2001). The first step of translation initiation is the binding of methionyl-tRNA to the 40 S ribosomal forming the 43 S ribosomal pre-initiation complex, a step facilitated by eukaryotic initiation factor 2 (eIf2) (Ma and Blenis, 2009; Sonenberg and Hinnebusch, 2009). The second step is the binding of the 43 S ribosomal to the 5' capped end of the RNA transcript mediated eukaryotic initiation factor E (eIF4E) and F (eIF4F) complex (Jackson et al., 2010). The inhibitory eIF4E binding protein 1 (4EBP1) suppresses the binding of eIF4E and eIF4G therefore inhibits translation initiation (Gingras et al., 1999). Activation of mTOR and the subsequent phosphorylation of the downstream target proteins, ribosomal protein S6 kinase 1 (S6K1) and 4E-binding protein 1 (4EBP1), the dissociation of eukaryotic initiation factor (eIF)4E•4EBP1, and the active formation of eIF4E•eIF4G, lead to enhanced translation initiation (Ma and Blenis, 2009).

Protein synthesis is regulated through insulin / insulin like growth factor-I (IGF-I) / PKB / mTOR signaling pathway (Regnault et al., 2005). Moreover, the role of mitogen activated protein kinase (MAPK) / Ras / ERK pathway in regulating transcription has also been well documented (Murphy and Blenis, 2006). The extracellular signal-regulated kinas (ERK) activated protein kinase MNK1 and MNK2 directly phosphorylate eIF4E (Pyronnet et al., 1999; Scheper et al., 2001). MNKs do not bind to and phosphorylate eIF4E directly, instead, these protein interact with eIF4G of the eIF4E•eIF4G complex, then bringing enzyme to phosphorylate eIF4E at Ser 209 (Pyronnet et al., 1999; Shveygert

et al., 2010). This suggests that MNK1/MNK2 links Ras / ERK pathway and PKB / mTOR signaling pathways (Ueda et al., 2004). To date, the role of Ras / ERK pathway in regulating protein synthesis and degradation in IUGR pigs remains unknown.

Branched-chain amino acids, particularly leucine stimulate protein synthesis via mTOR dependent mechanism (Wilson et al., 2010), and enhance protein synthesis (Wilson et al., 2011). However, whether leucine has the same stimulatory effect in IUGR pigs is still unclear. Therefore, the aim of this study was to investigate whether BCAA supplementation would enhance protein synthesis in neonatal IUGR pig.

**Material and Methods**

*Animal and surgeries*. At birth, pigs were weighed and defined as NBWT when the weight was within 0.5 SD, and IUGR 2 SD below the average of the litter (D'Inca et al., 2010). Each IUGR pig was matched with a corresponding NBWT sex-matched from the same litter. Newborn pigs were allowed to suckle colostrum for 24 h, then were housed in separated cages and fed a sow milk replacer (Soweena Litter Life; Merrik's, Middleton, WI) (Table.1). NBWT pigs (n = 9) were fed control diet (Table.2) resembling sow milk (Klobasa et al., 1987) to meet 80% of the NRC (2012 ) requirements. IUGR pigs were fed either control diet (IUGR, n = 6) or the same diet supplemented with 0.68% branched-chain amino acids (1:0.68:0.5 leucine: isoleucine: valine) (IUGRAA, n = 6). At 14 days of age, pigs were surgically fitted under general anesthesia with indwelled vascular catheters in a carotid artery, jugular vein and the inferior vena cava, and an ultrasonic flow probe around caudal aorta (Transonic Systems, Ithaca, NY). Pigs were allowed to recover for 4 days before tracer infusion and sampling. All experimental procedures were

approved by Virginia Tech Institutional Animal Care and Use Committee.

*Body composition by Dual-energy X-ray absorptiometry*. Lean tissue weight, total body fat mass, body bone mineral content (BMC) and bone mineral density (BMD) were measured using a Lunar Prodigy Dual-energy X-ray absorptiometry (DXA) whole body scanner (GE Medical systems, Milwaukee, WI). Body composition was determined before and 18 d after initiation of feeding. Pigs were fasted overnight and lightly sedated before each DXA scan. Feed conversion was calculated as the total amount of milk (g) consumed by each pig divided by total body weight gained (g) during the feeding period. Total body protein was calculated from DXA lean by using the equation: protein (g) = -1.062 + (0.2 · DXA lean) (Mitchell et al., 1998). The total deposition of fat and protein was determined by the difference between initial and final DXA fat and lean amount. Energy deposition were calculated from the ratio of deposition and intake assuming values of 23.765 MJ/kg for protein and 39.581 MJ/kg for fat (Sainz and Wolff, 1988).

*Tracer infusion and tissue sampling*. Amino acid kinetics was accessed using a continuous infusion of [$^{2}H_{5}$] phenylanaline and [$^{2}H_{2}$] tyrosine (Cambridge Isotope Laboratories, Andover, MA). Tracers were infused at a rate of 10 μmol/kg $\cdot h^{-1}$ into the jugular vein for 8 h on day 21 of feeding. During the last 4 hours of tracer infusion, 1.5 ml of arterial and venous blood samples were simultaneously taken every 30 min. Blood flow in the caudal aorta was measured during each blood sampling using ultrasonic flow probes. To a known weight of plasma was added an equal weight of a [U-$^{13}$C, $^{15}$N] amino acids internal standard and samples were stored at -20$^{o}$C until analysis. Concentration and enrichment of amino acids were determined by gas chromatography/mass spectrometry (GC 7890 with 5975 mass selective detector; Agilent Technologies,

Wilmington, DE) using procedures described elsewhere (El-Kadi et al., 2012). At each sample point, heparinized blood was transferred to microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) and filled to about 3/4 capacity. The blood to plasma ratio was determined directly by reading hematocrit value form the scale plate after centrifugation. After the last blood sample was taken, pigs were fed and sacrificed one h later. Longissimus dorsi, gastrocnemius and liver were isolated, weighted and snap frozen in liquid nitrogen for further analysis.

*Amino acids kinetics*. Phenylalanine tracer was used because it is neither catabolized nor synthesized in muscles. Phenylalanine net removal by the hindquarters occurs when protein synthesis exceeds protein degradation, while appearance occurs when degradation is higher. Amino acids kinetics were calculated as previously described (El-Kadi et al., 2012).

*Whole body flux and Phe hydroxylation*. Phenylalanine and tyrosine whole body flux, and phenylalanine hydroxylation was calculated using following equations in non-steady state condition when the concentration and flux of amino acids were not constant (Bos et al., 2003; Proietto et al., 1987).

$$\text{Whole body flux} = (IR - (pV \times C(t) \times dE / dt)) / E(t)$$

where IR is the phenylalanine and tyrosine tracer infusion rate ($\mu mol \cdot kg^{-1} \cdot h^{-1}$), p is the pool fraction for instant mixing ($p = 0.25$), V is the volume of distribution ($V = 0.5$ 1/kg), C(t) is arterial phenylalanine or tyrosine concentration (μmol/ml), dE/dt is the phenylalanine or tyrosine enrichment variation, and E(t) is the mean of enrichment of phenylalanine or tyrosine between two time points. Phenylalanine hydroxylation to tyrosine was calculated to estimate phenylalanine oxidation rate by using the following

equations (Clarke and Bier, 1982).

$$\text{Hydroxylation of Phenylalanine} = E_{Tyr} / E_{Phe} \cdot Q_{Tyr}$$

where $Q_{Tyr}$ is the whole body flux of tyrosine ($\mu mol \cdot kg^{-1} \cdot h^{-1}$); $E_{Tyr}$ and $E_{Phe}$ are the enrichments of phenylalanine $^2H_5$ and tyrosine $^2H_2$ respectively.

*Protein synthesis and degradation*. Pig hindquarter muscle protein synthesis and degradation ($-\mu mol\ kg^{-1}$) was calculated using the following equations (Fujita et al., 2006).

$$\text{Protein synthesis} = (([A_{Phe}] \cdot EA / EV)-[V_{Phe}]) \cdot PF$$

$$\text{Protein degradation} = [A_{Phe}] \cdot (EA / EV -1) \cdot PF$$

$$\text{Protein deposition} = \text{Synthesis} – \text{Degradation}$$

where, $[A_{Phe}]$ or $[V_{Phe}]$ is arterial or venous concentration of phenylalanine (μmol), EA or EV is arterial or venous enrichment of phenylalanine $^2H_5$, and PF is plasma flow (ml/h).

*Insulin and IGF-I*. Plasma insulin concentrations were measured using a commercially available porcine insulin ELISA kit (R&D System, Minneapolis, MN). Muscle homogenate and 30 min plasma IGF-I concentrations were determined using a porcine IGF-I ELISA kit (Biotang, Lexington, MA). The quantification protocols were performed accordingly to manufacturers' instructions.

*Quantitative real-time PCR*. Muscle mRNA expression of several genes involved in protein synthesis were assayed by real-time qPCR. Briefly, gastrocnemius muscle RNA was extracted using a commercial available RNA isolation kit (ZYMO Research, Orange, CA). First strand cDNA was synthesized from 1 μg RNA using random hexanucleotide-primed cDNA synthesis. ABI 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA) was used to perform quantitative real-time PCR. Primer sequences used in qPCR are listed in Table 3. Data were normalized to β-actin and

expressed in arbitrary units.

*Protein expression*. Frozen gastrocnemius muscle samples were homogenized by TissueLyser II (Qiagen, Valencia, CA) and centrifuged at 10,000 g for 5 min. Protein concentration was determined using Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL). Equal amount of protein in each sample were separated on polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane (Thermo Scientific, Waltham, MA). Membranes were incubated with appropriate primary antibodies at 4 ℃ overnight, followed by 1 h incubation with secondary antibody (Bio-Rad, Richmond, CA) at room temperature. The primary antibodies used in immunoblotting were α-tubulin (Cell Signaling Technology, Danvers, MA), PKB (Cell Signaling Technology, Danvers, MA), phospho-PKB (Ser473; Cell Signaling Technology, Danvers, MA), S6K1 (Cell Signaling Technology, Danvers, MA), phosphor-S6K1 (Thr389; Millipore, Temecula, CA), 4EBP1(Bethyl Laboratories, Montgomery, TX), phospho-4EBP1(Thr46; Life Technologies, Gaithersburg, MD). All blots were developed using Amersham ECL Detection kits (GE Healthcare, Piscataway, NJ), imaged and analyzed using Gel Doc XR+ Imaging System (Bio-Rad, Hercules, CA). Protein abundance of each target was normalized to α-tubulin in all samples.

*Statistical analysis*. Data were analyzed using with SAS version 9.3 (SAS Inst. Inc., Cary, NC). All data were considered in repeated measure analyses where appropriate using the PROC MIXED procedure. When a significant treatment effect was detected, means were compared using Tukey-Kramer Multiple Comparison Test. Data are expressed as the least squares mean ± SEM and differences considered significance at $P < 0.05$, unless otherwise noted.

**Results**

*Growth performance*. Mean body weights in IUGR and IUGRAA groups were 60% ($P \leq 0.001$) that of in NBWT group at birth. IUGR and IUGRAA piglets grew at a slower rate during the 21 d feeding period as compared with their NBWT littermates ($P \leq 0.001$) (Fig.4-1). Branched-chain amino acid supplementation did not affect body weight gain. Feed conversion and protein efficiency were not different among the three groups; however, the efficiency of energy deposition in NBWT pigs was 25 % ($P \leq 0.05$) higher than those in IUGR and IUGRAA groups (Fig.4-2). On day 21, longissimus dorsi, gastrocnemius and liver weights were 46%, 51% and 35% lower ($P \leq 0.001$) in pigs from IUGR groups compared to NBWT pigs (Fig.4-3). No differences in muscle and liver weights were observed between IUGR and IUGRAA groups.

*Fat and Lean deposition*. Body composition measurement by DXA showed a reduction ($P \leq 0.001$) in fat percentage accompanied by an increase ($P \leq 0.001$) in lean tissue in all groups at the end of study (data not shown). Fat and lean deposition and bone mineral content were significantly greater ($P \leq 0.01$; $P \leq 0.01$ and $P \leq 0.001$ respectively) in NBWT pigs compared to their IUGR siblings (Fig.4-4). Yet, no effect of BCAA supplementation on body composition was observed in IUGRAA group.

*IGF-I and insulin*. There was a rise ($P \leq 0.001$) in insulin concentrations 30 min after feeding in pigs of all groups, followed by gradual decline returning to baseline at 180 min postfeeding (Fig.4-5). There were no differences in insulin concentrations at any time point among three groups. In addition plasma and muscle concentration of IGF-I showed no difference among the three groups.

*Amino acid concentration and amino acid transporters*. Plasma amino acids (valine, leucine, isoleucine, phenylalanine, tyrosine and lysine) concentration increased after feeding and peaked between 60 min and 90 min postfeeding, returning to prefeeding level by 240 min in all groups (Fig.4-6). Branched-chain amino acids supplementation in IUGRAA group increased valine, leucine and isoleucine concentrations during 240 min postprandial period compared to NBWT and IUGR pigs ($P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.01$ respectively). The mRNA expression of large neutral amino acid transporter 1 and 2 (LAT1/2), proton-coupled amino acid transporter 1 (PAT1), sodium-coupled neutral amino acid transporter 2 and 3 (SNAT2/3) were not different among groups (appendix, Fig. 6-3).

*Hindquarters amino acid net flux*. The net balances of BCAA, phenylalanine, tyrosine and lysine were similar in all groups (Fig.4-7). The net removal of amino acids by the hindquarters was not different during the sampling period. Amino acid net flux was highest between 30 min and 60 min, returning to baseline by 240 min.

*Whole body flux of phenylalanine and tyrosine and hydroxylation of phenylalanine*. Phenylalanine and tyrosine whole body flux peaked between 30 and 60 min postfeeding, returning to baseline by 240min in all three groups (Fig.4-8). The hydroxylation of phenylalanine to tyrosine was highest at 90 min and returned to baseline at 150min in all three groups. No group differences were observed in phenylalanine and tyrosine whole body flux and phenylalanine hydroxylation.

*Hindquarters protein turnover*. Temporal protein synthesis rate of NBWT pigs were not different from those of IUGR and IUGRAA pigs at 0 min, but higher ($P \leq 0.01$) at 30 min and 60 min after feeding compared to their IUGR siblings (Fig.4-9). No difference in protein degradation rates were observed among all groups during 240 min sampling

period. As a result, protein deposition exhibited the same pattern as protein synthesis and total protein accretion was 48% higher ($P \leq 0.01$) in NBWT compared to IUGR and IUGRAA pigs. There was no effect of BCAA supplementation on muscle protein synthesis in IUGRAA pigs.

*Protein synthesis signaling*. IGF-I receptor mRNA expression in gastrocnemius muscle was higher ($P \leq 0.01$) in IUGR and IUGRAA pigs compared to their NBWT littermates (Fig.10). However insulin receptor, IGF-I binding protein 3 (IGFIBP3), IGF-I binding protein 5 (IGFIBP5), PKB, S6K1 and 4EBP1 mRNA expression was not different among the three groups (appendix, Fig. 6-2). MNK1 mRNA expression was higher ($P = 0.07$) in IUGR and IUGRAA pigs (Fig.4-10). The total eIF4E protein expression in the gastrocnemius was 30% lower ($P = 0.01$), while phosphorylation of eIF4E protein was lower ($P = 0.07$) in IUGR and IUGRAA groups (Fig.4-12). No differences were observed in total and phosphorylation of PKB, 4EBP1 and S6K1 (Fig.4-11). Total and phosphorylated MNK1 were higher ($P \leq 0.05$ and $P \leq 0.01$, respectively) in IUGR and IUGRAA groups (Fig.4-13).

**Discussion**

*Experimental aspects*. As a model for LBW human infants, IUGR pigs were used for skeletal muscle protein synthesis and degradation measurement using stable isotope tracer infusion. This approach allows us to measure the dynamic changes of protein synthesis and degradation in skeletal muscle, thus provides us potential information about LBW human neonates skeletal muscle growth. Further, supplementing pigs with BCAA has been proved

to enhance protein synthesis (Wilson et al., 2011), however it remains unclear whether BCAA supplementation has similar effects on protein synthesis in IUGR pigs. Our research may provide dietary approaches for feeding LBW human infants and improving their muscle growth.

*Body composition and asymmetric growth*. First, our current study provides knowledge of IUGR neonatal pig muscle growth and body composition changes on restricted feeding compared to NBWT. Therefore, all pigs finished the food that was offered at each meal before more being offered at the next meal. In spite of the amino acid supplementation in IUGRAA group, our data suggested that lower average daily body weight gain and less lean mass deposition in IUGR pigs which agree with previous studies (Milligan et al., 2002; N Quinioua, 2002; Rehfeldt and Kuhn, 2006).

Animal and human studies have shown that IUGR alters energy metabolism. In humans, it has been suggested that the risk of childhood and adult obesity and metabolic syndromes are associated with LBW (Gluckman et al., 2005; Harding, 2001). Similarly, IUGR in pigs causes compromised muscle growth but increased body fatness during puberty and adulthood (Lin et al., 2012; Rehfeldt and Kuhn, 2006; Wu et al., 2006). Compared to NBWT, higher fat deposition in juvenile and adult IUGR pigs may be due to lower fat and high carbohydrate oxidation (Krueger et al., 2014). In our study, neonatal IUGR pigs had reduced muscle growth as well as less fat deposition. However, the underling mechanism alter energy metabolism in IUGR animal has not been well established, further studies are still needed.

In our study, the ratio of lean mass between IUGR and NBWT increased during 21 days feeding period (appendix. 6-1). Moreover, on d 21 liver weight of IUGR : NBWT

was relatively higher than the ratio of LD and gastrocnemius muscles (see appendix). Disproportional growth of liver and muscle in IUGR pigs was probably due to higher protein accretion in internal organs which is referred as asymmetrical growth. Typical asymmetrical growth in IUGR animals was also reported by other studies in which muscle growth is largely affected but visceral organs growth is relatively protected (Bauer et al., 2003; Rehfeldt and Kuhn, 2006). In our study, IUGR pigs tend to distribute the limited nutrients and energy toward lean but not fat tissue.

*Protein synthesis*. Compromised postnatal lean growth in IUGR animals are well documented however the mechanism has not been well established. Davis and her colleagues found similar fractional protein synthesis rate in IUGR and NBWT newborn pigs (Davis et al., 1997). De Boo *et al* also reported no effect of IUGR on protein synthesis in the ovine fetus (de Boo et al., 2005). De Boo *et al* used an artificial induced IUGR model in ovine by embolization which can only produce relatively mild IUGR fetus. In human infant studies, the effect of IUGR on protein turnover rates has been less conclusive. In one study lower protein synthesis rates but normal protein accretion were reported in small (SGA) compared to average for gestation age infants (AGA) (Cauderay et al., 1988). However, authors reported higher protein synthesis and degradation in SGA than AGA infants (Pencharz et al., 1981). In our study, pigs were fed equal amounts of energy and protein per kg body weight, and protein turnover was measured using primed continuous stable isotope infusion. Our data show a reduction in protein synthesis but not degradation resulting in lower protein accretion in IUGR pigs. This result can also be supported by DXA data showing less total lean deposition in IUGR pigs.

*BCAA supplementation*. The beneficial effects of BCAA supplementation on

protein synthesis and animal growth via mTOR pathway is well documented in pigs, rats and human (Anthony et al., 2000a; Anthony et al., 2000b; Dreyer et al., 2008; Escobar et al., 2005; Wilson et al., 2010; Wilson et al., 2011). Leucine supplementation in human and pigs increase plasma leucine concentration and enhance protein synthesis in skeletal muscle (Boutry et al., 2013; Escobar et al., 2005; Fujita et al., 2007; Katsanos et al., 2006). Interestingly, in our study, IUGR neonatal pigs did not respond to BCAA supplementation, despite an increase in plasma concentrations of BCAA in IUGRAA pigs indicating successful delivery of BCAA supplementation through diet. All other amino acids level in both atrial and venous blood were similar among all three groups suggesting that amino acid availability may not have been a factor limiting protein synthesis in IUGR pigs. Supplementation of BCAA raised plasma leucine concentrations significantly (up to 800 μmol/L) at the peak after meal compared to non-supplementated IUGR pigs (500 μmol/L). Muscle mRNA expression of amino acid transporters, especially LAT1 which delivers BCAA did not differ among groups which further supported the normal delivery of BCAA in IUGR pigs. These data raise a question why BCAA did not stimulate protein synthesis in IUGR pig? One possible reason is that the stimulation of leucine is mediated by mTORC1 (Suryawan et al., 2008), and it is possible that activation of downstream effectors of mTORC1 may be a limiting step in IUGR pigs.

*Signaling pathways*. The regulation of protein synthesis involve two major signaling pathways: mTOR and MAPK (Corradetti and Guan, 2006; Ellederova et al., 2008). Both pathways are activated by IGF-1 via PKB in mTOR and Ras/Raf in MAPK pathways (Bibollet-Bahena and Almazan, 2009; Weigl, 2012). Our results show that there were no differences among the three groups upstream (PKB) and downstream

(4EBP1 and S6K1) of mTOR suggesting reduced muscle protein synthesis in IUGR pig was not caused by changes in mTOR signaling. Phosphorylation of 4EBP1 releases eIF4E which becomes available to form eIF4E•eIF4G complex. Binding of eIF4E•eIF4G to eukaryotic initiation factor 3 (eIF3) and its phosphorylation enhances translation initiation (Sonenberg and Hinnebusch, 2009; Walsh and Mohr, 2014). Our results document for the first time that eIF4E total protein is down regulated in IUGR pig muscle which maybe an explanation for the low protein synthesis observed in these animals. Moreover, the major effect of BCAA on protein synthesis is largely through mTOR complex 1 activation (Anthony et al., 2002; Suryawan et al., 2008). However, no activation of proteins downstream of mTOR (4EBP1 and S6K1) was observed in IUGR pigs supplementing BCAA which suggests BCAA did not have stimulatory effect in these animals. Interestingly, we also observed increased IGF-I receptor and MNK1 mRNA level, as well as increased total and phosphorylated MNK1 protein level in muscle of IUGR pigs. MNK1 is recruited by eIF3 subunit e and allows it to bind to eIF4G and phosphorylates eIF4E in response to upstream p38MAPK or ERK activation (Walsh and Mohr, 2014). Accordingly, the up-regulation of IGF-I receptor and MNK1 activation, and reduced eIF4E expression might be the mechanism responsible for the lower protein synthesis in IUGR pigs.

**Conclusion**

In conclusion, our data suggest that lower protein synthesis in IUGR pigs paralleled reduction in eIF4E protein expression. Although, IGF-I receptor expression increased and MNK1 in MAPK/ERK pathway was up-regulated, protein synthesis was not enhanced

in IUGR pigs. It may be proposed that down-regulation of protein synthesis in IUGR pigs may be in part due to a less responsive translation initiation. However, the mechanisms that underlie the link between MAPK/ERK pathway and translation initiation signaling in IUGR pigs need further investigation.

Table 4-1. Ingredients and nutrients composition of the Soweena milk replacer

| Ingredient, (g/100 g, as fed) | Percent or amount |
|---|---|
| Water | 87.5 % |
| Crude Protein , minimum | 25.0 % |
| Lysine, minimum | 2.4 % |
| Crude Fat, minimum | 10 % |
| Crude Fiber, maximum | 0.15 % |
| Calcium, minimum | 0.7 % |
| Calcium, maximum | 1.2 % |
| Phosphorus, minimum | 0.8 % |
| Selenium, minimum | 0.3 ppm |
| Zinc, minimum | 120 ppm |
| Vitamin A, minimum | 25,000 IU/lb |
| Vitamin D3, minimum | 2,500 IU/lb |
| Vitamin E, minimum | 150 IU/lb |
| M.E. Swine, kj/kg | 11874 |

Table 4-2. Ingredients and nutrients composition of the experimental milk formulas

| Ingredient, (g/100 g, as fed) | Control | AA supplementation |
|---|---|---|
| Water | 87.53 | 87.21 |
| Whey protein isolate (80% CP)[1] | 6.075 | 6.07 |
| Lactose[2] | 0.9 | 0.9 |
| Corn oil[3] | 3.42 | 3.024 |
| Animal fat[4] | 0.675 | 0.725 |
| Vitamin mix[2] | 0.2 | 0.2 |
| Mineral mix[2] | 0.9 | 0.9 |
| Xanthan gum[2] | 0.2 | 0.2 |
| L-Leucine[1] | - | 0.3 |
| L-Isoleucine[1] | - | 0.22 |
| L-Valine[1] | - | 0.15 |
| Dicalcium Phosphate[2] | 0.2 | 0.2 |
| Nutrient composition | | |
| Crude protein, % | 44.7 | 43.4 |
| Fat, % | 34.15 | 29.19 |
| Lactose, % | 9.17 | 9.17 |
| M.E. Swine, kj/kg | 12033 | 12016 |

1: Nutrabio, Middlesex, NJ. 2: Dyets, Bethlehem, PA. 3. Crisco, Orrville, OH. 4: Milk Specialties Global Animal Nutrition, Carpentersville, IL. Vitamin mix provided (in g/kg): thiamine HCl, 0.1; riboflavin, 0.375; pyridoxine HCl, 0.1; niacin, 1; calcium pantothenate, 1.2; folic acid, 0.13; biotin, 0.02; cobalamin B12. 1.5; retinyl palmitate, 0.8; cholecalciferol, 0.05; tocopheryl acetate, 8.8; menadione sodium bisulfite, 0.08. Mineral mix provided (in g/kg): calcium phosphate dibasic, 187; calcium carbonate, 279; sodium chloride, 85; potassium phosphate monobasic, 155; magnesium sulfate anhydrous, 44; manganous carbonate, 0.93; ferric citrate, 10; zinc carbonate, 1.84; cupric carbonate, 0.193; potassium iodate, 0.005; sodium selenite, 0.007.

Table 4-3. Nucleotide sequences of primers used for qPCR

| Gene | Direction | Primer sequence | Accession No. |
|---|---|---|---|
| β-actin | Forward | 5’-CCA TCC AAT CGG TAG TAG CG-3’ | U_07786 |
| | Reverse | 5’- CTA ACC CGT TGA ACC CCA TT-3’ | |
| Insulin receptor | Forward | 5’-GAAAGGGGGCAAGGGTCTAC-3’ | XM_005654749.1 |
| | Reverse | 5’-CTCGGGTGCTTTGTTCTCCT-3’ | |
| IGF-I receptor | Forward | 5’-GTCTTCACCACGCACTCTGA-3’ | NM_214172.1 |
| | Reverse | 5’-TACTGTTTGCACGGGATCGG-3’ | |
| MNK1 | Forward | 5’-TAAAGCAGAGGCCGATGAGG-3’ | NM_001143719.1 |
| | Reverse | 5’-CAGTTCCGAGGTCAGCTTGT-3’ | |

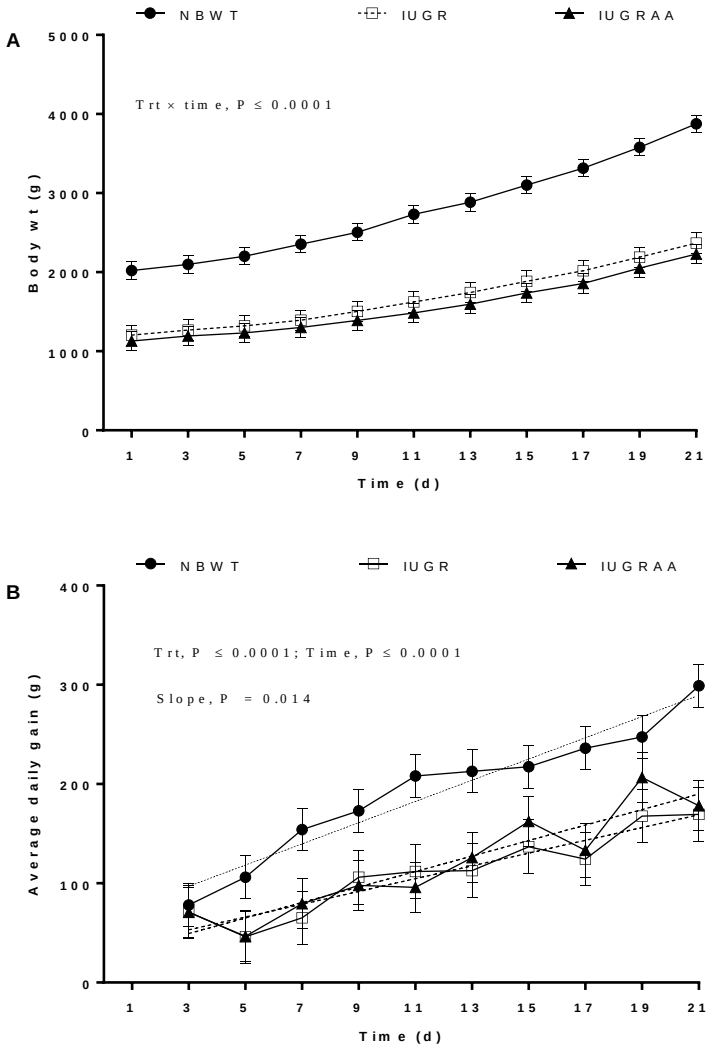


Fig. 4-1. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Body weight (*A*) and average daily body weight gain (*B*) of NBWT, IUGR or IUGRAA pigs. Values are means ± SEM; $n = 6$.

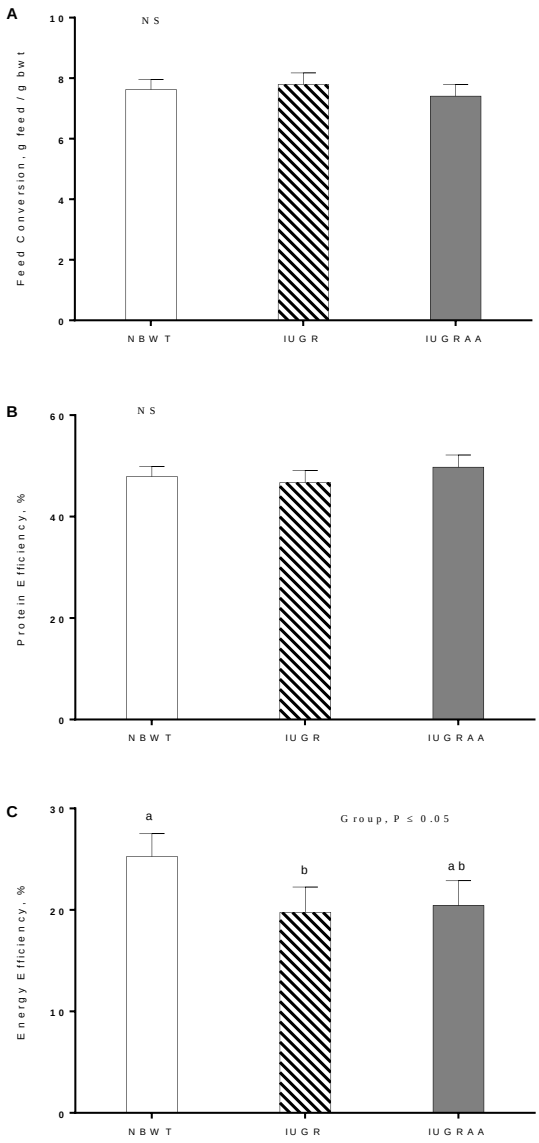


Fig. 4-2. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Feed conversion ratio (*A*), protein efficiency (*B*) and energy efficiency (*C*) of NBWT, IUGR or IUGRAA pigs during 21 d feeding period. Values are expressed as means ± SEM; $n = 6$; different letter indicate significantly difference of means; NS = not significant.

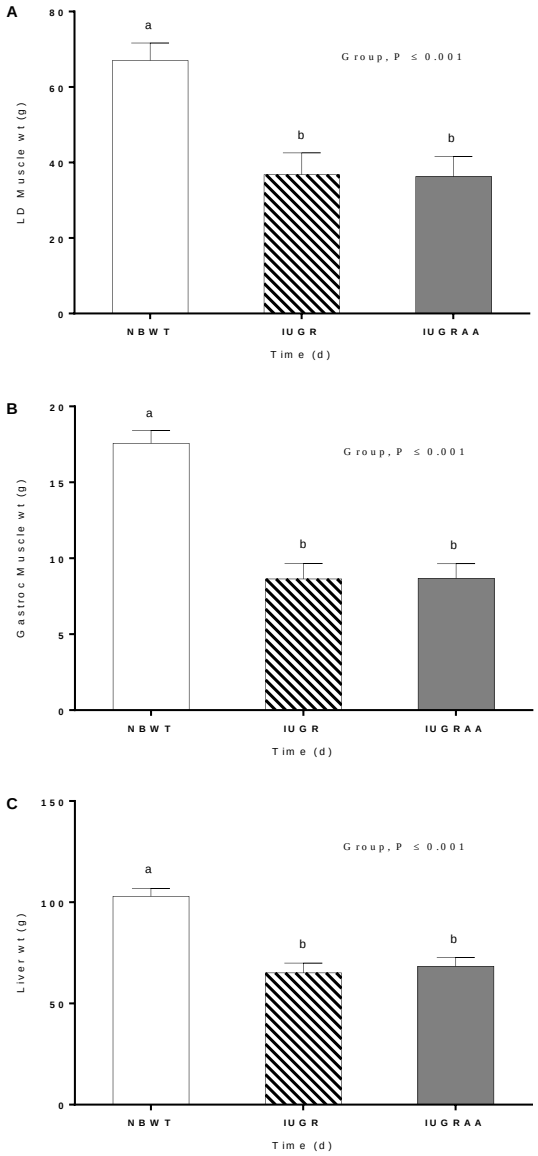


Fig. 4-3. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Longissimus dorsi (LD) muscle weight (*A*), gastrocnemius muscle (gastroc) weight (*B*) and liver weight (*C*) of NBWT, IUGR or IUGRAA pigs of d 21. Values are means ± SEM; $n$ = 6; different letter indicate significantly difference of means.

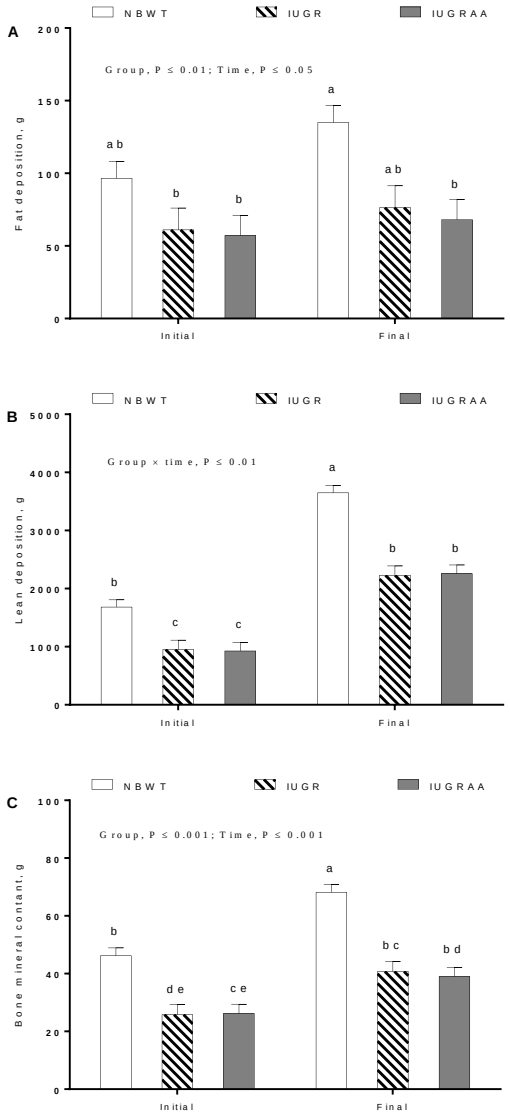


Fig. 4-4. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Fat deposition (*A*), lean deposition (*B*) and bone mineral content (*C*) on d 1 (initial) and d 21 (final) of the feed trail of NBWT, IUGR or IUGRAA pigs were measured by dual-energy X-ray absorptiometry. Values are means ± SEM; $n = 6$; different letter indicate significantly difference of means.

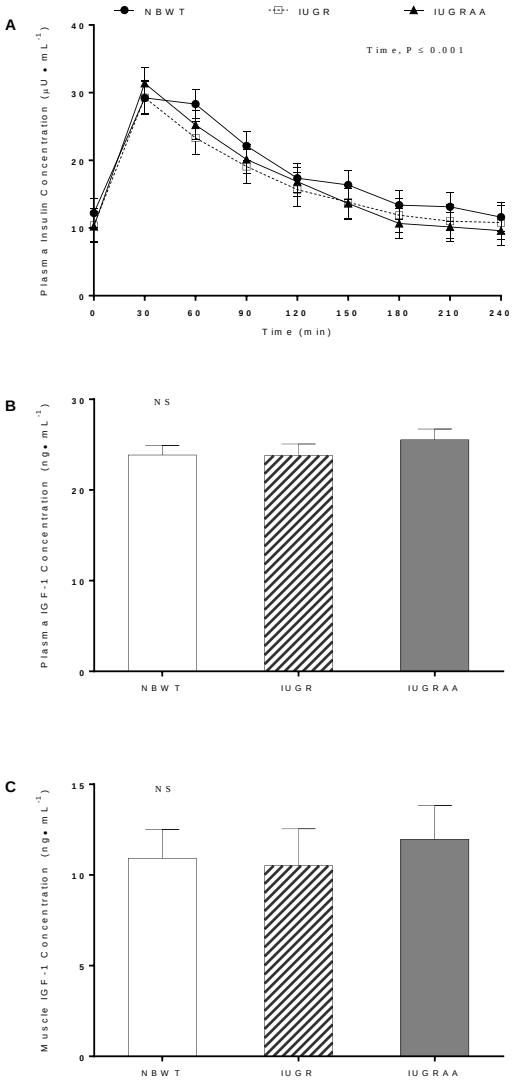


Fig. 4-5. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Plasma insulin concentration during 240 min after feeding (*A*), plasma insulin like growth factor-I (IGFI) concentration at 30 min after feeding (*B*) and muscle IGF-I concentration 30 min after feeding (*C*) in NBWT, IUGR or IUGRAA pigs of d 21. Values are means ± SEM; $n = 6$; NS = not significant.

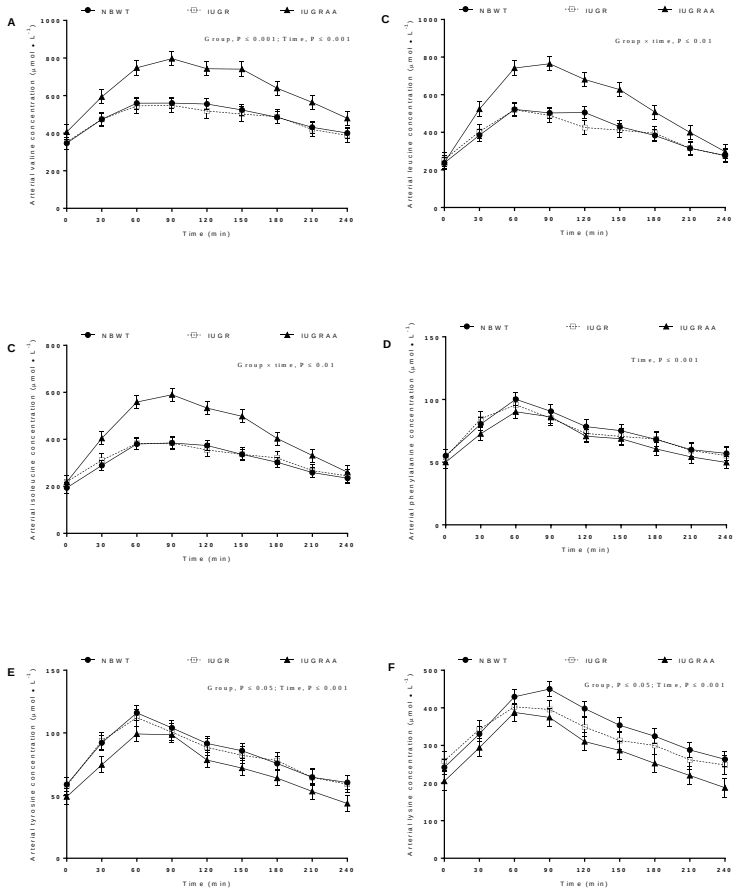


Fig. 4-6. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Arterial plasma concentration of valine (*A*), leucine (*B*), isoleucine (*C*), phenylalanine (*D*), tyrosine (*E*) and lysine (*F*) in NBWT, IUGR or IUGRAA pigs during 240 min after feeding on d 21. Values are means ± SEM; $n = 6$.

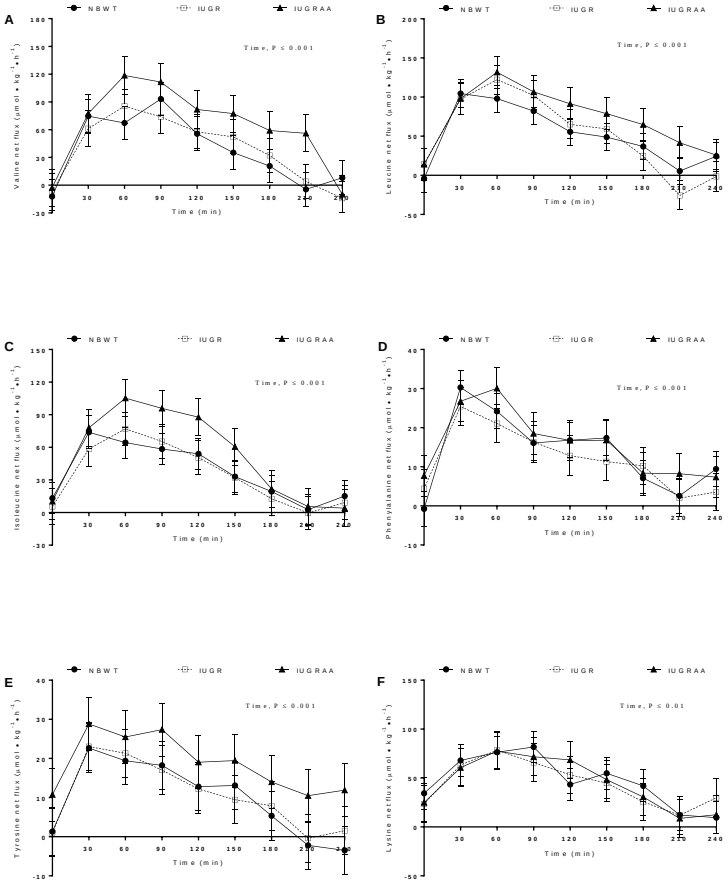


Fig. 4-7. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Hindquarters net balance of valine (*A*), leucine (*B*), isoleucine (*C*), phenylalanine (*D*), tyrosine (*E*) and lysine (*F*) in NBWT, IUGR or IUGRAA pigs during 240 min after feeding on d 21. Values are means ± SEM; $n = 6$.

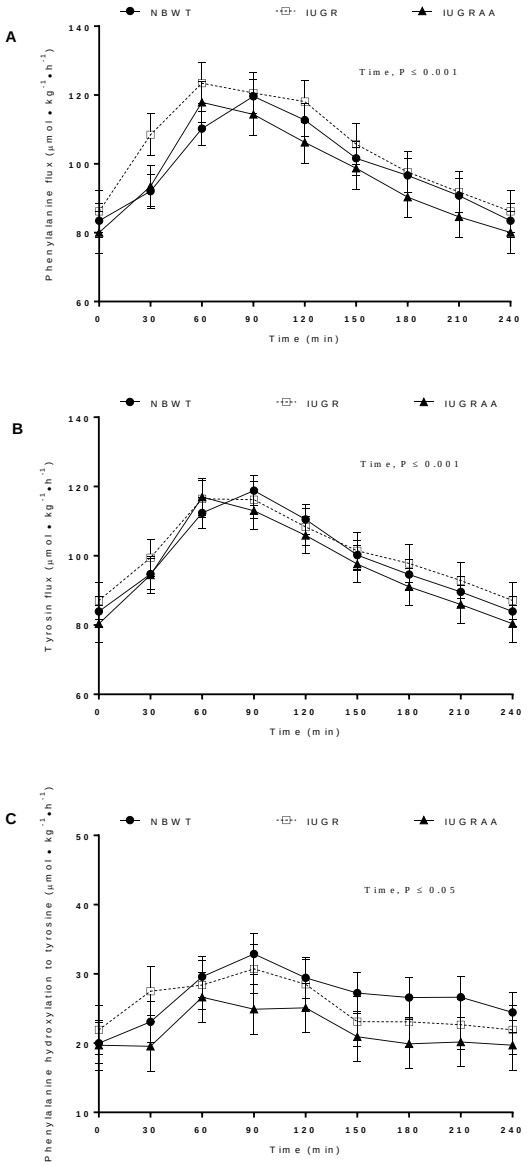


Fig. 4-8. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Whole body flux of phenylalanine (*A*) and tyrosine (*B*), and phenylalanine hydroxylation to tyrosine (*C*) in NBWT, IUGR or IUGRAA pigs during 240 min after feeding on d 21. Values are means ± SEM; $n = 6$.

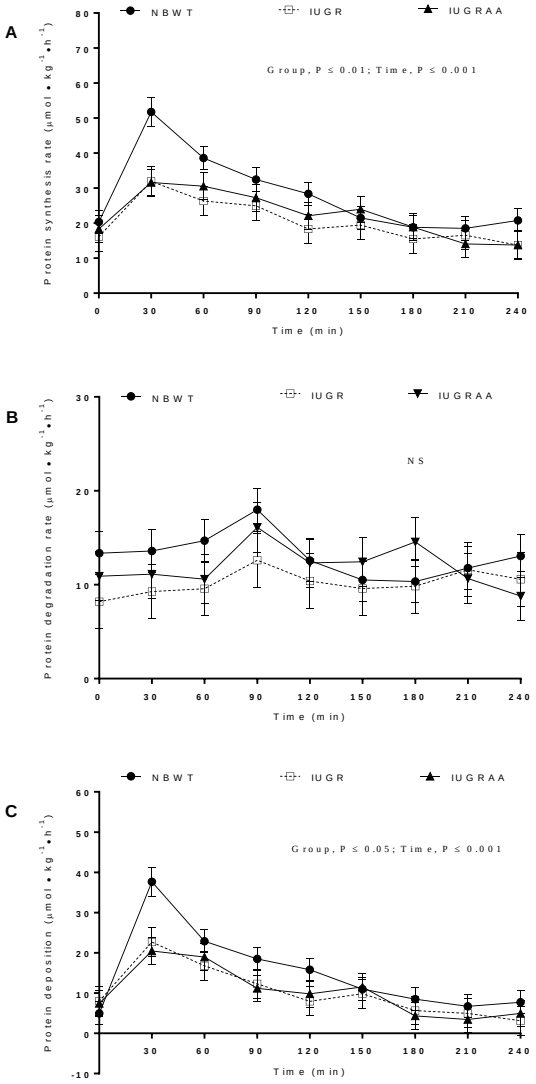


Fig. 4-9. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Hindquarters muscle protein synthesis rate (*A*), degradation rate (*B*) and deposition rate (*C*) in NBWT, IUGR or IUGRAA pigs during 240 min after feeding on d 21. Values are means ± SEM; $n = 6$.

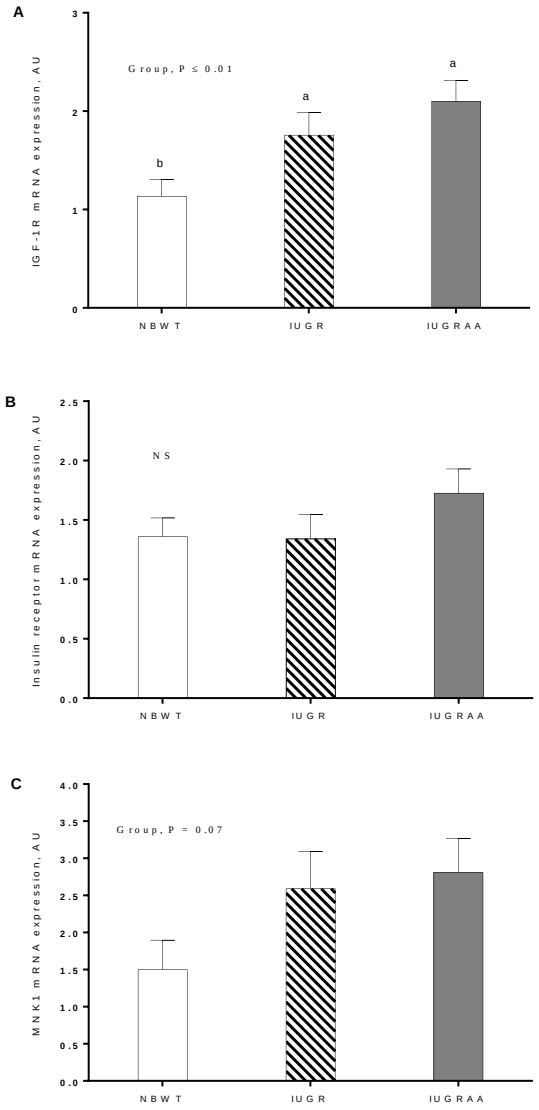


Fig. 4-10. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Gastrocnemius muscle mRNA expression of insulin like growth factor-I (IGF-I) receptor (*A*), insulin receptor (*B*) and mitogen activated protein kinase interacting kinase 1 (MNK1) (*C*) in NBWT, IUGR or IUGRAA pigs of 2 21. Values are means ± SEM; $n = 6$; different letter indicate significantly difference of means, NS = not significant.

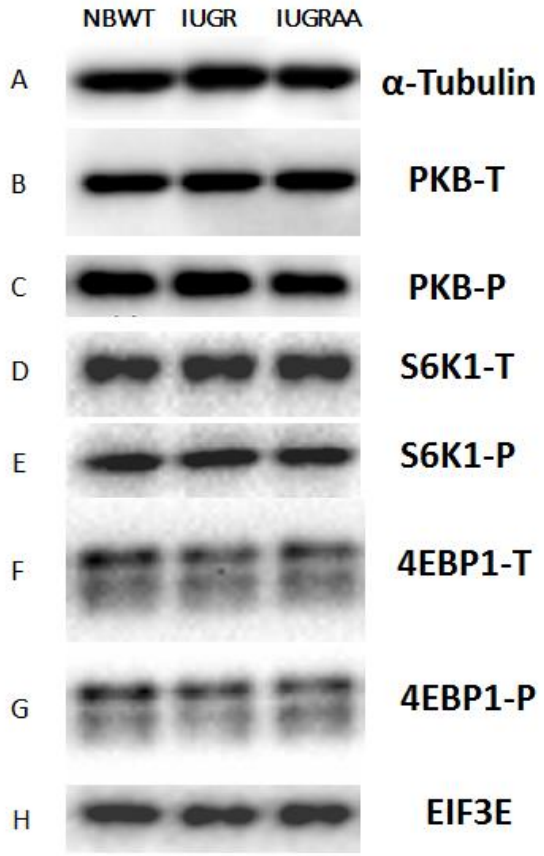


Fig. 4-11. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Gastrocnemius muscle protein expression of α-tubulin (*A*), total protein kinase B (PKB) (*B*), phosphorylated PKB (*C*), total ribosomal protein S6 kinase 1 (S6K1) (*D*), phosphorylated S6K1 (*E*), total eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (*F*), phosphorylated 4EBP1(G) and eukaryotic translation initiation factor 3 subunit E (EIF3E) in NBWT, IUGR or IUGRAA pigs of d 21.

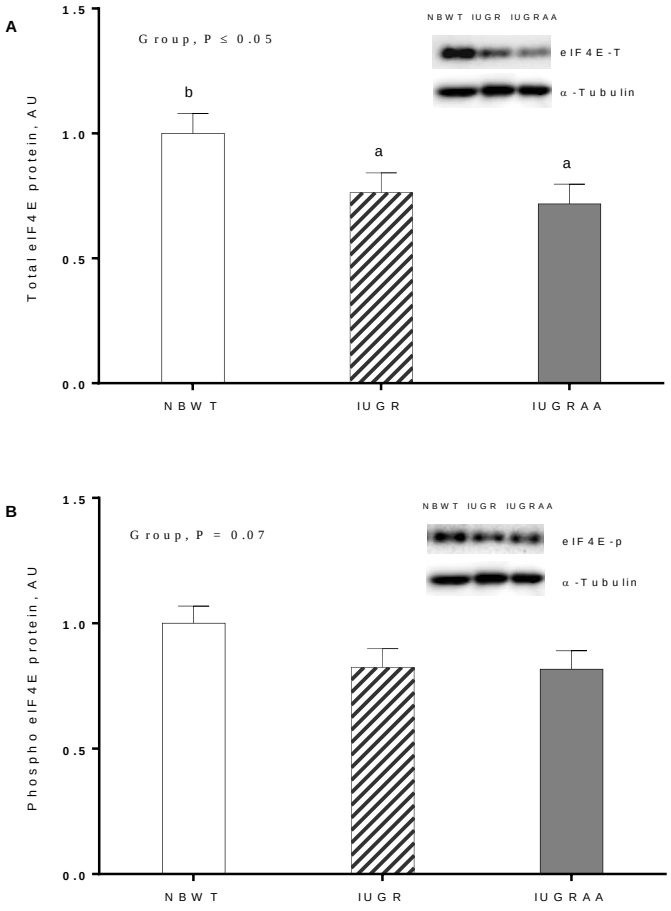


Fig. 4-12. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Gastrocnemius muscle protein expression of total eukaryotic translation initiation factor 4E (eIF4E) (*A*) and phosphorylated eIF4E (*B*) in NBWT, IUGR or IUGRAA pigs of d 21. Values are means ± SEM; $n = 6$; different letter indicate significantly difference of means.

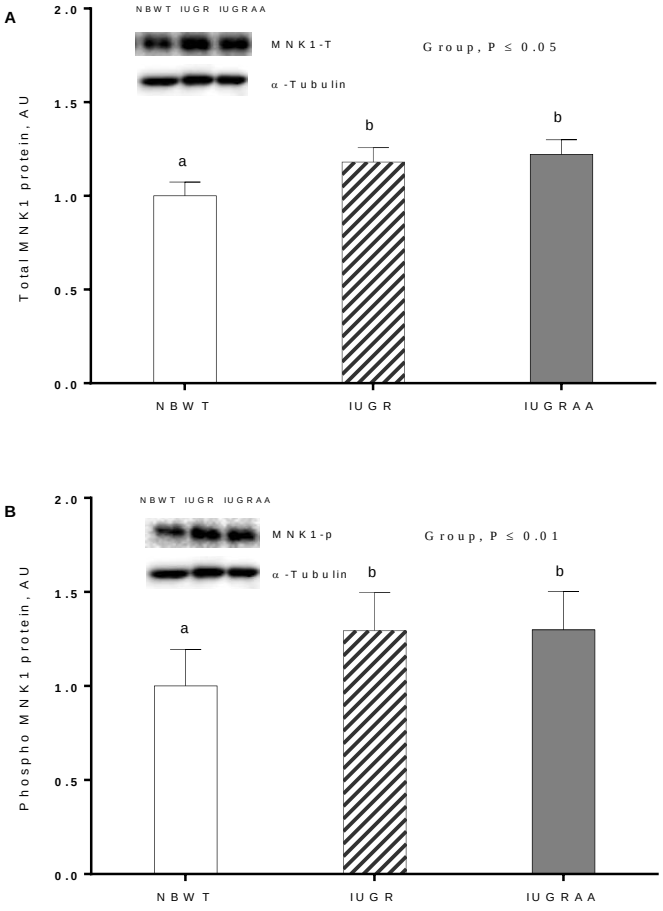


Fig. 4-13. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Gastrocnemius muscle protein expression of total mitogen activated protein kinase interacting kinase 1 (MNK1) (*A*) and phosphorylated MNK1 (*B*) in NBWT, IUGR or IUGRAA pigs of d 21. Values are means ±SEM; $n = 6$; different letter indicate significantly difference of means.

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## Chanpter 5.

## Summary

In the neonatal period, muscle growth is fastest than any other period in life, driven by high muscle protein turnover rates. Intrauterine growth restricted animals often fail to grow at the same rate of their normal body weight littermates. Our aim was to examine muscle growth in IUGR neonatal pigs compared to their sex matched NBWT siblings.

Intrauterine growth restriction reduces muscle satellite cell numbers per fiber which suggest impaired prenatal myogenic precursor cell proliferation and/or migration. This results in diminished myogenic precursor cell pool in IUGR pig muscle and in turn may influence prenatal muscle fiber formation and postnatal muscle hypertrophy. Intrauterine growth restriction not only affects muscle satellite cell number, but also alters cell activity intrinsically, indicated by reduced ultimate fusion percentage in IUGR pig derived cultures.

Muscle protein synthesis rates were lower in IUGR pigs, which may be a leading cause for the reduced growth observed in these animals. A closer examination of the signaling pathways involved in protein synthesis revealed that the low expression of eIF4E may be the cause for a reduction in translation initiation and thus limiting protein synthesis rate. IGF1 receptor and MNK1 were both up-regulated in IUGR pig muscle, suggesting MAPK pathway may be activated. However, the mechanism regulating MAPK pathway under IUGR condition needs further investigation.

In conclusion, the compromised muscle hypertrophy in neonatal IUGR pigs maybe

a result of smaller stem cells pool size and reduced activity, and lower protein deposition rates in these animals.

## Appendix

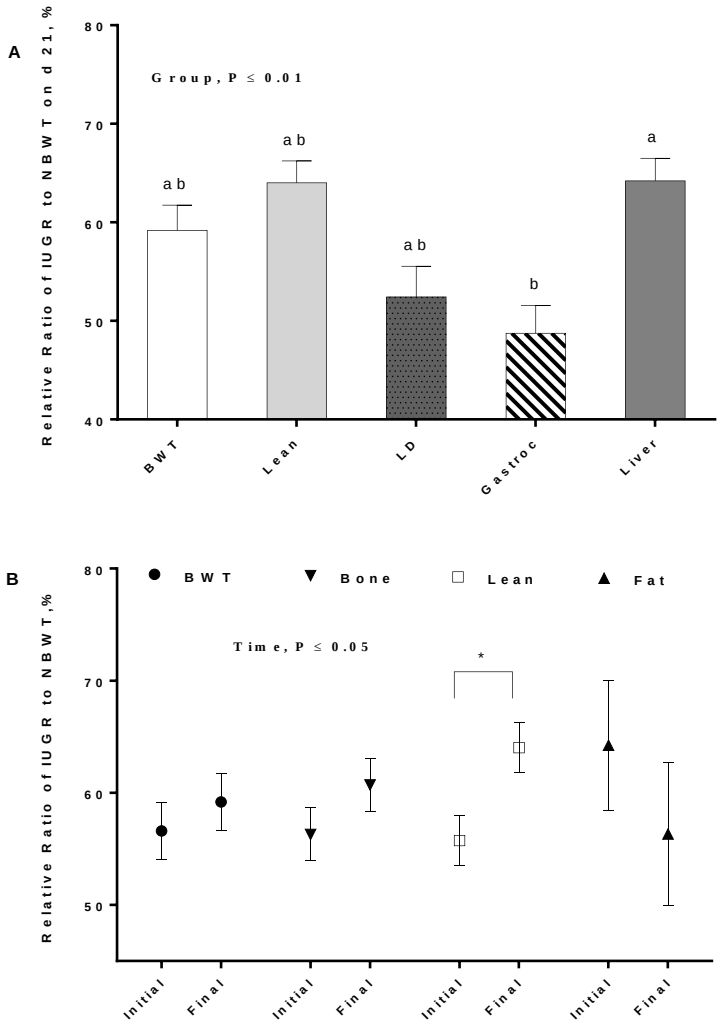


Fig. A-1. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were maintained for 21 days. Ratio of body weight, lean tissue, muscle and liver of IUGR to NBWT on d 21 (*A*) and initial and final ratio of body weight, bone, lean and fat tissue of IUGR to NBWT (*B*). Values are means ± SEM; $n$ = 6; different letter indicate significantly difference of means, * is $P \leq 0.01$.

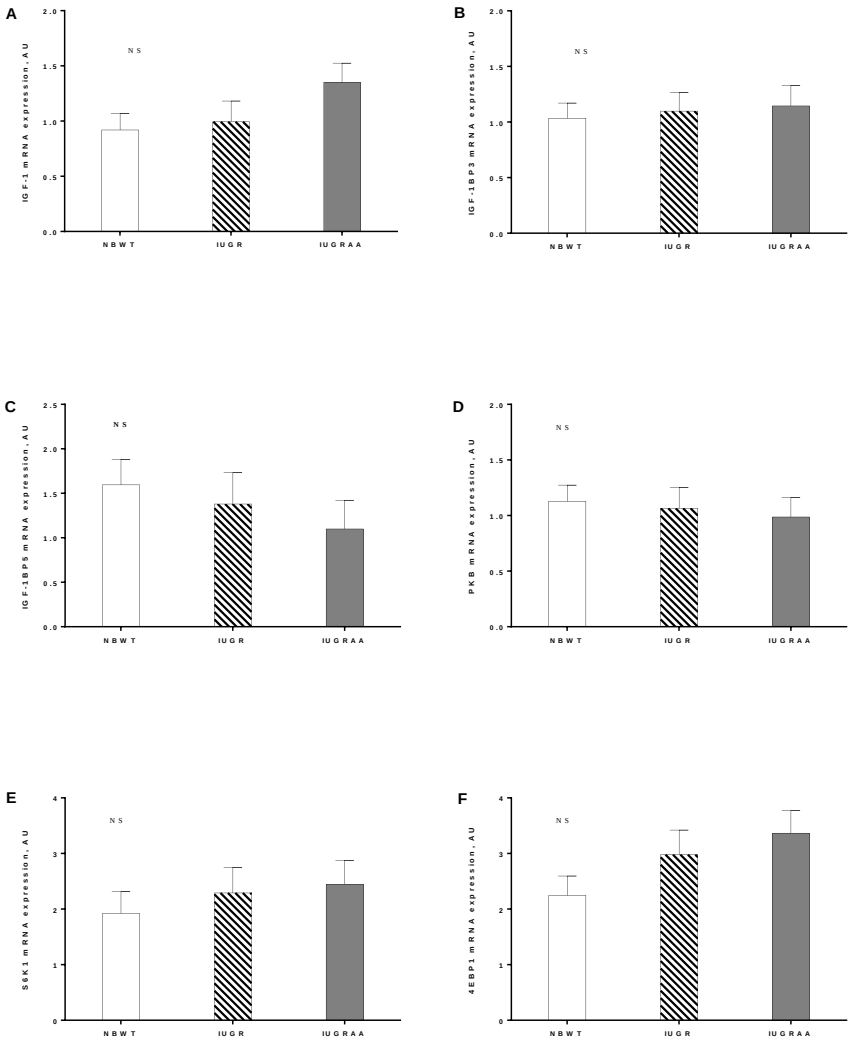


Fig. A-2. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were housed for 21 days. Gastrocnemius muscle mRNA expression of insulin like growth factor-I (IGF-I) (*A*), IGF-I binding protein 3 (IGFIBP3) (*B*), IGF-I binding protein 5 (IGFIBP5) (*C*), protein kinase B (PKB) (*D*), ribosomal protein S6 kinase 1 (S6K1) (E) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (*F*) in NBWT, IUGR or IUGRAA pigs of d 21. Values are means ± SEM; $n$ = 6; NS = not significant.

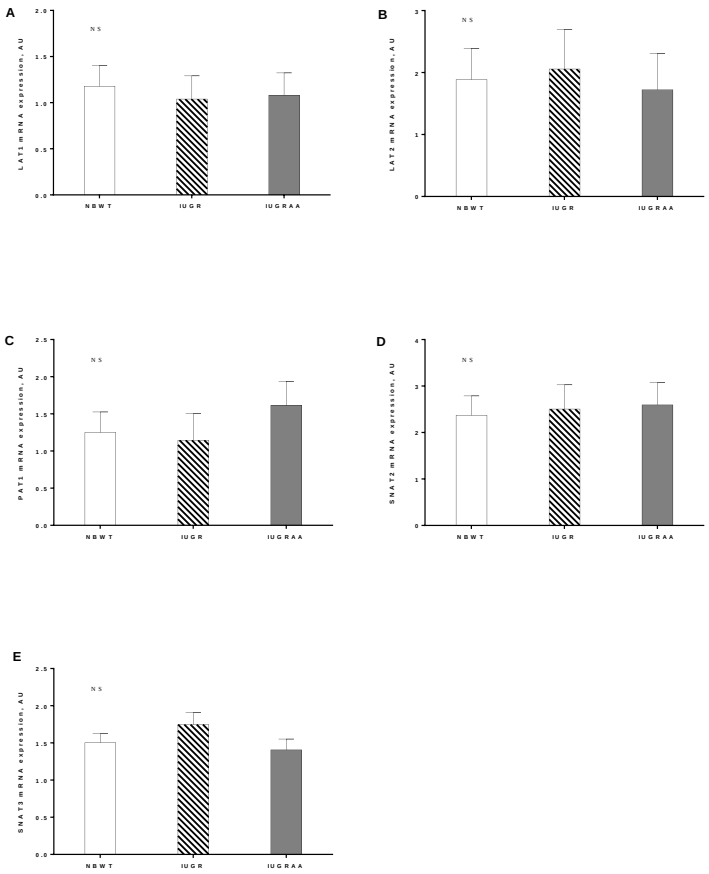


Fig. A-3. Normal body weight pigs fed control diet (NBWT), intrauterine growth restricted pigs fed control diet (IUGR) and intrauterine growth restricted pigs fed control diet supplemented with branch-chained amino acids (IUGRAA) were housed for 21 days. Gastrocnemius muscle mRNA expression of large neutral amino acid transporter 1 (LAT1) (*A*), large neutral amino acid transporter 2 (LAT2) (*B*), proton-coupled amino acid transporter 1 (PAT1) (*C*), sodium-coupled neutral amino acid transporter 2 (SNAT2) (*D*), sodium-coupled neutral amino acid transporter 3 (SNAT3) (E) in NBWT, IUGR or IUGRAA pigs of d 21. Values are means ± SEM; $n$ = 6; NS = not significant.