

COMPARISON OF THE MOLECULAR PHENOTYPES OF PIGS CARRYING DIFFERENT *IGF2* ALLELES
AT FOUR DEVELOPMENTAL TIME POINTS

BY

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THESIS

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Abstract

A single nucleotide polymorphism in *insulin-like growth factor 2* (*IGF2* intron3-G3072A) is associated with greater muscle mass and decreased subcutaneous fat deposition in pigs carrying a paternal copy of the A allele (A^{pat}). While many studies have focused on the differences in between the gross phenotypes of these animals, relatively little has been done to understand how these animals differ at the molecular level. It was the objective of this study to compare the molecular phenotypes of animals carrying the A^{pat} allele and those carrying the G^{pat} allele in order to gain insight into how those differences may result in the differences seen at the whole-animal level. RNA was extracted from *longissimus* (LM) samples taken from six animals of each genotype (A^{pat} and G^{pat}) at four different time points, fetal day 90 (d90), birth (0d), weaning (21d), and market weight (176d). In total 46 samples were sequenced on the Illumina HiSeq 2000 with a target depth of 20 million reads per sample. Analysis of the RNA-seq data using tweedEseq determined differentially expressed genes between A^{pat} and G^{pat} pigs at each time point. Additionally, IPA analysis was used to determine if there were any differentially activated pathways between the two phenotypes. IGF2 was not found to be upregulated in A^{pat} pigs compared to G^{pat} at d90 or 0d; however, IGF2 expression was found to be increased in A^{pat} pigs compared to G^{pat} pigs at 21d and 176d. Though there was not differential expression of IGF2 at d90 and 0d, there was still differential expression of several other genes at these time points. At d90, A^{pat} pigs tended to have an increase in the expression anti-apoptotic genes and a decrease in pro-apoptotic genes, possibly indicating enhanced myoblast survival, while G^{pat} pigs had greater expression of genes associated with protein synthesis. At 0d, there was an increased expression of genes associated with proliferation and

protein synthesis in A^{pat} pigs, and G^{pat} pigs appeared to have upregulation of genes involved in inflammation. At both 0d and 21d A^{pat} and G^{pat} pigs had displayed upregulation of markers of myogenesis, suggesting different stages of muscle development. At 176d, A^{pat} animals had decreased expression of genes involved in adipose signaling, including inflammation, stress, and autophagy and greater expression of genes associated with tissue development and the cell cycle. Thus, throughout the developmental timepoints, there appear to be markedly different processes taking place in the muscle tissue of A^{pat} pigs and G^{pat} pigs.

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Chapter1

Review of Literature

Introduction

A goal of livestock producers is to improve the production efficiency of animals while providing consumers with a high quality product. Over the past 60 years, the pork industry has selected heavily for pigs with reduced backfat thickness and increased lean percentage in response to changing consumer demands. Selection for leaner animals has not only reduced the deposition of subcutaneous fat in commercial lines but also has increased the capacity for muscle accretion. Consequently, the commercial swine population now exhibits a more muscular phenotype providing consumers with a leaner product. Due to lean accretion being less energetically expensive than fat accretion, the heavier muscled hogs of today are also more feed efficient than those of past decades.

In order to build upon the advances made by the commercial swine industry over the past 60 years, it is beneficial to understand muscle growth on a molecular level and how it differs in these leaner, heavier muscled pigs. Muscle growth is a polygenic trait controlled by many genes called quantitative trait loci (QTL). Muscle growth is a complex, multistep process that, during embryogenesis, involves commitment of embryonic progenitor cells to a myogenic lineage, migration and proliferation of myoblasts, differentiation of myoblasts to myocytes, and the fusion of myocytes into myofibers. Postnatally, muscle growth involves the proliferation, differentiation, and fusion of satellite cells with the existing myofibers, thereby increasing their size. The added muscle growth that has resulted from the intensive selective breeding by pork producers suggests that one or more of these processes involved in muscle growth have been

altered. Thus, it is likely that many of the genes and gene networks responsible for muscle growth have been affected.

Identifying the affected genes and understanding their role in muscle growth and development has been the focus of much research. For the purpose of QTL mapping, researchers have generated large intercross swine populations and conducted genome-wide scans for the detection of loci influencing phenotypes. Currently, one QTL with the capability of explaining significant phenotypic differences in muscle mass and backfat thickness has been described in detail (Jeon et al., 1999; Nezer et al., 1999). This QTL is found on chromosome 2p near *IGF2*, a maternally imprinted gene known to be involved in myogenesis (Florini et al., 1991; Jeon et al., 1999; Nezer et al., 1999). Further investigation of the candidate QTL led to sequence comparison of pigs differing in QTL genotype, determined through marker-assisted segregation. This comparison uncovered a single nucleotide substitution (G to A) in a regulatory region of *IGF2* (intron3-3072) that segregates perfectly between the two QTL genotypes, indicating that this is a quantitative trait nucleotide (QTN) underlying the QTL effect (Van Laere et al., 2003). Furthermore, maternal imprinting of the gene results in only the paternal allele being expressed in the skeletal muscle, meaning that it is the allele containing the IGF2 intron3-G3072A substitution received from the sire that results in the muscular phenotype (Van Laere et al., 2003).

To date, a considerable amount of research has been done to characterize the effect of this QTN on economically important traits of pigs. Studies indicate that the allele associated with greater leanness, the paternal A allele (A^{pat}), increases the weights of heavy muscled cuts, such as the ham, tenderloin, shoulder, and loin, as a percentage of the carcass weight (Burgos

et al., 2012; Clark et al., 2014) while decreasing backfat thickness (Burgos et al., 2012; Clark et al., 2014; Estellé et al., 2005; Oczkiewicz et al., 2009). This is done with little to no effect on meat quality measures (Burgos et al., 2012; Clark et al., 2014; Van den Maagdenberg et al., 2008). Additionally, there are studies that show greater average daily gain (Oczkiewicz et al., 2009; Fontanesi et al., 2010), reduced feed intake (Oczkiewicz et al., 2009), and greater carcass weight (Estellé et al., 2005) for certain breeds carrying the A^{pat} allele. These data suggest that the A^{pat} allele confers an economically advantageous phenotype that results in a greater production of lean meat and improved feed efficiency at the expense of fat deposition.

To elucidate the molecular basis for the observed increase in lean meat accretion, studies have attempted to understand how the QTN alters IGF2 expression over the course of swine development. Pigs carrying the A^{pat} allele have been shown to have a three-fold increase of IGF2 expression in *gluteus* (Van Laere et al., 2003), *trapezius* (Gardan et al., 2008), and *longissimus* (Clark et al., 2015) muscles at three weeks of age. This increase in postnatal IGF2 expression persists in market-aged hogs (Clark et al., 2015; Gardan et al., 2008). This information combined with the finding that cross-sectional area (CSA) of myofibers tended to be larger in A^{pat} pigs (Gardan et al., 2008) indicate that greater lean meat accretion could be due, in part, to postnatal muscle hypertrophy. However, other studies indicate that the CSA of myofibers from A^{pat} pigs were not different or even had a tendency to be slightly smaller than those from G^{pat} pigs (Clark et al., 2015; Van den Maagdenberg et al., 2008), suggesting that muscle hyperplasia is likely the cause of greater lean meat accretion. The results of Clark et al. (2015) supports the possibility of increased prenatal myogenesis in A^{pat} pigs, as well; their study indicated that A^{pat} fetuses tended to have greater IGF2 expression at day 90 of gestation

compared to G^{pat} fetuses, and this increase in IGF2 was associated with increased expression of the myogenic regulatory factors (MRFs) myogenic factor 5 (MYF5) and myogenin (MYOG). Thus, there is data to suggest that differential expression of IGF2 occurs late in fetal development and carries through to postnatal development possibly affecting both prenatal myogenesis as well as postnatal muscle hypertrophy.

The current information pertaining to IGF2 expression and the role that its QTN plays in muscle hyperplasia and/or hypertrophy has just begun to scratch the surface. Understanding how the expression of IGF2 is altered as well as the downstream pathways that are affected as a result of the QTN would allow us to more completely understand how the accretion of lean muscle may be enhanced at the molecular level. Such information could be used to make meaningful improvements to the performance of other production animals. Thus, a more comprehensive approach to studying the molecular differences between A^{pat} and G^{pat} pigs is needed.

Muscle Growth

Myogenesis

Skeletal muscle is derived from the mesodermal germ layer of the developing embryo. During embryogenesis, the paraxial mesoderm condenses into paired blocks on either side of the midline forming somites. It is the dorsal portion of the somite, the dermamyotome, which develop into the skeletal muscles of the trunk and limbs (reviewed in Parker et al., 2003).

The cells of the dermamyotome, characterized by their expression of paired-box transcription factors PAX3 and PAX7 (Kassar-Duchossoy et al., 2005), are encouraged to produce early myogenic regulatory factors (MRFs) by sonic hedgehog signaling from the

notochord and floor plate combined with WNT signaling from the dorsal neural tube and ectoderm (Munsterberg and Lassar, 1995). These early MRFs, myoblast determination protein (MYOD) and myogenic factor 5 (MYF5), are essential for the cells to acquire myogenic identity (Rudnicki et al., 1993). This activity is mediated by the beta helix-loop-helix (bHLH) domain of the MRFs, which facilitates transcription of muscle specific genes by binding both DNA and E proteins, transcription factors that recognize to the genomic E boxes in the promoters of the muscle specific genes (Massari and Murre, 2000).

After commitment of the mesodermal cells to a myogenic lineage, the myoblasts must migrate throughout the trunk of the embryo and into the developing limb buds. This is accomplished by the induction of MET proto-oncogene, receptor tyrosine kinase (MET) expression by PAX3 (Dietrich et al., 1999). As the developing limb bud grows, scatter factor (SF) is secreted and binds to the MET receptor; this elicits a response that causes the cells to migrate towards the SF source, allowing myoblasts to move into the limb bud (Dietrich et al., 1999).

Once the myoblasts have migrated throughout the body of the embryo, proliferation must occur to establish a pool of myoblasts that will eventually differentiate and fuse to form mature myofibers. Growth factors encourage proliferation by initiating pathways that prevent cell cycle exit during the G₁ phase and promote entry into S phase (Jones and Kazlauskas, 2001). In addition to encouraging cellular proliferation, growth factors can also inhibit differentiation by promoting the activation of pathways that prevent the activity of MRFs, promoters of terminal differentiation, and cyclin-dependent kinase (CDK) inhibitor proteins that are

important for progression through the cell cycle (Rommel et al., 1999; Winter and Arnold, 2000).

Cells continue to proliferate until they reach confluency. At this point, extracellular cues such as cadherin-mediated cell adhesion, initiate withdrawal from the cell cycle and the expression of genes important in myogenic differentiation, such as the secondary MRFs *MYOG* and *MRF4* (Goichberg and Geiger, 1998; Hinterberger et al., 1991). IGF signaling strengthens this response by activating downstream targets that will halt the proliferative pathways in myoblasts and encourage differentiation (Lee et al., 2002). Importantly, the resulting signal transduction cascades stabilize MRF transcripts for translation, activate MYOD, and inhibit CDK activity, resulting in the expression of myogenic genes and the halt of the cell cycle (Briata et al., 2005; Lluís et al., 2005; Héron-Milhavet et al., 2006; Gherzi et al., 2010). Fusion of myocytes immediately follows differentiation, resulting in multinucleated myotubes. Continued transcription of muscle-specific genes, including contractile proteins, increases the diameter of the myotubes and pushes nuclei to the periphery of the sarcoplasm.

Myogenesis generally occurs in two waves (Ashmore et al., 1972; Ashmore et al., 1973). Primary fiber formation occurs from day 30 to day 60 of gestation in pigs and produces myofibers up to six times larger than secondary fibers (Wigmore and Stickland, 1983; Christensen et al., 2000). Secondary fiber formation occurs from day 54 to day 90 of gestation and produces roughly 20 secondary myofibers for each primary myofiber (Wigmore and Stickland, 1983). Additionally, there is evidence of a third wave of myofiber development in larger animals (Mascarello et al., 1992). This wave is smaller compared to the second wave of

myofiber development and continues for the first three weeks of the pig's life and (Bérard et al., 2011)

Muscle Hypertrophy

Postnatally, skeletal muscle does not grow through the addition of new myofibers but through the increase in size of existing fibers; this is mediated by satellite cells located between the myofibers and the surrounding basal lamina (Muir et al., 1965). Satellite cells are undifferentiated mononucleated cells that remain quiescent until activated by signals from the surrounding muscle tissue, causing them to re-enter the cell cycle. These cells then proliferate and fuse with existing fibers, allowing for increased protein accretion.

Quiescent satellite cells are marked by their expression of PAX7 (Beauchamp et al., 2000; Seale et al., 2000). Additionally, these cells express the receptor MET which binds SF released by muscle tissue to induce reentry into the cell cycle (Allen et al., 1995). Upon reentry into the cell cycle, satellite cells undergo asymmetric division, yielding daughter cells with two distinct fates; one daughter cell maintains satellite cell identity, replacing the original satellite cell, while the other daughter cell becomes a myoblast and undergoes multiple rounds of proliferation (Kuang et al., 2007).

The fate of the satellite cell-derived myoblast is very similar to that of myoblasts in embryonic development. Following activation, expression of MYF5 and MYOD is induced, giving the daughter cells a myogenic character (Cornelison and Wold, 1997). The myoblasts then undergo proliferation induced by FGF and IGF signaling, followed by the expression of secondary MRFs MYOG and MRF4 during the onset of differentiation (Allen and Boxhorn, 1989; Cornelison and Wold, 1997). During this time, proliferative pathways are inhibited, and IGF

signaling triggers pathways responsible for the expression of myogenic genes, the translation of protein, and the halt of the cell cycle (Allen and Boxhorn, 1989; Briata et al., 2005; Lluís et al., 2005; Héron-Milhavet et al., 2006; Gherzi et al., 2010). Differentiated myocytes then fuse with the existing myofibers, enhancing their ability for protein accretion.

Insulin-like Growth Factor 2

Insulin-like growth factors (IGFs) are highly conserved proteins that are necessary for normal growth and development in vertebrates, as they play critical roles in cellular proliferation, survival, and differentiation (Liu et al., 1993). The IGF family includes three hormones, IGF1, IGF2, and insulin; three receptors, insulin-like growth factor 1 receptor (IGF1R), insulin-like growth factor 2 receptor (IGF2R), and insulin receptor (IR); and six insulin-like growth factor binding proteins (IGFBP1-6). The mature IGF2 polypeptide consists of 67 amino acids and shares 47% identity with the mature insulin polypeptide (reviewed in O'Dell and Day, 1998).

The *IGF2* gene is imprinted; thus, only one allele of the gene is expressed. In this case, it is the paternal allele that remains active due to maternal imprinting. This is controlled by a differentially methylated region (DMR) downstream of *IGF2*. This region on the maternal allele is in an unmethylated state, allowing an insulator protein, CCCTC binding factor (CTCF), to bind to the DMR and suppress the activity of the allele. Methylation of this DMR on the paternal allele abrogates CTCF binding, allowing for expression to be maintained (reviewed in Chao and D'Amore, 2008).

Generally, IGF2 expression is highest in prenatal animals, with peak levels being 3 fold greater than that of IGF1 (Constancia et al., 2002; reviewed in Fowden, 2003). Mice inheriting a

nonfunctional paternal *Igf2* allele are 40% smaller than wild-type, likely owing to reduced proliferation (DeChiara et al., 1991). However, paternal *Igf2* nulls are still viable and postnatal growth appears to be unaffected, underscoring Igf2's importance as a predominantly prenatal growth factor (Baker et al., 1993).

The biological effects of IGF2 are mediated through IGF1R. Upon binding to this receptor, the phosphoinositide 3-kinase (PI3K)-AKT cascade and/or the mitogen-activated protein kinase kinase (MEK)-extracellular-regulated kinase (ERK) cascade are initiated. Depending on other extracellular cues and growth factor signaling that the cell is being subjected to, the IGF2 signal transduction cascade may result in cellular survival, proliferation, or differentiation.

The MEK-ERK pathway initiates proliferation by preventing the cell cycle exit during the G₁ phase and promoting entry into S phase (Jones and Kazlauskas, 2001). This is accomplished by increasing the expression of critical cell cycle regulators, such as cyclin D1, cyclin-dependent kinase 4 (CDK4), and cyclin D2, and decreasing the expression of CDK inhibitors, such as p21 (Engert et al., 1996; Rommel et al., 1999; Rosenthal and Cheng 1995). Additionally, this signal transduction cascade inhibits differentiation. In skeletal muscle, this is accomplished by preventing the nuclear accumulation of MEF2 and decreasing the expression of MYOD, transcription factors necessary for the expression of myogenic genes (Lassar et al., 1989; Winter and Arnold, 2000).

The PI3K-AKT cascade is involved in cellular survival. Prevention of apoptosis is achieved through the inhibition of several pro-apoptotic pathways and transcription factors. For instance, this cascade results in the phosphorylation and inactivation of the bcl-2-associated

death promoter (BAD) protein, which prevents them from binding and inhibiting the anti-apoptotic proteins of the BCL-2 family (Datta et al., 1997). This pathway also inactivates caspases, specifically caspase 9, a protease that plays a critical role in cellular degradation during programmed cell death (Kermer et al., 2000). Pro-apoptotic transcription factors, such as the forkhead box O (FOXO) proteins, are also inhibited, reducing the expression of genes which initiate programmed cell death, such as *FASL* (Brunet et al., 1999).

In addition to preventing apoptosis the PI3K-AKT pathway is also critical to differentiation, which is marked by withdrawal from the cell cycle, the expression of tissue-specific genes, and protein synthesis by the activation of mTOR. The cell cycle is halted through the increased expression of the CDK inhibitor p21 as well as the inhibition of the MEK-ERK pathway by inactivating the RAF protein, a protein which lies near the beginning of the signal transduction cascade (Lawlor and Rotwein, 2000; Rommel et al., 1999). As the cell withdraws from the cell cycle, the expression of tissue-specific genes increases; in skeletal muscle these include secondary MRFs, *MYOG* and *MRF4* (Musaró and Rosenthal, 1999).

Though IGF2 is crucial to normal development and growth efficiency, an overabundance causes fetal overgrowth and death (Lau et al., 1994). Thus IGF2 availability is balanced by its binding to IGF2R and IGFBPs. In addition to limiting the availability of IGFs, IGFBPs also increase the half-lives of the proteins; thus, the vast majority of IGFs found in the body are not independently circulating, but they are bound to IGFBPs, particularly IGFBP-3 (reviewed in LeRoith and Roberts, 2003). The IGF2R receptor limits IGF2 availability through sequestration and degradation. Once IGF2 is bound to its receptor, the protein is internalized in an endosome and transferred to the lysosome for degradation (Jones and Clemmons, 1995).

IGF2 as a QTL

Due to its role in development and myogenesis, *IGF2* was a likely candidate when a genome wide analysis of 677 Large White X Piétrain F₂ individuals identified microsatellite markers located at the distal end of swine chromosome 2p associated with three of the phenotypes measuring muscularity and three of the phenotypes measuring fat deposition (Nezer et al., 1999). Similar results were found with a QTL analysis of an intercross population between the European Wild Boar and Large White (Jeon et al., 1999). Fluorescent *in situ* hybridization (FISH) of a BAC clone containing *IGF2* with porcine metaphase chromosomes, confirmed its location on chromosome 2p. This QTL accounted for 15-30% of the phenotypic variation in muscle mass as well as 10-20% of the variation in backfat thickness (Jeon et al., 1999; Nezer et al., 1999).

This QTL effect is the result of a single nucleotide substitution, *IGF2*-intron3-G3072A, which occurs in a highly conserved CpG island (Van Laere et al., 2003). Electrophoretic mobility shift assays (EMSAs) have shown that a repressor protein binds to the hypomethylated CpG island of *IGF2*-intron3 on the G allele; this interaction was neither present in the A allele nor the wild-type allele that had been methylated at the QTN site (Van Laere et al., 2003). Additionally, a threefold increase in *IGF2* mRNA was found in postnatal skeletal muscle of pigs carrying the paternal A allele (A^{pat}) compared to those carrying the paternal G allele (G^{pat}), which is likely the result abrogated repressor binding site in the A^{pat} pigs (Van Laere et al., 2003). The function of this repressor, zinc finger BED-containing protein 6 (ZBED6) was later confirmed. Silencing of *Zbed6* in mouse C2C12 myoblasts resulted in a significant increase in Igf2 expression, leading to

increased proliferation and faster myotube formation; whereas, overexpression of Zbed6 decreased Igf2 expression (Markljung et al., 2009).

Carcass and Meat Quality

The *IGF2* A allele is economically valuable, as it has the capacity to increase average daily lean meat growth and lean meat percentage (Van den Maagdenberg et al., 2008).

However, muscle yield is not the only important aspect of pork production; meat quality also needs to be considered. Thus, studies have been carried out with the aim of determining how the carcass quality of the two *IGF2* genotypes differ and whether the increase in lean meat associated with the *IGF2* A allele is also accompanied with any decrease in meat quality measures.

These studies indicate that while ending live weight, hot carcass weight, dressing percentage, and chilled side weight are not significantly different between A^{pat} and G^{pat} pigs, weights of individual cuts of meat and the percentage of the carcass that they comprise are significantly different between the two genotypes (Clark et al., 2014; Van den Maagdenberg et al., 2008). Clark et al. (2014) found that the trimmed loin, boneless Canadian back loin, back rib, bone-in and boneless Boston butt, and whole and trimmed ham weights were increased in A^{pat} pigs compared to G^{pat} pigs. Additionally, these cuts made up a larger percentage of the chilled side weight in A^{pat} pigs (Clark et al., 2014). Similar results were found in previous studies with heavier muscled cuts, such as the loin, ham, shoulder, and tenderloin, making up a larger percentage of the carcass weights in A^{pat} pigs (Burgos et al., 2012; Van den Maagdenberg et al., 2008). These studies also found that A^{pat} pigs had reduced backfat thickness and reduced weights of fattier cuts, such as the jowl and belly, comprised a smaller percentage of the

carcass weight in these animals (Burgos et al., 2012; Clark et al., 2014; Van den Maagdenberg et al., 2008).

With regards to meat quality, there seem to be a few differences between A^{pat} and G^{pat} pigs. Studies have shown that meat from A^{pat} pigs tends to be lighter in color, i.e. have a larger L* value, than meat from G^{pat} pigs (Burgos et al., 2012; Clark et al., 2014; Van den Maagdenberg et al., 2008). These results were consistent for the *longissimus dorsi*, a muscle that is more glycolytic, and the *triceps brachii*, which tends to be more oxidative (Van den Maagdenberg et al., 2008). Additionally, the meat may have a less red color, i.e. a lower a* value (Van den Maagdenberg et al., 2008). However, these differences have not resulted in significantly different subjective color scores (Clark et al., 2014). Cooking loss may also be greater in A^{pat} pigs (Clark et al., 2014), yet these results did not agree with those of previous studies (Burgos et al., 2012; Van den Maagdenberg et al., 2008). Interestingly, studies have indicated that A^{pat} may have more intramuscular fat in the *longissimus dorsi* (Burgos et al., 2012; Clark et al., 2014), but contradictory results have been found in other studies (Oczkiewicz et al., 2012).

While there are some differences in meat quality between the two genotypes, many measures of meat quality remain the same. No significant differences between ultimate pH, drip loss, subjective measures of firmness and marbling, and shear force of heated and raw meat have been identified between A^{pat} and G^{pat} pigs (Burgos et al., 2012; Clark et al., 2014; Van den Maagdenberg et al., 2008). These results indicate that consumers will likely perceive meat from A^{pat} pigs to be of similar quality as meat from G^{pat} pigs, especially since there were no significant differences in the subjective measures between the two genotypes.

Gene Expression Analysis

The somatic cells that make up an organism all share a common genome; it is the expression of different subsets and combinations of genes that allows cellular identity to change from tissue to tissue, enabling cells to perform specialized tasks. Changes in gene expression also occur during cellular development as immature cells proliferate and differentiate. Additionally, gene expression may be altered by growth factor signaling or extracellular cues, such as cell-cell contact. To understand the relationship between alterations in cellular phenotypes and the expression of different gene complements, researchers have often focused their attention on the transcriptional activity of genes. It is by comparing how the complement of transcriptionally active genes within cells differs between phenotypes, that researchers can begin to understand the functional roles of individual genes and how they contribute to the overall phenotype of the cell.

Early research methods focused primarily on the comparison of transcriptional activity for a single or few genes of interest between experimental conditions. For this purpose, the Northern blot has been the standard method for gene expression comparison for decades as it is capable of allowing for direct comparison of cellular RNA content without enzymatic manipulation. This method requires extracted RNA to be denatured and separated by gel electrophoresis. The separated RNA is then transferred to a nylon membrane where it is immobilized and washed with buffer containing labeled probes that are complementary to the RNA species of interest. After the probes are allowed to hybridize with the RNA, the membrane is washed to diminish any nonspecific binding. During visualization, the labels included in the probes emit a signal, and relative abundances of RNA can be compared between samples by

looking at the relative intensity of the signals (reviewed in Brown and Mackey, 2001; reviewed in Meinkoth and Wahl, 1984; Thomas, 1983).

Though Northern blotting is reliable and capable of discerning between relatively small changes in RNA expression, the amount of information that can be gained from the Northern blot is extremely limited (Taniguchi et al., 2001). Since there is no amplification step, this method is not likely to detect low to moderately abundant transcripts, as at least 100,000 copies of a transcript are necessary for its detection by blot hybridization (Cooper, 1997). Also, the Northern blot is typically used to gain information about a single transcript at a time, as using multiple probes to detect transcripts on a single blot requires the membrane to undergo a time-consuming stripping process to remove the first probe before the next probe can be applied (Brown et al., 2004). As information can only be gained on a single transcript at a time, this approach does not offer researchers the ability to easily detect relationships between the expression of different transcripts that could possibly indicate cellular pathways or cause-effect relationships within the cell.

Thus, the ability to survey the entire complement of RNA present in the cell at one time, the transcriptome, would be more useful in describing cellular phenotypes. By gaining information on such a large number of transcripts at once, it is possible for researchers to detect possible cause-effect relationships of gene expression as well as identify gene networks. The microarray, like the Northern blot, also relies on the hybridization of RNA transcripts to a probe for identification, but a single microarray slide is capable of containing probes for tens of thousands of genes; thus, it has the ability to give researchers much more information about the RNA profile of the cell compared to the Northern blot (Schena et al., 1995). For this method

of transcript comparison, cDNA libraries must be created from the RNA of the two conditions to be compared. The cDNA libraries are then labeled with two different fluorescent dyes, allowing differential detection on two cDNA libraries at once. The libraries are then flooded onto the microarray slide, which contains hundreds to thousands of spots of immobilized probes with each spot corresponding to single transcript. As the cDNA libraries wash over the slide, the probes hybridize to their complementary transcripts, allowing for their detection. The abundance of the transcripts within each cDNA library is then determined by the color and intensity of the fluorescent signal (Schena et al., 1995; reviewed in Trevino et al., 2007).

The amount of information given by microarrays makes them an attractive choice for researchers looking to detect differentially expressed genes and explain phenotypic differences between two conditions; however, this method of transcript detection still presents several limitations. One such limitation is the relatively small window of relative transcript abundance that can be detected. Rare transcripts produce a weak signal which can be drowned out by background noise, nonspecific binding of transcripts to probes, and abundant transcripts are prone to signal saturation, a point where the signal exceeds detection threshold of the visualization software (Hsiao et al., 2002). Furthermore, the design of microarray and the high cost of producing spotted slides make pooling of biological replicates for comparison on a single slide attractive to researchers, who wish to limit cost and technical variation; however, this common practice makes it impossible to identify biological variation between replicates of the same condition and remove outliers (Kendzierski et al., 2005).

Advancements in sequencing technology, along with its increased availability have enabled researchers to use next-generation sequencing (NGS) as a method of transcriptome

interrogation. NGS technology offers ultra high-throughput sequencing by making use of massively parallel sequencing, where millions of sequence reads can be processed during a single experiment (Mardis, 2008). In response to growing amount of sequence data being produced, advancements in bioinformatics have also been made. With ultrahigh-throughput approaches available and the improved ability to analyze large amounts of sequence data, much more information can be gathered about cellular transcriptomes than ever before. This change to a global approach of transcriptome analysis frees researchers from the task of deciding which genes to include in an experiment and also allows for the opportunity to get data on new transcripts that have not yet been discovered. Data gathered through NGS can then be used to gain new information regarding gene networks and cause-effect relationships within cells.

The great advantages of NGS combined with dropping costs have caused high-throughput RNA sequencing (RNA-seq) to replace microarray as the preferred method of transcriptome analysis (Wang et al., 2009). To prepare samples for sequencing on an NGS platform, total RNA extracted from cells is purified and may be depleted of ribosomal RNA at the discretion of the researcher. This RNA is then fragmented into 200-500 bp pieces in order to be compatible with deep-sequencing technologies. Following fragmentation, a cDNA library is created from the RNA, and adapters and indices are attached to both ends of the cDNAs to allow for hybridization and identification of the molecule. These libraries are then flowed onto the flow cell, a glass slide containing eight lanes and immobilized oligos complementary to the adapter sequence. Once flooded onto the flow cell the cDNAs hybridize with immobilized oligos via the adapters and then are amplified by repeated replication until thousands of copies

of the original cDNAs have been produced. Following amplification, the reverse strands are cleaved and washed away leaving the forward strand, which is identical to the original cDNA, to be sequenced (reviewed in Wang et al., 2009; reviewed in Zhong et al., 2011).

The sequences of the strands are determined in a massively-parallel manner through sequencing by synthesis (SBS). During this process, a sequencing primer anneals to the strand and differentially fluorescently labeled base pairs compete for complementary binding to the strand. After the addition of each new nucleotide, the fluorescent label is excited by a light source and the characteristic emitted signal is captured by a high-speed camera. This process is repeated anywhere from tens to a couple hundred times depending on the needs of the researcher. At the end of this forward strand sequencing, the complementary sequence is washed away, and an index primer is annealed so that the index sequence can be determined for library and strand identification. If paired-end reads are desired, then following the reading of the first strand and its index, the molecule blocking the 3' end of the strand is removed; thus, the adaptor sequence is free to fold over and bind the second oligo, creating another bridge. Another complementary strand is created through the elongation of this second oligo, and the double-stranded bridges are denatured. The forward, original strand is then cleaved from the oligo and washed away so that the reverse strand may be indexed and sequenced through SBS. The resulting sequence data can be assembled *de novo* or aligned to a reference sequence, and transcript abundance can be quantified and compared between conditions (reviewed in Hart et al., 2010).

RNA-seq eliminates many problems associated with Northern blots and microarray. Since the only primers being used are for ligated adapter sequences, sequence data that will be

obtained is not limited by our current knowledge of expression profiles or genome annotation. Thus, in addition to known transcripts novel isoforms and previously unknown transcripts can be detected. RNA-seq also offers an extremely broad range for the detection of dynamic transcript expression, as there is no upper limit for transcript quantification and an increasing number of rare transcripts can be detected at greater read depths. For instance, 16 million reads in *Saccharomyces cerevisiae* resulted in a transcript expression range greater than 9,000-fold while 40 million reads in the mouse offered a range of five orders of magnitude (Mortazavi et al., 2008; Nagalakshmi et al., 2008). This thorough interrogation of the transcriptome offers researchers more complete data for use in pathway analyses, which could lead to greater understanding of transcript expression profiles and cause-effect relationships within cells. Furthermore, RNA-seq results have been shown to be highly reproducible in both technical and biological replicates (Cloonan et al., 2008; Mortazavi et al., 2008).

Regardless of the vast benefits offered by RNA-seq, this method still has associated challenges. For instance, it is relatively common for sequences to map to more than one location in the genome. Typically, 15-20% of reads generated in humans are incapable of being uniquely mapped to the genome because the sequence lacks enough specificity (Wold and Myers, 2008). Also, since RNA-seq relies on the construction of a cDNA library prior to amplification, artificial differences between transcript abundance could be introduced due to bias inherent to PCR amplification. Nonetheless, RNA-seq still offers the most comprehensive method of transcriptome interrogation along with quantitative results, which makes it arguably the most valuable tool for understanding the molecular phenotype of cells.

Differential gene expression identified by high-throughput methods like RNA-seq and microarray can be further validated by real-time quantitative PCR (qPCR) (Kogenaru et al. 2012). This method is often relied upon as a confirmatory tool because it is a fast, sensitive, reproducible, and quantitative approach for the comparison of gene expression (Ding et al., 2007). To prepare samples for qPCR, cDNA is amplified from several target RNA transcripts using specific primers, and as amplification occurs, fluorescent dyes are incorporated into the PCR product. The incorporation of these fluorescent dyes into the product allows for the quantification of product accumulation after every round of amplification, as the fluorescent signal is directly proportional to the abundance of the PCR product. The ease of use of qPCR and its ability to generate reproducible, quantitative information makes it ideal for use as a confirmatory tool. Since a typical analysis only includes dozens of target transcripts of known identity, it does not readily lend itself to transcriptome interrogation.

Pathway Analysis

High-throughput gene expression analysis has the ability to provide expression information on tens of thousands of gene products, and subsequent statistical analysis allows researchers to determine which of these gene products are differentially expressed between conditions or phenotypes. Typically, statistical analysis yields a list of tens or hundreds of genes that are differentially expressed. However, a gene list has little explanatory power when it comes to describing how the underlying biology differs between phenotypes. To make biological sense of gene expression data, the differentially expressed genes must be considered within the context of the biological pathways in which they are involved.

There are several methods of analyzing and explaining the biological significance of gene expression data, and they are constantly evolving as our knowledge base of biological pathways continues to grow. Over-representation analysis (ORA) has been a popular method of analyzing high-throughput expression data (Khatri and Drăghici 2005). This method counts the number of times a pathway is represented by a gene in a list differentially expressed genes. Then, a count of pathways represented is determined for all genes measured in the experiment. Hypergeometric distribution, chi-square, binomial distribution, or Fisher's exact test is used for statistical analysis to determine if any pathways are over- or under-represented in the list of differentially expressed genes (Curtis et al., 2005; Khatri and Drăghici 2005). Unfortunately, the amount of information to be gained from ORA is severely limited. Regardless of the statistical test used, an assumption made during analysis is that the genes and pathways counted are independent from others in the list, which is not the case (Tian et al. 2005). This false assumption reduces the biological significance of the data, as the relationships between genes and pathways are not fully considered. Additionally, measurements associated with gene expression, such as fold-change in expression or p-value, are not taken into account during ORA, except to select differentially expressed genes (Khatri et al., 2012). Excluding this data fails to give weight or directionality to expression of genes or the pathways with which they are associated.

Functional class scoring (FCS) improves upon some of the shortcomings associated with ORA. For this method, all genes measured in the experiment are used to determine which pathways are represented, and the data associated with these genes, fold change and p-value, are used to assign a pathway-level statistic (Pavlidis et al. 2009). Thus, researchers are not

required to select a subset of genes for the analysis using arbitrary cutoff values, such as $p\text{-value} \leq 0.05$ or $\text{fold change} \geq 1.5$, allowing for the detection of smaller, coordinated changes in gene expression within pathways that would have been missed by ORA (Khatri et al., 2012). Though FCS does consider the interdependence of gene expression within pathways, this analysis, like ORA, still examines each pathway independently of others (Tian et al. 2005). This assumption of independence ignores the fact that pathways may overlap or may ultimately lead to the activation of another pathway.

An increasingly popular method of pathway analysis is the pathway topology (PT)-based method. This method works similarly to FCS, as it considers all of the genes and their associated data before assigning a pathway-level statistic; however, unlike FCS, PT also takes into account the position of gene products within pathways, the interactions they have with other gene products, and the location of these interactions within the cell (Drăghici et al. 2007). Thus, PT does not simply treat pathways as gene lists as ORA and FCS do; rather, it takes advantage of the broader knowledge contained in databases concerning the interaction of gene products (Tarca et al. 2009). However, pathway annotations are far from being complete and likely contain incorrect information. Additionally, pathway topology is likely to be cell-specific, that is gene product interactions are dependent upon tissue-type and developmental stage of the cell (Khatri et al., 2012).

Unfortunately, all methods of pathway analysis are limited by our current knowledge about gene products and their activity within the cell; therefore, we cannot expect for any single method to provide a complete picture of the biological significance of differentially expressed genes. Moreover, these methods of pathway analysis may provide a researcher with

information regarding coordinated up or down regulation of gene products involved in a particular pathway, but depending upon the role these gene products play in the pathway, this may offer little to no biological insight. For instance, if the pathway is not activated due to the lack of signaling by a growth factor or the absence of a receptor, then the up regulation or down regulation of gene products downstream will likely have little or no impact on the physiology of the cell. These short-comings limit the amount of meaning that can be obtained from a list of differentially expressed genes, but that is not to imply that pathway analysis is not important. Applying the most current experimental data to a list of differentially expressed genes and considering the context of the experiment (i.e. the tissue and developmental stage) is critical for extracting biological relevance; however, it is equally important to recognize the limitations of these bioinformatics tools and to be aware of the incompleteness of the data they provide.

Objectives

The objective of the proposed research is to more completely describe the molecular phenotype of pigs carrying the A^{pat} allele of *IGF2* to better understand the effect this mutation has on the proliferation of myoblasts and/or hypertrophy of developed muscle fibers.

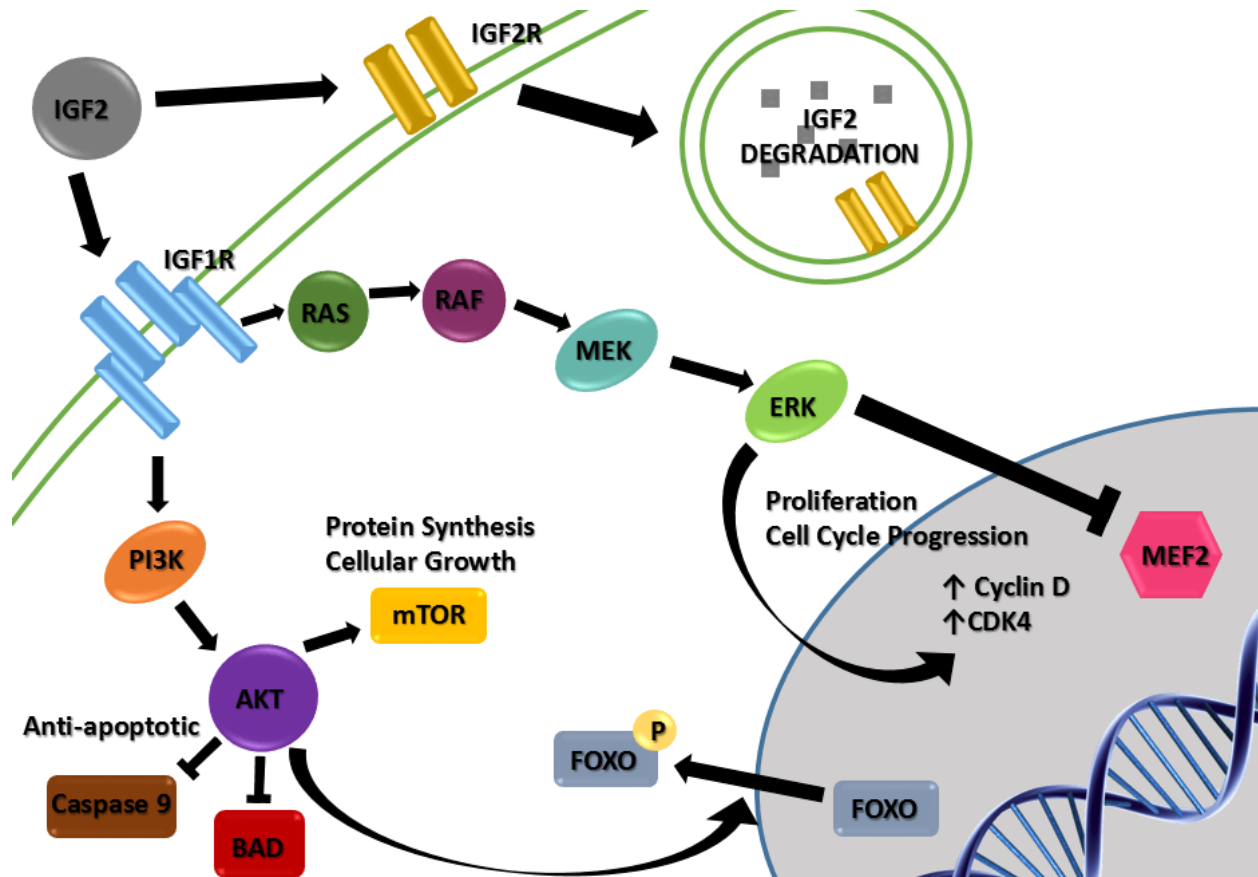
To date, much of the research regarding the *IGF2* mutation has focused on the differential expression of *IGF2*, among a handful of other genes, between genotypes. Relatively little work has been done to define the molecular phenotype of individuals carrying the A^{pat} allele of *IGF2* and determine how it differs from the phenotype of the individuals carrying the G^{pat} allele outside the limited scope of real-time quantitative polymerase chain reaction (qPCR). RNA-seq offers the ability to characterize the molecular phenotype of the *IGF2* genotypes in a

more detailed manner. In addition to detecting regulatory, non-coding RNAs, RNA-seq allows for the discovery of differential expression in genes which were not anticipated to be altered between genotypes. Furthermore, pathway analysis can add meaning to the differential expression data by indicating signal transduction cascades affected by alterations to the transcriptome. The global approach that RNA-seq and pathway analysis offers is advantageous and essential to determining the effects that the *IGF2* mutation has on a trait as complex as muscle growth.

The information obtained from this research will help shed light the pathways affected by the economically valuable *IGF2* A allele and advance our knowledge of networks involved muscle growth and development. Such information is critical to gaining a deeper understanding of the signal pathways involved in muscle growth and necessary as we look for ways to further improve carcass yield, meat quality, and feed efficiency of pigs and other livestock species.

Figure

Figure 1. IGF2 pathway overview



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Chapter 2

Comparison of the Molecular Phenotype of Pigs Carrying Different *IGF2* Alleles at Four Developmental Time Points

Introduction

Insulin-like growth factor 2 (*IGF2*) remains one of the most well-studied quantitative trait loci (QTL) in commercial swine to date. Jeon et al. (1999) and Nezer et al. (1999) found this QTL to explain up to 20% of the variation in backfat and up to 30% of the variation in muscle mass within their crossbred lines. Further interrogation of the QTL identified that a single nucleotide polymorphism SNP, a G to A substitution in intron 3 (intron3-G3072A) of *IGF2*, was responsible for these observed phenotypic differences in fat deposition and muscle mass (Van Laere et al., 2003). Specifically, it is the paternal A allele (A^{pat}) that confers the lean phenotype (Van Laere et al., 2003) as *IGF2* is maternally imprinted; thus, only the paternal allele is expressed in the skeletal muscle.

Many studies with the aim of describing the phenotypic differences between the A^{pat} and G^{pat} pigs have focused on carcass characteristics, economic measures, and meat quality measures. It is well established that A^{pat} pigs have an economically valuable phenotype that is more feed efficient (Oczkiewicz et al., 2009) and has a greater capacity for increased muscle mass (Burgos et al., 2012; Clark et al., 2014; Van den Maagdenberg et al., 2008). Additionally, fattier cuts of meat, such as the jowl and belly, comprise a smaller portion of the carcass weight in these animals as a result of reduced fat deposition (Burgos et al., 2012; Clark et al., 2014; Van den Maagdenberg et al., 2008). However, relatively few studies have attempted to elucidate

the phenotypic differences of A^{pat} and G^{pat} pigs at the molecular level. Understanding how these genotypes differ in their muscle development and the time points at which their muscle development differs may offer insight to further the improvement of commercial swine genetics. Additionally, such information could be applied to enhance the growth of other livestock species.

The objective of the current study was to use next generation sequencing (NGS) technology to characterize the transcriptomes of both A^{pat} and G^{pat} pigs at four developmental time points in order to determine how the molecular phenotype of A^{pat} and G^{pat} pigs differs over the course of the development.

Materials and Methods

Animals

A heterozygous Berkshire boar (AG at *IGF2* intron3-G3072A) was bred to homozygous (AA) Yorkshire-cross sows, guaranteeing that all heterozygous offspring would have inherited the G allele from the sire. At day 90 of gestation (d90), two sows were slaughtered to obtain fetuses. The 13 remaining sows were allowed to farrow. Pigs farrowed from these litters were harvested at birth (0d), weaning (21d), and market weight (176d). Samples from the *longissimus* (LM) muscle were collected immediately from harvested pigs, and stored at -80°C. Of these samples, 46 were used for RNA-seq, six samples per genotype (A^{pat} and G^{pat}) from d90, 21d, and 176d and five samples per genotype from 0d. All samples with the exception of four, two A^{pat} 0d samples and two G^{pat} 0d samples, were placed in RNeasy lysis buffer (Qiagen) to preserve the integrity of the RNA.

RNA-seq

Frozen LM tissue was removed from RNAlater (Ambion-Life Technologies, Grand Island, NY), rinsed with water (Optima grade, Fisher Scientific, Pittsburgh, PA) and added to 1.5 ml of TRIzol (Ambion-Life Technologies, Grand Island, NY) for total RNA isolation according to the manufacturer's instructions. Samples were homogenized using the TissueLyzer II (QIAGEN, Valencia, CA) and acid phenol:chloroform pH 4.5 (Ambion-Life Technologies, Grand Island, NY) was used to remove any remaining genomic DNA. Following RNA isolation, total RNA samples were purified using the RNeasy Mini Kit (QIAGEN, Valencia, CA), and the integrity of each sample was evaluated using the Agilent 2100 Bioanalyzer. All 46 samples had an RNA integrity number (RIN) greater than or equal to 8 and were considered to be of a high enough quality for RNA-seq. The Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) (Illumina, San Diego, CA) was used to deplete rRNA from 1 µg of total RNA for all samples, and TruSeq Stranded Total RNA sample Prep Kit (Illumina, San Diego, CA) was used to barcode the samples for identification in preparation for RNA sequencing.

The 46 samples were divided into two pools, and pools were balanced with regard to genotype and time point. Each pool was sequenced on the Illumina HiSeq2000 for 101 cycles. There was a target of 20 million reads per sample with each read having a length of 100 nucleotides.

Differential Expression Analysis

Raw Illumina sequencing reads were trimmed of adapters and low-quality bases using Trimmomatic (v0.32) (Bolger et al., 2014). These reads were then aligned to the Sscrofa 10.2 reference genome using TopHat2 (v2.0.13) (Kim et al., 2013), and the alignments were used by

Cufflinks (v2.2.1) (Trapnell et al., 2010) to create transcript assemblies for each sample.

Cuffmerge was used to merge the transcript assemblies for each sample to create a reference transcriptome in the form of a GTF file for use during differential expression analysis by Cuffdiff.

Differential expression analysis was conducted between the two genotypes, A^{pat} and G^{pat}, at each individual time point, d90, 0d, 21d, and 176d. Furthermore, the 0d samples were analyzed in two different groups: all samples (n=10) and those samples that were stored in RNAlater (n=6).

Additionally, TopHat2 alignments were used by HTSeq (Anders et al., 2014) for the quantification of transcripts mapped to each gene or feature in the Sscrofa10.2 reference genome. Feature count data produced by HTSeq were used for differential expression analysis between genotypes at each time point in three R packages, edgeR (McCarthy et al., 2012; Robinson et al., 2010; Robinson and Oshlack, 2010), DESeq2 (Love et al., 2014), and tweedEseq (Esnaola et al., 2013). Dam information was included as a covariate for analysis in edgeR and DESeq2 to improve the performance of the negative binomial model of the software. However, the addition of a covariate was not an option while using Cuffdiff and resulted in the loss of important statistical information needed for pathway analysis while using tweedEseq; thus, dam information was excluded while using these tools.

Trimmed sequence reads were additionally aligned to reference genomes for human (GRCh37), mouse (NCBIM37), and cow (Btau 4.0) using STAR 2-pass (v2.4.0j) (Dobin et al., 2012) in order to gain information about conserved genes missing from the pig genome assembly (Sscrofa 10.2). STAR 2-pass was also used to realign reads to the pig genome (Sscrofa 10.2) in order to gain any additional information that may have not been available through the use of

TopHat2. These alignments were used to produce feature count data using HTSeq, and differential expression analysis within each species were performed using tweedEseq. TweedEseq data resulting from TopHat2 and STAR 2-pass alignments were pooled for each time point, and duplicates were removed from each list.

Functional and Pathway Analyses

Two groups of differentially expressed genes were taken from the pooled data at each time point, those with a false discovery rate (FDR) ≤ 0.05 and FDR ≤ 0.10 . Pathway analyses for both groups of differentially expressed genes at each time point were carried out using the core analysis option of Ingenuity Pathway Analysis (IPA) software (Qiagen). Fisher's exact test was used by IPA in order to determine over-represented canonical pathways.

Results

Reads and Alignments

Across the 46 samples a total of 1,266,552,669 100-nt single-end reads were generated with an average of 27,533,754 reads per sample. Mean quality scores per base were all above 30 on the Solexa scale. The reads were mapped to Sscrofa10.2 using TopHat2 and STAR with an average of 84.54% and 72.03% uniquely mapped reads, respectively. Transcripts were also aligned to GRCh37, NCBIM37, and Btau 4.0 using STAR 2-pass with average of 36.25%, 20.63%, and 37.45% uniquely mapped reads, respectively.

Differential Expression Analysis

The number of differentially expressed features (FDR ≤ 0.05) at each time point varied greatly between analyses carried out with Cuffdiff, edgeR, DESeq2, and tweedEseq. Cuffdiff analysis, which counts differentially expressed transcripts rather than differentially expressed

genes (DEGs), resulted in the most differentially expressed features at each time point, and thus, overall. Of the software used detect DEGs from feature counts generated with HTSeq, tweedEseq was able to yield the greatest number of DEGs at each time point (Table 1).

DEGs with $FDR \leq 0.05$ (Table 1) resulting from all genome alignments were pooled at each time point for pathway analysis. Additionally, DEGs with an $FDR \leq 0.10$ were also pooled for submission to IPA (Table 2). The resulting number of DEGs, both with $FDR \leq 0.05$ and $FDR \leq 0.10$, used as input for IPA (Qiagen) are given in Table 3. The overlap of the pooled DEGs with an $FDR \leq 0.10$ is illustrated in Figure 2.1. There were no DEGs common to all four time points, yet there were several common DEGs yielded from pairwise comparisons between the individual time points. The only comparison that resulted in no common DEGs was d90 and 21d. The greatest number of common DEGs across time points was 32 DEGs shared between 176d and 0d RNAlater samples. All time points had common DEGs except for d90 and 21d. DEGs common to d90 samples and 0d samples tended to have a $\log_2$ fold change in opposite directions (Table 4).

The top ten up- and down-regulated genes from each time point tended to be weakly expressed in both A^{pat} and G^{pat} pigs (Table 5). The top ten highly expressed (≥ 100 counts for either A^{pat} or G^{pat} pigs) are given in Table 6.

Differential Expression of IGF2

Overall, IGF2 expression decreased over time from d90 to 176d, and differential expression between A^{pat} and G^{pat} pigs became more pronounced after 0d (Figure 2.2). $\log_2$ fold change in IGF2 expression for 21d pigs yielded from alignments to Btau 4.0, NCBIM37, and GRCh37 were -1.35, -1.40, and -1.45, respectively, indicating decreased expression in G^{pat} pigs.

Log₂ fold change in IGF2 expression for 176d pigs yielded from alignments to Btau 4.0, NCBI37, and GRCh37 were -1.66, -1.47, and -0.80, respectively. Thus, average fold change across all alignments for did not change considerably from 21d to 176d with respective values of -1.40 and -1.31. Pigs at time point d90 consistently showed slightly less IGF2 expression in G^{pat} pigs; alignments to Btau 4.0, NCBI37, and GRCh37 resulted in a log₂ fold change of -0.15, -0.13, and -0.05, respectively, with an average of -0.11 fold change, but difference in expression was not significant. None of the 0d analyses yielded a fold change indicating lower IGF2 expression in G^{pat} pigs (Table 7).

Discussion

Variation at *IGF2* intron3-G3072A has resulted in marked phenotypic differences between A^{pat} and G^{pat} pigs at the organismal level, and extensive research has focused on understanding the extent of those phenotypic differences between A^{pat} and G^{pat} pigs on a macro level, including measures of live pigs and carcass measures of animals after slaughter. A^{pat} pigs have been shown to have reduced subcutaneous fat deposition compared to G^{pat} pigs, resulting in decreased backfat thickness (Burgos et al., 2012; Clark et al., 2014; Estellé et al., 2005; Oczkiewicz et al., 2009) and reduced yield of fattier cuts, like the jowl and belly (Burgos et al., 2012; Clark et al., 2014; Van den Maagdenberg et al., 2008). Additionally, the heavily muscled cuts of A^{pat} pigs are generally heavier and comprise a larger proportion of the carcass weight when compared to heavily muscled cuts from G^{pat} pigs (Burgos et al., 2012; Clark et al., 2014). Not surprisingly, it has also been shown that A^{pat} pigs have a greater average daily gain of muscle compared to G^{pat} pigs (Van den Maagdenberg et al., 2008).

Although carcass differences between A^{pat} and G^{pat} pigs are well established, little has been revealed about the phenotypic differences between A^{pat} and G^{pat} pigs on a molecular level. Van Laere et al. (2003) concluded that there is a threefold greater expression of IGF2 in the skeletal muscles of three week old pigs carrying the A^{pat} allele compared to pigs carrying the G^{pat} allele. Furthermore, increased IGF2 expression persisted in pigs aged two months, four months, and six months, but no significant differences in IGF2 muscle expression was detectable in fetal pigs (gestational day unknown, Van Laere et al., 2003). Given that IGF2 expression is mainly increased at postnatal ages, these results suggest that postnatal muscle hypertrophy accounts for the organismal phenotypic differences between A^{pat} and G^{pat} pigs at slaughter. Other evidence suggests that there is differential expression of IGF2 at as early as fetal day 90, lending credence to the possibility that hyperplasia may account for the differences in muscularity between A^{pat} and G^{pat} pigs (Clark et al., 2015). The characterization of the molecular phenotype is incomplete; thus, the full consequence of the *IGF2* intron3-G3072A SNP is not well understood. Moreover, it is unclear whether the *IGF2* intron3-G3072A SNP affects muscle development and growth over the entire course of the animal's lifetime or only at specific time points of the animal's life. Therefore, the goal of the current study was to understand how the molecular phenotype of the skeletal muscle differs between A^{pat} and G^{pat} pigs over the course of four developmental time points.

Molecular Characteristics at d90

This study failed to detect any significant differential expression of IGF2 in the LM between A^{pat} pigs and G^{pat} pigs at d90. This tends to support the results obtained by Van Laere et al. (2003), as that study also found no difference in IGF2 expression between the two

genotypes during prenatal development. However, the results of both of these studies contradict the data yielded by Clark et al. (2015), which indicated a 1.4- to 1.5-fold increase in IGF2 expression in the LM of A^{pat} pigs compared to G^{pat} pigs. Therefore, if there is a tendency for prenatal hyperplasia in the skeletal muscle of A^{pat} pigs, it is yet unclear if it is the consequence of increased expression of IGF2.

Despite the failure to detect increased IGF2 expression in A^{pat} pigs at this time point, there appears to be data independent of IGF2 measures that indicate the possibility of prenatal skeletal muscle hyperplasia in A^{pat} pigs. Previous studies that have indicated that there is a tendency for A^{pat} pigs to have a greater number of muscle fibers in the *semitendinosus* (ST) muscle when compared to the G^{pat} pigs, yet this increase in fiber number did not reach statistical significance ($p \leq 0.05$) (Clark et al., 2015; Van den Maagdenberg et al., 2008). Nevertheless, the ST of A^{pat} pigs was found to be heavier despite having muscle fibers with a reduced cross sectional area (CSA) (Clark et al., 2015). Additionally, the LM of A^{pat} pigs has a greater weight compared to G^{pat} pigs without any significant differences in CSA of the muscle fibers between the two genotypes (Clark et al., 2015; Van den Maagdenberg et al., 2008). These data tend to support the idea that A^{pat} pigs have a greater number of muscle fibers with similar or even smaller cross sectional area compared to G^{pat} pigs, rather than A^{pat} pigs having similar number of fibers with a greater CSA. Accordingly, the greater muscle mass found in A^{pat} pigs would be the result of skeletal muscle hyperplasia in fetal pigs during one of the waves of muscle fiber production, as opposed to hypertrophy of the skeletal muscle during postnatal development.

The development of skeletal muscle fibers occurs in two waves (Ashmore et al., 1972; Ashmore et al., 1973). During the first wave primary fibers are formed, and they comprise a scaffold for the attachment of secondary fibers during the subsequent wave of fiber development (Ashmore et al., 1973). Wigmore and Stickland (1983) found that primary fibers were already present at day 38 of fetal development, and secondary fiber formation took place from day 54 to day 90 of gestation. At the end of secondary fiber formation, at day 100 of gestation secondary fibers account for over 90% of the total muscle fibers comprising a muscle (Christensen et al., 2000). Therefore, it is most likely that hyperplasia would occur during this second wave of muscle development and any significant differences in the molecular phenotype at d90 between A^{pat} and G^{pat} pigs may indicate the underlying mechanism for hyperplasia in A^{pat} pigs during fetal development.

For skeletal muscle hyperplasia to occur, there must be increased myoblast proliferation and/or myoblast survival in order to establish a larger pool of progenitor cells for the creation of more myofibers. Because proliferating myoblasts are more susceptible to apoptosis compared to differentiated myotubes, cell survival is important for the maintenance of myoblast populations (Walsh, 1997). The current study found expression of IGF1R was greater in A^{pat} pigs at d90 than G^{pat} pigs, and it is through this pathway that IGFs confer their proliferative and anti-apoptotic effects. Therefore, although there is not a greater expression of IGFs in A^{pat} pigs, it is likely that IGFs' effect is greater because of increased receptor expression.

The present study also found the products of IGF signaling were upregulated in A^{pat} pigs. Kruppel-like factor 6 (KLF6) is one such protein. This anti-apoptotic zinc finger protein is upregulated by IGF signaling (Bentov et al., 2008) and also transactivates the promoter of

IGF1R, increasing IGF1R expression (Rubenstein et al., 2004). Interference of KLF6 expression with small interfering RNAs (siRNAs) results in increased cell death and diminished proliferation; thus, it may play an important role in myoblast survival and proliferation (Rubenstein et al., 2004). It has been shown that solute carrier family 23 (ascorbic acid transporter), member 2 (*SLC23A2*) expression may be regulated by IGF signaling, as well (Chothe et al., 2013). The *SLC23A2* gene encodes the sodium coupled vitamin C transporter 2 (SVCT2), which allows for the uptake of ascorbic acid (vitamin C) into the cell. Evidence suggests that vitamin C inhibits apoptosis in myoblasts in a dose-dependent manner and may promote myoblast proliferation (Liu et al., 2010). Furthermore, it has been shown that cellular vitamin C concentrations within the muscles peak during secondary myogenesis and decay thereafter, so vitamin C may also be important for the early fusion of myoblasts into myotubes (Wilson, 1990).

A^{pat} myoblasts and developing myotubes may also be protected by the increased expression of the anti-apoptotic genes glutathione S-transferase pi 1 (*GSTP1*) and MAX dimerization protein 1 (*MXD1*). While *GSTP1* is able to prevent apoptosis in proliferating cells (Ishii et al., 2003), *MXD1* prevents apoptosis while also inhibiting the cell cycle, which would protect differentiating myocytes (Gehring et al., 2000). This is compounded by the possibility of enhanced myoblast migration in A^{pat} pigs indicated by increased expression of exocyst complex component 2 (*EXOC2*) and LIM domain containing preferred translocation partner in lipoma (*LPP*). *EXOC2* is activated by RAS-Like protein A (*RALA*) downstream of *IGF1R* signaling (Moskalenko et al., 2002). *RALA* is essential to myoblast migration (Suzuki et al., 2000), while its effector, *EXOC2*, appears to be necessary for cells to meet their migratory potential (Wu et al.,

2010). LPP localizes to focal adhesions (Petit et al., 2000) and also binds cytoskeleton structural protein α -actinin (Li et al., 2003). These focal adhesions are points of traction for the migrating myoblasts, as they allow the cells to make contact with the extracellular matrix.

The developing muscles of fetal A^{pat} pigs may be better able to support additional myoblasts due to the increased availability of resources for the developing cells. These animals were shown to have increased expression of protein kinase, AMP-activated, gamma 2 non-catalytic subunit (PRKAG2). Expression of this gene can be induced when an energy deficit is detected within the cell and results in increased mitochondrial biogenesis (Zong et al., 2002) for greater ATP production and increased glucose uptake by muscle cells (Merrill et al., 1997).

Cell survival in A^{pat} pigs is also likely enhanced by the decreased expression of the pro-apoptotic genes *G0/G1 switch 2 (G0S2)* and *SIVA1 apoptosis-inducing factor (SIVA1)*, as well as *aryl hydrocarbon interacting protein (AIP)*, which has some pro-apoptotic qualities. G0S2 binds anti-apoptotic protein BCL-2 which prevents it from forming a heterodimer with pro-apoptotic protein BAX, freeing up BAX to induce cell death (Welch et al., 2009). Similarly, SIVA1 overexpression alone has been shown to induce apoptosis (Yoon et al., 1999), as it is able to inhibit the effects of anti-apoptotic proteins BCL-XL and BCL-2 (Chu et al., 2004). AIP is a negative regulator of the alpha estrogen receptor (ER α) (Cai et al., 2011). ER α signaling has the ability to activate the IGF1R signal transduction cascade leading to enhanced cell survival (Kahlert et al., 1999); thus, the downregulation of AIP in A^{pat} pigs may lead to additional IGF1R signaling through interaction with ER α .

Surprisingly, the downregulation of genes involved in RNA translation and processing in A^{pat} pigs compared to G^{pat} pigs might indicate that there is faster or more efficient proliferation

occurring in G^{pat} pigs; these include genes coding for ribosomal proteins (*RPS12*, *RPLP0P2*, *RPL37A*, *RP11-383610.3*, *RPS11P5*, and *RPS26*) and small non-coding RNAs (*SNRPD2*, *SNORA66*, *ACA64*, *SNORA64*, *RNU1-120P*, *RNU1-7P*, *U1*, and *SCARNA10*). The enrichment in pathways involved in protein synthesis (EIF2 signaling, regulation of eIF4 and p70S6K signaling, and mTOR signaling) in fetal G^{pat} pigs compared to A^{pat} animals, reflects the increase in ribosomal proteins seen in G^{pat} pigs. Though these enriched pathways indicate greater myoblast proliferation in G^{pat} pigs, it fails to indicate whether these myoblasts survive. In fact, the enriched aryl hydrocarbon signaling in the G^{pat} pigs may negatively affect myoblast survival. Furthermore, expression of proliferative markers H3 histone, family 3A (H3F3A) and histone cluster 1, H2ad (HIST1H2AD) offers support to the possibility that there is greater proliferation in G^{pat} animals.

These results may be explained by the inhibition of RPTOR independent component of mTOR, complex 2 (RICTOR) in G^{pat} pigs, which was predicted by IPA upstream analysis (data not shown). RICTOR activation is necessary for AKT phosphorylation (Sarbasov et al., 2005), and AKT activity has been implicated in the upregulation of IGF1R (Tanno et al., 2001). This apparent differential activation of RICTOR may translate to the observed differential expression of IGF1R. Furthermore, mTORC2, which relies on the component RICTOR, has been shown to be essential for cell migration and cytoskeleton reorganization (Lang et al., 2010), both of which seem to be upregulated in A^{pat} pigs compared to G^{pat} pigs.

Molecular Characteristics at 0d

Though much of the emphasis for hyperplastic muscle growth is placed on secondary myotube formation, there is evidence of a third wave of myotube formation after birth

(Mascarello et al., 1992). Postnatal development of tertiary muscle fibers in pigs continues until postnatal day 28 and may account for much of the muscle growth during the first weeks of a pig's life (Bérard et al., 2011). Thus, this third wave of myogenesis may also contribute to any hyperplastic skeletal muscle growth observed in A^{pat} pigs.

As with the fetal d90 pigs, there was no evidence for differential expression of IGF2 at Od for either sample subsets (i.e. Od All and Od RNAlater). This is in contrast to the findings of Clark et al. (2015) whose results show a 1.5-fold increase in IGF2 expression in neonatal A^{pat} pigs compared to G^{pat} neonates. Thus, it remains unclear if IGF2 contributes to the differing phenotype between A^{pat} and G^{pat} pigs at this time in their development.

Nonetheless, A^{pat} pigs may be experiencing postnatal hyperplastic skeletal muscle growth, as indicated by the upregulation of markers for RNA translation (RPS12L1, RPS29, and CH242-511J12.1) and non-coding RNAs responsible for RNA processing (SNORA43, SNORA60, SNORA71, SNORA73, SNORD112, SNORD113, SCARNA1, and U1). These genes, which play a role in ribosome biogenesis and RNA processing are necessary for cell growth and subsequent proliferation (Donati et al., 2012). Proliferation in A^{pat} pigs may even be greater in postnatal muscle as eukaryotic initiation factor 2 (EIF2) signaling, which plays an important role in the initiation of protein synthesis, appears to be more greatly enriched in A^{pat} pigs compared to G^{pat} pigs. This could be partially due to the decreased expression of diacylglycerol lipase alpha (DAGLA) in A^{pat} pigs. This protein hydrolyzes diacylglycerol to 2-arachidonoyl glycerol, which activates cannabinoid receptor 1 (CB1). Signal transduction through CB1 decreases activity of mammalian target of rapamycin (mTOR), reducing protein synthesis (Pekkala et al., 2015).

The upregulation of several markers of proliferation (YRNA, HIST1H3B, SRGN, SHCBP1, BUB1B, and ANLN) yields support for the possibility that rapid myoblast proliferation is occurring in A^{pat} pigs. YRNA expression is required for DNA replication (Christov et al., 2006), histone cluster 1, H3B (HIST1H3B) is upregulated during S phase of the cell cycle (Medina et al., 2012), and BUB1 mitotic checkpoint serine/threonine kinase B (BUB1B) expression is important for spindle assembly during mitosis and is important for checkpoint signaling (Bolanos-Garcia and Blundell, 2011). Meanwhile, the expression of actin binding protein anillin (ANLN) is upregulated during cellular division as it is a structural component of the cleavage furrow during cytokinesis (Oegema et al., 2000). SHC SH2-domain binding protein 1 (SCHBP1) appears to be necessary for the completion of cytokinesis, or abscission, and is degraded after mitosis has finished (Asano et al., 2014). Serglycin (SRGN), a proteoglycan involved in protein storage and secretion, has a role in myoblast proliferation that is less clear; however, it has been established that SRGN expression in skeletal muscle peaks in proliferating myoblast and decreases once differentiation has begun (Hjorth et al., 2015).

Not only are markers of proliferation increased in A^{pat} pigs, inhibitors of proliferation (let-7g and SUFU) are also downregulated in these animals with respect to G^{pat} pigs. MicroRNA let-7g represses the translation of many cell cycle related genes, which delays or even prevents the ability of cells to transition from G₁ phase of the cell cycle to S phase (Johnson et al., 2007). Suppressor of fused (SUFU) inhibits sonic hedgehog (SHH) mediated proliferation by binding to the SHH signal mediators glioma-associated oncogenes (GLIs) and preventing the activation proliferation associated genes (Liu et al., 2014).

In addition to actively proliferating myoblasts, A^{pat} pigs also appear to have a large population of myoblasts undergoing differentiation. This is indicated by the upregulation genes specific to differentiating myoblasts such as *myosin light chain 6 (MYL6)*, which is part of the contractile unit within the sarcomere, and *myocyte enhancer factor 2c (MEF2C)*, a transcription factor exclusively expressed in myotubes. Genes associated with the progression of the differentiation program of myoblasts (*MIR433*, *ASPN*, and *SKIL*) also lend support to this. MicroRNA-433(MIR433) is able to allow myoblast differentiation to progress by binding to and repressing the activity of secreted frizzled-related protein 2 (SFRP2) (Snyder et al., 2013), a protein that has been shown to be an inhibitor of terminal differentiation in developing muscle (Descamps et al., 2008). Asporin (ASPN) and SKI-like proto-oncogene (SKIL) also inhibit the activity of proteins that prevent terminal differentiation of myoblasts. ASPN able to block the transforming growth factor-beta receptor type 2 (TGFβR2) (Nakajima et al., 2007). This stops the activation of its effectors, the mothers against decapentaplegic proteins (SMADs), which interfere with the expression of muscle-specific genes (Liu et al., 2001). SKIL, on the other hand, binds directly to SMAD proteins, inhibiting their ability to prevent myoblast differentiation (Wrighton et al., 2007).

Myoblast differentiation and fusion into myotubes occurs nearly simultaneously, and, as may be expected, genes necessary for myoblast fusion (*NPNT*, *POSTN*, and *F2RL2*) were upregulated in A^{pat} pigs alongside the aforementioned markers of myoblast differentiation. Nephronectin (NPNT) and periostin (POSTN) are both extra-cellular matrix proteins associated with the fusion of myoblasts (Sunadome et al., 2011; Özdemir et al., 2014), and coagulation

factor II (thrombin) receptor-like 2 (F2RL2) is an intracellular protein responsible for the movement of the nuclei within the myotube after fusion (Cadot et al., 2012).

Compared to A^{pat} pigs, G^{pat} pigs have a more modest increase in genes linked to myoblast differentiation. G^{pat} pigs have increased expression of *nuclear factor 1* genes (*NFIA* and *NFIX*), which are able to associate myogenin (MYOG) and enhance the transcription of muscle-specific genes (Funk and Wright, 1992). Additionally, G^{pat} pigs have greater expression of PR domain containing 1 (PRDM1) which promotes slow-twitch muscle fiber identity by repressing genes associated with fast-twitch myofibers (von Hofsten et al., 2008). These animals also have increased expression of genes involved in cytoskeleton reorganization (*WASF1* and *NEK3*). NIMA-related kinase 3 (NEK3) interacts with Vav 2 Guanine Nucleotide Exchange Factor (VAV2) to activate RAC1, a Rho family GTPase involved in myoblast fusion (Benjamin et al., 2011), and WAS protein family member 1 (WASF1) acts downstream of RAC1, resulting in nucleation of actin filaments (Eden et al., 2002). Despite the increased expression of these genes, Rho family GTPase signaling and actin cytoskeleton signaling appears to be more enriched in A^{pat} pigs compared to G^{pat} pigs, possibly indicating a larger population of cells undergoing myoblast fusion and cytoskeleton reorganization.

Unlike proliferating myoblasts which rely primarily on glycolysis, these differentiating myotubes rely on mitochondria as their primary source of ATP, requiring oxidative phosphorylation to provide roughly 60% of the energy used by the cell (Leary et al., 1998). Therefore, mitochondrial biogenesis is greatly increased in upon differentiation of muscle cells (Remels et al., 2010). Thus, the enrichment of the oxidative phosphorylation pathway in A^{pat} pigs may reflect a greater number of muscle fibers compared to G^{pat} pigs. The mitochondrial

dysfunction pathway was also enriched in A^{pat} pigs; however, the gene list associated with this pathway was nearly identical to the list associated with oxidative phosphorylation. Thus, the inclusion of mitochondrial dysfunction likely reflects the increased expression of mitochondrial genes in A^{pat} pigs rather than any actual pathology associated with the molecular phenotype of A^{pat} pigs.

Interestingly, a gene associated with mitochondrial dysfunction is nearly doubled in G^{pat} pigs compared to A^{pat} pigs. *Nuclear receptor subfamily 2 group F member 2 (NR2F2)* is upregulated in G^{pat} pigs and expression of this gene is inversely correlated with the expression of genes associated with the electron transport chain (Wu et al., 2015). Additionally, NR2F2 inhibits myogenic differentiation by binding MYOD and its cofactor P300, preventing them from promoting the expression of muscle-specific genes (Bailey et al., 1998). NR2F2 also appears to play a crucial role in adipogenesis, as haploinsufficiency of NR2F2 has been associated with the reduction of white adipose tissue by up to 30% (Li et al., 2009).

Greater adipogenesis in G^{pat} pigs is also supported by the increased expression of ubiquitin-conjugating enzyme E2O (UBE2O), mediator complex subunit 14 (MED14), and acetyl-CoA carboxylase beta (ACACB). UBE2O allows bone morphogenetic protein 7 (BMP7) induced adipocyte differentiation to progress uninhibited by inactivating SMAD6, a protein which would otherwise interfere with BMP7 signaling (Zhang et al., 2013). Meanwhile, MED14 interacts directly with peroxisome proliferator-activated receptor gamma (PPAR γ), a transcription factor for genes involved in adipogenesis, and is necessary for the activation of adipogenic genes (Grøntved et al., 2010). Knockout of *Acacb* in mice increases fatty acid oxidation and reduces

accumulation of adipose tissue, indicating an important role for this gene in the storage of fat (Abu-Elheiga et al., 2001).

Greater adiposity in G^{pat} pigs could be linked to increased inflammation in their muscle tissue. Increased expression of T-cell surface glycoprotein CD4 (CD4), zeta-chain-associated protein 70 (ZAP70), and ring finger protein 213 (RNF213), as well as the enrichment of pathways involved in T-cell activation, indicate that there is likely greater inflammation occurring in the skeletal muscle of G^{pat} pigs compared to A^{pat} pigs. CD4 is an antigen on the surface on T-cells, and ZAP70 is important for the activation of T-cells in the immune response (Reviewed in Smith-Garvin et al., 2009). While RNF213 expression has been shown to be linked to many cellular processes, it is also sharply upregulated in response to the release of pro-inflammatory cytokines (Ohkubo et al., 2015).

Molecular Characteristics at 21d

Unlike the previous time points, IGF2 was found to be differentially expressed in the LM between A^{pat} and G^{pat} pigs at 21d. This is in agreement with the results found by Clark et al. (2015) that indicated increased IGF2 expression in the LM of A^{pat} pigs compared to G^{pat} pigs at 21d. Similarly, Van Laere et al. (2003) also described increased IGF2 expression in the muscle of postnatal A^{pat} pigs compared to G^{pat} pigs. This increased expression IGF2 in A^{pat} animals coincides with the third wave of myogenesis that has been hypothesized to exist in larger mammals, a process that is suggested to last until postnatal day 28 in pigs (Bérard et al., 2011). Therefore, it is possible that any hyperplasia observed in the muscles of A^{pat} pigs is greatly impacted by the increased IGF2 expression during this time point while tertiary myofibers are being formed.

Along with greater IGF2 expression in A^{pat} pigs, there is also upregulation of synaptotagmin-like 5 (SYTL5), a gene whose product may be important in IGF2 signaling. SYTL5 is an effector of the RAS oncogene family member RAB27A (Kuroda et al., 2002). RAB27A plays a role in exosome secretion and has been shown to be involved in the cellular secretion of IGF2 (Wang et al., 2008). Thus, SYTL5 is likely a player in the autocrine signaling of IGF2 in muscle cells during this time point.

IGF2 signaling has a positive effects on myoblast proliferation (reviewed in Florini et al., 1991) and myoblast survival (Stewart and Rotwein, 1996), and A^{pat} pigs have increased expression of several markers associated with both proliferation and survival (STK26, GAB2, KV1.5, KCNE3, and AK4) at this time point. Serine/threonine protein kinase 26 (STK26) and GRB2-associated binding protein 2 (GAB2) can activate the MEK-ERK pathway (Lin et al., 2001; Meng et al., 2005). Enrichment of this pathway is essential to continued myoblast growth and proliferation, whereas, disruption of this pathway could result in premature cell cycle exit and differentiation (Heller et al., 2001; Jones and Kazlauskas, 2001; Volonte et al., 2005). The voltage-gated potassium channel genes, *potassium voltage-gated channel, subfamily A, member 5 (KV1.5)* and *potassium voltage-gated channel, subfamily E, regulatory subunit 3 (KCNE3)*, are also greatly expressed in proliferating myoblasts, and KV1.5 expression, in particular, is a driver of myoblast growth and proliferation (Villalonga et al., 2008). While adenylate kinase 4 (AK4) expression is associated with enhanced proliferation, it is likely that this is a result of its anti-apoptotic properties. AK4 is restricted to the mitochondrial matrix within cells (Takafumi et al., 2001) and knockdown of its expression has been associated with increased cell death and reduced proliferation (Kong et al., 2013; Liu et al., 2009).

Despite the proliferative edge that A^{pat} pigs appear to have, G^{pat} pigs do have increased expression of one marker of proliferation, centromere protein I (CENPI), and one possible marker of proliferation, ROS proto-oncogene 1, receptor tyrosine kinase (ROS1). The role of CENPI in proliferation is relatively clear, as it associates with kinetochores within dividing cells (Liu et al., 2003). However, the role of expression of ROS1 in skeletal muscle is less clear as it has been shown to encourage proliferation, differentiation, survival, and immune response, depending upon other cellular signaling (Charest et al., 2006; Schreck et al., 1992).

Compared to A^{pat} pigs, G^{pat} pigs appear to have an upregulation of a larger number of genes associated with myogenic differentiation and myotube development. Cellular processes such as autophagy and endoplasmic reticulum (ER) stress are necessary for myoblast differentiation and fusion (Fortini et al., 2016; Nakanishi et al., 2005), and genes associated with these processes (*TP53INP2*, *TRIM63*, *FBXO43*, *ASB11*, *BST1*, and *NFE2L3*) were found to be increased in G^{pat} pigs. Tumor protein p53 inducible nuclear protein 2 (TP53INP2) is required for the formation of autophagosomes in cells, and thus, is a chief regulator of autophagy in skeletal muscle (Nowak et al., 2009). Tripartite motif containing 63 (TRIM63) and F-box protein 40 (FBXO40) are E3 ubiquitin ligases, and the increased expression of these genes are associated with skeletal muscle atrophy (Bodine et al., 2001; Ye et al., 2007). While ankyrin repeat and SOCS box containing 11 (ASB11) is also an E3 ubiquitin ligase, it localizes to the endoplasmic reticulum where it is able to elicit the degradation of ribophorin 1 (RPN1), a proteasome subunit (Andresen et al., 2013). It is the inhibition of the proteasome that results in the accumulation of misfolded proteins and subsequent ER stress (Ding et al., 2007). Bone marrow stromal cell antigen 1 (BST1), an ER-associated protein, is necessary for the transport of

misfolded proteins to the Golgi apparatus (Vashist et al., 2001), and while downregulation of BST1 expression promotes cytoprotective effects (Labunskyy et al., 2014), upregulation of this gene likely maintains ER stress. Prolonged ER stress results in the upregulation of nuclear factor, erythroid 2-like 3 (NFE2L3) (Pepe et al., 2010), leading to the buildup of toxic reactive oxygen species (ROS) within the cell (Santos et al., 2009). Taken together, these genes may point toward the later stages of myogenesis taking place to a greater extent in G^{pat} pigs compared to A^{pat} pigs at this time point.

A^{pat} pigs do have upregulation of at least one gene that is clearly associated with autophagy, *F-box and leucine-rich repeat protein 6 (FBXL6)*. The FBXL6 protein is a part of the SKP1-CUL1-F-box (SCF) E3 ubiquitin ligase complex, which is important for cell cycle regulation (Peters, 1998; Skowyra et al., 1997), rather than a modulator of skeletal muscle differentiation. Despite this lack of upregulation of genes associated muscle autophagy and ER stress in A^{pat} pigs, there appears to be an increased expression of a few myogenic genes (*CHRNE*, *ARG1*, *CRABP1*, and *COL19A1*) in these animals. The acetylcholine subunit, cholinergic receptor nicotinic, epsilon subunit (CHRNE), is preferentially expressed in postnatal skeletal muscle (Mishina et al., 1986; Witzemann et al., 1987; Gu and Hall, 1988) and restricted to the endplates of motor neuron synapses (Schuetze and Role, 1987). Arginase 1 is highly expressed by M2a macrophages, a group of macrophages that are important in promoting terminal differentiation in myoblasts and the fusion of myoblasts into myotubes (Sacrier et al., 2013). Likewise, retinoic acid can act as a driver of myogenic differentiation (Halevy and Lerman, 1993), and cellular retinoic acid binding protein 1 (CRABP1), a regulator of retinoic acid signaling, is upregulated in cells undergoing myogenesis, with its greatest expression occurring

in myotubes (Zhao et al., 2014). In order to create an extracellular matrix that is capable of supporting the fusion of myoblasts into myotubes, differentiating myoblasts increase expression of collagen, type XIX, alpha 1 (COL19A1) (Sumiyoshi et al., 2001). Together, these genes offer evidence that the A^{pat} pigs may still have a large number of differentiating myoblasts or developing myotubes this time point; however, myogenesis in A^{pat} at this point is likely in a different stage of differentiation or myotube development compared to G^{pat} pigs.

With increased expression of myosin heavy chain 7 (MYH7); WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2 (WFIKKN2); and genes associated with innervation of skeletal muscle (*VAMP1*, *ALS2*, and *ADCY2*) in G^{pat} pigs, it is possible that these animals are in later stages of myogenesis compared to A^{pat} animals. MYH7 is the major adult myosin filament present in slow, oxidative type I skeletal muscles (Tajsharghi et al., 2003), and upregulation of MYH7 expression is an indicator of myotube maturation (Chaturvedi et al., 2015). Furthermore, WFIKKN2 allows for the progression of myogenesis by binding to and inhibiting myostatin (MSTN) (Hill et al., 2003), a negative regulator of myoblast proliferation of terminal myoblast differentiation (Langley et al., 2002). These genes are indicative of myotube development and maturation. In large mammals, myotube development is restricted to sites of innervation (Duxson and Sheard, 1995), and G^{pat} pigs have an increase of expression of genes associated with motor neurons. Vesicle-associated membrane protein 1 (VAMP1) is preferentially expressed in motor neurons in skeletal muscles (Tajika et al., 2007), while alsin rho guanine nucleotide exchange factor (ALS2) is important for neurite outgrowth (Tudor et al., 2005) and motor neuron protection (Kanekura et al., 2005). As adenylate cyclase 2 (ADCY2) expression is found to be decreased in denervated skeletal muscle (Suzuki et al., 1998),

indicating that the increased expression of this gene in G^{pat} animals likely reflects the greater innervation of skeletal muscle in these pigs.

Molecular Characteristics at 176d

At 176d, A^{pat} pigs had greater expression of *IGF2* in the LM compared to G^{pat} pigs; these results reflect the findings by Clark et al. (2015). At this time point, the number of myofibers found in the muscles is set, yet muscle cells are still able to undergo hypertrophy. Energy intake by the animal may also go towards hyperplasia and hypertrophy of adipose tissue. At this time point, evidence has shown that A^{pat} pigs have decreased development of subcutaneous adipose tissue (Burgos et al., 2012; Clark et al., 2014; Estellé et al., 2005; Oczkiewicz et al., 2009) and greater muscle mass (Burgos et al., 2012; Clark et al., 2014; Van den Maagdenberg et al., 2008) compared to G^{pat} pigs. While skeletal muscle hyperplasia early in development of A^{pat} pigs is a likely contributor to the phenotypic differences between the two genotypes, altered metabolism within skeletal muscle and delayed development of adipose tissue in market weight pigs could add to the differences between A^{pat} and G^{pat} pigs, as well.

The A^{pat} pigs at this time point have increased expression of genes associated with muscle repair (*IL1RL1*, *LOXL3*, *PBX3*, and *S100A10*) and angiogenesis (*S100A10*, *CCDC23*, and *MYH11*). Interleukin 1 receptor-like 1 (*IL1RL1*) is ubiquitously expressed in regulatory T cells found in damaged skeletal muscle, and recruitment of regulatory T cells to sites of muscle damage is critical for comprehensive muscle regeneration after injury (Kuswanto et al., 2016). Lysyl oxidase-like 3 (*LOXL3*) is an extracellular matrix protein that is localized at the ends of myofibers (Kraft-Sheleg et al., 2016), and expression of *LOXL3* has been shown to be upregulated in response to exercise-induced hypertrophy of skeletal muscle (Palstra et al.,

2014). Pre-B-cell leukemia homeobox 3 (PBX3) appears to be important driver of the transcription of MYOD target genes during the differentiation of fast-twitch skeletal muscle (Berkes et al., 2004; Maves et al., 2007), and the increased expression of PBX3 is a possible indicator of satellite cell differentiation. While the upregulation of S100 calcium binding protein A10 (S100A10) expression has been found to take place during skeletal muscle repair (Warren et al., 2007), it also appears to play a role in angiogenesis (Surette et al., 2011). Angiogenesis is able to be controlled by genes such as *coiled-coil domain containing 23* (CCDC23). The product of this gene is a chaperone protein that allows the secretion of angiogenic and anti-angiogenic factors, thereby regulating the process of creating new blood vessels (Suzuki et al., 2010; Xue et al., 2013). The walls of the blood vessels are composed of vascular smooth muscle whose differentiation is marked by the expression of myosin heavy chain 11 (MYH11), a smooth muscle specific myosin (Miano et al., 1994).

The continued development and repair of muscular and vascular tissues in the skeletal muscles of A^{pat} pigs at this time point might be the cause of the increased expression of genes associated with the cell cycle (*RMDN1*, *NUSAP1*, *DYRK2*, *PGRMC1*, and *7SK*). Regulator of microtubule dynamics 1 (*RMDN1*) and nucleolar and spindle associated protein 1 (*NUSAP1*) are crucial for proper chromosome segregation (Oishi et al., 2007; Raemaekers et al., 2003), and dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (*DYRK2*) and progesterone receptor membrane component 1 (*PGRMC1*) control the progression of the cell cycle. *DYRK2* is able to activate p53, if necessary, to induce apoptosis (Taira et al., 2007), whereas *PGRMC1* promotes cell cycle progression and confers anti-apoptotic effects (Lin et al., 2015). While *7SK* small nuclear RNA (*7SK*) is not necessarily involved in the cell cycle, it may modulate the cell

cycle and diminish proliferation by its ability to indirectly inhibit the activity of RNA polymerase II and suppress the transcription of genes (Nguyen et al., 2001).

The activity of IGF2 in A^{pat} pigs is perhaps somewhat dampened by the increased expression of insulin-like binding protein 6 (IGFBP6) and the enrichment of the PTEN signaling pathway indicated by IPA analysis. IGFBP6 preferentially binds IGF2 (Bach et al., 1993) and thereby has the ability to inhibit IGF2 activity (Bach et al., 1994; Bach, 1999). Furthermore, IGF2 enhances the expression of phosphatase and tensin homolog (PTEN), an inhibitor of IGF activity, acting as a negative feedback loop of IGF2 expression (Moorehead et al., 2003).

Some of the differences seen in the molecular phenotype of A^{pat} pigs at this time point may also be the result of decreased secretion of cytokines from adipose tissue (aka adipokines), as these animals have a significantly less amount of subcutaneous fat than G^{pat} animals. Adipose tissue releases interleukin 6 (IL-6) (Mohamed-Ali et al., 1997), adiponectin (Maeda et al., 1996), tumor necrosis factor- α (TNF- α) (Hotamisligil et al., 1993), and other molecules that alter the gene expression and pathways in nearby tissues. IPA analysis indicated that G^{pat} animals have enriched pathways that are associated with the increased expression of adipokines (acute phase response signaling and ciliary neurotrophic factor (CNTF) signaling) compared to A^{pat} pigs, indicating that these pathways are diminished in A^{pat} animals. IL-6 and TNF- α are capable of inducing acute phase response signaling, a pathway marked by systemic inflammation (Emery and Salmon, 1991; Castell et al., 1989). Additionally, IL-6 can upregulate the expression of CNTF (Shuto et al., 2001), possibly contributing the enrichment of this pathway in G^{pat} animals compared to A^{pat} animals. These adipokines released by subcutaneous adipose tissue may also increase expression of associated genes in the muscles of G^{pat} pigs. For

example, IL-6 has been shown to induce the expression of interleukin 6 signal transducer (IL6ST) (Fujisawa et al., 2002). Evidence suggests that adiponectin has been able to induce the expression of adiponectin receptor 1 (ADIPOR1) in the skeletal muscle of healthy individuals (McAinch et al., 2006). Also, TNF- α has been found to increase expression of pro-inflammatory molecule *CD83* (Thurnher et al., 1997).

Chronic inflammation, like that associated with greater adiposity and acute phase response signaling, leads to greater expression of p53 and p21 (Hofseth et al., 2003). The cell cycle genes upregulated in G^{pat} pigs (*MKRN1* and *ARID3A*) appear to have roles in cell cycle arrest specific to the expression of p53 and p21. Under stress conditions, the pro-apoptotic protein p53 induces cell cycle arrest by increasing the cyclin-dependent kinase inhibitor p21, which represses apoptosis, but under lethal stress conditions, cell cycle arrest genes, such as p21, are not induced (Martinez et al., 2002; Rinaldo et al., 2007). AT rich interactive domain 3A (*ARID3A*) can be upregulated by increased p53, and together, *ARID3A* and p53, promote the transcription of p21 during stress conditions (Lestari et al., 2012). Makorin ring finger protein 1 (*MKRN1*) is an E3 ubiquitin ligase that controls the degradation of p53 and p21 (Lee et al., 2009). Under normal cellular conditions, *MKRN1* promotes cell cycle progression and suppresses apoptosis by degrading p53; however, during cellular stress, *MKRN1* promotes cell cycle arrest and apoptosis through the degradation of p21 (Lee et al., 2009). The greater expression of these genes may reflect the chronic inflammation that is associated with larger amounts of subcutaneous adipose tissue; therefore, the reduction of the expression of these genes in A^{pat} pigs may indicate an underlying mechanism for the reduced development of subcutaneous adipose tissue compared to G^{pat} pigs.

Furthermore, G^{pat} pigs have upregulation of genes associated with cellular stress and autophagy (*MAPKAPK3*, *SQSTM1*, and *MYBPH*). Mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3) is a part of the stress-activated protein kinase signaling pathway (Cargnello and Roux, 2011) and contributes to stress-induced autophagy (Wei et al., 2015). Similarly, *sequestosome 1* (*SQSTM1*) expression is induced by cellular stress (Jain et al., 2010), and it associates with autophagosomes (Shvets et al., 2008). Less is known about the role of myosin binding protein H (MYBPH); however, some evidence has shown that it may play a role in autophagosome maturation (Mouton et al., 2015).

Apart from stress- and inflammation-related genes, G^{pat} pigs have an increased of wingless-type MMTV integration site family, member 5B (WNT5B) expression. This gene may play roles in the regulation of skeletal muscle and the development of adipose tissue. WNT5B is expressed in myoblasts and myofibers of regenerating adult muscle (Polesskaya et al., 2003); therefore, it likely plays a role in skeletal muscle hypertrophy. However, it is also able to block canonical WNT signaling pathway in order to promote adipogenesis (Kanazawa et al., 2005). Thus, if WNT5B is able to act as a adipokine, then it may be able to induce adipogenesis in nearby tissue.

There were, in fact, genes associated with adipocyte differentiation upregulated in the skeletal muscle of the G^{pat} pigs (*NR6A1*, *FLOT1*, and *MRPS9*). Nuclear receptor subfamily 6, group A, member 1 (NR6A1) is a repressor of octamer-binding transcription factor 4 (OCT4), an inhibitor of differentiation (Fuhrmann et al., 2001). Flotillin-1 (FLOT1) functions as a membrane-associated protein that contributes to the trafficking of molecules via caveolae in many tissues (Bickel et al., 1997). Mitochondrial ribosomal protein S9 (MRPS9) is a subunit of a mitochondrial

ribosome. Though these proteins function in many different tissues, the expression of these genes have been shown to be increased during adipocyte differentiation (Bickel et al., 1997; Fu et al., 2005; Yin et al., 2014). The upregulation of these genes may indicate that there were more adipocytes in the G^{pat} LM samples compared to the A^{pat} LM samples.

Despite indicators of increased inflammation, autophagy, and possible increase of adipogenic genes in G^{pat} pigs, IPA analysis indicated that mTOR signaling, p70S6K signaling, and growth factor signaling were enriched in G^{pat} skeletal muscle. In part, this prediction could be due to the lower expression of IGFBP6 in G^{pat} pigs. However, there are genes associated with mTOR and p70S6K (*EIF3L* and *EEF2*) upregulated in G^{pat} pigs. Eukaryotic translation initiation factor 3, subunit L (*EIF3L*) is one of the 13 subunits that comprise the EIF3 complex, an integral component in the initiation of translational initiation (reviewed in Dong and Zhang, 2006), and eukaryotic translation elongation factor 2 (*EEF2*) is a regulator of peptide-chain elongation during protein synthesis (reviewed in Kaul et al., 2011). The upregulation of these genes may suggest that G^{pat} pigs do have increased protein synthesis in their skeletal muscle tissue at this time point.

Conclusion

Increased expression of IGF2 in A^{pat} pigs was not detected until 21d; this increase in IGF2 expression in A^{pat} pigs persisted at 176d. Although no differential expression of IGF2 was detected between A^{pat} and G^{pat} pigs at d90 and 0d, there was still marked differential expression of other genes that may contribute to their differences in phenotype. These differentially expressed genes at d90 and 0d may contribute to hyperplastic skeletal muscle growth in A^{pat} pigs. Furthermore, increased IGF2 expression in A^{pat} pigs may, in fact, delay the

progression of myogenesis in favor of greater myofiber development during tertiary myogenesis, as the G^{pat} pigs appeared to be farther along in their stages of muscle growth. At 176d, A^{pat} and G^{pat} pigs show marked differences in skeletal muscle metabolism. This is likely due to the combined effects of differential expression of IGF2 expression and the differences in quantity of adipokine signaling between the two genotypes.

Tables and Figures

Table 1. Number of differentially expressed features between A^{pat} and G^{pat} pigs with FDR ≤ 0.05

Alignment Software	Genome Used	Analysis Software	d90	0d All	0d RNAlater	21d	176d
TopHat2	Sscrofa10.2	Cuffdiff	146	1142	N/A	279	560
		edgeR (no covariate)	16	0	1	9	3
		edgeR (covariate)	55	2	19	11	18
		DESeq2 (covariate)	60	3	13	11	16
		tweeDEseq	38	77	291	65	142
STAR	Sscrofa10.2	tweeDEseq	29	82	299	42	146
STAR	GRCh37	tweeDEseq	20	64	264	27	111
STAR	NCBIM37	tweeDEseq	19	45	158	11	49
STAR	Btau4.0	tweeDEseq	23	49	214	19	119

Table 2. Number of DEGs yielded by tweeDEseq analysis with FDR ≤ 0.10

Alignment Software	Genome Used	d90	0d	0d RNAlater	21d	176d
TopHat2	Sscrofa10.2	55	148	394	79	255
STAR	Sscrofa10.2	45	122	409	70	255
STAR	GRCh37	52	106	420	43	187
STAR	NCBIM37	46	85	289	11	113
STAR	Btau4.0	36	114	326	34	203

Table 3. Number of pooled DEGs at each time point for FDR ≤ 0.05 and FDR ≤ 0.10 used in IPA

Time Point	Number of DEGs	FDR
d90	57	0.05
d90	138	0.1
0d All	137	0.05
0d All	289	0.1
0d RNAlater	516	0.05
0d RNAlater	867	0.1
21d	64	0.05
21d	110	0.1
176d	228	0.05
176d	468	0.1

Table 4. Top ten DEGs by FDR at each time point

Time Point	Associated Gene Name	Full Gene Name	Counts A ^{pat}	Counts G ^{pat}	Log ₂ Fold Change	p-value	FDR
d90	<i>KLF6</i>	Kruppel-Like Factor 6	1944.5	1721.833	-0.1754537	3.15E-17	3.91E-13
	<i>LPP</i>	LIM Domain Containing Preferred Translocation Partner In Lipoma	1004.5	865.333	-0.2151497	1.61E-12	2.55E-08
	<i>RPS12</i>	Ribosomal Protein S12	6562.667	7872.167	0.2624786	1.32E-11	8.19E-08
	<i>ST8SIA2</i>	ST8 Alpha-N-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 2	59.667	126.667	1.0860398	1.90E-10	7.86E-07
	<i>PYGB</i>	Phosphorylase, Glycogen; Brain	152.667	73.833	-1.0480409	3.22E-10	2.07E-06
	<i>RPLP0P2</i>	Ribosomal Protein, Large, P0 Pseudogene 2	685.667	797	0.2170723	3.10E-09	9.84E-06
	<i>RPL37A</i>	Ribosomal Protein L37a	1932.667	2353	0.2839085	1.31E-07	0.0003253
	<i>SNRPD2</i>	Small Nuclear Ribonucleoprotein D2	437.167	514	0.233585	1.08E-07	0.000343
	<i>DOPEY2</i>	Dopey Family Member 2	297.333	234	-0.3455727	2.46E-07	0.00051
	<i>MCM3AP</i>	Minichromosome Maintenance Complex Component 3 Associated Protein	972.667	865.667	-0.1681339	4.83E-07	0.0007495
Od All	<i>NFIA</i>	Nuclear Factor I/A	219.4	288	0.3925053	5.19E-11	4.14E-07
	<i>HECTD4</i>	HECT Domain Containing E3 Ubiquitin Protein Ligase 4	264.4	444.4	0.7491366	1.01E-08	0.000051
	<i>AGO1</i>	Argonaute RISC Catalytic Component 1	80.8	117.4	0.5390052	6.13E-09	0.0000727
	<i>RPS12L1</i>	Ribosomal protein S12-like 1	105	64.4	-0.7052567	9.28E-09	0.000077
	<i>ZAP70</i>	Zeta-chain-associated protein 70	145	200.6	0.4682687	1.32E-08	0.000078
	<i>SCN4A</i>	Sodium Channel, Voltage Gated, Type IV Alpha Subunit	505	974.6	0.9485268	2.40E-08	0.0000947
	<i>SUFU</i>	Suppressor Of Fused Homolog	31	54.8	0.8219077	3.64E-08	0.0001078
	<i>NR2F2</i>	Nuclear Receptor Subfamily 2, Group F, Member 2	72.4	126.6	0.8062158	5.62E-08	0.0001111
	<i>CH242-511J12.1</i>	Ribosomal Protein L27A Pseudogene	236.2	159.8	-0.5637416	1.63E-07	0.0002719
	<i>MED14</i>	Mediator Complex Subunit 14	304.8	418.4	0.45701995	1.73E-07	0.0002933
Od RNAlater	<i>F2RL2</i>	Coagulation Factor II (Thrombin) Receptor-Like 2	277.667	167.333	-0.7306291	1.32E-19	7.51E-16
	<i>SKIL</i>	SKI-Like Proto-Oncogene	551.667	356.333	-0.6305694	1.57E-17	5.94E-14
	<i>COX5B</i>	Cytochrome C Oxidase Subunit Vb	3260	2815.333	-0.2115662	1.01E-14	2.86E-11
	<i>MYL6</i>	Myosin, Light Chain 6	442	306	-0.5305147	1.30E-14	5.07E-11
	<i>MAN2C1</i>	Mannosidase, Alpha, Class 2C, Member 1	170.333	264	0.6321771	3.85E-13	7.29E-10
	<i>CECR2</i>	Cat Eye Syndrome Chromosome Region, Candidate 2	58.333	123	1.0762659	1.26E-12	1.59E-09
	<i>KCNN3</i>	Potassium Channel, Calcium Activated Intermediate/Small Conductance Subfamily N Alpha, Member 3	64	179.667	1.489179	1.14E-12	1.59E-09
	<i>MEF2C</i>	Myocyte Enhancer Factor 2C	3313	2298.667	-0.5273409	2.00E-12	2.27E-09

Table 4 (cont.)

	<i>NR2F2</i>	Nuclear Receptor Subfamily 2, Group F, Member 2	64	122.333	0.9346738	3.48E-12	3.59E-09
	<i>RPS12L1</i>	Ribosomal protein S12-like 1	102.333	52.333	-0.96747	3.32E-12	3.76E-08
21d	<i>RAVER2</i>	Ribonucleoprotein, PTB-Binding 2	235.667	173	-0.4459757	3.55E-11	4.17E-07
	<i>FRMD8</i>	FERM Domain Containing 8	154	105.167	-0.5502528	1.95E-10	9.88E-07
	<i>STK26</i>	Serine/Threonine Protein Kinase 26	124.667	80.167	-0.6370014	2.52E-10	9.88E-07
	<i>ASB11</i>	Ankyrin Repeat And SOCS Box Containing 11, E3 Ubiquitin Protein Ligase	211	325.5	0.6254145	2.52E-09	0.0000074
	<i>TP53INP2</i>	Tumor Protein P53 Inducible Nuclear Protein 2	198.833	285.833	0.5236145	5.08E-08	0.0001081
	<i>SYTL5</i>	Synaptotagmin-Like 5	15.5	5.167	-1.5849625	2.84E-07	0.0004774
	<i>AGO4</i>	Argonaute RISC Catalytic Component 4	1085.833	1515.5	0.4809912	4.31E-07	0.0006333
	<i>ALS2</i>	Alsin Rho Guanine Nucleotide Exchange Factor	240	290.5	0.2755038	7.33E-07	0.0009542
	<i>ADCY2</i>	Adenylate Cyclase 2	1286.667	1630.667	0.3418236	1.25E-06	0.0016391
	<i>FAM180A</i>	Family With Sequence Similarity 180, Member A	24	10	-1.2630344	2.08E-06	0.0024419
176d	<i>GM13050</i>	Eukaryotic Translation Elongation Factor Pseudogene	1744	2184.833	0.3251232	2.97E-18	3.14E-14
	<i>EEF2</i>	Eukaryotic Translation Elongation Factor 2	24794	29823.833	0.2664747	4.05E-15	4.46E-11
	<i>EIF3L</i>	Eukaryotic Translation Initiation Factor 3, Subunit L	1653	1908	0.2069744	1.74E-10	6.39E-07
	<i>ADIPOR1</i>	Adiponectin Receptor 1	618.5	711.5	0.2020902	4.20E-10	1.16E-06
	<i>FLOT1</i>	Flotillin 1	818.667	1102	0.4287762	1.70E-09	3.12E-06
	<i>MAPKAPK3</i>	Mitogen-Activated Protein Kinase-Activated Protein Kinase 3	374.833	484	0.3687578	2.05E-09	3.23E-06
	<i>IL6ST</i>	Interleukin 6 Signal Transducer	1567	1759	0.1667503	6.85E-09	9.45E-06
	<i>RMDN1</i>	Regulator Of Microtubule Dynamics 1	235	184	-0.352955	8.53E-09	0.0000105
	<i>DYRK2</i>	Dual-Specificity Tyrosine-(Y)-Phosphorylation Regulated Kinase 2	296.833	227.5	-0.3837866	3.14E-08	0.0000346
	<i>MKRN1</i>	Makorin Ring Finger Protein 1	425.167	560.167	0.3978276	4.27E-08	0.0000428

Table 5. Top ten up- and down-regulated genes by log₂ fold change for DEGs with an FDR ≤ 0.10

Time Point	Expression with Respect to G ^{pat} Pigs	Gene Symbol	Full Gene Name	Counts A ^{pat}	Counts G ^{pat}	Log ₂ Fold Change	p-value	FDR
d90	Down	<i>APOL5</i>	Apolipoprotein L, 5	6.833	2.167	-1.657112286	0.000139	0.068822
		<i>NYX</i>	Nyctalopin	14	6.167	-1.182864057	4.11E-05	0.034284
		<i>PYGB</i>	Phosphorylase, Glycogen; Brain	152.667	73.833	-1.0480409	3.22E-10	2.07E-06
		<i>ZNF536</i>	Zinc Finger Protein 536	18.5	10.333	-0.840219556	0.000212	0.074054
		<i>STK10</i>	Serine/Threonine Kinase 10	39	24.5	-0.670692375	0.000307	0.087385
		<i>ZFP30</i>	Zinc Finger Protein 30	29.667	19.333	-0.617752436	0.000299	0.089412
		<i>VWF</i>	Von Willebrand Factor	207.333	138.333	-0.583803244	1.20E-06	0.00255
		<i>MXD1</i>	MAX Dimerization Protein 1	39.167	27.167	-0.527788792	0.000307	0.095625
		<i>RFX1</i>	Regulatory Factor X, 1	62.833	44.167	-0.508572164	0.000223	0.084394
		<i>SPTY2D1</i>	Suppressor Of Ty, Domain Containing 1	47.167	33.167	-0.508033622	0.000367	0.087385
d90	Up	<i>RNU1-120P</i>	RNA, U1 Small Nuclear 120, Pseudogene	2.5	7.667	1.61667136	0.000198	0.076465
		<i>SNORA66</i>	Small Nucleolar RNA, H/ACA Box 66	4.167	11.5	1.464668267	0.00023	0.082138
		<i>GM20721</i>	Predicted Gene, 20721	4.5	12.333	1.454565863	0.000351	0.098152
		<i>ACA64</i>	Small nucleolar RNA ACA64	13.333	34	1.350497247	7.54E-05	0.030948
		<i>SIVA1</i>	SIVA1 apoptosis-Inducing Factor	5.5	13.833	1.330645312	0.000115	0.068822
		<i>SNORA64</i>	Small Nucleolar RNA, H/ACA Box 64	5.167	12.833	1.31259023	0.000225	0.084394
		<i>RNU1-7P</i>	RNA, U1 Small Nuclear 7, Pseudogene	6	14.5	1.273018494	0.000281	0.092971
		<i>RP11-383G10.3</i>	Ribosomal Protein L17 (RPL17), Pseudogene	4.833	11.167	1.208108195	0.000162	0.071563
		<i>U1</i>	Small Nuclear Ribonucleoprotein Polypeptide C	12.667	28	1.144389909	0.00037	0.087385
		<i>ST8SIA2</i>	ST8 Alpha-N-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 2	59.667	126.667	1.086039831	1.90E-10	7.86E-07
Od All	Down	<i>CFAP44</i>	Cilia And Flagella Associated Protein 44	5.4	1	-2.432959407	0.00015	0.041845
		<i>HIST1H3B</i>	Histone Cluster 1, H3b	5.6	1.4	-2	0.000694	0.086049
		<i>SHCBP1</i>	SHC SH2-Domain Binding Protein 1	8.4	2.6	-1.691877705	0.000106	0.034595
		<i>NPNT</i>	Nephronectin	15.6	5	-1.641546029	0.000773	0.082595
		<i>BUB1B</i>	BUB1 Mitotic Checkpoint Serine/Threonine Kinase B	18.4	6	-1.61667136	0.000503	0.075542
		<i>MIR433</i>	microRNA 433	20.8	7	-1.571156701	1.85E-06	0.00223
		<i>B3GALT2</i>	UDP-Gal:BetaGlcNAc Beta 1,3-Galactosyltransferase, Polypeptide 2	11.6	4.2	-1.465663572	7.99E-05	0.021021
		<i>EGFL6</i>	EGF-Like-Domain, Multiple 6	34.6	12.8	-1.434628228	8.45E-05	0.021021
		<i>GM26163</i>	Predicted Gene, 6163, snoRNA	11.8	4.6	-1.359081093	7.10E-05	0.026752
		<i>SNORD112</i>	Small Nucleolar RNA, C/D Box 112	41.8	16.4	-1.349807127	0.000758	0.08168

Table 5 (cont.)

Od All	Up	<i>WASF1</i>	WAS Protein Family, Member 1	1	5	2.321928095	0.000338	0.063926
		<i>CD4</i>	T-Cell Surface Glycoprotein CD4	4.8	22.2	2.209453366	0.000145	0.028579
		<i>DBP</i>	D Site Of Albumin Promoter (Albumin D-Box) Binding Protein	3.4	9.2	1.436099115	0.000517	0.082757
		<i>FAM86A</i>	Eukaryotic Elongation Factor 2 Lysine Methyltransferase	3.2	8.4	1.392317423	0.00083	0.090658
		<i>RTN4RL2</i>	Reticulon 4 Receptor-Like 2	4.8	12.4	1.36923381	7.03E-05	0.022123
		<i>PAR6G</i>	Par-6 Family Cell Polarity Regulator Gamma	4.8	12.2	1.345774837	0.00061	0.081462
		<i>PLXNA2</i>	Plexin A2	130.8	320	1.290709364	4.64E-07	0.000541
		<i>DAGLA</i>	Diacylglycerol Lipase, Alpha	22.2	53.8	1.277046496	7.21E-05	0.020159
		<i>CH242-280I8.1</i>	Ferritin, Heavy Polypeptide 1 (FTH1) Pseudogene	6	14.4	1.263034406	3.20E-05	0.011163
		<i>PRDM1</i>	PR Domain Containing 1, With ZNF Domain	5	11.8	1.23878686	0.000717	0.086135
Od RNAlater	Down	<i>RN7SL16P</i>	RNA, 7SL, Cytoplasmic 16, Pseudogene	7	0.667	-3.392317423	0.000685	0.042025
		<i>9430073C21RIK</i>	Riken cDNA 9430073C21 gene	9.333	1	-3.222392421	0.000185	0.022335
		<i>RN7SL782P</i>	RNA, 7SL, Cytoplasmic 782, Pseudogene	8	1	-3	9.73E-05	0.010505
		<i>RNU2-14P</i>	RNA, U2 Small Nuclear 14, Pseudogene	7.333	1	-2.874469118	0.000407	0.028866
		<i>H2-Q7</i>	Histocompatibility 2, Q region locus 7	6.667	1	-2.736965594	0.00052	0.041786
		<i>MLLT11</i>	Myeloid/Lymphoid Or Mixed-Lineage Leukemia (Trithorax Homolog, Drosophila); Translocated To, 11	13.333	2.333	-2.514573173	0.001326	0.070626
		<i>ANLN</i>	Anillin, Actin Binding Protein	10	2	-2.321928095	0.000825	0.054256
		<i>SHCBP1</i>	SHC SH2-Domain Binding Protein 1	6.667	1.333	-2.321928095	0.001091	0.054865
		<i>EGFL6</i>	EGF-Like-Domain, Multiple 6	11	2.333	-2.237039197	0.000501	0.033235
		<i>GM1848</i>	Na K-ATPase beta-3 Subunit Pseudogene	10.333	2.333	-2.146841388	9.89E-05	0.014665
Od RNAlater	Up	<i>NEK3</i>	NIMA-Related Kinase 3	0	4.333	INF	0.000312	0.031461
		<i>WASF1</i>	WAS Protein Family, Member 1	0.333	5	3.906890596	0.000532	0.034673
		<i>RP11-277I20.3</i>	Processed Pseudogene	0.333	4.667	3.807354922	0.001793	0.076525
		<i>ZFP286</i>	Zinc Finger Protein 286	0.333	4.333	3.700439718	0.001527	0.076076
		<i>PROM2</i>	Prominin 2	0.667	6.333	3.247927513	0.00026	0.020731
		<i>HPS1</i>	Hermansky-Pudlak Syndrome 1	0.667	5.667	3.087462841	0.000719	0.050718
		<i>TMEM234</i>	Transmembrane Protein 234	0.667	5.667	3.087462841	0.000719	0.050718
		<i>LET-7G</i>	MicroRNA Let-7g	1	7.333	2.874469118	0.000196	0.016743

Table 5 (cont.)

		<i>HOXA10</i>	Homeobox A10	2.333	12.333	2.402098444	0.000996	0.059856
		<i>PARD6G</i>	Par-6 Family Cell Polarity Regulator Gamma	2.667	13.333	2.321928095	3.86E-06	0.00087
21d	Down	<i>SLC6A13</i>	Solute Carrier Family 6 (Neurotransmitter Transporter), Member 13	26.667	6.5	-2.036525876	0.000233	0.055921
		<i>CU633166.1</i>	Tumor necrosis factor receptor superfamily member 11B	22.833	7	-1.70571466	4.23E-05	0.022591
		<i>SYTL5</i>	Synaptotagmin-Like 5	15.5	5.167	-1.584962501	2.84E-07	0.000477
		<i>GSTM5P1</i>	Glutathione S-Transferase Mu 5 Pseudogene 1	15	5.167	-1.537656786	5.62E-06	0.008031
		<i>FBXL6</i>	F-Box And Leucine-Rich Repeat Protein 6	10	3.5	-1.514573173	1.47E-05	0.015422
		<i>ARG1</i>	Arginase 1	12.167	4.5	-1.434937057	0.000268	0.093757
		<i>CRABP1</i>	Cellular Retinoic Acid Binding Protein 1	9.833	3.833	-1.359081093	7.02E-05	0.041388
		<i>IGF2</i>	Insulin-like Growth Factor 2	693	272.167	-1.348364967	4.75E-06	0.00556
		<i>FAM180A</i>	Family With Sequence Similarity 180, Member A	24	10	-1.263034406	2.08E-06	0.002442
		<i>CHRNE</i>	Cholinergic Receptor, Nicotinic, Epsilon (Muscle)	17	8.5	-1	5.24E-05	0.01968
21d	Up	<i>AKR1C1</i>	Aldo-Keto Reductase Family 1, Member C1	5.667	13.167	1.216317907	0.000303	0.098514
		<i>VAMP1</i>	Vesicle-Associated Membrane Protein 1 (Synaptobrevin 1)	4.167	9.667	1.214124805	0.000292	0.097094
		<i>KLHL29</i>	Kelch-Like Family Member 29	6.167	13.333	1.112474729	0.000295	0.093374
		<i>ROS1</i>	ROS Proto-Oncogene 1 , Receptor Tyrosine Kinase	25.833	54.667	1.081427599	0.000252	0.093046
		<i>CHST9</i>	Carbohydrate (N-Acetylgalactosamine 4-0) Sulfotransferase 9	7.833	16.167	1.045323991	3.09E-05	0.025989
		<i>BST1</i>	Bone Marrow Stromal Cell Antigen 1	29	56.833	0.970684433	3.60E-05	0.020162
		<i>WFIKN2</i>	WAP, Follistatin/Kazal, Immunoglobulin, Kunitz And Netrin Domain Containing 2	26.667	52	0.963474124	5.22E-05	0.023626
		<i>GSTM3</i>	Glutathione S-Transferase Mu 3 (Brain)	12.667	23.667	0.901819606	0.00029	0.093374
		<i>MYH7</i>	Myosin, Heavy Chain 7	3823.833	7102.833	0.89337499	0.000152	0.042455
		<i>NFE2L3</i>	Nuclear Factor, Erythroid 2-Like 3	37.667	63.333	0.749676646	0.000347	0.067915
176d	Down	<i>HLA-K</i>	Major Histocompatibility Complex, Class I, K (Pseudogene)	6	1.5	-2	9.56E-05	0.025612
		<i>IGF2</i>	Insulin-like Growth Factor 2	106	33.5	-1.661831264	3.71E-05	0.007756
		<i>SBSPON</i>	Somatomedin B And Thrombospondin, Type 1 Domain Containing	18.333	6.167	-1.571906348	3.12E-07	0.000181
		<i>CCDC23</i>	Coiled-Coil Domain Containing 23	7.667	2.667	-1.523561956	0.001144	0.074091

Table 5 (cont.)

		<i>7SK</i>	RNA, 7SK Small Nuclear	12.167	4.667	-1.382469637	0.001439	0.076654
		<i>IL1RL1</i>	Interleukin 1 Receptor-Like 1	12.333	4.833	-1.351472371	0.001238	0.071459
		<i>LOXL3</i>	Lysyl Oxidase-Like 3	17.167	7	-1.294183104	7.64E-05	0.012039
		<i>MPC1</i>	Mitochondrial Pyruvate Carrier 1	8.167	3.333	-1.292781749	0.000481	0.067829
		<i>TRPT1</i>	TRNA Phosphotransferase 1	57.167	23.667	-1.272317647	0.000362	0.036948
		<i>NUSAP1</i>	Nucleolar And Spindle Associated Protein 1	9.833	4.167	-1.23878686	0.000208	0.025364
176d	Up	<i>SLC26A7</i>	Solute Carrier Family 26 (Anion Exchanger), Member 7	16.167	122.333	2.919723411	3.45E-07	0.00019
		<i>KCNN1</i>	Potassium Channel, Calcium Activated Intermediate/Small Conductance Subfamily N Alpha, Member 1	1.167	5.5	2.237039197	0.00015	0.040608
		<i>KLHL22</i>	Kelch-Like Family Member 22	8.667	17.667	1.027480736	1.74E-05	0.004546
		<i>EXD1</i>	Exonuclease 3'-5' Domain Containing 1	9.833	19.167	0.962847002	2.20E-05	0.00571
		<i>MYBPH</i>	Myosin Binding Protein H	6199.833	11363.5	0.874105921	0.001997	0.090982
		<i>ARID3A</i>	AT Rich Interactive Domain 3A (BRIGHT-Like)	10.167	18.333	0.850622376	0.001522	0.089507
		<i>THEM7</i>	Thioesterase Superfamily Member 7, Pseudogene	10.167	18.333	0.850622376	0.000229	0.049518
		<i>NXT2</i>	Nuclear Transport Factor 2-Like Export Factor 2	10.667	18.667	0.807354922	0.000297	0.045305
		<i>NR6A1</i>	Nuclear Receptor Subfamily 6, Group A, Member 1	16.333	27.833	0.768994448	2.25E-05	0.005388
		<i>WNT5B</i>	Wingless-Type MMTV Integration Site Family, Member 5B	10.833	18.333	0.7589919	0.001378	0.077746

Table 6. Top ten up- and down- regulated highly expressed genes (≥ 100 read counts for A^{pat} or G^{pat} pigs) by log₂ fold change for DEGs with an FDR ≤ 0.10

Time Point	Expression with Respect to G ^{pat} Pigs	Gene Symbol	Full Gene Name	Counts A ^{pat}	Counts G ^{pat}	Log ₂ Fold Change	p-value	FDR
d90	Down	<i>PYGB</i>	Phosphorylase, Glycogen; Brain	152.667	73.833	-1.0480409	3.22E-10	2.07E-06
		<i>VWF</i>	Von Willebrand Factor	207.333	138.333	-0.583803244	1.20E-06	0.00255
		<i>GSTP1</i>	Glutathione S-Transferase Pi 1	380.667	290.167	-0.391646448	5.34E-05	3.69E-02
		<i>IGF1R</i>	Insulin-Like Growth Factor 1 Receptor	427	329.667	-0.37322805	9.46E-05	0.050489
		<i>SLC23A2</i>	Solute Carrier Family 23 (Ascorbic Acid Transporter), Member 2	219.167	171.333	-0.355222535	0.000177	0.073432
		<i>PRKAG2</i>	Protein Kinase, AMP-Activated, Gamma 2 Non-Catalytic Subunit	234.5	183.5	-0.35380786	0.000164	0.075536
		<i>DOPEY2</i>	Dopey Family Member 2	297.333	234	-0.34557268	2.46E-07	0.00051
		<i>PHC1</i>	Polyhomeotic Homolog 1	129.833	105	-0.3062715	0.000215	0.074054
		<i>EXTL3</i>	Exostosin-Like Glycosyltransferase 3	187.667	152	-0.304101098	0.000112	0.068822
		<i>EXOC2</i>	Exocyst Complex Component 2	116.5	95	-0.294330536	0.000293	0.089412
d90	Up	<i>ST8SIA2</i>	ST8 Alpha-N-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 2	59.667	126.667	1.086039831	1.9E-10	7.86E-07
		<i>GM25704</i>	Predicted Gene, 25704	90.333	167.833	0.893698927	6.66E-05	0.04516
		<i>RPS11P5</i>	Ribosomal Protein S11 Pseudogene 5	170	233.333	0.456857675	3.65E-05	0.032147
		<i>GM21957</i>	Predicted Gene, 21957	134.167	182.833	0.446502837	3.48E-04	0.098152
		<i>G0S2</i>	G0/G1 Switch 2	148.5	200.667	0.434338055	1.58E-06	0.002175
		<i>HIST1H2AD</i>	Histone Cluster 1, H2ad	269	362.833	0.431700829	0.000172	0.053038
		<i>SCARNA10</i>	Small Cajal Body-Specific RNA 10	2053	2765	0.429545853	0.000188	0.074545
		<i>H3F3A</i>	H3 Histone, Family 3A	100.667	134.667	0.419806743	1.4E-06	0.00256
		<i>RPS26</i>	Ribosomal Protein S26	1447	1919.167	0.407415083	2.34E-06	0.002397
		<i>AIP</i>	Aryl Hydrocarbon Receptor Interacting Protein	84.5	111.833	0.404327019	1.18E-05	1.04E-02
Od All	Down	<i>SNORD113</i>	Small Nucleolar RNA, C/D Box 113-1	140.6	55.8	-1.333259567	6E-07	0.000646
		<i>SNORA43</i>	Small Nucleolar RNA, H/ACA Box 43	174.8	80.6	-1.116853441	0.000183	0.033552
		<i>SRGN</i>	Serglycin	117.4	59.8	-0.973215019	4.31E-06	0.00269
		<i>SCARNA1</i>	Small Cajal Body-Specific RNA 1	342	180.2	-0.924397314	0.000675	0.086049
		<i>SNORA71</i>	Small Nucleolar RNA, H/ACA Box 71C	119	63.2	-0.91296511	5.7E-06	0.003366
		<i>SNORA60</i>	Small Nucleolar RNA, H/ACA Box 60	101.2	55.2	-0.874469118	8.08E-06	0.005549
		<i>RNU1-77P</i>	RNA, U1 Small Nuclear 77, Pseudogene	261	144.6	-0.851982255	2.46E-04	0.053043
		<i>RN7SL444P</i>	RNA, 7SL, Cytoplasmic 444, Pseudogene	161.2	94	-0.778119082	2.44E-04	0.053043
		<i>RPS12L1</i>	Ribosomal Protein S12-like 1	105	64.4	-0.705256734	9.28E-09	0.000077

Table 6 (cont.)

		<i>RPS29</i>	Ribosomal Protein S29	138.2	87.2	-0.664357576	0.000503	0.08093
Od All	Up	<i>PLXNA2</i>	Plexin A2	130.8	320	1.290709364	4.64E-07	0.000541
		<i>KCNN3</i>	Potassium Channel, Calcium Activated Intermediate/Small Conductance Subfamily N Alpha, Member 3	67.4	143.6	1.091235253	0.001036	0.088225
		<i>TLE1</i>	Transducin-Like Enhancer Of Split 1	72.6	150.8	1.054594975	2.29E-05	0.011914
		<i>SCN4A</i>	Sodium Channel, Voltage Gated, Type IV Alpha Subunit	505	974.6	0.948526835	2.4E-08	9.47E-05
		<i>RNF213</i>	Ring Finger Protein 213	80.4	154.6	0.943272913	8.67E-05	0.021021
		<i>ATP2A1</i>	ATPase, Ca++ Transporting, cardiac muscle, Fast Twitch 1	11087.6	20839.2	0.910352778	0.000187	0.033552
		<i>PCNXL3</i>	Pecanex-Like 3	75.4	135.4	0.84459131	1.86E-05	0.007907
		<i>NR2F2</i>	Nuclear Receptor Subfamily 2, Group F, Member 2	72.4	126.6	0.806215802	5.62E-08	0.000111
		<i>SOGA1</i>	Suppressor Of Glucose, Autophagy Associated 1	70.6	123.2	0.803262167	3.75E-04	0.065984
		<i>ACACB</i>	Acetyl-CoA Carboxylase Beta	685.6	1190.2	0.795765008	2.19E-06	0.0015
Od RNAlater	Down	<i>POSTN</i>	Periostin	2094	662.667	-1.659906186	0.000349	0.020022
		<i>PCDH15</i>	Protocadherin-Related 15	103.333	34	-1.603699063	0.000214	0.013805
		<i>SNORA73</i>	Small Nucleolar RNA, H/ACA Box 73	2659	1055.333	-1.333185022	1.95E-03	0.064989
		<i>GM23153</i>	Predicted Gene, 23153	17854.667	7765.667	-1.201119513	1.48E-05	0.004234
		<i>ASPN</i>	Asporin	885.333	387.333	-1.192645078	0.000877	0.038917
		<i>SNORA71</i>	Small Nucleolar RNA, H/ACA Box 71C	107.667	48.667	-1.145565796	6.7E-06	0.001104
		<i>COQ10B</i>	Coenzyme Q10 Homolog B	481.333	218.667	-1.138303022	0.003205	0.092694
		<i>U1</i>	Small Nuclear Ribonucleoprotein Polypeptide C	3007.667	1370.667	-1.133766921	2.6E-09	1.85E-06
		<i>RN7SL444P</i>	RNA, 7SL, Cytoplasmic 444, Pseudogene	142.333	65	-1.130761946	1.78E-07	0.000113
		<i>Y RNA</i>	Y RNA	221.333	103.667	-1.094268661	1.02E-03	0.041931
Od RNAlater	Up	<i>KCNN3</i>	Potassium Channel, Calcium Activated Intermediate/Small Conductance Subfamily N Alpha, Member 3	64	179.667	1.489178962	1.14E-12	1.59E-09
		<i>PLXNA2</i>	Plexin A2	126.333	313	1.308927309	1.54E-05	0.001865
		<i>POLR2A</i>	Polymerase (RNA) II (DNA Directed) Polypeptide A	115	248.667	1.112579269	0.00061	0.0295
		<i>CECR2</i>	Cat Eye Syndrome Chromosome Region, Candidate 2	58.333	123	1.076265894	1.26E-12	1.59E-09
		<i>SCN4A</i>	Sodium Channel, Voltage Gated, Type IV Alpha Subunit	508	1027.667	1.016471987	2.95E-08	1.34E-05
		<i>TLE1</i>	Transducin-Like Enhancer Of Split 1	78.333	157.333	1.006126103	0.000026	0.003631
		<i>SUMF2</i>	Sulfatase Modifying Factor 2	52.667	105	0.99542727	3.35E-09	2.24E-06

Table 6 (cont.)

		<i>NFIX</i>	Nuclear Factor I/X (CCAAT-Binding Transcription Factor)	152.667	293.333	0.942155925	2.36E-04	0.019048
		<i>NR2F2</i>	Nuclear Receptor Subfamily 2, Group F, Member 2	64	122.333	0.934673752	3.48E-12	3.59E-09
		<i>UBE2O</i>	Ubiquitin-Conjugating Enzyme E2O	70.333	134.333	0.93353684	0.001155	0.057098
21d	Down	<i>IGF2</i>	Insulin-Like Growth Factor 2	693	272.167	-1.348364967	4.75E-06	0.00556
		<i>COL19A1</i>	Collagen, Type XIX, Alpha 1	111	56.5	-0.974236904	9.25E-05	0.030999
		<i>KV1.5</i>	Potassium Channel, Voltage Gated Shaker Related Subfamily A, Member 5	103.167	56.667	-0.864404663	3.33E-04	0.067915
		<i>SPON1</i>	Spondin 1, Extracellular Matrix Protein	354.333	197.5	-0.843254538	5.83E-04	0.097901
		<i>KCNE3</i>	Potassium Channel, Voltage Gated Subfamily E Regulatory Beta Subunit 3	76	45.667	-0.734857931	9.98E-06	0.007333
		<i>AK4</i>	Adenylate Kinase 4	122.667	74.333	-0.722662056	0.0001	0.031792
		<i>STK26</i>	Serine/Threonine Protein Kinase 26	112	68.167	-0.71636039	6.63E-14	8E-10
		<i>STAC</i>	SH3 And Cysteine Rich Domain	182.667	123.833	-0.560813682	1.61E-04	0.038847
		<i>FRMD8</i>	FERM Domain Containing 8	154	105.167	-0.550252846	1.95E-10	9.88E-07
		<i>GAB2</i>	GRB2-Associated Binding Protein 2	108.667	75	-0.534946963	4.82E-06	0.00472
21d	Up	<i>MYH7</i>	Myosin, Heavy Chain 7	3823.833	7102.833	0.89337499	0.000152	0.042455
		<i>ASB11</i>	Ankyrin Repeat And SOCS Box Containing 11, E3 Ubiquitin Protein Ligase	211	325.5	0.625414544	2.52E-09	7.4E-06
		<i>FBXO40</i>	F-Box Protein 40	2327.333	3514.833	0.594778425	4.44E-05	0.022724
		<i>PDGFC</i>	Platelet Derived Growth Factor C	303.667	446	0.554555157	0.000271	0.04963
		<i>CENPI</i>	Centromere Protein I	92.833	135.667	0.547351467	6.70E-05	0.024889
		<i>TP53INP2</i>	Tumor Protein P53 Inducible Nuclear Protein 2	198.833	285.833	0.523614533	5.08E-08	0.000108
		<i>FAM214A</i>	Family With Sequence Similarity 214, Member A	487	693.667	0.510320787	5.76E-05	0.024889
		<i>AGO4</i>	Argonaute RISC Catalytic Component 4	481.333	685.333	0.509769522	1.5E-08	0.000035
		<i>RP11-421N8.1</i>	Known Processed Pseudogene	78.333	111.167	0.505026005	0.000192	0.082078
		<i>TRIM63</i>	Tripartite Motif Containing 63, E3 Ubiquitin Protein Ligase	1289.167	1781.167	0.466383727	0.00012	0.036078
176d	Down	<i>IGF2</i>	Insulin-like Growth Factor 2	106	33.5	-1.661831264	3.71E-05	0.007756
		<i>GM5908</i>	Predicted Gene, 5908	188.167	109	-0.787682945	4.73E-04	0.067648
		<i>IGFBP6</i>	Insulin-like Growth Factor Binding Protein 6	212.167	127.167	-0.738477457	1.53E-03	0.079723
		<i>S100A10</i>	S100 Calcium Binding Protein A10	162.333	98.5	-0.720763642	0.000928	0.060651
		<i>RP11-475C16.1</i>	Known Processed Pseudogene	177	110.5	-0.679702991	4.65E-06	0.002168

Table 6 (cont.)

		<i>PGRMC1</i>	Progesterone Receptor Membrane Component 1	144.667	93.5	-0.629694272	0.002257	0.098037
		<i>MYH11</i>	Myosin, Heavy Chain 11	153.667	101.667	-0.595957508	4.01E-04	0.049115
		<i>PLN</i>	Phospholamban	1399.5	935.5	-0.581101928	4.28E-05	0.008203
		<i>PBX3</i>	Pre-B-Cell Leukemia Homeobox 3	181	123.333	-0.553426927	0.000117	0.016085
		<i>LSM5</i>	LSM5 Homolog, U6 Small Nuclear RNA Associated	165.667	114.333	-0.535037275	0.000215	0.024995
176d	Up	<i>SLC26A7</i>	Solute Carrier Family 26 (Anion Exchanger), Member 7	16.167	122.333	2.919723411	3.45E-07	0.00019
		<i>MYBPH</i>	Myosin Binding Protein H	6199.833	11363.5	0.874105921	0.001997	0.090982
		<i>SCMH1</i>	Sex Comb On Midleg Homolog 1	91.333	140	0.616213435	1.49E-04	0.019792
		<i>CD83</i>	CD83 Molecule	72.333	109.333	0.596000772	2.08E-03	0.092849
		<i>ASCC3</i>	Activating Signal Cointegrator 1 Complex Subunit 3	72	106.333	0.562525112	1.94E-07	0.000141
		<i>STARD10</i>	StAR-Related Lipid Transfer (START) Domain Containing 10	152	220.333	0.535616447	1.36E-07	0.000088
		<i>SQSTM1</i>	Sequestosome 1	7935	11454.5	0.529612357	0.001283	0.072929
		<i>MRPS9</i>	Mitochondrial Ribosomal Protein S9	484.167	694.5	0.520470936	0.001655	0.082404
		<i>FN3K</i>	Fructosamine 3 Kinase	257.333	366.667	0.510830771	2.08E-03	0.092849
		<i>RP11-54I5.1</i>	Known Processed Pseudogene	95.5	133.167	0.479660364	0.000221	0.039873

Table 7. Differential expression of *IGF2* at each time point resulting from alignment to Btau4.0, NCBIM37, and GRCh37

Time point	Genome Used	Log ₂ Fold Change	FDR
d90	Btau 4.0	-0.153322372	0.940031
	NCBIM37	-0.132757556	0.968236
	GRCh37	-0.048943934	0.982465
0d All	Btau 4.0	0.189293988	0.999377
	NCBIM37	0.026875706	1
	GRCh37	0.209468674	1
0d RNAlater	Btau 4.0	0.626550539	1
	NCBIM37	0.658531719	0.995091
	GRCh37	0.44735522	1
21d	Btau 4.0	-1.348364967	0.00556
	NCBIM37	-1.398066304	0.001778
	GRCh37	-1.454378391	0.004247
176d	Btau 4.0	-1.661831264	0.007756
	NCBIM37	-1.467358534	0.030943
	GRCh37	-0.797811149	0.311453

Table 8. Enriched canonical pathways yielded by IPA analysis

Time Point	FDR	Ingenuity Canonical Pathways	Molecules	z-score	-log(p-value)	Adjusted p-value
d90	0.05	EIF2 Signaling	7	2	6.7	2.00E-07
		Estrogen Receptor Signaling	3	--	2.51	0.00309
		Aryl Hydrocarbon Receptor Signaling	3	--	2.39	0.00407
		Regulation of eIF4 and p70S6K Signaling	3	--	2.34	0.00457
		mTOR Signaling	3	--	2.04	0.00912
		Glycogen Degradation II	1	--	1.64	0.02291
		Glucocorticoid Receptor Signaling	3	--	1.6	0.02512
		Glycogen Degradation III	1	--	1.57	0.02692
		Assembly of RNA Polymerase III Complex	1	--	1.53	0.02951
		Hereditary Breast Cancer Signaling	2	--	1.45	0.03548
		Granzyme A Signaling	1	--	1.35	0.04467
d90	0.1	EIF2 Signaling	12	2.646	9.82	1.51E-10
		mTOR Signaling	6	--	3.43	0.00037
		Aryl Hydrocarbon Receptor Signaling	5	--	3.15	0.00071
		Regulation of eIF4 and p70S6K Signaling	5	--	3.07	0.00085
		Estrogen Receptor Signaling	4	--	2.41	0.00389
		Glutamine Biosynthesis I	1	--	2.3	0.00501
		Cell Cycle Control of Chromosomal Replication	2	--	2.09	0.00813
		Formaldehyde Oxidation II (Glutathione-dependent)	1	--	2	0.01000
		Glucocorticoid Receptor Signaling	5	--	1.9	0.01259
		IGF-1 Signaling	3	--	1.89	0.01288
		Nucleotide Excision Repair Pathway	2	--	1.87	0.01349
		tRNA Charging	2	--	1.79	0.01622
		Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	2	--	1.65	0.02239
		Hereditary Breast Cancer Signaling	3	--	1.56	0.02754
		Regulation of Cellular Mechanics by Calpain Protease	2	--	1.48	0.03311
		Glioblastoma Multiforme Signaling	3	--	1.43	0.03715

Table 8 (cont.)

		Remodeling of Epithelial Adherens Junctions	2	--	1.34	0.04571
		Glycogen Degradation II	1	--	1.31	0.04898
OdAll	0.05	Heme Biosynthesis from Uroporphyrinogen-III I	1	--	1.7	0.01995
		Biotin-carboxyl Carrier Protein Assembly	1	--	1.7	0.01995
		Glycine Cleavage Complex	1	--	1.53	0.02951
		Calcium-induced T Lymphocyte Apoptosis	2	--	1.39	0.04074
		Calcium Transport I	1	--	1.36	0.04365
		Heme Biosynthesis II	1	--	1.36	0.04365
OdAll	0.05	EIF2 Signaling	9	-1.633	3.55	0.00028
		UVA-Induced MAPK Signaling	5	1.342	2.47	0.00339
		Calcium Transport I	2	--	2.35	0.00447
		Calcium-induced T Lymphocyte Apoptosis	4	2	2.21	0.00617
		Oxidative Phosphorylation	5	--	2.08	0.00832
		CDP-diacylglycerol Biosynthesis I	2	--	1.85	0.01413
		Estrogen Receptor Signaling	5	--	1.82	0.01514
		TR/RXR Activation	4	--	1.79	0.01622
		Phosphatidylglycerol Biosynthesis II (Non-plastidic)	2	--	1.76	0.01738
		CNTF Signaling	3	--	1.67	0.02138
		N-acetylglucosamine Degradation I	2	--	1.47	0.03388
		Retinoic acid Mediated Apoptosis Signaling	3	--	1.44	0.03631
		Actin Cytoskeleton Signaling	6	--	1.41	0.03890
		Heme Biosynthesis from Uroporphyrinogen-III I	1	--	1.35	0.04467
		Myo-inositol Biosynthesis	1	--	1.35	0.04467
		Biotin-carboxyl Carrier Protein Assembly	1	--	1.35	0.04467
		N-acetylglucosamine Degradation II	1	--	1.35	0.04467
		Mitochondrial Dysfunction	5	--	1.33	0.04677
OdRNAlater	0.05	Oxidative Phosphorylation	17	--	10.9	1.26E-11
		Mitochondrial Dysfunction	18	--	8.62	2.40E-09
		Actin Cytoskeleton Signaling	15	--	4.96	0.00001
		EIF2 Signaling	13	-2.714	4.45	0.00004

Table 8 (cont.)

	Breast Cancer Regulation by Stathmin1	13	--	4.3	0.00005
	Phospholipase C Signaling	14	-0.577	3.88	0.00013
	Regulation of Actin-based Motility by Rho	8	-1.414	3.6	0.00025
	RhoGDI Signaling	11	--	3.47	0.00034
	Actin Nucleation by ARP-WASP Complex	6	-0.816	3.28	0.00052
	Thrombin Signaling	11	-0.905	3.11	0.00078
	Signaling by Rho Family GTPases	12	-0.577	2.9	0.00126
	Gα12/13 Signaling	8	-2.121	2.87	0.00135
	RhoA Signaling	8	-1.134	2.75	0.00178
	Protein Kinase A Signaling	16	--	2.7	0.00200
	Estrogen Receptor Signaling	8	--	2.64	0.00229
	Cellular Effects of Sildenafil (Viagra)	8	--	2.6	0.00251
	Molecular Mechanisms of Cancer	15	--	2.53	0.00295
	Integrin Signaling	10	-1	2.41	0.00389
	GM-CSF Signaling	5	1	2.27	0.00537
	PAK Signaling	6	-0.816	2.24	0.00575
	phagosome maturation	7	--	2.19	0.00646
	Polyamine Regulation in Colon Cancer	3	--	2.15	0.00708
	ILK Signaling	9	-1.414	2.15	0.00708
	Gap Junction Signaling	8	--	2.12	0.00759
	Glioma Signaling	6	0.816	2.11	0.00776
	Citrulline Biosynthesis	2	--	2.07	0.00851
	p53 Signaling	6	1.633	2.05	0.00891
	Germ Cell-Sertoli Cell Junction Signaling	8	--	2.04	0.00912
	CDK5 Signaling	6	1.342	2.02	0.00955
	UDP-N-acetyl-D-galactosamine Biosynthesis II	2	--	1.96	0.01096
	Tight Junction Signaling	8	--	1.94	0.01148
	HER-2 Signaling in Breast Cancer	5	--	1.9	0.01259
	Assembly of RNA Polymerase II Complex	4	--	1.89	0.01288
	Axonal Guidance Signaling	15	--	1.87	0.01349

Table 8 (cont.)

	Superpathway of Cholesterol Biosynthesis	3	--	1.86	0.01380
	Regulation of eIF4 and p70S6K Signaling	7	-1	1.76	0.01738
	Glioblastoma Multiforme Signaling	7	-0.378	1.76	0.01738
	Epithelial Adherens Junction Signaling	7	--	1.76	0.01738
	Cardiac Hypertrophy Signaling	9	-1	1.67	0.02138
	CXCR4 Signaling	7	-1.89	1.67	0.02138
	Synaptic Long Term Potentiation	6	--	1.66	0.02188
	Mevalonate Pathway I	2	--	1.65	0.02239
	Huntington's Disease Signaling	9	--	1.61	0.02455
	Superpathway of Citrulline Metabolism	2	--	1.59	0.02570
	Antiproliferative Role of Somatostatin Receptor 2	4	--	1.56	0.02754
	PCP pathway	4	--	1.56	0.02754
	Glucocorticoid Receptor Signaling	10	--	1.53	0.02951
	PI3K Signaling in B Lymphocytes	6	--	1.53	0.02951
	Granzyme B Signaling	2	--	1.48	0.03311
	Docosahexaenoic Acid (DHA) Signaling	3	--	1.47	0.03388
	Cardiac β -adrenergic Signaling	6	2.236	1.46	0.03467
	Remodeling of Epithelial Adherens Junctions	4	--	1.45	0.03548
	Macropinocytosis Signaling	4	--	1.45	0.03548
	Glutamine Degradation I	1	--	1.44	0.03631
	Relaxin Signaling	6	1	1.43	0.03715
	Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	2	--	1.43	0.03715
	Renal Cell Carcinoma Signaling	4	--	1.39	0.04074
	Chemokine Signaling	4	1	1.39	0.04074
	Melanoma Signaling	3	--	1.39	0.04074
	Rac Signaling	5	-0.447	1.38	0.04169
	STAT3 Pathway	4	--	1.36	0.04365
	Sertoli Cell-Sertoli Cell Junction Signaling	7	--	1.35	0.04467
	Calcium Signaling	7	1	1.35	0.04467
	Role of NFAT in Cardiac Hypertrophy	7	1.134	1.34	0.04571

Table 8 (cont.)

		Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	10	--	1.33	0.04677
		cAMP-mediated signaling	8	1.633	1.32	0.04786
OdRNAlater	0.1	Oxidative Phosphorylation	25	--	14.3	5.01E-15
		Mitochondrial Dysfunction	26	--	10.4	3.98E-11
		EIF2 Signaling	22	-2.5	6.93	1.17E-07
		Actin Cytoskeleton Signaling	23	--	6.3	5.01E-07
		Signaling by Rho Family GTPases	22	0.218	5.19	6.46E-06
		Thrombin Signaling	18	0	4.37	4.27E-05
		Breast Cancer Regulation by Stathmin1	18	--	4.37	4.27E-05
		Integrin Signaling	18	0	4.08	8.32E-05
		RhoA Signaling	13	-0.302	3.85	0.00014
		Regulation of eIF4 and p70S6K Signaling	14	0.378	3.61	0.00025
		Cellular Effects of Sildenafil (Viagra)	13	--	3.61	0.00025
		Axonal Guidance Signaling	28	--	3.46	0.00035
		Gα12/13 Signaling	12	-1.155	3.44	0.00036
		Huntington's Disease Signaling	18	--	3.38	0.00042
		PAK Signaling	10	0	3.27	0.00054
		Germ Cell-Sertoli Cell Junction Signaling	14	--	3.21	0.00062
		Estrogen Receptor Signaling	12	--	3.12	0.00076
		PI3K Signaling in B Lymphocytes	12	--	3.09	0.00081
		Assembly of RNA Polymerase II Complex	7	--	3.01	0.00098
		Gap Junction Signaling	13	--	2.85	0.00141
		Phospholipase C Signaling	17	-0.775	2.75	0.00178
		Rac Signaling	10	0	2.75	0.00178
		Chemokine Signaling	8	0.707	2.73	0.00186
		Dopamine-DARPP32 Feedback in cAMP Signaling	13	1.508	2.7	0.00200
		Cardiac Hypertrophy Signaling	16	-0.5	2.66	0.00219
		UDP-N-acetyl-D-galactosamine Biosynthesis II	3	--	2.63	0.00234
		Regulation of Actin-based Motility by Rho	9	-1.667	2.61	0.00245
		Sphingosine-1-phosphate Signaling	10	1.265	2.59	0.00257

Table 8 (cont.)

	ILK Signaling	14	0.277	2.58	0.00263
	Protein Kinase A Signaling	23	--	2.49	0.00324
	Glioma Signaling	9	1	2.48	0.00331
	CXCR4 Signaling	12	-1.155	2.45	0.00355
	RhoGDI Signaling	13	--	2.43	0.00372
	B Cell Receptor Signaling	13	--	2.37	0.00427
	p70S6K Signaling	10	0.632	2.32	0.00479
	phagosome maturation	10	--	2.29	0.00513
	PI3K/AKT Signaling	10	-0.632	2.22	0.00603
	FAK Signaling	8	--	2.18	0.00661
	Molecular Mechanisms of Cancer	21	--	2.15	0.00708
	RANK Signaling in Osteoclasts	8	--	2.15	0.00708
	Lymphotoxin β Receptor Signaling	6	2.236	2.14	0.00724
	Tight Junction Signaling	12	--	2.13	0.00741
	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	18	--	2.13	0.00741
	Docosahexaenoic Acid (DHA) Signaling	5	--	2.12	0.00759
	Actin Nucleation by ARP-WASP Complex	6	-0.816	2.06	0.00871
	Relaxin Signaling	10	1.633	1.95	0.01122
	Role of NFAT in Cardiac Hypertrophy	12	1.155	1.91	0.01230
	p53 Signaling	8	1.414	1.88	0.01318
	CD28 Signaling in T Helper Cells	9	1	1.88	0.01318
	PTEN Signaling	9	-0.707	1.88	0.01318
	CDK5 Signaling	8	1.134	1.86	0.01380
	Ceramide Signaling	7	-0.816	1.86	0.01380
	GM-CSF Signaling	6	1.342	1.86	0.01380
	Aryl Hydrocarbon Receptor Signaling	10	--	1.84	0.01445
	Glucocorticoid Receptor Signaling	16	--	1.81	0.01549
	Prostate Cancer Signaling	7	--	1.8	0.01585
	mTOR Signaling	12	0.707	1.75	0.01778
	Ephrin A Signaling	5	--	1.75	0.01778

Table 8 (cont.)

	Glioblastoma Multiforme Signaling	10	0.632	1.73	0.01862
	NGF Signaling	8	1.89	1.67	0.02138
	Remodeling of Epithelial Adherens Junctions	6	--	1.67	0.02138
	Macropinocytosis Signaling	6	--	1.67	0.02138
	CREB Signaling in Neurons	11	1	1.67	0.02138
	fMLP Signaling in Neutrophils	8	0	1.65	0.02239
	iCOS-iCOSL Signaling in T Helper Cells	8	2.121	1.65	0.02239
	Interferon Signaling	4	--	1.65	0.02239
	Melatonin Signaling	6	-1.633	1.62	0.02399
	CNTF Signaling	5	0.447	1.61	0.02455
	Endometrial Cancer Signaling	5	--	1.61	0.02455
	Citrulline Biosynthesis	2	--	1.6	0.02512
	Renal Cell Carcinoma Signaling	6	--	1.59	0.02570
	Androgen Signaling	8	--	1.59	0.02570
	Cardiac β -adrenergic Signaling	9	2.121	1.57	0.02692
	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	11	0.905	1.53	0.02951
	Calcium Transport I	2	--	1.5	0.03162
	Polyamine Regulation in Colon Cancer	3	--	1.5	0.03162
	14-3-3-mediated Signaling	8	--	1.47	0.03388
	CCR3 Signaling in Eosinophils	8	--	1.47	0.03388
	IL-8 Signaling	11	-0.905	1.47	0.03388
	HER-2 Signaling in Breast Cancer	6	--	1.47	0.03388
	Glioma Invasiveness Signaling	5	0.447	1.46	0.03467
	PKC θ Signaling in T Lymphocytes	8	--	1.45	0.03548
	Clathrin-mediated Endocytosis Signaling	11	--	1.45	0.03548
	T Cell Receptor Signaling	7	--	1.45	0.03548
	Synaptic Long Term Potentiation	8	--	1.43	0.03715
	ERK/MAPK Signaling	11	0.905	1.42	0.03802
	Telomerase Signaling	7	1.342	1.41	0.03890
	Nitric Oxide Signaling in the Cardiovascular System	7	-0.378	1.39	0.04074

Table 8 (cont.)

		Induction of Apoptosis by HIV1	5	1.342	1.38	0.04169
		ErbB4 Signaling	5	1	1.38	0.04169
		Paxillin Signaling	7	1.342	1.37	0.04266
		Melanoma Signaling	4	--	1.36	0.04365
		Epithelial Adherens Junction Signaling	9	--	1.35	0.04467
21d	0.05	Hepatic Fibrosis / Hepatic Stellate Cell Activation	4	--	2.93	0.00117
		Autophagy	2	--	2.43	0.00372
		eNOS Signaling	3	--	2.25	0.00562
		Regulation of eIF4 and p70S6K Signaling	3	--	2.22	0.00603
		AMPK Signaling	3	--	1.98	0.01047
		EIF2 Signaling	3	--	1.94	0.01148
		Serine Biosynthesis	1	--	1.9	0.01259
		Superpathway of Serine and Glycine Biosynthesis I	1	--	1.75	0.01778
		VEGF Signaling	2	--	1.64	0.02291
		Glioma Signaling	2	--	1.62	0.02399
		Amyotrophic Lateral Sclerosis Signaling	2	--	1.59	0.02570
		Sphingosine-1-phosphate Signaling	2	--	1.51	0.03090
		DNA Double-Strand Break Repair by Non-Homologous End Joining	1	--	1.46	0.03467
		Cellular Effects of Sildenafil (Viagra)	2	--	1.37	0.04266
		Cardiomyocyte Differentiation via BMP Receptors	1	--	1.31	0.04898
21d	0.1	Arginine Degradation I (Arginase Pathway)	2	--	3.93	0.00012
		Hepatic Fibrosis / Hepatic Stellate Cell Activation	5	--	2.85	0.00141
		Pyrimidine Ribonucleotides Interconversion	2	--	2.13	0.00741
		PAK Signaling	3	--	2.12	0.00759
		VEGF Signaling	3	--	2.09	0.00813
		Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	3	--	2.07	0.00851
		Salvage Pathways of Pyrimidine Ribonucleotides	3	--	2.07	0.00851
		Pyrimidine Ribonucleotides De Novo Biosynthesis	2	--	2.07	0.00851
		Proline Degradation	1	--	2.05	0.00891
		4-hydroxyproline Degradation I	1	--	2.05	0.00891

Table 8 (cont.)

		Autophagy	2	--	1.94	0.01148
		Sphingosine-1-phosphate Signaling	3	--	1.89	0.01288
		Fatty Acid β -oxidation III (Unsaturated, Odd Number)	1	--	1.87	0.01349
		Serine Biosynthesis	1	--	1.65	0.02239
		Insulin Receptor Signaling	3	--	1.65	0.02239
		eNOS Signaling	3	--	1.58	0.02630
		Urea Cycle	1	--	1.57	0.02692
		Arginine Degradation VI (Arginase 2 Pathway)	1	--	1.57	0.02692
		Regulation of eIF4 and p70S6K Signaling	3	--	1.55	0.02818
		Protein Kinase A Signaling	5	--	1.52	0.03020
		Superpathway of Serine and Glycine Biosynthesis I	1	--	1.51	0.03090
		Pyridoxal 5'-phosphate Salvage Pathway	2	--	1.47	0.03388
		Hypoxia Signaling in the Cardiovascular System	2	--	1.46	0.03467
		Citrulline Biosynthesis	1	--	1.45	0.03548
		GABA Receptor Signaling	2	--	1.44	0.03631
		Growth Hormone Signaling	2	--	1.42	0.03802
		Chemokine Signaling	2	--	1.39	0.04074
		RhoGDI Signaling	3	--	1.37	0.04266
		B Cell Receptor Signaling	3	--	1.35	0.04467
		AMPK Signaling	3	--	1.33	0.04677
		IL-8 Signaling	3	--	1.3	0.05012
176d	0.05	Amyloid Processing	6	--	4.99	1.02E-05
		ILK Signaling	10	0.333	4.81	1.55E-05
		Integrin Signaling	10	1	4.52	0.00003
		PTEN Signaling	7	-1.89	3.78	0.00017
		Regulation of Cellular Mechanics by Calpain Protease	5	2	3.62	0.00024
		Mouse Embryonic Stem Cell Pluripotency	6	0.816	3.46	0.00035
		IGF-1 Signaling	6	2.236	3.41	0.00039
		IL-17 Signaling	5	--	3.15	0.00071
		Role of --OG in Mammalian Embryonic Stem Cell Pluripotency	6	1.342	3.1	0.00079

Table 8 (cont.)

	Actin Cytoskeleton Signaling	8	--	2.86	0.00138
	IL-22 Signaling	3	--	2.79	0.00162
	FAK Signaling	5	--	2.78	0.00166
	CNTF Signaling	4	2	2.78	0.00166
	Endometrial Cancer Signaling	4	--	2.78	0.00166
	IL-2 Signaling	4	--	2.74	0.00182
	Role of JAK family kinases in IL-6-type Cytokine Signaling	3	--	2.74	0.00182
	EGF Signaling	4	2	2.66	0.00219
	IL-17A Signaling in Airway Cells	4	--	2.44	0.00363
	IL-15 Signaling	4	--	2.4	0.00398
	Oncostatin M Signaling	3	--	2.35	0.00447
	IL-6 Signaling	5	1.342	2.24	0.00575
	Type II Diabetes Mellitus Signaling	5	--	2.23	0.00589
	PDGF Signaling	4	2	2.16	0.00692
	PI3K/AKT Signaling	5	1.342	2.14	0.00724
	Melanoma Signaling	3	--	2.1	0.00794
	Regulation of the Epithelial-Mesenchymal Transition Pathway	6	--	2.01	0.00977
	FGF Signaling	4	2	2.01	0.00977
	G Beta Gamma Signaling	4	--	1.96	0.01096
	RAR Activation	6	--	1.95	0.01122
	VEGF Signaling	4	1	1.89	0.01288
	Epithelial Adherens Junction Signaling	5	--	1.84	0.01445
	UVB-Induced MAPK Signaling	3	--	1.82	0.01514
	Nitric Oxide Signaling in the Cardiovascular System	4	1	1.77	0.01698
	ErbB2-ErbB3 Signaling	3	--	1.73	0.01862
	Germ Cell-Sertoli Cell Junction Signaling	5	--	1.68	0.02089
	Tight Junction Signaling	5	--	1.61	0.02455
	Pyridoxal 5'-phosphate Salvage Pathway	3	--	1.6	0.02512
	Wnt/ β -catenin Signaling	5	0	1.59	0.02570
	Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	3	--	1.57	0.02692

Table 8 (cont.)

		IL-15 Production	2	--	1.54	0.02884
		Diphthamide Biosynthesis	1	--	1.54	0.02884
		Coenzyme A Biosynthesis	1	--	1.54	0.02884
		p70S6K Signaling	4	2	1.53	0.02951
		Agrin Interactions at Neuromuscular Junction	3	--	1.52	0.03020
		Sertoli Cell-Sertoli Cell Junction Signaling	5	--	1.51	0.03090
		AMPK Signaling	5	0	1.5	0.03162
		Caveolar-mediated Endocytosis Signaling	3	--	1.49	0.03236
		IL-3 Signaling	3	--	1.49	0.03236
		JAK/Stat Signaling	3	--	1.47	0.03388
		EIF2 Signaling	5	0	1.45	0.03548
		Estrogen Receptor Signaling	4	--	1.44	0.03631
		Role of BRCA1 in DNA Damage Response	3	--	1.38	0.04169
		MIF-mediated Glucocorticoid Regulation	2	--	1.38	0.04169
		Acute Myeloid Leukemia Signaling	3	--	1.37	0.04266
		Insulin Receptor Signaling	4	--	1.37	0.04266
		Inhibition of Angiogenesis by TSP1	2	--	1.36	0.04365
		IL-17A Signaling in Fibroblasts	2	--	1.34	0.04571
		Prostate Cancer Signaling	3	--	1.33	0.04677
		Eumelanin Biosynthesis	1	--	1.32	0.04786
		Lysine Degradation II	1	--	1.32	0.04786
		Lysine Degradation V	1	--	1.32	0.04786
		Autophagy	2	--	1.32	0.04786
		Melanocyte Development and Pigmentation Signaling	3	--	1.3	0.05012
176d	0.1	Integrin Signaling	15	0.832	4.76	1.74E-05
		Amyloid Processing	7	--	4.12	7.59E-05
		ILK Signaling	13	1.155	3.91	0.00012
		EGF Signaling	7	1.134	3.86	0.00014
		Regulation of Cellular Mechanics by Calpain Protease	7	1.342	3.81	0.00015
		FAK Signaling	8	--	3.39	0.00041

Table 8 (cont.)

		Role of --OG in Mammalian Embryonic Stem Cell Pluripotency	9	1.633	3.34	0.00046
		Actin Cytoskeleton Signaling	13	--	3.26	0.00055
		IL-6 Signaling	9	1.667	3.2	0.00063
		CNTF Signaling	6	2.449	3.19	0.00065
		IL-17 Signaling	7	--	3.18	0.00066
		PTEN Signaling	9	-2.333	3.15	0.00071
		Mouse Embryonic Stem Cell Pluripotency	8	0.707	3.14	0.00072
		Autophagy	5	--	3.11	0.00078
		IGF-1 Signaling	8	2.449	3.08	0.00083
		PDGF Signaling	7	1.134	3	0.00100
		NRF2-mediated Oxidative Stress Response	11	0.447	2.91	0.00123
		Role of JAK family kinases in IL-6-type Cytokine Signaling	4	--	2.81	0.00155
		Melanocyte Development and Pigmentation Signaling	7	1.134	2.78	0.00166
		G Beta Gamma Signaling	7	--	2.66	0.00219
		Glioblastoma Multiforme Signaling	9	-0.333	2.5	0.00316
		Caveolar-mediated Endocytosis Signaling	6	--	2.48	0.00331
		Colorectal Cancer Metastasis Signaling	12	1.508	2.46	0.00347
		Endometrial Cancer Signaling	5	--	2.39	0.00407
		Nitric Oxide Signaling in the Cardiovascular System	7	0.378	2.35	0.00447
		IL-2 Signaling	5	2	2.35	0.00447
		Estrogen Receptor Signaling	8	--	2.34	0.00457
		Gap Junction Signaling	9	--	2.33	0.00468
		Regulation of the Epithelial-Mesenchymal Transition Pathway	10	--	2.33	0.00468
		Paxillin Signaling	7	0.816	2.33	0.00468
		Cellular Effects of Sildenafil (Viagra)	8	--	2.3	0.00501
		Reelin Signaling in Neurons	6	--	2.26	0.00550
		Germ Cell-Sertoli Cell Junction Signaling	9	--	2.24	0.00575
		RAR Activation	10	--	2.23	0.00589
		TR/RXR Activation	6	--	2.1	0.00794
		FGF Signaling	6	1.633	2.1	0.00794

Table 8 (cont.)

	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	13	--	2.06	0.00871
	Antiproliferative Role of Somatostatin Receptor 2	5	0	2.03	0.00933
	RANK Signaling in Osteoclasts	6	--	2.03	0.00933
	IL-17A Signaling in Airway Cells	5	--	2.01	0.00977
	Type II Diabetes Mellitus Signaling	7	--	1.99	0.01023
	Epithelial Adherens Junction Signaling	8	--	1.98	0.01047
	Melanoma Signaling	4	--	1.98	0.01047
	Hypoxia Signaling in the Cardiovascular System	5	--	1.98	0.01047
	p70S6K Signaling	6	1.134	1.95	0.01122
	IL-15 Signaling	5	--	1.95	0.01122
	VEGF Signaling	6	0.816	1.94	0.01148
	AMPK Signaling	9	0.378	1.94	0.01148
	Role of NFAT in Cardiac Hypertrophy	9	0.333	1.94	0.01148
	IL-22 Signaling	3	--	1.9	0.01259
	CXCR4 Signaling	8	0	1.89	0.01288
	UDP-N-acetyl-D-galactosamine Biosynthesis II	2	--	1.87	0.01349
	Clathrin-mediated Endocytosis Signaling	9	--	1.85	0.01413
	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	10	--	1.83	0.01479
	Antiproliferative Role of TOB in T Cell Signaling	3	--	1.81	0.01549
	JAK/Stat Signaling	5	1.342	1.8	0.01585
	Cardiac Hypertrophy Signaling	10	1.897	1.78	0.01660
	STAT3 Pathway	5	--	1.78	0.01660
	GNRH Signaling	7	1.134	1.77	0.01698
	Thrombin Signaling	9	0	1.77	0.01698
	Human Embryonic Stem Cell Pluripotency	7	--	1.69	0.02042
	UVB-Induced MAPK Signaling	4	--	1.64	0.02291
	Acute Phase Response Signaling	8	2.828	1.64	0.02291
	Wnt/ β -catenin Signaling	8	1.134	1.64	0.02291
	Cleavage and Polyadenylation of Pre-mRNA	2	--	1.62	0.02399
	Lymphotoxin β Receptor Signaling	4	2	1.61	0.02455

Table 8 (cont.)

	Sphingosine-1-phosphate Signaling	6	0.816	1.61	0.02455
	Prostate Cancer Signaling	5	--	1.58	0.02630
	eNOS Signaling	7	1.633	1.57	0.02692
	ErbB2-ErbB3 Signaling	4	--	1.54	0.02884
	Sertoli Cell-Sertoli Cell Junction Signaling	8	--	1.52	0.03020
	PPAR α /RXR α Activation	8	-1.134	1.51	0.03090
	G α q Signaling	7	0.378	1.5	0.03162
	Oncostatin M Signaling	3	--	1.5	0.03162
	Inhibition of Angiogenesis by TSP1	3	--	1.5	0.03162
	p38 MAPK Signaling	6	0	1.48	0.03311
	P2Y Purigenic Receptor Signaling Pathway	6	1.633	1.45	0.03548
	Apoptosis Signaling	5	0	1.45	0.03548
	Protein Ubiquitination Pathway	10	--	1.43	0.03715
	IL-1 Signaling	5	2	1.41	0.03890
	mTOR Signaling	8	2.646	1.4	0.03981
	ERK5 Signaling	3	1	1.4	0.03981
	Estrogen-Dependent Breast Cancer Signaling	4	1	1.4	0.03981
	PI3K/AKT Signaling	6	1.633	1.39	0.04074
	Notch Signaling	3	--	1.38	0.04169
	PPAR Signaling	5	0.447	1.36	0.04365
	CD40 Signaling	4	2	1.36	0.04365
	Non-Small Cell Lung Cancer Signaling	4	1	1.36	0.04365
	Docosahexaenoic Acid (DHA) Signaling	3	--	1.35	0.04467
	Glioma Signaling	5	0.447	1.34	0.04571
	Angiopoietin Signaling	4	--	1.34	0.04571
	Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	4	--	1.34	0.04571
	Adipogenesis pathway	6	--	1.33	0.04677
	Erythropoietin Signaling	4	--	1.32	0.04786

Figure 2. Overlap of DEGs with $FDR \leq 0.10$ for each time point

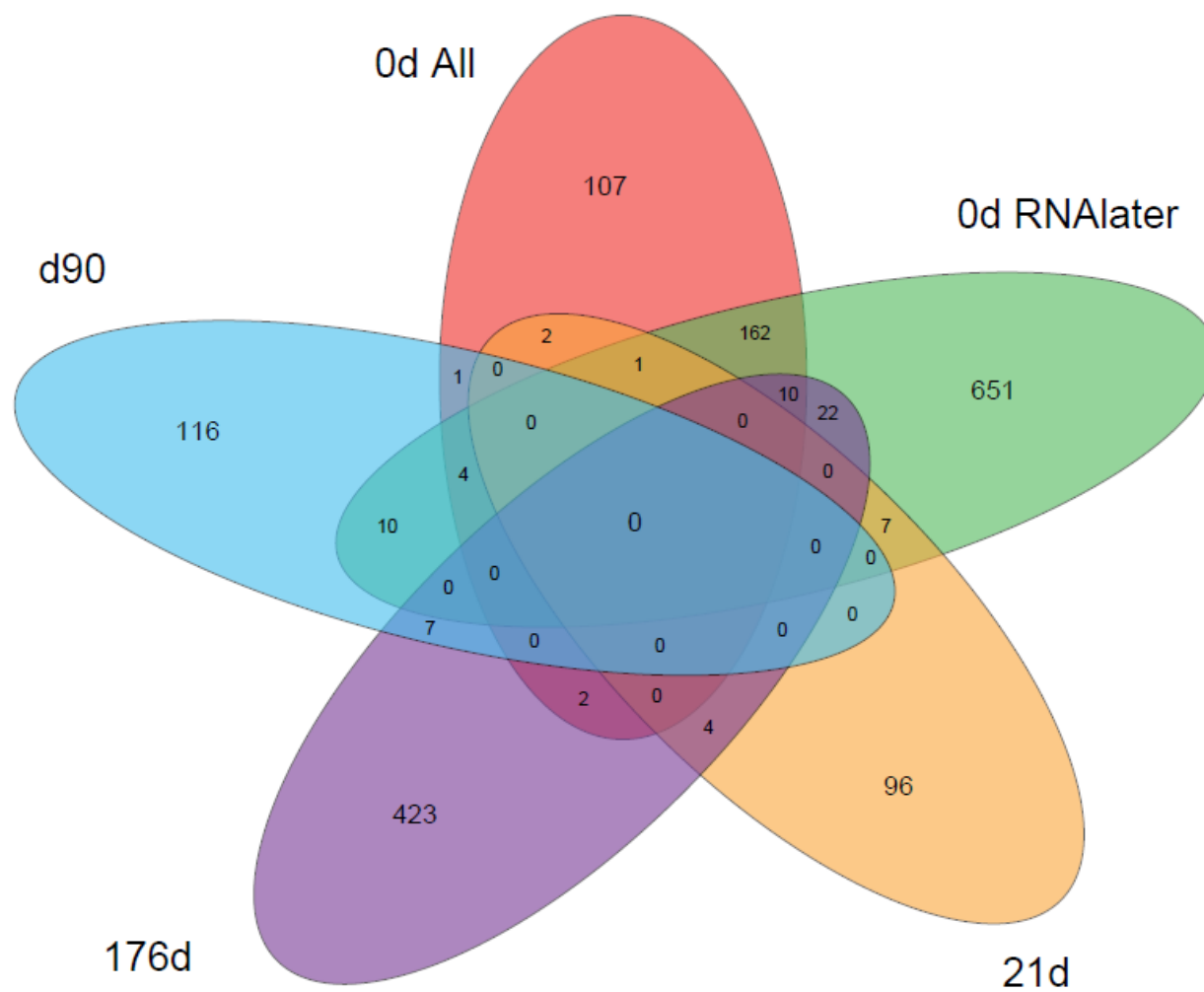
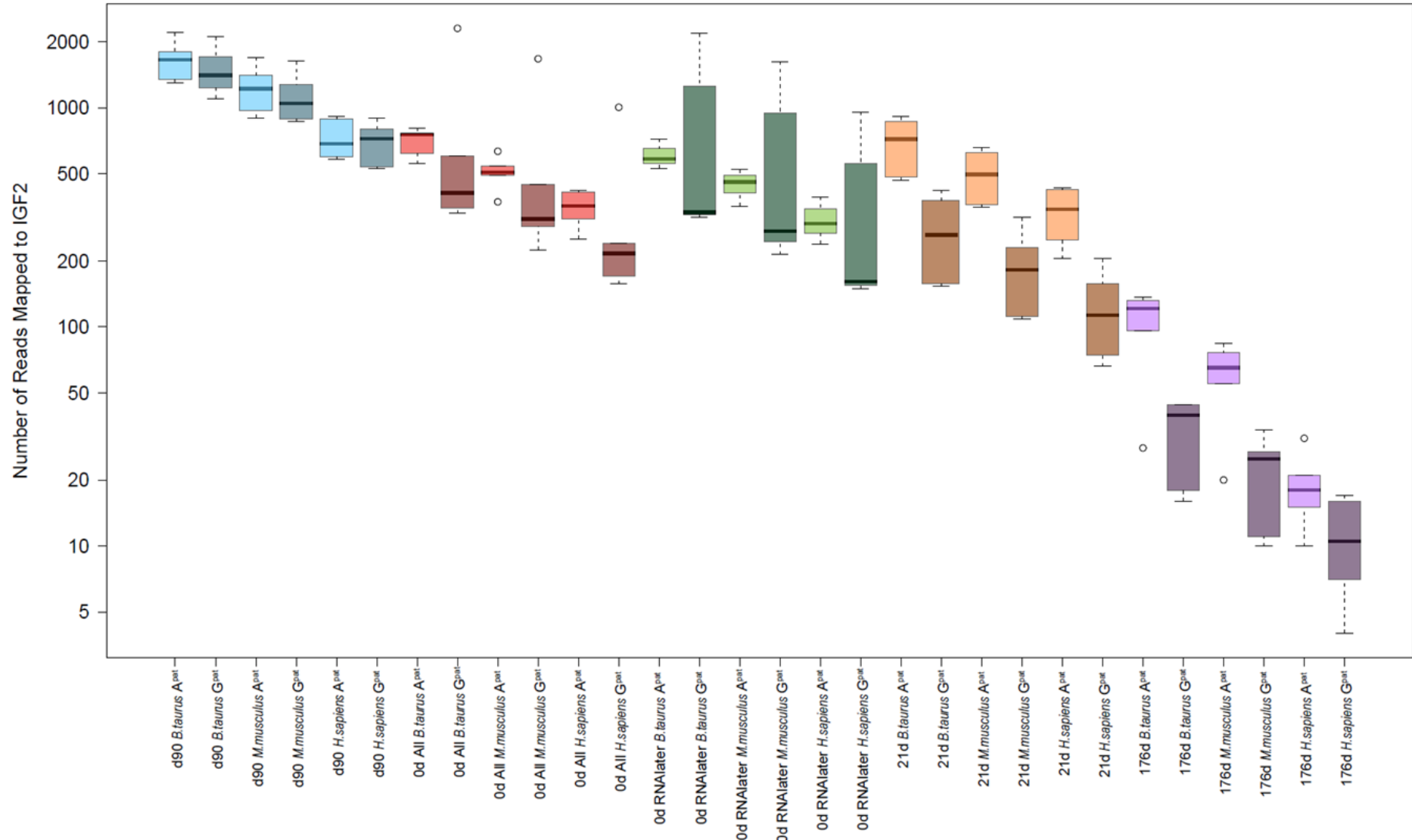


Figure 3. Boxplot of IGF2 expression at each time point for yielded from Btau4.0, NCBI37, and GRCh37 alignments



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