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Genes differentially expressed at 1 day, 6 weeks, and 6 months of age in aortas of spontaneously atherosclerotic White Carneau pigeons

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GENES DIFFERENTIALLY EXPRESSED AT 1 DAY, 6 WEEKS, AND 6 MONTHS OF AGE IN AORTAS OF SPONTANEOUSLY ATHEROSCLEROTIC WHITE CARNEAU PIGEONS

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DEDICATION

This thesis is dedicated to all the people that have supported me, cheered me on, and believed in me, even when I doubted myself.

Life is not a destination
It is not a race to the finish
Nor a process to be taken lightly
Life is truly, a journey

I came here to learn
I came here to understand the science of the human body
I came here to understand how nutrition influences health
I came here to better myself
I came here to challenge myself
I got much more than I ever bargained for,
……..And then some……..
I am not the same person I was when I walked through the door
I am grateful for all of life's lessons, experiences, and rewards
Thank YOU ALL!

Mostly to my husband and very best friend, Douglas for which there are no words

And to my mentors, Sam, Janet, and Betty
For inviting me into your world
My life has never been the same
I will cherish these experiences always

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GENES DIFFERENTIALLY EXPRESSED AT 1 DAY, 6 WEEKS, AND 6 MONTHS OF AGE IN AORTAS OF SPONTANEOUSLY ATHEROSCLEROTIC WHITE CARNEAU PIGEONS

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University of New Hampshire, May, 2011

Genetics is reported to be the primary causative factor for individuals diagnosed with atherosclerosis, in the absence of known risk factors. The development of atherosclerosis in White Carneau (WC) pigeons is of genetic origin, making it an excellent model to study genetic factors.

Representational Difference Analysis (RDA) was used to determine genes differentially upregulated between three ages, at the celiac bifurcation of the aorta in WC pigeons. Genes responsible for spontaneous initiation of atherosclerosis were hypothesized as being differentially expressed at 1 day, while those differentially expressed at 6 weeks and 6 months were related to progression.

Multiple candidate genes were upregulated at 1 day, although they were not definitively assigned to initiation. Genes upregulated at 6 weeks reflected increases in protein synthesis, loss of cellular integrity, and changes in muscle contraction. By 6 months, increases in lipid metabolism and changes in energy metabolism from oxidative phosphorylation to glycolysis were apparent.

INTRODUCTION

Atherosclerotic heart disease continues to be the number one cause of morbidity and mortality in the United States and other developed countries (1, 2). Atherosclerosis is a chronic multifactorial disease that results from both environmental and genetic factors, as well as their interactions (3-5). Diet, lack of exercise, high blood cholesterol levels, smoking, gender, and high blood pressure are known risk factors that contribute to approximately half of the cases in the United States (1, 2, 6). Upon clinical diagnosis of the disease, treatments modifying these factors are successful in only about 50 % of the cases (1). There is a dichotomy here because many people who have some or all of these known risk factors never develop atherosclerosis (1), while others who do develop atherosclerosis never exhibit any of these known risk factors. Heritability studies reveal an increased familial relationship for death from coronary heart disease. Interestingly, monozygotic twins have an even greater risk than dizygotic twins, suggesting a strong genetic component (7).

Heredity is therefore becoming more widely accepted as the underlying factor, particularly in those individuals who do not respond to prevention or treatment strategies, which focus on risk factor modification (1-3, 8, 9). Unfortunately, current research has not been successful in identifying the underlying human genetic factors that contribute to the development of atherosclerotic lesions. In addition, research conducted on humans is difficult due to ethical issues. This emphasizes the need for using animal models,

particularly those that exhibit atherosclerotic development similar to that observed in human cases.

White Carneau (WC) pigeons have been used for many years to study atherosclerosis and are an appropriate model for multiple reasons (8, 10-12). In the WC pigeon, lesions occur at branch points along the aorta, and progress into full blown plaques which pose a striking resemblance to those found in humans (8, 10-14). Also, the development of atherosclerosis in WC pigeons occurs spontaneously (non-induced) in the absence of any known risk factors (8, 10, 12) making it easier to identify underlying genetic components. In contrast, the Show Racer (SR) pigeon is resistant to the disease, providing an experimental control to compare differences in genetic expression (8). Research also shows that atherosclerosis in the WC pigeon is inherited as a single-gene, autosomal recessive trait (15) potentially facilitating efforts to identify the gene responsible for the disease in pigeons. Once the gene contributing to atherosclerosis in the WC pigeon is identified, it can then be compared with the human genome to assist in understanding the mechanisms responsible for atherosclerosis in humans.

While the precise mechanisms of initiation and progression of atherosclerosis are not clear in humans, biochemical changes in the aorta of WC pigeons occur by 6 weeks of age while morphological changes are observed by 6 months of age (2, 16, 17). These predictable changes provide specific time-frames in which to search for genetic changes in the WC pigeon to better understand stages of lesion development. Comparing the differential expression between these ages in the WC pigeon will help to distinguish genes responsible for initiation and progression of atherosclerosis.

Representational Difference Analysis (RDA) was developed to identify differentially expressed genes between two similar populations (18). This method couples Subtractive Hybridization (SH) with Polymerase Chain Reaction (PCR), to exponentially amplify only those genes that are differentially expressed between two similar populations. Compared to other methods, isolation of genes using RDA is both cost effective and efficient in a small laboratory setting, when the genome under investigation (pigeon) has not yet been characterized (18, 19). RDA has the potential to capture unknown genes even when expressed in low copy number. Once these genes are sequenced, they can be identified by comparing them with known sequences of other species due to genetic homology (18). This work described herein utilized RDA to identify genes differentially upregulated in the WC pigeon at ages known to reflect biochemical and morphological changes associated with atherosclerosis.

CHAPTER I

REVIEW of LITERATURE

Human Atherosclerosis

Cardiovascular disease (CVD) is the major cause of morbidity and mortality in the United States with atherosclerosis being the primary cause of this pathology (20-23). It is estimated that one in three adults has CVD, which on average translates into one death every 37 seconds from that cause (24). Atherosclerosis is considered a chronic multifactorial disease (25) caused by individual environmental and genetic factors, as well as synergistic environmental and genetic interactions (3-5). Chronic multifactorial diseases collectively account for the greatest burden on health care services in the U.S. (26).

Multiple risk factors associated with the clinical expression of atherosclerosis include age, gender, hypertension, hypercholesterolemia, diabetes, obesity, smoking, lack of physical activity, and stress (22, 27, 28). These risk factors alone, however, cannot always account for the development of atherosclerosis (20). Many individuals who develop atherosclerosis do so in the absence of any of these known risk factors. In fact, approximately 50% of men and women who die suddenly from this disease have no obvious risk factors or prior symptoms (24). For example, many individuals who have what are considered healthy cholesterol levels, still suffer heart attacks (29).

Adding to the complexity, often people who have some or all of these known risk factors never develop clinical atherosclerosis (1, 30). Once diagnosis of the disease is

made, however, treatments used to modify these factors are only successful in approximately 50% or less of the cases (1, 30).

In spite of the fact that millions of dollars have already been spent to study atherosclerosis, little is known about the early mechanism(s) of this disease (31). One explanation is that the ability to distinguish between lesion initiation and progression continues to elude current research. Another issue is that atherosclerosis in humans develops very slowly, and at different rates depending on the individual, making it difficult to map out a consistent pattern. In addition, humans develop a thickened intima before clinical "atherosclerosis" presents itself, and there has been much disagreement whether this thickened intima is actually the precursor or earliest stage of the disease. There are further questions of whether or not the accumulation of extracellular proteoglycans and lipid precede the infiltration of macrophage cells (31). Given that atherosclerosis is a chronic disease exhibiting many multifactorial contributions there is a surprising shortage of information dealing with the earliest stages of atherogenesis (28).

Post-mortem studies are helpful in that they provide evidence of the sub-clinical stages of atherosclerosis, revealing visible lesions, that often do not manifest clinically until the third or fourth decade of life (20, 29). These visible manifestations are considered the earliest stage of atherosclerosis and present as foam cells or fatty streaks due to the collection of lipid droplets within the cytoplasm of cells (22).

Autopsies conducted from 1987 – 1994 on victims who died from accidents revealed that atherosclerosis begins in youth (32). Strong et al., found that intimal lesions were present in all aortas, and in more than half of the right coronary arteries of 15 – 19 year olds with increasing prevalence and extent through 30 – 34 years of age. They also

found that all the age groups contained fatty streaks in their aortas while fibrous plaques appeared in coronary arteries of only a few individuals under the age of 20 (32). The researchers concluded that while fatty streaks are usually innocuous, if they persist, and in the presence of specific conditions, they can progress to the clinical disease state (32). Additional human autopsies reveal that some regions in the vessel wall have a propensity for early atherosclerotic lesions while others are resistant (11). These same autopsies also reveal a very distinct pattern in the distribution of early atherosclerotic lesions, which is in agreement with Caro's low sheer stress theory (11).

Stary's work supports the research conducted on these autopsies and states, while advanced lesions may result in clinical symptoms, the early lesions that pave the way are silent (33). Given these findings, it is essential to understand the earliest molecular and cellular mechanisms responsible for atherosclerotic development in order to establish approaches that will delay onset as well as progression of the disease before it produces adverse clinical outcomes (34).

Additional histological studies of coronary arteries from human autopsies of various ages, from infants to adults, demonstrate regions that are susceptible to atherosclerosis are abundant in smooth muscle cells (SMCs), while regions that are resistant to its development contain very few SMCs (33-35). Some animal experiments also demonstrate that SMCs are integral components that increase during the initial stage of lesion formation (33). Studies also show that intimal SMCs express lower levels of proteins associated with contractile function, and have an increased capacity for producing extracellular matrix, proteases and cytokines, compared with medial SMCs (34, 36, 37). In addition, intimal

SMCs, also known as synthetic SMCs have a higher proliferative index, and can synthesize up to 46 times more collagen than medial (contractile) SMCs (34, 36-38).

Until recently, it has not been clear whether extracellular lipid and proteoglycan accumulation, occurs before macrophage infiltration in the human disease (31). It is suggested that SMCs play a key role in initiation and early progression of atherosclerosis, since alterations of their extracellular matrix can cause SMCs to proliferate and increase their lipid content (31, 33). This lipid accumulation by SMCs then contributes to foam cell formation in a pattern that is analogous to the pattern observed with macrophages, which is believed to then accelerate the formation of lesions (34, 39).

It is now widely agreed that the SMCs of human atherosclerotic lesions originate from the medial layer and that most of these intimal SMCs contain lipid droplets (33). One of the challenges with determining which cell type is ultimately responsible for lesion development and progression is that many of the functions that are attributable to SMCs during early lesion development are known to occur in other cell types such as macrophages (34).

The current paradigm as laid out by the American Heart Association (AHA) (**Table 1**) proposes an orderly, linear pattern of lesion development where an initial lesion or fatty streak progresses into an advanced lesion (40).

Table 1: Current AHA Classification

Current AHA Classification			
Terms for Atherosclerotic Lesions According to Histological Classification		***Other Terms for the Same Lesions Often Based on Appearance to the Unaided Eye***	
Type I lesion	Initial lesion	Fatty dot or streak	Early lesion
Type IIa lesion	Progression-prone type II lesion	Fatty dot or streak	Early lesion
Type IIb lesion	Progression-resistant type II lesion	Fatty dot or streak	Early lesion
Type III lesion	Intermediate lesion (pre-atheroma)		
Type IV lesion	Atheroma	Atheromatous plaque, fibrolipid plaque, fibrousplaque, plaque	Advanced lesions, raised lesions
Type Va lesion	Fibroatheroma (type V lesion)	Atheromatous plaque, fibrolipid plaque, fibrousplaque, plaque	Advanced lesions, raised lesions
Type Vb lesion	Calcific lesion (type VII lesion)	Calcified plaque	Advanced lesions, raised lesions
Type Vc lesion	Fibrotic lesion (type VIII)	Fibrous plaque	Advanced lesions, raised lesions
Type VI lesion	Lesion with surface defect and/or hematoma/hemorrhage and/or thrombotic deposit	Complicated lesion, complicated plaque	Advanced lesions, raised lesions

(40)

Studies used to formulate this paradigm were conducted on animals. One of the problems with this is that lesions in induced animal models differ from lesions found in humans (31, 32, 40). To complicate matters further, lesions from human autopsy studies demonstrated a wide array of morphological variations that are not entirely consistent with the current AHA paradigm (40). Work by Virmani et al., found that there is insufficient direct experimental evidence from humans and animal models to clearly explain lesion formation and progression as laid out by the AHA (40). Nakashima also found that the morphological features of early atherosclerosis differ between humans and animal models (31). For example, the site of lipid deposition differs between animals and humans (41). He found that the narrow subendothelial space was the site of lipid deposition in animal models unlike in humans where the earliest lipid deposits occurred in the deep layer of the

thickened intima (31). As a result, Virmani et al., devised a more thorough classification (**Table 2**) that is consistent with the AHA paradigm, yet which more accurately classifies the morphological variations that exist in such models of atherosclerotic research (40).

Table #2: Virmani's Modified AHA Characterization

Modified AHA Classification Based on Morphological Description		
Nonatherosclerotic intimal lesions		
Intimal thickening (prone)	The normal accumulation of smooth muscle cells (SMCs) in the intima in the absence of lipid or macrophage foam cells	Thrombus absent
Intimal xanthoma, or "fatty streak" (resistant)	Luminal accumulation of foam cells without a necrotic core or fibrous cap. Based on animal and human data, such lesions usually regress.	Thrombus absent
Progressive atherosclerotic lesions		
Pathological intimal thickening	SMCs in a proteoglycan-rich matrix with areas of extracellular lipid accumulation without necrosis	Thrombus absent
Erosion	Luminal thrombosis; plaque same as above	Thrombus mostly mural and infrequently occlusive
Fibrous cap atheroma	Well-formed necrotic core with an overlying fibrous cap	Thrombus absent
Erosion	Luminal thrombosis; plaque same as above; no communication of thrombus with necrotic core	Thrombus mostly mural and infrequently occlusive
Thin Fibrous cap atheroma	A thin fibrous cap infiltrated by macrophages and lymphocytes with rare SMCs and an underlying necrotic core	Thrombus absent; may contain intraplaque hemorrhage/fibrin
Plaque rupture	Fibroatheroma with cap disruption; luminal thrombus communicates with the underlying necrotic core	Thrombus usually occlusive
Calcified nodule	Eruptive nodular calcification with underlying fibrocalcific plaque	Thrombus usually nonocclusive
Fibrocalcific plaque	Collagen-rich plaque with significant stenosis usually contains large areas of calcification with few inflammatory cells; a necrotic core may be present	Thrombus absent

(40)

Virmani reorganized the AHA classification of type I and II lesions as non-atherosclerotic, intimal xanthomas that do not develop into progressive atherosclerotic lesions in animal models (40). He also found that intimal xanthomas are consistently prevalent in all populations especially when exposed to a Western diet (40). According to

human, as well as animal studies, intimal xanthomas usually regress. On the other hand, intimal thickenings include SMCs that are clonal in nature which can readily progress to "pathological" intimal thickenings that include a proteoglycan rich matrix along with some areas of extracellular lipid accumulation (40). Intimal lesions are characterized by the infiltration of mononuclear cells in the absence of foam cells thus outlining the earliest stage of atherosclerotic lesion development (28). Doran et al., found that SMCs are the first cells present at sites destined to develop into atherosclerotic lesions (34). Recent studies reveal that intimal thickenings are in fact the precursors for advanced atherosclerotic lesions and that intimal proteoglycans are actively involved in the disease process of atherosclerosis in humans (31, 41, 42).

While human and animal models contribute to the understanding and characterization of the morphological features that present in early atherosclerosis, they raise many more questions regarding the underlying pathology of the disease. Challenging these classifications, however, will provide a valuable framework from which to develop a more complete understanding of the processes involved in this complex multifactorial disease (22).

Research thus far, emphasizes the need to look deeper to explore the genetic factors that contribute to susceptibility to atherosclerosis in some individuals while others maintain resistance (21). Collectively, clinical genetic studies have established that genetic factors do play a major role in susceptibility to atherosclerosis (5), but because of the phenotypic complexity of atherosclerosis in humans it has been increasingly difficult to characterize genetic mechanisms responsible for the disease (15). While examining heritability of atherosclerosis in both family and twin studies, inheritance was a reliable

factor for determining atherosclerotic risk, in most cases exceeding 50% of those evaluated (43). This was further demonstrated in a study by Marenberg et al., which found the concordance rate among monozygotic twins to be twice as high (8.1:3.8 ♂; 15:2.6 ♀, with a 95% confidence interval) as that of dizygotic twins (7).

Given this understanding, atherosclerosis does not behave like a single gene Mendelian disease (5). In general, atherosclerosis in humans is believed to be a complex polygenic disorder that results from a combination of numerous genes and environmental factors (5). In addition, atherosclerosis is also a heterogeneous disease in that different individuals express various manifestations of the disease with respect to genetic and environmental contributions (5, 30, 43). As a result, understanding the various contributing factors of the disease may determine the therapy or therapies best suited to treat it (43). Understanding Mendelian diseases and known Mendelian forms of atherosclerosis could also provide an understanding into the pathogenesis of common non-Mendelian forms of atherosclerosis (5).

Familial Hypercholesterolemia (FH) and Tangier disease are primary examples of how studying Mendelian disorders have contributed to the effective treatment and understanding of monogenic diseases (43) that ultimately lead to atherosclerotic complications (5). The study of FH has also contributed to the determination of the molecular pathways that regulate cholesterol metabolism, thus leading to drug treatment for many individuals affected by this major risk factor, associated with atherosclerosis (43, 44). It is thus becoming increasingly clear that understanding the role of inheritance is crucial to be able to reduce mortality from this disease. In order to effectively identify genetic factors responsible for atherosclerosis, it will be necessary to incorporate complementary

approaches such as the use of animal models that facilitate functional genetic experiments (43). When it comes to candidate genes, Mendelian diseases provide a logical basis for the study of atherosclerosis (43). Many candidate genes have already been identified, but because of the multiple factors implicated in human atherosclerosis, it is difficult to directly examine the role of these genes (43, 44). Consequently, there has been limited success in providing reliable explanations for disease susceptibility (3). Because atherosclerosis involves the interactions between arterial cells, blood elements, and genetic factors acting at the level of the arterial wall, each of these may likely influence the genetic regulation of susceptibility to the disease process (43, 45). In addition, the heterogeneity of human populations, as well as the growing likelihood that many genes are involved in the disease process, makes it increasingly challenging to identify genes involved in atherosclerosis (3). For these reasons, animal models can be particularly useful to identify genes, that may be involved in human atherosclerosis (43).

Pigeon Models

When selecting an animal model it is essential to choose one that best approximates the pathophysiology of the disease in humans (4). It is also important, whenever possible, to choose a model that is low in cost, provides ease of housing, is manageable in size, breeds relatively quickly and easily, and has a well-defined genetic background (4). Specifically, when selecting a model, one needs to look for a model that develops atherosclerotic lesions in a pattern similar to that in humans as described in previous autopsies, and histology reports.

There is no one model that meets all these criteria when designing an experiment; therefore, one must select the best model given the available options when testing a particular hypothesis (4). A systematic examination of the sequence of events using a model with minimal environmental influences is therefore essential to determine the genetic contributions, which phenotypically resembles the disease in humans (21, 26).

Studies already reveal that the genetic scaffolding of humans is shared with many other organisms (46). The completion of the Human Genome Project is an asset in that it provides a foundation for determining functional parts of genomes in order to improve the health of individuals (47). Once the gene or genes responsible for atherosclerosis in an animal model are identified, they can then be matched against the human genome to enhance the understanding of the multifactorial process involved in human atherosclerosis (26). The comparative analyses of genome sequences are of great value because common functions between two organisms are often conserved, and thus encoded within the DNA (47).

The pigeon (*Columba livia)* originated from Europe, Asia and North Africa, giving rise to hundreds of varieties (48). The use of the pigeon as a model is exceptional because, both resistant breeds as well as susceptible breeds, which spontaneously develop atherosclerosis, exist (49). Clarkson as well as others have described the development of spontaneous atherosclerotic lesions in the White Carneau (WC) pigeon as nearly identical with those in humans, both grossly, microscopically, (8, 13, 14, 50, 51) and biochemically (52).

Kjaernes reported that the results of atherosclerotic research in animal models often contradicts the effects found in humans, while the events in WC pigeons regarding lesion

properties and location are quite similar to those in humans (11). Kjaernes observed that the spontaneous lesions found in WC pigeons, develop primarily at sites of low sheer stress such as the celiac bifurcation of the aorta, which supports Caro's low sheer stress hypothesis (at sites of turbulent flow) (11). This same pattern was also observed in human autopsies (11, 49). It was therefore suggested that the pigeon may be the best model to study the development of atherosclerosis in humans (4, 11).

The care and feeding of pigeons is such that they can be maintained in a wide variety of seasonal climates as long as water is kept from freezing and that they are permitted limited exercise in the form of a flypen (48). In general, a 12-hour light and dark cycle is recommended, although a 14-hour light cycle will enhance breeding (48). Pigeons achieve sexual maturity by 6-7 months and achieve peak breeding between 3-6 years (48) .

Some of the advantages of using the pigeon as a model to study heart disease include; susceptibility to atherosclerosis, lesions that are similar to those in humans, based on location, histology, and progression, low cost, easy handling, sufficient size, and relatively long lifespan (4). Some disadvantages of using pigeons are that they are difficult to sex; they mate for life and often live well into their twenties. If separated from their partner, it can take up to a year before pigeons will select a new mate and reproduce at a maximum rate of two eggs every 6 weeks, from spring to early fall (15). Pigeons are nonmammalian (although both sexes produce a crop milk secretion to feed their young) (53); they are at risk of developing a herpes virus associated with atherosclerosis (50, 54); and they undergo many changes in lipoprotein metabolism during egg-laying and through the first few days after hatching (4).

In addition, all pigeons, unlike humans, lack apo-E, apo-B-48, and chylomicron formation (52). Because both breeds share the same commonalities of cholesterol transport explained later in this report, the lack of apo-E and apo-B48 cannot explain susceptibility in the WC and resistance in the SR, and therefore removes Apo-E and Apo-B-48 as risk factors for the development of atherosclerosis (13, 50).

The pigeon has been the most widely used avian species in atherosclerotic research (8, 52). The primary site of plaque development in WC pigeons is at the celiac bifurcation (branch point) of the aorta (1, 52). As time progresses, lesions develop at other sites in the aorta and in the coronary arteries (50). Clarkson et al., first reported that WC pigeons develop spontaneous atherosclerosis while other breeds such as the Show Racer (SR) pigeons maintain resistance (49). The SR pigeons originated from racing homers about 185 years ago and have remained a closed breeding colony for more than 45 years (52). Clarkson et al., suggested that atherosclerosis in the WC pigeon is the result of a genetic factor, independent of other risk factors (8, 13, 55).

Morphological analysis of aortas in WC pigeons revealed that lesions are present in all WC pigeons with no difference between genders (8). The lesions appear as raised yellow plaques that project into the lumen and are mostly found at branch points (sites of bifurcation). Although some lesions do develop along the straight segments of the aorta, a few plaques practically occlude the lumen of the aorta (8). Most of the spontaneous lesions are primarily fibrous (8). The predictability of these spontaneous lesions at the celiac bifurcation of the aorta make the WC pigeon a good model system for the study of metabolic parameters associated with the disease (10).

Paired muscular intimal thickenings are present at the celiac bifurcation of 10-day embryos and very young birds, with cushions of SMCs protruding into the lumen (10). In squabs, ridge-like thickenings were observed prior to the presentation of lipid along the lateral edges of the celiac orifice (10). By 4-6 months, the earliest lipid accumulation can be seen on the surface of these intimal cushions (10). According to Santerre, aggregates of SMCs appear to be a prerequisite for lipid accumulation and appear to dominate metabolism in the healthy state, as well as in all lesions and at all stages of the spontaneous diseased state (10). Vacuoles appear to develop from the dilated granular endoplasmic reticulum in modified SMCs of very young WC pigeons (14). The appearance of thickened basement membrane envelopes on modified SMCs, disorganization of elastic laminae, and excess collagen in WC pigeons are strikingly similar to those features seen in the early lesions found in humans (14).

Lesions that develop in WC pigeons increase in size with age, and progress through various stages of plaque development (49). The activation of cells involved in the disease process, relies not only on local effects, cytokines, and growth factors, but also on intrinsic genetic properties (49). Advanced lesions contain intra- and extracellular lipids, cholesterol clefts, fibrous tissue, SMCs, and what appears to be macrophage foam cells (52). Only after atherosclerotic lesions appear is there an increase in the influx of LDL at specific sites of arterial lesions (50). As lesions progress, ulcerations, hemorrhage, mineral deposition, and even bone formations are common (52). Complications of thrombosis and complete occlusion in the WC pigeon develop only during the later stages of the disease (8). A unique feature in WC pigeons, in contrast to other animal models is that myocardial

infarctions occur in older pigeons displaying the most severe stage of atherosclerosis (50).

Richards (as well as Santerre) studied the spontaneous development of lesions in WC pigeons from 1 month to 3 years of age (and 1 month to 6 years respectively) to document their location and stages in development (10, 12). Richards observed that in hatchlings, lesions progressed as age increased, though the final distribution pattern was apparent by 5 months of age (56). In other words, no new lesions developed after this age (12).

Comparing and contrasting susceptible and resistant breeds are useful to examine the biochemical functions involved in atherosclerosis (49). When comparing lipid content in the celiac bifurcation at 1 day, 6 weeks, 12 weeks and 6 months, Hajjar et al., found that total lipid content in the WC pigeon was significantly greater by 6 weeks of age and 6 months of age when compared to the SR pigeon (57). A dramatic increase in non-esterified fatty acids (NEFA) was also observed by 6 weeks of age in the WC when compared to the SR and remained significantly greater as age increased. Also apparent was a marked increase in cholesterol at celiac sites by 6months in the WC pigeon as compared to the SR (57).

In another study, Hajjar et al., found that by 6 months of age there was a dramatic decrease in the P/O ratios of ATP synthesis and oxygen consumption in the celiac cushions of the WC as compared to the SR (17). They suggested that the reduced ability of the WC to produce ATP was of a genetic origin (17). Work conducted by Curwen et al., on quantitative electrophoretic profiles of glycosaminoglycans (GAG) in WC and SR at 1 day,

6 weeks, 6 months and 3 years of age revealed that the WC contained more total glycosaminoglycans than the SR at all ages in the celiac bifurcation (16). After 6 months, changes were noted in specific GAGs between WC and SR, suggesting GAGs play a role in the pathogenesis of atherosclerosis (16).

While examining aortic tissue of both WC and SR Hajjar et al., did not notice any ultrastructural differences between the two breeds until 6 months of age (57). It was at that point that they consistently found more debris-like material in the celiac cushions of the WC when compared to the SR pigeon (57). Some of this extracellular membranous material appeared to be uniformly round while other vesicles were more heterogeneous in shape, but both appeared to originate from SMCs (57).

Both SR and WC pigeons, unlike humans, maintain typical cholesterol levels of approximately 300 mg/dl. Approximately, two thirds of their cholesterol is transported in the form of HDL as part of their normal cholesterol pathway in contrast to humans, which are LDL carriers (45, 52, 58). Since most animals do not develop spontaneous arterial lesions, they have been historically placed on high-cholesterol diets for months at a time prior to examination of their arteries (11). Upon feeding a 0.5% cholesterol diet, both SR and WC pigeons develop marked hypercholesterolemia of approximately 1,000 to 2,000 mg/dl (52). The marked increase in cholesterol concentrations interestingly causes the primary carriers of cholesterol to shift from HDL to B-VLDL and LDL.
Apo-B becomes the predominant apolipoprotein in this induced state, and acyl-CoA-cholesterol acyl transferase (ACAT) activity increases to facilitate transport, which is similar to humans and other animals (50).

Examination of WC pigeons to determine whether blood pressure (BP) plays a major role in the progression of atherosclerosis, showed no significant effect of season or age on BP between 6-12 months when early atherosclerosis begins; nor was there a major difference in BP between SR and WC (59). Wagner did find, however, that after 6 months on an atherosclerosis-inducing diet, BP increased as a result of atherosclerosis (59) making it therefore a secondary effect.

While examining the lumen of the aorta, Wagner found that the WC pigeon has a larger lumen compared to the SR pigeon although there were no differences between wall thicknesses. He also found that the WC wall contained greater amounts of elastin but little difference in collagen (49). These differences were not found to occur in response to plaque development but were the result of intrinsic properties within the aorta attributable to each specific breed of pigeon (49).

In addition, when spontaneously atherosclerotic WC pigeons are fed an atherogenic diet, lesion development is accelerated (1, 52). These induced lesions contain lipid engorged macrophage cells, that form fatty streaks, which differ from those that develop spontaneously (1, 50, 60). This diet-induced state not only exacerbates, but alters the progression of the spontaneous disease (1, 52, 61, 62), no longer making it a valid model to study the underlying early mechanisms of the disease (60). In addition, upon examination of the induced disease, locations of these lesions are typically found at sites of high physical stress, which correlates with Fry's high shear stress hypothesis, unlike lesions found in non-induced states (11, 49). Once the atherogenic diet is replaced by a typical non-inducing pigeon chow diet, cholesterol concentrations in both SR and WC pigeons return to normal levels within a few weeks (52). In the presence of an atherogenic diet,

resistance to atherosclerosis is maintained in the SR pigeons unlike the WC pigeons. This difference appears to be genetically controlled (55), and the absence of lesions in the SR pigeons suggests that genetic factors are responsible for resistance to atherosclerosis (8, 15).

Breeding experiments present the most convincing evidence regarding genetic influence on the development of specific atherogenic lesions (55). After crossbreeding F1 WC and SR to produce F2 progeny, Goodman and Nash suggested that genetic factors play a major role in atherosclerosis (9). They also reported that genetic factors for initiation were largely independent of those responsible for lesion progression (9). Smith et al., reported that a limitation of Goodman and Nash's study was that the pigeons were placed on a high cholesterol diet which altered and accelerated the spontaneous disease progression, and that the progeny were never backcrossed to determine the inheritance pattern (15).

In 2001, after completing a 15-year study Smith, Smith and Taylor reported the mechanism of inheritance for susceptibility to spontaneous atherosclerosis in WC pigeons (15). They demonstrated this inheritance pattern through crossbreeding inbred strains of susceptible WC and resistant SR pigeons to produce F1 and F2 progeny and finally backcrossing the F1 progeny with parental WC and SR pigeons (15). Upon examination of lesions visible at the celiac bifurcation of the various progeny, they determined that atherosclerosis was inherited as a Mendelian single-gene, autosomal recessive pattern (15). Smith et al thus concluded that the WC pigeon is an effective model to study genetic factors that lead to the development of lesions at the level of the arterial wall (15). They

therefore determined that the genetic patterns of spontaneous lesion development in the WC pigeon makes it a simplest case scenario due to the absence of risk factors (15).

To better understand genetic factors responsible for both susceptibility and resistance, Anderson compared differential gene expression between WC and SR pigeons (63). Her work on SMCs (from the celiac bifurcation) in-vitro (representing a compressed time- frame of approximately 2-3 years in-vivo) showed that genetic differences do in fact exist between both breeds. Anderson found for example, ribosomal biogenesis was clearly upregulated in the SR (63). While 7 genes were unique to the WC (27copies) only 1 gene (1 copy) was unique to the SR. Five genes were differentially upregulated in both the WC and the SR, yet the number of copies in the SR greatly exceeded those in the WC (81:10). The primary contributor of this regulation was Ribosomal Protein L3 (RPL3) that was differentially upregulated 69 times in the SR (63). Given the number of genes that were differentially upregulated between both breeds, the regulation in the SR was more than twice that of the WC.

Anderson also reported a marked difference in energy metabolism between both breeds. While the genes Cyctochrome C oxidase subunit I (COI), NADH dehydrogenase subunit 4 (ND4), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10 (NDUFA10), and ATP synthase subunit 4B (ATP4B) representing oxidative phosphorylation were upregulated in the SR, Enolase 1 (ENO1), Glucose 6 phosphate Isomerase (GPI), and Lactate Dehydrogenase (LDHA) which participate in glycolysis, were upregulated in the WC pigeon (63). Additional metabolic differences reported by Anderson revealed that genes expressed by the SR were indicative of a healthy smooth muscle contractile phenotype (63) while the susceptible WC pigeons expressed genes that represented a

synthetic phenotype (or loss in smooth muscle regulation). Anderson also found that the genes expressed by the resistant breed indicated that they were much better at organizing and maintaining the extracellular matrix while genes expressed in the susceptible WC indicated a lack of structural integrity of the matrix (63). Anderson concluded the differences in genetic expression patterns between both breeds helped to explain factors responsible for both susceptibility and resistance however, she was unable to discern the chronological sequence of events for initiation and progression of atherosclerosis (63). Based on the available research, it is reasonable to suggest that the WC pigeon is one of the best models to study the genetic effects at the level of the arterial wall that are associated with human atherosclerosis (4).

Identification of Differentially Expressed Genes

Expression patterns of genes are continuously changing during the growth and differentiation of tissues (19). The expression of a given gene in a cell at any one point in time defines the characteristics of the cell and impacts the downstream effects which can ultimately invoke a disease state (19). Given that atherosclerosis is a disease that develops slowly over time presenting various characteristics, it is important to choose a method or methods that will capture genetic material and effectively identify genes of interest, particularly those that are different between known ages of development.

Research conducted on WC pigeons shows that in the aorta chemical changes occur by 6 weeks of age while morphological changes are observed by 6 months of age (2, 16, 57, 64). Given these results, it is necessary to utilize a method that examines differences in genetic expression between these known ages of development.

Many methods are available to target differential expression between two similar systems. Some of the methods include Serial Analysis of Gene Expression (SAGE), Differential Display (DD), Subtractive Hybridization (SH), Microarray and Representational Difference Analysis (RDA). One of the main advantages of all of these methods except for Microarray is that they do not require prior knowledge of the sequences to be analyzed (65). Although Microarray can only analyze known genes it does allow for large numbers of genes to be compared in a single assay (66, 67). An advantage of both SAGE and DD is that both methods only require small amounts of total RNA as their starting material (19, 65, 68). A disadvantage is that primers are attached to the 3' end of transcripts in both SAGE and DD which may hinder their identification and characterization following Polymerase Chain Reaction (PCR) (69).

Although DD is versatile, easy to monitor and offers ready access to sequence information (19, 70), a major disadvantage is the number of false positives when determining actual differences between transcripts (68-70). This is because determination of differential expression relies totally on "visual" examination of the bands from agarose gels (69). One study found that 19 out of 20 amplified bands that were analyzed were false positives (70). While SAGE amplifies thousands of sequence tags, because they are small, they offer little sequence information for novel genes (69). In addition the large numbers of transcripts that are generated make it cumbersome and rather time consuming to detect genes that are differentially expressed (69).

The method of SH involves the removal of genes that are shared between two similar populations. Genes that do not hybridize with one another are therefore differentially expressed and remain single stranded (ss). Subsequent steps therefore require

additional methods such as the preparation of libraries to store the ss DNA which can be a cumbersome and costly process (19).

Representational Difference Analysis is a positive enrichment technique that couples both SH and PCR to amplify only those genes that differ in their expression between two populations (69, 71, 72). The method of combining two populations includes the primer ligated target population known as the "testers" and the non-primer attached "drivers" which are greatly in excess of testers (69, 71, 72). Following the hybridization reaction, three types of populations result; driver:driver (non-amplified), tester:driver populations that experience linear amplification, and finally the differentially expressed target populations known as, tester:tester that are exponentially amplified, diluting non-differentiated genes beyond detection (69, 71, 72). Tyson and Shanahan successfully used this technique to compare differences between cDNA populations from human atherosclerotic plaques and normal segments of the aorta (69).

One of the benefits associated with RDA include, its ability to detect transcripts of low copy number due to the combination of hybridization kinetics and exponential enrichment (71). This is especially important given that in a mammalian cell, 15,000 genes on average are expressed at any one time and of that approximately 83% of transcripts are present in only one copy per cell (71, 73). Also, only a small amount (100-250ug) of total RNA is needed (69) as the starting material. Unlike other methods, RDA produces longer expressed sequence tags (ESTs) (256bp on average) that are more likely to originate from their parent cDNAs (72) ultimately optimizing the number of positive outcomes. Another advantage of RDA is its ability to detect novel transcripts (72). In addition, RDA is cost effective, sensitive, relatively quick and yields a minimum number of false positives (<

5%), along with the fact that once the method is complete only differentially expressed genes remain (72).

In light of the many advantages of RDA, some limitations remain. For example, differences between genes expressed at the moment that total RNA is extracted represent only a snapshot in time and therefore does not capture all the possible genes expressed in a cell. Because the method selects for expressed differences, it is possible that many of these genes have not yet been annotated, and results in genes being classified as "unknowns" (72). Low yield can result from poor techniques so it is essential to maintain meticulous care in planning as well as execution of techniques throughout the experiment to optimize recovery of material (71). In addition, any cDNA fragments that are less than 100-150 base pair in length are removed, which may potentially remove key genes of interest (72). An additional limitation can occur when an EST of interest lacks two sticky ends on the 5' prime ends of the double stranded cDNA. In this event, the tag is amplified linearly at best, and therefore washed out. The current use of a four-cutter enzyme in place of the previously used six-cutter optimizes the number of manageable sizes of cDNA that can be used with this technique. The subsequent use of an additional four-cutter can further optimize the recovery of genes of interest (72). Anderson's work included optimization of the RDA method that was provided by Pastorian et al., therefore reducing many of the limitations experienced with the method (63). When compared to other methods, RDA presents fewer limitations, making this an excellent method of choice.

Work conducted by Anderson utilizing the optimized RDA method in WC and SR pigeon aortic cell cultures identified 165 genes that were differentially upregulated in SMCs (63). Seventy-four genes were differentially upregulated in the WC while 63 genes

were uniquely upregulated in the SR pigeon. While Anderson was able to identify many of the genes, because of the compressed developmental time-frame associated with her in-vitro system, she was unable to determine their chronological sequence of expression associated with susceptibility or resistance. Anderson's findings emphasize the need to conduct future in-vivo experiments that compare selected ages, in order to reveal the chronological sequence of differential gene expression. Once the metabolic roles of the differentially expressed genes are understood, the sequence of events in the disease process can perhaps be elucidated.

Research Objective

To distinguish between genes that are responsible for initiation of spontaneous atherosclerosis in the pigeon model and those responsible for progression by identifying genes that are differentially expressed at the celiac bifurcation of the aorta between 1 day, 6 weeks, and 6 months of age in White Carneau (WC) pigeons.

Genes that are differentially expressed early (1 day) will be considered to be involved in initiation of the disease while those that are differentially expressed later (6 weeks and 6 months) will be considered to be involved in the progression of the disease.

Hypothesis

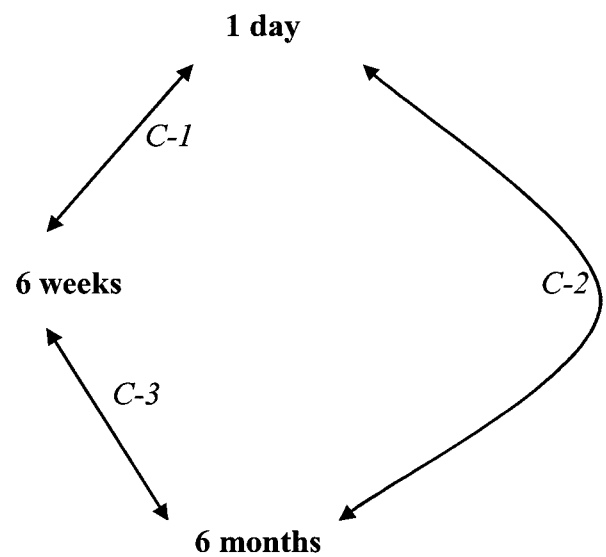
Genes responsible for initiation and progression of atherosclerosis in the WC pigeon will be differentially expressed at 1 day, 6 weeks and 6 months of age.

Experimental Design

1. Representational Difference Analysis (RDA) will be conducted to compare genes differentially expressed in-vivo at the celiac bifurcation of the aorta in atherosclerosis susceptible WC pigeons.

2. Vertical comparisons between three age groups (outlined in Figure 1) will be performed using RDA in order to examine differently expressed genes:
comparison 1 (C-1):1 day versus 6 weeks, comparison 2 (C-2): 1 day versus 6 months, and finally comparison 3 (C-3): 6 weeks and 6 months.

Figure 1: RDA Experiment of WC Pigeon Comparisons Between the Ages



CHAPTER II

METHODS AND MATERIALS

Pigeon Colonies

The WC pigeons were obtained from University of New Hampshire (UNH) colonies that were housed in fly coops at ambient temperature and allowed free access to water, Purina Pigeon Chow Checkers, and Kaytee Red Grit. The origin of the WC population began from four pairs of birds that were purchased in 1915. They were then mated with a few outsiders and a few more additions to complete the entire inbred WC population by 1916 at the Palmetto Pigeon Plant in Sumter, South Carolina (8). The UNH colony was established in 1962 from WC pigeons obtained from the Palmetto Pigeon Plant and have remained inbred since that time (63). The WC pigeons that are housed in the UNH colonies are maintained under the guidelines of the UNH Animal Care and Use Committee (Approval # 050601).

Whole Tissue Extraction Followed by cDNA Synthesis from Total RNA

Previous to this current research project RNA was extracted from aortic tissue at the celiac bifurcation of WC pigeons at 1 day (experiment #137), 6 weeks (experiment #190a-2), and 6 months of age (experiment #162) by Anderson in this lab. Surgical collection of the celiac bifurcation of six WC pigeons at 1 day of age was conducted on two separate occasions and stored in RNA*later*® Solution (Applied Biosystems), (a tissue collection RNA isolation stabilization solution), according to the standard protocol provided with this

product (74). Once 62mg of whole tissue was collected, the tissue was frozen in liquid nitrogen and ground by mortar and pestle, which was then combined with 1ml of Quantum Prep® Master Blaster (BioRad) an RNA extraction reagent, according to the standard protocol provided with the product (75). Master Blaster separates RNA from DNA and cellular debris resulting in the collection of total RNA (experiment #137).

At 6 weeks of age, the celiac bifurcation of two female and one male WC pigeons was surgically removed using the same protocol as described for the WC pigeons at 1 day of age except, RNA*later*® was not used. The reason for this was simply due to the fact that tissue at 6 weeks of age is larger, making tissue collection easier. This allowed for the celiac bifurcation of the birds to be collected on the same day and was followed immediately by RNA extraction using TRIzol® (Invitrogen) instead of Master Blaster, according to the standard protocol provided with Trizol® (76). The result was the successful collection of total RNA (experiment #190a-2). At 6 months of age, the celiac bifurcation was surgically removed from 3 males and 2 females according to the same procedures outlined at 6 weeks of age resulting in sufficient amounts of total RNA (experiment #162).

Complementary DNA synthesized from total RNA of 2 similar populations, is the starting material for RDA (69, 71). Once 1 ug of total RNA was prepared at each age as previously laid out the process of synthesizing cDNA ensued (63). The BD SMART ™ PCR cDNA Synthesis Kit (BD Biosciences Clontech Division #K1052-1), which includes special oligo dT primers, was used to synthesize single stranded (ss) DNA from mature mRNA with Poly-A tails by reverse transcriptase. This was followed by second strand synthesis of DNA to yield full length double stranded (ds) cDNA. An advantage of the

SMART ™ program is that as little as 50ng of total RNA are needed to select for mRNA in order to produce and amplify cDNA in sufficient amounts for RDA (63). Finally, the ds cDNA was placed in a -80° C freezer for storage awaiting the following RDA experiments.

Quantitative Analysis, Matching and Size Distribution of cDNA

Eighteen samples of cDNA (6 samples from each age 1 day, 6 weeks, and 6 months) were transferred from the -80° C freezer to the -20° C freezer for short-term use. Standard aliquots of 2ul from each sample of cDNA in a total volume of 70ul Tris-EDTA (TE) buffer (pH 8.0) were quantitated using spectrophotometry at 260nm (A260) to determine yield. Next, 0.7ug of cDNA from each sample was run on a 1.2% surface tension gel with 10ul ethidium bromide (0.01mg/ml) at 85 volts for 30 minutes. A photograph of the cDNA samples under UV light using the MultiDoc-It Digital Imaging System (UVP Model M-20) revealed a poor pattern of fluorescing as most of the lanes appeared blank. Each of the cDNA samples should appear at this stage as a smear (as opposed to distinct banding patterns) that range between 300 and 7000 base pairs (bp) with the greatest intensity between 2000 to 3000 base pairs to be considered for RDA (63, 72). It was then decided to rerun the samples using a slightly thinner gel and to increase the ethidium bromide to 15ul to ensure fluorescing of any cDNA smears that are present. In addition, the volume was set at 1ul therefore increasing the amount of the cDNA in each sample from 0.7ug to at least 1.0ug. These changes resulted in quality smears in 88% of the cDNA samples. Precise matching of cDNA smears according to bp size distributions is essential to ensure that differentially expressed products are reflective of two similar

comparisons (72). The three selected ages were visually matched, and stored at -20° C to await RDA.

Representational Difference Analysis (RDA)

As described previously in the Literature Review, RDA is a multistep enrichment technique that couples SH with PCR to discriminately select and amplify only transcripts that are differentially expressed between two populations of cDNA. The RDA protocol, provided by Pastorian et al., (72) was systematically modified in this laboratory by Anderson (63) to minimize sample losses during multiple chemical and physical manipulations.

A. Amplicon Preparation of cDNA by Restriction Digest

Five micrograms from previously matched 1 day and 6 week WC pigeon cDNA samples (comparison one), were each combined with 4.44ul DpnII enzyme (10U/ul; New England BioLabs R05431), 10ul 10X DpnII buffer and 18.2 Mohm water (in a total volume of 100ul), and digested in the MasterCyler Gradient Thermal Cycler (TC) (77) (Eppendorf #5331; 115V), for 18 hours at 37° C. DpnII is a four-cutter enzyme that recognizes and cuts at the 5 prime end of the GATC site of double stranded cDNA leaving "sticky" ends that allow for ligation of primers and amplification during subsequent steps. In addition, DpnII cuts the cDNA into amplicons that are on average 256bp, (ranging in length from 200 to 1000bp) making them manageable in length which ensures amplification by PCR (69, 72).

Following digestion, both samples were transferred to Phase Lock Gel tubes (PLG) (Eppendorf # 955154151) and extracted first with 100ul of phenol-chloroform-isoamyl

alcohol (PCI) and centrifuged (Eppendorf Model #5415D) at 14,000xg for 5 minutes followed by the addition of 100ul of chloroform and repeated centrifugation. The advantage of the PLGs is that they separate proteins and organics from the aqueous cDNA phase and optimize recovery of cDNA.

The recovered 100ul aqueous cDNA was run through Micro-spin S-300 HR Spin columns from Sephacryl® and centrifuged at 735xg for two minutes to remove small pieces of cDNA less than 100-150bp in length and any remaining restriction reagents. The samples were incubated overnight at -20° C in a mixture of 1ul glycogen (20 mg/ml), 0.1 volume (10ul) 3M sodium acetate (pH 5.3) and 2.5 volumes (250ul) of 100% molecular grade ethanol to precipitate the cDNA out of solution further purifying the pellets.

The following day both tubes were centrifuged at 16,000xg for 18 minutes to pellet the cDNA. Each pellet was washed with 100ul of 70% ethanol for an additional 2 minutes to precipitate the glycogen and salt, purifying the cDNA. The supernatant was carefully, vacuumed off from the cDNA using a 10ul pipette tip to help prevent loss of the pellet. The rim of the tubes were wicked dry with a paper towel and laid on their side to air dry. Drying each cDNA pellet as much as possible facilitates the resuspension of cDNA in 20ul of 1X TE buffer. Resuspended cDNA was stored at -20° C for no more than 2 days or was used immediately in the ligation of primers.

B. Preparation of Primers for Ligation to Drivers

The 12 and 24 primer sets presented in **Table 3** purchased from Genosys (a subdivision of Sigma), were recommended by Pastorian (72) and successfully used by Anderson (63). The non-complementary interchangeable primer sets allow for ligation of

primers to targeted cDNA populations, which prevent the unintentional amplification of cDNA populations from previous rounds of RDA. This results in specific amplification of true difference products.

Table 3: 12 / 24 RDA Primer Sets

Primer Set	**12mer (5'-3')**	**24mer (5'-3')**
A Driver	GATCAATAACTA	TGACGGACCGGTTGCGTAGTTATT
B Tester Round 1	GATCTTATGGCT	AGACAGTGCCGGATGTAGCCATAA
C Tester Round 2	GATCCTATTGAC	ATCTCAGGGGACCTGAGGCAATAG
D Tester Round 3	GATCCAGATGTA	ATACGTGCAGGCTGGTTACATCTG
E Tester Round 4	GATCTTATACTA	TAACCTCGGCCCTCGGTAGTATAA
F Tester Round 5	GATCTACGTACT	TCACATCGCCCCCTATAGTACGTA

(63)

To prevent the hydrolysis of lyophilized primers from frequent thawing and refreezing throughout RDA, each of the (12mers and 24mers) primers were dissolved in specified amounts of 1X TE buffer according to the molecular weight (MW) provided by Genosys. The absorbance (A260) of each of the primers was determined and calculated with the manufactures MW, along with the dilution factor to determine the actual molecular weight of each primer dissolved in 1X TE buffer. Two different sets of aliquots were then prepared in single use tubes, one set for the A primers and another set for primer sets B-F and are as follows. Primer set A included: 12/24Amers (4.5ug and 9.0ug) and

24Amers (25ug). Each of the primer sets B-F included aliquots of 12/24 primers (4.5ug and 9.0ug), 24 primers (4.5ug) and 24 primers (18ug) that were stored at -20° C, ready for use. Primer sets A 12/24mers were used to prepare drivers for each of the three comparisons. Primer sets B through D 12/24mers were used to prepare testers for each of the 3 rounds in each of the three comparisons to prevent unintentional amplification from previous rounds throughout the RDA experiments. Primer sets E and F were not used during the following steps however, were prepared in the event that additional rounds were conducted, or as alternate primers during the preparation of drivers or testers.

C. Ligation of Primers to Drivers

The12/24mer primer set A mix (4.5 ug/9.0ug) was removed from the -20° C freezer, thawed, pulse spun, and kept on ice. An aliquot of 9ul of 10X ligation buffer (New England Biolabs) was thawed (kept on ice) and added directly to the 12/24mer mix followed by enough Mohm water to bring the total volume of this core mix up to 45ul. Both cDNA samples were removed from the -20° C freezer and thawed, and the total contents (20ul) were transferred to thin-walled 0.5ml PCR tubes. Twenty microliters of the core mix was then added to the PCR tubes containing the cDNA to bring the total volume to 40ul. The PCR tubes were placed in the preheated TC at 55 ° C, preset to the "Slow Cool" program, designed and optimized by Anderson (63). The program runs for approximately 40 minutes and holds at 8° C until samples are removed. During this step, the 12mers form temporary bridges with the 3 prime blunt ends of ds cDNA while the GATC site on the 12mer anneals with the CTAG overhang on the 5'end of cDNA forming hydrogen bonds. This allows the 24mers to temporarily anneal with nucleotides of the

12mer and hold them in place in preparation for ligation of the 24mers to the GATC sticky ends of the cDNA in the next phase.

A core mix containing 11ul 10X ligation buffer, 90ul Mohm water, and 9ul T4 DNA ligase enzyme (New England Biolabs # M0202S) was made. Fifty microliters of this mixture was transferred directly into the PCR tubes that contained the cDNA mixture, and was incubated at 15° C overnight. During this step, the 24mer is ligated to the CTAG 5' sticky end of the cDNA forming a covalent bond that was previously restricted by DpnII. Following the ligation step, the cDNA products were incubated at 65° C for 10 minutes to deactivate the ligase. Since the 12mers are not phosphorylated they easily dissociate from the 3' end of the cDNA when incubated at 65° C (18).

The cDNA samples were run through S-300 spin columns as previously described to remove ligase, buffer, 12mers, and any tags less than 100-150bp in length. The recovered volume of the eluate primer-ligated cDNA products was increased by 10ul of Mohm water and the 100ul total volume was placed on ice.

D. PCR Amplification of Drivers

A core mix of 250ul 10X PCR buffer, 400ul 25mM $MgCl_2$, 62.5ul 10mM dNTPs and 1487.5ul Mohm water was prepared. Of that, 240ul was transferred into another tube and placed on ice to use with Taq DNA Polymerase (5U/ul Promega Corporation M1865). To the remaining core mix, 15ul of the same 24mer set-A (25ug) used to ligate primers onto drivers was added with 85ul of Mohm water to bring the total volume to 2060ul. The core mix was divided equally between the two ligated cDNA populations and then 180ul was transferred to 6 new PCR tubes for each population. The 12 PCR tubes were placed in

the preheated TC at 72° C, and incubated for 1-2 minutes to fully extend the cDNAs, and to create primer-binding sites on the 3' ends. Thirteen microliters of Taq polymerase was added to the 240ul of core mix sitting on ice, and 20ul was added to each of the 12 PCR tubes incubating in the TC. The PCR driver program was run for an additional 5 minutes at 72° C to fully extend the double stranded 24mer cDNA amplicons followed by 25 cycles that alternated between denaturing at 95° C for 45 seconds, followed by 4 minutes at 72° C to amplify the cDNA. A final incubation at 72° C for an additional 10 minutes ensured extension of the cDNA drivers. The populations were pooled, extracted with PCI and chloroform, precipitated, washed, vacuumed as previously described, and then resuspended in 100ul of TE buffer.

E. Removal of Driver PCR Primers by DpnII

After the completion of amplified drivers, a restriction digest comprised of 90ul of cDNA driver, 10.5ul of 10X DpnII buffer and 4.5ul DpnII enzyme was incubated overnight at 37° C to remove the primers, regenerating the 5' sticky ends. Both cDNA populations were phase extracted with PCI and chloroform, purified with S-300 spin columns, followed by an overnight precipitation, ethanol wash, vacuuming, and finally resuspended in 50ul of 1X TE buffer.

The yield of amplified restricted drivers was determined by measuring absorbance at A260 using an aliquot of 1.5ul of cDNA in a total volume of 70ul TE buffer. Aliquots (0.5ug) of restricted cDNA drivers and unrestricted drivers were run on a 2.0% agarose gel at 75V for 60 minutes, stained with 5ul ethidium bromide (0.01mg/ml), and photographed under UV light. Size distributions of the driver amplicons should appear as a smear as

opposed to distinct bands, ranging between 150-1000bp (72). While bands are not usually visible until follow up rounds, which reflects the removal of cDNA lengths common between both populations, visual analysis revealed that distinct bands were present in all of the driver populations. The banding patterns ranged mostly between 250-750 bp with the brightest intensity around 500 bp (well within the recommendations), therefore verifying successful amplification and restriction of drivers and an abundance of differentially expressed transcripts. Visual comparison between primer-ligated cDNA and restricted cDNA revealed successful restriction digest by DpnII. Drivers were frozen at -20° C until the following step.

F. Preparation of Testers

Each of the driver products were used to create the tester population of round one. The ligation procedure is primarily the same as that performed for drivers except, that a new interchangeable set of primers was used in each successive round of RDA to prevent the unintentional amplification of transcripts from previous rounds. One microgram of driver, along with a new set of B 12/24mers, was used to perform ligation in a total volume of 60ul using the "Slow Cool" program. Following overnight enzymatic ligation at 15° C, the TC was heated to 65° C for 10 minutes to deactivate the ligase. The newly ligated cDNA products served as the testers in the subsequent steps of subtractive hybridization.

G. Subtractive Hybridization of Species Common to both cDNA Populations

In each round of SH, the amount of driver used remained constant (5.0ug). The amount of tester on the other hand was reduced in each consecutive round (1:10, 1:100 and 1:5000) to ensure that only differentially upregulated products between the comparisons will result (72). Thirty microliters (0.5ug) from the newly ligated tester populations was transferred into new tubes. Five micrograms from each driver was then added to the tester populations. The total volume in each tube was adjusted to 100ul by adding 1X TE buffer resulting in two reciprocal SH reactions. Both tester:driver populations (C-1, 1Day:6weeks and 6weeks:1Day) were then extracted with PCI and chloroform, precipitated for a minimum of 4 hours or overnight at -20° C, washed, vacuumed, and resuspended in 4ul of hybridization buffer which helps to facilitate the hybridization reaction of like proteins (72).

One microliter of 5M sodium chloride (NaCl) was added to two new PCR tubes, and then placed in the TC. Both of the resuspended (4ul) tester:driver populations were placed in the TC at 95° C, with the PCR tubes containing NaCl for one minute, to denature the double stranded cDNA of the tester and driver populations and heat the NaCl. The contents (4ul) of the tester:driver populations were quickly transferred into the tubes containing NaCl (facilitates hybridization reaction), followed by the addition of 20ul of mineral oil to prevent dehydration and loss of material. Both of the PCR tubes were returned to the TC and incubated at 95°C for an additional 3 minutes, to ensure that all the cDNA species were denatured. The TC was reset to 67° C and hybridization was allowed to proceed for 24 hours followed by the addition of 45ul of Mohm water to each of the completed hybridization reactions. Both tubes were placed in the -20° C freezer for

storage, or an aliquot of 10ul from each SH population was transferred into 2 new PCR tubes to be used for amplification.

H. PCR Amplification of Round One Subtractive Hybridization Products

Amplification of the SH products results in three possible outcomes. The first experiences no amplification because hybridized driver:driver populations lack primers on both of their 5'ends. The second products, tester:driver ideally have primers ligated to the 5' end of the tester and therefore experience linear amplification at best. Finally the target population, tester:tester contains primers on both the 5' ends of hybridized cDNA which are amplified exponentially. Although drivers are much in excess of testers, the absence of primers and the exponential amplification of targeted testers cause the drivers to become diluted beyond detection due to such low copy number.

A dilution step added during the extension step of the 7^{th} cycle of PCR at 72° C effectively replaced the use of mung bean nuclease (72) ensuring that any linearly amplified single stranded cDNA populations were reduced below the point of detection. This was carried out by the addition of 4 new PCR tubes for each tester:driver population which contained 180ul of core mix and included the same B 24mers that were used during the ligation step along with the addition of 20ul Taq mix. Finally, aliquots of 10ul were transferred from the tester:driver populations already in the TC to each of the newly added PCR tubes for a total volume of 210ul. The TC was resumed for an additional 20 cycles to ensure that only exponentially amplifiable differentially upregulated products would be present. Following PCR, the products were pooled, extracted with PCI and chloroform,

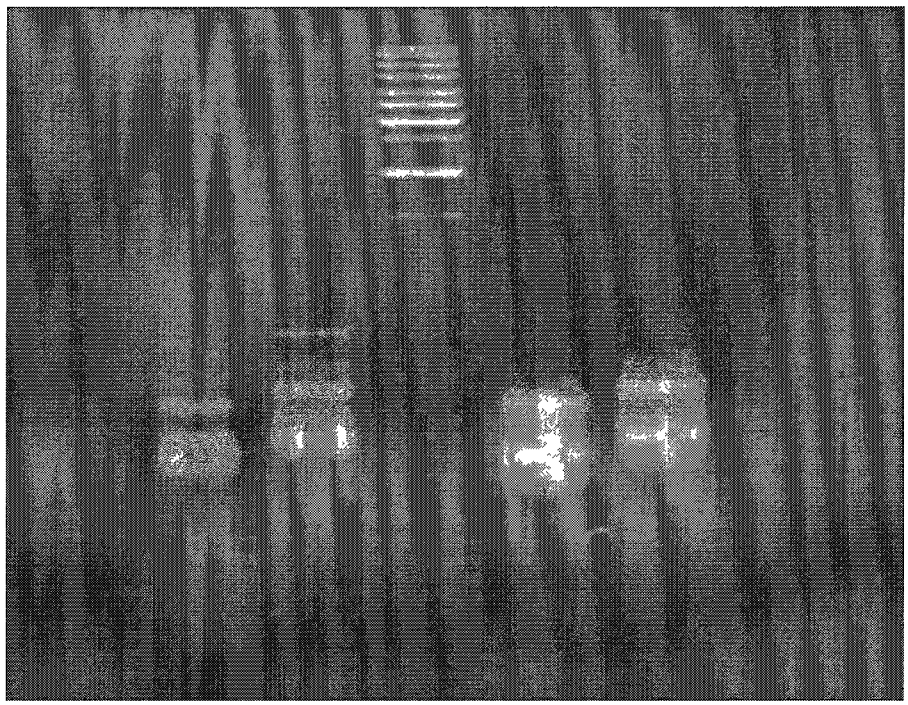
precipitated, washed, vacuumed, and resuspended in TE buffer to yield 100ul, that were labeled as Round One Difference Products (DP1).

I. Removal of PCR Primers by DpnII

Eighty-seven microliters of amplified DP1 was transferred into new tubes. Ten microliters of 10X DpnII buffer and 3ul DpnII enzyme were added for a total volume of 100ul in each tube. The tubes were incubated overnight at 37° C to restrict the 24Bmers from the tester populations and regenerate 5' sticky ends. The next day the round one, difference products were extracted, purified, precipitated, washed, vacuumed and resuspended in a total volume of 50ul 1X TE buffer.

Both difference products were quantified by measuring absorbance at 260nm using 5ul from each DP1 in a total volume of 500ul 1X TE buffer. Calculations revealed the amount of cDNA ranged between 30-45ug for each of the rounds throughout the 3 comparisons, which was well above the recommended 10-20 ug as suggested by Pastorian (63, 72). Five micrograms from the DP1 products were separated on a 2% gel at 75V for 60 minutes, and stained with 5ul ethidium bromide (0.01mg/ml) to assess size distribution patterns. Although bands are not expected until the second or third rounds of RDA (72) visual analysis revealed distinct bands (**Figure 2**) ranging between 250 and 750 bp, well within the recommended 150-1000 bp. This was the case in all rounds of the three comparisons.

Figure 2: Round One Difference Products (DP1) 1 Day and 6 Weeks



1 day (digested), 1day *10kb* 6 weeks (digested), 6 weeks

J. Rounds Two and Three of RDA

In each successive round of RDA, the amplified differentially upregulated products from the previous round of SH served as the tester for the next round. For example, the amplified difference products from round one (DP1) became the tester for round two, and the products from round two became the tester for round three. In rounds two and three the procedure used for the ligation of primers onto the 5'end of testers for SH and amplification of difference products was the same as for round one except in round 2, 12/24mer C primers were used, and for round 3, 12/24mer D primers were used. In each round of SH, the amount of driver (5.0ug) remained constant, while the amount of tester was decreased from 0.5ug in round one, to .05ug in round two, to .001ug in round three. During round two of PCR, the samples were subjected to the same number of cycles as in

round one; however, in round three, during the second half of PCR, the number of cycles was increased from 20 cycles to 25 cycles. Following PCR, the amplified hybridized testers went through the same restriction and purification steps as in previous rounds. Determination of yield and gel analysis also followed the same steps as in the previous rounds. The yield for each round was well within the recommendations as was the size distribution patterns of each of the gels.

Storage of digested cDNA for more than two weeks can result in the ligation of sticky ends to one another (63). Frequent digests can result in the loss of cDNA yield. Therefore, since the round three products (DP3) were to be cloned simultaneously, DpnII digestion of the DP3 products from all three comparisons were conducted together just before cloning.

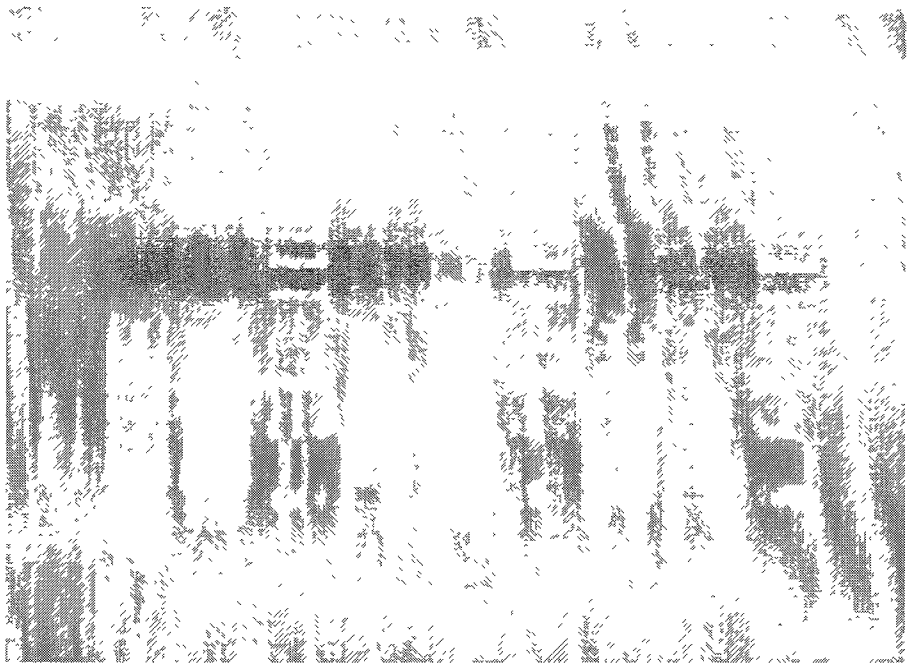
Cloning of Subtracted Round Three Difference Products

The pBluescript II SK+ (pBS) Vector (Stratagene #212205) was selected due to its easy ligation of cDNA inserts at the BamHI site (63). A restriction digest using BamHI enzyme (10U/ul, Fermentas), as well as dephosphorylation with calf intestine alkaline phosphatase enzyme (CIAP, Stratagene #600015), was necessary to prepare the vector for the cDNA inserts. Five microliters of the pBS vector, 5ul 10X BamHI buffer, and 5ul BamHI enzyme (10U/ul) in a total volume of 50ul was digested at 37° C for 1 hour. This step was followed by dephosphorylation with 30ul CIAP enzyme, and 10ul 10X CIAP buffer in a total volume of 100ul that was incubated in the TC for 30 minutes at 37° C and then adjusted to 68° C for an additional 15 minutes. The CIAP catalyzes the removal of phosphates from the 5' end of cDNA, which prevents re-ligation to the 3' end (78). This

mixture was extracted with 100ul PCI and chloroform, precipitated, purified with S-300 spin columns, washed, and vacuumed as explained in previous methods. The pBS vector was resuspended in 5ul of 1X TE buffer, and stored at -20° C until its use. One microliter of pBS vector in a total volume of 50ul 1X TE buffer was used to determine yield at A260 followed by running 0.3ug of vector on a 1.2% gel with 6ul ethidium bromide at 85V for 25 minutes. The gel revealed a successful dephosphorylated linearized pBS Vector that appeared as a single band located at 3KB.

The final digestion of the DP3 products from the three comparisons was then carried out using 3ul of DpnII enzyme in a total volume of 100ul in the same manner as in previous ligations. Digested DP3 products were followed up with extraction using PCI and chloroform, precipitation, purification, wash, and vacuum of populations followed by, the resuspension of each DP3 product in 50ul 1X TE buffer. Five microliters of each DP3 in a total volume of 500ul TE buffer was used to determine yield at A260. Aliquots of 0.5ug of cDNA from each sample were run on a 1.2% gel at 75V for 60 minutes, and stained with 5ul ethidium bromide (0.01mg/ml) as previously described. Results in **Figure 3** revealed distinct banding patterns and appropriate size distributions to proceed forward with the method.

Figure 3: Round Three DpnII Digestion of All Three Comparisons



1 day:6 weeks *10kb* 1 day:6 months *10kb* 6 weeks:6 months *10kb*

Stratagene recommended that the concentrations of vector and inserts should be 0.1ug/ul (78). These concentrations were determined by Anderson to work best when in a ratio of 1:2 (insert: vector) during the ligation reaction (63). Rather than dilute the whole cDNA DP3 population and risk degradation, 2ul was aliquoted from the total volume (63). This aliquot was then diluted to the recommended concentration of 0.1ug/ul of which 0.5ul was then combined with 1.0ul of vector, 0.5ul T4 DNA ligase, and 1.0ul T4 buffer in a 10ul total volume that was incubated at 4° C overnight.

An LB/agar bacterial growth broth comprised of 10g NaCl, 10g Tryptone, and 5g Yeast, in a total volume of 1 liter of Mohm water at a pH of 7.0 was then mixed with 15g Agar and autoclaved. The broth was cooled to 55° C followed by the addition of 8ml X-gal (10mg/ml, Promega), 0.5ml IPTG (100mM, Promega), 1.0ml ampicillin (50mg/ml) and

2.5ml tetracycline (5mg/ml). This was poured into plates and allowed to set overnight at room temperature according to the XL1-Blue MRF' Supercompetent Cells protocol (79).

The following day the pBS vector-ligated difference products were transformed into the XL1-Blue MRF' Supercompetent Cells (XL1-cells). A vial of SOC Medium (is a nutrient rich medium that improves transformation efficiency) was transferred from -20° C to a 42° C water bath to slowly thaw the SOC. Six 14ml tubes were chilled on ice while the XL1-cells were removed from -80° C and were slowly thawed on ice. Once thawed, 100ul of the XL1-cells were transferred into each of the 14ml tubes, followed by 1.7ul B-Mercaptoethanol (serving as a reducing agent to cleave disulfide bonds). The contents were stirred gently every 2 minutes for 10 minutes (while on ice) so not to damage the cells. Next 2ul of pBS vector-ligated cDNA was transferred into the respective transformation tubes, which were stirred and incubated for an additional 30 minutes. All six transformation tubes were placed in a rack and heat pulsed in a 42° C water bath for precisely 45 seconds. Next, 900ul of the pre-warmed (42° C) SOC was transferred into each of the six 14ml transformation tubes, which were then shaken, at 230 rpm for 1 hour at 37° C to allow the cells to produce approximately one generation. Next, the Agar treated growth plates were warmed at 37° C and labeled to include 5 plates for each transformation, with one serving as a titer plate. Five microfuge tubes for each corresponding transformation reaction were labeled, and 2ul of transformation mixture was added to the 6 tubes that were labeled as titer tubes, while 40ul was added to the rest of the tubes (4 for each transformation). Next, 198ul of SOC was added to the 6 allocated titer tubes, while 160ul was added to the rest of the tubes for a total of 200ul in each tube. Finally, 200ul from the tubes were plated onto their respective plates, and allowed to soak

in for 15-20 minutes. The plates were then inverted, and incubated at 37° C to allow the colonies to multiply exponentially, overnight. Refrigeration of the plates followed to enhance the blue/white selection of the clones.

Colonies of pBS vectors containing inserts will remain white because the inserts disrupt the coding region on the lacZ gene fragment of the vector, and those without inserts will produce blue colonies (78). The $lacI^{q}Z\Delta M15$ lac gene is carried on the F' episome region of the XL1-Blue MRF' Supercompetent Cells and allows for this blue/white selection (78). Ampicillin together with tetracycline, further selects for colonies that contain both the pBS Vector and F' episome respectively to help reduce the incidence of false positives (78). The use of tetracycline was reported by Anderson to reduce the incidence of false positives after she experienced a large number of false positives following the hand selection of (white) clones, only to find they contained no inserts (63).

A total of 2304 clones were picked by hand (768 for each comparison) using sterile toothpicks and placed one in each of the 96-deep well plates containing 400ul Ampicillin-2XYT media. These were sealed with Air Pore tape (Rainin) to allow respiration of the clones, while shaken at 180rpm for 17-18 hours and incubated at 37° C. The next day, 140ul of 60% glycerol was added to each well, and the plates were then sealed, wrapped and placed in the -80° C freezer to await Rolling Circle Amplification (RCA).

Positive and negative controls were conducted using the XL1-cells and pUC18 control plasmids supplied by Stratagene to evaluate the blue white selection of the XL1-cells and to ensure their quality. Since the pUC18 plasmid does not contain inserts, the colony forming units (cfu), should remain blue for the positive control. This was the case, with too many cfu present to count (**Table 4**). As anticipated, the negative control resulted

in no cfu since pUC18 vector was not added to the XL1-cells. Titer plates (presented in **Table 4)** were also conducted to estimate the number of cfu for blue (pBS-no cDNA) and white (pBS-cDNA) inserts, and for the potential number of inserts in each transformation reaction. The controls correlated well with the outcomes described by Stratagene.

Table: 4: Controls for Blue White Selection of XL1-Cells, pBS Vector & Inserts

Titer Plates and Positive and Negative Control								
Comparison	Age	# Blue Colonies in Titer plate	# White Colonies in Titer Plate	Total # Colonies in Titer Plate	# Potential Blue Colonies in Transformation	# Potential White Colonies in Transformation	Total # Potential Colonies in Transformation	% Contain Inserts for Titer and Transformation
C-1	1Day	9	76	85	4517	38141	42657	89%
C-1	6Weeks	4	56	59	2007	28104	29609	95%
C-2	1Day	3	99	102	1506	49683	51189	97%
C-2	6Months	5	78	83	2509	39144	41653	94%
C-3	6Weeks	2	36	38	1004	18067	19070	95%
C-3	6Months	2	16	18	1004	8030	9033	89%
Average:								93%
Negative Control: Cells without vector resulted in zero colonies								
Positive Control: Cells with vector were blue and resulted in too many colonies to count								

The results for the titer plates in **Table 4**, revealed that on average, 93% of XL1-cells contained pBS-cDNA inserts. These findings allowed proceeding to the next step of purifying and amplifying the cDNA inserts by Rolling Circle Amplification (RCA).

Rolling Circle Amplification (RCA) of Bacterial Cultures

The RCA Tempi Phi 500 Amp kit (Amersham Biosciences, #25-640-50) was selected to prepare the cDNA inserts for sequencing by releasing them from the XL1-cells followed by amplification. The protocol was adjusted by Anderson to use half the amount of the recommended reactants while providing the same results, and doubling the number of reactions that could be conducted with a single kit (63). The following procedures were the same throughout for each of the 24, 96-well plates. A cloned 96-deep well plate was

removed from -80° C and slowly thawed at room temperature for two hours or overnight at 4° C.

With a multi-channel pipetter, 2.5ul of Sample Buffer (5X 0.5ml) and 0.8ul of the cloned XL1-cells were added to each well of a new shallow 96-well plate. The plate was sealed with strip caps, and pulse spun in a plate spinning head in the Beckman/Coulter Allegra 25R centrifuge, for 25 seconds at 5700 rpm and then placed in the preheated TC at 95° C for precisely 3 minutes to lyse the XL1-cells and release plasmid vector-cDNA inserts from the cells, without denaturing DNA. The plate was removed and immediately placed on ice while preparing a master mix of 250ul Reaction Buffer (5X 0.5ml) and 10ul Enzyme Mix (5X 20ul). An aliquot of 2.5ul from the master mix was then added to each well of the cooled plate bringing the total volume to 5.8ul. The plate was again sealed with the strip caps, and pulse spun for 25 seconds and placed in the preheated TC at 30° C for 16 hours. The temperature was adjusted to 65° C for 10 minutes to denature the enzyme, and was then adjusted to 4° C until removal of the plate from the TC. The plate was then pulse spun for 25 seconds to collect the contents. Ten microliters of Mohm water were added to each well bringing the total volume to 15.8ul, and the plate was pulse spun again.

The following restriction digestion reaction was conducted to determine whether the vector was in fact released from the XL1-cells, and to assess whether the pBS vector contained an insert. The TC was preheated at 37° C while a master mix of 11ul Bovine Serum Albumin (BSA) (10mg/ml), 110ul 10X Multi Core Buffer, 329ul Mohm water, 50ul Hind III enzyme (10U/ul), and 50ul XbaI enzyme (12U/ul) were added to equal a total volume of 550ul. The Hind III and XbaI are six cutter enzymes that recognize and cut 24 bp upstream and 8 bp downstream from the BamHI site respectively. Using a new shallow

96-well plate, 5ul of the master mix was pipetted into each well with the multi-channel pipetter. Strip caps were removed from the RCA plate and 5ul from the total volume of 15.8ul was transferred from each RCA well into the respective wells of the new plate containing the master mix. Strip caps were used to seal the wells, and the plate was pulse spun for 25 seconds and then placed in the preheated TC at 37° C for 2 hours.

The digestion plate was removed from the TC after 2 hours and pulse spun to collect the contents. The strip caps were removed to pipette 4ul of gel loading dye into each well for a total volume of 14ul. The wells were again covered and pulse spun. Using the multichannel pipetter, 7ul aliquots were transferred row by row from the 96 digested samples, and were run on a 1.2% gel, with 7.5ul of ethidium bromide in a large electrophoresis gel bed at 85V for 30 minutes. The gel was photographed under UV light as previously explained to assess the location of XL1-cells, pBS Vector and the digested cDNA inserts. Acceptable gels revealed three main components. The XL1-cells were located at the wells, while the pBS Vector formed a distinct band at 3000bp. A third band, the cDNA inserts, was located below 1000bp. To proceed, at least 88 of the 96 pBS Vectors had to contain cDNA inserts visualized on the gel below 1000bp before the entire plate could be submitted for sequencing (63).

A new 96-well shallow plate was used to prepare cDNA inserts for sequencing. A master mix of, 4.8ul M13 reverse primer (M13R) (2uM) was prepared in 451.2ul MOhm (nuclease free) water. Of the 456ul total volume, 3.8ul aliquots were pipetted individually into each well. Aliquots of 1.2ul from the thawed and pulsed RCA plate were pipetted into each well using the multi-channel pipetter. The wells were sealed and the plate was pulse spun for 30 seconds, wrapped in cellophane and aluminum foil, labeled with a pre-assigned

ID number and submitted on dry ice to the Hubbard Center for Genome Studies (HCGS) at the University of New Hampshire for sequencing of the differentially expressed RDA transcripts in the ABI3130 genetic analyzer. The M13 Reverse primer (5' GGAAACAGCTATGACCATG 3') was chosen to amplify the target inserts from the pBS Vector (63). The results were provided from the Genome Center in digital file format, which included the individual sequences, quality scores in "fasta" format, and chromatograms to help determine the quality of the sequences (63).

Sequence Analysis and Characterization of Differentially Expressed Products

To begin analysis of the 2626 sequences provided by the Hubbard Genome Center, the VecScreen program within the National Center for Bioinformatics (NCBI) data base, provided the ability to flank nucleotide sequences for vector contamination or M13R primer and to determine the quality of the nucleotide sequences. The process began with uploading a sequence into VecScreen to look for vector contamination within the nucleotide sequences. The sequences were matched with known vectors in the data base and reported as strong, moderate, weak, or suspect. Strong matches were always cut at the recommended nucleotide sequence sites, most often removing both sides of the retained quality sequence.

DNAClub is an open freeware program created by Xiongfong Chen at http://128.84.203.244 while a student at Cornell University. This program was used to highlight and cut the identified vector nucleotide sequences that were flanked by VecScreen. This program also allowed one to search for the GATC restriction sites and verify a match with VecScreen before removing nucleotide sequences. In addition, this

program provided the ability to search for all GATC sites throughout the sequence to determine whether more than one sequence was ligated together at the sticky ends. This was the case more often than not, and allowed for individual analysis in a later step to search for and identify multiple genes within a single sequence.

Matches that were suspect in VecScreen, were cross evaluated using FinchTV Version 1.4.0. located at www.geospiza.com. Finch TV is a very useful program that provides a chromatogram of the nucleotide sequence patterns displaying peaks and changes that help to further confirm the quality of a sequence that is suspect. Patterns that consistently revealed strong peaks, yet were identified as suspect in VecScreen, were retained for further analysis. Sequences that revealed small or rounded peaks or many changes in the size and shape of peaks were considered poor quality and were discarded.

Once sequences were evaluated and determined to be quality sequences, they were trimmed and saved in a file later to be queried against a database of known gene sequences. The Basic Local Alignment Search Tool (BLAST) or BLASTn located within NCBI's data base was used to match quality nucleotide sequences with known genes and identify potential proteins for the sequences. E-values established in the database were used to indicate a level of confidence between matches. A typical cutoff used in this lab was e-4 or less. After much discussion a more conservative cutoff of

e-10- or less was adopted for this research, to indicate greater confidence that a match was not by chance. Gene sequences meeting the criteria were accepted as quality sequences. Those that were matched with a known gene sequence and met the e-value were "identified" genes. Those that were quality sequences but didn't meet the e-value criteria of a quality match in BLASTn remained "unidentified".

Sequences that were labeled as "null" had been either Vec-screened and/or underwent BLASTn and were identified as vector or nothing at all, had a poor chromatogram, and or a high e-value.

Annotation, Placement, Regulation and Interactions of Genes

The MetaCore Software Program located at www.Genego.com is a licensed online database that incorporates a systems biology approach to fully annotate and link genes of interest to hundreds of available metabolic pathway maps. This approach, provides visual analysis of the placement of genes, their interactions and the cascade of events that result in a given pathway. Detailed descriptions of the pathways are linked directly to the pathway maps. In addition, the individual genes in a given pathway map are linked to a wealth of information regarding annotation, gene-gene interactions, and metabolic functions within a given pathway. Some of the additional benefits available with MetaCore include, detailed descriptions of multiple functions that many of the genes are known to participate in, links to known diseases, pharmaceutical research, NCBI, and Pub Med's database, as well as the use of E-Z search engines to access gene information. MetaCore also offers the ability to build direct networks (which include all the genes known to participate between the selected genes of interest) or indirect networks (only displays selected genes of interest), and the ability to create custom pathway maps between selected genes (not accessed during this project).

Once genes were identified in NCBI's BLASTn database, they were then retained in excel to be later analyzed further for their potential role in initiation and progression. MetaCore was utilized to fully annotate genes of interest, and to determine any gene-gene

interactions, and regulation within metabolic pathways in order to, distinguish between genes potentially involved in initiation and/or progression of atherosclerosis from those of normal development. Some of the candidate genes were also compared with unpublished in-vitro and in-vivo work conducted by Anderson (63) in this laboratory and are presented in Appendices Q – T (pages 147 – 150).

CHAPTER III

RESULTS

A total of 2304 cloned DPs were selected for further determination of their nucleotide sequences. **Table 5,** includes the raw sequence data, as well as Vector-Trimmed and BLASTed quality sequences from each of the three RDA comparisons of differentially upregulated DPs. Of the 2304 possible clones, 70 of the pBluescript (pBS) vectors lacked cDNA inserts. The remaining 2234 clones, resulted in a total of 2556 quality sequences. The unusual find of 111% quality sequences was due to occasions when more than one insert was ligated together at their GATC sticky ends within a pBS vector. Of the total number of quality sequences that were accepted, 2270 differentially upregulated sequence tags (ESTs) were identified using BLASTn leaving the remaining 286 sequences as unidentified.

Table 5: Raw Data and BLAST Results Summary

Pigeon Clones			Raw Sequence Data	Vector-Trimmed and BLASTed Quality Sequences							
	Breed & Age	Plate	Mean # bp raw	# NULL	%	# Accepted	%	Mean # bp query	# Identified	%	# Unidentified	%
	WC 1day	A	558	6		107		282	73		34	
		B	803	2		109		305	76		33	
		C	889	3		115		285	93		22	
		D	673	3		119		249	99		20	
RDA Comparison C_1	Total	384	731	14	4%	450	117%	280	341	76%	109	24%
	WC 6wks	A	672	2		103		311	97		6	
		B	665	5		105		294	104		1	
		C	667	2		113		314	107		6	
		D	641	0		116		312	111		5	
	Total	384	661	9	2%	437	114%	308	419	96%	18	4%
	WC 1day	A	635	1		109		316	89		20	
		B	668	4		96		290	75		21	
		C	649	1		97		253	73		24	
		D	702	6		98		274	76		22	
RDA Comparison C_2	Total	384	664	12	3%	400	107%	283	313	78%	87	22%
		A	649	2		100		260	92		8	
		B	672	4		94		255	84		10	
		C	676	1		104		284	92		12	
		D	707	9		96		247	89		7	
	Total	384	676	16	4%	394	103%	262	357	91%	37	9%
	WC 6wks	A	647	3		101		296	96		5	
		B	663	2		103		283	99		4	
		C	651	5		107		297	104		3	
		D	655	3		114		303	107		7	
RDA Comparison C_3	Total	384	654	13	3%	425	111%	295	406	96%	19	4%
		A	699	1		104		273	102		2	
		B	635	1		114		281	111		3	
		C	657	1		117		270	111		6	
		D	659	3		115		259	110		5	
	Total	384	663	6	2%	450	117%	271	434	96%	16	4%
Totals		2304	675	70	3%	2556	111%	275	2270	89%	286	11%

Table 5 represents the three ages that were compared against one another. Comparison one (C-1) 1day verses 6 weeks (1day:6wks), comparison two (C-2) 1day verses 6 months (1day:6mos), and comparison three (C-3) 6 weeks verses 6 months (6wks:6mos).

KEY: Raw data and BLAST results
Plate = ideal total # inserts per 384 wells/clones per age per comparison
Mean # bp +/- SEM raw = average # bp before Vec trimmed
NULL: is # of wells or clones that lack inserts
%Null: is % based on # absent inserts / # wells/clones
Accepted: = # of quality inserts accepted (after Vec trimmed and BLASTed)
% accepted: is based on actual # inserts accepted / # wells/clones
Mean # bp +/- SEM query: average # bp after Vec Trimmed & before BLASTed
indentified is # inserts Vec trimmed & BLASTed & identified
% identified: is % based on # accepted (# identified/ # accepted)
unidentified: is # inserts Vec trimmed & BLASTed but remained unidentified (is a quality insert with an e-value of > e-10) these are novel sequences not in data base
% unidentified is % based on # accepted

Presented in the following six tables, are lists of the differentially expressed genes from each of the three comparisons. Each of the tables, T**able 6a and 6b,** C-1 (1 day:6 weeks), T**able 7a and 7b**, C-2 (1 day:6 months) and T**able 8a and 8b**, C-3 (6 weeks:6 months) include the combined data from the four plates that were analyzed for each age

comparison, using NCBI's BLAST program, and presents the genes that were differentially upregulated between the ages compared. The colored asterisks in each of the six tables represent the rare instances when difference products were upregulated in both ages within a comparison. Gene's upregulated in this manner are color coded by an asterisk next to their gene name, so they can be easily tracked between the tables.

Totals for each age compared are located at the bottom of each column. The first represents the mean base pair length of all the quality sequences that were Vec-Screened and BLASTed. The "identified" represents the total number of quality sequences that were identified in BLAST (meeting the e-value cut off of e-10 or <) and includes annotated as well as non-annotated genes. The unidentified quality ESTs followed since they did not meet the e-value criteria of acceptance in NCBI. Since the unidentified ESTs did not meet the e-value criteria, it is not known whether any of the ESTs are the same or are all different genes. Additional efforts required to identify these ESTs were beyond the scope of this thesis. The total number of ESTs for an age includes the combined totals of the identified as well as the unidentified difference products. The total number of identified, unique, non-redundant sequence tags is represented in each of the tables as, "total number of identified non-redundant transcripts". Finally, in the instances when the actual "gene name" for the identified genes was not available, NA was used in the appropriate column.

Table 6a: Differentially Expressed Genes, Comparison 1 (1 Day Versus 6 Weeks)

Ortholog (C-1 1day Merged plates A-D)	Gene Name	Copy #
lumican (LUM)	LUM *	159
clone ChEST85g22	FBLN5	87
collagen, type I, alpha 2 (COL1A2)	COL1A2, COLA	26
telokin	MYLK	13
actin, alpha 2, smooth muscle, aorta (ACTA2)	ACTA2	10
similar to SEC61 gamma (LOC776639)	SEC61G	7
clone ChEST874k20	clone ChEST874k20	5
1B-3866 cytochrome c oxidase subunit I (COI)	COI *	4
BAC clone CH261-10P22	BAC CH261-10P22	3
leucine rich repeat containing 17	LRRC17	3
ATPsynthase FO subunit 8 (ATP8) gene	ATP8	2
dermatan sulfate proteoglycan decorin	DCN	2
fdx1 gene and 4 orf' s	NA	2
hypothetical LOC424163 (LOC424163)	424163	1
vascular smooth muscle alpha-actin (ACT-4)	ACT-4	1
ATP synthase F0 subunit 8 and 6 (ATPase 8, 6),	ATPase 6	1
BAC clone TGMCBa-23H12	BAC TGMCBa-23H12	1
klra17 mRNA for lectin-like transmembrane protein, Ly49q1 isoform (CDR-1)	CDR-1	1
clone 0064p0012D12 putative ribosomal protein	clone 0064p0012D12	1
collagen, type VIII, alpha I (COL8A1)	COL8A1	1
fatty acid binding protein 4, adipocyte (FABP4)	FABP4	1
hypothetical LOC424163	FASTKD1	1
fk506 binding protein 9, 63 kDA (FKBP9)	FKBP9	1
myoglobin gene	MB	1
NADH dehydrogenase subunit 4 gene	ND4 *	1
neomycin resistance cassette pNeo4 NeoTet (neoTet)	NEOTET	1
microsatellite marker vemos44	NA	1
CR1 gene for chicken repeat 1	NA	1
has operon deletion mutant	NA	1
Gene encoding methionine sulfoxide reductase	NA	1
mitochondrial DNA	NA	1
	MEAN (BP)	**280**
	[illegible]	**341**
	UNIDENTIFIED	**109**
	TOTAL (EST) of 384 wells	**450**
	TOTAL # identified non-redundant transcripts	**31**

Table 6b: Differentially Expressed Genes, Comparison 1 (6 Weeks Versus 1 Day)

Ortholog (C-1 6wks Merged plates A-D)	Gene Name	Copy #
heat shock protein 90 alpha	HSP90AA1	67
BAC clone CH261-126j23	BAC CH261-126j23	49
gelsolin (GSN)	GSN	43
clone 0064P0021C06 spermidine/spermine N1-acetyltransferase	SAT1	34
similar to ribosomal protein L3 (LOC771432)	RPL3	39
hypothetical LOC415891 (LOC415891)	*415891*	*13*
myosin heavy chain II smooth muscle (MYH11)	MYH11	9
clone ChEST398a16 (RPL32)	RPL32	9
ribosomal protein L4 (RPL4)	RPL4	9
mitochondrion	16S rRNA	7
clone ChEST189k11	clone ChEST189k11	6
clone ChEST118j5	clone ChEST118j5	5
eukaryotic translation initiation factor 3 subunit H (EIF3H)	EIF3H	5
rho-associate coiled-coil forming kinase 1 (ROCK1)	ROCK1	5
chromogranin B (secretogranin 1) (CHGB)	CHGB	4
DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 (DDX5)	DDX5	4
hemoglobin alpha-globin chain	HBA	4
lymphocyte antigen 6 complex locus E (LY6E)	LY6E	4
similar to NPD014 protein (NPD014)	NPD014	4
ribosomal protein S3A (RPS3A)	RPS3A	4
splicing factor arginine/serine-rich 10 (transformer 2 homolog drosophila) (SFRS10)	SFRS10	4
splicing factor arginine/serine-rich 6 (SFRS6)	SFRS6	4
beta2-microglobulin	B2M	3
cyclin-dependent kinase inhibitor p27 Kip1	CDKN1B	3
clone ChEST1007e10	clone ChEST1007e10	4
mitochondrial DNA	COII	3
mitochondrial DNA	ND4 *	3
similar to yeast ribosomal protein S28 homologue (LOC427323)	RPS28	3
alpha-tropomyosin gene exons 9c 9d and complete cds alternatively spliced	TPM1	3
clone 0062P0001C01 putative high-mobility group nucleosome binding domain 1 variant 1	clone 0062P0001C01	2
beta-actin	ACTB	2
acidic (leucine-rich) nuclear phosphoprotein 32 family member A (ANP32A)	ANP32A	2
alpha thalassemia/mental retardation syndrome X-linked (ATRX)	ATRX	2
BAC clone CH261-114A20	BAC CH261-114A20	2
clone ChEST398a16	clone ChEST398a16	2
destrin (actin depolymerizing factor) (DSTN)	DSTN	2
jagged 1 (Alagille syndrome) (JAG1)	JAG1	2
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10 42kDa (NDUFA10) nuclear	NDUFA10	2
similar to B-aggressive lymphoma 2B	PARP14	2
RPS6 mRNA for ribosomal protein S6	RPS6	2
vimentin (VIM)	VIM	2
similar to collagen alpha-2(IV) chain (LOC418752)	418752	1
hypothetical LOC423192 (LOC423192)	423192	1
hypothetical LOC423602 (LOC423602)	423602	1
hypothetical protein LOC770909	770909	1
similar to SDF3 (LOC771608)	771608	1
clone 0058P0011D09 putative beta 1 integrin binding protein 2 variant 1	clone 0058P0011D09	1
12S ribosomal RNA gene	12S rRNA	1
B-cell translocation gene 1 anti-proliferative (BTG1)	BTG1	1
calmodulin 1 (phosphorylase kinase delta) (CALM1)	CALM1	1
chaperonin containing TCP1 subunit 8 (theta) (CCT8)	CCT8	1
MHC class II associated invariant chain (CD74)	CD74	1
clone ChEST608i22	clone ChEST608i22	1
clone ChEST751d6	clone ChEST751d6	1
clone ChEST984n15	clone ChEST984n15	1
cytochrome oxidase subunit I (COI)	COI *	1
sp 28150kinawa mitochondrial genomic DNA	COIII	1
cleavage stimulation factor 3 pre-RNA subunit 1 50kDa (CSTF1)	CSTF1	1
eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	EEF1A1	1
eukaryotic translation initiation factor 4A isoform 2 (EIF4A2)	EIF4A2	1
eukaryotic translation initiation factor 5 (EIF5)	EIF5	1
FYN oncogene related to SRC FGR YES (FYN)	FYN	1
hect domain and RLD 2 (HERC2)	HERC2	1
heterogeneous nuclear ribonucleoprotein D-like (HNRPDL)	HNRPDL	1
low density lipoprotein receptor-related protein associated protein 1 (LRPAP1)	LRPAP1	1
lumican (LUM)	LUM *	1
mitochondrial ribosomal protein L27 (MRPL27)	MRPL27	1
pumilio homolog 2 (drosophila) (PUM2)	PUM2	1
ring finger protein 11 (RNF11)	RNF11	1
ribosomal protein L5 (RPL5)	RPL5	1
ribosomal protein L6 (RPL6)	RPL6	1
clone 0058P0031F07 putative ribosomal protein S3	RPS3	1
splicing factor arginine/serine-rich 1 (SFRS1)	SFRS1	1
T cell receptor delta chain (TCRD) pseudogene	TCRD	1
WD repeat domain phosphoinositide interacting 2 (WIPI2)	WIPI2	1
clone 0058P0047C07 putative SNF7 domain containing 2 variant 2	clone 0058P0047C07	1
clone 0069P0004A06 transcriptional regulator Id2-like	clone 0069P0004A06	1
clone 0064P0010H05 high-mobility group nucleosome binding domain 1 variant 1-like	clone 0064P0010H05	1
clone 0061P0031D11 matrix Gla-like	clone 0061P0031D11	1
steroid dehydrogenase	NA	1
invariant chain (Ii)	NA	1
encoding methionine sulfoxide reductase	NA	1
	MLAN (UP)	308
	[illegible]	419
	UNIDENTIFIED	18
	TOTAL (EST) of 384 wells	437
	TOTAL # identified non-redundant transcripts	82

Table 7a: Differentially Expressed Genes, Comparison 2 (1 Day Versus 6 Months)

Ortholog (C-2 1day plates A-D)	Gene Name	Copy #
collagen, type I, alpha 2 (COL1A2)	COL1A2	158
clone ChEST874k20	clone ChEST874k20	21
fibulin 5 (FBLN5)	FBLN5	15
similar to SEC61 gamma(LOC776639)	SEC61G	12
keratin sulfate proteoglycan lumican	LUM	10
nephroblastoma overexpressed (NOV)	NOV	9
FK506 binding protein 9, 63 kDa (FKBP9)	FKBP9	7
telokin myosin, light chain kinase (MYLK)	MYLK	5
clone 0062P0005D06 putative 40S ribosomal protein S8	RPS8	4
fibronectin 1 (FN1)	FN1	4
clone 0063P0029G01 putative RHoA GTPase variant 3	RhoA GTPase-like	4
ATP synthase F0 subunit 8 (ATP8)	ATP8	3
cytochrome c oxidase subunit I (COI)	COI	3
mitochondrial genomic DNA	COIII	3
collagen, type III, alpha 1 (COL3A1)	COL3A1	3
leucine rich repeat containing 17 (LRRC17)	LRRC17	3
ras homolog gene family, member A (RHOA)	RHOA	3
clone 0058P0007G11 putative peroxiredoxin 1 variant 3	clone 0058P0007G11	2
16S ribosomal RNA	16SrRNA	2
calmodulin 1 (phosphorylase kinase, delta) (CALM1)	CALM1	2
carboxypeptidase E (CPE)	CPE	2
mitochondrion	ND4	2
PG-M mRNA for proteoglycan	PG-M	2
ribosomal protein L7 (RPL7)	RPL7	2
hypothetical LOC424163	424163	1
clone 0061P0024E07 putative sm like protein U6 snRNA-associated Sm-like protein	clone 0061P0024E07	1
clone 0063P0030D07 putative solute carrier family 25 member 6 variant 1	clone 0063P0030D07	1
clone 0063P0009H02 putative proteosome maturation protein	clone 0063P0009H02	1
clone 0058P0011D09 putative beta 1 integrin binding protein 2 variant 1	clone 0058P0011D09	1
BAC clone CH261-10P22	BAC CH261-10P22	1
BAC clone CH261-147F20	BAC CH261-147F20	1
BAC clone CH261-79I12	BAC CH261-79I12	1
non-muscle caldesmon	CALD1	1
cell division cycle 2, G1 to S and G2 to M (CDC2)	CDC2	1
similar to Methylosome subunit pIC1n (chloride conductance regulatory protein IC1n)	CLNS1A	1
clone ChEST433n1	clone ChEST433n1	1
clone ChEST587b19	clone ChEST587b19	1
type VI collagen alpha-2 subunit	COL6A2	1
dermatan sulfate proteoglycan decorin	DCN	1
destrin (actin depolymerizing factor) (DSTN)	DSTN	1
clone 0062P0005H11 putative eukaryotic translation initiation factor 1 variant 1	EIF1	1
H3 histone, family 3B (H3.3B)	H3.3B	1
similar to otoancorin (LOC771927)	KDELR2	1
clone 0063P0009H02 putativee proteosome maturation protein	clone 0063P0009H02	1
ornithine decarboxylase antizyme 1 (OAZ1)	OAZ1	1
similar to yeast ribosomal protein s28 homologue (LOC427323)	RPS23	1
ribosomal protein S3A (RPS3A)	RPS3A	1
actin, alpha 2, smooth muscle, aorta (ACTA2)	ACTA2	1
cytochrome oxidase II	COII	1
	MEAN (BP)	283
	[illegible]	313
	UNIDENTIFIED	87
	TOTAL (EST) of 384 wells	400
	TOTAL # identified non-redundant transcripts	**49**

Table 7b: Differentially Expressed Genes, Comparison 2 (6 Months Versus 1 Day)

Ortholog (C-2 6mos plates A-D)	Gene Name	Copy #
heat shock protein 90 alpha	HSP90AA1	213
similar to myosin-11 (Myosin heavy chain, gizzard smooth muscle) (LOC777204)	MYH11	27
BAC clone CH261-117C7	BAC CH261-117C7	23
calnexin (CANX)	CANX	9
actin, alpha 2, smooth muscle, aorta (ACTA2)	ACTA2	7
lumican (LUM)	LUM *	7
myosin, light chain kinase (MYLK)	MYLK	7
16S ribosomal rna	16S rRNA	6
CD36 molecule (thrombospondin receptor) (CD36)	CD36	6
mrna for tau-crystallin/alpha enolase	ENO1	5
clone 0057P0001H01 calmodulin variant 1-like	clone 0057P0001H01	3
cytochrome oxidase subunit II (COII)	COII *	3
Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)	ROCK1	3
12S ribosomal rna	12S rRNA	2
ATP synthase F0 subunit 8 (ATP8)	ATP8 *	2
clone 0058P0017H07	clone 0058P0017H07	2
similar to CD59 protein (LOC423148)	CD59	2
mitochondrial genomic DNA	COIII	2
mitochondrial DNA	ND4 *	2
phosphodiesterase 5A, cGMP-specific (PDE5A)	PDE5A	2
clone 0058P0057E07 ribosomal protein L32-like	RPL32	2
similar to bullous pemphigoid antigen 1, 230/240kDa (LOC421884)	421884	1
acyl-CoA synthetase long-chain family member 1 (ACSL1)	ACSL1	1
beta-actin (ACTB)	ACTB	1
acidic (leucine-rich) nuclear phosphoprotein 32 family, member A (ANP32A)	ANP32A	1
similar to asporin (LOC415954)	ASPN	1
BAC clone CH261-138K4	BAC CH261-138K4	1
BAC clone CH261-187N23	BAC CH261-187N23	1
clone Pgp29 microsatellite sequence	clone Pgp29	1
similar to CLIP-associating protein 1; multiple asters 1 (LOC420724)	CLASP2	1
clone ChEST163a24	clone ChEST163a24	1
clone ChEST189k11	clone ChEST189k11	1
clone ChEST398a16	clone ChEST398a16	1
clone ChEST417p21	clone ChEST417p21	1
clone IMAGE:8236308	clone IMAGE:8236308	1
cytochrome c oxidase subunit I (COI)	COI *	1
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, x-linked (DDX3X)	DDX3X	1
eukaryotic translation initiatin factor 4A, isoform 2 (EIF4A2)	EIF4A2	1
fatty acid binding protein 4, adipocyte (FABP4)	FABP4	1
mitochondrion	ND5	1
similar to phospholipase C beta 4, transcript variant 2 (LOC416730)	PLCB4	1
UNK clone TGMCBa-14H12	UNK clone TGMCBa-14H12	1
	MEAN	**262**
		357
	UNIDENTIFIED	**37**
	TOTAL (EST) of 384 wells	**394**
	TOTAL # identified non-redundant transcripts	**42**

Table 8a: Differentially Expressed Genes, Comparison 3 (6 Weeks Versus 6 Months)

Ortholog (C-3 6wk A-D compared)	Gene Name	Copy #
clone 0064P0021C06 spermadine/spermine N1-acetyltransferase variant 1-like	SAT1	74
BAC clone CH261-126J23 from chromosome ul	BAC CH261-126J23	34
similar to yeast ribosomal protein S28	RPS28	25
ribosomal protein S3A (RPS3A)	RPS3A	25
clone 0063P0030D07 putative solute carrier family 25 member 6 variant 1	SLC25A6	21
gelsolin (GSN)	GSN	20
similar to ribosomal protein L3 (LOC771432)	RPL3	18
prohibitin 2 (PHB2)	PHB2	17
similar to coactosin-like 1 (dictyostelium) (LOC768420)	COTL1	16
nucleoside diphosphate kinase	NDPK	15
finished cDNA, clone ChEST14214	SERPINF2	15
NADH hydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa (NDUFA10),	NDUFA10	14
ras homolog gene family, member A (RHOA)	RHOA	9
ribosomal protein L9 (RPL9)	RPL9	9
S-phase kinase-associated protein 1 (SKP1)	SKP1	6
eukaryotic translation initiation factor 3, subunit H (EIF3H)	EIF3H	5
clone 0058P0007G11 putative peroxiredoxin 1 variant 3	PRDX1	5
similar to SDF3 (LOC771608)	SERPINF1	5
BAC clone CH261-77K20	BAC CH261-77K20	4
cytochrome oxidase subunit II (COII) , mitochondrial	COII	4
collagen, type I, alpha 2 (COL1A2)	COL1A2	4
clone 0061P0016C08 putative actin related protein 2/3 complex subunit 2	100190509	3
actin, beta (ACTB)	ACTB	3
acidic (leucine-rich) nuclear phosphoprotein 32 family, member A (ANP32A)	ANP32A	3
clone ChEST611b2	clone ChEST611b2	3
clone 0062P0005H11 putative eukaryotic translation initiation factor 1 variant 1	EIF1	3
similar to B-aggressive lymphoma 2B (LOC424266)	PARP14	3
clone 0061P0001E09 putative Yip1 domain family member 1	100190677	2
mitochondrion	16S rRNA	2
chaperonin containing TCP1, subunit 8 (theta) (CCT8)	CCT8	2
cyclin-dependent kinase inhibitor p27 Kip1	CDKN1B	2
clone 0061P0031D11 matrix Gla-like	clone 0061P0031D11	2
ribosome binding protein 1 homolog 180kDa (dog) (RRBP1)	RPBP1	2
ribosomal protein L4 (RPL4)	RPL4	2
ribosomal protein S23, 11 days embryo whole body cDNA, RIKEN full-length	RPS23	2
vimentin (VIM)	VIM	2
hypothetical protein LOC770045	770045	1
clone 0061P0022F12 putative creatine kinase B variant 1	100190470	1
clone 0064P0012G12 RIKEN cDNA 0610039A15-like	100190503	1
similar to adenylate kinase (EC 2.7.4.3) (LOC428227)	AK2	1
chromosome 12 open reading frame 49 (c12orf49)	C12orf49	1
chaperonin containing TCP1, subunit 6A (zeta 1) (ccT6A)	CCT6A	1
clone 0058P0030G01	clone 0058P0030G01	1
clone 0065P0012D05	clone 0065P0012D05	1
clone ChEST1004o7	clone ChEST1004o7	1
clone ChEST5e12	clone ChEST5e12	1
similar to collagen alpha-2 (IV) chain	COL4A2	1
DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX5)	DDX5	1
DnaJ (Hsp40) homolog, subfamily A, member 1 (DNAJA1)	DNAJA1	1
destrin (actin depolymerizing factor) (DSTN)	DSTN	1
GNAS complex locus, transcript variant 2 (GNAS)	GNAS	1
similar to glycogen synthase kinase 3 beta (LOC418335)	GSK3B	1
lectin associated matrix protein (hLAMP-1)	hLAMP-1	1
similar to microfibrillar associated protein 5 (LOC418256) MFAP5	MFAP5	1
proline-rich nuclear receptor coactivator 1 (PNRC1)	PNRC1	1
ring finger protein 11 (RNF11)	RNF11	1
ribosomal protein L5 (RPL5)	RPL5	1
ribosomal protein S6 (RPS6)	RPS6	1
transmembrane emp24 protein transport domain containing 5 (TMED5)	TMED5	1
alpha-tropomyosin, clone cC401	TPM1	1
	MEAN (bp)	295
		406
	UNIDENTIFIED	19
	TOTAL (EST) OF 384	425
	TOTAL # identified non-redundtant transcripts	**60**

Table 8b: Differentially Expressed Genes, Comparison 3 (6 Months Versus 6 Weeks)

Ortholog (C-3 6mos A-D compared)	Gene Name	Copy #
heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSP90AA1)	HSP90AA1	175
lumican (LUM)	LUM	104
eukaryotic translation initiation factor 4A, isoform 2 (EIF4A2)	EIF4A2	35
actin, alpha 2, smooth muscle, aorta (ACTA2)	ACTA2	32
myosin, heavy chain 11, smooth muscle (MYH11)	MYH11	32
fibroblast myosin light chain kinase	MYLK	21
enolase 1, (alpha) (ENO1)	ENO1	6
BAC clone CH261-117C7	BAC CH261-117C7	4
acyl-CoA synthetase long-chain family member 1 (ACSL1)	ACSL1	3
ATP synthase F0 subunit 8 (ATP8)	ATP8	2
BAC clone CH261-126J23	BAC CH261-126J23	2
clone ChEST85g22	FBLN5	2
16S ribosomal RNA gene, partial sequence; tRNA-leu gene, complete sequence; NADH dehydrogenase subunit 1 (ND1) gene	ND1	2
similar to 0TTHUMP00000028984 (LOC418017)	418017	1
hypothetical LOC419017, transcript variant 1 (LOC419017)	419017	1
BAC clone CH261-16J12	BAC CH261-16J12	1
BAC clone CH261-19B3	BAC CH261-19B3	1
BAC clone TAM31-16o5	BAC TAM31-16o5	1
BAC clone TGMCBa-49H12	BAC TGMCBa-49H12	1
CD151 molecule (Ralph blood group) (CD151)	CD151	1
clone 0061P0022F12 putative creatine kinase B variant 1	CKB	1
mitochondrion	COII	1
mitochondrial genomic DNA	ND4	1
ZMUC 125434 ornithine decarboxylase (ODC)	ODC	1
odd-skipped related 1 (drosophila) (OSR1)	OSR1	1
GTP-specific succinyl-CoA synthetase alpha subunit (SCS)	SCS	1
clone 0063P0006F01 ubiquitin C variant 5-like	UBC	1
	MEAN (BP)	**271**
		434
	UNIDENTIFIED	**16**
	TOTAL (EST) of 384	**450**
	TOTAL # identified non-redundant transcripts	**27**

Chi square analysis was initially used to determine statistical differences (using a p-value of < 0.05) and was required to establish regulation patterns between the ages compared, prior to uploading into MetaCore. Because the RDA method in and of itself enriches only for sequences that are differentially expressed, genes that were upregulated between the comparisons were significant by nature of the method. This ability of the RDA method to detect and selectively enrich transcripts of low copy number speaks for the level of sensitivity of the method (71) and negated any further reason for using chi square analysis to assist in the interpretation of the results.

The largest number of identified genes (presented in **Table 9**) was observed at 6 months of C-3 with 434 gene transcripts followed by 419 genes at 6 weeks of C-1. The greatest number of unidentified transcripts, (109) was observed at 1 day of C-1, followed by 87 transcripts at 1 day of C-2. In C-3, only 16 unidentified transcripts were found at 6 months, which was the lowest number of unidentified transcripts of all the ages compared. This is perhaps because more work to identify genes, has been completed on older vertebrates as compared to younger vertebrate animals. In C-1 and C-3, both 1 day and 6 months respectively contained the greatest number (450) of identified and un-identified expressed sequence tags (ESTs) combined. The largest number (83) of unique, non-redundant transcripts was observed at 6 weeks in C-1 when compared to 1 day. In contrast, 27 genes at 6 months of C-3 was the lowest number of non-redundant transcripts found in an age group compared.

Table 9: Number of Transcripts from Greatest to Lowest by Comparison and Age

Comparision	Age	Total Number of Identified Genes
C-3	6 months	434
C-1	6 weeks	419
C-3	6 weeks	406
C-2	6 months	357
C-1	1 day	341
C-2	1 day	313
Comparison	**Age**	**Total Number of Unidentified Transcripts**
C-1	1 day	109
C-2	1 day	87
C-2	6 months	37
C-3	6 weeks	19
C-1	6 weeks	18
C-3	6 months	16
Comparison	**Age**	**Total Number of Expressed Sequence Tags (ESTs)**
C-1	1 day	450
C-3	6 months	450
C-1	6 weeks	437
C-3	6 weeks	425
C-2	1 day	400
C-2	6 months	394
Comparison	**Age**	**Total Number of Identified Non-Redundant Gene Transcripts**
C-1	6 weeks	82
C-3	6 weeks	60
C-2	1 day	49
C-2	6 months	42
C-1	1 day	31
C-3	6 months	27

When distinguishing between genes involved in the disease process, and those that were expressed as a result of normal development, the genes that were expressed in the highest number of copies were not necessarily the primary focus. Copy number provides one perspective into the understanding of which genes may be key players participating in atherosclerosis. In fact, as demonstrated in monogenic diseases, a single gene may be responsible for kicking off a cascade of events. Given the extensive time required to evaluate each of the 291 differentially expressed, non-redundant transcripts in this experiment, it became necessary to develop criteria to effectively address the current hypothesis and select the most likely candidates involved in atherosclerotic disease.

Genes that were differentially upregulated at 1 day, 6 weeks, and 6 months when compared to each of their respective ages are presented in **Tables 10, 11, and 12,** and comprised the *first criteria* of distinguishing between genes that are potentially involved in initiation and those that participate in progression. Genes that were differentially upregulated specifically at 1 day when compared to 6 weeks and 6 months are regarded as representing the earliest stage of the disease. This may in fact be the most important stage when distinguishing initiation from that of progression. **Table 10** provides a combined list of the 55 genes that were differentially upregulated at only 1 day when compared to 6 weeks and 6 months. The 7 genes that were differentially upregulated at 1 day in both C-1 and C-2 may actually represent the gene or genes that are related to initiation of atherosclerosis. The genes that were exclusive to 1 day in this case include, SEC61 Gamma (SEC61G), FK 506 binding protein9 (FKBP9), clone ChEST874k20, BAC CH261-10P22, Leucine rich repeat containing 17 9LRRC17), Decorin (DCN), and hypothetical protein LOC424163.

Table 10:

Genes Differentially Upregulated at 1 Day When Compared to 6 Weeks & 6 Months

Ortholog (C-1 1day versus 6weeks)	Gene Name	Copy #	Ortholog (C-2 1day versus 6months)	Gene Name	Copy #
similar to SEC61 gamma (LOC776639)	SEC61G	7	clone ChEST874k20	clone ChEST874k20	21
clone ChEST874k20	clone ChEST874k20	5	similar to SEC61 gamma(LOC776639)	SEC61G	12
BAC clone CH261-10P22	BAC CH261-10P22	3	nephroblastoma overexpressed (NOV)	NOV	9
leucine rich repeat containing 17	LRRC17	3	fk506 binding protein 9, 63 kDA (FKBP9)	FKBP9	7
dermatan sulfate proteoglycan deconn	DCN	2	clone 0062P0005D06 putative 40S ribosomal protein S8	RPS8	4
hypothetical LOC424163 (LOC424163)	424163	1	fibronectin 1 (FN1)	FN1	4
vascular smooth muscle alpha-actin (ACT-4)	ACT-4	1	clone 0063P0029G01 putative RHoA GTPase variant 3	RhoA GTPase-like	4
ATP synthase F0 subunit 8 and 6 (ATPase 8, 6),	ATPase 6	1	collagen, type III, alpha 1 (COL3A1)	COL3A1	3
BAC clone TGMCBa-23H12	BAC TGMCBa-23H12	1	leucine rich repeat containing 17 (LRRC17)	LRRC17	3
kdra17 mRNA for lectin-like transmembrane protein, Ly49q1 isoform (CDR-1)	CDR-1	1	clone 0058P0007G11 putative peroxiredoxin 1 variant 3	clone 0058P0007G11	2
clone 0064p0012D12 putative ribosomal protein	clone 0064p0012D12	1	carboxypeptidase E (CPE)	CPE	2
collagen, type VIII, alpha I (COL8A1)	COL8A1	1	PG-M mRNA for proteoglycan	PG-M	2
hypothetical LOC424163	FASTKD1	1	ribosomal protein L7 (RPL7)	RPL7	2
fk506 binding protein 9 63 kDA (FKBP9)	FKBP9	1	hypothetical LOC424163	424163	1
myoglobin gene	MB	1	clone 0061P0024E07 putative sm like protein U6 snRNA-associated Sm-like protein	clone 0061P0024E07	1
neomycin resistance cassette pNeo4 NeoTet (neoTet)	NEOTET	1	clone 0063P0030D07 putative solute carrier family 25 member 6 variant 1	clone 0063P0030D07	1
microsatellite marker vemos44	NA	1	clone 0063P0009H02 putative proteosome maturation protein	clone 0063P0009H02	1
CR1 gene for chicken repeat 1	NA	1	clone 0058P0011D09 putative beta 1 integrin binding protein 2 variant 1	clone 0058P0011D09	1
fdx1 gene and 4 orf' s	NA	2	BAC clone CH261-10P22	BAC CH261-10P22	1
has operon deletion mutant	NA	1	BAC clone CH261-147F20	BAC CH261-147F20	1
Gene encoding methionine sulfoxide reductase	NA	1	BAC clone CH261-79I12	BAC CH261-79I12	1
mitochondrial DNA	NA	1	non-muscle caldesmon	CALD1	1
			cell division cycle 2, G1 to S and G2 to M (CDC2)	CDC2	1
			similar to Methylosome subunit pIC1n (chloride conductance regulatory protein IC1n) (I(C1n)) (chloride channel, nucleotide sensitive 1A) (LOC425500)	CLNS1A	1
			clone ChEST433n1	clone ChEST433n1	1
			clone ChEST587b19	clone ChEST587b19	1
			type VI collagen alpha-2 subunit	COL6A2	1
			dermatan sulfate proteoglycan deconn	DCN	1
			H3 histone, family 3B (H3 3B)	H3 3B	1
			similar to otoanconn (LOC771927)	KDELR2	1
			clone 0063P0009H02 putativee proteosome maturation protein	clone 0063P0009H02	1
			ornithine decarboxylase antizyme 1 (OAZ1)	OAZ1	1
			similar to yeast ribosomal protein s28 homologue (LOC427323)	RPS23	1

Table 11 represents the 60 genes that were differentially upregulated at 6 weeks when compared to 1 day and the 47 genes differentially upregulated at 6 weeks in C-3. Since these genes were not found to be upregulated until 6 weeks of age, they are likely contributors in the progression of atherosclerosis. Of the 107 genes that were differentially upregulated at 6 weeks, only 16 were differentially upregulated at 6 weeks when compared to both ages in C-1 and C-3. They appear to be distinct only to 6 weeks and include Gelsolin,(GSN), Spermidine/spermine N1-acetyltransferase (SAT1), Ribosomal protein L3

(RPL3), Ribosomal protein L4 (RPL4), Eukaryotic translation initiation factor 3, subunit H (EIF3H), Dead (Asp-Glu-Ala-Asp) box polypeptide 6 (DDX5), Cyclin-dependant kinase inhibitor p27 kip1 (CDKN1B), Ribosomal protein S28 (RPS28), Tropomyosin-alpha (TPM1), Similar to B-aggressive lymphoma / Poly (ADP-ribose) polymerase (PARP14), Ribosomal protein S6, (RPS6), Vimentin (VIM), Chaperonin containing TCP1, subunit 8 (CCT8), Ring finger protein 11 (RNF11), Ribosomal protein L5 (RPL5), and clone 0061P0031D11.

Table 11:

Genes Differentially Upregulated at 6 Weeks When Compared to 1 Day & 6 Months

Ortholog (C-1 6weeks versus 1day)	Gene Name	Copy #	Ortholog (C-3 6weeks versus 6months)	Gene Name	Copy #
gelsolin (GSN)	GSN	43	clone 0064P0021C06 spermidine/spermine N1-acetyltransferase variant 1-like	SAT*	74
clone 0064P0021C06 spermidine/spermine N1-acetyltransferase	SAT1	34	similar to yeast ribosomal protein S28 homologue (LOC427323)	RPS28	25
similar to ribosomal protein L3 (LOC771432)	RPL3	39	clone 0063P0030D07 putative solute carrier family 25 member 6 variant 1	SLC25A6	21
hypothetical LOC415891 (LOC415891)	415891	13	gelsolin (GSN)	GSN	20
ribosomal protein L4 (RPL4)	RPL4	9	similar to ribosomal protein L3 (LOC771432)	RPL3	18
clone ChEST118j5	clone ChEST118j5	5	prohibitin 2 (PHB2)	PHB2	17
eukaryotic translation initiation factor 3 subunit H (EIF3H)	EIF3H	5	similar to coactosin-like 1 (dictyostelium) (LOC768420)	COTL1	16
chromogranin B (secretogranin 1) (CHGB)	CHGB	4	nucleoside diphosphate kinase	NDPK	15
DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 (DDX5)	DDX5	4	finished cDNA, clone ChEST14214	SERPINF2	15
hemoglobin alpha-globin chain	HBA	4	ribosomal protein L9 (RPL9)	RPL9	9
lymphocyte antigen 6 complex locus E (LY6E)	LY6E	4	S-phase kinase-associated protein 1 (SKP1)	SKP1	6
similar to NPD014 protein (NPD014)	NPD014	4	eukaryotic translation initiation factor 3 subunit H (EIF3H)	EIF3H	5
splicing factor arginine/serine-rich 10 (transformer 2 homolog drosophila) (SFRS10)	SFRS10	4	clone 0058P0007G11 putative peroxiredoxin 1 variant 3	PRDX1	5
splicing factor arginine/serine-rich 6 (SFRS6)	SFRS6	4	similar to SDF3 (LOC771608)	SERPINF1	5
clone ChEST1007e10	clone ChEST1007e10	4	BAC clone CH261-77K20	BAC CH261-77K20	4
beta2-microglobulin	B2M	3	clone 0061P0016C08 putative actin related protein 2/3 complex subunit 2	clone 0061P0016C08	3
cyclin-dependent kinase inhibitor p27 Kip1	CDKN*B	3	similar to B-aggressive lymphoma 2B (LOC424266)	PARP14	3
similar to yeast ribosomal protein S28 homologue (LOC427323)	RPS28	3	clone 0061P0001E09 putative Yip1 domain family member 1	clone 0061P0001E09	2
alpha-tropomyosin gene, exons 9c 9d, and complete cds alternatively spliced	TPM*	3	chaperonin containing TCP1 subunit 8 (theta) (CCT8)	CCT8	2
clone 0062P0001C01 putative high-mobility group nucleosome binding domain 1 variant 1	clone 0062P0001C01	2	cyclin-dependent kinase inhibitor p27 Kip1	CDKN1B	2
alpha thalassemia/mental retardation syndrome X-linked (ATRX)	ATRX	2	clone 0061P0031D11 matrix Gla-like	clone 0061P0031D11	2
BAC clone CH261-114A20	BAC CH261-114A20	2	ribosome binding protein 1 homolog 180kDa (RPBP1)	RPBP1	2
jagged 1 (Alagille syndrome) (JAG1)	JAG1	2	ribosomal protein L4 (RPL4)	RPL4	2
similar to B-aggressive lymphoma 2B	PARP 4	2	vimentin (VIM)	VIM	2
RPS6 mRNA for ribosomal protein S6	RPS6	2	hypothetical protein LOC770045	770045	1
vimentin (VIM)	VIM	2	clone 0061P0022F12 putative creatine kinase B variant 1	clone 0061P0022F12	1
similar to collagen alpha-2(IV) chain (LOC418752)	418752	1	clone 0064P0012G12 RIKEN cDNA 0610039A15-like	clone 0064P0012G12	1
hypothetical LOC423192 (LOC423192)	423192	1	similar to adenylate kinase (EC 2 7 4 3) (LOC428227)	AK2	1
hypothetical LOC423602 (LOC423602)	423602	1	chromosome 12 open reading frame 49 (c12orf49)	C12orf49	1
hypothetical protein LOC770909	770909	1	chaperonin containing TCP1, subunit 6A (zeta 1) (ccT6A)	CCT6A	1
similar to SDF3 (LOC771608)	771608	1	clone 0058P0030G01	clone 0058P0030G01	1
B-cell translocation gene 1 anti-proliferative (BTG1)	BTG1	1	clone 0065P0012D05	clone 0065P0012D05	1
chaperonin containing TCP1, subunit 8 (theta) (CCT8)	CCT8	1	clone ChEST1004o7	clone ChEST1004o7	1
MHC class II associated invariant chain (CD74)	CD74	1	clone ChEST5e12	clone ChEST5e12	1
clone ChEST608i22	clone ChEST608i22	1	similar to collagen alpha-2 (IV) chain	COL4A2	1
clone ChEST751d6	clone ChEST751d6	1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX5)	DDX5	1
clone ChEST984n15	clone ChEST984n15	1	DnaJ (Hsp40) homolog subfamily A member 1 (DNAJA1)	DNAJA1	1
cleavage stimulation factor, 3' pre-RNA, subunit 1 50kDa (CSTF1)	CSTF1	1	GNAS complex locus transcript variant 2 (GNAS)	GNAS	1
eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	EEF1A1	1	similar to glycogen synthase kinase 3 beta (LOC418335)	GSK3B	1
eukaryotic translation initiation factor 5 (EIF5)	EIF5	1	lectin associated matrix protein (hLAMP-1)	hLAMP-1	1
FYN oncogene related to SRC FGR, YES (FYN)	FYN	1	similar to microfibrillar associated protein 5 (LOC418256) MFAP5	MFAP5	1
hect domain and RLD 2 (HERC2)	HERC2	1	proline-rich nuclear receptor coactivator 1 (PNRC1)	PNRC1	1
heterogeneous nuclear ribonucleoprotein D-like (HNRPDL)	HNRPDL	1	ring finger protein 11 (RNF11)	RNF11	1
low density lipoprotein receptor-related protein associated protein 1 (LRPAP1)	LRPAP1	1	ribosomal protein L5 (RPL5)	RPL5	1
mitochondrial ribosomal protein L27 (MRPL27)	MRPL27	1	ribosomal protein S6 (RPS6)	RPS6	1
pumilio homolog 2 (drosophila) (PUM2)	PUM2	1	transmembrane emp24 protein transport domain containing 5 (TMED5)	TMED5	1
ring finger protein 11 (RNF11)	RNF11	1	alpha-tropomyosin clone cC401	TPM*	1
ribosomal protein L5 (RPL5)	RPL5	1			
ribosomal protein L6 (RPL6)	RPL6	1			
clone 0058P0031F07 putative ribosomal protein S3	RPS3	1			
splicing factor, arginine/serine-rich 1 (SFRS1)	SFRS1	1			
T cell receptor delta chain (TCRD) pseudogene	TCRD	1			
WD repeat domain, phosphoinositide interacting 2 (WIPI2)	WIPI2	1			
clone 0058P0047C07 putative SNF7 domain containing 2 variant 2	clone 0058P0047C07	1			
clone 0069P0004A06 transcriptional regulator Id2-like	clone 0069P0004A06	1			
clone 0064P0010H05 high-mobility group nucleosome binding domain 1 variant 1-like	clone 0064P0010H05	1			
clone 0061P0031D11 matrix Gla-like	clone 0061P0031D11	1			
steroid dehydrogenase	NA	1			
invariant chain (Ii)	NA	1			
encoding methionine sulfoxide reductase	NA	1			

Thirty-seven genes were differentially upregulated at 6 months when compared to 1 day (C-2) and 6 weeks (C-3) of age respectively and are listed in **Table 12**. These are related to disease progression since they were not differentially upregulated in the previous ages. Of the 37 genes only 3, BAC CH261-117C7, Enolase 1 (ENO1) and Acyl-coA synthetase long chain family member 1 (ACSL1) were differentially upregulated exclusively at 6 months when compared to both 1 day and 6 weeks.

Table 12:

Genes Differentially Upregulated at 6 Months When Compared to 1 Day & 6 Weeks

Ortholog (C-2 6months versus 1day)	Gene Name	Copy #	Ortholog (C-3 6months versus 6weeks)	Gene Name	Copy #
BAC clone CH261-117C7	BAC CH261-117C7	23	enolase 1, (alpha) (ENO1)	ENO1	6
calnexin (CANX)	CANX	9	BAC clone CH261-117C7	BAC CH261-117C7	4
CD36 molecule (thrombospondin receptor) (CD36)	CD36	6	acyl-CoA synthetase long-chain family member 1 (ACSL1)	ACSL1	3
mrna for tau-crystallin/alpha enolase	ENO1	5	16S ribosomal RNA gene, partial sequence; tRNA-leu gene, complete sequence; NADH dehydrogenase subunit 1 (ND1) gene	ND1	2
clone 0057P0001H01 calmodulin variant 1-like	clone 0057P0001H01	3	similar to 0TTHUMP00000028984 (LOC418017)	418017	1
clone 0058P0017H07	clone 0058P0017H07	2	hypothetical LOC419017, transcript variant 1 (LOC419017)	419017	1
similar to CD59 protein (LOC423148)	CD59	2	BAC clone CH261-16J12	BAC CH261-16J12	1
phosphodiesterase 5A, cGMP-specific (PDE5A)	PDE5A	2	BAC clone CH261-19B3	BAC CH261-19B3	1
similar to bullous pemphigoid antigen 1, 230/240kDa (LOC421884)	421884	1	BAC clone TAM31-16o5	BAC TAM31-16o5	1
acyl-CoA synthetase long-chain family member 1 (ACSL1)	ACSL1	1	CD151 molecule (Ralph blood group) (CD151)	CD151	1
similar to asporin (LOC415954)	ASPN	1	clone 0061P0022F12 putative creatine kinase B variant 1	CKB	1
BAC clone CH261-138K4	BAC CH261-138K4	1	ZMUC 125434 ornithine decarboxylase (ODC)	ODC	1
BAC clone CH261-187N23	BAC CH261-187N23	1	odd-skipped related 1 (drosophila) (OSR1)	OSR1	1
clone Pgp29 microsatellite sequence	clone Pgp29	1	GTP-specific succinyl-CoA synthetase alpha subunit (SCS)	SCS	1
similar to CLIP-associating protein 1; multiple asters 1 (LOC420724)	CLASP2	1	clone 0063P0006F01 ubiquitin C variant 5-like	UBC	1
clone ChEST163a24	clone ChEST163a24	1			
clone ChEST417p21	clone ChEST417p21	1			
clone IMAGE:8236308	clone IMAGE:8236308	1			
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, x-linked (DDX3X)	DDX3X	1			
mitochondrion	ND5	1			
similar to phospholipase C beta 4, transcript variant 2 (LOC416730)	PLCB4	1			
UNK clone TGMCBa-14H12	UNK clone TGMCBa-14H12	1			

The following bar graphs, **Figures 2a – 2af** provide a pictorial representation of genes in a manner that was similar to those listed in **tables 10, 11, and 12**. In addition, however, the bar graphs were developed to visually display the regulation patterns of only

those genes that were differentially expressed in more than one age comparison, either at the same age or at different ages therefore comprising the *second criteria*. They were then grouped as participating in either initiation or progression of atherosclerosis. Given the three age comparisons, seven different patterns of regulation were possible and are laid out in **Table 13**.

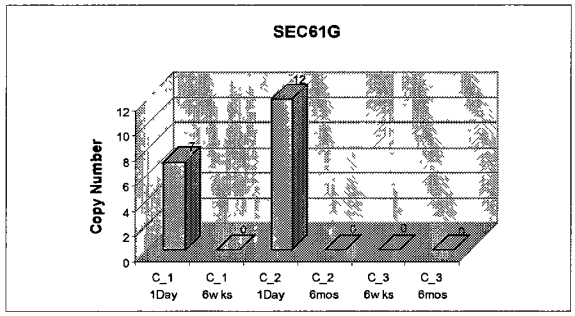
Table 13: Patterns of Genes Differentially Expressed in Age Comparisons

Genes Differentially Expressed in More Than One Age Comparision					
Pattern #	**1day**	**6weeks**	**6months**	**# of Genes**	**Figures**
1	↑	—	—	7	3a-3g
2	—	↑	—	15	4h-4w
3	—	—	↑	3	5x-5z
4	↑	↑	—	10	6aa-6jj
5	↑	—	↑	5	7kk-7oo
6	—	↑	↑	10	8pp-8yy
7	↑	↑	↑	6	9zz-9af

Of the 291 genes that were originally identified by BLASTn, 57 genes were grouped into one of the seven patterns. While it is interesting to look at the change in patterns of expression throughout the three comparisons, it is important to keep in mind that the differential expression is unique between the ages compared and cannot necessarily be compared to another comparison (age group) or across comparisons. The following **Figures 4a – 4af** represent the upregulation pattern of a given gene within an age group compared, as well as patterns of expression across comparisons.

Figures 4a – 4af: Upregulation of Genes Within and Across Comparisons:

Figure 4a: Sec61 Gamma Subunit (SEC61G) **Figure 4b:** Clone ChEST874k20



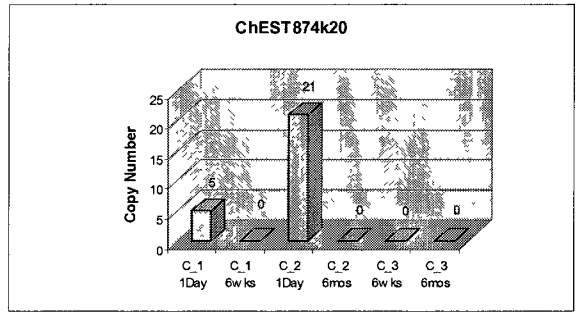
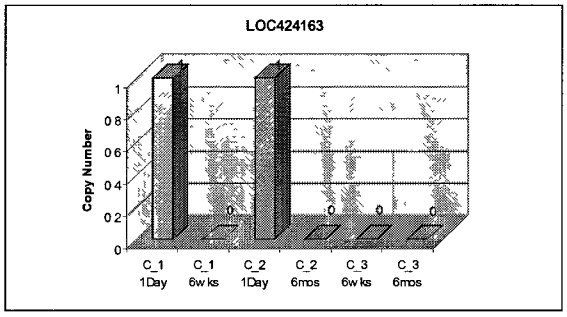


Figure 4c: Hypothetical LOC424163

Figure 4d: Decorin (DCN)



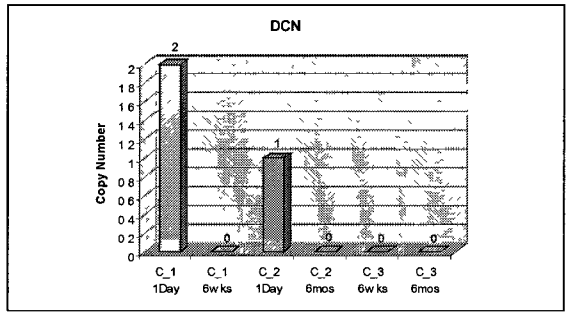
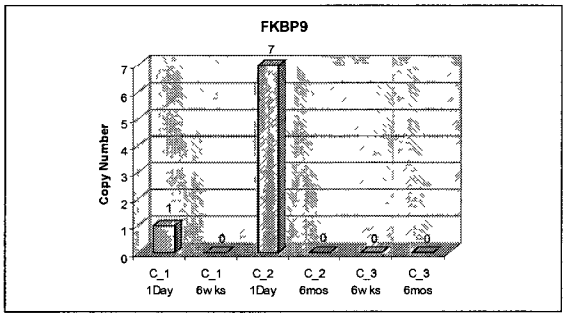


Figure 4e: FK506 Binding Protein 9 (FKBP9)

Figure 4f: Leucine Rich Repeat Containing 17 (LRRC17)



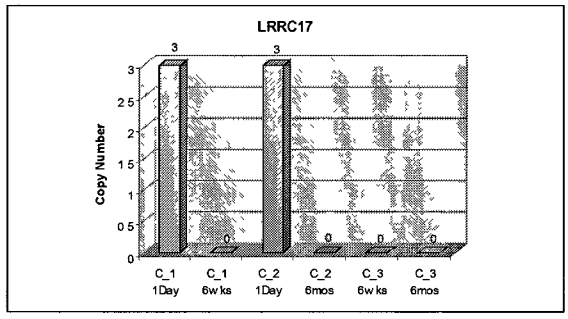


Figure 4g: BAC CH261-10P22

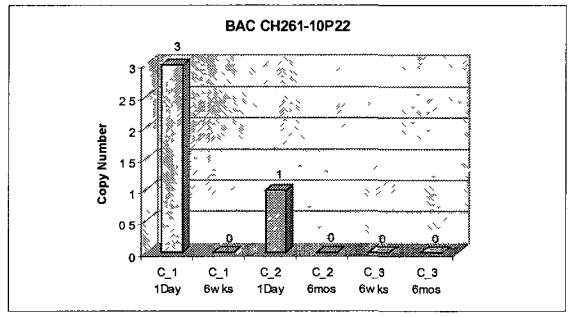


Figure 5h: Gelsolin (GSN)

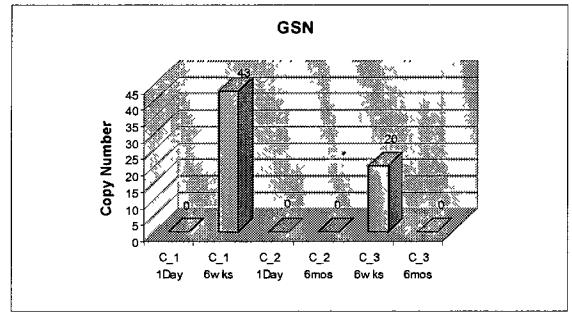


Figure 5i: Ribosomal Protein L3 (RPL3)

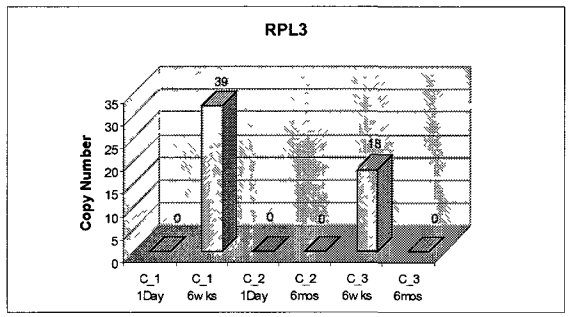


Figure 5j: Spermidine/Spermine N1-actetyltransferase (SAT1)

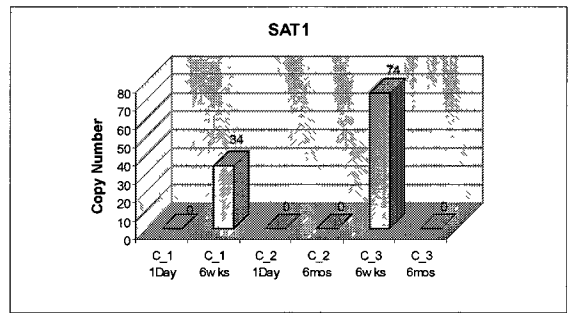


Figure 5k: Ribosomal Protein S28 (RPS28)

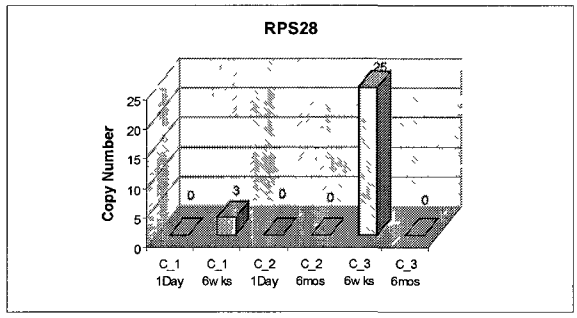


Figure 5l: Chaperone Containing TCP1, subunit 8 (theta) (CCT8)

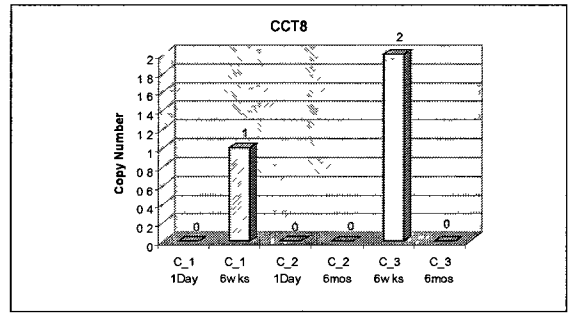


Figure 5m: DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX5)

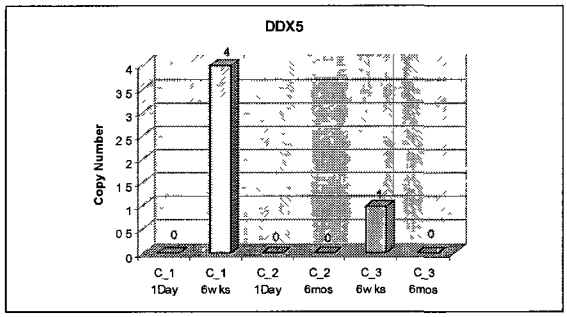


Figure 5n: Eukaryotic Translation Initiation Factor 3, Subunit H (EIF3H)

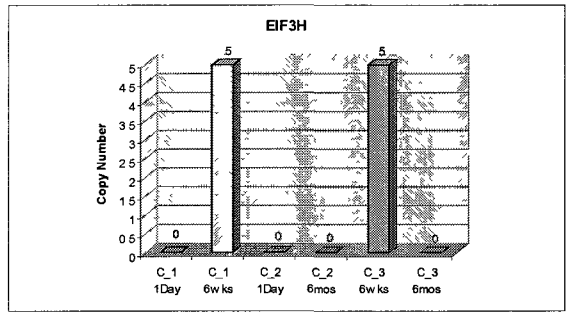


Figure 5o: B-aggressive Lymphoma 2B (PARP14)

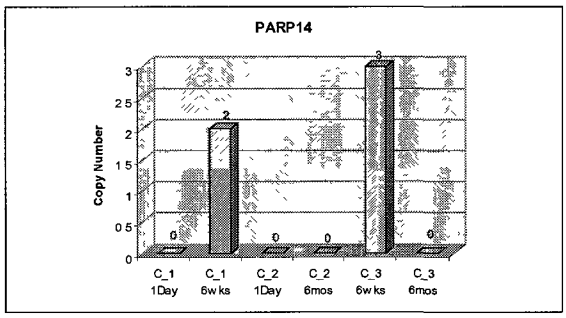


Figure 5p: Ring Finger Protein (RNF11)

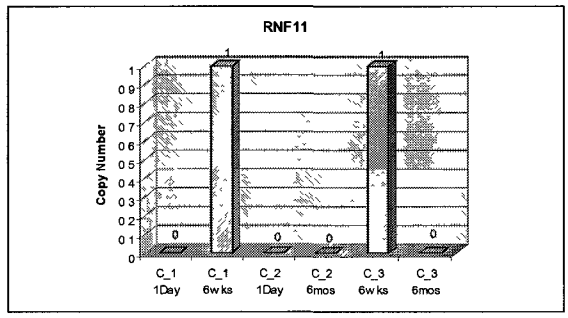


Figure 5q: Ribosomal Protein L4 (RPL4)

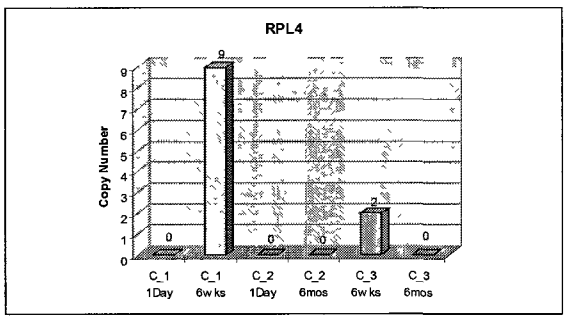


Figure 5r: Ribosomal Protein L5 (RPL5)

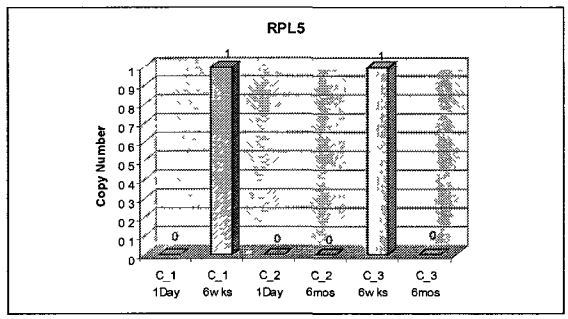


Figure 5s: Ribosomal Protein S6 (RPS6)

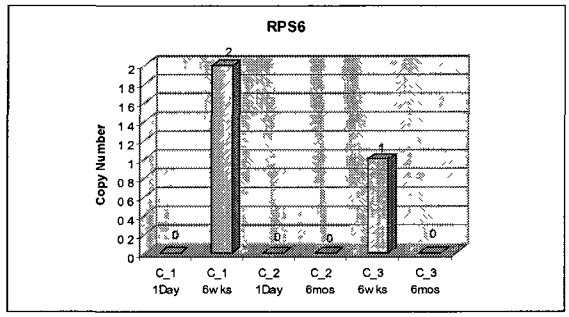


Figure 5t: Alpha-Tropomyosin 1 (TPM1)

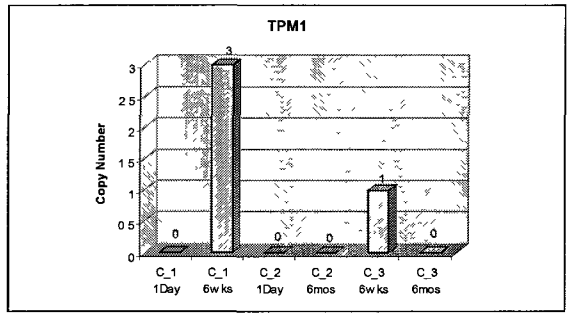


Figure 5u: Vimentin (VIM)

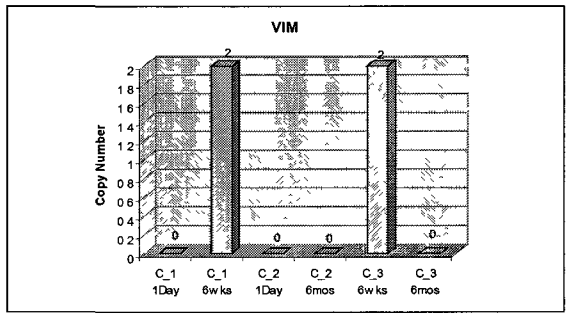


Figure 5v: Cyclin Dependant Kinase Inhibitor p27 Kip1 (CDKN1B)

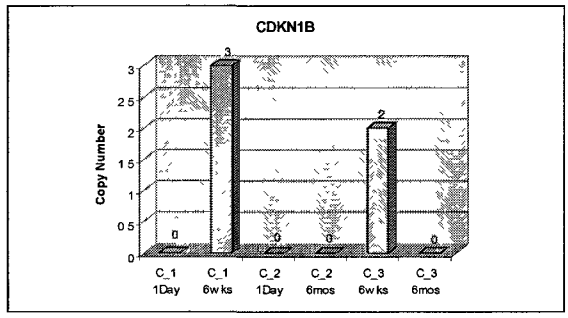


Figure 5w: Clone 0061P0031D11

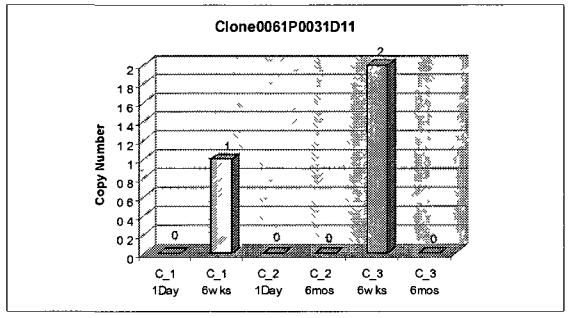


Figure 6x: BAC clone CH261-117C7

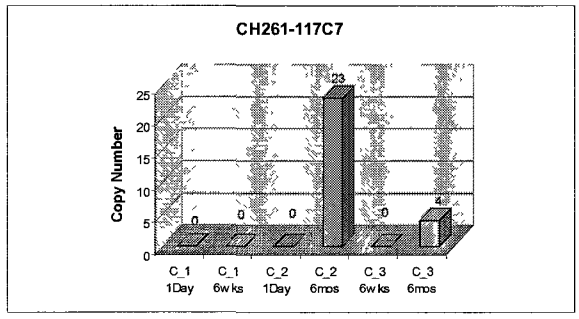


Figure 6y: Enolase (ENO1)

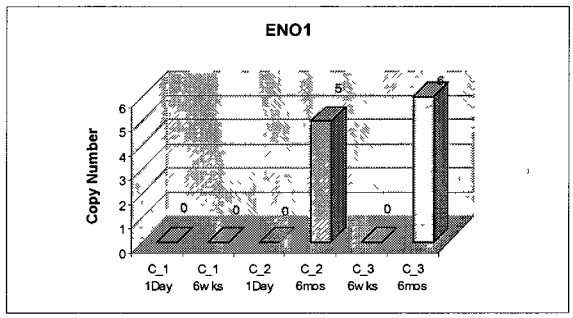


Figure 6z: Acyl-CoA synthetase long-chain family member 1 (ACSL1)

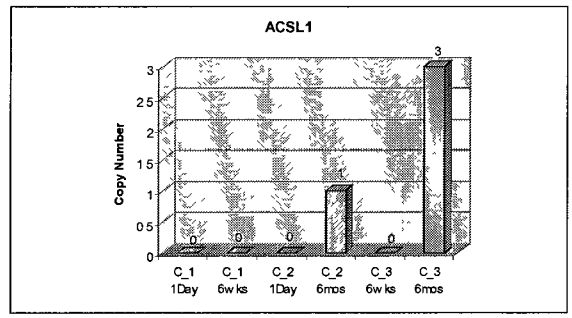


Figure 7aa: Collagen, type 1, alpha 2 (COLA1A2)

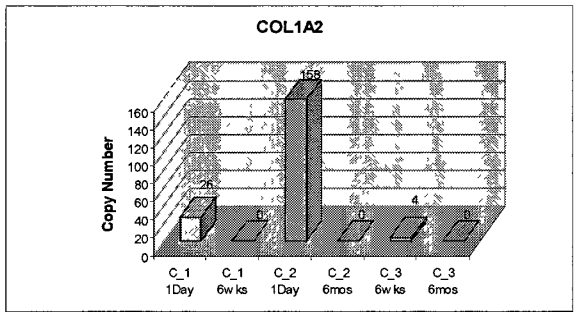


Figure 7bb: NADH hydrogenase (ubiquinone) 1 alpha subcomplex, 10 (NDUFA10)

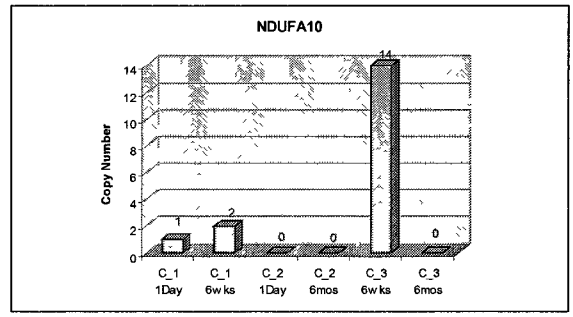


Figure 7cc: Eukaryotic translation Initiation Factor 1 (EIF1)

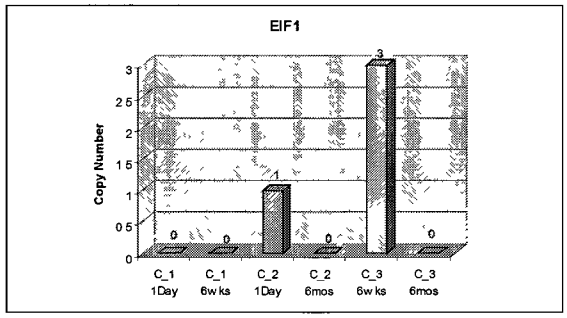


Figure 7dd: Ras Homolog Gene Family, Member A (RHOA)

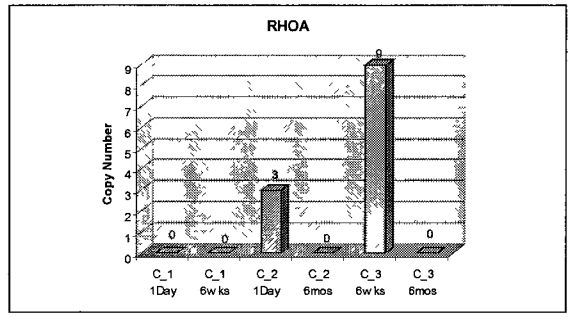


Figure 7ee: Ribosomal Protein S23 (RPS23)

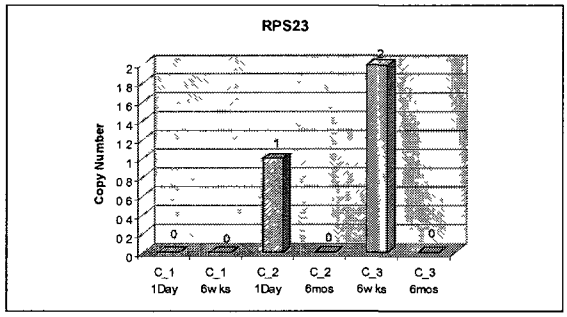


Figure 7ff: Clone 0058P0011D09

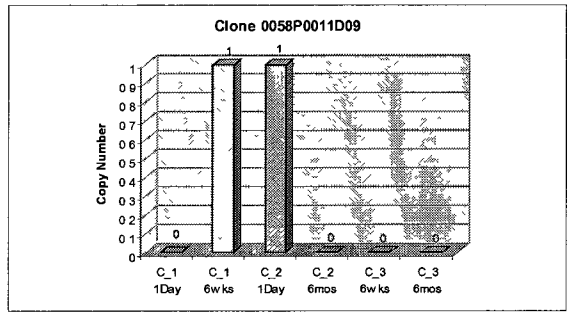


Figure 7gg: Calmodulin 1 (CALM1)

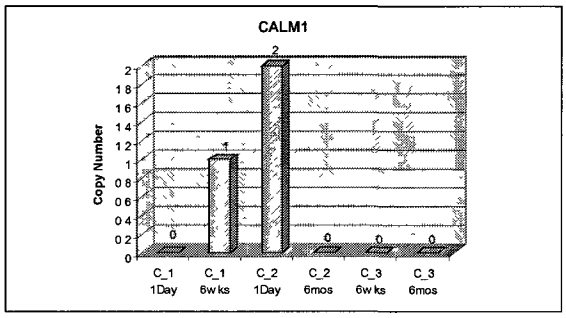


Figure 7hh: Ribosomal Protein S3A (RPS3A)

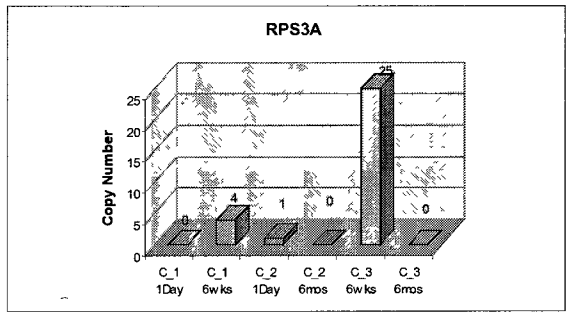


Figure 7ii: Destrin (DSTN)

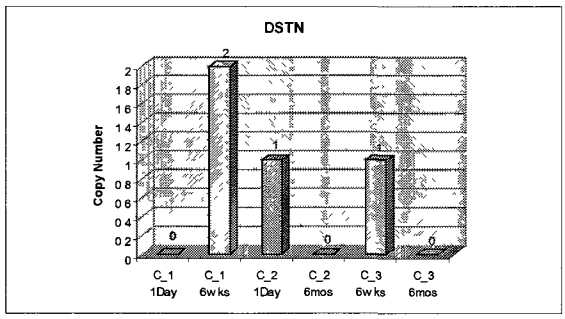


Figure 7jj: Acidic (leucine rich) nuclear phosphoprotein 32, member A (ANP32A)

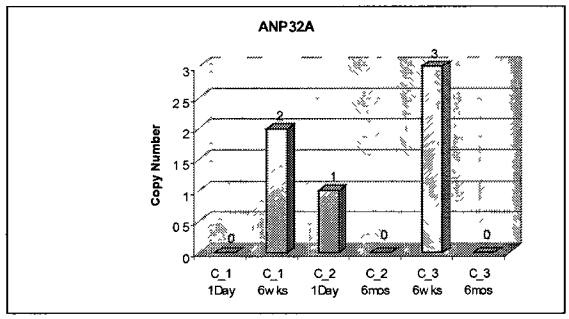


Figure 8kk: Fatty Acid Binding Muscle, Protein 4 (FABP4)

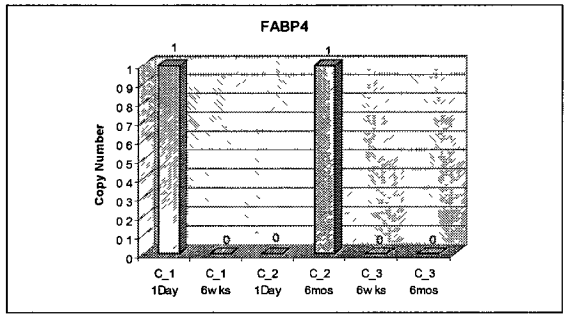


Figure 8ll: Fibulin 5 (FBLN5)

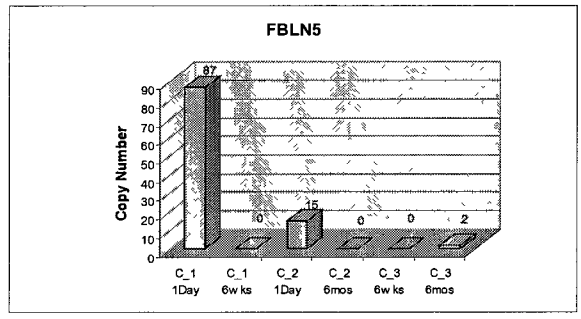


Figure 8mm: Myosin Light Chain Kinase (MYLK)

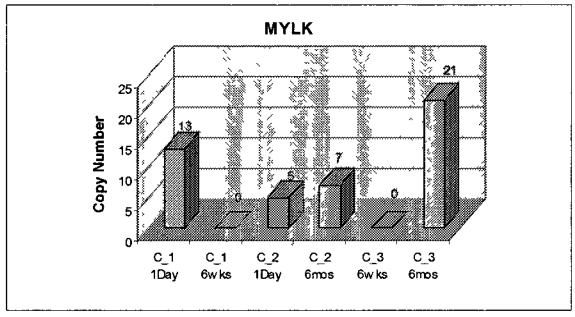


Figure 8nn: ATP Synthase F0 Subunit 8 (ATP8)

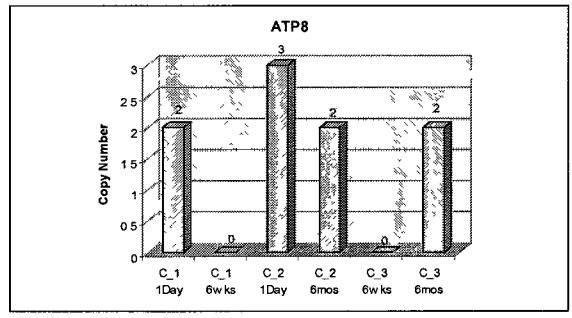


Figure 8oo: Actin, alpha 2, Smooth Aorta (ACTA2)

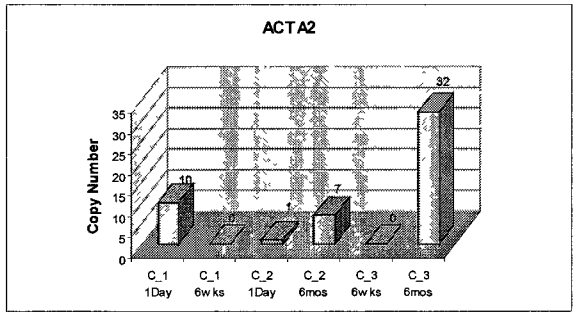


Figure 9pp: Actin, Beta (ACTB)

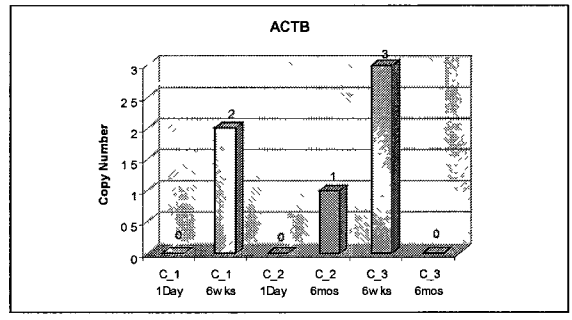


Figure 9qq: Ribosomal Protein L32 (RPL32)

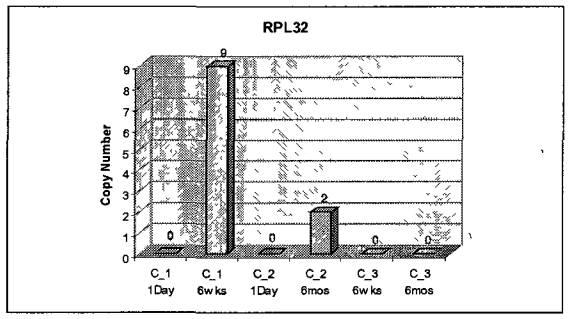


Figure 9rr: 12S Ribosomal rRNA (12S rRNA)

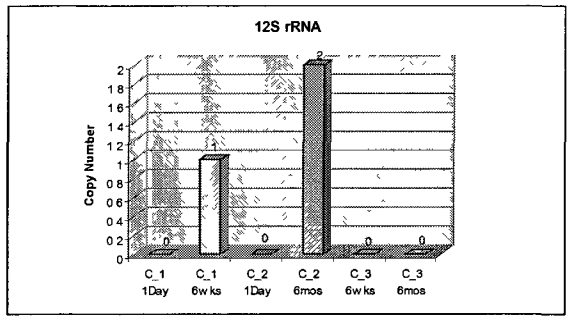


Figure 9ss: Clone ChEST 189k11

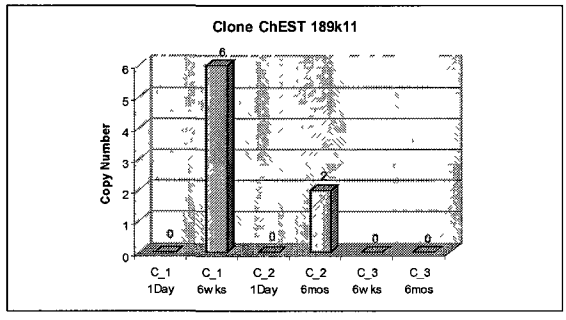


Figure 9tt: RHO Associate Coiled-Coil Forming Kinase 1 (ROCK1)

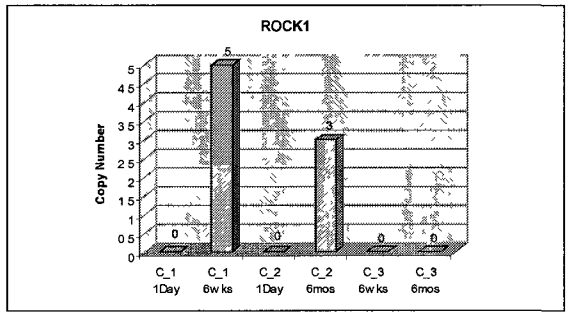


Figure 9uu: Clone ChEST 398a16

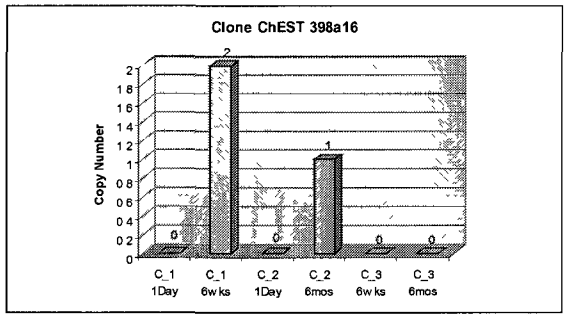


Figure 9vv: Figure 2uu: Heat Shock Protein 90 AA1 (HSP90AA1)

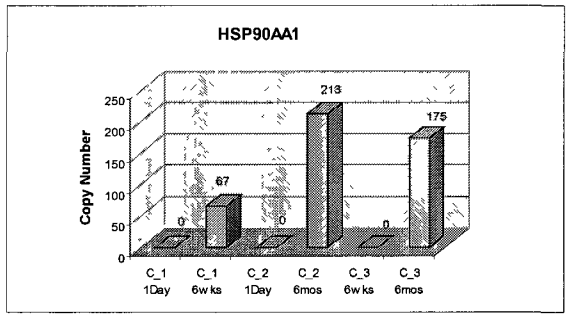


Figure 9ww: Eukaryotic Translation Initiation Factor 4A, Isoform 2 (EIF4A2)

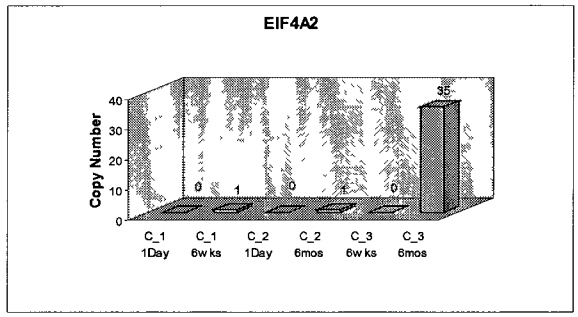


Figure 9xx: Myosin Heavy Chain 11, Smooth Muscle (MYH11)

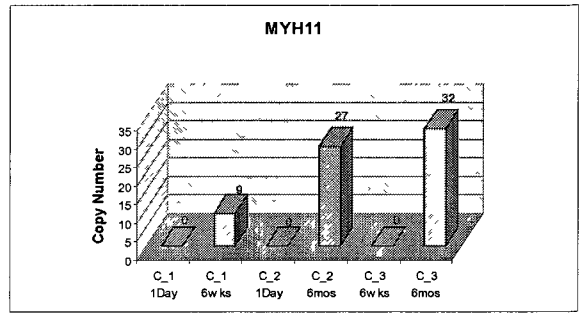


Figure 9yy: BAC Clone CH261-126j23)

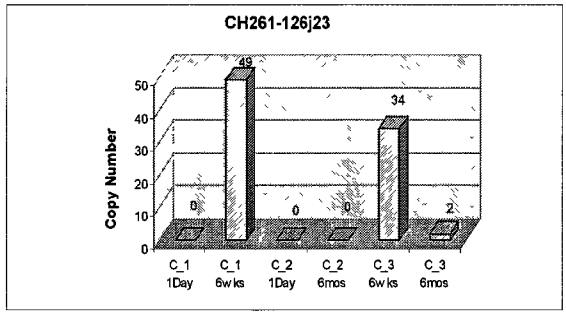


Figure 10zz: Cytochrome Oxidase III (COIII)

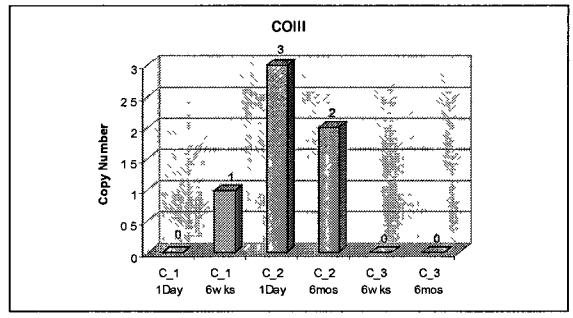


Figure 10ab: 16S Ribosomal rRNA

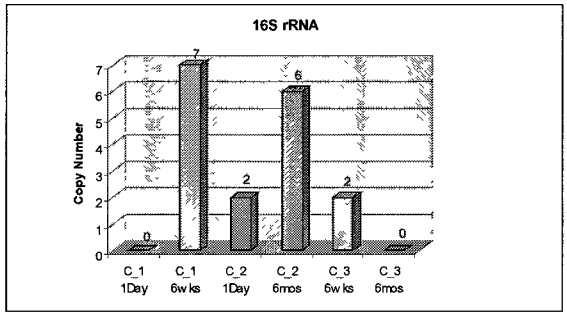


Figure 10ac: NADH Dehydrogenase Subunit 4 (ND4)

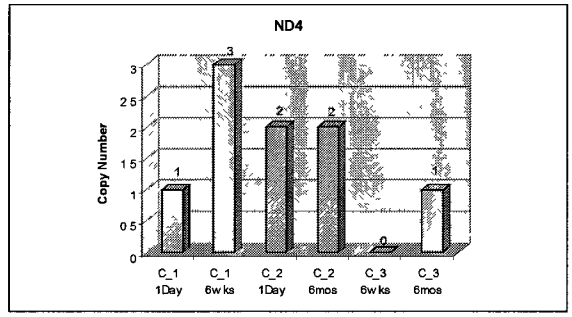


Figure 10ad: Cytochrome Oxidase II (COII)

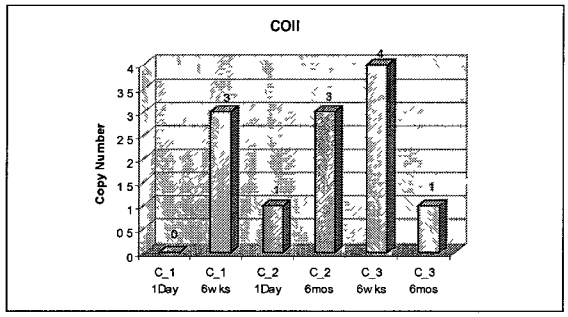


Figure 10ae: Lumican (LUM)

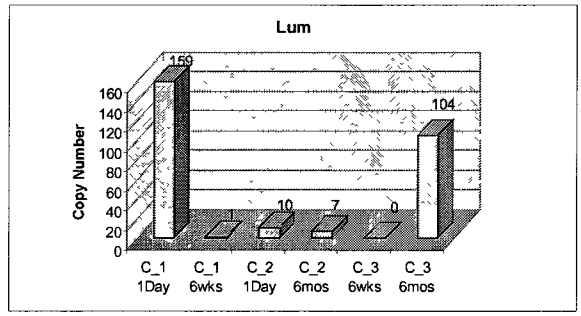
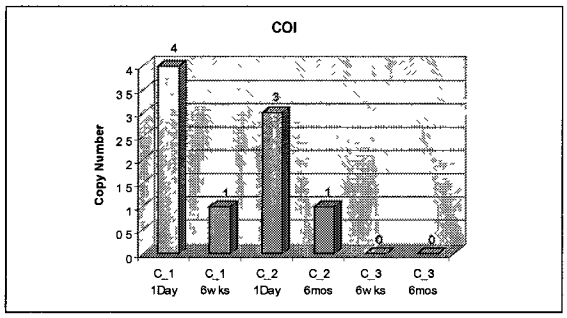


Figure 10af: Cytochrome Oxidase 1 (COI)



While examining the differential upregulation of genes within the comparisons, the seven patterns of expression that were possible when distinguishing between initiation and that of progression can be explained as follows. The first pattern, reflects genes that were differentially upregulated exclusively at 1 day when compared to the other two ages. Genes exhibiting this pattern are presented in **Figures 4a-4g** and may be the most important genes when looking at whether these genes play a role in initiation of the disease. The second pattern of expression was observed in **Figures 5h-5w.** In this pattern, genes were differentially upregulated at 6 weeks when compared to both 1 day and 6 months, suggesting early progression. The third pattern observed in **Figures 6x-6z,** include genes that were differentially upregulated exclusively at 6 months, which is suggestive of later progression. A fourth pattern that was observed in **Figures 7aa-7jj** involved genes that

were differentially upregulated at both 1 day and 6 weeks. Although this pattern may be slightly ambiguous, since they were upregulated at both 1 day and 6 weeks it is possible that they serve in initiation of the disease and may participate in early progression. The fifth pattern of expression presented in **Figures 8kk-8oo** reflected genes that were differentially upregulated at both 1 day and 6 months. This was rather interesting suggesting several possibilities. The differential upregulation at 1 day suggests genes are involved in initiation. Since they were upregulated again at 6 months, however, this may indicate that these genes are involved in both initiation and progression, or that their role was simply that of normal development. Since this pattern does not fit the pattern laid out in the objectives, these genes were not considered further for purposes of this thesis. The sixth pattern of expression **(Figures 9pp-9yy)** in which genes were differentially upregulated at both 6 weeks and at 6 months of age, indicated involvement in progression of the disease. The final and seventh pattern of expression included genes that were differentially upregulated in all of the three the ages (**Figures 10zz-10af**). The regulation of these genes, presented an ambiguous situation and resulted in not placing them in either initiation or progression.

When looking more closely at the regulation of the genes, beginning with the first pattern of expression, in **Figure 4a**, SEC61G gene was differentially upregulated at 1 day when compared to 6 weeks and 6 months of the first two comparisons respectively. This was also the case for ChEST874k20, LOC424163, DCN, FKBP9, and LRRC17 and BAC CH261-10P22 in **Figures 4b-4g**.

Genes fitting the second pattern, included GSN, RPL3, SAT1, RPS28, CCT8, DDX5, EIF3H, PARP14, RNF11, RPL4, RPL5, RPL6, TPM1, VIM, CDKN1B, and clone

0061P0031D11 represented in **Figures 5h-5w** respectively and were differentially upregulated at 6 weeks when compared with both 1 day and 6 months (in C_1 and C_3).

In the third pattern, **(Figures 6x-6z)**, CH261-117C7, ENO1, and ACSL1 were differentially upregulated at 6 months when compared to both 1 day and 6 weeks respectively. In the fourth pattern, Collagen type 1, alpha 2 (COL1A2) in **Figure 7aa**, was upregulated at 1 day when compared to both 6 weeks and 6 months in C_1 and C_2. It was also differentially upregulated at 6 weeks when compared to 6 months in C_3. **Figure 7bb** shows that NADH hydrogenase (ubiquinone) 1 alpha subcomplex, 10 (NDUFA10) was upregulated in both reciprocal ages at 6 weeks when compared to 1 day, and at 1 day when compared to 6 weeks in C-1. It was also upregulated at 6 weeks when compared to 6 months. **Figures 7cc-7ee** revealed the differential upregulation of, Eukaryotic translation initiation factor 1(EIF1), Ras homolog gene family, member A (RHOA) and Ribosomal protein S23 (RPS23) at 1 day when compared to 6 months in C-2, and at 6 weeks, when compared to 6 months in C-3 yet no difference in upregulation was observed in C-1. **Figures 7ff and 7gg** showed Clone 0058P0011D09, and Calmodulin 1 (CALM1), were differentially upregulated at 6 weeks in C-1 as well as at 1 day in C-2. **Figures 7hh-7jj** reflect the upregulation of Ribosomal protein S3A (RPS3A), Destrin (DSTN), and Acidic (leucine rich) nuclear phosphoprotein 32, member A (ANP32A), at 6 weeks when compared to both 1 day and 6 months respectively, and were also upregulated at 1 day when compared to 6 months in C-2 thus concluding genes that fit the fourth pattern of expression. In the fifth pattern of expression, **Figure 8kk**, Fatty acid binding muscle protein 4 (FABP4) was differentially upregulated at 1 day when compared to 6 weeks, and at 6 months when compared to 1 day, yet no difference in regulation appeared between 6

weeks and 6 months in C-3. Fibulin 5 (FBLN 5) **(Figure 8ll)** was differentially upregulated at 1 day when compared to both 6 weeks and 6 months and was also differentially upregulated at 6 months when compared to 6 weeks. This same pattern of regulation that was exhibited by FBLN 5 was also observed for Myosin light chain kinase (MYLK), ATP synthetase F0 subunit 8 (ATP8), and Actin, alpha 2, smooth aorta (ACTA2) (**Figures 8mm-8oo**) except that, they were also upregulated at 6 months when compared to 1 day in C-2.

Genes that fit the sixth pattern of expression included, Actin, beta (ACTB,) Ribosomal protein L32 (RPL32), 12S ribosomal rRNA (12SrRNA), Clone ChEST 189k11, RHO associate coiled-coil forming kinase 1 (ROCK1), Clone ChEST 398a16, Heat shock protein 90 AA1 (HSP90AA1), Eukaryotic translation initiation factor 4A (EIF4A2), Myosin heavy chain 11, smooth muscle (MYH11) and CH261-126j23 (**Figures 9pp-9yy**). In **Figure 9pp,** ACTB was differentially upregulated at 6 weeks when compared to both 1 day and 6 months, and at 6 months when compared to 1 day in C-2. The genes in **figures 9qq-9uu** were all upregulated at 6 weeks when compared to 1 day, and were up again at 6 months when compared to 1 day, yet there was no difference in regulation between 6 weeks and 6 months in C-3. HSP90AA1, EIF4A2, and MYH11 (**Figures 9vv-9xx**), were all upregulated at 6 weeks when compared to 1 day, and at 6 months when compared to both 1 day and 6 weeks.

The final pattern of regulation, exhibited by genes in **Figures 10zz-10af** was rather ambiguous as stated previously, and therefore prevented their placement in either initiation or progression of the disease. Genes that comprised this seventh pattern included Cytochrome oxidase III (COIII), 16S ribosomal rRNA (16S rRNA), NADH dehydrogenase

subunit 4 (ND4), Cytochrome oxidase II (COII), Lumican (LUM), and Cytochrome oxidase I (COI).

Once the 57 genes fitting the 7 different patterns of regulation were made known, they were then entered into MetaCore to determine their primary function. Although genes' fitting patterns five and seven were excluded from further analysis based on the hypothesis, they were included in this step with the hope of providing some understanding, as to why the seven groups of genes were differentially upregulated at the time points reflected by the seven patterns of regulation. These genes were laid out in table format according to the 7 types of expression patterns in **Table 14**.

Table 14: Primary Function of Genes Fitting 7 Patterns of Differential Upregulation

Seven Patterns of Expression: Gene Function According to MetaCore			
Pattern # & Age Differentially Upregulated	**Figure**	**Gene Name**	**Function**
#1: ↑ 1 day	2a	SEC61G	Transporter/Translocator
	2b	ChEST874k20	?
	2c	LOC424163	?
	2d	DCN	Proteoglycan
	2e	FKBP9	muscle contration, receptor, immunity
	2f	LRRC17	?
	2g	BAC CH261-10P22	?
#2: ↑ 6 weeks	3h	GSN	calcium modulation dependent & regulating proteins, muscle contraction
	3i	RPL3	Ribosomal production
	3j	SAT1	Transporter/transloactor
	3k	RPS28	Ribosomal production
	3l	CCT8 (TCP theta)	Chapterone
	3m	DDX5	Mitochondrial energy production, mRNA splicing, translation
	3n	Eif3H	Translation
	3o	PARP14	Transcription & translation
	3p	RNF11	Protein-protein interactions
	3q	RPL4	Ribosomal production
	3r	RPL5	Ribosomal production
	3s	RPS6	Ribosomal production
	3t	TPM1	Muscle contraction, calcium modulation dependent & regulating proteins
	3u	VIM	Cytoskeletal elements
	3v	CDKN1B (p27 KIP1)	cell cycle, kinase
	3w	Clone 0061P0031D11	?
#3:↑ 6 months	4x	CH261-117C7	?
	4y	ENO1	Glycolysis, cell growth, regulation, differentiation, immunity
	4z	ACSL1	Lipid metabolism
#4: ↑ 1 day & ↑ 6 weeks	5aa	COL1A2	Connective tissue / matrix integrity
	5bb	NDUFA1D	Mitochondrial energy production
	5cc	EIF1	Translation
	5dd	RHOA	Signal transduction, muscle contraction
	5ee	RPS23	Ribosomal production
	5ff	Clone 0058P0011D09	?
	5gg	CALM1	Calcium modulation dependent & regulating proteins, muscle contraction
	5hh	RPS3A	Ribosomal production
	5ii	DSTN	Cytoskeletal elements & organization, muscle contraction
	5jj	ANP32A (PHAP1 (pp32	Cell growth, regulation & differentiation, tumor suppressor, transcription
#5: ↑ 1 day & ↑ 6 months	6kk	FABP4 (A-FABP)	Lipid metabolism
	6ll	FBLN-5	Adhesion molecule
	6mm	MYLK	Calcium modulation dependent & regulating proteins, muscle contraction, kinase
	6nn	ATP8	Mitochondrial energy production
	6oo	ACTA2	Cell motility, muscle contraction, cytoskeletal elements
#6: ↑ 6 weeks & ↑ 6 months	7pp	ACTB	Cell motility, muscle contraction, cytoskeletal elements
	7qq	RPL32	Ribosomal production
	7rr	12SrRNA	Ribosomal production
	7ss	Clone ChEST 189k11	?
	7tt	ROCK1	Adhesive molecule, kinase
	7uu	Clone ChEST398a16	?
	7vv	HSP90AA1	Chaperone, protein folding
	7ww	EIF4A2	Translation
	7xx	MYH11	Muscle contraction
	7yy	CH261-126j23	?
#7: ↑ 1 day, ↑ 6 weeks & ↑ 6 months	8zz	COIII	Mitochondrial energy production
	8ab	16SrRNA	Ribosomal production
	8ac	ND4	Mitochondrial energy production
	8ad	COII	Mitochondrial energy production
	8ae	LUM	Proteoglycan
	8af	COII	Mitochondrial energy production

According to MetaCore, of the original 57 genes, 46 of them that fit patterns one, two, three, four, and six, were annotated and remained genes of interest. Given the

extensive amount of time that would be necessary to fully characterize each of these candidate genes, and in light of the expectations of this project, it was decided that only genes that were differentially upregulated exclusively as laid out in **Tables 10, 11, and 12** would be further analyzed with the assistance of MetaCore's database.

Of the 55 genes that were differentially upregulated at 1day (**Table 10**), only the 7 genes that were differentially upregulated exclusively at 1 day when compared to both 6weeks and 6months respectively were analyzed further for their potential role in initiation of atherosclerosis in the discussion. In addition, 107 genes were differentially upregulated at 6weeks, while 37 genes were differentially upregulated at 6months (**Tables 11 and 12,**). Of those, only the 16 genes that were differentially upregulated exclusively at 6weeks when compared to both 1day and 6months respectively (**Table 11**), and the 3 genes that were differentially upregulated exclusively at 6months when compared to both 1day and 6weeks (**Table 12**), were analyzed further for their potential role in progression. Genes fitting this criterion were then researched further in hopes of understanding their potential role in initiation and progression of atherosclerosis, and are presented in **Table 15.** These genes will be discussed further in Chapter four of this report. Some of these annotated genes were placed in available pathway maps in MetaCore, and are located in the Appendix section.

Table 15: Genes Upregulated Exclusively at 1 Day, 6 Weeks, and 6 Months When Compared to Their Respective Ages.

Annotation of Genes Differentially Upregulated Exclusively When Compared to Both Respective Ages		
Age	**Gene Name**	**Function According to MetaCore**
1 day		
	SEC61 G	Protein transport protein, necessary for protein translocation in the endoplasmic reticulum and is the central component of the protein translocation apparatus of the endoplasmic reticulum (ER) membrane
	FKBP9	Associates with ryanodine receptor (RYR-2) in cardiac muscle SR & may play unique physiological role in excitation-contraction coupling in cardiac muscle
	DCN	Bone proteoglycan II may affect the rate of fibril formation Small cellular or pericellular matrix proteoglycan that is closely related in structure to biglycan protein Is a component of connective tissue, binds to type I collagen fibrils plays a role in matrix assembly & is capable of suppressing the growth of various tumor cell lines
6 weeks		
	RPL3	The L3 protein is a component of the large 60S subunit of cytoplasmic ribosomes, that catalyze protein synthesis Ribosomes consist of a small 40S subunit and a large 60S subunit Together these subunits are composed of 4 RNA species and approximately 80 structurally distinct proteins
	RPL4	The L4 is a componet of the large 60S ribosomal subunit of cytoplasmic ribosomes
	RPL5	This gene encodes a ribosomal protein that is a component of the large 60S ribosomal subunit The protein belongs to the L18P family of ribosomal protein that interacts specifically with the beta subunit of casein kinase II Variable expression of this gene in colorectal cancers, compared to adjacent normal tissues has been observed,
	RPS6	This gene is a componet of the 40S ribosomal subunit of cytoplasmic ribosomes May play an important role in controlling cell growth and proliferation through the selective translation of particular classes of mRNA Belongs to the S6E family of ribosomal proteins It is the major substrate of protein kinases in the ribosome, with subsets of five C-terminal serine residues phosphorylated by different protein kinases
	RPS28	The S28 gene encodes a ribosomal protein that is a component of the 40S subunit located in the cytoplasm
	SAT1	Is a transporter that binds and undergoes conformational changes Catalysis of 2ndary active transfer of sulfate from one side of membrane to the other up its concentration gradient
	EIF3H	Is involved in translation of proteins in the cytoplasm
	DDX5	RNA-dependent ATPase activity The rate of ATP hydrolysis is highly stimulated by single-stranded RNA May be involved in pre-mRNA splicing DEAD box proteins are characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), are putative RNA helicases They are implicated in a number of cellular processes involving alteration of RNA secondary structure such as translation initiation, nuclear and mitochondrial splicing and ribosome and spliceosome assembly Some members of this family are believed to be involved in embryogenesis spermatogenesis and cellular growth and division
	CDKN1B	Is an important regulator of cell cycle progrssion Involved in G1 arrest Potent inhibitor of cyclin E- and cyclin A-CDK2 complexes Positive regulator of cyclin D-dependent kinases such as CDK4 Regulated by phosphorylation and degradation events Positive regulation of cellular and developmental processes
	TPM1	Binds to actin filaments in muscle and non-muscle cells Plays a central role in association with the troponin complex, in the calcium dependent regulation of vertebrate striated muscle contraction Smooth muscle contraction is regulated by interaction with caldesmon In non-muscle cells is implicated in stabilizing cytoskeleton actin filaments This gene is a member of the tropomyosin family of highly conserved widely distributed actin-binding proteins involved in the contractile system of striated and smooth muscles and the cytoskeleton of non-muscle cells
	PARP14	Enhances STAT6-dependent transcription (By similarity) Has ADP-ribosyltransferase activity Poly(ADP-ribosyl)ation is an immediate DNA damage-dependent posttranslational modification of histones and other nuclear proteins that contributes to the survival of injured proliferating cells
	VIM	Vimentins are class-III intermediate filaments found in various non-epithelial cells, especially mesenchymal cells Along with actins and microtubules (tubulins) the intermediate filaments represent a third class of well-characterized cytoskeletal elements
	CCT8	Mmolecular chaperone assist the folding of proteins upon ATP hydrolysis Known to play a role in vitro in the folding of actin and tubulin Also, known as chaperonin containing TCP1, subunit 8 (theta)
	RNF11	Contains a RING-H2 finger motif which is known to be important for protein-protein interactions Has been shown to be induced by mutant RET proteins (MEN2A/MEN2B) The germline mutations in RET gene are known to be responsible for the development of multiple endocrine neoplasia (MEN)
	GSN	Calcium-regulated actin-modulating protein that binds to the plus (or barbed) ends of actin monomers or filaments preventing monomer exchange (end-blocking or capping) It can promote the assembly of monomers into filaments (nucleation) as well as sever filaments already formed
6 months		
	ENO1	Multifunctional enzyme that, as well as its role in glycolysis plays a part in various processes such as growth control hypoxia tolerance and allergic responses May also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons Stimulates immunoglobulin production May be a tumor suppressor
	ACSL1	Activation of long-chain fatty acids for both synthesis of cellular lipids and degradation via beta-oxidation is an isozyme of the long-chain fatty-acid-coenzyme A ligase family Converts free long-chain fatty acids into fatty acyl-CoA esters and thereby plays a key role in lipid biosynthesis and fatty acid degradation

CHAPTER IV

DISCUSSION

In the WC pigeon, spontaneous atherosclerosis develops in the absence of known risk factors (49) making it an excellent model in which to study underlying genetic factors associated with atherosclerosis. In this study, two hundred and ninety one non-redundant genes were differentially upregulated at 1 day, 6 weeks, and 6 months of age in the celiac bifurcation of the WC pigeon. When considering the placement of genes that were differentially upregulated at each of the three ages, previous research demonstrates that initiation occurs early on. In fact, Santerre reported that in the WC pigeon, spontaneous atherosclerosis occurs as early as 1 day (80). Therefore, genes that were differentially upregulated at 1 day were considered to participate in initiation of atherosclerosis.

While the actual point at which initiation occurs is less defined, by 6weeks of age the biochemical and morphological hallmarks demonstrate that progression of the disease is well under way (57). In addition, reports suggest that factors responsible for initiation are independent of those that are responsible for progression (81). With that said, the initiating event in the WC sets in motion the documented cellular, biochemical, and molecular changes that ensue marking the disease progression (50). Based on these studies, genes that were differentially upregulated at 6 weeks and 6 months of age were considered more likely to participate in the progression of atherosclerosis.

Only a subset of the genes most likely to be involved in the disease, that were upregulated exclusively and annotated in MetaCore, were chosen from each of the three age groups to be analyzed further for their potential role in initiation and progression of atherosclerosis. It is important, not to lose sight of the fact that many of the genes of interest may actually be involved in normal development. In light of this, efforts were made to distinguish between genes involved in normal healthy development and those involved in the pathogenic state.

Of the 7 genes that were differentially upregulated exclusively at 1 day, only 3 genes were fully annotated in MetaCore and include, SEC61G, FKBP9, and DCN. According to MetaCore, SEC61G is the central component of the protein translocation apparatus which is necessary for translocation of proteins in the endoplasmic reticulum (82). FKBP9 associates with ryanodine receptor (RYR-2) in cardiac smooth muscle sarcoplasmic reticulum and may play a unique role in excitation contraction coupling in cardiac muscle. FKBP9 also has the potential to contribute to the immunosuppressive and toxic effects of FK506 (an immunosuppressive drug) and rapamyacin (an immunosuppressant) (82, 83). In a study conducted to explore novel genetic causes of inherited cardiomyopathies in one kindred (4 generations, 32 individuals), linkage was established to a novel locus on chromosome 7 (7p12.1-7q21) which included the candidate genes SEC61G and FKBP9, although no disease causing mutations were identified (84).

The third gene fully annotated in MetaCore, DCN, is recognized as bone proteoglycan II (82). DCN is a component of connective tissue and binds to type I collagen fibrils (COL1A2). The gene COL1A2 which codes for Type I collagen fibrils was also differentially upregulated at 1 day when compared to 6 weeks and again when compared to

6 months, and is also known to play a role in matrix assembly (82). DCN is also capable of suppressing the growth of various tumor lines (82). A study, conducted to assess the relationship between proteoglycans and calcification of cultured bovine aortic SMCs revealed that there was a significant increase in DCN, peaking by 7-10 days and decreasing by day 14, although it remained elevated when compared to control cells (85). The researchers also reported that over expression of DCN enhanced the calcification of tissues as seen in sheets of arterial SMCs and in colocalized regions of atherosclerotic plaques in arteries (85).

Immunofluorescence of human carotid artery segments, obtained from autopsy or from surgery, revealed that in non-atherosclerotic arteries, DCN was detected only in the adventitia (86). On the other hand, in arteries that contained atherosclerotic lesions, DCN was present primarily in the layer of the intima close to the media with less in the subendothelial part of the intima (86). In addition, this study found that while type I and III collagen in the arterial intima associates with DCN, group II secretory nonpancreatic phospholipase A2 (snpPLA2) binds preferentially to DCN which enhances snpPLA2 activity, and may contribute to the pathogenesis of atherosclerosis by modifying lipoproteins and releasing inflammatory lipid mediators at sites of lipoprotein accumulation (86). While it is not known precisely whether SEC61G and FKBP9 are responsible or involved in initiation of atherosclerosis, research strongly implicates DCN as a major player in atherosclerosis, although a specific role in initiation remains unclear at the present time.

At 6 weeks, 15 of the 16 genes that were exclusive to this age were annotated in MetaCore. Five of these included genes responsible for ribosomal production. The first

three, RPL3, RPL4, and RPL5, are all components of the large 60S subunit of cytoplasmic ribosomes while RPS6 and RPS28 belong to the small 40S subunit of cytoplasmic ribosomal proteins. RPL3 belongs to the L3P family of ribosomes and is suggested to contribute to tat-mediated transactivation by binding to RNA which then activates translation (82) [Appendix D]. While RPL4 belongs to the L4E family of ribosomal proteins (82), a study by Zengel et al., found that RPL4 regulates the S10 operon of Escherichia coli by inhibiting translation of the proximal gene and by stimulating premature termination of transcription within the S10 leader (87). According to MetaCore, ribosomal protein L5 belongs to the L18p family of ribosomal proteins in the cytoplasm (82). Ribosomal S6 belongs to the S6E family of ribosomal proteins and may be key in controlling cell growth and proliferation through selective translation of particular classes of mRNA. It is a major substrate of protein kinases in the ribosome, with subsets of five C-terminal serine residues that are phosphorylated by different protein kinases (82). RPS28 belongs to the S28E family of ribosomal proteins (82) [Appendices D, and K].

Interestingly, RPS28 and RPL3 were also differentially upregulated exclusively at 6 weeks in the WC when compared to the SR as shown in Anderson's unpublished in-vivo RDA work (63) [Appendix S]. While RPL3 was upregulated differentially at 6 weeks in both this work and in the in-vivo work, it was also downregulated by 6 months of age in the WC in both bodies of work. This was also the case in Anderson's in-vitro work (which represents that of a compressed time-frame of approximately 2-3 years) [Appendix Q]. There, RPL3 was upregulated significantly in the SR when compared to the WC. These patterns appear to support the ultrastructural work conducted by Cooke where he found that

free ribosomes gradually decreased in the aorta of the spontaneously atherosclerotic WC as they developed unlike those of the SR (88).

In support of Cooke's findings, while many different ribosomal genes were differentially upregulated at each of the three ages throughout the three comparisons, most of the ribosomal genes that were upregulated at 6 weeks in this study were down by 6 months of age in the WC. More specifically, 10 different ribosomal genes were upregulated at 6 weeks of age when compared to 1 day [Appendix D], and 8 different ribosomal genes were upregulated at 6 weeks when compared to 6 months in the WC. However, in the reciprocal age comparisons, there was no increased regulation of ribosomal genes at 1 day or at 6 months when compared to 6 weeks. It was suggested by Anderson that, although the changes in regulation could not be explained, it is possible that the WC is attempting to compensate for ribosomal degradation by increasing synthesis by 6 weeks, followed by sharp a decline as the WC ages (63). This seems reasonable, given that this was the case in this current work and was also reported by Cooke, although future work will be needed to discern whether the changes in regulation by 6 weeks are due to the disease progression or other mechanisms that are unrelated to the disease.

Also of interest is that many of the genes that were differentially upregulated at 6 weeks were also involved in both transcription and translation, protein folding and transport. EIF3H was also upregulated exclusively at 6 weeks. While the exact role of EIF3H was not determined it is involved in translation of proteins following transcription (82). It also seems reasonable to suggest that the increased regulation at 6 weeks may be due at least in part to some of the increased synthesis of extracellular materials that occur during early progression of atherosclerosis. For example, DDX5 was also upregulated

exclusively at this age. It is an RNA helicase that is implicated in a number of cellular processes involved in altering the RNA secondary structure such as translation initiation, nuclear, and mitochondrial splicing, as well as ribosome and spliceosome assembly (82). DDX5 is known to encode a dead box protein, which is an RNA-dependent ATPase, and a proliferation-associated nuclear antigen, that is specifically known to react with the simian virus 40tumor antigen, and may also be involved in pre-mRNA splicing (82).

Inhibitor p27 Kip1 (CDKN1B) is a positive regulator of cellular and developmental processes that impact cell cycle progression (82). It is involved in G1-arrest and is a potent inhibitor of cyclin-E and cyclin-A CDK2 complexes. It is also a positive regulator of cyclin-D-dependant kinases such as CDK4 that is regulated by phosphorylation and degradation events (82). According to Lauter et al., CDKN1B is a dependant kinase inhibitor which serves to repress the function of cyclin-dependent kinases, necessary for cellular progression through G1 to S-phase of the cell cycle (89). CDKN1B expression has been shown to be decreased in various tumor types including colon cancer (89) and may explain the decreased regulation by 6 months.

Molatore et al., also reported that CDKN1B acts as a tumor suppressor to regulate the suppression of G1 to S-phase by regulating the activity of cyclin E and cyclin A CDK2 (90). In addition to its negative role in cell cycle progression, it is involved in cell migration, neuronal differentiation and apoptosis (90). A study by Nowel et al., found that CDKN1B was downregulated while p52 was upregulated in early stage fallopian tube carcinoma (FTC) when compared to normal fallopian tube tissues (91).

Immunohistochemistry results revealed that the decreased protein expression of CDKN1B in FTCs continued as the cancer progressed (91). In addition, when SMCs are

bound to fibronectins and proteoglycans in atherosclerotic plaques, CDK2 inhibitors such as P27Kip1 are downregulated to promote SMC proliferation (34). Based on these studies, there may actually be a relationship between the decreased regulation of CDKN1B by 6 months of age in the WC, the progression of atherosclerosis, and the mechanisms involved in tumor progression although future work will be needed to fully discern this.

Poly (ADP-ribose) polymerase or PARP14 was also exclusively upregulated at 6 weeks of age. PARP14 is known to enhance STAT6-dependent transcription factor, while STAT6 acts in response to cytokine and growth factor activity (82). PARP14 responds immediately to DNA damage following posttranslational modification of histones and other nuclear proteins in order to collectively contribute to the survival of injured proliferating cells (82). Research by Agarwal et al., demonstrated the enzymatic DNA repair activity of PARP14 in male germ cells, during spermatogenesis (92). There, increased levels of PARP14 were present in mature spermatozoa, in response to strand breaks in DNA as a result of oxidative stress, chromatin remodeling and cell death (92). This response of PARP14 to DNA damage was associated particularly with inflammatory cytokines, and with oxidative stress that was connected with protein modification due to biological or chemical stressors (92). During atherosclerosis, cytokine production is dramatically increased. Cells such as T-cells, macrophages, endothelial cells, and SMCs in particular, produce such cytokines as platelet derived growth factor, macrophage inhibitory factor (MIF+), interferon gamma (IFNγ) and monocyte chemoattractant protein (MCP-1) (34). It is therefore possible that, the increase in PARP14 by 6 weeks of age, may be in response to an increase in cytokine production known to occur during atherosclerosis.

Ring finger protein (RNF11) contains a RING-H2 finger motif and a PY motif, which is known to be important for protein-protein interactions (82). RNF11 has been shown to be induced by mutant rearranged transformation (RET) proteins, also known as multiple endocrine neoplasia (MEN2A/MEN2B). The germline mutations in the RET gene are known to be responsible for the development of multiple endocrine neoplasias (82). Immunohistochemical analysis of RNF11 protein revealed that RNF11 was markedly overexpressed in breast cancer, and to a lesser extent in pancreatic and prostate cancers (93). It is believed that RNF11 may function as an adaptor molecule for the recruitment of specific substrates to E3 ligase complexes for ubiquitination and subsequent degradation by 26S-proteosome (93). Li et al., concluded that RNF11 is likely involved in multiple cancer related cellular processes such as gene transcription, protein ubiquitination and ubiquitination associated events related to receptor endocytosis and protein sorting (93).

Gelsolin (GSN) is a calcium-regulated, actin-modulating protein that binds to the plus (or barbed) ends of actin monomers or filaments, preventing monomer exchange (end-blocking or capping). It can promote the assembly of monomers into filaments as well as sever filaments already formed (82). Nishio et al., found that gelsolin has multiple regulatory activities, including cytoskeletal remodeling and ion channel regulation (94). They also found that GSN mRNA levels are elevated while plasma concentration levels of GSN are decreased in patients with ischemic heart disease and in animal models of heart failure. As a result, they suggested that GSN plays a key role in cardiac remodeling (94). Work conducted by Li et al., suggests a link between GSN and induction of apoptosis following MI (95). They detected enhanced expression of gelsolin in human heart failure, and in dilated and ischemic cardiomyopathy (95). Li et al., also found that cells with

gelsolin are likely to produce more actin-barbed ends and actin monomers, which bind to deoxyribonuclease (DNase I), a key enzyme responsible for DNA degradation following the stimulus generated by MI, which tries to attenuate enhanced activation of promoting cell death by apoptosis (95). Following microarray gene expression analysis of 22,283 human genes, it was found that GSN was markedly increased in human heart failure of both dilated and ischemic cardiomyopathy phenotypes (95). Immunoblotting revealed that GSN protein was also increased following MI in mouse models (95). In mice, COL1A1 and COL3A1 were also higher in GSN +/+ hearts than GSN-/- hearts which is believed to be pro-apoptotic(95). This relationship was not the case however, for Gelsolin at 6 weeks in that COL1A1 and COL3A1 were down by this age in the WC, possibly suggesting loss of regulation of apoptosis. Also, GSN, which is a substrate of caspase-3, -7+ -9 is frequently downregulated in human cancers, that are able to escape apoptosis (95). Caspase-3 is considered a key mediator of apoptosis in mammalian cells (96). In cells that were exposed to Fas receptor, there was an increased presence of GSN cleaved fragments through caspase-3 which then led to apoptosis of the cells (96).

Chaperonin containing TCP1, subunit 8 (CCT8) according to MetaCore is a cytosolic chaperone protein that assists in protein folding upon ATP hydrolysis (82). In-vitro it was shown to be involved in the folding of tubulin and actin, which interestingly ACTB was also upregulated at this age (82). The presence of CCT8 at 6weeks of age may actually be in response to the increased regulation of the many non-redundant genes that were presented in **Table 9** of the results. CCT8 may also be associated with the folding of Vimentin (VIM), which was also exclusively upregulated at this age. According to MetaCore, VIM, like actins and microtubules, are class III intermediate cytoskeletal

filaments, which are all associated with one another in various capacities (82) [Appendices B and L]. VIM is found primarily in mesenchymal cells, displaying tissue specific patterns of expression associated with cell integrity (97). Unlike actins and microtubules however, VIM provides resiliency to cells during times of mechanical stress. (97).

In a study by Goldman et al, Vim-free cells were shown to be more fragile, pointing to the structural role that VIM plays (97). It appears that VIM in an attempt to deal with stress inducing alterations associated with disease progression, is attempting to offset the morphological alterations that are observed in the cytoskeleton (98). As noted in the literature, these changes are thought to be in response to the shift in SMCs to the synthetic state, which is also known to increase the proliferative index of ECM, further compromising the integrity of atherosclerotic tissue (34). These changes were followed by increases in Chondroitin 6-sulfate and Chondroitin 4-sulfate, both glycosaminoglycans (GAG) that remain elevated at 6months. This is believed to precede and be at least partially responsible for the uptake of lipid in the cells, and further progression of atherosclerosis (16, 34). Each of these findings are believed to facilitate lipid accumulation and hypoxic stress, both contributors to the disease progression (16, 57). Given all that is in transition during this stage, it seems reasonable to suggest that the increased regulation observed by all these genes at 6 weeks, occurs in response, at least in part to some of the regulatory changes and stressors placed on aortic tissues during early progression.

Tropomyosin (TPM1) was also exclusively upregulated at 6weeks. It functions in both muscle and non-muscle cells and is a member of the tropomyosin family of highly conserved actin binding proteins(82) [Appendices B and C]. TPM, which is regulated by caldesmon binds to and stabilizes cytoskeletal actin filaments during SMC contraction (82).

Rong reported a decrease in both TPM1 as well as ACTA following cholesterol loading in SMCs, which he reported to be the result of a shift from the healthy contractile state to the synthetic state (39). It is well established that ACTA levels as well as other SMC differentiation markers, decrease in-vivo in response to atherosclerosis and or vascular injury (36, 99). Work conducted by Kashiqada et al., reported that a shift from differentiated SMCs, to dedifferentiated SMCs led to the phenotypic modulation of TPM1 causing it to shift from a SMC type to a fibroblastic type 1 and 2 (100) .

A shift in regulation between ACTA and ACTB was observed at 6weeks in this current research. Here ACTA was up at 1day and again by 6months, while ACTB was not upregulated until 6weeks and remained up at 6months. Interestingly, Anderson found an inverse relationship between ACTA and ACTB (63). The results from her work suggest that higher levels of ACTA represent normal contractility, while increased regulation of ACTB represents a loss of contractility in the SMC (63). She proposed that decreased ACTA and TPM1, in parallel with increased ACTB, signal a loss of muscle contraction (63).

It is possible that the upregulation of TPM1 at 6weeks in the current results had not yet caught up with the changes in regulation between ACTA and ACTB. The increased regulation that was observed by TPM1 exclusively at 6weeks may actually reflect a compensatory mechanism of the WC, to at least initially maintain some sort of regulation during the transition from the contractile state to the synthetic state (100).

Spermidine/spermine N1-acetyltransferase (SAT1) was also upregulated exclusively at 6weeks and is a member of the family of sulfate/anion transporters. According to MetaCore, SAT1 functions as a sodium-dependent amino acid transporter,

which participates in many reactions such as the uptake of nutrients, detoxification, energy production, and neurotransmitter cycling (82) [Appendices M and N]. In multiple studies, SAT1 was upregulated during times of stress. Babbar reported that, increases in the cytokine, tumor necrosis factor alpha (TNFalpha), resulted in increases in SAT1 which encodes a rate-limiting polyamine catabolic enzyme, reducing intracellular polyamine contents in lung cancer cells (101). SAT1 has also been associated with decreased cell growth and apoptosis (101). A study by Wang et al showed that, over expression of SAT1 resulted in rounding up and loss of cell anchoring, along with alterations in the morphology of actin-containing filopodia (102). In another study SAT2, not SAT1, was shown to have a high affinity for I-proline transport in vascular SMCs while SAT1 was found exclusively in brain cells (103). These findings conflict with Babbar's work, as well as the results of this current research, and Anderson's work, which all found that SAT1 was upregulated in the WC when compared to the SR. Perhaps the increased cytokine production in response to disease progression of atherosclerosis, as reported by Doran, may explain the increased regulation of SAT1 at 6 weeks.

While it is interesting that many of the genes that were upregulated at 1 day and at 6 weeks were associated with transcription, translation and changes in cell integrity, a number of the genes were also associated with decreased immune function, increases in cytokine production, inflamation, and progression of some tumor lines and cancers. It is also interesting that 6 of the genes that were differentially upregulated in the WC, were reportedly expressed in multiple tumor lines and cancers. While it is not known at this point, whether this is due to the large amount of work that has been conducted on tumors and cancers, this pattern does seem to raise interest in Benditt's monoclonal theory of

atherogenesis (104). It is also possible that the increased regulation at 6 weeks was an early compensatory mechanism to control some of the negative downstream affects following initiation and even early progression, especially those in response to stress and cytokine production. It is also important to note that 6 of the 15 genes that were upregulated exclusively in the WC and analyzed here, were also upregulated in the WC when compared to the SR in Anderson's unpublished in-vivo work. Those genes include CCT8, VIM, TPM1, RPL3, RPS28, and SAT1, which are presented in Appendix A. Future work will be needed to fully understand the mechanisms, in which these genes are involved.

At 6 months of age, genes that were differentially upregulated exclusively include ENO1 and ACSL1. The first gene, ENO1 is a multifunctional enzyme that participates in the later steps of glycolysis to convert 2-phosphoglycerate to phosphorenolpyruvate (82) [Appendices E, F and J]. It is also known to participate in many additional metabolic functions such as the regulation of growth, response to hypoxic conditions, and immunoglobulin production (82). Unpublished in-vitro SMC work conducted previously in this lab revealed that ENO1 was differentially upregulated (30:0) in the WC when compared to the SR (63) [Appendix Q]. Additional in-vivo work revealed however, that ENO1 was not differentially upregulated at 6months when compared to the SR [Appendix T]. This appeared to confound ENO1 as a major gene of interest in the progression of atherosclerosis, although it is quite possible that this type of regulation of ENO1 in-vitro indicates that ENO1 is acting at the level of the SMC. Since this was in-vitro, it may also indicate that the increased regulation of ENO1 occurs much later than 6months of age. Research shows that SMCs have been shown to be the first cells present in locations

destined to develop atherosclerotic plaques (34, 39, 105), and that intrinsic properties of SMCs are intimately implicated with the regulation of the disease (49). Since ENO1 was also associated with hypoxic stress according to MetaCore, and given that one of the characteristics of atherosclerosis is that of hypoxic stress, coupled with the role of SMCs, ENO1 appears to remain a primary gene of interest involved in atherosclerotic progression.

In regards to energy production, Hajjar et al showed that the WC totally lacked ATP regulation of NADH transhydrogenation by 6weeks of age, and by 6months had lower P/O ratios as compared to the SR, suggesting uncoupled respiratory-chain phosphorylation (17), which may in part explain the increased regulation of ENO1. While examining the differences in energy production between both breeds, Zemplenyi revealed there were differences in the amount of arterial enzymes that were produced between WC and SR pigeons at 5-8 weeks of age. His findings demonstrated that there was in fact a clear shift in the WC from oxidative phosphorylation to glycolysis at the level of the arterial wall (106). Zemplenyi suggested that this hypoxic shift predisposed the WC to atherosclerosis. He also found that lipoamide dehydrogenase, a component of the pyruvate dehydrogenase complex was significantly lower in the WC when compared to the SR (106). The decreased amount of lipoamide dehydrogenase in the presence of acetyl CoA slowed down the conversion of pyruvate to oxaloacetate markedly slowing down the Krebs cycle. There was also an increase in the amount of phosphofructose kinase, which is the rate-limiting step of glycolysis in the WC at this age. This effect was however believed to be a secondary effect resulting from the decreased rate of the Krebs cycle (106).

Work conducted by Santerre found that respiratory control ratios in the WC were high at 1day, dropping to low levels by 3-6 months unlike the SR, where respiratory ratios

increase as the SR grows and develops (80). Zemplenyi also found that tissue hypoxia not only forces glycolysis to become the primary energy pathway but it also contributes to the accumulation of lipids and the synthesis of arterial connective tissue apparatus (106). Hajjar et al showed that tissue hypoxia occurs later, following a rise in GAGs (64). By 12-24 weeks, they were able to demonstrate that the O2 tension in the medial layer of WC was lower than that in the SR and in younger WC pigeons (64). By 6 months, increases in chondroitin 6-sulfate and other GAGs were present which have been correlated with lipid accumulation (16).

As stated previously, acyl-CoA synthetase long-chain family member 1 (ACSL1) was also differentially upregulated exclusively by 6months. ACSL1s are membrane bound long chain synthetases that trap fatty acids on the cytosolic side following passive diffusion across the plasma membrane (107) [Appendices G and I]. This timing of the increased regulation of ACSL1, according to MetaCore, occurs in response to an increase in the synthesis of lipids, and appears to correlate well with the build up of lipids observed by Zemplenyi, and the rise in GAGs reported by Hajjar et al. Increases in ACSL1 are also known to occur in response to beta-oxidation of fatty acids, which must first be converted to fatty acyl-CoA in the cytosol using ACSL1 [Appendix H]. These are then transported into the mitochondria assisted by the carnitine shuttle to then undergo beta-oxidation to their two carbon units. Un-published in-vitro work in this lab revealed that ACSL1 was also differentially upregulated when compared to the SR, which correlates well with the energy changes that occur as a result of the disease progression. This loss of regulation in energy metabolism represents a shift from oxidative phosphorylation to glycolysis as the

dominant energy pathway by 6 months in the WC, and that it may actually be at the level of the SMC.

Limitations

While the RDA method is quite effective at presenting only genes that are differentially expressed between two similar populations, limitations do exist within the context of this project. Given the narrow selection process of analyzing only genes differentially upregulated exclusively at 1 day, a number of potential genes of interest were excluded, reducing the chances of finding a gene responsible for initiation.

Such a small amount of starting material that was used from the original pool of cDNA at the onset of RDA, may also have limited the selection process of potential genes throughout, especially since only 1ng was used during the third rounds of SH. While the goal of the RDA method was to remove genes common between both populations, it was observed following Rounds 3, that electrophoresis gels revealed some of the bands were less defined than in the previous round. As a result, questions were raised whether actual quality difference products were removed, therefore reducing potential genes of interest at all of the ages. The small amount of cDNA that was used originally could further reduce the chances of finding a gene of interest. The other point to keep in mind is that, the amount of total RNA that was collected prior to RDA represents only a snapshot in time. Despite these limitations, the level of sensitivity of the RDA method is impressive in that it can select for genes differentially upregulated, of low copy number and then amplify them, without prior knowledge of their sequence, and with a low number of false positives (72).

In addition, of the 286 quality sequences that were unidentified, 68% were

upregulated at 1 day alone [Appendices O and P]. This fact alone makes it likely that the gene(s) associated with initiation remain unknown. Given the RDA method, the parameters of selection, and the large number of unidentified genes that remain, it is quite probable that additional gene(s) of interest associated with initiation and progression remain elusive.

Future work that encompasses a wider scope of analysis, and includes more complex and detailed investigation of additional candidate genes may be helpful in identifying genes responsible for atherosclerosis. Additional research is also needed to identify the 196 unidentified quality ESTs that were upregulated at 1 day. It is also quite possible that the gene (s) of interest lie in this group of ESTs. Given all these limitations, and in light of the technology that is currently available, the RDA method is still the best method we have at this point in time.

When considering the genes that were not placed in either initiation or progression, new research demonstrates that genes share dual roles. It is also possible that the two processes of initiation and progression, may not be distinct from one another (or at least for some of the genes) as reported by Herndon et al. Some of the genes that were differentially upregulated at 1 day, and again at 6 months may actually share this duality. For example, genes that were differentially upregulated at 1 day may reflect initiation, yet when they were differentially upregulated at 6 months, they may in fact reflect normal development, or progression of the disease. According to Seroude, changes in gene regulation are associated with the rate of physiological aging as opposed to chronological aging (108). Interestingly, aging is associated with the same highly dynamic regulatory changes observed during development, and these same regulatory changes that occur during aging,

also occur in response to stress (108). In fact, both the up and downregulation of genes whose expression changed during aging, also changed when exposed to oxidative stress (109). All told, it is the combination of genes and their regulation that impacts the downstream effects (108). In light of this and the current findings, future work will be required to sort through all the genes that were differentially upregulated at more than one age.

CONCLUSIONS

A number of candidate genes were differentially upregulated at 1 day. More importantly seven genes were differentially upregulated exclusively at 1 day, therefore placing them as potentially being involved in initiation of atherosclerosis in the WC pigeon under the conditions evaluated in the hypothesis. However, given the tight parameters of this hypothesis, while SEC61G, FKBP9 and DCN were correlated with atherosclerosis they could not be definitively assigned a significant role in initiation.

At 6 weeks and at 6 months, seventeen candidate genes were differentially upregulated exclusively, and were analyzed for their role with progression of atherosclerosis. The genes included RPL3, RPL4, RPL5, RPS6, RPS28, EIF3H, DDX5, CDKN1B, PARP14, RNF11, GSN, CCT8, VIM, TPM1, SAT1, ENO1, and ACSL1. The events associated with these genes include increases in ribosomal biogenesis in response to increased protein synthesis, transcription, translation, splicing associated with transcription and translation, regulation of cell cycle progression, immune and inflammatory functions, stress associated with protein translation and modification. Several genes were also involved in ubiquitination and degradation, cytoskeletal remodeling and apoptosis, increases in chaperonin protein involved in the folding of newly translated proteins, decreases in cellular integrity, changes in muscle contraction, and increases in a transporter gene that was also upregulated in response to cellular stress, and increased regulation of cell cycle.

Finally, changes in energy metabolism were apparent by 6 months of age reflecting a shift from oxidative phosphorylation to glycolysis in the WC pigeon, as well as increases in lipid synthesis and degradation. These findings concur with previous studies which strongly suggest that this energy shift is a major event in the development of atherosclerosis in the WC pigeon.

Future work will be necessary to fully analyze the functional role for each of the major candidate genes associated with initiation and progression of atherosclerosis, as well as for the many additional genes of interest that were not analyzed based on the current hypothesis. Also, many more unidentified quality transcripts were differentially upregulated at 1 day than at any other age according to the e-value criteria of acceptance. It will therefore be essential to identify and further annotate the quality sequences that were retained so that they can then be analyzed for their potential role in initiation of atherosclerosis.

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APPENDICES

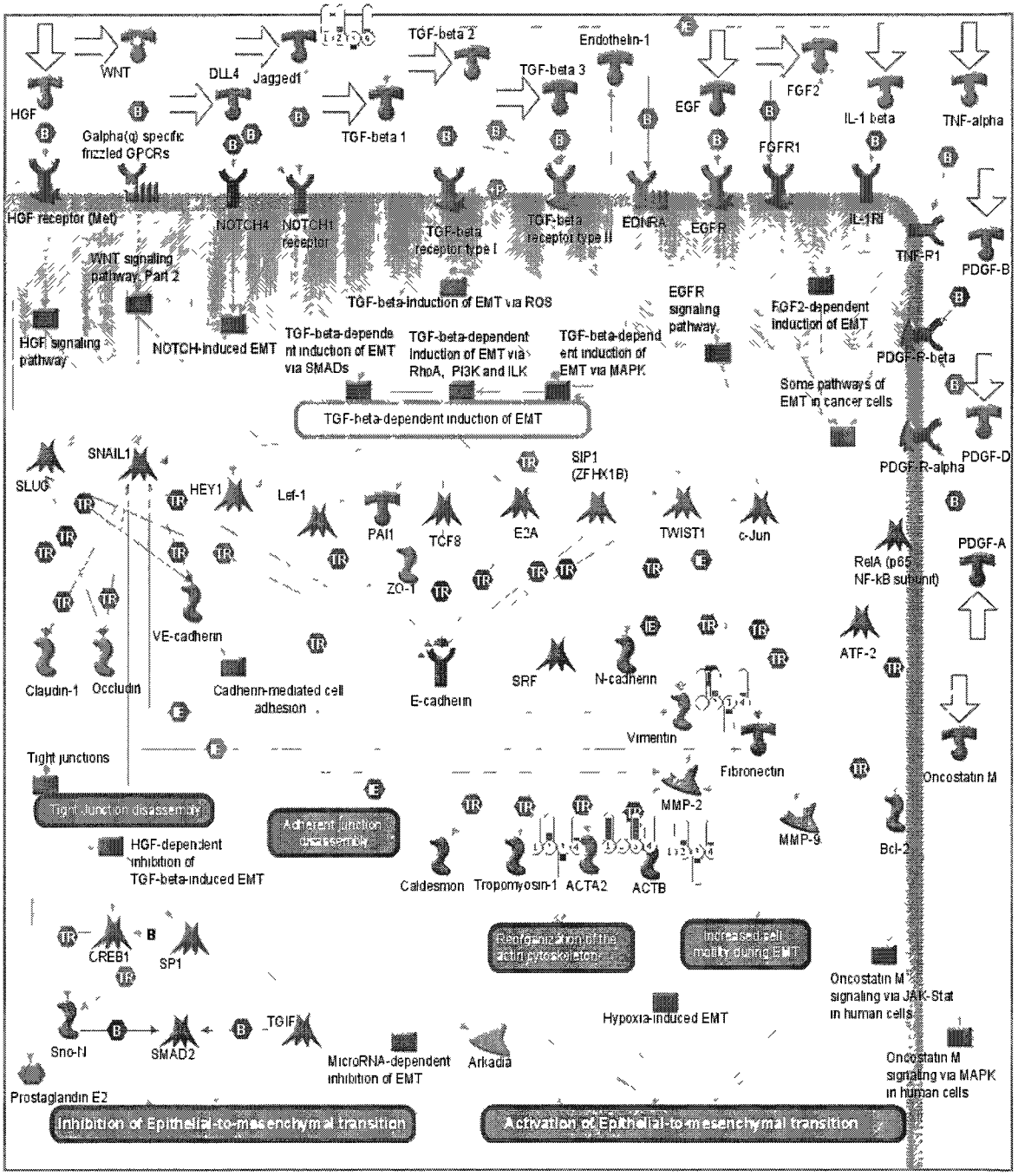
APPENDIX A: GENE FUNCTION ACCORDING TO METACORE

Table 1: Genes Upregulated or Downregulated Exclusively in WC and in WC When Compared to SR

Genes Upregulated or Downregulated exclusively in the WC in this Current Body of Work and in Anderson's Work			
Function According to MetaCore			
Age	Gene Name	Ortholog	Function(s)
Upregulated			
6weeks	RPL3	Ribosomal Protein L3	The L3 protein is a member of the larges 60S subunit of cytoplasmic ribosomes responsible for catalyzing protein synthesis.
6weeks	RPS28	Ribosomal Protein S28	The S28 is a component of the small 40S subunit of cytoplasmic ribosomes responsible for catalyzing protein synthesis.
6weeks	CCT8	Chaperonin Containing TCP1, subunit 8	Assists in the folding of proteins upon ATP hydrolysis. Known to play a role, in-vitro, in the folding of actin and tubulin
6weeks	VIM	Vimentin	Are class II intermediate filaments found in various non-epithelial cells, especially mesenchymal cells. Along w/ actins and tubulins, it represents a third class of well characterized cytoskeletal elements
6weeks	TPM1	Tropomyosin, Alpha	Binds to actin filaments in both muscle and non-muslce cells. Plays a role, in association with the troponin complex, in the calcium dependent regulation of vertebrate striated muscle contraction. In SMC contraction is regulated by interaction with caldesmon. In non-muscle cells is implicated in stabilizing cytoskeleton actin filaments.
6weeks	SAT1	Spermadine/Spermine N1-Acetyltransferase variant 1-like	Is a member of a family of sIfate/anion transporter genes. Functions as a sodium-dependent amino acid transporter. Plays an essential role in the uptake of nutrients, production of energy, chemical metabolism, detoxification, and neurotrnsmitter cycling.
6months	ENO1	Enolase, Alpha	Multifunctional enzyme, that in addtion to its role in glycolysis, participates in regulation of growth, hypoxic conditions, and immunity. Has the ability to serve as a receptor and activator of plasminogen on the cell surfaces of such cell-types as leukocytes and neurons. Stimulates immunoglobulin production.
6months	ACSL1	Acyl CoA Synthetase Long Chain Family Member 1	Is an isozyme of the long-chain fatty-acid-coenzyme A ligase family. Converts free long-chain fatty acids into fatty acyl-CoA esters, and thereby plays a key role-in lipid biosynthesis and fatty acid degredation.
Downregulated			
6months	EIF1	Eukaryotic Translation Initiation Factor 1	Is necessary for scanning and is involved in initiation site selection. Promotes the assembly of 48S ribosomal complexes at the authentic initiation codon of a conventional capped mRNA
6months	RPS3A	Ribosomal Protein S3A	The S3A protein is a member of the small 40S subunit located in the cytoplasm responsible for protein synthesis

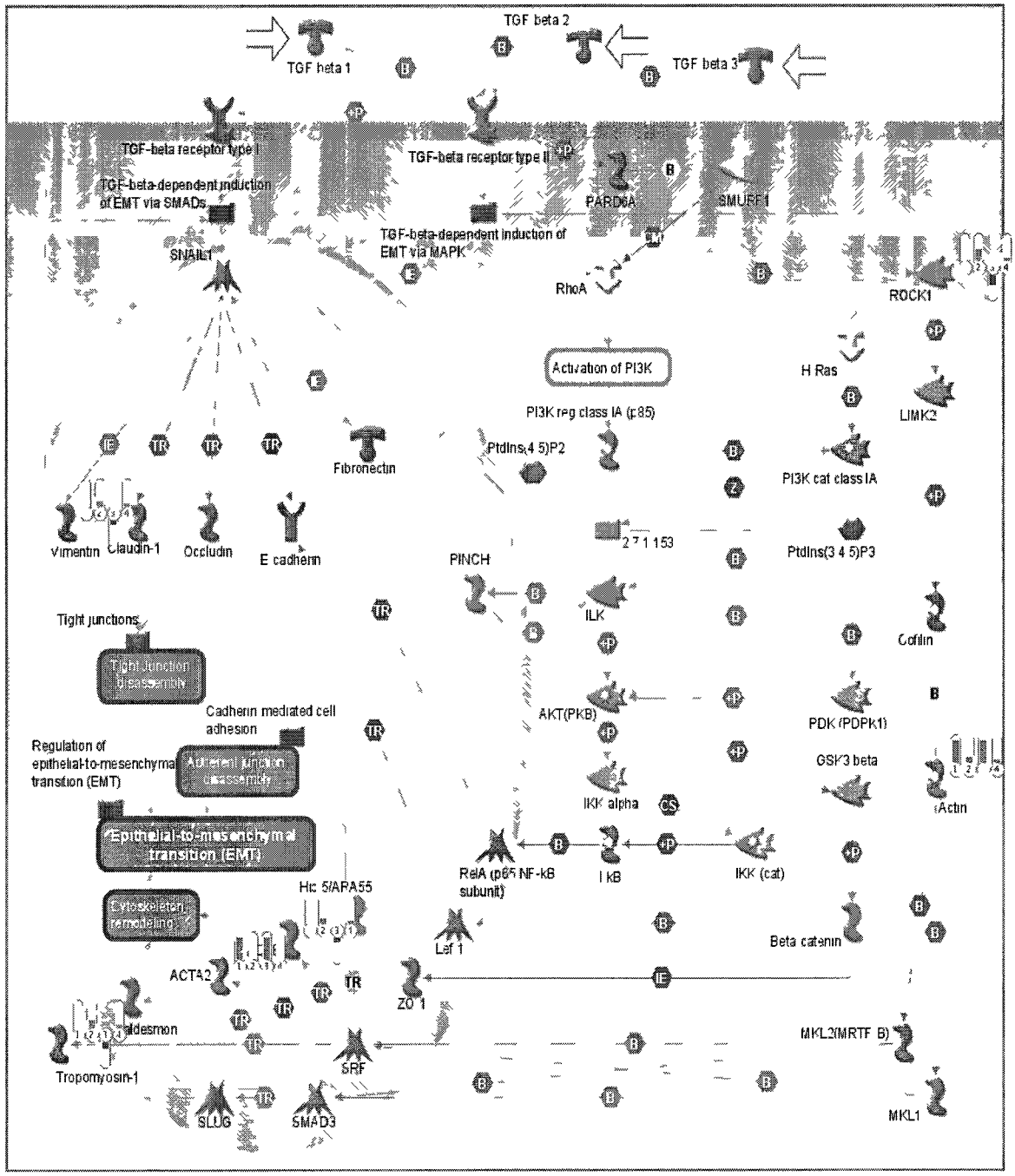
APPENDIX B: METABOLIC PATHWAY - VIM & TPM1

Figure B.1: C-1 (1day-red versus 6weeks-blue)
VIM & TPM1 Upregulated at 6weeks
Development-Regulation of Epithelial-to-Mesenchymal Transition (EMT)



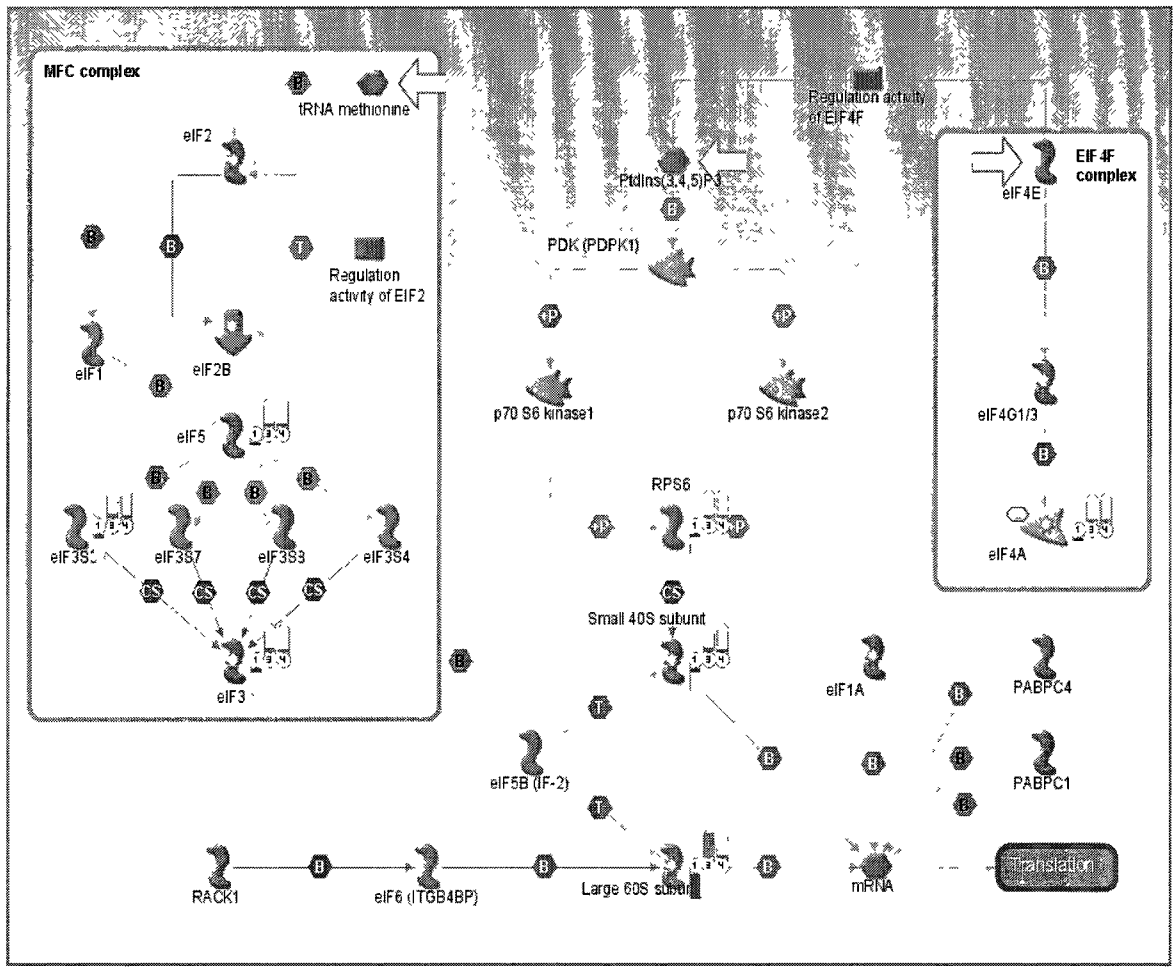
APPENDIX C: METABOLIC PATHWAY - TPM1

Figure C.2: C-1 (1day-red versus 6weeks-blue)
TPM1 upregulated at 6weeks
Development-TGF-Beta Dependant Induction of EMT via RHO, PI3K, & ILK



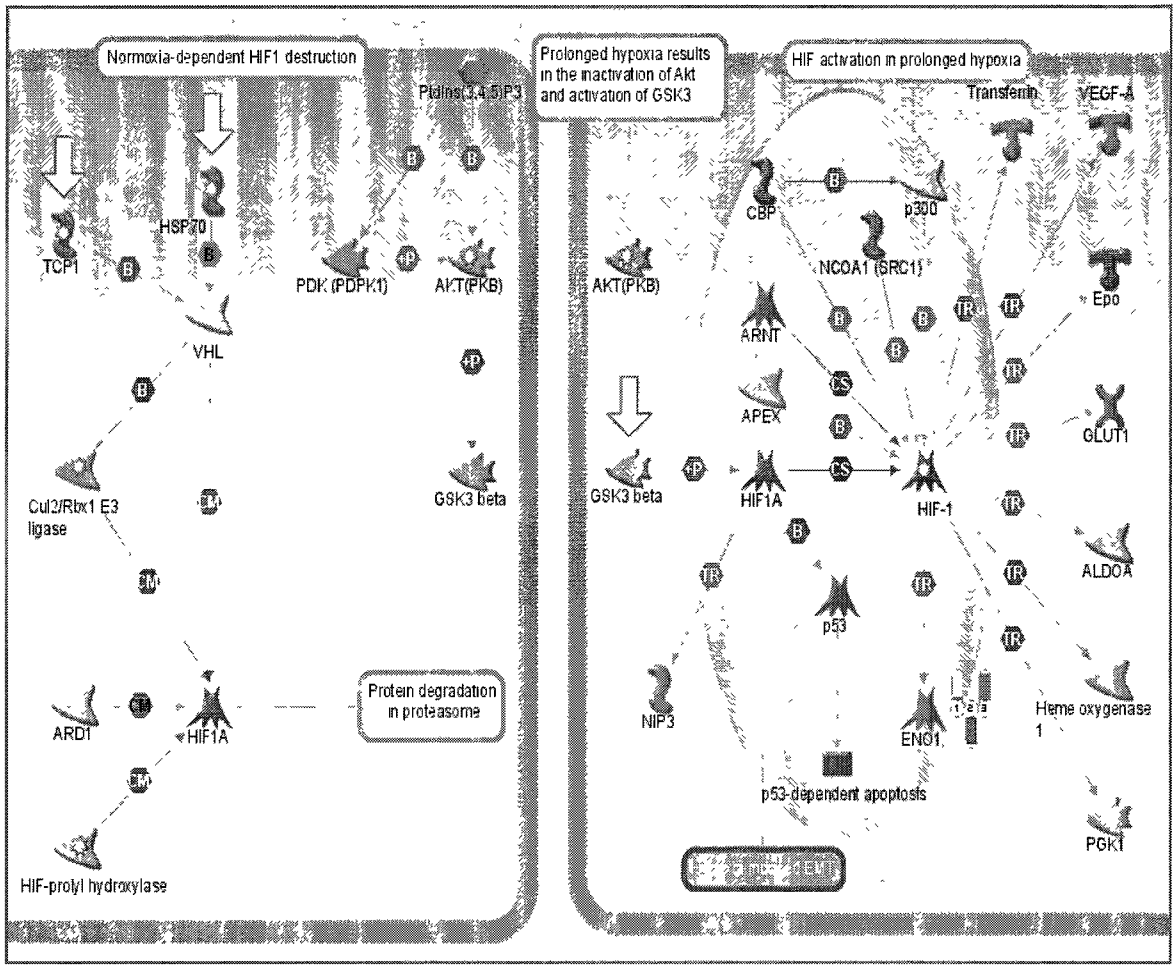
APPENDIX D: METABOLIC PATHWAY - RPL3 & RPS28

Figure D.3: C-1: (1day-red versus 6weeks-blue)
RPL3 & RPS28 Upregulated at 6weeks
Translation-Regulation of Translation Initiation



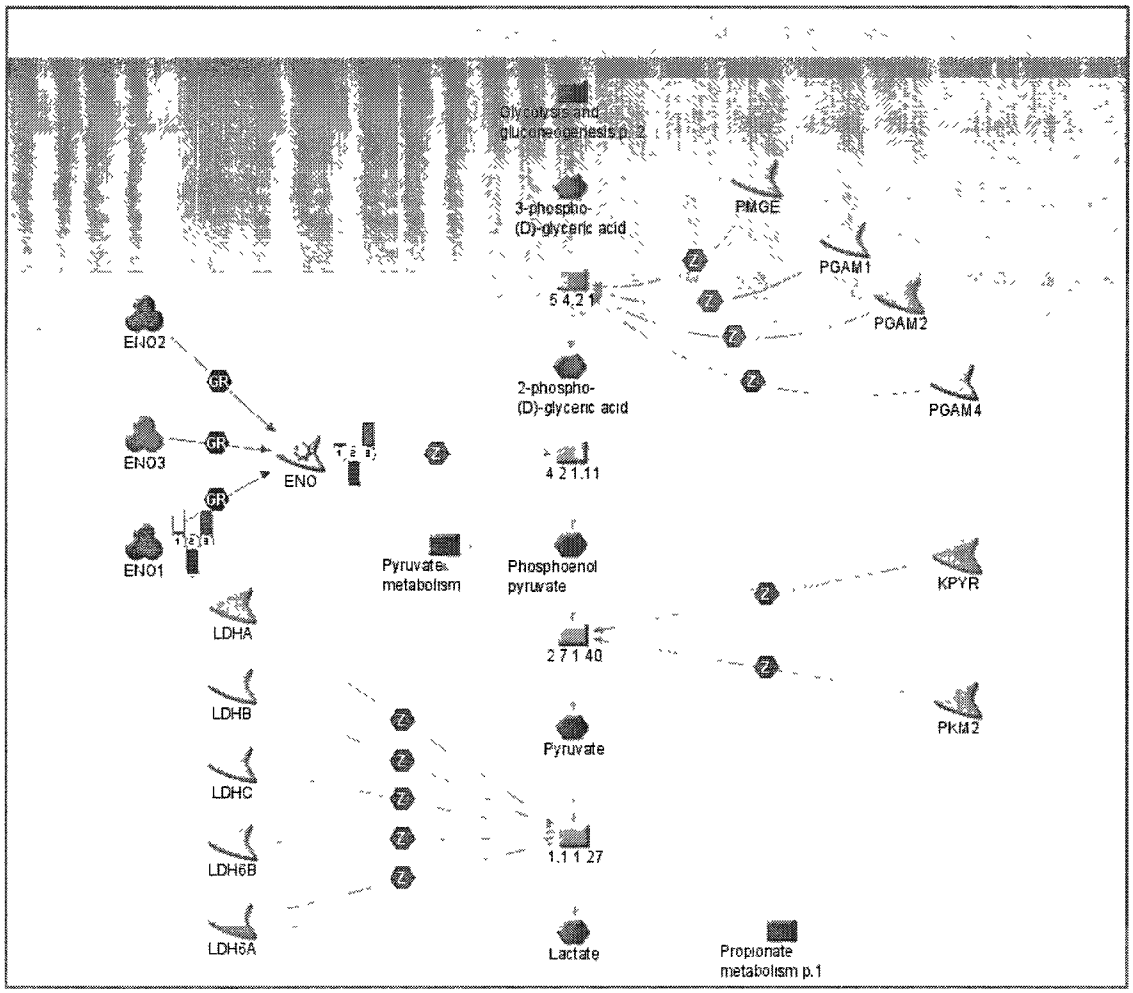
APPENDIX E: METABOLIC PATHWAY - ENO1

Figure E.4: C-2: (1day-red versus 6months-blue)
ENO1 Upregulated at 6months
Transcription Role of Akt in Hypoxia Induced HIF1 Activation



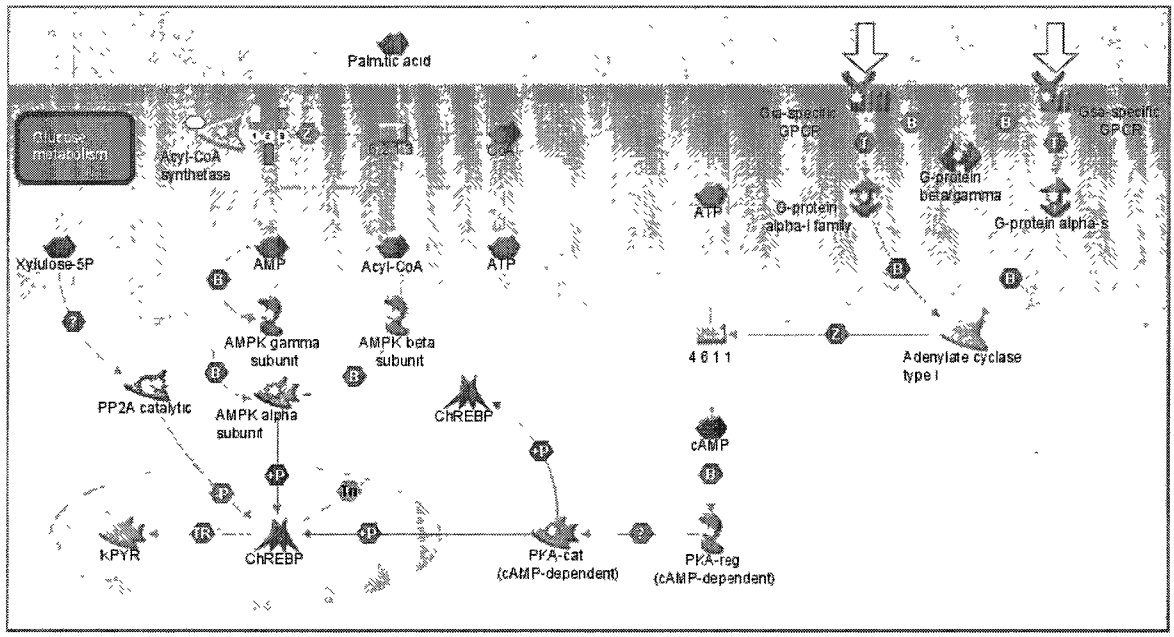
APPENDIX F: METABOLIC PATHWAY - ENO1

Figure F.5: C-2: (1day-red versus 6months-blue)
ENO1 Upregulated at 6months
Glycolysis



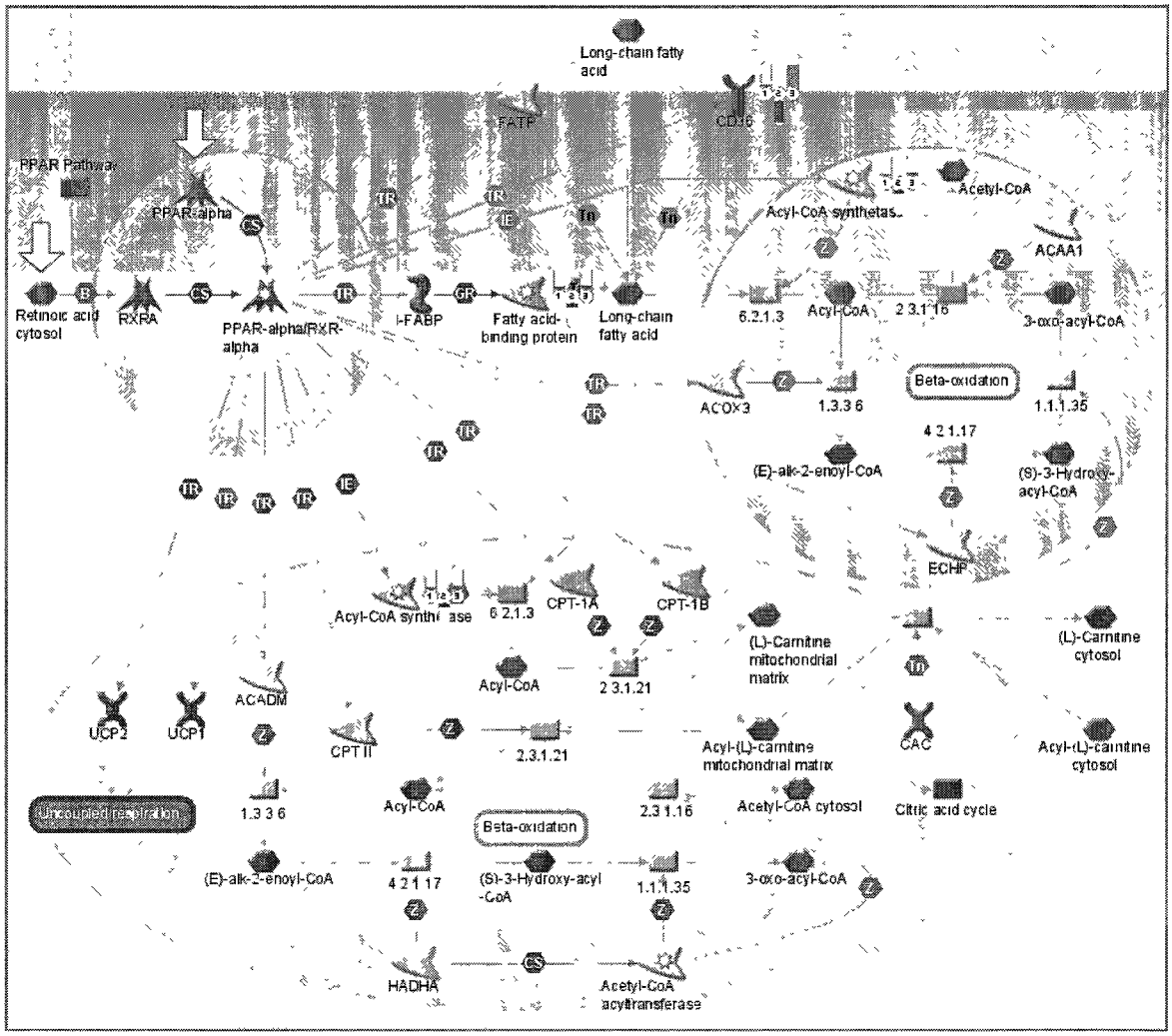
APPENDIX G: METABOLIC PATHWAY – ACSL1

Figure G.6: C-2: (1day-red versus 6months-blue)
ACSL1 Upregulated at 6months
Glucose Metabolism



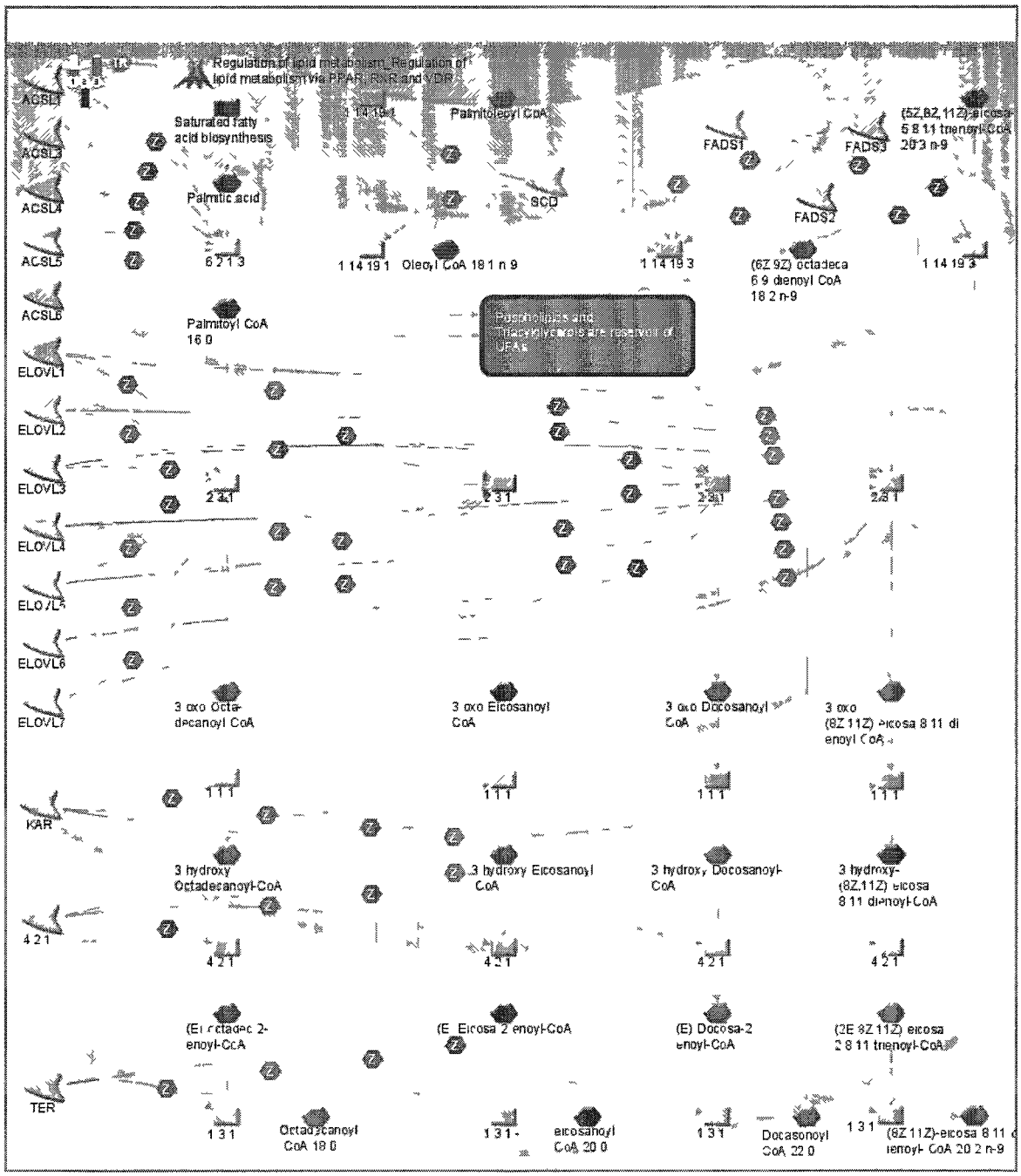
APPENDIX H: METABOLIC PATHWAY – ACSL1

Figure H.7: C-2: (1day-red versus 6months-blue)
ACSL1 Upregulated at 6months
Beta Oxidation



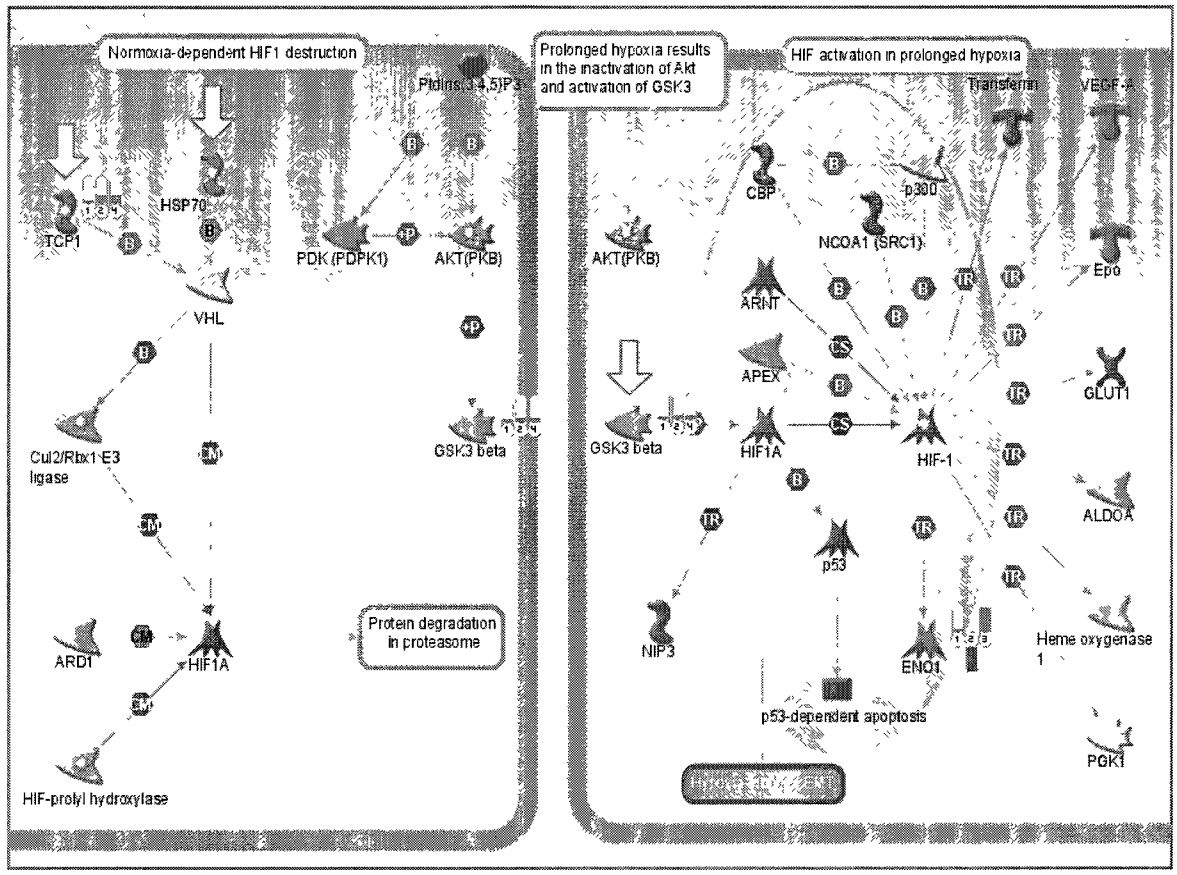
APPENDIX I: METABOLIC PATHWAY – ACSL1

Figure I.8: C-2: (1day-red versus 6months-blue)
ACSL1 Upregulated at 6months
Regulation of Lipid Metabolism



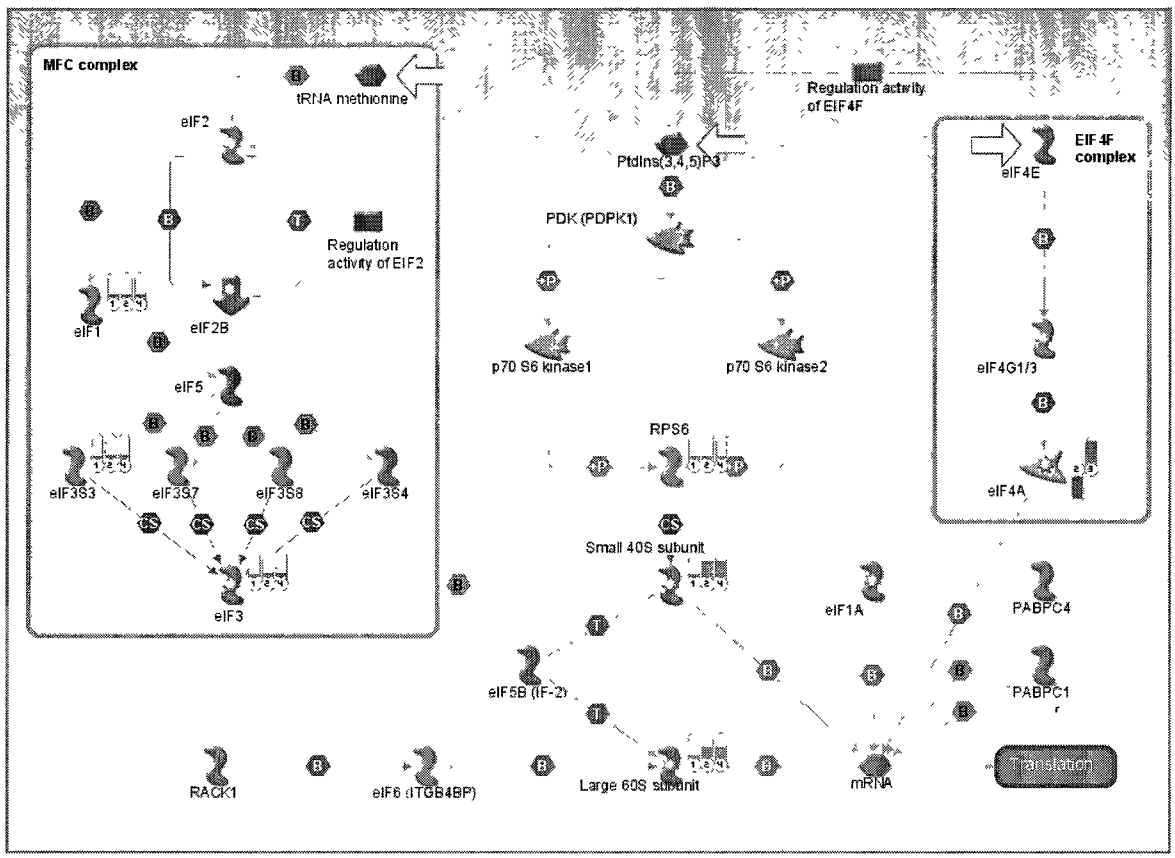
APPENDIX J: METABOLIC PATHWAY - ENO1

Figure J.9: C-3 (6weeks-red versus 6months-blue)
ENO1 Upregulated at 6months
Transcription Role of Akt in Hypoxia Induced HIF1 Activation



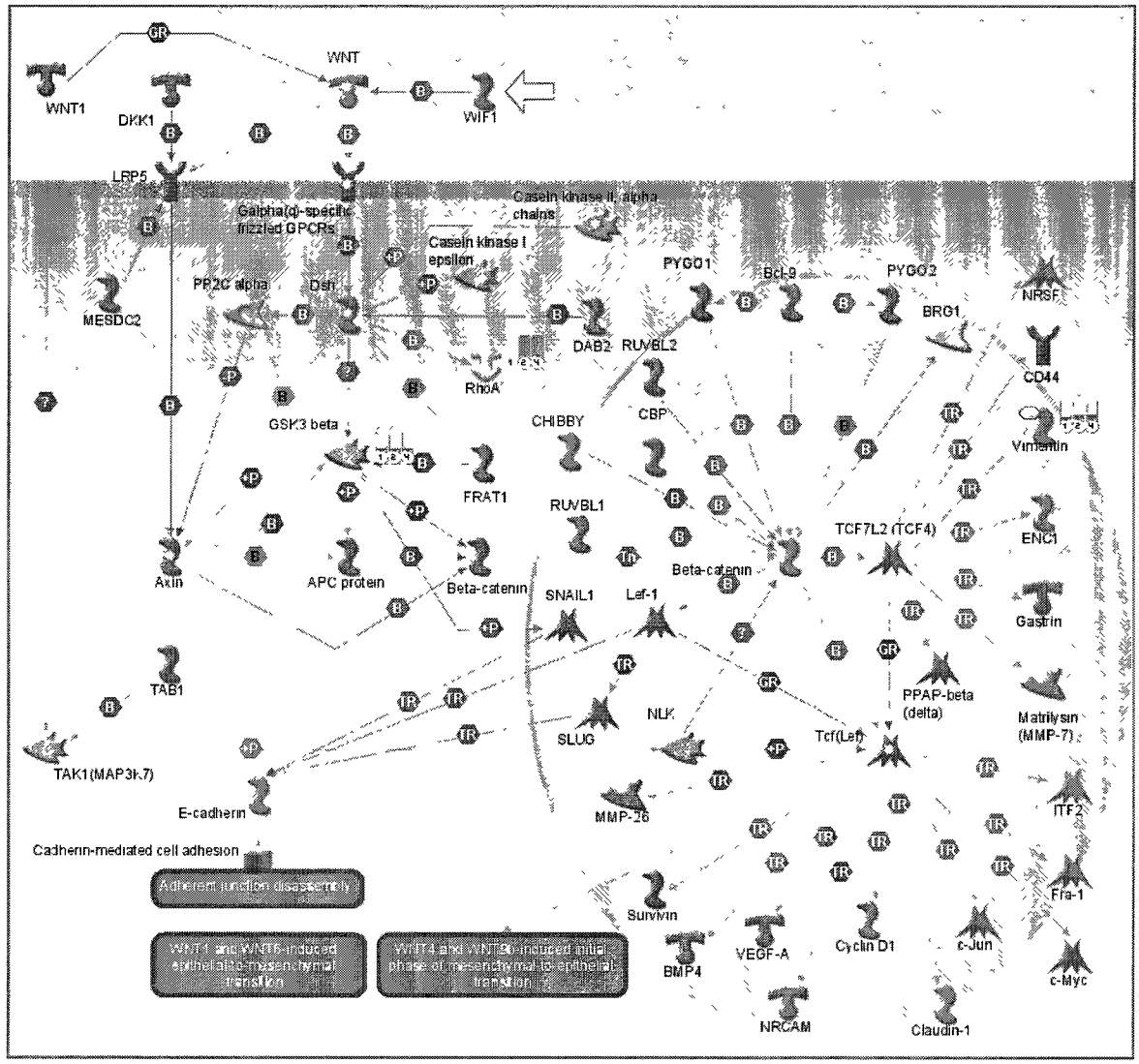
APPENDIX K: METABOLIC PATHWAY - RPL3, RPS28,

Figure K.10: C-3 (6weeks-red versus 6months-blue)
RPL3, RPS28, Upregulated at 6weeks
Translation-Regulation of Translation Initiation



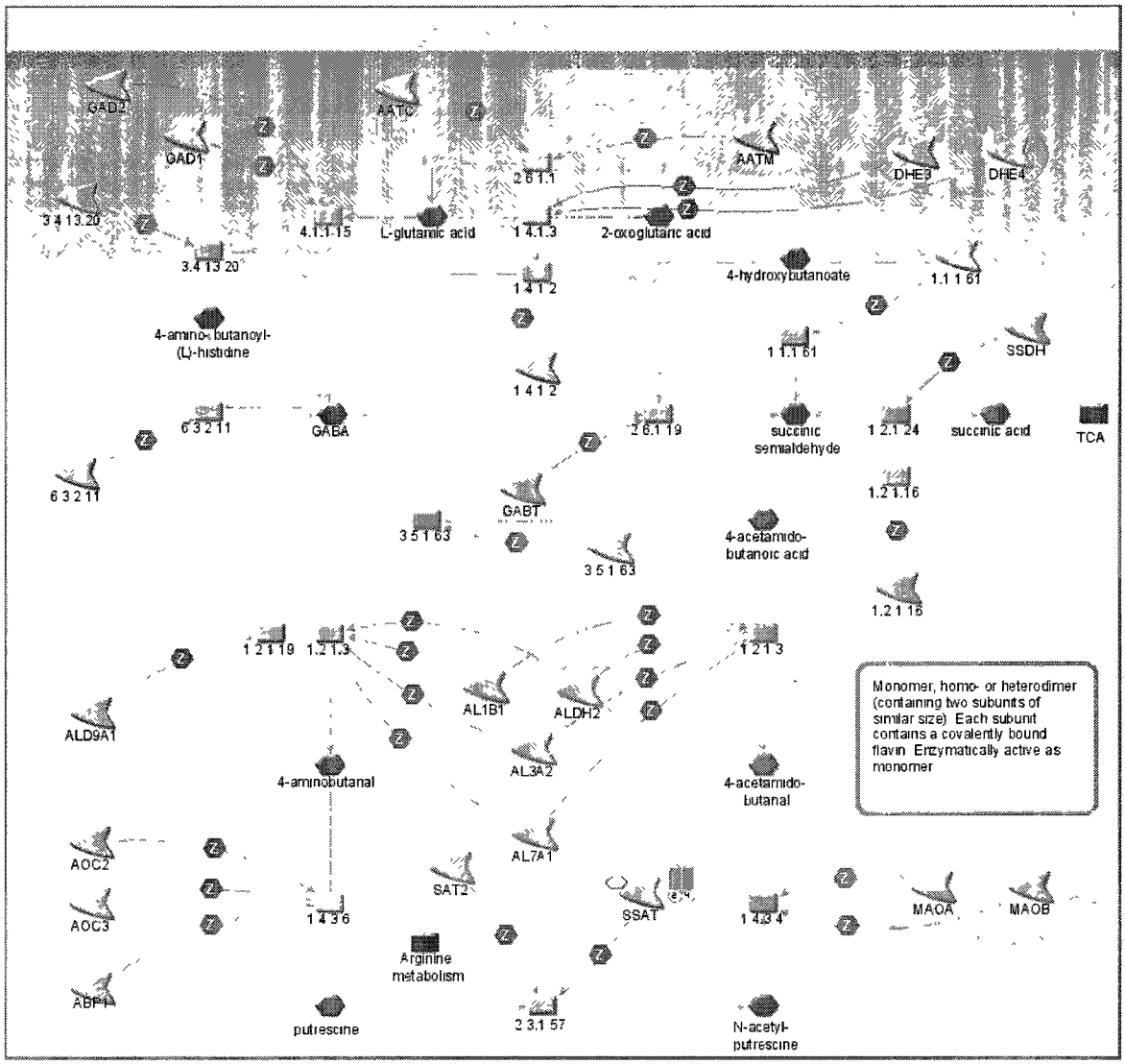
APPENDIX L: METABOLIC PATHWAY - VIM

Figure L.11: C-3 (6weeks-red versus 6months-blue)
VIM Upregulated at 6weeks
Development-Wingless-Type MMTV Integration Site Family (WNT) Signaling Pathway Part 2



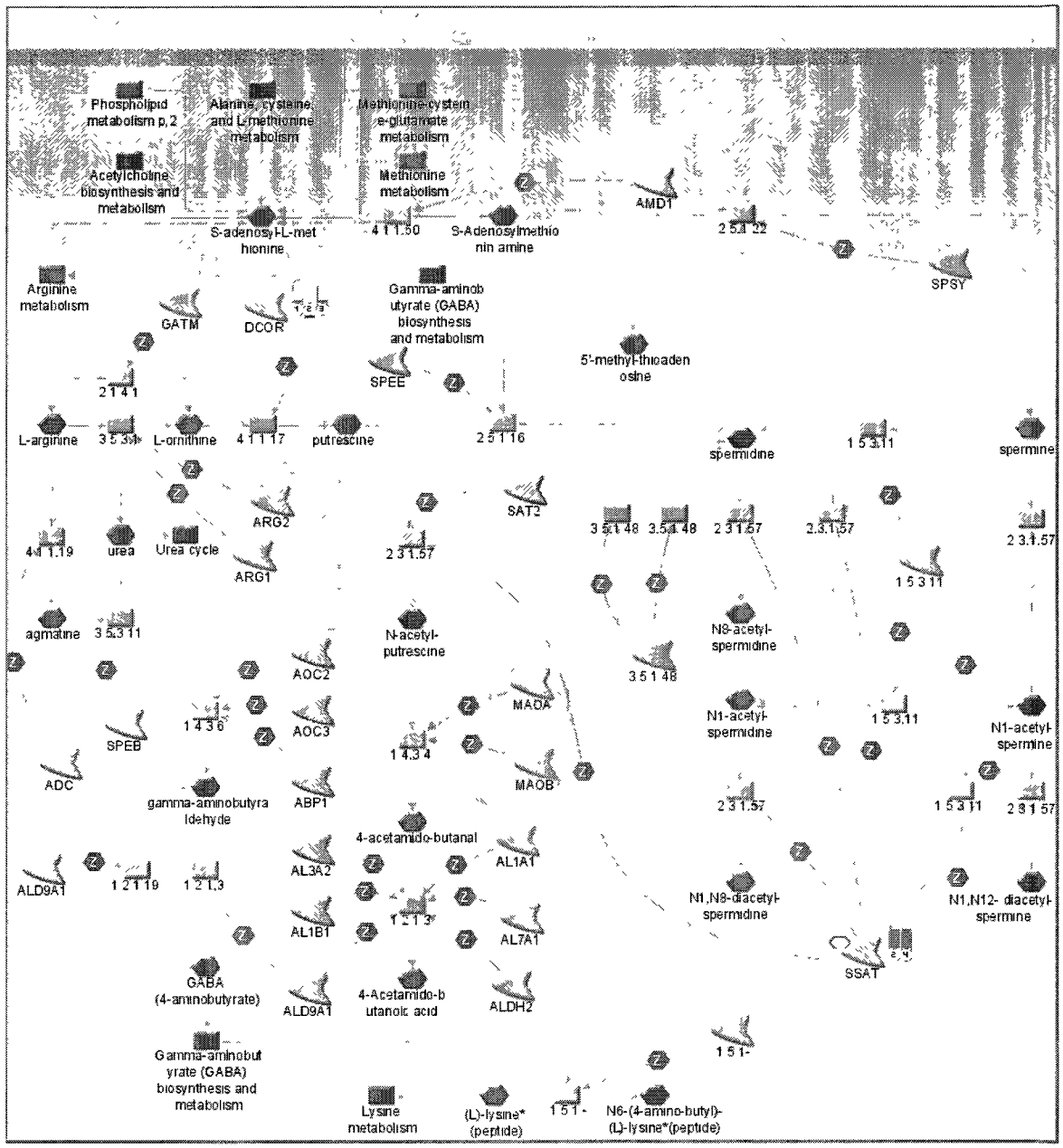
APPENDIX M: METABOLIC PATHWAY - SAT1

Figure M.12: C-3: (6weeks-red versus 6months-blue)
SAT1 (SSAT) Upregulated at 6weeks
Gamma-aminobutyrate (GABA) Biosynthesis and Metabolism



APPENDIX N: METABOLIC PATHWAY - SAT1

Figure N.13: C-3: (6weeks-red versus 6months-blue)
SAT1 (SSAT) Upregulated at 6weeks
Polyamine Metabolism



APPENDIX O: Table 2: C-1 Unidentified Transcripts 1day versus 6weeks

Comparison	Query		
Age		Size (#bp)	Sequences
C_1 1Day A	A01	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGGAAGTTGGACGCTATGA GAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAGCACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTG TTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATTGGTAGATATATCAGTTTATA TATGACCTCCGAGGATC
C_1 1Day A	A06a	355	GATCAACCAAAGCCTTAAGGAGAACATAGGGAACGAAAATATGAATTATACTGCATCACTTGTAATTCAACCAAATACGTGGGAAATTCAGTTGGCAAGGAA AGGGAGTAGAATAATGTGTGAAATGTTCGACTACGTATCCCCTTTCCAACCATTAAAGCTAATGTCAGTAAATTTTGGTCAACCCAAGAAGAATATTCATTCA TCCAGCCTGATGATGGCTTCTTTTAGGCTGCTTTAAATTTTTGTTTTCAACAGACGTCTAATTTGCTGTGTGCCAAGTCACATCTTCCAGACTGTGTGCTGC ATGTACGTGCACAATGGAGGCTGGAAGATGAAAGGAAGGCACAGGATC
C_1 1Day A	A06b	195	GATCAAGTGCTTATATTGGAAACATAAATCTACTTCCAGTCCTGTTCCTAATATATTTGTGTAAATGAAGAATGCTGCATTTCAGTCTCTTGTGGCCATATTTT TGGTGGTGGCCTGAAAGGCTCCACTAAAGGAGACATCAGCAGTGAATGTCAGAGCATCAGAGAGTGAAATATCCCTGGAATGTGAAGTGCC
C_1 1Day A	A07	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGCGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATG TATGACCTCCAAAGATC
C_1 1Day A	A08	322	GATCCCTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATACTTCTTTCCATTAGGT CCCAGCTGTGGTATAGAACACATTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTGGGTGGGGGATGGCATATGAAGTCTAATTT TGCCGTCCATGAGGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCTACTCATCTCGAATTCAGAA GCAAACAAACTGATC
C_1 1Day A	B01b	247	GAGGTGAACCCACTGATTCCGTATTCAAATCACCCTATCGCTACCGGGGACATTAAGGGGGGGCCCGAACCAATTCGCCCCTAGAGATCGCAATACTCAC GCTCACTTGGCTCGTTTTCAAGGCCGGTTGGGAAACCCGGCGTGCCCACTAACCCCCCCCGCCATCCCCTTTCCCAGCGGGCGTAAATTCAATTTGCCCC ATCCCATCCCTCCCCAAAGCGGCTCACCCTAAAAGCAAAGGGCACGG
C_1 1Day A	B02	183	GATCCTCTATGTTTCGTTTTGGCACACACTTGTTGAGAGTGGAAAGGTGGAGTGAGGCCACTGTTACATTAGACAAGTGAAAAGTAAGAAGAGGATAATAC TTAGGTTCGCACTTTAATCTCAGAATTAAACCAAACCTTGCATTCAAAGTAGATTTTTCTTTTTAAGTGGTGGTAGTTGATC
C_1 1Day A	B03	430	ATTCAAAGGGCGAATTAACCCTCACTAAAGGGACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAAAAAGTGGAGCAATGTTACTCGCAGCC CTACCTCTTAAGCTAGGCGGCTATGGCATCATGCGAGTTACCCTCCTAATAAACCCCACCGCCAACCACCTATACTACCCATTCATTACCTTAGCCCTATGG GGCGCCCTAATAACTAGCTCAATCTGCTTGCGCCAAACGGACCTAAAGTCCCTCATTGCCTACTCTTCCGTTAGCCATATAGGCCTAGTCATCGCTGCAAG CATAATTCAAACCCACTGAGCATTCTCAGGCGCAATACTCCTTATAATCTCACACGGACTAACCTCTTCCATACTATTCTGCCTGGCTAACACAAACTACGAA CGAACACATAGCCGAATCTTGATC
C_1 1Day A	B05	307	GATCAAGTGCTTATATTGGAAACATAATCTACTTCCAGTCCTGTTCCTAATATATTTGTGTAAATGAAGAATGCTGCATCTCAGTCTCTTGTGGCCGTATTTT TGGTGGTGGCCTGAAAGGCTCCTCTAAAGGAGACATCAGCAGTGAATGTCAGAGCATCAGAGAGTGAAATATCCCTGGAATGAGAAGTGCCAAGGGGTTT TAACTTCCCCAGATTTCTCTGCATGTGCCATGATGCATGGGAGATGTCAGGTTACCCTTTGTTCCAAACTTATCTTCTCAGATGATAAACTTTTCTGTCTGAT C
C_1 1Day A	B07	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATTATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGTCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTGT TCTATACCACAGCCGGGACCTAATGGAAAGAAATATATCCGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATA TATGACCTCCAAGGATC
C_1 1Day A	B10	259	GATCAGACTAACTCCCCAACACAGTTCAGTCTTTTTTTCTTCTCTTTCTCTGCTAGCTATAAGTAAGTGCTGAGTATAGGCCTACCCATCCAGATTTCTGATA GTTAAAATGAAAGAAACTGAATGCCACCCTCATTGACTAGCCTCAGAAATTTGTTTTCAACTTTTCACTGCCTTCCTTGGATGCACAGAGGAATTTAGAAGC CCAGACTCTAAGATAAAGAAAAATCACTTTGAAATGGGACACAAGCTGTGGATC
C_1 1Day A	B11	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGTGGTTTAACATAACTTTTGCAGGAGTGAGAAAGCTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCCCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATA TATGACCTCCAAGGATC
C_1 1Day A	C04b	243	GATCAGTTTGGTTTGCTTCTGATTCGAGATGAGTGGATTAGTGAAAATCATGGGGAGGTTAACATACTTTTGCAGGAGTGAGAAAGTTGGACCTATGAGAG CTTCATGACAGCAAATTAGACTTCTATGCCATCCCCTACCACCACTGCGAATTCCATGGACTCTTAAGAGGAGCTCTCAGAGCGATGTGTTTCTTACCCAGC TGGACCTAAGGAAAAAAAAACGTATCTCAAATTTCTATTT
C_1 1Day A	C05	147	GATCCTCCAGAAGAAAGATTGGAGACGCCCAGGGTCACTATGGCGGCAGAGGAACCTGGGTGTGCCGGCTCTGTTGTCTTTCATCGCAAACTTAAGGCCA TAGTCCGATGCACACTTCTGGGGGCAGTTCCCAGCCCTTTGCTGATC

APPENDIX O: C-1 Unidentified Transcripts 1day versus 6weeks (continued)

Comparison			
Age		Size (#bp)	Sequences
C_1 1Day A	C09	323	GATCCTTGGAGGTCATATATAAACAGGTATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGGTATATTTCTTTCCATTAGGT CCCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGTTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATT TTGCTGTCCACGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTTAGA AGCAAACAAACTGATC
C_1 1Day A	C11	555	GATCTATTCACCTGCTGTTCTGCTGCTCCACCTGCCCCGTCGCCTCACTACCTGGTCCAGCCGTTCAGCTGGGATGACTCATCTCTGCACGAGTACATTGT TAAATGATGTATTTTCAGGGGTTTGTTTCTGCAAAAGCAGAAGGAAGGATAAAGACCTTTCCTTAATACCACATCACTTACTTTGTTTGCCTCCCTCCTTTGC CTTTTTTGAAGCAGAAAAAGCACAGTGGCTGAATCCTTCAGGCTTCTGAATGCCAGGCTAATGTGCAATTCCCCGCTTCGTAATGAAAGGCATCATAAGTCA AGGGCATCTGCATCATCCTCCCCCAGATAACAGTTTACTGAAGCAGTGAAAGGGAAAGAGCTTTCAAAGTTTGTTGCAAAAATTTCATGATCTTAGCCACTGG AATACAGGCACAAACTTCCCTTTATGTGTGACCTTGCTGGGTGCCAGCTGGTATACTATCAGATGCTATTTAAAAAAGAAACAAACAAACAAAAACAAAGCC ATGGCTTTTTTTTCCCACCTGAGACCATTGTCTTTGAAGTTGATC
C_1 1Day A	D01	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTC CCAGCCGTGGTATAGAACACATCCGCTCTGAGTGGCTTCCTCTTTCAGAGTTCCATGGTAATTCGCAGTGTTGGTAGGGGATGGCATATGAGGTCTAATTT TGCTGTCCATGAAGCCCTCATAGTGTCCAACTTTCTCACTCCTGCGAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAA GCAAACAAACTGATC
C_1 1Day A	D04	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCCTTCCATTAGGTC CCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCCCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTACGGGATGGCATATGAAGTCTAATTT TGCCGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAA GCAATCAAACTGATC
C_1 1Day A	D10b	323	GATCAGTTTGTTTGCTTCTGAATTCGAGGTGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAAAGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATAT ATGACCTCCAAGGATC
C_1 1Day A	E01b	243	GATCAACAACTAGTTGCTTATCCAGGTTTCAGTCTCCACAGCAGAGTTTTGCTTTCTCCTTTATTTGTGCACTAGAAAGTGAAGTTGTTTTCTTCCCCTGCTT CCTCCAAGAAGAAGAATGGAAGGCAAATAAATAGTAGAGAATACAGCCATCAAGTTTGAGAGATGACCTTTACACTTCTATGGCATTTGCAACCTACAACCT TGCCTTCCCCACTTCCACTGGCTACAGCTAACATGATC
C_1 1Day A	E03	323	GATCAGTTTGTTTGCTTCTGAGTTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGATTTAACATAACTTTTGCAGGAGTGAGAAAATTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGT TCTGTACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATA TATGACCTCCAAGGATC
C_1 1Day A	E07a	227	GATCTTCACCATACCTTGATATTTCATTCTTGGGACTAGGCTGATAGATGATTTCAACTGCTCTGACAGGGCCATGCTAGATGTATCTGTGCTCTCTACCTG AAGCACACACTCGGGGGATGCTTCCAATTTTCTCTGTTTATTGTGGATTTGCAAGGATAAACAAGGACACTGACCGCTTCCCCGGGGGTGCAGGAATCTTT AAAGCCCTGTAAGAATACCGCAACAACTCAATAGGGGGCCCGGCATCCACCTTGCCCTATGGGGAGTCGTATTACTTGCTGAGTGCGGGCGTCGTTTTGA GGCCTGCAGAGGGGAAAAAGCCTGGGGCTACCCAAATGGTGGCCCTTTAAGTACACCCCCCATTCACCCAGTGGCTCAATCACGAAGACGGCCTCCTTA CTCAAACATTTATACAGTTGTGCCACCTGAATGGCCAGTTCCACACTCCCTGAAGGGGCAAGATATCTCATCCCCGGGGTGGGTTACATTCAATATCAACG CTACGATGCCAGCGCCCTCGAGGGGGGGTCCTGTACCCA
C_1 1Day A	E07b	147	GATCTTCACCATACCTTGATATTTCATTCTTGGGACTAGGCTGATAGATGATTTCAACTGCTCTGACAGGGCCATGCTAGATGTATCTGTGCTCTCTACCTG AAGCACACACTCGGGGGATGCTTCCAATTTTCTCTGTTTATTGTGGATTTGCAAGGATAAACAAGGACACTGACCGCTTCCCCGGGGGTGCAGGAATCTTT AAAGCCCTGTAAGAATACCGCAAC
C_1 1Day A	E08	327	AAGGGAGCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCACTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATT AGGTCCCAGCTGTGGTATAGAACACATTTGCTCTGAGGGGCTTCCTCTTTCAGAGTCCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCT AATTTTGCTGTCCATGAAGCTCTCATAGTGTCCAACATTCTCACTCCTGCAAAAGTTGTGTTAAACCTCCCATGATTTTCACTAGTCCAACTCATCTCGAATG CAGAAGCAAACAAACTGATC
C_1 1Day A	E10	323	GATCAGTTTGTTTGCTTCTGAATTCGAGAAGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCGTGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAACGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTGGATATATCTGTTTATA TATGACCTCCAAGGATC
C_1 1Day A	F03	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGTACTCTGAAAGTGGAAGCCTCTCAGAGCAATTGTGTT CTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTACCCAAGTCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATAT ATGACCTCCAAGGATC
C_1 1Day A	F06	433	GATCAGCAAAGGGCTGGGAACTGCCCCCAGAAGTGTGCACTGGACTATGGCCTTAAGTTCGCGATGGAAGACAACAGAGCCAGCACACCCAGGTTCCTC TGCCACCATAGTGACCCTGGGCATCTCCAATCTTTCTTCTGGAGAGTCAGGCTGGACAGCAGAGCAATACTGTCCCACCTTACTTCCCACGATTCTTCTGG ACCTGGCAGCTGTGAAGTGCTCCCATTTTCTGCATTTTACATGCACCCTGTAGACCAAGCAGACTGCAGTCATTGAGCAAATCTATGATAGAAATGGGAAT GGGACTGCAGGAGGCTTTCAGGTGAATAGTTTCTAACTAGTTGTGAAATTCTTCAGCGGGAAGGGAACGTGCAAGTGCTGCAGAGGCAATATAGCCAGCT TTACATCATTGCCCAAGCCCATAACCATGATC
C_1 1Day A	F10	23	GATCCTTGGAGGTCGTATATGAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATGTATTTCTTTCCATTAGGT CCCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATT TTGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAA AGCAAACAAACTGATC
C_1 1Day A	G07	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATGGAGATACAGATATATTTCTTTCCATTAGGTC CCAGCTGTGGTATAGAGCACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTT TGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACACCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAA GCAAACAAACTGATC
C_1 1Day A	G08	237	GATCCAGAGGAGAGCTCACAAAGAACAGATAATGTTAGATGAGTGTAGATAACAGCCTTCTTTCTTTGACAAAAATACCAGTTTTCATTTTTTAAATGTGCCT GAAAGCATTCTTTCACTATCATCAGCTAGGGACCTGGGCTATTTTCATTAAGTTTTCATTTTTGATATGAATATTGTATGAATATTTTTGTTTAAAATATGTTAA ATGAAGATAAGAAAATGTCCATTTTGATC

APPENDIX O: C-1 Unidentified Transcripts 1day versus 6weeks (continued)

Comparison			
Age		Size (#bp)	Sequences
C_1 1Day A	G11	323	GATCCTTGGAGGTCATATATAAACAGATATATCTGCCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTC CCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTTT GCTGTCCATGGAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATAATTTTCACTAACCCAACTCATCTCGAATTCAGAAG CAAACAAACTGATC
C_1 1Day A	H02b	320	GATCCCTAGGGGTTGAGGGTATCCGACATGGGTGACAGAATAGCACTTTTCAAATGAACTAAATATAGGAGAGTTTACTTATTTTACTCTGTTCCTGCTGTC CCTGGTGTGGACAAAAACGCATGGGCTGAGGGAGGACCGCAAGATCAGAGATCAATGGCAGGTCTCATGAGGAATTGGAAAGGGAATATAAGGTCTGAAT TTGCTGTTCTTGCTGCTCTCATTTCTCCAAATCATTTCACTCATTTTCTCGGTATTCAGATGCTCTGTGCTATTGGGTATTGAACACTATGGTCTATACAGAA GCTACTGCAATAACC
C_1 1Day A	H07	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTC CCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCCTATGAAGTCTAATCT TGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCTCTCCTGCAAAAGTTATGTTAGACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAA GCAAACAAACTGATC
C_1 1Day A	H08	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCAATCCATTTGTCTATAGACATAGATATGGATAGAGATATAGATACATTTCTTTCCATTAGGTC CCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTTT GCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCGAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAAG CAAACAGGCTGATC
C_1 1Day B	A01	323	GATCAGTTTGTTTGCTTCTGAACTCGAGATGAGTTGGATTAGAGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGTGAAAGTTGGACACTATGAG AGCTTCATGGACTGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGTT CTATACCACAGCTGGGACCTAGAGGAAAGAAATATATCCGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATAT ATGACCTCCAAGGATC
C_1 1Day B	A06b	261	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCCCAGAGCGAATGTGT TCAATACCACAGCCGGGACCTAATGGGAAAGAAATATATCTGTATCTCTATCCATATC
C_1 1Day B	A07	197	GATCCAAATGTGGCTGTAAACTGCGACAGGGTTAGGATGGAGGTACTCAAAAGGAAATGGAGGATGTCTTCACGGTTCCCACCAGACATCTGAATGTATGT CTATCTGTCTTCATCTGTGCTAAAGGGCACGTTGCCGTAAGACATAGCGTGGGGTTGGTGTTGTTTTTATAACTCCACTCAAAGACATTGTTGATC
C_1 1Day B	A08	302	GATCAGATTGCAGGAAGGTGACTGCTACTGTGCATTCTTGAACGCAATGGTATGTGATGCCATCTCTGAGCTGTGACAGTTTCTGTTGCCTATGTGTTGAG TATATGCACCAGCTGCAGCATGGCCTTCCACTGATTGCTCCTCCAGTGAGATGGGAACCCCAGTGGAGATTCCTTACTGTGTTGGCTATGGTTTTTCAGAA CGCATTATTTTATGTGTGTGTACGTAAAGGAAAATGCAATAGCTATGATAGCACCCTGAGCTGTGTTTTCCTAGTAGCTGTCTCTGTCAGCAATTTGATC
C_1 1Day B	A11	309	GATCAGACAGAAAAGTTTATCATCTGAGAAGATAAGTTTGGAACGAAGGGTAACCTGACATCTCCCATGCATCATGGCACATGCAGAAGAAATCTGGGGAA GTTAAAACCCCTTGGCACTTCTCATTCCAGGGATATTTCACTCTCTGATGCTCTGACATTCACTGCTGATGTCTCCTTTAGAGGAGCCTTTCAGGCCACCAC CAAAAATATGGCCACAAGAGACTGAAATGCAGCATTCTTCATTTACACAAATATATTAGGAACAGGACTGGAAGTAGATTTATGTTTCCAATATAAGCACTTG ATC
C_1 1Day B	B02	323	GATCCTTGGAGGTCATGTATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATACGGATAGAGATACAGATATATTTCTTTCCATTAGGT CCGAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATT TTGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGA AGCAAACAAACTGATC
C_1 1Day B	B03	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTACGAG AGCTTCATGGTCAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGTT CTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTACCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGGTATATCTGTTTATAT ATGACCTCCAGGGATC
C_1 1Day B	B04	246	GATCTCAGCCTGAGGGCAAAGGTAAGTATGAGACAGGGAAGAATATATCCATGGTGTATAGCACTAAGGGGACACTGTTTTTGAGTGTGCTCTGGCAAGG CTAAGGGGAAATAGTTCAGCATTTACATATTTTTGCATCATTGTGTAAGAATAGCCCAAGATGTCGTACTGATTCTCTACCCATGTTGTGTACAGGCAGAAAT CCGGGTTTGCCAGCCTGACAGAGCATAGATTTAGCCCTGGATC
C_1 1Day B	B07b	490	GATCATTAGAATTAAATATCTGAGTAATGGGGCTTCCCACATTCCGTTTTTTAAAAACTATATCTGATTTAACACCTCTAAAAATTCTTCTTGCATCTCAATAC CTTTGTTTAATCTGATATTTAGAGAATTTCGTATGATAGGTAATCAACAGCAAATATAACTGTGGTTGAGTTCTGTACTTAGAAATCATGTAAAGCTAAGAAAT GGTGGAGTATACAATGGGCCTCATTTCTCAAAGAGAACAAACAGACTAATTCATGTGTCTTTAAAATCAAGGCCAAAATTTTTAAAGGAAACAAATTAAGCCT GCATTTGTTTGTTTTTAAAAAGTCTTAAAATCCTTTGGGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCAACCACAAGAGGGCATCCATGCTTGCAAC CTGATCACCCATAAAGCCCATAATGTTTTTCTTCTAGTTCGAGTAGGTGAAGTTTGATGATATACATTCTGTGGGG
C_1 1Day B	B10	322	GATCCTTGGAGGTCATGTATGAACAGACATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGT CCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTT TGCTGTCCATGAGGCTCTCATAGTGTCCGACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAA GCAAACAAACTGATC
C_1 1Day B	D03b	590	GATCAAAAACCCTAAACTCCATCCTAAAGCCACAGCGCATCTTTTCTCAACAAAAAATGACGAAAGAAATTTCTAAAGCCTATCAGAGTTTTTTCTTGCATAT GTTATTTAATAGCAGATGTTAACTCAGTTATATCAGTTGACAAAGTGTTGGGTTTTTTTTTTCTGTAAACCGTGCCAAAACACCATTATTATATTATTTTACCA AGAAATAAAGGCAGTTGTGTTCAACTGTAGGGCAATGGCCTTTTATTTTTACTTGTAGTCAGAGTTAGAAAGCTGTTACTGTTACTCACAAGGAACAAAACA GTTGTTATTATTACCCAAGAAAAAAACTGATGTTGATTTTTAGCGCTGTTATGCTGGATGAGTTTTTGATTTTGGATC
C_1 1Day B	D06	237	GATCATAAACAAGTTACCCCCTGCAATGTGCTTTCTGCCTCCATTTTGCAGAATTGGCAGTTCTAACTCAATTTGTTGAACAACAGCAGGCAGCTAATACTA TTATTATTTTACAATCAGCTGGAAAAATAAATGACTTCTTTGATACTGCATTGTATGATACTGATGGTGTGGTTGAAATTAGTTCAGATAGAAACCCTCTAAAG CTTAGGGGCGGAGGCAGGGGTGGTTAAGATC

APPENDIX O: C-1 Unidentified Transcripts 1day versus 6weeks (continued)

Comparison			
Age		Size (#bp)	Sequences
C_1 1Day B	D10b	610	GATCAACCAAAGCCTTAAGGAGAACATAAGGAACGAAAATATGAATTATACTGCATCACTTGCAATTCAACCAAGTACGTGGGAAATTCAGCTGGCAAGGAA AGGAAGTAGAATAATGTGTGAAATGTTCGGCTACGTATCCCCTTTCCAACCATTAAAGTTAATGTCAGTAAATTTTGGTCAACCCAAGAAAAATATTCATTCA TCCAGCCAGATGATGACTTCTTTTAGACTGCTTTAAATTTTTGTTTTCAACAGACGTCTAATTTGCTGTGTGCCAAGTCACATCTTCCAGACTGTGTGCTGCA TGTACGTGCACAATGGTGGCTGGAAGATGAAAGGAAGGCACAGGATCAGACAGAAAAGTTTATCATCCGAGAAGATAAGTTTGGAACGAAGGGTAACCTG ACATCTCCCATGCATCATGGCACATGCAGAAGAAATCTGGGGAAGTTAAAACCCCTTGGCACTTCTCATTCCAGGGATATTTCACTCTCTGATGCTCAGACA TTCACTGCTGATGTCTCCTTTAGGGGAGCCTTTCGGGCCACCACCAAAAATGTGGCCACAAGAGACTGAAATGCAGCATTCTTCATTTACACAAATATAT
C_1 1Day B	E06b	323	GATCAGTTTGTTTGCTCCTGAATCCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGGAAGTTAGACACTATGA GAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTG TTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGAATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTAT ATATGACCTCCAAGGATC
C_1 1Day B	E07	309	GATCAAGTGCTTATATTGGAAACATAAATCTACTTCCAGTCCTGTTCCTAATATATTTGTGTAAATGAAGAATGCTGCATTTCAGTCTCTTGTGGCCATATTTT TGGTGGTGGCCTGAAAGGGTCCTCTAAAGGAGACGTCAGCAGTGAATGGCAGAGCATCAGAGAGTGAAATATCCCTGGAATGAGAAGCGCCAAGGGGTT TTAACTTCCCCAGATTTCTTCTGCATGTGCCATGATGCATGGGAGGTGTCAGGTTACCCTTTGTTCCAAACTTATCTACTCAGATGATAAACTTTTCTGTCTG ATC
C_1 1Day B	E08	323	GATCCTTGGAAGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTC CCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTT TGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAA GCAAACAAACTGATC
C_1 1Day B	E10	322	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGCTGGATTAGTGAAAATCATGGGAGGGCAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTCCGTATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCCATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATA TATGACCTCCAAGGATC
C_1 1Day B	E11	322	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGTGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAGA GCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCCACCAACACTGGGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTGTT CTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATAACTGTTTATAT ATGACCTCCAAGGATC
C_1 1Day B	F02	237	GATCCAGAGGAGGGCTCACAAGGAACAGATAATGTTAGATGAATGTAGATAACAGCCTTCTTTCTTTGACAAAAATACCAGTTTTCATTTTTTAAATGTGCCA GAAAGCATTCTTTCACTATCATCAGCTAGGAACCTAGGCTATTTTCATTAAGTCTTCATTTTTGATATGAATATTGTATGAATATTTTTGTTTAAAATATGTTAA ATGAAGATAAGAAAATGTCCATTCTGATC
C_1 1Day B	F03	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCTTCCCCTACCAGCACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGT TCTATACCACAGCTGAGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTTTAGACAAATGGATAGATAGGTAGATATATCTGTTTATAT ATGACCTCCAAGGATC
C_1 1Day B	F10	321	GATCCTTTTGGGCGCTTCTGAATTCGAGATGACCCGGATTGGTGAAAATCATGGGAGGTTTAACATGCCTTTTGCAGGAGTGAGAAAGTTGGGCACTATGA GAGCTTCACGGAAGTGGAATTAATACTCATATGCCATCCCCTATGTGACGGCGAAATACCATGAAAATCTGAAAGAGGAAGCCTCTCACATCAAATGTGTTA TATACCACGCTGGGGACCTAATGGAAAAAAATATATCTGTATCTCTATCCATAAGGGGGGGTATGGACCCATATTTAGATAAGTAGATATATCTGTTTATATA TGACCTGGAAGGATCCCCCCGGCTGCATGAATGGGAAAACAATGTTATTAATACCGTTAACC
C_1 1Day B	F11	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGGC CCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCGGAGGTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGGATATGAAGTCTAATTT CGCTGTCCATGAAGCTCTCCTAGTGTCCAACTTTCTCACTCCGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAA GCAAACAAACTGATC
C_1 1Day B	G01a	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCGTGGGAGGTTTAACATAACTTTTGCAGGAGTGAGGAAGTTGGGCACTATGA GAGCTTCGTGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTG TTCTATACCACAGCTGGGACCTAATGGAAAGAAATGTATCTGTATCCCTATCCATATCTATGACTATAGACAAATGGATAGATAGGCAGATATATCTGTTTAT ATATGACCTCCAAGGATC
C_1 1Day B	G06b	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTC CCAGCTGTAGTATAGAACATATTCGCTCTGAGAGGCTTCCTCCTTCAGAGTTTCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTTT GCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAAG CAAACAAATTGATC
C_1 1Day B	G08	243	GATCAACAACTAGTTGCTTATCCAGGTTTCAGTCTCTACAGCAGAGTTTTGCTTTCTCCTTTATTTGTGTACTAGGAAGTGAAGTTGTTTTCTTCCCCTGCTT CCTCCAAGAAGAAGAATGGAAGGGAAATAAATAGTAGAGAGTACAGCCATCAAGTTTGAGAGATGACCTTTACACTTCTACGGCATTTGCAACCTACAACCT TGCCTTCCCCACTTCCACTGGCCACAGCTAACATGATC
C_1 1Day B	G09	323	GATCAGTTTGTTTGGTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGGAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATAT ATGACCTCCAAGGATC
C_1 1Day B	G11	323	GATCAGTTTATTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAACACCATGGAACTCTGAAAGAGGAAGCCTCTCAGGGCAAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAAACAAATGGATAGATAGGTAGATATATCTGTTTATAT ATGACCTCCAAGGATC
C_1 1Day B	H01a	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATAAGTTGGATTAGTGAAAATCATGGGAAGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGGAAGAGGAAGCCTCTCAGAGCGAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCCGTATCTCTATTCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCCGTTTATA TATGGCCTCCAAGGATC

APPENDIX O: C-1 Unidentified Transcripts 1day versus 6weeks (continued)

Comparison			
Age		Size (#bp)	Sequences
C_1 1Day B	H06	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGACTAGTGAAAATCATGGGAGGTTTAACGTAACTTTTGCAGGAGTGAGTAAGTTGGACACTATGAG AGCTTCATGGACAGCGAAATTAGACTTCATATGCCATCCCCTACCAGCACTGCGAAATACCATGGAACCCTGAAAGAGGAAGCCTCTCAGAATGAATGTGT TCTATACCACAGCCGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATA TATGACCTCCAAGGATC
C_1 1Day B	H08	247	GATCAATAGTCAATATTGCCAGGGCTTAGCTGCCTTGCTTCTACCTAATTTTTACCCTTGCATGTCACAATAAAACCCAATGTGGAAGGCCCTCCAGGATAT GGAGGGGGAGAAAGCACGAGAAACCATACCTACTGTCAATGTTGTCTGCTTTTTAATAAACAATATGTTTTGAAAATTCAAGCACTCAGGTAGGTTTGTAAA TTGGATGGAGATGTCTAAGTCCCTGTAAAATTGAGCATTGATC
C_1 1Day B	H09a	290	GATCAATCAAACCCTTAAGGACAACAAGGGCTCTGAAGGGTTAATTACTATCTCTCTGTCATTACCCTGCGAAGAAGGTAGGAAGCTCAGGTGGTAGGAGA CGCATGCACAATGAAAAGGGAAAGGAATTCCACTTTTCTGGCATAAATAAATCCATTTGTGTGCATTGAAGAGAATTGCAGCAGAGGAAGAGGTCTTCACAT ACAACCCCAGACTGAACAGTGTAAGGTGTGCTCCCTGTAAGAGGAAGCTAGCAGCCCCTCCAGAGGGAAAGTGGCAGCTTTAGGATC
C_1 1Day B	H10a	170	GATCCTACTTGTAGCTACAACCTCTGACGAACAGAGGAGCAGTGTCTCTCATACAGCAAGGCAAGCTGATGTCTATAAGCCAAAAGGGTATGTGAAAAGGG TATTCAGCTCTTTTCACACACACACACACACACACACACTAAAAGGACTCAAGTCTGCTTTCTGAGATC
C_1 1Day B	H10b	167	GATCACCAAAAGAAAACCTACCTTCCTGGCTGAAGTCCATTAGGGAAGCCCCCAATAAAACCTTCCAAGTACCTGTCATCTCTGGTGTCTAGAGCTTTATAC AGGACCCAAGAGTCCAGGCTGCAGATGCGTGATATGGGCTGCTCATGGAAGAGTAGGCTAGGATC
C_1 1Day C	A01	322	GATCAGTTTGTTTGCTTCTGAATTGGAGATGAGCTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAATATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTATT CTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATGGGTAGATATATCTGGTTATAT ATGACCTCCGAGATC
C_1 1Day C	A05	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTACGAG AGCTTCATGGTCAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGTT TTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTGTAT GTGACCTCCAAGGATC
C_1 1Day C	A11b	241	GATCTTAACCTTATCTGCCTCCACCCCCAAGCTTTAGAGGGTTTCTATCTGAACTAATTTAAACCACACCATCGAAGTCATACAATGCATTATCAAAGAAGTC CTTACTTTTTCCAGCTGATTAAAAAAAAATAATATTATTATCTGCTTGCTGTTGGTCAACAAATTGAGTAATAACTGCCAATTCTGCAAAATGGAGGCAGAAAA CACTTTGCCCGGGGTAACTTGGAAATGATTGATC
C_1 1Day C	C03	44	GATCCACAAGGCCCCCAAGAGCACCAGGGTTGACTTTTGTGATC
C_1 1Day C	C06b	309	GATCAAGTGCTTATATTGGAAACATAAATCTACTTCCAGTCCTGTTCCTAGTATATTTGTGTAGATGAAGAATCCTGCATTTCAGTCTCTTGTGGCCATATTT TTGGTGGTGGCCTGAAAGGCTCCTCTAAAGGAGACATCAGCAGTGAATGTCAGAGCATCAGAGAGTGAAATATCCCCGGAATGAGAAGTGCCAAGGGGTT TTAACTTCCCCAGATTTCTTCTGCATGTGCCATGATGCATGGGAGATGTCAGGTTACCCTTTGTTCCAAACTTATCTTCTCAGATGATAAACTTTTCTGTCCG ATC
C_1 1Day C	C07	323	GATCAGTTTGTTTGCTTCCGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACCTTTGCAGGAGTGAGAAAGTTGGACACTATGA GAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCCACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTG TTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATA TATGACCTCCAAGGATC
C_1 1Day C	D05	287	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTAACTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTC CCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTTT GCTGTCCATGAGGCTCTCATAGCGTCCAACTTTCTCACTCCTGCAGAAGTTATGTTAAACCTCCCATGATTTTCACTGATC
C_1 1Day C	D09	238	GATCAAAATGGACATTTTCTTATCTTCATTTAACATATTTTAAACAAAAATATTCATACAATATTCATATCAAAAATGGAGACTTAATGAAAATAGCCTAGGTTC CTAGCTGATGATAGTGAAAGAATGCTTTCTGGCACATTTAAAAAAATGAAAACTGGTATTTTTGTCAAAGAAAGAAGGCTGTTATCTACATACATCTAACATT ATCTGTTCTTTGTGAGCTCTCCTCTGGATC
C_1 1Day C	E07a	369	GATCTTAAATGGTTTATTTGAGGAAACTGCAAGTTAATTGCCACCAGACATATCAGGCAAGTCTTTCTCCAAGGGGTTTTCCCTGTGATGAGACTGACATCA AATTTTACTGTGAGCTATTTATTCTACTCCTTTGAAAATTCAGTCCTATATGCAAACTGAAGAAAAAGAAAACTTCCCATTGAAAATGGGTGTTGCACTCTGA ATAATGTACTCAGAACATTTTCTTCAACAGACAGGGTTACCAGCTGTAGACAGGCACATTTCCAAATAACATGACATTAAAGGAACCCAGTCACATGAAACT GCAATTGTTAACAATGTTACAGGCAAGTTACTGAACTTGTATTGGTCAAATCATTTAAGATC
C_1 1Day C	F03a	31	GATCATATCTGTGTGGGCAGAAAGCCAGTTTTTCCTCGGAAAGTTTCCAGCCAACCCTTTGGGGCAACAGTTATCTTACTGGGATGGTGGTGTTACGGTGC CTGATC

APPENDIX O: C-1 Unidentified Transcripts 1day versus 6weeks (continued)

Comparison			
Age		Size (#bp)	Sequences
C_1 1Day C	F05	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAGTCGCGGGAGGTTTAACATAACCTTTGCAGGAGTGAGAAAGTTGGACACTATGA GAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCCACCAACACTGCGAAATACCATGGAACCTGAAAGAGGAAGCCTCTCGGAGCAAATGTG CTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTTTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATA TATGACCTCCAAGGATC
C_1 1Day C	F10	277	GATCCTTGGAGGTTATATATAAACAGATATATCAACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTC CCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTTT GCTGTCCATGAAGCTCTCATAGCGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTC
C_1 1Day C	G01	643	GATCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTCC CGGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTACAGAGTTCCATGGTATTTCGCAGTGTAGGTAGGGGATGGCATATGAAGTCTAATTCT GCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAAACTCATCTCGAATTCAGAAG CAAACAAACTGATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACTGATATATTTCTTT CCATTAGGTCCCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTACTTCGCAGTGTTGGTAGGGGATGGCATATGA AGTCTAATTTTGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCTCATGATTTTCACTAATCCAAACTCATCTC GAATTCAGAAAGCAAACAAACTGATC
C_1 1Day C	G01a	322	GATCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTCC CGGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTACAGAGTTCCATGGTATTTCGCAGTGTAGGTAGGGGATGGCATATGAAGTCTAATTCT GCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAAG CAAACAAACTGATC
C_1 1Day C	G01b	325	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACTGATATATTTCTTTCCATTAGGTC CCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTACTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTT TGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCTCATGATTTTCACTAATCCAAACTCATCTCGAATTCAGA AAGCAAACAAACTGATC
C_1 1Day C	G03	119	GATCTTCTGGGCTAGCCTTAAGAGTCCTCATTTTCTGTGAGGGAATGTTCATATTGACTCACCAACAAGTCAAGGTCTCTCAAACTTTGAGGAACTTTCCTT TGGGGGCACCCCTGATC
C_1 1Day C	G08a	323	GATCAGTTTGTTTGCTCCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACCTCATATGCCATCCCCTACCGACACTGCGAAATACCATGGAACCCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATAT ATGACCTCCAAGGATC
C_1 1Day C	G10	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGGTATATTTCTTTCCATTAGGTC CCAGCTGTGGTATAAAACACATTCGATCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGAATTGTTGGTAGGGGATGGCATATGAAGTCTAATTTT GCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTTACTCCTGCAAAAGTTATGTTAAACTTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCACAAG CAAACAAACTGATC
C_1 1Day C	G11a	321	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGGGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACGGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGGACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTGT TCTATACTACAGCTGGGACCTAATGGAAAGAAATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATATAT GACCTCCAAGGATC
C_1 1Day C	G11c	234	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGTGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTATCAACACTGCGAAATACCATGGTACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGTT CTATACCACAGCTGGGACCTAATGGAAAGA
C_1 1Day C	H02	247	GATCAATAGTCAATATTGCCAGGGCTTAGCTGCCTTGCTTCTACCTAATTTTTACCATTGCATGTCACAATAAAACCCAATGTGGAAGGCCCTCCAGGATAT GGAGGGGGAAAAAGCACAAGAAACCATACCCACCGTCAATGTTGTCTGCTTTTTAATAAACAATATGTTTTGAAAATTCAAGCACTCAGGTAGGTTTGTAAA TTGGATGGAGATGTCTAAGTCCCTGTAAAATTGAGCATTGATC
C_1 1Day C	H12	83	GATCTGCGATTTCTAAGGAAAGGCTTTTTGTTGAAATGCTTTTTGTTTGTTCATTTGTTTCACACCTCAGCTTTTATTTGATC
C_1 1Day D	A03	308	GATCAAGTGCTTATATTGGAAACATAAATCTACTTCCAGTCCTGTTCCTAACATATTTGTATAAATGAAGAATGCTGCATTCCAGTCTCTTGTGGCCGTATTT TTGGTTGTGGCCTGAAAGGCTCCTCTAAAGAGACATCAGCAGTGAATGTCAGAGAATCAGAGAGTGAAATATCCCTGGCCCGAGAAGTGCCAATGGGTTT TAACTTCCCCAGATTTCTTCTGCATGTGCCATGATGCGGGGGAGATGTCAGCTTACCCTTTGTTCCAAACTCATCTTCTCAGATGATAAACTTTTCTGTCTG ATC
C_1 1Day D	A12	193	GATCAGGTCCTCCAAGGCAGTCATGATAAATCAATTACTTGCAATCGTGTGTGCTAGCTATTCATATATAGGTGGGACAGTACCAGTTTTTAGCCAAATATT TACTGATTTTATTTCTGATACCAAAACATTTCTCAGCTGAATATAATTCAACGCAGCTTAAAGATGCAGCACATCTTTCTACCACCAGATC
C_1 1Day D	B01	360	GATCCACATGTAAGGAGGTAAGTATGTAAAGTAGTCAGTTAGAATGCAACCATCTATGTATAATTATTTTCTTGCTTCCTATTTTCAATTGTCATTGTCTAGAA ATGTGTCAGAGGCAGTAGTTACCTGGCAACATGCTATAGTGTTGATAAGCTCTGGTTTGCTGCTTGGTCTAATGGGTAAAAGCAGACAAATCTACTACCTG GATGAAGGGTGAGTAGCCAGGCCAGCAGCTACCCAAATTACCACAGCCAGTTCTCAGTCAGTGGGGCTCTGAGAGTTTATTCCAACAGATATATTGTACCA GATAGAGTATGTGATGGGTCTATGATGGTGTAATTATTTTATTCTGCCGTGATC
C_1 1Day D	B02	431	GATCAGCAAAGGGCTGGGAACTGCCCCCAGAAGTGTGCATTGGACTATGGCCTTAAGTTTGCGATGAAAGACAACAGAGCCAGCACACCCAGGTTCCTCT GCCACCATAGTGACCCTGGGCATCTCCAATCTTTCTTCTGGAGAGTCAGGCTGGACAGCAGAGCAATACTGTCCCACCTTACTTCCCACGATGCCCCTGG ACCTGGCAGCTGTGAAGTGCTCCCATTTTCTGCATTTTACATGCACCCTGTAGACCAAGCAGACCGCAGTCATTGAGGAAATCTATGATAGAAATGGGAAT GGATTGCAGAAGGCTTTCAGGTGAATAGTTTGTAACTAGTTGTGAAATTCTTCAGCGGAAGGGAATGTGCAAGTGCTGCAGAGGCAATATAGCCAGCTTTA CATCATTGCCCAAGCCCTTAACCATGATC

APPENDIX O: C-1 Unidentified Transcripts 1day versus 6weeks (continued)

Comparison			
Age		Size (#bp)	Sequences
C_1 1Day D	B05	237	GATCAAAATGGACATTTTCTTATCTTCATTTAACATATCTTAAACAAAAATATTCATACAATATTCATATCAAAAATGAAGACTTAATGAAAATAGCCTAGGTCC CTAGCTGATGTTAGTGAAAGAATGCTTTCTGGCACATTTAAAAAATGAAAACTGGTATTTTTGTCAAAGAAAGAAGGCTGTTATCTACATTCATCTAACATTAT CTGTTCTTTGTGAGCTCTCCTCTTGATC
C_1 1Day D	B09	322	GATCATTTTGTTTGCTTCTGATTCAAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCGGGAGCGAGAAAGTTGGACACTATGAGG GCCTCATGGACATCAAAATTACACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGTTC TATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATGGACAAATGGATAGATAGGTAGATATATCTGTTTATATA TGACCTCCAAGGATC
C_1 1Day D	B11	237	GATCCAGAGGAGAGCTCACAAAGAACAGATAATGTTAGATGAATGTAGATAACAGCCTACTTTCTTTGACAAAAATACCAGTTTTCATTTTTTAAATGTGCCA GAAAGCATTCTTTCACTATCATCAGCTAGGAACCTAGGCTATTTTCATTTAGTCTTCATTTTTGATATGAATATTGTATGAATATTTTTGTTTAAAATATGTTAA ATGAAGATAAGAAAATGTCCATTTTGATC
C_1 1Day D	D02c	96	GATCCGTCGACTCGAGGTGGCCAGATACCAATCGACCTATATGAGTCGAATACGGCGCTCACGGCGTTGTTTTCAAGCCGTGCTGGGAAACCGGGT
C_1 1Day D	D05b	108	GATCAATGCTAAAGGTGATAAGAAAGTTCACAGTCACCCTGCAGCGAACGGTCTTACATGTCCCTGGGAGAAGATTAGAAATTGAGAACTAAGAAGCCCGT GCGGATC
C_1 1Day D	D05c	253	GATCCCCCTGGCGAGAATCATAAGAGGTCAAGCTTTTCCATACGTCGACTCAATGGGGGGCCTGTACTCAATTCGACTTTATTGAGTCATTTACAGCACTC ATTGGACGTCGATTACACGTCGTGCTGGAACACCGTGTGCTAGCATCTAATTAGCTGAGCACATCGCCATTCAGGTGGGCCATATCCAATGCCCTATGTAG CCTATCAAAGGGGCAAAAGCGGGATATGTAACGTTCGGGCCTGGTAAACCC
C_1 1Day D	D09c	109	GATCGGCGTCCAGGTTCGTGGCAGGGCCACAGCGCAGAGAGAGAAGCCTAAGTATTGCACCGGCTGGATGCAAGGGTTTCCGTTGTACATTCATTCAAT CTGGCCGATC
C_1 1Day D	E12	323	GATCAGTTTGTTTGCTTCTGAATTCGTGATGAGTTGGATTAGTGAAAATCATGGGAGGGTTAACTTAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG GGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGGAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATA TACGACCTCCAGGGATC
C_1 1Day D	F03	323	GATCCTTGGAGGTCACATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTC CCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGGAGTCTAATTT TGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAGTTCAGAA GCAAACAAACTGATC
C_1 1Day D	G04b	309	GATCAAGTGCTTATATTGGAAACATAAACCTACTTCCAGTCCTGTTCCTAATATATTTGTGTAAATGAGGAATGCTGCACTTCAGTCTCTTGTGGCCACATTT TTGGTGGTGGCCTGAAAGGCTCCTCTAAAGGAGACATCAGCAGTGAATGTCAGAGCATCAGAGAGTGAAATATCCCTGGCCTGAGAAGTGCCAAGGGGTT TTAACTTCCCCAGATTTCTTCTGCATGTGCCATGATACATGGGAGATGTCAGCTTACCCTTTGTTCCAAACTTATCTTCTCAGATGATAAACTTTTCTGTCTG ATC
C_1 1Day D	G04c	116	GATCAGTTTGTTTGCTTCTGAAATTCGAGATGAGTTGGATAAGTGAAAAATCATGGGAGGTTTAAACATAACTTTTGCAGGAGTGAGAAAAGTTTGGACACT ATGAGAGCTTCAGG
C_1 1Day D	G08	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAGATGGATAGATAGGTAGATATATCTGTCTATA TATGACTTCCAAGGATC
C_1 1Day D	G11	323	GATCAGTTTGTTTGCTACTGAATTCGAGATGAGTTGGATTAGTGTAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAG AGCATCACGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCGAATGTGT TCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGGTAGATAGGTAGATATATCTGTTTATA TATGACCTCCAAGGATC
C_1 1Day D	G12	99	GATCTGCATAGCTACCTGACTCACTGGGGGAAATCTGATGTAGGGCTGTCTCACAGAAATACTGCCTGGTTTCAGAACAATTTAGTATCTCACTTGATC
C_1 1Day D	H09c	144	GATCTCACTCTTACGGGTCTAACGTATATCTCCTGGATATTGAACCCGTTGCTGGGAGGGGAGGGGAGGTCTTGTTCCATCATGAATCTGGCACGTCTGTA TCGCAGGAGGTTAGCCGAGGCCATACGAATAGAACAGGCGGTA
C_1 1Day D	H10	570	GATCCTCCCCGTTTGTGGGTGTAAACTCCCCTATTCTCCTGGGATATTTAAATTTGGAAGATGCAATGTGCTCGTGGGGATTCTGGTTTCACATTCATTCAT AAGATGGCAGCATGTGCTAACCGCCCGGAAGTTATGTCCCTCAGGCTGGATACGGGGGGTGAAAACTAGACCCCCTCGCCCGCTCGGACTCGCAAAGAA GCTTAGCGAAGCTGCCTACCTCCATGGCAGGACCGTGACCTAAATCAGCCTCCAGTTAGTCGGATAGTCCCCGCTGCTGAGCAGTCAACTACAGCGACTG AACTGGGCAAAGCGAGCGGTTTTAATTGATCATCTTGCACTCTTGCCCTGTCCCCAGGGGTGTAGAATTCAGATGACGCTCTGAATACCGTCACATCGTGG GGGGCCTGATGCAAATCGGCTCTACTCAGACTGTACACTAAGTCACGGGGCGTGGTGTTACCGCATGATGCGAAACTTGCGTGCCAATACGCCTGTAGCA TTTCCTTCTCATGCGATACGAATGCGGGCGGTCATCGAATCTACGTAAGTATGGTACGCCTTGACGCA

APPENDIX P: Table 3: C-2 Unidentified Transcripts 1day versus 6months

Comparison				
Age		Size (#bp)		Sequences
C_2 1Day A	B03	379		GATCAAAAAGGGAAAATGCTTTAAAATGTGGGGGACTTGTTAGGTTGAAACTTGTTTATAAGTGACTTTGACTTGTTGTCTGAAAATTCATATATACCA TACTGGGACTCTTTTTTTGTTATACTCAAATTGCTGCAGGTGTAAAAGTGTCTCTTTGAAGTGTATTGTGGCCTCGAGGAAGTTTTAATCAATGAGGTGA ACTTCATTAAAACAAAAGACTGCTACTTTTATGATGCCCAGTCCCAGTGTCTGGCTTACCTAGAAGCACTGACAAGTAGTGTTGTGCCAAAAGACTGA TTTATATCTTCCCTCTTGATGTCACACCCAGCTGTCAGCCAGGGAGAATGGTAGGGGAAAACACACTTATCCTGTCTTGGATC
C_2 1Day A	B07	388		GATCCCAATGAGCTGTCACAGGAGCACCAACGTATTACAGCATAACGCCAGATGCTGCTTCTTATGCCTATAGAGAGCGGTTGAATTCAGTATGGTA TGGCATCTTTTCACTGTGAACCTTCATGAGTTTTCCTAGATATTCTTCCAGTCGCATTACCACAAACCTTTTGGTTCCAAAGTGTAGATGAAAATTGCA ACTGGCTTCAATAAAAAGCACATGATAAAGGCAGGATATAACCACTTTGAAGGAATTGCAGCATGGGTTTGAAAAATGTCTTAATATCCTCCTCCTCTT ACTGCCTGCCTAAAACTACTTCTTTTTTTTAAGGTTTCTTACATAAAGATGGTCTCAATCAGAAAACTGGAAGAACTGGAAGGAAGTCTGATC
C_2 1Day A	C02b	317		GATCAAATGAACACAGTTCCTGCAGTGTAGGCAAGAACTATTCACCTCAGGTTATGATTAAATCCTCTAGACAATGGATTTATTAAACTGTAATCCCCA TACCTCTATTGGCAGTCTCTTCTCTTTGCTGCCTCAACATTCTCATCCGTACAGCATTTATATTTTCAAAACAAATAAAAACAAAAAAACCCTCCCTTTG TTTCCATATTTATTTCTTGGGTTTTTTTGGAGTTCACAAGTACTCAAAATTAAGGGCGTAAAATAGAGATTTCTCATTCTGACATGGCAGACCTTTCTAT TTTTCCCTTAAAGAAAGT
C_2 1Day A	C06b	365		CAAAACTTAGAATGTCCTGCCTCACCCACAGCTTAAGCATGTGGATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGAGTAGTGAAAATCATGGG AGGTTTAACATAACTTTCGCAGGAGTGAGAAAGTTGGACGCTATGAGAGCTTCATGGACAGCGAAATTAGACTTCATATGCCACCCCCTACCAACACT GCGAAATACCATGGAATTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGTTCTATACCACAGCTGGACCTAATGGAAAGAAATATATCTGTACCTCTA TCCATATCTATGCCTATAGACAAATGGGTAGATAGGTAGATATATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day A	C07b	292		GATCCTCGGAGGCCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTA GGTCCCAGCCGTGGTATAGAACACATTCGCCCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTAGTAGGGGATGGCATATGAAG TCTAATTTTGCTGTCCACGAAGCTCTCAAAGTGTCCAACTTTCTCTCTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACT
C_2 1Day A	C10	323		GATCAGTTTGTTCGCTTCTGAATTCGTGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAGCTTTCGCAGGAGTGAGAAAGTTGGACACTA CGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAGCTCTGAAAGAGGAAGCCTCTCAGGGC AAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACATATGGATAGATAGGTAGATA TATCTGTCCATATTTGACCTCCAAGGATC
C_2 1Day A	C12b	259		GATCAGCTTGTTTGCTTCTGAATTCGAGATGAGATGGATTAGTGAAAATCGTGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAATACTGCGAAATACCATGGAACTCTGAAAGAGAAAGCCTCTCAGAGCG AATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATAT
C_2 1Day A	D04	323		GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGATGCCTCTCAGAGG AAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCTATATCTATGTCTATAGACAAATGGATAGATAGGTAGATA TATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day A	D05	323		GATCAGTTTGTCTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAAACATGGGAGGTTTTACATAACTCTTACAGGAGCGAGAAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATTGGACTTCATATGCCATCCCCTACCAACACTGCGAAACACCATGGAACTCTGAGAGAGGAAGCCTCTCAGAGC AAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATGGATAGGTAGATA TATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day A	F01	323		GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTGGCAGGAGTGAGAAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATTAGGCTTCATATGCCATCCCCTACCAACACTGCGAAATACCACGGAACTCTGAAAGAGGAAGCCTCTCAGAGC AAATGTGTCCTATACCACAGCTGGGACCTAATGGGAAGAGATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATA TAACTGTTTATATATGACCTCCAAGGATC
C_2 1Day A	F02	323		GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTA GGTCCCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTCCGCAGTGTTGGTAGGGGATGACATATGAAG TCTAATTTTGCTGTCCATGAAGCTCTCACAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCT CGAATTCTGAAGCTAACAAACTGATC
C_2 1Day A	F03	265		GATCAGGCCAGGTCTTTCCTACATGAAGAGCAAGGAGGCAGGCAGCACTCTTCAGGACTGTTCCAAGAGACAGAACTTTCCTTGAAGGCATCATCCT AGATGCAGAAGGTTCATTTAAAACATTGCAAAAGAGCTTAAGTCTTCCAGCAATAAATCCTGTGAGTTCTTGCAGAATCACACACATCCTCAGCATGTA AAAGAAAGGACAGTCAGGTCCACGTATTAGTTGATTCCTTACACCATCTCTCCAGCATAAAAGGGGATC
C_2 1Day A	F05	322		GATCATCCTGAGTATTATAGAATCTGTAAGCAGTATGATACTAATAAAGATGAAAAATTTGTTATTAGTTTGTCCCTTCCCACTTGTTAATGTCAGGAAT TTTTGTTTTATATCTGTCTGTTATATACTTACTTCTTTTACAACAAGTGGACCAACGCAGTATTTATAACAAATAGACAGTATGTTTTATAACAAATAGGC CAAAACGGTTTTTATAAATGTATCCTGAAAACAGTAGTGTAGAATCTTAAGCTCCATGGTTGGTTTTATTTAAGACTACATATCATAACAACATACAGAT TATTGTACTAACTGGAAGATC
C_2 1Day A	F07b	403		AAAACATAGAATGTCCTGTCTTACCCGCTGCTTAAGCATGTGGATCAGTTTGTTTGCTTCTGAATTCGAGATAAGTTGGATTAGTGAAAATCATGGGA GGTTTAACATAACTTCTGCAGGAGTGAGGAAGTTGGACACTATGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCCACCGACACT GCGAAATACCATGGAACTCAGAATGAGGAAGCCTCTCAGAGCAAGTGTGTTCTATACCACAGCTGGGGCCTAATGGAAAGAAATATATCTGTATCTC TATCCATATCTATGTCTATAGACAAATGGGTAGATAGGTAGATATATCTGTTTATATATGACCTCCAAGGATCCTGTGAATGTACACAGAGACTCTGGA ATACTGTAAAGC
C_2 1Day A	F10b	363		GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAACCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGC GAATGTGTTCTATACCACAGCTGGGACCTAATGGAGAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGACAGGTAGATA TATCTGTTTATATATGACTCCAAGGATCCACATGCTTAAGCTGCGGGTGAGACAGGACATTCTAAGTTT
C_2 1Day A	G02b	436		AAAACATAGAATGTCCTGTCTTACCCGCTGCTTAAGCATGTGGATCAGTTTGTTTGCTTCTGAATTCGAGATAAGTTGGATTAGTGAAAATCATGGGA GGTTTAACATAACTTCTGCAGGAGTGAGGAAGTTGGACACTATGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCCACCGACACT GCGAAATACCATGGAACTCAGAATGAGGAAGCCTCTCAGAGCAAGTGTGTTCTATACCACAGCTGGGGCCTAATGGAAAGAAATATATCTGTATCTC TATCCATATCTATGTCTATAGACAAATGGGTAGATAGGTAGATATATCTGTTTATATATGACCTCCAAGGATCCTGTGAATGTACACAGAGACTCTGGA ATACTGTAAAGCCAGATGATCAACCATTCACCGTCTTGTACAGAG

APPENDIX P: C-2 Unidentified Transcripts 1day versus 6months (continued)

Comparison				
Age		Size (#bp)		Sequences
C_2 1Day A	G05	421		GATCAGGTTGCAAGCATGGATGCCCTCTTGTGGTTGAGATTGCCAAGATATTGGTTTGATTTTATAGGAACCCAAAAGATTTTAAGACTTTTTAAAAAC AAACAAATGCAGGCTTAATTTGGTTCCTTTAAAAATTTTGGCCTTGATTTTAAAGACACATGAATTAGACTGCTTGTTTTCTTTGAGAAATGAGGCCCAT AGTATAACTCCACCATTTCTTAGCTTTACATGATTTCTAAGTACAGAACTCAACCACAGTTATATTTGCTGTTGATTACCTATCATACGAAATTTTCTAAA TATCAGATTATACAAAGGTATTGAGATGCAAGAAGAATTTTTAGAGGTGTTAAATCAGATATAGTTTTTAAAAAACAGAATGTGGGAAGCCTCATTACT CAGATATTTAATTCTAATGATC
C_2 1Day A	G09b	428		AAAACTTAGAATGTCCCGTCTCACCCGCAGCTTAAGCATGTGGATCCAGATGTAACCGGGGTGGGGGTGGAGGGGGGTTGGGTGGTTTGAGCTGG ACTTCCATGCAACAGAGAAGTGATACCAGGAAAGCTCGGGGTCGGTGCTAGTTCAGGTCCACCGAGGGAGTCTGAGGAGAAAGCCAGACAGGTGA AAGGTATGGGGAGAGCCCGAAAACGATCGAGAGGGACGGCGGGGGGGGGGGGAAAAACATCTATATAAAGAAAGGGAGGAGAGTGAGAGTTGGAA AACGTAGCAGAAAATAATCAATATTATTGGTAATCGCCACAATCCATCCAAGAAAGTAACCATTTTGTATCCGGAATTATAATACCCTCACAAACTCTAC CCTCTCCAATAGCAAGGAAGAAATATAACAGATTTTACCAACAC
C_2 1Day A	G12	94		GATCAGACAGAAAAGTTTATCATCTGAGAAGTTAAGTTTGGAACAAAGGGTAAGCTGACATCTCCCATACATCATGGCACACGCAGAAGAGATC
C_2 1Day A	H06	182		GATCTATAAATGTAGCAGGCTGCTTAATAGAAAATACACTAAACAGACGCGATTTTCTGCATCCCCCTCGGTACGCTCCATGTAGAGAGCACAGATAC ATATAGTGCGGCACTGTCAGAGAAGTAGAAACGAGTTATGTTGCCATTACCAGCTTCATGCCTCGTTCCTGTCTTTTCTGGATC
C_2 1Day B	A11	322		GATCAGTTTGTTTGCTTCTGAATTCGGGATGAGTTGGATTAGTGAAAATCATGGGAGGCTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGC GAATGTGTTCTATACCACAGCTGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCAAGTCTATGTCTATAGACAAATGGATAGATAGGTAGATAT ATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day B	A12	421		GATCATTAGAATCAAATATCTGAGTAATGAGGCTTCCCACATTCTGTTTCTTAAAAACTATATCTGATTTAACACCTCCAAAAATTCTTCTTGCATCTCA ATACCTTTGTTTAATCTGATATTTAGAAAATCTCGTATGAAAGGTAATCAACAGCAAATATAACTGTGATTGAGTTCTGTACTTAGATATCATGTAAAAC TAAGAAATGGTGGAGTTATACAATGGGCCTCATTTCTCTAAGAGAACAAACAGACTAATTCATGTGTCTTTAAAATCAAGGCCAATATTTTTAAGGGAA ACAAATTAAGCCTGCATTTGTTTGTTTTAAAAAGTCTTAAAATCTTTTGGGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCGACCACAGGAGGG CATCCATGCTTGCAACCTGATC
C_2 1Day B	B02	421		GATCATTAGAATTAAATATCTGAGTAATGAGGCTTCCCACACTCTGTTTTTTAAAAACTATATCTGATTTAACGCCTCTAAAAATTCTTCTCGCATCTCA ATACCTTTGTTTAATCTGATATTTAGAAAATTTCGTATGATAGGTAATCAACAGCAAATATAACTGAGGTTGAGTTCTGTACTTAGAAATCATGTAAAAC TAAGAAATGGTGGAGTTATACAATGAGCCTCATTTCTCAAAGAAAACAAACAGACTAATTCATGTGTCTTTAAAATCAAGGCCAAAATTTTTAAAGGAAA CAAATTTAGCCTGCATTTGGTTGTTTTTAGAAAGTCTTAAAGTCTTTTGGGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCAACCACAAGAGGG CATCCATGCTTGCAACCTGATC
C_2 1Day B	B04	322		GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTA TGAGAGCTCCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGTAGCCTCTCAGAGT AAATGTGTTCTATACACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATGGGTAGATAT ATCTGTTTATATATGACCTCCAGGGATC
C_2 1Day B	B08b	421		GATCATTAGAATTAAATATCTGAGTAATGAGGCTTCCCACATTCTGTTTTTTAAAAACTATATCTGATTTAACACCTCTAAAGATTCTTCTTGCATCTCAA TACCTTTGTTTAATCTGATATTTAGTAAATTTCGTATGATGGGTAATCAACAGCAAATATAACTGTGGTTGAGTTCTGTACTTAGAAATCATGTAAAACT AAGAAATGGTGGAGTTATACAATGGGCCTCATTTCTCAAAGAAAACAGACAGACTGATTCATGTGTCTTTAAAATCAAGGCCAAAATTTTTAAAGGAAA CAAATTAAGCCTACATTTGTTTGTTTTTAAAAAGTCTTAAAATCTTTTGGGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCAACCACAAGAGGGC ATCCATGCTTGCGACCTGATC
C_2 1Day B	C01	250		GATCCATTCACCGTGTTTATAACCACTGCTTCTCGGAGTACTTTGTAAAGCTTTTAATGCCAACAGTAATGATGAGAGCTCTAGCACTGACAGGTAGG AATTTGTTGGCTTTTTAGCTACCACGCTGACCACTTGTAATAGCTGGGGAACTCAAGGGGAAAAAAAATGTCTCCTATAGACTAAGCTGTTAGAAAGGT ATTTATGATATGAAACATAAGTGCAGAGCAACTAGGAAGACTGCAGCTTTGATC
C_2 1Day B	C07	322		GATCAGTTTGTTTGCTTCTGAACTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTGCAGGAGTGAGAAAGTTGGACACTAT GAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATGCCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCA AATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCACTATCCATGTCTATGTCTATAGACAAATGGACAGATAGGTAGATAT ATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day B	C08	389		GATCAGACTTCCTTCCAGTTCTTCCAGTTTTCTGATAGAGACCATCTTTATGTAGGAAACCTTAAAAAAAAGAAGTAGTTCTAGGCAGGCAGTAAGAG GAGGAGGATATTAAGACATTTTTCTAACCCATGCTACAATTCCTTCAGAGTGGTTATATCCTGCCTTTATCATGTGCTTTTTATAAAAGCCAGTTACAAT TTCCATCTACACTTTGGAACCAATAGGTTTGTGGTAAATGCGACTGGAAGAATATCTAGGAAAACTCAAGAAGATTCACAGTGAAAAGATGCCATGCC ATACTGAATTCAGTCGCTCTCTATAAGCATAAGAAGCAGTATCTGGTGTTGTGCTGTAACACGTTGGTGCTCCTGTGACAGCTCATTGGGATC
C_2 1Day B	C12	640		GATCAGTTTGTTTGCTTCTGAATTCGAGATGAATTGGATTAGTGAAAATCATGGGGGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTA CGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCGTCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGC AAATGTGTTCTATACCACAGCTGGGACCTAATGGGAAGAAATATATCTGTATCTCTATCCATGTCTATGTCTATAGACAAATGGATAGATAGGTAGATA TATCTGTTTATATATGACCTCCAAGGATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGGAAATCATGGGAGGTTTAACATAACTTTTG CAGGAGTGAGAAAGTTGGTCACTATGAGAGCTTCATGGACAGCAAAACTTGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACT CTGGAAGAGGAAGCCTCTCAGAGCGAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTAT AGACAAATGGATAGATAGGTAGATATATCTGTTTATATATGACCTCCAAGGA

APPENDIX P: C-2 Unidentified Transcripts 1day versus 6months (continued)

Comparison	[illegible]		
Age		Size (#bp)	Sequences
C_2 1Day B	D01	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACTGATATATTTCTTTCCATTA GGTCCCAGCCGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTACAGAGTTCCATGGCATTTCGCAGTGTTGATAGGGGATGGCATATGAAG TCTAATTTTGCTGTCCATGAAGCTCTCATAGTGTCCAACTTCCCCACCCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATC TCGAATTCAGAAGCAAACAGACTGATC
C_2 1Day B	D02	323	GATCAGTTTGTTTGCTTCTGAATTCGAGACGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGGCACTA TGAGAGCTTCATGGACAGCAAAATTAGACTTCGTACGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGGAGCCTCTCAGAGC AAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACGAATGGATAGATAGGTAGATA TATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day B	E02	322	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTAT GAGAGCTTCATGGGCAGCAAAATTAGACTTCATGTGCCATCCCCTACCAACACTGCGAAATACCGTGGAACTCTGAAGGAGGTAGCCTCTCAGAGCG AATGTGTTTTATACCACAGCTGGGACCTAATGGAAAGAGATATATCTGTATCTCTATCCATATCTATGTCCATAGACAAATGGATAGATAGGTAGATAT ATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day B	E04	388	GATCAGACTTCCTTCCAGTTCTTCCAGTTTTCTGATTGAGACCATCTTTATGTACGAAACCTTAAAAAAAGAAGTAGTTTTAGGCAGGCAGTAAGAGGA GGAGGATACTAAGACATTTTTCTAACCCATGCTGCAATTCCTTCAAAGTGGTTATATCTTGCCTTTATCACGTGCTTTTTATAGAAGCCAGTTACAACT TTCATCTACACTTTGGAACCAATAGGTTTGTGGTAAATGCGACTGGAAGAATATCTAGGAAAACTCAAGAAGATTCACAGTGAAAAGATGACATACCAT ACTGAATTCAATCGCTCTCTATAAGCATAAGAAGCAGCATCTGGTGTTGTGCTGTAATACGTTGGTGCTCCTGTGACAGCTCATTGGGATC
C_2 1Day B	E10	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCCTTCCACTA GGTCCCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATAGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAG TCTAATTTTGCTGTCCATGAAGCTCTCATAGTGCCCAACTTTCCCACTCCTGCAAAAGTTATGTTAAACCTCCCTTAATTTTCACTATTACAACACATCT CGAATTCGAAGGCAAACAAACTGATC
C_2 1Day B	E12	251	GATCCCAGTGATGATAGAAACGTCTGTGTGCAGCTGCATTTGTAATCCCTGAGTCATAACACAGCTGGTTTGGATTTAAAAATGATAGTTTATGCCAC TATGCATGTTTGAAGACGAACCTACATAGCCAGCCTTTATTTAGCTGCACGGCAGCTTCCAGTTCAACCAGAGGTCTTGCTAGGCTGAAGCTATACTC CTACCAAGGTCCAGAAATAGCTGGAGGTATTAAGTTTCTAGACACGACTGTGATC
C_2 1Day B	F05	290	GATCTGGCCACATAAGCTTCACTAGACAATCAATATTCTCAACACCAAAAATATTTACTTAACTGGGCACTCTGAGCTAGTAGAGCTCTGTACTAGTTA TGGAGGTGAGTTGAGCATGGCACAAGTTCCGATTCCACGTATGCACTATGACCCCAGGCACTAAGACCTAGATATGATTCCTATGAAAACTGCAGGT GATGTTAACCAACAGGATGTGCCCACAGCTCTGTTAAATGAAGGCTAACTCTCCCCTGAGAAGTATACAACCCATCCTAATAGCATGGATGATC
C_2 1Day B	F06b	351	GATCCAAGTGCTTTCTGCTTTCAGCAGTGCTAAAGACTTAGAGCTCGCACTGAAATCCAAACCACTCATGACTCAAGCTTACAGATGAGAGTAGCTAC TGCTTTTGTATTTTGACAGTCTGTGGGTAGGGTTTAGGTGGATGTGAAGAGCTGCAGCAGCAATTTCAGACAGGGCTCATTGGAGCTGTTTTTTGCT GCTAAATCCATAACTGAAGTACAGAAAAAATATGCTCAGGCTGAAAAAGAATAGTTGGCAGTGAGTGTAATGTGGTAAGCTCAGTAGACAATTGCACA GACCCCAAACACTCCATCTACTCCATAAACAGGCAGCAAATGTACGCTGTCATTGATC
C_2 1Day B	F07b	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTATAGCATAACTTTTGCAGGAGTGAGAAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATTAGACTTCATATACCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCA AATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTAACCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATAT ATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day B	H01	323	GATCATTGGAGGTGCCTCTGAATTCGAGTTGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGCTGGACACT ATGAGAGCTACATGGACAGCAAAATTAGACTTCTTATGCCACCCCCTACCAACCCTGCCAATTCCCTTGAAACTCCGAGGGGGGAAGCCTCTCAAAT CGAATGTGTCCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCGTATCTATGTCTATAGACCAATGGTTAGATAGGTAGAT ATATCTGTTTATATATGACCTCCGAGGATC
C_2 1Day B	H05	321	GATCCTAGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCCTTCCATTA GGTCCCAGCTGTGGTATAGAACACATTCGCTCTGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTC TAATTTTGCTGTCCATGTAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAGAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACCCATCTC GAATTCAGATGCAAACAAACTGATC
C_2 1Day B	H06	123	GATCTTGCCATTTTGGTCAGGTAACAATATTTACCACTGCCAATTTCAAACCTGTATTTAAAGCAGCAGGTGAGTCTGAGGGGCGGGTTGTAAGGCAC ACCAAACAGTTTGTGTTAAGTGATC
C_2 1Day C	A06	216	GATCACATAACCATCTAGAGAGGAGCTTGAGACTCCAATGTTGTGATATTACTTCAGCAATTTAACAAATTTGTGAAAATCATCATGAGACAATGAACA ACTGTGACGTCCATAAACAGTGTCATTTTTATAAGGTCATAGAAGAACTGTGGTGAAATATTTTACATCTTCCAGTTGAAGATAATGAAGTCTAAGTGT TAGAATCCGGCGGAGATC
C_2 1Day C	A09	422	GATCATCAGAATTAAATATCTGAGTAATGAGGCTTCCCACATTCTGTTTTTTAAAAAACTATATCTGATTTAACACCTCTAAAAATTCTTCTTGCATCTCA ATACCTTTGTTTAATGTGATATTTAGAAAATTTCGTATGATAGGTAATCAACAGCAAATATAACTGTGGTTGAGTTCTGTACTTAGAAATCATGTAAAAC TAAGAAATGGTGGAGTTATACAATGGGCCTCATTTCTCAAAGAAAACAAACAGACTAATTCATGTGTCTTTAAAATCAAGGCCAAAATTTTTAAAGGGA ACAAATTAAGCCTGCATTTGTTTGTTTTTAAAAAGTCTTAAAATCTTTTGGGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCTACCACAAGAGGG CATCCATGCCTGCAACCTGATC
C_2 1Day C	B02	308	GATCCTCACTCCATGTCACCTAGTGGAAATTTTGTTCATTTTCTGGTCGGTTGAACTTTGACGACTGTTTCCTCTTTCTACAATTCTGTAATTGGGCAA GAGCATCACCATAGTGGGTCTCCAGTGAACACTTTATTGTTGCCCAGGTACCGCATTTTGAGACATAACGAGCAGAGGGTGGTATTTCAGTGTAACC TAATCCTCAGAAACAAAAGCAGATTTATAAGTAAATATAGTCCTGCAGAGACCATCTCAGAGACTTGAGAGCTAAAGCAATGTGCTCCCATTAAGCAAT GCATTTTGTGATC
C_2 1Day C	B09	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTA GGTCCCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAG TCTAATTTTGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCGTGATTTTCACTAATCCAACTCATCT CGAATTCAGAAGCAAACAAACTGATC
C_2 1Day C	C04	322	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAACCATGGGAGGTTAGCATAACTTTCGCGGGAGCGAGAAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATCGGACTTCATACGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGC GTATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATA TATCTGTTTATATATGACCTCCAAGGATC

APPENDIX P: C-2 Unidentified Transcripts 1day versus 6months (continued)

Comparison			
Age		Size (#bp)	Sequences
C_2 1Day C	C10	284	GATCCCTGCCATTGATATAGCCTGGAAATTTAGAGTCTTCATCATCCTCCCACTACTCTCTATGAATGCTCTTACCTAAATATCCTACTGAATTGATTTTAAGACATGTTCTAAACAGCTCTTAATTGGCCACTTTTAGAACCAATCACGTCCTGAAAGTCTTGTGGGATTTACTCCAAGCAGAAAGACTGAAGTAAAGGTTGGTATGAGAATATCCTGTATAGTATGTCATTTATAAGTTGCTGCTTGCTTGTGAGTTGATTTATTTCAAGTGTTTCTTGATC
C_2 1Day C	C11	323	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTCCCAGCTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTTTGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCGTGATTTTCACTAATCCAACTCATCTCGAATTCAGAAGCAAACAAACTGATC
C_2 1Day C	D04	251	GATCGCAGTTGTGTCTAGAAACTTTATACCTCCAGCTATTTCTGGACCTTGGTAGGAGCATTGCTTCAGCCTAGCAAGACCTCCGGTTGAACTGGAAGCTGCTGTGCAGCTAAATAAAGGCTGGCTATGTAGGTTCGTCTTCAAACATGCATAGTGGCATAAACTATCATTTTTAAATCCAAACCAGCTGTGTTACGACTCAAGGATTACAAATGCAGCTGCACACAGACGTTTCTACCATCACTGGGATC
C_2 1Day C	D06	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAAACATGGGAGGTTTAACATAACTTTTACGGGAGTGAGAAAGTTGGACACTATGAGAGCTTCATGGACGGCAAAATTAGACTTCATATGCCATTCCCTACCAACGCTGCGAAATACCATGGAACTCTGAAAGAGGAGGCCTCTCAGAGCGAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATACCTATGTCCATAGACAAATGGATAGATAGGTAGATATATCTGTTTATATATGACCTCCAGGGATC
C_2 1Day C	D09	419	GATCATTAGAATTAAATATCTAAGTAATGAGGCTTCCCACACTCTGTTTTTTAAAAACTATATCTGATTCAACACCTCTAAAAATTCTTCTTGCATCTCAATACCTTTGTTTAATCTGATATTTAGAAAATTTCGTATGATAGGTAATCAACAGCAAATATAACTGTGGTTGAGTTCCGTACTTAGAAGTCATGTAAAACTAAGAAATGGCGGAGTTATACAATGGACCTCATTTCTCAAAGAAAACAAACAGACTAATACATGTGTCCTTAAAATCAAGGCCAATTTTTAAAGGAAGCAAATTAAGCTTGCATTTGTTTGTTTTTAAGAAGTCTTAAAATCTTTTGGGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCAACCACAAGAGGGCATCCATGCTTGCAACCTGATC
C_2 1Day C	E02	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCACGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTAGACACTATGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCGTCCCCTACCAGCACTGCGAAATACCATGGAACTCCGAACGAGGAAGCCTCTCAGAGCAAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATAACTATGTCTATGGACAAATGAATAGGTAGGTAGATATATCTGTTTATACATGACCTCCAATGATC
C_2 1Day C	E06	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTAGGATTAGTGAAAATCATGGGAGGTTAAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTATGAGAGCTACATGGACAACAAAATTAGATTTCGTATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAGGCCCCTCAGAGCGAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATGGACAAATGGATGGATAGGTAGATATATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day C	E08	276	GATCATTAGAATTAAATATCTGAGTAATGAGGCTTCCCACAGTCTGTTTTTTAAAAACTATATCTGATTTAACACCTCTAAAAATTCTTCTTGCAACTCAATACCTTTGTTTAATCTGATATTTAGAAAGTTTCGTATGATAGGTATCAACAGCAAATATAACTGTGGTTGAGTTCTGTACTTAGAAATCGTGTAAAACTAAGAGATGGTGGAGTTATACAATGGGCCTCATTTCTCAAAGAAAACAAACAGGCTAATTCATGTGTCTTTAAGATC
C_2 1Day C	E09b	413	GATCAAGAATGGGGAAAAACTTCCTTTACCAGGAGAGAAGAAAAGAAGCATTGAGTATCTTCCATCTGGAAAGAAGAATGAAGGGACATCTAATCAAGATTTGCAAAATCAGGAATGTGTGAATAAGGTTTATCCAGAGGTTGCTTTCATCAATACTTCCAATACTTCAATTACAGTAGAACAGTAAGTAATCAATTTCAAACAAAGTACAGAACTTTTTCATATAGGTGACGTGTGAATTTCAATTCCCTGCTTATTTTGTCATTCACTCAAATGCAAATGGAACCACAACCACAAAGATGCTAAACTTTAGTAGCTTCAAACAGTATGGGTGACAAATTGATATATATTTTCTTAAAAAAAAGAGCATTTTTTTTGTTTTCAAGATCCAGATGTAACCAGCCTGCACGTAT
C_2 1Day C	E10	323	GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGACTAGTGAGAATCATGGGAGGTTTAACATAACTTCTGCAGGAGTGAGAAAGTTGGACACTACGAGAGCTGCATAGACAGCAAAATTAGACTTCATAAGCCGTCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGAGGAAGCCTCTCAGAGCAAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTGTCCATATCCATGTCTATAGACAAATGGATAGATAGGTAGATATATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day C	F10	250	GATCCCAGTGATGATAGAAACGTCTGTGTACAGCTGCGTTTGTAATCCTTGAGTCAAACACAGCTGGTTTGGATTTAAAAATGATAGTTTATGCCATTATGCATGTTTGAAGACGAACCTACATAGCCAGCCTTTATTTAGCTGCACAGCAGCTTCCAGTTCAACCAGAGGTCTTGCTAGGCTGAAGCAATACTCCTACCAAGGTCCAGAAATAGCTGGAGGTATTAAGTTTCTAGACACAACTGTGATC
C_2 1Day C	G02	321	GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTACAGACATGGATGTGGATAGAGATACAGATATATTTCTTTCCATTAGGTCCCAGCTGTGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTTTGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCTGGTTTTCACTAATCCAACTCATCTCGAATTCAGAAGCAAACAAACTGATC
C_2 1Day C	G03	323	GATCCTTGGAGGCCATATACAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTAGGTCCCAGCTGTGGTGTAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAGTCTAATTTTGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCTCGAATTCAGAGGCAACCAAACTGATC
C_2 1Day C	G06	281	GATCAGGTTGCAAGCATGGATGCCCTCCTGTGGTTGAGAATGCCAAGATATTGGTTTGATTTTATAGGGACCCAAAGGATTTTAAGACTTTTTAAAAACAACCAAATGCAGGCTTAATTGTTTCCTTTAAAAATTTTGGCCTTGATTTTAAAGACACATGAATTAGTCTGTTTGTTTTCTTTGAGAAATGAGGCCATCGTATAACTCCACCATTTCTTAGTTTTACATGGTTTCTAAGTACAGAACTCAACCACAGTTATATTTGCTGTTGATTACCGATC
C_2 1Day C	G08	320	GATCTTCCGGTTAGTACAATAATCTGTATGTTGTTATGATATGTAGTCTTAAATAAAACCAACCATGGAGCTTAAGATTCTACACTACTATTTTCAGGAAACATTTATAAAAACCGTTTTGGTCTATTTGTTGTAAAATATACTGTCTATTTGTTATAAATACTGTGTTGGTCTACTTGCTGTAAAAGAAGTAAGTATAACAGATAGATATAGAACAAAAATTCCTGACATTAACAAGTGGGAAGGGACAAACTAATAACAAATTTTTCATCTTTATTAGTATACTGTTTACAGATTCTGTAATACTCAGGATGATC
C_2 1Day C	H07b	402	GATCAAGAGATTGCTATGTAACCTGTGTCTCATTTGGGTAGGAGATACTTAATTCCAAGCTTGAGGGGAGCTTCAGTTCCTGTAGGTTGTGTGATGACCTTTAAGCCTCTGTTGTGATGAGGAACCCCTGTGGAAAAAGAAGAATCTTATCGGTACATGTCTTGGTAACAACTTGTGTCTAGCTGCGCATGAGAATGTGAGTAAAGGATGCCCAGTTACACCAGCTCGGGTGTTTATGACTGTTCGAAAGGCTGAGACACGTAACACAAAACACGCTCTGGGTCTAGAAGCAATGACGCAGTTTTCAACAGAACCAGGTAGAATCGAAACAAACAGTATAAAATGAATTATAACAAGACACTGCTGGCAGGTGGGCAAATGGTTATAGGGGTGTTTCTGATC

APPENDIX P: C-2 Unidentified Transcripts 1day versus 6months (continued)

Comparison	Query			
Age		Size (#bp)		Sequences
C_2 1Day C	H08	267		GATCATCATTTTCCTCTGAAGTCAGATGTTTTACCTGCAATTCCTGTGTTGTAAATACACCGGCAGTACAGCGCCAACACTTACCAATAAACACATCGC ATTTGCCTGCTAAGAGACAGATGAGTAGGACAAGGATTTTTAAGTATTACTTTGAGAATCCATTGTATTCTTTCTTTAACTTAGACACAAAGAATCTGTT GAATACCTTATAAAATTCTTGGTCATTTTCTAAATCAATGTAAGGCATATGAATCTGGAAAACTGATC
C_2 1Day C	H09	336		GATCAGATGTAACCAGCCTGCACGTATTTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGA GAGTTGGACACTATGAGAGCCTCACGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCGACACTGCGATATACCATGGAACTCTGAAAGGGGA AGCCTCTCAGAGCAAATGTGTTCTATACCACAGCTGGAACCTAATGGAAAGAAATATATCGGTATCTCTATCCATATCTATGTCTATAGACAAATGGAT AGATAGGTAGATACATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day C	H11	309		GATCCTCATTCCATGTCACCTAGTGGAAATTTTGTTCATTTTCTGGTTGGTTGAACTTTGACAACTGCTTCCTCTTTTCTACAATTCTGTAATTGGGCAA GAGCGTCATCATAGTGGGTCTCCAGTGAACACTTTATTGTTGCCCAGGTACTGCATTTTGAGACATAACGAGCGGAGAGTGGTATTTCAGTGTAACC TAATCCTCAGAAACAAAAGCAGATTTATAAGTAAATATAGTCCTGCAGAGACCATCTCAGAGACTTGAGAGCTAAAGCAATGTGCTCCCTTTAAGCAA CGCATTTTGTGATC
C_2 1Day D	A04	323		GATCCTTGGAGGTCATATATAAACAGATACATCTACCTATCAATCCATTTGTCTATAGACATAGATATGGATAGAAATACAGATATATTTCTTTCCATTA GGTCCCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTTGTAGGGGACGGCATATGAAG TCTAATTTTGCAGTCCATGAAGCTCTCATAATGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCT CGAATTCAGAAGCAAACAAACTGATC
C_2 1Day D	A09	385		GATCCACAGCACCAGCGCTGCCCATCGAAACCGTATGAGCTCTCCTGGAGCAGCACCAAGTGTAGGGGTGAAAGCCCTTGCTGTAGACTGAGGATA CATCCAAATGGGCAGAGAGACTGGTCTCAGATTAAGTCATCGATGCTATCCCAGTTTCCACCACAGCAAAAAAACTCCTTTGAACATAGCTTTATGTT TTAGGTCAAGAAAATAAATGTATCATTCCAGTAGTGTTGGTAGGTCTTTGGAAGTGCTAATTAATGAGCATTCAGTTTTCCCTGTGGTGAAGATAGATT TGCTATAGACTTTTTAGGAATATTTTTGGCCTCTCATGGCTACTCAATTATTACTTCTTATTCTGAGATTTGTGTCAAAATAAATCCTGATC
C_2 1Day D	A12	322		GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGGGGATTAACTTAACTTCTGCGGGAGTGAGAAAGTTGGACACTA TGGGAGCTTCATGGACAGCAAGATTAGACTTCATATGGCATCCCCTACCAACACTGCGAAATACACGGAACTCTGAAAGGGGAAGCCTCTCAGAGCA AATGTGTTCTACACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTGTCCATATCTATGTCTACAGATAAATGGATAGATAGGTAGATAT ATCTGGTTATATATGACCTCCAGGGATC
C_2 1Day D	B03	322		GATCCTTGGAGGTCGTATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATACGGATAGAGATATAGATATATTTCTTTCCATTA GGTCCCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCGGTGTTGATAGGGGATGGCATATGAAG TCTAATTTTGCTGTCCATGAAGCTCTCATAGTGTCAACTTTCTCACTCCCGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACACGTCTC GAATTCGGAAGCAAACAAACTGATC
C_2 1Day D	B06b	323		GATCCCTGGAGGCCATATATAAACAGATATATCTACCTATCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTA GGTCCCAGCTGTGGCATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCAGAGTTCCATGGTATTTCGCAGTGTTGGTGGGGGATGGCGTACGAA GTCTAATTTTGCTGTCCATGAAGCTCTCATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATC TCGAAATCAGAAGCAAACAAACTGATC
C_2 1Day D	B08	421		ATCAGGTTGCAAGCATGGATGCCCTCTTGTGGTTGAGAATGCCAAGATATTGGTTTGATTTTATAGGAACCCAAAAGATTTTATGACTTTTTAAAAAAC AAACAAATGCAGGCTTAATTTGTTTCCTTTAAAAATTTTGGCCTTGATTTTAAAGACACATGGATAAGTCTGTTTGTTTTCTTTGAGAAATGAGACCCAT TGTATAACTCCACCATTTCTTAGTTTTACATGATTTCTAGGTACAGAACTCAGCCACAGTTATATTTGTTGTTGATTACCTATCATACGAAATTTTCTAAA TATCAGATTAAACAAAGGTATTGAGATGCAAGAAGAATTTTTAGAGGTGTTAAATCAGATATAGTTTTTAAAAAACAGAATGTGGGAAGCCTCATTACT CAGATATTTAATTCTAATGATC
C_2 1Day D	B10	607		GATCTGTTGCTATGCTGTTACCTTAGTTTAAGACTATACTAATAACAGTAATATCCCAGAACATAACAATGGTAAAATAGAGCTGGTTTTCAAAATATTT ATCATTAATGTACTGGAAAAACTGTGAGAGTGTTTTGTAATCATCCCAAAAGCATCCATTATATATCAGATAATAATTGATTCTGAAATGGTATTCTACT TCCAGTTATGTGATCATTAGAATTAAATATCTGAGTAATGAGGCTTCCCACATTCTGTTTTTAAAAACTATATCTGATTTAACACCTCTAAAAATTCTTCT TGCATCTCAATACCTTTGTTTAATCTGATATTTAGAAAATTTCGTATGATAGGTAATCAACAGCAAATATAACTGTGGTTGAGTTCTGTACTTAGAAGTC ATGTAAAACTAAGAAATGGTGGAGTTATACAATGGGCCTCATTTCTCAAAGAAAACAAACAGACTAATTCATGTGTCTTTAAAATCAAGGCCAAAATTTT TAAAGGAAACAAATTAAGCCTGCATTTGTTTGTTTTAAAAAGTCTTAAAATCTTTTGGGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCAACCAC AAGAGA
C_2 1Day D	B11	468		GATCATTAAAAGTAAATATGTTACTTTAGAGGCTGCCCTCTCCCCGTTGCCCTCAACTATATCGGATTTAACATCTCTGAAAATTCCTCCTGCCTCTCC ATATGTTAGGGTAATCTGTTTTTTATAACATTTCTTGTGATAGGTAATCAACCTCAAATAACACTGTGGTTGAGTTCTGTACACTGCTGACGAGGGCCA CTAAGGAAAGGTGGAGTTGTACGAAGGGCCTCATTTCTCAAAGAAAACAACCGACTAATTCAGGGGGCCCGGAACCCAATGCCCCCATTTTTGAAGG AAATAAATCAAGCCCGCTGTTGTTTGTTTTTCAAAAGTCTTACTGGGAAAACCGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCCCCCACAAGA GGGCATCCATGCTTGCAACCTGATCCCCCGGGCTGCCCCTTCCCAATATCAAGCTTATCTGAATGGTCAACCTC
C_2 1Day D	B12b	322		GATCCTTGGAGGTCATATATAAACAGATATATCTACCTATCTATCCACTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTA GGTCCCAGCTGTGGTATAGAACACATTTGCTCTGAGAGGCTTCCTCTTTCCGAGTTCCATGGTATTTCGCAGTGTTGGTAGGGGATGGCATATGAAG TCTACTTTTGCTGTCCATGAAGCTCACATAGTGTCCAACTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCGCTAATCCAACTCAACT CGAATTCAGAAGCAAACAACTGATC
C_2 1Day D	C04	150		GATCTCAGAGAAGAGGAACACACGGCTTGCCAAGGAGAAGTAAGCAAGGTCAAGCCTGGGTTTTACGAAGTCATTTATTACCCCAAAGAGAGAACAT GGTATCACTAAAACCTGCTCAGCATCAATCTGAAGTCGTGACTGTCGGAGATC
C_2 1Day D	D01	419		GATCATTAGAATTAAATCTGAGTAATGAGGCTTCCCACATTCTGTTTTTTAAAAACTATATCTGATTTAACACCTCTAAAAATTCTTCTTGCATCTCAATA CCTTTGTTTAATCTGATATTTAGAAAATTTCGTATGATAGGTAATCAACAGCAAATGTAATTGTGGTTGAGTTCTGTACTTAGAAATCATGTAAAACTAA GAAATGGTGGAGTTATACAATGGGCCTCATTTCTCAAAGAAAACAAACAGACTAATTCATGTGTCTTTAAAATCAAGGCCAAAATCTTTAAAGGAAACA AATTAAGCCTGCATTTGTTTGTTTTTAAAAAGTCTTAAAATCTTTTGGGTTCCTATAAAACCAAACCAATATCTTGGCATTCTCAACCACAAGAGGGCAT CCACGCTTGCAACCTGATC

APPENDIX P: C-2 Unidentified Transcripts 1day versus 6months (continued)

Comparison				
Age		Size (#bp)		Sequences
C_2 1Day D	D02	321		GATCCTTGGAGGTCATATATAAACAGATGTATCTACCTACCTATCCATTTGTCTATAGACATAGATATGGATAGAGATACAGATATATTTCTTTCCATTA GGTCCCGACTGTGGTATAGAACACATTCGCTCTGAGAGGCTTCCTCCTTCAGAGTTCCATGGTATTTCGCGGTGTTGGTAGGGGATGCCATATGAAA TCTAATTTTGCTGTCATGAAGCTCTCATGGTGTCCAGCTTTCTCACTCCTGCAAAAGTTATGTTAAACCTCCCATGATTTTCACTAATCCAACTCATCA CGAATTCAGAGCAACCAAACTGATC
C_2 1Day D	D03b	421		GATCATTAGAATTAAATATCTGAATAATGAGGCTTCCCACATTCCGTTTTTTAAAAACTATATCTGACTTAATACCTCTAAAAATTCTTCTTGCATCTCAA TACCTTTGTTTAATCTGATAGTTAGAAAATTTCGTATGATAGGTAATCAACAGCAAATATAACTGTGGTTGAGTTCTGTACTTAGAAATCATGTAGAACT AAGAAATGGTGGAGTTATACAATGGGCCTCATTTCTCAGAGAAAACAAACAGACTAATTCATGTGTCTCTAAAATCAAGGCCAAAATTTTTAAAGGAAA CAAATTAAGCCTGCATTTGTTTGTTCTTAAAAAGTCTTAAAATCTTTTGGGTTCCTATAAAATCGAACCAATATCTTGGCATTCTCAACCACAAGAGGGC ATCCATGCTTGCAACCTGATC
C_2 1Day D	D08	322		GATCTGAAGGGAAACCAACTAACCATGTAGCATCTTTTTGAATTTATCCCCCACTGTCACCTTCGAAACAACTAGATGGCTATAGATAGATTAGAGACT ACAACAAGTGATATTTCACAACTCGTAAGGCAAGTGAATCCGTAAGGCCAAGGAGAGGAGCTGTTAGACATTGTGAAAGACTAAATAAAATTGAAGCC ATTATCTGGTGTGTTTTCATTGCGTATGCCTCAATATCTTAAAAAATACAAACAAACAAAACCCCAGCAACATCAAAATGCACAAAGCTTTTAAAATATG TTAGAATAAGACATTGGTTGTGATC
C_2 1Day D	D12b	359		GATCCTCACATCTTTCTGGGACTAAACATTAGGATTCTCCTGGTGTATTAAAACAGTTCCTCTGCAGAGAACGGGTGTTTGGAATGGTTTCACATCTG ATGATATAAATATCTTTGCTGCTATCGGGTGCTCTTTATAATCCAAATGGCGCTTCCAGGAAGGGTAACCTTGCCAATTCAACCATTCACGCTGGATTA GGGACTCTGAGAAGCTGAAAAGATTTTTCTGATGATTTATAACTCCATCAGATTCCAAAACGAAGACAGGTCTACACTTTCTGATAAAACAGCCGCCA GAATAGACACAAGTGTGTGTGCAGGGAAATGCACTTTCTCATTCATTCACATCTAGATATGATC
C_2 1Day D	F04b	323		GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGGAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATTAGACTTCGTATGCTATCCCCTACCAACACAGCGGAATATCATGGGACTCTGAAAGAGGAAGCCTCTCAGAGC GAATGTGTTCTATACCACAGCTGGGACCTTATGGAAAGAGATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATA CATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day D	F07	322		GATCAGTTTGTTTGCTACTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAAGAGTGAGTAAGTTGGACACTA TGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTACGAAATACCATGGAACTCTGAAAGAGGAAGCCTCGCAGAGC GAATGTGTTCTATACCACAGCTGGGACCTATGGAAAGAGATATATCTGTATCTCTATCTACATCTATGCCTATAGACAAATGGATAGATAGGTAGATAT ATCTGTTTATATATGACCTCCAAGGATC
C_2 1Day D	F09	170		GATCCATGGTATTTCGCAGTGGTGGTAGGGGATGGTATATGAGGTCTAATTTTGCTGTCCATGAAGCTCCCATAATGTCCAACTTTCTCGCTCCTGCA AAAGTTATGTTAAACCTCCCATGATTCTCACTAATCCAACTCATCTCGAATTCTGAAGCAAACAAACTGATC
C_2 1Day D	F10	421		GATCATTATAATTAAATATCTGAGTAATGAGGCTTCCCACATTCTGGTTTTTAAAAACTATATCTGATTTAACACCTCTAAAAATTCTTCTTGCATCTCAA TACCTTTGTTTAATCTGATATTTATAAAATTTCCTATGATACGTAATCAACAACGAATATAACTGTGGTTGAGTTCTGTACTTACAAATCATGTAAAACTA AAAAATGGTGGAGTTATACAATAGGTCCCATTTCCCAAAGAAAACAAACAGACTAATCCATGTGTCTTTAAAATCAATGCCAATATTTTTAAAGGAAACA AATTAAGCCTGCATTTGTTTGTTTTTAAATAGTCTTTAAATCTTTTGGGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCAACCACAAGAGGGCAT CCATGCTTGCAACCTGATC
C_2 1Day D	G01	422		GATCATTAGAATTAAATATCTGAGTAATGAGGCTTCCCACATCCTGTTTTTTTAAAGACTATATCTGATTTAACACCTCTAAAAATTCTTCTTGCATCTCA ATACCTTTGTTTTATCTGATATTTAGAAAATTTCGTATGATAGGTAATCAACAGCAAATATAACTGTGGTTGAGTTCTGTACTTAGAAATCATGTAAAAC TAGGAAATGGTGGAGTTATACAATGGGCCTCATTTCTCAAAGAAAACAAACAGACTAATTCATGTGTCTTTAATATCAAGGCCAAAATTCTTAAAGGAA ACAAATTAAGCCTGCATTTGATTGTTTTTAAAAAGTCTTAAAATCTTGTGGGTTCCTATAAAATCAAACCAATATCTTGGCATTCTCAACCACAAGAGGG CATCCATGCTTGCAACCTGATC
C_2 1Day D	G07	323		GATCAGTTTGTTTGCTTCTGAATTCGAGATGAGTTGGATTAGTGAAAATCATGGGAGGTTTAACATAACTTTTGCAGGAGTGAGAAAGTCGGACACTA TGAGAGCTTCATGGACAGCAAAATTAGACTTCATATGCCATCCCCTACCAACACTGCGAAATACCATGGAACTCTGAAAGGGGAAGCCTCTCAGAGC AAATGTGTTCTATACCACAGCTGGGACCTAATGGAAAGAAATATATCTGTATCTCTATCCATATCTATGTCTATAGACAAATGGATAGATAGGTAGATA TATCTGTTTATATACGACCTCCAAGGATC
C_2 1Day D	H07	421		GATCATTAGAATTAAATATCTGAGTAATGAGGCTTCCCACATTCTGTTTTTCAAAAACTATATCTGATTTAACACCCCTAAGAATTCTTCTTGCATCTCA ATACCTTTGTTTAATCTGATATTCAGAAAATTTCGTATGATAGGTAATCAACAGCAAATATAACTGTGGCTGAGTTCTGTACTTAGAAATCATGTTAAAC TAAGAAATGGTGGAGTTATACAATGGGCCTCATTTCTCAAAGAAAACAAACAGACTAATTCATGTGTCTTTATAATCAAGGCCAAAATTTTTAAAGAAAA CAAATTAAGCCTGCATTTGTTTGTTTTTAAAAAGTCTTAAAATCTTTTGGGTTCCTATAAAATCAAACCAATATCTTGTCATTCTCAACCACAAGAGGGC ATCCATGCTTGCAACCTGATC

APPENDIX Q: Table 4: Genes Differentially Upregulated Exclusively at 6 Months and In-vitro in WC Versus SR

Gene Name	Ortholog	C-1 (WC)		C-2 (WC)		C-3 (WC)		SMC In-vitro	
		1day copy #	*6weeks copy #*	*1day copy #*	*6months copy #*	*6weeks copy #*	*6months copy #*	*WC copy #*	*SR copy #*
ACSL1	acyl-CoA synthetase long-chain family member 1				1		3	1	0
ACTA2	actin, alpha 2, smooth muscle, aorta	10		1	7		32	0	6
ACTB	beta-actin		2		1	3		9	1
ATP6	ATP synthase FO subunit 6	1						2	3
CANX	calnexin				9			0	1
CCT6A	chaperonin containing TCP1, subunit 6A					1		1	0
CCT8	chaperonin containing TCP1, subunit 8		1			2		1	0
COII	mitochondrial DNA, cytochrome oxidase subunit II		3	1	3	4	1	11	28
COL1A2	F07 putative ribosomal protein S3	2		158		4		6	1
COTL1	similar to coactosin-like 1 (dictyostelium) (LOC768420)					16		0	5
DCN	dermatan sulfate proteoglycan decorin	2		1				2	1
EEF1A1	eukaryotic translation elongation factor 1 alpha 1		1					1	0
EIF4A2	eukaryotic translation initiation factor 4A, isoform 2				1			8	19
ENO1	mrna for tau-crystalin/alpha enolase				5		6	30	0
FABP4	fatty acid binding protein 4, adipocyte	1			1			2	3
FBLN5	fibulin 5	87		15			2	0	30
FKBP9	FC506 binding protein 9, 63 kDa	1		7				0	1
FN1	fibronectin 1			4				0	29
LUM	lumican	159	1	10	7		104	2	47
MRPL27	mitochondrial ribosomal protein L27		1					18	0
MYH11	myosin heavy chain II, smooth muscle		9		27		32	0	12
MYLK	myosin light chain kinase telokin	13		5	7		21	0	7
ND4	Mitochondrial DNA - NADH dehydrogenase subunit 4 gene	1	3	2	2		1	4	19
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10 , 42kDa, nuclear gene encoding mitochondrial protein		2			14		0	1
PGM5	PG-M mRNA for proteoglycan			2				0	1
PHB2	prohibitin 2					17		2	3
PRDX1	clone 0058P0007G11 putative peroxiredoxin 1 variant 3					5		8	20
RHOA	ras homolog gene family, member A			3		9		0	1
RPL3	similar to ribosomal protein L3 (LOC771432)		32			18		6	73
RPL32	clone ChEST398a16		9		2			9	0
RPS3	clone 0058P0031		1					2	0
SEC61G	similar to SEC61 gamma (LOC776639)	6		12				0	3
SFRS1	splicing factor, arginine/serine-rich 1		1					0	1
SLC25A6	clone 0063P0030D07 putative solute carrier family 25 member 6 variant 1					21		5	3
TPM1	alpha- tropomyosin gene, exons 9c, 9d, and complete cds, alternatively spliced		3			1		0	9
VIM	vimentin		2			2		1	0
						upregulated at 6months & In-vitro in WC			
						downregulated at 6months & In-vitro in WC			

APPENDIX R: Table 5: Genes Differentially Upregulated Exclusively at 1 Day and at 1 Day in WC Versus SR

Gene Name	Ortholog	C-1 (WC)		C-2 (WC)		C-3 (WC)		1-3 day In-vivo	
		1day copy #	*6weeks copy #*	*1day copy #*	*6months copy #*	*6weeks copy #*	*6months copy #*	*WC copy #*	*SR copy #*
16S rRNA	mitochondrial 16S ribosomal RNA		7	2	6	2		2	6
ACTA2	Actin, alpha 2, smoth muscle, aorta	10		1	7		32	6	4
ACTB	Beta-actin		2		1	3		1	0
ATPase6	ATP synthase FO subunit 6	1						3	4
ChEST751d6	clone ChEST751d6		1					1	0
COI	Cytochrome c oxidase subunit II	4	1	3	1			3	0
COII	Cytochrome c oxidase subunit II		3	1	3	4	1	2	3
DSTN	Destrin		2	1		1		1	0
EIF1	Eukaryotic translation initiation factor 1 variant 1			1		3		1	1
EIF4A2	Eukaryotic translation initiation factor 4A isoform 2		1		1		35	2	2
FBLN5	Fibulin 5	87		15			2	1	0
LUM	Lumican	159	1	10	7		104	12	2
MYLK	Myosin light chain kinase or telokin	15		5	7		21	0	1
MRPL27	Mitochondrial ribosomal protein L27		1					0	1
MYH11	Myosin, heavy chain II, smooth muscle		9		27		32	2	0
MG	Myoglobin	1						1	0
NADH1	NADH dehydrogenase subunit 1						2	5	11
NADH4	NADH dehydrogenase subunit 4	1	3	2	2		1	9	6
PRDX1	Peroxiredoxin 1 variant 3					5		2	3
RPL7	Ribosomal protein L7			2				0	2
RPL3	Ribosomal protein L3		32					1	0
RPS3A	Ribosomal protein small subunit 3A		4	1				0	1
SERPIN1	similar to SDF3 (LOC771608)					5		1	0
SLC25A6	clone 0063P0030D07 putative solute carrier family 25 member 6 variant 1					21		3	1
TPM1	Tropomyosin, alpha					1		1	0
VIM	Vimentin		2			2		0	1
							upregulated at 1day in WC		
							downregulated at 1day in WC		

APPENDIX S Table 6: Genes Differentially Upregulated Exclusively at 6 Weeks and at 6 Weeks in WC Versus SR

Gene Name	Ortholog	C-1 (WC)		C-2 (WC)		C-3 (WC)		6weeks In-vivo	
		1day copy #	*6weeks copy #*	*1day copy #*	*6months copy #*	*6weeks copy #*	*6months copy #*	*WC copy #*	*SR copy #*
ACTA2	actin, alpha 2, smooth muscle, aorta	10		1	7		32	0	6
ACTB	beta actin		2		1	3		1	0
ATP6	ATP synthase FO subunit 6	1						0	4
CCT8	chaperonin containing TCP1, subunit 8		1			2		6	1
COI	cytochrome c oxidase subunit I	4	1	3	1			0	4
COTL1	similar to coactosin-like 1 (dictyostelium) (LOC768420)					16		3	0
EIF4A2	eukaryotic translation initiation factor 4A, isoform 2		1		1		35		74
EIF5	eukaryotic translation initiation factor 5		1					1	0
ENO1	enolase 1, alpha				5		6	0	2
FABP4	fatty acid binding protein 4, adipocyte	1			1			0	2
FN1	fibronectin 1			4				2	0
GNAS	GNAS complex locus, transcript variant 2					1		1	0
LUM	lumican	159	1	10	7		104	0	30
MYH11	myosin heavy chain 11, smooth muscle		9		27		32	0	13
ND	NADH dehydrogenase subunit 1 gene						2	0	1
ND4	NADH dehydrogenase subunit 1 gene, mitochondrial DNA	1	3				1	0	7
PHB2	prohibitin 2					17		1	0
PRDX1	clone 0058P0007G11 putative peroxiredoxin 1 variant 3							13	1
RHOA	ras homolog gene family , member A			3		9		8	1
RPL23	clone 0058P0057E07 ribosomal protein L32-like					2		0	1
RPL3	similar to ribosomal protein L3 (LOC771432)		32			18		13	0
RPS23	ribosomal protein S23, 11 days enbryo whole body cDNA, RIKEN full-length enriched library			1		2		2	0
RPS28	similar to yeast ribosomal protein S28		3			25		3	0
RPS3A	ribosomal protein S3A		4	1		25		9	0
SAT1	clone 0064P0021C06 spermadine/spermine N1-acetyltransferase variant 1-like		34			74		3	1
SKP1	S-phase kinase-associated protein 1					6		1	0
SLC25A6	clone 0063P0030D07 putative solute carrier family 25 member 6 variant 1					21		1	0
TPM1	alpha-tropomyosin gene, exons 9c, 9d, and complete cds, alternatively spliced		3			1		2	0
VIM	vimentin		2			2		1	0
								upregulated at 6weeks in WC	
								downregulated at 6weeks in WC	

APPENDIX T: Table 7: Genes Differentially Upregulated Exclusively at 6 Months and at 6 Months in WC Versus SR

Gene Name	Ortholog	C-1 (WC)		C-2 (WC)		C-3 (WC)		6 months In-vivo	
		1 day copy #	*6 weeks copy #*	*1 day copy #*	*6 months copy #*	*6 weeks copy #*	*6 months copy #*	*WC copy #*	*SR copy #*
?	BAC CH261-126J23		49			34	2	0	3
?	BAC CH261-16J12						1	0	1
?	BAC CH261-117C7 (WAG-65N20)				23			37	0
16SrRNA	mitochonrial genome		7	2	6	2		3	0
ACTA2	Actin, alpha 2 smooth muscle, aorta	10		1	7		32	0	3
ACTB	Beta-actin		2		1	3		0	4
CDKN1B	Cyclin-dependent kinase inhibitor p27 Kip1		3			2		0	1
COTL1	Coactosin-like 1					16		2	1
COI	Cytochrome c oxidase subunit I	4	1	3	1			2	0
COII	Cytochrome c oxidase subunit II		3	1	3	4	1	19	3
COIII	Cytochrome c (mitochondrial genome) III		1	3	2			1	0
DDX5	DEAD box polypeptide 5, RNA helicase		4			1		0	1
EEF1A1	Eukaryotic elongation factor 1-alpha 1		1					0	1
EIF1	Eukaryotic translation initiation factor 1			1		3		0	1
EIF4A2	Eukaryotic translation initiation factor 4A isoform 2		1		1		35	0	3
ENO1	Enolase 1 alpha				5		6	0	1
GSN	Gelsolin		43			20		0	6
HSP90AA1	Heat Shock Protein 90 alpha		67		213		175	5	9
LUM	Lumican	159	1	10	7		104	0	6
MYH11	Myosin, heavy chain II smooth muscle		9		27		32	0	1
MYLK	Myosin light chain kinase	13		5	7		21	0	2
ND1	NADH dehydrogenase subunit 1						2	5	3
ND4	NADH dehydrogenase subunit 4	1	3	2	2		1	5	0
NDUFA10	Ubiquinone		2					1	5
PHB2	Prohibitin 2					17		0	6
PRDX1	Peroxiredoxin 1, variant 3					5		0	2
RPL3	Ribosomal protein L3		32			18		0	4
RPL4	Ribosomal protein L4		9			2		0	2
RPL9	Ribosomal protein L9					9		0	1
RPS3A	Ribosomal protein S3A			1		25		0	6
SAT1	Spermidine/spermine N1-acetyl transferase		34			74		0	8
SERPINF2	Serpin peptidase inhibitor clade F alpha-2 antiplasmin (clone ChEST14214)					15		0	4
SLC25A6	Solute carrier protein family 25 member 6					21		2	4
TPM1	Tropomyosin		3			1		1	2
							upregulated at 6months in WC		
							downregulated at 6months in WC		