THE GROWTH AND DEVELOPMENT OF MUSCLE AND FAT CELLS

By

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AND FAT CELLS

Abstract

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Twelve compounds commonly touted as having ergogenic properties were tested on ovine satellite cells for their ability to elicit satellite cell activity (proliferation and/or differentiation) *in vitro*. The compounds were tested at levels ranging from pharmocologic to physiologic. None of the compounds tested were able to induce satellite cell proliferation ($P<0.05$) or differentiation ($P<0.05$) under the cell culture system.

Purified cultures of adipofibroblasts, derived from the dedifferentiation of bovine adipocytes, were used to evaluate their "plasticity" and redifferentiative ability *in vitro*. Adipofibroblasts proliferated and overgrew the culture dishes when exposed to fetal bovine serum-containing medium, but demonstrated protracted adipogenesis when exposed to horse serum-containing medium. Protracted adipogenesis was evident through the adipofibroblast accumulation of cytoplasmic vesicles that first appeared 5 d after a uniform monolayer was established and remained for up to 60 d *in vitro*. These vesicles demonstrated little/no lipid incorporation despite positive immunocytochemistry staining, immunoblot, and isolation of mRNA for peroxisome proliferator activated receptor γ. The cytoplasmic vesicles did not possess glycogen, and lipid uptake was not enhanced by the addition of insulin, lipid, induction

medium, or thiazolidinedione to the serum-containing medium. Examination of the ultrastructure of cytoplasmic vesicles with transmission electron microscopy revealed a discrete structure with a monophosphate layer, and immunofluorescence demonstrated the lipid-droplet protein perilipin surrounding the vesicle. Collectively, these data suggest that mature adipocytes dedifferentiate to form proliferative-competent progeny cells, that dedifferentiated adipofibroblasts retain their adipocyte lineage characteristics and possess the ability to undergo *in vitro* adipogenesis without complicated induction procedures or through the use of exogenous chemicals, and that the adipofibroblasts do not experience *in vitro* transformation. The protracted nature of cellular conversion from adipofibroblasts to adipocytes may now be exploited in studies to determine the cellular/molecular regulation of adipogenesis or progressive regulation of lipid metabolism.

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Dedication

To e.b.e. …for getting me up at 4:30 every

morning…whether I wanted to or not.

CHAPTER ONE

INTRODUCTION

The objective of this research project is two-fold: to determine the influence of nutritional supplement components on regulating specific activities of the postnatal skeletal muscle stem cell (satellite cell) and to define the cellular and molecular regulation of fat cell (adipocyte) dedifferentiation and "plasticity" of progeny cells. All of the experiments, data, discussions and/or other material contained within this dissertation have either already been published or have been submitted for publication.

Satellite cells (SC) are cells residing adjacent to postnatal skeletal muscle fibers (myofibers) and are unique in that they possess the ability to proliferate and differentiate, and progeny cells may fuse with the myofibers [4, 18, 19, 26, 104, 116, 143]. By donating their cellular constituents, SC are involved in the regulation of myofiber protein synthesis, since protein accretion relies on DNA from additional SC nuclei [113, 114]. In instances of myofiber damage, SC also facilitate muscle regeneration [136]. Thus, determining the factors that independently regulate SC is important to deciphering the overall mechanism(s) involved in normal and exercise-induced muscle growth and regeneration. A possibility that circulating nutrient compounds (nutraceuticals) alter SC to proliferate or differentiate exists, but the potential (direct) regulatory effects of nutrients on SC activity is novel. These studies were undertaken to gain knowledge about the mechanisms through which individual ergogenic dietary supplements, commonly used by athletes for obtaining increases in skeletal muscle hypertrophy and strength, regulate SC activity.

The prevalence of obesity, or the excess storage of triglycerides in adipocytes, is increasing in the Unites States (US) [51, 52, 109, 117, 121]. It is estimated that obesity rates in

adults will reach 30 % by 2015 and exceed 40 % by 2025 [64]. The cost of obesity makes this disease the third most costly in the US health system [64, 142, 147]. Obesity has been attributed to nearly 10% of the total health care expenditures in the US [35] and is associated with numerous weight-related problems, including untreated type II diabetes and heart disease [50, 117, 124, 147]. Excess adipose tissue also is associated with social problems such as lost productivity (workdays) and disability [148] and also may be related to early death of humans [9, 35, 50, 53, 54, 155]. A key to combating obesity is gaining insight into the regulation of the adipocyte, which may provide for alternative treatments of obesity.

Numerous variables of adipose tissue development are of concern in the production of economically important (quality) traits of food-producing animals including fat depth and distribution, marbling, and the overall efficiency of nutrient use [65]. Decreasing subcutaneous fat (second largest fat depot in ruminants) of only a few percentage points in beef cattle would save up to an estimated $4.4 billion dollars for U.S. beef producers – $2 billion in production costs and $2.4 in shipping and removal costs. Additionally, marbling extent influences consumer preferences for beef [38, 81, 82, 119, 126, 127]. Understanding the mechanisms underlying adipogenesis could help producers modulate the characteristics of adipose tissues and promote the occurrence of marbling deposition without the excess accumulation of subcutaneous or visceral fat [65]. The carcass composition of beef animals is a direct function of the 'cellularity' of cells committed to form adipocytes [31]. Beef steers given a high-concentrate diet commonly possess fewer adipocytes in any given fat depot, but most of the cells are filled with lipid to capacity, whereas steers on a high-roughage diet seem to possess more fat cells, but fewer of them are filled to capacity with storage lipid [105, 135]. The

specific mechanisms involved in directing cellularity of any specific fat depot, however, are poorly understood.

Muscle Cells

The muscle cell research of this dissertation is centered on the use of a model to assess the ability of individual nutrient compounds to regulate aspects of myogenic satellite cell proliferation and differentiation *in vitro*. In order to appreciate the complexity of this system, a review of specific areas of satellite cell research is provided, and until recently, most muscle research was focused on events of muscle regeneration. The earliest reports of skeletal muscle regeneration appeared in the second half of the eighteenth century [48]. Although it was still generally believed that adult skeletal muscle cells (myofibers) possess little regenerative ability [60], observations began to be published regarding skeletal muscle repair/regeneration after injury [48, 59, 70, 73, 90, 108, 133, 144] from a variety of damage including transection [59, 73, 90, 92], disease [55], heat [48], freezing [48], and injection with a toxic substance [48, 78]. The earliest investigations into muscle regeneration were performed by simple observation [48], whereas later work was conducted with the use of the light microscope by observing changes over time in muscle following injury using histological preparations of muscle tissue [2, 36, 59, 78, 90, 91, 133, 141]. The light microscope remained as the mainstay research tool for this type of research until the early to mid 1950's, which is the time that more sophisticated electron microscopes were produced and available to researchers at many universities [11, 15, 57, 58, 66, 71, 83, 86, 89, 94, 104, 129, 139, 145].

While research was being conducted regarding muscle repair, a budding field emerged dealing with the physiology and regulation of normal skeletal muscle cell growth. The bulk of myofiber growth occurs postnatally through hypertrophy, or the increase in size, [47, 102,

106, 112], since the complement of myofibers that an animal possesses is essentially fixed at birth [45, 47, 74, 101, 106, 110, 112, 115]. The number of myofiber nuclei (myonuclei) was also believed fixed at birth [93], as there was no available method to determine otherwise at the time. However, through the use of histometrics and enumeration of total DNA content of the muscle, it was subsequently demonstrated that the number of myonuclei do increase during postnatal growth in the rat [46, 47, 102, 113, 114] and in the chicken [112, 115]. These results suggested that there was no increase in myofiber number, but as the muscle weight increased from growth so did the number of myonuclei [47, 112]. In a 1968 paper, Moss suggested that during myofiber growth, the cross-sectional area of the cell and the myonuclei number maintain a constant proportion [112]. The requirement of skeletal muscle cells to maintain a constant myonuclei to cytoplasm ratio for proper function was confirmed by Cardassis and Cooper [28].

Based on this early research, several theories regarding the source of myonuclei needed for myofiber growth and repair were expressed [146]. The myonuclei might come from mitosis of the existing myonuclei without fission of the cytoplasm resulting in a multinucleate cell [90, 102, 146]. A second theory, based on the lack of mitotic figures within the regenerating muscle fiber histological preparations [27, 36, 48, 128], suggested that the myonuclei arise from amitosis or simple cleavage of the myonucleus to form two myonuclei [7, 27, 36, 48, 59, 60, 90, 91, 108, 128, 133, 146] and that any mitotic figures belonged to macrophages or fibroblasts [36, 48, 133, 141]. Volkmann (1893) described plasmodial plaques in regenerating muscle tissue as "produced by fusion of individual cells or failure of cytoplasm to divide after nuclear division" and was considered to be amitosis [48]. Other theories included lymphocytes as a potential source of "non-specialized" nuclei that can be transformed [48], metaplasia of

connective tissue resulting from induction [95], and independent muscle cells that are round and spindle shaped [40, 55, 56]. As it was subsequently demonstrated, none of these ideas were physiologically relevant. Instead, an alternative theory for nuclei accretion suggested that myonuclei were derived from the mitosis of mononucleated myoblast cells [27, 69, 90, 146] and the subsequent fusion of these cells to combine with a multinucleate myotube [27, 69, 90, 146]. If this mechanism were valid, then an independent cell that resides in skeletal muscle (other than myofibers) needed to be identified.

This mysterious "mononucleated" cell, or the "myoblast," is a cell whose first evidence was seen in electron microscopy studies by Mauro in 1961 [104]. Mauro named the previously unknown (but myogenic) cell a SC for its location next to, but not within, the myofiber. The SC, as described by Mauro, is "wedged between the plasma membrane of the muscle fiber and the basement membrane" [104]. Given the proximity to the myofiber, it would be difficult to discern SC from regular myonuclei with regular light microscopy [104]. Mauro noted that the discovery of such a cell could help answer the "vexing problem of muscle regeneration" (and normal skeletal muscle growth), as well as explain observations from those researching in that area [104]. Mauro offered three hypotheses as to the origin of his newfound cell: the SC comes from a multinucleate muscle cell giving rise to single cells by "gathering up" cytoplasm from the sarcoplasm of the muscle cell, the SC is a remnant cell from embryogenesis and thus is a dormant myoblast, and, lastly, the SC is a wandering cell that is waiting to be induced to mobilize [104]. Shortly after Mauro published his findings, a second electron micrographic study of skeletal muscle was published by Allbrook. In this study [2] the ultrastructure of regenerating skeletal muscle in mice and rabbits [2] was documented, and a cell "commonly found in the position of a sarcolemma nucleus" in which the sarcoplasm is being removed, as

well as mononucleate (and binucleate) cells in the "tissue spaces," was identified [2]. While Allbrook does not address the SC by name, these cells most likely are SC, and he concluded that it is these cells in the tissue spaces that undergo mitosis and fuse to form myotubes [2].

In 1970, Moss and LeBlond addressed the question of the source of myonuclei for postnatal skeletal muscle growth and repair [113]. Their objective was to reconcile reports up to that point of 1) an increase in myonuclei during growth [47, 112], 2) the inability of myoblasts to divide once incorporated into myotubes [16, 146], 3) the lack of tritiated thymidine uptake in the myonuclei of regenerating muscle [16], and 4) the presence of mitotic figures within the myofibers of growing rats [102]. Their experiment, involving the injection of tritiated thymidine into rats and examining autoradiographs of muscle sections, demonstrated the inability of myonuclei to incorporate tritiated thymidine (indicating a lack of DNA synthesis), whereas the SC (clearly evident outside the plasmolemma but inside the basement membrane of the myofiber) had an overlay of silver staining indicating premitotic DNA synthesis [113]. This experiment was followed by a second experiment [114] using a lower dose of radioactivity, one not likely to produce radiation damage. The results of the second experiment validated the first: no myonuclei were labeled with ^{3}H-thymidine (only SC) and at 24 hr after the injection of ^{3}H-thymidine, the resulting labeled myonuclei were attributed to fusion of SC into the myofiber [114]. The growth of a muscle fiber occurs through the addition of myonuclei obtained from its SC population [26, 30, 42, 114].

Richard Bischoff published a method by which myogenic SC could be liberated from their entrapment between the myofiber and basement membrane [17], in an attempt to ascertain whether these cells were also present in healthy skeletal muscle. While myogenic cells had been obtained from adult skeletal muscle explants [85, 125], Bischoff noted that the

presence of co-isolated connective tissue cells made quantification of myogenic cell numbers difficult [17]. Bischoff's experiments tested several enzymatic solutions for their efficacy in the removal of SC, but not fibroblasts, from rat skeletal muscle tissue and examined the viability of the isolated cells and their ability to fuse to form myotubes [17]. Several results were generated from this research: 1) high numbers of myogenic cells can be released from adult muscle tissue through the use of enzymes that digest the basement membrane of the myofibers (trypsin and pronase), 2) these cells are viable and can be propagated in culture, 3) the liberated myogenic cells will fuse to form myofibers, and 4) the myogenic cells cultured from the enzymatic digestion of muscle tissue are satellite cells [17]. Satellite cells are the source of nuclei for skeletal muscle growth [113, 114] and for muscle regeneration after injury [17].

In addition to SC, it has been proposed that skeletal muscle tissue contains other populations of myogenic cells [10, 23, 153]. Using a fluorescence-activated cell sorter (FACS), John Blanton, Jr. demonstrated two populations of myoblasts (small and large) in porcine skeletal muscle [23]. The small myoblasts, termed side population (SP) cells, shared the same characteristics as the "self-renewing myoblasts" described by Barrafio [13, 23]. A similar SP cell was found in mouse skeletal muscle [63]. Gussoni et al. found that a majority of the isolated SP cells remained spherical and failed to adhere to the culture plate and those that did attach required over two weeks before differentiating *in vitro* [63]. Additionally, Gussoni determined that skeletal muscle SP cells readily differentiate (*in vitro* and *in vivo*) into a cell with functional and phenotypic properties similar to hematopoietic stem cells from bone marrow [63]. Asakura et al. further demonstrated that muscle SP cells are different from SC and that there appears to be two populations of SP cells: one that forms hematopoietic cells and one that can differentiate into other mesenchymal cells when induced, the regulation of which is unknown [10]. Recent

experiments by Uezumi suggested that another (minor) population of SP cells existed that may actually possess the greatest differentiation potential of the other myogenic cell populations [153]. Collectively, these findings suggest that skeletal muscle SP cells and SC represent distinct cellular populations, that may be co-isolated from adult skeletal muscle [10, 14, 23, 63, 153].

When first isolated, SC from adult healthy skeletal muscle are quiescent and are responsive to a select few extracellular signals (extrinsic factors) [3] that induce activation [19, 20] and subsequently promote proliferation [4]. This protracted lag time between first isolation and re-entry of the cells into the cell cycle [41, 137] is not seen in SC isolated from young rats or from SC isolated from regenerating skeletal muscle [72] but is seen in SC cultures isolated from older animals [41]. Bischoff reported that SC associated with undamaged myofibers remain quiescent [19, 20], but when an extract of crushed skeletal muscle is applied to the fiber, the quiescent SC become activated [21]. The specific "crushed muscle mitogen" was shown to be hepatocyte growth factor (HGF) [6]. This finding was supported by Cornelison and Wold, who determined that the receptor for HGF (*c-met*) was expressed on quiescent SC *in vivo* and in cultures of proliferating and differentiating SC [37].

Satellite cells of the normal adult animal are essentially dormant and require some activation signal (such as a growth factor) to proliferate or differentiate [4-6, 29, 39, 151]. As such, it would not necessarily be expected that a simple nutrient taken orally would stimulate satellite cell activation. However, examples do exist of orally consumed compounds that alter satellite cell proliferative/differentiative activity. For example, β-agonists are orally active and do alter SC (and muscle growth) dynamics, which result in larger skeletal muscles [32, 130]. In an athlete who is experiencing moderate to heavy training, satellite cells are likely activated to some degree, as bouts of resistance exercise may lead to muscle damage, as well as increased

blood flow (hypermeia) to the damaged muscle [43, 75, 76]. The hyperemia to the muscles under these conditions might bring increased levels of systemic growth factors and other blood-borne nutrients [154]. It is plausible that this scenario is at play in athletes who consume oral ergogenic compounds and indicate that they see muscle gains as a result. Through the screening of a variety of putative substances touted for their effects on increasing muscle mass of exercised individuals, the results of this project may demonstrate that SC respond directly to four to five specific dietary supplement compounds. This occurrence may be interpreted to suggest that under specific *in vivo* physiology, altered SC activity might lead to an increase in skeletal muscle hypertrophy. While this area of SC research is in its infancy, the information from this project provides some scientific evidence to an area that is overwhelmed by vanity, confusion, and unbelievable commercial promotions.

Fat Cells

As the prevalence of obesity rises, so does interest in determining the physiology of adipose tissue. A new role for adipose tissue is presently emerging that will provide new ideas about the regulation of carcass composition and the promotion of lean muscle growth and will lead to decreasing energetically inefficient fat accumulation. One method to accomplish this is by altering the regulation of the adipocyte. Cell culture systems offer an efficient mechanism to elucidate variables of adipocyte metabolism (lipogenesis and lipolysis) and adipogenesis (formation of new fat cells from precursor cells). Numerous cell culture systems have used stromal vascular (SV) cells, as well as other lipogenic cell lines, and much of what is understood about adipogenesis is from the study of both of these groups of cells (chapter three). The use of these cells, though, is not without problems (chapter three). To truly understand the role of adipocytes *in vivo*, the use of primary cells would seem to be the most suited, as they are a

homogenous population of adipocytes (chapter three). Relevant to this discussion are the few reports in which mature adipocytes were shown to possess the ability to form proliferative-competent daughter cells *in vitro* (chapters three and four; appendix D).

The research of this dissertation provides evidence that mature adipocytes may form proliferative-competent progeny cell (adipofibroblasts), and that these cells possess the ability to undergo protracted processes of adipogenesis (see chapter five). The data provide interpretation for several possibilities for mature adipocyte dedifferentiation. First, mature fat cells may retain the capability to proliferate. Second, lipid-laden adipocytes may not necessarily be fully differentiated cells and may actually retain proliferative ability. Third, the lipid-containing cells that we have documented as possessing the ability to proliferate may represent a preadipocyte, adipofibroblast, or fibroblast that possesses the ability to assimilate large quantities of lipid while remaining a non-differentiated adipocyte. A fourth possibility is that these cells are more "stem cell-like," and that they not only are able to proliferate but might be more "plastic" (have the capacity to vary in developmental pattern or phenotype, according to environmental conditions) and have the ability to form other cell types (myocytes, chondrocytes, osteocytes) [77]. Lastly, the possibility exists that we have identified a cell type residing in adipose tissue that may be an entirely new cell of the mesodermal lineage. Regardless of the determination of the final cell type, we believe that knowledge of the regulation of the dedifferentiation event, *in vitro*, will add to our understanding of adipose tissue biology. However, methods to conduct single cell research with mature adipocytes were impractical to attempt, during the short time of the conduct of dissertation research. As such, I examined the progeny cells for their ability to undergo adipogenesis *in vitro* as demonstrated by specific markers of adipogenesis.

At each step of the adipogenesis process, cellular and molecular markers have been described and characterized [84]. As one of the earliest markers of adipocyte differentiation [120], lipoprotein lipase (LPL) is an important enzyme in the deposition of triacyglycerides (TAG) into adipocytes [49]. Lipoprotein lipase works with the very low density lipoprotein receptor of adipocytes to recognize and subsequently hydrolyze lipoproteins (such as chylomicrons) thereby releasing fatty acids (FA) and free glycerol for transport into the adipocytes [107, 122]. In adipocytes, lipoprotein lipase is encoded by a single gene containing a peroxisome proliferator response element (PPRE) site and is subject to regulation by peroxisome proliferator activated receptor γ (PPARγ) [134]. The upregulation of the LPL gene, and its subsequent protein expression, demonstrate the ability of the adipocytes to package TAG into lipid storage droplets. The mature adipocyte expresses several markers, but the definitive marker for differentiation is PPARγ [131] . Although PPARγ is not expressed early in the differentiation process [84], it is a powerful regulator of the mature phenotype and induces the expression of many mature adipocyte genes (LPL, hormone sensitive lipase, Ap2, perilipin, insulin receptor, insulin receptor substrate, leptin) [84]. Peroxisome proliferator activated receptors are a class of ligand-dependant nuclear receptor transcription factors [80]. Three homologous PPARs have been described thus far: PPARα, PPARβ/δ, and PPARγ [12]. PPARα is found in a variety of tissues including liver, heart, and skeletal muscle and functions in fatty acid oxidation [80], whereas PPARβ/δ is found in numerous tissues and has a function similar to that of PPARγ [80]. Two isoforms of PPARγ (PPARγ1 and PPARγ2), arising from alternate gene splicing [12, 132, 149], have been described in most mammals, and, in humans, a separate PPARγ (PPARγ3) originates from a separate promoter [12]. Both PPARγ1 and

PPARγ2 are expressed in adipocytes [132, 149] but PPARγ2 is the form that functions in adipocyte differentiation through gene regulation [132, 149]. The third isoform, PPARγ3, has been localized in macrophages and the large intestine only [12]. The ability of PPARγ2 to regulate gene expression occurs through the binding of specific ligands, the forming of heterodimers with retinoid X receptors (RXR) and then by the binding of specific PPREs in enhancer regions of target genes [12, 123, 152]. These genes, many of which are associated with lipid metabolism, include LPL [12, 134] and perilipin [8, 118, 140]. Therefore, PPARγ2 may be considered to be a prime marker of the differentiation of adipocytes [131]. Perilipin was first localized to the periphery of lipid droplets using immunofluorescence [61] in differentiated 3T3 L1 cells. This finding was later confirmed through the use of electron microscopy [22]. To date, perilipin has been isolated from adipocytes in several mammalian species including the mouse [62], rat [61, 97], human [62, 79], and hamster [1], as well as from steroidogenic cells [96, 97, 138] and sebocytes [1]. Through the isolation of cDNA sequences, four isoforms of perilipin (perilipin A, B, C, D) have been described [62, 97, 100]. Perilipin A (58 kDa) and perilipin B (46 kDa) are found in both adipocyte and steroidogenic cells [62], whereas perilipin C and D are found only in steroidogenic cells [100]. A single gene gives rise to all of the isoforms of perilipin, and the structural differences are due to alternate splicing of mRNA [62, 100]. The perilipin gene contains a PPRE site, and, therefore, its expression is directly regulated by PPARγ2 [8, 118, 140]. In the differentiated adipocyte, perilipin replaces adipocyte differentiation-related protein (a protein found on coalescing lipid droplets; ADRP) early in the formation of the lipid droplet to suppress lipolysis of neutral lipids [99]. Perilipin accomplishes this through sterically blocking access to the lipid droplet of hormone sensitive lipase (HSL)

[24, 25, 97, 98, 150]. Lipolysis, when required, is achieved by the phosphorylation of perilipin through the activation of protein kinase A (PKA) in response to elevation of the levels of cAMP [24, 25, 33, 97, 98, 103]. In this way, the conformational change in perilipin allows access to the lipid droplet by phosphorylated HSL [97, 98, 111]. Hormone sensitive lipase is a cytosolic enzyme thought to be key in the mobilization of stored TAG; indeed, it has a wide range of substrates (all acetylglycerols and numerous esters) [67, 68, 97]. In adipocytes, this 84-86 kDa enzyme functions to hydrolyze intracellular TAG and release FA [87, 97, 156]. To date, three isoforms of HSL have been reported arising from a single gene [67, 68]. The HSL gene is regulated by two TATA-less and CAAT-less promoter regions [67], but the factors regulating these promoters remain largely unknown. Lipolysis by HSL is upregulated by catecholamines and strongly downregulated by insulin [67, 68]. In response to the binding of an agonist to a β-adrenergic receptor coupled to G_s-protein, PKA is activated through an increase in cAMP [67, 88, 97, 98]. Protein kinase A phosphorylates HSL at three locations, thereby allowing the translocation of HSL to the lipid droplet [33, 34, 44, 97, 98]. In addition, PKA phosphorylates perilipin [24, 25, 150] to allow for the access of the lipid droplet by activated HSL. For the studies of this project, I used four well-characterized markers of adipogenesis (PPARγ, perilipin, HSL, LPL) in an attempt to demonstrate the differentiation status of the adibofibroblasts, as well as to show that these cells possess the machinery to process lipid in a protracted manner into and out of lipid droplets.

DISSERTATION OUTLINE

The research of this project is divided into two separate experimental lines, examining the hypotheses that postnatal skeletal muscle stem cells (SC) may be regulated by individual

nutrient components, and that mature adipocytes dedifferentiate and form proliferative-competent progeny cells possessing adipogenic capabilities. This body of work is comprised of seven self-contained chapters. Chapter One is a brief review of the literature covering major observations in both fields, and an overview of the presentation and importance of this research is outlined. Chapters Two and Three cover of the influence of nutritional supplements on the proliferation and differentiation of SC *in vitro*. Chapter Four is a literature review for the fat cell portion of this dissertation. In Chapter Five there is a description of the techniques developed to isolate and purify the adipocyte cells used for the remainder of the research, as well as descriptive documentation of the ability of mature adipocytes to dedifferentiate into proliferative-competent progeny cells *in vitro*. Chapter Six examines of the regulation and mechanism of lipid storage vesicle formation in adipofibroblasts (pure populations of progeny cells of dedifferentiated adipocytes). Chapter Seven is a discussion about difficulties in the culture system and the possibilities for the origins of the adipofibroblast. One appendix is included. Appendix A consists of a discussion of dedifferentiation of mature adipocytes and its implications. Chapters Two and Three are written in *Research in Sports Medicine* format, Chapter Four in the format of the journal *Adipocytes*, Chapter Five in *Methods in Cell Science* format, and Chapter Six in *Differentiation* format. Chapter Seven is formatted for the journal *Cells, Tissues, Organs*. Appendix A is formatted for *Tissue & Cell*. At the time of this dissertation submission, Chapters Two and Three has been published in *Research in Sports Medicine* (2004; 12(2):71-93; 12(3):161-190), Chapter Four has been published in *Adipocytes* (2005; 1:17-24), Chapter Five has been published in *Cytotechnology* (2004; 46:163-172), Chapter Six has been submitted to *Differentiation*, and Chapter Seven has been submitted to *Cells, Tissues, Organs*. Appendix B has

been published in *Tissue & Cell* (2005; 37:335-338). All the manuscripts are copyrighted by their respective publishers and are reprinted with permission.

NOVEL RESEARCH FINDINGS

Data from this work have provided new knowledge in the area of muscle growth and development. Specifically, this research has:

1) Tested 12 different nutritional components.
2) Demonstrated that a nutrient component can directly act on satellite cell activiy *in vitro*.
3) Shown that a viable, reliable, and repeatable cell culture system can be used to assay nutrient components *in vitro*.

The work in the area of adipocyte growth and development has:

1) Developed a viable and repeateble system to isolate, culture, and purify mature adipocytes for primary culture.
2) Demonstrated the ability of mature, lipid-laden adipocytes to divide *in vitro* (dedifferentiation).
3) Demonstrated the ability of the dedifferentiated adipocytes to form proliferative-competent cells (adipofibroblasts).
4) Demonstrated that the adipofibroblasts form cytoplasmic vesicles when exposed to HS.
5) Demonstrated that these cells have a protracted adipogenic program through biochemical and molecular biological techniques.

While this research has opened up new avenues for future research in diverse systems (muscle and fat), the basic outcome of this research demonstrates the integrated contribution of both

cell types, which should not be forgotten when studying cellular or developmental aspects of muscle growth and development.

References

1. Akimoto, N., T. Sato, C. Iwata, M. Koshizuka, F. Shibata, A. Nagai, M. Sumida, and A. Ito. Expression of perilipin A on the surface of lipid droplets increases along with the differentiation of hamster sebocytes in vivo and in vitro. *J Invest Dermatol*, 2005. 124: 1127-33.

2. Allbrook, D. An electron microscopic study of regenerating skeletal muscle. *J Anat*, 1962. 96: 137-52.

3. Allen, R.E., M.V. Dodson, L.K. Boxhorn, S.L. Davis, and K.L. Hossner. Satellite cell proliferation in response to pituitary hormones. *J Anim Sci*, 1986. 62: 1596-601.

4. Allen, R.E., M.V. Dodson, and L.S. Luiten. Regulation of skeletal muscle satellite cell proliferation by bovine pituitary fibroblast growth factor. *Exp Cell Res*, 1984. 152: 154-60.

5. Allen, R.E. and L.L. Rankin. Regulation of satellite cells during skeletal muscle growth and development. *Proc Soc Exp Biol Med*, 1990. 194: 81-6.

6. Allen, R.E., S.M. Sheehan, R.G. Taylor, T.L. Kendall, and G.M. Rice. Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. *J Cell Physiol*, 1995. 165: 307-12.

7. Altschul, R. On nuclear division in damaged skeletal muscle. *Rev Canad de Biol*, 1947. 6: 485-95.

8. Arimura, N., T. Horiba, M. Imagawa, M. Shimizu, and R. Sato. The peroxisome proliferator-activated receptor gamma regulates expression of the perilipin gene in adipocytes. *J Biol Chem*, 2004. 279: 10070-6.

9. Aronne, L.J. Epidemiology, morbidity, and treatment of overweight and obesity. *J Clin Psychiatry*, 2001. 62 Suppl 23: 13-22.

10. Asakura, A., P. Seale, A. Girgis-Gabardo, and M.A. Rudnicki. Myogenic specification of side population cells in skeletal muscle. *J Cell Biol*, 2002. 159: 123-34.

11. Ashley, C.A., K.R. Porter, D.E. Philpott, and G.M. Hass. Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate. *J Exp Med*, 1951. 94: 9-20.

12. Auwerx, J. PPARgamma, the ultimate thrifty gene. *Diabetologia*, 1999. 42: 1033-49.

13. Baroffio, A., J.P. Aubry, A. Kaelin, R.M. Krause, M. Hamann, and C.R. Bader. Purification of human muscle satellite cells by flow cytometry. *Muscle Nerve*, 1993. 16: 498-505.

14. Baroffio, A., M.L. Bochaton-Piallat, G. Gabbiani, and C.R. Bader. Heterogeneity in the progeny of single human muscle satellite cells. *Differentiation*, 1995. 59: 259-68.

15. Bennett, H.S. and K.R. Porter. An electron microscope study of sectioned breast muscle of the domestic fowl. *Am J Anat*, 1953. 93: 61-105.

16. Bintcliff, S. and B.E. Walker. Radioautographic study of skeletal muscle regeneration. *Am J Anat*, 1960. 106: 233-46.

17. Bischoff, R. Enzymatic liberation of myogenic cells from adult rat muscle. *Anat Rec*, 1974. 180: 645-61.

18. Bischoff, R. Regeneration of single skeletal muscle fibers in vitro. *Anat Rec*, 1975. 182: 215-35.

19. Bischoff, R. Proliferation of muscle satellite cells on intact myofibers in culture. *Dev Biol*, 1986. 115: 129-39.

20. Bischoff, R. A satellite cell mitogen from crushed adult muscle. *Dev Biol*, 1986. 115: 140-7.

21. Bischoff, R. Interaction between satellite cells and skeletal muscle fibers. *Development*, 1990. 109: 943-52.

22. Blanchette-Mackie, E.J., N.K. Dwyer, T. Barber, R.A. Coxey, T. Takeda, C.M. Rondinone, J.L. Theodorakis, A.S. Greenberg, and C. Londos. Perilipin is located on

the surface layer of intracellular lipid droplets in adipocytes. *J Lipid Res*, 1995. 36: 1211-26.

23. Blanton, J.R., Jr., A.L. Grant, D.C. McFarland, J.P. Robinson, and C.A. Bidwell. Isolation of two populations of myoblasts from porcine skeletal muscle. *Muscle Nerve*, 1999. 22: 43-50.

24. Brasaemle, D.L., D.M. Levin, D.C. Adler-Wailes, and C. Londos. The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. *Biochim Biophys Acta*, 2000. 1483: 251-62.

25. Brasaemle, D.L., B. Rubin, I.A. Harten, J. Gruia-Gray, A.R. Kimmel, and C. Londos. Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J Biol Chem*, 2000. 275: 38486-93.

26. Campion, D.R. The muscle satellite cell: a review. *Int Rev Cytol*, 1984. 87: 225-51.

27. Capers, C.R. Multinucleation of skeletal muscle in vitro. *J Biophys Biochem Cytol*, 1960. 7: 559-66.

28. Cardasis, C.A. and G.W. Cooper. An analysis of nuclear numbers in individual muscle fibers during differentiation and growth: a satellite cell-muscle fiber growth unit. *J Exp Zool*, 1975. 191: 347-58.

29. Carlson, B.M. and J.A. Faulkner. The regeneration of skeletal muscle fibers following injury: a review. *Med Sci Sports Exerc*, 1983. 15: 187-98.

30. Charge, S.B. and M.A. Rudnicki. Cellular and molecular regulation of muscle regeneration. *Physiol Rev*, 2004. 84: 209-38.

31. Cianzio, D.S., D.G. Topel, G.B. Whitehurst, D.C. Beitz, and H.L. Self. Adipose tissue growth and cellularity: changes in bovine adipocyte size and number. *J Anim Sci*, 1985. 60: 970-6.

32. Claeys, M.C., D.R. Mulvaney, F.D. McCarthy, M.T. Gore, D.N. Marple, and J.L. Sartin. Skeletal muscle protein synthesis and growth hormone secretion in young lambs treated with clenbuterol. *J Anim Sci*, 1989. 67: 2245-54.

33. Clifford, G.M., C. Londos, F.B. Kraemer, R.G. Vernon, and S.J. Yeaman. Translocation of hormone-sensitive lipase and perilipin upon lipolytic stimulation of rat adipocytes. *J Biol Chem*, 2000. 275: 5011-5.

34. Clifford, G.M., D.K. McCormick, R.G. Vernon, and S.J. Yeaman. Translocation of perilipin and hormone-sensitive lipase in response to lipolytic hormones. *Biochem Soc Trans*, 1997. 25: S672.

35. Colditz, G.A. Economic costs of obesity and inactivity. *Med Sci Sports Exerc*, 1999. 31: S663-S7.

36. Constance, T.J. An experimental study of the reaction of skeletal muscle to injury. *Aust J Exp Biol Med Sci*, 1955. 33: 257-74.

37. Cornelison, D.D. and B.J. Wold. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev Biol*, 1997. 191: 270-83.

38. Crouse, J., H. Cross, and S. Seideman. Effects of a grass or grain diet on the quality of three beef muscles. *Journal of Animal Sciences*, 1984. 58: 619-25.

39. Darr, K.C. and E. Schultz. Exercise-induced satellite cell activation in growing and mature skeletal muscle. *J Appl Physiol*, 1987. 63: 1816-21.

40. Dawson, J.W. Changes in cross-stripped muscle in the healing of incised wounds. *J Path Bact*, 1908-1909. 13: 174-84.

41. Dodson, M.V. and R.E. Allen. Interaction of multiplication stimulating activity/rat insulin-like growth factor II with skeletal muscle satellite cells during aging. *Mech Ageing Dev*, 1987. 39: 121-8.

42. Dodson, M.V., D.C. McFarland, A.L. Grant, M.E. Doumit, and S.G. Velleman. Extrinsic regulation of domestic animal-derived satellite cells. *Domest Anim Endocrinol*, 1996. 13: 107-26.

43. Ebbeling, C.B. and P.M. Clarkson. Exercise-induced muscle damage and adaptation. *Sports Med*, 1989. 7: 207-34.

44. Egan, J.J., A.S. Greenberg, M.K. Chang, S.A. Wek, M.C. Moos, Jr., and C. Londos. Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet. *Proc Natl Acad Sci U S A*, 1992. 89: 8537-41.

45. Eliot, T.S., R.C. Wiggington, and K.C. Corbin. The number and size of muscle fibres in the rat soleus in relation to age, sex and exercise. *Anat Rec*, 1943. 85: 307-8.

46. Enesco, M. and C.P. Lablond. Increase in cell number asa factor in the growth of the organs and tissues of the young male rat. *J Embryol Exp Morphol*, 1962. 10: 530-62.

47. Enesco, M. and D. Puddy. Increase in the Number of Nuclei and Weight in Skeletal Muscle of Rats of Various Ages. *Am J Anat*, 1964. 114: 235-44.

48. Field, E.J., *Muscle regeneration and repair*. Structure and function of muscle, III, ed. G.H. Bourne. 1960, New York: Academic Press. 139-70.

49. Fielding, B.A. and K.N. Frayn. Lipoprotein lipase and the disposition of dietary fatty acids. *Br J Nutr*, 1998. 80: 495-502.

50. Flegal, K.M. Epidemiologic aspects of overweight and obesity in the United States. *Physiol Behav*, 2005. 86: 599-602.

51. Flegal, K.M. Commentary: the epidemic of obesity--what's in a name? *Int J Epidemiol*, 2006. 35: 72-4; discussion 81-2.

52. Flegal, K.M., M.D. Carroll, C.L. Ogden, and C.L. Johnson. Prevalence and trends in obesity among US adults, 1999-2000. *Jama*, 2002. 288: 1723-7.

53. Flegal, K.M., B.I. Graubard, and D.F. Williamson. Methods of calculating deaths attributable to obesity. *Am J Epidemiol*, 2004. 160: 331-8.

54. Flegal, K.M., D.F. Williamson, E.R. Pamuk, and H.M. Rosenberg. Estimating deaths attributable to obesity in the United States. *Am J Public Health*, 2004. 94: 1486-9.

55. Forbus, W.D. Pathologic changes in voluntary muscle I. Degeneration and regeneration of the rectus abdominus in pneumonia. *Arch Path*, 1926. 2: 318-39.

56. Forbus, W.D. Pathological changes in voluntary muscle II. Experimental studies of the degeneration and regeneration of striated muscle with vital stains. *Arch Path*, 1926. 2: 486-99.

57. Franziniarmstrong, C. Sarcolemmal Invaginations and the T-System in Fish Skeletal Muscle. *Nature*, 1964. 202: 355-7.

58. Franzini-Armstrong, C. and K.R. Porter. The Z Disc of Skeletal Muscle Fibrils. *Z Zellforsch Mikrosk Anat*, 1964. 61: 661-72.

59. Gay, A.J., Jr. and T.E. Hunt. Reuniting of skeletal muscle fibers after transection. *Anat Rec*, 1954. 120: 853-71.

60. Godman, G.C. On the regeneration and redifferentiation of mammalian striated muscle. *J Morph*, 1957. 100: 27-81.

61. Greenberg, A.S., J.J. Egan, S.A. Wek, N.B. Garty, E.J. Blanchette-Mackie, and C. Londos. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J Biol Chem*, 1991. 266: 11341-6.

62. Greenberg, A.S., J.J. Egan, S.A. Wek, M.C. Moos, Jr., C. Londos, and A.R. Kimmel. Isolation of cDNAs for perilipins A and B: sequence and expression of lipid droplet-associated proteins of adipocytes. *Proc Natl Acad Sci U S A*, 1993. 90: 12035-9.

63. Gussoni, E., Y. Soneoka, C.D. Strickland, E.A. Buzney, M.K. Khan, A.F. Flint, L.M. Kunkel, and R.C. Mulligan. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature*, 1999. 401: 390-4.

64. Hambrecht, R. and S. Gielen. Essay: Hunter-gatherer to sedentary lifestyle. *Lancet*, 2005. 366 Suppl 1: S60-S1.

65. Hansen, C., A. Fu, C. Li, W.T. Dixon, R. Christopherson, and S.S. Moore. Global gene expression patterns spanning 3T3-L1 preadipocyte differentiation. *Can. J. Ani Sci.*, 2004. 84: 367-76.

66. Hodge, A.J., H.E. Huxley, and D. Spiro. Electron microscope studies on ultrathin sections of muscle. *J Exp Med*, 1954. 99: 201-6.

67. Holm, C. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem Soc Trans*, 2003. 31: 1120-4.

68. Holm, C., T. Osterlund, H. Laurell, and J.A. Contreras. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annu Rev Nutr*, 2000. 20: 365-93.

69. Holtzer, H., J.M. Marshall, Jr., and H. Finck. An analysis of myogenesis by the use of fluorescent antimyosin. *J Biophys Biochem Cytol*, 1957. 3: 705-24.

70. Horn, J.S. and S. Sevitt. Ischaemic necrosis and regeneration of the tibialis anterior muscle after rupture of the popliteal artery. *J Bone Joint Surg*, 1951. 33B: 348-58.

71. Huxley, H.E. Electron microscope studies of the organisation of the filaments in striated muscle. *Biochim Biophys Acta*, 1953. 12: 387-94.

72. Johnson, S.E. and R.E. Allen. Proliferating cell nuclear antigen (PCNA) is expressed in activated rat skeletal muscle satellite cells. *J Cell Physiol*, 1993. 154: 39-43.

73. Jones, D.S. Effects of various incisions on the rectus abdominus muscle. *Anat Rec*, 1949. 103: 473.

74. Joubert, D.M. An analysis of the factors influencing post-natal growth and development of the muscle fibre. *J Agric Sci Camb*, 1956. 47: 59-102.

75. Joyner, M.J. and J.R. Halliwill. Neurogenic vasodilation in human skeletal muscle: possible role in contraction-induced hyperaemia. *Acta Physiol Scand*, 2000. 168: 481-8.

76. Joyner, M.J. and D.N. Proctor. Muscle blood flow during exercise: the limits of reductionism. *Med Sci Sports Exerc*, 1999. 31: 1036-40.

77. Justesen, J., S.B. Pedersen, K. Stenderup, and M. Kassem. Subcutaneous adipocytes can differentiate into bone-forming cells in vitro and in vivo. *Tissue Eng*, 2004. 10: 381-91.

78. Kellner, A. and T. Robertson. Selective necrosis of cardiac and skeletal muscle induced experimentally by means of proteolytic enzyme solutions given intravenously. *J Exp Med*, 1954. 99: 387-404.

79. Kern, P.A., G. Di Gregorio, T. Lu, N. Rassouli, and G. Ranganathan. Perilipin expression in human adipose tissue is elevated with obesity. *J Clin Endocrinol Metab*, 2004. 89: 1352-8.

80. Kiec-Wilk, B., A. Dembinska-Kiec, A. Olszanecka, M. Bodzioch, and K. Kawecka-Jaszcz. The selected pathophysiological aspects of PPARs activation. *J Physiol Pharmacol*, 2005. 56: 149-62.

81. Killinger, K.M., C.R. Calkins, W.J. Umberger, D.M. Feuz, and K.M. Eskridge. Consumer sensory acceptance and value for beef steaks of similar tenderness, but differing in marbling level. *J Anim Sci*, 2004. 82: 3294-301.

82. Killinger, K.M., C.R. Calkins, W.J. Umberger, D.M. Feuz, and K.M. Eskridge. Consumer visual preference and value for beef steaks differing in marbling level and color. *J Anim Sci*, 2004. 82: 3288-93.

83. Knappeis, G.G. and F. Carlsen. Electron microscopical study of skeletal muscle during isotonic (afterload) and isometric contraction. *J Biophys Biochem Cytol*, 1956. 2: 201-11.

84. Kokta, T.A., M.V. Dodson, A. Gertler, and R.A. Hill. Intercellular signaling between adipose tissue and muscle tissue. *Domest Anim Endocrinol*, 2004. 27: 303-31.

85. Konigsberg, I.R. Cellular differentiation in colonies derived from single cells platings of freshly isolated chick embryo muscle cells. *Proc Natl Acad Sci U S A*, 1961. 47: 1868-72.

86. Kono, T., F. Kakuma, M. Homma, and S. Fukuda. The Electron-Microscopic Structure and Chemical Composition of the Isolated Sarcolemma of the Rat Skeletal Muscle Cell. *Biochim Biophys Acta*, 1964. 88: 155-76.

87. Kraemer, F.B. and W.J. Shen. Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis. *J Lipid Res*, 2002. 43: 1585-94.

88. Lafontan, M. and M. Berlan. Fat cell adrenergic receptors and the control of white and brown fat cell function. *J Lipid Res*, 1993. 34: 1057-91.

89. Laguens, R. Satellite Cells of Skeletal Muscle Fibers in Human Progressive Muscular Dystrophy. *Virchows Arch Pathol Anat Physiol Klin Med*, 1963. 336: 564-9.

90. Lash, J.W., H. Holtzer, and H. Swift. Regeneration of mature skeletal muscle. *Anat Rec*, 1957. 128: 679-97.

91. Le Gros Clark, W.E. An experimental study of the regeneration of mammalian striped muscle. *J Anat*, 1946. 80: 24-40.

92. Le Gros Clark, W.E. and L.B. Blomfield. The efficiency of intramusclular anastomoses with observations on the regeneration of devascularized muscle. *J Anat*, 1945. 79: 15-36.

93. Leblond, C.P. and B.E. Walker. Renewal of cell populations. *Physiol Rev*, 1956. 36: 255-76.

94. Lee, J.C. and R. Altschul. Electron Microscopy of the Nuclei of Denervated Skeletal Muscle. *Z Zellforsch Mikrosk Anat*, 1963. 61: 168-82.

95. Levander, G. Tissue induction. *Nature*, 1945. 155: 148-9.

96. Londos, C., D.L. Brasaemle, J. Gruia-Gray, D.A. Servetnick, C.J. Schultz, D.M. Levin, and A.R. Kimmel. Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells. *Biochem Soc Trans*, 1995. 23: 611-5.

97. Londos, C., D.L. Brasaemle, C.J. Schultz, D.C. Adler-Wailes, D.M. Levin, A.R. Kimmel, and C.M. Rondinone. On the control of lipolysis in adipocytes. *Ann N Y Acad Sci*, 1999. 892: 155-68.

98. Londos, C., D.L. Brasaemle, C.J. Schultz, J.P. Segrest, and A.R. Kimmel. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin Cell Dev Biol*, 1999. 10: 51-8.

99. Londos, C., C. Sztalryd, J.T. Tansey, and A.R. Kimmel. Role of PAT proteins in lipid metabolism. *Biochimie*, 2005. 87: 45-9.

100. Lu, X., J. Gruia-Gray, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, C. Londos, and A.R. Kimmel. The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. *Mamm Genome*, 2001. 12: 741-9.

101. MacCallum, J.B. On the histogenesis of the striated muscle fibre and the growth of the human sartorius muscle. *Johns Hopkins Hosp Bull*, 1898. 9: 208-15.

102. Macconnachie, H.F., M. Enesco, and C.P. Leblond. The Mode of Increase in the Number of Skeletal Muscle Nuclei in the Postnatal Rat. *Am J Anat*, 1964. 114: 245-53.

103. Marcinkiewicz, A., D. Gauthier, A. Garcia, and D.L. Brasaemle. The phosphorylation of serine 492 of perilipin A directs lipid droplet fragmentation and dispersion. *J Biol Chem*, 2006.

104. Mauro, A. Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol*, 1961. 9: 493-5.

105. May, S.G., J.W. Savell, D.K. Lunt, J.J. Wilson, J.C. Laurenz, and S.B. Smith. Evidence for preadipocyte proliferation during culture of subcutaneous and intramuscular adipose tissues from Angus and Wagyu crossbred steers. *J Anim Sci*, 1994. 72: 3110-7.

106. McKeekan, C.P. Growth and development in the pig, with special reference to carcass quality characteristics. *J Agric Sci*, 1940. 30: 276-343.

107. Merkel, M., W. Velez-Carrasco, L.C. Hudgins, and J.L. Breslow. Compared with saturated fatty acids, dietary monounsaturated fatty acids and carbohydrates increase atherosclerosis and VLDL cholesterol levels in LDL receptor-deficient, but not apolipoprotein E-deficient, mice. *Proc Natl Acad Sci U S A*, 2001. 98: 13294-9.

108. Millar, W.G. Regeneration of skeletal muscle in young rabbits. *J Path Bact*, 1934. 38: 145-51.

109. Montague, M.C. The physiology of obesity. *Abnf J*, 2003. 14: 56-60.

110. Montgomery, R.D. Growth of human striated muscle. *Nature*, 1962. 195: 194-5.

111. Moore, H.P., R.B. Silver, E.P. Mottillo, D.A. Bernlohr, and J.G. Granneman. Perilipin targets a novel pool of lipid droplets for lipolytic attack by hormone-sensitive lipase. *J Biol Chem*, 2005. 280: 43109-20.

112. Moss, F.P. The relationship between the dimensions of the fibers and the number of nuclei during normal growth of skeletal muscle in the domestic fowl. *Am J Anat*, 1968. 122: 555-64.

113. Moss, F.P. and C.P. Leblond. Nature of dividing nuclei in skeletal muscle of growing rats. *J Cell Biol*, 1970. 44: 459-62.

114. Moss, F.P. and C.P. Leblond. Satellite cells as the source of nuclei in muscles of growing rats. *Anat Rec*, 1971. 170: 421-35.

115. Moss, F.P., R.A. Simmonds, and H.W. McNary. The growth and composition of skeletal muscle in the chicken 2. The relationship between muscle weight and the number of nuclei. *Poult Sci*, 1964. 43: 1086-91.

116. Muir, A.R. Normal and regenerating skeletal muscle fibres in Pietrain pigs. *J Comp Pathol*, 1970. 80: 137-43.

117. Must, A., J. Spadano, E.H. Coakley, A.E. Field, G. Colditz, and W.H. Dietz. The disease burden associated with overweight and obesity. *Jama*, 1999. 282: 1523-9.

118. Nagai, S., C. Shimizu, M. Umetsu, S. Taniguchi, M. Endo, H. Miyoshi, N. Yoshioka, M. Kubo, and T. Koike. Identification of a functional peroxisome proliferator-activated receptor responsive element within the murine perilipin gene. *Endocrinology*, 2004. 145: 2346-56.

119. Neely, T.R., C.L. Lorenzen, R.K. Miller, J.D. Tatum, J.W. Wise, J.F. Taylor, M.J. Buyck, J.O. Reagan, and J.W. Savell. Beef customer satisfaction: role of cut, USDA quality grade, and city on in-home consumer ratings. *J Anim Sci*, 1998. 76: 1027-33.

120. Ntambi, J.M. and K. Young-Cheul. Adipocyte differentiation and gene expression. *J Nutr*, 2000. 130: 3122S-6S.

121. Ogden, C.L., K.M. Flegal, M.D. Carroll, and C.L. Johnson. Prevalence and trends in overweight among US children and adolescents, 1999-2000. *Jama*, 2002. 288: 1728-32.

122. Olivecrona, G. and T. Olivecrona. Clearance of artificial triacylglycerol particles. *Curr Opin Clin Nutr Metab Care*, 1998. 1: 143-51.

123. Osumi, T., J.K. Wen, and T. Hashimoto. Two cis-acting regulatory sequences in the peroxisome proliferator-responsive enhancer region of rat acyl-CoA oxidase gene. *Biochem Biophys Res Commun*, 1991. 175: 866-71.

124. Overweight, obesity, and health risk. National Task Force on the Prevention and Treatment of Obesity. *Arch Intern Med*, 2000: 898-904

125. Peterson, E.R. and S.M. Crain. Regeneration and innervation in cultures of adult mammalian skeletal muscle coupled with fetal rodent spinal cord. *Exp Neurol*, 1972. 36: 136-59.

126. Platter, W.J., J.D. Tatum, K.E. Belk, P.L. Chapman, J.A. Scanga, and G.C. Smith. Relationships of consumer sensory ratings, marbling score, and shear force value to consumer acceptance of beef strip loin steaks. *J Anim Sci*, 2003. 81: 2741-50.

127. Platter, W.J., J.D. Tatum, K.E. Belk, S.R. Koontz, P.L. Chapman, and G.C. Smith. Effects of marbling and shear force on consumers' willingness to pay for beef strip loin steaks. *J Anim Sci*, 2005. 83: 890-9.

128. Pogogeff, I.A. and M.R. Murray. Form and behavior of adult mammalian skeletal muscle *in vitro*. *Anat Rec*, 1946. 95: 321-35.

129. Price, H.M. The Skeletal Muscle Fiber in the Light of Electron Microscope Studies. A Review. *Am J Med*, 1963. 35: 589-605.

130. Ricks, C.A., R.H. Dalrymple, P.K. Baker, and D.L. Ingle. Use of a β-agonist to alter fat and muscle deposition in steers. *Journal of Animal Sciences*, 1984. 59: 1247-55.

131. Rosen, E.D. The transcriptional basis of adipocyte development. *Prostaglandins Leukot Essent Fatty Acids*, 2005. 73: 31-4.

132. Rosen, E.D. and B.M. Spiegelman. Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol*, 2000. 16: 145-71.

133. Saunders, J.H. and H.A. Sissons. The effect of denervation on the regeneration of skeletal muscle after injury. *J Bone Joint Surg Br*, 1953. 35-B: 113-24.

134. Schoonjans, K., J. Peinado-Onsurbe, A.M. Lefebvre, R.A. Heyman, M. Briggs, S. Deeb, B. Staels, and J. Auwerx. PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *Embo J*, 1996. 15: 5336-48.

135. Schoonmaker, J.P., F.L. Fluharty, and S.C. Loerch. Effect of source and amount of energy and rate of growth in the growing phase on adipocyte cellularity and lipogenic enzyme activity in the intramuscular and subcutaneous fat depots of Holstein steers. *J Anim Sci*, 2004. 82: 137-48.

136. Schultz, E., D.L. Jaryszak, and C.R. Valliere. Response of satellite cells to focal skeletal muscle injury. *Muscle Nerve*, 1985. 8: 217-22.

137. Schultz, E. and B.H. Lipton. Skeletal muscle satellite cells: changes in proliferation potential as a function of age. *Mech Ageing Dev*, 1982. 20: 377-83.

138. Servetnick, D.A., D.L. Brasaemle, J. Gruia-Gray, A.R. Kimmel, J. Wolff, and C. Londos. Perilipins are associated with cholesteryl ester droplets in steroidogenic adrenal cortical and Leydig cells. *J Biol Chem*, 1995. 270: 16970-3.

139. Shafiq, S.A., M.A. Gorycki, and A. Mauro. Mitosis during postnatal growth in skeletal and cardiac muscle of the rat. *J Anat*, 1968. 103: 135-41.

140. Shimizu, M., A. Takeshita, T. Tsukamoto, F.J. Gonzalez, and T. Osumi. Tissue-selective, bidirectional regulation of PEX11 alpha and perilipin genes through a common peroxisome proliferator response element. *Mol Cell Biol*, 2004. 24: 1313-23.

141. Sissons, H.A. and G.J. Hadfield. The effect of cortisone on the regeneration of skeletal muscle after injury. *J Bone Joint Surg Br*, 1953. 35-B: 125-30.

142. Smyth, S. and A. Heron. Diabetes and obesity: the twin epidemics. *Nat Med*, 2006. 12: 75-80.

143. Snow, M.H. An autoradiographic study of satellite cell differentiation into regenerating myotubes following transplantation of muscles in young rats. *Cell Tissue Res*, 1978. 186: 535-40.

144. Speidel, C.C. Studies of living muscles I. Growth, injury and repair of striated muscle, as revealed by prolonged observations of individual fibers in living tadpoles. *Am J Anat*, 1938. 62: 179-235.

145. Spicer, S.S. and G. Rozsa. An electron microscope study of contractile muscle proteins. *J Biol Chem*, 1953. 201: 639-44.

146. Stockdale, F.E. and H. Holtzer. DNA synthesis and myogenesis. *Exp Cell Res*, 1961. 24: 508-20.

147. Sturm, R. The effects of obesity, smoking, and drinking on medical problems and costs. *Health Aff (Millwood)*, 2002. 21: 245-53.

148. Sturm, R., J.S. Ringel, and T. Andreyeva. Increasing obesity rates and disability trends. *Health Aff (Millwood)*, 2004. 23: 199-205.

149. Sundvold, H., A. Brzozowska, and S. Lien. Characterisation of bovine peroxisome proliferator-activated receptors gamma 1 and gamma 2: genetic mapping and differential expression of the two isoforms. *Biochem Biophys Res Commun*, 1997. 239: 857-61.

150. Tansey, J.T., C. Sztalryd, E.M. Hlavin, A.R. Kimmel, and C. Londos. The central role of perilipin a in lipid metabolism and adipocyte lipolysis. *IUBMB Life*, 2004. 56: 379-85.

151. Tatsumi, R., J.E. Anderson, C.J. Nevoret, O. Halevy, and R.E. Allen. HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev Biol*, 1998. 194: 114-28.

152. Tugwood, J.D., I. Issemann, R.G. Anderson, K.R. Bundell, W.L. McPheat, and S. Green. The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. *Embo J*, 1992. 11: 433-9.

153. Uezumi, A., K. Ojima, S. Fukada, M. Ikemoto, S. Masuda, Y. Miyagoe-Suzuki, and S. Takeda. Functional heterogeneity of side population cells in skeletal muscle. *Biochem Biophys Res Commun*, 2006. 341: 864-73.

154. Vierck, J., B. O'Reilly, K. Hossner, J. Antonio, K. Byrne, L. Bucci, and M. Dodson. Satellite cell regulation following myotrauma caused by resistance exercise. *Cell Biol Int*, 2000. 24: 263-72.

155. Visscher, T.L. and J.C. Seidell. The public health impact of obesity. *Annu Rev Public Health*, 2001. 22: 355-75.

156. Yeaman, S.J. Hormone-sensitive lipase--new roles for an old enzyme. *Biochem J*, 2004. 379: 11-22.

CHAPTER TWO

Commonly consumed oral herbal supplements do not influence satellite cell activity*

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Abstract

This study evaluated the ability of common herbal components in ergogenic dietary supplements to alter satellite cell activity *in vitro*. Herbal compounds studied were banaba leaf extract (1 μg•ml^{-1}, 10 μg•ml^{-1}, 100 μg•ml^{-1}, 1 mg•ml^{-1}), foenugreek seed extract (1 μg•ml^{-1}, 10 μg•ml^{-1}, 100 μg•ml^{-1}, 1 mg•ml^{-1}), and *Cystoseira canariensis* extract (10 pg•ml^{-1}, 1 ng•ml^{-1}, 100 ng•ml^{-1}, 10 μg•ml^{-1}, 100 μg•ml^{-1}, 1 mg•ml^{-1}, 10 mg•ml^{-1}). Additionally, *Ephedra sinenis* extract and *Citrus aurantium* extract were re-evaluated at lower levels (0.0000001 %, 0.00001 %, 0.001 %). The effects of selected concentrations of these components were studied in combination with 0.1% creatine monohydrate, which we had previously determined possessed some capability to induce satellite cell differentiation. Ovine satellite cells were exposed to the four treatment levels of the herbal compound for a specified amount of time and analyzed by counting mononucleated and multinucleated cells. None of the studied compounds, either alone or in combination with creatine monohydrate, altered satellite cell proliferation or differentiation over that of control cultures ($P>0.05$). These results suggest that the ergogenic compounds examined do not directly influence satellite cell activity *in vitro*.

Key Words: muscle stem cells, dietary supplements, nutriceuticals, herbs

Introduction

Regardless of their type of exercise, most competitive athletes use some variety of oral ergogenic supplements (Slater, Tan and Teh 2003; Sobal and Marquart 1994a; Ziegler, Nelson and Jonnalagadda 2003). Whether it is caffeine, sports drinks, or more complex compounds, oral supplements are here to stay. The popularity of supplements with athletes is high (46 %; Sobal and Marquart 1994b), and the annual sales of "botanical" (herbal) products in the United States was estimated to be $1.6 billion in 1994 (Brevoort 1996) and almost $4 billion in 1998 (Brevoort 1998). Approximately 25% of these sales can be attributed to orally consumed sports supplements if products containing the herb Ma Huang for weight management are considered, as is customary. As scientists, we can either ignore these supplements, since the specific composition of these products is not regulated as drugs are, or we can attempt to provide the scientific basis for the utility of specific components.

Our laboratory is attempting to determine how the specific components of orally consumed supplements work. Do they influence the body to synthesize and secrete hormones and/or growth factors? Alternatively, do some of these agents directly regulate the desired effects for which athletes are striving? For example, can orally consumed ergogenic compounds directly influence muscle growth dynamics in bodybuilders?

Should a specific oral ergogenic compound facilitate muscle growth, one likely mechanism would be regulation of myogenic satellite cell (Mauro 1961) activity. Satellite cells are postnatal muscle stem cells sandwiched between the basal lamina and the sarcolemma of the myofiber (Mauro 1961; Muir, Kanji, and Allbrook 1965). These cells are capable of proliferating, differentiating, and fusing (Allen and Boxhorn 1987; Bischoff 1986; Bischoff 1975; Campion 1984; Mauro 1961; Muir 1970; Snow 1978) in response to signals (Allen and

Boxhorn 1989; Allen and Boxhorn 1987; Allen, Dodson and Luiten 1984; Allen and Rankin 1990; Allen, Sheehan, Taylor et al. 1995; Dodson, McFarland, Grant et al. 1996; Vierck, O'Reilly, Hossner et al. 2000) in order to facilitate muscle repair (Byrne, Vierck and Dodson 2000; Carlson and Faulkner 1983; Stewart, Foss, Carraro et al. 1997) and myofiber hypertrophy (Allen, Merkel and Young 1979; Schultz 1989). The focus of the present project is to determine the effects of popular herbal compounds found in products designed for bodybuilders on the proliferation and differentiation of myogenic stem cells. Our hypothesis tested whether herbal compounds contain components that might directly influence the proliferative and differentiative activity of cultured satellite cells.

Methods

Materials

Purified herbal compounds, in the same form in which they would be placed into processed (commercially available) supplements, were obtained from companies that supply manufacturers of ergogenic dietary supplement products. Banaba leaf extract (*Lagerstroemia speciosa*; PE 1 %; colosolic acid; Pure World Botanicals, Inc.), foenugreek seed extract (*Trigonella foenum-graecum*; PE 2 %; 4-hydroxyisoleucine; Pure World Botanicals, Inc.), phenalgin extract (*Cystoseira canariensis;* sulfopolysaccharide; Pharmline Inc.), *Ephedra sinenis* extract (6 % alkaloids; Ma Huang) and *Citrus aurantium* extract (6 % alkaloids; Bitter Citrus; Zhi Shi) were all obtained by Weider Nutrition International and shipped to Washington State University for analysis. Dulbecco's Modified Eagle Medium (DMEM), horse serum (HS), fetal bovine serum (FBS), penicillin/streptomycin, and gentamicin were purchased from Invitrogen/Life Technologies. Pigskin gelatin (porcine skin, type A), Giemsa stain (Accustain), trypsin, KCl, KH_2PO_4, NaCl, Na_2HPO_4, and Na_2 EDTA•$2H_2O$ (all PBS components) were

purchased from Sigma Chemical Company. Dimethyl sulfoxide (DMSO) and methanol were obtained from Fischer Scientific. The cell culture plates were purchased from Nalge Nunc™.

Cell culture methods

Satellite cells used for the present studies were exactly as described previously (Vierck, Icenoggle, Bucci et al. 2003). In brief, the basal medium was composed of DMEM and antibiotics, supplemented with either horse serum (HS) or fetal bovine serum (FBS). Ovine muscle satellite cells were thawed, placed into a 75 cm^2 flask that had been previously coated with 0.1 % pigskin gelatin, and allowed to proliferate for 72 hr in a humidified environment at 37° C in an atmosphere of 95 % air and 5 % CO_2. Viable, proliferative cells were harvested and plated into 24-well culture plates that had been coated with 0.1 % pigskin gelatin (Vierck, Icenoggle, Bucci et al. 2003). The cells were allowed to attach for 24 hr in DMEM + 10 % FBS. Next, the medium was removed, the cultures were thoroughly washed three times with DMEM (without serum) to remove serum components, and the cells were then exposed to appropriate treatments. The treatment medium was changed every 24 hr until a pre-determined endpoint was reached (Vierck, Icenoggle, Bucci et al. 2003). At this time, the cultures were removed from incubation, fixed with methanol, stained with Giemsa, and then counted to determine the effects of treatment on cell proliferation and/or differentiation. Differentiation (measured as morphological fusion) was determined when three or more nuclei were detected within one continuous cell membrane, forming an elementary myotube (Vierck, Icenoggle, Bucci et al. 2003). Total and fused nuclei densities were ascertained by counting the number of nuclei in ten random fields in each well at 400 X magnification on a Nikon research-grade phase contrast (inverted) microscope (Vierck, Icenoggle, Bucci et al. 2003).

Test compound preparations

Note on intent: All compounds were handled in the same manner as that of a product production line. That is, all compounds were tested at levels that would be found in the blood of an athlete consuming such a product. Compound purity was the same as that which went directly into commercially available products. While one potential problem of the present research was that we did not evaluate the identity of carrier/filler compounds, we tried to mimic what might be found in any orally consumed product available to athletes. Using such a process, we have previously determined that one compound (creatine monohydrate; CM) possessed some ability to alter satellite cell activity *in vitro* (Vierck, Icenoggle, Bucci et al. 2003). As such, in the present study, when we determine if a compound had the potential to alter satellite cells, we further evaluated the compound in additive studies with CM. None of our present studies is intended to provide specific mechanisms of how the compounds to alter satellite cell physiology, but such studies would have been conducted if significant effects of specific compounds were observed.

4-Hydroxyisoleucine and colosolic acid were dissolved in DMEM at 1.0 % w/v (6.75 X 10^{-3} M and 2.11 X 10^{-3} M respectively). The solutions were then filtered twice, to remove particulate matter, first using a Whatman # 2 filter and then using a 0.45 μm syringe filter. The pH of the treatment solutions was adjusted to pH = 7.08, and the media were sterile filtered using a 0.2 μm syringe filter. These stocks were then diluted in basal media using 1:10 dilutions of each treatment resulting in a lowest treatment level of 1 μg/ml (2.11 X 10^{-6} M). Phenalgin was dissolved in DMEM at 10 mg/ml and then centrifuged for 10 min at 2,700 rpm (1,500 x G using a horizontal rotor; Beckman TJ-6 centrifuge). The supernatant was removed and centrifuged again for 15 min at 10,000 rpm (19,000 x G using a RC-5B HB6 rotor; Sorvall

centrifuge). The final supernatant was filtered with a 0.45 μm syringe filter, the pH of the solution was returned to pH=7.08, and then sterile filtered through a 0.20 μm syringe filter. The resulting sterile solution was the highest level of treatment (10 mg/ml). The remaining treatment levels (1 mg/ml, 100 μg/ml, and 10 μg/ml) consisted of serial 1:10 dilutions. The herbs Ma Huang and Zhi Shi were suspended in DMEM at 1.0 % w/v and then centrifuged for 10 min at 2,700 rpm (1,500 x G using a horizontal rotor; Beckman TJ-6 centrifuge). The supernatants were removed and centrifuged for 15 min at 10,000 rpm (19,000 x G using a RC-5B HB6 rotor; Sorvall centrifuge), diluted 1:10 in DMEM (resulting in a 0.1 % solution), and filtered with a 0.45 μm syringe filter. The solutions were adjusted to pH=7.08, and then sterile filtered through a 0.20 μm syringe filter. The treatment levels of Ma Huang and Zhi Shi were three serial 1:100 dilutions of the stock solutions for final treatment levels of 0.001 %, 0.00001 %, and 0.0000001 %. The controls for all the assays consisted of maintenance media without the test compound. Concentrations of all herbal compounds ranged from below physiologically relevant to toxic levels.

Additive studies with creatine monohydrate (CM)

Compounds used as treatments for the additive studies were selected based upon morphological observation (no or minimal cell deatah, no abnormal cell physiology) and cell counts of their effects in screening assays. The selected treatments for the proliferation experiments with 0.1 % CM (6.7 X 10^{-3} M) included 1 μg/ml colosolic acid (2.11 X 10^{-6} M), and 100 μg/ml 4-hydroxyisoleucine (6.75 X 10^{-4} M). Studies involving CM and an added test compound were prepared as indicated above with the addition of 0.1 % CM to media prior to the first filtration. The control for assays involving CM consisted of maintenance media + 0.1

% CM without the test compound. Additionally, cells were exposed to serum-containing medium (alone) as 0 level controls.

Positive Controls

Over the course of the past year, we have been developing assay systems for evaluating the ability of test compounds to influence satellite cell proliferation and differentiation. The proliferation test system was developed by exposing relatively few satellite cells to test compounds, and the endpoint was designed to be any change in cell numbers. In such a system, satellite cell numbers between 0 and slightly more than 500 cells per mm^2 can be enumerated, and such an elementary system has been used for nearly twenty years to evaluate satellite cell proliferation (Allen and Boxhorn 1987; Allen, Dodson and Luiten 1984; Allen, Dodson, Luiten et al. 1985). For the differentiation induction test system, it is a common practice to add more satellite cells to the test system, reduce the serum level, and evaluate satellite cell fusion into myotybes *in vitro* as a measure of differentiation (Allen and Boxhorn 1989; Vierck, Icenoggle, Bucci et al. 2003; Vierck, McNamara, Hossner et al. 1995). Since we saw little ability of the test compounds to alter either proliferation or differentiation of satellite cells, we conducted positive control experiments with fibroblast growth factor (FGF) and insulin-like growth factor-I (IGF-I).

For proliferation assay validation, satellite cells were plated at 10 nuclei/mm^2 and grown for 96 h in varying levels of FGF contained in either a chemically-defined treatment medium (ovine defined medium; ODM; Vierck, McNamara, and Hossner et al. 1995) or DMEM + 2 % HS medium. The controls were ODM or DMEM + 2 % HS with no FGF added. The levels of FGF added to basal media were 0 (control), 1, 10, 25, 50, 100, and 125 $ng \bullet ml^{-1}$. For validation of differentiation assays, cells were plated at 100 nuclei/mm^2 and

exposed for 120 h to varying levels of IGF-1 in either ITTC defined treatment medium (Vierck, Icenoggle, Bucci et al. 2003) or DMEM + 0.5 % HS. The differentiation controls consisted of ITTC or DMEM + 0.5% HS without IGF-1. The levels of IGF-1 added were 0 (control), 1, 10, 25, 50, 100, and 125 $ng \bullet ml^{-1}$. The intention behind using a positive control was to determine if (under conditions of the experiments) known growth factors could elicit an appropriate response. As FGF is a proliferative agent (Dodson and Mathison 1988; Doumit, Cook and Merkel 1993; Greene and Allen 1991; McFarland, Pesall and Gilkerson 1993) and IGF-I both a proliferative and differentiative agent, (Doumit, Cook and Merkel 1993; Duclos, Wilkie and Goddard 1991; Greene and Allen 1991; McFarland, Pesall and Gilkerson 1993; Venkateswaran, Brackett, Vierck et al. 1995), both compounds performed as expected when provided to the system under conditions used to examine supplement components. FGF induced a dose-dependent proliferative response but there was considerable room in the culture well for additional proliferation to occur before counts would not be successful. A similar rationale should be used for differentiation and IGF-I.

Statistical Analyses

All screening experiments were performed and evaluated exactly as previously described (Vierck, Icenoggle, Bucci et al. 2003). In brief, experiments consisted of three wells per treatment point and were performed twice for a total of six wells per treatment. Experiments with CM contained four wells per treatment and were repeated twice for a total of eight wells per treatment. At the termination of the experiments, all cultures were counted, data were collated, and a one-way ANOVA was performed. Separation of treatment means was accomplished through the use of the Tukey-Kramer Multiple Comparisons Test (Motulsky 1999). Data were presented as Mean $\pm$ SE and considered significant when ($P<0.05$).

Results

Proliferation assays

The results from the proliferation assays are summarized in Table 3. Satellite cells exposed to treatments of 1 mg•ml^{-1}, 100 μg•ml^{-1}, and 10 μg•ml^{-1} of colosolic acid, 1 mg•ml^{-1} of 4-hydroxyisoleucine, and 10 mg•ml^{-1} and 1 mg•ml^{-1} of phenalgin showed significant decreases in cell number ($P<0.05$), as compared to controls. Although treatments of 1 μg/ml of colosolic acid, 100 μg•ml^{-1}, 10 μg•ml^{-1}, and 1 μg•ml^{-1} of 4-hydroxyisoleucine, and 100 μg•ml^{-1} and 10 μg•ml^{-1} of phenalgin elicited significant proliferation over the other treatment groups ($P<0.05$), they were not significant when compared to control cultures ($P>0.05$). Treatments of 100 ng•ml^{-1}, 1 ng•ml^{-1}, and 10 pg•ml^{-1} of phenalgin and all treatments of Ma Huang and Zhi Shi resulted in similar cell densities when compared to control cultures ($P>0.05$).

Morphological evaluation of the treatment cultures in which colosolic acid was exposed to satellite cells showed cells with healthy appearances at all levels. Cells exposed to 1 mg•ml^{-1} of 4-hydroxyisoleucine remained "stem cell-like," displaying small rounded nuclei with scant cytoplasm (Figure 2A). Treatments of 100 μg•ml^{-1}, 10 μg•ml^{-1}, and 1 μg•ml^{-1} of 4-hydroxyisoleucine in these assays produced healthy, actively proliferating cells. Cultures under a treatment of 10 mg•ml^{-1} and 1 mg•ml^{-1} phenalgin had sparse populations of cells with dark staining nuclei (quiescent), and small darkly staining granules within the cytoplasm were seen in some cells. While the cell densities in phenalgin treatments of 100 μg•ml^{-1} and 10 μg•ml^{-1} were less than in control cultures, they appeared morphologically healthy. Cell cultures under proliferative treatments of 100 ng•ml^{-1}, 1 ng•ml^{-1}, and 10 pg•ml^{-1} of phenalgin produced

healthy cells. All cells exposed to Ma Huang and Zhi Shi treatments exhibited healthy morphology.

Differentiation assays

The results from differentiation screening assays are summarized in Table 3. At the magnification used for counting cells (400 X), only the 0.00001 % Ma Huang, 0.001 % Zhi Shi, and 100 ng•ml^{-1} phenalgin treatment levels promoted a small increase in measurable fusion ($P<0.05$). None of the remaining treatments tested in the differentiation assays produced detectable satellite cell fusion at any treatment level ($P>0.05$). Morphological evaluation of treatment cultures resulted in the following observations. Cells exposed to 1 mg•ml^{-1} of 4-hydroxyisoleucine remained "stem cell-like" and possessed small rounded nuclei with scant amounts of cytoplasm (Figure 2A), while levels of 100 μg•ml^{-1}, 10 μg•ml^{-1}, and 1 μg•ml^{-1} of 4-hydroxyisoleucine produced cells with healthy morphologies. Using traditional counting methods at 400 X, fusion was not observed in the 4-hydroxyisoleucine treatments. However, when scanning the culture plates under low power (100 X), fusion was seen in all wells at all levels of treatment and seemed to be greater in the 100 μg•ml^{-1} treatment than the other two treatment levels (Figure 2B). Assays with colosolic acid produced healthy cells at all treatment levels. Colosolic acid at 100 μg•ml^{-1}, 10 μg•ml^{-1}, and 1 μg•ml^{-1} elicited levels of cell fusion equal to or greater than control wells (observed under 100 X). Phenalgin at 10 mg•ml^{-1} and 1 mg•ml^{-1} yielded small pyknotic nuclei, darkly staining cytoplasms, and a high degree of stress fibers and pseudopodia. Some vacuolation was noted. Cultures exposed to phenalgin at 100 μg•ml^{-1} displayed sparse, but healthy, populations of cells. Some cells contained dark granules within the cytoplasm. Phenalgin at a concentration of 10 μg•ml^{-1} produced healthy cells,

which, when the wells were scanned, demonstrated fusion at higher levels (Figure 2C) when compared to control cultures (under 100 X), while exposure to 100 ng•ml^{-1}, 1 ng•ml^{-1}, and 10 pg•ml^{-1} of phenalgin produced healthy cells. All cells exposed to Ma Huang and Zhi Shi treatments displayed healthy morphology.

Additive studies with creatine monohydrate (CM)

Assays involving basal media + 0.1 % creatine monohydrate and a test compound are summarized in Table 4. Only colosolic acid displayed any significant changes in proliferation or differentiation over those of control cultures ($P<0.05$). Proliferation with treatment of colosolic acid at 1 μg•ml^{-1} + 0.1 % CM exhibited a significant decrease in cell numbers when compared to control values ($P<0.05$). This may be attributed to low sample numbers and two treatment wells that displayed high variability to other wells. All studies with CM produced morphologically healthy cells, and no differentiation was observed under differentiative treatments.

Discussion

Do commercially available ergogenic compounds influence the activity of postnatal muscle stem cells (satellite cells; Dangott, Schultz and Mozdziak 2000; Vierck, Icenoggle, Bucci et al. 2003; Fernyhough unpublished observations), whose activity is necessary for regulating aspects of postnatal myofiber hypertrophy (Allen, Merkel and Young 1979; Rosenblatt, Yong and Parry 1994)? Think of the potential physiology that is found in muscles of those who are active in resistance training (bodybuilding; Armstrong, Oglivie and Schwane 1983; Campbell 1995; Friden 1984; Friden, Sjostrom and Ekblom 1983). Muscle damage results in the liberation of the satellite cells from their encapsulated position adjacent to skeletal muscle fibers (Armstrong 1990; Carlson and Faulkner 1983). These cells are then capable of

responding to whatever compound is found in the fluid that is bathing the damaged area (Carlson and Faulkner 1983; Ebbeling and Clarkson 1989; Joyner and Halliwill 2000; Joyner and Proctor 1999; Vierck, O'Reilly, Hossner et al. 2000). Blood-borne compounds, including agents that are consumed, are likely to be found in the damaged muscle. Many of these compounds are normal growth regulators, but we suggest that agents other than hormones or growth factors might influence satellite cell activity.

This area of research is in its infancy. However, over the course of the past two years we have evaluated twenty-nine different compounds for their ability to alter satellite cell activity *in vitro* (Vierck, Icenoggle, Bucci et al. 2003; Fernyhough unpublished observations). The compounds were of diverse types, including agents that might directly regulate satellite cells (androstenedione, DHEA, phenalgin), serve as energy-sparing alternatives (biotin, chromium picolinate, conjugated linoleic acids, lipoic acid, Ma Huang, omega-3 fatty acids, ornithine alpha-ketoglutarate, pinitol, sulbutiamine, ubiquinone, Zhi Shi), act as metabolic intermediates (arginine, calcium alpha-ketoglutarate, citrulline, creatine, glutamine, glutathione, beta-hydroxybutyrate, beta-hydroxy-beta-methylbutyrate, pyruvate), or facilitate the activity of other regulatory compounds (cinnamic acid, colosolic acid, evodiamine, ferulic acid, 4-hydroxyisoleucine, magnesium pidolate, phenalgin). Of these test agents, only creatine monohydrate has directly influenced satellite cell activity (Vierck, Icenoggle, Bucci et al. 2003). While we still do not understand the specific mechanism involved in its actions, one possible explanation for these effects is that creatine released from damaged muscle cells adjacent to satellite cells act as a specific signal of sarcolemmal damage, thus initiating or reinforcing proliferation and/or differentiation of satellite cells.

In the present study, we were interested in examining whether commonly available herbal extracts possessed the ability to alter satellite cells. Herbal extracts are readily available and (for years) have been utilized for their medicinal (real or perceived) properties (Craig 1999; Winslow and Kroll 1998). Bodybuilders commonly associate ginseng with increased performance because it elevates the mood and increases concentration as the bodybuilder prepares for rigorous exercise. Problems exist with the use of herbal compounds, however. Plant health, extract potency, and other potential problems limit confidence in the consumption of herbal agents (Barnes 2003a, Barnes 2003b, Harkey, Henderson, Gershwin et al. 2001). Even so, a number of these compounds are found in commercially available products, designed to increase performance of bodybuilders and athletes. In a highly competitive field filled with a large number of similar products and enthusiastic consumers, any perceived advantage of one product over another is desired by both manufacturers and consumers. After protein and creatine products flourished and then became a commodity with budget pricing (and thus, smaller profit margins), many manufacturers turned to other ingredients to position their products as unique. Most products available to bodybuilders combine several different ingredients in an effort to affect multiple hypothetical mechanisms of action for muscle growth. Few ingredients or combinations other than creatine have been tested for effects on muscle growth or on the consumer targets – young male bodybuilders.

The world-wide increase in published research on herbal extracts and their active ingredients (Barnes 2003a, Barnes 2003b, Brevoort 1996, Brevoort 1998, Bucci 2000, Craig 1999, Winslow and Kroll 1998) enabled dietary supplement producers in the United States to utilize dozens of previously obscure nutrients and herbal extracts (see tables 1 and 2). Results from mechanistic, in vitro, animal, or human disease studies have been extrapolated to the

claimed enhancements for muscle strength and hypertrophy (see Table 1 for examples). This investigation has endeavored to provide data useful to help interpret the appropriateness of the extrapolated and hyperbolic claims seen in Table 1.

The specific compounds used in this study were selected because of a suggested ability to positively influence muscle growth, as claimed by manufacturers. For example, studies using foenugreek (4-hydroxyisoleucine) and banaba (colosolic acid) extracts have suggested that these two agents mimic insulin, decreasing blood glucose levels and increasing tissue uptake of glucose (Broca, Gross, Petit et al. 1999; Kakuda, Sakane, Takihara et al. 1996; Vats, Grover and Rathi 2002; Zia, Hasnain and Hasan 2001). In theory, since insulin is known to enhance creatine uptake into muscles, any agent that enhances insulin action might enhance creatine uptake into muscles, a condition known to improve weightlifting performance.

Although our results do not demonstrate a direct action of the tested herbs on satellite cells, there are many instances where nutrients, not necessarily needed for cell health and viability, will positively induce satellite cell activity *in vitro*. For example, satellite cells can be induced to proliferate in serum-free medium with the addition of additional "nutrients" such as insulin, FGF, epidermal growth factor, IGF-1, and dexamethasone (Allen, Dodson and Luiten 1985; Doumit, Cook and Merkel 1993; McFarland, Pesall and Gilkerson 1993). Such components as insulin, triiodothyronine, corticosterone, and IGF-1, when added to a serum-free basal medium, will induce satellite cell differentiation (Allen, Dodson and Luiten 1985; Erickson, Welter, Calkins et al. 1988; Vierck, McNamara, Hossner et al. 1995). Thus, herbal extracts that have an influence on these agents have potential to enhance satellite cell proliferation and/or differentiation, and thus, muscle hypertrophy. As much is still unknown about the metabolism of herbal products, it is not beyond the realm of possibility that an herbal

supplement would contain a compound capable of directly influencing satellite cells. For example, phenalgin has been postulated to interfere with binding of myostatin on muscle cell receptors (see Table 1). This action would reduce signals inhibitory to muscle growth, and lead to increased muscle mass without resistance training, as seen in experimental situations where the myostatin signal is blocked or removed (Sharma 2001). It is, however, more likely that these supplements act indirectly on satellite cells through any number of *in vivo* systems. For example, herbal compounds might alter an effective endocrine axis involving an agent that alters satellite cell activity or promotes the production of as yet unknown paracrine factors in the tissue bed by ancillary cells, or by the muscle fibers themselves.

It is well understood that satellite cells respond to a variety of growth factors and hormones. If a dietary compound were discovered that regulated satellite cells, our entire understanding of muscle cell regulation (of growth and development) would change. For example, it is feasible that there might be times when nutrient status might be involved in regulating cell growth. Certainly, this comes into play in the restriction point of the cell cycle, whereby a cell must have the appropriate building blocks at hand (in G_1) prior to passage of the cell into the S-phase. Certainly, nutrient status, as well as specific nutrient types, are involved in the regulation of the GH/IGF-I axes, which would suggest that an orally consumed compound might influence satellite cell growth and development via an indirect (secondary) mechanism (Hossner, McCusker and Dodson 1997).

A goal of this research is to delineate specific compounds that might be involved in the direct regulation of satellite cell growth and development. While the results of the present study suggest that none of the tested compounds in the present study influenced satellite cell growth and development, we have reported that creatine monohydrate and other compounds

could influence satellite cells (Vierck, Icenoggle, Bucci et al. 2003). As such, future research designs of parallel studies will include testing compounds together or with creatine monohydrate to assess whether any synergistic or additive effects exist.

Another avenue might be testing these ergogenic compounds on adipocyte and/or fibroblast cell cultures. Fibroblasts secrete growth factors (Clemmons, Elgin, Han, et al. 1986), and are the major cells responsible for the production of connective tissue (structural) components. Alternatively, adipocytes do not play a role in strength, but if fat cells can be induced to release their energy stores, it would provide additional energy for muscle growth and alter the makeup of the overall muscle towards one that is leaner (*i.e.* a more desirable composition; "more cut"). We are presently testing these two systems as models for evaluating ergogenic test compounds. Why? Direct cell responsiveness to ergogenic substances could greatly influence other areas of physiology such as fat reduction or sarcopenia in the elderly/infirmed. Research in both of these areas is important in determining whether dietary compounds are capable of positively influencing connective tissue cells residing within muscle tissue. As muscle mass decreases with advancing age, the utilization of agents that can alter satellite cells, fat cells, or fibroblasts, could benefit the treatment of senile muscle atrophy.

In summary, the research presented does not link the five examined herbal extracts with the ability to regulate satellite cell activity directly. However, we do not exclude the possibility that other mechanisms for these herbs or other herbal agents might be capable of exerting an effect. With this in mind, we suggest that the present research effort be expanded to include other herbal supplements to build up a reasonable database of compounds and their effects in order to make determinations on the regulatory effects of herbal extracts on myogenic satellite cells.

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References

Allen RE, Boxhorn LK (1989) Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. Journal of Cell Physiology 138:311-15.

Allen RE, Boxhorn LK (1987) Inhibition of skeletal muscle satellite cell differentiation by transforming growth factor-beta. Journal of Cell Physiology 133:567-72.

Allen RE, Dodson MV, Luiten LS (1984) Regulation of skeletal muscle cell proliferation by bovine pituitary fibroblast growth factor. Experimental Cell Research 152:157-60.

Allen RE, Dodson MV, Luiten LS, Boxhorn LK (1985) A serum-free medium that supports the growth of skeletal muscle satellite cells. In Vitro Cell Developmental Biology 21:636-40.

Allen RE, Merkel RA, Young RB (1979) Cellular aspects of muscle growth: Myogenic cell proliferation. Journal of Animal Science 79:115-27.

Allen RE, Rankin LL (1990) Regulation of satellite cells during skeletal muscle growth and development. Proceedings of the Society for Experimental Biology and Medicine 197:81-86.

Allen RE, Sheehan SM, Taylor RG, Kendall TL, Rice CM (1995) Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. Journal of Cell Physiology 165:307-12.

Armstrong RB (1990) Initial events in exercise induced muscular injury. Medicine in Science and Sports Exercise 22:429-35.

Armstrong RB, Oglivie RW, Schwane, JA (1983) Eccentric exercise-induced injury to rat skeletal muscle. Journal of Applied Physiology 54:80-93.

Barnes J (2003a) Quality, efficacy and safety of complementary medicines: fashions, facts and the future. Part I. Regulation and quality. British Journal of Clinical Pharmacology 55:226-33.

Barnes J (2003b) Quality, efficacy and safety of complementary medicines: fashions, facts and the future. Part II. Efficacy and safety. British Journal of Clinical Pharmacology 55:331-40.

Bell DG, Jacobs I, Ellerington K (2001) Effect of caffeine and ephedrine ingestion on anaerobic exercise performance. Medicine in Science and Sports Exercise 33:1399-1403.

Bischoff R (1986) A satellite cell mitogen from crushed adult muscle. Developmental Biology 115:129-39.

Bischoff R (1975) Regeneration of single skeletal muscle fibers in vitro. The Anatomy Record 82:215-05.

Brevoort P (1996) The US botanical market. Herbalgram 36:49-57.

Brevoort P (1998) The booming US botanical market: A new overview. Herbalgram 44:33-46.

Broca C, Gross R, Petit P, Sauvaire Y, Manteghetti M, Tournier M, Masiello P, Gomis R, Ribes G (1999) 4-Hydroxyisoleucine: experimental evidence of its insulinotropic and antidiabetic properties. American Journal of Phsiology 277:E617-23.

Bucci L (1998) Dietary supplements as ergogenic aids. In: Wolinksy I, ed. Nutrition in Exercise and Sport, 3rd ed. Boca Raton FL: CRC Press 315-368.

Bucci LR (2000) Selected herbals and human exercise performance. American Journal of Clinical Nutrition 72:624-36S.

Byrne KM, Vierck J, Dodson MV (2000) In vitro model of equine muscle regeneration. Equine Vet Journal 32:401-05.

Campbell KP (1995) Three muscular dystrophies: Loss of cytoskeleton extracellular matrix linkage. Cell 80:675-79.

Campion DR (1984) The muscle satellite cell: A review. International Review of Cytology 87:225-51.

Carlson BM, Faulkner JF (1983) The regeneration of skeletal muscle fibers following injury: A review. Medicine and Science in Sports and Exercise 15:187-98.

Carpene C, Galtozky J, Fontana E, Atgie C, Lafontan M, Berlan M (1999) Selective activation of beta3-adrenoreceptors by octopamine: comparative studies in mammalian fat cells. Archives of Pharmacology 359:310-21.

Clemmons DR, Elgin RG, Han VK, Casella SJ, D'Ercole AJ, Van Wyk JJ (1986) Cultured fibroblast monolayers secrete a protein that alters the cellular binding of somatomedin-C/insulinlike growth factor I. Journal of Clinical Investigation 77:1548-56.

Craig WJ (1999) Health-promoting properties of common herbs. American Journal of Clinical Nutrition 70:491S-99S.

Dangott B, Schultz E, Mozdziak PE (2000) Dietary creatine monohydrate supplementation increases satellite cell mitotic activity during compensatory hypertrophy. International Journal of Sports Medicine 21:13-16.

Dodson MV, Mathison BA (1988) Comparison of ovine and rat-derived satellite cells: response to insulin. Tissue and Cell 20:909-18.

Dodson MV, McFarland DC, Grant AL, Doumit ME, Velleman SG (1996). Extrinsic regulation of domestic animal-derived satellite cells. Domestic Animal Endocrinology 13:107-26.

Doumit ME, Cook DR, Merkel RA (1993) Fibroblast growth factor, epidermal growth factor, insulin-like growth factors and platelet-derived growth factor-BB stimulate proliferation of clonally derived porcine myogenic satellite cells. Journal of Cell Physiology 157:326-32.

Duclos MJ, Wilkie RS, Goddard C (1991) Stimulation of DNA synthesis in chicken muscle satellite cells by insulin and insulin-like growth factors: Evidence for exclusive mediation by a type-1 insulin-like growth factor receptor. Endocrinology 128:35-42.

Erickson S, Welter C, Calkins D, Vierck J, Krabbenhoft L, Byrne K, Greene E, Dodson M (1988) Fusion of equine myogenic satellite cell strains in vitro. Eastern Oregon Science Journal 14:10-14.

Ebbeling CB, Clarkson PM (1989) Exercise-induced muscle damage and adaptation. Sports Medicine 7:207-34.

Friden J (1984) Changes in human skeletal muscle induced by long-term eccentric exercise. Cell and Tissue Research 206:b365-72.

Friden J, Sjostrom M, Ekblom B (1983) Myofibrillar damage following intense eccentric exercise in man. International Journal of Sports Medicine 4:170-76.

Greene EA, Allen RA (1991) Growth factor regulation of bovine satellite cell growth in vitro. Journal of Animal Science 69:146-52.

Harkey MR, Henderson GL, Gershwin ME, Stern JS, Hackman RM (2001) Variability in commercial ginseng products: an analysis of 25 preparations. American Journal of Clinical Nutrition 73:1101-06.

Hossner KL, McCusker RH, Dodson MV (1997) Insulin-like growth factors and their binding proteins in domestic animals. Journal of Animal Sciences 64:1-15.

Joyner MJ, Halliwill JR (2000) Neurogenic vasodilation in human skeletal muscle: possible role in contraction-induced hyperaemia. Acta Physiologica Scandinavica 168:481-88.

Joyner MJ, Proctor DN (1999) Muscle blood flow during exercise: the limits of reductionism. Medicine and Science in Sports in Exercise 31:1036-40.

Kakuda T, Sakane I, Takihara T, Ozaki Y, Takeuchi H, Kuroyanagi M (1996) Hypoglycemic effect of extracts from Lagerstroemia speciosa L. leaves in genetically diabetic KK-AY mice. Bioscience Biotechnology and Biochemistry 60:204-08.

Liu F, Kim J, Li Y, Liu X, Li J, Chen X (2001) An extract of Lagerstroemia speciosa L. has insulin-like glucose uptake-stimulatory and adipocyte differentiation-inhibitory activities in 3T3-L1 cells. Journal of Nutrition 131: 2242-47.

Mauro A (1961) Satellite cells of skeletal muscle fibres. The Journal Biophysical and Biochemical Cytology 9:493-96.

McFarland DC, Pesall JE, Gilkerson KK (1993) The influence of growth factors on turkey embryonic myoblasts and satellite cells in vitro. General and Comparative Endocrinology 89:415-24.

McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387:83-90.

Motulsky H (1999) Analyzing Data with Graph Pad Prism® San Diego CA: GraphPad Software Inc. 68-69.

Muir A (1970) The structure and distribution of satellite cells. In: Mauro A, Shafiq S, Milhorat A eds. Regeneration of striated muscle and myogenesis. Amsterdam: Exterpta Medica 91-100.

Muir AR, Kanji AH, and Allbrook D (1965) The structure of the satellite cells in skeletal muscle. Journal of Anatomy 99:435-44.

Rosenblatt JD, Yong D, and Parry DJ (1994) Satellite cell activity is required for hypertrophy of overloaded adult rat muscle. Muscle & Nerve 17:608-13.

Sharma M, Langley B, Bass J, Kambadur R (2001) Myostatin in muscle growth and repair. Exercise and Sports Science Reviews 29:155-58.

Shugarman AE, Askew EW, Stadler DD, Luetkemeier MJ, Bullough RC, Bucci LR (1999) Effect of thermogenic dietary supplements on resting metabolic rate in healthy male and female volunteers. Medicine in Science and Sports Exercise 31:S164.

Shultz E (1989) Satellite cell behaviour during skeletal muscle growth and regeneration. Medicine in Science and Sports Exercise 21:S181-86.

Slater G, Tan B, Teh KC (2003) Dietary supplementation practices of singaporean athletes. International Journal of Sports Nutrition and Exercise Metabolism 13:320-32.

Snow MH (1978). An autoradiographic study of satellite cell differentiation into regenerating myotubes following transplantation of muscles in young rats. Cell and Tissue Research 186:535-40.

Sobal J, Marquart LF (1994a) Vitamin/mineral supplement use among high school athletes.

Adolescence 29: 835-43.

Sobal J, Marquart LF (1994b) Vitamin/mineral supplement use among athletes: A review of the literature. International Journal of Sport Nutrition 4:320-34.

Stewart NT, Foss M, Carraro U, Cantini M, Byrne K, Vierck JL, Chen Y, Greene EA, Dodson MV (1997) Muscle regeneration is modulated by satellite cell-macrophage interactions at the site of muscle injury: prospective clinical applications. Journal of Equine Veterinary Science 17:172-77.

Vats V, Grover JK, Rathi SS (2002) Evaluation of anti-hyperglycemic and hypoglycemic effect of Trigonella foenum-graecum Linn, Ocimum sanctum Linn and Pterocarpus marsupium Linn in normal and alloxanized diabetic rats. Journal of Ethnopharmacology 79:95-100.

Venkateswaran V, Brackett EJ, Vierck J, Cloud JG, Dodson MV (1995) Substratum is an important determinant in growth factor regulation of trout-derived satellite cells. Basic & Applied Myology 5:297-304.

Vierck JL, Icenoggle DL, Bucci LR, Dodson MV (2003) The effects of ergogenic compounds on the proliferation and differentiation of myogenic satellite cells. Medicine in Science and Sports Exercise 35:769-76.

Vierck JL, O'Reilly B, Hossner K, Antonio J, Byrne K, Bucci L, Dodson M (2000) Satellite cell regulation following myotrauma caused by resistance exercise. Cell Biology International 24:363-72.

Vierck JL, McNamara JP, Hossner KL and Dodson, MV (1995) Characteristics of ovine skeletal muscle satellite cell strains in a defined medium formulated to enhance differentiation: Fusion and the IGF-I system. Basic & Applied Myology 5:11-20.

Winslow LC, Kroll DJ (1998) Herbs as medicines. Archives of Internal Medicine 158:2192-99.

Zia T, Hasnain SN, Hasan SK (2001) Evaluation of the oral hypoglycaemic effect of Trigonella foenum-graecum L. (methi) in normal mice. Journal of Ethnopharmacology 75:191-95.

Ziegler PJ, Nelson JA, Jonnalagadda SS (2003) Use of dietary supplements by elite figure skaters. International Journal of Sports Nutrition and Exercise Metabolism 13:266-76.

Table 1: Examples of commercially available sports nutrition products containing the compounds tested in the present study.

Company	Product Name[a]	Ingredients	Amount per serving	Size Serving Size	SRP[b]	Product Claims
Colosolic Acid						
Met-Rx	Glycemet IGF (Part of the Brand New Anabolic Drive Series from Met-Rx!)	Colosolic acid Alpha lipoic acid, Chromium, Total carbohydrates (Glucosol, Fibersol)	0.48 mg	12.7 ounces 12 g/serving	33.99	"It provides more stable bloodstream sugar levels, thereby enhancing thermogenesis and increased muscle sythesis through the regulation of nutrient digestion and absorption."
Pinnacle Bodyonics	Insulene Poppers	Colosolic acid Chromium (as Carnosinate), D-Pinitol, Proprietary GlucoXcel™ Blend containing lipoic acid	0.3 mg	60 tablets 2 tabs/serv	39.99	"New safe, natural Insulene Poppers are scientifically designed to enhance glucose uptake for natural powerful anabolic effects."
Pinnacle Bodyonics	Insulene	Colosolic acid Pinitol, GlucoPlex Proprietary Blend (*Gymnema silvestre*), Chromium, α-Lipoic acid, Phosphatidylserine	0.9 mg	30 capsules 1 cap/serv	39.99	"Insulene is a phyto-insulin-like formula designed to support greater anabolic gains as part of an overall diet, exercise, and nutrition program."
4-Hydroxyisoleucine						
EAS	Insuload™ HP	4-Hydroxyisoleucine	375 mg	60 capsules 3 caps/serv	39.99	Augment insulin secretion to boost nutrient delivery to muscle tissue Maximize glycogen and creatine storage Increase fuel stores for maximal workout intensity
EAS	Phosphagen™ XT	4-Hydroxyisoleucine Creatine, carbohydrates, creatine, taurine, phosphates	600 mg	39 oz 62 g/serv	59.99	"This product is a powerful combination of EAS Phosphagen creatine, a three-stage carbohydrate blend, and the breakthrough insulin potentiator 4-hydroxyisoleucine."
Kaizen®	Creatine Transport™	4-Hydroxyisoleucine (in Proprietary blend) Creatine, L-glutamine, carbohydrate	Not specified	4.4 lbs 100 g/serv	34.95	Insulin mimicker Loads MORE creatine into your system, than creatine monohydrate alone
Pinnacle	MascuLean	4-Hydroxyisoleucine Magnolia bark extract, *Rhodiola rosea* root extract, Thermo Complex (cocoa, yerba mate, green tea extracts), lycopene, Saw palmetto extract, *Myrica ceritera* extract	1 mg	60 capsules 2 caps/serv	39.99	"Helps tighten up and get lean" "Fat burning" "Insulin enhancing" "Glucose stabilizer" "Fast-track fat mobilizer"
VPX Vital Pharmaceuticals	Creaject™	4-Hydroxyisoleucine, Creatine, carbohydrates, Proprietary blend (arginine α-	Proprietary blend	480 ml 20 ml/serv	59.95	"Out Performs Creatine!"

		ketoglutarate, arginine pyroglutamate, ATP)				
Phenalgin						
Biotest Laboratories	Myostat™	CSP-3 *Cystoseira canariensis* sulfo-polysaccharide	500 mg	80 capsules 1 cap/serv	79.95	"THERE ARE NO LIMITS!™ Neutralize Myostatin and… Grow and Grow and Grow and Grow and Grow! Formation of new muscle Permanent muscle gains Automatic decrease in body fat
Champion Nutrition	MyoStim	*Cystoseira canariensis* Fraction C extract (>90% sulfo-polysaccharide)	1200 mg	120 caplets 4 caps/serv	99.99	"We believe myostatin binding is something you should start using as soon as possible in your bodybuilding life and stay on it until you are so big that you can't wipe yourself…then back off just a little…please."
Cytodyne Technologies	Myo-Blast™ CSP3™	MyoZap™ CSP3 (*Cystoseira canariensis*) *Rhodiola rosea* extract, *Rhaponticum carthomoides* extract (20-hydroxyecdysterone), Anabolic amino acid complex (taurine, carnosine, L-leucine)	600 mg	120 caps 2 caps/serv	79.95	"MyoBlast CSP3 represents without question the most stunning breakthrough for growing and building awe-inspiring muscle ever invented. Now, this amazing muscle-maximizing technology gives everyone the power to reach championship size and muscularity."
Pinnacle Bodyonics	Juiced Protein with MyoZap™	MyoZap (*Cystoseira canariensis* derived from marine algae) Protein	200 mg	2 lbs 27 g/serv	49.99	"KFC breeding gigantic chickens three times normal size! McDonald's raising massive cows yielding 6 times more meat!" "You can bust right through your genetic ceiling."

[a] Information obtained from company websites or other internet sales companies from public domain.
[b] SRP = Suggested Retail Price. All products are routinely available in retail and internet sales outlets for 10-70% less than the SRP.
[c] Serv = Serving

Table 2 – Ergogenic compounds tested in the present study and desired effect(s) by athletes.

Supplement	Desired Effects and Results	References
Colosolic acid	Involved in glucose transport/uptake and glycogen synthesis Increase insulin-facilitated creatine uptake into muscle cells without carbohydrates Increase anabolism via increased insulin action Increase muscle mass and strength	Kakuda, Sakane, Takihara et al. 1996; Liu, Kim, Li et al. 2001
4-Hydroxyisoleucine	Involved in glucose transport/uptake and glycogen synthesis Increase insulin-facilitated creatine uptake into muscle cells without carbohydrates Increase anabolism via increased insulin action Increase muscle mass and strength	Broca, Gross, Petit et al. 1999; Vats, Grover and Rathi 2002; Zia, Hasnain and Hasan 2001
Ma Huang	Increase motivation to train Increase thermogenesis to reduce body fat, increasing muscle definition Increase anaerobic exercise performance Increase beta-adrenergic responses	Bell, Jacobs and Ellerington 2001; Bucci 1998; Shugarman, Askew, Stadler et al. 1999
Zhi Shi	Increase motivation to train Increase thermogenesis to reduce body fat, increasing muscle definition Increase beta-adrenergic responses	Carpene, Galtozky, Fontana et al. 1999; Shugarman, Askew, Stadler et al. 1999
Phenalgin	Inhibit mysotatin receptor binding Increase muscle cell size Increase muscle mass and strength	McPherron, Lawler and Lee 1997; Sharma, Langley, Bass et al. 2001

Table 3 – Results from ergogenic compound assays conducted for the present study.

	Proliferation	Differentiation[b]	
Compound	**Nuclei/mm^2**	**Nuclei/mm^2**	**% Fusion**
Colosolic acid			
Level 0	164.4 ± 32.7	121.0 ± 68.2	1.4 ± 0.6
1 μg•ml^{-1}[c, d] (2.11 X 10^{-6} M)	131.6 ± 19.3	89.3 ± 35.6	0.8 ± 0.4
10 μg•ml^{-1} (2.11 X 10^{-5} M)	80.5 ± 23.4[a]	100.1 ± 31.6	0.4 ± 0.3
100 μg•ml^{-1} (2.11 X 10^{-4} M)	38.2 ± 10.5[a]	16.7 ± 11.0[a]	0.5 ± 0.5
1 mg•ml^{-1} (2.11 X 10^{-3} M)	6.0 ± 6.8[a]	0[a]	0
4-Hydroxyisoleucine			
Level 0	164.4 ± 32.7	121.0 ± 68.2	1.4 ± 0.6
1 μg•ml^{-1} (6.75 X 10^{-6} M)	133.8 ± 6.2	82.5 ± 32.4	0.3 ± 0.2
10 μg•ml^{-1} (6.75 X 10^{-5} M)	74.7 ± 13.5[a]	83.4 ± 11.5	0.6 ± 0.6
100 μg•ml^{-1} (6.75 X 10^{-4} M)	136.5 ± 21.6	34.1 ± 26[a]	0.5 ± 0.3
1 mg•ml^{-1} (6.75 X 10^{-3} M)	0[a]	0[a]	0
Phenalgin			
Level 0[f]	57.7 ± 2.4	199.2 ± 20.0	2.1 ± 0.4
10 pg•ml^{-1}	66.3 ± 24.9	168.3 ± 29.7	2.7 ± 0.8
1 ng•ml^{-1}	130.9 ± 30.5	190.3 ± 20.7	2.0 ± 0.6
100 ng•ml^{-1}	91.7 ± 35.9	161.4 ± 16.8	6.0 ± 1.5[e]
Level 0[f]	119.3 ± 23.6	44.2 ± 10.6	1.21 ± 0.8
10 μg•ml^{-1}	56.6 ± 12.6[a]	14.4 ± 13.1	3.5 ± 1.4
100 μg•ml^{-1}	31.7 ± 9.8[a]	0[a]	0
1 mg•ml^{-1}	0[a]	0[a]	0
10 mg•ml^{-1}	0[a]	0[a]	0
Ma Huang			
Level 0	57.7 ± 2.4	199.2 ± 20.0	2.1 ± 0.4
0.0000001 %	70.7 ± 22.3	189.1 ± 24.5	2.3 ± 0.7
0.00001 %	111.0 ± 37.1	211.1 ± 23.2	5.5 ± 0.8[e]
0.001 %	85.0 ± 29.3	178.4 ± 19.3	2.3 ± 0.6
Zhi Shi			
Level 0	57.7 ± 2.4	199.2 ± 20.0	2.1 ± 0.4
0.0000001 %	78.1 ± 17.4	168.3 ± 45.0	3.7 ± 0.7
0.00001 %	94.3 ± 14.0	215.4 ± 24.7	4.2 ± 1.1
0.001 %	94.6 ± 22.1	203.5 ± 15.0	4.8 ± 0.8[e]

[a] Significantly lower than control mean ($P<0.05$).

[b] No morphological differentiation was observed under the present *in vitro* conditions with current counting methods.

[c] Levels have been detailed in methods.

[d] Bolded treatments were selected for additive studies with CM.

[e] Percent fusion significantly different than control cultures.

[f] These levels are identical but represent controls from two separate experiments.

Table 4 – Results from additional screening of specific ergogenic compounds with the addition of 0.1% creatine monohydrate.

Compound	Nuclei/mm^2	
	Proliferation	Differentiation[†]
Colosolic acid		
0.1% CM + 1 μg•ml^{-1} colosolic acid[‡]	141.1 ± 14.9[*]	0
0.1 % CM	156.7 ± 19.1	0
2 % HS	211.5 ± 20.7	0
4-Hydroxyisoleucine		
0.1 % CM + 1 mg•ml^{-1} 4-hydroxyisoleucine[‡]	201.0 ± 22.0	0
0.1 % CM	191.8 ± 24.2	0
2 % HS	218.9 ± 22.0	0

[*] This value is significantly lower than all other values in this assay ($P<0.05$).

[†] No morphological differentiation was observed under the present *in vitro* conditions with current counting methods.

[‡] Levels have been detailed in methods.

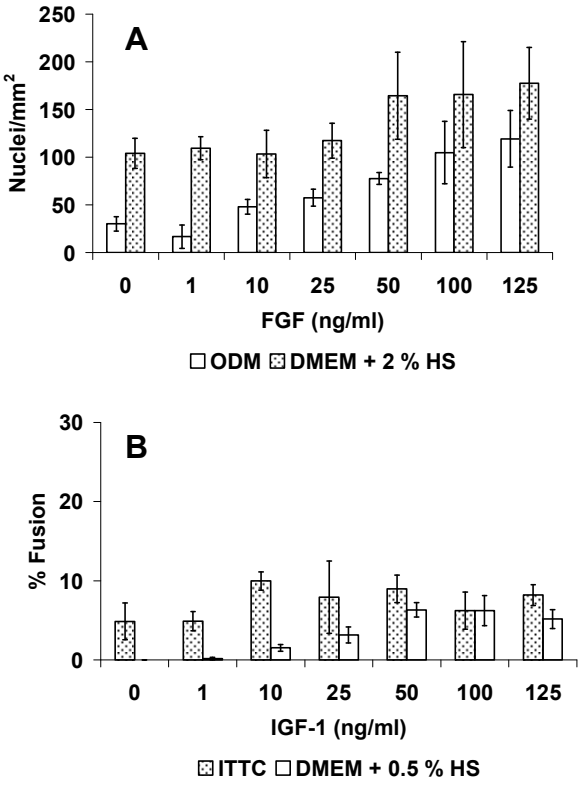

Figure 1 – The effects of known proliferative and differentiative agents on satellite cell proliferation (1A) and % fusion (1B). Cells were plated at 10 nuclei/mm^2 and grown for 96 h in varying levels of FGF and either ODM or DMEM + 2 % HS for proliferation (1A). Differentiation assays consisted of cells plated at 100 nuclei/mm^2 and exposed for 120 h to varying levels of IGF-1 in either ITTC defined media or DMEM + 0.5 % HS. Percent fusion was determined by the total number of nuclei in multinucleated cells (greater than three nuclei per cell) over the total number of nuclei. The levels of treatment (FGF or IGF-1) added to basal media were 0 (control), 1, 10, 25, 50, 100, and 125 ng•ml^{-1}. The controls consisted of basal media with no treatment added.

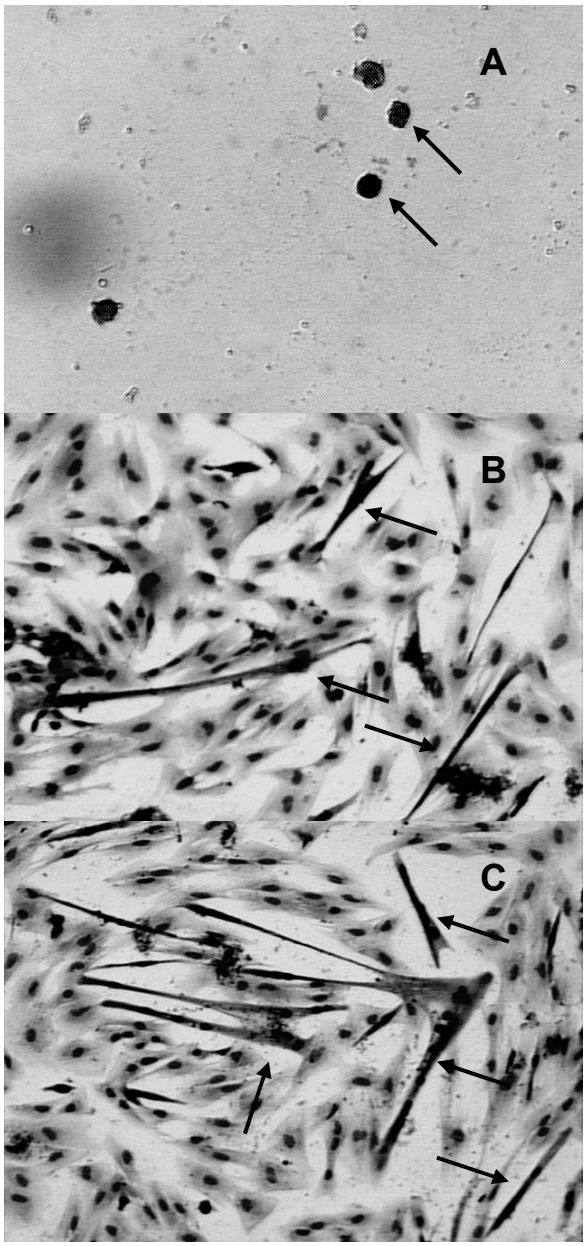

Figure 2 – Morphology of cells exposed to treatment medium. The effect of 1 mg•ml^{-1} of 4-hydroxyisoleucine (Figure 2A) on both proliferative and differentiative cell cultures. Note the rounded nuclei and small amounts of cytoplasm (arrows). Low power (100 X) observation of fusion (arrows) in differentiative cultures exposed to treatments of 100 μg•ml^{-1} of 4-hydroxyisoleucine (Figure 2B) and phenalgin at a concentration of 10 μg•ml^{-1} (Figure 2C).

CHAPTER THREE

Myogenic satellite cell proliferative and differentiative responses to components of common oral ergogenic supplements*

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Abstract

This study evaluated the ability of common ergogenic supplement components to alter satellite cell proliferative activity *in vitro*. Compounds studied were cinnamic acid, ferulic acid, L-glutathione, β-hydroxybutyric acid, calcium-β-hydroxy-β-methylbutyrate monohydrate, DL-thioctic acid (α-lipoic acid), and ornithine α-ketoglutarate – all supposed builders of muscle size. Satellite cells were exposed to different levels of ergogenic test compound for a specified amount of time and analyzed by counting mononucleated and multinucleated cells. At the levels evaluated, none of these compounds altered satellite cell proliferation over that of control cultures ($P>0.05$). Four of the compounds were shown to alter satellite cell differentiation over control cultures ($P<0.05$), but due to the small amounts of fusion, the biological relevance is in question (*e.g.* differences in small numbers). These data suggest that a few of the ergogenic compounds examined by this laboratory do influence satellite cell activity *in vitro*. However, additional studies are vital in order to define the biological relevance of our observations.

Key words: muscle stem cells, dietary compounds, nutriceuticals, cell culture

Introduction

Oral ergogenic supplements are commonly consumed by many individuals, including athletes, in an effort to increase muscle mass and strength (Ahrendt 2001; Applegate 1999; Bazzare 1998; Bucci 1998; Silver 2001). While many orally active supplements rely on the provision of elevated protein levels, a large number of oral supplements contain a wide variety of other compounds purported to assist in the accretion of muscle mass. Examples of commercially available products containing the oral ergogenic supplement compounds examined in this study are summarized in Table 1. Although some of these compounds may have an overall positive physiologic effect in increasing muscle mass, there is little evidence to suggest that these agents act directly on myogenic stem cells that specifically influence postnatal muscle growth dynamics (*i.e.* satellite cells; Figure 1).

Satellite cells are independent cells residing adjacent to postnatal skeletal muscle fibers (myofibers) and are unique in that they possess the ability to proliferate, differentiate, and progeny cells may fuse with the myofibers (Allen, Dodson and Lutein 1987; Bischoff 1986; Bischoff 1975; Campion 1984; Mauro 1961; Muir 1970; Snow 1978). By donating their cellular constituents, satellite cells are involved in the regulation of myofiber protein synthesis, since protein accretion relies on satellite cell-derived DNA accretion (Moss and LeBlond 1971; Moss and LeBlond 1970). In instances of myofiber damage, satellite cells also facilitate muscle regeneration (Shultz 1989). Thus, determining the factors that independently regulate satellite cells is important to deciphering the overall mechanism(s) involved in normal and exercise-induced muscle growth and regeneration.

It is generally accepted that satellite cells are controlled by a variety of hormones, growth factors, and metabolites (Allen and Boxhorn 1989; Allen and Boxhorn 1987; Allen,

Merkel and Young 1979). However, little evidence is available to suggest that satellite cells may be regulated by specific dietary components (Dangott, Schultz, and Mozdziak 2000). We have initiated *in vitro* studies aimed at understanding the direct effects of dietary ergogenic supplement components on altering satellite cell activity (Figure 1). Results from these types of studies would be of some importance to those in the exercise field, as optimizing muscle growth is a goal of a variety of exercise regimens.

In an earlier study, we provided preliminary evidence that an oral dietary supplement (creatine monohydrate; CM) possesses some ability to influence satellite cell differentiative activity *in vitro* (Vierck, Icenoggle, Bucci et al. 2003). The present study extends the concept of the previous report to include additional commercially available ergogenic compounds (see methods) screened in the satellite cell culture system. The ergogenic compounds utilized in this study were selected because of their purported ability to positively impact skeletal muscle tissue growth *in vivo*, and because of their presence in dietary supplements marketed to weightlifters (Table 2). For example, β-hydroxybutyric acid, one of the common ketones, is a metabolic fuel and energy source for muscles especially in stressful situations or in low carbohydrate supply (Birkhahn and Border 1981; Lestan, Walden, Schmaltz et al. 1994; Mitchell, Kassovska-Bratinova, Boukaftane et al. 1995; Ricks, Dalrymple, Baker et al. 1984). Infusion of β-hydroxybutyric acid during ischemic forearm exercise led to less ammonia, lactate and purines in the blood, indicating reduced breakdown of ATP in normal humans (Lestan, Walden, Schmaltz et al. 1995). Ferulic acid and HMB have been linked to increased muscle mass and strength in individuals during resistance exercise (Bucci, Blackman, Defoyd et al. 1990; Gallagher, Carrithers, Godard et al. 2000; Jowko, Ostaszewski, Jank et al. 2001;

Nissen and Sharp 2003; Nissen, Sharp and Ray 1996). Furthermore, HMB is thought to protect muscle against injury after exercise (Gallagher, Carrithers, Godard et al. 2000; Knitter, Panton, Rathmacher et al. 2000; Nissen and Sharp 2003). Alpha-lipoic acid has been shown to enhance glucose transport/uptake in normal animal muscles and cells *in vitro* (Estrada, Ewart, Tsakiridis et al. 1996; Haugaard and Haugaard 1970; Henriksen 2002) and in insulin resistant, but not normal, humans (Henriksen, Jacob, Streeper et al. 1997; Henriksen and Saengsirisuwan 2003; Jacob, Rett, Henriksen et al. 1999). In general, antioxidants are proposed to reduce oxidative damage caused by exhaustive exercise, leading to improved recovery or performance, which would permit greater results from training programs (Bucci 1998; Ji 1999; Ji 1996; Ji 1995; Sastre, Asensi, Gasco et al. 1992; Sen and Packer 2000). The phenolic compounds cinnamic acid and ferulic acid exhibit such antioxidant effects in biological systems (Castelluccio, Bolwell, Gerrish et al. 1996; Imamichi, Nakamura, Hayashi et al. 1990; Perez-Alvarez, Bobadilla and Muriel 2001; Toda, Kumura and Ohnishi 1991; Yagi and Ohishi 1979). L-glutathione is the major intracellular antioxidant and routinely becomes oxidized during intense exercise, such that the ratio of oxidized to reduced glutathione is an accepted marker of oxidative stress and/or free radical burden during exercise (Ji 1999; Ji 1996; Ji 1995; Sastre, Asensi, Gasco et al. 1992; Sen 1999; Sen and Packer 2000). One study in which glutathione supplemented with vitamin C was administered daily for seven days to athletes found that oxidation of glutathione caused by treadmill exercise was prevented (Sastre, Asensi, Gasco et al. 1992). Glutathione administered as acute or chronic intraperitoneal injections to rats increased swim time to exhaustion (Novelli, Falsini and Bracciotti 1991).

Alpha-lipoic acid apparently protects cells and tissues against oxidative stress from exercise by several mechanisms, such as sparing glutathione, generating reducing equivalents

and preserving insulin signaling (Henriksen and Saengsirisuwan 2003; Ji 1999; Ji 1996; Ji 1995; Khanna, Atalay, Laaksonen et al. 1999; Packer, Witt and Tritschler 1995; Sen 1999; Sen and Packer 2000). Lipoic acid supplementation has been shown to reduce markers of oxidative damage (Androne, Gavan, Veresiu et al. 2000; Bailey and Davies 2001; Marangon, Devaraj, Tirosh et al. 1999). There is some evidence that suggests that OKG decreases skeletal muscle protein catabolism while increasing the levels or effects of glutamine, insulin, and growth hormone (Cynober 1999; Cynober 1991; Le Boucher 1998). In another experiment in which OKG was given to weight-trained males, there were significant improvements in muscular size, strength, training volume and acute insulin response compared to a placebo group, suggesting that OKG has beneficial effects for weightlifters (Chetlin, Yeater, Ullrich et al. 1996). Table 1 lists examples of commercially available products that contain these ergogenic compounds. Table 2 lists the specific compounds we tested and summarize the physiological effects that the compounds are intended to produce.

We are in the process of screening a variety of putative ergogenic substances for their effects on altering satellite cell proliferation and/or differentiation. This area of satellite cell research is in its infancy. However, we believe that information of this nature is needed in order to provide some scientific evidence to an area that is overwhelmed by confusing, and commercial, promotions. In the present study, one of our working hypotheses was that ergogenic compounds accelerate satellite cell proliferation and differentiation, which ultimately may increase skeletal muscle hypertrophy.

Methods

Materials

Ergogenic supplement components were obtained from companies that normally

supply these agents to commercial manufacturers of dietary products. Natural cinnamic acid (Weinstein Nutritional Products), ferulic acid (Seltzer Chemicals, Inc.), L-glutathione (Schweizerhall, Inc.), β-hydroxybutyric acid (organoleptic grade; Wiley Organics), calcium-β-hydroxy-β-methylbutyrate (HMB) monohydrate (79.99 %; Wiley Organics), alpha-lipoic acid (DL-thioctic acid; Schweizerhall, Inc.), and Cetornan® (ornithine alpha-ketoglutarate; OKG; Laboratories Jacques Logeais) were all obtained by Weider Nutrition International and shipped to Washington State University for screening. Dulbecco's Modified Eagle Medium (DMEM), horse serum (HS), fetal bovine serum (FBS), penicillin/streptomycin, and gentamicin were purchased from Invitrogen/Life Technologies (Carlsbad, CA). Pigskin gelatin (porcine skin, type A), Giemsa stain (Accustain), trypsin, KCl, KH_2PO_4, NaCl, Na_2HPO_4, and sodium EDTA•$2H_2O$ (all PBS components) were purchased from Sigma Chemical Company (St. Louis, MO). Dimethyl sulfoxide (DMSO) and methanol were obtained from Fischer Scientific Corporation (Fairlawn, NJ). The cell culture plates were purchased from Nalge Nunc™ (Rochester, NY).

Cell culture assay system

Satellite cells used for the present studies were exactly as described previously (Vierck, Icenoggle, Bucci et al 2003). In brief, the basal medium was composed of DMEM and antibiotics, supplemented with either 2 % or 0.5 % HS. Satellite cells were thawed, placed into a 75 cm^2 flask that had been previously coated with 0.1 % pigskin gelatin, and allowed to proliferate for 72 h in a humidified environment at 37° C in an atmosphere of 95 % air and 5 % CO_2. Cells were harvested and plated into 24-well culture plates that had been coated with 0.1 % pigskin gelatin (Vierck, Icenoggle, Bucci et al 2003). The cells were allowed to attach for 24

h in DMEM + 10 % FBS. Next, the medium was removed, the cultures were thoroughly washed three times with DMEM to remove serum components, and the cells were then exposed to appropriate treatments. The treatment medium was changed every 24 h until a pre-determined endpoint was reached (Vierck, Icenoggle, Bucci et al 2003). At this time, the cultures were fixed with methanol, stained with Giemsa stain, and then counted to determine the effects of treatment on cell proliferation and differentiation. Total nuclei densities were ascertained by counting the number of nuclei in ten random fields in each well at 400 X magnification, using a Nikon Diaphot phase-contrast research-grade microscope (Vierck, Icenoggle, Bucci et al 2003). Differentiation (measured as morphological fusion) was determined when three or more nuclei were detected within one continuous cell membrane, forming an elementary myotube (Vierck, Icenoggle, Bucci et al 2003).

Ergogenic test compound preparations

Cinnamic acid, L-glutathione, HMB, β-hydroxybutyric acid and OKG were diluted in basal media to levels of 1.0 %, 0.5 %, 0.25 %, and 0.10 % $w \bullet v^{-1}$ (for molar concentrations refer to Tables 3 and 4). The pH of each treatment medium was normalized (pH = 7.08), and the treatments were filtered with a 0.2 μm syringe filter. Ferulic acid and α-lipoic acid were suspended in DMSO (2.5 g in 5 ml DMSO) and added to DMEM using a 1:100 dilution to create a stock solution of 5 $mg \bullet ml^{-1}$. The first treatment level of 50 $\mu g \bullet ml^{-1}$ (2.42×10^{-4} M) was made by adding stock solution to basal media at a 1:100 dilution. Three subsequent levels of treatments were made by serially diluting each treatment 1:100. Levels of DMSO were equalized by adding an additional 1μl of DMSO per 10 ml medium to all treatment medium and basal medium (for controls). The controls for the remainder of the assays consisted of

maintenance media without the test compound. After the initial screenings, it was determined that concentrations of some of the compounds (originally produced as % $w \bullet v^{-1}$) were toxic to cultures. Alternatively, impurities in the preparations could have caused cell death. Subsequent assays were designed to test these compounds at lower (non-toxic) levels, consistent with a concentration range recommended by a manufacturer for commercial products containing the compound (for molar concentrations see Tables 3 and 4).

Statistics

All screening experiments contained three wells per treatment point and were performed twice (on separate occasions) for a total of six wells per treatment. To determine significant proliferation or differentiation over that of respective control cultures, all wells were counted, data were collated and reduced, and a one-way ANOVA was performed at the termination of the experiments, whereby proliferation or differentiation (effects) were considered dependent variables and treatment concentration and type were independent variables. Separation of treatment means was accomplished using the Tukey-Kramer Multiple Comparisons Test (Motulsky 1999). Data were presented as Mean $\pm$ SE and considered significant when ($P<0.05$).

Results

Proliferation assays

The results from all proliferation assays are summarized in Table 3. The data from assays involving ferulic acid, α-lipoic acid, and OKG suggest that these compounds were not capable of enhancing satellite cell proliferation greater than control values ($P>0.05$). Satellite cells exposed to the 0.10 % OKG treatment possessed proliferative values similar to control cultures, however. Morphological evaluation of the treatment cultures in which ferulic acid, α-

lipoic acid, and OKG were exposed to satellite cells showed cells with healthy appearances at all levels.

Satellite cells exposed to treatment levels of 1.0 %, 0.5 %, and 0.25 % β-hydroxybutyric acid and 1.0 %, 0.5 %, and 0.25 % HMB exhibited a reduction in cell numbers when compared to control cultures ($P<0.05$). The 0.1 % treatment level of β-hydroxybutyric acid and the 0.1 % treatment level of HMB treatment expressed proliferation similar to control cultures. It appeared as though the 1 %, 0.5 %, and 0.25 % treatments of HMB and β-hydroxybutyric acid precipitated in solution, which may have compromised cell growth. Cells in these cultures had small nuclei and scant cytoplasm, and some cell death and abnormally shaped cells were observed. However, HMB and β-hydroxybutyric acid treatments at 0.1 % produced cells with healthy morphologies. As high treatment levels of these latter two compounds likely resulted in the precipitation, we repeated the experiments with lower levels of these compounds.

Satellite cell cultures to which cinnamic acid was added at treatment levels of 1 % and 0.5 % exhibited cellular death, while cells exposed to treatment level 0.25 % showed a decrease ($P<0.05$) in cell numbers but not total cell death. L-glutathione induced significant decreases in cell numbers at all concentrations tested ($P<0.05$), but treatment cultures exposed to 1.0 % L-glutathione produced "stem-like" cells with small rounded nuclei and scant cytoplasm. Alternatively, culture wells with proliferation treatment levels of 0.5 % and 0.25 % L-glutathione displayed sparse populations of cells, many of which had pyknotic nuclei on microscopic examination, scant amounts of cytoplasm, and ragged cell borders. Few healthy appearing cells were noted in the 1.0 %, 0.5 %, and 0.25 % L-glutathione treatment cultures.

Proliferation treatments with 0.1 % L-glutathione produced a sparse population of cells, which, although small, appeared otherwise healthy. Cells exposed to cinnamic acid at 1.0 % and 0.5 % failed to grow, while those cultures exposed to 0.25 % and 0.1 % displayed healthy morphologies.

Differentiation assays

The results from all differentiation-screening assays are summarized in Table 4. Counting ten random fields at the microscope magnification used for counting cells (400 X), which is a traditional magnification for enumerating these cells (Allen and Boxhorn 1989; Allen and Boxhorn 1987; Allen, Dodson, and Luiten 1987), no differentiation was detected at any treatment level ($P>0.05$). Cinnamic acid induced cell death at treatments 1 % and 0.5 % and decreased ($P<0.05$) in cell numbers at treatments of 0.25 % and 0.1 %. Morphological evaluation of treatment cultures resulted in the following observations: As described previously, when exposed to satellite cells at 37° C, HMB and β-hydroxybutyric acid treatments produced a precipitate, which prohibited the evaluation of cell morphology. Assays with ferulic acid, lipoic acid, and OKG produced healthy cells at all treatment levels. One percent L-glutathione in differentiation assays produced "stem-like" cells with small rounded nuclei and scant cytoplasm. Wells exposed to differentiative treatment levels of 0.5 % and 0.25 % L-glutathione had a sparse population of cells, many of which displayed pyknotic nuclei, scant amounts of cytoplasm, and ragged cell borders. Few healthy appearing cells were noted. L-glutathione at 0.1 % produced a sparse population of cells, which although small, appeared otherwise healthy. Cinnamic acid at 1.0 % and 0.5 % induced cell death, while a level of 0.25 % cinnamic acid produced sparse but healthy cultures and 0.1 % produced healthy cells with minimal fusion.

Assays testing ergogenic compounds at low levels

Because some of the compounds (cinnamic acid, L-glutathione, β-hydroxybutyric acid, HMB, OKG) were toxic to satellite cells when initially provided to cultures at relatively high levels, we examined whether lower levels of these compounds effected a change in satellite cell proliferative and differentiative activity. In the proliferation assays, none of the compounds significantly increased cell numbers when compared to control cultures ($P>0.05$), but all of these cultures exhibited a healthy morphology. Results of these assays are summarized in Tables 3 and 4. Differentiation treatments of cinnamic acid at 6.75×10^{-8} M (Figure 2A), L-glutathione at 3.25×10^{-8} M (Figure 2B), HMB at 7.95×10^{-8} M (Figure 2C), OKG at 3.59×10^{-10} M (Figure 2D), and β-hydroxybutyric acid at 3.25×10^{-6} M induced a small, but significant ($P<0.05$), increase in satellite cell differentiation. HMB at 7.95×10^{-10} M also induced an increase in cell numbers over that of control cultures ($P<0.05$). To determine if any additive effects would occur with these compounds, cinnamic acid at 6.75×10^{-8} M, L-glutathione at 3.25×10^{-8} M, HMB at 7.95×10^{-8} M, and OKG at 3.59×10^{-10} M were added to a single treatment medium and exposed to satellite cell cultures. The addition of these compounds was unable to induce significant amounts of differentiation (data not shown).

Discussion

Satellite cells of the normal adult are essentially dormant and require an activation signal to proliferate or differentiate (Allen, Dodson, and Luiten 1987; Allen and Rankin 1990; Allen, Sheehan, Taylor, et al. 1995; Carlson and Faulkner 1983; Darr and Schultz 1987; Tatsumi R, Anderson JE, Nevoret et al. 1998). As such, it would not necessarily be expected that a simple nutrient taken orally would stimulate satellite cell activation. However, examples

do exist of compounds that are orally consumed altering satellite cell proliferative/differentiative activity. For example, beta-agonists are orally active and do alter satellite cell (and muscle growth) dynamics, which result in larger muscles (Claeys, Mulvaney, McCarthy et al. 1989; Ricks, Dalrymple, Baker et al. 1984). In an athlete who is experiencing moderate to heavy training, satellite cells are likely activated to some degree, as bouts of resistance exercise may lead to muscle damage and hyperemia (Ebbeling and Clarkson 1989; Friden 1984; Joyner and Halliwill 2000; Joyner and Proctor 1999). The increased blood flow to the muscles under these conditions would likely bring increased levels of growth factors and blood-borne nutrients (Vierck, O'Reilly, Hossner et al. 2000). Therefore, there is a possibility that (as yet) unknown circulating (nutrient) compounds might alter satellite cells to become more active (*i.e.* while not directly activating satellite cells, these compounds may lead to greater satellite cell proliferation or differentiation). These studies were undertaken to gain knowledge about the mechanisms through which ergogenic dietary supplements, commonly used by athletes for gaining muscle hypertrophy and strength, operate at the cellular level. To date, we have tested over seventeen different compounds in our satellite cell assay system, and only creatine monohydrate was partially effective in altering satellite cell physiology (Vierck, Icenoggle, Bucci et al 2003).

These assay systems were designed and validated to provide for healthy and viable satellite cell cultures that would still allow for measured effects from added compounds with minimal proliferation and/or differentiation in the control cultures (Vierck, Icenoggle, Bucci et al 2003). While these experiments described herein utilized this same system, our control wells exhibited some increase in cell numbers at the termination of the experiments. Control experiments, utilizing increasing levels of fibroblast growth factor (FGF) and insulin-like

growth factor 1 (IGF-1), induced proliferation and differentiation in a dose-dependent manner (data not shown), suggesting that reasonable proliferation and differentiation induced by test compounds could have been detected. The specific reason for the increased proliferation in the control cultures is unknown but has led us to re-evaluate our assay system for future application (Vierck, Icenoggle, Bucci et al 2003). Further, although serum-containing media were used in the present study to mimic physiological conditions, this certainly is not the most optimal way to test these ergogenic compounds (Zimmerman, Vierck, O'Reilly et al. 2000). Future investigations, or more mechanistic studies, will utilize defined environmental conditions (defined medium; Vierck, Icenoggle, Bucci et al 2003). Through the continued examination of numerous other potential oral ergogenic agents and their combinations, we suspect that, like creatine monohydrate, we will discover other nutrients that may alter satellite cell activity.

The cell death observed in the present study was not a result of general cell culture conditions. This is supported by positive control experiments in which FGF/IGF-1 elicited the appropriate proliferative/differentiative response from the cultured satellite cells (data not shown). This suggests that some of the test compounds (at high levels) induced satellite cell death. Morphological observations of treatment wells are consistent with this idea, as these wells contained few viable cells. Additionally, under certain treatments (L-glutamine), satellite cells displayed "stem-like" morphologies similar to those at first isolation from muscle tissue. If these (isolated) satellite cells are induced to become "stem-like," then these treatment conditions might be used to facilitate the study of activation of satellite cells. Any mechanistic knowledge about this possibility is important and could lead to a valuable *in vitro* model system.

International attention about use of anabolic steroids and pro-hormone dietary supplements by high-profile athletes has generated headlines and renewed interest in non-

pharmaceutical and non-hormonal means to safely increase muscle mass and strength. Developing an *in vitro* method to assess the activity of reputed ergogenic compounds for enhancing muscle strength and mass is important in and of itself. Such a system would provide scientific information to a field that is lacking in mechanistic details on how ergogenic compounds operate. This laboratory will continue to probe whether oral supplement compounds individually, or in combination with creatine monohydrate (or other agents), influence a change in satellite cell activity. It is logical to speculate that nutrients may augment the effects of specific growth factors or hormones, or that orally active compounds may, themselves, trigger the ability of satellite cells to initially interact with extrinsic agents (Bonner, Warren and Bucci 1990; Chromiak and Antonio 2002; Dangott, Schultz and Mozdziak 2000; Wellbourne 1995; Wideman, Weltman, Patrie et al. 2000).

In addition to evaluating how satellite cells might respond to potential ergogenic compounds, screening of other cells such as cells of the fat lineage (preadipocytes, adipocytes) and fibroblasts should be conducted. Ergogenic compounds may elicit an effect on fat cells (proliferation or differentiation of preadipocytes, or lipid filling of adipocytes) in such a way as to alter adipogenesis. Alternatively, because fibroblasts synthesize and secrete growth factors, as well as components of the connective tissue that surrounds and supports muscle structure/function, it may be important to determine if dietary compounds possess the ability to influence these connective tissue cells residing within muscle tissue. As muscle mass decreases with advancing age, the identification of agents which may alter satellite cells, fat cells, or fibroblasts, could potentially benefit the treatment of senile muscle atrophy (sarcopenia).

In summary, the present study demonstrates that specific ergogenic compounds do not influence satellite cell proliferation or differentiation *in vitro*. This study represents a partial sampling from the long list of compounds that we intend to evaluate in our satellite cell culture system. We will continue to tabulate the results from our experiments into a master list of compounds, along with their effects on satellite cells *in vitro*. We also intend to screen the same compounds in other cell systems, such as preadipocyte, adipocyte and fibroblast cell systems. Collective data will be recorded and available as scientific evidence for/against compound activity. The present study has also provided some positive data, including the possibility that some compounds possess the ability to revert satellite cells to a stem cell-like morphology. Additional experiments to examine this area will be forthcoming.

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References

Ahrendt DM (2001) Ergogenic aids: counseling the athlete. American Family Physician 63:872-73.

Allen RE, Boxhorn LK (1989) Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. Journal of Cell Physiology 138:311-15.

Allen RE, Boxhorn LK (1987) Inhibition of skeletal muscle satellite cell differentiation by transforming growth factor-beta. Journal of Cell Physiology 133:567-72.

Allen RE, Dodson MV, Luiten LS (1987) Regulation of skeletal muscle cell proliferation by bovine pituitary fibroblast growth factor. Experimental Cell Research 152:157-60.

Allen RE, Merkel RA, Young RB (1979) Cellular aspects of muscle growth: Myogenic cell proliferation. Journal of Animal Science 79:115-27.

Allen RE, Rankin LL (1990) Regulation of satellite cells during skeletal muscle growth and development. Proceedings of the Society for Experimental Biology and Medicine 197:81-86.

Allen RE, Sheehan SM, Taylor RG, Kendall TL, Rice CM (1995) Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. Journal of Cell Physiology 165:307-12.

Androne L, Gavan NA, Veresiu IA, Orasan R (2000) In vivo effect of lipoic acid on lipid peroxidation in patients with diabetic neuropathy. In Vivo 17:327-30.

Applegate E. Effective nutritional ergogenic aids (1999) International Journal of Sports Nutrition 9:229-39.

Bailey DM, Davies B (2001) Acute mountain sickness; prophylactic benefits of antioxidant vitamin supplementation at high altitudes. High Altitude Medicine & Biology 2:21-29.

Bazzare TL (1998) Nutrition and strength. In: Wolinksy I, ed. Nutrition in Exercise and Sport, 3rd ed. Boca Raton FL: CRC Press, 369-719.

Birkhahn RH, Border JR (1981) Alternate or supplemental energy sources. Journal of Parental and Enteral Nutrition 5:27-31.

Bischoff R (1986) A satellite cell mitogen from crushed adult muscle. Developmental Biology 115:129-39.

Bischoff R (1975) Regeneration of single skeletal muscle fibers in vitro. The Anatomy Record 82:215-05.

Bonner B, Warren B, Bucci L (1990) Influence of ferulate supplementation on postexercise stress hormone levels after repeated exercise stress. Journal of Applied Sports Science Research 7:110-11.

Bucci L (1998) Dietary supplements as ergogenic aids. In: Wolinksy I, ed. Nutrition in Exercise and Sport, 3rd ed. Boca Raton FL: CRC Press 315-368.

Bucci, LR, Blackman G, Defoyd W, Kaufmann R, Mandel-Tayes C, Sparks WS, Stiles JC, Hickson JF (1990) Effect of ferulate on strength and body composition of weightlifters. Journal of Applied Sports Science Research 7:107.

Burke DG, Chilibeck PD, Parise G, Tarnopolsky MA (2001) The effect of α-lipoic acid supplementation on resting muscle creatine during acute creatine loading. The FASEB Journal 15:A814.

Campion DR (1984) The muscle satellite cell: A review. International Review of Cytology 87:225-51.

Carlson BM, Faulkner JF (1983) The regeneration of skeletal muscle fibers following injury: A review. Medicine and Science in Sports and Exercise 15:187-98.

Castelluccio C, Bolwell GP, Gerrish C, Rice-Evans C (1996) Differential distribution of ferulic acid to the major plasma constituents in relation to its potential as an antioxidant. Biochemistry Journal 316:691-94.

Chetlin R, Yeater R, Ullrich I, Hornsby G, Malanga C, Bryner R (1996) Effect of ornithine alpha-ketoglutarate on weight trained men. Journal of Strength and Conditioning Research 10:284.

Chromiak JA, Antonio J (2002) Use of amino acids as growth hormone-releasing agents by athletes. Nutrition 18:657-61.

Claeys MC, Mulvaney DR, McCarthy FD, Gore MT, Marple DN, Sartin JL (1989) Skeletal muscle protein synthesis and growth hormone secretion in young lambs treated with clenbuterol. Journal of Animal Science 67:2245-54.

Cynober LA (1999) The use of alpha-ketoglutarate salts in clinical nutrition and metabolic care. Current Opinion in Clinical Nutrition and Metabolic Care 2:33-37.

Cynober L (1991) Ornithine alpha-ketoglutarate in nutritional support. Nutrition 7:313-22.

Dangott B, Schultz E, Mozdziak PE (2000) Dietary creatine monohydrate supplementation increases satellite cell mitotic activity during compensatory hypertrophy. International Journal of Sports Medicine 21:13-16.

Darr KC, Schultz E (1987) Exercise-induced satellite cell activation in growing and mature skeletal muscle. Journal of Applied Physiology 63:1816-21.

Ebbeling CB, Clarkson PM (1989) Exercise-induced muscle damage and adaptation. Sports Medicine 7:207-34.

Estrada DE, Ewart HS, Tsakiridis T, Volchuk A, Ramlal T, Tritschler H, Klip A (1996) Stimulation of glucose uptake by the natural coenzyme alpha-lipoic acid/thioctic acid: participation of elements of the insulin signaling pathway. Diabetes 45:1798-1804.

Friden J (1984) Changes in human skeletal muscle induced by long-term eccentric exercise. Cell and Tissue Research 206:b365-72.

Gallagher PM, Carrithers MP, Godard MP, Schulze KE, Trappe SW (2000) β-hydroxy-β-methylbutyrate ingestion, Part I: Effects on strength and fat free mass. Medicine and Science in Sports and Exercise 32:2109-15.

Gorewit RC (1983) Pituitary and thyroid hormone responses of heifers after ferulic acid administration. Journal of Dairy Science 66:624-29.

Haugaard N, Haugaard ES (1970) Stimulation of glucose utilization by thioctic acid in rat diaphragm incubated in vitro. Biochimica et Biophysica Acta 222:583-86.

Henriksen EJ (2002) Therapeutic effects of lipoic acid on hyperglycemia and insulin resistance. In: Cadenas E, Packer L eds. Handbook of Antioxidants. Second Edition Revised and Expanded. New York NY: Marcel Dekker 535-47.

Henriksen E, Jacob S, Streeper R, Fogt D, Hokama J, Tritschler H (1997) Stimulation by α-lipoic of glucose transport activity in skeletal muscle of lean and obese Zucker rats. Life Sciences 61:805-12.

Henriksen, EJ, Saengsirisuwan V (2003) Exercise training and antioxidants: relief from oxidative stress and insulin resistance. Exercise and Sport Sciences Reviews 31:79-84.

Hiraide A, Katayama M, Sugimoto H, Yoshioka T, Sugimoto T (1991) Effect of 3-hydroxybutyrate on posttraumatic metabolism in man. Surgery 109:176-81.

Imamichi T, Nakamura T, Hayashi K, Kaneko K, Koyama J (1990) Different effects of cinnamic acid on the O2- generation by guinea pig macrophages stimulated with a chemotactic peptide and immune complex. Journal of Pharmacobiodynamics 13:344-52.

Jacob S, Rett K, Henriksen EJ, Haring HU (1999) Thioctic acid – effects on insulin sensitivity and glucose metabolism. Biofactors 10:169-74.

Ji LL (1999) Antioxidants and oxidative stress in exercise. Proceedings of the Society for Experimental Biology and Medicine 222:283-92.

Ji LL (1996) Exercise, oxidative stress, and antioxidants. American Journal of Sports Medicine 24:S20-S24.

Ji LL (1995) Oxidative stress during exercise: implication of antioxidant nutrients. Free Radical Biology & Medicine 18:1079-86.

Jowko E, Ostaszewski P, Jank M, Sacharuk J, Zieniewicz A, Wilczak J, Nissen S (2001) Creatine and β-hydroxy-β-methylbutyrate (HMB) additively increase lean body mass and muscle strength during a weight-training program. Nutrition 17:558-66.

Joyner MJ, Halliwill JR (2000) Neurogenic vasodilation in human skeletal muscle: possible role in contraction-induced hyperaemia. Acta Physiologica Scandinavica 168:481-88.

Joyner MJ, Proctor DN (1999) Muscle blood flow during exercise: the limits of reductionism. Medicine and Science in Sports in Exercise 31:1036-40.

Khanna S, Atalay M, Laaksonen DE, Gul M, Roy S, Sen CK (1999) α-Lipoic acid supplementation: tissue glutathione homeostasis at rest and after exercise. Journal of Applied Physiology 86:1191-96.

Knitter AE, Panton L, Rathmacher JA, Petersen A, Sharp R (2000) Effects of β-hydroxy-β-methylbutyrate on muscle damage after a prolonged run. Journal of Applied Physiology 89:1340-44.

Langfort J, Pilis W, Zarzeczny R, Nazar K, Kaciuba-Uscilko H (1996) Effect of low-carbohydrate-ketogenic diet on metabolic and hormonal responses to graded exercise in men. Journal of Physiology and Pharmacology 47:361-71.

Le Boucher J, Cynober LA (1998) Ornithine α-ketoglutarate: The puzzle. Nutrition 14:870-73.

Lestan B, Walden K, Schmaltz S, Spychala J, Fox IH (1994) Beta-Hydroxybutyrate decreases adenosine triphosphate degradation products in human subjects. The Journal of Laboratory and Clinical Medicine 124:199-209.

Marangon K, Devaraj S, Tirosh O, Packer L, Jialal I (1999) Comparison of the effect of alpha-lipoic acid and alpha-tocopherol supplementation on measures of oxidative stress. Free Radical Biology & Medicine 27:1114-21.

Mauro A (1961) Satellite cells of skeletal muscle fibres. The Journal Biophysical and Biochemical Cytology 9:493-96.

Mitchell GA, Kassovska-Bratinova S, Boukaftane Y, Robert MF, Wang SP, Ashmarina L, Lambert M, Lapierre P, Potier E (1995) Medical aspects of ketone body metabolism. Clinical and Investigative Medicine 18:193-216.

Moss FP, Leblond CP (1971) Satellite cells as the source of nuclei in muscles of growing rats. Anatomy Record 170:421-35.

Moss FP, Leblond CP (1970) Nature of dividing nuclei in skeletal muscle of growing rats. Journal of Cell Biology 44: 459-62.

Motulsky H (1999) Analyzing Data with Graph Pad Prism® San Diego CA: GraphPad Software Inc. 68-69.

Muir A (1970) The structure and distribution of satellite cells. In: Mauro A, Shafiq S, Milhorat A eds. Regeneration of striated muscle and myogenesis. Amsterdam: Exterpta Medica 91-100.

Nissen, SL, Sharp RL (2003) Effect of dietary supplements on lean mass and strength gains with resistance exercise: a meta-analysis. Journal of Applied Physiology 94:651-59.

Nissen S, Sharp R, Ray M (1996) Effect of leucine metabolite β-hydroxy-β-methylbutyrate on muscle metabolism during resistance-exercise training. Journal of Applied Physiology 81:2095-04.

Novelli GP, Falsini S, Bracciotti G (1991) Exogenous glutathione increases endurance to muscle effort in mice. Pharmacology Research 20:149-55.

Packer L, Witt EH, Tritschler HJ (1995) Alpha-lipoic acid as a biological antioxidant. Free Radical Biology & Medicine 19:227-50.

Perez-Alvarez V, Bobadilla RA, Muriel P (2001) Structure-hepatoprotective activity relationship of 3, 4-dihydroxycinnamic acid (caffeic acid) derivatives. Journal of Applied Toxicology 21:527-31.

Ricks CA, Dalrymple RH, Baker PK, Ingle DL (1984) Use of a β-agonist to alter fat and muscle deposition in steers. Journal of Animal Sciences 59:1247-55.

Sastre J, Asensi M, Gasco E, Pallardo FV, Ferrero JA, Furukawa T, Vina J (1992) Exhaustive physical exercise causes oxidation of glutathione status in blood: prevention by antioxidant administration. American Journal of Physiology 263:R992-95.

Sen CK (1999) Glutathione homeostasis in response to exercise training and nutritional supplements. Molecular and Cellular Biochemistry 196:31-42.

Sen CK, Packer L (2000) Thiol homeostasis and supplements in physical exercise. American Journal of Clinical Nutrition 72:653S-69S.

Shultz E (1989) Satellite cell behaviour during skeletal muscle growth and regeneration. Medicine and Science in Sports and Exercise 21:S181-86.

Silver MD (2001) Use of ergogenic aids by athletes. Journal of American Academy of Orthopedic Surgeons 9:61-70.

Snow MH (1978). An autoradiographic study of satellite cell differentiation into regenerating myotubes following transplantation of muscles in young rats. Cell and Tissue Research 186:535-40.

Tatsumi R, Anderson JE, Nevoret CJ, Halevy O, Allen RE (1998) HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Developmental Biology 194:114-28.

Toda S, Kumura M, Ohnishi M (1991) Effects of phenolcarboxylic acids on superoxide anion and lipid peroxidation induced by superoxide anion. Planta Med 57:8-10.

Varnier M, Leese GP, Thompson J, Rennie MJ (1995) Stimulatory effects of glutamine on glycogen accumulation in human skeletal muscle. American Journal of Physiology 269:E309-15.

Vierck JL, Icenoggle DL, Bucci LR, Dodson MV (2003) The effects of ergogenic compounds on the proliferation and differentiation of myogenic satellite cells. Medicine and Science in Sports and Exercise 35:769-76.

Vierck J, O'Reilly B, Hossner K, Antonio J, Byrne K, Bucci L, Dodson M (2000) Satellite cell regulation following myotrauma caused by resistance exercise. Cell Biology International 24:263-72.

Wellbourne TC (1995) Increased plasma bicarbonate and growth hormone after an oral glutamine load. American Journal of Clinical Nutrition 61:1058-61.

Wideman L, Weltman JY, Patrie JT, Bowers JT, Bowers CY, Shah N, Story S, Veldhuis JD, Weltman A (2000) Synergy of L-arginine and GHRP-2 stimulation of growth hormone in men and women: modulation by exercise. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 279:R1467-77.

Yagi K, Ohishi M (1979) Action of ferulic acid and its derivatives as antioxidants. J Nutr Sci Vitaminology (Tokyo) 25:127-30.

Zimmerman AM, Vierck JL, O'Reilly BA, Dodson MV (2000) Formulation of a defined medium to maintain cell health and viability in vitro. Methods in Cell Science 22:43-49.

Table 1: Examples of commercially available sports nutrition products containing the compounds tested in the present study.

Company	Product Name[a]	Ingredients	Amount per serving	Size Serving Size	SRP[b]	Product Claims
Cinnamic Acid						
Numerous	*Panax ginseng* root powders & extracts	Cinnamic acid	Not specified			Listed as normally occurring component of ginseng root (herbalremedies.com)
Action Labs	Ginseng Power Max 4x	Cinnamic acid Red Chinese ginseng root Korean ginseng root Siberian ginseng root American ginseng root	Not listed 350 mg 350 mg 150 mg 150 mg	100 caps 2 caps/serv[c]	26.99	"Boost your energy to the max with Action Labs Ginseng Power Max 4x."
Ginsana® Sport	Pharmaton®	Cinnamic acid Asian ginseng extract (*Panax ginseng*) standardized to 4% ginsenosides	Not listed 200 mg	40 softgels 1 softgel /serv	15.99	Helps maintain aerobic capacity during workouts Promotes efficient oxygen utilization
Ferulic Acid						
Allsports International	Pure Ferulic Acid	Ferulic acid	50 mg	60 capsules 1 cap/serv	£9.30	
American Body Building	Blue Thunder™	Transferulic acid Total Carbohydrates, Protein, Proprietary Energy Blend	Not specified	22 fl oz	3.39	Super Activators
Body & Fitness	PrePower	Ferulic acid Branched chain amino acids, cytochrome C, calcium & potassium aspartates, inosine	75 mg	120 caps 3 caps/serv	33.99	"Pre-Power help boosts the efficiency of oxygen usage in muscle cells, decrease lactic acid build-up for less burning and cramping with faster recuperation."
Met-Rx	APM 60™	Ferulic acid Protein, ARS Proprietary blend (6 ingredients)	Not specified	12 packs 1 pack/serv	39.99	"An ultra high protein source with advanced support components engineered to meet the demands of athletes."
Source Naturals	Trans-Ferulic Acid	Trans-Ferulic acid	500 mg	60 tablets 2 tabs/serv	19.75	
SportsQuest Direct.com	Interphase	Ferulic acid Protein, carbohydrates, multiple vitamin/mineral, creatine, amino acids, OKG, L-carnitine, colostrum, phosphates	24 mg	2.6 lbs 50 g/serv	34.95	fast muscle gains and recovery increase and maintain lean muscle
Ultra Labs	Sterobol Suspension	Ferulic acid *Cissus quadrangularis*, Shilajit, *Cyanotis*	50 mg	8 fl oz	64.95	

		vaga (Ecdysterone), Kre-Alkalyn, *Mucuna pruriens*				
Vitamin Research Products	Performance Plus	Ferulic acid DMAE, Xylitol, L-ornithine, L-glutamine, L-tyrosine, *Mucuna pruriens*, Creatine pyruvate, choline citrate, citric acid	200 mg	415 g 28 g/serv	42.95	
Vitol	Russian Bear Tabs	Ferulic acid (15 other ingredients)	600 mg	140 tablets 8 tabs/serv	28.99	"Russian Bear Tabs combines Russian and American technology to form an anabolic formula for increased muscle mass; strength; endurance; and energy."
L-Glutathione						
Immunotec Research	Immunocal®/HMS90	Whey protein	9 g	30 packs 1 pack/serv	99.00	Patented with published research showing increased GSH levels
Physiologics	Liposomal Glutathione	Glutathione	25 mg	80 sprays 1 spray/serv	6.49	
Solaray	L-Glutathione	Glutathione	50 mg	60 tablets 1 tab/serv	15.09	
Twinlab	Mega L-Glutathione	Glutathione	250 mg	60 capsules 1 cap/serv	35.95	
Twinlab	Antioxidant Fuel	Glutathione Beta carotene, Vitamins C & E, Selenium, Coenzyme Q10, N-Acetyl-L-cysteine, α-lipoic acid	33 mg	60 capsules 1 cap/serv	26.95	Performance enhancer
VPX Sports	Medivin™	Glutathione Multiple vitamin/mineral/herbal	8 mg	480 ml 4 ml	121.95	
HMB						
EAS	HMB	HMB	1 g	200 caps 4 caps/serv	39.95	Minimize protein breakdown and damage to muscle cells Help the body maintain an anabolic state Improve body composition
EAS	Betagen® HP	HMB Carbohydrates, creatine, taurine, L-glutamine	1 g	930 g 10 g/serv	69.95	Increase lean muscle mass and strength Suppress muscle protein breakdown Enhance muscle energetics and recuperation
Met-Rx87	Mass Action	HMB Carbohydrates, creatine, L-glutamine, trimethylglycine	1.5 g	1.5 lbs 26 g/serv	48.95	"Mass Action® from Met-Rx® is a powerful nutritional supplement which contains ingredients that can help gain lean

						body mass and improve muscular strength and power."
Optimum Nutrition	HMB	HMB	1 g	90 caps 1 cap/serv	59.99	
Twinlab	HMB Fuel	HMB	1 g	120 caps 4 caps/serv	33.99	
Weider Nutrition	HMB	HMB	1 g	60 caps 4 caps/serv	16.99	"These results suggest that HMB supports results of strenuous exercise in both young and long-lived adults for both weightlifting and endurance exercises."
α-Lipoic Acid						
AST Sports Science	ALA 200	α-Lipoic acid	200 mg	90 caps 1 cap/serv	39.95	"ALA will help enhance the absorption of creatine into the muscle cell by providing a transport vehicle by its unique ability to mimic insulin."
Met-Rx	Glycemet IGF (Part of the Brand New Anabolic Drive Series from Met-Rx!)	α-Lipoic acid Carbohydrates (Glucosol, Fibersol), chromium, colosolic acid, xanthan gum, citric and malic acids	100 mg	12.7 ounces 12 g/serving	33.99	"It provides more stable bloodstream sugar levels, thereby enhancing thermogenesis and increased muscle sythesis through the regulation of nutrient digestion and absorption."
MuscleTech	Cell Tech	α-Lipoic acid Carbohydrates, creatine	Not specified	2.2 lbs 98.5 g/serv	32.99	"Muscletech's Cell Tech also has Alpha Lipoic Acid which mimics the actions of insulin in the body. Thus more creatine is shuttled into the muscle cell and elicits a more pronounced volumizing effect than with any other creatine based product on the market."
Performance Biomedical Labs	Effervescent Creatine Plus	α-Lipoic acid Creatine, L-glutamine, taurine	100 mg	1 lb 14 g	44.95	"In addition, Effervescent Creatine plus contains an advanced effervescent delivery system which includes 100 mg of Alpha Lipoic Acid to induce an insulin response without unwanted sugars."
S.A.N. (Scientifically Advanced Nutrition)	Loaded	α-Lipoic acid R-Lipoic acid Guanidinoacetic acid, pinitol, vanadyl sulfate	100 mg 100 mg	60 caps 2 caps/serv	29.99	**"LOADED** is comprised of the most potent glycemic agents in existence. Unsurpassed by its competition, **LOADED** effectively shuttles nutrients into the muscle cell getting them primed for peak performance.

						LOADED also inhibits the storage of insulin fat that accompanies high glycemic/carbohydrate diets."
OKG						
Champion Nutrition	Met Max Metabolol Maximum™	OKG Protein, carbohydrate, amino acids, polylactate, creatine, succinate, 14 others	Not specified	2.7 lbs 62 g/serv	35.00	"OKG and L-glutamine to volumize muscle and prevent exercise induced catabolism."
Eclipse	Eclipse 2000 Hard Trainer Pak	OKG Protein, carbohydrates, multiple vitamin/mineral, amino acids, 42 others	850 mg	30 packs 1 pack/serv (11 pills)	32.75	"The Hard Trainer pack may regulate cortisol production and promote anabolism by feeding the muscle free form amino acids, OKG, and a host of other powerful compounds."
Source Naturals	OKG	OKG	3.5 g	4 oz 1 tsp	48.98	"It is used by athletes and those who want to improve performance."
Universal Nutrition	OKG	OKG	750 mg	90 capsules 1 cap/serv	30.00	"Research has shown that supplementation of OKG assists with increased protein synthesis, decreased protein catabolism, improvements in insulin and somatotropin secretion and increased uptake of amino acids and glucose to the muscles."

[a] Information obtained from company websites or other internet sales companies from public domain.

[b] SRP = Suggested Retail Price. All products are routinely available in retail and internet sales outlets for 10-70% less than the SRP.

[c] Serv = Serving

Table 2 – Ergogenic compounds tested in the present study and desired effect(s) by athletes.

Supplement	Desired Effects and Results	References
Cinnamic acid	Cytoprotective against oxidative stress induced by exercise Increase brain dopamine levels and/or turnover Enhance exercise-mediated pituitary hormone release (somatotropin) Improve anabolism Increase muscle mass and strength	Imamichi, Nakamura, Hayashi et al. 1990; Perez-Alvarez, Bobadilla and Muriel 2001; Toda, Kumura and Ohnishi 1991
Ferulic acid	Cytoprotective against oxidative stress induced by exercise Increase brain dopamine levels and/or turnover Enhance exercise-mediated hormone release (β-endorphins, somatotropin) Improve anabolism Increase muscle mass and strength	Bonner, Warren and Bucci 1990; Bucci, Blackman, Defoyd et al. 1990; Castelluccio, Bolwell, Gerrish et al. 1996; Gorewit 1983; Toda, Kumura and Ohnishi 1991; Yagi and Ohishi 1979
L-glutathione	Cytoprotective against oxidative stress induced by exercise Improve recovery from intense exercise Improve gains from training Increase muscle mass and strength	Ji 1995; Khanna, Atalay, Laaksonen et al. 1999; Sastre, Asensi, Gasco et al. 1992; Sen 1999; Sen and Packer 2000
β-hydroxybutyric acid	Non-carbohydrate cellular energy source (spare carbohydrate use during exercise) Improve muscle energetics and performance during training Improve gains from training Increase muscle mass and strength	Birkhahn and Border 1981; Hiraide, Katayama, Sugimoto et al. 1991; Langfort, Pilis, Zarzeczny et al. 1996; Lestan, Walden, Schmaltz et al. 1994; Mitchell, Kassovska-Bratinova, Boukaftane et al. 1995; Ricks, Dalrymple, Baker et al. 1984
HMB	Spares leucine and indispensable amino acids in muscle Improve exercise recovery Enhance exercise performance Increase muscle mass and strength	Gallagher, Carrithers, Godard et al. 2000; Jowko, Ostaszewski, Jank et al. 2001; Nissen and Sharp 2003; Nissen, Sharp and Ray 1996
α-Lipoic acid	Involved in glucose transport/uptake and glycogen synthesis Cytoprotective against oxidative stress induced by exercise Increase insulin-facilitated creatine uptake into muscle cells without carbohydrates Increase anabolism via increased insulin action Improve recovery from intense exercise Improve gains from training Increase muscle mass and strength	Burke, Chilibeck, Parise et al. 2001; Estrada, Ewart, Tsakiridis et al. 1996; Haugaard and Haugaard 1970; Henriksen 2002; Henriksen, Jacob, Streeper et al. 1997; Henriksen and Saengsirisuwan 2003; Jacob, Rett, Henriksen et al. 1999; Ji 1996; Khanna, Atalay, Laaksonen et al. 1999; Packer, Witt and Tritschler 1995; Sen 1999; Sen and Packer 2000
OKG	Increase or spare intracellular glutamine Decrease skeletal muscle protein catabolism (anabolic) Increase the systemic concentration of insulin and growth hormone Increase recovery from intense exercise Increase gains from training Increase muscle mass and strength	Chromiak and Antonio 2002; Cynober 1999; Cynober 1991; Le Boucher and Cynober 1998; Varnier, Leese, Thompson et al. 1995; Wellbourne 1995

Table 3 – Results from ergogenic compound proliferation assays.

Compound	Nuclei/mm^2
Cinnamic acid	
Level 0[a]	33.0 ± 7.4
0.00000001 % (6.75×10^{-10} M)	31.6 ± 8.6
0.000001 % (6.75×10^{-8} M)	42.1 ± 8.8
0.0001 % (6.75×10^{-6} M)	42.8 ± 8.0
Level 0[a]	173.1 ± 25.9
0.1 % (6.75×10^{-3} M)	82.3 ± 22.7[b]
0.25 % (1.69×10^{-2} M)	4.9 ± 4.9[b]
0.5 % (3.37×10^{-2} M)	0[b]
1.0 % (6.75×10^{-2} M)	0[b]
Ferulic acid	
Level 0	94.0 ± 8.7
50 pg•ml^{-1} (2.57×10^{-10} M)	75.7 ± 8.9
5 ng•ml^{-1} (2.57×10^{-8} M)	59.5 ± 8.7
0.5 μg•ml^{-1} (2.57×10^{-6} M)	67.4 ± 17.5
50 μg•ml^{-1} (2.57×10^{-4} M)	98.4 ± 8.5
L-glutathione	
Level 0[a]	33.0 ± 7.4
0.00000001 % (3.25×10^{-10} M)	19.3 ± 4.5
0.000001 % (3.25×10^{-8} M)	30.7 ± 4.3
0.0001 % (3.25×10^{-6} M)	37.2 ± 10.7
Level 0[a]	160.1 ± 18.7
0.1 % (3.25×10^{-3} M)	45.8 ± 8.1[b]
0.25 % (8.13×10^{-3} M)	82.6 ± 13.7[b]
0.5 % (1.63×10^{-2} M)	100.1 ± 22.2[b]
1.0 % (3.25×10^{-2} M)	38.8 ± 2.9[b]
β-hydroxybutyric acid	
Level 0[a]	33.0 ± 7.4
0.00000001 % (9.61×10^{-10} M)	31.6 ± 8.6
0.000001 % (9.61×10^{-8} M)	42.1 ± 8.8
0.0001 % (9.61×10^{-6} M)	42.8 ± 8.0
Level 0[a]	173.1 ± 25.9
0.1 % (9.61×10^{-3} M)	104.1 ± 41.1
0.25 % (2.40×10^{-3} M)	0[b]
0.5 % (4.80×10^{-2} M)	0[b]
1.0 % (9.61×10^{-2} M)	0[b]
HMB (79.99 %)	
Level 0[a]	33.0 ± 7.4
0.00000001 % (7.95×10^{-10} M)	18.8 ± 5.0
0.000001 % (7.95×10^{-8} M)	48.0 ± 10 7
0.0001 % (7.95×10^{-6} M)	38.9 ± 7.4
Level 0[a]	173.1 ± 25.9
0.1 % (7.95×10^{-3} M)	122.4 ± 39.4
0.25 % (1.99×10^{-2} M)	0[b]
0.5 % (3.98×10^{-2} M)	0[b]
1.0 % (7.95×10^{-2} M)	0[b]
α-Lipoic acid	
Level 0	94.0 ± 8.7
50 pg•ml^{-1} (2.42×10^{-10} M)	82.3 ± 19.2
5 ng•ml^{-1} (2.42×10^{-8} M)	41.4 ± 11.9
0.5 μg•ml^{-1} (2.42×10^{-6} M)	68.4 ± 11.1
50 μg•ml^{-1} (2.42×10^{-4} M)	78.5 ± 14.1
OKG	
Level 0[a]	33.0 ± 7.4

0.00000001 % (3.59×10^{-10} M	22.9 ± 3.7
0.000001 % (3.59×10^{-8} M	39.0 ± 5.2
0.0001 % (3.59×10^{-6} M	48.5 ± 12.0
Level 0[a]	160.1 ± 18.7
0.1 % (3.59×10^{-3} M)	149.0 ± 30.2
0.25 % (9.98×10^{-3} M)	62 ± 20[a]
0.5 % (1.80×10^{-2} M)	40.6 ± 22.1[a]
1.0 % (3.59×10^{-2} M)	33.9 ± 6.4[a]

[a] These levels are identical but represent controls from two separate experiments.

[b] Significantly lower than control mean ($P<0.05$).

Table 4 – Results from ergogenic compound differentiative assays conducted for the present study.

Compound	Differentiation	
	Total nuclei/mm^2	% Fusion
Cinnamic acid		
Level 0[*]	113.1 ± 32.7	0.1 %
0.1 % (6.75×10^{-3} M)	48.5 ± 31	0[b]
0.25 % (1.69×10^{-2} M)	-83.8 ± 37.7[a]	0[b]
0.5 % (3.37×10^{-2} M)	-79.6 ± 50.6[a]	0[b]
1.0 % (6.75×10^{-2} M)	-80.5 ± 49.8[a]	0[b]
Ferulic acid		
Level 0[†]	169.3 ± 25.8	0.8 %
50 pg•ml^{-1} (2.57×10^{-10} M)	148.5 ± 19.9	0.2 %
5 ng•ml^{-1} (2.57×10^{-8} M)	143.1 ± 21.8	0[b]
0.5 µg•ml^{-1} (2.57×10^{-6} M)	110.8 ± 13.7	0.1 %
50 µg•ml^{-1} (2.57×10^{-4} M)	120.3 ± 13.2	0.1 %
α-Lipoic acid		
Level 0[†]	169.3 ± 25.8	0.8 %
50 pg•ml^{-1} (2.42×10^{-10} M)	195.5 ± 42.8	0.4 %
5 ng•ml^{-1} (2.42×10^{-8} M)	129.7 ± 11.6	0[b]
0.5 µg•ml^{-1} (2.42×10^{-6} M)	114.4 ± 13.3	0.7 %
50 µg•ml^{-1} (2.42×10^{-4} M)	137.9 ± 23.8	0.2 %
OKG		
Level 0[‡]	114.5 ± 42.2	1.0 %
0.1 % (3.59×10^{-3} M)	124.8 ± 21.7	0.3 %
0.25 % (9.98×10^{-3} M)	59.9 ± 29.1	0.1 %
0.5 % (1.80×10^{-2} M)	63.6 ± 23.1	0[b]
1.0 % (3.59×10^{-2} M)	77.0 ± 9.4	0[b]
L-glutathione		
Level 0[‡]	114.5 ± 42.2	1.0 %
0.1 % (3.25×10^{-3} M)	-153.7 ± 10.1[a]	0[b]
0.25 % (8.13×10^{-3} M)	-182.9 ± 2.5[a]	0[b]
0.5 % (1.63×10^{-2} M)	-170.0 ± 6.0[a]	0[b]
1.0 % (3.25×10^{-2} M)	-155.2 ± 5.9[a]	0[b]
β-hydroxybutyric acid		
Level 0	79.5 ± 20.7	0.7 %
0.00000001 % (9.61×10^{-10} M)	59.3 ± 29.9	1.7 %
0.000001 % (9.61×10^{-8} M)	146.1 ± 32.0	2.6 %
0.0001 % (9.61×10^{-6} M)	127.9 ± 20.5	2.8 %
Level 0[*]	113.1 ± 32.7	0.1 %
0.1 % (9.61×10^{-3} M)	-134.0 ± 10.2[a]	0[b]
0.25 % (2.40×10^{-3} M)	-134.0 ± 10.2[a]	0[b]
0.5 % (4.80×10^{-2} M)	-134.0 ± 10.2[a]	0[b]
1.0 % (9.61×10^{-2} M)	-134.0 ± 10.2[a]	0[b]
HMB (79.99 %)		
Level 0[*]	113.1 ± 32.7	0.1 %
0.1 % (7.95×10^{-3} M)	-134.0 ± 10.2[a]	0[b]
0.25 % (1.99×10^{-2} M)	-134.0 ± 10.2[a]	0[b]
0.5 % (3.98×10^{-2} M)	-134.0 ± 10.2[a]	0[b]
1.0 % (7.95×10^{-2} M)	-134.0 ± 10.2[a]	0[b]

[a] Significantly lower than control mean ($P<0.05$).

[b] No morphological differentiation was observed under the present *in vitro* conditions with current counting methods.

[c] Levels have been detailed in methods.

[*, †, ‡] These assays were conducted at the same time with same control cultures.

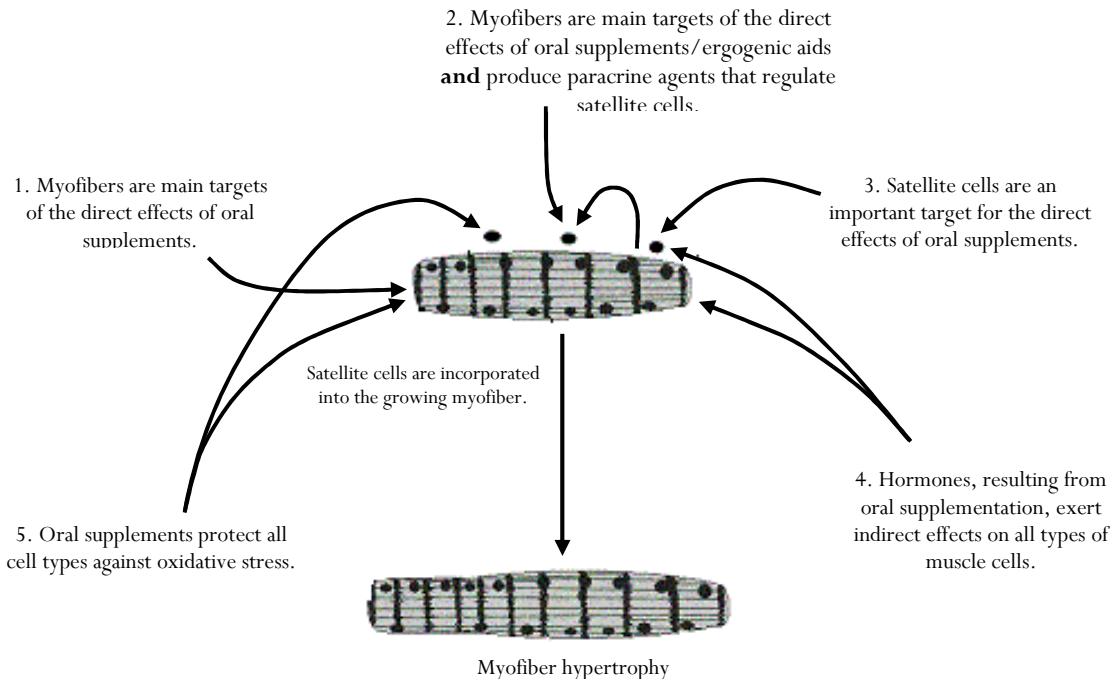

Figure 1 – Potential mechanisms by which oral supplements may influence muscles. Muscles may be directly affected by ergogenic compounds taken orally (1). Muscles may be directly influenced by oral ergogenic supplements and produce paracrine agents, which affect satellite cells (2). Oral supplements may influence satellite cells directly (3). Oral supplementation may produce hormones that influence muscles and satellite cells (4). Oral supplements (antioxidants) protect all cell types against oxidative damage (5).

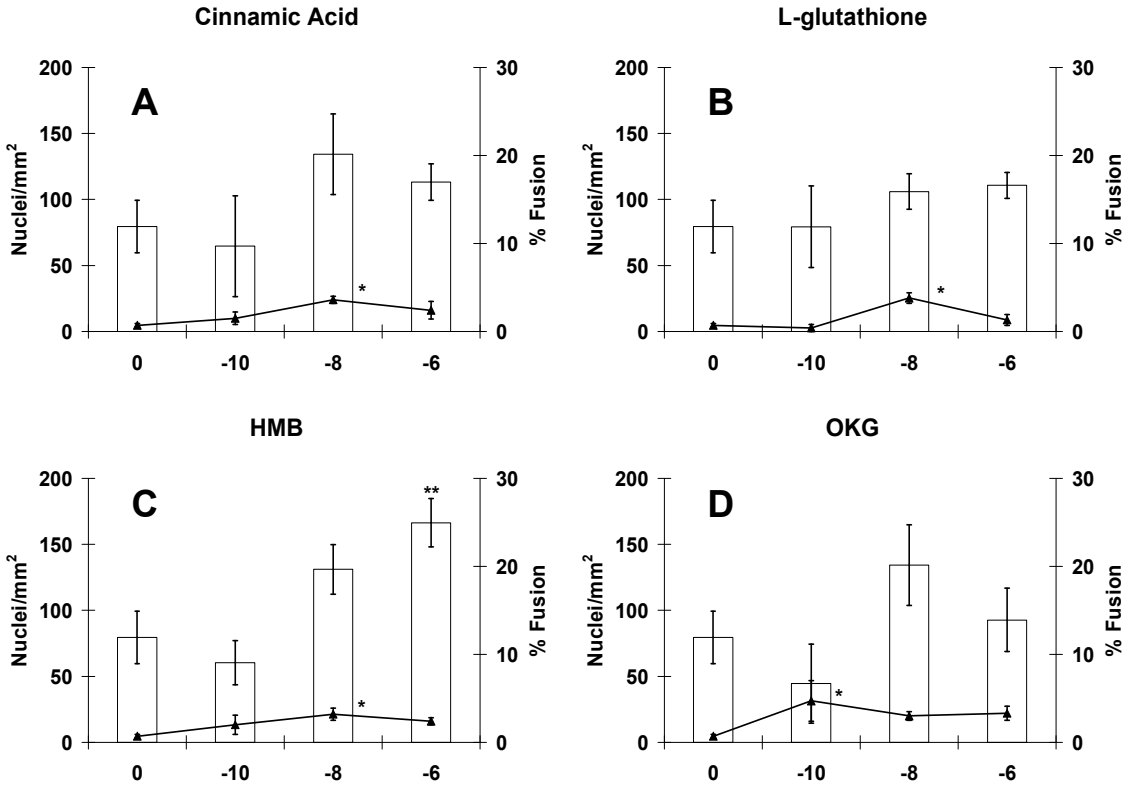

Figure 2 – Effects of (A) cinnamic acid, (B) L-glutathione, (C) HMB, and (D) OKG on the differentiation of satellite cells cultured under differentiative conditions. Satellite cells (two experiments per compound) were plated at a density of 100 nuclei•mm^{-2} and cultured for 120 h in a medium containing DMEM + 0.5 % HS + antibiotics and either: (A) 6.75×10^{-10} M, 6.75×10^{-8} M, 6.75×10^{-6} M cinnamic acid; (B) 3.25×10^{-10} M, 3.25×10^{-8} M, 3.25×10^{-6} M L-glutathione; (C) 7.95×10^{-10} M, 7.95×10^{-8} M, 7.95×10^{-6} M HMB; or (D) 3.59×10^{-10} M, 3.59×10^{-8} M, 3.59×10^{-6} M OKG. The controls for all experiments consisted of basal media with no treatment compound. The graph for each compound represents the total nuclei number and the percent fusion at the end of 120 h for three treatment levels and control. Each *bar* represents the total nuclei Mean ± SE of six wells (controls consisted of 12 wells). Each *line point* represents the total percent fusion Mean ± SE of six wells (controls consisted of 12 wells). *Percent fusion was significantly different from the 0.5 % HS control ($P<0.05$). **Total nuclei count was significantly different from the 0.5 % HS control ($P<0.05$).

CHAPTER FOUR

Dedifferentiation of mature adipocytes to form adipofibroblasts: More than just a possibility*

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ABSTRACT

Little attention has been focused on the mechanisms underlying the dedifferentiation of mature, committed adipocytes to form adipofibroblasts. We hypothesize that at some point a mature fat cell that is undergoing active lipolysis is capable of re-entering the cell cycle and expressing genes that regulate cell passage through to a proliferative state. Development of mature adipocyte culture methods could provide a viable system to determine the cellular changes in mature adipocytes as they initiate the dedifferentiation program to revert to proliferative-competent adipofibroblasts.

INTRODUCTION

Adipogenesis is commonly thought of as the process of proliferation, differentiation, and maturation of stem-like (mesodermal) cells to form committed cells of the fat cell lineage capable of conducting lipogenesis and lipolysis. Traditionally, scientific studies on the mechanisms of adipogenesis commonly utilize cell lines (*i.e.* 3T3-L1, 3T3-F442A, ob1771 cells) or cultures of stromal-vascular cells (all other cells from fat tissue excluding mature adipocytes). For years, mature adipocytes were considered as being terminally differentiated, rather innocuous cells that primarily functioned as energy storing cells of connective tissue. However, a diametrically opposite picture is presently unfolding about mature adipocytes. These cells produce specific signaling agents, such as leptin, that systemically regulate energy substrate utilization and thus affect body composition. In addition, mature adipocytes appear capable of losing lipid (the specific amount needed to be lost for efficient conversion is not known) and reverting (commonly termed dedifferentiation) into proliferation-capable preadipocytes (also referred to as adipofibroblasts; Adebonojo 1975a; Adebonojo 1975b; Sugihara et al., 1986; Sugihara et al., 1987; Sugihara et al., 1989; Justesen et al., 2004).

Adipogenesis, lipogenesis, and lipolysis occur through the interactions of endogenous genetic mechanisms (mediated through gene expression and regulated by intrinsic factors), external controls (endocrine agents, extrinsic factors and nutritional metabolites), as well as local interactions within cells in a fat depot (reviewed in Ailhaud et al., 1992; Cornelius et al., 1994; Houseknecht et al., 1998; Rosen and Spiegelman, 2000; Gregoire, 2001; Fajas, 2003; Kokta et al., in press). Adipocytes develop from primordial cells termed preadipocytes (some refer to these cells as adipofibroblasts to denote their "stem cell-like" character; Adebonojo, 1975a; Johnson and Francendese, 1985; Vierck et al., 1996a; Vierck et al., 1996b; Vierck et

al., 2001), and the extent of their postnatal cellular differentiation is both genetically and environmentally controlled. These fibroblastic cells (*i.e.* fibroblasts, adipofibroblasts, or preadipocytes) may be induced to assume a new morphology and to increase the expression of enzymes fundamental to lipid deposition, including acetyl CoA carboxylase, fatty acid synthetase, and glycerol phosphate dehydrogenase (Lee and Kauffmann, 1974a; Wise and Green, 1979; Bjorntorp et al., 1980; Serrero and Khoo, 1982; Broad and Ham, 1983; Gaben-Cogneville et al., 1983; Sugihara et al., 1987; May et al., 1994; Eguinoa et al., 2003). The rate of cell conversion (from a fibroblast-like cell to a cell of the fat lineage) is high in young animals and decreases with age, while the accumulation of lipid previously converted cells increases with age (Van and Roncari, 1978; Robelin, 1981; Vernon, 1986; Hausman et al., 1989). This pattern is alterable by providing a positive or negative energy balance, the former decreasing both hyperplasia and hypertrophy and the latter increasing both processes (Vernon, 1980). Genetic differences also manifest in varying fat deposition patterns (Eguinoa et al., 2003). For example, large-framed, fast growing animals display qualitatively different patterns of adipocyte cellularity and adipose growth compared to early-maturing, slower growing animals (Lee and Kauffman, 1974b; Deslex et al., 1987; Smith and McNamara, 1987; Eguinoa et al., 2003). Similar gross differences are recognized in swine, mice, and humans (Ng et al., 1971; Lee and Kauffman, 1974b; Dixon-Shanies et al., 1975; Ailhaud et al., 1992).

In most adipose depots, the number of adipocyte-like cells with the capability of lipid synthesis and storage does not appear fixed at birth. Rather, postnatal adipocyte growth is both hyperplastic and hypertrophic, the extent of each changing with depot location (Hirsch and Han, 1969; Greenwood and Hirsch, 1974; Lee and Kauffman, 1974c; May et al., 1994; Huerta-Leidenz et al., 1996; Eguinoa et al., 2003). Different adipose tissue depots possess

unique cellular properties (Smith and Crouse, 1984; Cianzio et al., 1985; Eguinoa et al., 2003), enzymatic activities (May et al., 1994; Eguinoa et al., 2003), response of the cells to hormones or growth factors, and expression of enzymatic activity *in vitro* (Wu et al., 2000). Differences also exist between animal breeds in terms of adipose depot characteristics (May et al., 1994, Huerta-Leidenz et al., 1996; Eguinoa et al., 2003). Numerous papers have been published documenting adipose depot differences in an assortment of animals (Dijan et al., 1983; Sztalryd et al., 1989; Kirkland et al., 1990; Bjorntorp, 1991; Pons et al., 1992; de la Hoz and Vernon, 1993; Barber et al., 2000; Caserta et al., 2001; Wu et al., 2001; Soret et al., 1999; Lelliot et al., 2002; Shahparaki et al., 2002; Altomonte et al., 2003).

A variety of different cell culture systems have been used to address questions dealing with adipogenesis, including the use of lines of Swiss mouse embryo 3T3-L1 cells (Green and Meuth, 1974; Green and Kehinda, 1976; Mackall et al., 1976; Wise and Green, 1978; Reed and Lane, 1980; Spiegelman and Ginty, 1983; Gamou et al., 1990; Hauner, 1990; Toscani et al., 1990; Sadowski et al., 1992; Tafuri, 1996; Mandrup and Lane, 1997) and stromal-vascular cells (the cells that pellet during adipocyte isolation; Ng et al., 1971; Pozanski et al., 1973; Dixon-Shanies et al., 1975; Van et al., 1976; Van and Roncari, 1977, Van and Roncari, 1978; Bjorntorp et al., 1980; Broad and Ham, 1983; Gaben-Cogneville et al., 1983; Cryer et al., 1984; Hausman et al., 1984; Hausman et al., 1985; Deslex et al., 1987; Novakofski, 1987; Ramsay et al., 1987; Nougues et al., 1988; Hausman, 1989; Ramsay et al, 1989; Hausman and Wright, 1990; Hausman et al., 1992; Akambi et al., 1994; Hausman et al., 1996; Sato et al., 1996; Suryawan and Hu, 1997; Suryawan et al., 1997; Boone et al., 2000; Wu et al., 2000). Both of these cell systems have provided information regarding the regulators involved in cell conversion from mononucleated (spindle-shaped) cells into lipid-filled adipocytes and the

dynamics of lipogenesis (well reviewed in Ailhaud et al., 1992; Cornelius et al., 1994; Houseknecht et al., 1998; Rosen and Spiegelman, 2000; Gregoire, 2001; Fajas, 2003). Unfortunately, use of these cells is not without controversy, as 3T3-L1 cells (and other cell lines) may or may not reflect physiology similar to cells derived from other animals. Further, the use of stromal-vascular cells in studies of adipogenesis may be flawed due to contamination in the cultures by numerous other cells. Indeed, May et al., (1994) suggested that although adipocyte precursor cells are evident in stromal-vascular cell populations derived from animals, their specific number (cellularity) could not be enumerated. If mouse cell lines are not necessarily a good model for adipogenesis and if the use of stromal-vascular cell numbers cannot be quantified, why not use the mature adipocyte fraction in scientific studies?

Methods to isolate mature adipocytes were reported in the scientific literature over twenty-five years ago (Adebonojo, 1975a), but few laboratories (worldwide) have attempted to develop a research effort with these cells in which the cells are maintained in culture for long durations (Ng, 1971; Van et al., 1976; Johnson and Francendese, 1985; Sugihara et al., 1986; Sugihara et al., 1987; Ailhaud, 1992; Suryawan and Hu, 1995; Zhang et al., 2000). Although the use of mature adipocytes seems logical for numerous studies, it is controversial due to the perception that mature fat cells are considered to be fully differentiated and incapable of dedifferentiation (unless dedifferentiation is truly an aberrant physiological event; Novakofski, 1987).

Reports of "plasticity" of numerous cellular developmental programs, as well as our own laboratory observations (Figure 1; Figure 2), have led us to embrace the mature adipocyte culture system. Recently, two reports suggested that mature adipocytes might dedifferentiate and assume a rudimentary (stem) cell phenotype (Shigematsu et al., 1999; Justensen et al.,

2004). In both reports, mature fat cells were isolated and allowed to lose lipid. The resultant cells appeared to assume stem cell morphology (small, scant cytoplasm) but then were determined to form cells of adipose lineage. Additionally, Justensen et al. (2004) induced these cells to form osteogenic cells as well. Even if mature, committed fat cells derived from meat-animals can only dedifferentiate and form proliferative-competent adipofibroblasts, it would be of some worth to define the regulatory mechanisms involved in the dedifferentiation process. These studies involving adipocytes are exciting, and as such, we believe that this line of research is important.

Presently, we are not aware of any other laboratory conducting long-term *in vitro* research with mature fat cells (or their progeny cells) from any fat depot in domestic animals. This is surprising, because data from this research area has 1) economic implications in terms of directing deposition of energy in meat animals (our main interest), 2) repercussions in obesity research and other biomedical dysfunctions involving adipose tissue/energy partitioning, and 3) potential ramifications that may aid/facilitate the efficiency of fitness regimens. Moreover, this line of research might provide knowledge for targeting cells of specific fat depots to make it impossible for immature cells to accumulate lipid, or to induce mature fat cells to release their lipid allowing for efficient targeting and manipulation of fat depots. The ability to achieve relatively low fat deposition in undesirable fat depots, while enhancing desirable fat deposition (as marbling fat), without the use of controversial (exogenous) regulators would go a long way toward enhancing the efficiency and sustainability of meat animal growth and reducing the financial cost and the environmental impact of producing meat animals.

Alternative possibilities

What do we really know about fat cell determination, differentiation, and/or development? Published reports with 3T3-L1 (and other) cell lines, stromal-vascular cells, and short-term mature fat cell cultures have provided a clearer understanding about preadipocyte differentiation into a lipid-filled fat cell, about the specific cellular and molecular markers that transiently appear and disappear during the process of preadipocyte differentiation into lipid-filled adipocytes (Kokta et al., in press), and about the metabolic properties/regulation of mature adipocytes. Other than a few observational reports from several years ago, there existed little data, until recently, to suggest that mature fat cells possessed the ability to dedifferentiate into less differentiated phenotypes (Figure 1) and possess the capability to undergo population expansion (Figure 2).

In reality, if mature fat cells do not possess the capability to dedifferentiate and form stem-like cells with the capability to proliferate and expand the population of potential lipid storing cells, there are other (equally important) explanations for the apparent "plasticity" of the mature adipocytes. One such possibility, which certainly goes against all dogma regarding most somatic cells, is that what we are currently describing as mature adipocytes may actually be bipotent fibroblast/preadipocyte cells (adipofibroblasts; Vierck et al., 1996a,b; 2001) that possess a propensity towards accumulation of lipid. Under this possible scenario, adipofibroblasts proliferate until the cell population is expanded to the correct number, and then without becoming terminally differentiated (precluding future proliferation), these cells are regulated with proper extrinsic signals (e.g., IGF, cortisol, etc.) to initiate lipid metabolism/accumulation. However, even though the cells may appear to be mature adipocytes, they retain their capacity to lose lipid and return to the proliferative mode when

required. Indeed, this has been suggested to occur with other cell types. Rupnick et al. (2002) proposes that the endothelial cells associated with blood vessels in adipose tissue, too, are immature because they respond to anti-angiogenic agents. Alternatively, what may appear to be a fully committed mature adipocyte with lipid storage ability may actually represent a cell type that we do not fully understand – one that is capable of expressing both a differentiated and a proliferative phenotype. In light of reports of asymmetric cell division by lipid-filled (apparently mature) fat cells (Sugihara et al., 1987; Zhang et al., 2000), as well as reports of mature adipocytes releasing their lipid and reverting to a more fibroblast/proliferating phenotype (Adebonojo 1975a; Adebonojo 1975b; Sugihara et al., 1986; Sugihara et al., 1987; Sugihara et al., 1989; Justesen et al., 2004), the traditional definition of adipogenic cell commitment may need to be revised.

Summary

If a mature fat cell, which possesses large lipid droplets, is capable of undergoing replication, what does this imply in terms of the cell being fully committed and terminally differentiated? We suggest that studies with mature adipocytes (or their progeny cells) will provide much information about the cellular and molecular mechanisms that regulate the cells of adipose depot fractions. Further, the developmental potential of cells of the adipocyte lineage may be further defined, which may offer alternative points for manipulating the fatness of an animal. This type of information is lacking in the current literature but may be addressed by research utilizing mature fat cell cultures, or progeny cell cultures. Validation of results may be accomplished using differentiation markers (e.g., PPARγ, C/EBPα), molecular biological techniques (RNA isolation and PCR, etc.), established cell lines, or stromal-vascular cell cultures. The use of parallel types of fat cell cultures would provide useful comparative

information about the characteristics and regulation of all cells residing in connective tissue, which may harbor an accumulation of different populations of adipocytes.

REFERENCES

Adebonojo, F.O. (1975a) Studies on human adipose cells in culture: Relation of cell size and cell multiplication to donor age. *The Yale Journal of Biology and Medicine* **48**, 9-16.

Adebonojo, F.O. (1975b) Monolayer cultures of disaggregated human adipocytes. *In Vitro* **11**, 50-54.

Aihaud, G., Grimaldi P. and Negrel R. (1992) Cellular and molecular aspects of adipose tissue development. *Annual Reviews in Nutrition* **12**, 207-233.

Akambi, K.A., Brodie, A.E., Suryawan, A. and Hu, C.Y. (1994) Effect of age on the differentiation of porcine adipose stromal-vascular cells in culture. *Journal of Animal Science* **72**, 2828-2835.

Altomonte, J., Harbaran, S., Richter, A. and Dong, H. (2003) Fat depot-specific expression of adiponectin is impaired in zucker fatty rats. *Metabolism* **52**, 958-963.

Barber, M.C., Ward, R.J., Richards, S.E., Salter, A.M., Buttery, P.J., Vernon, R.G. and Travers, M.T. (2000) Ovine adipose tissue monounsaturated fat content is correlated to depot-specific expression of stearoyl-CoA desaturase gene. *Journal of Animal Science* **78**, 62-68.

Bjorntorp, P. (1991) Adipose tissue distribution and function. *International Journal of Obesity* **15**, 67-81.

Bjorntorp, P., Karlsson, M., Patterson, P. and Sypniewska, G. (1980) Differentiation and function of rat precursor cells in primary culture. *Journal of Lipid Research* **21**, 714-723.

Boone, C., Gregoire, F. and Remacle, C. (2000) Culture of porcine stromal-vascular cells in serum-free medium: Differential action of various hormonal agents on adipose conversion. *Journal of Animal Science* **78**, 885-895.

Broad, T.E. and Ham, R.G. (1983) Growth and adipose differentiation of sheep preadipocyte fibroblasts in serum-free medium. *European Journal of Biochemistry* **135**, 33-39.

Caserta, F., Tchkona, T., Civelek, V.N., Prentki, M., Brown, N.F., Mcgarry, J.D., Forse, R.A., Corkey, B.E., Hamilton, J.A. and Kirkland, J.L. (2001) Fat depot origin affects fatty acid handling in cultured rat and human preadipocytes. *American Journal of Physiology Endocrinology and Metabolism* **280**, E238-E247.

Cianzio, D.S., Topel, D.G., Whithurst, G.B., Beitz, D.C. and Self, H.L. (1985) Adipose tissue growth and cellularity: Changes in bovine adipocyte size and number. *Journal of Animal Science* **60**, 970-976.

Cornelius, P., MacDougald, O.A. and Lane, M.D. (1994) Regulation of adipocyte development. *Annual Review of Nutrition* **14**, 99-129.

Cryer, A., Gray, B.R. and Woodhear, J.S. (1984) Studies on the characterization of bovine adipocyte precursor cells and their differentiation in vitro, using an indirect-labeled-second-antibody cellular immunoassay. *Journal of Developmental Physiology* **6**, 159-176.

De La Hoz, L. and Vernon, R.G. (1993) Endocrine control of sheep adipose tissue fatty acid synthesis: Depot specific differences in response to lactation. *Hormone and Metabolic Research* **25**, 214-218.

Deslex, S., Negrel, R. and Ailhaud, G. (1987). Development of a chemically defined serum-free medium for differentiation of rat adipose precursor cells. *Experimental Cell Research* **168**, 15-30.

Djian, P., Roncari, D.A.K. and Hollenberg, C.H. (1983). Influence of anatomical site and age on the replication and differentiation of rat adipocyte precursors in culture. *Journal of Clinical Investigation* **72**, 1200-1208.

Dixon-Shanies, D., Rudick, J. and Knittle, J.L. (1975) Observations on the growth and metabolic functions of cultured cells derived from human adipose tissue. *Proceedings of the Society for Experimental Biology and Medicine* **149**, 541-545.

Eguinoa, P., Brocklehurst, S., Arana, A., Mendizabal, J., Vernaon, R.G. and Purroy, A. (2003) Lipogenic enzyme activities in different adipose depots of Pireniacan and Holstein bulls and heifers taking into account adipocyte size. *Journal of Animal Science* **81**, 432-440.

Fajas, L. (2003) Adipogenesis: A cross-talk between cell proliferation and differentiation. *Annuals of Medicine* **35**, 79-85.

Gaben-Cogneville, A.M., Aron, Y., Idriss, G., Jahchan, T., Pello, J.Y. and Swierczewski, E. (1983) Differentiation under the control of insulin of rat preadipocytes in primary culture: Isolation of homogenous cellular fractions by gradient centrifugation. *Biochemica et Biophysica Acta* **762**, 437-444.

Gamou, S., Shimizu, Y. and Shimizu, N. (1990) Adipocytes. In: Pollard, J.W. and Walker, J.M. (Eds), Methods in Molecular Biology (Clifton: New Jersey: Humana Press Vol 5), pp. 197-207

Green, H. and Meuth, M. (1974) An established pre-adipose cell line and its differentiation in culture. *Cell* **3**, 127-133.

Green, H. and Kehinda, O. (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell* **7**, 105-113.

Greenwood, M. and Hirsch, J, 1974. Postnatal development of adipocyte cellularity in the normal rat. *Journal of Lipid Research* **15**, 474-483.

Gregoire, F.M. (2001) Adipocyte differentiation: From fibroblast to endocrine cell. *Experimental Biology and Medicine* **226**, 997-1002.

Hauner, H. (1990) Complete adipose differentiation of 3T3 L1 cells in a chemically defined medium: Comparison to serum-containing culture conditions. *Endocrinology* **127**, 865-872.

Hausman, G.J. Novakofski, J.E., Ramsay T. and Martin R.J. (1985) Adipocyte development in primary rat cell cultures, effect of cell density and serum source. *Journal of Animal Science* **60**, 1553-1561.

Hausman, G.J. (1989) The influence of insulin, triiodothyronine (T3) and insulin-like growth factor-I (IGF-I) on the differentiation of preadipocytes in serum-free cultures of pig stromal-vascular cells. *Journal of Animal Science* **67**, 3136-3143.

Hausman, G.J., Jewell D.E. and Hentges E.J. (1989) Endocrine regulation of adipogenesis. In: Campion, D.J., Hausman G.J. and and Martin R.J. (Eds), Animal Growth Regulation. (New York: New York: Plenum Press).

Hausman, G.J., Novakofski, J.E., Martin, R.J. and Thomas, G.B. (1984) The development of adipocytes in primary stromal-vascular culture of fetal pig adipose tissue. *Cell and Tissue Research* **236**, 459-464.

Hausman, G.J. and Wright, J.T. (1990) Genetic, endocrine and paracrine/autocrine aspects of porcine adipocyte differentiation. *Reciprocal Meat Conference Proceedings* **43**, 73-80.

Hausman, G.J., Hausman, D.B. and Martin, R.J. (1992) Biochemical and cytochemical studies of preadipocyte differentiation in serum-free culture of porcine stromal-vascular cells: interaction of dexamethasone and growth hormone. *Acta Anatomica* **143**, 322-329.

Hausman, G.J., Wright, J.T. and Richardson, R.L. (1996) The influence of extracellular matrix substrata on preadipocyte development in serum-free cultures of stromal-vascular cells. *Journal of Animal Science* **74**, 2117-2128.

Hirsch, J. and Han, P. (1969) Cellularity of rat adipose tissue; effects of growth starvation and obesity. *Journal of Lipid Research* **10**, 77-82.

Houseknecht, K.L., Baile, C.A., Matteri, R.L. and Spurlock, M.E, (1998) The biology of leptin: A review. *Journal of Animal Science* **76**, 1405-1420.

Huerta-Leidenz, N.O., Cross, H.R., Savell, J.W., Lunt, D.K., Baker, J.F. and Smith, S.B. (1996) Fatty acid composition of subcutaneous adipose tissue from male calves at different stages of growth. *Journal of Animal Science* **74**, 1256-1264.

Johnson, P.R. and Francendese, A.A. (1985) Cellular regulation of adipose tissue growth. *Journal of Animal Science* **61**(suppl 2), 57-75.

Justesen, J, Pedersen, S.B., Stenderup, K. and Kassem, M. (2004) Subcutaneous adipocytes can differentiate into bone-forming cells *in vitro* and *in vivo*. *Tissue Engineering* **10**, 381-391.

Kirkland, J.L., Hollenberg, C.H. and Gillon, W.S. (1990) Age, anatomical site and the replication and differentiation of adipocyte precursors. *American Journal of Physiology* **258**, C206-C210.

Kokta, T., Dodson, M.V., Gertler, A. and Hill, R.A. (in press) Intercellular signaling between adipose tissue and muscle tissue. *Domestic Animal Endocrinology*

Lee, Y.B. and Kauffman, R.G. (1974a) Cellular and enzymatic changes with animal growth in porcine adipose tissue. *Journal of Animal Science* **38**, 532.

Lee Y.B. and Kauffmann, R.G. (1974b) Cellularity and lipogenic enzyme activities of porcine intramuscular adipose tissue. *Journal of Animal Science* **38**, 538-544.

Lee Y.B. and Kauffmann, R.G. (1974c) Cellular and enzymatic changes with animal growth in porcine adipose tissue. *Journal of Animal Science* **38**, 532.

Lelliot, C.J., Logie, L., Sewter, C.P., Berger, D., Jani, P., Blows, F., O'Rahilly. S. and Vidal-Puig, A. (2002) Lamin expression in human adipose cells in relation to anatomical site and differentiation state. *The Journal of Clinical Endocrinology and Metabolism* **87**, 728-734.

Mackall, J.C., Student, A.K., Polakis, S.E. and Lane, M.D. (1976) Induction of lipogenesis during differentiation in a preadipocyte cell line. *The Journal of Biological Chemistry* **251**, 6462-6464.

Mandrup, S. and Lane, M.D. (1997) Regulating Adipogenesis. *The Journal of Biological Chemistry* **272**, 5367-5370.

May, S.G., Savell, J.W., Lunt, D.K., Wilson, J.J., Laurenz, J.C. and Smith, S.B. (1994) Evidence for preadipocyte proliferation during culture of subcutaneous and intramuscular adipose tissues from Angus and Wagyu crossbred steers. *Journal of Animal Science* **72**, 3110-3117.

Ng, C.W., Poznanski, W.J., Borowiecki, M. and Reimer, G. (1971) Differences in growth in vitro of adipose cells from normal and obese patients. *Nature* **231**, 445.

Nougues, J., Reyne, Y. and Dulor, J.P. (1988) Differentiation of rabbit adipocyte precursors in primary culture. *International Journal of Obesity* **12**, 321-333.

Novakofski, J. (1987) Primary cell culture of adipocytes. In: Hausman, G.J., Martin, R. (Eds), Biology of the adipocyte: Research Approaches (New York: New York: Van Nostrand Reinhold Company), pp 160-197.

Pons, C.M., Mattacks, C.A. and Sadler, D. (1992) The effects of exercise and feeding on the activity of lipoprotein lipase in nine different adipose depots of guinea pigs. *International Journal of Biochemistry* **24**, 1825-1831.

Pozanski, W.J., Waheed, I. and Van, R. (1973) Human fat cell precursors. Morphologic and metabolic differentiation in culture. *Laboratory Investigation* **29**, 570-576.

Ramsay, T.G., Hausman, G.J. and Martin, R.J. (1987) Effects of fetal versus postnatal sera upon adipose tissue stromal-vascular cells in primary culture. *Cell and Tissue Research* **250**, 185-190.

Ramsay, T.G., White, M.E. and Wolverton, C.K. (1989) Glucocorticoids and the differentiation of porcine preadipocytes. *Journal of Animal Science* **67**, 2222-2229.

Reed, B.C. and Lane, M.D. (1980) Insulin receptor synthesis and turnover in differentiating 3T3-L1 preadipocytes. *Proceedings of the National Academy Science* **77**, 285-289.

Robelin, J. (1981) Cellularity of bovine adipose tissues: Developmental changes from 15 to 65 percent mature weight. *Journal of Lipid Research* **22**, 452-457.

Rosen, E.D. and Spiegelman, B.M. (2000) Molecular regulation of adipogenesis. *Annual Reviews in Developmental Biology* **16**, 145-171.

Rupnick, M.A., Panigrahy, D., Zhang, C.Y., Dallabrida, S.M., Lowell, B.B., Langer, R. and Folkman, M.J. (2002) Adipose tissue mass can be regulated through the vasculature. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 10730-5.

Sadowski, H.B., Wheeler, T.T. and Young, D.A. (1992) Gene expression during 3T3-L1 adipose differentiation. *The Journal of Biological Chemistry* **266**, 4722-4731.

Sato, K., Nakanishi, N. and Mitsumoto, M. (1996) Culture conditions supporting conversion of stromal-vascular cells from bovine intramuscular adipose tissues. T*he Journal of Veterinary Medical Science* **58**, 1073-1078.

Serrero, G. and Khoo, J.C. (1982) An in vitro model to study adipose differentiation in serum-free medium. *Analytical Biochemistry* **120**, 351-359.

Shahparaki, A., Grunder, L. and Soriski, A. (2002) Comparison of human abdominal subcutaneous versus omental preadipocyte differentiation in primary culture. *Metabolism* **51**, 1211-1215.

Shigematsu, M., Watanabe, H. and Sugihara, H. (1999) Proliferation and differentiation of unilocular fat cells in the bone marrow. *Cell Structure and Function* **24**, 89-100.

Smith, S.B. and Crouse, J.D. (1984) Relative contributions of acetate, lactate and glucose to lipogenesis in bovine intramuscular and subcutaneous adipose tissue. *The Journal of Nutrition* **114**, 782-800.

Smith, T.R. and McNamara, J.P. (1987) Adipocyte cellularity and body growth in two crossbred lines of beef cattle. *Proceedings of the Western Section of the Society of Animal Science* **38**, 127-129.

Soret, B., Lee, H-J., Finley, E., Lee, S.C. and Vernon, R.G. (1999) Regulation of differentiation of sheep subcutaneous and abdominal preadipocytes in culture. *Journal of Endocrinology* **161**, 517-524.

Spiegelman, B.M. and Ginty, C.A. (1983) Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell* **35**, 657-666.

Sugihara, H., Yonemitsu, N., Miyabara, S. and Yun, K. (1986) Primary cultures of unilocular fat cells: Characteristics of growth in vitro and changes in differentiation properties. *Differentiation* **31**, 42-49.

Sugihara, H., Yonemitsu, N., Miyabara, S. and Toda, S. (1987) Proliferation of unilocular fat cells in primary culture. *Journal of Lipid Research* **28**, 1038-1045.

Sugihara, H., Funatsumaru, S., Yonemitsu, N., Miyabara, S., Toda, S. and Hikichi, Y. (1989) A simple culture of fat cells from mature fat tissue fragments. *Journal of Lipid Research* **30**, 1987-1995.

Suryawan, A. and Hu, C.Y. (1995) The primary cell culture system for preadipocytes. In: Smith S.B. and Smith D.R. (Eds), The Biology of Fat in Meat Animals (Champaign: Illinois: American Society of Animal Science), pp 78-92.

Suryawan, A. and Hu, C.Y. (1997) Effect of retinoic acid on differentiation of cultured pig preadipocytes. *Journal of Animal Science* **75**, 112-117.

Suryawan, A., Swanson, L.V. and Hu, C.Y. (1997) Insulin and hydrocortisone, but not triiodothyrnine, are required for the differentiation of pig preadipocytes in primary culture. *Journal of Animal Science* **75**, 105-111.

Sztalryd, C., Levacher, C. and Picon, L. (1989) Acceleration by triiodothyronine of adipose conversion of rat preadipocytes from two anatomical locations. *Cellular and Mollecular Biology* **35**, 81-88.

Tafuri, S.R. (1996) Troglitazone enhances differentiation, basal glucose uptake and glut-1 protein levels in 3T3-L1 adipocytes. *Endocrinology* **137**, 4706-4712.

Toscani, A., Soprano, S.R. and Soprano, K.J. (1990) Sodium butyrate in combination with insulin or dexamethasone can terminally differentiate actively proliferating Swiss 3T3 cells into adipocytes. *The Journal of Biological Chemistry* **265**, 5722-5730.

Van, R. and Roncari, D.A. (1977) Isolation of fat cell precursors from adult rat adipose tissue. *Cell and Tissue Research* **181**, 197-203.

Van, R.L. and Roncari, D.A. (1978) Complete differentiation of adipose precursors. A culture system for studying the cellular nature of adipose tissue. *Cell and Tissue Research* **195**, 317-329.

Van, R., Bayliss, C.E. and Roncari, D.A. (1976) Cytological and enzymological characterization of adult human adipocyte precursors in culture. *The Journal of Clinical Investigatio*n **58,** 699-704.

Vernon, R.G. (1980) Lipid metabolism in the adipose tissue of ruminant animals. *Progress in Lipid Research* **19**, 23-106.

Vernon, R.G. (1986) The growth and metabolism of adipocytes. In: Buttery, P.J., Lindsay D.B. and Haynes, N.B. (Eds) Control and Manipulation of Animal Growth. (London: England: Butterworths).

Vierck, J.L., McNamara, J.P. and Dodson, M.V. (1996a) Two alternative procedures to isolate adipofibroblasts from sheep skeletal muscle. *Methods in Cell Science* **18**, 309-314.

Vierck, J.L., McNamara, J.P. and Dodson, M.V. with technical assistance by Mathison, B.D. (1996b) Proliferation and differentiation of progeny of ovine unilocular fat cells (adipofibroblasts). *In Vitro Cellular &Developmental Biology* **32**, 564-572.

Vierck, J.L., Dal Porto, D. and Dodson, M.V. (2001) Induction of preadipocyte differentiation by a defined treatment medium without DMI. *Basic and Applied Myology* **11**, 99-104.

Wise, L.S. and Green, H. (1978) Studies of lipoprotein lipase during the adipose conversion of 3T3 cells. *Cell* **13**, 233-242.

Wise, L.S. and Green, H. (1979) Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells. *The Journal of Biological Chemistry* **254**, 273-275.

Wu, K.S., Suzuta, F., Hikasa, Y. and Kagota, K. (2000) Effects of lipid-related factors on adipocyte differentiation of bovine stromal-vascular cells in primary culture. *The Journal of Veterinary Science* **62**, 933-939.

Wu, X., Hoffsted, T.J., Deeb, W., Singh, R., Sedkova, N., Zilbering, A., Zhu, L., Park, P.K., Arner, P. and Goldstein, B.J. (2001) Depot-specific variation in protein-tyrosine phosphatase activities in human omental and subcutaneous adipose tissue: A potential contribution to differential insulin sensitivity. *The Journal of Clinical Endocrinology and Metabolism* **86**, 5973-5980.

Zhang, H.H., Kumar, S., Barnett, A.H. and Eggo, M.C. (2000) Ceiling culture of mature human adipocytes: use in studies of adipocyte functions. *The Journal of Endocrinology* **164**, 119-128.

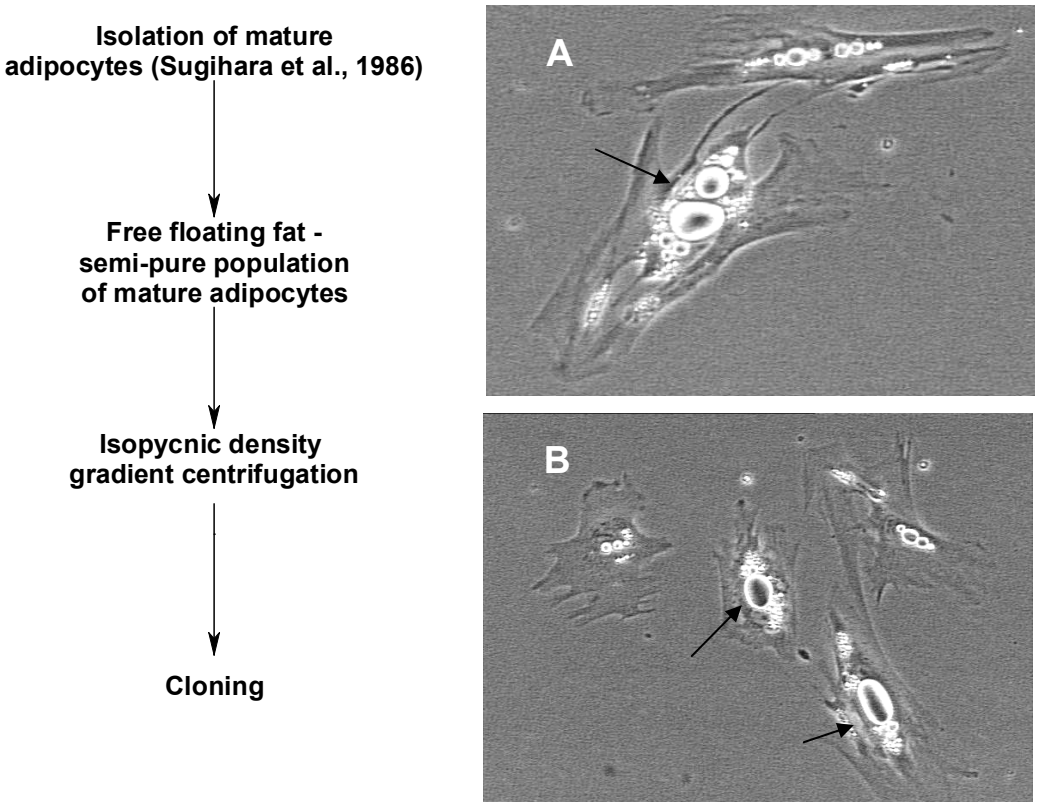

Figure 1: A flow diagram illustrating one procedure used to isolate (representative) mature adipocytes (Panel A: initial division to produce two progeny cells in Panel B). Isopycnic density gradient centrifugation was performed using 3 ml OptiPrep® mixed into 11 ml serum-containing medium. The gradient was preformed by centrifuging in a Beckman TJ-6 centrifuge with a TH-4 horizontal rotor for 30 min at 800 X g. The free-floating fat layer (approximately 1 ml) was removed during the last step in the traditional collagenase isolation procedure, layered onto the top of the preformed gradient, and centrifuged for 30 min at 800 X g. The easily discernable floating cell layer was removed from the gradient and placed into ceiling culture (see chapter 4) for 7 d on DMEM + 10 % horse serum (HS) before inverting. After inversion of the flask, any remaining stromal-vascular cells, fibroblasts, adipofibroblasts or undetermined mononuclear cells (without lipid) were removed, the basal medium was changed to DMEM + 10 % fetal bovine serum (FBS), and the existing adipocytes were evaluated with photomicroscopy for 8 d. (Panel A) The morphology of two lipid-containing mature adipocytes after a total of 8 d in culture and 24 h on a basal medium of DMEM + 10 % FBS (40 X magnification). The multilocular appearance of these cells may be attributed to the length of time needed before cultures can be manipulated (approximately 7 d) and the cells' ability to dedifferentiate. (Panel B) After 48 h of exposure to DMEM + 10 % FBS, the two original cells had divided to produce daughter cells. Each daughter cell possessed half the complement of lipid of the parental cell (20 X magnification). The arrows point to the parent cell (Panel A) and the two daughter cells (Panel B). Photomicrographs were taken with a NIKON inverted Diaphot microscope, equipped with Sony RGB (0.6 in chip) camera and OPTIX image analysis system.

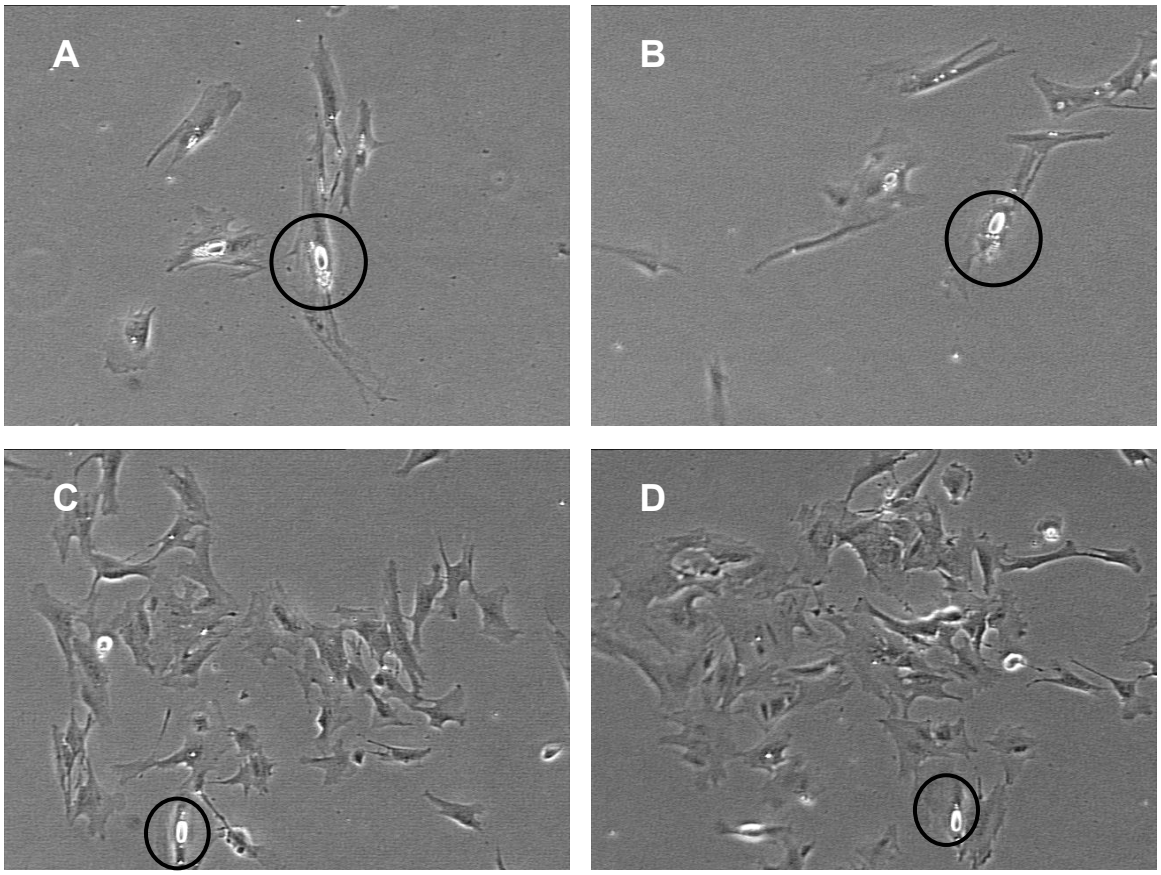

Figure 2: Photomicrographs of a colony of proliferative adipofibroblast cells derived from the dedifferentiation of mature adipocytes in Figure 1. (Panel A) Ten total days after isolation and following a 72 h exposure to DMEM + 10 % FBS, the cells now numbered six (20 X magnification). (Panel B) The same colony after 96 h of exposure to DMEM + 10 % FBS (20 X magnification). (Panel C) After 7 total days exposure to DMEM + 10 % FBS, the colony numbered 35 (10 X magnification). (D) On the last day of monitoring, the colony contained 43 cells (10 X magnification). Each subsequent division of cells resulted in a lower amount of detectable lipid in each daughter cell with the exception of the one cell (circled in all Panels). This cell was one of the original two cells cloned (refer to Figure 1) and appeared to withdraw from the cell cycle after only a few rounds of cell division.

CHAPTER FIVE

Cytotechnology

Primary adipocyte culture: Adipocyte purification methods may lead to a new understanding of adipose tissue growth and development*

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Abstract

In the present manuscript, the methods required to generate purified cultures of mature adipocytes, as well as stromal vascular cells, from the same isolation are detailed. Also, we describe the *in vitro* conditions for the dedifferentiation of the isolated mature adipocytes. These two types of cells may be used to reevaluate differences between presently available cellular models for lipogenesis/lipolysis and might provide a new cellular physiological system for studies utilizing the proliferative progeny from mature adipocyte dedifferentiation. Alternative possibilities to the dedifferentiation phenomenon are proposed, as this new area of research is novel.

Key Words: Adipofibroblasts, ceiling culture, cell culture, dedifferentiation, mature adipocytes

Abbreviations: DMEM = Dulbecco's modified Eagle medium; DMEM/F12 = 1:1 ratio; Dulbecco's modified Eagle medium + Ham's F12; FBS = fetal bovine serum; HBSS = Hank's balanced salt solution; HS = horse serum; PBS = phosphate buffered saline, pH 7.08; PSG = pigskin gelatin; SC = satellite cell

1. Introduction

The cellularity of adipogenesis has been questioned, since mature adipocytes possess the ability to proliferate and form populations of proliferative-competent progeny cells (Fernyhough et al., 2005a). This previous communication has led to descriptive papers, which discuss the physiological significance of such plasticity displayed by the seemingly differentiated mature adipocyte (Dodson et al., 2005; Fernyhough et al., 2005b). However, exact methods that might allow researchers to isolate absolutely pure populations of mature adipocytes for subsequent research endeavors have not been published. As such, in the present communication, we provide detailed methods, which will allow for the isolation of purified cultures of mature adipocytes from beef-derived fat tissue, and their subsequent progeny cell cultures. These same isolation procedures may be used for adipose tissue from any other animal.

2. Materials

A. Major equipment

1. Autoclave, gravity air remover type, model P-89501-091.[1]
2. Balance, model A-160.[6]
3. Centrifuge, model TJ-6.[4]
4. CO_2 water-jacketed incubator, model NU-4500.[14]
5. Dry heat gravity oven, model 1370 GM.[18]
6. Laminar flow, biological safety cabinet, Labguard Class II, Type A/B3, model NU-425-4000.[14]
7. Microscope, Diaphot-TMD phase inverted.[13]
8. Peristaltic pump, model 7015-72.[11]

9. pH electrode, model 47636.[5]

10. pH meter, digital, model 430.[5]

11. Pipet Aid, model 174.[7]

12. Propane torch, Bernz-o-matic model TS2000.[17]

13. Water bath stainless steel, model 185.[15]

B. Media, reagents, and chemicals

1. Bovuminar protease-free BSA, product number 3100-01.[8]

2. Collagenase type I, product number 17100-017.[8]

3. Dulbecco's modified Eagle's medium (DMEM), product number 12800-017.[8]

4. Dulbecco's modified Eagle's medium (DMEM)/ Ham's F12 (F12), product number 12500-062.[8]

5. Ethylenediamine tetraacetic acid (EDTA), product number 15576-028.[8]

6. Fetal bovine serum (FBS), product number 26140-079.[8]

7. Hank's balanced salt solution (HBSS), product number H-1387.[16]

8. HCl, product number H-1758.[16]

9. Horse serum (HS), product number 16050-114.[8]

10. Gentamicin, 10 mg/ml gentamicin sulfate, product number 15710-064.[8]

11. Giemsa stain, product number GS-1L. [17]

12. D-(+)-Glucose, product number G-7021.[17]

13. KCl, product number P-5405.[17]

14. KH_2PO_4, product number P- 5655.[17]

15. NaCl, product number S-5886.[17]

16. $NaHCO_3$, product number S-5761.[17]

17. NaOH, pellet, product number 3722-I. [9]

18. Na_2PO_4, product number S-5136.[17]

19. OptiPrep®, product number D1556.[17]

20. Penicillin-streptomycin (pen/strep), liquid; contains 10,000 units of penicillin G (sodium salt) and 10,000 µg of streptomycin sulfate/ml in 0.85 % saline, product number 15140-122.[8]

21. Trypsin, product number T-4549.[17]

C. Glassware

1. Media bottles

   a. 125 ml with cap, product number 219715.[20]

   b. 250 ml with cap, product number 219717.[20]

   c. 500 ml with cap, product number 219719.[20]

2. Pipettes

   a. 2 ml, glass disposable, product number 72120-21100.[10]

   b. 5 ml, glass disposable, product number 53283-774.[19]

   c. 10 ml, glass disposable, product number 53283-776.[19]

   d. Pasteur, non-plugged, glass, 5 ¾" X 1/10", product number 14672-200.[19]

3. Volumetric flask, 1000 ml, product number 2813-1000.[10]

D. Plastic supplies

1. Beakers

   1000 ml, polypropylene, product number 1201-1000.[12]

2. Centrifuge tubes

   a. 50 ml, product number 352070.[2]

b. 15 ml, product number 352096.[2]

3. Tissue culture flask, 25 cm^2, product number 83.1810.001.[16]

4. Tissue culture flask, 12.5 cm^2, product number 353018.[2]

3. Procedures

A. Preparation of reagents and media used in cell culture

1. Preparation of PBS

   a. Dissolve 10.0 g NaCl, 0.25 g KCl, 1.44 g Na_2HPO_4 in 900 ml of glass distilled H_2O.

   b. Adjust to pH to 7.08 with HCl/NaOH.

   c. Bring volume to 1 L in a volumetric flask.

   d. Aliquot into 2-500 ml bottles and autoclave 30 minutes at 121° C at 15 psi.

   e. Store at 4° C.

2. Preparation of trypsin solution

   a. Prepare PBS/EDTA by adding 0.168 g $Na_2EDTA \cdot 2H_2O$ to 1 liter of PBS.

   b. Sterilize by autoclaving for 30 minutes at 121° C at 15 psi.

   c. Add 10 ml sterile trypsin to 90 ml sterile PBS/EDTA.

   d. Store frozen.

3. Preparation of DMEM

   a. Dissolve a 1-liter envelope of DMEM and 3.7 g $NaHCO_3$ in 900 ml of glass distilled H_2O.

   b. Adjust pH to 7.08 with HCl/NaOH.

   c. Bring the volume to 1 liter in a volumetric flask.

d. Filter sterilize in the laminar flow hood by passing the medium through a 0.22 µm pore filter into a sterile 1-liter bottle.

e. Store at 4° C.

4. Preparation of DMEM/F12

a. Dissolve a 1-liter envelope of DMEM/F12 and 2.438 g $NaHCO_3$ in 900 ml of glass distilled H_2O.

b. Adjust pH to 7.08 with HCl/NaOH.

c. Bring the volume to 1 liter in a volumetric flask.

d. Filter sterilize in the laminar flow hood by passing the medium through a 0.22 µm pore filter into a sterile 1-liter bottle.

e. Store at 4° C.

5. Preparation of HBSS

a. Dissolve a 1-liter container of HBSS and 0.35 g $NaHCO_3$ in 900 ml of glass distilled H_2O.

b. Adjust pH to 7.08 with HCl/NaOH.

c. Bring the volume to 1 liter in a volumetric flask.

d. Filter sterilize in the laminar flow hood by passing the medium through a 0.22 µm pore filter into a sterile 1-liter bottle.

e. Store at 4° C.

6. Preparation of HBSS + antibiotics/antimycotics

In the laminar flow hood, supplement the HBSS with antibiotics and antimycotics by adding 1 % Pen/Strep, 0.5 % ml Gentamicin, and 0.001 % (1:1000 dilution) of antimycotic to sterile HBSS. Store at 4° C.

7. Basal medium + 10 % serum (FBS or HS)

   In the laminar flow hood, prepare 500 ml of serum-containing medium by adding 50 ml of serum, 5.0 ml of pen/strep, 2.5 ml gentamicin to 442.5 ml of basal medium. Store at 4° C.

8. Preparation of the enzyme solution

   Measure 100 ml of PBS into a glass beaker and place on a stir plate. Add 1.5 g BSA and 90.1 g mg glucose. For this procedure, any good quality BSA would likely be acceptable. As we are eventually going to devise a completely defined system to evaluate fractions of isolated cells, we used a form of BSA that was protease and fatty acid free. Stir until all BSA is dissolved then filter through a 0.22 μm pore vacuum filter. Just before use, add 0.25 g collagenase to warmed (37° C) PBS solution and sterile filter through a 0.22 μm pore vacuum filter. The final concentrations of the solution are 0.25 % collagenase, 5 mM glucose, and 1.5 % BSA in PBS.

Experimental Procedures

Cell isolation. Adipocytes and cells possessing similar buoyant densities are initially isolated and cultured as a modification of an earlier method (Sugihara et al., 1986; Figure 1a).

1. Transport the adipose tissue slice from the abattoir (since this isolation is for beef-derived fat tissue; if smaller animals are used, then the tissue isolation may be obtained in the tissue culture laboratory) to the cell culture laboratory in sterile 37° C HBSS supplemented with antibiotics and antimycotics.

2. In a laminar flow hood, place the tissue in a sterile 150 mm dish, bathe in HBSS, and cut into approximately 1-cm^2 pieces using sterile scissors and thumb forceps.
3. Place approximately 5 g of tissue into each of the four 50 ml centrifuge tubes (20 g tissue total). Pipette or pour 25 ml sterile enzyme solution to each tube. Place the tubes on a rocker and incubate 1 hr at 37 °C.
4. After enzymatic cell dispersal, filter the tissue in each tube through a sterile 1000 μm plastic mesh in a sterile funnel into a clean sterile 50 ml centrifuge tube.
5. Spin the filtrate in a centrifuge for 10 min at 186 × g. In the laminar flow hood, remove and discard the underlying pellet (containing preadipocytes, fibroblasts, and erythrocytes) and media. If the stromal vascular cells are wanted, remove the underlying pellet and transfer to a tissue culture flask containing serum-containing medium. Re-suspend the remaining fat layer in 20 ml HBSS and centrifuge for 10 min at 186 × g. Repeat this step two additional times.
6. After the last centrifugation step, transfer the fatty layer (containing the mature adipocytes) to 12.5 cm^2 cell culture flasks (one flask per tube). Fill the flasks completely with a 1:1 mix of DMEM/F12 + 10 % HS.
7. Invert the flasks so that the bottom of the flask is on top. This allows the floating unilocular adipocytes to attach to the upper portion of the flask and any remaining fibroblast-like cells to sink to the bottom. Incubate the flasks at 37 °C in a 5 % CO_2 incubator and monitor daily for cell attachment. There will be residual cells that contaminate the floating cell compartment, and these will need to be removed from the cultures in order to insure a homologous population of cells for research

purposes. The removal of the contaminating cells is vital for subsequent success in culturing mature fat cells and determining that mature fat cells are capable of dedifferentiating to form proliferative-competent cells.

8. After sufficient attachment of the cells (usually 5-7 d) remove the medium, replace with 5 ml fresh medium, and re-invert the flasks. This allows for the normal observation and subsequent manipulation of the cultures.

Purification of isolated cells

Differential plating. Two methods of differential plating are employed to insure the purity the mature adipocyte cultures. The first method (early differential plating; Figure 1b) exploits the extended time needed for the unilocular adipocytes to attach to the cell culture surface, as compared to any preadipocyte and fibroblast that might have been co-isolated and cultured. The second method of differential plating (late differential plating; Figure 1c) involves cultures that are firmly adhered to the flask (*i.e.* greater than 4 d in culture).

Early Differential Plating

1. Remove the medium from each flask and transfer to a clean flask.
2. Add additional serum-containing medium (if needed) to fill the flask.
3. Place each flask once more in ceiling culture. This transfer will allow the more tenuously attached mature adipocytes to detach (and be removed with the medium) and the cells without lipid (preadipocytes and fibroblasts) to remain attached to the discarded flask.

Late Differential Plating

1. Enzymatically detach the cells from the culture flask by removing the medium from each flask and adding 2 ml of a 10 % trypsin solution. Place the flask into the incubator.
2. After 4-5 min, monitor cell detachment under a phase contrast microscope. When the cells have all detached, remove the cell suspension, and pipette into a sterile 50 ml centrifuge tube.
3. Rinse the flask with 5 ml serum-containing medium and add to the 50 ml centrifuge tube.
4. Neutralize any remaining trypsin by adding 10 ml serum-containing medium to the 50 ml centrifuge tube.
5. Centrifuge for 10 min at 186 × g.
6. Gently pipette the top 15 ml of supernatant into a clean 12.5 cm^2 flask and fill with DMEM/F12 + 10 % HS.
7. Place the flasks in ceiling culture (*i.e.* invert the flasks). Cells that are lipid- laden will float and adhere to the culture surface whereas non-lipid containing cells, which had not previously discarded in the pellet, will sink and adhere to the non-culture surface. The cells usually attach within 24 h, after which time the excess medium can be removed and the flask re-inverted.
8. If, after the cells have attached, many non lipid-containing cells are seen in the flask, a second differential plating may need to be performed to purify the cultures.

Isopycnic density gradient centrifugation. This method has been used for many years to separate not only different cell types from each other (especially in hematological studies) but

also to separate intracellular organelles for experimentation. Herein, we describe a method to use Optiprep® to separate lipid-filled cells.

1. Pipette Optiprep® into a 15 ml centrifuge tube containing DMEM/F12 + 10 % HS. Four different ratios are used in this experiment: 1 ml Optiprep® + 13 ml serum-containing medium, 2 ml Optiprep® + 12 ml serum-containing medium, 3 ml Optiprep® + 11 ml serum-containing medium, and 4 ml Optiprep® + 10 ml serum-containing medium (Figure 4).
2. Centrifuge the solution for 30 min at 800 × g to pre-form the gradient.
3. After the last step during initial adipocyte isolation (before placing in ceiling culture; Step 5), layer the uppermost 1 ml of supernatant on the preformed density gradient and centrifuge for 30 min at 800 × g.
4. Once the centrifugation cycle is complete, transfer the upper 1 ml of supernatant to a flask and fill with a 1:1 mix of DMEM/F12 + 10 % HS. Invert the flask and place in ceiling culture.

Cloning. The above methods will remove almost all of the non lipid-containing cells from the cultures. Any remaining fibroblastic cells must be removed through culture surgery and cloning techniques.

1. After sufficient cell attachment in ceiling culture (1 – 5 d), followed by a primary method of purification, remove all but 5 ml of the medium from the flask.
2. Using a phase contrast inverted microscope, in a laminar flow hood mark the cells containing lipid with an indelible marker on the bottom of the flask for daily monitoring (Refer to Figure 3). Also, mark non lipid-containing cells for removal

(with a non permanent ink). Use different color inks for lipid-filled and non-lipid filled cells.

3. While looking through the microscope eyepieces, scrape the cells previously marked for removal off the flask with a sterile Pasteur pipette.
4. Wash the cultures 3 X with DMEM + 10 % HS and add 5 ml of medium to the flask.
5. Monitor the cultures daily and remove any non-lipid containing cells. The purified cultures are ready for experimentation.

4. Results and Discussion

The methods describe here have been found to generate pure populations of mature adipocytes. Although this laboratory has employed all the described techniques in various different combinations, the cells displayed in Figure 2 were obtained with an early differential plating 2 d after first isolation and two sequential late differential platings. After the cultures were purified (*i.e.* without any fibroblastic/preadipocyte cells) the cultures were exposed to a traditional growth medium (DMEM + 10 % FBS). The cells were monitored daily for proliferative activity, which subsequently occurred. In fact, a previous communication (Fernyhough et al., 2005b) showed mitotic figures in mature adipocytes just prior to mitosis. These cells may be studied for regulation of lipolysis as well as for mechanisms underlying their propensity to proceed from the mature phenotype to a proliferative-competent (presumably less differentiated) cell type (preadipocyte or adipofibroblast – termed reverse differentiation or dedifferentiation). In addition, the progeny cells are homologous and may be used for detailed study of the regulation of lipogenesis and dynamics of lipid accumulation.

The photomicrographic results obtained through these procedures clearly demonstrate the ability of these mature adipocytes to proliferate *in vitro* (Figure 5; Dodson et al., 2005; Fernyhough et al., 2005a; Fernyhough et al., 2005b). In this communication, we have presented additional evidence that mature adipocytes proliferate *in vitro*. This concept has previously been disputed, stemming from the idea that once the cells accumulated intracellular lipid, adipocytes were considered "terminally differentiated". Our overall hypothesis is that that the accumulation of lipid is not a terminal event for these cells. Our next set of objectives include: a) attempting to determine the specific number of cells, in any adipose depot, that are capable of dedifferentiating, b) Evaluating this phenomenon in young versus old animals, animals of different sexes and breeds, as well as c) other physiological states that may influence the dedifferentiation process. As we have not defined the specific cellular and biochemical/molecular characteristics of the cells that are capable of dividing, it may be possible that the isolated lipid-filled cells in Figure 5 represent a different cell form, such as a fibroblast, which may accumulate and release lipid in a similar manner to an adipocyte. In addition, other, yet unknown, cells, that are capable of metabolizing lipid similar to adipocytes, might also reside in these depots. However, we suggest that this area of research is of sufficient interest such that a re-evaluation of the cell composition of adipose tissue depots may be needed. Collectively, this research area may lead to a new understanding of adipose tissue growth and development.

Application of Methods

The many processes regulating adipose tissue development are of considerable interest due not only to their physiological effects, but also to their potential effects on current medical concerns. For example, obesity is of great concern as adults classified as overweight topped

64.5 % (Flegal et al., 2002) and has been deemed a predisposing factor in other medical conditions such as diabetes and heart disease. Indeed, both diabetes and hypertension has been steadily increasing every year in the U.S. The percentage of adults who have been ever been diagnosed as having diabetes was 6.6% in 2003 (up from 5.1% in 1997; Schiller et al., 2005) and the percent of adults with hypertension from 1999-2002 was 30.1 % (Schiller et al., 2005).

5. In addition to the biomedical field, adipose development is of concern in the economically important traits of food-producing animals such as cattle. These traits include fat depth and distribution (including marbling) as well as the overall efficiency of nutrient use (Hansen et al., 2004). For example, a reduction in subcutaneous fat (waste fat) would be of tremendous economic savings to producers. The total cost of excess fat to the U.S. beef industry has been shown to be $ 4.4 billion; $ 2 billion in production costs and $ 2.4 in shipping and removal costs (Ritchie et al., 1993). Additionally, marbling has been shown to influence consumer preferences for beef (meat) products. Understanding the mechanisms underlying adipocyte differentiation and adipogenesis could eventually help producers to modulate the characteristics of adipose tissues and promote the occurrence of marbling deposition without the excess accumulation of subcutaneous or visceral fat (Hansen et al., 2004)

6. Notes on Suppliers

1. American Sterilizer Company, Erie, PA, USA
2. BD Falcon, Bedford, MA, USA
3. Beckman Coulter Inc., Fullerton, CA, USA
4. Beckman Instruments Inc., Palo Alto, CA, USA
5. Corning Incorporated Life Sciences, Acton, MA USA
6. Denver Instrument Co., Arvada, CO 80004, USA
7. Drummond Scientific Co., Brownhill, PA, USA
8. Invitrogen/Life Technologies, Carlsbad, CA USA
9. JT Baker, Paris, KY, USA
10. Kimble/Kontes, Vineland, NJ, USA
11. Millipore Corp., Bedford, MA, USA
12. Nalge Nunc™, Rochester, NY
13. Nikon, Nippon K. K., Tokyo, Japan
14. Nu Aire Inc., Plymouth, MN, USA
15. Precision Scientific, Chicago, IL, USA
16. Sarstedt, Newton, NC, USA
17. Sigma Chemical Company, St Louis, MO, USA
18. The Newell Group, Medina, NY, USA
19. VWR Scientific Products Corporation, Chester, PA, USA
20. Wheaton Scientific Products, Millville, NJ, USA

Acknowledgements

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References

Dodson MV, Fernyhough ME, Vierck JL & Hausman GJ (2005): Adipocytes may not be a terminally differentiated cell type: Implications for animal production. Anim. Sci. 80:239-240.

Fernyhough ME, Bucci L, Hausman GJ, Antonio J, Vierck J & Dodson MV (2005a): Gaining a Solid Grip on Adipogenesis. Tissue & Cell 37:79-82.

Fernyhough ME, Helterline DL, Vierck JL, Hausman GJ, Hill RA & Dodson MV (2005b): Dedifferentiation of mature adipocytes to form adipofibroblasts: More than just a possibility. Adipocytes 1:17-24.

Flegal KM, Carroll MD, Ogden CL & Johnson CL (2002): Prevalence and trends in obesity among US adults, 1999-2000. Jama 288:1723-7.

Hansen C, Fu A, Li C, Dixon WT, Christopherson R & Moore SS (2004): Global gene expression patterns spanning 3T3-L1 preadipocyte differentiation. Can. J. Ani Sci. 84:367-376.

Ritchie HD, Rust SR, Merkel RA & Bergen WG (1993): Getting rid of excess fat is not an easy task. Feedstuffs:13.

Schiller JS, Martinez M & Barnes P (2005): Early release of selected estimates based on data from the 2004 National Health Interview Survey. National Center for Health Statistics http://www.cdc.gov/nchs/nhis.htm.

Sugihara H, Yonemitsu N, Miyabara S & Yun K (1986): Primary cultures of unilocular fat cells: characteristics of growth in vitro and changes in differentiation properties. Differentiation 31:42-9.

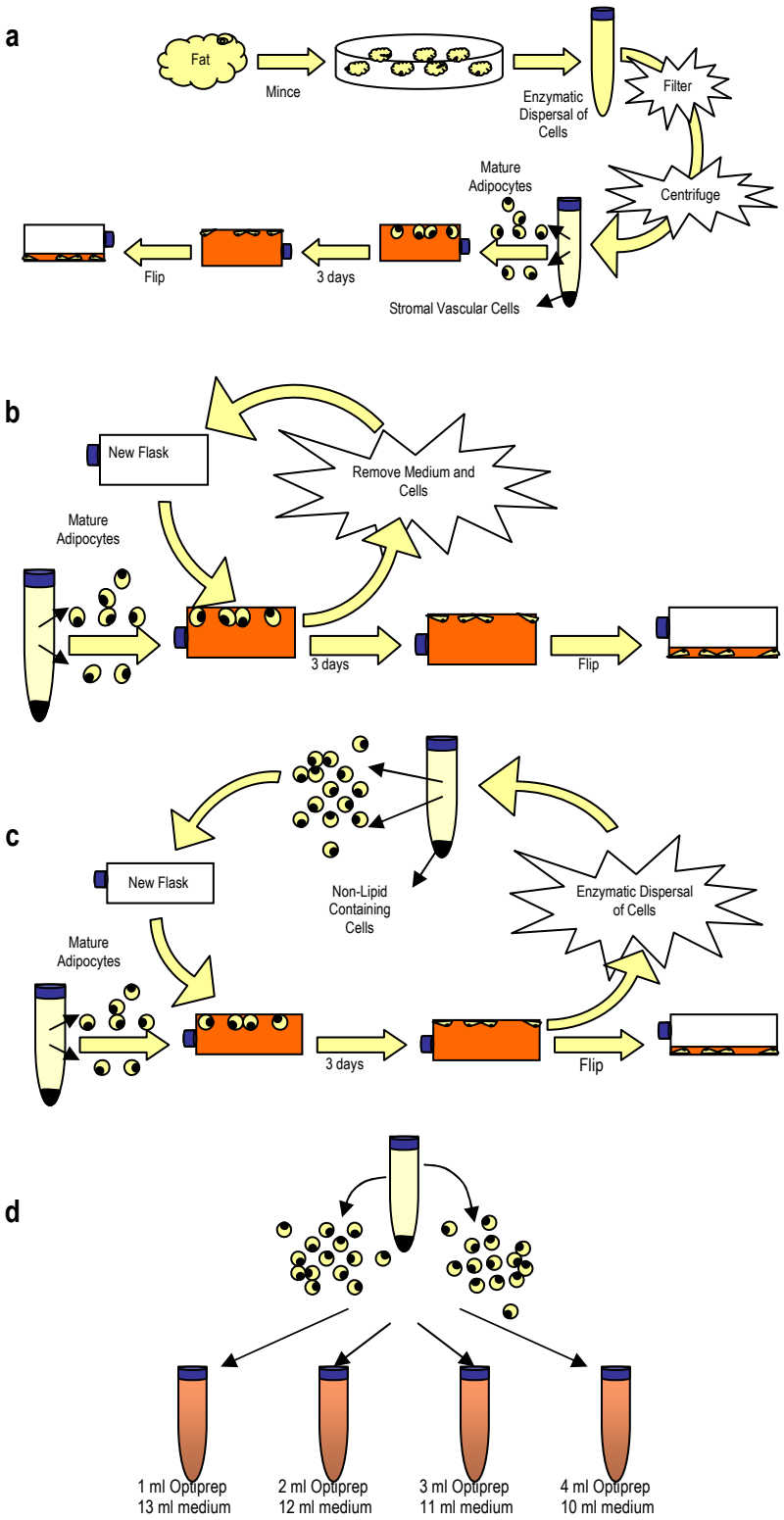

Figure 1: A flow diagram outlining the steps in (a) primary adipocyte isolation, (b) early differential plating, (c) late differential plating, and (d) isopycnic density gradient centrifugation.

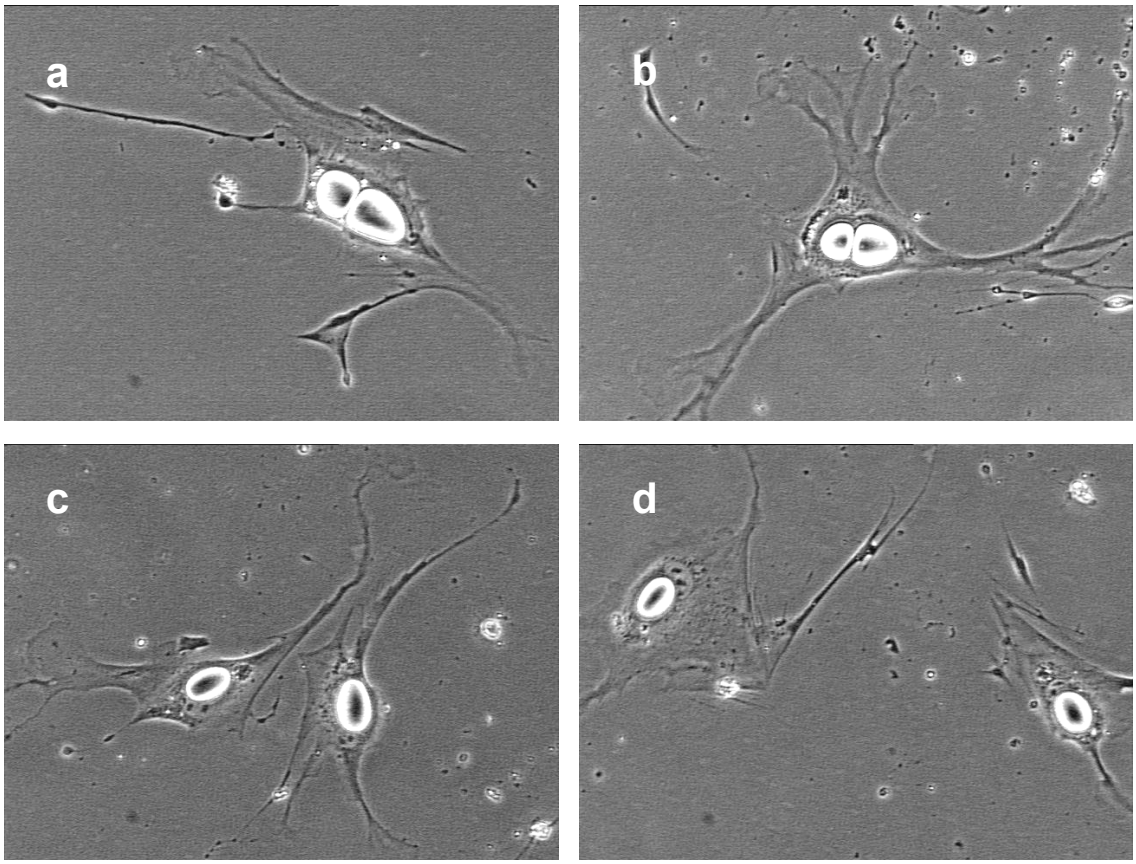

Figure 2: Photomicrograph of a bilocular bovine adipocyte undergoing proliferation *in vitro*. The culture was purified using the early differential plating protocol within 2 d of isolation and two serial late differential platings. Once the cultures were pure (no fibroblastic cells), the cultures were exposed to a traditional growth medium (DMEM + 10 % FBS). The cultures were monitored daily for non-lipid laden cells and, once the culture was pure, the culture flask was exposed to a proliferative medium (DMEM + 10 % FBS) for 5 d before cell division resulted: (a) the appearance of the maternal cell 24 h after the proliferative treatment was applied. Panel (b) shows the same cell after 3 d. Five days after the proliferative medium was applied (c), the cell divided, through symmetric cell division, into two daughter cells. Panel (d) shows the two daughter cells after 6 d. All photomicrographs were captured at 20 X magnification with a NIKON inverted Diaphot microscope, equipped with Sony RGB (0.6 in chip) camera and OPTIX image analysis system.

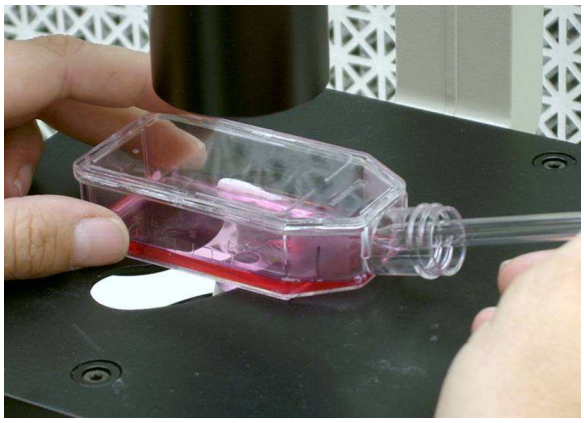

Figure 3: Photograph of a flask during the cloning procedure. The microscope has been placed in a laminar flow hood and the non-lipid containing cells have been marked on the bottom of the flask. In the hood, the cap was removed from the flask and the Pasteur pipette was inserted into the flask. While looking through the eyepieces of the microscope, the marked cells are scraped off the plate.

TROUBLESHOOTING TABLE

PROBLEM	SOLUTION
Low cell yield from isolation This can occur from a number of problems: (1) the time from removal of tissue from animal to processing is too long; (2) the tissue was left in the enzyme solution for too long or (3) not long enough; (4) the collagenase used in the enzyme solution has lost its activity; or (5) not enough of the supernatant was removed during the last step in isolation.	(1) Shorten the time from removal of tissue to processing. Keep the HBSS solution at 37 ° C. (2) When removing the tissue from the enzyme solution check the turbidity of the liquid. If it is clear or only slightly turbid, you may want to increase the incubation time. (3) Leaving the tissue in the enzyme solution for too long will have a detrimental effect on the cells – shorten the incubation time. (4) If the collagenase is out dated or appears to have lost activity a replacement with fresh collagenase is warranted. (5) Remove more of the supernatant to place in ceiling culture.
Large numbers of mononucleated cells in the flask after isolation	(1) Increase the centrifugation time during initial isolation to pellet the heavier non lipid-containing cells. (2) Perform an early differential plating.
Large numbers of mononucleated cells in the flask after late differential plating	(1) Check the centrifuge settings. (2) Increase the amount of centrifuge time to allow the heavier mononucleated cells to pellet at the bottom of the tube. (3) Remove less of the supernatant taking care to remove the uppermost supernatant. (4) Perform a second late differential plating.
Low adipocyte cell yield from late differential platings Spinning too fast or for too long will disrupt the membranes of the lipid-laden cells causing a low cell yield.	(1) Check the centrifuge settings. (2) Decrease the amount of centrifuge time allowing the lipid-laden cells to remain suspended. This solution may yield more fibroblastic cells than desired. Perform a second differential plating.
Low numbers of fibroblastic cells in the cultures after late differential plating	(1) With a small number of fibroblastic cells on the culture surface, it is easy to perform cloning techniques. Alternatively (2) another late differential plating may be performed.
Low adipocyte cell yield from isopycnic density gradient centrifugation	(1) Check the centrifuge settings. (2) Increase the spin time when pre-forming gradient. (3) Increase the amount of Optiprep® placed in culture after the final spin.

CHAPTER SIX

Protracted adipogenesis of pure cultures of adipofibroblasts*

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ABSTRACT

Pure cultures of progeny adipofibroblasts, derived from the dedifferentiation of cloned mature (beef-derived) fat cells, were used to evaluate their ability to redifferentiate into lipid-filled adipocytes *in vitro*. The adipofibroblasts proliferated and overgrew the culture dishes when exposed to Dulbecco's Modified Eagle Medium + 10% fetal bovine serum but demonstrated protracted adipogenesis when exposed to media containing 10 % horse serum. Protracted adipogenesis was evident through the adipofibroblast accumulation of cytoplasmic vesicles with no/little lipid incorporation, even though immunocytochemistry for peroxisome proliferator activated receptor γ was positive. The vesicles first appeared five d after a uniform monolayer was established, but remained for up to 60 d *in vitro* without the cells undergoing proliferation. The adipofibroblast vesicles did not possess glycogen, and lipid uptake was not enhanced by the addition of insulin and/or lipid to serum-containing medium. Examination of the ultrastructure of cytoplasmic vesicles with transmission electron microscopy revealed a discrete structure and immunofluorescence demonstrated perilipin surrounding the vesicle. Induction medium ± thiazolidinedione did not enhance lipid uptake, and vesicle formation was delayed compared to control cultures. Expression of peroxisome proliferator activated receptor γ was demonstrated through mRNA isolation and PCR. Lastly, peroxisome proliferator activated receptorγ, perilipin, hormone sensitive lipase, and lipoprotein lipase proteins were demonstrated in the adipofibroblasts with an immunoblot. Collectively, these data suggest that mature adipocytes dedifferentiate to form proliferative-competent progeny cells, that dedifferentiated adipofibroblasts retain their adipocyte lineage characteristics and possess the ability to undergo *in vitro* adipogenesis without complicated induction procedures or

through the use of exogenous chemicals, and that the adipofibroblasts do not experience *in vitro* transformation. The protracted nature of cellular conversion from adipofibroblasts to adipocytes may now be exploited in studies to determine the cellular/molecular regulation of adipogenesis or progressive regulation of lipid metabolism.

Key words: dedifferentiation, adipofibroblast, adipogenesis, lipid vesicles, thiazolidinedione, rosiglitazine, bovine adipocyte

INTRODUCTION

Several cell models are available for studying adipogenesis: established cell lines such as 3T3-L1 cells (Green and Kehinde, 1975), stromal vascular cells (SV; Hausman and Richardson, 1998; Boone et al., 2000a; Hausman and Poulos, 2004), and primary cells (Fernyhough et al., 2004; Cancello et al., 2005; Dodson et al., 2005; Fernyhough et al., 2005a; Fernyhough et al., 2005b; Tholpady et al., 2005). 3T3-L1 cells, and other immortalized cell lines, although widely studied, may not be the best models for the study of adipogenesis, since the immortality of these cells does not mimic the state of cells *in vivo*, in which a cell possesses a finite ability to divide (Campisi et al., 1996). Some established cell lines are aneuploid (Rosen and Spiegelman, 2000), but these cells do not undergo apoptosis like primary cells would. Also, established cell lines such as 3T3-L1 cells are already committed to the adipocyte lineage and provide little information about the regulation of determination (Rosen, 2005). Furthermore, different cell lines are halted at different stages of differentiation, which makes comparing data between cell lines or cell models difficult.

The use of primary cells offers advantages over the established cell lines. One such advantage is the ability to select the depot from which the cells are obtained. Cells isolated from different adipose depots appear to be dissimilar not only in cellularity but also in regulation and endocrine function (Rosen and Spiegelman, 2000; Wu et al., 2000; Caserta et al., 2001; Stolic et al., 2002; Li et al., 2003; Boucher et al., 2005; Tchkonia et al., 2005). As such, using cells that originate from only one adipose depot to represent the adipose lineage may not give a true picture of the entire adiposity of the animal. Of the primary cell systems, SV cells are highly utilized in studies of adipogenesis (Ramsay et al., 1992; Prunet-Marcassus et al., 2006), but the use of these cells also has drawbacks (Ntambi and Young-Cheul, 2000).

Stromal vascular cells are a mixture of preadipocytes, fibroblasts, epithelial cells, blood cells, and haemopoetic stem cells (Marques et al., 1998; Kim et al., 2001; Granneman et al., 2004). Thus, the exact numbers of adipocyte precursor cells within the SV fraction from any adipose depot are difficult to ascertain (May et al., 1994). If the cell proportion cannot be enumerated, then it is hard to identify the contributions an individual cell population has towards the cumulative, overall cell physiology observed *in vitro* (Ntambi and Young-Cheul, 2000). Furthermore, as it is almost impossible to identify the factors produced by individual cell populations, the mixture of cells in the same culture well makes identification of the regulation of the adipocyte fraction very complex, especially if additional growth regulators are added to the medium (Hausman, 2005).

Although primary adipocytes are difficult to isolate, purify, and propagate *in vitro* (Shigematsu et al., 1999), the benefits of using homogeneous purified cultures of primary cells may outweigh the inherent difficulties in obtaining and culturing them (Boone et al., 1999; Zhang et al., 2000). We have previously described a method by which we purified mature adipocytes and descriptively monitored their dedifferentiation physiology *in vitro* (Fernyhough et al., 2004; Dodson et al., 2005; Fernyhough et al., 2005a; Fernyhough et al., 2005b).These purified progeny cells (adipofibroblasts) are committed to the adipose lineage and retain their capacity to proliferate but little else is known about them (Fernyhough et al., 2004; Dodson et al., 2005; Fernyhough et al., 2005a; Fernyhough et al., 2005b). Regardless of the cell type used, the conversion of cells committed to the adipose lineage and the subsequent lipid assimilation into early (differentiated) adipocytes appear to be mediated by environmental (extrinsic) agents such as hormones, growth factors (cortisol, GH, insulin, IGF-1), and free fatty acids (FFA; McNeel and Mersmann, 2003; McNeel et al., 2003; Madsen et al., 2005;

Matsubara et al., 2005), to ultimately mediate intrinsic factors such as PPARγ (Rosen, 2005). A typical extrinsic differentiation medium consists of insulin, dexamethasone, and a phosphodiesterase inhibitor such as methylisobutylxanthine (Rosen, 2005). This program "induces" a preadipocyte to begin to express the cellular and molecular markers associated with lipid synthesis, assimilate/store triglycerides, and perform endocrine functions (Kokta et al., 2004) at which time, the cell is termed an adipocyte (Boone et al., 2000b). The events associated with preadipocyte proliferation are thought to be diametrically opposite to those occurring when the preadipocyte differentiates into an adipocyte (Gregoire, 2001; Fajas, 2003; Rosen, 2005), and terminal (the adipocyte appears to be incapable of further proliferation; Russell and Ho, 1976).

Preadipocytes passing through this differentiation process *in vitro* display characteristic changes in cell morphology and specific molecular/cellular markers, such as PPARγ, are evident (Boone et al., 2000a). Morphologically, nascent lipid droplets initially appear as a perinuclear halo (Boone et al., 2000a), which will later coalesce into larger lipid droplets (Boone et al., 2000a). Ultimately, the cell contains a peripherally located nucleus and a unilocular lipid droplet (Russell and Ho, 1976), which can be stained with oil red O (Kutt and Tsaltas, 1959; Kinkel et al., 2004). The specific physiological transitions of adipofibroblasts through the adipogenesis differentiation program are unknown.

The objective of this research was to examine the events in the conversion of the adipofibroblast cells into lipid-assimilating adipocytes, including the expression of adipogenic markers like PPARγ. This paper addresses several hypotheses: the adipofibroblasts were capable of redifferentiation into viable lipid-assimilating cells *in vitro*, that redifferentiation

would be almost 100 % due to the homogeneous population dynamics of the initial cell preparation, that the redifferentiation time would parallel that observed with other adipogenic cell models, and that the cytosolic vesicles observed in the adipofibroblasts exposed to a horse serum induction procedure were lipid storage vesicles. Our results are supportive of the adipofibroblasts representing a unique adipogenic model for further studies.

MATERIALS & METHODS

Animals, research experimental design, and data analyses

Fat samples, taken from 15-24 mo old beef cattle during routine slaughter at the AALAC accredited Washington State University (WSU) abattoir, were excised from the ventral midline brisket fat (subcutaneous) depot, immediately placed into sterile buffer (with antibiotics) and transported to the cell culture laboratory (Vierck et al., 1996). The subcutaneous depot was selected because of ease of sample removal, the size of the depot (second largest in the body), and the cellularity is constant during this time in the growth of hte animal. The WSU Animal Care and Use Committee screened the use of animals in this research, and the animal use met the standards imposed by both the USDA and PHS. Because each experiment was designed to provide either a positive or a negative answer, experiments were observed for morphological responses to treatments such as proliferation, dedifferentiation, vesicle formation, lipid accumulation, and cell death. All controls, positive and negative, are described in the methods.

Mature adipocyte isolation

Adipose tissue was used for both the isolation of RNA (detailed later) and cells. Mature bovine adipocytes were isolated by collagenase dispersion and placed in ceiling culture as previously described (Sugihara et al., 1986; Fernyhough et al., 2004; Fernyhough et al., 2005b). In brief, adipose tissue was minced and subjected to enzyme action (Sugihara et al., 1986; Vierck et al., 1996). After 1 h, the tissue/cell suspension was filtered to remove undigested material and centrifuged to pellet any non-lipid containing cells (traditional stromal vascular cell fraction; this fraction was discarded). The floating fatty layer (mature adipocytes) was transferred to cell culture flasks, the flasks were filled completely with a 1:1 mixture of

Dulbecco's Modified Eagle Medium and Ham's F-12 (DMEM/F12) + 10 % serum, inverted (so that the bottom of the flask was on top; this process is termed ceiling culture), and placed into an incubator (37 °C and 5 % CO_2).

Mature adipocyte culture

After sufficient time for the attachment of the mature adipocytes (5 to 7 d), the medium was changed, and the flasks were re-inverted. The lipid-laden cells were purified from any remaining fibroblast-like cells using a combination of differential plating, isopycnic density gradient centrifugation, and cell culture surgery/cloning techniques (Fernyhough et al., 2004; Tholpady et al., 2005). The cloned mature adipocytes were allowed to dedifferentiate (Fernyhough et al., 2004; Dodson et al., 2005; Fernyhough et al., 2005a; Fernyhough et al., 2005b) and proliferate, and the progeny adipofibroblasts (Fernyhough et al., 2004; Dodson et al., 2005; Fernyhough et al., 2005a; Fernyhough et al., 2005b) were frozen in liquid nitrogen for future use.

Comparison of serum type on mature adipocytes

A variety of media containing fetal bovine serum (FBS) and various supplements are commonly used when isolating, propagating, or inducing the differentiation of adipogenic cells (Russell and Ho, 1976; Student et al., 1980; Stewart et al., 2004). When bovine-derived adipofibroblasts were exposed to FBS-containing media, unexpected cell physiology was observed. To determine the specific effects of different sera on mature adipocyte dedifferentiation and proliferation, parallel cultures of mature adipocyte isolates (three separate isolations; 2-12.5 cm^2 flasks per treatment) were maintained in ceiling culture on a basal medium of either DMEM/F-12 + 10 % FBS or DMEM/F-12 + 10 % horse serum (HS) from initial isolation. Once the cells had sufficiently attached, the flasks were re-inverted, the media

were changed, and all the cell cultures were monitored for cell death, aberrant morphology, potential lipid loss, general cell health, and any morphological suggestion of proliferation (Vierck and Dodson, 2000). In a second experiment, parallel isolates of mature adipocytes (4-12.5 cm^2 flasks) were maintained on DMEM/F12 + 10 % HS until the ceiling culture flasks were re-inverted (approximately 7 d), and the basal medium was either changed to DMEM + 10 % FBS (2-12.5 cm^2 flasks) or kept on DMEM + 10 % HS (2-12.5 cm^2 flasks). Cells were monitored daily as described previously.

Adipofibroblast cell culture

When needed, frozen adipofibroblasts were thawed, placed into a 75 cm^2 flask, and allowed to proliferate for 72 h in a humidified environment at 37 °C in an atmosphere of 95 % air and 5 % CO_2. When the cells were approximately 60 % confluent, they were enzymatically released from the flask and either plated into a 24-well culture plate for use in an experiment or into a propagation culture. Only cells of passage numbers 1-11 were used for these studies. The cells were allowed to attach for 24 h in Dulbecco's Modified Eagle Medium (DMEM) + 10 % serum, the medium was removed, the cultures were thoroughly washed three times with DMEM to remove serum components, and the cells were then exposed to appropriate treatments. The cultures were monitored daily, and the treatment medium was changed every 2 d. At the end of each treatment regimen, the cultures were fixed and evaluated.

Comparison of serum type on adipofibroblasts

The adipofibroblasts were exposed to two different sera to assess the effect of the serum type on cell health, proliferation ability, or extent of differentiation. The adipofibroblasts were plated at a density of 100 nuclei/mm^2 on uncoated 24-well cell culture plates and exposed to DMEM + 10 % HS. At confluence, the wells were washed with DMEM

and either 1 ml DMEM + 10 % FBS or DMEM + 10 % HS was added. The cells were monitored daily for cell health and lipid uptake. The treatment medium was changed every 2 d. After 12 d , the cells were fixed and stained (Kutt and Tsaltas, 1959; Kinkel et al., 2004), and the cell number and percent differentiation were determined.

Evaluation of sera components

Because the different sera induced different physiological responses in both the mature cell cultures and the adipofibroblast cultures, we suspected that the sera contained different proportions of adipogenic regulatory agents. Therefore, both HS and FBS samples were sent to the Michigan State University College of Veterinary Medicine Diagnostic Center for Population and Animal Health for evaluation of cortisol, insulin, glucose, and insulin-like growth factor -1 (IGF-1) levels, and fatty acid profiles.

Oil red O staining

The adipofibroblast cultures were stained for lipid content as previously described (Kutt and Tsaltas, 1959; Kinkel et al., 2004). Briefly, the cultures were rinsed in phosphate buffered saline (PBS) to remove medium components and fixed in 10 % formalin for 10 min. Next, the cultures were rinsed well with distilled water and stained with oil red O in an isopropanol carrier for 10 min. The cultures were then rinsed with distilled water for 30 s, counterstained with hematoxylin for 10 min, and rinsed with tap water for 30 s to blue the hematoxylin. The cultures were air-dried and observed/analyzed with light microscopy (Kinkel et al., 2004). Photomicrographs were taken with a NIKON inverted Diaphot microscope, equipped with a Sony RGB (0.6 in chip) camera and an OPTIX image analysis system.

Peroxisome proliferator activated receptor γ immunocytochemistry

Immunocytochemistry was performed on cultures by first removing the media and then fixing the cells in the wells with 4 % paraformaldehyde for 30 min. Following three 1 min washes in PBS, the cell membranes were permeablized in 3 % Nonidet® P-40 (NP-40) for 30 min. The permeablized cells were rinsed three times with PBS for 1 min, and then 3 % hydrogen peroxide was added to the wells for 5 min. These cells were next washed with PBS, exposed for 1 h to 1 % normal goat serum (NGS) in Tris-buffered saline-Tween 20 (TBS-Tween 20; 0.05 M Tris, 0.15 M NaCl, 0.05% Tween 20, pH 7.4), and then washed again. To block any non-specific binding, an avidin/biotin blocking kit (Vector Laboratories) was used following the manufacturer's instructions. The blocked cells were washed again and then incubated at room temperature with a 1:200 dilution of the peroxisome proliferator activated receptor γ (PPARγ) antibody (rabbit polyclonal anti-human/mouse/rat; Research Diagnostics Inc.) in 1 % NGS in TBS-Tween-20. After washing, a 1:200 dilution of a biotinylated secondary antibody (goat anti-rabbit; Vector Laboratories) in 1 % NGS in TBS-Tween-20 was applied and incubated for 1 h. The cells were washed again with PBS, and Elite® ABC reagent (Vector Laboratories) was applied for 30 min. After a final wash with PBS, a color substrate was applied (diaminobenzadene [DAB] or NovaRed®) to the cells according to the manufacturer's instructions. Finally, the nuclei were counterstained with hematoxylin. Four control wells were used for each immunocytochemistry experiment: (1) the primary antibody only, (2) the secondary antibody only, (3) the omission of both primary and secondary antibody (substrate and colorizer only), and (4) colorizer only. Photomicrographs were taken with a

NIKON inverted Diaphot microscope, equipped with a Sony RGB (0.6 in chip) camera and an OPTIX image analysis system.

Periodic acid Schiff staining

The cells were stained for the presence of glycogen within cytoplasmic structures with periodic acid Schiff (Sigma) following the manufacturer's directions with minor alterations. The medium was removed from the wells, and the cells were fixed for 10 min with 10 % formalin and then gently rinsed for 1 min in running tap water. Periodic acid was added to the wells for 5 min. The wells were rinsed 4 X with distilled water, and Schiff's reagent was applied to the wells and left for 15 min. The wells were rinsed in running tap water for 5 min, the nuclei were counterstained with hematoxylin for 90 s, and the wells were rinsed in tap water for 30 s. The wells were then air-dried and evaluated with light microscopy. Photomicrographs were taken with a NIKON inverted Diaphot microscope, equipped with a Sony RGB (0.6 in chip) camera and an OPTIX image analysis system.

Effects of insulin and lipid added to a serum-containing medium

Bovine adipofibroblasts were plated at a density of 100 nuclei/mm^2 on a 24-well plate (3 wells per treatment for a total of 12 wells). After 24 h, the medium was removed and 1 ml of treatment medium was applied. The treatments consisted of DMEM + 10 % HS (control), DMEM + 10 % HS + 0.5 μM insulin, DMEM + 10 % HS + 0.5 μM insulin + 5 ml/L defined lipid mixture (2 μg/ml arachidonic and 10 μg/ml each linoleic, linolenic, myristic, oleic, palmitic and stearic acid, 0.22 mg/ml cholesterol, 70 μg/ml tocopherol acetate; Sigma), and DMEM + 10 % HS + 5 ml/L lipid mixture. The media were changed every 2 d. The cells

were monitored daily for 12 d, then co-stained for PPARγ and for oil red O reactive lipid, and evaluated through the use of light microscopy.

Transmission electron microscopy

The adipofibroblasts were grown in a 75 cm^2 flask until they were confluent and until approximately 90 % contained cytosolic vesicles (20 d). The cultures were then fixed with 2.5 % gluteraldehyde/2 % paraformaldehyde in PBS for 2 h (Robenek et al., 2005a; Robenek et al., 2005b). All the adipofibroblasts were scraped off the flask with a rubber policeman, placed into microcentrifuge tubes and subjected to an overnight post fixation with 1 % osmium tetroxide, followed by a stepwise dehydration in ethanol (Robenek et al., 2005a). After dehydration, the cells were embedded in SPURRs resin and cured overnight. The blocks were then sectioned on a microtome, the sections were placed on grids, and the grids were stained with 2 % uranyl and 1 % lead citrate. The cells were visualized with a transmission electron microscope (JOEL; JEM 1200 EX), and the images were captured with a MegaView III digital camera.

Perilipin immunofluorescence

The adipofibroblasts were grown on Thermanox® cell culture coverslips (Nunc) until the cells were confluent and had formed intracellular vesicles. The coverslips were then removed from the culture wells, washed 3 X for 5 min with cold PBS, fixed for 20 min in 2 % paraformaldehyde + 0.5 % Triton X in PBS, rinsed with cold PBS 3X for 5 min, and then incubated in blocking buffer (1 % NGS in PBS) for 1 h at room temperature. After being washed three times for 5 min in cold PBS, the coverslips were incubated with the primary antibodies (1:100 dilution of guinea pig anti-perilipin and 1:100 dilution of rabbit anti-

vimentin) in 1 % normal goat serum (NGS) at 4 °C overnight. The coverslips were subsequently washed 3 X with cold PBS, and the secondary antibody was added (Texas Red®goat anti-guinea pig [Vector] and Oregon Green goat anti-rabbit [Molecular Probes] in 1 % NGS) and incubated for 1 h at room temperature in the dark. After incubation, the coverslips were rinsed 3 X for 5 min in PBS, mounted on a glass slide with Vectashield®, and visualized with a Nikon inverted microscope (TE300) with a laser confocal attachment (BioRad MRC 1024). The image was captured with LaserSharp 2000™ (BioRad). The control consisted of the omission of both primary antibodies.

Induction of adipogenesis

The effectiveness of a traditional adipogenesis induction protocol, (Gamou et al., 1990), as well as the effect of a thiazolidinedione compound (rosiglitazone; PPARγ ligand) on adipofibroblast gene regulation, was evaluated using the following procedure. Three 150 cm^2 cell culture flasks were plated with 5.0 x 10^5 adipofibroblasts (passage number 11), and the cells were grown to confluence in DMEM + 10 % HS. At confluence (approximately 6 d post plating) one of three treatments was applied. Flask 1 remained on DMEM + 10 % HS, flask 2 received a traditional induction cocktail for 2 d (Gamou et al., 1990) of 0.25 mM MIX, 0.5 mM dexamethasone and 1 μg/μl insulin, and flask 3 received the same induction cocktail as flask 2 with the addition of 0.25 μM rosiglitazone. After 2 d, the media on flasks 2 and 3 were changed to DMEM + 10 % HS (flask 2) and DMEM + 10 % HS + 0.25 μM rosiglitazone (flask 3). The media were changed every other day. The adipofibroblasts were monitored for mitosis, vesicle formation, cell health, and lipid accumulation by phase microscopy. At 11 d post-induction, RNA was isolated from the cells.

RNA isolation from cells and adipose tissue

The treatment medium was removed from the flasks, and the cultured adipofibroblasts were rinsed with RNase-free PBS. One ml of RNA*later* (Ambion) was added to each flask, and the adipofibroblasts were scraped off the flask surface with a rubber policeman. The cell suspensions were then removed from the flasks and placed in 50 ml centrifuge tubes and stored at -20 °C. Total RNA was isolated from the adipofibroblasts using a Qiagen RNeasy mini prep kit following the manufacturer's instructions. Briefly, the adipofibroblasts stored in RNA*later* were pelleted by adding 15 ml RNase-free PBS to each centrifuge tube and centrifuging for 10 min at 1500 X g. The supernatant was removed, and lysis buffer was added to the tube to disrupt the cells. The cells were homogenized by placing the lysate in a Qiashredder spin column (Qiagen) and centrifuging in a microfuge for 2 min at maximum speed. Ethanol (70 %) was added to the lysate, and the lysate was mixed well, pipetted into an RNeasy Mini Spin Column, and centrifuged at $\geq$8000 X g for 15 s. This step was repeated with any additional lysate. Buffer RW1 was added to the RNeasy Mini Spin Column, which was then centrifuged for 15 s at $\geq$8000 X g to wash the column. Buffer RPE was then pipetted onto the RNeasy Spin Column, and the column was centrifuged for 15 s at $\geq$8000 X g to wash the column a second time. This step was repeated. To elute the RNA, 30-50 µl RNase-free water was pipetted onto the RNeasy silica-gel membrane, followed by centrifugation for 1 min at $\geq$8000 x g. The purity and concentration of the RNA samples were determined using a Molecular Devices Spectromax Plus microplate spectrophotometer and Softmax Pro software. The total RNA samples were stored at -20 ° C.

Adipose tissue was transported from the abattoir to the cell culture laboratory and two 1-cm^3 pieces were removed from the sample, placed immediately in RNAlater, and stored at -20°C for RNA isolation. The tissue was removed from storage in RNA*later* and blotted to remove traces of RNA*later*. Total RNA was then isolated using a Qiagen RNeasy mini prep kit, following the manufacturer's instructions with minor alterations. An 85 mg piece of adipose tissue was added to 1 ml of TRIzol Lysis Reagent (Invitrogen) in a 2 ml microfuge tube and immediately homogenized with a Tissue-Tearor homogenizer (BioSpec Products, Inc.) until the sample was uniformly homogenous. The tube was incubated at room temperature for 5 min, and 200 µl of chloroform was added. The tube was shaken vigorously for 15 s and then was allowed to incubate at room temperature for an additional 2 min. The sample was then centrifuged at 12,000 X g for 15 min at 4 °C. The colorless, upper aqueous phase (~ 600 µl) was pipetted into a second microfuge tube. Ethanol (70 %) was added to the lysate, and the lysate was mixed well. The sample was pipetted into an RNeasy Mini Spin Column and centrifuged at $\geq$8000 x g for 15 s. All remaining isolation and detection methods were similar to those described previously for the isolation of RNA from the adipofibroblasts.

RT-PCR

First strand synthesis of cDNA from RNA was performed with a First Strand Synthesis for RT-PCR kit (Invitrogen) following the manufacturer's instructions. Two µl of isolated total RNA from the adipofibroblasts were used for the reverse transcriptase (RT) reactions. RNA isolated from adipose tissue was used as a positive control for the detection of markers in adipofibroblast cultures, and β-actin was used as an internal control. One µl of the RT product (cDNA) was used in the 50 µl PCR amplification reaction for the detection of PPARγ RNA

using the following primers designed against bovine PPARγ (accession No. NM181024): left, 5'-CCCTGGCAAAGCATTTGTAT-3'; right 5'-GGATATGAGGACCCCATCCT-3'; product 389 bases. PCR was performed using a PCR master mix (Platimun *Taq* Supermix; Invitrogen) and a Perkin Elmer Gene Amp 2400 thermalcycler, under the following conditions for 40 cycles: denature at 94°C for 1 min; anneal at 47°C for 30 sec; and extension at 72°C for 25 s. Amplification with β actin primers served as internal controls. The negative control consisted of the reaction with no reverse transcriptase added. The amplification products of each RT-PCR reaction were then separated by electrophoresis in 1% agarose DNA gels and stained with GelStar (Cambrex Biosciences) for nucleic acids. The results were viewed with fluorescent light and captured with GeneSnap software (Spectronics Corp.).

PCR product cleanup and sequencing

Prior to sequencing, excess primers and nucleotides were removed from the PCR products with an ExoSAP-it PCR clean up kit (Amersham). Then the cDNA was submitted to the WSU Center for Reproductive Biology Molecular Biology Core for sequencing. The templates were sequenced with an ABI automated sequencer using the original primers. The nucleotide sequences were analyzed with an NCBI BLASTnn search to determine sequence homology (Benson et al., 2006; Wheeler et al., 2006).

Protein isolation and detection

Nuclear and cytosolic proteins were isolated from the cells (d 45 in culture) with an NE-Per protein extraction kit (Pierce), and membrane proteins were isolated using a Mem-Per protein extraction kit (Pierce), both following manufacturer's instructions. Specific proteins were detected with an immunoblot (Krabbenhoft et al., 1997). Briefly, 0.45 μm nitrocellulose

was placed in TBS-Tween 20 for 25 min and then placed in the Hybridot™ apparatus (BRL). For each of the proteins of interest, 10 μl of protein isolate (nuclear for PPARγ; cytosolic for perilipin and hormone sensitive lipase [HSL]; membrane for lipoprotein lipase [LPL]) was diluted in 100 μl of TBS-Tween 20 and triplicate samples were blotted on nitrocelluose using the Hybridot™ apparatus. The nitrocellulose was then cut into appropriate strips, and the reactions were completed in 100 mm sterile cell culture dishes. Following two 5 min washes in TBS-Tween 20, the nitrocellulose pieces were blocked with 5 % powdered milk in TBS-Tween 20, incubated for 1 h, and then washed again (2 X 5 min in TBS-Tween 20). The blocked nitrocellulose pieces were then incubated at room temperature with a 1:2000 dilution of either the PPARγ antibody, the perilipin antibody, the HSL antibody (Cayman # 10006371), or the LPL antibody (RDI-PRO61081) in TBS-Tween-20. After washing (2 X 5 min in TBS-Tween 20), a 1:2000 dilution of alkaline phosphatase conjugated secondary antibody (goat anti-guinea pig for perilipin, goat anti-rabbit for PPARγ and HSL, goat anti-mouse for LPL; Abcam) in TBS-Tween-20 was applied and allowed to incubate for 1 h. The nitrocellulose pieces were washed, and 1-Step™ NBT/BCIP was added to each dish containing a nitrocellulose piece. Three controls were used for each immunoblot experiment: (1) the secondary antibody only, (2) the primary antibody only, and (3) colorizer only.

RESULTS

Mature adipocyte isolation and culture

Mature lipid-laden adipocytes were successfully isolated from adipose tissue. Based on the numbers obtained from depot characterization studies (Smith and Crouse, 1984; Cianzio et al., 1985), it is estimated that approximately 1.0 X 10^6 adipocytes/gram of adipose tissue may be isolated. Of these, we estimate that 1/100 original isolated adipocytes from the adipose tissue pieces displayed the ability to become proliferative-competent (maternal) adipofibroblasts. Considering that the subcutaneous adipose depot of beef cattle may possess 10-15 kg of tissue at 1 yr of age (Robelin, 1981; Cianzio et al., 1985), which would yield approximately 1.2- 1.5 X 10^{10} cells (total subcutaneous depot; Robelin, 1981; Cianzio et al., 1985), the number of dedifferentiation-competent cells (1.2- 1.5 X 10^8) is physiologically relevant.

Comparison of the effects of serum type on mature adipocytes

The liberated mature adipocytes remained viable, attached to the cell cultureware (ceiling culture), were subsequently purified from any non lipid-containing cells, and dedifferentiated to form proliferative-competent progeny adipofibroblasts. Ceiling cultures of freshly isolated adipocytes exposed to DMEM/F12 + 10 % FBS lost their mature phenotype, dedifferentiated into proliferative-competent cells, and proliferated more quickly than those isolates that were maintained on DMEM/F12 + 10 % HS. Additionally, since the cells in ceiling culture had not yet been purified, there were increased levels of proliferation of non lipid-containing cells in the flasks exposed to DMEM/F12 + 10 % FBS compared to flasks containing DMEM/F12 + 10 % HS. Of the 12.5 cm^2 flasks (n =24) from three isolations, eleven received DMEM/F12 + 10 % FBS as ceiling culture media. Isolates that had been

allowed to attach in ceiling culture and exposed to DMEM/F12 + 10 % HS before changing the medium to DMEM/F12 + 10 % FBS retained their lipid stores until the basal medium was changed, after which the cells began dividing and lost appreciable lipid.

Comparison of the effects of serum type on adipofibroblasts

The adipofibroblasts exposed to DMEM + 10 % HS proliferated slowly (when compared to DMEM + 10 % FBS) and routinely developed cytoplasmic vesicles, beginning at approximately 3-5 d post confluence (Figure 2A). The cells exposed to DMEM + 10 % FBS proliferated rapidly and began forming foci after 10 d, whereas foci formation and cell lifting occurred only rarely after 21 d in cultures exposed to DMEM + 10 % HS. Foci formation was defined as the proliferation of cells to the point where they began to grow on top of one another and the individual cell characteristics were difficult or impossible to discern (Vierck and Dodson, 2000). This growth pattern resulted in large numbers of cell loss when the foci lifted off the cultureware. Subsequent experiments used DMEM + 10 % HS as a basal medium.

Evaluation of sera components

Due to the disparity in cell responsiveness to the serum-containing media, the sera were evaluated to quantify potential adipogenic agents that might be influencing cell physiology. Analysis of the two sera revealed: HS = 146 nmol/L cortisol, 23 pmol/L insulin, 2.0 mmol/L glucose, 18 nmole IGF-1; FBS = no detectable cortisol, 19 pmol/L insulin, 5.2 mmol/L glucose, 10 nmole IGF-1. The results of the fatty acid analyses are summarized in Table 2.

Oil red O staining

When stained with oil red O, the majority of the adipofibroblasts did not assimilate the stain, suggesting that little/no lipid accumulation was occurring in the cells. On a rare

occasion, however, a cell would display one or more stained vesicles (Figure 2B). Moreover, the surrounding cells were a mixture of cells that contained vesicles and showed no uptake of lipid stain, as well as cells without any vesicles at all (Figure 2B).

PPARγ immunocytochemistry

Cultures exposed to DMEM + 10 % HS for 17 d contained both cytoplasmic vesicles and positive staining for the presence of nuclear PPARγ (Figure 2C). Cells that were PPARγ negative did not contain cytoplasmic vesicles (Figure 2C). The control wells did not exhibit PPARγ reactivity (Figure 2D).

Periodic acid Schiff staining

If the cytoplasmic vesicles were not accumulating lipid, it was possible that the vesicles were acting as glycogen storage vehicles (Hausman, personal communication). Therefore, the adipofibroblasts were cultured for 18 d in the presence of DMEM + 10 % HS and then stained with periodic acid Schiff. The adipofibroblast vesicles did not incorporate any stain, suggesting that the vesicles were not for storing glycogen (Figure 2E).

Addition of insulin and lipid to serum-containing medium

Since the cytoplasmic vesicles were not acting as glycogen storage sites, and since some lipid incorporation into individual vesicles was detected on rare occasions, an experiment was designed to attempt to actively induce lipid uptake by the cells (as viewed by incorporation by the vesicles). In the adipofibroblast cell cultures that were exposed to insulin (± lipid), cytoplasmic vesicles appeared in all wells (including control) but at different rates (10 % HS + insulin > 10 % HS + insulin + lipid > 10 % HS + lipid > 10 % HS). The adipofibroblasts exposed to insulin in any treatment proliferated at a greater rate than the cells exposed to 10 %

HS + lipid or 10 % HS (control) and subsequently developed foci after 16 d of treatment. Adipofibroblasts exposed to DMEM + 10 % HS + lipid mixture exhibited greater amounts of lipid filling than did adipofibroblasts exposed to insulin + lipid, insulin only, and control treatments. After 17 d of treatment, the adipofibroblasts were immunostained (NovaRed®, Vector Laboratories) for PPARγ, followed by oil red O staining for lipid. All wells contained cells that stained for PPARγ as well as those that did not stain for PPARγ (Figures 2F, G). Some cells appeared to stain for PPARγ but contained no lipid or vesicles (Figures 2F, G). The cells exposed to DMEM + 10 % HS + 0.5 μM insulin showed low levels of lipid accumulation (Figures 2F, G). The negative control consisted of the omission of the primary antibody and showed no staining for PPARγ (Figure 2H).

Transmission Electron Microscopy

Cells were elongate due to the method of cell harvesting, but the vesicles were clearly recognizable within the cell (Figure 3A, B). The vesicles were surrounded by a phospholipid monolayer, but no filamentous structure was noted in any of the preparations.

Perilipin Immunofluorescence

Perilipin staining (Texas Red) was visible around the periphery of the cytoplasmic vesicles (Figure 3C). Nonspecific staining for vimentin (Oregon green) was evident in the nucleus and cytoplasm (Figure 3C). No fluorescent staining was evident in the control (data not shown).

Induction of adipogenesis

The cells on all three adipogenesis-induction treatments remained healthy and morphologically normal. The cells exposed to DMEM + 10 % HS formed cytoplasmic vesicles,

and it was estimated that approximately 40 % of the cells contained vesicles at the time of termination of the culture (11 d). Vesicle formation was delayed in the two induction treatments until d 11 of treatment, when minute numbers of the cells contained vesicles in the "no rosiglitazone" induction treatment. Lipid assimilation was not noted in any of the treatments. The cells exposed to induction treatments (± rosiglitazone) displayed increased levels of mitosis when compared to the DMEM + 10 % HS treatment (alone). By d 11, the number of mitotic figures was equal in all three treatments. During the second experiment, all the cells remained healthy. Cells treated with the induction/lipid mixture displayed increased proliferation (4 d post treatment) compared to those cells on DMEM + 10 % HS. By d 6 post treatment, the cells exposed to the induction/lipid mixture had lost the cytoplamic vesicles, which had not reappeared by the termination of the experiment (d 17; unpublished observations). Lipid assimilation was not noted in any of the treatments.

RNA isolation

The concentrations of RNA isolated were as follows: adipose tissue 0.26 μg/μl, pre-induction cells on 10 % HS 0.31 μg/μl, induced cells 0.47 μg/μl, induced cells + TZD 0.26 μg/μl, and cells on DMEM + 10 % HS 0.42 μg/μl.

RT-PCR

The primers were validated and the PCR protocol was optimized using RNA from adipose tissue. PPARγ and β actin (internal control) were visualized on a 1 % agarose DNA gel (Figure 4). The PCR product from the cells on 10 % HS, induction + TZD, and induction (no TZD) exhibited some non-specific products. Therefore, the PCR protocol was re-optimized for annealing temperature, annealing time, and primer concentration using RNA from the

cells. Most of the non-specific PCR product was reduced with re-optimization (data not shown). A band was visualized at approximately 390 bases using PPARγ specific primers and RNA from adipose tissue, pre-induction cells, cells on 10 % HS, cells on induction + TZD, and cells on induction (no TZD). All the negative controls displayed no bands and all the reactions for β actin (internal control) demonstrated a band at ~ 500 bases. Cell cultures, still in a proliferative state before induction, were found to contain some expression of PPARγ.

PCR product cleanup and sequencing

The low temperatures required in the PCR protocol prompted sequencing of the PCR product. The results from the PCR product sent to the WSU Center for Reproductive Biology Molecular Biology Core were retrieved and, when a BLAST search was conducted, homologous sequences were returned. Specifically, when the PCR product for PPARγ was analyzed, the sequence resulted in species homology for PPARγ.

Protein isolation and detection

Immunoblot reactions were positive for the proteins of interest (Figure 4). All controls, except one, exhibited no color. Minor non-specific background staining was noted in the first control (no primary antibody) using the goat-anti mouse secondary antibody indicating some cross reactivity with the nitrocellulose or the buffer used (unpublished data). When the control was compared to the treatment, there still existed positive staining for LPL protein.

DISCUSSION

We present evidence to suggest that mature beef-derived adipocytes possess the ability to revert to the adipofibroblast stage of development, as supported by the basic observation that adipofibroblasts derived from cloned mature adipocytes were capable of redifferentiation into viable lipid-assimilating cells *in vitro*. A long held hypothesis has been that mature lipid-laden adipocytes are "terminally" differentiated and thus incapable of proliferation (Crescenzi et al., 1995; Boone et al., 2000b; Latella et al., 2001). Evidence to support this non-proliferation conclusion was hampered by the inherent difficulties in culturing mature adipocytes or their progeny cells (Fernyhough et al., 2004; Tholpady et al., 2005). To address this problem, we used an extended period of ceiling culture at lower initial adipocyte densities (along with cell cloning) to insure the uniformity of the mature adipocytes in culture (Fernyhough et al., 2004; Tholpady et al., 2005). We estimate that we can isolate 1/100 mature adipocytes that possesses the ability to convert to a proliferative-competent progeny adipofibroblast. It is likely that some of the mature cells are destroyed during the isolation process and initial culture manipulations (Etherton et al., 1977; Etherton and Chung, 1981). Therefore, we are likely underestimating the actual numbers of mature adipocytes capable of dedifferentiating *in vivo*. Additional research will be required to assess whether adipocytes from different adipose depots differ in their abilities to dedifferentiate and form proliferative-competent progeny adipofibroblasts.

We did not observe that redifferentiation in any cell culture was 100 %, our expected rate, despite the homogeneous population dynamics of the initial cell preparation. Are there other (alternative) explanations for the cellularity of these cells? One possibility is that the mature adipocyte fraction that is characteristically isolated never loses its ability to proliferate

and that terminal differentiation may not be a true occurrence at all. Alternatively, the transition of mature adipocyte to adipofibroblast may simply be an abnormal event that occurs in an *in vitro* environment (Fernyhough et al., 2004; Dodson et al., 2005; Fernyhough et al., 2005a; Fernyhough et al., 2005b; Tholpady et al., 2005). Similar occurrences have been noted in a three-dimensional cell culture system (Sugihara et al., 1988; Shigematsu et al., 1999), but even if it is determined to be an *in vitro* artifact, this culture system could prove valuable as a developmental biology cell model system for adipogenesis.

Dedifferentiated adipofibroblasts appeared capable of redifferentiation to form lipid-containing adipocytes. Under our culture conditions, however, redifferentiation was a protracted event, unlike the differentiation observed by other adipogenic cell models. Some of the first structures observed in the redifferentiating adipofibroblast cultures were non-lipid containing (cytosolic) vesicles. These vesicles appeared 3-5 d post confluence when the cells were exposed to DMEM + 10 % HS. When stained with conventional oil red O (for lipid), some of the vesicles possessed lipid, whereas others did not. Moreover, these vesicles did not stain positive for PAS (Figure 2E), suggesting that they were not glycogen storage vesicles.

Differences in the effects of the sera were also seen when serum was added to the cell culture medium of adipofibroblasts. The HS-containing medium always prompted the adipofibroblasts towards redifferentiation, whereas the FBS-containing medium resulted in increased cell proliferation with little or no differentiation. Both HS and FBS were analyzed for levels of cortisol, insulin, IGF-1, and fatty acids. Cortisol, the endogenous corticosteroid, and other glucocorticoids are efficient inducers of adipogenesis (Rosen and Spiegelman, 2000). In the present study, the cortisol level in HS was greater than the cortisol level in FBS, which could support the finding that adipocytes in 10% HS retain their mature phenotype longer than

those cells exposed to FBS. Additionally, this finding might explain why adipofibroblasts exposed to 10 % HS differentiated and formed vesicles. The levels of insulin and IGF-1 were similar between the HS and FBS samples; therefore, the level of insulin and/or IGF-1 was not likely a factor in the differences in the reaction of the cells to the different sera. The fatty acid profiles demonstrate a similarity between HS and FBS, but HS had markedly more linoleic acid (C18:2n6c; Table 1) than FBS. It has been reported that fatty acids induce adipocyte gene expression and differentiation, not only through PPARγ upregulation, but also as PPARγ ligands (MacDougald and Lane, 1995). PPARγ prefers polyunsaturated fatty acids like linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid as ligands (Schoonjans et al., 1996b; Clarke and Jump, 1997; Jump and Clarke, 1999; Moller and Berger, 2003; Al-Hasani and Joost, 2005; Lehrke and Lazar, 2005).

An artificial differentiation induction "cocktail" has been used to promote the differentiation of preadipocytes, *in vitro*, and consists of combinations of IGF-1 or insulin, dexamethasone or cortisol, and methylisobutylxanthine (MIX; Green and Kehinde, 1975; 1979; Gamou et al., 1990). These components ultimately function to upregulate PPARγ. PPARγ may be the major participant in cellular responsiveness to insulin through the expression of the glucose transporter GLUT-4 (Figure 1; Kokta et al., 2004; Rosen, 2005), the insulin receptor substrate, and the insulin receptor (Figure 1; Rosen, 2005). Dexamethasone, a member of the glucocorticoid family, appears to mimic the effects of endogenous cortisol (Rosen and Spiegelman, 2000) and may influence adipocyte differentiation by binding to the cytosolic glucocorticoid receptor (Floyd and Stephens, 2003), which, in turn, binds to glucocorticoid response elements (GRE) in the nucleus (Rosen and Spiegelman, 2000). The

GRE drives the expression of C/EBPδ early in the differentiation process to activate the expression of PPARγ (Rosen and Spiegelman, 2001). MIX is supplied in the medium as an inducer of intracellular cAMP (Ntambi and Young-Cheul, 2000), which phosphorylates the cAMP responsive element binding protein (CREB; Reusch et al., 2000; Zhang et al., 2004). Activation of CREB has been shown to induce adipogenesis in 3T3-L1 cells (Reusch et al., 2000; Zhang et al., 2004) through its association with several adipogenic CREB response elements (Reusch et al., 2000; Zhang et al., 2004). At the nuclear level, treatment with MIX results in the activation of the related transcription factor C/EBPδ. C/EBPβ and δ induce the transcription of C/EBPα and PPARγ (Rosen and Spiegelman, 2000). When exposed to HS-containing medium (alone), or a traditional induction treatment, the adipofibroblasts expressed PPARγ, a marker of mid-differentiation, which is similar to the response of 3T3-L1 cells to a traditional induction medium. In numerous cases, we observed cytosolic vesicle formation prior to the cell beginning to accumulate lipid, and in parallel with the expression of PPARγ. However, the adipofibroblasts did not accumulate lipid in all of the vesicles. As such, we altered the induction procedure to include a PPARγ agonist, thiazoladinedione (TZD). TZDs are members of a class of antidiabetic compounds that are direct ligands for PPARγ (Lehmann et al., 1995; Spiegelman, 1998; Rosen and Spiegelman, 2000; Perrey et al., 2001; Bogacka et al., 2004; Tan et al., 2005) and have strong adipogenic effects. Induction of the adipofibroblasts with insulin, dexamethasone, and MIX did not have an effect on lipid accumulation (unpublished observations), and vesicle formation was delayed in these cultures (unpublished observations). The addition of TZD to the induction medium also did not have any effect on

lipid incorporation (unpublished observations) and was similar to the HS-containing media in differentiation promotion.

Because the adipofibroblasts were found to express PPARγ, we asked the question – do the adipofibroblasts possess incomplete lipid processing machinery? Two lipases were assayed; LPL, which is responsible for lipid entry into the cell, and HSL, which allows mobilization of lipid. LPL is an important enzyme in the deposition of triacylglycerides (TAG) into adipocytes (Fielding and Frayn, 1998). In the adipocytes, LPL is encoded by a single gene containing a PPRE site and is subject to regulation by PPARγ2 (Schoonjans et al., 1996a). The upregulation of the LPL gene, and its subsequent protein expression, demonstrate the ability of the adipocytes to package TAG into lipid storage droplets. Our results demonstrate the presence of LPL protein in the adipofibroblasts exposed to DMEM + 10 % HS. HSL is a cytosolic enzyme thought to be key in the mobilization of stored TAG; indeed, it has a wide range of substrates (Holm et al., 2000; Holm, 2003). In adipocytes, this enzyme functions to hydrolyze intracellular TAG and release FFA (Kraemer and Shen, 2002). Lipolysis by HSL is upregulated by catecholamines and strongly downregulated by insulin (Holm et al., 2000; Holm, 2003). Protein kinase A (PKA) phosphorylates HSL at three locations, thereby activating HSL (Egan et al., 1992; Clifford et al., 1997; Clifford et al., 2000). Our results demonstrate the presence of HSL protein (Figure 4B) in the adipofibroblasts exposed to DMEM + 10 % HS.

Regardless of LPL or HSL production by the cell, the vesicles were not accumulating lipid. Therefore, we proposed that the vesicle might, itself, be defunct. One of the major components of the lipid vesicle in active adipocytes is the lipid droplet-associated protein, perilipin (0.25 – 0.5 % of total cell protein; Londos et al., 1999). The perilipin gene contains a

PPRE site and, therefore, its expression is directly regulated by PPARγ (Arimura et al., 2004; Nagai et al., 2004; Shimizu et al., 2004). In the differentiated adipocyte, perilipin coalesces early in the formation of the lipid droplet to suppress lipolysis of neutral lipids (Londos et al., 2005) by blocking access to the lipid droplet by HSL (Brasaemle et al., 2000a; Brasaemle et al., 2000b; Tansey et al., 2004). Lipolysis, when required, is achieved by the phosphorylation of perilipin by PKA in response to elevation of the levels of cAMP (Brasaemle et al., 2000a; Brasaemle et al., 2000b; Clifford et al., 2000). The conformational change in perilipin, from phosphorylation, allows activated HSL access to the lipid droplet. In the present studies, perilipin protein was found to be associated with the cytosolic vesicles, suggesting that we need to delve further into the mechanisms associated with delayed lipid storage in the redifferentiated adipofibroblasts.

In summary, our data suggest that mature adipocytes may dedifferentiate to form proliferative-competent adipofibroblasts, but that the adipofibroblasts redifferentiate in a protracted manner. While major markers of differentiation, like PPARγ, are expressed in the conversion of adipofibroblasts to adipocytes, we have not yet deduced the major regulator of lipid filling of the cytosolic lipid storage vesicles. Elucidation of the specific mechanism through which lipid is assimilated into adipofibroblast vesicles to form adipocytes may result in adding knowledge to our understanding of adipogenesis. Redifferentiated adipofibroblasts appear novel for another reason-- the cells are from a different source than that used for most adipogenic studies (cell lines or SV cells). Taken together, the results of this study may provide new insights into the cellular and molecular regulation of adipogenesis.

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REFERENCES

Al-Hasani, H., and Joost, H.G. (2005) Nutrition-/diet-induced changes in gene expression in white adipose tissue. Best Pract Res Clin Endocrinol Metab 19:589-603.

Arimura, N., Horiba, T., Imagawa, M., Shimizu, M., and Sato, R. (2004) The peroxisome proliferator-activated receptor gamma regulates expression of the perilipin gene in adipocytes. J Biol Chem 279:10070-10076.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Wheeler, D.L. (2006) GenBank. Nucleic Acids Res 34:D16-20.

Bogacka, I., Xie, H., Bray, G.A., and Smith, S.R. (2004) The effect of pioglitazone on peroxisome proliferator-activated receptor-gamma target genes related to lipid storage in vivo. Diabetes Care 27:1660-1667.

Boone, C., Gregoire, F., and Remacle, C. (1999) Regulation of porcine adipogenesis in vitro, as compared with other species. Domest Anim Endocrinol 17:257-267.

Boone, C., Gregoire, F., and Remacle, C. (2000a) Culture of porcine stromal-vascular cells in serum-free medium: differential action of various hormonal agents on adipose conversion. J Anim Sci 78:885-895.

Boone, C., Mourot, J., Gregoire, F., and Remacle, C. (2000b) The adipose conversion process: regulation by extracellular and intracellular factors. Reprod Nutr Dev 40:325-358.

Boucher, J., Castan-Laurell, I., Daviaud, D., Guigne, C., Buleon, M., Carpene, C., Saulnier-Blache, J.S., and Valet, P. (2005) Adipokine expression profile in adipocytes of different mouse models of obesity. Horm Metab Res 37:761-767.

Brasaemle, D.L., Levin, D.M., Adler-Wailes, D.C., and Londos, C. (2000a) The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. Biochim Biophys Acta 1483:251-262.

Brasaemle, D.L., Rubin, B., Harten, I.A., Gruia-Gray, J., Kimmel, A.R., and Londos, C. (2000b) Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. J Biol Chem 275:38486-38493.

Campisi, J., Dimri, G.P., Nehlin, J.O., Testori, A., and Yoshimoto, K. (1996) Coming of age in culture. Exp Gerontol 31:7-12.

Cancello, R., Pietri-Rouxel, F., and Clement, K. (2005) Spontaneous lipid accumulation in primary cultures of dedifferentiated human adipocytes. Adipocytes 1:73-78.

Caserta, F., Tchkonia, T., Civelek, V.N., Prentki, M., Brown, N.F., McGarry, J.D., Forse, R.A., Corkey, B.E., Hamilton, J.A., and Kirkland, J.L. (2001) Fat depot origin affects fatty acid handling in cultured rat and human preadipocytes. Am J Physiol Endocrinol Metab 280:E238-247.

Cianzio, D.S., Topel, D.G., Whitehurst, G.B., Beitz, D.C., and Self, H.L. (1985) Adipose tissue growth and cellularity: changes in bovine adipocyte size and number. J Anim Sci 60:970-976.

Clarke, S.D., and Jump, D. (1997) Polyunsaturated fatty acids regulate lipogenic and peroxisomal gene expression by independent mechanisms. Prostaglandins Leukot Essent Fatty Acids 57:65-69.

Clifford, G.M., Londos, C., Kraemer, F.B., Vernon, R.G., and Yeaman, S.J. (2000) Translocation of hormone-sensitive lipase and perilipin upon lipolytic stimulation of rat adipocytes. J Biol Chem 275:5011-5015.

Clifford, G.M., McCormick, D.K., Vernon, R.G., and Yeaman, S.J. (1997) Translocation of perilipin and hormone-sensitive lipase in response to lipolytic hormones. Biochem Soc Trans 25:S672.

Crescenzi, M., Soddu, S., and Tato, F. (1995) Mitotic cycle reactivation in terminally differentiated cells by adenovirus infection. J Cell Physiol 162:26-35.

Dodson, M.V., Fernyhough, M.E., Vierck, J.L., and Hausman, G.J. (2005) Adipocytes may not be a terminally differentiated cell type: Implications for animal production. Anim. Sci. 80:239-240.

Egan, J.J., Greenberg, A.S., Chang, M.K., Wek, S.A., Moos, M.C., Jr., and Londos, C. (1992) Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet. Proc Natl Acad Sci U S A 89:8537-8541.

Etherton, T.D., and Chung, C.S. (1981) Preparation, characterization, and insulin sensitivity of isolated swine adipocytes: comparison with adipose tissue slices. J Lipid Res 22:1053-1059.

Etherton, T.D., Thompson, E.H., and Allen, C.E. (1977) Improved techniques for studies of adipocyte cellularity and metabolism. J Lipid Res 18:552-557.

Fajas, L. (2003) Adipogenesis: a cross-talk between cell proliferation and cell differentiation. Ann Med 35:79-85.

Fernyhough, M.E., Bucci, L.R., Hausman, G.J., Antonio, J., Vierck, J.L., and Dodson, M.V. (2005a) Gaining a solid grip on adipogenesis. Tissue Cell 37:335-338.

Fernyhough, M.E., Helterline, D.L., Vierck, J.L., Hausman, G.J., Hill, R.A., and Dodson, M.V. (2005b) Dedifferentiation of mature adipocytes to form adipofibroblasts: More than just a possibility. Adipocytes 1:17-24.

Fernyhough, M.E., Vierck, J.L., Hausman, G.J., Mir, P.S., Okine, E.K., and Dodson, M.V. (2004) Primary adipocyte culture: Adipocyte purification methods may lead to a new understanding of adipose tissue growth and development. Cytotechnology 46:163-172.

Fielding, B.A., and Frayn, K.N. (1998) Lipoprotein lipase and the disposition of dietary fatty acids. Br J Nutr 80:495-502.

Floyd, Z.E., and Stephens, J.M. (2003) STAT5A promotes adipogenesis in nonprecursor cells and associates with the glucocorticoid receptor during adipocyte differentiation. Diabetes 52:308-314.

Gamou, S., Shimizu, Y., and Shimizu, N. (1990) Adipocytes. In: Pollard, J.W., and Walker, J.M. (eds) Methods in Molecular Biology. Humana Press, New Jersey, pp. 197-207.

Granneman, J.G., Li, P., Lu, Y., and Tilak, J. (2004) Seeing the trees in the forest: selective electroporation of adipocytes within adipose tissue. Am J Physiol Endocrinol Metab 287:E574-582.

Green, H., and Kehinde, O. (1975) An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5:19-27.

Green, H., and Kehinde, O. (1979) Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. J Cell Physiol 101:169-171.

Gregoire, F.M. (2001) Adipocyte differentiation: from fibroblast to endocrine cell. Exp Biol Med (Maywood) 226:997-1002.

Hausman, D.B. (2005) Proliferative activity of adipose tissue conditioned media correlates with fat cell size in animal models of obesity. Adipocytes 1:25-34.

Hausman, G.J., and Poulos, S. (2004) Recruitment and differentiation of intramuscular preadipocytes in stromal-vascular cell cultures derived from neonatal pig semitendinosus muscles. J Anim Sci 82:429-437.

Hausman, G.J., and Richardson, R.L. (1998) Newly recruited and pre-existing preadipocytes in cultures of porcine stromal-vascular cells: morphology, expression of extracellular matrix components, and lipid accretion. J Anim Sci 76:48-60.

Holm, C. (2003) Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. Biochem Soc Trans 31:1120-1124.

Holm, C., Osterlund, T., Laurell, H., and Contreras, J.A. (2000) Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. Annu Rev Nutr 20:365-393.

Jump, D.B., and Clarke, S.D. (1999) Regulation of gene expression by dietary fat. Annu Rev Nutr 19:63-90.

Kim, H.S., Hausman, G.J., Hausman, D.B., Martin, R.J., and Dean, R.G. (2001) The expression of cyclin D1 during adipogenesis in pig primary stromal-vascular cultures. Obes Res 9:572-578.

Kinkel, A.D., Fernyhough, M.E., Helterline, D.L., Vierck, J.L., Oberg, K.S., Vance, T.J., Hausman, G.J., Hill, R.A., and Dodson, M.V. (2004) Oil red-O stains non-adipogenic cells: a precautionary note. Cytotechnology 46:49-56.

Kokta, T.A., Dodson, M.V., Gertler, A., and Hill, R.A. (2004) Intercellular signaling between adipose tissue and muscle tissue. Domest Anim Endocrinol 27:303-331.

Krabbenhoft, E.A., Shultz, K., Brannon-O'Reilly, B.A., Chen, Y., Stewart, N.T., and Dodson, M.V. (1997) A simplified method of analysis of cell--conditioned medium for IGF activity. Methods in Cell Science 19:129-135.

Kraemer, F.B., and Shen, W.J. (2002) Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis. J Lipid Res 43:1585-1594.

Kutt, H., and Tsaltas, T.T. (1959) Staining properties of oil red O and a method of partial purification of the commercial product. Clin Chem 5:149-160.

Latella, L., Sacco, A., Pajalunga, D., Tiainen, M., Macera, D., D'Angelo, M., Felici, A., Sacchi, A., and Crescenzi, M. (2001) Reconstitution of cyclin D1-associated kinase activity drives terminally differentiated cells into the cell cycle. Mol Cell Biol 21:5631-5643.

Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M., and Kliewer, S.A. (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). J Biol Chem 270:12953-12956.

Lehrke, M., and Lazar, M.A. (2005) The many faces of PPARgamma. Cell 123:993-999.

Li, Y., Bujo, H., Takahashi, K., Shibasaki, M., Zhu, Y., Yoshida, Y., Otsuka, Y., Hashimoto, N., and Saito, Y. (2003) Visceral fat: higher responsiveness of fat mass and gene expression to calorie restriction than subcutaneous fat. Exp Biol Med (Maywood) 228:1118-1123.

Londos, C., Brasaemle, D.L., Schultz, C.J., Segrest, J.P., and Kimmel, A.R. (1999) Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. Semin Cell Dev Biol 10:51-58.

Londos, C., Sztalryd, C., Tansey, J.T., and Kimmel, A.R. (2005) Role of PAT proteins in lipid metabolism. Biochimie 87:45-49.

MacDougald, O.A., and Lane, M.D. (1995) Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 64:345-373.

Madsen, L., Petersen, R.K., and Kristiansen, K. (2005) Regulation of adipocyte differentiation and function by polyunsaturated fatty acids. Biochim Biophys Acta 1740:266-286.

Marques, B.G., Hausman, D.B., and Martin, R.J. (1998) Association of fat cell size and paracrine growth factors in development of hyperplastic obesity. Am J Physiol 275:R1898-1908.

Matsubara, Y., Sato, K., Ishii, H., and Akiba, Y. (2005) Changes in mRNA expression of regulatory factors involved in adipocyte differentiation during fatty acid induced adipogenesis in chicken. Comp Biochem Physiol A Mol Integr Physiol 141:108-115.

May, S.G., Savell, J.W., Lunt, D.K., Wilson, J.J., Laurenz, J.C., and Smith, S.B. (1994) Evidence for preadipocyte proliferation during culture of subcutaneous and intramuscular adipose tissues from Angus and Wagyu crossbred steers. J Anim Sci 72:3110-3117.

McNeel, R.L., and Mersmann, H.J. (2003) Effects of isomers of conjugated linoleic acid on porcine adipocyte growth and differentiation. J Nutr Biochem 14:266-274.

McNeel, R.L., Smith, E.O., and Mersmann, H.J. (2003) Isomers of conjugated linoleic acid modulate human preadipocyte differentiation. In Vitro Cell Dev Biol Anim 39:375-382.

Moller, D.E., and Berger, J.P. (2003) Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. Int J Obes Relat Metab Disord 27 Suppl 3:S17-21.

Nagai, S., Shimizu, C., Umetsu, M., Taniguchi, S., Endo, M., Miyoshi, H., Yoshioka, N., Kubo, M., and Koike, T. (2004) Identification of a functional peroxisome proliferator-activated receptor responsive element within the murine perilipin gene. Endocrinology 145:2346-2356.

Ntambi, J.M., and Young-Cheul, K. (2000) Adipocyte differentiation and gene expression. J Nutr 130:3122S-3126S.

Perrey, S., Ishibashi, S., Yahagi, N., Osuga, J., Tozawa, R., Yagyu, H., Ohashi, K., Gotoda, T., Harada, K., Chen, Z., Iizuka, Y., Shionoiri, F., and Yamada, N. (2001) Thiazolidinedione- and tumor necrosis factor alpha-induced downregulation of peroxisome proliferator-activated receptor gamma mRNA in differentiated 3T3-L1 adipocytes. Metabolism 50:36-40.

Prunet-Marcassus, B., Cousin, B., Caton, D., Andre, M., Penicaud, L., and Casteilla, L. (2006) From heterogeneity to plasticity in adipose tissues: Site-specific differences. Exp Cell Res 312:727-736.

Ramsay, T.G., Rao, S.V., and Wolverton, C.K. (1992) In vitro systems for the analysis of the development of adipose tissue in domestic animals. J Nutr 122:806-817.

Reusch, J.E., Colton, L.A., and Klemm, D.J. (2000) CREB activation induces adipogenesis in 3T3-L1 cells. Mol Cell Biol 20:1008-1020.

Robelin, J. (1981) Cellularity of bovine adipose tissues: developmental changes from 15 to 65 percent mature weight. J Lipid Res 22:452-457.

Robenek, H., Robenek, M.J., Buers, I., Lorkowski, S., Hofnagel, O., Troyer, D., and Severs, N.J. (2005a) Lipid droplets gain their PATs by interaction with specialized plasma membrane domains. J Biol Chem.

Robenek, H., Robenek, M.J., and Troyer, D. (2005b) PAT family proteins pervade lipid droplet cores. J Lipid Res 46:1331-1338.

Rosen, E.D. (2005) The transcriptional basis of adipocyte development. Prostaglandins Leukot Essent Fatty Acids 73:31-34.

Rosen, E.D., and Spiegelman, B.M. (2000) Molecular regulation of adipogenesis. Annu Rev Cell Dev Biol 16:145-171.

Rosen, E.D., and Spiegelman, B.M. (2001) PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. J Biol Chem 276:37731-37734.

Russell, T.R., and Ho, R. (1976) Conversion of 3T3 fibroblasts into adipose cells: triggering of differentiation by prostaglandin F2alpha and 1-methyl-3-isobutyl xanthine. Proc Natl Acad Sci U S A 73:4516-4520.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A.M., Heyman, R.A., Briggs, M., Deeb, S., Staels, B., and Auwerx, J. (1996a) PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. Embo J 15:5336-5348.

Schoonjans, K., Staels, B., and Auwerx, J. (1996b) Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. J Lipid Res 37:907-925.

Shigematsu, M., Watanabe, H., and Sugihara, H. (1999) Proliferation and differentiation of unilocular fat cells in the bone marrow. Cell Struct Funct 24:89-100.

Shimizu, M., Takeshita, A., Tsukamoto, T., Gonzalez, F.J., and Osumi, T. (2004) Tissue-selective, bidirectional regulation of PEX11 alpha and perilipin genes through a common peroxisome proliferator response element. Mol Cell Biol 24:1313-1323.

Smith, S.B., and Crouse, J.D. (1984) Relative contributions of acetate, lactate and glucose to lipogenesis in bovine intramuscular and subcutaneous adipose tissue. J Nutr 114:792-800.

Spiegelman, B.M. (1998) PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. Diabetes 47:507-514.

Stewart, W.C., Baugh, J.E., Jr., Floyd, Z.E., and Stephens, J.M. (2004) STAT 5 activators can replace the requirement of FBS in the adipogenesis of 3T3-L1 cells. Biochem Biophys Res Commun 324:355-359.

Stolic, M., Russell, A., Hutley, L., Fielding, G., Hay, J., MacDonald, G., Whitehead, J., and Prins, J. (2002) Glucose uptake and insulin action in human adipose tissue--influence of BMI, anatomical depot and body fat distribution. Int J Obes Relat Metab Disord 26:17-23.

Student, A.K., Hsu, R.Y., and Lane, M.D. (1980) Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. J Biol Chem 255:4745-4750.

Sugihara, H., Yonemitsu, N., Miyabara, S., and Yun, K. (1986) Primary cultures of unilocular fat cells: characteristics of growth in vitro and changes in differentiation properties. Differentiation 31:42-49.

Sugihara, H., Yonemitsu, N., Toda, S., Miyabara, S., Funatsumaru, S., and Matsumoto, T. (1988) Unilocular fat cells in three-dimensional collagen gel matrix culture. J Lipid Res 29:691-697.

Tan, G.D., Fielding, B.A., Currie, J.M., Humphreys, S.M., Desage, M., Frayn, K.N., Laville, M., Vidal, H., and Karpe, F. (2005) The effects of rosiglitazone on fatty acid and triglyceride metabolism in type 2 diabetes. Diabetologia 48:83-95.

Tansey, J.T., Sztalryd, C., Hlavin, E.M., Kimmel, A.R., and Londos, C. (2004) The central role of perilipin a in lipid metabolism and adipocyte lipolysis. IUBMB Life 56:379-385.

Tchkonia, T., Tchoukalova, Y.D., Giorgadze, N., Pirtskhalava, T., Karagiannides, I., Forse, R.A., Koo, A., Stevenson, M., Chinnappan, D., Cartwright, A., Jensen, M.D., and Kirkland, J.L. (2005) Abundance of two human preadipocyte subtypes with distinct capacities for replication, adipogenesis, and apoptosis varies among fat depots. Am J Physiol Endocrinol Metab 288:E267-277.

Tholpady, S.S., Aojanepong, C., Llull, R., Jeong, J.H., Mason, A.C., Futrell, J.W., Ogle, R.C., and Katz, A.J. (2005) The cellular plasticity of human adipocytes. Ann Plast Surg 54:651-656.

Vierck, J.L., and Dodson, M.V. (2000) Interpretation of cell culture phenomena. Methods Cell Sci 22:79-81.

Vierck, J.L., McNamara, J.P., and Dodson, M.V. (1996) Proliferation and differentiation of progeny of ovine unilocular fat cells (adipofibroblasts). In Vitro Cell Dev Biol Anim 32:564-572.

Wheeler, D.L., Barrett, T., Benson, D.A., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M., DiCuccio, M., Edgar, R., Federhen, S., Geer, L.Y., Helmberg, W., Kapustin, Y., Kenton, D.L., Khovayko, O., Lipman, D.J., Madden, T.L., Maglott, D.R., Ostell, J., Pruitt, K.D., Schuler, G.D., Schriml, L.M., Sequeira, E., Sherry, S.T., Sirotkin, K., Souvorov, A., Starchenko, G., Suzek, T.O., Tatusov, R., Tatusova, T.A., Wagner, L., and Yaschenko, E. (2006) Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 34:D173-180.

Wu, P., Sato, K., Suzuta, F., Hikasa, Y., and Kagota, K. (2000) Effects of lipid-related factors on adipocyte differentiation of bovine stromal-vascular cells in primary culture. J Vet Med Sci 62:933-939.

Zhang, H.H., Kumar, S., Barnett, A.H., and Eggo, M.C. (2000) Ceiling culture of mature human adipocytes: use in studies of adipocyte functions. J Endocrinol 164:119-128.

Zhang, J.W., Klemm, D.J., Vinson, C., and Lane, M.D. (2004) Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein beta gene during adipogenesis. J Biol Chem 279:4471-4478.

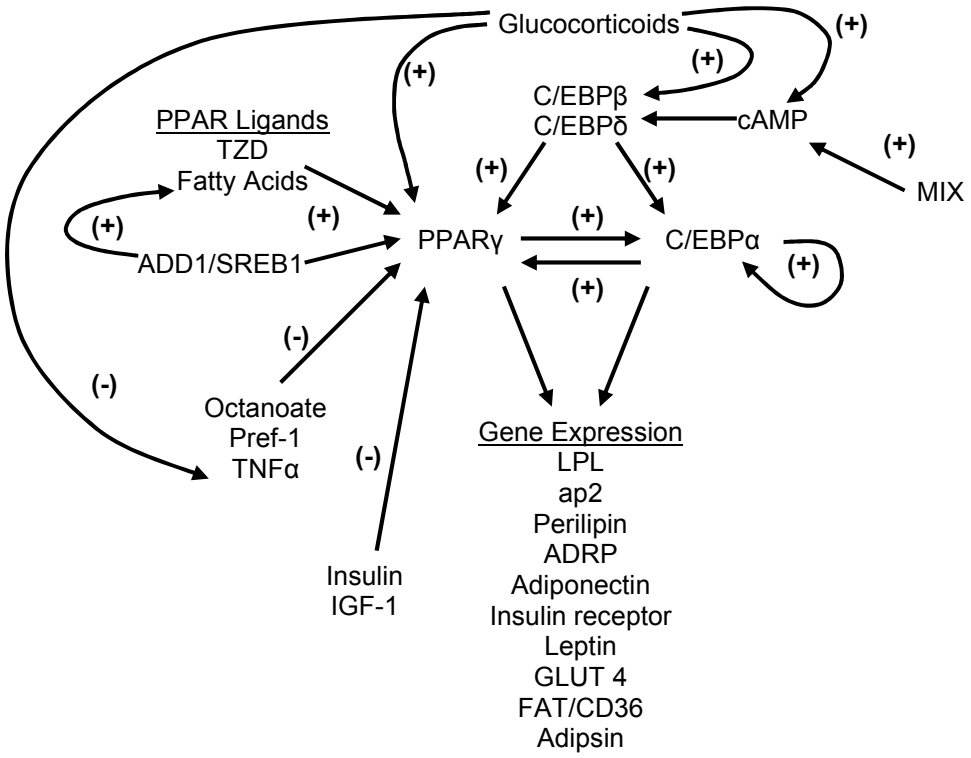
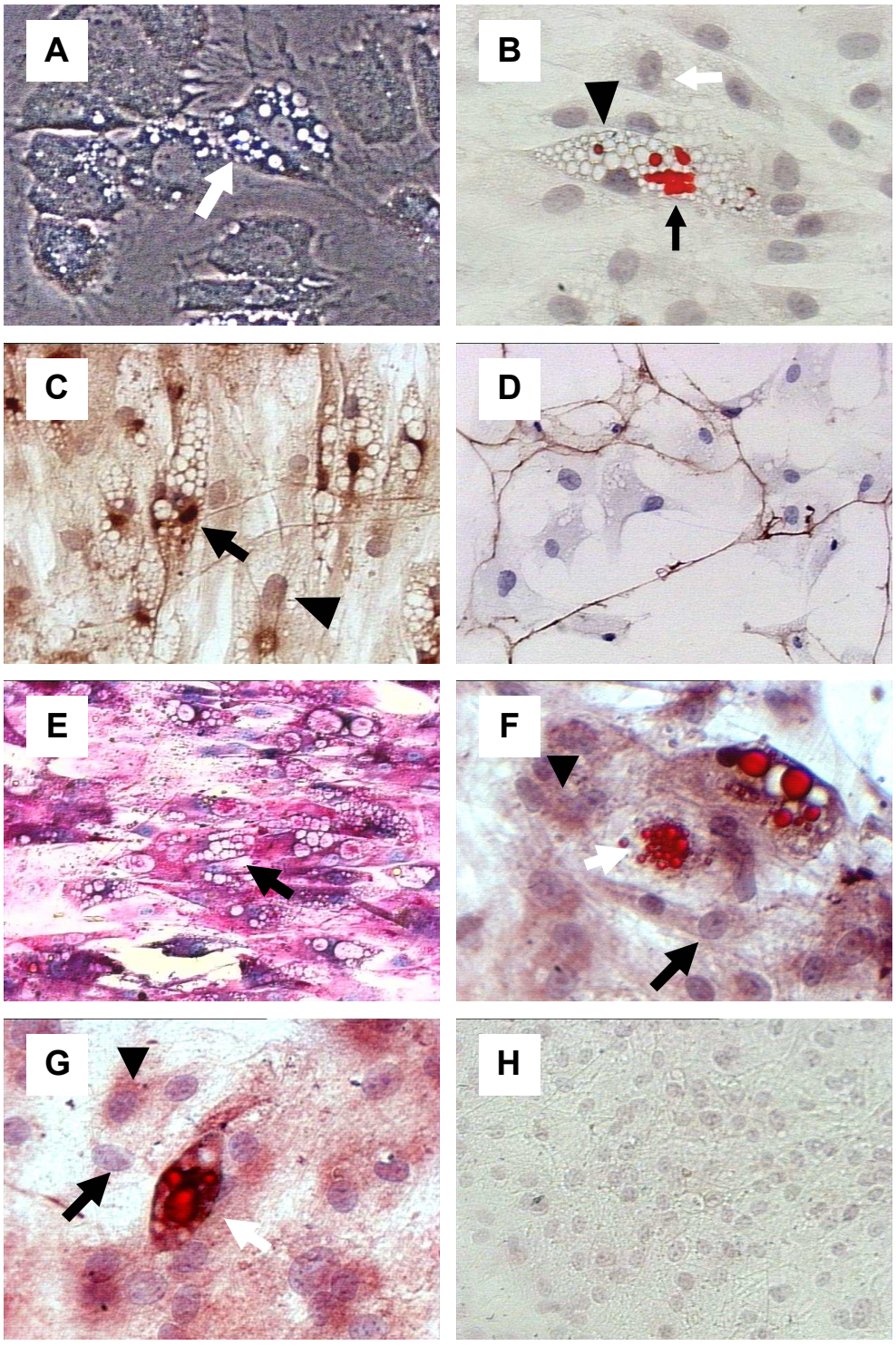


Figure 1: The complex mechanisms involved in adipogenesis involve a key player in the differentiation of adipocytes: PPARγ.

Figure 2: (A) Phase contrast photomicrograph of adipofibroblasts on DMEM + 10 % HS for 10 d displaying the cytoplasmic vesicles (open arrow; 400 X). (B) Photomicrograph of oil red O stained adipofibroblasts on a basal medium of DMEM + 3 % HS for 14 d (400 X). Some vesicles stain with oil red O (closed arrow) indicating lipid within vesicles whereas other vesicles in the same cell do not (arrowhead). Other cells in the same field have cytoplasmic vesicles but do not stain with oil red O (open arrow). (C) Photomicrographs of immunocytochemistry staining of bovine adipofibroblasts for PPARγ (400 X). Cells that stained with DAB also have cytoplasmic vesicles (arrow) whereas cells that did not stain with DAB do not have cytoplasmic vesicles (arrowhead). (D) The negative control for PPARγ immunostaining consisted of an omission of the primary antibody (400 X magnification). (E) Periodic acid Schiff staining for glycogen within the vesicles (400 X). Vesicles did not stain with PAS (arrow) indicating a lack of glycogen. (F, G) Staining adipofibroblasts for PPARγ (NovaRed) and lipid (oil red O). (F, G) Adipofibroblasts demonstrating lipid uptake (oil red O) and staining with NovaRed indicating the presence of PPARγ (open arrows; 400 X). Other cells stained with NovaRed but did not stain with oil red O (arrowheads) whereas some cell did not stain with NovaRed (closed arrows) demonstrating a lack of PPARγ. (H) Negative control for experiments in panels F and G consisted of an omission of the primary antibody (200 X).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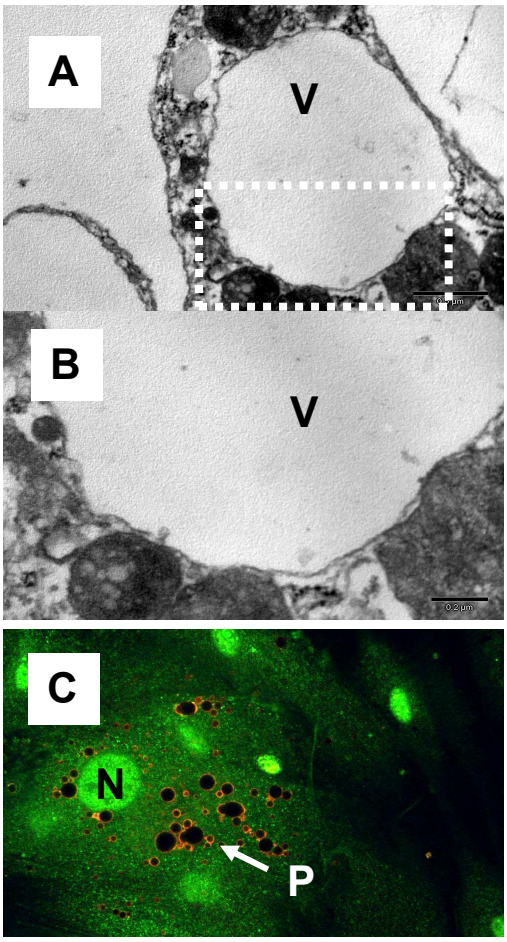

Figure 3: (A, B) Transmission electron microscopy of adipofibroblasts. (A) Cross section of an adipofibroblast containing a vesicle (V). (B) An enlargement of the outlined portion in (A). M = mitochondrion, V = vesicle, Arrow = vesicle membrane. (C) Immunofluorescence for vimentin and perilipin of adipofibroblasts 600X. Cells were stained for vimentin (Oregon green) and perilipin (Texas red) and viewed under a confocal microscope. N = nucleus, arrow = perilipin

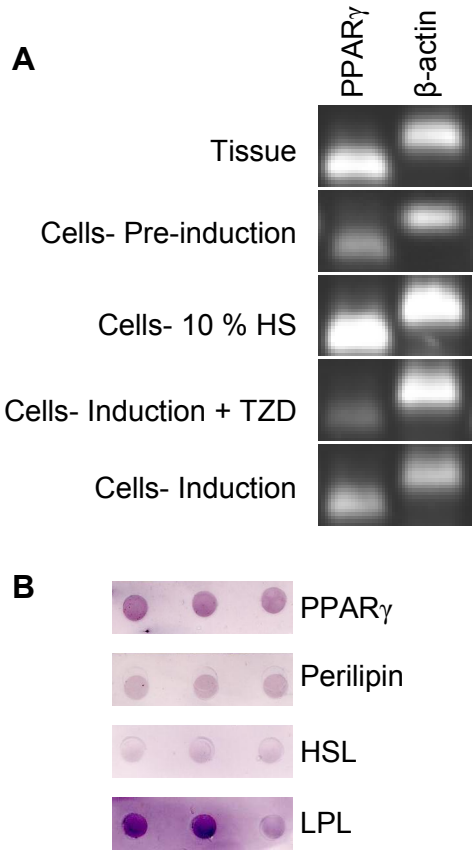

Figure 4: (A) Electrophoresis of RT-PCR for PPARγ as a marker of differentiation and β actin as an internal control. (B) Immunoblot of proteins (PPARγ, perilipin, HSL, and LPL) isolated from adipofibroblasts maintained on DMEM + 10 % HS for 45 d. Three blots for each protein of interest were used.

Table 1. Fatty acid profiles of the two serum types.

Fatty acid		Serum type	
		HS*	FBS*
Caproic acid	C6:0	1.7 %	1.99 %
Caprylic acid	C8:0	3.93 %	7.52 %
Capric acid	C10:0	1.85 %	2.5 %
Lauric acid	C12:0	4.56 %	5.4 %
Myristic acid	C14:0	2.35 %	3.89 %
Palmitic acid	C16:0	12.38 %	14.67 %
Palmitoleic acid	C16:1n7c	1.58 %	2.25 %
Steric acid	C18:0	3.74 %	3.7 %
Linoleic acid	C18:2n6c	33.74 %	8.44 %
Linolelaidic acid	C18:2n6t	10.96 %	9.76 %
Linolenic acid	C18:3n3c	5.79 %	0 %
cis-11, 14, 17-Eicosatrienoic acid	C20:3n3c	0 %	4.08 %

* Percentages do not add to 100.

CHAPTER SEVEN

Assessing a non-traditional view of adipogenesis: adipocyte dedifferentiation – mountains or molehills?*

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Abstract

Based on our studies we propose the following hypothesis: mature, lipid-containing adipocytes possess the ability to undergo symmetrical or asymmetrical cell division, without losing lipid. While our research to discern the mechanism(s) involved in what we have termed "dedifferentiation" of adipocytes is ongoing, we have identified several roadblocks to our work in this area. However, due to the newness of this research area, we believe that none of these problems discounts the potential importance of our initial observations, or the excitement of contributing knowledge in the area. In this manuscript, we address some of these problems and suggest possible solutions in an attempt to make “molehills” out of “mountains.”

On several occasions, we have described the ability of lipid-containing adipocytes, obtained from the subcutaneous fat depot in beef cattle, to proliferate *in vitro* [Dodson, et al., 2005, Fernyhough, et al., 2005b, Fernyhough, et al., 2005c, Fernyhough, et al., 2005d].We have termed this physiological transition, adipocyte dedifferentiation [Vierck, et al., 1996] into adipofibroblasts [Vierck, et al., 1996]. The plasticity of progeny cells to re-form lipid-containing adipocytes or other cell types is presently being evaluated by us [Dodson, et al., 2005, Fernyhough, et al., 2005b, Fernyhough, et al., 2005c] and others [Cancello, et al., 2005, Justesen, et al., 2004, Zhang, et al., 2000]. We think that this may prove to be an emerging area of cell physiology and may amend present thinking about tissue renewal capabilities. However, this research focus is not without its problems, which will need to be rectified prior to suggesting definitively that the classical idea of the terminally- differentiated state of adipocytes needs revision.

One such research problem that will need to be experimentally corrected is our analysis of cells derived from only one depot (subcutaneous) of adipose tissue rather than from other body depots. This observation stems from work demonstrating multiple differences between the regulation and cellularity of adipose depots [Altomonte, et al., 2003, Arvidsson, et al., 2004, Berndt, et al., 2005, Boucher, et al., 2005, Dicker, et al., 2004, Giorgino, et al., 2005, Hishikawa, et al., 2005, Laplante, et al., 2003, Oliver, et al., 2003, Pujol, et al., 2005, Ren, et al., 2002, Rodriguez, et al., 2004, Rodriguez-Cuenca, et al., 2005, Ross, et al., 2005, Schoof, et al., 2004, Tan, et al., 2004, Tchkonia, et al., 2002, Tchkonia, et al., 2005, Van Harmelen, et al., 2004, Vidal 2001]. While we are aware of these differences, the reasoning behind the use of one depot, presently, is two-fold. The subcutaneous depot in the beef animal is the largest and easiest depot from which to remove adipose tissue during slaughter.

Additionally, while there exists some preliminary evidence that adipocytes isolated from other fat depots possess a similar ability to dedifferentiate *in vitro* [Cancello, et al., 2005, Justesen, et al., 2004, Shigematsu, et al., 1999, Sugihara, et al., 1987, Sugihara, et al., 1986, Sugihara, et al., 1988], the focus of the previous work cannot address the specific population dynamics of mature adipocyte dedifferentiation. Consequently, while we would like to extrapolate our work to other adipose depots, we will not be in a scientific position to do so until we have actually performed a careful study of cells derived from other depots.

Another problem that will need to be resolved is the suggestion that we are looking at cell physiology transitions in an abnormal *in vitro* environment. In other words, is the dedifferentiation that we observe an artifact of the two-dimensional cell culture system being employed? Further, would such physiologic reversion ever occur with mature (differentiated) adipocytes *in vivo*? The closest culture environment to that experienced by adipocytes in vivo is the three-dimensional culture system [Shigematsu, et al., 1999, Sugihara, et al., 1988], which has similarly showed results suggesting that dedifferentiation of unilocular adipocytes may occur. However, in all cell culture systems, there is no blood supply, nervous interaction or exposure to all of the chemical mediators that mature cells may be exposed to in vivo. Consequently, our observation may be relegated to the in vitro environment, only. However, even if the dedifferentiation process only occurs *in vitro*, such a system would provide knowledge of the regulation of such events that might be (eventually) useful in alleviating the problems associated with the general accumulation of excess body fat. The elucidation of similar cell reversions occurring *in vivo* must wait until we have generated cellular assessment tools (markers) that might be employed in either *in vivo* or, possibly, in an *in situ* system.

A further possible drawback with our cell culture system is that, despite the constant exposure of the adipocytes to classical, high serum growth medium (10%), we may be starving the cells of some component present *in vivo*; consequently they have no recourse but to lose some lipid and divide *in vitro*. We propose that a "starving" adipocyte would do one of three things: remain in the same physiological state, utilize the intracellular lipid stores for energy (delipidation), or undergo apoptosis. Many of the dedifferentiating adipocytes do so without losing lipid [Dodson, et al., 2005, Fernyhough, et al., 2005b, Fernyhough, et al., 2005c, Fernyhough, et al., 2005d]. However, we are constantly evaluating our *in vitro* environment for the presence/absence of regulators and metabolites required to maintain cell health.

Because adipose tissue contains different populations of cells, there may be a chance that our cell cultures never achieve the purity required in order to convince anyone of the homogeneous nature of the cell population. Whereas this observation may hold true with earlier observations [Adebonojo 1975a, b, Shigematsu, et al., 1999, Sugihara, et al., 1987, Sugihara, et al., 1986, Sugihara, et al., 1988, Van, et al., 1976], recent work on methods to purify the primary adipocyte cultures in our laboratory, as well as in at least one separate laboratory, has led to remarkably similar procedures and results [Fernyhough, et al., 2005d, Tholpady, et al., 2005].

Our observations to date, albeit descriptive, lead us to several possible explanations regarding possibilities for adipocyte dedifferentiation. First, mature fat cells may not necessarily be fully differentiated cells and may actually be preadipocytes, adipofibroblasts, or fibroblasts that possess the ability to assimilate large quantities of lipid while remaining non-differentiated adipocytes. Previous research has demonstrated that normal preadipocyte differentiation into lipid-laden mature adipocytes can be depot dependent [Shigematsu et al. 1999]. Why, then, is

it not possible for some of these cells to contain lipid, but not be fully differentiated? This is for additional research to elucidate, and may actually be the physiology at play in our observations. A second possibility is that these cells are more "stem cell-like" and that they not only are able to proliferate but might be more "plastic" and have the ability to form other cells types (myocytes, chondrocytes, osteocytes) [Justesen, et al., 2004]. Lastly, the possibility exists that we have identified a cell type residing in adipose tissue that may be an entirely new cell of the mesodermal lineage. Regardless of the determination of the final cell type, we believe that knowledge of the regulation of the dedifferentiation event, *in vitro*, will add to our understanding of adipose tissue biology.

We are excited to bring this research area to the attention of others. By so doing, regardless of present barriers, we hope to encourage other researchers to commit adequate personnel and resources to focus on this potentially vital (but relatively ignored) part of adipocyte physiology-the ability of "mature" adipocytes to retain their ability to proliferate. We consider this research relevant for a number of human medical diseases including obesity, diabetes, lipodystrophy, and hypertension.

References

Adebonojo, F.O. (1975a) Monolayer cultures of disaggregated human adipocytes. In Vitro *11:* 50-54

Adebonojo, F.O. (1975b) Studies on human adipose cells in culture: relation of cell size and multiplication to donor age. Yale J Biol Med *48:* 9-16

Altomonte, J., S. Harbaran, A. Richter, H. Dong (2003) Fat depot-specific expression of adiponectin is impaired in Zucker fatty rats. Metabolism *52:* 958-963

Arvidsson, E., L. Blomqvist, M. Ryden (2004) Depot-specific differences in perilipin mRNA but not protein expression in obesity. J Intern Med *255:* 595-601

Berndt, J., N. Kloting, S. Kralisch, P. Kovacs, M. Fasshauer, M.R. Schon, M. Stumvoll, M. Bluher (2005) Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. Diabetes *54:* 2911-2916

Boucher, J., I. Castan-Laurell, D. Daviaud, C. Guigne, M. Buleon, C. Carpene, J.S. Saulnier-Blache, P. Valet (2005) Adipokine expression profile in adipocytes of different mouse models of obesity. Horm Metab Res *37:* 761-767

Cancello, R., F. Pietri-Rouxel, K. Clement (2005) Spontaneous lipid accumulation in primary cultures of dedifferentiated human adipocytes. Adipocytes *1:* 73-78

Dicker, A., M. Ryden, E. Naslund, I.E. Muehlen, M. Wiren, M. Lafontan, P. Arner (2004) Effect of testosterone on lipolysis in human pre-adipocytes from different fat depots. Diabetologia *47:* 420-428

Dodson, M.V., M.E. Fernyhough, J.L. Vierck, G.J. Hausman (2005) Adipocytes may not be a terminally differentiated cell type: Implications for animal production. Anim. Sci. *80:* 239-240

Fernyhough, M.E., L.R. Bucci, G.J. Hausman, J. Antonio, J.L. Vierck, M.V. Dodson (2005a) Gaining a solid grip on adipogenesis. Tissue Cell *37:* 335-338

Fernyhough, M.E., D.L. Helterline, J.L. Vierck, G.J. Hausman, R.A. Hill, M.V. Dodson (2005b) Dedifferentiation of mature adipocytes to form adipofibroblasts: More than just a possibility. Adipocytes *1:* 17-24

Fernyhough, M.E., J.L. Vierck, G.J. Hausman, P.S. Mir, E.K. Okine, M.V. Dodson (2005c) Primary adipocyte culture: Adipocyte purification methods may lead to a new understanding of adipose tissue growth and development. Cytotechnology *46:* 163-172

Giorgino, F., L. Laviola, J.W. Eriksson (2005) Regional differences of insulin action in adipose tissue: insights from in vivo and in vitro studies. Acta Physiol Scand *183:* 13-30

Hishikawa, D., Y.H. Hong, S.G. Roh, H. Miyahara, Y. Nishimura, A. Tomimatsu, H. Tsuzuki, C. Gotoh, M. Kuno, K.C. Choi, H.G. Lee, K.K. Cho, H. Hidari, S. Sasaki (2005) Identification of genes expressed differentially in subcutaneous and visceral fat of cattle, pig, and mouse. Physiol Genomics *21:* 343-350

Justesen, J., S.B. Pedersen, K. Stenderup, M. Kassem (2004) Subcutaneous adipocytes can differentiate into bone-forming cells in vitro and in vivo. Tissue Eng *10:* 381-391

Laplante, M., H. Sell, K.L. MacNaul, D. Richard, J.P. Berger, Y. Deshaies (2003) PPAR-gamma activation mediates adipose depot-specific effects on gene expression and lipoprotein lipase activity: mechanisms for modulation of postprandial lipemia and differential adipose accretion. Diabetes *52:* 291-299

Oliver, P., C. Pico, F. Serra, A. Palou (2003) Resistin expression in different adipose tissue depots during rat development. Mol Cell Biochem *252:* 397-400

Pujol, E., A. Proenza, I. Llado, P. Roca (2005) Pregnancy effects on rat adipose tissue lipolytic capacity are dependent on anatomical location. Cell Physiol Biochem *16:* 229-236

Ren, M.Q., J. Wegner, O. Bellmann, G.A. Brockmann, F. Schneider, F. Teuscher, K. Ender (2002) Comparing mRNA levels of genes encoding leptin, leptin receptor, and lipoprotein lipase between dairy and beef cattle. Domest Anim Endocrinol *23:* 371-381

Rodriguez, E., J. Ribot, A.M. Rodriguez, A. Palou (2004) PPAR-gamma2 expression in response to cafeteria diet: gender- and depot-specific effects. Obes Res *12:* 1455-1463

Rodriguez-Cuenca, S., M. Monjo, A.M. Proenza, P. Roca (2005) Depot differences in steroid receptor expression in adipose tissue: possible role of the local steroid milieu. Am J Physiol Endocrinol Metab *288:* E200-207

Ross, J.W., T.K. Smith, C.R. Krehbiel, J.R. Malayer, U. DeSilva, J.B. Morgan, F.J. White, M.J. Hersom, G.W. Horn, R.D. Geisert (2005) Effects of grazing program and subsequent finishing on gene expression in different adipose tissue depots in beef steers. J Anim Sci *83:* 1914-1923

Schoof, E., A. Stuppy, F. Harig, R. Carbon, T. Horbach, W. Stohr, W. Rascher, J. Dotsch (2004) Comparison of leptin gene expression in different adipose tissues in children and adults. Eur J Endocrinol *150:* 579-584

Shigematsu, M., H. Watanabe, H. Sugihara (1999) Proliferation and differentiation of unilocular fat cells in the bone marrow. Cell Struct Funct *24:* 89-100

Sugihara, H., N. Yonemitsu, S. Miyabara, S. Toda (1987) Proliferation of unilocular fat cells in the primary culture. J Lipid Res *28:* 1038-1045

Sugihara, H., N. Yonemitsu, S. Miyabara, K. Yun (1986) Primary cultures of unilocular fat cells: characteristics of growth in vitro and changes in differentiation properties. Differentiation *31:* 42-49

Sugihara, H., N. Yonemitsu, S. Toda, S. Miyabara, S. Funatsumaru, T. Matsumoto (1988) Unilocular fat cells in three-dimensional collagen gel matrix culture. J Lipid Res *29:* 691-697

Tan, G.D., G.H. Goossens, S.M. Humphreys, H. Vidal, F. Karpe (2004) Upper and lower body adipose tissue function: a direct comparison of fat mobilization in humans. Obes Res *12:* 114-118

Tchkonia, T., N. Giorgadze, T. Pirtskhalava, Y. Tchoukalova, I. Karagiannides, R.A. Forse, M. DePonte, M. Stevenson, W. Guo, J. Han, G. Waloga, T.L. Lash, M.D. Jensen, J.L. Kirkland (2002) Fat depot origin affects adipogenesis in primary cultured and cloned human preadipocytes. Am J Physiol Regul Integr Comp Physiol *282:* R1286-1296

Tchkonia, T., Y.D. Tchoukalova, N. Giorgadze, T. Pirtskhalava, I. Karagiannides, R.A. Forse, A. Koo, M. Stevenson, D. Chinnappan, A. Cartwright, M.D. Jensen, J.L. Kirkland (2005) Abundance of two human preadipocyte subtypes with distinct capacities for replication, adipogenesis, and apoptosis varies among fat depots. Am J Physiol Endocrinol Metab *288:* E267-277

Tholpady, S.S., C. Aojanepong, R. Llull, J.H. Jeong, A.C. Mason, J.W. Futrell, R.C. Ogle, A.J. Katz (2005) The cellular plasticity of human adipocytes. Ann Plast Surg *54:* 651-656

Van Harmelen, V., K. Rohrig, H. Hauner (2004) Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects. Metabolism *53:* 632-637

Van, R.L., C.E. Bayliss, D.A. Roncari (1976) Cytological and enzymological characterization of adult human adipocyte precursors in culture. J Clin Invest *58:* 699-704

Vidal, H. (2001) Gene expression in visceral and subcutaneous adipose tissues. Ann Med *33:* 547-555

Vierck, J.L., J.P. McNamara, M.V. Dodson (1996) Proliferation and differentiation of progeny of ovine unilocular fat cells (adipofibroblasts). In Vitro Cell Dev Biol Anim *32:* 564-572

Zhang, H.H., S. Kumar, A.H. Barnett, M.C. Eggo (2000) Ceiling culture of mature human adipocytes: use in studies of adipocyte functions. J Endocrinol *164:* 119-128

APPENDIX A

Gaining a Solid Grip on Adipogenesis*

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ABSTRACT

Obesity is presently being combated by fitness regimens, drugs and diet. Increasing our understanding of the physiology of adipocytes, by deducing the regulatory pathways involved in lipid metabolism and all aspects of adipogenesis, will provide alternative strategies to reduce adverse problems of obesity. Research has suggested that mature fat cells may dedifferentiate to form proliferative-competent fat cell precursors. Knowledge of the dedifferentiation process will allow us to gain a solid grip on adipogenesis.

INTRODUCTION

The cost of obesity

Americans are becoming obese; whether young, or old, our society is getting fatter (11, 21). Obesity is associated with numerous dysfunctions, including type II diabetes and heart disease (19). Nearly 10% of the total health care expenditures in the United States have been attributed to obesity (6). The cost of obesity can also be felt in other ways such as lost productivity (work days) and early death (4, 6, 25). Americans spend billions of dollars on therapists, weight loss programs, exercise machinery and dietary products in an attempt to lose weight. To some degree, many of these may work, but Americans seem incapable of staying with any one program. Consequently, most Americans fall back to their old behaviors of consuming more energy than is expended, thereby regaining whatever weight they were initially successful in losing. A key to combating obesity is gaining insight into the regulation of the adipocyte.

Physiology of adipocytes

At the cellular level, obesity involves two different physiological components. The first, lipid metabolism, is the energy flow into or out of adipocytes (lipogenesis and lipolysis respectively). Numerous review articles have been written summarizing the detailed components of both of these processes (7, 14, 16-18). The second physiological component, termed adipogenesis, is (collectively) the discernable cellular transitions through which a spindle-shaped fibroblastic cell proceeds, first forming a preadipocyte, then a multilocular adipocyte, and, finally, a mature (unilocular) adipocyte (3, 9, 13, 20). Whereas countless scientific papers are published each year regarding both of these areas (lipid metabolism and adipogenesis), little gains have been made to either formulate an effective exogenous treatment

for inducing an overall reduction in body lipid or for altering (decreasing) the cellular conversion to form adipocytes. Indeed, the majority of published articles in the adipogenesis field suggest that once a preadipocyte accumulates lipid, then the cell is a terminally differentiated adipocyte – with the only option to metabolize lipid from that point onward (reviewed in 10). It is interesting to note that, according to traditional thought, should additional adipocytes be required in any specific fat depot, then the fibroblast-like cells that reside in the connective tissue fraction have been proposed to be converted into the requisite number of adipocytes.

Dedifferentiation

It is unfortunate that, because of the current belief in the terminality of mature adipocytes, little research has been conducted on an alternative and potentially important area: the dedifferentiation of mature adipocytes to form proliferative-competent cells. Not only is it apparently possible for adipocyte number to increase through the activity of fibroblasts/preadipocytes, but a limited number of scientific observations exist in the literature to suggest that mature adipocytes may dedifferentiate to form proliferative-competent cells (1, 2, 5, 10, 15, 22-24). It is our belief that this new area of research may provide several potentially new targets for weight loss. It might be possible to devise pharmacological treatments to induce mature cells to revert back to proliferative-competent cells. Once the cells resemble the lesser differentiated cell form, it may then be possible to devise a treatment to terminate the cells, thereby disallowing further fat storage by the cells. Alternatively, there may be devised a regimen to disallow the mature fat cells to proliferate (at all), thereby decreasing the fat cell number that might be capable of accumulating lipid. In both instances, there would be a net decrease in the cells responsible for lipid storage. Further, as the progeny

of proliferative-competent mature adipocytes have, themselves, received little attention, we suggest that deciphering the "plasticity" characteristics of these daughter cells may lead to greater advances in our current idea of "traditional" adipogenesis. Indeed, our collaborative efforts have resulted in a repeatable method to purify mature adipocytes, documentation of the cells as they dedifferentiate, and initiation of experiments to determine the ability of progeny cells to re-differentiate into lipid-filled adipocytes (10, Figure 1). This phenomenon of reverse adipogenesis by mature adipocytes, along with progeny cell adipogenesis, will allow scientists to gain a new and solid grip on adipogenesis.

Alternative possibilities

As we are observing the physiology of cells *in vitro*, we must allow for a bit of caution in our results. For example, might there be different explanations for the dedifferentiation seen in our cultures? From a traditional view, mature adipocytes are incapable of dedifferentiating, or even returning to proliferative competency. As we have documented, mature, lipid-laden adipocytes do initiate proliferation and do form daughter cells (many of which possess equal amounts of lipid, whereas others display asymmetrical distributions of lipid). One possible explanation for the dedifferentiation and subsequent proliferation may actually be that some fibroblast-type cells have the ability to accumulate lipid, but not to terminally differentiate. Similarly, what we have long assumed regarding the developmental potential of preadipocytes may need some revision. Preadipocytes may be capable of both proliferation and lipid metabolism – without terminal differentiation. Thus, despite the seemingly outward mature appearance of these two cell types, they may be capable of retaining their ability to return to the proliferative mode when required. Lastly, what may seem to be a fully committed mature adipocyte may, in fact, represent a cell that we do not fully understand – a cell type that is able

to express both differentiated and proliferative phenotypes. Regardless of the true nature of these cells, the traditional definition of adipocyte cell commitment may need to be revised in order to gain a solid grip on the complete picture of adipogenesis.

Another caveat that must be taken into consideration is the *in vitro* environment in which the dedifferentiation of mature adipocytes has been recorded. At best, *in vitro* conditions are not *in vivo* conditions, and the artificial milieu in which these cells are cultured must be critically evaluated. One criticism of the dedifferentiation research has been that cultured cells are not supplied with all the required nutrients for survival in a differentiated state – in essence, the cells are "starved." However, the medium used *in vitro* to induce the dedifferentiation of mature adipocytes is medium that is classically referred to as "growth" medium (8, 12, 22, 23). This implies that the medium contains sufficient nutrients to induce proliferation of cells – a process known to have a high requirement for energy and other metabolic building blocks. Another potential criticism of this type of research is that mature adipocyte cells have been removed from their three-dimensional *in vivo* environment and placed in two-dimensional plastic cell cultureware. When placed in a three-dimensional culture environment, mature adipocytes exhibit dedifferentiation in a similar manner to that in Figure 1 (24).

In summary, we are proposing that alternative treatments of dysfunctions such as obesity may involve defining the regulation of a new, and more complete, version of adipogenesis. If mature adipocytes dedifferentiate and divide the resulting daughter cells will then proceed to differentiate into a greater numbers of mature adipocytes. If this subsequent differentiation (and accumulation of lipid) is not controlled, this may be an associative factor in making one fatter. Experiments with mature adipocytes (or whatever they are determined to

actually represent) may provide a new strategy for regulation of the cellularity (or metabolism) of cells capable of being involved in adipose tissue formation and growth.

REFERENCES

1. Adebonojo, F.O., 1975. Studies on human adipose cells in culture: Relation of cell size and cell multiplication to donor age. Yale J. Biol. Med. 48, 9-16.

2. Adebonojo, F.O., 1975. Monolayer cultures of disaggregated human adipocytes. In Vitro 11, 50-54.

3. Ailhaud, G., Grimaldi, P., Negrel, R., 1992. Cellular and molecular aspects of adipose tissue development. Ann. Rev. Nutr. 12, 207-233.

4. Aronne, L.J., 2001. Epidemiology, morbidity and treatment of overweight and obesity. J. Clin. Psychiatry 62, 13-22.

5. Cancello, R., Pietri-Rouxel, F., Clement, K., 2005. Spontaneous lipid accumulation in primary cultures of dedifferentiated human adipocytes. Adipocytes 1, 73-78.

6. Colditz, G.A., 1999. Economic costs of obesity and inactivity. Med. Sci. Sports Exerc. 31, S663-667.

7. Cornelius, P., MacDougald, O.A., Lane, M.D., 1994. Regulation of adipocyte development. Ann. Rev. Nutr. 14, 99-129.

8. Cousin, B., Munoz, O., Andre, M., Fontanilles, A.M., Dani, C., Cousin, J.L., Laharrague, P., Casteilla, L., Penicaud, L., 1999. A role for preadipocytes as macrophage-like cells. FASEB J. 13, 305-12.

9. Fajas, L., 2003. Adipogenesis: A cross-talk between cell proliferation and differentiation. Ann. Med. 35, 79-85.

10. Fernyhough, M.E., Helterline, D.L., Vierck, J.L., Hausman, G.J., Hill, R.A., Dodson, M.V., 2005. Dedifferentiation of mature adipocytes to form adipofibroblasts: More than just a possibility. Adipocytes 1, 17-24.

11. Flegal, K.M., Carroll, M.D., Ogden, C.L., Johnson, C.L., 2002. Prevalence and trends in obesity among US adults, 1999-2000. JAMA. 288:1723-1727.

12. Gamou, S., Shimizu, Y., Shimizu, N., 1990. Adipocytes. In: *Methods in Molecular Biology Vol. 5, Animal Culture.* J. W. Pollard, and J. M. Walker (Eds.) Clifton, NJ: The Humana Press, 197-207.

13. Gregoire, F.M., 2001. Adipocyte differentiation: From fibroblast to endocrine cell. Exp. Biol. Med. 226, 997-1002.

14. Houseknecht, K.L., Baile, C.A., Matteri, R.L., Spurlock, M.E., 1998, The biology of leptin: A review. J. Anim. Sci. 76, 1405-1420.

15. Justesen, J., Pedersen, S.B., Stenderup, K., Kassem M., 2004. Subcutaneous adipocytes can differentiate into bone-forming cells *in vitro* and *in vivo*. Tissue Eng. 10, 381-391.

16. Kersten, S., 2001 Mechanisms of nutritional and hormonal regulation of lipogenesis. EMBO Rep. 2, 282-286.

17. Kokta, T., Dodson, M.V., Gertler, A., Hill, R.A., 2004. Intercellular signaling between adipose tissue and muscle tissue. Domest. Anim. Endocrinol. 27, 303-331.

18. Large, V., Peroni, O., Letexier, D., Ray, H., Beylot, M., 2004. Metabolism of lipids in human white adipocyte. Diabetes Metab. 30, 294-309.

19. National Task Force on the Prevention and Treatment of Obesity, 2000. Overweight, obesity, and health risk. Arch. Intern. Med. 160, 898-904.

20. Rosen, E.D., Spiegelman, B.M., 2000. Molecular regulation of adipogenesis. Annu. Rev. Cell Dev. Biol. 16, 145-171.

21. Sturm, R., 2003. Increases in clinically severe obesity in the United States, 1986-2000. Arch. Intern. Med. 163, 2146-2148.

22. Sugihara, H., Yonemitsu, N., Miyabara, S., Toda., S., 987. Proliferation of unilocular fat cells in primary culture. J. Lipid Res. 28, 1038-1045.

23. Sugihara, H., Yonemitsu, N., Miyabara, S., Yun, K., 1986. Primary cultures of unilocular fat cells: Characteristics of growth in vitro and changes in differentiation properties. Differentiation. 31, 42-49.

24. Sugihara, H., Yonemitsu, N., Toda, S., Miyabara, S., Funatsumaru, S., Matsumoto, T., 1988. Unilocular fat cells in three-dimensional collagen gel matrix culture. J. Lipid Res. 29, 691-7.

25. Visscher, T.L., Seidell., J.C., 2001. The public health impact of obesity. Annu. Rev. Public Health. 22, 355-375.

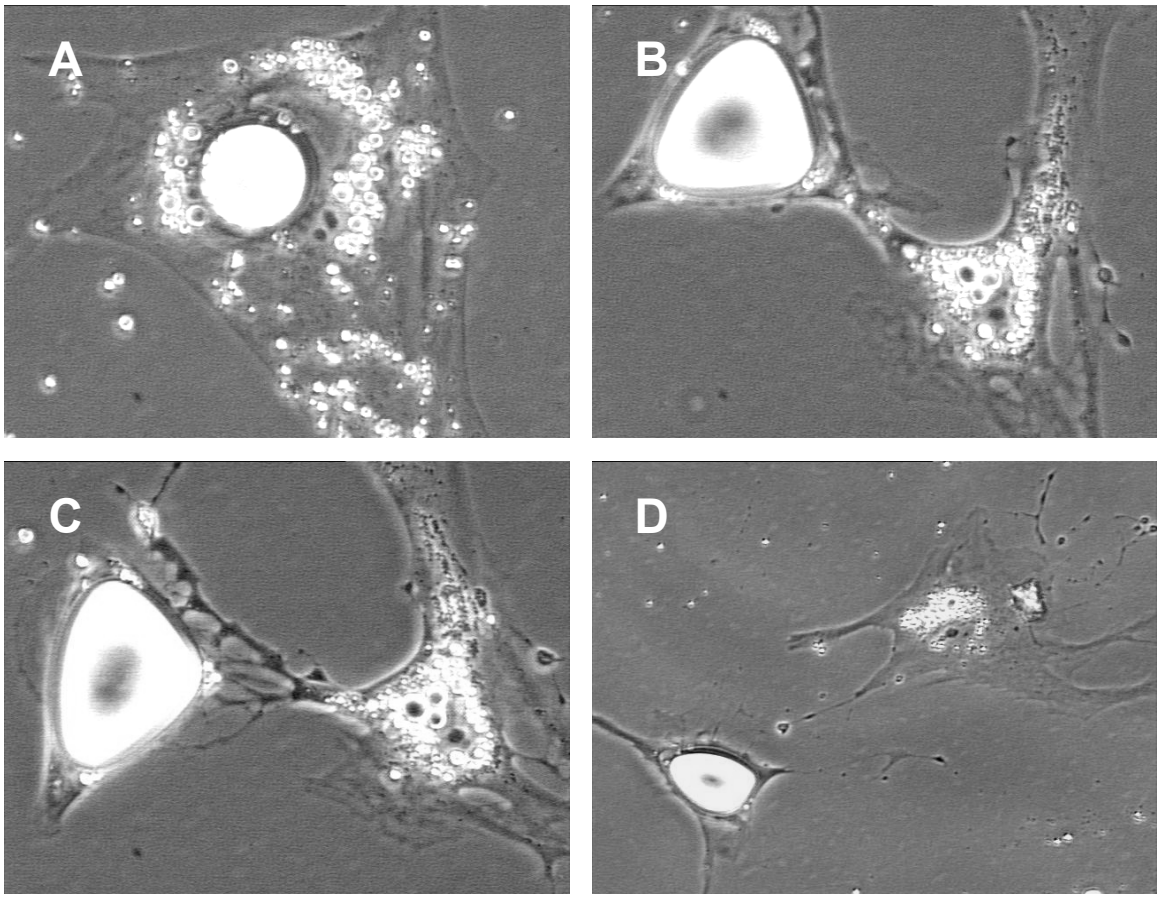

Figure 1: Photomicrographs of a mature lipid-containing adipocyte asymmetrically dividing into proliferative competent cells. Panel A shows a purified mature adipocyte in culture two days after a differential plating (200X). Five days after purification (panels B and C) the cell is actively dividing into two cells. The photomicrographs were taken 3 h apart at 200X magnification. Panel D shows the resulting daughter cells (100X) from the cell in panel A. The large lipid drop in the parent cell can be easily followed to one of the daughter cells.