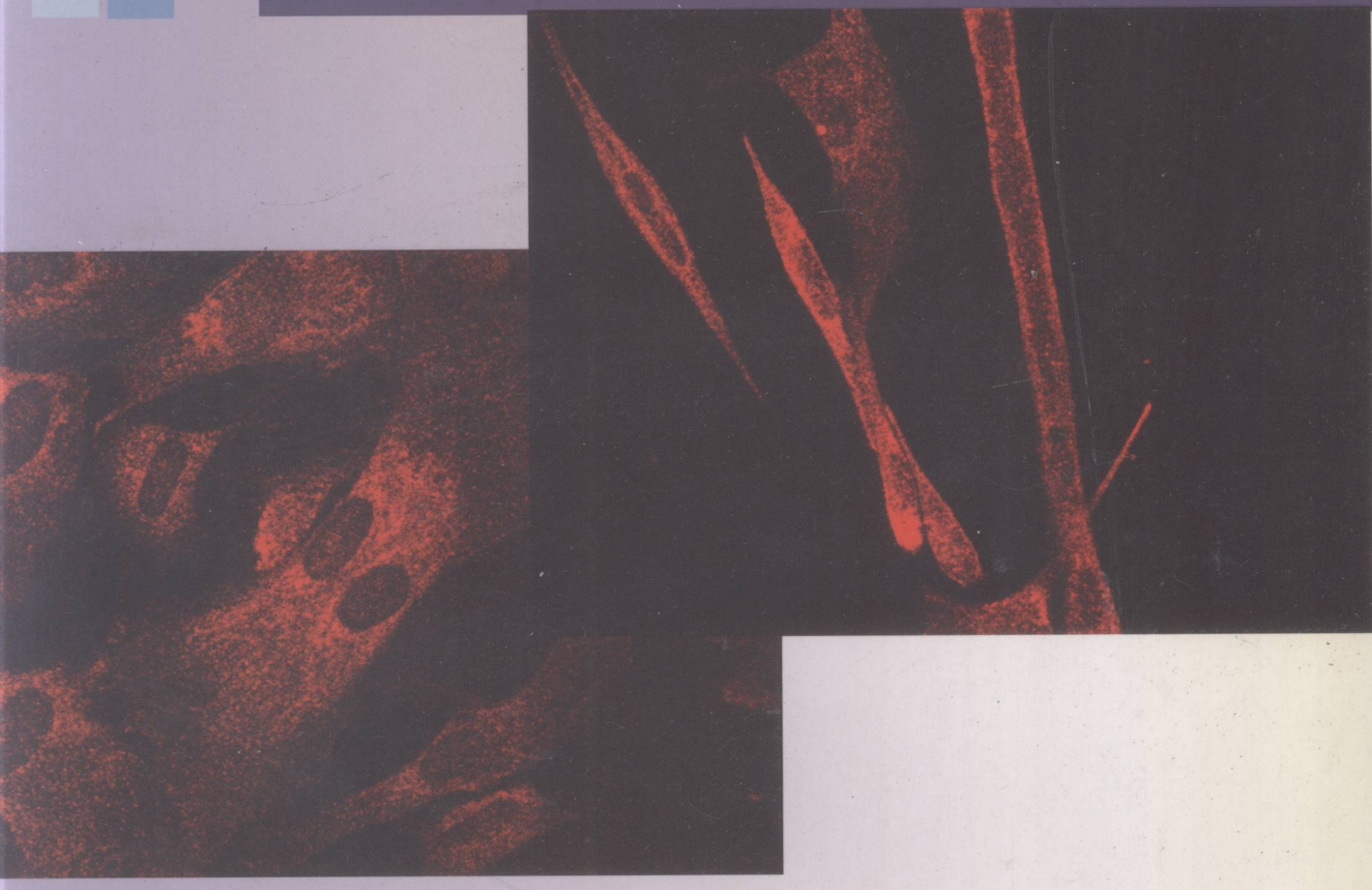

動物生長生物學研究技術專輯

Proceedings of Research Technology Related to Animal Growh Biology

編著：丁詩同、陳保基、鄭登貴

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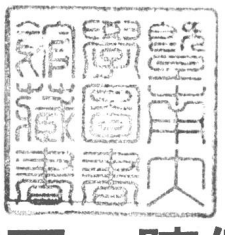

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編者 序

肌肉細胞之增生與分化程度，直接影響動物生長與瘦肉產出，對畜牧生產之影響甚巨。雖然動物生長與發育經多年之研究，已有眾多發現，但對胞外基質與其在肌肉細胞生長與分化之角色，則為近年的研究重點。本專輯中 Dr. Sandra G. Velleman 針對動物肌肉細胞增生與分化之分子調控機制詳加闡述；歐柏榮教授對 Myostatin 在肌肉發育中的角色有精彩的解說；而林名釗博士對可調控式轉基因系統的介紹則有助於研究的推展。Dr. Michael S. Lilburn 則對腸道的生長與腸道多肽對其發育的調控有所探討。本專輯亦囊括多種目前在細胞生物學研究領域中常被使用之技術，包括：建立肌肉衛星細胞、肌肉細胞、脂肪前身細胞與脂肪細胞等之體外培養系統、各相關基因產物諸如 mRNA 之測定方法（即時 RT-PCR）；惟本專輯鑑於篇幅之限，並未對各項原理有較深入之解說，而著重於各試驗技巧之實作描述，遂更能符合實際從事該相關領域研究工作者之需求，請讀者依個人需要取捨各章節參考閱讀之。

本專輯除蒙各位作者努力編撰外，亦賴教育部「提升科技系所師資計畫」之經費補助，始克順利付梓，謹此併致謝忱。文中用詞或有不妥，校對或有不週，尚祈時賢不吝指正，俾便更正，是所至盼感激。

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民國九十三年十一月二十三日

動物生長生物學研究技術專輯
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目錄

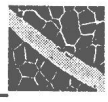
1. Molecular Regulation of Muscle Growth

Sandra G. Velleman, The Ohio State University/OARDC, Department of Animal Sciences, 1680 Madison Ave., Wooster, OH 44691

(Prepared to accompany the seminar, "Molecular Regulation of Muscle Growth")

Skeletal muscle comprises the largest proportion of animal mass. Understanding the mechanisms that regulate muscle growth has both biomedical and agricultural implications. From a biomedical perspective muscling is important for regulating muscle mass (an area of intense importance to body builders), the repair and regeneration of muscling with injury, maintenance of muscle mass with aging, and the maintenance of muscle in the advent of muscle wasting diseases like muscular dystrophy. The maintenance of muscle mass with aging is becoming a primary issue with increased longevity. The loss of muscle mass as one ages is termed sarcopenia. A sedentary 70 year old can lose up to 40% of their muscle mass. This leads to a sedentary lifestyle as mobility is decreased. A key issue is people with osteoporosis and sarcopenia. An osteoporoetic individual has increased bone fragility and if they also have sarcopenia have more difficulty in righting themselves if they lose their balance. Thus, creating a situation of increased risk of falling and bone fractures. Many elderly people do not recover from a bone fracture in this situation and die from pneumonia. In the United States, The National Institute of Aging has placed sarcopenia research as one of their top research priorities. With regard to agriculture, muscle is the major food product. One of the goals of the domestic animal industries has been to select animals for growth rate and muscle mass while maintaining meat quality.

Muscle cell proliferation, migration, adhesion, and fusion are processes involved with the formation of multinucleated myotubes that will further differentiate into mature muscle fibers (Swartz et al., 1994). Not all muscle fibers form simultaneously during the prenatal period of muscle development. The first set of embryonic muscle fibers to form is termed "primary fibers" that have centrally located nuclei. Surrounding the primary fibers are mononucleated myogenic cells that will differentiate into the secondary muscle fibers. The secondary muscle fibers position is based on the location of the primary fibers. After the secondary fibers form, the Z band of the sarcomeres, the contractile unit of muscle, will line up forming a mature muscle fiber. The sarcomere is comprised of overlaps of myosin and actin. During contraction, the myosin head will attach to the



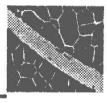
actin filaments and the actin filament mediated by the movement of the myosin head will move towards the center of the sarcomere. This results in a shortening of the sarcomere.

In almost all animals and birds, muscle fiber formation is complete at birth or hatch. Hence, after birth or hatch, there is no new muscle fiber formation. Neonatal animals have dramatic muscle growth so additional mechanisms beyond increases in muscle fiber number must be involved in postnatal muscle formation.

Mechanisms of Muscle Growth

Muscle growth can be separated into two phases, hyperplasia and hypertrophy. Hyperplasia refers to the prenatal increase in myoblast cell number. During the prenatal period of muscle development, myoblasts are proliferating and then differentiating into myotubes. Through the prenatal formation of multinucleated myotubes, nuclei number is set at birth. However, for postnatal muscle growth to occur there must be an increase in protein synthesis which is the direct consequence of more DNA resulting in increased transcription and translation. To acquire more DNA, there is a required increase in nuclei number. Since nuclei number is set at birth, there must be an additional mechanism leading to continued postnatal muscle growth. Alexander Mauro (1961) identified the presence of a cell "wedged" between the plasma membrane and the basement membrane of skeletal muscle fibers. These were called "satellite cells" as they have little cell cytoplasma. Mauro hypothesized that the satellite cells may be involved in postnatal muscle growth. In 1971, Moss and LeBlond demonstrated that when ^{3}H-thymidine was given to rats the label incorporated into the nuclei of the satellite cells for the first hour. After this time, it was found in the nuclei of the muscle fiber. Allen et al. (1979) reported that most of the nuclei in a mature muscle fiber are from satellite cells. After birth, the satellite cells fuse with the existing muscle fibers causing an increase or hypertrophy in muscle fiber size. This postnatal muscle growth through satellite cell activation is termed hypertrophy. It is also the satellite cell population that is responsible for the repair of muscle with injury in humans. With aging, the satellite cells have reduced activation resulting in the sarcopenia of muscle.

Simply incorporating more satellite cell nuclei into a muscle fiber alone does not hypertrophy muscle. But it is the balance between protein synthesis and degradation. To have an increase in muscle fiber size requires protein synthesis rates being higher than the rate of protein degradation. Degradation of muscle is largely regulated by the calpain system (for review Goll et al., 2003). The calpains, μ-calpain and m-calpain, are calcium dependent proteases that do not degrade proteins to amino acids but disassemble the



myofibril to the point of the Z-band. The further degradation of the myofibrillar structure is through the activity of the proteasome system. Calpastatin is a protein whose only known function is to inhibit the activity of the calpains.

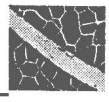
Factors Regulating the Growth Muscle

Muscle growth is regulated by growth factors, muscle-specific transcriptional regulatory factors, cell surface integrin receptors, and the extracellular matrix. Growth factors are strong stimulators or inhibitors of muscle cell proliferation and differentiation. The following growth factors have been shown to affect muscle growth properties: hepatocyte growth factor, fibroblast growth factor 2 (FGF2), insulin-like growth factor, transforming growth factor beta (TGF-β), and myostatin. These growth factors all impact the activity of satellite cells in their ability to proliferate and differentiate. Table I summarizes how each of these growth factors influences satellite cell function.

Table I: The Effect of Growth Factors on Myogenic Satellite Cell Behavior

Growth factor	Activation	Proliferation	Differentiation
Hepatocyte Growth Factor	Stimulates	Stimulates	Inhibits
Fibroblast Growth Factor	No effect	Stimulates	Inhibits
Insulin-like Growth Factor	No effect	Stimulates	Stimulates
Transforming Growth Factor Beta	No effect	Inhibits	Inhibits
Myostatin	No effect	Inhibits	Inhibits

In recent years, myostatin has received significant research attention from both the biomedical and agricultural communities. Myostatin is a member of the TGF-β family and is a strong inhibitor of both satellite cell proliferation and differentiation. Inhibiting the expression of myostatin results in a double muscling condition in cattle which significantly increases muscle mass. In addition to the increase in muscle mass, meat quality is good from these animals with a decrease in intramuscular fat and connective tissue, and the animals exhibit improved feed efficiency. For the domestic animal



industries, these myostatin regulated changes in muscling are viewed as a positive. How the domestic animal industries uses inhibiting myostatin expression to achieve increased muscle mass is a question which still needs to be resolved. It is clear that decreasing the amount of myostatin expressed has beneficial aspects, but in the United States consumer acceptance of a product is a critical factor. Many consumers are convinced that genetically engineered products are not healthy. Thus, consumer education and how a product is introduced to the market are critical in its acceptance.

Although enhanced muscling is a morphological characteristic that is selected for by the domestic animal industries, increased muscling does not always lead to a desirable meat product. For example, the callipyge condition in lambs visually appears to produce well developed muscling with acceptable fat levels. The resulting meat product is extremely tough and not desirable. The important message from the callipyge condition is that selection for muscle mass alone is not sufficient.

The development and growth of muscle is regulated by muscle-specific transcription factors. These factors permit the expression of genes necessary for muscle cell proliferation and differentiation. The muscle-specific transcriptional regulatory factors are members of a superfamily of basic helix-loop-helix transcription factors and consist of MyoD (Davis et al., 1987), Myf5 (Braun et al., 1989), myogenin (Edmondson and Olson, 1989; Wright et al., 1989), and MRF4 (Rhodes and Konieczny, 1990). MyoD and Myf5 are required for commitment of the proliferating somatic cells to a myogenic lineage whereas myogenin and MRF4 are required for the committed cells to further differentiate into myocytes and mature myofibers. Satellite cells have a similar myogenic regulatory expression pattern to that of embryonic myoblasts, with the expression of MyoD and Myf5 upregulated in activated satellite cells during proliferation and the upregulation of myogenin and MRF4 in satellite cells entering differentiation (reviewed in Seale and Rudnicki, 2000). Although not well understood, it is likely that the developmental timing and amount of expression of the muscle transcriptional regulatory factors will affect muscle growth potential through the regulation of the processes of proliferation and differentiation.

The formation of muscle requires cell to cell contact and adhesion between muscle cells or satellite cells with existing muscle fibers. The contact between cells is mediated through cell receptors, termed integrins, which influence cell adhesion, migration, proliferation, and differentiation (Blaschuk and Holland, 1994; Boettiger et al., 1995; Chon et al., 1998). Integrins are a widely expressed family of heterodimeric cell surface receptors containing α- and β-subunits that provide a means for bidirectional transmission of signal information between cells or cells and their extracellular

environment. The α- and β- subunits contain cytoplasmic, transmembrane, and extracellular domains. There are numerous isoforms of each integrin subunit with tissue-specific distributions and unique functional properties. The β1 integrin subunit is expressed by both myoblasts and myotubes. Investigations have shown that β1-containing integrins are important in the adhesion of muscle cells (Neff et al., 1982; Decker et al., 1984) and myoblast fusion into multinucleated myotubes (Rosen et al., 1992). Myoblasts cultured in the presence of the β1 integrin cell substrate attachment antibody do not align and fuse to form multinucleated myotubes but remain as proliferative single cells (Menko and Boettiger, 1987). Thus, the appropriate expression of integrin is required for the cell to cell adhesion necessary for the formation of muscle fibers.

The formation of muscle requires the migration of muscle cells to align and then fuse into multinucleated myotubes. Without the formation of multinucleated myotubes, mature muscle fibers will not be able to form. For cells to migrate, they must attach and spread across the extracellular matrix environment. This interaction of the integrins with the extracellular matrix is not a passive mechanical association but activates focal adhesion kinase and growth factor signaling pathways (Ruoslahti, 1997) modulating cell proliferation and differentiation. The integrin receptors are not randomly located across the plasma membrane but form focal adhesion clusters regulating cell shape. As illustrated in Figure 1, Chen et al. (1997) showed the binding of integrins to the extracellular matrix caused the cells to extend over a large surface area, whereas rounded cells exhibited a loss of extracellular matrix contact and had reduced survival and proliferation. The reduction in cell survival is referred to as the process of programmed cell death or apoptosis.

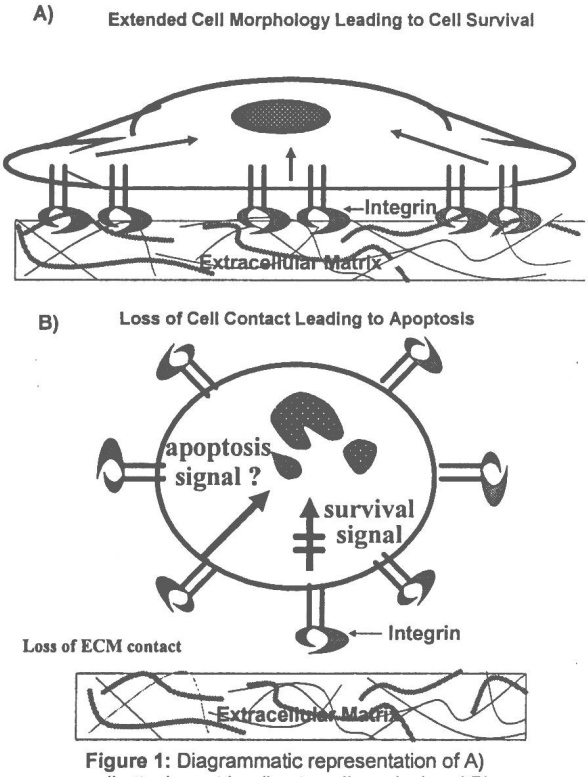
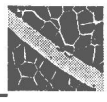


Figure 1: Diagrammatic representation of A) cell attachment leading to cell survival and B) loss of cell attachment leading to apoptosis.

When cell migration is complete, the newly migrated cells begin to interact with one another and reorganize into a functional tissue. Appropriate expression of the extracellular matrix and interaction of extracellular matrix macromolecules with each other plays a pivotal role in the formation and maintenance of tissues. The extracellular

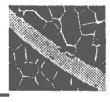


matrix is a dynamic structure whose macromolecular composition changes with tissue needs and governs cellular responses through its interaction with growth factors, adhesion to cell receptors like the integrins, and interaction with other extracellular matrix macromolecules. The extracellular matrix is composed of all secreted molecules immobilized outside the cell. In muscle, there are three layers of connective tissue composed of extracellular matrix macromolecules. These layers are the epimysium, perimysium, and endomysium. The major macromolecular components of the extracellular matrix include collagens, proteoglycans, and noncollagenous glycoproteins.

Collagen biosynthesis is an extremely complex process. There are over 20 different vertebrate collagens with tissue-specific distributions and unique functional properties. These unique collagen types can be subdivided into the following classes based on function or size: fibrillar; fibril-associated; network forming; filamentous; short chain; and long chain (van der Rest and Garrone, 1991). Bone contains collagen Type I; cartilage contains collagen Type II, and skeletal muscle contains collagens Type I and III. These collagens are fibrillar in nature. The fibrillar collagens such as Types I and III contain a single triple-helical domain consisting of three separate peptide chains. The three chains wrap around each other, forming an alpha helix, and are linked together by interchain disulfide bonds. After the collagen molecules are synthesized, they are secreted into the extracellular space and align in a quarter-stagger array; crosslinking between the microfibrils is initiated and larger diameter fibrils forms. As an animal ages, collegen crosslinking is progressive, and fiber size increases (Reiser et al., 1992). Collagen crosslinking is an issue of utmost importance with regard to meat tenderness with tougher meat usually having higher levels of collagen crosslinking.

Extracellular matrix proteoglycans are proteins that contain carbohydrates called glycosaminoglycans, which are covalently attached to a central core protein. Typical glycosaminoglycans attached to the proteoglycan central core protein include chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate. Based on this definition, the proteoglycans are a diverse family of macromolecules that exhibit both developmental and tissue specificity in terms of their expression. Unlike the collagens with a unique triple helical domain, there is no common structural feature associated with the proteoglycans.

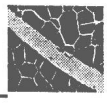
In skeletal muscle, the proteoglycans play a major role in regulating muscle cell responsiveness to the growth factors like FGF2 and TGF-β. FGF2 is a potent stimulator of muscle cell proliferation and a strong inhibitor of differentiation. The interaction of FGF2 with its high affinity receptor is mediated by the interaction of FGF2 with the heparan sulfate chains of heparan sulfate containing proteoglycans like syndecan and



glypican. Yayon et al. (1991) demonstrated that cells deficient in heparan sulfate proteoglycan levels and transfected to express a FGF2 receptor were unable to bind FGF2. Furthermore, treatment of cells with chlorate to prevent glycosaminoglycan sulfation decreased binding of FGF2 to its high affinity receptor (Olwin and Rapraeger, 1992). These and other studies have clearly demonstrated that heparan sulfate functions as a low affinity co-receptor for FGF2. Since domestic animals have been selected for enhanced muscling based on phenotype not by biological mechanisms involved in regulating muscle growth, it is unclear how growth selection has affected the expression of extracellular matrix macromolecules critical to the regulation of muscle growth muscle.

To investigate how selection for growth and muscling has affected the expression of the heparan sulfate proteoglycans, studies are in progress comparing heparan sulfate proteoglycan expression in a turkey line not selected for growth (RBC2) and one selected from the RBC2 line for only increased 16 wk body weight (F line). During both embryonic and posthatch periods of age, heparan sulfate proteoglycan expression was higher in the growth selected F line compared to the unselected RBC2 line (Liu et al., 2002). The expression of FGF2 mRNA was higher earlier in embryonic development for the F line compared to the RBC2 line (Liu et al., 2003). This suggests that the growth selected F line has the potential for increased FGF2 signaling which would stimulate the proliferation of muscle cells leading to enhanced muscle development and growth. In a related study, McFarland et al. (2003) showed that fast growing turkey muscle cell populations were more responsive to FGF2, expressed more FGF2 mRNA, and had higher levels of heparan sulfate proteoglycans compared to slower growing muscle cell populations.

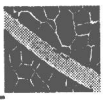
In muscle, the two predominant groups of heparan sulfate proteoglycans expressed are the syndecans and glypicans. The syndecan family is composed of four members, syndecan-1 through 4 that are all found in skeletal muscle. The syndecans have a membrane spanning core protein possessing a highly conserved cytoplasmic domain and transmembrane domain, and a diverse ectodomain to which the glycosaminoglycan chains are attached (Carey, 1997; Rapraeger, 2001). Glypicans, 1 through 6, have a core protein that contains conserved cysteine residues and glycosaminoglycan attachment sites, and they are attached to the cell plasma membrane through a glycosylphosphatidyl-inositol anchor (David et al., 1990). Only glypican-1 (glypican) has been reported in skeletal muscle tissue. *In vitro* studies indicated that syndecan-1, 3, and 4 expression are down regulated during skeletal muscle differentiation (Larraín et al., 1997; Fuentealba et al., 1999), whereas syndecan-2 remains unchanged (Brandan and Larraín, 1998). In contrast, glypican expression increases significantly



during cell differentiation (Brandan et al., 1996). Both syndecans and glypicans mediate FGF2 binding to fibroblast growth factor receptors and regulate FGF2 activity (Steinfeld et al., 1996; Filla et al., 1998). The different expression patterns of syndecan-1 and glypican imply that these two proteoglycans may have functional differences in regulating cellular responsiveness to FGF2 signaling. In order to investigate the expression of syndecan-1 and glypican as it relates to muscle development, studies have been ongoing in Dr. Velleman's laboratory measuring the expression patterns of syndecan-1 and glypican in the F and RBC2 lines during both embryonic and posthatch development (Liu et al., 2004). Through *in situ* hybridization, syndecan-1 expression was detected through embryonic days 14 through 24 but no during posthatch development in both the F and RBC2 lines with no measurable difference between the lines. In contrast, glypican expresssion was only measurable beginning at embryonic day 18 and was present during posthatch stages through 16 weeks of age. These initial results support the findings that syndecan-1 and glypican are differentially expressed. Syndecan-1 expression coincides with the period of muscle cell proliferation and maybe playing a role in presenting FGF2 to its receptor permitting the stimulatory affect of FGF2 on muscle cell proliferation. Glypican appears to be expressed later in muscle development when differentiation is occurring. It is possible that glypican functions by sequestering FGF2 from its receptor to permit differentiation. Currently, expression vector constructs for both syndecan-1 and glypican are being prepared in Dr. Sandra Velleman's laboratory to transfect muscle cells to determine the effect of altered expression of these heparan sulfate proteoglycans on muscle cell proliferation, differentiation, and responsiveness to FGF2.

Responsiveness to TGF-β is also mediated through proteoglycan dependent cell signaling. Decorin a chondroitin/dermatan sulfate containing proteoglycan interacts with TGF-β. Riquelme et al. (2001) showed in myoblasts in which the expression of decorin was inhibited were less sensitive to TGF-β-dependent inhibition of myogenesis. Thus, the suppression of decorin results in an acceleration of muscle terminal differentiation.

In summary, a number of factors are involved in the molecular regulation of muscle growth. A comprehensive understanding of these mechanisms and their impact on muscle growth through the processes of hyperplasia and hypertrophy is of importance to both the biomedical and agricultural communities.



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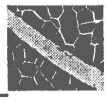

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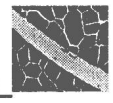

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