Overview of Molecular, Cellular and Genetic Mechanisms Regulating Myogenesis

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Introduction

Those of you who are working in the area of muscle growth or myogenesis acknowledge the challenge in presenting this topic because of the complexities of myogenesis. The title infers a tall order but the objective of this presentation is to inventory, by way of overview, some of the new and emerging aspects of myogenesis while providing an introduction to the topics for the following presentations. The intent is to keep the topic as conceptual as possible; but in the process, many important details from the primary sources of literature have been intentionally kept out.

One can hypothesize that some of the quantity and quality problems facing meat animal production may be addressed through studies of the molecular and cellular mechanisms of myogenesis and muscle growth. Some of the items that will be discussed here may sound a little bit foreign to those not directly working on myogenesis.

As meat scientists, we tend to look at muscle as having a very narrow role, and that being an agricultural or a dietary role to fulfill. In reality, skeletal muscle has a much larger and fundamental biological role; that is to provide locomotion and to enable us to perform various functions and work. It is from these external demands that skeletal muscle has evolved. Skeletal muscle is a beautiful tissue, being highly organized in terms of its architecture, arrangement and development (Muntz, 1990). It has unique differentiation characteristics and distinct proteins. Myogenesis, the formation of muscle, requires extensive orchestration of many factors which affect commitment of cells to a lineage, movement/migration of cells, proliferation, interaction of different cell types, differentiation and, ultimately, morphogenesis into a functional organ. Consequently, it remains a tissue that is highly sought after and highly studied by scientists around the world as a model for cell biology. Recent literature proves that it has become an important model for studies of the extra-cellular, cellular and sub-cellular molecular mechanisms governing tissue-specific gene regulation, proliferation and differentiation.

In the past several years, there has been renewed and increased interest in the study of some of the genetic and molecular events, controls of basically "determinants" of muscle growth (Buckingham, 1992; Sassoon, 1993; Olson and Klein, 1994). This has been particularly important at the very early stages of embryonic development, because this is when the somites are formed and when cells derived from the somites are proliferating and migrating to form skeletal muscle tissue. We also are in an era where skeletal muscle cells are being examined as plausible delivery systems for gene therapy (Barr and Leiden, 1991) and mutagenic studies (Hill and Wurst, 1993).

Animal agriculture offers several examples of genetic conditions of exaggerated muscling around the world (e.g., double muscling in cattle, Pietran breeding in pigs and the muscular hypertrophy gene in sheep), and also some other examples of models where one could talk about animals modified physiologically to express greater amounts of muscle (e.g., use of somatotropins and beta-agonists). These diverse conditions of enhanced muscle growth all present interesting and intriguing opportunities and questions for meat animal scientists and biologists for continued study of myogenesis. Key questions about the genetic models are: How do these kinds of diversity result? Secondly, from a molecular and cellular basis, what do we know about these diverse conditions?

Conventional Dogma of Myogenesis

If one went back 25 to 30 years, one would find that the literature on myogenesis began expanding as embryonic muscle cells were initially cloned. As a measuring stick, we could go back about 10 years and talk about what we would have described as myogenesis at that time. One may have talked about the events depicted in Figure 1. One could have talked about a few key hormones, selected growth factors, certainly some of the contractile protein genes, etc. We would have talked about the sequence of events where cells would depart from a mesenchymal mesoderm forming presumptive muscle cells, being able to proliferate to increase in their cell number and upon receiving the proper stimuli or signals, would withdraw from the proliferative stages of the cell cycle, initiate expression of tissue-specific proteins, such as contractile protein genes. Accompanying these events based on cell culture, we would find these differentiated mononucleated muscle cells (myoblasts) fusing with each other to form multi-nucleated myotubes and bulk synthesis of contractile proteins would occur. Eventually, the myofiber would develop from the myotube as myofibrils are assembled and nuclei take on a peripheral location. One could have talked about some of the roles of selected growth factors that would be involved in those various stages. This dogma was enabled because myoblasts are easily cultured and maintained in undifferentiated, proliferative states in the presence of high serum or mitogens found

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Figure 1
Conceptualized Sequence of Myogenesis

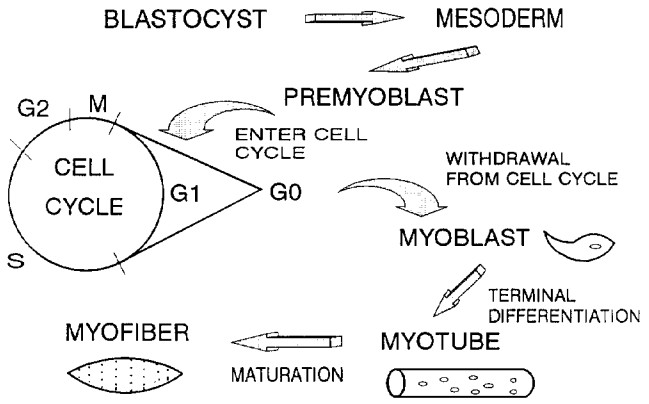
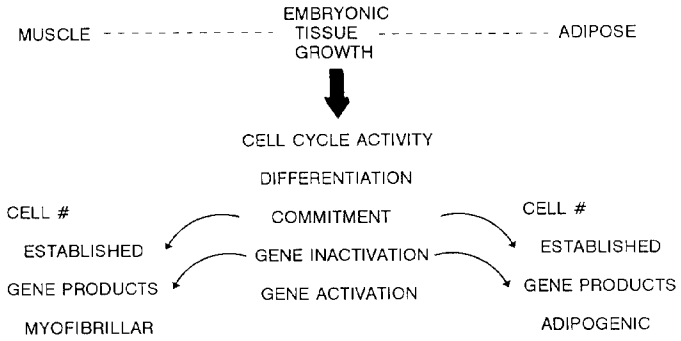


Figure 2
Summary of Processes Important in Embryonic Tissue Development



in serum. Removal of serum from the culture environment favors the differentiation stages.

Over the years, scientists have used many cell culture experiments to examine the physiological controls of myogenesis. One realizes that biological activities of muscle cells are easily manipulated in vitro. Without question, employment of cell culture techniques have led to major advancements in understanding myogenesis. However, it has become clear that the environment that cell culture provides cannot replicate or reproduce some of the spatial and chemical kinds of environments or complexity of cell-to-cell interactions that you would find in an embryo. And one can't really mimic or orchestrate exactly many of the interactions of various factors involved in regulating cell division and differentiation and even tissue formation observed in a developing embryo.

In embryonic and fetal tissue development, commitment and differentiation are important events (Figure 2). Establishment of skeletal muscle during embryonic development involves commitment of mesodermal progenitors to myogenic cell lineages. When we talk about commitment, we mean heritable changes in the genetic material within cells. These changes define the lineage that the cell is going to remain in, and the tissue that will arise from that sequence of cells. It describes the genetic changes which conceptually restrict the fate of the cell (Emerson, 1993; Konieczny, 1991).

During terminal differentiation, undifferentiated cells become functional muscle cells. This differentiation involves events which are distinct from commitment because at this point we're getting activation of lineage-specific genes, such as α-actin or contractile protein genes, etc. This occurs as the cells are forced or signalled to exit the cell cycle as a result of specific growth factors from the localized cell environment. In the process of embryonic muscle cell formation, events which affect decision of cells to withdraw from a proliferative stage of the cell cycle are considered critical to determining the ultimate number of muscle cells in a muscle of an animal. The

Figure 3
Summary of Processes Important in Postnatal Tissue Development

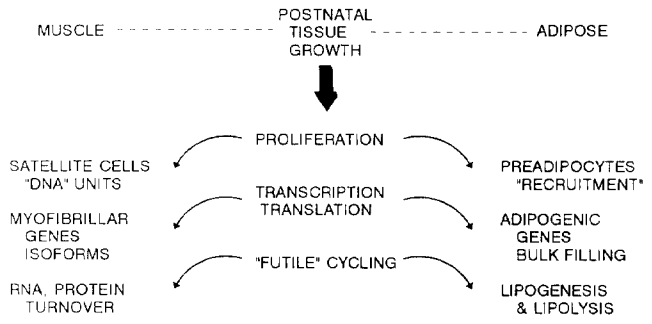
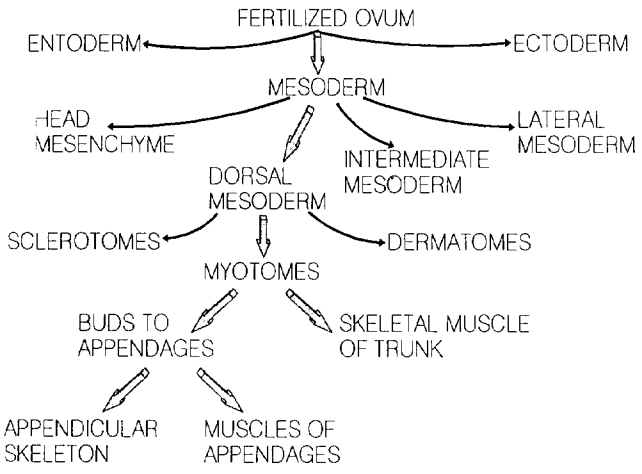


Figure 4
Formation of Primary Germ Layers and Cell Lineages



next phase of differentiation involves maturation and specialization. Cells begin to express selected members or isoforms of multi-gene families in a developmental pattern from embryonic, neonatal and adult stages of the myofiber development (Young and Brown, 1990).

Also on the post-natal side, (Figure 3) one should discuss the events that affect satellite cell proliferation, which endow skeletal muscle with an increased enucleation and DNA units, allowing for greater hypertrophy later on in post-natal growth. Along with these events, several factors which influence bulk hypertrophy are primarily under transcriptional control, and in some cases, translational control. They involve activation of contractile protein gene families which make the muscle fiber more adapted to the demands which may be placed on the muscle (Muntz, 1990). Again, there may be switching of different isoforms of muscle genes. Lastly, the involvement of protein turnover or the continual synthesis and degradation of existing proteins are critical control points for the biological efficiency of protein accretion (Bergen et al., 1985).

Establishment of Lineages

Events important to differentiation are now believed to occur many divisions prior to a final mitosis. The developmental paths that all cells in the body are derived from are summarized in Figure 4. Basically, all the cells are derived from a single, totipotent cell — the fertilized egg or ovum. As cells enter a development path, their differentiative options become limited and this may have impact on the extent of expansion through division or development through the points at which a terminal phenotype is achieved. Cells at developmental points make up a lineage. The three primary germ layers that we should be interested in are the ectoderm, endoderm and mesoderm. Cells from the mesoderm give rise to the dermal myotome layer which will give rise then to myotomes and all the muscle tissue that would be present in the mature animal (Buckingham, 1992). As cells progress down this lineage, what are the signals that tell the cell to depart or to be at a branch point? Another question one could ask is: Are there opportunities for controlling any of these kinds of cellular decisions? It is clear that during muscle development, there is a hierarchal sequence of events which dictate specialization and even memory of cells (Miller and Smith, 1994). The differentiation status of a cell is dependent on appearance and stoichiometry of many intracellular and intranuclear proteins which are induced by the localized cell environment (Olson, 1992).

While it is well accepted that early embryonic fiber formation is largely neural independent, work by Holtzer (1978) and more recently by Williams and Ordahl (1994) shows that cell lineages in early myotome (Ordahl and LeDouarin, 1992) are not fixed and are sensitive to neural influence. Other studies show that isolated blastodermal cells can give rise to muscle in culture indicative of an inhibitor being induced by cell-cell interaction. In contrast to early embryonic cells, fetal and post-natal fiber formation and changes in fiber type are extremely sensitive to innervation (Hughes and Ontell, 1992; Landon, 1992).

A legitimate hypothesis is that skeletal muscle could be influenced by manipulating events which control normal muscle and even potential of growth of muscle post-natally. This hypothesis also suggests that the events that are crucial would occur within the first 28 days of embryonic development. Another opportunity would be presented when precursors of secondary myofibers are proliferating and forming the secondary myofibers. With certainty, there are other factors that can influence muscle growth, degree of muscling and hypertrophy.

Master Gene Concept

Is it possible that a single molecular event could initiate cell fate? The basis for this hypothesis is that members of a family of muscle-specific regulatory factors which include MyoD (Davis et al., 1987), myogenin (Edmundson and Olson, 1989; Cheng et al., 1993), Myf5 (Braun et al., 1989a and 1989b) and MRF4 (Rhodes and Konieczny, 1989) or herculin (Miner and Wold, 1990), also referred to as basic helix-loop-helix proteins (bHLH) discovered several years ago, could independently activate the gene programs in a variety of non-muscle cells. There was much excitement that genes might be activated very early in development in terms of a "master gene" concept. This activation would be responsible for subsequent activation of other sets of genes or families of genes, and then we could get a cascade or hierarchy of gene expression. It was conceivable that there may be master switches, much like a light switch. Critical questions have been: 1) What are these master switches? and, 2) What are the mechanisms of activation of these master switches, if they exist and are important in determining whether a cell is going to become a muscle cell or some other cell type? As an era of biology, this area of study of myogenic regulatory genes represents a real paradigm shift (Olson, 1990) in the way one thinks about how tissue develops. This was, without question, a very important stage in terms of biology and has important relevance to skeletal muscle development as well as other tissue types, such as neural and adipose. It involved the concepts that certain genes could switch on a whole program and then determine the fate of a cell type very early in embryonic development. The result is a cell that has very stale heritable changes and subsequent heritable changes within the lineage.

Over the past several years, a number of genes in the myogenic regulatory factor family have been shown to induce myogenesis (Aurade et al., 1994). As already mentioned, the products of these genes are bHLH proteins, which have unique characteristics as well as similarities and are a family of proteins. The bHLH type proteins have 80% homology in structure (Olson and Klein, 1994). They bind DNA as heterodimers with other bHLH proteins, such as E-box proteins (Blau, 1992). They also form complexes with many non-HLH proteins such as proto-oncogenes and tumor suppressor proteins as well as HLH proteins, which lack domains for DNA binding (Johnson and McKnight, 1989).

A key point is that when reports of these genes were first published, they showed that the protein or these genes were introduced into basically fibroblast-like cells, they would make those cells become muscle cells (Olson and Klein, 1994). So they were a candidate then for genes that would prescribe the lineage (Grieshammer et al., 1992; Kirschhofer et al., 1994) or basically the commitment or determination to a particular tissue. From a practical sense, and of relevance to meat scientists, Coutinho et al. (1993) reported that fast-growing quail

Figure 5
Example of Binding of Myogenic Regulatory Protein Dimers to Regulatory Regions of a Gene

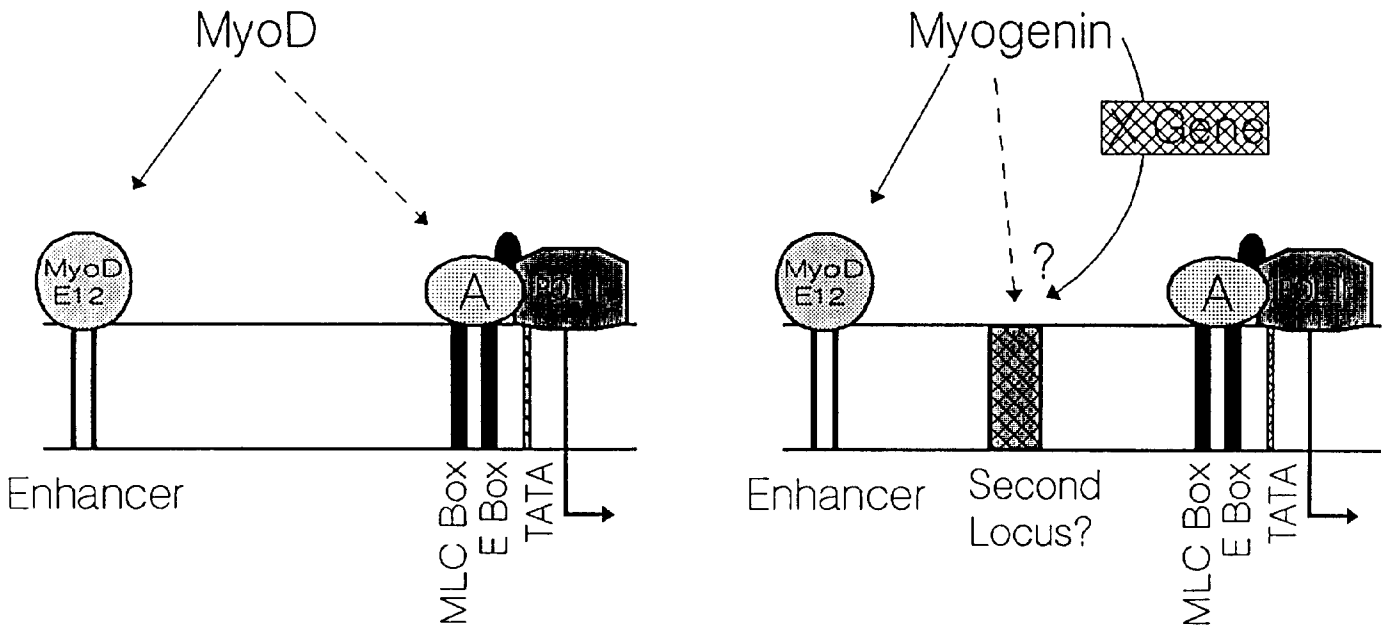
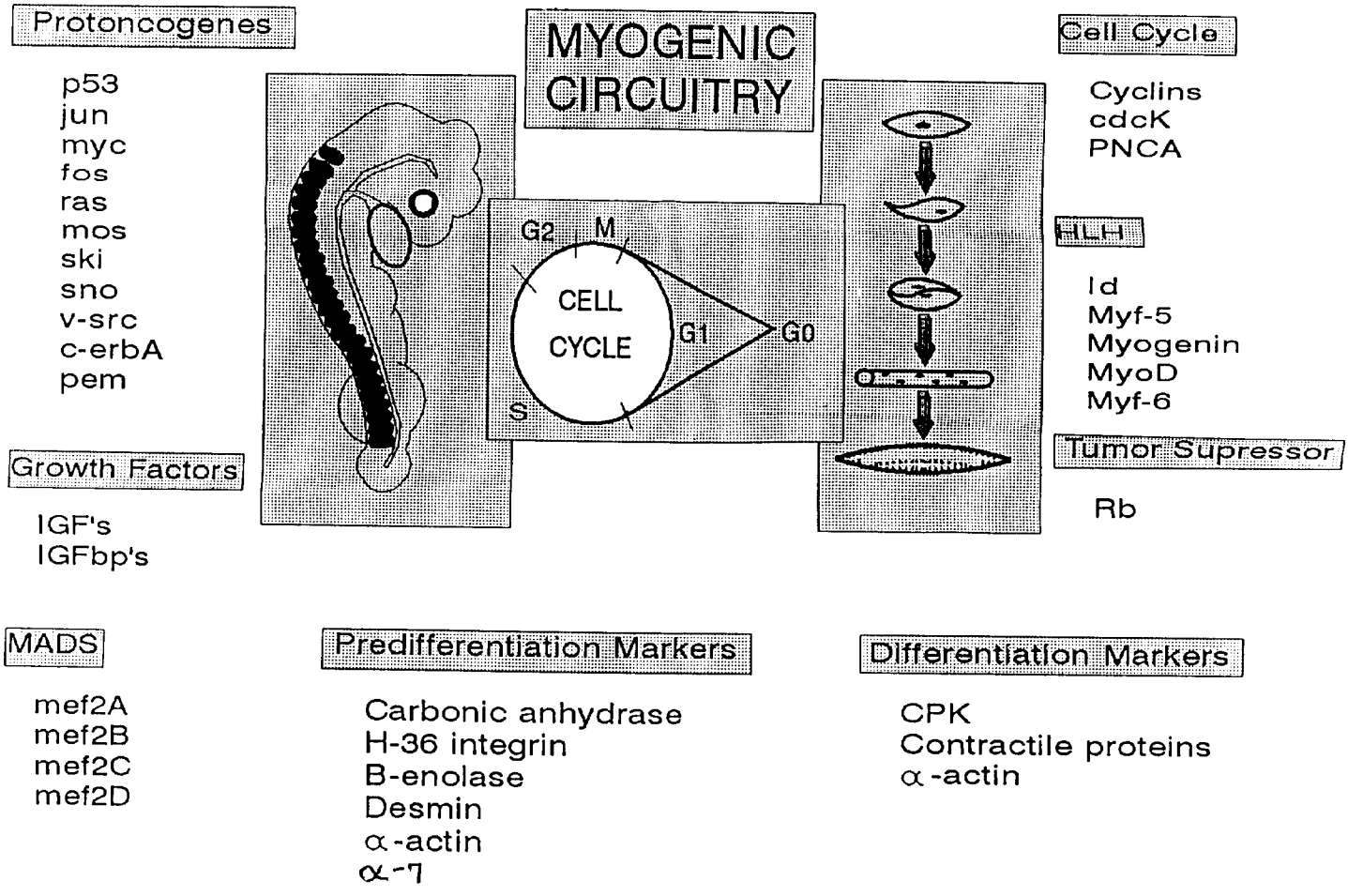


Figure 6
Selected Key Players Involved in the Myogenic Circuitry



had delayed expression of MRFs and myosin heavy chain compared to a slow line. Key questions are: What are the molecular mechanisms for these differences? and, Do these differences exist in animal models which exhibit even greater diversity in myofiber number and muscle mass?

As it turns out now, the consensus of recent literature is that these MRF genes or products are considered to be more transcriptional factors rather than a true commitment or determination type of a gene (Sassoon, 1993). Activities of these genes appear to have an important role in skeletal muscle development and myogenesis (Buckingham, 1992). In some cases, they are involved in maintenance of committed states. Certainly, they're involved in the differentiation process and fusion or preceding the fusion process.

All genes have some important working parts, and for us to discuss this whole area of biology, this is worth covering. One could talk about a sequence of DNA which codes for proteins ultimately — RNA first, then proteins. On the upstream portion of a gene or sets of genes, one finds the coding portion of the genes and an important part of a gene that turns the gene on — or is responsible for turning it on. This is the promoter region or sequence. Within those promoter regions, and sometimes enhancer regions outside of the promoter, are sequences of DNA that are important recognition sites for proteins and protein complexes. Binding of proteins to these sites can result in a facilitated or enhanced activation of genes through this promoter region.

It just so happens that the genes mentioned, the MRFs, recognize a very specific region of DNA and this is called a motif, consensus site or a recognition box on the strand of DNA (Skerjanc and McBurney, 1994). Furthermore, most of the myofibrillar or contractile genes have this CANNTG (E box) sequence of DNA in their promoter regions; therefore, when an MRF protein complex binds this sequence, there can be activation of virtually all myofibrillar genes.

An example of how this might work is presented in Figure 5. This is an example of one of the MRFs, the myogenin, and there is also a protein that is found virtually in all cells, E box type proteins. The E12 protein likes to bind with the myogenin protein, or one of the other MRFs, to form a dimer which can then recognize this very specific sequence of DNA, and then facilitate or enhance the activation of a gene (Murre et al., 1989; Lassar et al., 1991). In this case, this gene or this protein can activate itself. Actually, once it gets cranked on, it will autoactivate and we get quite a bit of activation of the particular gene. On the other hand, this MRF could bind with E12 and then bind to the consensus site for many of the muscle genes, such as actin, etc.

Key Players in the Game of Myogenesis

To fully appreciate and begin to understand contemporary views of myogenesis, one needs to become aware of the key players that are involved. Using the analogy of football, think about a playing field with opposing goals and all the needed players. The actions of cells may be like a football game inside a cell and inside a nucleus. Using that analogy, one can talk about a lot of players. Some have very specialized roles. There is a goal. Some factors have a functional reason for being present. The plays are highly orchestrated. Occasionally, there are some players or fans that want to come in from off the field, get involved and mess things up. There may be some referees that sometimes try to get in the way. One can use examples where the play is to go for the bomb, the long or the short yardage. There are some cases where if one doesn't do it, another one will. So there is what is referred to as redundancy — a back-up system. One could give examples where one protein player interacts with another one and it is the combined activity which accomplishes the objective. The bottom line is that there are both positive and negative regulators present all the time within the cell. Some view myogenesis and differentiation in general as requiring continuous control by key players, transcriptional factors (Blau, 1992; 1993; Lobe, 1992).

While not exhaustive in coverage, many of the significant players involved in some way with the myogenic circuitry are listed in Figure 6. While there is a category of the HLH of regulatory genes, some are not MRFs. There are several other transcriptional regulators such as Id, which stands for differentiation inhibitor. Visualized in the cartoon are the three very important components of a myogenic circuitry (Bober et al., 1994). During somite formation, there is a lot of regulation of cell cycle activity. On the left-hand side of the figure there is a category or group of genes called proto-oncogenes that are intimately involved and are very important in cell cycle regulation of skeletal muscle progenitor cells and many cell types.

Another category includes tumor suppressors, such as Rb or retinoblastoma protein, a member of a viral onco-protein pocket protein family (Schneider et al., 1994). Rb encodes a nuclear phosphoprotein which functions as a negative regulator of cell cycle progression (DeCaprio et al., 1992). It is present in normal functioning cells, normal myogenesis and normal growth. Cell-type specific of Rb likely results from interaction with other regulatory proteins (more later).

The myogenic regulatory factors are HLH proteins, but there is another HLH, Id (designated differentiation inhibitor) that is implicated in regulation of this circuitry and the regulation of the regulators (Benezra et al., 1991). Expression of Id is high during periods of high mitogen concentration, decreases when cells are signalled to differentiate and an abundance of Id appears to inhibit differentiation along with down-regulation of expression of MRFs (Christy et al., 1991; Evans and O'Brien, 1993). While it does not bind DNA, it binds MRFs as heterodimers and prevents their interaction with DNA. Interaction of Id with Rb has been implicated as a molecular pathway for synchronous changes in growth and differentiation of skeletal muscle (Iavarone et al., 1994). The interaction of Rb with c-myc (a proto-oncogene protein), MRF's and Id may be important.

A great deal of time could be spent on specific genes that are involved in the cell division cycle — cyclins. There is a whole family of cyclins. There also are kinases, the cell cycle-dependent kinases, that are attaching phosphate groups onto a variety of other proteins, thereby regulating their activity.

On the bottom right of Figure 6 are differentiation markers. As cells fuse, there is the onset of activation of genes such as creatine kinase. There will be appearance of products of contractile proteins genes, alpha actin and other myofibrillar genes.

There are some muscle-specific genes that are observed only in muscle but their expression precedes other well-char-

Figure 7
Example of How Transcription Factors Can Form Dimers

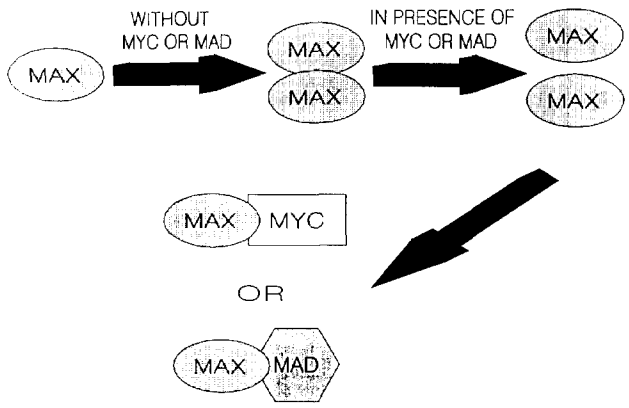
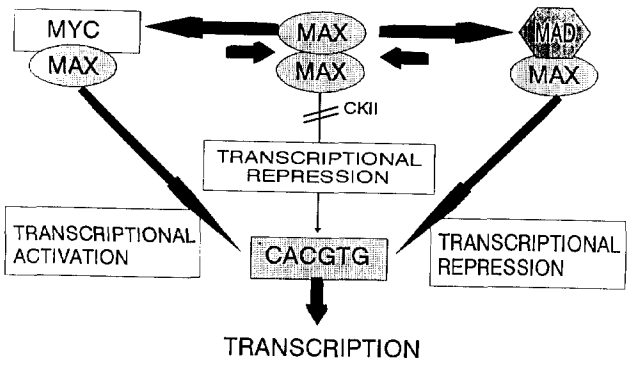


Figure 8
Example of How Transcription Factors May Control Gene Expression

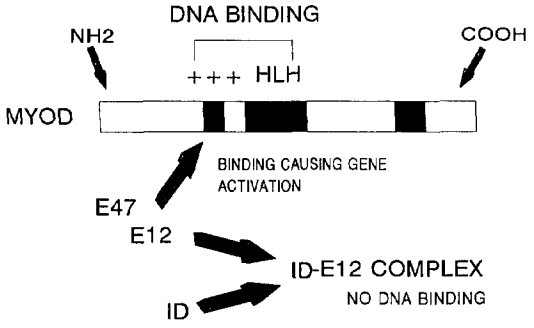


acterized muscle genes which are activated at terminal differentiation. Carbonic anhydrase (Edwards et al., 1992), membrane or protein integrin (Kaufman et al., 1991), beta enolase (Peterson et al., 1992), desmin (Ewald et al., 1988), α-actin (Babai et al, 1990), and one called alpha 7 (George-Weinstein et al., 1993) are observed to be expressed in vitro before the onset of full differentiation or overt differentiation observed as fusion. One could use these as markers of muscle cell lineage prior to observing differentiation.

On the bottom left of Figure 6 is a very important grouping that is unfolding in the literature. It is a family of genes called MADS (MCM1 aquamous deficiens and serum response element; Martin et al., 1994). They have MADS recognition sites on their DNA. Note and remember some of these MADS proteins as myogenic-enhancing factors (MEF). There's a family of these, and it looks like they're all implicated; but certainly 2A, 2B and 2D are prime candidates for serving as determination or commitment-type factors. They can induce non-muscle cells to engage in myogenic activity when ectopically introduced (Breitbart et al., 1993). However, they are found in skeletal, cardiac and smooth muscle cells as well as neuronal cells (Stockdale, 1994). The MRF family can be regulated by negative control mechanisms which change the distribution of protein factors between the nucleus and cytoplasm. MEFs are also transcription factors which bind AT-rich motifs of the promoters of muscle-specific genes (Gossett et al., 1989). They regulate E box-dependent and E box-independent muscle-specific gene activity (Kaushl et al., 1994). Binding of MRFs, MEFs and RB appear to regulate myogenesis. Rb binds both MRF and MEF proteins in regions which would otherwise bind DNA. As with MRFs, the MEFs are sequentially expressed during development (Olson and Kriel, 1994). The association of MRFs with MEFs (Edmondson et al., 1992) appears to provide the great specificity required for exclusive activation of muscle-specific genes.

Another grouping of genes which should be discussed are homeobox genes. These control the pattern of formation and morphogenesis. Disruption of selected members of the Hox family results in developmental defects. Pax genes encode a family of vertebrate transcription factors with highly conserved regions (Gruss and Walther, 1992; Goulding et al., 1994;

Figure 9
Illustration of Relationships of a Myogenic Regulatory Factor (MyoD), Differentiation Inhibitor Protein (Id) and E Box Proteins

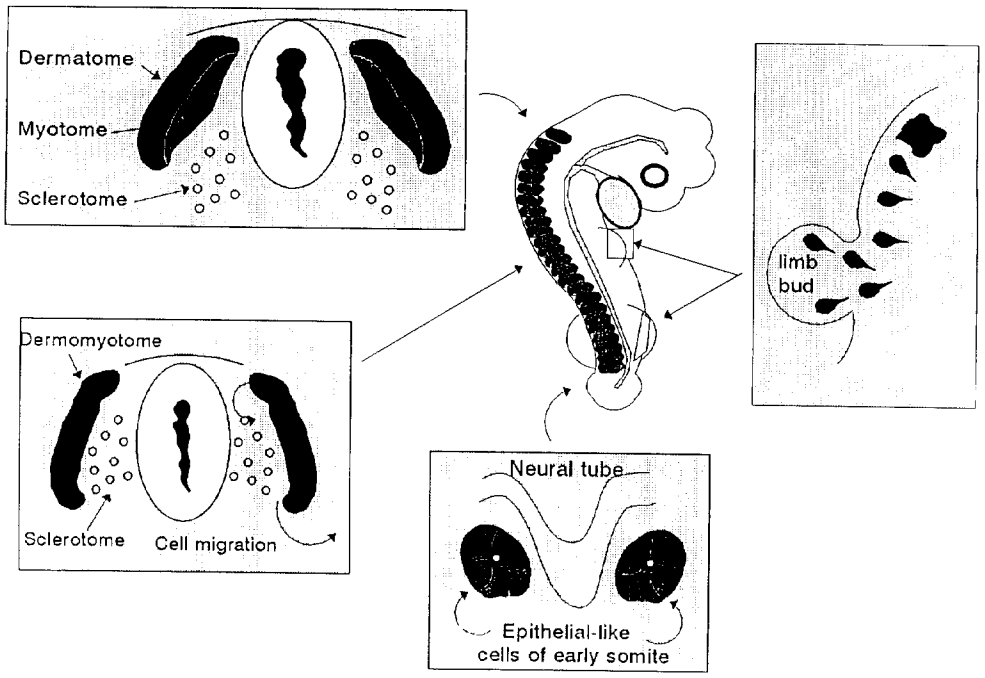


Kioussi and Gruss, 1994). It is thought that pax 7 and pax 3 may play a pivotal role of signalling myogenic precursor cells in somites of developing embryos (Bober et al., 1994b; Williams and Ordahl, 1994; Stockdale, 1994). Recently, other regulatory proteins, MHox (Cserjesi et al., 1992), Hox-7 (Hill et al., 1989) and S8 (Opstelten et al., 1991) were identified in mesodermal and muscle cells exclusively. Forced expression of Hox-7 in myogenic cells resulted in loss of MRFs and failure to differentiate (Song et al., 1992). It is possible that genes like Hox 7.1, Id and Mhox are important in preventing differentiation.

Some Key Plays and Interactions

In order to simplify how some of this works in terms of regulation of regulators, here's a short story. Figure 7 is a cartoon about only three players of transcriptional regulation: Mad, Max and Myc (Ayer and Eisenman, 1993). Max is around in virtually all cells, it is constituatively expressed. Myc is highly regulated in terms of close link to cell cycle entry. In other words, the decision to go on and enter into—from G1 phase into an S phase. It would have to be down-regulated if the cell was going to depart from the cell cycle.

Figure 10
Illustration of Somite Formation During Embryonic Development



(adapted from Buckingham, 1992)

Mad also is very highly regulated and it likes to team up with some of the other proteins as well. Max is usually in excess with regard to Myc (Blackwood et al., 1992). Both homo- and hetero-dimers recognize E box sequences (Blackwood and Eisenman, 1991). Because of the structure of these proteins, one would infer then that they are potentially partners some of the time with the myogenic regulatory factors that we've already discussed. So we can tell a more complicated story than what we're about to tell.

Max is constituatively expressed; without Myc or Mad, one could form a homo dimer — two partners — and they could bind DNA and cause selected cellular effects. If Myc or Mad is present, they will disassociate and form partners (Figure 8). In summary, one could have a Max:Myc or a Max:Mad combination. Conceptually, this is a simplified version of how proteins can interact to determine cellular activity, such as how muscle is formed. If we assume that Myc:Max and Mad:Max dimers are preferred over the Max:Max dimer, and if Mad and Max are together, it shuts things down (Ayer and Eisenman, 1993). Again, here is a model of negative control of growth or proteins that are involved in proliferation.

On the other hand, if Myc and Max are together, they are going to activate those proteins that are involved in proliferation, particularly of muscle cells. One could tell now of how these genes may be involved in partnering up with the myogenic regulatory factors to affect in our myogenesis process. One can add complexity by factoring in the relationships mentioned with Id:MRF and MRF:Mef complexes. For example, Figure 9 shows one of the myogenic regulatory factors, myo D. As mentioned with myogenin, it can combine with the E12 protein. E12 can also bind with the protein called Id. If E12 and myo D or one of the other family members get together, they can form a dimer and activate the muscle gene program. However, if Id is around, it binds up E12, takes it away, and so the activation of those genes is delayed along with differentiation. High serum concentrations, a lot of growth factors, mitogens favor the binding of Id to E box proteins. There will not be activation of myogenic regulatory factors, nor the contractile protein gene families as any MRF genes are inactivated. Remove the serum, take away the growth factors and Id falls off. Now MRFs can partner up with E12 and this dimer may bind to the consensus region on the contractile gene promoter, kicks on the gene (Benezra, 1991).

Another example of a single gene influencing the development of muscle is SKI. It's in a family of viral oncogenes and was originally from avian species (Colmenares and Stavnezer, 1989). While ski is not an HLH, in transgenic model of both mice and pigs, the transgene is only expressed in skeletal muscle and these animals have exaggerated muscle mass and very little fat.

How does one definitively show that genes have a crucial role in myogenesis or development? One approach is to perform gene deletion experiments or knock-out studies. These strategies might involve the creation of embryos that have deficiencies in the gene for some of the myogenic regulatory factors.

If one knocks out myoD, animals have normal muscle mass. They will tend to have some abnormalities of some selected muscles, but basically they're fertile and they can grow to adulthood. The other thing to notice in that model is that Myf5 is cranked up. So there appears to be a fall-back system and Myf is taking over (Hughes, 1993).

A knockout of Myf results in fairly normal kinds of growth of muscle as the embryos develop. They do have some abnormalities in the diaphragm muscles, some intercostal muscles and certainly some problems with anterior rib cage development. These animals usually die at birth (Braun et al., 1994; Hughes, 1993).

Another knockout experiment involved myogenin; these animals basically do not develop muscle, they develop skeletal tissue but the muscle mass is very, very deficient, indicating an important role of myogenin in myogenesis and muscle formation (Hasty et al., 1993).

Spatial and Temporal Patterns of Expression in Embryos

There is a great deal of interest in studying embryonic development and using in-situ hybridization techniques to study the sequence or timing of expression along with the spatial pattern of expression of numerous players/genes important in myogenesis. This includes the relationships of growth factor genes, receptors and target response genes.

The cartoon in Figure 10 (adapted from Buckingham, 1992) represents an embryo. One could spend a great deal of time discussing the formation of somites. It is from these structures that muscles of the trunk and limbs are formed. Somites appear out of the mesenchymal unsegmented plate (Rugh, 1977). With segmentation from the plate, somites form dorsal and ventral structures, the dermamytome and sclerotome, respectively (Buckingham, 1992). Identification of genes along with their targets which are involved in development is currently an active area of biology. An emerging list of mesoderm-inducing factors (e.g., activins) elicit redundant effects on differentiation of cells of mesoderm (Cornell and Kimelman, 1994; Labonne and Whitman, 1994). This induction likely involves a multiple-step process involving sequential activation of numerous intercellular signalling mechanisms, particularly growth factors (e.g., IGF-I and FGF) and their receptors. MRFs are found to follow expression of mesodermal-inducing factors (Hopwood et al., 1989). In vertebrates, presomitic mesoderm is made up of somitomeres (Rugh, 1977). The latter mature into somites in a rostral-to-caudal direction along the cranial/anterior or anterior-posterior axis. Somites in the caudal region represent the one most recently formed (Rugh, 1977). In the middle then would be a more advanced, later stage or earlier-formed somite, and the most cranial one would be one of the earliest somites formed. Somites possess cell types which can give rise to the myotome, which will develop skeletal muscle, sclerotome, which gives rise to cartilage in the ribs and vertebral column, and dermatome, which forms the dorsolateral dermis and some muscle (Sassoon, 1993). Myotome is the earliest skeletal muscle and after forming centrally in the somite, will separate from the dermatome and give rise to the muscle of the trunk (axial) and progenitor, migratory cells of the limb bud. If one looks at this very early form or most recently-formed somite, we can see they are balled-up epithelial-like cells. One may see some subtle activations of some of the myogenic genes — particularly Myf and mNotch (Nye et al., 1994) — for brief moments. It is expression of the MRF's which appear to play a role in dictating the fate of presomitic cells to a muscle lineage (Pinney et al., 1988; 1990). However, expression of MRF's MRNA in the presomitic mesoderm before muscle commitment appear to be under some inhibition by a constituitively-expressed protein mNotch (Kopan et al., 1994; Nye et al., 1994).

Migration to an earlier somite or an older somite, one can see then the transformations into some tissues of the dermal myotome, which would give rise to skin, the sclerotome, which would give rise to some of the cartilage and vertebrae. Molecular events leading to decisions of cells to enter these lineages are largely unknown. However, expression of Hox genes along the antero-posterior axis appears to be critical for formation of sclerotome (Buckingham, 1992). One can also see that cells are migrating out of this dermal myotome or the eventual portion of that dermal myotome and migrating out. One may find that Myf protein or genes are expressed throughout this arrangement at different times (Buckingham, 1992).

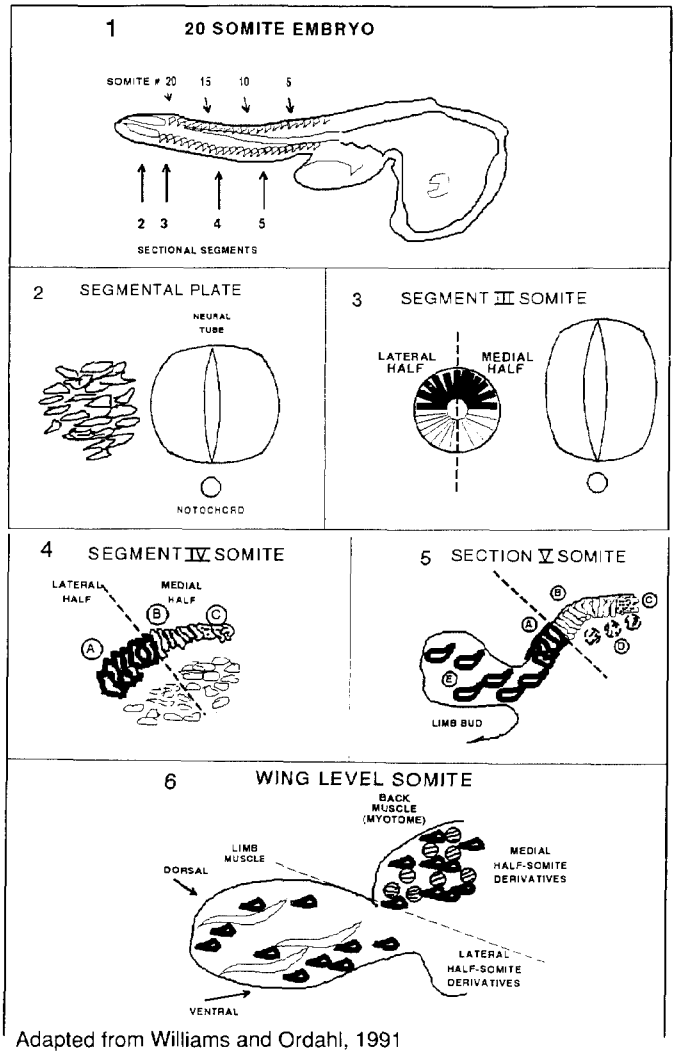
In a very early formed somite, one can also examine the myotome which will give rise to most of the muscle cells that the animal is ultimately going to have, and then you can find some expression patterns of Myf (Ott et al., 1991) and some of the other myogenic regulatory factors in that region. Interestingly, the earliest muscle-specific genes to be detected in myotome (remember the first skeletal muscle to form), are desmin, titin and cardiac α-actin (Babai, 1990).

Let's put some things together in a package. Let's look at another embryo in Figure 11. Now we have the most recently somite at the tail. As we move cranially we look at a reasonably early formed somite, one that's a little bit older and one that's considerably older. Then we are going to look at what is happening in a forming limb bud. The reason we are talking about this is to describe how it may be possible for a candidate gene to influence the decision of cells to become muscle cells. The dark staining regions in every case are going to refer to a gene called Pax 3 (Williams and Ordahl, 1994) or possibly MEF (Breitbart et al., 1993). Pax is a member of the homeo box gene family, which describes how the pattern of bodies form. Pax is heavily expressed in this very early embryo or early somite, and with movement to a stage I somite, one can see some changes in Pax expression, in the dorsal, and then on the lateral and medial half of this somite, a lot of Pax expression. However, it is down-regulated, turned off and eventually expression becomes undetectable (Williams and Ordahl, 1994).

As one moves to another stage somite, a little bit older, the ventral portion of that somite, the cells have changed into mesenchymal cells. There is a region of very high, intense Pax expression, down regulation, and then some cells, regions exhibit a lot of myogenic regulatory factor expression. This pattern suggests that Pax has roles in communicating decisions of cells to become muscle cells (Goulding et al., 1994).

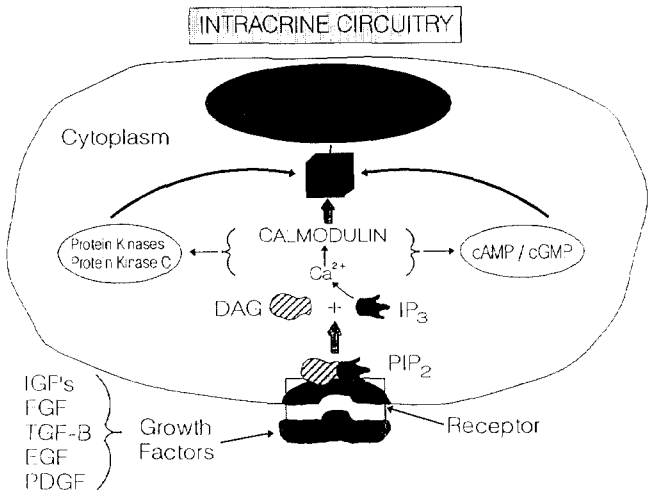
Moving a little further, one may see at least five regions of different kinds of expression of genes. A lot of cells are beginning to migrate into a limb bud region, all of which, or some of which, will be expressing the gene Pax. Then there will be a region of cells where there are a lot of myogenic regulatory factors, such as Myf or even myo D. It is suggested that only myf-5 or MEF is adequately expressed early to play a role in specifying progenitor cells of skeletal muscle (Stockdale, 1994). One could show further that the parent cells are actu-

Figure 11
Illustration of Somite Formation and Migration of Muscle Precursor Cells During Embryonic Development



Adapted from Williams and Ordahl, 1991

Figure 12
Illustration of an Intracrine Circuitry Transduced by Growth Factors



ally beginning to express the contractile protein genes, as in region 6 of Figure 12. If one looks a little further, there will be some mixtures of PAX expressed in waves, little pulses of expression of PAX throughout structures that look like myotube structures, and even some cells that are just beginning to express myofibrillar proteins.

Growth Factors and an Intracrine Circuitry

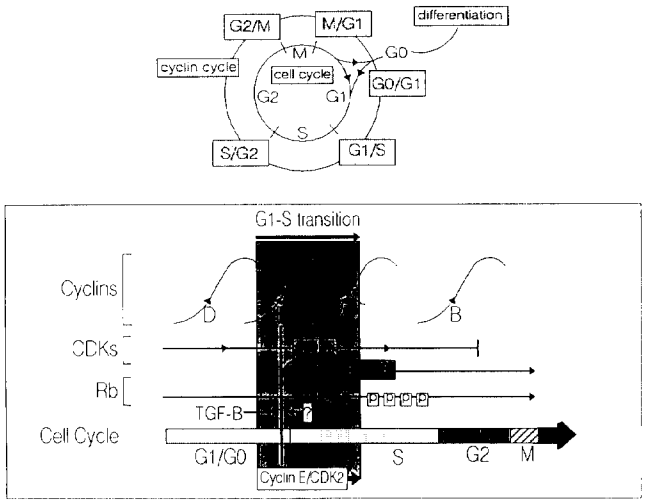
Many of the events are controlled by growth factors, and these are listed in Figure 12. They send signals through an intracrine circuitry that adds new intracellular and intranuclear players into the arena. So how are regulatory genes turned on and off? How are they affected by growth factors? To fully integrate the myogenesis story, one should discuss families of growth factors, particularly, IGFs, FGFs and TGF-β. The interactions of these growth factors are critical to the myogenic process (Greene and Allen, 1991). IGF's have been reviewed extensively (Florini and Ewton, 1992; Florini et al., 1991; Magri et al., 1991) and will be discussed by Dr. Quinn. IGF-I induces differentiation through a MRF mechanism (Florini et al., 1991). There are super families of FGF (Hauschka, 1994) and TGF-β (Kingsley, 1994). Based on in-vitro observations, these growth factors play significant synergistic roles in myogenesis. Based on their expression in somites and presomitic tissue in vivo, one should acknowledge potential powerful roles in regulating cell activity.

FGFs are potent inhibitors of differentiation. Recent reports (Fox and Swain, 1993) showed that FGFs can auto- and trans-regulate their own expression in muscle and FGF is required for proliferation of myoblasts (McFarland et al., 1993). Additional observations have shown that the extracellular matrix is crucial for FGF influence on myogenesis (Olwin and Rapraeger, 1992). It also is suggested that FGF inhibits differentiation by inhibiting IGF-II expression (Rosenthal et al., 1991).

Inhibition of myogenic differentiation by TGF-β (Koff et al., 1993) is mediated through an inhibition of MRF, increased production of extracellular matrix and possibly through regulation of the phosphorylation of Rb (Laiho et al., 1990; Massague et al., 1991). Addition of TGF-β to muscle cultures decreases c-myc expression and slows the cell cycle. The growth inhibition is associated with increased differentiation even in mitogen-rich environments (Zentella and Massague, 1992). Recent data showed that non-dividing embryonic myoblasts do not respond to TGF-β can differentiate and fetal myoblasts were inhibited by TGF-β (Cusella-De Angelis et al., 1994). Once formed, primary fibers may activate proliferation of fetal myoblast to increase the number of cells for secondary fiber formation. Additional studies (McLennan, 1993) showed that myotubes which formed prior to expression of TGF-β gave rise to slow fibers whereas those formed adjacent to TGF-β containing connective tissue matured into fast fibers. TGF-β represses expression of MRFs through a post-translational mechanism which renders the basic HLH regions nonfunctional. If growth factors are examined in combination, one sometimes gets different responses than with a growth factor added singly.

The growth factor TGF-β is important in affecting some of the decisions of cells to become during secondary fiber formation (Cusella-De Angelis et al., 1994). It also affects the

Figure 13
Relationships of Key Players in Regulation of the Cell Cycle



Adapted from Stein et al., 1994

characteristics of the extracellular matrix and this affects cell migration and positioning of various myogenic cells.

Interrelationships During the Cell Cycle

Figure 13 summarizes some of the events in the cell cycle (Murray and Kirschner, 1991; Stein et al., 1994). The cell cycle is much like an automotive engine which powers the cell through stages of G1, S, G2 and M but there are several regulatory brakes put in place. As we progress around a cell cycle from G1 all the way to M or mitosis, we have the cycle. One can linearize this to illustrate points more effectively. In terms of G1, cells are not dividing and could be resting or residing in a protracted G1 period or G0 state. The latter is characteristic of mature, fully-differentiated muscle cells. If competent, cells could progress into *S* for synthesis of DNA; then there would be a gap period (G2) preceding the actual mitosis (Murray and Kirschner, 1991; Hatakeyama et al., 1994).

There may be eight cyclin genes (A, B1, B2, C, D1, D2, D3 and E) expressed in a transient fashion during G1 and G2 (Hatakeyama et al., 1994). Those gene products are important in the regulation of the cell cycle in both normal and abnormal cell growth. In particular, cancers seem to have dysfunction of cyclin D. All but cyclins A, B1 and B2 appear to be expressed and active at the mid-to-late G1 portion of the cell cycle which also corresponds to the time critical for Rb phosphorylation (Kato et al., 1993). However, this area is unclear.

There is a class of genes called cdc kinases or CDK's which at present have five members (CDC2, CDK2, CDK3, CDK4 and CDK5) and appears to be a main part of the engine in our analogy (Norbury and Nurse, 1992). Some CDK's are expressed constituitively, others may not. One that is not represented in the figure would be CDK4. The interaction between cyclin D and CDK4 is probably a major explanation for many kinds of abnormal growth situations which materialize as tumors (Weinberg, 1991). There are many dimensions of cell cycle regulation and these are beyond the scope of this presentation. For example, one could envision that there are specific protein regulators which seem to provide surveillance of each stage of the cycle. Several gene products seem to be involved in the regulation of the transition of cells from G1 to S, either as activators or brakes.

To get back to the RB protein, an interesting emerging story relative to myogenesis is revealed. One thing that happens to RB is that it gets phosphorylated at different times during late G1 and shortly after the G1 period (DeCaprio et al., 1992). It will continue to be phosphorylated through division. During G1, it is hypophosphorylation that is important because if RB stays hypophosphorylated very long, into the late G1 period, basically that cell would differentiate. However, if the conditions are right where a kinase can tack on a phosphorous group to the RB protein and make it hyperphosphorylated, it will catapult that cell into the cell cycle and achieve yet another round of division. Recent data suggests that permanent terminal differentiation, withdrawal from the cell cycle and maintenance of the differentiated state of mammalian muscle requires RB (Stockdale, 1994). Another protein p107 can substitute for RB as a cofactor for differentiation but unlike RB, conditions of high growth factor concentrations reverse the differentiation (Schneider et al., 1994). One assumes that because the hypophosphorylated form of Rb is only found in G0 or G1, it plays a key regulatory role in proliferation. Phosphorylation sites are detected when Rb is coincubated with CDK and selected cyclins (Hatakeyama et al., 1994).

The interesting thing about RB is that it may have direct interaction with muscle-specific bHLHs controlled by IGFs. In Dr. Quinn's presentation, we will hear about IGFs. We acknowledge that IGFs have a dual role in regulation of myogenesis. In some cases, they stimulate proliferation while in some cases they stimulate differentiation (Florini et al., 1991). In a very early exposure of myogenic cells to IGF, the phosphorylated state will be perpetuated and those cells will continue to proliferate in a timely fashion. During that period of high phosphorylation, one can observe a down regulation of messenger RNA for many of the MRF's by approximately 90% to 100% (Schneider et al., 1994). With longer term exposure of IGF's, the cells will change in terms of phosphorylation state and actually will favor the low or hypophosphorylated state; therefore, inducing the expression of myogenic regulatory genes and differentiation. Interestingly, the IGF-I gene itself has a region in its promoter that will recognize or bind RB, the protein itself. RB is not only involved at the IGF gene level, it is involved in cell cycle regulation of myogenic cells which are affected by IGF. It affects IGF expression and this may serve as partial explanation for the pronounced expression of IGFs in many embryonic and fetal muscle cells (Florini et al., 1991; Gerrard, personal communication).

Myogenesis in 1994

Let's now come back about 10 years from where we started, and talk about what holes we might be able to fill in for myogenesis. One could talk about some determination gene candidates, but we still aren't sure if we have any. In other words, genes that actually describe or define that cells are going to become muscle cells very early on — a decision which is made before one could know that they are muscle cells.

Present candidates for major switches during early embryonic development are the homeobox genes and MEF family of genes. Expression of these would likely precede MRFs. These are working in concert with the MRFs, protooncogene products, other non-MRF HLH proteins such as Id, Rb and cell-cycle specific/regulated genes. The stoichiometry of intracellular and intranuclear proteins is likely governed by key growth factors such as IGF-I, IGF-II, FGFs and TGF-β's, along with growth factor binding proteins and receptor populations for these growth factors. While elevated expression of MRFs leads to terminal differentiation, increased expression of protooncogenes, Hox genes and Id prevent differentiation.

The MRF genes are certainly involved in various states of the withdrawal from the cell cycle, fusion, differentiation, (there's quite a bit of evidence that they're involved) and they're quite important but the involvement is more complex than this and involves additional players. One could talk about the sequence of activation of these genes — which one comes on first, which one's later. In most cases, I think we could say that in the meat animal species, the Myf5 would be a very early-activated gene, followed by either myogenin or myo D. Then the gene that's called MRF4 or also called Myf6 (Bober et al., 1991), may have some real relevance from the human standpoint, in terms of satellite cell activation later on in post-natal growth (Smith et al., 1994).

Other growth factors that may synergistically impinge on muscle precursor cells and muscle cells directly can affect the presence or activity of key regulatory genes or the expression of other growth factor genes. In some cases, phosphorylation of the gene product itself. One could mention a few cases where we know endocrine factors influence the expression patterns. We don't have much evidence about nutrition, but we could also consider the contention that any perturbations of nutritional status during gestation, even subtle changes or an insult to nutrition, may have some effects on these kind of events.

Development of primary and secondary fibers is orchestrated by multiple mechanisms (see George-Weinstein et al., 1993). Primary myofibers serve as scaffolds for a larger number of late-forming secondary fibers (Evans et al., 1994). By formation of limb buds, progenitor cells for primary fibers are in place but are not expressing α7 integrin or desmin. Eventually, these cells are capable of expressing desmin but not α7 integrin. These cells will then undergo terminal differentiation. After this differentiation, slow myosin heavy chain are expressed. In contrast, cells which give rise to secondary fibers will develop the ability to express either desmin or α7 integrin and population of replicating cells capable of expressing both proteins. Upon differentiation of these cells, both markers are increased. Along with TGF-β, α7 integrin and laminin may play a significant role in secondary myoblasts and fiber formation.

Manipulation of Embryonic Development and Myogenesis

Some of the work that we have done for the last couple of years (Kelly et al., 1995) involves the examination of expression of MRF genes and myogenesis in porcine embryos and in cultured muscle cells. We have been examining myogenesis at these very early stages of embryos of somite formation to examine some of the expression of these genes and even manipulate the expression of these genes during this critical period of time during embryonic development.

In addition to developmental changes in expression, a model that we have been employing involves gestational manipulation where we administer porcine somatotropin to the gestating animal at very strategic or key windows of development, trying to alter then the milieu of growth factors that embryos are exposed to in the utero-placental units. We followed pigs through various embryonic stages, as well as postnatal observations, all the way to market weight, and certainly observed some interesting events. We saw that we can alter the pattern of expression of some of these myogenic regulatory. A consistent down regulation of Myf5, which is one of the early expressed genes that I mentioned.

We can also report some real kinds of productivity having relevance to the industry in that selected muscles are increased in a dose-dependent fashion, increased in their mass. We have consistently observed 25% increases in loin eye area in pigs at market weights, and 25% to 30% reduction in carcass 10th-rib fatness.

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