**Regulation of Muscle Mass – Notes** (Musculoskeletal System, LCRS)

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## SLIDES 2-11

1. Why do we need to know how muscle mass and phenotype is regulated?

Cachexia or loss of muscle mass occurs in many chronic diseases including cancer, AIDS, heart disease, and chronic obstructive pulmonary disease (COPD).

Muscle mass is an important regulator of survival/mortality in chronic illness and loss of muscle mass is more closely associated with death than many standard prognostic indicators. For example, COPD is a lung disease in which smoking results in damage to the lung tissue and restricts the amount that people can breathe. In this disease unsurprisingly FEV1 (forced expiratory volume), as a % of the expected normal value, is a classic marker of disease progression and severity. So you might expect that it would be a good predictor of how well people survive. In the limiting cases it is predictive (i.e. if something else doesn’t get you the inability to breathe surely will). However, this relationship affects only a few people and something else is important in determining survival in many others. One much better predictor of mortality is muscle mass, in this case measured by quadriceps strength. The amount of muscle is also an important feature of quality of life. If you think about the daily tasks you need to do to live sufficient muscle mass to be able to get up walk around and so on is important. You need to have enough to get on. You can see that muscle size can become limiting in slide 7 where if you measure the cross sectional area of the mid thigh region it is directly correlated to the distance a person can walk in 6 minutes. This distance is also related to their general feeling of wellbeing and thus their quality of life.

But the amount of muscle present is not the end of the story. Muscle is not a uniform tissue and is composed of different fibre types that contract at different rates and are used for different things. They range from slow fibres that contract slowly and use oxidative metabolism as their energy source through to fast fibres that contract quickly and use glycolytic metabolism as their energy source. Different muscles have different contributions of these different fibres with the muscles that work for long periods of time (e.g. anti-gravity muscles and diaphragm) containing large amounts of slow fibres and muscles that are required for short bursts of rapid activity (e.g. eye moving muscles) having large amounts of fast fibres. If you look at the quadriceps which myosin you have is an important correlate of how far you can walk so if you have a higher proportion of type I fibres you can walk further in 6 mins than if you have a low proportion. It is also true that the more physical activity you do the higher the proportion of type I fibres you will have and the higher your expression of type I MHC. So it is not only how much muscle you have that is important but also the type of muscle that you have in the locomotor system that is an important contributor to your quality of life.

**SLIDES 12-15**

1. How does a cell become a muscle cell?

To understand how muscle mass is regulated it is useful to understand where skeletal muscle cells come from and the basics about how their differentiation is controlled.

* If we look at adult skeletal muscle cells they are multinucleate fibres with the nuclei at the edge of the fibre, but this is not the way they start out. So the question is how do they get to this state?
* Well if we look at the skeletal muscle in an embryo we can see that the nuclei are down the middle of the fibre but we still have one cell with lots of nuclei.
* But like all other cells skeletal muscle cells start life as individual cells with a single nucleus. There are two ways we could go from here to a multinucleated cell and they are
  1. to divide the nucleus multiple times without dividing the cell as happens in the formation of megakaryocytes (which produce platelets), OR
  2. to fuse cells together. In fact, this is how the skeletal muscle syncitium (=one cell with many nuclei) is formed.

So we start with several skeletal muscle **myoblasts** and under the appropriate conditions these will stop proliferating, start to express markers of the differentiated skeletal muscle phenotype and fuse together to form a **myotube**. This process is followed by the maturation of these myotubes into myofibres giving us our skeletal muscle..

* + - These steps in differentiation are principally controlled by a set of transcription factors, which are proteins of the basic-helix-loop-helix family. There are 4 of these transcription factors in total and the first discovered was the protein **MyoD** which was identified using a differential screen for genes that were up-regulated when a particular line of fibroblasts was treated with aza-cytidine (which causes de-methylation of DNA and allowed these cells to convert into other cell types particularly myocytes).
    - Transfection of MyoD into a number of different cells converts them into myoblasts which can subsequently fuse into myotubes.
    - Subsequent studies identified 3 similar proteins, **Myf5, MRF-4** and **myogenin**, which have subsequently, using gene targeting, been shown to regulate specific parts of the differentiation of skeletal muscle cells as shown in this slide.
    - MyoD and Myf 5 play redundant roles in the differentiation of myoblasts into myocytes. These then require myogenin to increase drive the formation of myotubes and finally MRF-4 is required for the maturation of these myotubes into myocytes.
    - Once expression of these transcription factors is activated there is a positive feedback system which maintains and enhances their expression.

**SLIDE 16**

Obviously we can control muscle mass and as importantly the type of muscle by regulating the **amount of message** made and the **amount of protein** made from each message and I am going to start by looking at the basics of regulating transcription. Regulating the amount of transcription is becoming more important in the analysis of cell phenotype but I am going to ignore that completely for the rest of this talk.

# SLIDES 17 -22

1. **Regulating the amount of message**

In determining how a cell becomes a skeletal muscle cell we have started to talk about the factors that control gene expression in skeletal muscle cells and we have talked about the myogenic basic helix loop helix proteins (MyoD, Myf5, MRF-4 and myogenin). The control of expression of a specific gene is likely to be a lot more complicated than that. So we need to introduce a few more of the players that regulate skeletal muscle specific gene expression, but in the context of why the system would be designed like that. Conceptually this approach can be used for any gene promoter as the questions are always

1. where does the gene need to be expressed
2. what level does it need to be expressed at under basal conditions
3. in response to what stimulus does expression need to change

So for skeletal muscle we need to consider what factors should be built in to the system to allow expression of the gene in skeletal muscle cells, and what factors are likely to increase or reduce the expression of such a gene.

The easiest place to start is to look at the control of one of the contractile proteins and consider when the gene needs to be expressed and what it needs to respond to.

* The first thing to consider is that we want the gene expressed in skeletal muscle cells once they have become skeletal muscle cells. The easiest way to do this is to use the same transcription factors that are involved in causing the cells to become skeletal muscle cells in the first place.
* The next thing to consider is that these proteins are contractile proteins and they need to be able to respond to changes in demand. These proteins make up a significant portion of the muscle cell and making them therefore consumes a significant portion of the cell's energy. We only want to have enough and not too much. The organism also needs to be able to grow and to respond to changes in demand so we need a system that will respond to that.

We will illustrate the regulation of skeletal muscle contractile protein expression using **skeletal -actin** as an example.

So we can regulate the amount of message that is made by regulating the amount of each transcription factor that is bound to each binding site and the activity of that factor (how active and whether activators or inhibitory).

So to run through some detail (not necessary to remember but illustrates the general point)

To determine that the protein is expressed in the right tissue (skeletal muscle only) we can use tissue-specific transcription factors that bind to the promoter regions of the gene. In skeletal muscle the bHLH (basic-helix-loop-helix) transcription factors that we have already described bind to a recognition sequence in the appropriate promoter and here in the skeletal actin promoter we have the appropriate binding sites for the myoD class of factors.

So what about increasing expression in response to increased muscle activity (as in exercise training)? Well one example of this type of regulation is the following: increasing contraction occurs alongside **increased average Ca++ concentration** in the cell as Ca++ is required for contraction to occur. This increase in Ca++ triggers an increase in the activity of two systems: the **CaMKIV** and **calcineurin** systems. Calcinuerin targets the transcription factor **NF-AT** present in the cytoplasm and causes it to translocate into the nucleus, where it targets the transcription factor **MEF-2**. CamKIV targets MEF-2. In both cases there is an increase in activity leading to an increase in the expression of the target gene in our case skeletal muscle -actin. The regulation of MEF-2 is not direct; it involves a second protein and that is a **histone deacetylases** (HDAC4 and 5). These proteins inhibit the expression of genes when they are in the nucleus by increasing the positive charge on histone residues causing the DNA to become more compact and therefore less accessible. **HDAC4 and 5** bind to MEF-2 when they are in the nucleus and inhibit the expression of genes to which MEF-2 is bound. Phosphorylation of these HDACs by CaMKIV causes them to exit the nucleus and bind to the 14-3-3 proteins in the cytoplasm. MEF-2 can then bind other factors that acetylate the histones and open up the chromatin and increase gene expression.

The other regulatory mechanism we will consider is coordinating the expression of different components of the contractile system. This is achieved in part by having similar promoters and common transcription factors. So in addition to the regulatory systems I have outlined above, the contractile protein genes have an element in them, which to all intents and purposes says “I am a contractile protein gene”. This element binds the transcription factor **serum response factor** (SRF). Binding sites for SRF are found in all or almost all of the contractile proteins and often exist in pairs (though not normally right next to each other).

When bound to DNA on its own, SRF is not very active and requires the presence of co-activators. One of these coactivators is called **Mal** or **MRTF-A** and it has two close relatives **myocardin** (only in the heart and vascular smooth muscle) and MRTF-B. These proteins are powerful t**ransactivators** and bind to SRF when they are in the nucleus. Myocardin is always found in the nucleus but both MRTFs translocate from the cytoplasm to the nucleus in response to the amount of free actin. So what does that mean? Although we think of actin filaments as relatively static things that interact with myosin fibres to allow for contraction, they are very dynamic (like almost everything in biology) and this allows for regulation. So actin is constantly being polymerised and depolymerised by adding a globular actin monomer (G-actin) onto one end of the actin filaments and removing G-actin from the other end of the filament. To allow this to occur we must have a pool of G-actin but we don’t want that pool to be too large. What we find is that the size of the G-actin pool regulates the amount of MRTF that is present in the nucleus. A large amount of G-actin causes the MRTF to become localised to the cytoplasm whereas a small amount causes the MRTF to be localised to the nucleus. Therefore when we need to make actin (there is little free in the pool) the MRTF moves into the nucleus and activates SRF which increases the expression of the components of the contractile apparatus. Now we get co-ordinated expression of contractile proteins in response to an increase in demand.

In addition to an increase in drive, alterations in the actin cytoskeleton also contribute to changes in inhibition of contractile protein expression and again this mechanism targets the SRF binding site. Many of the SRF binding sites are composite SRF – YY1 binding sites. YY1 is a transcription factor that can act as both an activator and an inhibitor of gene expression. At most SRF binding sites in actin genes it acts as an inhibitor. Like the MRTFs the control of YY1 localisation is regulated by the actin cytoskeleton. However, the movement of YY1 occurs in the opposite direction to the movement of the MRTFs so that when there is a lot of filamentous actin YY1 becomes localised to the cytoplasm but in the presence of G-actin it becomes located in the nucleus. This gives us an exquisite method for regulating actin gene expression in response to the demand on the system.

Then by varying the amount and activity of different transcription factors in the muscle cell we can modulate its phenotype and drive it to become the particular type required. So changing the proportions and activities of these proteins can influence fibre type.

# SLIDES 23-26

1. **Regulating the amount of protein**

There are two sides to regulating the amount of protein the first is regulating the amount of **protein synthesis** and the second is regulating the amount of **protein breakdown** and it is the **net turnove**r that is important. Once you are an adult these are in reasonable equilibrium so that we don’t keep growing and the amount of muscle we have stays relatively constant. However, if you increase the amount of exercise you do the amount of protein synthesis and degradation increases but synthesis increases more that degradation so that we make more muscle. In chronic disease the amount of breakdown decreases but so does the amount of synthesis and this occurs to a greater degree resulting in muscle loss. These changes in protein synthesis and breakdown can reflect changes in the expression of growth factors caused by disease processes and an important growth factors involved in protein synthesis is IGF-1 and one involved in protein breakdown is known as myostatin.

**SLIDES 27- 34**

1. **Regulation of protein synthesis**

The role of IGF-1 I am going to look at is its effect on protein synthesis. This should be seen as just as important and increasing RNA synthesis.

But first we probably need a little **reminder** of some of the factors involved in the protein synthesis machinery.

As you all know proteins are made by translating the mRNA code into a polypeptide chain one amino acid at a time. We can consider two phases of this process: one is the **initiation phase** where a ribosome binds to the 5’ end of an mRNA molecule and the second is the **elongation phase** where the ribosome moves along the mRNA adding more amino acids as it goes. We are most interested in the initiation phase because the rate of protein synthesis can be more effectively increased by increasing the rate of initiation than by increasing the rate of elongation. This does not mean that elongation isn’t also a target.

IGF-1 is a small peptide hormone that is part of the insulin family. The predominant source of circulating IGF-1 is the liver where it is made in response to growth hormone. However, some tissues, including skeletal muscle can produce IGF-1 locally and in muscle changes in IGF-1 expression occur in response to stretch and load on the muscle.

IGF-1 signals by binding to the IGF-1 receptor, a tyrosine kinase that exists in the membrane as a preformed homodimer. Binding of IGF-1 to this receptor causes a conformational change that activates the tyrosine kinase portion of the protein leading to phosphorylation of IRS-1. One of the immediate targets of IGF-1 signalling is **PI3Kinase** which in turn phosphorylates and activates protein kinase B (also known as **Akt**). Here we have the activation of a classic protein kinase cascade which allows for amplification of a signal as one kinase can phosphorylate more than one target at each step in the chain.

In skeletal muscle there are two Akt isoforms that we need to consider Akt1 and Akt2. These proteins appear to be coupled to different components because knockout of Akt1 in mice leads to growth retardation whereas knockout of Akt2 leads to type II diabetes. It appears in part from this, but also from direct assays of IGF-1 and insulin function, that Akt1 is a target of the IGF-1 receptor whereas Akt2 is a target of the insulin receptor.

For the moment we will consider two targets of Akt that are involved in increasing protein synthesis as part of the hypertrophy signalling cascade. The first is glycogen synthase kinase (**GSK3** and the second is the **mTOR** complex (mammalian target of rapamycin).

So where in this system do mTOR and GSK3 function?

GSK3 inhibits the activity of one of the initiation factors, **eIF-2B,** so inhibits protein synthesis by blocking the loading of Met-tRNA into the ribosome. Inactivation of GSK3 by Akt releases this block leading to increased RNA synthesis

mTOR on the other hand phosphorylates S6 kinase leading to increased phosphorylation of the ribosomal protein s6.

One further target of this system is the eIF4E binding protein, which sequesters eIF4E. Phosphorylation of eIF4E binding protein reduces its binding of eIF4E allowing eIF4E to participate in initiation of protein synthesis.

# SLIDES 35-44

1. **Regulation of protein breakdown**

So that is a brief introduction to the control of muscle protein synthesis and hypertrophy. The flip side of this coin is **muscle protein breakdown and atrophy**. It must be remembered that although we think of this as a pathological process it is a normal and important system that has evolved to allow us to use the large amount of stored energy present in skeletal muscle protein during times of starvation.

I am going to illustrate this by looking at **myostatin** a regulator of protein turnover in mammals. Myostatin was identified initially as the product of a gene that when mutated was associated with a double muscle phenotype in animals and here you can see the piedemontese cow and the bully whippet. There are also people with mutations in myostatin and one child is shown in slide 34. He was born to a mother from a family renowned for their strength and a father who was a sprinter.

So what happens to myostatin in disease well in COPD what you find is an increase in myostatin in patients who do the least activity and these are the weakest patients as well so that myostatin expression is inversely correlated with strength.

So what does myostatin do, well one of the things it does is to increase the expression of two proteins **MuRF**-1 and **atrogin**-1 proteins that have been shown to be increased in a range of atrophic conditions.

MuRF1 and atrogin are both **ubiquitin ligases** and as such their job was to add ubitquitin moieties to proteins. Ubiquitin is a small protein that functions in the cell primarily as a label that indicates that a protein is to be degraded by the proteosomal system. So an increase in the expression of MuRF1 and atrogin suggests that we will see increased protein degradation. This raises the question of how the expression of these proteins is controlled.

FOXO1 and FOXO3 are a pair of transcription factors that are important in the regulation of the expression of the genes for MuRF1 and atrogin. The **transcription factors FOXO1 and FOXO3** and their activity are regulated by protein phosphorylation and nuclear localisation. The active form of each protein is not phosphorylated and is present in the nucleus, whereas the inactive form of each protein is phosphorylated and becomes localised to the cytoplasm.

The regulation of the activity of Foxo transcription factors is therefore an important aspect of the regulation of atrophy; so what is involved?

There are two important components. The first is **Akt**, which phosphorylates and inhibits Foxo, and the second is AMP kinase, which is involved in activating Foxo.

AMP kinase is a protein that is central to the "energy sensing system". It is activated by AMP a molecule that is present at a low concentration but is in equilibrium with ADP. This equilibrium allows the cell to measure its energy status in the following way. In a muscle contraction leads to the conversion of ATP into ADP; most (but not all) of the ADP is turned back into ATP by the creatine kinase reaction. The resulting small increase in ADP after a period of contractile activity is measurable by NMR spectroscopy of contracting muscle. Thus a sign of heavy ATP use is the increase in ADP, which results the conversion of 2 molecules of ADP into 1 ATP and 1 AMP. This does not have a big effect on ATP concentrations but increases the levels of AMP markedly as the amount of AMP in the cell is very low. You should remember that it is not the absolute concentration change that is important in triggering a response but the relative change so the percentage reduction in ATP is small but the percentage increase in AMP is very large. Hence when energy levels are low ADP increases and AMP increases by a much larger margin. This increase in AMP is then picked up by **AMPK**, which becomes activated. Activation of AMPK can be both allosteric and through post translational modification (phosphorylation).

The stimulation of Foxo activity by AMPK may seem surprising as it is by phosphorylation and we have just said that phosphorylation of Foxos leads to inhibition through nuclear exclusion. However AMPK phosphorylates a different part of the protein and this phosphorylation leads to activation of the transcription promoting activity of Foxo.

AMPK has two other affects that are related to energy metabolism in muscle cells. Firstly it inhibits protein synthesis by inactivating the mTOR complex. Given the expense of making protein this is a sensible short term move and can be seen to be closely associated with protein breakdown. The second effect of AMPK activation is an increase in mitochondriogenesis. This has the effect of increasing the energy available from each glucose molecule, as the energy yield from each glucose is higher when it is fully oxidised by the Krebs cycle rather than by glycolysis. Mitochondriogenesis is obviously a much longer term effect and may be restricted to a subset of fibres.

In addition to activation by energy status, a number of other systems are also involved in the nucler factor kappa B (NF-B) system which also activates MuRF expression and the regulation of atrophy including the muscle equivalent of apoptosis in which individual nuclei are lost to maintain the nuclear cytoplasmic ratio.

**SLIDE 45**

How do these signalling pathways interact?

You will not be surprised to see that these pathways are interrelated. Indeed as you have seen there are at least 2 nodal points mTOR and Foxo with activation of Akt increasing the activity of mTOR and protein synthesis and inhibiting the activity of Foxo. Conversely AMPK increases the activity of Foxo but suppresses the activity of mTOR.

**SLIDE 46-48**

One problem with changing the amount of protein in a cell is that it changes size. A nucleus can only generate a certain amount of mRNA and control a maximum cell volume. As muscle cell nuclei don’t divide how do we increase the number of nuclei to allow the muscle cell to grow? The muscle has a set of stem like cells called satellite cells that exist next to the myofibre. One effect of growth factors like IGF-1 is to stimulate the proliferation of these cells. so as the myofibre grows it can recruit satellite cells to make sure that the nuclear domain size is not exceeded. When the muscle shrinks the opposite is true. This results in too many nuclei and too many organelles. Factors like myostatin therefore drive autophagy a process that breaks down organelles and apoptosis to reduce the number of nuclei.

**SLIDE 49**

Can you alter myostatin activity in people? Well the answer is yes and there have been a number of agents that have got to varying stages in clinical trials. These include agents that block the binding of myostatin to its receptor and agents that inhibit myostatin signal transduction. At least two of these agents have entered clinical trials for congenital muscular dystrophies.