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Jason White
Gayle Smythe *Editors*

Growth Factors and Cytokines in Skeletal Muscle Development, Growth, Regeneration and Disease

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Jason White • Gayle Smythe
Editors

Growth Factors and Cytokines in Skeletal Muscle Development, Growth, Regeneration and Disease

Springer

Editors
Jason White
Faculty of Veterinary Science
University of Melbourne
Parkville, VIC, Australia

Murdoch Childrens Research Institute
Royal Childrens Hospital
Parkville, VIC, Australia

Gayle Smythe
Faculty of Science
Charles Sturt University
Albury, NSW, Australia

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Introduction

Skeletal muscle is a highly dynamic and adaptive tissue, constantly changing in response to biomechanical and metabolic demand. Such adaptation, spanning hypertrophy, atrophy, and regeneration require remodelling of muscle fibres, extracellular matrix, and microvasculature, and involves complex interplay between soluble and insoluble regulatory factors. Understanding the role of soluble factors in particular is attractive to potential therapeutic approaches to diseases and conditions in which muscle function is compromised. This volume focuses on the critical role of soluble factors, including growth factors and cytokines, in regulating and maintaining muscle tissue function and adaptive change.

Chapter 1 (Anderson) provides a detailed review of the history and biology of the role of hepatocyte growth factor (HGF) in regulating satellite cell activity. HGF was one of the earliest identified growth factors in satellite cell regulation and acts at multiple stages in the myogenic program. HGF is predominantly sequestered to the muscle fibre basement membrane and extracellular matrix and is liberated in response to physical stimuli, such as stretch and injury. Thus it is expressed extremely early, almost immediately, upon damage to muscle fibres, making it essential in the initial stages of satellite cell activation. The reliance of release from the matrix on nitric oxide synthase-1 (NOS-1) and the role of this relationship in certain disease states is reviewed. This chapter also emphasises that most studies on growth factor function in muscle biology relate to effects on single cell types (e.g. muscle cells, fibroblasts, endothelial cells, nerve fibres). It suggests that there is a significant need to utilise advancing technologies to develop a detailed understanding roles of growth factors such as HGF in highly complex tissues such as skeletal muscle.

Chapter 2 by Joanisse and Paris focuses primarily on the role of cytokines in regulating muscle satellite cell activity in response to exercise, ageing, disease and repair following injury. Central to this chapter is the role of the inflammatory response to muscle remodelling following exercise, damage/injury, ageing, and in certain disease states. Inflammation is a well-established normal reaction to any tissue injury type, and cytokines with direct roles in this process have been shown to have roles beyond inflammation through direct interactions with muscle cells

themselves, regulating their activity. The chapter focuses largely on interleukins, with several isoforms being implicated both directly and indirectly on the myogenic program, and muscle hypertrophy following exercise. This chapter also highlights the potential role of muscle tissue as a direct source of cytokine ("myokine") secretion, with evidence indicating muscle production of several interleukins which may have both local and systemic effects. The work presented in this chapter clearly identifies gaps in the literature base while emphasising the high level of complexity of interactions and cross-talk between the immune and musculoskeletal systems.

The pleiotropic roles of Leukaemia Inhibitory Factor (LIF) are described in Chap. 3 by Hunt and White. Cytokines of the LIF receptor family are pleiotropic cytokines which can influence muscle in a number of ways including via direct interaction with the LIF receptor complex on the surface of muscle cells and indirect effects mediated through other cells. Originally described as a mitogenic cytokine for isolated myoblasts, the list of myogenic effects of LIF has grown since then to include development, adult muscle homeostasis, metabolic, and regulation of regeneration through its anti-inflammatory properties.

An often overlooked factor in the regulation of satellite cell activity is the extracellular matrix, and this is considered by Song in Chap. 4. Traditionally, the ECM has been thought of as a structural scaffold in which cells reside; in reality, components of the ECM directly regulate cell behaviour. Many molecules in the ECM play a critical role in regulating satellite cell activities including activation, proliferation, and differentiation. Of particular importance are membrane-associated heparin sulphate proteoglycans which function as co-receptors for many growth factors to enhance their binding affinity to their primary receptors and initiate downstream signal transduction. The heparin-bound growth factors including FGF2, HFG and TGF-β have been reported to modulate satellite cell activation, migration, proliferation, and differentiation.

While many studies describe the positive regulation of cellular activity in skeletal muscle, negative regulators are equally critical. Of particular interest are those affecting muscle mass and function in pathological conditions. Many readers will be familiar with myostatin, which is a key regulator of muscle development, and more recent evidence is emerging that other TGF-β proteins act in concert with myostatin to regulate the growth and remodelling of skeletal muscle. Chen et al. in Chap. 5 consider the growing understanding of TGF-β biology in muscle and its potential to advance the development of therapeutics for muscle-related conditions is reviewed here. The complexity of the TGF-β network provides the means for this system to finely regulate a variety of different cellular processes in many different cell types and organ systems, including skeletal muscle.

Cross-talk between adipocytes and skeletal muscle via correct control by adipokines is important in controlling energy homeostasis during rest and exercise and can help prevent metabolic disease. Adipose tissue not only functions as a reserve to store energy but has become of major interest as an endocrine organ, releasing signalling molecules termed adipokines which impact on other tissues, such as skeletal muscle. Adipocytes, within skeletal muscle and adipose tissue, secrete

adipokines to finely maintain the balance between energy intake and expenditure. Chapter 6 by Coles focuses on the three adipokines, adiponectin, leptin, and IL-6, which have potent effects on skeletal muscle at rest and during exercise. Similarly, adiponectin, leptin, and IL-6 enhance glucose uptake and increase fatty acid oxidation in skeletal muscle. Whilst the last decade has revealed a vast amount of knowledge about these adipokines, there is much more to understand, particularly in the search for drugs that might reduce lipotoxicity in skeletal muscle for the treatment of metabolic syndrome, obesity, and diabetes.

Growth factor-mediated regulation of microvascular growth and remodelling is the focus of Chap. 7 (Smythe). This chapter discusses changes in the muscle microvasculature in response to exercise, atrophy due to ageing and disuse, regeneration following injury, and disease, and highlights both the redundancy and balance of pro- and anti-angiogenic growth factors regulating capillary growth and remodelling. This chapter also investigates the evidence in the literature for potential for growth factor-based therapies in promoting vascular supply, and thus compromised muscle function, such as in certain diseases and following significant muscle trauma.

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Chapter 1
Hepatocyte Growth Factor and Satellite Cell Activation

Judy E. Anderson

Abstract Satellite cells are the "currency" for the muscle growth that is critical to meat production in many species, as well as to phenotypic distinctions in development at the level of species or taxa, and for human muscle growth, function and regeneration. Careful research on the activation and behaviour of satellite cells, the stem cells in skeletal muscle, including cross-species comparisons, has potential to reveal the mechanisms underlying pathological conditions in animals and humans, and to anticipate implications of development, evolution and environmental change on muscle function and animal performance.

Keywords c-met receptor • HGF • Skeletal muscle • Growth • Regeneration • Nitric oxide • Muscle atrophy • Cytoskeleton • DGC • Zebrafish

1.1 Introduction

As the building blocks for skeletal muscle during development, growth and regeneration (Anderson 2006; Mauro 1961; Mauro et al. 1970; Yablonka-Reuveni 2011), satellite cells play a fundamental role in muscle biology and animal function. They can respond dramatically and continuously to alterations in physiological demands on muscle, and to injuries and changes in nutritional constraints during adaptation. It is well-established that growth factors and cytokines have important roles in the repair of skeletal muscle (Karalaki et al. 2009), and that they have differential effects at various stages of myogenesis. Hepatocyte growth factor (HGF) for instance, is a protein made in myocytes and myoblasts (Charge and Rudnicki 2004; Sheehan et al. 2000; Tatsumi et al. 1998) and it promotes their proliferation while inhibiting myotube formation and differentiation in culture and regeneration.

J.E. Anderson (✉)
Department of Biological Sciences, University of Manitoba, Winnipeg, Canada
e-mail: Judith.Anderson@umanitoba.ca

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HGF binds to the c-met receptor expressed on myoblasts and on satellite cells; that binding inhibits expression of the muscle regulatory genes, MyoD and myogenin, and the structural protein, myosin heavy chain (Anastasi et al. 1997; Gal-Levi et al. 1998; Leshem et al. 2000; Miller et al. 2000). By comparison, other growth factors also promote proliferation of myogenic cells, for example insulin-like growth factor (IGF-1) and fibroblast growth factor (FGF-2) and then promote myotube differentiation (Allen and Boxhorn 1989; Florini and Magri 1989; Hayashi et al. 2004).

Distinctions among growth factors in their activity in skeletal muscle were first explored in now-classic *in vitro* culture studies of proliferation and differentiation (Allen et al. 1995b; 1979) to understand the regulation of muscle growth. Studies of HGF, FGF and transforming growth factor beta (TGF-β) on proliferation and differentiation (Allen et al. 1995b; Allen and Boxhorn 1989; Allen and Rankin 1990; Johnson and Allen 1993; 1995) opened the field to studies of different stages in the myogenic regulatory program. As revealed at the time, the sequence of regulatory gene expression was delayed in cultures of satellite cells from muscle of old rats, pending a latent period between isolation of the quiescent satellite cells from muscle and their activation and proliferation in culture (Smith et al. 1994). Bischoff used a crushed-muscle extract (CME) preparation to induce activation of satellite cells on isolated fibers prepared for culture, and that extract was also shown to enhance muscle regeneration and satellite cell activity *in vivo* (Bischoff 1986a, b; Bischoff and Heintz 1994).

Early *in situ* hybridization experiments demonstrated that HGF was regulated during muscle development and then transiently expressed following ischemia-induced muscle injury and repair (Jennische et al. 1993). However, it was the notion that live and dead muscle fibers have distinctive interactions with their resident satellite cells and that living, intact fibers produce a factor that activates satellite cells and stimulates their transition from G0 to the cell cycle (Bischoff 1990). This was confirmed later, in studies of activation on fibers exposed to CME or the myotoxin, marcaine (Anderson and Pilipowicz 2002); those studies led to the identification of HGF as the activating factor in CME (Tatsumi et al. 1998). Subsequently, HGF-c-met interactions were examined in the context of paracrine signaling (Anastasi et al. 1997). More recently, an engineered high-affinity HGF with two c-met binding sites called Magic-Factor 1, has been shown to stimulate hypertrophy through anti-apoptotic pathways (Cassano et al. 2008). Current literature gives HGF a very prominent profile in processes mediating the balance between the activation of satellite cells and their return to quiescence (bou-Khalil et al. 2010; Chazaud 2010; Yamada et al. 2010), although other gene products and genetic background clearly impact satellite cell populations and their functionality in healthy and diseased muscle (Fukada et al. 2010; 2013).

Satellite cell activation is initiated through a cascade of calcium-dependent signals that release HGF from the extracellular matrix (Hara et al. 2012; Tatsumi et al. 2009b; Tatsumi 2010). Injury-induced activation leads to those processes involved in regeneration of muscle tissue, including angiogenesis, nerve sprouting and innervation, matrix remodelling and myogenesis, itself. All these processes impact

directly on the outcome of tissue repair and the level of function attained by that tissue. Since the identification of nitric oxide as a potent mediator of satellite cell activation in mouse muscle (Anderson 2000; Wozniak and Anderson 2007; 2009), many roles of HGF and HGF-mediated signaling during the regeneration of muscle tissue have been reported. However, the scope of differences in HGF-mediated processes during activation and regeneration in different species, and many of the key aspects of chemical, mechanical, growth-factor- and chemokine-mediated signaling that affect muscle plasticity and function are not as well established across species, despite their importance in shaping muscle adaptation and the wide range of life-history challenges met by different species.

Capabilities of research using the single-fiber culture model to study HGF-mediated activation in mammalian and zebrafish muscle provide a strong platform for future exploration of HGF-induced signaling in development and muscle-tissue repair. This chapter provides a focused review of literature that relates to HGF-mediated mechanisms important in muscle growth and regeneration. Many studies demonstrate the interdependence of muscle structure, development and phenotype by modeling their combined impact on the activation of satellite cells. Evolutionary distinctions in activation by nitric oxide signaling (Anderson 2000) and possible species-dependent contributions of satellite cell regulation may contribute to determining the phenotypic pattern of muscle tissue expansion.

1.2 Satellite Cell Activation

The cytoskeletal transmembrane dystroglycan complex (DGC) anchors nitric oxide synthase (NOS)-1 close to the muscle fiber membrane. The DGC stabilizes the membrane of contracting muscle fibers by linking the actin cytoskeleton through the membrane to the surrounding matrix (Barresi and Campbell 2006; Campbell and Stull 2003; Durbeej and Campbell 2002; Ervasti and Campbell 1991; 1993; Matsumura et al. 1993). Changes in dystrophin, NOS-1 and transmembrane β-dystroglycan in the DGC, between muscles of wild-type, dystrophin-deficient and old mice accompany changes in satellite cell activation and cycling in muscle development, atrophy and regeneration (Janke et al. 2013; Leiter et al. 2012; Wozniak et al. 2003; Wozniak and Anderson 2007). We recently identified that the DGC develops in relation to muscle loading (Janke et al. 2013).

Satellite cells are precursor or stem cells on muscle fibers (Anderson 2006; Seale and Rudnicki 2000; Yablonka-Reuveni 2011) and are the source of cells for growth and regeneration (Collins et al. 2005; Collins and Partridge 2005). In adult mice, satellite cells are mitotically quiescent (G0); once activated to cycle, they form myoblasts that commit to the muscle lineage, divide, differentiate and fuse to extend existing fibers or form new ones. By asymmetrical division, some myoblasts return to a satellite position on fibers to renew the stem-cell niche (Collins et al. 2005; Kuang et al. 2007; Rudnicki et al. 2008). As fiber nuclei are post-mitotic, satellite cell activation is *critical* for muscle regeneration and plasticity.

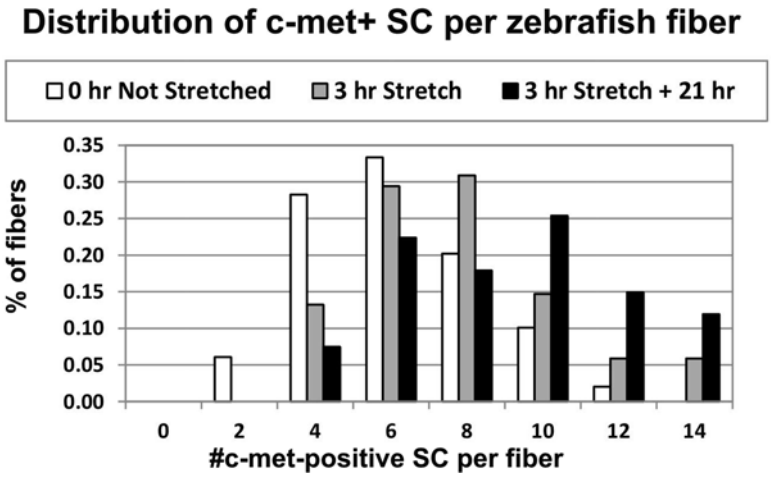

Fig. 1.1 ***Satellite cell heterogeneity on fibers.*** Zebrafish fibers were isolated, plated on collagen and incubated with or without cyclical stretching using a FlexCell vacuum-system apparatus. Fibers were fixed after 0 h, 3 of stretching or after 24 h (3 h stretching plus 21 h further incubation) before immunostaining for c-met protein. The number of c-met-positive satellite cells on each fiber was plotted as a frequency distribution for control fibers (*without stretch*) and fibers immediately or 21 h after stretch (Zhang and Anderson 2014). Graphs show significant heterogeneity for the distribution of c-met-positive satellite cells as a population on fibers (at least 67 fibers per group)

Satellite cells are activated to cycle via signaling through the potent gaseous signal of nitric oxide release (Anderson 2000). Time-course studies of satellite cell activation using single muscle fibers that were mechanically stretched in culture revealed functional satellite-cell heterogeneity related to mRNA expression of c-met, the receptor for HGF (Fig. 1.1), and that loss of dystrophin appears to deplete particular subpopulations (Anderson and Wozniak 2004; Beauchamp et al. 1999; Collins et al. 2007; Heslop et al. 2000; Wozniak et al. 2003; Wozniak and Anderson 2007; 2009). Changes in NOS-1 expression or activity reduce or delay activation by injury, and then perturb the muscle-regeneration cascade (Anderson 2000; Wozniak and Anderson 2007). NOS-1 down-regulation in dystrophic muscle (Brenman et al. 1995) occurs with muscle loading in development and is not a direct result of the genetic loss of dystrophin (Janke et al. 2013). The mechano-sensitive NOS-1 protein releases nitric oxide after stretch; this liberates HGF from the fiber matrix. HGF binds to the c-met receptor on satellite cells, initiating satellite cell cycling and movement (Siegel et al. 2009; 2011; Tatsumi et al. 1998; 2002; Wozniak et al. 2003; 2005; Wozniak and Anderson 2007; 2009). Satellite cells also express dystrophin (Anderson et al. 1991), loss of which was shown recently to particularly impact satellite cell polarity in mitotic division and disease progression and regeneration in muscular dystrophy (Dumont et al. 2015).

The level of c-met expression in muscle and the number of c-met expressing satellite cells on fibers increases after activating satellite cells in wild-type mouse

muscle (Anderson and Wozniak 2004; Wozniak et al. 2003). Interestingly, HGF is expressed in many types of cells in muscle tissue including skeletal, smooth and cardiac muscle cells (Fig. 1.2). HGF is also expressed by infiltrating mononuclear inflammatory cells in regenerating mouse muscle (Sakaguchi et al. 2014) and in human muscle of patients with polymyositis/dermatomyositis (Sugiura et al. 2010). Interestingly, macrophages were reported to activate myogenic cells as early as 1999 (Merly et al. 1999). In cultures of CD56-expressing myoblasts from those patients, the level of HGF was reduced by treatment with TGFβ and anti-inflammatory treatment, and increased by treatment with interleukin-1α (Sugiura et al. 2010). HGF treatment of those cells in culture reduced their synthesis of pro-collagen 1, while interferon-gamma increased the expression of c-met, suggesting the use of HGF to promote regeneration of muscle in inflammatory conditions (Sugiura et al. 2010). HGF-mediated muscle cell proliferation was also suggested as an approach to promoting muscle growth for treatment of urinary incontinence (Sumino et al. 2007). Molecules including miRNA489 (Brack et al. 2009), FoxO1, TGFβ3 and myostatin promote quiescence and inhibit growth and cycling (Argiles et al. 2012; Huang et al. 2012; Reed et al. 2012; Thomas et al. 2000). The balance between quiescence and activation states in myogenic cells is influenced by epigenetic effects of the surrounding matrix that are mediated by signaling through the Wnt pathway and the methylation state of chromatin that is mediated in part by mixed lineage leukemia 5 (MLL5) (Dhawan and Rando 2005; Sebastian et al. 2009; Sellathurai et al. 2013; Srivastava et al. 2010; Subramaniam et al. 2013).

1.3 Myogenic Cell Motility

Syndecans, especially syndecan 3 among the heparan sulphate proteoglycans (Casar et al. 2004; Cornelison et al. 2001; 2004; Pisconti et al. 2010; Rapraeger 2000), CD34, Eph-ephrin and Notch-delta interactions, Wnt, FGF2 and possibly Hedgehog (Xie et al. 2013) promote motility and cycling after activation (Alfaro et al. 2011; Ieronimakis et al. 2010; Luo et al. 2005; O'Brien et al. 2004; Stark et al. 2011). Syndecan-3 and dermatan sulfate are required for muscle regeneration and activation signaling (Casar et al. 2004; Villena and Brandan 2004). Interestingly, HGF induces the formation of lamellipodia and cell migration in cultures of C2C12 cells in a 3-dimensional assay. This is reported to occur through a balance of PI3-kinase-dependent signals in the WAVE2 N-WASP pathway that involve the formation of branched actin filaments and polarization of the plasma membrane (Kawamura et al. 2004). Such regulation likely plays a role in the fascinating HGF-dependent travel by activated satellite cells along muscle fibers that can be so beautifully demonstrated in fiber cultures (Siegel et al. 2009; 2011). This HGF-mediated migration may also interact with CD44-dependent regulation of the speed of satellite cell migration (Mylona et al. 2006). Involvement of glypican-1 in stabilizing HGF-c-met interactions via mechanisms related to lipid-raft formation is required for the

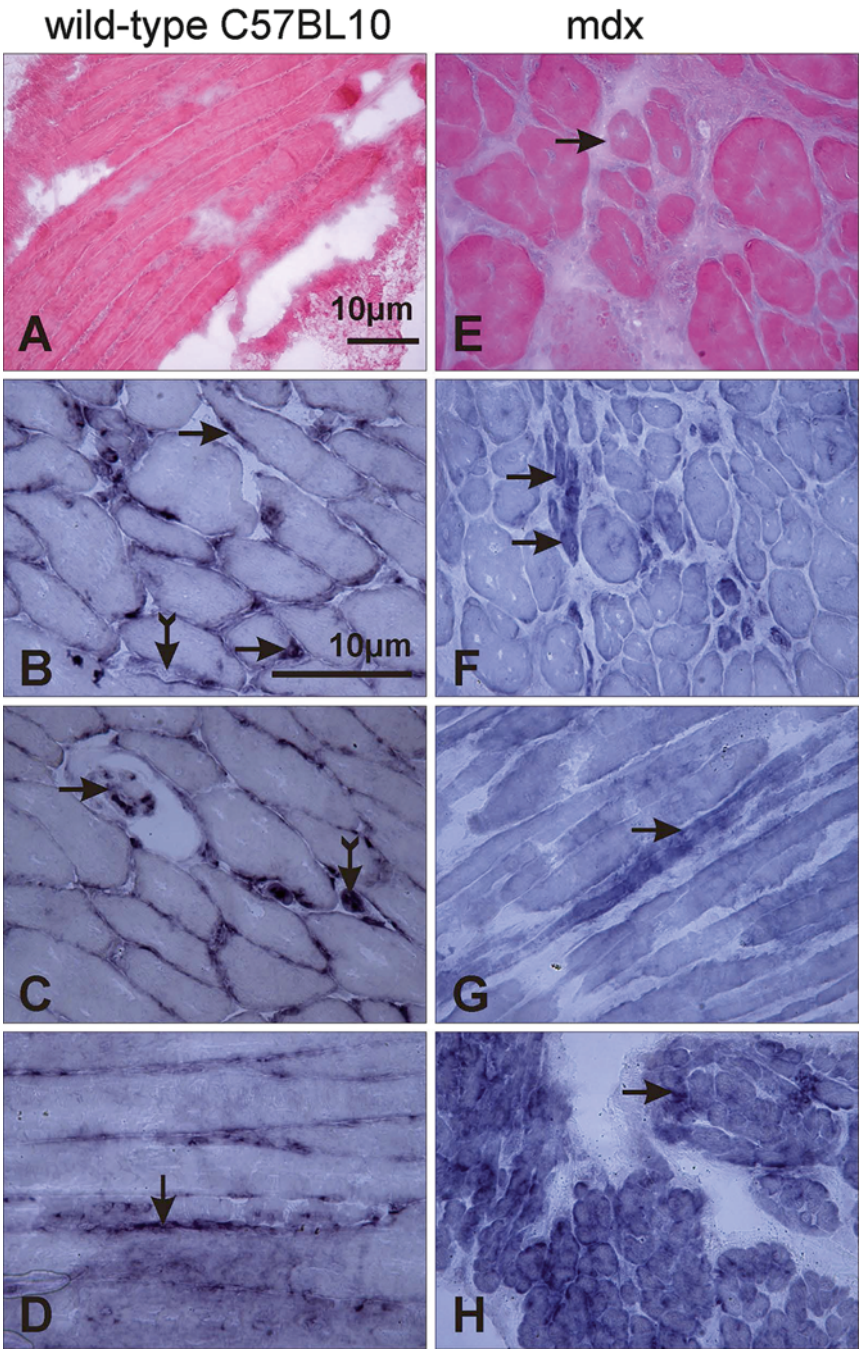

Fig. 1.2 ***Hepatocyte growth factor (HGF) expression in control and dystrophic muscle.*** Muscle histology viewed by hematoxylin and eosin staining (**a**, **e**) and in situ hybridization for HGF) using digoxigenin-labeled riboprobe to identify mRNA (**b–d**, **f–h**) from wild-type C57BL10 mice (**a**–**d**) and dystrophin-deficient *mdx* mice (**e–f**). (**a**) Muscle fibers in control diaphragm. (**b**) Two satellite

optimal response of mouse C2C12 cells to HGF signaling and to stabilize CD44 (Gutierrez et al. 2014). HGF also induces expression of caveolin-1 which inhibits cell proliferation, and is proposed to regulate the activity of the ERK pathway in satellite cell mobility and proliferation during regeneration (Volonte et al. 2005).

Another distinction among different growth factor-mediated processes is that HGF appears not to influence fibrinolysis required for invasion of myogenic cells into a matrix, although the migration process is dependent on urokinase plasminogen activator activity which is in turn, regulated by FGF-2 and TGFβ (Fibbi et al. 2002). Possible similarities among different types of cells (e.g., myogenic cells and keratinocytes) suggest that many features of migration, may show distinctions based on the surrounding matrix or particular features of how factors are applied in an experiment (e.g., separately or in combination), as illustrated by use of a wound-assay culture system (Peplow and Chatterjee 2013). In a review of heparanase and syndecan-1 in tumours, the up-regulation of HGF expression by heparanase, and subsequent binding of HGF to syndecan-1 for degradation, was highlighted as a mechanism regulating growth factor activity (Ramani et al. 2013). It is possible that similar interactions during HGF-mediated responses by satellite cells play a role in the timing or dose-response of activation and migration during growth and repair of muscle and could point the way to designing novel therapeutics to promote those processes.

1.4 Zebrafish Single-Fiber Studies of Satellite Cell Activation

Temperature, environment and life-history events such as migratory behaviour in fish and birds and hibernation patterns in mammals, influence muscle plasticity and metabolic adaptation (Corrigan et al. 2011; Johnston 2006; Johnston and Hall 2004; Meyerrochow and Ingram 1993; Price et al. 2011; Wund et al. 2008). For instance, hibernation affects muscle mass or growth in some vertebrates and not in others. Interestingly, hibernation can protect muscle from atrophy that ensues with starvation, disuse/immobilization or even denervation. Hibernation apparently changes the neural regulation of catabolic pathways, such that decreases in muscle mass and fiber cross-sectional area in hibernating bears after denervation are only one third to

Fig. 1.2 (continued) cells (*arrows*) in tibialis anterior muscle contain cytoplasm densely stained for HGF transcript; less intense staining for HGF transcripts is visible around the periphery of myofibers. A nerve bundle is indicated by the *crossed vertical arrow*. (**c**) HGF expression is demonstrated in intrafusal fibers of a muscle spindle (*arrow*) and in smooth muscle around a blood vessel (*crossed vertical arrow*). (**d**) A small myotube (*vertical arrow*) is regenerating between intact fibers in the diaphragm of a control mouse. (**e**) Regenerating fibers (*arrow*) within sections of dystrophic tibialis anterior muscle. (**f** and **g**) Small regenerating fibers (*arrows*) are intensely stained for HGF mRNA in an inter-nuclear distribution in longitudinal section (**g**). (**h**) Cardiac myocytes from the atrium of a dystrophic mouse are stained for HGF transcripts (*arrow*). Bar = 10 μm (original magnification X200); bar in panel **b** applies to **b–h** (original magnification X400)

one half as pronounced as in non-hibernating bears (Lin et al. 2012). Hibernation is also accompanied by down-regulation of autophagic and proteolytic pathways mediated by serum- and glucocorticoid-inducible kinase-1 (GSK1) (Andres-Mateos et al. 2013). Moreover, in studies of the 13-striped ground squirrel, while the level of myostatin, which typically represses satellite cell proliferation in non-hibernating animals and typically increases in starvation and disuse, it is surprising that in hibernating animals, myostatin levels do not change. Myostatin stays at normal pre-hibernation levels well into hibernation, and the marked increase only happens when the animal is beginning to *arouse* from topor, toward the end of hibernation (Brooks et al. 2011). Regulation of muscle atrophy is clearly overlain by complex metabolic changes somewhat separable from neural and other activity and/or endocrine influences. Such fascinating observations encourage in-depth comparative studies of muscle satellite-cell biology and the impact of various life-history events.

A key distinction in muscle-growth capacity between mammals and teleosts is the regulation of fiber number. In mammals, fiber number does not increase after birth; by comparison, in teleost fish, the number of fibers, especially fast fibers, rises nearly continuously with body size and muscle mass, as part of the process termed indeterminate growth (Hall et al. 2004; Johnston 2006; Johnston and Hall 2004). A leader in the field, IA Johnston, notes the major gap in our understanding of the impact of indeterminate growth and ectothermy on the regulation of muscle growth in teleosts versus mammals (Johnston et al. 2011).

On the basis of the growing body of work on mammalian muscle fibers, and long-standing questions about the regulation of muscle growth in fish, we modified the protocol for isolating mouse-muscle fibers to enable culture studies of zebrafish muscle fibers (Anderson et al. 2012) prepared with careful attention to Bischoff's technique (Bischoff 1986b). Using single-fiber cultures from zebrafish, experiments revealed that satellite cell activation on zebrafish fibers is mediated by nitric oxide, HGF and mechanical-stretch signals (Zhang and Anderson 2014) (Fig. 1.3a, b), similar to fiber cultures from mouse (Anderson and Pilipowicz 2002; Wozniak et al. 2003; Wozniak and Anderson 2007). However, although the cascade of activation involved similar signals, peak activation in zebrafish fiber cultures occurred at a distinctly higher concentration of nitric oxide and a lower concentration of HGF, than in our earlier findings on activation in mouse-fiber cultures; values in fish vs. mouse fiber experiments were: 1 mM isosorbide dinitrate (2 molar equivalents of nitric oxide released) vs. 100 nM of the NOS substrate L-arginine (1 molar equivalent of nitric oxide produced); and 10 ng/mL vs. 25 ng/mL HGF in medium, respectively (Anderson and Pilipowicz 2002; Wozniak and Anderson 2007; Zhang and Anderson 2014). [Unfortunately, the direction of the comparative fish-mouse difference for response to nitric oxide is reversed in Zhang and Anderson 2014.] While the levels of nitric oxide and HGF extant in the two types of fibers is not known, the relative differences indicate there is a differential "tuning" of activation by the HGF-mediated cascade in mouse and fish fibers. The dose-response of HGF-induced activation is shifted to a lower peak concentration of exogenous HGF by stretching in culture (Fig. 1.3c), due to release of HGF from the low-affinity receptors in the extracellular matrix surrounding fibers (Tatsumi et al.

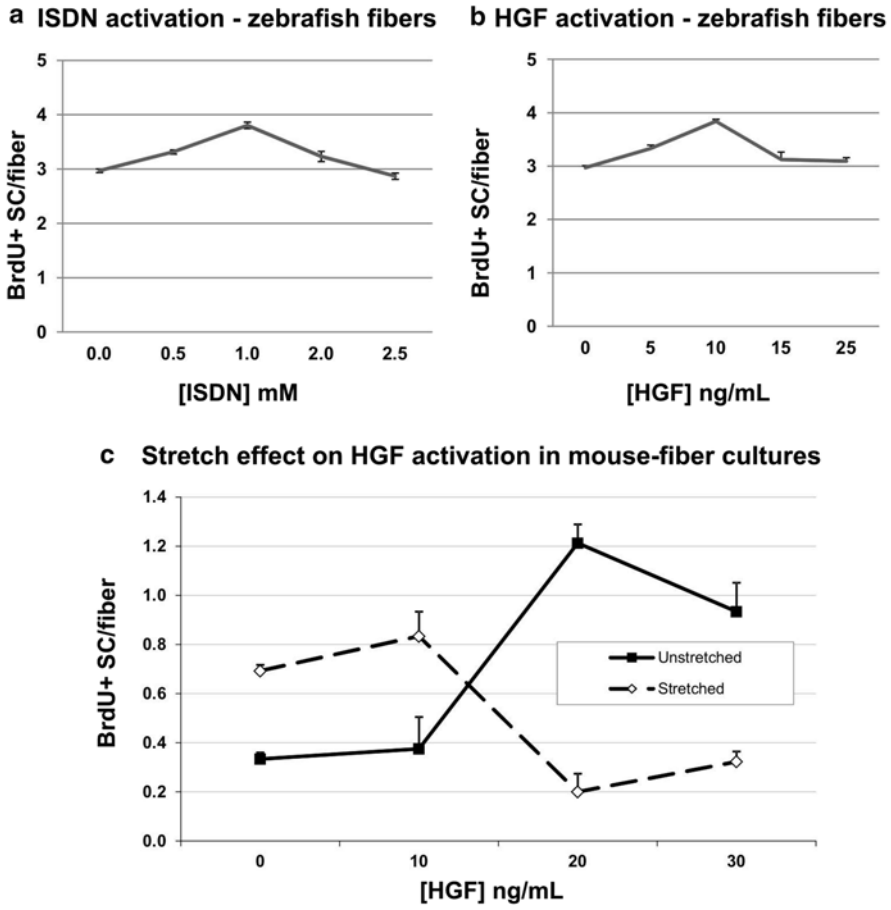

Fig. 1.3 ***Dose-response curves for satellite cell activation on fibers in culture.*** Muscle fibers were isolated from zebrafish (**a**, **b**) and mouse (**c**) and cultured in basal growth medium containing bromodeoxyuridine (BrdU) for 24 h in the presence of: (**a**) the NO donor isosorbide dinitrate, ISDN (0–2.5 mM); (**b**) HGF (0–25 ng/mL); and (**c**) HGF (0–30 ng/mL) with or without cyclical stretching. After 24 h incubation, fibers were fixed and immunostained for BrdU, the dose-response of satellite cell activation was determined as the number of BrdU-positive satellite cells per fiber. Note that peak activation by HGF occurred at a lower concentration in unstretched zebrafish than mouse fiber cultures (**c** vs. **b**), and that HGF-induced activation on mouse fibers shifts to a lower concentration of HGF in the presence of 3 h of stretching

2001; 2002; Tatsumi and Allen 2004). Activation by exposure to HGF and by mechanical stretching in the fish fibers is additionally mediated by temperature. Results of studies using inhibition of HGF-c-met binding and NOS activity demonstrated that activation of satellite cells on fibers in culture is reduced at 21 °C compared to the level observed at 27 °C, the typical temperature for rearing zebrafish (Zhang and Anderson 2014).

The interesting finding of a novel temperature-sensitive pattern of satellite cell activation on zebrafish fibers was accompanied by observation that the population of satellite cells identified by immunostaining studies for the c-met receptor protein, increased in size only 3 h after stretch (Zhang and Anderson 2014). This finding indicated that c-met is acting as an immediate early gene in satellite cell activation on zebrafish fibers, consistent with findings from mouse-fiber cultures (Wozniak et al. 2003). As well, there were more c-met-positive satellite cells on fish fibers, and they cycled faster than in mouse (Zhang and Anderson 2014). Very recent extension of these fiber-culture studies is now examining developmental aspects of interactions between fibers and their resident satellite cells.

The study demonstrated the sensitivity of the fiber-model system for testing the impact of brief or subtle changes in the fiber environment, on muscle growth and adaptive capacity. While there is clear conservation of regulatory pathways between zebrafish and mouse muscle, at least in the fiber-culture model system, these recent studies provide fresh insights on possible mechanisms of indeterminate muscle growth in fish that utilize fiber hyperplasia and hypertrophy, in comparison to post-natal muscle growth by hypertrophy of fibers in mammalian species. Such information could be a strong asset to aquaculture and food-meat production industries, and assist in further studies of the epigenetic interactions of the fiber cytoskeleton with satellite cell activity as they relate to understanding how the growth and repair of muscle tissue shape functional muscle plasticity.

1.5 Re-defining the Impact of Age on Satellite Cell Activation and Muscle-Growth Capacity

Previous research in this laboratory tracked the concurrent loss of muscle mass, strength, proliferation and fiber size in aging mice, with changes in NOS-1 and β-dystroglycan in the DGC and membrane function. Exercise increased levels of the protein NOS-1 in skeletal muscle and increased the proliferation of satellite cells when the intervention was given at eight but not 18 months-of-age, and the response was muscle-specific rather generalized in all muscles (Leiter et al. 2011). We also found that satellite cells were not activated in old muscle by the level of stretching that effectively activates them when younger muscle is stretched in culture, and this refractoriness to stretching increased with greater age (Leiter and Anderson 2010). The novel finding was that in muscles from old mice, proteins of the DGC were relocated and reduced in concentration (Leiter et al. 2012), and that remarkably, combined treatment with voluntary-running exercise and the nitric oxide-donor compound called isosorbide dinitrate, partly restored the beneficial muscle-building effects of exercise in the old mice, toward effects that are more typical of exercise effects on muscle in younger animals. This was despite the significant decline in the number of satellite cells expressing transcripts for myoD, myogenin or pax7 found in the gastrocnemius muscle of animals at 18 months-of-age, the age at which the

combined treatment with ISDN plus exercise had started, compared to muscle at 3, 8 or 12 months-of-age (Fig. 1.4, Leiter & Anderson, *unpublished data*). Treatment also shifted the subcellular pattern of the β-dystroglycan and NOS-1 in muscle from the old mice, toward the localization pattern seen in muscle from much younger mice. This was concurrent with decreased permeability to serum immunoglobulins in the muscle of old mice; overall, the combined treatment of exercise and the nitric oxide donor resulted in a 25 % increase in muscle mass and increased fiber diameter in the old mice (Leiter et al. 2012). Without the increase in nitric oxide provided by the NO-donor treatment, exercised old mice displayed the typical loss of muscle mass and the distribution of the two proteins in the DGC had the pattern that was observed in muscle of old mice. There may be potential for application of therapy based on nitric oxide, to improve the functional state of muscle growth capacity in aging or to assist in "pre-habilitation" of muscle prior to surgical procedures. This study shows the growth capacity of satellite cells in old muscle is retained. A comprehensive examination of the capacity for muscle regeneration in response to three different types of injury (freeze, crush and myotoxin) in very old mice, showed that the capacity of satellite cells in muscle of old or very old mice to provide fully functional regenerated muscle tissue can be retained if muscle damage has not affected the neurovascular supply (Lee et al. 2013). The possibility of progressive changes in HGF expression and HGF-mediated signaling in satellite cell activation has not been fully explored through the aging process.

1.6 Integrating Growth, Satellite-Cell Behaviour and Cytoskeletal Development

Cytoskeletal NOS-1 and β-dystroglycan develop earlier in mouse diaphragm than limb muscle. This pattern changes with loss of dystrophin from the DGC (Janke et al. 2013). Such findings support the idea that differential muscle loading, which is higher and earlier in diaphragm than in limb muscle, and the structure of the DGC that differs in important but seemingly subtle ways from the DGC in the central nervous system, may explain muscle-specific variations in muscular dystrophy (Janke et al. 2013; Snow et al. 2013a; b). Previous reports on c-met regulation of cell functions, such as migration of invasive tumour cells, limb muscle precursors in development, and tubule formation by epithelial cells, in regeneration of liver and proliferation of liver and placental cells (Trusolino et al. 2010), and the outcome of muscle satellite-cell activation (Leiter et al. 2012; Webster and Fan 2013; Wozniak and Anderson 2007), clearly demonstrate the importance of the cytoskeleton in mediating that regulation.

Semaphorin3A (Sema3A) is a paracrine neuro-repellant protein, novel to muscle biology since 2009 (Tatsumi et al. 2009a). Sema3A is implicated in an axon-guidance role during the regeneration of skeletal muscle. HGF induces Sema3A secretion by activated satellite cells, but interestingly this does not occur through

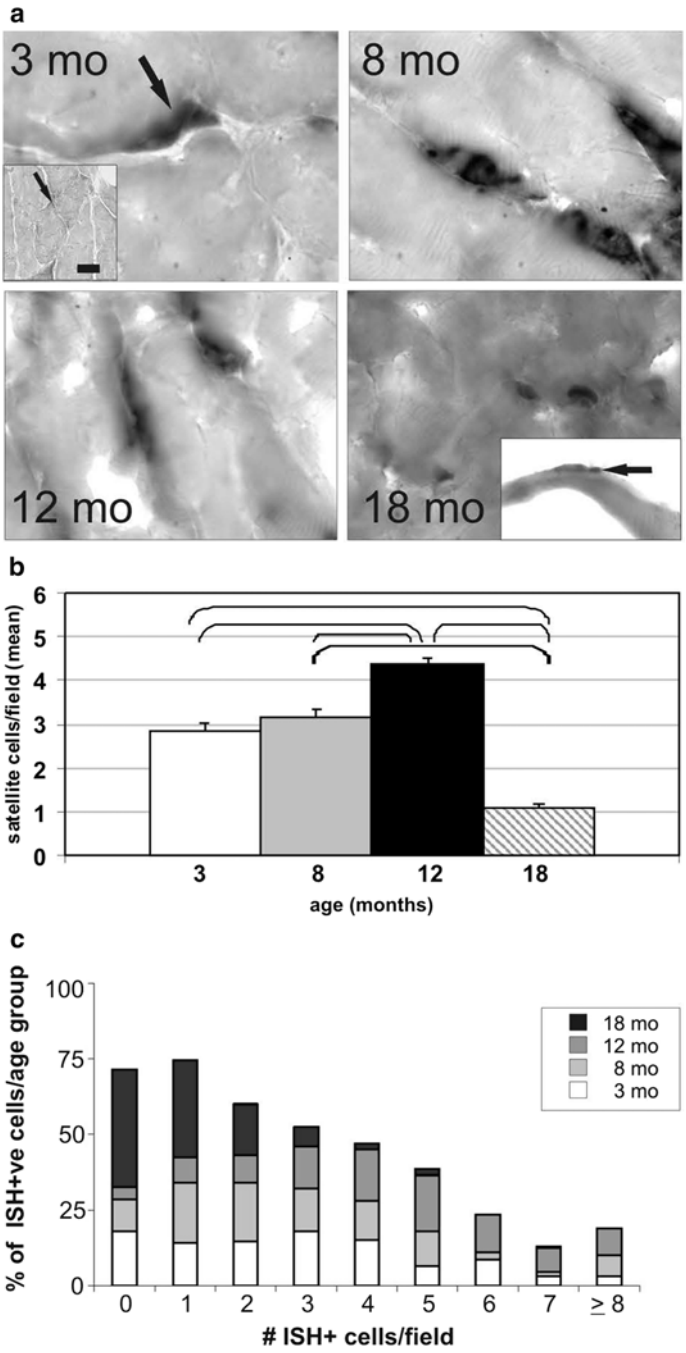

Fig. 1.4 ***Satellite cell populations decline with increasing age.*** (**a**) Gastrocnemius muscle from mice aged 3, 8, 12, and 18 months, showing satellite cells stained positive by combined *in situ* hybridization (ISH) using a cocktail of riboprobes for pax7 (a muscle-specification gene, expressed in quiescent and activated satellite cells), and myoD and myogenin RNA (muscle-regulatory genes

HGF binding to c-met. These findings suggest that satellite cells serve to collect and integrate activating and quiescence factors and the functional capacity for nerve-independent and nerve-dependent repair via Sema3A (Do et al. 2011; 2012; Suzuki et al. 2013). Sema3A is up-regulated by HGF, peaks early in myoblast differentiation and could mediate the timing of axonal ingrowth to regenerating fibers. It is intriguing that Sema3A may act through a receptor in the plexinA2-myogenin pathway that is particularly active in slow muscle (Suzuki et al. 2013; Tatsumi et al. 2009a). This field of study opens exciting new ways to study nerve-muscle interplay during muscle development. For example, it would be fascinating to examine the expression and behaviour of satellite cells on developing fibers at different life-stages from a number of species, as the stage that is particularly critical to organism survival may differ in different species. Satellite cell activity could be explored using an architectural approach, by examining the expression of HGF, Sema3A and other regulatory and structural proteins by satellite cells and their activation status, according to their distance from vessels or nerve-muscle junctions along a fiber in culture or *in vivo*. Such studies could help identify factors that contribute to producing a heterogeneous population of stem cells in a muscle, and how the behaviour of satellite cells during development and activation in growth, may influence the muscle phenotype that develops in different species.

Characterization of the satellite cell populations *in vivo* on adult and developing muscle fibers on non-mammalian species is an important and achievable goal, given the acceleration of the satellite cell field that has resulted in the past three decades of focused research using rodent models of human disease. Understanding how satellite cell populations vary in size and activation state by species, fiber type and through development can now be examined in relation to differences in the environment, function and behaviour of an organism. A good "baseline" understanding is still missing information about cross-species comparisons of satellite cell populations in adult and developing muscle that explain the differential responses by muscle to activating stimuli and environmental change, and the distinctive capacity for nearly life-long formation of new fibers in some species. Studies of the differential growth by fish developing in cold versus warm water, and the genes expressed by muscle tissue or fibers (Johnston 2006; Johnston et al. 2009; Johnston and Hall 2004; Steinbacher et al. 2011) have not fully explained the important biology of

Fig. 1.4 (continued) expressed by activated satellite cells). Satellite cells are directly juxtaposed to the sarcolemma of unstained fibers (*arrows* indicate satellite cells with a prominent nucleus). *Insets* show a negative-control section (*arrow* points to an unstained satellite cell) and a satellite cell on an atrophic fiber (at 3 and 18 months, respectively). Bar = 10 μm. (**b**) Satellite cell density (number of ISH-positive mononuclear cells in the satellite position per field, mean ± SEM, 247–303 fields per group) was highest at 12 months. *Brackets* indicate significant differences ($p < 0.001$). (**c**) The percentage of satellite cells detected by in situ hybridization for each age group, plotted against satellite cell density (the number of ISH+ cells per field). The density of satellite cells expressing at least one of myoD, myogenin or pax7 declined with increasing age ($p < 0.01$, Chi-square statistics); 18-mo gastrocnemius muscles had the highest frequency of fields without any satellite cells (Leiter and Anderson, unpublished)

muscle satellite cells, the proteins they make or bind and their response to changing conditions. That information is crucial to anticipating animal responses to climate change, toxic habitat or tissue injury.

By studying the regulation of satellite cell activation signaling on single fibers *in vitro*, experiments could be conducted to examine species-specific differences in the regulation of satellite cell activation and how they may relate to features of the DGC. A synthesis of information on how the DGC and internal cytoskeleton shape nuclear position and development is also missing from the literature, despite the critical roles of those proteins in regulating satellite cell activation (for example, see Janke et al. 2013; Wozniak and Anderson 2007) and muscle fiber integrity.

A further gap in our knowledge relates to understanding the basis for central nucleation in regenerated fibers. Typically in development, as myotubes differentiate and receive innervation, nuclei shift from the central region of myotubes toward the periphery of fibers (just inside the DGC). Nesprin1 may be involved in that relocation. Nesprins are part of a complex that links the outer nuclear envelope with the actin cytoskeleton, and preliminary observations showed nesprin1 distribution differed between the peripheral nuclei in normal adult fibers and those centrally located nuclei in regenerated fibers in the absence of dystrophin (Kaluzny and Anderson, *unpublished*). The positioning of satellite cells in proximity to in-growing nerves and blood vessels (Duxson and Sheard 1995; Harris et al. 1989), particularly at the nerve-muscle junction or endplate regions, is also reported to relate to the location of giant nesprinG proteins (>1000 kb) (Burke and Roux 2009; Crisp et al. 2006; Roux et al. 2009; Starr and Fischer 2005; Tzur et al. 2006; Zhang et al. 2007). It would be fascinating to examine nerve-muscle junctions on fibers in culture, in relation to the position and activity of the resident satellite cells and myonuclei at important stages in development and regeneration, and also the interaction of satellite cells with angiogenic processes and vascular endothelial growth factor through those processes.

Since muscle stem-cell activity on fibers is so strongly linked with the ultimate outcome of muscle growth, physiology and adaption to neurovascular regulation (Allen et al. 1995a), it will be important to take new advantage from the fiber-model system to interrogate the relationship between satellite cell activation, behaviour and muscle phenotype. Fiber cultures could be used to study whether growth factors such as HGF or cytokines derived from the many tissue types in a muscle belly, direct satellite cell populations toward regenerating fibers that take up a particular fast or slower phenotype after the repair. Alternatively, satellite cells may be the prime organizers of key stages in the regenerative response through their integration of incoming signals and subsequent release of factors that actively signal to the local or systemic environment. For example, an interesting report examined systemic interactions mediating HGF expression, and found that within an hour of a muscle injury, both liver and spleen had up-regulated HGF expression and production of the inactive 90 kDa form of HGF protein. Serum from rats undergoing muscle regeneration was also able to induce expression of HGF transcripts in cells cultured from rat spleen, suggesting a role for an endocrine-level regulation of satellite cell behaviour (Suzuki et al. 2002). Fiber cultures could be used to screen for myogenic or

growth-promoting effects of a range of compounds or extracts from natural or engineered tissues. Another report noted the importance of HGF in recruiting myogenic cells into injured muscle by mediating their interactions with vascular endothelial cells (Corti et al. 2001). With the recent discovery that satellite cells make Sema3A during activation and regeneration (Do et al. 2011; Suzuki et al. 2013; Tatsumi et al. 2009a), it is time to open the window wide for study of this novel aspect of muscle biology and nerve-muscle interaction.

Experiments on HGF-induced Sema3A production by satellite cells on single fibers, classified by immunostaining (Anderson et al. 1988; Merrifield and Atkinson 2000) and structure from mouse and zebrafish could be designed to manipulate satellite cell activation by HGF and nitric oxide, inhibit NOS-1 or block c-met and other cytokine and growth-factor receptors. Time-course studies such as those that tracked repair (Anderson et al. 1998; Grounds 1987; Grounds et al. 1992; Grounds and McGeachie 1987; 1992; McGeachie and Grounds 1987; McIntosh et al. 1998a, b, c; Moor et al. 2000; Smythe et al. 2008) may identify further fiber-type (Suzuki et al. 2013) or differential species effects on activation and Sema3A synthesis. Studies of larval muscle development in various fish species may identify species-specific patterns of HGF and Sema3A signaling that influence receptor clustering at nerve-muscle junctions (Lomo 2003), motor synapse elimination (Busetto et al. 2003; Jansen and Fladby 1990; Missias et al. 1996) and satellite cell positioning near synapses and vessels (Duxson et al. 1986; Duxson and Sheard 1995; Paul et al. 2004). HGF is known to increase blood vessel formation if it is incorporated in local-release acellular grafts after nerve sectioning (Li et al. 2008; Watanabe and Okada 1967). HGF also promotes angiogenesis and muscle-fascicle organization, as studied in a gel matrix around engrafted myoblasts (Barbero et al. 2001), and HGF expression increases during hyperbaric oxygen treatment to improve muscle repair after severe ischemia (Asano et al. 2007). Both findings are consistent with a recent report on the impact of oxygen levels on HGF-promoter activity and satellite cell-mediated angiogenesis (Flann et al. 2014).

Interestingly, acetylcholine receptors cluster on the muscle membrane before, not after developing fibers are innervated, so the pattern of fiber formation may influence the growth of motor axons and synapse formation (Arber et al. 2002). There are also distinct innervation patterns in parasitic and free-living lamprey (Atkins and Pezzementi 1993; Pezzementi and Chatonnet 2010) that have not been studied in relation to satellite cells on those muscle fibers. Such research could pursue the impact of satellite cell-derived factors such as HGF on driving angiogenesis (Ferrara et al. 2003; Williams and Annex 2004) or axon-guidance, and provide an exciting revision of classical thinking that is untested at the level of satellite-cell biology on fibers.

The precise state of satellite cells in a particular model system during assay, is critically important in interpreting findings. Satellite cells can be examined in many model systems: single-cell primary cultures, cell homogenates after expansion or differential plating, myoblasts derived from bulk cultures of mixed muscle cells, fibers cultured either free-floating or attached to a substrate, and in sections of normal, developing or regenerating muscle. In each system, the previous activities of

those cells, the experimental conditions, and the way satellite cells or fibers are isolated as reagents for use in each assay *all* affect research findings and their interpretation. For instance, the mechanism of fiber isolation impacts the activation status of their resident satellite cells. Isolation to study satellite cell motility in response to HGF can effectively use cultures in which the preparation protocol activates the satellite cells (Siegel et al. 2009). By comparison, studies of the transition from quiescence to activation need preparations that avoid shaking or other activating stimuli (Anderson et al. 2012; Wozniak and Anderson 2005). The status of satellite cells, quiescent, activated, mobile and/or proliferative, needs to be verified in a control group under the same conditions (Wozniak et al. 2003; Wozniak and Anderson 2007). An understanding the status of satellite cells, such as their expression of muscle-regulatory genes (therefore activated), genes such as pax7 that specify muscle (Seale et al. 2000) or identify satellite cells (e.g., c-met, CD44, or CD56/NCAM) and their proliferation state, is as critical as experimental design in interpreting *in vivo* experiments. A recent report suggesting that HGF-c-met signaling may not be required for activation or proliferation of satellite cells (Webster and Fan 2013) did not control for a number of important features of experimental design: the interval between tamoxifen-knockdown of c-met gene activity and experimental assay; the possibility of residual c-met in satellite cells that were most stem-like or quiescent; satellite cell activation in different-aged mice at the time of injury; uncertain half-life of c-met; the activity of c-met as an immediate early gene (Wozniak and Anderson 2007; Zhang and Anderson 2014); and the complex implications of satellite-cell heterogeneity. Thus, the conclusions of that report regarding satellite cell activation are not well founded. Satellite cell behaviour following injury, stretching and isolation reflects the events of activation, including entry into the cell cycle and mobilization (Anderson 2000); the many molecular-genetic pathways and epigenetic and feedback mechanisms that regulate activation and/or are impacted by it, are yet to be fully established.

1.7 Conclusion

HGF activates satellite cells; its release from the muscle extracellular matrix is initially mediated via nitric oxide release, calcium-calmodulin signaling and matrix metalloproteinase activity after mechanical or injury-induced signals. Satellite cells respond by proliferating and making HGF and Sema3A; they also underpin the multi-directional signaling process between muscle fibers and proteins and cells in the large volume of tissue outside the endomysium. By using the single-fiber model system, single-cell physiology is accessible to comparative study and can give insights into the integral role of a heterogeneous population of satellite cells. Such research has potential to improve our ability to promote human muscle growth and repair, to prevent conditions such as age-related atrophy and to improve management and treatment of neuromuscular diseases. For animal and comparative physiologists, such basic muscle-cell biology studies could test new approaches to aquaculture and species conservation.

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Chapter 2
Cytokine Mediated Control of Muscle Stem Cell Function

Sophie Joanisse and Gianni Parise

Abstract Skeletal muscle stem cells, known as satellite cells (SC), are an absolute requirement for muscle regeneration and contribute significantly to post-natal muscle growth. This stem cell population is governed by a network of transcription factors collectively referred to as the myogenic regulatory factors. These factors are responsible for the progression of a SC from the quiescent state through activation, proliferation and terminal differentiation in a process referred to as the myogenic programme. At each stage in this process, cytokines and growth factors have been shown to play a role in directing the myogenic response. The myogenic programme is complex and requires input from a host of factors that provide both stimulatory and inhibitory signals that regulate SC. Despite years of work in this field, there remains a paucity of information on the precise factors that drive the myogenic programme. In recent years, factors, such as IL-6, have been shown to be critical factors in promoting SC proliferation. In fact, a complete absence of IL-6 in skeletal muscle substantially impairs muscle SC proliferation. These observations highlight the potential importance of the inflammatory response and the cross-talk between inflammatory cells and SC in promoting muscle repair and growth. This chapter will focus on recent advances in cytokine (and some growth factors) regulation of SC. Work from cell, animal and human models will be discussed.

Keywords Satellite cells • Myogenic programme • Muscle repair • Muscle growth • Inflammation • IL-6

S. Joanisse • G. Parise (✉)
Department of Kinesiology, McMaster University, Hamilton, Canada
e-mail: pariseg@mcmaster.ca

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2.1 Introduction

2.1.1 Skeletal Muscle

Skeletal muscle is a rather unique tissue in that it is composed of multinucleated cells, also known as muscle fibres that run the entire length of a muscle linking tendon to tendon (Fig. 2.1). Working together, muscle fibres are capable of generating significant forces allowing us to initiate and complete all tasks of daily living. Additionally, skeletal muscle fibres can withstand significant mechanical strain during cycles of contraction and relaxation. Vital for the appropriate function of skeletal muscle is that it retains the ability to adapt and remodel in response to physiological cues. For example, one who assumes a regular exercise regimen would expect to undergo adaptation to the exercise as to avoid damage to the muscle upon subsequent exercise bouts. The capacity of skeletal muscle to adapt is determined by both existing nuclei that populate a muscle fibre and by a specialized population of muscle stem cells referred to as satellite cells (SC). The contribution of new nuclei from the SC pool into existing muscle fibres is essential for muscle repair and regeneration and is part of the response to regular exercise.

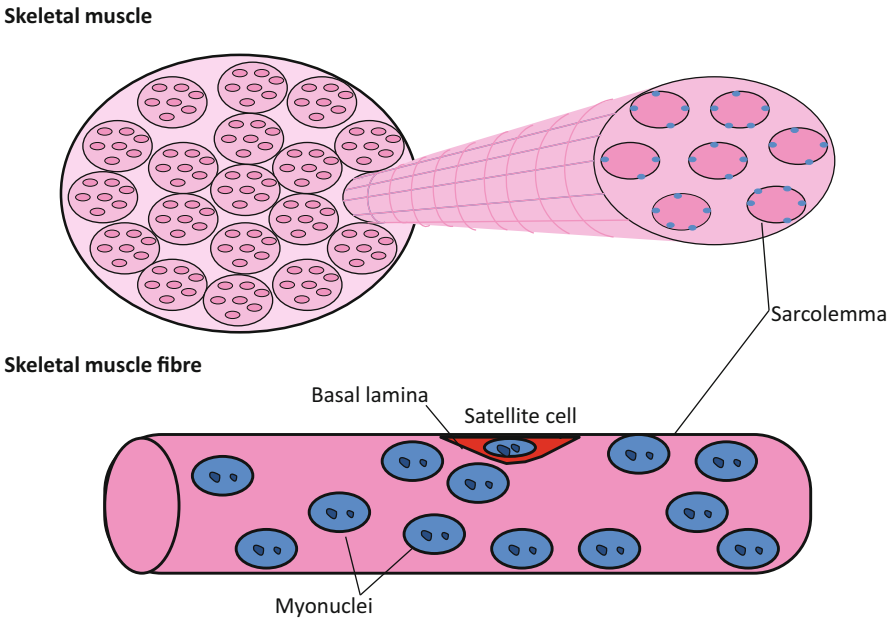

Fig. 2.1 Skeletal muscle is composed of many post-mitotic muscle fibres which are unable to divide. Muscle fibres are terminally differentiated multinucleated cells. Quiescent satellite cells (SC) are mononucleated cells located between the sarcolemma (plasma membrane) and the basal lamina of muscle fibres. When SC become activated they can migrate to the site of injury and contribute to muscle repair

2.2 Satellite Cells

Although skeletal muscle is post-mitotic it retains the ability to remodel and regenerate. This is due to muscle SC, which are mononucleated cells located on the periphery of multinucleated muscle fibres, specifically between the sarcolemma (cell membrane) and the basal lamina (Mauro 1961) (Fig. 2.1). Maintenance of the SC pool is essential in maintaining muscle health throughout adulthood. SC are located in a region referred to as the SC niche (Kuang et al. 2008). The location of the SC within its niche allows for their visualization and quantification in response to various stimuli such as exercise and injury. The muscle fibre to which a SC is associated composes part of the SC niche, and various signals released from the given muscle fibre are able to regulate SC activity (Kuang et al. 2008). Additionally, SC are located in close proximity to capillaries and are influenced by various factors such as, insulin like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF-BB) and vascular endothelial growth factor (VEGF), produced by endothelial cells (Christov et al. 2007). Therefore the microvasculature is also an important part of the SC niche (Fig. 2.2).

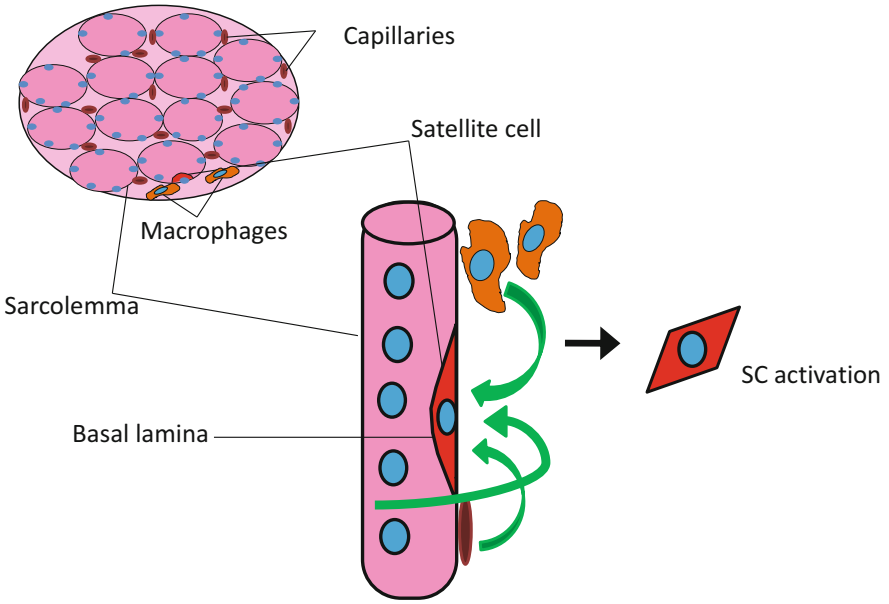

Fig. 2.2 The satellite cell (SC) resides between the sarcolemma and the basal lamina, its anatomical location is referred to as the SC niche. SC are influenced by various signals released by infiltrating immune cells, like macrophages, circulating systemic factors reaching the SC via the microvasculature (capillaries) and also by signals produced by the muscle fibre itself. These signals have the ability to activate quiescent SC and promote muscle repair by mediating SC proliferation and differentiation

SC have been implicated in playing an important role in growth, regeneration and have even been identified as being affected in several disease states. The following section will discuss the various roles and function of SC.

2.3 The Role of Satellite Cells

2.3.1 Muscle Regeneration and Growth

The importance of SC in early development have been highlighted by Pax7 deficient mouse models. Pax7 is a paired box transcription factor expressed by quiescent and activated SC and is widely used as a marker of SC in skeletal muscle (McKay et al. 2010). Mice with a targeted null mutation of Pax7 (Pax7 -/-), generally lack SC and fail to thrive with only a small number living to adulthood, while most are not post-natally viable (Mansouri et al. 1996; Oustanina et al. 2004). Additionally, these mice were smaller than heterozygous littermates, had reduced muscle mass and contained more small fibres (Mansouri et al. 1996; Oustanina et al. 2004). In order to study the necessity of SC in adult skeletal muscle, several groups explored other models allowing mice to thrive into adulthood while rendering the SC pool non-functional. Irradiation of mouse skeletal muscle inhibits SC activity and any other mononuclear dividing cell resident in skeletal muscle. Irradiation studies have explored the potential role of SC in mediating gains in muscle mass following hypertrophic stimuli by essentially rendering the SC non-functional. Several studies using irradiated rodent skeletal muscle demonstrated that the tissue was unable to hypertrophy even when challenged with an overload stimulus which lead to significant hypertrophy in control animals (Adams et al. 2002; Rosenblatt and Parry 1992; Rosenblatt et al. 1994). Hypertrophy was inhibited following 3 months of functional overload in rats suggesting that SC play a crucial role in mediating skeletal muscle hypertrophy (Adams et al. 2002).

Although irradiation studies have highlighted the importance of SC in the hypertrophic response more recent work suggests that SC may be redundant in mediating gains in muscle mass. A novel mouse model was developed in which there is near complete ablation of SC in mature mouse skeletal muscle. Two weeks of functional overload led to hypertrophy even in SC depleted skeletal muscle (McCarthy et al. 2011).

Although rodent models have provided compelling evidence that SC are not necessary in mediating skeletal muscle hypertrophy, a concomitant increase in muscle fibre cross sectional area (CSA) and expansion of the SC pool has consistently been reported in humans (Bellamy et al. 2014; Kadi and Thornell 2000; Petrella et al. 2006, 2008). Therefore under normal physiological conditions SC do seem to contribute to muscle hypertrophy; this is evident in both rodent and human models.

The contribution of SC in mediating increases of muscle mass following hypertrophic stimuli in rodents is debatable but, their presence is necessary in skeletal muscle regeneration. Following traumatic injury, skeletal muscle is able to repair and regenerate; this process is possible due to appropriate function of SC. Following injury to skeletal muscle, immune cells, namely neutrophils and macrophages, infiltrate the tissue and secrete factors promoting SC activation and proliferation in order to initiate the regenerative process (Deng et al. 2012). The expression of factors such as leukemia inhibitory factor (LIF) and IGF-1 are up-regulated following injury and their presence has been shown to stimulate SC proliferation (Edwall et al. 1989; Kurek et al. 1996). FGF-6 has also been demonstrated to play a role in mediating SC activity during regeneration. Muscle regeneration is severely impaired in mutant FGF-6 mice (FGF-6 -/-) and is accompanied by a reduced number of proliferating and differentiating SC (Floss et al. 1997). HGF also plays an important role in SC proliferation as evidenced by *in vitro* experiments in which cultured rat SC treated with HGF increased proliferation in a dose dependent manner (Allen et al. 1995). *In vivo* models have also demonstrated a role for HGF in SC proliferation, as evidenced by increased expression in skeletal muscle following stretch-induced activation of SC (Tatsumi et al. 2006). Interestingly, HGF is a potent regulator of SC and is involved in more than just inducing proliferation. In fact, HGF has been shown to be a potent chemoattractant in myoblast cultures (Bischoff 1997). This is a rather important observation since migration of SC to the sites of injury is an absolute necessity in promoting muscle repair.

Given the extent of inflammation following injury it has been hypothesized that cross-talk between inflammatory cells and SC might be an important event in promoting muscle repair. Cytokines released from macrophages have been shown to promote SC proliferation (Cantini et al. 1994). Gene expression of interleukin-6 (IL-6), a pro-inflammatory cytokine, is increased following injury in mouse skeletal muscle (Kurek et al. 1996), and rodent studies have demonstrated a direct effect of IL-6 on SC, which strongly supports a role for cytokines in mediating the SC response to injury.

When skeletal muscle injury is induced via injection of toxin into muscle devoid of SC, muscle regeneration is impaired (Lepper et al. 2011; McCarthy et al. 2011; Sambasivan et al. 2011). Furthermore, the transplantation of SC into Pax7-/- mouse muscle rescues the ability of the muscle to regenerate following injury (Sambasivan et al. 2011). Collectively, these findings provide evidence of the essential role that SC play in mediating skeletal muscle regeneration. There is also evidence to suggest a role for SC in muscle repair/adaptation following exercise. Following 2 weeks of exercise, SC deficient mice experienced a loss of muscle fibres, a decrease in muscle mass and an increase in inflammation and fat infiltration into the muscle. The exercise program did not negatively affect muscle of control animals who had an intact and functional SC pool (Sambasivan et al. 2011). This suggests that SC are necessary in maintaining muscle health in more physiologically relevant injury or exercise models (Sambasivan et al. 2011).

2.3.2 Exercise and Adaptation

Although skeletal muscle regeneration has been a primary model used to understand SC biology, it is also well established that SC are responsive to exercise. This suggests that exercise induced adaptation is, at least in part, regulated through SC activity. As already mentioned in this chapter mice lacking SC were not able to repair muscle following damage induced via exercise (Sambasivan et al. 2011). In humans, following damage inducing exercise there is a significant expansion of the SC pool (Crameri et al. 2004; McKay et al. 2010; O'Reilly et al. 2008; Toth et al. 2011). Additionally, following weight bearing physical activity SC become activated (Crameri et al. 2004; Darr and Schultz 1987) and several studies have highlighted the association of SC pool expansion and hypertrophy (Petrella et al. 2006, 2008; Sinha-Hikim et al. 2003). Resistance training leading to increases in muscle mass is often accompanied by a concomitant increase in the number of myonuclei per fibre, this increase is mediated by the contribution of new nuclei via SC to fibres undergoing hypertrophy (Kadi and Thornell 2000; Petrella et al. 2006, 2008).

The theory of the myonuclear domain highlights that each nuclei governs a defined volume of cytoplasm within a muscle fibre and, when a given cytoplasmic volume expands beyond a certain threshold the addition of new nuclei, via the SC, is necessary in order to support growth (Allen et al. 1999). The maintenance of the myonuclear domain following hypertrophic conditions highlights a role for SC in exercise. During responses to hypertrophic stimuli, SC activate, proliferate and fuse to existing fibres donating their nuclei in order to support muscle fibre growth – a process very similar to muscle regeneration and maintenance. Recently, it has been demonstrated that there is a positive relationship between an acute increase in circulating IL-6 following a bout of resistance exercise and the extent of muscle hypertrophy following resistance training (Mitchell et al. 2013), suggesting that the acute increase in circulating cytokines following exercise may play an important role in activating SC and contributing to muscle growth.

2.3.3 Muscle Pathology

Various myopathies are characterized by a compromised cytoarchitechture of the muscle fibre. Myopathic skeletal muscle is constantly undergoing cycles of degeneration-regeneration. Duchenne muscular dystrophy (DMD) is one of the most common myopathies and is characterized by the absence the cytoskeletal protein dystrophin leaving muscle fibres in a fragile state. SC from DMD patients are required to undergo a greater number of cell divisions compared to those in healthy muscle. For this reason the SC pool becomes exhausted as the SC are constantly mediating muscle regeneration (Cossu and Mavilio 2000; Hawke and Garry 2001).

Cerebral palsy (CP) is a common movement disorder in children. Patients with CP have a restricted range of motion around joints thus limiting their mobility

(Smith et al. 2013). CP is characterized by impaired muscle growth and reduced CSA of skeletal muscle. Further analysis of skeletal muscle of CP patients reveals that they have a reduced number of SC compared to healthy controls (Smith et al. 2013). The reduced pool of SC in skeletal muscle of CP patients may contribute to a hallmark characteristic of CP - reduced muscle mass.

Although not a disease per se, aging is accompanied by alterations in muscle function and structure. Aging is associated with a reduction in CSA of type II muscle fibres which is accompanied by a reduction in type II associated SC (McKay et al. 2012). The acute response of SC from aged skeletal muscle to respond to a bout of resistance exercise is also impaired (McKay et al. 2013, 2012). The number of proliferating and differentiating SC following exercise is reduced in aging muscle which may contribute to the reduced muscle mass associated with aging (McKay et al. 2012). Additionally the response of SC to acute exercise in aged adults seems to be dysregulated. The dysregulation associated with aging is further supported by the fact that there is a reduction in the number of SC expressing IL-6 in old adults compared to young following exercise (McKay et al. 2013). Collectively, although still debatable, the evidence suggests that an age-related loss in skeletal muscle may be associated with dysfunction of the SC population.

Whether related to myopathy, muscle regeneration following injury, or exercise induced adaptation SC appear to play a critical role in the maintenance of muscle health. However, the precise factors responsible for SC regulation remain somewhat less clear.

2.4 Myogenic Regulatory Factors

Although normally quiescent, the activation of SC and their progression through the myogenic programme is essential in muscle repair and growth. This process is governed by a transcriptional network collectively referred to as the myogenic regulatory factors (MRF) (Holterman and Rudnicki 2005; Le Grand and Rudnicki 2007; Rudnicki et al. 2008; Seale and Rudnicki 2000; Ten Broek et al. 2010). Four MRF are involved in myogenesis Myf5, MyoD, MRF4 and myogenin. During SC proliferation both Myf5 and MyoD are synergistically expressed, as observed by an increase in the proportion of SC co-localized with both MRF during muscle regeneration in rodents (Cooper et al. 1999). Additionally *in vitro* studies have demonstrated that very few myoblasts from MyoD deficient rodents fail to express myogenin indicating impaired differentiation potential (Yablonka-Reuveni et al. 1999) (Fig. 2.3). Following SC activation in humans there is an increase in the proportion of SC expressing IL-6 during the proliferative phase (McKay et al. 2009). Additionally, the mRNA expression of mechano growth factor (MGF) in whole muscle, a splice variant of IGF-1, is correlated with Myf5 mRNA expression in whole muscle following SC activation in humans (McKay et al. 2008). The down-regulation of Myf5 followed by MyoD and the subsequent up-reguation of MRF4 and myogenin induces SC differentiation (Kassar-Duchossoy et al. 2004). In human

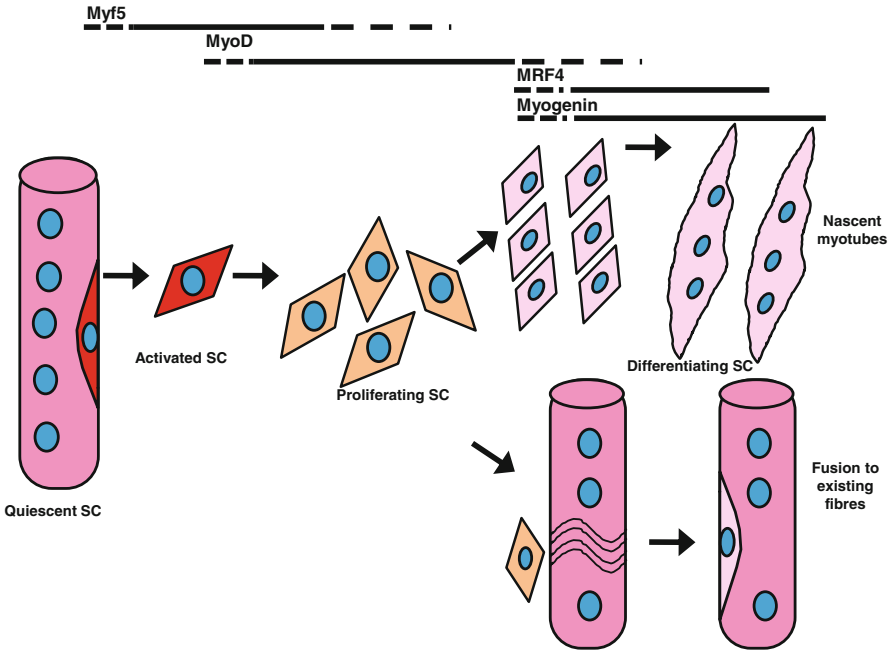

Fig. 2.3 Myogenic regulatory factors (MRF) regulate the progression of the SC through the myogenic programme. SC will become activated following various stimuli such as weight baring physical activity and injury. Myf5 is the first MRF to be expressed, it occurs shortly after activation and indicates myogenic commitment of the SC. Proliferating SC will begin to express MyoD and down-regulate Myf5 expression. MyoD expression will persist until early differentiation of the SC. The expression of MyoD becomes down-regulated during late differentiation where the expression of both MRF4 and myogenin are observed

skeletal muscle, gene expression of MRF4 a marker of terminal differentiation in SC is correlated to the gene expression of both IGF-1Ea and b in whole muscle following SC activation (McKay et al. 2008). More specifically the proportion of SC co-localized with IGF-1 in muscle cross sections is significantly increased 72 h following SC activation via resistance exercise in humans (Grubb et al. 2014). Together this implicates various isoforms of IGF-1 in specific phases of the myogenic programme. The upstream factors regulating up- and down-regulation of the MRF are relatively unknown but specific cytokines, such as IL-6, appear closely related to MRF gene expression suggesting that cytokines and other growth factors may influence SC progression through the myogenic programme.

Notch signaling also plays an important role in SC regulation. It was originally demonstrated to play an important role in SC activation and proliferation (Conboy and Rando 2002; Luo et al. 2005) and more recently was shown to play an important role in maintaining quiescence in the SC pool (Bjornson et al. 2012). A reduction in Notch signaling in necessary in order to promote SC differentiation When SC differentiation is induced there is a reduction in Notch signaling and an increase in

Wnt signaling. The coordination between Notch and Wnt singling results in the regulation of various MRF also contributing to the progression of a SC through the myogenic programme.

2.5 Interleukins and Their Role in Muscle Growth and Regeneration

Although interleukins normally act on immune cells to regulate immune and inflammatory responses, it is now becoming clear that at least a subset of interleukins can substantially influence SC function. Interleukins and their traditional role as mediators of the immune response are now implicated to play a role in skeletal muscle repair and regeneration.

There is now a critical mass of evidence in humans suggesting that cytokines, such as the interleukin family (IL- 1 to 35) (Della Gatta et al. 2014), are responsive to exercise. The IL-6 response to exercise has been most well characterized with a significant increase in muscle IL-6 expression (Febbraio and Pedersen 2002). There is also a very substantial increase in plasma IL-6 levels following exercise which is dependent upon the intensity and duration (Pedersen 2007). In addition to IL-6 other interleukins are also associated with exercise. Following 6 weeks of resistance training there was an increase in anti-inflammatory cytokines IL-4 and IL-13 gene expression in skeletal muscle (Prokopchuk et al. 2007). Acute bouts of aerobic, concurrent and resistance exercise have also been shown to increase IL-1 and IL-6 mRNA expression in skeletal muscle (Donges et al. 2014). Protein expression of IL-6, IL-8 and IL-10 are all increased acutely in skeletal muscle following a bout of resistance exercise. IL-4 protein expression is up-regulated acutely following resistance exercise but only following 12 weeks of resistance exercise training, protein expression failed to increase when subjects were untrained (Della Gatta et al. 2014). IL-6 mRNA expression was increased in skeletal muscle following a resistance training program that led to significant hypertrophy in rats (Begue et al. 2013). The acute increases in cytokines as described above following exercise are likely important in mediating skeletal muscle adaptation as described previously SC also become activated acutely following exercise. Therefore, the acute increases in cytokines are likely important in regulating SC activity.

IL-6 has also been shown to co-localize with SC in response to resistance exercise. Following resistance exercise in humans and rodents there is an increase in the proportion of the SC pool that is positive for IL-6 protein (Begue et al. 2013; McKay et al. 2009). More specifically, following resistance exercise STAT3 signals to the SC via IL-6 likely mediating SC proliferation (Begue et al. 2013; Toth et al. 2011). Although these results do not directly link interleukins to muscle growth their response to hypertrophic stimuli, like resistance exercise, suggests a role in mediating gains in muscle mass.

As described previously growth factors and cytokines govern specific phases of the myogenic programme. Interleukins also play a role in muscle growth and repair as evidenced by their necessity in mediating both of these active processes in skeletal muscle. Many rodent models utilizing ablation of specific interleukins such as IL-4 (Horsley et al. 2003), IL-6 (Serrano et al. 2008) and IL-10 (Deng et al. 2012) all demonstrate impaired muscle growth following injury (IL-4) or during periods of overload-induced hypertrophy (IL-6, IL-10). Additionally, when recombinant IL-15 is administered to mice an increase in soleus muscle weight results as compared to controls (Carbó et al. 2001). *In vitro* models have also demonstrated a potential role for IL-15 in mediating skeletal muscle hypertrophy; over-expression of IL-15 in C2C12 cells as well as in human myoblasts leads to a greater accumulation of myosin heavy chain protein compared to control (Furmanczyk and Quinn 2003; Quinn et al. 2002).

Infiltrating macrophages are reduced in IL-6 deficient mice following injury which impacts SC proliferation thereby impairing muscle regeneration (Zhang et al. 2013). IL-6 acts via STAT3 in macrophages to promote the production of other cytokines which can also impact muscle regeneration by stimulating SC proliferation (Zhang et al. 2013). Various cytokines are able to influence muscle growth and regeneration; both processes that require the progression of the SC through the myogenic programme.

2.6 Interleukins and Myogenesis

2.6.1 Proliferation

As mentioned above interleukins play an important role in myogenesis (Della Gatta et al. 2014). IL-4 is an anti-inflammatory cytokine and its role in the immune system has been well described (Brown 2008). Some work suggests that IL-4 may also play an important role in myogenesis. Serum response factor (SRF) is a transcription factor that plays a role in SC proliferation. When SRF expression was eliminated SC proliferation and myonuclear accretion was impaired in mouse skeletal muscle undergoing compensatory hypertrophy (Guerci et al. 2012). Specifically, SRF positively regulates IL-4 expression via Cox2 and IL-4 expression is necessary for the SRF-controlled fusion of myoblasts (Guerci et al. 2012).

IL-6 is a cytokine with various immune functions (Kishimoto 2005) however some evidence suggest that it also plays an important role in myogenesis. Compensatory hypertrophy is impaired in IL-6 deficient rodents, this impairment is also accompanied by an impairment in SC activation (Serrano et al. 2008). The necessity of IL-6 signaling via STAT3 for myoblast proliferation was also confirmed *in vivo* (Serrano et al. 2008). Treatment of rat SC cultures with IL-6 resulted in an increase in proliferation, the number of SC staining for phosphorylated-STAT3 (p-STAT3) and MyoD/cyclin D1 also increased (Kurosaka and Machida 2013). These results are consistent with studies exploring the role of IL-6 in humans.

An increase in the number of SC expressing IL-6, 4 and 24 h following damaging eccentric exercise has been reported (McKay et al. 2009). The increase in SC expressing IL-6 is accompanied by a significant increase in the total number of SC 24 h following damage implying a role for IL-6 in mediating SC proliferation in humans (McKay et al. 2009). It also appears that IL-6 signals through STAT3 in humans to promote SC proliferation as the number of SC expressing p-STAT3 following damaging exercise is also increased (Toth et al. 2011).

2.6.2 Differentiation

An indispensable component for successful muscle regeneration is the recruitment of SC to the site of damage and subsequent fusion to existing myotubes or with other myoblasts to form new myotubes. In addition to mediating SC proliferation IL-4 has also been implicated in SC differentiation. IL-4 deficient mice have a reduced muscle fibre cross sectional area compared to their wild type counterparts (Horsley et al. 2003) implying it has a role in muscle growth. More specifically the authors demonstrate that IL-4 acts in promoting the fusion of myoblasts to nascent myotubes in periods of growth and regeneration (Horsley et al. 2003). In human myoblasts an increase in IL-4 expression is observed during differentiation (Lafreniere et al. 2006). In accordance with the rodent literature IL-4 has been shown to be essential in myonuclear accretion in differentiating human myoblasts. When cells are treated with an IL-4 blocking antibody myonuclear accretion is reduced suggesting a role in muscle growth (Lafreniere et al. 2006). When differentiation is induced in myoblasts prepared from chick embryos an increase in IL-4 concentration is observed in the conditioned media (Possidonio et al. 2011). Additionally, myotubes treated with recombinant IL-4 following induction of differentiation had greater myotube thickness compared to untreated cells, again implicating a role for IL-4 in differentiation (Possidonio et al. 2011). Additionally, IL-13, another anti-inflammatory cytokine of the interleukin family, is secreted in response to insulin like growth factor-1 (IGF-1) treatment, via nuclear factor of activated T-cells, cytoplasmic calcineurin-dependent 2 (NFATc2), and stimulates fusion of myoblasts (Jacquemin et al. 2007). Thus, anti-inflammatory interleukins −4 and −13 appear to play a role in SC differentiation.

In addition to playing a role in SC differentiation IL-4 has also been shown to increase myoblast migration both *in vitro* and *in vivo*. When human myoblasts are incubated with IL-4 migration is increased. This has also been confirmed in an *in vitro* model where human myoblasts were co-injected with IL-4 into mouse skeletal muscle and showed a greater migration distance than myoblasts transplanted without IL-4 (Lafreniere et al. 2006).

Not only does IL-6 play a significant role in inducing proliferation of SC, evidence also suggests a role for IL-6 in SC differentiation. Differentiating C2C12 myoblasts have an increased IL-6 expression. Ablation of IL-6 expression led to a decrease in differentiation while over-expression of IL-6 increased differentiation

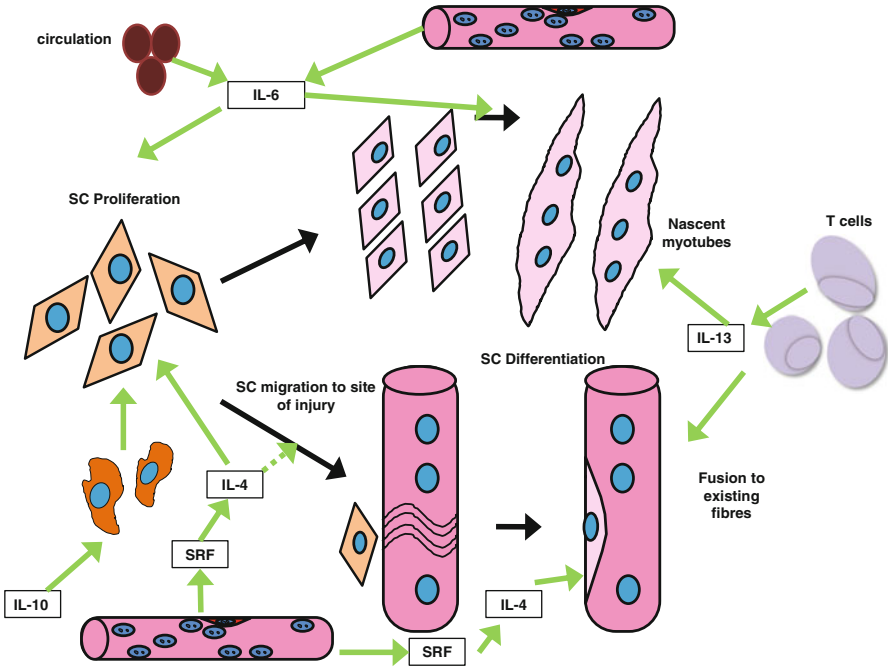

Fig. 2.4 Various interleukins play in different phases of the myogenic programme. IL-4, IL-6 and IL-10 are associated with SC proliferation. Additionally IL-4 and IL-6 also play a role in SC differentiation as does IL-13. Following exercise there is an acute increase in plasma and muscle expression of IL-6, IL-6 has been associated with both SC proliferation and differentiation. Following acute exercise there is an increase in IL-10, when macrophages are stimulated with IL-10 SC proliferation is increased. Similarly to IL-6, IL-4 is involved in both SC proliferation and differentiation, its expression is increased in muscle following exercise training. The secretion of IL-13 by activated T-cells stimulated SC fusion

(Baeza-Raja and Muñoz-Cánoves 2004). Additionally myoblasts isolated from IL-6 deficient mice had a decreased propensity for differentiation and fusion (Hoene et al. 2013). Although IL-6 has an important role in promoting differentiation the activation of its downstream signalling molecule STAT3 is also necessary to induce differentiation of myoblasts (Hoene et al. 2013). IL-6 is able to activate various signaling cascades ultimately leading to different outcomes, either proliferation or differentiation. Although this notion seems contradictory an increase in IL-6 can affect various phases of myogenesis; it may be possible that negative feedback regulators of IL-6, like suppressor of cytokine signalling (SOCS)-3, also influence its effect in either proliferation or differentiation. IL-6 plays an important role in myoblast proliferation and differentiation and is therefore a key cytokine in driving the myogenic programme. The role of interleukins in the myogenic programme is summarized in Fig. 2.4.

2.6.3 Interleukins Derived Macrophages and Myogenesis

Certain interleukins have the ability to act directly upon SC but are also able to act on other cell types residing within skeletal muscle, such as macrophages, to indirectly stimulate SC. Mice deficient in IL-10, an anti-inflammatory cytokine, have impaired muscle regeneration (Deng et al. 2012). When mouse myoblasts were directly stimulated with IL-10, proliferation was not affected. However, when myoblasts were co-cultured with M2 macrophages, activated with IL-10, proliferation was increased (Deng et al. 2012). Together this highlights that IL-10 plays an indirect role in myogenesis by inducing macrophage activity.

Although IL-6 production by skeletal muscle is greatly increased following exercise and its production plays a role in SC regulation (Steensberg et al. 2000); the production of IL-6 by inflammatory cells also mediates many processes throughout the body. Mice deficient in RING finger protein 13 (RNF13) exhibit improved muscle regeneration compared to wild type following muscle injury induced via toxin injection due to increase in SC proliferation (Meng et al. 2014). It was demonstrated that the increase in SC proliferation was due to the release of IL-4/IL-6 from macrophages rather than the skeletal muscle itself (Meng et al. 2014). Together these results highlight the importance of IL-6 in mediating SC proliferation whether it be produced by the muscle itself or by various cell types resident in the muscle.

2.7 Conclusion

Undoubtedly, muscle stem cells play a critical role in skeletal muscle health. Their contribution to post natal growth, muscle regeneration following trauma and muscle adaption following exercise is well documented. Although the transcriptional networks that drive muscle stem cell from quiescence through proliferation and into differentiation are relatively well described there is a lack of information regarding the upstream signals that induce the myogenic programme. Over the years great strides have been made to identify growth factors and other cytokines that contribute to the regulation of muscle stem cells. There is much research that still needs to be completed in order to elucidate their specific roles in SC regulation. It is, however, becoming more clear that growth factors, interleukins and other cytokines, whether derived from inflammatory cells or from skeletal muscle itself have a an important role to play in this process (Tables 2.1 and 2.2).

Table 2.1 Interleukins involved in exercise

Interleukin	Type	Tissue	Increased expression
IL-1	Pro-inflammatory	Muscle	Acute increase following exercise Donges et al. (2014).
IL-4	Anti-inflammatory	Muscle	Increase following training Prokopchuk et al. (2007)
			Essential in myonuclear accretion Guerci et al. (2012)
IL-6	Anti/pro-inflammatory	Plasma, muscle	Acute increase following exercise Febbraio and Pedersen (2002), Pedersen (2007), McKay et al. (2009), Toth et al. (2011), Begue et al. (2013), Donges et al. (2014)
			Increase following training Begue et al. (2013)
IL-8	Anti-inflammatory	Muscle	Acute increase following exercise Della Gatta et al. (2014)
IL-10	Anti-inflammatory	Muscle	Acute increase following exercise Della Gatta et al. (2014)
IL-13	Anti-inflammatory	Muscle	Increase following training Prokopchuk et al. (2007)
IL-15	Pro-inflammatory	N/A	Increase in muscle weight when recombinant IL-15 administered to mice Carbó et al. (2001)

Summary of interleukins involved in muscle growth, the tissue in which the interleukin was measured and when its expression was increased

Table 2.2 Interleukins and myogenesis

Interleukin	Type	Stage of myogenesis	Effect
IL-4	Anti-inflammatory	Proliferation, differentiation, migration	Essential for SRF-controlled fusion of myoblasts Guerci et al. (2012)
			Increased expression during myoblast differentiation Lafreniere et al. (2006)
			Incubation of myoblasts with IL-4 increases migration Lafreniere et al. (2006)
IL-6	Anti/pro-inflammatory	Proliferation, differentiation	IL-6 signals via STAT3 during proliferation and differentiation Serrano et al. (2008), McKay et al. (2009), Toth et al. (2011), Hoene et al. (2013), Kurosaka and Machida (2013)
IL-10	Anti-inflammatory	Proliferation	Macrophages activated via IL-10 increase myoblast proliferation Deng et al. (2012)
IL-13	Anti-inflammatory	Differentiation	Secreted by activated T-cells to stimulate myoblast fusion Jacquemin et al. (2007)

Summary of interleukins involved in myogenesis, which phase of the myogenic programme they play a role and their effect

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Chapter 3
The Role of Leukemia Inhibitory Factor Receptor Signaling in Skeletal Muscle Growth, Injury and Disease

Liam C. Hunt and Jason White

Abstract Cytokines are an incredibly diverse group of secreted proteins with equally diverse functions. The actions of cytokines are mediated by the unique and sometimes overlapping receptors to which the soluble ligands bind. Classified within the interleukin-6 family of cytokines are leukemia inhibitory factor (LIF), oncostatin-M (OSM), cardiotrophin-1 (CT-1) and ciliary neurotrophic factor (CNTF). These cytokines all bind to the leukemia inhibitory factor receptor (LIFR) and gp130, and in some cases an additional receptor subunit, leading to activation of downstream kinases and transcriptional activators. LIFR is expressed on a broad range of cell types and can generate pleiotropic effects. In the context of skeletal muscle physiology, these cytokines have been shown to exert effects on motor neurons, inflammatory and muscle cells. From isolated cells through to whole organisms, manipulations of LIFR signaling cytokines have a wide range of outcomes influencing muscle cell growth, myogenic differentiation, response to exercise, metabolism, neural innervation and recruitment of inflammatory cells to sites of muscle injury. This article will discuss the shared and distinct processes that LIFR cytokines regulate in a variety of experimental models with the common theme of skeletal muscle physiology.

Keywords Leukemia inhibitory factor • Leukemia inhibitory factor receptor • Oncostatin M • Cardiotrophin-1 • Ciliary neutrophic factor • Skeletal muscle

L.C. Hunt (✉)
Murdoch Childrens Research Institute, Royal Childrens Hospital, Parkville, VIC, Australia
e-mail: Liam.Hunt@stjude.org

J. White
Faculty of Veterinary Science, University of Melbourne,
Flemington Road, Parkville, VIC 3010, Australia

Murdoch Childrens Research Institute, Royal Childrens Hospital, Parkville, VIC, Australia

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3.1 Introduction

Leukemia inhibitory factor (LIF) is the archetypal cytokine of the leukemia inhibitor factor receptor (LIFR) signaling family. The gene for LIF was first cloned from a cDNA library with the help of partial peptide sequence from a protein purified with M1 myeloid leukemia inhibitory properties (Gearing et al. 1987). Shortly thereafter, oncostatin M (OSM) was cloned in a similar fashion (Malik et al. 1989). Both genes show significant similarity (likely due to branching from a conserved ancestral gene), were placed in the same cytokine family and share the same receptor (Gearing et al. 1991, 1992; Rose and Bruce 1991; Gearing and Bruce 1992). At a similar time, ciliary neurotrophic factor (CNTF) was cloned and found to share comparable neurotrophic properties with LIF and also utilize the same receptor components (Lin et al. 1989; Davis et al. 1993a). The receptor components identified capable of binding LIF, OSM and CNTF and generating intracellular signaling were the beta receptor subunits LIF receptor (LIFR) and gp130, the interleukin-6 (IL-6) transducing receptor subunit.

Formation of the receptor complex leads to phosphorylation of janus kinases (JAKs) associated with the cytoplasmic domain of the receptor and allows for intracellular communication (Heinrich et al. 1998). Signal transducers and activators of signaling (STATs) are recruited and phosphorylated forming homodimers that translocate into the nucleus and initiate gene transcription. In addition, cytokine signaling may activate mitogen activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways through JAKs or the tyrosine phosphatase SHP2. In this way LIFR signaling can induce a wide range of effects within a cell depending on the particular machinery possessed by that cell to process the downstream signal.

After the elucidation of these three similar cytokines (LIF, OSM and CNTF) and the mechanisms by which they initiate intracellular signaling, there have been additional LIFR signaling ligands and receptor components identified. CNTF, in addition to utilizing LIFR and gp130 was found to recruit a third receptor subunit referred to as CNTFR (Davis et al. 1993b). Cardiotrophin-1 (CT-1) was found to induce hypertrophy of cardiac myocytes *in vitro* (Pennica et al. 1995a) and compete for the same receptor as LIF as well as induce similar effects including inhibition of M1 myeloid growth and neurotrophic effects (Pennica et al. 1995b).

A similar signaling mechanism for CT-1 as CNTF was observed. However, the third component of the tripartite receptor has yet to be identified (Robledo et al. 1997). OSM has also been shown to utilize a distinct receptor complex, the OSM receptor (OSMR) and gp130 (Mosley et al. 1996). Although reports have suggested that OSMR/gp130 may be specific to mouse OSM and may not bind LIFR/gp130 unlike human OSM (Ichihara et al. 1997), there is evidence that OSM can induce effects in OSMR deficient mice via LIFR indicating that usage of both receptor complexes is likely to occur in both species (Walker et al. 2010). Finally a novel ligand, cardiotrophin-like cytokine (CLC) also binds CNTFR and gp130 along with the soluble receptor cytokine-life factor (CLF) (Elson et al. 2000), because these

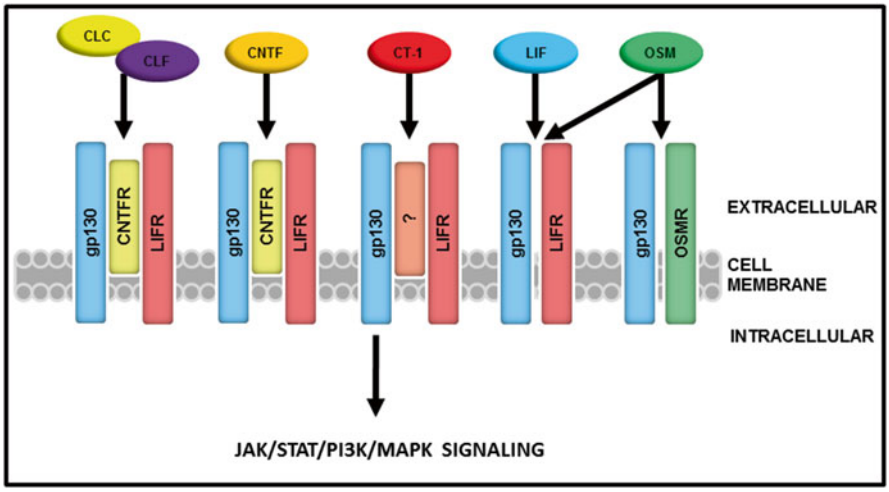

Fig. 3.1 Illustration of LIF receptor signaling complexes and ligands utilized to initiate intracellular signaling

ligands have more recently been dicovered there is not much known about them in the context of skeletal muscle. Figure 3.1 illustrates the different ligand and receptor components for this group of cytokines.

This article will discuss how the unique group of LIFR cytokines can affect muscle physiology. The potential cellular sources and stimuli which give rise to these cytokines and the direct and indirect effects on muscle cells in conditions of normal function, growth and wasting are highlighted. This discussion emphasises the broad range of effects that these cytokines can produce and suggests potential for treating disorders involving muscle degeneration, such as Duchenne Muscular Dystrophy.

3.2 Varied Sources of LIFR Cytokines Regulating Muscle and Peripheral Homeostasis

Cytokines that influence muscle directly can be produced from within the muscle cells as an autocrine, by neighboring cells within the muscle tissue (which includes non-muscle cells such as adipose, fibroblasts, endothelial cells and more) as a paracrine or from a distant tissue which then circulates as an endocrine. In addition, cytokines may exert functions by binding other cells or tissues and affecting muscle cells indirectly. The sources of cells which can produce LIFR cytokines that are capable of exerting effects on muscle appear to include most of these possibilities; one such example is that macrophages can produce LIF and this acts as a chemotactic factor for myogenic precursor cells (Robertson et al. 1993). We observed LIF

immunoreactivity in injured muscle deriving from mononucleated cells, which could include inflammatory cells such as macrophages or neutrophils (Hunt et al. 2013). Nerve injury increases LIF expression and therefore the neurons or glial Schwann cells that comprise the nerve could be a source of LIF that can regulate muscle homeostasis (Ito et al. 1998).

Cultured myotubes also express LIF, which is increased with mimetics of muscle stimulation and activity including electrical stimulation and calcium influx by an ionophore; moreover human muscle also expresses increased amounts of LIF mRNA shortly after (6 h) a bout of exercise (Broholm et al. 2008, 2011). The term myokine was coined to describe peptide factors produced by skeletal muscle in response to stimuli (for example exercise or nutrients) that can act as an autocrine, paracrine or endocrine. Fulfilling this definition, LIF and other LIFR cytokines are indeed myokines. Whilst monitoring the administration of a LIF mutant, we detected a significant increase in circulating endogenous LIF in the control group shortly after muscle injury (1 day) (Hunt et al. 2013). This suggested that muscle damage may be one such stimulus, in addition to the aforementioned muscle stimulation or exercise, which induces LIF and myokine expression capable of acting as an endocrine. OSM is also a verified myokine (Hojman et al. 2011); cultured myotubes that were electrically stimulated secreted OSM and it is up-regulated in muscles of mice after exercise and increased in circulation too. The production of endocrine factors from muscle following exercise has become an exciting area of development recently with discoveries indicating myokines can have profound impacts on systemic metabolism (Boström et al. 2012). Many of the reported health benefits of exercise (Pedersen and Fischer 2007) could indeed be mediated by the effects of myokines on peripheral tissues; for example OSM was shown to be a component of serum from exercised mice that could block the growth of breast cancer cells (Hojman et al. 2011). Both the sources and targets of LIFR cytokines may be varied and this article will discuss the effects of these cytokines which can mediate muscle cell growth and homeostasis via direct and indirect effects.

3.3 LIFR Is Involved in Muscle Development Predominantly Via Neurotrophic Effects

The individual cytokines that signal through LIFR may be partially redundant during development because individual deficiency of LIF, CT-1, CNTF and OSM does not affect viability and causes only minimal defects in mice, yet combining deficiency of multiple genes can be severe (Holtmann et al. 2005; Morikawa et al. 2004), whilst loss of LIFR causes perinatal lethality with massive loss of motor neurons (Ware et al. 1995; Li et al. 1995). This indicates an essential role for this group of cytokines as a whole in development. The majority of developmental phenotypes reported in mice deficient singly for LIF, CT-1, CNTF and OSM demonstrate defects in neuronal development. In particular LIF, CT-1 and

CNTF are deficient in motor neuron development whilst OSM deficient mice have nociceptive impairment.

LIF mRNA is present at high levels in the muscle of embryonic mice at E17 but decreases dramatically by birth (Kwon et al. 1995). E17 coincides with the end of secondary myogenesis, which is when all myofibres that would be present in postnatal life have been formed, whilst decreases in LIF expression by birth coincide with the time that neonatal synaptic withdrawal occurs. Treatment of neonatal mouse tensor fasciae latae limb muscles with recombinant LIF caused a transient delay in synaptic withdrawal; that is the maturation of the motor units innervating muscle. LIF deficient mice also have reduced area of the postsynaptic motor end plate. In combination with CNTF and CT-1, LIF deficiency reduces motor function, whilst age dependent loss of function is most pronounced with LIF deficiency alone (Holtmann et al. 2005), suggesting a requirement for LIF in the maintenance of the neuromuscular junction both at the presynaptic and postsynaptic ends. Although LIF may be important for development and maintenance of motor neurons there is little evidence that LIF is directly required for muscle formation during development. Muscles from LIF deficient mice appear phenotypically normal unless stressed (Kurek et al. 1997). In fact, no defects in muscle formation and function in young mice without a stressor have been reported for deficiency of CT-1, CNTF and LIF in studies examining muscle histologically (Holtmann et al. 2005; Miyake et al. 2009). These studies indicate that the LIFR cytokines are critical for motor neuron development and maintenance of the neuromuscular junction. Although there has been one study that reported that the intraperitoneal administration of CNTF to neonatal rats (from 1 to 6 days) increased muscle area and myofibre number, there is little evidence however that the endogenous loss of these cytokines directly influences developmental myogenesis.

3.4 Indirect and Direct Effects of LIFR Cytokines Regulate Adult Muscle Cell Homeostasis

Deficiency of LIFR and ligands does not appear to alter developmental myogenesis to a significant degree to induce a phenotype; however LIFR cytokines can influence the proliferation, differentiation and myogenesis of muscle precursor cells *in vitro* (Hunt et al. 2010, 2011a; Austin and Burgess 1991). LIF induces a growth effect in cultured myoblasts (Austin and Burgess 1991), the precursors of syncytial muscle fibres, which is largely mediated through suppression of apoptosis by inhibiting caspase-3 activation and activity (Hunt et al. 2010). In conjunction, LIF also inhibits differentiation via reduction in caspase-3 activity, which is required for differentiation (Hunt et al. 2011a). Indeed, most members of this cytokine subgroup including CT-1 (Miyake et al. 2009), OSM (Xiao et al. 2010) and CNTF (Wang et al. 2008; Chen et al. 2005; Hiatt et al. 2012) inhibit myogenic differentiation and promote the growth of myoblasts. In the majority of cases, MAPK

extracellular regulated kinase (MEK) activity was necessary for cytokine induced inhibition of differentiation. PI3K dependent mechanisms have also been identified that promote the survival of muscle precursor cells (Hunt et al. 2010; Hiatt et al. 2012). Together these indicate that LIFR cytokines promote cell growth and viability, whilst inhibiting differentiation and thus may play important roles in controlling the muscle precursor pool present during myogenesis.

In addition to myogenesis that occurs developmentally, myogenesis can also occur in adults in response to injury to regenerate damaged muscles. Studies with mice deficient in LIFR cytokines or with additional exogenous cytokines have demonstrated roles during adult muscle regeneration. In a revascularization and denervation model of muscle injury, local administration of CNTF promoted the regeneration of new myofibres in the muscle (Marques and Santo Neto 1997). Although the authors were dismissive of a neurotrophic effect allowing more rapid innervation and subsequent growth they could not exclude this possibility, but suggested in their opinion it was most likely due to a direct effect on myogenic cells. Further studies suggested that LIF also promotes skeletal muscle regeneration via direct growth effects on myogenic cells (Kurek et al. 1997; Barnard et al. 1994).

LIF null mouse muscles had a reduced regenerative response to crush injury, and the opposite could be achieved by local administration of LIF (Kurek et al. 1997). Interestingly, though it was speculated that LIF induced stimulation of regeneration was caused by promoting proliferation of myogenic precursors, they did not see the same effect when other factors capable of inducing proliferation such as interleukin-6 and transforming growth factor-α were administered. In contrast to these studies, when CT-1 was overexpressed systemically by adenovirus in mice myogenic differentiation and regeneration of muscle cells (induced by cardiotoxin) was inhibited (Miyake et al. 2009). This was also attributed to a direct effect on myogenic cells with CT-1 inhibiting differentiation. Similarly, transgenic expression of OSM in muscle injured by electroporation inhibited regeneration of muscle fibres but did not affect the number of MyoD positive myoblasts during the early stages of regeneration, which suggested no effect on the myogenic pool (Xiao et al. 2010).

Whilst it may be tempting to speculate that these cytokines could have differential effects during regeneration it becomes difficult to compare between the different studies using different methodologies for administration, genetic manipulation, timing and modes of regeneration. Therefore we attempted to provide clearer insight into the role of LIFR signaling by utilizing a broad antagonist of the LIFR and study its effects during the different phases of muscle regeneration (inflammatory and myogenic differentiation phases) (Hunt et al. 2013). We employed the LIF mutant MH35-BD, which is shown to compete with and antagonize at least LIF and OSM (Upadhyay et al. 2009). MH35-BD was administered systemically (via intraperitoneal injection at 1 mg/kg) to mice either at the same time as induction of regeneration with notexin or after 3 days (Hunt et al. 2013). This protocol allowed for robust circulating levels of MH35-BD within a specific time frame that was maintained at high levels for around 3–4 days and thus we could target the processes of inflammatory infiltration of the muscle (1–3 days) and myogenic proliferation and differentiation (3–7 days). When the inflammatory phase was targeted with LIFR inhibition there

was a large increase (~three–four fold increase with MH34-BD compared to vehicle injected mice) in mRNA expression of the pro-inflammatory cytokines tumor necrosis factor (TNF-α) interleukin-1B (IL-1B) and IL-6. Coincidentally there was also an increase in neutrophils infiltrating the muscle but not macrophages.

This pro-inflammatory response resulted in a subsequent delay in regeneration of the muscle fibres. No effect on regeneration was observed when LIFR was targeted during 3–7 days, which corresponds to a large increase in the number of myoblasts that support myogenic differentiation and fusion into syncytial muscle. Taken together, this indicated that LIFR signaling was predominant during the early phase of regeneration and relates to inflammatory infiltration, thus it is not likely to be necessary for myogenesis *in vivo*. LIFR signaling during muscle injury therefore is necessary to limit pro-inflammatory cytokines that inhibit myogenic differentiation. Consistent with roles for LIFR cytokines in modulating inflammation following injury, endogenous expression of these cytokines coincides with the early stages of injury and inflammation (Hunt et al. 2013; Xiao et al. 2010). Indeed, LIF is shown to suppress peripheral inflammation; OSM is also suggested to act as an anti-inflammatory cytokine (Wahl and Wallace 2001). Therefore LIFR cytokines may influence muscle regeneration indirectly by modulating inflammatory cell responses.

Like IL-6, LIF and related cytokines have been suggested to also act in pro- as well as anti-inflammatory fashions (Sugiura et al. 2000; Kerr and Patterson 2004; Gadient and Patterson 1999). It may be possible that the pro- vs anti-inflammatory effects could be highly context specific and relate to particular tissue or model systems studied or even the modality of altered signaling (eg. complete endogenous loss vs partial inhibition vs exogenous administration above physiological levels). IL-6 inflammatory potential has been suggested to relate to the specific receptor interaction, that is trans-signaling (via a soluble IL-6 receptor) is pro-inflammatory whereas classic signaling (via the membrane bound receptor) is anti-inflammatory (Scheller et al. 2011). Whilst many studies have demonstrated these pro- and anti-inflammatory effects of LIFR, none have differentiated and elucidated what mediates these distinct effects. It may be possible that a similar mechanism to IL-6 signaling exists for LIFR cytokines as a soluble receptor has been discovered (Layton et al. 1992) though very little is known.

These studies have demonstrated the effects of LIFR cytokines on the mononucleate inflammatory cells which infiltrate muscle during regeneration as well as the myogenic precursors which can respond to muscle injury. There are few studies though that have examined either the presence of receptors for or direct effects of LIFR cytokines on syncytial muscle cells. Following muscle contusion, LIFR mRNA was observed by in situ hybridization in cells surrounding syncytial muscle fibres such as myogenic precursors and endothelium as well as in the myofibres themselves (Kami et al. 2000). CNTFR was also observed in the nascent myotubes. We have also demonstrated the presence of LIFR protein by immunohistochemistry in mononuclear cells, which includes macrophages (Hunt et al. 2013). LIFR immunoreactivity was also present in nascent myotubes and around the edges of myofibres, in a non-uniform pattern and did not appear to be related to myofibre type (Hunt et al. 2011b). There did appear to be a close association between the LIFR

protein and localization of neuromuscular junctions; intense LIFR immunoreactivity co-localized with bungarotoxin immunofluorescence. Though we could not distinguish whether this was present on the pre-synaptic neurons or post-synaptic muscle, but it is consistent with a role for LIFR cytokines in synaptic signaling between the muscle and nerve.

The presence of LIFR protein on the sarcolemma of myofibres suggests that this family of cytokines may be able to directly influence muscle fibre maintenance. Both LIF and CT-1 induce cardiac hypertrophy (Pennica et al. 1995a; Kodama et al. 1997), yet research on the hypertrophic effects of LIFR cytokines on skeletal muscle is limited. A study examining the interplay between LIF and β-adrenoceptor agonist clenbuterol found that daily systemic administration of LIF for 4 weeks in healthy adult rats increased muscle size and force producing capacity synergistically with clenbuterol in both fast and slow twitch muscle, but LIF alone only produced these effects in the slow twitch soleus muscle (Gregorevic et al. 2002). Therefore LIF and related cytokines may bind receptors on the sarcolemma of myofibres and induce hypertrophic responses that work independently from and in conjunction with beta agonists. Further evidence that LIF may be involved in maintenance of muscle mass is demonstrated with increased LIF mRNA expression in human cases of chronic muscle atrophy compared to healthy controls (Reardon et al. 2001). Compensatory hypertrophy in skeletal muscles in response to increased loading requires LIF as LIF deficient mice show either no or delayed hypertrophic responses (Spangenburg and Booth 2006). Contrarily, it was proposed that LIF could induce muscle atrophy via activation of JAK and subsequent STAT transcriptional activity (Seto et al. 2015). LIF was secreted in large quantities by the cachexia inducing C26 tumour cells and shown to reduce the size of cultured myotubes. Although inhibition of STAT3 induced hypertrophy and prevented C26 tumour induced muscle wasting, whether or not elevated circulating LIF could induce atrophy, or conversely muscle specific loss of LIFR could prevent atrophy, was not tested in vivo. Because of the limitations of these studies, whether LIF could influence muscle hypertrophy by direct effects on myofibres or by influencing the motor neurons innervating the muscle is not known, but this research favors the likelihood that LIFR cytokines and downstream mediators could mediate muscle hypertrophy and atrophy.

3.5 Metabolic Functions of LIFR Cytokines

IL-6 also shares many similar attributes with LIFR cytokines including being produced by varied cellular sources and pleiotropic effects that include altering muscle and peripheral metabolism (Febbraio and Pedersen 2005). IL-6 has been suggested to induce lipolysis and could be an important myokine mediating exercise dependent increases in insulin sensitivity indirectly by acting in an anti-inflammatory manner (Pedersen et al. 2004). LIFR cytokines also appear to influence metabolism; for example LIFR knockout mice, which die perinatally, show increased glycogen content in livers during the fetal stages (Ware et al. 1995).

Because LIFR cytokines may be produced by muscle and effect peripheral tissues, their influence on metabolism may be quite broad like that of IL-6, regulating

homeostasis of important metabolic tissues such as liver and adipose. LIF alters lipid metabolism in cultured adipocytes, inhibiting lipoprotein lipase activity (Marshall et al. 1994). LIFR is necessary for formation of fat storing adipocytes from embryonic stem cells and LIF promoted adipogenic differentiation via MAPKs of certain preadipocyte cells (Aubert et al. 1999). Conversely, OSM appears to inhibit adipocyte differentiation also through MAPK and STAT dependent pathways (Miyaoka et al. 2006). Further studies have suggested that LIF has no adipogenic effects in 3T3-L1 preadipocytes, but may regulate specific transcripts related to adipogenesis and have minor effects on insulin stimulated glucose uptake (Hogan and Stephens 2005). CT-1 ubiquitous knockout induces an obese insulin resistant phenotype in mice, whilst CT-1 administration induces fatty acid oxidation, lipolysis and adipose remodeling (Moreno-Aliaga et al. 2011). CNTF has also been shown to influence adipoctyes; increasing insulin sensitivity, decreasing fatty acid synthesis (Zvonic et al. 2003) and increasing production of the anti-obesogenic adipokine leptin (Ott et al. 2004). CNTF has received a great deal of attention for these anti-obesogenic properties with trials of a human recombinant variant successfully demonstrating weight loss (White and Stephens 2011). Inflammation has also been linked with insulin resistance and metabolic syndromes such as diabetes and therefore because LIFR cytokines can act as inflammatory mediators may be myokine mediators of metabolism similar to IL-6 (Dandona et al. 2004). Effects of LIFR cytokines on these metabolic tissues such as adipose and the liver may influence skeletal muscle metabolism by altering the availability of fuel stores such as glucose and fatty acids and manipulating endocrines that influence systemic metabolism. Thus the production of LIFR cytokines whether from the muscle or from other tissues may influence skeletal muscle metabolism indirectly.

Directly, LIFR cytokines may also influence muscle metabolism. Systemic CNTF administration reduced circulating glucose levels and this was related to direct effects of CNTF increasing muscle glucose uptake (Steinberg et al. 2009). This effect required the PI3K signaling pathway but was not affected with loss of adenosine monophosphate activated kinase (AMPK). There have also been reports of AMPK dependent CNTF functions that signal via gp130, CNTFR and IL-6R (instead of LIFR) (Watt et al. 2006). CNTF in this instance increased fatty acid oxidation and improved insulin sensitivity within skeletal muscle. Whether other LIFR cytokines apart from CNTF directly affect muscle metabolism has not yet been examined. However present data suggests that LIFR cytokines may be capable of influencing muscle metabolism, either indirectly or directly, and link muscle activity, cytokine production, inflammation and metabolism.

3.6 Potential for LIFR Cytokines in Treating Muscular Dystrophies

Muscular dystrophies are a group of pathologies that can cause severe muscle wasting which can impair basic muscle function and cause premature death with the most severe types. Several genetic models of mice that recapitulate the human

pathologies are used to study muscular dystrophies; one of the most common is the mdx mouse. Mdx mice lack the protein dystrophin, an integral membrane protein that links the sarcolemma of myofibres to the surrounding extracellular matrix. Without this protein myofibres are not stable, undergo cell death and the muscles of the mice experience constant cycles of degeneration and regeneration. LIFR cytokines have been implicated as modulators of pathogenesis, with LIF itself being the most studied by far.

Following initial studies that showed LIF induced growth of muscle precursor cells (Austin and Burgess 1991) there was much excitement over the possibility that stimulating the myogenic pool capable of regenerating muscle could alleviate symptoms of muscle loss in mdx mouse. Methods were trialed to perfuse LIF into the muscles of mdx mice and it was found that LIF infusion for 1 week stimulated muscle regeneration and reduced morphological parameters that correlate with pathology in the treated vastus lateralis muscle (Kurek et al. 1996). Indeed sustained release of LIF over a period of 3 months in the diaphragm of mdx mice also improved morphological parameters closer to those of wild-type non-dystrophic muscles; moreover LIF treatment improved force producing capacity of the muscles (Austin et al. 2000). A study to establish the possible mechanism that LIF may increase myoblast replication to enhance regeneration confirmed that LIF infusion into the diaphragm increased tritiated thymidine incorporation in myotube nuclei, suggesting that myoblasts were more proliferative prior to fusing into post-mitotic myotubes, however regeneration was not enhanced in this study (White et al. 2002). Interestingly Evan's Blue Dye incorporation into damaged muscle fibres was reduced in LIF treated mdx muscles compared with untreated mdx muscles indicating that the myofibres may have been protected against cell death. A subsequent study also confirmed that LIF treatment sustains myoblasts proliferation (note that LIF treatment did not actually increase but prevented decreases in proliferation over time) in a crush injury model (White et al. 2001a).

The best interpretation of this data that reconciles all evidence that LIF and related cytokines inhibit myogenic differentiation yet may be beneficial to regeneration is that whilst exogenous LIF may maintain the myogenic pool in a proliferative state and delay differentiation, in the long-term differentiation still occurs and ultimately the growth and regeneration of muscle fibres is not affected. It is most likely that the effect of exogenous LIF on regenerating muscle is also influenced by the action of LIF on inflammatory cells as described earlier wherein we found that LIFR blockade had pro-inflammatory effects during regeneration. LIF limiting an inflammatory response in the chronically inflamed dystrophic muscle could well account for the observed benefits of exogenous LIF administration; in particular it may explain how dystrophin deficient fibres were protected from necrosis similar to inhibition of the pro-inflammatory cytokine TNF with Remicade (Grounds and Torrisi 2004).

In addition to direct administration, LIF has been considered in conjunction with stem cell therapies that attempt to engraft dystrophin expressing myoblasts into dystrophin deficient muscle. One such study found that LIF administration enhanced dystrophin expression following myoblast transplantation (White et al. 2001b).

We examined the survival of myoblasts transplanted into dystrophic muscle and found that LIF treatment transiently aided the survival of transplanted cells (Hunt et al. 2011b) in a manner similar to depletion of innate immune cells such as neutrophils and natural killer cells (Sammels et al. 2004); again illustrating the role of LIF in modulating inflammatory responses.

There are no studies examining the potential of additional LIFR cytokines such as CT-1, OSM or CNTF as therapeutics for muscular dystrophies; however links between the signaling pathways induced in muscle by these cytokines and pathways that can modulate pathogenesis are apparent. An example is that CNTF induces activation of AMPK in muscle and AMPK activation has been shown to improve muscle function in dystrophic mice (Ljubicic et al. 2011). Because administration of protein factors is limited by oral bioavailability and even despite engineering platforms such as slow releasing alginate rods for cytokine delivery, gene therapy methods to directly target the genetic lesions or simple small molecule drugs that have good oral bioavailability and may slow disease progression are the simplest candidates to consider for dystrophy therapies. However, studying the pathways by which these cytokines may influence muscle can shed light on the endogenous processes by which muscle adapts to growth, exercise, injury and disease. Summarised in Fig. 3.2 is a diagram of the proposed modes by which LIFR cytokines (using LIF as an example) can influence muscle directly, indirectly as well as peripheral tissues due to muscle specific stimuli.

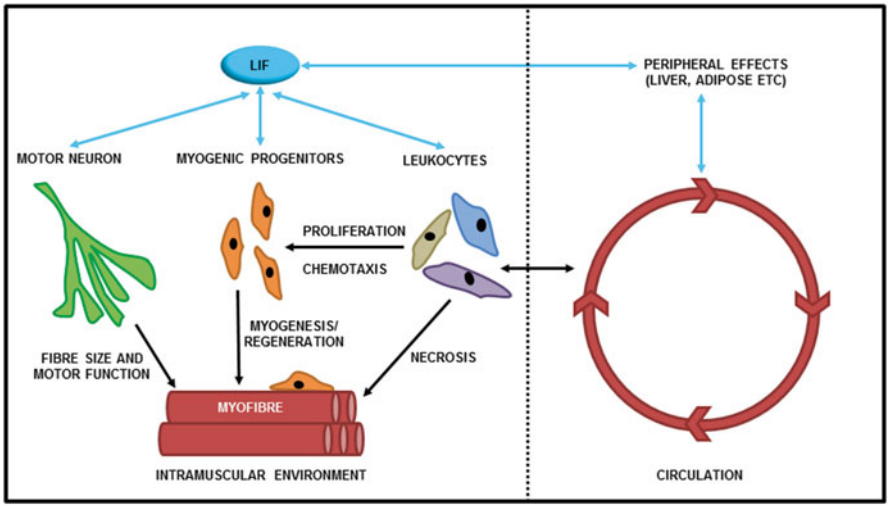

Fig. 3.2 Schematic of mechanisms by which LIF and related cytokines influence muscle directly, indirectly and contribute to peripheral homeostasis

3.7 Conclusions

LIFR cytokines are pleiotropic cytokines which can influence muscle in a number of ways including via direct interaction with the LIF receptor complex on the surface of muscle cells and indirect effects mediated through other cells. Our understanding of how LIFR cytokines may influence mature syncytial myofibre cells directly is incomplete, but evidence points towards possible hypertrophic and metabolic effects. The use of genetic models to ablate LIFR expression specifically within myofibres may however allow these properties to be elucidated. The direct effects of LIFR cytokines inhibiting myogenic differentiation of muscle precursor cells are now well established *in vitro*. However, developmentally this does not appear to occur and there is a lack of consensus as to the effects during adult muscle regeneration. We suggest that LIFR signaling may actually influence adult muscle regeneration indirectly via regulation of inflammation following injury. The anti-inflammatory properties of LIFR cytokines may also be useful in the treatment of muscle diseases in which chronic inflammation mediates the pathogenesis. Indirect actions of LIFR cytokines that can influence muscle homeostasis are becoming more widely recognized with neurotrophic and inflammatory regulating actions linked to muscle function. The discovery that LIFR cytokines may be produced in response to muscle stimulation and are distributed systemically to act as endocrines opens a range of new and exciting possibilities for muscle derived cytokines to influence not only muscle but peripheral and systemic homeostasis.

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Chapter 4
Function of Membrane-Associated Proteoglycans in the Regulation of Satellite Cell Growth

Yan Song

Abstract Muscle growth can be divided into embryonic and postnatal periods. During the embryonic period, mesenchymal stem cells proliferate and differentiate to form muscle fibers. Postnatal muscle growth (hypertrophy) is characterized by the enlargement of existing muscle fiber size. Satellite cells (also known as adult myoblasts) are responsible for hypertrophy. The activity of satellite cells can be regulated by their extracellular matrix (ECM). The ECM is composed of collagens, proteoglycans, non-collagenous glycoproteins, cytokines and growth factors. Proteoglycans contain a central core protein with covalently attached glycosaminoglycans (GAGs: chondroitin sulfate, keratan sulfate, dermatan sulfate, and heparan sulfate) and N- or O-linked glycosylation chains. Membrane-associated proteoglycans attach to the cell membrane either through a glycosylphosphatidylinositol anchor or transmembrane domain. The GAGs can bind proteins including cytokines and growth factors. Both cytokines and growth factors play important roles in regulating satellite cell growth and development. Cytokines are generally associated with immune cells. However, cytokines can also affect muscle cell development. For instance, interleukin-6, tumor necrosis factor-α, and leukemia inhibitory factor have been reported to affect the proliferation and differentiation of satellite cells and myoblasts. Growth factors are potent stimulators or inhibitors of satellite cell proliferation and differentiation. The proper function of some cytokines and growth factors requires an interaction with the cell membrane-associated proteoglycans to enhance the affinity to bind to their primary receptors to initiate downstream signal transduction. This chapter is focused on the interaction of membrane-associated proteoglycans with cytokines and growth factors, and their role in satellite cell growth and development.

Keywords Cytokine • Growth factor • Proteoglycan • Satellite cell • Skeletal muscle

Y. Song (✉)
Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02215, USA
e-mail: yan_song@dfci.harvard.edu

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Abbreviations

C1	constant region 1
C2	constant region 2
ECM	extracellular matrix
FGF	fibroblast growth factor
FGF2	fibroblast growth factor 2
FGFR	fibroblast growth factor receptor
GAGs	glycosaminoglycans
GPI	glycosylphosphatidylinositol
HGF	hepatocyte growth factor
HSPG	heparan sulfate proteoglycan
IGF	insulin-like growth factor
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IL-6	interleukin-6
LIF	leukemia inhibitory factor
MAP	mitogen-activated protein
N-glycosylated chain	N-linked glycosylated chain
PIP_2	phosphatidylinositol 4, 5-bisphosphate
PKC	protein kinase C
PKCα	protein kinase C alpha
TGF	transforming growth factor
TGF-β	transforming growth factor-beta
TGF-β1	transforming growth factor-beta 1
TGF-β2	transforming growth factor-beta 2
TNF-α	tumor necrosis factor-alpha
V	variable region
VEGF	vascular endothelial growth factor

4.1 Introduction

Skeletal muscle composes about 50 % of an animal body mass (Huard et al. 2002) and it is the contractile tissue of an animal which forms force and movements. Multinucleated myofibers are derived from mesenchymal stem cells, myoblasts. During the embryonic period, the number of myofiber number increases and after birth skeletal muscle grows by the enlargement of existing muscle fiber size (hypertrophy). Satellite cells are responsible for hypertrophy and muscle regeneration. They are undifferentiated mononuclear myogenic precursor cells. They have the ability to self-renew in order to maintain their population pool; to proliferate, differentiate and fuse with existing muscle fibers in order to maintain, repair

and increase muscle fiber size (Moss and LeBlond 1971; Kuang et al. 2007). Satellite cells are normally quiescent in adults (Schultz et al. 1978; Cornelison and Wold 1997). However, when skeletal muscle tissue is injured or heavily used, the satellite cells become active and re-enter the cell cycle (Chargé and Rudnicki 2004; Dhawan and Rando 2005) and then proliferate, fuse with damaged myofibers or form new myofibers through a process similar to fetal muscle formation (Parker et al. 2003).

The activity of satellite cells can be regulated by their extracellular matrix (ECM). The ECM is composed of collagens, proteoglycans, non-collagenous glycoproteins, cytokines, and growth factors. Proteoglycans contain a central core protein with covalently attached glycosaminoglycans (GAGs: chondroitin sulfate, keratan sulfate, dermatan sulfate, and heparan sulfate) and N- or O-linked glycosylated chains. Membrane-associated proteoglycans attach to the cell membrane either through a transmembrane domain of the core protein or a glycosylphosphatidylinositol (GPI) anchor. The GAGs can bind to and regulate the activity of cytokines and growth factors which play important roles in regulating satellite cell growth and development.

4.2 Skeletal Muscle Growth and Development

In the early stages of embryonic development, ectoderm, mesoderm, and endoderm layers form through a process called gastrulation. Typical mesoderm derived tissues include blood, blood vessels, bones, cartilage, connective tissue, and muscle. On both sides of the neural tube, the mesoderm divides into four different layers: axial mesoderm, intermediate mesoderm, paraxial mesoderm, and the lateral plate mesoderm (Christ and Ordahl 1995). Paraxial mesoderm is the source for almost all of the embryonic skeletal muscle. Paraxial mesoderm gives rise to somites which are clusters of cells. Cells from the dorsal part of the somites form the dermomyotome. Muscle progenitor cells delaminate from the four edges of the dermomyotome and then migrate to the areas that muscle will form (Gros et al. 2004). Through the regulation of myogenic regulatory factors, these muscle progenitor cells become myoblasts. The myoblasts will further differentiate into myocytes, fuse with each other and then mature into muscle fibers.

Postnatal muscle growth continues by the proliferation, differentiation, and fusion of satellite cells. Satellite cells were first described by Mauro (1961) and were named due to their location between basement membrane and the sarcolemma. The progenitor cells from the central dermomyotome give rise to most satellite cells (Armand et al. 1983; Gros et al. 2005). They are responsible for postnatal muscle growth and regeneration. The activity of satellite cells is high during the neonatal stage of development and decreases with age. Satellite cells are usually quiescent or in the G0 phase of the cell cycle in adults (Bischoff 1990a). However, the activity can be up-regulated in case of muscle injured or heavily used. Once activated, satel-

lite cells re-enter the cell cycle, proliferate, and fuse with adjacent myofibers or form new ones to reconstitute the muscle. The number of satellite cells decreases in aged animals, but the capacity for self-renewal, proliferation and differentiation remains the same (Shefer et al. 2006). It has been reported that satellite cells from aged animal have enhanced activation when they are grafted to a young animal whereas satellite cells from young animal showed decreased activation when they are grafted to an aged animal (Zacks and Sheff 1982; Carlson and Faulkner 1989). This also indicates that the satellite cell niche is important for the activity of satellite cells. Bischoff (1990b) demonstrated that the behavior of satellite cells can be regulated by their ECM. The ECM not only provides extracellular cues to allow satellite cells to survive, but also dynamically regulates their behavior to maintain a balance between quiescence, self-renewal, and differentiation (Fuchs et al. 2004; Morrison and Spradling 2008). The ECM regulates satellite cell behavior through modulating interactions between cell membrane receptors and growth factors and cytokines.

Skeletal muscle regeneration is important for maintaining skeletal muscle mass and function. Regeneration occurs not only in response to major injury and stimuli, but also due to daily wear and tear (Tidball and Villalta 2010). During regeneration, there are three overlapping phases: inflammation dominated by the invasion of macrophages, tissue formation by the activated satellite cells, and tissue remodeling. When muscle gets injured, the myofibers undergo necrosis and degeneration, and leukocytes invade from damaged blood vessels. Neutrophils are the first inflammatory cells to enter, and then macrophages to phagocytose muscle debris (Chargé and Rudnicki 2004). Both neutrophils and macrophages release cytokines and chemokines which can amplify the inflammatory response and recruit satellite cells to the site of injury. Leukaemia inhibitory factor (LIF) and interleukin-6 (IL-6) are two cytokines which strongly influent muscle myoblast proliferation. IL-6 stimulates fibroblast growth and suppresses fusion of myoblast to form myotubes whereas LIF has no effect on fibroblast growth but promotes myoblast fusion.

Except for cytokines, many growth factors are also recruited into the wound area, including fibroblast growth factor (FGF), transforming growth factor (TGF) families, insulin-like growth factors (IGF-I, IGF-II), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF). Growth factors control the proliferation and differentiation of satellite cells thus is essential in muscle regeneration process (Chargé and Rudnicki 2004; Ten Broek et al. 2010). Among them, IGF-1 is critical for muscle growth and myoblast proliferation; HGF stimulates satellite cell activation, proliferation, and differentiation; and transforming growth factor beta-1 (TGF-β1) inhibits satellite cell proliferation and differentiation, and myoblast recruitment. Under the regulation of cytokines and growth factors, the activated satellite cells migrate to the site of injury to repair damaged myofibers or form new myofibers (Chen and Goldhamer 2003; Chargé and Rudnicki 2004; Buckingham 2006; Shi and Garry 2006).

4.3 Extracellular Matrix

The ECM is an extracellular environment that surrounds cells. It is not only a substrate for the cells to adhere to, a scaffold to support cells, but also an organized dynamic structure that regulates intracellular communication and cell behavior. It is secreted by cells and feeds back information to the cell by sending signals into the cell. The composition of the ECM varies from tissue to tissue: each tissue has its own specific composition of matrix molecules that is adapted to the functional requirements of that tissue. In the same tissue, it is also changes with developmental stage, age, and health conditions, etc. The ECM modulates the production, degradation, and remodeling of its components to support tissue development, function, and repair (Uldbjerg and Malmström 1991; Page-McCaw et al. 2007; Lu et al. 2011).

The ECM is composed of fibrous proteins including collagens, non-fibrous proteins like glycoproteins and proteoglycans. Proteoglycans are glycosylated proteins with a high net negative charge that can attract water to keep cells hydrated. Proteoglycans are involved in a variety of biological processes by modulating growth factor activity, organizing the extracellular environment, and maintaining tissue structure and function. They are composed of a central core protein and covalently linked GAG chains (Hardingham and Fosang 1992) and N-linked glycosylated chains (N-glycosylated chain) attached to a center core protein. Glycosaminoglycan chains are long unbranched polysaccharides consisting of a repeating disaccharide unit which is covalently attached to the core protein at Ser-Gly repeats. There are four types of GAGs: heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate. Accordingly, the proteoglycans bearing these GAGs are named as heparan sulfate proteoglycans (HSPG), chondroitin sulfate proteoglycans, dermatan sulfate proteoglycans, and keratan sulfate proteoglycans (Table 4.1).

Chondroitin sulfates are major components of the ECM, especially in the tissues such as cartilage, tendons, and aorta walls. They interact with macromolecules in the ECM and contribute to tissue structural integrity. Versican and aggrecan are two large chondroitin sulfate proteoglycans. Their chondroitin sulfate chains are composed of two alternative residues of D-glucuronic acid and *N*-acetylgalactosamine. Versican has been reported to play important roles in cell adhesion, migration and proliferation (Zhang et al. 1998). Aggrecan is a major structural proteoglycan in cartilage. It functions in mediating chondrocyte-chondrocyte and chondrocyte-matrix interactions.

Dermatan sulfates, also named as chondroitin sulfate B previously, are composed of a repeating disaccharide unit of L-iduronate and *N*-acetylglucosamine. Members in this family include decorin and biglycan. They are mainly expressed in skin, blood vessels, heart valves, tendons, and lung. Depending on the tissue, they can bear either chondroitin sulfates or dermatan sulfates. As dermatan sulfate proteoglycans, they play important roles in coagulation, cardiovascular disease, carcinogenesis, infection, wound repair, and fibrosis.

Table 4.1 Proteoglycan classification

(a) According to the glycosaminoglycan attached:	
Chondroitin sulfate	
Aggrecan	Cartilage
Betaglycan	Fibroblasts
Biglycan	Muscle
Decorin	Connective tissue
Perlecan	Basement membrane
Serglycin	Mast cells, hematopoietic cells
Syndecan 1, 3	Epithelial cells, fibroblasts
Versican	Blood vessels, skin
Dermatan sulfate	
Biglycan	Muscle
Decorin	Connective tissue
Heparan sulfate	
Agrin	Basement membrane
Betaglycan	Fibroblasts
CD44 V3	Keratinocytes, activated monocytes
Collagen XVIII	Epithelial cells, basement membrane
Glypican 1-6	Epithelial cells, fibroblasts
Perlecan	Basement membrane
Serglycin	Mast cells, hematopoietic cells
Syndecan 1-4	Epithelial cells, fibroblasts
Keratan sulfate	
Aggrecan	Cartilage
Fibromodulin	Extracellular matrix
Lumican	Corneal stroma
(b) According to cell localization:	
Extracellular	
Aggrecan	Cartilage
Agrin	Basement membrane
Biglycan	Muscle
Collagen XVIII	Epithelial cells, basement membrane
Decorin	Connective tissue
Fibromodulin	Extracellular matrix
Lumican	Corneal stroma
Perlecan	Basement membrane
Versican	Blood vessels, skin
Membrane-associated	
Betaglycan	Fibroblasts
CD44v3	Lymphocytes
Glypican 1-6	Epithelial cells, fibroblasts
Neuropilin-1	Endothelial cells
Syndecan 1-4	Epithelial cells, fibroblasts
Intracellular	
Serglycin	Mast cells, hematopoietic cells

Table 4.2 Cell membrane-associated heparan sulfate proteoglycan binding growth factors and cytokines

		Function in muscle	References
Growth Factors	FGF2	Promotes satellite cell proliferation, inhibits satellite cell differentiation	Allen et al. (1984)
			DiMario and Strohman (1988)
			Greene and Allen (1991)
			Dollenmeier et al. (1981)
			McFarland (1999)
	HGF	Activates satellite cells, regulates myogenesis, muscle regeneration, and would healing	Allen et al. (1995)
			Anastasi et al. (1997)
			Gallagher and Lyon (2000)
	TGF-β	Inhibits satellite cell proliferation, differentiation, and muscle-specific gene expression	Allen and Boxhorn (1987)
			Florini and Magri (1989)
	IGF	Induces satellite cell proliferation, promote muscle hypertrophy	Adams and Haddad (1996)
			Adams and McCue (1998)
			Adams and Haddad (1996)
			Rosenblatt and Parry (1992)
Cytokines	IL-6	Promotes satellite cell proliferation, remodels muscle extracellular matrix	Austin et al. (1992)
			Baeza-Raja and Muñoz-Cánoves (2004)
			Al-Khalili et al. (2006)
			Serrano et al. (2008)
			White et al. (2009)
	LIF	Stimulates satellite cell proliferation, muscle regeneration and hypertrophy	Rosenblatt et al. (1994)
			Hawke and Garry (2001)
			Mitchell and Pavlath (2001)
			Spangenburg and Booth (2006)
	TNF-α	Regulate muscle cell proliferation and differentiation, regeneration	Layne and Farmer (1999)
			Thaloor et al. (1999)
			Guttridge et al. (2000)
			Warren et al. (2002)
			Li (2003)

FGF2 fibroblast growth factor 2, *HGF* hepatocyte growth factor, *IGF* insulin-like growth factor, *IL-6* interleukin-6, *LIF* leukemia inhibitory factor, *TGF-β* transforming growth factor-beta, *TNF-α* tumor necrosis factor-alpha

Keratan sulfates are mainly expressed in cornea, cartilage, and bone. Keratan sulfates are composed of a repeating disaccharide unit of galactose and *N*-acetylglucosamine. They can absorb large amounts of water and act as a cushion in joints to absorb mechanical stress and as a buffer of corneal hydration. The main family members in keratan sulfate proteoglycans are fibromodulin and lumican. Fibromodulin is involved in TGF-β activity regulation and ECM assembly (Hildebrand et al. 1994) whereas lumican mainly functions in corneal transparency and ECM assembly. To date, there are no keratan sulfate proteoglycans have been found in skeletal muscle.

Heparan sulfate proteoglycans are present at the surface of all adherent animal cells. They are linear polysaccharides that are composed of a repeat disaccharide unit of glucuronic acid or iduronic acid attached to a glucosamine. Family members of HSPG include syndecan, glypican, betaglycan, CD44v3, perlecan, agrin, and collagen XVIII. They can interact with growth factors, chemokines, cytokines, ECM molecules, morphogens, and clotting factors (Esko and Selleck 2002), and regulate a wide variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumor metastasis. Heparan sulfate proteoglycans are also involved in assembling the ECM (Iozzo 2005; Peretti et al. 2008), modulating enzymes activity (Yu and Woessner 2000; Nascimento et al. 2005), and regulating activity of growth factors and cytokines. Furthermore, HSPG can promote growth factor signal transduction pathways by function as a receptor or co-receptor.

Heparan sulfate proteoglycans can bear one or several heparan sulfate chains to their core protein. They may bind to growth factors and enhance their binding affinity to their high-affinity tyrosine kinase receptors. This model was first and extensively studied in fibroblast growth factor (FGF2; Rapraeger et al. 1991), and extended to other growth factors, such as HGF (Rubin et al. 2001; Kemp et al. 2006), VEGF (Gitay-Goren et al. 1992), platelet-derived growth factor (Rolny et al. 2002; Abramsson et al. 2007), and placenta growth factor (Athanassiades and Lala 1998). Other growth factors can be regulated by HSPGs including TGF-β and bone morphogenetic protein (Chen et al. 2006; Rider 2006).

In skeletal muscle, HSPGs are very important regulators of muscle growth and development. The expression of HSPGs is significantly higher in growth selected turkey breast muscle than those from non-growth selected ones (Liu et al. 2002), which indicates an important role of HSPGs in skeletal muscle growth and development. Sporer et al. (2011) in microarray analysis showed that HSPG, syndecan-4 and glpican-1, are differentially expressed with growth selection. Several *in vitro* experiments showed altered myogenic progression when proteoglycan sulfation is inhibited in cultured C2C12 cells and myofibers (Melo et al. 1996; Osses and Brandan 2001; Cornelison et al. 2001). Moreover, the adding of heparan sulfate mimicker, synthetic polymers, can stimulate muscle regeneration and reinnervation (Desgranges et al. 1999)

According to the location, proteoglycans can be divided into three groups: extracellular proteoglycans, intracellular proteoglycans, and membrane-associated proteoglycans (Table 4.1). Extracellular proteoglycans include aggrecan, perlecan, agrin, and collagen XVIII etc. They play important roles in define matrix structure

and provide space for cell adehesion, proliferation, and migration. Intracellular proteoglycans, serglycin, plays an important role in forming vesicles to package and store granular contents (Tantravahi et al. 1986; Stevens et al. 1988), and regulating cell apoptosis and immune responses (Metkar et al. 2002; Raja et al. 2002; Kolset and Pejler 2011).

The cell membrane-associated proteoglycans attach to the cell membrane either through a GPI anchor or a transmembrane domain. They can interact with integrins and ECM molecules including fibronectin, laminins, vitronectin, and fibrillar collagens to organize the cytoskeletal structure, regulate cell shape, cell adhesion, migration, and cell signal transduction (Bernfield et al. 1999). Membrane-associated proteoglycans can function as co-receptors for various growth factors. For example, FGF2 and HGF are the two growth factors whose signaling can be markedly enhanced by cell membrane-associated proteoglycans (Rapraeger et al. 1991; Yayon et al. 1991; Derksen et al. 2002).

4.4 Cell Membrane-Associated Proteoglycans

Almost all of the cell membrane-associated proteoglycans are heparan sulfate proteoglycans. Syndecans and glypicans are the two main families of cell membrane-associated proteoglycans. Syndecans bind to the cell membrane through a highly conserved transmembrane domain whereas glypicans bind to the cell membrane by a GPI anchor. They have been reported to bind to cytokines such as IL-6 and tumor necrosis factor-alpha (TNF-α) and growth factors including FGF (Gallagher 2001; Wu et al. 2003), HGF (Lyon and Gallagher 1994), and VEGF (Neufeld et al. 1999) to regulate cell signal transduction (Bernfield et al. 1999; Tumova et al. 2000). Other membrane-associated proteoglycans are betaglycan, CD44, and neuropilin all of which are part-time HSPGs due to them containing heparan sulfate chains in some proportion or under certain conditions.

4.4.1 Syndecans

The syndecans have four family members, all of which have been found to be expressed in skeletal muscle (Brandan and Larraín 1998; Larraín et al. 1998; Fuentealba et al. 1999; Cornelison et al. 2001; Liu et al. 2004, 2006). Syndecan-1 is predominantly expressed in epithelial cells; syndecan-2 is in fibroblasts whereas syndecan-3 is predominantly expressed in neural tissue. Syndecan-4 is expressed ubiquitously in developing embryo and adult tissues (Kim et al. 1994). Based on sequence homology, syndecan-1 and syndecan-3 are classified to one subfamily whereas syndecan-2 and syndecan-4 belong to another (Bernfield et al. 1999; Couchman et al. 2001). They are type I membrane glycoproteins that usually have heparan sulfates attached to the core protein, and some contain chondroitin or

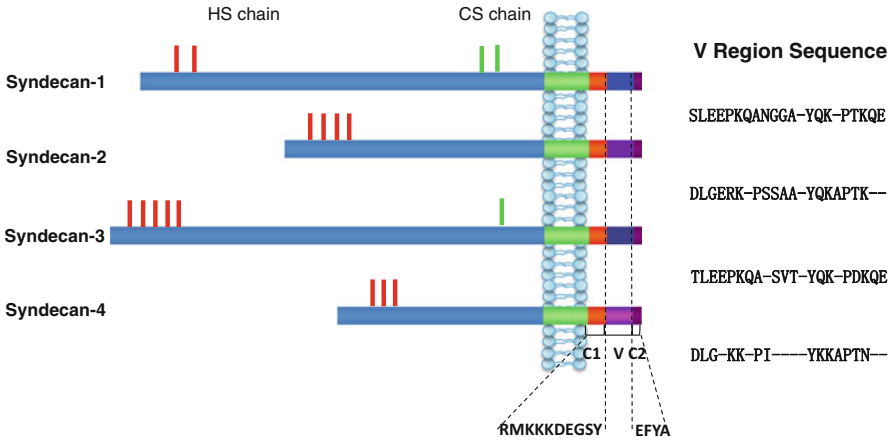

Fig. 4.1 Syndecan structure. Syndecan family has 4 members, syndecan −1 to −4. All of them are composed of a transmembrane core protein and covalently attached heparan sulfate (HS) chains. Syndecan-1 and syndecan-3 also can also bear chondroitin sulfate (CS) chains. Syndecan cytoplasmic domain is divided into three domain, constant region 1(C1) and 2 (C2), and variable region (V). The C1 and C2 domains are highly conserved among all of the four syndecans, but not the V domain

dermatan sulfate chains (Bernfield et al. 1999; Deepa et al. 2004). All of the syndecans have an extracellular domain, a hydrophobic transmembrane domain, and a short cytoplasmic domain (Fig. 4.1). Syndecans have a unique extracellular domain, a highly conserved transmembrane domain, and a cytoplasmic domain. The cytoplasmic domain has two constant regions, C1 and C2, separated by a unique variable region, V. At the C terminus of the cytoplasmic domain, the conserved amino acid sequence (EFYA) provides a binding region for postsynaptic density protein, disc-large, zonulin-1 domain containing proteins, syntenin, synectin, and the Ca2/calmodulin associated serine/threonine kinase (Zimmermann et al. 2005; Naccache et al. 2006; Jemth and Gianni 2007; Beekman and Coffer 2008; Boukerche et al. 2008). This structure allows syndecans to function as cell surface receptors (Choi et al. 2011).

Syndecan-1 was first discovered in a mouse epithelial cell line (Rapraeger and Bernfield 1983). It is abundantly expressed in epithelial cells and lowly expressed in the fibroblast and endothelial cells (Elenius et al. 1991, 1992; Kojima et al. 1992). Syndecan-1 core protein can be attached with both heparan sulfates and chondroitin sulfates (Rapraeger et al. 1985). Haparin binding growth factors have the potential to bind to syndecan-1 heparan sulfate chains. It has been reported that syndecan-1 can mediate FGF2 binding and activity (Filla et al. 1998) and regulate fibroblast growth factor receptor (FGFR) signaling (Steinfeld et al. 1996). In muscle cells, syndecan-1 is highly expressed during cell proliferation and down-regulated during differentiation (Larraín et al. 1997). In growth selected turkeys and high-growth mice, there is increased syndecan-1expression (Summers and Medrano 1997; Liu

et al. 2004), which may indicate a period of increased cell proliferation and growth (Velleman et al. 2012).

Syndecan-2 is primarily expressed in fibroblasts, and also in endothelial, mesenchymal and carcinoma cells (Marynen et al. 1989; Essner et al. 2006; Fears et al. 2006). Syndecan-2 has important roles in embryonic development (Chen et al. 2002; Kramer and Yost 2002; Arrington and Yost 2009) and matrix assembly (Klass et al. 2000). Wang et al. (2010) reported that the inhibiting syndecan-2 expression causes defects in actin skeleton formation and fibronectin deposition. The extracellular domain promotes integrin mediated cell focal adhesion formation in mesenchymal cells independent of the heparan sulfate chains (Whiteford et al. 2007). The cytoplasmic domain of syndecan-2 may play a role in TGF-β signal transduction (Chen et al. 2004). Brandan and Larraín (1998) and Liu et al. (2006) have shown the expression of syndecan-2 during skeletal muscle proliferation and differentiation. However, the mechanism of how syndecan-2 functions in skeletal muscle is still unclear.

Syndecan-3 is mainly expressed in the nervous system and its role in nervous system development has been well studied (Raulo et al. 1994; Lauri et al. 1999; Paveliev et al. 2008). Syndecan-3 is the receptor for the ECM component heparin-binding growth-associated molecule and it is required for the survival of primary sensory neurons (Paveliev et al. 2008). Cultured dorsal root ganglion neurons from syndecan-3 deficient mice had massive cell death compared to the wild type controls. Furthermore, anti-syndecan-3 antibodies added to the culture media can also inhibit heparin-binding growth-associated molecule induced neurite outgrowth (Raulo et al. 1994).

Syndecan-3 is also expressed in skeletal muscle cells (Fuentealba et al. 1999). The expression of syndecan-3 is down-regulated during skeletal muscle differentiation. Syndecan-3 –null satellite cells have an aberrant cell cycle which causes reduced proliferation, increased cell death and delayed differentiation (Pisconti et al. 2010). Using antisense syndecan-3 mRNA, Fuentealba et al. (1999) found that the inhibition of syndecan-3 expression accelerated skeletal muscle cell differentiation through an FGF2-dependent mechanism. Syndecan-3 binds to FGF2 through its heparan sulfate chains (Chernousov and Carey 1993). It is has also been suggested to function as a co-receptor with FGFRs and integrins (Woods and Couchman 2000).

Syndecan-4 is ubiquitously expressed during development and in most adult tissues (Oh and Couchman 2004). Syndecan-4 transmembrane core protein is composed of an extracellular domain, a transmembrane domain and a short cytoplasmic domain (Fig. 4.2a). There are three GAG chain attached to the core protein at the N-terminus and two N-glycosylated chains attached to the core protein close to the cell membrane. Song et al. (2011) reported that syndecan-4 GAG chains and N-glycosylated chains are required during turkey satellite cell proliferation. The extracellular domain of syndecan-4 can be shed from the cell surface and function as an effector in maintaining the proteolytic balance during inflammation (Kainulainen et al. 1998). The cytoplasmic domain of syndecan-4 is critical in syndecan-4 regulated cell proliferation, FGF2 responsiveness, (Song et al. 2012a),

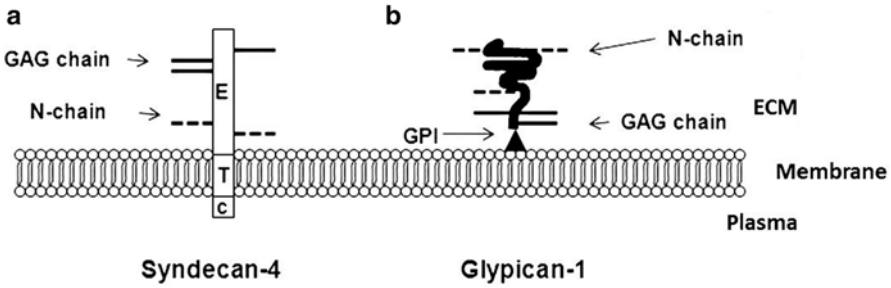

Fig. 4.2 Structure of cell membrane-associated heparan sulfate proteoglycan syndecan-4 and glypican-1. A, syndecan-4 attaches to the cell membrane through a transmembrane core protein. The core protein is composed of an extracellular domain (E), a transmembrane domain (T) and a short cytoplasmic domain (C). There are three glycosaminoglycan (GAG) chains attached to the core protein at the N-terminus and two N-linked glycosylated chains (N-chain) attached to the core protein close to the cell membrane. B, glypican-1 attaches to the cell membrane via a glycophosphatidylinositol (GPI) anchor. Glypican-1 globular domain-containing core protein has three GAG chains attached to the core protein close to the cell membrane and three N-chains spread into the extracellular matrix (ECM)

focal adhesion formation (Saoncella et al. 1999), cell migration (Echtermeyer et al. 2001), and apoptosis (Jeong et al. 2001).

Focal adhesions are the points of strong attachment of cells to the ECM or other cells. They are composed of a large number of proteins including ECM molecules that cells attach to, cell membrane receptors including integrins and syndecan-4, and cytoplasmic proteins including but not limited to vinculin, talin, alpha actinin, paxillin, focal adhesion kinase, SrC, and protein kinase C (PKC) (Burridge and Chrzanowska-Wodnicka 1996; Clark and Brugge 1995). The heparan sulfate chains of sydencan-4 bind to the heparin-binding domain of fibronectin in the ECM and together with integrins promote the formation of focal adhesion and stress fibers (Saoncella et al. 1999; Lyon et al. 2000; Woods et al. 2000). The number, size, and stability of focal adhesions influence cell migration. Gopal et al. (2010) reported that syndecan-4-null fibroblasts have altered actin cytoskeleton formation, and that multiple heparan sulfate chains are required in this process. The overexpression of syndecan-4 in Chinese hamster ovary cells leads to increased focal adhesion formation and decreased cell motility whereas the overexpression of a truncated syndecan-4 without the V region of the cytoplasmic domain decreased cell spreading and focal adhesion (Longley et al. 1999). This indicates the critical role of the syndecan-4 cytoplasmic domain V region in the formation of focal adhesions.

Syndecan-4 also plays an important role in muscle maintenance and regeneration (Cornelison et al. 2001, 2004). During muscle development, more syndecan-4 is expressed in turkey embryonic pectoralis major muscle in growth selected turkeys compared to unselected turkeys (Liu et al. 2006). Furthermore, syndecan-4 knockout mice lose the ability to regenerate damaged muscle (Cornelison et al. 2004). Syndecan-4 participates in signaling transduction by transmitting signals from the

ECM into the cell as a co-receptor and by activating protein kinase C alpha (PKCα) signal transduction (Volk et al. 1999; Oh et al. 1997, 1998).

Syndecan-4 functions as a co-receptor of FGF2. Volk et al. (1999) reported that syndecan-4 cytoplasmic domain is required in FGF2-induced cell proliferation and migration in endothelial cells. In this process, syndecan-4 GAG chains may bind to FGF2 and present FGF2 to its high affinity receptor, FGFR (Kojima et al. 1996; Steinfeld et al. 1996). The binding of FGF2 phosphorylates FGFR, and subsequently activates Raf-1 and mitogen-activated protein (MAP) kinase which enters nucleus to regulate gene transcription involved in growth and migration (reviewed by Wilcox-Adelman et al. 2002). However, Zhang et al. (2008) showed that syndecan-4 GAG chains are not required for syndecan-4 function in turkey satellite cell proliferation and differentiation. In the same cell system, the deletion of the cytoplasmic domain of syndecan-4 increased cellular responsiveness to FGF2 (Song et al. 2012a). How syndecan-4 modulates FGF2 responsiveness may be cell type specific (Velleman et al. 2012).

In the presence of phosphatidylinositol 4, 5-bisphosphate (PIP_2), the cytoplasmic domain of syndecan-4 can translocate PKCα to the cell membrane and activate it (Fig. 4.3; Oh et al. 1997, 1998; Horowitz et al. 1999). The activated PKCα will activate RhoA and its targets, the Rho kinase to regulate gene expression (Dovas et al. 2006). The binding of PIP_2 to the syndecan-4 cytoplasmic domain V region at the sequence of KKPIYKK promotes and stabilizes the oligomerization of syndecan-4 (Oh et al. 1998; Lee et al. 1998). The oligomerization of syndecan-4 promotes the binding and activation of PKCα to its cytoplasmic domain (Horowitz and Simons 1998a, b) whereas the monomeric syndecan-4 is not capable of potentiating PKCα activity. The phosphorylation status of the Ser residue in the C1 region of the syndecan-4 cytoplasmic domain is critical in regulating the affinity to PIP_2 and oligomerization status of syndecan-4. Fibroblast growth factor 2 can be the regulator in this process. Without FGF2, Ser can be phosphorylated which will block oligomerization of the syndecan-4 cytoplasmic domain, thus decrease the binding of PIP_2 and activation of PKCα (Horowitz and Simons 1998a). When FGF2 is added, it may bind to syndecan-4 GAG chains, and dephosphorylate Ser (Horowitz and Simons 1998b), thus forming oligomers and activate PKCα activity and downstream signaling pathways (Rybin et al. 1999). The activation of PKCα can regulate both cell migration (Harrington et al. 1997) and proliferation (Schonwasser et al. 1998; Besson and Yong 2000). Furthermore, Ser residue in the cytoplasmic domain can regulate cellular responsiveness to FGF2 in turkey satellite cells (Song et al. 2012b).

4.4.2 Glypican-1

Six glypicans have been identified, but only glypican-1 has been shown to be expressed in skeletal muscle (Campos et al. 1993). The glypicans are attached to the cell membrane by a GPI anchor (Fig. 4.1b). Glypican-1 globular domain-containing

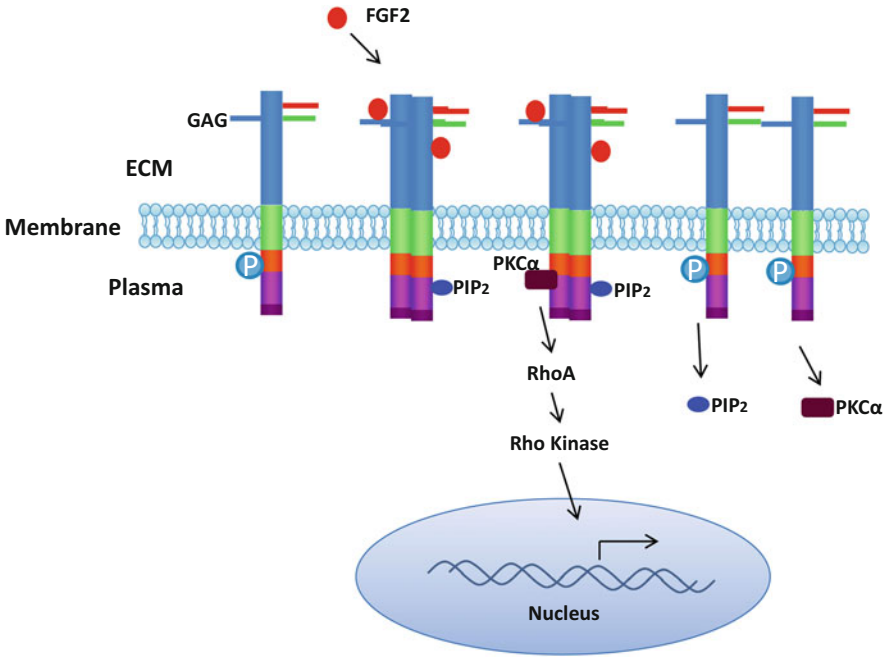

Fig. 4.3 The phosphorylation status of the Ser residue in the constant region 1 (C1) of the syndecan-4 cytoplasmic domain is critical in regulating the affinity to phosphatidylinositol 4, 5-bisphosphate (PIP_2) and oligomerization status of syndecan-4. Without fibroblast growth factor 2 (FGF2), Ser can be phosphorylated which will block oligomerization of the syndecan-4 cytoplasmic domain. In presence FGF2, it binds to syndecan-4 glycosaminoglycan (GAG) chains, and dephosphorylates Ser which enhances the binding of PIP_2 to the syndecan-4 cytoplasmic domain, promotes and stabilizes the oligomerization of syndecan-4. The oligomerization of syndecan-4 promotes the binding and activation of protein kinase C-alpha (PKCα) to its cytoplasmic domain. The activated PKCα activates the downstream pathway leading to the activation of RhoA and its targets, the Rho kinase to regulate gene expression

core protein has three GAG chains attached close to the cell membrane and three N-glycosylated chains extending into the ECM. The GPI anchor allows glypican-1 to diffuse in the outer leaflet of the cell membrane and affiliate with lipid rafts (Taylor et al. 2009; Gutiérrez and Brandan 2010). Lipid rafts are the membrane domain rich in sphingolipids and cholesterols (Brady et al. 2004; Brown and London 1998). The localization of glypicans into the lipid rafts may allow them to bind more directly and efficiently with a number of morphogens located in lipid rafts (Rietveld et al. 1999; Zhai et al. 2004).

The membrane-associated glypican-1 has been hypothesized to increase FGF2 binding to its signaling receptor (Steinfeld et al. 1996; Brandan and Larraín 1998). During muscle cell differentiation, the expression of glypican-1 is increased (Brandan et al. 1996; Liu et al. 2004; Velleman et al. 2006). The increased glypican-1 sequesters FGF2 away from its receptor by being shed from the cell surface and having FGF2 bound to its heparan sulfate GAG chains (Velleman et al. 2013). In

addition, glypican-1 can sequester FGF2 in lipid raft and prevent its binding and dependent signaling (Gutiérrez and Brandan 2010).

The glypican-1 GAG chains are critical in binding and regulating growth factor activities. The GAG chains are attached to the core protein at Ser residues near the C termini. Zhang et al. (2007) reported that glypican-1 GAG chains are required for its function to regulate turkey myogenic satellite cell differentiation and response to FGF2 during proliferation. The N-glycosylated chains are attached to the core protein at the Asn residue of the sequence of Asn-Xaa-Ser/Thr; Xaa can be any amino acid except Pro (Kornfeld and Kornfeld 1985). Glypican-1 N-glycosylated chains play important roles in satellite cell proliferation and differentiation (Song et al. 2010). The deletion of both glypican-1 N-glycosylated chains and GAG chains increased satellite cell proliferation, differentiation and cellular responsiveness to FGF2 (Song et al. 2010). However, the deletion of glypican-1 GAG chains had no effect on satellite cell proliferation, which suggests that glypican-1 GAG and N-glycosylated chains interact with each other in regulating turkey satellite cell proliferation, differentiation, and responsiveness to FGF2.

4.4.3 Betaglycan

Betaglycan (also named as TGF-β receptor III) is a part-time membrane-associated proteoglycan (López-Casillas et al. 1991). It has a high affinity for TGF-β superfamily members and FGF2. The interaction with TGF-β and FGF2 suggests a role of betaglycan in skeletal muscle satellite cell proliferation and differentiation. The betaglycan core protein is composed of an extracellular domain, a transmembrane domain, and a short cytoplasmic tail. The extracellular domain has several potential attachment sites for chondroitin sulfate and heparan sulfate chains. Betaglycan binds to TGF-β superfamily members via its core protein independent of its glycosaminoglycan chains. However, the binding of betaglycan to FGF2 requires its heparan sulfate chains (Andres et al. 1992). Betaglycan binds to TGF-β and increased the affinity of TGF-β to its serine/threonine kinase receptors, type II and type I receptor, to initiate downstream signaling (Blobe et al. 2001; Eickelberg et al. 2002). The betaglycan extracellular domain can be cleaved by membrane type-matrix metalloproteinase as a soluble receptor (Arribas and Borroto 2002; Velasco-Loyden et al. 2004) to inhibit TFG-β activity (López-Casillas et al. 1994; Mythreye and Blobe 2009).

4.4.4 CD44

CD44 is a cell membrane-associated glycoprotein (Brown et al. 1991). It has 19 exons with many splice sites that result in numerous CD44 isoforms. CD44 is a primary receptor for hyaluronic acid (Ponta et al. 2003), and can also interact with

other ECM molecules, such as osteopontin, fibrinogen, fibronectin, collagen, and laminin (Naor et al. 2002). CD44 participates in various cellular functions including lymphocyte activation (Seth et al. 1991; Galandrini et al. 1994), cell-cell and cell-matrix interactions (Lazaar et al. 1994), cell migration (Legg et al. 2002; Thorne et al. 2004), and tumor metastasis (Herrera-Gayol and Jothy 1999; Bourguignon et al. 2001; Wong et al. 2003). The function of CD44 is dependent on its posttranslational modifications. CD44 isoforms can be modified with chondroitin sulfate, heparan sulfate, and keratan sulfte. Heparan sulfate can only attach to the v3 exon of v3-containing CD44 isoform and will bind to many growth factors including HGF, FGF2, fibroblast growth factor 4, and fibroblast growth factor 8 (Bennett et al. 1995; Sherman et al. 1998; Grimme et al. 1999; van der Voort et al. 1999, 2000; Jones et al. 2000; Yu et al. 2002).

CD44 has been shown to be expressed in muscle cells (Yu and Toole 1996). It may regulate myoblast migration and differentiation (Mylona et al. 2006). The CD44 deficient myoblasts showed defects in cell motility and differentiation *in vitro*. Furthermore, the CD44 null mice had delayed regeneration of muscle tissue following injury. However, the mechanism of how CD44 regulating myoblast migration and differentiation needs further study.

4.4.5 Neuropilin

The neuropilins have 2 family members. Neuropilin1 is mainly expressed in heart and placenta whereas neuropilin2 is expressed in liver hepatocytes, kidney distal and proximal tubules. Both neuropilins are transmembrane glycoproteins with a large N-terminal extracellular domain, a transmembrane domain, and a short cytoplasmic domain. The N-terminal domain contains a complement-binding, coagulation factor V/VIII, and meprin domains (Pellet-Many et al. 2008) which allow neuropilins to bind various ligands and co-receptors.

Neuropilin1 can bind to VEGF and semaphorin family members to regulate vascular and neuronal development, respectively (He and Tessier-Lavigne 1997; Kitsukawa et al. 1997; Kolodkin et al. 1997; Soker et al. 1998; Kawasaki et al. 1999; Gu et al. 2002). Neuropilin1 knockout mice are embryonic lethal with severe defects in the vasculature and nervous system. Both neuropilin1 and neuropilin2 have been reported to be expressed in satellite cell cultures from the soleus and extensor digitorum longus muscle and regulate myogenin expression activity (Suzuki et al. 2012). Furthermore, the upregulated expression of neuropilin1 enhances VEGF signaling in exercise-trained skeletal muscle (Ji et al. 2007). The enhanced VEGF signaling promotes myotube hypertrophy and myogenic differentiation (Bryan et al. 2008). The mechanism of how neuropilin1 functions in skeletal muscle and satellite cells are still unclear.

4.5 Cytokines and Growth Factors Affecting Muscle Development and Growth

Cytokines are a diverse group of diffusible, soluble proteins, peptides, or glycoproteins that function as mediators between cells. They are generally associated with immune cells and hematopoietic cells. However, some cytokines exhibit growth factor activities and has been reported to be secreted and function in all sorts of other cells and tissues. For example, cytokines can also be expressed by skeletal muscle cells and affect different stages of myocyte development, including proliferation, differentiation, and regeneration. Pedersen (2013) suggested that peptides and cytokines produced by muscle cells and exert autocrine, paracrine, or endocrine effects should be named as 'myokines'. Interleukin-6, TNF-α, and LIF have been reported to affect the proliferation and differentiation of satellite cells and myoblasts. Both syndecan-4 and glypican-1 have the potential to bind to heparin-binding domain containing cytokines including Macrophage inflammatory protein, interleukin-3, interleukin-7, interleukin-8, interferon gamma, and granulocyte macrophage colony-stimulating factor (Tanaka et al. 1998). In fact, many cytokines, such as interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-10, and interferon gamma have been reported to bind to heparin/heparan sulfate (Lortat Jacob and Grimaud 1991; Clarke et al. 1995; Lipscombe et al. 1998; Spillmann et al. 1998; Borghesi et al. 1999; Mummery and Rider 2000; Salek-Ardakani et al. 2000). According to Tanaka et al. (1998) some cytokines bind to the HSPGs in order to: 1. accumulate cytokines at high concentration; 2. to induce cytokine conformational changes; 3. to promote appropriate conformation changes for initiating signal transduction; and 4. to protect cytokines from degradation. The binding of cytokines to HSPGs are highly specific which indicates the pivotal role of HSPGs in regulating the functions of heparin-binding cytokines.

Growth factors are naturally occurring polypeptides that are capable of regulating cell proliferation, division, and survival. They are usually small proteins less than 200 amino acids. Growth factors in the ECM play a key role in regulating cell functions. They share many similarities with cytokines in regulating cell activities, and the distinction between growth factors and cytokines is arbitrary. They are sometimes interchangeable. Both cytokines and growth factors are potent stimulators or inhibitors of satellite cell proliferation and differentiation. Growth factors can be produced by muscle cells and secreted into the ECM where they are bound to proteoglycans. Heparin-binding growth factors FGF2, HFG, and VEGF all can bind to HSPGs. This binding increases the affinity to interact with their primary receptors and initiate downstream signal transduction (Hartmann et al. 1998; Sperinde and Nugent 1998; Kan et al. 1999; Lin et al. 1999; Clayton et al. 2001). Growth factors that are involved in regulating satellite cell growth and differentiation include but are not limited to TGF-β, IGF, HGF, and FGF2 (Hawke and Garry 2001).

Transforming growth factor-beta inhibits satellite cell differentiation to a myogenic lineage (Allen and Boxhorn 1987). Insulin like growth factor 1 increases proliferation and differentiation by increasing the expression of cell-cycle progression factors during proliferation (Engert et al. 1996) and induces myogenic regulatory factor expression during differentiation (Musaro and Rosenthal 1999). Hepatocyte growth factor can stimulate quiescent satellite cells to enter the cell cycle, increase cell proliferation and inhibit satellite cell differentiation (Allen et al. 1995; Gal-Levi et al. 1998; Miller et al. 2000). Fibroblast growth factor 2 is a strong stimulator of satellite cell proliferation and inhibitor of satellite cell differentiation (Allen and Boxhorn 1989; Yablonka-Reuveni et al. 1999).

4.5.1 Interleukin-6

Interleukin-6 functions in modulating immune responses and regulating energy metabolism (Kishimoto 2005; Pedersen 2012). Skeletal muscle is an important source of IL-6 (Pedersen and Febbraio 2008). Interleukin-6 is expressed in skeletal muscle at a very low levels during the resting stage, but increases rapidly after exercise (Pedersen and Febbraio 2008; Puppa et al. 2012) and during muscle regeneration (Kurek et al. 1996; Kami and Senba 1998). It has been reported that IL-6 promotes myogenic satellite cell proliferation (Austin et al. 1992; Serrano et al. 2008), and myotube formation (Baeza-Raja and Muñoz-Cánoves 2004; Al-Khalili et al. 2006). Additionally, Tsujinaka et al. (1996) reported that that IL-6 transgenic mice showed atrophy and enhanced muscle proteolysis. Protein degradation was also accelerated by IL-6 treatment in C2C12 myotubes (Ebisui et al. 1995). Furthermore, IL-6 may be involved in muscle ECM remodeling (White et al. 2009). Mummery and Rider (2000) reported that IL-6 can bind to heparin and heparan sulfate to retain IL-6 close to its secretion site and function in a paracrine manner. During muscle regeneration period, IL-6 may be secreted and bind to cell membrane-associated proteoglycans at the site of injury to degrade muscle cell debris and promote satellite cell proliferation and form new myotubes.

4.5.2 Leukemia Inhibitory Factor

Leukemia Inhibitory Factor is a myokine that belongs to the IL-6 cytokine superfamily (Broholm et al. 2008). Leukemia Inhibitory Factor is involved in a wide variety of biological activities including regulating embryonic stem cell differentiation and stimulating myoblasts proliferation. Leukemia Inhibitory Factor is expressed in skeletal muscle at a low level and up-regulated during muscle regeneration process (Kurek et al. 1998; Schoser et al. 1998; Sakuma et al. 2000). It has been shown to affect muscle cell growth, differentiation, and muscle regeneration (Kami and Senba 1998; Sakuma et al. 1998, 2000; Austin et al. 2000; Reardon et al. 2000; Jo et al. 2005; Spangenburg and Booth 2006). Spangenburg and Booth (2002)

reported that LIF can induce skeletal muscle satellite cell proliferation through activation of the JAK2-STAT3 signaling pathway. Satellite cell proliferation is essential for skeletal muscle regeneration and hypertrophy (Rosenblatt et al. 1994; Hawke and Garry 2001; Mitchell and Pavlath 2001). Leukemia Inhibitory Factor functions as an indispensable factor in both processes (Barnard et al. 1994; Spangenburg and Booth 2006). However, it is still unclear if membrane-associated HSPGs are involved in LIF regulated satellite cell growth and regeneration.

4.5.3 Tumor Necrosis Factor-α

Tumor Necrosis Factor-α is a central mediator of cellular inflammatory and apoptotic signaling pathways. It is primarily produced by activated macrophages. In addition to macrophages, muscle cells are also a source of TNF-α. Muscle cells constitutively express TNF-α (Saghizadeh et al. 1996), especially during the regeneration process. In response to TNF-α, the skeletal muscle myocytes undergo protein loss (Garcia-Martinez et al. 1993; Li et al. 1998; Li and Reid 2000) and promote muscle muscle regeneration. Mice deficient of TNF-α have been shown to have decreased MyoD expression and myogenesis after injury. Muscle cell proliferation and differentiation can also be regulated by TNF-α (Layne and Farmer 1999; Guttridge et al. 2000; Warren et al. 2002). In mouse C2 cells, TNF-α enhanced myoblast proliferation and inhibited differentiation (Szalay and Duda 1997), however, in rat L8 myoblasts, TNF-α inhibited both cell proliferation and fusion (Ji et al. 1998).

The interaction between TNF-α and the membrane-associated proteoglycans is still not clear. Tumor Necrosis Factor-α has been shown to enhance syndecan-4 expression in the endothelium-like EAhy926 cells (Okuyama et al. 2013). On the other hand, TNF-α expression can be up-regulated by HSPG in murine microglia (Bussini et al. 2005). In the intestinal epithelium, syndecan-1 can down-regulate inflammatory cytokines, such as TNF-α and IFN-gamma, to maintain the intestinal epithelial barrier integrity. Bode et al. (2008) proposed that syndecan-1 plays a direct role in sealing the gaps between epithelial cells to prevent protein leakage during this process.

4.5.4 Hepatocyte Growth Factor

Hepatocyte growth factor is a cytokine produced by mesenchymal cells that functions in regulating cellular growth, motility and morphogenic activities in many tissues and organs. The activation and proliferation of hepatocytes, renal tubule cells, endothelial cells, and epithelial cells have been reported to be regulated by HGF (Michalopoulos 1995). In skeletal muscle, HGF is involved in myogenesis, regeneration, and wound healing (Gallagher and Lyon 2000). Takayama et al.

(1996) reported that the overexpression of HGF in transgenic mice results in the abnormal development of skeletal muscle. Furthermore, quiescent satellite cells can be activated by HGF to enter cell cycle for myogenesis (Allen et al. 1995; Anastasi et al. 1997) by interacting with the c-met proto-oncogene receptor (Gonzatti-Haces et al. 1988).

Hepatocyte Growth Factor is located in the ECM in uninjured muscle and released from the ECM and presented to the c-met receptor to initiate downstream signal transduction upon stress stimulation (Tatsumi et al. 2001, 2002). The rapid increase in HGF concentration in response to muscle injury or stretch may come from the muscle fiber and satellite cell ECM (Tatsumi et al. 1998; Tanaka et al. 2006), and be synthesized by activated satellite cells (Sheehan et al. 2000). Heparan sulfate appears to play a role in forming the HGF-c-met complex. Derksen et al. (2002) reported that syndecan-1 can bind to HGF and strongly promotes HGF-mediated signaling by activating its receptor tyrosine kinase, Met. The binding of syndecan-1 also promotes activation of phosphatidylinositol (PI) 3-kinase/protein kinase B and RAS/MAP kinase pathways to regulate cell proliferation and survival.

4.5.5 Insulin-Like Growth Factors

Insulin-like growth factors share high sequence similarity with insulin (Froesch and Zapf 1985). They are small single chain peptides consist of closely related two forms: IGF-I and IFG-II. Both IGF-I and IGF-II are both important in regulating cell proliferation, differentiation, and metabolism (Allen and Boxhorn 1989). IGF-I has been shown to induce satellite cell proliferation (Adams and Haddad 1996; Adams and McCue 1998), and muscle hypertrophy (Rosenblatt and Parry 1992; Adams and Haddad 1996). The IGF-I knockout mice have decreased muscle mass and development which indicates a role of IGF-I in muscle growth and development (Powell-Braxton et al. 1993). Insulin-like growth factor-I contributes to skeletal muscle hypertrophy by enhancing satellite cell activation and proliferation, muscle specific gene expression and protein synthesis (Florini et al. 1996; Chakravarthy et al. 2000; Chargé and Rudnicki 2004; Philippou et al. 2007). IGF-1 binds to the tyrosine kinase IGF-1 receptor to initiate PI3-kinase and MAP kinases pathways to regulate cell survival, proliferation, and growth (Coffer et al. 1998; Delafontaine et al. 2004). IGF-I has been shown to bind to ECM molecules to affect cell survival and migration (Kricker et al. 2003; Kashyap et al. 2011).

4.5.6 Transforming Growth Factor-Beta

The TGF-β superfamily contains more than 30 members in mammals. They are involved in diverse biological activities, regulating cell proliferation, differentiation, and migration in many cell types. In adult muscle cells, TGF-β negatively affects cell regeneration by inhibiting satellite cell proliferation, differentiation, and

muscle-specific gene expression (Allen and Boxhorn 1987; Florini and Magri 1989). Many TGF-β superfamily members including transforming growth factor-beta 1(TGF-β1), transforming growth factor-beta 2 (TGF-β2), and bone morphogenetic protein-2, -4 and -7 have been shown to bind to heparin and heparan sulfate (Ruppert et al. 1996; Lyon et al. 1997; Ohkawara et al. 2002; Irie et al. 2003; Takada et al. 2003). The membrane-associated proteoglycan betaglycan, also known as TGF-β receptor type III, functions as a co-receptor to present TFG-β ligands to type II receptor (Blobe et al. 2001; Eickelberg et al. 2002). The binding of TGF-β to heparin or HSPGs retain them at the sites of secretion within the tissues. However, the TGF-β superfamily members do not share a conserved heparin and heparan sulfate binding sites (Rider 2006). Lyon et al. (1997) reported that the binding of heparan sulfate to TGF-β1 and -β2 has no effect on receptor signaling, instead it protects the growth factor from inactivation by α2-macroglobulin. The mechanism of membrane-associated proteoglycan modulation by the TGF-β superfamily responsiveness remains to be studied.

4.6 Conclusions

Satellite cells are skeletal muscle stem cells responsible for postnatal muscle growth and regeneration. The activation of satellite cells decreases with age and remains quiescent in adults. An important factor to regulate satellite cell activity is the ECM. The ECM not only functions as a structural scaffold for cells to reside in, but also directly regulating cell behavior. Many molecules in the ECM play a critical role in regulating satellite cell activities including activation, proliferation, and differentiation (Bentzinger et al. 2010). Extracellular matrix mediated signal transduction includes growth factors and cytokines.

Upon damage, the ECM component of satellite cells changes to activate the satellite cells. The activated satellite cells contribute to muscle regeneration and hypertrophy. These two processes include the interactions of satellite cells with the immune system and growth factors. The immune system leads to inflammation at the site of damage in order to clean up the waste products in the injured area and repair the damage. Both neutrophils and macrophages phagocytose muscle debris and secret chemokines and cytokines to amplify the inflammatory response. Cytokines serve as the directors of immune cells to stimulate the arrival of lymphocytes, nuetrophils, monocytes, and also satellite cells to the site of injury. During this process, cytokines may bind to the HSPGs in order to accumulate a high concentration and function properly without degradation (Tanaka et al. 1998). Growth factors are the most powerful factor regulating satellite cells activities (McFarland 1999). Heparin-binding growth factors including FGF2, HFG, and TGF-β have been reported to modulate satellite cell activation, migration, proliferation, and differentiation (Hawke and Garry 2001). In these processes, membrane-associated HSPGs function as a co-receptor for many growth factors to enhance their binding

affinity to their primary receptors and initiate downstream signal transduction (Hartmann et al. 1998; Sperinde and Nugent 1998; Kan et al. 1999; Lin et al. 1999; Clayton et al. 2001). Future research will need to address how the membrane-associated proteoglycans binding to growth factors, especially cytokines in regulating satellite cell behavior. Understanding the mechanism of how membrane-associated proteoglycans interact with growth factors and cytokines in satellite cells will shed light on muscle regeneration and excise related muscle hypertrophy.

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Chapter 5
The TGF-β Signalling Network in Muscle Development, Adaptation and Disease

Justin L. Chen*, Timothy D. Colgan*, Kelly L. Walton, Paul Gregorevic, and Craig A. Harrison

Abstract Skeletal muscle possesses remarkable ability to change its size and force-producing capacity in response to physiological stimuli. Impairment of the cellular processes that govern these attributes also affects muscle mass and function in pathological conditions. Myostatin, a member of the TGF-β family, has been identified as a key regulator of muscle development, and adaptation in adulthood. In muscle, myostatin binds to its type I (ALK4/5) and type II (ActRIIA/B) receptors to initiate Smad2/3 signalling and the regulation of target genes that co-ordinate the balance between protein synthesis and degradation. Interestingly, evidence is emerging that other TGF-β proteins act in concert with myostatin to regulate the growth and remodelling of skeletal muscle. Consequently, dysregulation of TGF-β proteins and their associated signalling components is increasingly being implicated in muscle wasting associated with chronic illness, ageing, and inactivity. The growing understanding of TGF-β biology in muscle, and its potential to advance the development of therapeutics for muscle-related conditions is reviewed here.

Keywords Skeletal muscle wasting • Neuromuscular disorders • Myostatin • Activin • TGF-β network

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*Author contributed equally with all other contributors.

J.L. Chen
Centre for Endocrinology and Metabolism, Hudson Institute of Medical Research, Clayton, VIC, 3168, Australia

Department of Molecular and Translational Sciences, Monash University, Melbourne, VIC, Australia

Muscle Research and Therapeutics Development, Baker IDI Heart and Diabetes Institute, Melbourne, VIC 3004, Australia

Department of Biochemistry and Molecular Biology, Monash University, Melbourne, VIC, Australia

T.D. Colgan
Muscle Research and Therapeutics Development, Baker IDI Heart and Diabetes Institute, Melbourne, VIC 3004, Australia

Department of Physiology, The University of Melbourne, Melbourne, VIC, Australia

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5.1 Introduction

Skeletal muscle is a highly adaptive tissue, capable of responding to imposed physiological stimuli, to tune its performance as a contractile tissue, a metabolic regulator, and a secretor of factors that influence other organs. Finely regulated processes controlling protein synthesis and degradation enable changes in muscle mass and function as demands require.

The adaptive properties of skeletal muscle also make it susceptible to loss of mass and functional capacity, when protein turnover is adversely affected in pathological contexts. Significant wasting of skeletal muscle is associated with many diseases, including (but not limited to) advanced progression of certain cancers, sepsis, organ failure, HIV/AIDS, anorexia, disuse, advanced ageing and neuromuscular disorders (Ciciliot et al. 2013; Dam et al. 2014; Jung et al. 2014; Mondello et al. 2015; Puthucheary et al. 2013; Tang et al. 2002; Visvanathan and Chapman 2009; Wall et al. 2014). As the debilitating effects of muscle wasting reduce quality of life and survival, placing significant burden on our healthcare system, it is necessary to understand the mechanisms that regulate muscle attributes, and use this information to advance the development of muscle-directed therapeutics.

Increasingly, muscle wasting observed in chronic illness is being linked with perturbed regulation of the transforming growth factor-β (TGF-β) signalling network. The TGF-β ligand family comprises 34 structurally-related proteins (Cusella-De Angelis et al. 1994; Wu and Hill 2009) broadly classified as: TGF-β isoforms, growth differentiation factors (GDFs), bone morphogenetic proteins

K.L. Walton
Centre for Endocrinology and Metabolism, Hudson Institute of Medical Research, Clayton, VIC 3168, Australia

Department of Molecular and Translational Sciences, Monash University, Melbourne, VIC, Australia

P. Gregorevic (✉)
Muscle Research and Therapeutics Development, Baker IDI Heart and Diabetes Institute, Melbourne, VIC 3004, Australia

Department of Biochemistry and Molecular Biology, Monash University, Melbourne, VIC, Australia

Department of Physiology, The University of Melbourne, Melbourne, VIC, Australia

Department of Neurology, School of Medicine, The University of Washington, Seattle, WA, USA
e-mail: Paul.Gregorevic@bakeridi.edu.au

C.A. Harrison (✉)
Centre for Endocrinology and Metabolism, Hudson Institute of Medical Research, Clayton, VIC 3168, Australia

Department of Molecular and Translational Sciences, Monash University, Melbourne, VIC, Australia

Department of Physiology, Monash University, Melbourne, VIC, Australia
e-mail: Craig.Harrison@hudson.org.au

(BMPs), activins and inhibins (Massague 1998). Other members include Nodal, lefty and anti-Müllerian hormone. Myostatin (also known as GDF-8) has been identified as a critical regulator of muscle development and homeostasis (Mcpherron et al. 1997), although subsequent studies have begun to identify other TGF-β proteins, such as TGF-β1, activins and several BMPs, as also having crucial roles in muscle. This review considers the significance of signalling regulated by the TGF-β network as a regulator of muscle attributes in relation to musculoskeletal disorders.

5.2 The TGF-β Signalling Network in Overview

In the human body, the vast majority of cell types express at least one TGF-β family ligand and their cognate receptors (Blobe et al. 2000). Together, TGF-β proteins and their signalling components exert physiological control over proliferation, differentiation, apoptosis, adhesion and extracellular matrix deposition, thereby controlling embryogenesis, organogenesis and adult tissue homeostasis (Buijs et al. 2007; Chang et al. 2002; Massague 1990; Shi and Massague 2003). While this review focuses on myostatin and other TGF-β family proteins with emerging roles in the biology and disease of skeletal muscle, it is beneficial to review the processes that control ligand synthesis and activity.

5.2.1 Ligand Synthesis and Secretion

Like most TGF-β family proteins, myostatin is synthesised as a precursor protein comprising an N-terminal prodomain and a C-terminal mature domain (Fig. 5.1). During biosynthesis, hydrophobic residues at the N-terminus of the prodomain bind to residues in the mature domain, an interaction that is critical for correct protein formation (Walton et al. 2010). Two monomers are linked covalently at a site within the mature domain (Husken-Hindi et al. 1994), and non-covalently between the two prodomains. Following complex assembly, proprotein convertases cleave between the pro- and mature domains (Dubois et al. 1995; Gentry et al. 1988), resulting in a covalently bound mature protein non-covalently associated to its prodomain dimer. From herein, TGF-β family proteins are referred to as to the ‘mature dimer’ and the resultant prodomain dimer is described as the ‘prodomain’. Following synthesis, the mature dimer and prodomain complex is secreted from the cell, and in many instances, localised to the extracellular matrix (ECM).

Some TGF-β proteins, such as myostatin, activins and TGF-β isoforms, bind to the ECM via specific interactions (Ramirez and Rifkin 2009; Rifkin 2005; Sengle et al. 2008a). The ECM network of glycoproteins plays essential roles in the structure, survival, migration and proliferation of cells (Assoian et al. 1983; Brunner et al. 1989; Gentry and Nash 1990), and can also act as an important intermediate reservoir for many members of the family prior to their activation (Fig. 5.2) (Sengle et al. 2008a). ECM components vary between organs as they are temporally and spatially expressed and secreted by proximal tissues, but the three major proteins

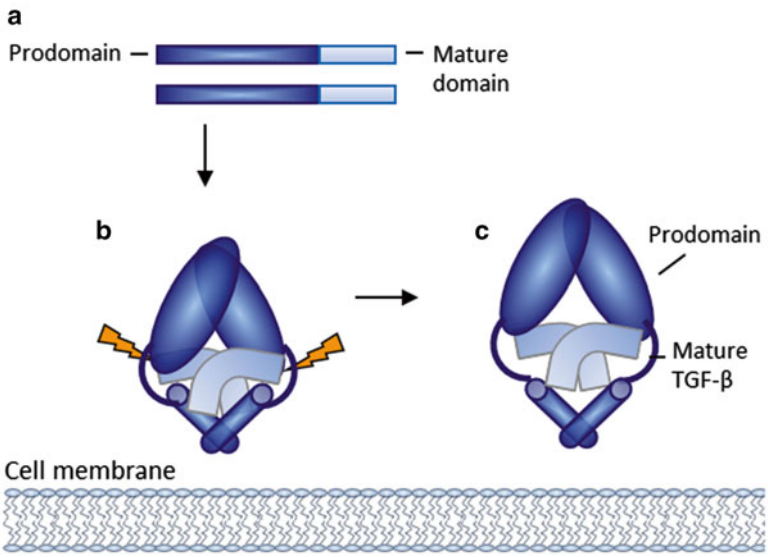

Fig. 5.1 Synthesis of TGF-β proteins. (**a**) TGF-β proteins are synthesised as precursor proteins consisting of an N-terminal prodomain and a C-terminal mature domain. Prodomains form tight contacts within their mature domains, which facilitate folding and dimerisation. (**b**) Dimeric precursors are cleaved between the pro- and mature domains by proprotein convertases, such as furin, to form (**c**) a complex comprising prodomain non-covalently associated to the mature TGF-β dimer

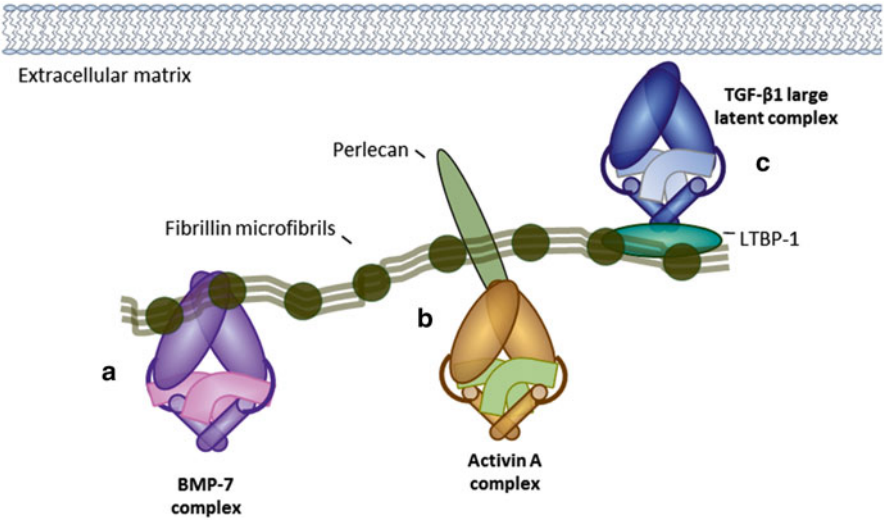

Fig. 5.2 Localisation of TGF-β proteins to the extracellular matrix. The prodomains target TGF-β proteins to the ECM in the vicinity of target cells. (**a**) Some TGF-βs, such as BMP-7 bind directly to fibrillin microfibrils in the ECM, (**b**) while others, such as activin A, bind fibrillin-associated proteins, such as perlecan. (**c**) The TGF-β isoforms bind to LTBPs, which then bind to fibrillin in the ECM

comprising the ECM are collagen, fibronectin and fibrillin (Vakonakis and Campbell 2007). Interestingly, TGF-β proteins are also critical regulators of ECM production, regulating collagen synthesis and deposition by fibroblasts (Akhurst and Hata 2012).

The prodomains of the TGF-β isoforms bind the ECM via an intermediate association with latent TGF-β-binding proteins (LTBPs). Prodomain binding to LTBPs promotes the formation of large latent complexes (LLCs). The C-terminal region of LTBPs binds to the N-terminal region of fibrillin, targeting LLCs to the ECM, an interaction that is essential for normal TGF-β signalling (Ramirez et al. 2008). Other TGF-β proteins, including BMP-2, -4, -7, -10 and GDF-5 can bind directly to fibrillin via their prodomains (Sengle et al. 2008a), whereas activin prodomains can bind heparin sulphate proteoglycans, such as perlecan, in the ECM (Li et al. 2010).

5.2.2 Prodomains Control TGF-β Ligand Activity and Localisation in the Extracellular Matrix

Prodomains can modulate the signalling potential of their associated TGF-β proteins. For the majority of TGF-β family members, the interaction between the mature protein and their associated prodomains is weak, allowing them to be readily displaced in the presence of their target receptors (Sengle et al. 2008b; Walton et al. 2009). However, myostatin, GDF-11, TGF-β1, TGF-β2, TGF-β3, BMP-10 and human GDF-9 bind their prodomains with high affinity and are secreted in a latent form (Simpson et al. 2012). The prodomains confer latency by shielding the receptor-binding epitopes (Bottinger et al. 1996; De Crescenzo et al. 2001; Ge et al. 2006; Hill et al. 2002; Thies et al. 2001).

The latency of some TGF-β proteins provides an additional barrier in activity regulation. Myostatin is expressed in skeletal muscle and is a potent negative regulator of muscle mass, therefore an additional level of regulation is necessary to constrain its effects. The BMP-1/tolloid family of metalloproteinases is hypothesised to activate latent myostatin, by cleaving after the aspartate-76 residue within the myostatin prodomain and releasing mature active myostatin.(Lee 2008; Wolfman et al. 2003). Other latent TGF-βs can be activated by thrombospondin-1 and integrins, which alter the conformation of the prodomain enabling the release of the mature active proteins (Fig. 5.3) (Annes et al. 2004; Ribeiro et al. 1999).

5.2.3 TGF-β Family Members Target Specific Receptor Complexes and Transcription Factors

The biological effects of myostatin are initiated upon engagement of the ligand with two specific transmembrane serine/threonine kinase receptors (Fig. 5.4). Binding of mature myostatin to the extracellular domain of a type II activin receptor (ActRII)

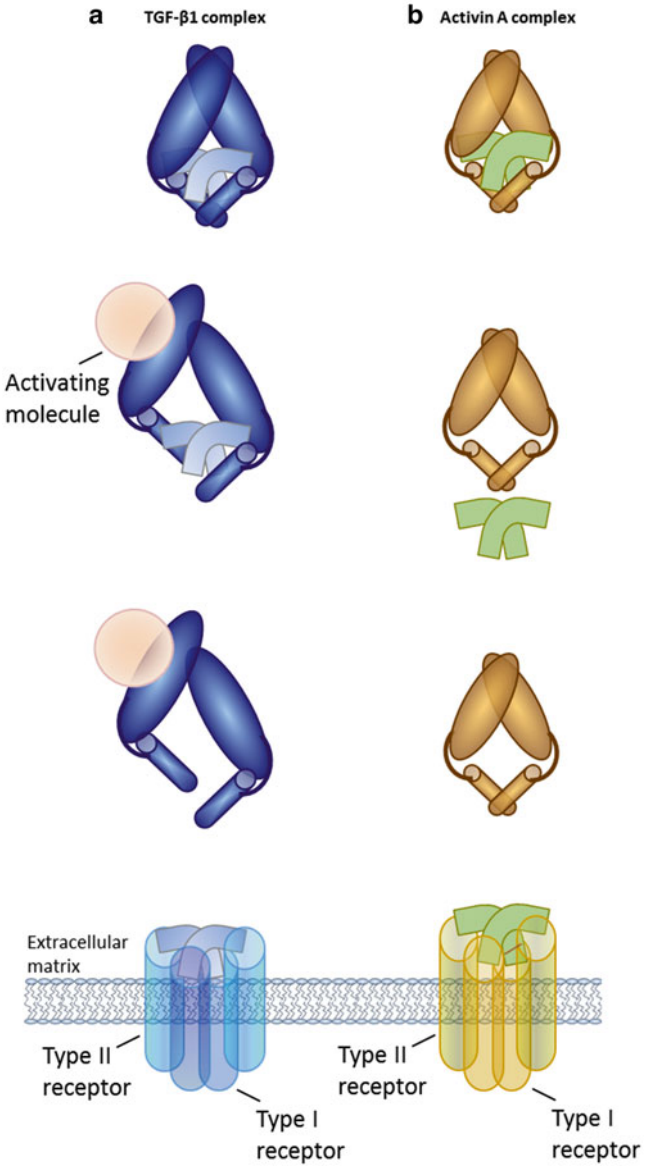

Fig. 5.3 TGF-β activation. (**a**) The TGF-β isoforms, myostatin and GDF-11 bind their prodomains with high affinity, with the prodomains shielding the receptor-binding epitopes. In order for these ligands to signal, they must be liberated from their prodomains via an activation mechanism, enabling them to bind to their receptors. (**b**) For the other TGF-β proteins, such as activin A, they are secreted in an 'active' form. These ligands have a higher affinity for their signalling receptors than their prodomains and do not require an activation step

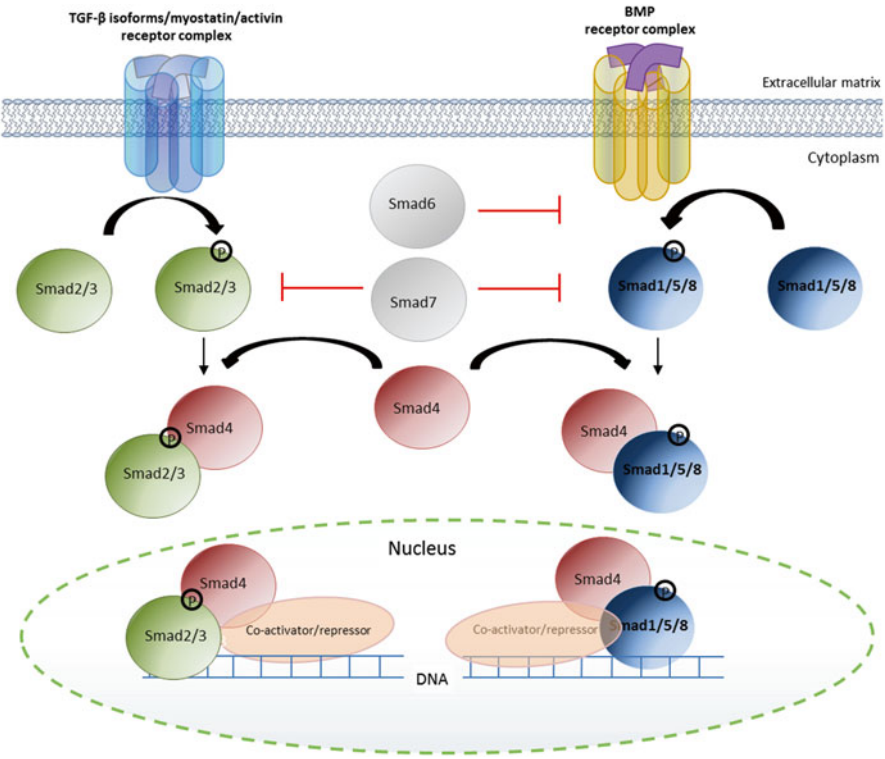

Fig. 5.4 TGF-β signalling pathway. TGF-β signalling is divided into two main intracellular pathways (the TGF-β and BMP pathways) according to the type I and II receptors the ligands target and the intracellular Smads they activate. The binding of a growth factor to a type II receptor leads to recruitment of a type I receptor. The TGF-β subgroup phosphorylate type I receptors. In turn, type I receptors phosphorylate Smads 2 or 3. Conversely, activation by the BMP subgroup leads to phosphorylation of Smads 1, 5 or 8. All Smads form complexes with Smad4, and translocate to the nucleus to regulate gene transcription with co-activators, co-repressors and additional transcription factors. Smad6 and 7 are inhibitory Smads that prevent Smads forming complexes with Smad4, and also bind to type I receptors

leads to recruitment, phosphorylation and activation of a type I activin receptor (ActRI), also known as an activin receptor-like kinase (ALK) (Ten Dijke and Arthur 2007). Type II and type I transmembrane receptors are classified by their structural and functional properties. There are five mammalian type II receptors: ActRIIA, ActRIIB, TβRII, BMPRII and AMHRII, and seven type I receptors: activin receptor-like kinases (ALK) 1-7 (Shimasaki et al. 2004). Predominantly, the type II receptors facilitate ligand engagement, whereas the type I receptors determine the signalling specificity by phosphorylating specific Smad proteins that modulate intracellular signalling and regulate target gene transcription (Ten Dijke and Arthur 2007).

Myostatin can signal through the type II receptors ActRIIA/B and BMPRII, and the type I receptors ALK4 and ALK5 (Sidis et al. 2006; Tsuchida et al. 2004). Activation of ALK4 and ALK5 receptors proceeds to phosphorylate intracellular Smad2 or 3 transcription factors (Wrighton et al. 2009b), which then bind to Smad4, and the resulting Smad complex translocates to the nucleus to regulate gene transcription (Massague and Gomis 2006; Wrighton et al. 2009a). Other TGF-β proteins that initiate Smad2/3 signalling include the TGF-β isoforms, activins and Nodal. Notably, in parallel with this ActRII-Smad2/3 signalling pathway, the TGF-β network supports signalling mediated by other ligands that activate ALKs1, 2, 3 and 6 to phosphorylate Smad1/5/8. BMPs, other GDFs and AMH can employ this axis via their binding to discrete type II receptors (Massague and Gomis 2006).

As there are only a handful of type I and type II receptors for the large number of TGF-β proteins, several TGF-β proteins can utilise the same receptor complexes. For example, activin A, activin B, BMP-4, BMP-7 and GDF-5 can bind ActRIIA/B (Greenwald et al. 2003; Harrison et al. 2004; Tsuchida et al. 2004). Similarly, BMP-2, BMP-4, BMP-7, BMP-15 and GDF-9 bind BMPRII (Shi et al. 2000). In some circumstances, more than one ligand can utilise the same receptor complex; both myostatin and activin A can initiate signalling via ActRIIA/B and ALK4 (Tsuchida et al. 2004). The interplay of numerous ligands and receptor combinations and the interaction of downstream Smad signalling with other pathways varies by cell type and context, thus creating a system for highly varied and nuanced effects of the TGF-β network.

5.2.4 Extracellular Regulation of TGF-β Signalling

Further regulation of TGF-β signalling is facilitated by extracellular, membrane-bound and intracellular proteins. Some members of the TGF-β family also require accessory receptors, such as betaglycan, endoglin and cripto, to enhance their interaction with signalling receptors. For example, betaglycan acts as a co-receptor for the TGF-β isoforms, most notably TGF-β2, and inhibin (Cheifetz et al. 1990; Lewis et al. 2000). Within cells, inhibitory Smad proteins 6 and 7 govern TGF-β signalling through a negative feedback loop. Smad6 and 7 bind to type I receptors, to inhibit the phosphorylation of receptor-regulated Smads (Gazzerro and Canalis 2006). In addition, Ski binds to Smad4 to prevent the formation of Smad complexes, as well as retaining existing Smad complexes bound to DNA to prevent binding of new complexes (Luo 2003).

Follistatin is an extracellular protein found in circulating and membrane-bound forms, and exhibits high affinity binding for activins, but also bind myostatin, GDF-11 and some BMPs (Lee and Mcpherron 2001; Nakamura et al. 1990; Tsuchida 2006). In the case of activins, follistatin neutralises activity by shielding approximately a third of the residues on the mature ligand, including both the type I and type II receptor-binding sites (Harrington et al. 2006). Follistatin-related gene (FLRG) is an inhibitor of TGF-β signalling that also contains a follistatin-binding

domain, which binds similar ligands as follistatin (Tsuchida et al. 2000). There are also specific extracellular BMP antagonists, such as noggin, that preferentially bind certain BMP family members and reduce their access to signalling receptors (Gazzerro and Canalis 2006; Shimasaki et al. 2004). Collectively, these extracellular proteins confer an additional layer of regulation to control the bioactivity of the TGF-β family of ligands.

5.3 The Role of TGF-β Signalling in Skeletal Muscle Development and Homeostasis

The formation of skeletal muscle (myogenesis) commences early during embryonic development. Pluripotent muscle progenitor cells originating from the somites give rise to myoblasts, the precursor cells of skeletal muscle fibres (Kubota et al. 1989; Mauro 1961). The fusion of myoblasts produces multi-nucleated muscle fibres, which, remarkably, can be maintained for life. In parallel, a population of progenitor cells persist as 'satellite cells', which reside in proximity with muscle fibres as a reservoir of cells for repair of muscle fibres. Proliferation and recruitment of the resident satellite cells occurs when damaged muscle fibres must be repaired (or regenerated). Post-natal adaptation of muscle mass occurs largely via changes in the size of existing muscle fibres, although in some instances recruitment of satellite cell may also provide a means of contributing additional nuclei to existing fibres during episodes of growth.

Myostatin, in particular, has been defined as a potent negative regulator of skeletal muscle mass (Mcpherron et al. 1997). Myostatin knockout mice (*Mstn*$^{-/-}$) exhibit dramatically increased muscle mass as a result of increased fibre numbers being formed during development, and increased fibre size in adulthood. The significance of myostatin in skeletal muscle is evident from its conservation across species, as loss of myostatin during development has been associated with a 'hypermuscular' phenotype in rodents (mice, rats), dogs, birds (chickens, turkeys), sheep, pigs, horses and humans. Although other members of the TGF-β family have also been found to exert unique effects upon muscle development and post-natal adaptation (Kollias and Mcdermott 2008; Lee et al. 2005), myostatin remains arguably the most dominant regulator of skeletal muscle phenotype in development.

5.3.1 Skeletal Muscle Development

Cells of somatic origin are directed down a program of myogenesis commencing early in embryonic development according to episodes of determination, differentiation and maturation. Determination begins with the formation of pluripotent

muscle progenitor cells (Mauro 1961) that give rise to myoblasts, the precursor cells of skeletal muscle fibres (Kubota et al. 1989). Myoblasts completing an active proliferative phase typically undergo differentiation, which entails irreversible withdrawal from the cell cycle, and the expression of early muscle-specific genes, including the MyoD and MEF2 family of transcription factors (Joulia-Ekaza and Cabello 2006). These myogenic factors support myoblast commitment and fusion, and the subsequent expression of late muscle-specific genes (Acharyya et al. 2004; Megeney et al. 1996; Wheeler et al. 1999). As myoblasts fuse into post-mitotic syncytial structures spanning from tendon to tendon, the specialised proteins required to assemble the excitation-contraction apparatus are synthesised, and support the maturation of muscle fibres. For most species, the number of skeletal muscle fibres is determined before birth and maintained largely until approaching senescence.

5.3.2 Myostatin as a Regulator of Myogenesis

During embryonic myogenesis, myostatin signalling initiates the Smad2/3 complex to translocate to the nucleus and block production of the muscle transcription factor MyoD. Inhibition of MyoD prevents myoblast proliferation, differentiation and fusion through cell cycle arrest (i.e., myoblasts accumulate in the G1 and G2 phase of the cell cycle) (Allen and Unterman 2007; Langley et al. 2002; Rios et al. 2002; Thomas et al. 2000; Zhu et al. 2004). This coincides with up-regulation of p21, a potent inhibitor of the cyclin-Cdk complexes (Thomas et al. 2000) that support cell cycle progression. Cyclin-Cdk complex inhibition results in reduced phosphorylation of Rb, a tumour suppressor protein that inhibits cell growth. Hypophosphorylated Rb inhibits cell cycle progression by binding and repressing the activity of the E2F family of transcription factors (Lam and La Thangue 1994), thereby preventing their transcription of S-phase-specific genes (La Thangue 1996).

5.3.3 Myostatin as a Regulator of Muscle Fibre Size

In mature muscle fibres, the maintenance of size is dependent on the balance between protein synthesis and protein degradation. While these processes are paramount to maintaining muscle health and homeostasis, the interaction of signalling pathways that influence protein turnover in muscle continues to be expanded upon. As with the process of muscle fibre formation, myostatin appears to play a prominent role in governing the processes controlling muscle fibre size.

Stimulation of the canonical Smad2/3 pathway by myostatin suppresses the transcription and activity of MyoD and the myogenic regulatory factor myogenin to limit protein synthesis. While these effects serve to maintain satellite cells in a quiescent state (Langley et al. 2002; Liu et al. 2001), the same process limits the growth

of muscle fibres by restricting expression of muscle proteins. Although myostatin has been shown to activate the canonical Smad pathway in muscle, crosstalk with other non-Smad pathways also contributes to the regulation of protein turnover in muscle fibres (Kollias and Mcdermott 2008; Mcpherron et al. 1997) (Fig. 5.5).

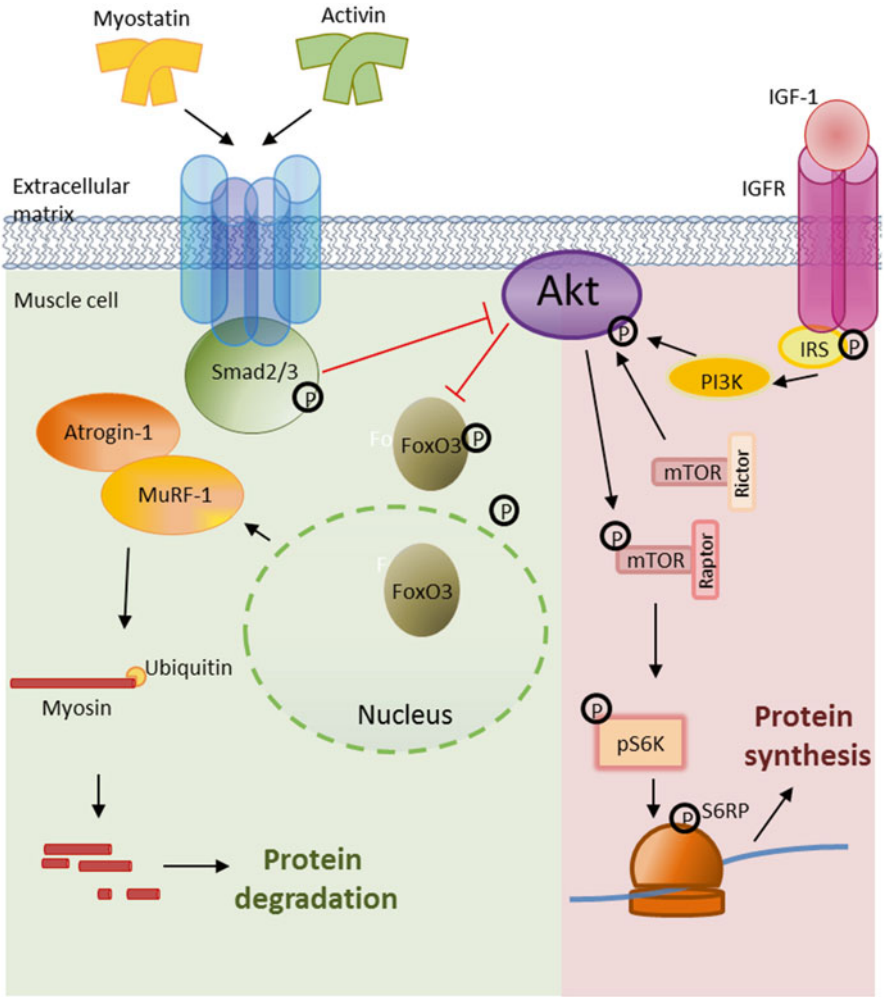

Fig. 5.5 Increased activation of the Smad2/3 signalling pathway results in muscle wasting. Heightened activation of the Smad2/3 signalling pathway, as when myostatin or activin A are overexpressed, leads to inactivation of Akt and additional dephosphorylation of the transcription factor FoxO3. Dephosphorylated FoxO3 can translocate to the nucleus and induce the expression of the muscle-specific ubiquitin ligases, atrogin-1 and MuRF-1. These ligases ubiquitinate myofibrillar proteins, such as myosin, targeting them for degradation via the ubiquitin-proteasome system, resulting in muscle wasting. Akt is also a central mediator of protein synthesis, where its phosphorylation by PI3K activates mTOR-mediated phosphorylation of S6 ribosomal protein to increase protein translation

The IGF-1/PI3K/Akt signalling cascade is a regulator of processes associated with cell cycle proliferation and cell survival, and in muscle, is also a key governor of protein synthesis and glucose metabolism (Chen et al. 2014; Morissette et al. 2009; Trendelenburg et al. 2009; Zdychova and Komers 2005). Phosphorylation of Akt results in increased protein synthesis through activation of mTOR, and subsequently 4E-BP1 and S6K (Bodine et al. 2001; Laplante and Sabatini 2012). mTOR is a key contributor to skeletal muscle growth and as such, can be regulated through a variety of signalling targets. *In vitro* and *in vivo* studies have demonstrated that the absence of myostatin results in increased Akt activity and therefore elevated mTOR signalling (Lipina et al. 2010). Prevention of muscle atrophy also occurs downstream of Akt through inhibition of apoptotic factors such as Bax, and the Forkhead box O (FoxO) transcription factor family (Fig. 5.5) (Hribal et al. 2003). During the differentiation of myoblasts, as myostatin expression increases, FoxOs are dephosphorylated and enter the nucleus, where they promote transcription of the muscle-specific E3-ubiquitin ligases, atrogin-1 and MuRF-1 (Zhao et al. 2007). These muscle-specific ligases mark muscle proteins for degradation via the ubiquitin-proteasome pathway. During homeostasis, the protein synthesis and degradation pathways are in balance to maintain muscle mass (Lokireddy et al. 2011; Sartori et al. 2009). In the *Mstn*$^{-/-}$ mouse, the IGF-1/PI3K/Akt pathway is de-repressed, increasing protein synthesis, while FoxO proteins remain in the cytoplasm in the phosphorylated form, unable to initiate transcription of the catabolic E3-ubiquitin ligases (Mcpherron et al. 1997). Thus, elevated expression of myostatin promotes increased atrogin-1 and MuRF-1 transcription, while myostatin inhibition has the opposite effect.

While the Smad/Akt/FoxO axis is a major regulator of muscle size, other cellular pathways intersect with this network, adding an additional layer of control to the system. Crosstalk exists between the Smad pathway and the mitogen-activated protein kinase (MAPK) pathways of the extracellular signal-related kinase 1/2 (ERK1/2), p38 and JNK (Hanafusa et al. 1999; Mulder 2000; Philip et al. 2005). TGF-β proteins can bind to MAPK receptors in a cell-specific and context-dependent manner, but MAPKs that are not activated by TGF-βs are capable of regulating Smad proteins and Smad complexes (Javelaud and Mauviel 2005; Rahimi and Leof 2007). To date, the role of ERK1/2 in skeletal muscle mass remains incompletely defined. Further research in this area could yield new insights into muscle regulation in the future.

5.3.4 Other TGF-β Proteins as Regulators of Muscle Development and Maturation

Although myostatin is well characterised as a negative regulator of muscle, other members of the TGF-β are also essential in guiding myogenesis and supporting muscle homeostasis. For instance, TGF-β1 expressed during myogenesis inhibits

the expression of muscle-specific mRNA and proteins *in vitro* (Massague et al. 1986), and inhibits differentiation of myoblasts (Cusella-De Angelis et al. 1994; Massague et al. 1986; Olson et al. 1986). TGF-β1 has also been shown to inhibit skeletal muscle satellite cell differentiation, proliferation and fusion *in vitro*, suggesting a role in regulating muscle regeneration (Allen and Boxhorn 1987; Li et al. 2004).

Other members of the TGF-β family are also implicated in muscle homeostasis. The broad spectrum TGF-β antagonist, follistatin, binds several TGF-β family members in addition to myostatin, including activins, GDF-11, and BMP-2, -4, -6, and -7 (Glister et al. 2004; Schneyer et al. 2008; Sidis et al. 2006). Transgenic mice over expressing follistatin exhibit dramatically increased muscle mass as a product of increased muscle fibre number and size(Lee and Mcpherron 2001). Similarly, transgenic overexpression of a dominant negative ActRIIB in mice, which binds similar TGF-β proteins as follistatin, also yields a hypermuscular phenotype (Lee and Mcpherron 2001). Importantly, when follistatin or the dominant negative ActRIIB were overexpressed in *Mstn*$^{-/-}$ mice, which already exhibit twice the muscle mass of wild type mice, a further increase in muscle mass was still observed (Lee 2007; Winbanks et al. 2012). As this additional increase occurred in the absence of myostatin, at least one other TGF-β protein capable of binding follistatin and ActRIIB must contribute to the negative regulation of muscle mass under homeostatic conditions. Recent studies have subsequently revealed that activin A and B contribute to the negative regulation of muscle mass (Chen et al. 2015).

Historically, research into the control of muscle homeostasis has focussed on the Smad2/3 signalling arm of the TGF-β family. This has largely left the role of the Smad1/5/8 pathway in skeletal muscle unchartered. However, recent reports have begun to show that the Smad1/5/8 axis is a positive regulator of muscle mass (Sartori et al. 2013; Winbanks et al. 2013). Increases in muscle mass and Smad1/5 phosphorylation were observed when BMP-7, or a constitutively active ALK3, were overexpressed in muscle (Winbanks et al. 2013), with growth mediated by increased mTOR activity. Interestingly, while blockade of Smad2/3 signalling results in muscle hypertrophy, overexpression of Smad6, an inhibitor of Smad1/5/8 signalling, had no effect on endogenous muscle mass (Lee 2007; Lee and Mcpherron 2001; Lee et al. 2005; Winbanks et al. 2013). Collectively, the studies to date suggest that the Smad2/3 arm of the TGF-β signalling pathway is prominent in regulating muscle mass, but that the parallel opposing arm of Smad1/5/8 is required for sustaining or promoting muscle hypertrophy. This latter aspect is noted from observations that muscles upregulate expression of BMPs and their signalling receptors to protect against muscle atrophy in models of denervation. Inhibiting BMP signalling in this context exacerbates muscle wasting (Sartori et al. 2013; Winbanks et al. 2013). Mechanistically, the BMP-Smad1/5/8 signalling axis was shown to negatively regulate the transcription of E3 ubiquitin ligases that drive proteasome-mediated protein breakdown (Sartori et al. 2013; Winbanks et al. 2013). Thus, the BMP-Smad1/5/8 signalling arm of the TGF-β network exerts positive effects on protein synthesis and degradation in muscle depending on the context. These findings suggest multiple TGF-β family members contribute simultaneously to the establishment of skeletal muscle attributes.

5.4 The Role of TGF-β Signalling in Post-natal Muscle Regeneration

The regeneration of muscle fibres after damage recapitulates many of the events involved in myogenesis during embryonic development. Myostatin tightly governs the activity of transcription factors involved with lineage progression within both satellite cells and myoblasts, and by doing so is a key regulator in muscle regeneration following injury (Cornelison et al. 2000; Langley et al. 2002; Mccroskery et al. 2003).

To better dissect the role of myostatin during regeneration, several injury models have been employed. Following injection with myotoxic agents, the muscles of 24-month-old senescent *Mstn*$^{-/-}$ mice regenerate similarly to their wild-type counterparts (Wagner et al. 2005). However, while wild-type muscles eventually established a similar histological profile, injured *Mstn*$^{-/-}$ muscles were able to attain larger myofibre diameters at an earlier phase of regeneration than was possible for wild-type muscles (Wagner et al. 2005). These findings provide evidence for myostatin playing a more complex role in muscle regeneration than solely governing lineage control within muscle itself, and highlight the possibility of non-muscle factors contributing to this regenerative phenotype.

Using a dry-ice injury-regeneration model in *Mstn*$^{-/-}$ and wild-type bovine muscle, it was demonstrated that myostatin can govern chemokine secretion following injury (Iwasaki et al. 2013). The injured muscles of *Mstn*$^{-/-}$ cows did not parallel the level of elevation of chemokines (CXCL1, CXCL2, CXCL6 and CCL2) found in wild-type counterparts, as measured by protein level and transcript abundance: a result that is mirrored *in vitro*. Whether these chemokines are typically all of muscle origin in an *in vivo* setting is yet to be established. These findings, however, highlight that a complete picture of myostatin and TGF-β signalling in muscle regeneration is still being pieced together. Similarly control of muscle size can be heavily influenced by the inflammatory response, which also plays a major role during muscle regeneration.

Inflammatory cells feature heavily in the regenerative process, and are also implicated in pro-fibrotic signalling, throughout which TGF-β is a major coordinator of fibrotic deposition (Bernasconi et al. 1999; Gosselin et al. 2004). After injury, migration of macrophages and fibroblasts increase the production of ECM components (Serrano and Munoz-Canoves 2010). TGF-β, in conjunction with other signalling molecules, including tumour necrosis factor-alpha (TNF-α), promotes the production of ECM components collagen and elastin (Gosselin and Martinez 2004; Gosselin et al. 2004). ECM components provide a support framework within the damaged muscle, which is then degraded during the regeneration process (Serrano and Munoz-Canoves 2010). While external factors can modulate TGF-β signalling during regeneration, myostatin expression is significantly down-regulated in satellite cells, enabling lineage progression and myofibre re-population (Cornelison et al. 2000). This tight regulation of TGF-β signalling is important in controlling muscle remodelling following trauma through injury or disease.

5.5 TGF-β Signalling in Skeletal Muscle Disease

The majority of the 34 TGF-β proteins play critical and overlapping roles in organogenesis and post-natal tissue homeostasis. Intriguingly, these potent signalling proteins only utilise a very small number of receptors. The canonical signalling pathway uses five type II receptors (TβRII, ActRII, ActRIIB, BMPRII and AMHRII) and seven type I receptors (ALKs 1-7) to finely regulate the inputs of the distinct TGF-β proteins. These receptors can interact with multiple TGF-β proteins. Therefore, the expression and activity of ligands is tightly regulated, and this strict control extends to all their associated components including signalling receptors, inhibitors and the downstream molecules that transduce their signals to the nucleus for gene expression.

Owing to these ligands' secreted nature, altered expression of specific TGF-β family proteins in disease settings can exert deleterious effects in either the tissue of origin or at distal sites. Given the diverse biological roles of TGF-β family proteins, it is not surprising that disruption within the signalling network has detrimental consequences for cell function. As examples, many mutations in TGF-β proteins or their associated signalling components have been identified in human disease (Table 5.1). Though mutations within the TGF-β signalling network often result in developmental disorders, vascular diseases and cancer (Gordon and Blobe 2008), ongoing research is revealing that altered TGF-β network signalling also exerts detrimental effects on tissue function, including in skeletal muscle. Consequently, manipulation of TGF-β signalling may offer the means to target TGF-β actions in muscle to improve specific disease states.

5.5.1 Muscular Dystrophy

Collectively, muscular dystrophies are a group of disorders characterised by progressive loss of functional muscle mass and an accumulation of non-functional fibrotic tissue. A large array of genetic mutations have been identified within dystrophic muscle fibres (Cohn and Campbell 2000), predominantly within genes encoding for proteins that make up the dystrophin-associated protein complex (DAPC) (Tsuchida 2006). The DAPC is a highly specialised and regulated network of scaffolding proteins anchoring the outside of the muscle fibre to the inside, which protects muscle fibres from injury under typical mechanical loading (Campbell and Kahl 1989; Ervasti et al. 1990). Proteins of the DAPC not only provide physical links, but also act to transmit signals regarding membrane stability and force transduction (Constantin 2014).

As the most common heritable neuromuscular disorder, Duchenne muscular dystrophy (DMD) is a severe and debilitating condition that typically affects young males, confining them to a wheelchair early in their youth (Engvall and Wewer 2003). In DMD, mutations in the dystrophin gene prevent the production of

Table 5.1 Mutations in the TGF-β signalling pathway in human disease

Protein/gene	Disease	References
TGF-β1	Atherosclerosis, Camurati-Engelmann disease, childhood asthma, cardiovascular disorders, fibrosis, hypertension, osteoporosis, otosclerosis, psoriasis; breast, colon, pancreatic, and prostate cancers; tumour invasion & metastasis	Baran et al. (2007), Border and Noble (1994), Gordon and Blobe (2008), Kinoshita et al. (2000), Li et al. (1999), Maehara et al. (1999), Salam et al. (2007), Thys et al. (2007), Yamada et al. (1998)
TGF-β2	Cleft palate, breast and pancreatic cancer	Gordon and Blobe (2008), Slayton et al. (2003)
TGF-β3	Cleft palate	Tanabe et al. (2000)
GDF-3	Microphthalmia, isolated type 7, Klippel–Feil syndrome type 3	Harrison et al. (2011)
GDF-5	Brachydacyly type A2 and C, Du Pan syndrome, Hunter-Thompson chondrodysplasia, Grebe chondrodysplasia, Proximal symphalangism	Harrison et al. (2011), Lehmann et al. (2007), Thomas et al. (1996, 1997)
GDF-6	Bilateral anophthalmia, microphthalmia	Harrison et al. (2011)
BMP-2	Breast cancer	Clement et al. (2005)
BMP-4	Breast cancer, microphthalmia syndromic type 6, Non-syndromic orofacial cleft type 11	Gordon and Blobe (2008), Harrison et al. (2011)
BMP-7	Breast cancer and ocular developmental disorders	Gordon and Blobe (2008), Harrison et al. (2011)
BMP-10	Cardiomyopathy	Gordon and Blobe (2008)
BMP-15	Premature ovarian failure	Harrison et al. (2011)
Lefty-1	Situs Ambiguus	Gordon and Blobe (2008)
Nodal	Situs Ambiguus, visceral heterotaxy autosomal type 5	Gordon and Blobe (2008), Harrison et al. (2011)
Inhibin-α	Premature ovarian failure	Gordon and Blobe (2008)
ALK1	Familial primary pulmonary hypertension, Hereditary haemorrhagic telangiectasia, gondadotroph tumour	Berg et al. (1997), D'abronzo et al. (1999), Loscalzo (2001)
ALK2	Fibrodysplasia Ossificans Progressiva	Groppe et al. (2007), Shore et al. (2006)
ALK3	Bannayan–Riley–Ruvalcaba, Cowden disease, juvenile polyposis	Howe et al. (2001), Zhou et al. (2001)
ALK5	Breast, cervical, head and neck, and pancreatic cancers; chronic lymphocytic leukaemia, familial thoracic aortic aneurysm syndrome, Furlong syndrome, Loeys–Dietz syndrome	Blobe et al. (2000), Loeys et al. (2005), Matyas et al. (2006), Pannu et al. (2006)
ALK6	Breast cancer, brachydactyly type A2	Lehmann et al. (2003)

(continued)

Table 5.1 (continued)

Protein/gene	Disease	References
AMH	Persistent Müllerian duct syndrome	Belville et al. (1999)
AMHR2	Persistent Müllerian duct syndrome	Belville et al. (1999)
BMPR2	Familial primary pulmonary hypertension	Lane et al. (2000)
TGFBR2	Breast, cervical, colon, endometrial, gastric, glioma, liver, lung, lymphoma, pancreatic and prostate cancers; atherosclerosis, Loeys–Dietz syndrome, familial thoracic aortic aneurysm syndrome, Sphrintzen–Goldberg syndrome	Baldwin et al. (1996), Chen et al. (2013, 2014), Goggins et al. (1998), Gordon and Blobe (2008), Grady et al. (1999), Knaus et al. (1996), Loeys et al. (2005), Matyas et al. (2006), Myeroff et al. (1995), Pannu et al. (2006), Villanueva et al. (1998)
Endoglin	Atherosclerosis, hereditary haemorrhagic telangiectasia	Mcallister et al. (1994), Mccaffrey et al. (1997), Shovlin et al. (1997)
Decreased betaglycan	Pancreatic and prostate cancer	Gordon and Blobe (2008)
Noggin	Multiple synostoses syndrome, proximal symphalangism, Stapes ankylosis	Brown et al. (2002), Gong et al. (1999)
Fibrillin-1	Marfan syndrome	Dean (2007), Ramirez and Dietz (2007)
Smad2	Colorectal, lung and hepatocellular cancers	Eppert et al. (1996), Riggins et al. (1997)
Smad3 mutations	Lung cancer	Gordon and Blobe (2008)
Increased Smad3 nuclear localisation	Prostate cancer	Gordon and Blobe (2008)
Smad4	Bladder, breast, colorectal, head and neck, hepatocellular, gastric, lung, pancreatic, prostate, oesophageal, ovarian and renal cancers	Hahn et al. (1996), Thiagalingam et al. (1996)
Smad4	Familial juvenile polyposis	Howe et al. (1998)
Increased Smad4 nuclear localisation	Prostate cancer	Gordon and Blobe (2008)

functional dystrophin protein (Hoffman et al. 1987; Jacobs et al. 1981; Koenig et al. 1987). Dystrophin is a pivotal member of the DAPC acting both as a large, central tether and as a marshalling molecule for many associated proteins that assemble in its proximity (Constantin 2014). The mutations that cause the loss of dystrophin, or similarly important structural proteins from the DAPC, weaken the overall integrity of muscle fibres. These more fragile fibres are susceptible to contraction-induced injury and subject to bouts of ongoing degeneration and regeneration. Repeated cycles of breakdown and repair are hypothesised to compromise the regenerative process over time (Jiang et al. 2014). Similarly, muscles comprised of structurally

impaired fibres are vulnerable to infiltration by adipose, fibrotic and inflammatory cells, which further impair the muscles' regenerative capacity (Acharyya et al. 2007; Andreetta et al. 2006; Jacobs et al. 1981).

During muscle regeneration after injury, TGF-β1 is increased to regulate a short inflammatory response in damaged muscles, enabling the removal of cellular debris from sites of new fibre formation (Serrano and Munoz-Canoves 2010). In dystrophic muscle, the repeated cycles of regeneration leaves the muscles persistently exposed to TGF-β1, inflammatory cells and their signalling molecules (Cohn et al. 2007). This aberrant signalling promotes the production of collagen and other ECM proteins, exacerbating fibrotic accumulation (Sabatelli et al. 2012; Zanotti et al. 2010). As TGF-β signalling is often associated with facilitating ongoing fibrotic infiltration, TGF-β inhibition has been proposed as potential therapeutic strategy in the dystrophic setting (Chen et al. 2005; Zanotti et al. 2010).

Several studies have investigated the potential outcomes of inhibiting the TGF-β signalling pathway in DMD. Administration of neutralising antibodies against TGF-β significantly resolved fibrosis in treated *mdx* mice, a model for DMD, when compared to their untreated littermates (Andreetta et al. 2006). While no change was observed regarding the ongoing cycles of degeneration and regeneration, there was an increase in inflammatory CD4+ lymphocytes, highlighting a potential hurdle when considering immunomodulation accompanying long-term treatment (Andreetta et al. 2006). As an alternate approach, the angiotensin II type 1 receptor blocker losartan, a widely-used FDA-approved drug for hypertension, has been demonstrated to inhibit TGF-β effects when administered to *mdx* mice, broadly replicating the effects of TGF-β inhibition by neutralising antibodies (Cohn et al. 2007). Losartan administration to *mdx* mice was reported to enhance limb grip strength compared to untreated *mdx* mice (Cohn et al. 2007), and reduce cardiac, diaphragm and skeletal muscle fibrosis (Spurney et al. 2011). Though effects on fibrotic signature have been significant, improvements in cardiac and limb muscle function to date appear relatively modest. These findings suggest that targeting TGF-β signalling may be beneficial in ameliorating fibrosis associated with dystrophy, but offers modest potential to enhance muscle strength.

Consequently, interventions that target other TGF-β members may be required. In this regard, several alternate strategies have been pursued to inhibit the actions of myostatin and other related TGF-β family members that repress muscle mass. These include treatment with a soluble form of the ActRIIB receptor, which comprises the extracellular domain fused to an IgG-Fc domain (Morine et al. 2010; Pistilli et al. 2011), myostatin-neutralising antibodies (Bogdanovich et al. 2002), and the administration of a myostatin prodomain fused to an IgG-Fc domain (Bogdanovich et al. 2005). Increased expression of soluble follistatin (as an inhibitor of myostatin and activins) via gene delivery using adeno-associated virus-based vectors (AAV) has also been pursued (Rodino-Klapac et al. 2009). Promising early studies in mice have supported clinical evaluation of viral vector-mediated administration of follistatin to treat Becker muscular dystrophy and sporadic inclusion body myositis (Mendell et al. 2015). Each of these intervention approaches has been demonstrated to increase muscle mass and functional capacity to an extent, thus supporting the

suggestion that strategic manipulation of TGF-β network activity in dystrophic muscles may hold promise for maintaining or restoring the functionality of musculature in this setting.

5.5.2 *Marfan Syndrome*

Marfan syndrome (MFS) is an autosomal dominant disease resulting from mutations in the fibrillin-1 gene, causing loss of this key protein in the ECM and a crucial regulator of TGF-β signalling (Dietz et al. 1991; Hollister et al. 1990; Kaartinen and Warburton 2003; Neptune et al. 2003). Patients with MFS exhibit a range of clinical symptoms, including defects in the musculoskeletal, cardiovascular and ocular systems. Altered ECM integrity contributes to reduced ability of the aortic wall to withstand intraluminal pressure, which can result in the formation of an aortic aneurysm, a leading cause of morbidity in patients with MFS (Judge and Dietz 2005). Although the most visible manifestation of the disease is the increase in bone growth that results in hyper-flexible joints and malformations of digits, limbs and the chest wall, respiratory muscle dysfunction, poor skeletal muscle development and clinical weakness are also observed (Behan et al. 2003).

The mutations in fibrillin-1 associated with MFS render the fibrillin-1 protein sensitive to proteolysis and degradation (Ramirez and Dietz 2007; Ramirez and Rifkin 2009; Reinhardt et al. 1997). As fibrillin-1 is required for TGF-β localisation via LTBPs (Kaartinen and Warburton 2003; Neptune et al. 2003), the lack of ECM structural integrity in patients with MFS enhances the level of active TGF-β that is released from the matrix (Kaartinen and Warburton 2003; Neptune et al. 2003). TGF-β protein levels that are significantly elevated in the sera of patients with MFS are hypothesised to contribute to MFS aetiology by disrupting signalling during tissue development and adaptation (Hillebrand et al. 2014; Neptune et al. 2003). Mouse studies have also identified TGF-β signalling to be a major contributor to the prolapsing of the mitral valve, a pathology common in MFS (Ng et al. 2004). The dysregulation of TGF-β that can manifest pathologies, such as those found in MFS, highlights the importance of regulating TGF-β signalling through ligand release and activation within the ECM production, as well as ligand production (Ramirez and Dietz 2007).

Administration of a TGF-β neutralising antibody or losartan to fibrillin-1-deficient mice modelling MFS can restore muscle fibre diameter (reduced in the diseased state) to wild-type dimensions, enhance the regenerative capacity of muscles, and slow the progression of aortic pathology (Cohn et al. 2007; Habashi et al. 2006). However, while murine studies show promise for the drug, the use of losartan in individuals with MFS has yielded mixed results. Depending on the study, clinical use of losartan has had modest effects on normalising circulating TGF-β levels (Franken et al. 2014; Groenink et al. 2013; Lacro et al. 2013, 2014; Pees et al. 2013). Moreover, there has been some discrepancy in the apparent effects of losartan treatment on measures of aorta morphology (Franken et al. 2014; Groenink et al. 2013;

Lacro et al. 2013, 2014; Pees et al. 2013). Collectively, these findings serve to provide a unique insight into treating MFS via TGF-β inhibition, highlighting that this disease may be more nuanced than originally anticipated and that researchers will require an even greater understanding of the disease aetiology. Whether TGF-β inhibition through losartan therapy will be clinically effective for all patients with MFS remains to be conclusively determined. While the mismatch between preclinical and clinical trial results could emphasise the innate differences that exist between the physiology of mouse and man, the findings are indicative of a field moving forward in their understanding of the complex relationship between TGF-β regulation and disease pathology.

5.5.3 Muscle Wasting Associated with Disuse

Muscle atrophy is a major problem in conditions of disuse, such as limb casting, immobilisation, or patients undergoing intensive care unit hospitalisation. In patients confined to extended bed rest, significant muscle atrophy leads to a poorer prognosis for recovery, increasing their hospital stay duration, and can also severely impair the successfulness of long-term rehabilitation post discharge.

Animal studies of limb immobilisation have demonstrated that ensuing disuse atrophy of muscles is a product of simultaneous reductions in protein synthesis and increases in protein degradation (Booth and Seider 1979). This contrasts with denervation-induced muscle atrophy, where both protein synthesis and degradation increase, though protein breakdown exceeds protein synthesis (Argadine et al. 2009).

Recently, Zhang *et al.* provided an important link between mechanical load-sensing via the dystrophin-associated protein complex and the protein synthesis/degradation pathways in unloaded muscles, which implicated the TGF-β network (Zhang et al. 2014). In wild-type mice, plantaris muscles comprising fast-twitch muscle fibres and soleus muscles comprising slow twitch muscle fibres lost differing amounts of mass and exhibited differing expression of dystrophin mRNA over the course of unloading (Zhang et al. 2014). Comparisons of unloading atrophy in wild-type and dystrophin-deficient mice revealed relative sparing of some dystrophin-deficient muscles, accompanied with reduced expression of the E3 ubiquitin ligases MuRF-1 and atrogin-1, responsible for controlling ubiquitin/proteasome degradation. These observations point to a role of the dystrophin-associated protein complex as a regulator of protein degradation pathways in unloaded muscles. Additionally, wild-type mice exhibited up-regulation of TGF-β1 and increased phosphorylation of Smad3 subsequent to unloading, while these proteins, which were expressed at a greater basal rate in *mdx* mice, were reduced after the unloading period. As the TGF-β1/Smad3 pathway influences protein synthesis and degradation processes, these findings suggest that regulation of TGF-β network signalling downstream of the dystrophin-associated protein complex contributes to changes in muscle mass with unloading. As a comparison, examination of denervated muscles has determined that myostatin transcription is increased in neurogenic atrophy

(Baumann et al. 2003). Subsequent studies have identified a peak in myostatin protein level anywhere between 3-28 days post-denervation, where Smad2 phosphorylation is at its highest level around 3 days (Shao et al. 2007; Zhang et al. 2006).

Collectively, these studies implicate increased myostatin and Smad2/3 signalling as contributing to muscle atrophy in mouse models of disuse or denervation. The involvement of myostatin in disuse atrophy in humans remains unclear. While some studies have reported unchanged myostatin mRNA levels following unloading (De Boer et al. 2007; Jones et al. 2004), increased myostatin transcripts have been observed in reduced limb use associated with chronic osteoarthritis (Reardon et al. 2001). Whether myostatin is elevated temporally or only in discrete models of disease is yet to be identified. As myostatin levels can be controlled independently of transcription, further investigation may need to pursue protein detection techniques to analyse myostatin activity in sera or in muscle itself.

5.5.4 Sarcopenia

Sarcopenia is defined as the loss of muscle mass and functional capacity specifically associated with advancing age (Roubenoff 2000). Hallmarks of sarcopenia include muscle fibre atrophy, reduced muscle fibre number, diminished satellite cell activity, and progressive deterioration of pre-synaptic structures of the neuromuscular junction. Rodent studies of sarcopenia have identified gene signatures associated with oxidative stress and motoneuron degeneration as early indicators of sarcopenia, but at the signalling level, there is also evidence that the TGF-β network plays a role in ageing-associated muscle wasting. Muscle biopsies from human septuagenarians have been noted to exhibit increased myostatin expression relative to the muscles of young adults (Leger et al. 2008). Aged mice and humans also demonstrate elevated levels of TGF-β1 protein with an accompanying elevation in Smad3 phosphorylation (Carlson et al. 2009), the sera of which can inhibit the myogenic potential of satellite cells *in vitro* (Carlson et al. 2009). These findings point to mechanisms by which elevated TGF-β1 and myostatin may contribute to the suppressed regenerative capacity of aged muscle.

Seeking to prevent or overcome sarcopenic muscle atrophy, other studies have identified the inhibition of myostatin-Smad2/3 signalling as a potential therapeutic target. Mice lacking myostatin have been reported to exhibit reduced susceptibility to ageing-associated atrophy of fast twitch muscle fibres compared with wild-type mice. Treatment of aged mice with neutralising antibodies that target myostatin has also been shown to preserve muscle mass and force-producing capacity, and reduce markers of apoptosis (Murphy et al. 2010). Similar effects have been achieved using recombinant viral vectors to deliver myostatin prodomain constructs to old animals (Collins-Hooper et al. 2014) or via the administration of soluble ActRIIB receptors as ligand traps (Chiu et al. 2013). In aged mice, attenuation of TGF-β signalling using a receptor kinase inhibitor has also proven beneficial for enhancing muscle regeneration after experimental injury (Carlson et al. 2009). Paralleling the benefits

of losartan treatment in mouse models of muscular dystrophy, administering losartan to aged mice confers improvements in muscle mass and function and reduced fibrosis (Burks et al. 2011). Combined, these studies support further investigation into blocking TGF-β signalling as a preventative or restorative intervention for sarcopenia.

5.5.5 Cachexia

As many as 80 % of all patients contending with the progression of advanced cancer exhibit symptoms of cachexia, a debilitating syndrome characterised by the loss of body mass and fatigue (Tisdale 2009). The loss of muscle mass is a leading contributing factor for increased morbidity and mortality among cancer patients, accounting for nearly 30 % of cancer-related deaths (Anker et al. 2003; Tisdale 2002, 2009). Perturbation of the autoimmune, neuromuscular and inflammatory systems has also been implicated in the manifestation of cachexia (Jespersen et al. 2006; Tan and Fearon 2008). The degree of weight loss experienced by patients varies by cancer type, with cachexia occurring especially frequently and being particularly severe in association with pancreatic and gastric cancers (Dewys 1986). Although a common side effect of cancer and chemotherapy is decreased appetite (Theologides 1979), cachexia is a hypercatabolic state that cannot be reversed by hyper-caloric food intake (Bosaeus et al. 2001). Importantly, cachexia is not unique to patients with cancer, as patients with other forms of chronic illness can present with similar degrees of wasting. A particularly striking example is that of cardiac cachexia diagnosed in patients with chronic heart failure. Severe loss of muscle mass in these individuals has similar implications as in patients with cancer, with significant weight loss representing a contributing factor to death in a significant percentage of patients with heart disease (Glass and Roubenoff 2010; Wigmore et al. 1997).

Our understanding of the aetiology of cancer cachexia has greatly improved in recent years, with the current model recognising the condition as a product of the combined insult of tumour- and host-derived factors (Fearon et al. 2013; Morley et al. 2006). Increased expression and excess signalling of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β are heavily implicated in the manifestation of cachexia (Iwase et al. 2004; Kuroda et al. 2007; Moses et al. 2009; Oliff et al. 1987; Scott et al. 1996), but in tandem with these pro-cachectic factors, a growing body of work has identified specific TGF-β family proteins as dominant players in pathogenesis of cachexia (Zhou et al. 2010).

TGF-β family members that have been associated with cancer cachexia include TGF-β1 (Ikushima and Miyazono 2010), activin A (Pirisi et al. 2000; Wildi et al. 2001), BMP-4 (Thawani et al. 2010), GDF-15 (Bauskin et al. 2006) and myostatin (Lokireddy et al. 2012). Several studies have also established direct effects of specific TGF-β proteins as cachectic factors. Administration of TGF-β1 into mice xenografted with human breast cancers induced severe weight loss and fibrosis (Zugmaier et al. 1991). Elevated levels of activin A and activin B induced in mice

as a consequence of inhibin knock-out promotes rapid loss of muscle and fat mass that is ultimately fatal (Matzuk et al. 1992, 1994). Systemic overexpression of myostatin or activin A in mice by implantation of genetically engineered Chinese hamster ovary cells also promotes extensive wasting of lean and fat mass (Zhou et al. 2010; Zimmers et al. 2002). Importantly, both activin and myostatin have also been shown to induce significant muscle wasting if over expressed in healthy mice in the absence of other tumour-derived factors (Chen et al. 2014). These findings illustrate that excessive levels of specific TGF-β family ligands can be considered as direct contributors to the development of cachexia, and thus may also serve as sensitive biomarkers of patient prognosis.

The growing interest in the TGF-β network as a factor in the etiology of cachexia has spurred considerable effort to evaluate interventions that might modulate the actions of excessive TGF-β ligand levels. SB431542, a small molecule inhibitor of TGF-β signalling via selective antagonism of ALK4/5/7 (Laping et al. 2002), has proven capable of reducing the tumourigenic effects of TGF-β (Halder et al. 2005; Watt et al. 2010). Treatment mice bearing activin-expressing tumours with antibodies against activin A was effective at reversing cachexia and prolonging survival (Zhou et al. 2010). Antibodies designed to neutralise myostatin have also demonstrated efficacy in protecting mice bearing cachexia-inducing carcinomas (Murphy et al. 2011). Arguably, the most potent effects have been observed in studies evaluating the administration of soluble ActRIIB receptors as 'ligand traps' for circulating TGF-β family members (Zhou et al. 2010). Promising results in mouse models have motivated the clinical evaluation of soluble ActRIIA and ActRIIB designs (Attie et al. 2013).

The use of modified receptors, however, has raised concerns about the potential off-target effects as they still retain their capacity to bind multiple ligands (Massague 1998), which could disrupt normal endogenous signalling. One such concern is the potential disruption of BMP-9 and BMP-10, which operate as regulators of angiogenesis (Allen and Unterman 2007; Vallese et al. 2013; Zhang and Bradley 1996). Concerns over adverse events have recently tempered enthusiasm for continued development of soluble ActRIIB receptors for long-term. However, the exploration of TGF-β network antagonism as a treatment for diseases associated with muscle wasting is still a field of in its infancy. Continued study will hopefully yield useful interventions for long-term patient benefits (Attie et al. 2013).

One alternative to soluble ActRII-based interventions currently showing promise is the use of antibodies directed against the endogenous ActRIIB receptor (Lach-Trifilieff et al. 2014). This intervention has been demonstrated to increase muscle mass in both animals and human subjects with conditions of muscle wasting. However, given the previously reported side-effect risks of using soluble ActRIIB receptors (Attie et al. 2013), it may also eventuate that targeting the endogenous ActRIIB receptor at the systemic level may present similar challenges. Consequently, ligand-specific inhibitors may prove the most effective strategy to mitigate muscle wasting with minimal risk of off-target effects. These may take the form of antibodies (Yaden et al. 2014) or proteins that mimic the prodomain of specific TGF-β ligands (Chen et al. 2015). The latter approach was recently shown to effectively

prevent activin-induced muscle wasting in mouse models (Chen et al. 2015). Ongoing research should help to identify the most promising approaches for modulating the TGF-β network to combat muscle wasting.

5.6 Concluding Remarks

The complexity of the TGF-β network provides the means for this system to finely regulate a variety of different cellular processes in many different cell types and organ systems, including skeletal muscle. Considerable effort has provided us with an appreciation for the role of the TGF-β network as a regulator of skeletal muscle development and adaptation, and when perturbed, as a contributor to muscle disease. However, it is clear that our understanding of the significance of the TGF-β network in muscle will be enhanced with continued examination of TGF-β family members and their signalling mechanisms. Novel approaches to modulating the TGF-β network are increasingly showing promise as prospective interventions for muscle disease. Though hurdles remain to be faced in realising the widespread clinical use of these approaches, continued dedication to better understanding the TGF-β network in muscle will hopefully aid the development of invaluable new therapeutics, and help us to more completely define the mechanisms that govern the attributes of skeletal muscle in health and disease.

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Chapter 6
Adipokines in Healthy Skeletal Muscle and Metabolic Disease

C.A. Coles

Abstract Adipose tissue not only functions as a reserve to store energy but has become of major interest as an endocrine organ, releasing signalling molecules termed adipokines which impact on other tissues, such as skeletal muscle. Adipocytes, within skeletal muscle and adipose tissue, secrete adipokines to finely maintain the balance between feed intake and energy expenditure. This book chapter focuses on the three adipokines, adiponectin, leptin and IL-6, which have potent effects on skeletal muscle during rest and exercise. Similarly, adiponectin, leptin and IL-6 enhance glucose uptake and increase fatty acid oxidation in skeletal muscle. Fatty acid oxidation is increased through activation of AMPK (adenosine monophosphate-activated protein kinase signalling) causing phosphorylation and inhibition of ACC (acetyl-coenzyme A carboxylase), decreasing availability of malonyl CoA. Leptin and adiponectin also control feed intake via AMPK signalling in the hypothalamus. Adipokines function to maintain energy homeostasis, however, when feed intake exceeds energy expenditure adipokines can become dysregulated causing lipotoxicity in skeletal muscle and metabolic disease can prevail. Cross-talk between adipocytes and skeletal muscle via correct control by adipokines is important in controlling energy homeostasis during rest and exercise and can help prevent metabolic disease.

Keywords Adipokines • Adipocytokines • Adiponectin • Leptin • Interleukin-6 • Skeletal muscle • Adipose tissue • Metabolism • Exercise • Insulin resistance • Inflammation

6.1 Introduction

Adipose tissue was originally believed to solely serve as an energy reserve, storing lipids in order to finely maintain the homeostatic balance between food intake and energy expenditure. In the last decade adipose tissue has prevailed as an endocrine

C.A. Coles (✉)
Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Rd, Parkville, Melbourne, VIC 3052, Australia
e-mail: chantal.coles@mcri.edu.au

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organ responsible for expression and release of signalling molecules in the circulation, having potent effects on other tissues involved in energy homeostasis including skeletal muscle, liver and hypothalamus. These signalling molecules are termed adipokines or adipocytokines, characterised as being expressed and secreted by adipocytes into the circulation to target receptors in other tissues. Skeletal muscle is the largest insulin-sensitive tissue in the body, responsible for the majority of insulin-stimulated glucose uptake and storage, in order to store energy during feeding and maintain energy demand during expenditure (Stump et al. 2006). Therefore cross-talk between energy reserves, such as adipose tissue and skeletal muscle, is vital to finely manage energy homeostasis. Adipokines have emerged as being fundamental in controlling the signals between tissues whilst maintaining energy balance. In times when food intake exceeds energy expenditure this finely tuned system becomes overwhelmed and metabolic disease prevails. Ultimately, adipokines become dysregulated contributing to insulin resistance in skeletal muscle and an increase in free circulating fatty acids. Exercise has been shown to improve insulin resistance in skeletal muscle. There are numerous adipokines; however this chapter aims to discuss three adipokines, adiponectin, leptin and interleukin-6 (IL-6) as their effects on skeletal muscle during energy homeostasis, including rest and exercise, have been well documented.

6.2 Function of Adipokines and Their Role in Healthy Skeletal Muscle

6.2.1 Adiponectin

6.2.1.1 Background and Metabolism

Adiponectin is a 30 kDa adipokine expressed and secreted exclusively from differentiated adipocytes and is important for energy homeostasis acting in skeletal muscle, brain and liver. Adiponectin is also referred to as *Acrp30, AdipoQ, apM1, Gbp28* (Kraemer and Castracane 2007) and is found pre-dominantly in circulating plasma at substantial levels, up to 5 to 10 μg/ml in healthy humans (Matsuzawa et al. 2004). There are two forms of adiponectin that exist in plasma, the full-length form and a proteolytic cleaved fragment of the globular C-terminal domain (Fruebis et al. 2001). Two receptors with affinity for adiponectin are AdipoR1 and AdipoR2. AdipoR1 is expressed in skeletal muscle and brain and AdipoR2 is mainly expressed in the liver, with the globular C-terminal cleavage fragment having high affinity with AdipoR1 and full-length adiponectin having higher affinity for AdipoR2 (Duntas et al. 2004; Kubota et al. 2007). Adiponectin was first identified as a being significantly low in adipose tissue in obese mice and humans (Hu et al. 1996), reduced in plasma of obese humans (Arita et al. 1999), reduced in patients with type II diabetes (Hotta et al. 2001) and decreased with increased body mass index (BMI) related to elevated adiposity (Matsuzawa et al. 2004). Insulin resistance associated with lipoatrophic mice was reversed by administration of adiponectin (and more potently when

combined with leptin) (Yamauchi et al. 2001). This distinct association of adiponectin, insulin resistance and metabolic disease (discussed in more detail in part II) lead to investigations into discovery that adiponectin is important in sensitising cells to insulin and increasing mitochondrial fatty acid oxidation in skeletal muscle (Fruebis et al. 2001; Yamauchi et al. 2001, 2002). Globular adiponectin enhances glucose uptake by GLUT4 translocation and decreases rates of glycogen synthesis, shifting glucose towards lactate production in skeletal muscle (Ceddia et al. 2005). Thus it is through this enhanced glucose uptake adiponectin is believed to increase insulin sensitivity. Increased fatty-acid oxidation in skeletal muscle is also found to be through globular adiponectin binding to AdipoR1 (and less potently AdipoR2) to activate AMP-kinase (Ceddia et al. 2005). Activation of AMP-K inactivates acetyl-coenzyme A carboxylase and decreases malonyl CoA (similarly like IL-6) to enhance fatty acid oxidation in skeletal muscle (Tomas et al. 2002; Ceddia et al. 2005; Yoon et al. 2006) (described in more detail Sect. 6.2.4). In addition to this, adiponectin stimulates AMPK (through receptor binding to AdipoR1) in the hypothalamus to increase food intake and decrease energy expenditure (Kubota et al. 2007).

6.2.1.2 Adiponectin and Exercise

Exercise is associated with enhancing insulin sensitivity (Tamura et al. 2005; Ikeda et al. 2013). In light of the findings that adiponectin has been involved in increasing insulin sensitivity it is thought adiponectin concentrations may be altered during exercise. However, investigations following acute (short-term $\leq$ 60 min and long-term $\geq$ 60 min) and chronic (short-term $\leq$ 12 weeks and long-term $\geq$ 12 weeks) have shown minimal adjustments in adiponectin concentrations. In a study where elite rowers performed a 6000-m ergometer test (20 min), plasma adiponectin was decreased immediately after exercise but elevated above pre-exercise levels following 30 min of recovery (Jurimae et al. 2005). Similarly, adiponectin was not altered immediately after exercise but increased above pre-exercise values after 30 min of recovery following 6.5 km of rowing by trained rowers. In contrast, no change in adiponectin has been found following acute short-term exercise where healthy male subjects ran at 79 % of VO_2 max for 30 min. Acute long-term exercise also did not alter plasma adiponectin levels in healthy males and females cycling at 60 % VO_2 max for 60 min (Ferguson et al. 2004) and males cycling at 50 % VO_2 max for 120 min (Punyadeera et al. 2005). Punyadeera et al. (2005) found gene expression *AdipoR1* and *AdipoR2* in muscle was not altered even though adipose tissue lipolysis and plasma free fatty acids were. These authors showed histologically adiponectin is present at the sarcolemma of skeletal muscle fibres. The authors believe discrepancies between studies and the increased adiponectin in the rowing experiments are possibly due to a large proportion of muscles being used during rowing, and the anaerobic nature of the exercise resulting in greater energy expenditure.

Contradicting results are also apparent for adiponectin concentrations in response to chronic short and long-term training. Young (mean age 22) and middle-aged (mean age 60) women who undertook a 10-week aerobic training program,

where they trained three times weekly at 70 VO_2 max displayed increased serum adiponectin (greater in the middle-aged women) with improved insulin sensitivity (Lim et al. 2008). However, Hulver et al. (2002) found no exercise-induced change in adiponectin where insulin action was improved, body weight was reduced. In addition, elite male rowers who trained 7 times weekly for 24-weeks at low intensity showed no difference in post-exercise adiponectin from pre-exercise levels (Jurimae et al. 2007).

In summary the effects of exercise on adiponectin in healthy individuals are not well understood. Overall, it is believed adiponectin increases where duration, frequency and intensity is sufficient enough to increase insulin sensitivity with body weight loss and when all muscles are being used in exercises of high intensity of great energy expenditure. Adiponectin may serve as a signal to utilise energy stores only when the body is at high energy demand.

6.2.2 Leptin

6.2.2.1 Background and Metabolism

Leptin is a 16 kDa protein found circulating in serum and is expressed primarily by adipocytes but additionally expressed in skeletal muscle, liver, gastric wall, vascular cells placenta and ovary (Bouassida et al. 2010). Gene expression and serum levels of leptin correlate with body fat, therefore are increased in obesity and reduced with weight loss (Boden et al. 1996; Considine et al. 1996). Leptin is encoded by the *Obese (ob)* gene, in mouse and humans, important for reducing food intake and increasing energy expenditure (Halaas et al. 1995) via interaction with the *Ob* receptor, which has numerous spliced variants (Ob-Ra: leptin transporter, Ob-Rb: long form with intracellular signalling domain, Ob-Re: soluble form of transmembrane receptor, Ob-Rc and Ob-Rd) (Meier and Gressner 2004; Koerner et al. 2005). In normal mice, leptin treatment reduces body weight (Halaas et al. 1995). Administration of recombinant leptin to mutant *Ob* mice, which display symptoms including obesity, increased fat deposition, hyperglycaemia and hyperinsulinemia, had reduced food intake, increased body weight loss and reduced body fat (Pelleymounter et al. 1995; Halaas et al. 1995). In addition, Pelleymounter et al. (1995) showed leptin's effects on body weight loss was dose dependent also decreasing serum concentrations of insulin and glucose in a dose dependent manner. Leptin expression is diurnal, increasing during the night for appetite suppression during sleep (Saladin et al. 1995; Sinha et al. 1996). In rats, leptin expression was also regulated by feeding patterns where night time levels were reduced after feeding (fasting) rats (Saladin et al. 1995). Fasting in humans causes large decreases in serum leptin levels. After 52 h of fasting in normal weighted and obese humans, leptin serum levels declined 64 % and 72 % respectively, this was prevented by IV administration of glucose to maintain basal plasma glucose and insulin concentrations (Boden et al. 1996). Leptin targets the arcuate nucleus (Arc) and ventromedial

nucleus (PVN) areas in the hypothalamus in reducing food intake and body weight (Sahu et al. 2011). Studies on leptin prove it be an important adipokine in regulating talk between adipose tissue, the hypothalamus and skeletal muscle to ensure adequate energy reserves are available, thus maintaining energy homeostasis.

In skeletal muscle, leptin interacts with Ob-R long and short form (Murakami et al. 1997) to stimulate fatty acid oxidation, increase glucose uptake and prevent accumulation of lipids in non-adipose tissue. Leptin treatment increases fatty acid uptake and oxidation in mice fed a high-fat diet (for 3-months) independent of leptin-induced energy intake. Treatment of leptin to the *soleus* muscle increased fatty acid oxidation by 42 % and decreased incorporation of fatty acids into triacylglycerol by 35 % (Muoio et al. 1997). In contrast to insulin administration in the *soleus* muscle which decreased fatty acid oxidation by 40 % and increased triacylglycerol synthesis by 70 % (Muoio et al. 1997). The *extensor digitorum longus* (EDL) muscle showed minimal effects in response to leptin treatment thought to be being a highly glycolytic muscle, with much less oxidative enzymes, mitochondria and a reduced capacity to store and utilise triacylglycerol as an energy reserve (Muoio et al. 1997). When administered with insulin, leptin reduced triacylglycerol synthesis by 50 % in both the EDL and *soleus* muscle. Leptin stimulates fatty acid oxidation through activation of AMPK, a potent inhibitor of ACC (described in detail in Sect. 6.2.4) attenuating lipid biosynthesis and thus activating fatty acid oxidation (Minokoshi et al. 2002; Suzuki et al. 2007). In addition to increasing fatty acid oxidation in skeletal muscle, leptin also stimulates glucose transport in skeletal muscle. This is supported by Yaspelkis et al. (1999) who also showed improvements in insulin-stimulated glucose transport in skeletal muscle after leptin administration in rats. Injection of leptin into the medial hypothalamus, making it accessible to ventromedial hypothalamic nucleus, increases glucose uptake in skeletal muscle. In summary, leptin is important in the regulation of glucose uptake in skeletal muscle via its action in the hypothalamus and to stimulate fatty acid oxidation directly in skeletal muscle through AMPK signalling.

6.2.2.2 Leptin and Exercise

The effect of acute short-term exercise on leptin concentrations in healthy subjects has been investigated. Leptin concentrations declined, immediately after and 30 min, following a 30-min ergometer rowing test (Jurimae et al. 2005). Plasma leptin concentrations also decreased in male and females immediately after 20 min of running at 70 % VO_2 max (following an overnight fast) (Legakis et al. 2004). Basal levels of leptin returned 1 h following the exercise. Zafeiridis et al. (2003) investigated the effect of three resistance training protocols (maximum strength, muscular hypertrophy and strength endurance training) on leptin levels. Plasma levels decreased following 30 min of exercise, however this was not different to control groups who did not exercise. The decline in leptin was believed to be due to an overnight fast, thus different protocols of resistance training did effect leptin concentrations in plasma.

In response to acute long-term exercise plasma leptin concentrations show varying alterations. In competitive endurance races with differing duration and energy expenditure, (1) half-marathon 21 km, energy expenditure (EE) 5852 kJ: (2) ski-alpinism 45 km, EE 20,900 kJ: (3) ultra-marathon 100 km, 29,269 kJ, plasma leptin levels were decreased (within 20 min post-race) in subjects who completed the ski-alpinism and ultra-marathon race but not the half-marathon. Plasma free fatty acids were increased significantly after each race displaying a negative correlation with per cent reduction in leptin. Leptin levels were found to be decreased following 60 min of moderate intensity (70 % VO_2 max) 24 h and 48 h post-exercise (subjects had 12 h fast prior to exercise) (Olive and Miller 2001). A trend for decreased leptin occurs following long-term acute exercise when energy expenditure is high.

Plasma leptin concentrations following chronic short-term ($\leq$12 weeks) and long-term training ($\geq$12 weeks) also show varying results. Males who completed a 14-day ski expedition where they were active for 10 h per day, carrying 25 kg and skiing between 12 and 30 km/day displayed reduced plasma leptin concentrations at the end of the 14-days. Leptin levels increased after 6 weeks of recovery but not to pre-race levels (Eriksson et al. 2008). Highly trained rowers who also performed chronic short-term training (2000 m ergometer test) following 3 weeks of heavy training had reduced leptin levels (Jurimae et al. 2003). In contrast, a 6-week resistance training protocol did not alter plasma leptin concentrations even though fat mass was reduced by 7 % (Ara et al. 2006). For long-term training studies, Desgorces et al. (2004) found leptin to be decreased in trained rowers who completed a 90 min exercise session at 70–75 % VO_2 max following a 36-week rowing protocol. Fatouros et al. (2005) found leptin not to be changed in healthy control subjects following a 24-week resistance training program (three times weekly for 60 min). In summary, the effects of exercise on circulating leptin following chronic short and long term exercise programs vary between studies, but show either no change or a decrease in plasma leptin levels. The decrease in leptin levels is attributed to exercises with high energy demand and where a large proportion of muscles are utilised.

6.2.3 Interleukin-6

6.2.3.1 Background and Metabolism

Interleukin-6 (IL-6) is well known for its regulation in pro-inflammatory signalling during immune responses and host defence mechanisms. During sepsis circulating IL-6 increases. Recently IL-6 has emerged as an important signalling molecule during exercise and metabolic processes to maintain energy homeostasis. Termed an adipokine and myokine, circulating plasma IL-6 can act on adipose tissue and skeletal muscle. At rest adipose tissue accounts for 10–35 % of the circulating plasma IL-6 levels (Mohamed-Ali et al. 1997), with adipocytes and non-adipocyte cells within the tissue extracellular matrix also secreting IL-6 (Hoene and Weigert 2008).

Interstitial concentrations of IL-6 in adipose tissue are 100-fold higher than in circulating plasma (Sopasakis et al. 2004) suggesting local effects on adipocytes and other cell types such as macrophages. IL-6 produced from visceral fat is greater than that released from subcutaneous adipose tissue (Fain et al. 2004). Direct effects of IL-6 on adipocytes include increased lipolysis and whole body fat oxidation (Hoene and Weigert 2008). The metabolic role of IL-6 is further supported in IL-6 deficient mice which develop mature-onset obesity, this can be rescued by treatment with IL-6 (Wallenius et al. 2002).

6.2.3.2 IL-6 and Exercise: The Energy Sensor in Skeletal Muscle

During physical activity, plasma IL-6 concentration increases dramatically, by up 100-fold during strenuous exercise (Ostrowski et al. 1998). During prolonged exercise (2 h marathon) IL-6 was detectable in plasma and skeletal muscle immediately (plasma concentration 100-fold greater than pre-exercise) and 2 h (plasma concentration 25-fold greater than pre-exercise) post-exercise (Ostrowski et al. 1998). Due to IL-6 being well established as a pro-inflammatory cytokine and the concomitant increase in creation kinase (marker of muscle damage), the spike in IL-6 during exercise was first believed to be secreted from inflammatory cells in response to exercise-induced muscle damage. However, elevations in plasma IL-6 are not related to muscle damage (Toft et al. 2002) and monocytes are not to be the source of IL-6 during exercise (Starkie et al. 2000, 2001). A study by Steensberg et al. (2000), whereby six healthy males performed one-legged dynamic knee extensor exercise (at 40 % peak power) for 5 h, were able to show that the contracting muscle (working leg) was the source of IL-6 release and responsible for elevations in IL-6 plasma concentration. Repetitive low-intensity exercise also results in a large increase in IL-6 but only locally within the contracting muscle, not within the arterial circulation (Rosendal et al. 2005). Steensberg et al. (2000) were also able to show that the exercise-induced elevation in IL-6 (both local and arterial) increased with duration of exercise. IL-6 release from contracting skeletal muscle is myofibre specific, predominantly to Type 2 myofibres (Hiscock et al. 2004). Adipose tissue is not believed to contribute to increased plasma IL-6 concentration during exercise (Lyngsø et al. 2002). Thus during exercise IL-6 serves more potently as a myokine rather than an adipokine which appears to just have local effects within adipose tissue. Nonetheless the role of IL-6 as circulating endocrine factor enables cross-talk between adipose tissue, skeletal muscle and the liver during energy homeostasis.

IL-6 has been termed the ‘energy sensor’ due to substantial increases in its gene expression and protein release during exercise when glycogen levels are low (Keller et al. 2001; Steensberg et al. 2002; Fischer et al. 2004). Ingestion of glucose during exercise has shown to attenuate the spike in IL-6 during exercise (Febbraio et al. 2003). The role of IL-6 when glycogen reserves are made low during exercise was made evident in a study where healthy untrained but active humans received treatment of recombinant human IL-6 to cause enhanced fatty acid concentration and turnover, independent of adrenaline, insulin or glucagon (Van Hall et al. 2003). This

demonstrates IL-6 increases lipolysis and is an important modulator of whole-body fat oxidation, supported by work of others (Bruce and Dyck 2004; Petersen and Pedersen 2005). Moreover, Bruce and Dyck (2004) showed IL-6 regulates fatty acid oxidation in skeletal muscle and attenuates insulin's ability to repress fatty acid oxidation and stimulation of lipogenesis. In light of this IL-6 exercise-induced response is found to be much lower in magnitude in a trained athlete, whose glycogen content is increased, has greater capacity to oxidise fat and less dependency to utilise plasma glucose and glycogen reserves as an energy substrate (Fischer et al. 2004; Pedersen 2012). Exercise training is also believed to sensitise IL-6 to the IL-6 receptor (IL-6R), this comes from the finding that IL-6R mRNA is increased in a trained skeletal muscle (Keller et al. 2005). IL-6 signals through AMP-activated protein kinase (AMPK) to increase basal glucose uptake and translocation of GLUT4 (Carey et al. 2006). This also evident in IL-6 KO mice which have diminished AMPK activity in muscle and adipose tissue (Ruderman et al. 2006).

6.2.4 Skeletal Muscle Fatty Acid Oxidation and Increased Glucose Uptake Via Adipokine Mediated AMPK Activation

The adipokines leptin, adiponectin and IL-6 function to increase glucose uptake and fatty acid oxidation in skeletal muscle. These metabolic processes are induced through activation of the enzyme AMPK. AMPK can increase glucose uptake through enhanced glucose transporter 4 (GLUT4) translocation to the plasma membrane (Ruderman et al. 2006; Friedrichsen et al. 2013). In a neutral state (when AMPK is not activated), malonyl-CoA (derived from glucose) inhibits carnitine palmitoytransferase-1 (CPT-1), the enzyme responsible for transfer of long-chain fatty acyl-CoA (FA-CoA from the cytosol into the mitochondria for fatty acid oxidation (through the *fatty-acid β-oxidation pathway*) (Ruderman et al. 2006). Suppression of CPT-1 makes more FA-CoA available in the cytosol for triglyceride and diacylglycerol synthesis (lipogenesis). Activation of AMPK by leptin, adiponectin and IL-6 leads to phosphorylation of AMPK, phosphorylating and inhibiting ACC to diminish malonyl-CoA concentration (Berggren et al. 2005). By decreasing malonyl-CoA, CPT-1 transports FA-CoA across the mitochondrial membrane for fatty acid oxidation. In addition to this, AMPK can decrease ACC through transcriptional regulation of SREBP1C and activate expression of PGC1α to increase direct synthesis of CPT-1 and enhance expression of genes regulating mitochondrial biogenesis and oxidative phosphorylation (Ruderman et al. 2006). Adiponectin and leptin were shown to be important for AMPK induced deacetylation of PGC1α (activating mitochondrial biogenesis) in C2C12 cell *in vitro* (Li et al. 2011).

Leptin and adiponectin can influence food intake through AMPK signalling in the hypothalamus. Adiponectin activates AMPK as described above during fasting to increase food intake and leptin can suppress food intake during feeding (Kubota

et al. 2007). Leptin functions to decrease Arc and PVN AMPK phosphorylation and subsequent ACC phosphorylation to activate ACC, increasing malonyl-CoA and thus fatty acid biosynthesis (Gao et al. 2007). Availability of cellular malonyl-CoA inhibits mitochondrial CPT-1 and therefore fatty acid oxidation (Minokoshi et al. 2008). Inhibition of CPT-1 activity in the hypothalamus has shown to suppress food intake (Obici et al. 2003). A rise in hypothalamic malonyl-CoA signals skeletal muscle to increase fatty acid oxidation via the sympathetic nervous system (SNS) and β-adrenergic receptors (Cha et al. 2006). Leptin also influences glucose uptake in skeletal muscle in the hypothalamus via the SNS and β-adrenergic receptors (Minokoshi et al. 2012). Other studies have shown AMPK is activated to increase fatty acid oxidation in skeletal muscle and hypothalamus independent of malonyl-CoA. Chronic treatment of leptin increased AMPK and ACC phosphorylation without reducing malonyl-CoA in the *soleus* and *gastrocnemius* muscle (Steinberg et al. 2003) and hypothalamus (Keung et al. 2011) Fig. 6.1.

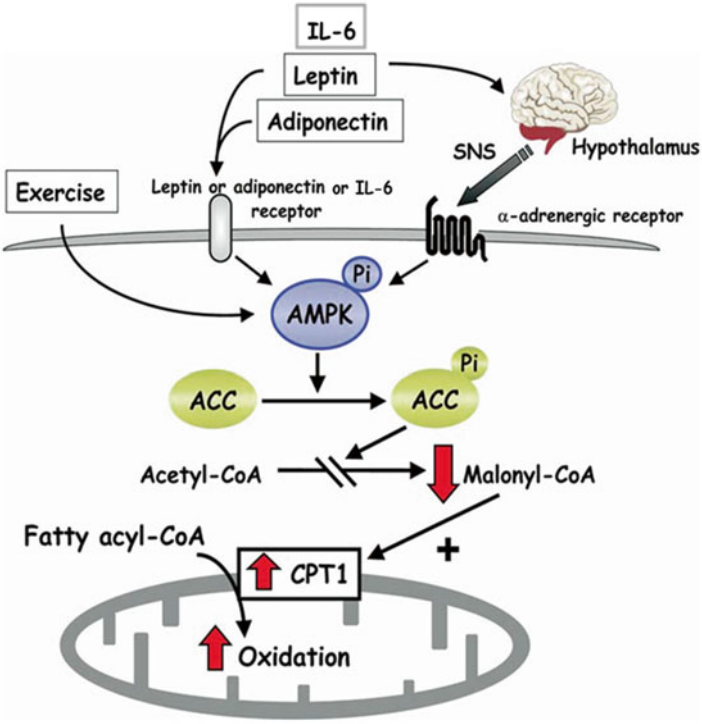

Fig. 6.1 Adiponectin, leptin and IL-6 induced-AMPK signalling. Leptin, adiponectin and IL-6 receptor binding causes phosphorylation of AMPK, phosphorylating ACC causing its inactivation. Inactivated ACC reduces availability of malonyl-CoA inhibiting lipogenesis in the cytosol, ultimately causing more FA-CoA to be available for transport across mitochondrial membrane for increased fatty acid oxidation. In skeletal muscle, this pathway has shown to be active independent of malonyl-CoA (AMPK: adenosine monophosphate-activated protein kinase, ACC: acetyl-coenzyme A carboxylase, FA-CoA: fatty acyl-CoA) (Figure part modified from Kahn et al. 2005)

6.3 Part II: Adipokines and Metabolic Disease

6.3.1 Metabolic Disease and Impact on Skeletal Muscle

Adipose tissue serves as an energy reserve to facilitate energy requirements whilst fasting and during times when energy expenditure is high, such as exercise. When the balance between fuel intake and energy consumption is not matched, thus food intake exceeds energy expenditure, lipids accumulate overloading mechanisms to balance energy homeostasis leading to the development of metabolic disease. Metabolic disease has become increasingly prevalent in developed industrialised countries. In a national health and nutrition examination survey in North America, metabolic syndrome was present in 22.8 % and 22.6 % of male and female adults respectively (Park et al. 2003). Insulin resistance is a common symptom of the metabolic diseases, diabetes, metabolic syndrome and obesity. Metabolic syndrome is characterised as displaying symptoms including high blood pressure, insulin resistance, hyperlipidemia and obesity. Obesity is defined in subjects with increased lipids in subcutaneous, visceral adipose tissue, ectopic lipid accumulation in non-adipose tissue including skeletal muscle and increased circulating fatty acids. Lipid overload, particularly in adipose tissue and skeletal muscle, affects insulin sensitivity and normal skeletal muscle maintenance and regeneration (Akhmedov and Berdeaux 2013). Mice fed a high fat diet for 8-months had impaired muscle regeneration after cardio-toxin muscle injury, whereby myofibres were smaller with increased interstitial collagen deposition (Hu et al. 2010). In addition, mice fed a high-fat diet for 3 weeks had reduced number of satellite cells following cold-induced injury (Woo et al. 2011). Muscle biopsies from obese people with type II diabetes mellitus have shown increased accumulation of intramyocellular lipids (IMCL: lipids within myofibres) (Goodpaster et al. 2000). Moreover, extramyocellular lipids (EMCL: lipids within adipocytes between myofibres) are associated with obese patients with insulin resistance and poor muscle performance (Hilton et al. 2008; Malenfant et al. 2001). Lipotoxicity whereby pools of saturated fatty acids are high can cause increased apoptosis of L6 and C2C12 muscle cells (Akhmedov and Berdeaux 2013). Endurance trained athletes have shown increased IMCL, not related to insulin resistance (Kiens 2006). However, the lipid metabolites ceramide, diacylglycerol and long chain fatty acyl CoA esters have been associated with causing skeletal muscle insulin resistance rather than intramyocellular triglyceride (Cooney et al. 2002; Kraegen and Cooney 2008; Hla and Kolesnick 2014). Adipokines have shown to be important in regulating these metabolites to healthy levels. The adiponectin receptors AdipoR1 and AdipoR2 were found to be fundamental in adiponectin mediating a reduction in ceramide (Holland et al. 2011). Recovery of insulin and leptin action in rats on a high fat diet by endurance exercise also restored ceramide to control levels (Ritchie et al. 2011). Skeletal muscle is important for energy homeostasis to balance energy intake and expenditure. Adipokines are vital for this balance to prevent an accumulation of intramuscular lipids which can cause excess lipid metabolites, development of insulin resistance and insulin resistance.

6.3.2 *Inflammation and Development of Insulin Resistance in Metabolic Disease*

In metabolic disease where obesity is a major contributing factor, white adipose tissue (WAT) expands through adipocyte hyperplasia and hypertrophy. Weight gain via expansion of WAT significantly impacts on whole body glucose homeostasis, particularly when adipocyte size increases (adipocyte hypertrophy). Larger adipocytes are linked to insulin resistance and increased risk of type 2 diabetes (Salans et al. 1968; Cotillard et al. 2014). Cotillard et al. (2014) were able to develop a model to predict a greater incidence of developing type 2 diabetes with increased adipocyte size in two cohorts of Caucasian women who were candidates for bariatric surgery. Adipocyte hypertrophy is characterised as large adipocytes containing a lipid volume greater than 800pL (Kloting and Bluher 2014). In adipose tissue where hypertrophic adipocytes predominate an inflammatory microenvironment prevails. Hotamisligil et al. (1993) were first to show TNFα is secreted from adipose tissue of obese mice. The source of TNFα comes from an infiltration of M1 macrophages (M1: pro-inflammatory macrophages, classically activated), also responsible for release of other pro-inflammatory cytokines including, MCP-1, IL-8, IL-18, inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS) (Weisberg et al. 2003; Xu et al. 2003; Rudich et al. 2007; Cao 2014). Obese mice deficient in TNFα have reduced free circulating fatty acids and were protected from insulin resistance (Uysal et al. 1997). Furthermore, activation of the NLRP3 inflammasome (Nod-like receptor, pyrin domain containing 3) contributes to release of the pro-inflammatory cytokines, IL-1β and IL-18, via induction of caspase-1 cleavage in obesity and type 2 diabetes (Masters et al. 2010; Vandanmagsar et al. 2011). This is concomitant with a decrease in anti-inflammatory cytokines, such as IL-10, from the M2 macrophage subtype (M2: anti-inflammatory macrophages, alternatively activated). Lumeng et al. (2007) showed that diet-induced obesity leads to a shift from M2 polarised state as seen in lean subjects to a pro-inflammatory M1 state. In addition to the development of an inflammatory microenvironment, stress sensing kinases (p38 mitogen-activated protein kinase (MAPK), Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK) and inhibitory-κB kinase (IKK)) are activated (Bashan et al. 2007; Rudich et al. 2007), ER (endoplasmic reticulum) stress is induced (Xu et al. 2010), autophagy (Kovsan et al. 2011) and apoptosis (Keuper et al. 2011) of adipocytes is exacerbated contributing further to a hypoxic environment.

Collectively these factors contribute to the development insulin resistance. TNFα is implicated in contributing to insulin resistance by binding to the TNF receptor (TNF-R55) (Hotamisligil et al. 1994, 1996; Peraldi et al. 1996). Activation of TNF-R55 induces the 'death domain', enhancing A-SMase activity to cause accumulation of the metabolite ceramide (Csehi et al. 2005). It is proposed ceramide activates serine kinases, such as JNK, to phosphorylate serine residues on IRS-1 (insulin-receptor substrate), inhibiting signals from the insulin receptor (IR) and thus insulin sensitivity (Csehi et al. 2005). Stuart et al. (2014) showed in skeletal muscle, serine phosphorylation (Ser363) of IRS-1 (phosphorylation site of JNK1)

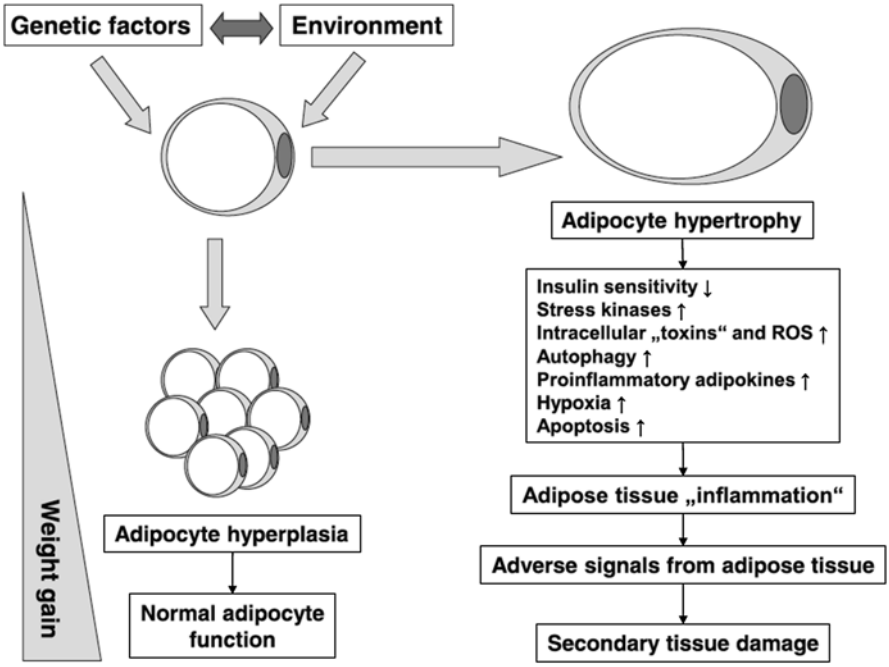

Fig. 6.2 Consequences of adipocyte hypertrophy in the development of adipose tissue inflammation and eventually secondary tissue damage in tissues such as skeletal muscle. When energy intake exceeds energy expenditure, weight gain increases, causing adipocyte hyperplasia (increase in the number of adipocytes) and adipocyte hypertrophy (increase in adipocyte size). Adipocyte hypertrophy is linked to impaired insulin signalling, reducing insulin sensitivity. Activation of stress kinases, pro-inflammatory cytokines, intracellular toxins (iNOS and ROS) lead to a hypoxic environment inducing autophagy, apoptosis, and adipose tissue inflammation. Collectively these factors contribute to insulin resistance in metabolic disease (such as obesity and type 2 diabetes) and can cause secondary tissue damage in tissues such as liver, brain, endothelium, vasculature, endocrine organs and skeletal muscle (Figure sourced from Kloting and Bluher 2014)

was upregulated in metabolic syndrome subjects to cause insulin resistance. IKKβ and JAK STAT are also involved in serine phosphorylation IRS-1 to inhibit action of the IR receptor and therefore insulin sensitivity (Gao et al. 2002; Johnston et al. 2003) Fig. 6.2.

6.3.3 Adiponectin and Metabolic Disease

In animal models of obesity and insulin resistance circulatory levels of adiponectin are reduced (Hu et al. 1996; Hotta et al. 2001). Mice deficient in adiponectin have insulin resistance and glucose intolerance (Kubota et al. 2002; Maeda et al. 2002; Nawrocki et al. 2006). Similarly in humans, plasma adiponectin levels are low and

negatively correlate with adiposity, insulin resistance, type 2 diabetes and metabolic syndrome (Rabe et al. 2008). Gene expression of the skeletal muscle specific adiponectin receptor (*AdipoR1*) is suppressed in genetically obese and diabetic (db/db) mice (Inukai et al. 2005). Continuous systemic administration of recombinant adiponectin reverses insulin resistance in lipoatrophic mice alleviating hyperglycaemia, hyperinsulinemia. Adiponectin acts by decreasing triglyceride content in skeletal muscle and serum free fatty acid levels (Yamauchi et al. 2001). The C-terminal globular region of adiponectin proving to be more potent in its effects than full-length adiponectin (Yamauchi et al. 2001). Adiponectin also reverses insulin resistance in obese mice. Globular and full-length adiponectin function to do this by increasing fatty acid oxidation and decreasing triglyceride content in skeletal muscle and reducing serum free fatty acids (Yamauchi et al. 2001). Intraperitoneal administration of adiponectin for seven days in obese mice reduced serum glucose, insulin, triglycerides and weight of visceral WAT (Masaki et al. 2003). Treatment of globular adiponectin to excised skeletal muscle from lean and obese non-diabetic females increased skeletal muscle glucose uptake and fatty acid oxidation through increased AMPK activity. The effects of globular adiponectin was blunted in excised muscle from obese patients, AMPK activity also reduced suggestive of adiponectin resistance in obese subjects (Bruce et al. 2005). This was also found in mice whereby globular adiponectin resistance developed in skeletal muscle of mice following a high fat diet, through loss of fatty acid oxidation by ACC signalling (Mullen et al. 2007). Endurance exercise training has proved to restore insulin response (in skeletal muscle) of insulin-resistant rats fed a high fat diet (Ritchie et al. 2011). However in a similar study, endurance exercise did not restore adiponectin resistance (to stimulate fatty acid oxidation) in skeletal muscle of rats fed a high fat diet even though insulin action was corrected (Gulli et al. 2012). Furthermore, exercised-induced restoration of glucose and insulin was recently found to be independent of adiponectin (Ritchie et al. 2014) even though treatment and overexpression of adiponectin improves insulin sensitivity (Yamauchi et al. 2001; Kandasamy et al. 2012). These results highlight the complexity of adiponectin signalling in adipose tissue and skeletal muscle during exercise and metabolic disease.

The importance of the adiponectin receptors, *AdipoR1* (skeletal muscle) and *AdipoR2* (liver), have been highlighted with the use of animal models, with reports of conflicting data. Targeted disruption of both *AdipoR1* and *AdipoR2* (*Adipor1-/-/ Adipo2-/- mice*) caused marked glucose intolerance and insulin resistance, proving to be more severe than adiponectin deficient mice suggestive other ligands may be involved (Yamauchi et al. 2007). Disruption of just *AdipoR1* influenced activation of AMPK pathways and *AdipoR2* was predominantly associated with activation of PPARα, reducing inflammation and oxidative stress (Yamauchi et al. 2007). Both pathways acting simultaneously to decrease triglyceride content and increase insulin sensitivity (Yamauchi et al. 2007). Bjursell et al. (2007) showed opposing effects of *AdipoR1-/-* and *AdipoR2-/-* mice. *AdipoR2-/-* mice were lean and resistant to diet-induced glucose intolerance whereas *AdipoR1-/-* mice showed increased adiposity and decreased glucose tolerance. In contrary, Liu et al. (2007) demonstrated *AdipoR2-/-* mice only reduced diet induced insulin resistance. Reports on adiponec-

tin, *AdipoR1* and *AdipoR2*, although some conflicting, prove them to be better candidates as a therapeutic for metabolic diseases, rather than targeting adiponectin as other compensatory ligands may be involved in adiponectin receptor signalling.

6.3.4 Leptin and Metabolic Disease

Levels of circulating leptin are elevated in obesity models, such as mice fed a high fat diet (Frederich et al. 1995). Circulating leptin and leptin mRNA in adipose tissue are elevated and strongly associated with body mass index and fat mass in obesity (Hamilton et al. 1995; Lonnqvist et al. 1995; Considine et al. 1996; Bastard et al. 2006). Maffei et al. (1996) screened 105 obese/diabetic patients for mutations in the human *Ob* gene, no mutations in the leptin gene were evident in any of the patients. Montague et al. (1997) did however, find a homozygous frame-shift mutation (single deletion of guanine in codon 133) in leptin gene of two severely obese children, displaying symptoms consistent with the leptin deficient mice, such as increased body weight and adiposity, hyperinsulinaemia and insulin resistance. These findings highlight the importance of leptin in regulating food intake and energy expenditure.

Deletion of the Ob gene (leptin) in mice causes obesity, displaying increased fat deposition, hyperglycaemia and hyperinsulinemia, reduced food intake, increased body weight loss and reduced body fat (Pelleymounter et al. 1995; Halaas et al. 1995). In skeletal muscle, deficiency of leptin in mice caused reduced muscle mass (in the gastrocnemius, extensor digitorum longus and soleus) and differential gene expression of 1127 genes in the gastrocnemius muscle (Sainz et al. 2009, 2010). Gene ontology analysis found these largely constituted genes associated with oxidative stress and inflammation. Skeletal muscle oxidative stress, muscle atrophy and inflammation was reversed in leptin deficient mice by leptin administration (Sainz et al. 2010). Administration of recombinant leptin to mutant *Ob* mice also increases weight loss by decreasing body fat, food intake and lowering serum concentrations of glucose and insulin (Pelleymounter et al. 1995; Halaas et al. 1995). Chronic treatment of leptin (2 weeks) in rats resulted in reduced fatty acid transport and uptake across the sarcolemmal membrane of red and white skeletal muscle fibres, reducing intramuscular triacylglycerol depots (Steinberg et al. 2002b) and improved fatty acid oxidation and hydrolysis of triacylglycerols (Steinberg et al. 2002a). This suggest leptin is important for reducing storage of intramuscular lipids.

Elevated leptin in metabolic disease is associated with leptin resistance, evident in diet-induced obesity model mice which had diminished response to peripherally administrated leptin (Van Heek et al. 1997) and mice fed a high fat-diet displayed reduced lipid oxidation in soleus muscle after leptin treatment (Steinberg and Dyck 2000). This confirmed in human studies where leptin increased fatty acid oxidation in lean skeletal muscle but not in obese subjects (Steinberg et al. 2002c). Human obesity subjects show an accumulation of intramuscular lipids (triacylglycerol and diacylglycerol, long chain fatty acid acyl-CoA) suggestive that fatty acid and lipid

metabolism is dysregulated in skeletal muscle (Consitt et al. 2009). Suppressor of cytokine signalling-3 (Socs3) is involved as a negative regulator of leptin sensitivity. Socs3 deficient mice are resistant to high fat diet-induced weight gain, hyperleptinemia, suppression of food intake and are insulin-sensitive (Howard et al. 2004; Mori et al. 2004). These mice show enhanced leptin-induced hypothalamic Stat3-tyrosine phosphorylation (Mori et al. 2004). Prolonged stimulation of the leptin receptor (Ob-Rb: long form), induces expression of Socs3, as a negative feedback mechanism by inhibiting Stat3 signal transduction to cause insulin resistance (Dunn et al. 2005). Deletion of muscle specific-Socs3 did not alter muscle development, body mass, adiposity or energy expenditure but did improve glucose tolerance and insulin sensitivity in high fat-diet induced-obese mice (Jorgensen et al. 2013). Leptin resistance is also accompanied with increased Socs3 in skeletal muscle (Steinberg et al. 2003). Endurance training in rats was able to reverse diet-induced leptin resistance in skeletal muscle, however, this appeared to be independent of Socs3 suggesting activation of alternate pathways inducing leptin resistance in skeletal muscle during exercise (Steinberg et al. 2004). This was accompanied by reduced triacylglycerol storage but not increased fatty acid oxidation (Steinberg et al. 2004). Inhibition of Socs3 as a therapy may be beneficial to improve leptin sensitivity and therefore insulin sensitivity, in patients with metabolic disease such as obesity and type 2 diabetes. Further studies are required to understand the mechanisms behind leptin resistance in skeletal muscle.

6.3.5 IL-6 and Metabolic Disease

Elevated levels of plasma IL-6 have been found in obese and type 2 diabetic patients associated with insulin resistance (Kern et al. 2001; Bastard et al. 2002; Spranger et al. 2003). Increased IL-6 insulin resistance has also been associated with hypertension in obese type 2 diabetic patients (Lukic et al. 2014). Elevated arterial and subcutaneous venous IL-6 in obese patients were negatively associated with forearm glucose uptake and glucose uptake in skeletal muscle was also decreased suggesting a role for IL-6 in skeletal muscle insulin resistance (Mitrou et al. 2011). Furthermore, a 3-week low calorie weight loss diet in obese subjects reduced IL-6 levels in adipose tissue and serum (Bastard et al. 2000). These findings are supported by the discovering the IL-6 174G>C gene variant is associated with metabolic syndrome displaying insulin resistance and increased fasting insulin (Fishman et al. 1998; Fernandez-Real et al. 2000; Stephens et al. 2007). Subjects homozygous for the C allele have reduced plasma IL-6, increased insulin sensitivity and reduced fasting insulin than those with G/G and G/C genotype (Fernandez-Real et al. 2000).

It has been controversial over the years the exact role IL-6 plays in metabolic disease. Elevated circulating IL-6 is associated with diabetes and insulin resistance and this is also associated with low-level chronic inflammation. IL-6 is well known as a pro-inflammatory cytokine and recently has been well accepted as a myokine important for insulin sensitivity in the working muscle during exercise as described

in previous sections of this chapter. Thus a role for IL-6 in regulating insulin resistance and inflammation in metabolic disease is still yet to be fully elucidated. However, so far evidence in mouse models has hinted, whilst some conflicting, IL-6 is important for insulin action and regulating leptin. Wallenius et al. (2002) found IL6-/- mice developed obesity by 9 months of age, with an increase predominantly in subcutaneous fat, increased leptin level, leptin insensitivity and altered carbohydrate and lipid metabolism. Treatment of IL-6 to IL-6-/- mice was successful in reducing body weight, total abdominal and intraperitoneal area (from CT scan) and leptin levels (Wallenius et al. 2002). Di Gregorio et al. (2004) also generated IL-6-/- mice which did not develop mature-onset obesity, elevated leptin levels or abnormal lipid metabolism, IL-6-/- mice fed a high fat diet did have high blood glucose following a glucose tolerance. Evidence IL-6 influences insulin resistance was shown in a study by Klover et al. (2003) whereby chronic exposure of IL-6 caused insulin resistance in the liver of mice. Depletion of IL-6 by neutralising antibody in obese mice improved hepatic insulin action (Klover et al. 2005). Secretion of IL-6 in an adipose stress signalling pathway (JNK-1) was thought to induce insulin resistance via increased expression of SOCS3 (Sabio et al. 2008).

More recently IL-6 has proved to be a positive regulator of insulin sensitivity and not a mediator of inflammation in metabolic disease. Human IL-6 (hIL-6) was overexpressed in the brain and lung of mice increasing insulin sensitivity and leptin action to prevent diet-induced obesity. Overexpression of hIL-6 in mice on a high-fat diet remained insulin sensitive and glucose tolerant, had reduced body weight, reduced daily food consumption and reduced serum leptin with no alteration in inflammatory cells observed by tissue histology or serum white blood cell counts (Sadagurski et al. 2010). These authors also crossed the *ob/ob* mouse with mice overexpressing hIL-6, showing reduced body weight, lower glucose and fasted insulin. Interestingly, administration of leptin to *ob/ob* mice overexpressing hIL-6 decreased body weight and feed intake suggesting IL-6 enhances leptin action to prevent obesity in mice. Moreover, inactivation of interleukin receptor alpha (*Ilra*) genes in myeloid cells caused insulin resistance and increased inflammation in diet-induced obesity supporting the notion IL-6 signalling is important for maintaining insulin sensitivity and is not pro-inflammatory in metabolic disease (Mauer et al. 2014). The diverse role IL-6 has in multiple tissues and cell types makes it very difficult to elucidate a definitive role for IL-6 in the metabolic diseased state. Thus the complexity of IL-6 and its multiple functions makes it a difficult candidate as a therapeutic for metabolic disease. Further studies are required to eliminate the controversy surrounding the role IL-6 has in metabolic disease.

6.3.6 The Anti-inflammatory Effect of Exercise in Metabolic Disease: Focus on Adipokines

Chronic systemic low-grade inflammation (two-four fold elevation in plasma of normal range) is associated with metabolic diseases such as obesity, diabetes and metabolic syndrome. Regular ‘non-damaging’ exercise is shown to suppress

macrophage infiltration into adipose tissue (Kawanishi et al. 2010) and reduce inflammatory biomarker concentrations (Bruunsgaard 2005; Beavers et al. 2010). These include the adipokines, TNFα and IL-6 (also inflammatory cytokines) whereby circulating levels are found to be elevated in metabolic diseases and are strongly associated with increased risk of other diseases including cardiovascular disease, cancer and disability (Beavers et al. 2010). Studies on elderly patients (>70 years), showed reduced levels of circulating IL-6 and TNFα where exercise was used as an intervention (Beavers et al. 2010). Aerobic exercise has shown to be more effective in reducing chronic inflammation than resistance exercise. For example, a study conducted on obese postmenopausal women with type 2 diabetes who undertook a 14-week aerobic training program showed a 15 % reduction in the inflammatory biomarker acute phase reactant C-reactive protein (CRP). In contrast, 12 weeks of high intensity progressive resistance strength training did not alter pro-inflammatory cytokines IL-1β, TNFα, IL-6 or IL-2 (Rall et al. 1996). Similarly, IL-6 and TNFα were not changed following resistance training in elderly women (65–80 years) (McFarlin et al. 2004). A combination of aerobic (40 % contribution to 1 h) and resistance (60 % contribution to 1 h) training over 12 weeks in patients with metabolic syndrome reduced the inflammatory cytokine IL-18 by 17.5 % (Troseid et al. 2009). Aerobic exercise is more likely to be effective as an intervention to reduce low-grade inflammatory biomarker concentrations in patients with metabolic disease.

Whilst IL-6 is known to be elevated in metabolic diseases, its role as a circulatory pro-inflammatory cytokine is questioned. IL-6 and TNFα are tightly linked whereby TNFα stimulates IL-6 production and during exercise IL-6 inhibits the production of TNFα (Schindler et al. 1990; Starkie et al. 2003). IL-6 also enhances plasma concentrations of the anti-inflammatory cytokine IL-10 (Steensberg et al. 2003). Thus IL-6 is thought to serve as an anti-inflammatory cytokine, particularly when considering its role in exercise-induced AMPK activation for enhanced glucose uptake and fatty acid oxidation.

6.3.7 Effect of Exercise on Regulating Adiponectin and Leptin in Metabolic Disease Patients

There is evidence to suggest exercise can be used as an intervention to regulate leptin and adiponectin back to healthy levels in patients with metabolic disease. Serum leptin was markedly reduced in obese females after long term (12 weeks) training of 3–4 times weekly at anaerobic threshold for 45 min (Ozcelik et al. 2004). Similarly, leptin levels were reduced in obese females who exercised for 45 min daily for 5 days weekly at 50 % VO_2 max (for 12 weeks) (Polak et al. 2006). Exercise has shown to increase adiponectin levels in obese females who completed 7 months of training (30–60 min, 4–5 days per week), leptin was also reduced along with body weight, body fat mass and TNFα (Golbidi and Laher 2014). Bruun et al.

(2006) were also able to show daily exercise (2–3 h of moderate intensity) in severely obese males who lost 1 % body weight per week caused adiponectin to be elevated. However, a study of 19 overweight and obese females who undertook 12 weeks of training (3 times weekly, 40 min per session) showed no difference in adiponectin and leptin even though insulin sensitivity was improved. Nonetheless, there is evidence to suggest circulating adipokine levels can be altered back to healthy levels through exercise, to assist reducing insulin resistance in adipose tissue and skeletal muscle.

6.4 Concluding Remarks

Cross-talk between adipose tissue and skeletal muscle is fundamental in maintaining energy homeostasis, in order to balance feed intake with energy expenditure. The adipokines adiponectin, leptin and IL-6 have shown to be potent mediators of glucose uptake and fatty acid oxidation, via AMPK signalling, in skeletal muscle. Serum IL-6, also termed a myokine, is significantly up regulated during exercise; these findings are consistent between large numbers of studies. However, studies on leptin and adiponectin following exercise are conflicting; some show no alterations in serum adiponectin or leptin post-exercise, others show leptin to decrease following exercise and adiponectin to increase not immediately but 30 min post-exercise. Consistency between studies for leptin and adiponectin alterations after exercise was found during exercises where whole-body muscles were utilised such as rowing. Experimental studies whereby exercise intensity, duration, type of exercise and energy expenditure are finely controlled may reduce inconsistences between studies to fully elucidate the effect exercise has on adiponectin and leptin.

These adipokines have also been fundamental in the development of insulin resistance in metabolic disease. Elevated levels of leptin and IL-6 and low levels of adiponectin have been associated with insulin resistance. These adipokines can develop their own resistance in metabolic disease contributing to an accumulation of lipids in skeletal muscle, impacting on the pathogenesis of insulin resistance. Exercise, in particular endurance training, is successful in reducing insulin resistance and adipokine resistance in skeletal muscle (in rodents on high fat diet), regulating these adipokines to optimal levels to maintain insulin sensitivity. Overall, whilst the last decade has revealed a vast amount of knowledge about these adipokines, there is much more to understand, particularly in the search for drugs to treat metabolic syndrome, obesity and diabetes to reduce lipotoxicity in skeletal muscle. However, the most obvious and cost-effective means to potentially help prevent and treat metabolic disease, such as obesity, may be as simple as a controlled diet and exercise so food intake does not exceed energy expenditure.

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Chapter 7
Role of Growth Factors in Modulation of the Microvasculature in Adult Skeletal Muscle

Gayle Smythe

Abstract Post-natal skeletal muscle is a highly plastic tissue that has the capacity to regenerate rapidly following injury, and to undergo significant modification in tissue mass (i.e. atrophy/hypertrophy) in response to global metabolic changes. These processes are reliant largely on soluble factors that directly modulate muscle regeneration and mass. However, skeletal muscle function also depends on an adequate blood supply. Thus muscle regeneration and changes in muscle mass, particularly hypertrophy, also demand rapid changes in the microvasculature. Recent evidence clearly demonstrates a critical role for soluble growth factors in the tight regulation of angiogenic expansion of the muscle microvasculature. Furthermore, exogenous modulation of these factors has the capacity to impact directly on angiogenesis and thus, indirectly, on muscle regeneration, growth and performance. This chapter reviews recent developments in understanding the role of growth factors in modulating the skeletal muscle microvasculature, and the potential therapeutic applications of exogenous angiogenic and anti-angiogenic mediators in promoting effective growth and regeneration, and ameliorating certain diseases, of skeletal muscle.

Keywords Angiogenesis • Vascular endothelial growth factor • Cytokine • Skeletal muscle • Myogenesis • Hypertrophy

7.1 Introduction

An efficient vasculature for the supply of oxygen and other nutrients to, and removal of metabolic waste from, skeletal muscle is essential for healthy tissue function. The plasticity of skeletal muscle in response to a range of normal environmental stimuli (e.g. injury, exercise) demands that the microvasculature is similarly dynamic and

G. Smythe (✉)
Faculty of Science, Charles Sturt University, Albury, NSW, 789, 2640, Australia
e-mail: gsmythe@csu.edu.au

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capable of rapidly adapting to tissue change. Such modifications to the microvasculature are regulated through a complex interplay between cellular, extracellular matrix and both blood-borne and local soluble growth factors. Defining the processes underlying modification of the muscle microvasculature is not only important in understanding normal muscle biology, but also in identifying potential therapeutic approaches in a range of muscle conditions and diseases.

In postnatal tissues, capillary growth generally occurs via angiogenesis. In this process, endothelial buds sprout (sprouting angiogenesis) or split (intussusceptive angiogenesis) from existing vessels, and grow toward the stimulus (De Spiegelaere et al. 2012). Sprouting angiogenesis is predominantly driven by growth factor-based stimuli, while intussusception is regulated by changes in haemodynamics and shear stress (Gianni-Barrera et al. 2011). This chapter focuses on the soluble growth factors and cytokines that contribute to angiogenic blood vessel growth in skeletal muscle, in response to a range of both normal and pathological conditions of muscle tissue. Therefore, the term "angiogenesis" hereafter refers to sprouting-type angiogenesis, while reference to intussusceptive angiogenesis will be specified as such.

In sprouting-type angiogenesis, a local stimulus causes small buds of endothelial cells to form and grow toward the stimulus (reviewed in Carmeliet 2000). The initial stimulus is typically a change in the local release of one or more soluble growth factor/s, and it is well established that angiogenesis is a highly regulated process that is governed largely by the balance of pro- and anti-angiogenic factors (reviewed in Bouis et al. 2006). The growth of endothelial buds is also regulated by changes in the basement membrane and extracellular matrix via the actions of matrix metalloproteinases (MMPs) and their inhibitors, and interactions of other cell types including pericytes and vascular smooth muscle cells.

7.2 Angiogenesis: A Function of the Pro- and Anti-angiogenic Growth Factor Balance

A range of soluble factors has been identified with pro- and/or anti-angiogenic functions. This section will focus on growth factors and morphogens with a known role in vascularisation of postnatal skeletal muscle, including vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), angiopoietins, thrombospondins, and the Sonic hedgehog (Shh) family. These key factors will be discussed in detail in relation to their general angiogenic roles, followed by a review of their roles in skeletal muscle function in a range of normal and pathological circumstances. An overview of the key factors regulating angiogenic mechanisms in skeletal muscle is provided in Table 7.1.

Table 7.1 Summary of key angiogenic growth factors relevant to skeletal muscle biology

Factor	Known roles in skeletal muscle[a,b]			Key references
	Exercise	Injury/repair/disease	Ageing	
VEGF[c]	Promotes vascular growth; potentiates Ang-2	Promotes vascular growth, cross-talk with myogenic factors; altered expression profile in some muscle diseases, potential therapeutic target	Decreases with ageing with reduced angiogenic potential in ageing muscle	Hoffner et al. (2003), Olenich et al. (2013), Hoier et al. (2010, 2012, 2013), Hoier and Hellsten (2014), Wagner (2011), Delavar et al. (2014), Rhoads et al. (2013), Palladino et al. (2013), Deasy et al. (2009), Volpi et al. (2013), Wagatsuma (2006)
TGF-β	Limited role compared with VEGF	Promotes inflammation and fibrosis; potential therapeutic target to reduce fibrosis in some muscle diseases	May increase with age, promoting anti-angiogenic and fibrotic phenotype	Carlson et al. 2009a, b; Orlova et al. 2011; Gustafsson and Kraus 2001;
FGF-2	Limited role compared with VEGF	Promotes angiogenesis and myogenesis	Expression and/or signaling pathways may decrease with age	Gustafsson and Kraus (2001), Stratos et al. (2011), Lieu et al. (2011), Amir et al. (2007), Chakkalakal et al. (2012)
Shh		Promotes both myogenic and angiogenic cellular activity; regulates VEGF and Ang-1/-2 expression	Decreases with ageing with reduced angiogenic potential in ageing muscle	Pola et al. (2001), Koleva et al. (2005), Elia et al. (2007), Straface et al. (2009), Fujii and Kuwano (2010), Renault et al. (2010), Palladino et al. (2011), Renault et al. (2013b), Piccioni et al. (2014b)
Dhh		Promotes expression of other angiogenic factors by peripheral nerves	Decreases with ageing with reduced angiogenic potential in ageing muscle	Renault et al. (2013a, b), Piccioni et al. (2014b)

(continued)

Table 7.1 (continued)

Factor	Known roles in skeletal muscle[a,b]			Key references
	Exercise	Injury/repair/disease	Ageing	
TSP-1/-2	TSP-1 is inhibitory; varied responses to exercises depending on acute vs chronic training type	TSP-1 elevated and contributes to limited revascularisation in some disease states (e.g. ischemia); role for overall regulation of capillary bed density in physiological and pathological states		Malek et al. (2013), Malek and Olfert (2009), Olfert et al. (2006), Roudier et al. (2013), Audet et al. (2013), Hoier et al. (2012)
Ang-1	Anti-angiogenic, balance shifts to increased Ang-2	Anti-angiogenic, high early expression with later balance shift to Ang-2; promotes satellite cell survival/quiesence	Does not appear impaired or altered with age	Lloyd et al. (2003), Gustafsson et al. (2007), Wagatsuma et al. (2005), Wagatsuma (2006, 2007, 2008), Qin et al. (2013), Dallabrida et al. (2005), Abou-Khalil et al. (2009), Wang et al. (2011)
Ang-2	Pro-angiogenic, potentiated by VEGF	Pro-angiogenic, induced later to promote vascularisation; promotes myogenic differentiation	Does not appear impaired or altered with age	Lloyd et al. (2003), Gustafsson et al. (2007), Wagatsuma et al. (2005), Wagatsuma (2006, 2007, 2008), Qin et al. (2013), Mofarrahi and Hussain (2011), Hoier et al. (2012)

[a]The growth factor roles summarised in this table align with those discussed in detail in this review chapter

[b]Where no information is entered for a growth factor, either limited or no literature was identified, or it was beyond the scope of this chapter

[c]Abbreviations used in this table: *VEGF* vascular endothelial growth factor, *TGF-β* transforming growth factor-β, *FGF-2* fibroblast growth factor-2, *Shh* sonic hedgehog, *Dhh* desert hedgehog, *TSP-1/-2* thrombospondin-1/-2, *Ang-1/-2* angiopoietin-1/-2

7.2.1 Vascular Endothelial Growth Factor (VEGF)

VEGF is a relatively ubiquitous growth factor family that regulates microvascular remodelling and growth, including during embryonic development, cyclic changes in the ovaries and uterus, and wound healing of otherwise healthy tissues (reviewed in Byrne et al. 2005). VEGF is also known as a key angiogenic growth factor in promoting tumour growth and metastasis, and, on secretion by developing tumour cells, it exerts not only paracrine effects on the local microvasculature, but also

autocrine effects on tumour cell survival (reviewed in Byrne et al. 2005). The VEGF gene and protein families, their receptors and signalling cascades have been reviewed extensively elsewhere (Byrne et al. 2005; Shibuya 2008) and will be covered here only briefly.

There are 6 known genes in the VEGF family, denoted VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF) (reviewed in Byrne et al. 2005). VEGF proteins involved in postnatal angiogenesis are predominantly encoded by the VEGF-A gene. Multiple isoforms are generated through alternative splicing from 8 exons, and these are denoted by their amino acid number with $VEGF_{165}$ being the most predominant isoform. This family constitutes both anti- and pro-angiogenic isoforms, of which the former are usually denoted as $VEGF_{xxxb}$. (reviewed in Byrne et al. 2005; Nowak et al. 2008). The expression pattern of pro- and anti-angiogenic VEGF isoforms is regulated largely through the local growth factor environment regulating alternative splicing of pre-mRNA (Nowak et al. 2008), with the angiogenic outcome being regulated at least in part through the ratio of positive and negative isoforms (Carter et al. 2011; Zhao et al. 2011). The pro-angiogenic VEGF isoforms promote vascular permeability, angiogenesis and inflammation and are expressed by a range of cell types, including those of the myogenic lineage (reviewed in Volpi et al. 2013). Proteins encoded by VEGF-C and VEGF-D have weaker effects on the blood vasculature, having a more significant role in lymphangiogenesis (reviewed in Byrne et al. 2005). VEGF-B is known to be specifically expressed in several tissues, including skeletal muscle where it is thought to contribute to angiogenesis (Olofsson et al. 1996).

7.2.2 Transforming Growth Factor-β

TGF-β promotes both early events of endothelial cell proliferation and migration, as well as the extracellular matrix synthesis and perivascular cell migration which correspond with the later stages of vessel stabilisation and growth cessation (Roberts et al. 1986; Madri et al. 1988; Pepper et al. 1990, 1993; reviewed in Bertolino et al 2005). *In vitro*, TGF-β exerts dose-dependent effects on endothelial cell mitosis, whereby low doses promote proliferation while high doses are inhibitory (Pepper et al. 1993). These multifunctional roles of TGF-β are differentially regulated through receptor- and downstream signalling pathway-dependent mechanisms (Orlova et al. 2011). Expression levels of the two key TGF-β receptors, activin receptor-like kinase-1 (ALK1) and ALK5, correspond with the phenotypic response of endothelial cells to growth factor exposure. ALK1 ligation and activation of the Smad1/5 signalling pathway occurs early in angiogenesis and promotes proliferation and migration. In contrast, ALK5-Smad2/3 activation occurs later and corresponds with matrix synthesis and vessel maturation. It has been proposed that imbalances or discordance in these signalling pathways may be a contributing factor in pathological forms of angiogenesis (reviewed in Jakobsson and van Meeteren 2013). However, TGF-β has also been strongly implicated in fibrosis and scar tissue

formation through fibroblast proliferation and matrix production, while also having the capacity to suppress the immune system (reviewed in Jakobsson and van Meeteren 2013). Therefore, up-regulation of TGF-β as a pro-angiogenic therapy may not be suitable for promoting tissue repair due to the dose-dependent effects on endothelial cells, and increased likelihood of fibrosis and poor tissue healing. However, as discussed below, inhibition of this growth factor has been widely considered as an anti-fibrotic treatment approach, including for promoting skeletal muscle repair following injury.

7.2.3 Fibroblast Growth Factors

While both acidic and basic fibroblast growth factors (FGF-1 and FGF-2 respectively) are known to promote angiogenesis, FGF-2 is the most potent and well characterised of the two. FGF-2 is readily stored in the extracellular matrix and endothelial cell basement membrane of most tissues by binding in an inactive form to heparan sulphate proteoglycan (Vlodavsky et al. 1987; Folkman et al. 1988). FGF-2 alone promotes endothelial cell activity, but there is also significant evidence for synergistic cross-talk of this growth factor with VEGF, angiopoietins, and platelet-derived growth factors in the regulation of angiogenesis (Lieu et al. 2011). FGF-2 also has a well-established role in promoting the formation of new skeletal muscle tissue (myogenesis), exerting direct effects on proliferation and differentiation of muscle precursor cells (myoblasts) (Stratos et al. 2011). Thus, this factor appears to exert multiple roles in muscle, having dual myogenic and angiogenic functions, and these are discussed in detail below.

7.2.4 Sonic Hedgehog Family

The Hedgehog (Hh) family includes Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh), and they act via the Patched1 receptor, subsequent derepression of the trans-membrane protein Smoothened and activation of the Gli transcription factors (reviewed in Villavivicencio et al. 2000). While originally identified for its role as a pre-natal myogenic and vasculogenic morphogen, Shh is now known to be up-regulated in adult skeletal muscle following injury where it promotes both angiogenic and myogenic processes (Pola et al. 2003; Straface et al. 2009). Shh promotes post-natal angiogenesis by direct effects on endothelial cells (Renault et al. 2010), and may have a role in controlling the expression of VEGF and the ratio of pro- and anti-angiogenic angiopoietin isoform expression (Pola et al. 2001; Straface et al. 2009; Fujii and Kuwano 2010). Exogenous administration of Shh promotes wound healing in diabetic skin ulcers by enhancing wound

revascularisation through direct effects on endothelial cell proliferation and migration, and capillary maturation/tube formation (Asai et al. 2006). Similar effects are shown in limb ischemia with direct administration of Shh alone (Palladino et al. 2011), and in combination with bone marrow-derived endothelial cell progenitors to increase the local pool of angiogenic cells (Palladino et al. 2012). Dhh also promotes revascularisation in ischemic muscle, by promoting survival and expression of angiogenic growth factors by local peripheral nerves, rather than through a direct effect on the microvasculature (Renault et al. 2013a).

During development, Shh promotes myogenic differentiation and hypertrophy (Duprez et al. 1998). There is evidence that this occurs at least in part via the Gli2-mediated transcriptional up-regulation of myogenic regulatory factors (e.g. *Myf5, MyoD*) (Gustafsson et al. 2002; Maves et al. 2007; Straface et al. 2009) and myosin expression (Duprez et al. 1998; Sacks et al. 2003). A role for Shh in postnatal myogenesis was first described in limb ischemia (Pola et al. 2003). This appears to be largely through regulation of myogenic cell proliferation and survival by inhibiting pro-apoptotic pathways regulated by caspase-3 (Koleva et al. 2005), and activating the phosphatidyl-inositol 3-kinase/Akt and mitogen-activated protein kinase signalling pathways (Elia et al. 2007). It has been hypothesised that these mechanisms are largely through autocrine secretion of Shh by myoblasts in injured muscle (Elia et al. 2007).

Thus there are clear roles for the Hh family of proteins in postnatal muscle injury and repair whereby Shh promotes both myogenesis and angiogenesis through direct effects on myoblasts and endothelial cells respectively, while Dhh up-regulates nerve-derived angiogenic factors to indirectly enhance revascularisation.

7.2.5 Thrombospondins (−1 and −2)

Thrombospondins (TSPs) were identified as being anti-angiogenic as they promote the latter phases of angiogenesis, including capillary stabilisation and maturation (Iruela-Arispe et al. 1991). TSPs appear to have a key role in regulating the skeletal muscle microvasculature. TSP-1 knockout mice show a significant increase in capillary density in muscle (Malek and Olfert 2009; reviewed in Lawler and Lawler 2012), and blocking TSP-1 activity can promote revascularisation of ischemic muscle (Roudier et al. 2013). In contrast, *in vivo* delivery of a TSP-1 mimetic causes reduced muscle capillary density (Audet et al. 2013). TSP-2 is not as well understood, but is known to inhibit the release and activity of pro-angiogenic factors such as VEGF and matrix-degrading MMPs (MacLauchlan et al. 2009). While the inhibitory effects of TSPs on angiogenesis have largely been characterised in tumour models for their potential therapeutic value (reviewed in Lawler and Lawler 2012), these factors are also up-regulated in skeletal muscle under certain conditions, and these are discussed in detail below.

7.2.6 *Angiopoietins*

Of the angiopoietin (Ang) family of proteins, Ang-1 and Ang-2 are the best characterised in relation to angiogenic roles. Ang-1 has pro-angiogenic effects, promoting endothelial cell activation, migration and survival, and recruitment of pericytes and vascular smooth muscle cells to newly formed vessels (Brindle et al. 2006; reviewed in Fagiani and Christofori 2013). The role of Ang-2 is more complex; when acting alone, it is potently anti-angiogenic, but in concert with VEGF it can potentiate endothelial cell sprouting (Holash et al. 1999; Visconti et al. 2002; reviewed in Fagiani and Christofori 2013). Angiogenic outcomes are often a function of the ratio of Ang-1 to Ang-2, with increased expression of both isoforms often occurring, but usually to a higher magnitude for one over the other (Fujii and Kuwano 2010). This appears to be regulated at least in part by other factors influencing angiogenesis, with Shh having the capacity to up-regulate Ang-1 expression, and FGF2 selectively inducing Ang-2 expression (Fujii and Kuwano 2010). Specific examples of angiogenesis being regulated by the Ang1/Ang-2 balance are outlined in the context of skeletal muscle biology in the following sections.

7.3 Angiogenesis in Skeletal Muscle: Exercise, Regeneration, Disease and Ageing

Skeletal muscle is responsible for all voluntary movement. It is highly responsive to environmental change, being able to rapidly hypertrophy to accommodate higher metabolic demand, while atrophying during periods of disuse. Tissue growth requires a concomitant expansion of the microvasculature to ensure adequate nutrient exchange, and therefore angiogenic growth factors are essential in coupling vascular supply with tissue mass. In addition, as in any wound-healing situation, revascularisation of injured skeletal muscle is essential in promoting repair and functional recovery. In small injuries, this is relatively efficient, but can become problematic with repetitive damage or extensive trauma. Consequent to these roles of angiogenesis in normal healthy muscle is that its' regulation can be disrupted through the normal ageing process, and in a range of disease states. A detailed understanding of the role of angiogenic growth factors in healthy muscle responding to metabolic changes, and the impact of ageing and disease on these factors, may provide a basis for developing "angiogenic therapies" for conditions affecting muscle function.

7.3.1 *Exercise-Induced Angiogenesis*

Exercise is a well-established stimulant of skeletal muscle angiogenesis, whereby expansion of the microvasculature occurs to match muscle hypertrophy and increased metabolic demand, and this has been well documented since the 1970s

(Andersen and Henriksson 1977; Tesch et al. 1984; Hudlicka 1990). This is a highly regulated process defined by the temporal expression of pro- and anti-angiogenic growth factors regulating microvascular dynamics in skeletal muscle in response to both acute and chronic exercise.

The most potent angiogenic factor clearly involved in exercise-induced angiogenesis in skeletal muscle is VEGF (reviewed in Hoier and Hellsten 2014). Acute exercise in mice (Olenich et al. 2013) and humans (Hoier et al. 2012) leads to a rapid increase in VEGF expression in skeletal muscles and a subsequent increase in capillary density. VEGF is stored within sub-sarcolemmal vesicles in skeletal myofibres and released upon contraction, making the protein available to stimulate local endothelial cell activity and angiogenesis (Hoffner et al. 2003; Hoier et al. 2010; Hoier et al. 2013). The essential role of VEGF in exercise-induced angiogenesis and the relationship of this to muscle function has been clearly demonstrated. Mice exhibiting a VEGF deficiency in their skeletal muscles showed reduced muscle capillary density following 8 weeks of exercise training, and this impacted significantly on overall running performance and muscle oxidative capacity (Wagner 2011; Delavar et al. 2014). This appears to be regulated at least in part through β-adrenergic-mediated up-regulation of peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α), and oestrogen-related receptor-α (ERRα) (Chinsomboon et al. 2009; Wallace et al. 2011). Oxygen supply, specifically hypoxia, promotes angiogenesis in skeletal muscle, and this may contribute to increased exercise (and thus oxygen) demand and similarly to the direct effects of exercise. Hypoxia-induced angiogenesis may similarly be controlled regulated by PGC-1α/ERRα/VEGF signaling (Arany et al. 2008). There is also evidence that hypoxia-inducible factors (HIFs) can also promote VEGF up-regulation (Ameln et al. 2005; Gustafsson et al. 1999; Flann et al. 2014; reviewed in Hoier and Hellsten 2014), although this may represent some redundancy, because while a genetic deficiency of VEGF significantly impairs exercise-induced angiogenesis, there is no similar effect of HIF deficiencies (Wagner 2011). Thus, while it is clear that VEGF has a significant angiogenic function in response to exercise, both directly in response to muscle activity and as a result of increased oxygen demand, the upstream regulation of VEGF activity is clearly complex and further signaling pathways may remain to be characterised.

The balance of Ang-1/Ang-2 expression is also modified in response to exercise in skeletal muscle, with a shift towards increased Ang-2 levels (Lloyd et al. 2003; Gustafsson et al. 2007). The corresponding increase in VEGF expression likely potentiates the pro-angiogenic effects of Ang-2 (Gustafsson et al. 2007). Interestingly, there is a decrease in both Ang-1 and Ang-2 expression in muscle disuse (Wagatsuma 2008) and in denervated muscle (Wagatsuma et al. 2005). In both situations, muscles atrophy and thus demand on the vascular supply decreases, allowing for vascular regression to occur.

Concomitant with increased pro-angiogenic factor expression is an initial decrease in expression of anti-angiogenic TSP-1 with exercise (Malek et al. 2013). TSP-1 null mice not only show a significant increase in capillary density, they also show an increased capacity to undertake exercise through increased maximal speed and time to exhaustion (Malek and Olfert 2009). However, the role of TSP-1 in

exercise-induced angiogenesis may be regulated through exercise type, oxygen demand, and the timing of sample analysis. A single bout of acute exercise induces TSP-1 up-regulation, but with chronic activity this effect is lost, and where exercise occurs in hypoxic conditions, TSP-1 induction capacity is significantly reduced (Olfert et al. 2006). The impact of chronic activity may explain why some studies report no effect of regular exercise training on TSP-1 expression levels (Hoier et al. 2012).

Following up-regulation of pro-angiogenic growth factors, subsequent negative regulation of angiogenesis to limit the extent of vessel growth to physiological requirements, comes not only through increased release of anti-angiogenic factors, but is also largely controlled by the p53 tumour suppressor, which blocks angiogenic factor expression while simultaneously up-regulating anti-angiogenic factors (Ma et al. 2012). Indirectly, through down-regulation of p53, other factors such as the E3 ubiquitin ligase murine double minute-2 (Mdm2), can also tip the balance of pro- and anti-angiogenic factor activity towards the promotion of new vessel growth (Ma et al. 2012; Roudier et al. 2012). Mdm2 expression is strongly induced by exercise, and its actions are partly via induction of VEGF-A (Ma et al. 2012; Roudier et al. 2012). Interestingly, while Mdm2 is a known negative regulator of p53 activity, recent studies show that Mdm2 can also promote angiogenesis via VEGF-A induction independently of p53 (Roudier et al. 2012; Xiong et al. 2014).

In summary, it is abundantly clear that angiogenesis is a critical component of the adaptability of skeletal muscle to exercise. The roles of both pro- and anti-angiogenic factors and the processes that govern their expression profiles are clearly complex and in many cases still remain to be fully elucidated. However, it is most evident that, as in all forms of physiological angiogenesis, the overall balance of pro- and anti-angiogenic growth factors, and their extremely tight spatio-temporal regulation, is critical in governing changes in the muscle microvascular network to ensure efficient nutrient supply based on demand. An understanding of compounds that have the capacity to modify the microvasculature and thus promote the muscle response to exercise has the potential to inform not only elite athletic performance, but also to promote exercise in those experiencing muscle fatigue or other bases for an inability to exercise. Recently the cocoa-derived (−)-epicatechin was shown to significantly promote VEGF and down-regulate TSP-1 expression, with an overall increase in capillary density, in skeletal muscle of low running capacity rats (Huttemann et al. 2013). This compound also promoted exercise performance in low running capacity animals (Huttemann et al. 2013) and allowed exercise capacity to be maintained following a non-training period (Huttemann et al. 2012).

7.3.2 Revascularisation of Injured Muscle

Efficient muscle regeneration requires a controlled inflammatory response, followed by combined muscle formation (myogenesis) and angiogenesis. These processes are highly inter-dependent and studies on the roles of growth factors in their

regulation are complex, because a number of growth factors have overlapping myogenic, fibrogenic, and angiogenic functions.

The detailed process of muscle regeneration *in vivo* has been recently and extensively reviewed elsewhere (see Jarvinen et al. 2014; Uezumi et al. 2014). Briefly, following injury of normal healthy muscle, a rapid inflammatory response leads to removal of tissue debris, and this is followed by activation of resident muscle precursor (satellite) cells, which undergo proliferation, fusion with one another and/or damaged myofibres, and terminal differentiation to form mature repaired myofibres. There is concomitant stabilisation of damaged capillaries, and growth of new capillary branches to re-supply the regenerating muscle tissue. An important aspect of regeneration is the resolution of the initial inflammatory process, since chronic inflammation impairs myogenesis and promotes a fibrogenic (scar) tissue phenotype. While young, healthy skeletal muscle can regenerate well after small injuries, there is evidence to suggest that pre-injury structure may not always be fully regained due to the formation of fibrotic scar tissue, thus making previously damaged muscle susceptible to re-injury (Kasemkijwattana et al. 2000). Therefore, exogenous delivery of growth factors to promote myogenesis and/or angiogenesis, or limit inflammation and fibrosis, has long been considered of potential therapeutic value in aiding post-injury muscle repair (Kasemkijwattana et al. 2000).

TGF-β is a key therapeutic target in tissue repair due to its known fibrogenic role, however, as outlined above, it also has complex roles in angiogenesis that require consideration. TGF-β transfection transforms skeletal myoblasts to a myofibrogenic phenotype (Li et al. 2004). A recent study reported that this phenotypic switch can be potentiated by the lymphangiogenic VEGF-C, which directly activates TGF-β to promote myofibroblast activation and tissue fibrosis in myocardial infarction (Zhao et al. 2014). The hormone relaxin can restore myogenic properties of TGF-β-transfected myoblasts, and enhances muscle regeneration *in vivo* (Negishi et al. 2005). More recent studies have demonstrated that relaxin promotes both myogenesis and angiogenesis, while reducing inflammation, in injured muscle (Mu et al. 2010). These effects of relaxin may be via up-regulation of pro-angiogenic VEGF isoforms (Unemori et al. 2000), and/or through up-regulation of MMPs, which enhance extracellular matrix degradation and facilitate migration of both myoblasts and capillary endothelial cells (Rivilis et al. 2002; Johnson et al. 2004; Mu et al 2010). Recent studies have demonstrated that the anti-fibrotic agent, losartan, promotes regeneration of injured muscle through limiting expression of TGF-β to reduce muscle fibrosis (Terada et al. 2013), and enhances angiogenesis in overloaded muscles by up-regulating VEGF expression (Gorman et al. 2014). Thus, a number of exogenous approaches to manipulating TGFβ and VEGF expression have been tested for their therapeutic benefits in the context of skeletal muscle regeneration.

Similarly to exercise, muscle injury and ischemia also induce VEGF expression (Rissanen et al. 2002), likely through release of this protein directly from storage sites in damaged myofibres (Hoffner et al. 2003; Hoier et al. 2010). As described above, VEGF up-regulation in ischemic conditions, resulting largely from restricted vascular and oxygen supply, may also be controlled through upstream signaling via

PGC-1α/ERRα (Arany et al. 2008). Therefore, not surprisingly, the VEGF family has been widely tested for its capacity to induce angiogenesis in skeletal muscle, largely through virus-mediated delivery (Smythe et al. 2002; Rissanen et al. 2003; Arsic et al. 2004; Messina et al. 2007; Frey et al. 2012; Long et al. 2013; Heikura et al. 2012; Kuwahara et al. 2013). Recent studies have focused on delivery modes and co-administration with other agents, including using endothelial progenitor cells over-expressing both the VEGF-A and heme oxygenase-1 genes, which promoted increased regeneration and muscle revascularisation to a greater extent than delivery of VEGF alone (Long et al. 2013). VEGF delivered in combination with pro-myogenic growth factors such insulin-like growth factor-1 (IGF-1) (Borselli et al. 2010) and hepatocyte growth factor (Makarevich et al. 2012) has been shown to enhance repair and reperfusion of ischemic skeletal muscles over and above the effects of single growth factor therapy alone. Interestingly, IGF-1 over-expression in muscle-derived stem cells promotes increased expression of VEGF, indicating cross-talk between potent myogenic and angiogenic growth factors (Chen et al. 2014).

The pre-transplantation therapeutic potential of angiogenic growth factors was shown by pre-loading mouse muscles with VEGF encoded by an adeno-associated viral vector (Smythe et al. 2002). This approach led to enhanced post-transplantation revascularisation and regeneration and has potential therapeutic applications in preparation for surgery-induced muscle trauma. More recently a liposome-based delivery system was utilised to treat major muscle injuries with $VEGF_{165}$ (Ye et al. 2010). VEGF therapy, including for intramuscular delivery, primarily to treat limb ischemia, has been trialed clinically in humans, with outcomes ranging from little or no effect, to significant improvements (reviewed in Giacca and Zacchiagna 2012). There has been some experimental success with drugs and other therapeutic regimes that indirectly impact on VEGF levels. Mechanical stimulation alone has been shown to enhance endothelial cell activity and this appears to occur by promotion of angiogenic growth factor expression in ischemic limbs, leading to a stronger neovascular response and more rapid functional recovery (Tepekoylu et al. 2013). The applicability of the use of mechanical stimulation may apply to combining massage therapies with pharmacological and/or cell-mediated approaches to promote muscle healing (Best et al. 2013).

Interestingly, while VEGF administration may be a potential therapeutic approach for a range of conditions, including promoting muscle healing after injury, it has been reported that the high doses required for functional improvement promote vascular growth through intussusceptive angiogenesis, rather than the sprouting form that is typically driven by changes in the extravascular environment (Gianni-Barrera et al. 2013). Thus, there may be a dose-dependent effect of VEGF on the cellular process/es by which revascularisation occurs, whereby high doses promote intravascular changes that drive vascular growth, compared with lower doses that directly impact on endothelial cells and promote sprouting events. Furthermore, there is evidence to suggest that VEGF delivered as the sole angiogenic growth factor may be problematic due to resultant poorly formed and abnormally permeable capillaries (Mujagic et al. 2013; Shimizu-Motohashi and Askaura 2014).

This appears to be due to a lack of pericyte recruitment, as a recent study demonstrated co-delivery of VEGF with platelet-derived growth factor, which activates pericyte migration, improves the morphology and function of newly formed capillaries in ischemic muscle (Banfi et al. 2012). Therefore, it is important to assess experimental angiogenic growth factor-based therapies not only for their capacity induce revascularisation, but to promote the formation of vessels with normal morphology and functional capacity.

Other factors contributing to angiogenesis and/or myogenesis in injured muscle include FGF2, angiopoietins, TSP-1, and Shh. As outlined above, FGF-2 also exerts both angiogenic and myogenic effects and may thus be of therapeutic use in enhancing regeneration of muscle following injury. Early studies reported that direct administration of FGF-2 into injured muscle did not improve the repair outcome (Mitchell et al. 1996), however more recent studies indicate that more robust modes of intramuscular delivery are more effective (Stratos et al. 2011). However, the relative effects of exogenous FGF-2 delivery on angiogenesis and/or myogenesis have not been investigated in detail, and it remains unclear if therapeutic benefits are due to single or dual effects on these processes.

Ang-1 and Ang-2 are both up-regulated in injured skeletal muscle, however similarly to exercise-induced expression, the fold-change in Ang-2 expression is much greater than that of Ang-1, leading to a ratio variation that favours the vessel destabilising effects of Ang-2 (Wagatsuma 2007). There is also evidence for tightly regulated temporal expression of angiopoietin isoforms throughout angiogenesis in muscle repair, with early high expression of Ang-1, followed by a later phase of high Ang-2 expression (Qin et al. 2013). As is the case with other angiogenic growth factors, angiopoietins also appear to exert a direct influence on cells of the myogenic lineage, with Ang-1 promoting satellite cell survival, renewal and quiescence (Dallabrida et al. 2005; Abou-Khalil et al. 2009), while Ang-2 appears to be involved in differentiation of skeletal muscle cells (Mofarrahi and Hussain 2011). However, the detailed time- and process-dependent roles of angiopoietin isoforms in muscle-based angiogenesis requires more in-depth investigation.

As described in detail above, the Hh factor family have dual roles in myogenesis and angiogenesis, and these roles have formed the basis for recent studies on the therapeutic potential of Hh protein administration to extensive muscle injuries. However, the benefits of Hh therapy may need to be regarded with caution because Hh signalling is also known to directly promote tumour cell survival (Delloye-Bourgeois et al. 2013), and dysfunctional Hh signalling has been linked with highly aggressive embryo-originating rhabdomyosarcoma (Rajurkar et al. 2013).

Taken together, these studies clearly demonstrate a highly complex interplay between myogenic, angiogenic and fibrogenic processes in the repair of skeletal muscle following injury. There are roles of other systems and cell types (e.g. the immune system) that are outside the scope of this chapter. What is abundantly clear is that there are multiple opportunities for therapeutic approaches that target overlapping roles of growth factors in these processes, and recent developments in this field of research are promising.

7.3.3 Muscle Disease

Many diseases of skeletal muscle are characterised by muscle weakness and susceptibility to injury and degeneration, and/or disruption to the subsequent regeneration process. Therefore, there are some parallels in therapeutic approaches for promoting repair and revascularisation of normal healthy muscle following injury (see above), and muscle disease. Duchenne muscular dystrophy (DMD) is one of the most common and severe muscle diseases, resulting from a genetic mutation in the dystrophin gene causing a deficiency in the muscle membrane-stabilising dystrophin protein (reviewed in Ennen et al. 2013). A range of therapeutic approaches, largely based on gene replacement utilising both cell- and non-cell-based vehicles, have been in experimental development for many years, and these have been extensively reviewed recently elsewhere (see Foster et al. 2012; Rodino-Klapac et al. 2013). Due to limitations of gene-based therapies relating to delivery, dissemination, and immunological responses and other side effects, alternative approaches are under consideration. Growth factor-mediated therapies to exogenously regulate the processes associated with muscle regeneration (myogenesis, angiogenesis, inflammation, fibrosis), may be of significant value, alone or in combination with gene therapy, in ameliorating the progression of DMD and other degenerative muscle diseases.

While muscle weakness, degeneration and limited capacity for repeated cycles of repair are the primary contributing factors to disease progression in DMD, the disease appears to be exacerbated by impaired angiogenic growth factor expression and abnormalities in the vascular endothelium itself. Expression of angiogenic growth factors, such as VEGF and HIF1α, by dystrophic muscle cells is significantly reduced, as is the overall potential of these cells to induce an angiogenic response (Rhoads et al. 2013). Dystrophic endothelial cells show an increased propensity to undergo apoptosis and a reduced capacity to contribute to angiogenesis (Palladino et al. 2013; reviewed in Shimizu-Motohashi and Askaura 2014). This is consistent with previous reports of significant abnormalities in the dystrophic brain endothelium, including increased permeability and disruption of the blood–brain barrier (Nico et al. 2003). The combined impacts of a dystrophin deficiency on the myogenic and angiogenic potential of dystrophic muscle have provided a basis for combining dystrophin and VEGF gene therapy utilising stem cells (Deasy et al. 2009). Interestingly, using mechanical stimulation in combination with muscle-derived stem cell therapy can also promote both myogenesis and angiogenesis, with the latter appearing to be largely due to up-regulation of VEGF expression (Beckman et al. 2013). Other approaches combining VEGF with myogenic factors to promote angiogenesis and myogenesis are discussed above in relation to muscle regeneration. VEGF-based therapies may also be of clinical significance in other muscle diseases, with evidence for a strong shift toward an anti-angiogenic VEGF profile through elevated $VEGF_{165b}$ expression in muscle biopsies from humans with inflammatory myopathies (Volpi et al. 2013).

Similarly to the repair of normal healthy muscle, the balance between myogenic, angiogenic and fibrotic processes need to be considered in growth factor-based therapeutic approaches to muscle disease. DMD is characterised by progressive replacement of large areas of muscle tissue with fibrotic scar tissue, and drugs that target fibrotic and inflammatory pathways have been shown to improve muscle pathology (Klingler et al. 2012). These may be of use in combination with angiogenic and other pharmacological approaches. There may also be significant value in considering non-invasive methods of potentiating the effects of pharmacologically-based treatments, such as light exercise and massage (Klingler et al. 2012). As discussed above, these may have the capacity promote endogenous angiogenic activity, and the responsiveness of muscle tissue to exogenously delivered factors.

7.3.4 Effects of Skeletal Muscle Ageing

Similarly to most tissues of the body, skeletal muscle undergoes certain changes with the normal ageing process, which can impact on deficits in muscle workload capacity, and in the ability to self-repair following injury. Compounding factors of older age, including a more sedentary lifestyle leading to muscle atrophy and loss of muscle mass (sarcopenia), can contribute to limitations in muscle function.

Many studies have investigated the capacity of aged skeletal muscle to regenerate following injury, with wide-ranging findings that can likely be attributed to variation in species, experimental approaches, sample sizes and sampling time points, and ages under investigation. The variation of data from very slight, to severe, effects of ageing on overall muscle repair is also likely due to the complexity of this process and involvement inflammatory, fibrotic, myogenic and angiogenic components (Grounds 1998; Carosio et al. 2011). There is evidence for an age-associated loss of the capacity of injured muscle to induce an angiogenic response (Shimada et al. 2004; Smythe et al. 2008). This may be due to altered levels of stored angiogenic factors, with endogenous VEGF expression in skeletal muscle declining with age (Wagatsuma 2006; Rhoads et al. 2013), combined with evidence that endothelial cells derived from aged skeletal muscle show a higher incidence of apoptosis (Wang et al. 2013). Aged muscle has a reduced capacity to induce VEGF expression in response to limb ischemia (Wang et al. 2011). In contrast, other factors such as Ang-1 and Ang-2 show both normal baseline expression levels (Wagatsuma 2006) and induction in response to ischemia (Wang et al. 2011) in old muscle. Ageing also appears to be associated with decreased expression of the pro-angiogenic Hedgehog family members, Dhh (Renault et al. 2013b) and Shh (Piccioni et al. 2014b). In particular impaired Shh expression appears to lead to limited revascularisation and regeneration of injured muscle, and intramuscular injection of plasmid-encoded Shh can ameliorate this effect of ageing (Piccioni et al. 2014b).

Ageing may also induce a shift in the microvascular environment to one that supports fibrosis, rather than myogenesis (Brack et al. 2007). This may be regulated through altered levels of TGF-β as inhibitors of this growth factor can promote

improved myogenic cell activity and limit the conversion of these cells to a fibrotic phenotype (Carlson et al. 2009a). Furthermore, muscle satellite cells express increased levels of TGFβ and Smad3 with age, which promotes higher levels of matrix synthesis and can thus contribute to a fibrotic (Carlson et al. 2009b) and anti-angiogenic (Orlova et al. 2011) phenotype. As outlined above, TGFβ may therefore be a key target for young and old muscles, and diseased muscles, in which reducing fibrosis is of therapeutic benefit.

In contrast to muscle injury, there does not appear to be an impact of age on exercise-induced angiogenesis, with aged mice exhibiting a similar capillary-to-fibre ratio following acute exercise to young animals (Gavin et al. 2006). Interestingly, the latter study showed that this was related to increased VEGF expression levels, and these results corresponded with data in humans (Gavin et al. 2007; Buford et al. 2012). These data suggest that within normal physiological conditions, old muscle retains the capacity to elicit angiogenic activity in response to exercise, but may be limited in the more extensive response required for injury repair. There may also be an effect of regular exercise and fitness level on angiogenic, myogenic and fibrotic growth factor expression profiles in ageing, since the capacity to induce expression of FGF-2 is increased in aged muscle of individuals with a higher level of fitness (Amir et al. 2007). Muscle satellite cells express high levels of the FGF inhibitor, Sprouty (Chakkalakal et al. 2012); while this was reported in the context of muscle regenerative capacity and satellite cell renewal, this may also indirectly impact on the capacity of aged muscle to induce an appropriate angiogenic response and maintain an adequate microvascular supply.

7.4 Conclusions and Recent Advances in Angiogenic Therapies

A dynamic microvasculature is essential for normal skeletal muscle function and its' capacity to respond to changes in functional demand, injury, ageing, and disease. Both anti- and pro-angiogenic therapies have been under investigation for some time, although the former is much more advanced, particularly in relation to anti-cancer treatment (reviewed in Katoh 2013). A number of promising anti-angiogenic drugs that specifically target VEGF have been developed for limiting tumour growth and metastasis, age-associated macular degeneration and other diseases that are potentiated by neovascular processes. Ramucirumab specifically targets the VEGF2 receptor and has been shown in phase III clinical trials to prolong life expectancy in cancer patients (Clarke and Hurwitz 2013), while tivozanib blocks VEGF receptor tyrosine kinase activity and has also shown positive effects through phase III clinical trials (Cowey 2013).

A number of therapies have been outlined in this chapter with specific relevance to treating conditions of skeletal muscle, which primarily focus on shifting the growth factor balance to one that favours angiogenesis. These include reducing

anti-angiogenic and pro-fibrotic effects of TGFβ (Terada et al. 2013), and promoting expression of VEGF (Smythe et al. 2002; Rissanen et al. 2003; Arsic et al. 2004; Messina et al. 2007; Frey et al. 2012; Long et al. 2013; Heikura et al. 2012; Kuwahara et al. 2013), FGF2 (Stratos et al. 2011), and Shh (Piccioni et al. 2014a, b) to enhance young, aged and diseased muscle regeneration. Studies have also manipulated expression of VEGF and TSP-1 activity to enhance capillary density and exercise capacity (Huttemann et al. 2012, 2013). Several studies have also investigated the efficacy of combination therapies to improve outcomes in overall muscle function, and in the quality of new vessels that are formed in response to angiogenic factor delivery (Borselli et al. 2010; Banfi et al. 2012; Makarevich et al. 2012; Long et al. 2013). These latter approaches have promising potential for treating a range of conditions affecting skeletal muscle.

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