3500 live male births (Emery, 1991). DMD causes progressive degeneration and regeneration of skeletal and cardiac muscles due to mutations in the dystrophin gene, which encodes a 427-kDa subsarcolemmal cytoskeletal protein (Hoffman *et al.*, 1987). DMD is associated with severe, progressive muscle weakness and typically leads to death between the ages of 20 and 35 years. Due to recent advances in respiratory care, much attention is now focused on treating the cardiac conditions suffered by DMD patients. Although various new therapeutic strategies, including new drug design, gene therapy and cell therapy, have been proposed, no effective treatment has yet been established (Table 1).

Impact of Exon-Skipping Therapy

Exon-skipping therapy is detailed in our previous reviews (Nakamura and Takeda, 2009; Miyagoe-Suzuki and Takeda, 2010). Here, we present an overview of the most specific impact of our approach. Dystrophin is essentially missing at the sarcolemma in DMD, although some dystrophin-positive fibers, called revertant fibers, are present. The number of revertant fibers increases with age due to the cycle of degeneration and regeneration (Wilton *et al.*, 1997). It is currently thought that the molecular mechanism underlying the revertant fibers is the skipping of the exon

Table 1. Clinical trials for DMD/BMD.*

Category	Drug/Genetic	Phase (ClinicalTrials.gov)
Drug interventions	Myostatin blocker	
	MYO-029	Completed; not effective
	Read-through	
	PTC124	Completed; not effective (Phase IIb)
	Gentamicin	Completed; not effective
	Others	
	Pentoxifylline	Completed; not effective
	Idebenone	Phase III
	Ramipril vs. Carvedilol	Phase VI
	CoQ10 and prednisone	Phase III
	Coenzyme Q10 and lisinopril	Phase II/III
	Debio-025 (cyclosporine analogue)	Phase IIb
Gene therapy	Exon skipping (systemic delivery)	
	PRO051 (21'-*O*-MePS AO)(exon 51 skipping)	Phase III
	PRO044 (2'-*O*-MePS AO)(exon 44 skipping)	Phase I/II
	AVI-4658 (PMO)(exon 51 skipping)	Phase IIb
	AAV vector	
	rAAV2.5-CMV-Mini-Dystrophin	Phase I
Cell therapy	Satellite cells (myoblasts)	Pending
	Mesoangioblasts	In preparation
	Mesenchymal stem cells	Ongoing
	Induced pluripotent stem (iPS) cells	Experimental

*Detailed information can be retrieved by using a registry of the clinical trials conducted in the United States and around the world (http://www.clinicaltrials.gov).

around the original mutation, which gives rise to the correction of the reading frame and expression of dystrophin. Consequently, exon skipping has attracted attention as a strategy for restoration of dystrophin expression in DMD. To induce skipping of specific exons during mRNA splicing, antisense oligonucleotides (AOs) against exonic and intronic splicing regulatory sequences have been generated. This use of these AOs has been shown to correct the open reading frame of the DMD gene and thus to restore truncated yet functional dystrophin expression *in vitro* (Takeshima *et al.*, 1995). In order to overcome the unstable disposition of single-stranded DNA or RNA, the AOs were designed using various chemical modifications of natural nucleic acid structure (Fig. 1).

Intravenous infusion of an antisense phosphorothioate oligonucleotide created an in-frame dystrophin mRNA via exon skipping in a 10-year-old DMD patient possessing an out-of-frame exon 20 deletion of the *dystrophin* gene (Takeshima *et al.*, 2006). Also, the adverse-event profile and local dystrophin-restoring effects of a single intramuscular injection of an antisense 2'-O-methyl phosphorothioate oligonucleotide (2OMeAO), PRO051, in patients with DMD were explored (Table 1). Four patients received a dose of 0.8 mg of PRO051 in the tibialis anterior (TA) muscle. Each

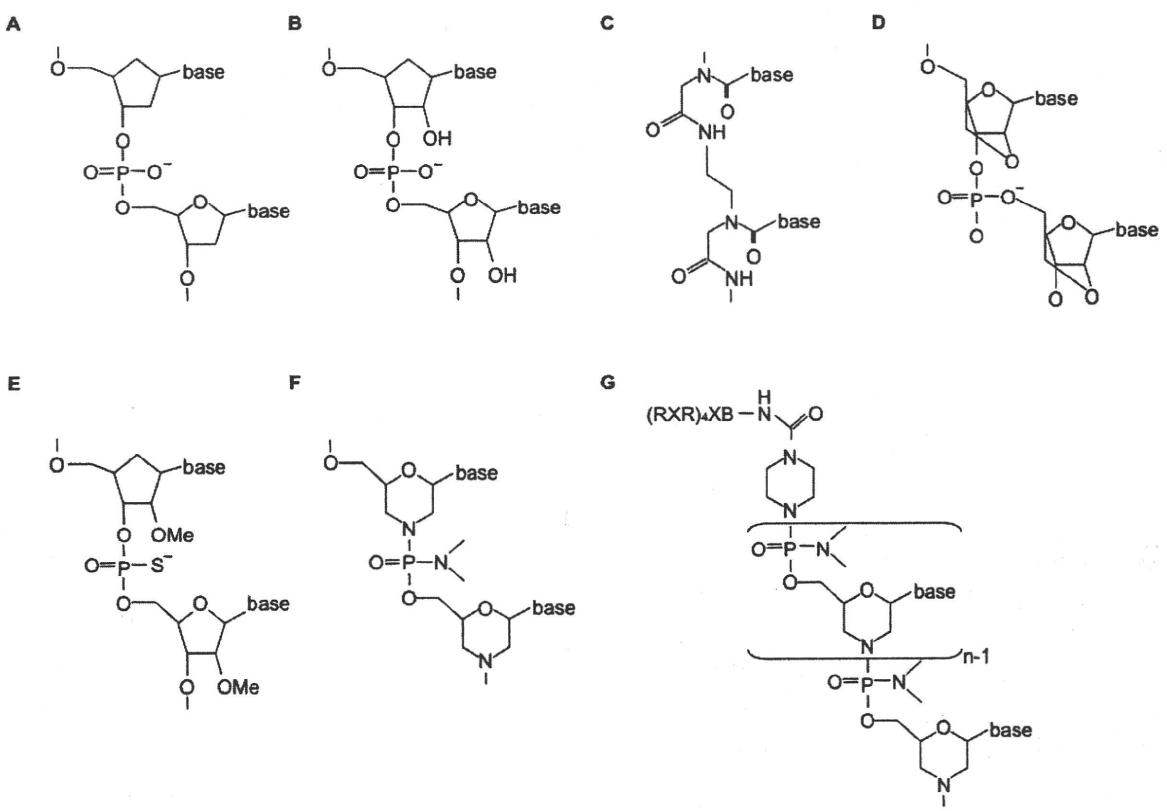

Fig. 1. Nucleic acid variations used for exon skipping. (redrawn from Nakamura and Takeda, 2009, with modifications). A, DNA; B, RNA; C, peptide nucleic acid; D, ethylene-bridged nucleic acid; E, 2′-O-methyl phosphorothioate oligonucleotide; F, phosphorodiamidate morpholino oligomer (PMO); G, peptide-linked PMO (PPMO).

patient showed specific skipping of exon 51 of dystrophin in 64 to 97% of myofibers, without clinically apparent adverse side effects (van Deutekom *et al.*, 2007).

Dystrophin-deficient canine X-linked muscular dystrophy was found in a golden retriever with a 3′ splice-site point mutation in intron 6 (Valentine *et al.*, 1988). The clinical and pathological characteristics of dystrophic dogs are more similar to those of DMD patients than are those of *mdx* mice. A beagle-based model of canine X-linked muscular dystrophy, which is smaller and easier to handle than the golden retriever-based muscular dystrophy dog (GRMD) model, has been established in Japan, and is referred to as $CXMD_J$ (Shimatsu *et al.*, 2005). The limb and temporal muscles of $CXMD_J$ show the effects of the disease starting at two months of age, which is the age corresponding to the second peak of serum creatine kinase. Using distinct multi-antisense oligonucleotides, we induced multi-exon skipping in $CXMD_J$ through muscular and intravenous injection of stable phosphorodiamidate morpholino oligonucleotides (PMOs). Treatment of the $CXMD_J$ dogs with the three PMOs targeting exons 6 and 8 resulted in the widespread rescue of dystrophin expression to therapeutic levels without signs of prominent toxicity (Yokota *et al.*, 2009).

Among the mutation hot spots, patients with various deletions within exons 45–55 account for 60% of DMD patients. We optimized the sequences of antisense PMOs targeting exon 51 of the mouse *DMD* gene to prepare for clinical trials involving patients with these hot-spot mutations. We designed 14 kinds of antisense PMOs targeting exon 51 of the mouse *DMD* gene and injected them separately or in combination into the muscles of *mdx*52 mice, in which exon 52 had been deleted by a gene targeting technique (Araki *et al.*, 1997). A combination of two PMOs showed an excellent restoration of sarcolemmal dystrophin in the injected muscle (Fig. 2;

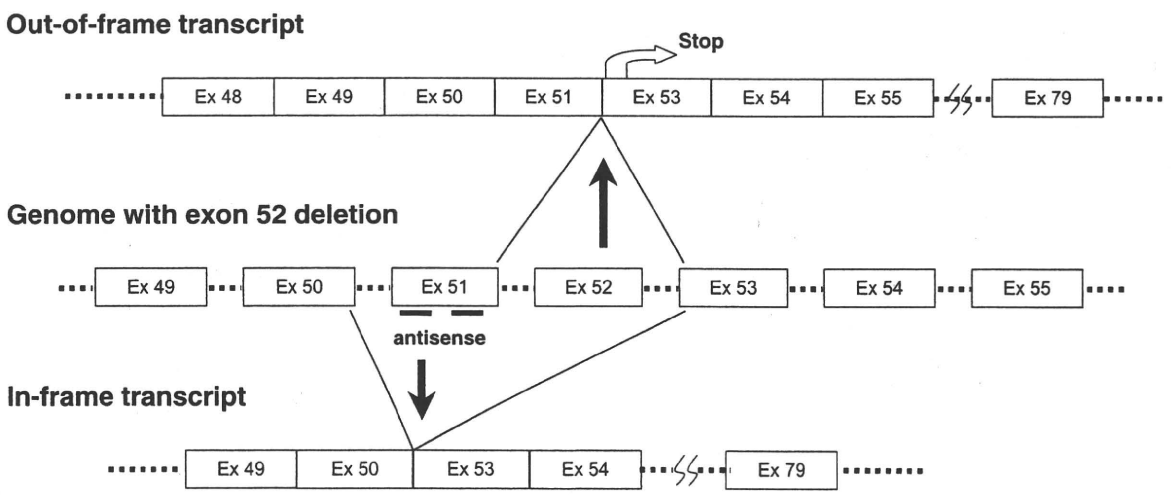

Fig. 2. Exon 51 skipping in the *mdx*52 mouse. Exon 52 (Ex 52) has been deleted in *mdx*52 mice and thus *mdx*52 mice harbor out-of-frame transcripts with a premature stop codon. The antisense oligomers targeting exon 51 (Ex 51) would induce exon 51 skipping, resulting in the production of in-frame transcripts.

Aoki *et al.*, 2010). We then continued to intravenously inject these PMOs into the *mdx*52 mice seven times weekly. Two weeks after the final injection, dystrophin was expressed at the sarcolemma throughout the body at an average of 10–50% of normal levels. This was accompanied by amelioration of dystrophic pathology as well as improvement in the contractile force of extensor digitorum longus muscle, grip power test, and treadmill performance. This study provides proof of concept for exon 51 skipping in a DMD animal model that can be applicable to up to 15% of *DMD*-deletion patients. Various unrelated patients with deletions of exons 45–55 harboring mild or asymptomatic skeletal muscle involvement have been reported (Beroud *et al.*, 2007; Nakamura and Takeda, 2009). If multi-exon skipping of exons 45–55 is possible, severe Becker muscular dystrophy (BMD) cases with a deletion in the hot spot may also be treatable. We have been currently investigating the use of mixtures of PMOs to confirm the feasibility of therapy based on exon 45–55 skipping.

One of the issues remaining for exon-skipping therapy is inconsistency of efficacy among different organs or tissues. For instance, systemic administration of AOs restored dystrophin expression, but expression levels were much lower in cardiac muscle compared with skeletal muscle (Yokota *et al.*, 2009). Since a number of DMD patients die of cardiac complications, including lethal arrhythmias, improvement of AO transfer in the heart is critical. It is still not clear why AO uptake into cardiac tissue is inefficient. Since cardiac muscle cells each harbor a single nucleus, in contrast to the multinucleated skeletal muscle cells, damaged cardiomyocytes may be immediately replaced with fibrous tissues before uptake of AOs (Nakamura and Takeda, 2009). To improve introduction efficiency into the heart, PMO injection using a microbubble contrast reagent under ultrasound has been proposed (Wang *et al.*, 2005). Also, PMOs with a designed cell-penetrating peptide have been developed to efficiently target a mutated *dystrophin* exon in cardiac muscles (Wu *et al.*, 2008). Long-term benefits can be achieved through the use of viral vectors expressing antisense sequences against regions within the *dystrophin* gene. The sustained production of dystrophin at physiological levels in entire groups of muscles as well as the correction of muscular dystrophy could be achieved by treatment with exon-skipping adeno-associated virus (AAV)-U7 (Goyenvalle *et al.*, 2004).

Gene-replacement Strategies using Adeno-Associated Virus Vectors

AAV-mediated gene therapy for DMD was updated in our previous review (Miyagoe-Suzuki and Takeda, 2010). Here, we highlight our own AAV-based effort aimed at expressing transgenic dystrophin in DMD patients. While various viral vectors have been considered for the delivery of genes to muscle fibers, the AAV-based vector is emerging as the gene transfer vehicle with the most potential for use in DMD gene therapy. The advantages of the AAV vector include the lack of pathology associated with the wild-type virus, the ability to transduce non-dividing cells,

and the long-term expression of the delivered transgene (see: Okada *et al.*, 2002). Serotypes 1, 6, 8, and 9 of recombinant AAV (rAAV) exhibit a potent tropism for striated muscles (Inagaki *et al.*, 2006). Since a 5-kb genome is considered to be the upper limit for a single AAV virion, a series of rod-truncated micro-dystrophin genes is used in this treatment (Yuasa *et al.*, 1998). To gain acceptance as a medical treatment, AAV vectors require a scalable and economical production method. A scalable method, using active gassing and large culture vessels, was developed to enable large-scale plasmid transfection in a closed system (Okada *et al.*, 2005). Recent developments in ion-exchange chromatography also suggest that vector production using transduction culture supernatant would be compatible with current good manufacturing practice and production on an industrial scale (Okada *et al.*, 2009).

Although recent studies suggest that vectors based on AAV are capable of body-wide transduction in rodents, translating this finding into large animals remains a challenge. Since increased permeability of the sarcolemma allows leakage of transgene products from the dystrophin-deficient muscle fibers, neo-antigens introduced by AAV vectors evoke significant immune reactions in DMD muscle (Yuasa *et al.*, 2002). Furthermore, an *in vitro* interferon-gamma release assay showed that canine splenocytes respond to immunogens or mitogens more strongly than do murine splenocytes. In fact, co-administration of the immunosuppressants cyclosporine (CSP) and mycophenolate mofetil (MMF) improved rAAV2 transduction. Immunohistochemical analysis revealed that the rAAV2-injected muscles showed higher rates of infiltration of CD4+ and CD8+ T lymphocytes in the endomysium than the rAAV8-injected muscles (Ohshima *et al.*, 2009). Our study also showed that mRNA levels of MyD88 and co-stimulating factors, such as CD80, CD86 and type I interferon, are elevated in both rAAV2- and rAAV8-transduced dog dendritic cells (DCs) *in vitro* (Ohshima *et al.*, 2009). Intravascular delivery can be performed as a form of limb perfusion, which might bypass the immune activation of DCs in the injected muscle. Administration of rAAV8-micro–dystrophin by limb perfusion produced extensive transgene expression in the distal limb muscles of $CXMD_J$ dogs without obvious immune responses for as long as eight weeks after injection (Ohshima *et al.*, 2009).

It is increasingly important to develop strategies to treat DMD that consider their effects on cardiac muscle. The pathology of the conduction system in $CXMD_J$ dogs was analyzed in order to establish a suitable therapeutic target for DMD (Urasawa *et al.*, 2008). Although dystrophic changes of the ventricular myocardium were not evident at the age of 1 to 13 months, Purkinje fibers showed remarkable vacuolar degeneration when dogs were as young as four months old. Because the dystrophin-deficient heart is highly sensitive to increased stress, increased activity by the repaired skeletal muscle provides the stimulus for heightened cardiac injury and heart remodeling. A single intravenous injection of AAV9 vector expressing micro-dystrophin efficiently transduces the entire heart in neonatal *mdx* mice, thereby ameliorating cardiomyopathy (Bostick *et al.*, 2008). The systemic delivery

of rAAV to transduce truncated dystrophin is predicted to ameliorate the symptoms of DMD patients. To realize gene transduction technologies in clinical practice, development of an effective delivery system with improved vector constructs as well as efficient immunological modulation must be established.

Prospects for Stem Cell Therapy and iPS Cells-mediated Gene Therapy

Stem cells-based cell therapy for DMD is another approach on which we are also focusing. Although initiated by intramuscular injection of allogeneic myoblasts in DMD patients (Law *et al.*, 1992; Huard *et al.*, 1992; Gussoni *et al.*, 1992; Karpati *et al.*, 1993), our ultimate goal is to deliver therapeutic stem cells into skeletal muscles via the vascular system as pioneered by Gussoni *et al.* with haematopoietic and muscle-derived stem cells on the *mdx* mouse model (1999). The main challenge is the identification and availability of the stem cell type able to efficiently perform this task. Mesoangioblasts are attractive candidates (Sampaolesi *et al.*, 1996) together with muscle satellite stem cells (Zammit *et al.*, 2002) and mesenchymal stem cells (MSCs) on which we have already a direct interest (Uezumi *et al.*, 2010; Kasahara *et al.*, 2010). However, we believe that patient-specific induced pluripotent stem (iPS) cells offer a unique therapeutic opportunity for DMD.

Since the breakthrough reprogramming of mouse (Takahashi and Yamanaka, 2006) and human (Takahashi *et al.*, 2007; Yu *et al.*, 2007) somatic cells into iPS cells, patient-specific iPS cells have been produced as disease-specific cellular models including DMD and BMD (Park *et al.*, 2008) and are envisioned as an obvious drive both for regenerative medicine (see Yamanaka, 2009) and stem cell gene therapy (e.g. Hanna *et al.*, 2007; Raya *et al.*, 2009). Of note, a first step in the proof-of-concept for iPS cells-mediated gene therapy for DMD has been recently published in which a human artificial chromosome has been used to correct iPS cells derived from a DMD patient and from *mdx* mice (Kazuki *et al.*, 2010). On the other hand, using directed differentiation and anti-satellite cell antibodies, stem/progenitor cells have been derived from wild-type mouse iPS cells that successfully engrafted in *mdx* mice (Mizuno *et al.*, 2010). The iPS cell breakthrough is thus moving straightforwardly toward the clinic. However, major safety concerns have still to be cleared before the initiation of the bench-to-bedside translation (Yamanaka, 2009).

Conclusions

The combination of gene and cell therapy is an optimal way to allow patient-specific custom-made therapies. Both gene and cell therapies for DMD have demonstrated promising results in animals, although their promise has not yet been translated into success in human patients. Investigations in large animal models are clarifying why it is hard to move from rodents to humans and are at the same time implying various possible solutions to these difficulties. The key to future accomplishment

would be cautious preclinical studies combined with well-planned clinical trials that concentrate on safety and efficacy issues.

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Chapter 4
Mechanobiology in Skeletal Muscle: Conversion of Mechanical Information into Molecular Signal

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4.1 Introduction

Overload leads to muscle hypertrophy. In the process, several events occur inside and outside the myofibers, including increased protein synthesis, change in gene expression, fiber-type transition, satellite cell activation, and angiogenesis (Bassel-Duby and Olson 2006; Blaauw et al. 2009). Interestingly, at the early phase of muscle hypertrophy, protein synthesis significantly increases (Baar et al. 2006), and later the transcription of growth-related genes follows (Carson 1997). Satellite cell activation is generally thought to be a critical component for increase in muscle mass; however, it is still a debated issue whether satellite cell incorporation into hypertrophying muscle fibers is required for muscle hypertrophy (O'Connor and Pavlath 2007; McCarthy and Esser 2007). A recent paper demonstrated that the rapid incorporation of BrdU seen in overloaded muscle reflects angiogenesis but not proliferation of satellite cells (Blaauw et al. 2009). Thus, muscle hypertrophy is a complicated and dynamic process, influenced by many factors (nutrients, blood flow, hormones, energy status, or oxidative status), making it difficult to elucidate the mechanism by which mechanical information is sensed by myofibers. IGF-1, anabolic steroids, or blockage of myostatin signaling increases muscle mass without mechanical stimulation via distinct mechanisms from mechanotransduction. For myostatin or steroids, please refer to other comprehensive reviews (Joulia-Ekaza and Cabello 2007; Kadi 2008).

The muscle atrophy process is characterized by suppressed protein synthesis, and increased rate of degradation of muscle proteins (Jackman and Kandarian 2004; Ventadour and Attaix 2006). Decreased muscle activity (i.e., denervation, prolonged bed rest, space flight, immobilization, etc.) and diseases (cancer, AIDS, muscular dystrophy, sepsis, chronic heart failure, diabetes, etc.), malnutrition, or drugs (glucocorticoids) lead to muscle atrophy. In chronic diseases, elevated levels

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of proinflammatory cytokines, glucocorticosteroids, tumor-derived factors, and endotoxins are responsible for the induction of muscle atrophy, whereas the upstream mediators which trigger catabolic signal cascades in unloaded muscle are largely unknown.

4.2 Mechanosensor in Skeletal Muscle

4.2.1 Experimental Models for Mechanotransduction

Experiments to study mechanotransduction in skeletal muscle employ in vivo and in vitro models (Table 4.1). As discussed below, experimental designs greatly influence the responses of the muscles to the mechanical stress.

Although it is not yet a consensus, it is reported that eccentric contraction more effectively activates p70S6K than centric contraction (Eliasson et al. 2006; Nader and Esser 2001). This difference might be because eccentric contraction accompanies stretching stimuli, and thereby strongly stimulates stretch-activated channels (SACs) or other stretch-sensitive mechanoreceptors. But this hypothesis needs more investigation.

In addition to the type of mechanical stimulation, the intensity and duration of stimulation must be taken into account on interpretation. Atherton et al. reported that aerobic exercise stimuli promoted specific adaptive responses toward mitochondrial biogenesis besides slow phenotypes, without activating mTOR and p70S6K. Resistance strength training stimuli, however, strongly activated mTOR and p70S6K (Atherton et al. 2005). But anther group did not observe such a difference (Nader and Esser 2001). As Zanchi and Lancha discuss in their review article (Zanchi and Lancha 2008), the difference might be due to the difference in the design of the experiment, i.e., in vivo versus in vitro, or in the duration of mechanical stimulation.

In overloaded muscle, the protein synthesis is initiated by mechanical stimuli, but later modulated by additional anabolic stimuli. IGF-1 is generally accepted to strongly

Table 4.1 Models to study mechanotransduction in skeletal muscle

Modes of mechanical load	
Overload	In vivo model
	Synergist ablation (functional overload)
	Electrical stimulation
	In vitro model
	Electrical stimulation in vitro
	Cell culture (stretch)
Unload	In vivo models
	Limb immobilization
	Hindlimb suspension
	Denervation

promote protein synthesis through the PI3K/Akt/mTOR pathway (Rommel et al. 2001; Bodine et al. 2001). Nonetheless, IGF-1-mediated PI3K/Akt/mTOR activation seems to be part of a late component of the hypertrophy process (Hameed et al. 2003; Haddad and Adams 2002). Furthermore, acute stimulation of skeletal muscle seems not to be always dependent on PI3K/Akt as a means to activate mTOR (Hornberger et al. 2004, 2006). Thus, acute phase might be more suitable than chronic phase to investigate the mechanotransduction system in skeletal muscle.

Lastly, skeletal muscle is composed of myofibers with different contractile and metabolic properties. For example, the soleus, which is tonic and postural, is a mixture of predominant oxidative slow-twitch (type I) fibers and glycolytic fast-twitch fibers (type II). The extensor digitorum longus (EDL) muscle, which is phasic, is composed of fast-twitch glycolytic fibers. Importantly, these two muscles respond to mechanical stimuli differently: the soleus shows a lower susceptibility to mechanical load compared with EDL (Widrick et al. 2002). The findings suggest either that different mechanosensing apparatus exists in these two muscles, or that different signal transduction pathways operate downstream of the mechanosensor in fast and slow muscles.

4.2.2 Mechanosensors in Skeletal Muscle

So far, many molecules have been proposed as a mechanosensing molecule in skeletal muscle, including SACs (Spangenburg and McBride 2006), the dystrophin-glycoprotein complex (Acharyya et al. 2005; Suzuki et al. 2007), integrins (discussed in Zanchi and Lancha 2008; Spangenburg 2009) or sarcomere structure (Gautel 2008) (Fig. 4.1), but none of them is definitive. It is quite plausible that skeletal muscle has multiple mechanosensors and integrates the mechanical information from all these sensors into anabolic or catabolic responses.

4.2.3 Stretch-Activated Channels

SACs were initially described in skeletal muscle (Franco and Lansman 1990a, b). SACs in skeletal muscle are permeable to both Na+ and Ca2+ and increase their open probability in response to stretch of the membrane. A previous report, using Ga3+ and streptomycin as specific blockers of SACs, suggested that SACs are activated during lengthening or stretch-induced contraction, activate the Akt/mTOR pathway, and induce muscle hypertrophy (Spangenburg and McBride 2006; Butterfield and Best 2009). However, since neither Ga3+ nor streptomycin completely abolished Akt/mTOR activities, the authors speculated that SACs is one of the molecules which sense mechanical load and promote protein synthesis. Currently, SACs are identified based on patch-clamping experiments, and channel activity is blocked with specific inhibitors. Cloning of genes encoding SACs and

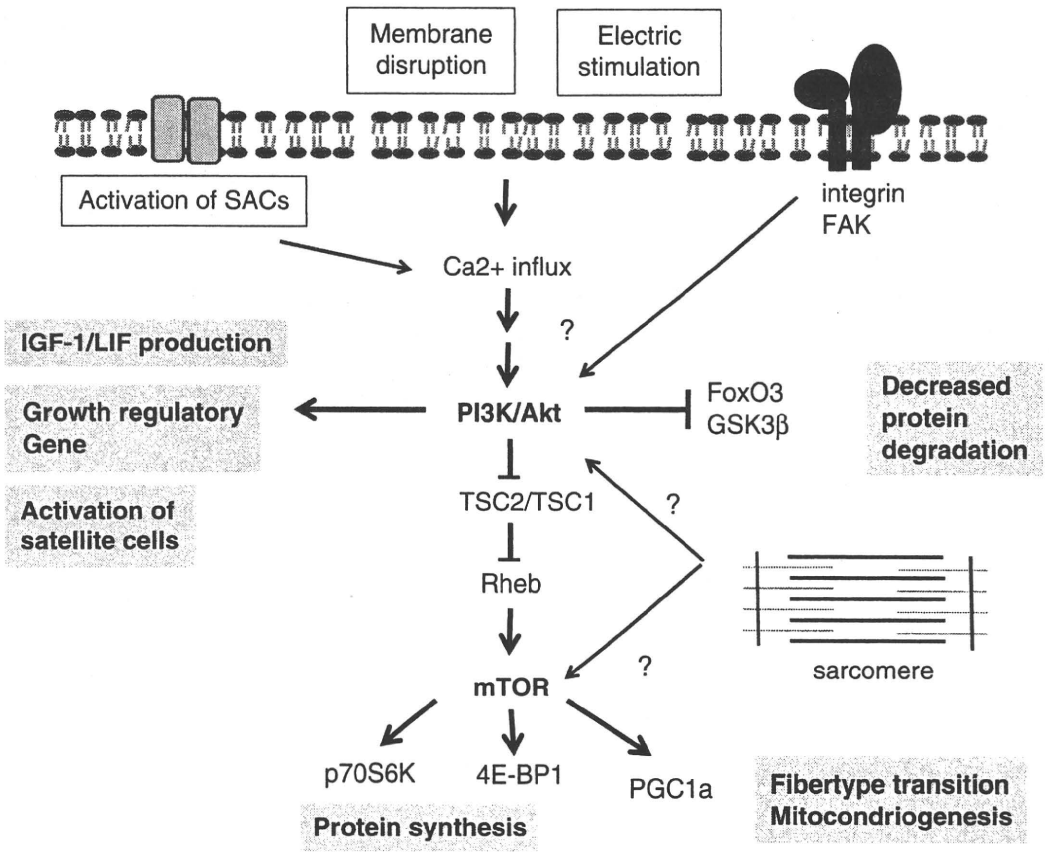

Fig. 4.1 Mechanical load and muscle hypertrophy. Stretching of myofibers activates sarcolemmal stretch-activated channels (SACs), disrupts the cytoplasmic membrane, starts a cascade of anabolic signal transduction, and through activation of mTOR increases its mass and contractile force. Active muscle contraction also activates the Akt/mTOR pathway. The sarcomere structure might work one of the sensors of muscle contraction, and convert the mechanical information into biochemical signals. Several studies suggest that a variety of mechanical information converge on mTOR, which in turn activates downstream targets (S6K, 4E-BP1, or PGC-1alph) to adapt the mechanical load. Note that some parts are not fully supported by experimental evidence

ablation of the gene in skeletal muscle would greatly accelerate the elucidation of the role of SACs in mechanotransduction in skeletal muscle, and would help develop pharmacological intervention of disuse-related muscle atrophy.

4.2.4 Neuronal Nitric Oxide Synthase and Mechanotransduction

Neuronal nitric oxide synthase (nNOS) is a peripheral member of the dystrophin–glycoprotein complex at the sarcolemma (Brenman et al. 1996), and especially concentrated at myotendinous junctions, which are specialized for force transduction across the muscle cell membrane (Chang et al. 1996).The expression and activity

of nNOS are upregulated by exercise (Roberts et al. 1999), mechanical loading, electrical stimulation of muscle, and passive stretching (Reiser et al. 1997; Tidball et al. 1998). Criswell and co-workers reported that inhibition of NOS by L-NAME (a wide inhibitor of NOS) during functional overload attenuated the increase of muscle mass and fiber-type transition (Smith et al. 2002; Soltow et al. 2006). The same group further showed that NO upregulated contractile protein mRNAs including α-actin and type 1 myosin heavy chain (Sellman et al. 2006). Steensberg et al. reported that L-NAME treatment inhibited exercise-induced mRNA expression of IL-6, HO-1 and PDK4 (Steensberg et al. 2007). Tidball et al. showed that talin and vinculin mRNA expressions induced by mechanical stimulation are mediated by NO (Tidball et al. 1999). Koh and Tidball showed that NO positively modulates sarcomere addition in immobilized–remobilized muscles (Koh and Tidball 1999). Together, these data strongly suggest that nNOS plays an important role in mechanotransduction in skeletal muscle.

Nitric oxide (NO) gas is reported to be released from myofibers when myofibers are stretched in vitro, and activate satellite cells via release of HGF (Tatsumi et al. 2002). Furthermore, Tatsumi et al. demonstrated that stretching of myofibers in vivo indeed activates satellite cells via production of NO (Tatsumi et al. 2006). The nitric gas is supposed to be generated by nNOS. Together, these findings suggest that the mechanosensor in skeletal muscle is directly or indirectly linked with nNOS and activate nNOS when the fibers are stretched. Alternatively, nNOS itself might sense the change in mechanical stimuli and be activated.

4.2.5 nNOS and Unloading

Suzuki et al. recently reported that nNOS acts as an upstream regulator of forkhead box O (FoxO) transcription factors in unloading-induced muscle atrophy (Suzuki et al. 2007) (Fig. 4.2). The study showed that during hind limb suspension, nNOS was lost from the sarcolemma, and cytoplasmic nNOS produced high levels of NO and activated FoxO3a, which in turn upregulated MuRF-1 and atrogin-1/MAFbx, two major muscle-specific E3 ligases. Although the data suggest that nNOS is a mediator of mechanotransduction in skeletal muscle, the mechanism by which nNOS is activated remains unknown. It is also still to be determined how NO gas activates FoxO transcription factors. One possibility is that NO controls FoxO transcription factors via S-nitrosylation. NO reacts with thiol of protein cysteine residues to form S-nitrosothiol. This posttranslational modification affects the functions of a wide range of proteins, and regulates protein–protein interactions and DNA-binding activity of transcription factors (Hess et al. 2005). Recent studies show that FoxO transcription factors are necessary and sufficient for induction of autophagy in skeletal muscle (Mammucari et al. 2007; Zhao et al. 2007). Whether nNOS activates the autophagy–lysosome system through activation of FoxO3a in unloaded muscle is to be determined.

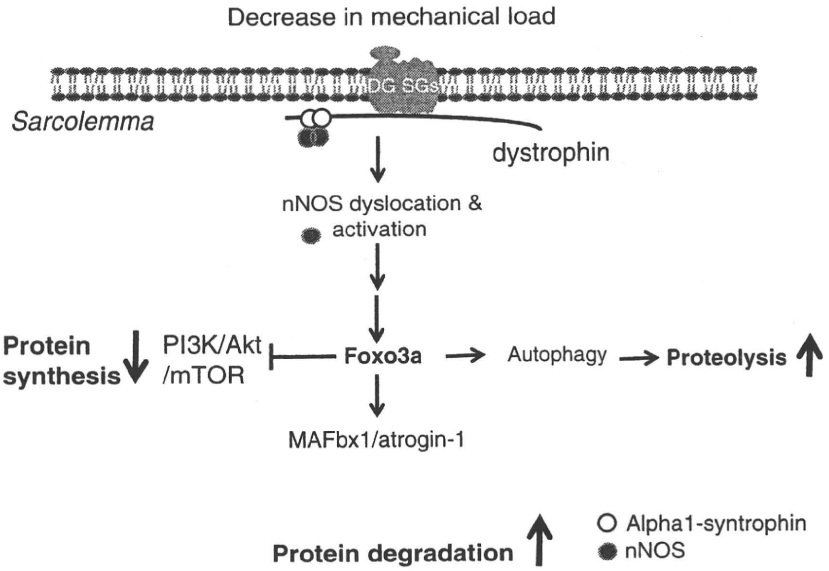

Fig. 4.2 A mechanism by which an unloaded signal activates nNOS and promotes muscle atrophy (hypothetical). In skeletal muscle, a major fraction of nNOS exists as a member of the dystrophin–glycoprotein complex at the sarcolemma. nNOS interacts directly with α1-syntrophin through its N-terminal PDZ domain. In unloading condition, nNOS is lost from the sarcolemma, and cytoplasmic nNOS is activated, produces nitric oxide and activates FoxO3a transcription factor (Suzuki et al. 2007). FoxO3a activates the ubiquitin-proteasome-mediated proteolysis and autophagy. How nNOS is activated and how it activates FoxO3a transcription factor remain to be determined in a future study

4.2.6 Phospholipase D and Phosphatidic Acid

Hornberger et al. showed that mechanical stimulation causes phospholipase D (PLD)-dependent increase in phosphatidic acid (PA), which binds to mTOR in the FRB domain and activates mTOR signaling (Hornberger et al. 2006). Although the observation is exciting, it remains to be determined whether elevation of PA concentration is necessary and sufficient for mechanical load-induced muscle hypertrophy.

4.2.7 Integrins/FAK Signaling and Mechanotransduction

The mechanical signal may be partly transmitted by focal adhesion complexes (FACs) in skeletal muscle. FACs connect cytoskeletal proteins to the extracellular matrix and therefore transmit force across the cell membrane. Beta-1 integrin in FACs is bound by focal adhesion kinase (FAK) and paxillin (Carson and Wei 2000; Flück et al. 1999). Mechanical loading on skeletal muscle results in phosphorylation of FAX or paxillin (Gordon et al. 2001). Furthermore, FAK is shown to affect

activation of Akt/mTOR independent of PI3K activity in fibroblasts (Xia et al. 2004). At present, however, there is no direct evidence that integrin/FAK signaling activates protein synthesis in mechanically stimulated skeletal muscle.

4.2.8 Sarcomere and Mechanotransduction

The sarcomere is a candidate mediator of mechanical transduction. The M-band is proposed to be a hub mainly for protein kinase-regulated ubiquitin signaling and protein turnover, and the I-band and Z-disk contain stretch-sensitive pathways involving transcriptional modifiers (Gautel 2008). It is highly likely that signals from the sarcomere modify mass and contractile properties of overloaded muscle.

4.2.9 Growth Factors and Overload

It has been suggested that a number of cytokines, including LIF (Spangenburg and Booth 2006), IGF-1 (Rommel et al. 2001; Ohanna et al. 2005), or a variant of IGF-1 termed mechano-growth factor (MGF) (Goldspink et al. 2008), are produced locally in overloaded muscle, and contribute to muscle hypertrophy. Since these cytokines activate the PI3K/Akt/mTOR/p70S6K pathway, mechanical loading is thought to induce the muscle hypertrophy, through production of growth-promoting autocrine/paracrine factors. For example, IGF-1 is shown to be a potent activator of the Akt/mTOR signaling pathway (Adams 2002; Glass 2005), and forced overexpression of IGF-1 in skeletal muscle increased muscle mass (Musarò et al. 2001; Barton 2006). If so, is IGF-1 production a trigger of overload-induced muscle hypertrophy? Upregulation of IGF-1 is, however, observed between 24 and 72 h after the onset of the mechanical load on muscle, whereas Akt/mTOR is activated a few hours after the onset of mechanical load. Furthermore, a recent study using transgenic mice expressing a dominant negative IGF-I receptor, suggested that IGF-1 signaling is dispensable in mechanical load-induced skeletal muscle hypertrophy (Spangenburg et al. 2008). Therefore, although IGF-1 would augment the hypertrophy by accelerating protein synthesis, it is not likely the trigger of increased protein synthesis. In contrast, the loss of leukemia inhibitory factor (LIF) is reported to result in a failed hypertrophic response after mechanical loading (Spangenburg and Booth 2006). The mechanisms by which LIF promotes muscle hypertrophy process remain to be determined.

4.3 Signaling Pathways in Mechanotransduction

Although how mechanical stimuli are converted into biochemical events in skeletal muscle remains to be determined, several downstream signaling pathways are shown to be involved in the mechanotransduction.

4.3.1 *mTOR Is a Key Signaling Molecule for Mechanical Overload-Induced Muscle Hypertrophy*

How mechanical loading on skeletal muscle activates the Akt/mTOR signaling pathway remains largely unknown. Nonetheless, the importance of mTOR in mechanical load-induced hypertrophy was generally accepted due to the findings that rapamycin administration (a potent and selective inhibitor of mTOR) completely abolished the hypertrophy under overload conditions (Bodine et al. 2001; Hornberger et al. 2003; Bodine 2006).

mTOR activates p70S6K (ribosomal protein kinase S6) and 4E-BP1. p70S6K phosphorylated by mTOR, increases the translation of mRNAs with a 5′-tract of pyrimidine, which is contained in all known ribosomal proteins. 4E-BP1 regulates the initiation of protein synthesis by sequestering eIF4E. When phosphorylated, 4E-BP1 releases eIF4E, which in turn complexes with eIF4G and eIF3 and initiates translation (Rennie et al. 2004). Once again, we emphasize that we have a very limited understanding of how physical loading of the muscle fibers activates the mTOR signaling pathway.

4.3.2 *Unload (Inactivity or Disuse) and the Catabolic Signaling Pathway*

When the activity of skeletal muscle is reduced by spaceflight (microgravity), prolonged bed rest, immobilization, denervation, or hindlimb suspension, skeletal muscle loses its weight and contractile force. Two major signaling pathways are reported to play major roles in unload-induced skeletal muscle atrophy: nuclear factor-kappa B (NF-κB) and FoxO pathways.

Genetically modified animal models confirmed that NF-κB is one of most important signaling pathways linked to the loss of skeletal muscle in both physiological and pathophysiological conditions. Hunter et al. reported that skeletal muscle inactivity leads to increased NF-κB transcriptional activity (Hunter et al. 2002). Later, it was reported that a specific NF-κB pathway (p50 and Bcl3), but not a classical p50–p65 NF-κB dimer, is required for disuse muscle atrophy (Hunter and Kandarian 2004; Judge et al. 2007). NF-κB activates the transcription of many genes, including skeletal muscle-specific E3 ligases, MuRF1. But how these target molecules induce muscle atrophy remains poorly defined.

Under atrophic conditions, it is proposed that the inhibition of FoxO activity by phosphorylation (possibly by Akt) is relieved, and the dephophorylated FoxO transcription factors accumulate in the nucleus, activate an atrophy-related ubiquitin E3 ligase, atrogin-1/MAFbx-1 and induce skeletal muscle wasting (Sandri et al. 2004; Stitt et al. 2004).

Although the upstream events of NF-κB or FoxO activation are not clarified, candidate molecules which link disuse and activation of NF-κB or FoxO are reactive

oxygen species (ROS) (Powers et al. 2007; Dodd et al. 2010). But, how ROS activate NF-κB or FoxO transcription factors is not clearly defined.

4.3.2.1 Downstream Targets of NF-κB and FoxO Transcription Factors

Downstream of these signaling pathways, three major proteolytic systems, (1) lysosomal proteases (cathepsins), (2) Ca^{2+}-activated proteases (i.e., calpains), and (3) the ubiquitin-proteasome system, are thought to contribute to the degradation of muscle proteins. Unloading skeletal muscle also causes caspase-3 activation and myonuclear apoptosis, maintaining a constant ratio of cytoplasm to nuclei (myonuclear domain) (reviewed in Powers et al. 2007).

NF-κB is reported to activate muscle RING finger protein 1 (MuRF1) E3 ligase (Cai et al. 2004; Mourkioti et al. 2006). FoxO3 is shown to activate atrogin-1/MAFbx to activate the ubiquitin-proteasome pathway (Sandri et al. 2004). Increased FoxO3 function also leads to decreased myocyte cell size through induction of autophagy pathway genes, including *LC3*, *Gabarapl1*, and *Atg12* (Zhao et al. 2007; Mammucari et al. 2007).

4.4 Conclusion

Skeletal muscle is sensitive to mechanical load, and adapts the demand by increasing or decreasing its mass and contractile force. Muscle hypertrophy and wasting are accompanied by changes in fiber compositions and metabolic properties. Thanks to advances in gene manipulation techniques in mice, major players in signal transduction regulating muscle hypertrophy and atrophy have been identified. But how myofibers sense change in mechanical load, and how the information is translated into biochemical signaling pathways, remain to be determined.

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