The ever-expanding myokinome: discovery challenges and therapeutic implications

Martin Whitham and Mark A. Febbraio

Abstract | Exercise reduces the risk of a multitude of disorders, from metabolic disease to cancer, but the molecular mechanisms mediating the protective effects of exercise are not completely understood. The realization that skeletal muscle is an endocrine organ capable of secreting proteins termed 'myokines', which participate in tissue crosstalk, provided a critical link in the exercise–health paradigm. However, the myokine field is still emerging, and several challenges remain in the discovery and validation of myokines. This Review considers these challenges and highlights some recently identified novel myokines with the potential to be therapeutically exploited in the treatment of metabolic disease and cancer.

VO_{2max}
The term given to the maximal oxygen uptake; an indicator of aerobic capacity and cardiorespiratory fitness.

Myokine
A cytokine or peptide that is produced by skeletal muscle cells and subsequently released into the circulation to exert paracrine or endocrine effects.

Cellular & Molecular Metabolism Laboratory, Diabetes & Metabolism Division, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, New South Wales 2010, Australia.
All authors contributed equally to this work.

Correspondence to M.A.F.
m.febbraio@garvan.org.au

doi:10.1038/nrd.2016.153
Published online 12 Sep 2016

It has been known for centuries that physical activity can help to prevent chronic disease. Even short periods of physical inactivity are associated with disrupted metabolic homeostasis (FIG. 1). It has become apparent that sedentary behaviour results in decreased insulin sensitivity, reduced postprandial lipid clearance, loss of muscle mass and accumulation of visceral adiposity[1–3]. These acute changes provide a link between physical inactivity and the increased risk of acquiring many diseases, including type 2 diabetes, cardiovascular disease, cancers such as colon and breast cancer, osteoporosis, osteoarthritis, erectile dysfunction and polycystic ovarian syndrome[1,2]. The benefits of physical activity have been attributed to various mechanisms, including reduced adiposity, increased cardiorespiratory fitness (VO_{2max}), reduced circulating lipids, maintenance of muscle mass[2,3] and decreased inflammation[4] (FIG. 1). Specifically, regular exercise is known to increase cerebral blood flow and brain oxygenation, increase adipose tissue lipolysis and reduce adipose tissue mass, allow for better heat dissipation via increased sweat rate, decrease liver fat accumulation and increase glucose uptake by skeletal muscle[3]. A rudimentary view of the benefits of physical activity on metabolic health is that greater energy expenditure simply counteracts positive energy balance and overfeeding, thereby assisting the maintenance of a 'healthy weight'. However, in contexts where participants are overfed, physical activity restores detriments in metabolic function, even when the energy expenditure of exercise is accounted for by increased intake to provide a net energy surplus[5,6].

Well over 50 years ago, Goldstein raised the possibility that skeletal muscle cells, in response to contraction, may liberate a factor that could affect metabolic processes[7]. However, this postulate was largely overlooked until just after the turn of the new millennium when researchers made a pivotal discovery that shed new light on the benefits of physical activity. Skeletal muscle was identified as an endocrine organ, with the observation that the cytokine interleukin-6 (IL-6) is released from skeletal muscle during exercise and leads to an increase in hepatic glucose production, which serves as an energy source for contracting muscles[8].

The term 'myokine' was coined to describe "a cytokine or peptide that is produced by skeletal muscle cells and subsequently released into the circulation to exert endocrine or paracrine effects in other cells, tissues or organs" (REF. 8). Since the identification of IL-6 as the prototypical myokine[9–11] (BOX 1), interest in the concept and the known muscle secretome, defined here as the 'myokinome', has steadily grown, with an exponential rise in the number of papers describing myokines in the past 3 years. Several recent reviews have discussed the proposed myokinome in detail[1,12–14]. However, myokine discovery is challenging, and proteins are often prematurely labelled as myokines before appropriate validation or an in-depth analysis of the proposed effects of the protein once it is released from muscle.

In this Review, we first highlight some of the common challenges faced in myokine discovery and validation, and consider possible methodological approaches to overcome them. Second, we discuss selected recent proteins that meet the criteria of a myokine (TABLE 1)

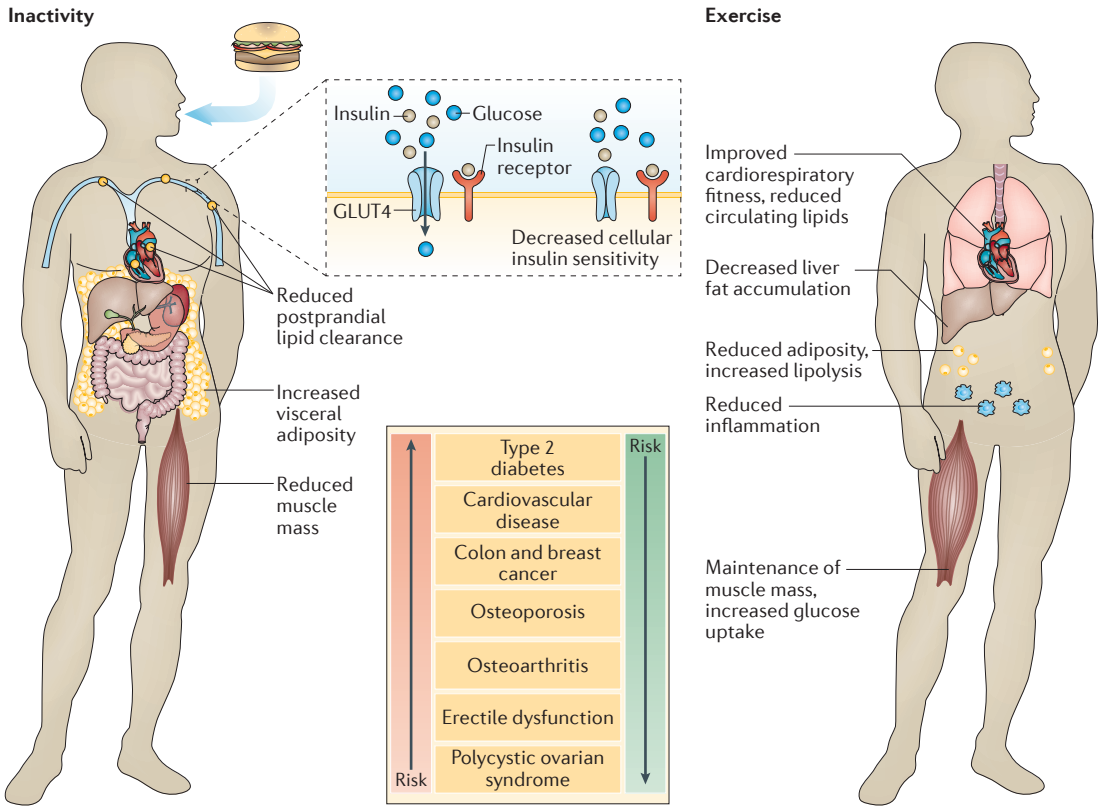

Figure 1 | **The opposing effects of physical inactivity and exercise on disease risk.** Inactivity is associated with decreased insulin sensitivity, reduced clearance of lipids following a meal, increased visceral adiposity and muscle atrophy. These effects can increase the risk of non-communicable diseases such as type 2 diabetes, cardiovascular disease, colon and breast cancer, osteoporosis, osteoarthritis, erectile dysfunction, and polycystic ovarian syndrome. Conversely, regular physical activity improves maximal aerobic capacity, which is a measure of cardiorespiratory fitness, and reduces circulating lipids, which are problematic in atherosclerosis. Individuals who exercise regularly present with lower liver fat, visceral adiposity and larger muscle mass than inactive individuals. Exercise improves glucose uptake and reduces the systemic, low-grade inflammation that is often associated with obesity. These combined effects are thought to reduce the risk of the aforementioned disease states that are associated with an inactive lifestyle. GLUT4, glucose transporter 4.

and warrant further investigation regarding their therapeutic potential in the treatment of obesity, metabolic disease and cancer.

Discovering the unidentified myokinome

Strictly speaking, in order to satisfy the definition of a myokine, a candidate protein needs to fulfil the following criteria: it must be derived from skeletal muscle; it must be secreted; and it must carry out a biological function in an endocrine or paracrine fashion[9]. Such is the transient nature of secreted protein expression — none of these criteria is easy to demonstrate. Despite this, research groups have adopted various models in an attempt to identify new candidate myokines. These studies are ambitious, as the potential number of secreted proteins is vast. Indeed, recent estimates predict the existence of at least 3,000 proteins with a known secretory signal peptide[15], and many additional proteins are known to be secreted by 'non-classical' means. Furthermore, an analysis of 32 different human tissues revealed that a larger fraction of tissue-enriched proteins are likely to be secreted or membrane-spanning than are intracellular[15].

'Omics' technologies in myokine discovery

An intuitive approach to discover novel myokines may therefore be to make use of the rapidly evolving 'omics' technologies. For example, microarray analyses have been applied to skeletal muscle biopsy samples from participants carrying out one-legged knee extension exercise[16]. This model has the advantage of a within-subject control sample in the form of the contralateral, non-exercising limb. Using this approach, significant changes in the expression of 938 genes in the exercising limb were identified, and this was reduced to just 23 candidate myokines following filtering for secreted products using gene ontology extracellular classification and SignalP prediction[16]. Of these, increased plasma protein concentrations were only observed for fractalkine (also known as CX3CL1) and monocyte chemoattractant protein 1 (MCP1; also known as CCL2). Such a modest return from a seemingly carefully designed experiment emphasizes the challenges faced in discovering new myokine candidates. Although there have been some recent successes using gene expression arrays (TABLE 1), there are limitations to combining transcriptomic assessments of changes in mRNA expression in

Omics
A term given to the comprehensive biological assessment of entities with the suffix 'ome', such as the geneome, transcriptome and proteome (or proteinome).

Box 1 | **IL-6 — the prototypical myokine**

The term 'myokine' arose from a series of observations regarding interleukin-6 (IL-6) by the Pedersen and Febbraio laboratories. Initially, increased circulating IL-6 levels following marathon running appeared to be a consequence of immune cell behaviour during exercise[120]; however, a series of studies identified skeletal muscle as the major source of this myokine[44,121–124].

It was well known that upon the onset of exercise, hepatic glucose production (HGP) rapidly and markedly increases[125]. Although much of this response was thought to be mediated by a change in the insulin/glucagon ratio, it was deemed that this change could not fully account for the magnitude of the increase in HGP during exercise[126]. By comparing HGP when IL-6 was increased by exercise or infusion, Febbraio *et al.*[8] were able to show that IL-6 is a factor that is released from skeletal muscle during exercise and that it contributes to HGP to meet the energy demands of the contracting muscle. Hence, the concept of a myokine was uncovered.

IL-6 is undoubtedly now the most well-characterized myokine. Despite being recognized as a mediator of pathogen-induced inflammation, studies have revealed that the regulation of IL-6 in contracting skeletal muscle is somewhat different[127], as it performs 'energy sensing' roles during exercise[124]. It is now well known that IL-6 is a potent activator of AMP-activated protein kinase (AMPK) in skeletal muscle[128–130], leading to enhanced glucose uptake and insulin sensitivity in healthy humans[130]. Moreover, IL-6 increases lipolysis and fatty acid oxidation both in skeletal muscle *in vitro* and in humans *in vivo*[131,132]. It appears that the effect of IL-6 on both muscle glucose uptake and fatty acid oxidation is AMPK-dependent, as infection of muscle cells with an AMPK-dominant negative adenovirus abrogates the effect of IL-6 on these metabolic processes[130]. In addition to its AMPK-mediated glucoregulatory effects, IL-6 can enhance glucose tolerance by activating glucagon like peptide 1 (GLP1) in intestinal L cells and pancreatic islets to adapt to changes in insulin demand[133]. GLP1 and its analogues also reduce food intake and body weight[134]. Interestingly, around the same time that IL-6 was identified as a myokine and subsequently found to improve metabolic homeostasis in obesity[130], Regeneron Pharmaceuticals was conducting clinical trials using Axokine, a recombinant variant of human ciliary neurotrophic factor (CNTF). CNTF, like IL-6, is a ligand of the gp130 transmembrane signal transduction protein that forms a subunit of the IL-6 family receptor complexes[119]. Axokine was modified to improve its plasma stability and was developed to reduce weight in overweight and obese individuals, particularly those with type 2 diabetes[135–137]. However, after showing early promise in Phase II and Phase III studies, the clinical development of Axokine was discontinued when a significant number of treated patients in the high-dose group developed neutralizing antibodies to Axokine, blocking its therapeutic effect[137]. Despite this setback, gp130 receptor ligands continue to be clinically developed for the treatment of metabolic disease[119,138].

muscle with targeted interrogation of plasma protein concentrations. First, the correlation between tissue protein and mRNA expression is surprisingly poor ($r = 0.2–0.6$)[17–20]. Second, 'resident' plasma protein concentration correlates poorly with mRNA expression abundance in the liver[21], implying that, particularly for secreted proteins, the examination of tissue mRNA may be misleading.

Myokine discovery by mass spectrometry

Considering some of the limitations described above, direct proteomic assessment of the skeletal muscle secretome is perhaps a more reliable approach to myokine discovery. Mass spectrometry (MS)-based proteomics[22,23], using algorithms to quickly match partial amino acid sequences with complete sequence data in vast databases, enables comprehensive identification of a sample's proteome within a timeframe of hours. A variety of quantitative and non-quantitative MS approaches have been applied to the examination of secreted proteins in cell culture models of muscle[24–30], complemented by similar proteomic studies using cytokine arrays[31,32]. However, although these studies have identified some interesting candidate myokines that warrant further investigation, they have not made considerable progress in comprehensively delineating the muscle secretome. This is no great criticism, as muscle and plasma offer perhaps the most challenging matrix with which to comprehensively characterize the proteome (FIG. 2). This is largely a consequence of the high-abundance proteins that hamper the identification of smaller, potentially more biologically relevant proteins. Indeed, such a broad dynamic range is primarily the consequence of contractile proteins in muscle (contributing ~50% of the total proteome)[33] and the 40–60 mg per ml abundance of albumin in plasma. Detecting an analyte at 10 pg per ml in plasma, in the midst of albumin molecules at 55 mg per ml, is much like trying to find a specific human being by searching through the population of the entire globe[34]. However, we know from the example of IL-6 (BOX 1) and other proteins that small changes of pg per ml quantities can exert significant biological changes. Complicating matters further is the fact that proteins exist in multiple proteoforms, including isoforms, splice variants and post-translational modifications. Over two-thirds of genes encoding secreted proteins have at least one splice variant with a different cellular location[15]. Finally, protein expression is largely dynamic with respect to time, so time-course sampling may be key to characterizing the biological importance of myokine candidates.

Although one option to simplify these analyses is to examine protein secretion, the validity of *in vitro* cell culture models must be considered. Indeed, comprehensive proteomic profiling of the commonly used mouse muscle cell line C2C12 and mouse skeletal muscle reveals extensive differences in their proteome[33] that perhaps limit their direct use in myokine discovery. Although differentiated C2C12 cells are capable of developing sarcomeric proteins and calcium transient-induced 'twitching', they lack the 3D structure characteristic of contractile muscle tissue[33]. However, as a consequence, the abundance of large proteins such as titin and myosin is considerably lower, which should favour the detection of lower-abundance proteins by liquid chromatography tandem mass spectrometry (LC–MS/MS). Deshmukh *et al.*[33] have exploited this by analysing mouse muscle tissue alongside C2C12 lysates, to identify peptides that would normally be hidden by tissue analysis alone. This resulted in the most comprehensive quantitative analysis of muscle to date, deriving abundance values for 10,218 proteins. This study highlights the potential of this approach to overcome the aforementioned problematic dynamic range issues associated with MS analysis of skeletal muscle and might well pave the way for systematic identification of novel myokines.

Plasma proteomics in myokine discovery

Myokine discovery research must distinguish between plasma proteins that reside in the circulating blood and proteins that are secreted from cells. This can be assisted with the use of quantitative, time-course information following varied exercise/stimulation protocols, which can characterize tightly controlled protein

Liquid chromatography tandem mass spectrometry (LC–MS/MS). A technique used to identify proteins in complex mixtures via ionization and manipulation of the resultant ions to derive amino acid sequence information.

Table 1 | **A summary of some recent candidate myokines, their purported effects and proposed mechanisms**

Myokine	Method of discovery	Validation as a secreted factor	Purported myokine function	Purported mechanism of action	Refs
Irisin	*In vitro* gene expression array and secreted protein bioinformatics of PGC1α-overexpressing muscle cells	Quantitative analysis of post-exercise plasma in mice (western blot) and humans (targeted mass spectrometry)	Browning of adipose tissue	PPARα-dependent upregulation of thermogenic genes (for example, UCP1)	64,70,71
Meteorin-like 1	Combined gene expression and mass spectrometry analysis of PGC1α4-overexpressing primary muscle cells	Delayed protein appearance in wild-type mice carrying out downhill running exercise	Browning of adipose tissue	IL-4 or IL-13-dependent stimulation of thermogenic genes via alternative M2 macrophage activation	72
Myonectin	Serendipitously, while characterizing metabolic function of CTRP family of proteins	Increased concentration of myonectin in serum of mice carrying out 2 weeks of voluntary running wheel activity	Improved hepatic fatty acid uptake	Upregulation of lipid binding and transport proteins such as CD36, FATP1, FABP4	86,87
Musclin	Screening of cDNA libraries specific for secreted proteins	Increased gene and plasma expression in mice subjected to daily treadmill exercise versus control mice. Systemic treatment of musclin rescued phenotype in *Ostn*-knockout mice	Increased mitochondrial biogenesis	PGC1α activation via co-stimulation (with ANP) of cGMP signalling	106,107
SPARC	Gene expression arrays of muscle derived from mice exercised for 4 weeks versus controls	Exercise time-course analysis in mice and humans. Secretion from primary muscle cells *in vitro*	Reduces precursor lesions of colon adenocarcinoma on the surface of the colon	Caspase 3- and caspase 8-mediated apoptosis	94,26

ANP, atrial natriuretic peptide; CTRP, complement C1q/TNF-related protein; FABP4, fatty acid-binding protein 4; FATP1, fatty acid transport protein 1; IL-4, interleukin-4; PGC1α, PPARγ coactivator 1α; PPARα; peroxisome proliferator-activated receptor-α; SPARC, secreted protein acidic and rich in cysteine; UCP1, mitochondrial brown fat uncoupling protein 1.

secretions and their temporal appearance in circulation. Considerable advances have been made in the analysis of the plasma proteome[34–38]. One approach to identify proteins secreted from cells and tissues is to focus on the mechanism of secretion. Many proteins appear to be secreted in extracellular vesicles (EVs), such as exosomes and microparticles, and these vesicles can be isolated from plasma using various methods such as ultracentrifugation, immunoaffinity isolation or gel filtration[39], and subsequently analysed[40,41]. Although it is difficult to determine what proportion of circulating proteins are contained within EVs, alignment of human protein-encoding genes with the manually curated database of proteins identified in vesicles[42] estimates that of the ~20,000 protein-encoding genes, as much as 70% are translated into proteins that have been identified in vesicles. Furthermore, Harel *et al.*[40] identified over 5,000 proteins in EV fractions of plasma from patients with prostate cancer and control subjects. This suggests that an unappreciated number of proteins are released into the circulation that may not be recognized as secreted proteins per se. This is particularly relevant in the context of omics approaches to myokine discovery when considering that bioinformatics analyses of skeletal muscle data sets are entirely reliant on reference databases such as UniProt, Swiss-Prot and their associated annotations. By searching for secreted proteins from skeletal muscle using Gene Ontology cellular location or secretory keyword annotations, one runs the risk of discarding important myokine candidates that are not annotated as 'secretory' or 'extracellular'. For example, the protein heat shock 70 kDa protein 1A (HSPA1A; also known as HSP70) does not contain a secretory signal sequence or have an extracellular GOCC annotation in the Uniprot database. However, we know that HSP70 is secreted into the circulation following exercise, most probably in exosomes[43].

A myokine candidate of interest must be shown to be produced and released from skeletal muscle in response to a stimulus. This is important to ascribe any benefits a myokine might exert to muscle stimulation and to examine the mechanisms by which the protein is expressed and released. A key finding in the work on IL-6, and indeed the myokine concept in general, involved a

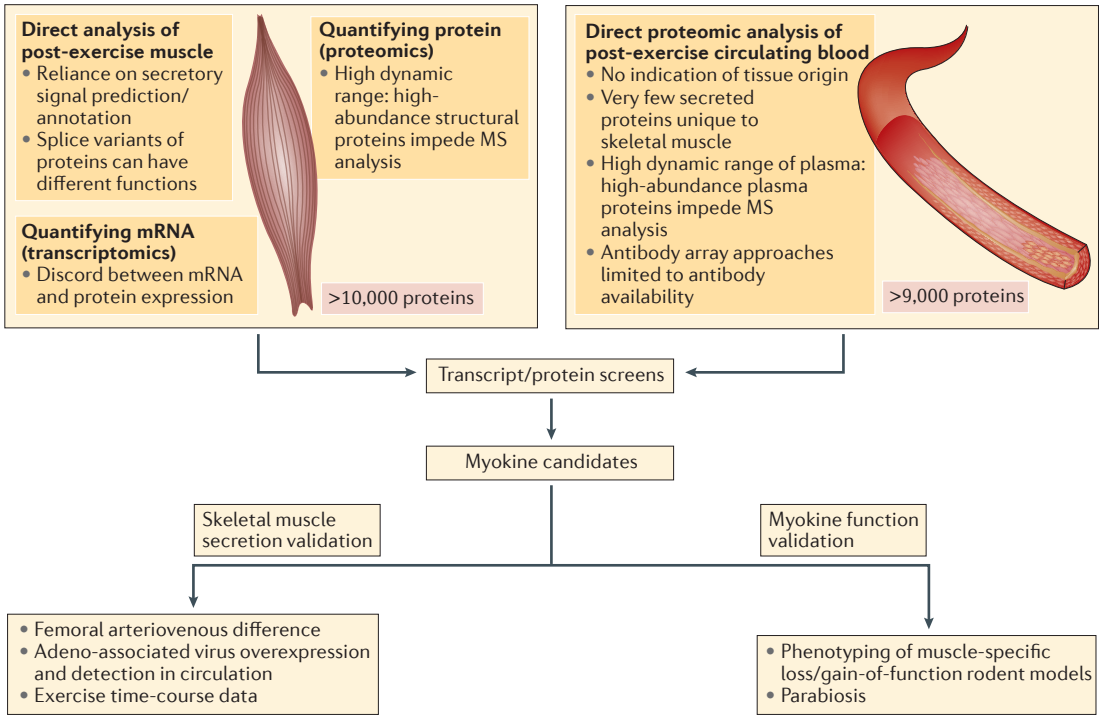

Figure 2 | **Challenges and approaches to myokine discovery.** The diagram presents a summary of some of the challenges in identifying new myokine candidates when sampling skeletal muscle itself or venous blood post-exercise. Although there is huge scope for the identification of important proteins, the range of proteins is vast; there are likely to be >9,000 to 10,000 proteins in skeletal muscle or the circulation. Some of the barriers to identifying novel myokines when using transcriptomic or proteomic analyses are shown. An approach to myokine identification is to combine comprehensive analyses of both muscle and serum/plasma to derive a selection of potential novel candidates and validate them for their tissue origin, their secretion during exercise and their biological function. Some currently available technologies and practices that can assist in this validation are listed. MS, mass spectrometry.

one-legged knee extension exercise model in human subjects, in whom the femoral artery and vein (a-v) of the exercising limb were carefully cannulated in order to measure and demonstrate IL-6 release[44]. Proteomic analysis of femoral a-v sampling is therefore a potentially powerful approach to identify novel myokine candidates. In addition, the recent advances in genetically modified mouse models by CRISPR technology[45], overexpression models using adeno-associated viruses[46] and the resurgence of the shared circulation parabiosis technique[47] allow specific examination of the effects of a secretory protein on rodent phenotype and provide, in our view, key approaches to myokine validation (FIG. 2).

Finally, a large proportion of myokine research suffers owing to low sample sizes and subsequent statistical power. This is particularly relevant in the context of exercise, where highly variable responses might be observed owing to inaccuracy in determining an individual participant's relative exercise work rate, or the lack of availability of precise measurement assays. Furthermore, as factors such as the frequency, duration, exercise type (resistance versus dynamic) and even environmental conditions are known to influence the biology of exercise, it is entirely conceivable that myokine responses are specific to one or a combination of these variables, adding an additional level of complexity to myokine discovery[9]. However, the aforementioned rise in omics technologies — in particular, targeted, data-independent proteomic techniques such as selective and parallel reaction monitoring[48,49], plus a wider consideration of statistical power and exercise variables in future experimental design — may serve to better validate putative myokine candidates.

Novel myokines and their therapeutic potential

Despite the challenges facing the identification and validation of myokines, several studies, often incorporating some of the aforementioned omic approaches as initial screening methods, have identified many novel candidates. Recent reviews have discussed the proposed myokinome in detail, so this will not be discussed in depth here[1,12]. For example, the cytokines IL-4, IL-7 and IL-15 have been reported to be myokines that affect lipolysis in visceral and subcutaneous fat depots, follistatin-like 1 protein has been reported to enhance endothelial function and revascularization, and insulin-like growth factor 1 and fibroblast growth factor 2 (FGF2) have been implicated in bone metabolism[32,50–53]. Furthermore, myostatin, a member of the transforming growth factor-β (TGFβ) family, regulates muscle growth as indicated by the striking skeletal muscle hypertrophy observed in myostatin-null animals[54]. Interestingly, myostatin expression in skeletal muscle correlates negatively with whole-body insulin sensitivity in mice and humans[55,56]. Although

CRISPR
A gene editing tool that facilitates the establishment of genetically modified rodent models.

Adeno-associated viruses
Methods of gene transfer, *in vivo*, used to create transgenic overexpression mouse models in specific tissues.

Parabiosis
A mouse model of shared circulation, whereby surgical suturing initiates skin-to-skin contact of paired mice. After a period of 2 weeks, inflammation induces the development of microcirculation between mice so that the effect of a circulating factor from one animal can be assessed in the partner animal.

this effect might be explained by increased muscle mass, manipulating circulating myostatin by recombinant protein injections[57] or by treatment with an anti-myostatin antibody[58] suggests that myonectin also functions as a myokine that affects insulin sensitivity. More recently, Moon *et al.*[59] identified cathepsin B in the conditioned media of AMP-activated protein kinase (AMPK)-agonist treated muscle cells. Intriguingly, levels of this protein are increased in circulation following exercise in mice, monkeys and humans; moreover, AMPK passes the blood–brain barrier and is associated with hippocampus-dependent memory function[59].

Preliminary studies within the field have begun to reveal potential opportunities in the therapeutic exploitation of myokines, particularly in the areas of metabolic disease and cancer. Below, we focus on some recently described secreted products of skeletal muscle, which we feel exhibit particularly promising therapeutic potential in these disease areas.

Myokines and metabolic disease

The World Health Organization (WHO) has reported that, worldwide, obesity has more than doubled since 1980. Given the often irreversible metabolic and cardiovascular consequences of obesity, there continues to be substantial investment in identifying novel ways to prevent and treat this disease.

There has been growing interest in the potential of therapeutically targeting brown fat[60]. In addition to classical brown fat cells largely located in subscapular and perirenal depots, brown-like or 'beige' adipocytes expressing mitochondrial brown fat uncoupling protein 1 (UCP1; also known as thermogenin) have been found to be present and can be upregulated in white adipose tissue[61,62]. As 'uncoupled' respiration in brown/beige adipocytes dissipates energy as heat, manipulation of brown adipose tissue (BAT) or promotion of white adipose tissue browning in the treatment of obesity has become an attractive therapeutic option[60]. Whether the induction of white adipose tissue browning can increase energy expenditure without a compensatory increase in food intake is yet to be experimentally confirmed in long-term clinical studies. However, several years ago nicotine was administered to obese mice to activate UCP1 in both brown adipocytes and what we now know as beige adipocytes. Such a treatment decreased rather than increased food intake[63]. Whether this was a direct side effect of nicotine is unclear, but this study nonetheless demonstrated that it is possible to activate browning pathways without increasing energy intake.

Several myokines, as discussed below, have recently been suggested to induce thermogenesis and promote browning in white adipose tissue, and may therefore provide novel therapeutic opportunities in the treatment of obesity and associated metabolic diseases.

Irisin. In 2012, Speigelman and colleagues[64] used gene expression array analysis of skeletal muscle from PPARγ coactivator 1α (PGC1α; encoded by *PPARGC1A*)-transgenic mice, which display many of the hallmarks of exercise-trained animals[65], to identify fibronectin type III domain-containing protein 5 (FNDC5) as a potential mediator of exercise-induced increases in the expression of thermogenic genes such as *UCP1* in adipose tissue[64]. FNDC5 was found to be cleaved at the carboxy terminal, resulting in the secretion of a protein the authors named irisin, levels of which were found to be increased in plasma in response to exercise in both mice and humans. Furthermore, overexpression of irisin in mouse models of obesity improved fasting insulin and glucose tolerance[64].

However, it was noted that human FNDC5 has an atypical translation start codon (that is, ATA rather than ATG), prompting claims that this represented a null mutation and, therefore, the protein could not be translated or produced[66]. Furthermore, a large body of work following the initial discovery of irisin relied chiefly on commercially available polyclonal antibodies. Interpretation of these data is challenging, as these antibodies demonstrate high degrees of cross-reactivity and nonspecific binding in human samples[67,68]. In addition, functional examinations of irisin have been hindered because, to date, a receptor for this peptide remains unidentified. The ligand–receptor complex may be difficult to decipher as irisin has been recently shown to form dimers in serum[69]. Importantly, however, a recent paper from the Spiegelman laboratory detected and quantified circulating irisin levels in humans using peptide labelling tandem MS[70], demonstrating that the peptide indeed exists. Furthermore, the absolute concentration of irisin was shown to be significantly increased in aerobically trained versus sedentary human participants. The availability of an accurate MS method now enables thorough examination of the duration, frequency and type of exercise that elicits the most favourable irisin response and the specific physiological conditions in which irisin might exert its effects. Further studies are therefore warranted to confirm the effect of exercise on irisin secretion and delineate the precise functions of this protein.

Although the discovery of irisin[64] has not yet been therapeutically exploited in the treatment of obesity or its related disorders, understanding precisely how brown fat is stimulated by muscle-secretory factors such as irisin may lead to the identification of novel therapeutic targets. Early indications suggest that irisin exerts browning of adipose tissue via peroxisome proliferator-activated receptor-α (PPARα)-dependent upregulation of the expression of thermogenic genes such as *UCP1* (REFS 64,71).

Meteorin-like protein. The Spiegelman group similarly reported the identification of meteorin-like protein (METRNL; also known as subfatin) as another circulating factor that is induced in muscle after exercise[72]. In particular, the authors were able to attribute the origin of METRNL to skeletal muscle as it was identified in muscle-specific PGC1α4 mice, the protein was identified in the culture media of myotubes with forced expression of PGC1α4, and both gene and protein levels of METRNL in muscle and plasma were upregulated following exercise. As PGC1α4 mice exhibited

AMP-activated protein kinase
(AMPK). A kinase that is considered to be a master regulator of metabolism by sensing changes in AMP:ATP ratio.

PPARγ coactivator 1α
(PGC1α). A transcription factor that regulates genes involved in energy metabolism, of which there are at least four variants (PGC1α1 to PGC1α4).

Box 2 | **Exercise and adipose tissue browning**

Exercise induces the secretion of irisin, meteorin-like and fibroblast growth factor 21 (FGF21) and production of the metabolite β-aminoisobutyric acid (BAIBA)[85], which may in turn induce browning and increase energy expenditure; these observations have resulted in a heightened focus on the complex effects of exercise on metabolism and created excitement in the field of obesity research.

However, it has been questioned why exercise, which mobilizes energy stores to meet ATP demand, would induce a programme of events that fundamentally lead to uncoupled respiration and reduced efficiency of substrate utilization at a time when the opposite might be more intuitive[139]. Indeed, Vosselman *et al.*[140] reported no improvements in brown/beige adipocyte recruitment in subcutaneous white adipose tissue in endurance-trained versus sedentary human participants. Interestingly, secretion of irisin and FGF21 is stimulated by cold exposure, and upregulation of brown fat thermogenesis is a concerted effect of both factors[71]. As irisin secretion via shivering was of a similar magnitude to exercise, the authors postulated that exercise-induced irisin secretion may have evolved from shivering that once served to assist cold-induced thermogenesis in concert with FGF21 (REF. 71). Although these data outline a complex interplay between muscle and adipose-derived factors in the browning of adipose tissue, the fact that most rodent studies in this area report data from mice housed below thermoneutrality (~30 °C) may be of significance in the interpretation of these findings.

thermogenic gene expression in adipose tissue, the authors hypothesised that METRNL might mediate this effect. Although recombinant METRNL-Fc was found to have no direct effect on UCP1 expression in the stromal vascular fraction of adipocytes *in vitro,* its release into the circulation indirectly increased the expression of thermogenic genes, including UCP1, through the stimulation of eosinophil-dependent increases in IL-4 and IL-13. These cytokines subsequently cause adipose tissue macrophages to adopt an M2-like phenotype, which is required for the increased expression of the thermogenic and anti-inflammatory gene programmes in adipose tissue[72]. Importantly, blocking the actions of METRNL *in vivo* significantly attenuated chronic cold-exposure-induced alternative macrophage activation and thermogenic gene responses; by contrast, increasing circulating levels of METRNL stimulated energy expenditure and improved glucose tolerance in mice[72]. Although greater insight is needed to elucidate the exercise conditions that derive the most robust METRNL responses, these findings support the concept of targeting beige fat thermogenesis as a potential treatment strategy for obesity.

FGF21. In a transgenic mouse model of muscle hypertrophy (mice overexpressing AKT), increases in strength[73] have been shown to be accompanied by an increase in the expression of FGF21. As FGF21 protein concentration was elevated in serum samples, and overexpression of AKT1 in C2C12 cells led to increased FGF21 secretion *in vitro*, the authors concluded that FGF21 is a myokine[74]. In support of this, protein levels of FGF21 have been reported to increase in serum following exercise in humans and mice[75].

Although the majority of FGF family members have largely been associated with development, FGF21 has been shown to have potent effects on metabolism, offering protection from diet-induced obesity when overexpressed in the liver of transgenic mice[76]. An intuitive assumption, therefore, is that if FGF21 is secreted from exercised skeletal muscle, its effects on key metabolic processes such as glucose uptake[76] and fat oxidation[77] might partially explain some of the positive effects of physical activity on metabolic health. However, the protein is also highly expressed in the liver. Recently, using invasive cannulation of the femoral artery and vein in human volunteers, Hansen *et al.*[78] demonstrated no uptake or release of FGF21 in the leg during and after an exercise bout that induced a 50% increase in circulating FGF21. Similar sampling across the hepatosplanchnic bed demonstrated a clear net release of FGF21 from the liver in fasting conditions and during exercise. These data are supported by the observation that in liver-specific *Fgf21*-knockout mice the protein does not circulate, at least in fasting conditions[79]. Therefore, although some of the positive effects of exercise on metabolic health might well be partially mediated by FGF21, these data suggest that contracting muscle may not be the source.

As FGF21 increases energy expenditure[76] and is expressed in BAT following cold exposure[80], Fisher *et al.*[81] reasoned that FGF21 might mediate adipose tissue browning and increased energy expenditure. Indeed, a 3-day infusion of FGF21 into mice increased the expression of thermogenic genes such as *UCP1* and deiodinase 2 (*DIO2*), as well as the expression of genes consistent with brown and beige fat cells in inguinal and perirenal adipose tissue[81]. Interestingly, FGF21 had an attenuated effect on thermogenic gene expression in *Ppargc1a*-knockout brown adipocytes, suggesting significant crosstalk between FGF21 and PGC1α in BAT metabolism[81]. Furthermore, FGF21 administration in UCP1-null mice failed to upregulate thermogenesis, as expected, but also resulted in other perceived benefits on metabolism such as reduced food intake and improved glycaemic control[82,83]. Data such as these have led to the development of FGF21 mimetics for the treatment of metabolic disease (reviewed in REF. 84).

However, despite the increasing evidence suggesting that exercise may stimulate adipose tissue browning (at least in animal models), perhaps through the secretion of factors such as FGF21, irisin and METRNL, as well as through the production of the metabolite β-aminoisobutyric acid (BAIBA)[85], this concept has been questioned (BOX 2).

Myonectin. Like many myokine candidates, myonectin was discovered serendipitously. When characterizing the metabolic function of the complement C1q/TNF-related protein (CTRP) family of proteins, Seldin *et al.*[86] cloned the cDNA of a secreted protein, CTRP15 (also known as myonectin). Mice given access to running wheels for 2 weeks demonstrated increased expression of myonectin in skeletal muscle and increased circulating levels of the protein. Given that myonectin is predominantly expressed in skeletal muscle and poorly expressed in other tissues, this implies that the source of myonectin associated with voluntary exercise is skeletal muscle[86]. Furthermore, the expression of myonectin was shown to be induced in muscle by a number

of compounds that raised either cAMP or calcium levels[86]. Recombinant administration of myonectin to mice reduced circulating free fatty acid (FFA) levels in the absence of changes in adipose tissue lipolysis, suggesting that FFA uptake mediated this decrease, which was confirmed *in vitro*[86]. Even though myonectin reduces circulating FFA levels, whether it is a viable therapeutic target to treat metabolic disease is questionable. Indeed, in a subsequent study, Seldin *et al.*[87] demonstrated that one mechanism of action of myonectin is to suppress autophagy in the liver via the activation of mechanistic target of rapamycin (mTOR). This may be of importance as previous studies in mice have shown that, in genetic and dietary models of obesity, hepatic autophagy is markedly downregulated, and that restoration of autophagic processes improves insulin resistance in the liver[88].

Myokines and cancer

It is now known that insufficient levels of physical activity may contribute to approximately 10% of breast and colon cancer cases in western countries[89]. Given the strong links between physical activity and reduced cancer risk[90], it comes as no surprise that voluntary running delays tumour progression and severity in mouse models of various cancers[91,92]. Furthermore, treatment of mammary cancer cells with serum from animals post-exercise inhibits proliferation and stimulates apoptosis, supporting the notion that secretory products of exercise can inhibit tumour cell growth[93]. As discussed below, such protective secretory products may include IL-6 (REF. 92) and SPARC (secreted protein acidic and rich in cysteine)[94]. Of note, although the protein decorin has yet to be proven as a bona fide myokine, its levels also increase in the circulation in response to acute resistance exercise in humans[95]. Decorin is a small proteoglycan that is associated with collagen fibrils in all connective tissues and has recently been found to have anti-metastatic effects in breast cancer[96,97].

IL-6. In an attempt to understand the importance of cytotoxic immune cells in the beneficial effects of voluntary running seen in mouse cancer models[91,92], Pedersen *et al.* recently analysed immune cell infiltration in the tumours of the B16F10 mouse model of melanoma with or without access to voluntary running wheels for 6 weeks[92]. Exercise was associated with an infiltration of natural killer (NK) cells, depletion of which induced a loss of the protective effects of voluntary running. Interestingly, exercise in this study was also accompanied by an increase in circulating IL-6, the prototypical myokine (BOX 1), and blocking IL-6 action by neutralizing antibodies inhibited the infiltration of NK cells into tumours of exercised mice. Notably, daily injections of IL-6 to mimic the circulating concentrations observed during exercise failed to influence NK cell infiltration or tumour growth. This suggests that a concerted effect involving IL-6 and other factors affecting immune cell recruitment, such as adrenaline, is essential for the positive effects of exercise in this animal model of tumour growth[92]. Given that caged mice without access to running wheels are a model of inactivity (as mice run freely when allowed to do so)[98], these findings may be interpreted as evidence that physical activity either promotes antitumour effects or it inhibits the tumorigenic effects of physical inactivity. Regardless, these results complement recent findings implying an important role of NK cells in tumour immunosurveilance[99] and suggest that exercise-induced secretory factors such as IL-6 may assist in creating an appropriate inflammatory tumour microenvironment.

SPARC. Several epidemiological studies have demonstrated that physical activity can reduce both the onset of and mortality associated with colorectal cancer[100,101], although the precise molecular mechanisms for such observations are unclear.

Using DNA microarray and bioinformatics tools, Aoi *et al.*[94] identified SPARC as a contraction-induced myokine in both mice and humans.Independent examinations of primary muscle cells derived from individuals who underwent strength-training also corroborated SPARC as a contraction-induced, secreted protein[26]. Importantly, exercise training reduced the formation of aberrant cryptic foci, precursor lesions of colon adenocarcinoma, on the surface of the colon in wild-type mice but not SPARC-deficient mice[94]. This study provided a molecular link between colon cancer prevention and regular physical activity.

SPARC itself has a myriad of functions as a prototypical matricellular protein, governing the fundamental processes affecting cell shape, adhesion and differentiation via its interaction with the extracellular matrix[102]. Although data showing enhanced pancreatic tumours in SPARC-null mice[103] support the therapeutic potential of SPARC, the multifunctional nature of this protein, like IL-6 (BOX 1) and decorin[104], limits direct therapeutic applications. However, further understanding of the pathways targeted by such molecules after exercise may lead to the identification of potential novel anticancer therapeutic targets. Indeed, apoptotic pathways have been implicated in the anticancer mechanism of action of SPARC and other post-exercise secretory factors, as exercise promotes apoptosis in colon mucosal and mammary cells and increases the cleavage of caspase 3 and caspase 8 in wild-type mice but not SPARC-deficient mice[93,94]. Furthermore, it has been demonstrated that the apoptotic effects of SPARC are attributed to the amino-terminal domain, and synthetically made peptides of this domain enhance apoptosis via inhibition of caspase–BCL-2 (B cell lymphoma 2) interactions[105].

Collectively, these studies highlight a surprising dearth of literature examining the relationship between exercise and cancer prevention, and highlight a possible role of post-exercise secretory factors in this relationship.

Myokines and exercise adaptation: musclin

Although musclin was identified over a decade ago as a peptide with high sequence homology to the natriuretic peptide family[106], it was only very recently found to meet all the criteria of a bona fide myokine. In a comprehensive series of experiments in mice, Subbotina and

PEGylation
Process of covalently adding repeating units of (polymerized) ethelene glycol to proteins with a view to improving stability, pharmacokinetics and therapeutic utility.

Fc fusion
Addition of the crystallizable fragment (Fc) domain of IgG molecules to therapeutic agents to improve pharmacokinetics and pharmacodynamics.

co-workers[107] first established that exercise stimulates musclin production and secretion via calcium-dependent activation of AKT. Interestingly, a mouse knockout of the musclin-encoded gene *Ostn* (*Ostn*-knockout mice) exhibited diminished maximal aerobic capacity, mitochondrial protein content and respiratory complex protein expression[107], a phenotype that was rescued when endogenous musclin was administered via an osmotic pump at similar concentrations to those observed in exercised mice. This implies a potential role of circulating musclin in mitochondrial biogenesis and describes a previously unrecognized mechanism of post-exercise skeletal muscle adaptation. This recent study adds to the growing list of biologically significant myokines. Given that exercise capacity is the most powerful predictor of mortality[108,109], the fact that this important facet of health might be influenced by secreted factors warrants further investigation.

Challenges in therapeutic exploitation

There are several challenges and limitations currently facing the therapeutic development of myokines or myokine secretagogues and/or analogues. First, myokines — by their very definition — are proteins or peptides, and therefore significant hurdles facing the use of biologics as therapies must be overcome. Protein biopharmaceuticals have become widely available after the rapid development of recombinant DNA technology over the past few decades[110]. The most important problems to be overcome in developing proteins for therapeutic administration include their physicochemical instability (in particular, aggregation), limited solubility, proteolytic instability, short half-life in plasma, immunogenicity and toxicity[111]. Although many different approaches have been applied to improve the performance of therapeutic proteins, there are two dominant technologies — PEGylation[112] and Fc fusion[113] — underpinning the biopharmaceuticals that have received marketing approval from regulatory authorities. By 2009 there were nine marketed PEGylated biopharmaceuticals. Seven Fc fusion-based drugs are currently on the market, and many more are in different stages of clinical trials, demonstrating that Fc fusion proteins have become credible alternatives to PEGylated proteins as therapeutics[114,115]. Therefore, although challenging, protein pharmaceuticals are viable options.

Another key limitation in the therapeutic exploitation of myokines is achieving cell- and/or tissue-specific targeting to avoid side effects. As discussed in this Review, although the vast majority of myokines are expressed in skeletal muscle and induced by muscle contraction, they are also expressed in other tissues and may have ubiquitous receptors[15]. However, in recent years the application of nanotechnology for drug delivery has begun to address this challenge[116]. Nanoparticles can provide long or short circulation times by controlling both the target surface and size[117]. Importantly, they can also target specific cell types within target organs[118].

Conclusion

Following the lead of the initial discovery of IL-6 as a myokine, research groups continue to uncover a range of factors secreted by skeletal muscle, which may lead to novel therapeutic approaches for the preventive treatment of non-communicable diseases such as diabetes[119] and cancer[92,94]. Perhaps the most promising therapeutic approach uncovered by myokines is to target the pathways activated by irisin[64] and METRNL[72] for the treatment of obesity. Further identification of novel myokines and a better understanding of their functions, mechanisms of action and downstream pathways is likely to lead to the identification of novel therapeutic approaches for a variety of diseases. Importantly, establishing molecular links between exercise and disease prevention can only strengthen the public health message that physical exercise results in disease prevention.

1. Pedersen, B. K. & Febbraio, M. A. Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nat. Rev. Endocrinol.* **8**, 457–465 (2012).
2. Booth, F. W., Roberts, C. K. & Laye, M. J. Lack of exercise is a major cause of chronic diseases. *Comp. Physiol.* **2**, 1143–1211 (2012).
3. Hawley, J. A., Hargreaves, M., Joyner, M. J. & Zierath, J. R. Integrative biology of exercise. *Cell* **159**, 738–749 (2014).
4. Starkie, R., Ostrowski, S., Jauffred, S., Febbraio, M. & Pedersen, B. Exercise and IL-6 infusion inhibit endotoxin-induced TNF-α production in humans. *FASEB J.* **17**, 884–886 (2003).
5. Walhin, J.-P., Richardson, J., Betts, J. & Thompson, D. Exercise counteracts the effects of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men. *J. Physiol.* **591**, 6231–6243 (2013).
6. Hagobian, T. A. & Braun, B. Interactions between energy surplus and short-term exercise on glucose and insulin responses in healthy people with induced, mild insulin insensitivity. *Metabolism* **55**, 402–408 (2006).
7. Goldstein, M. S. Humoral nature of the hypoglycemic factor of muscular work. *Diabetes* **10**, 232–234 (1961).
The first paper to propose that skeletal muscle might secrete 'exercise factors'.
8. Febbraio, M. A., Hiscock, N., Sacchetti, M., Fischer, C. P. & Pedersen, B. K. Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction. *Diabetes* **53**, 1643–1648 (2004).
A significant finding in the identification of IL-6 as a secreted product of skeletal muscle that carries out endocrine functions.
9. Pedersen, B. K. & Febbraio, M. A. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol. Rev.* **88**, 1379–1406 (2008).
10. Febbraio, M. A. & Pedersen, B. K. Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *FASEB J.* **16**, 1335–1347 (2002).
11. Pal, M., Febbraio, M. A. & Whitham, M. From cytokine to myokine: the emerging role of interleukin-6 in metabolic regulation. *Immunol. Cell Biol.* **92**, 331–339 (2014).
12. Benatti, F. B. & Pedersen, B. K. Exercise as an anti-inflammatory therapy for rheumatic diseases-myokine regulation. *Nat. Rev. Rheumatol.* **11**, 86–97 (2015).
13. Görgens, S. W., Eckardt, K., Jensen, J., Drevon, C. A. & Eckel, J. Exercise and regulation of adipokine and myokine production. *Prog. Mol. Biol. Transl. Sci.* **135**, 313–336 (2015).
14. Schnyder, S. & Handschin, C. Skeletal muscle as an endocrine organ: PGC-1α, myokines and exercise. *Bone* **80**, 115–125 (2015).
15. Uhlen, M. *et al.* Tissue-based map of the human proteome. *Science* **347**, 1260419 (2015).
16. Catoire, M., Mensink, M., Kalkhoven, E., Schrauwen, P. & Kersten, S. Identification of human exercise-induced myokines using secretome analysis. *Physiol. Genom.* **46**, 256–267 (2014).
17. Geiger, T. *et al.* Initial quantitative proteomic map of twenty-eight mouse tissues using the SILAC mouse. *Mol. Cell. Proteom.* **12**, 1709–1722 (2013).
18. Su, A. I. *et al.* A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc. Natl Acad. Sci. USA* **101**, 6062–6067 (2004).
19. Azimifar, S. B., Nagaraj, N., Cox, J. & Mann, M. Cell-type-resolved quantitative proteomics of murine liver. *Cell. Metab.* **20**, 1076–1087 (2014).
20. Williams, E. G. *et al.* Systems proteomics of liver mitochondria function. *Science* **352**, aad0189 (2016).
21. Kawamoto, S., Matsumoto, Y., Mizuno, K., Okubo, K. & Matsubara, K. Expression profiles of active genes in human and mouse livers. *Gene* **174**, 151–158 (1996).
22. Steen, H. & Mann, M. The ABC's (and XYZ's) of peptide sequencing. *Nat. Rev. Mol. Cell. Biol.* **5**, 699–711 (2004).
23. Matthiesen, R. & Bunkenborg, J. Introduction to mass spectrometry-based proteomics. *Methods Mol. Biol.* **1007**, 1–45 (2013).

24. Hartwig, S. *et al.* Secretome profiling of primary human skeletal muscle cells. *Biochim. Biophys. Acta* **1844**, 1011–1017 (2014).
25. Henningsen, J., Rigbolt, K. T., Blagoev, B., Pedersen, B. K. & Kratchmarova, I. Dynamics of the skeletal muscle secretome during myoblast differentiation. *Mol. Cell. Proteom.* **9**, 2482–2496 (2010).
26. Norheim, F. *et al.* Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. *Am. J. Physiol. Endocrinol. Metab.* **301**, E1013–E1021 (2011).
27. Henningsen, J., Pedersen, B. K. & Kratchmarova, I. Quantitative analysis of the secretion of the MCP family of chemokines by muscle cells. *Mol. Biosyst.* **7**, 311–321 (2011).
28. Yoon, J. H. *et al.* Comparative proteomic analysis of the insulin-induced L6 myotube secretome. *Proteomics* **9**, 51–60 (2009).
29. Yoon, J. H. *et al.* Proteomic analysis of the palmitate-induced myotube secretome reveals involvement of the annexin A1-formyl peptide receptor 2 (FPR2) pathway in insulin resistance. *Mol. Cell. Proteom.* **14**, 882–892 (2015).
30. Chan, C. Y., McDermott, J. C. & Siu, K. W. Secretome analysis of skeletal myogenesis using SILAC and shotgun proteomics. *Int. J. Proteom.* **2011**, 329467 (2011).
31. Raschke, S., Eckardt, K., Bjørklund Holven, K., Jensen, J. & Eckel, J. Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells. *PLoS ONE* **8**, e62008 (2013).
32. Haugen, F. *et al.* IL-7 is expressed and secreted by human skeletal muscle cells. *Am. J. Physiol. Cell Physiol.* **298**, C807–C816 (2010).
33. Deshmukh, A. S. *et al.* Deep proteomics of mouse skeletal muscle enables quantitation of protein isoforms, metabolic pathways, and transcription factors. *Mol. Cell. Proteom.* **14**, 841–853 (2015). **The deepest quantitation of the skeletal muscle proteome thus far and an insight into the power of discovery-based proteomics.**
34. Anderson, N. L. & Anderson, N. G. The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteom.* **1**, 845–867 (2002).
35. Omenn, G. S. *et al.* Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics* **5**, 3226–3245 (2005).
36. States, D. J. *et al.* Challenges in deriving high-confidence protein identifications from data gathered by a HUPO plasma proteome collaborative study. *Nat. Biotechnol.* **24**, 333–338 (2006).
37. Pernemalm, M., Lewensohn, R. & Lehtio, J. Affinity prefractionation for MS-based plasma proteomics. *Proteomics* **9**, 1420–1427 (2009).
38. Liu, X. *et al.* Mapping the human plasma proteome by SCX-LC-IMS-MS. *J. Am. Soc. Mass Spectrom.* **18**, 1249–1264 (2007).
39. Pocsfalvi, G. *et al.* Mass spectrometry of extracellular vesicles. *Mass Spectrom. Rev.* **35**, 3–21 (2015).
40. Harel, M., Oren-Giladi, P., Kaidar-Person, O., Shaked, Y. & Geiger, T. Proteomics of microparticles with SILAC Quantification (PROMIS-Quan): a novel proteomic method for plasma biomarker quantification. *Mol. Cell. Proteom.* **14**, 1127–1136 (2015).
41. Forterre, A. *et al.* Proteomic analysis of C2C12 myoblast and myotube exosome-like vesicles: a new paradigm for myoblast-myotube cross talk? *PLoS ONE* **9**, e84153 (2014).
42. Kalra, H. *et al.* Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol.* **10**, e1001450 (2012).
43. Lancaster, G. I. & Febbraio, M. A. Exosome-dependent trafficking of HSP70: a novel secretory pathway for cellular stress proteins. *J. Biol. Chem.* **280**, 23349–23355 (2005).
44. Steensberg, A. *et al.* Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *J. Physiol.* **529**, 237–242 (2000).
45. Wiles, M. V., Qin, W., Cheng, A. W. & Wang, H. CRISPR–Cas9-mediated genome editing and guide RNA design. *Mamm. Genome* **26**, 501–510 (2015).
46. Yang, Y. *et al.* A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. *Nat. Biotechnol.* **34**, 334–338 (2016).
47. Kamran, P. *et al.* Parabiosis in mice: a detailed protocol. *J. Vis. Exp.* http://dx.doi.org/10.3791/50556 (2013).
48. Chambers, A. G., Percy, A. J., Simon, R. & Borchers, C. H. MRM for the verification of cancer biomarker proteins: recent applications to human plasma and serum. *Exp. Rev. Proteom.* **11**, 137–148 (2014).
49. Peterson, A. C., Russell, J. D., Bailey, D. J., Westphall, M. S. & Coon, J. J. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol. Cell. Proteom.* **11**, 1475–1488 (2012).
50. Horsley, V., Jansen, K. M., Mills, S. T. & Pavlath, G. K. IL-4 acts as a myoblast recruitment factor during mammalian muscle growth. *Cell* **113**, 483–494 (2003).
51. Nieman, D. C. *et al.* Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run. *J. Appl. Physiol.* **94**, 1917–1925 (2003).
52. Ouchi, N. *et al.* Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism. *J. Biol. Chem.* **283**, 32802–32811 (2008).
53. Hamrick, M. W., McNeil, P. L. & Patterson, S. L. Role of muscle-derived growth factors in bone formation. *J. Musculoskelet. Neuronal Interact.* **10**, 64–70 (2010).
54. McPherron, A. C., Lawler, A. M. & Lee, S. J. Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. *Nature* **387**, 83–90 (1997).
55. Hittel, D. S., Berggren, J. R., Shearer, J., Boyle, K. & Houmard, J. A. Increased secretion and expression of myostatin in skeletal muscle from extremely obese women. *Diabetes* **58**, 30–38 (2009).
56. McPherron, A. C. & Lee, S. J. Suppression of body fat accumulation in myostatin-deficient mice. *J. Clin. Invest.* **109**, 595–601 (2002).
57. Wilkes, J. J., Lloyd, D. J. & Gekakis, N. Loss-of-function mutation in myostatin reduces tumor necrosis factor α production and protects liver against obesity-induced insulin resistance. *Diabetes* **58**,1133–1143 (2009).
58. Camporez, J-. P. G. *et al.* Anti-myostatin antibody increases muscle mass and strength and improves insulin sensitivity in old mice. *Proc. Natl Acad. Sci. USA* **113**, 2212–2217 (2016).
59. Moon, H. Y. *et al.* Running-induced systemic cathepsin B secretion is associated with memory function. *Cell. Metab.* http://dx.doi.org/10.1016/j.cmet.2016.05.025 (2016).
60. Bartelt, A. & Heeren, J. Adipose tissue browning and metabolic health. *Nat. Rev. Endocrinol.* **10**, 24–36 (2014).
61. Cousin, B. *et al.* Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J. Cell Sci.* **103**, 931–942 (1992).
62. Barbatelli, G. *et al.* The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *Am. J. Physiol. Endocrinol. Metab.* **298**, E1244–E1253 (2010).
63. Yoshida, T. *et al.* Nicotine induces uncoupling protein 1 in white adipose tissue of obese mice. *Int. J. Obes. Relat. Metab. Disord.* **23**, 570–575 (1999).
64. Boström, P. *et al.* A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* **481**, 463–468 (2012).
65. Lin, J. *et al.* Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. *Nature* **418**, 797–801 (2002).
66. Raschke, S. *et al.* Evidence against a beneficial effect of irisin in humans. *PLoS ONE* **8**, e73680 (2013).
67. Albrecht, E. *et al.* Irisin – a myth rather than an exercise-inducible myokine. *Sci. Rep.* **5**, 8889 (2015).
68. Erickson, H. P. Irisin and FNDC5 in retrospect: an exercise hormone or a transmembrane receptor? *Adipocyte* **2**, 289–293 (2013).
69. Schumacher, M. A., Chinnam, N., Ohashi, T., Shah, R. S. & Erickson, H. P. The structure of irisin reveals a novel intersubunit β-sheet fibronectin type III (FNIII) dimer: implications for receptor activation. *J. Biol. Chem.* **288**, 33738–33744 (2013).
70. Jedrychowski, M. P. *et al.* Detection and quantitation of circulating human irisin by tandem mass spectrometry. *Cell. Metab.* **22**, 734–740 (2015). **An example of how targeted proteomics can validate myokine expression in exercise contexts.**
71. Lee, P. *et al.* Irisin and FGF21 are cold-induced endocrine activators of brown fat function in humans. *Cell. Metab.* **19**, 302–309 (2014).
72. Rao, R. R. *et al.* Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. *Cell* **157**, 1279–1291 (2014).
73. Izumiya, Y., Hopkins, T., Morris, C., Sato, K. & Zeng, L. Fast/glycolytic muscle fiber growth reduces fat mass and improves metabolic parameters in obese mice. *Cell. Metab.* **7**,159–172 (2008).
74. Izumiya, Y. *et al.* FGF21 is an Akt-regulated myokine. *FEBS Lett.* **582**, 3805–3810 (2008).
75. Kim, K. H. *et al.* Acute exercise induces FGF21 expression in mice and in healthy humans. *PLoS ONE* **8**, e63517 (2013).
76. Kharitonenkov, A. *et al.* FGF-21 as a novel metabolic regulator. *J. Clin. Invest.* **115**, 1627–1635 (2005).
77. Coskun, T. *et al.* Fibroblast growth factor 21 corrects obesity in mice. *Endocrinology* **149**, 6018–6027 (2008).
78. Hansen, J. S. *et al.* Glucagon-to-insulin ratio is pivotal for splanchnic regulation of FGF-21 in humans. *Mol. Met.* **4**, 551–560 (2015). **An insightful examination of the source of circulating FGF21 during exercise.**
79. Markan, K. R. *et al.* Circulating FGF21 is liver derived and enhances glucose uptake during refeeding and overfeeding. *Diabetes* **63**, 4057–4063 (2014).
80. Hondares, E. *et al.* Thermogenic activation induces FGF21 expression and release in brown adipose tissue. *J. Biol. Chem.* **286**, 12983–12990 (2011).
81. Fisher, F. M. *et al.* FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev.* **26**, 271–281 (2012).
82. Samms, R. J. *et al.* Discrete aspects of FGF21 *in vivo* pharmacology do not require UCP1. *Cell Rep.* **11**, 991–999 (2015).
83. Véniant, M. M. *et al.* Pharmacologic effects of FGF21 are independent of the "browning" of white adipose tissue. *Cell. Metab.* **21**, 731–738 (2015).
84. Degirolamo, C., Sabbà, C. & Moschetta, A. Therapeutic potential of the endocrine fibroblast growth factors FGF19, FGF21 and FGF23. *Nat. Rev. Drug. Discov.* **15**, 51–69 (2016).
85. Roberts, L. D. *et al.* β-Aminoisobutyric acid induces browning of white fat and hepatic β-oxidation and is inversely correlated with cardiometabolic risk factors. *Cell. Metab.* **19**, 96–108 (2014).
86. Seldin, M. M., Peterson, J. M., Byerly, M. S., Wei, Z. & Wong, G. W. Myonectin (CTRP15), a novel myokine that links skeletal muscle to systemic lipid homeostasis. *J. Biol. Chem.* **287**, 11968–11980 (2012).
87. Seldin, M. M. *et al.* Skeletal muscle-derived myonectin activates the mammalian target of rapamycin (mTOR) pathway to suppress autophagy in liver. *J. Biol. Chem.* **288**, 36073–36082 (2013).
88. Yang, L., Li, P., Fu, S., Calay, E. S. & Hotamisligil, G. S. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell. Metab.* **11**, 467–478 (2010).
89. Leitzmann, M. *et al.* European Code against Cancer 4th Edition: physical activity and cancer. *Cancer Epidemiol.* **39** (Suppl. 1), 46–55 (2015).
90. Brown, J. C., Winters-Stone, K., Lee, A. & Schmitz, K. H. Cancer, physical activity, and exercise. *Comp. Physiol.* **2**, 2775–2809 (2012).
91. Goh, J. *et al.* Exercise training in transgenic mice is associated with attenuation of early breast cancer growth in a dose-dependent manner. *PLoS ONE* **8**, e80123 (2013).
92. Pedersen, L. *et al.* Voluntary running suppresses tumor growth through epinephrine- and IL-6-dependent NK cell mobilization and redistribution. *Cell. Metab.* **23**, 554–562 (2016). **A comprehensive demonstration that voluntary wheel running in mouse models of cancer is a beneficial effect partially mediated by the myokine IL-6.**
93. Hojman, P. *et al.* Exercise-induced muscle-derived cytokines inhibit mammary cancer cell growth. *Am. J. Physiol. Endocrinol. Metab.* **301**, E504–E510 (2011).
94. Aoi, W. *et al.* A novel myokine, secreted protein acidic and rich in cysteine (SPARC), suppresses colon tumorigenesis via regular exercise. *Gut* **62**, 882–889 (2013).
95. Kanzleiter, T. *et al.* The myokine decorin is regulated by contraction and involved in muscle hypertrophy. *Biochem. Biophys. Res. Comm.* **450**, 1089–1094 (2014).

96. Soria-Valles, C. *et al.* The anti-metastatic activity of collagenase-2 in breast cancer cells is mediated by a signaling pathway involving decorin and miR-21. *Oncogene* **33**, 3054–3063 (2014).
97. Araki, K. *et al.* Decorin suppresses bone metastasis in a breast cancer cell line. *Oncology* **77**, 92–99 (2009).
98. Laye, M. J. *et al.* Cessation of daily wheel running differentially alters fat oxidation capacity in liver, muscle, and adipose tissue. *J. Appl. Physiol.* **106**, 161–168 (2009).
99. Carotta, S. Targeting, N. K. Cells for anticancer immunotherapy: clinical and preclinical approaches. *Front. Immunol.* **7**, 152 (2016).
100. Morikawa, T. *et al.* Prospective analysis of body mass index, physical activity, and colorectal cancer risk associated with β-catenin (CTNNB1) status. *Cancer Res.* **73**, 1600–1610 (2013).
101. Je, Y., Jeon, J. Y., Giovannucci, E. L. & Meyerhardt, J. A. Association between physical activity and mortality in colorectal cancer: a meta-analysis of prospective cohort studies. *Int. J. Cancer* **133**, 1905–1913 (2013).
102. Murphy-Ullrich, J. E. & Sage, E. H. Revisiting the matricellular concept. *Matrix Biol.* **37**, 1–14 (2014).
103. Brekken, R. A. *et al.* Enhanced growth of tumors in SPARC null mice is associated with changes in the ECM. *J. Clin. Invest.* **111**, 487–495 (2003).
104. Neill, T., Schaefer, L. & Iozzo, R. V. Decorin: a guardian from the matrix. *Am. J. Pathol.* **181**, 380–387 (2012).
105. Rahman, M., Chan, A. P. K. & Tai, I. T. A peptide of SPARC interferes with the interaction between caspase8 and Bcl2 to resensitize chemoresistant tumors and enhance their regression *in vivo*. *PLoS ONE* **6**, e26390 (2011).
106. Nishizawa, H. *et al.* Musclin, a novel skeletal muscle-derived secretory factor. *J. Biol. Chem.* **279**, 19391–19395 (2004).
107. Subbotina, E. *et al.* Musclin is an activity-stimulated myokine that enhances physical endurance. *Proc. Natl Acad. Sci. USA* **112**, 16042–16047 (2015).
108. Myers, J. *et al.* Exercise capacity and mortality among men referred for exercise testing. *N. Engl. J. Med.* **346**, 793–801 (2002).
109. Korpelainen, R. *et al.* Exercise capacity and mortality - a follow-up study of 3033 subjects referred to clinical exercise testing. *Ann. Med.* http://dx.doi.org/10.1080/07853890.2016.1178856 (2016).
110. Lua, L. H. L. & Chuan, Y. P. in *Biopharmaceutical Production Technology* Vol. 1 & Vol. 2 43–77 (Wiley-VCH Verlag GmbH & Co. KGaA, 2012).
111. Pisal, D. S., Kosloski, M. P. & Balu-Iyer, S. V. Delivery of therapeutic proteins. *J. Pharm. Sci.* **99**, 2557–2575 (2010).
112. Harris, J. M. & Chess, R. B. Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug. Discov.* **2**, 214–221 (2003).
113. Jazayeri, J. A. & Carroll, G. J. Fc-based cytokines: prospects for engineering superior therapeutics. *Biodrugs* **22**, 11–26 (2008).
114. Huang, C. Receptor-Fc fusion therapeutics, traps, and MIMETIBODY technology. *Curr. Opin. Biotechnol.* **20**, 692–699 (2009).
115. Rath, T. *et al.* Fc-fusion proteins and FcRn: structural insights for longer-lasting and more effective therapeutics. *Crit. Rev. Biotechnol.* **35**, 235–254 (2015).
116. Panyam, J. & Labhasetwar, V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv. Drug. Deliv. Rev.* **55**, 329–347 (2003).
117. Davis, M. E., Chen, Z. G. & Shin, D. M. Nanoparticle therapeutics: an emerging treatment modality for cancer. *Nat. Rev. Drug. Discov.* **7**, 771–782 (2008).
118. Popielarski, S. R., Hu-Lieskovan, S., French, S. W., Triche, T. J. & Davis, M. E. A nanoparticle-based model delivery system to guide the rational design of gene delivery to the liver. 2. *In vitro* and *in vivo* uptake results. *Bioconjug. Chem.* **16**, 1071–1080 (2005).
119. Febbraio, M. A. gp130 receptor ligands as potential therapeutic targets for obesity. *J. Clin. Invest.* **117**, 841–849 (2007).
120. Ostrowski, K., Rohde, T., Zacho, M., Asp, S. & Pedersen, B. Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *J. Physiol.* **508**, 949–953 (1998).
121. Febbraio, M. A. *et al.* Hepatosplanchnic clearance of interleukin-6 in humans during exercise. *Am. J. Physiol. Endocrinol. Metab.* **285**, E397–E402 (2003).
122. Hiscock, N., Chan, M., Bisucci, T., Darby, I. & Febbraio, M. Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fiber type specificity. *FASEB J.* **18**, 992–994 (2004).
123. Starkie, R. L., Arkinstall, M. J., Koukoulas, I., Hawley, J. A. & Febbraio, M. A. Carbohydrate ingestion attenuates the increase in plasma interleukin-6, but not skeletal muscle interleukin-6 mRNA, during exercise in humans. *J. Physiol.* **533**, 585–591 (2001).
124. Steensberg, A. *et al.* Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J. Physiol.* **537**, 633–639 (2001).
125. Richter, E. A. & Galbo, H. Diabetes, insulin and exercise. *Sports Med.* **3**, 275–288 (1986).
126. Wasserman, D. H. Regulation of glucose fluxes during exercise in the postabsorptive state. *Annu. Rev. Physiol.* **57**, 191–218 (1995).
127. Whitham, M. *et al.* Contraction-induced interleukin-6 gene transcription in skeletal muscle is regulated by c-Jun terminal kinase/activator protein-1. *J. Biol. Chem.* **287**, 10771–10779 (2012).
128. Kelly, M. *et al.* AMPK activity is diminished in tissues of IL-6 knockout mice: the effect of exercise. *Biochem. Biophys. Res. Comm.* **320**, 449–454 (2004).
129. Al-Khalili, L. *et al.* Signaling specificity of interleukin-6 action on glucose and lipid metabolism in skeletal muscle. *Mol. Endocrinol.* **20**, 3364–3375 (2006).
130. Carey, A. L. *et al.* Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation *in vitro* via AMP-activated protein kinase. *Diabetes* **55**, 2688–2697 (2006).
131. Petersen, A. M. & Pedersen, B. K. The anti-inflammatory effect of exercise. *J. Appl. Physiol.* **98**, 1154–1162 (2005).
132. van Hall, G. *et al.* Interleukin-6 stimulates lipolysis and fat oxidation in humans. *J. Clin. Endocrinol. Metab.* **88**, 3005–3010 (2003).
133. Ellingsgaard, H. *et al.* Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. *Nat. Med.* **17**, 1481–1489 (2011).
134. Shirazi, R. *et al.* Glucagon-like peptide 1 receptor induced suppression of food intake, and body weight is mediated by central IL-1 and IL-6. *Proc. Natl Acad. Sci. USA* **110**, 16199–16204 (2013).
135. ALS CNTF Treatment Study Group. A double-blind placebo-controlled clinical trial of subcutaneous recombinant human ciliary neurotrophic factor (rHCNTF) in amyotrophic lateral sclerosis. *Neurology* **46**, 1244–1249 (1996).
136. Duff, E. & Baile, C. A. Ciliary neurotrophic factor: a role in obesity? *Nutr. Rev.* **61**, 423–426 (2003).
137. Ettinger, M. P. *et al.* Recombinant variant of ciliary neurotrophic factor for weight loss in obese adults: a randomized, dose-ranging study. *J. Am. Med. Assoc.* **289**, 1826–1832 (2003).
138. Kraakman, M. *et al.* Targeting gp130 to prevent inflammation and promote insulin action. Diabetes Obes. *Metab.* **15** (Suppl. 3), 170–175 (2013).
139. Tsiloulis, T. & Watt, M. J. Exercise and the regulation of adipose tissue metabolism. *Prog. Mol. Biol. Transl. Sci.* **135**, 175–201 (2015).
140. Vosselman, M. J. *et al.* Low brown adipose tissue activity in endurance-trained compared with lean sedentary men. *Int. J. Obes.* **39**, 1696–1702 (2015).

Acknowledgements
M.W. and M.A.F. are supported by the National Health and Medical Research Council of Australia (NHMRC Project Grant APP1062436 to M.A.F and M.W; Research Fellowship APP11021168 to M.A.F.).

Competing interests statement
The authors declare competing interests: see Web version for details.