Leibniz-Gemeinschaft

Final report

Title of the project:

Adipokines and Myokines - Common Language of Muscle and Fat? Establishing a Methodical Platform for Muscle Research in Human, Model and Farm Animals

Leibniz-Institute:	Leibniz Institute for Farm Animal Biology (FBN) Dummerstorf
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1. Executive summary

Body fat is stored mainly in two major depots as visceral or subcutaneous adipose tissue. Different metabolic conditions or a different genetic background may also cause fat storage in skeletal muscle tissue. Intramyocellular triglycerides are accumulated in muscle cells and adipocytes may be deposited between muscle cells as intramuscular fat (IMF). IMF is a major determinant of meat quality in farm animals because of major effects on tenderness and palatability. Breeding selection on increased muscularity led to a decrease of IMF in many farm animal species. This is also true for many cattle breeds. However, some breeds exist where very high intramuscular fat deposition can occur under energy-rich feeding regimens. Metabolic consequences of this fat deposition have not been investigated in detail. Consequently, the high variability in IMF makes cattle an interesting model for other species. In contrast, fat deposition in muscle is highly relevant for obesity-associated metabolic diseases in humans. Here, the amount of fat in skeletal muscle correlates with metabolic impairments such as insulin resistance and diminished metabolic flexibility in inactive individuals. Consequently, research in humans and farm animal targets the same tissue with different aims. Besides endocrine factors from peripheral organs proteins secreted from adipocytes and myocytes contribute to the high variability of fat deposition. These factors are called adipokines, myokines, and adipo-myokines and mediate the cross-talk in skeletal muscle tissue. The identification and characterization of new proteins of those classes was in the focus of the current project.

This goal was accomplished by using experimental and published data from farm animals, laboratory rodents, and humans. Specific adipo- and myokines were tested on their regulation and molecular function on lipid metabolism. A total of 119 myokines, 79 adipokines and 22 adipo-myokines was identified by this approach after several filtering and verification steps. Subsequent experiments involved cell cultures, animal models and a broad variety of molecular and biochemical methods to characterize the function of selected factors regarding intramuscular fat deposition in cattle and metabolic characteristics in human and mice. For two adipokines, adiponectin and adipsin, the molecular steps of their secretion were clarified. Furthermore, models and methods were exchanged by the partners. The generated list of adipo-, myo- and adipo-myokines comprises a valuable tool for further research of all project partners.

Results from this project have been published in 19 peer-reviewed articles. Different aspects of the studies were presented at national and international conferences in the fields of animal science, human metabolism, diabetes research and exercise physiology. The results contributed to 3 defended and 1 ongoing Ph.D. theses and 2 master theses.

2. Background and aims of the project

Endogenous fat is mainly stored as triacylglycerol in subcutaneous and visceral adipose tissue. However, depending on metabolic conditions and genetic background, fat is also accumulated in other organs. In the skeletal muscle, fat can either be stored in muscle cells as intramyocellular triglycerides or in adipocytes situated between the muscle fibers as intramuscular fat (IMF). IMF is a major determinant of meat quality in farm animals because of major effects on tenderness and palatability. Breeding selection on increased muscularity led to a decrease of IMF in many farm animal species. This is also true for many cattle breeds. However, some breeds exist where very high intramuscular fat deposition can occur under energy-rich feeding regimens. Metabolic consequences of this fat deposition have not been investigated in detail. Consequently, the high variability in IMF makes cattle an interesting model for other species. In contrast, fat deposition in muscle is highly relevant for obesity-associated metabolic diseases in humans. Here, the amount of fat in skeletal muscle correlates with metabolic impairments such as insulin resistance and diminished metabolic flexibility in inactive individuals.

The deposition of fat in skeletal muscle is under endocrine regulation but crosstalk between the affected tissues – skeletal muscle and related adipocytes – is likely to contribute to its high variability. Myocytes as well as adipocytes secrete numerous signaling molecules with potential auto-, para-, and endocrine effects. According to their origin these molecules were termed myokines and adipokines. There is an overlap between both groups comprising so-called adipo-myokines. Interestingly, some of them exert essentially opposite metabolic effects depending on the secreting and target tissue. Several hundreds of putative adipokines have been described in human and rodents whereas the knowledge about myokines is rather limited so far. In farm animals only few adiopokines and myokines have been identified so far and only a handful of these molecules have been investigated in some detail leaving a substantial gap of knowledge.

We hypothesized that a crosstalk between adipocytes and muscle cells via adipokines and myokines plays a major role in the manifestation of IMF and thereby in the quality of beef. Furthermore, ectopic lipid deposition in skeletal muscle is also relevant for metabolic diseases in mice and humans as it is closely associated with insulin resistance in inactive individuals.

The aim of this project was to identify new adipokines, myokines and adipomyokines by using experimental and published data from farm animals, laboratory rodents, and humans. Furthermore, specific adipo- and myokines should be tested whether and how they modulate the IMF deposition by studying their pattern in different models (cattle, human cells, mouse models), in respect to differences in fat distribution and metabolic flexibility, and by investigating their molecular function on lipid metabolism.

Following work packages (WP) were agreed:

WP1 Co-ordination and exchange of methods and materials
WP2 Adipokine and myokine signatures in farm animals, mice and humans
WP3 Bi-directional crosstalk between fat and muscle in vitro
WP4 Physical exercise and the muscle-fat connection in vivo

This approach should result in the identification of novel candidate genes and potential biomarkers that can be used for future breeding selection and might serve as drug targets for human metabolic diseases. Although the research of the partners ultimately aimed at different goals – optimization of the meat quality in farm animals and identification of potential drug targets for metabolic diseases in humans – they involved the same tissues and underlay common molecular mechanisms. The project aimed at a better understanding of signaling between skeletal muscle and adipose tissue across species by unifying research from different disciplines and made the network beneficial for all partners.

3. Development of the project

Two working groups were established during the kick-off meeting of the project in March, 2013. One was responsible for generating a virtual platform unifying the methodological competencies and resources of the partner. These involved an experimental cattle population which was extensively phenotyped including biochemical measures as well as specific mouse models, cell culture systems, laser microdissection technology, metabolomics, mitochondrial bioenergetics, and different in vivo exercise challenge approaches. These were introduced to all project partners. Another group collected and viewed data from numerous experiments to provide a basis for the development of adipokine and myokine signatures in farm animals, mice and humans. This group developed a methodology to combine the data from highly divergent experiments in different species and contributed to one major achievement of the project – the development of weighted lists of putative adipo-, myo-, and adipomyokines.

Annual meetings of all project partners (Potsdam, October 2013; Tübingen, March 2014; Düsseldorf, March 2015) were organized as scientific conferences with presentations of the current results from the project. Furthermore, information on existing methods and protocols were exchanged and facilities and resources of the respective hosts were presented. One short-term exchange mission was used for method's transfer between partners. Further communication during the project was facilitated by regular mailings and telephone conferences at principal investigator's level as well as between Ph.D. students and post-doctoral researchers of the project partners. A final project meeting took place in February 2016 in Dummerstorf to conclude the results of the project and to discuss opportunities for further collaboration. As a result further short-term methodical trainings and joint use of resources (animal models) were agreed between some of the partners.

The excellent communication between the project partners was a basis to follow the planned project outline. The identification of myokines and adipokines by consolidation of knowledge and results from numerous sources was in the focus during the first part of the project. Existing experimental data from the partners were collected and a bioinformatics pipeline for their exploitation was developed. The meetings in 2014 and 2015 were used to agree on selected myokines and adipokines for further investigations by the project partners. These subsequent investigations were done in responsibility of single partners and were embedded in ongoing research of the respective groups. In the case of myokine selection different interests of the partners led to deviation from original planning. The groups focusing on human research were mostly interested in identification of exercise-induced myokines as potential mediators of positive effects of exercise on metabolism. However, this group of myokines was of lower relevance in farm animals since physical activity is no major determinant of divergent body composition. Instead, myokines potentially affected by other exogenous factors like nutrition were of greater importance.

Partner FBN aimed additionally at establishing bovine primary cell culture models for further functional analyses. Based on earlier developments, a standardized protocol for bovine primary skeletal muscle cell was established. In contrast, a high number of methodological experiments with bovine primary adipocytes did not result in a sufficiently reproducible culture protocol. Instead a method for temporary cultivation of tissue explants was adopted. This method has the advantage to allow for investigations on adipocytes in an environment closer to *in vivo* conditions than traditional cultures. This cultivation is only suited for short-termed investigations up to 4 days but enlarged the spectrum of methods for further research.

Although, not in the primary focus of the project, the newly emerged, putative myokine irisin was included into the research of several project partners. The obvious ability of irisin to be induced by exercise and to cause "browning" of white adipose tissue (WAT) along with its apparent positive relationship to high muscle mass made it of potential interest in humans as well as in farm animals. Research in this topic resulted in publications with high impact in the controversial discussion of this putative myokine.

4. Results and discussion

Adipose tissue and skeletal muscle are organs that respond strongly to obesity and physical exercise and exhibit high secretory activity. To identify novel putative adipokines, myokines and adipomyokines, comparative expression studies of skeletal muscle and adipose tissue of different mouse models were available. Data from lean (C57BL/6J) and obese (C57BL/6J on a high-fat diet and NZO) mice, of sedentary and endurance trained C57BL/6J mice, and a genetically modified (UCP1-tg) mouse were provided. Expression profiles of cattle characterized by different amounts of intramuscular fat (Japanese Black with very high, Holstein with moderate and Charolais with low intramuscular fat; segregating F_2-population derived from Charolais and Holstein) were added. The bovine data sets were made available for the public by publication of two data reports (Komolka et al. 2016, Albrecht et al. 2016). Finally, experimental data from previous analyses of the secretome of primary human skeletal muscle cells (Hartwig et al. 2014) and human primary adipocytes (Lehr et al. 2012) were used for comparison with murine and bovine expression data (**WP1**). The secretome of primary human skeletal muscle cells was profiled in cells from healthy, adult donors combining three different mass spectrometry-based non-targeted approaches as well as one antibody-based method. This allowed a comprehensive profiling of skeletal muscle-derived secreted proteins and peptides. A total of 548 proteins were identified with 305 assigned as potentially secreted by stringent consecutive filtering. Twelve proteins containing a secretory signal peptide were not previously described as myokine (Hartwig et al. 2014). In a previous study on human primary adipocytes, 263 proteins were identified and predicted to be secreted. Forty-four proteins were identified as novel adipokines (Lehr et al. 2012).

Differently regulated and annotated transcripts from murine and bovine experiments were combined in two matrices. Those with a score ≥5 were defined as transcripts with "high score" (1074 in the muscle and 578 in the adipose tissue). After comparison with human secretome data we identified 119 myokines, 79 adipokines and 22 adipomyokines. Functional network analysis of these candidates revealed remodeling of extracellular matrix and tissue fibrosis as relevant functions of several of these candidates. Considering the pathophysiological relevance of fibrosis for adipose-muscle-crosstalk in obesity and type 2 diabetes mellitus and its physiological role in exercise adaptation and meat quality of farm animals, the identified putative myokines, adipokines and adipo-myokines represent new candidates for further investigations in different species (**WP2**). A detailed description of the applied methodology together with the results of the analysis is given in Schering et al. (2015).

Based on the data of the cross-species analysis a number of candidates were selected for further investigations by the project partners. However, candidates with high scores were not necessarily selected. Instead the partners focused on putative adipokines and myokines which fitted best to specific research interests. In the following section specific investigations of the partners are described and discussed.

Partner FBN

Since data on bovine adipokines and myokines are rather scarce, a selection of genes for initial characterization was done on the basis of their potential function and significance in humans and mice on the one hand and, on the other hand on the basis of existing reports on quantitative trait loci for fatness traits. Furthermore, genes considered as functional candidates in the literature were chosen. In a first step, mRNA expression of putative candidates with no data in cattle so far was measured: Annexin A1 (ANXA1), growth differentiation factor 15 (GDF15), WNT inhibitory factor 1 (WIF1), cell death-inducing DNA-fragmentation-factor like effector c (CIDEC). For *ANXA1* a differential expression could not be verified in a set of samples from bulls with high and low IMF, whereas *CIDEC* expression was significantly increased in cattle with higher IMF. This putative adipo-myokine comprises an interesting candidate for further analyses. In contrast, *GDF15* and *WIF1* were only marginally expressed in cattle muscle samples and not further pursued.

Two further candidates, thrombospondin 4 (THBS4) and thyroid hormone responsive protein (THRSP) were further investigated using molecular biological and protein biochemical methods to extend knowledge of gene expression and regulation in cattle. THBS4 was identified as putative myokine in mouse and cattle. There were no data in cattle regarding expression and function. In a first step the expressing cell type in skeletal muscle tissue was determined by dissection of fat cells and muscle fibers by laser microdissection. The purity of the samples was ensured by measuring simultaneously specific marker genes. Immunohistochemical stainings and western blot analyses using appropriate primary antibodies allowed protein localization in various bovine tissues. Collectively, the results suggest that THBS4 as classically secreted protein can be termed as adipo-myokine. However, expression differences related to fatness traits in cattle were not observed. This may be due to the fact that THBS4 in mice is exercise-induced. Since myokines induced by physical activity are of less relevance in farm animals THBS4 may have no impact on body composition in cattle (**WP3**). Instead, diet-inducible factors are of greater interest.

THRSP was selected due to its location in a bovine QTL region and numerous reports naming it a candidate for IMF deposition in cattle. THRSP is known to be involved in lipogenic processes in rodents. In cattle, THRSP could be a potential molecular marker for IMF deposition since mRNA abundance was frequently found to be increased in skeletal muscle with high IMF content compared to those with low IMF. The aim was to elucidate the background of this differential expression and to evaluate the role of THRSP as candidate for increased IMF content in cattle. By combination of mRNA and protein analyses, it could be demonstrated that THRSP is present mainly in nuclei of adipocytes and associated cells, and in cells of the portal triad of liver, whereas muscle cells did not express THRSP. Besides, no single nucleotide polymorphisms (SNPs) could be found in putative regulatory sequences or microRNA binding sites as explanation for the observed expression differences between muscle samples from bulls with high or low IMF content. Additionally, *in silico*-analyses showed that putative upstream regulatory elements described for the human and rat genes are not conserved in cattle. It was concluded that expression differences are not due to structural variation at the THRSP locus. Cell culture analyses revealed furthermore that THRSP is expressed in mature adipocytes rather than in early stages of adipogenesis. (Schering et al. 2017). In summary, the data indicate that an increased expression of *THRSP* in skeletal muscle reflects the result of an elevated intramuscular adipogenesis, is not involved in the early events, and is consequently not a reason of varying IMF content in MLD of cattle. The localization of *THRSP* mRNA and protein in nuclei of adipocytes provides experimental support for the suggested role of THRSP as in transcriptional co-regulator of lipid metabolism and exclude its putative function as secreted adipokine (**WP3**).

In a next step methodical prerequisites were created to conduct functional studies with selected adipokines and myokines (**WP1**). In order to find out how adipokines influence the development of bovine skeletal muscle cells, a primary cell culture model should be established. For this purpose three different media were tested. All used media were suitable to induce myotube formation. The highest differentiation index was determined after incubation with a serum-free differentiation medium containing dexamethasone, linoleic acid and insulin (Will et al. 2015). The primary bovine culture system provides a good *in vitro* model for studying proliferation and differentiation processes of bovine satellite cells under defined conditions. Since numerous tested cultivation protocols for primary bovine preadipocytes did not lead to optimal differentiation and desired reproducibility, an alternative model, termed explant culture, is currently tested.

Partner DIfE I

As several adipokines have been described as central players within the complex network of organ crosstalk there was also a big interest in the secretory machinery that is required for an appropriate release of adipokines. The partner DIfE foregrounded the post-Golgi vesicular trafficking machinery and used an adipose-tissue specific inducible knockout model in which the ubiquitously expressed GTPase ARFRP1 (ADP-ribosylation factor related protein 1) is

deleted (designated as *Arfrp1*$^{iAT-/-}$). ARFRP1 locates to trans-Golgi membranes and is implicated in the targeting of intracellular cargo to downstream destinations. A screening of several adipokines demonstrated a specific reduction of adiponectin and adipsin in the plasma of *Arfrp1*$^{iAT-/-}$ in comparison to control mice. Other adipokines such as leptin, WISP1, DPP4 were not affected. On the basis of secretion and recycling assays performed in HeLa cells, ARFRP1 was found to be critically involved in protein trafficking that avails the endosomal compartment for cell surface delivery, whereas constitutive secretion was principally functioning. In the absence of ARFRP1 endosomal recycling of the transferrin/ transferrin receptor complex appeared to be defective, most likely at the level of cargo exocytosis rather than endosomal internalization. A subcellular delocalization of the SNARE protein SNAP-23 which has been observed in Arfrp1-depleted 3T3-L1 adipocytes might contribute to inadequate fusion of endosomal cargo with the plasma membrane (**WP3**). The markedly reduced adiponectin and adipsin levels in *Arfrp1*$^{iAT-/-}$ mice were associated with detrimental effects on adipocyte metabolism including impaired insulin responsiveness, an elevation in basal lipolytic activity accompanied by increased plasma lipid levels and compromised adipose tissue expandability as observed in *Arfrp1*$^{iAT-/-}$ mice. Presumably due to impaired adiponectin secretion, *Arfrp1*$^{iAT-/-}$ mice showed deteriorated hepatic insulin sensitivity, an increase in hepatic glucose production and elevated fasting blood glucose levels (**WP4**).

In conclusion, the data of this mechanistic part of the study – which is currently prepared for submission - demonstrates that specific post-Golgi proteins act as part of the vesicle trafficking machinery largely determining adipocyte function in terms of adipokine secretory capacity, thereby preserving metabolic health.

Partner DIfE II

Targeted mitochondrial uncoupling is considered as an interesting therapeutic approach for the treatment of metabolic disorders such as obesity and type 2 diabetes (Ost et al. 2017). In this respect, UCP1-tg mice with targeted ectopic expression of the mitochondrial uncoupling protein UCP1 in skeletal muscle were established as a model of healthy aging at the project partner DIfE.

Despite of a reduced muscle mass and strength these mice show a resistance to adverse metabolic effects of high fat diet feeding which is linked to a recruitment of brown adipocytes within white fat depots (“browning”). This suggested a crosstalk between skeletal muscle and adipose tissue possible through the induction of myokines. A comparative transcriptome analysis (table 1) revealed the strong induction of fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15) whose plasma levels were also highly increased in UCP1-tg mice. In fact, FGF21 and GDF15 were among the 10 most highly induced genes in skeletal muscle of UCP1-tg mice (Ost et al. 2015).

Table 1: Microarray data showing relative gene expression of known myokines in skeletal muscle of UCP1-tg (TG) compared to wild type (WT mice). FC: fold change.

Gene	ProbeID	Description	WT	TG	FC	*P*-value
Fgf21	**A_52_P235347**	**Fibroblast growth factor 21**	**6.87**	**11.11**	**+19.88**	**1.47E-07**
Gdf15	**A_55_P1960735**	**Growth differentiation factor 15**	**6.20**	**10.24**	**+16.50**	**5.00E-08**
Ostn	**A_52_P374960**	**Osteocrin (Musclin)**	**9,23**	**10,69**	**+2.77**	**4.87E-04**
Fgf1	**A_55_P2047188**	**Fibroblast growth factor 1**	**10.01**	**10.58**	**+1.49**	**6.40E-03**
Metrnl	**A_52_P355084**	**Meteorin, glial cell differentiation regulator-like**	**8.17**	**8.35**	**1.13**	**ns**
Dcn	**A_51_P334104**	**Decorin**	**14.69**	**14.84**	**1.11**	**ns**
Mstn	**A_51_P384901**	**Myostatin**	**9.40**	**8.98**	**-1.35**	**1.35E-02**

FGF21 and GDF15 are two secreted proteins that are induced by cellular stress in various tissues and can be considered as myokines released under pathophysiological conditions

related to mitochondrial myopathies. Both show very low expression in wildtype skeletal muscle and are almost 20-fold induced in UCP1-tg skeletal muscle (table 1). At first attention was turned on the role of FGF21 as a myokine which was still a novel finding at the start of this project. As a pleotropic hormone-like circulating protein FGF21 is known to function as a major metabolic regulator of glucose and lipid metabolism. It is mainly produced by liver but also by adipocytes which are also considered as the main targets of FGF21. Its secretion from skeletal muscle has only been demonstrated recently. Interestingly, FGF21 is currently evaluated as novel diabetes therapeutic. Therefore, it was hypothesized that the healthy aging phenotype of UCP1-tg mice could be due to an organ crosstalk mediated by FGF21. It is generally accepted that multiple organ crosstalk involves both cell-autonomous and cell-non-autonomous mechanisms. In order to explore the role of FGF21 in the mice model, FGF21 ablated UCP1-tg mice were generated in which cell-autonomous and cell-non-autonomous effects were analyzed in detail (Ost et al. 2016a). Genetic ablation of FGF21 fully counteracted the cell-non-autonomous metabolic remodeling and browning of subcutaneous WAT thus confirming WAT as the major target of endocrine acting FGF21. But surprisingly, FGF21 signaling proofed to be dispensable for the healthy aging phenotype of UCP1-tg mice. Improvements of obesity resistance, glycemic control, and hepatic lipid homeostasis were still present in UCP1-tg mice with FGF21 ablation. AMP activated protein kinase (AMPK) as a key regulator of cellular energy homeostasis and cell-autonomous stress adaption was still activated in skeletal muscle in the absence of FGF21. Also the protective cell-autonomous muscle mitohormesis and metabolic stress adaptation did not require the presence of FGF21. Thus, although it could be clearly shown that FGF21 drives WAT remodeling (browning), the adaptive pseudo-starvation response under elevated muscle mitochondrial stress conditions apparently operates independently of both WAT browning and FGF21 action (**WP4**). These findings challenge FGF21 as key metabolic mediator of the muscle mitochondrial stress adaptation and suggest that other myokines might by responsible for the metabolic improvements in UCP1-tg mice (Ost et al. 2016a).

Using a double transgenic mouse model it could further be shown that activation of AMPK is important for maintenance of skeletal muscle integrity during mitochondrial respiratory stress. However, it is not necessary for stress-induced, myokine-mediated effects on whole body metabolism (Ost et al. 2014).

Currently, the focus is on the role of GDF15 as a myokine which is still not well explored. GDF15 belongs to the transforming growth factor beta (TGFβ) superfamily. In humans, circulating GDF15 is increased in a number of diseases (many of those age related) such as cancer and cardio vascular disease. In general, GDF15 is considered to play a protective role in various organs such as heart and adipose tissue, but similar to other cytokines, GDF15 can also have detrimental effects in pathophysiological conditions. A role of GDF15 in energy metabolism was demonstrated by overexpression of GDF15 in mice. They were found to be resistant to diet induced obesity and showed an improved glucose homeostasis and increased lifespan by activating adipose tissue as well as systemic energy metabolism. Therefore the hypothesis is that GDF15 is responsible for the decreased muscle mass and metabolic remodeling of skeletal muscle observed in UCP1-tg mice and also contributes to the metabolic improvements. This will be investigated by performing in vivo studies using UCP1-tg mice lacking GDF15 (UCP1/GDF15-/- mice) and by in vitro studies using murine and human myoblasts which will be treated with recombinant GDF15.

A further interesting outcome of our transcriptome analysis was the realization that skeletal muscle mitochondrial uncoupling is not an exercise mimetic although both exercise and mild mitochondrial uncoupling have similar beneficial health effects. Using the UCP1-tg mouse model a detailed metabolic reprogramming profile associated with mitochondrial perturbations in skeletal muscle was identified, triggering an increased protein turnover and amino acid metabolism with induced biosynthetic pathways of serine, one-carbon and glycine (SOG) and the longevity promoting polyamine spermidine as well as the transsulfuration pathway. This is related to an induction of NADPH generating pathways and glutathione metabolism as an adaptive mitohormetic response and defense against increased oxidative

stress. Strikingly, consistent muscle retrograde signaling profiles were observed in acute stress states such as muscle cell starvation and lipid overload, muscle regeneration as well as heart muscle inflammation but not in response to exercise (Ost et al. 2015). The induction of specific myokines such as FGF21 and GDF15 is apparently part of this skeletal muscle integrated stress response induced by ER-Stress. Interestingly, FGF21 and GDF15 are not exercise myokines under normal physiological conditions. Myokine profiles and skeletal muscle integrated stress response thus differ between metabolic/mitochondrial stress conditions and exercise (**WP4**). However, the role of free radicals, reactive oxidants, and ER-stress in myokine regulation is still poorly investigated and understood as concluded in a review paper on the role of exercise and cellular stress in the regulation of myokine expression (Ost et al. 2016b).

Partner DDZ

Metabolic diseases are often associated with inflammatory processes. As inflammation plays an important role in the development of peripheral insulin resistance in type 2 diabetes the partner DDZ is interested in the anti-inflammatory effects of putative myokines. Elevated levels of the pro-inflammatory cytokine tumor necrosis factor α (TNFα) are found in plasma, adipose tissue and skeletal muscle in patients with whole body insulin resistance. Further, this cytokine has been shown to directly induce insulin resistance in skeletal muscle. Thus, the identification and characterization of novel molecules which are able to reduce TNFα-mediated inflammation will certainly be an important approach to prevent the development of peripheral insulin resistance.

Chitinase-3-like protein 1 (CHI3L1; also known as YKL-40) is a heparin- and chitin-binding glycoprotein and was originally discovered in mouse breast cancer cells. Immune response studies have linked CHI3L1 to a down-regulation of the inflammatory mediators matrix metalloprotease (MMP) 1, MMP3 and interleukin-8 (IL-8), suggesting a protective influence under innate immune response condition. CHI3L1 was identified as a novel myokine and supposed to play a protective role involving auto- and/or paracrine pathways (Görgens et al. 2014). In this study, it was found that myotubes express CHI3L1 in a differentiation-dependent manner. Furthermore, pro-inflammatory cytokines up-regulate CHI3L1 expression (6-fold) and release (3-fold). Importantly, CHI3L1 treatment blocked TNFα-induced inflammation by inhibiting nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) activation in skeletal muscle cells. It was shown that this effect is mediated via protease activated receptor 2 (PAR2). In addition, CHI3L1 treatment diminished the TNFα-induced expression and secretion of IL-8, monocyte chemoattractant protein 1 (MCP1) and IL-6. Also, impaired insulin action at the level of Akt and glycogen synthase kinase (GSK) 3α/ß phosphorylation and insulin-stimulated glucose uptake was normalized by CHI3L1. In conclusion, the novel myokine CHI3L1 which is induced by pro-inflammatory cytokines can counteract TNFα-mediated inflammation and insulin resistance in human skeletal muscle cells, potentially involving an auto/paracrine mechanism (**WP3**).

The receptor for CHI3L1, namely proteinase-activated receptor 2 (PAR-2), is expressed in human and rodent skeletal muscle cells, as reported in the above mentioned study. Interestingly, it was shown that activation of PAR-2 mediates a proliferative response of murine skeletal myoblasts, which is essential for muscle hypertrophy and regeneration. However, the physiological role and main sources of circulating CHI3L1 under various conditions remain largely unknown. It was hypothesized that contraction-regulated CHI3L1 production and CHI3L1/PAR-2 signaling may impact skeletal muscle growth and repair. Furthermore, potential differences of CHI3L1 and PAR-2 expression in skeletal muscle and adipose tissue from control and dysglemic subjects and the effect of exercise intervention were assessed (Görgens et al. 2016). Three human exercise studies were used to measure CHI3L1 plasma levels. In addition, muscle and adipose tissue *CHI3L1* mRNA expression was measured in response to acute and long-term exercise. Primary human skeletal muscle cells were differentiated *in vitro* and electrical pulse stimulation was applied. In addition, myoblasts were incubated with CHI3L1 protein and activation of MAP kinase signaling as well as proliferation

was measured. It was found that circulating CHI3L1 levels and muscle *CHI3L1* mRNA were increased after acute exercise. In addition, *CHI3L1* mRNA expression as well as CHI3L1 secretion was enhanced in electrically stimulated cultured myotubes. Incubation of cultured human myoblasts with CHI3L1 protein leads to a strong activation of p44/42, p38 MAPK and Akt as well as enhanced myoblast proliferation. The findings suggest that CHI3L1 is induced by acute exercise and that CHI3L1/PAR-2 signaling activates myocyte proliferation, which is important for restructuring of skeletal muscle in the response to exercise training (**WP3 + 4**).

Partner UKT

The partner UKT laid the focus on the examination of exercise-induced myokines and adipokines as potential mediators of positive effects of exercise on metabolism. Physical activity is a keystone of lifestyle intervention programs to prevent and treat metabolic disorders such as type 2 diabetes. However, differences exist in the individual response to regular training, but the molecular basis for these differences in the improvement in metabolic parameters (glucose tolerance, insulin sensitivity, body fat distribution) are unclear. For that reason the mechanisms of an impaired exercise response and involvement of myokines in 20 individuals at high risk of developing type 2 diabetes have been studied (Bohm et al. 2016). The subjects performed 8 weeks of controlled cycling and walking exercise at 80 % of their individual VO_2peak. Participants who did not show improvement in insulin sensitivity based on their Matsuda index as surrogate parameter were identified as nonresponders. They did not differ in preintervention parameters, adherence to training or energy expenditure compared with good responders. Nonresponders in insulin sensitivity also showed a comparable improvement in fitness measured as individual aerobic threshold. Transcriptome analysis of skeletal muscle biopsies taken before and after intervention revealed activation of TGFβ and TGFβ target genes in the muscle of nonresponders after the intervention. TGFβ plays an important role in inflammatory processes in regenerating muscle and regulates the function of macrophages in injured skeletal muscle, and extracellular matrix production and reorganization, e.g. by regulating skeletal muscle fibroblasts. In addition, TGFβ blocks differentiation of skeletal muscle cells to myotubes, suppresses expression of regulators and enzymes necessary for glucose and fatty acid oxidation, and reduces insulin signal transduction (Bohm et al. 2016). This action of TGFβ can explain the reduced metabolic adaptation in the muscle of nonresponders. Moreover, it shows the local importance of secreted factors in skeletal muscle tissue that may never reach systemic circulation. Transcriptional and proteomics profiling of the skeletal muscle following exercise revealed a large number of regulated transcripts associated with extracellular matrix regulation and extracellular matrix components (Hoffmann et al. 2017). Some of these extracellular matrix components may have local signaling properties, and the dynamic nature of muscle connective tissue is e.g. relevant for mechanotransduction and storage and release of growth factors (**WP4**). The relevance of these local factors regulating the composition and function of the extracellular matrix was also highlighted in the study of Schering et al. (2015) mentioned above.

To investigate regulation of the lipid pattern by acute exercise, lipidomics analysis of oxidative soleus and glycolytic gastrocnemius muscle of mice after one bout of treadmill running have been performed and compared with liver lipids (Hoene et al. 2016). Lipid profiles of soleus and gastrocnemius muscle showed pronounced differences with soleus muscle more comparable to liver tissue. Soleus muscles showed a marked increase in acetylcarnitine after run. A $^{13}C_{16}$-palmitate tracer applied immediately after run was detectable after 10 min in several acylcarnitines and triacylglycerol species in both muscles. The data indicate the relevance of single-muscle analyses when studying lipid metabolism in mice (**WP4**). Another lipidomics study revealed strong differences in the lipid pattern of brown and WAT (Hoene et al. 2014).

For many myokines and adipokines the contribution of the respective tissue to systemic concentrations is uncertain. Only for a few exercise-regulated myokines it has been shown that skeletal muscle accounts for a major part of systemic elevation following exercise. FGF21 represents a current candidate for the assumption to be an exercise-regulated myokine with

potential effects in mice with mitochondrial disorders. The analysis of human flux of FGF21 over the exercising muscle and over the hepatosplanchnic bed reveals that liver, rather than skeletal muscle, is the main contributor to increase in FGF21 plasma concentrations following exercise (Hansen et al. 2015). These data indicate that studies aiming to validate the release of a certain factor from a specific tissue are needed to proof the concept of an inter-organ crosstalk (**WP4**).

Taken together, the joint analysis of data obtained from different breeds and species with subsequent pathway analysis proved a valuable tool for expanding the list of putative candidates for secretory proteins from skeletal muscle and adipose tissue. All partners benefitted from this approach by receiving new impulses for their own research. Numerous specific methods and models were contributed, and partly exchanged by the partners allowing for more comprehensive investigations in the future. A large number of putative adipokines, myokines and adipo-myokines still await detailed characterization in human, mice and farm animals.

Research on the putative myokine irisin

Boström et al. (2012) suggested a novel mechanism for the induction of "brite" (brown in white) adipocytes in WAT depots after exercise in mice. They showed that the transmembrane protein fibronectin type III domain containing 5 (FNDC5), which is upregulated by exercise, is cleaved and the extracellular protein part is released by transfected HEK293 cells, which acts as novel molecule called irisin. Viral delivery of *FNDC5* in mice caused a browning of subcutaneous fat, stimulated oxygen consumption, and diminished diet-induced weight gain and metabolic dysfunction. Thus, irisin induced a thermogenic mechanism in WAT, which improved whole body energy balance in mice. This initial report of irisin linked the *FNDC5* gene to browning in mice.

Due to this surprising, apparent link between a hitherto disregarded gene and a central metabolic mechanism some of the partners in this project started investigations at several levels.

Partner DDZ performed a bioinformatic analysis of the human *FNDC5* gene (Raschke et al. 2013) and revealed that two divergent sequences have been published. In an earlier study, human *FNDC5* was described as a gene with a mutation in the start codon to ATA, encoding isoleucine. In contrast, Boström et al. (2012) published a sequence with a downstream methionine, encoded by the conserved ATG translation site. Although, the initial description of irisin was focused on mice, the data raised the hope that exogenously administered irisin might have a therapeutic potential in the treatment of obesity and diabetes in humans. Therefore, the human *FNDC5* gene was analysed and explored its function in the human system. Analyses of genomic DNA, mRNA and expressed sequence tags revealed that *FNDC5*, the gene encoding the precursor of irisin, is present in rodents and most primates, but shows in humans a mutation in the conserved start codon ATG to ATA. HEK293 cells transfected with a human FNDC5 construct with ATA as start codon resulted in only 1% full-length protein compared to human *FNDC5* with ATG. Additionally, *in vitro* contraction of primary human myotubes by electrical pulse stimulation induced a significant increase in peroxisome proliferator-activated receptor γ coactivator 1α (*PGC1α*) mRNA expression. However, *FNDC5* mRNA level was not altered. *FNDC5* mRNA expression in muscle biopsies from two different human exercise studies was not changed by endurance or strength training. Preadipocytes isolated from human subcutaneous adipose tissue exhibited differentiation to brite human adipocytes when incubated with bone morphogenic protein 7 (BMP7), but neither recombinant FNDC5 nor irisin were effective (**WP3**). These findings suggested that it is rather unlikely that the beneficial effect of irisin observed in mice can be translated to humans (Raschke et al. 2013).

Partner FBN investigated the bovine gene for FNDC5 at DNA, mRNA, and protein level. A correct annotation of the bovine locus was achieved along with the description of at least 2 full length transcripts and further 5' – and 3' – variants. Although, FNDC5 protein was abun-

dantly expressed in skeletal muscle, no circulating irisin was found. This indicated a different regulation of FNDC5/irisin in cattle compared to mice (Komolka et al. 2014).

Investigations in mice demonstrated that the proposed Pgc1α-Fndc5/irisin pathway did not respond to mild exercise. However, acute exercise increased circulating irisin immediately (Brenmoehl et al. 2014). Here, irisin was detected with a commercial antibody in serum of mice.

Conflicting data published in the meantime led to an international collaborative effort to assess the reliability of irisin-antibodies used in commercial ELISA kits. Led by partner FBN, plasma and serum samples from humans, farm and wild animals were tested on circulating irisin. Although, a specific FNDC5/irisin signature could be found by mass spectrometry in human serum, the data have shown that all results based on ELISAs have to be called into question since unspecific binding of proteins was common to all tested antibodies (Albrecht et al. 2015). These data supported the notion of partner DDZ that effects of irisin initially seen in mice may not be readily transferable to humans (**WP3**). The physiological role of irisin remains still under debate (Jedrychowsky et al. 2015).

References (*articles published by project partners; articles mainly or partly based on funding by the Leibniz Association are listed under point 8.)

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5. Commercial utilization of results

The results of the project are part of basic research and will be or have been published in peer-reviewed, scientific journals of different disciplines. A direct commercialization of results is not predictable at the moment.

6. National and international cooperation

Different parts of the research were done in collaboration with national and international partners listed below:

Department of Cell Biology, Duke University, Durham, USA;

Department of Nutrition and The Biotechnology Centre of Oslo, University of Oslo, Norway;

Vetsuisse Faculty, University of Berne, Switzerland;

The Centre of Inflammation and Metabolism and the Centre for Physical Activity Research, Department of Infectious Diseases and CMRC, and the Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark;

Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China;

Norwegian School of Sports Sciences, Oslo, Norway;

R&D Diabetes Division, Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany;

Helmholtz Diabetes Center, Helmholtz Zentrum München, Neuherberg, Germany;

Department of Human and Animal Physiology, Wageningen University, Wageningen, Netherlands;

Biomedical Research Institute, University of Lleida, Lleida, Spain

7. Qualifications

Doctoral theses:

Diplom Ernährungswissenschaftler Mario Ost (DIfE, Potsdam)

"Mitochondrial uncoupling induced integrated stress response and metabolic remodeling in skeletal muscle."
Doctoral Thesis, University of Potsdam, Institute of Nutritional Science (October 2014).

M.Sc. Sven Görgens (German Diabetes Center, Düsseldorf)

"Identification and characterization of novel myokines"
Doctoral Thesis, Heinrich Heine University Düsseldorf (June 2015)

M. Sc. Lisa Schering (Leibniz Institute for Farm Animal Biology [FBN], Dummerstorf)

“Identifizierung und Charakterisierung von Myokinen und Adipokinen mit Bedeutung für die Körperzusammensetzung beim Rind.“
Doctoral Thesis, Martin-Luther-University Halle-Wittenberg (December 2016)

In preparation:

M.Sc. Lisa Kappler (Division of Pathobiochemistry and Clinical Chemistry, University Hospital Tübingen)

“Method development for valid high-resolution mitochondrial profiling and Omics investigation of the mitochondrial adaptions to excess energy intake and physical exercise”
(Doctoral Thesis expected in December 2017)

Master theses:

Verena Donner (DIfE, Potsdam)

"Untersuchung der endokrinen Funktion von FGF21 am Modell der HSA-UCP1xFGF21-/- transgenen Maus."
Master thesis, University of Potsdam, Institute of Nutritional Science (October 2014).

Sebastian Ringel (DIfE, Potsdam)

“Untersuchung regulatorischer Funktionen von FGF21 im murinen Leberstoffwechsel nach Hochfettdiät-Interventionen.”
Master thesis, University of Potsdam, Institute of Nutritional Science (February 2016).

8. Publications

Albrecht E, Norheim F, Thiede B, Holen T, Ohashi T, Schering L, Lee S, Brenmoehl J, Thomas S, Drevon CA, Erickson HP, Maak S. Irisin – a myth rather than an exercise-inducible myokine. Sci Rep 5 (2015) 8889

Bohm A, Hoffmann C, Irmler M, Schneeweiss P, Schnauder G, Sailer C, Schmid V, Hudemann J, Machann J, Schick F, Beckers J, Hrabe dA, Staiger H, Fritsche A, Stefan N, Niess AM, Haring HU, Weigert C. TGFbeta contributes to impaired exercise response by suppression of mitochondrial key regulators in skeletal muscle. Diabetes 65 (2016) 2849-2861

Brenmoehl J, Albrecht E, Komolka K, Schering L, Langhammer M, Hoeflich A, Maak S. Irisin is Elevated in Skeletal Muscle and Serum of Mice Immediately after Acute Exercise Int J Biol Sci 10 (2014) 338-349

Eckardt K, Görgens SW, Raschke S, Eckel J. Myokines in insulin resistance and type 2 diabetes. Diabetologia 57 (2014) 1087-1099

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Ost M, Coleman V, Voigt A, van Schothorst EM, Keipert S, van der Stelt I, Ringel S, Graja A, Ambrosi T, Engst W, Kipp AP, Jastroch M, Schulz TJ, Keijer J, Klaus S. Muscle mitochondrial stress adaptation operates independently of endogenous FGF21 action. Mol Metabol. 5 (2016a) 79-90

Ost M, Coleman V, Kasch J, Klaus S. Regulation of myokine expression: Role of exercise and cellular stress. Free Radic Biol Med 98 (2016b) 78-89

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Schering L, Albrecht E, Komolka K, Kühn C, Maak S. Increased expression of thyroid hormone responsive protein (THRSP) is the result but not the cause 1 of higher intramuscular fat content in cattle. Int J Biol Sci (2017 in press)

9. Data management

Data from this project were made freely available to the scientific community. Besides published lists of putative adipo-, myo-, and adipomyokines derived from cross species analysis (Schering et al. 2015, primary data were appropriately deposited and partly described in open access publications:

- *Data of bovine adipose tissue transcriptomes:*

NCBI GEO database Acc. #: GSE39006

- *Data of bovine muscle transcriptomes:*

Albrecht et al., Genom Data 7 (2016) 109-111; NCBI GEO database Acc. #: GSE75348
Komolka et al., Genom Data 7 (2016) 131-133; NCBI GEO database Acc. #: GSE75347

- *Data of wildtype and UCP1-tg mice skeletal muscle transcriptomes:*

Ost et al., FASEB J 29 (2015) 1314-1328; NCBI GEO database Acc. #: GSE45991

- *Data of wildtype, FGF21-/-, UCP1-tg, UCP1-tg/FGF21-/- mice adipose tissue transcriptomes:*
Ost et al., Mol Metabol 5 (2016) 79-90; NCBI GEO database Acc. #: GSE71749

10. Press releases and media coverage

Due to the controversial discussion following the initial description of the putative myokine irisin and its potential therapeutic implications articles on this topic were well received by the scientific community. The work done by partner DDZ prior to this project (Raschke et al. 2013) was cited more than 165 times and the article of Albrecht et al. (2015) received more than 95 citations as of March, 2017. Latter article ranks 949th among a total of 197,730 articles monitored by Altmetric and ranks ranked 11th of the 903 tracked articles of a similar age in Scientific Reports.

The article was extensively covered by online-media and scientific news outlets:

Press releases:

- Das „Fitnesshormon“ Irisin ist ein Mythos. Medieninformation, Leibniz-Institut für Nutztierbiologie Dummerstorf, March 17, 2015

- 'Exercise Hormone' Irisin Is More Myth Than Reality. Duke Today, Duke University Durham, USA, March 23, 2015

Covered by:

- Investigating Irisin. The Scientist Magazine®, March 10, 2015

-Irisin, The 'Exercise Hormone' Capable Of Burning Fat, Does Not Exist. Medical Daily, March 10, 2015

- 'Exercise hormone' irisin may be a myth. MNT, March 12, 2015

- 'Exercise Protein' Irisin Fades to Myth — Faulty assays undermine results of dozens of published studies. MedPage Today® and American Association of Clinical Endocrinologists, March 16, 2015

- Treningshormon kan være en myte. Forskning.no, March 16, 2015

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