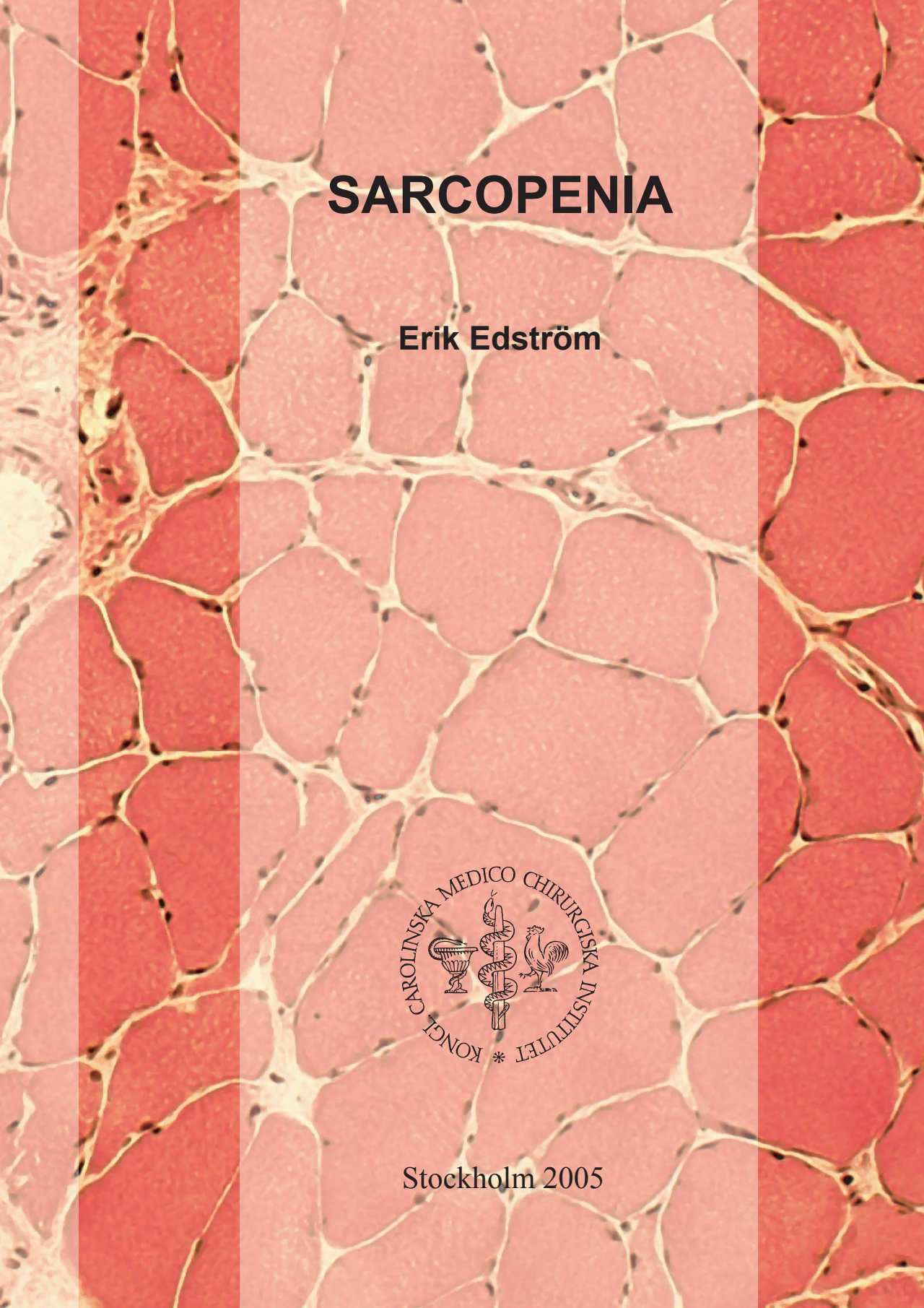
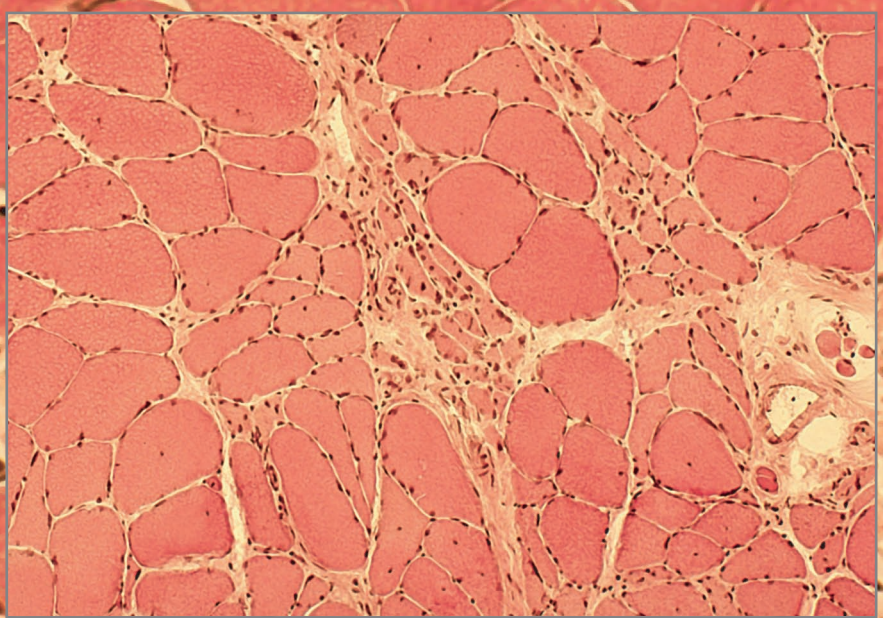


SARCOPENIA

Erik Edström

KONGL CAROLINSKA MEDICO CHIRURGISKA INSTITUTET

Stockholm 2005



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SARCOPENIA

Erik Edström

Stockholm 2005

Cover illustration: Soleus muscle from 30 month-old rats displaying characteristic features of sarcopenic skeletal muscle, including grouped atrophy, connective tissue increase, central nuclei, and angulated fibers. The counterstained pictures were captured at 10X magnification but the inserted picture is shown at 33%. The main picture represents a well preserved rat with discrete histological changes while the insert (back) represents a poorly preserved rat with pronounced behavioral symptoms and marked histological changes. The pictures have been color adjusted for illustration purposes.

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To Susanna

ABSTRACT

Sarcopenia (Greek; sarcos: flesh, and penia: poverty) is the pronounced muscle loss that affects the aging population. Sarcopenia of the leg muscles results in loss of independence and increased morbidity in the elderly. Emphasizing hind limb motor function, this thesis investigates different aspects of aging and sarcopenia in a rodent model.

After characterizing sarcopenia in a rodent model, the long standing notion that sarcopenia results from reduced IGF-1 signaling was addressed. Sarcopenia was not associated with lack of IGF-1 signaling components; instead the data indicate a regenerative phenotype, which was most pronounced in the most sarcopenic cases. These cases not only expressed high levels of myogenic transcription factors related to the activation, proliferation and maturation of muscle stem cells (satellite cells), but also large numbers of fibers expressing embryonic myosin showing that the initial steps of a regenerative program are activated.

To identify mechanisms underlying these changes, the proteomic and genomic profile of sarcopenia was studied. A multitude of adaptations occur in skeletal muscle with aging; these changes are consistent with a model of compensatory regenerative activity where oxidative stress, disturbed innervation and DNA damage are likely to contribute to the imbalance between buildup and breakdown of skeletal muscle.

These results were extended by testing if a signaling pathway common to muscular atrophy induced by a variety of conditions such as denervation, disuse and disease, was activated in sarcopenia as well. This pathway regulates Atrogin-1/MAFbx and MuRF1, which are ubiquitin ligases involved in ubiquitination steps necessary for proteasomal degradation of myofibrillar proteins during atrophy. Atrogin-1/MAFbx and MuRF1 transcripts were reduced in sarcopenia and the changes could be explained by changes in the levels and activity of signaling components (PI3K, AKT, FOXO) regulating their expression. This lead to the conclusion that sarcopenia is not to equate with muscle atrophy induced by denervation, disuse or disease. Furthermore, the data suggest that Shc may be involved in this signaling. Shc is strategically involved in several pathways of relevance for aging, including the signaling pathways of IGF-1, the nerve growth factor (NGF) family of neurotrophins and the GDNF family. The ShcA p66 isoform affords cells sensitivity to oxidative stress, and genetic removal of SchA p66 results in a prolonged lifespan. Increased levels of ShcA (all isoforms), suggest an amplified trophic and mitogenic signaling, which may reflect a response to oxidative stress as well as repair processes in sarcopenia.

Pursuing the data indicating disturbed innervation, GDNF signaling components were studied in sarcopenia. Reflecting the interdependency of nerve and muscle, a number of signals are exchanged at the neuromuscular junction. GDNF provides trophic support to motoneurons and is active in the establishment and maturation of neuromuscular connections. The results suggest a compensatory increase in the muscle to nerve GDNF signaling during aging, which may help to explain why motor neurons are fairly well preserved in senescence despite the gradual loss of target muscle with advancing age. However, in contrast to young adult denervated skeletal muscle, aged skeletal muscle becomes depleted of GDNF protein in spite of the transcriptional upregulation. A key event in restoring function to regenerated muscle is reestablishment of innervation, which is necessary for the switch from embryonic to adult myosin isoforms. Thus, data previously interpreted as resulting solely from denervation, may also reflect the failing reinnervation of regenerated fibers.

LIST OF PUBLICATIONS

I **Erik Edström** and Brun Ulfhake. Sarcopenia is not due to lack of regenerative drive in senescent skeletal muscle. Aging Cell 2005, 4:65-77.

II Mikael Altun, **Erik Edström**, Eric Spooner, Amilcar Flores-Moralez, Esbjörn Bergman, Petra Tollet-Egnell, Gunnar Norstedt, Benedikt M. Kessler and Brun Ulfhake. Proteomic and genomic profiling of aged rat skeletal muscle. Submitted

III Xiaogang Jiang, **Erik Edström**, Mikael Altun and Brun Ulfhake. Differential regulation of Shc adapter proteins in skeletal muscle, spinal cord and forebrain of aged rats with sensorimotor impairment. Aging Cell, 2003, 2(1):47-58

IV **Erik Edström**, Mikael Altun, Martin Hägglund, Brun Ulfhake. Atrogin-1/MAFbx and MuRF1 are downregulated in aging related loss of skeletal muscle. Submitted

V Ming, Y., Bergman, E., **Edström, E**. and Ulfhake, B. Evidence for increased GDNF signaling in aged sensory and motor neurons. NeuroReport, 1999, 10:1529-1535.

VI **Erik Edström,** Andreas Fahlström, Gustavo Paratcha, Brun Ulfhake. GDNF, GFRα1 and NCAM in sarcopenic and denervated skeletal muscle. Manuscript

CONTENTS

ABBREVIATIONS

2DE	Two-dimensional electrophoresis
ABC	Avidin biotin complex
AKT	Protein kinase B
ANOVA	Analysis of variance
Atrogin-1	Atrophy gene 1 (a.k.a. MAFbx)
CGRP	Calcitonin gene related peptide
CR	Caloric restriction
DAB	Diaminobenzidine
DR	Dietary restriction
ECF	Enhanced chemifluorescence
ECL	Enhanced chemiluminescence
FGF	Fibroblast growth factor
FGFR	FGF receptor
FITC	Fluorescein iso-thiocyanate
FOXO	Forkhead box O
GAB	GRB2 associated binding protein
GAP43	Growth associated protein 43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell line derived neurotrophic factor
GFR	GDNF family receptor
GH	Growth hormone
GHBP	GH binding protein
GHR	GH receptor
GRB2	Growth factor receptor bound protein-2
HGF	Hepatocyte growth factor
HRP	Horse radish peroxidase
IGF-1	Insulin-like growth factor
IGF-1R	IGF-1 Receptor
IL-6	Interleukin-6
IR	Insulin receptor
IRS	Insulin receptor substrate
LC	Liquid chromatography
LIF	Leukemia inhibitory factor
LIFT	Laser induced fragmentation technology
MAFbx	Muscle atrophy F-box (a.k.a. Atrogin-1)
MALDI	Matrix assisted laser desorption/ionisation
MAPK	Mitogen activated protein kinase
MHC	Myosin heavy chain
MPC	Muscle precursor cell
MS	Mass spectrometry
MuRF1	Muscle specific ring finger-1
NCAM	Neural cell adhesion molecule
NEPHGE	Non-equilibrium pH-gradient SDS-PAGE
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PMF	Peptide mass fingerprinting
PVDF	Polyvinylidene difluorid
QTOF	Quadrupole TOF
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT	Reverse transcription
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
Shc	Src homology 2 domain containing
SI	Sarcopenia index
SOD	Superoxide dismutase
TBS	Tris buffered saline
TIFF	Tagged image file format
TOF	Time of flight
UPP	Ubiquitin proteasome pathway

1 INTRODUCTION

1.1 SARCOPENIA AND AGING

Sarcopenia (Greek, sarcos: flesh, and penia: poverty) is the pronounced loss of skeletal muscle that affects the aging population. Sarcopenia of the leg muscles results in loss of independence and increased morbidity in the elderly. Emphasizing hind limb motor function, this thesis investigates aspects of aging and sarcopenia in a rodent model.

Standardized criteria for diagnosis of sarcopenia have not been established (Lauretani, Russo et al. 2003). Nevertheless, several estimates of the prevalence of sarcopenia and proportion of muscle lost with aging have been performed (recently reviewed in Karakelides and Sreekumaran Nair 2005). The prevalence of sarcopenia increases with age (Baumgartner, Koehler et al. 1998; Melton, Khosla et al. 2000), and age related muscle loss, progressing at an approximate rate of 5% per decade, seems to begin already in the fourth decade of life (Short, Vittone et al. 2003). Loss of muscle mass is accompanied by a strength loss that exceeds what would be expected from the reduced muscle mass. Thus, aging results not only in a loss of muscle mass, but has a detrimental effect on the quality of the remaining skeletal muscle as well (Frontera, Suh et al. 2000). The reduced functional muscle mass (amount and quality) is associated with increased morbidity (Evans 1995; Jette and Jette 1997). Thus, sarcopenia and poor walking ability in the elderly is associated with the dreaded fall related injuries often resulting in hip fractures and hospitalization (Evans 1995; Kinney 2004). However, it is worth noting that disability increases with sarcopenia also independently of morbidity (Baumgartner, Koehler et al. 1998).

Sarcopenia is part of aging, and to study sarcopenia one must engage in a parallel study of aging. Aging is a poorly understood phenomenon; in fact, there is no consensus on why or how we grow old. Instead, there are a number of theories, and to provide a backdrop to the subsequent discussion of sarcopenia, these theories will be briefly outlined below.

1.1.1 Aging theories

Ever since symbiotic bacteria assumed the function of mitochondria within eukaryotic cells, oxygen has been necessary for cellular respiration (Martin and Russell 2003; Searcy 2003). Oxidative stress, mediated via reactive oxygen species (ROS), is the by-product of our cells' dependence on the highly reactive oxygen molecule. Oxidative stress can damage DNA, proteins and lipids, and represents the canonical "wear and tear" theory of aging, often referred to as "the free radical theory of aging" (reviewed in Beckman and Ames 1998; Finkel and Holbrook 2000).

Cellular damage inflicted by ROS, is postulated to accumulate over time, and to result in the gradual breakdown of cellular functions in senescence (reviewed in Beckman

and Ames 1998; Finkel and Holbrook 2000). While, mitochondrial oxidative phosphorylation provides most of the ROS in the body, oxidants are also generated by several other enzymatic reactions, and may themselves serve important functions. For example, in inflammatory reactions, immune cells use targeted release of ROS to attack invading microorganisms (Beckman and Ames 1998). Since ROS are a necessary part of cellular functions, several systems have evolved to safeguard against oxidative damage. Catalase, superoxide dismutase and glutathione peroxidase convert ROS to less reactive molecules. However, ROS seems to interact with cellular signaling pathways, and several signaling molecules, such as the p66 isoform of ShcA, reportedly respond to oxidative stress levels (reviewed in Beckman and Ames 1998; Finkel and Holbrook 2000). In fact, among the life-span regulating genes discovered in the nematode *C. elegans* (see below), a common feature seems to be that they have an impact on the organism's sensitivity to stressors such as ROS (reviewed in Finkel and Holbrook 2000).

While wear and tear theories focus on possible detrimental effects of interaction with the environment, other theories focus on the endogenous regulation of life processes. Genes predetermine embryonal development, postnatal growth, puberty and along the same line may dictate aging and maximum life-span. Thus, these theories require genes or genetic programs to operate as timers, independently or in interaction with environmental cues.

At the ends of chromosomes are repetitive stretches of noncoding DNA, telomeres, that are thought to serve as protective buffers for the gene-carrying regions of the chromosomal DNA (reviewed in e.g. Ahmed and Tollefsbol 2001; Chang 2005). Telomeres become shortened with cell division but can be maintained or elongated by the enzyme telomerase. However, the transcription of telomerase ceases in most cells already during embryogenesis (reviewed in e.g. Ahmed and Tollefsbol 2001). Thus, telomeres will become shorter with each successive cell division and telomere shortening has been suggested to be the definitive life-span limiting timer (reviewed in Ahmed and Tollefsbol 2001; Chang 2005). Available evidence suggests that this may well be true when discussing cellular senescence, but the importance of telomeres in determining life span at the organism level remains to be shown. In fact, when following successive generations of telomerase deficient mice, no effect on longevity could be seen until the sixth generation (Rudolph, Chang et al. 1999). Thus, during a normal life-span it is probably safe to assume that the individual dies long before cells cease to replicate due to telomere shortening. Furthermore, in the case of sarcopenia no telomere shortening can be detected (Renault, Thornell et al. 2002).

1.1.1.1 Insights from studies on Caenorhabditis elegans

The intriguing finding that DAF-2, a homologue of the mammalian insulin/IGF-1 receptor, regulates lifespan in the nematode *C. elegans* (Kenyon, Chang et al. 1993; see also Kimura, Tissenbaum et al. 1997), provided a new perspective on IGF-1 actions in senescence (reviewed in Guarente and Kenyon 2000; Wolkow, Kimura et al. 2000). Spurred by this novel concept, subsequent research efforts have identified a larger set of molecules influencing life span. Many of these molecules are related to the IGF-1 signaling pathway.

The putative DAF-2 ligand, called Ceinsulin, has been identified and is described as a hybrid between insulin and IGF-1(Kawano, Ito et al. 2000). Mutants with reduced DAF-2 or Ceinsulin function have an increased longevity, even beyond twice the normal lifespan (Kenyon, Chang et al. 1993; Kawano, Ito et al. 2000).

AGE-1/PI-3-kinase, AKT and DAF-16 (homologous to mammalian forkhead box O, FOXO) were also found to be part of this life span regulating pathway in *C. elegans* (Friedman and Johnson 1988; Morris, Tissenbaum et al. 1996; Ogg, Paradis et al. 1997; Lin, Hsin et al. 2001; Wolkow, Munoz et al. 2002). AGE-1/PI-3-kinase signals to AKT, which can phosporylate DAF-16 at four phosphorylation sites, to induce its exclusion from the nucleus (Lin, Hsin et al. 2001). The transcription factor DAF-16, was found necessary for DAF-2 to have an effect on lifespan (Kenyon, Chang et al. 1993). Collectively, the *C. elegans* data provided a model where insulin/IGF-1 signaling via DAF-2, inhibits DAF-16 to induce normal maturation into adulthood and in consequence limits life-span (Riddle and Albert 1997). Thus, insulin/IGF-1 signaling, related to aging also in mammals (reviewed in Kenyon 2001; Kenyon 2005), seems to illustrate the theory of "antagonistic pleiotropy" (Williams 1957), which suggests that factors that are beneficial in youth become detrimental in adulthood (reviewed in Clark 2004; Leroi, Bartke et al. 2005).

1.1.2 Current concepts of sarcopenia

1.1.2.1 Histological changes and neurogenic origin of sarcopenia

Sarcopenic skeletal muscle is characterized by a dominance of slow type fibers (type I), loss of fiber type fidelity (hybrid fibers), fiber atrophy that often occurs in clusters (grouped atrophy), and fiber type grouping (Fig. 1, Gutman and Hanzlikova 1972; Tomonaga 1977; Larsson 1978; Caccia, Harris et al. 1979; Ansved and Larsson 1990; Klitgaard, Zhou et al. 1990; Lexell 1995; Porter, Vandervoort et al. 1995; Andersen, Terzis et al. 1999; Welle, Bhatt et al. 2000; Williamson, Godard et al. 2000; Balagopal, Schimke et al. 2001). Commonly, these aging-related changes have been attributed to an impaired innervation (sarcopenia etiologies reviewed in e.g.: Greenlund and Nair 2003; Marcell 2003).

The loss of muscle fibers in conjunction with fiber type grouping has provided the rational for the theory that sarcopenia is of neurogenic origin, caused by loss of spinal motoneurons (Gutman and Hanzlikova 1972; Larsson 1995). However, the data on motoneuron loss underlying these conclusions has been questioned based on methodological uncertainties, and awaiting unbiased approaches to motoneuron counting this issue remains unresolved. Nevertheless, available numbers suggest that the age related loss of neurons is too small (10-20%) to account for muscle loss (Johnson, Mossberg et al. 1995; Johnson and Duberley 1998). In addition, there seems to be no relation between motor neuron numbers and behavioral disturbances (Johnson, Mossberg et al. 1995). Aged motoneurons have a preserved cholinergic phenotype and cell body size (Johnson, Mossberg et al. 1995; Johnson, Hökfelt et al. 1999), but also upregulated CGRP and GAP43, a pattern typical of growth and regeneration (Johnson, Mossberg et al. 1995). Thus, motoneurons may be preserved, but lacking in functional connectivity with their target muscle fibers. Possibly reflecting poor neuromuscular connectivity, aged

motoneurons are less successful than young motoneurons at reinnervating a target muscle (Marsh, Criswell et al. 1997). Moreover, disturbed innervation, can be a peripheral process primarily involving the motor axon's capacity to maintain myofiber innervation or to innervate regenerating myofibers (See Cowen et al., in Dyck and Thomas 2005).

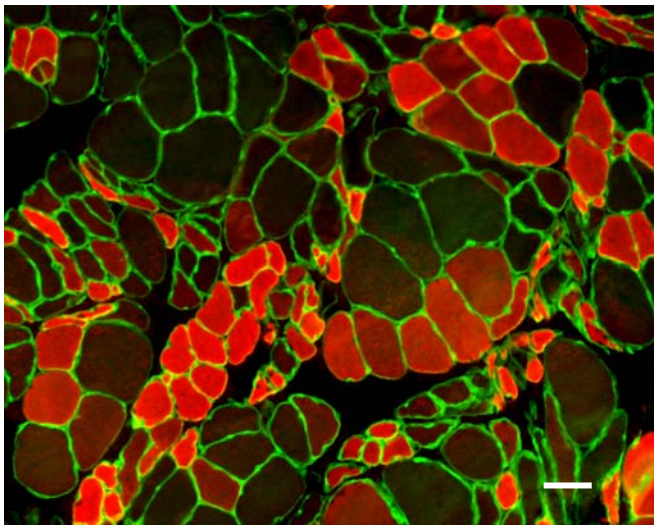


Fig. 1

Soleus muscle from sarcopenic 30 month-old rat with pronounced behavioral motor impairment. The transverse section, double stained with antibodies against laminin (green) and fast type MHC (red), provides typical examples of fiber type grouping and grouped atrophy. Scale bar indicates 50μm.

Senescence is associated with dystrophy of peripheral nerve axons. Interestingly, the distribution pattern of the axonal changes coincides with that of behavioral motor deficits. Namely, aging-related axon lesions are more prevalent in ventral roots and peripheral nerves of the lumbar than the cervical spinal cord (Johnson, Mossberg et al. 1995, and references therein), and more severe distally than proximally (Sharma, Bajada et al. 1980; Krinke 1983). In this context collateral reinnervation has been proposed to work as a rescue mechanism, where less severely affected motor units increase in size to prevent loss of abandoned muscle fibers (Caccia, Harris et al. 1979; Pestronk, Drachman et al. 1980). Support of this hypothesis is also found in the grouping of fibers (Fig. 1), seen in aged muscle (Tomonaga 1977; Caccia, Harris et al. 1979; Ansved and Larsson 1990).

Local growth factor signaling is needed in skeletal muscle adaptation to challenges such as altered load, denervation or experimental damage (McMahon and Priestley 1995; Fu and Gordon 1997). Growth factors are cytokines that play critical roles in governing cell survival, differentiation and growth during development (Barde 1989). Mature tissues and cells remain dependent on growth factors for adaptive changes, maintenance and regeneration. An important event in the transition of new fibers to a mature functional phenotype is to establish neuromuscular connectivity. This involves interactions between neuron, terminal Schwann cell and muscle; the signaling from the muscle to attract innervation is yet not fully understood but involves neurotrophic factors such as the glial cell line derived neurotrophic factor, GDNF. GDNF signals via its cognate receptor GFRα1 (GDNF receptor α1) and c-Ret (Jing, Wen et al. 1996; Trupp, Arenas et al. 1996), to support motor neurons (Henderson, Phillips et al. 1994; Zurn, Baetge et al. 1994; Li, Wu et al. 1995).

Since adult skeletal muscle fibers contain post-mitotic and terminally differentiated cells, growth factors are needed to activate quiescent satellite cells (muscle stem cells) to a proliferate state. Several growth factors such as IGF-1, hepatocyte growth factor (HGF),

fibroblast growth factor (FGF), interleukin-6 (IL-6) and leukemia inhibitory factor (LIF), have been shown to stimulate satellite cell proliferation (reviewed by Hawke and Garry 2001). Among these, IGF-1 has attracted special attention since it is involved in regulation of precursor cells, myofibers, muscle metabolism, protein turnover and also induces intramuscular nerve sprouting (Caroni and Grandes 1990). Frequently sarcopenia has been explained as the result of an inadequate stimulus to maintain muscle tissue during aging. Somatopause, with reduced circulating levels of growth hormone (GH) and liver produced insulin-like growth factor-1 (IGF-1), has a long standing acceptance as an explanation for the failure to sustain muscle mass in senescence.

1.1.2.2 Somatopause

The growth hormone axis comprises the hypothalamic growth hormone releasing hormone, which drives the pituitary release of growth hormone (GH) into the circulation. GH acts on specific receptors (GHR/GHBP) in peripheral tissues (reviewed in Kostyo and Reagan 1976; Carter-Su, Schwartz et al. 1996). One of the main targets is the liver, which responds through release of insulin-like growth factor (IGF-1) into the circulation (reviewed in e.g. Laron 1982; Li 1982). The age related reduction of circulating GH and, consequently, of liver produced IGF-1 has been referred to as the "somatopause". Based on the importance of the GH-axis for body and skeletal muscle growth (reviewed in Florini, Ewton et al. 1996), somatopause has been associated with many of the symptoms that accompany aging, and foremost with sarcopenia (reviewed in e.g. Binnerts, Deurenberg et al. 1992; Lamberts 2000; Toogood 2004). Numerous clinical trials have been undertaken in an effort to counteract somatopause and sarcopenia of aging. However, extrapolating data from *C. elegans* (see above), suggests that such therapies will fail, and the large number of studies performed in clinical settings support this conclusion; GH-secretagogues, GH and IGF-1 in systemic monotherapies or in different combinations have all been tested, and although pharmacologically successful, these studies have generally produced side effects (see e.g. Cohn, Feller et al. 1993), and failed to achieve the desired effects on skeletal muscle function (see also Papadakis, Grady et al. 1996; reviewed in Lieberman and Hoffman 1997; Kamel, Maas et al. 2002; Merriam, Schwartz et al. 2003).

1.1.2.3 Muscle protein turnover

Body proteins undergo a continuous buildup and breakdown, and turnover of total protein in the human (70kg male) amounts to about 280g per day (reviewed in Mitch and Goldberg 1996). Consequently, small changes in rates of synthesis or degradation will quickly result in a manifest loss or gain of protein. Skeletal muscle can serve as an amino acid reservoir and provide substrates for energy metabolism and protein synthesis needs (Tawa and Goldberg 1994). Loss of skeletal muscle mass is a clinically important problem in disease as well as in normal aging (Gutman and Hanzlikova 1972; Rosenberg 1997; Jagoe and Goldberg 2001; Attaix, Mosoni et al. 2005; Glass 2005). Sarcopenia implies atrophy and loss of muscle fibers, and consequently much attention has been directed towards the protein degradation machineries, which include the endocytic cathepsins, calpains and the

ubiquitin-proteasome pathway, UPP (reviewed in Attaix, Combaret et al. 2001; Jagoe and Goldberg 2001; Glass 2003; Attaix, Ventadour et al. 2005). There is little evidence to support a role for the endocytic pathway in the breakdown of myofibrilar proteins; instead the endosomal cathepsins seem be involved in degradation of membrane proteins, including receptors, ligands, channels, and transporters (reviewed by Turk, Turk et al. 2001; Jackman and Kandarian 2004). The calpains may serve key roles in myofibrillar disassembly, in making these proteins available for subsequent UPP-mediated degradation (Jackman and Kandarian 2004). The UPP is activated in catabolic states associated with atrophy (Mitch and Goldberg 1996), and recently two muscle specific ubiquitin ligases, atrogin-1/MAFbx and MuRF1, were identified and cloned based on their association with disease, disuse and denervation induced muscle atrophies (Bodine, Latres et al. 2001; McElhinny, Kakinuma et al. 2002; Sandri, Sandri et al. 2004). Studies of the UPP in aged skeletal muscle have, however, not given clear evidence for an increased specific activity (Attaix, Mosoni et al. 2005).

1.1.2.4 Thesis strategy

In summary, a number of questions in sarcopenia remain unanswered. This thesis represents an effort to address some of the key hypotheses in sarcopenia. To enable this, sarcopenia was characterized in a rodent model; the characterization included behavioral assessment of the motor performance of animals as well as hind limb muscle and body weights at several time points throughout the rodent life span. The characterization made it possible to subgroup animals; well preserved animals were contrasted against ill-fated animals, to provide an insight into the features of successful and unsuccessful aging. The rodent model also facilitated the study of the sarcopenic phenotype at the transcriptional and translational level: proteomic and gene array tools were used to provide comprehensive information, while immunohistochemistry, western blotting and reverse transcription PCR were used to address more specific questions. To further probe the model, well established interventions based on dietary restriction and muscle denervation were used.

Using the rodent model of sarcopenia and the methodology outlined above this thesis addresses issues related to the IGF-1 hypothesis, denervation and regeneration phenomena in sarcopenia and the regulation of signaling pathways involved in protein degradation.

2 AIMS OF THE STUDY

- To establish a rodent model for sarcopenia, including characterization of
    - the behavioral motor performance of the sarcopenic rodent
    - the histological alterations of sarcopenic skeletal muscle
    - the transcriptional and translational phenotype of sarcopenia
- To examine local aspects of the IGF-1 hypothesis in sarcopenic skeletal muscle
- To test if sarcopenia depends on the same atrophy mechanisms as do disuse, disease and denervation
- To test the hypothesis of compensatory GDNF signaling in the neuromuscular system perturbed by experimental denervation or sarcopenia
- To investigate the potential of dietary restriction to impede sarcopenia, and attenuate age related changes that may influence sarcopenia

3 EXPERIMENTAL PROCEDURES

3.1 ANIMAL MODEL

Sprague-Dawley rats (strain Bkl; Harlan Sprauge-Dawley, Houston, TX), were delivered by a local breeder (B&K, Stockholm, Sweden) at two months of age and were thereafter kept in standardized barrier housing conditions, in which the median life span is 30 months (Bergman 1999). Thus, 30 months old rats are herein defined as aged.

All rats were subjected to an extensive behavioral testing protocol, and staged in relation to symptoms of impaired muscle function, according to a previously described protocol (Johnson, Mossberg et al. 1995; Bergman and Ulfhake 1998). In paper I the parts of this testing protocol that deals with the motor function of the animals is described in detail (Edstrom and Ulfhake 2005). In paper I animals were dichotomized based on symptoms: animals were classified as having no or only minimal behavioral impairments (low symptom group, representing a 'successful' pattern of aging) or severely impaired (high symptom group; representing an unsuccessful pattern of aging), including hind limb muscle atrophy (sarcopenia), adduction insufficiency, ataxia, disturbed gait cycle and signs of muscle paralysis (Johnson, Mossberg et al. 1995). In paper II animals were further subcategorized.

All experiments were approved by the Local Ethical Committee (Stockholms Norra Djurförsöksetiska Nämnd; proj. no. N263/95, N54/00, N90/97 and N122/03) and conducted in accordance with Swedish law and regulations

3.1.1 Sarcopenia index

The relation between body weight and the mass of the postural soleus muscle was used to evaluate the adaptation of hind limb muscle to its weight bearing demands at chosen points during the rodent life span. The ratio, referred to in the text as sarcopenia index (SI), between soleus muscle weight (mg) and whole body weight (g) was created and is presented in fig. 2.

3.1.2 Behavioral testing

As indicated above, all animals used in this thesis were subjected to a standardized set of behavioral tests as well as a global assessment of hind limb locomotor function (Johnson, Mossberg et al. 1995; Bergman and Ulfhake 1998). The tests that were used to evaluate motor performance are summarized below.

Open field activity: Explorative behavior was examined with the open field test (Dorce and Palermo-Neto 1994; Peng, Lin et al. 1994; Drago, Coppi et al. 1996). In dim light, the animal was placed in the center of the arena (70 x 70cm), divided into 25 equally large squares (14 x 14cm), and allowed to freely explore for 180s, during which the following behavioral characteristics were recorded: a) number of squares entered with all

four paws, b) rearing frequency, c) immobility frequency; d) instances of urination and defecation. The last measures (d) were recorded to provide a measure of anxiety level.

Crossing a wire mesh screen: In dim light, the animal was placed at one end of a 70 cm long wire mesh (2.5 cm) screen. A 60W light source was directed to this spot. The "home cage" with litter mates was placed at the other, darker, end of the screen. Each animal was given 90s to cross the screen. Records included distance, time, and number of errors, i.e. instances of misplaced hind paws (slips).

Beam balance: A 2.5cm wide wooden beam was suspended 0.5m above a soft surface. The rat was placed on the beam for a maximum of 60s, and the performance was ranked according to the following scale (Clifton, Jiang et al. 1991): (1) balances with steady posture and keeps its paws on top of the beam; (2) grasps sides of beam and/or has shaky movement; (3) one or more paws slip off beam; (4) attempts to balance on beam but falls; (5) drapes over beam and/or hangs on beam and falls off; (6) falls off beam with no attempt to balance or hang on. Each animal was subjected to three consecutive trials, and the mean score of these trials was calculated.

Walking track analysis: For this test, the animals' feet were immersed in non-toxic acrylic color (fore paws with red and hind paws with black color) and they then had to walk through an 8.5 × 42cm transparent Plexiglas runway with the "home cage" at the other end. High-quality paper was placed on the runway floor and taken out for analysis after the animal had crossed the path. The following records were made from the walking tracks: (a) stride length; (b) gait width (distance between left and right hind paws), (c) placement of hind paw relative to fore paw (distance between hind paw-fore paw in each step cycle).

Righting response: The rat was held in the examiner's hand approximately 30 cm above a soft surface, and the righting reflex was elicited by quickly turning the rat over on its back. The rat's attempt to right itself during the drop was studied and a score of 2 was given if the animal showed a normal righting response, i.e. counter to the roll direction; a score of 1 was given if the righting response was weak, delayed or in the direction of the roll; a score of 0 indicated no righting attempt (Gale, Kerasidis et al. 1985; von Euler, Akesson et al. 1996).

3.2 SURGICAL PROCEDURES AND TISSUE COLLECTION

For tissue removal, chloral hydrate (300 mg/kg i.p.) was used to induce deep anesthesia. Before tissue removal, body weights were recorded. Triceps surae muscles were quickly dissected out and gastrocnemius and soleus muscles were frozen separately in liquid nitrogen. Special care was taken to remove the soleus muscle from tendon to tendon, for subsequent weighing (see Sarcopenia index above). After decapitation the lower lumbar spinal cord segments, mid-thoracic spinal cord segments and forebrains (corresponding to a hemisection 1.7 to –0.3 mm relative to Bregma, Paxinos 1982) used in paper III, were quickly dissected out and immediately frozen on dry ice or propane cooled by liquid nitrogen for histological or mixed tissue analyses, respectively. In paper VI, ten young adult animals were unilaterally axotomized, which included mid thigh Sciatic nerve

transection and ligation of proximal as well as distal stumps, performed on chloral hydrate anaesthetized animals.

3.3 RNA ANALYSIS

3.3.1 Preparation of RNA

Total RNA was isolated from tissue according to the Trizol protocol (Trizol, GibcoBRL, Life Technologies, Täby, Sweden), using a rotor homogenizer (Ultra-Turrax T8, IKA Labortechnik, Staufen, Germany). RNA amount and purity was measured spectrophotometrically. In papers II, IV and VI, all RNA samples used for real-time PCR were DNAse treated (DNA-free™, Ambion Inc., TX) resulting in OD 260/280 above 1.9.

3.3.2 Reverse Transcription

Reverse transcription was typically conducted in a reaction volume of 10µl containing 25ng of total RNA, 25 units MuLV reverse transcriptase (Perkin Elmer, Applied Biosystems, CA, USA), 2.5µM Oligo d(T)16, 10 units RNase inhibitor (Perkin Elmer), 1mM of each dNTP (dATP, dCTP, dGTP, dTTP), 5mM MgCl2 and 1×PCR Buffer II (Perkin Elmer). The RT-reaction mixture was incubated for 10 minutes at 25°C, brought to 42°C in a GeneAmp 2400 (Perkin Elmer) or PC960G PCR thermal cycler (Corbett Research, Mortlake, Australia) for 15 minutes and finally terminated by 5 minutes incubation at 99°C.

3.3.3 Conventional PCR

3.3.3.1 PCR

Polymerase chain reaction in papers III, V and VI (FGF and FGFR pilot) was carried out by addition of a PCR master mix to the RT-reaction mixture (5µl), yielding a reaction volume of 25µl containing the following components: 0.2µM of each oligonucleotide primer, 5 units AmpliTaq Gold DNA polymerase (Perkin Elmer), 2mM MgCl2, 200µM of each dNTP and 1×PCR Buffer II (Perkin Elmer). Hot start PCR was performed in a GeneAmp PCR system 2400 (Perkin Elmer) and included an initial template denaturation and DNA polymerase activation step at 95°C for 12 minutes.

For each experimental sample in papers III and V, three PCR reactions were prepared and subjected to three different numbers of PCR cycles, to cover a range of 5 cycles. Due to practical limitations (semi-quantification, see below) the pilot study analysis in paper VI was performed at only one chosen number of cycles. The number of cycles used, was chosen according to preceding experiments to ensure that for each combination of primer pairs and tissue, the PCR amplifications were halted within the range of exponential progression. Note that since the abundance of the examined mRNAs varied, the number of PCR cycles used varied accordingly. Each cycle consisted of denaturation at 95°C (15 seconds) and primer annealing/extension at 60°C (30 seconds, with an automatic increment

of 3 seconds/cycle). At the end of the last cycle, the reaction was kept at 72°C for 7 minutes and then brought to 4°C.

In all experiments PCR was performed simultaneously on compared samples, with the internal control (β-Actin, GAPDH or 18SrRNA) run in parallel with the examined mRNAs. All PCR studies included negative controls, where template RNA or reverse transcription was omitted, as well as a positive control to analyze failures during the PCR process (positive control: construct pAW109 and primer set DM151/DM152, Perkin Elmer).

3.3.3.2 Semi-quantification

Ten µl of the PCR reaction product was electrophoretically run on a 1.5% agarose gel containing ethidium bromide. The gels were visualized in a UV-transilluminator and images were captured using an 8-bit CCD camera. Subsequent analysis using Optimas software (Optimas Co., Bothell, WA, USA) included measurement of the integrated gray levels of each band, correction for local background and normalization against internal control levels (β-Actin, GAPDH or 18SrRNA). In papers III and V, the mean of the normalized values for the three cycle points, are presented as the percentage difference of the unit ratio between aged and young adult rats. The pilot study analysis of FGFs and FGF-receptors in paper VI, was done on a larger number of animals requiring the analysis of single reactions (triplicates would exceed the capacity of the electrophoresis equipment) from each case. Saturation problems occasionally occurred when differences between cases or groups were large; when these problems could not be solved by titration of the number of PCR cycles, cycle points that indicated a lack of exponential progression in any sample were disregarded in the quantitative evaluation and comparison of samples.

3.3.4 Realtime PCR

Realtime PCR was carried out on 2µl of the RT product (cDNA transcribed from total RNA), with standard SYBR-green mastermix (Applied Biosystems, Stockholm, Sweden or Qiagen, Crawley, UK) and the appropriate primer pairs in an ABI-Prism 7000 instrument (Applied Biosystems, Stockholm, Sweden). Analysis of results in papers I, III and V relied on the use of β-actin, 18SrRNA or GAPDH as internal controls, respectively, while high purity RNA quantification (RNA purification, see above) was used as single standardization in papers II, IV and VI (see also below: methodological considerations on housekeeping genes).

Realtime analysis of SYBR green chemistry allows relative quantification of template (cDNA) amount through comparison of number of cycles needed to reach a defined signal level, typically the detection threshold. Thus, data for comparison of expression levels are given in numbers of cycles at detection threshold. These cycle numbers represent the exponential growth from cycle to cycle and should be treated as log-values. Normalization to internal controls is consequently carried out through subtraction of their values from the target template cycles at detection threshold.

Correct melting temperature and size of the amplified products were confirmed using melting curves and electrophoresis (semi-quantification, see above), respectively.

The melting temperatures of a given product can be assessed in the ABI prism 7000 instrument through a gradual increase in temperature with concomitant signal detection. SYBR green has intense fluorescence only when DNA products are double stranded. When the melting temperature is reached the strands will dissociate and the signal intensity drops. Consequently, the drop in signal occurs at the melting point allowing confirmation of the same melting curve for all compared products.

3.3.5 Microchip array analysis

Complementary DNA chips were manufactured (CMM, KI) as described previously (Tollet-Egnell, Flores-Morales et al. 2000), from a collection of about 6400 cDNA clones selected from the TIGR Rat GENE Index (www.tigr.org), Research Genetics and an in-house collection.

In all hybridizations, pooled cDNA from aged rats was compared to an equal amount of cDNA from young adults. Each comparison was performed in triplicate to account for technical variability. Fluorescence-labeled cDNA was synthesized with oligo-dT primer (New England Biolabs Inc.) by reverse transcription reaction using Superscript II (Life Technologies Inc.) in the presence of fluorescently labeled (Cy3-UTP and Cy5-UTP) nucleotides (Amersham Pharmacia Biotech). Swapping of dyes was used to control for possible problems associated with efficiency of fluorophore labeling and detection. After hybridization, the chip was washed and dried before scanning with a GMS418 scanner (Affymetrix, CA, USA).

GenePix Pro 5.0 software (Axon Instruments, CA, USA) was used for fluorescent image analysis. The signal of each spot was calculated as the average intensity of the spot minus the background. Spots with intensity that were at least 1.6 times above the background were included in the study (paper II).

Normalization between the two fluorescent images was performed using the "LOWESS" method in the SMA (Statistic for Microarray Analysis) package (Dudoit and Fridlyand 2002; Yang, Dudoit et al. 2002). SMA is an add-on library written in the public domain statistical language R (www.r-project.org). The expression ratio for each group was calculated as the average of replicated determinations. Genes with missing data in more than one of the three triplicates within each group were excluded from further analysis. The identification of genes with significant variation between old and young animals (disregarding the stage) was performed using SAM (significance analysis of microarrays) statistical technique (Tusher, Tibshirani et al. 2001), with a false discovery rate threshold of 5%. This data analysis protocol has been used with the same microchip array in a number of studies, and results have been reproduced with methods such as Northern Blot, RNAse protection and realtime PCR (Flores-Morales, Stahlberg et al. 2001; Tollet-Egnell, Flores-Morales et al. 2001).

3.3.6 Methodological considerations on housekeeping genes

A growing body of evidence suggests that there are no true housekeeping genes (reviewed in Bustin 2000). This poses a problem, since editors and reviewers commonly ask for normalization of data against housekeeping genes. If housekeeping genes are regulated in relation to the experimental situation, normalization will introduce a bias. The normalization procedure used in the microchip gene array experiments allowed analysis of

gene expression patterns in relation to the total mRNA pool rather than in relation to individual "housekeeping genes". The analysis shows regulations of a number of commonly used housekeeping genes, including GAPDH, which was also detected in the 2D protein separation and mass spectrometry analysis (paper II). A number of housekeeping genes have been tested and discarded in relation to aging and sarcopenia. Among the ones tested β-actin has proven to be among those least affected by aging and sarcopenia. Nonetheless, the use of housekeeping genes may introduce systematic errors, and in consequence data from real-time PCR experiments in papers III, IV and VI rely solely on total RNA content for normalization of samples. To ensure the use of equal RNA amounts, RNA quality and integrity was carefully characterized (see Bustin 2000). After homogenization, RNA samples were diluted to equal concentrations based on optical density (OD) measurements. The samples were then treated to remove DNA and protein contaminations that can interfere with quantification, and ODs were remeasured for final dilution. The integrity of the total RNA samples subsequent to this procedure has been established using RNA gel electrophoresis.

3.4 PROTEIN ANALYSIS

3.4.1 Preparation of Protein

For the western blot analysis in paper III, the tissues were homogenized on ice in a 50mM sodium acetate buffer containing 0.1% triton X100, 1mM NaF, 1mM Na_3O_4V and protease inhibitors (Roche Molecular Biochemicals). Debris was removed through centrifugation at 12000g for 15min at 4°C.

For the proteomic analysis in paper II , fresh frozen gastrocnemius muscle was homogenized with one equivalent volume of glass beads (Sigma, Stockholm, Sweden) in Tris lysis buffer (50 mM Tris base pH 7.4, 5 mM $MgCl_2$, 250 mM sucrose, and freshly prepared 2 mM ATP and 1 mM DTT) on ice. Membrane fractions, nuclei, and cell debris were removed by centrifugation at 12,000g for 15 minutes at 4°C and an additional ultracentrifugation of the supernatant at 100,000g for 1 hour at 4°C (Beckman, Fullerton, CA, USA).

For the western blot analysis in papers IV and VI, fresh frozen gastrocnemius muscle tissue was homogenized, on ice, in RIPA buffer (pH 7.4: 50mM Tris, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1mM NaF, 1mM, Na_3O_4V, 1mM PMSF, 10 μg/ml leupeptin, and protease inhibitors (Roche Molecular Biochemicals)). Debris was removed through centrifugation at 12000g for 15min at 4°C.

3.4.2 Quantification

The protein concentrations for papers II-IV and VI were determined by Bradford assay (Bio-Rad, Hercules, CA, USA), using a spectrophotometer (Beckman) at 595 nm and bovine serum albumin diluted in the appropriate buffer for the standard curves.

3.4.3 Western Blot

3.4.3.1 Electrophoresis and blotting

Samples were denatured at 95°C for 5 minutes in a reducing loading buffer containing 5% β-mercaptoethanol. Equal amounts of protein from tissue lysates were separated by SDS-PAGE and transferred onto a PVDF membrane (Amersham Biosciences, Buckinghamshire, UK) and incubated (overnight at 4°C) with primary antibody (Table 1) diluted in 5% milk and 0.1% tween20.

3.4.3.2 Detection and analysis

Detection was performed using the enhanced chemiluminescence (ECL, paper III) or enhanced chemifluorescence detection (ECF, papers II, IV, VI) methods according to the manufacturer's protocols (Amersham Biosciences, Piscataway, NJ, USA). Briefly, the membranes were incubated for 90 minutes with horse radish peroxidase (ECL) or alkaline phosphatase (ECL) conjugated secondary antibody (1:10000; Amersham Biosciences). ECF or ECL-detection reagents (enzyme substrate) were applied during 1 to 5 minutes and the chemifluorescence was detected using a Storm™ fluorescence scanner (Amersham Biosciences), or (paper III) appropriate film and chemicals in a dark room.

Detection of NCAM in paper VI relied on the Odyssey infrared scanner (Li-Cor biotechnology; Westburg, Leusden, The Netherlands), and secondary antibodies conjugated with infrared spectrum fluorophores (Alexa680 conjugated goat anti-rat, 1:10000; Molecular Probes/Invitrogen, Eugene, OR, USA).

Relative protein levels were measured from the resulting scans (TIFF) by densitometric analysis of integrated gray levels corrected for local background, using Optimas software.

3.4.4 Proteomics

3.4.4.1 Sample preparation and two dimensional NEPHGE analysis

For analysis by two-dimensional gel electrophoresis (nonequilibrium pH gradient and SDS-PAGE; NEPHGE), the samples were prepared by adding urea (8 mol/L final concentration) and NEPHGE sample buffer with Pharmacia ampholyte (pH 3.5-9.5). Two-dimensional NEPHGE SDS-PAGE analysis was done as described previously (Kessler, Tortorella et al. 2001). Samples were resolved by 12.5% reducing SDS-PAGE and visualized by silver staining as described (Mortz, Krogh et al. 2001). Relative expression levels of individual spots were analyzed by densitometric analysis of gel scans using PDQuest software (Biorad, Hercules, CA, USA).

3.4.4.2 Protein Identification by Mass Spectrometry

Individual gel spots were excised and subjected to trypsinolysis (Hanna, Sherman et al. 2000). Protein digests were analyzed using a MALDI-TOF/TOF mass spectrometer (Ultraflex, Bruker Datonics, Bremen, Germany). Proteins were identified by peptide mass fingerprinting (PMF), in which MS spectra were searched against Swissprot and NCBInr

using Mascot (Matrixscience, London, UK, Cottrell et al., Electrophoresis 1999) or Protein Prospector (MS-fit, UCSF, USA; http://128.40.158.151/mshome4.0.htm). Unidentified proteins were further analyzed by MALDI-MS/MS (laser induced fragmentation technology, LIFT) and by electrospray LC-MS/MS using a QTOFmicro (Waters, Micromass, Manchester, UK), or a high-capacity iontrap (HCTplus, Bruker Daltonics) tandem mass spectrometer. Individual MS/MS spectra were searched against Swissprot/NCBInr using Mascot. Identification was based on the presence of at least two peptides, Mascot scores above 50 and Protein Prospector above 300 (for PMF).

3.4.5 Methodological considerations on tissue analysis

Measurements of transcriptional and translational changes are performed to provide information about the regulatory state of a cell or tissue. In this thesis work measurements of mRNA and protein levels have been used extensively. In contrast to the histological techniques, the measurements based on PCR, gene arrays, proteomic profiling and western blot are all done on lysates. While histological techniques benefit from interpretational prudence, it is an absolute requirement when analyzing data derived from comparison of lysates.

After homogenization, total mRNA is easily extracted from tissues, and available for further analysis. However, homogenization of the tissue presupposes loss of proportionality to the cellular level, and results from comparison of mRNA levels in different tissue specimens should not be inferred to represent regulatory events occurring in single cells. To understand the changes in individual cells however, is usually the motivation behind analysis of mRNA-levels. Messenger RNAs are blueprints for the synthesis of proteins, but the relationship between changes in levels of mRNA and output of functional proteins is not always straight forward. Pretranslational factors that will affect the proportionality between mRNA and protein levels include the efficiency of mRNA processing, splicing, turnover rate and availability for translation. These factors are generally not controlled for.

On the protein side of things, lysate analysis is closer to the true activity of the cell, or rather, tissue. Still, a number of problems remain. In contrast to mRNA extraction, total tissue protein is not as easily harvested. Proteins in extracellular, cellular and subcellular compartments may require different methodological approaches to become soluble and represented in the lysate. Thus, quantitative differences found between samples may reflect changes in a protein's tissue or cell distribution, rather than changes in amount of protein in the tissue.

Finally, a general issue of relevance to all comparisons of lysates, and yet to be resolved, is the problem of normalization. To ensure correct comparison of samples they should somehow be adjusted to ensure comparability. Thus, preparation and analysis of samples is standardized in different ways: equal tissue amounts or equal amounts of mRNA or protein are compared and in many instances this is accompanied by the use of "house keeping genes", genes which are supposed to be unresponsive to changes affecting the target genes. House keeping genes serve as internal controls and data are adjusted relative to their levels. This may seem like a sound approach, and it is indeed widely used, but it is not foolproof (see above, methodological considerations on housekeeping genes). Since

different approaches produce different results, one must choose the method best suited to the experimental conditions (i.e. producing the least bias). In this thesis work different house keeping genes have been used as well as analyses based solely on comparison of equal amounts.

When comparing equal amounts of mRNA or protein, one must accept a certain, unknown, loss in precision of the measurement. For a single mRNA or protein target (species) to be measured as increased in one sample compared to others, one must accept that all other, unchanged, mRNA or protein species will be reduced. If they were not, one would no longer be comparing equal amounts of sample. When one considers concomitant increases and decreases of species in a sample, it becomes obvious that the larger the proportion of regulated species the more likely it is that they may represent the aforementioned "equal amount" effects.

To summarize, when comparing equal total amounts of mRNA or protein from tissues the sum of all changes must be zero at the level of individual nucleotides (for mRNA comparisons) or amino acids (for protein comparisons), since nucleotides or amino acids are what the initial measurements of amounts are based on.

3.5 IMMUNOHISTOCHEMISTRY AND COUNTERSTAINING

3.5.1 Tissue preparation

The soleus muscles of adult (4-month-old) and aged (30-month-old) rats were transversely sectioned in a cryostat and thawed onto gelatin/chrome-alun coated slides. All sections except those used for embryonic MHC quantification (ABC-technique, see below), were then processed according to the indirect immunofluorescence technique.

3.5.2 Immunohistochemistries

3.5.2.1 Fluorescence based techniques

After drying at room temperature, the sections were rehydrated in PBS and then incubated for 18-72 hours at 4˚C in a humid chamber with the primary antibodies diluted in PBS containing Triton-X100 (0.3%). After incubation with the primary antibodies (Table 1), the sections were rinsed in PBS, transferred to a humid chamber and incubated at 37˚C for 30 minutes with FITC- or Rhodamine-conjugated goat anti-rabbit or goat anti-mouse (1:100; Jackson Immuno Research Laboratories Inc., West Grove, USA) antibodies. Counterstaining for nuclei was performed by immersion (5 min) in 0.001% propidium iodide (Sigma Aldrich) in distilled water, and subsequent rinsing in distilled water. The sections were mounted in glycerol/PBS (3:1) containing 1,4-Diazabicyclo[2,2,2]octane in order to retard fading.

3.5.2.2 HRP conjugated secondaries and the ABC technique

For quantification of MHCe positive fibers (paper I) the non-fluorescent Vectastain elite® avidin-biotin complex (ABC, Vector Laboratories Inc., Burlingame, USA) technique with diaminobenzidine (DAB, Sigma, Saint Louis, USA) was used. The protocol was run

according to the (above) standard protocol up to the addition of the secondary antibody. A biotin conjugated donkey-anti mouse secondary antibody was used (1.5h incubation at RT). During this time the ABC components (vial A and B of the kit) containing horseradish peroxidase (HRP) were mixed (both diluted 1:500) and left at room temperature until application (1.5h incubation at RT) to the sections after secondary antibody incubation and rinsing. Detection was subsequently performed through preincubation of the rinsed sections with DAB solution (10mg tablet / 25 ml solution; Sigma, Saint Louis, USA) for 5min followed by incubation using DAB solution with H_2O_2 to catalyze the conversion by HRP of DAB to an insoluble detectable product. After rinsing, the sections were dehydrated in a series of alcohol dilutions and mounted with Entellan® (Merck, Darmstadt, Germany).

Table 1

Primary antisera used for immunohistochemistry and western blot

Host	Antigen (catalogue nr)	Usage	Dilution	Supplier
Rabbit	AKT (9272)	WB	1:1000	Cell Signaling
Rabbit	Cu/ZnSOD/SOD1 (SOD-101)	WB	1:5000	Stressgen Biotechnology Inc
Mouse	ED1/CD68 (MCA341)	IHC	1:500	Serotec Ltd
Mouse	ED2/CD163 (MCA342)	IHC	1:500	Serotec Ltd
Rabbit	FOXO1 (9462)	WB	1:1000	Cell Signaling
Rabbit	FOXO4 (9472)	WB	1:1000	Cell Signaling
Rabbit	GAB1(06-579)	WB	1:1000	Upstate Biotechnology
Rabbit	GAB2 (06-967)	WB	1:1000	Upstate Biotechnology
Rabbit	GDNF	WB	1:1000	Professor Carlos Ibañes
Rabbit	GFRα1	WB	1:1000	Professor Carlos Ibañes
Rabbit	Grb2 (sc-255)	WB	1:1000	Santa Cruz Biotechnology
Rabbit	IGF-1Rβ (3027)	WB	1:1000	Cell Signaling
Rabbit	IRβ (3025)	WB	1:1000	Cell Signaling
Rabbit	Laminin (L9393)	IHC	1:800	Sigma
Mouse	MHC Embryonic isoform (NCL-MHCd)	IHC	1:50	Novocastra Laboratories Ltd
Mouse	MHC Fast type isoform (NCL-MHCf)	IHC	1:50	Novocastra Laboratories Ltd
Mouse	MHC Slow type isoform (NCL-MHCs)	IHC	1:10	Novocastra Laboratories Ltd
Rabbit	MnSOD/SOD2 (SOD-111)	WB	1:5000	Stressgen Biotechnology Inc
Rat	NCAM(MAB310)	WB	1:1000	Chemicon
Rabbit	p(ser256/ser193)-FOXO1/4 (9461)	WB	1:1000	Cell Signaling
Rabbit	p(ser473)-AKT (9271)	WB	1:1000	Cell Signaling
Rabbit	PI3K p85 (06-195)	WB	1:1000	Upstate Biotechnology
Rabbit	ShcA (06-203)	WB	1:1000	Upstate Biotechnology
Rabbit	ShcA (06-203)	WB	1:1000	Upstate Biotechnology

Abbreviations: IHC, immunohistochemistry; WB, western blot. **Supplier details**, Cell Signaling (Beverly, MA, USA), Chemicon (Temecula, CA, USA), Novocastra Laboratories Ltd (Newcastle uponTyne, UK), Santa Cruz Biotechnology (Santa Cruz, CA, USA), Sigma (Saint Louis, USA), Serotec Ltd (Oxford, UK), Upstate Biotechnology (Lake Placid, NY, USA), Stressgen Biotechnology Inc (San Diego, CA, USA),

3.5.3 Microscopy and Imaging

The immunofluorescence tissue sections were examined with a Nikon Microphot-FX epifluorescence microscope (×10/0.45 and ×20/0.75 dry planapochromate objectives) equipped with the proper filters for FITC, Rhodamine or propidium iodide-fluorescence. Images were captured with a Nikon DXM1200 or with a Bio-Rad radiance plus confocal microscope (×10/0.45 or ×20/0.75 dry planapochromate objectives or X60/1.4 oil immersion planapochromate objective).

For MHCe quantification, overview images of whole soleus muscle cross sections were captured using a ×2/0.1 planapochromate objective. The overviews were used as templates and opened in Illustrator 10 (Adobe). Immunopositive fibers were then identified, confirmed microscopically and marked in the software to allow summation when all fibers had been identified.

Negative control experiments where primary antibodies were omitted were routinely performed and made it possible to detect unspecific secondary antibody signals.

3.5.4 Considerations on the histological analysis

Histological examination is performed on transversely or longitudinally sectioned skeletal muscle, which is then stained to visualize general or specific morphological features. Counterstains such as hematoxylin and eosin staining (H&E) is commonly used to provide a general staining of the tissue, and allows for the identification of many typical features of senescent skeletal muscle. To address changes in fiber types however, requires the use of enzyme based methods such as myosin ATPase or Succinate dehydrogenase staining, or antibodies which can differentiate isoforms of myosin heavy chains. Both approaches are valid and commonly used, but due to the more quantitative nature of enzyme based techniques, and more qualitative nature of antibody based techniques, they do not produce identical results. In this thesis, the antibody-based approach has been used. Antibodies specific for fast, slow and developmental (embryonic) myosin heavy chain isoforms have been used.

Histological examination of skeletal muscle sections includes the search for and identification of changes, to separate the abnormal from the normal, the pathological from the non-pathological. Reducing the need for complex descriptions and thus simplifying the transfer of information, histological changes are typically described with a certain nomenclature.

However, nomenclature carries with it inherent problems. Nomenclature helps us to group, classify and simplify only when we accept a certain loss of information quality, or descriptive resolution. In addition, histological changes are often associated with specific pathologies, and although the knowledge of underlying events may be limited, the specific terms tend to become associated with the etiology as well. As a consequence, etiological hypotheses can be carried by nomenclature onto new situations with histopathological similarity. Nomenclature then provides the basis for grouping different situations into the same etiological category, even when the etiology is only hypothesized.

In the case of skeletal muscle however, there seems to be a limited response repertoire, and hallmark features of certain pathologies occur also in instances when the etiology seems to be completely different.

3.6 STATISTICS

Statistics were performed using Prostat (Poly Software International, Pearl River, NY) and Statistica 6.1 (Statsoft, Tulsa USA). Comparisons of groups were carried out with parametric or nonparametric testing depending on the data type. Interval and ratio variables with an approximately normal distribution (sarcopenia index, realtime PCR and western blot results and MHCe fiber counts) were tested using ANOVA (analysis of variance), and when significant differences were found, Bonferroni's post hoc test was used for pair-wise comparisons. Log-transformation was performed when needed to meet the criteria of the ANOVA method.

Ordinal data (behavior analysis data) were tested using Kruskal Wallis nonparametric analysis of variance. When the initial test was significant, pair wise comparisons using Statistica's multiple comparisons were performed (comparison of mean ranks, Siegel 1988). Statistical significance levels were set to: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

In papers III and V, the PCR and WB data are presented as the percentage difference of the unit ratio between aged and young adult rats. In the evaluation of the results, arbitrary limits were set such that differences of >100% were considered significant and >50%-100% were considered as tendencies. This approach was chosen since pooled control samples (young adult) were used, resulting in loss of variance information and consequently the possibility to perform true statistical testing.

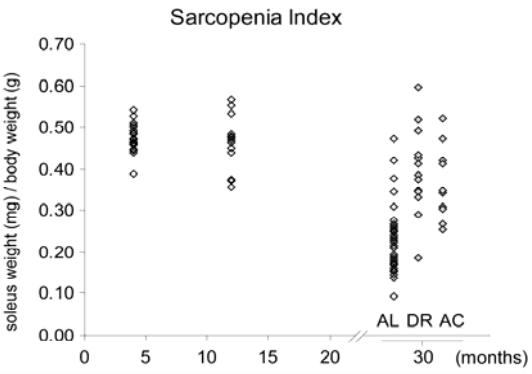
4 RESULTS AND DISCUSSION

4.1 THE RODENT MODEL, SARCOPENIA AND BEHAVIOR

Combined with other aspects of human aging, sarcopenia results in clinically relevant behavioral impairments, which result in disability and increased morbidity (Evans 1995; Jette and Jette 1997). Our data show that rats also become sarcopenic and behaviorally impaired in senescence. In fact, the results indicate a strong association between degree of sarcopenia and the extent of motor behavior disturbances (paper I).

The sarcopenia index (Fig. 2) illustrates the failure to maintain muscle mass in relation to body weight in the aged animals. Most likely reflecting the relatively rapid growth of young animals, the SI remains relatively low during adolescent development, up to an age of 3-4 months after which it appears to be stable over at least the first year of life (paper I). This observation stresses the importance of a multiple time point design in studies of aging and sarcopenia

Our results show that the rodent model of sarcopenia shares strong similarity with available data on the human counterpart (Dutta and Hadley 1995; Jette and Jette 1997; Rosenberg 1997; Roubenoff and Castaneda 2001), and should therefore be a useful model for experimental research on aging-related loss of skeletal muscle tissue.



Sarcopenia Index

	Category	SI	SI-SD	n
1	4mo AL	0.48	0.03	20
2	12mo AL	0.46	0.06	14
3	30mo AL	0.23	0.08	35
4	30mo DR	0.40	0.11	13
5	30mo AC	0.37	0.09	10

ANOVA

	2	3	4	5
1	ns	***	*	**
2		***	ns	*
3			***	***
4				ns

Fig. 2

The sarcopenia index has proven useful in its simplicity, relating the weight of the postural soleus muscle to the weight it supports. Abbreviations: mo, months of age; AL, *ad libitum* food intake; DR, dietary restriction (70% of AL); AC, activity cage, increased activity through improved housing and treadmill access. Aged animals with unrestricted access to food and no incentive for activity display a dramatic reduction in the sarcopenia index. Modified from Edstrom et al., submitted.

4.2 HISTOLOGY OF AGED SKELETAL MUSCLE

In line with the vast literature on changes in skeletal muscle with aging, the soleus muscles studied here showed numerous histological alterations including fiber type grouping, type I fiber dominance, isoform hybrid fibers, a wide distribution of fiber sizes, angulated fibers, rounded fibers, accumulation of nuclei and centrally located nuclei. These changes seem to relate more to the degree of motor impairment of the animal than the chronological age.

Thus, changes described as age-related are most prominent in unsuccessfully aged animals and may be very discrete or even lacking in successfully aged animals.

Fiber type grouping is a well characterized phenomenon of senescent muscle (Fig. 1), and is generally interpreted as a reflection of partial denervation and compensatory reinnervation by sprouting of adjacent motor axons (Gutman and Hanzlikova 1972; Larsson 1995; Lexell 1995). However, selective preservation of type I fibers and fiber type grouping is also seen in experimental denervation without reinnervation (Borisov, Dedkov et al. 2001), which suggests that these phenomena can occur without collateral sprouting and reinnervation. Instead, many of the phenomena seen in aged muscle may be interpreted as secondary to regenerative activity. This hypothesis was substantiated by the analysis of embryonic myosin in sarcopenic skeletal muscle (paper I). In sarcopenic skeletal muscle groups of small as well as large embryonic myosin positive fibers could be seen. This interpretation also suggested that activation and proliferation of muscle precursor cells (satellite cells and their progeny) could explain the increased numbers of myonuclei seen in sarcopenic skeletal muscle (paper I, Gallegly, Turesky et al. 2004). In line with these data, this (paper I) and other studies have provided evidence that aged sarcopenic muscle is not just passively slipping away but shows signs of regeneration, such as increased expression of myogenic regulatory factors (papers I and II, Musaro, Cusella De Angelis et al. 1995; Dedkov, Kostrominova et al. 2003), transcription factors essential for the development and regeneration of skeletal muscle (reviewed in Ludolph and Konieczny 1995; Sabourin and Rudnicki 2000).

4.3 THE TRANSCRIPTIONAL AND TRANSLATIONAL PHENOTYPE OF SARCOPENIA

To further characterize the rat model, including the shift in tissue phenotype described above, the proteomic and genomic phenotype of sarcopenia was investigated (paper II). Comparative two dimensional electrophoresis (2DE) coupled with mass spectrometry was used to identify skeletal muscle proteins that were differentially regulated between young adult and aged animals. In parallel, the transcriptional differences were investigated with a microchip gene array approach. A large number of proteins and transcripts were differentially regulated, and included changes in the protein synthesis and degradation machineries, transcripts of muscle-specific proteins and transcription factors, decreased levels of energy producing enzymes, signs of increased DNA repair and a response to oxidative stress (paper II). In agreement with other transcriptional profiles (reviewed in Weindruch, Kayo et al. 2001; Park and Prolla 2005), sarcopenic skeletal muscle seems to be in a compensatory mode attempting to counteract stressors which may include oxidative stress, impaired neuromuscular connectivity and DNA damage.

The loss of muscle mass that characterizes sarcopenia continues despite the compensatory and regenerative efforts, suggesting that the regenerative machinery fails in senescence. Due to the post-mitotic nature of skeletal muscle fibers, muscle regeneration includes recruitment and proliferation of muscle precursor cells (MPC), which must mature and fuse into myotubes and subsequently undergo an innervation dependent final

differentiation. Since the most sarcopenic muscles contained the largest numbers of fibers expressing embryonic myosin, which can be maintained without innervation, our findings suggest that part of the regeneration failure may be due to impaired innervation of newly formed fibers (Edstrom and Ulfhake 2005).

4.4 THE IGF-1 HYPOTHESIS OF SARCOPENIA

Growth factors are critical for muscle cell survival, differentiation and growth during development (Hawke and Garry 2001). Mature tissues and cells remain dependent on growth factors for adaptive changes, maintenance and regeneration. As indicated above, MPCs must be recruited, when mature skeletal muscle responds to challenges such as overload, denervation or experimental damage to the muscle. In these situations, changes in the local environment, including the production of growth factors, activate quiescent satellite cells to a proliferate state. Several growth factors such as IGF-1, hepatocyte growth factor (HGF), fibroblast growth factor (FGF) interleukin-6 (IL-6), leukemia inhibitory factor (LIF), have been shown to stimulate satellite cell proliferation (reviewed by Hawke and Garry 2001). Among these, IGF-1 has attracted special attention since it can act on satellite cells and myofibers to regulate muscle growth, metabolism and protein turnover. In addition, IGF-1 can induce intramuscular nerve sprouting (Caroni and Grandes 1990).

Somatopause, with reduced circulating levels of growth hormone (GH) and liver produced insulin-like growth factor-1 (IGF-1), has gained wide support as an explanation for the failure to sustain muscle mass in senescence. However, several animal models as well as clinical trials with humans, have found no role for systemic GH-IGF-1 in the shaping of the sarcopenic phenotype. Mutant mice instead support the negative impact of the GH-IGF-1 axis on longevity. Mutants lacking the GH receptor are long lived (Coschigano, Holland et al. 2003), as are dwarf mice, with pituitary deficiencies and low GH and IGF-1 levels (Brown-Borg, Borg et al. 1996; Flurkey, Papaconstantinou et al. 2002). In the context of sarcopenia, interest has instead come to focus on auto- and paracrine IGF-1 signaling. To test local aspects of the IGF-1 hypothesis, the transcriptional regulations of IGF-1 and its receptor (IGF-1R) were analyzed in skeletal muscle (Paper I). No reduction in expression of IGF-1 or its receptor, could be detected in senescent skeletal muscle (Hamilton, Marsh et al. 1995; Edstrom and Ulfhake 2005). In fact, among individuals with a less successful pattern of aging, local IGF-1 expression was significantly increased (paper I).

However, data from mice over-expressing IGF-1 isoforms in skeletal muscle, clearly demonstrate the potential of IGF-1 (Barton-Davis, Shoturma et al. 1998; Musaro, McCullagh et al. 2001). These animals display muscle hypertrophy that counteracts sarcopenia as the animals grow old. An interpretation to reconcile these data with those from the clinical setting is that increased myotrophic and neurotrophic support can produce a better starting point and consequently postpone or reduce the impact of mechanisms driving sarcopenia.

4.5 ShcA-p66 AND LIFE-SPAN DETERMINING GENES IN SARCOPENIA

As mentioned in the introduction, members of the IGF signaling pathway are well conserved in evolution and seem to be involved in basic mechanisms of life span regulation, in simple organisms and mammals alike (reviewed in Kenyon 2005). The IGF-1R belongs to the large group of receptor tyrosine kinases (RTKs). Through RTK-signaling, growth factors can influence protein and gene expression. As discovered when testing local correlates of the IGF-1 hypothesis in sarcopenic skeletal muscle, transcript levels of the IGF-1R were found to increase, and to explore these data further we investigated signaling components coupled to the IGF-1R.

Adapter proteins typically mediate activation pathways downstream of RTKs. The Shc proteins represent one important family of adapter proteins. Of the three Shc genes, ShcA is ubiquitously expressed and found in muscle (Pelicci, Dente et al. 1996). The ShcA gene encodes 3 transcripts (p52Shc, p46Shc and p66Shc, Pelicci, Dente et al. 1996; Migliaccio, Giorgio et al. 1999).

While ShcA-p46/p52 is associated with the proliferation promoting Ras-MAPK cascade, the p66 isoform has been shown to effectively inhibit this pathway, which is a necessary step to allow for terminal differentiation (Migliaccio, Mele et al. 1997; Okada, Kao et al. 1997; Natalicchio, Laviola et al. 2004). In addition ShcA-p66 is important for the cellular response to oxidative stress (Migliaccio, Giorgio et al. 1999), and although the mechanisms are yet to be fully elucidated, this mechanism involves phosphorylation of FOXO transcription factors (Nemoto and Finkel 2002). Mice lacking the p66 isoform of ShcA have an increased life span and increased resistance to environmental ROS (reviewed in Purdom and Chen 2003). All isoforms of ShcA, including p66, were found to be upregulated in aged skeletal muscle (Papers III and IV). Thus, the increased levels of ShcA may represent a response to oxidative stress, but may also reflect a compensatory role in growth factor signaling (Ravichandran 2001; Natalicchio, Laviola et al. 2004).

In summary, ShcA may be faced with a difficult task in sarcopenia. Regeneration requires proliferation as well as differentiation, and while ShcA-p46/52 may be involved in the recruitment and proliferation of satellite cells, ShcA-p66 may limit proliferation, perhaps in response to oxidative stress, to allow for differentiation (Migliaccio, Mele et al. 1997; Okada, Kao et al. 1997; Natalicchio, Laviola et al. 2004). Since the underlying data are derived from mixed tissue samples, it is impossible to know if these processes occur in single cells or represent different events taking place in separate cells. Nonetheless, it is tempting to speculate that the ShcA regulation reflects what may be at the core of sarcopenia: the need for regeneration in a non-permitting environment.

4.6 SARCOPENIA AND ATROPHY IN DISUSE, DISEASE AND DENERVATION

As stated in the introduction, sarcopenia implies atrophy and loss of muscle fibers. In skeletal muscle the UPP is responsible for degradation of the vast majority of proteins, including those of the contractile apparatus (reviewed in Attaix, Combaret et al. 2001; Jagoe and Goldberg 2001; Glass 2003; Attaix, Ventadour et al. 2005). The UPP consists of

a series of events initiated by the ubiquitination of proteins. Ubiquitin is a small protein cofactor (reviewed in Hershko and Ciechanover 1992; Ciechanover 1994), which is activated by E1 enzymes that subsequently transfer it to carrier proteins termed E2 proteins. Ubiquitin protein ligases, termed E3 ligases, then couple the activated carboxyl end of ubiquitin to lysines of the target protein (reviewed in Mitch and Goldberg 1996). Repeating this process results in the formation of ubiquitin chains that are recognized by the degradation machinery, the large multi-subunit proteolytic complex referred to as the proteasome (Mitch and Goldberg 1996). In most atrophy models studied, *in vitro* rates of ubiquitin conjugation are increased (Lecker, Solomon et al. 1999; Jagoe, Lecker et al. 2002; Stevenson, Giresi et al. 2003; Lecker, Jagoe et al. 2004). The E3 ligases are one of the largest functional families of mammalian proteins, and provide specificity to the UPP (Mitch and Goldberg 1996). However, data on the specificity of E3 ligases are scarce. The muscle specific ubiquitin ligases, Atrogin-1/MAFbx and MuRF1 are dramatically upregulated in atrophy caused by disease, disuse as well as denervation (Bodine, Latres et al. 2001; McElhinny, Kakinuma et al. 2002; Sandri, Sandri et al. 2004). In sarcopenia available evidence on the role of the UPP as well as Atrogin-1/MAFbx and MuRF1 are conflicting (Attaix, Mosoni et al. 2005).

Atrogin-1/MAFbx and MuRF1 are regulated by IGF-1 signaling, mediated via PI3K and AKT (Stitt, Drujan et al. 2004; Latres, Amini et al. 2005; Schulze, Fang et al. 2005; Skurk, Izumiya et al. 2005). In skeletal muscle PI3K-AKT can be activated by insulin or insulin-like growth IGF-1, signaling via their cognate receptors (IR and IGF-1R, respectively), and their subsequent interaction with either IRS-1 or the Shc-GRB2-GAB adaptor protein pathway (Gu, Maeda et al. 2000; Ravichandran 2001; Glass 2003).

While, IGF-1R and ShcA proteins were found to increase in sarcopenic skeletal muscle, little data support a role for increased IR or insulin receptor substrate (IRS-1) signaling in sarcopenia (paper IV, Carvalho, Brenelli et al. 1996; Arias, Gosselin et al. 2001; Lima, Ueno et al. 2002; Zhu, de Cabo et al. 2004). Consequently the levels of GRB2-GAB were analyzed and found to increase, suggesting a role in signaling from Shc to PI3K (paper IV, see also Gu, Maeda et al. 2000).

Increased activation of AKT results in phosphorylation and inactivation of FOXO (reviewed in Van Der Heide, Hoekman et al. 2004), and reduced Atrogin-1/MAFbx and MuRF1 levels (Bodine, Latres et al. 2001; Sacheck, Ohtsuka et al. 2004; Sandri, Sandri et al. 2004; Stitt, Drujan et al. 2004). Based on the transcriptional regulation of FOXOs, where only FOXO4 seemed to correlate with sarcopenia (paper IV), and the proposed relative importance of this factor in skeletal muscle (Schulze, Fang et al. 2005), focus was placed on protein content and phosphorylation status of FOXO4. FOXOs are inactivated by phosphorylation at three sites, and ser193 seems to be the key site for exclusion of FOXO4 from the nucleus (Brownawell, Kops et al. 2001; Rena, Prescott et al. 2001; Zhang, Gan et al. 2002). Increased FOXO4 phosphorylation at this site suggests its inactivation in sarcopenia, which would explain the reduced transcript levels of Atrogin-1/MAFbx (paper IV). Thus, we conclude that the loss of muscle mass in sarcopenia occurs through a mechanism that is different from that of atrophies induced by disease, disuse and denervation. In agreement with the regenerative phenotype, there is little evidence to

suggest an orderly disassembly of the contractile machinery in senescence. An alternative explanation that has gained some experimental support is that muscle fibers are lost through apoptosis (Whitman, Wacker et al. 2005).

4.7 GDNF SIGNALING IN SARCOPENIA

Thus, the provided evidence suggest a model where regeneration and blocked atrophy signaling characterize the sarcopenic phenotype; the data also indicate that oxidative stress may cause some of the aging-related damage, but does not explain why regeneration is failing.

While neuron loss is too small to account for the massive loss of skeletal muscle seen in sarcopenia (reviewed in Ulfhake, Bergman et al. 2000), senescent motoneurons may suffer an impaired capacity to maintain and reestablish myofiber innervation (See Cowen et al., in Dyck and Thomas 2005). Electromyographic analyses indicate an age-related decrease in the number of active motor units (Campbell, McComas et al. 1973; Brooks and Faulkner 1988), while low-threshold motor units increase in size (Sperling 1980). This is also supported by data based on glycogen depletion techniques, finding increased innervation ratios, and larger motor unit territories (reviewed in Larsson 1995). Based on EMG amplitudes and fiber densities, about a fourth of the motoneurons have been estimated nonfunctional (Stalberg and Fawcett 1982).

An important event in regeneration is the transition of newly formed immature fibers to a mature functional phenotype. This transition requires innervation, which involves the neuron, terminal Schwann cell and myotubes; the signaling from the muscle to attract innervation is not yet fully understood, but involves neurotrophic factors such as the glial cell line derived neurotrophic factor, GDNF. GDNF signals via its cognate receptor GFRα1 (GDNF receptor α1) and c-Ret (Jing, Wen et al. 1996; Trupp, Arenas et al. 1996), to support motor neurons (Henderson, Phillips et al. 1994; Zurn, Baetge et al. 1994; Li, Wu et al. 1995). To investigate the trophic support to the motoneuron, GDNF signaling components were analyzed in sarcopenia. Transcript levels of GDNF were found to be dramatically increased in sarcopenic skeletal muscle (Paper V). Coupled with a parallel increase in motoneuron receptor expression, these data suggest an increased GDNF signaling from muscle to neuron in aging (paper V, Bergman, Kullberg et al. 1999).

The importance of GDNF in senescence is emphasized by the transcriptional downregulation of other factors that support motoneurons, such as of the nerve growth factor (NGF) family of neurotrophins (Ming, Bergman et al. 1999) and fibroblast growth factor family (paper VI).

In seeking further support of this hypothesis, sarcopenic and denervated animals were compared. Although both displayed a dramatic transcriptional upregulation of GDNF, only the young animals were able to maintain their GDNF protein levels (paper VI). Aged sarcopenic animals failed to maintain their GDNF protein levels, suggesting increased removal or turnover of GDNF (paper VI). The transcriptional regulation GFRα1 was also different between sarcopenic and denervated muscles. In denervation GDNF and GFRα1 are co-upregulated, suggesting a possible bias towards signaling *in trans*. In the sarcopenic

situation, where GFRα1 was not upregulated, the protein data indicate the possibility that GFRα1 is provided by the motoneuron, suggesting signaling *in cis*.

The cell surface adhesion molecule NCAM, offers yet another possibility. NCAM, can act as an alternative signaling component for GDNF (Paratcha, Ledda et al. 2003), and in contrast to c-Ret, NCAM is abundantly expressed in skeletal muscle: on satellite cells and myofibers as well as the Schwann cells and terminal axons at the neuromuscular junction, NMJ (Covault and Sanes 1985). NCAM levels were increased not only in denervated and sarcopenic skeletal muscle (paper VI), but also in successfully adapting muscle (contralateral to denervation in adult and in aged DR animals; see paper VI).

Thus, in light of the data on increased GDNF in exercise (Wehrwein, Roskelley et al. 2002), polymyositis and muscular dystrophy (Suzuki, Hase et al. 1998), our findings support a model of autocrine GDNF action (Yang and Nelson 2004), where GDNF and GFRα1 may interact locally with NCAM in supporting skeletal muscle regeneration.

4.8 THE BENEFIT OF DIETARY RESTRICTION IN SARCOPENIA

Dietary restriction (DR) has established itself firmly in aging research as one of the most reproducible interventions to extend life-span across a range different species (McCay, Crowell et al. 1935; reviewed in Kirkwood and Shanley 2005). In this thesis, dietary restriction made it possible to differentiate aging per se (chronological), from degree of sarcopenia and motor disturbances (biological aging). In the majority of investigations, including those in this thesis (papers IV and VI), DR has been successful in attenuating or even normalizing the effects of aging. This is an important finding, since increased life-span without increased well-being, at least in a human population may create more problems than it solves. DR reduces insulin and IGF-1 levels (Dhahbi, Mote et al. 2001), and may mediate its effect via an impact of the longevity signaling pathways previously discussed. Alternatively, the effects may be mediated via altered metabolism and the resulting reduction in ROS generation (Gredilla and Barja 2005). A third concept suggests that DR acts as a low level stressor, which induces compensatory mechanisms and increases the organism's resistance to more harmful stressors (Masoro 2005). Our data suggest that DR attenuates the enhanced IGF-1 signaling drive as well as altered expression of oxidative stress responsive genes in sarcopenia. Since metabolism is the main source of ROS, these theories may in fact represent different perspectives on the same underlying biology.

4.9 CONCLUSIONS

Rodent sarcopenia as investigated in this thesis bears strong similarity to available data on the human counterpart (Dutta and Hadley 1995; Jette and Jette 1997; Rosenberg 1997; Roubenoff and Castaneda 2001), and has proven to be a useful model for experimental research on aging-related loss of skeletal muscle tissue.

The large variability in degree of sarcopenia and behavioral impairments between same age animals living in a standardized environment emphasizes the importance of the genetic background (paper I). Thus, genetic make up probably serves a fundamental role in determining the pace and extent of sarcopenia, while environmental influences and epigenetic modifications represent modulators in this process (cf. Mori 1997).

A recurring theme in sarcopenia research is the rejuvenation therapies based on treating senescent hormonal paucities. Clinical studies targeting the GH-IGF-1 axis, sex steroids and adrenal sex steroid precursors seem to provide the same results: manifest sarcopenia does not respond to increased "supportive" signaling (Borst 2004; Karakelides and Sreekumaran Nair 2005). This thesis has investigated aspects of somatopause in sarcopenia, and found no support for deficiencies of IGF-1 or its receptor in skeletal muscle.

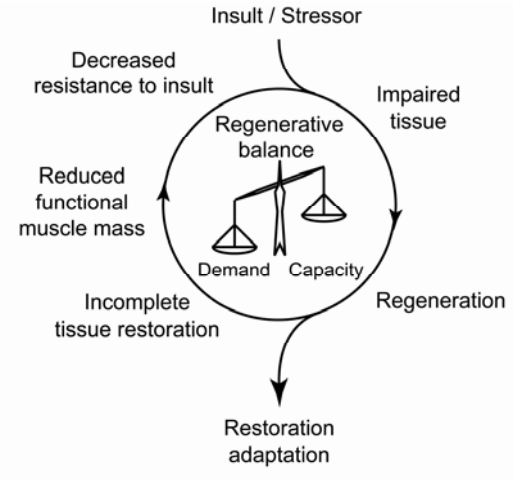


Fig. 3.

The importance of balance between regenerative demand and capacity in the progression of sarcopenia is illustrated in a schematic representation of the regenerative outcome after an insult. Failure to adapt in response to a stressor or insult and consequently to restore the functional capacity will produce a more vulnerable tissue, which produces an impaired tissue with an increased vulnerability.

Instead, driven by local growth factor signaling, sarcopenic skeletal muscle seems to expend its reserves in trying to regenerate. This signaling also seems to inhibit the machinery which normally disposes of myofibrillar proteins in states of muscle atrophy.

Sarcopenic skeletal muscle seems to accumulate immature fibers, perhaps as a result of failure to make the final innervation-dependent transition to the mature phenotype. In this context GDNF has been investigated, and although mRNA levels were found to be dramatically upregulated, there was a net loss of protein. Thus the data accumulated in this thesis tell of a failing struggle, not lack of effort.

In conclusion, sarcopenic skeletal muscle seems trapped in a state of continuous repair. Sarcopenia progresses in spite of a prominent regenerative activity, in cases with an unsuccessful pattern of aging. The combined effect of fibers in a state of degeneration, regeneration or maturation implies a functionally impaired muscle with a decreased resistance to insult (Renault, Piron-Hamelin et al. 2000). This vulnerability can perhaps partly explain why the regenerative activity is out-matched by a counteracting breakdown of muscle tissue in cases with pronounced sarcopenia.

Thus, regenerative activity in sarcopenic muscle is part of a downward spiral, initiated by an imbalance between tissue breakdown and tissue regeneration (Fig. 3). As a consequence of this process, an increasing proportion of the total number of muscle fibers are degenerating or regenerating rather than being in a functional state. The remaining

intact fibers have, according to this scheme, to take-on increased demands and the imbalance is compounded (Fig. 3).

But there is hope. While dietary interventions seem to improve longevity and impede sarcopenia through an organism effect, physical activity is the preferred stimulus for skeletal muscle and exercise interventions in the elderly have shown dramatic effects (Fig. 2, reviewed in Borst 2004). However, the importance of genetic make up in determining sarcopenia suggests that future efforts should be directed towards identifying genes that underlie unsuccessful aging and sarcopenia. Thus, based on identification of the most susceptible individuals, targeted measures may be taken to minimize the effects of sarcopenia in the population.

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