TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl und Poliklinik für Präventive und Rehabilitative Sportmedizin

Exercise Associated Genes and
Their Involvement in the Prevention of Colon Cancer –
A Voluntary Running Experiment in a Rat Model

Katja Bühlmeyer

Kein Weg ist zu lang für den, der langsam

und ohne Eile vorwärts schreitet

Jean de La Bruyère

To my family…

and my uncle, who lost his fight.

Table of contents

List of Figures

List of Tables

1 Introduction

Colon cancer is the third most common cancer worldwide in both men and women. Possible causes of colon cancer are for example tobacco consumption and excessive alcohol consumption. Additional influencing factors are the level of physical activity and nutrition or energy intake. Moderate physical activity can reduce the risk of colon cancer and an increase in exercise volume can further enhance the risk reduction. Obesity also seems to increase the risk of developing colon cancer and this association seems to be stronger in men. Not only the energy amount but also variations in nutrient composition of the diet are known to influence the risk of colon cancer. Consumption of vegetables and fruits is correlated with a decreased risk of colon cancer whereas high intake of processed and/or red meat has been shown to increase the risk. Therefore the American Cancer Society has set guidelines for cancer prevention with a focus on the improvement of lifestyle particularly with regard to exercise and nutrition (American Cancer Society, 2007).

Apart from lifestyle-related factors the risk of colorectal cancer increases with age. About 90% of the patients are 50 years or older. Hereditary factors are also known to affect the development of colon cancer. Genetic mutations of familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) are linked to an increased risk of colorectal cancer. Risk factors are also family or personal histories of colorectal cancer or polyps and a personal history of chronic inflammatory bowel disease (American Cancer Society, 2007).

The frequency of colorectal tumour cases varies greatly between developed countries and e.g. India. The development of colon cancer is highly influenced by the environment as it has been shown that after immigration the incidence of colon cancer reaches the values of the host country. This correlation might reflect the influence of nutritional aspects present in a particular country. In addition to varied societies and altered genetic background economic factors need to be considered as well. One has to take into account that preventive strategies are very expensive. Appropriate health care is much too cost-intensive for people who live below the poverty line. For example in the US 11% Whites, 24% African Americans and 23% Hispanics/Latinos live below the poverty line. If we compare this with the incidence rate of tumours it does become apparent that not only genetic factors but also the economic situation plays an important role in the development of e.g. colon cancer.

African Americans for example have an increased incidence and mortality rate of colon cancer (American Cancer Society, 2007).

1.1 Colorectal tumorigenesis

There are several well-known genetic events, which are involved in the development of colorectal cancers. It is a stepwise process whereas each step involves genetic alterations, which ultimately lead to the progression of normal cells into malignant cells. Almost two decades ago Fearon and Vogelstein (1990) proposed a model for colorectal tumorigenesis which has been widely accepted and confirmed. Since then a defined sequence of genetic alterations in the process of colorectal tumour development is known. **Figure 1** gives an overview of colorectal tumorigenesis: it describes the progression of colon cancer with its different stages and molecular abnormalities.
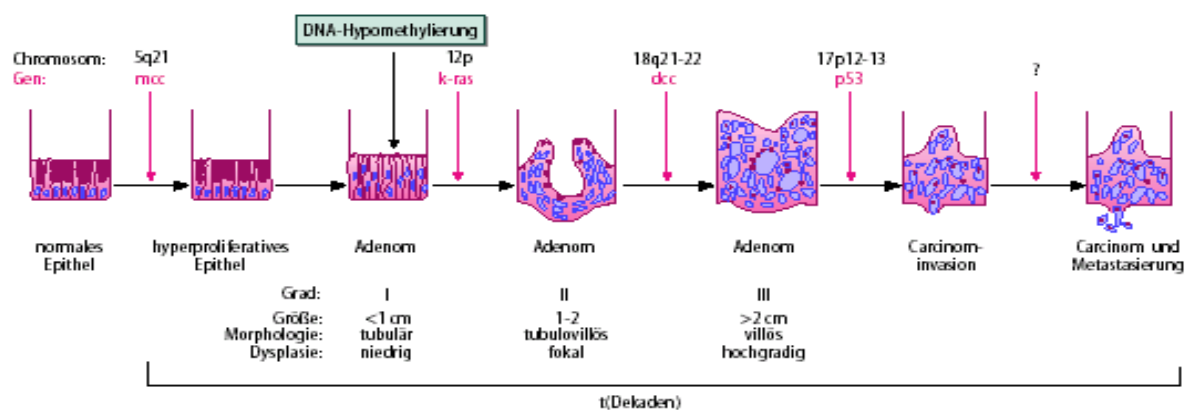
Figure 1: Progression of colorectal tumorigenesis (Loeffler and Pedrides, 2003); © with kind permission of Springer Science+Business Media

In addition to these established risk genes at least four pathways have been shown to be involved in colon cancer progression: Wnt signalling pathway, K-ras pathway, p53 pathway and the TGF-β pathway (Kinzler and Vogelstein, 1996).

An early tumour suppressor gene which affects the development of colon cancer is the gene mutated in colorectal cancer (mcc) (Greenwald et al., 1995). Further events in the early stages of colon cancer progression are hypomethylation and mutations of k-ras, an oncogene. DNA global hypomethylation is frequently seen in early adenomas. During progression of the disease DNA methyltransferase activity is enhanced and there is an increase in the occurrence of hypermethylated sites

(Hernandez-Blazquez et al., 2000). Changes in the methylation status might be responsible for instability of the genome which is also affected by the disorganization of the chromatin compaction (Hernandez-Blazquez et al., 2000). K-ras mutations are found in 50% of colorectal cancers in the early stages. The resulting protein activates the MAP (mitogen-activated) kinase.

A key function in the initial phase of colon cancer development has the APC tumour-suppressor gene which is inactivated in over 80% of colorectal tumours (Tejpar and Van Cutsem, 2002). APC prevents the accumulation of catenins, molecules associated with cancer development (Su et al., 1993, Rubinfeld et al., 1993). Loss of its function leads to abnormal crypt homeostasis. APC has been suggested to be the gatekeeper for colorectal development. It might have a role in cell migration, maturation and death and therefore multiple theories of how this protein might contribute to colorectal tumorigenesis have been proposed. The protein is located in the upper part of the crypts of the colon (Tejpar and Van Cutsem, 2002). A loss of function leads to an increase of catenins. The latter can lead to the transcription of the oncogenes c-*myc* and cyclin DI. As mentioned above, APC regulates the cellular levels of β-catenin, which functions as a mediator in the Wnt (= secreted glycoprotein) signalling pathway. Wnts act as ligands and mediate gene expression and cell fate together with other pathways (FGF-pathway, TGF-β-pathway). Wnt indirectly decreases the degradation of β-catenin which binds to TCF/LEF resulting in the expression of several target genes, e.g.: c-*myc*, cyclin DI, metalloproteinase-7, nuclear-receptor PPARdelta, T (brachyury), homeobox proteins engrailed-2, siamois, and twinned (Tejpar and Van Cutsem, 2002). In the normal adult colon tissue the Wnt signalling pathway should be suppressed. APC is furthermore discussed to be involved in chromosomal instability (CIN). A loss of the function seems to promote CIN (Tejpar and Van Cutsem, 2002).

Two genes, which are involved in the later processes of colon cancer development, are DCC (Deleted in colorectal cancer) and p53. The exact order might vary (Tejpar and Van Cutsem, 2002). P53 mutations are found in nearly all colorectal tumours. The protein might induce the conversion of a benign to a malignant stage **(Fig. 1).**

In order to maintain homeostasis of the colorectal tissue, genes regulating the cellular proliferation, differentiation and cell death have to act in concert. The normal adult colon presumably contains 5×10^{10} epithelial cells of which one third to one sixth is shed into the lumen every day. Enterocytes are supplied continuously from

stem cells located in the base of the crypt. Normally stem cells divide asymmetrically so that they produce one stem cell and one migrating and differentiating daughter cell. Under stress conditions symmetrical divisions can occur producing two daughter stem cells. This event leads to an increase in the stem cell population and might contribute to tumour growth (Tejpar and Van Cutsem, 2002). Beside genetic alterations, an inherent genetic instability (resulting in fast accumulation of cancerous mutations) occurs in colorectal cancers. The reasons for this are chromosomal instability (found in 80% of colorectal cancers) or microsatellite instability (Tejpar and Van Cutsem, 2002).

Hereditary non-polyposis colorectal cancer (HNPCC) and some sporadic colorectal cancers show Microsatellite Instability (MIN) instead of CIN. This results in an accumulation of somatic mutations after loss of the cell's DNA repair mechanisms (Chang et al., 2001, Schmutte and Fishel, 1999). Predominantly genes with a repetitive sequence (microsatellite) within a coding region are affected (Markowitz et al., 1995).

In about 5-10% of colorectal cancers hereditary mutations are the cause. The most common ones are HNPCC and familial adenomatous polyposis (FAP). HNPCC is caused by a germ-line mutation in a mismatch repair (MMR) gene and FAP is an autosomal dominant disorder with germ-line mutations in the APC gene (Tejpar and Van Cutsem, 2002).

1.2   Epidemiological findings – physical activity and colon cancer prevention

Currently, there is a large number of epidemiological studies (more than a hundred) regarding the positive effects of physical activity on cancer prevention (Lee, 2003). For example Friedenreich and Orenstein (2002) presented a review article incorporating nearly 170 observational epidemiological studies. They found convincing evidence for the positive effects of physical activity on the risk reduction of colon cancer. This type of cancer shows the most convincing epidemiological evidence with an average risk reduction of about 40-50%. 43 of the 51 considered studies showed a reduction in the risk of developing colon cancer with increased physical activity. Additionally 25 of 29 studies support a dose-response relationship (Friedenreich and Orenstein, 2002). These findings were also described by Lee

(2003) who reported in her review article that physical active men and women have a risk reduction of about 30-40% and also found a dose-response relationship. In general the spectrum of colon cancer risk reductions reaches from a 80% risk reduction to a 60% increase of risk (Lee, 2003). The scale and direction of the influence of exercise strongly depends on the type, duration and frequency of physical activity. The great variations in the results of the different studies are supposedly caused by the different types of exercise studied. But on the other hand the findings about the positive and therefore preventive effect of physical activity on colon cancer development gain more emphasis against the background of the variety and in parts ill-conceived different approaches, which resulted almost invariably in risk reductions of colon cancer. Additionally the investigators took into account that the effects of physical activity might be influenced or superposed by other life-style factors like nutrition and body weight. Nevertheless these factors are also known as being preventive with regards to colon cancer development (Friedenreich and Orenstein, 2002). This aspect will be discussed later in section 1.5.

A huge amount of overview articles exist not only on cancer in general but also on colon cancer prevention by physical activity (Colditz et al., 1997, Colbert et al., 2001, Quadrilatero and Hoffman-Goetz, 2003, Slattery et al., 2003, Slattery, 2004, Samad et al., 2005). The interested reader is referred to these articles for further information concerning epidemiological data on colon cancer prevention by physical activity. Within this section only a brief overview about the current knowledge of epidemiological studies can be given.

It was shown (Garabrant et al., 1984) very early in a study including 2,950 males that an increased job activity level reduced colon cancer risk. Samad et al. (2005) included 47 studies from all over the world, e.g. China, Japan, New Zealand, the US and some European countries. There have been 19 cohort- and 28 case-control studies with occupational as well as recreational activity. The risk reduction seemed to be more pronounced in males (preventive effect in 40 studies of 47 studies) than in females (preventive effect in 18 studies of 47 studies). Some cohort studies of occupational exercise showed this inconsistence between males and females, while others showed a gender independent risk reduction. In few studies only the exercise amount, intensity and duration was analysed. One of these was presented by Lee et al. (1991). They followed a cohort of 17,148 men for 26 years - 225 cases of colon cancer were observed. The amount of exercise was taken into account in this study.

Those subjects who burned more than 1000 kcal/week in physical activities had lower colon cancer rates in comparison to the sedentary colleagues (Lee et al., 1991). The same result was also shown in a study including 67,803 women that were followed for 6 years with 212 colon cancer cases. Women expending 11-21 MET* h/week in moderate or vigorous recreational activities had a 33% risk reduction compared to those expending less than 2 MET*h/week. Women with more than 21 MET*h/week had a statistically significant risk reduction of about 46% (Martinez et al., 1997). Little is known regarding the intensity and duration of the physical activity. Slattery et al. (1997) showed in a case-control study with both genders that vigorous-intensity activities but not moderate-intensity activities resulted in a risk reduction of colon cancer. Irrespective of the lack with respect to the amount, intensity and duration of exercise, the list of studies generally observing a risk reduction of colon cancer in physically active subjects is very long. This cannot be explained here in more detail – I again refer to the overview articles mentioned previously.

1.3   Animal models – physical activity and colon cancer prevention

Animal models are very important tools to understand the mechanistic background of how physical activity plays a part in colon cancer prevention.

Currently only a few animal models exist which allow to study the influence of physical activity on colon cancer development or prevention (Andrianopoulos et al., 1987, Reddy et al., 1988, Thorling et al., 1993, Thorling et al., 1994, Colbert et al., 2000, Colbert et al., 2003, Demarzo and Garcia, 2004). In general these models can be divided into two different categories: chemical-induced carcinogenesis models which are the most common ones or preventive models using mice with a predisposition to neoplasia.

The first experiment studying the effect of physical activity in an animal colon tumour model was done by Adrianopoulos et al. (1987). To date there are still only 5 comparable experiments (Andrianopoulos et al., 1987, Reddy et al., 1988, Thorling et al., 1993, Thorling et al., 1994, Demarzo and Garcia, 2004) in total available. In these studies the colon tumour was chemically induced with Azoxymethane (AOM) (Reddy et al., 1988, Thorling et al., 1993, Thorling et al., 1994) or 1,2 dimethylhydrazine (DHM) (Andrianopoulos et al., 1987, Demarzo and Garcia, 2004). Both chemicals are potent inducers of carcinomas in the large intestine and are commonly used in preclinical models for assessing the influence of chemopreventive

substances on colon tumour development (Reddy, 2004). Colbert et al. (2000) have been the first group to use the multiple intestinal neoplasia (MIN) mouse in the context of physical activity. This mouse model carries an APC mutation, which leads to adenomas in the small intestine and colon. It was first described in 1990 by Moser et al. (1990). Despite the mutation only a small number of adenomas can be detected in the colon. Therefore it is a limited model for investigations concerning colon cancer development.

Most investigations using chemical-induced (AOM or DHM) colon tumour models have shown a significant reduction in the number of colon tumours or precursors in animals with enhanced activity levels (Andrianopoulos et al., 1987, Reddy et al., 1988, Thorling et al., 1993, Thorling et al., 1994). The exercise schedule varied between these studies. 2 investigations dealt with voluntary exercise (Andrianopoulos et al., 1987, Reddy et al., 1988). In one study there was no information about the exercise volume, the other (Reddy et al., 1988) reported a mean exercise distance of about 6000 m per night during week one to five. This decreased to 2600 m per night during week twelve to 32 and till the end they reported a decline down to 500 m per night on average. Thorling et al. (1993, 1994) investigated electric motor-driven exercise of about 2000 m per night. Another study reported an increase in the number of aberrant crypt foci (ACF) following an exhaustive swimming endurance test (Demarzo and Garcia, 2004). The only studies with a primary preventive approach were undertaken by Colbert et al. (2000, 2003) and revealed no significant effects in the number and size of colon polyps of both male and female mice. There were however significant fewer colon and total polyps in the exercising males (Colbert et al., 2000) and fewer jejunal polyps in the exercise group (Colbert et al., 2003). The corresponding exercise intensity was estimated as approx. 55-65% VO_{2max} and the animals ran not more than 1-2 km per day in one study (Colbert et al., 2000) and about 3 km per day in the other study (Colbert et al., 2003). The motivation to run was ensured by gentle hand prodding and free access to a wheel, which was permanently attached to the cage.

The results of the animal model studies differ in a way, which reflects the different amounts and types of exercise investigated. With the exception of enforced swimming in one case, running activities were used in the majority of the investigations. This running activity was voluntary or motor-driven.

Besides variations in the exercise schedule and exercise amount and intensity the use of different animal species and strains can further explain the big differences between the results. In three experiments Fischer F344 rats were used (Reddy et al., 1988, Thorling et al., 1993, Thorling et al., 1994), one research group used Wistar (Demarzo and Garcia, 2004) and one Sprague Dawley rats (Andrianopoulos et al., 1987). Colbert et al. (2000, 2003) used C57BL/6 mice. Only one group mentioned a very important aspect: they used an inbred strain for their experiments (Reddy et al., 1988). Another important aspect is the age of the animals. There is no consistent approach at which age the animals were studied. The youngest animals were three weeks old (Colbert et al., 2000) and the oldest nine and a half weeks old (Reddy et al., 1988) when the exercise treatment was started. The other research groups used animals of 4 (Demarzo and Garcia, 2004), 6 (Andrianopoulos et al., 1987), 7 (Thorling et al., 1993) and 8,5 (Colbert et al., 2003) weeks.

1.4 Supposed mechanisms of colon cancer prevention by physical activity

Even though the reduction of colon cancer risk by increased physical exercise is well established through epidemiological data, little is known about the underlying biological processes of this association. The proposed mechanisms can be divided into two classes: on the one hand known physiological/morphological alterations in the context of colon cancer development are discussed to be influenced by physical activity. On the other hand specific molecular variations are known to influence the development of colon cancer and are now discussed to be influenced by physical activity including protein and gene alterations.

Physiological mechanisms such as enhanced immune functions, decreased gut transit time, lowered bile acid secretion, variations in serum cholesterol and altered gut flora are discussed to be influenced by physical activity and might mediate the risk reduction of developing colon cancer.

Further mechanisms influenced by physical activity are variations in prostaglandin levels, alterations of insulin/insulin-like growth factor signalling, modulation of intestinal/pancreatic hormone profiles and improved antioxidative defence.

1.4.1 Enhancing immune functions

The colon is normally in a permanent low-grade inflammatory response induced by the endogenous microbial flora. Colonic mucosal immunity is discussed to be the driving force in the development of cancer (Campbell et al., 2001). Inflammatory bowel diseases are accompanied by an increased production of pro-inflammatory cytokines such as TNF-α, IL-6 or COX-2. This process is mediated by NFκB (Jobin and Sartor, 2000). There are several distinct differences between ulcerative colitis and Crohn´s disease but an increase in COX-2 and/or lipoxygenase is associated with both conditions. The similar glygosylation changes in inflammatory bowel disease and sporadic colon cancer might indicate that these alterations in the inflammatory response represent an important factor in colon cancer development. Campbell et al. (Campbell et al., 2001) described this in a model, which is shown in **Fig. 2**.

Campbell, Yu and Rhodes
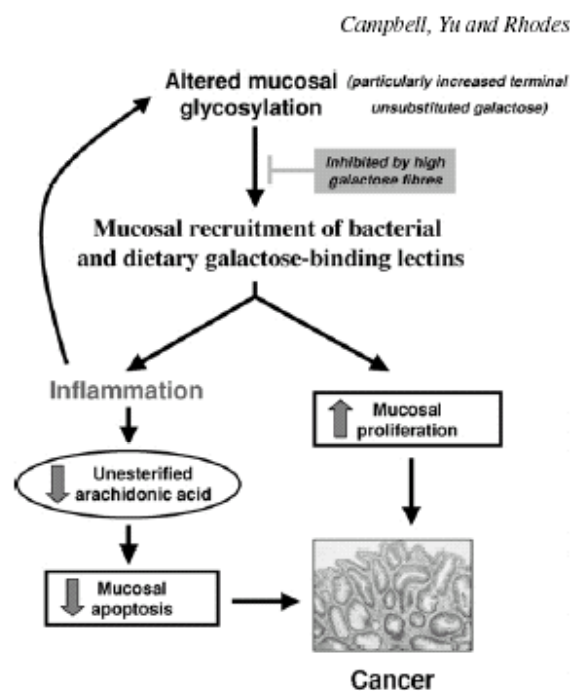
Figure 2: Hypothesis of altered mucosal glycosylation as a cause of colonic inflammation and cancer (Campbell et al., 2001); © with kind permission of Springer Science+Business Media

It is well known that physical activity can modulate several parameters of the immune system. This is described in detail for example by Hoffman-Goetz and Husted (Hoffman-Goetz and Husted, 1996). There is such a tremendous amount of studies regarding the effect of physical activity on the immune system that I refer to the

existing overview articles (Shephard and Shek, 1995, Hoffman-Goetz and Husted, 1996, Shephard RJ, 1996, Pedersen and Hoffman-Goetz, 2000, Nieman, 2003). Nevertheless the exact mechanisms are still not known in detail. The majority of the studies investigated the amount and activity of the immune cells in the blood and therefore concentrated on the peripheral immune status. A common hypothesis is that moderate physical activity increases and excessive physical activity decreases the immune functions (Nieman, 2003). Physical activity can influence for example the number and activity of macrophages, natural killer (NK) cells, lymphokine-activated killer cells and cytokines (Nieman, 2003, Woods et al., 1999). Those have the potential to destroy cancer cells. However, until now there is still a lack of studies investigating the immune response of the colonic mucosa following physical activity. Experimental data come from other organs than the colon. In an animal experiment of 9 weeks of voluntary wheel running and forced treadmill running NK cell cytotoxicity was enhanced in vivo and in vitro (MacNeil and Hoffman-Goetz, 1993). Eight weeks of treadmill or voluntary wheel running in mice increased the activity of the splenic lymphokine activated killer cells (Hoffman-Goetz et al., 1994). Rats undergoing 5 weeks of voluntary wheel running had a significant increase in the percentage of splenic NK cells and radiolabeled YAC-1 lymphoma cell clearance compared with sedentary controls (Jonsdottir et al., 1996). 17 days of moderate treadmill running led to higher in vitro phagocyte activity compared to sedentary mice (Woods et al., 1994). Bacurau et al. (2000) showed an increase in the function of peritoneal macrophages and mesenteric lymph node lymphocytes in rats (bearing a tumour) investigating 8 weeks of moderate intensity training. Besides the data from animal experiments some human studies observing the influence of exercise on immunological parameters in cancer patients are available. Physical activity lead to significantly greater in vitro NK cell cytotoxicity activity against K562 target cells (Na et al., 2000), significant increase in vitro NK cytotoxicity (Peters et al., 1994), significant decrease in the percentage of lymphocytes and monocytes and significant increase in granulocytes and phagocytotic activity of monocytes against srb cells (Peters, 1995), decrease in absolute CD3+, CD4+, CD8+ and CD25+ cell counts and increase in spontaneous and IL-2 stimulated cytolytic activity, the latter were not significant (Shore and Shepard, 1999).

The lack of immunological data on the colonic mucosa following physical activity cannot be explained. This is even more astonishing since there are many immune

cells of the colon which act against malignant cells and therefore against cancer development. This emphasizes the importance of the colonic mucosa especially with respect to the influence of physical exercise. The gastrointestinal lymphoid tissue with its numerous immune cells (**Fig. 3**) is the largest part of the body's immune system and the intraepithelial lymphocytes (IELs) of the mucosa play a major role in the first line of immune defence. They destroy infected mucosal enterocytes and foreign pathogens (Vaupel and Ewe, 1997). IELs have cytotoxic functions against tumour cells (Tagliabue, 1981) together with NK cells and cytotoxic T-lymphocytes (CTLs). The NK cells and CTLs are activated by a subgroup of IELs, the χδT-cells. There have been investigations of χδT-cells and αβT-cells in mice and the studies revealed that variations in those cell types in particular influence the development of adenoma and adenocarcinoma in the colon. Currently human and animals studies show evidence that altered immune parameters of the gastrointestinal tract influence the development of cancer. Nevertheless, the role of physical activity is still not understood with respect to variations of the gastrointestinal immune system. As it is well known that peripheral immune parameters are influenced by physical activity it can be assumed that the mucosal immune functions can be influenced by physical activity as well.
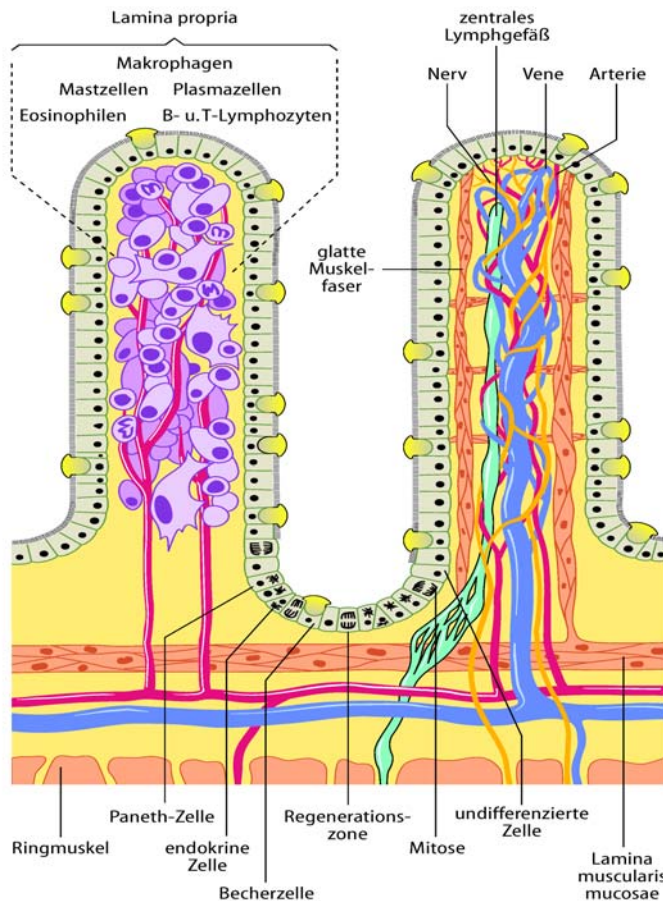
Figure 3: Structure and cell types of the gastrointestinal mucosa (Schmidt et al., 2005); © with kind permission of Springer Science+Business Media

1.4.2 Decreased gut transit time

The gut transit time is discussed as being shortened by physical activity via stimulation of the autonomous nervous system. Physical activity might increase the vagal tone and enhance peristalsis which in turn decreases the gut transit time and thereby might prevent colon cancer development (Holdstock et al., 1970, Cordain et al., 1986, Koffler et al., 1992, Liu, 1993). As a result of the decreased gut transit time carcinogens like bile acids have shorter contact with the colonic mucosa (Reddy and Wynder, 1977, Zaridze, 1983). In an animal experiment Endo et al. (2001) showed that an increased carcinogen exposure resulted in an increased number of colon cancer tumours. Inactive bedridden patients tend to get constipation and it was shown that colonic motor activity increases with walking after a period of sleep (Sarna, 1991). A study on healthy elderly persons showed an almost doubled mean colonic transit time in an akinetic period of two weeks (Liu, 1993). Other studies confirmed a decrease in transit time with body strength training (Koffler et al., 1992),

cycling and jogging (Oettle, 1991). However, a comparison between soccer players and radiology student technicians revealed no significant difference in colonic transit time (Sesboue et al., 1995). A comparable result was seen in 16 male health care workers with sedentary lifestyle. They were studied during one week of rest and during one week of exercise and there was no difference in the gastrointestinal transit time (Robertson et al., 1993). The same was shown with jogging, running and cycling by Bingham and Cummings (Bingham and Cummings, 1989), Coenen et al. (Coenen et al., 1992), Kayaleh et al. (Kayaleh et al., 1996) and Scott and Scott (Scott and Scott, 1994). The current knowledge on gastrointestinal transit time is largely inconsistent and the variety of the different studies makes it difficult to draw conclusions. Some other epidemiological studies also do not support the hypothesis of a decreased colon transit time and a reduced risk of colon cancer. They compared transit times in different populations with the related colon cancer risks and could not see any significant difference between the transit time in high-risk and low-risk persons (Glober et al., 1977, Maclennan and Jensen, 1977, Dukas et al., 2000a, Dukas et al., 2000b). The hypothesis of preventive effect of a reduced transit time needs to be studied in more detail, as the differences in the results reflect the variability of the study approaches. With respect to the different exercise interventions in terms of intensity, duration, and amount, there is a need for better-controlled and comparable studies to investigate whether there is a lower threshold of defined physical exercise that has to be achieved for a preventive effect. It should also be taken into account that the effects might strongly depend on the age and general health status. Elderly people and persons with gastrointestinal problems like obstipation might have benefits from a physically active lifestyle, whereas young and healthy persons might not notice any improvement. The list of aspects, which should be taken into account, might be longer but the need of better-controlled studies has clearly been shown and an analysis of the most inconsistent factors should be done.

1.4.3 Lowered bile acid secretion

Bile acids are known to act as cancer promoting agents in the colon. Epidemiological studies revealed an association between increased colon cancer risk and elevated bile acid excretion (Quadrilatero and Hoffman-Goetz, 2003). Exhausting treadmill running resulted in an increase of serum bile acid levels in male Wistar rats (Villa et al., 1993). Chronic voluntary running increased bile acid flow, bile acids, cholesterol

and phospholipid excretion (Yiamouyiannis et al., 1993). Prior to adjustments for dietary fibre intake, faecal bile acid concentrations were significantly lower in male distance runners compared to sedentary controls (Sutherland et al., 1991). The results of the available studies are quite different which is not surprising given the various experimental designs and species studied. This again highlights a prominent shortcut of medical research in the field of sport science. There are still not enough and mostly not stringent enough studies to draw general conclusions. A concept, which takes the current knowledge into account and deals with clear restricted exercise schedules, might be of importance in order to improve the current knowledge. New exercise regimens should be avoided as long as the existing studies still need more confirmation. The sport science as a very small field in the research community should focus on the current basic knowledge and deliberately establish the findings step by step.

1.4.4 Variation in serum cholesterol

Variations in cholesterol levels have also been shown to be associated with colon cancer risk (Quadrilatero and Hoffman-Goetz, 2003). The findings are inconsistent with compelling positive (Yamada et al., 1998, Jarvinen et al., 2001) as well as negative (Nomura et al., 1991, Eichholzer et al., 2000) associations between increased colon cancer risk and elevated serum cholesterol levels in the blood. Reduced blood cholesterol levels have been reported after regular aerobic exercise (Durstine et al., 2001) which might result in reduced bile acid secretion (Quadrilatero and Hoffman-Goetz, 2003). This in turn would be in line with the findings on bile acid secretion and colon cancer risk. Nevertheless after taking into account confounding factors like for example body weight or caloric intake the significant associations disappeared. Only about 25% of the publications reviewed by Durstine et al. (2001) showed lowered cholesterol levels after exercise training. It has also been shown that physical activity increases the levels of high-density lipoprotein (HDL) (Hsieh et al., 1998) and decreases the levels of low-density lipoprotein (LDL). Franklin et al. (2002) confirmed this improvement in HDL:LDL ratio after a moderate exercise program. It has clearly been shown that regular exercise alters blood lipids and protects against cardiovascular diseases but the effects of regular physical activity on total cholesterol and hereof affected pathways are currently unknown. This should be studied in more detail in the future.

1.4.5 Altered gut flora

The gut flora might influence the colon cancer development (Guarner and Malagelada, 2003). The state of the mucosal glycosylation (intracellular, cell-surface and secreted glycoconjugates) varies and effects colon cancer development (Campbell et al., 2001). Physical activity has various impacts on the gastrointestinal tract. Heavy exercise might induce heartburn, chest pain, nausea, vomiting, abdominal cramps, side ache and diarrhoea. Moderate physical activity has protective effects regarding cholelithiasis, diverticular disease and of course colon cancer (Simren, 2002). There is a clear link between exercise and gastrointestinal disorders. Nevertheless little is known about the underlying mechanisms. The interaction of nutrition, body weight and physical activity might be very important for the composition of the gut flora. Therefore food-based gut flora variations might give interesting hints with respect to the underlying mechanisms. The food industry for example recommends special nutrients with prebiotic or probiotic effects on the gut flora that might reduce the risk of colon cancer development (von Wright and Salminen, 1999). Beside the rare existing studies on the effects of nutrition and exercise on the gut flora there is still a wide knowledge gap concerning the exact alterations and the underlying mechanisms. Currently there is only one study available that investigated the molecular or histological alterations of the gut flora as a consequence of physical activity (Mc Tiernan et al. 2006). This should be addressed in the future because it has the potential of decreasing the risk of developing colon cancer.

1.4.6 Variations in prostaglandin levels

Prostaglandins (PGs) are products of phospholipids, which in a first step are hydrolysed by phospholipases to arachidonic acid (AA). This bioactive product is then further converted to different PGs by cyclooxygenase enzymes (Kudo and Murakami, 2002). Some of the PGs have inflammatory and some have anti-inflammatory effects – the inflammatory reaction depends on the relation of the PGs to each other. Apart from these inflammatory processes they regulate the blood vessels, contribute to the muscle contraction and inhibit hormones of the fat metabolism. The different PGs have several distinguished roles. PGE_2 is known to increase the proliferation rate of colonic cells and decreases colonic motility whereas PGF acts as an antagonist (Quadrilatero and Hoffman-Goetz, 2003). The role of

some PGs in colon cancer development is well known. Colonic mucosal PGE_2 levels were shown to be higher in cancer patients or individuals with polyps than in controls (Pugh and Thomas, 1994). There are several human and animal studies which show that the use of aspirin and nonsteroidal anti-inflammatory drugs reduce the risk of colon cancer. This is of importance because the drugs are inhibitors of PG synthesis (Giovannucci et al., 1994). There are already some studies, which looked at the influence of exercise on PG levels. One study showed that physical activity has the potential to lower mucosal PGE_2 levels (Martinez et al., 1999). A strenuous marathon run on the other hand resulted in significantly elevated plasma PGE_2, $PGE_2\alpha$ and 6-keto $PGF_1\alpha$ levels (Demers et al., 1981). A low intensity exercise decreased PGE_2 levels but this reduction was not significant (Vapaatalo et al., 1984). An increase in plasma PGE_2 levels was also observed after an exhaustive treadmill run (Venkatraman et al., 2001). In another experiment treadmill running significantly decreased the renal $PGF_2\alpha$ levels measured in urine (Zambraski et al., 1986). During muscle contraction there is a release of Ca^{2+} (Quadrilatero and Hoffman-Goetz, 2003) which is an important factor for the activity of phospholipases. This leads to the production of AAs and further on of PGs. It was also shown that exercise might influence the dialysis of PGE_2 and PGI_2 to skeletal muscle interstitial fluid (Karamouzis et al., 2001a, Karamouzis et al., 2001b). Trappe et al. (2001) showed that eccentric exercise significantly elevates skeletal levels of $PGF2\alpha$ but not PGE_2. Apart from several studies about the influence of physical exercise on PG levels in different tissues there are currently no experiments that assessed PG levels in the colonic mucosa with respect to exercise modulations.

1.4.7 Insulin/insulin-like growth factor signalling

The insulin/insulin-like growth factor signalling cascade is thought to be the most interesting pathway concerning colon cancer risk reduction by physical activity (Quadrilatero and Hoffman-Goetz, 2003, Samad et al., 2005). Physical activity may contribute to colon cancer prevention by direct (Regensteiner et al., 1991, McKeown-Eyssen, 1994, Giovannucci, 1995) or indirect reduction of insulin levels. Chronic hyperinsulinaemia might be a risk factor for several cancer types amongst others colon cancer (Kaaks and Lukanova, 2001). Moreover, insulin has been associated with colon cancer development in vivo but the exact mechanisms are still unclear (Kiunga et al., 2004, Samad et al., 2005). The hormone is known to increase the

amount of normal and carcinoma colonic cells in vitro (Koenuma et al., 1989, Watkins et al., 1990, Bjork et al., 1993). In an experiment the injection of insulin in Fischer 344 rats was directly linked to the increased growth of aberrant crypt foci in the colon (Corpet et al., 1997). It was shown that there was an elevation of insulin receptors (Kiunga et al., 2004), IGF-1 receptors (Koda et al., 2004) or maybe corresponding hybrid receptors of both insulin and IGF-1 (Giovannucci, 2001) in colon tumour tissue compared to normal tissue. IGF-1 is also commonly known as a risk factor for colon cancer (Yu and Rohan, 2000). By influencing growth hormones and IGFBP-1 and -2 insulin indirectly increases bioactive IGF-1 (Giovannucci, 2001). Even though the majority of IGF-1 is bound to IGFBP-3, both are mainly produced in the liver (Jones and Clemmons, 1995). The free protein inhibits apoptosis and is involved in the cell cycle process (Aaronson, 1991). Several studies showed that IGF-1 might be the critical factor influencing carcinogenesis of various cancer types (Ruggeri et al., 1989, Klurfeld et al., 1991, Dunn et al., 1997, Hursting et al., 1993) and it was shown that a somatostatin analogue that reduces serum IGF-1 levels can slow down colonic tumour growth (Pollak et al., 1989, Dy et al., 1992). Patients suffering from acromegaly a disease that is accompanied by overproduction of IGF-1 and growth hormones have a higher risk of developing colon cancer (Giovannucci, 2001). A case-control study showed that increased levels of circulating IGF-1 and IGF-2 as well as decreased levels of IGFBP-3 are associated with colon cancer risk (Manousos et al., 1999). Another study revealed increased IGF-1 and IGFBP-3 levels with colon cancer risk (Palmqvist et al., 2002). Two studies showed an association of colon cancer risk with increased levels of IGF-1 and decreased levels of IGFBP-3 (Ma et al., 1999, Giovannucci et al., 2000).

Insulin sensitivity is described to improve with physical activity (Kriska and Bennett, 1992) and physical activity has the potential to decrease plasma IGF-1 (Colditz et al., 1997). Several human studies confirm the latter hypothesis investigating extremely different exercise types such as nordic ski race, soccer practice, marathon run, cross-country ski race and a wrestling practice session (Suikkari et al., 1989, Koistinen et al., 1996, Ngyen et al., 1998, Scheett et al., 1999, Nehmet et al., 2002). Nevertheless there also exists a large amount of investigations which showed an increase in circulating IGF-1 (Cappon et al., 1994, Schwarz et al., 1996, Hornum et al., 1997, Ngyen et al., 1998, Wallace et al., 1999, Elias et al., 2000, Kostka et al., 2003, Ehrnborg et al., 2003, Kraemer et al., 2004). Animal experiments mostly done

in rats revealed similar inconsistent findings. Matsakas et al. (2004) showed a decrease in circulating IGF-1 after 12 weeks of voluntary running whereas Yeh et al. (1994) found an increase after moderate exercise training. Three studies reported no effect (Cooper et al., 1994, Banu et al., 1999, Bravenboer et al., 2001). Anthony et al. (2001) conducted a motor-driven treadmill running experiment and showed a lower IGF-1 level in the exercise group compared to the control group. Yet, after 12 weeks the IGF-1 level in the exercise group was above the level of the control group. Physical activity also affects the levels of circulating IGFBP-1 (Ngyen et al., 1998, Chadan et al., 1999, Wallace et al., 1999) and IGFBP-3 (Ngyen et al., 1998, Chadan et al., 1999, Wallace et al., 1999, Bravenboer et al., 2001, Kraemer et al., 2004) but the findings for these proteins are much more inconsistent than for IGF-1.

1.4.8 Modulation of intestinal/pancreatic hormone profiles

Gastrointestinal-pancreatic hormones influence large intestine and gall-bladder motility and might thus indirectly alter colonic transit time and bile acid secretion (Bartram and Wynder, 1989, Quadrilatero and Hoffman-Goetz, 2003). Human males and females show elevated levels of circulating vasoactive intestinal polypeptide, gastrin, secretin, pancreatic polypeptide neurokinin A, pancreastatin, and glucagon-like peptide I after a marathon race (O'Connor et al., 1995). Vasoactive intestinal polypeptide was also shown to be elevated after a short-term ergometer exercise (Woie et al., 1986). Three other studies reported elevations of several hormones after different exercise bouts: They showed higher plasma gastrin (MacLaren et al., 1995, Banfi et al., 1996, Sliwowski et al., 2001) and intestinal polypeptide levels (MacLaren et al., 1995) after a treadmill run of 90 min (MacLaren et al., 1995), a marathon (Banfi et al., 1996) or a incremental cycle ergometer exercise (Sliwowski et al., 2001). Nevertheless another study showed no alterations in either plasma gastrin, motilin, glucagon, pancreatic polypeptide or vasoactive intestinal polypeptide after graded exercise (Soffer et al., 1993). Based on these variations and the lack of further studies the question how physical activity might alter intestinal or pancreatic hormone levels still remains unclear. It also poses the question how hormone alterations caused by physical activity indirectly act as a modulator of bile acid secretion or colon motility.

1.4.9 Improved antioxidative defence

Free radical scavenger like glutathione and polyamines (e.g., spermidine and spermine) can inhibit the action of reactive oxygen species (von Deutsch et al., 2005). Physical activity is discussed to improve free radical defences in the muscle by up-regulation of endogenous antioxidative enzymes. Several studies confirmed this hypothesis, showing that acute exhaustive exercise or intensive endurance exercise up-regulates antioxidative enzymes like for example superoxide dismutase as well as glutathione and hemoxygenase-1. The stimulation of the antioxidative defence in plasma shows less consistent findings, yet there is currently only a small number of studies available (Fehrenbach and Northoff, 2001). This emphasises the need for further studies to complete the existing knowledge. Experiments in rats revealed that the antioxidative protection in the intestinal mucosa decreases from proximal to distal leaving the colon less protected. Taken together it can be assumed that physical activity helps to prevent colon cancer by an enhanced antioxidative defence. To our knowledge there have no studies been performed so far investigate the oxidative variations in the colon mucosa after physical activity. Therefore in addition to completing the current knowledge gained on other tissues the colonic mucosa should be the focus of future studies.

1.5   Nutritional influence and interaction with physical activity

It is a well known fact that lifestyle factors such as physical activity and nutrition have an impact on the risk of colon cancer development as reviewed in detail in a number of scientific publications (Friedenreich and Orenstein, 2002, Quadrilatero and Hoffman-Goetz, 2003).

Before starting to discuss the influence of energy intake, two very important factors should be taken into account regarding the effect of physical activity or/and nutrition on colon cancer development. This was discussed in detail in a review article by Slattery (2004) on physical activity and colorectal cancer: the link between colon cancer risk and physical activity can only be fully assessed considering confounding factors and effect modifications (Slattery, 2004). Taking into account confounding factors hereby means to keep in mind that physically active people in general have a healthier lifestyle resulting in lower body weight, healthier nutrition, less or no smoking, etc. Therefore it is possible that not the physical activity itself mediates the effect but for example the nutrition. Many epidemiological studies that have been

adjusted for these confounders still revealed a risk reduction of colon cancer with increased physical activity (Slattery, 2004). The other important factor mentioned by Slattery (2004) is effect modification, which can be investigated only in large sample sizes. This includes that e.g. in observational studies the interaction between physical activity and for example dietary factors or body weight in relation to colon cancer risk was assessed. As a result the largest risk reduction of colon cancer caused by physical activity occur in persons with high levels of energy intake (Slattery, 2004). Another example is that people with a family history of colorectal cancer might have a lower risk reduction mediated by physical activity than those without a family history of colorectal cancer (Slattery et al., 1997).

If one discusses the impact of nutrition on colon cancer development not only nutrients but also energy intake itself should be taken into account. Obesity is one of the discussed risk factors in the development of colon cancer (Samad et al., 2005). Obese Zucker rats treated with N-methyl-*N*-nitrosurea had a colon carcinoma incidence of 13,3 % (Lee et al., 2001). Variations in the energy balance influence insulin levels and might therefore contribute to colon cancer development. A huge amount of human retrospective and prospective as well as case-control studies (Caan et al., 1998) underline the association between obesity and colon cancer risk. For an overview see for example Giovannucci (2001). An association between physical inactivity and excessive body mass was shown as overweight and inactive people had the highest risk of colon cancer (Giovannucci et al., 1995). Nevertheless several studies showed that physical activity after controlling for BMI can independently influence the risk of colon cancer development in a positive way (Colditz et al., 1997, Martinez et al., 1997, Slattery et al., 1997).

Also energy restriction by itself shows a strong evidence of inhibiting carcinogenesis in varies animal models (Giovannucci, 2001). It is currently well known that caloric restricted rats have a significant longer life-time span compared to their normal fed counterparts (Holloszy, 1997, Holloszy and Schechtman, 1991). Nevertheless the exact mechanisms behind this are not clear yet.

Nutrition might influence colon cancer development through differences in nutrient composition of the diet for example fat content. In developed and rich countries the high colon cancer incidence is discussed to be due to a high fat and low fibre diet (McTiernan et al., 1998). Hyperinsulinemia resulting from high-fat and refined-sugar consumption might also be a reason for colon cancer development (Giovannucci,

2003, Komninou et al., 2003). Diets with saturated fatty acids and omega-6 polyunsaturated fatty acids are described to enhance the concentration of colonic luminal secondary bile acids (Reddy, 1995). Secondary bile acids might induce protein kinase C, cell proliferation and ornithine decarboxylase (ODC) (Craven et al., 1987, Rao and Reddy, 1993, Davidson et al., 1994, Rao et al., 1996). The latter is a rate-limiting enzyme in polyamine biosynthesis. An inverse relationship between fibre intake and colon cancer risk has been shown (Bingham et al., 2003, Peters et al., 2003). Nevertheless there are also studies which did not detect a relationship (Fuchs et al., 1999, Pietinen et al., 1999, Terry et al., 2001) or obtained inconsistent results (Platz et al., 1997, Park et al., 2005). Slattery et al. (2002) reported a positive association between a Western-style diet rich in refined sugar and red meat and p53 mutations.

2 The aim of this work

The aim of the current thesis was to investigate the physiological and molecular alterations of the colon following physical activity in order to get a better understanding of the risk reduction of colon cancer by physical activity. The scientific evidence for the association between physical activity and cancer prevention in general is convincing and especially the risk of colon cancer can be reduced by about 40-50% (Friedenreich and Orenstein, 2002). Despite varied and often crude physical activity assessment methods used in the various studies, a very consistent risk reduction was found irrespective of the different designs and populations studied. The most profound link comes from epidemiological studies (Friedenreich and Orenstein, 2002) but there are also some animal experiments (Andrianopoulos et al., 1987, Reddy et al., 1988, Thorling et al., 1993, Thorling et al., 1994, Colbert et al., 2003, Colbert et al., 2000) confirming this effect. However there is to date only one study investigating the influence of physical activity on the colonic mucosa (McTiernan et al., 2006). This gap should be closed and attention should be paid to alterations of the mucosal cells in particular where the first events in the development of colon cancer take place.

To examine the alterations of the colon mucosa and to get an insight into possible molecular mechanisms we designed our experimental model (**Fig. 4**). In addition to the biometric data collection and the IGF-serum analysis gene expression was studied. Overall three different approaches were applied: Real-time RT-PCR to test the discussed and suggested mechanisms, IGF-pathway related approaches to clarify the currently most evident mechanism and cDNA micro-array analysis to identify currently unknown target genes.

With respect to the above mentioned genes for which an association to induce preventive pathways against development of colon cancer has already been drawn, we did a literature search to select a number of genes which might be interesting in the current context. Therefore not only genes involved in colon cancer development have been chosen. We were also interested in genes for which a correlation to physical activity has been shown by other research fields, e.g. muscle research. Genes were selected based on their proven correlation to physical activity or their involvement in the process of colon cancer development. The following mechanisms were addressed: vascularisation (VEGF, HIF-1α, ODC-1), prostaglandin synthesis
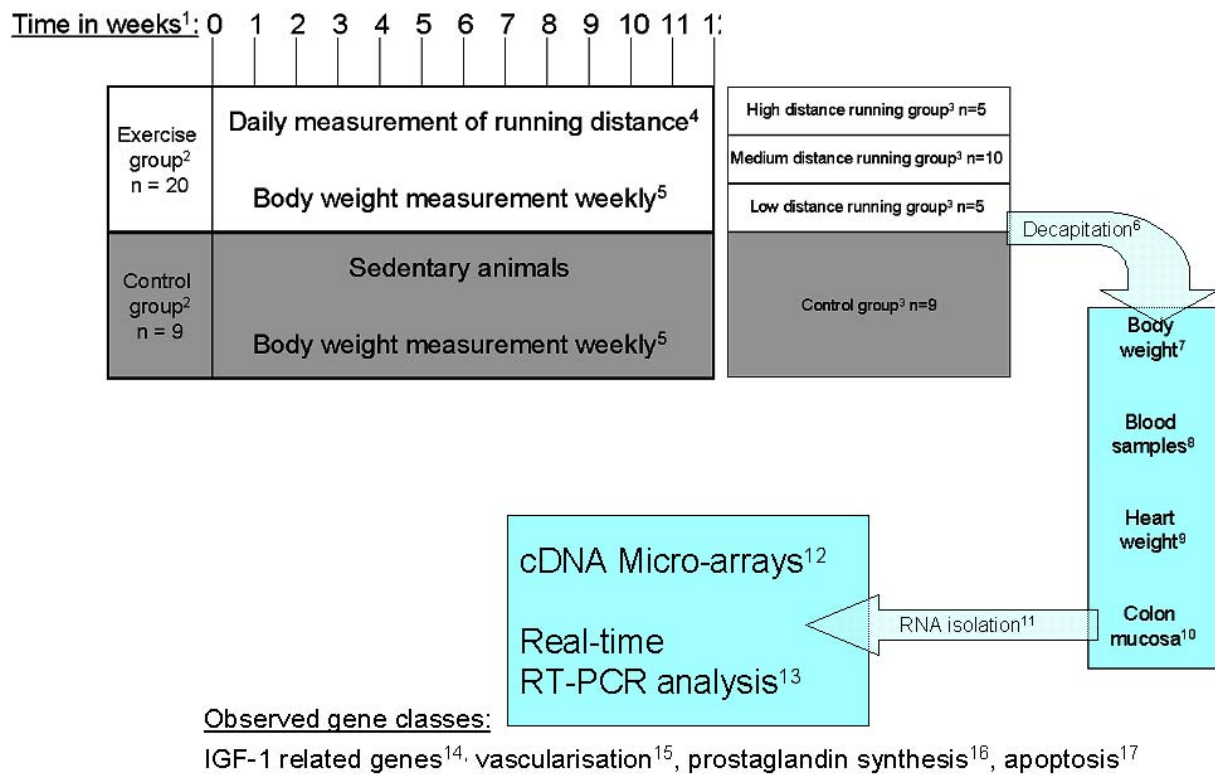
Figure 4: Scheme of the experimental period and procedures: Based on two experimental groups[2] the duration of the activity phase was 12 weeks[1] and there were daily activity measurements[4] and weekly body weight[5] measurements. At the end of the activity phase the exercise group was divided into three groups[3] dependent on the observed mean exercise-volume during the last 12 experimental weeks. After decapitation[6] final body weight[7] and heart weight[9] was assessed. Blood samples[8] and the colon mucosa[10] were collected. The RNA[11] was further isolated for cDNA micro-array analysis[12] and real-time RT-PCR[13] analysis to investigate different gene classes[14-17].

Indices and related chapters: [1]see 4.1.2; [2]see 4.1.2; [3]see 5.1.1 and 5.1.2; [4]see 4.1.2; [5]see 4.1.2; [6]see 4.1.3; [7]see 4.1.2 and 5.1.2; [8]see 4.2 and 5.2; [9]see 4.1.3 and 5.1.3; [10]see 4.1.3; [11]see 4.3; [12]see 4.5 and 5.4; [13-17]see 4.4 and 5.3.

(COX-2) and apoptosis (Bcl-2, PPARγ). Genes involved in vascularisation processes are induced by low oxygen conditions. VEGF as a key angiogenic factor is produced by ischaemic tissue and growing tumours (Ferrara et al., 1992), e.g. human colon cancer produces VEGF which is up-regulated in tumour cells by both COX-2 and PGE_2 (Calviello et al., 2004). The prostaglandin pathway has been taken into account because prostaglandin synthesis is affected in cancer development (Pugh and Thomas, 1994) as well as following physical activity (Martinez et al., 1999). Physical activity also affects apoptotic pathways. In this context physical activity may induce the apoptotic cascade in different tissues, such as skeletal muscle, heart muscle as well as in cancer cells (Leung et al., 2003). In contrast to this a growing tumour tissue fails to induce apoptosis or results in promoting cell survival (Fehrenbach and Northoff, 2001).

In our second approach we studied IGF-1 pathways following physical activity. The relation between insulin-like growth factors and physical activity is currently the most probable mechanism to explain the preventive potential. Hormonal variations in the blood after exercise have often been analysed but not in connection with mucosal variations. Blood levels and mucosal gene expression of IGF-1 were investigated. It is unlikely that the preventive effect is mediated by one gene alone therefore IGF-1R and IGFBP-3 were also analysed.

Nowadays high-throughput screening methods such as gene-arrays in genomics and 2-D-gele-electrophoresis in proteomics are widely available to get new insights into the preventive character of physical activity on the development of colon carcinoma. Nevertheless, only a few groups have reported a micro-array experiment in connection with physical exercise treatment especially in animal models (Irwin, 2001, Tong et al., 2001, Molteni et al., 2002, Bronikowski et al., 2003, Diffee et al., 2003, Colombo et al., 2005, Hagg et al., 2005, Iemitsu et al., 2005, Maeda et al., 2005, Perreau et al., 2005, Strom et al., 2005, Chow et al., 2006, Lee et al., 2006). Gene expression analyses with respect to exercise and alterations in the colon have not been published yet. On the basis of a genome-wide expression analysis a micro-array study was performed to detect new genes that are differentially expressed in response to physical activity.

Based on the previous shown figure we propose the following hypotheses:

1) There should be exercise-dependent variations in the body and heart weight of the different experimental groups (Kingwell et al., 1998, Allen et al., 2001).

2) Vascularisational processes should be varied in physically active animals in comparison to sedentary animals with the knowledge about genes which are involved in vascularisation processes and induced by low oxygen conditions (Ferrara et al., 1992).

3) It is already known that physical exercise influences mucosal (Martinez et al. 1999), renal (Zambraski et al., 1986), peripheral (Demers et al., 1981, Vapaatalo et al., 1984, Venkatraman et al., 2001) and skeletal (Trappe et al., 2001) prostaglandin levels. This leads to the hypothesis that genes involved in prostaglandin synthesis might be modulated by a long-term period of physical exercise.

4) Genes of apoptotic processes in the colonic mucosa should be differentially expressed in running animals compared to sedentary animals. Physical activity may induce the apoptotic cascade in different tissues, such as skeletal muscle, heart muscle as well as in cancer cells (Leung et al., 2003).

5) Peripheral IGF-1 is well known to be modulated by physical activity, nevertheless the direction of modulation is extremely dependent on the amount, type and intensity of physical activity (Suikkari et al., 1989, Cappon et al., 1994, Koistinen et al., 1996, Schwarz et al., 1996, Colditz et al., 1997, Hornum et al., 1997, Ngyen et al., 1998, Scheett et al., 1999, Wallace et al., 1999, Elias et al., 2000, Nehmet et al., 2002, Ehrnborg et al., 2003, Kostka et al., 2003, Kraemer et al., 2004).

6) The gene expression of IGF-related genes is suggested to be influenced by exercise interventions. Data from other tissues indicate an influence of exercise on the genes (Zanconato et al., 1994, Eliakim et al., 1997, Matsakas et al., 2004, Matsakas et al., 2005).

7) cDNA micro-array analysis should support the existing hypotheses (Friedenreich and Orenstein, 2002, Quadrilatero and Hoffman-Goetz, 2003) and reveal new approaches in the context of the influence of physical activity on the gene expression in the colon mucosa.

3 Materials

3.1 Equipment

Besides common laboratory equipment the following specific tools were used: Affymetrix 428^{TM} array scanner (Affymetrix, Santa Clara, CA, USA), Anthos 2000 photometer (Anthos, Salzburg, Austria), Biophotometer (Eppendorf, Hamburg, Germany), DasyLab 5.0 data collection system software (Datalog, Mönchengladbach, Germany), FastPrep$^{®}$ FP120A Instrument (Q-BIOgene, Irvine, CA), LightCycler (Roche, Mannheim, Germany), PCR Sprinter, PCR Express and Hybridization oven Shake´n´ Stack (all from Hybaid, Middlesex, UK), Speed vacuum centrifuge RC 10.10 (Jouan, Saint-Herblain, France), Plastic cages (Tecniplast, Hohenpeißenberg, Germany).

3.2 Biochemicals and consumables

Unless otherwise specified all chemicals used (pro analysis quality) were from Sigma (Taufkirchen, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Fermentas (St. Leon-Rot, Germany) and Invitrogen (Karlsruhe, Germany).

4 Methods

4.1 Animal model

4.1.1 Animals

Twenty-nine male Wistar Rats (outbred strain) with an age of 6 weeks (194 ± 9 g body weight) were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed under controlled environmental conditions (21°C, 12:12-h light-dark cycle). Food (standard rodent chow from Ssniff; Soest, Germany) and water were given ad libitum. The animals were maintained according to the Guide for the Care and Use of Laboratory Animals (published by the U.S. Department of Health and Human Services). The study design was approved by the Regional Administration of the City of Cologne (Bezirksregierung Köln).

4.1.2 Voluntary running model

After 3 days of acclimatisation the animals were randomly divided into two groups: an exercise group (n = 20) and a control group (n = 9). The exercising animals were housed in cages with free access to a running wheel to assure a stress-free running model. The wheels are not commercially available. **Figure 5** shows the construction comprising of a running wheel, which is connected to a standard plastic cage by a small hole. Connection to a computer software allowed on-line recording of running distances 24 hours a day (DasyLab 5.0 data collection system software from Datalog; Mönchengladbach, Germany). The voluntary physical exercise training period lasted for 12 weeks. During this period, the control animals were housed individually in standard plastic cages. The body weight of both groups was monitored weekly. For details see also Matsakas et al. (2004).

Figure 5: Voluntary running model – with computer-based data collection (kindly provided by Dr. T. Schulz)

4.1.3 Tissue collection

After completion of the 12-week trial, all animals were decapitated in approximately the same time window (9-11am). Wheels and food had been removed from the cages 12h earlier to minimize the influence of the last exercise bout and the last feeding on the targets of interest. The heart (without the great vessels) was removed and the heart weight was determined immediately (Matsakas et al., 2004). The whole colon was separated, rinsed with Ringer's solution and prepared to be free of fat and faeces. Subsequently the colon was turned inside out to scrape off the mucosa (**Fig. 6**). The isolated mucosa was transferred directly to liquid nitrogen and stored at – 80°C for further analysis.
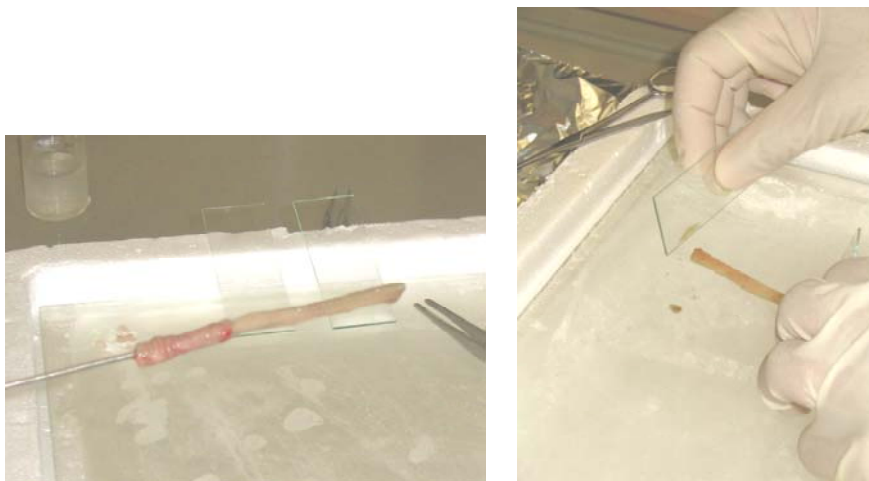
Figure 6: Preparation of the colon, turning it inside out and scraping off the mucosa

4.2 Hormone analysis

Blood samples were collected after decapitation and processed immediately. Samples were stored in an upright position for 30 min at room temperature. To obtain the serum the blood was centrifuged for 10 min at 1500 g. The supernatant was transferred to a sterile tube and stored at –20°C. Further analysis was done by enzyme immunoassay with the use of kits from DRG (Marburg, Germany) for IGF-1. Measurements were taken in an Anthos 2000 photometer (Salzburg, Austria). The sensitivity was about 30 ng ml^{-1} and the intra- and inter-assay coefficients of variation were 7.4 and 9.5 %, respectively. For details see Matsakas et al. (2004).

4.3 Total RNA isolation

Frozen tissue was homogenised using 2 ml Lysing Matrix D tubes (Q-BIOgene, Irvine, CA) filled with provided 1.4 mm ceramic spheres and additionally filled up with buffer for the cell lysis (Macherey-Nagel, Düren, Germany). For the pulverisation process a FastPrep® FP120A Instrument (Q-BIOgene, Irvine, CA) was used. In the following steps total RNA was isolated using the NucleoSpin®RNA II-Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. A DNA digestion step was included. Isolated RNA was diluted in 60 µl of RNAse-free water and stored at –80°C. RNA concentration was measured photometrically and purity was checked assessing the optical density (OD) ratio of 260/280 nm (Biophotometer Eppendorf; Hamburg, Germany). In addition, RNA-gel- electrophoresis under denaturating conditions (1% agarose, formaldehyde) was performed to determine the quality of the isolated RNA by interpreting the 18S and 28S bands. Only high quality material was accepted and used for further analysis.

4.4 Real-time RT-PCR

4.4.1 Primer design
Highly purified salt-free primers from MWG (Ebersberg, Germany) were used. The concentration of the working solutions was 20 µM. The primer sets were designed based on the LightCycler Probe Design Software, Version 1.0 (Idaho Technology Inc., 2001). Optimal annealing temperatures and quantification temperatures were established using the LightCycler (Roche, Germany). Melting curve analysis and agarose gel electrophoresis of the PCR products were used to validate the

experimental conditions. The quantification temperature or each primer pair was set below the specific melting temperature of its product.

4.4.2 Reverse transcription of RNA into cDNA

Total RNA was reversely transcribed into cDNA as follows: a mix of 0.5 µg RNA, 8 µl 5-x-MMLV reaction buffer (Promega, Mannheim, Germany), 6 µl dNTP´s (300 µM; Fermentas, Germany) together with an adequate amount of nuclease-free water was prepared to get a final volume of 30 µl. For denaturation the mix was incubated for 5 min at 65°C. Afterwards 0.4 µl random hexamers (0.2 µg/µl; Fermentas, Germany), 0.63 µl RNAse inhibitor (20 U/µl; Fermentas, Germany), 1 µl M-MLV Reverse Transcriptase (200 U/µl; Promega, Mannheim) and 7.37 µl nuclease-free water were added. The samples were incubated for 1 h at 37°C with a final step of 1 min at 99°C.

4.4.3 Primer validation

A cDNA-pool was prepared by combining the same volume of each cDNA sample (control and exercise animals). This pool was used for establishing the optimal annealing and quantification temperatures for each individual primer pair. The reason for using a cDNA pool is to ensure optimization over the entire sample pool. After denaturation of the cDNA for 5 min at 65°C a standard reaction mix for each sample was prepared as follows: 1 µl cDNA (12.5 ng), 6.4 µl nuclease-free water, 1.2 µl MgCl$_2$ (final concentration: 4 mM), 0.2 µl of each primer (final concentration: 4 pmol) and 1 µl 10 x LCM-reaction mix (Roche, Germany). Samples were analysed with the LightCycler instrument (Roche, Germany). The following protocol was used for the specific gene amplification: the initial polymerase activation step was 10 min at 95°C, the denaturation step was 15 sec at 95°C, the annealing step was 10 sec at 60°C, the elongation step was 20 sec at 72°C and the quantification step was 5 sec at 79°C. To evaluate the specificity of the amplification a final melting curve analysis (60 - 99°C) under continuous fluorescence measurements was added and gel-electrophoresis (1.5% agarose) of the amplification products was performed. Replicates were done for each new primer pair. Different annealing and quantification temperatures were tested to determine the optimal conditions. After setting the annealing and quantification temperatures (± 0.1 up to 4°C less or more) a dilution series was prepared with 2-fold, 1-fold, 1/10, 1/100 and 1/1000 of the normal (12.5 ng) cDNA amount to test the amplification efficiency of the primer pairse. Criteria for

an optimal primer pair were: a) reliability, b) sharp melting peak, c) distinct and single product after gel electrophoresis and d) correct dilution series. **Table 7** (addendum) shows established primer pairs for rat colonic mucosa. **Table 8** (addendum) shows those primer pairs, which failed in rat colonic mucosa. The reason for the failure is stated.

4.4.4 Real-time RT-PCR

Real-time RT-PCR was performed using the SYBR Green I - Kit (Roche, Germany). After denaturation of the cDNA for 5 min at 65°C a standard reaction mix was prepared for each sample: 1 µl cDNA (12.5 ng), 6.4 µl nuclease-free water, 1.2 µl MgCl$_2$ (final concentration: 4 mM), 0.2 µl of each primer (final concentration: 4 pmol) and 1 µl 10-x-LCM-reaction mix (Roche, Germany). Samples were analysed with the LightCycler instrument (Roche, Germany). A standard protocol was used for the amplification of specific gene products using specific annealing (AT) and quantification (QT) temperatures for each primer pair: initial polymerase activation step (95°C, 10 min), denaturation (95°C, 15 sec), annealing (AT, 10 sec), elongation (72°C, 20 sec) and quantification (QT, 5 sec). To evaluate the specificity of the amplification a final melting curve analysis (AT up to 99°C) was added under continuous fluorescence measurements.

4.4.5 Relative quantification of real-time RT-PCR data

Relative quantification was done based on sample crossing points as described earlier (Rasmussen, 2001) and analysed by the LightCycler Software 3.5 (Roche Diagnostics). The method of choice was the "second derivative maximum" method (Rasmussen, 2001). Two different Excel-based applications were applied for further data analysis to ensure the reliability of the results. Using the two different programs BestKeeper (Pfaffl et al., 2004) and Rest© (Pfaffl et al., 2002b), the data were processed and tested for statistical significance, normality and reliability.

4.5 MWG Rat 10K micro-array

4.5.1 RNA preparation

60 µg total RNA of eight individual animals within each group were pooled. Out of the 20 running animals we pooled the RNA of eight individuals with a running activity in the range of 5747 ± 2800 m to 8446 ± 3434 m per night (**Table 9**, addendum). We

randomly selected the RNA of 8 control animals for the control pool. In order to increase the RNA concentration each pool was subjected to a precipitation step (according to Macherey-Nagel, Düren, Germany): 3 M NaCl (1/10 volume) and ethanol (abs., 2 volumes) were added and samples were stored at –20°C over night. Samples were centrifuged at 18,400 X g for 10 minutes at 4°C and the supernatant was discarded. The pellet was covered with a layer of 500 µl of 70% ethanol and centrifuged again at 18,400 X g for 7 minutes at 4°C. Again the supernatant was discarded and the pellet was dried via speed vacuum centrifugation (model RC 10.10, Jouan Inc., Winchester, VA). The total RNA was resuspended in 40 µl RNAse free water. Quality, purity and concentration of the two sample pools were checked as described previously (see 4.3 Total RNA isolation).

4.5.2 cDNA micro-array

Oligonucleotide arrays on glass slides containing 9,715 gene-specific oligonucleotide probes (50 mer) were obtained from MWG Biotech AG (Ebersberg, Germany). Pooled and concentrated total RNA-samples of the exercise and control group were obtained as described above (see 4.3 Total RNA isolation and 4.5.1 RNA preparation). Reverse transcription, labelling and hybridisation were performed according to the manufacturer's manual. For the fluorescence detection Cy3- and Cy5-labelled dCTP (Amersham Bioscience Europe, Freiburg, Germany) were used to produce fluorescence labelled first-strand cDNAs. Arrays were scanned (Affimetrix 428 Array Scanner, Santa Clara, CA) under dried conditions. Data were normalised and analysed using the ImaGene 4.2 software (BioDiscovery, Los Angeles, CA). Three independent hybridisations were carried out. Genes were considered as differentially expressed if: a) the expression ratio was more than 2-fold in at least two hybridisations and b) signal intensity values were at least 2-fold above overall background signal intensity in each group.

4.6 Calculations and statistics

Based on normality tests, data are shown as the mean of the group ± standard deviation unless stated otherwise. Calculations were done using the SigmaStat 3.0 (SPSS Inc.) software. Results were considered significantly different at a p-value < 0.05.

4.6.1 Animal data and hormone analysis

One-way analysis of variance (factor: group) was used to compare the subgroups with the control group in the cases of serum IGF-1, heart mass and body mass. A Holm-Sidak post-hoc test was used to assess significant differences at a p-value < 0.05.

4.6.2 Relative quantification

The BestKeeper version was used to determine the best fitting housekeeping genes according to Pfaffl et al. (2004). Therefore the investigated housekeeping genes were compared to each other and the BestKeeper index was calculated. The BestKeeper index represents the average of the included housekeeping genes. A further pair-wise correlation analysis between the housekeeping genes and their calculated BestKeeper index validates the results and supports to recognise inadequate housekeeping genes. By using the REST© software (Pfaffl et al., 2002) the raw gene expression data for a particular gene were transformed into a normalised[1] x-fold expression ratio. This ratio gives the relative expression of a target gene between two groups normalized to the expression of the housekeeping genes. The testing for statistical significance (P < 0.05) in this case was done by a Pair Wise Fixed Reallocation Randomisation Test© as described elsewhere (Pfaffl et al., 2002).

4.6.3 Correlation analysis

Correlation analysis was performed by Pearson product moment correlation test.

4.6.4 cDNA micro-array

Criteria for the gene array analysis were: a) an expression ratio of more than 2-fold in at least two hybridisations and b) signal intensity values of at least 2-fold above overall background signal intensity in each group.

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[1] The normalized x-fold expression ratios are given without a standard deviation since calculation of standard deviations is not intended in this procedure.

5 Results

5.1 Animal data

5.1.1 Running activity

Fig. 7 shows the mean running distances (m) per night for individual animals. Running distances ranged from 1168 ± 880 m up to 10766 ± 3869 m.
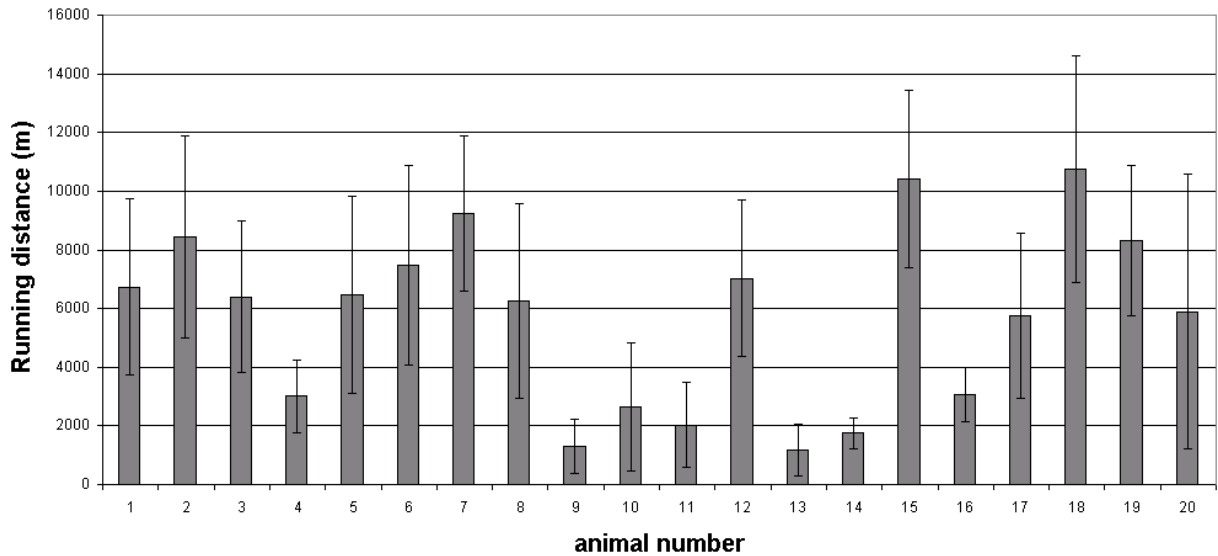
Figure 7: Exercise volume per night in meters (m) for individual animals

In an additional step, the exercise group was subdivided into a low (2629 m/night; n = 5: animal number 9, 10, 11, 13, 14), a medium (3003-7458 m/night; n = 10: animal number 1, 3, 4, 5, 6, 8, 12, 16, 17, 20) and a high (>8314 m/night; n = 5: animal number 2, 7, 15, 18, 19) distance running group.

Table 1: Running distances (m/night) of individual animals (mean ± SD)

Group	Running distance (m/night)	
	Mean	SD
H-EX	*10766*	*3869*
H-EX	*10411*	*3007*
H-EX	*9239*	*2665*
H-EX	*8446*	*3434*
H-EX	*8315*	*2550*
M-EX	7458	3403
M-EX	7023	2663
M-EX	6732	3000
M-EX	6472	3372
M-EX	6393	2573
M-EX	6245	3325
M-EX	5897	4672
M-EX	5747	2800
M-EX	3065	930
M-EX	3003	1258
L-EX	**2628**	**2186**
L-EX	**2031**	**1435**
L-EX	**1743**	**514**
L-EX	**1308**	**938**
L-EX	**1168**	**880**

Running distances (m/night) are shown as the mean ± standard deviation (SD) of the 12-weeks voluntary running exercise period. Bold letters: low distance running group **(L-EX)**, bold italics letters: high distance running group *(H-EX)*, normal letters: medium distance running group (M-EX).

Data were further analysed considering the exercise group as a whole and subdivided. The progression of the mean exercise volume showed similar patterns in all groups, the whole group as well as the subgroups, with an increase in the mean exercise volume in each group up to week 3. From week 5 onwards a decrease in the exercise activity was observed (**Fig. 8**).
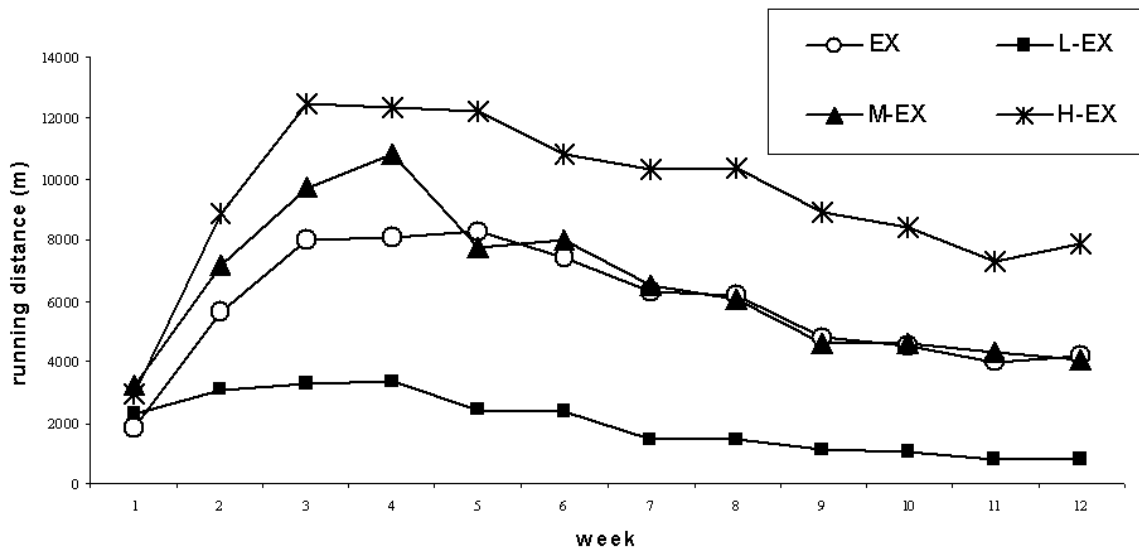
Figure 8: Mean running distances per night. Black circles (O) reflect the whole exercise group (EX, n=20), small black squares (■) the low distance runners (L-EX, n=5), black triangles (▲) the medium distance runners (M-EX, n=10) and black stars (٠) the high distance runners (H-EX, n=5). See also Buehlmeyer et al. 2007b.

5.1.2 Body weight

Fig. 9 shows the progression body weights during the course of the experiment. Although all animals gained weight throughout the program, the body weights of the medium and high running distance groups were consistently and significantly lower than those in the control group from week four until week twelve of the voluntary running exercise (p < 0.05). The corresponding mean body weights at the day of decapitation for the different groups were 477 ± 31 g for the low distance running group, 446 ± 29 g for the medium distance running group, 442 ± 39 g for the high distance running group and 499 ± 48 g for the control group.

5.1.3 Heart mass

Animals in the medium (3.1 ± 0.2 g/kg body weight) and high distance running groups (3.4 ± 0.1 g/kg body weight) had a significantly (p < 0.05) higher heart mass compared to the control group (2.7 ± 0.3 g/kg body weight) as shown in **Fig. 10**. The heart mass per kg body weight of the low distance running group was 2.7 ± 0.3 g/kg body weight. Absolute heart masses showed slight but not significant differences between the most exercising animals (high distance running group: 1.5 ± 0.1 g; medium distance running group 1.4 ± 0.2 g) and the least exercising animals (low distance running group: 1.3 ± 0.1 g) and the control group (1.3 ± 0.2 g), respectively.
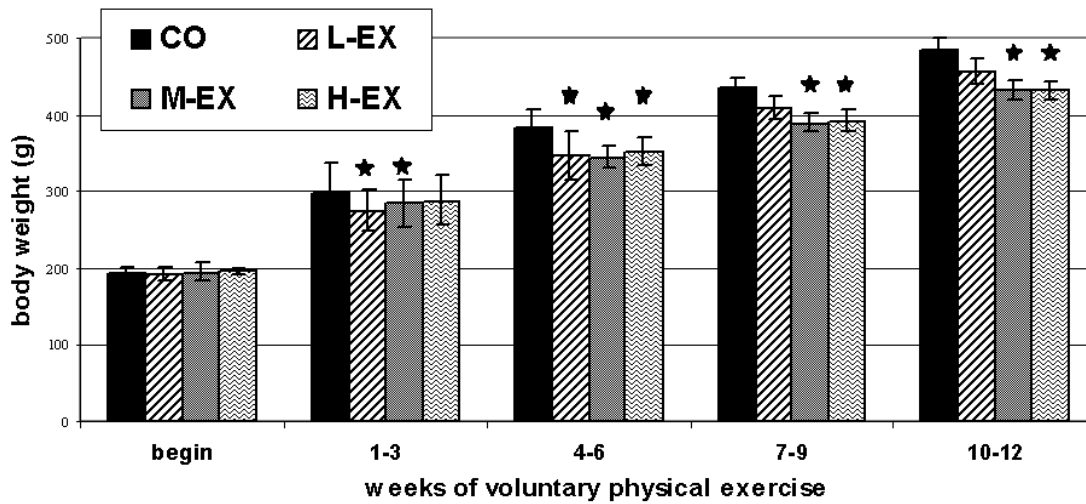
Figure 9: Mean body weight over the course of the experiment. Black bars represent the control group (CO, n=9), hatched bars the low distance runners (L-EX, n=5), checkered bars the medium distance runners (M-EX, n=10) and corrugated bars the high distance runners (H-EX, n=5); asterisks indicate significant ($p < 0.05$) changes compared to the CO. See also Buehlmeyer et al. 2007b.
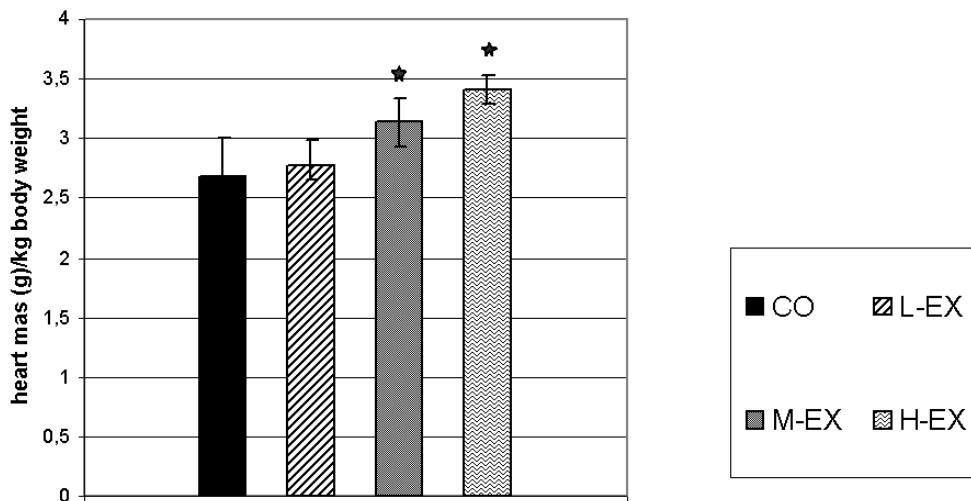
Figure 10: Mean heart mass/kg body weight. Control group (CO, n=9), low distance runner (L-EX, n=5), medium distance runner (M-EX, n=10), high distance runner (H-EX, n=5). Asterisks indicate significant ($p < 0.05$) changes compared to the CO.

5.3 Gene Expression Data

5.3.1 Real-time RT-PCR

Several genes, which are proposed to mediate the effects of exercise on colon cancer, were selected and primer pairs for those genes were established for further real-time RT-PCR in the rat colon mucosa. **Table 7** (addendum) gives a detailed summary of the investigated genes. In addition we measured the mRNA steady-state levels of the following genes via quantitative real-time RT-PCR: BCL-2, COX2, IGF-1, IGF-1R, IGFBP-3, ODC-1, HIF-1α, PPARγ, and VEGF. The following genes were used as housekeeping genes: 18S, ALDA, GAPDH and ß-AKTIN. IGF-1, IGF-1R and IGFBP-3 were analysed separately because of their discussed relevance in the context of physical activity and colon cancer prevention. The IGF-related genes were analysed together because there might be interactions between them, which might be of biological importance without detecting significant changes.

5.3.1.1 Calculation of a BestKeeper index, choice of housekeeping genes and descriptive statistics of target genes

The BestKeeper software tool (Pfaffl et al., 2004) was used to select the most stable housekeeping genes. **Table 2** shows the studied housekeeping genes with their calculated BestKeeper index. The BestKeeper index represents the average of the most stable housekeeping genes. The housekeeping gene 18S was excluded from the quantification process because it differed in many aspects from the three other housekeeping genes, namely lower crossing points (CPs), higher minimum and maximum values of the CPs (**Table 2**) and low correlation coefficient (r< 0.6, data not shown) as compared to the BestKeeper index.

Table 2: Descriptive statistics of the studied housekeeping genes

	ß-actin	*GAPDH*	*ALDA*	*18S*	*BestKeeper**
n	29	29	29	29	29
Mean [CP]	21,00	20,97	19,57	12,94	20,50
Min [CP]	19,74	20,24	18,84	12,23	19,85
Max [CP]	22,70	22,35	21,03	14,64	21,86
SD [± CP]	0,48	0,42	0,45	0,24	0,40
Min [x-fold]	-2,38	-1,47	-1,53	-1,63	1,54
Max [x-fold]	3,27	2,09	2,37	3,27	2,50
SD [± x-fold]	1,40	1,34	1,36	1,18	1,30

Sample number (n), mean of the crossing points (CP), minimum (Min) and maximum (Max) of the CPs and standard deviation (SD) of the CPs are shown. X-fold variations between the CPs were calculated and shown as min and max x-fold changes regarding the mean CPs and finally x-fold SDs are shown.
*The Best Keeper index (Pfaffl et al., 2004) was calculated including ß-actin, GAPDH and ALDA.

The housekeeping genes ß-actin, GAPDH and ALDA were found to be constitutively expressed and were used for further analysis. The mean and standard deviation of the BestKeeper index are 20.5 ± 0.4 CPs. The pair-wise correlation analysis shows significant ($p < 0.003$) correlations between the three included housekeeping genes and highly significant ($p < 0.001$) correlations between each included housekeeping gene and the BestKeeper index. The corresponding Pearson correlation coefficients (r) reached from 0.82 to 0.91. **Table 3** shows the Pearson correlation coefficients (r) of the regression analysis of each target gene to the BestKeeper index. Highly significant correlations for PPARγ, VEGF and HIF-1α were found. In contrast, no correlations were found for Bcl-2, COX-2, and ODC-1 and, therefore, these genes were used for further analysis.

Table 3: Descriptive statistics of the studied target genes

	Bcl-2	COX-2	HIF-1α	VEGF	ODC-1	PPARγ
n	29	29	29	29	29	29
Mean [CP]	28,27	30,12	24,49	25,44	23,83	24,47
Min [CP]	25,98	28,18	23,75	24,52	22,95	23,45
Max [CP]	29,28	32,49	26,54	26,62	25,32	26,67
SD [± CP]	0,53	0,84	0,48	0,42	0,44	0,60
Min [x-fold]	-4,32	-4,16	-1,67	-1,80	-1,67	-1,91
Max [x-fold]	1,93	5,89	4,15	2,13	2,41	4,14
SD [± x-fold]	1,41	1,86	1,40	1,31	1,30	1,47
coeff. of corr. [r]	-0,16	-0,05	0,88	0,6	0,19	0,87
p-value	0,397	0,797	0,001	0,001	0,336	0,001

Sample number (n), mean of the crossing points (CP), minimum (Min) and maximum (Max) of the CPs and standard deviation (SD) of the CPs are shown. X-fold variations between the CPs were calculated and shown as min and max x-fold changes regarding the mean CPs and finally x-fold SDs are shown. The Pearson correlation coefficients (coeff. of corr.[r]) of the regressions analysis of each target gene against the BestKeeper index (compare **Table 2**) (Pfaffl et al., 2004) are shown together with their corresponding p-value.

5.3.1.2 Data analysis via REST®

The REST® software (Pfaffl et al., 2002) was employed for data analysis. Each gene was normalised to each housekeeping gene as well as to the BestKeeper values. **Table 4** shows the normalised x-fold changes of the target genes between the exercise and the control group. There was no indication of any significant regulation apart from ODC-1.

Table 4: Relative quantification using REST© software (Pfaffl et al., 2002)

Normalized to	Bcl-2	COX-2	HIF-1α	ODC	PPARγ	VEGF
18S	0,98	1	0,93	1,18	0,93	0,82
ALDA	1,25	1,26	1,17	1,48	1,16	1,03
BestKeeper	1,24	1,25	1,16	1,46	1,15	1,02
ß-actin	1,16	1,16	1,08	1,36	1,07	0,95
GAPDH	1,25	1,26	1,17	1,47	1,16	1,03
Mean	1,18	1,19	1,10	1,39	1,09	0,97
SD	0,12	0,11	0,10	0,13	0,10	0,09
Mean (without 18S)	1,23	1,23	1,15	1,44	1,14	1,01
SD (without 18S)	0,04	0,05	0,04	0,06	0,04	0,04

X-fold regulation factors of the target genes normalised to the housekeeping genes are shown. Lower lines show mean and standard deviation (SD) of all x-fold regulation factors (with and without 18S). Grey marked values are significantly ($p=0.03$) regulated.

In a next step, COX-2, ODC-1 and BCL-2 were analysed subdivided regarding the three different running distance groups. **Fig. 11** displays the results for ODC-1, COX-2 and BCL-2 as the ratio between the target and the housekeeping gene (BestKeeper index) while the control group was set at one. No significant alterations of mRNA expression levels in the case of BCL-2 were found (**Fig. 11a**) whereas COX-2 showed a tendency of down-regulation in the high distance running group but this difference did not reach the level of significance (**Fig. 11b**). The only gene exhibiting significant changes while looking at exercise subgroups was ODC-1, showing a 1.8-fold up-regulation in the high distance running group (**Fig. 11c**).

a)

Bcl-2

b)

COX-2
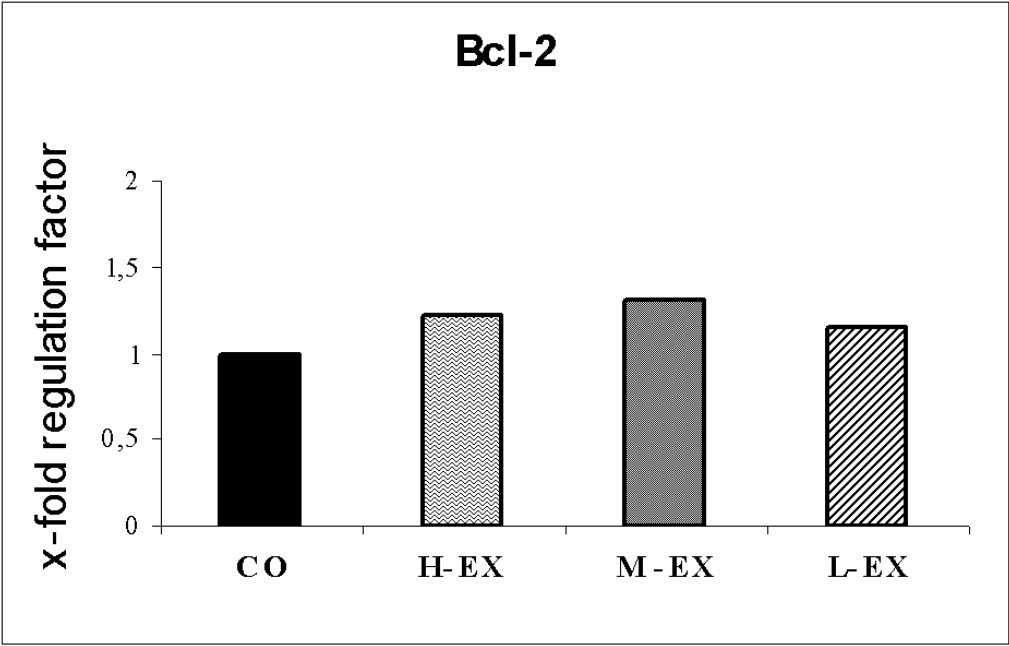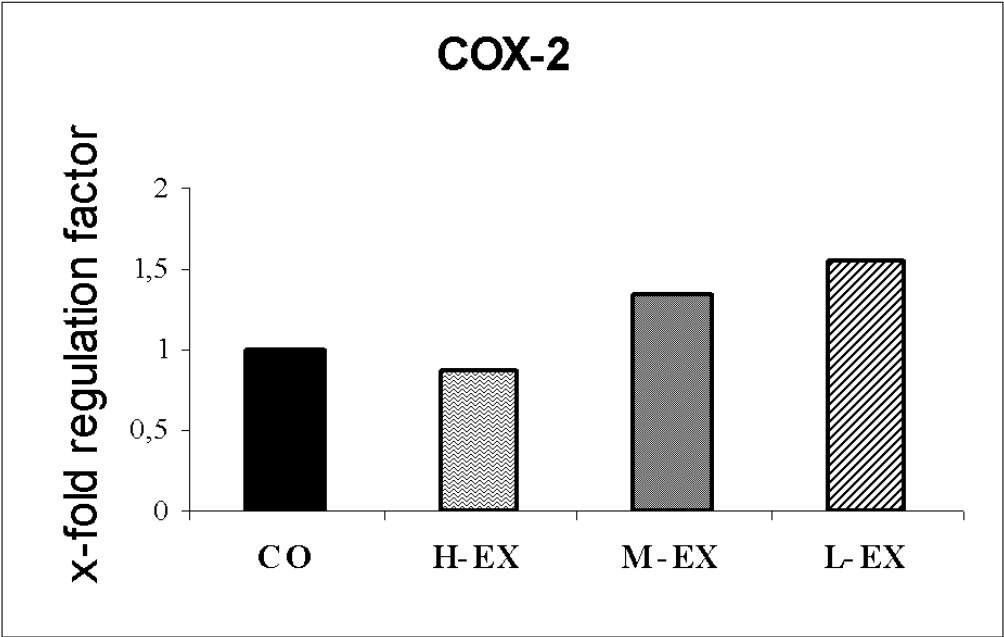
c)
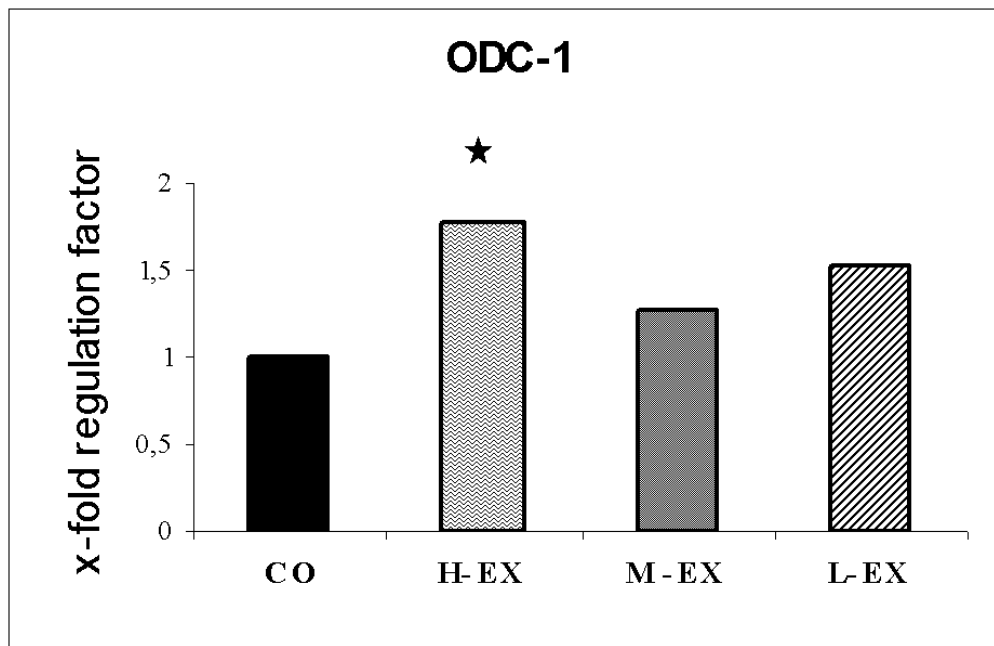
Figure 11 a-c: X-fold regulation factors – control group (CO) is set as 1 – exercise groups are shown in relation to the CO. CO (n=9), low distance runners (L-EX, n=5), medium distance runners (M-EX, n=10) and high distance runners (H-EX, n=5). Significant (p<0.05) changes are indicated by an asterisk. See also Buehlmeyer et al. 2007b.

Fig. 12 displays the normalised data for IGF-1, IGF-1R and IGF-BP3 as the ratio of the target to the housekeeping gene (BestKeeper) by assigning the control group a factor of 1. Even though we were not able to find any significant differences or correlations, the following results are worth mentioning: Firstly, a linear negative trend between running distances and IGF-BP3 mRNA levels was observed. Secondly, IGF-1R mRNA levels tended to be lower in the L-EX and M-EX groups. Thirdly, in comparison to the control group, we observed 1.8- to 2.2-fold higher expression levels of IGF-1-mRNA in colon mucosa in the L-EX and M-EX groups.
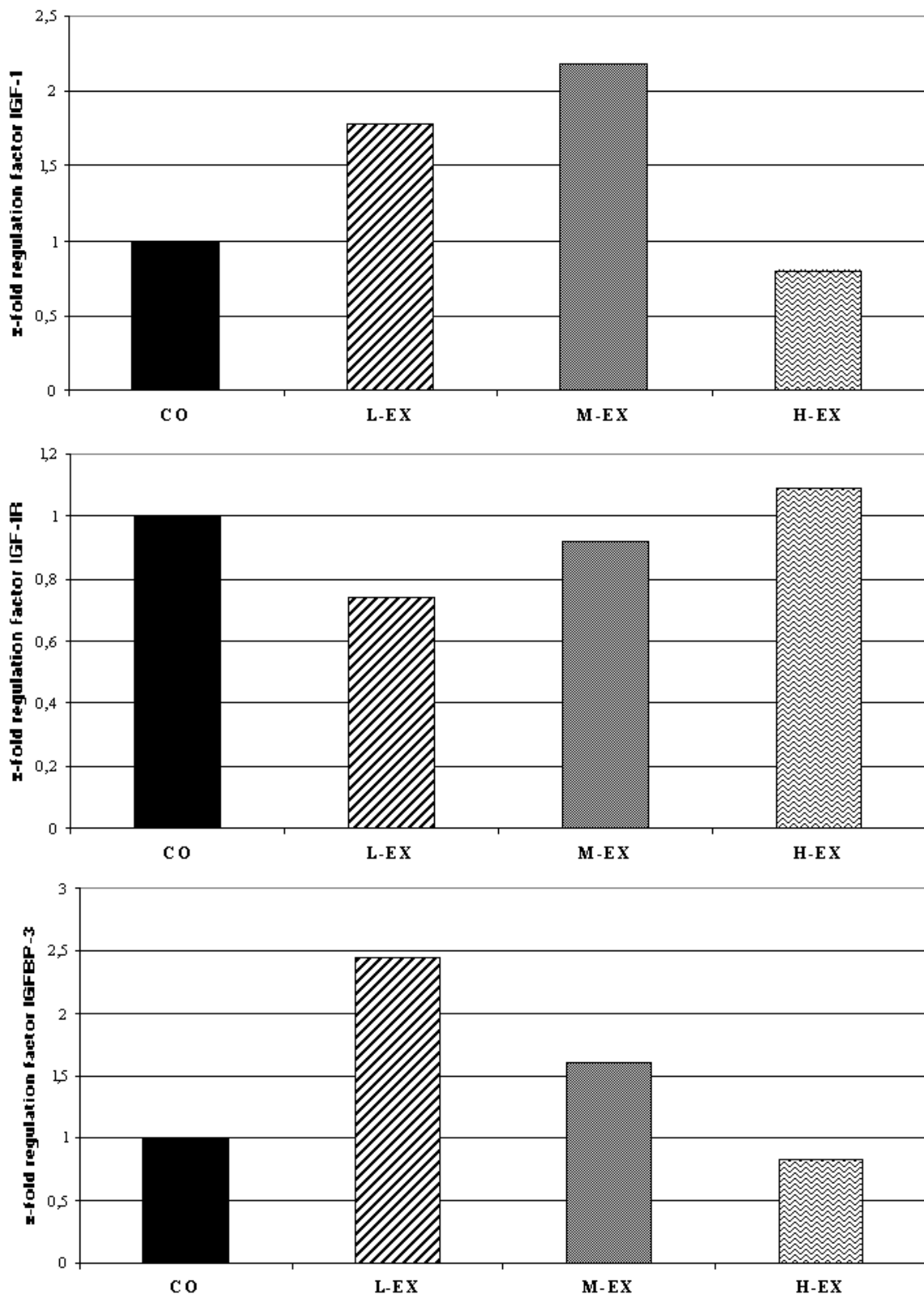
Figure 12 a-c: X-fold regulation factors – control group (CO) is set as 1 – exercise groups are shown in relation to the CO. CO (n=9), low distance runners (L-EX, n=5), middle distance runners (M-EX, n=10) and high distance runners (H-EX, n=5).

5.2 Hormone results

Serum IGF-1 concentrations were significantly lower in the low, medium and high distance running groups as compared to the control group after the exercise period (**Fig. 13**). In addition, there was a negative but statistically not significant trend in the relationship between mean running distances and serum IGF-1 concentrations. The mean IGF-1 serum concentration of the exercise group as a whole is significantly lower compared to the control group. See therefore Matsakas et al. (2004).
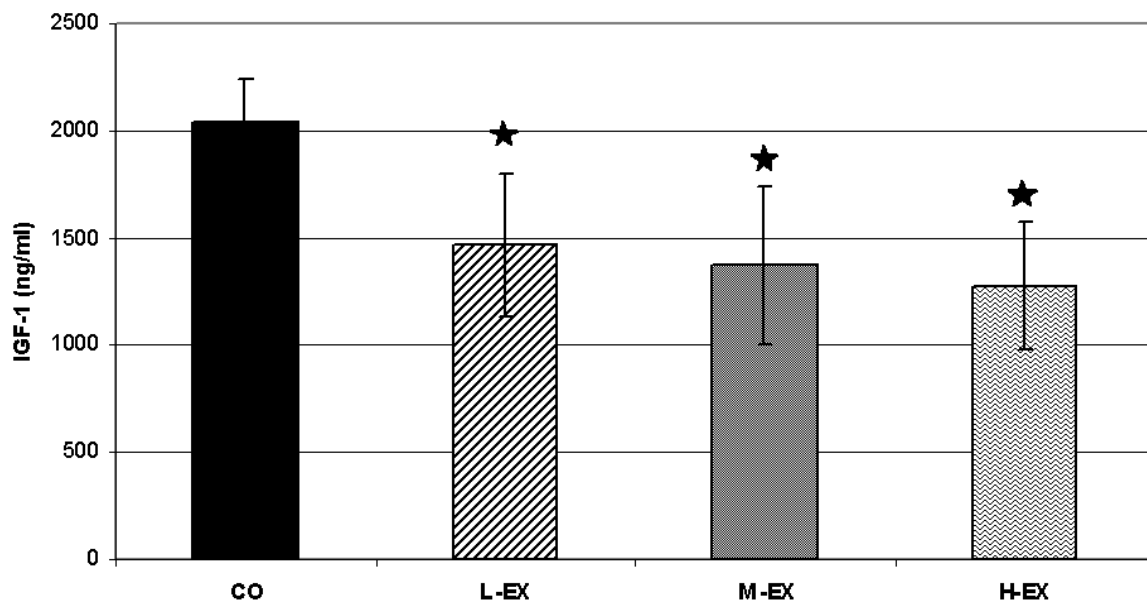
Figure 13: IGF-1 serum concentrations of the control (CO), low distance running (L-EX), medium distance running (M-EX) and high distance running (H-EX) groups.

5.3 Correlations between exercise volume, body weight, heart mass and serum IGF-1

In all rats, heart masses per kg body weight showed a significant negative correlation to serum IGF-1 levels (r = -0,66; P < 0.001) and body weights (r = -0,55). IGF-1 serum levels showed a significant positive correlation to body weights (r = 0.50). Exercise volumes revealed a significant negative correlation to body weight (r = -0.50) and a highly significant positive correlation to heart masses per kg body weight (r = 0.77; P < 0.001).

5.4 MWG Rat 10K array

5.4.1 Running activity, weight progression and heart mass alterations of the pooled samples

The pooled exercise group showed a similar running distance pattern compared to the whole exercise group (Matsakas et al., 2004; Buehlmeyer et al., 2006) besides higher values during the first 9 weeks as shown in **Fig. 14**. The peak mean running distances in the pooled exercise group were seen after 4 weeks with 12600 ± 1600 m per night. The following decline lay between 10300 ± 1900 and 8600 ± 770 m per night (week 5), 9800 ± 2100 m and 7200 ± 2100 m per night (week 6), 8800 ± 1300 m and 7200 ± 1700 m per night (week 7), 6800 ± 2100 m and 4600 ± 2000 m per night (week 8, 9). Between weeks 10 to 12 mean running distances remained steady and were nearly at the same level as those of the whole exercise group (Matsakas et al., 2004; Buehlmeyer et al., 2006).
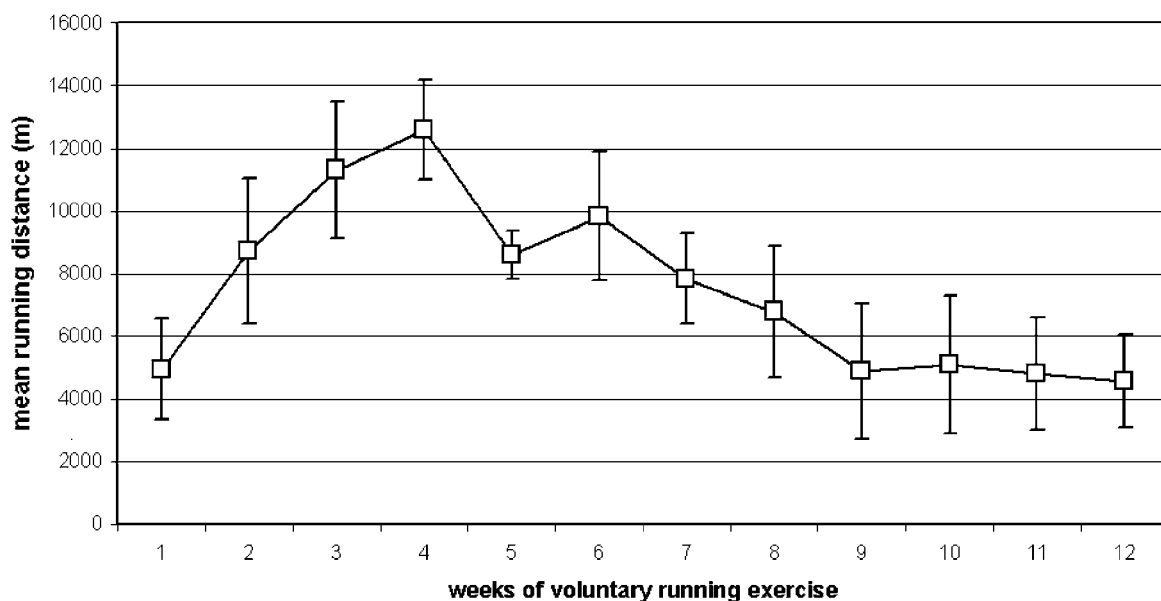
Figure 14: Mean running distances (m) of the pooled exercise group. See also Buehlmeyer et al. 2008.

Fig. 15 shows the progression of body weight of the pooled exercise and the pooled control group over the course of the experiment. There was a slight increase in body weight in each group over time. During the fifth week until the end of the study there was a significant difference between the pooled exercise and the pooled control group ($p < 0.05$) reflecting a higher body weight increase in the pooled control group.

In **Table 2** the mean heart masses (g) per kg body weight of the pooled exercise and the pooled control group are shown. There was a significantly (p < 0.05) higher heart mass per kg body weight in the pooled exercise group compared to the pooled control group. Absolute heart masses of the pooled exercise group and the pooled control group showed no significant differences.
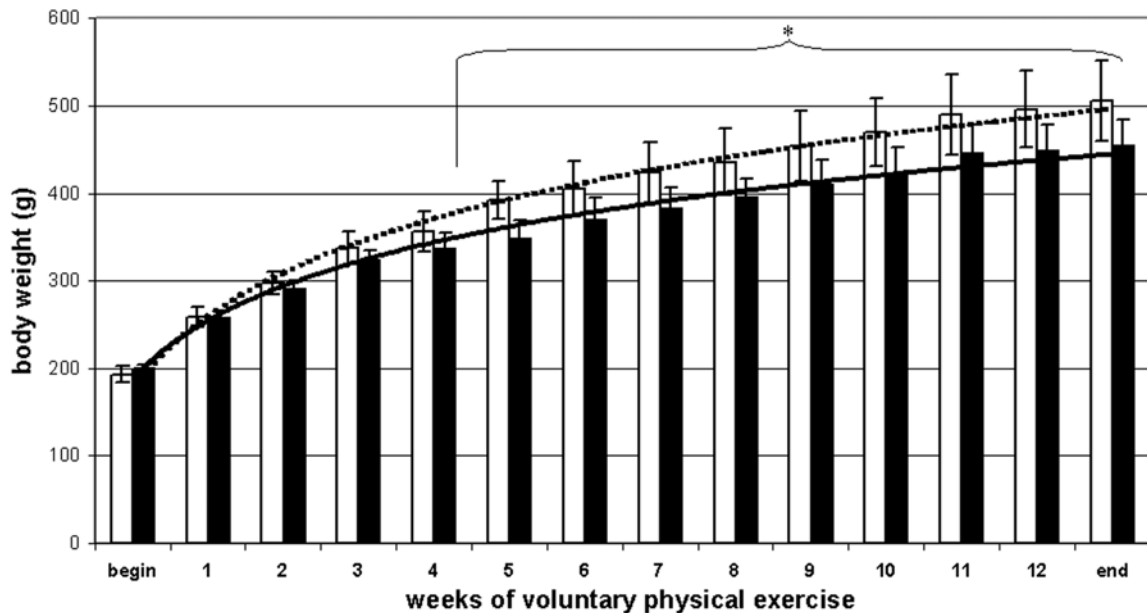
Figure 15: Body weight (g) of the pooled exercise (black bars) and control (white bars) group. See also Buehlmeyer et al. 2008

Table 5: Group heart mass (g) per kg body weight

Group	Heart mass per kg BW (mean±SD)
pEX	3,2 ± 0,2 *
pCO	2,7 ± 0,3

Heart mass per kg body weight (BW) of the **pooled exercise (pEX)** and the **pooled control group (pCO).** Values are shown as mean ± standard deviation (SD). Significant differences (p < 0.05) compared to the pCO are indicated with an asterisk.

5.4.2 Gene expression data
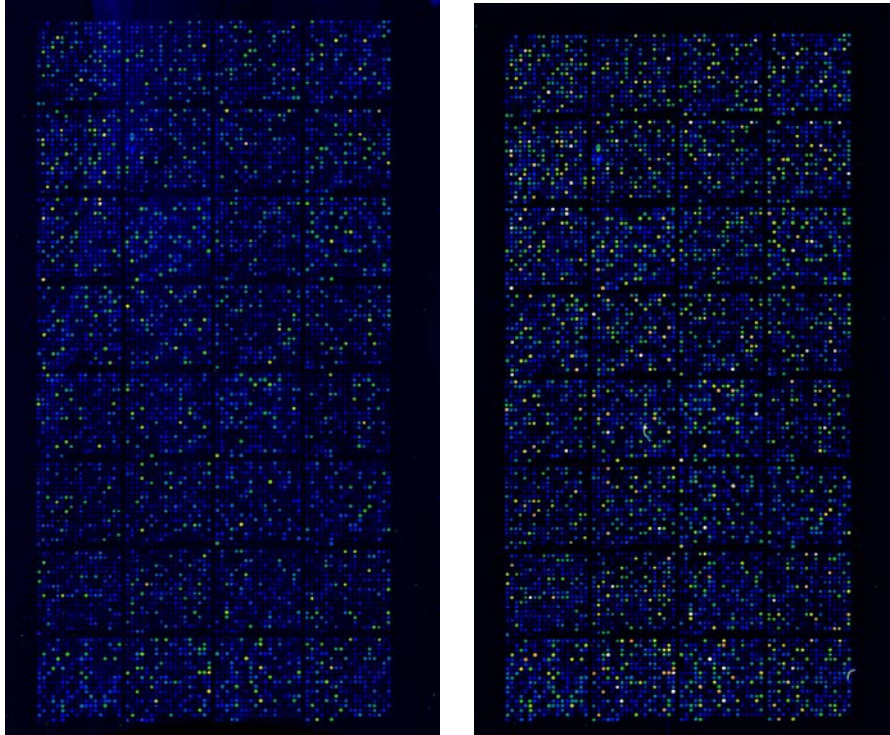
Figure 16: Micro-array experiments with two different fluorescent dyes: the left picture shows cy3 labeling of the pooled control group sample, the right picture shows the cy5-labeling of the pooled exercise group sample

In order to identify colon-expressed genes responsive to chronic exercise, total RNA from exercise and control animals were isolated and samples were pooled to get homogenous exercise and control mRNA pools. **Fig. 16** shows two micro-array experiments. Cy3 reflects the pooled control group, Cy5 the pooled exercise group. The scatter plots show logarithmised Cy3 (pooled control group) and Cy5 (pooled exercise group) fluorescent signal values of the three independent experiments indicating a small number of regulated (> 2-fold) genes. The regulated genes are reflected by the dots lying above or below the indicated line.
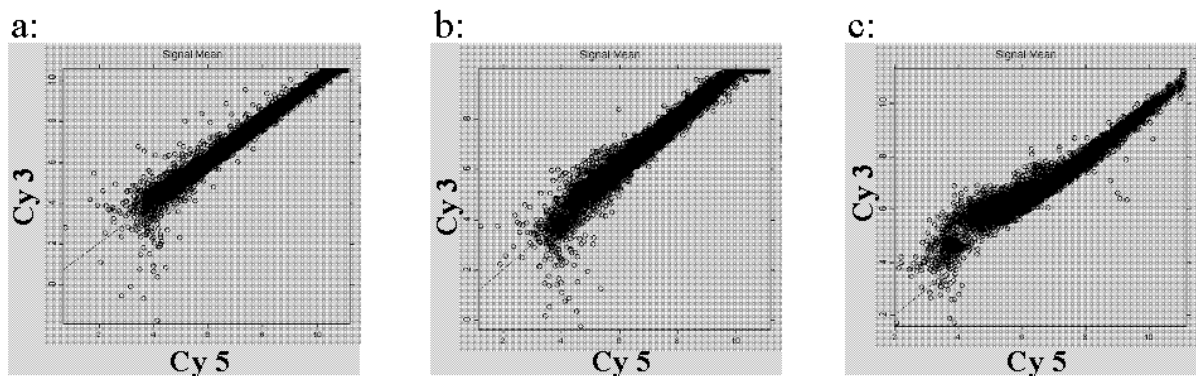
Figure 17: Scatter plots of the three different cDNA micro-array experiments.

Based on our criteria 8846 out of the 9715 genes spotted on the array were considered as being expressed. A complete list of all genes has been deposited in GEO under accession number GSE3342 (http://www.ncbi.nlm.nih.gov/geo/). Table 3 summarises the encoded proteins, gene IDs (GenBank accession number or manufacturer's specification), mean signal ratios of the pooled exercise versus the pooled control group, the corresponding protein function and the regulation factors. 50 genes were considered as being regulated (> 2-fold): Chronic exercise caused a decrease in the expression of 47 genes at a threshold-factor of 2.0. 3 genes were found to be up-regulated in response to chronic exercise. Classification based on the gene product functions resulted in nine groups (**Table 6, Fig. 18**). The identified genes encode proteins involved in signal transduction, transport, immune system, cytoskeleton, protein targeting, metabolism, transcription, and vascularisation. The functions of two genes are unknown. Four genes involved in signal transduction processes might influence colon cancer development: calcium-independent phospholipase a2, epithelial membrane protein 1, n-methyl d-aspartate 1 receptor, and inositol polyphosphate multikinase. Among the genes encoding proteins for transport processes there are 2 candidates with a suggested involvement in colon cancer development, named sodium-calcium exchanger and large-conductance calcium-activated potassium channel. It is a well known fact that the immune system plays an important role in cancer development and therefore all genes related to immune functions are potentially interesting candidates with respect to our approach. The most profound changes in mRNA expression levels were detected for betaine-homocysteine methyltransferase 2. This gene is involved in metabolic processes, which are linked to colon cancer. Both genes which play a role in vascularisation (angiopoietin-2 and vascular endothelial growth factor) are of particular interest in the context of colon cancer development.
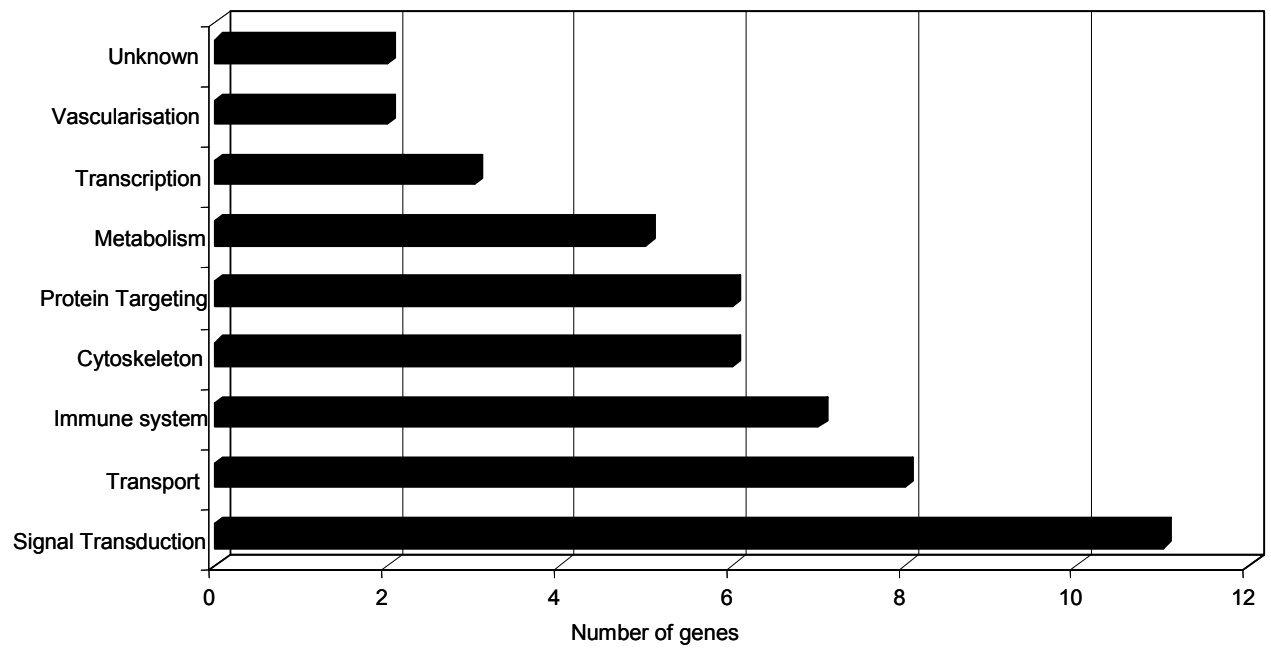
Figure 18: Clustering of the differentially expressed genes into functional groups. See also Buehlmeyer et al. 2008

Table 6: Classification of the differentially expressed genes. See also Buehlmeyer et al. 2008

Gene ID	Mean signal pEX/pCO	RF	Function	Name
Similar to BC006901	0.29	-3.41	Signal transduction	protein kinase nyd-sp15
NM_031657_1	0.32	-3.11	Signal transduction	g protein-coupled receptor kinase 6
NM_030871_1	0.33	-3.02	Signal transduction	phosphodiesterase 1a
NM_030857_1	0.34	-2.98	Signal transduction	lyn protein non-receptor kinase
NM_013006_1	0.41	-2.46	Signal transduction	calcium-independent phospholipase a2
NM_012843_1	0.43	-2.32	Signal transduction	epithelial membrane protein 1
AF271042_1	0.43	-2.31	Signal transduction	Odorant receptor
NM_017010_1	0.43	-2.31	Signal transduction	Glutamate receptor, ionotropic, n-methyl d-aspartate 1
AY014898_1	0.44	-2.29	Signal transduction	inositol polyphosphate multikinase
NM_053816_1	0.44	-2.26	Signal transduction	calcitonin receptor
NM_013088_1	0.46	-2.15	Signal transduction	protein tyrosine phosphatase, non-receptor type 11 (Ptpn11)
X68812_1	0.35	-2.90	Transport	Sodium-calcium exchanger
AJ223355_1	0.43	-2.34	Transport	mitochondrial dicarboxylate carrier
AF150102_1	0.22	-4.52	Transport	small zinc finger-like protein
NM_053427_1	0.27	-3.66	Transport	differentation-associated na-dependent inorganic phosphate cotransporter
NM_058211_1	0.29	-3.40	Transport	solute carrier family 4 member 7
AF081367_1	0.33	-3.03	Transport	atp-regulated k+ channel romk3.1 isoform
NM_017137_1	0.34	-2.93	Transport	chloride channel protein 2
AF135265_1	0.37	-2.68	Transport	large-conductance calcium-activated potassium channel
AF118854_1	0.30	-3.28	Immune system	immune suppressor factor
S79311_1	0.31	-3.22	Immune system	immunoglobulin light chain
NM_019127_1	0.31	-3.22	Immune system	interferon beta 1
AF062038_1	0.33	-3.00	Immune system	glycoprotein-39 precursor
M34097_1	0.40	-2.47	Immune system	rat natural killer cell protease 1
NM_053373_1	0.44	-2.29	Immune system	peptidoglycan recognition protein
NM_053372_1	2.63	+2.63	Immune system	secretory leukocyte protease inhibitor
NM_053474_1	0.25	-3.99	Protein targeting	spinophilin, neurabin
NM_031692_1	0.32	-3.11	Protein targeting	prenylated snare protein ykt6-pending

Gene ID	Mean signal pEX/pCO	RF	Function	Name
AF130121_1	0.38	-2.62	Protein targeting	Wd-containing protein
AB038495_1	0.39	-2.54	Protein targeting	Rat homolog of karp-1-binding protein 1
NM_052829_1	0.41	-2.41	Protein targeting	Rab3 effector rim
NM_017063_1	0.43	-2.34	Protein targeting	Importin beta; karyopherin
NM_053395_1	0.35	-2.88	Cytoskeleton	small muscle protein
X51531_1	0.35	-2.87	Cytoskeleton	atrial myosin light chain 1
NM_053986_1	0.35	-2.87	Cytoskeleton	myosin I heavy chain
NM_012698_1	0.38	-2.63	Cytoskeleton	Dystrophin
NM_017212_1	0.40	-2.52	Cytoskeleton	microtubule-associated protein tau
NM_021681_1	0.49	-2.02	Cytoskeleton	erythrocyte protein band 4.1-like 1 (Epb4.1l1), transcript variant 2
Similar to BC013515	0.10	-9.81	Metabolism	betaine-homocysteine methyltransferase 2
NM_080576_1	0.46	-2.17	Metabolism	Apolipoprotein
NM_031533_1	0.48	-2.07	Metabolism	UDP glycosyltransferase 2 family, polypeptide B (Ugt2b)
NM_017272_1	2.57	+2.57	Metabolism	Aldehyde dehydrogenase
NC_001665_6	2.80	+2.80	Metabolism	Atp synthase subunit 6
NM_013075_1	0.36	-2.80	Transcription factor	homeo box A1
AF277903_1	0.42	-2.37	Transcription factor	Zinc finger protein
NM_021742_1	0.46	-2.16	Transcription factor	nuclear receptor subfamily 5, group A, member 2 (Nr5a2)
AF030378_1	0.26	-3.91	Vascularisation	Angiopoietin-2
NM_031836_1	0.46	-2.19	Vascularisation	vascular endothelial growth factor
AF010442_1	0.35	-2.84	Unknown	marrlc7a
similar to AK007514	0.40	-2.50	Unknown	Unnamed

Gene ID, mean signal ratio of the pooled exercise versus pooled control group (mean signal pEX/pCO), regulation factor (RF), functional classification (function) and name of the regulated genes.

6 Discussion

Evidence-based risk reduction of colon cancer by physical activity (Friedenreich and Orenstein, 2002) poses many questions concerning the underlying biological mechanisms. The aim of our study was to evaluate the effects of voluntary exercise in terms of hormonal changes and alteration of gene expression in rat colon mucosa. 20 male Wistar rats completed 12 weeks of voluntary exercise compared to sedentary controls. A stress-free running model was established to minimise the influence of stress on the hormonal axis.

As shown in **Table 1 and Fig. 7** the mean running distances per night showed great variations between animals. This might at least partly be explained by the fact that an outbred rodent strain was used which usually shows larger interindividual variations compared to an inbred rodent strain (Tou and Wade, 2002). The advantage of an outbred strain is that it is more useful for drawing conclusions that are generally valid because the genetic background is more manifold. An inbred strain would lead to repetitions of the same experiment with several different inbred strains in order to show generalisability of the results. Otherwise one would not know whether the seen effects might only occur in one particular genetic background. Moreover only a multivariate genetic background reflects the situation in humans. Nevertheless, the given activity-versus-time curve of all exercise groups (**Fig. 8**) displayed a trend already described in similar experiments (Reddy et al., 1988, Allen et al., 2001, Nikolaidis et al., 2004), i.e. a rapid increase in the amount of exercise during the first experimental weeks, followed by a plateau phase and a decrease in the mean activity per night.

We further studied exercise volume-dependent variations of body mass, heart mass, hormone concentrations and gene expression. As suggested (hypotheses no. 1) we saw exercise-influenced variations of body and heart weight. The observed increase of heart masses per kg body mass in the high distance running and the medium distance running groups reflected (for more details see also Matsakas et al. 2004) the expected adaptation of the heart to exercise in accordance with previously reported data (Kingwell et al., 1998, Allen et al., 2001). The progression of body weights reflected the growth of the animals but also showed group-specific changes with highest and significant differences in the high distance running and the medium distance running groups as compared to the control animals (**Fig. 9**).

These biometric changes give rise to the assumption that this experimental design is suitable to unravel long-term alterations in the gene expression pattern of colon mucosa and therefore is able to provide insights into the mechanistically background of the colon cancer prevention by physical activity.

6.1 Selected genes - Real-time data and hormone measurements

It was one aim of this study to assess changes in the expression levels of target genes in the rat colon mucosa for which it has been proposed that they either contribute to or mediate the effects of exercise on tissue homeostasis, renewal and apoptosis. We therefore chose genes involved in vascularisation (VEGF, HIF-1α), induction of apoptosis (Bcl-2, PPARγ) or prostaglandin synthesis (COX-2). Genes involved in vascularisation processes are induced by low oxygen conditions. VEGF as a key angiogenic factor is produced by ischaemic tissue and growing tumours (Ferrara et al., 1992), e.g. human colon cancer produce VEGF whose expression is up-regulated in tumour cells by both COX-2 and PGE_2 (Calviello et al., 2004). Prostaglandin synthesis is affected in cancer development (Pugh and Thomas, 1994) as well as after physical activity (Martinez et al., 1999). In addition, physical activity may induce the apoptotic cascade in different tissues, such as skeletal muscle and heart muscle as well as in cancer cells (Leung et al., 2003). In contrast, a growing tumour fails to induce apoptosis and promotes cell survival (Fehrenbach and Northoff, 2001).

Gene expression analysis was performed by real-time RT-PCR. This method is characterised by high sensitivity, good reproducibility and a wide quantification range (Pfaffl et al., 2002). To avoid variations due to unequal sample amounts, normalisation to housekeeping genes was the method of choice. As previously reported (Thellin, 1999) the expression of housekeeping genes is not always stable and unaffected by treatment. In addition, the influence of physical exercise on housekeeping gene expression is not well investigated (Murphy et al., 2003, Jemiolo and Trappe, 2004, Mahoney et al., 2004,). This stresses the importance of choosing a valid housekeeping gene for the normalisation process. One possibility is the inclusion of more than one housekeeping gene. We decided to normalise to several genes (ß-actin, ALDA, GAPDH) presumed to be not affected by physical activity. Hence the BestKeeper tool was used (Jemiolo and Trappe, 2004, Pfaffl et al., 2004). We found that three (GAPDH, ß-actin, ALDA) of our four housekeeping genes

remained unaffected by exercise and identified 18S rRNA as the most unstable housekeeping gene (**Table 2**). This result is in contrast to previous studies in colon adenoma, cancers or normal colonic mucosa which have frequently employed 18S rRNA as a reference gene and identified it as the most stable one, e.g. Tsuji et al. (2002) and also Bryner et al. (Bryner et al., 2004) used 18S for normalisation in a muscle mRNA expression study. The effect of exercise on mRNA expression levels of housekeeping genes has not been systematically studied and appears to vary in different tissues (Murphy et al., 2003, Jemiolo and Trappe, 2004, Mahoney et al., 2004). We therefore decided to normalise target gene expression to the three housekeeping genes that we had identified as the most stable ones by application of the BestKeeper tool (Jemiolo and Trappe, 2004, Pfaffl et al., 2004), an established tool that allows to calculate BestKepper-values from several housekeeping genes.

Out of six selected target genes only the gene encoding ODC-1 revealed a significant change in mRNA expression levels (**Table 3 and 4**). None of the other genes involved in the processes of vascularisation (VEGF, HIF-1α), induction of apoptosis (Bcl-2, PPARγ) or prostaglandin synthesis (COX-2) were significantly affected.

Even though there is existing evidence of the influence of exercise on prostaglandin synthesis (hypothesis no. 3) and apoptosis (hypothesis no. 4) we could not observe any modulation of the investigated genes. Vascular processes were suggested to be influenced after physical activity (hypothesis no. 2) and we could confirm this by showing that ODC-1, which is involved in vascularisation processes, was regulated following physical exercise. Activity of ODC-1 is known to be increased after various stimuli including food intake and selected hormones. In addition this enzyme is being discussed as a regulator and modulator of gastrointestinal cell growth. The enzyme displays an early response to a variety of growth-promoting stimuli (reviewed in detail by Schipper and Verhofstad 2002) and measurements of ODC-1 in rat muscle following endurance and resistance training revealed increased activity (Turchanowa et al., 2000). To date there aren't any experimental studies reporting changes in colon ODC-1 following exercise (neither mRNA, nor protein, nor activity level) and therefore the observation that ODC-1 as a target of an altered energy turnover is differentially expressed in the colon as a result of enhanced physical activity is novel. With respect to the proposed role of ODC-1 as a marker of tissue damage and remodelling (Schipper and Verhofstad, 2002) our finding may be taken as an indicator that physical activity leads to alterations in energy turnover or to metabolic

changes which in turn induce a remodelling of the colonic mucosa. Polyamines are produced in a pathway in which ODC-1 acts as a rate-limiting enzyme and have been shown to possess an indirect protective effect on nuclear DNA after oxidative stress (von Deutsch et al., 2005). Therefore it may be speculated that an increased transcription of ODC-1 may contribute to the protection of DNA in the colonic mucosa. This is especially important in running animals, which are exposed, to higher oxidative damage as a result of physical activity. It remains to be determined whether indeed physical activity or nutritional variations (e.g. different amount of food intake, altered exposure of specific nutrients) are responsible for the changes of gene expression in rat colon mucosa. Yet unpublished data from follow-up experiments indicate that food intake strongly increases with physical activity compared to sedentary animals. Nevertheless, body weight of active animals remains lower.

IGF-regulated processes display the most profound processes with respect to the influence of physical activity and colon cancer prevention. Therefore it is not amazing that peripheral IGF concentrations are regulated by exercise as suggested before (hypothesis no. 5) but it is remarkable that we could not confirm the hypothesis (no. 6) and none of the most important genes involved in IGF pathways seem to be affected in our approach.

With regards to the serum IGF-1 concentration, there were exercise volume-dependent variations showing lower values at higher running activities (**Fig. 13**). The relevant literature describes predominantly increased or constant circulating IGF-1 concentrations in rats (Anthony et al., 2001, Bravenboer et al., 2001, Cooper et al., 1994, Yeh et al., 1994) and humans (Ngyen et al., 1998, Chadan et al., 1999, Wallace et al., 1999, Kraemer et al., 2004) after various exercise periods. Nevertheless, some human studies indicate that long-term exercise can decrease circulating IGF-1 (Suikkari et al., 1989, Koistinen et al., 1996, Nehmet et al., 2002) and also a number of animal experiments showed constant or slightly decreased serum IGF-1 following physical exercise (Banu et al., 1999, Colbert et al., 2003, Matsakas et al., 2004). These inconsistent findings may be due to the variety of activity models, ages, rat strains and genders studied. This makes it almost impossible at the moment to compare the studies and explain the results in detail. However, our data showed a significant positive correlation between circulating IGF-1 and body weight. This finding is in accordance with a human study which detected significantly lower body mass indexes and IGF-1 levels in the activity group

compared to the control group (Barnard et al., 2003, Leung et al., 2003). The negative correlation between physical exercise and body weight is widely accepted and well documented (Tou and Wade, 2002). Nutrition studies provide evidence for a correlation between decreased circulating IGF-1 and energy restriction (Kritchevsky, 1999). Taken together, we hypothesise that in our approach physical exercise reduced body weight and hence decreased IGF-1 levels in the blood. This might provide a preventive mechanism of colon cancer development by physical activity, since it is known that clinical conditions characterised by elevated blood IGF-1 concentrations (acromegaly) increase the risk of colon cancer (Giovannucci, 2001, Kaaks and Lukanova, 2002, Sandhu et al., 2002).

The mRNA steady-state levels of IGF-1, IGF-1R and IGF-BP3 in rat colon mucosa were analysed by real-time RT-PCR. To our knowledge, no other study has examined the influence of exercise on the expression of the genes encoding IGF-1 and the proteins mediating its action in rat colon mucosa. Similar to the study in different muscle types (Matsakas et al., 2004, Matsakas et al., 2005), we found no exercise-dependent regulation of IGF-1 mRNA levels in colon mucosa. This is in accordance with the sparse literature in this field (Zanconato et al., 1994, Eliakim et al., 1997). Based on the major role of the liver in IGF-1 synthesis (>80% of the circulating IGF-1 is derived from the liver) (Kaaks and Lukanova, 2002), the observed colon-specific IGF-1 gene expression is presumably not related to alterations of circulating IGF-1 levels. Recent studies in mice confirm this by showing that predominantly hepatic IGF-1 expression is responsible for alterations in IGF-1 serum levels as hepatic IGF-1 gene deletion reduced circulating IGF-1 by about 75% (Yakar et al., 1999, Yakar et al., 2001). Therefore gene expression analysis in the liver after exercise would be of great interest. Nevertheless, Lund et al. (1986) reported the presence of IGF-1R mRNA in rat intestine and suggested that IGF-1 and its receptor play an important role in the growth and function of this tissue. Given the role of the IGF system in colon tumour growth (Grimberg and Cohen, 2000), we considered it of scientific value to examine whether physical activity influences the expression of genes related to this system in the colon mucosa. The gene expression analysis in our model revealed no significant differences in the steady-state levels of IGF-1, IGF-1R and IGF-BP3 mRNA between the exercise and control groups. Since there is evidence that gene expression at the mRNA level varies widely in the different parts

of the colon (Zhang et al., 1998), future gene expression studies might have to take this into account.

6.2 cDNA micro-array data

As suggested in the beginning (hypothesis no. 7) we found several regulated pathways by cDNA micro-array analysis. Some of them have already been connected to colon cancer prevention others not but all results holds promise for new insights in the genetic regulation of colonic mucosal variations following physical activity.

Traditionally, the regulation of biochemical and genetic pathways by physical activity has been studied on a gene-by-gene basis. The majority of the gene expression analyses focused on variations in the skeletal muscle. In this part of the study, we employed cDNA micro-arrays for large-scale gene expression profiling in the rat colon mucosa. This approach holds promise for the discovery of new genes and biochemical pathways involved in the preventive effect of physical activity on the development of colon cancer. After the screening procedure a data base search (Pubmed/Medline, http://www.ncbi.nlm.nih.gov/sites/entrez) was performed to functionally classify the identified regulated genes (**Table 6, Fig. 18**). Some of the regulated genes we found in our experiment have already been described in earlier studies. This is the case for Ptpn11, which encodes protein tyrosin phosphatase non-receptor type II. Kim et al. (1999) found no alteration of the mRNA in rat skeletal muscle after a 9 weeks training session. It is well known from muscle research that physical activity has several effects on gene expression and therefore influences a broad range of processes like vascularisation, growth, and metabolism (Zambon et al., 2003, Kojda and Hambrecht, 2005). Therefore it is not remarkable that our long-term exercise model alters several mucosal genes involved in vascularisation, cellular transport, signal transduction, metabolism, protein targeting, immune function, transcription, and cytoskeleton. A further comparison of published speculated mechanisms involved in the risk reduction of colon cancer by physical activity with the identified gene classes from this study and additional possible influences of exercise resulted in the following hypothesis: the genes involved in immune functions, metabolic processes, vascularisation and signal transduction seem to be of great interest.

6.2.1 Immune system

The immune system is thought to be influenced directly by physical activity (Fehr et al., 1989, Nieman, 1994, Shephard and Shek, 1995, Pedersen and Hoffman-Goetz, 2000) and therefore may contribute to cancer prevention. The mucosal immune response is discussed in a number of publications (Friedenreich and Orenstein, 2002, Quadrilatero and Hoffman-Goetz, 2003, Slattery, 2004) to be potentially affected after physical exercise. In our study, we identified differentially expressed genes that belong to the immune system: a) immune suppressor factor J6B7-like protein (ISF-J6B7), b) immunoglobulin light chain (IG-κ), c) interferon beta 1 (IFN-ß1), d) glycoprotein-39 precursor (GP-39p), e) sectretory leucocyte protease inhibitor (SLPI), f) rat natural killer cell protease 1 (RNKP-1), a homologue to granzyme B (Fell et al., 2002), and g) the peptidoglycan recognition protein (PGlyRP). Data with regard to the effect of exercise on these genes or proteins are sparse. To our knowledge a link between ISF-J6B7, GP-39p and PglyRP and physical activity has never been described before. In some publications, blood samples were used to detect the protein of interest (e.g. granzyme B, IGs, IFNs) (Viti et al., 1985, Nehlsen-Cannarella et al., 1991, Kohut et al., 2004) but there are no studies yet looking at these targets in the colonic mucosa. It is well known that the immune system plays an important role in the tumour defence (Fairey et al., 2002, Philpott and Ferguson, 2004) and that exercise influences a huge range of peripheral immune parameters (Peters et al., 1994, Nieman and Pederson, 1999, Pedersen and Hoffman-Goetz, 2000). This is the first published study showing a local immune response in the colon mucosa following exercise. The presented regulation of mucosal immunological factors by physical activity supposable contributes to a reduced risk of developing colon cancer.

6.2.2 Metabolic processes

Physical exercise leads to various metabolic changes in a broad range of tissues including adipose tissue, skeletal muscle or heart (Petridou et al., 2005). Hence it is not surprising that voluntary physical activity in our study modulates 5 genes of rat colon mucosa encoding metabolic proteins: Betaine-homocysteine methyltransferase 2 (BHMT2), ATP synthase subunit 6 (ATPsynthase), Aldehyde dehydrogenase (ADH), Apolipoprotein (ALP), and UDP glycosyltransferase 2 family, polypeptide B (UGT2b). BHMT2 is the gene with the most profound changes in transcript levels

(about 10-fold) and the only one in this group for which we found a link to colon cancer. It is a zinc-dependent enzyme linked to the synthesis of dimethylglycine and methionine (Castro et al., 2004). The latter is an important methyl donor for DNA and therefore involved in DNA synthesis/repair and epigenetic processes. Aberrant DNA methylation plays a role in cancer development. This has been observed in a variety of tumours amongst others human colon cancer. Not only genomic aberrations take place in human neoplastic tissues, but also site specific alterations in methylation (hypo- and hypermethylation) appear (Choi and Mason, 2000). Increased DNA methylation is a common event in cancer development (Muller et al., 2004). There is additional evidence that DNA methylation in tumours probably increases mutational rates. Two pathways lead to the methyl-donor methionine: the folate-dependent way with homocysteine methyltransferase as the corresponding enzyme or the BHMT-reaction. Nutritional studies described that the BHMT-pathway is more active in rodents compared to humans (Choi and Mason, 2000). Thus in our animal experiment the down-regulation of BHMT2 in the colon mucosa may result in decreased methylation of DNA. This might be an adaptation following physical exercise and presumably diminishes DNA damage. Recently a decreased level of 8-hydroxydeoxyguanosine in the DNA of colonocytes has been shown after endurance training of dogs indicating enhanced DNA repair capacity (Okamura et al., 1997). Many references show that regular physical exercise has the potential to activate compensatory mechanisms against oxidative stress (Radak et al., 2001). Accordingly, it is possible that long-term physical activity protects against aberrant methylation by repressing BHMT2 and thus contributes to a reduced risk of developing colon cancer.

6.2.3 Vascularisation

We have also identified vascular endothelial growth factor (VEGF) and angiopoietin-2 (ANG-2) both with markedly reduced transcript levels in the mucosa of active rats. These genes play an important role in angiogenesis. According to several studies VEGF may lead to increased levels of ANG-2 and this cooperation might be a critical point in tumour neovascularisation (Hideyasu et al., 1999, Zhang et al., 2003). Recent data show a link between increased ANG-2 and VEGF levels and enhanced tumour angiogenesis and tumour growth (Ahmad et al., 2001a). Both genes show higher expression levels in colon tumour tissue compared to normal tissue (Yoshida

et al., 1999, Ahmad et al., 2001b). As shown in our experiment voluntary running activity seems to have the potential to reduce mRNA expression levels of both genes. Steady-state mRNA levels of VEGF and ANG-2 were found to be about 2-fold and about 4-fold respectively down-regulated in the colon mucosa of long-term exercising rats. However when more animals included we failed to find regulation of VEGF in rat colon mucosa via quantitative real-time RT-PCR. This apparent discrepancy between these two methods has to be further clarified. One obvious reason seems to be splice-variants of the VEGF-gene (e.g. ENSRNOT00000026516, ENSRNOT00000026559, ENSRNOT00000026637; see http://www.ensembl.org). Therefore the results must be carefully interpreted and further experiments have to consider these known splice variants. Nevertheless, the down-regulation of these two genes as observed here may contribute to the reduction in the development of colon cancer by physical activity.

6.2.4 Signal transduction

Evidence exists that prostaglandin (PG) variations play a role in the prevention of colon cancer (Martinez et al., 1999, Quadrilatero and Hoffman-Goetz, 2003). Several studies showed changes of PG levels in the blood, urine, and muscle following physical exercise (reviewed by Quadrilatero and Hoffman-Goetz 2003). According to findings in clinical research differences in prostaglandine levels were also observed between normal and colon cancer tissue samples. Higher PG levels of the E series were shown in the neoplastic specimens (Pugh and Thomas, 1994). Furthermore an aggressive tumour progression is associated with a tumour derived increase in PG production (Futura et al., 1988). More importantly nonsteroidal anti-inflammatory drugs have a protective effect against colon cancer development. This effect is based on the inhibition of cyclooxigenase (with the isoenzymes COX-1 and COX-2), an enzyme involved in PG synthesis (Giovannucci et al., 1994). Arachidonic acid (AA) is metabolised by both COX isoforms to PGs which are further metabolised to eicosanoids which act as proinflammatory mediators (Akiba and Sato, 2004). We already showed via real-time RT-PCR analysis that there is a tendency towards decrease of COX-2 mRNA in rat colonic mucosa following intensive physical exercise. Also involved in these processes is the calcium-independent phospholipase A2 (iPL-A2), however the cytosolic and secretory PL-A2s seem to be more important for AA release (Akiba and Sato, 2004). Nevertheless, the participation of the different

iPL-A2s is known to be cell-type and stimulus dependent (Akiba and Sato, 2004). Apart from the PG synthesis, the iPL-A2 induced AA variation influences apoptosis. This proapoptotic potential of AAs also varies from tissue to tissue (Kudo and Murakami, 2002). The 2.5-fold down-regulation of iPL-A2 in rat colonic mucosa following physical activity might reduce the AA levels in the mucosal cells and as a consequence might influence apoptotic pathways and decrease prostanoid and leukotriene production. The iPL-A2 reduction might reflect the cell's reaction in response to external factors to maintain cellular phospholipid homeostasis. To our knowledge this is the first study showing a down-regulation of iPL-A2 in the colonic mucosa in response to prolonged exercise. Our data indicate an influence of exercise on AA levels by decreased iPL-A2 expression. Hence this presents an important link between colon cancer and physical activity even though we are currently not able to speculate about positive or negative effects of exercise in this context.

7 Summary

The development of colon cancer is highly influenced by life style factors such as nutrition and physical activity. Epidemiology confirms that physical activity has the potential of reducing the risk of colon cancer. The underlying biological mechanisms are still to be unravelled. The purpose of this study was to investigate the effects of chronic wheel running on the gene expression in rat colon mucosa by real-time RT-PCR and cDNA micro-array analysis. Therefore six-week-old male Wistar rats completed a stress free voluntary running exercise period of 12 weeks. Sedentary rats served as a control group. Running distances, body weights and heart weights as measures of physical adaptations were recorded. The serum IGF-1 levels were analysed exercise volume-dependent.

As the running distances showed great inter-individual differences we divided the animals of the running group in three subgroups of low (L-EX< 2629 m/night; n = 5) medium (M-EX, 3003-7458 m/night; n = 10), and high (H-EX, >8314 m/night; n = 5) distance running. The M-EX and H-EX groups revealed significant ($p < 0.05$) adaptive changes with an increase in heart mass per kg body weight and a decrease in mean body weight. Changes in mRNA steady-state levels of marker genes involved in vascularisation (VEGF, HIF-1α, ODC-1), apoptosis (Bcl-2, PPARγ) and prostaglandin synthesis (COX-2) were determined by qRT-PCR since these processes have been hypothesized as being influenced by physical activity. The four housekeeping genes GAPDH, ß-actin, 18S and ALDA served as reference genes. Amongst the marker genes studied by mRNA expression analysis only ODC-1 was differentially expressed at mRNA level. Its 1.8-fold increase in mRNA level in the H-EX group suggests, that synthesis of polyamines may be enhanced by physical activity. This finding could provide a new mechanism to explain the protective effect of physical activity on colon cancer development.

There is increasing evidence for a close link between the insulin/insulin-like growth factor system and colon cancer prevention by physical exercise. In a rat model with voluntary long-term exercise we have assessed the resulting changes in serum insulin-like growth factor (IGF) and of colonic target genes of the IGF-system. Gene expression of IGF-1, IGF-1 receptor and IGF-binding protein 3 were quantified by real-time RT-PCR. Circulating IGF-1 was measured by ELISA. Based on the three subgroups of low, medium and high exercise intensity, we observed lower serum

IGF-1 levels (P < 0.05) in all exercise groups as compared to the control group. Moreover there was a negative correlation between IGF-1 levels and running distances. A significant (P < 0.05) positive correlation was found between IGF-1 concentration and body mass (r = 0.50) and a significant negative correlation exists between body mass and running distances (r = -0.50). Significant changes in colonic mRNA levels of IGF-1, IGF-1R and IGF-BP3 could not be observed. Based on our results we propose that the activity-induced body mass reduction leads to a decrease in circulating IGF-1 that might contribute to protection against colon cancer development.

In the colonic mucosa of exercise and control animals, steady-state mRNA expression levels of approximately 10,000 genes were compared between both groups by cDNA micro-array analysis (MWG rat 10k array). 8,846 mRNAs were detected with a signal intensity above background level. Chronic exercise led to a decreased expression of 47 genes at a threshold-factor of 2.0. Three genes were found to be up-regulated in the exercise group. The identified genes encode proteins involved in signal transduction (n=11), transport (n=8), immune system (n=7), cytoskeleton (n=6), protein targeting (n=6), metabolism (n=5), transcription (n=3), and vascularisation (n=2). Among the genes regulated by chronic exercise, the betaine-homocysteine methyltransferase 2 (BHMT2) seems to be of particular interest. Physical activity may protect against aberrant methylation by repressing the BHMT2 gene and thus contribute to a decreased risk of developing colon cancer. We have also identified vascular endothelial growth factor (VEGF), angiopoietin-2 (ANG-2) and calcium-independent phospholipase A2 (iPL-A2), all of which showed markedly reduced transcript levels in the mucosa of active rats. In summary, our experiment presents the first comprehensive screening of gene expression changes in rat colon mucosa in response to chronic wheel running and represents an important step in understanding the molecular mechanisms responsible for the preventive effect of physical activity on the development of colon cancer.

8 Zusammenfassung

Die Darmkrebsentstehung wird stark von Lebensstilfaktoren, wie der Ernährung und der körperlichen Aktivität beeinflusst. Epidemiologische Erkenntnisse bestätigen, dass körperliche Aktivität das Risiko, an Darmkrebs zu erkranken beträchtlich reduziert. Die zugrundeliegenden biologischen Mechanismen sind jedoch derzeit noch unklar. Das Ziel dieser Studie war es daher, die Effekte von langandauernder körperlicher Aktivität auf die Genexpression der Darmmukosa in Ratten zu untersuchen. Dies geschah mittels real-time RT-PCR und cDNA Micro-Array-Analyse. In diesem Zusammenhang wurden sechs Wochen alte Ratten über 12 Wochen einem stressfreien Lauftraining unterzogen. Tiere, die keine Möglichkeit zu körperlicher Aktivität hatten, dienten als Kontrollgruppe. Die Laufdistanzen, Körpergewichte und Herzgewichte aller Tiere wurden als Marker für die körperliche Aktivität erfasst. Aktivitätsabhängig wurden zudem die Serum-IGF-1-Level gemessen.

Die Laufdistanzen der einzelnen Tiere zeigten immense inter-individuelle Unterschiede. Anhand dieser wurden die aktiven Tiere im weiteren Verlauf der Analyse in drei verschiedene Laufgruppen eingeteilt. Dadurch erhielt man eine Gruppe mit Tieren, die geringe Distanzen (L-EX, < 2629 m/Nacht, n = 5), die mittlere Distanzen (M-EX, 3003-7458 m/Nacht, n = 10) und die lange Distanzen (H-EX, >8314 m/Nacht, n = 5) absolvierte. Die M-EX und die H-EX-Gruppen zeigten signifikante adaptive Veränderungen des Herz- und Körpergewichts, welches sich in einem signifikanten Anstieg des Herzgewichts pro kg Körpergewicht zeigte. Ebenso konnte eine signifikante Reduktion des Körpergewichts in diesen beiden Gruppen im Vergleich zur Kontrollgruppe gezeigt werden. Anhand der quantitativen real-time RT-PCR wurden Veränderungen der mRNA-Level von Markergenen erfasst, welche in Vaskularisierungs- und Apoptoseprozesse, sowie Veränderungen der Postaglandinsynthese involviert sind, da Literaturdaten darauf hin deuten, dass diese Prozesse durch körperliche Aktivität beeinflusst werden. Die vier Housekeepinggene GAPDH, ß-actin, 18S und ALDA wurden als Referenzgene in Betracht gezogen. Von den Markergenen, welche mittels mRNA-Expressionsanalyse untersucht wurden zeigte lediglich ein Gen signifikante Veränderungen. Die 1,8-fach gesteigerte Expression der ODC-1 mRNA innerhalb der H-EX-Gruppe lässt vermuten, dass als Folge der körperlichen Aktivität die Synthese von Polyaminen in dieser Gruppe

erhöht sein könnte. Diese Erkenntnis eröffnet neue Ansätze, den Einfluss von körperlicher Aktivität auf die Darmkrebsentstehung biologisch und mechanistisch zu untersuchen.

Die Evidenzen, dass das Insulin/Insulin-like growth factor (IGF)-System massgeblich am Einfluss von körperlicher Aktivität auf das Darmkrebsrisiko beteiligt ist verdichten sich. Darum wurden anhand des dargestellten Modells eines freiwilligen, langfristigen Laufexperiments die resultierenden Veränderungen im Serum-IGF-Spiegel sowie die IGF-Zielgene in der Mucosa des Kolons untersucht. Die Genexpression von IGF-1, IGF-1 receptor and IGF-binding protein 3 wurden mittels quantitaitver real-time RT-PCR bestimmt. Das zirkulierende IGF-1 wurde anhand eines ELISA-Tests gemessen. Es konnten signifikant ($P < 0.05$) geringere Serum-IGF-Level in allen Läufergruppen (L-EX, M-EX and H-EX) im Vergleich zur Kontrollgruppe festgestellt werden. Die IGF-Spiegel zeigten eine negative Korrelation zur Laufdistanz. Zwischen der IGF-Konzentration in der Peripherie und dem Körpergewicht konnte eine positive Korrelation festgestellt werden ($P < 0.05$, $r = 0,50$), wohingegen das Körpergewicht und die Laufleistung eine signifikant negative Korrelation aufwiesen ($P < 0.05$, $r = -0,50$). Es wurden keine signifkanten Veränderungen in der mRNA-Expression von IGF-1, IGF-1R und IGF-BP3 in der Mukosa des Kolons gefunden. Basierend auf den dargestellten Daten wird vermutet, dass die aktivitätsinduzierte Körpergewichtsreduktion zu einem Abfall des zirkulierenden IGF-1 führt. Dies könnte eine Verknüpfung zur Darmkrebsprävention durch Sport darstellen.

Zusätzlich wurde das zeitpunktabhängige Expressionverhalten von nahezu 10,000 Genen von Läufer- und Kontrolltieren anhand einer cDNA Micro-array-Analyse (MWG rat 10k array) untersucht. Dabei wurden 8,846 mRNAs detektiert, deren Signal über dem Fluoreszenzhintergrund hervortrat. Langzeitaktivität führte dabei zu einer Reduktion der Genexpression von 47 Genen bei einem gesetzten Schwellenwert von mindestens 2-facher Regulation. Drei Gene wurden in der körperlich aktiven Gruppe im Vergleich zur Kontrollgruppe verstärkt exprimiert. Die identifizierten Gene kodieren dabei für Proteine, welche eine Rolle bei Signaltransduktionsprozessen (n = 11), Transportprozessen (n = 8) und im Immunsystem (n = 7) spielen. 6 Gene stellen Strukturgene des Cytoskeletts dar. Weitere 6 Gene sind in das Proteintargeting eingebunden. Die einzelne Gene lassen sich folgenden Funktionsklassen zuordnen: Metabolismus (n = 5), Transkription (n = 3) und Vaskularisierung (n = 2). Von all diesen Genen, die durch die langfristige

körperliche Aktivität reguliert wurden, scheint vor allem die betaine-homocysteine methyltransferase 2 (BHMT2) von besonderer Bedeutung zu sein. Körperliche Aktivität scheint hierbei das Potential zu haben, gegen aberrante Methylierung zu schützen, indem die Expression von BHMT2 supprimiert wird. Dies könnte das Risiko einer Darmkrebsentwicklung senken. Zudem wurde eine veränderte Expression des vascular endothelial growth factor (VEGF), des angiopoietin-2 (ANG-2) und der calcium-independent phospholipase A2 (iPL-A2) gefunden. Jedes dieser 3 Gene zeigte deutlich reduzierte Transkriptionsspiegel in der Mukosa von körperlich aktiven Ratten. Zusammenfassend zeigte dieser Teil der Untersuchung zum ersten Mal ein umfassendes Genexpressionmuster der Darmmukosa von körperlich aktiven Ratten im Vergleich zu nicht aktiven Kontrollratten. Dies repräsentiert daher einen überaus wichtigen Schritt im Verständnis der molekularen Mechanismen der Darmkrebsprävention durch körperliche Aktivität.

9 Addendum

9.1 Primers

Table 7 summarises primer sequences established in rat colon mucosa with their specific annealing (AT) and quantification temperatures (QT).

Table 7: Primer sequences used for quantitative real-time RT-PCR

Name	Forward 5´ to 3´	Reverse 5´ to 3´	AT	QT
ß-actin	CTA CGT CGC CCT GGA CTT CGA GC	GAT GGA GCC GCC GAT CCA CAC GG	60°C	79°C
AldolaseA	ATG CCC CAC CCA TAC CCA GCA CT	AGC AGC AGT TGG CGG TAG AAG CG	64°C	79°C
Bcl2	CTC GTC GCT ACC GTC GCG ACT TTG	CAG ATG CCG GTT CAG GTA CTC AGT C	59°C	79°C
COX2	TGA ACA CGG ACT TGC T	TGG GAT TAC CCA TAA GGC	58°C	81°C
Emp1	CTC GTC CAT AGG GCT TT	ACA CTC CAA TCA GAA TGC	60°C	79°C
GAPDH	GTC TTC ACT ACC ATG GAG AAG G	TCA TGG ATG ACC TTG GCC AG	60°C	79°C
HIF1α	GTC TGG GTT GAG ACT CAA G	AGT TTC GT GTC ATC GCT G	56°C	84°C
IGF-1	AAG CCT ACA AAG TCA GCT CG	GGT CTT GTT TCC TGC ACT TC	58°C	79°C
IGF-1R	AAA ACC ATC GAT TCT GTG ACG	GGT TCT TCA GGA AGG ACA AGG	60°C	79°C
IGFBP-3	CGC TAC AAA GTT GAC TAT GAG	CGT CTT TCC CCT TGG T	60°C	79°C
ODC-1	CCT GAG ACC TTC GTG C	GCT GAT GCG ACG TAG T	60°C	79°C
PPARγ	GCC CAC CAA CTT CGG A	AGA CTC GGC ACT CGA T	58°C	79°C
RNKP-1	CAG ACG CAA CGT CAA AG	CCG TGG AGT TGA ACC AT	60°C	79°C
SLPI	TCC CTC TCA TGG TGC T	ACC ACA GGT ATC TTG GC	60°C	79°C
Ubiquitin	CGC TGG GCG TTT GTT CCT TC ATC	TCT GGA TGT TGT AGT CAG AGA GGG	58°C	79°C
VEGF	CAC CCA CGA CAG AAG G	GAC GTT GCT CTC CGA C	56°C	79°C
18S	AAG TCT TTG GGT TCC GGG	GGA CAT CTA AGG GCA TCA CA	58°C	82°C

AT = annealing temperature, QT = quantification temperature, COX2 = cyclooxigenase 2, Emp1 = epithelial membrane protein 1, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, HIF-1α = hypoxia-inducible factor 1α, IGF-1 = insulin-like growth factor 1, IGF-1R = insulin-like growth factor 1 receptor, IGFBP-3 = insulin-like growth factor binding protein 3, ODC-1 = ornithin decarboxylase-1, PPARγ = peroxisome proliferator-activated receptor γ, RNKP-1 = rat natural killer protease-1, SLPI = secretory leukocyte protease inhibitor, VEGF = vascular endothelial growth factor

Table 8: Primer sequences which failed to establish in real-time RT-PCR

Name	Forward 5´ to 3´	Reverse 5´ to 3´	Reliability	MC	PCR-gel	Dilution
Agpt2	CGG CGA GGA GTC TAA C	GTT GAA CTT ATT CGT ATT CTG CT	fine	fine	fine	0
Bax	CGG CGA ATT GGA GAT GA	GGT CCC GAA GTA GGA G	0	OK	0	--
Bcl-xα	CACTGTGCGTGGAAAGC	GTCATGCCCGTCAGGA	0	fine	fine	--
BHMT2	GCA GTT CGT CAA CTT CAC	GAT GTC TGG CAG ATG CC	OK	0	0	--
FAS	CCTTAGTAGTGCGTGG	AATTTGCCGATCTCTAGGA	0	fine	0	--
MPtx	GAATCGTCCACTGCCTATGTGTCCC	CCAGAGGCAGACTCCCAGTTCACAC	OK	0	0	--
ncx	AAC AAT ATC AGT CAA GGT AAT CG	TGG TGT GTT CGC CTA GA	OK	fine	0	--
PCNA	TCGACACATACCGCTG	GGTCCCGGCATATACG	fine	0	fine	--
Pfk	AGG TGA CCA AAG ACG TGA CC	TAT GTT GGC ACT GAT CTG TTC C	fine	OK	OK	--
Slc25a10	AAC TAC TCT CAC GCC C	CAC AAT GGA AAA CAC CCT	fine	fine	0	--

Reliability: good reproducibility, MC: clear melting curve, PCR-gel: clear single band after gel electrophoresis, dilution: optimal dilution series;

Explanation of the test results:　fine　=　optimal results in repeated experiments,
　　　　　　　　　　　　　　　OK　=　optimal to suboptimal results with need for further optimisation,
　　　　　　　　　　　　　　　0　=　nonsense results which can´t be used for further analysis,
　　　　　　　　　　　　　　　--　=　test omitted because of previous results

Agpt2 = angiopoietin-2, Bax = Bcl2-associated x protein, BHMT2 = betaine-homocysteine methyltransferase 2, FAS = fatty acid synthase, MPtx = mucosal pentraxin, ncx = sodium-calcium exchanger, PCNA = proliferating cell nuclear antigen (cyclin), Pfk = phosphofructokinase, Slc25a10 = mitochondrial dicarboxylate carrier.

9.2 Individual running distances

Table 9: Running distances (m) of animals used for sample pooling

Animal number	Running distance (m/night)	
	Mean	SD
1	8446	3434
2	8315	2550
3	7458	3403
4	6732	3000
5	6393	2573
6	6245	3325
7	5897	4672
8	5747	2800

Running distances in meters (m) of individual animals are shown as the mean ± standard deviation (SD) over the 12 weeks voluntary running exercise period. Animals were included in the pool based on RNA purity and quantity as well as the running distance.

9.3 List of abbreviations

AA	Arachidonic acid
AT	Annealing temperature
COX2	Cyclooxygenase 2
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
Emp1	Epithelial membrane protein 1
FGF	fibroblast growth factor
Fig.	Figure
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hour(s)
HIF-1α	Hypoxia-inducible factor 1α
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IGFBP-3	Insulin-like growth factor binding protein 3
IL-6	Interleukin-6
LEF	lymphoid enhancing factor
m	Meter(s)
MET	The amount of energy expended sitting quietly at rest adjusted to body weight (1 MET = 3.5 ml oxygen consumed/kg of body weight/minute). Also equal to 1 kcal/kg/hour.
min.	Minute(s)
no.	number
ODC-1	Ornithin decarboxylase-1
PCR	Polymerase chain reaction
PG	Prostaglandin
PPARγ	Peroxisome proliferator-activated receptor γ
QT	Quantification temperature
RNA	Ribonucleic acid
RNKP-1	Rat natural killer protease-1
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
sec.	Second(s)
SLPI	Secretory leukocyte protease inhibitor

TCF   T-cell factor

TGF   Transforming growth factor

TNF-α Tumour necrosis factor α

VEGF   Vascular endothelial growth factor

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Lebenslauf

Name:	Katja Bühlmeyer
Geburtsdatum:	03.04.1979
Geburtsort:	Miltenberg
Familienstand:	ledig
Staatsangehörigkeit:	deutsch

Schul-/Ausbildung

1985-1989	Besuch der Grundschule Amorbach
1989-1998	Besuch des Karl-Ernst Gymnasiums Amorbach
26.07.1998	Erlangung der allgemeinen Hochschulreife Abiturnote: 1,9
1998-2002	Studium der Sportwissenschaft an der Deutschen Sporthochschule Köln
2000-2002	Biologiestudium an der Universität zu Köln
22.10.2002	Erhalt des Diploms im Studiengang Sportwissenschaft – Studienschwerpunkt Prävention und Rehabilitation an der Deutschen Sporthochschule Köln Note des Diploms: 1,9
Okt. 2005-Sept. 2006	Beendigung des Biologiestudiums an der Technischen Universität München
30.09.2006	Erhalt des Akademischen Grades Bachelor of Science nach erfolgreichem Abschluss im Studiengang Biologie mit der Gesamtnote: 1,8

wissenschaftlicher Werdegang

01.06.2000-31.12.2002	studentische Hilfskraft am Institut für Morphologie und Tumorforschung der Deutschen Sporthochschule Köln

01.06.2000-31.03.2002	studentische Hilfskraft am Institut für Kreislaufforschung und Sportmedizin der Deutschen Sporthochschule Köln
01.04.2002-31.09.2002	studentische Hilfskraft am Institut für Biochemie der Deutschen Sporthochschule Köln
01.10.2002-31.09.2005	wiss. Mitarbeiterin am Lehrstuhl für Sport- und Gesundheitsförderung der Technischen Universität München

beruflicher Werdegang:

01.02.2006-14.04.2007	Selbstständige Tätigkeit im Bereich Sport und Gesundheit
seit 15.04.2007	Mitarbeiterin in der Vorentwicklung der Firma GRAMMER

Parts of this work have already been published:

Original published or accepted papers:

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit

Exercise Associated Genes and their Involvement in the Prevention of Colon Cancer - A Voluntary Running Experiment in a Rat Model

selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die den benutzten Quellen wörtliche oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Dies Arbeit hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen.

Amberg, 29.04.2008

Katja Bühlmeyer