CANCER PREVENTIVE MECHANISMS BY EXERCISE: ACTIVATION OF P53 AND P53-RELATED IGF-1 PATHWAY REGULATORS

by

MIAO YU

B.E., Jinan University, P. R. China, 2010

M.E., Jinan University, P. R. China, 2013

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College of Human Ecology

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Approved by:

Major Professor
Weiqun Wang

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Abstract

Exercise has been previously reported to lower cancer risk through reducing circulating IGF-1 and IGF-1-dependent signaling in mouse skin cancer models. This study is to investigate the underlying mechanisms by which exercise might impact IGF-1 pathway regulated by p53 and p53-related proteins in mouse skin epidermis. Female SENCAR mice were pair fed an AIN-93 diet with or without 10-week treadmill exercise at 20 m/min for 60 min daily. Animals were topically treated with TPA or vehicle control 2 hours before sacrifice and the target proteins in the epidermis were assessed by immunohistochemistry and Western blotting. Under TPA or vehicle treatment, MDM2 was significantly reduced in exercised mice compared with sedentary control. Meanwhile, p53 was significantly increased. In addition, p53 transcription target proteins p21, IGFBP-3, and PTEN were elevated in response to exercise. An interaction between exercise and TPA was observed on the decrease of MDM2 and increase of p53, but not p53 down-regulated proteins. Taken together, exercise appears to activate p53 by reducing MDM2 suppression, resulting in enhanced expression of p21, IGFBP-3 and PTEN that might further induce a negative regulation of IGF-1 pathway and therefore contribute to the observed cancer prevention by exercise in this mouse skin cancer model.

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Chapter 1 - Literature Review

Overview of cancer and cancer prevention

American Cancer Society estimated that the U.S. new cancer cases would increase to approximately 1.69 million in 2016 (American Cancer Society, 2016). Moreover, of all the cancer patients, about 1630 were predicted to die on a daily basis, representing nearly 1 in 4 deaths in the U.S. (American Cancer Society, 2016). Cancer is characterized as uncontrolled growth and spread of abnormal cells (American Cancer Society, 2016). Cancer is a multifactor disease resulted from both non-modifiable factors such as age, gender, genetic family history, and modifiable factors such as tobacco use, dietary factors, and overweight (Kollarova et al., 2014; Brown et al., 2012). Over the past decades, research regarding the impact of overweight on cancer is accumulating. It has been reported that 25% excess of normal body weight might contribute to a 33% higher cancer risk (Kritchevsky, 2003). Moreover, high body mass index (BMI) over 40 is associated with 50-60% higher cancer mortality rate compared to that in normal BMI (Calle et al., 2003). As such, weight control appears critical for cancer risk reduction.

Research to date has developed physical activity and dietary caloric restriction as major weight control strategies with respect to cancer prevention. Physical activity and dietary caloric restriction seem to share no similarity, but they are both related to energy balance. A negative energy balance achieved by increasing energy expenditure via physical activity and/or reducing energy intake via dietary caloric restriction may contribute to cancer primary prevention.

Physical activity refers to skeletal muscle movement with the classification of four subgroups, occupational activity, household activity, transport activity, and recreational or leisure-time activity (Caspersen et al., 1985). Exercise is under the subgroup of recreational or leisure-time activity, and can be identified into light, moderate, and vigorous exercise based on

the intensity (Thompson, et al., 2010). Scientific evidence of cancer prevention by physical activity is accumulating promptly. To date, numerous studies have demonstrated an inverse relationship between physical activity and the risk of various cancers, including breast cancer (Friedenreich, 2010), colon and colorectal cancer (Harriss et al., 2009; Slattery et al., 2003), pancreatic cancer (O'Rorke et al., 2010), prostate cancer (Patel et al., 2005; Liu et al., 2011), endometrial cancer (Voskuil et al., 2007), ovarian cancer (Olsen et al., 2007), lung cancer (Mao et al., 2003; Kubik et al., 2004), and others (Lee, 2003; Karim-Kos et al., 2008; Friedenreich and Orenstein, 2002). Despite the large number of scientific studies, the mechanisms by which physical activity may prevent cancer are not well established.

Evidence of cancer prevention by physical activity

Epidemiological studies

The majority of epidemiological studies investigating the role of physical activity demonstrated a reverse association between physical activity and cancer risk, with a reduction rate of 10-50% (Kruk and Czerniak, 2013). As suggested by Friedenreich and Orensterin, the evidence of physical activity in cancer prevention is convincing for colon and breast cancer, probable for prostate cancer, possible for lung and endometrium cancer, and limited for other types of cancers (Friedenreich and Orensterin, 2002). A cohort study conducted in the U.S. with 45,631women and an average follow-up of 8.9 years observed statistically significant decrease in breast cancer risk among those who exercised moderately (>10 hours per week of hiking or walking), when compared to non-exercised group (Howard et al., 2009). In another study among 4,722 elder participants that developed colorectal cancer, those who engaged in moderate or vigorous exercise at least five times a week showed reduced colon cancer risk versus counterpart

control (Howard et al., 2008). In regard to the intensity of exercise, most studies reported a dose-response effect that increased intensity and longer duration provided a greater protection effect (Friedenreich, 2010; Lynch et al., 2011). For example, the highest level of leisure-time physical activity reduced endometrial cancer risk by 27% versus the lowest level of physical activity (Voskuil et al., 2007). However, it remains unclear what type of physical activity and in which life period to start exercise are optimal for cancer prevention. It is suggested that 4-7 hours/week of moderate to vigorous physical activity is necessary to reduce cancer incidence (Thune and Furberg, 2001). In addition, an observational study predicted vigorous exercise at least 3 hours/week might be sufficient to reduce prostate cancer mortality (Kenfield et al., 2011).

Clinical trials

The majority of observational studies have described a potential association between physical activity and cancer risk reduction, further clinical trials are necessary to establish causality. However, measuring cancer endpoints are challenging for investigators because the latency of cancer development requires a long follow-up duration. An alternative is to measure the changes of biomarkers related to cancer risk. The frequently examined biomarkers include, but not limited to, free estradiol, estradiol, estrone, testosterone, androstenedione, glucose, IGF-1, IGFBP-3, leptin, and colon crypt (Winzer et al, 2011). Physical activity guidelines set up by American College of Sports Medicine (ACSM) suggested healthy adults, as well as cancer survivors, perform at least 30 min moderate intensity exercise daily for 5 days per week (Haskell et al., 2007; Schmitz et al., 2010). The American Institute for Cancer Research (AICR) and the World Cancer Research Fund (WCRF) recommend a minimum of 60 min moderate intensity or 30 min vigorous intensity exercise per day to achieve cancer risk reduction (World Cancer Research Fund / American Institute for Cancer Research, 2007). The exercise interventions in

randomized clinical trials are developed based on the ACSM or AICR/WCRF guidelines; the control groups in general received “usual care”. Plausible results from clinical trials reported a preventive effects of exercise on cancer through modifications of sex hormones (Friedenreich et al., 2010; Campbell et al., 2012). For example, a two-center, two-arm randomized controlled trail including 320 postmenopausal, sedentary women showed significant decrease in free estradiol and estradiol and increase in SHBG compared to control group after exercise intervention for 12 months (Friedenreich et al., 2010). However, there exists trials that reported no significant difference in selective sex hormones after exercise treatment. McTiernan et al. did not observe overall differences in estrone, estradiol, and free estradiol between exercise and control groups after a 12-month exercise intervention; significance were only found among individuals who lost at least 2% of baseline body fat (McTiernan et al., 2004). Thus, further studies are warrant to assess the effectiveness and mechanisms of physical activity on cancer prevention.

Animal studies

As a complement to clinical trials, the utilization of animal models facilitates the investigation of exercise-induced cancer prevention mechanisms. Exercise has been associated with the inhibition of chemically induced carcinogenesis in plenty of animal studies. Voluntary wheel running plus swimming for 21days remarkably decreased Walker 256 tumor weight among male Wistar mice (Hoffman et al., 1962). Zielinski et al. observed delayed lymphoma tumor growth in Female BALB/c mice following strenuous treadmill exercise in comparison to sedentary control mice (Zielinski et al., 2004).The onset and size of tumors were inhibited in an ultraviolet B induced mouse skin cancer model after voluntary running wheel exercise (Michna et al., 2006). In another study, F344 male rats subjected to treadmill exercise (120 min/d, 10 m/min, and 5 d/week) developed significantly less colon tumors compared to non-exercise group

(Fuku et al., 2007). A very recent study found treadmill running (60 min/day, 5 days/week for 32 weeks) decreased the number and volume of liver tumor in male hepatocyte-specific PTEN-deficient (AlbCrePtenflox/flox) mice (Piguet et al., 2015). However, no significant effect on DMH induced colon tumor incidence was found in male Wistar rats under swim training 20 min/d, 5 d/week for 35 week and those in control group (Lunz et al., 2008). There were also studies reported increased cancer risk after exercise training. Sprague-Dawley rats induced by breast carcinogenesis experienced a 200% increase in tumor growth rate versus sedentary control after intense swimming for 38-65 days, despite no significance in overall survival time (del Carmen Sáez et al. 2007). The mixed results may be attributed to the differences in exercise protocols and the gender of animals. As supported by Mehl et al., the crypt depth-to-villus height ratio decreased significantly for mice exercise by treadmill running rather than wheel running (Mehl et al. 2005). It was also found that male but not female ApcMin/+ mice showed significant decrease in the number of large polyps after treadmill exercise. Additionally, the intensity of exercise may also impact the results. It has been hypothesized that extreme high level of exercise may lead to oxidative stress that increase DNA damage and inflammation (Poulsen et al. 1996; Poulsen et al. 1999; Cooper et al. 2002). The impact of physical activity on cancer prevention in animal studies is less consistent than that in epidemiological studies and clinical trials.

Potential cancer prevention mechanisms by physical activity

Alteration of growth factors

Growth factors such as insulin-like growth factors (IGFs) have received increasing attention in physical activity promoted cancer prevention due to their significant role in cell growth and anti-apoptosis. IGF-1 is a polypeptide that has sequence highly similar to insulin.

The level of circulating IGF-1 is modulated by its binding proteins (IGF-BPs). When IGF-1 is bound to corresponding receptors, the downstream auto-phosphorylation is activated, resulting in activation of PI3K-Akt-anti-apoptosis cascades (Jiang and Wang, 2008). It has been reported that high circulating IGF-1 levels in serum were associated with higher premenopausal breast cancer risk in Chinese women (Yu et al., 2002). Moreover, a prospective case-control study nested in the Physicians' Health Study found men in the highest IGF-I quintile had an elevated risk of colorectal cancer compared to those in the lowest quintile (Ma et al., 1999).

Given the fact that increased IGF-1 level is associated with higher risk of cancer, reducing bioavailable IGF-1 may be critical in cancer prevention. Rosendal et al. demonstrated an inverse relationship between physical training and circulating IGF-1 and IGF binding proteins (IGFBPs) in a human study (Rosendal et al. 2002). In this study, seven untrained and twelve well-trained young men received a 10-week intensive exercise training. As a result, total IGF-I, circulating IGF-I, and IGFBP-4 were decreased in both groups. Previous study in our lab observed significant decrease in IGF-1 level following treadmill exercise in skin carcinogenesis mice (Xie et al., 2007). These results supported that reduced IGF-1 levels and the corresponding IGF-1 down-regulation may be responsible for anti-tumorigenic mechanisms.

Reduction of oxidative stress

It is well known that oxidative stress is associated with aging and various chronic diseases. The increased reactive oxygen species are the cause of protein and lipid damage, as well as DNA mutation, which may further lead to tumor cell proliferation (Westerlind, 2003). Many studies have pointed moderate physical activity to reduced oxidative stress. In a study using mouse model, moderate treadmill exercise was reported to significantly prevent aging-

associated oxidative stress by preventing decrease in bioactivity of antioxidant enzymes and mitochondrial NADH-cytochrome-c reductase (Navarro et al., 2004).

Regulation of sex hormones

The level of serum sex hormones, such as oestrogens and the protein sex hormone-binding globulin (SHBG), are related to risk of post-menopausal breast cancer. It has been consistently observed that postmenopausal women with highest sex hormone levels showed up to two-fold elevated breast cancer risk compared with those in the lowest quintile (Key et al., 2002; Kaaks et al., 2005). A recent intervention trial on postmenopausal overweight and physical inactive women observed significant decrease in serum sex hormones and SHBG after 16-week intensive exercise (van Gemert et al., 2015). In an animal study, five out of sixteen female Harlan Sprague-Dawley rats showed reduced estradiol levels (Caston et al., 1995). Exercise is concluded to be able to alter anterior pituitary gonadotrope, thus impact estrous cycles of rats.

Reduction of inflammation

Chronic inflammation has been revealed to increase cancer incidence by promoting abnormal cell growth and tumor development (Brown et al., 2012). Biomarkers associated with cancer risk include C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). An analysis involved in 14,461 U.S. adults estimated those who reported to participate in vigorous intensity physical activity (≥500 MET·min·wk-1) have significantly lower rate in CRP increase compared to physical inactive adults (Richardson, et al., 2015). In an intervention study, 120 premenopausal and obese women were assigned to low-energy diet, exercise, and control group, respectively, a 2-year intervention resulted in decreased circulating levels of IL-6, IL-18 and CRP when compared to control group (Esposito et al., 2003).

Proteins critical for cancer prevention

There are several proteins that play a key role in cancer induction and development. The development of cancer is caused by the mutation of genes such as oncogenes and tumor suppressor genes, which encode a variety of proteins that regulate cell growth and proliferation. P53 is a well-known tumor suppressor protein that is critical in genomic stability, DNA damage repair, and cell cycle arrest (Feng and Levine, 2010). Under normal condition, the p53 protein is maintained at low level due to the negative regulation by MDM2, an E3 ligase that promotes the degradation of p53 (Moll and Petrenko, 2003). However, when DNA damage occur, the p53 protein can be activated with an increase in level and elevation in ability to transcript down-stream proteins such as p21, IGFBP-3, and PTEN (Lakin and Jackson, 1999). P21 is a cyclin/CDK cascade inhibitor. The p53 works together with p21 to arrest cell cycle at G1 (Waldman et al., 1995), therefore prevent cell growth during cancer development. IGFBP-3 and PTEN are regulators of IGF-1-dependent pathways. IGFBP-3 is a binding protein that regulates bioavailable IGF-1 levels. In human body, IGFBP-3 is the most abundant in IGF binding protein family and accounts for approximately 90% of IGF-1 binding in serum, forming a large complex that prevents IGF-1 transporting out of bloodstream (Jiang et al., 2008). A reduction in bioavailable IGF-1 has been associated with reduced cancer risk (Xie et al., 2007). PTEN is known to retain IGF-1-PI3K-Akt pathway by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) back to phosphatidylinositol 4,5-bisphosphate (PIP2) (Lu et al., 2006). A negative regulation of IGF-1-Akt pathway is related to enhanced apoptosis of abnormal cells.

Conclusion

Cancer is developed due to uncontrolled growth and spread of abnormal cells. Many modifiable factors including overweight may contribute to cancer incidence. Human and animal studies have suggested an association between physical activity and cancer risk reduction. However, the underlying mechanisms remain unclear. Hypothesized molecular mechanisms include regulation of growth factors and sex hormones, mitigation of oxidative stress, DNA repair, and reduction of inflammation. Study by others and in our lab found a potential role of exercise in decreasing bioavailable IGF-1 and IGF-1-dependent pathway, but the regulators involved in this pathway have not been identified. Tumor suppressor proteins play a critical role in cancer prevention and seem to be associated with IGF-1 network. Therefore, the aimed of this thesis study is to identify critical proteins involved in IGF-1 pathway regulation, thus provide further insight into the mechanisms of exercise-promoted cancer prevention.

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Chapter 2 - Experiment

Abstract

Exercise has been previously reported to lower cancer risk through reducing circulating IGF-1 and IGF-1-dependent signaling in mouse skin cancer models. This study is to investigate the underlying mechanisms by which exercise might impact IGF-1 pathway regulated by p53 and p53-related proteins in mouse skin epidermis. Female SENCAR mice were pair fed an AIN-93 diet with or without 10-week treadmill exercise at 20 m/min for 60 min daily. Animals were topically treated with TPA or vehicle control 2 hours before sacrifice and the target proteins in the epidermis were assessed by immunohistochemistry and Western blotting. Under TPA or vehicle treatment, MDM2 was significantly reduced in exercised mice compared with sedentary control. Meanwhile, p53 was significantly increased. In addition, p53 transcription target proteins p21, IGFBP-3, and PTEN were elevated in response to exercise. An interaction between exercise and TPA was observed on the decrease of MDM2 and increase of p53, but not p53 down-regulated proteins. Taken together, exercise appears to activate p53 by reducing MDM2 suppression, resulting in enhanced expression of p21, IGFBP-3 and PTEN that might further induce a negative regulation of IGF-1 pathway and therefore contribute to the observed cancer prevention by exercise in this mouse skin cancer model.

Introduction

Physical inactivity, together with smoking and high calorie diet, is a main modifiable factor that contributes to cancer development (Friedenreich and Orenstein, 2002). Scientific evidence for primary cancer prevention through physical activity is accumulating promptly. To date, large epidemiological studies and clinical trials have demonstrated that physical activity is

effective in reducing the risk of various cancers, including breast cancer, colon and colorectal cancer, pancreatic cancer, prostate cancer, endometrial cancer, ovarian cancer, and lung cancer, with a reduction rate of 10- 50% (Friedenreich et al., 2010; Winzer et al., 2011; Kruk and Czerniak, 2013). A cohort study with 45,631 U.S. women and an average follow-up of 8.9 years observed statistically significant decrease in breast cancer risk among those who exercised moderately (>10 hours per week of hiking or walking), when compared to no hiking or walking group (Howard, et al, 2009). In addition to human studies, exercise intervention studies conducted in animal models also showed protective effects against PTEN-deficient mouse liver tumors (Piguet et al., 2015), DNH-induced rat colon cancer (Lunz et al., 2008), and UVB-induced mouse skin cancer (Michna et al., 2006). Previous study in our lab observed reduced skin cancer risk in exercised mice fed with iso-caloric diet comparable to sedentary control (Xie et al., 2007).

Although exercise has been associated with cancer prevention, the underlying mechanisms are not well established. The current hypothesized mechanisms through which exercise might reduce cancer risk include hormone regulation, inflammation reduction, immune function enhancement, and DNA repair, etc. (Rundle et al., 2005). Specifically, mitogenic hormones such as IGF-1 have been related to elevated risk of multiple cancers (Yu et al., 2002; Ma et al., 1999). IGF-1 is known to trigger cell proliferation and inhibit apoptosis. When binding to corresponding receptors, IGF-1 is able to activate downstream networks including Ras-MAPK proliferation and PI3K-Akt anti-apoptosis, resulting in cancer development (Benito et al., 1996). In animal study, such activation can be stimulated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a phorbol ester commonly used for skin carcinogenesis promotion in mouse model (DiGiovanni, 1992). When applied to mice epidermis, TPA is accepted by a calcium-activated,

phospholipid dependent serine/threonine kinase protein kinase C (PKC) (Ashendel, 1985). The PKC isoforms then activate the Ras-regulated downstream networks, particularly the MAPK cascade, leading to tumor cell growth (Schönwasser et al., 1998; Corbit et al., 2000). Studies in our laboratory found exercise plus an iso-caloric diet with counterpart control significantly decreased circulating IGF-1 level and IGF-1 signaling in a TPA-induced skin cancer mouse model (Xie et al., 2007; Standard et al., 2014). Due to the significant role of IGF-1 in cell growth and survival, inhibiting IGF-1 signaling appears to be a critical target for cancer prevention.

However, the possible up- and down- stream proteins that regulate IGF-1 signaling and the IGF-1 pathways with respect to exercise were not identified. Studies have demonstrated a potential connection between p53 protein and IGF-1 pathways in a caloric restriction-induced anti-aging study (Tucci, 2012). P53 is a tumor suppressor gene essential for DNA repair and gene stabilization. Furthermore, as a transcription factor, p53 has a sequence-specific DNA-binding domain in the central region and a transcription activation domain at N terminus (Ko and Prives, 1996). In normal cells, p53 is maintained at a low concentration by its negative regulator MDM2 protein, a ring finger ubiquitin ligase that bind with p53, forming a complex that triggers p53 degradation (Fang et al., 2000; Moll and Petrenko, 2003). However, upon DNA damage, p53 is released from MDM2 through phosphorylation (Ashcroft et al., 2000). Activated p53 then selectively stimulates the transcription of its target genes, such as p21, IGFBP-3, and PTEN to initiate cellular apoptosis and cell cycle arrest (Stambolic et al., 2001; Grimberg, 2000; Waldman et al., 1995). It has been shown that p53 gene is effective in protecting mice against UV-induced cancer (Jiang et al., 1999).

The current study investigated moderate exercise-induced cancer prevention by p53 activated down-regulation of IGF-1 pathway in TPA promoted SENCAR mice. P53 up-

regulation protein and transcription proteins involved in IGF-1 pathway modulation were assessed by immunohistochemistry and Western blotting. The aim of this study is to provide further insight into the cancer prevention mechanisms through exercise.

Materials and methods

Animals and treatment

Six-week old female SENCAR mice from National Institutes of Health (Frederick, MD) were housed individually at 24 ± 1 ˚C with a 12:12 light-dark cycle. Mice were randomly assigned into one of two groups: *ad libitum* feeding sedentary control and exercise but pair feeding of the same amount as sedentary control (AIN-93 diet). After a two-week adaptation period for the new environment or treadmill exercise accordingly, animals in exercise group were trained on a zero-grade adjustable-speed mouse 5-lane treadmill (Harvard Apparatus, Holliston, MA) at 20 m/min, 60 min/day and 5 days/week for 10 weeks. At the end of the experiment, the mice dorsal skin was shaved and treated with TPA at 3.2 nmol in 200 μL of acetone or acetone only. The mice were sacrificed two hours after TPA or vehicle acetone treatment. Skin tissues were then snap-frozen in liquid nitrogen and stored at -80 ˚C for further analysis of targeted proteins by immunohistochemistry (IHC) and Western blotting.

Protein analysis by IHC

The frozen mice dorsal skin tissues were fixed in 3.7% formalin for 22 hours at -70 ˚C and then switched to 70% ethanol. The skin tissues were set on edge in paraffin blocks, sectioned at a thickness of 4 micrometers, and then placed on slides. For immunostaining, sections were deparaffinized twice in Master Clear (American MasterTech Scientific) for 10 min, followed by rinsing in absolute alcohol for 1 min. Slides were subsequently placed in 30% H_2O_2 in methanol

for endogenous peroxidase quenching for 20 min. Sections then underwent rehydrated through 100%, 95%, 75%, and 50% ethanol, and were finally placed in distilled water. Antigens were retrieved by steaming in citrate buffer for 20 min. After PBST washes, sections were incubated overnight at 4 ˚C, with 1: 160 dilution in PBST for rabbit p53 primary monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and 1: 50 dilution for other rabbit against mouse MDM2, IGFBP-3, PTEN antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA), respectively. Control sections were incubated with 5% BSA in PBST only. Alexa Fluor 555 goat anti-rabbit IgG secondary antibody (Life Technologies, Carlsbad, CA) were applied to both samples and negative controls with a dilution of 1:500 at 37 ˚C for 60 min. Images were obtained using a LSM-5 confocal microscopy and analyzed using Image J software (Jena, Germany). The IHC images were quantified by deducting fluorescence intensity of controls from that of samples to eliminate the background stain. The sample size for each group was four.

Protein analysis by Western blotting

Mouse skin tissues were sliced and placed in 600 μL ice-cold PBS containing PMSF (1:100). After sonicating for 15s, samples were centrifuged at 7,000 rpm for 5 min at 4 ˚C. The supernatants were removed and 250 μL of RIPA buffer (50 mM Tris HCL at pH 8.0, 150 mM NaCl, 0.5% Sodium Deoxycholate, 0.1% SDS, and fresh PMSF) were added to samples for incubation for 30 min with vortexing every 5 min. The tissue samples were then centrifuged at 13,000 rpm for 15 min at 4 ˚C. The supernatants containing soluble proteins were transferred to a new tube. The protein extraction from pellet was performed one more time with 100 μL of RIPA, The supernatants from the first and second extractions were combined. Protein concentrations were measured using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Whole cell protein 30 μg was electrophoresed on mini-protein TGX gels (Bio-Rad Laboratories,

Hercules, CA) at a voltage of 90 V for 90 min. The protein bands were then transferred to a nitrocellulose membrane, where the transferred bands corresponding to MDM2 at 90 kDa, p53 at 53 kDa, p21 at 21 kDa, IGFBP-3 at 40/44 kDa, PTEN at 55 kDa, and β-actin at 43 kDa were respectively bound to rabbit monoclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Subsequently, the bound proteins were incubated with Thermal Scientific Pierce anti-rabbit secondary antibody (Rockford, IL). Protein bands were visualized and quantified using FluorChem™ 8900 Advanced Imaging System (Alpha Innotech, San Leandro, CA). The band density was standardized to the loading control β-actin. Afterwards, the band density of all four experimental groups were divided by that of Acetone sedentary control for adjustment and reported as relative density. Sample size for each group was four.

Statistical analysis

Student t-test was performed to analyze the significance of protein expression between sedentary control and exercise group. Two-way analysis of variance (ANOVA) was performed to assess the interaction between exercise and TPA induction. Minimum significance level was set at 0.05.

Results

Effects of exercise on the expression of protein MDM2 and p53

IHC Images of protein MDM2 fluorescence intensity quantified by Image J were shown in Figure 2-1A. The protein levels were calculated by the fluorescence intensity with respect to the brightness and amount of red stain. When SENCAR mice were treated with acetone vehicle only, MDM2 fluorescence intensity of exercised mice was significantly decreased by 44% in comparison with sedentary control. Similarly, exercised mice with TPA treatment showed a

decrease of 47% compared to the counterpart control group. This significant decrease of MDM2 determined by IHC was confirmed by Western blotting results (Figure 2-1B). A significant decrease of MDM2 expression by 41% and 34% was observed within exercise group treated with acetone and TPA, respectively. Opposite to MDM2, the IHC fluorescence intensity of p53 protein showed prominently increase (59%) in exercise group treated with acetone only (Figure 2-2A). Furthermore, this increase became dramatic after TPA treatment, with a large increase by 159% (exercised vs. sedentary mice). Western blotting showed a similar enhancement of p53 expression in exercise group under both Acetone and TPA treatment (Figure 2-2B).

Effects of exercise on the expression of p53 transcription proteins

The expression of p53 downstream proteins p21, IGFBP-3, and PTEN were also assessed by both IHC and Western blotting. Elevated levels of all the three proteins were observed in exercise group when compared with the corresponding sedentary control with or without TPA treatment. As is shown in Figure 2-3, the p53 direct transcription target protein p21showed a statistical enhance by 121% in IHC and 29% in Western blotting in exercised mice treated with acetone. Similarly, p21 was increased by 30% in IHC and 39% in Western blotting in exercised mice treated with TPA. For IGFBP-3 (Figure 2-4), a slight increase of 15% by IHC and 25% by Western blotting was observed among acetone treated exercised mice. After TPA treatment, the increase of IGFBP-3 in exercise group was not significant as detected by IHC, but significant by Western blotting. In terms of PTEN protein level (Figure 2-5), a significant increase of 207% by IHC but not Western blotting was found in exercised animal with acetone treatment. Under TPA treatment, both IHC and Western blotting showed significant increase by 89 % and 18%, respectively.

Interactive effects of Exercise and TPA on p53 regulator and transcription proteins

The exercise and TPA interactive effects were further investigated on the five targeted proteins, MDM2, p53, p21, IGFBP-3 and PTEN. To analyze this interaction, protein levels between sedentary control under Acetone vehicle treatment (Sed+Ace) and exercise group under TPA treatment (Ex+TPA) were compared statistically. A significant decrease of MDM2 and increase of p53 was found under the intervention of exercise and TPA. However, the p53 downstream proteins p21, IGFBP-3 and PTEN showed a potential but not significant increase under this intervention.

Discussion

The aim of this study was to illustrate a potential mechanism of cancer prevention by exercise. Previous work found exercise might reduce cancer risk through decreasing circulating IGF-1 and IGF-1signaling pathway in skin carcinogenesis mice (Xie et al., 2007; Standard et al., 2014). To further investigate this mechanism, this study was focused on the impact of moderate exercise on selective IGF-1 related regulators in mice treated with pair-feeding, moderate exercise, and TPA. In this study, isocaloric diet was utilized to eliminate diet impact on test results, as energy expenditure from exercise might be compensated by free access to diet. Previous study in our lab (Xie et al., 2007; Standard et al., 2014) on weight-loss-induced cancer prevention by dietary calorie restriction and/or exercise found that exercise with *ad libitum* feeding was not sufficient in reducing body weight, therefore had limited or no effect on biomarkers involved in cellular growth regulation. When dietary intake in exercise mice was limited to the same amount as sedentary control, however, significant results were observed. Furthermore, exercise intensity and duration were also taken into consideration in this study.

Most studies reported a dose-response effect that increased intensity and prolonged duration provide greater protection against cancer risk (Friedenreich et al., 2010; Lynch et al., 2011). A moderate intensity treadmill exercise at 20 m/min, 60 min/day and 5 days/week for 10 weeks was employed to ensure the sufficiency and accessibility of exercise. In addition, a 2-hour TPA promotion period was selected, as optimal induction in AP-1: DNA binding and c-Jun mRNA was observed in a previous time-course study (Przybyszewski et al., 2001).

To better understand the regulation of IGF-1 pathway, protein p53 and its down-stream proteins were examined. As expected, a significant increase in p53 protein and decrease in MDM2 protein were observed in exercised mouse compared to sedentary control. Particularly, the exercise-induced p53 enhancement increased largely to 159% after TPA promotion. This is consistent with a recent study by Higgins et al. that reported a dramatic increase of p53 protein (1103%) in mice lung tumors after a four-week wheel running exercise (Higgins et al., 2014). The reduction of MDM2 together with increase of p53 indicated that p53 might be activated following exercise. The activation of p53 was further confirmed by the increase of its direct-regulated downstream protein p21, a cyclin/CDK cascade inhibitor (Xiong et al., 1993). The p53 together with p21 has been reported to contribute to G1 cell cycle arrest (Waldman et al., 1995), therefore prevent cell growth in cancer development.

In addition to p53, two critical p53 transcription proteins IGFBP-3 and PTEN showed significant increase after treadmill exercise. IGFBP-3 is the most abundant in IGF-1 binding protein family and accounts for approximately 90% of IGF-1 binding in serum, forming a large complex that prevents IGF-1 from transporting out of bloodstream (Jiang et al., 2008). Based on the current results, we assume that the competition binding of IGFBP-3 with IGF-1 to IGF-1 receptor might contribute to reduced IGF-1signaling. Although the IGF-1 levels were not

detected in this study, previous studies from our laboratory observed significant decrease in IGF-1 in exercised mice (Xie et al., 2007). The p53-dependent enhancement of IGFBP-3 has been supported in a p53 knockout study (Buckbinder et al., 1995). In addition to IGFBP-3, the increase of phosphoprotein PTEN appears to bridge p53 with IGF-1 pathway following exercise. PTEN is known to retain IGF-1-PI3K-AKT pathway by dephosphorylating PIP3 back to PIP2 (Lu et al., 2006). The p53 regulated PTEN elevation has also been reported (Stambolic et al., 2001). As such, exercise-activated p53 might inhibit IGF-1 pathway through promoting IGFBP-3-IGF-1 binding as well as increasing PTEN negative regulation.

The impact of exercise on target protein expression was compared under both TPA treatment and basal level. Considering the significant roles of p53 and p53 related proteins in tumor suppression, it would be of interest to know the effect not only in a TPA induced cancer development circumstance, but also in a basal level of normal condition. In general, all the target proteins showed significant change following exercise in both TPA promotion and basal level. Furthermore, an intervention between exercise and TPA was observed on the decrease of MDM2 and increase of p53. The significant change in MDM2 and p53 confirmed the critical role of p53 after DNA damage particularly during cancer development, suggesting a larger protective effect of exercise on high cancer risk than that in normal or low cancer risk.

In this study, each protein was quantified using the same primary antibody by both IHC and Western blotting methods. Consistency in protein change was demonstrated for protein MDM2, p53, and p21 by IHC and Western blotting, whereas the change for IGFBP-3 and PTEN was not consistent between the two methods. IHC did not show a significant increase of IGFBP-3 under TPA treatment, but it provided a general distribution of each protein in addition to quantification. The basal level of PTEN expression, on the other hand, did not significantly

detected by Westerns blotting. Although Western blotting is generally more quantitative and sensitive than IHC, it should be noted the skin epidermis might be diluted by other types of cells and thus cause some variation.

In conclusion, the current study demonstrated a potential protective effects of exercise on mouse skin cancer by modulation of p53-IGF-1 signaling pathway. As summarized in Figure 2-6, exercise might exert its function through activating p53 by reducing MDM2, subsequently leading to over-expression of p21, IGFBP-3 and PTEN. Both IGFBP-3 and PTEN may further reduce circulating IGF-1 and IGF-1-dependent pathway, respectively. A negative regulation of IGF-1 pathway through p53 activation and p53-related regulators may thus inhibit cell proliferation and promote apoptosis, as a result, contribute to the exercise-induced cancer prevention in this mouse skin cancer model.

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Figures

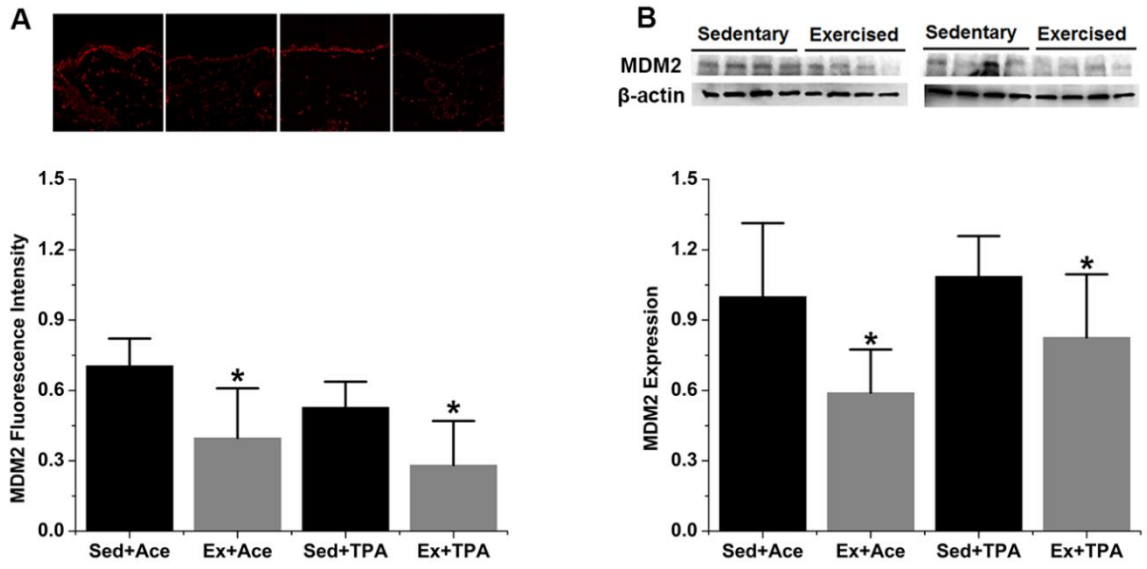

Figure 2-1 MDM2 protein expression

(A). MDM2 protein fluorescence intensity measured by IHC. (B). MDM2 protein expression measured by Western blotting. Sed = Sedentary; Ex = Exercised; Ace = Acetone; TPA = 12-O-tetradecanoylphorbol-13-acetate. Data presented are mean ± SD. *$p<0.05$ as compared to corresponding sedentary control under acetone or TPA, respectively.

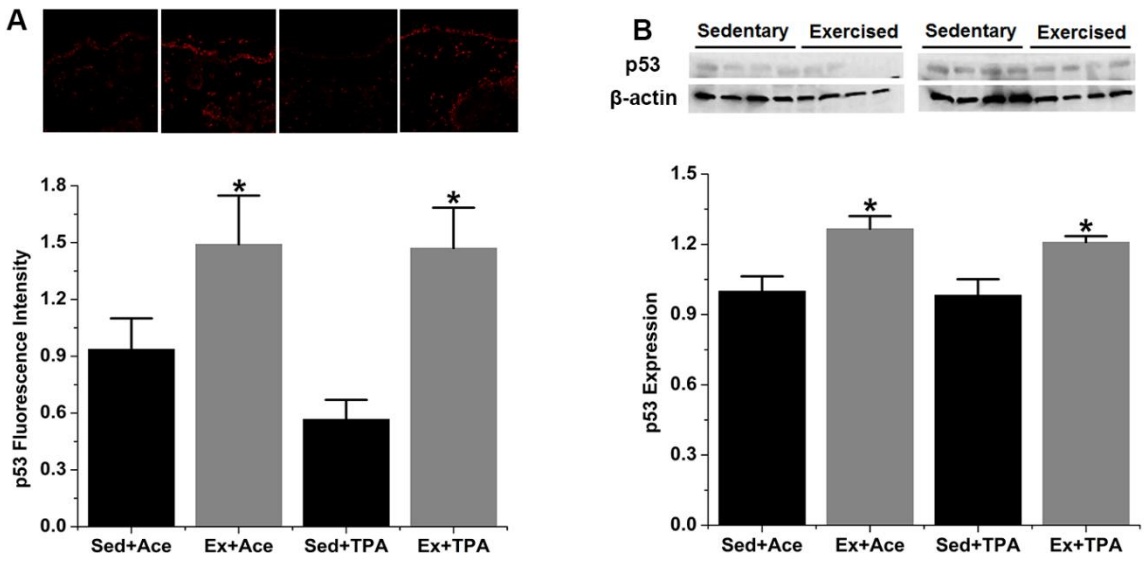

Figure 2-2 p53 protein expression

(A). p53 protein fluorescence intensity measured by IHC. (B). p53 protein expression measured by Western blotting. Sed = Sedentary; Ex = Exercised; Ace = Acetone; TPA = 12-O-tetradecanoylphorbol-13-acetate. Data presented are mean ± SD. *$p<0.05$ as compared to corresponding sedentary control under acetone or TPA, respectively.

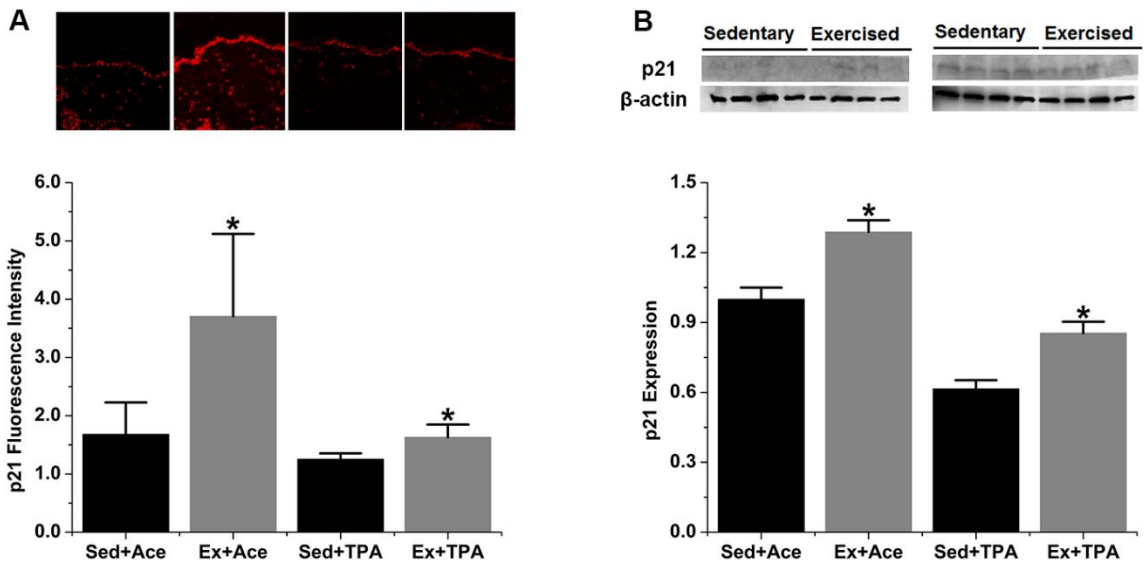

Figure 2-3 p21 protein expression

(A). p21 protein fluorescence intensity measured by IHC. (B). p21 protein expression measured by Western blotting. Sed = Sedentary; Ex = Exercised; Ace = Acetone; TPA = 12-O-tetradecanoylphorbol-13-acetate. Data presented are mean ± SD. *$p<0.05$ as compared to corresponding sedentary control under acetone or TPA, respectively.

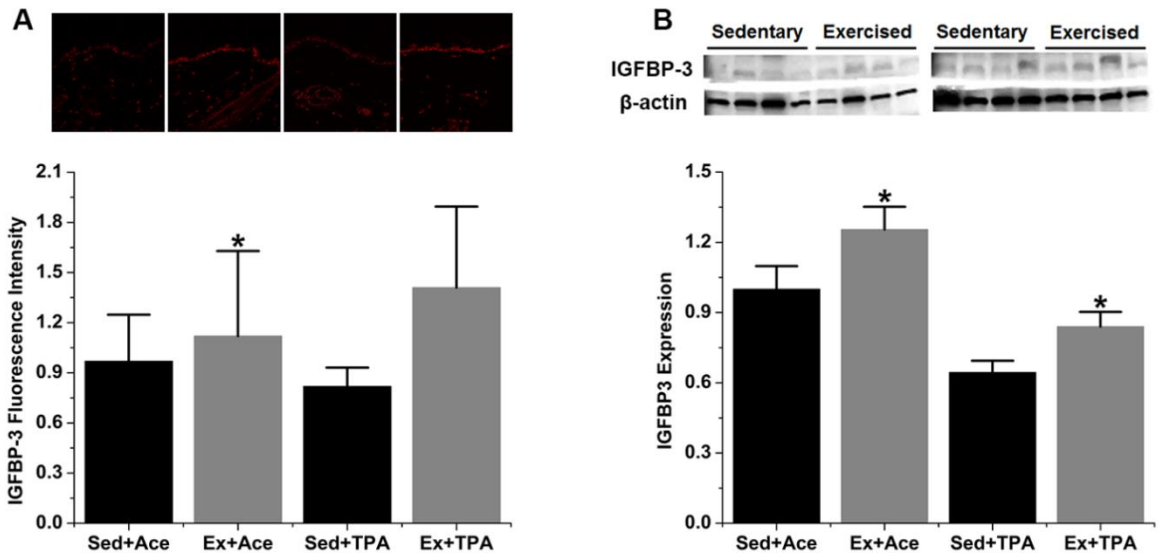

Figure 2-4 IGFBP-3 protein expression

(A). IGFBP-3 protein fluorescence intensity measured by IHC. (B). IGFBP-3 protein expression measured by Western blotting. Sed = Sedentary; Ex = Exercised; Ace = Acetone; TPA = 12-O-tetradecanoylphorbol-13-acetate. Data presented are mean ± SD. *$p<0.05$ as compared to corresponding sedentary control under acetone or TPA, respectively.

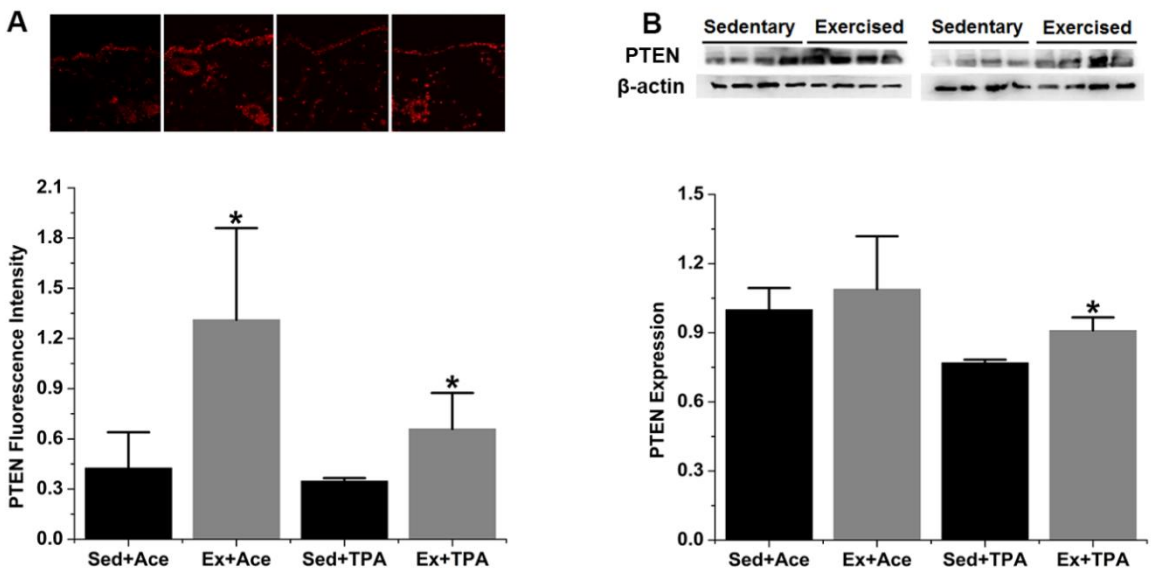

Figure 2-5 PTEN protein expression

(A). PTEN protein fluorescence intensity measured by IHC. (B). PTEN protein expression measured by Western blotting. Sed = Sedentary; Ex = Exercised; Ace = Acetone; TPA = 12-O-tetradecanoylphorbol-13-acetate. Data presented are mean ± SD. *$p<0.05$ as compared to corresponding sedentary control under acetone or TPA, respectively.

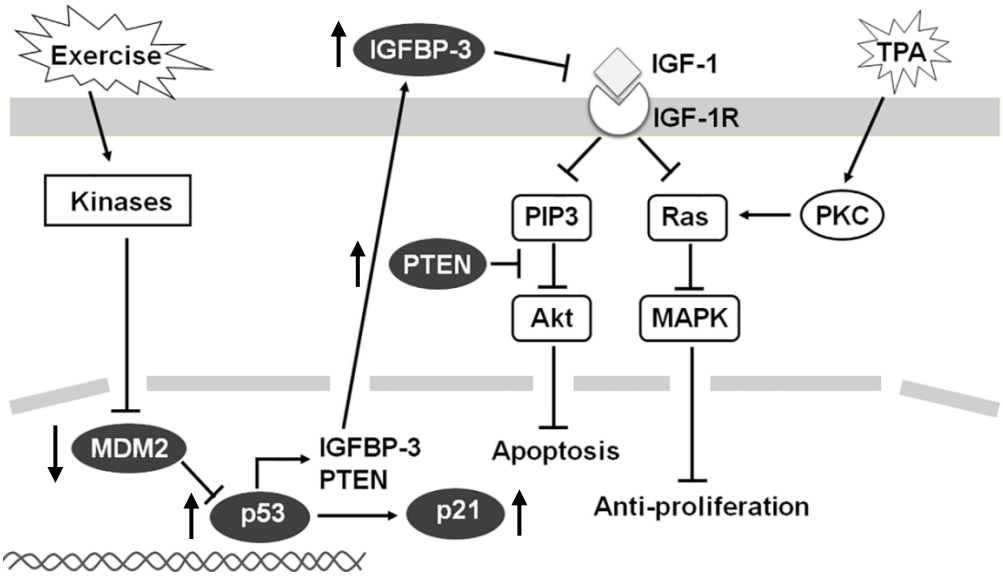

Figure 2-6 Molecular mechanism of cancer prevention via p53-IGF-1 pathway

Exercise may activate p53 by inhibiting MDM2 suppression, subsequently enhance the expression of p21, IGFBP-3 and PTEN. Both IGFBP-3 and PTEN may further reduce circulating IGF-1 and IGF-1-dependent pathway, respectively. A negative regulation of IGF-1 pathway through p53 activation and p53-related proteins may thus inhibit TPA induced cell proliferation and promote cell apoptosis, therefore contribute to the exercise-induced cancer prevention in this mouse skin cancer model.