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Title	Senescence marker protein-30欠乏は虚血時血管新生を障害する(本文)
Author(s)	山内, 宏之
Citation	
Issue Date	2015-09-28
URL	http://ir.fmu.ac.jp/dspace/handle/123456789/475
Rights	This is the pre-peer reviewed version. Published version "Free Radic Biol Med. 2016 May;94:66-73. doi: 10.1016/j.freeradbiomed.2016.02.020. © 2016 Elsevier Inc."
DOI	
Text Version	ETD

This document is downloaded at: 2024-04-19T19:11:29Z

**Senescence marker protein-30 deficiency impairs
angiogenesis under ischemia in aging**

加齢に伴う Senescence marker protein-30 の欠乏は
虚血時の血管新生を障害する

山内 宏之

循環・血液病態情報学

Senescence marker protein-30 deficiency impairs angiogenesis under ischemia in aging

(Brief title: SMP30 and angiogenesis under ischemia)

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Total words: 5175 words (Abstract: 197 words)

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Key words:

senescence marker protein-30 (SMP30)

angiogenesis

oxidant stress

nitric oxide

ischemia

Abbreviations:

SMP30 KO mice, senescence marker protein-30 knockout mice

WT mice, wild-type mice

CBF, cutaneous blood flow

NO, nitric oxide

eNOS, endothelial nitric oxide synthase

GSH, reduced glutathione

GSSG, oxidized glutathione

Abstract

Aging causes collateral rarefaction in ischemic conditions; however, the underlying mechanism is unknown. Senescence marker protein-30 (SMP30) decreases with aging. Mice with SMP30 deficiency, a model of aging, have a short life span with senility and with increased oxidant stress. To elucidate the effect of SMP30 on ischemia-induced collateral growth, we examined the recovery of cutaneous blood flow (CBF) after femoral artery ligation using laser Doppler imaging in SMP30 knockout (KO) mice. The CBF and tissue capillary density recoveries were suppressed in SMP30 KO mice compared to those in wild-type mice. Nitric oxide (NO) generation induced by L-arginine and GSH/GSSG in the aorta of SMP30 KO mice were lower than those in wild-type mice. The levels of NADPH oxidase activity and superoxide production in the aorta were higher in SMP30 KO mice than in wild-type mice. The phosphorylated eNOS and Akt levels and VEGF levels in ischemic muscle were lower in SMP30 KO mice than in wild-type mice. Thus, SMP30 deficiency exacerbates oxidant stress related to NADPH oxidase activity enhancement and impairs eNOS activity, which leads to rarefaction of angiogenesis induced by ischemia. These results suggest that SMP30 plays a key role in disrupting collateral growth under ischemia in aging.

Introduction

The occurrence of coronary and peripheral artery diseases increases with age, even in a population without other major risk factors (1, 2). Additionally, aging augments the severity of tissue injury after acute and chronic ischemia (3). The impairment of collateral growth would be a major exacerbating factor in the prognosis of ischemic

disease. However, whether aging reduces the extent of the collateral circulation is unclear particularly because the mechanisms of age-associated decline in vascular function are complicated. Senescence marker protein-30 (SMP30), a 34-kDa protein, is a novel molecule whose expression decreases with age in a sex-independent manner. In humans, the SMP30 gene is located in the p11.3-q11.2 segment of the X chromosome. In mice, SMP30 transcripts are detected in various organs, including the liver, kidney, cerebrum, testis, and lung. SMP30 protects cellular functions from age-associated deterioration in several organs, such as lung and brain, and SMP30 knock-out (KO) mice with senility have a short life span (4, 5, 6). Recently, we reported that the decrease of SMP30 impairs endothelium-dependent vasodilation and endothelial nitric oxide synthase (eNOS) activity in mice (7, 8). Therefore, SMP30 has a protective role in maintaining coronary circulation against oxidant stress as well as a cytoprotective role in a cell line (4, 9). Groleau et al. have reported that oxidant stress increases with aging and that the deficiency of superoxide dismutase impairs angiogenesis in a senescence model (10). We hypothesized that SMP30 plays an important role in collateral growth under ischemia that is related not only to angiogenetic factors, including eNOS, but also to defense against oxidant stress in aging. Therefore, we investigated the effect of SMP30 on angiogenesis with limb ischemia and the impact of oxidant stress on collateral development in SMP30 KO mice.

Results

Collateral flow recovery. We examined the effect of SMP30 deficiency on blood flow recovery after the induction of hind limb ischemia in a mouse model using laser Doppler flow imaging. The collateral flow recovery was impaired in the ischemic hind limb of SMP30 KO mice compared to that of WT mice (Figure 1A and B). The impairment of blood flow recovery reflected a decrease in capillary density determined by CD 31 staining of the muscle tissue (Figure 1C and D). These data support the notion that SMP30 stimulates angiogenesis.

Generation of superoxide and NADPH oxidase activity in the aorta. Superoxide generation (Figure 2A) and NADPH oxidase activity (Figure 2B) were greater in the aorta of SMP30 KO mice than in those in wild-type mice. These data suggest that SMP30 deficiency increases reactive oxygen species through NADPH oxidase and that SMP30 has a protective role in oxidant stress in the vessel.

Total thiol and glutathione levels in the aorta. The oxidation of the thiol group, including cysteine, is involved in many biological processes. Thiol oxidation induces protein conformation changes by converting free thiols (-SH) into sulfenic acids (SO-), sulfinic acids (SOO-), sulfonic acids (SOOO-), and disulfide bridges (S-S) (11). Thiol oxidation is involved in many cellular processes, such as eNOS glutathionylation (12), and regulation of vascular tone through reactive oxygen species (11). To determine how SMP30 deficiency affects thiols, total thiols and reduced (GSH) and oxidized glutathione (GSSG) levels were measured in the aortic tissue of SMP30 KO and WT mice. In the aortic tissue of SMP30 KO mice, there were large decreases in total thiols, GSH level, and GSH/GSSG compared to those in WT mice (Figure 3A, B, and C,

respectively). The results demonstrated that the depletion of SMP30 may affect the level of total thiols in the arterial tissue.

Nitric oxide (NO) production induced by L-arginine. We measured L-arginine-induced NO production in the isolated aortic strip. L-arginine-induced NO production detected by DAF-2DA fluorescence was markedly reduced in the aorta of the SMP30 KO mice (Figure 4A and B). This result suggests the likelihood that SMP30 deficiency may impact L-arginine metabolism of eNOS and that bioactive NO requires the stimulation of angiogenesis.

VEGF, phospho-Akt, phospho-eNOS levels in the hind limb. Several angiogenic factors, including VEGF, are known to modify eNOS phosphorylation in an Akt-dependent and Akt-independent fashion, and the increased endothelium-derived NO, in turn, regulates the VEGF bioactivity in the vascular smooth muscle cells (13, 14, 15). According to previous reports, excessive oxidative stress can impair VEGF-induced angiogenesis in endothelial cells (16, 17). Moreover, Akt phosphorylation is enhanced or inhibited by oxidant stress in the skeletal muscle depending on differential thiol oxidation of the signaling protein of Akt (18). Thus, we examined the VEGF, phospho-Akt, Akt, phospho-eNOS, and eNOS levels in the ischemic muscle of the hind limb considering the hypothesis that excessive oxidant stress in the SMP30 KO mice induces the impairment of Akt-eNOS/VEGF. The VEGF level in the non-ischemic muscle did not differ between SMP30 KO (215.4 ± 38.4 pg/ml, n=16) and WT mice (186.2 ± 32.2 pg/ml, n=16) at 14 days after femoral artery ligation. The VEGF ratio of ischemic to non-ischemic muscle was lower in the SMP30 KO mice than in the WT

mice (Figure 5A). In Western blotting, phospho-Akt, eNOS and phospho-eNOS levels were lower in the ischemic femoral muscles of the SMP30 mice compared to those of the WT mice in 14 days post-surgery (Figure 5B and C), suggesting that SMP30 plays an important role in angiogenesis through the Akt-eNOS/VEGF axis against oxidant stress under ischemia.

Discussion

Aging is a major factor in increasing oxidant stress and impairing collateral growth under ischemia (19, 20). SMP30 is a novel molecule whose expression decreases with age. We have reported that SMP30 is closely associated with the increase in oxidant stress that occurs with aging (7). In the present study, we investigated the role of SMP30 in angiogenesis under ischemia because acute limb ischemia induced by femoral artery ligation in mice has been widely used as a model for studying angiogenesis (21). To the best of our knowledge, this study is the first to indicate that SMP30 deficiency impairs angiogenesis under ischemia with inhibition of bioavailable NO production and disability of the Akt-eNOS/VEGF pathway due to excessive oxidant stress. First, the deficiency of SMP30 enhanced the activity of NADPH oxidase in the aorta and increased superoxide production, according to the report by Mizukami et al. (7), indicating that SMP30 deficiency increased NADPH oxidase activity in cardiomyocytes. Therefore, SMP30 KO mice are suitable chronic oxidant stress models. Second, bioavailable NO production in the aorta responded to L-arginine reduction in SMP30 KO mice, as demonstrated in our previous report showing that acetylcholine-induced

NO production was reduced and that thiol oxidation appeared in the coronary artery of SMP30 KO mice (8). Additionally, the GSH/GSSG ratio decreased in the aorta of SMP30 KO mice. The protein thiol can undergo thiol oxidation, a reversible protein modification involved in cellular signaling and adaptation. Under oxidant stress, thiol oxidation, such as S-glutathionylation in cysteine residues of eNOS, occurs through a thiol-disulfide exchange with GSSG, which can only occur when the cellular GSH/GSSG ratio is low, as shown in SMP30 KO mice (22, 23, 24), or when a reaction of oxidant-induced protein thiyl radicals occurs with reduced glutathione (25). Recently, it has been reported that s-glutathionylation of eNOS reversibly decreases NOS activity with an increase in superoxide generation primarily from reductase. In this state, two highly conserved cysteine residues are identified as sites of s-glutathionylation and are found to be critical for redox-regulation of eNOS function (12). GSH synthase inhibition without oxidation by buthionine-(S, R)-sulfoximine has been reported to have no effect on NO bioactivity (26). However, thiol depletion in vivo greatly reduces NO generation from eNOS (27, 28, 29). The decrease in NO bioactivity with GSH depletion under oxidant stress has been reported (30), and thiol oxidizing agents, such as diamide, decreased both the GSH level and the NO bioactivity (26). Therefore, we speculate that decreases in GSH and total thiols, including cysteine residues in the eNOS domain by oxidative stress, may contribute to the impairment of NO generation in SMP30 KO mice. SMP30 deficiency results in a deletion of vitamin C biosynthesis (31). In this study, we fed the animals chow with vitamin C, and we observed that the vitamin C level was not different among SMP30 KO and WT mice. Thus, the collateral growth

under ischemia was unaffected by the vitamin C level in our setting. However, the long-term effect of vitamin C treatment remains unknown because previous data have suggested that there was improvement in NOS activity with an overdose chronic administration of vitamin C (32). Thus, additional study is needed to clarify the effect of vitamin C on thiol oxidation. Third, VEGF and phospho-Akt expression levels in the ischemic muscle were reduced in SMP30 KO mice compared to those in WT mice. Angiogenesis is a complex process that includes endothelial proliferation, migration, and tube formation, involving several growth factors and related signaling networks. Among them, VEGF signaling is a crucial step (33); eNOS is critical for VEGF-triggered postnatal angiogenesis because NO stimulates VEGF, and eNOS is also an essential downstream effector of VEGF signaling in promoting postnatal angiogenesis (14, 34). Additionally, Jozkowicz et al. proposed the existence of a paracrine loop between endothelial cells, a source of eNOS, and vascular smooth muscle cells generating VEGF (35). Whether VEGF regulates the NO signal as the net effector of angiogenesis or whether a reciprocal relation between VEGF and NO contributes to regulating angiogenesis in our model is unclear. Under SMP30 deficiency with a low NO concentration, the stimulation of neovascularization by NO might be due to the upregulation of local VEGF expression, as previously described in studies demonstrating that a small amount of NO induces activation of VEGF synthesis in vascular smooth muscle cells (36, 37). Additionally, activated Akt in endothelial cells is known to be closely related to ischemia, and phospho-Akt is a key signaling molecule for mediating the action of VEGF and eNOS in ischemic limbs (38). Thus, we infer that

a low level of phospho-Akt following deletion of SMP30 contributes to impairment in the generation of bioactive NO through a decrease in eNOS phosphorylation. Taken together, Akt would be a good target to estimate angiogenesis, and SMP30 deficiency impairs the Akt-eNOS pathway. Augmentation of NADPH oxidase activity is reported to be required for the hypoxia-stimulated increase in VEGF expression and retinal neovascularization (39), and Nox2-containing NADPH oxidase is shown to play an important role in VEGF-induced angiogenesis (40) and in neovascularization after hind limb ischemia (41). Additionally, low levels of ROS, including superoxide and hydrogen peroxide, can serve as intracellular signals in response to ischemia, stimulating mechanisms that prevent tissue injury and promote angiogenesis (42). These studies, however, were performed in young healthy animals, a situation that does not reflect the state of increased oxidative stress encountered in old patients with advanced ischemic cardiovascular disease. The excessive production of ROS typically leads to cellular toxicity and has been associated with impaired angiogenesis in different models (17, 43). Nox2^{-/-} mice demonstrated faster blood flow recovery, increased capillary density in the ischemic muscles, and improved endothelial progenitor cell functional activities compared to Nox2^{+/+} mice, in addition to a preserved expression of eNOS in the ischemic tissues (44). According to the results in our study, the overproduction of superoxide impairs Akt-eNOS/VEGF-dependent angiogenesis associated with the decrease in SMP30. In conclusion, the reduction of SMP30 causes rarefaction of the collateral circulation resulting in severe ischemic injury, which would be a pivotal factor in complicating the prognosis of ischemic heart disease in elderly patients.

Methods

Animals. SMP30 KO mice were created from C57BL/6 mice using the gene-targeting technique previously described (45). WT C57BL/6 and SMP30 KO male mice (age, 8 weeks; body weight, 22.8 ± 2.8 g; heart weight, 166 ± 22 mg) were housed and bred in a room at $22 \pm 3^\circ\text{C}$, with relative humidity of $50 \pm 10\%$ and a 12-h light-dark cycle. The mice were given food that included vitamin C (21 mg/100 g, CLEA Japan, Tokyo, Japan) and water *ad libitum*. The experiments were conducted according to the Guidelines on Animal Experiments of Fukushima Medical University, the Japanese Government Animal Protection and Management Law (No. 105), and *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85–23, revised 1996).

Hind limb ischemia. The mice were anesthetized with 1.25% isoflurane/O₂, and the hind limbs were depilated. The rectal temperature was maintained $37.0 \pm 0.5^\circ\text{C}$. The left femoral artery was exposed aseptically and ligated distal to the inguinal ligament and proximal to the saphenous-popliteal bifurcation using 7–0 suture. The artery and all of the branches were carefully dissected free from the veins and nerves and were excised with ligation. The wound was irrigated with saline and closed; cefazolin (50 mg/kg im), furazolidone (topical) and pentazocine (10 mg/kg im) were administered.

Laser Doppler perfusion imaging. Under 1.125% isoflurane/O₂ anesthesia, scanning laser Doppler perfusion imaging (Moor Instruments, Wilmington, DE, USA) was used to record the hind limb perfusion before ligation and on postoperative days 0, 1, 7, 10 and 14. Hair was removed using depilatory cream before imaging, and the mice

were placed on a heating pad at 37°C to minimize temperature variation. Average perfusion was recorded from the plantar surface, which indexes overall limb blood flow, and is expressed as the ischemic/nonischemic ratio for assessing the variables, including ambient light and temperature (46).

Capillary density measurement. After the laser Doppler imaging on day 14 post-surgery, the gastrocnemius tissue was excised from the ischemic hind limb, and the snap was frozen in O. C. T. compound (Sakura Finetek, Tokyo, Japan) for cryosectioning. For mouse capillary density analysis, the hind limb sections from each mouse were stained using a mouse-specific CD31 (Santa Cruz Biotechnology, Dallas, TX, USA), followed by Alexafluor-594 secondary antibody. For quantification, the numbers of CD31-positive cells were counted in 10 randomly selected transverse sections in each animal. The results were averaged, and capillary density is expressed in terms of the number of CD31-positive cells per high-power field (47).

Detection of superoxide anions and NADPH oxidase activity in the aorta. Intracellular superoxide anions were quantified using a high-performance liquid chromatography/fluorescence assay that employs dihydroethidium as a probe (48). A stable fluorescent product, 2-hydroxyethidium, is formed from the reaction between dihydroethidium and superoxide anions. After the laser Doppler imaging on day 14 post-surgery, descending aortas were opened longitudinally and were incubated in Krebs-HEPES buffer containing 50 µmol/L of dihydroethidium (Molecular Probes, Eugene, OR) at 37°C for 15 minutes. The samples were washed to remove the free probe and were incubated in Krebs-HEPES buffer for 1 additional hour at 37°C. The

arteries were homogenized in 4°C cold methanol and centrifuged at 12 000 rpm. The supernatant was analyzed using high-performance liquid chromatography/fluorescence (Beckman Coulter, Brea, CA) in 37.0% acetonitrile in 0.1% aqueous trifluoroacetic acid solution. The data were normalized against tissue protein levels. NADPH oxidase activity was quantified using lucigenin-enhanced chemiluminescence (49). NADPH (100 µmol/L, Sigma-Aldrich, St. Louis, MO, USA) was added to the buffer containing the aorta (30 µg protein in 500 µL), and lucigenin was injected automatically at 5 µM to avoid known artifacts when used at higher concentrations. NADPH oxidase activity was calculated by subtracting the basal values from those in the presence of NADPH.

Total thiol and tissue glutathione concentrations in the aorta. Total thiols were determined in the descending aorta homogenates by measuring the absorbance of 5-thio-2-nitrobenzoic acid, the reaction product of sulfhydryl groups with 5, 5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent). An equal volume of 10% metaphosphoric acid was added to the samples, the resulting precipitated proteins were pelleted by centrifugation, and the supernatant was neutralized with 50 µL/mL of 4 mol/L triethanolamine. Thiols were measured by adding 200 µL of Ellman's reagent from a commercially available assay to 50 µL of the neutralized supernatant. The absorbance of the Ellman's reagent adduct was measured at 405 nm (50). Tissue concentrations of glutathione (total, reduced, and oxidized) were measured in tissue homogenates (10% wt/vol) after deproteinization using metaphosphoric acid in an enzymatic recycling method with glutathione reductase provided by a commercially available assay (Cayman Chemical, Ann Arbor, MI, USA) (51). The values were

normalized to protein concentration in the homogenate.

Evaluation of intracellular nitric oxide (NO) level in the aorta. To examine the production of NO at 14 days after femoral artery ligation, the mice were anesthetized with pentobarbital sodium (50 mg/kg ip), and the descending aorta was removed and rinsed with cold physiological salt solution (PSS) composed of the following (in mM): 119 NaCl, 4.7 KCl, 1.17 MgSO₄, 1.6 CaCl₂, 1.18 NaH₂PO₄, 24 NaHCO₃, 0.026 EDTA, and 5.5 glucose. After removal of any adhering connective tissue, the aorta was cut into several segments. Each segment was opened with a fine scissors and was pinned onto a piece of rubber with the endothelium facing up, so that the endothelial cells on the inside surface of the aorta were exposed to the solution. After they were affixed to the rubber, the segments of the aorta were placed into the PSS at 37°C and were aerated for 2 h with a gas mixture containing 21% O₂, 5% CO₂, and 74% N₂. The NO levels in the vessels were assessed using 4, 5-diaminofluorescein (DAF-2DA, Sekisui Medical, Tokyo, Japan) (52, 53). The aortic segments were loaded with 5 μM DAF-2DA for 30 min at 37°C in HEPES buffer (pH 7.4). Once loading was completed, the vessels were rinsed three times with HEPES buffer and were placed in a chamber containing HEPES buffer maintained at 37°C with a water bath. L-arginine (100 μM) was placed into the chamber during measurements to ensure adequate substrate availability for NOS. Fluorescent images were recorded using an Olympus IX71 inverted microscope (Olympus CCD, Tokyo, Japan) and were analyzed for fluorescence intensity using fluorescein isothiocyanate excitation/emission spectra from digitized images and were normalized to the vessel area (expressed as intensity/100 μm²). All of the camera

settings were maintained constant throughout the image analysis.

VEGF immunoassays. The VEGF content in the gastrocnemius muscles from the ischemic hind limb (left) and the non-ischemic hind limb (right) was quantified using Quantikine Mouse VEGF Immunoassay kits (IBL, Fujioka, Japan) according to the manufacturer's instructions at 14 days post-surgery (54). The change in the VEGF level is expressed as the ratio of the VEGF value from the ischemic muscle to that from the non-ischemic muscle.

Western blotting. Total protein was extracted from the snap-frozen ischemic muscles dissected from the mice on day 14 after surgery using Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) with Protease Inhibitor Cocktail (BD Biosciences, San Jose, CA, USA). Western blotting analyses with antibodies for e-NOS, phospho-eNOS (Ser 1177), Akt, and phospho-Akt (Ser 473) (Cell Signaling Technology, Danvers, MA, USA) were performed. Band intensities were quantified with Image J software and normalized by β -actin (20, 55).

Statistics. The data (mean \pm SEM values) were subjected to ANOVA followed by Dunn-Bonferroni corrected *t* tests for preplanned comparisons or Student's *t* test.

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Figure legends.

Figure 1

Deficiency of SMP30 impaired blood flow restoration in mouse ischemic hind limbs.

Representative laser Doppler perfusion imaging after inducing hind limb ischemia in SMP30 knockout (SMP30 KO) and wild-type (WT) mice (**A**). Ischemic (left)/non-ischemic (right) limb blood flow ratio used for quantitative analysis (**B**).

Immunofluorescent staining of cross sections from the ischemic muscle at 14 days after inducing ischemia, stained for the endothelial marker CD31. Scale bars, 100 μm (**C**).

Quantitative analysis of capillary density expressed in terms of the number of CD31-positive cells/high-power field (HPF) 14 days after the hind limb ischemia (**D**).

The values are expressed as the mean \pm SEM; n=16 for each experimental group;

* $P < 0.01$ vs. wild-type (WT) mice.

Figure 2

Effect of SMP30 deficiency on the generation of superoxide and activity of NADPH oxidase in the aorta.

The levels of superoxide generation (**A**) and NADPH oxidase activity (**B**) were greater in the aorta of SMP30 KO mice compared to that of WT mice. The values are expressed as the mean \pm SEM; n=16 for each experimental group; * $P < 0.01$ vs. WT mice.

Figure 3

Aortic tissue total thiols and glutathione levels.

In the aortic tissue, the levels of total thiols (**A**) and reduced glutathione (GSH) (**B**)

decreased in SMP30 KO mice compared to those in WT mice. The deficiency of SMP30 led to a decrease in the reduced/oxidized glutathione (GSH/GSSG) ratio (C). The values are expressed as the mean \pm SEM; n=16 for each experimental group; * P <0.01 vs. WT mice.

Figure 4

NO production induced by L-arginine examined in the aortic tissue.

Immunofluorescent staining of NO induced by L-arginine from the aortic strip, stained for DAF-2DA. Scale bars, 10 μ m. (A). Quantification of NO production in response to L-arginine (B). NO production was impaired in the aortic tissue with SMP30 deficiency. The values are expressed as the mean \pm SEM; n=16 for each experimental group; * P <0.01 vs. WT mice.

Figure 5

Levels of VEGF, phospho-Akt, Akt, phospho-eNOS, eNOS in ischemic and non-ischemic muscle 14 days post-surgery.

The VEGF concentration ischemic/non-ischemic muscle ratio was lower in SMP30 KO mice than that in WT mice at 14 days post-surgery (A), which suggests that SMP30 deficiency impairs post-ischemic VEGF upregulation in the ischemic limb muscle. The protein expressions of total Akt, phospho-Akt (B), eNOS, and phospho-eNOS (C) in the ischemic muscle on day 14 after ischemia were quantified using the Western blotting. Expression of eNOS and phosphorylation of Akt and eNOS were suppressed in the

ischemic muscle of SMP30 KO mice than those of WT mice. The values are expressed as the mean \pm SEM; n=16 for each experimental group; *P<0.01 vs. WT mice.