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Pro-protein convertase subtilisin/kexin type 9 promotes intestinal tumor development by activating Janus kinase 2/signal transducer and activator of transcription 3/SOCS3 signaling in Apc$^{Min/+}$ mice

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Abstract

Introduction: Pro-protein convertase subtilisin/kexin type 9 (PCSK9) regulates lipoprotein homeostasis in humans. Evolocumab is a selective PCSK9 inhibitor that can reduce low-density lipoprotein cholesterol (LDLC) level and decrease hypercholesterolemia. The current study aimed to explore whether PCSK9 increases the risk of colorectal cancer.
Methods: First, we utilized the classic intestinal tumor Apc$^{Min/+}$ mouse model and PCSK9 knock-in (KI) mice to establish Apc$^{Min/+}$PCSK9(KI) mice. Then, we investigated the effect of PCSK9 overexpression in Apc$^{Min/+}$PCSK9(KI) mice and PCSK9 inhibition using evolocumab on the progression of intestinal tumors *in vivo* by hematoxylin and eosin (HE) staining, Western blot, and immunohistochemistry (IHC) assay.
Results: Apc$^{Min/+}$PCSK9(KI) mice had higher numbers and larger sizes of adenomas, with 83.3% of these mice developing adenocarcinoma (vs. 16.7% of Apc$^{Min/+}$ mice). However, treatment with evolocumab reduced the number and size of adenomas and prevented the development of adenocarcinomas in Apc$^{Min/+}$ mice. PCSK9 overexpression reduced tumor cell apoptosis, the Bax/bcl-2 ratio, and the levels of cytokine signaling 3 protein (SOCS3) suppressors, but activated Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling in intestinal tumors. In contrast, evolocumab treatment had the opposite effect on Apc$^{Min/+}$mice.
Conclusion: PCSK9 might act as an oncogene or have an oncogenic role in the development and progression of colorectal cancer *in vivo* via activation of JAK2/STAT3/SOCS3 signaling.

Keywords

Colorectal cancer, PCSK9, evolocumab, Apc$^{Min/+}$ mice, PCSK9(KI) mice

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Introduction

Colorectal cancer (CRC) is the second and third most common cancer in women and men in 2020, respectively.[1] The incidence of colorectal cancer in China has increased over the past five years, from the fifth most common cancer in 2015 to the second most common cancer in 2020.[1] Colorectal cancer treatment still relies on surgery, with or without neoadjuvant radiotherapy and chemotherapy, although novel treatment options such as immune and targeted therapy are increasingly used.[2-4] Unfortunately, the 5-years survival rate of patients with CRC is very low, and

there is, therefore, a significant need to identify novel strategies and effective agents that can control CRC development and progression.

The *Apc* gene is a major tumor suppressor that helps regulate colorectal carcinogenesis and mutations this gene contributes to malignant transformation of colorectal cells. Thus, *Apc* can be thought of as "the gatekeeper gene" for colon mucosae.[5] The C57BL/6J $Apc^{Min/+}$ mouse model has a point mutation in the *Apc* gene, which causes the development of numerous intestinal adenomas, thus providing a useful *in vivo* system to investigate colorectal tumorigenesis, prevention, and treatment.

The pro-protein convertase subtilisin/kexin type 9 (PCSK9) was first reported in 2003 by Abifadel et al.,[6] who showed that two gain-of-function (GOF) mutations (S127 R and F216 L) in the PCSK9 gene were associated with autosomal dominant hypercholesterolemia. Since then, research on PCSK9 has focused mostly on the regulation of lipoprotein homeostasis and has largely involved studying how PCSK9 promotes the internalization and degradation of low-density lipoprotein receptor (LDLR) and in turn reduces the LDLR number and recycling thereof in hepatocytes.[7] In addition to regulating the homeostasis of low-density lipoprotein cholesterol (LDLC), PCSK9 also participates in many non–cholesterol-related processes, such as endothelial function, inflammation, and platelet activation.[8] Although mainly synthetized and released by the liver, PCSK9 is also expressed in many tissues (e.g., intestine, kidney, and brain) and cell types (e.g., macrophages).[9] Altered PCSK9 expression has been reported in liver, gastric, and thyroid cancers.[10-12] On one hand, preclinical and clinical studies have confirmed that manipulating cholesterol metabolism can inhibit tumor growth, reshape the immune landscape, and enhance anti-tumor immunity[13]; On the other, research data have demonstrated that PCSK9 participates in cell proliferation and apoptosis.[8] A nanoliposome anti-PCSK9 vaccine was reported to moderately reduce tumor growth and prolong the life span of mice with colorectal cancer.[14] These findings raise the question of whether PCSK9 plays a role in enhancing colorectal cancer risk. This study aimed to explore the relationship between PCSK9 and CRC, as well as the relevant underlying mechanisms of action. Evolocumab, a selective PCSK9 inhibitor, can reduce LDLC levels and decrease hypercholesterolemia.[15]

We used the classic intestinal tumor mouse model $Apc^{Min/+}$ and PCSK9 knock-in (KI) mice to establish $Apc^{Min/+}$PCSK9(KI) mice. Using $Apc^{Min/+}$PCSK9(KI) mice and evolocumab, we investigated the effects of PCSK9 overexpression and PCSK9 inhibition on *Apc* mutation-mediated intestinal tumor development *in vivo*. This study provides insight into the role of PCSK9 in the regulation of colorectal cancer development and progression, as well as the utility of evolocumab in controlling colorectal cancer.

Materials and methods

Animals and animal care

This study was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the School of Pharmaceutical Sciences, Shandong University (Jinan, China) (approval #SYXK (LU) 20100418). The experiments followed the Guidelines for the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research. C57BL/6J-$Apc^{Min/+}$ mice were purchased from Nanjing Biomedical Research Institute, Nanjing University (Nanjing, China), and C57BL/6J PCSK9(KI) mice were obtained from the Institute of Laboratory Animal Sciences, Peking Union Medical College, Chinese Academy of Medical Sciences (Beijing, China). These mice were maintained in a specific pathogen-free (SPF) in-house animal facility under controlled temperature and humidity, with alternating 12-h light and dark cycles. The mice received SPF mouse chow and were allowed to drink sterile water *ad libitum*. The mice were acclimated to laboratory conditions for at least one week before the experiments and/or breeding. All surgical procedures were performed under anesthesia (4% isoflurane), and the mice were sacrificed via exposure to CO_2 and cervical dislocation.

Generation of $Apc^{Min/+}$PCSK9(KI) mice

We first established $Apc^{Min/+}$PCSK9(KI) mice using C57BL/6J-$Apc^{Min/+}$ and C57BL/6J PCSK9(KI) mice. In brief, male $Apc^{Min/+}$ mice were bred with female PCSK9(KI) mice to generate $Apc^{Min/+}$PCSK9(KI) littermates, and male $Apc^{Min/+}$ mice were bred with female C57BL/6J mice to generate $Apc^{Min/+}$ littermates. The littermates were genotyped using PCR and Southern blotting

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according to protocols provided by the institutes where the mice were purchased.[16]

Drug treatment

$Apc^{Min/+}$ mice (12 females and 12 males) were assigned into two groups ($Apc^{Min/+}$ mice and evolocumab-treated $Apc^{Min/+}$ mice) based on body weight. The number of mice was determined according to previous literatures.[17,18] At two months old, before their intestinal adenomas were apparent, mice in the evolocumab-treated $Apc^{Min/+}$ group were treated with evolocumab (also called Repatha; Amgen Manufacturing Limited State Road 31, Km 24.6, Juncos, PR 00777, America) at a dose of 10 mg/kg via a single flank subcutaneous injection on a 3-month schedule, as previously described.[19] $Apc^{Min/+}$ and $Apc^{Min/+}$PCSK9(KI) mice were treated with normal saline in the same manner. Mice were monitored daily and their body weights measured every three days. Mice exhibiting physical symptoms were treated based on the animal protocol, and all mice were euthanized with CO_2 at the age of six months. The small and large intestines were removed immediately following sacrifice and flushed free of debris with phosphate-buffered saline (PBS). A single observer without any knowledge of the experimental groups counted and measured the tumors under a dissecting microscope equipped with a micrometer. The intestinal tissues and tumors were fixed in 10% formalin for 24 h and then processed.

Hematoxylin and eosin (HE) staining

Mouse tissue specimens were processed and embedded in paraffin blocks and prepared into 4–5 μm thick sections on coated slides. Tissue sections were deparaffinized in xylene, rehydrated in a series of ethanol solutions, and stained with HE using an in-house laboratory protocol. The stained sections were scanned using a NanoZoomer scan system (Hamamatsu Photonics, Hamamatsu, Japan). A senior pathologist without knowledge of the experimental groups reviewed and evaluated the histology of these tissue sections. Adenomas were characterized by an increase in tissue volume and disappearance of the intestinal mucosal structure.

Western blot assay

A BCA assay kit (Thermo Scientific Pierce, Rockford, IL, USA) was used to measure protein concentration. The protein samples (30 μg total protein from each mouse) were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (IPVH00010; Merck Millipore, Billerica, MA). The PVDF membranes were then blocked with 5% nonfat milk for 1–2 h and incubated with primary antibodies overnight at 4°C. The next day, the PVDF membranes were washed with 0.1% Tween-20/TBS buffer. After incubation with horseradish peroxidase-conjugated secondary antibody for 1–2 h, the bands were visualized with enhanced chemiluminescence reagent and quantified by densitometry using a ChemiDoc XRS+ image analyzer. Image J software (1.52q, Wayne Rasband, National Institutes of Health, USA) was used to perform a densitometry analysis and determine the relative levels of target proteins normalized to β-actin protein. All experiments were performed in triplicate ($n = 4$).

Immunohistochemistry (IHC) assay

Mouse tissue sections were deparaffinized in xylene, rehydrated in a series of ethanol solutions, and placed first in distilled water and then in PBS. The sections were first incubated with normal goat serum at room temperature for 30 min and then with a primary antibody at 4°C overnight, as previously described.[20,21] The primary antibodies used were anti-p38 MAPK (Cat. #14064-1-AP), JAK2 (Cat. #17670-1-AP), SOCS3 (Cat. #14025-1-AP; Proteintech Group, Chicago, IL, USA), anti-phosphorylated (p)-STAT3 (Tyr705; Cat. #AF3293; Affinity Biosciences, Beijing, China), anti-p-p38 MAPK (Thr180/Tyr182; Cat. #4511), Bax (cat. #2774; Cell Signaling Technology, Danvers, MA, USA), and anti-Bcl-2 (Cat. #ab182858; Abcam, Cambridge, UK). The following day, the sections were washed three times with Tris-buffered saline (TBS) and incubated at room temperature for 30 min with a secondary antibody from the Vector VECTASTAIN Elite ABC kit (Cat. #PK-6100; Vector Laboratories, Burlingame, CA, USA). The color reaction was conducted using 3,3-diaminobenzidine (DAB) solution, and the sections were counterstained with Mary's hematoxylin. The immune-stained sections were reviewed under a microscope and scored independently by two investigators (Jie Zhu and Huan-hua Luo) following a previously described protocol.[21]

Statistical analysis

Data are presented as mean ± standard deviation (SD). Student's t-test was used to analyze paired samples. All statistical analyses were performed using the SPSS/Win 13.0 software (SPSS, Chicago, IL, USA). Different statistical significance levels are considered as follows: * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$ between $Apc^{Min/+}$ mice and evolocumab-treated $Apc^{Min/+}$ mice and between $Apc^{Min/+}$ and $Apc^{Min/+}$PCSK9(KI) mice.

Results

PCSK9 induces intestinal tumor development in $Apc^{Min/+}$ mice

We used transgenic mouse models to assess the role of PCSK9 in the regulation of tumor growth. First, male

$Apc^{Min/+}$ mice were crossed with female PCSK9(KI) mice, and the genotypes of their offspring determined at weaning (Table 1). There was no neonatal mouse with physical defects and no significant differences in the weights of neonatal mice in each group (Figure 1(a)). We conducted our experiments using littermates with the same genetic background, that is, we sacrificed them after treatment and assessed the growth of intestinal tumors. Before euthanasia, 83.3% of $Apc^{Min/+}$PCSK9(KI) mice suffered from rectocele, hematochezia, weight loss, and poor physical shape (e.g., dry hair and thin bodies). In contrast, 91.7% of the evolocumab-treated $Apc^{Min/+}$ mice appeared healthy, active, and gained more weight (Figure 1(a)).

Table 1. Genotypes of the progeny of the cross between female $APC^{Min/+}$ mice and male PCSK9(KI) mice.

Genotype	Observed*	Predicted
Wild type mice	32 (27.6%)	29 (25.0%)
$Apc^{Min/+}$ mice	30 (25.9%)	29 (25.0%)
$Apc^{Min/+}$ PCSK9(KI) mice	18 (15.5%)	29 (25.0%)
PCSK9(KI) mice	36 (31.0%)	29 (25.0%)

*Observed progeny number (percentage of total based on 116 mice).

Overexpression of PCSK9 in $Apc^{Min/+}$PCSK9(KI) mice led to an increase both in the tumor size and the number of intestinal tumors (Figure 1(c)). 16.8, 14.3, and 20.2 small intestinal tumors and 18.3, 16.0, and 22.7 colon tumors were extracted from $Apc^{Min/+}$, evolocumab-treated $Apc^{Min/+}$, and $Apc^{Min/+}$PCSK9(KI) mice, respectively (Figure 1(b)). The mean tumor sizes were 2.6 mm, 1.9 mm, and 3.0 mm in the small intestines and 2.9 mm, 2.5 mm, and 3.3 mm in the colons of $Apc^{Min/+}$ mice, evolocumab-treated $Apc^{Min/+}$ mice, and $Apc^{Min/+}$PCSK9(KI) mice, respectively (Figure 1(d)). These data indicate that PCSK9 plays an important role in the induction of tumor development in the mouse model.

PCSK9 promotes intestinal tumor progression and malignant transformation in $Apc^{Min/+}$ mice

Previous studies have shown that $Apc^{Min/+}$ mice contain only benign polyps, which are rarely malignant.[22,23] Our current study revealed a decrease in goblet cells but a high degree of cell aggregation and severe inflammation in the colonic mucosa of $Apc^{Min/+}$ mice (Figure 2(a)). Two (16.7%) of the $Apc^{Min/+}$ mice developed malignant colonic transformation. In the evolocumab-treated $Apc^{Min/+}$ group, the degree of cell aggregation was low, tissue inflammation was mild, and none of the 12 mice developed

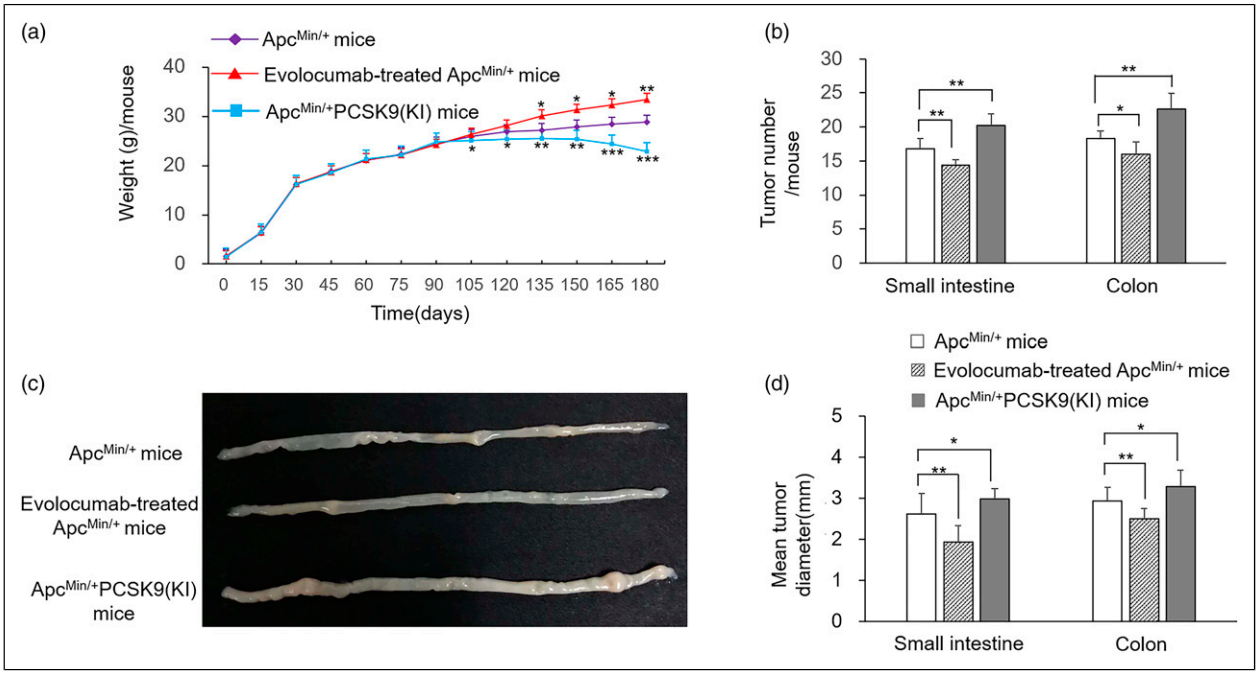

Figure 1. PCSK9 induces intestinal tumor development in $Apc^{Min/+}$ mice. (a) Body weight of mice. (b) Tumor numbers. (c) Illustration of intestinal macroscopic photos. (d) Tumor size. The data are described as mean ± SD (n = 12 mice for each group). Significant differences are shown between $Apc^{Min/+}$ mice and evolocumab-treated $Apc^{Min/+}$ mice and between $Apc^{Min/+}$ mice and $Apc^{Min/+}$PCSK9(KI) mice. *p < 0.05, **p < 0.01, and ***p < 0.001.

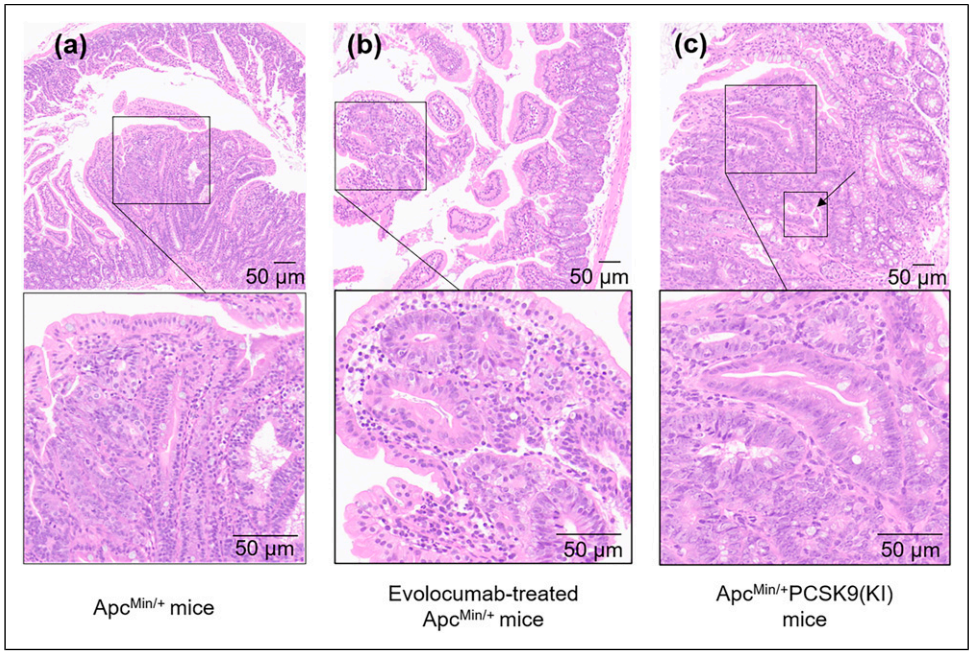

Figure 2. PCSK9 promotes intestinal tumor progression and malignant transformation in $Apc^{Min/+}$ mice by HE staining. (a) The colonic mucosae of $Apc^{Min/+}$ mice showed a high degree of cell aggregation and severe inflammation. (b) A low degree of cell aggregation and mild inflammation in colonic mucosae of evolocumab-treated $Apc^{Min/+}$ mice. (c) The colonic mucosae of $Apc^{Min/+}$PCSK9(KI) mice showed carcinogenic cytology and nuclear necrosis (the arrow). The stained sections were scanned by the NanoZoomer scan system, and representative photos are shown (100× and 300×). Scale bar = 50 μm.

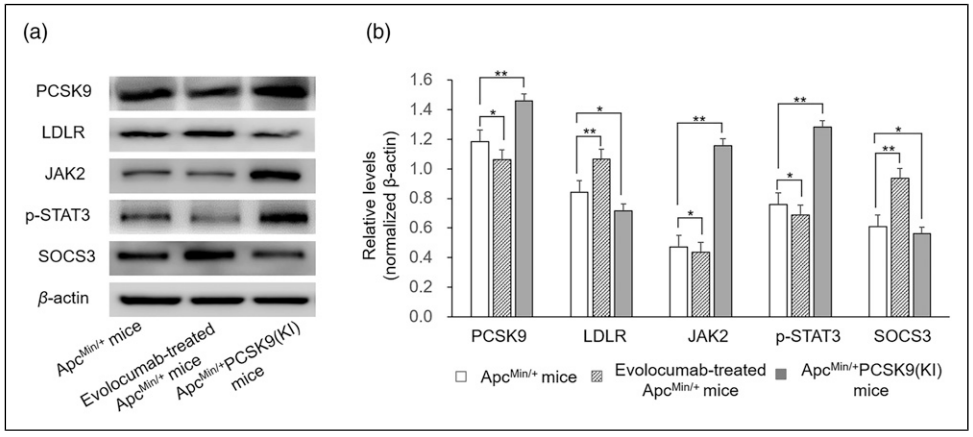

Figure 3. Western blot assay showed PCSK9 regulated LDLR level and JAK2/STAT3/SOCS3 signaling. (a) Expression levels of PCSK9, LDLR, JAK2, p-STAT3, and SOCS3. β-actin served as a loading control. (b) The bar graph indicates the relative density calculated by Image J software. The results represent experiments performed in triplicate. *$p < 0.05$ and **$p < 0.01$.

adenocarcinoma (Figure 2(b)). In contrast, 83.3% of $Apc^{Min/+}$PCSK9(KI) mice developed adenocarcinoma. The intestinal tract of these mice contained tumors with localized mucosal ulcerations, irregular glands, thickened chromatin, hyperchromatic nuclei, an increased nuclei-cytoplasm ratio, and nuclear necrosis (Figure 2(c)). Based on these results, we concluded that inhibition of PCSK9 using evolocumab inhibited colorectal tumor development, whereas its overexpression promoted intestinal tumor progression and malignant transformation against the *Apc* mutation background.

PCSK9 activates JAK2/STAT3/SOCS3 signaling against the Apc mutation background in mice

First, we analyzed the expression levels of PCSK9 and LDLR in colon tumor tissues. PCSK9 overexpression reduced the level of LDLR, while its inhibition increased

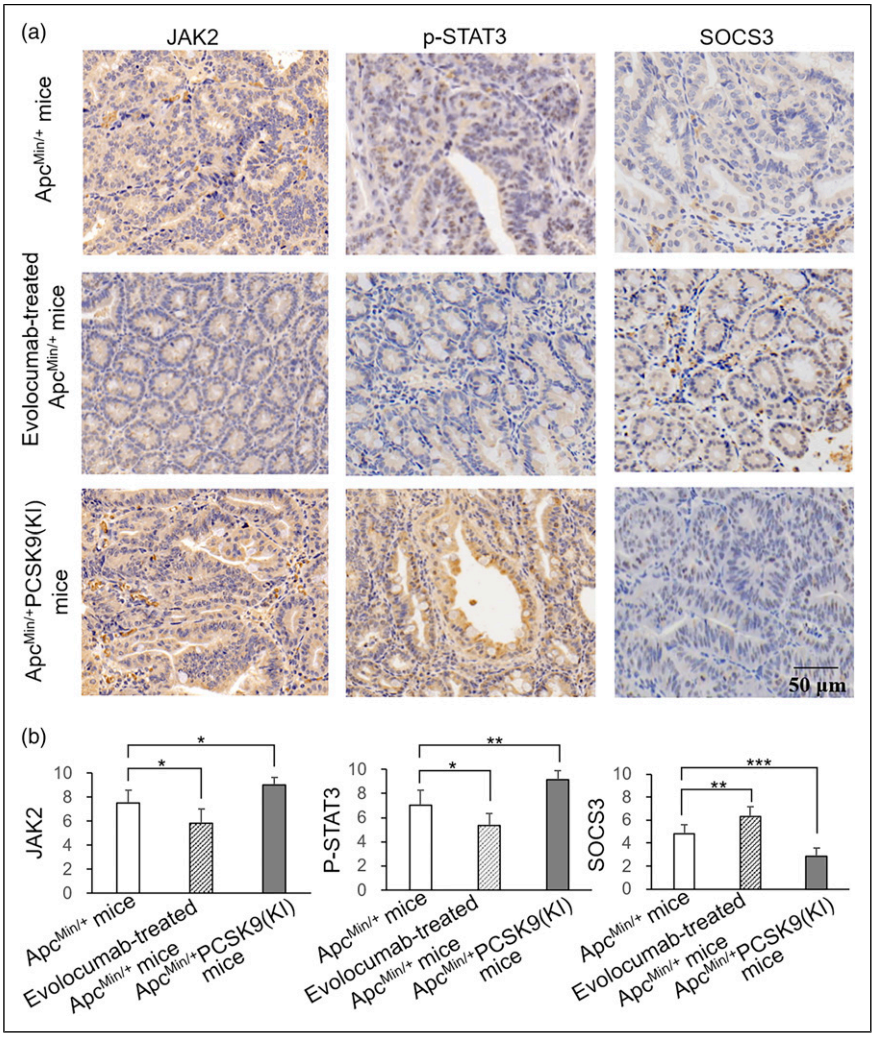

Figure 4. Immunohistochemistry (IHC) assay. (a) Representative photos are shown (200×). Scale bar = 50 μm. (b) The bar graph indicates IHC staining scores. Data are presented as the mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

the level of LDLR in evolocumab-treated $Apc^{Min/+}$ mice compared with $Apc^{Min/+}$ mice (Figure 3). We then investigated whether PCSK9 interacts with the JAK2/STAT3 signaling pathway to promote mouse intestinal tumor development and malignant transformation. As shown in Figure 3, inhibition of PCSK9 using evolocumab increased the levels of SOCS3 proteins, leading to the inhibition of JAK2/STAT3 signal activation. On the other hand, the levels of SOCS3 were lower in adenomas of $Apc^{Min/+}$PCSK9(KI) mice, even though the levels of phosphorylated-STAT3 (p-STAT3) were higher. In addition, we detected the expression of JAK2, p-STAT3, and SOCS3 using IHC assay and obtained similar results (Figure 4). These results demonstrate that PCSK9 can activate JAK2/STAT3/SOCS3 signaling to promote intestinal tumor progression and malignant transformation in $Apc^{Min/+}$ mice.

PCSK9 inhibits intestinal tumor cell apoptosis in $Apc^{Min/+}$ mice

We next assessed changes in the levels of tumor cell apoptosis in the intestinal tissues using Western blot analysis and found that the Bax/bcl-2 ratio was higher in the intestinal tumors of $Apc^{Min/+}$mice after treatment with evolocumab than in $Apc^{Min/+}$ mice, although the opposite trend was observed in the Bax/bcl-2 ratio in $Apc^{Min/+}$PCSK9(KI) mice (Figure 5). These findings are consistent with results from the IHC assay (Figure 6). Thus, these results indicate that PCSK9 overexpression inhibits intestinal tumor cell apoptosis and promotes tumor progression and malignant transformation in $Apc^{Min/+}$ mice, whereas evolocumab might inhibit the effect of PCSK9.

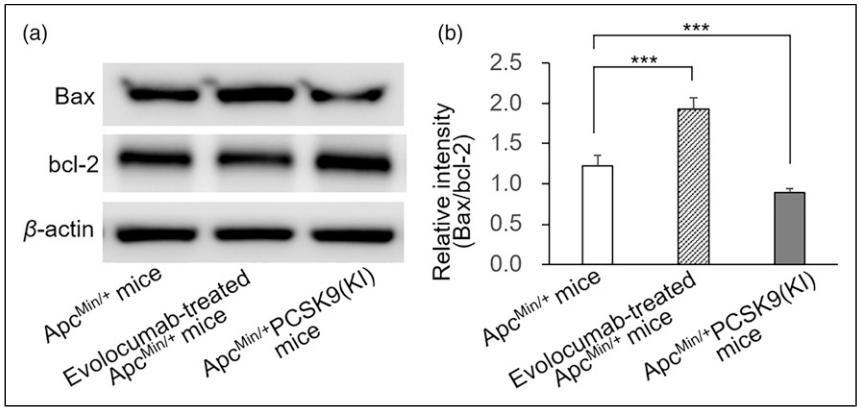

Figure 5. Levels of Bax/bcl-2 by Western blot assay. (a) PCSK9 inhibits intestinal tumor cell apoptosis in $Apc^{Min/+}$ mice. The β-actin protein served as a loading control. (b) The bar chart shows the ratios of Bax/bcl-2. The data were quantified with Image J software. ***$p < 0.001$.

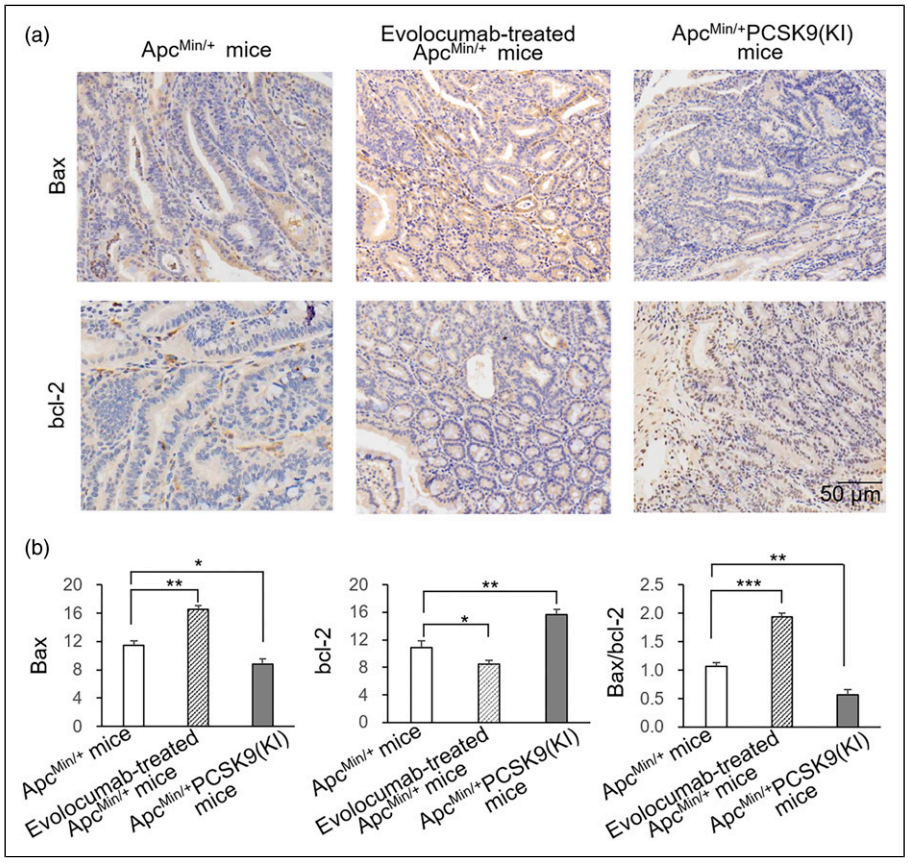

Figure 6. Levels of Bax/bcl-2 by IHC assay. (a) Representative photos are shown (200×). Scale bar=50 μm. (b) The bar graph indicates IHC staining scores. Data are presented as the mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Discussion

Colorectal cancer development is a multi-stage, multi-factor, and multi-gene-altered molecular and pathological processes.[24] Previous studies have revealed that chronic intestinal inflammation,[25] a high-fat and low-fiber diet,[26] and hereditary factors[27] are all closely associated with colorectal carcinogenesis. The "adenoma-to-carcinoma" consequence theory from Muto et al. is widely accepted, and thus, colorectal adenoma is considered a precancerous lesion.[28] Seong[29] et al. confirmed that hyper-LDLC is associated with the presence of colorectal adenomas,

especially in the proximal colon. It is well known that PCSK9 plays a crucial role in regulating the level of LDLC *in vivo*. In the current study, we examined the role of PCSK9 expression and evolocumab treatment in the regulation of intestinal tumor development using the classic $Apc^{Min/+}$ mouse model. We found that both the number and size of adenomas in $Apc^{Min/+}$PCSK9(KI) mice increased, and 83.3% of the mice developed adenocarcinoma. However, evolocumab treatment significantly reduced the number and size of adenomas. PCSK9 has multiple biological functions, including regulation of cell apoptosis and the tissue inflammation response, in addition to regulating blood lipid *in vivo*.[30,31] The inhibition of PCSK9 induced the apoptosis of the lung cancer cells, A549, and the glioma cells, U251,[32,33] prevented liver metastasis in mice with melanoma,[34] and prolonged the survival of BALB/c mice inoculated with the colon cancer cells, CT26.[14]

Over the past 10 years, a considerable amount of literature has accumulated on the role of PCSK9 in hypercholesterolemia and human malignancies.[35,36] Recently, researchers have begun to assess anti-PCSK9 vaccines against breast cancer and melanoma in mice,[34,37] and a previous study reported that a nano liposomal anti-PCSK9 vaccine has potential anti-tumor effect in colorectal cancer.[14] This study directly linked PCSK9 with the development and progression of mouse adenomas and adenocarcinomas in transgenic mice, supporting previous studies which examined an anti-PCSK9 vaccine against colorectal cancer.

Furthermore, we explored the underlying molecular mechanisms through which PCSK9 overexpression induces alterations in the expression of various proteins. Our results showed that the level of p-STAT3 in $Apc^{Min/+}$PCSK9(KI) mice was higher than in $Apc^{Min/+}$ and evolocumab-treated $Apc^{Min/+}$ mice. STAT3 activation promotes chronic inflammation, which increases the susceptibility of normal cells to carcinogenesis, and the continuous activation of STAT3 drives various oncogenic pathways, including cell cycle progression, proliferation, inhibition of apoptosis, tumor angiogenesis, invasion, and metastasis. Therefore, STAT3 is an important signaling pathway regulator and transcriptional mediator of carcinogenic signals.[38-40] STAT3 participates mainly in the regulation of JAK2/STAT3 signaling pathway, and STAT3 molecules are activated by the binding of growth factors or cytokines such as IL-6, IL-10, and IL-11 via JAK-induced cell surface receptors.[41] In fact, STAT3 is also involved in mediating leptin- and resistin-driven PCSK9 activation.[42] This study suggests that PCSK9 overexpression might activate JAK2/STAT3 signaling, at least in $Apc^{Min/+}$ mice. Interestingly, the activation of the JAK2/STAT3 pathway led to a significant increase in bcl-2, an anti-apoptotic downstream target of the STAT pathway, and a reduction in Bax, a pro-apoptotic protein. Our results are consistent with these observations. We hypothesized that PCSK9 first activated JAK2/STAT3 signaling, leading to a decrease in the Bax/bcl-2 ratio, which inhibited intestinal tumor cell apoptosis in $Apc^{Min/+}$ mice. Moreover, our data showed that the levels of SOCS3 were lower in $Apc^{Min/+}$PCSK9(KI) mice and higher in evolocumab-treated $Apc^{Min/+}$ mice compared with $Apc^{Min/+}$ mice. The SOCS3 protein, an important member of the SOCS family, was reported to induce PCSK9 expression in hepatic HepG2 cell line[43] and inhibit the JAK2/STAT3 signaling pathway and plays a negative regulatory role.[44] Therefore, PCSK9 could activate JAK2/STAT3 signaling partly by downregulating SOCS3 levels. In conclusion, this study strongly indicates that PCSK9 is an oncogene or has an oncogenic role in the development and progression of colorectal cancer via activation of the JAK2/STAT3/SOCS3 signaling pathway. Indeed, we confirmed that PCSK9-led JAK2/STAT3 signaling was able to differentially regulate the expression of proliferation- and apoptosis-related proteins. In contrast, the selective anti-PCSK9 antibody, evolocumab, induced effects opposite to that of PCSK9 in mouse adenoma and adenocarcinoma development and progression.

The inhibition of PCSK9 was reported not to influence the proliferation of mouse colon cancer cells, CT26, *in vitro*, but inhibit tumor growth in mice.[45] PCSK9 inhibition significantly enhanced the anti-tumor immune responses of cytotoxic T cells.[45] Therefore, PCSK9 plays important roles in colorectal cancer development, not only through JAK2/STAT3 signaling, but also via its anti-tumor immunity effects. In summary, our data demonstrate that PCSK9 expression moderately accelerated tumor progression in an $Apc^{Min/+}$ mouse model. PCSK9 acts as an oncogene or at least plays an oncogenic role in the development of colon cancer by inhibiting SOCS3 expression and activating JAK2/STAT3 signaling. Evolocumab has been widely used in clinical settings, and PCSK9 inhibitors have been reported to enhance immune checkpoint blockade therapy without additional side effects.[45] Further preclinical and clinical trials are needed to verify the efficacy and safety of PCSK9 inhibitors on tumor incidence and progression. Evolocumab may be useful as a novel treatment option for the control of human colorectal cancers.

Some limitations of this study should be noted. First, our data on PCSK9 inhibition are limited to evolocumab and whether deletion of SOCS3, JAK2, and STAT3 could influence the observed effects of PCSK9 overexpression and/or PCSK9 inhibition need to be explored. Second, in this study, we did not focus on the influences of PCSK9-mediated cholesterol level in colorectal tumor progression. The relationship between PCSK9-regulated cholesterol level and progression of colorectal tumor need to be further studied.

Conclusions

PCSK9 might promote growth and malignant transformation of intestinal tumors in mice with *Apc* mutation by activating the JAK2/STAT3/SOCS3 signaling pathway. Evolocumab, a selective inhibitor of PCSK9, could inhibit the formation and carcinogenesis of intestinal adenomas in $Apc^{Min/+}$ mice.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethics approval

Ethical approval for this study was obtained from *Institutional Animal Care and Use Committee (IACUC) of the School of Pharmaceutical Sciences, Shandong University (Jinan, China) (approval #SYXK (LU) 20100418) *.

Animal welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

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References

1. WHO. *Global Cancer Observatory* (2020). https://gco.iarc.fr/.
2. Cunningham D, Atkin W, Lenz HJ, et al. (2010) Colorectal cancer. *Lancet* 375: 1030–1047. DOI: 10.1016/S0140-6736(10)60353-4. 2010/03/23.
3. Li Y, Wang J, Ma X, et al. (2016) A review of neoadjuvant chemoradiotherapy for locally advanced rectal cancer. *Int J Biol Sci* 12: 1022–1031. DOI: 10.7150/ijbs.15438. 2016/08/05.
4. Boland PM and Ma WW (2017) Immunotherapy for colorectal cancer. *Cancers* 9. DOI: 10.3390/cancers9050050. 2017/05/12.
5. van Heemst D, den Reijer PM and Westendorp RG (2007) Ageing or cancer: a review on the role of caretakers and gatekeepers. *Eur J Canc* 43: 2144–2152. DOI: 10.1016/j.ejca.2007.07.011. 2007/09/04.
6. Abifadel M, Varret M, Rabes JP, et al. (2003) Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet* 34: 154–156. DOI: 10.1038/ng1161. 2003/05/06.
7. Yamamoto T, Lu C and Ryan RO (2011) A two-step binding model of PCSK9 interaction with the low density lipoprotein receptor. *J Biol Chem* 286: 5464–5470. DOI: 10.1074/jbc.M110.199042. 2010/12/15.
8. Macchi C, Ferri N, Sirtori CR, et al. (2021) PCSK9: a view beyond the canonical cholesterol lowering impact. *Am J Pathol*; 191(8): 1385–1397. DOI: 10.1016/j.ajpath.2021.04.016.
9. Zaid A, Roubtsova A, Essalmani R, et al. (2008) Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. *Hepatology* 48: 646–654. DOI: 10.1002/hep.22354. 2008/07/31.
10. Bhat M, Skill N, Marcus V, et al. (2015) Decreased PCSK9 expression in human hepatocellular carcinoma. *BMC Gastroenterol* 15: 176. 2015/12/18. DOI: 10.1186/s12876-015-0371-6.
11. Marimuthu A, Subbannayya Y, Sahasrabuddhe NA, et al. (2013) SILAC-based quantitative proteomic analysis of gastric cancer secretome. *Proteomics Clin Appl* 7: 355–366. DOI: 10.1002/prca.201200069. 2012/11/20.
12. Ozkan C, Akturk M, Altinova AE, et al. (2015) Proprotein convertase subtilisin/kexin type 9 (PCSK9), soluble lectin-like oxidized LDL receptor 1 (sLOX-1) and ankle brachial index in patients with differentiated thyroid cancer. *Endocr J* 62: 1091–1099. DOI: 10.1507/endocrj.EJ15-0308. 2015/10/23.
13. Huang B, Song BL and Xu C (2020) Cholesterol metabolism in cancer: mechanisms and therapeutic opportunities. *Nat Metab* 2: 132–141. DOI: 10.1038/s42255-020-0174-0. 2020/07/23.
14. Momtazi-Borojeni AA, Nik ME, Jaafari MR, et al. (2019) Potential anti-tumor effect of a nanoliposomal antiPCSK9 vaccine in mice bearing colorectal cancer. *Arch Med Sci* 15: 559–569. DOI: 10.5114/aoms.2019.84732. 2019/05/22.
15. Sheridan C (2013) Phase 3 data for PCSK9 inhibitor wows. *Nat Biotechnol* 31: 1057–1058. DOI: 10.1038/nbt1213-1057. 2013/12/10.
16. Jacoby RF, Cole CE, Tutsch K, et al. (2000) Chemopreventive efficacy of combined piroxicam and difluoromethylornithine treatment of Apc mutant Min mouse adenomas, and selective toxicity against Apc mutant embryos. *Cancer Res* 60: 1864–1870. 2000/04/15.
17. Lin S, Lee SJ, Shim H, et al. (2010) The absence of LPA receptor 2 reduces the tumorigenesis by ApcMin mutation in the intestine. *Am J Physiol Gastrointest Liver Physiol* 299: G1128–G1138. DOI: 10.1152/ajpgi.00321.2010. 2010/08/21.
18. Xing S, Shen D, Chen C, et al. (2014) Early induction of oxidative stress in a mouse model of Alzheimer's disease with heme oxygenase activity. *Mol Med Rep* 10: 599–604. DOI: 10.3892/mmr.2014.2252. 2014/05/27.
19. Ason B, van der Hoorn JW, Chan J, et al. (2014) PCSK9 inhibition fails to alter hepatic LDLR, circulating cholesterol,

and atherosclerosis in the absence of ApoE. *J Lipid Res* 55: 2370–2379. DOI: 10.1194/jlr.M053207. 2014/09/27.
20. Zhang X, Liu D, Li M, et al. (2017) Prognostic and therapeutic value of disruptor of telomeric silencing-1-like (DOT1L) expression in patients with ovarian cancer. *J Hematol Oncol* 10. DOI: 10.1186/s13045-017-0400-8.
21. Liu Z, Wang Y, Dou C, et al. (2018) Hypoxia-induced up-regulation of VASP promotes invasiveness and metastasis of hepatocellular carcinoma. *Theranostics* 8: 4649–4663. DOI: 10.7150/thno.26789. 2018/10/04.
22. Guillen-Ahlers H, Suckow MA, Castellino FJ, et al. (2010) Fas/CD95 deficiency in ApcMin/+ mice increases intestinal tumor burden. *PLoS One* 5: e9070. DOI: 10.1371/journal.pone.0009070. 2010/02/09.
23. Halberg RB, Katzung DS, Hoff PD, et al. (2000) Tumorigenesis in the multiple intestinal neoplasia mouse: redundancy of negative regulators and specificity of modifiers. *Proc Natl Acad Sci U S A* 97: 3461–3466. DOI: 10.1073/pnas.050585597. 2000/03/15.
24. Ku G, Tan IB, Yau T, et al. (2012) Management of colon cancer: resource-stratified guidelines from the Asian Oncology Summit 2012. *Lancet Oncol* 13: e470–481. DOI: 10.1016/S1470-2045(12)70424-2. 2012/11/03.
25. Hisamatsu T (2014) [Inflammatory bowel disease and colorectal cancer]. *Nihon Rinsho* 72: 56–62. 2014/03/07.
26. Lee J, Jeon JY and Meyerhardt JA (2015) Diet and lifestyle in survivors of colorectal cancer. *Hematol Oncol Clin N Am* 29: 1–27. DOI: 10.1016/j.hoc.2014.09.005. 2014/12/06.
27. Mundade R, Imperiale TF, Prabhu L, et al. (2014) Genetic pathways, prevention, and treatment of sporadic colorectal cancer. *Oncoscience* 1: 400–406. DOI: 10.18632/oncoscience.59. 2015/01/17.
28. Muto T, Bussey HJ and Morson BC (1975) The evolution of cancer of the colon and rectum. *Cancer* 36: 2251–2270. DOI: 10.1002/cncr.2820360944. 1975/12/01.
29. Hong SH, Cha JM, Lee JI, et al. (2014) Association of hyper-LDL cholesterolemia with increased risk of colorectal adenoma. *Hepato-Gastroenterology* 61: 1588–1594. 2014/12/02.
30. Wu CY, Tang ZH, Jiang L, et al. (2012) PCSK9 siRNA inhibits HUVEC apoptosis induced by ox-LDL via Bcl/Bax-caspase9-caspase3 pathway. *Mol Cell Biochem* 359: 347–358. DOI: 10.1007/s11010-011-1028-6. 2011/08/19.
31. Dwivedi DJ, Grin PM, Khan M, et al. (2016) Differential Expression of PCSK9 Modulates Infection, Inflammation, and Coagulation in a Murine Model of Sepsis. *Shock* 46: 672–680. DOI: 10.1097/SHK.0000000000000682. 2016/07/13.
32. Piao MX, Bai JW, Zhang PF, et al. (2015) PCSK9 regulates apoptosis in human neuroglioma u251 cells via mitochondrial signaling pathways. *Int J Clin Exp Pathol* 8: 2787–2794.
33. Xu X, Cui Y, Cao L, et al. (2017) PCSK9 regulates apoptosis in human lung adenocarcinoma A549 cells via endoplasmic reticulum stress and mitochondrial signaling pathways. *Exp Ther Med* 13: 1993–1999. DOI: 10.3892/etm.2017.4218. 2017/06/02.
34. Momtazi-Borojeni AA, Nik ME, Jaafari MR, et al. (2020) Effects of immunisation against PCSK9 in mice bearing melanoma. *Arch Med Sci* 16: 189–199. DOI: 10.5114/aoms.2020.91291. 2020/02/14.
35. Canuel M, Sun X, Asselin MC, et al. (2013) Proprotein convertase subtilisin/kexin type 9 (PCSK9) can mediate degradation of the low density lipoprotein receptor-related protein 1 (LRP-1). *PLoS One* 8: e64145. DOI: 10.1371/journal.pone.0064145. 2013/05/16.
36. Athavale D, Chouhan S, Pandey V, et al. (2018) Hepatocellular carcinoma-associated hypercholesterolemia: involvement of proprotein-convertase-subtilisin-kexin type-9 (PCSK9). *Canc Metabol* 6: 16. 2018/11/06DOI: 10.1186/s40170-018-0187-2.
37. Momtazi-Borojeni AA, Nik ME, Jaafari MR, et al. (2019) Effects of immunization against PCSK9 in an experimental model of breast cancer. *Arch Med Sci* 15: 570–579. DOI: 10.5114/aoms.2019.84734. 2019/05/22.
38. Lin Y, He Z, Ye J, et al. (2020) Progress in understanding the IL-6/STAT3 pathway in colorectal cancer. *OncoTargets Ther* 13: 13023–13032. DOI: 10.2147/OTT.S278013. 2020/12/31.
39. Loh CY, Arya A, Naema AF, et al. (2019) Signal transducer and activator of transcription (STATS) proteins in cancer and inflammation: functions and therapeutic implication. *Front Oncol* 9: 48. 2019/03/09. DOI: 10.3389/fonc.2019.00048.
40. Ji K, Zhang M, Chu Q, et al. (2016) The Role of p-STAT3 as a prognostic and clinicopathological marker in colorectal cancer: a systematic review and meta-analysis. *PLoS One* 11: e0160125. DOI: 10.1371/journal.pone.0160125. 2016/08/10.
41. Chalikonda G, Lee H, Sheik A, et al. (2021) Targeting key transcriptional factor STAT3 in colorectal cancer. *Mol Cell Biochem*. DOI: 10.1007/s11010-021-04156-8.2021/04/19.
42. Macchi C, Greco MF, Botta M, et al. (2020) Leptin, Resistin, and Proprotein Convertase Subtilisin/Kexin Type 9: The Role of STAT3. *Am J Pathol* 190: 2226–2236. DOI: 10.1016/j.ajpath.2020.07.016.2020/08/18.
43. Ruscica M, Ricci C, Macchi C, et al. (2016) Suppressor of cytokine signaling-3 (SOCS-3) induces proprotein convertase subtilisin kexin type 9 (PCSK9) expression in hepatic HepG2 cell line. *J Biol Chem* 291: 3508–3519. DOI: 10.1074/jbc.M115.664706.2015/12/17.
44. Durham GA, Williams JJL, Nasim MT, et al. (2019) Targeting SOCS proteins to xontrol JAK-STAT signalling in disease. *Trends Pharmacol Sci* 40: 298–308. DOI: 10.1016/j.tips.2019.03.001.2019/04/06.
45. Liu X, Bao X, Hu M, et al. (2020) Inhibition of PCSK9 potentiates immune checkpoint therapy for cancer. *Nature* 588: 693–698. DOI: 10.1038/s41586-020-2911-7.2020/11/13.