MALAT1 affects atherosclerosis by regulating endothelial cell's microautophagy

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Abstract: Autophagy of vessels endothelial cells is the critical pathological process in atherosclerosis (AS). Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is a kind of long non-coding RNA (lncRNA) that regulates the autophagy of vessels endothelial cells, including microautophagy. However, the relationship between AS and *MALAT1* is not completely understood, and microautophagy has been ignored. In this study, I designed the experiments to research the impact of *MALAT1* in endothelial cells, which could regulate the progression of AS. In the present study, I design to establish the AS model mice with low *MALAT1* level. The expression level of *MALAT1* needs to be detected to verify the mouse model. Then, the intensity of microautophagy of endothelial cells of mouse model and normal mouse are detected by RT-qPCR, immunofluorescence assay, and observation directly with electron microscope. The atherosclerosis progression and plaque stability are detected by comparing the rations of macrophage/vascular smooth muscle cell and collagen/lipid. This paper only provides theoretical experiment design and possible results about how *MALAT1* affects AS by regulating microautophagy of vascular endothelial cells (VECs) which needs additional research in the pathology of atherosclerosis. This paper provided the possibility that *MALTA1* regulates the microautophagy in VECs and *MALTA1* may be the target to cure AS.

1. Introduction

Atherosclerosis (AS) can lead to cardiovascular disease. Cardiovascular disease is already responsible for the most deaths worldwide, killing an estimated 17.6 million people each year[1]. Vascular endothelial cells (VECs) line all blood vessels[2]. VECs' autophagy impairs the AS process while defective autophagy in cells enhances AS[3]. Autophagy, the process which cells transfer their components to lysosomes and digest them, is divided into three types: macroautophagy, chaperon-mediated autophagy (CMA) and microautophagy [4]. Microautophagy is the process by which late endosomes and lysosomes take up autophagic cargoes directly by membrane protrusion and invagination, and the autophagic cargoes are degraded in the endolysosomal lumen later[5]. Most of the microautophagy currently studied in mammalian cells is fission-type microautophagy, microautophagy has two types: fusion-type microautophagy and fission-type microautophagy, mediated by Endosomal sorting complexes required for transport (ESCRT)[5]. ESCRT proteins are highly conserved proteins machinery and key components for microautophagy and required for many types of microautophagy by direct roles[6,7]. ESCRT machinery directly functions in scission of the lysosomal membrane to complete the microautophagy[6]. CHMP2A is a kind of subunit of ESCRT[8]. In recent years, long non-coding RNA (lncRNA) has been regarded as an essential regulatory factor in the pathogenesis of atherosclerosis[3, 9]. LncRNAs have been well known as mRNA-like RNA polymerase II transcripts that have strong evolutionary conservation and are also the most complex and largest class of ncRNAs, ranging in size from 200nt to more than 100kb, and numerous express in type-specific cell such as regulating protein activities, altering RNA processing events, modulating transcriptional patterns, serving as precursors to small RNAs, and serving organizational or structural roles[10-12]. Evidence shows that lncRNA can regulate the autophagy process[3, 13]. Most autophagy depends on the role of lncRNA on key genes of autophagy[3]. Although some lncRNA such as Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) has been causally linked to the autophagy of VECs[13], the relationship between AS and *MALAT1* is not completely understood, and microautophagy has been ignored. Therefore, it is crucial to discover the relationship between AS and *MALAT1* by regulating VECs' microautophagy.

In this study, I investigated the effect of *MALAT1* in microautophagy in VECs. To discover the relationship between AS and *MALAT1* by regulating endothelial cells' microautophagy, I recently designed a kind of experiment about the role of *MALAT1* in VECs. In the kind of experiments, I used RT-qPCR, immunofluorescence assay, observation directly with electron microscope, aortic oil red detection and comparing the rations of macrophage/vascular smooth muscle cell and collagen/lipid to measure the expression of *MALAT1*, the key genes of microautophagy, the content of related

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proteins and factors and observe lysosome or vacuole protrusion, invagination, endosomatic invagination and the phenotypic of atherosclerosis. With the research articles about the mechanism of endothelial cells in AS development, I expect that the low expression of MALTA1 may enhance endothelial microautophagy and further inhibit the progression of AS.

2. Method

To test whether *MALAT1* affects the progression of AS through regulating endothelial microautophagy, I established AS model mice with low *MALAT1* levels. (Figure 1. The technology roadmap)

2.1. Establishment of AS model mice with low *MALAT1* level

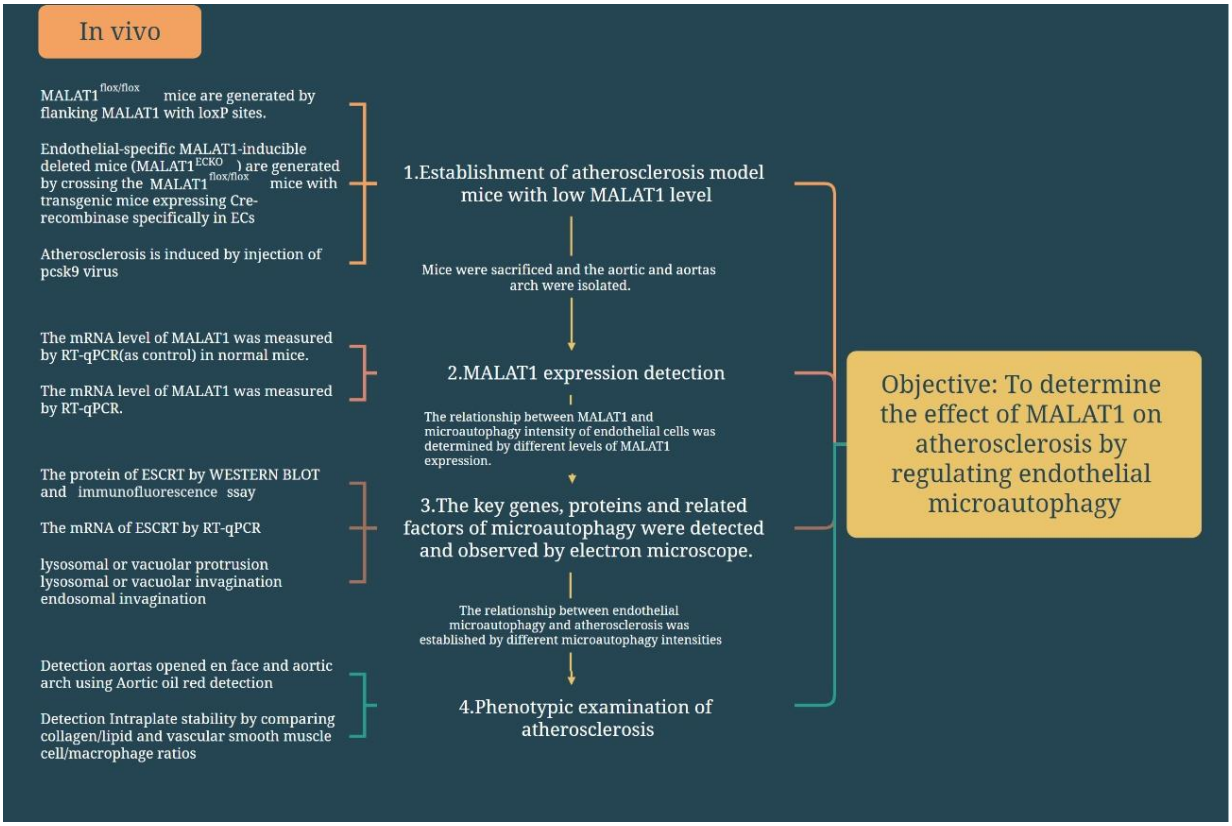

Figure 1. The technology roadmap

MALAT1$^{flox/flox}$ mice are generated by flanking *MALAT1* with loxP sites. Next, I establish the Endothelial-specific *MALAT1*-inducible deleted mice (*MALAT1*^{ECKO}) by crossing the *MALAT1*$^{flox/flox}$ mice with transgenic mice that express in VECs specifically Cre-recombinase. Then, PCSK9 adeno-associated virus (rAAV-D377Y-mPCSK9/ rAAV-D374Y-hPCSK9) is packaged and injected into mice about 8 weeks of age with 5.0×1011 vector genomes. The samples are then subjected to a 12-week high-fat diet for subsequent detection. (Figure 2. The establish of model mice)

2.2. RT-qPCR to detect MALAT1 expression

To make sure the model mice are established successfully and discover the *MALAT1's* impact in VECs. I isolate the mice's aortic of *MALAT1*^{ECKO} to measure the expression of *MALAT1* and some key landmarks of microautophagy and AS by comparing them with normal control mice.

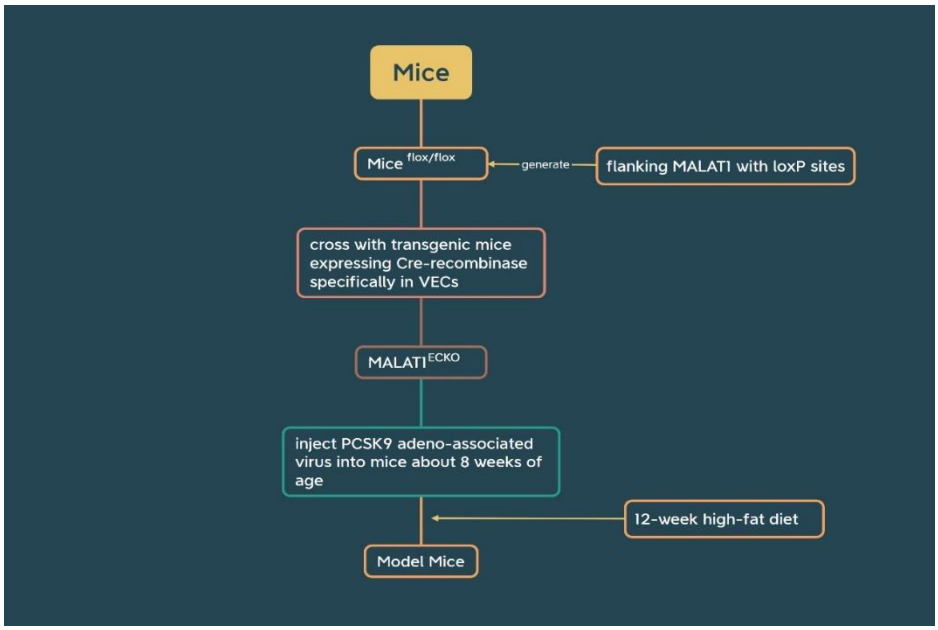

Figure 2. The establish of model mice

First, RT-qPCR is used to detect the mRNA expression level of *MALAT1*. From VECs, I isolate total RNA. I use reverse-transcribed to make the total RNA into cDNA and use it in RT-qPCR. The internal control is β-ACTIN and the *MALAT1's* mRNA level is evaluated with $2^{-\Delta\Delta Ct}$ method. (Figure3. The primer of *MALAT1*)

My predicted result is that *MALAT1*^{ECKO} has lower *MALAT1* expression.

forward 5′-TGCAATGCACTCAGCATGC-3′,

reverse 5′-CCGACATTACGACGTATTCG-3′

Figure 3. The primer of *MALAT1*

2.3. Microautophagy intensity of endothelial cells intensity detection

The present studies have found that the downregulation of *MALAT1* expression enhances the autophagy in VECs[3, 4]. Microautophagy, as a kind of autophagy, is a process by which eukaryotic cells degrade autophagic cargoes similar to other forms of autophagy and can be regulated by similar stimuli[5]. The relationship gives me a new way to discover the relationship between *MALAT1* and microautophagy intensity of VECs.

To discover the relationship between *MALAT1* and microautophagy intensity of VECs. I examine the expression of several key genes, proteins, and factors in VECs with different levels of *MALAT1* expression in the mice's aortic of *MALAT1*^{ECKO} and normal mice.

2.3.1. RT-qPCR to detect ESCRT expression

forward 5′- CGCGAGCGACAGAAACTAGAG-3′

reverse 5′- CCCGCATCAATACAAACTTGC-3′

Figure 4. The primer of gene of CHMP2A

ESCRT is a kind of key complex of microautophagy[5]. CHMP2A is the critical component protein of ESCRT[14]. To measure the microautophagy intensity of endothelial cells, I design the primer of CHMP2A to detect ESCRT expression. From VECs, I isolate total RNA. I use reverse-transcribed to make the total RNA into cDNA and use it in RT-qPCR. The internal control is glyceraldehyde 3-phosphate dehydrogenase (GADPH) and the mRNA level of *MALAT1* is evaluated with the $2^{-\Delta\Delta Ct}$ method. (Figure4. The primer of gene of CHMP2A)

Guess based on literature reading; my predicted result is that *MALAT1*^{ECKO} has higher ESCRT expression. In other word, the mice with lower *MALAT1* expression have higher microautophagy intensity.

2.3.2. Western blot to detect ESCRT expression

In addition, the cell lysates of mice's aortic of *MALAT1*^{ECKO} and normal mice are used to western blot (WB) to detect CHMP2A directly to detect the expression of ESCRT. The primary antibody is anti-ISG15 serum. The secondary antibody, enhance chemiluminescence (ECL), is anti-mouse IgG-horseradish peroxidase (HRP).

Guess based on literature reading; my predicted result is that *MALAT1*^{ECKO} has higher ESCRT expression. In other word, the mice with lower *MALAT1* expression have higher microautophagy intensity.

2.3.3. Immunofluorescence assay to detect ESCRT expression and location

In addition, to further detect ESCRT by quantitative positioning and prepare for further research, I designed the experiment by Immunofluorescence assay.

The mice aortic endothelial cells are isolated and cultured to detect the ESCRT. The cultured mice aortic endothelial cells are fixed with 4% formaldehyde for 10 min at room temperature, then the endothelial cells are incubated with 0.3% Triton™ X-100 to seal the membrane

of the cells, washed with PBS, incubated with the antibody of CHMP2A. The laser confocal scanning microscope is used to generate images and calculate fluorescent intensity.

Guess based on literature reading; my predicted result is that *MALAT1*^{ECKO} has more fluorescence. In other word, the mice with lower *MALAT1* expression have higher microautophagy intensity.

2.3.4. Observed by electron microscope the behaviors reflect microautophagy

Some behaviors of lysosomal, vacuolar, or endosomal are a reflection of microautophagy. An Electron microscope provides a new way to detect microautophagy intensity directly. For the electron microscope, the mice's aortic of *MALAT1*^{ECKO} and normal mice are fixed and washed with PBS. Next, samples are permeabilized before blocking and incubation. After washing, samples are post-fixed and treated with the silver enhancement kit. Tissues are finally processed with a short osmication step.

Guess based on literature reading; my predicted result is that *MALAT1*^{ECKO} has more lysosomal or vacuolar protrusion or invagination and endosomal invagination. In other word, the mice with lower *MALAT1* expression have higher microautophagy intensity.

2.4. Phenotypic examination of atherosclerosis

Based on previous reports indicating that autophagy in VECs protects themselves and against AS progression [4]. Considering the aforementioned relationship, our prediction is that *MALAT1*$^{flox/flox}$ mice with lower *MALAT1* expression will exhibit increased intensity of microautophagy and reduced extent of AS in our study.

To further explore whether *MALAT1* influences atherosclerosis progression by influencing VECs microautophagy, I examined some of the typical phenotypes of atherosclerosis. By examining some of the typical phenotypes of atherosclerosis, I can determine the extent of AS in mice. I designed an experiment about Oil red O staining to quantify the AS lesion areas and another experiment about the comparison of macrophage/vascular smooth muscle cell and collagen/lipid ratios in mice.

2.4.1. Oil red O staining

The mice's aortic of *MALAT1*^{ECKO} and normal mice are fixed and washed twice with PBS, and transferred to 20% sucrose solution for preservation. The samples were rinsed with PBS solution, and the external connective tissue and fat are recovered, the working fluid was rinsed 3 times in oil red O working solution, at room temperature for 24 h, and isopropanol 60%.

Guess based on literature reading; my predicted result is that *MALAT1*^{ECKO} has less AS lesion areas. In other word, the mice with lower *MALAT1* expression have less extent of AS.

2.4.2. Compares the ratios of macrophage/vascular smooth muscle cell and collagen/lipid

The rations of collagen/lipid and vascular smooth muscle cells/macrophage reflect the instability of plaques. The higher the ratio, the less instability the plaques are, and the less dangerous the AS is.

The macrophage/vascular smooth muscle cell ratios

The mice aortic endothelial cells are isolated and cultured to detect the ESCRT. The cultured mice aortic endothelial cells are fixed with 4% formaldehyde for 10 min at room temperature, then the endothelial cells are incubated with 0.3% Triton™ X-100 to seal the membrane of the cells, washed with PBS, incubated with the antibody of CHMP2A. The laser confocal scanning microscope is used to generate images and calculate fluorescent intensity.

Guess based on literature reading; my predicted result is that *MALAT1*^{ECKO} has lower macrophage/vascular smooth muscle cell ratio. In other word, the mice with lower *MALAT1* expression have less extent of AS because the less instability of the plaques.

The collagen/lipid ratio

I detected collagen and compared the collagen/lipid in combination with the oil red O experiments that have been conducted. Masson staining is performed on serial sections of mice's aortic of *MALAT1*^{ECKO} and normal mice to detect collagen.

Guess based on literature reading; my predicted result is that *MALAT1*^{ECKO} has a lower collagen/lipid ratio. In other word, the mice with lower *MALAT1* expression have less extent of AS because the less instability of the plaques.

3. Discussion

Recent studies have reported that IncRNA has a critical function in the autophagy of VECs, but the *MALAT1*, a kind of LncRNA, is not fully understood to discover the influence of microautophagy in VECs regulated by *MALAT1* to AS[3, 13]. In the current study, I established AS model mice with low *MALAT1* levels. Endothelial-specific *MALAT1*-inducible deleted mice (*MALAT1*^{ECKO}) are generated by crossing the *MALAT1*$^{flox/flox}$ mice with transgenic mice expressing Cre-recombinase specifically in VECs and AS is induced by injection of pcsk9 virus. *MALAT1* expression in mice's aortic of *MALAT1*^{ECKO} and normal mice is detected by RT-qPCR to determine whether the MALAT1^{ECKO} is established successfully. Indicators of some key substances in different levels of MALAT1 expression are detected by RT-qPCR, western blot, and immunofluorescence assay to reflect the intensity of microautophagy. In addition, I observed microautophagy directly by electron microscope in different levels of *MALAT1* expression. The extent of AS in mice is detected by the comparison of collagen/lipid and vascular smooth muscle cell/macrophage ratios by oil red O staining, Masson staining, and immunofluorescence. It has been reported that autophagy including microautophagy can impair AS process[3, 4].

Zhu et al. reported the lower MALAT1 expression can enhance the autophagy of VECs of mice[3, 4]. But the

relationship between MALAT1 and microautophagy has not been discovered. Microautophagy, as a kind of autophagy, is a process in which eukaryotic cells degrade autophagic cargoes like all forms of autophagy and can be regulated by similar stimulant[5]. In the present study, the *MALAT1*$^{flox/flox}$ mice are applied as a kind of model with lower MALAT1 expression. Therefore, I predict the lower *MALAT1* expression may enhance endothelial microautophagy. The future impact of lower microautophagy intensity of VECs promotes the extent of AS. In this relationship, I predict the result shows the lower expression of MALAT1 may enhance the microautophagy of VECs. It has been reported that the autophagy in VECs can protect themselves and the atherosclerosis progression is inhibited[4]. The potential protection ability of microautophagy is not been discovered. Given the relationship we've already mentioned, it is predicted that the *MALAT1*$^{flox/flox}$ mice with lower MALAT1 expression have higher microautophagy intensity and have less extent of AS in the present study. Therefore, my study about *MALAT1* may provide a new option to cure AS by regulating *MALAT1* expression.

4. Conclusion

This paper only provides theoretical experiment design and possible results about how *MALAT1* affects AS by regulating endothelial cells' microautophagy which needs additional research in the pathology of atherosclerosis. This paper provided the possibility that MALTA1 regulates the microautophagy in VECs and MALTA1 may be the target to cure AS.

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