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ORIGINAL

ROLE OF THE AHNAK2/ERK PATHWAY IN REGULATING VASCULAR SMOOTH MUSCLE CELL DYNAMICS: IMPLICATIONS FOR RECOVERY FROM VASCULAR INJURY AND ENHANCING CARDIOVASCULAR RESILIENCE IN SPORTS MEDICINE

Baozhu Wang[1], Yan Li[2], Dan Zhang[1], Xiang Ma[1],Yitong Ma[1,*]

[1] Department of Cardiology of the First Affiliated Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang, China.
[2] Department of Intensive Care Unit of the Fifth Affiliated Hospital, Xinjiang Medical University, Urumqi 830000, Xinjiang, China.
E-mail: xjmayitong@163.com

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ABSTRACT

Background: The AHNAK2/ERK signaling pathway has been implicated in regulating vascular smooth muscle cell (VSMC) dynamics, which are crucial for vascular repair and the progression of coronary artery disease (CAD). This study aimed to investigate the role of the AHNAK2/ERK pathway in promoting VSMC proliferation and migration and its potential implications for vascular recovery and cardiovascular health, particularly in enhancing physical resilience. **Methods:** Proliferation and migration of HA-VSMC cell lines were evaluated using CCK-8, MTT, and scratch assays. AHNAK2 expression was manipulated through gene knockdown and overexpression methods. Relative expression levels of AHNAK2 and ERK mRNA were quantified using PCR assays to determine their interaction and signaling effects. **Results:** Overexpression of AHNAK2 significantly enhanced VSMC proliferation and migration, as evidenced by increased cell viability in MTT and CCK-8 assays and greater wound closure in scratch assays. Conversely, AHNAK2 knockdown reduced these effects, demonstrating its critical role in regulating VSMC activity. Mechanistically, AHNAK2 influenced the activation of the ERK1/2 pathway, with ERK1/2 levels significantly upregulated in AHNAK2-overexpressing cells. This suggests that AHNAK2 facilitates VSMC dynamics through ERK signaling,

promoting vascular repair processes. **Conclusion:** This study reveals that AHNAK2 effectively promotes VSMC proliferation and migration via the ERK1/2 signaling pathway, underscoring its potential role in vascular recovery and the treatment of endothelial injury and CAD. These findings have important implications for cardiovascular rehabilitation, as enhancing vascular resilience may improve physical performance and recovery in individuals with CAD or related conditions. Future research should focus on in vivo models to further elucidate the downstream molecular mechanisms of the AHNAK2/ERK pathway and its broader applications in sports and rehabilitation medicine

KEYWORDS: Vascular Smooth Muscle Cells; AHNAK2/ERK Pathway; Proliferative Activity; Cellular Migration

1. INTRODUCTION

Coronary artery disease (CAD) remains one of the leading causes of morbidity and mortality worldwide, posing a significant burden on healthcare systems and affecting the quality of life for millions(Lechartier et al., 2022). A key pathological feature of CAD is vascular endothelial injury, which leads to the proliferation and migration of vascular smooth muscle cells (VSMCs), processes essential for vascular repair and remodeling. While these cellular activities are critical for maintaining vascular integrity, their dysregulation can exacerbate atherosclerosis and contribute to the progression of CAD(Katta, Loethen, Lavie, & Alpert, 2021). Understanding the molecular mechanisms that regulate VSMC behavior is therefore critical for developing targeted therapies to treat vascular injury and improve cardiovascular outcomes. Recent studies have identified the AHNAK2 protein as a significant regulator of cellular processes, including proliferation, migration, and survival, particularly in cardiovascular tissues. AHNAK2 is a large scaffold protein known to interact with various signaling pathways, including the extracellular signal-regulated kinase (ERK) pathway, a critical regulator of cell growth, differentiation, and migration(Group, 2020). The AHNAK2/ERK signaling axis has been implicated in promoting the dynamic behavior of VSMCs, suggesting its potential role in vascular repair and remodeling(Owens, Kumar, & Wamhoff, 2004; Shi, Yang, Cheng, Xu, & He, 2020). However, the precise mechanisms by which AHNAK2 influences the ERK pathway to regulate VSMC activity remain poorly understood. The ERK pathway plays a pivotal role in mediating responses to extracellular stimuli, including growth factors and mechanical stress, making it a central player in vascular biology. Activation of ERK signaling promotes the proliferation and migration of VSMCs, processes that are essential for repairing endothelial injury and restoring vascular homeostasis(Félétou, Köhler, & Vanhoutte, 2010). (Incalza et al., 2018). Dysregulation of this pathway, however, can lead to pathological vascular remodeling, contributing to the progression of CAD and other vascular diseases(Esse, Barroso, Tavares de Almeida, & Castro, 2019), (Liu & Wu, 2022). Exploring the interplay between AHNAK2 and the ERK

pathway offers an opportunity to uncover novel therapeutic targets for regulating VSMC activity and improving vascular health. In the context of sports and rehabilitation medicine, vascular health is paramount for optimizing physical performance and recovery. Vascular remodeling and repair are critical for maintaining blood flow and tissue oxygenation during physical activity. Dysregulated VSMC activity not only impairs vascular function but also limits physical resilience and endurance(Hennigs, Matuszcak, Trepel, & Körbelin, 2021; Ribatti, Tamma, & Annese, 2021). Targeting molecular pathways, such as the AHNAK2/ERK axis, that influence vascular repair processes could have significant implications for enhancing cardiovascular health and physical performance in individuals with CAD or those recovering from vascular injuries. This study investigates the role of the AHNAK2/ERK pathway in regulating VSMC proliferation and migration, with a focus on its potential applications in treating vascular endothelial injury and CAD(Cong & Kong, 2020; Marziano, Genet, & Hirschi, 2021), (Abdullahi, Tripathi, & Ronaldson, 2018). By elucidating the molecular mechanisms underlying AHNAK2's effects on VSMC dynamics, this research aims to provide insights into novel therapeutic strategies that can enhance vascular repair, promote cardiovascular resilience, and support physical recovery(Jensen, Bentzon, & Albarrán-Juárez, 2021). Additionally, the findings may contribute to developing multidisciplinary approaches that integrate sports and rehabilitation medicine to improve outcomes for patients with vascular diseases. AHNAKs are massive proteins (> 600kDa) that are mainly expressed in muscular cells and are discovered in numerous cellular locations (Shtivelman, Cohen, & Bishop, 1992). Two members make up the AHNAK protein family; they are AHNAK and AHNAK2 (Komuro et al., 2004). AHNAK protects in vascular regeneration after injury (Haase et al., 2017). AHNAK2 was originally discovered in cardiac tissue extracts from mice and has gradually been described recently as being an involvement in cellular migration and repair (Komuro et al., 2004). Kirov et al. identified AHNAK2 as an essential component of the non-classical pathway of FGF1, which modulates angiogenesis and the inflammatory process (Kirov, Kacer, Conley, Vary, & Prudovsky, 2015). However, the role of the AHNAK2/ERK pathway in CHD and vascular endothelial injury has not been clarified.

2. Methods

2.1 Cell culture

The base medium of HA-VSMC cells consists of 90% DMEM (DMEM, GIBCO) and 10% good quality fetal bovine serum. The incubation conditions were 95% air, 5% CO2, temperature at 37°C and incubator humidity of 70%-80%. If the cell density reaches 80-90%, the culture can be passaged and the culture medium changed once every 2-3 days.

2.2 Lentiviral transfection and Cell transfection

Human AHNAK2 was synthetic and cloned into the expressed vector pcDNA3.1. The small hairpin RNA (shRNA) of AHNAK2 was produced and obtained by cloning into the pLVX-shRNA1 vector. AHNAK2 siRNAs were generated by Gene-Pharma (US). siRNA was designed and synthesized by Ambion (US). MicroRNA mimics and inhibitors were purchased from RiboBio (China). Plasmid vectors and siRNAs were transfected into HA-VSMC cells using Lipofectamine 2000. All siRNA sequences are listed in Table S1.

Table S1: Result of Forward and Reverse.

	FORWARD (5'-3')	**REVERSE(5'-3')**
SI-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
SIRNA AHNAK2#1	CCAAGUGGAUGUGAAACUUTT	AAGUUUCACAUCCACUUGGTT
SIRNA AHNAK2#2	GCGGACAAGGAUGUGACUATT	UAGUCACAUCCUUGUCCGCTT

2.3 CCK-8 assay

The CCK8 test kit was employed to assess cellular proliferation (Beyotime). In 96-well microplate, VSMCs and VSMC cells produced under varied environments were planted at a density of 5 x 10^3 cells/well. CCK8 reagents (10μL) was then supplied at a concentration of ten L/well and maintained at 37°C in a 5% CO_2 environment for 0.5 hours. The absorption of each sample was then quantified using such a microplate reader at 450 nm.

2.4 Wound Healing

HA-VSMC cells were seeded in six-well plate and incubated to cell monolayer formulation. The monolayers of cells were manually scraped with a 10μL- micropipette tip to cause injury. Cells were then grown for 48 hours in supplemented serum-free medium either with or without the specified concentrations. The wound restoration was measured using Image J software to calculate the wounded area covered by the cells from the border of the wound.

2.5 MTT assay

MTT assays were prepared following a manufacturer's protocol by applying the MTT kit. The cells were received to 70% to 80% confluence by being seeded at 5 x 10^4 cells/mL in 96-well plates. 150μL of 1 mg/mL MTT reagent was pipetted into the cells and incubated for 4 hours, after which 100μL of DMSO (Sigma) was supplemented and incubated for 1 hour. Absorbance was measured at 590 nm.

2.6 The qRT-PCR test

Whole RNA from HA-VSMC cells was prepared with Trizol (Takara

Biotechnology Co.) starting with the manufacturer's manual. cDNA was subsequently generated from the complete RNA by synthesis using a reverse transcription kit. The response volume was 15 µl and the reversal conditions were 68°C for 5 min, 38°C for 5 min and 40°C for 60 min. The tests were performed using the SYBR kit, following the parameters under (93°C for 3 minutes, 36 cycles, 95°C for 30 seconds, 55°C for 30 seconds, 73°C for 30 seconds) and gene expression was performed using the 2-ΔΔCt method (Table S2).

Table S2: The primers of qRT-PCR

GENE_NAME	SEQUENCES(5'-3')
AHNAK2	F: CGCGATGTGCGACTGC; R: TGGTCATCTTCCGTTTCTGC
ERK1	F: TGGCAAGCACTACCTGGATCAG; R: GCAGAGACTGTAGGTAGTTTCGG
ERK2	F: ACACCAACCTCTCGTACATCGG; R: TGGCAGTAGGTCTGGTGCTCAA
GAPDH	F: CGGAGTTGTTCGTATTCGG; R: TACTAGCCGATGATGGCATT

2.7 Statistical analyses

There were at least three biological and technical replicates in every trial. A Graph Pad Prism 9.4 software was chosen for the calculation of the data. Individual tests were replicated on three separate occasions and values were presented as mean ± standard deviation (SD). Student's t-test was used to establish a distinction between various interventional conditions (unpaired, 2-tailed). A difference was deemed statistically significant when $p<0.05$.

3. Results

3.1 AHNAK2 accelerates the VSMC proliferations

We looked at the expression of AHNAK2 in proliferating VSMCs stimulated with 10% FBS in order to determine whether AHNAK2 is involved in the proliferation of VSMCs. Following starving with serum-free medium, VSMCs were given 10% FBS-containing medium for 0, 6, 12, and 24 hours. In comparison to the serum-free treatment group, the results demonstrated that 10% FBS dramatically elevated the expression of AHNAK2 in VSMCs in a time-dependent manner (Fig.1a). This finding suggests that VSMC proliferation and AHNAK2 overexpression are closely related. Then, using lentiviral transfection, we created a stable VSMC-AHNAK2 line to confirm the effects of AHNAK2 on VSMC growth. In comparison to VSMC transfected with an empty vector (VSMC-vector), the mRNA levels of AHNAK2 in VSMC-AHNAK2 were much higher, proving that a stable VSMC line overexpressing AHNAK2 had been effectively produced (Fig.1b). According to results of MTT and CCK8 assays, overexpression of AHNAK2 significantly induced the proliferation of VSMC-AHNAK2 cells when compared to the VSMC-vector group, as seen in Fig 1c&d.

These findings imply that AHNAK2 may encourage the growth of VSMCs.

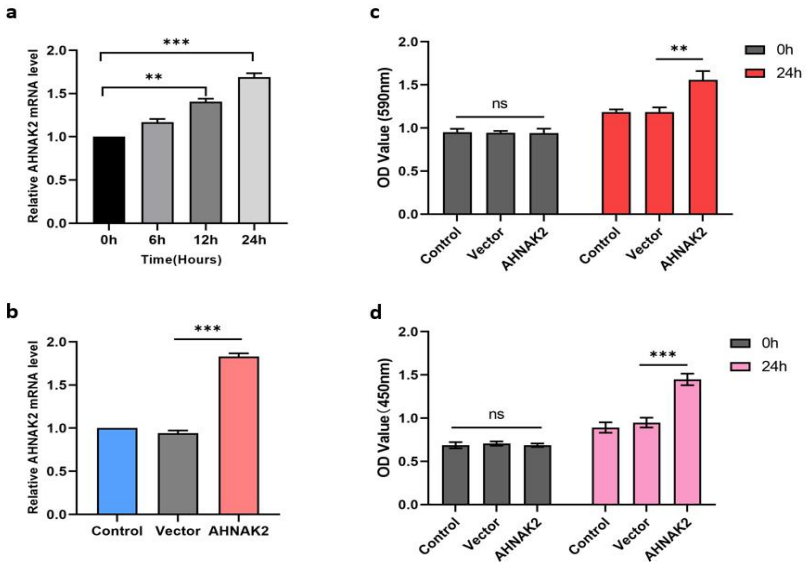

Figure 1: Influence of AHNAK2 overexpression on VSMC proliferation. (a) AHNAK2 mRNA expression in VSMC in medium containing 10% FBS for 0, 6, 12 and 24 h; (b) overexpressed AHNAK2 in VSMCs. The cellular viability and prefoliation were examined by (c) MTT and (d)CCK8 assays.

3.2 AHNAK2 accelerates the VSMC migrations

We further looked at the effect of AHNAK2 in migrating VSMCs. Following starving with serum-free medium, the areas of wound exposure and wound closure at 0h and 48h respectively were recorded and calculated. Next, we built a knockdown AHNAK2-expressing to fully validate the impacts of AHNAK2 on VSMC migration (Fig.2a). This finding suggested that VSMC migration and expressed level of AHNAK2 are closely related. It was displaying larger wound closure, indicating that overexpressed AHNAK2 can strengthen migrative activity of VSMCs. In addition, smaller wound closure was also observed in si-AHNAK2 group, implying that silencing AHNAK2 can weaken the migrative ability of VSMCs.

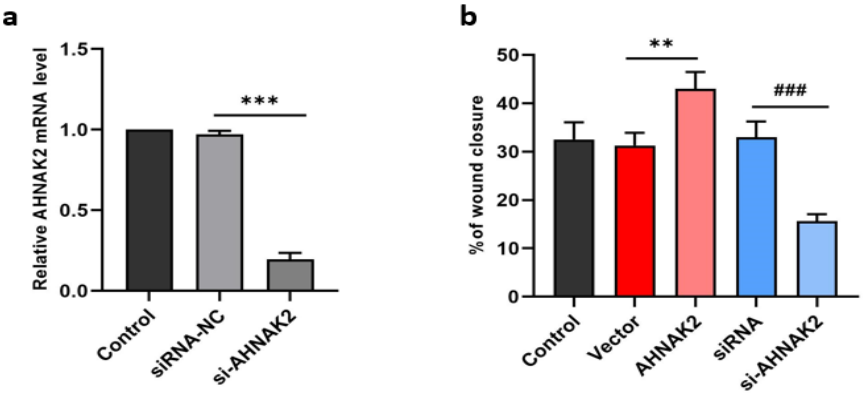

Figure 2: (a) Knockdown AHNAK2 mRNA in VSMCs; (b) the percentages of 48-hour wound closure in the overexpressed and silencing AHNAK2 in VSMCs.

3.3 Knockdown of AHNAK2 suppresses the proliferation of VSMCs as well

Next, we applied the knockdown AHNAK2-expressing VSMC line to further validate the impacts of AHNAK2 on cellular proliferations. The results suggested that silencing AHNAK2 can weaken the proliferative ability of VSMCs. In both MTT and CCK8 assays, lower and weaken growth of VSMCs was also observed in si-AHNAK2 group (Fig.3 a and b).

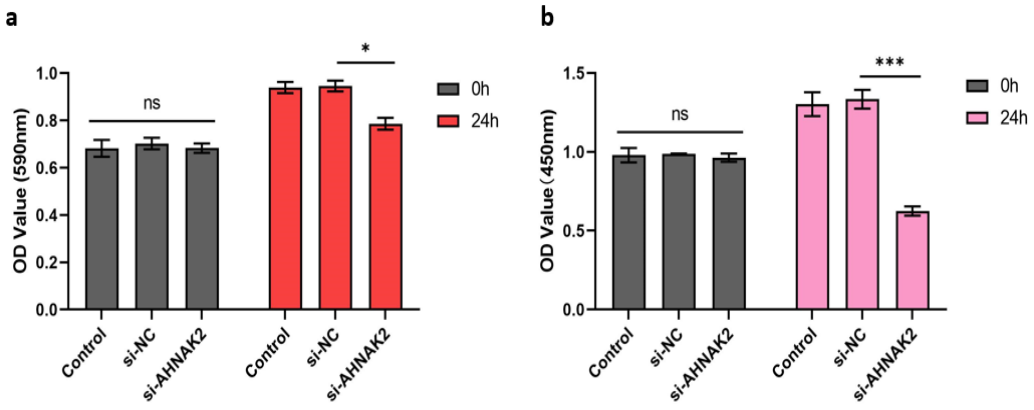

Figure 3: The cellular viability and prefoliation were examined by (c) MTT and (d)CCK8 assays when silencing AHNAK2 in VSMCs.

3.4 ERK1/2 acted as a player for AHNAK2 pathway and was up-regulated by AHNAK2 in VSMCs

We subsequently investigated the possibility that the AHNAK2/ERK signaling pathway contributed to the growth of VSMCs. ERK expression increased in VSMC-AHNAK2 cells, as seen in Fig. 4, indicating that AHNAK2 elevated ERK expression in VSMCs. In contrast, AHNAK2 knockdown might counteract the increase in ERK expression caused by AHNAK2 in VSMC-AHNAK2 cells (Fig. 4).

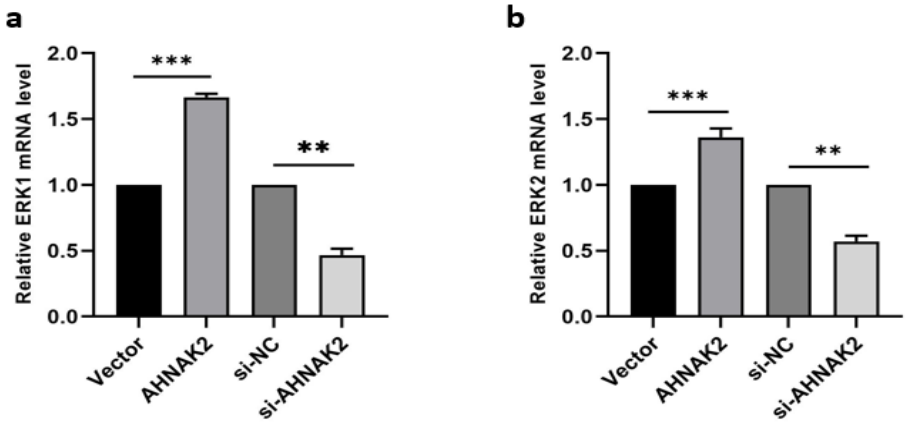

Figure 4: (a) The ERK1 mRNA when overexpression and knockdown of AHNAK2 in VSMCs; (b) the ERK2 mRNA when overexpression and knockdown of AHNAK2 in VSMCs.

4. Discussion

The biological functionality of maturing VSMCs, which have extremely poor proliferative and synthesis activity and which exhibit particular constriction proteins, involves predominantly vasoconstriction and blood pressure modulation (Bennett, Sinha, & Owens, 2016; Grootaert & Bennett, 2021). The highly plastic properties of VSMCs are evident in vascular growth and in damage recovery. VSMCs possess heightened capacity for proliferations, migrations and generations of the extracellular matrix components in vessel formation (Frismantiene, Philippova, Erne, & Resink, 2018). Likewise, responding to vessel damage, VSMCs considerably boost their capacity for multiplication, emigration and synthesis. The plasticity of VSMCs is, nevertheless, vulnerable to abnormal microenvironments, resulting in phenotypic transformation and vascular disorder advancement (Zhang et al., 2016). VSMC over-proliferation has a major part to play in the pathogenesis of vascular disorders (Huynh & Heo, 2021; Rombouts et al., 2022). In contrast to Ahnak1, Ahnak2 is preferentially labelled on internal smooth muscle cells, whereas endothelium is not Aknak2-positive (Haase et al., 2017). So far, it has not been reported on the functionality of Ahnak2 in smooth muscle. Our finding suggests that proliferation and migration of VSMCs are closely related to the various expressed level of Ahnak2 by regulation of ERK1/2. The costameric system, which is situated with both Z-disks and the combination of characteristics and serves as a modulator of the lateral transfer of stress from sarcomeres across the sarcolemma to the extracellular matrix, is made up of two proteins called AHNAK2 and AHNAK (Jaka, Casas-Fraile, de Munain, & Saenz, 2015; Marg, Haase, Neumann, Kouno, & Morano, 2010). Mice research looks at wound healing after vertebral arteries wire injury revealed endothelium-specific expression of AHNAK and AHNAK2, with cytoplasmic expression of AHNAK2 only in endothelial and intermediate cells (Haase et al., 2017). Following AHNAK was knocked down, it showed a slowdown in healing from trauma with no alteration in AHNAK2 expression, showing that both peptides were not duplicated in naive arteries and that AHNAK2 had a unique localization in smooth muscle cells (Haase et al., 2017). Here, our results confirm the results of other previous studies from another perspective. Prior research has discovered that interleukin-1 receptor-associated kinase-1 (IRAK1) stimulates VSMC multiplication and epithelial hypertrophy in a PKC-dependent way via external signal-regulated ERK1/2 (Jain, Singh, Singh, & Barthwal, 2015). PKC-dependent stimulation of ERK1/2 increased VSMC growth and epithelial hyperplasia (Jiang et al., 2017). PKC was found to be upregulated in oxygen starvation rat arterial blood smooth muscle cells (PASMCs). PKC stimulated ERK1/2 phosphorylation, resulting in PASMC multiplication. In contrast, inhibiting the PKC-ERK1/2 signaling pathway decreased VSMC growth due to high hyperglycemia (Yang et al., 2011). Our data further recommended AHNAK2 accelerates the VSMC proliferations and migration by ERK1/2 signaling. In comparison to VSMC-vector group, the mRNA levels of AHNAK2

in VSMC-AHNAK2 were much higher, proving that a stable VSMC line overexpressing AHNAK2 had been effectively produced. According to results of MTT and CCK8 assays, overexpression of AHNAK2 significantly induced the proliferation of VSMC-AHNAK2 cells when compared to the VSMC-vector group, implying that AHNAK2 may encourage the growth of VSMCs. It was displaying larger wound closure, indicating that overexpressed AHNAK2 can strengthen migrative activity of VSMCs. In addition, smaller wound closure was also observed in si-AHNAK2 group, implying that silencing AHNAK2 can weaken the migrative ability of VSMCs. We subsequently investigated the possibility that the AHNAK2/ERK signaling pathway contributed to the growth of VSMCs. ERK1/2 acted as a player for AHNAK2 pathway and was up-regulated by AHNAK2 in VSMCs. This study highlights the pivotal role of the AHNAK2/ERK signaling pathway in regulating the proliferation and migration of vascular smooth muscle cells (VSMCs), processes essential for vascular repair and remodeling. The findings demonstrate that AHNAK2 significantly enhances VSMC activity by activating the ERK1/2 pathway, a mechanism that is central to the cellular response to vascular injury. Overexpression of AHNAK2 promoted both proliferation and migration of VSMCs, while its knockdown attenuated these processes, further underscoring its critical role in maintaining vascular homeostasis. The ability of AHNAK2 to modulate VSMC dynamics through ERK signaling has important therapeutic implications, particularly for the treatment of vascular endothelial injury and coronary artery disease (CAD). Vascular injury and impaired endothelial function are key drivers of CAD progression, and the findings of this study suggest that targeting the AHNAK2/ERK axis could enhance vascular repair while mitigating pathological remodeling. By promoting controlled proliferation and migration of VSMCs, therapies targeting this pathway could aid in restoring vascular integrity, preventing the progression of atherosclerosis, and improving overall cardiovascular health. From a sports and rehabilitation medicine perspective, these findings also emphasize the importance of vascular health in physical performance and recovery. Vascular remodeling and repair are critical for maintaining blood flow, oxygen delivery, and tissue recovery during and after physical activity. Dysregulated VSMC activity can lead to impaired vascular function, limiting endurance and resilience. By targeting the AHNAK2/ERK pathway, it may be possible to develop therapeutic strategies that not only address CAD but also enhance vascular recovery, supporting better physical performance and rehabilitation outcomes for individuals with vascular-related health challenges. Future research should focus on translating these findings into clinical applications, exploring the role of AHNAK2 in in vivo models of vascular injury and CAD. Additionally, investigating the downstream molecular mechanisms of the AHNAK2/ERK pathway could uncover further therapeutic targets for enhancing vascular health. Long-term studies evaluating the safety and efficacy of interventions targeting this pathway in diverse patient populations, including athletes and individuals undergoing rehabilitation, will be

crucial for validating its broader implications. In conclusion, the AHNAK2/ERK signaling pathway represents a promising target for therapies aimed at enhancing vascular repair, mitigating endothelial injury, and treating CAD. By bridging molecular biology with clinical applications, this research provides a foundation for innovative approaches to improving cardiovascular health and supporting physical resilience, aligning with the multidisciplinary goals of sports and rehabilitation medicine.

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