Title: A Narrative Review on the FSTL-1 Protein and its Current Known Impact in Cardiovascular Ischaemic Disease

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Discussion Points: Did you know cardiac ischaemic disease is one of the leading causes of death and morbidity worldwide? It is not currently possible to completely reverse ischaemic cardiac disease, but for how long will it remain that way? In the last few years, novel therapeutic pathways have been discovered and worked at a molecular and animal-model level with convincing results. This article shows the properties and potential of one of these routes, namely by a glycoprotein called Follistatin-like 1 (FSTL-1) and highlight some of its forthcoming challenges.

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ABSTRACT.

Myocardial infarction (MI) is one of the leading causes of death worldwide, and even though modern medicine has reduced considerably the number of deaths due to MI, patients still undergo serious cardiac issues that dramatically affects their well-being. Since the start of the century, massive efforts have been employed in exploring stem cell therapy, however, it is believed that it has failed to live up to expectations and further work must done. Alongside this, FSTL-1 has been an emerging protein which seems to offer a multitude of benefits in post-MI. A considerable number of studies with FSTL-1 have been conducted, always with a very satisfactory level of success, especially considering that research is still in its preliminary phases. In this study, a general evaluation is done to 1) the known mechanisms regulated by FSTL-1, 2) the recognized effects of FSTL-1 in cardiac tissue and cells, 3) and what work can be done to clarify questions and further expand our knowledge in order to advance FSTL-1 as a potential therapeutic agent.

Key Words: Myocardial Ischemia, Coronary Artery Disease, FSTL-1, Follistatin-Related Proteins, Follistatin-Related Protein 1 (Source: MeSH-NLM).

INTRODUCTION.

Introduction

In 54 countries belonging to the European cardiology society, there were 19.9 million new cases of cardiovascular disease (CVD) and 108.6 million people suffering with CVD in 2017. (1) Ischaemic heart disease (IHD) was the most usual expression of CVD, with 3.6 million new cases and 34.9 million people living with IHD. (1) CVD is the most common cause of death in Europe, accounting for 4.1 million deaths each year; corresponding to 47% of all deaths among women and 39% among men. (1)

Within the scientific community, for most of the time it was believed that cardiac cells didn't have regenerative capacity, until fairly recent studies have shown otherwise. (2) This has motivated a new wave of research into the field. Since the start of the century, cardiac cell therapy has been held as the most promising research area in order to find clinically applicable therapeutic techniques. (3) Considerable amounts of resources and effort (4) have been deployed in generating what can be considered as promising results (5) and which has motivated a quick pre-clinical implantation. (3) However, these investigations have yet to reach unanimity among the experts. A well composed article by Emmert et al. covers the reasons into why cell-based cardiac regeneration has failed, in many senses, to meet expectations. (6)

Ischaemic Heart Disease

As mentioned before, IHD has shown to be a severe pathology with a high mortality rate. Within a simplistic approach, IHD arises due to the acute occlusion of one or multiple sizable epicardial coronary arteries for more than 20 minutes, which can lead to an acute myocardial infarction. Typically, the necrosis spreads from the sub-endocardium to the sub-epicardium region. Depending on the territory affected by the infarction, the cardiac function is typically compromised, and current treatment options are limited. Due to the minor renewal capacity of the myocardium, the infarcted area heals by scar formation, and often, the heart is remodeled characterized by dilation, segmental hypertrophy of remaining viable tissue, and cardiac dysfunction.

The initial effects of oxygen deprivation will result in disruption of the sarcolemma arrangement in heart muscle tissue and a relaxation of myofibrils, which are shortly followed by alterations in mitochondrial ultrastructure. These changes will then lead to mitochondrial dysregulation, having grave effects on energy availability. (7) More advanced stages of prolonged ischaemic will result in liquefactive necrosis of heart tissue, especially in the myocardium(8). The deposition of collagen type I and type III in fibrosis is essential in the short term to stop the rupture of ventricular walls, however this mechanism makes it increasingly difficult for the injured to maintain its functional capacity. This is due to it ill effects on the ventricle's geometry, resulting in an accentuated loss of contractile and pump function. (9) Alongside this, an inflammatory reaction will also occur by motivating the migration of macrophages to the myocardium, mainly M1 and M2. (10) Macrophage activity will be rich in several different types of growth factors and cytokines. (10) These factors have been proven to have an overall positive net impact on the heart after MI, and have been identified to release proangiogenic factors such as TGFβ and VEGF (11).

[FIGURE 1]

METHODS

In the methodological framework of this narrative review, we employed a comprehensive search strategy to identify pertinent literature sources. The primary databases utilized for the literature search included PubMed, Scopus, and Web of Science, among others, with a focus on articles published between January 2000 and May 2022. We systematically screened titles and abstracts to select studies that aligned with the overarching narrative of our review, which centered on the role of FSTL-1 in heart tissue regeneration. Inclusion criteria encompassed both observational and interventional studies, as well as reviews and meta-analyses, written in English and encompassing molecular and animal models. The synthesis of findings was carried out through a narrative approach, providing a qualitative analysis of the existing literature to draw comprehensive insights into the relationship between FSTL-1 and cardiovascular regeneration

FSTL-1 in Ischaemic Injury

1) FSTL-1 in the heart

The most prominent mechanism of regulation by FSTL-1 is the serine/ threonine protein kinase (AKT), also known as phosphatidylinositol 3-kinase (PI3K). AKT has been identified as a key piece in myocardial growth induced by stress. (12) (13) Investigation of AMP-activated protein kinase (AMPK) has also been conducted and discovered to safeguard cardiomyocytes from apoptosis during MI (14) (15). This is due to FSTL1's capacity to stimulate the phosphorylation of AMPK Thr172. (14) Research proved that FSTL-1 overexpression would lead to an up regulation of AKT (14) as well as ERK signalling in cardiac myocytes, which resulted in better survival rates under hypoxic condition and induced apoptosis. (16) This correlates with previous research as both AKT and ERK had been identified in cellular survival. (17)(18)(19) This is further sustained by experimentation with PI3K and ERK inhibitors, which successfully inhibited the antiapoptotic effect of FSTL-1. Additionally, a reduction in FSTL-1 resulted in decrease in AKT phosphorylation and an increase in apoptosis. (16) Other pro-survival factors include Pim-1, hypoxia-inducible factor-1α and heme-oxygenase-1 which are also involved in the AKT signalling mechanism, although, their relationship and function are still not understood in its entirety. (20)(21)

ERK signalling seems to occur mainly in cardiac fibroblasts, being its central purpose the proliferation and migration of the same cells. Maruyama et al. hypothesized that the controlled fibrotic reaction offered by FSTL-1derives from an early activation and migration of cardiac fibroblasts, that in turn will lead to a greater myofibroblasts build-up in the infarcted area. (22) It is believed that this is what allows for an improved synthesis and maturing of extracellular matrix in the affected zone. (14) This reasoning is well based, as FSTL-1 resembles in large part with the SPARC family, which functions as an initial controller of extracellular matrix maturation after MI. (23) Although, contrasting information to that of Murayama et al. exists regarding the activation of FSTL-1. As a study by Dong et al. demonstrated that fibroblasts are responsible for the activation of Smad2/3 signalling via TGF-β1 which will cause a fibrotic response due to the presence of FSTL-1. Murayama et al. stated that FSTL-1 was not involved with TGF-β1 and its subsequent signals. Currently, there is not a consensual answer to this query, although the results by Murayama et al. seem to have a greater theoretical depth behind them. (22)

Bone morphogenic protein-4 (BMP4) has been shown to boost the apoptosis of cardiomyocytes. (23) BMP4 is one of the commonly released proteins during an inflammatory response to MI (24)(25) and is related to an enhanced phosphorylation of Smad1/5/8 signalling. Reports have been conducted where they showed that FSTL-1would bind to BPM4 (26), which would inhibit further activity. (14) Being this one of the principal reasons behind the antiapoptotic behaviour of FSTL-1. The inhibition of BMP4 by FSTL-1 proved to reduce myocardial infarct size and apoptosis after MI in murine models. (27)

Due to the quick inflammatory after MI, an unleashing of macrophages occurs. They are the main source of proinflammatory cytokines during myocardial MI. (28)(29) Some cytokines such as IFNγ and IL-1β will increase the secretion of FSTL-1. Other cytokines are responsible for an increase in levels of tumour necrosis factor-

α (TNF-α) and interleukin-6 (IL-6). BMP4 is largely known for its increase in the expression of TNF-α and IL-6 after MI, resulting in an exaggerated and prejudicial inflammation of cardiac tissue. Due to the inhibition of BMP4 by FSTL-1, inflammatory processes are considerably decreased, thus proving FSTL-1 as a strong anti-inflammatory in post-MI. (14) AMPK signalling, dependant on FSTL-1, has also been linked to an inhibition of macrophage migration. (15) FSTL-1 also decreased lipopolysaccharide-stimulated expression of proinflammatory genes via activation of AMPK. (14)

2) The function of FSTL-1

After a MI, a quick and vigorous response from the epicardial tissue occurs, as there is a necessity to reactive the gene pool. (30) Activated genes include Tbx18, Wt1 and Realdh2. (31) Currently, there isn't a consensus on which cells are responsible for the secretion of FSTL-1 in cardiac tissue. One of the pioneer studies in the field stated that FSTL-1was secreted by cardiac myocytes, which went in accordance to previous literature. (16) While Maruyama et al., defended that FSTL-1 was predominantly expressed by myofibroblasts after MI. (22) Interestingly macrophages have also been reported to secrete FSTL-1. (22)

In a forerunner investigation by Wei et al., FSTL-1 was introduced to the epicardium through a nanofibrillar collagen patch. (34) It proved some different short-term effects, including reduced fibrosis and increased vascularization beneath and surrounding the epicardial patch. Measurements showed an increase in the number and size of blood vessels. It believed one of the processes behind revascularization is dependent on a nitric-oxide process which is most likely regulated by a paracrine mechanism. (30) Cardio protection also occurred, as embryonic stem cell-derived cardiomyocytes didn't undergo apoptosis provoked by the hypoxic environment, which is in correlation with previous studies. (30)(16)

Wei et al. also proved that FSTL-1could have mid-term effects, as after a 4-week period, the area beneath the patch showed striated myocytes, and in the border zones of the patch cardiomyocytes had also undergone cell division, which proved that FSTL-1 had successfully induced cell cycle entry and cytokines release. (33) However, Chen et al. 2016. proved that FSTL-1 failed to incentivize the proliferation of mature adult ventricular cardiomyocytes, as it did not induce synthesisation of DNA or division as well as hypertrophy, showing some limitations in this aspect. (34) Other gaps in knowledge existed, as investigators did not know if these cells originated from *de novo* or from previously present cardiomyocytes. Some studies have been conducted in this area, as fate mapping indicated that resident cardiomyocytes were the main source of regeneration in myocardium. Although, during the investigation there was a small percentage of cardiomyocytes that didn't undergo labelling, which suggests that there was an alternative source of cardiomyocytes. (35) Current evidence points that this unknown source is most likely made up of ckit+ cells, as it was found that they can also contribute to the proliferation and regeneration of cardiomyocytes after MI. (36)

Probably the most pertinent issue that came Wei et al. was of the difference of FSTL-1expression in the myocardium *vs.* epicardium. The overexpression of myocardial FSTL-1varied in its role in comparison to the overexpression of FSTL-1 in the epicardium. The initial idea behind this discrepancy, was that there were differences in the migration rates of the cells due to differing glycosylation processes. FSTL-1 produced from

different cellular sources are most likely exposed to differences in the post-translational glycosylation, that will inevitably result in varying isoforms. (37) FSTL-1 derived from the myocardium demonstrated cardioprotective functions, but not cardio regenerative. (38) While FSTL-1 derived from the epicardium demonstrated a cardio regenerative capacity. (38) Other studies showed that non-glycosylated FSTL-1 increases proliferation of cardiomyocytes, while glycosylated FSTL-1 protects cardiomyocytes from peroxidase-induced apoptosis. (38) However, other factors might also be influencing the activation processes and subsequent role of FSTL-1. Maruyama et al. in a more recent investigation explored this topic, as they evaluated the effect of glycosylation of FSTL-1 in relation to cardiac fibroblast activation. (39) For this they used insect, mammalian, and bacteria cells. Although, the glycosylation mechanisms varied substantially between the three, there were no statistically significant differences in the capacity of each FSTL-1 protein to promote activation of cardiac fibroblasts and their role. (39)

Other areas of interest revolve around the relationship of FSTL-1 with other peptides such as thymosin β4 due to their similarities, such as the production of epicardial derived cells and a strong driving force of angiogenesis and mobilization. (38) Thymosin β4 has already been reported as a strong pro-vasculogenic factor. (40) Following MI, thymosin β4 has been shown to induce epicardial derived cells to form vascular precursors and prompt angiogenesis in the human heart. (41) Further investigation has established a relationship between thymosin β4 and the capacity of Wt1+ cells to undertake cardio myogenesis. (40)(42)

Alongside this, FSTL-1 proved to inhibit the entrance into apoptotic mode of cardiomyocytes. (34) Akt/GSK-3β signalling was verified in hypoxic- FSTL-1 cells, being currently held as the main mechanism behind anti-apoptosis in hypoxic conditions. More technical analysis have also shown that the heart upregulated Fstl-1 expression under mechanical stresses such as pressure (34). Due to these cardioprotective roles, cardiac tissue under the presence of Fstl-1 can was able to positively regulate the cardiac microenvironment and induce the importation or production of pro-regenerative substances. (34) This regulation post-MI is managed by elements such retinoic acid, hypoxia-inducing factor-1α, fibroblast growth factors, transforming growth factor-beta (TGFβ), insulin-like growth factor, BMP among many others. (43)

FSTL-1 produced outside the heart is also essential, being skeletal muscle the main producer of circulating FSTL-1 , as it has been linked to transgenic AKT-1 overexpression. (44) FSTL-1 will resemble a myokine, by maintaining its previously reported cardioprotective role through an endocrine method. (45) Increased myocardium angiogenesis and an overall increase in the heart's performance was reported due to an augmented TGFβ-Smad2/3 signalling, thus proving that circulating FSTL-1 also had a cardioprotective role. (45)(46) Reduced levels of FSTL-1 in the blood also result in more prominent inflammatory reactions in arterial injury, thus showing FSTL-1 as a potentially important factor in the therapeutics of peripheral vascular disease. (46) FSTL-1 will also inhibit proliferation and migration of smooth muscle cells in damaged blood vessels, which will lower the effect of neointimal hyperplasia after vascular injury. (45) This will result in the control of smooth muscles cells through the stimulation of AMPK mechanisms, through an increase in phosphorylation. (45) Exercise is essential as it increases FSTL-1 expression in skeletal muscle, especially through intermittent aerobic exercise. (46) This is furthered emphasised as circulating FSTL-1 would increase its expression in the heart's myocardium heart, which is a strong indicator to regulation through positive feedback. (45)

FSTL-1 has also been tested in conjunction with mesenchymal stem cells, due to the inherent characteristics FSTL-1 possess. (30) This was an attempt at addressing some of the issues currently associated with cardiac stem cell therapy mainly low survival percentage and difficulty in retention and engraftment of donor cells in ischemic tissue. (7) A study by Shen et al. 2019 should promise in this hypothesis, as Fstl-1 successfully enhanced the resistance of mesenchymal stem cells to hypoxia, a reduction in fibrosis, inflammatory cell infiltration and an increase in neovascularization. (30) All very strong indicators and in concordance with what literature has referenced up to date. The production of extracellular matrix was also considerably modulated, as tested by western blot analysis, showed that mesenchymal stem cells injected in conjunction with FSTL-1 decreased the transcription rates of collagen type I and fibronectin in peri-infarcted zones. Other pro-fibrogenesis cytokines such as connective tissue growth factor decreased in ischaemic myocardium. This resulted in a diminution of fibrous tissue in post-MI. (30)

At an immune level, efforts have been conducted to better understand the signalling related to FSTL-1. It is believed that Fstl-1 is responsible for the activation of AMPK and inhibition of Smad1/5/9 signaling (40) which is linked to an overall reduction of pro-inflammatory gene expression of M2 cells, cardiomyocytes and mediators. (47)

3) The future of FSTL-1

I believe the potential adjacent to FSTL-1 will be best utilized in junction with other therapies such as those of mesenchymal stem cells, as the epicardium can be used as a targeted for cell therapy. However, for this to occur a mapping of the previously mentioned mechanisms and how they link to each other is essential for a complete and comprehensible approach to be established. It's clear that these networks are highly complex and can have both up or down regulating effects on each other. This also applies to the relationship that Fstl-1 established with other cardio protective or cardio regenerative proteins that also undergo expression in the myocardium. With this said, a lack of consensus still exists in different topics, for example an understanding of the glycosylation processes is vital in the functioning of these proteins, however, the existing data is contradictory to each other. A precise study would be advised to understand which cells specialize in the secretion of FSTL-1, as it is most likely that there is a joint effort of myofibroblast/fibroblast and cardiomyocytes. However, for precise therapeutics techniques to be enabled it is vital to know the proportions of secretion of each type of cells. Currently it seems that we have a very good grasp of which mechanisms FSTL-1 regulates, although, the mechanism that activates FSTL-1 remains unclear and would be imperative to close this gap in knowledge. Even though very recent studies have been published in this area, there was a limited number of studies investigating the effects of Fstl-1 in immune responses, and vice-versa. Considering its importance in the modulation of cardiac tissue in post-MI its vital that further efforts are employed in this direction.

[FIGURE 2]

It is also imperative that this research can link up with other innovative exploration in the area of cardiovascular sciences. In the last years, a lot of quality investigations have been conducted in stem cell therapy, c-kit+ cells, Wt1+ cells among many other. These new avenues can definitely help overcome current challenges associated

with stem cell therapy such as low engraftment of donor cells in hypoxic environments. (48) A noteworthy investigation would also include a more thorough look at the secretion of FSTL-1 in skeletal muscle and its importance in cardiac rehabilitation in post-MI or even post-op.

Conclusion

FSTL-1 has consistently proven to be a stable and effective cardio protective and regenerative factor. It's clear that any type of cardiac regenerative technique in MI will require a rapid and vigorous approach in order to extract any sort of fruitful results. MI treatment must tackle a wide range of issues such as effective clearance of deceased tissue, restoration of lost cardiomyocytes, regeneration of electric capacity, and end of inflammation and collagen/fibrin accumulation. The way FSTL-1 is implanted is also a crucial step, would it be intravenously or through nanofibrillar collagen patch, it will inevitably affect the result and the quality of engrafted cells. We are still far from ideal solution to MI treatment, however, with FSTL-1 I believe we are a step closer to offering a clinically viable solution.

SUMMARY - ACCELERATING TRANSLATION

Follistatin-like 1 and its application in Ischaemic Heart Disease

FSTL-1 is a protein that has gained significant attention in the field of cardiac rejuvenation due to its crucial role in promoting cardiac tissue repair and regeneration. This study has as its main aim to identify, understand and summarize the molecular and immune-physiological mechanisms underpinning this molecule and how it they might apply to cardiac regeneration. Through this narrative review, the aim was to synthesize information from various sources on this topic in a descriptive and/or qualitative manner, with a more flexible and holistic overview of the existing literature.

FSTL-1 has been identified as a key player in this subject because of its ability to stimulate the growth and repair of heart muscle tissue. This is of great importance because the heart has limited regenerative capacity, and injuries or diseases can lead to irreversible damage. FSTL-1 has also been found to activate cardiac stem cells and promote their differentiation into functional cardiac muscle cells (cardiomyocytes). This process is vital for replenishing damaged or dead cells in the heart after injury, such as a heart attack. FSTL-1 exhibits anti-inflammatory properties by modulating the immune response. Inflammation is a major contributor to heart damage following cardiac events. By reducing inflammation, FSTL-1 helps create a more favorable environment for cardiac repair. Another critical aspect of cardiac regeneration is the formation of new blood vessels (angiogenesis) to supply oxygen and nutrients to regenerating tissue. FSTL-1 has been shown to promote angiogenesis in the heart, facilitating the healing process. FSTL-1 may also help reduce fibrosis, the excessive formation of scar tissue in the heart, which can impair cardiac function. By limiting fibrosis, FSTL-1 aids in preserving and restoring the heart's contractile function.

Understanding the importance of FSTL-1 in cardiac regeneration holds significant promise for the development of novel therapeutic approaches for heart disease treatment. Researchers are exploring ways to harness the regenerative potential of FSTL-1, such as through gene therapy or the development of pharmaceutical agents that mimic its effects.

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FIGURES AND TABLES.

Figure 1. *Common Complications that Arise from MI within the General Population.*

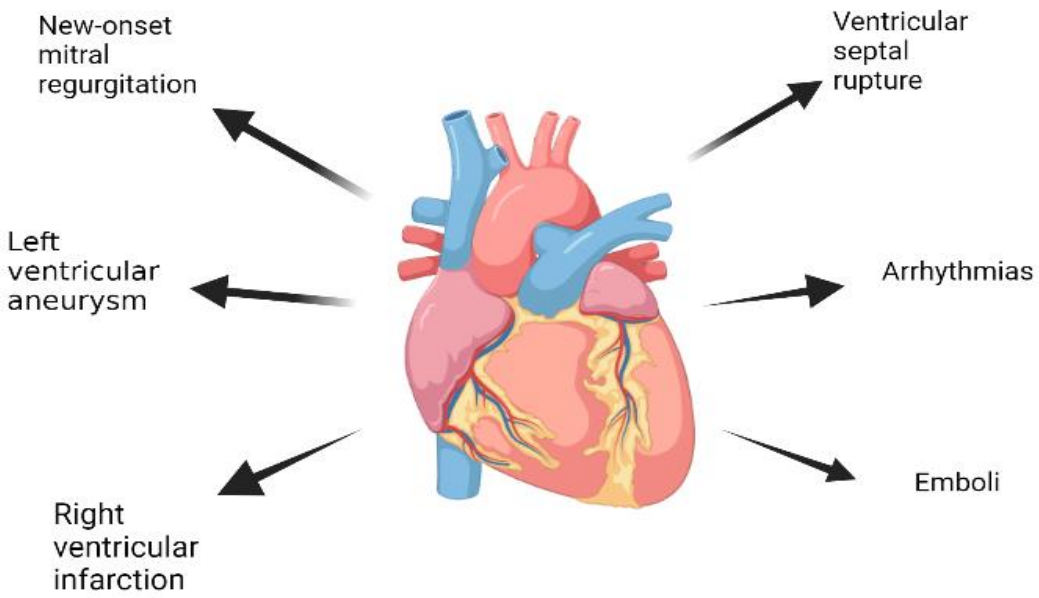

Figure 2. Figure 2- *Visual Representation of the Discussed FSTL-1 Stimulated Pathways. AMPK, AMP-Activated Protein Kinase; PI3K, Phosphoinositide 3-Kinase; TSC1/2, Tuberous Sclerosis Complex 1/2; mTORC1/C2, Mammalian Target of Rapamycin Complex1/2; DIP2A, Disco-Interacting Protein 2 Homolog A; Akt, Protein Kinase B; BAX, Bcl-2 Associated X-protein; eNOS, Endothelial Nitric Oxide Synthase; NO, Nitric Oxide; ERK, Extracellular Signal-Regulated Kinase; BMP-4, Bone Morphogenetic Protein-4.*