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Follistatin-like 1 and its paralogs in heart development and cardiovascular disease

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Abstract

Cardiovascular diseases (CVDs) are a group of disorders affecting the heart and blood vessels and a leading cause of death worldwide. Thus, there is a need to identify new cardiokines that may protect the heart from damage as reported in GBD 2017 Causes of Death Collaborators (2018) (The Lancet 392:1736–1788). Follistatin-like 1 (FSTL1) is a cardiokine that is highly expressed in the heart and released to the serum after cardiac injury where it is associated with CVD and predicts poor outcome. The action of FSTL1 likely depends not only on the tissue source but also post-translation modifications that are target tissue- and cell-specific. Animal studies examining the effect of FSTL1 in various models of heart disease have exploded over the past 15 years and primarily report a protective effect spanning from inhibiting inflammation via transforming growth factor, preventing remodeling and fibrosis to promoting angiogenesis and hypertrophy. A better understanding of FSTL1 and its homologs is needed to determine whether this protein could be a useful novel biomarker to predict poor outcome and death and whether it has therapeutic potential. The aim of this review is to provide a comprehensive description of the literature for this family of proteins in order to better understand their role in normal physiology and CVD.

Keywords

FSTL1; FSTL4; FSTL5; Heart failure; Inflammation

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Author contribution

The whole team participated in the preparation of the manuscript, while Martin Horak conceived the idea and prepared the initial draft, Piia Kokkonen and David Bednar provided the database analyses and provided valuable insight into the functional aspects of FSTL1 and its paralogs. DeLisa Fairweather critically reviewed the manuscript and provided valuable insight into the practical implications of use of FSTLs as biomarkers in cardiology. Julie Bienertova-Vasku provided guidance for manuscript preparation by Martin Horak and critically reviewed the manuscript.

Conflict of interest

The authors declare no competing interests.

Introduction

Follistatin-like 1 (FSTL1) is a secreted cardiokine discovered in 1993 in murine osteoblasts as a protein regulated by transforming growth factor (TGF)1, and thus, it was initially named TGF1-stimulated clone 36 [1, 2]. FSTL1 was simultaneously identified in rat glioma cells where it was named follistatin-related protein (FRP) based on its homology to follistatin (FST) [3]. Over time, many different names were used for FSTL1 so that some early publications mistakenly referred to FSTL1 as FSTL3 [4]. FSTL1 has been reported to be upregulated in the sera of patients with various cardiovascular diseases (CVDs) [5], yet FSTL4 and FSTL5 are essentially uncharacterized [6–12]. FSTL4 was initially identified in chick retina development as a clone of an unknown secretory protein termed D/Bsp1201 [13]. Because of its homology to secreted protein acidic and cysteine rich (SPARC), the protein was later named SPARC-related protein containing immunoglobulin domains 1 (SPIG1), and this name is currently used together with FSTL4 [14]. FSTL5 was first cloned in 2008 as a novel gene differentially expressed during early development of mouse dorsal spinal cord that encodes a highly expressed secreted protein [15].

CVDs are a group of disorders affecting the heart and blood vessels that are a leading cause of death worldwide. Thus, there is a need to identify new serum biomarkers to better predict heart failure and develop novel therapies to reduce cardiac damage and prolong life [16]. FSTL1 is elevated in the serum and myocardium in a number of cardiovascular conditions/CVDs that include hypertrophy, HFpEF, dilated cardiomyopathy, acute coronary syndrome (ACS), MI, and stroke and in children with acute Kawasaki disease [17–19]. Importantly, serum levels of FSTL1 have been found to correlate with heart disease severity and mortality for some CVDs [20, 21]. Overwhelmingly, research in small animal models reports a protective effect for FSTL1 for a variety of CVDs where it has been found to improve angiogenesis and reduce apoptosis, hypertrophy, inflammation, cardiac remodeling, and fibrosis [20]. Overall, data support that FSLT1 may be useful as a novel biomarker to predict heart failure and serve as a novel therapy to reduce CVDs.

Structural characterization

The human FSTL1 gene is present at cytogenetic band 3q13.33, has a size of around 59 kb, and consists of 11 exons with exon one being non-coding [22, 23]. Intriguingly, the primary transcript of FSTL1 encodes not only for a protein but also for a microRNA (miRNA) miR198 which is encoded in exon 11 and belongs to a group of non-coding RNAs (ncRNAs) that negatively regulate gene expression by binding to their corresponding mRNA [24, 25]. FSTL1 has a 5.9 kb long transcript that is processed into a~35 kDa protein consisting of 308 amino acid (AA) residues with a 20 residue signal peptide on the N-terminus [22, 23]. FSTL4 and FSTL5 are more related to each other than to FSTL1 (Fig. 1), share 96% nucleotide sequence homology, and have a protein homology according to PSI-BLAST of 60% [26]. FSTL1 has nucleotide sequence homology of 88% and 84% with FSTL4 and FSTL5, respectively, and a protein homology (PSI-BLAST) of 30% and 29%. FSTL4 is located at 5q31.1 and FSTL5 at 4q32.2 with a size of 647 kb and 780 kb, respectively. Both genes have 16 exons with exon 1 being non-coding [22, 23]. FSTL4 and FSTL5 transcripts have a length of 5.4 kb and 4.8 kb and are processed and translated into proteins of similar

weight ~ 93.1 kDa and~95.8 kDa, respectively. Mature FSTL4 and FSTL5 have a length of 842AA and 847AA, and both proteins possess an N-terminal sequence of 22 and 20 residues, respectively.

FSTL1, FSTL4, and FSTL5 undergo post-translational modifications with potential disulfide bonds and glycosylation sites in their sequence, while only FSTL1 has a predicted phosphorylation site (Fig. 2) [27]. Murine Fstl1 has two isoforms based on glycosylation status but no observed functional difference. It is speculated that glycosylation status strongly influences the hydrophilic properties of the protein, which may also affect its interaction with ligand although cell type-specific glycosylation patterns have also been described [28]. FSTL1 isoforms have been reported to have different molecular weights 50–55 kDa in one study and 40–48 kDa in another [3, 29]. However, the discrepancy in size may be attributed to the fact that one study analyzed FSTL1 from human serum, while the other study examined protein released from a tumor cell line, which may lead to different post-translational modifications. In a study mapping N-glycoproteins from human blood plasma, only one glycosylated isoform of FSTL1 was identified using 2 out of 3 predicted residues, and no glycosylated forms of FSTL4 or FSTL5 were detected [30].

Evolutionary, FSTL1 was found to be highly conserved among species from zebrafish and frogs to humans [31]. Aside from its signal peptide, FSTL1 contains five identified domains, namely, follistatin-N-terminal domain-like (FOLN domain, also known as follistatin-like domain), Kazal-like domain, two EF-hand domains, and a von Willebrand factor type C domain (Fig. 3) [27, 32]. The follistatin domain (FS domain) consisting of FOLN and Kazal-like domains is represented by successive~70AA long sequences including ten conserved cysteine residues [33, 34]. Interestingly, the FSTL4 sequence was first analyzed using BLAST searches of the GenBank database where it was concluded that FSTL4 contains, among others, a FS domain [13]. Even though FSTL4 and FSTL5 contain conserved cysteine residues occurring within the FS domain, the current output from InterPro, ProteomicsDB, and UniProt databases shows that chick and human FSTL4 and FSTL5 lack the FS domain [27, 32, 35]. Although this is probably due to a missing N-terminal sequence of the FS domain (i.e., FOLN domain) in both proteins, a functional analysis is needed in order to make a definite conclusion. Thus, FSTL4 and FSTL5 both contain a signal peptide, Kazal-like domain, EF-hand domain, two IG-like domains, and a WD40/YVTN domain.

FSTL1 was grouped into the follistatin family of proteins because it contains the characteristic FS domain [2]. Based on its domain similarities, namely, the FS domain and the extracellular calcium binding domain, FSTL1 is also included in the SPARC protein family which contains seven members [36, 37]. Although FSTL4 was originally named SPIG1 because of its homology to SPARC, it is rarely included in the SPARC family of proteins. This categorization is noteworthy due to the fact that FSTL1 is more similar to its two paralogs than to other members of the SPARC family and it is the only member of the SPARC family with a non-functional extracellular calcium binding domain [28].

Functional characterization

The DNA regulatory elements controlling the transcription of FSTL1, FSTL4 and FSTL5 are not well understood. The only report is from Wu et al., who described inhibition of

the murine Fstl1 promoter after binding of Kruppel-like factor 15 (KLF15) transcription factor in vitro using 3T3-L1 preadipocytes [38]. However, post-transcriptional regulation carried out by miRNAs has received more attention. Several studies report that FSTL1 is subjected to regulation by miRNAs of which some have already been experimentally verified including miR206, miR137, miR32–5p, miR29a-3p, miR27a-3p, and miR9–5p [39–44]. Of these, miR206 plays a vital role during myogenesis, miR137 is a tumor suppressor that also inhibits Notch and WNT signaling, miR32–5p and miR27a-3p are implicated in inflammation, and miR29a-p3 and miR9–5p are involved in cardiovascular homeostasis. In addition, many more miRNAs targeting FSTL1, FSTL4, or FSTL5 were predicted or detected by microarray or nextgeneration sequencing [45, 46]. miR198, which is encoded within the FSTL1 transcript, is listed in many databases as a miRNA that targets FSTL1 mRNA; however, Mouillet et al. reported virtually no decrease in FSTL1 expression using a FSTL1-luciferase reporter construct in HTR-8/SVneo cells suggesting that miR198 does not directly target FSTL1 mRNA [45–47].

FSTL1, FSTL4, and FSTL5 possess a signal peptide. FSTL1 encodes a protein secreted from the skeletal muscle and heart through the classical secretory pathway, and according to the Human Protein Atlas data, both FSTL1 and FSTL4 have been identified as secretory proteins [1, 14, 15, 48, 49]. The half-life of FSTL1 secreted from 3T3-L1 preadipocytes in vitro is approximately 3 h, while its stability at room temperature in serum or whole blood has been estimated as 48 h [38, 50]. Intracellular FSTL1 was found to localize to the cytosol, vesicles, and Golgi apparatus, while FSTL4 was mainly localized to the mitochondria [51]. Interestingly, it was reported that intracellular murine Fstl1 is able to bind the mitochondria in certain conditions and positively modulate cellular bioenergetics [52]. According to the subcellular localization prediction tools MitoProt II and TargetP, the probability of FSTL1, FSTL4, or FSTL5 being exported to mitochondria ranges between 5.9–66.1%, 2.7–5.5%, and 1.3–1.7%, respectively, and the probability of being secreted is 92.7%, 94.4%, and 93.5% [53, 54]. Thus, further studies examining the subcellular localization of FSTL1, FSTL4, and FSTL5 are warranted.

Another functional aspect affecting FSTL1 is glycosylation status [55]. Myocardial Fstl1 has been found to migrate more slowly in SDS–polyacrylamide gels compared to epicardial Fstl1, suggesting cell-specific post-translational modifications. Subsequent analysis of FSTL1 from eukaryote vs. bacteria, which are glycosylated vs. non-glycosylated, respectively, showed a similar pattern indicating different posttranslational modifications. Importantly, un-glycosylated FSTL1 promotes immature cardiomyocyte proliferation in the rat, while glycosylated FSTL1 promotes cell survival by inhibiting apoptosis. Those findings support the recent report that ablation of a single N-glycosylation site within FSTL1 enhances adult mouse and neonatal rat cardiomyocyte proliferation in vitro and in vivo [56]. Since the glycosylation status can vary between different tissues and model organisms, it is crucial to keep in mind that differentially glycosylated FSTL1 can exert distinct molecular effects, especially when trying to understand conflicting results between studies [5].

Although FSTL1 possesses the FS domain similar to the FST protein, there are several structural and functional differences. For example, FSTL1 contains only the first of three consecutive FS domains presented in FST, and it lacks the N-terminal domain and heparin-

binding motif which significantly enhances FST bioactivity [32, 33]. As mentioned above, the FS domain of FST contains ten conserved cysteine residues and the N-terminal domain with hydrophobic residues essential for binding and neutralizing activin, a TGF superfamily member [33, 57]. It was shown that the N-terminal domain itself is unable to bind activin or inhibit activin-mediated transcription; moreover, it was observed that deletion of the first or second FS domain of FST or mutations in their highly conserved C-terminal regions abolished activin binding and biological activity [33]. Based on the partially crystalized murine Fstl1, it was observed that its FS domain is similarly folded as the FS domain of FST [58]. Although FSTL1 contains only one FS domain, it was demonstrated using recombinant tick Fstl1 that it is capable of binding human activin and bone morphogenetic protein (BMP)2 [59]. A later study demonstrated that FSTL1 binds not only to activin but also to other proteins and receptors of the TGF superfamily [60]. Although the extracellular calcium binding domain with EF-hand motifs is shared among FSTL1, FSTL4, and FSTL5, FSTL1 lacks the calcium binding sequence that is present in FSTL4 and FSTL5 [32]. Additionally, it was demonstrated by Hambrock et al. that FSTL1 EF-hand motifs are non-functional and unable to bind calcium [28].

In addition to activin protein binding, FSTL1 also directly binds the activin receptor, activin A receptor type 2B (ACVR2B), preventing its signaling [60]. FSTL1 was also found to bind to disco-interacting protein 2 homolog A (DIP2A), which is thought to be a primary receptor for FSTL1 because knockdown of DIP2A reduced binding of FSTL1 to cells. Importantly, FSTL1 was also found to bind multiple TGF superfamily receptors, CD14, Toll-like receptor 4 (TLR4), and ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) indicating that FSTL1 has broad physiological activity [60–64].

Due to its role in receptor binding, it is not surprising that FSTL1 has been implicated in multiple signaling pathways including AKT serine/threonine kinase (AKT), AMP-activated protein kinase (AMPK), and mitogen-activated protein kinase (MAPK also known as ERK), all pathways important in the pathogenesis of CVDs. Specifically, FSTL1 induces AKT phosphorylation via DIP2A and is upregulated by AKT forming a positive feedback loop [1, 49, 65]. Importantly, FSTL1 has been found to have a cardioprotective effect via activation of both AKT and ERK [1]. FSTL1 knockdown reduced baseline phosphorylation of AKT but not ERK. FSTL1 activation of AMPK was able to prevent ischemic injury by reducing apoptosis and the inflammatory response [66], preventing cardiac hypertrophy, and improving the vasculature and energy metabolism, which could be reversed using an AMPK inhibitor [67–69]. Additionally, FSTL1 was found to induce JAK/STAT3 and NFκB signaling via TLR4 and TGF1-SMAD2/3 signaling through direct interaction with TGF1, mediate WNT signaling by blocking the Wnt family member 7A (WNT7A), and disrupt BMP signaling by directly interacting with BMP4 leading to BMP4-SMAD1/5/8 pathway inhibition [61, 70, 71]. FSTL5 overexpression or administration of recombinant FSTL5 has also been found to inhibit WNT signaling [72].

Physiological role in cardiac development

The cellular distribution of FSTL1 is restricted primarily to cells of mesenchymal origin with expression reported in adipocytes, chondrocytes, osteocytes, fibroblasts,

epicardial mesothelial cells, cardiomyocytes, skeletal and smooth muscle cells, and vascular endothelial cells of myocardial vessels and neurons [8, 49, 55, 62, 73, 74]. FSTL4 and FSTL5 have so far only been studied in the murine retina and central nervous system, respectively [14, 75]. Fstl4 was found in retinal ganglion cells, while Fstl5 mRNA was restricted to the olfactory system, hippocampal CA3 area, and granular cell layer of the cerebellum.

Interestingly, ExAC browser data associating exome sequences of 60,706 individuals showed no homozygous loss-of-function (LoF) mutations for FSTL1, FSTL4, or FSTL5 [76]. Furthermore, according to the determined probability of LoF intolerance, both FSTL1 and FSTL4 belong among the extremely LoF-intolerant set of genes (probability≥0.9) with their LoF intolerance probability being 0.96 and 0.98, respectively, further indicating their important physiological role in humans. During development, FSTL1 was found to be essential for proper organogenesis because Fstl1 knock-out mice develop multiple organ defects resulting in neonatal lethality, which was attributed to the ability of Fstl1 to inhibit BMP signaling [77]. In the course of mouse development, ubiquitous expression of Fstl1 in the early embryo is progressively excluded from the epithelium and, at the same time, restricted predominantly to cells of mesenchymal origin in multiple organs [77, 78]. In the adult mouse, the primary sites of Fstl1 mRNA expression include subcutaneous white adipose tissue, the lungs, and the heart [38]. Intriguingly, the overexpression of Fstl1 in frog, zebrafish, or mouse appears not to affect the development indicating that Fstl1 does not only inhibit BMP signaling but its action is more complex [79–81]. Compared to Fstl1-defcient mice, Fstl4 knock-in (Fstl4gfp/gfp) mice with no Fstl4 expression are healthy, fertile, and apparently normal [14]. Morphological abnormalities of retinal neurons and aberrant spine formation were observed in Fstl4 knockout animals [82]. The analysis of circular RNA (circRNA) suggest a role for FSTL1, FSTL4, and FSTL5 during human fetal development with changing expression patterns in multiple tissues at various time points [83].

The heart functions not only to pump blood but also as an endocrine organ secreting proteins, lipids, and genetic material including DNA, mRNA, and non-coding RNA, such as miRNA and circRNA, via extracellular vesicles [84, 85]. The proteins secreted from heart cells like cardiomyocytes, cardiac fibroblasts, vascular endothelial cells, smooth muscle cells, and progenitor cells are referred to as cardiokines. Cardiokines are responsible for proper cell and tissue communication during cardiac development and during physiological and pathological conditions. FSTL1 has been identified as a cardiokine [1]. In early mouse and chick fetal development, Fstl1 is expressed ubiquitously, but its expression becomes regionalized to non-myocardial elements during mid-gestation where it persists during adulthood [55, 78, 86]. In contrast, a recent study found that Fstl1 was expressed in the adult mammalian heart by cardiac fibroblasts, but no Fstl1 was detected in the epicardium [87]. This developmental pattern suggests a regulatory role in initial as well as late heart muscle cell formation. An Fstl1 knock-out mouse exhibited an overall enlargement of the heart in neonates compared to controls; however, it is not clear whether this was the result of cardiomyocyte hyperplasia or hypertrophy [5, 77].

Based on analysis of circRNA from 35 human fetal cardiac tissue samples collected between 10 and 20 weeks of gestation, FSTL1 is already significantly expressed at week 10, and

this expression is doubled at week 11 and then drops to its original level which is retained up to week 20. FSTL4 and FSTL5 circRNA levels peaked at week 10, slowly decrease at week 11, and were subsequently maintained at low levels up to week 20 suggesting their possible effect on early cardiac development [83]. Although FSTL4 and FSTL5 were not studied in the mature heart so far, both proteins and their mRNAs were detected in cardiac tissue using mass spectrometry and microarray, but their expression is significantly lower than FSTL1 [35, 88, 89]. WNT signaling is also needed for proper heart development where strictly controlled activation and inhibition are required in a time- and space-specific manner [90]. Importantly, FSTL1 and FSTL5 have been found to modulate the WNT pathway [71, 72].

Conditional ablation of Fstl1 from the endocardial/endothelial lineage in mice caused the mitral valves to be abnormally long and thick, which in turn resulted in mitral regurgitation, cardiac dilation, heart failure with preserved ejection fraction (HFpEF), and subsequent death of mice in the second to fourth week after birth [91]. Mitral valve leaflet deformation was caused by sustained BMP and TGF1 signaling, which led to delayed postnatal attenuation of proliferation and endocardial to mesenchymal transition. One study explored the FSTL1 gene of patients with mitral valve defects, which were similar to those of transgenic mice and concluded that this phenotype is unlikely to cause pathogenic variants in this gene [92]. On the other hand, neither a specific Fstl1 knock-out in cardiomyocytes or cardiac fibroblasts nor Fstl1 overexpression in cardiac and skeletal muscle in mice showed any alteration in phenotype or cardiac function at baseline under normal conditions, yet it had a significant impact after cardiac injury in both cases [69, 87, 93, 94].

Biomarker of CVD and heart failure

Elevated plasma and serum FSTL1 levels were observed in patients with the acute coronary syndrome (ACS), where elevated FSTL1 was found to be a prognostic biomarker indicating a higher risk of cardiovascular death [50, 95]. A recent study also reported higher FSTL1 levels in plasma collected prior to elective percutaneous coronary intervention (PCI) [96]. FSTL1 levels were used as an independent predictor of the occurrence of major adverse cardiac or cerebrovascular events (MACCE) defined as a composite of cardiac death, non-fatal myocardial infarction (MI), ACS, cerebrovascular event or stroke, target vessel revascularization, and/or hospitalization for heart failure [96]. Circulating FSTL1 levels were also higher in patients with a metabolically unhealthy state, which is a risk factor for CVD and in patients with coronary artery plaques (i.e., atherosclerosis) [97]. Plasma FSTL1 was upregulated in patients with acute Kawasaki disease, which is a primary cause of acquired coronary artery aneurysms (CAA) in childhood, compared to age-matched controls, and its levels were significantly higher in patients who developed CAA than patients without CAA [19]. FSTL1 also exhibits a high sensitivity and specificity as a predictive biomarker for the development of CAA. Moreover, Fstl1 was elevated in the plasma, serum and/or heart tissue of mouse and rat models of CVD in response to various experimentally-induced cardiac injuries such as thoracic aortic banding, transverse aortic constriction (TAC), HFpEF, ischemia/reperfusion (I/R) injury, and left anterior descending (LAD) ligation-induced MI [1, 8, 66, 69, 93, 94, 98]. Taken together, FSTL1 appears to have diagnostic and predictive value as a biomarker of CVD.

Protective role during CVD

Evidence that FSTL1 could play a protective role in CVD is supported by a number of studies. Cardiac fibroblasts or cardiomyocytes that are deficient in Fstl1 have worse damage in response to injury [69, 93, 94]. Cardiac fibroblast-specific knock-out of Fstl1 has been found to increase heart failure twofold (from 27% in control to 47% in Fstl1 knock-out) in a mouse model of cardiac rupture induced by MI [93]. Similarly, conditional Fstl1 deletion in cardiac fibroblasts causes death within 5 days due to post-MI cardiac rupture [87]. Cardiomyocyte-specific deletion of Fstl1 exacerbates cardiac hypertrophy, fibrosis, ventricular performance, and myocardial capillary density 4 weeks post-TAC and increases cardiomyocyte hypertrophy and diastolic dysfunction in a HFpEF model compared to controls [69, 94]. Additionally, circulating Fstl1 is elevated in mice with renal injury where it has been found to be secreted from the heart [99]. In this renal injury mouse model, cardiomyocyte-specific deficiency of Fstl1 exacerbated kidney injury after subtotal nephrectomy indicating that FSTL1 released from the heart is important not only in protecting the heart but also the kidney from damage. One study in patients with CVD also suggests a protective effect for FSTL1. Patients with dilated cardiomyopathy that had higher myocardial levels of FSTL1 at the time of left ventricle assist device implantation showed significantly better recovery with improved ejection fraction 1 year after implantation [8].

Although there are currently no studies that have examined the role of FSTL4 or FSTL5 in CVD, several genomewide association studies (GWAS) suggest FSTL4 or FSTL5 may be risk factors for CVD. For instance, single-nucleotide polymorphisms (SNPs) within the Fstl5 gene have been associated with the development of dilated cardiomyopathy and the severity of mitral valve disease in dogs [100, 101]. In humans, SNPs in FSTL1 and FSTL5 were linked to cardiac arrest and sudden cardiac death, 2 SNPs in FSTL4 and 1 SNP in FSTL5 were identified as potential candidate loci involved in coronary artery calcification, and FSTL4 was associated with hypertension and abnormal cardiac morphology [102–104].

In addition to its potential value as a novel biomarker for heart failure, FSTL1 may also be a novel therapeutic target for heart disease due to data suggesting it has a protective role. Recently, gold nanoparticles were used for fast, accurate, and inexpensive detection of FSTL1 using real-time PCR to detect highly expressed FSTL1 30 min after heart failure in patients [12]. Additionally, FSTL1 was found to improve CVD in small and large preclinical animal models using mice, rats, pigs, and dogs. For example, intravenous administration of an adenoviral vector encoding murine Fstl1 to mice led to local overexpression of FSTL1 in the liver and subsequent upregulation of circulating Fstl1 that reduced myocardial infarct size and improved survival after I/R injury [1]. Similar results were reported after injection of human recombinant FSTL1 in a murine model either before ischemia or after reperfusion and in a porcine model 10 min after ischemia that resulted in a significant reduction in infarct size and improved cardiac function after reperfusion [66]. The beneficial effect of FSTL1 was accompanied by improved cell survival, a decrease in the plasma heart damage marker troponin I in mice and troponin I and creatine kinase MB (CKMB) in pigs and downregulation of proinflammatory cytokines. Furthermore, attachment of a collagen patch seeded with bacterial-synthesized FSTL1 to the heart immediately after MI induced by permanent LAD coronary artery ligation in mice improved survival, cardiac

function, and vascularization and reduced fibrosis compared to control patches within 2 weeks [55]. Similar results were obtained when a FSTL1-enriched patch was grafted 1 week after I/R, resulting in nearly complete recovery of cardiac function compared to nontreated controls where cardiac function progressively declined [55]. These observations were recapitulated using a porcine model, where addition of an epicardial FSTL1-enriched patch 1 week after I/R led to recovery of cardiac function and reduced fibrosis. Intriguingly, even though myocardial transgenic Fstl1 overexpression in a mouse model of acute I/R is anti-apoptotic, it did not improve cardiac function leading to the conclusion that epicardial but not myocardial FSTL1 is needed for recovery [105]. However, transgenic overexpression of a protein may be physiologically different than FSTL1 released from a collagen patch.

Intermittent aerobic exercise (IAE) or mechanical vibration training (MVT) exercise was able to induce FSTL1 expression which was found to mediate the protective effect of exercise after MI by improving angiogenesis and cardiac function and by reducing fibrosis [98]. In a murine model of TAC-induced hypertrophy, Fstl1-specifc overexpression in cardiac and skeletal muscle reduced decline in LV performance, hypertrophy, and fibrosis and increased myocardial capillary density [69]. Furthermore, acute systemic delivery of an adenoviral vector overexpressing Fstl1 protected both control and Fstl1 knock-out mice from pressure overloadinduced hypertrophy and heart failure [99]. Another study focusing on the action of Fstl1 using a murine model of hypertension-induced HFpEF caused by aldosterone administration demonstrated that treatment with adenoviral-delivered Fstl1 significantly reduced cardiac hypertrophy and improved diastolic function [94]. In a model of dogs with tachypacinginduced heart failure, cardiac energy substrate consumption pathologically switched from fatty acids to glucose, modeling the cardiac metabolic switch that occurs during heart failure in patients [68, 106]. In this model, both acute and chronic 2-week administration of glycosylated human recombinant FSTL1 reversed the pathologic switch and improved diastolic and contractile function; however, once FSTL1 was depleted from the blood, the substrate consumption switched back again to the pathologic state [68]. In vivo silencing of miR9–5p (a FSTL1-suppressing miRNA) reduced inflammation and fibrosis, improved cell survival, and preserved post-MI cardiac function in mice [43]. In a rat model of heart allograft tolerance, donor-specific blood transfusion before transplantation stably increases Fstl1 in recipients' allografts compared to nontreated controls [107]. The involvement of Fstl1 in graft tolerance was confirmed by intravenous administration of adenoviral vector-producing rat Fstl1 on the day of transplantation, which was accompanied by significantly prolonged survival and decreased proinflammatory cytokines in the graft. Moreover, transplantation of Fstl1-overexpressing mesenchymal stem cells (MSCs) into the peri-infarct zone of a mouse with MI induced by LAD ligation reduced scar formation and inflammation and enhanced neovascularization and cardiac function after injury as well as significantly prolonging the retention of MSCs after injection compared to non-modified MSCs [108].

Location in the heart during CVD

Several studies have tried to locate the source of FSTL1 during CVD. During physiological conditions, Fstl1 is restricted to the epicardium, but during ischemic injury, Fstl1 becomes abundantly expressed in the myocardium and absent from non-myocardial areas [55]. After

LAD-induced MI in mice, Fstl1 levels were increased in the ischemic zone compared to remote areas of the heart [87, 93, 109]. Fstl1 levels peaked 1 week post-MI, progressively declined to low levels at 2 weeks, and became nearly undetectable 1 month after injury in the remote area while maintaining expression in the ischemic zone. Since the ischemic area seems to be a primary source of Fstl1, this could explain its correlation with disease severity and the higher risk of death in patients with CVD; nevertheless, its secretion from other organs remains a possibility [5]. Maruyama et al. identified Fstl1-producing cells using immunohistochemistry in post-MI hearts and found that Fstl1 did not co-localize with sarcomeric actin in positive cells but rather with non-cardiomyocyte cell types including cardiac fibroblasts and myofibroblasts, concluding that these cells are the primary producers of FSTL1 in the heart [93]. In contrast, cardiomyocytes were the primary source of Fstl1 in murine models of TAC-induced hypertrophy and aldosterone infusion-induced HFpEF, although Fstl1 levels were markedly lower than those observed in the MI model [69, 94]. In addition, human myocardial samples from patients with dilated cardiomyopathy clearly showed staining for FSTL1 in cardiomyocytes [8], which was also observed in a rat model of LAD ligation-induced MI [98]. Taken together, these findings suggest that heart-derived FSTL1 is induced in a cell-specific manner depending on the type of cardiac injury.

Apoptosis and autophagy

One way that FSTL1 may protect against CVD is by influencing apoptosis. Several independent signaling pathways downstream of FSTL1 have been found to inhibit apoptosis. In rat cardiomyoblast H9c2 cells and in cultured neonatal rat cardiomyocyte overexpression or addition of recombinant FSTL1 to culture prevented apoptosis in response to hypoxia/reoxygenation (H/R) conditions or excessive sodium nitroprusside toxicity [1, 65, 66, 110]. FSTL1 was found to prevent apoptosis by activating (1) AMPK-acetyl-CoA carboxylase alpha (ACACA) signaling; (2) DIP2A-phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)-AKT axis and its downstream targets mechanistic target of rapamycin kinase (mTOR) and forkhead box O1/3 (FOXO1/3); (3) the mitogen activated protein kinase kinase 1/2 (MEK1/2)-ERK pathway; and (4) inhibition of pro-apoptotic BMP4 which stimulates apoptosis by downstream signaling of SMAD1/5/8 [1, 65, 66, 110]. The Fstl1-regulated pro-apoptotic pathway is independent of SMAD2/3 signaling because blocking it using SB525334, a selective inhibitor of TGF receptor 1 (TGF R1), fails to abrogate the pro-survival effect of FSTL1 [110]. The cytoprotective action of FSTL1 was also diminished after the ablation or inhibition of FSTL1 or other individual signaling proteins involved in these pathways in vitro [66]. Furthermore, FSTL1 inhibits apoptosis in endothelial cells by upregulating the antiapoptotic protein BCL2 apoptosis regulator (BCL2) over the PIK3CA-AKT-NFκB axis, and so the loss of FSTL1 cytoprotection in endothelial cells may, in turn, promote CVDs such as atherosclerosis [65, 80]. Also, the pretreatment of H9c2 cells with FSTL1 prior to H/R reduces apoptosis and the amount of secreted CKMB, which is a diagnostic marker of myocardial tissue injury [111].

In contrast to FSTL1, both overexpression and addition of recombinant human FSTL5 promote apoptosis in various hepatocellular carcinoma (HCC) cell lines by inhibiting WNT/β-catenin signaling, a pathway associated with HCC progression [72]. Administration of FSTL5 to HCC cell lines elevated phosphorylation of β-catenin and glycogen synthase

kinase 3 beta (GSK3B) and elevated cleaved poly(ADP-ribose) polymerase 1 (PARP1), a marker of apoptosis. A similar effect was observed where FSTL5 promoted apoptosis in HCC cell lines in a caspase-dependent manner by downregulating the anti-apoptotic protein BCL2 and upregulating the pro-apoptotic proteins BCL2-associated agonist of cell death (BAD), BCL2-associated X apoptosis regulator (BAX), and BCL2 binding component 3 (BBC3) [112]. It should be noted that in both cases, the mechanism was studied only in oncogenic cell lines, and therefore, the results should not be generalized, and the confirmation using a non-oncogenic cell line is warranted [72, 112].

FSTL1 has also been found to affect autophagy. Cells pretreated with FSTL1 were examined for autophagy-associated proteins, and it was observed that the level of beclin 1 (BECN1) was higher, the level of the sequestosome 1 (SQSTM1) was lower, and the microtubule-associated protein 1 light chain 3 beta (MAP1LC3)/MAP1LC3 ratio was higher, suggesting a proautophagic role for FSTL1 in rat cardiomyocyte injury [111]. In addition, when H9c2 cells were treated with the autophagy activator rapamycin or autophagy inhibitor 3-methyladenine (3-MA), there was increased or decreased viability, respectively, and the addition of FSTL1 to either of these had no effect on final survival, suggesting that the cardioprotective effect of FSTL1 involves autophagy [111].

Hypertrophy

Cardiac hypertrophy is characterized by abnormal heart muscle enlargement or thickening caused by individual cardiomyocyte volume increase [113]. Cardiac hypertrophy can be either physiological or pathological since it is initially an adaptive response induced by increased mechanical workload that helps the heart to maintain its function and efficiency by reducing ventricular wall stress but can became maladaptive if it persists significantly increasing the risk of heart failure. Evidence that FSTL1 reduces hypertrophy comes from animal models. Overexpression or administration of recombinant FSTL1 to cultured neonatal rat ventricular cardiomyocytes promotes resistance to pressure overload/aldosterone-induced myocardial hypertrophy in vivo and reduces phenylephrine/aldosterone-induced hypertrophy in vitro, respectively [69, 94]. Cardiomyocyte-specific Fstl1 ablation in a murine model of TAC/aldosterone-induced hypertrophy reduces NOS3 phosphorylation and natriuretic peptide A (NPPA) and NPPB gene expression, which are molecular markers of hypertrophy produced by heart ventricles in response to increased mechanical workload and wall stretch, indicating that FSTL1 reduces hypertrophy [69, 94, 114]. Similarly, infection with an adenoviral vector expressing Fstl1 or treatment with recombinant Fstl1 to rat cardiomyocytes in vitro decreases phenylephrine or aldosterone-induced increases in NPPA and NPPB expression [69, 94]. The benefcial efect of Fstl1 on hypertrophy is mediated by its ability to promote phosphorylation of AMPK and its downstream target ACACA in cultured cardiomyocytes since AMPK inhibition reversed the protective efect of Fstl1 on hypertrophy [69].

Angiogenesis

Gradual disruption of coordinated cardiac signaling during prolonged adaptive hypertrophy contributes to impaired coronary angiogenesis and subsequent contractile dysfunction, which promotes the transition from cardiac hypertrophy to heart failure [115]. Thus, the

improvement of myocardial angiogenesis and restoration of normal cardiac performance is a therapeutic target in CVD. FSTL1 has been found to positively correlate with platelet and endothelial cell adhesion molecule 1 (PECAM1) in patients with heart failure, suggesting that it may improve angiogenesis [8]. Several other studies have described a proangiogenic effect for FSTL1 including a study by van Wijk et al., who observed that ectopic expression of FSTL1 reduces scar size in post-MI and improves cardiomyocyte survival by inducing revascularization rather than promoting new cardiomyocyte formation [55, 93, 109]. Shimano et al. reported that cardiac FSTL1 activates AMPK and nitric oxide synthase 3 (NOS3) in the TAC model in mice leading to production of NO, a mediator that is critical in mediating myocardial angiogenesis [69]. Similarly, adenoviral overexpression of Fstl1 in skeletal muscle using a murine model of hind limb ischemia stimulated revascularization and flow recovery and increased phosphorylation of NOS3 via PIK3CA-AKT [1]. Treatment with AKT, PIK3CA, or NOS3 inhibitors prevented the Fstl1-induced NOS3 phosphorylation and subsequent endothelial cell survival, migration, and differentiation into network-like vascular structures. Consistent with these observations, intramuscular injection of an adenoviral vector carrying Fstl1 did not affect the vasculature in ischemic tissue in NOS3 knock-out mice suggesting that Fstl1 promoted revascularization in vivo is NOS3-dependent [49]. Fstl1 overexpression in normoxic muscle had no noticeable impact on the capillary density [49]. Recently, exogenous FSTL1 was found to induce post-MI angiogenesis in a rat model via TGF1-SMAD2/3 signaling [98]. However, in smooth muscle cells, FSTL1 has an opposite effect on vascular remodeling where muscle-specific Fstl1 overexpression in mice or treatment of cultured human aortic smooth muscle cells with recombinant FSTL1 suppresses proliferation and migration via activation of AMPK-ACACA and inhibition of ERK phosphorylation [67]. Overall, data to date indicate that FSTL1 promotes angiogenesis.

Inflammation

Inflammation plays a critical role in healing the heart in response to cardiac injury; its early activation sets in motion the transition to later healing remodeling and regeneration [116]. On the other hand, prolonged, dysregulated, spatially expanded, or overactive inflammatory responses promote pathology including death of cardiomyocytes, degradation of extracellular matrix, and damaging fibrosis that can impair cardiac function [117]. FSTL1 has been found to both promote and inhibit inflammatory responses in various diseases and animal models. Elevated serum levels of FSTL1 are present in many systemic autoimmune diseases including rheumatoid arthritis and Sjögren's syndrome, where FSTL1 has been shown to bind to TLR4/CD14 and promote an inflammatory response increasing IL-1 and interleukin (IL)-6 levels, for example [5].

Most often, FSTL1 has been reported to reduce CVD by suppressing inflammation and proinflammatory cytokines [66]. FSTL1 activation of AMPK prevents ischemic injury by reducing apoptosis and the inflammatory response [66]. FSTL1 was found to induce JAK/STAT3 and NFκB signaling via TLR4 and TGF1-SMAD2/3 signaling through direct interaction with TGF1 [61, 70, 71]. Treatment of neonatal rat ventricular myocytes or cultured macrophages with human recombinant FSTL1 reduced the proinflammatory cytokines tumor necrosis factor (TNF) and IL-6 by suppressing BMP4-SMAD1/4/5

signaling and AMPK and ACACA phosphorylation [118]. Consistent with these results, muscle-specific Fstl1 knock-out mice subjected to arterial injury have increased macrophages based on a threefold increase in MOMA-2 (a marker of monocytes and macrophages) and CD68 and proinflammatory cytokines/chemokines including TNF, IL-1, and C–C motif chemokine ligand 2 (CCL2) [67].

Inflammation also plays a critical role during heart transplantation, where Fstl1 was found to be overexpressed in tolerated allografts, specifically in graft-infiltrating CD8+ T cells, suggesting that Fstl1 could be an active component during both the induction and maintenance phase of graft tolerance [107]. Moreover, intravenous administration of an adenovirus encoding rat Fstl1 on the day of transplantation resulted in prolonged allograft survival that was associated with a reduction in proinflammatory cytokines IL6, IL17A, and interferon (IFN). Overall, FSTL1 can increase or decrease inflammation but appears to mainly play a regulatory role in animal models of CVD.

Proliferation

The proliferative capacity of the myocardium is quite limited. The number of cardiomyocytes remains constant in the course of a lifespan, and only about 40% are renewed over the entire lifetime in a healthy heart, and so cardiomyocyte loss after cardiac injury is largely irreversible [119, 120]. Additionally, the renewal rate continuously declines with age as it was calculated that during young adulthood, human cardiomyocytes renew at a rate of~ 1%, but this rate drops by more than half to <0.5% in older individuals. Although the renewal of mesenchymal cells in the heart is higher, the rate is still <4% in adults. Therefore, improving cardiomyocyte proliferation and regeneration is an important target for therapy in post-myocardial injury. FSTL1 could serve as a good candidate therapy. However, it should be kept in mind that glycosylated myocardial and non-glycosylated epicardial FSTL1 exert different effects; glycosylated myocardial FSTL1 promotes cell survival, while non-glycosylated epicardial FSTL1 enhances immature cardiomyocyte proliferation [1, 55]. Glycosylated FSTL1 induces proliferation of cardiac fibroblasts via ERK1/2 but not SMAD2/3 signaling, suggesting a role for FSTL1 in acute cardiac repair [93]

Fibrosis

Due to the minimal regenerative capacity of the mammalian heart, cardiomyocytes lost in response to ischemic or other injury are replaced by fibroblasts and myofibroblasts forming a nonmuscular fibrotic scar [116]. The purpose of this adaptive process is to prevent rupture of the ventricular wall; however, extensive fibrosis leads to thickening and stiffening and progressive impairment of myocardial contractility, cardiac output, and ultimately to heart failure. Although many studies report that FSTL1 reduces myocardial fibrosis, the underlying molecular mechanism remains unclear [43, 55, 69, 98, 108]. In TAC and HFpEF models in mice, cardiomyocyte-specific deletion of Fstl1 was found to increase cardiac fibrosis compared to controls, indicating that FSTL1 typically reduces remodeling and fibrosis [69, 94]. Fibrosis was also reduced after administration of a collagen patch that had been seeded with bacterialsynthesized FSTL1 to the heart immediately after MI compared to control patches [55]. In a murine model of TAC-induced hypertrophy, Fstl1-specifc overexpression in cardiac and skeletal muscle reduced decline in LV performance,

hypertrophy, and fibrosis and increased myocardial capillary density [69]. In vivo silencing of miR9–5p (a FSTL1-suppressing miRNA) reduced inflammation and fibrosis, improved cell survival, and preserved post-MI cardiac function in mice [43]. Overall, many studies that examine the role of FSTL1 in the heart report a protective role.

Matrix metalloproteinases (MMPs) are a group of endopeptidases involved in collagen degradation and deposition that are critical in the progression to fibrosis after cardiac injury [116]. About half of the 25 MMPs identified to date have been associated with post-MI tissue remodeling with MMP2 and MMP9 being the most frequently studied members of the family [121]. However, identification of the specific role for MMPs in remodeling and fibrosis is difficult because of the continuous remodeling cycle. For example, the inhibition of one MMP can increase other MMPs, and multiple MMPs compete for the same substrate. Therefore, even though FSTL1 has been shown to modulate MMPs involved in myocardial remodeling such as MMP1, MMP2, MMP3, MMP9, and MMP13, it remains difficult to determine the causative effect of individual MMPS [70, 71, 122–128]. Also, most research on this topic has examined the effect of FSTL1 on MMPs in arthritis and cancer, for example, but not in CVDs. A study by Zabala et al. provides evidence that FSTL5 interacts with MMPs [129].

Energy metabolism

At rest, free fatty acid (FFA) oxidation is a primary myocardial energy source responsible for up to 80% of high-energy phosphate production, while the rest is covered by glucose metabolism which is more rapid but less efficient in terms of ATP produced per unit of glucose consumed [130]. During heart failure, the cardiac energy metabolism is commonly dysregulated leading to an increase in glucose consumption at the expense of FFA in cardiomyocytes causing progressive impairment of high-energy phosphate production, which subsequently activates other maladaptive processes, further exacerbating heart failure progression. In a dog model of tachypacing-induced heart failure, acute or chronic 2-week administration of recombinant FSTL1 reversed the pathologic switch in substrate consumption, reduced cardiac and systemic respiratory quotient, and improved oxygen consumption through activation of AMPK signaling, while administration of the AMPK inhibitor compound C abrogated these effects [68]. Interestingly, the promotion of oxygen consumption by FSTL1 in vitro was significantly stronger in primary mouse cardiomyocytes than in primary canine skeletal muscle myocytes showing a variable susceptibility to FSTL1 in a cell-type-dependent manner. That is in line with a previous in vitro study that reported that murine Fstl1 is capable of binding mitochondria and enhancing cellular energy metabolism [52]. Specifically, Fstl1 inhibition by short hairpin RNA (shRNA) resulted in the significant suppression of the oxidative phosphorylation (OXPHOS) complexes I and III, and conversely, the transfection of cells that do not express Fstl1 under normal conditions with FSTL1 cDNA or FSTL1 led to an increase in ATP production. Other indirect evidence was provided by recent epigenetic studies focusing on ncRNA describing that depletion of miR9–5p or miR29a, miRNAs that directly target FSTL1, enhances ATP production and in the latter case also promotes OXPHOS complex I activity and basal oxygen consumption [40, 43, 131]. Although it was reported that intracellular FSTL4 is

mainly bound to mitochondria, to date, no study describes whether there is any effect on energy metabolism resulting from its subcellular localization [51].

Conclusions and future directions

FSTL1 is a secreted glycoprotein and cardiokine primarily expressed by cells of mesenchymal origin that is required during organ development. Cardiac FSTL1 is involved in the maintenance of normal heart function, homeostasis, and metabolism. Its levels become elevated with cardiac stress and injury causing FSTL1 to be released to the circulation where serum levels are associated with worse CVD and poor outcome. For this reason, FSTL1 may be a useful serum biomarker reflecting cardiac damage and predicting poor outcome (i.e., mortality). Mainly using animal models FSTL1 has been found to protect against many different types of CVD by reducing apoptosis, hypertrophy, revascularization, inflammation, cell proliferation, OXPHOS, activation of cardiac fibroblasts, and remodeling/fibrosis. Emphasizing the importance of stress/injury in the induction of FSTL1, neither specific ablation nor overexpression of Fstl1 alters the normal phenotype in healthy animals, while its absence under pathological conditions significantly exacerbates cardiac injury.

Preclinical animal data suggest that recombinant FSTL1 may reduce CVD; however, several important points should be taken into account when interpreting the literature and designing future experiments. First, FSTL1 can exert varied effects depending on its tissue source and post-translational modifications. For example, although the myocardial-derived glycosylated FSTL1 can be used in the early treatment of acute cardiac damage due to prosurvival benefits, it is not sufficient for a full recovery; instead, the bacterial-derived or epicardial-derived non-glycosylated FSTL1 that promotes proliferation is needed. Secondly, FSTL1 affects multiple signaling pathways such as TGF/BMP, AMPK, ERK, and/or AKT, and these pathways are regulated in a tissue- and cell-type-specific manner. Thus, reports describing the effect of FSTL1 under specific conditions should not be generalized [5]. Additionally, FSTL1 may differ according to sex as has been found to be the case for other heart failure biomarkers soluble ST2 and IL-17A[132]. Several studies have found that serum FSTL1 levels are elevated in women with rheumatoid arthritis or osteoarthritis compared to men [133]. However, studies examining whether sex differences exist in FSTL1 during CVD are largely lacking.

It is not surprising that FSTL1 has been extensively studied over the last 15 years because of its strong potential as a diagnostic and prognostic biomarker and potential novel therapeutic tool to prevent CVD. GWAS suggest that the paralogs of FSTL1, FSTL4, and FSTL5 may also be worthwhile to investigate. Future studies should determine whether sex differences exist in this cardiokine and whether FSTL1 on its own or added to the current panel of markers/biomarkers for heart failure could more powerfully predict who is at risk of death from CVD.

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Abbreviations

AA	Amino acid
ACS	Acute coronary syndrome
ACVR2B	Activin A receptor type 2B ATP1A1
ATPase	Na+/K+ transporting subunit alpha 1
BMP	Bone morphogenetic protein
CAA	Coronary artery aneurysms
circRNA	Circular RNA
CVD	Cardiovascular disease
DIP2A	Disco-interacting protein 2 homolog A
FS	Follistatin
FST	Follistatin
FSTL	Follistatin-like
GWAS	Genome-wide association studies
HFpEF	Heart failure with preserved ejection fraction
I/R	Ischemia/reperfusion
LAD	Left anterior descending
LoF	Loss-of-function
LV	Left ventricular
MI	Myocardial infarction
miRNA	MicroRNA
ncRNAs	Non-coding RNAs
PCI	Percutaneous coronary intervention
SPARC	Secreted protein acidic and cysteine rich
SPIG1	SPARC-related protein containing immunoglobulin domain 1
TAC	Transverse aortic constriction
TGF	Transforming growth factor

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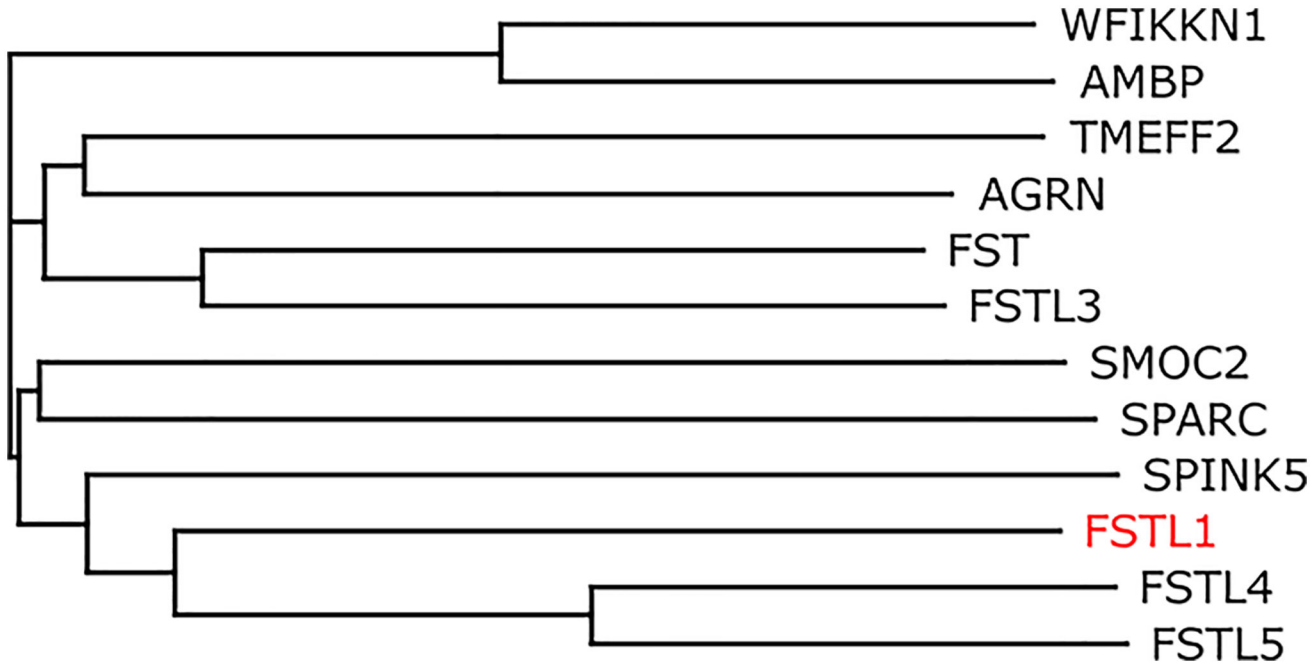

Fig. 1. The phylogenetic tree of homologous sequences with the FSTL1 protein.

A FASTA sequence of FSTL1 was used as a query for BLASTp search on human sequences. Only unique sequences with protein identity higher than 25% and e value lower than 0.1 are represented. The fnal phylogram was generated using the Interactive Tree Of Life (iTOL) online tool (Ref. Letunic, I., and Bork, P. (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 47, W256–W259.

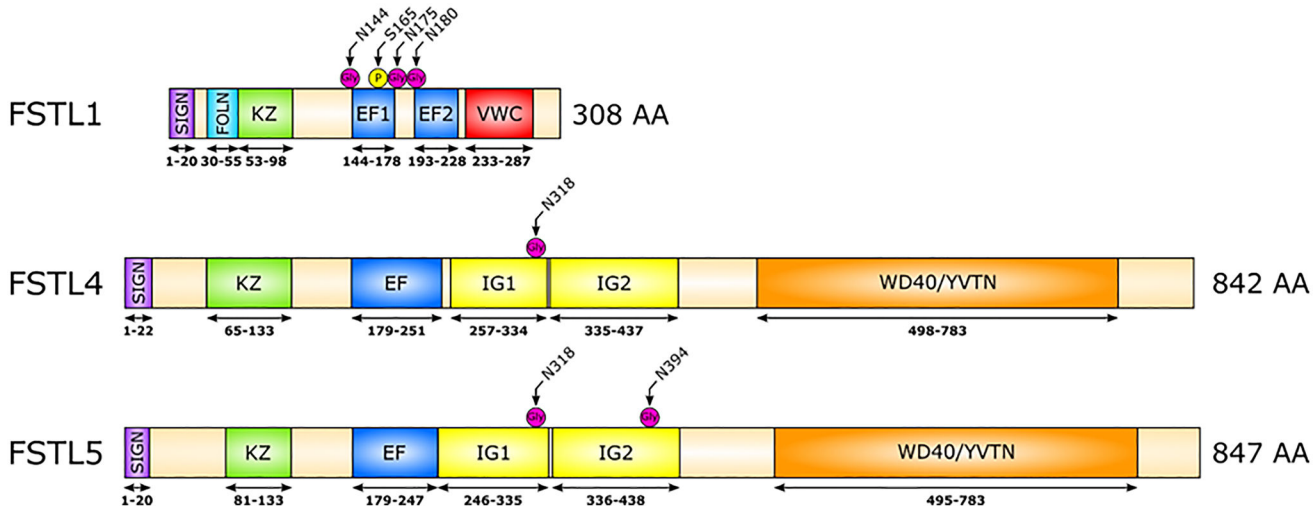

Fig. 2. Structural organization of the FSTL1, FSTL4, and FSTL5 proteins.

The circle represents post-translation modification, and the text above arrow represents specific amino acid residue and its position. The bottom arrows represent amino acid numbers in the specific domains. SIGN, signal sequence; FOLN, follistatin-like domain; KZ, Kazal-like domain; EF, EF-hand domain; VWC, von Willebrand factor type C domain; IG, immunoglobulin-like domain; WD40/YVTN, WD40/YVTN repeat-like-containing domain; Gly, glycosylation; P, phosphorylation; N, asparagine; S, serine.

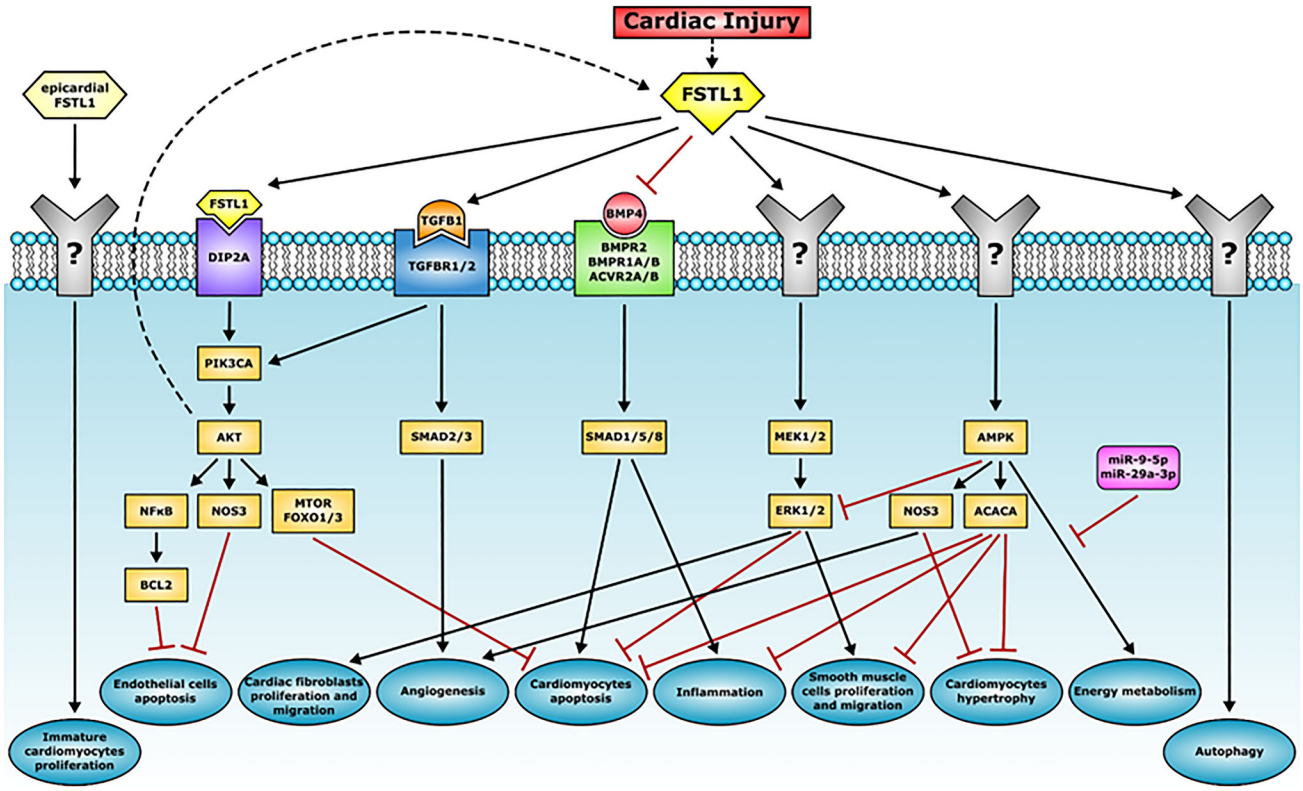

Fig. 3. FSTL1 signaling pathways in cardiovascular diseases.

Schematic representation of signaling pathways modulated by FSTL1 in response to cardiac injury. The black arrows indicate promotion/activation, and broken red arrows indicate inhibition. The question marks represent unknown receptors.