Follistatin-like 1 in development and disease

Mattiotti, A.

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Chapter 1

Introduction and scope of the thesis

Andrea Mattiotti
Cardiovascular disease

Cardiovascular disease (CVD), such as myocardial infarction (MI), is one of the leading causes of death in Western countries. During an MI the perfusion of the heart muscle is temporarily or permanently hampered, causing massive cardiomyocyte death in the affected area. As a result of the minimal regenerative capacity of the heart muscle cells, lost cardiomyocytes are not replenished but become replaced by fibrous tissue. Current therapy aims at restoring the perfusion of the ischemic area usually then followed-up by pharmacological treatment to reduce restenosis, hopefully improving quality of life and long-term survival. This therapy, however, is not a cure for the underlying problem; a lack of functional, working myocardium. Therefore, it is paramount that alternative therapies need to be developed.

Follistatin-like 1

Follistatin-like 1 (FSTL1) is a secreted glycoprotein initially identified as a Transcription Growth Factor-β (TFG-β) induced factor and involved in skeletal and heart development. It displays expression changes during several pathological conditions including CVD. It is highly and transiently induced after MI and serum concentration levels of FSTL1 have been suggested to be a good prognostic biomarker in human CVD. Its cardioprotective role has been widely studied in recent years. However, little is known about its regulation and mechanism of action during cardiac development and after MI. FSTL1 expression is highly regulated at the transcriptional, translational and post-translational level. Multiple microRNA binding sites have been identified in the 3’UTR of FSTL1 mRNA and their role in inhibition of protein expression has been shown in several models. In primates an additional level of post-transcriptional regulation has been identified: the primary transcript serves in a mutually exclusive way as a precursor for FSTL1 protein or as pre-microRNA198. At the post-translational level FSTL1 is regulated by glycosylation and at the level of secretion. The structure of the oligosaccharide chain shows species- and cell-specificity and determines the effects of FSTL1 in vitro and in vivo.
Introduction

Scope of this thesis:

Follistatin-like 1 in development and disease

We consider FSTL1 a promising target for therapy in CVD, therefore we investigated its expression, regulation and role during heart development and in cardiac disease.

It has been reported that FSTL1 is involved in multiple human diseases, including cancer progression and immune disorders. Because common biological processes, such as cell proliferation and migration, angiogenesis and immune inflammatory response, are involved in these diseases, we considered it important to expand our view beyond the cardiac field. The current knowledge on the clinical function of FSTL1 in human disease and its role during mouse embryonic development has been reviewed in Chapter 2. This review emphasizes the signalling pathways and biological processes regulated by FSTL1 in cardiovascular disease, cancer, and arthritis as well as during embryonic development.

To assess if loss of function mutation in FSTL1 can underlie genetically unresolved congenital skeletal or heart defects, in Chapter 3 sixty-nine patients present with anomalies resembling the phenotype observed in Fstl1-deficient mice were Sanger-sequenced with respect to the FSTL1 coding sequence and on key regulatory regions in the 3’UTR of the gene. Analysis of the exomes of over 60,000 individuals revealed that homozygous loss of function mutations are never observed and heterozygous ones have only been described in thirty-five individuals (<0.05%). For this reason, patients were selected based on phenotype of both full- and conditional-Fstl1 deficient mice. We studied two groups of a total of sixty-nine patients, who present with (1) skeletal defects, such as campomelic dysplasia, small patella syndrome and BILU syndrome, and with (2) cardiac anomalies, valve defects in the majority, in combination with or without kyphoscoliosis.

To gain insight in the cardiac role of FSTL1, in Chapter 4 we describe the expression pattern of Fstl1 during murine cardiac development and in the adult healthy heart as well as during cardiac ischemia and hypertrophy. MI was induced by permanent ligation of the left anterior
descending coronary artery and hypertrophy was induced by TAC. The pattern of expression of Fstl1 mRNA was determined using non-radioactive *in situ* hybridization and of the protein using immunofluorescent antibody labelling. The levels of expression were determined using quantitative polymerase chain reaction (qPCR) and Western blot analysis. Because the limited and fragmented data on the expression pattern of Fstl1 protein are often conflicting, the specificity of commercially available antibodies was determined.

The qPCR analyses are often hampered by the selection of reference genes to normalize the expression levels of the genes of interest, therefore a set of stable reference genes for normalization is paramount for accurate qPCR analyses. In **Chapter 5** different sets of reference genes were selected by analysing 119 different murine cardiac tissues and cardiac related samples in combination with 9 candidate references genes. We determined different sets of stable reference genes that can be used in different fields of heart research.

In **Chapter 6** the transcriptional regulation of Fstl1 expression was studied with special focus on the identification of the genetic regulatory region responsible for gene activation upon MI (manuscript in preparation). An *in silico* analysis of existing cardiac-related Hi-C, Chip-seq, ATAC-seq and STAR-seq data was performed, resulting in the identification of twelve candidate genomic regions. These regions were cloned into a luciferase reporter vector and tested *in vitro* in three different murine cell lines: NIH/3T3 fibroblasts, C2C12 myoblasts and HL1 cardiomyocytes. The potential regulatory regions were tested during normal culture conditions and under different experimental conditions. In particular, we noted a TGF-β response via cotransfection with a constitutively active TGF-β type I receptor kinase (ca-Alk5), R-Smad3 and Co-Smad4 and upon chemically-induced hypoxia stress. Two interesting regions were selected to create GFP-reporter mouse lines. The activity of these elements was evaluated *in vivo* during embryonic development, and in healthy adult heart and after MI.

A single-cell sequencing analysis performed on proliferating cells in the regenerating murine hearts, revealed post-damage fibroblast expressing Fstl1 that closely resemble neonatal cardiac fibroblasts. In **Chapter 7**,
we used mice in which Fstl1 was conditionally deleted using the tamoxifen-inducible fibroblast-specific Colla1-CreERT2 mouse strain (Fstl1-ifKO) to study the mechanism underlying the cardioprotective role of Fstl1 after MI. Deleting Fstl1 two weeks prior the induction of a MI using this mouse line revealed that Fstl1-ifKO mice all die within the first week after surgery due to cardiac rupture.

In Chapter 8 our observations and findings are summarized in the context of the current knowledge and future directions are discussed.