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Characterization of the novel non-coding RNA *Zeb2os* as a potential therapeutic target in cardiac disease

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1. Abstract

Cardiovascular diseases remain the leading cause of death worldwide. Mechanisms of disease genesis and progression are therefore of great clinical interest but have yet to be entirely comprehended. The heart comprises various cell types that contribute to homeostasis and disease. Being the most abundant immune cell in the human heart, cardiac resident macrophages (crMΦs) were only recently identified as a crucial cell population and have since come into focus. It has become evident that analyzing protein-coding regions alone cannot sufficiently explain pathway regulations and the pathogenesis of cardiovascular disease. Therefore, we chose to investigate the role of long non-coding RNAs (lncRNA) in this study. The aim of this project was to first identify and then elucidate the significance and function of a long non-coding RNAs in crMΦs both in health and disease. Not only are macrophages a promising cell population for intervention during disease, the intervention with lncRNAs in general offers the possibility to manipulate molecules that are expressed in a very cell- and tissue-specific manner. Therefore, potential side-effects after lncRNA depletion are avoided in other cell types or organs.

Through RNA sequencing and single cell sequencing of cardiac cells, we identified *Zeb2os,* a highly expressed and specific lncRNA in cardiac resident macrophages. *Zeb2os* is a lncRNA that acts as an antisense to Zeb2, a zinc-finger E homeobox-binding transcription factor, which is known to play an important role in the development and survival of tissue-resident macrophages. By using loss-of-function assays, we could show that *Zeb2os* affects the migration capabilities of macrophages while also influencing their response to a chemoattractant. These results suggest that *Zeb2os* could be a crucial component in the regulation of inflammation and subsequently wound healing abilities. Furthermore, we could observe that Zeb2os alters the expression of Zeb2 by regulating its transcription, which infers a potential synergistic effect between the two factors.

Taken together, our findings demonstrate that Zeb2os may have a significant role in the pathogenesis of cardiac disease by influencing migration, wound healing capacity, chemotaxis, and the maintenance of crMΦs, hence presenting a promising opportunity for therapeutic intervention. Further insight into the characterization of Zeb2os in an in vivo model is necessary to better understand its role in cardiac health and disease.

2. Introduction

2.1. Cardiovascular disease as the leading cause of death

Cardiovascular diseases (CVDs) are causing one in four deaths worldwide, representing the leading cause of death in industrialized and developing countries [1]. In the past thirty years, numbers have been climbing due to an aging and growing global population suggesting an even bigger socio-economic burden for the future [2]. Resulting from their high prevalence, CVDs like coronary heart disease and myocardial infarction play a major role in public health. Therefore, it is of great importance to understand how cardiac tissue is injured, it regenerates and the underlying mechanisms. Cardiovascular diseases (CVDs) refer to a range of pathological conditions that affect the heart or blood vessels. The pathogenesis of cardiac diseases is based on varies stimuli affecting almost any cell type of the heart. Even though great advances have been made in research, many underlying mechanisms remain unknown due to their complexity.

2.2. Response to cardiac injury

After cardiac injury, an intricate spectrum of local and systemic mechanisms is initiated and contributes to cardiac remodeling, including the activation of immune cells. Among others, it serves with two important functions: response to invading pathogens or environmental damage (e.g., ischemia or hemodynamic overloading) and repair/regeneration through stimulation of tissue growth.

It has been shown that these processes have a two-sided effect on the heart: they are beneficial but can also be detrimental [3]. An inflammatory response leads to drastic changes in geometry, structure and function of the heart and thus may have severe effects on cardiac function in the long term [4,5].

In the event of acute injury, dying cardiac myocytes release a variety of factors that act as danger signals. These signals lead to the activation of toll-like receptors, the complement system and eventually to the release of pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-α, interleukin-1 and CCL2 [6]. Via these cytokines and chemokines, leukocytes are recruited e.g., from the spleen and bone marrow and infiltrate the damaged tissue. Neutrophils and inflammatory monocytes

then digest components of the necrotic cardiac cells and their segregations. Their tasks range from degrading cell debris, to coordinating the healing of the tissue by repairing myocardial extracellular matrix and channeling angiogenesis. These tasks are finely modulated and composed of different stages. After a blunt and intense response of the immune system follows a reparative phase. The reparative phase includes replenishment of (myo)fibroblasts, scar formation and neovascularization. The prospects of success of the repair process are defined by the critical balance of tissue inflammation and then subsequently by its timely suppression and resolution [7,8].

Consequently, especially monocytes and macrophages seem to be of great relevance in the healing stages of the heart. It has been shown that these cells influence the ability of myocardial tissue to recover after injury. Interestingly both, increased and insufficient macrophage expansion, impair infarct healing, demonstrating that inflammation is harmful after injury, but also essential for tissue repair [9].

The immune system is not only one of the key players when it comes to acute injury like myocardial infarction but has also proven to be of great importance for the progression of chronic inflammatory diseases like atherosclerosis. Here, maladaptive immune response leads to an accumulation of cholesterol-laden macrophages (foam cells) which further drive the recruitment of monocytes through secretion of pro-inflammatory mediators resulting in inflammation and disease progression [10]. Nevertheless, recent studies have shown that monocytes as well as other leukocytes are also vital for plaque regression and inflammation resolution [8].

One explanation for this phenomenon might be that there are distinct macrophage populations that mediate different functions.

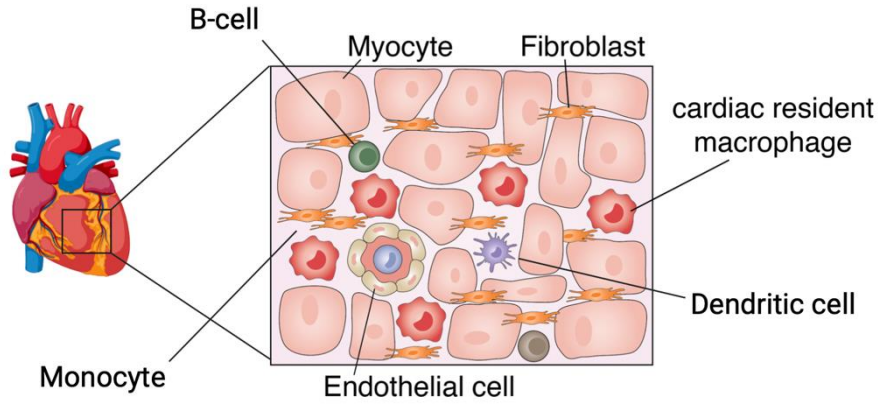


Figure 1: Cell types in the myocardium

The heart is composed of many different cell types, major cell types including cardiomyocytes, fibroblasts, and endothelial cells. Recently, cardiac resident macrophages were discovered to present the most abundant immune cell in the heart suggesting a critical role in cardiac health and disease.

2.3. Origin and function of macrophages

Macrophages were originally identified in the early 19th century by Elie Metchnikoff as cells that ingested bacteria and other microbes [11]. Over the years it has been shown that macrophages are multifunctional white blood cells and contribute an irreplaceable part to the immune system. They have functions in almost every aspect of an organism's biology by aiding to development, homeostasis, and immune responses. They are distributed throughout the body where they display great anatomical and functional diversity based on the tissue in which they reside from the earliest stages of development [12,13].

Macrophages play a crucial role in the immune response and tissue homeostasis by engulfing cell debris, pathogens, and cancerous cells. Furthermore, as part of the innate immune system, they signal lymphocytes (B- and T-cells) about the presence of pathogens and activate a specific response [14]. Macrophages can both boost and attenuate inflammation and thus regulate normal physiology. Additionally, they contribute to many pathological processes making them attractive targets for therapeutic intervention. This, however, requires an even more detailed understanding about macrophage biology and their involvement in the development and maintenance of disease.

Based on their functions, macrophages have been divided into several subsets. Historically, these subsets include the division into M1- and M2- macrophages. In general understanding M1- macrophages used to be the classical activated ones. They are activated during inflammation processes and lead the defense against pathogens.

M2-macrophages are alternatively activated. They perform quite the opposite role by resolving the inflammation phase and aiding in tissue repair [15]. Meanwhile, macrophage phenotypes have presented themselves to be much more diverse. There is a great heterogeneity across the macrophage lineage, suggesting different subtypes, but also exhibiting different developmental stages with distinct physiological roles [16].

Historically, it has been the prevailing view that all macrophages originate from circulating bone-marrow derived monocytes that colonize in different tissues. Recently though, it has become evident that most adult tissue macrophages already originate during embryonic development and not exclusively from circulating monocytes. [17].

One hypothesis is that macrophages are generated in the yolk-sac (YS) in two consecutive waves. In mice, the first "primitive" macrophage progenitors appear around E8 in the YS. Most of them migrate to the brain. Afterwards multipotent 11erythro-myeloid progenitors (EMP) develop from E8.25 onwards and are a major source of tissue resident macrophages. From E10.5 on, hematopoietic stem cells (HSC) emerge from the aorto-gonado-mesonephros (AGM) region and circulate to the liver where they start fetal hematopoiesis. Later on, hematopoiesis takes place in the bone marrow (BM) where myeloid cells can be continuously replaced from [18]. Tissue macrophages are independent of the BM potentially throughout life due to their ability to self-renew. They are maintained by *in situ* proliferation and can expand dramatically if needed [19].

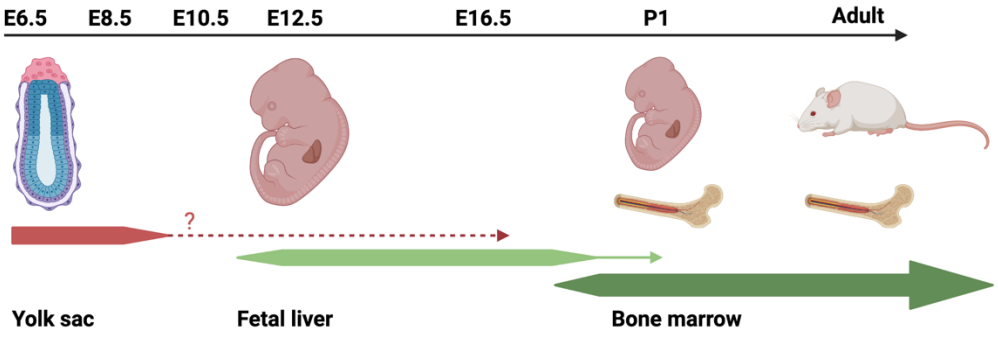


Figure 2: Embryonic and adult macrophages, modified from Epelman et al., 2014

This sketch visualizes schematically the origin of embryonic and adult macrophages lineages. Embryonic macrophages mainly derive from the yolk sac, while later on in development they first are fetal-liver-monocyte-derived and then in adult life generated from bone marrow precursors.

2.4. Tissue-resident macrophages

Most organs and tissues in the body harbor unique tissue-resident macrophage populations, exhibiting remarkable heterogeneity based on their very organ-specific, specialized functions.

Several transcription factors are responsible for the development of tissue macrophages and respectively for the evolution of hematopoietic stem cells. For instance, *PU.1* is required for the development of macrophages and *Myb* for HSCs, while they are each dispensable for the other [20]. Furthermore, a study showed that tissue macrophages are composed of different cell pools based on the expression of certain markers. F4/80^{hi} macrophages were assumed to descend from YS precursors with the ability to self-renew in their respective tissues, while it was indicated that CD11b^{hi} macrophages belong to a group that is continuously replaced by BM-generated monocytes [21].

Among representants of tissue macrophages are e.g., Kupffer cells (liver), microglia (brain), peritoneal, lung, splenic red pulp, and bone marrow macrophages. They are characterized by their tissue and niche specific functions and their ability to self-renew [22].

Embryonically derived macrophages fulfil a multitude of functions both in the developing embryo and later in the adult. Mice lacking tissue macrophages show increased mortality (perinatal and postnatal), stunted growth and defects in embryonic vascular development in numerous tissues including the central nervous system. Even more importantly, tissue macrophages of the brain, microglia, are essential for neuronal survival, releasing growth factors, aiding the development of several brain structures and synaptic patterning[23]. Kupffer cells are responsible to remove microorganisms, cell debris and aged erythrocytes from the blood from the liver [24]. Alveolar macrophages in the lung are chaperones of the immune system and examine inhaled pathogens. They are also part of the homeostatic regulation of tissue function, e.g., the clearance of surfactant [25]. Langerhans cells in the skin are also part of immune surveillance by interacting with T-lymphocytes [26]. Red pulp macrophages in the spleen eliminate defect erythrocytes and take part in the iron metabolism [27]. Recent studies now suggested that also the heart is in possession of specified resident tissue macrophages.

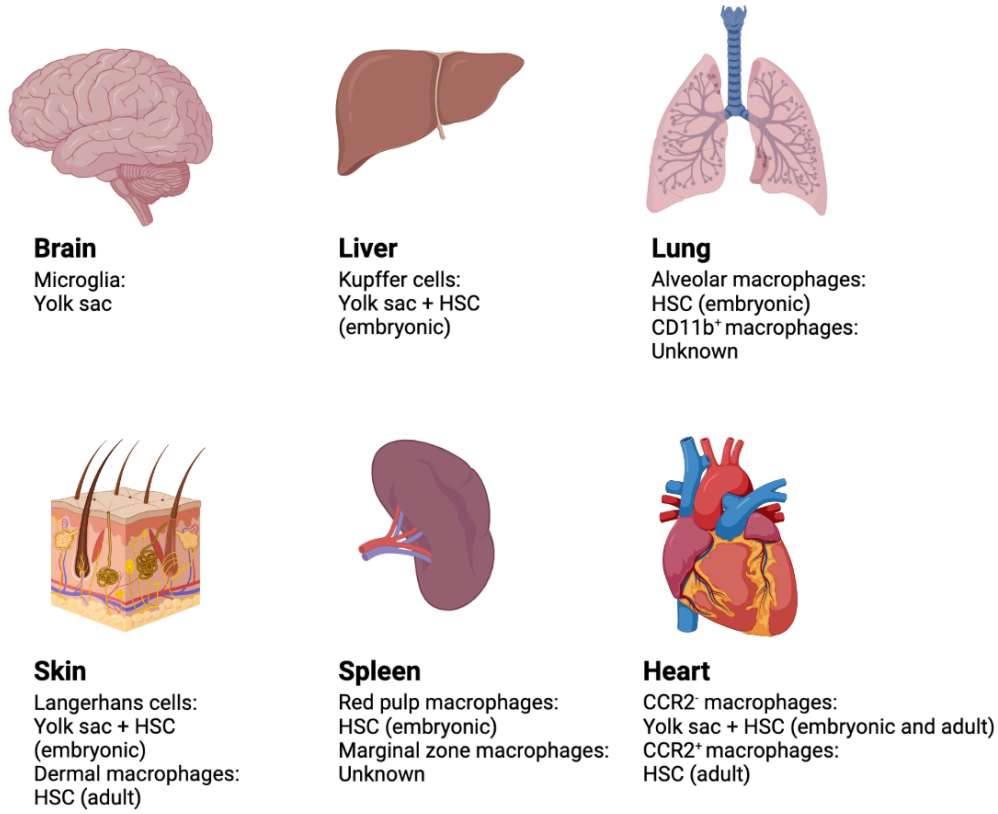


Figure 3: Tissue-resident macrophages and their origin, modified from Epelman et al., 2014

Depiction of contribution of macrophage lineages to various populations of specific resident macrophages in different organs.

2.5. Resident cardiac macrophages

Recent studies looking at *in vitro* and *in vivo* data from mice show that also most cardiac macrophages were established prior to birth, with significant contribution from yolk sac progenitors. These cardiac resident macrophages (crMΦs) populate the heart during embryogenesis and comprise roughly 6-8% of the non-cardiomyocyte population in the heart, however they are only poorly understood up to now.

CrMΦs maintain through local proliferation and seem to be independent of bone marrow derived blood monocytes [28]. They reside interspaced between myocytes, fibroblasts and endothelial cells and are the most abundant immune cell type in the heart. Among other things, they control homeostasis, tissue damage and repair. Recent studies found them to be also part of facilitating electrical conduction in the heart in the atrioventricular node. Here, macrophages expressing connexin 43 reside next to conducting cells. When coupled via gap junctions, cardiac macrophages

depolarize in synchrony with cardiomyocytes. Also, macrophages influence the resting membrane potential of cardiomyocytes and thus accelerate their repolarization. [29].

Initially, one can divide macrophages in the heart and their response to cardiac injury in those that are resident in the tissue when injured and those that are recruited to the myocardium after an injury occurs. Recent studies show that the healthy adult myocardium contains at least four subsets of macrophages. These four subsets consist of one that is maintained without the influence of blood monocytes (TIMD4^{+}LYVE1^{+}MHC^{-}IIloCCR2^{-}), one that is partly replenished by peripheral monocytes (TIMD4^{-}LYVE1^{-}MHC^{-}IIhiCCR2^{-}) and two which are completely refilled by monocytes (CCR2^{+}MHC^{-}IIhi) [30]. Following the disruption of homeostasis, e.g., through cardiac injury, changes are brought upon these subsets.

Emerging evidence has shown that resident macrophages are fundamental players in this setting by modulating inflammatory responses and tissue remodelling. Among other diverse functions like efferocytosis, cardiac development and cardiac conduction, they mediate the recruitment of circulating immune cells and contribute to the production of pro- and anti-inflammatory cytokines [31], [29], [32], [33]. Resident cardiac macrophages possess a unique role with their ability to either stimulate an inflammatory response or, at the same time, repress it. While these processes after injury are not fully understood yet, they are partly based on the great heterogeneity of resident macrophages and their ability to adapt to their environment. They do so by modifying their gene expression [34]. Resident cardiac macrophages inherit special genes that are responsible for critical parts in e.g. tissue remodelling [32]: For instance, there is LYVE1 which plays a big part in vascular homeostasis which makes it invaluable for healing processes [35]. Furthermore, cardiac macrophages seem to secrete growth factor IGF1 which promotes angiogenesis [30]. Recruited macrophages from bone marrow are failing to compensate for their unique functions. Interestingly, after tissue injury resident cardiac macrophages were subsequently replenished by blood monocyte-derived macrophages [36]. The depletion of cardiac resident macrophages compromised cardiac function and seemed to badly affect wound healing. The ability of the murine heart to regenerate after MI seems to be dependent on the expansion of embryonic macrophages [37].

While these findings all point towards a pivotal role of cardiac macrophages in homeostasis in diseases of the heart, finding specific targeting strategies remains

challenging. This is where long non-coding RNAs come into play which are expressed and regulated in a very tissue-specific manner.

2.6. Long non-coding RNAs

About 80% of the human genome is biochemically active in at least one cell type [38]. Contrary to the general perception only about 2% of the genome is transcribed into protein-coding mRNAs [39]. Over the years, it has become apparent that analyzing these protein-coding regions do not suffice to explain pathway regulations and the pathogenesis of many diseases. It has also become evident that in the course of evolution more complex organisms obtained a growing non-coding part of the genome which suggests a great impact on development, organization and molecular mechanisms [40].

The majority of the genome is transcribed into RNAs with no apparent coding potential. Non-coding RNAs can be further divided into small non-coding RNAs such as microRNAs, snoRNAs, tRNAS and snRNAs and transcripts longer than 200 nucleotides also known as long non-coding RNAs (lncRNAs). Although the most common RNA species, very little is known about them yet. LncRNAs can be further classified based on their location with respect to protein-coding genes and their functions. Location wise they can be further divided in two major categories: nuclear and cytoplasmic. LncRNAs located in the nucleus interact with chromatin-modifying complexes and have structural and regulatory roles, controlling nuclear architecture and gene transcription [41]. Cytoplasmic lncRNAs are modulating mRNA stability, translation, post-translational modifications and protein localization and turnover [42, 43]. Secondly, lncRNAs can be categorized by their function. LncRNAs can act as enhancers of transcription at promoter and enhancer regions where they interact via chromosomal loops in a tissue-specific manner [44]. They also regulate transcription through binding and segregating RNA-binding proteins or miRNA targets. Additionally, they can act as signaling molecules in responses to cellular or other stimuli. LncRNAs can operate as molecular chaperones to guide functional proteins to target gene loci. Scaffold lncRNAs link multiple proteins to form ribonucleoprotein complexes (RNPs) e.g. to initiate transcriptional programs [45].

LncRNA loci exist in various orientations with respect to coding genes. There are antisense transcripts of protein-coding genes, long intergenic non-coding RNAs, sense

overlapping transcripts containing a coding gene within an intron on the same strand, sense intronic transcripts residing within introns of a coding gene, but that do not intersect any exons and processed transcript that do not contain and open reading frame and cannot be placed in any of the other categories [46].

Increasing evidence depicts lncRNAs as regulators in various cellular processes. Their expression is regulated in physiological state, but also in disease [47]. This also applies for the cardiovascular system where they play crucial roles in cardiovascular development and CVD-related pathogenesis [48].

However, many lncRNAs in the cardiovascular field and their mechanisms of action are yet to be explored.

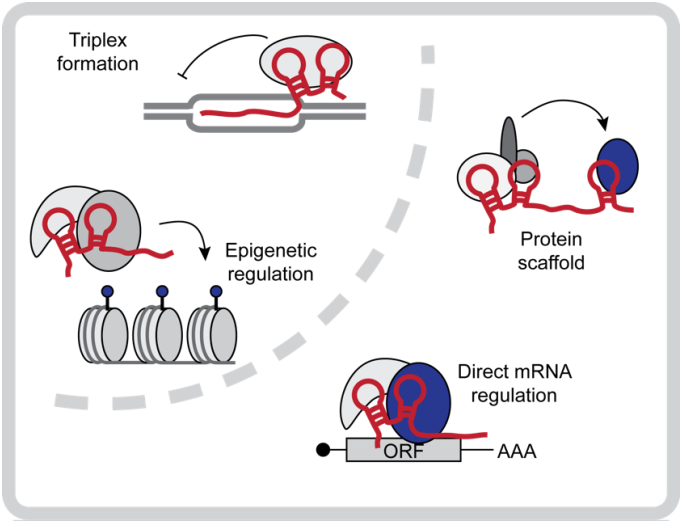


Figure 4: Selection of lncRNA functions

Long non-coding RNAs perform a variety of functions in a genetic and epigenetic context by participating in direct mRNA regulation or protein scaffold in the cytoplasm or in triplex formation and epigenetic regulation in the nucleus.

2.7. Long non-coding RNAs in the cardiovascular system

LncRNAs are implicated in the genesis, maintenance, and progression of various CVDs by affecting processes like cardiac hypertrophy and fibrosis, atherosclerosis, myocardial infarction, and heart failure. They are believed to be novel regulators of

cardiovascular risk factors and cell functions by controlling gene expression programs [49,50].

It was shown that lncRNAs seem to be critical involved in cardiac developmental processes partly because of their tissue/cell-specific expression and their regulation [51]. Klattenhoff et al. demonstrated that lncRNAs in embryonic stem cells are essential for lineage commitment. They identified the heart-associated lncRNA *braveheart* in mouse which is required for progression of nascent mesoderm into a cardiac fate [52].

Also, they seem to play an important role in cardiovascular disease. Many lncRNAs go through specific expression changes in response to either cardiac stress or pathological heart conditions and therefore act as key regulators in homeostasis and disease.

Pressure overload induced hypertrophy after transaortic constriction (TAC) in mice led to the identification of a cluster of lncRNAs which play a role in the development of cardiac hypertrophy [53]. Han et al. could show that the lncRNA *Mhrt* is downregulated in this disease model and that the rescue of *Mhrt* by over-expression prevents mice from eventually developing pathological hypertrophy and heart failure. *Mhrt* is a lncRNA localized in the nucleus. There, it binds to a factor called BRG1 and prevents it from remodeling chromatin and hereby its repression of its targets.

In the vascular system, lncRNA *Malat1* was discovered to play a role the context of atherosclerosis. Decreased *Malat1* levels lead to the augmentation of lesion formation in mice by the enhancement of an inflammatory response in the vascular wall. *Malat1* deficiency also increased monocyte adhesion and heightened expression of tumor necrosis factor [54 55].

A polymorphism in the gene of *Malat1* (rs619586AG/GG genotype) was shown to be associated with a decreased risk of coronary atherosclerotic disease [56].

Although recent studies allow first insights in the function and regulation of lncRNAs in the cardiovascular system, the function of many lncRNAs still remains unknown. In addition, the research of lncRNAs is challenging - among other things due to the fact that they are often not conserved among species and are expressed as many transcript variants. Nonetheless, they lead the way into the future as promising therapeutic targets and diagnostic and prognostic biomarkers. Up until now functions of lncRNAs in crMΦs haven't been identified and described.

2.8. Zeb proteins

Zeb1 and Zeb2 are part of a protein family of zinc-finger E homeobox-binding transcription factors. These proteins contain a N-terminal and a C-terminal zinc finger cluster which can independently bind to e.g., promoter regions. The Zeb proteins are mainly functioning as transcriptional repressors by interacting with activated SMAD proteins, recruitment of C-terminal binding protein or histone deacetylase complexes [57]. Also, they are known to play an important role in epithelial mesenchymal transition (EMT). EMT is a process which occurs in wound healing, fibrosis and in invasive phenotypes of epithelial tumors. Furthermore, EMT is crucial for embryonic processes [58]. Zeb proteins induce EMT through repression of epithelial genes. Amongst others they bind the promoter of E-cadherin and thereby downregulates its expression. Epithelial cells thereupon acquire a rounder shape, lose their cell-cell contacts, and gain the ability to migrate [59].

Recent studies have also demonstrated a role of Zeb1 and Zeb2 in other cellular processes like maintaining stemness and initiating cell-cycle arrest [60]. Zeb2 is required for embryonic development and has an impact on melanocyte, neuronal and oligodendrocyte cell fate. Mice with Zeb2 deficiency die around E12.5, patients with heterozygous abnormalities often develop Hirschsprung's disease or a syndrome called Mowat-Wilson which is a genetic disease characterized by a variety of health defects including epilepsy, intellectual disability, delayed growth and motor development and congenital heart disease [61,62]. It could also be shown that Zeb2 is an important regulator in adult hematopoiesis by influencing the differentiation of multiple cell lineages [63]. Many immune cells express Zeb2 demonstrating its important function for maintenance, function, and regulation of these cells. Deletion of Zeb2 led to cell-intrinsic reduction of monocytes in the bone marrow, spleen, and blood [64]. Looking at the only 67 genes that are conserved across the tissue-resident macrophage lineage, Zeb2 is among them. Scott et al. found that tissue-resident macrophages lacking Zeb2 disappeared from their respective tissues, most likely through necroptosis, followed by their replenishment from bone-marrow precursors. This finding led to the conclusion that Zeb2 functions to maintain tissue-specific identities of macrophages [65].

2.9. Zeb2os

Zeb2os is a long non-coding RNA which is located antisense to the Zeb2 gene.

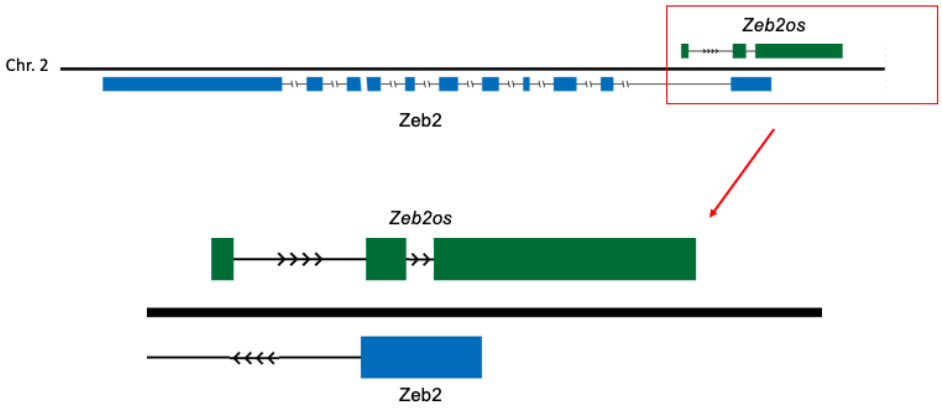


Figure 5: Genomic locus of Zeb2 and *Zeb2os*

Schematic depiction of the genomic locus of Zeb2 (blue) and *Zeb2os* (green). *Zeb2os* is located antisense to Zeb2.

The function of this long non-coding RNA is yet to be determined. Recent studies suggest *Zeb2os* might be involved in the regulation of Zeb2 expression [66], [67], [68].
One study proposes a mechanism that involves the internal ribosome entry site (IRES) of Zeb2. This IRES is situated within a sizable intron in the 5' untranslated region and crucial for the expression of Zeb2. *Zeb2os* prevents the splicing of this intron and thus upregulates the levels of Zeb2 protein. Hence, there is a strong correlation between *Zeb2os* presence, the conservation of the 5'-UTR and the abundance of Zeb2 [67]. In mesenchymal cells the presence of Snail1 leads to an upregulation of *Zeb2os* resulting in an RNA-RNA duplex conformation that prevents the recognition of the spliceosome. This leads to the inclusion of said intron which contains the IRES [69]. In macrophages, this mechanism or similar ones could not be detected yet. Other studies have suggested a role of *Zeb2os* as an oncogene by promoting the proliferation and metastasis of breast cancer cells [66]. Additionally, *Zeb2os* was placed in the context of hepatocellular carcinoma (HCC). It was demonstrated that *Zeb2os* levels were upregulated in HCC tissues and led to decreased tumor growth and metastasis [68]. In the context of bladder cancer, *Zeb2os* was discovered to be positively correlated with Tgfβ1 mRNA expression which suggests a Tgfβ1-*Zeb2os*-Zeb2 axis that leads to the induction of EMT [70]. Potential roles of *Zeb2os* have been predominantly described in

the cancer field. Other mechanisms of action remain in question. *Zeb2os* seems to be quite unexplored, especially in other contexts than cancer. The location of this lncRNA in striking distance to Zeb2 and their correlating expression suggests a direct influence on Zeb2 and its functions. Therefore, it is of great interest to investigate the role of *Zeb2os* in tissue resident macrophages and the cardiovascular system.

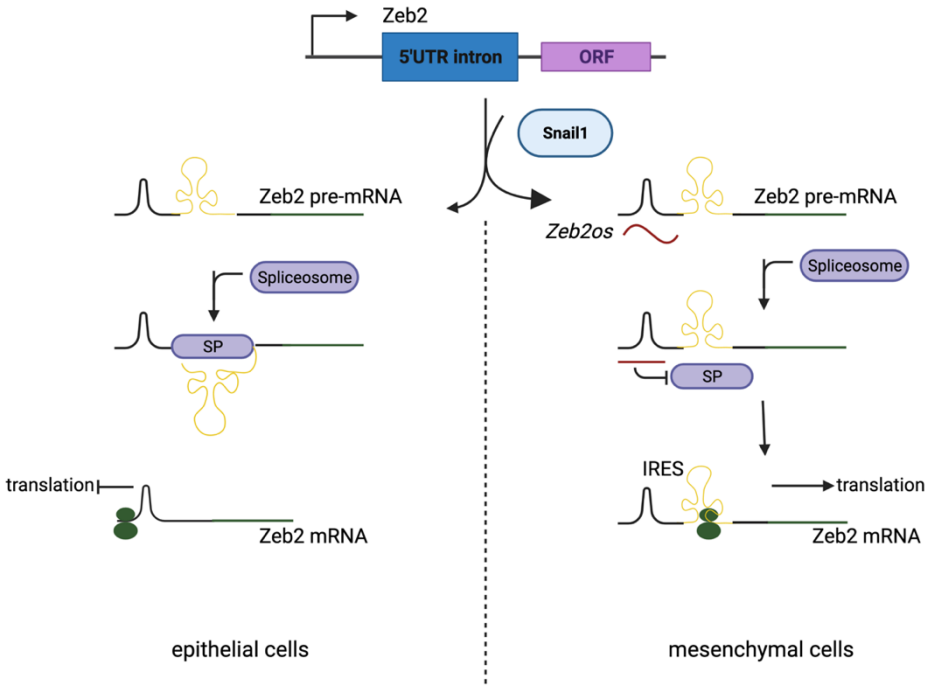


Figure 6: RNA-RNA interference of *Zeb2os* with Zeb2 pre-mRNA, modified from Romero-Barrios et al., 2018

In mesenchymal cells, it has been shown that presence of *Zeb2os* prevents the splicing of an internal ribosomal entry site of Zeb2 pre-mRNA. This eventually leads to the translation of Zeb2 mRNA and therefore to a positive regulation of expression of Zeb2 by *Zeb2os.*

3. Materials and Methods

3.1. Culture and treatment of cellular models

To study our lncRNA candidate we used several different *in vitro* cell systems. Most cardiac resident macrophages originate from embryonic tissue. Therefore, we used a new cell line of yolk sac derived macrophages (YSDMs) out of the lab of Christian Schulz. They are generated from HoxB8-SCF-progenitor YS cells differentiating into macrophages in the presence of macrophage colony stimulating factor (M-CSF). We also used bone marrow derived macrophages (BMDMs) obtained from murine bone marrow cells since they are easy to cultivate and represent one of two macrophage populations in the heart. To create a knockout cell line of our candidate lncRNA *Zeb2os,* we used a Hoxb8-Flt3l-BM-progenitor cell line.

Cultivation of mouse yolk sac cells

Mouse yolk sac (YS) suspension culture was maintained in RPMI 1640 medium (Gibco), supplemented with 10% fetal calf serum (FCS), 1% Penicillin G (100IU/ml) and Streptomycin (100IU/ml), 6% stem cell factor supernatant, 1µM estradiol (Sigma) and 30 µM ß-mercaptoethanol. For passaging the cells, the medium was removed, and cells washed once with PBS (Dulbecco's Phosphate Buffered Saline). Cells were counted with an automated cell counter (Invitrogen Countess). Cell suspension was centrifuged at 400 g for 5 minutes, 10^5-10^6 cells were seeded in 12 ml proliferation medium described above and incubated at 37 °C and 5% CO_2. According to cell density, cells were usually passaged every 2-3 days. To differentiate the yolk sac cells to YS-derived macrophages, cells were seeded 150000 per 10 cm dish in RPMI 1640 medium supplemented with 10 FCS, 1% Penicillin G - Streptomycin and 10 ng/ml recombinant macrophage colony-stimulating factor (rMCSF, PeproTech). Cells were reseeded at day four, completed differentiation and were harvested on day eight.

The differentiation time frame of YSDMs was previously analyzed and established in the lab by others, using flow cytometry and published marker genes only expressed by fully differentiated macrophages.

Isolation and cultivation of murine bone marrow macrophages (BMDMs)

The BMDMs were isolated from bone marrow of C57BL6/N wild type mice. The tissue-free femurs were stored in PBS on ice until disinfection in 80% ethanol followed by drying in a petri dish under the tissue culture hood. After cutting of both ends of the femora the bone marrow was flushed with Roswell Park Memorial Institute 1640 medium (RPMI, Life Technologies) with 1% (v/v) penicillin/streptomycin and 10% (v/v) FCS (RPMI complete) using a syringe with a 26-gauge needle (Braun). The cell solution was centrifuged (1,200 rpm, 5 min) and the cell pellet was resuspended in RPMI complete. In a 10-cm petri dish (Thermo Fisher Scientific) 2.0 to 3.0 $\times$ 10^6 cells were seeded with addition of 10% Macrophage Colony-Stimulating Factor (M-CSF) and a total volume of 10 ml.

After three days of cultivation (day 3) the plating volume was doubled by addition of fresh RPMI complete and 10% M-CSF, assuming initially added M-CSF has been used up. On day 6, half of the medium is replaced by RPMI complete and fresh 10% M-CSF, again under previous assumption. The macrophage culture was considered as fully differentiated after eight days of cultivation.

Cultivation of Hoxb8FL progenitor cell line

The Hoxb8-FL progenitor cell line is a growth-factor dependent cell line, which is an *in vitro* model to study immune-cell differentiation and inflammatory mechanisms. In the presence of estrogen and Flt3 ligand (Flt3L), the cells can be maintained as progenitor cells retaining the potential to differentiate into lymphoid and myeloid lineages. Upon addition of M-CSF (macrophage colony stimulating factor), the cells can be driven into monocyte lineage [71].

Thaw cells

To start a new batch of Hoxb8-FL progenitors, thaw an aliquot of Hoxb8-FL-progenitors in the water bath (37°C) and transfer it into a falcon. Add roughly 5-10ml of proliferation medium and centrifuge the cells (5min, 200g, 20°C) to pellet them. Remove supernatant and resuspend the cells in 6ml proliferation medium and culture them in a small suspension-flask at 37°C and 5% CO_2.

Passaging cells

Hoxb8-FL cells are cultured in suspension flask and must be passaged every 2 or 3 days. Without washing, the medium containing the cells is transferred from the flask in a 15ml or 50ml tube and centrifuged (5min, 400g, 20°C).
The supernatant is removed, and the pellet is resuspended in 5ml PBS and centrifuged again. Cells are resuspended in 5ml proliferation medium (depending on pellet-size) and counted.
For keeping cells in culture for two days, use 1 million cells. For keeping cells in culture for three days, transfer 200.000 cells. Transfer and cultivate cells in 12ml proliferation medium in T75 flasks (suspension, green cap) at 37°C, 5% CO_2.

Differentiation procedure

Before starting the differentiation, freshly add 20% M-CSF to differentiation medium.
Take off the medium containing the cells from the flask and transfer it in a 15ml or 50ml tube and centrifuge (5min, 400g, 20°C). Wash cells with 5ml PBS (Hoxb8FL proliferation medium contains estradiol), resuspend in 2-5ml PBS and count cells.
Plate 100.000 cells in 10ml differentiation medium in 10cm bacteria-culture plate (non-treated plates). To remove residual PBS, transfer a multiple amount of x µl of cell suspension required for 100.000 cells in a falcon and fill up with PBS. After centrifugation, resuspend cells in 1ml differentiation medium and take required volume for 100.000 cells. (E.g.: transfer 182µl of cell suspension containing 500.000 cells in a falcon and fill up to 2ml. After centrifugation, resuspend the pellet in 1ml medium and take 200µl containing 100.000 cells for the differentiation.)
Add fresh medium at day 3 (replate at day 6, and harvest mature cells at day 8),
OR
Day 4: Remove medium containing the cells from the plate and transfer in 15ml tube, centrifuge cells (5min, 400g, 20°C). Remove supernatant, dissolve pellet in 5ml fresh differentiation medium and plate cells in a total of 10ml differentiation medium in 10cm tissue-culture plates.
At day 6, remove medium from the plate and wash cells carefully with 5ml PBS. Scrape off cells from the plate in PBS or a respective buffer of the downstream experiment.
The cells can be considered as mature macrophages, based on FACS F4/80 staining.

3.2. Cellular and molecular biological methods

siRNA knockdown

The Lipofectamine RNAimax reagent (Thermo Fisher Scientific) was used with Opti-MEM medium (Gibco) according to the manufacturer's instructions with certain modifications. Cells were kept in 2 ml medium on 3.5 cm tissue culture plates. Mix A containing 100 µl Opti-MEM and 5.5 µl siRNA (20 µM, final concentration 50 nM) and mix B containing 100 µl Opti-MEM and 5 µl RNAimax were mixed and incubated at room temperature for 20 minutes. The mixture was added to the cells drop-by-drop. *Zeb2os* siRNA (#) and non-target control siRNA (#D-001320-01-05) are from Dharmacon.

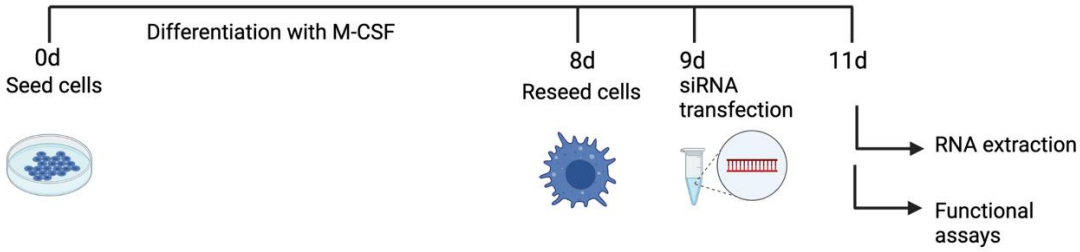


Figure 7: Timeline of siRNA transfection

RNA Extraction from cultured cells

Cells were harvested by scraping off, trypsination or centrifugation. They were subsequently washed with cold PBS and immediately lysed in peqGOLD TriFast (peqlab) reagent following the manufacturer's instruction. After incubation at room temperature for 5 to 10 min, 200 µl of chloroform per milliliter of reagent were added, the components were mixed and phases were separated by centrifugation (10-15 min, 12000*g*, 4°C). The aqueous phase containing the RNA was precipitated by the addition of isopropanol to a final concentration of 45% (v/v) over night at - 20°C. When expecting a low amount of RNA, 20 µg of glycogen (RNA grade, Fermentas) per milliliter of initial reagent were added. The RNA was recovered by centrifugation (17000*g*, 4°C, 30 min), followed by a washing step (1 ml ice-cold 80% EtOH). After another centrifugation step (17000*g*, 4°C, 15 min), the RNA pellet was air-dried for 2-

3 min and solved in ultra-pure water (5 min, 70°C, shaking at 1000 rpm). RNA was stored at -80°C.

cDNA synthesis

For the reverse transcription-polymerase chain reaction (RT-PCR) 1 µg RNA was used, whereas the concentration determination of the isolated RNA sample was analyzed with the NanoDrop ND-1000 spectrophotometer (peqlab). All steps with RNA samples were carried out on ice.

1 µg of RNA solution, 1 µl of 50 mM random hexamer primer (Thermo Fisher Scientific) and 1 µl of 10 mM dNTPs (Invitrogen) were mixed with RNase free water to a total volume of 12 µl. The RNA/primer mixture was incubated at 70 °C for 10 minutes. 8 µl of reaction mixture, containing 2 µl 5x Protoscript II Reaction buffer (NEB), 2 µl 0.1 M DTT (dithiothreitol, NEB), 1 µl murine RNase inhibitor (NEB) and 1 µl Protoscript II Reverse Transcriptase (NEB) was added to each sample.

After mixing, samples were incubated at 25°C for 10 minutes, 42 °C for 60 minutes and 60 °C for 10 minutes in a PCR machine (Eppendorf vapo.protect Mastercycler pro).

Reverse transcription and mRNA detection by quantitative real-time PCR

With the online tool Primer3Plus [72] suitable primers for qPCR analysis were designed, which resulted in a product size of 100-150 base pairs. Prior to RNA analysis, used primer pairs were tested based on a dilution series of a cDNA sample (1:1, 1:2, 1:8) by qPCR and the efficiency as well as the melting curves were determined. Good primers yielded efficiencies of 0.9-1.1 and only one distinct maximum in the melting temperature curve.

The qPCR amplifies synthesized cDNA to detectable levels and is used for analysis of long non-coding RNA. Thereby, technical duplicates or triplicates of the qPCR samples as well as biological replicates (n=2-3) of the RNA-isolates from different cell types (total myocardial cells after Langendorff perfusion [73], BMDMs day 0/day 8, resident cardiac macrophages, RAW 264.7 cell line) were conducted. DNase-RNase-free water and the control of the RT-reaction served as controls for the used primer pairs. For a single reaction, 2x Fast Start Universal SYBR Green Master (Roche), 0.4 µM forward primer, 0.4 µM reverse

primer and 10ng cDNA were filled up with DNase-RNase-free water to a total volume of 12.5 µl in a FastGene Fast 96-well PCR plate (Nippon Genetics). After sealing with an adhesive foil and centrifugation, the qPCR was undertaken within the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). Following program was chosen:

- Holding stage: 95 °C – 10 min
- Cycling stage: 40 cycles with [95 °C – 15 sec, 60 °C – 60 sec]
- Holding stage: 65 °C – 90 sec
- Melting curve: 95 °C – 15 sec, 60 °C – 60 sec, +0,3 °C steps till 95 °C, 95 °C – 15 sec

Analysis of the qPCR-data was carried out analog to the C_T-method [74].

cDNA was diluted 1:10 in water to get a concentration of approximately 5 ng/µl, assuming a 1:1 RNA to cDNA efficiency of the reverse transcription reaction.

For each well, 6.25 µl FastStart Universal SYBR Green Master (Sigma), 2.25 µl nuclease-free water and 1-1 µl of the 5 µM forward and reverse primers (sequence in Supplementary) and 2 µl diluted cDNA was mixed. For each sample, triplicate was prepared. Non-template control was run for each primer pair and primer efficiency was tested before use. The StepOnePlus Real Time PCR System and the StepOne software (Applied Biosystems) were used for q-RT PCR analyses. Used primers are listed in the table below.

Used qRT-PCR primers

Primer	Sequence (5'>3')
Rpl32 fwd	ACATCGGTTATGGGAGCAAC
Rpl32 rv	GGGATTGGTGACTCTGATGG
Zeb2os fwd	GTCCCTACACCCTGCACCTA
Zeb2os rv	CGGCTTCTTCATGCTTTTTC
Zeb2 fwd	CCAGAGGAAACAAGGATTTCAG
Zeb2 rv	AGGCCTGACATGTAGTCTTGTG
Malat1 fwd	AGGCAGAATGCCTTTGAAGA
Malat1 rv	CAGCTCAAGTCCAATGCAAA

Scratch assay

Scratch assay was performed following the manufacturer's protocol.

Chemotaxis assay

Chemotaxis assay was performed following the manufacturer's instructions with following modifications. Both sides of the membrane were coated with 50 µg/ml Matrigel® solved in cold medium (RPMI +10% FBS), adding 20 µl to the insert wells (reverse pipette) and 150 µL to the reservoir wells. The ClearView™ cell migration plate was placed at 37°C and incubated for 30 minutes. Immediately prior to macrophage addition, Matrigel® Matrix coating was aspirated from both the reservoir plate and insert. Then, 200 µl D-PBS was added to the reservoir plate and then the insert was gently put into the reservoir.

Differentiated macrophages were harvested by scraping (HT1080 with Trypsin) and a cell count was performed (e.g., trypan blue staining + hemacytometer). The cell suspension was centrifuged (350 x g, 4 minutes) and the cell pellet resuspended in respective medium with low FBS at 33,333 cells per ml (for HT1080 16,666 cells/ml). By using a manual multi-channel pipette and reverse pipetting technique, cells were seeded (60 µl per well, 2,000 cells per well) into every well of the insert plate.

Using a manual multi-channel pipette, we added 200 µl of the chemoattractant (for Macrophages we recommend C5a as a positive control) and control medium to the appropriate wells of the second reservoir plate. We then carefully transfered the insert plate containing the cells into the pre-filled second reservoir plate containing medium ± chemoattractant. Afterwards, we placed the IncuCyte™ ClearView™ cell migration plate into the IncuCyte ZOOM® instrument and allowed the plate to warm to 37°C for (at least) 15 minutes. In the IncuCyte ZOOM® software, we scheduled 72-hour repeat scanning (10x) for every 45 minutes.

Cell fractionation

To analyze the cellular localization of *Zeb2os*, macrophages were stimulated with LPS or not stimulated as a control according as described above. Cellular fractionation into nuclear and cytoplasmatic fraction of BMDMs was performed using a published protocol [75]. Cells were washed twice with cold PBS and harvested in PBS by scraping. One third of the cells was centrifuged separately at 200×*g* for 10 min for overall expression samples. The remaining cells were centrifuged at 200 g for 10 min. Pellets were resuspended in 220 µl lysis buffer A (10 mM Tris pH=8, 140 mM NaCl, 1.5 mM MgCl2, 0.5% Nonidet P-40) and incubated on ice for 5 min. Then the lysate was centrifuged at 1000×*g* for 3 min, 4°C. The supernatant containing cytoplasmic RNA was mixed with 1 ml peqGOLD (peqlab). Pellets containing the nuclear fraction were washed twice more with buffer A and once with buffer B (buffer A supplemented with 1% Tween-40, 0.5% deoxycholic acid). Finally, pellet was resuspended in peqGOLD (peqlab). RNA extraction, cDNA synthesis and q-RT PCR analysis were performed according to the description above. *RPL32* known to have almost fully cytoplasmatic localization and *Malat1* known to have almost fully nuclear localization were used to normalize the results.

Generation and testing of single cell clones from CRISPR-Cas mutated cells (Seren Baygun, Schmidt-Supprian lab)

Firstly, we made a serial dilution down to 0.5 cells/100µl (equals 60 cells in 12ml, which is enough for 1x 96well plate). Then, we used a U-bottom plate and plated 2x 96well plates per deletion variant. After seeding the cells, we checked with the help of a microscope how many cells were seeded per well. We added or changed the medium after approximately 10 days. We then let the cells grow for 2-3 weeks for achieving enough cells to test. We used 1/3 to ½ of one well to perform a cell lysis and PCR. We performed a 1st PCR with primers testing for the mutant allele and then checked with the positive ones if they were hetero- or homozygous. To sequence the clones, we performed a PCR with Phusion and sequenced them using one PCR primer.

Extraction genomic DNA with QuickExtract™ DNA Extraction Solution (Lucigen)

We used 100,000 cells in a 50 µl quick extract. We then pelleted the cells by centrifuging them for 5 to 10 minutes at 200g. Then we took of the supernatant. Afterwards, we washed with PBS and then centrifuged again in a PCR tube centrifuge. We then added quick extract (10-30ul depending on the pellet size) and ran our PCR protocol. After, we measured the DNA concentration on nanodrop. Each sample was then diluted to 50ng/ul concentration, or we directly used the volume required for ~50ng.

Primers for *Zeb2os* knockout

Primer	**Sequence**
Zeb2os sg1 fwd (SB58)	GATCGGCTGCTTCATTGATAAG
Zeb2os sg6 rev (SB61)	TTCTTCTCACCATTTCTGGCC
Zeb2os sg3 fwd (SB62)	AAGTCTGGAAGGTGTGGTG
Zeb2os sg4 rev (SB60)	GTACCTGTCCATTCAGGTAACC

3.3. Next generation sequencing

RNA cloning and sequencing

RNA cloning and sequencing was performed with the Illumina TruSeq RNA Library Prep Kit according to the manufactures protocol except SuperScript III was used and protocol was adapted to 50°C reaction temperature.

Databases, software, and statistics

DNA sequence data was downloaded from the UCSC Genome Browser and from Ensembl. Graphpad Prism 9 was used for data analysis and figure generation. MacVector12, Primer3Plus and Tm Calculator (Thermo Fisher Scientific) were used to design constructs and optimize PCR conditions. IncuCyte® ZOOM software was used for automated image analysis of Scratch and Chemotaxis assays.

To analyze gene ontologies terms and pathway networks we used DAVID bioinformatics resources [76] and Cytoscape with the plug-in tool ClueGO [77,78].

Zeb2os and Zeb2 gene expression microarray data was obtained from GEO (Gene Expression Omnibus). The profile IDs of the used data sets were the following:

90385895, 120873595, 92766795, 79456095, 110104895, 130507795, 66791195, 125380895, 110733495, 63973595, 118938195, 110733495, 67586695, 115857095, 122038195, 126823995, 124752895, 115526995, 109163695, 84873795, 107183795, 104694795, 104694795, 125344195, 87191595, 117264495, 115773895, 92730095, 118591795, 124202795, 87705695, 92126830, 97814595, 103913995, 117966795, 106432595, 126591395, 113881812, 103095895, 132023595, 102626895, 92126829, 95914595, 116055895, 118288195, 111873495, 118901495, 109269995, 103132595, 86972195, 123372295, 94192695, 114081795, 116143295, 129614795, 83607495, 98486895, 131903995, 105246595, 84636095, 87496495, 67503195, 122337395, 84719295, 129416195, 108094995, 102890495, 132403295, 129744495, 88084495, 124004495, 110532895,

105060395, 92336595, 89908495, 117301195, 111836795, 73138995, 110779295, 102543412, 113209112, 84599395, 108855495, 109306695, 11106319

Galaxy [79] was used to perform biomedical analyses of the RNA Sequencing data. Galaxy (https://usegalaxy.eu) is a web-based platform for biomedical analyses.

Images were created with the help of BioRender.com.

4. Results

The aim of this project was to detect and characterize a long non-coding RNA in cardiac resident macrophages and to further analyze its role in cardiac health and disease. For this purpose, we used new sequencing techniques such as single cell sequencing (scRNA-seq) to detect specific and regulated lncRNAs. In recent years, lncRNAs have been receiving more and more attention due to their newly discovered important regulatory functions and their cell-type specificity which could be of great use when developing new therapeutic targets. As I will show with the results of this thesis, the lncRNA *Zeb2os* is promising target that regulates macrophage function.

4.1. Selection of disease-related lncRNA in cardiac macrophages

As a first step, I picked a suitable and promising long non-coding RNA candidate for further studies. RNA sequencing (RNA-seq) and scRNA-seq (Figure 8) on cells of murine hearts in health and cardiac disease were previously performed in the lab and provided the basis for these studies. Transverse aortic constriction (TAC) was used as a model for cardiac disease. In this model, cardiac hypertrophy is induced by aortic banding leading to a chronic hemodynamic overload on the heart and eventually to cardiac dilatation and heart failure [80]. RNA-seq was performed on cardiac macrophages that were isolated using Langendorff perfusion in resting state and 6, 14 and 28 days after treatment with TAC. ScRNA-seq after 6days of TAC (representing the time point of maximal inflammation and macrophage activity) complemented these data sets that allowed for the detection of thousands of long non-coding RNAs in cardiac macrophages. Further analysis of these data sets revealed many lncRNAs that were highly abundant in cardiac resident macrophages and regulated during different time points of disease. As our aim was to find a lncRNA that is present in cardiac resident macrophages and plays a role in the course of cardiac disease, these were the genes we took into consideration for our studies.

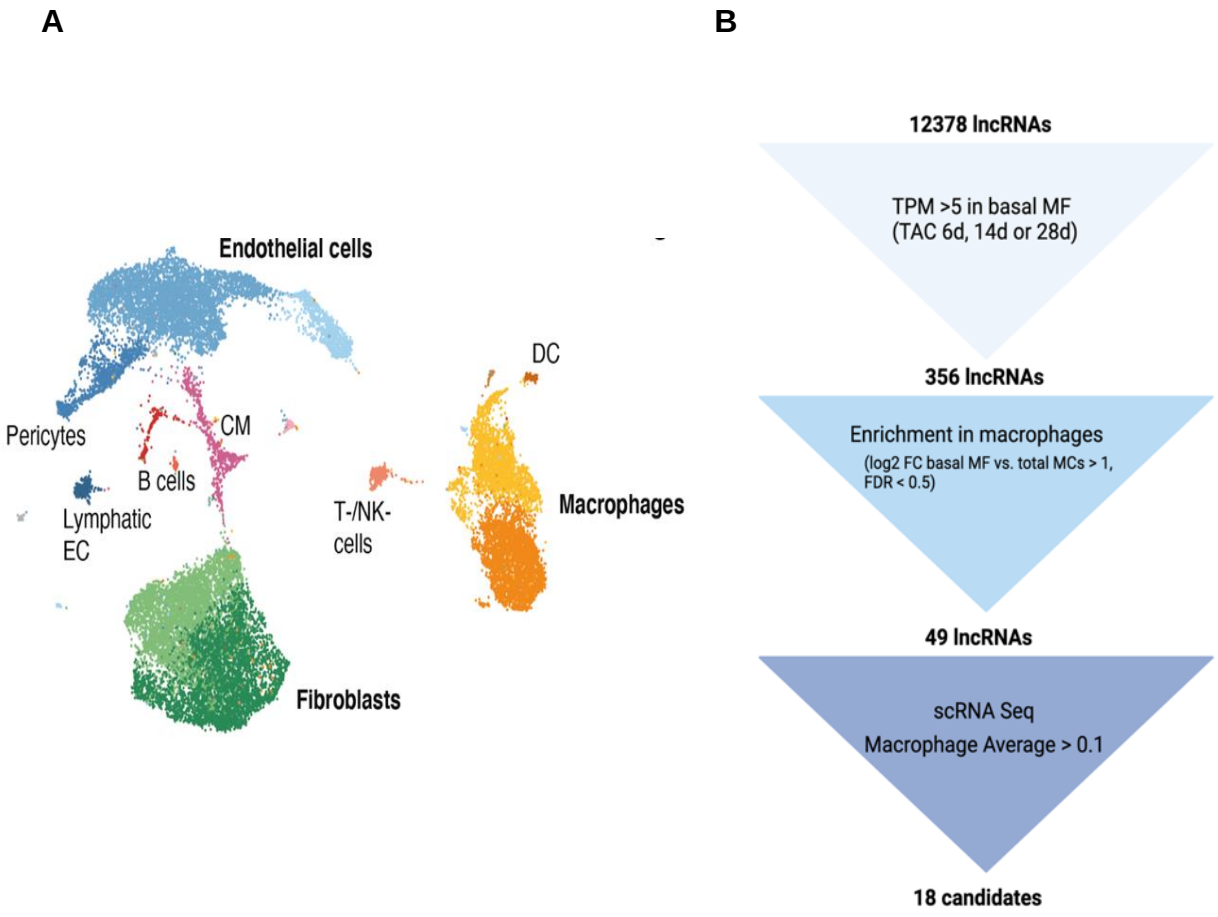


Figure 8: Single cell sequencing of total myocardial cells

(A) Single cell sequencing of total myocardial cells. Abbreviations: EC=endothelial cell, CM=cardiomyocytes, DC=dendritic cells, NK-cell=natural killer cell

(**B)** Selection process of candidate lncRNA: The lncRNAs detected in RNA Sequencing data of total myocardial cells and cardiac macrophages was analyzed and filtered based on regulation during cardiac disease and enrichment in cardiac macrophages. Remaining lncRNAs were further narrowed down based on regulation during cardiac disease and enrichment in cardiac macrophages. Remaining lncRNAs were further narrowed down based on detection in single cell Sequencing data. Abbreviations: TPM=transcripts per million, MC=monocytes, basal MF=basal macrophages

By choosing cut-off values (Figure 8) to guarantee high abundance of the respective lncRNAs in cardiac resident macrophages and regulation during cardiac disease, we narrowed down our list of lncRNA candidates. To start with, we only picked lncRNAs that were abundant and highly regulated after intervention with TAC (transcripts per million (TPM) >5) to ensure an involvement in cardiac disease. As a next step, we chose to include only lncRNAs that were enriched in cardiac resident macrophages when compared

to total myocardial cells (log2 Fold change (FC) basal MF vs. total myocardial cells > 1, false discovery rate (FDR) < 0.05). Also, we wanted to make sure that our lncRNA would be detectable in the scRNA-seq data. This marked another step to ensure high expression. Here, one could also sort for lncRNAs that marked specifically in the macrophage cluster demonstrating macrophage enrichment. After applying all these criteria, we could find 18 remaining lncRNAs that were enriched in cardiac resident macrophages and regulated during cardiac disease. To further investigate the remaining candidates, we looked at genome data (ensembl.org) and already existing literature. A factor of great significance when looking at the genomic locus of the lncRNAs was their conservation in the human genome since this represents a crucial condition for future therapeutic approaches. Based on the criteria outlined in the paragraph above, we chose the lncRNA *Zeb2os* out of 18 remaining candidates to further investigate. Additionally, it is conserved in the human genome. Taken together, these characteristics could hint towards an important role of *Zeb2os* in macrophages of the heart and make it an interesting target to investigate in more detail. As outlined in the introduction, *Zeb2os* is an antisense lncRNA to Zeb2. Zeb2 is mainly known and described in previous studies as a transcriptional inhibitor of E-cadherin and its role in epithelial-mesenchymal transition [67]. More recent studies detected that Zeb2 is a conserved feature among macrophages and that loss of Zeb2 resulted in a loss of resident macrophages from their respective tissues [65]. Little is known of possible modes of interaction between Zeb2 and *Zeb2os* and resulting effects.

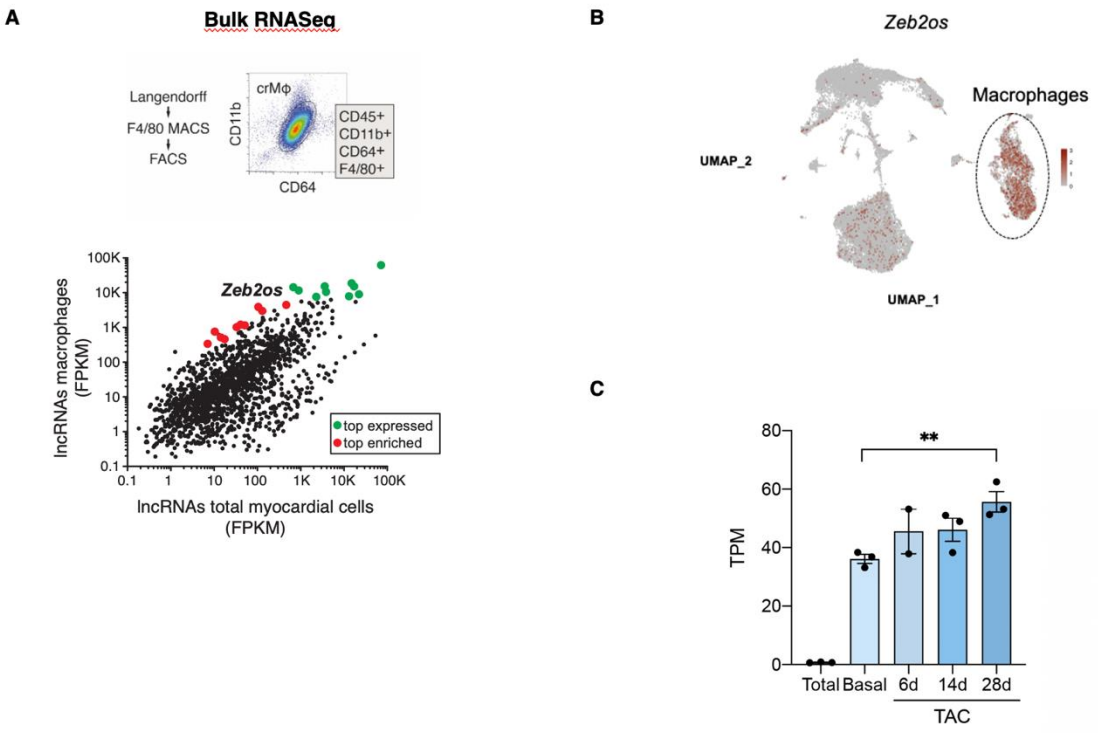


Figure 9: Selection process of *Zeb2os* as candidate lncRNA

(A) Analysis of RNA Sequencing data of total myocardial cells and cardiac macrophages: Highlighted in green are lncRNAs that are top expressed in total myocardial cells. Highlighted red are lncRNAs that are highly enriched in cardiac resident macrophages. *Zeb2os* is among the top expressed and enriched lncRNA candidates.

(**B)** Analysis and clustering of single cell data of myocardial cells showed for once detectability of *Zeb2os* and secondly a strong enrichment of this lncRNA in the macrophage cluster.

(C) Expression levels of *Zeb2os* (in TPM) measured via RNA-seq in different conditions show a significant enrichment of *Zeb2os* in macrophages (basal) compared to total myocardial cells (total) and a constant increase of its expression during cardiac disease (TAC on day 6, 14 and 28)

The Gene Expression Omnibus (GEO) is a public platform that combines, and archives high-throughput gene expression data submitted by the scientific community. These data include gene expression measurements from different organisms in different biological processes (www.ncbi.nlm.nih.gov/geo). Next, I analyzed if the two neighbored genes Zeb2 and *Zeb2os* are co-expressed in a correlated or anti-correlated way giving a hint of a synergistic or antagonistic function by extracting and plotting their gene expression profiles. The analysis of their gene expression levels in various organs like e.g., the lung, the colon and the liver investigating multiple pathways like response to infection or influence on different cells showed a positive

correlation of expression ($R^2 = 0.7055$) suggesting possible synergistic effects of these two genes.

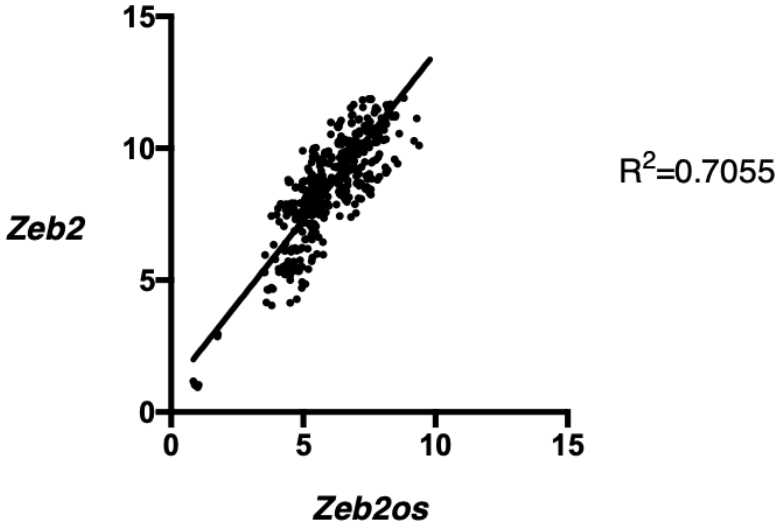


Figure 10: Analysis of GEO profiles of Zeb2 and *Zeb2os*

Gene expression levels of Zeb2 and *Zeb2os* in different conditions showed a positive correlation of both genes suggesting potential synergistic effects.

4.2. Functional characterization of *Zeb2os in vitro*

4.2.1. Knockdown with siRNA to perform loss-of function assays

To further investigate the functions of *Zeb2os* in macrophages, we started by performing a knockdown with small interfering RNAs (siRNAs) in bone-marrow-derived macrophages (BMDMs) to be able to perform loss-of-function assays. SiRNAs are designed to bind specifically and with full complementarity to their target sequence and decrease its expression by guiding the endonuclease Ago2 to its target leading to its degradation [81]. Successful knockdown of *Zeb2os* was a first crucial requirement for further experiments.

Cells treated with siRNA targeting the sequence of *Zeb2os* showed significantly reduced expression levels of *Zeb2os* in a qPCR analysis compared to non-treated (mock) cells and cells treated with a control siRNA (Figure 7). The relative RNA expression level was normalized to *rpl32*.

A **B**

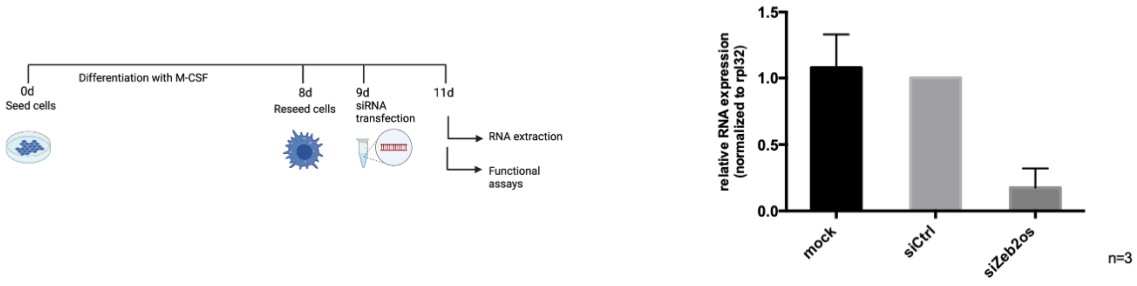


Figure 11: Expression levels of *Zeb2os* in BMDMs after siRNA-mediated knockdown

(A) Experimental set up

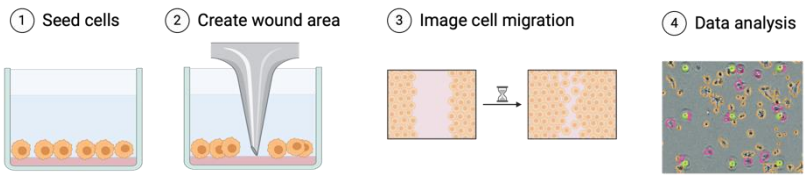
(B) qPCR analysis of expression levels of *Zeb2os* in BMDMs. Cells treated with a siRNA targeting *Zeb2os* showed reduced expression levels of *Zeb2os* compared to a siCtrl and mock cells. BMDMs were transfected with 50 nM siRNA for 72h

4.2.2. Role of *Zeb2os* in migration capabilities of macrophages

As previously stated, Zeb2 has been found to play a role in the maintenance of tissue specific identities of macrophages. Knockout of Zeb2 resulted in a disappearance of tissue resident macrophages from their respective tissue and a replenishment with unspecific bone-marrow precursors [65]. A mechanism for this finding and its significance in the context of disease is yet to be explored. Conceivable would be a process such as apoptosis or a process of resident macrophages leaving their respective tissues by losing their tissue specific identities. One important characteristic of macrophages is their ability to migrate. Also, they are essential in processes of wound healing. To see whether migration of macrophages is affected by a knockdown of Zeb2 or *Zeb2os* knockdown, we performed a Scratch assay to simulate wound healing and migration. Cells were seeded in a well, a scratch in the middle of the well was introduced and cell density dependent migration was measured over a period of 24 hours using an Incucyte System (Sartorius).

Both, cells with a knockdown of *Zeb2os* and cells with a knockdown of Zeb2, showed a reduced capability to migrate. A paired t-test confirmed the significancy of results for knockdowns of Zeb2 and knockdowns of *Zeb2os* (p value < 0.0001)

A



B

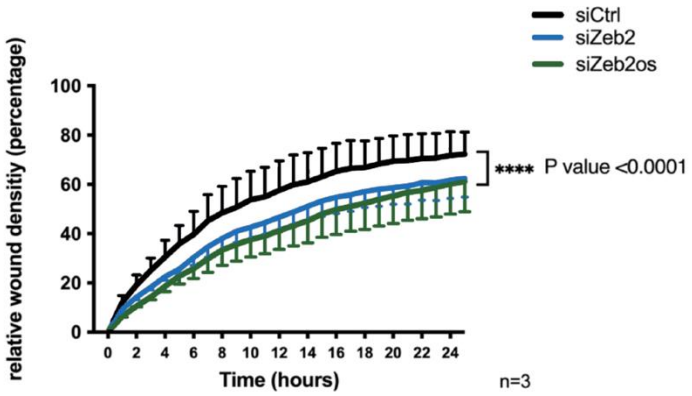


Figure 12: Macrophages depleted in Zeb2os show reduced capacity to migrate

(A) Experimental set up

(B) BMDMs treated with si*Zeb2os*, siZeb2 and a control for 48 h were seeded in different wells. An artificial wound was added into each well with a specific wound maker tool. Relative wound density was measured over a period of 24 hours. BMDMs treated with si*Zeb2os* and siZeb2 show a tendency towards reduced wound density compared to a control group. This might suggest a reduced capacity of these cells to migrate.

4.2.3. Influence of *Zeb2os* on susceptibility of macrophages to chemoattractant

Macrophages need to be motile and move to sites of injury or inflammation where they perform tasks such as initiation and regulation of adaptive immune responses or phagocytosis. Often, they are attracted to these sites by a chemoattractant. Therefore, we wanted to include a Chemotaxis assay in our studies to investigate the moving capabilities of cardiac resident macrophages under different conditions. Non-treated macrophages and macrophages treated with siRNAs (siZeb2, si*Zeb2os*, control) were seeded on a porous membrane and chemotaxis was triggered using complement component 5a (C5a). C5a is an activated part of the complement system which participates in the innate immune response and acts as a chemotactic agent for macrophages [82]. Chemotaxis was measured by analyzing the cell count moving from the top side of the membrane to the bottom side – towards a higher concentration of chemoattractant.

Shown in **Fehler! Verweisquelle konnte nicht gefunden werden.**, the cells with a k nockdown of Zeb2 and *Zeb2os* showed an impaired ability to move towards the chemoattractant.

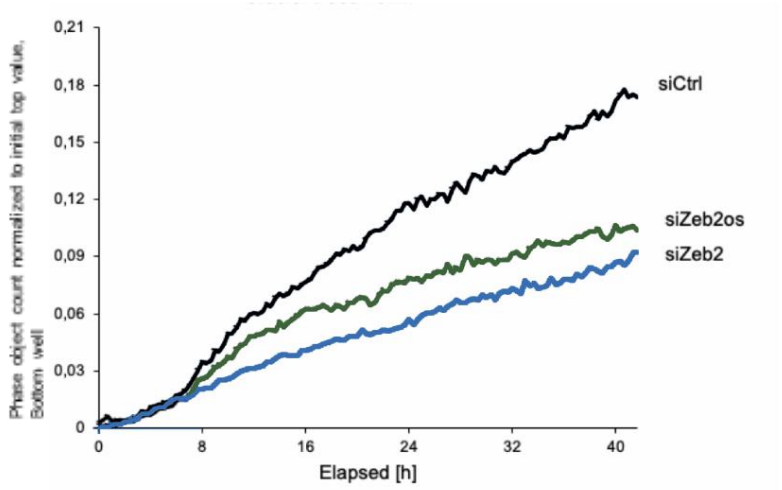


Figure 13: Macrophages with a knockdown of *Zeb2os* are less responsive to chemoattractant

BMDMs with a knockdown of *Zeb2os* as well as Zeb2 showed less migration towards the chemoattractant C5a compared to a control group. Cells migrated towards a gradient of C5a of 40 nM.

4.2.4. Discovering the cellular location of *Zeb2os* by using cell fractionation

As mentioned above, there is little known about possible mechanism of interaction between Zeb2 and *Zeb2os.* Yet, there is one hypothesis already outlined in the introduction. In these studies, the authors suggest that the presence of *Zeb2os* prevents the splicing of Zeb2 pre-mRNA. This leads to the preservation of an internal ribosomal entry side of Zeb2 which then induces an increased expression of Zeb2. This mechanism has been shown for mesenchymal cells, but has yet to be confirmed to happen in other cell types [69].

To see if Zeb2 and *Zeb2os* coexist in the same cell department in macrophages – building the foundation for this mechanism of interaction to work, we performed a cell fractionation and analyzed it using qPCR (**Fehler! Verweisquelle konnte nicht g efunden werden.**). As a mRNA we would expect Zeb2 to mainly be present in the cytoplasm. *Zeb2os* as a lncRNA would be expected to mainly reside where it executes its function. Furthermore, we investigated if we could detect any changes concerning the localization of Zeb2 and *Zeb2os* by stimulation with Lipopolysaccharide (LPS), a pro-inflammatory stimulus. LPS is part of the membrane of Gram-negative bacteria and a potent activator of monocytes and macrophages. Measurement of the mRNA *Rpl32* and the lncRNA *Malat1* showed that the fractionation assay had been performed successfully as Malat1 is known to be primarily present in the nucleus (Figure 14A).

Our results show that both, Zeb2 and *Zeb2os,* are primarily located in the cytoplasm, but are also present in the nucleus where beforementioned mechanism takes place. The stimulation with LPS induced no significant change of location for neither one of them.

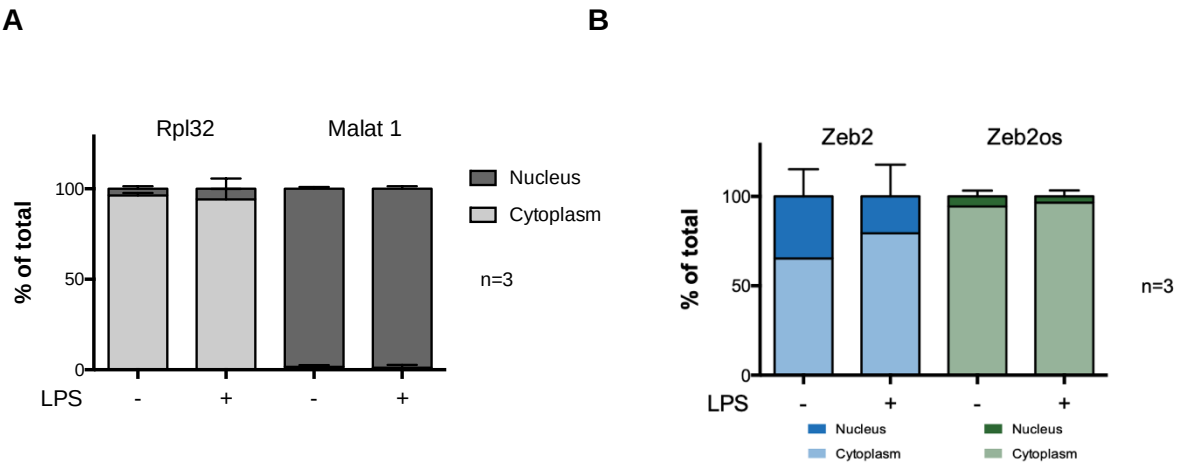


Figure 14: *Zeb2os* lncRNA localizes mainly to the cytoplasm

(A) Controls for cellular localization. *Rpl32* is a mRNA considered to be almost entirely located in the cytoplasm. *Malat1* is a lncRNA considered to be almost entirely present in the nucleus. Expression levels are shown with and without stimulation with LPS.

(**B)** Total expression levels of Zeb2 and *Zeb2os.* There is no major change of location upon stimulation with LPS.

4.3. Generation of a *Zeb2os* knockout in murine Hoxb8 macrophages using CRISPR/Cas

The siRNA knockdown performed with siZeb2os in BMDMs worked well, but didn't last for a longer period of time, so as a next step was to further elucidate the functions of the lncRNA *Zeb2os,* we performed a knockout of *Zeb2os* in a macrophage progenitor cell line using CRISPR/Cas [71]. To decide on the right targeting strategy, it was important to carefully examine the genomic locus of *Zeb2os.* As already mentioned, *Zeb2os* is an antisense lncRNA to Zeb2. As one can see in Figure 15, Zeb2 and *Zeb2os* share some common DNA stretches which could lead to an effect on Zeb2 when targeting *Zeb2os.* Therefore, we decided not to target the whole sequence of *Zeb2os,* but rather target Exon 1 of *Zeb2os* as it is the only exon that solely overlaps with only intronic regions of Zeb2.

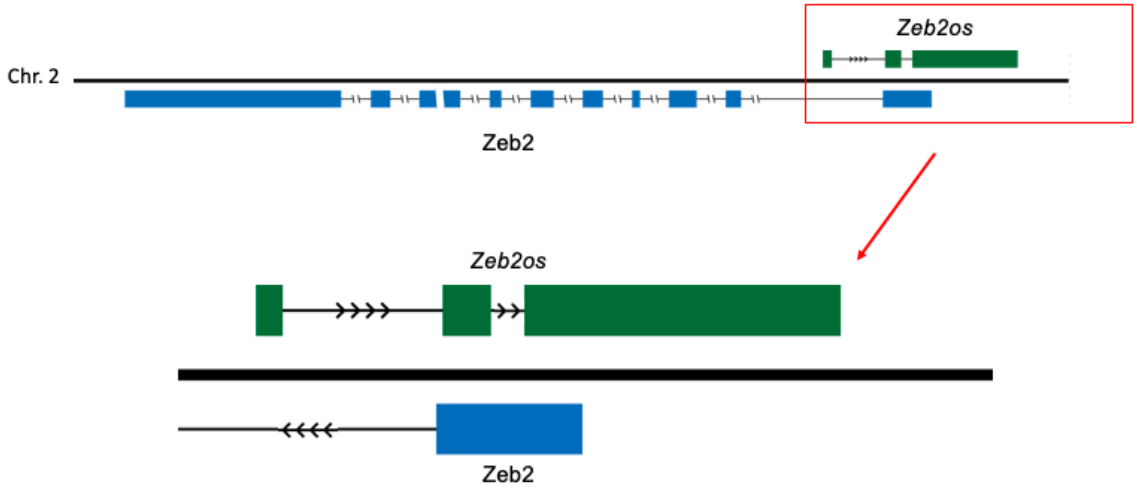


Figure 15: Genomic locus of Zeb2 and *Zeb2os*

The genomic locus shows that lncRNA *Zeb2os* is located antisense to Zeb2. Only exon 1 of *Zeb2os* shows no overlapping with exons of Zeb2.

To make sure effective gene silencing of *Zeb2os* is still achieved, the promoter region of *Zeb2os* was included in the targeted region for the deletion approach.
Pairs of sgRNAs were designed to target exon 1 and intronic promoter regions (Figure 16A) and then electroporated into Hoxb8-FL macrophages. This procedure was performed by our cooperation partner Seren Baygün from the Marc Schmidt-Supprian lab, bulk cells were transported to our lab, and I performed subsequent steps to generate cell lines from single clones.

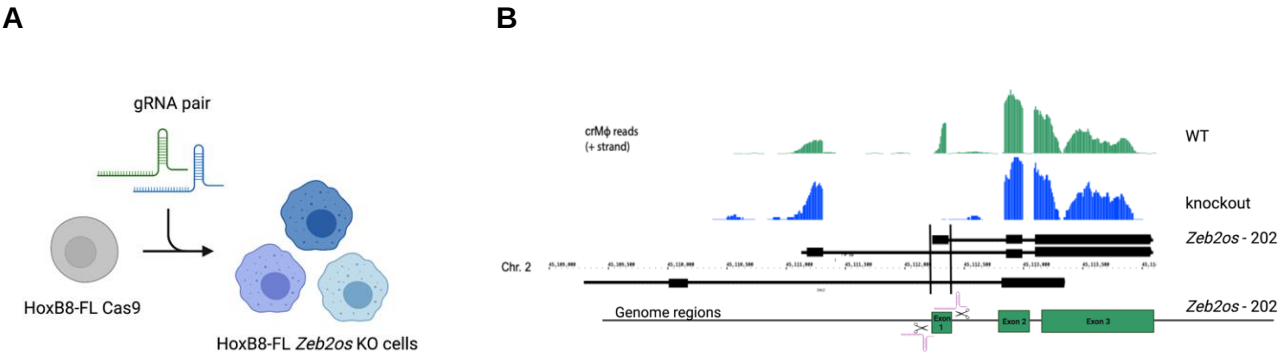


Figure 16: Targeting of *Zeb2os* and its genomic locus

(A) Targeting of *Zeb2os* via gRNA pair and electroporation

(B) Visualization of the genomic locus and targeting strategy of *Zeb2os* before and after knockout of *Zeb2os*.

After delivery of sgRNA via electroporation into Cas9-expressing cells, single cell clones from the CRISPR-Cas9 mutated cells were picked and propagated until around 50,000 cells per clone had grown. Genomic DNA was extracted from an aliquot of each picked clone and subjected to PCR to investigate which clones had the correct band size corresponding to the desired deletion. This approach yielded 25 clones that were successfully propagated, with 11 homozygous, 5 heterozygous and 9 wildtype clones. Sanger sequencing was used to confirm the correct excision of the Zeb2os exon (for homozygous clones) and no change of sequence for the wildtype control cell lines. Finally, two knockout clones and one wildtype control were further used for downstream analyses (Figure 17) all other successfully created clones were stored as backup in liquid nitrogen.

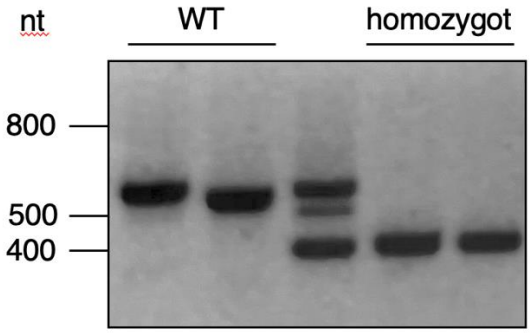


Figure 17: PCR of single cell clones

A PCR was performed on the genomic DNA extracted from CRISPR-Cas mutated cells to confirm band size.

4.4. Transcriptomic changes in macrophages caused by *Zeb2os* knockout

The knockout cell lines of *Zeb2os* allowed us to investigate the long-lasting perturbations of a *Zeb2os* loss. First, we performed deep RNA-seq on differentiated macrophages with and without *Zeb2os* and analyzed transcriptomic changes in the knockout cells compared to wild type cells. Total RNA of *Zeb2os* knockout Hoxb8 macrophages and Cas9 single cell clones as a wildtype control were isolated and used to prepare RNA-seq libraries (two different KO clones with 3 biological replicates). After sequencing, RNA-seq reads were mapped to the mouse reference genome (mm10) and abundances of annotated genes were determined. Principial component analysis of RNA-seq samples showed that *Zeb2os* KO clones and wildtype clones clustered according to sample type (Figure 18), also the samples that were stimulated with Il-4 demonstrated separative clustering. IL-4 is acting as an anti-inflammatory cytokine. Numerous studies have shown that Il-4 is involved in the regulation of immune and inflammatory processes. In the context of macrophages, it is known to stimulate the activation of the M2 phenotype which is among other things involved in tissue remodeling and immunoregulatory functions [83-85]. This shows for one thing that stimulation with Il-4 results in a different phenotype but also indicates that the stimulation with Il-4 did not interfere with the knockout of *Zeb2os* since WT and *Zeb2os* clones still cluster separately.

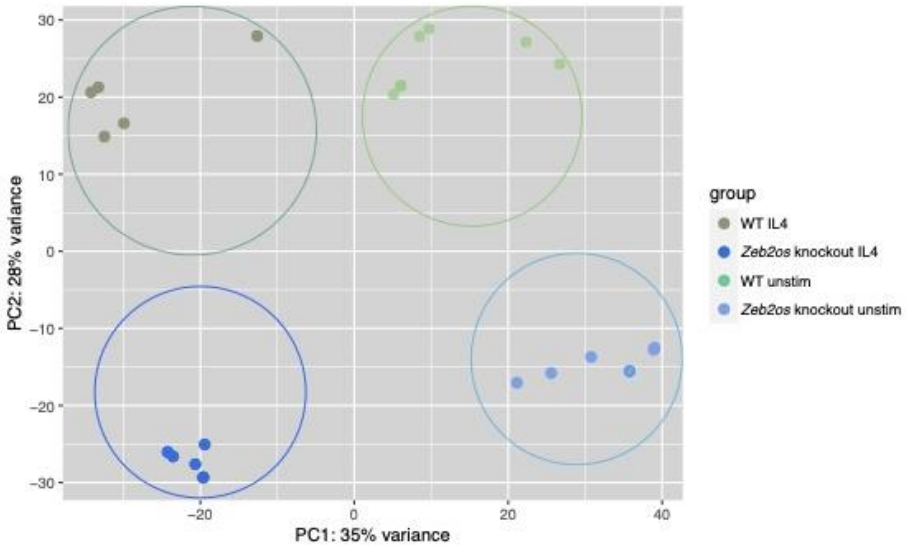


Figure 18: Principal component analysis of RNA-seq samples

Two different knockout clones with 3 biological replicates and two different WT clones with 2-3 biological replicates were sequenced via RNA-seq and clustered via principal component analysis. Abbreviations: WT=wild type, IL4=stimulated with IL4 (10ng/ml for 24h), unstim=unstimulated.

Our results indicate that *Zeb2os* knockout cells exhibited a significant decrease in *Zeb2os* expression (17% reduction) without affecting Zeb2 mRNA abundance, as shown in Figure 19. Nevertheless, residual expression of *Zeb2os* was expected as we were only able to target exon 1 of the gene. Taken together, I successfully generated knockout cells for *Zeb2os,* which resulted in significantly reduced expression levels without affecting the expression of the opposing strand's gene, Zeb2.

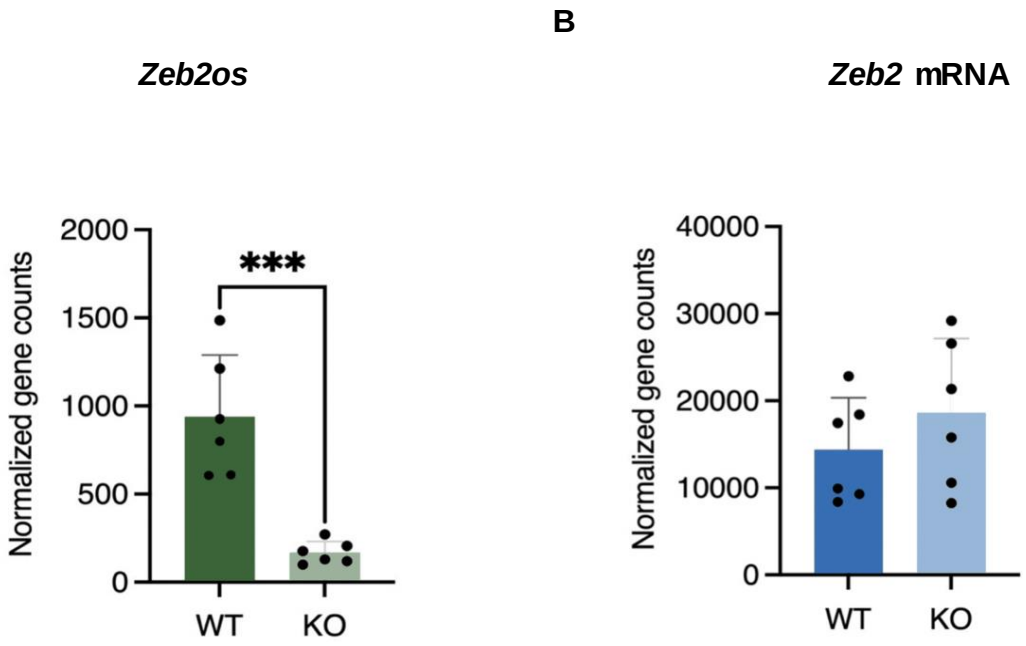


Figure 19: RNA-seq shows that the knockout of *Zeb2os* works successfully and does not affect the expression of Zeb2

(A) Normalized gene counts for *Zeb2os* in WT compared to KO cells show a significant decrease of expression for *Zeb2os* in knockout cells. *** p-value < 0.001

(B) Normalized gene counts for Zeb2 in WT and KO cells. There was no significant decrease in the expression of Zeb2 in the KO cells.

To determine which genes are differentially expressed upon *Zeb2os* depletion, we used the tool DESeq2 [86] on the Galaxy platform (Ref Galaxy) to calculate differential gene expression. Next, we filtered the resulting list of genes to obtain the strongly impacted genes (log2FC > |2|, p-value < 0.05) and subjected this list to a gene ontology analysis. A gene ontology analysis (GO) assigns genes to categories of biological processes and reports potential enrichment of terms to uncover possible biological functions. Cytoscape is an open source software that helps to perform and visualize analyses of interaction networks with high-throughput expression data [77]. Deregulated genes with previously defined cut-offs were entered in Cytoscape's plug-in tool ClueGO, which visualizes these biological processes in clusters of functionally grouped networks by integrating Gene ontology terms and KEGG/BioCarta pathways [78]. We chose to look at the GO terms of biological processes and chose an additional cut-off to specify shown pathways by setting the p-value to < 0,01.

Figure 20 visualizes the networks of affected biological processes when looking at downregulated genes (A) and upregulated genes (B) in *Zeb2os* KO cells when

compared to wildtype cells. 405 genes were downregulated with a log2FC less than or equal to -2 in the *Zeb2os* KO cells when compared to wildtype cells. 375 genes were upregulated in the KO cells with a log2FC bigger than or equal to 2.

A Upregulated **B Downregulated**

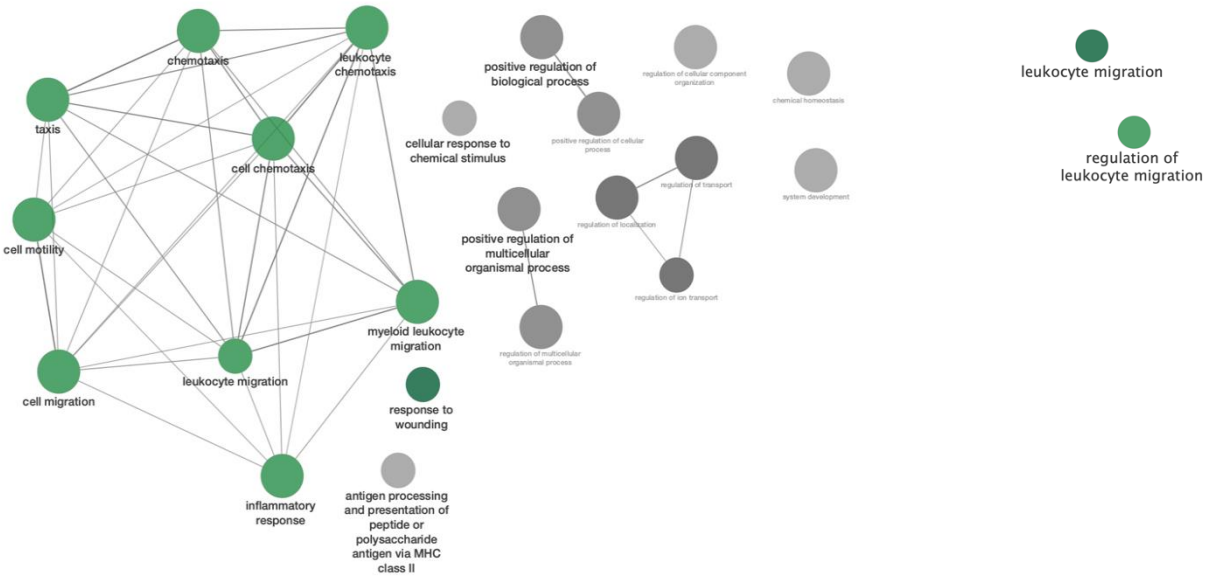


Figure 20. Gene ontology analysis of deregulated genes with Cytoscape

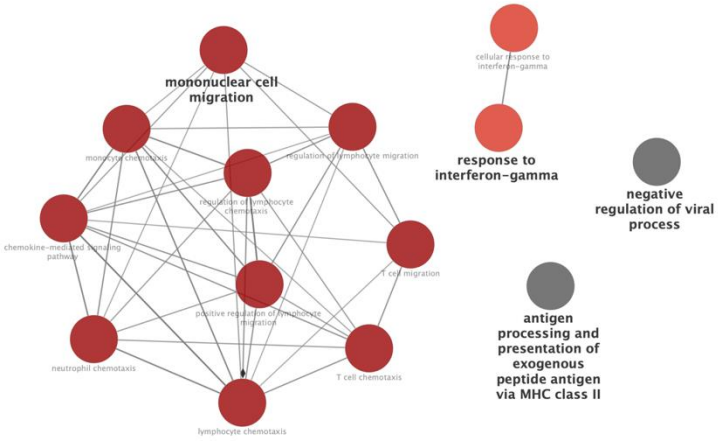
(A) GO terms of genes deregulated with a log2FC<-2 and a p-value <0.01 (additional cut-off to specify shown pathways) in RNA-seq data comparing *Zeb2os* KO cells with wildtype cells

(B) GO terms of genes deregulated with a log2FC>2 and a p-value <0.01 (additional cut-off to specify shown pathways) in RNA-seq data comparing *Zeb2os* KO cells with wildtype cells

To complete our studies, we also did RNA-seq on *Zeb2os* KO cells and wildtype cells after treating them with interleukin-4 (IL-4). Since this is a phenotype which is promoted in the course of cardiac disease, we also wanted to investigate the influence of a *Zeb2os* knockout in this condition. Again, we looked at differentially expressed genes when comparing KO and wildtype cells. Because analysis of downregulated genes with ClueGO showed an overwhelming number of regulated pathways, we applied even stricter cut-off values with a log2FC less than or equal to -4. All other restrictions stayed identical. For upregulated genes, we applied the same cut-off values used for the analysis of unstimulated cells. Figure 21 illustrates pathways of deregulated genes after stimulation of *Zeb2os* KO cells stimulated with IL-4. Consistent with prior findings,

Zeb2os significantly affects the processes of migration and chemotaxis, as well as antigen processing and presentation and the regulation of the immune response.

A Upregulated pathways



B Downregulated pathways

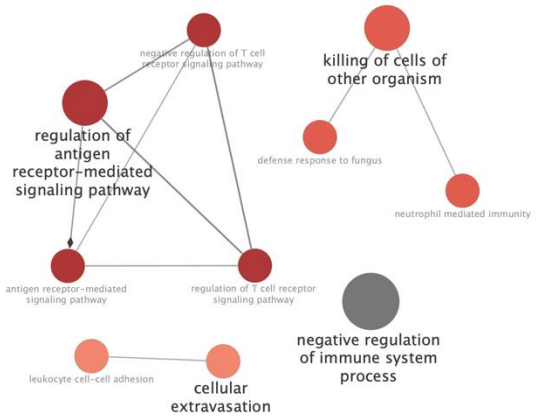


Figure 21: Gene ontology analysis of deregulated genes with Cytoscape after stimulation of *Zeb2os* KO cells with wildtype cells with IL-4

(A) GO terms of genes deregulated with a log2FC<-4 and a p-value <0.01 (additional cut-off to specify shown pathways) in RNA-seq data comparing *Zeb2os* KO cells with wildtype cells. Cells were treated with Interleukin-4 prior to RNA-seq.

(B) GO terms of genes deregulated with a log2FC>2 and a p-value <0.01(additional cut-off to specify shown pathways) in RNA-seq data comparing *Zeb2os* KO cells with wildtype cells. Cells were treated with Interleukin-4 prior to RNA-seq.

Using RNA-seq, we were able to demonstrate the successful knockout of *Zeb2os.* Subsequently, the *Zeb2os* knockout cell line allowed us to study the persistent defects resulting from *Zeb2os* deficiency. Using DESeq2 on the Galaxy platform, we were able to determine which genes are differentially expressed upon Zeb2os depletion. With further analysis using gene ontology analysis and Cytoscape, we were able to illustrate and support our previous results showing that Zeb2os is involved in processes of migration and chemotaxis. This strongly supports our hypothesis that Zeb2os plays a crucial role in regulating inflammatory processes in cardiac disease.

5. Discussion

Despite significant progress in cardiovascular research, cardiovascular diseases continue to be the primary cause of death worldwide [87]. Although the development and course of cardiac diseases have been the subject of investigation for decades, numerous processes are still unclear. Among the many cell types in the heart, cardiac resident macrophages were only recently identified as the most abundant immune cell indicating their crucial role in cardiac homeostasis and disease [88]. Long non-coding RNAs represent a significant and cutting-edge field of biomedical research. They serve as vital players in numerous biological processes, occupying multiple functions in essential cellular pathways, and are characterized by their cell-type specificity. Nonetheless, the majority of lncRNAs have not been thoroughly characterized due to the versatility and intricacy of their mechanisms-of-action.

The objective of this project was to identify and describe a lncRNA that is highly expressed in cardiac resident macrophages and regulated during cardiac disease. This was done to enhance our understanding of the pathophysiology of cardiac disease and to identify a novel target for future therapeutic approaches.

As previously demonstrated, the utilization of RNASeq and scSeq facilitated the identification of *Zeb2os* as a long non-coding RNA highly expressed in cardiac resident macrophages linked to the development and course of cardiac disease. *Zeb2os* is an antisense lncRNA that corresponds to the protein-coding gene Zeb2. Only a limited number of studies have been carried out on the potential functions of *Zeb2os* and its impact on Zeb2. To obtain a better understanding of the putative interplay between *Zeb2os* and Zeb2, we performed a cell fractionation experiment to identify the lncRNA's primary cellular localization. If Zeb2 mRNA and Zeb2os are localized in the same cell department, it may suggest a direct interaction, whereas localization in different cell departments may indicate an interaction at an epigenetic level with other interaction partners, or no interaction at all. Our findings indicate that *Zeb2* mRNA and *Zeb2os* primarily reside in the same cell department: the cytoplasm. The stimulation of macrophages by the inflammation-promoting stimulus LPS before cell fractionation did not result in significant changes of localization. These results neither prove nor contradict previous studies suggesting a mechanism by which *Zeb2os* inhibits splicing of Zeb2 mRNA's internal ribosomal entry site (Beltran et al., 2008), which occurs in the nucleus. Beltran et al. and Romero-Barrios have reported that *Zeb2os* affects the

abundance of Zeb2 mRNA by controlling its splicing mechanism through an internal ribosomal entry site. This mechanism has been proposed for mesenchymal cells but has not been confirmed in macrophages. However, these results are not direct evidence for this mechanism, and it will need to be validated in the future to be true for macrophages. Nevertheless, there may be a completely different, independent mechanism of action for *Zeb2os*. This needs to be investigated in further experiments. To gain a more complete understanding of *Zeb2os*, we generated a *Zeb2os* knockout cell line for our research. We decided to use CRISPR/Cas9 for this purpose. However, the application of CRISPR/Cas to lncRNAs faces numerous obstacles due to their complex loci and their non-coding nature. For example, frameshift mutations are not a viable option because lncRNAs are a part of the non-coding genome. In the case of our candidate lncRNA *Zeb2os,* it is antisense to its sense gene Zeb2. Furthermore, it can be described as one of three types of antisense lncRNAs: exons that overlap with a part of the sense gene [51]. This bears the difficulty that targeting *Zeb2os* might also disrupt the expression of its host gene *Zeb2*. Since we didn't want to interfere with the expression of *Zeb2*, we took this into account when planning our targeting strategy. *Zeb2os* has three exons. Only one of them, exon 1, is antisense to only intronic parts of Zeb2, so we decided to target only this one exon with gRNAs. This has the risk of not diminishing the whole lncRNA, but the advantage of not affecting Zeb2. To make sure that the removal of one exon was sufficient to silence the entire *Zeb2os* gene, we also tried to target the promoter region of *Zeb2os*. Since attempts to deliver Cas9 and the corresponding sgRNAs with the help of a viral vector failed, our collaborator Seren Baygün (Marc Schmidt-Supprian lab) successfully tried to deliver carefully designed sgRNAs to the cells using electroporation. For ultimate validation of a *Zeb2os* knockout and a continuous expression of Zeb2, we prepared RNA-seq libraries of knockout and wild-type cells. Our results show that the knockdown of *Zeb2os* works well. Compared to wild-type cells, the *Zeb2os* knockout cells showed a significantly reduced expression of *Zeb2os.* However, we could still see some residual expression of *Zeb2os* since we only targeted one exon. This is acceptable when considering the challenges of targeting lncRNAs in general and considering the lack of suitable genetic manipulation tools. According to a study by Goyal et al., up to two-thirds of all lncRNAs are considered "non-CRISPRable", mostly due to potential effects on neighboring genes [89]. However, as demonstrated by RNA-seq, this targeting method served the purpose of not affecting Zeb2 expression. This is especially important since we want to make

sure that potential phenotypes in executed experiments can only be ascribed to *Zeb2os* and not to Zeb2. In addition, to validate an independent effect of Zeb2os, it is necessary to examine its effects not only on transcription but also on translation. To confirm or refute the previously reported splicing mechanism of the IRES of Zeb2 by *Zeb2os*, the protein expression levels of both Zeb2 and *Zeb2os* should be analyzed, for example by Western blot analysis. If it is found that Zeb2 protein levels are unaffected by *Zeb2os* knockout, then it can be concluded that the previously mentioned published mechanism is not applicable to macrophages. It would also confirm that the observed effects are solely dependent on *Zeb2os*.

We used RNA-seq not only to confirm that the knockout of *Zeb2os* had worked, but also to gain further information on about the genomic changes and the resulting potential functions. Knockout of *Zeb2os* resulted in a large number of highly deregulated genes, highlighting its importance for biological processes in macrophages. Analysis of these deregulated genes revealed that genes downregulated in the *Zeb2os* knockout cells are primarily involved in processes of chemotaxis and migration. This finding suggests a function of *Zeb2os* in the regulation of migration and chemotaxis and confirms our previous findings in loss-of-function assays.

CrMΦs are the most abundant immune cell type in the heart and are maintained by local proliferation. This, among other findings, suggests a central role of cardiac macrophages in the homeostasis of cardiac diseases. However, finding specific targeting strategies remains a challenge. As mentioned above, studies show that Zeb2 is required for the maintenance of tissue resident macrophages in their respective tissues. This has been shown for almost every organ that contains tissue resident macrophages like spleen, brain, liver, colon, and lung, but has not yet been demonstrated for the heart [65]. However, it is highly likely that this is also true for cardiac resident macrophages, making Zeb2 an interesting target when it comes to the regulation of inflammatory responses and homeostasis in cardiac disease. Also, increasing evidence points towards a regulation of Zeb2 via *Zeb2os.* Specific targeting of Zeb2 using *Zeb2os* could present a chance to influence the fate of cardiac resident macrophages in the course of cardiac disease. Coincidentally, during our experiments, we observed that the Hoxb8 cell line with a knockout of *Zeb2os* was less able to differentiate into macrophages than the cell lines without this knockout, suggesting that Zeb2os may be critical for the differentiation of monocytes into macrophages. Taken

together, Zeb2 and *Zeb2os* appear to have critical functions in the localization and homeostasis of crMΦs. Further experiments, such as a pulldown to identify protein interaction partners, are needed to explore the detailed mechanism of action of *Zeb2os.*

Cardiovascular disease, whether acute or chronic, is associated with systemic inflammation. After cardiac injury, the death of cardiomyocytes leads to a blunted immune response. This inflammatory response after cardiac events such as myocardial infarction or in more chronic processes such as cardiac hypertrophy or atherosclerosis is a necessary and natural response, but eventually leads to further destruction of cardiac tissue. Therefore, peripheral monocyte infiltration is thought to be primarily responsible for adverse outcomes such as left ventricular dysfunction and progression of atherosclerosis (Dutta et al., 2012). Macrophages are essential for inflammatory responses, proper wound healing and adequate scar formation. Nevertheless, among other cardiac cells, cardiac resident macrophages release pro-inflammatory cytokines and chemokines that amplify inflammation [90]. In addition, they participate in the recruitment of monocytes to the heart [91]. Therefore, we decided to investigate the role of crMΦ, and, moreover, the role of lncRNA in crMΦ in inflammatory responses and thus in disease progression. Using RNA-seq and scSeq and transverse aortic constriction (TAC) as a disease model, we identified *Zeb2os* as a potentially valuable therapeutic target in this context. We also performed a knockdown of this gene in an *in vitro* model to investigate its role in the aforementioned processes. For this purpose, we used siRNAs to repress the translation of *Zeb2os,* as well as that of Zeb2 for comparative studies. SiRNAs have the ability to specifically knock down genes, but do not result in a complete knockout of the target gene. Therefore, some residual expression cannot be excluded and should be taken into account when analyzing the results. Also, variations in the strength of respective knockdowns in different experiments cannot be avoided. Nevertheless, we were able to achieve efficient knockdown of *Zeb2os* in BMDMs to make valuable predictions about *Zeb2os* functions. As an *in vitro* cell model, we used bone marrow derived macrophages (BMDMs). We chose this cell line because these cells are easy to maintain and differentiate and knockdown with siRNAs works well. In addition, they are well established as an *in vitro* model for macrophages. In terms of organ specificity, a more precise cell model should be considered for future experimental approaches, as BMDMs do not function as tissue resident macrophages in the heart. BMDMs were

treated with siRNAs targeting *Zeb2os*, Zeb2 or a control siRNA prior to the execution of experiments. Knockdown was validated by RNA extraction followed by qPCR. Since Zeb2 is known to be critical for the tissue identity of resident macrophages [65], and - as a transcriptional inhibitor of E-cadherin [92] – is involved in cell adhesion and mobility, we first wanted to take a closer look at the influence of *Zeb2os* and Zeb2 on the migratory abilities of macrophages. Analysis of macrophages under conditions of wound healing and migration, showed that cells with a knockdown of Zeb2os as well as Zeb2 led to less migration. Furthermore, we could see that the treated macrophages had an impaired ability to completely fill the wound area when compared to the control group. It should be kept in mind that the wound area is inflicted on the well by a "wound maker tool" and cells cannot be seeded identically in each well. This leads to a range of variability in the results, resulting in relatively wide error bars. However, these error bars represent a rather conservative estimate. The progression of the three curves shows a clear tendency towards reduced migration and thus impaired wound closure in *Zeb2os* and Zeb2 knockdown cells, suggesting an involvement of *Zeb2os* in migration and wound healing processes. To gain further insight into how migration is affected, we looked at cell migration in the context of immune and inflammatory responses by using chemoattractant. Our initial experiments showed a similar trend to the Scratch assay. Macrophages treated with siRNAs for *Zeb2os* and Zeb2 showed a slower and impaired ability to migrate towards the chemoattractant. However, further experiments are needed to validate this trend. Taken together, *Zeb2os* appears to influence macrophage migration to inflamed and injured sites and participate in wound healing. Studies suggest that inflammatory macrophages that migrate to injured cardiac tissue may dammage myocytes by producing cytokines that lead to apoptosis (Haudek et al., 2007). Our experiments show that targeting *Zeb2os* can lead to reduced migration and inflammatory response in an in vivo model of cardiac disease. This manipulation has the potential to alter the development and progression of cardiac disease. Targeting *Zeb2os* could potentially attenuate migration and reduce the intensity of the inflammatory response, leading to a decrease in tissue fibrosis and scar formation. As a result, this approach could help preserve cardiac contractility and function. As mentioned above, cardiac resident macrophages participate in the recruitment of monocytes to the heart after cardiac injury. In addition, they appear to have an anti-inflammatory effect by producing anti-inflammatory cytokines and inhibiting the recruitment of circulating immune cells [32,33] . In this way, they drive a

carefully orchestrated immune response with a balance of pro- and anti-inflammatory factors.

A next step in our studies should be the characterization of *Zeb2os* in an *in vivo* model of cardiac health and disease. Analysis of tissue samples and extraction of cell samples at different time points of the disease could help to gain further insights into the exact effects of *Zeb2os* on the development and course of cardiovascular diseases.

In conclusion, I characterized the long non-coding RNA *Zeb2os* in macrophages and showed that depletion of *Zeb2os* led to widespread transcriptomic changes resulting in impaired migration and chemotaxis. Furthermore, as a putative regulator of the transcription factor Zeb2, *Zeb2os* poses as a promising target to influence scar formation and inflammatory response and therefore disease progression.

6. Summary

Among the various cell types in the heart, cardiac resident macrophages (crMΦs) have only recently been identified as a vital cell population in the course of cardiac disease. Merely 2% of the genome is transcribed into protein-coding mRNAs. Consequently, it has become evident that that a majority of the genome is transcribed into RNAs without any discernible coding potential. Secondly, an analysis of solely protein-coding regions is insufficient to explain pathway regulations and the pathogenesis of numerous diseases. The non-coding regions of the genome can be further classified as small non-coding RNAs (microRNAs), and long non-coding RNAs (lncRNAs), which consist of transcripts greater than 200 nucleotides. LncRNAs represent the most prevalent RNA species; however, their functions and mechanisms of operation are largely unknown. They are expressed in a tissue-specific manner, making them a compelling therapeutic target for the development of new approaches. Using large data sets based on RNASeq and scSeq, we identified *Zeb2os,* a highly expressed lncRNA that is enriched in crMΦ and regulated during cardiac disease. A look at its genomic locus showed that *Zeb2os* is an antisense transcript to Zeb2, a zinc-finger E homeobox-binding transcription factor. This caught our attention because Zeb2 is not only known to be a transcriptional inhibitor of E-cadherin and its involvement in epithelial-mesenchymal transition, but also to play an important role in the maintenance of tissue-resident macrophages. When analyzing the expression data of Zeb2 and *Zeb2os,* we observed a correlation in the expression patterns suggesting a synergism in the function of these two genes. This is supported by previous studies suggesting a mechanism of interaction where the presence of *Zeb2os* leads to an increase in the expression of Zeb2. To gain further insight into the functions of *Zeb2os* itself, we used loss-of-function assays after achieving *Zeb2os* knockdown by using siRNAs on BMDMs. Our experimental results suggest that *Zeb2os* may play a role in macrophage migration and chemotaxis. Macrophages deficient in *Zeb2os* exhibited a diminished ability to migrate and participate in wound healing. They also showed an impaired ability to migrate toward a chemotactic stimulus, indicating a reduced immune and inflammatory response. To support our previous findings and to gain a deeper understanding of the functions of *Zeb2os*, we generated *Zeb2os* knockout cells using CRISPR/Cas9. We performed RNASeq on these *Zeb2os* knockout cells and compared them to wild-type controls. Differentially expressed genes were analyzed using

Summary

Cytoscape and ClueGO, tools for gene ontology analysis. Deregulated genes, especially downregulated genes, turned out to be mainly involved in processes of migration, chemotaxis and other immune responses when comparing Zeb2os knockout cells with wild-type cells. This corroborated our findings in in vitro experiments and may indicate an essential function of *Zeb2os* in the pathways of migration, wound healing, and chemotaxis. Our findings require further investigation and confirmation in an in vivo model. Our studies identified *Zeb2os*, a long non-coding RNA that is highly abundant in cardiac resident macrophages and is regulated during cardiac disease. We showed that *Zeb2os* plays a critical role in regulating migration and chemotaxis. In addition, it may control the fate of cardiac resident macrophages by interacting with Zeb2. This highlights a potential target for future therapeutic intervention by selectively targeting macrophages in the heart to control and reduce the inflammatory response to cardiac injury.

7. Acknowledgements

For my family and friends who endured with me and supported me the entire time.

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