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der Ludwig-Maximilians-Universität zu München
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MicroRNA-associated regulatory mechanisms in human mesenchymal stem cells and endothelial cells

Kumulative Habilitationsschrift

Zur Erlangung der Venia Legendi
für Klinische Pathobiochemie
in der Medizinischen Fakultät.

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15. 04. 2024

Dedicado a Diego y Santi

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2 Summary

At the sites of inflammation mesenchymal stem cells (MSCs) are found aiding in tissue repair and immunomodulation. MSCs are in the focus of international research efforts aiming to understand their characteristics and potential for cell- and secretome-based therapeutic applications in the context of various diseases. MSCs migrate from different origin tissues to sites of injury or inflammation in a complex process that involves chemokine-mediated migration and the release of bioactive factors that modulate the behavior of surrounding cells. MicroRNAs (miRNAs) are a class of non-coding RNAs which are pivotal in post-transcriptional regulation of gene expression. Fluctuations in miRNA expression result in changes of protein expression, thereby influencing numerous biological processes and contributing to a variety of human diseases. Additionally, miRNAs can be transferred between cells, thereby regulating gene expression remotely from their cell of origin. It is therefore not surprising that miRNAs modulate major MSC functions such as differentiation, migration and paracrine immunomodulation.

The central aim of the habilitation project was ultimately to investigate the role of miRNAs in the process of chemokine-driven migration of MSCs towards sites of inflammation, as well as their paracrine impact on target cells in tumor or atheromatous tissues. Interestingly, although members of the let-7 miRNA family typically function as tumor suppressors by restraining growth and invasion in cancer cells, let-7f appears to exhibit a contrasting role in MSCs. In our studies, we explored how this unique characteristic could potentially prove advantageous in the development of therapeutic approaches. Through four distinct yet complementary studies, we were able to underscore the importance of the miRNA let-7f in MSC biology. Our findings consistently demonstrated that overexpression of let-7f enhances the migratory capacity of MSCs. Let-7f upregulates cell surface receptors such as CXCR4 and FPR2, which in turn improves MSCs migratory response to stimuli like SDF-1 and LL-37. Finally, at the tumor site, MSC-derived exosomes containg let-7f are able to reach and enter the cancer cells exhibiting an anti-tumoral effect. These results were confirmed by *in vitro* and *in vivo* results in both glioma and breast cancer tumor models. The aforementioned properties were also evaluated *ex vivo* using an arterial perfusion model of atherosclerosis in a non-tumoral context. During our studies we also identified a remarkable non-canonical example of miRNA functionality in endothelial cells demonstrating that miR-126-5p inhibits the function of caspase 3 through direct biophysical interaction. This ultimately modulates crucial aspects of cardiovascular biology including the integrity of endothelial cells.

The findings presented here have been achieved during my postdoctoral tenure at the Department of Clinical Chemistry and Biochemistry, led by Prof. Dr. rer. nat. Marianne Jochum, and Institute for Cardiovascular Research (IPEK), led by Prof. Dr. Christian Weber, at LMU Munich.

3 Introduction

The biology of stem cells is highly intriguing to the scientific community due to their remarkable capacity for self-renewal through symmetric cell division and their ability to differentiate into various specialized cell types through asymmetric division. Consequently, there is hope that stem cells will play a crucial role in the treatment of many human pathophysiologies. Various tissues harbor adult stem cells, which are essential in maintaining tissue homeostasis and repair. However, increasing evidence suggests that stem cells may also be a source of cancer initiation. Therefore, a detailed understanding of the regulatory mechanisms controlling stem cell functions represents one of the foremost challenges for future science.

3.1 Mesenchymal stem cells

MSCs are adult multipotent stem cells giving promise for various pharmaceutical applications (Pittenger et al 1999, Salem & Thiemermann 2010). These cells have been harvested from a variety of tissues, including umbilical cord, adipose tissue, dental tissue, and bone marrow. MSCs typically exhibit a fibrous, spindle-shaped morphology and adhere to surfaces, proliferating well *in vitro* (Fig.2) (Pittenger et al 1999).

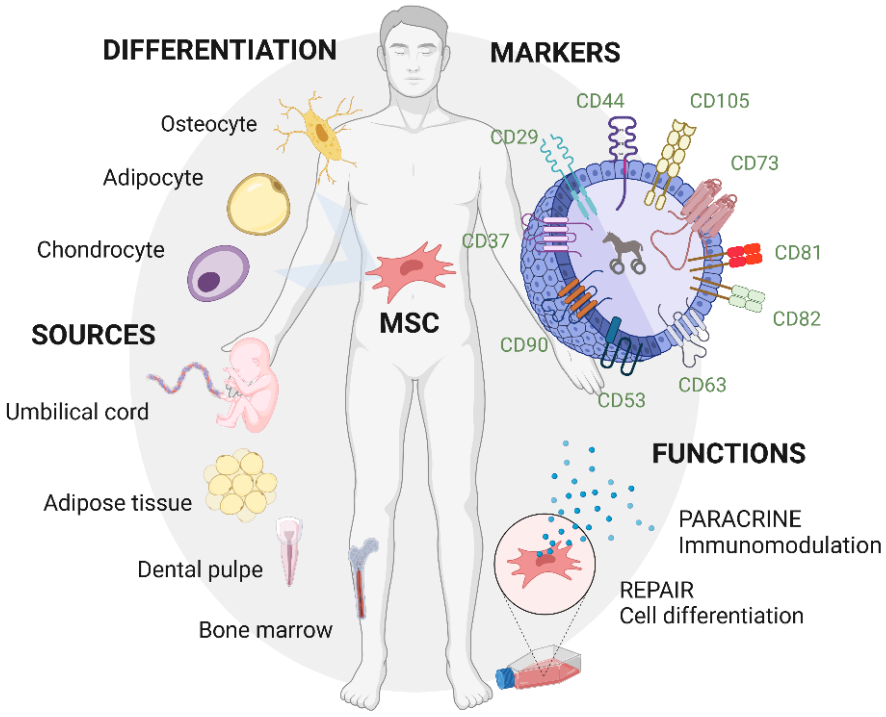


Figure 1: MSC properties and functions.

MSCs can be harvested from various tissues and have the capacity to differentiate into diverse cell types. Due to the absence of a specific biomarker, a combination of multiple markers is utilized for their identification.

Additionally, MSCs are recruited to sites of inflammation for tissue repair and can act as immune-modulators in a paracrine manner via release of cytokines, chemo-kines, and exosomes. They can therefore be used as trojan horses delivering therapeutic agents to the site of inflammation.

Figure by V. Egea.[1]

[1] All figures in this document were created with BioRender.com

MSCs proliferation potential is significant, yet it is influenced by the donor's age, tissue source, and culture conditions such as cell density and composition of growth media (Marquez-Curtis & Elliott 2024, Pittenger et al 1999).

There is no specific biomarker for MSCs. Hence, the International Society for Cellular Therapy (ISCT) established a combination of biomarkers required for the phenotypic characterization of MSCs with ≥95% of the cells to be positive for markers including CD105, CD73, and CD90, and negative for lineage-specific surface molecules (Fig.1). A defining characteristic of MSCs is their capacity for osteogenic, chondrogenic, and adipogenic differentiation. Moreover, MSCs have the ability for transdifferentiation into neuroectodermal (neurons) and endodermal (hepatocytes) lineages. Because of their multipotency, MSCs have been proposed as a promising therapeutic option for a wide range of diseases, including orthopedic, cardiovascular, and neurodegenerative disorders (Jiang et al 2002). Furthermore, MSCs are known to respond to inflammation in their microenvironment and modulate both adaptive and innate immune responses. They influence the proliferation, activation, and effector functions of T-cells, antigen-presenting cells (dendritic cells, macrophages, B-cells), natural killer cells (NK cells), invariant NKT cells, and granulocytes through the secretion of paracrine mediators and/or cell contact signals (Jasim et al 2022, Singer & Caplan 2011). The paracrine activity of MSCs, particularly through the secretion of various factors via extracellular vesicles like exosomes, plays a crucial role in their effectiveness (Lotfy et al 2023). MSC-derived exosomes (MSCs-Exos) transmit biological information by directly transferring intra-exosomal content, including messenger RNA (mRNA) and microRNA (miRNA), into recipient cells through fusion with the cell membrane.

As MSCs exhibit a natural attraction to areas of inflammation, including solid tumors and atherosclerotic plaques, these cells can be used as carriers of therapeutic substances to these specific sites, a strategy known as the Trojan horse approach. (Levy et al 2016).

Consequently, the efforts of many researchers have focused on the potential clinical application of MSCs and MSC-Exos for treating not only cancer but also immune and inflammation-related diseases, such as inflammatory bowel diseases, liver disorders, diabetes, and cardiovascular diseases (Lin et al 2015, Salem & Thiemermann 2010).

3.2 miRNAs in cell physiology

In the late 1993, Lee et al. and Wightmann et al. made the initial discovery of lin-4, a non-coding gene. The 21-nucleotide transcript had sequence complementarity to the 3’ untranslated region (3’-UTR) of the specific mRNA of lin-14 (Lee et al 1993, Wightman et al 1993). The pairing of lin-4 with lin-14 mRNA UTR caused significant repression led to a strong inhibition of its translation. Shortly after, another short non-coding RNA, namely let-7 (also known as lethal-7), was identified in Caenorhabditis elegans (Reinhart et al 2000). Initially, let-7 was believed to be specifically involved in the

developmental timing of Caenorhabditis elegans. These findings led to the classification of this new type of regulator as stRNAs, which stands for small temporal RNAs. Later, hundreds of small non-coding RNAs have been discovered in worms, flies, and human cells, and they were given the name microRNAs (miRNAs) (Lee & Ambros 2001).

miRNAs are a class of small non-coding RNAs, approximately 22 nucleotides in length, that modulate gene expression by interfering with mRNA translation (Bartel 2018). They are key regulators of gene expression in both normal and neoplastic tissues. miRNAs have attracted attention as potential biomarkers, and targets for diagnostic and therapeutic applications (Rupaimoole & Slack 2017). Many miRNAs are highly conserved across different species, particularly in their "seed" region which is crucial for determining target gene specificity (Kehl et al 2017).

The post-transcriptional control of gene expression by miRNAs is a significant regulatory mechanism involved in most cellular processes, including the function of stem cells and their therapeutic applications. At the molecular level, miRNAs are versatile regulators of gene expression, sparking considerable interest in the fields of cancer and cardiovascular research. They regulate the expression of specific target mRNAs by either inhibiting mRNA translation or promoting mRNA decay within the RNA-induced silencing complex (RISC) (Kehl et al 2017).

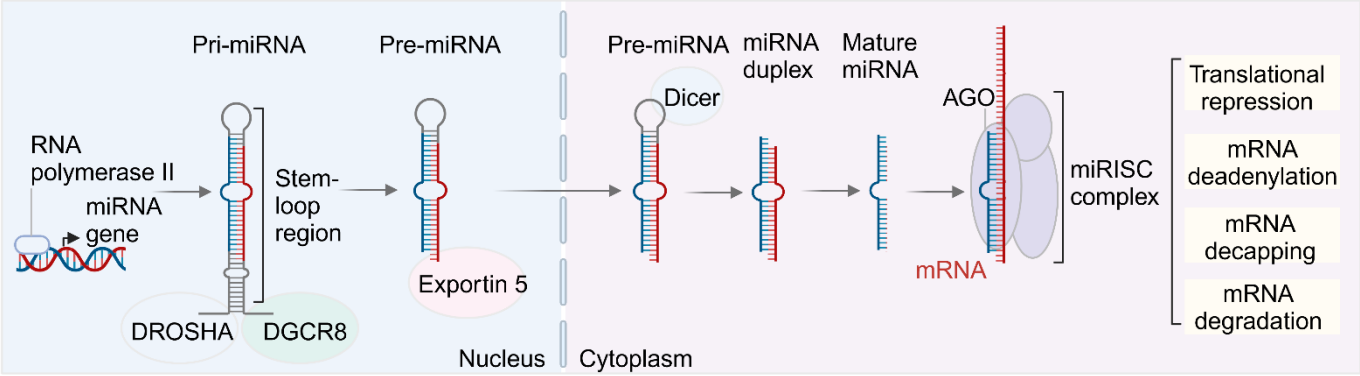
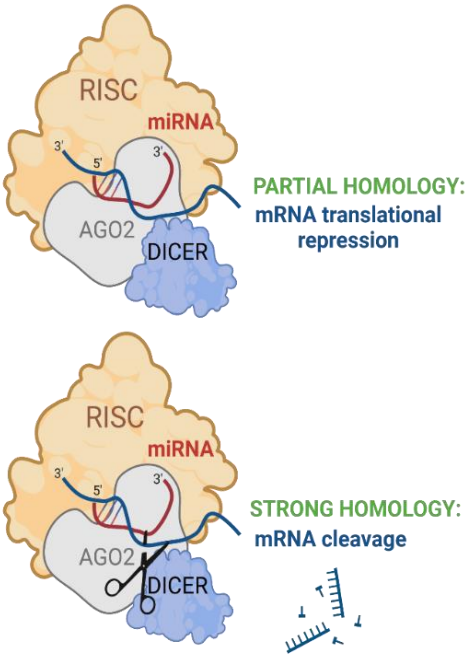


Figure 2: MiRNA biogenesis.

MiRNA genes undergo transcription into primary miRNAs (pri-miRNAs) by RNA polymerase II. The microprocessor complex component DGCR8 binds to these pri-miRNAs, which are then processed by Drosha's ribonuclease III (RNase III) enzyme into precursor miRNAs (pre-miRNAs) with hairpin structures. These pre-miRNAs are transported from the nucleus to the cytoplasm by Exportin 5. In the cytoplasm, the RNase III enzyme Dicer removes the loop from the hairpin, resulting in a miRNA duplex. This duplex separates, and the mature miRNA strand combines with Argonaute (AGO) proteins and additional accessory proteins to create the miRNA-induced silencing complex (miRISC). This complex is responsible for the translational inhibition and increased degradation of target mRNAs. The mature miRNA, in conjunction with an AGO protein, constitutes the miRISC's essential component. The AGO protein attracts additional complexes that inhibit translation and promote the deadenylation of the target mRNA. This process eventually leads to the decapping and degradation of the mRNA. Figure by V. Egea.

The intricate process of miRNA biogenesis commences in the nucleus and concludes in the cytoplasm (Fig.2). Initially, miRNA genes are transcribed predominantly by RNA polymerase II, followed by modifications including capping, splicing, and polyadenylation. This results in the formation of a primary miRNA (pri-miRNA) that features one or more hairpin structures.

The pri-miRNA undergoes processing by the Drosha enzyme and its partner DGCR8 in the nucleus, yielding a precursor miRNA (pre-miRNA) of 70 to 100 nucleotides. This pre-miRNA is exported to the cytoplasm via Exportin-5 through nuclear pores.



In the cytoplasm, Dicer, another ribonuclease, cleaves the pre-miRNA into a double-stranded RNA, which includes the miRNA strand and its complementary strand. A helicase then separates these strands, producing a mature miRNA that is incorporated into the RNA-induced silencing complex (RISC) containing an Argonaute protein (AGO2). The RISC complex, guided by the mature miRNA, binds to the 3′ untranslated region (3′UTR) of target mRNAs, leading to mRNA degradation or translational inhibition, the latter being more common in mammals (Fig.3) (Ha & Kim 2014).

Figure 3: Canonical functions of miRNAs. miRNAs, bind to the 3′UTR of target mRNAs. High degree of binding homology results in mRNA degradation whereas partial homology leads to inhibition of translation. Figure by V. Egea.

In addition to this canonical pathway, miRNAs can also be generated from introns of protein-coding genes through a Drosha/DGCR8-independent mechanism involving the pre-mRNA splicing machinery. These intron-derived miRNAs, known as miRtrons, are associated with the expression of their host genes and are located within introns or at splice site junctions. miRtrons are similarly exported to the cytoplasm and processed by Dicer (Westholm & Lai 2011).

Interestingly, for intercellular communication miRNAs are being released from cells. Free soluble miRNAs, so-called circulating miRNAs, can be detected in body fluids including blood and urine (Weber et al 2010). Frequently, they form complexes with proteins such as AGO2, NPM1, and HDL, protecting miRNAs from enzymatic degradation. miRNAs are also released from cells within extracellular vesicles such as exosomes (Rayner & Hennessy 2013, Vickers et al 2011). Upon fusion of exosomes with recipient cells, miRNAs are delivered into the cytoplasm where they influence gene expression in the target cells remotely from their cells of origin (Jy et al 2010, Kalluri & LeBleu 2020). The composition of exosomes reflects the physiological or pathological state of the originating cell. Analysis of circulating and exoxomal miRNAs provides an innovative non-invasive diagnostic and prognostic tool (Hamam et al 2017, Kalluri & LeBleu 2020). The natural communication mechanism mediated by exosomes can be utilized to deliver therapeutic miRNAs, thereby overcoming the challenges associated with miRNA stability and delivery (Jurga et al 2021).

3.3 miRNAs in disease

Altered expression of miRNAs can lead to multiple diseases including cancer (Rupaimoole & Slack 2017). In carcinogenesis miRNAs promoting tumor growth and invasion are referred to as 'oncomirs' (Esquela-Kerscher & Slack 2006). Other miRNAs such as let-7, miR-15, and miR-16, can act as tumor supressors negatively regulating the RAS oncogenes and BCL2. These miRNAs have been demonstrated to be decreased in many tumors and are therefore promising candidates for cancer treatment (Ma et al 2021, Michael et al 2003, Mizuno et al 2018).

Not only in cancer but also in the overall mantainance of the cardiovascular system function are miRNAs key players. One example is miR-126-5p, which supports the growth of endothelial cells by inhibiting the production of the Dlk1 protein (Schober et al 2014). This mechanism allows miR-126-5p to maintain the normal growth of endothelial cells even under conditions of high lipid levels, thus protecting against the formation of arterial lesions. Regions of the arterial tree which are prone to lesion formation due to disturbed blood flow, showed reduced levels of miR-126-5p associated with increased expression of Dlk1 and reduced endothelial cell growth under high lipid conditions. Interestingly, treatment with miR-126-5p was able to reverse this inadequate endothelial cell growth and reduce plaque formation at these vulnerable sites. These findings suggested that miR-126-5p could be a promising therapeutic target for the treatment of atherosclerosis (Schober et al 2014).

4 Aims of the study

Our studies aimed to understand how MSCs are recruited to tissue sites of inflammation. The main goal was to develop new strategies to enhance the ability of MSCs to target primary tumors or atherosclerotic plaques for therapeutic interventions. We investigated this recruitment pattern *in vitro, in vivo*, and *ex vivo* using different cell and animal models, focusing on glioma, breast tumors, and atherosclerotic plaques (Fig.4).

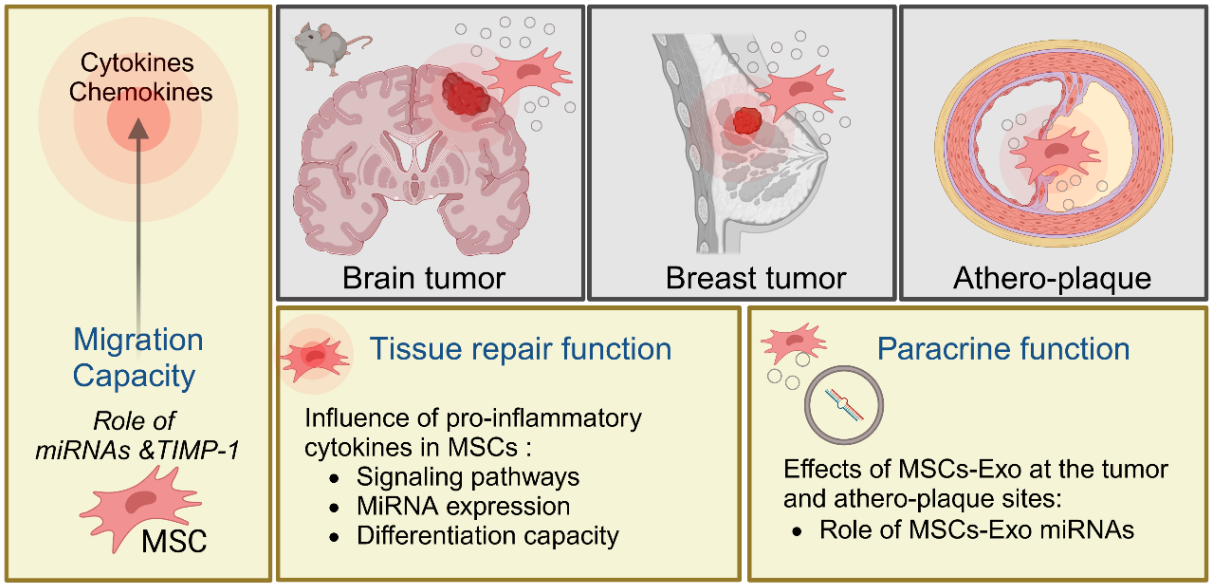


Figure 4: Aims of the study: Our studies were conducted with the purpose of comprehending the fundamental mechanisms that govern the migration capacity of MSCs in their recruitment to inflammation sites, such as glioma, breast tumor, or atherosclerotic plaques. Additionally, our objective was to gain insight into the subsequent role of these cells at these sites, specifically in terms of tissue repair and paracrine functions.

The aims of our studies were therefore:

- **MSC ACTIVATION**: To uncover the role of inflammatory cytokines in MSC activation for migration. This included exploring MSCs signaling pathways, miRNA expression as well as differentiation capacity. Here we also aimed to understand the role of TIMP-1 in MSCs biology.
- **MSC RECRUITMENT**: To investigate the processes behind MSCs migration towards glioma, breast tumors and atherosclerotic plaques.
- **MSC AT TARGET SITE**: To analyze the paracrine effects of MSCs at the target site via exosomal release (MSCs-Exo) in glioma, breast tumors and atherosclerotic plaques.
- **MIRNAs THERAPEUTICS**: To explore the potential of miRNA mimics and antagomiRs in MSCs related therapeutic approaches.

5 Essential findings

5.1 Migratory properties of MSCs and their therapeutic potential

Egea et al. 2010

In this study, we demonstrated that pre-treatment of MSCs for two weeks with 50 ng/ml of Tumor Necrosis Factor-alpha (TNF-α) triggers cellular expression of the chemokine receptor CXCR4 resulting in an enhanced capacity of the cells for directed migration towards Stromal cell-Derived Factor 1 (SDF-1) alpha *in vitro*. TNF-α-pre-treated MSCs also show an increased ability to infiltrate glioma cell spheroids *in vitro*. Furthermore, TNF-α-pre-treated MSCs exhibited enhanced tropism towards intracranial malignant gliomas as demonstrated by use of an *in vivo* mouse model. Concluded from our findings, pre-treatment of MSCs with TNF-α might be useful to improve efficacy in cell therapeutical applications in the context of neurological disorders, such as malignant gliomas.

Egea et al. 2012

Tissue Inhibitor of Metalloproteinases 1 (TIMP-1) has been recently shown to have cytokine-like functions. In this study, we demonstrated that endogenous TIMP-1 suppresses proliferation, metabolic activity, and the capacity of MSCs for osteogenic differentiation. This is triggered by the binding of TIMP-1 to CD63 on the surface of MSCs mediating cellular effects through the Wnt/β-catenin signaling pathway. Furthermore, we identified the miRNA let-7f to be crucial for the regulation of β-catenin activity and osteogenic differentiation by targeting Axin-2, an antagonist of β-catenin stability. Our findings indicate that TIMP-1 is a direct regulator of MSC functions involving a molecular network where let-7f modulates Wnt/β-catenin activity.

Egea et al. 2021

In this study, the function of let-7f in MSCs was further explored with a particular focus on its influence on the cells' invasive behavior towards tumor tissue and subsequent paracrine effects. The expression of let-7f in MSCs was found to be upregulated upon exposure of the cells to SDF-1α, various inflammatory cytokines, or hypoxic conditions. Levels of endogenous let-7f correlated with MSC motility and invasion by modulating cellular autophagy, a molecular recycling process activated during cellular stress such as inflammation and hypoxia. Moreover, MSC-derived let-7f packaged in exosomes reduced the proliferative and invasive capacity of breast cancer cells.

Furthermore, *in vivo* studies using 3D spheroids composed of MSCs and breast cancer cells implanted into a mouse model revealed that elevated let-7f expression in MSCs resulted in improved suppression of tumor growth. These findings confirmed a pivotal role of let-7f in the regulation of MSC

tropism in response to inflammatory and hypoxic stimuli, and suggest that let-7f delivered through exosomes exert anti-tumor effects in a paracrine manner.

Egea et al. 2023

In this study, we further explored the role of let-7f in MSCs that are recruited from the circulation into atherosclerotic plaques. Our results demonstrated that endogenous let-7f promotes MSC tropism towards atheromas by activation of the LL-37/FPR2 signaling transduction pathway. Upon contact with human plaque, let-7f also mediated an atheroprotective signature in MSCs favouring their myogenic differentiation.

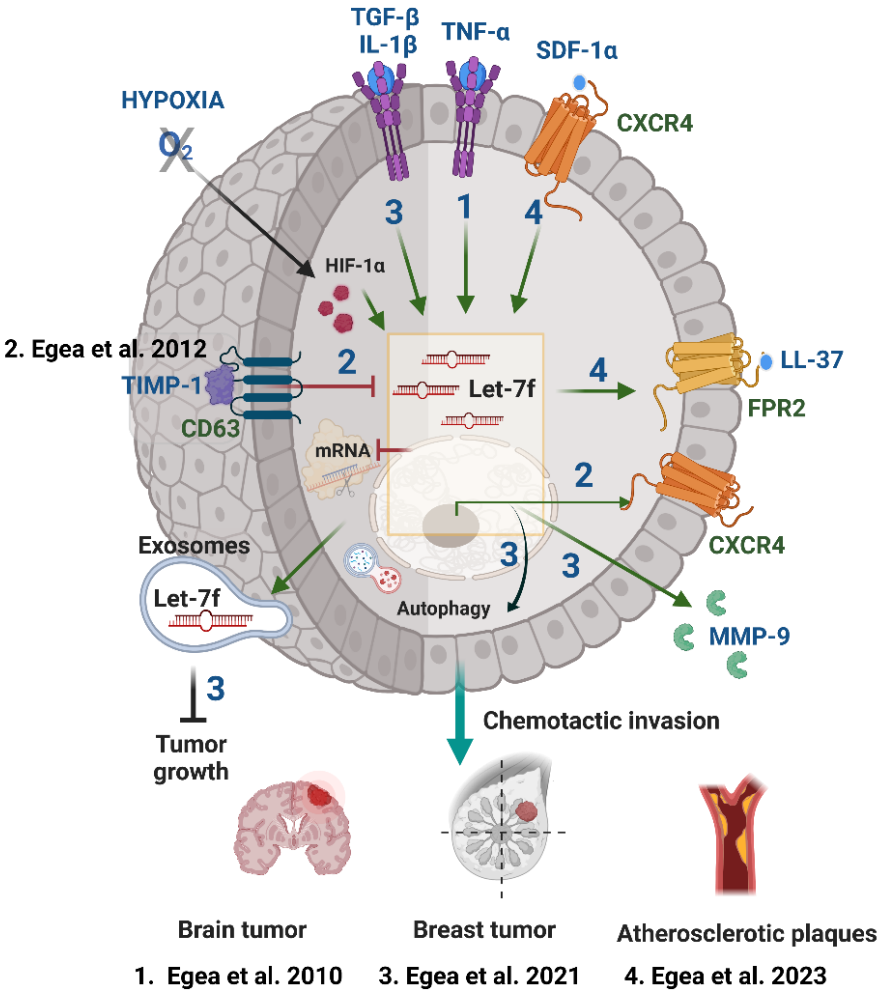


Fig. 5: Graphical abstract of the results obtained in MSCs.

The miRNA let-7f is a powerful player in MSC biology. Let-7f is upregulated in MSCs when exposed to inflammatory cytokines or hypoxic conditions. When overexpressed, let-7f effectively augments the migratory ability of MSCs and impairs tumor growth in a paracrine manner being released in exosomes. Let-7f plays also a key role in MSC recruitment to the atherosclerotic plaques where it might confer a stabilizing effect. Consequently, the overexpression of let-7f in MSCs represents a promising therapeutic strategy to improve the delivery of genetically engineered MSCs to tumors or atherosclerotic sites. Figure by V. Egea.

5.2 Non-canonical effects of miRNA

Egea et al. 2020

In the course of this study, we identified a remarkable non-canonical example of miRNA functionality, where miRNA miR-126-5p inhibits the protein function of caspase 3 through a direct biophysical interaction, ultimately modulating crucial aspects of cardiovascular biology, such as endothelial integrity. In endothelial cells we observed that, miR-126-5p is translocated from cytoplasm into the nucleus in a complex with AGO2 and the RNA-binding protein MEX3A. In the nucleus, miR-126-5p dissociates from AGO2 and then from MEX3A to interact with effector caspase 3. Binding to miR-126-5p inhibits the catalytic activity of caspase 3 and limits apoptosis to preserve endothelial integrity.

5.3 Summary

In summary, the results of the research presented in my postdoctoral thesis suggest that let-7f may promote the development of MSC-based targeted therapeutic strategies (Fig.5). Furthermore, a novel mode of miRNA action was identified that preserves endothelial integrity during atherosclerosis. In the future, my motivation is to further investigate the relevance of non-canonical functions of miRNAs in cardiovascular homeostasis and pathology, and to contextualize how the discovery of these unconventional properties can expand the scope of translational research in the cardiovascular field and beyond.

6 Subprojects and significance for the field

6.1 Migratory properties of MSCs and their therapeutic potential

MSCs are at the forefront of global efforts not only to understand their nature and unique properties but also to develop cell and secretome-based therapies for a variety of diseases. While the paracrine role of MSCs is increasingly acknowledged, the mechanisms of their migration from the bloodstream to targeted lesions with compromised vascular integrity are not fully understood.

6.1.1 TNF-α respecifies MSCs to a neural fate and promotes migration toward experimental glioma.

(Egea V, et al. 2011)

TNF-α is involved in a wide range of physiological and pathological processes that regulate the function and development of various cell types, including neural stem/progenitor cells (Gonzalez Caldito 2023). In MSCs, TNF-α can act as a chemoattractant and stimulate the expression of matrix metalloproteinases (MMPs) and TIMPs (Ries et al 2007). In this study, we demonstrated that long term culture of MSCs in the presence of TNF-α leads to a significant change in cell morphology, resembling the star-shaped appearance of astrocytic glial cells in the brain and spinal cord. Indeed, after treatment with TNF-α, MSCs stained positive for GFAP and showed a reduced transcription rate for Nestin and Vimentin (Jurga et al 2021), confirming the astroglial nature of these cells.

Stem cells, both endogenous and transplanted, must possess the ability to migrate directionally towards areas of damage, chronic disease, or inflammation to promote tissue repair and regeneration. SDF-1 is present in elevated concentrations in the CNS tissues during development and injury, acting as a chemotaxin that attracts neuronal stem cells, which typically synthesize high levels of CXCR4, thus enabling the mobilization and recruitment of these cells along SDF-1 gradients (Imitola et al 2004). Similarly, we discovered that MSCs treated with TNF-α exhibit increased CXCR4 expression, enhancing their capability for SDF-1α-driven invasion through the human ECM *in vitro*, facilitated by MMP activity. These findings are consistent with other studies showing that TNF-α incubation enhances the migration of MSCs towards SDF-1 (Ponte et al 2007).

A notable characteristic of MSCs is their pronounced tropism for malignant gliomas (Nakamizo et al 2005). Following intravascular or local administration, MSCs have been shown to specifically integrate into gliomas, attracted by cytokines and growth factors released from tumor cells (Sasportas et al 2009). Glioblastoma multiforme, the most aggressive form of gliomas, has a median survival time of only one year post-conventional therapy, including resection, chemotherapy, and radiotherapy (Hanif

et al 2017). Consequently, innovative therapeutic strategies targeting the invasive tumor cells that have penetrated deep into the surrounding normal tissue are urgently needed. An increasing number of *in vitro* and *in vivo* studies have indicated the utility and therapeutic efficacy of MSCs as cellular vectors for targeted treatment of malignant gliomas (Bexell et al 2010, Sasportas et al 2009). MSCs engineered to produce immunotherapeutic cytokines such as TNF-related apoptosis-inducing ligand or interferon-β have demonstrated potent antitumor effects and significantly increased survival when specifically embedded in gliomas, as shown in animal models (Iosif et al 2008). The success of these novel therapeutic strategies critically depends on the effective recruitment of MSCs to glioma cells and tissues (Hanif et al 2017). Therefore, enhancing the potential of MSCs to migrate to and within invasive solid tumors could improve the clinical application of these cells as vector systems for the selective treatment of highly aggressive tumors.

In this study, we investigated the effects of TNF-α pretreatment on the glioma tropism of MSCs. We observed that invasion into glioma spheroids was enhanced in MSCs previously incubated with TNF-α. The enhanced infiltration of TNF-α-pre-primed MSCs into glioma spheroids may be explained by the adoption of an expression profile and characteristics resembling neural cells, such as neural progenitor cells, also known for their tropism to brain tumors. Moreover, our approach demonstrated that MMP activity appears crucial for the penetration of MSCs and glioma cells, as evidenced by the use of a broad-spectrum MMP inhibitor.

By combining multi-photon microscopy and a chronic cranial window in nude mice (allowing for noninvasive serial detection of brain tumors and metastases *in vivo* at cellular resolution) (Winkler et al 2009), we could monitor the tropism of MSCs to experimental gliomas in mice. Importantly, the pretreatment of MSCs with TNF-α enhanced the glioma-specific recruitment of MSCs after intravenous injection. It was previously assumed that local implantation of MSCs, requiring surgical access to the tumor, is a suitable strategy to enable therapeutic infiltration of MSCs into the tumor. However, improving the tumor tropism of intravenously administered MSCs through TNF-α pretreatment could represent a less invasive alternative in the treatment of malignant gliomas.

Conclusion and future perspective

In summary, our data indicate that the culture expansion of MSCs in the presence of TNF-α generates cells with neuroglial properties and enhances the ability for CXCR4/SDF-1-directed migration through alterations in the expression level of various neural genes. These insights could improve the development of MSC-based therapeutic strategies for the treatment of neurodegenerative diseases and malignancies.

6.1.2 TIMP-1 regulates mesenchymal stem cells through let-7f miRNA and Wnt/β-catenin signaling.

(Egea et al. 2012)

Already in 2007 (Ries et al 2007), we could demonstrate that the constitutive expression of matrix metalloproteinase 2 (MMP-2), membrane type 1 MMP (MT1-MMP), TIMP-2 but not TIMP-1 essentially contribute to the ability of bone marrow–derived MSCs to traverse human reconstituted basement membranes. Now in this study, we were able to describe a mechanism by which TIMP-1 regulates essential stem cell functions. Inhibition of TIMP-1 expression in MSCs significantly enhanced cell growth and differentiation into the osteogenic lineage, suggesting that TIMP-1 is an endogenous inhibitor of these processes. Previous reports already have shown that TIMP-1 inhibits the proliferation of epithelial cells and osteoblastic differentiation, but stimulates the differentiation of hematopoietic progenitor cells, B cells, and Burkitt lymphoma cells (Fata et al 1999, Schiltz et al 2008). However, none of these studies provided information on the underlying molecular mechanisms. Given the high secretion of TIMP-1 in MSCs and the absence of its main target protease MMP-9, our hypothesis was that TIMP-1 could be involved in non-proteolytic functions in these cells.

Our studies demonstrated that reducing TIMP-1 production in MSCs increases the stability, nuclear translocation, and promoter activity of β-catenin, indicating that TIMP-1 is an inhibitor of the β-catenin-signaling pathway. In our study, both endogenous TIMP-1 and exogenously added recombinant TIMP-1 acted as negative regulators of β-catenin activity in MSCs, thereby preventing the upregulation of β-catenin target genes Cyclin D1 and MT1-MMP (Quiescent state, Fig.6).

We also shown that TIMP-1 colocalizes with CD63 at the surface of MSCs, suggesting an extracellular mechanism by which TIMP-1 reassociates with the plasma membrane of MSCs. Functional depletion of CD63 in MSCs significantly increased Wnt/β-catenin activity. This result is consistent with previous reports showing that CD63 can regulate the activity of phosphatidylinositol-3-kinase, focal adhesion kinase, Src, and Akt, all of which have been associated with the anti-apoptotic activity of TIMP-1 (Liu et al 2003). The addition of TIMP-1 to CD63-depleted MSCs reduced the increase in Wnt/β-catenin activity in these cells, suggesting that CD63 is not the only structure that interacts with TIMP-1 and thereby influences gene expression in these cells.

To further investigate the intracellular molecular mechanism by which TIMP-1 suppresses the stability and activity of β-catenin in MSCs, we investigated the role of miRNAs in this process. Functional disruption of the miRNA processing machinery significantly inhibited β-catenin activity in TIMP-1-depleted MSCs, indicating that precise amounts of mature miRNAs are critical for the TIMP-1-mediated modulation of β-catenin. This finding is consistent with other studies showing that various miRNAs can act as positive or negative regulators of β-catenin transcriptional activity (Huang et al 2010). Among the miRNAs that were upregulated following the knock down of TIMP-1 biosynthesis

in MSCs, let-7f showed the most significant increase. Let-7 family of miRNAs is considered as "pro-differentiation factors" with "anti-stem cell properties" (Peter 2009). For instance, let-7 is undetectable in embryonic stem cells but become highly enriched following the onset of differentiation. (Peter 2009, Viswanathan et al 2008). In MSCs, let-7 is upregulated during osteogenic differentiation and presumably downregulate factors that inhibit osteogenesis (Oskowitz et al 2008). Previous studies already described Axin-2, a negative regulator of the Wnt/β-catenin pathway, as a let-7f target (Jho et al 2002). Here, we could demonstrate that let-7f targets Axin-2 in the absence of TIMP-1 favouring an activation of the Wnt/β-catenin signaling in MSCs (Active state, Fig.6).

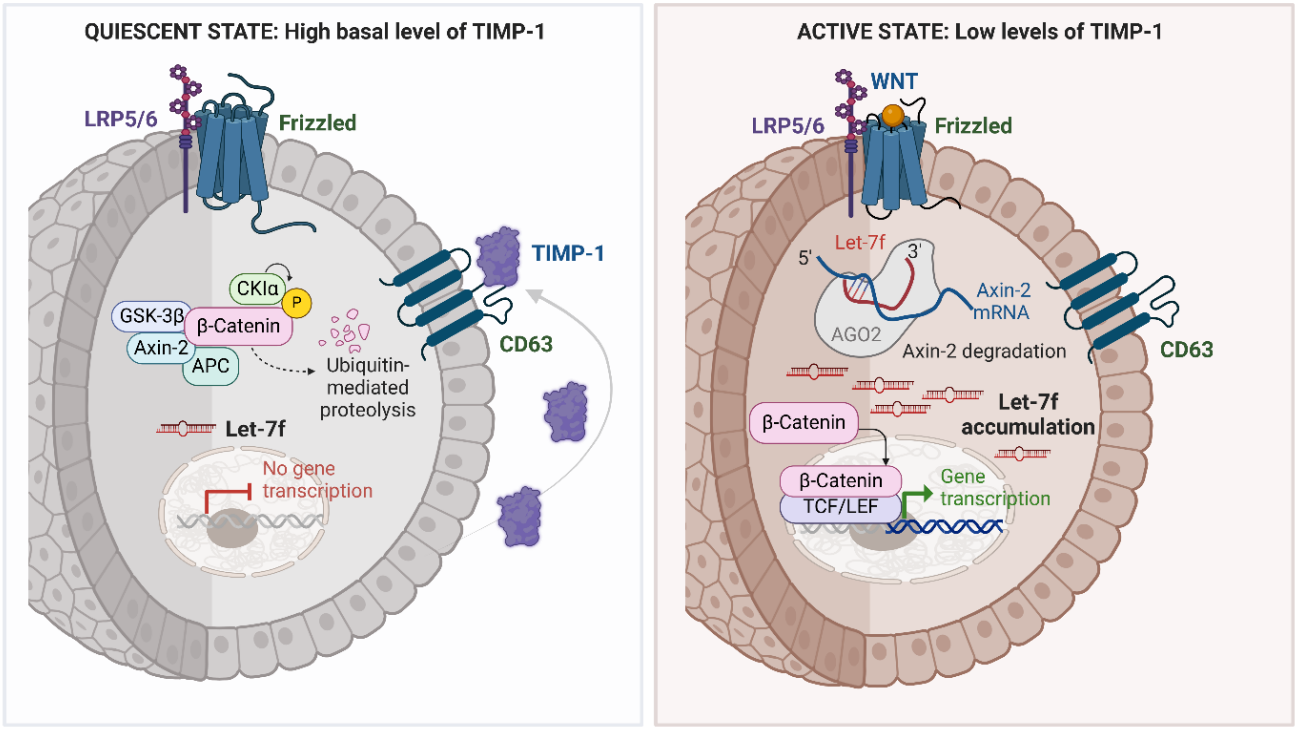


Fig.6: Proposed model for TIMP-1-mediated suppression of the Wnt/β-catenin signaling pathway via let-7f in human mesenchymal stem cells. (**QUIESCENT STATE**) MSCs secrete TIMP-1, which can bind to CD63 at the cell membrane. This action leads to the degradation of β-catenin through a complex containing Axin 2. (**ACTIVE STATE**) In the absence of TIMP-1, intracellular let-7f miRNA accumulates, which inhibits the translation of its target gene Axin-2, allowing the stabilization of β-catenin. This results in (TCF/LEF)-dependent transcription of Wnt/β-catenin target genes. Consequently, proliferation and differentiation into bone cells are promoted in TIMP-1-depleted MSCs. Figure by V. Egea.

Conclusion and future perspective

Over an extended period, TIMPs have primarily been studied for their role as MMP inhibitors. The results of our studies, however, suggest that TIMP-1 possess additional biological functions as signaling molecule. These functions are independent of its ability to inhibit metalloproteinases and instead involves direct binding to CD63 receptor at the cell surface. This cytokine-like activities of TIMP-1 are in line with other studies showing TIMPs to encompass the regulation of cell proliferation, apoptosis, differentiation, and angiogenesis (Liu et al 2003). In MSCs, TIMP-1/CD63-induced signaling promotes quiescence by attenuating let-7f-controlled Wnt/β-catenin activity. This quiescence is reversed in the absence of TIMP-1, facilitating the transition of these cells into an active state with an increased capacity for differentiation. These findings underscore the significance of post-transcriptional and post-translational mechanisms in TIMP-1's regulation of cellular functions.

Moreover, the interaction between TIMP-1 and cell surface receptors, as well as subsequent gene regulation, is greatly influenced by the levels of proMMP-9 in the pericellular environment. ProMMP-9 is the primary target protease of TIMP-1. The dual nature of TIMP-1, acting as both a cytokine and a metalloproteinase inhibitor, highlights its crucial role in facilitating communication between intracellular signaling networks and the extracellular matrix (ECM). Further investigations into the molecular mechanisms underlying TIMP's cytokine activities in different cell types would enhance our understanding of TIMP-mediated cellular processes in both physiological and pathological conditions. This knowledge may prove valuable in the development of rational, mechanism-based therapies for the treatment of cancer and other chronic diseases.

Crucially, the findings indicate that either reducing TIMP-1 levels or augmenting let-7f expression can enhance osteogenic differentiation in MSCs. Gaining a more comprehensive understanding of this regulatory mechanism could potentially aid in creating new treatments aimed at stimulating bone growth in conditions characterized by pathological bone loss.

6.1.3 Let-7f miRNA regulates SDF-1α- and hypoxia-promoted migration of MSCs and attenuates mammary tumor growth upon exosomal release.

(Egea et al. 2021)

Previously, we demonstrated that inflammatory cytokines and SDF-1α enhance MSC invasion through increased MMP production (Egea et al 2011). In this study, we introduced let-7f to be a crucial regulator of MSC invasion in response to these factors and a potential candidate for therapeutic interventions against cancer.

Overexpression of let-7f in MSCs led to an upregulation of the SDF-1α receptor CXCR4, an increase in MMP-9 release, and elevated pericellular proteolysis thereby promoting MSC invasion. Remarkably, while let-7 family members often act as tumor suppressors by inhibiting growth and MMP-dependent invasion in cancer cells (Noruzi et al 2018, Sun et al 2016), we could demonstrate an opposite role for let-7f in MSCs, a characteristic that may be beneficial for therapeutic strategies.

Hypoxia (Luo et al 2022), is a common feature of myocardial ischemia and cancer (Eltzschig et al 2014, Semenza 2003), being known to attract MSCs (Liu et al 2011). Preconditioning MSCs with hypoxia has been shown to enhance their migration, survival, and immunosuppressive capabilities (Liu et al 2011). In this study, we could demonstrate that hypoxia-induced MSC migration is also modulated by let-7f. This effect is similar to the role of let-7b in hypoxia-mediated cell proliferation and cell cycle regulation in zebrafish (Huang et al 2017). Additionally, hypoxia activated autophagy in MSCs, a process that is crucial for cellular stress responses and is cytoprotective, in contrast to apoptosis, which is cytodestructive (Marino et al 2014). Our research results demonstrated that let-7f itself, can as well activate autophagy, thereby increasing the invasion capabilities of MSCs. This aligns with findings in neural cells where let-7 regulates migration through autophagy via the mTOR signaling pathway (Petri et al 2017).

The secretome of MSCs is thought to have therapeutic effects in multiple diseases (Kalluri & LeBleu 2020, Roszkowski 2024). In this study, we could shown that SDF-1α, hypoxia, and autophagy activation not only increased intracellular let-7f levels but also promoted its secretion in exosomes. MSC-Exos are considered potential anti-cancer agents due to their ability to transfer their contents to host cells in tumor tissue (Roszkowski 2024). Our study showed that MSC-Exos enriched with let-7f are uptaken by breast cancer cells impairing their proliferation and invasion more effectively than exosomes without let-7f enrichment. This is consistent with the known tumor suppressor function of let-7 in cancer (Johnson et al 2005).

Conclusion and future perspective

The ability of MSCs to home to tumors is crucial for their use in cancer management. Our findings demonstrated that let-7f plays a significant role in the cytokine/chemokine-mediated tumor tropism of MSCs by positively regulating MMP-9 and CXCR4. Using a mouse breast tumor model, we could demonstrated that MSCs overexpressing let-7f exhibit anti-tumorigenic functions at the tumor site, through the exosomal delivery of let-7f. Let-7f overexpressing MSCs or their exosomes are therefore a potential way of intervention in order to replenish the let-7 pool in cancer cells.

Despite their anti-tumorigenic functions, MSCs can also contribute to tumor-promoting processes such as angiogenesis and therapy resistance, necessitating further research to optimize MSC-based therapies and the use of MSC-Exos in cell-free treatments. Factors such as the source of MSCs and their culture conditions need to be thoroughly investigated, as they have been shown to influence the functional properties of the exosomes.

6.1.4 Properties and fate of human mesenchymal stem cells upon miRNA let-7f-promoted recruitment to atherosclerotic plaques.

(Egea et al. 2023)

Atherosclerosis (AS) is a chronic inflammatory reaction of the blood vessel wall caused by dyslipidemia (Weber & Noels 2011). Inflammation is important in all stages of atherosclerosis, from plaque formation to rupture (Libby 2021). When the endothelium is dysfunctional, it disrupts the balance between pro-inflammatory and protective pathways, leading to the accumulation of atherogenic lipoproteins. This triggers the release of chemotactic factors that recruit immune cells and promote the formation of atherosclerotic plaques. Despite the availability of appropriate pharmacological and surgical treatment modalities, AS remains the leading cause of cardiovascular death worldwide (Vaduganathan et al 2022). Current treatment strategies aim to stabilize plaque, suppress inflammation, and lower serum lipid levels. Interestingly, stem cells exhibit a range of effects, including the ability to regulate lipid levels, suppress inflammation, repair damaged tissues, and support hematopoiesis, offering an innovative approach to the treatment of AS (Li et al 2017, Nauta & Fibbe 2007).

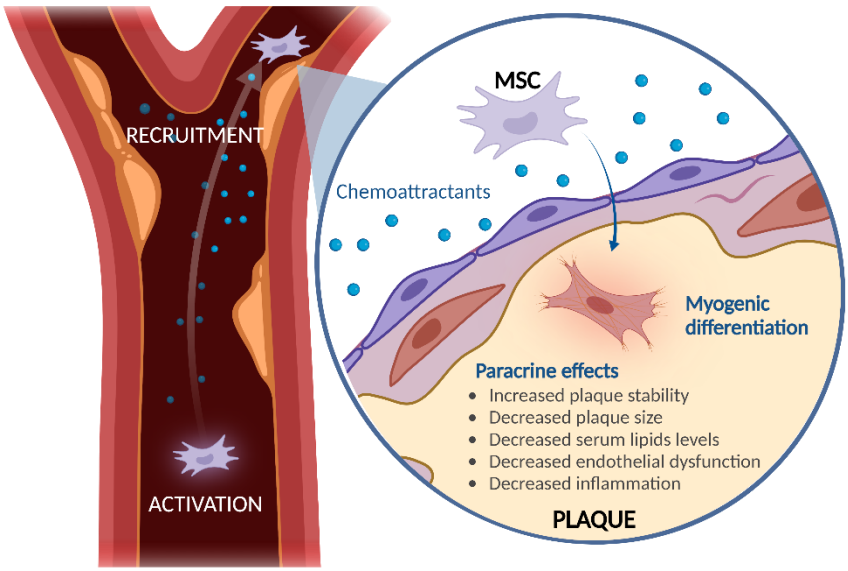


Fig.7: Recruitment of human MSCs to atherosclerotic plaque. *MSCs* exhibit a natural tendency to migrate to sites of inflammation, including atherosclerotic plaques. Once at these sites, MSCs display a protective role primarily through paracrine signaling, which helps in reducing endothelial dysfunction, hyperlipidemia, and inflammation. This results in an overall increase in plaque stability and decrease in plaque size. Furthermore, our studies have shown that MSCs are stimulated by the surrounding atherosclerotic plaque to differentiate into myogenic cells (Egea 2024). Figure by V. Egea.

Atherosclerotic plaques, being inflammatory lesions, also generate high levels of cathelicidin antimicrobial peptide LL-37, a small peptide derived from neutrophils which is believed to contribute to disease progression (Zhang et al 2015). LL-37 has been demonstrated to elevate early growth response factor 1 (*EGR1*) expression and stimulate mitogen-activated protein kinase (MAPK) activation, thereby augmenting MSC functions such as cell proliferation, cell motility, and paracrine activities. These regulatory effects could prove beneficial for tissue regeneration applications, particularly in the context of implantation (Yang et al 2016). In this study, we could demonstrate that LL-37, known to be prevalent in the plasma and plaques of atherosclerosis patients, serves as a

chemoattractant for MSCs. Our investigation also identified miRNA let-7f as a pivotal regulator in the LL-37 mediated trafficking of MSCs to inflamed tissues. LL-37 influences cells through formyl peptide receptor 2 (FPR2), a prevalent G protein-coupled receptor that triggers the expression of miRNA let-7f. This, in turn, enhances FPR2 on the cell surface in a positive feedback loop, implying an indirect regulatory function of let-7f by targeting a suppressor of FPR2 expression in these cells. A similar mechanism of let-7f indirectly boosting CXCR4 expression in MSCs was also described in Egea et al. 2021. Therefore, let-7f not only enhances LL-37/FPR2-mediated chemotaxis towards plaques but also upregulates CXCR4, and induces the expression and release of ECM-degrading MMP-9, thereby efficiently improving MSC invasion (Egea et al 2021).

Upon recruitment to the plaque site, MSCs are subjected to the influence of the new environment, which potentially jeopardizes their stemness and triggers differentiation. In this study, we could provide evidence that human plaque components elicit myogenic differentiation of MSCs into smooth muscle-like cells. However, understanding their role in plaque development is more complex. Early in plaque formation, MSCs could contribute to inflammation and foam cell formation, while in later stages, they are expected to produce extracellular matrix proteins and collagen fibers, which help stabilize the plaques (Bennett et al 2016). Interestingly, vulnerable plaques prone to rupture are characterized by only few smooth muscle cells in a thin cap (Finn et al 2010). Thus, myogenic differentiation of MSCs in plaque environment might confer a stabilizing effect in vulnerable plaques in later stages of AS. In fact, transplanted MSCs were shown to stabilize vulnerable plaques in an animal model of AS by strengthening the fibrous cap (Wang et al 2015). Consistently, we found that human plaque lysates up-regulated endogenous levels of miR-335 in MSCs, a miRNA shown to promote overall plaque stability.

Conclusion and future perspective

Numerous research efforts have highlighted the propensity of MSCs to migrate towards atheromatous tissues, contributing to atheroprotection through the secretion of paracrine factors and their maturation into cells that stabilize plaques. The differentiation potential, paracrine effects, exosomal release, and direct-contact modulatory functions of MSCs have been the focus of extensive investigation. Each of these mechanisms plays a role in the holistic process of MSC therapy in AS. Nevertheless, the protective mechanisms of MSCs warrant further exploration. Understanding the function of MSCs in varying stages of atherosclerosis, the disparities among MSC sources, and the efficiency of MSC recruitment to the plaque are all crucial areas for further study to enhance the safety, efficacy, and outcomes of MSC-based therapy (Egea 2024).

6.2 Non canonical effects of miRNAs

The majority of studies on miRNAs, are based on their canonical way of function where they target mRNAs for inhibition of translation or degradation of mRNA (Fig.3). However, our research suggests that miRNAs can also function beyond this canonical paradigm, exhibiting novel unconventional regulatory roles that may play a pathophysiological role in cardiovascular diseases.

Fig.8: Breaking the miRNA dogma.

The artwork by Banksy, initially known as "Girl with Balloon," experienced a metamorphosis when it was partially shredded during an auction; this event paradoxically increased its worth (Badshah 2021). This image serves as a metaphor for the disruption of the established model of miRNA function, suggesting that miRNAs have roles beyond the traditional framework, thereby adding a substantial layer of complexity and amplifying the significance of miRNAs.

Figure by V. Egea.

6.2.1 Noncanonical inhibition of caspase-3 by a nuclear microRNA confers endothelial protection by autophagy in atherosclerosis.

(Egea et al. 2020)

In this study, we explored the function of miRNA-126-5p in the autophagy of endothelial cells within the context of AS. We found that increased shear stress led to the nuclear translocation of miR-126-5p in endothelial cells, where it exerted antiapoptotic effects by directly interacting with caspase-3. This pathway was also identified in the aortas of atherosclerotic mouse models and in human vascular disease tissue samples. Our findings revealed an unconventional role of miR-126-5p in safeguarding against endothelial cell dysfunction (Santovito et al 2020).

Our research centered on endothelial cells and was able to show that miR-126-5p, when complexed with AGO2 and the RNA-binding protein MEX3A, is atypically translocated into the nucleus of the cell. Once in the nucleus, miR-126-5p dissociates from AGO2 and MEX3A to interact with effector caspase 3 (Fig.9). This interaction requires both an intact seed sequence of miR-126-5p and the substrate-binding pocket as well as the interface for caspase-3 p17-p12 heteromer assembly. The binding of miR-126-5p to caspase 3 inhibits its catalytic activity and thus apoptosis in the cells, which promotes endothelial integrity.

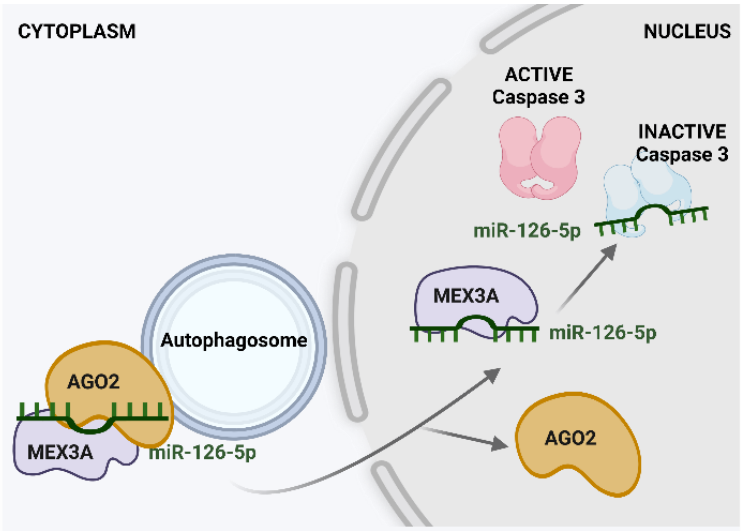


Fig.9: The noncanonical role of miR-126-5p within the nucleus of endothelial cells:

Under conditions of shear stress and active autophagy, a complex consisting of miR-126-5p, Mex3A, and AGO2 forms in the cytosol. This complex is subsequently directed to the extraluminal surface of the autophagosome, which shields it from degradation and aids in its nuclear transport. Upon entry into the nucleus, miR-126-5p dissociates from AGO2 and Mex3a, rendering it free to interact with its target, Caspase 3. Figure by V. Egea.

Remarkably, this non-canonical function of miR-126-5p coexists with the miR-126-3p-mediated silencing of Dlk1 and synergistically protects endothelial integrity by reducing apoptosis of endothelial cells and increasing their proliferation (Schober et al 2014). Although the structural aspects of this non-canonical function of miR-126-5p require further investigation, MEX3A is not strictly necessary for the inhibitory function but facilitates the interaction between miR-126-5p and caspase 3. Therefore, MEX3A's role may be to be responsible for unloading miR-126-5p from AGO2 and enabling aptamer-like miRNA functions for miR-126-5p and possibly other miRNAs.

Autophagy is an integral part of well-orchestrated cellular stress response programs. Many autophagy triggers can also induce apoptosis, with both processes often occurring in the same cell. Apoptosis refers to a type of programmed cell death that is regulated by specific cascades of proteases. These cascades ultimately lead to the activation of effector caspases, such as caspase-3, which play a crucial role in causing cell death. Given the antagonistic roles of the two processes, where autophagy is cytoprotective and apoptosis is cytodestructive, it is conceivable that an inhibitory interaction exists. For instance, the removal of damaged mitochondria or proapoptotic proteins during autophagy can inhibit apoptosis, while caspase-3 can cleave autophagy-specific proteins to impair autophagy (Marino et al 2014). Our findings reveal a mechanism by which autophagy can inhibit apoptosis through miR-126-5p. This inhibition mainly affects caspase cleavage, not expression, and its rapid onset precedes transcriptional changes, suggesting that miR-126-5p acts directly on effector caspases. Among these, caspase-3 is essential for nuclear changes during apoptosis and localizes to the nucleus upon activation and substrate recognition, regardless of their cleavage (Kamada et al 2005). In this compartment, interaction with miR-126-5p could prevent the catalysis of nuclear components to maintain cell viability. Typically, the inhibition of apoptosis through direct control of caspase-3's catalytic activity occurs via protein-protein interactions. Our results integrate and expand the pathophysiological relevance of related findings that show:

(i) miRNAs have the ability to form a stable secondary structure that resembles aptamers. Aptamers are short sequences of oligonucleotides that exhibit a high affinity for specific molecules, including proteins; (ii) miRNAs and AGO2 are often localized in low molecular weight complexes without functional RISC components, forming in vivo miRNA reservoirs; and (iii) the conserved non-coding RNA vtRNA1-1, known as Riboregulator, can prevent the oligomerization of the protein p62, thereby impairing its function in autophagy (Horos et al 2019).

Conclusion and future perspective

miR-126-5p is present in ECs in a remarkable abundance compared to other cell types and likely serves as an important switch to control programmed cell death in ECs. Maintaining endothelial integrity is crucial in AS. Our results add a new layer of complexity for miR-126 in the manteinance of endothelial integrity.

Promoting the nuclear accumulation of miR-126-5p could be therapeutically relevant to prevent endothelial dysfunction and the development of AS. Tailored overexpression of Mex3a could be achieved using endothelial-specific constructs in viral microparticles. Our data reinforce the notion that proteins relevant to cell viability, such as caspase-3 or p62, previously not recognized as RNA-binding proteins, are indeed involved in such processes. Modulating this pathway could provide therapeutic options in vascular disease and beyond.

7 Perspective

The research projects presented here have demonstrated the significant role of miRNAs in the diverse functions of MSCs and endothelial cells. Our findings emphasize the significance of let-7f in the field of MSC-biology and its relevance in the context of MSC-based therapies for cancer and AS. Our studies explored the positive potential of let-7f in both MSCs and MSC-Exos in different disease models. MSC-Exos, in particular, could offer significant advantages over MSCs themselves as they effectively reduce adverse effects associated with cell infusion. Certainly, MSC-Exos are emerging as a promising cell-free therapeutic tool, with a growing number of clinical studies examining their therapeutic effects in different diseases.

The differentiation potential, paracrine effects, exosomal release, and direct-contact modulatory functions of MSCs have been investigated in our studies, as each of these mechanisms plays a role in the comprehensive process of MSC therapy. Nonetheless, further exploration is needed to fully understand the protective mechanisms of MSCs. It is crucial to study the function of MSCs in different stages of disease, the differences among MSC sources, and the effectiveness of MSC recruitment to the tumor or plaque. These areas of research are essential for enhancing the safety, efficacy, and outcomes of MSC-based therapy.

We also explored the noncanonical role of miR-126-5p within the nucleus of endothelial cells. Investigating whether other miRNAs have similar mechanisms of action could greatly enhance our understanding of the underlying causes of various disorders. This, in turn, could facilitate the development of innovative miRNA-based therapeutic approaches for the treatment and prevention of these diseases.

In the future, my research interests are focused on exploring the relevance of non-canonical miRNA functions in cardiovascular homeostasis and pathology. The goal is to incorporate these newly discovered properties of miRNAs into the development of innovative translational therapeutic approaches.

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