GENOME-WIDE MICRORNA EXPRESSION ANALYSIS IN TWO CLINICALLY RELEVANT MODEL SYSTEMS

PhD thesis

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List of abbreviations

3′-UTR: 3′-Untranslated region

BMMC: Bone marrow-derived mast cell

CCL: Chemokine ligand

CREB: Cyclic AMP-response-element-binding protein

CTMC: Connective tissue-type mast cells

CXCL: Chemokine (C-X-C motif) ligand

DNP-HSA: Dinitrophenyl-human serum albumin

FAM: Fluorescein amidite

FBS: Fetal bovine serum

Fc∈RI: Fc-epsilon receptor I

FGF: Fibroblast growth factor

FYN: Oncogene related to SRC, FGR, YES

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GSEA: Gene Set Enrichment Analysis

HBEGF: Heparin-binding EGF-like growth factor

HER2: Human epidermal growth factor receptor 2

ICAM: Intercellular Adhesion Molecule

IL: Interleukin

KIT: KIT Proto-Oncogene, Receptor Tyrosine Kinase

LFA: Lymphocyte function-associated antigen

LPS: Lipopolysaccharide

LYN: v-yes-1 Yamaguchi sarcoma viral related oncogene homolog

MeCP2: Methyl CpG-binding domain protein-2

miR/miRNA: microRNA

MMC: Mucosal mast cells

mMcpt1: Mouse Mast Cell Protease-1

NGF: Nerve growth factor

ncRNA: Non-coding RNA

PAF: Platelet-activating factor

PLC-γ: Phospholipase C-γ

RISC: RNA-induced silencing complex

SCF: Stem cell factor

SYK: Spleen tyrosine kinase

TGF-β: Transforming growth factor beta

TLR: Toll-like receptor

VEGF: Vascular endothelial growth factor

1. Introduction

1.1. MicroRNAs as a layer of gene expression regulation

1.1.1. Non-coding RNA world

Recognition of the control of gene expression by small, noncoding RNA molecules dated nearly 30 years ago represents a fundamental step for molecular genetics (Lee et al. 1993). The non-coding RNAs (ncRNAs) and their functions were out of focus of investigations, primarily due to the fact that they are not converted into proteins and therefore the interpretation of their function is difficult. These signals were thought to come from "junk" regions of the genome that needed to be distinguished from "transcriptional noise." (Costa 2007). According to estimates, non-protein-coding RNA accounts for 97% of a human cell's transcriptional output, and large-scale sequencing have revealed that the majority of transcriptomes have little or minimal protein-coding ability (Mattick 2001).

After the discovery of RNA-interference and microRNAs (miRNAs), and with improved methods to explore the transcriptome, in recent years there have been great advances in identifying and understanding non-coding RNAs. The approach to non-protein-coding genomic sections changed fundamentally and life science began to puzzle out a new-defined layer of gene expression regulation (Bartel 2004). Meanwhile investigations of small non-coding RNAs unfolded many, yet unpredictable regulatory relationships. It is now clear that functions of non-coding kingdom involve gene silencing, gene transcription, DNA imprinting, DNA demethylation, chromatin structure dynamics, and RNA interference as well. At the same time, several efforts were induced to unveil and annotate new super-families of non-coding genes in the genomes with known sequence (Uszczynska-Ratajczak et al. 2018). Non-coding RNAs are summarized as transcripts that are not translated (synonym: non-protein-coding RNA), that are typically divided into small and medium/large size molecules (i.e. long non-coding RNAs that are longer than 200 nt). Another classification of RNA families integrates the well-known ribosomal, transfer, and messenger RNA as housekeeping and is distinguished from the regulatory class. The relevance of microRNAs in regulating gene expression by interacting with messenger RNA (mRNA) — either by inhibiting mRNA translation or by inducing mRNA degradation — should be highlighted among non-protein coding RNAs (Ambros 2004, Jonas and Izaurralde 2015, Krutzfeldt et al. 2005, Lim et al. 2005).

1.1.2. MicroRNAs are post-transcriptional regulators of cell fate decisions

Mature forms of miRNAs are a class of conserved, approximately 21-25 nucleotide long RNAs, which are coded in their own transcription units (they can be intergenic, intronic or exonic). The primary or pri-miR transcripts are substantially longer than the mature miRNA and create secondary structures with a hairpin structure enclosing the mature sequence. After a characteristic multi-stage maturation process, miRNAs integrate into the RISC complex and guide it to the 3'UTR of target messenger RNAs and induce its degradation or inhibit the translation of the protein **(Figure 1)**. The interaction between miRNAs and target mRNA depends on the sequence complementarity.

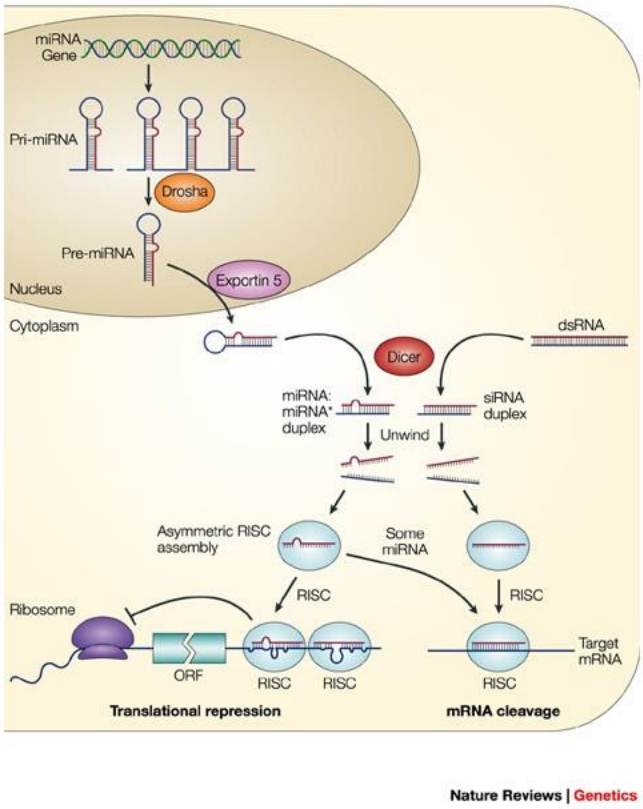


Figure 1. Overview of biogenesis of miRNA. Firstly, the pri-miRNA transcripts are converted into pre-miRNAs which are transported to the cytoplasm and processed into miRNA:miRNA* duplexes by Dicer like the siRNA duplexes. One strand of duplex is assembled into the RNA-induced silencing complex (RISC), which subsequently acts on its target by translational repression or mRNA cleavage, depending on the complementarity between the miRNA and its mRNA target Adapted from (He and Hannon 2004). A number of non-canonical ways of miRNA synthesis have been reported. For instance, a subset of intronic miRNAs known as mirtrons skip the Drosha step, and the splicing machinery itself creates pre-miRNAs. On the other hand, a Dicer independent mechanism is used to generate some miRNAs, such as miR-451.

MiRNAs bind to partially complementary sequences of target mRNAs. Imprecise complementarity allows miRNAs to bind to hundreds of targets, thus a single miRNAs can have much larger than anticipated effects on whole transcriptome, as the spectrum of targets is much wider than that of coding genes.

MiRNAs have been identified in both plants and animals, acting as key regulators of multicellular differentiation. Many miRNAs are conserved across species and their expression is either tissue-specific or restricted to certain stages of development. (Guo et al. 2014, Lagos-Quintana et al. 2002).

In accordance with miRNAs control a range of developmental events, such as timing of cell-fate decision, stem cell maintenance, apoptosis and organ morphogenesis, they are suggested to play a role in the appropriate establishment of the tissue or cell type specific expression patterns along the development. It is now already established that miRNAs contribute not only to developmental processes related functions, as they are able to adjust the concentration of many proteins in a synchronized and rapid manner, miRNAs are thought to play an important role in cell responses and adaptation to the environment. Intriguingly, miRNAs may also participate in intercellular communication as they can be identified as a component of exosomal shuttled RNA in exosomes secreted by mast cells. It is suggested that this may allow the donor cell to directly influence gene expression in target cells at the post-transcriptional level (Valadi et al. 2007).

MiRNAs also play a major role in the regulation of hematopoietic differentiation, where hematopoietic stem cells are responsible for the lifelong regeneration of all mature blood cells. Proper stem cell function is regulated by interplay of extrinsic factors secreted by the supporting cells in the stem cell niche of the bone marrow and by cell-intrinsic regulatory programs, involving also miRNAs. In addition to maintaining self-renewal, several studies have demonstrated that miRNAs influence the fate of hematopoietic stem cells by targeting essential lineage-specific transcription factors (Bissels et al. 2012, O'connell R. M., Chaudhuri, et al. 2010).

The key role of miRNAs is illustrated by knockout of Dicer, the key enzyme for the processing of pre-miRNAs into mature miRNAs. Firstly, the in vivo impact of deficiency can be only investigated in conditional knockouts, as Dicer knockouts are embryonic lethal. The global deficiency of miRNA regulatory layer leads to defective differentiation, however, it is not constant throughout the entire process. With the conditional inactivation

of Dicer at the early stage of T-cell development using Lck-Cre transgenic mouse, it was demonstrated that Dicer is not essential for lineage commitment, whereas it is involved in the process later, in the development of regulatory T cells (Cobb et al. 2006, Cobb et al. 2005).

1.1.3. Target recognition by microRNA

The regulatory potential of miRNAs is enormous: more than 60% of protein-coding genes have been computationally predicted as targets. This estimation is based on a conserved base pairing between the 3′ UTR and a mostly heptametrical sequence positions situated 2-7 from the miRNA 5′-end, known as seed region (Friedman et al. 2009). Although the binding sites of many miRNAs and their targets are deeply conserved, suggesting an important function, a typical miRNA-target interaction results in only a subtle (less than 2-fold) decrease in protein levels (Baek et al. 2008), and many miRNAs can be deleted without producing any obvious phenotype.

As the region of complementarity is typically short and the base pairing imprecise between miRNA and their targets, a given miRNA can have hundreds of targets. The result of miRNA-mediated regulation can be detected mainly on protein level in animals, and for this purpose application of high-throughput experimental techniques are needed. To resolve this problem, several computational algorithms were developed, which are able to predict more or less reliably the putative targets of miRNAs. These algorithms search seed sequences to represent complementary motifs in the 3'-UTR of genes.

Over the past two decades, a number of in silico, more or less reliable solutions have been attempted to map miRNA targets (Peterson et al. 2014). Although the criteria for target prediction differ between different calculation methods, they generally take into account the following aspects:

1. Watson-Crick base pairing rules between nucleotides 2–8 of the 5 ′ end of the miRNA and the corresponding linked region of the 3′UTR of the target mRNA.
2. Minimum free energy (MFE) value of the RNA-RNA duplex formed by miRNA and the predicted mRNA target
3. Site accessibility, AU flanking content, position of the target site within the 3′ UTR, and UTR length
4. Evolutionary conservation of the miRNA binding site.

Basically, these algorithms are working with parameters including sequence complementarity, stability of heteroduplex, and evolutionary conservation, however, the even small differences of algorithms can cause a great shift in the list of targets. In fact, the extent of overlap among the target sets predicted by different algorithms is typically low and among the predicted hundreds of targets only a small proportion were experimentally validated (Rajewsky 2006).

Prediction of interactions between RNA molecules is possible even by utilizing only sequence data. It is which is particularly remarkable compared to the in silico predictability of interactions between transcription factors and their binding sites on DNA (protein and nucleic acid!). However, additional, partly unknown factors must be taken into account, as the algorithms predicting miRNA targets are barely acceptable with their high (typically 2/3) false positive rate (Baek et al. 2008, Selbach et al. 2008).

1.2. Stress-responsive miR-132 as a homeostatic regulator in various tissues and functions

MiRNA-132 is a member of the MiR-212/132 family, that are expressed from the same cluster and have identical seed regions, suggesting that they might share the same mRNA targets. The miR-212/132 family is highly conserved in vertebrates. The similarity of the mature sequences of miR-212 and miR-132 and, more importantly, their identical core sequences, suggest that these miRNAs may have evolved from a gene duplication event. MiR-132 and MiR-212 are both expressed in the brain; however, MiR-212 has also been shown to be expressed in the heart and mammary glands. Despite the identical seed, there is also growing evidence that the 3′ non-seed nucleotides of miRNAs, in where there are divergent nucleotides between the sequences of miR-132 and miR-212, play a role in target recognition and may complement or compensate for single nucleotide bulges or divergence in the seed region. In addition, the relations of expression levels may be important as they are apparently different in cell types and conditions due to a presumed differential processing of miR-132 and miR-212.

The regulatory role of miR-132 in the nervous system is well established first (Klein et al. 2007). Later, miR-132/-212 dysregulation has been associated with several brain-related disorders, found to be deregulated in various neurodegenerative conditions, such as Huntington's disease, Parkinson disease, Alzheimer disease (Haviv Rotem et al. 2018). Also there are remarkable similarities comparing mice and humans in terms of miR-132

regulation and function as suggested by enrichment analysis identified co-regulation of stress-related targets in inflammatory, metabolic and neuronal growth pathways (Haviv Rotem et al. 2018).

Several miR-132-driven mechanisms have been revealed, even beyond the brain and immune system. Some, without claiming completeness, may be worth mentioning due to the similar context or other link to our findings. The miR-212/132 locus is transcriptionally active in cortical neurons stimulated by neurotrophins, it was first identified in neuronal cells as a target of the cAMP-response element binding (CREB) protein transcription factor. Hence, various stimuli lead to the transcription of the miR-212/132 locus through CREB activation, then upregulated miRNAs repress the expression of several mRNA targets, e.g. by decreasing the levels of p250GAP ((Rho GTPase-activating protein 32). In this model, miR-132 promotes neuronal outgrowth and sprouting as well as the resetting of the circadian clock, and it regulates the expression of the glutamate receptors involved in synaptic functions (Wanet et al. 2012).

A fascinating feedback loop mechanism involving miR-132 has been revealed that is responsible for the homeostatic control of MeCP2 (methyl-CpG-binding protein 2) expression. As a dosage-sensitive gene, related to Rett syndrome and MECP2 duplication syndrome, maintaining the level of MeCP2 expression is critical, and over- or underexpression of this regulator causes neurodevelopmental defects. Intriguingly, it was found that MeCP2 induces the neuronal miRNA miR-132 via BDNF (brain-derived neurotrophic factor), which then feeds back to repress MeCP2 (Klein et al. 2007).

The immune system has been the other area of mir-132 driven regulation discovered. An early profiling work identified miR-132 (together with miR-146, miR-155) as an upregulated microRNA in the LPS-stimulated human acute monocytic leukemia cell line THP-1 (Taganov et al. 2006). Notably, LPS failed to induce miR-132 in our IgE/antigen activated mast cells model. Furthermore, miR-132 induction is not detectable even in LPS-stimulated primary mouse bone marrow-derived macrophages (Taganov et al. 2006).

In adaptive immune responses, upregulation of miR-132 reported in CD4+ T cells in response to activation by cognate interaction with dendritic cells. In this work, the phosphatidylinositol 3-kinase mRNA (pik3r1) was identified as a target of miR-132 and

found to be regulated by a combination of miRNAs that control that PI3K signaling is precisely dosed during T cell activation (Gutiérrez-Vázquez et al. 2017).

In a tissue with cells playing multiple roles, the regulatory loops can be even more complicated. Transplantation experiments have demonstrated the essential role of the miR-212/132 family for proper ductal outgrowth during pubertal development of mammary glands in mice. The miR-212/132 cluster is found to be exclusively active in mammary stromal cells. In case of miR-212/132 deficient knockout mice, the level of its target MMP-9 increased and subsequently activated latent TGF-β in the periductal stroma of mutant glands. It resulted in hyperactivation of TGF-β pathway and in turn stimulated the production and secretion of MMP9. In this case, the role of this miRNA-driven regulation may be to suppress this positive feedback loop (Ucar et al. 2010).

It is remarkable that the application of miR-132 is nearing clinical translation in cardiology. Increased expression of the miR-212/132 family was reported in cardiomyocytes during pathological cardiac conditions (Xu et al. 2021). Furthermore, it was demonstrated that transgenic mice overexpressing the miR-212/132 cluster developed pathological myocardial remodeling that led to premature death due to progressive heart failure. The modulation of miR-132 provided proof that it is necessary and sufficient for pathological cardiomyocyte growth in a mouse model of left ventricular pressure overload (Ucar et al. 2012). Genes involved in sarcomere shortening of ventricular cardiomyocytes, Foxo3 (Forkhead Box Protein O3), Serca2a (Sarcoplasmic/Endoplasmic Reticulum Ca2+ ATPase 2) , both is under control of miR-132. By applying locked nucleic acid based antisense inhibitor of miR-132 (antimiR-132) in heart failure mice and in a clinically highly relevant (by the way mangalica breed) pig models demonstrated that systemic administration successfully normalized miR-132 levels in cardiomyocytes and not only safe and well-tolerated but improved heart failure reflected by multiple endpoints as well (Foinquinos et al. 2020).

1.3. Mast cells: versatile sentinels orchestrating response

Mast cells are tissue-resident hematopoietic cells that are located at the boundaries between tissues and the external environment. They as sentinels stand guards in a strategic location at barrier tissues for months or years and are able to detect potential threats through expression of a wide range of receptors. They respond to triggers by producing a wide range of mediators. Their high heterogeneity and plasticity with different biological

activities ensure their multi-purpose and coordinating functions. However, synthesis and storage of preformed mediators ensuring an immediate response makes them indeed potentially dangerous and a central player of many human diseases. Besides mast cell-driven pathologies like urticaria, mastocytosis and many allergic diseases, plenty of (and frequently conflicting) evidence support the role for mast cells in chronic inflammatory diseases and cancer (namely mast cell-associated diseases) as well (Gilfillan et al. 2011, Gurish and Boyce 2006).

1.3.1. Differentiation and heterogeneity of mast cells

Mast cells are derived from CD34+ pluripotent bone marrow stem cells and their differentiation is highly dependent on the presence of the ligand of c-Kit, stem cell factor (SCF).

The real mystery is around the origin of mast cells. A landmark study in 1977 showed that mouse bone marrow reconstitutes mast cell compartments from irradiated mice, implicating the bone marrow origin of mast cells in adult mice (Kitamura et al. 1977). The early controversial results on mast cell origin with relation to other lineages were probably due to the different experimental models used in the previously described studies. Unipotent committed mast cell progenitors were later identified in fetal blood and later in other adult tissues as well (Chen C. C. et al. 2005, Jamur et al. 2005)

However, the exact definition of a cell population based on surface markers was delayed and could not clarify the differentiation trajectory as well. Different concepts have been proposed ranging from the shared basophil/eosinophil/mast cell differentiation or bipotent mast cell/monocyte progenitors to an alternative scenario in which mast cells develop immediately from hematopoietic stem cells. Finally, single-cell RNA sequencing experiments may provide insights into the structure of the differentiation landscape and suggest the involvement of the CD34+ c-Kit int/hi FcεRI+ population in the bone marrow for mast cell progenitors (Grootens et al. 2018).

During their maturation, precursor cells exit from the bone marrow and migrate to the peripheral tissues, meanwhile finishing their maturation depending on the tissue environment. Depending on the tissue environment, their differentiation is completed as morphologically and functionally distinct mature mast cell types.

In rodents at least two subpopulations of mast cells, namely connective tissue-type and mucosal mast cells (CTMC and MMC) can be distinguished by the set of proteases

[CTMC: high levels of Mcpt1 and 2, MMC: Mcpt4, 5 (chymase1, Cma1), 6 (tryptase beta2, Tpsb2), 7 (tryptase alpha / beta1) and carboxypeptidase], their localization in the body, staining properties, mediator content, and dependence on T-cell-derived cytokines. In humans, mast cell subgroups can generally be classified according to their protease content: MCT type produces only tryptase, MCTC cells also produce tryptase and chymase, two protease families that are involved in a number of pathological conditions, like proinflammatory and protective actors (e.g., parasitism and bacterial infections, allergic inflammations, arthritis, etc.). The MCT type corresponds to the rodent MMC, while the MCTC corresponds to the CTMC type (Galli, Grimbaldeston, et al. 2008, Gilfillan et al. 2011, Gurish and Boyce 2006, Pejler et al. 2010). Mast cells and basophils share many similarities, notably their role in allergy, their activation through the high-affinity IgE-receptor and their secretion of histamine and lipid mediators following activation, however, mast cells are tissue-resident, while basophils are blood-born and recruited to tissues in an activated state.

1.3.2. Mast cells are a source of a wide array of mediators

Mast cells are primarily known as central effector cells for allergic inflammation and also play an important role in coordinating host defense mechanisms, influencing both innate and adaptive responses. Mast cell activation results in the release of a number of mediators during the allergic reaction, which is triggered by the interaction of IgE and the high-affinity receptor (FcεRI) on the surface of mast cells. This process is initiated by cross-linking of Fcε receptors, which is mediated by IgE molecules bound by the antigen to its receptor. This is followed by rapid phosphorylation of kinases such as FYN, LYN, or SYK on tyrosine, resulting in the induction of PLC-γ, which catalyzes the release of inositol triphosphate from the plasma membrane, thus mobilizing cellular calcium stores to increase intracellular calcium itself. With the opening of the sensitive cell surface calcium channels, the process is further intensified and the exocytosis of its mediators (preformed) previously accumulated in granules in mast cells.

The most well-known activation process of mast cells is the crosslink of the high-affinity FcεRI-bound IgEs, which characterizes allergic disorders. In allergy, allergen-specific IgE antibodies are generated, which are captured by receptors on mast cells and pre-activated (sensitized) as a result **(Figure 2)**. Upon repeated contact with the allergen, mast cells are activated (technically degranulated) and undergo immediate mediator release

(Moon et al. 2014). Additionally, mast cells can also become activated via Fc receptors for IgA and IgG, complement receptors and toll-like receptors (TLRs) as well. Accordingly, beyond the interaction of IgE and specific antigen, various stimuli can activate mast cells to release a diverse array of biologically active products, many of which can potentially mediate pro-inflammatory, anti-inflammatory and/or immunosuppressive functions.

Followed on a different timescale, activation of mast cells can result in both degranulation and de novo cytokine synthesis. Mediators released shortly after antigen- and IgE-induced mast cell degranulation induce a response termed an immediate hypersensitivity involving the rapid (seconds to minutes) release of pre-packaged mediators into the surrounding tissue.

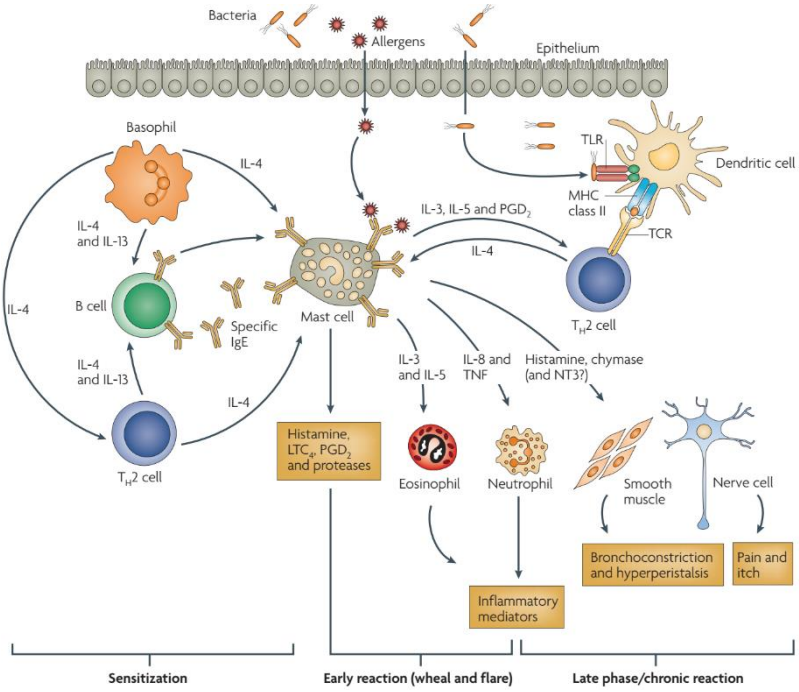


Figure 2. Mast cells in allergic reaction. Adapted from (Bischoff 2007). Mast-cell activation by IgE crosslinking with allergen induces the release of mast-cell mediators such as histamine, leukotriene C4 and prostaglandin D2 leads to an early reaction ("wheal and flare"). Other mediators, such as IL-3, IL-5, IL-8 and proteases contribute to the initiation of a facultative late phase reaction by recruiting and activating eosinophils, neutrophils and TH2 cells, and by interaction with tissue cells.

Depending on species and mast cell subtype the following "granular" mediators can be mentioned: biogenic amines, such as histamine, serotonin; heparin and chondroitin-sulphate peptidoglycans, pre-synthetized proteases, such as tryptase, chymase, Cpa3 and TNF-α, VEGF and FGF2. After stimulation, mast cells also begin to produce lipid-derived eicosanoids (e.g. LTC4, LTB4, PGD2 PGE2) and platelet-activating factor in the initial minutes of activation, as transcription is not required for these mediators to be converted to an active form. As a second wave they will be followed by cytokines (promoting recruitment of innate immune cells: TNF-α, IL-1α/β, IL-6, favoring Th2: IL-4, IL-5, IL-13, or Th1: IL-12, IFN-γ responses or suppress

both: IL-10, TGF-β), growth factors with angiogenic (VEGF and FGF2) or other, for example autocrine (SCF, GM-CSF) potential, chemokines (eg. CCL2, CCL3, CXCL8 etc.) and miscellaneous others (nitric oxide, antimicrobial peptides). The latter group also includes several mediators that increase the division of fibroblasts and smooth muscle cells: activin A, amphiregulin and HBEGF (Abraham and St John 2010, Galli and Tsai 2012).

Mast cells are a crucial source of mediators contributing to the initiation of the second phase of inflammation (late phase reaction) by secreting mediators that promote tissue infiltration and functional activation of circulating leukocytes, monocytes and T cells **(Figure 3)**. In fact, in addition to the development of inflammation, the airways of individuals with asthma also show structural changes, airway remodeling, which include epithelial cell mucus metaplasia, smooth muscle hypertrophy/hyperplasia, subepithelial fibrosis, and angiogenesis (Calvén et al. 2020, Galli and Tsai 2012).

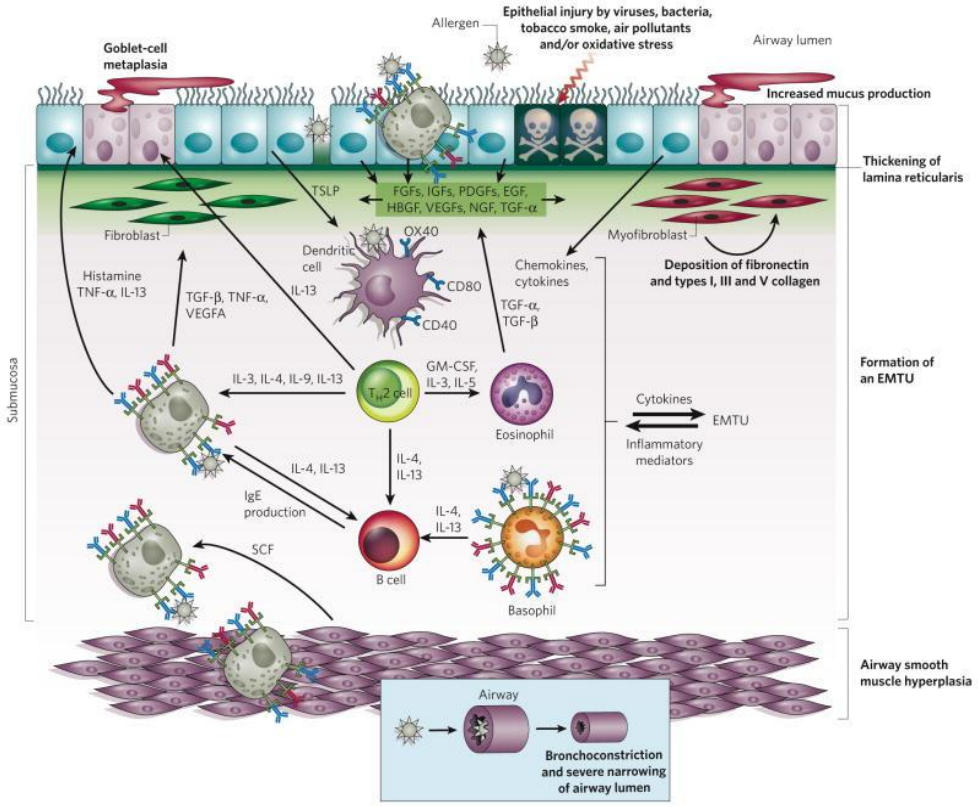


Figure 3. Chronic stage of allergen-induced airway inflammation. Adapted from (Galli, Tsai, et al. 2008). In the case of chronic allergic inflammation, repeated or prolonged exposure to allergens has a number of consequences. Innate and adaptive immune cells persistently colonize the affected tissues, and more mast cells migrate and become present with high levels of FcεRI-bound IgE. There are complex interactions between recruited and tissue resident immune cells, epithelial and structural cells (e.g. fibroblasts, myofibroblasts and airway smooth muscle cells), blood and lymphatic vessels and nerves. The recurrent epithelial injury that results from chronic allergic inflammation can be exacerbated by exposure to pathogens or environmental factors, and the resulting repair response results in the formation of an epithelial-mesenchymal trophic unit (EMTU).

1.3.3. Experimental models for studying mast cell functions

Mast cell-targeted interventions enable characterization of specific functions in human diseases, ranging from blockade of released mediators, inhibition of activation and decrease the number of cells by depletion strategies. Non-human models, like murine mast cells and in vivo mouse models provide valuable tools for mimicking different aspects of the pathophysiology of human diseases. Despite the similarities between human and rodent mast cells, differences in specific features (e.g. IL-4 dependent FcεRI expression or tissue-distribution and enzyme expression) may limit the extrapolation of the observations to humans. Mast cell-deficient mouse models, either KIT-dependent (Kit mutant, like KitW/KitW-v and KitW-sh mice), and -independent (using Cre-recombinase under the control of mast cell-specific promoters, like in Chm-Cre;Mcl-1fl/fl mice which specifically deficient for mucosal mast cells) manner, are able to contextualize molecular mechanisms at the the level of the organism.

To overcome the biasing effect of constitutive mast cell deficiency, reflected by e.g. sterility of KitW/KitW-v mice as well, next generation of mouse models were developed in where mast cells can be selectively and inducibly depleted (by using a transgenic mouse that expresses a receptor for diphtheria toxin only in specific endophenotypes of mast cells)(Kolkhir et al. 2022).

The limited amount of mature mast cells in tissues has led to the development of a large variety of in vitro models. Although mast cell lines are easy to expand and maintain in a culture with homogeneous phenotype, they show immature phenotypes and the lack of key characteristics of mast cells (e.g. HMC-1 cannot be activated by IgE-crosslinking). Bone marrow-derived mast cells (BMMCs) are widely used as a model system for examination of mouse mast cell function in vitro. These cells are easy to obtain from total bone marrow cells by culturing in the presence of IL-3 or SCF. After 3 weeks a cell population with nearly homogenous phenotype is obtained, expressing high-affinity receptor for IgE and stained positive by classical mast cell-staining dyes. Phenotypically they are considered as immature, relating mostly to connective tissue mast cells (CTMC) in mice. The KIT (CD117) expression is utilized to measure purity and differentiation state in in vitro cultures. Phenotypically BMMCs have no identifiable physiological equivalent in tissues. Nevertheless, these immature cells can repopulate both the mucosal and serosal mast cell compartments when adoptively transferred to mast cell-deficient

mice. Thus BMMCs may represent precursor cells coming from the bone marrow that may require additional site-specific signals to develop into mature tissue resident mast cells (Galli et al. 2011, Wolters et al. 2005).

Consistently, BMMCs represented immature cells of the mast cell lineage, also reflected by low levels of specific CTMC-specific proteases, including Mcpt4 and Mcpt6. However, virtually lacking expression of mucosal MC specific proteases Mcpt1 and Mcpt2 associate them to CTMC more closely. Recently, a comparative transcriptome analysis of cultured BMMC and isolated peritoneal mast cells reported a detailed picture about significant similarities, which occurs at least at resting conditions and quantitative rather than decisive differences (Akula et al. 2020).

Recently, a new emerging technique for the rapid and efficient differentiation of human induced pluripotent stem cell (hiPSC)-derived mast cells allowed the generation of phenotypically mature and functional mast cells from hiPSC-derived mast cells from different patients to better understand disease-specific characteristics (Luo et al. 2022).

1.4. HBEGF: a growth factor need to be tightly controlled

Heparin-binding EGF-like growth factor (HBEGF) is a member of the epidermal growth factor family and contains besides its EGF-like domain also a heparin-binding domain that allows interactions with heparin and cell-surface heparan sulfate. HBEGF is initially synthesized as a transmembrane protein (pro-HBEGF) which has been identified as the receptor for diphtheria toxin. All the EGFR ligands are known as inactive transmembrane precursors which require to be processed and cleaved to active secretory and surface proteins **(Figure 4)**.

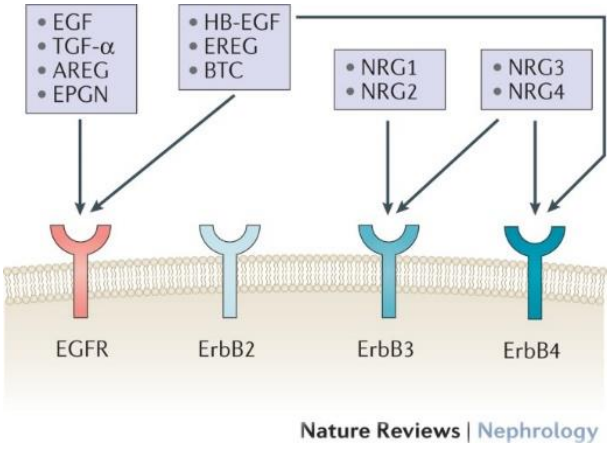


Figure 4. The EGFR/ErbB family members and their ligands, the EGF-related peptide growth factors. Adapted from: (Harskamp et al. 2016). EGF: epidermal growth factor, AREG: amphiregulin, BTC: betacellulin, NRG: neuregulins. The second group of ligands includes heparin-binding EGF which exhibit dual specificity for both EGFR (also known as ErbB1) and ErbB4. Interestingly, different EGFR ligands may differentially regulate not only EGFR signaling but also the EGFR turnover. Indeed, HBEGF induces sustained receptor phosphorylation, ubiquitination and degradation, whereas TGF-α binding rather leads to transient EGFR phosphorylation, minimal ubiquitination and thus almost complete recycling from endosomes (Roepstorff et al. 2009).

Accordingly, pro-HBEGF is then cleaved by matrix metalloproteinases (the process is called ectodomain shedding), resulting in the release of the mature, soluble form. HBEGF binds to its human receptors HER1 (EGFR) and HER4 (ErB4) in the presence of heparan sulfate as cofactor. Many cell types, including epithelial cells, skeletal muscle cells, macrophages, keratinocytes and T cells express HBEGF and it shows potent mitogenic and chemotactic activity on fibroblasts and epithelial cells. Activation of the HER tyrosine kinase receptors simultaneously triggers a series of signaling cascades, like MAPK or protein kinase C pathways (Chen J. et al. 2016).

HBEGF is implicated in various tissue/organ context (e.g. skin, liver, lung, intestine, brain) as a factor promoting repair and regeneration after organ injuries, as well as biological processes, like wound healing, blastocyst implantation, heart function and diseases, such as cancer and arteriosclerosis. The critical role of HBEGF in growth was first recognized by the high mortality rate observed in mice expressing HBEGF, where death typically occurred in the first postnatal week.

Mice carrying a keratinocyte-specific HBEGF deletion had significantly reduced epithelialization and wound closure but no difference in cell proliferation at the wound edge, emphasizing that HBEGF plays a role in cell migration rather than proliferation during skin wound healing (Shirakata et al. 2005). Overexpression of HBEGF, unlike the other EGFs paradoxically decreases keratinocyte proliferation, although induces an epithelial-mesenchymal transition-like phenotype of highly motile keratinocytes with markedly increased invasive potential (Dao et al. 2018, Stoll et al. 2012). The antiproliferative effect of HBEGF on keratinocytes can be explained by the fact that HBEGF stimulation tends to induce lysosomal degradation of internalized EGFR, whereas TGFα, AR, EREG and EGF stimulation leads to EGFR recycling (Roepstorff et al. 2009).

Infiltrating immune cells, as well as epithelial cells, have been demonstrated to be a source of HBEGF in the airway remodeling that occurs with chronic allergic inflammation. For example, the functional involvement of HBEGF in airway remodeling in a mouse allergic asthma model was established by employing a Cre-lox technique to selectively delete the HBEGF gene in CD4 T cells. The HBEGF protein promotes the proliferation of airway smooth muscle, and EGFR signaling is also critical in goblet cell metaplasia. Moreover, HBEGF deficiency in CD4 T cells was associated with increased

Bcl-6 binding to the IL-5 gene, which results in lower IL-5 mRNA expression and, as a result, less eosinophil recruitment. It revealed a novel immune regulatory role for HBEGF produced by CD4 T cells (Farahnak et al. 2019).

1.5. Adrenocortical tumors

Primary adrenal cortical carcinoma, known as adrenocortical carcinoma (ACC) represents a rare endocrine malignancy with significant clinical heterogeneity and high mortality. Differentiation of adrenal incidentalomas with diverse biological behaviors poses a significant diagnostic challenge for clinical practice due to their high prevalence alone. In most patients with carcinoma, the disease is in an advanced stage and is typically recognized with mass effect symptoms. Even after the recognition of the lesion, adrenal carcinoma demonstrates significant clinical, morphological, and molecular heterogeneity, which can make accurate diagnosis difficult in some cases. Even by considering histological features, it is often difficult to differentiate ACC from benign cortical adenoma or borderline cortical tumor (Lam 2021).

Most cases are sporadic, however, some cases are associated with an underlying hereditary syndrome, such as Li-Fraumeni, Lynch syndrome, as well as neurofibromatosis type 1, multiplex endocrine neoplasia type 1, Carney complex, familial adenomatous polyposis and Beckwith–Wiedemann syndrome (De Krijger and Papathomas 2012). Key molecular events like the IGF2 overexpression seem to be essential in adrenal cortical carcinoma tumorigenesis in general. Several additional genes as potential drivers involved in sporadic adrenocortical tumorigenesis, including p53 and Wnt/beta-catenin signaling pathways. Interestingly, in the encoding gene of the β-catenin, gain-of-function mutations are evident in approximately 25% of both benign and malignant sporadic adrenocortical neoplasms. Multi-omics studies revealed signatures of different tumor types, as like mutations of cAMP/PKA pathway genes are typical for cortisol-secreting adenomas (Bonnet-Serrano and Bertherat 2018, Szabó et al. 2010)

The hypothesis that benign adenomas show a multistep progression towards carcinoma is debated. The resolution is complicated by with supporting (for instance, data of case studies with clonality assessment enabling microsatellite allelotyping and the findings that some genetic alterations are present also in adenomas and carcinomas as well) and contradicting (epidemiology, distinct molecular signature) observations as well (Di Dalmazi et al. 2020).

Integrative application of multiple levels of molecular data may give a better insight into the pathomechanism of adrenocortical tumorigenesis as well as patterns that provide greater predictive value for treatment decisions. Dysregulation of gene expression may reflect biological behavior more precisely. It is proven that integration of expression profiling data is beneficial in identifying subgroups of malignancies with different molecular alterations and clinical outcomes.

Obtaining the omics landscape of tumors with different biological behavior creates opportunities to use genomic information to address clinical questions. Based on the pan-genomic profiling of surgical samples, it was shown that a "targeted classifier" combining molecular data ranging from methylation, chromosome alterations to expression or mutational profile has been shown to outperform a traditional approach based on purely clinical data. It has been demonstrated that it is also helpful for ACC, for instance (Assié et al. 2019).

An approach limited to benign adrenocortical lesions, involving several genomic technologies showed miRNA patterns defining specific molecular groups, and direct regulation of steroidogenic enzyme expression by methylation (Faillot et al. 2021). Specifically, abnormal levels of miRNAs in tumors are implicated in oncogenesis by downregulating tumor suppressor genes, others are shown to contribute in expression of their targeted oncogenes by downregulation.

Simultaneous profiling of miRNA and mRNA may be more promising as the rules of the relationship between the two levels (namely the miRNAs and their targets identified on the basis of sequence complementarity) are relatively consistent and easily mappable. Moreover, by considering data of tissue-specific expressions and aggregation of signatures up to pathways may increase robustness and specificity of data. Based on these assumption, our working group has developed a strategy by combining publicly available data with our own measurements from a representative set of adrenocortical lesions (Tömböl et al. 2009).

2. Objectives - aims

Early studies of microRNA expression in a wide range of tissues and cell types revealed distinct patterns of microRNA expression at different developmental stages and in activated states. The ability of miRNAs to regulate cellular processes by simultaneously regulating multiple targets illustrates their potential as robust biomarkers or viable therapeutic tools. In the case of mast cells, previously only custom-made and difficult to compare datasets on the microRNAs they express have been published. With the availability of the first version of standard gene expression microarray technology adapted to microRNAs, the question arose whether a characteristic microRNA expression could be associated to different phases of mast cell fate.

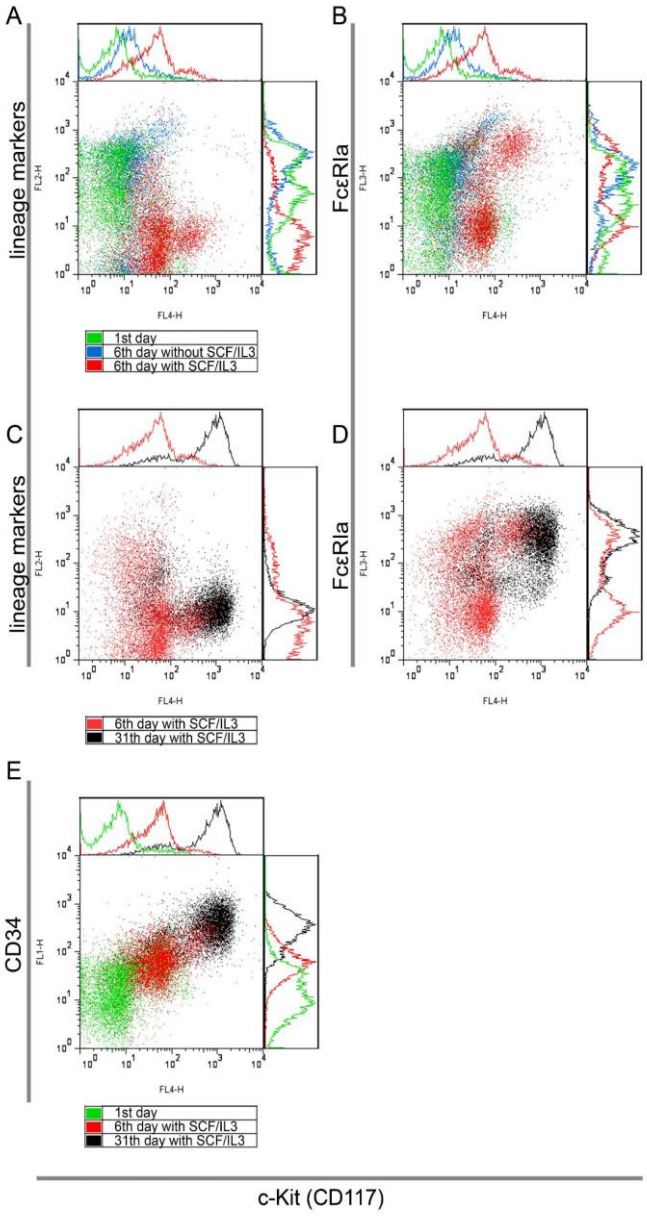
The aim of this work was to determine how microRNA signatures are formed during mast cell differentiation and activation, and to prioritize the mRNA targets of miRNAs identified on this basis. Optimally, selection of targets may consider multiple in silico and own experimental data resources, the latter case not only allowed the restriction of analysis to tissue-relevant mRNA but also the more probable targets can be ranked according to presumed anti-correlation in individual miRNA-mRNA sample pairs. It is then necessary to verify whether the putative miRNA-driven mechanism indeed controls a given phenotypic trait of the cell.

Therefore the overall objective is broken down into four main aims:

- To uncover comprehensive microRNA signatures that characterize distinct stages of a mouse mast cell in vitro culture,
- To apply an approach supporting optimal identification of the possible targets of differentially expressed microRNA(s),
- To design and adapt a methodology to exploit miRNA-mRNA profile pairs in real-life clinical samples, ranging from normal adrenocortical tissues to benign adenomas and adrenocortical carcinomas, in where our aim was (1) providing a landscape of miRNA expression patterns with a (2) functional representation in adrenocortical tumors with diverse histological and presumably biological behavior.
- To verify the hypothesized mechanism involving miR-132 and HBEGF both in human and mouse mast cells.

3. Results

3.1. In vitro model of mouse mast cell differentiation and activation



To describe microRNA expression changes associated with mast cell differentiation and IgE-dependent activation, bone marrow cells isolated from mice were cultured in the presence of SCF and IL-3. A large number of murine bone marrow-derived mast cells (BMMCs) can be produced by differentiation and expansion of hematopoietic progenitor cells over 3-4 weeks (**Figure 5 A-D)**.

Figure 5. Flow cytometric analysis of representative stages of mast cell differentiation. Cultures established from bone marrow cells on day 1 represent the initial mixed cell population and they were compared to the next stage of differentiating cultures, which were maintained with or without 20 ng/ml SCF and 5ng/ml IL-3 for 6 days. (a) A distinct c-Kit+ lineage- can be observed in the SCF/IL-3 group (red); (b) c-Kit+ cells appear to be more FcεRIa+ (red). (c, d) c-Kit and FcεRIa positivity on day 31 compared to data from day 6. (e) CD34 cell surface expression during the differentiation process. Data of a representative experiment. (Molnar et al. 2012). Channels and fluorochromes for labeling of antibodies: FL1: FITC, FL2: PE, FL3: PerCP, FL4: APC.

On day 6 of the differentiating bone marrow culture, the committed lineage+ cells can be clearly distinguished from c-Kit+ (CD117+) cells by flow cytometry. Importantly, the c-Kit+ cells on day 6 of the culture under differentiating conditions also showed an intermediate CD34+ phenotype, which is suggestive that the commitment is indeed initiated and established a separable progenitor population that mast cells later derived from. CD34 is a cell surface sialomucin glycoprotein expressed by hematopoietic stem cells, mast cells, and vascular endothelial cells. It is thought to be involved in mast cell adhesion, and contributes to mast cell progenitor cell influx into the circulation by inhibiting mast cell progenitor cell adhesion in the bone marrow. In our study, the intermediate expression of CD34 indirectly confirms that the population constituting the majority of c-Kit+ cells at day 6 is committed to mast cell differentiation (**Figure 4 E)**. This is consistent with the observation that CD34 is expressed at high levels only by mast cells in mature mouse hematopoietic cells and is considered a specific marker for these cells (Drew et al. 2002).

Therefore, day 6 was chosen as the starting point and putative committed mast cell progenitors were positively isolated with magnetic beads to label the cell surface protein c-Kit. Other cultures started in parallel and continued their developmental program for an additional 4 weeks to obtain differentiated but immature BMMCs. Flow cytometric measurements showed a c-Kit positivity of over 95%, of which c-Kit+ / FcεRIα+ double positive cells were present in 70% of events within the live cell gate.

To induce final differentiation of BMMCs toward mucosal MCs in vitro, IL-9 and TGF-β were added to the culture for an additional 5 days. Completed maturation was confirmed by measuring the upregulation of mast cell protease-1 (mMcpt1) chymase gene (Kakinoki et al. 2019).

To establish a model for antigen-specific, IgE-mediated activation of murine mast cells, BMMCs were pre-sensitized with anti-DNP IgEs for 2 hours and then stimulated with DNP-HSA antigen for an additional 2 hours. Successful activation was verified by measuring the expression of the IL13 gene by RT-PCR **(Figure 6)** and the activity of the released β-hexosaminidase in the conditioned medium (data not shown).

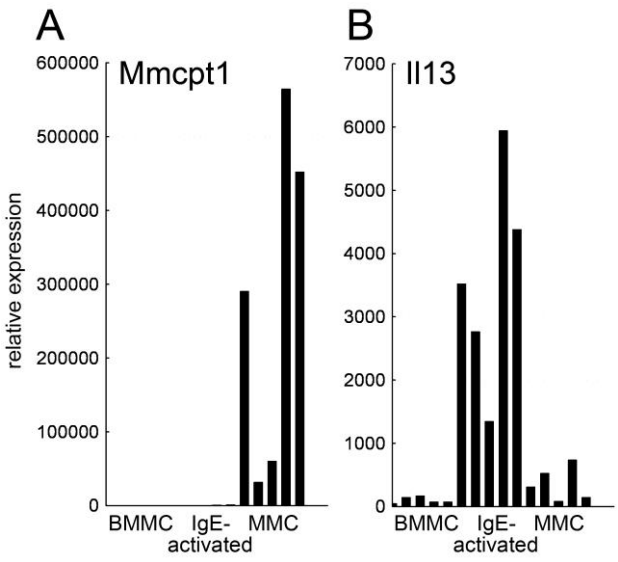
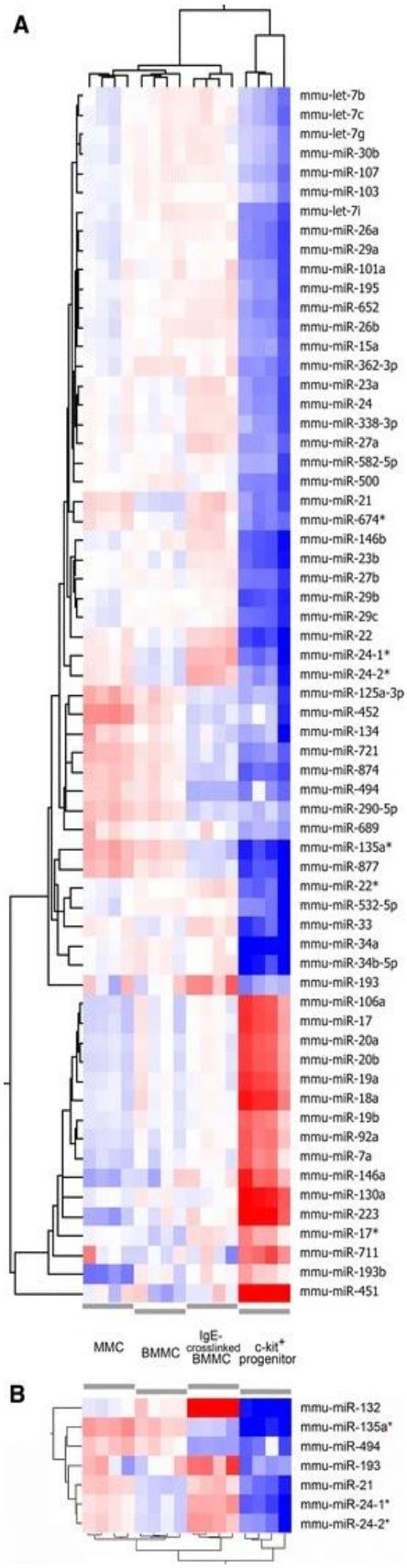


Figure 6. Uniformity of Il13 (activation) and Mcpt1 (maturation to mucosal type) mRNA levels in individual BMMC cultures representing different experimental groups which underwent differentiation. Expression of Mmcpt1 (a) and Il13 (b) measured by qRT-PCR. The y-axis shows the relative values, where the average expression level of BMMC biological parallel cultures was defined as 1 (one-way ANOVA, Il13: $p<0.001$, Mcpt1: $p<0.05$) (n=5/group) On the y-axis the groups are the following: BMMC= BMMC unstimulated, IgE-activated= BMMC from the same culture after two hours after IgE-crosslinking by DNP antigen and MMC=mucosal mast cells. (Molnar et al. 2012).

3.2. Reprogramming of microRNA expression characterizes mast cell differentiation and activation stages

To determine the miRNA expression profile that is characteristic for differentiation and activation of murine mast cells, the following experimental groups with four biological parallels were defined:

- **c-kit+ progenitors:** early putative progenitor cells, which have already been committed to mast cell differentiation, defined as c-Kit + cells isolated on day 6 of BMMC culture by magnetic bead separation;
- **BMMCs:** primary cultures of immature yet differentiated mast cells generated by culturing in the presence of SCF and IL-3 cytokines and primarily composed of c-Kit+/FeRI+ cells;
- **MMCs:** primary cultures of mature, end-differentiated mucosal mast cell analogues were obtained by exposing BMMCs to extra IL-9 and TGF- for an additional 5 days; and
- **IgE-crosslinked / activated BMMC:** BMMCs pre-treated with DNP-specific IgE and then stimulated with DNP, followed by the incubation time required to reach the state of IgE-dependent activation.



Profiling and comparison of these experimental groups was performed on a single-color Agilent standard microarray platform capable of quantitative measurement of 567 miRNA levels. A clustered heat map was created to visualize the relative expressions of differentially expressed miRNAs. The list of these selected miRNAs displayed is determined by the following rules: (1) a signal is detected as "present" according to the corresponding probe(s) (by summing up several qualitative features of the signal), and (2) the signal intensity is above the threshold in at least one sample of each group, and (3) the average fold change is greater than two, and (4) the difference is statistically significant (by analysis of variance using Benjamini-Hochberg multiple comparison p-value correction) between any pairwise compared conditions. Analysis of the microarray data revealed significant differences in the expression level of 63 miRNAs **(Figure 7)** .

Figure 7. Heat map of the differentially expressed miRNAs during mast cell differentiation and activation, downregulation (blue), upregulation (red). (a) Hierarchical clustering was done by considering genes that reached the minimum 2-fold change between BMMCs and c-Kit progenitors (entities and conditions, Euclidean metric centroid linkage). (b) Most significantly changed genes upon mast cell activation are shown and clustered the same way. (n=4/group) (Molnar et al. 2012).

SystematicName	Corrected p-value	FCAbs	log2(FC)	Regulation
mmu-miR-34a	4,78E-07	14,8	3,9	↑
mmu-miR-877	1,62E-03	12,2	3,6	↑
mmu-miR-135a*	9,27E-06	11,9	3,6	↑
mmu-miR-34b-5p	2,00E-05	9,7	3,3	↑
mmu-miR-532-5p	3,67E-02	7,9	3,0	↑
mmu-miR-22*	8,87E-03	7,4	2,9	↑
mmu-miR-33	1,14E-03	6,6	2,7	↑
mmu-miR-146b	1,61E-05	5,8	2,5	↑
mmu-miR-22	8,29E-06	5,7	2,5	↑
mmu-miR-29b	6,00E-06	5,2	2,4	↑
mmu-miR-874	4,78E-07	5,1	2,4	↑
mmu-miR-29c	1,62E-05	4,6	2,2	↑
mmu-miR-23b	1,96E-05	4,5	2,2	↑
mmu-miR-500	3,11E-05	4,2	2,1	↑
mmu-miR-362-3p	1,61E-05	3,9	2,0	↑
mmu-miR-27b	1,61E-05	3,9	2,0	↑
mmu-let-7i	3,11E-05	3,8	1,9	↑
mmu-miR-29a	4,03E-05	3,8	1,9	↑
mmu-miR-26a	7,46E-05	3,7	1,9	↑
mmu-miR-721	8,29E-06	3,7	1,9	↑
mmu-miR-24-1*	5,88E-04	3,7	1,9	↑
mmu-miR-125a-3p	1,05E-03	3,3	1,7	↑
mmu-miR-134	1,39E-02	3,3	1,7	↑
mmu-miR-101a	4,42E-04	3,2	1,7	↑
mmu-miR-652	1,63E-04	3,2	1,7	↑
mmu-miR-26b	3,17E-04	3,1	1,6	↑
mmu-miR-195	7,52E-04	3,1	1,6	↑
mmu-miR-23a	1,42E-04	2,9	1,5	↑
mmu-miR-494	1,57E-03	2,9	1,5	↑
mmu-miR-24	1,73E-04	2,9	1,5	↑
mmu-miR-674*	9,27E-06	2,7	1,5	↑
mmu-miR-15a	3,52E-04	2,7	1,4	↑
mmu-miR-107	5,04E-04	2,7	1,4	↑
mmu-miR-338-3p	1,14E-03	2,6	1,4	↑
mmu-miR-582-5p	2,12E-03	2,6	1,4	↑
mmu-miR-27a	9,79E-05	2,6	1,4	↑
mmu-miR-452	3,91E-03	2,6	1,4	↑
mmu-let-7c	1,93E-03	2,5	1,3	↑
mmu-let-7b	1,65E-03	2,5	1,3	↑
mmu-miR-24-2*	2,46E-03	2,5	1,3	↑
mmu-miR-290-5p	1,61E-05	2,4	1,2	↑
mmu-miR-21	3,17E-05	2,3	1,2	↑
mmu-miR-30b	1,57E-03	2,3	1,2	↑
mmu-miR-193	4,84E-02	2,2	1,2	↑
mmu-let-7g	2,14E-03	2,2	1,1	↑
mmu-miR-103	4,42E-03	2,1	1,1	↑
mmu-miR-689	1,14E-04	2,1	1,1	↑
mmu-miR-193b	6,12E-05	1,7	-0,7	↓
mmu-miR-17*	1,92E-03	2,7	-1,4	↓
mmu-miR-19b	4,62E-04	2,8	-1,5	↓
mmu-miR-7a	1,09E-03	3,0	-1,6	↓
mmu-miR-92a	1,14E-04	3,4	-1,8	↓
mmu-miR-20a	7,46E-05	4,0	-2,0	↓
mmu-miR-20b	6,51E-05	4,2	-2,1	↓
mmu-miR-711	4,95E-03	4,2	-2,1	↓
mmu-miR-19a	5,15E-05	4,5	-2,2	↓
mmu-miR-106a	1,03E-04	4,6	-2,2	↓
mmu-miR-146a	8,33E-04	4,6	-2,2	↓
mmu-miR-17	8,52E-05	4,8	-2,3	↓
mmu-miR-18a	3,84E-05	5,7	-2,5	↓
mmu-miR-130a	1,61E-05	7,4	-2,9	↓
mmu-miR-223	1,61E-05	8,9	-3,2	↓
mmu-miR-451	1,62E-05	66,0	-6,0	↓

Table 1. Significantly differentially expressed miRNAs in BMMCs compared to c-Kit+ progenitors. FCAbs means the absolute value of the mean fold change (Molnar et al. 2012).

When comparing c-Kit+ progenitors, BMMCs 63 miRNAs showed significant changes (Table 1). The most pronounced differences were observed between early precursors and BMMC cultures of immature mast cells. However, contrary to our expectations, despite the characteristic gene expression (e.g., proteases) profile and phenotype of mucosal-type mast cells, only minimal miRNA pattern changes were associated with their differentiation process. Interestingly, only 3 miRNAs expressed differently between BMMCs and MMCs **(Table 2)**.

Table 2 Significantly differentially expressed miRNAs in BMMCs compared to in vitro differentiated mucosal-type mast cells. FCAbs means the absolute value of the mean fold change (Molnar et al. 2012).

SystematicName	Corrected p-value	FCAbs	log2(FC)	Regulation
mmu-miR-452	2,68E-04	2,1	1,1	↑
mmu-miR-21	2,62E-05	2,0	1,0	↑
mmu-miR-193b	4,26E-04	3,0	1,6	↑

As a result of the IgE-crosslinking, the activation program led to up- or downregulation of 7 miRNA **(Table 3).**

Table 3 Significantly differentially expressed miRNAs in IgE/antigen-activated BMMCs compared to unstimulated BMMCs. FCAbs means the absolute value of the mean fold change (Molnar et al. 2012).

SystematicName	Corrected p-value	FCAbs	log2(FC)	Regulation
mmu-miR-132	7,30E-06	9,5	3,2	↑
mmu-miR-193	0,013195	2,6	1,4	↑
mmu-miR-24-1*	7,76E-05	2,4	1,3	↑
mmu-miR-24-2*	1,45E-04	2,4	1,3	↑
mmu-miR-21	1,45E-04	2,0	1,0	↑
mmu-miR-135a*	1,45E-04	2,2	-1,2	↓
mmu-miR-494	5,22E-05	2,4	-1,2	↓

Several miRNAs that showed alteration between c-Kit+ progenitor cells (6th day of culture) and BMMCs are known to be involved in the differentiation or function of other hematopoietic cells**.** Taken together, these results show that mast cells coordinatively downregulate many miRNAs during their differentiation which are specific for other cell lineages and probably participate in shaping differentiation programs. For instance, miRNAs of the miR-17~92 and miR-106a~363 polycistronic clusters which are well known for their central role in the lymphoid differentiation, showed an intensive and coordinated downregulation. In our model the expression of the myeloid lineage-specific miR-223 was also downregulated during mast cell differentiation. Similarly, miR-451

which is characteristic for the erythroid cells, showed a strikingly lower (~66-fold) expression in BMMCs compared to the progenitors.

To validate results quantitative real-time RT-PCR was applied for selected miRNAs which showed strong correlation with the microarray data **(Figure 8).**

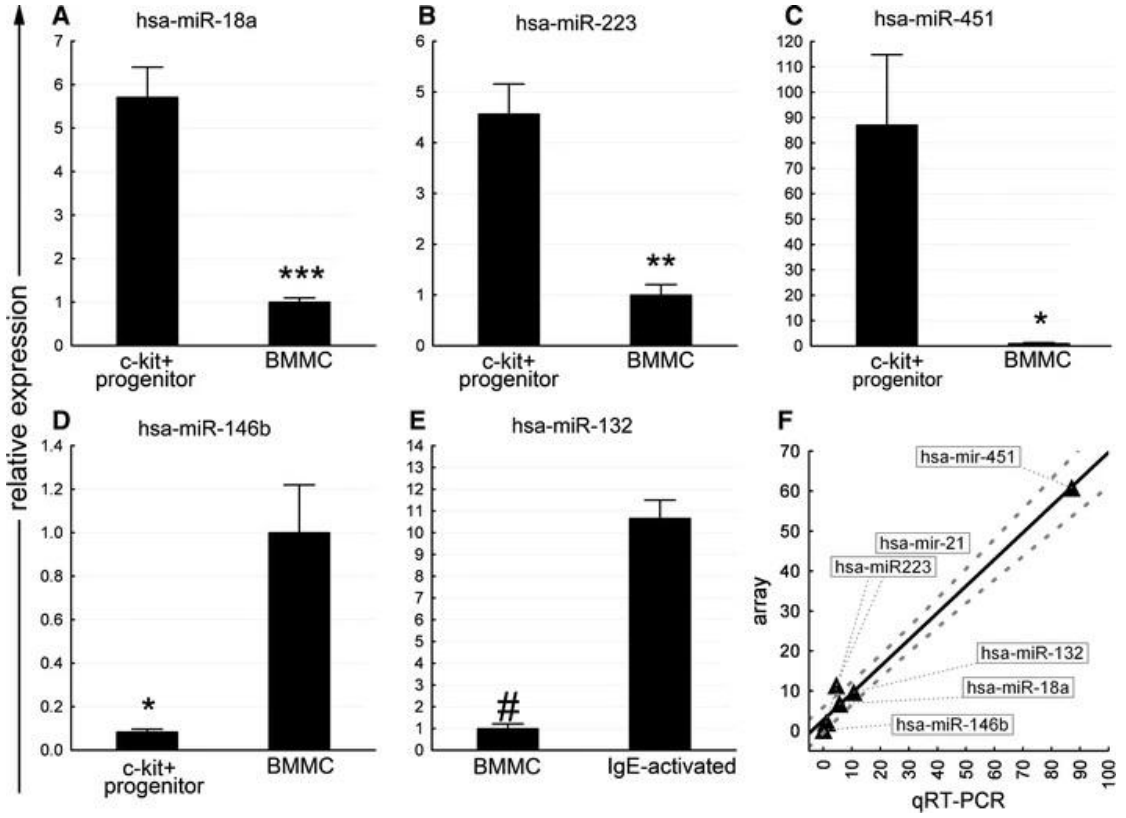


Figure 8. Real-time RT-PCR validation of the expression of selected miRNAs. Relative expressions of specific miRNAs are represented which were determined by comparing to snoRNA135 (a)-(e) mean + SEM; n = 4–6 samples per group. (f) Correlation of miRNA microarray and qRT-PCR data. Dashed line represents 95% confidence interval (Spearman rank order correlation R = 0.857, $p < 0.05$). Please note that, due to strong conservation, the human sequences for many mature miRNAs are identical to the mouse sequences. Here, the prefix "hsa" (Homo sapiens) indicates that human stem loop primers can be used in mouse samples (Molnar et al. 2012).

3.3. IgE-mediated mast cell activation is characterized by upregulation of miR-132

Because miR-132 microRNA showed the greatest change during activation, and its regulatory role in the innate immune response is also emerging, we focused on miR-132 in our further studies.

Previously, miR-132 has been identified as an endotoxin-sensitive gene in human THP-1 monocytes, and since the major receptor for LPS, TLR4 can also be detected on the surface of BMMCs, there is a possibility that the LPS contamination of the added reagents causes the detected change in our activation model. To rule this out, a control experiment was performed in which cells from BMMC cultures were stimulated through TLR4 or FcεRI, and then the relative expression of miR-132 was determined. IgE and DNP specifically upregulated miR-132, whereas LPS did not act in any of the concentrations used **(Figure 9)**.

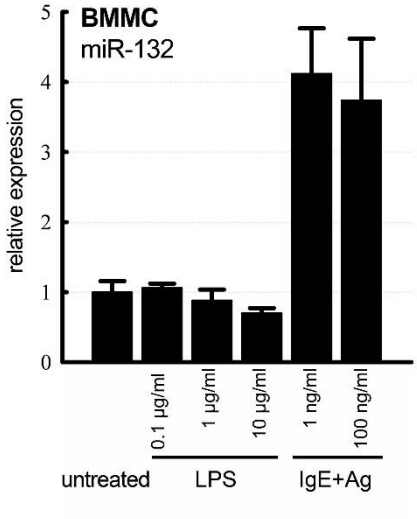


Figure 9. miR-132 cannot be induced by LPS treatment. Compared to IgE+antigen stimulation, miR-132 was not induced by LPS treatment in BMMCs following the treatment (n=3, mean+SEM) (Molnar et al. 2012).

The efficacy of LPS treatment or Fcε-receptor cross-linking was verified by determining the amount of interleukin-1beta (II1b), interleukin-6 (II6) and tumor necrosis factor-alpha (Tnfa) at the RNA level using real-time RT-PCR in same samples. LPS treatment induced the production of the measured proinflammatory cytokines at all applied concentrations, while, as expected, this effect was absent in case of IgE-DNP antigen stimulation in J774.2 cells. However, for BMMCs, both LPS and IgE cross-linking led to the induction of proinflammatory markers **(Figure 10)**.

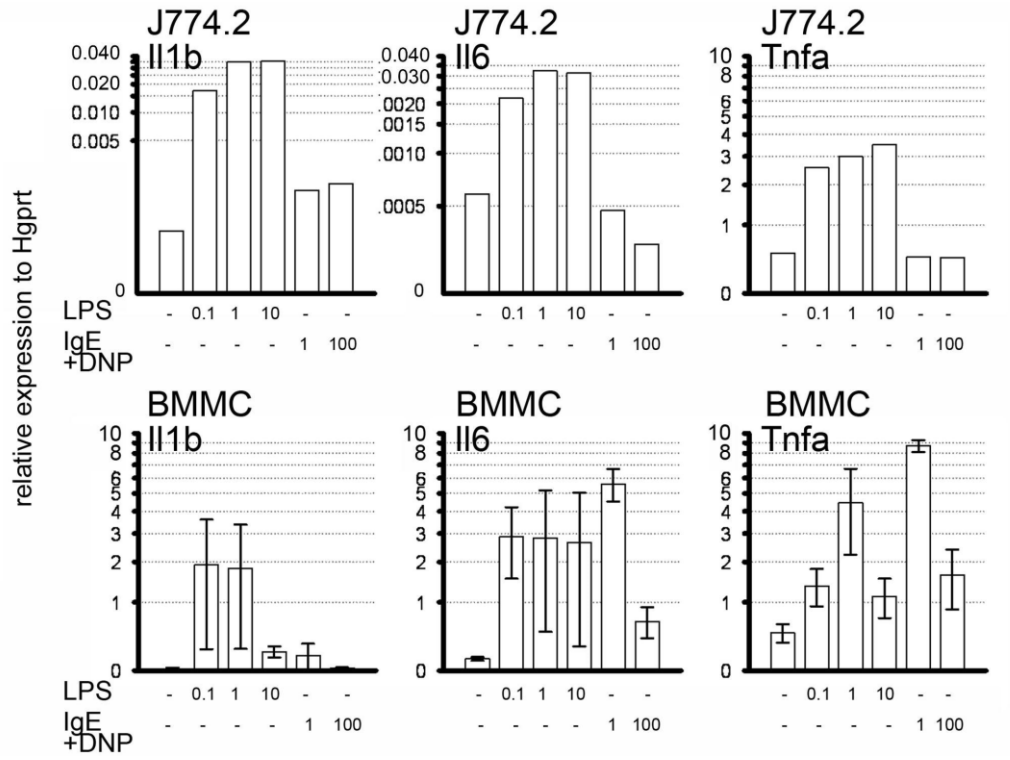


Figure 10. Differential expression of proinflammatory cytokines in J774.2 macrophage cell line and BMMC through TLR4 and FcεRI at 2 h after stimulation. The expressions of interleukin-1beta (Il1b), interleukin-6 (Il6) and tumor necrosis factor-alpha (Tnfa) were determined at the RNA level by real-time RT-PCR. The same RNA samples were used, which were applied to disclose the possible upregulation of miR-132 upon LPS treatment in BMMCs (n=3). The relative expressions to Hgprt are plotted on an independent (log-shifted) scale on y-axes to make the relative amounts visible and comparable among the different cell types and targets on a single panel (mean+SEM) (Molnar et al. 2012).

An increased cytoplasmic Ca++ concentration has also been implicated in the activation response accompanying IgE cross-linking that also involves miR-132 induction. To examine whether the Ca++ influx is able to mimic IgE/Ag-specific activation, miR-132 expression kinetics was recorded upon Ca-ionophore ionomycin-induced activation in the MC/9 mouse mast cell line. MiR-132 expression detectably began to increase 30 min after activation and peaked at 2 hours **(Figure 11 A)**.

To analyze and validate the expression of miR-132 in the human system as well, human cord blood-derived mast cells (CBMC) were generated. The ethical approval for using human biological samples was given by the national regulatory body, the Scientific and Research Ethics Committee of the Medical Research Council (Functional analysis of human mast cell progenitors and mast cells differentiated from umbilical cord blood, Principal Investigator: Zoltán Wiener, Department for Genetics, Cell- and Immunobiology, Semmelweis University). Umbilical cord blood was taken following

healthy non-complicated deliveries, then mononuclear cells were isolated with density centrifugation and labeled with anti-CD34 magnetic beads. After the magnetic separation CD34+ cells were seeded and cultured for 8 weeks in the presence of 100 ng/ml SCF, 50 ng/ml IL-6 and 3 μM lysophosphatidic acid. To induce the activation of CBMCs, myeloma derived human IgE was added to the culture overnight, the next day anti-human IgE was used for subsequent activation for 2h. Similarly to the murine mast cells, activation induced upregulation was observed in the human mast cells **(Figure 11 B)**. These results raise the possibility that mir-132 has an important role in the consequences of mast cell activation.

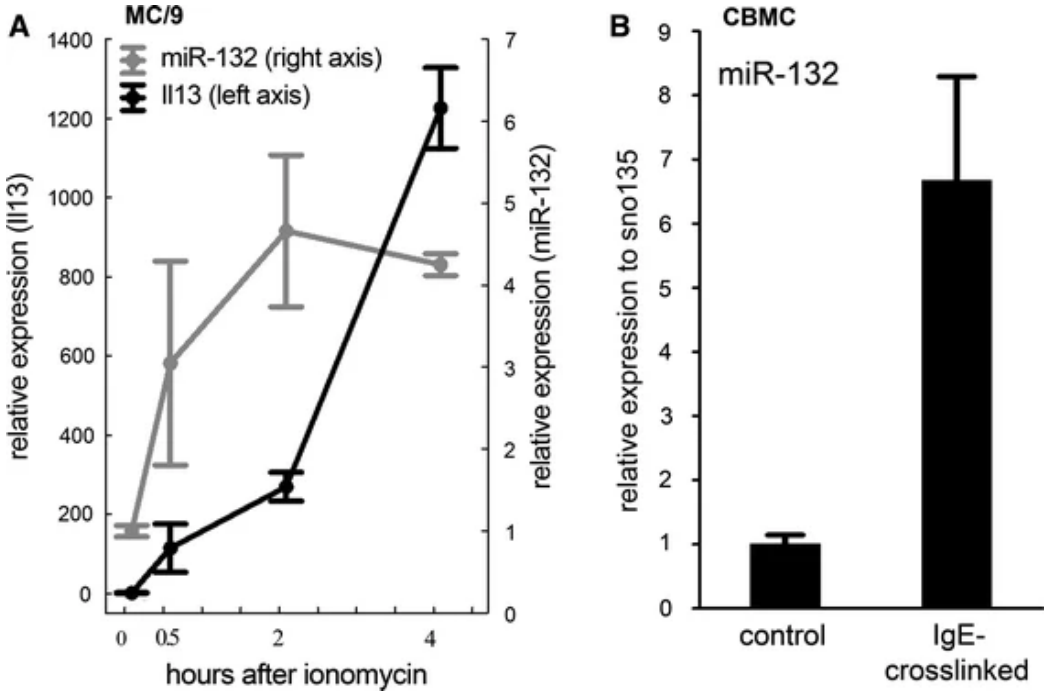


Figure 11. Mir-132 expression is upregulated upon activation in a cell line and in human cord blood derived mast cells. (A) The expressions of miR-132 and IL13 genes were tracked after provoking immediate activation response by ionomycin. (B) Human mast cells were differentiated from cord blood and were activated by anti-human IgE for two hours followed by adding human IgE. The relative expression of miR-132 and IL13 was determined by real-time RT-PCR and normalized to sno135 and HGPRT housekeeping genes, respectively ($n = 4$, $p < 0.05$, unpaired T test; mean+SEM) (Molnar et al. 2012).

The only miRNAs that were previously demonstrated to elevate in the comparison of resting and ionomycin-activated BMMCs are miR-221 and -222 (Mayoral et al. 2009). In our own experimental setup, we did not identify miR-221/222 as a significant variable, which may be due to several factors. One of these may be the different activation process: activation provoked by IgE cross-linking is a milder, but much more specific and

physiological stimulus for mast cells, and in our experiments, the induction of miR-221/222 was also observed in response to ionomycin, however this increase in expression lagged behind miR-132 **(Figure 12)**.

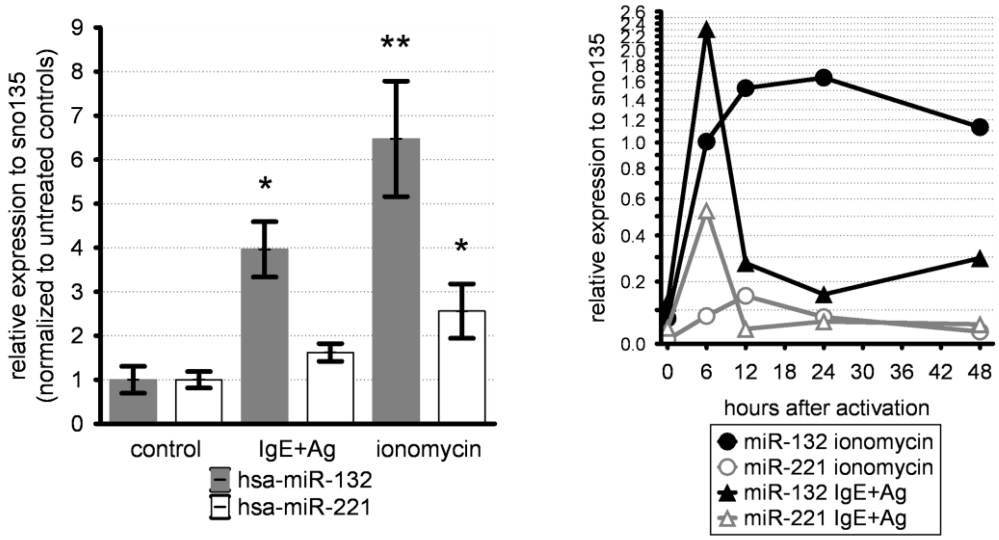


Figure 12. Comparison of the fold changes induced by IgE+Ag or ionomycin in BMMCs. qRT-PCR expression data (relative to sno135) were normalized to controls (mean+SEM). n=3, One-way ANOVA followed by Tukey-HSD post-hoc test: *p<0.05, **p<0.001. Expressions of miR-132 and miR-221 show similar kinetics after stimulation by either FcεR-crosslinking or Ca-ionophore treatment. Single BMMC culture was split and stimulated by IgE+Ag or by ionomycin (1μg/ml) for the indicated periods and relative expressions (normalized to sno135) were determined by qRT-PCR. Note that the baseline expression level of miR-221 is 3-6 fold lower and it is upregulated also (4-20 fold) upon stimulation by either IgE+Ag or ionomycin (Molnar et al. 2012).

3.4. Target prediction of miR-132

After discovering that miR-132 was activated during the activation of both human and murine mast cells, we sought to identify its potential mRNA targets.

In order to increase the robustness of the target prediction, lists of potential targets produced by various algorithms (PicTar, MiRBase, TargetScan) were compiled. The target mRNAs in the list with different identifiers were converted using the BioMART database. A score from 0 to 100 (most likely target is 100) was ranked from the different binding probability scores **(Figure 13)**. The aim was to identify a possible target implicated in mast cell-related or mast cell-driven biological processes. Because of two conserved binding sites for miR-132 in the 3'UTR of the Hbegf mRNA **(Figure 14)**, HBEGF was shown to be a promising target, and it has previously been discovered in isolated human skin mast cells. (Artuc et al. 2002).

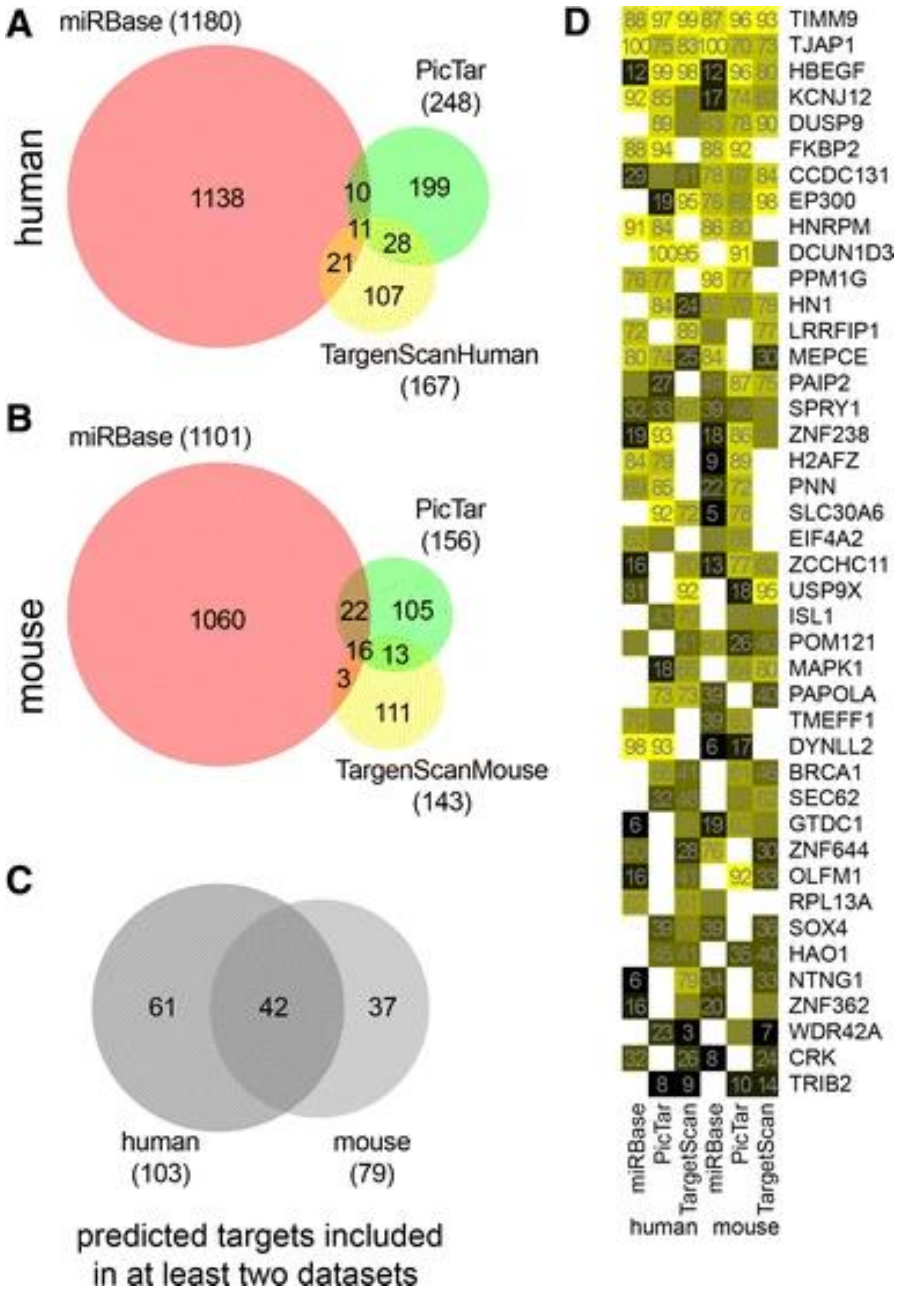


Figure 13. Target prediction of miR-132 using mouse and human datasets of three different target prediction databases. The size of outputs from the predictions and their overlap for the human (a) and mouse (b) database show surprisingly minimal overlap. After setting slightly less stringent conditions (it was enabled with Targetscan), focused on the predictions, that are (1) obtained in both mice and humans, and (2) those were mentioned at least 2 of the 3 prediction algorithms. c Targets included in at least two outputs. d The 42 prioritized genes from c were graded according to the sum of ranked scores. The color intensity indicates the relative probability of the interaction between miR-132 and the respective targets. Values in each box show the fractionated ranks in the given dataset with HUGO identifiers on the right side (Molnar et al. 2012).

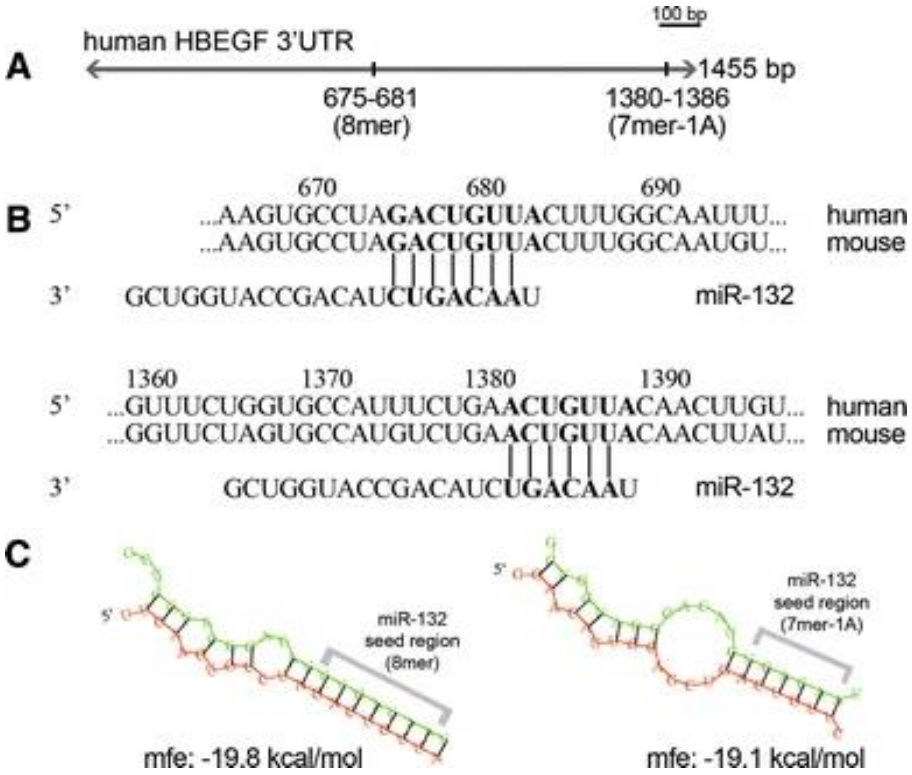


Figure 14. MiR-132 has two predicted binding sites in the 3'UTR of mouse and human HBEGF. a The target prediction database TargetScan (version 5.1) shows an 8-mer and a 7-mer-1A predicted pairing between human HBEGF 3'UTR and miR-132-3p. b The predicted target sites are identical in the mouse and human HBEGF 3'UTRs. c Minimum free energy values and structures of the hybridized miRNA/target duplexes calculated by RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/ ; date of download November 2, 2011) (Molnar et al. 2012).

3.5. The predicted target HBEGF is upregulated during mast cell activation

To assess the applicability of the target prediction result, HBEGF mRNA was measured using real-time RT-PCR and protein expression was measured using Western blot in BMMCs after FcRI-mediated activation at different time points. The activation increased HBEGF mRNA levels by 100-fold, whereas protein expression increased by a maximum of 2-fold with a peak at 2 h. Within 24 hours, the mRNA and protein levels of expression were normalized **(Figure 15 A-C)**. We observed comparable results in CBMCs, where HBEGF mRNA expression was increased after 2 hours of stimulation **(Figure 15 D)**.

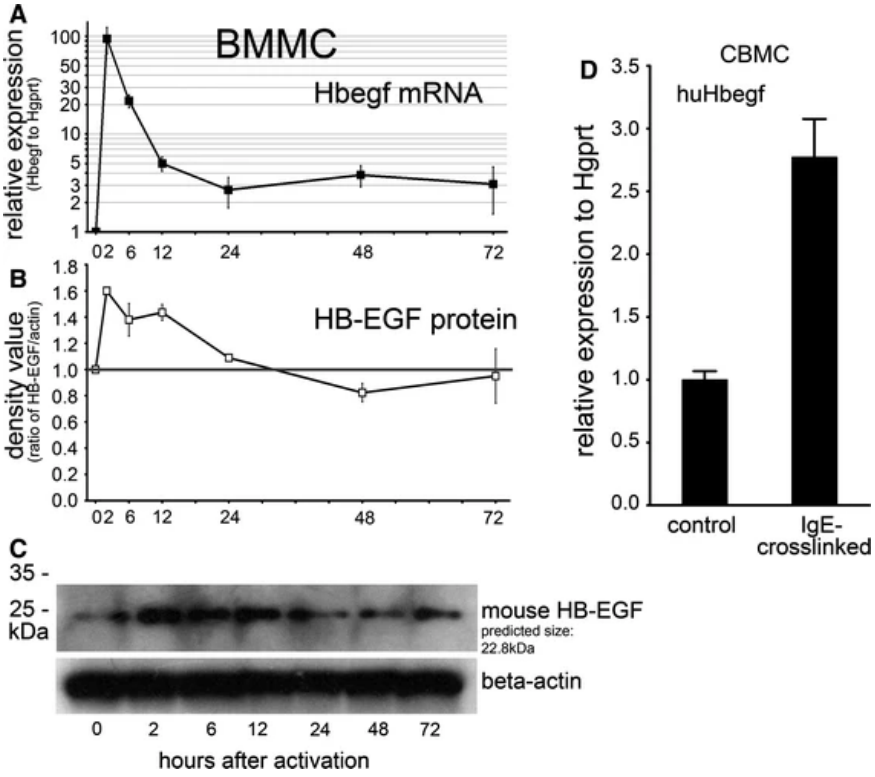


Figure 15. Analysis of the expression of HBEGF both in mouse and in human primary mast cells. mRNA (a, d) was isolated at different time points, thus, the kinetics of the activation-induced expressional changes was monitored (n = 5, unpaired T test, mean+SEM). (b, c) Protein was also isolated from the same samples at each time point and the expression was followed by Western blot. Representative Western blot image (c) and the result of the densitometry (b) are shown (mean+SEM), (Molnar et al. 2012).

3.6. HBEGF is a target gene for miR-132

In order to prove that HBEGF is a true target of miR-132, silencing and overexpression experiments were set up. BMMCs and MC/9 mouse mast cell line were transfected by electroporation with either a miR-132 precursor (Pre-miR-132) or neutralizing (Anti-miR-132) oligonucleotides and we used negative control vectors as well. Transfected BMMC cells then were activated by IgE/antigen and the expression of miR-132 parallel to HBEGF was detected. As indicated in the figures, pre-miR-132 significantly increased the level of miR-132, whereas anti-miR effectively decreased the activation-induced increase in endogenous miR-132. In the same cells, the mRNA expression of HBEGF was also investigated. The activation-induced elevation of HBEGF in the presence of pre-miR-132 was similar to the negative control transfected group, which could be due to the maximal suppression already attained by the endogenous miR-132, indicating that the extra amount could not have a significant effect. Importantly, cells that received anti-miR-132 oligos showed a much higher activation induced expression of HBEGF, thus the

miR-132 induced silencing was indeed abolished or at least was significantly affected. Interestingly, the kinetics of the expression of HBEGF was not affected either by the mimics or the inhibition of miR-132. To further validate these results, protein expression of HBEGF was also investigated in BMMCs upon IgE/antigen activation with Western blot. In the presence of pre-miR-132 the kinetics of HBEGF expression was influenced, the maximal expression shifted from 2 h to 24 h, however, this maximum was also much lower than in the control group. As expected from the mRNA data, the anti-miR-132 facilitated a significantly higher expression of HBEGF. Moreover, the drop in increased HBEGF expression lasts longer in activated mast cells and the high protein level was maintained beyond 72 hours, although it should have been downregulated to the non-activated level at 24 hours. **(Figure 16)**.

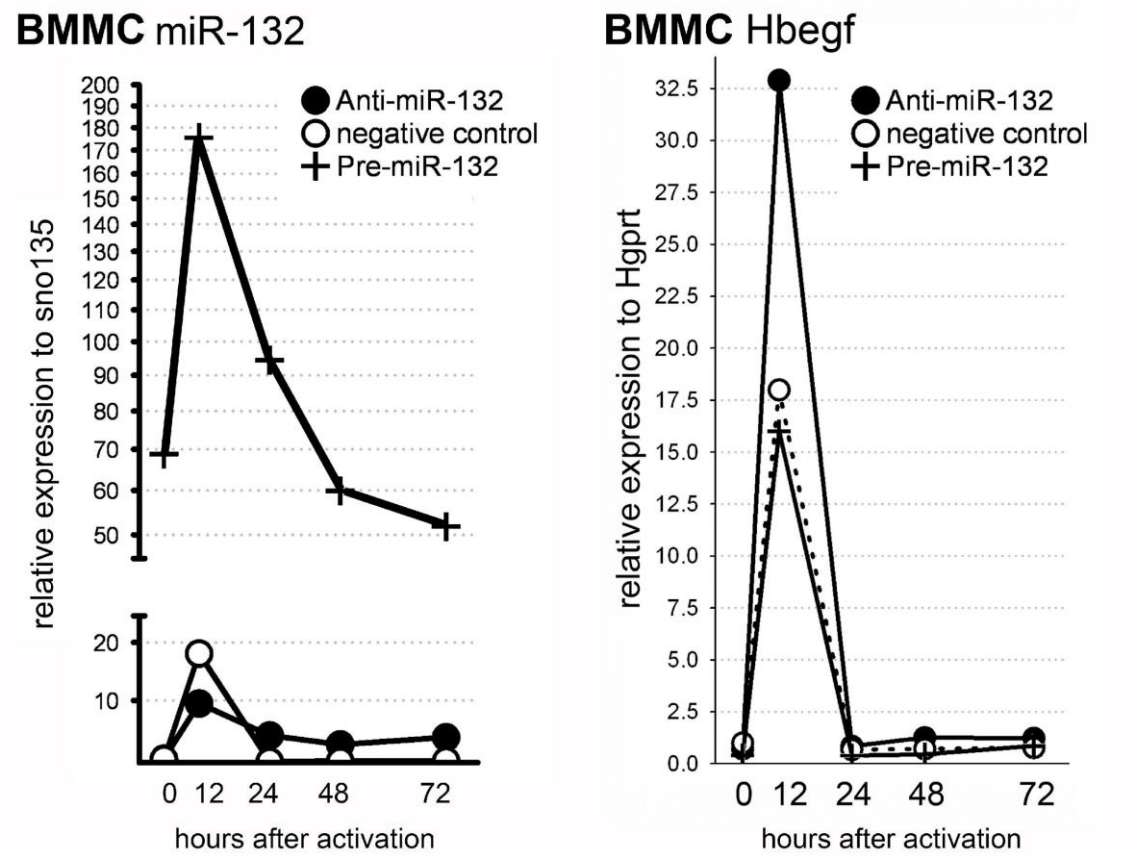


Figure 16. Kinetics of miR-132 and Hbegf expression in BMMCs transfected with miR-132 mimicking (Pre-miR) or neutralizing (Anti-miR) oligonucleotides. The x-axis shows the time after IgE/antigen activation. Data from a representative experiment. (Molnar et al. 2012).

To validate the direct binding of miR-132 to human HBEGF 3'UTR region, CHO cells were transfected either with a luciferase vector containing HBEGF 3′UTR or with a

scrambled vector containing a random genomic sequence (non-target 3′UTR), and these cells were also transfected with the pre-miR-132 or with control oligos. According to our findings, in the presence of pre-miR-132, the luciferase activity of the HBEGF 3′UTR containing vector was significantly reduced at all concentrations tested, whereas the luciferase activity of the control 3'UTR vector was comparable to the vector only group. **(Figure 17)**.

Collectively, these data clearly demonstrate that HBEGF is a true and direct target of miR-132 during the activation process of mast cells, both in humans and in mice.

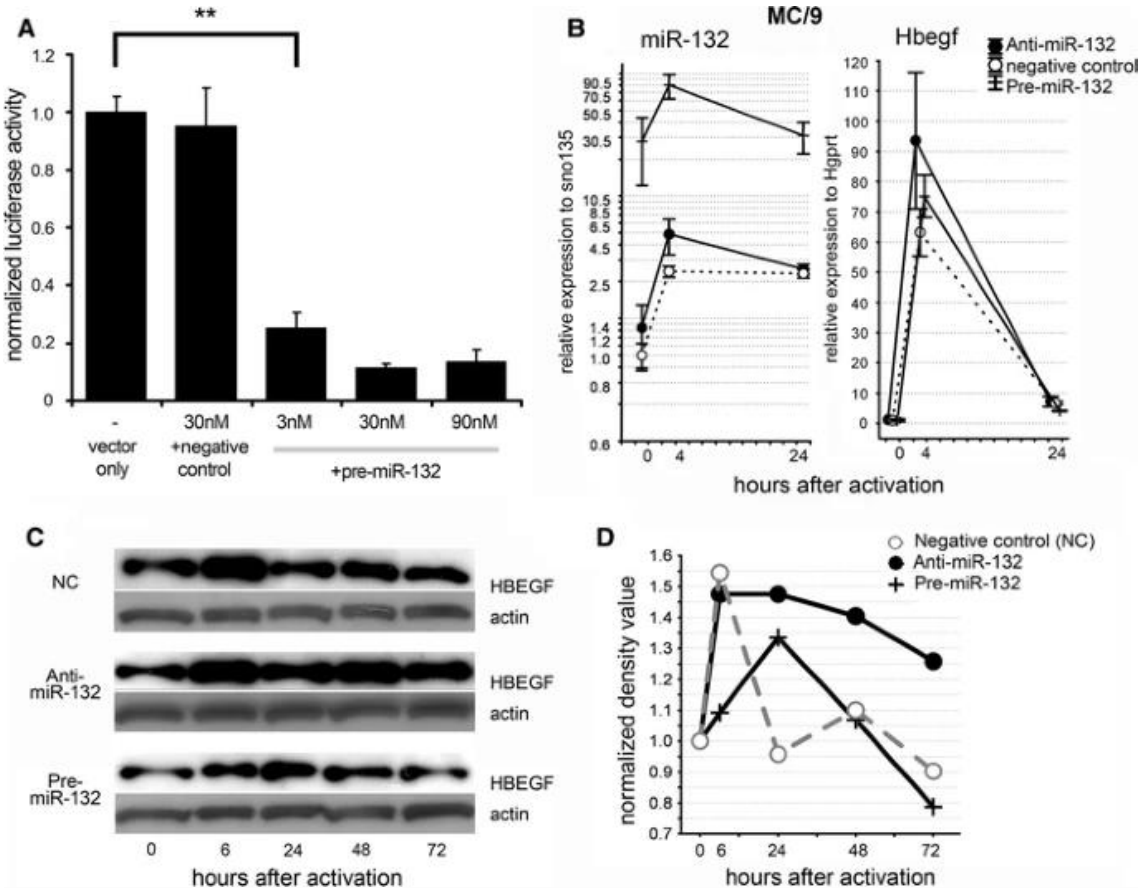


Figure 17. MiR-132 regulates HBEGF expression at protein level. (a) CHO cells were co-transfected with a luciferase vector containing HBEGF 3′UTR or control sequence and negative control precursor oligos or pre-miR-132. The luciferase activity values were normalized to the vector only samples (n = 3, mean+SEM are shown, ANOVA and Tukey-HSD post-hoc). (b) Expression analysis of miR-132 and HBEGF in the miR-mimicking and neutralizing vector samples of activated MC/9 (n = 3, mean+SEM). (c) HBEGF expression is shown in the transfected cells upon IgE crosslinking at different time points in BMMCs. Representative image. (d) Bands were quantified with ImageJ and HBEGF/actin density values were plotted after normalization to the corresponding controls (Molnar et al. 2012).

3.7. Tissue-specific target prediction strategy in biomarker discovery

To apply our approach for combining target predictions from different prediction algorithms that has been successfully used in mast cells, we chose a clinically relevant problem. We focused on biomarkers which are able to correlate with biological behavior of adrenocortical lesions.

Utilization of microRNAs as possible biomarkers is well established in cancer (Bracken et al. 2016). Moreover, several lines of evidence indicate that miRNAs are involved in the pathogenesis of various cancer types in a tissue and context specific manner. Changes in expression patterns observed in tissue samples and minimally invasive liquid biopsies may indicate the problem at an early stage of solid cancer, when it is still curatively approached.

Biological samples from different lesions represent the biological behavior and clinical outcomes ranging from normal adrenocortical tissues, the quite common benign adenomas and adrenocortical carcinomas characterized by poor prognosis.

By using these groups of samples we aimed to identify the presumably miRNA-regulated pathways. The ethical approval for using human biological samples was given by the national regulatory body, the Scientific and Research Ethics Committee of the Medical Research Council (Functional genomics studies on the pathogenesis of adrenocortical tumours – focusing on the expression of cytokines and cytokine receptors, ETT 089/2006, Principal Investigator: Péter Igaz, Department of Internal Medicine and Oncology, Semmelweis University).

To achieve this aim, the differentially expressed genes were specified by concomitant analysis of miRNA and transcriptome profiles (**Figure 18**). Some other preparatory steps were applied to enhance specificity of the readouts. Undetectable mRNAs were subtracted from the pool of potential miR targets, thus, only expressed (tissue-relevant) targets had been considered.

After that, a combined list (i.e. union) was prepared of the targets predicted in silico by three (mirbase, pictar, targetscan) different algorithms of miRNAs showing difference between tumor types. Finally, the union of these predicted target mRNA sets were prioritized according to the degree of inverse expression alterations by their regulatory miRNAs (Kozomara et al. 2019, Krek et al. 2005, Lewis et al. 2005).

For interpreting gene expression data, Gene Set Enrichment Analysis (GSEA) was applied on potential mRNA targets selected by tissue relevance (or specificity), in silico predictions and inverse expression profile concerning their regulatory miRNAs. As the traditional gene expression analysis focuses on genes with significant differences in expression, biologically relevant, however, subtle, but consistent alterations of functionally related gene expression sets may remain unnoticed (Subramanian et al. 2005). GSEA determines whether a priori or a user-defined set of genes shows statistically significant, concordant differences between two biological states (e.g. phenotypes). GSEA analyzes gene expression data by rank statistics, and determines whether a particular set of genes is over- or underrepresented between the groups.

As a final step, GSEA-filtered tissue-specific miRNA targets and significant mRNA microarray results in all pairwise comparisons were analyzed and visualized by using Ingenuity Pathway Analysis. This approach revealed possible involvement of the Cell cycle: G2/M DNA damage checkpoint regulation, as it was found to be the top canonical pathway altered in malignant adrenocortical carcinomas (Tömböl et al. 2009).

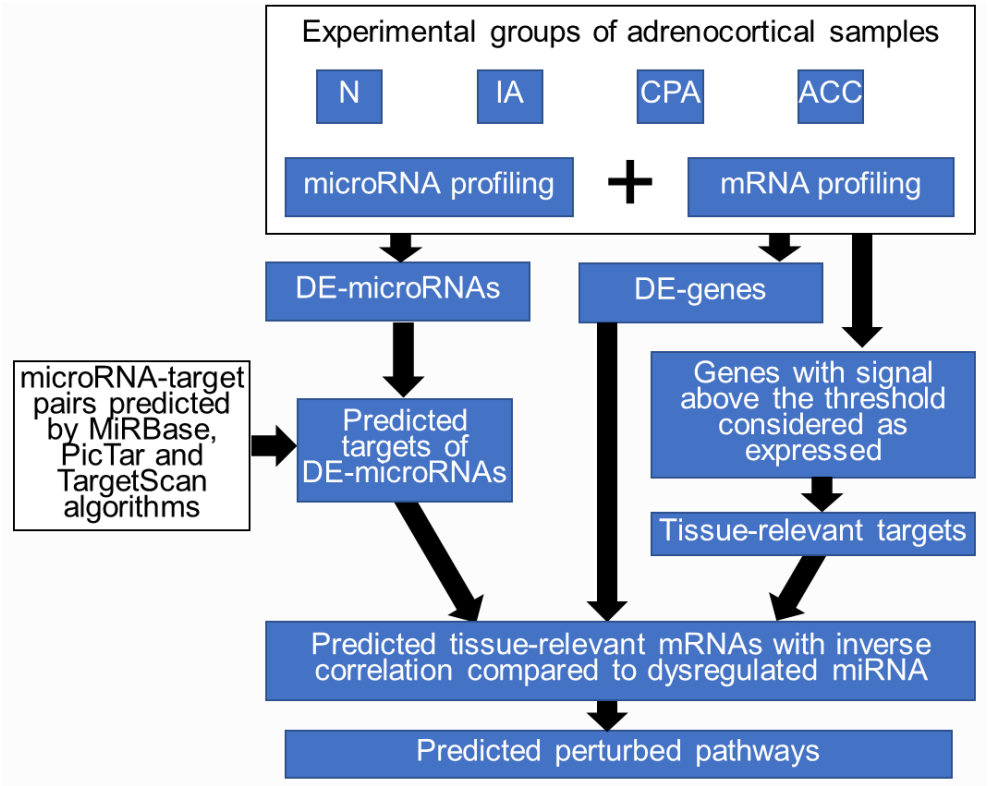


Figure 18. Data analysis pipeline for analyzing expressions of adrenocortical lesions by using tissue-specific target prediction to identify miRNA-regulated pathways. N, normal adrenocortical tissue; IA, inactive adenoma; CPA, cortisol-producing adenoma; ACC, adrenocortical carcinoma; DE: differentially expressed. Based on (Tömböl et al. 2009).

4. Discussion

A comprehensive landscape of miRNA expression patterns was obtained in our in vitro model representing steady-state stages of the mast cell life cycle. Of course, this first-line approach has its drawbacks and limitations. For instance, the model may disregard the frequently considerable temporal variations in expression levels or the interaction of multiple cell types in a tissue context. Historical or publicly available datasets can be useful to provide indirect evidence or to test a hypothesis. However, profiling of targets on RNA or more complicated on protein level for exploring anti-correlating sets or enrichment of miRNA binding motifs among differentially expressed gene sets is practical in a hypothesis-driven manner. Briefly, to collect experimental data on target selection, a transgenic model or modulating the expression of specific miRNA by either transiently (e.g. by PremiR or AntimiR, together with plenty of designed chemically modified miRNA modulators) or permanently (e.g. by viral vector-based overexpression) can be required to achieve a clear interpretation without unwanted summation and crosstalk of numerous effects. However, our goal was not to map the complete interaction network of miRNA-driven processes, but to identify a mechanism that may be important for better understanding mast cell function and/or universal in different species, tissue context, cell types etc. and/or may be indicative of a targetable process.

4.1. MicroRNA profiling of differentiation of murine mast cells

Given their importance in participating in the immune responses, it is essential to understand the molecular basis of mast cell's development, proliferation and functions. Since microRNAs play a role in differentiation and adaptation to a changing environment in virtually all cell types of the organism studied previously, it seemed inevitable to investigate the genome-wide profile using the first version of the available standard array-based platform.

In the regulation of hematopoietic and immune functions in mammals, the gene regulatory network formed by microRNAs complements the well-described signaling processes. For instance, Oh et al. demonstrated the importance of miRNA-mediated regulation when they developed a mast-cell-specific knock out of Dicer in mice (Mcpt5-Cre Dicer fl/fl) and reported that this condition is associated with severe loss of tissue

mast cells (Oh et al. 2014). Some miRNAs are expressed primarily by certain groups of immune cells, such as miR-142, miR-150, miR-155, miR-181, miR-221/222, and miR-223, suggesting a role in their differentiation. Of these, miR-181a and miR-150 are mainly required in cells of the adaptive immune response, while others much more widely required for normal function like miR-155 in T and B cells, as well as in monocyte-macrophage lineage cells (Bissels et al. 2012, Chen C. Z. et al. 2004, Montagner et al. 2014, Petriv et al. 2010). Importantly, the effect of microRNAs is not limited to fine-tuning longer-term expression patterns, as their role in modifying rapid cell-function-related responses has been demonstrated in several cases.

Monticelli et al. published the first comprehensive overview about the expression profile of murine hematopoietic cells, including BMMCs as the only representants of myeloid lineage, and established a coarse, prototypic skeleton for switch of miRNA expression pattern that is accompanied to lineage commitments (Monticelli et al. 2005). Importantly, all of the highlighted miRNAs by them were successfully reproduced in our model.

Similarly, we found a significant overlap of miRNA profiles in the process of hematopoietic differentiation of mast cells between our data set and the analogous data set of Xiang et al. From week 2 to week 6, bone marrow-derived cell mixtures were subjected to a weekly time-series analysis as they differentiated into mast cells. Among the 45 up- and 41 downregulated miRNAs, which are showing at least 5-times fold change, several of them shows consistent regarding direction of change after matching to differentially expressed miRNA sets of our experiment (e.g. miR-223, -451 down, -34a up). To explore miRNA-driven biological pathways, outputs of in silico target prediction program Targetscan were used to identify putative regulators and relevant targets showing correlation. For instance, KIT is suggested to be a target of miR-223 which is rapidly and markedly upmodulated during progression of differentiation (Xiang et al. 2014).

A recent study presented a systematic overview of miRNAs (“miRNAome”) from 63 mouse immune cell types that were sorted using immune phenotype-based cell sorting. MiRNA complexity was determined to be quite low, with >90% of each population's miRNA compartment consisting of 75 miRNAs; yet, each cell type exhibited a distinct miRNA signature (Rose et al. 2021). An undeniable advantage of similar studies involving a representative sample of differentiated immune cells is the ability to directly

compare the signatures of different lines. Some of the highlighted miRNAs that showed pronounced change in our differentiation model are associated to different cell fate decisions during hierarchical lineage commitment **(Table 4)**.

Table 4 Differentially expressed miRNAs involved in the hematopoietic differentiation and functions (research in literature).

Differently expressed miRNAs between c-Kit$^+$ progenitor cells (6th day of culture) and BMMCs				Involvement in differentiation or function of hematopoietic cells			References
miRNAs		Fold change	Regulation	Cells (with direction of alteration)	Relevant function	Target	
		BMMC vs. c-Kit+ progenitors					
	miR-34a	14.8	up	dendritic cell (up) megakaryocyte (up) B cells	- upregulated during monocyte-derived dendritic cell differentiation - promotes phorbol esther-induced megakaryocyte differentiation - constitutive expression perturbs B cell development by causing an increase in cells at the pro-B to pre-B cell transition	JAG1, WNT1 MYB FOXP1	(Hashimi et al. 2009) (Navarro et al. 2009) (O'connell R. M., Rao, et al. 2010)
	miR-21	2.26	up	monoc/macroph (up) dendritic cell (up)	- induced in three mouse models of asthma largely through an IL-13Rα1-independent pathway, expressed in cells of monocyte/macrophage lineage - upregulated during monocyte-derived dendritic cell differentiation	IL12-p35 JAG1, WNT1	(Lu T. X. et al. 2009) (Hashimi et al. 2009)
	miR-451	66	down	erythroid cells (up)	- promotes erythroid differentiation	gata2 (zebrafish)	(Pase et al. 2009)
	miR-223	8.91	down	neutrophil granulocytes (up)	- myeloid cell-specific expression - enhances granulocytic differentiation, negative regulation of maturation - enhances T cell differentiation	MEF2c NFI-A	(Fazi et al. 2005)
	miR-146a	4.65	down	monocytes (up)	- response to LPS as part of TLR–NFκB signalling	IRAK1,IRAK2 and TRAF6	(Taganov et al. 2006)
	miR-146b	5.83	up				
miR-17~92 *cluster*	**miR-17**	4.77	down	B cells T cells monocytes (down)	- mice deficient for miR-17~92 die shortly after birth (lung hypoplasia and ventricular septal defect) - essential for fetal and adult B-cell development (progression from the pro-B to pre-B cell stage), overexpressed cluster results in the expansion of CD4+ T cells - miR-17-5p-20a-106a downregulation unblock *AML1* translation (causes M-CSFR upregulation) in an *in vitro* monocytic differentiation model - functionally cooperates with miR-17~92 cluster in regulating mouse embryonic development	BIM PTEN AML1 (RUNX1)	(Ventura et al. 2008) (Xiao et al. 2008) (Fontana et al. 2007)
	miR-18a	5.67	down				
	miR-19a	4.47	down				
	miR-19b	2.8	down				
	miR-20a	3.96	down				
	miR-92a	3.43	down				
miR-106a~363 *cluster*	**miR-106a**	4.58	down				
	miR-20b	4.19	down				

4.2. Activation-induced miRNAs in mast cells

The activity miR-132 in the immune system is known to be functionally involved in psychological stress responses. For instance, miR-132 is overexpressed in bacterial lipopolysaccharide LPS-stimulated primary human macrophages (Taganov et al. 2006). Importantly, its role may not be restricted to myeloid cells, as miR-132 is found to be induced in B cells in response to BCR signaling. Moreover, it has also been shown to have an effect on B-cell development, presumably via the validated target, SOX4 (Mehta et al. 2015).

In case of mast cells it is well known, that if resting cells have already been preconditioned by an external factor (e.g. a mediator or cell-cell contact), this may even change the magnitude of the immediate response. Exactly that happened according to the observation of Bao et al. that miR-126 by modulating PI3K/Akt signaling pathway enhanced Ca2+ influx which leads to accelerated IgE-mediated mast cell degranulation in rats (Bao et al. 2018). For example, the activation process can be decomposed as a resting, already

sensitized and activated state of mast cells, as in the study by Just et al. In human CD133+ stem cell-derived mast cell culture, a significant increase in miRNA-210 expression was detected immediately as a result of IgE-mediated sensitization, at a similar level that peaks later, upon activation. This is in contrast to the dynamics of miRNA-132/212 clusters, which was again confirmed to increase only after activation. Interestingly, predicted target genes of miRNA-210/132/212 were enriched in several pathways known to be involved in activation (e.g. IL-4 and IL-13 pathway) suggesting a potential role in modulating activation (Just et al. 2019).

Interestingly, our observation that despite of the artificially elevated level of miR-132 by Pre-miR-132, we could not detect any quantitative changes in commonly activation readouts (data not shown; hexosaminidase assay, cytokine production profiling using antibody array: Proteome Profiler Mouse Cytokine Array, manufacturer: R&D Systems). For studying activation Teng et al. published data about miRNA profile from BMMCs which were compared resting to stimulated at 1 or 6 h hours following IgE-FcεRI crosslinking with antigen. Despite the application of a more advanced version of microarray platform (version 19.0 contains capture probes for a total of 1,247 mouse miRNAs; our experiment run on the version 1.0 with probes directed to 567, the actual version is 21.0 with 1,881 unique mature miRNAs) there is an overlap with our gene list in 7 upregulated miRNAs (including miR-132 and its close relative -212, and -21), although they found 10 miRNAs downregulated (the majority of these miRNAs are not included in previous versions) under similar experimental setup to our work (Teng et al. 2015). A recent paper used another platform and resulted in less overlap (Li Y. et al. 2020).

Experimental settings, such as strain, genetic background of the mice, purity of the culture, mode of stimulation or incubation time, are all likely to have varying degrees of control over gene expression patterns. Indeed, if we use a different type of stimulation instead of IgE cross-linking, we can observe different aspects of phenotypes or molecular events. For instance, Mayoral et al. reported that miR-221-222 showed the most pronounced increase in BMMC stimulated with PMA and ionomycin. In this study they hypothesized that it may act on cell cycle checkpoints in mast cells in response to acute stimulation, through the involvement of the cell cycle regulator p27Kip1 and the pro-survival c-KIT (Mayoral et al. 2009).

Following the identification of differently expressed miRNAs via profiling, the next goal is to determine the functional significance of relevant miRNAs. This can be accomplished by identifying regulated targets as well as the pathways and associated processes that are influenced.

4.3. Target prediction

Target prediction allows exploring the combinatorial regulation of miRNAs on numerous target mRNA transcripts in a sequence dependent manner. It is known that miRNAs usually bind to specific sites in the majority of cases in the 3'UTR region of targeted mRNA transcripts. It is possible to estimate the putative binding sites by calculating the weights of different descriptor parameters such as seed match (namely a Watson-Crick match between a miRNA and its target in the seed sequence, which is defined as the first 2–8 nucleotides starting at the 5′ end), free energy of the heteroduplex, accessibility, presence of a flanking AU and sequence conservation (Peterson et al. 2014). In fact, even the early high-throughput validation experiments showed that miRNA binding sequences tend to be overrepresented in sets of miRNA regulated genes compared to a random selection of genes (Baek et al. 2008, Selbach et al. 2008).

Despite efforts, the accurate identification of miRNA targets remains a challenge because mammalian miRNAs are characterized by a poor, imperfect homology toward their target sequence except in the conserved "seed" region.

Complexity is further complicated by a hierarchy of evidence that provides the validation with different qualities of a miRNA-target relationship and they are ranging from pure computational, through high-throughput to the focused functional assays. Experiments using massively parallel sequencing to identify sequences of miRNA-mRNA sequences purified from interacting proteins, such as Argonaute proteins, offer a new perspective in terms of both quality and quantity.

A single miRNA can dampen levels of hundreds of proteins by impeding their translation, thus it exerts combined effect on many different targets may be what determines the final phenotypical outcome of miRNA expression (Baek et al. 2008, Selbach et al. 2008)

Consequently, a consistently dysregulated miRNA may reflect perturbation of biological processes involved in the pathogenesis of certain disorders. Assaying miRNAs has plenty of advantages from clinical viewpoints such as stability and coordinated action on a large

number of genes. Not surprisingly, biomarker discovery investigations prefer to focus on these small non-coding regulators with the promise of interpretability.

Indeed, exploration of the miRNA-regulated processes rely on the in silico identification of genes regulated by specific miRNAs. Machine learning-based methods can handle the calculation of multi-parameter similarity using validated data as a training set. A comprehensive pathway analysis of miRNA-targeted genes showed that ubiquitous, basic metabolic processes are relatively underrepresented, while genes involved in cell growth and death, development (especially in certain gene sets, e.g. axon guidance pathways), transcriptional regulation and intercellular communication are more exposed to miRNA-driven control. Possibly, the miRNA-mediated regulation can be necessary in processes when beside the fundamental regulation, a fine-balancing is also required for driving the cell to the most proper response for different signals (Gaidatzis et al. 2007).

There are several algorithms available based on principles of miRNA–target interaction, indicating the potential for computation (N.B. largely based on alignment of nucleic acid sequences) and a high demand of research for them in prioritization of miRNA-regulated candidates. Each tool has a unique set of learning attributes and each of them has its strengths and weaknesses, generally showing poor accuracy and sensitivity, and the generated miR:target pairs vary even more between tools. To date, no single method consistently outperforms the others (Liu and Wang 2019), each tool has its own strengths and limitations. It supports the idea that combining the content of databases is an effective way to compensate for the limitations of each feature alone.

The binding sites of a given miRNA are present in more, functionally related transcripts, and this co-regulatory principle may allow for miRNAs to harmonize the elements of a pathway or related pathways. There is some evidence of the coordinate action, while different miRNAs own independent binding sites in the 3'-UTR of the same mRNA. In this case, miRNAs are able to influence the level of their targets coordinatively, increasing the degree of translational repression synergistically. In accordance with the observation that the number of binding sites correlates with the effectiveness of translational repression (Doench et al. 2003). Furthermore, the overlapping binding sequences in the 3' UTR can result in a competition between the respective miRNAs. Finally, the more independent binding sites do not mean that all of the silencers are expressed and present at the same time. Under certain circumstances and in different cell types can be observed

a given combination of miRNAs, which provide a variety of choices in setting up the proper level of target. This latter principle is supposed to be named as pattern differential regulation (Hua et al. 2006). The various recognized phenomena can be the basis of interpretation of the experimental results concerning miRNAs and the integration as a new layer to the knowledge of regulation of gene expression.

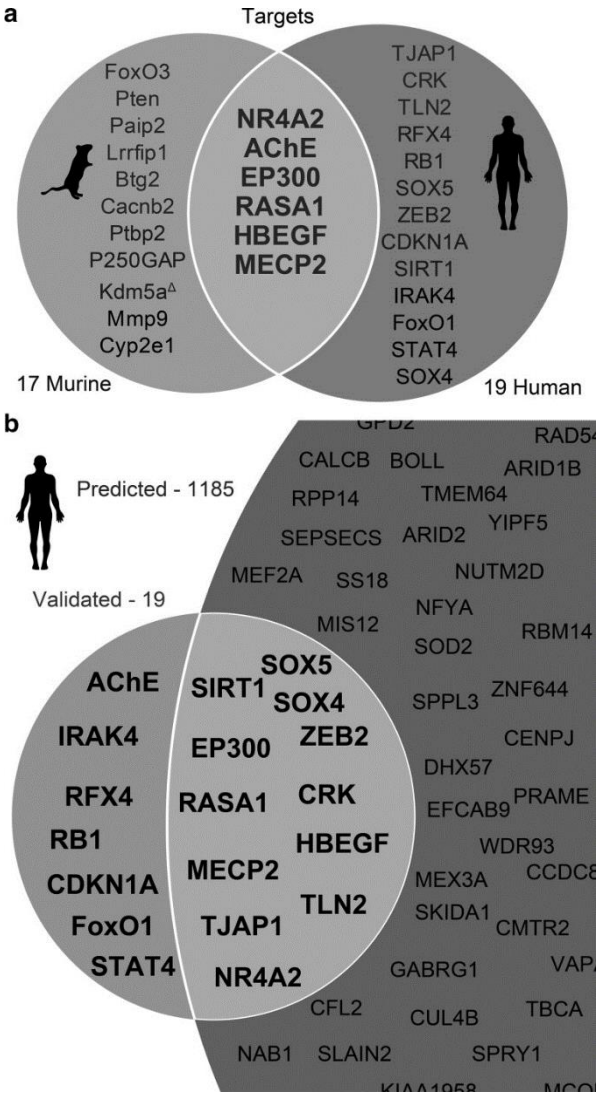


Figure 19. Validated and predicted miR-132-3p targets in mice and humans. a Venn diagram of validated miR-132-3p targets in mice and humans and b Venn diagram of validated and predicted targets of miR-132-3p in human, respectively. Predicted transcripts were retrieved from Diana microT-CDS using a default threshold of 0.7 Adapted from (Haviv R. et al. 2018).

It is a common method for dealing with similar complexity and a large number of influencing factors to combine the outputs of several non-redundant assumptions to boost the model's robustness and precision. Similar ideas drive variant classification in uncommon hereditary disorders diagnostics or variable selection approaches for hierarchical integration of multi-omics data (incorporating data across multiple levels of disease phenotype from methylation through mRNA, protein to the clinical outcome) (Nicora et al. 2022).

Nevertheless, the growing experimental data with the emergence of a plethora of new sequencing-based techniques also show that computational predictions is far from omnipotent. There are numerous "exceptions" that demonstrate the potential pitfalls associated with overestimation of evolutionary conservation **(Figure 19 A)** or the sensitivity of target prediction systems **(Figure 19 B)**.

To address the limits of individual methods, there is a growing interest in integrated tools that incorporate various features from miRNA target identification tools (Vejnar et al. 2013). The miRabel software, like our approach, aggregates the results of more algorithms and experimental datasets by converting outputs to a rank-scale (Quillet et al. 2019). Furthermore, certain integrative tools, such as ComiR, are designed to predict the targets of a group of miRNAs by using miRNA expression levels as an integral component of the target decision process and allow expression data to be used in the ranking of miRNA target predictions (Coronnello and Benos 2013). One way to circumvent shortcomings in miRNA targeting data is to address tissue specificity in the prediction of miRNA targets. Incorporating tissue-specific data such as miRNAs abundance, tissue-specific mRNA transcript variants, or in a certain experiment, the highly upregulated or downregulated genes (e.g. in miTALOS v2) improve the biological relevance of predictions (Preusse et al. 2016).

All widely used target prediction algorithms were trained utilizing a wide range of high-throughput profiles, such as microarray profiling data or crosslinking and immunoprecipitation (CLIP) sequencing data (DIANA micro-T). A complementary approach would be to use datasets from experiments in which the resulting downregulated transcripts are examined in the presence of a specific miRNA overexpression (Liu and Wang 2019). However, these are only available on a small scale, generally focused only on a few miRNAs in any given study, in varying quality, and frequently in artificial in vitro models that cannot reflect physiological situations. Because of these variables, training sets are suboptimal for developing a general target prediction model and emphasizing the significance of incorporating own experimental datasets.

The opportunity of target prediction establish a strategy to increase specificity by fusing experimental data and a priori knowledge. Moreover, simultaneous miRNA and mRNA profiling allows to effectively focus on relevant, tissue-specific targets and to interpret the miRNA-mediated regulation not only on single interactions but also even on level of pathways and networks. This approach can help uncover microRNA co-expression patterns in cancer, which can provide a personalized description of molecular signatures to predict tumor biological behavior (Dugo et al. 2018, Szabó et al. 2010, Tömböl et al. 2009).

4.4. Growth factor rheostat by activation-induced miRNAs

The application of a customized approach for target prioritization for miR-132, we identified a target and outlined a miRNA-driven mechanism. Because both HBEGF and its negative regulator, miR-132, are overproduced in mast cells, they are thought to contribute to the regulation of appropriate levels of growth factor, which may be of particular interest in physiological responses and diseases that cross-section of tissue remodeling and mast cell inflammatory activity such as wound healing and asthma, nasal polyposis and psoriasis. Similar auto-regulatory circuit reported that induction of miR-132 and miR-212 in IL-12-stimulated primary human natural killer cells negatively regulates the IL-12 signalling pathway resulting in decreasing IFN-γ expression through the repression of signal transducer and activator of transcription 4 (STAT4) expression (Huang et al. 2011). In such scenario, target protein output is decoupled from mRNA input through an incoherent feed-forward loop in which miRNA and target mRNA are transcriptionally coinduced. Molecular networks that control the initiation, peak magnitude, and resolution of inflammation must all be properly tuned for optimal health. Several examples are known how microRNAs can impact the magnitude of inflammatory responses. Specific miRNAs expressed in inflammatory cells, miR-155 and miR-146a were originally identified as inflammatory response miRNAs that are upregulated by NF-κB, a master transcription factor that regulates the strength of the inflammatory signal.

The need for precise adjustment is undoubtedly critical in an immune system that handles dangerous weapons and recruits them in exponential numbers. Optimally a transient inflammatory response that clears infection without causing host tissue damage. The deficiency of certain miRNAs, as we learned in case of miR-155 from knockout models, can result in an immunodeficiency. However, one can have too much of a good thing, overexpression of miR-155 can result in a hyperactive response to infection (endotoxemia), as can the absence of miR-146a. Moreover, constant overexpression of miR-155 or deletion of miR-146a can cause a chronic inflammatory state that does not resolve (O'connell Ryan M. et al. 2012).

The functional link between miR-132 and HBEGF was later confirmed in other cell types, models and species. Because of the close interconnectedness of our focus, we highlight one of these, which seems to be a continuation of our results, and places the mechanism in vivo, in the context of wound healing. In a human in vitro wound healing model, Li et al.

discovered that keratinocyte-derived miR-132 was strongly increased during the inflammatory phase of wound repair, peaked in the subsequent proliferative phase, and declined 14 days after wounding. They sought to investigate the mechanism and discovered: (1) TGF-ß was shown to increase the expression of miR-132 in keratinocytes. (2) Gene expression analysis on miR-132 overexpressing keratinocytes found that hundreds of mRNAs containing miR-132–binding sites are preferentially changed. (3) Pathway analysis showed for down- and upregulated target mRNAs were "Positive regulation of immune system process," "Positive regulation of cell communication," "Cytoskeletal organization," and "M phase," "Cell cycle phase," and "Cell cycle," respectively. (4) In fact, miR-132 suppressed keratinocyte chemokine production under inflammatory conditions mimicked by treatment with TNF-α or IL-1β (consistently, NF-κB pathway target genes were significantly enriched among the genes downregulated by miR-132) and miR-132 is found to promote keratinocyte growth by regulating STAT3 and ERK pathways. (5) Remarkably, silencing of the validated target of miR-132, Hbegf growth factor mimics the functional consequences of miR-132 overexpression in keratinocytes. (6) Later on, the in vivo administration of Anti-miR-132 into wounds of WT mouse or human ex vivo skin tissue delayed wound healing or reepithelialization, respectively that further confirmed the fidelity of the mechanism.

Altogether, based on these observations upregulation of miR-132 may mediate (a TGF-ß induced) coordinate message in keratinocytes by facilitating the transition from the inflammatory to the proliferative phase of wound healing process. (For supplementary information, according to our gene set enrichment analysis for predicted targets of human miR-132-3p found that TGF-beta signaling pathway is the top-enriched KEGG pathway.) These findings may raise the possible application of miR-132 as an attractive therapeutic target due to its capacity to alleviate inflammation and promote keratinocyte proliferation in chronic wounds (Li D. et al. 2015, Li X. et al. 2017).

4.5. Potential of miRNAs in clinical practice

Observations of miRNA expression profiles have shown that miRNAs tend to anticorrelate with target gene expression in related developmental stages or tissues. In general, miRNA may play primarily a reinforcing role, by repressing leaky transcripts, by adjusting transcriptional patterns to sharpen developmental transitions and establish cell identity. In addition, certain miRNAs may buffer fluctuations in gene expression. Remarkably, changes

in miRNA patterns in cancer can accurately predict developmental lineage and differentiation state, outperforming messenger RNA profiles in the classification of poorly differentiated tumors (Lu J. et al. 2005).

An miRNA's ability to pleiotropically target potentially hundreds or even thousands of genes is a common feature, and some operate in an organ- or cell-specific manner. As a result, one miRNA candidate could be capable of regulating entire cellular pathways that have been pathogenically altered in a patient.

Following the discovery that miRNA could be identified in both extracellular and intracellular contexts, their potential application as biomarkers became a major focus of current research (Condrat et al. 2020). Nevertheless, the possible application of miRNAs is promising as they fulfill several properties of non-invasive and good biomarkers, such as their stable availability in various body fluids among other viewpoints (Cherradi 2015). For instance, it would be beneficial in early recognition of cancer, through utilization of liquid biopsies as a non-invasive screening method that enables the quantification of blood-borne molecular biomarkers in a targeted manner. In addition, there is a fundamental clinical demand to predict recurrence and the need for adjuvant therapy based on molecular signatures. For example, cell-type-specific expression of miRNAs can reflect histological types and can effectively complement the classification of tumors beyond classical morphology-based methods.

Beyond application of miRNAs as a biomarker, they are also promising therapeutic candidates in diseases, that may not be provoked by a single major genetic event as in case of monogenic disorders. In fact, small molecule drugs can be designed to pleiotropically target multiple targets or pathways. Due to the development of bioinformatic techniques for identifying miRNA-binding sites in target genes and the associated biological pathways, as well as an expanding repertoire of in vitro and in vivo preclinical research models, tools (miRNA mimics and repressors) and strategy for experimental validation of miRNA-mRNA interactions (Riolo et al. 2021), miRNAs may be used in therapeutic practice very soon. Several pharmaceutical and biotechnology companies have already started to develop miRNA-based diagnostics and therapeutics research in their development pipeline (Bonneau et al. 2019, Hanna et al. 2019). The framework for these breakthroughs in the near future can be established by characterizing multiple cells and states, discovering molecular pathways, and optimizing the development strategy.

5. Conclusion

- By using genome-wide microRNA expression analysis in mast cells of an in vitro mouse model we have been among the first research groups to study and describe microRNA patterns that characterize the different phases of mast cell life cycle.
- During differentiation and maturation of mast cells we observed downregulation of certain miRNAs being specific for other haemopoietic lineages that presumably play a role in shaping the mast cell commitment.
- IgE/antigen-driven activation of mast cells found to associate with upregulation of miR-132 which is also confirmed in a human umbilical cord blood-derived mast cell.
- We identified a negative feedback mechanism in which the activation-induced miR-132 suppress the protein production of the concomitantly activated HBEGF gene. Importantly, HBEGF is a potent growth factor for epithelial cells in various pathological contexts, however, require a tight control, as e.g. overexpression of HBEGF was showed to paradoxically decrease proliferation of keratinocytes.
- We have successfully used a novel strategy for prioritizing predicted miRNA targets by combining the results of multiple algorithms to uncover a regulatory mechanism with contribution of miR-132. This approach was translated and enhanced for a clinical problem linked to diagnostics of adrenocortical cancers in order to rank a large number of reverse correlating and possibly miRNA-regulated in silico predicted targets that also filtered based on tissue-specific expression to reduce false-positives.

6. Summary

In the present study, we explored the genome-wide changes in the microRNA pattern during the process of differentiation of mature mucosal mast cells and in IgE-mediated activation by applying microarray technology and a widely accepted in vitro model, bone marrow derived mast cells (BMMCs). Target prediction in silico methods were used to investigate the range of regulated mRNAs by miR-132 that is increased in activated mast cells. In order to exploit the results of multiple target predictions, ranked with different weights for calculations, the output lists were assembled into a jointed dataset. All the three used algorithm predicted with high probability a growth factor as a target, namely HBEGF, which is also relevant in the context of allergic inflammation. The regulatory role of miR-132 was confirmed by determining the functional ability of miR-132 to bind to the binding sites in the 3'UTR of the HBEGF mRNA and the changes in the of RNA and protein levels of this growth factor following mast cell activation. We demonstrated that miR-132 induction is able to post-transcriptionally regulate HBEGF expression in IgE/antigen-activated mouse and human mast cells. This unraveled mechanism may prevent the excessive production of mast cell-secreted growth factor, which plays a critical role in cell proliferation, cell migration, wound healing and a variety of other biological processes.

To improve the robustness of biomarker identification in adrenocortical tumors, an analysis approach was designed (1) to incorporate the results of experimentally obtained miRNA and mRNA profiles, including (a) reverse alteration between pairs as well as (b) raw expression levels for delineation of tissue-relevant transcripts, and (2) to consider outputs of multiple target prediction algorithms with united ranking for convertibility across algorithm-specific scores, applied successfully in previous mast cell relating works. These advancements have aided in the exploration of pathways with potential miRNA-driven regulation and in identification of biomarkers that enable a sensitive and specific discrimination between malignant and benign adrenocortical tumors.

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