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

Ph.D. thesis

Viktor Molnár

Molecular Medicine Doctoral School
Semmelweis University

Supervisor:	András Falus, DSc
Official reviewers:	Tamás Németh MD, PhD Péter Engelmann MD, PhD
Head of the Examination Committee:	Vásárhelyi Barna, MD, DSc
Members of the Examination Committee:	Márta Medvecz MD, PhD Barbara Uzonyi MD, PhD

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1. Introduction

Mature forms of miRNAs are a class of conserved, approximately 21-25 nucleotide long RNAs, representing a new layer regulating gene expression. 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. Imprecise complementarity allows miRNAs to bind to hundreds of targets.

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.

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. 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.

Along with their important involvement in the control of hematopoietic differentiation, miRNAs also play a significant role in the regulation of inflammation and immune response in mature cells. For instance, they are implicated as factors ensuring negative feedback regulation to block aberrant protein expression or defining a narrow protein concentration range for normal cellular 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. If appropriate, they respond to triggers by producing a wide range of mediators.

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). 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 can be distinguished by the set of proteases they produce, their localization in the body, staining properties, mediator content, and dependence on T-cell-derived cytokines.

The most well-known activation process of mast cells is the crosslink of the high-affinity FcεRI-bound IgEs, which characterizes allergic disorders.

Mast cell activation results in the release of a number of mediators, ranging from immediately released preformed mediators such as biogenic amines, proteoglycans, proteases, to de novo synthetized lipid eicosanoids and a wide array of proteins, like cytokines, chemokines and growth factors. 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 evidence support the role for mast cells in chronic inflammatory diseases and cancer (namely mast cell-associated diseases) as well.

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 in mice.

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. 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. 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.

Primary adrenal cortical carcinoma, known as adrenocortical carcinoma (ACC) represents a rare endocrine malignancy with significant clinical heterogeneity and high mortality. 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. 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.

2. Objectives

The aims of this work were 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

In vitro model of mouse mast cell differentiation and activation was applied 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).

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+/FcRI+ 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.

Reprogramming of microRNA expression characterizes mast cell differentiation and activation stages, where 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. During early commitment to mast cells, progenitors downregulate several miRNAs that are specific for other cell lineages and most likely help in determining differentiation programs.

Upregulation of MiR-132 is characteristic of IgE-mediated mast cell activation as miR-132 showed the most pronounced alteration in IgE cross-linked activated cells. Although miR-132 has been previously identified as an endotoxin-sensitive gene in monocytes, we showed that lipopolysaccharide failed to induce miR-132 in mast cells. An elevation in cytoplasmic Ca++ concentration has also been linked to the activation response, which may also be involved in miR-132 induction, as demonstrated by a comparable response to Ca-ionophore ionomycin treatment. To analyze and validate the expression of miR-132 in the human system as well, human cord blood-derived mast cells (CBMC) were applied.

Target prediction of miR-132 was applied to explore the range of regulated mRNAs. 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 predicted target HBEGF is upregulated during mast cell activation and showed quite significant 100-fold elevation on RNA, 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.

HBEGF is a target gene for miR-132 as it was demonstrated by silencing and overexpression experiments. Briefly, BMMCs were transfected by electroporation with either a miR-132 precursor (Pre-miR-132) or neutralizing oligonucleotides (Anti-miR-132). Transfected BMMC cells then were activated by IgE/antigen and the expression of miR-132 parallel to HBEGF was detected. 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. Moreover, the modulation of miR-132 had an consistent impact on the time course of the HBEGF response. Specifically, for the treatment with inhibitory anti-mir-oligos, 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.

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 at these cells were also transfected with the pre-miR-132 or with control oligos. We found that pre-miR-132 significantly reduced the luciferase activity of the HBEGF 3′UTR containing vector in a specific manner.

In summary, miR-132 induction is able to post-transcriptionally regulate HBEGF expression in IgE/antigen-activated mouse and human mast cells. This unravelled 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. Our suggested mechanism about the negative feedback regulation by miR-132 to buffering protein concentration to within a narrow concentration range has later been confirmed by others in different models and biological contexts, as well. By targeting the HBEGF mRNA, miR-132 involved in modifying response to mast cell activation and may limit through this mechanism the pathological remodeling and promoting wound healing in contexts where mast cell-derived growth factors are essential, e.g. in case of chronic allergen exposure.

Tissue-specific target prediction strategy in biomarker discovery

A biomarker discovery approach, successfully applied to a basic research problem and leads to the recognition of miR-132-driven HBEGF regulation, was further developed for a clinical question. 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.

4. Conclusions

- 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.

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