195 CRISPR/CAS9 GENE EDITING REVEALS NOVEL TERTIARY CONSTRAINTS IN CLUSTERED MIRNA PROCESSING

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Introduction MicroRNAs (miRNAs) play an important role in the cellular function. They often form families, with members sharing high sequence homology, a property that hampers miRNA research as there is a lack of elegant tools for specific miRNA manipulation.

Objective To establish a reliable workflow for miRNA inhibition using genome editing.

Methods and Results This study focused on miR-195, a member of the miR-15 family and employed the CRISPR-Cas9 system. To this end we generated mouse vascular smooth muscle cells (VSMCs) stably expressing Cas9 nuclease. Cells were then transfected with an *in vitro* transcribed single guide RNA targeting the miR-195 stem loop. T7 endonuclease I assay (T7EI) and Sanger sequencing confirmed efficient editing. QPCR demonstrated effective decrease of miR-195 but not of miR-15a and miR-16, two highly expressed members of the miR-15 family in VSMCs. Surprisingly the expression of miR-497 was also decreased in edited cells. Noteworthy, miR-195 and miR-497 form a miRNA cluster and are co-transcribed as a primary miRNA. No gene editing was detected by T7EI and sequencing of the mir-497 genomic locus. Computational simulation predicted that mutations of the miR-195 stem loop led to changes in the three dimensional structure of the primary miR-497∼195 transcript that could affect its processing to mature miRNAs.

Similar findings were obtained the miR-143∼145 cluster that encodes miR-143 and miR-145a, two miRNAs that do not belong to the same family, show no sequence homology and play a pivotal role in vascular biology. Specific targeting of the mir-145a locus effectively inhibited the expression of both miR-143 and miR-145a while no genomic editing was observed for the mir-143 locus. Noteworthy, the expression of Carmn, a long non coding RNA in the vicinity of the miR-143∼145 cluster that constitutes an independent transcription unit did not differ in miR-145a edited cells confirming that only the primary miRNA transcript is affected. On the contrary, gene editing in the miR-17∼92 and miR-106b∼25 clusters, two miRNA clusters with a key function in the cardiovascular system, resulted in targeted miRNA inhibition. MiR-18a and miR-25 were targeted on each cluster, respectively. Specific editing only for the intended miRNA locus was observed and QPCR quantification indicated inhibition of the edited miRNA. No effect on the expression of other miRNAs occurred, both for cluster miR-17∼92 and miR-106b∼25.

Detailed analysis of the gene editing in the four clusters revealed that the unintended inhibition of miRNA expression in the cluster coincides with disruption of sequence motifs of the terminal loop of the targeted hairpin, suggesting that these elements are critical for the maturation not only of individual hairpins but the entire primary transcript in miRNA clusters.

Conclusions CRISPR/Cas9 emerged as a powerful tool that can offer novel insights into the role of miRNAs in cardiovascular diseases.

196 FUNCTIONAL HIGH-THROUGHPUT SCREENING IDENTIFIES MICRORNA-26B AS PRO-SURVIVAL AND ANGIOGENEIC FACTOR FOR ENDOTHELIAL CELLS

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Critical limb ischaemia (CLI) is a serious form of peripheral artery disease where patients suffer from reduced arterial blood flow and subsequent loss of tissue viability. Therapeutic angiogenesis uses pro-angiogenic factors to stimulate new blood vessels formation to bypass existing blockages. Despite promising pre-clinical data, the outcome of clinical trials using single proangiogenic growth factors remains inconclusive. MicroRNAs (miRNAs) are small non-coding RNAs that orchestrate genetic networks by modulating simultaneous gene expression, thus suitable for therapeutic regulation of post-ischaemic angiogenesis. The aim of this study is to identify novel miRNAs that can influence vascular endothelial cells (EC) function and identify their relevant target genes. High content screening (HCS) of human miRNA mimic library is an emerging approach that utilises robotic microscopy platforms along with unbiased image analysis algorithms to generate quantitative data. Therefore, we used HCS to identify miRNAs that regulate EC proliferation and establish their potential as a therapeutic target for CLI. High throughput screening of over 1500 unique microRNA mimics revealed miR-26b as a top candidate for regulating EC functions. The overexpression of miR-26b significantly increased the proliferation and migration of ECs. Furthermore, it increased cell survival and importantly enhanced EC tube formation and branching morphogenesis. Stimulating ECs with the growth factor VEGFA increased EC proliferation associated with increased miR-26b expression. Expression analysis revealed an increase in endothelial cell tip enrichment genes including FLT4, ESM1, Ang-2 and PDGFB in EC overexpressing miR-26b. *In-vivo*, Q-PCR analysis confirmed an increase level of miR-26b expression in EC sorted form limb muscles 3 days post-ischaemia. Furthermore, we tested the effects on *in vivo* angiogenesis using the Matrigel implant model. Histological analysis at day 10 with the implants confirmed miR-26b mimics increased the vessel density. After confirming the important pro-survival and pro-angiogenic role miR-26b has in ECs, we used HCS to identify downstream target genes. We found that Phosphatase and Tensin homolog (PTEN) and phosphatase gene PPP2R2A are direct targets of miR-26b and their regulation determined the phenotype of miR-26b in ECs. MicroRNA-26b was for the first time identified as a pro-survival and pro-angiogenic factor for EC by the use of novel HCS techniques. We believe miR-26b is an excellent therapeutic target for the treatment of CLI and we continue to investigate and characterise its role in post-ischaemic angiogenesis.

197 THE CARDIAC STRESS RESPONSE PROTEIN MS1- A NEW TRANSCRIPTION FACTOR REGULATING HYPERTROPHY?

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