Editorial

Circular non-coding RNAs in diabetic retinopathy

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Diabetes mellitus is a pandemic disease currently affecting more than 400 million people worldwide (1). The common symptom of Diabetes is hyperglycemia. Chronically elevated blood glucose concentrations result in severe morbidities such as cardiac infarction, stroke, nephropathy, neuropathy and retinopathy primarily caused by micro- and macrovascular dysfunctions (1). Diabetic retinopathy (DR) is a multi-stage disease with a complex pathogenesis comprising changes in function and/or viability of several cell types such as pericytes, vascular endothelial cells, glial cells, neuronal cells and immune cells (2,3). Clinically the first symptoms of a beginning DR are presented as microaneurysms of the retinal vasculature and neuronal dysfunctions with not yet detectable neurodegeneration (2,3). The underlying cause of this disease is still subject of research and involves, amongst other reasons, formation of reactive oxygen species (ROS), reactive metabolites, activation of protein kinase C and the hexosamine pathway (3,4). However, given the complexity of retinal retinopathy, which currently affects ~one-third of people with type 2 diabetes and its temporal disease stages other players, such as non-coding RNAs (ncRNAs) might also play a role.

Transcribed RNAs can be grouped into protein-coding messenger RNAs (mRNAs) and ncRNAs. The ncRNAs are nowadays subdivided by their size into small ncRNAs (<200 nt) and long ncRNAs (>200 nt, lncRNAs). However, there is no standardized classification. The extensively studied, ~20 nt long microRNAs (miRNAs) represent one class of small ncRNAs that repress the expression of their targets on the post-transcriptional level and exert functions for e.g., development and pathogenesis (5-7). In contrast, knowledge about the heterogeneously composed lncRNAs is limited but next generation sequencing techniques and array technologies brought first insights elucidating their potential roles. A special class of lncRNAs represent circular RNAs (cricRNAs), which are presumably generated from pre-mRNAs by back-splicing of exons, resulting in the exclusion of a covalently closed, circular RNA molecule (5,8). A special feature of circRNAs is their relatively high stability because they lack "open" ends that would be prone to nucleolytic degradation (9). Earlier it was supposed that back-splicing is only a very seldom event but this has been revised due to the findings that circRNAs were abundantly expressed in different cell types. The majority of circRNAs seem to be expressed at low levels, however, there are also genes like *HIPK3* where circRNAs are more abundant than their linear isoforms in special cell types (8-11). Mechanistically, inverted repeats, like e.g., ALU elements, or proteins binding to the pre-mRNA seem to be able to facilitate the generation of circRNAs from their linear isoforms. First studies revealed essential physiological functions of circRNAs for the brain in mice and humans (2,12,13). As an example circRNA *ciRS*-7 (also named *CDR1as*) is conserved in mouse and man with a preferential expression in the brain. *CiRS*-7 contains >60 miR-7 target sites and is localized in the cytoplasm where it functions as a miRNA sponge by competing with miR-7 targets for miR-7 binding (2,14). Overexpression of *ciRS*-7/*CDR1as* in zebrafish, which had lost the *cdr1* locus, resulted in a phenotype of impaired midbrain development comparable to a miR-7 knockout (14). However, a large number of circRNAs does not contain miRNA target sites (15) and thus do not function as a miRNA sponge. It was suggested that these circRNAs potentially act as scaffolds for the assembly of macromolecular complexes or act via other mechanisms (5,8). Until today only sparse

knowledge exists about the exact mechanisms, regulations, expression patterns and functions of the circRNA family within the lncRNAs.

In a recent issue of circulation Shan *et al.* (11) reported that a circular non-coding RNA from the *HIPK3* gene has important roles for the development of retinal dysfunctions in diabets. The authors initially screened the expression of predicted circRNAs of the *HIPK3* gene in human and murine endothelial cells. Subsequently they focused on a conserved circRNA isoform (specified as *circHIPK3*) expressed in samples from both species. The expression of *circHIPK3* was verified in various mouse tissues and different human endothelial cells such as retinal vascular endothelial cells (HRVEC). Strongly expressed cricRNAs from the *HIPK3* gene were also identified by two earlier studies in endothelial cells and fibroblasts (9,16). In *in vitro* cultured HRVECs challenged with high glucose, inflammatory cytokines or oxidative stressors exhibited an increased expression of *circHIPK3*, indicating roles for this circRNA during DR. Most importantly, *circHIPK3* expression was also increased in a time-dependent manner in STZ-induced diabetic mice.

The authors detected the transcription factor c-myb as an upstream regulator of *HIPK3/circHIPK3* expression by promoter-luciferase assays. Using HRVECs they demonstrated that the expression level of *circHIPK3* influenced the cellular properties; overexpression increased viability, proliferation and accelerated migration and tube formation, while silencing had the opposite effects. The *circHIPK3* was predominantly found in the cytoplasm of HRVEC, which is in line with earlier reports (9,16). The localization and *in silico* predicted miR target sites for some miR-30 family members, but also other miRNAs, suggested that *circHIPK3* might function as miRNA sponge. Worth mentioning, the predicted miR binding sites do not congruently match the data from a previous study (16).

Shan and co-workers could now show that *circHIPK3* acted as a miRNA sponge for some but not all members of the miR-30 family, all sharing identical seed sequences. Overexpression of miR-30a-3p by transfecting mimics yielded repressive effects on viability, proliferation, migration and the tube formation ability of HRVECs, while inhibitors had the opposite effects. The repressive effects of miR-30a-3p mimic transfection could be rescued by additional overexpression of *circHIPK3*, confirming the role of its sponging function. MiR-30a has been described as the most abundant miRNA of the miR-30 family in human endothelium representing ~2.6% of the miRnome (17). MiR-30a regulates angiogenic cell behavior through interaction with the Notch signaling component delta-like ligand 4 (DLL4) (17). Now *VEGFC*, *WNT2*, and *FZD2* were additionally detected as important miR-30a-3p targets effecting viability, proliferation, migration and the tube formation ability of HRVECs. Truly, the presence of VEGFC counteracted the repressive effects of *circHIPK3* silencing. To test these results *in vivo* the authors developed an adeno-associated viral (AAV) shRNA delivery system to silence *circHIPK3* in diabetic mice. With this system the down-regulation of *circHIPK3* could partially reduce the negative effects on retinal vascular functions that occurred under hyperglycemia, while overexpression worsened the negative effects. The retinas of diabetic mice also exhibited higher expression levels of *VEGFC*, *FDZ4* and *WNT2*, which could be decreased by shRNA-mediated knockdown of *circHIPK3* as already seen in their *in vitro* experiments. Remarkably was the 1–2 weeks delay of experimentation after AAV delivery due to problems with the gene expression detection immediately after virus injection. Furthermore, the authors showed that also miR-30a-3p overexpression, similar to *circHIPK3* downregulation, could reduce the diabetes-induced retinal vascular leakage. This indicates that a miR-30a-3p intervention may overcome the *circHIPK3* sponge to decrease diabetes-associated retinal vascular dysfunctions. Finally, the authors identified upregulated *circHIPK3* concentrations in the blood plasma of diabetic patients. This is, however, conflicting with a recent study in which patients with type 2 diabetes mellitus (T2DM) were sub grouped into patients with or without DR and compared to healthy controls. Thirty differentially expressed circRNAs were identified by microarrays in patients with DR but circHIPK3 was not included in this list (18).

So far only a limited number of miRNAs were discovered that play roles in DR. For miR-126 it has been shown that this miRNA inhibits the expression of VEGF and MMP9, both important for DR, as well as that its expression decreases under hypoxic conditions (19). In line with this T2DM patients exhibited decreased miR-126 concentrations in the plasma (20). Along with the finding that downregulation of miR-126 could induce endothelial injury as well as participate in development/progression of diabetic vascular complications (21,22) these points indicate important roles of miR-126 in hypoxia-induced angiogenesis. MiR-146 was linked to a negative feedback regulatory mechanism on thrombin-induced GPCR-mediated NF-κB activation, a decreased

adenosine deaminase-2 expression and subsequently lower inflammatory responses (23), thereby suggesting a crucial regulatory role during the pathogenesis of DR. More recently, also a SNP in miR-146a was reported to be associated with DR and diabetic macular edema (24). *CircHIPK3* is now the first circRNA connected to the pathogenesis of DR. Out of three reasons this finding is of particular interest for potential translations into the clinic. First, *circHIPK3* expression was detected in the serum of diabetic patients, which potentially allows establishing *circHIPK3* as a novel biomarker. Second, *circHIPK3* is involved in the regulation of proliferation and survival of vascular endothelial cells, a key cell type during the early stages of vasoregression in non-proliferative retinopathy as well as later during vascular remodeling in the stage of proliferative retinopathy. Third, *circHIPK3* seems to act via sponging miR-30a-3p. Thus, *circHIPK3* or mir-30a-3p might represent important therapeutic targets in the future. However, this is the first report involving circRNAs and their roles in DR. Future studies should confirm these findings especially because in the data obtained by Gu and colleagues (18) *circHIPK3* was not one of the 30 differentially expressed circRNAs when comparing T2DM patients with or without DR and healthy controls. Nevertheless, this study indicated an additional valuable result because it identified *VEGFC*, *FDZ4* and *WNT2* as possible markers and effectors of DR. The exact mechanisms how these three genes are involved in the complex and multi-stage DR should be further clarified.

In summary the authors identified *circHIPK3* as part of the circHIPK3/miR-30a-3p/VEGFC-FDZ4-WNT2 network that influences retinal endothelial cell functions *in vitro* and *in vivo*. Furthermore, *circHIPK3* might be a biomarker for DR and its involvement as miRNA sponge for miR-30a-3p allows envisioning *circHIPK3* and miR-30a-3p as therapeutic targets in the future. More detailed pre-clinical studies would be required to characterize how the described network integrates into the complex and, multi-stage process of DR, which affects next to the endothelial cells also a variety of other cell-types.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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