

# Treating the diabetic wound through miR inhibitor cocktails: A question of timing?

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Type 2 diabetes (T2D) and the associated hyperglycemia-led microvascular alterations challenge the integrity of the microvascular endothelia through the weakening of both intercellular junctions and angiogenesis. These hyperglycemia-driven impairments increase vascular permeability, corrode wound healing, promote chronic ulceration and loss of tissues, and possibly lead to amputation, making the treatment of peripheral artery diseases (PAD) difficult. Unsurprisingly, the increasing prevalence of diabetes and hypertension led to an increase of 31% in the global disability owing to diabetes-related amputation between 1990 and 2016.<sup>1</sup> In recently published work, Kujawa et al.<sup>2</sup> show that inhibitions of microRNA (miR)-200 and miR-466 regulate the permeability of endothelial cells (ECs) subjected to hyperglycemia and improve wound healing in diabetic db/db mice. miR-200 and miR-466 may act on different stages of wound healing. Indeed, miR-466 inhibition was most efficiently restoring the expression of the tight junction protein claudin-5, while miR-200 inhibition improved angiogenesis. The synergistic effect of combining miR-200 and miR-466 inhibitions proposed by Kujawa et al.<sup>2</sup> was most obvious in the first week of healing, but remains minimal at later time points. Through their work, Kujawa et al. provide additional evidence that miR-based therapies represent a promising avenue to treat non-healing wounds with the goal of delivering better outcomes for patients at high risk of amputation. Yet, their work stresses some of the challenges inherent to miR targeting for treating wound healing: delivering effective doses of miR inhibitors at the right time (Figure 1A).

Restoring the usually well orchestrated process of wound healing might, indeed, require

a perfect timing of miR targeting. The transient downregulation of miR-200b, one member of the miR-200 family, supports the angiogenic switch in wound healing.<sup>3</sup> In healthy mice, the decrease in miR-200b 3 days after wound enhanced the expression of the transcription factor GATA-binding factor 2 (GATA2) and vascular endothelial growth factor receptor 2 (VEGFR2), generating a pro-angiogenic profile in the tissue in the following days. Db/db mice lacked this capacity to downregulate miR-200b; therefore, the silencing of GATA2 and VEGFR2 remained in place during wound healing.<sup>3</sup> In work by Kujawa et al.,<sup>2</sup> miR-200 inhibition alone had no impact on the early stage of wound healing (first week) in db/db mice. Yet, the authors observed an increased capillary density in the wounded tissues of db/db mice treated with miR-200 inhibitors, further validating miR-200 as a valuable target to trigger angiogenesis. The db/db mice treated with both miR-200 and miR-466 inhibitors did not show significant differences in wound closure compared with db/db mice treated with miR-200 inhibition only. It is plausible here that the cocktail of miR inhibitors used by Kujawa et al. could not normalize all phases of wound healing.

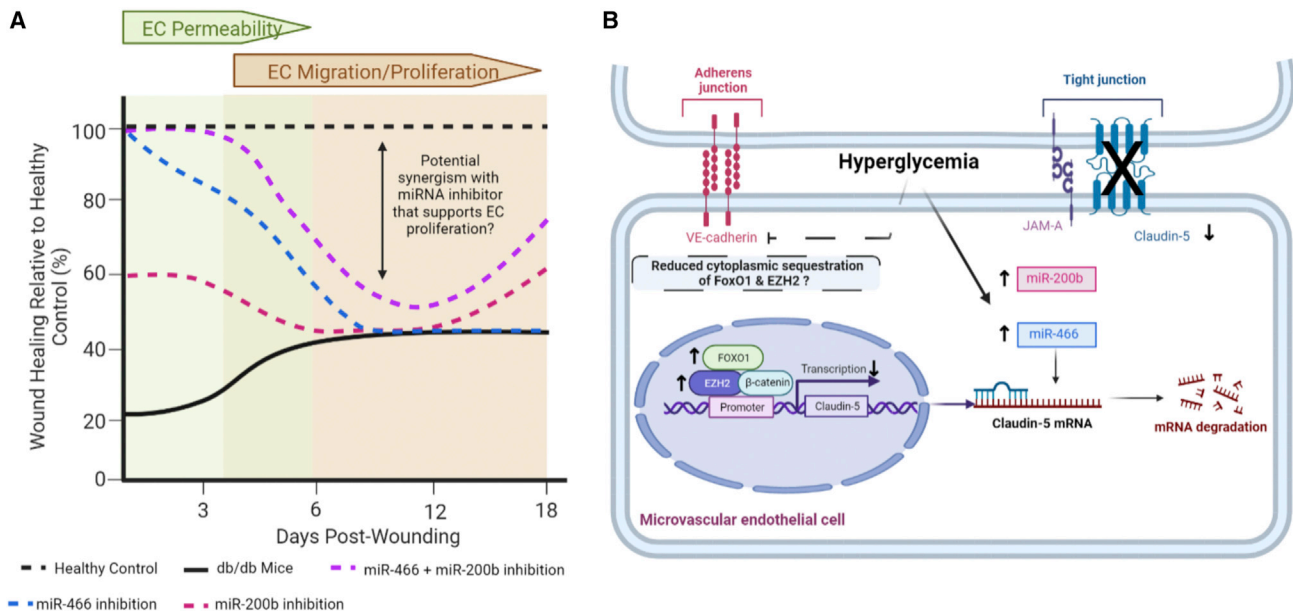
The activity of microvascular ECs (MECs) is staggered over multiple phases of wound healing (i.e., permeability and angiogenesis). In the early stage, the MEC plays a crucial role in regulating permeability, acting as an active barrier that coordinates the paracellular movements of ions, nutrients, plasma proteins, and cells between the blood and the tissue. A key determinant of microvascular permeability and resulting tissue inflammation are inter-endothelial junctions. Adherens

junctions (AJ) and tight junctions (TJ) support inter-endothelial interactions through transmembrane protein complexes organized around adhesive proteins, vascular endothelial (VE)-cadherin and claudin proteins, respectively. Work by Kujawa et al.<sup>2</sup> brings new insight regarding the mechanisms through which hyperglycemia-driven miR silencing controls the expression of claudin-5, the most abundant claudin in ECs and a key component of TJ. *In vitro*, precursors of miR-200 and miR-466 efficiently silence claudin-5 expression in aortic ECs (AECs) under hyperglycemia and the use of miR inhibitors confirm a role of both miRs in regulating permeability.<sup>2</sup> However, in MECs, only miR-466 inhibition efficiently restored claudin-5 levels under hyperglycemia, while both inhibitors efficiently decreased permeability. This questions whether miR-200 acts on permeability only through a claudin-5-dependent mechanism during hyperglycemia. Thus, Kujawa et al. report noticeable differences in the response of AECs and MEC to miR inhibition. EC heterogeneity might help to explain what could appear as a discrepancy, as the expression and regulation of inter-endothelial junctions differs depending on their origin in the vasculature.<sup>4</sup> As permeability varies along the vascular tree, the number of TJ and expression of claudin-5 follows.<sup>4</sup> *In situ*, claudin-5 is highly expressed in artery compared with vein, while claudin-5 expression decreases gradually when moving along the microvascular tree from arterioles to venules.<sup>4</sup> Interestingly, the data reported by Kujawa et al.<sup>2</sup> regarding the *in vivo* impact of miR-200 and miR-466 inhibitions on permeability fit better with their *in vitro* data collected on MECs compared with those collected on AEC. This highlights the importance of using MECs when studying permeability and the role of claudin-5 in wound healing.

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**Figure 1. The timing the treatment of miR-inhibitors to normalize TJ and angiogenesis in the diabetic wound**

(A) Diabetes attenuates the capacity for wound healing. The synergistic inhibition of miR-200 and miR-466 leads to an improved wound healing response in diabetic db/db mice. miR-466 inhibition seems to be most beneficial on the early stage of wound healing where alterations of permeability and migration might prevail in diabetic MECs. miR-200 inhibition might have a broader range of action correcting the capacity of MECs to migrate and to form tube in the early and later stage of wound healing, respectively. Yet, the observed lack of full restoration suggests that the cocktail of miR-200 and miR-466 inhibitors might not normalize the proliferative capacity of MECs during the diabetic state. This may contribute to a negated normalization of wound healing to control levels. This stresses the difficulty of designing miR-targeted therapies that influence all aspects of wound healing to achieve a high degree of efficacy in restoring these processes. (B) Within a physiological context, the expression of claudin-5 is influenced by the integrity of both AJ- (VE-cadherin) and TJ-related (Junctional adhesion molecule A [JAM-A]) proteins. Hyperglycemia lowers claudin-5 expression in MECs. The work of Kujawa et al. indicates that hyperglycemia-inducible miRNAs, miR-200 and miR-466, negatively influence claudin-5 expression through translational repression by directly binding to the 3' untranslated region of claudin-5 messenger RNA (mRNA). This brings another layer of intricacy to claudin-5 regulation in MECs. Indeed, multiple mechanisms may contribute to decreasing claudin-5 levels in diabetic MECs. Future studies will need to address whether hyperglycemia leads to decreased cytoplasmic sequestration of EZH2 and FoxO1 by VE-cadherin, resulting in PRC2-mediated epigenetic silencing of claudin-5 mRNA. This will help to uncover how hyperglycemia destabilizes TJs through transcriptional and translational gene silencing.

The data presented by Kujawa et al.<sup>2</sup> suggest that miR-200 and miR-466 may alter different stages of wound healing. miR-200 was a more potent negative regulator of the non-proliferative angiogenic processes than miR-466 in AECs.<sup>2</sup> Increased expression of miR-200 using a miR-specific precursor negatively regulated both migration and tube formation in cultivated AECs, while miR-466 precursor only impacted migration. The inhibitors of miR-200 and miR-466 used alone or in combination prevented hyperglycemia-induced permeability in both AECs and MECs. The inhibition of miR-466, and not miR-200, restored claudin-5 protein expression in MECs exposed to hyperglycemia. *In vivo*, the topical application of these inhibitors led to a significant improvement in the wound healing process in a T2D db/db mouse model. miR-466 inhibition was more efficient than miR-200 in restoring claudin-5 positive area in

wounded skin. While miR-466 inhibition seems to be beneficial in the early phase of wound healing to restrain hyperpermeability through the maintenance of claudin-5 expression, combining it with miR-200 inhibition might enhance MEC migration and capillary formation to support angiogenesis throughout the whole process.

It is noteworthy that the work by Kujawa et al.<sup>2</sup> complements the existing literature regarding how endothelial claudin-5 expression is silenced when intercellular junctions are altered in human diseases. The cross-talk between the AJ and TJ in ECs helps to establish and maintain a stable microvasculature that retains its barrier function. When AJs are destabilized by VE-cadherin inhibition or knock-down, the coordinated action of the repressor  $\beta$ -catenin and the transcription factor Forkhead Box protein O1 (FoxO1) sup-

presses claudin-5 transcription. This mechanism of claudin-5 silencing appears to be further supported by epigenetic regulation through the polycomb repressive complex 2 (PRC2).<sup>5</sup> In the absence of functional AJs, FoxO1 supports the recruitment of the Enhancer of Zeste Homology 2 (EZH2), the catalytic subunit of PRC2 on the promoter of claudin-5. The resulting enrichment in histone 3 trimethylation on lysine 27 (H3K27<sup>me3</sup>) around claudin-5 transcription start site leads to epigenetic silencing.<sup>5</sup> In a healthy quiescent microvascular endothelium, the silencing of claudin-5 is circumvented as AJs and TJs remain functional. First, clustering of VE-cadherin in AJs restricts the nuclear localization of both  $\beta$ -catenin/FoxO1 and EZH2. Additionally, the presence of the Junctional adhesion molecule A, a transmembrane component of TJ, supports claudin-5 expression by enhancing the transcriptional

activity of CCAAT Enhancer Binding Protein- $\alpha$  and restricting the repressive action of  $\beta$ -catenin.<sup>6</sup> Kujawa et al.<sup>2</sup> add a further layer of intricacy to the regulation of claudin-5, where miR-200 and miR-466 could act through translational repression in human ECs. Transcriptional silencing of claudin-5 expression might be highly relevant in ovarian cancer, where alterations of endothelial junctions are frequent.<sup>5,6</sup> So, one may question whether translational repression of claudin-5 by miRs further support silencing in the tumor microvasculature. It will be equally interesting to study whether the transcriptional repression of claudin-5 induced by FoxO1/ $\beta$ -catenin/EZH2 acts in conjunction with miR-translation repression to alter wound healing. In preclinical models of PAD (i.e., skeletal muscle ischemia) and in pre-T2D conditions, the increased expression of endothelial FoxO1 seems to restrict angiogenesis.<sup>7,8</sup> And, intermittent high glucose levels could enhance the nuclear localization of EZH2 in EC.<sup>9</sup> It is tempting to hypothesize that hyperglycemia uses these multilayered and potentially overlapping processes to repress of claudin-5 expression and to drive T2D-related microvascular alterations (Figure 1B). This remains to be ascertained. Therefore, further investigations are necessary to determine the best approach to normalize the endothelial junctions during T2D.

Recurrent exposure to hyperglycemia fragilizes the microvascular endothelium by un-tightening the inter-endothelial junctions. This may be viewed as an overflowing dam where inflammatory molecules and infiltrating cells might just flood the wounded

tissue. Here, Kujawa et al. provide promising evidence that this dam could be kept under control by combining multiple miR inhibitors. By normalizing permeability and the non-proliferative angiogenic processes, the combined inhibition of miR-200 and miR-466 supports better wound healing. However, the absence of a full wound closure observed by Kujawa highlights the difficulty of designing and developing miR-targeting therapies that achieve a high degree of efficacy and normalize wound healing. Their work provides a good rationale for further investigating how to treat the diabetic wound using miR inhibitors and draws attention to the necessity of delivering the most effective cocktail at the right time.

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