Diabetic Complications Consortium

Application Title: Nanotechnology-based solutions for diabetic peripheral arterial disease

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1. Project Accomplishments:

Cell therapies have emerged as a promising strategy for diabetic complications. Angiotrophic cell therapies, for example, have shown great potential for addressing vascular deficiencies[4, 1-8]. Current approaches to cell therapies under diabetes, however, face multiple hurdles, including the use of scarce or functionally impaired cell sources (*e.g.,* endothelial progenitors), and the need for cumbersome and/or immunogenic/carcinogenic *ex vivo* pre-processing steps (*e.g.*, induced pluripotency, expansion)[8-13]. Advances in cell reprogramming *in vivo* could potentially overcome these limitations by utilizing readily available cells (*e.g.,* fibroblasts), and bypassing the need for *ex vivo* handling[14]. Current reprogramming methodologies, however, are fraught with caveats, including heavy reliance on viral vectors[15, 16]. While promising, safety concerns hamper clinical implementation, and although adeno-associated viruses (AVV) are less pathogenic, AVV-host interactions and immunity are still a major concern[17]. Moreover, the stochasticity of status quo methods (viral/non-viral) poses additional limitations further highlighting the need for safer and more deterministic technologies. Tissue nano-transfection (**TNT**) is a more deterministic technology with single cell resolution co-developed by PI Gallego-Perez[18-21]. Such an approach allows direct reprogramming factor delivery in a rapid (~100ms) and non-invasive manner by applying a highly focused electric field through arrayed nanochannels, causing localized poration and electrophoretic delivery of reprogramming factors.

Diabetic tissues possess inherent dysfunctionalities that could hamper reprogramming. Here we used the TNT nanotechnology to evaluate key pathway components mediating the conversion of diabetic skin stroma into functional vascular parenchyma, and the use of such induced vasculature (iVas) in the resolution of critical limb ischemia in murine models of type 2 diabetes (T2D). Experiments in non-diabetic mice established that nanochannel-based delivery of *Etv2*, *Foxc2*, and *Fli1* (*EFF*) converts stromal cells/tissue into vascular cells/parenchyma *in vitro* and *in vivo.* Experiments with diabetic mice, however, indicate that the *EFF* reprogramming cocktail needs to be modified in order to induce vasculogenic reprogramming under diabetes. Additional studies were conducted establishing that TNT can be used to tailor the plasticity of diabetic skin stroma in order to facilitate diabetic skin reprogramming for therapeutic applications. Tcf3/ß-catenin/Sox9 emerged as potentially targetable links in the reprogramming cascade towards a vasculogenic lineage, with TNT'd *Tcf3* stimulating focal activation of ß-catenin and Sox9 in skin stroma. Both of these factors have been known to play a role in blood vessel formation, and as such could conceivably be used to potentiate iVas derivation from diabetic skin stroma, with the intent to resolve vascular deficiencies that lead to diabetic complications. Future studies should therefore

consider developing and testing new/advanced reprogramming cocktails towards a vasculogenic lineage by introducing *Tcf3* plasmids into the reprogramming gene mixture.

2. Specific Aims:

Specific Aim 1. Identify pathway components mediating efficient derivation of iVas in diabetic mice

Results: We developed a robust TNT-based methodology to reprogram skin fibroblasts into vascular cells (non-virally) in a fast and efficient manner. We first identified and validated a novel cocktail of reprogramming factors, *Etv2*, *Foxc2*, and *Fli1* (*EFF*), which effectively converted human (HDAF) and mouse (MEF) primary fibroblasts into vascular cells remarkably rapidly (<<1 week) upon nanochannel-based delivery of these factors. Newly formed vascular cells were able to assemble into blood vessels *in vitro* and *in vivo* following flank injection. Studies in non-diabetic mice showed that TNT-based delivery of *EFF* into the skin at a 1:1:1 ratio, promoted successful iVas derivation (*e.g.,* ~300% increase in vascular markers). Laser speckle imaging (LSI) showed increased blood flow to the treated area (*e.g.,* ~70% increase with respect to control at day 7 post-TNT). Ultrasound imaging confirmed the presence of superficial blood vessels, which are absent in the control skin. These newly formed vessels also exhibited a pulsatile behavior, thus suggesting successful anastomosis with the parent circulatory system of the mouse.

While these data established that TNT-based delivery of equimollary mixed *EFF* in to the skin can convert non-diabetic stroma into iVas, diabetic tissues possess inherent dysfunctionalities that could hamper reprogramming. We identified deficiencies in Fli1, intrinsic to diabetic tissues, that need to be overcome to trigger iVas derivation. Immunohistochemistry (IHC) and qRT-PCR analyses suggest that diabetic tissues exhibit Fli1 deficiencies at the protein and gene expression level (*e.g.,* ~50% decrease). Successful TNT-based derivation of iVas from diabetic skin required compensation for Fli1 deficiencies in murine models of T2D. Experiments in db/db mice indicate that increasing the molar ratio of *Fli1* in the *EFF* cocktail led to enhanced iVas formation, as evidenced by increased vWF immunoreactivity (*e.g.,* ~200%). The db/db mouse exhibits many of the hallmarks seen in T2D humans, such as vascular/cardiovascular disorders and associated complications[22-25]. The "baseline" 1x recipe, proven to work in non-diabetic skin, did not result in significant iVas formation under diabetes. Additional *in vitro* experiments show that nanochannel-based delivery of *Fli1* alone can induce angiogenic tube formation in matrigel, with tube-like structures extending over ~650 µm for *Fli1* alone vs. ~600 µm for *EFF*. *Fli1* transfection also resulted in increased endogenous expression of *Foxc2* (*e.g.,* ~25-fold increase). *Etv2* transfection, on the other hand, did not lead to enhanced *Foxc2* expression. These observations indicate that Fli1 alone may be able to modulate iVas morphogenesis *in vivo*.

In addition, recently we started to look into Tcf3 as a target transcription factor for the regulation of diabetic skin plasticity. Tcf3 is a key regulator of embryonic/adult skin progenitors, which promotes gene expression patterns associated with embryonic and

postnatal stem cells, and represses transcriptional programs that lead to skin-specific fate determination[26-28]. Tcf3 modulation may therefore facilitate diabetic skin stroma conversion into different types of parenchymata. Pilot studies revealed that *Tcf3* TNT on adult mice initially led to increased ß-catenin stimulation and early enhanced folliculogenesis[26, 27]. Interestingly, folliculogenesis partially occurred through invagination of epidermis into dermis, following a pattern only seen in embryonic skin[26]. Supportive of the notion that *Tcf3* TNT may impart a more plastic state to diabetic skin, IHC showed widespread expression of Sox9, a stem cell marker typically expressed only in the bulge and outer root sheet cells. Sox9 has also been shown to play a role in blood vessel formation under hypoxic conditions[29]. Likewise, ß-catenin overexpression/activation has been known to augmented angiogenesis[30].

Specific Aim 2. Evaluate the extent to which iVas counteracts CLI in a diabetic murine model.

Results: Once we established an adequate *EFF* cocktail for iVas derivation from diabetic skin stroma (*i.e., EFF* with 3x *Fli1* with respect to *Etv2* and *Foxc2* or 3x-*EFF*), we proceeded to evaluate the extent to which iVas can be used to resolve CLI in murine models of T2D. CLI was induced in db/db mice by transecting the femoral artery through a small incision. LSI was used to confirm decreased limb perfusion right after CLI induction. Decreased perfusion was more evident at the paw level compared to the non-ischemic contralateral limb. The underlying subcutaneous fat and associated vasculature in upper portions of the hindlimb impeded LSI-based verification of CLI. Nevertheless, IHC analysis at the muscle level clearly showed reduced vascularization as a result of the CLI surgery. 3x-*EFF* was TNT'd on the hindlimb skin 3 days post-CLI induction. LSI confirmed significantly improved paw perfusion 2-3 weeks following TNT intervention with 3x-*EFF* compared to controls (*e.g.,* ~140% increase). IHC analysis of muscle tissue revealed increased vascularization. Subcutaneous fat pads were also more vascularized in response to 3x-*EFF* TNT. These observations suggest that TNT-derived iVas can contribute to the resolution of limb ischemia under diabetes. Additional studies, however, would need to be conducted to determine the extent to which iVas derived from diabetic stroma exhibits endothelial dysfunctionalities typical of diabetes[31], which are also conducive to a number of complications related to vascular and cardiovascular deficiencies. Moreover, While preliminary findings suggest that TNT-based derivation of iVas from healthy and diabetic skin can reperfuse infarcted tissue, the extent to which the reprogrammed cell population needs to rely upon, and/or modulate secondary mechanisms important for establishing a functional vasculature (*e.g.,* paracrine angiogenesis), has yet to be established. Future studies need to evaluate whether endothelial cells derived from diabetic skin through reprogramming play an integral structural role in establishing functional iVas.

Along the same lines, in addition to evaluating the role of TNT in the reperfusion of ischemic limbs, we found that skin TNT'd with *EFF* releases extracellular vesicles (EVs) loaded with cDNA/mRNA copies of these factors, which can then be dispatched locally to induce vasculogenic reprogramming/remodeling in the ischemic tissue niche. Such EVs

were isolated from dorsal skin at different timepoints and analyzed via Nanosight. TNT'd *EFF* led to increased release of EVs, which were also smaller in size, on average, compared to EVs isolated skin TNT'd with mock/control plasmids. This potentially suggests increased presence of exosomal components (~40-120 nm) rather than microvesicles (~50-1,000 nm)[6]. EV released peaked around 24h for the *EFF* group compared to control (*e.g.,* ~10^{14} vs 10^{13} particles per 12 mm diameter biopsy. *Ex vivo* experiments in which mouse fibroblasts were exposed to *EFF*-laden EVs resulted in the formation of CD31+ cellular pockets not seen for control EVs. Injecting *EFF*-laden EVs into normoxic skin led to a noticeable increase in cellular CD31 and vWF expression. LSI analysis, however, showed only a modest (~20%) increase in skin perfusion compared to control EVs, which is possibly due to the action of angiostatic mechanisms responsible for modulating homeostasis in normoxic tissues[7]. Nevertheless, additional experiments in non-diabetic murine models of CLI indicate that such EVs could modulate muscle tissue revascularization following injection into the ischemic limb, and an overall increase in limb perfusion as determined by LSI (*e.g.,* ~190% increase). These observations clearly establish a potential role for secondary/paracrine signals, partially mediated by EVs, in the resolution of vascular deficiencies following TNT.

3. Publications:

- This project provided partial support to:

  Gallego-Perez, D. *et al*. Topical tissue nano-transfection mediates non-viral stroma reprogramming and rescue. Nat Nano advance online publication (2017).

- This project provided full support to:

  Higuita-Castro N *et al.*, Gallego-Perez D. Nanotechnology-based Reprogramming of Diabetic Tissue Resolves Local Vasculopathy. **In preparation**.

4. References:

1. Cho, S.W. et al. Improvement of postnatal neovascularization by human embryonic stem cell derived endothelial-like cell transplantation in a mouse model of hindlimb ischemia. *Circulation* **116**, 2409-2419 (2007).
2. Rufaihah, A.J. et al. Endothelial cells derived from human iPSCS increase capillary density and improve perfusion in a mouse model of peripheral arterial disease. *Arterioscler Thromb Vasc Biol* **31**, e72-79 (2011).
3. Lian, Q. et al. Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. *Circulation* **121**, 1113-1123 (2010).
4. Iwase, T. et al. Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. *Cardiovasc Res* **66**, 543-551 (2005).

5. Kinoshita, M. et al. Long-term clinical outcome after intramuscular transplantation of granulocyte colony stimulating factor-mobilized CD34 positive cells in patients with critical limb ischemia. *Atherosclerosis* **224**, 440-445 (2012).
6. Al-Khaldi, A., Al-Sabti, H., Galipeau, J. & Lachapelle, K. Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model. *Ann Thorac Surg* **75**, 204-209 (2003).
7. Humpert, P.M. et al. Locally applied mononuclear bone marrow cells restore angiogenesis and promote wound healing in a type 2 diabetic patient. *Exp Clin Endocrinol Diabetes* **113**, 538-540 (2005).
8. Tamarat, R. et al. Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment. *Am J Pathol* **164**, 457-466 (2004).
9. El-Ftesi, S., Chang, E.I., Longaker, M.T. & Gurtner, G.C. Aging and diabetes impair the neovascular potential of adipose-derived stromal cells. *Plast Reconstr Surg* **123**, 475-485 (2009).
10. Rodrigues, M. et al. Progenitor cell dysfunctions underlie some diabetic complications. *Am J Pathol* **185**, 2607-2618 (2015).
11. Lerman, O.Z., Galiano, R.D., Armour, M., Levine, J.P. & Gurtner, G.C. Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia. *Am J Pathol* **162**, 303-312 (2003).
12. Lee, A.S., Tang, C., Rao, M.S., Weissman, I.L. & Wu, J.C. Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies. *Nat Med* **19**, 998-1004 (2013).
13. Cunningham, J.J., Ulbright, T.M., Pera, M.F. & Looijenga, L.H. Lessons from human teratomas to guide development of safe stem cell therapies. *Nat Biotechnol* **30**, 849-857 (2012).
14. Heinrich, C., Spagnoli, F.M. & Berninger, B. In vivo reprogramming for tissue repair. *Nat Cell Biol* **17**, 204-211 (2015).
15. Grande, A. et al. Environmental impact on direct neuronal reprogramming in vivo in the adult brain. *Nat Commun* **4**, 2373 (2013).
16. Morita, R. et al. ETS transcription factor ETV2 directly converts human fibroblasts into functional endothelial cells. *Proc Natl Acad Sci U S A* **112**, 160-165 (2015).
17. Daya, S. & Berns, K.I. Gene Therapy Using Adeno-Associated Virus Vectors. *Clinical Microbiology Reviews* **21**, 583-593 (2008).
18. Gallego-Perez, D. et al. Deterministic transfection drives efficient nonviral reprogramming and uncovers reprogramming barriers. *Nanomedicine* **12**, 399-409 (2016).
19. Chang, L. et al. Dielectrophoresis-assisted 3D nanoelectroporation for non-viral cell transfection in adoptive immunotherapy. *Lab Chip* **15**, 3147-3153 (2015).
20. Chang, L. et al. Controllable Large-Scale Transfection of Primary Mammalian Cardiomyocytes on a Nanochannel Array Platform. *Small* **12**, 5971-5980 (2016).
21. Gallego-Perez, D. et al. Topical tissue nano-transfection mediates non-viral stroma reprogramming and rescue. *Nat Nano* **advance online publication** (2017).
22. Entabi, F. et al. Hind limb ischemia-reperfusion in the leptin receptor deficient (db/db) mouse. *J Surg Res* **139**, 97-105 (2007).
23. Rees, D.A. & Alcolado, J.C. Animal models of diabetes mellitus. *Diabet Med* **22**, 359-370 (2005).

24. Hummel, K.P., Dickie, M.M. & Coleman, D.L. Diabetes, a new mutation in the mouse. *Science* **153**, 1127-1128 (1966).
25. Yan, J. et al. Recovery from hind limb ischemia is less effective in type 2 than in type 1 diabetic mice: roles of endothelial nitric oxide synthase and endothelial progenitor cells. *J Vasc Surg* **50**, 1412-1422 (2009).
26. Nguyen, H., Rendl, M. & Fuchs, E. Tcf3 governs stem cell features and represses cell fate determination in skin. *Cell* **127**, 171-183 (2006).
27. Lien, W.H. et al. In vivo transcriptional governance of hair follicle stem cells by canonical Wnt regulators. *Nat Cell Biol* **16**, 179-190 (2014).
28. Merrill, B.J., Gat, U., DasGupta, R. & Fuchs, E. Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. *Genes Dev* **15**, 1688-1705 (2001).
29. Eshkar-Oren, I. et al. The forming limb skeleton serves as a signaling center for limb vasculature patterning via regulation of Vegf. *Development* **136**, 1263-1272 (2009).
30. Kim, K.-i. et al. β-catenin overexpression augments angiogenesis and skeletal muscle regeneration through dual mechanism of vascular endothelial growth factor–mediated endothelial cell proliferation and progenitor cell mobilization. *Arteriosclerosis, thrombosis, and vascular biology* **26**, 91-98 (2006).
31. Bakker, W., Eringa, E.C., Sipkema, P. & van Hinsbergh, V.W. Endothelial dysfunction and diabetes: roles of hyperglycemia, impaired insulin signaling and obesity. *Cell Tissue Res* **335**, 165-189 (2009).