Journal Pre-proof

Defining the activity of pro-reparative extracellular vesicles in wound healing based on miRNA payloads and cell type-specific lineage mapping

Dong Jun Park, Wooil Choi, Sakeef Sayeed, Robert A. Dorschner, Joseph Rainaldi, Kayla Ho, Jenny Kezios, John P. Nolan, Prashant Mali, Todd Costantini, Brian P. Eliceiri

Please cite this article as: Park DJ, Choi W, Sayeed S, Dorschner RA, Rainaldi J, Ho K, Kezios J, Nolan JP, Mali P, Costantini T, Eliceiri BP, Defining the activity of pro-reparative extracellular vesicles in wound healing based on miRNA payloads and cell type-specific lineage mapping, *Molecular Therapy* (2024), doi: https://doi.org/10.1016/j.ymthe.2024.02.019.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Untreated diabetic wound

Epidermis

Dermis

Adipose

Macrophage EVs

**Pro-resolution phenotype
in treated diabetic wound**

miR-425-5p-
loaded EVs

Fibroblasts

Proliferation Basal
Keratinocytes

Adiponectin ↑

1

2

Defining the activity of pro-reparative extracellular vesicles in wound healing based on miRNA payloads and cell type-specific lineage mapping

6

7

8

Dong Jun Park[1], Wooil Choi[1], Sakeef Sayeed[1], Robert A. Dorschner[2], Joseph Rainaldi[3], Kayla Ho[1],

Jenny Kezios[1], John P. Nolan[4], Prashant Mali[3], Todd Costantini[1], Brian P. Eliceiri[1,2*]

[1]Department of Surgery, University of California San Diego, La Jolla, CA 92093, USA

[2]Department of Dermatology, University of California San Diego, La Jolla, CA 92093, USA

[3]Department of Bioengineering, University of California San Diego, La Jolla, CA 92093, USA

[4]Scintillon Institute, San Diego, CA 92121, USA

*Correspondence: beliceiri@health.ucsd.edu (B.P.E.)

17

18

19

20 **ABSTRACT**

21 Small extracellular vesicles (EVs) are released by cells and deliver biologically active payloads to coordinate the response

22 of multiple cell types in cutaneous wound healing. Here we used a cutaneous injury model as a donor of pro-reparative

23 EVs to treat recipient diabetic obese mice, a model of impaired wound healing. We established a functional screen for

24 miRNAs that increased the pro-reparative activity of EVs and identified a down-regulation of miR-425-5p in EVs *in vivo*

25 and *in vitro* associated with the regulation of adiponectin.   We tested a cell type-specific reporter of a tetraspanin CD9

26 fusion with GFP to lineage map the release of EVs from macrophages in the wound bed, based on the expression of miR-

27 425-5p in macrophage-derived EVs and the abundance of macrophages in EV donor sites. Analysis of different

28 promoters demonstrated that EV release under the control of a macrophage-specific promoter was most abundant and

29 that these EVs were internalized by dermal fibroblasts.   These findings suggested that pro-reparative EVs deliver

30 miRNAs such as miR-425-5p that stimulate the expression of adiponectin that has insulin-sensitizing properties. We

31 propose that EVs promote intercellular signaling between cell layers in the skin to resolve inflammation, induce

32 proliferation of basal keratinocytes, and accelerate wound closure.

33

INTRODUCTION

Healthy repair of cutaneous wounds is a coordinated response of hemostasis, immune cell recruitment, angiogenesis, and re-epithelialization,[1,2] however, dysregulation of these normal processes in diabetes, obesity, aging and infection presents a risk for chronic wounds.[3,4] Recent studies have identified extracellular vesicles (EVs), especially small EVs (50-120 nm), as the most abundant EV mediators of signaling crosstalk between mammalian cells.[5-7] In the context of wound healing, we have previously shown that small EVs comprise the vast majority of all EVs in the wound bed,[8-10] and deliver biologically active nucleic acid and protein payloads demonstrating their physiological relevance in intercellular signaling in skin injury.[8,11] Currently, many EV studies include the use of *in vitro* cultured cells as EV donors that often test activity of human cell-derived EVs in mouse models.[12] The use of human derived EVs in mouse models represents a significant limitation of the translational potential because of rejection considerations. An additional limitation is the overall lack of *in vivo* studies that address the heterogeneity of *in vivo* EV donors and the cell type of origin of EVs released into the wound microenvironment.[11,13,14]

Since pro-reparative activity of EVs that is generally associated with their ability to promote tissue repair by horizontal transfer of nucleic acids and proteins to recipient cells,[7,15] we developed an allograft model where EVs were harvested from subcutaneous implantations of sterile polyvinyl alcohol (PVA) sponges.[9,10] PVA sponge implants were originally developed as an animal model of foreign body response that we adapted for the efficient recovery of cells that release EVs relevant to the immune component of the injury response and for *in vivo* gene delivery to modify the activity of infiltrating cells.[16-18] The key advantages are that high concentrations of biologically active EVs are recovered using non-destructive approaches without the complications of blood products and culture media components used *in vitro*.[8] We previously used this model to identify the mobilization of human myeloid cells to PVA sponge implants in humanized mouse models, defined the activity of specific EV biogenesis genes that uncouple the production of pro-reparative EVs in wound healing, and used vesicle flow cytometry (vFC) to quantify EV heterogeneity *in vivo*.[9,10] Another key limitation of many wound healing studies of EVs as therapeutics is the lack of translationally relevant animal models of impaired tissue repair. We addressed this by focusing on testing of genetically-defined mouse models of impaired wound healing such as the Leptin receptor knockout mouse, referred to as the db/db mouse, that has an onset of obesity and hyperglycemia at 12 weeks of age.[19-22] db/db mice are characterized by impaired wound healing kinetics and are an important genetic model for the study of injury-related complications in diabetes. Protease inhibitors were identified as

61 down-regulated in EVs isolated from db/db mice. In the context of functional testing of the biological activity of specific

62 pro-reparative EV payloads, we have recently engineered the protein payload of the EVs.   Specific protease inhibitors

63 were over-expressed in engineered EVs and used to reverse the impaired wound healing phenotype.[8] Together, we

64 identified the importance of using genetically-defined donor mice for allograft studies of the biological activity of donor

65 EVs to address a major challenge in the translational relevance of EVs as therapeutics and engineered EV payloads in

66 cutaneous injury.

67   While the relative importance of EVs released from various cell types *in vivo* remains poorly understood, recent

68 advances in the development of cell type-specific transgenic models have demonstrated the utility of tracking fluorescent

69 EV reporters.[23-26] For example, expression of EV-associated proteins such as the tetraspanins CD9, CD63 and CD81 as

70 fusion proteins with fluorescent reporter proteins can be used to identify EV distribution in the circulation and tumor

71 microenvironment.   Since these three tetraspanins are among the most highly enriched EV markers, they have been used

72 to monitor EV trafficking *in vitro* in release and uptake studies.[25,26]   In the context of cutaneous injury and our

73 development of defined allograft models of EV release,[8-10] we used single cell RNA sequencing (scRNAseq) to define

74 the cellular landscape from where EVs are harvested in combination with transgenic mice expressing CD9 with a C-

75 terminal green fluorescent protein (GFP) tag to determine the relative contributions of specific cell types in the donor

76 site microenvironment.[24]   These reporter approaches address key questions regarding the relative contributions of EVs

77 released from different cell types, as well as being useful tools to assess the uptake of GFP[+] EV populations in recipient

78 cells.   In addition to GFP-based reporter models, we used single vFC to quantify the size, number, expression of specific

79 endogenous proteins presents on the surface of EVs, as well as tracking of epitope tagged EV proteins.

80   In the field of cutaneous injury, intercellular communication can regulate differentiation and tissue injury

81 responses between adipose, dermal, and epidermal cell layers.   Directional movement of cells can also be controlled by

82 persistent release of EVs that conditions the microenvironment, promotes adhesion, and regulates cell polarization.[25]

83 Therefore, we focused on testing the pro-reparative activity of EVs isolated from wildtype (WT) vs. db/db animals

84 implanted with PVA sponges that were used to collect donor site EVs.   We identified EV-mediated differences in EVs

85 from WT vs. db/db donors that regulated wound repair kinetics, changes in microRNA (miRNA) payloads and tested the

4

86   activity of specific miRNAs by loading EVs and testing their capacity to restore tissue repair in the impaired wound

87   healing model db/db recipient mice.[13]

88   Together, these studies take advantage of recent technological advances in vFC,[27] EV payload profiling by

89   miRNAseq,[28] transgenic reporters to identify EV source,[29] and uptake in cell types relevant to wound repair.[6]   Based

90   on the distribution of macrophages interspersed in subcutaneous adipose tissue,[30]   we propose that macrophage-derived

91   EVs can be internalized by overlying fibroblasts, leading to production of adipokines such as adiponectin that are pro-

92   reparative and associated with increased cell proliferation of basal keratinocytes. Our data supports a model in which

93   EV-mediated acceleration of wound closure is regulated by specific miRNA payloads released by donor cells such as

94   macrophages to affect the activity of overlying cell layers of the skin.

95

96   **RESULTS**

97   **Diabetic obese mice drive a transcriptional reprogramming of immune cell subsets recruited into sites of**

98   **cutaneous injury.**

99   To determine the source of EVs and identify biologically active payloads in impaired models of wound healing

100   in the analysis of EVs from biological fluids it was essential to have model systems with defined cell profiles. Therefore,

101   we used scRNAseq to identify the activation state of cell types infiltrating sterile subcutaneous PVA sponges implanted

102   in the dorsum of WT mice (Figure 1A), a model that we and others have shown reflects the recruitment of macrophages

103   and neutrophils observed in wound healing.[8-10,31] scRNAseq was performed at 7 days post-implantation, a timepoint that

104   we previously showed was associated with peak of EV release,[8] to identify cells recruited to the PVA sponge. These

105   cells were primarily macrophages and neutrophils, along with lower levels of dendritic cells and lymphocytes, as seen

106   on the UMAP projection (Figure 1B and Supplementary Figures S1A and B). Expression of canonical genes associated

107   with each cell type (Log2Max visualization of multiple genes based on Loupe Browser; Figure 1C and Table S1) were

108   used as the basis to identify cell type-specific changes in gene expression mediated by the loss of the leptin receptor in

109   the db/db mouse model (Figure 1D). Cell type designations were based on canonical genes for each cell type (i.e., *Trem2*

110   for macrophages, *S100a8* for neutrophils, *Ccr7* and *Zbtb46* for DCs, and *CD3* for lymphocytes).[32-42] Additional genes

111   (i.e., *H2-dmb1* and *Ms4a4c*) were also identified as highly expressed in macrophages infiltrating PVA sponges (Table

5

112 S1).[43,44] We identified changes in gene expression that were common among several cell types such as increased levels

113 of Apolipoprotein E (*ApoE*), Cathepsin L (*Ctsl*), and Prostaglandin synthase (*Ptgs2*), and Cystatin domain proteins

114 (*Cstdc4*). Cell type-specific changes in gene expression of the top 10 genes upregulated vs. down-regulated genes of WT

115 vs. db/db PVA sponges were observed in each major cell type. For example, we noted changes of specific genes in

116 macrophages (*Stfa2l1* and *Trf*), neutrophils (*Egr*), DCs (*Lyz2* and *Ccl17*), and lymphocytes (*Mmp12* and *Il1r2*).

117 scRNAseq analysis identified additional changes in gene expression associated with the db/db mouse model that were

118 primarily comprised of metabolic factors associated with diabetic obesity (Supplementary Figure S1D). Importantly,

119 regardless of the genetic background of the donor mice, similar numbers of macrophages, neutrophils, DCs and

120 lymphocytes were recruited to the PVA sponge in db/db and WT mice (Supplementary Figure S1E and F), and were

121 consistent with analyses of cell types recruited to the PVA sponge based on antibody-dependent flow cytometry studies.[8]

122 These findings identified macrophages and neutrophils as the predominant cell types in the PVA sponge model used as

123 *in vivo* EV donors. In addition, this data showed that although there were differences in the gene profile of cells harvested

124 from WT vs db/db mice, these changes were related to their physiology rather than affecting pathways directly related

125 to EV release.

126

127 **Characterization of EVs released into biological fluid of cutaneous injury site.**

128   Our previous studies established the efficacy of PVA sponge implants as an *in vivo* source of highly concentrated

129 EVs relevant to wound healing.[8] We used these established standard parameters to analyze EVs purified from PVA

130 implants in WT and db/db mice. EVs harvested from the PVA sponge implants in the wound fluid were subjected to

131 serial centrifugation followed by size exclusion chromatography (SEC) (Figure 2A). We have previously shown that the

132 most numerous EVs in subcutaneous implants of PVA sponges were 100-120 nm in diameter and comprised the vast

133 majority of all EVs observed in this biological fluid, with relatively few larger EVs being observed.[8] Each fraction of

134 the SEC was analyzed for EV concentration using vesicle flow cytometry (vFC) as assessed by staining with the

135 fluorescent lipophilic membrane dye, vFRed (Figure 2B, column), and compared in parallel with protein concentration

136 in each fraction (Figure 2B, line) as detailed in the Methods and Materials. Each fraction of the SEC was further validated

137 for EV content by immunoblotting for a canonical EV marker like the tetraspanin CD9. We identified of high levels of

138 CD9 protein in the EV containing fraction 7 by immunoblot (Figure 2C) and by vFC with a fluorescently-labeled anti-

6

139 CD9 antibody (Supplementary Figure S2). Low levels of CD9 protein were detected in later fractions that lacked

140 significant numbers of small EVs (i.e., fractions 15-20; Figure 2C). Immunoblotting of whole cell lysates (WCL)

141 compared to purified EVs from the same WT PVA sponges demonstrated that CD9, CD63, CD81, and Alix were all

142 expressed in mouse PVA sponge EVs (Figure 2D). Enrichment of CD9 and Alix in EVs vs. WCLs was noted in the

143 analyses of mouse PVA sponge EVs, suggesting that these proteins may be more EV-specific. For the characterization

144 of EVs isolated from db/db mice, we established cohorts of 12–16-week-old WT and db/db mice where db/db mice used

145 for the collection of EVs were significantly more hyperglycemic (Figure 2E) and obese (Figure 2F) compared to WT

146 mice. These two parameters were the hallmark of the diabetic obese phenotype that is characteristic of the db/db mouse

147 model. The concentration of EVs isolated from PVA sponges was in the range of 5-7 x 10^6 PVA EVs/µL (Figure 2G).

148 EVs isolated from WT and db/db mice had similar size distributions with the mean diameter of EVs detected being 116.7

149 ± 8.79 nm (n=6) from db/db donors and 119.8 ± 6.8 nm (n= 6) from WT donors (Figure 2H). Similar sizing analysis of

150 each of the other SEC fractions did not reveal any substantial numbers of larger EVs in later fractions (Figure 2B and

151 data not shown). Transmission electron microscopy established that the EVs purified from WT and db/db donors had a

152 similar size and shape (Supplementary Figure S3A). To monitor for the potential of lipoprotein contamination of EV

153 fractions collected by SEC we performed immunoblotting of SEC fractions with an antibody to detect lipoproteins such

154 ApoE that could be present in EV fractions. We confirmed that the EVs collected in early fractions of the SEC (i.e.,

155 fractions 6-9; See Figure 2B) were well-separated from lipoproteins observed collected in late fractions (i.e., fractions

156 21-23; Supplementary Figure S3B and C). We also observed that the levels of ApoE expression were unchanged between

157 WT and db/db EVs (Supplementary Figure S3D).[45,46] Together, these analyses established the purification, expression

158 of canonical protein markers, size, and concentration from an *in vivo* EV donor model that is known to exhibit a well-

159 defined phenotype of impaired wound healing.[8]

160

161 **Identification of changes in EV proteins isolated from diabetic obese donor model.**

162   To determine the profile of proteins expressed on the EV surface we used a combination of batch and single EV

163 analysis (i.e., vFC; Figure 3A). WT EVs were purified from cutaneous implants and subjected to a multiplex analysis

164 (Figure 3B) that identified proteins associated with leukocytes (i.e., CD45, MHCII, and CD20), leukocyte activation

165 (CD44, CD66a) and cell adhesion (CD49e, CD11b, CD61). To address the heterogeneity of EVs in this biological fluid,

7

166 we performed vFC to determine the expression of individual tetraspanins that are generally used as EV markers. We

167 observed high levels of CD9 and CD63 expression on the surface of WT EVs (Figure 3C). Further, vFC analysis

168 identified several immune cell-relevant proteins expressed on the surface of WT EVs such as MHC I, CD29 (ITGB1),

169 CD274 (PD-L1), and CD39 (ENTPD1) (Figure 3D). These assays on WT EVs formed the basis for the vFC analysis of

170 proteins expressed on biological replicates of WT vs db/db EVs (n=5 for each genotype). For example, we observed that

171 expression levels of tetraspanins CD9 and CD63 measured by vFC were unchanged between WT and db/db EVs thus

172 providing a control for the levels of EVs collected from each genotype using canonical tetraspanin markers (Figure 3E).

173 Based on the importance of integrins in mediating binding to the extracellular matrix, we next measured proteins levels

174 of integrins by vFC. We noted reductions of in the number of EVs expressing detectable CD11b (ITGAM) (0.6-fold

175 decrease, $p < 0.0026$) and CD49e (ITGA5) (0.69-fold decrease, $p < 0.0074$) in db/db vs. WT EVs, but no significant

176 change for CD29 (ITGB1) (Figure 3F). We observed significant reductions in the number of EVs expressing detectable

177 immune-related proteins CD45 (PTPRC) (0.77-fold decrease, $p < 0.0285$), CD44 (hyaluronic acid receptor) (0.83-fold

178 decrease, $p < 0.0246$), and CD54 (ICAM1) (0.75-fold decrease, $p < 0.0221$). In contrast, levels of CD274 (PD-L1) were

179 increased in db/db vs WT EVs (1.47-fold increase, $p < 0.0345$). Levels of MHC I-positive EVs were unchanged (Figure

180 3G). We observed no significant changes in the number of EVs positive for other EV markers implicated in injury

181 models including CD326 (EPCAM), CD39 (ENTPD1), CD66a (CEACAM1), CD24 (HAS), or CD126 (IL6R) (Figure

182 3H).[47-51] Taken together, these findings suggested that the quantitative differences in the expression of select integrins

183 and other immune-related factors between WT and db/db EV donors may affect EV binding and activity in the wound

184 bed.

185

186 **EVs from diabetic obese donors have impaired wound healing activity.**

187 We previously reported that the pro-reparative phenotype of EVs in wound healing can be uncoupled by

188 interference with specific EV biogenesis pathways,[9] and that the EV profile (i.e., expression of proteins on the surface

189 of EVs) is regulated by the genetic background of immune-deficient and db/db donor models.[10] To define the activity of

190 EVs from db/db vs. WT donors, we purified EVs from PVA sponge implants from WT and db/db mice as described

191 above and applied these EVs to naïve wounds (Figure 4A). Specifically, EVs were applied topically in a single dose (5-

192 10×10^6 EVs/50µl/wound) to freshly prepared splinted full thickness wounds into naive recipient db/db mice, the standard

193   mouse model for impaired wound healing (Figure 4B). We observed that db/db donor EVs had a significant reduction

194   of pro-reparative activity in wound healing compared to the treatment with WT control EVs at Days 5, 7, 10 and 13

195   (n=10; Figure 4C, p values: **<0.005, *<0.05). Analysis of the kinetics of db/db EV-mediated wound closure was

196   comparable to saline-treated controls (Figure 4C). Histological analysis revealed decreases in wound closure (Figure

197   4D), and statistically significant reductions in epidermal thickness (Figure 4E; p <0.0001), and dermal cellularity in the

198   margins (Figure 4F; refer to brackets in Figure 4D for regions of analysis; p <0.0001) of wounds treated with db/db vs.

199   WT EVs. To determine whether there were differences in epithelial cell proliferation in EV-treated wounds, a hallmark

200   of the wound repair process,[52,53] we performed immunostaining with an anti-Ki67 antibody to localize the effects of EVs

201   on proliferation. We observed that treatment with WT EVs promoted proliferation of basal keratinocytes as detected by

202   the increase in Ki-67^{+} cells, a molecular endpoint that is physiologically relevant for wound closure, whereas there was

203   an absence of Ki67^{+} cells in basal keratinocytes of db/db EV-treated wounds was observed (Figure 4G). Given this effect

204   of WT EV treatment stimulating the proliferation of basal keratinocytes, we focused on an EV tagging strategy to assess

205   the distribution of EVs in the wound bed to better understand what cells may uptake EVs based on localization of the

206   EV tag. We designed a FLAG-tagged tetraspanin CD63 that would express the FLAG tag on the outside of the EV that

207   took advantage of the transient transfection properties of a cell line like HEK293 to rapidly prepare high purity EVs for

208   biological testing. Since HEK293 cells are also widely used in the EV field for engineering and production,[54-56] we could

209   purify EVs from the conditioned media of cultured HEK293 cells that either over-expressed human CD63 or CD63-

210   FLAG (Figure 4H, top). We determined that the FLAG epitope was displayed on 29% of all EVs collected from the

211   conditioned media (Figure 4H, bottom). FLAG expression was confirmed by immunoblotting of EVs from CD63-FLAG

212   transfected cells vs. control CD63-transfected cells (Figure 4I). Next, CD63-FLAG or CD63 expressing EVs were added

213   to full thickness wounds and incubated for 24 hours. Upon harvest and immunohistochemical staining to detect the

214   FLAG tag, we observed uptake of FLAG-tagged EVs in cells of the dermis, where especially in the higher

215   magnifications, FLAG-positive cells were observed primarily in the dermis, a cell layer characterized by an abundance

216   of fibroblasts (Figure 4J). Wounds treated with untagged CD63 or saline treated wounds used as negative

217   immunohistochemical controls (Figure 4J). Based on the substantial localization of FLAG-tagged EVs in the dermis,

218   we further assessed the uptake of CD63-FLAG-expressing EVs into cultured primary fibroblasts using mouse embryonic

219   fibroblasts (MEFs). MEFs were treated with CD63-FLAG tagged EVs or control CD63 EVs and immune-stained with

220 an anti-FLAG antibody and imaged by immunofluorescence (Figure 4K). We observed dose dependent EV uptake into

221 MEFs (Figure 4L). These findings showed that the activity of EVs administered to full thickness wounds can be

222 monitored by assessing cell proliferation as a molecular endpoint for the activity of pro-reparative EVs and that the

223 uptake of EVs can be localized using molecular tags such as the FLAG tag.

224

225 **Regulation of miRNA EV payloads isolated from diabetic obese donor model.**

226 Studies of miRNAs in EVs in diabetic wounds have established their translational relevance in wound healing.[13]

227 Therefore, we analyzed changes in miRNA payloads from WT and db/db mice in our model. We performed miRNA

228 sequencing (miRNAseq) on EVs purified from WT and db/db donors harvested from the PVA sponge model and

229 identified statistically significant changes in miRNAs from three biological replicates from WT and db/db EVs (Data has

230 been archived at NCBI #GSE242496) (Figure 5A). While many EV miRNAs were similar between WT and db/db EVs

231 (Figure 5B), consistent with the concept many miRNAs could be considered housekeeping miRNAs.[57,58] However, of

232 the miRNAs that were down-regulated more than 2 fold in db/db vs. WT EVs, changes in the following miRNAs were

233 statistically significant: miR-425-5p (2.68 fold decrease), 361-3p (3.15 fold decrease), 3068-3p (3.15 fold decrease), and

234 186-5p (2.02 fold decrease). The only miRNA that was significantly up-regulated in EVs from db/db vs. WT donors

235 more than 2-fold was miR-409-5p (2.38-fold increase) (Figure 5C). KEGG pathway analysis (Figure 5D) of the overall

236 changes of these db/db-regulated miRNAs were associated with signaling pathways relevant in diabetic wound healing

237 and complications such as stem cell regulation and AGE-RAGE signaling in diabetic complications that are relevant in

238 impaired wound healing in a diabetic obese model.[59] To better understand the potential targets of individual miRNAs

239 identified, we analyzed potential targets using miRPathDB v2.0 and performed a literature search as summarized in Table

240 S2. With this approach, we identified miR-425-5p as the lead candidate as it had also been recently reported to mediate

241 endothelial survival relevant to EV action in a streptozoicin-induced diabetic mouse model,[60] stimulate cell proliferation

242 relevant to the Ki-67 readouts of the wound healing model[60,61] and that miR-425-5p was the only miRNA predicted from

243 miRPathDB to mediate dysregulation of insulin signaling[62,63]; all highly relevant to the db/db model used here. GO Term

244 analysis also provided additional candidate targets for miR-425-5p action suggesting roles in regulating hypoxia

245 inducible factor, cyclin-dependent kinase and CD44 (Supplementary Figure S4). Therefore, to better understand the

246 relevance of a specific miRNA we focused on a relevant *in vitro* model. Since we observed that myeloid cell types

10

247   comprised the vast majority of cells in PVA sponges as identified by scRNAseq (Figure 1), and that the collection of EVs

248   from cultured primary macrophages yields biologically active EVs,[8] we used M-CSF to differentiate cells isolated from

249   PVA sponge implants from WT vs. db/db mice from which EVs were then collected for further analysis (Figure 5E).

250   Equivalent numbers of EVs were released into the conditioned media from db/db and WT macrophages (Figure 5F).

251   These EVs were subjected to qRT-PCR to measure changes in the levels of miR-425-5p in EVs from db/db vs. WT

252   macrophages and identified a significant decrease in miR-425-5p levels (15-fold decrease, $p < 0.0051$) in db/db EVs

253   compared to WT mouse EVs (Figure 5G).   These *in vitro* findings focused on EVs collected from cultured macrophages

254   were consistent with *in vivo* miRNAseq studies of PVA sponge-derived EVs where both showed a down-regulation of

255   miR-425-5p levels in db/db vs. WT EVs.   Therefore, we next focused on identifying a biological activity of miR-425-

256   5p-loaded EVs in the db/db wound model.

257

258   **Functional activity of miR-425-5p-loaded EVs in diabetic wounds.**

259   Having shown that db/db EVs isolated from cultured macrophages have reduced levels of miR-425-5p compared

260   to WT EVs (Figure 5G), we tested the *in vivo* biological activity of miR-425-5p-loaded EVs on wound healing compared

261   to a negative control miRNA. We used cel-miR-67 as a negative control as it is derived from *C. elegans* and associated

262   with minimal effects on eukaryotic cell signaling.[57,63,64] In addition several control studies using fluorescently labeled

263   miRNA controls were performed to optimize the concentrations for loading EVs with specific miRNAs and validating

264   by vFC that the surface profile of canonical tetraspanins CD9 and CD63 were unchanged by treatment with Exofect

265   reagent (Supplementary Figure S5).   We focused on using WT EVs for miRNA loading since although WT EVs are

266   known to be pro-reparative, our goal was to identify a miRNA payload that would substantially improve upon the known

267   pro-reparative activity of WT EVs from naïve mice. Therefore, to determine whether treatment with miR-425-5p-loaded

268   EVs would stimulate a pro-reparative phenotype in a wound healing assay, we treated full thickness wounds in recipient

269   db/db mice, observed the wounds over 14 days and performed image analysis over 14 days (Figure 6A). We observed

270   that treatment with miR-425-5p-loaded EVs significantly accelerated wound closure on Day 7 compared to standard WT

271   EVs from naïve mice (no loading with any miRNA) (Figure 6B; $p < 0.005$), consistent with the hypothesis that pro-

272   reparative activity of WT EVs was increased by loading EVs with miR-425-5p. Additional controls comparing miR-

273   425-5p-loaded EVs with negative control miRNA, Mock-loaded EVs (i.e., treated with the Exofect reagent but lacking

274 miRNA), or saline alone demonstrated the pro-reparative activity of miR-425-5p-loaded EVs in wound healing on Days

275 7, 10 and 14 (Figure 6C; p < 0.0001). Based on our observation that treatment with WT EVs led to an increased number

276 of Ki67^{+} basal keratinocytes in wound healing (Figure 4), we analyzed the effect of miR-425-5p-loaded EVs on cell

277 proliferation in treated wounds as a molecular endpoint for the accelerated wound healing. We observed an increase in

278 Ki67^{+} basal keratinocytes (Figure 6D) that was quantified and statistically significant (5.2-fold increase, p < 0.0024)

279 compared to a negative control miRNA mimic-loaded EVs (Figure 6E). Analysis of H&E histology following treatment

280 with miR-425-5p-loaded EVs, negative control EVs, or saline controls further defined the effects of EV treatment (Figure

281 6F). Treatment with miR-425-5p-loaded EVs led to a statistically significant increase in the number of cells (Figure 6G,

282 based on blue box on margins of Figure 6F; 1.6-fold increase, p < 0.0001), but no significant changes in epidermal

283 thickness (Figure 6H, centered on the wound bed), or collagen staining based on a Masson's Trichrome stain (Figure 6I;

284 Image J analysis of blue staining). We did observe an overall increase in the differentiation of the underlying dermis that

285 was associated with increased vascularity based on H&E staining (Supplementary Figure S6A and B). However, the

286 density of CD31^{+} blood vessels per high powered field was similar between treatment with miR-425-5p-loaded EVs vs.

287 Neg-miR-loaded EVs (Supplementary Figure S6C and D). Therefore, the effects of treatment with miR-425-5p-loaded

288 EVs on blood vessels in this model may be indirect because it was associated with a robust pro-reparative phenotype or

289 may be direct by promoting endothelial survival as recently proposed.[60] Based on the translational relevance of our

290 studies of miR-loaded EVs on wound healing, we next determined whether loading of EVs with miRNA using Exofect

291 would lead to a significant amount of miRNA attached to the surface or would miRNAs be internalized and protected by

292 the lipid membrane. Therefore, we tested the effect of an *in vitro* nuclease treatment on isolated EVs using benzonase.[65]

293 qRT-PCR was performed to measure changes in levels of miR-425-5p from vehicle vs. benzonase-treated EVs. We

294 observed no substantial differences, consistent with miRNAs being present within the EV and thus protected from

295 benzonase activity (Supplementary Figure S7).

296   Based on the pro-reparative effect miR-425-5p-loaded EVs on wound healing (Figure 6C) associated with the

297 increased proliferation of basal keratinocytes (Figure 6D and E), we focused on the identification of soluble mediators

298 such as cytokines and adipokines that could mediate signaling between cell layers in the skin. Therefore, we performed

299 a cytokine analysis testing a panel of 24 cytokines/adipokines to quantify relevant changes in inflammation mediators.[66]

300 Whole cell lysates of wound tissue treated with miR-425-5p-loaded EVs were compared with tissues treated with

301 negative control miRNA-loaded EVs as described above. We observed significant increases in several factors, including

302 Adiponectin (2.4-fold increase, $p < 0.0009$), IL-1α (2.3-fold increase, $p < 0.002$), and Serpin E1 (1.9-fold increase, $p <$

303 0.037), along with many other factors that were unchanged (Supplementary Figure S8). Based on the relevance of

304 Adiponectin as an important mediator of glucose metabolism in diabetic obese models,[67,68] the uptake of FLAG-tagged

305 EVs into fibroblasts (Figure 4), and the general abundance of fibroblasts in the wound bed, we focused on testing whether

306 macrophage-derived EVs containing miR-425-5p (Figure 5G) could stimulate fibroblasts *in vitro*. Therefore, we

307 assessed whether treatment of MEFs with miR-425-5p-loaded EVs would stimulate Adiponectin expression *in vitro*

308 (Figure 6K), as predicted from *in vivo* treatments with miR-425-5p-loaded EVs stimulating Adiponectin expression

309 (Figure 6J). We subjected cultures of MEFs to 48 h treatment with miR-425-5p-loaded EVs compared to negative control

310 miRNA-loaded EVs as prepared for the *in vivo* studies above. We observed by immunoblotting of whole cell lysates

311 that miR-425-5p-loaded EVs led to a 1.4-fold increase in Adiponectin expression compared to treatment of MEFs with

312 control EVs (Figure 6L), using actin levels as a loading control. Although it remains unclear whether miR-425-5p has a

313 direct effect on the Adiponectin mRNA based on predicted binding sites of miR-425-5p, these findings suggest that

314 treatment of wounds *in vivo* or MEFs *in vitro* with miR-425-5p-loaded EVs leads to increases in Adiponectin expression.

315 In a model where macrophage-derived EVs promote intercellular signaling such as the release of EVs from macrophages

316 that stimulate fibroblasts, defining cell type-specific EV release with a genetic tool would provide important insights.

317

318 **Cell type-specific tracking of EV release and uptake.**

319 To address the question of cell type-specific EV release, we used a Cre-lox system for the regulated expression

320 of a fluorescent EV reporter, the tetraspanin CD9 as a fusion to GFP. Cell profiling of PVA sponge donor site using

321 scRNAseq showed high numbers of macrophages and related cell types (Figure 1), while immunohistochemistry of full

322 thickness wounds showed high numbers of F4/80[+] macrophages distributed in the adipose layer (Supplementary Figure

323 S9). Therefore, we selected transgenic mice that expressed the Cre recombinase in macrophages for comparison with

324 transgenic mice expressing Cre in other skin-relevant cell types such as endothelium (TEK) and keratinocytes (KRT14).

325 Each tissue-specific transgenic Cre mouse line was crossed with mice expressing the CD9:GFP, termed the TIGER

326 reporter (Transgenic inducible GFP EV reporter; Figure 7A).[24] Tissue specific expression of CD9-GFP was under the

327 control of an upstream lox-STOP-lox cassette and crossed with transgenic mice expressing Cre under the control of the

328 LysM promoter (LysM-Cre) to assess the expression and release of GFP+ EVs from myeloid immune cells like

329 macrophages and monocytes. Crosses with TEK-Cre and CD9-GFP mice were performed in parallel with crosses with

330 TEK-Cre x CD9: GFP and KRT14-Cre x CD9-GFP. We first established the expression of GFP in each of the transgenic

331 mouse lines by analyzing cells recruited into the PVA sponge implants in parallel with the collection of donor EVs as

332 described above (Figure 7B). In addition, laser scanning confocal microscopy of PVA sponge implants was performed

333 for each genotype to establish positive controls for each of the mouse models (i.e., TEK-CD9-GFP, LysM-CD9-GFP and

334 KRT14-CD9-GFP; Supplementary Figure S10). For each donor genotype, cells (Figure 7B) and EVs (Figure 7C) were

335 purified from the PVA sponge implants as described above. Standard cell flow cytometry was performed for cells

336 collected where we observed that LysM-CD9-GFP and TEK-CD9-GFP mice expressed GFP. Since the surgical

337 placement of PVA sponge implants was between the adipose and dermal layers, few KRT14+ cells migrated or infiltrated

338 the PVA sponge implant in KRT14-CD9-GFP mice (Figure 7B and Supplementary Figure S10), although KRT14+

339 keratinocytes were present in intact overlying skin (data not shown). Analysis of EVs purified from PVA sponge implants

340 of each genotype focused on LysM-CD9-GFP mice that released high numbers of bright GFP+ EVs. In contrast, there

341 were few TEK-CD9-GFP+ or KRT14-CD9-GFP EVs (Figure 7C). We proceeded to further assess the distribution of

342 macrophage-derived EVs (i.e., LysM-CD9-GFP EVs) based on their abundance relative to other cell type-specific

343 promoters tested (Figure 7B) by treatment of full thickness wounds of db/db mice (Figure 7D). We observed that GFP+

344 EVs or accumulations of GFP+ EVs could be observed by confocal microscopy in wounds treated with LysM-CD9-GFP

345 EVs compared to the lack of signal in images of wounds treated with non-fluorescent controls EVs isolated from sibling-

346 matched controls lacking the Cre driver genes (Figure 7E). To determine whether LysM-CD9-GFP EVs could be

347 localized in fibroblasts based on a model of EV uptake by fibroblasts in the wound bed, we first established the

348 distribution of fibroblasts in the wound site by immunostaining full thickness wound sites with an anti-vimentin antibody

349 (Figure 7F). Imaging of both the left and right sides of the wound show the distribution of vimentin+ fibroblasts on the

350 wound margin (Figure 7F, sides of the images of the left and right panels) and the wound bed (Figure 7F, bottoms of the

351 images of the left and right panels). These low magnification images provide landmarks for the wound margins (i.e.,

352 sides of the wound) vs. the wound bed (i.e., bottom of the wound), and the negative control images for wounds treated

353 with WT non-fluorescent EVs (Figure 7F) vs. wounds treated with LysM-CD9-GFP EVs (Figure 7G). These

354 representative low magnification images (Figure 7F and G) were further analyzed at higher magnification to localize the

355 accumulation of LysM-CD9-GFP EVs in replicate high power fields of the wound margin (Figure 7H, based on red box

356 corresponding to Figure 7D), and in the wound bed (Figure 7I, based on blue boxes corresponding to Figure 7D).   These

357 imaging studies established that populations of LysM-CD9-GFP EVs were co-localized with vimentin[+] fibroblasts in the

358 wound margin (Figure 7H) and in the wound bed (Figure 7I). While these co-localization analyses did not exclude the

359 possibility of CD9-GFP uptake into other cell types, these studies provided insights into the distribution of EV uptake in

360 a complex microenvironment. Furthermore, EVs purified from a specific cell type such as macrophages express surface

361 proteins that may be relevant to their tropism and activity.

362

363 **DISCUSSION**

364   The healthy wound healing response is characterized by coordinated phases of hemostasis, inflammation,

365 proliferation, and remodeling, however, many aspects of the molecular and cellular basis of this response remain poorly

366 understood. The dysregulation of the coordinated response that is associated with impaired wound healing has led us to

367 identify defects in intercellular signaling between cell layers in the skin with a focus on EVs and their payloads as

368 mediators of these processes. We propose that macrophage-derived EVs from resident macrophages have biologically

369 active payloads that are internalized by skin fibroblasts to stimulate signaling, and specific adipokine expression that

370 leads to a pro-reparative response that includes proliferation of overlying keratinocytes.

371   The composition and function of EVs released into biological fluids and cultured media, which are heterogenous

372 in origin, is dependent on the cells that produce them. There are few *in vivo* models that have mapped the landscape of

373 cells and EVs in a well-defined microenvironment with genetic tools. Here, we focused on wound healing in db/db mice

374 as a genetic model of impaired wound healing to show that EVs enriched from db/db donors have impaired wound

375 healing activity and reduced capacity to signal specific molecular endpoints in fibroblasts that was associated with

376 stimulating proliferation of basal layer epithelial cells.   miRNAseq of EVs isolated from db/db vs. WT mouse donors

377 revealed a reduction in the miR-425-5p. miR-425-5p, especially when loaded into EVs, has been associated with

378 dysregulated insulin signaling in some models[61] or as a pro-survival endothelial factor in db/db EVs in other models.[60]

379 We show that miR-425-5p was differentially expressed in macrophage-derived EVs isolated from db/db vs. WT mice,

380 that wounds treated with miR-425-5p-loaded EVs promoted wound closure, and identified a miR-425-5p-mediated

381 upregulation of Adiponectin in the wound bed *in vivo* and in cultured fibroblasts *in vitro*.   Based on our findings that

382 macrophage-derived EVs signal to other cell types in the wound, we used a cell type-specific CD9-GFP reporter model

383 to define the distribution and map uptake of macrophage-derived EVs into fibroblasts. Together, these studies defined a

384 population of macrophage-derived EVs that are internalized by dermal fibroblasts to regulate Adiponectin expression

385 associated with promoting wound healing and cell proliferation.

386   Recent work from our lab has focused on the identification of quantitative changes in protein payloads in EVs

387 isolated from db/db vs. WT donor mouse models with a goal of engineering EVs to deliver these pro-reparative payloads

388 to the wound bed. For example, we identified a down-regulation of proteins associated with extracellular matrix

389 remodeling and innate immunity[8] and re-expressed select serine protease inhibitors to reverse the impaired wound

390 healing phenotype of db/db recipients. Here we focused on miRNA analysis of db/db vs. WT EV payloads and identified

391 several miRNAs that were down-regulated in db/db EVs and others that were up-regulated. To establish the function of

392 a specific miRNA as an example of how to validate the activity of candidate miRNA, we selected miR-425-5p for testing,

393 along with negative controls for activity, and positive controls for EV loading. While several miRNA profiling studies

394 have identified miRNAs that are relevant in impaired wound healing, comparatively fewer have assessed the activity of

395 specific EV-loaded miRNAs in wound healing.[69] Therefore, we used a combination of target pathway analysis databases

396 and literature review to prioritize specific miRNAs for functional testing. While our studies were limited to the

397 identification of miRNAs dysregulated in the db/db PVA sponge model, further studies with antagomirs, miRNA

398 knockout mice and miRNA activity reporter tools will be important to better understand loss of function phenotypes for

399 specific miRNAs.[70-73] One of the limitations of functional testing of endogenous miRNA-loaded EVs in the field is the

400 poorly understood nature of miRNA abundance and distribution in a population of EVs.[74,75] For our analysis of miRNA

401 activity, we focused on testing a miRNA that was down-regulated in db/db EVs that could then be delivered in an EV to

402 restore a pathway(s) in a wound bed treated with EVs loaded with that down-regulated miRNA. To this end we identified

403 miR-425-5p as one of the most relevant miRNAs based on the obese hyperglycemic phenotype of the db/db mice from

404 which EVs were collected and analyzed by miRNAseq, and reports that linked miR-425-5p action to the regulation of

405 insulin responsiveness.[62,63] We suggest that miR-425-5p regulated insulin signaling may be linked to the miR-425-5p-

406 mediated changes in the expression of an adipokine like Adiponectin in the regulation glucose sensitivity.[76] We proposed

407 that miR-425-5p-loaded EVs may have a role in stimulating secreted factors in adjacent cell types that promote wound

408 healing. In addition to Adiponectin, future studies may focus on other candidates such as IL-1α and Serpin E1, also

16

409 known as plasminogen activating inhibitor, that are relevant in inflammation and angiogenesis, respectively. Therefore,

410 with recent studies identifying an activity for miR-425-5p in endothelial cells as a pro-survival regulator of endothelial

411 cells that promotes wound healing in a streptozoicin model of impaired diabetic wound healing[60] we examined the effects

412 of miR-425-5p-loaded EVs in a genetic db/db model. Although we did not observe a significant difference in CD31^{+}

413 blood vessel density, systematic approaches that test miRNA action in complex tissues will be necessary to better define

414 cell type-specific effects of miRNAs.

415   With a significant interest of the EV field focused on how to define EV source and uptake, we focused on an *in*

416 *vivo* model where the EV source is defined by cell types relevant to cutaneous wound healing, like macrophages. We

417 show that macrophage-derived EVs can be tracked and purified for adoptive transfer studies using cell type-specific

418 promoters to follow GFP fluorescence by flow cytometry and microscopy. Fluorescent reporter systems utilizing CD9-

419 GFP fusions,[24] as we have done for cutaneous injury models, along with recent studies using fusion reporters with CD81

420 for EV tracking from blood, brain, liver and ovary,[26] provide important insights into the relevant cell types and

421 biodistribution *in vivo*. The development of pH-dependent fluorescent EV reporters that distinguish between EVs in

422 acidic late endosomal MVBs vs. the release of EVs in neutral extracellular space and for tracking EV uptake provide

423 further support for the utility of tetraspanin:fluorescent reporters in the understanding of EV release and uptake.[77] These

424 tetraspanin:fluorescent reporters are important tools to understand the biodistribution and activity of nucleic acid

425 payloads like functional miRNAs and guide RNAs that direct CRISPR/Cas9 machinery for gene editing target cells.[78]

426 We have also shown here that direct tagging of tetraspanins, like the FLAG tagging of CD63 on EVs takes advantage of

427 a well-established molecular tool that also has utility in purification strategies.[79]

428   The overall novelty of our studies is that we establish an *in vivo* system for the efficient and high yield

429 purification of EVs that can be applied to various animal models to define the biological activity and assess molecular

430 endpoints of engineered EVs. These engineered EVs can deliver pro-reparative payloads identified by -omic approaches

431 that are most relevant in accelerating the resolution of inflammation and promoting the proliferation of specific cell types

432 relevant in tissue repair. With the application of fluorescent, genetic and other advanced EV tracking technologies,

433 lineage mapping of the source of EV release and uptake of EVs into recipient cells can lead to a molecular understanding

434 of intercellular signaling mediated by EVs between skin layers in wound healing.

17

435

MATERIALS AND METHODS

Mouse model for EV collection from PVA sponges

All mouse studies were conducted in accordance with the Institutional Animal Care and Use Committee of the University of California San Diego. 12-16 week old WT and db/db mice (B6.BKS(D)-Lepr[db/db]/J; The Jackson Laboratory #000697, Bar Harbor, Maine) where db/db mice had a blood glucose level > 300 mg/dL and body weight >45 g, criteria for the diabetic obese model[80,81]. Mice were prepared for the subcutaneous implantation of three Polyvinyl Alcohol (PVA) sponges (Cat# SQ5000, PVA Unlimited Inc., Warsaw, IN) by shaving and topical treatment with depilatory cream of dorsal skin. Following PVA sponge implantation, skin was closed with nylon monofilament sutures and incubated for 7 days. PVA sponges were then harvested by direct transfer of all three sponges into 1 mL of phosphate-buffered saline for the recovery of cells infiltrating the sponges and associated fluid flushed from the sponges that contained EVs. Centrifugation at $3,000 \times g$ for 5 min separated cells into a pellet that was used for scRNAseq, while the supernatant of the PVA sponge fluid contained EVs for further analysis.

For the goal of identifying cell type-specific sources of EVs, we used transgenic mice expressing a CD9-TurboGFP reporter targeted to extracellular vesicles under the control of a lox-STOP-lox cassette to facilitate cell type specific expression based on promoters driving the expression of Cre in specific cell types, termed TIGER knock-in mice.[24] The following three crosses were performed with these CD9-GFP mice (B6;129S1-Gt(ROSA)26Sor[tm1(CAG-CD9/GFP)Dmfel]/J; Jackson Laboratories #033361). For expression of the CD9-GFP reporter in macrophages/monocytes mice were crossed with a LysM-Cre mice (B6.129P2-Lyz2[tm1(cre)Ifo]/J; Jackson Laboratories #004781). For the expression of the CD9-GFP reporter in endothelial cells, mice were crossed with TEK-Cre mice (B6.Cg-Tg(TEK-cre)12Flv/J; Jackson Laboratories #004128). For the expression of CD9-GFP reporter in keratinocytes, mice were crossed with KRT14-Cre mice (B6N.Cg-Tg(KRT14-cre)1Amc/J; Jackson Laboratories #018964) (Table S2).

scRNAseq

scRNAseq was performed on cells recovered from PVA sponges implanted into each of 3 different WT mice that were then pooled and compared with similarly pooled cells from each of 3 different db/db mice. scRNAseq data was

18

461 archived at NCBI (GSE242497). In brief, 1 x 10^5 cells were collected from the PVA sponges from each mouse, pooled

462 and then 1 x 10^4 cells were loaded on the 10x Chromium Next GEM using the Single Cell 3' Reagent (v3.1) with gel

463 beads and master mix for cell capture and GEM generation (Cat # 1000147, 10X Genomics, San Francisco, CA).

464 Subsequently, samples underwent GEM reverse transcription cleanup, cDNA amplification, and 3' gene expression

465 library construction according to the manufacturer's instructions (10x Genomics). Constructed libraries were then

466 sequenced on HiSeq sequencers (Illumina, San Diego, CA) using paired end reads at the University of California, San

467 Diego Institute for Genomic Medicine (IGM) (Table S3 and S7). scRNAseq data was demultiplexed, giving rise to 2

468 FASTQ files per sample (4 FASTQ files in total), and aligned to the reference murine genome GRCm38 (mm10, v2020-

469 A) into single cells using the Cell Ranger Count pipeline (10x Genomics, v7.0.0) with the following settings for each

470 sample, independently— Library Type: Single Cell 3' Gene Expression; check library compatibility: true; chemistry:

471 auto; include introns: true; no_bam: false; no secondary analysis: false. Cell Ranger Count outputs for each sample were

472 then aggregated and normalized into a single gene expression matrix using the Cell Ranger Aggr pipeline (10x Genomics,

473 v7.0.0) with the follow settings— no secondary analysis: false; normalization mode: mapped. Running Cell Ranger Aggr

474 yielded approximately 18,000 post-normalizations mean reads per cell. Further data filtering and analysis were conducted

475 using Loupe Browser (10x Genomics, v6.1.0). Quality control included omitting cells with >15% Mitochondrial UMIs

476 per barcode (Linear) or <9.185 Genes per Barcode (Log2); cells that passed these quality control filters were included in

477 downstream analysis. The top 10 principal components were used for graph-based clustering, and the following settings

478 were applied for dimensionality reduction via uniform manifold approximation and projection (UMAP) analysis—

479 Minimum Distance: 0.1; Number of Neighbors: 15.

480

481 **EV isolation and analysis**

482 EV studies addressed the methodological recommendations of the Minimal Information for Studies of

483 Extracellular Vesicles 2018 (MISEV 2018)[82] including nomenclature, collection/pre-processing, EV

484 separation/concentration, EV characterization, functional studies, and reporting that are all archived at EV-TRACK

485 (evtrack.org; #EV230979). For the isolation of EVs from PVA sponge implants, the cell-free supernatant was subjected

486 to two 10,000 × g spins for 30 min at 4º C followed by size exclusion chromatography (SEC) (Cat # ICI-70, IZON,

487   Medford, MA) and collection of 22 fractions of 700 µL each.   EVs from cultured cell media were enriched using

488   Exoquick reagent (Cat # EQULTRA-20A-1, System Biosciences, Palo Alto, CA) following the manufacturer's protocol.

489

490 **Single vesicle flow cytometry (vFC)**

491   EV concentration, size, and analysis of surface proteins and fluorescent proteins were measured by single

492   vesicle flow cytometry (vFC) using a commercial assay based on a fluorescent lipophilic membrane dye, vFRed (vFC

493   Assay kit, Cat # CBS4HP-1PE, Cellarcus Biosciences, San Diego, CA), using a CytoFLEX flow cytometer (Model S,

494   V4-B2-Y4-R3, Beckman Coulter, Indianapolis, IN) (Table S7). The flow cytometer was calibrated for vesicle size and

495   immunofluorescence (IF) using fluorescent intensity standard beads (nanoRainbow, Cellarcus) and antibody capture

496   beads (nanoCal, Cellarcus),[83-85] and showed a size (diameter) limit of detection (LOD) of ~80 nm and an IF LOD of ~25

497   PE MESF. Samples were diluted (optimal dilution determined in preliminary experiments), stained with vFRed and PE-

498   conjugated antibodies (See Table S5 and S6), subjected to a 1000-fold post-stain dilution, and 100 µL measured on the

499   flow cytometer at a flow rate of 60 µL/min. Data were analyzed using FCS Express (Dotmatics/Denovo Software) and a

500   standardized layout used to apply gating, compensation, and calibration (Cellarcus). Single vesicle flow cytometry data

501   was archived at flowcytometry.org (ID: FR-FCM-Z749) with a MIFlowCyt Score of 95%.

502 **EV characterizations by immunoblotting, electron microscopy, and multiplex analysis**

503   Lysates of EVs isolated from PVA sponges, paired along with whole cell lysates (WCLs) from the sponge

504   implants, were prepared in RIPA lysis buffer. Loading of WCLs were normalized by protein quantification with a BCA

505   assay (Cat# 23225, ThermoFisher, Carlsbad, CA) while loading of EVs was normalized to EV counts based on vFC

506   analysis as described above. Nonfat Dry Milk (Cell Signaling Technology, Denver, MA) was used for blocking in Tris-

507   buffered saline with 0.05% Tween 20 and primary antibodies incubated overnight at 4 °C. Table S4 details primary and

508   secondary antibodies used for immunoblotting. Immunoblots were detected with horseradish peroxidase-conjugated

509   secondary, incubated with enhanced chemiluminescent reagent (Cat# 32209, ThermoFisher) and detected with an IVIS-

510   Lumina Imager (Perkin Elmer). For imaging of EVs by transmission electronic microscopy, EV samples were applied

511   onto EM grids, washed, and stained with uranyl acetate and images obtained with a Jeol 1400 plus transmission electron

512   microscope at 80 KeV. For the multiplex analysis of EVs present on EVs, a bead-based screen for 37 EV surface proteins

513   was used (MACSPlex Exosome Kit, Cat#130-122-211, Miltenyi-Biotec, San Diego, CA), and followed manufacturer's

514   recommendations. Data was analyzed with data analysis template using MACSQuant (ver. 2.12.2) (Miltenyi-Biotec).

515

516   **EVs studies to assess wound healing, signaling and uptake *in vivo*.**

517   To assess the activity of EVs upon the kinetics of wound closure, EVs were purified from PVA sponge implants

518   as described above and used to treat full thickness splinted 4 mm wounds as previously described.[86,87] Briefly, a silicone

519   ring (Cat# GBLRD476687, Grace Bio-labs, Bend, OR) was immobilized with 4-0 nylon sutures (Cat # 50-118-0628,

520   ThermoFisher) around each wound and immediately treated with $5\text{-}7 \times 10^6$ EVs in a volume of 50 μl of PBS per wound

521   and covered with 3M Tegaderm (Cat #, 264435, Mckesson, Irving, TX).[86] Wounds were imaged with a Galaxy S10e

522   (1200 pixels, AF, F1.5/F2.4 super speed dual pixel, Samsung) and analyzed by Image J (1.53e version). Tissues were

523   harvested for histology analysis by fixation of skin wound samples in paraformaldehyde into paraffin at the UCSD Tissue

524   Technology Shared Resource (TTSR) that prepared slides stained with Hematoxylin/Eosin and Masson's Trichrome

525   stains, to detect collagen staining in blue. Immunohistochemical staining to localize Ki-67 (1:50; Cat #,16667, Genetex,

526   Irvine, CA) immunohistochemical staining was performed with a Intellipath Automated IHC Stainer (Biocare, Pacheco,

527   CA) by the TTSR, while immunostaining with anti-FLAG antibody (1:100; Cat # 14793, Cell Signaling Technologies)

528   and anti-F4/80 (1:100, Cat# 70076, Cell Signaling Technologies) was performed with HRP detection SignalBoost

529   reagents (Cat# 8114 and 8059, Cell Signaling Technologies). Tissues harvest for analysis for cytokines/adipokines was

530   performed with a Proteome Profiler (Cat # ARY028, R&D Systems, Minneapolis, MN) and followed manufacturers

531   recommendations for detection and quantification with an IVIS-Lumina imaging system. Analysis of wounds treated

532   with CD9-GFP$^+$ EVs was performed by cryosectioning treated wounds with a cryostat (Model CM1850, Leica,

533   Davisburg, MI), imaged with a Nikon Confocal microscope (Model AXR, Tokyo, Japan). Counterstaining of CD9-GFP$^+$

534   EVs was performed with an anti-vimentin antibody (1:200, Cat # 5741, Cell Signaling Technologies) to localize

535   fibroblasts and detected with an Alexa 546 secondary antibody (1:1,000, Cat #A11010, ThermoFisher). All H&E,

536   Masson's Trichrome and immunofluorescence images were analyzed with Image J software.

537

Generation and testing of FLAG-tagged EVs

An epitope tagged variant of CD63 was created by cloning the FLAG sequence (DYKDDDK) at amino acid 1397 on the second extracellular loop of CD63. Following transient transfection of HEK293T cells (Cat # CRL-1573, ATCC, Manassas, VA) with Lipofectamine 3000 (ThermoFisher) per manufacturer's directions, the expression on the cell surface was validated by immunoblot with an anti-FLAG antibody (Cat# F-1804, Sigma, St. Louis, MO). vFC was performed to detect expression of FLAG-tagged CD63 on EVs isolated from the conditioned media of transfected cells using a PE-conjugated anti-FLAG antibody (Cat# CBS18-PE-100T, Cellarcus Biosciences, San Diego, CA). Uptake of FLAG-tagged EVs into primary MEFs was assessed by treatment of $1e^6$ cultured primary MEFs (Cat# SCRC-1008, ATCC) with 2×10^9 EVs in a volume of 100 μl, incubated for 48 h, fixed, and stained with an anti-FLAG tag antibody (1:100, Cat#14793, Cell Signaling Technologies) and a fluorescent anti-Rabbit Alex Fluor 488 (Cat# A-11008, ThermoFisher).

In vitro analysis of macrophage-derived EV miRNAs and Adiponectin expression

Primary macrophages prepared from PVA sponges were cultured for the collection of macrophage-derived EVs to measure levels of miR-425-5p. Briefly, PVA sponges were implanted into mice, incubated for 7 days and cells harvested as described above. Cells were cultured in RPMI media supplemented with 10% fetal bovine serum and 25 ng/ml M-CSF (Cat # 14-8983-80, Life Technologies, Carlsbad, CA) for 7 days, with a media change at 3 days. Flow cytometry was performed on cells incubated for 7 days in M-CSF with an anti-F4/80 antibody, which was used to verify >90% F4/80^{+} cells. After the 7 day incubation, cells were transferred to RPMI media supplemented with 10% exosome-depleted FBS (SBI) and conditioned media collected after an additional 7 day incubation. From this conditioned media, EVs were harvested using Exoquick per manufacturer's recommendation, and EVs analyzed for qPCR for miR-425-5p as described below. The activity of miR-425-5p-loaded EVs upon the stimulation of MEFs was determined by incubation of EVs for 48 h, then measured by immunoblotting for adiponectin with an anti-adiponectin antibody (Cat #MA1-054, ThermoFisher) and followed by detection with an IVIS-Lumina Imaging system.

miRNA sequencing, analysis, EV loading and RT-PCR

563   For the analysis of miRNA EV payloads, EVs were purified by SEC from 7 day PVA sponge implants of WT

564   vs. db/db mice.   To obtain a more concentrated sample for RNA extraction, purified EVs were subjected to

565   ultracentrifugation (259,000 x g, for 70 minutes, Beckman Optima, Rotor TLA 120.2, k-factor 8), the supernatant

566   discarded, and the pellet extracted to obtain RNA using Trizol (ThermoFisher). RNA quality and quantity were analyzed

567   with a Bioanalyzer 2100 (Agilent, CA, USA), and 1 μg of RNA used to prepare a small RNA library using a TruSeq

568   Small RNA Sample Prep Kit (Illumina, San Diego, CA). Single-end 50 bp sequencing was performed on an Illumina

569   HiSeq 4000 (LC Sciences, Houston, TX) with initial processing of raw reads using proprietary software ACGT101-miR

570   (LC Sciences) to remove adapter dimers, foreign sequences, low-complexity fragments, common RNA families (e.g.

571   rRNA, tRNA, snRNA, snoRNA) and repetitive sequences. Sequences were mapped against species-specific miRNA

572   precursor sequences available in miRBase 21.0 using NCBI BLAST to identify known and potentially novel miRNAs,

573   with the alignment process allowing for length variation at both the 3' and 5' ends of the sequence and a tolerance for

574   one mismatch within the sequence. Identification of known miRNAs involved recognition of unique sequences aligned

575   with the mature miRNAs of a specific species located on the hairpin arm. At the same time, sequences aligning with the

576   opposite arm of a known species-specific precursor hairpin without an annotated mature miRNA were classified as

577   candidates for new 5p- or 3p-derived miRNAs. Unmapped sequences were aligned against the precursor sequences of

578   selected species (excluding certain species) within miRBase 21.0 and subjected to further analysis. These mapped pre-

579   miRNAs were then cross-referenced with the genome of a specific species to confirm their genomic location and

580   classified as known miRNAs. The remaining unmapped sequences were subjected to BLAST searches against the

581   genomes of specific species. Hairpin RNA structures containing these sequences were predicted using RNA fold software

582   (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) using the 80 nucleotides flanking the sequence.[88-90] For

583   the analysis of differentially expressed miRNAs, a normalization based on deep-sequencing counts. To predict the genes

584   targeted by most abundant miRNAs, two computational target prediction algorithms (TargetScan 50 and Miranda 3.3a)

585   were used to identify miRNA binding sites. Finally, the data predicted by both algorithms were combined and the

586   overlaps were calculated. The GO terms and KEGG Pathway of these most abundant miRNAs, miRNA targets were also

587   annotated. For loading miRNAs into EVs isolated from WT PVA sponge donors, 100 μL of EVs at a concentration of 6

588   x 10^6 PVA EVs/μL were mixed with 200 pmoles of miR-425-5p (Cat# C-310988-01-0050, Horizon, San Diego, CA) or

589   a negative control cel-miR-67 (Cat# CN-001000-01-50, Horizon) in a volume of 200 μL following manufacturer's

590   recommendations for Exofect kit (Cat# EXFT20A-1, System Biosciences, SBI, Palo Alto, CA). Conditions for EV

591   loading with miRNAs were optimized with a Cy3-labeled miR control (SBI).   miR-loaded EVs and followed

592   manufacturer's recommendations for purification. qRT-PCR was performed on CFX96 (Bio-Rad) using the TaqMan™

593   Fast Advanced Master Mix for qPCR (Cat# 4444556, ThermoFisher) and TaqMan™ Advanced miRNA Assay

594   (mmu481161_mir) (Cat# A25576, ThermoFisher). To confirm the expression of miR-425-5p in EVs collected from M-

595   CSF-differentiated PVA macrophages, we extracted RNA using a mirVana™ miRNA Isolation Kit (Cat#AM1560,

596   ThermoFisher, Carlsbad, CA) following manufacturer's protocol.   cDNA was synthesized for each sample using the

597   TaqMan™ Advanced miRNA cDNA Synthesis Kit (Cat# A28007, ThermoFisher).   and qPCR performed with a

598   TaqMan™ Fast Advanced Master Mix (Cat# 4444556, ThermoFisher) and the TaqMan™ Advanced miRNA Assay

599   specific for mouse miR-425-5p (mmu481161_mir; '3-AAUGACACGAUCACUCCCGUUGA-5') (Cat# A25576,

600   ThermoFisher).

601

Statistical analysis

603   All statistical analyses were performed with Prism 6.0 (Graphpad Software). Data were expressed as the mean

604   ± standard deviation (SD). Differences between different groups were compared by Student t-test (i.e., vFC) and two-

605   way ANOVA with multiple comparisons (i.e, wound healing assays), with statistically significant p-values indicated as

606   ****<0.0001, ***<0.001, **<0.005, *<0.05. All statistical analyzes and representative images presented and observed

607   in at least 3 independent experiments.

608

609

610 **Detailed Reagent Supporting Information**

611 Additional details on reagents, mice, software, kits, antibodies, and software are provided in Supplementary Tables S3-

612 8.

613

614 **DATA AVAILABILITY STATEMENT**

615 scRNAseq (#GSE242496) and miRNAseq data (#GSE242497) produced in this study are accessible via GEO archives

616 maintained by the NCBI. Vesicle flow cytometry data is deposited into flowrepository.org (#FR-FCM-Z749).

617

618 **ACKNOWLEDGMENTS**

626

627 **AUTHOR CONTRIBUTIONS**

632

633 **DECLARATION OF INTERESTS**

636

637 **KEYWORDS**

639
640

References

1. Koh, T.J., and DiPietro, L.A. (2011). Inflammation and wound healing: the role of the macrophage. Expert Rev Mol Med *13*, e23. 10.1017/S1462399411001943.

2. Minutti, C.M., Knipper, J.A., Allen, J.E., and Zaiss, D.M. (2017). Tissue-specific contribution of macrophages to wound healing. Semin Cell Dev Biol *61*, 3-11. 10.1016/j.semcdb.2016.08.006.

3. Novak, M.L., and Koh, T.J. (2013). Phenotypic transitions of macrophages orchestrate tissue repair. Am J Pathol *183*, 1352-1363. 10.1016/j.ajpath.2013.06.034.

4. Larouche, J., Sheoran, S., Maruyama, K., and Martino, M.M. (2018). Immune Regulation of Skin Wound Healing: Mechanisms and Novel Therapeutic Targets. Adv Wound Care (New Rochelle) *7*, 209-231. 10.1089/wound.2017.0761.

5. Hoshino, A., Costa-Silva, B., Shen, T.L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., Molina, H., Kohsaka, S., Di Giannatale, A., Ceder, S., et al. (2015). Tumour exosome integrins determine organotropic metastasis. Nature *527*, 329-335. 10.1038/nature15756.

6. Pluchino, S., and Smith, J.A. (2019). Explicating Exosomes: Reclassifying the Rising Stars of Intercellular Communication. Cell *177*, 225-227. 10.1016/j.cell.2019.03.020.

7. Jeppesen, D.K., Fenix, A.M., Franklin, J.L., Higginbotham, J.N., Zhang, Q., Zimmerman, L.J., Liebler, D.C., Ping, J., Liu, Q., Evans, R., et al. (2019). Reassessment of Exosome Composition. Cell *177*, 428-445 e418. 10.1016/j.cell.2019.02.029.

8. Park, D.J., Duggan, E., Ho, K., Dorschner, R.A., Dobke, M., Nolan, J.P., and Eliceiri, B.P. (2022). Serpin-loaded extracellular vesicles promote tissue repair in a mouse model of impaired wound healing. J Nanobiotechnology *20*, 474. 10.1186/s12951-022-01656-7.

9. Qin, S., Dorschner, R.A., Masini, I., Lavoie-Gagne, O., Stahl, P.D., Costantini, T.W., Baird, A., and Eliceiri, B.P. (2019). TBC1D3 regulates the payload and biological activity of extracellular vesicles that mediate tissue repair. FASEB J *33*, 6129-6139. 10.1096/fj.201802388R.

10. Qian, J., Park, D.J., Perrott, S., Patel, P., and Eliceiri, B.P. (2021). Genetic Background and Kinetics Define Wound Bed Extracellular Vesicles in a Mouse Model of Cutaneous Injury. Int J Mol Sci *22*. 10.3390/ijms22073551.

11. Silva, A.M., Teixeira, J.H., Almeida, M.I., Goncalves, R.M., Barbosa, M.A., and Santos, S.G. (2017). Extracellular Vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration. Eur J Pharm Sci *98*, 86-95. 10.1016/j.ejps.2016.09.017.

12. Veerman, R.E., Teeuwen, L., Czarnewski, P., Gucluler Akpinar, G., Sandberg, A., Cao, X., Pernemalm, M., Orre, L.M., Gabrielsson, S., and Eldh, M. (2021). Molecular evaluation of five different isolation methods for extracellular vesicles reveals different clinical applicability and subcellular origin. J Extracell Vesicles *10*, e12128. 10.1002/jev2.12128.

27

676   13.   Prasai, A., Jay, J.W., Jupiter, D., Wolf, S.E., and El Ayadi, A. (2022). Role of Exosomes in Dermal Wound
677   Healing: A Systematic Review. J Invest Dermatol *142*, 662-678 e668. 10.1016/j.jid.2021.07.167.

678   14.   Sarcinella, A., Femmino, S., and Brizzi, M.F. (2023). Extracellular Vesicles: Emergent and Multiple Sources in
679   Wound Healing Treatment. Int J Mol Sci *24*. 10.3390/ijms242115709.

680   15.   Pitt, J.M., Kroemer, G., and Zitvogel, L. (2016). Extracellular vesicles: masters of intercellular communication
681   and potential clinical interventions. J Clin Invest *126*, 1139-1143. 10.1172/JCI87316.

682   16.   Deskins, D.L., Ardestani, S., and Young, P.P. (2012). The polyvinyl alcohol sponge model implantation. J Vis
683   Exp. 10.3791/3885.

684   17.   Baird, A., Deng, C., Eliceiri, M.H., Haghi, F., Dang, X., Coimbra, R., Costantini, T.W., Torbett, B.E., and
685   Eliceiri, B.P. (2016). Mice engrafted with human hematopoietic stem cells support a human myeloid cell
686   inflammatory response in vivo. Wound Repair Regen *24*, 1004-1014. 10.1111/wrr.12471.

687   18.   Crane, M.J., Daley, J.M., van Houtte, O., Brancato, S.K., Henry, W.L., Jr., and Albina, J.E. (2014). The
688   monocyte to macrophage transition in the murine sterile wound. PLoS One *9*, e86660.
689   10.1371/journal.pone.0086660.

690   19.   Michaels, J.t., Churgin, S.S., Blechman, K.M., Greives, M.R., Aarabi, S., Galiano, R.D., and Gurtner, G.C.
691   (2007). db/db mice exhibit severe wound-healing impairments compared with other murine diabetic strains in
692   a silicone-splinted excisional wound model. Wound Repair Regen *15*, 665-670. 10.1111/j.1524-
693   475X.2007.00273.x.

694   20.   Zhao, G., Hochwalt, P.C., Usui, M.L., Underwood, R.A., Singh, P.K., James, G.A., Stewart, P.S., Fleckman, P.,
695   and Olerud, J.E. (2010). Delayed wound healing in diabetic (db/db) mice with Pseudomonas aeruginosa
696   biofilm challenge: a model for the study of chronic wounds. Wound Repair Regen *18*, 467-477.
697   10.1111/j.1524-475X.2010.00608.x.

698   21.   Park, S.A., Teixeira, L.B., Raghunathan, V.K., Covert, J., Dubielzig, R.R., Isseroff, R.R., Schurr, M., Abbott,
699   N.L., McAnulty, J., and Murphy, C.J. (2014). Full-thickness splinted skin wound healing models in db/db and
700   heterozygous mice: implications for wound healing impairment. Wound Repair Regen *22*, 368-380.
701   10.1111/wrr.12172.

702   22.   Dhall, S., Do, D., Garcia, M., Wijesinghe, D.S., Brandon, A., Kim, J., Sanchez, A., Lyubovitsky, J., Gallagher,
703   S., Nothnagel, E.A., et al. (2014). A novel model of chronic wounds: importance of redox imbalance and
704   biofilm-forming bacteria for establishment of chronicity. PLoS One *9*, e109848.
705   10.1371/journal.pone.0109848.

706   23.   McCann, J.V., Bischoff, S.R., Zhang, Y., Cowley, D.O., Sanchez-Gonzalez, V., Daaboul, G.D., and Dudley,
707   A.C. (2020). Reporter mice for isolating and auditing cell type-specific extracellular vesicles in vivo. Genesis
708   *58*, e23369. 10.1002/dvg.23369.

709   24.   Neckles, V.N., Morton, M.C., Holmberg, J.C., Sokolov, A.M., Nottoli, T., Liu, D., and Feliciano, D.M. (2019).
710   A transgenic inducible GFP extracellular-vesicle reporter (TIGER) mouse illuminates neonatal cortical

28

711   astrocytes as a source of immunomodulatory extracellular vesicles. Sci Rep *9*, 3094. 10.1038/s41598-019-
712   39679-0.

713 25.   Sung, B.H., Ketova, T., Hoshino, D., Zijlstra, A., and Weaver, A.M. (2015). Directional cell movement through
714   tissues is controlled by exosome secretion. Nat Commun *6*, 7164. 10.1038/ncomms8164.

715 26.   Fordjour, F.K., Abuelreich, S., Hong, X., Chatterjee, E., Lallai, V., Ng, M., Saftics, A., Deng, F., Carnel-Amar,
716   N., Wakimoto, H., et al. (2023). Exomap1 mouse: a transgenic model for in vivo studies of exosome biology.
717   bioRxiv. 10.1101/2023.05.29.542707.

718 27.   Nikoloff, J.M., Saucedo-Espinosa, M.A., Kling, A., and Dittrich, P.S. (2021). Identifying extracellular vesicle
719   populations from single cells. Proc Natl Acad Sci U S A *118*. 10.1073/pnas.2106630118.

720 28.   Giraldez, M.D., Spengler, R.M., Etheridge, A., Godoy, P.M., Barczak, A.J., Srinivasan, S., De Hoff, P.L.,
721   Tanriverdi, K., Courtright, A., Lu, S., et al. (2018). Comprehensive multi-center assessment of small RNA-seq
722   methods for quantitative miRNA profiling. Nat Biotechnol *36*, 746-757. 10.1038/nbt.4183.

723 29.   Sung, B.H., and Weaver, A.M. (2023). Visualization of Exosome Release and Uptake During Cell Migration
724   Using the Live Imaging Reporter pHluorin_M153R-CD63. Methods Mol Biol *2608*, 83-96. 10.1007/978-1-
725   0716-2887-4_6.

726 30.   Blandin, A., Amosse, J., Froger, J., Hilairet, G., Durcin, M., Fizanne, L., Ghesquiere, V., Prieur, X., Chaigneau,
727   J., Vergori, L., et al. (2023). Extracellular vesicles are carriers of adiponectin with insulin-sensitizing and anti-
728   inflammatory properties. Cell Rep *42*, 112866. 10.1016/j.celrep.2023.112866.

729 31.   Daley, J.M., Brancato, S.K., Thomay, A.A., Reichner, J.S., and Albina, J.E. (2010). The phenotype of murine
730   wound macrophages. J Leukoc Biol *87*, 59-67. 10.1189/jlb.0409236.

731 32.   Rani, M., and Schwacha, M.G. (2017). The composition of T-cell subsets are altered in the burn wound early
732   after injury. PLoS One *12*, e0179015. 10.1371/journal.pone.0179015.

733 33.   Engin, A.B. (2017). Adipocyte-Macrophage Cross-Talk in Obesity. Adv Exp Med Biol *960*, 327-343.
734   10.1007/978-3-319-48382-5_14.

735 34.   Ellett, F., Pazhakh, V., Pase, L., Benard, E.L., Weerasinghe, H., Azabdaftari, D., Alasmari, S., Andrianopoulos,
736   A., and Lieschke, G.J. (2018). Macrophages protect Talaromyces marneffei conidia from myeloperoxidase-
737   dependent neutrophil fungicidal activity during infection establishment in vivo. PLoS Pathog *14*, e1007063.
738   10.1371/journal.ppat.1007063.

739 35.   Wang, S., Song, R., Wang, Z., Jing, Z., Wang, S., and Ma, J. (2018). S100A8/A9 in Inflammation. Front
740   Immunol *9*, 1298. 10.3389/fimmu.2018.01298.

741 36.   Wei, J., and Gronert, K. (2019). Eicosanoid and Specialized Proresolving Mediator Regulation of Lymphoid
742   Cells. Trends Biochem Sci *44*, 214-225. 10.1016/j.tibs.2018.10.007.

743 37.   Pan, R.Y., Chu, M.T., Wang, C.W., Lee, Y.S., Lemonnier, F., Michels, A.W., Schutte, R., Ostrov, D.A., Chen,
744   C.B., Phillips, E.J., et al. (2019). Identification of drug-specific public TCR driving severe cutaneous adverse
745   reactions. Nat Commun *10*, 3569. 10.1038/s41467-019-11396-2.

746 38. Wang, X., Balaji, S., Steen, E.H., Li, H., Rae, M.M., Blum, A.J., Miao, Q., Butte, M.J., Bollyky, P.L., and
747   Keswani, S.G. (2019). T Lymphocytes Attenuate Dermal Scarring by Regulating Inflammation,
748   Neovascularization, and Extracellular Matrix Remodeling. Adv Wound Care (New Rochelle) *8*, 527-537.
749   10.1089/wound.2019.0981.

750 39. Amon, L., Lehmann, C.H.K., Baranska, A., Schoen, J., Heger, L., and Dudziak, D. (2019). Transcriptional
751   control of dendritic cell development and functions. Int Rev Cell Mol Biol *349*, 55-151.
752   10.1016/bs.ircmb.2019.10.001.

753 40. Johnson, J., Jaggers, R.M., Gopalkrishna, S., Dahdah, A., Murphy, A.J., Hanssen, N.M.J., and Nagareddy, P.R.
754   (2022). Oxidative Stress in Neutrophils: Implications for Diabetic Cardiovascular Complications. Antioxid
755   Redox Signal *36*, 652-666. 10.1089/ars.2021.0116.

756 41. Hong, W., Yang, B., He, Q., Wang, J., and Weng, Q. (2022). New Insights of CCR7 Signaling in Dendritic Cell
757   Migration and Inflammatory Diseases. Front Pharmacol *13*, 841687. 10.3389/fphar.2022.841687.

758 42. Kim, K., Park, S.E., Park, J.S., and Choi, J.H. (2022). Characteristics of plaque lipid-associated macrophages
759   and their possible roles in the pathogenesis of atherosclerosis. Curr Opin Lipidol *33*, 283-288.
760   10.1097/MOL.0000000000000842.

761 43. Deming, Y., Filipello, F., Cignarella, F., Cantoni, C., Hsu, S., Mikesell, R., Li, Z., Del-Aguila, J.L., Dube, U.,
762   Farias, F.G., et al. (2019). The MS4A gene cluster is a key modulator of soluble TREM2 and Alzheimer's
763   disease risk. Sci Transl Med *11*. 10.1126/scitranslmed.aau2291.

764 44. Guo, L., Liu, Y., Chen, Y., Xu, J., and Liu, Y. (2023). Liver macrophages show an immunotolerance phenotype
765   in nonalcoholic fatty liver combined with Porphyromonas gingivalis-lipopolysaccharide infection. Hua Xi Kou
766   Qiang Yi Xue Za Zhi *41*, 385-394. 10.7518/hxkq.2023.2023111.

767 45. Boing, A.N., van der Pol, E., Grootemaat, A.E., Coumans, F.A., Sturk, A., and Nieuwland, R. (2014). Single-
768   step isolation of extracellular vesicles by size-exclusion chromatography. J Extracell Vesicles *3*.
769   10.3402/jev.v3.23430.

770 46. Sodar, B.W., Kittel, A., Paloczi, K., Vukman, K.V., Osteikoetxea, X., Szabo-Taylor, K., Nemeth, A., Sperlagh,
771   B., Baranyai, T., Giricz, Z., et al. (2016). Low-density lipoprotein mimics blood plasma-derived exosomes and
772   microvesicles during isolation and detection. Sci Rep *6*, 24316. 10.1038/srep24316.

773 47. McFarland-Mancini, M.M., Funk, H.M., Paluch, A.M., Zhou, M., Giridhar, P.V., Mercer, C.A., Kozma, S.C.,
774   and Drew, A.F. (2010). Differences in wound healing in mice with deficiency of IL-6 versus IL-6 receptor. J
775   Immunol *184*, 7219-7228. 10.4049/jimmunol.0901929.

776 48. LeBlanc, S., Arabzadeh, A., Benlolo, S., Breton, V., Turbide, C., Beauchemin, N., and Nouvion, A.L. (2011).
777   CEACAM1 deficiency delays important wound healing processes. Wound Repair Regen *19*, 745-752.
778   10.1111/j.1524-475X.2011.00742.x.

779 49. Bourke, C.D., Prendergast, C.T., Sanin, D.E., Oulton, T.E., Hall, R.J., and Mountford, A.P. (2015). Epidermal
780   keratinocytes initiate wound healing and pro-inflammatory immune responses following percutaneous
781   schistosome infection. Int J Parasitol *45*, 215-224. 10.1016/j.ijpara.2014.11.002.

782 50. Shapira, S., Ben-Amotz, O., Sher, O., Kazanov, D., Mashiah, J., Kraus, S., Gur, E., and Arber, N. (2015).
783 Delayed Wound Healing in Heat Stable Antigen (HSA/CD24)-Deficient Mice. PLoS One *10*, e0139787.
784 10.1371/journal.pone.0139787.

785 51. Huang, X., Gu, S., Liu, C., Zhang, L., Zhang, Z., Zhao, Y., Khoong, Y., Li, H., Gao, Y., Liu, Y., et al. (2022).
786 CD39(+) Fibroblasts Enhance Myofibroblast Activation by Promoting IL-11 Secretion in Hypertrophic Scars.
787 J Invest Dermatol *142*, 1065-1076 e1019. 10.1016/j.jid.2021.07.181.

788 52. Noguchi, F., Nakajima, T., Inui, S., Reddy, J.K., and Itami, S. (2014). Alteration of skin wound healing in
789 keratinocyte-specific mediator complex subunit 1 null mice. PLoS One *9*, e102271.
790 10.1371/journal.pone.0102271.

791 53. Prabhu, V., Rao, B.S.S., Rao, A.C.K., Prasad, K., and Mahato, K.K. (2022). Photobiomodulation invigorating
792 collagen deposition, proliferating cell nuclear antigen and Ki67 expression during dermal wound repair in
793 mice. Lasers Med Sci *37*, 171-180. 10.1007/s10103-020-03202-z.

794 54. Wang, L., Jiang, J., Zhou, T., Xue, X., and Cao, Y. (2021). Improvement of Cerebral Ischemia-Reperfusion
795 Injury via Regulation of Apoptosis by Exosomes Derived from BDNF-Overexpressing HEK293. Biomed Res
796 Int *2021*, 6613510. 10.1155/2021/6613510.

797 55. Wang, C., Li, N., Li, Y., Hou, S., Zhang, W., Meng, Z., Wang, S., Jia, Q., Tan, J., Wang, R., et al. (2022).
798 Engineering a HEK-293T exosome-based delivery platform for efficient tumor-targeting
799 chemotherapy/internal irradiation combination therapy. J Nanobiotechnology *20*, 247. 10.1186/s12951-022-
800 01462-1.

801 56. Zhang, J., Song, H., Dong, Y., Li, G., Li, J., Cai, Q., Yuan, S., Wang, Y., and Song, H. (2023). Surface
802 Engineering of HEK293 Cell-Derived Extracellular Vesicles for Improved Pharmacokinetic Profile and
803 Targeted Delivery of IL-12 for the Treatment of Hepatocellular Carcinoma. Int J Nanomedicine *18*, 209-223.
804 10.2147/IJN.S388916.

805 57. Qu, Y., Zhang, Q., Cai, X., Li, F., Ma, Z., Xu, M., and Lu, L. (2017). Exosomes derived from miR-181-5p-
806 modified adipose-derived mesenchymal stem cells prevent liver fibrosis via autophagy activation. J Cell Mol
807 Med *21*, 2491-2502. 10.1111/jcmm.13170.

808 58. Katakowski, M., Buller, B., Zheng, X., Lu, Y., Rogers, T., Osobamiro, O., Shu, W., Jiang, F., and Chopp, M.
809 (2013). Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. Cancer Lett *335*,
810 201-204. 10.1016/j.canlet.2013.02.019.

811 59. Wang, Q., Zhu, G., Cao, X., Dong, J., Song, F., and Niu, Y. (2017). Blocking AGE-RAGE Signaling Improved
812 Functional Disorders of Macrophages in Diabetic Wound. J Diabetes Res *2017*, 1428537.
813 10.1155/2017/1428537.

814 60. Fernandes, H., Zonnari, A., Abreu, R., Aday, S., Barao, M., Albino, I., Lino, M., Branco, A., Seabra, C.,
815 Barata, T., et al. (2022). Extracellular vesicles enriched with an endothelial cell pro-survival microRNA affects
816 skin tissue regeneration. Mol Ther Nucleic Acids *28*, 307-327. 10.1016/j.omtn.2022.03.018.

31

817   61.   de Almeida Oliveira, N.C., Neri, E.A., Silva, C.M., Valadao, I.C., Fonseca-Alaniz, M.H., Zogbi, C., Levy, D.,

818   Bydlowski, S.P., and Krieger, J.E. (2022). Multicellular regulation of miR-196a-5p and miR-425-5 from

819   adipose stem cell-derived exosomes and cardiac repair. Clin Sci (Lond) *136*, 1281-1301.

820   10.1042/CS20220216.

821   62.   Kwon, D.N., Chang, B.S., and Kim, J.H. (2014). MicroRNA dysregulation in liver and pancreas of CMP-

822   Neu5Ac hydroxylase null mice disrupts insulin/PI3K-AKT signaling. Biomed Res Int *2014*, 236385.

823   10.1155/2014/236385.

824   63.   Yuan, J., Wu, Y., Li, L., and Liu, C. (2020). MicroRNA-425-5p promotes tau phosphorylation and cell

825   apoptosis in Alzheimer's disease by targeting heat shock protein B8. J Neural Transm (Vienna) *127*, 339-346.

826   10.1007/s00702-019-02134-5.

827   64.   Lou, G., Song, X., Yang, F., Wu, S., Wang, J., Chen, Z., and Liu, Y. (2015). Exosomes derived from miR-122-

828   modified adipose tissue-derived MSCs increase chemosensitivity of hepatocellular carcinoma. J Hematol

829   Oncol *8*, 122. 10.1186/s13045-015-0220-7.

830   65.   Lee, H.K., Lee, B.R., Lee, T.J., Lee, C.M., Li, C., O'Connor, P.M., Dong, Z., and Kwon, S.H. (2022).

831   Differential release of extracellular vesicle tRNA from oxidative stressed renal cells and ischemic kidneys. Sci

832   Rep *12*, 1646. 10.1038/s41598-022-05648-3.

833   66.   Spiegel, A., Brooks, M.W., Houshyar, S., Reinhardt, F., Ardolino, M., Fessler, E., Chen, M.B., Krall, J.A.,

834   DeCock, J., Zervantonakis, I.K., et al. (2016). Neutrophils Suppress Intraluminal NK Cell-Mediated Tumor

835   Cell Clearance and Enhance Extravasation of Disseminated Carcinoma Cells. Cancer Discov *6*, 630-649.

836   10.1158/2159-8290.CD-15-1157.

837   67.   Achari, A.E., and Jain, S.K. (2017). Adiponectin, a Therapeutic Target for Obesity, Diabetes, and Endothelial

838   Dysfunction. Int J Mol Sci *18*. 10.3390/ijms18061321.

839   68.   Yanai, H., and Yoshida, H. (2019). Beneficial Effects of Adiponectin on Glucose and Lipid Metabolism and

840   Atherosclerotic Progression: Mechanisms and Perspectives. Int J Mol Sci *20*. 10.3390/ijms20051190.

841   69.   Lou, R., Chen, J., Zhou, F., Wang, C., Leung, C.H., and Lin, L. (2022). Exosome-cargoed microRNAs:

842   Potential therapeutic molecules for diabetic wound healing. Drug Discov Today *27*, 103323.

843   10.1016/j.drudis.2022.07.008.

844   70.   Barman, B., Sung, B.H., Krystofiak, E., Ping, J., Ramirez, M., Millis, B., Allen, R., Prasad, N., Chetyrkin, S.,

845   Calcutt, M.W., et al. (2022). VAP-A and its binding partner CERT drive biogenesis of RNA-containing

846   extracellular vesicles at ER membrane contact sites. Dev Cell *57*, 974-994 e978.

847   10.1016/j.devcel.2022.03.012.

848   71.   Tang, Y., Yang, L.J., Liu, H., Song, Y.J., Yang, Q.Q., Liu, Y., Qian, S.W., and Tang, Q.Q. (2023). Exosomal

849   miR-27b-3p secreted by visceral adipocytes contributes to endothelial inflammation and atherogenesis. Cell

850   Rep *42*, 111948. 10.1016/j.celrep.2022.111948.

851   72.   Turk, M.A., Chung, C.Z., Manni, E., Zukowski, S.A., Engineer, A., Badakhshi, Y., Bi, Y., and Heinemann, I.U.

852   (2018). MiRAR-miRNA Activity Reporter for Living Cells. Genes (Basel) *9*. 10.3390/genes9060305.

32

853   73.   Ying, W., Riopel, M., Bandyopadhyay, G., Dong, Y., Birmingham, A., Seo, J.B., Ofrecio, J.M., Wollam, J.,
854   Hernandez-Carretero, A., Fu, W., et al. (2017). Adipose Tissue Macrophage-Derived Exosomal miRNAs Can
855   Modulate In Vivo and In Vitro Insulin Sensitivity. Cell *171*, 372-384 e312. 10.1016/j.cell.2017.08.035.

856   74.   Albanese, M., Chen, Y.A., Huls, C., Gartner, K., Tagawa, T., Mejias-Perez, E., Keppler, O.T., Gobel, C.,
857   Zeidler, R., Shein, M., et al. (2021). MicroRNAs are minor constituents of extracellular vesicles that are rarely
858   delivered to target cells. PLoS Genet *17*, e1009951. 10.1371/journal.pgen.1009951.

859   75.   Chevillet, J.R., Kang, Q., Ruf, I.K., Briggs, H.A., Vojtech, L.N., Hughes, S.M., Cheng, H.H., Arroyo, J.D.,
860   Meredith, E.K., Gallichotte, E.N., et al. (2014). Quantitative and stoichiometric analysis of the microRNA
861   content of exosomes. Proc Natl Acad Sci U S A *111*, 14888-14893. 10.1073/pnas.1408301111.

862   76.   Qi, R., Wang, J., Wang, Q., Qiu, X., Yang, F., Liu, Z., and Huang, J. (2019). MicroRNA-425 controls
863   lipogenesis and lipolysis in adipocytes. Biochim Biophys Acta Mol Cell Biol Lipids *1864*, 744-755.
864   10.1016/j.bbalip.2019.02.007.

865   77.   Sung, B.H., von Lersner, A., Guerrero, J., Krystofiak, E.S., Inman, D., Pelletier, R., Zijlstra, A., Ponik, S.M.,
866   and Weaver, A.M. (2020). A live cell reporter of exosome secretion and uptake reveals pathfinding behavior of
867   migrating cells. Nat Commun *11*, 2092. 10.1038/s41467-020-15747-2.

868   78.   de Jong, O.G., Murphy, D.E., Mager, I., Willms, E., Garcia-Guerra, A., Gitz-Francois, J.J., Lefferts, J., Gupta,
869   D., Steenbeek, S.C., van Rheenen, J., et al. (2020). A CRISPR-Cas9-based reporter system for single-cell
870   detection of extracellular vesicle-mediated functional transfer of RNA. Nat Commun *11*, 1113.
871   10.1038/s41467-020-14977-8.

872   79.   Einhauer, A., and Jungbauer, A. (2001). The FLAG peptide, a versatile fusion tag for the purification of
873   recombinant proteins. J Biochem Biophys Methods *49*, 455-465. 10.1016/s0165-022x(01)00213-5.

874   80.   Fajardo, R.J., Karim, L., Calley, V.I., and Bouxsein, M.L. (2014). A review of rodent models of type 2 diabetic
875   skeletal fragility. J Bone Miner Res *29*, 1025-1040. 10.1002/jbmr.2210.

876   81.   Lee, Y., Berglund, E.D., Yu, X., Wang, M.Y., Evans, M.R., Scherer, P.E., Holland, W.L., Charron, M.J., Roth,
877   M.G., and Unger, R.H. (2014). Hyperglycemia in rodent models of type 2 diabetes requires insulin-resistant
878   alpha cells. Proc Natl Acad Sci U S A *111*, 13217-13222. 10.1073/pnas.1409638111.

879   82.   Thery, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R., Antoniou, A., Arab,
880   T., Archer, F., Atkin-Smith, G.K., et al. (2018). Minimal information for studies of extracellular vesicles 2018
881   (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the
882   MISEV2014 guidelines. J Extracell Vesicles *7*, 1535750. 10.1080/20013078.2018.1535750.

883   83.   Welsh, J.A., Van Der Pol, E., Arkesteijn, G.J.A., Bremer, M., Brisson, A., Coumans, F., Dignat-George, F.,
884   Duggan, E., Ghiran, I., Giebel, B., et al. (2020). MIFlowCyt-EV: a framework for standardized reporting of
885   extracellular vesicle flow cytometry experiments. J Extracell Vesicles *9*, 1713526.
886   10.1080/20013078.2020.1713526.

887 84.   Shpigelman, J., Lao, F.S., Yao, S., Li, C., Saito, T., Sato-Kaneko, F., Nolan, J.P., Shukla, N.M., Pu, M., Messer,
888   K., et al. (2021). Generation and Application of a Reporter Cell Line for the Quantitative Screen of
889   Extracellular Vesicle Release. Front Pharmacol *12*, 668609. 10.3389/fphar.2021.668609.

890 85.   Welsh, J.A., Arkesteijn, G.J.A., Bremer, M., Cimorelli, M., Dignat-George, F., Giebel, B., Gorgens, A.,
891   Hendrix, A., Kuiper, M., Lacroix, R., et al. (2023). A compendium of single extracellular vesicle flow
892   cytometry. J Extracell Vesicles *12*, e12299. 10.1002/jev2.12299.

893 86.   Li, M., Wang, T., Tian, H., Wei, G., Zhao, L., and Shi, Y. (2019). Macrophage-derived exosomes accelerate
894   wound healing through their anti-inflammation effects in a diabetic rat model. Artif Cells Nanomed Biotechnol
895   *47*, 3793-3803. 10.1080/21691401.2019.1669617.

896 87.   Hsu, H.H., Wang, A.Y.L., Loh, C.Y.Y., Pai, A.A., and Kao, H.K. (2022). Therapeutic Potential of Exosomes
897   Derived from Diabetic Adipose Stem Cells in Cutaneous Wound Healing of db/db Mice. Pharmaceutics *14*.
898   10.3390/pharmaceutics14061206.

899 88.   Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-
900   seq data with DESeq2. Genome Biol *15*, 550. 10.1186/s13059-014-0550-8.

901 89.   Sahraeian, S.M.E., Mohiyuddin, M., Sebra, R., Tilgner, H., Afshar, P.T., Au, K.F., Bani Asadi, N., Gerstein,
902   M.B., Wong, W.H., Snyder, M.P., et al. (2017). Gaining comprehensive biological insight into the
903   transcriptome by performing a broad-spectrum RNA-seq analysis. Nat Commun *8*, 59. 10.1038/s41467-017-
904   00050-4.

905 90.   Ye, Z., Wang, S., Huang, X., Chen, P., Deng, L., Li, S., Lin, S., Wang, Z., and Liu, B. (2022). Plasma
906   Exosomal miRNAs Associated With Metabolism as Early Predictor of Gestational Diabetes Mellitus. Diabetes
907   *71*, 2272-2283. 10.2337/db21-0909.

908 **List of Figure Captions**

909 **Figure 1. Diabetic obese mice drive a transcriptional reprogramming of immune cell subsets recruited into sites**

910 **of cutaneous injury.**

911 (A) Schematic of PVA implant model for the harvest of EVs from cutaneous site. (B) scRNAseq of cells from PVA

912 sponge implants in WT vs. db/db mice (C) Expression of genesets mapping to macrophages, neutrophils, dendritic cells

913 (DCs), and lymphocytes based on supporting references in Table S1. (D) Analysis of changes in gene expression of top

914 10 up-regulated vs. down-regulated genes in cells from WT vs. db/db donors (GSE #242496).

915

916 **Figure 2. Identification and characterization of EVs derived from the PVA sponges.**

917 (A) Schematic of EV purification and profiling using size exclusion chromatography (SEC). (B) Quantification of SEC

918 fractions by determining EV concentration using vFRed staining as detailed in the Materials and Methods (bars) and

919 soluble protein using a BCA assay (line) from a representative WT EV sample. EV concentration of WT vs. db/db EVs

920 from each SEC fraction is quantified in replicates in Supplementary Figure S2A. (C) Immunoblotting of SEC fractions

921 to detect CD9 (bottom) and a Memcode protein stain (top) for total protein from each SEC fraction. (D) Immunoblotting

922 of EV markers of whole cell lysates (WCLs) and EVs from a representative WT PVA donor. (E) Blood glucose and (F)

923 body weight measurements that define the pathophysiology of the db/db mouse model. (G) EV concentration and (H)

924 size distributions of WT vs. db/db EVs in fraction 7 (n=10). (p values: *<0.05, ****<0.0001).

925

926 **Figure 3. vFC of EVs isolated from PVA sponge implants.**

927 (A) Schematic of EV analyses by batch vs. vesicle flow cytometry (vFC). (B) Quantification of a bead-based EV protein

928 screen of inflammation-related proteins of WT EVs. (C) Representative vFC analysis of isotype and tetraspanin levels

929 on WT PVA EVs, and (D) immune-related proteins. vFC analysis of WT vs. db/db EVs for (E) tetraspanins, (F) integrins,

930 (G) immune-related proteins, and (H) other EV-related inflammation proteins. (n=5 for each group; p values: **<0.005,

931 *<0.05).

932
933
934
935

936 **Figure 4. Adoptive transfer of EVs from diabetic obese donors have impaired wound healing activity.**

937 (A) Schematic of EV adoptive transfer strategy. (B) Representative images of recipient wound beds on each day after

938 treatment with saline control, WT EVs, or db/db EVs (a volume of 50 µL of EVs at 5-7 x 10^6 PVA EVs/µL) and (C)

939 quantification of wound closure kinetics (n=10 per group; p values: ****<0.0001, **<0.005, *<0.05). (D) Representative

940 hematoxylin and eosin (H&E) stained wounds on Day 14 after treatment with WT vs. db/db EVs (inset on right = high

941 magnification). (E) Quantification of epidermis thickness (µm) based on H&E images indicated with brackets in Panel

942 D. (F) Quantification of cell count per area (mm^2) based on H&E-stained images. (G) Localization of Ki67$^+$ cells by

943 immunohistochemistry on Day 14 after treatment with WT or db/db EVs. (H) Schematic of WT CD63 and CD63-FLAG

944 tag (top) and vFC analysis of surface levels FLAG tag (bottom). (I) Immunoblotting to detect FLAG tag expression. (J)

945 Localization of FLAG-tagged EVs in wound bed after a 24 hour treatment with saline (top), CD63-expressing EVs

946 (middle), or CD63-FLAG expressing EVs. (green line: edge, blue box: wound margin, red box: wound bed). High

947 magnification images of wound margin (middle) and wound bed (red) are shown. (K) Uptake of control CD63 (top) vs.

948 CD63-FLAG-tagged (bottom) EVs into MEFs. (L) Quantification of FLAG-tagged EV uptake into MEFs.

949

950 **Figure 5. Regulation of miRNA EV payloads isolated in diabetic obese mouse model.**

951 (A) miRNAseq analysis of fold changes in WT vs db/db EVs. (B) Distribution of miRNA profile between WT and db/db

952 EVs. (C) Analysis of fold change (FC) vs. significance (P) of miRNAs identified as described in the Materials and

953 Methods. (D) KEGG pathway analysis of miRNAs identified. (E) Schematic of EV collection from cultured sponge-

954 derived macrophages. (F) Quantification of EV yield from cultured macrophages. (G) qRT-PCR analysis of miR-425-

955 5p in EVs isolated from the conditioned media from db/db vs. WT macrophages (n=3 from each group. p < 0.005).

956

957 **Figure 6. Biological testing of miR-425-5p-loaded EVs.**

958 (A) Schematic of EV collection, loading with specific miRNAs *in vitro*, followed by treatment of splinted wounds *in*

959 *vivo* to assess miR-425-5p biological activity. (B) Comparison of wound closure kinetics of normal WT EVs (i.e., no

960 miRNA loading) vs. WT EVs loaded with miR-425-5p to show that EVs loaded with miR-425-5p are more pro-reparative

961 than WT EVs. (C) Analysis of wound closure kinetics with controls including saline (black), mock-treated EVs (i.e., EVs

962 treated with Exofect reagent without miRNA; green), negative control miRNA-loaded EVs (i.e., cel-miR-67; blue), or

963   miR-425-5p-loaded EVs (red) (a volume of 50 µL of EVs at 5-7 x 10^6 PVA EVs/µL). (D) Localization and (E)

964   quantification of Ki67$^+$ cells in the basal epidermal skin layer to assess the general pro-reparative effects of miR-425-5p-

965   loaded EV treatment. (F) Representative H&E (top) and Masson's Trichrome stained images (bottom) on Day 14 after

966   treatment with saline, neg-miRNA-loaded EVs or miR-425-5p-loaded EVs (Red dotted line = wound margin; blue box

967   = area used for cell counts and epidermis thickness measurements). Quantification of the effects of treatment with neg-

968   miRNA-loaded EVs vs. miR-425-5p-loaded EVs on (G) cell count, (H) epidermis thickness, and (I) collagen as a

969   percentage of the dermis area (mm^2) based on Masson's Trichrome staining. (J) Protein panel quantifying changes in

970   adipokine/cytokine expression in wound beds treated with miR-425-5p-loaded EVs vs. negative control miRNA-loaded

971   EVs at 14 days (n=2 for each treatment group). Supplementary Figure S8 has the complete profile. (K) Schematic of *in*

972   *vitro* of PVA sponge-derived EVs loaded with negative control miRNA vs. miR-425-5p used to treat MEFs. (L)

973   Immunoblotting of Adiponectin protein of MEFs treated with control vs. miR-425-5p-loaded EVs. (p values:

974   ****<0.0001, ***<0.001, **<0.005, *<0.05)

975

976   **Figure 7. Transgenic mice expressing the tetraspanin CD9-GFP to assess cell type specific EV release and track**

977   **EV uptake.**

978   (A) Schematic of lineage mapping mouse lines using CD9-GFP TIGER model and cell-type specific expression of Cre.

979   (B) Analysis of GFP expression in cells and (C) EVs from PVA sponge implants from transgenic mice expressing CD9-

980   GFP under the control of LysM, TEK, or Krt14 promoters, as detailed in the Materials and Methods. (D) Schematic of

981   adoptive transfer of CD9-GFP EVs into splinted wounds of db/db mice. (a volume of 50 µL of EVs at 5-7 x 10^6 PVA

982   EVs/µL) (E) Detection of CD9-GFP$^+$ EVs (left; green fluorescence) vs. WT EVs (right; non-fluorescent EVs from CD9-

983   GFP$^-$ mice) in the wound bed. Representative low magnification images of (F) WT non-fluorescent EVs vs. (G) CD9-

984   GFP$^+$ EVs (white arrowheads) in the splinted wound (left and right images comprise the full wound site), counterstained

985   with vimentin for fibroblasts (red) and DAPI for nuclei (blue). (H) Representative high magnification images of CD9-

986   GFP$^+$ EVs from two different fields (top and bottom) detected in the wound margin (based on red box from Panel D) and

987   (I) wound bed (based on blue box from Panel D). CD9-GFP$^+$ EVs colocalized with vimentin indicated with white arrows.

988

A

in vivo EV donors

cell profiling

Sponges implanted in WT vs. db/db mice

Cells → scRNAseq → Identify cells releasing EVs into sponges

EVs

B

UMAP_2 / UMAP_1

Lymphocytes
DCs
Neutrophils
Macrophages

Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8

C

Macrophages (IGF1, TREM2, H2-DMB1, MS4A4C)

Log2 Max Exp

Cluster 1, Cluster 2, Cluster 3, Cluster 4, Cluster 5, Cluster 6, Cluster 7, Cluster 8

Neutrophils (CSF3R, S100A8, S100A9)

Log2 Max Exp

Cluster 1, Cluster 2, Cluster 3, Cluster 4, Cluster 5, Cluster 6, Cluster 7, Cluster 8

Classic DCs (CCR7, ZBTB46)

Log2 Max Exp

Cluster 1, Cluster 2, Cluster 3, Cluster 4, Cluster 5, Cluster 6, Cluster 7, Cluster 8

Lymphocytes (CD3D, CD3E, CD3G, LTB)

Log2 Max Exp

Cluster 1, Cluster 2, Cluster 3, Cluster 4, Cluster 5, Cluster 6, Cluster 7, Cluster 8

D

Macrophages

Stfa2l1, Cstdc4, Ctsk, Atp6v0d2, Trf, Gpnmb, Zdhhc14, Hgsnat, Ccl8, Selenop, Ly6c2, Cxcl10, Ifi205, Ly6a, Ms4a4c, Isg15, H2-Q7, Plac8, Ifit1, Zbp1

db/db / WT

Neutrophils

Egr1, Apoe, Stfa1, Ctsl, Cxcl3, Plin2, Cdkn1a, Noct, Ptgs2, Fos, Isg15, Rsad2, Ifi27l2a, Ifitm3, Bri3, Oasl2, Esd, Ctsz, Ifi204, Ltb

db/db / WT

Classic DCs

Cstdc4, Apoe, Ptgs2, Stfa2l1, Ctsl, Lyz2, Fn1, S100a8, Abca1, Cebpb, Isg15, Ccl17, Tuba1a, Rnasel2i, Ass1, Ddt, Avpi1, Tubb2a, H2-T23, Bst2

db/db / WT

Lymphocytes

Mmp12, Stfa2l1, Il1r2, Ptgs2, H2-Aa, H2-Ab1, H2-Eb1, Cstdc4, Mt1, Cd74, Gzmc, Klra7, Nkg7, Gzmb, H2-T23, Gzma, Prf1, Klra4, H2-Q7, Ly6c2

db/db / WT

A

in vivo EV donors

EV profiling

Sponges implanted in WT vs. db/db mice

EVs

Cells

Purification

F22

F1

Size Exclusion Chromatography (SEC)

B

C

Protein

CD9

D

CD9   CD81   CD63   Alix

WCL EVs   WCL EVs   WCL EVs   WCL EVs

E

Blood Glucose (mg/dL)

*

WT   db/db

F

Body weight (g)

****

WT   db/db

G

EV concentration (10⁶ EVs/µL)

ns

WT EVs   db/db EVs

H

WT EVs

119.8±6.8 nm

db/db EVs

116.7±8.7 nm

Diameter (nm)   Diameter (nm)

A *in vivo* EV donors

Sponges implanted in WT vs. db/db mice → SEC-purified EVs → Batch bead-based multiplex analysis · Rapid · Screening

Single vesicle flow analysis · Heterogeneity · Quantitation

B

Median fluorescence intensity

REA control, Rat IgG1, CD44, CD49e, CD45, CD66a, CD11b, CD61, CD9, MHC II, CD20, CD29, CD115, EphA2, CD31, CD40, CD81, MHC I, CD63, CD69, CD146, CD3, CD24, CD140a, CD133, CD11c, CD41, AN2, CD19, CD2, CD326, CD8a, CD205, CD25, CD49b, CD86, CD62P, CD4, CD105

C

Isotype 0 % | CD9 20.0±4.3 % | CD63 23.0±10.9 % | CD81 3.4±2.1 %

MFI: 0 | MFI: 397±16.8 | MFI: 395.4±15.4 | MFI: 223±102.4

D

MHC I 35.7±10.4% | CD29 17.0±6.5% | CD274 2.7±1.3% | CD39 3.5±1.6%

MFI: 761.8±13.1 | MFI: 326.4±14.4 | MFI: 208.2±59.8 | MFI: 297.4±24.4

E CD9 (TSPAN9) ns | CD63 (TSPAN63) ns

F CD11b (ITGAM) ** | CD49e (ITGA5) ** | CD29 (ITGB1) ns

G CD45 (PTPRC) * | CD44 * | CD54 (ICAM1) * | CD274 (PD-L1) * | MHC I ns

H CD326 (EpCAM) ns | CD39 (ENTPD1) ns | CD66a (CEACAM1) ns | CD24 (HAS) ns | CD126 (IL6R) ns

WT EVs | db/db EVs

A *in vivo* EV donors

B Cells

C EVs

D EV recipients

E CD9-GFP EV (fluorescent) — WT EVs (non-fluorescent)

F Wound margin + Wound bed (x20)

G Wound margin + Wound bed (x20)

H Localization of CD9-GFP EVs in Wound margin (x60)

I Localization of CD9-GFP EVs in Wound bed (x60)

Eliceiri and colleagues identify a novel intercellular signaling axis mediated by the release of pro-reparative extracellular vesicles from macrophages to accelerate wound healing. We identify biologically active payloads and establish genetic models that define the source and uptake of vesicles into specific cell types in the wound bed.