

5 **Induced pluripotent stem cell-derived endothelial cells promote angiogenesis and**
6 **accelerate wound closure in a murine excisional wound healing model**

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9 **Word count:** 5675

10 **Brief title:** iPSC-ECs promote wound healing

11 **Disclosures:** None

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24 **Perspectives**

25 i) Ischaemia-mediated angiogenesis is impaired in patients with cardiovascular
26 disease and diabetes, which leads to the development of intractable wounds and
27 ulcers.

28 ii) Here we show that human induced pluripotent stem cell derived endothelial cells
29 (iPSC-ECs) promote angiogenesis, increase collagen deposition and accelerate
30 healing in a murine wound healing model.

31 iii) Further development of human iPSC-ECs for therapeutic angiogenesis is a
32 promising strategy to reduce the burden of chronic wounds in patients with
33 peripheral arterial disease and diabetes.

34 **Keywords**

35 Angiogenesis, wound healing, stem cells, endothelial cells

36 **Author Contribution Statement**

37 ZEC and RPT contributed significantly and equally to the design and execution of this study.
38 ZEC conceived and designed the study, developed the research plan, performed experiments,
39 assisted with data analysis and wrote the manuscript. RPT conducted all *in vivo* and histology
40 experiments, performed data analysis and contributed to manuscript writing. MMM and KL
41 performed histological staining and quantitative PCR preparation and analysis. CAB
42 provided guidance on wound healing procedures. CAB, JPC, SGW and SP supervised the
43 project. All authors discussed the results, gave feedback and approved the final manuscript.

44

45

46 **Abstract**

47

48 Chronic wounds are a major complication in patients with cardiovascular diseases. Cell
49 therapies have shown potential to stimulate wound healing, but clinical trials using adult stem
50 cells have been tempered by limited numbers of cells and invasive procurement procedures.
51 Induced pluripotent stem cells (iPSCs) have several advantages of other cell types, for
52 example they can be generated in abundance from patients' somatic cells (autologous) or
53 those from a matched donor. iPSCs can be efficiently differentiated to functional endothelial
54 cells (iPSC-ECs). Here, we used a murine excisional wound model to test the pro-angiogenic
55 properties of iPSC-ECs in wound healing. Two full-thickness wounds were made on the
56 dorsum of NOD-SCID mice and splinted. iPSC-ECs (5×10^5) were topically applied to one
57 wound, with the other serving as a control. Treatment with iPSC-ECs significantly increased
58 wound perfusion and accelerated wound closure. Expression of endothelial cell (EC) surface
59 marker, PECAM-1 (CD31), and pro-angiogenic EC receptor, Tie1, mRNA was upregulated
60 in iPSC-EC treated wounds at 7 days post-wounding. Histological analysis of wound sections
61 showed increased capillary density in iPSC-EC wounds at day 7 and day 14 post-wounding,
62 and increased collagen content at day 14. Anti-GFP fluorescence confirmed presence of
63 iPSC-ECs in the wounds. Bioluminescent imaging showed progressive decline of iPSC-ECs
64 over time, suggesting that iPSC-ECs are acting primarily through short-term paracrine
65 effects. These results highlight the pro-regenerative effects of iPSC-ECs and demonstrate that
66 they are a promising potential therapy for intractable wounds.

67 **Introduction**

68 Due to poor circulation and prolonged tissue ischaemia, many patients with peripheral arterial
69 disease develop chronic wounds or ulcers on their lower limbs, which can become infected
70 and often necessitate amputation of the affected foot or leg. The complications associated
71 with infection and amputation also lead to increased mortality in these patients, particularly in
72 diabetics. Revascularisation therapies improve wound healing in patients with critical limb
73 ischaemia, yet chronic wounds remain a major individual and societal burden, with estimated
74 global cost of care in excess of US\$22 billion each year (1, 2). Current treatments for chronic
75 wounds are largely focused on cleaning, infection control and debridement of dead tissue.
76 The gold-standard in chronic wound care is the split-thickness autograft, which is a patch of
77 skin taken from a healthy region and grafted onto the wound (3). Other treatment options
78 include bioactive dressings and donor keratinocytes, but these have limitations and additional
79 therapies are required to better address the microvascular deficiency, which underlies the
80 development of these wounds and contributes to their persistence and re-occurrence.

81 Acute wound healing is comprised of four overlapping stages; haemostasis, inflammation,
82 proliferation and remodelling (4). The inflammation and cellular proliferation phases take
83 place between hours to several weeks after an injury and involve the recruitment of
84 inflammatory cells to the wound site, formation of extracellular matrix (ECM) proteins and
85 granulation tissue, keratinocyte migration, contraction and wound closure. Angiogenesis, the
86 growth of new blood vessel networks, is a vital component of these processes and is
87 dependent upon pro-angiogenic growth factors released by supporting cells, proliferation and
88 migration of local endothelial cells into the wound bed, and recruitment of bone marrow-
89 derived stem cells/endothelial progenitor cells (EPCs) (4-7). The pathophysiology of chronic
90 wounds is complex and disordered, but can be largely attributed to persistent inflammation
91 and a lack of adequate tissue perfusion in the region, exacerbated by failure of ischaemia-

92 mediated angiogenesis in the wound. Collagen deposition is also reduced in diabetic/PAD
93 patient wounds and the collagen that is present is often glycated, which impairs the adherence
94 of new keratinocytes to the ECM (8). Inflammation is also known to be the primary driving
95 force behind excessive scar formation when a wound does eventually heal (9). Meanwhile,
96 the inability of EPCs and supporting cells to form new blood vessel networks to clear
97 necrotic debris and deliver oxygen and nutrients to the granulation tissue maintains the
98 wound's inflammatory status. Therapies that can effectively resolve either or both
99 pathological processes are key to breaking the vicious cycle of failed healing in chronic
100 wounds.

101 Stem cell therapy is a promising strategy to enhance angiogenesis and promote healing of
102 chronic wounds. Adult stem and progenitor cells have been studied extensively in animal
103 models of wound healing and appear to exert their beneficial effects via multiple
104 mechanisms, including recruitment of other cells types, such as keratinocytes, macrophages
105 and endothelial progenitor cells (10-12). They have also been shown to produce collagen
106 types I and III, which are critical for regeneration of the ECM and are the source of structural
107 integrity and tensile strength in the healing tissue (13). Clinical trials with bone marrow
108 derived stem cells are underway and there are a handful of published studies in both acute
109 (surgical) and chronic (venous insufficiency and diabetic) wounds, which have reported
110 consistent decreases in wound size, particularly with repeat treatments (14-17). EPCs and
111 embryonic stem cell derived endothelial cells have also been tested in animal models and
112 improve wound healing via increased vascularisation (18, 19). Indeed, a common beneficial
113 feature of all stem cell therapies seems to be their pro-angiogenic effect.

114 Induced pluripotent stem cells (iPSCs) are derived by reprogramming somatic cells, such as
115 dermal fibroblasts. They have unlimited self-renewal capacity and are therefore an abundant
116 potential source of autologous or donor matched cells for therapy and may be more clinically

117 translatable than other stem cell types. iPSCs can be differentiated to functional endothelial
118 cells (iPSC-ECs) with high efficiency and reproducibility. We have shown previously that
119 iPSC-ECs enhance angiogenesis and improve perfusion recovery in a mouse model of
120 peripheral arterial disease (20, 21). The objective of the current study was to determine
121 whether the pro-angiogenic properties of iPSC-ECs can be harnessed to promote wound
122 healing. We demonstrate for the first time that iPSC-ECs enhance wound angiogenesis and
123 perfusion, promote physiological collagen deposition and accelerate wound closure in a
124 murine excisional wound healing model. These findings suggest that iPSC-ECs have
125 potential as a treatment for chronic wounds and support further development of clinical grade
126 iPSC-ECs for therapeutic angiogenesis.

127

128 Methods

129 *iPSC reprogramming and differentiation to iPSC-ECs*

130 Human iPSCs were generated via retroviral overexpression of Oct4, Klf4, Sox2 and c-Myc
131 (OKSM) transcription factors in human dermal fibroblasts derived from healthy subjects. The
132 iPSCs were differentiated to endothelial cells using previously described methods (20-
133 23)(Supplementary figure 1). The cells were transduced with a double fusion reporter
134 construct encoding GFP and firefly luciferase, to enable *in vivo* fluorescence and
135 bioluminescence imaging (BLI).

136 *Wound healing procedure*

137 The wounding procedure was adapted from that previously described by Galiano et al. and
138 Dunn et al. (24, 25). Male NOD/SCID mice were used at 8-10 weeks of age. The operative
139 region of the mouse's back was prepared by removing the fur with clippers and a light
140 depilatory cream, and two wound outlines were made, using a sterilised 5mm biopsy punch.

141 The skin in the middle of the outline was lifted using serrated forceps and full-thickness
142 wounds were cut and excised using iris scissors. Silicone splints (approx. 10mm diameter)
143 were used to prevent wound closure via contraction. An adhesive was applied sparingly to
144 one side of the splint and the splints were centred over the wounds. The splints were then
145 secured in place using interrupted 6-0 PROLENE™ sutures (8805H, Ethicon LLC, San
146 Lorenzo, Puerto Rico). After splinting, the mice were scanned with a laser Doppler (MOOR-
147 LMD V192, Moor Instruments, UK) for wound perfusion measurement. They were placed on
148 a heat mat in the prone position and the wound area was scanned three times per mouse per
149 timepoint. Doppler scans were performed on alternate days post-wounding, up to and
150 including day 14. Subsequent to the initial Doppler scan, cell treatments (5×10^5 iPSC-ECs
151 suspended in vehicle containing a 1:1 ratio of endothelial basal media to growth factor
152 reduced Matrigel) were injected into the wounds and the wounds were covered with adhesive
153 Opsite™ dressings (66000041, Smith & Nephew, London, UK). All wound healing
154 experiments and associated procedures were conducted in accordance with National Health
155 and Medical Research (NHMRC) guidelines for the care and use of animals for scientific
156 purposes and were approved by the Sydney Local Health District Animal Welfare
157 Committee, Protocol #2014-004A.

158

159 *Bioluminescent imaging (BLI)*

160 BLI was used for longitudinal tracking of iPSC-EC survival *in vivo* and was performed with
161 an IVIS Lumina XRMS and Living Image software (Version 4.5, Perkin Elmer, Waltham,
162 MA 02451, USA). The mice were anaesthetised with 2% isoflurane and D-luciferin (100 μ L,
163 375mg/kg) was administered by subcutaneous injection at a medial position immediately
164 inferior to the wound sites. Bioluminescence intensity was calculated as the maximum mean

165 radiance (photons/second/cm²/steradian) recorded in pre-defined regions of interest centred
166 over the wounds. BLI was performed on alternate days post-wounding, up to and including
167 day 14.

168 *Histological analysis of explanted wounds*

169 Wound explants were fixed in 4% paraformaldehyde for up to 4 hours at room temperature
170 then changed to 70% ethanol for at least 24 hours. Paraffin infiltration was performed
171 overnight by an automated tissue processor (Leica TP1020, Leica Biosystems Nussloch
172 GmbH, Heidelberger Straße 17-19 69226 Nussloch, Germany). The infiltrated samples were
173 then embedded in paraffin blocks for sectioning. Tissue samples were cut into 5 µm thick
174 transverse sections using a rotary microtome, deparaffinised, and stained. Milligan's
175 Trichrome stain was performed to visualise collagen content. For immunohistochemistry
176 analysis, paraffin sections were stained using immunohistochemistry techniques with primary
177 antibodies anti-CD31 (Abcam, USA) for endothelial cells and anti-CD68 (Abcam, USA) for
178 macrophages. Fluorescence microscopy was then used to visualise cell markers using Alexa
179 Fluor 594 conjugated secondary antibodies. Anti-GFP staining was done using
180 cryosectioning. Briefly, tissue was fixed in 4% paraformaldehyde for up to 4 h at room
181 temperature then changed to 30% sucrose for at least 24 h. Tissue was then fixed in optimum
182 cutting temperature (OCT) compound and kept at -80°C until cryosectioning. Samples were
183 sectioned into 40 µm thick transverse sections and dropped into PBS in order for the residual
184 O.C.T. to dissolve. Sections were then stained using standard free-floating techniques with a
185 FITC-conjugated anti-GFP antibody (GeneTex). Both paraffin and cyrosections were then
186 mounted and coverslipped with DAPI-containing mounting media (Vectashield, Vector
187 Laboratories Inc., California, USA).

188

189 *Analysis of host angiogenic gene expression in wounds*

190 Total RNA was isolated from explanted control and iPSC-EC treated wounds with Trizol
191 reagent. Real-time quantitative polymerase chain reaction analysis was performed to evaluate
192 the expression of murine vascular endothelial cadherin (VE-cadherin; sense ‘5’-
193 TCCTCTGCATCCTCACTATCACA-‘3’, antisense: ‘5’-
194 GTAAGTGACCAACTGCTCGTGAAT-‘3’) , vascular endothelial growth factor receptor-1
195 (Flt-1; sense: ‘5’-GAGGAGGATGAGGGTGTCTATAGGT-‘3’, antisense: ‘5’-
196 GTGATCAGCTCCAGGTTTGACTT-‘3’), vascular endothelial growth factor receptor-2
197 (KDR; sense ‘5’- GCCCTGCTGTGGTCTCACTAC-‘3’, antisense: ‘5’-
198 CAAAGCATTGCCATTCGAT-‘3’), platelet endothelial cell adhesion molecule (PECAM-
199 1; sense: ‘5’-GAGCCCAATCACGTTTCAGTTT-‘3’, antisense: ‘5’-
200 TCCTTCCTGCTTCTTGCTAGCT-‘3’), tyrosine kinase with immunoglobulin-like and
201 EGF-like domains-1 (Tie-1; sense: ‘5’- CAAGGTCACACACACGGTGAA-‘3’, antisense:
202 ‘5’- GCCAGTCTAGGGTATTGAAGTAGGA-‘3’), and vascular endothelial growth factor
203 (VEGF; sense: ‘5’- TGCCAAGTGGTCCCAG-‘3’, antisense: ‘5’-
204 GTGAGGTCTTGATCCG-‘3’). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH;
205 sense: ‘5’- GGGGCTCTCTGCTCCTCCCTGT-‘3’, antisense: ‘5’-
206 CGGCCAAATCCGTTACACCGA-‘3’) was used as a loading control. Relative gene
207 expression was calculated as fold change compared to paired control wounds.

208

209 *Quantitative analysis of bioluminescence and histological images*

210 Quantification of bioluminescence images was performed using LivingImage 4.5 (Perkin
211 Elmer) software. Histological and immunohistochemical sections were imaged using a Zeiss
212 Upright Olympus fluorescence multi-channel microscope, captured with a Nikon DP

213 Controller 2.2 (Olympus, Japan). Immunohistochemical and histopathological analysis was
214 done using ImageJ. Briefly, regions of interest (ROIs) were drawn around the wound site. For
215 endothelial cells and fibroblasts, positive staining was quantified as individual particles
216 counted based on a common threshold intensity. All cell types were quantified from an n=6
217 sections per group per time point.

218

219 *Statistics*

220 Data are expressed as mean \pm SEM and indicated in figures as * $p < 0.05$, ** $p < 0.01$. The data
221 were compared using paired student t-tests followed by Bonferoni's post-hoc test using
222 GraphPad Prism version 5.00 (Graphpad Software, San Diego, California) for PC. iPSC-EC
223 experimental samples were paired to control wounds within the same mouse.

224

225 Results

226 *Engraftment of iPSC-ECs within wounds decreases following transplantation*

227 Bioluminescence imaging was used to quantify the levels of iPSC-EC engraftment within
228 wounds over time. Transfection of iPSC-ECs with a dual-report construct containing both the
229 enhanced green fluorescent protein (eGFP) and the firefly luciferase enzyme enabled
230 visualization and quantification of transplanted iPSC-ECs. Intramuscular injection of D-
231 luciferin substrate catalyzed a bioluminescent reaction through the firefly luciferase enzyme
232 within viable iPSC-ECs that could be visualized and quantified using the IVIS apparatus
233 (Figure 1A). Bioluminescence quantification over 14 days showed that iPSC-EC presence in
234 wounds decreases steadily over time (Day 0 $1.39 \times 10^7 \pm 5.61 \times 10^6$ vs. Day 14 $4.04 \times 10^4 \pm$
235 1.53×10^4 photons/cm²/s/steradian). The largest decrease in bioluminescence occurred

236 between days 2-4 (Day 2 $8.36 \times 10^6 \pm 5.44 \times 10^6$ vs. Day 4 $7.66 \times 10^5 \pm 3.65 \times 10^5$
237 photons/cm²/s/steradian, Figure 1B). Immunohistochemistry staining of the eGFP marker
238 identified iPSC-ECs engrafted within the wound site at day 7, and a smaller proportion
239 remaining at 14 days post-transplantation (Figure 1C).

240

241 *Engraftment of iPSC-ECs correlates with upregulation of host angiogenic gene expression*

242 Functional angiogenesis is a complex process regulated by numerous cells types and growth
243 factors. Ischemic/injured tissues undergoing revascularization processes are often identified
244 by the upregulation of key angiogenic genes including VE-cadherin, VEGF, Flt-1, KDR,
245 PECAM, and Tie-1. Wounds were explanted at days 7 and 14 for qPCR analysis to determine
246 levels of these classical angiogenic genes. Increasing trends were observed in the expression
247 of VE-Cadherin, Flt-1, and KDR in iPSC-EC treated wounds when compared to control
248 wounds at both time points, however no statistical significance was obtained. At day 7,
249 PECAM and Tie-1 genes were found to be significantly upregulated by $350 \pm 89\%$ and $534 \pm$
250 134% in iPSC-EC treated wounds compared to control wounds (Figure 2). At day 14, VEGF
251 expression was significantly upregulated by $12 \pm 4\%$ in iPSC-EC treated wounds compared
252 to controls (Figure 2).

253

254 *Wounds treated with iPSC-ECs exhibit increased angiogenesis*

255 The anatomical hallmarks of angiogenesis include increased blood perfusion and high density
256 of neo-capillary formation. Non-invasive infrared laser Doppler imaging was conducted at
257 the wound sites over 14 days to visualize and quantify levels of blood perfusion for the
258 duration of wound healing (Figure 3A). Laser Doppler analysis revealed a maximum of 2-
259 fold increase in wound perfusion compared to control within the first 4 days following

260 treatment in iPSC-EC treated wounds compared to control (Figure 3B). This gradually
261 decreased back to baseline control wound perfusion levels by day 10. The subcutaneous
262 layers of iPSC-EC treated, and control wounds were photographed following explant. A
263 higher density of observable blood vessels was observed in iPSC-EC treated wounds
264 compared to control (Figure 3C). To assess neo-capillary formation, cross-sections of wound
265 explants were stained using the CD31 endothelial cell marker. At day 7, iPSC-EC treated
266 wounds showed a 16-fold increase in capillary density compared to control wounds, which
267 decreased to a 4-fold increase by day 14 (Figure 3D).

268

269 *iPSC-EC treatment enhances the native wound healing process*

270 Native wound healing processes involve numerous cell phenotypes responsible for rebuilding
271 the tissue architecture necessary for cell repopulation. Of these cell types, fibroblasts are
272 largely implicated as essential matrix remodelling cells which facilitate neo-collagen
273 deposition as a temporary matrix for cell growth and tissue regeneration. Using trichrome
274 staining, wound cross-sections were quantified for levels of collagen deposition. iPSC-EC
275 treated wounds showed a $20 \pm 4\%$ increase in collagen deposition at day 14 compared to
276 control wounds (Figure 4B). Similar trends were observed with macrophage infiltration. At
277 day 14, iPSC-EC treated wounds showed a $174 \pm 6\%$ increase in macrophage numbers
278 compared to control wounds (Figure 5). The functional outcomes of wound healing were
279 measured by taking three diameter measurements of wounds over 14 days and represented as
280 percentage of total wound closure. iPSC-EC treated wounds show accelerated wound closure
281 from day 2, which was significant on days 4 and 10 post-wounding and achieved complete
282 wound closure 4 days earlier than control wounds (Figure 4A).

283

284 Discussion

285 Chronic wounds and ulcers persist in a state of pathological inflammation, characterised by
286 increased proteolytic activity, increased production of reactive oxygen species (ROS),
287 senescent or dysfunctional fibroblasts, neutrophils and macrophages, and reduced pro-
288 angiogenic growth factors and cytokines (4, 7). The aetiology of chronic wound formation in
289 diabetes and peripheral arterial disease is multifactorial, but a major cause is underlying
290 vasculopathy and diminished angiogenesis. The angiogenic response to ischaemia declines
291 naturally with age and is further impaired in patients with vascular disease, who have reduced
292 number and functionality of circulating EPCs (26-30). The importance of angiogenesis in
293 wound healing has been highlighted by interventional studies, which have shown that topical
294 application of pro-angiogenic factors, such as VEGF or bFGF, is beneficial in accelerating
295 diabetic wound healing in mice, while neutralising them has the opposite effect (31-33).
296 However, clinical trials using single growth factors to boost angiogenesis have been largely
297 unsuccessful, which likely reflects the complex regulation of angiogenesis *in vivo* and the
298 need for a multifactorial approach (34).

299 Bone marrow and adipose tissue derived MSCs and EPCs have been in development for
300 therapeutic angiogenesis for the past two decades, and have shown promise in promoting
301 wound healing , however limited availability of healthy adult stem cells, and invasive
302 aspiration procedures have tempered their use. Induced pluripotent stem cells and their
303 derivatives have several advantages, such as their relatively abundant supply, their non-
304 controversial origins and the lack of immunogenicity if used for autologous transplantation
305 (35). Furthermore, the development of iPSC banking, the creation of multiple iPSC lines
306 representing commonly present HLA allele combinations, means that patients could receive
307 non-autologous cells without evoking an immune rejection, increasing the throughput while
308 simultaneously reducing the time, cost and invasiveness of pluripotent cell therapies (36).

310 Here, we demonstrate the pro-angiogenic and wound healing capabilities of iPSC-ECs in a
311 murine excisional wound model. Angiogenesis is vital for the formation and maintenance of
312 granulation tissue in the first 2-4 days post wounding. Neovessel formation typically begins
313 around this time, peaking around day 7 before giving way to remodelling and maturation of
314 the newly formed vasculature. We observed significantly accelerated wound closure in iPSC-
315 EC treated wounds, as well as increased wound perfusion in the first week of healing, which
316 was associated with a significant increase in endothelial cell surface marker PECAM-1
317 (CD31) staining in the wounds at both 7 and 14 days post-wounding. Consistent with
318 previously described temporal patterns of angiogenesis in wound healing, absolute laser
319 Doppler perfusion measurements decreased in both groups during the second week,
320 approaching the those of the surrounding healthy skin, as new dermis progressively covered
321 exposed capillaries at the wound surface. Expression of PECAM-1 and Tie-1 mRNA was
322 significantly upregulated in iPSC-EC wounds on day 7 but was similar to expression in
323 control wounds by day 14. Tie 1 is an endothelial cell specific orphan receptor, which has
324 been shown to promote sprouting angiogenesis via regulation of Tie 2 receptor signalling
325 (37).

326 iPSC-EC treatment also increased wound collagen deposition; sections from wounds treated
327 with iPSC-ECs had significantly higher collagen content than the control wounds on day 14.
328 Collagen deposition is vital for healthy wound healing, but the implication of increased
329 collagen depends on the stage of healing as well as the types of collagen present; in the
330 proliferative stage of healing collagen provides a matrix to support inflammatory and
331 vascular cells as well as forming a stronger barrier than the fibrin clot (38). In the late stages
332 (remodelling), a reduction in collagen content is desirable to limit scarring. Collagen
333 deposition begins within the first 24 hours after wounding and peaks between 1-3 weeks

334 later, depending on the wound site and size. As these wounds were still actively healing and
335 in the proliferative phase at day 14, the observation of increased collagen is most likely
336 indicative of a more advanced stage of healing compared to the controls, rather than
337 excessive scarring. We also observed increased macrophage infiltration in iPSC-EC treated
338 wounds at day 14 post-wounding, as evidenced by significantly increased CD68+ staining. In
339 normal wound healing, macrophage infiltration peaks during the inflammatory phase (day 1-3
340 post-wounding) before subsiding. We observed no difference in CD68+ staining between
341 control and iPSC-EC treated wounds at 7 days post-wounding. However, by day 14 iPSC-EC
342 treated wounds had significantly greater CD68+ staining compared to their respective
343 controls, suggesting sustained macrophage activity. This observation may be due to increased
344 recruitment of macrophages in response to iPSC-ECs.

345 Poor cell survival and engraftment remains a major limitation to the long-term efficacy of cell
346 therapies and therefore their suitability for clinical use. Most cells are lost within the first 48h
347 after administration, due to poor retention and cell death due to excessive inflammation,
348 ischaemia or anoikis, the programmed death that occurs in endothelial cells and other
349 anchorage-dependent cell types when they lose their interaction with an extracellular matrix
350 (39). Very few previous wound healing studies included longitudinal tracking of the
351 implanted cells to determine their eventual fate and those that did reported low rates of
352 engraftment (40, 41). We used a firefly luciferase reporter gene construct and BLI for
353 longitudinal tracking of iPSC-EC survival *in vivo* and although iPSC-EC fluorescent and
354 bioluminescent signal was still detectable in the wounds 14 days post-treatment, we observed
355 a substantial and progressive decline over the two-week period. This data is consistent with
356 previous *in vivo* survival of iPSC-ECs and suggests that the pro-angiogenic and pro-healing
357 effects of our cells were primarily mediated via secretion of paracrine factors rather than
358 engraftment or proliferation of the cells themselves (42). Despite low rates of engraftment,

359 stem cell treatments consistently evoke a beneficial response. This suggests that even small
360 increases in retention could translate to sizeable improvements in tissue regeneration and
361 long-term recovery and we are conducting further studies seeding iPSC-ECs on biomaterial
362 scaffolds to determine if this can increase their *in vivo* longevity and potentiate their
363 beneficial effects.

364 *Limitations*

365 NOD/SCID mice are widely used in human haematopoietic cell studies and are an ideal strain
366 for human cell transplantation because they lack functional B and T lymphocytes, lack the
367 ability to mount an antibody mediated response, and additional deficiencies in their innate
368 immune system allow for increased donor cell engraftment (43). However, the blunted
369 immune response to the cells creates a somewhat artificial environment in comparison to the
370 situation in an immune competent organism. That being said, it is not possible to conduct pre-
371 clinical, mechanistic investigations using human cells in other species without modulating the
372 immune response. It is anticipated that the use of autologous or donor matched cells clinically
373 would also mitigate the effects of immune rejection of the engrafted cells by the recipient,
374 therefore we considered the NOD/SCID mouse to be the most suitable and appropriate model
375 for these studies. It should also be noted that these mice are neither aged nor diabetic, so most
376 innate wound healing capabilities are intact. This means that treatment effects are more
377 difficult to detect, thus we anticipate an even more pronounced improvement after iPSC-EC
378 treatment if we were able to use these cells in such a model.

379 Finally, we acknowledge that the iPSC-ECs used in this study were differentiated from iPSCs
380 that were generated using integrating viral vectors. These cells may not be suitable for use in
381 a clinical setting because of the possibility of foreign DNA integrating into the host genome.

382 However, the development of reprogramming protocols using small molecules means that
383 iPSC-ECs for clinical applications can be generated without the use of viral vectors.

384 *Conclusions*

385 In this study, we have demonstrated for the first time that human iPSC-ECs have pro-
386 angiogenic functionality in wound healing, promote fibroblast infiltration and collagen
387 deposition, and accelerate wound closure. Longitudinal tracking of iPSC-ECs *in vivo*
388 revealed a progressive decline in surviving iPSC-ECs over time, indicating that the cells are
389 acting primarily through short-term paracrine effects. These findings have provoked further
390 studies optimising iPSC-EC delivery to improve *in vivo* survival and engraftment rates, and
391 also make a strong case for the development of clinical grade induced pluripotent stem cells
392 and their derivatives for therapeutic angiogenesis in patients with vascular diseases.

393 Funding Sources

394 SP was supported by a National Health and Medical Research Council (NHMRC) Early
395 Career Fellowship (grant number GNT0633283). The study was funded by the Sydney
396 Medical School Foundation.

397

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520 **Figure titles and legends**

521 Figure 1. iPSC-EC engraftment in wounds. iPSC-ECs were transduced with a double fusion
522 reporter construct encoding GFP for fluorescence imaging and firefly luciferase for
523 bioluminescent imaging. A. Representative IVIS images, showing bioluminescent signal
524 present in iPSC-EC-treated wounds (right) on days 0, 6 and 14 post-wounding. B. Declining
525 bioluminescent signal in wounds over time, relative to control wound background signal. C.
526 GFP staining (green) with DAPI nuclear stain (blue) in control and iPSC-EC treated wounds
527 at on days 7 and 14 post-wounding (n=6 per treatment group).

528 Figure 2. iPSC-EC treatment increases expression of pro-angiogenic genes. QPCR analysis
529 of wound mRNA revealed a significant upregulation of PECAM (CD31) and endothelial cell
530 receptor, Tie-1, expression in iPSC-EC wounds on day 7 post wounding. This resolved by
531 day 14. No significant differences in endothelial cell surface marker VE-Cadherin (CD144),
532 VEGF, VEGF receptor 1 (Flt1) or VEGF receptor 2 (KDR) expression were measured.
533 (* $p < 0.05$ compared to controls, $n = 6$ per treatment group)

534 Figure 3. iPSC-EC treatment increases vascular density and wound perfusion. A.
535 Representative laser Doppler images, showing perfusion in control and iPSC-EC treated
536 wounds on days 0, 4, 8 and 18 post-wounding. B. Wound perfusion in iPSC-EC treated
537 wounds, relative to their respective control wounds. Increased perfusion in iPSC-EC treated
538 wounds was most pronounced during the first week of healing. C. Representative
539 photomicrographs of wounds, showing increased neovessel formation in iPSC-EC wounds
540 relative to controls. D. Capillary density, as measured by CD31+ staining, was significantly
541 increased in iPSC-EC wounds at both early and late timepoints. (* $p < 0.05$, $n = 6$ per treatment
542 group)

543 Figure 4. iPSC-EC treatment increases collagen deposition and accelerates wound closure. A.
544 Representative photomicrographs, showing progressive wound closure over a 14 day period.
545 Rate of closure was significantly increased in iPSC-EC treated wounds compared to controls.
546 B. Milligan's Trichrome staining for collagen content. No differences were observed on day
547 7; by day 14 iPSC-EC treated wounds had significantly higher collagen content. ($p < 0.05$, $n = 6$
548 per treatment group)

549 Figure 5. iPSC-EC treatment increases macrophage infiltration A. Anti-CD68 staining for
550 macrophage infiltration. No differences were observed on day 7; by day 14 iPSC-EC treated
551 wounds had significantly macrophage infiltration. ($p < 0.05$, $n = 6$ per treatment group)

552 Supplementary figure 1. Generation of induced pluripotent stem (iPSCs) and induced
553 pluripotent stem cell derived endothelial cells (iPSC-ECs) from dermal fibroblasts was
554 achieved by retroviral overexpression of Oct4, Sox2, Klf4 and cMyc, followed by culture in
555 endothelial lineage specific growth factors.

556

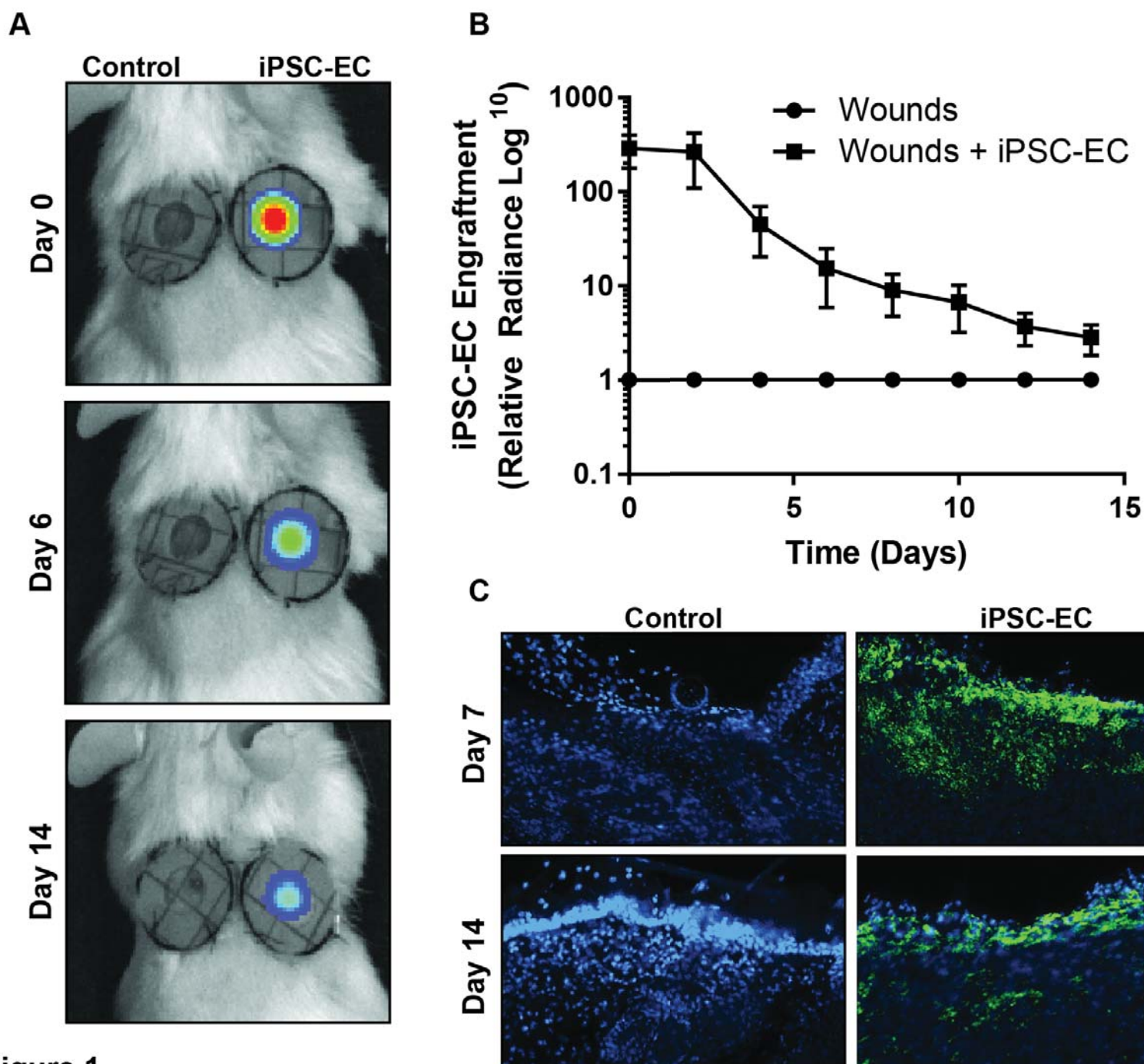


Figure 1

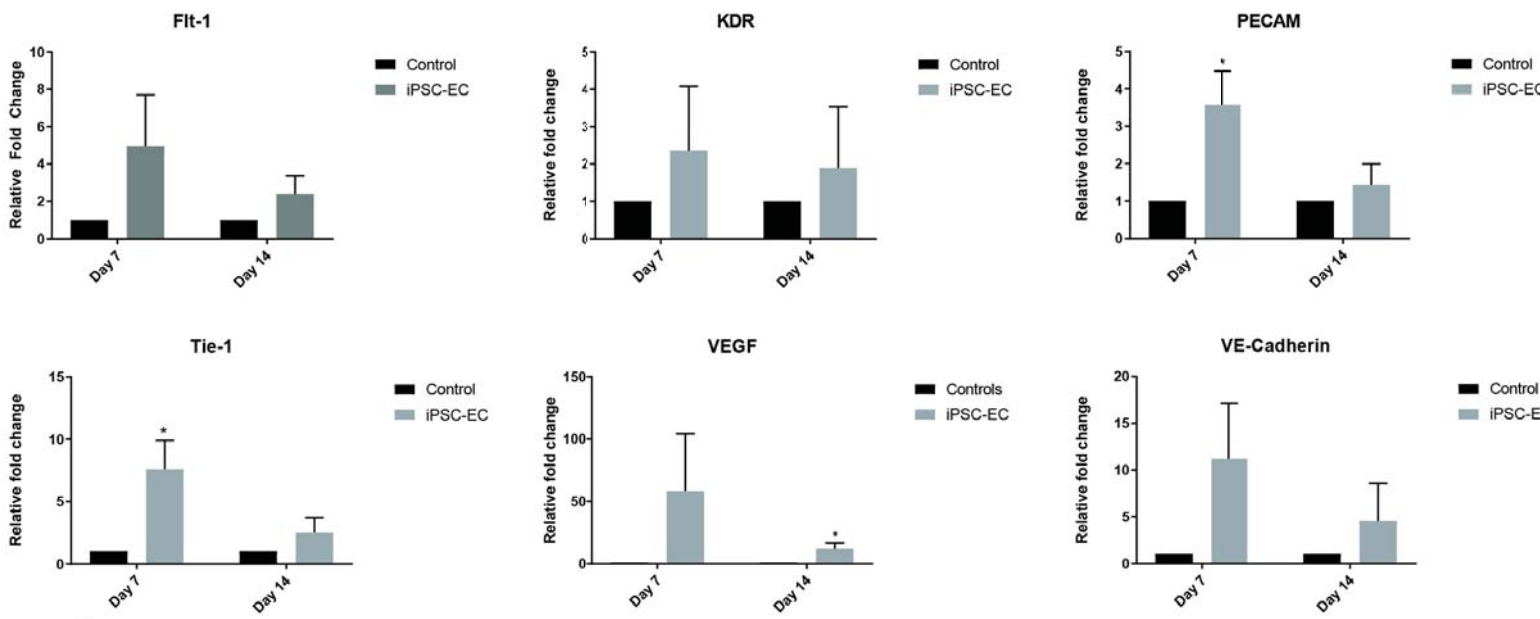


Figure 2

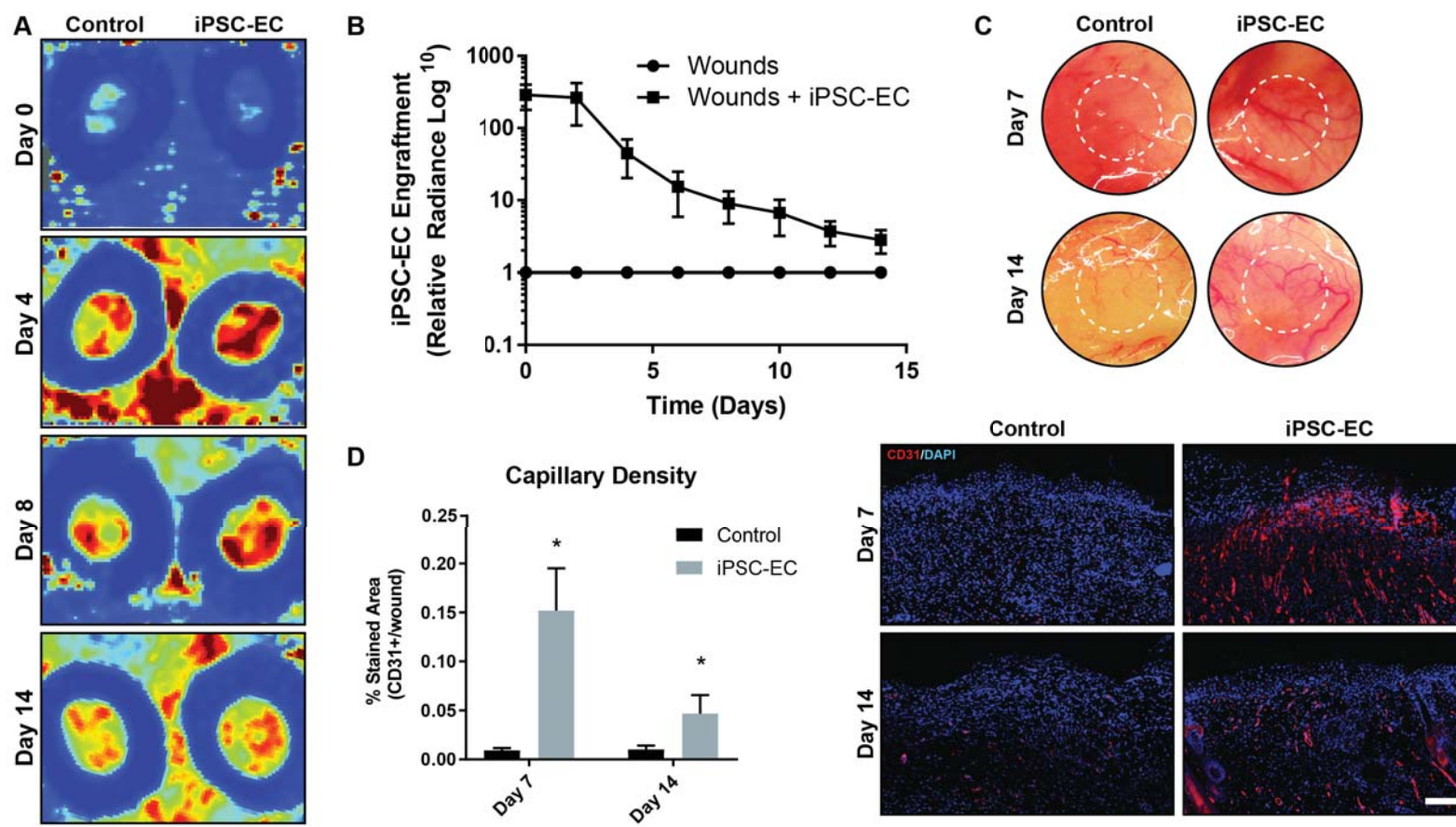


Figure 3

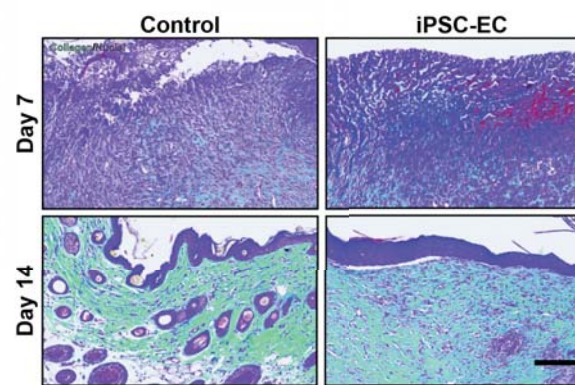
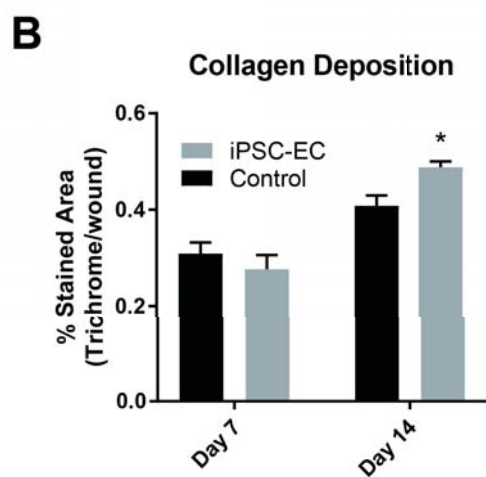
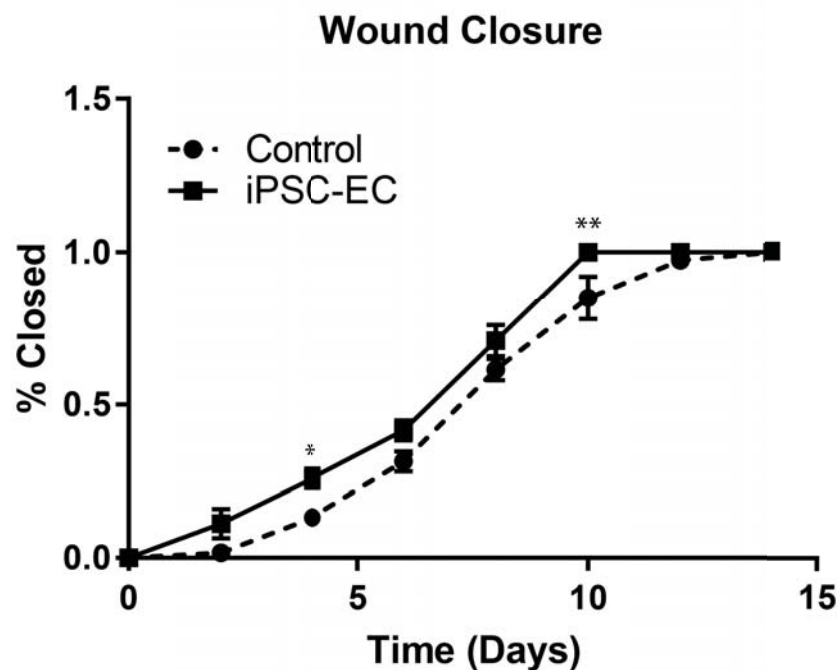
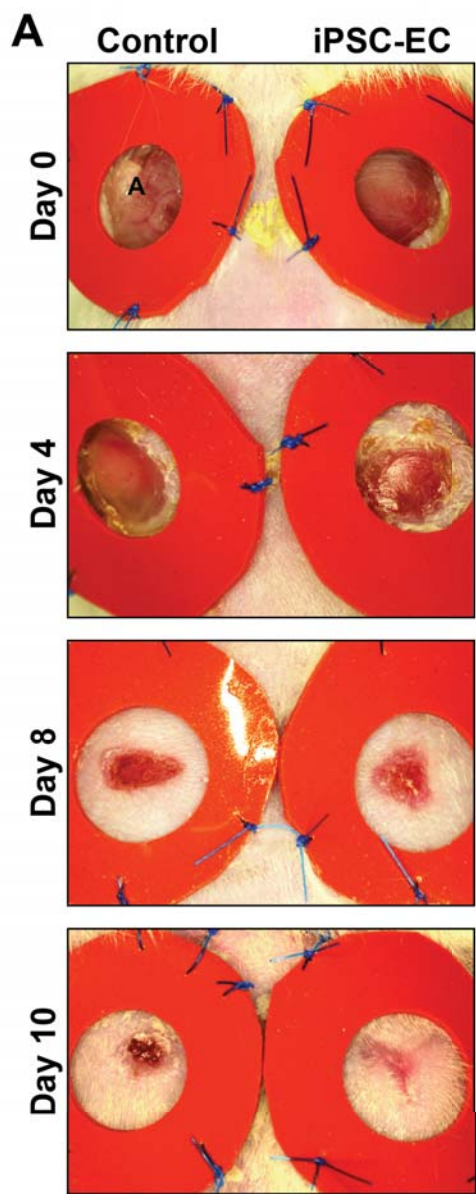


Figure 4

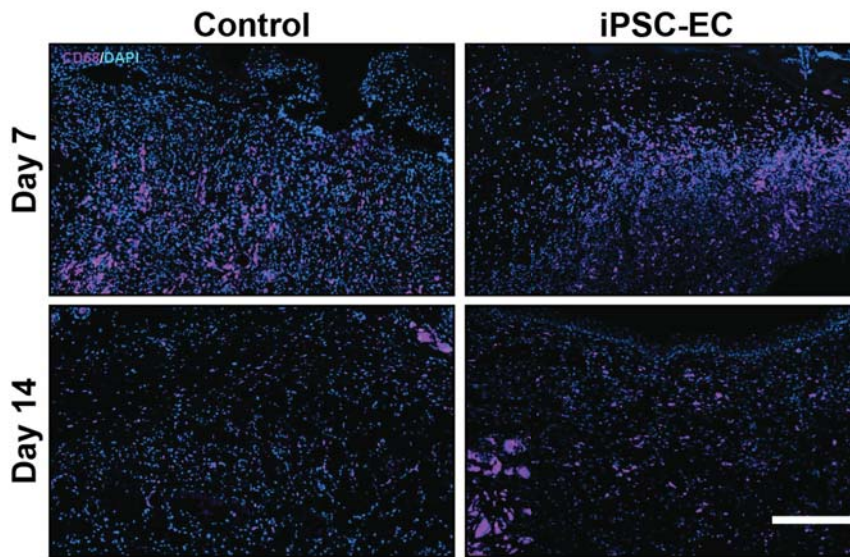
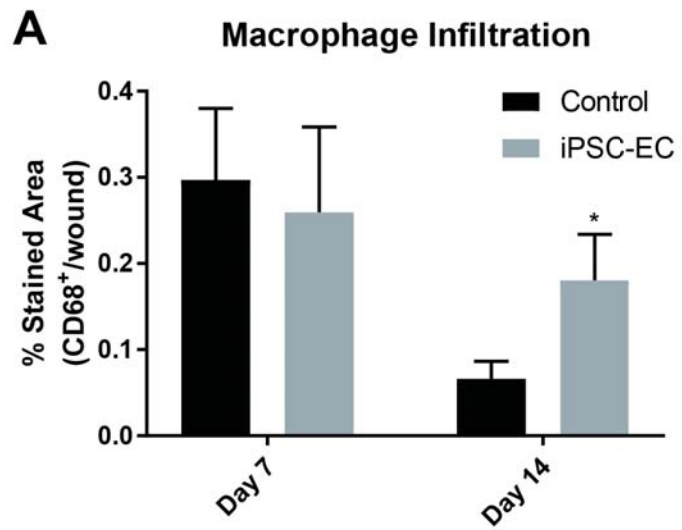
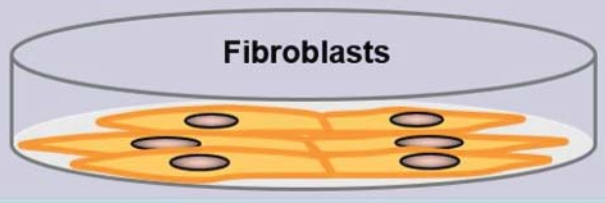


Figure 5

Dermal fibroblasts

Induced Pluripotency



Reprogramming (14 days)

INTEGRATING VECTORS

- Retrovirus (OKSM)



Induced pluripotent stem cells (iPSCs)



Differentiation (14 days)

Differentiation media:

- VEGF
- bFGF
- BMP4

Endothelial growth medium

Lentivirus LV-pUb-Fluc-GFP



Endothelial cells

