1 **A Single Trophoblast Layer Acts as the Gatekeeper at the Endothelial-Hematopoietic**

2 **Crossroad in the Placenta**

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Abstract

During embryonic development the placental vasculature acts as a major hematopoietic niche, where endothelial to hematopoietic transition ensures emergence of hematopoietic stem cells (HSCs). However, the molecular mechanisms that regulate the placental hematoendothelial niche are poorly understood. Using a parietal trophoblast giant cell (TGC)-specific knockout mouse model and single-cell RNA-sequencing, we show that the paracrine factors secreted by the TGCs are critical in the development of this niche. Disruptions in the TGC-specific paracrine signaling leads to the loss of HSC population and the concomitant expansion of a KDR+/DLL4+/PROM1+ hematoendothelial cell-population in the placenta. Combining single-cell transcriptomics and receptor-ligand pair analyses, we also define the parietal TGC-dependent paracrine signaling network and identify Integrin signaling as a fundamental regulator of this process. Our study elucidates novel mechanisms by which non-autonomous signaling from the primary parietal TGCs maintain the delicate placental hematopoietic-angiogenic balance and ensures embryonic and extraembryonic development.

Introduction

Trophoblast cells of the placenta establish a vascular connection between the mother and the fetus and express hormones that are essential for the successful progression of pregnancy. Placenta also acts as one of the major organs for hematopoietic stem cell generation, and the mid-gestation mouse placenta plays a significant role in the HSC development where it provides a temporary niche for definitive HSC pool [1-3]. Defective development of placental hematopoiesis and vasculogenesis leads to serious pathological conditions such as preeclampsia and intra uterine growth restriction (IUGR)/ fetal growth restriction (FGR). These disorders result in pregnancy-related complications, maternal, prenatal and neonatal mortality, and affect ~2–8% of pregnant women worldwide [4]. The pathogenesis in preeclamptic patients is believed to be a response of vasculature to abnormal placentation [5]. Thus, to define

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45 therapeutic modalities against these pregnancy-associated disorders, it is crucial to

46 understand the molecular mechanisms that are associated with the proper development of

47 placental hematopoiesis and vasculogenesis.

48 Trophoblast cell differentiation in the placenta involves mechanisms by which secreted

49 paracrine factors within the placenta, and from the fetus and the mother regulate embryonic and

50 extraembryonic development [6]. Embryonic hematopoietic sites are characterized by the

51 interlinked developments of the vascular and hematopoietic systems. Several studies have

52 shown that the hemangioblasts and hemogenic endothelium act as the presumptive precursors

53 to emerging hematopoietic cells [7]. It has been indicated that the definitive hematopoiesis is

54 autonomously initiated in the placenta, which subsequently generates HSCs from hemogenic

55 endothelium and provides a niche for expansion of aorta-derived HSCs [3,8]. Moreover, secreted

56 pro-angiogenic factor, like Placental Growth Factor (PLGF), a member of the Vascular

57 Endothelial Growth Factor (VEGF) family, and anti-angiogenic factors, like soluble Vascular

58 Endothelial Growth Factor receptor-1 (sFLT1) and Endoglin (ENG), are expressed in the mouse

59 placenta, implying autocrine or paracrine actions [9,10]. How these signaling mechanisms

60 influence the development of the maternal-fetal interface are poorly understood.

61 Several studies have implicated GATA family of transcription factors in the development of

62 HSCs in other organs, and previously we have shown that GATA2 and GATA3 are involved in

63 the trophoblast development and differentiation [11-15]. They are implicated in the regulation of the

64 expression of several trophoblast-specific genes, including prolactin hormone Placental

65 lactogen I (*Prl3d1*, also known as *Pl1*) and the pro-angiogenic factor Proliferin (*Prl2c2*, also

66 known as *Plf*) [16-18]. Recently we demonstrated that placenta-specific redundant functions

67 of *Gata2* and *Gata3* are important in trophoblast lineage development [19]. The simultaneous

68 knockout of *Gata2* and *Gata3* resulted in significant developmental defects in the placenta as

69 well as the embryo proper, leading to very early embryonic lethality [19]. These developmental

70 defects were accompanied by severe blood loss in the placenta, yolk sac, and the embryo

71 proper. Moreover, we established dual conditional *Gata2* and *Gata3* knockout trophoblast stem

72 cells and used ChIP-Seq and RNA-Seq analyses to define independent and shared global

73 targets of GATA2 and GATA3. We found that several pathways associated with the embryonic

74 hematopoiesis and angiogenesis are targets for both transcription factors[19]. However, very little

75 is known about how these two master regulators operate in a spatiotemporal manner within the

76 mature trophoblast during placental development and dictate placental hematopoiesis and

77 angiogenesis.

78

79 In a mouse conceptus, parietal trophoblast giant cells (parietal TGCs), which line the border of

80 the growing placenta and the maternal decidua, is thought to be a major source of endocrine

81 and paracrine signals during mouse placentation [20,21]. Parietal TGCs are initially developed from

82 the trophoectoderm (primary parietal TGCs) and subsequently from the secondary

83 differentiation of precursors in the ectoplacental cone (secondary parietal TGCs). Gene

84 expression analyses reveled that both the primary and secondary parietal-TGCs specifically

85 express prolactin 3d1 gene (*Prl3d1*), also known as placental lactogen 1 (PL-1). Therefore, to

86 understand how GATA2/GATA3 functions could dictate endocrine and paracrine functions in a

87 developing placenta, we specifically deleted both *Gata2* and *Gata3* in the parietal TGC using

88 *Prl3d1tm1(cre)Gle* (*Pl1-Cre*) mouse line [20,22,23] that drive Cre expression specifically within the

89 parietal TGCs. We noticed in-utero death of *Gata2/3* conditional double knockout

90 *Gata2$^{f/f}$;Gata3$^{f/f}$;Pl1$^{Cre/wt}$* (GATA-Pl1 KO) embryos starting from embryonic day 12.5 (e12.5). A

91 fraction of the GATA-Pl1 KO embryos survived to birth. However, majority of the surviving

92 GATA-Pl1 KO pups showed severe growth restriction. Remarkably, we noticed that along with

93 defect in trophoblast development, the delicate balance between hematopoietic vs angiogenic

94 differentiation is lost in the developing GATA-Pl1 KO placentas. The GATA-Pl1 KO placentas

95 showed severe defect in hematopoiesis due to reduced hematopoitic stem and progenitor cells

96 and disorganized placental vasculature due to defective differentiation of endothelial

4

97 progenitors. Thus, our study highlight the importance of a GATA-mediated transcriptional

98 program within a single trophoblast subtypes that fine-tunes the hematopoietic and endothelial

99 development during placentation.

100

101 **Results**

102 **GATA deletion in the trophoblast giant cell layer in mouse embryos display embryonic**

103 **and extraembryonic hematopoietic defects**

104 Our previous studies have shown that the loss of GATA factors in the trophoblast lineage cells

105 results in the gross phenotypic abnormality in the placenta and the embryo proper in a mouse

106 model [19]. The placenta contains distinct layers of differentiated trophoblast cells, each having

107 specialized functions to support a pregnancy. These include TGCs, spongiotrophoblasts (SpT),

108 glycogen trophoblasts, and labyrinthine trophoblasts. Each subclass is characterized by their

109 unique gene expression signatures [24]. Thus, it is imperative to analyze how GATA factors act in

110 different subclasses of trophoblast cells in the context of placental development and function. As

111 TGCs have been reported to express both GATA2 and GATA3, we chose to delete *Gata2* and

112 *Gata3* together in the TGCs [16]. Placental TGCs are marked by the expression of three prolactin

113 family of proteins Prl2c2 (PLF), Prl3b1 (PL2), Prl3d1 (PL1) and Cathepsin Q (CTSQ)[20]. Out of

114 these four genes, PL1 expression is restricted to the parietal TGCs only . Thus, to restrict GATA

115 deletion exclusively to the parietal TGCs, we used a previously established *Prl3d1^{tm1(cre)Gle}*

116 (*Pl1^{Cre}*) mouse model [22] , where Cre recombinase is selectively expressed in the *Pl1* expressing

117 cells (**Fig. S1A**). We validated the *Pl1*-specific Cre expression in this mouse model by LacZ

118 staining (**Fig. S1B).** We also developed a murine model in which the parietal trophoblast giant

119 cell layers were fluorescently labeled. We used *Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}/J*, (also

120 known as *mT/mG*) mice, which possess loxP flanked membrane-targeted tdTomato (mT)

121 cassette and express strong red fluorescence in all tissues [25]. Upon breeding with the *Pl1*-Cre

122 recombinase expressing mice, the resulting offsprings have the mT cassette deleted in the Cre

5

123   expressing cells(s), allowing expression of the membrane-targeted EGFP (mG) cassette located

124   in-frame immediately downstream. Microscopic analyses of the conceptuses and their

125   cryosections from a cross between *Pl1^Cre* male and *mT/mG* female confirmed the selective

126   EGFP fluorescence only in the parietal TGC layers (**Fig. S1C**, **Fig. 1A, B**). This expression of

127   *Pl1-Cre* is consistant with the recent publication which clearly illuminates the PL1 protein

128   expression exclusively in the P-TGCs [23].

129   Using *Gata2^f/f*;*Gata3^f/f* mouse model reported in an earlier study from our laboratory, we

130   established a conditional GATA knockout mouse model *Gata2^f/f*;*Gata3^f/f*;*Pl1^Cre/wt* (GATA-Pl1 KO)

131   [19]. Although a significant number of GATA-Pl1 KO embryos showed lethality and abnormal

132   embryonic and extraembryonic phenotypes, a small percentage of them were viable. However,

133   almost all of them showed visible growth restriction at birth (**Fig. S2**). These viable growth

134   restricted pups were fertile and were chosen to function as breeders. For the embryo analyses,

135   we used these males for mating with non-Cre females to restrict the effect of GATA deletion

136   exclusively to the placental tissue. These mice were used to selectively knockout both *Gata2*

137   and *Gata3* in the parietal trophoblast giant cells. PCR with *Gata2* and *Gata3* deletion specific

138   primers confirmed the gene deletions specifically in the placenta in the GATA-Pl1 KO and not in

139   the embryos (**Fig. S3A**). In addition, using laser captured microdissection (LCM) we excised

140   GFP-positive pTGCs from *Gata2^f/f*;*Gata3^f/f*;*Pl1^Cre/wt* *mT/mG* placental sections. Again, PCR with

141   *Gata2* and *Gata3* deletion specific primers confirmed efficient deletion of the *Gata2* and *Gata3*

142   in these cells compared to the cells excised from adjacent labyrinth zone (**Fig. S3B**). Thus, we

143   validated that *Gata2* and *Gata3* could be efficiently deleted in a highly pTGC-specific manner in

144   the *Gata2^f/f*;*Gata3^f/f*;*Pl1^Cre/wt* mouse model.

145   Previous studies have shown that the major expansion of the hematopoietic stem cell (HSC)

146   population in the mouse placenta takes place between E11.5 and E13.5 [1]. Thus

147   *Gata2^f/f*;*Gata3^f/f*; *Pl1^Cre /wt* males were crossed to *Gata2^f/f*;*Gata3^f/f* females and the embryos were

148   analyzed between E10.5 and E13.5 (**Fig. 1C**). Resulting phenotypic abnormalities were

149   observed mostly at E12.5 and E13.5. For all subsequent analyses, E12.5 and E13.5

150   conceptuses were chosen.

151   Two major groups of embryos with distinct phenotypic abnormalities were observed in the *Pl1^Cre*

152   positive embryos (**Fig. 1D, E**). One group showed very early embryonic death and was

153   associated with extremely small fetal (necrotic) and placental tissues (**Fig. 1E, F**). The other

154   group showed significant growth restriction, developmental defects, and blood loss in the

155   embryo proper, while their placentae showed significant anomalies in size and weight and were

156   associated with apparent blood loss (**Fig. 1E, G, H**). These placentae showed appernt loss of

157   vasculature (**Fig. 1G**). These defects were most prominent at E12.5 and E13.5 (**Fig. S2**). Non-

158   Cre embryos from the same littermates were treated as controls (**Fig. 1E, G**). Along with these

159   gross abnormalities, significant alterations in the placental architecture were observed in the

160   GATA-Pl1 KO samples. A marked increase in the junctional zone (marked by the

161   spongiotrophoblast/ glycogen trophoblast marker *Tpbpa*) was observed compared to the control

162   (**Fig. 1I-J**).

163   Interestingly, the fetal liver in the GATA-Pl1 KO embryos showed blood loss (**Fig. 1G**), which is

164   consistent with the hypotheses that along with the hematopoietic stem and progenitor cells

165   (HSPCs) from the yolk sac, the placental HSCs migrate to and seeds fetal liver for

166   hematopoiesis [26].

167   Thus, our study showed that the GATA factor-loss in the parietal TGCs of the placenta was

168   sufficient to significantly impair embryonic and extraembryonic growth and also result in blood

169   loss and vasculature defects.

170

171   **GATA factor functions in parietal trophoblast giant cells regulate trophoblast progenitor**

172   **developments**

173   Some of the critical tasks of TGCs are the secretion of autocrine and paracrine factors that are

174   involved in the trophoblast outgrowth and placental development process [27,28]. Genetic knockout

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175   models targeting TGC specific genes have been shown to affect lineage-specific trophoblast

176   differentiation and thereby results in abnormal development of the placenta [22,27,29,30]. As mid-

177   gestation mouse placenta contains numerous cell types, including several different trophoblast

178   subtypes, endothelial cells, hematopoietic cells (both HSCs, multi-lineage cells, and terminally

179   differentiated cells), stromal cells, it is challenging to analyze the effect of a trophoblast-specific

180   gene knockout on placental cell subpopulations. To analyze the effect of GATA deletion in the

181   TGCs, we performed Single-cell RNA-Sequencing (scRNA-seq) analyses of E13.5 placenta

182   from a pregnant *Gata2^{f/f}*;*Gata3^{f/f}* female crossed with *Gata2^{f/f}*;*Gata3^{f/f}; Pl1^{Cre/wt}* male. Two

183   individual GATA-Pl1 KO placentae and two individual control littermate placentae were used for

184   the sequencing. The genotypes were confirmed by PCR using tissue from the embryo

185   proper(genotype for GATA Pl1-KO sample 1 is shown in **Fig. S3A**). The knockout placentae

186   where the apparent growth defects and blood loss phenotypes were most severe were chosen

187   for the scRNA-seq analysis (**Fig. 1G**).

188   T-distributed Stochastic Neighbor Embedding (t-SNE) plot of the aggregated hierarchical

189   clustering of the four samples revealed 33 distinct clusters (**Fig. 2A**). The clustering of both the

190   control samples showed significant similarity to each other, while significant clustering

191   similarities were observed in both the knockout samples, indicating a high degree of homology

192   between the placentae of the same genotypes (**Fig. S4**). To categorize the clusters, we

193   identified the top upregulated genes from each cluster (**Dataset 1**). Alongwith that, we used

194   "single-cell Mouse Cell Atlas (scMCA) analysis" pipeline previously described in a single-cell

195   RNAseq study on murine embryonic and extraembryonic tissues at E14.5 [31]. We individually

196   fed digital gene expression (DGE) matrix for each cluster to scMCA which in turn used a

197   previously defined set of gene signatures [31] and predicted nature of that cluster with a

198   probability score. Alongwith that, it also listed top markers associated with that cluster. Finally,

199   we cross-matched the scMCA top markers from **Dataset 2** with the top markers we derived

200   earlier (**Dataset 1**). In this way we broadly identified five different cluster types in our samples.

201   They include trophoblast cells (clusters 1, 6, 7, 8, 9, 11, 16, 20, 21, 22, 24, 26, 33),

202   hematopoietic cells (cluster 2, 4, 5, 10, 12, 14, 17, 18, 28, 30, 31, 32), endothelial cells (clusters

203   15, 19, 29), endodermal cells (clusters 13, 27), and stromal cells (clusters 23, 25) (**Fig. 2B,**

204   **Dataset 2**). As scMCA could not predict the cell type for cluster 3, we looked at the top marker

205   expression for this cluster. It showed significant enrichment of several mitochondrial genes

206   (**Dataset 1**). Although contributions for the cluster 3 population came almost entirely from the

207   GATA PI1-KO samples (**Fig. 2C, S4**), the high level of mitochondrial gene enrichment in this

208   cluster implied dead cells captured during the library preparation for scRNA-seq. Thus Cluster 3

209   was excluded for subsequent analyses. However, we can not exclude the possibility of cluster 3

210   being a yet unknown. Aggregates of two control samples and GATA-PI1 KO placentae are used

211   and represented as Control and GATA-PI1 KO henceforth. Major alterations and rearrangement

212   of the trophoblast cell populations, which was associated with significant loss of the

213   hematopoietic cell populations, were evident in the GATA-PI1 KO placentae compared to the

214   control (**Fig. 2C**).

215   We also examined the enrichment of different trophoblast markers in individual trophoblast-

216   clusters based on their log2-fold expression ratio of normalized mean gene UMI counts with a p-

217   value < 0.05 (**Dataset 2, 3**). Based on this analysis, we detected considerable gain in the

218   spongiotrophoblast population, including invasive spongiotrophoblasts, clusters 1, 7, 9, and 11,

219   which further validates our *in situ* hybridization observation above in **Fig. 1I-J** (**Fig. 2D**). We also

220   observed a significant increase in the spiral artery-associated TGCs, clusters 8, 20, and 26 (**Fig.**

221   **2D**). This was accompanied by a significant loss in the high level of *Gjb3* expressing trophoblast

222   progenitor population, clusters 6 and 24 (**Fig. 2D**). Cluster 16, which showed strong expression

223   of syncytiotrophoblast markers *Dlx3*, *Tfeb*, *Hsd11b2*, gap junction protein *Gjb2*, PDGF receptor

224   α (*Pdgfa*), *Pparg*, *Slc16a1*, did not show significant changes in the GATA-PI1 KO placentae.

225   Although the scMCA analyses predicted cluster 33 to be of mixed nature including invasive

226   spongiotrophoblasts, spiral artery trophoblast giant cells and progenitor trophoblasts (**Dataset**

9

227   **2**), we observed enrichment of several trophoblast giant cell associated genes in that cluster

228   (**Dataset 1**). They include pregnancy specific glycoproteins (*Psg23*, *Psg18*, *Psg19*,), several

229   prolactin family members (*Prl8a9*, *Prl7d1*, *Prl3b1*), Trophoblast-specific protein alpha (*Tpbpa*),

230   Endothelial protein C receptor (*Procr*), LIF receptor alpha (*Lifr*), Fibronectin 1 (*Fn1*). Moreover,

231   along with strong *Tpbpa* expression, cluster 33 also contained cells positive for *Prl3d1*, *Prl2c2*,

232   *Hand1*, *Prl3b1,* all markers for parietal trophoblast giant cells, spiral artery-associated

233   trophoblast giant cells, canal trophoblast giant cells, and sinusoidal trophoblast giant cells [32]

234   (**Fig. 2E**). Thus we identified cluster 33 as the TGC-cluster. Significantly, out of all the 33

235   clusters, only this cluster showed increased coexpression of *Prl3d1*, *Gata2* and *Gata3* (**Fig.**

236   **S5**). Interestingly, the loss of *Gata2* and *Gata3* in these cells did not seem to negatively affect

237   the parietal TGC population (**Fig. 2E, F**).

238   A comparison between the control sample and the knockout sample populations showed a

239   significant increase in the Trophoblast-specific protein alpha (*Tpbpa*) expressing cell population

240   (**Fig. 2E, F**). These cells were also found to be positive for Prolactin family member *Prl3b1*. On

241   the other hand labyrinth trophoblast progenitor cells marked by the expression of *Ly6a* (*Sca1*)

242   and high levels of Epcam (*Epcam*[Hi]) [33] and *Hand1* expressing cell population did not show any

243   significant alterations (**Fig. 2E, F**).

244   We also noticed defective syncytiotrophoblast (SynT) development in GATA-PI1 KO placentae.

245   The mouse labyrinth contains two layers of SynTs, namely SynT-I and SynT-II. which express

246   two distinct moncarboxylate transporters (MCTs). The SynT-I expresses MCT1 [also known as

247   Solute Carrier Family 16, Member 1 (SLC16A1)] and the SynT-II expresses MCT4 [also known

248   as Solute Carrier Family 16, Member 3 (SLC16A3)]. Recent studies revealed that the labyrinth

249   of a developing mouse placenta contains distinct progenitors for SynT-I and SynT-II layers[15,23].

250   The progenitors for SynT-I can be identified from the mRNA expression of *Glis1, Snap91, Stra6,*

251   *Tfrc, Epha4* and *Slc16a1*, whereas the progenitors of SynT-II can be identified from the mRNA

252   expression of *Igf1r, Egfr* and *Slc16a3*. From our scRNA-seq analyses, we noticed that the

253 relative abundance of The SynT-I progenitors were not altered in E12.5 GATA-Pl1 KO

254 placentae (**Fig. S6A**). However, a significant increase in SynT-II progeniors was noticed in

255 GATA-Pl1 KO placentae. We also noticed increased number of *Gcm1* expression is gradually

256 suppressed in SynTs after E9.5 and by E12.5 only a few SynT-II cells express *Gcm1* (**Fig.**

257 **S6B**). Thus, increased abundance of SynT-II precursors and *Gcm1* expressing cells indicate an

258 skewed SynT differentiation program in GATA-Pl1 KO placentae. Together, these findings

259 revealed that the parietal TGC-specific loss of GATA factors skews the trophoblast

260 differentiation process and thereby alters the distribution of trophoblast subpopulations in the

261 developing placenta and affects gross placental architecture. Remarkably, these data also

262 indicate a novel mechanism whereby the parietal TGC-specific paracrine signaling dictates the

263 differentiation of the different trophoblast progenitors and regulates the development of distinct

264 placental layers.

265

266 **Loss of GATA factors disrupts hematopoietic-endothelial cell lineage segregation and**

267 **affects fetal hematopoiesis**

268 The placenta is one of the major sites for *de novo* hematopoiesis in an embryo [1]. It not only

269 supports the expansion of the nascent HSC population but also protects the HSCs from

270 premature differentiation cues. Several studies have implicated primary and secondary

271 trophoblast giant cells in secreting paracrine and endocrine factors [34], which are thought to

272 regulate placental hematopoiesis. These include prolactin/ placental lactogen class of hormones

273 [35], interferon [28], vasodilators [36], anticoagulants [37] and several angiogenic factors [29,38,39]. In order

274 to analyze the effect of PL1-specific GATA factor loss on the placental hematopoietic cell

275 development, we used our scRNA-seq data. Ingenuity Pathway Analysis (IPA) was performed

276 to compare the physiological functions of the *Prl3d1+* cells between the control and the KO

277 samples. Our results indicated that major physiological functions related to the hematopoiesis

278 and angiogenesis were downregulated in the *Prl3d1+* cells in the GATA-Pl1 KO samples (**Fig.**

11

279   **S7**).

280   We identified major hematopoietic subpopulations in our samples that include B-cells, T-cells,

281   Granulocytes, Macrophages, Erythroblasts, Dendritic cells, Basophils, Megakaryocytes,

282   Macrophages, and Natural Killer cells (**Fig. 3A, Dataset 2)**. Placental HSCs are localized in the

283   labyrinth, and umbilical blood vessels [40] and have been shown to express surface markers KIT,

284   CD34, and Ly6A [2]. We found two major clusters which harbored Kit^+ $Cd34^+$ $Ly6a^+$ cells, cluster

285   15 and cluster 17 (**Fig. 3A, B**). We calculated the relative percentages of cluster 15 and 17 cells

286   with respect to the total cell numbers per sample. While cells in the cluster 17 show gross

287   reduction in the GATA PI1-KO, cluster 15 displayed opposite trend. Almost all cells in cluster 15

288   were the contribution from the GATA PI1-KO placentae (**Fig. 3C**). Moreover, when we

289   calculated the relative percentages of the Kit^+ $Cd34^+$ $Ly6a^+$ HSC population, we found marked

290   reduction of these cells in the GATA PI1-KO compared to the control (**Fig. 3D)**. Interestingly,

291   unlike 17, cluster 15 is marked by high level of expression of vascular endothelial growth factor

292   receptor- 2 (*Vgfr2/Kdr*) (**Fig. 3E**).

293   A hallmark of fetal hematopoiesis is the interrelated development of vascular and hematopoietic

294   systems where hemogenic endothelium and hemangioblasts serve as the precursors to the

295   hematopoietic stem cell populations [41]. Multiple studies have shown that the embryonic

296   hematopoiesis is intricately connected to the vascular development where hemogenic

297   endothelium, a part of the vascular endothelial cells, gives rise to the definitive hematopoietic

298   precursors during mammalian development [42-44]. Thus, the genetic signature of the cells in

299   cluster 15, which is unique to the knockout samples, indicates a hematoendothelial cell

300   population that still retains the HSC lineage markers. This cell population was also found to

301   express hematoendothelial markers *Cdh5*, *Icam2*, *Cd40*, confirming the bipotent nature of these

302   cells [45-47] (**Fig. S8**). As a high expression of the arterial-specific marker *Delta-like, 4* (*Dll4*) is

303   essential for the segregation of the endothelial lineage from the hematopoietic lineage [48,49], we

304   looked at the *Dll4* expression in this subset, and found that this cluster expresses a high level of

12

305 *Dll4* (**Fig. 3E**). Curiously, cells in cluster 15 also showed expression of a recently identified

306 placental hemogenic endothelium marker *Prom1* [50,51], further confirming the hematoendothelial

307 nature of cluster 15 (**Fig. 3E-F**).

308 We further looked at the hematoendothelial population by further subjecting the placental cell

309 suspension to flow analysis. We screened single-cell suspension from control and GATA-PI1

310 KO placentae and filtered them against other hematopoietic lineage cells using antibody

311 specific for CD45, a pan-hematopoietic surface marker that appears on the more mature HSCs

312 [52], and a cocktail of hematopoietic lineage marker antibodies (Lin). These cells were further

313 screened for cells expressing CD34 and KDR simultaneously (**Fig. S9**). The percentage of this

314 hematoendothelial Lin$^-$ CD45$^-$ CD34$^+$ KDR$^+$ population showed significant increase in the GATA-

315 PI1 KO sample than the controls (**Fig. 3F, G**).

316 The result from the flow analysis mirrors the data from the scRNA-seq experiments (**Fig. 3C**)

317 and further confirms that the loss of GATA factors in the parietal TGCs skews the

318 hematopoietic-endothelial lineage specification in a developing placenta and leads to the arrest

319 of hematoendothelial progenitor population compared to the control.

320 In the next step, we used the three surface markers CD34, KIT (c-KIT), and Ly6A (SCA-1),

321 which together define the placental HSCs, as the determinant for the long-term reconstituting

322 (LTR) HSC population. Again, the cells were selected for CD45$^-$ and Lin$^-$ markers. These cells

323 were further subjected to flow analysis and were selected for KIT$^+$ CD34$^+$ Ly6A/E$^+$ cells. In the

324 GATA-PI1 KO placental samples, the Lin$^-$ CD45$^-$ KIT$^+$ CD34$^+$ Ly6A/E$^+$ cell population was

325 significantly reduced compared to the control (**Fig. 4A**). In comparison to the total number of

326 cells, the percentage of the HSC population in the KO placenta was significantly lower than that

327 in the control population (**Fig. 4B**) which resembles the results from the scRNA-seq analyses

328 (**Fig. 3D**).

329 Next, we evaluated the differentiation potential of the GATA-PI1 KO placental HSCs by using

330 the colony-forming assay. Placental single-cell suspension from the control and GATA-PI1 KO

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331 placentae were plated on methylcellulose medium containing a cocktail of Stem Cell Factor

332 (SCF), IL-3, IL-6, and Erythropoietin. The placental samples gave rise to mostly granulocyte and

333 mixed lineage erythroid, macrophage colonies. We observed a significant reduction in the

334 number of colonies generated from the GATA-PI1 KO placentae compared to the control (**Fig.**

335 **4C, D**).

336 It is speculated that the placental HSCs migrate and seed fetal liver along with the HSCs (Lin⁻

337 Ly6A/E⁺ KIT⁺ (LSK)) from the aorta-gonad-mesonephros (AGM) [1,26,53,54]. In order to analyze the

338 fetal liver HSC population, we performed FACS analysis of the E13.5 fetal liver of the GATA

339 PI1-KO embryos and compared them to the control. We found significant depletion of the LSK

340 population in the GATA PI1-KO fetal livers compared to the controls (**Fig. S10**), which validated

341 our observation that the GATA PI1-KO embryos show blood loss in their fetal liver (**Fig. 1G**).

342 Collectively these data prove that the GATA factor KO in the trophoblast giant cells negatively

343 affects hematopoiesis in the placenta and fetal liver and results in the apparent blood loss

344 phenotype. The loss of the HSC population was also accompanied by the incomplete

345 segregation of the hematopoietic and endothelial lineage, indicating a loss of signaling network

346 that balances and fine-tunes the hematopoietic versus endothelial cell lineage development in

347 the placenta.

348

349 **TGC-specific loss of GATA factors affects placental vasculature development**

350 Embryonic hematopoiesis and angiogenesis are tightly linked. Hemogenic endothelium in the

351 vascular labyrinth gives rise to both the endothelial cell population as well as the hematopoietic

352 cell population. As our study revealed a defective hematopoietic and endothelial lineage

353 segregation due to TGC-specific GATA factor loss, we used our GATA-PI1 KO placentae model

354 to test placental angiogenesis. Anti-CD31 (PECAM-1) antibody, which marks early and mature

355 endothelial cells, was used to stain the vasculature in the placental sections at E13.5.

356 Compared to the control samples, the KO placentae showed gross disruption of the blood

14

357   vessel architecture in the labyrinth (**Fig. 5A, B)**

358   Embryonic angiogenesis relies on a delicate balance of several key pro-angiogenic and anti-

359   angiogenic factors [55,56]. Among them anti-angiogenic factor soluble Vascular Endothelial Growth

360   Factor (VEGF) receptor FLT1 (sFLT1) has been shown to play essential roles in the placental

361   vascularization and angiogenesis [57,58]. Our results showed upregulation of *Flt1* in the GATA-PI1

362   KO placentae compared to the control (**Fig. 5C**). The sc-RNAseq data also showed that the

363   highest upregulation of *Flt1* in the GATA PI1-KO placentae was associated with cluster 33,

364   previously identified in this study as the TGC cluster (**Fig. 5D, E**). Other prominent sources of

365   Flt1 expression were the hematoendothelial cells (cluster 15), spongiotrophoblast cells in

366   clusters 1, 9, 11, and spiral artery associated TGCs in cluster 26 (**Fig. 5D**).

367   To functionally test the increase in the *Flt1* level, we performed matrigel based vascular tube

368   formation assays using Human Uterine Microvascular Endothelial Cells (HUtMEC). As

369   differentiated trophoblast stem cells express antiangiogenic sFLT1, we harvested conditioned

370   media from the differentiated *Gata2/Gata3* double knockout (GATA DKO) trophoblast stem cells

371   and differentiated control trophoblast stem cells described in our earlier study [19]. These

372   conditioned media were added to the assays individually. Interestingly, HUtMECs in the

373   presence of the conditioned media from the control trophoblast stem cells readily formed tubular

374   structure while they failed to do so in the presence of conditioned media from GATA DKO

375   trophoblast stem cells (**Fig. 5F**).

376   These findings indicate that the loss of GATA factors in the TGC layer results in the disruption of

377   the delicate balance between the secreted angiogenic and anti-angiogenic factors by direct

378   upregulation of *Flt1* expression in the TGC cells and by increasing the numbers of *Flt1*

379   expressing trophoblast cells. This overall rise in the FLT1 level, in turn prevents proper

380   development of the labyrinth vasculature in the placenta.

381

382   **GATA loss alters trophoblast giant cell-mediated paracrine signaling**

15

383   TGCs are known to be a major source of autocrine and paracrine factors in the placenta [32,59].

384   These factors, in turn, influence the development of the placenta as well as regulate numerous

385   placental functions. In order to analyze the TGC-mediated signaling mechanism that regulates

386   the development of placental trophoblast subpopulations and dictate hematoendothelial

387   differentiation, we employed a recently reported bioinformatic tool PyMINEr [60]. PyMINEr

388   identifies receptor-receptor and ligand-receptor pairs by filtering out cell type-enriched receptors

389   or secreted ligand genes. It then builds up a network of protein level interactions within and

390   across all cell types by cross-referencing gene-gene pairs for physical protein-protein

391   interactions. Finally, PyMINEr uses pathway analyses to identify the autocrine or paracrine

392   signaling mechanism for these cell types. We used all the 33 clusters as input to PyMINEr to

393   ensure correlation with the rest of our analyses. We identified several cross-cluster ligand-

394   receptor pairs across our identified clusters. As we were interested in the paracrine signaling

395   emanating from *Prl3d1+* cluster 33, we evaluated all extracellular ligand pairs for corresponding

396   receptor matches in other clusters. PyMINER analysis indicated the HSCs and HSPCs (cluster

397   17), and the hematoendothelial population (cluster 15), among the major paracrine signaling

398   targets for extracellular factors expressed by the *Prl3d1+* cluster 33 (**Fig. 6A**). This analysis also

399   suggested laminin subunits *Lamb1*, *Lamb2*, *Lamc3*, *Lama5*, Collagen type IV alpha 1 (*Col4a1*),

400   Fibronectin 1 (*Fn1*), and *Ccl27a* as possible major paracrine regulators acting on cluster 17

401   (**Dataset 4**). Similarly, our data suggested *Lamb1*, *Lamb2*, *Lamc3*, *Lama5*, Collagen type IV

402   alpha 1 (*Col4a1*), Fibronectin 1 (*Fn1*), Lymphocyte antigen 96 (*Ly96*) and *Wnt11* to be the major

403   potential paracrine factors acting on the hematoendothelial cluster 15 (**Fig. 6A**). When these

404   genes were subjected to PANTHER pathway analysis, we observed significant enrichment of

405   the Integrin signaling pathway in both the cases (**Fig. S11A**). The integrin signaling pathway has

406   been implicated in both mouse and human placental development [61].

407   Interestingly, it has been shown that knocking out Integrin Subunit Alpha 5 (*Itga5*) in mice

408   results in embryonic lethality for a large number of the embryos and shows poor development of

16

409   the placental labyrinth and poor interdigitation of fetal and maternal vessels [62]. Also, knocking

410   out Integrin Subunit Alpha 7 (*Itga7*) in mice results in defective placental structures, including

411   infiltration of the spongiotrophoblast layer into the placental labyrinth [63].

412   Moreover, our data pointed to two trophoblast clusters, progenitor trophoblasts (cluster 6), and

413   labyrinthine trophoblasts (cluster 16) as the potential targets for 28 paracrine factors expressed

414   by cluster 33 (**Fig. 6A**). These groups of paracrine factors included the Integrin signaling

415   pathway members identified above, along with 16 prolactin family of hormones and Inhibin beta

416   B (*Inhbb*) (**Dataset 4**). While the Prolactin family of genes has been studied extensively in the

417   context of placental development and function [64,65], *Inhbb* has been implicated in preeclampsia

418   [66].

419   We analyzed these genes for GATA factor occupancy using our previously published data [19].

420   Interestingly, we found about half of these genes (*Col4a1*, *Col4a2*, *Fn1*, *Inhbb*, *Lama5*, *Lamb1*,

421   *Lamb2*, *Lamc3*, *Ly96*, *Prl2a1*, *Prl7d1, Wnt11*) to be putative GATA targets (**Fig. S11B-C**).

422   Further analysis with the *Prl3d1*-positive cells in the cluster 33 showed upregulation of *Col4a1*,

423   *Col4a2*, *Fn1*, *Inhbb*, *Lama5*, *Lamb1*, *Lamb2*, *Lamc3*, while downregulation of *Prl2a1*, *Prl2c2*,

424   *Prl2c5*, *Prl4a1*, *Prl7d1* (**Fig. 6B**).

425   Among the suggested paracrine factors associated with the pTGCs in the GATA PI1-KO

426   placentae, we tested the expression level of LAMB1 and LAMB2. Immunostaining revealed

427   increased expression levels of these two factors in the KO samples compared to the controls

428   (**Fig. 6C**).

429   Collectively, these results suggest that inhibition of the Integrin signaling pathway in the parietal

430   TGCs might be critical for hematoendothelial differentiation and maintaining hematopoietic-

431   angiogenic balance in the developing mouse placenta. Our data also indicated that repression

432   of the integrin signaling pathway along with the activation of the prolactin signaling might play a

433   crucial part in the trophoblast lineage differentiation and helps maintain the proper ratio of

434   placental trophoblast subtypes (**Fig. 7**).

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435

Discussion

GATA factor function in trophoblast differentiation and function has been subjected to numerous studies. Previously, we showed how GATA2 and GATA3 have overlapping functions during placental development, where the simultaneous loss of both the factors in all trophoblast cells leads to early embryonic death and placental defect with impaired hematopoiesis [14,19]. It also revealed the loss of placenta layers, including the labyrinth region. Interestingly, the pan-trophoblast-specific knockout did not show significant change in the trophoblast giant cell numbers at the junctional zone.

As the placenta is a complex tissue consisting of diverse trophoblast subtypes as well as hematopoietic and endothelial cell populations, it is of utmost importance to categorize these cellular subtypes and define cell-cell interactions at the single-cell resolution. A recent study in mouse placenta employed single-nuclei RNAseq to highlight labyrinth development, but left out the role of the parietal TGCs [67]. Although due to the inherent technical limtations of the 10X genomics sc-RNAseq platform we could only capture a small fraction of the syncytiotrophoblasts and TGCs, our analyses successfully identified the TGC cluster and the syncytial layers. Our sc-RNASeq data not only predicted the genetic signatures of the major trophoblast subtypes but also showed how the pTGC-specific transcriptional program governs the differentiation of the progenitor population and thereby altered the distribution of cells at different placental layers. Data from our *Gata2^f/f^;Gata3^f/f^; Pl1^Cre/wt^* mouse models showed that the loss of the GATA factors in the parietal TGC population severely reduced the placental size and also led to fetal growth restriction with significant phenotypic abnormality between E12.5 and E13.5. Importantly, we observed significant structural changes in the GATA-Pl1 KO placentae compared to the control. Molecular and morphological analyses revealed gross redistribution of different trophoblast progenitor cells, including *Prl2c2* expressing giant cells, *Tpbpa* expressing spongiotrophoblast progenitor, *Prl3b1* expressing secondary giant cell progenitors. While the

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461   number of *Epcam* expressing labyrinth progenitor cells showed a marked increase, we did not

462   observe any significant enlargement of the labyrinth regions in the GATA-PI1 KO placentae

463   when normalized against the total placental area. In total, these findings reveal how different

464   layers of trophoblast cells interact during placental development and influence the differentiation

465   of trophoblast progenitors.

466   TGCs in the placenta secrete a lot of cytokines and hormones, which are not only crucial for

467   trophoblast differentiation and placental development but are also critical for placental

468   angiogenesis and hematopoiesis. Our study predicted possible mechanisms through which

469   TGC-specific GATA factors dictate TGC-specific paracrine signaling and, in turn, regulate

470   placental hematoendothelial niche. Curiously, the loss of TGC-specific GATA factors resulted in

471   a unique cell population marked by the expression of *Kdr*, as well as *Kit*, *Cd34,* and *Ly6A*. The

472   expression of both the endothelial gene and hematopoietic stem cell markers indicated towards

473   the emergence of a hematoendothelial cell cluster. The complete absence of this cell population

474   in the control placentae also indicated differentiation of these cells in the absence of TGC-

475   specific GATA factors.

476   The GATA-PI1 KO placentae were further characterized by the lack of proper angiogenic

477   reoganization in the labyrinth. Our observation about the increased level of *Flt1* gives us a

478   possible explanation about the angiogenic defect. The concomitant expansion of the *Tpbpa*

479   expressing spongiotrophoblast in the GATA-PI1 KO could also contribute to the impairment of

480   the labyrinth development. We speculate that this loss in the surface area of exchange may

481   result in the reduction of the nutrient supply and finally result in fetal growth restriction.

482   The integrin signaling pathway has been extensively studied in relation to the placental

483   development as well as angiogenesis in both rodents and humans. Our novel finding suggests

484   potential mechanisms by which inhibition of the integrin signaling pathway promotes placental

485   hematoendothelial differentiation. This brings a new insight into how a small number of parietal

486   trophoblast giant cells at the maternal-fetal interface can dictate the hematopoietic and

19

487   angiogenic development in the placenta and thereby can affect fetal development.

488   Mouse embryogenesis involves vascularization on both the placental side as well as the

489   decidual side. TGCs secrete a lot of angiogenic and anti-angiogenic paracrine factors, and in

490   turn, regulate the decidual vascularization. However, how these factors dictate decidual

491   vascularization, is poorly understood. It would be interesting to study how TGC-specific loss of

492   GATA factors affect decidualization and associated vascular development. In this aspect, our

493   TGC-specific GATA knockout mouse model holds great promise to help investigate the

494   paracrine signaling from the TGC layers involved in the decidual angiogenesis.

495   GATA2 and GATA3 are evolutionarily conserved among mammals, and they are expressed in

496   the human placenta. However, unlike the mouse placenta, human placental layers do not have

497   trophoblast giant cells. In the absence of defined TGC subtypes, it is impossible to analyze the

498   role of GATA factors in the placental development in humans. Moreover, ethical and logistical

499   issues make it impossible to examine the role of GATA factors in human placental development

500   *in vivo*. Nonetheless, the recent establishment of a human trophoblast cell line [68] has presented

501   us with a new tool to examine the role of GATA factors in human placental development. As

502   these cells can be readily differentiated *in vitro* to extravillous trophoblast and

503   syncytiotrophoblast subtypes, they open the possibility of serving as models to study the role of

504   GATA factors in the context of human placental development.

505   GATA2 and GATA3 are evolutionarily conserved among mammals, and they are expressed in

506   the human placenta[19,69]. Although a human placenta does not have a Junctional zone, the TGC

507   population resembles with the extravillous trophoblast cells (EVTs) of a human placenta. EVTs

508   arise at the anchoring villi (which anchor the placenta with the uterine tissue) within a

509   developing human placenta and invades into the uterine compartment[69]. A subset of EVT

510   population migrates deeply into the uterus and upto the first third of the myometrium, where they

511   differentiate to a giant cell population [70,71], which undergo endoreduplication, similar to the

512   mouse parietal TGCs. These human invasive EVT-derived giant cells also mediate paracrine

513   signaling to modulate the uterine microenviroment [71]. Intriguingly both GATA2 and GATA3 are

514   higly expressed in invasive EVTs at the human maternal-fetal interface. Thus, it will be

515   interesting to find out how GATA2 and GATA3 regulate gene expression dynamics in human

516   EVTs. Successful establishment of bona-fide human trophoblast stem cell lines [68] has

517   presented us with a new tool to examine this aspect and opens up the possibility of serving as

518   models to better understand the role of GATA factors in the context of human placental

519   development.

Materials and methods

Generation of conditional knockout mice strains

522   All procedures were performed after obtaining IACUC approvals at the Univ. of Kansas Medical

523   Center. Female *Gata2flox/flox* (*Gata2$^{f/f}$*) mice [72] were mated with *Prl3d1tm1(cre)Gle* (*Pl1^{Cre}*)

524   male in order to generate *Gata2$^{f/+}$; Pl1$^{Cre/wt}$*. In the next step, *Gata2$^{f/+}$; Pl1$^{Cre/wt}$* female mice were

525   bred with *Gata2$^{f/+}$; Pl1$^{Cre/wt}$* males to generate *Gata2$^{f/f}$; Pl1$^{Cre/wt}$*. Similarly female *Gata3flox/flox*

526   (*Gata3$^{f/f}$*) mice [73] were used to generate *Gata3$^{f/f}$; Pl1$^{Cre/wt}$*. In the next step, *Gata2$^{f/f}$; Pl1$^{Cre/wt}$* and

527   Gata3$^{f/f}$; *Pl1$^{Cre/wt}$* mice were crossed to generate Gata2$^{f/+}$;*Gata3$^{f/+}$*; *Pl1$^{Cre/wt}$*. Later

528   *Gata2$^{f/+}$;Gata3$^{f/+}$; Pl1$^{Cre/wt}$* males and females were crossed to generate Gata2$^{f/f}$;Gata3$^{f/f}$; Pl1$^{Cre/wt}$

529   strain. Further crosses with Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J, (also known as

530   mT/mG) mouse strain was used to establish *Gata2$^{f/f}$;Gata3$^{f/f}$;mT/mG* mouse line.

Embryo harvest and tissue isolation

532   Animals were euthanized on desired day points, as indicated in the main text. Uterine horns and

533   conceptuses were photographed. Conceptuses were dissected to isolate embryos, yolk sacs,

534   and placentae. All embryos and placentae were photographed at equal magnification for

535   comparison purposes.

536   Uteri containing placentation sites were dissected from pregnant female mice on E12.5 and

537   E13.5 and frozen in dry ice-cooled heptane and stored at −80°C until used for histological

538   analysis. Tissues were subsequently embedded in optimum cutting temperature (OCT) (Tissue-

539   Tek) and were cryosectioned (10μm thick) for immunohistochemistry (IHC) studies using Leica

540   CM-3050-S cryostat.

541   Placenta samples were carefully isolated, ensuring the decidual layer was peeled off. Individual

542   samples were briefly digested in the presence of collagenase and were made into single-cell

543   suspensions by passing them through a 40μm filter. These cell suspensions were further used

544   for Flow analysis or FACS. Corresponding embryonic tissues were used to confirm genotypes.

545   For scRNA-seq, these samples were further processed using Debris Removal Solution and

546   Dead Cell Removal Kit (Miltenyi Biotec). Red blood cell depletion from the placental

547   suspensions were carried out using anti-Mouse Ter-119 antibody (BD Biosciences) was used.

548   **Single Cell RNA-Sequencing and analysis**

549   The transcriptomic profiles of mouse placental samples from two control (biological replicates)

550   and two *Gata2/Gata3* double knockout (biological replicate) specimens were obtained using the

551   10x Genomics Chromium Single Cell Gene Expression Solution (10xgenomics.com). The

552   primary analysis of the scRNA-seq data was performed using the 10x Genomics Cell Ranger

553   pipeline (version 3.1.0). This pipeline performs sample de-multiplexing, barcode processing, and

554   single-cell 3' gene counting. The quality of the sequenced data was assessed using the FastQC

555   software [74]. Sequenced reads were mapped to the mouse reference genome (mm10) using the

556   STAR software [75]. Individual samples were aggregated using the "cellranger aggr" tool in Cell

557   Ranger to produce a single feature-barcode matrix containing all the sample data. This process

558   normalizes read counts from each sample, by subsampling, to have the same effective

559   sequencing depth. The Cell Ranger software was used to perform two-dimensional PCA and t-

560   SNE projections of cells, and k-means clustering. The 10x Genomics Loupe Cell Browser

561   software (v 4.1.0) was used to find significant genes, cell types, and substructure within the

562   single-cell data.

563 Digital Gene Expression matrices for each clusters identified using Cell Ranger, were

564 individually fed into the scMCA pipeline (http://bis.zju.edu.cn/MCA/blast.html). scMCA output

565 consisted of top markers for each clusters, predicted probabilities for the clusters alongwith their

566 *p*-values. Clusters were identified using the smallest *p*-value. Where multiple equal probabilities

567 existed, we calculated the abundance of the top markers in the respective clusters and also

568 looked at the rest of the genes expressed in that cluster to attribute cell types in that cluster.

569 Using Ingenuity Pathway Analysis (Qiagen) software, we performed a core analysis of the

570 significantly ($p \leq 0.05$) upregulated transcripts in the *Prl3d1+* cell clusters from the control and

571 the GATA PI1-KO scRNA-seq transcriptomes. The core analyses results were then compared

572 and filtered for physiological functions related to hematopoiesis and angiogenesis to generate

573 the heatmap.

574 PyMINEr analysis was performed using methods described by Tyler *et al.* [60]. Clusters defined

575 by the Cell Ranger were used as input. The receptor-ligand pairs were sorted on a PyMINER

576 score. Pairs with a score more than 800 were retained to introduce high stringency. PANTHER

577 pathway analysis was done using the predicted paracrine secreted ligands from cluster 33.

578 Corresponding gene list were supplied as input.

579 **Cell culture and reagents**

580 Mouse trophoblast stem cells (TSCs) were cultured with FGF4, Heparin, and MEF-conditioned

581 medium (CM) according to protocol [76]. *Gata2* and *Gata3* floxed alleles were efficiently excised

582 from *Gata2f/f;Gata3f/f;UBC-cre/ERT2* (GATA DKO) TSCs by culturing the cells in absence of

583 FGF4 and MEF-conditioned media and in the presence of tamoxifen (1 μg/ml) [19]. Conditioned

584 media was harvested upon removal of tamoxifen and was used for subsequent experiments.

585 **Flow analysis and sorting**

586 For analyzing HSC population, placental single-cell suspensions were stained with APC-

587 conjugated anti-mouse CD117 (c-Kit) (BioLegend), PerCP/Cy5.5-conjugated anti-mouse CD34

588 (BioLegend), PE-conjugated anti-mouse Ly-6A/E (Sca-1) (BioLegend), PE/Cy7 anti-mouse

23

589 CD45 (BioLegend) and Pacific Blue-conjugated anti-mouse Lineage cocktail (BioLegend)

590 monoclonal antibodies. Unstained, isotype and single-color controls were used for optimal

591 gating strategy. Samples were run on either an LSRII flow cytometer or an LSRFortessa (BD

592 Biosciences), and the data were analyzed using FACSDiva software.

593 Similarly, E13.5 fetal liver cells were also stained with APC-conjugated anti-mouse CD117 (c-

594 Kit) (BioLegend), PE-conjugated anti-mouse Ly-6A/E (SCA-1) (BioLegend), and Pacific Blue-

595 conjugated anti-mouse Lineage cocktail (BioLegend) monoclonal antibodies.

596 **Colony Formation Assay**

597 Placenta cell suspensions were suspended in Iscove's MDM with 2% fetal bovine serum and

598 cultured using a MethoCult GF M3434 Optimum kit (STEMCELL Technologies). 10,000 cells

599 from each sample were plated in 35-mm culture dishes (STEMCELL Technologies) and

600 incubated at 37°C in a humidified, 5% CO_2 environment for 14 days. Colonies were observed

601 and counted using an inverted microscope.

602 *In-Vitro* **Endothelial Network Assembly Assay on Matrigel**

603 Endothelial network assembly was assayed by the formation of capillary-like structures by

604 HUtMECs on Matrigel (BD Biosciences). Matrigel was diluted 1:1 with a supplement-free M200

605 medium, poured in 12-well plates, and allowed to solidify at 37 °C. Sub confluent HUtMECs

606 were harvested and preincubated for 1 hour in growth supplement-free M200 medium in

607 microcentrifuge tubes. An equal volume of M200 medium containing FGF2/EGF was added. In

608 addition, conditioned medium from differentiated *Gata2^{f/f}*;*Gata3^{f/f}*;*UBC^{CreERT2}* trophoblast stem

609 cells cultured in the presence (control) and absence of tamoxifen (GATA DKO) were added. The

610 cells were plated on Matrigel (1.5 × 105 cells/well) and incubated at 37 °C and photographed at

611 different time intervals.

612 **Quantitative RT-PCR**

24

613   Total RNA from cells was extracted with the RNeasy Mini Kit (Qiagen). Samples isolated using

614   FACS were further processed using the PicoPure RNA Isolation kit. cDNA samples were

615   prepared and analyzed by qRT-PCR following procedures described earlier [14].

616   **Genotyping**

617   Genomic DNA samples were prepared using tail tissues or embryonic tissues from the mice

618   using the REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich). Genotyping was done using

619   REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich) and respective primers. Respective primers

620   are listed in the materials and methods section.

621   **Immunofluorescence**

622   For immunostaining with mouse tissues, slides containing cryosections were dried, fixed with

623   4% PFA followed by permeabilization with 0.25% Triton X-100 and blocking with 10% fetal

624   bovine serum and 0.1% Triton X-100 in PBS. Sections were incubated with primary antibodies

625   overnight at 4 °C, washed in 0.1% Triton X-100 in PBS. After incubation (1:400, one hour, room

626   temperature) with conjugated secondary antibodies, sections were washed, mounted using an

627   anti-fade mounting medium (Thermo Fisher Scientific) containing DAPI and visualized using

628   Nikon Eclipse 80i fluorescent microscope.

629   *mT/mG* positive embryos and cryosections were imaged directly under a Nikon Eclipse 80i

630   fluorescent microscope.

631   **In situ hybridization**

632   E13.5 whole conceptus cryosections were subjected to staining using RNAscope 2.5 HD

633   Detection Kit (ACD Bio, Newark, CA). RNAscope probe for *Tpbpa* was used to detect the

634   junctional zone of the mouse placenta, while hematoxylin was used as the counterstain.

635   **Statistical analyses**

636   Independent data sets were analyzed using GraphPad Prism software. Two-tailed Student's t-

637   tests were performed for significabce and the data are presented as mean±s.e.m.

638

25

639 **Primer List**

640 **Primers used for genotyping**

Gata2-floxed and Gata2-null	GCCTGCGTCCTCCAACACCTCTAA	TCCGTGGGACCTGTTTCCTTAC
Gata3-flox	CAGTCTCTGGTATTGATCTGCTTCTT	GTGCAGCAGAGCAGGAAACTCTCAC
Gata3-null	TCAGGGCACTAAGGGTTGTTAACTT	GTGCAGCAGAGCAGGAAACTCTCAC
Cre	AAAATTTGCCTGCATTACCG	ATTCTCCCACCGTCAGTACG

641

642 **Antibody list**

643 **Immunostaining**

Primary antibodies	Species raised in	Vendor	Catalog number	Dilutions used
anti-mouse CD31	Rat	BD Pharmingen	553369	1:100
anti-Laminin beta 1 [LT3]	Rat	Abcam	ab44941	1:100
Anti-Laminin beta 2	Mouse	Developmental Studies Hybridoma Bank	C4	1:1
Secondary antibodies	Species raised in	Vendor	Catalog number	Dilutions used
Alexa Fluor 488 anti-mouse	Donkey	Abcam	ab150105	1:400

26

IgG				
Alexa Fluor 568 anti-rat IgG	Donkey	Abcam	ab175475	1:400

Antibodies used in FACS	Species raised in	Vendor	Catalog number
APC anti-mouse CD117 (c-kit)	Rat	BioLegend	105812
PerCP/Cy5.5 anti-mouse CD34	American Hamster	BioLegend	128607
PE anti-mouse Ly-6A/E (Sca-1)	Rat	BioLegend	108108
PE/Cy7 anti-mouse CD45	Rat	BioLegend	103113
Pacific Blue anti-mouse Lineage Cocktail	Rat	BioLegend	133310
Alexa Fluor 488 anti-mouse CD309 (VEGFR2, Flk-1)	Rat	BioLegend	121908

644

645 **Data availability**

646 Processed data can be accessed from the NCBI Gene Expression Omnibus database

647 (accession number GSE163286).

648 **Acknowledgment**

27

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650   Histology Core facility, and the Bioinformatics Core of the University of Kansas Medical Center.

651

652   **Conflict of interest**

653   The authors declare no competing interests.

654

655   **Funding**

662

663   **Data availability**

664   All the sequencing data are available and will be uploaded in a public database upon

665   acceptance of this manuscript.

666

667   **Contribution**

668   R.P.K. and S.P. conceived, designed and performed the initial study. P.H., RPK and A. Ghosh,

669   performed the main experiments. P.H., A. Ghosh., R.P.K., and S.R. analyzed data. S.G., P.H.

670   and R. K analyzed genomics data. P.H. wrote the manuscript. PH and RPK Revised the

671   manuscript. S.P. edited the final manuscript.

References

1.   Gekas, C., Rhodes, K.E., Van Handel, B., Chhabra, A., Ueno, M., and Mikkola, H.K. (2010). Hematopoietic stem cell development in the placenta. Int J Dev Biol *54*, 1089-1098. 10.1387/ijdb.103070cg.

2.   Ottersbach, K., and Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. Developmental Cell *8*, 377-387. DOI 10.1016/j.devcel.2005.02.001.

3.   Rhodes, K.E., Gekas, C., Wang, Y., Lux, C.T., Francis, C.S., Chan, D.N., Conway, S., Orkin, S.H., Yoder, M.C., and Mikkola, H.K. (2008). The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. Cell Stem Cell *2*, 252-263. 10.1016/j.stem.2008.01.001.

4.   Duley, L. (2009). The global impact of pre-eclampsia and eclampsia. Seminars in perinatology *33*, 130-137. 10.1053/j.semperi.2009.02.010.

5.   Lindheimer, M.D., Roberts, J.M., Cunningham, F.G., and Chesley, L.C. (2009). Chesley's hypertensive disorders in pregnancy, 3rd Edition (Academic Press/Elsevier).

6.   Iliodromiti, Z., Antonakopoulos, N., Sifakis, S., Tsikouras, P., Daniilidis, A., Dafopoulos, K., Botsis, D., and Vrachnis, N. (2012). Endocrine, paracrine, and autocrine placental mediators in labor. Hormones (Athens) *11*, 397-409.

7.   Nishikawa, S.I. (2001). A complex linkage in the developmental pathway of endothelial and hematopoietic cells. Curr Opin Cell Biol *13*, 673-678.

8.   Koushik, S.V., Wang, J., Rogers, R., Moskophidis, D., Lambert, N.A., Creazzo, T.L., and Conway, S.J. (2001). Targeted inactivation of the sodium-calcium exchanger (Ncx1) results in the lack of a heartbeat and abnormal myofibrillar organization. FASEB J *15*, 1209-1211.

9.   Hirashima, M., Lu, Y., Byers, L., and Rossant, J. (2003). Trophoblast expression of fms-like tyrosine kinase 1 is not required for the establishment of the maternal-fetal interface in the mouse placenta. Proceedings of the National Academy of Sciences of the United States of America *100*, 15637-15642. 10.1073/pnas.2635424100.

10.   Achen, M.G., Gad, J.M., Stacker, S.A., and Wilks, A.F. (1997). Placenta growth factor and vascular endothelial growth factor are co-expressed during early embryonic development. Growth Factors *15*, 69-80. 10.3109/08977199709002113.

11.   Tsai, F.Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W., and Orkin, S.H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature *371*, 221-226.

12.   Pandolfi, P.P., Roth, M.E., Karis, A., Leonard, M.W., Dzierzak, E., Grosveld, F.G., Engel, J.D., and Lindenbaum, M.H. (1995). Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. Nature genetics *11*, 40-44. 10.1038/ng0995-40.

13.   Ray, S., Dutta, D., Rumi, M.A.K., Kent, L.N., Soares, M.J., and Paul, S. (2009). Context-dependent function of regulatory elements and a switch in chromatin occupancy between GATA3 and GATA2 regulate Gata2 transcription during trophoblast differentiation. The Journal of biological chemistry *284*, 4978-4988. 10.1074/jbc.M807329200.

14.   Home, P., Ray, S., Dutta, D., Bronshteyn, I., Larson, M., and Paul, S. (2009). GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. J Biol Chem *284*, 28729-28737. 10.1074/jbc.M109.016840.

15.   Ghosh, A., Kumar, R., Kumar, R.P., Ray, S., Saha, A., Roy, N., Dasgupta, P., Marsh, C., and Paul, S. (2024). The GATA transcriptional program dictates cell fate equilibrium to

721   establish the maternal-fetal exchange interface and fetal development. Proc Natl Acad
722   Sci U S A *121*, e2310502121. 10.1073/pnas.2310502121.

723 16. Ng, Y.K., George, K.M., Engel, J.D., and Linzer, D.I.H. (1994). Gata Factor Activity Is
724   Required for the Trophoblast-Specific Transcriptional Regulation of the Mouse Placental-
725   Lactogen-I Gene. Development *120*, 3257-3266.

726 17. Ma, G.T., Roth, M.E., Groskopf, J.C., Tsai, F.Y., Orkin, S.H., Grosveld, F., Engel, J.D.,
727   and Linzer, D.I. (1997). GATA-2 and GATA-3 regulate trophoblast-specific gene
728   expression in vivo. Development (Cambridge, England) *124*, 907-914.

729 18. Jackson, D., Volpert, O.V., Bouck, N., and Linzer, D.I. (1994). Stimulation and inhibition
730   of angiogenesis by placental proliferin and proliferin-related protein. Science *266*, 1581-
731   1584. 10.1126/science.7527157.

732 19. Home, P., Kumar, R.P., Ganguly, A., Saha, B., Milano-Foster, J., Bhattacharya, B., Ray,
733   S., Gunewardena, S., Paul, A., Camper, S.A., et al. (2017). Genetic redundancy of
734   GATA factors in the extraembryonic trophoblast lineage ensures the progression of
735   preimplantation and postimplantation mammalian development. Development *144*, 876-
736   888. 10.1242/dev.145318.

737 20. Simmons, D.G., Fortier, A.L., and Cross, J.C. (2007). Diverse subtypes and
738   developmental origins of trophoblast giant cells in the mouse placenta. Dev Biol *304*,
739   567-578. 10.1016/j.ydbio.2007.01.009.

740 21. Bany, B.M., and Cross, J.C. (2006). Post-implantation mouse conceptuses produce
741   paracrine signals that regulate the uterine endometrium undergoing decidualization. Dev
742   Biol *294*, 445-456. 10.1016/j.ydbio.2006.03.006.

743 22. Ouseph, M.M., Li, J., Chen, H.Z., Pecot, T., Wenzel, P., Thompson, J.C., Comstock, G.,
744   Chokshi, V., Byrne, M., Forde, B., et al. (2012). Atypical E2F repressors and activators
745   coordinate placental development. Dev Cell *22*, 849-862. 10.1016/j.devcel.2012.01.013.

746 23. Jiang, X., Wang, Y., Xiao, Z., Yan, L., Guo, S., Wang, Y., Wu, H., Zhao, X., Lu, X., and
747   Wang, H. (2023). A differentiation roadmap of murine placentation at single-cell
748   resolution. Cell Discovery *9*, 30. 10.1038/s41421-022-00513-z.

749 24. Simmons, D.G., and Cross, J.C. (2005). Determinants of trophoblast lineage and cell
750   subtype specification in the mouse placenta. Developmental biology *284*, 12-24.
751   10.1016/j.ydbio.2005.05.010.

752 25. Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-
753   fluorescent Cre reporter mouse. Genesis *45*, 593-605. 10.1002/dvg.20335.

754 26. Mikkola, H.K., and Orkin, S.H. (2006). The journey of developing hematopoietic stem
755   cells. Development *133*, 3733-3744. 10.1242/dev.02568.

756 27. El-Hashash, A.H.K., Warburton, D., and Kimber, S.J. (2009). Genes and signals
757   regulating murine trophoblast cell development. Mechanisms of development *127*, 1-20.
758   10.1016/j.mod.2009.09.004.

759 28. Bany, B.M., and Cross, J.C. (2006). Post-implantation mouse conceptuses produce
760   paracrine signals that regulate the uterine endometrium undergoing decidualization.
761   Developmental Biology *294*, 445-456. 10.1016/j.ydbio.2006.03.006.

762 29. Carney, E.W., Prideaux, V., Lye, S.J., and Rossant, J. (1993). Progressive expression of
763   trophoblast-specific genes during formation of mouse trophoblast giant cells in vitro. Mol
764   Reprod Dev *34*, 357-368. 10.1002/mrd.1080340403.

765 30. Parisi, T., Beck, A.R., Rougier, N., McNeil, T., Lucian, L., Werb, Z., and Amati, B. (2003).
766   Cyclins E1 and E2 are required for endoreplication in placental trophoblast giant cells.
767   EMBO J *22*, 4794-4803. 10.1093/emboj/cdg482.

768 31. Han, X., Wang, R., Zhou, Y., Fei, L., Sun, H., Lai, S., Saadatpour, A., Zhou, Z., Chen,
769   H., Ye, F., et al. (2018). Mapping the Mouse Cell Atlas by Microwell-Seq. Cell *173*, 1307.
770   10.1016/j.cell.2018.05.012.

771 32. Simmons, D.G., Fortier, A.L., and Cross, J.C. (2007). Diverse subtypes and
772   developmental origins of trophoblast giant cells in the mouse placenta. Developmental
773   biology *304*, 567-578. 10.1016/j.ydbio.2007.01.009.
774 33. Natale, B.V., Schweitzer, C., Hughes, M., Globisch, M.A., Kotadia, R., Tremblay, E., Vu,
775   P., Cross, J.C., and Natale, D.R.C. (2017). Sca-1 identifies a trophoblast population with
776   multipotent potential in the mid-gestation mouse placenta. Sci Rep *7*, 5575.
777   10.1038/s41598-017-06008-2.
778 34. Muntener, M., and Hsu, Y.C. (1977). Development of trophoblast and placenta of the
779   mouse. A reinvestigation with regard to the in vitro culture of mouse trophoblast and
780   placenta. Acta Anat (Basel) *98*, 241-252.
781 35. Linzer, D.I., and Fisher, S.J. (1999). The placenta and the prolactin family of hormones:
782   regulation of the physiology of pregnancy. Mol Endocrinol *13*, 837-840.
783   10.1210/mend.13.6.0286.
784 36. Yotsumoto, S., Shimada, T., Cui, C.Y., Nakashima, H., Fujiwara, H., and Ko, M.S.H.
785   (1998). Expression of adrenomedullin, a hypotensive peptide, in the trophoblast giant
786   cells at the embryo implantation site in mouse. Developmental Biology *203*, 264-275.
787   DOI 10.1006/dbio.1998.9073.
788 37. WeilerGuettler, H., Aird, W.C., Rayburn, H., Husain, M., and Rosenberg, R.D. (1996).
789   Developmentally regulated gene expression of thrombomodulin in postimplantation
790   mouse embryos. Development *122*, 2271-2281.
791 38. Lee, S.J., Talamantes, F., Wilder, E., Linzer, D.I.H., and Nathans, D. (1988).
792   Trophoblastic Giant-Cells of the Mouse Placenta as the Site of Proliferin Synthesis.
793   Endocrinology *122*, 1761-1768. DOI 10.1210/endo-122-5-1761.
794 39. Cross, J.C., Hemberger, M., Lu, Y., Nozaki, T., Whiteley, K., Masutani, M., and
795   Adamson, S.L. (2002). Trophoblast functions, angiogenesis and remodeling of the
796   maternal vasculature in the placenta. Mol Cell Endocrinol *187*, 207-212.
797 40. Gekas, C., Dieterlen-Lievre, F., Orkin, S.H., and Mikkola, H.K.A. (2005). The placenta is
798   a niche for hematopoietic stem cells. Developmental Cell *8*, 365-375. DOI
799   10.1016/j.devcel.2004.12.016.
800 41. Bollerot, K., Pouget, C., and Jaffredo, T. (2005). The embryonic origins of hematopoietic
801   stem cells: a tale of hemangioblast and hemogenic endothelium. APMIS *113*, 790-803.
802   10.1111/j.1600-0463.2005.apm_317.x.
803 42. Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L.,
804   and Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-
805   deficient mice. Nature *376*, 62-66. 10.1038/376062a0.
806 43. Shalaby, F., Ho, J., Stanford, W.L., Fischer, K.D., Schuh, A.C., Schwartz, L., Bernstein,
807   A., and Rossant, J. (1997). A requirement for Flk1 in primitive and definitive
808   hematopoiesis and vasculogenesis. Cell *89*, 981-990. Doi 10.1016/S0092-
809   8674(00)80283-4.
810 44. Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J.C., and Keller, G. (1998). A
811   common precursor for hematopoietic and endothelial cells. Development *125*, 725-732.
812 45. Kim, I., Yilmaz, O.H., and Morrison, S.J. (2005). CD144 (VE-cadherin) is transiently
813   expressed by fetal liver hematopoietic stem cells. Blood *106*, 903-905. 10.1182/blood-
814   2004-12-4960.
815 46. Nasrallah, R., Fast, E.M., Solaimani, P., Knezevic, K., Eliades, A., Patel, R.,
816   Thambyrajah, R., Unnikrishnan, A., Thoms, J., Beck, D., et al. (2016). Identification of
817   novel regulators of developmental hematopoiesis using Endoglin regulatory elements as
818   molecular probes. Blood *128*, 1928-1939. 10.1182/blood-2016-02-697870.
819 47. Kubo, A., Chen, V., Kennedy, M., Zahradka, E., Daley, G.Q., and Keller, G. (2005). The
820   homeobox gene HEX regulates proliferation and differentiation of hemangioblasts and

821   endothelial cells during ES cell differentiation. Blood *105*, 4590-4597. 10.1182/blood-
822   2004-10-4137.
823 48. Park, M.A., Kumar, A., Jung, H.S., Uenishi, G., Moskvin, O.V., Thomson, J.A., and
824   Slukvin, II (2018). Activation of the Arterial Program Drives Development of Definitive
825   Hemogenic Endothelium with Lymphoid Potential. Cell Rep *23*, 2467-2481.
826   10.1016/j.celrep.2018.04.092.
827 49. Marcelo, K.L., Goldie, L.C., and Hirschi, K.K. (2013). Regulation of endothelial cell
828   differentiation and specification. Circ Res *112*, 1272-1287.
829   10.1161/CIRCRESAHA.113.300506.
830 50. Pereira, C.F., Chang, B., Gomes, A., Bernitz, J., Papatsenko, D., Niu, X., Swiers, G.,
831   Azzoni, E., de Bruijn, M.F., Schaniel, C., et al. (2016). Hematopoietic Reprogramming In
832   Vitro Informs In Vivo Identification of Hemogenic Precursors to Definitive Hematopoietic
833   Stem Cells. Dev Cell *36*, 525-539. 10.1016/j.devcel.2016.02.011.
834 51. Azevedo Portilho, N., and Pelajo-Machado, M. (2018). Mechanism of hematopoiesis and
835   vasculogenesis in mouse placenta. Placenta *69*, 140-145.
836   10.1016/j.placenta.2018.04.007.
837 52. North, T.E., de Bruijin, M.F.T.R., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M.,
838   Dzierzak, E., and Speck, N.A. (2002). Runx1 expression marks long-term repopulating
839   hematopoietic stem cells in the midgestation mouse embryo. Immunity *16*, 661-672. Doi
840   10.1016/S1074-7613(02)00296-0.
841 53. Lee, L.K., Ueno, M., Van Handel, B., and Mikkola, H.K.A. (2010). Placenta as a newly
842   identified source of hematopoietic stem cells. Curr Opin Hematol *17*, 313-318.
843   10.1097/MOH.0b013e328339f295.
844 54. Mahony, C.B., and Bertrand, J.Y. (2019). How HSCs Colonize and Expand in the Fetal
845   Niche of the Vertebrate Embryo: An Evolutionary Perspective. Front Cell Dev Biol *7*.
846   UNSP 34

847 10.3389/fcell.2019.00034.
848 55. Breier, G. (2000). Angiogenesis in embryonic development--a review. Placenta *21 Suppl
849   A*, S11-15.
850 56. Drake, C.J., and Fleming, P.A. (2000). Vasculogenesis in the day 6.5 to 9.5 mouse
851   embryo. Blood *95*, 1671-1679.
852 57. Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y.,
853   Bono, F., Devy, L., Beck, H., et al. (2001). Synergism between vascular endothelial
854   growth factor and placental growth factor contributes to angiogenesis and plasma
855   extravasation in pathological conditions. Nat Med *7*, 575-583. 10.1038/87904.
856 58. Palmer, K.R., Tong, S., and Kaitu'u-Lino, T.J. (2017). Placental-specific sFLT-1: role in
857   pre-eclamptic pathophysiology and its translational possibilities for clinical prediction and
858   diagnosis. Mol Hum Reprod *23*, 69-78. 10.1093/molehr/gaw077.
859 59. Hu, D., and Cross, J.C. (2010). Development and function of trophoblast giant cells in
860   the rodent placenta. Int J Dev Biol *54*, 341-354. 10.1387/ijdb.082768dh.
861 60. Tyler, S.R., Rotti, P.G., Sun, X., Yi, Y., Xie, W., Winter, M.C., Flamme-Wiese, M.J.,
862   Tucker, B.A., Mullins, R.F., Norris, A.W., and Engelhardt, J.F. (2019). PyMINEr Finds
863   Gene and Autocrine-Paracrine Networks from Human Islet scRNA-Seq. Cell Rep *26*,
864   1951-1964 e1958. 10.1016/j.celrep.2019.01.063.
865 61. Lee, C.Q.E., Turco, M.Y., Gardner, L., Simons, B.D., Hemberger, M., and Moffett, A.
866   (2018). Integrin alpha2 marks a niche of trophoblast progenitor cells in first trimester
867   human placenta. Development *145*. 10.1242/dev.162305.
868 62. Bader, B.L., Rayburn, H., Crowley, D., and Hynes, R.O. (1998). Extensive
869   vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all
870   alpha v integrins. Cell *95*, 507-519. 10.1016/s0092-8674(00)81618-9.

871 63. Welser, J.V., Lange, N.D., Flintoff-Dye, N., Burkin, H.R., and Burkin, D.J. (2007).
872   Placental defects in alpha7 integrin null mice. Placenta *28*, 1219-1228.
873   10.1016/j.placenta.2007.08.002.
874 64. Wiemers, D.O., Shao, L.J., Ain, R., Dai, G., and Soares, M.J. (2003). The mouse
875   prolactin gene family locus. Endocrinology *144*, 313-325. 10.1210/en.2002-220724.
876 65. Simmons, D.G., Rawn, S., Davies, A., Hughes, M., and Cross, J.C. (2008). Spatial and
877   temporal expression of the 23 murine Prolactin/Placental Lactogen-related genes is not
878   associated with their position in the locus. BMC Genomics *9*, 352. 10.1186/1471-2164-9-
879   352.
880 66. Johnson, M.P., Brennecke, S.P., East, C.E., Goring, H.H., Kent, J.W., Jr., Dyer, T.D.,
881   Said, J.M., Roten, L.T., Iversen, A.C., Abraham, L.J., et al. (2012). Genome-wide
882   association scan identifies a risk locus for preeclampsia on 2q14, near the inhibin, beta
883   B gene. PLoS One *7*, e33666. 10.1371/journal.pone.0033666.
884 67. Marsh, B., and Blelloch, R. (2020). Single nuclei RNA-seq of mouse placental labyrinth
885   development. Elife *9*. 10.7554/eLife.60266.
886 68. Okae, H., Toh, H., Sato, T., Hiura, H., Takahashi, S., Shirane, K., Kabayama, Y.,
887   Suyama, M., Sasaki, H., and Arima, T. (2018). Derivation of Human Trophoblast Stem
888   Cells. Cell Stem Cell *22*, 50-63 e56. 10.1016/j.stem.2017.11.004.
889 69. Paul, S., Home, P., Bhattacharya, B., and Ray, S. (2017). GATA factors: Master
890   regulators of gene expression in trophoblast progenitors. Placenta *60 Suppl 1*, S61-S66.
891   10.1016/j.placenta.2017.05.005.
892 70. Pollheimer, J., Vondra, S., Baltayeva, J., Beristain, A.G., and Knofler, M. (2018).
893   Regulation of Placental Extravillous Trophoblasts by the Maternal Uterine Environment.
894   Front Immunol *9*, 2597. 10.3389/fimmu.2018.02597.
895 71. Arutyunyan, A., Roberts, K., Troule, K., Wong, F.C.K., Sheridan, M.A., Kats, I., Garcia-
896   Alonso, L., Velten, B., Hoo, R., Ruiz-Morales, E.R., et al. (2023). Spatial multiomics map
897   of trophoblast development in early pregnancy. Nature *616*, 143-151. 10.1038/s41586-
898   023-05869-0.
899 72. Charles, M.A., Saunders, T.L., Wood, W.M., Owens, K., Parlow, A.F., Camper, S.A.,
900   Ridgway, E.C., and Gordon, D.F. (2006). Pituitary-specific Gata2 knockout: effects on
901   gonadotrope and thyrotrope function. Mol Endocrinol *20*, 1366-1377. 10.1210/me.2005-
902   0378.
903 73. Zhu, J., Min, B., Hu-Li, J., Watson, C.J., Grinberg, A., Wang, Q., Killeen, N., Urban, J.F.,
904   Guo, L., and Paul, W.E. (2004). Conditional deletion of Gata3 shows its essential
905   function in T(H)1-T(H)2 responses. Nature Immunology *5*, 1157-1165.
906 74. Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data.
907   Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc.
908 75. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P.,
909   Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner.
910   Bioinformatics *29*, 15-21. 10.1093/bioinformatics/bts635.
911 76. Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., and Rossant, J. (1998).
912   Promotion of trophoblast stem cell proliferation by FGF4. Science *282*, 2072-2075.

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Figure legends

Fig. 1: Trophoblast giant cell-specific GATA deletion leads to embryonic lethality and developmental defects. (A) Mating strategy to identify the Prl3d1 positive parietal giant cell layer. (B) Cryosections of the *mT/mG Pl1^Cre* conceptus show exclusive EGFP expression in the parietal TGCs, indicating the specific nature of the *Pl1^Cre* expression. (C) Mating strategy to define the importance of GATA factors in the trophoblast giant cells. (D) E12.5 uterine horn harvested from the above mating contains apparent embryonic resorption sites. (E) Isolated conceptuses associated with GATA Pl1-KO embryos show severe developmental defects (∗) and size differences (∗, ∗) compared to the control (∗). (F) Embryos isolated from one of the

942 extremely small conceptuses (∗) reveal gross developmental defects and embryonic death

943 accompanied by small and thin placental tissue. Scale bar 2 mm. (G) Comparison between a

944 GATA PI1-KO embryo (∗) and non-PI1^{Cre} littermate (*) indicates fetal growth reduction and

945 apparent blood loss , and defective vasculature in both the embryo proper and the placenta.

946 Fetal liver (marked by red arrow) of the GATA PI1-KO embryo also showed reduced

947 hematopoiesis compared to the control. Scale bar 2 mm. (H) GATA PI1-KO placentae shown in

948 G (∗) were of significantly smaller weight compared to the controls (∗) (Mean±s.em., n=21 for

949 the control, n=18 for the KO, **P≤0.01, analyzed by two-tailed Student's t-test). (I) RNAScope

950 labeling of implantation sites using the *Tpbpa* probe marks the spongiotrophoblast layers. Scale

951 bar 1 mm (J) Quantitative comparison of the relative ratio of Tpbpa+ junctional zone area and

952 labyrinth area between the control and GATA PI1-KO implantation sites (Mean±s.em., n=5 for

953 each samples, ***P≤0.001, analyzed by two-tailed Student's t-test).

954

955 **Fig. 2: Single Cell RNA-Seq analyses of the TGC-specific GATA factor loss show altered**

956 **placental trophoblast subpopulation.** (A) A t-SNE plot of the aggregate of the hierarchical

957 clustering of 2 control and 2 GATA PI1-KO placental samples shows 33 distinct clusters. (B) t-

958 SNE plots of the aggregate samples show major cell types. (C) Individual t-SNE plots of the

959 control (aggregate of two control samples) and the KO (aggregate of two KO samples) samples

960 show differences in the major cell subtypes. (D) Individual clusters representing trophoblast

961 subtypes show major differences between the control and the KO placentae. Cluster 3,

962 identified as dead cells/ unidentified cell type, were excluded. (E) Comparative t-SNE plot of

963 cells marked by the expression (log 2-fold expression > 0, p-value <0.05) of trophoblast lineage

964 markers *Prl3d1*, *Prl2c2*, *Tpbpa*, *Prl3b1*, *Hand1* in the control (green box) vs GATA PI1-KO (red

965 box) placentae. Expression in only the trophoblast cells are represented. (F) Quantitative

966 analyses of the relative cell percentages for the *Prl3d1*, *Prl2c2*, *Tpbpa*, *Prl3b1, Hand1, Ly6a,*

35

967 *and Epcam*^{Hi} positive cells. For all markers except *Epcam*^{Hi}, log 2-fold expression > 0; for

968 *Epcam*^{Hi} log 2-fold expression > 3. All cell numbers were normalized using total trophoblast cell

969 numbers corresponding to the sample. (Mean±s.em., n=2 for each sample type, ****P≤0.0001,

970 analyzed by two-tailed Student's t-test).

971

972 **Fig. 3. TGC-specific GATA factor function is essential for the differentiation of the**

973 **hematoendothelial niche.** (A) Comparative t-SNE plots representing the hematopoietic

974 (orange) and endothelial (purple) cell populations in control vs. GATA PI1-KO placentae are

975 shown. Further cluster distribution of these two populations are shown with the orange box

976 (hematopoietic cells) and the purple box (endothelial cells). These subclusters are described in

977 Fig. 2. (B, C) scRNA-seq analyses show cluster 15 and 17 to harbor the *Kit+ Cd34+ Ly6A+*

978 population of HSCs. The t-SNE plots show a marked reduction in the overall cell numbers in

979 cluster 17 and huge increase in the cluster 15 of the GATA PI1-KO placentae compared to the

980 control (Mean±s.e.m., n=2 for each sample type, **P≤0.01, analyzed by unpaired Student's t-

981 test, Two-stage step-up (Benjamini, Krieger, and Yekutieli)). (D) Quantification of the HSC

982 population in cluster 17 of the control vs. GATA PI1-KO placentae. The total number of cells in

983 each sample type were used to calculate the percentage ((Mean±s.e.m., n=2 for each sample

984 type). (E) Endothelial marker *Kdr* is highly expressed in cluster 15, unlike cluster 17, indicating

985 hematoendothelial nature of the cells belonging to cluster 15. This cluster also harbors cells

986 positive for Arterial-specific marker *Dll4* and placental hemogenic endothelium marker *Prom1*.

987 (F) Flow cytometry analyses of Lin⁻ CD45⁻ CD34⁺ KDR⁺ cells show significant increase in the

988 absence of pTGC-sepcific GATA factors. (G) Box plot of the flow analysis in the panel F. The

989 percentages were calculated against total number of viable cells per sample (Mean±s.em., n=19

990 for the control and n=18 for the GATA PI1-KO, **P≤0.01, analyzed by two-tailed Student's t-

991 test).

992

993 **Fig. 4: GATA factor loss in the TGCs results in the loss of the hematopoietic progenitor**

994 **population.** (A) Flow analysis data shows a significant reduction in the HSC population in the

995 knockout placenta. E12.5 placental samples from GATA PI1-KO and control littermate placentae

996 were subjected to flow analysis. Samples were gated for viable cells, followed by gating for

997 single cells. This was followed by selecting cells negative for lineage markers (Lin$^-$) and

998 subjecting them to CD45 negative selection. The Lin$^-$ CD45$^-$ population were further gated for

999 CD34$^+$ and Ly6A/E$^+$ fraction. Finally these Lin$^-$ CD45$^-$ CD34$^+$ Ly6A/E$^+$ cells were gated for KIT$^+$

1000 population. The sample gating schematics are depicted using cell numbers representative of the

1001 respective panels. Black arrows show the flow of the steps. (B) Quantitation of the Lin$^-$ CD45$^-$

1002 CD34$^+$ KIT$^+$ Ly6A/E$^+$ population reveals significant loss of the HSC population in the KO sample.

1003 The counts are normalized against the number of viable cells and are presented as percentages

1004 (Mean±s.e.m., n=26 for the control and n=24 for the KO, **P≤0.01, analyzed by two-tailed

1005 Student's t-test). (C) Micrographs show the formation of hematopoietic cell colonies on a

1006 methylcellulose plate. Colonies included were of CFU-G/M, BFU-E, and CFU-GEMM in nature.

1007 (D) Quantitation of the number of colonies in the control vs. GATA PI1-KO samples. The counts

1008 are normalized against the number of cells plated for each sample (10,000 cells) (Mean±s.e.m.,

1009 n=7 for each sample type, *P<0.05, analyzed by two-tailed Student's t-test).

1010

1011 **Fig. 5: GATA factor-functions in the trophoblast giant cells are essential for the**

1012 **vasculature development at the maternal-fetal interface.** (A-B) Immunostaining of the control

1013 and GATA PI1-KO placenta with the endothelial cell marker CD31 shows abnormal vasculature

1014 development in the GATA PI1-KO in the TGC layer (Scale Bar 200μm). (C) Violin plot shows

1015 *Flt1* expression increases in the GATA PI1-KO placentae compared to the control. The plot

1016 (*log2* expression) and the values are derived from the scRNA-seq data and calculated using

1017 Loupe Browser (v 4.1.0) (10x Genomics). For the control, mean=1.63, median=1; for the KO

1018 mean=2.16, median=1.58. n=2 for both sample tytpes. (D) t-SNE plots for *Flt1* expression
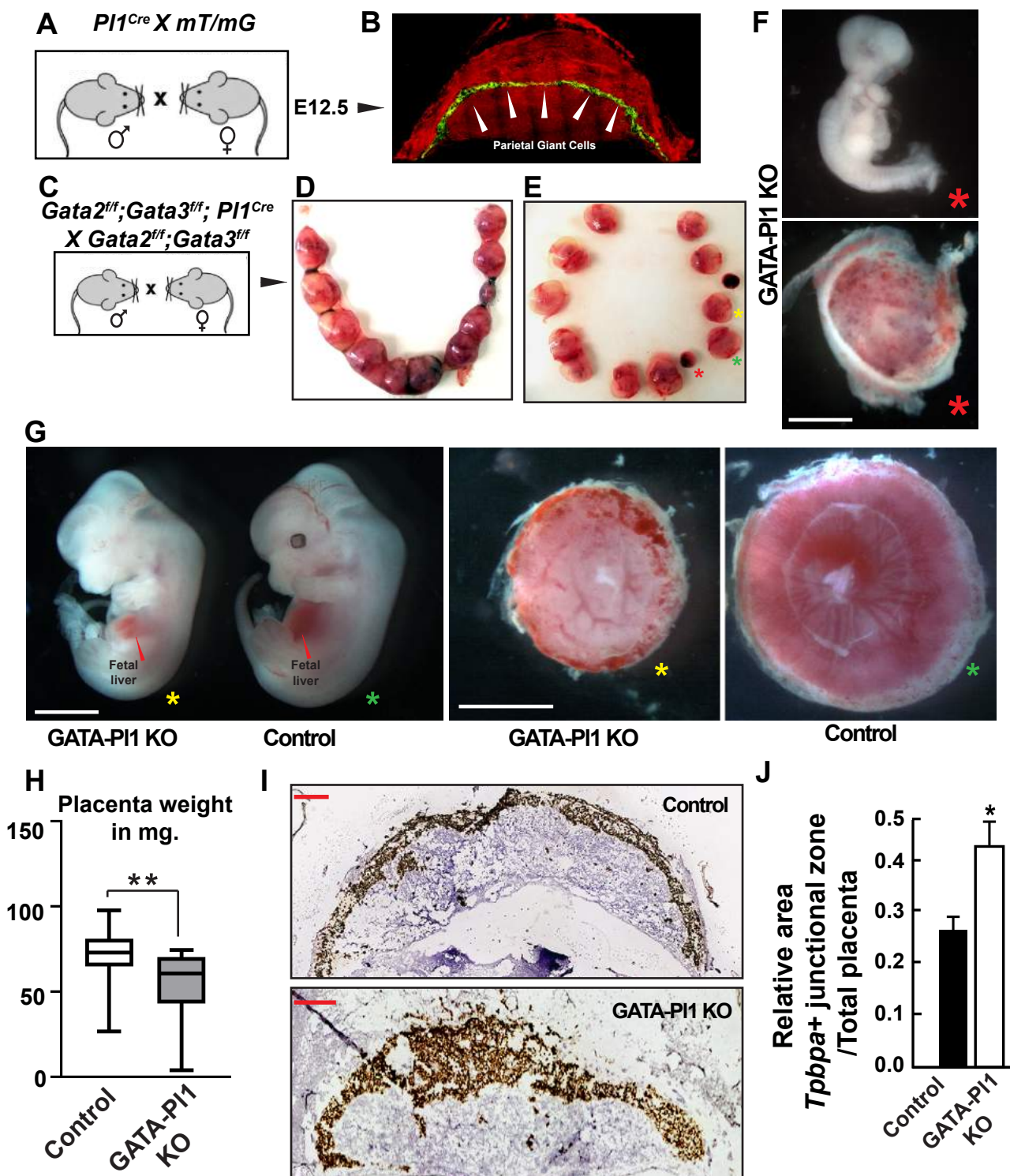
1019   across the clusters show marked increase in the number of *Flt1* expressing cells in the GATA

1020   PI1-KO placentae than control. Some of the major source of *Flt1* expression appears to be the

1021   TGC cluster 33, spiral artery associated TGC cluster 26, hematoendothelial cluster 15 and

1022   spongiotrophoblast trophoblast clsuters. (E) Heatmap of cluster-specific *Flt1* expression shows

1023   that the largest change in the *Flt1* expression occurs in the TGC cluster 33 (n=2 for each

1024   sample type). (F) In the presence of conditioned medium from GATA2/GATA3 double knockout

1025   (GATA DKO) trophoblast stem cells, HUtMECs fail to form vascular ring-like structures in a

1026   matrigel based vascular tube formation assays unlike when treated with conditioned medium

1027   from control trophoblast stem cells (n=3, Scale Bar 200μm).
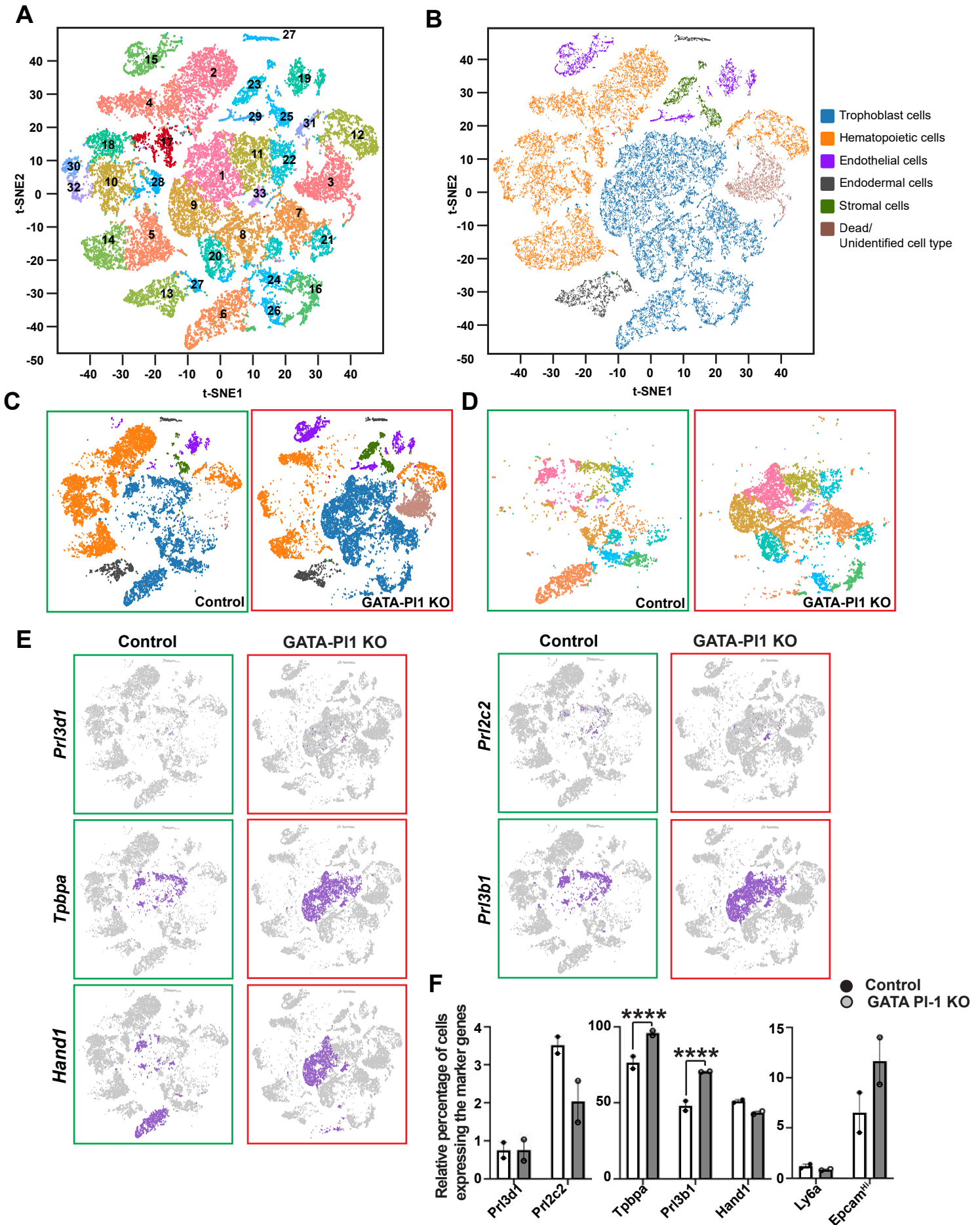
1028

1029   **Fig. 6: The GATA factors regulate parietal TGC-specific paracrine factors and help**

1030   **maintain hematopoietic-angiogenic balance.** (A) PyMINEr based prediction of the enriched

1031   paracrine ligands expressed by cells in cluster 33 shows receptor matched targets in four major

1032   clusters that include HSPC/ HSC containing cluster 33, hematoendothelial cluster 15,

1033   trophoblast progenitor cluster 6 and labyrinthine trophoblast cluster 16. Major signaling

1034   pathways involved Integrin signaling pathway members lamin and collagen group 4 of proteins

1035   and prolactin family of hormones. (B) *Prl3d1* expressing cells in cluster 33 were further analyzed

1036   for the level of paracrine factors expression. While almost all the Integrin pathway members

1037   were upregulated, all the prolactin family members showed downregulation in the GATA PI1-KO

1038   samples compared to the control. Average log2 fold change expression values are plotted. (C)

1039   Immunostaining of E13.5 placental sections with LAMBI and LAMB2 antibodies show increased

1040   expression for both the proteins in the pTGCs of the KO samples than controls (Scale Bar
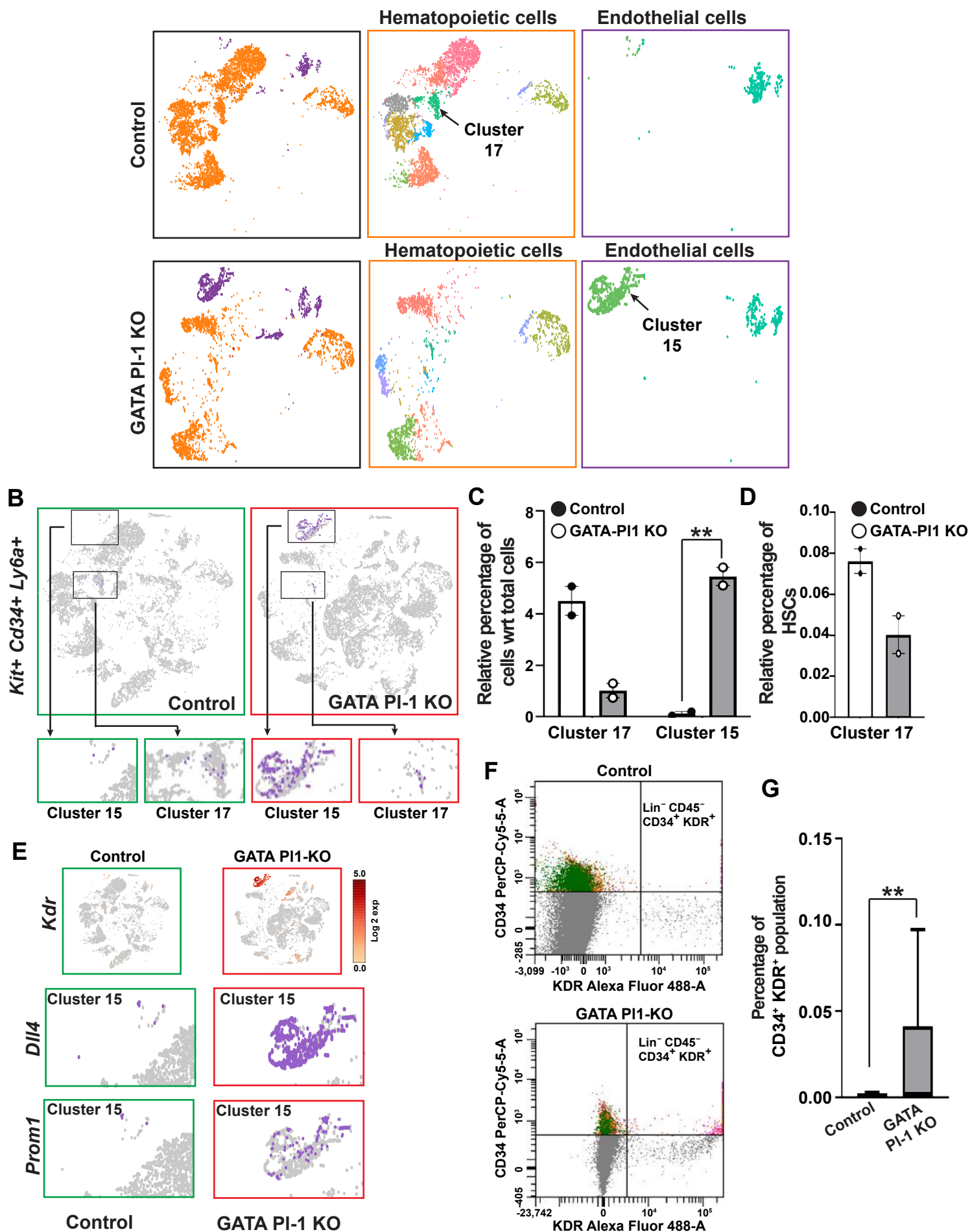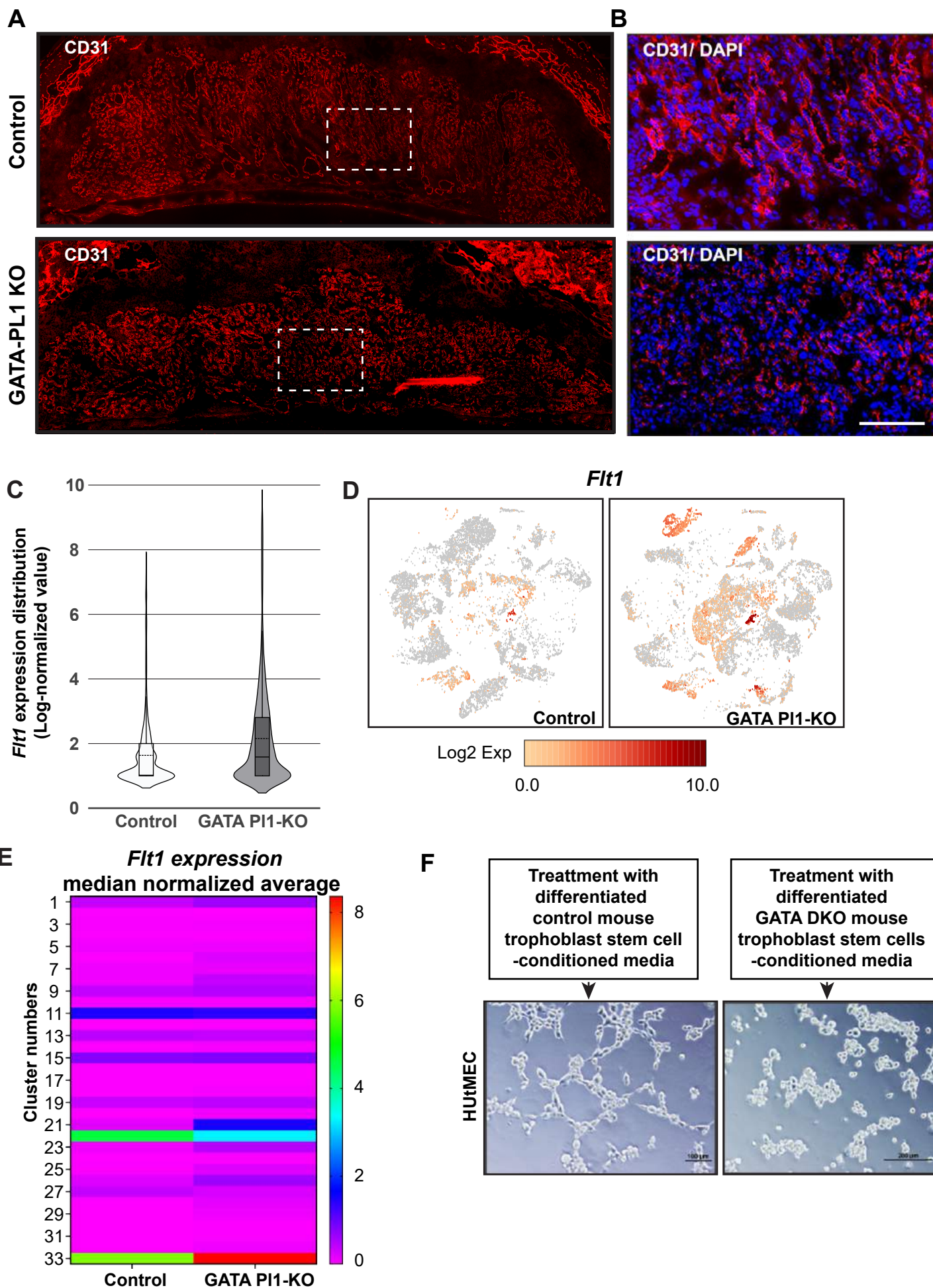
1041   100μm).

1042

1043 **Fig. 7: TGC-specific Integrin signaling and Prolactin signaling govern placental**

1044 **hematoendothelial differentiation and placental development.** Schematic representation

1045 mid-gestation mouse placenta shows GATA factors regulate parietal-TGC-specific Integrin and

1046 Prolactin signaling. Repression of Integrin signaling and activation of Prolactin signaling by the

1047 GATA factors are achieved in the TGCs, which in turn helps angiogenic development, HSC

1048 development, and proper trophoblast differentiation in a control mouse placenta. In the absence

1049 of the GATA factors, an increase in the Integrin signaling and repression of the Prolactin

1050 signaling trigger loss of angiogenic branching and HSC population. This is accompanied by

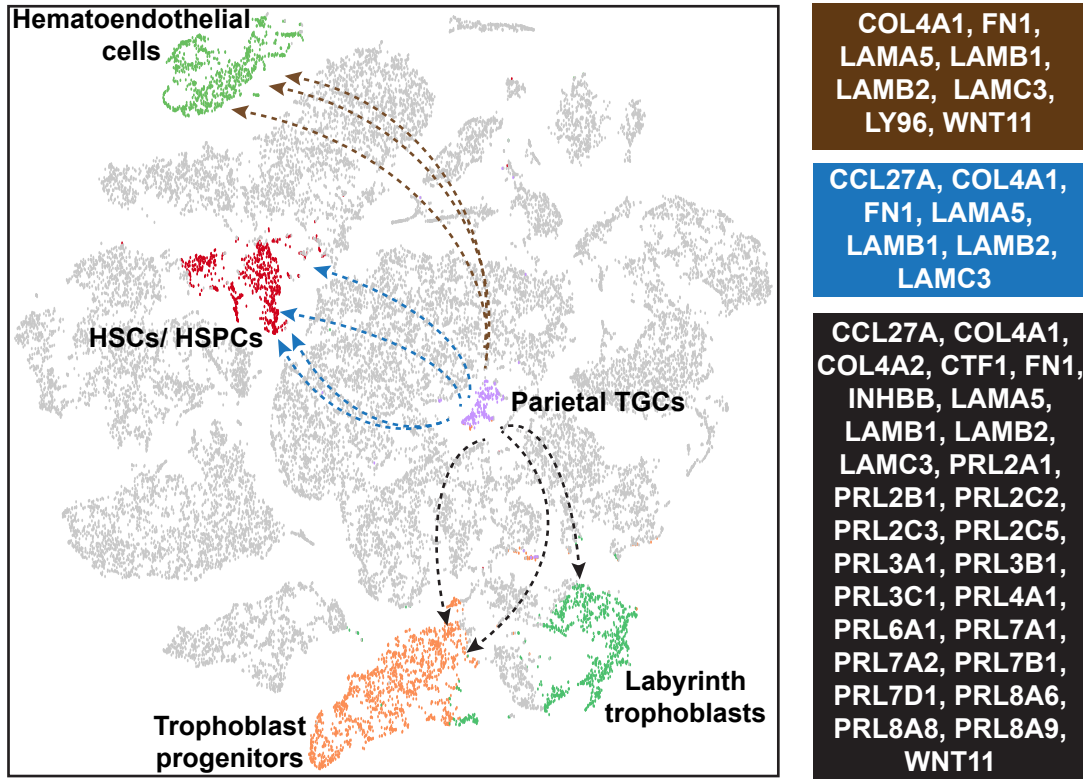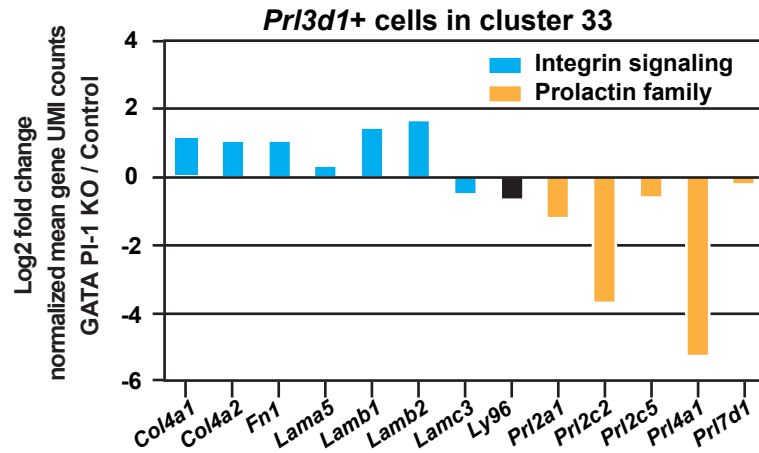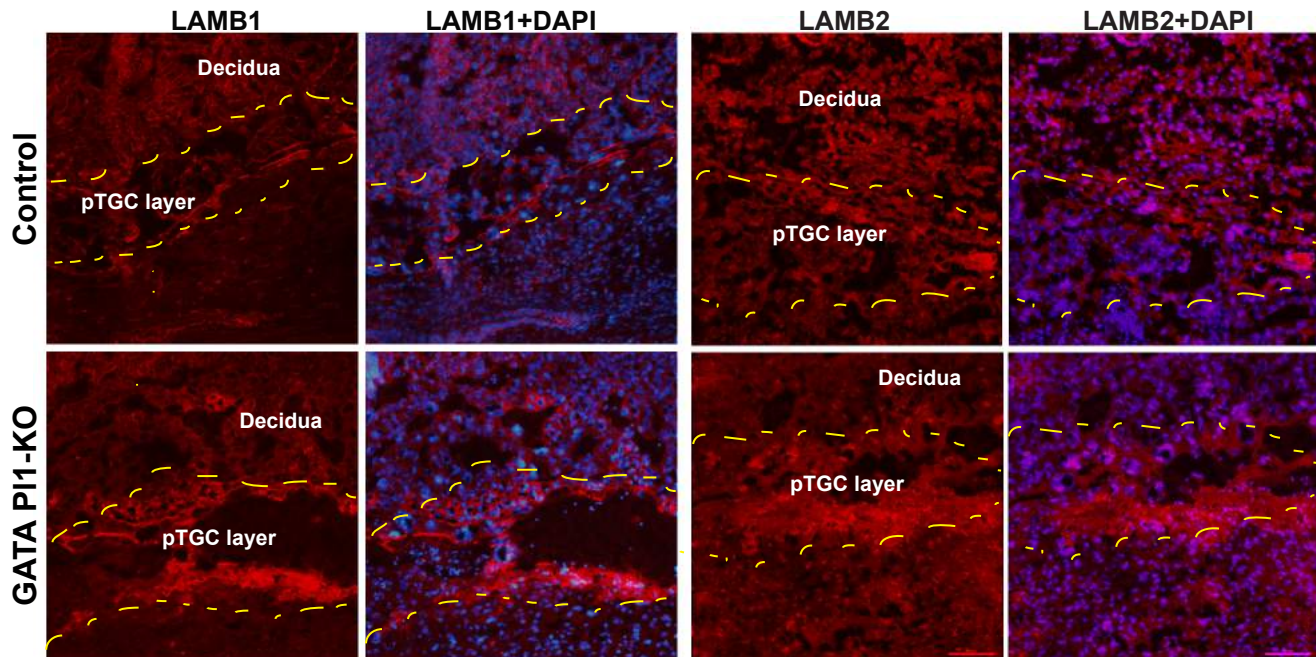1051 abnormal trophoblast differentiation and trophoblast subtype distribution.

A *PI1^{Cre} X mT/mG*

B E12.5 ▶ Parietal Giant Cells

C *Gata2$^{f/f}$;Gata3$^{f/f}$; PI1^{Cre} X Gata2$^{f/f}$;Gata3$^{f/f}$*

D

E

F GATA-PI1 KO

G GATA-PI1 KO — Fetal liver — Control — Fetal liver — GATA-PI1 KO — Control

H Placenta weight in mg.

I Control — GATA-PI1 KO

J Relative area *Tpbpa*+ junctional zone /Total placenta

Home_Fig. 2

A

Hematoendothelial cells

HSCs/ HSPCs

Parietal TGCs

Trophoblast progenitors

Labyrinth trophoblasts

COL4A1, FN1, LAMA5, LAMB1, LAMB2, LAMC3, LY96, WNT11

CCL27A, COL4A1, FN1, LAMA5, LAMB1, LAMB2, LAMC3

CCL27A, COL4A1, COL4A2, CTF1, FN1, INHBB, LAMA5, LAMB1, LAMB2, LAMC3, PRL2A1, PRL2B1, PRL2C2, PRL2C3, PRL2C5, PRL3A1, PRL3B1, PRL3C1, PRL4A1, PRL6A1, PRL7A1, PRL7A2, PRL7B1, PRL7D1, PRL8A6, PRL8A8, PRL8A9, WNT11

B

Prl3d1+ cells in cluster 33

Log2 fold change normalized mean gene UMI counts GATA PI-1 KO / Control

Integrin signaling
Prolactin family

Col4a1, Col4a2, Fn1, Lama5, Lamb1, Lamb2, Lamc3, Ly96, Prl2a1, Prl2c2, Prl2c5, Prl4a1, Prl7d1

C

| | LAMB1 | LAMB1+DAPI | LAMB2 | LAMB2+DAPI |

Control

Decidua

pTGC layer

GATA PI1-KO

Decidua

pTGC layer

A

B

C Control   GATA PI-1 KO

D

Home_Fig. 7