Ccl2/Ccr2 signalling recruits a distinct fetal microchimeric population that rescues delayed maternal wound healing

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Foetal microchimeric cells (FMCs) traffic into maternal circulation during pregnancy and persist for decades after delivery. Upon maternal injury, FMCs migrate to affected sites where they participate in tissue healing. However, the specific signals regulating the trafficking of FMCs to injury sites had to be identified. Here we report that, in mice, a subset of FMCs implicated in tissue repair displays CD11b\textsuperscript{+} CD34\textsuperscript{+} CD31\textsuperscript{+} phenotype and highly express C-C chemokine receptor 2 (Ccr2). The Ccr2 ligand chemokine ligand 2 (Ccl2) enhances the recruitment of FMCs to maternal wounds where these cells transdifferentiate into endothelial cells and stimulate angiogenesis through Cxcl1 secretion. Ccl2 administration improves delayed maternal wound healing in pregnant and postpartum mice but never in virgin ones. This role of Ccl2/Ccr2 signalling opens new strategies for tissue repair through natural stem cell therapy, a concept that can be later applied to other types of maternal diseases.
Foetal microchimeric cells (FMCs) enter maternal circulation, engraft into various organs and persist for decades after delivery, probably for the mother’s remaining lifespan. Foetal cells nest in maternal bone marrow (BM) and remain well tolerated by the maternal immune system. FMCs have been reported to contain progenitor cells, including lymphoid, hematopoietic, mesenchymal and cardiomyocyte progenitors. Upon various types of maternal injury, these cells can be triggered to migrate to various maternal tissues in which they will adopt the phenotype of the damaged organ. Indeed, FMCs are able to differentiate into neurons, hepatocytes, endothelial cells, thyroid, cervix and gut cells in concerned maternal tissues.

Wound healing is an intricate, interactive biological process. There is a well-coordinated interplay between tissue structures and residential cells as well as distant cells from BM that are essential for the healing process. Studies using chimeric mice with tagged BM identified various types of BM-derived endothelial progenitor cells (EPCs) participating in wound angiogenesis. The first reported one is a CD34+/CD31+/VEGFR2+/CD31+/CD45− population that can form blood vessels. Interestingly, others identified a CD34+/VEGFR2+/CD31+/CD45− population in maternal wounds during and after delivery. We have also investigated the Ccr2-mediated chemotactic cues in skin wounds. We next investigated the Ccr2-mediated chemotactic cues in skin wounds.

Monocytes secrete Ccl2 early in skin wound healing. We next investigated the Ccr2-mediated chemotactic cues in skin wounds. Ccl2 mRNA and protein levels increased strongly in skin wounds 1 day after injury, subsequently decreasing on day 3 (Fig. 1h,k). Ccl2 mRNA levels followed a parallel pattern, increasing on day 1 then decreasing on day 3 (Fig. 1i). Meanwhile, the levels of Ccl8, another Ccr2 ligand, peaked later in the wound, on day 3 (Fig. 1i). Thus Ccl2/Ccr2 signalling corresponds with the observed early peak of postwounding fetal cells.

Ccl2 recruits fetal cells to maternal wounds. Given the overexpression of Ccr2 in FMCs upon wounding, the high percentage of FMCs expressing Ccr2 on their surface and the overexpression of Ccl2 in wound tissue during early stages of healing, we hypothesized that Ccl2 mediates the early recruitment of FMCs to wounds. We therefore injected physiological dose of Ccl2 (50 ng) or PBS into the wound bed on days 0 and 2 after wounding in E15.5 pregnant or virgin mice (Fig. 2a). The numbers of eGFP+ cells in blood did not differ between the mice receiving Ccl2 and PBS on day 7 (Supplementary Fig. 3a). In contrast, there were 2.03-folds more eGFP+ cells in skin wound tissues into which Ccl2 had been injected as in control wounds (Fig. 2b,c). Consistent with our FACS results, by performing immunofluorescence staining, we detected 3.38-folds more as many eGFP+ cells in the sections of mice receiving Ccl2 as in those receiving PBS.

Results

Maternal skin wounding recruits FMCs through Ccr2 signalling.

We investigated the molecular mechanisms that selectively recruit fetal cells into maternal skin wounds in mice. We find that C-C chemokine receptor 2 (Ccr2) was overexpressed on FMCs upon maternal wounding. Ccr2 is a chemokine receptor expressed on BM progenitor cells as well as monocytes. Chemokine ligand 2 (Ccl2), its main ligand, is secreted by endothelial cells and macrophages. It triggers the recruitment of Ccr2+ various cell types from the marrow to the secreting site. Here Ccl2, given at physiological doses, was able to induce maternal neangiogenesis and to improve skin healing by recruiting FMCs. Importantly, such effect of Ccl2 was restricted to pregnant and postpartum mice and was dependent on Ccr2 expression on FMCs. Our results pave the way for natural stem cell therapy for tissue repair in diseased females.
Figure 1 | Maternal wounding activates FMCs and induces Ccr2 production. Quantification by FACS of fetal cells in the bone marrow (a), blood (b) and skin/wound (c) after maternal skin injury (n = 4–9). (d) PCR array analysis of cytokine and chemokine gene expression in FMCs sorted from maternal bone marrow in mice with or without wounds on day 3 (n = 6). White bars represent the ligand genes and black bars represent the receptor genes. (e) RNA sequencing of circulating FMCs in maternal blood during pregnancy with or without wounds. (f) Labelling for Ccr2 (red) and natural eGFP (green) fluorescence of the wounds (day 1 after wounding) of pregnant female mice carrying eGFP+ foetuses. White arrowheads indicate colocalization. Scale bars: 50 μm. (g) FACS analysis of Ccr2+ cells among eGFP+ FMCs in the peripheral blood, with and without maternal wounding (n = 3). Quantitative RT-PCR analysis for Ccr2 (h) and its ligand Ccl2 (i) and Ccl8 (j). Levels of mRNA normalized against mRNA levels for Gapdh in normal skin and wounds (n = 3). (k) Western blot of Ccr2 in normal skin and wounds at different time points. Representative results from two independent experiments are shown. (l) Dual labelling for Ccl2 (red) and F4/80 (green) in the wound on day 1. White arrowheads indicate colocalization. Dual labelling for GR-1 (green) and Ccl2 (red) in a wound on day 1, with white arrowheads showing single staining for GR-1. Dual labelling for Ccl2 (green) and CD31 (red) in the wound. Scale bars: 50 μm. (m) Quantitative RT-PCR analysis for Ccl2 in sorted leukocytes from day 1 wounds. Student’s t-test, *P<0.05; mean ± s.e.m.
receiving PBS (Fig. 2d,e). The enhanced mobilization of eGFP⁺ cells in wounds upon Ccl2 administration was also found at days 3 and 5 (Supplementary Fig. 4). Therefore, Ccl2 is able to recruit FMCs to wound sites throughout the cutaneous healing process.

**Ccl2 improves wound healing by enhancing neovascularization.** We then analysed the effect of local Ccl2 treatment on maternal wound healing. On days 3, 5 and 7 after wounding, the neo-epidermal tongue, which reflects the healing without...
We analysed virgin female Cer2KO/KO mice, female Cer2KO/KO mice mated with eGFP+ males and female Cer2KO/KO mice mated with eGFP+ male mice bearing Cer2KO/KO foetuses, Ccl2 injections do not enhance wound healing at any day (Fig. 3c,d). In contrast, when Cer2KO/KO female mice bear Cer2KO/KO foetuses, Ccl2 decreases wounded area by 49.08, 28.96 and 57.58% at days 2, 5 and 7, respectively (Fig. 3e,f). Interestingly, this ratio is similar to the ratio we found with Cer2 in WT mice. In addition, only Cer2KO/KO mice bearing Cer2KO/WT/KO foetuses displayed an increase in fetal cell infiltrate in granulation tissue upon Ccl2 local injections (Fig. 3g,h). Finally, the virgin Cer2KO/KO mice did not show any change when treated with Ccl2 (Fig. 3a,b). Therefore, all these data demonstrate that Cer2 enhances wound healing through Cer2-dependent fetal cell recruitment to wound bed.

### Figure 2 | Ccl2 recruits FMCs to maternal wounds and improve skin wound healing in pregnant mice.

(a) Experimental design: an 8 mm wound was created in pregnant female mice carrying eGFP+ foetuses. We injected Ccl2 or PBS into the wound immediately and 2 days after skin excision. (b) FACS analysis showed that there were significantly larger numbers of eGFP+ cells in the wounds of pregnant mice treated with Ccl2 than in those treated with PBS (n = 3). (c) Quantification of eGFP+ cells in wounds on day 7 for pregnant mice receiving injections of PBS or Ccl2 (n = 3). (d) Wound sections from pregnant mice carrying eGFP+ foetuses after the injection of PBS or Ccl2. Representative micrographs of the spontaneous fluorescence of eGFP+ (green) cells in granulation tissue, indicated by white arrowheads. Scale bars: 50 μm. (e) Quantification of eGFP+ cells in sections of wounds from pregnant mice carrying eGFP+ foetuses after the injection of PBS or Ccl2. Representative micrographs of the spontaneous fluorescence of eGFP+ (green) cells in granulation tissue, indicated by white arrowheads. Scale bars: 50 μm. (f) Time course of skin wound healing. Scale bars: 1 mm. (g) Planimetry of the wound area relative to the initial wound area at various time points (n = 5). (h) Anti-Ki14 (red) labelling of neoeidermal tongues and gaps in the wound. Scale bars: 1 mm. (i) Measurement of neoeidermal tongues and gaps at wound sites (n = 5). (j) Dual labelling for CD31 (red) and Lyve1 (green) in the wound. Scale bars: 50 μm. (k) Quantification of the relative vessel area per 20 field by fluorescence densitometry (n = 4). (l) Quantification of the number of vessel types per 20 field (n = 4). (m) Representative micrograph of a wound section labelled for vWF (red) and displaying spontaneous eGFP fluorescence (green), demonstrating the recruitment of FMCs to wound sites to form a vessel wall. Scale bars: 50 μm. (n) Quantification of vWF+ eGFP+ double-positive vessels in 4 × field (n = 4). (o) FACS analysis showed that there were significantly larger numbers of eGFP+ CD31+ cells in the wounds of pregnant mice treated with Ccl2 than in those treated with PBS (n = 3). Student’s t-test, *P* < 0.05; mean ± s.e.m.
**Figure 3 | FMCs are recruited to maternal wounds through Ccl2/Ccr2 pathway.** An 8 mm wound was created in pregnant female mice carrying eGFP + foetuses. We injected Ccl2 or PBS into the wound immediately and 2 days after skin excision. (a) Experimental design and time course of skin wound healing from Ccr2 KO/KO virgin mice. (b) Planimetry of the wound area relative to the initial wound area at various time points from Ccr2 KO/KO virgin mice (n = 5). (c) Experimental design and time course of skin wound healing from Ccr2 KO/KO female mice mated with eGFP KO Ccr2 KO male mice. (d) Planimetry of the wound area relative to the initial wound area at various time points from Ccr2 KO/KO female mice mated with eGFP KO Ccr2 KO male mice (n = 5). (e) Experimental design and time course of skin wound healing from Ccr2 KO/KO female mice mated with eGFP male mice. Scale bars, 1 mm. (f) Planimetry of the wound area relative to the initial wound area at various time points (n = 5) from Ccr2 KO/KO female mice mated with eGFP male mice. (g) Representative micrographs of the spontaneous fluorescence of eGFP + (green) cells in granulation tissue indicated by white arrowheads. Scale bars, 50 μm. (h) Quantifications of eGFP + cells in sections of wounds from pregnant mice after injections of PBS or Ccl2 (n = 3). Student's t-test, *P < 0.05; mean ± s.e.m.
Peripheral mononuclear blood cells (PBMCs) were collected from pregnant female mice carrying eGFP+ foetuses or from virgin control mice with and without wounds, on days 0–3 for FACS analysis. (a,b) Representative FACS results for CD34/CD31 staining in the CD11b+ gate for virgin mice, with quantification (n=3). (c,d) Representative FACS results for CD34/CD31 staining in the CD11b+ gate in maternal cells (eGFP−) from pregnant mice, with quantification (n=3). (e,f) Representative FACS results for CD34/CD31 staining in the CD11b+ gate for fetal cells (eGFP+) from pregnant mice (n=3). (b,d,f) Percentage of CD11b+ CD34+ CD31+ cells. Pregnant female mice carrying eGFP+ foetuses were wounded and PBS or Ccl2 was injected into the wound immediately and 2 days after wounding. PBMCs and wounds were collected 7 days later for FACS analysis. (g,h) PBMCs and (i,j) wound tissues were analysed to determine the levels of maternal CD11b+ CD34+ CD31+ MPCs (eGFP− gate) and fetal CD11b+ CD34+ CD31+ MPCs (eGFP+ gate) present after the administration of PBS or Ccl2. (g,i) Representative FACS results for CD34/CD31 staining in the CD11b+ /eGFP−/+ gate. (h,j) Percentage of CD11b+ CD34+ CD31+ cells in the eGFP−/+ gate (n=4). Student’s t-test, *P<0.05; mean ± s.e.m.
virgin wounded transgenic eGFP⁺ female mice (Fig. 5a). The cells were injected, on day 1, into the wounds of WT C57BL/6 virgin mice. At day 7, wounds injected with eGFP⁺ fetal MPCs contained vWF⁺ endothelial cells and were able to form a full vessel (Fig. 5b and Supplementary Fig. 11). In addition, some eGFP⁺ fetal MPCs-derived cells expressed smooth muscle cell actin (α-SMA) (Fig. 5c) but never macrophage markers (F4/80) (Fig. 5d). By contrast, following the injection of eGFP⁺ adult
MPCs into wounds, no endothelial or myofibroblastic eGFP+ cells were detected (Fig. 5b,c). Thus only fetal MPCs have the specific properties required for differentiation into endothelial and mural cells in maternal wounds.

We also investigated how fetal MPCs enhance angiogenesis in maternal wounds. We sorted fetal and maternal MPCs from wounded tissues of mice pregnant with eGFP+ foetuses on day 3 after wounding (Fig. 5h). We studied the expression profiles of genes associated with angiogenesis pathways by PCR array analysis (Fig. 5i,k). Five mRNA transcripts were upregulated in the fetal MPCs, including Cxcl1, Sphk1 and TGF-β2, Cxcl1 being the highest increased one. Immunofluorescence analysis on wound sections into which fetal or adult MPCs were injected showed that only injected fetal MPCs in mice expressed Cxcl1 (Fig. 5j). Foetal MPCs expressed lower levels of Thbs2 and Bat1, two angiogenesis inhibitors (Supplementary Data 2).

Foetal MPCs form proliferative clusters and express Ccr2. The question of the clonal origin of the fetal population has repeatedly been raised and is beyond the scope of this article. We nevertheless examined wounds from virgin adult mice into which either adult or fetal MPCs had been injected. At day 7, wound sections into which we had injected adult MPCs contained isolated cells adult MPCs (Fig. 5e,f), whereas those injected with fetal cells contained clusters of eGFP+ Ki67+ cells (Fig. 5e–g). Interestingly, nearly all fetal but <1% of the adult MPCs expressed Ccr2 (Supplementary Fig. 12a–c). Furthermore, using FACS analysis performed on the blood of pregnant mice carrying eGFP+ foetuses, Ccr2 was expressed by 85.77 ± 1.048% of fetal MPCs and by only 0.313 ± 0.128% of their maternal counterparts (Supplementary Fig. 12e,f).

Ccl2 rescues delayed wound healing in postpartum mice. When present, wound-healing disorders affect adult females years after delivery. Since fetal cells persist in maternal BM throughout life1, we investigated the effects of Ccl2 in a delayed wound-healing model, in the early and late postpartum periods. Two weeks or 6 months after delivery, females that had given birth to eGFP+ pups were treated daily with a topical application of 0.05% clobetasol on the lower back for 12 days. At that stage, the skin was atrophic. Excisional skin wounds were made in the clobetasol-treated areas. Ccl2 or PBS was injected into the wounds on days 0 and 2 after wounding (Fig. 6a). Ccl2 injections improved wound closure kinetic, neopelidermal tongues, epidermal and dermal cell proliferation and blood vessel angiogenesis in these mice (Fig. 6b–k). By contrast, lymphangiogenesis, as measured by LYVE1+ lymphatic vessel density and VEGF-C and VEGFR3 gene expression, and inflammation, as measured by the number of GR1+ and F4/80+ cells in granulation tissue, did not change after Ccl2 injections (Fig. 6h–k and Supplementary Fig. 13a–c). Importantly, Ccl2 recruited more fetal cells to the wound bed than PBS, as demonstrated by FACS analysis (Fig. 6l,m). Therefore, Ccl2 recruits FMCs and improves delayed wound healing in the early and late postpartum in mice (Supplementary Fig. 14a–h). Finally, Ccl2 treatment did not induce any systemic effect, with no impact on blood cells count, glycaemia and gamma-glutamyltransferase dosage (Supplementary Table 1).

Discussion

We and others have shown that fetal progenitor cells are involved in maternal repair, including wound-healing processes, as well as in the liver, thyroid, myocardial infarction and brain excitotoxic lesions in humans and/or mouse models10–13. Here we purified fetal cells specifically involved in wound healing and determined their transcriptional profiles. These cells appeared to overexpress chemokine receptor Ccr2. We found that local injections of physiological doses of Ccl2, a Ccr2 ligand, improved skin healing only in pregnant and postpartum mice (Fig. 7). These data provide the first demonstration that a specific pathway involving physiological doses of Ccl2 acts exclusively through the mobilization of FMCs. Moreover, we have been able to show that Ccl2’s ability to recruit FMCs in maternal wound healing was dependent upon Ccr2 expression on fetal cells. Previous studies have shown that, even at higher doses, Ccl2 has no effect on normal wound healing in virgin mice30. Ccl2 injections enhance wound healing in db/db mice, in which Ccl2 secretion is impaired31. Ccl2 plays a role in tissue repair, because complete Ccl2 deficiency results in an impairment of myeloid cell recruitment and skin healing32. Thus our data show for the first time the selective recruitment of fetal progenitor cells through low doses of Ccl2 into maternal injured tissue. This translates into enhanced maternal healing both during pregnancy and much later after delivery, in conditions of normal or delayed skin healing.

One key question concerns the advantages associated with the recruitment of fetal rather than adult cells. We identified the fetal cells mobilized by Ccl2 and recruited to wounds as CD11b+ CD34+ CD31+ cells. These proliferated in clusters and differentiated into vWF+ endothelial cells and α–SMA+ mural cells within the wound. By contrast, adult cells of the same phenotype never formed clusters; they had a low proliferative index and did not differentiate into vascular cells of any type. Our data indicate that such fetal progenitors, even when persisting in an adult, are more pluripotent and proliferative than adult progenitors in accordance with others33. Our results are consistent with the findings of several studies reporting an absence of adult EPCs22,23, whereas such progenitors are found in the fetal compartment. Furthermore, fetal CD11b+ CD34+ CD31+ cells have an mRNA profile different from that of their adult counterparts, as they overproduce various proangiogenic molecules, including Cxcl1. Cxcl1/Cxcr2 signalling plays a role in the regulation of fetal progenitor cell mobilization and their recruitment to maternal wounds where they participate in tissue repair.

Figure 5 | In vivo integration of transplanted fetal MPCs into the wound and fetal MPCs overproduce Cxcl1 in wounds. (a) Experimental design: female Cag-eGFP mice or pregnant female mice carrying eGFP+ foetuses were wounded and eGFP+ CD11b+ CD34+ CD31+ myeloid progenitor or fetal MPCs were isolated from blood on the day after wounding. The recipient mice were normal virgin females with the same genetic background as the donor mice. We transplanted 1 × 10⁶ adult MPCs into the wounds of recipient mice on day 1 after wounding, and the wound was harvested on day 7. Cryosections showing (b) anti-vWF (red) labelling, (c) anti-α-SMA (red) labelling, (d) anti-F4/80 (red) labelling, (e) anti-Ki67 (red) labelling and spontaneous eGFP (green) fluorescence. White arrowheads indicate colocalization. Cryosections showing (f) spontaneous eGFP (green) fluorescence after the transplantation of adult MPCs or fetal MPCs in the wounds of virgin mice. Scale bars: 50 μm. (g) Quantification of eGFP+ cells in the granulation tissue after the transplantation of MPCs or fetal MPCs. Experimental design: (h) pregnant female mice carrying eGFP+ foetuses were wounded and eGFP+ CD11b+ CD34+ CD31+ fetal MPCs and maternal eGFP+ CD11b+ CD34+ CD31+ MPCs were isolated from wound tissue on day 3. We extracted mRNA from these cells and performed a high-throughput PCR array analysis. (i) PCR array analysis of angiogenesis-associated gene expression in fetal MPCs (black bars) and MAPCs (white bars) from mice (n = 3). Cryosections showing (j) anti-Cxcl1 (red) and displaying spontaneous eGFP (green) fluorescence. Scale bars: 50 μm. (k) Quantitative RT-PCR validation of Cxcl1 mRNA levels normalized against mRNA levels for Gapdh (n = 3). Student’s t-test, *P < 0.05; mean ± s.e.m.
crucial role in neovascularization during skin repair. Foetal progenitors persisting in the adult therefore reach maternal tissues and improve angiogenesis via the formation of new vessels derived from fetal cells and the stimulation of maternal angiogenesis.

The injection of as few as $10^4$ fetal cells led to clusters of proliferating fetal cells in the granulation tissue of injected wounds. Similarly, the maternal wounds displaying an improvement of healing after Ccl2 injection contained only $6.365 \pm 0.332\%$ fetal cells. Thus only very small numbers of fetal
cells are sufficient to improve both normal and delayed maternal wound healing. By contrast, stem cell therapy with adult cells requires the injection of millions of cells into wounds or myocardial infarcts. Our results are in line with previous findings showing that, even in very low numbers, FMCs can rescue maternal defects. This efficiency of small number of active fetal progenitors is consistent with the findings of Tamai et al., who showed that Hmgb1 administration led to the recruitment of small numbers of BM Pdgfrα+ Lin− cells to skin injury sites, thereby improving delayed healing. These findings can be extended to humans, as allogeneic BM transplantation rescues dystrophic epidermolysis bullosa in children through the migration of this population of cells to sites of skin injury and its differentiation into appropriate cell types.

In conclusion, this study lays the foundations for a new concept natural fetal stem cell therapy. We found that, due to the higher levels of Ccr2 expression on particular fetal progenitors, these cells could rescue delayed wound repair in parous mice and that this advantage persisted for a prolonged period after delivery. This strategy may provide an interesting new alternative to induced pluripotent stem cell, embryonic stem cell or allogeneic therapy. The mobilization of natural fetal stem cells results in the recruitment of cells well tolerated by the maternal immune system. Being semi-allogeneic, these cells should have beneficial effects in mothers with genetic or acquired diseases, such as diabetes or sickle cell disease. In addition, fetal cells have several advantages over adult cells for this purpose, in terms of their pluripotency and proliferation capacity. Senescence levels are lower in fetal stem cells than in their adult counterparts, limiting the effects of aging. Finally, the mobilization of such cells avoids the need for various steps associated with risks of cell selection and amplification. This approach could, theoretically, be extended to other organs, such as heart, liver or even the nervous system.

**Methods**

**Mice.** Male mice transgenic for eGFP were obtained from Riken Laboratories (CD57BL/6-Tg(CAG-eGFP)1Ob/s1J), namely, eGFP and mated with wild-type (WT) 6-to-8-week-old C57BL/6 females from Harlan (Harlan). Female mice inactivated for Ccr2 (B6.129S4-Ccr2tm1Ifc/J), namely, Ccr2KO and male mice transgenic for eGFP gene inserted in Ccr2 gene (8B6.6-3Tg(Ccr2tm1.1Cln))J, namely, eGFPKOCcr2KO were obtained from Jackson Laboratory. Ccr2KO female were mated with male mice eGFPKOCcr2KO. Animal experiments were performed according to experimental protocols following European Community Council guidelines and approved by our Institutional Animal Care and Use Committee (approval number 01161.02 and #8127).

**Flow cytometry.** The skin on the back of the mice was shaved and wounds were harvested and incubated overnight at 4 °C in 0.05% trypsin-EDTA (Invitrogen) for...
mechanical separation of the epidermis. Tissues were digested by incubation in collagenase IV for 60 min at 37 °C, with vortexing every 10 min, and the resulting suspension was filtered through a cell strainer with 60 μm pores and then a cell strainer with 40 μm pores (BD Pharmingen), to obtain a single-cell suspension. Blood was collected from the heart of the mouse and peripheral mononuclear blood cells were separated from erythrocytes and platelets by the Ficoll 1.088 method (HealthCare). The cells were harvested and the resulting suspension was washed with PBS (Life Technologies) and filtered through a cell strainer with 40 μm pores (BD Pharmingen). Bones from the legs were collected and BMs were flushed, after sectioning the two heads of the bone, with 1 ml of PBS with a syringe 1 ml and a needle of 25G. The resulting suspension was washed with PBS and filtered through a cell strainer with 40 μm pores (BD Pharmingen). The antibodies used for cytometry were CD34+Fluor660 (1:100; ebioscience), CD11b+PERCP-Cy5.5 (1:100; ebioscience), CD31-PE-Cy7 (1:100; ebioscience) and CCR2 (1:100; Santa Cruz) crossed with an anti-goat-Alexa 555 (1:1,000 Invitrogen). Flow cytometric data were acquired with a BD LSRII (BD Pharmingen) machine and sorting was performed on a MoFlo cell sorter (Beckton Coulter). The cells were then analysed with the FlowJo software (Treestar, San Carlos, CA).

In order to assess the specificity and sensitivity of FACS technique, we performed a dilution curve of eGFP + splenocytes in WT splenocytes ranging from 100% to 0.0001%. FACs analysis always showed the expected amount of eGFP cells, even in very diluted specimens. (Supplementary Fig. 2a,b). The correlation curve between the expected percentage of eGFP cells and the detected percentage on the standard curve was perfectly linear (correlation factor $R^2 = 1$). Furthermore, we checked the purity of the sorted eGFP + cells from the dilution curve using quantitative PCR that showed 92–100% purity in all samples except for the most diluted one that had 81% purity. ApoB was used as a reference gene (Supplementary Fig. 2c).

**Immunostaining.** We cut 5 μm sections of frozen tissue. These sections were incubated with cold acetone for permeabilization and then blocked by incubation with 2% BSA (Sigma-Aldrich). The primary antibodies used included rat anti-mouse CD31 (1:40; BD Biosciences), rabbit anti-mouse K14 (1:1,000; Covance), rabbit anti-mouse LVVEI (1:200; Abcam), rat anti-mouse F4/80 (1:250; Abcam), rat anti-mouse GR-1 (1:250; ebioscience) rabbit anti-mouse Ki67 (1:200; Abcam), goat anti-mouse Ccr2 (1:200; Santa Cruz Biotechnology), goat anti-mouse Ccl2 (1:200; Santa Cruz) and rabbit anti-mouse vWF (1:800; Abcam). For immunofluorescence, we used the following secondary antibodies: goat-anti-rabbit IgG labelled with Alexa 488 or Cy3 (Life Technologies), donkey anti-rat IgG labelled with Alexa 488 or Cy3 (Life Technologies), goat anti-mouse Ccr2 (1:500; goat polyclonal; Santa Cruz). They were then incubated with Horseradish peroxidase-conjugated anti-goat antibody (1:5,000; Santa Cruz). Immunofluorescence sections were obtained using a MoFlo cell sorter (Beckton Coulter). The cells were then analysed with the FlowJo software (Treestar, San Carlos, CA).

**Microscopic scoring and measurements.** We used a Nikon Eclipse 90i fluorescence microscope equipped with a Nikon DS-FiC digital camera (Nikon, Tokyo, Japan). For cell scoring, we took photographs of three different fields and counted the labelled cells by fluorescence densitometry, reporting the number of cells as a percentage of the total (assessed as the total number of nuclei). The mean percentage of labelled cells was calculated for each specimen. Measurements were made with the ImageJ software (NIH, Bethesda, MD).

**RNA extraction quantitative PCR and RNA sequencing.** Total RNA was extracted from cells or tissues with Trizol reagent, in accordance with the manufacturer’s (Invitrogen) instructions. For low amount of cells, RNA was extracted with the NucleoSpin RNA XS Kit, in accordance with the manufacturer’s (Macherey–Nagel) instructions. It was then reverse-transcribed with the Script cDNA Synthesis Kit (Bio-Rad). The resulting cDNA was used for PCR with the SYBR-Green Master PCR Mix (Roche). PCR and data collection were performed on a LightCycler 480 (Roche). The levels of gene expression in the samples were normalized against that of the housekeeping gene (β-actin). All the primers were from Qiagen (Quantitect).

**RNA sequencing** was performed on sorted fetal cells from the peripheral blood obtained from wounded and unwounded pregnant mice. Parameters used were 2 reads × 25 million fragments, paired-end 2 × 100 nt. Each sample was a pool of six mice, leading to >500 fetal cells per sample.

**RNA sequencing analyses** were performed at ICM, Hospital La Pitié Salpêtrière, with an Illumina Hi-Seq device.

**Surgical wounds.** Mice were anesthetized by the inhalation of 4.9% isoflurane delivered at a flow rate of 300 ml min⁻¹ in ambient air. The backs of the animals were shaved, and punch biopsy devices were used to create four 6 mm surgical wounds or a single 8 mm surgical wound. All tissues above the panniculus carnosus were excised. Wounds were left uncovered until harvesting. Standardized images of the wounds were obtained at various times postwounding. With a Sony Cybershot 10.1-megapixel DSC-W180 digital camera (Sony, Tokyo, Japan). Wound tissues were harvested and either snap-frozen in liquid nitrogen or stored at −80 °C.

**Corticoid treatment.** Four days after delivery, mice were treated by the topical application of 200 μl clobetasol (Dermoval) per day onto shaved dorsal skin over a period of 10 or 12 days depending on age.

**Chemokine/cell injections.** Following the generation of an 8 mm wound, 100 μl of Ccl2 (Chemicon, Naperville, IL) was injected into the four cardinal points of the wound bed on days 0 and 2, at a concentration of 0.5 ng μl⁻¹. We therefore injected 50 ng of Ccl2 after the surgery and 48 h later. Alternatively, 10,000 cells were injected into the wound after FACS sorting and resuspension in PBS in accordance with the procedure used before, on day 1.

**Western blotting.** Wound samples were homogenized in RIPA buffer (Bio-Rad, Hercules, CA) supplemented with Complete Protease Inhibitor Cocktail (Roche) and centrifuged to obtain lysates. Equal amounts of extracted protein (20 μg) were subjected to SDS–polyacrylamide gel electrophoresis in a NuPAGE 4–12% Bis-Tris Gel (Novex, Invitrogen) and transferred to nitrocellulose membranes (GE Healthcare, Glattbrugg, Switzerland). The membranes were incubated with antibodies directed against β-lactin (1:2,000; rabbit polyclonal; Cell Signaling) or against Ccr2 (1:500; goat polyclonal; Santa Cruz). They were then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:2,500; Cell Signaling) and horseradish peroxidase-conjugated anti-goat antibody (1:5,000; Santa Cruz). Immune complexes were visualized with ECL prime (GE Healthcare) and signals were captured on ChemiDoc (Bio-Rad). Protein bands were analysed by densitometry with ImageJ. The uncropped Ccr2 membrane is shown in Supplementary Fig. 15.

**PCR array for chemokines and receptors and angiogenesis.** Changes in chemokines and receptors or angiogenesis levels were measured with an RT² Profiler PCR Array for mouse angiogenic and receptors PAMM-0222A or mouse angiogenesis PAMM-0242R (Qiagen, Hilden, Germany). Total RNA was extracted from sorted eGFP + cells from BM. The expression of 86 cytokine and chemokine genes was analysed with the Lightcycler 1536 system (Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions. The data obtained were analysed with the RT² Profiler PCR Array Data Analysis Template (Qiagen). Data were normalized against five housekeeping genes (Aabi, B2m, Gapdh, Gusb, Hspa9b1), and relative expression levels were calculated by the 2⁻ΔΔCt method.

**PCR array analyses** were performed at the Hospital La Pitié Salpêtrière ICM, with a Lightcycler 1536 (Roche) and RT² Profiler PCR Arrays.

**Statistical analysis.** Statistical analysis was performed with the Graphpad Prism software. The results are expressed as mean ± s.e.m. Pairs of groups were performed with unpaired, two-tailed Student’s t-tests. Differences were considered statistically significant if $P<0.05$.

**Data availability.** The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request.

**References**

1. O’Donoghue, K. et al. Microchimerism in female bone marrow and bone decades after fetal mesenchymal stem-cell trafficking in pregnancy. _Lancet_ 364, 179–182 (2004).

2. Khosrutehrani, K. et al. Pregnancy allows the transfer and differentiation of fetal lymphoid progenitors into functional T and B cells in mothers. _J. Immunol._ 180, 889–897 (2008).

3. Bianchi, D. W., Zickwolf, G. K., Weil, G. J., Sylvester, S. & DeMaria, M. A. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. _Proc. Natl Acad. Sci. USA_ 93, 705–708 (1996).

4. Fujiki, Y., Johnson, K. L., Peter, I., Tighiouart, H. & Bianchi, D. W. Fetal cells in the pregnant mouse are diverse and express a variety of progenitor and differentiated cell markers. _Biol. Reprod._ 81, 26–32 (2009).

5. Osada, H. et al. Detection of fetal HPCs in maternal circulation after delivery. _Transfusion_ 41, 499–503 (2001).

6. Campagnoli, C. et al. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. _BLOOD_ 98, 2396–2402 (2001).

7. O’Donoghue, K. et al. Identification of fetal mesenchymal stem cells in maternal blood: implications for non-invasive prenatal diagnosis. _JAMA._ 294, 947–952 (2003).

8. Kara, R. J. et al. Fetal cells traffic to injured maternal myocardium and undergo cardiac differentiation. _Circ. Res._ 110, 82–93 (2012).

9. Khosrutehrani, K., Johnson, K. L., Cha, D. H., Salomon, R. N. & Bianchi, D. W. Transfer of fetal cells with multilineage potential to maternal tissue. _JAMA._ 292, 75–80 (2004).
33. Guillot, P. V., Gotherstrom, C., Chan, J., Kurata, H. & Fisk, N. M. Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. *Stem Cells* 23, 1443–1452 (2005).

34. Khosrotehrani, K. et al. Fetal cells participate over time in the response to specific types of murine maternal hepatic injury. *Hum. Reprod.* 22, 654–661 (2007).

35. Nguyen Huu, S. et al. Maternal neangiogenesis during pregnancy partly derives from fetal endothelial progenitor cells. *Proc. Natl Acad. Sci. USA* 104, 1871–1876 (2007).

36. Násar, D. et al. Fetal progenitor cells naturally transferred through pregnancy participate in inflammation and angiogenesis during wound healing. *FASEB J.* 26, 149–157 (2012).

37. Cha, D. et al. Cervical cancer and microchimerism. *Obstet. Gynecol.* 102, 774–781 (2003).

38. Gurtner, G. C., Werner, S., Barrandon, Y. & Longaker, M. T. Wound repair and regeneration. *Science* 327, 1759–1765 (2010).

39. Singer, A. J. & Clark, R. A. Cutaneous wound healing. *N. Engl. J. Med.* 341, 738–746 (1999).

40. Asharha, T. et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964–967 (1997).

41. Fakhoury, C. et al. Bone marrow-derived cells to skin: collagen deposition and wound repair. *Stem Cells* 22, 812–822 (2004).

42. Yoder, M. C. Is endothelium the origin of endothelial progenitor cells? *Arterioscler. Thromb. Vasc. Biol.* 30, 1094–1103 (2010).

43. Timmermans, F. et al. Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. *Arterioscler. Thromb. Vasc. Biol.* 27, 1572–1579 (2007).

44. Case, I. et al. Human CD34+ AC133+ VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. *Exp. Hematol.* 35, 1109–1118 (2007).

45. Okuno, Y., Nakamura-Ishizu, A., Kishi, K., Suda, T. & Kubota, Y. Bone marrow-derived cells serve as proangiogenic macrophages but not endothelial progenitors. *Blood* 117, 5264–5272 (2011).

46. Purhonen, S. et al. Bone marrow-derived circulating endothelial progenitors do not contribute to vascular endothelium and are not needed for tumor growth. *Proc. Natl Acad. Sci. USA* 105, 6620–6625 (2008).

47. Seppanen, E. et al. Distant mesenchymal progenitors contribute to skin wound healing and produce collagen: evidence from a murine fetal microchimerism model. *PLoS ONE* 8, e62662 (2013).

48. Nguyen Huu, S. et al. Fetal microchimeric cells participate in tumour angiogenesis in melanomas occurring during pregnancy. *Am. J. Pathol.* 174, 630–637 (2009).

49. Charo, I. F. et al. Molecular cloning and functional expression of two monocyte chemoattractant protein-1 receptors reveals alternative splicing of the carboxy-terminal tails. *Proc. Natl Acad. Sci. USA* 91, 2752–2756 (1994).

50. Si, Y., Tsou, C. L., Croft, K. & Charo, I. F. CCR2 mediates hematopoietic stem and progenitor cell trafficking to sites of inflammation in mice. *J. Clin. Invest.* 120, 1192–1203 (2007).

51. Charo, I. F. & Taubman, M. B. Chemokines in the pathogenesis of vascular disease. *Circ. Res.* 95, 858–866 (2004).

52. Salcedo, R. et al. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 96, 34–40 (2000).

53. Dipietro, L. A., Reinjens, M. G., Low, Q. E., Levi, B. & Gamelli, R. L. Modulation of macrophage recruitment into wounds by monocyte chemoattractant protein-1. *Wound Repair Regen.* 9, 28–33 (2001).

54. Wood, S. et al. Pro-inflammatory chemokine CCL2 (MCP-1) promotes healing in diabetic wounds by restoring the macrophage response. *PLoS ONE* 9, e91574 (2014).

55. Lin, Q. et al. Impaired wound healing with defective expression of chemokines and recruitment of myeloid cells in TLR3-deficient mice. *J. Immunol.* 186, 3710–3717 (2011).

56. Guillot, P. V., Goetherstrom, C., Chan, J., Kurata, H. & Fisk, N. M. Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. *Stem Cells* 25, 646–654 (2007).

57. Devalaraja, R. M. et al. Delayed wound healing in CCR2 knockout mice. *J. Invest. Dermatol.* 115, 234–244 (2000).

58. Binato, R. et al. Stability of human mesenchymal stem cells during in vitro culture: considerations for cell therapy. *Cell Prolif.* 46, 10–22 (2013).