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WNT7b mediates macrophage-induced programmed cell death in patterning of the vasculature

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Abstract

Macrophages have a critical role in inflammatory and immune responses through their ability to recognize and engulf apoptotic cells¹. Here we show that macrophages initiate a cell-death programme in target cells by activating the canonical WNT pathway. We show in mice that macrophage WNT7b is a short-range paracrine signal required for WNT-pathway responses and programmed cell death in the vascular endothelial cells of the temporary hyaloid vessels of the developing eye. These findings indicate that macrophages can use WNT ligands to influence cell-fate decisions—including cell death—in adjacent cells, and raise the possibility that they do so in many different cellular contexts.

In most systems, it has largely been assumed that macrophage involvement in programmed cell death comes after the apoptotic event, and is a response to the presence of membrane-tethered or soluble 'eat-me' signals from dead and dying cells. However, in some circumstances phagocytes actively induce programmed cell death. In mice, macrophages are required for the programmed regression of temporary capillary networks within the

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developing eye^{2,3}. In the worm *Caenorhabditis elegans*, phagocyte recognition pathways can act as a backup stimulus for the induction of programmed cell death when the autonomous caspase-driven pathway is deficient^{4,5}. These data suggest that there are evolutionarily conserved pathways for phagocyte-induced programmed cell death.

Using ablation and repletion experiments^{2,3}, it has been shown that resident macrophages are necessary and sufficient for the programmed cell death that drives the regression of the temporary ocular capillary networks. To determine whether mice deficient in the lymphomyeloid transcription factor *PU.1*, which lack macrophages⁶, are useful for studying programmed vascular regression, we confirmed that resident ocular macrophages were absent in these animals (Fig. 1a–d) and assessed ocular vessel network regression postnatally, when the hyaloid vessels would normally regress⁷. As expected^{2,3}, the pupillary membrane (on the anterior surface of the lens, Supplementary Fig. 1) and the tunica vasculosa lentis (on the posterior surface of the lens, data not shown), as well as the hyaloid vessels (between the lens and retina, Fig. 1e–h), appeared to persist postnatally in *PU.1*-deficient mice. Counts of vessel numbers at postnatal day 8 (P8) (Fig. 1i) confirmed this, and also revealed that *PU.1* heterozygotes had a partial regression phenotype. TdT-mediated dUTP nick end-labelling (TUNEL) analysis showed that *PU.1*-null mice have a defect in apoptotic cell clearance (data not shown) that precluded the meaningful quantification of apoptotic vascular endothelial cells (VECs). These data establish that *PU.1*-mutant mice are a useful model for studying macrophage function in programmed vascular regression.

The WNT signalling pathway has a crucial function in developmental cell fate decisions^{8–11} and, when aberrantly activated, in the development of cancer¹². In vertebrates, the canonical WNT response requires a receptor complex comprising the coreceptor low-density lipoprotein receptor-related protein (LRP)5 or LRP6 (ref. 13) and a multiple-pass transmembrane receptor of the Frizzled (FZD) family¹⁴. When activated by a WNT ligand, this complex initiates a cascade of events that culminates in the stabilization of β -catenin, its association with transcription factors of the Lef/Tcf family¹⁵ and the regulation of target genes including some that stimulate cell cycle entry⁸. Notably, as indicated by the phenotype of the homozygous null *Lrp5^{lacZ/lacZ}* mice^{16,17} (Fig. 2a–d, g), the coreceptor gene *Lrp5* is required for the regression of the hyaloid vessels as a consequence of reduced VEC apoptosis¹⁷ (Fig. 2h). As LRP5 may have activities outside the canonical WNT pathway⁹, we assessed the consequences of mutating the *Lef1* gene¹⁸ and again found persistent hyaloid vessels (Fig. 2e–g) and reduced levels of cell death (Fig. 2h). As the WNT pathway is often a stimulus for proliferation, we also performed 5-bromodeoxyuridine (BrdU) labelling and showed that in capillary cells of both the *Lrp5* and *Lef1* mutants, the rate of cell cycle entry is reduced (Fig. 2i). Taken together, these data indicate that canonical WNT signalling is required for VEC cell cycle entry, apoptosis and programmed capillary regression.

To determine which cell type was WNT-responsive, we took advantage of canonical WNT pathway reporter mice (*TOPGAL*) that carry a transgene with Lef/Tcf binding sites and a minimal promoter upstream of an open reading frame encoding β -galactosidase (β -gal)¹⁹. Staining of hyaloid vessels from *TOPGAL* hemizygous mice with 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) revealed β -gal-positive VECs adjacent to the vessel lumen

(Fig. 2j). The intensity of X-gal staining in VECs increased from P3 (Fig. 2l) to P5 and P7 (Fig. 2m, k). Macrophages could be identified morphologically (Fig. 2j–m, dashed circles) and did not stain. The X-gal staining pattern in P7 hyaloid vessels from *TOPGAL* and *Lrp5^{lacZ/lacZ}* mice were quite distinct, with *TOPGAL* mice showing sporadic intensely stained VECs (Fig. 2j, k) and the *Lrp5^{lacZ/lacZ}* mice showing weak general staining (Fig. 2n). In compound *Lrp5^{lacZ/lacZ};TOPGAL* mice, the intense sporadic VEC labelling was lost (Fig. 2o), consistent with the expectation that LRP5 is required for WNT signalling. When combined with the reduced cell death and regression failure of the *Lrp5*- and *Lef1*-mutant mice, these data indicate that the WNT response of VECs is critical for scheduled vascular regression.

One way to explain the requirement for macrophages in scheduled vascular regression would be if they were a source of WNT ligands. To investigate this possibility, we determined whether the macrophage-deficient *PU.1*-null mice showed defects that were characteristic of perturbations in the WNT pathway. BrdU labelling showed that, like the *Lrp5* and *Lef1* mutants (Fig. 2i), *PU.1*-null mice show a reduced rate of S-phase labelling in cells of primary and secondary hyaloid capillary branches (Supplementary Fig. 2a). Crosses between *PU.1* and *TOPGAL* mice produced macrophage-deficient *TOPGAL* animals that showed a 70% reduction in the number of X-gal-stained cells in the hyaloid vessels (Supplementary Fig. 2b). These data suggest that macrophages can stimulate WNT pathway activation in hyaloid VECs.

To determine whether macrophages might express a set of *Wnt* genes associated with vascular development²⁰, we performed reverse transcription–polymerase chain reaction (RT–PCR) analysis on purified populations of hyaloid macrophages gathered from wildtype P5 hyaloid preparations using the laser-capture microdissection technique (Supplementary Fig. 2). This revealed that of a small number of *Wnt* genes expressed in macrophages, *Wnt7b* seemed to be specific for hyaloid macrophages (Supplementary Fig. 2). To investigate further the expression of *Wnt7b*, we performed X-gal staining on hyaloid vessel preparations from *Wnt7b^{+lacZ}* mice²¹ (Fig. 3a–c), which confirmed that *Wnt7b^{lacZ}* expression was restricted to macrophages. Furthermore, the level of X-gal staining increased dramatically from P1 to P5 (Fig. 3a–c) and correlated with increasing *TOPGAL* expression in VECs over the same time interval (Fig. 2k–m). These data suggested that macrophages are required for scheduled vascular regression because they are a source of WNT7b.

To determine whether this was the case, we generated a hypomorphic *Wnt7b^{d1}* gene-targeted allele (Supplementary Fig. 3). It was necessary to generate this allele for analysis as previously generated *Wnt7b* mutations, including the *Wnt7b^{lacZ}* allele used above²¹, result in lethality too early to permit analysis of hyaloid vessel regression. Interestingly, *Wnt7b^{d1/d1}* mice show persistence of the hyaloid vessels (Fig. 3d–h). Immunolabelling of wild-type and *Wnt7b^{d1/d1}* hyaloid vessels for the macrophage marker F4/80 showed that normal numbers of macrophages were present in mutant mice (Fig. 3i, j), eliminating the possibility that hyaloid vessel persistence was a consequence of a lack of macrophages at these sites. It should be noted that *Wnt7b^{d1/d1}* mice also showed levels of apoptosis (Fig. 3k) and BrdU labelling (Fig. 3l) that were much reduced from wild-type levels but comparable to the levels observed in *Lrp5*-mutant mice. As *Wnt7b* expression is restricted to macrophages,

these data suggest that WNT7b is a macrophage product critical for inducing cell death in hyaloid VECs.

To determine which FZD receptor might mediate WNT7b signalling, we assessed the activity of FZD3–FZD8 using the *SuperTOPFLASH* WNT reporter cell line¹¹ and a luciferase reporter assay (Supplementary Fig. 4). Although we could not exclude the participation of multiple FZDs, the expression of *Fzd4* in hyaloid capillaries, its activity in mediating WNT7b signalling (Supplementary Fig. 4a, b) and the persistence of hyaloid vessels in *Fzd4*-mutant mice¹¹ all implicated FZD4. For this reason, we also examined the specificity of the interaction using a mixed-cell assay in which we tested the ability of FZD4 ligand-binding domain mutants (see Methods) to mediate paracrine signalling by WNT7b (Supplementary Fig. 4c). This showed that subtle mutations in FZD4 prevent paracrine WNT7b signalling, and further support a model in which WNT7b produced by macrophages signals through FZD4 expressed on endothelial cells.

If macrophage WNT7b was signalling to hyaloid VECs and eliciting a WNT pathway response, we would predict that in *TOPGAL;Wnt7b^{d1/d1}* compound mice, the *TOPGAL* response would be reduced. To assess this, we generated X-gal-stained P8 hyaloid vessel preparations from control *TOPGAL* and *TOP-GAL;Wnt7b^{d1/d1}* mice (Fig. 4a, b) and counted the numbers of stained cells. The data were normalized to take into account the greater number of vessels in the *Wnt7b^{d1/d1}* mutant; this showed that the *Wnt7b* mutation reduced the *TOPGAL* response to 29% of the control value (Fig. 4c). This corresponds very closely to the reduced *TOPGAL* response observed when macrophages are absent (in *PU.1*-mutant mice, Supplementary Fig. 2b) and further argues that macrophage WNT7b elicits a WNT pathway response in hyaloid VECs.

WNT ligands can be lipid-modified and are therefore highly insoluble²². This suggests that macrophage WNT7b might elicit VEC responses only through cell–cell contact. Therefore, we used a technique described previously¹¹ to assess the range of action of WNT7b using populations of producer and responder cells that were either closely apposed or widely separated in the culture dish. The *SuperTOPFLASH* cell line¹¹ transfected with *Fzd4* and *Lrp5* expression plasmids was used to assess WNT pathway responses. Transfected 293 cells, containing a *Wnt7b* expression plasmid, were used as a producer cell line. Significant activity was detected only when WNT7b-producer cells were mixed with FZD4-expressing responder cells (Fig. 4d), suggesting that WNT7b had a short range of action.

Despite the reduced *TOPGAL* responsiveness in *TOP-GAL;Wnt7b^{d1/d1}* mice, it remained possible that another source of WNT7b in addition to the macrophage might be critical to initiate the hyaloid VEC death programme. To investigate this possibility, we took advantage of *PU.1*-mutant mice and asked whether injected macrophages could rescue the hyaloid vessel regression failure. Bone-marrow-derived macrophages were differentiated in the presence of CSF-1 (colony-stimulating factor 1) as described previously³ and were allowed to mature to 14 ± 3 days before injection. Intravitreal injection into the right eye was performed on the day of birth; the contralateral eye was used as an uninjected control. As expected, no macrophages were observed in uninjected *PU.1*-mutant eyes and the hyaloid vessels were persistent (Fig. 4e). Injected wild-type macrophages were localized to

the hyaloid capillaries (Fig. 4f), and by P8 had rescued regression completely (Fig. 4f, h) according to the number of hyaloid vessels remaining at P8 in wild-type mice (Fig. 3h). Notably, when bone marrow macrophages from *Wnt7b^{dl/dl}* mice were injected, they also localized to hyaloid vessels (Fig. 4g) but completely failed to mediate hyaloid vessel regression (Fig. 4g, h). When combined with the range-of-action experiments and the results from the *TOPGAL;Wnt7b^{dl/dl}* compound mice, these data indicate that the critical, paracrine source of WNT7b required for scheduled vascular regression is the macrophage.

Here we have established that macrophages actively regulate life-and-death decisions in adjacent cells through the WNT pathway. In the context of the hyaloid vessel system, the critical mediator of cell death is WNT7b. Although it is possible that a more complex signalling pattern exists, the data support a model in which macrophage WNT7b activates the WNT pathway in adjacent vascular endothelial cells, most likely through cell–cell contact. As the WNT pathway can stimulate cell cycle entry in this and other systems⁸, there is a possibility that programmed cell death is dependent on cell cycle entry. Coupling of cell death and cell cycle entry has been noted in other systems²³.

The current study defines a role for the WNT pathway in regulating vascular regression. Given the broad distribution of macrophages, they may be more generally involved in regulating vascularity through the activation of the WNT pathway in vascular structures^{10,11}. In addition, tumour-associated macrophages are known to express WNT genes²⁴ and can extrinsically regulate the growth of the tumour vasculature, and also perhaps of the tumour cells themselves²⁵. Thus, the finding that macrophages can produce WNT ligands has important implications given the many circumstances in which the WNT pathway has a critical function. The short-range action of macrophage WNT7b is a mechanism for controlling the activity of this potent mediator.

Beyond vascular development, the major question that arises from this analysis is whether the ability of macrophages to actively signal cell death is a unique adaptation in the eye or a more general phenomenon. Although these alternatives need to be tested rigorously, there is some evidence to suggest the latter. For example, in mammalian systems, macrophage-related microglia can promote cell death in the retina through the production of nerve growth factor²⁶. Recently, ablation experiments in a model of liver injury have shown that inflammatory macrophages promote myofibroblast proliferation and apoptosis as a mechanism of resolution and repair²⁷. It has also been shown that phagocytes can promote programmed cell death outside of mammalian systems. In *C. elegans*, the machinery required for the recognition and engulfment of dead cells can promote apoptosis when CED3 (caspase) activity is compromised, suggesting that phagocytes provide a backup mechanism for disposing of superfluous cells^{4,5}. All this would suggest that phagocytes may have a conserved function in signalling cell death in a variety of contexts.

METHODS

Mouse breeding and genotyping

Genotyping of *Lrp5^{lacZ/lacZ}* (ref. 17), *Lef1^{-/-}* (ref. 18), *PU.1^{-/-}* (ref. 6) and *TOPGAL* (ref. 19) mice was performed as previously described. When *Lef1*-mutant mice were produced as

C57BL/6 $\times$ 129Sv F₁ hybrids they showed enhanced survival that allowed the analysis of hyaloid vessel regression postnatally.

Dissections, immunostaining and imaging

Hyaloid vessel preparations were generated as previously described¹⁷ with the exception that 5% (w/v) gelatin was used at 56 °C and allowed to set on ice before completion of the dissection. X-gal staining was done according to established protocols²¹. Indirect immunofluorescent staining and BrdU labelling were performed as previously described²⁸. Primary antibodies used were anti-vascular-endothelial-cell-cadherin (Santa Cruz), anti-BrdU (Dako) and anti-F4/80 (Caltag laboratories), all at a 1:100 dilution. Secondary antibodies labelled with Alexa fluorochromes (Molecular Probes) were used at a 1:500 dilution. TUNEL labelling of apoptotic cells was performed using an in situ cell death detection kit (Roche).

Plasmids

All *Fzd*, *Wnt* and *Norrin* complementary DNAs used were mouse. The plasmid encoding the Alkaline phosphatase (AP)–Norrin fusion protein has been described elsewhere¹¹. WNT7b was expressed from pCDNA3.1 as a V5- and His-epitope-tagged fusion protein. In addition, we generated *Fzd4^{m1}* and *Fzd4^{m2}* in pCDNA3.1 using a PCR-based mutagenesis strategy. In *Fzd4^{m1}*, the sequence encoding the cysteine-rich domain (residues 54-QNLGYNV-60) was modified to encode AALAYAA. In *Fzd4^{m2}*, the sequence encoding residues 105-MCT-107 was modified to encode ACA. These alanine mutations in the equivalent regions of murine FZD8 were shown to eliminate binding of *Xenopus* WNT8–AP (ref. 29). These expression plasmids were used in the paracrine and range-of-action signalling assays (Supplementary Fig. 4).

Laser-capture microdissection

To purify hyaloid macrophages, we performed laser-capture microdissection from whole-mount hyaloid vessel preparations using the PixCell II laser-capture microdissection system (Arcturus). The macrophages shown in Supplementary Fig. 2c encircled in red are shown after they have been lifted from the preparation (Supplementary Fig. 2d) on the laser-melted polymer membrane.

Reverse transcription–polymerase chain reaction

RNA from isolated macrophages was purified using PicoPure RNA isolation kit (Arcturus) and subsequent RT–PCR performed using the OneStep RT–PCR kit (Qiagen). Nested primers were used for a second round of PCR-amplification using Takara LA Taq (Takara). Primer nucleotide sequences used were: *Gapdh*, forward (F) 5′ -ACT CCACTCACGGCAAATTC-3′, reverse (R) 5′ -CACATTGGGGGTAGGAACAC-3′; *Wnt2*, F 5′ -CGGCCCTTTGTTTACGCCATC-3′, R 5′ -TGAATACAGTAGTCTG GAGAA-3′; *Wnt7b*, F 5′ -AAGAACTCCGAGTAGGGAGTCG-3′, R 5′ -TGCG TTGTA CTCTCCTTGAGC-3′; *Wnt7b*, second round F 5′ -CCGAGTAGGG AGTCGAGAGG-3′, R 5′ -CACACCGTGACACTTACATTCC-3′; *Wnt10b*, F 5′ -GTGGTAACGGAAAACCTGAAGC-3′, R 5′ -CTCATCACACAGCACATAA CAGC-3′;

Wnt10b, second round F 5' -GCCAATTCAAGACCTGTTGG-3', R 5' -CATAACAGCACCAAGTGGAAACG-3'; *Fzd4*, F 5' -GCTACAACGTGACCAA GATGC-3', R 5' -CAAACCCAAATTCTCTCAGGAC-3'; *Fzd4*, second round F 5' -AACTTAGTGGGACACGAGCTG-3', R 5' -CAGCGTCTCTTGACTGAAAGG-3'; *Norrin*, F 5' -GAGAAATCATGTACTAGCTGCATCC-3', R 5' -TGTACCGG TAAGTGGCAGTAAG-3'; *Norrin*, second round F 5' -GGCCATAATGGGAGATA CAGAC-3', R 5' -GAGGACAGTGCTGAAGGACAC-3'; *Lef1*, F 5' -CTACAGCG ACGAGCACTTTTC-3', R 5' -AGGATCTGGTTGATAGCTGCAC-3'.

Macrophage rescue

Mouse bone-marrow-derived macrophages were isolated as previously described³. Approximately 4,000 macrophages from wild-type or *Wnt7b^{dl/dl}* animals were injected into the vitreous at P1 in a volume of 200 nl using a modification of the trans-corneal technique described previously³. Animals were killed at P8 and hyaloid vessels dissected.

Luciferase and range-of-action assays

For the Frizzled receptor activity screen, *SuperTOPFLASH* cells¹¹ (which carry a reporter plasmid with Lef/Tcf binding sites and a minimal promoter upstream of an open reading frame encoding luciferase) were plated into 60-mm dishes and transfected after 24 h with combinations of 1 µg *Lrp5*-Flag, 1 µg *Wnt7b*-V5His and 0.1 µg *Fzd* plasmid made up to 4 µg with *pIRES*-GFP using Fugene 6 (Roche). Forty-eight hours after transfection, cells were washed with PBS and luciferase activity measured with a luciferase assay kit (Promega). For the mixed-cell paracrine signalling assay, 293 cells were transfected with WNT pathway ligands and overlaid on *SuperTOPFLASH* responder cells that were separately transfected with plasmids encoding LRP5 and a FZD receptor. Luciferase activity was measured as indicated above. WNT7b range-of-action experiments were carried out as described¹¹.

Statistical analysis

Vessel number was quantified using established methods⁷. At least four hyaloid vessel preparations were quantified for each experiment. All data are presented with standard error bars. Student's *t*-test and one-way analysis of variance were used to assess statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Regression of the hyaloid vessels is macrophage-dependent

a–d, Hyaloid vessel preparations from wild-type (**a**, **c**) and *PU.1*^{-/-} (**b**, **d**) mice at P5. **a**, **b**, Differential interference contrast illumination indicating the presence of macrophages in wild-type mice (**a**, with red dots adjacent) and the absence of macrophages in *PU.1*^{-/-} mice (**b**). **c**, **d**, Fluorescent immunostaining for macrophages (F4/80, a macrophage-specific marker, green) and nuclei (blue). **e–h**, Hyaloid vessels from wild-type (**e**, **f**) and *PU.1*^{-/-} (**g**, **h**) animals of the indicated ages stained with Hoechst 33258. **i**, Hyaloid vessel number in wild-type, *PU.1*^{-/-} and *PU.1*^{+/-} mice at P8. All error bars are standard errors. Significance levels: three asterisks, $0.0001 < P < 0.001$; four asterisks, $P < 0.0001$. Original magnification: $\times 50$ (**e–h**); $\times 400$ (**a–d**). WT, wild type.

Figure 2. The WNT pathway response in VECs is required for hyaloid vessel regression
a–f, Hoechst-33258-labelled hyaloid vessel preparations from mice of the indicated ages and genotypes. **g–i**, Vessel number at P8 (**g**), number apoptotic events at P5 (**h**) and BrdU labelling index in primary (1°) and secondary (2°) capillary branches at P5 (**i**) in the hyaloid vessels of mice with the indicated genotypes. *Lrp5*^{lacZ/lacZ} is an abbreviated reference to the *Lrp5*^{lacZ/lacZ} allele. **j–o**, X-gal staining (blue) in hyaloid vessels from mice of the indicated ages and genotypes. Intensely stained *TOPGAL*-expressing cells (red arrowheads) and resident macrophages (red dashed circles) are apparent. All error bars are standard errors. Significance levels: two asterisks, 0.001 < *P* < 0.01; three asterisks, 0.0001 < *P* < 0.001; four asterisks, *P* < 0.0001. Original magnification: ×50 (**a–f**); ×630 (**k–o**); ×1,000 (**j**). WT, wild type.

Figure 3. WNT7b is required for hyaloid vessel regression and is expressed in macrophages
a–c, X-gal staining (blue) of hyaloid vessels from *Wnt7b^{+/lacZ}* mice of the indicated ages. Macrophages are indicated by dashed red circles. **d–g,i,j**, Hyaloid vessels from wild-type (**d,e,i**) and *Wnt7b^{d1/d1}* (**f,g,j**) animals at the indicated ages, stained with Hoechst 33258 (**d–g**) or anti-F4/80 (**i,j**). **h,k,l**, Quantification, in hyaloid vessels from mice with the indicated genotypes, of vessel number at P8 (**h**), apoptotic events at P5 (**k**) and BrdU labelling index in primary (1°) and secondary (2°) capillary branches at P5 (**l**). All error bars are standard errors. Significance levels: two asterisks, $0.001 < P < 0.01$. Original magnification: $\times 50$ (**d–g,i,j**); $\times 630$ (**a–c**). WT, wild type.

Figure 4. Macrophages are a critical paracrine source of WNT7b required for hyaloid vessel regression

a, b, X-gal staining (blue) of P8 hyaloid vessel preparations in *TOPGAL* (**a**) and *TOPGAL; Wnt7b^{d1/d1}* (**b**) mice. Red arrowheads indicate stained cells. **c**, Relative number of X-gal-stained cells in *TOPGAL* (grey bar) and *TOPGAL; Wnt7b^{d1/d1}* (green bar) hyaloid preparations at P8. The data are normalized for the increased vessel number of the *Wnt7b^{d1/d1}* mutants. **d**, Quantification of WNT responsiveness in *SuperTOPFLASH* cells transfected with *Fzd4* and *Lrp5* and either mixed with WNT7b producer cells or separated from them by restricting *SuperTOPFLASH* responder cells and WNT7b producer cells to each half of a culture dish. **e–g**, Hyaloid vessel preparations at P8 from uninjected *PU.1^{-/-}* mice (**e**), *PU.1^{-/-}* mice injected with wild-type macrophages (**f**) and *PU.1^{-/-}* mice injected with *Wnt7b^{d1/d1}* macrophages (**g**). Preparations are labelled for the F4/80 marker (red), vascular endothelial cell cadherin (green) and nuclei (blue). Original magnification: $\times 200$. **h**, Vessel number at P8 in uninjected *PU.1^{-/-}* mice (yellow bar), *PU.1^{-/-}* mice injected with wild-type macrophages (yellow/grey bar) and *PU.1^{-/-}* mice injected with *Wnt7b^{d1/d1}* macrophages (yellow/green bar). All error bars are standard errors. Significance levels: one asterisk, $0.01 < P < 0.05$; two asterisks, $0.001 < P < 0.01$; four asterisks, $P < 0.0001$. WT, wild type; Mac, macrophages.