Cell and Tissue Research (2019) 378:441–456
https://doi.org/10.1007/s00441-019-03068-w

REGULAR ARTICLE

A population of nonneuronal GFRα3-expressing cells in the bone marrow resembles nonmyelinating Schwann cells

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Received: 13 November 2018 / Accepted: 1 July 2019 / Published online: 14 July 2019

Abstract

Artemin is a neurotrophic factor that plays a crucial role in the regulation of neural development and regeneration and has also been implicated in the pathogenesis of inflammatory pain. The receptor for artemin, GFRα3, is expressed by sympathetic and nociceptive sensory neurons, including some that innervate the bone marrow, but it is unclear if it is also expressed in other cell types in the bone marrow. Our goal in the present study was to characterise the expression of GFRα3 in nonneuronal cells in the bone marrow. Immunohistochemical studies revealed that GFRα3-expressing cells in the bone marrow are spatially associated with blood vessels and are in intimate contact with nerve fibres. We used various combinations of markers to distinguish different cell types and found that the GFRα3-expressing cells expressed markers of nonmyelinating Schwann cells (e.g. GFAP, p75NTR, nestin). Analysis of bone marrow sections of Wnt1-reporter mice also demonstrated that they originate from the neural crest. Further characterisation using flow cytometry revealed that GFRα3 is expressed in a population of $CD51^{+}Sca1^{-}PDGFR\alpha^{-}$ cells, reinforcing the notion that they are neural crest-derived, nonmyelinating Schwann cells. In conclusion, there is a close association between peripheral nerve terminals and a population of nonneuronal cells that express GFRα3 in the bone marrow. The nonneuronal cells have characteristics consistent with a neural crest-derived, nonmyelinating Schwann cell phenotype. Our findings provide a better understanding of the expression pattern of GFRα3 in the bone marrow microenvironment.

Keywords Artemin · GFRα3 · Nonmyelinating Schwann cells · Bone marrow · Peripheral nervous system

Introduction

Artemin is a member of the glial cell line-derived neurotrophic factor (GDNF) family of ligands that is involved in the development of the peripheral nervous system (Andres et al. 2001; Honma et al. 2002), axonal regeneration (Harvey et al. 2010; Wang et al. 2008) and in pain signalling (Albers et al. 2014; Malin et al. 2006; Nencini et al. 2018). It acts through a core receptor tyrosine kinase RET and an accessory GDNF family receptor alpha-3 (GFRα3) which confers ligand specificity for the receptor complex (artemin/GFRα3) (Baloh et al. 1998). The GFRα3 receptor is expressed in developing sympathetic postganglionic neurons (Baloh et al. 1998) and a subpopulation of small-diameter peptidergic sensory neurons (Ernsberger 2008; Orozco et al. 2001). Artemin- and GFRα3-deficient mice have abnormalities in the migration and axonal projection pattern of sympathetic neurons (Honma et al. 2002; Nishino et al. 1999). There are also a number of reports of GFRα3 expression in nonneuronal cells across many different tissue systems. For example, Widenfalk et al. (1998) reported GFRα3 mRNA expression on Schwann cell precursors, olfactory ensheathing cells, chromaffin cells in the adrenal gland and small clusters of cells in the intestinal epithelium. Baloh et al. (1998) provided a broader expression pattern, including in the digestive tract, appendix and kidneys. It has also been reported in a subset of epithelial cells in the human digestive and reproductive systems (Yang et al. 2006). The functional significance of GFRα3 expression in nonneuronal cells remains poorly understood.

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In our previous studies, we have reported that GFRα3 is expressed in approximately 40% of sensory neurons that innervate the bone marrow, and that artemin/GFRα3 is involved in the pathogenesis of inflammatory bone pain (Nencini et al. 2018, 2019). However, there are many other nonneuronal and/or stromal cell types that are essential components of the bone marrow microenvironment (Anthony and Link 2014; Boulais and Frenette 2015; Isern et al. 2014; Wang et al. 2013). Mesenchymal stem cells (MSCs) represent a heterogeneous population of multipotent cells capable of giving rise to various mesenchymal tissues, including bone, cartilage and adipose (Caplan 1991; Pittenger et al. 1999). Perivascular cells, endothelial cells and Schwann cells also form a complex and supportive niche for haematopoietic stem cells (HSCs) and cells of the immune system. Many of these cell types are spatially associated with HSCs and provide regulatory signals to preserve and maintain aspects of stem cell function, both by cell-cell interactions and release of soluble factors (Morrison and Scadden 2014). It is unknown if GFRα3 is expressed in any of the nonneuronal cells in the bone marrow microenvironment, and importantly, if artemin/GFRα3 is involved in the regulation of haematopoiesis or other bone marrow functions.

In the present study, we have identified and characterised the expression of GFRα3 in nerve terminal endings and nonneuronal cells in the bone marrow with a view to better understanding the role of artemin/GFRα3 in the bone marrow microenvironment. In particular, we describe the morphology, distribution and molecular profile of GFRα3-expressing nonneuronal cells.

Materials and methods

Animals

Immunohistochemistry and fluorescence-activated cell sorting (FACS) were used to identify the expression of GFRα3, and a variety of cell specific markers for nonneuronal cells, in the bone marrow of long bones taken from 8- to 10-week-old C57BL/6 mice. To examine whether GFRα3-expressing cells in the bone marrow also expressed the intermediate protein nestin, we immuno-labelled the bone marrow of long bones taken from 9- to 13-week-old Tg(*Nes*-cre)1Kln mice, hereafter termed *Nes*-cre mice. *Nes*-cre mice expressed the Cre recombinase enzyme under the control of the nestin promoter gene and were crossed to *Rosa26-loxP-stop-loxP*-enhanced yellow fluorescent protein (*R26-stop*-EYFP; B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J)(*Nes*-cre; *EYFP*). To identify neural crest-derived cells in the bone marrow, we used postnatal (P4) and 7-week-old *Wnt1*-cre mice crossed to Ai32(RCL-ChR2(H134R)/EYFP) mice, which expressed *ChR2-EYFP* fusion protein after exposure to Cre recombinase (*Wnt1*-cre; *ChR2-EYFP*). Animals were killed by cervical dislocation or by cardiac perfusion with saline. All experiments performed were in accordance with ethical standards of the University of Melbourne Animal Experimentation Ethics Committee, St. Vincent's Health Animal Ethics Committee and the Animal Welfare Committee of Flinders University of South Australia.

Tissue preparation and immunohistochemistry

The femurs and tibias of mice were dissected and cleaned of surrounding soft tissue. They were fixed with 4% paraformaldehyde at 4 °C overnight and immersed for 7 days in a decalcifying solution containing 10% ethylenediaminetetraacetic acid (EDTA; pH 7.3) in 0.1 M phosphate-buffered solution (PBS). The solution was replaced every 2 days. After 7 days, the bones were cryoprotected in PBS containing 30% sucrose overnight at 4 °C. On the following day, they were embedded in optimal cutting temperature compound and sectioned at 30 μm using a cryostat. Sections were collected on gelatinised glass slides (1% gelatin and 0.1% chrome alum), air-dried for 1 h and processed for immunolabelling.

Sections were washed three times in PBS and blocked for 1 h in PBS containing 10% normal horse serum and 1% Triton X-100. They were incubated in primary antibody (see Table 1) overnight at room temperature. All antibodies were diluted in PBS containing 0.3% Triton X-100 and 0.1% sodium azide. Sections were immuno-labelled to identify coexpression of GFRα3, nestin-EYFP, the neurotrophic receptor p75 (p75NTR), glial fibrillary acidic protein (GFAP) and/or Wnt1-EYFP. Double labelling was also used to detect association of GFRα3-expressing cells with nerve terminals labelled with the panneuronal marker protein gene product 9.5 (PGP9.5), the sympathetic neuron marker tyrosine hydroxylase (TH) or the peptidergic sensory neuron marker calcitonin gene-related peptide (CGRP). On the following day, sections were washed three times in PBS and then incubated in secondary antibody (see Table 1) for 2 h at room temperature. Following another three washes in PBS, the slides were coverslipped using DAKO fluorescence mounting medium (Carpentaria, CA) and air-dried before imaging. Some sections were counterstained during the last PBS wash with DAPI (1:1000). Controls were prepared by incubating sections with an isotype-matched control antibody (normal goat IgG) or by omission of primary antibody.

The anti-GFRα3 antibody (R&D Systems, #AF2645) is a goat polyclonal antibody raised against the purified recombinant mouse GFRα3 extracellular domain (Glu34-Arg379). Using direct ELISA, this antibody shows less than 2% crossreactivity with recombinant mouse GFRα2 or GFRα4 or recombinant rat GFRα1 (manufacturer's information). Immuno-labelling of dorsal root ganglion (DRG) neurons

Table 1 Details of the primary and secondary antibodies used for immunohistochemistry

Primary antibody antigen	Immunogen	Manufacturing details	Dilution	Specificity/characterization
Calcitonin gene-related peptide (CGRP)	Synthetic CGRP (rat) conjugated to keyhole limpet hemocyanin	Sigma; rabbit polyclonal; #C8198	1:1000	(Lorenzo et al. 2008, Taylor et al. 2009) Manufacturer's information
Glial fibrillary acidic protein (GFAP)	GFAP isolated from cow spinal cord tissue	Dako; rabbit polyclonal; #Z0334	1:500	(Hanbury et al. 2003, Yamazaki et al. 2011) Manufacturer's information
Green fluorescent protein (GFP), also detects EYFP	GFP protein isolated directly from *Aequorea Victoria*, purified using ion-exchange chromatography	Invitrogen; rabbit polyclonal; #A11122	1:500; 1:5000	(Moldrich et al. 2010) Manufacturer's information
GDNF family receptor alpha 3 (GFRα3)	*S. frugiperda* insect ovarian cell line *Sf* 21-derived recombinant mouse GFRα3 (34-379)	R&D Systems; goat polyclonal; #AF2645	1:300	(Fasanella et al. 2008, Malin et al. 2006) Manufacturer's information
Normal goat IgG control		R&D Systems; goat polyclonal IgG #AB108C	1:300	
P75 neurotrophin receptor (p75NTR)	Recombinant fusion protein produced in *E. coli*	Promega; rabbit polyclonal; #G323A	1:100	(Paul et al. 2004) Manufacturer's information
Platelet-derived growth factor receptor alpha (PDGFRα)	Mouse myeloma cell line NS0-derived recombinant mouse PDGFRα Leu25-Clu524	R&D Systems; goat; polyclonal; #AF1062	1:50	(Koh et al. 2012, Kurahashi et al. 2013) Manufacturer's information
Protein gene product (PGP) 9.5	Human PGP9.5 protein purified from pathogen-free human brain	Cedarlane Labs; rabbit polyclonal; #CL95101	1:1000	(Chidlow et al. 2011, Doran et al. 1983) Manufacturer's information
Tyrosine hydroxylase (TH)	Denatured TH from rat pheochromocytoma	Millipore; rabbit polyclonal; #AB152	1:1000	(Brown et al. 2011) Manufacturer's information
Stem cell antigen-1 (Sca-1)	IL-2-dependant mouse T cell line	Biolegend; rat; #108102	1:1000	(English et al. 2000) Manufacturer's information
α-Smooth muscle actin (α-SMA)	Synthetic peptide corresponding to N-terminus of actin from human smooth muscle	Abcam; rabbit polyclonal; #AB5694	1:100	(Farahani et al. 2012) Manufacturer's information
Secondary antibody		Manufacturing details		Dilution
Donkey α Chicken Dylight 488		Jackson; #703-485-155		1:200
Donkey α Chicken Dylight 594		Jackson; #703-515-155		1:200
Donkey α Goat Alexa Flour 488		Molecular Probes; #A11055		1:200
Donkey α Goat IgG Texas Red		Jackson; #705-075-147		1:200
Donkey α Rabbit Alexa Flour 488		Molecular Probes; #A21206		1:200
Donkey α Rabbit Alexa Flour 594		Molecular Probes; #A21207		1:200
Donkey α Rat Alexa Flour 488		Molecular Probes; #A21208		1:200
Streptavidin, Alexa Flour 488 conjugate		Molecular Probes, #S11223		1:200

with this antibody is not present in GFRα3 knockout mice, but is present in wild-type controls (Fasanella et al. 2008). We have also performed experiments to show that preadsorption with the manufacturer's peptide (tested at 0.01, 0.1, 1 and 10 μg/ml; Recombinant Mouse GFR alpha-3 Fc Chimera Protein; R&D Systems; #2645-FR-050) completely abolishes staining of GFRα3 profiles in the bone marrow of mice at 1 μg/ml (data not shown).

Anterograde tracing of sensory nerve terminals in bone

C57BL/6 mice were anaesthetised with isoflurane (induction 4%, maintainence 1.5%, in oxygen). A 1–2-cm incision was made at the dorsal surface of the lumbar vertebrae, and into the musculature over the intervertebral foramina, to identify the DRG. L3-5 DRG were exposed and injected with 50–100 nl dextran biotin (10–20% in saline; Molecular Probes #D1956, Eugene, Oregan, USA), using a custom made nitrogen-delivered drug spritzer system. The musculature was sutured with 4.0 suture (Dynek, Australia), and the skin incision was closed with fine suture. Mice were given a 7-day recovery period to allow for the tracer to be transported from DRG soma to nerve terminals in bone (Spencer et al. 2014). Seven days post surgery, mice were anaesthetised with sodium pentobarbitone (60 mg/ml; 0.1 ml/100 g; i.p.) and perfused via the aorta with 100 ml of heparinised saline, followed by 100 ml of 4% paraformaldehyde. The femurs and tibias were removed, prepared and processed for GFRα3 immuno-labelling as above. Streptavidin-Alexa Flour 488 conjugate (Molecular Probes, #S11223, 1:200) was included in the secondary antibody incubation solution to bind to and reveal the extent of anterograde labelling of sensory nerve terminals with dextran biotin.

Imaging

High-power images of immuno-labelled sections were captured using a confocal microscope (Airyscan LSM800 confocal microscope with Zen 2.1 imaging software, Carl Zeiss MicroImaging, Oberkocken, Germany). Alexa Fluor 488 (AF488), Rhodamine and DAPI filter sets were used to discriminate cells labelled with AF488 and 594 fluorophores and the DAPI counterstain, respectively. Optical z-stack projections were generated using a maximal intensity algorithm to examine nerve terminals and cell profiles through the full thickness of bone marrow sections at multiple magnifications (× 20, × 40 and × 63 objectives). For each objective, the pinhole was set to 1 Airy unit to obtain optimal slice thickness. Lower-power images of whole sections were imaged using a Zeiss Axioscope.Z1 fluorescence microscope (Carl Zeiss Microscope, Oberkocken, Germany) using × 20 objective magnification and stitched together using ImageJ software (National Institutes of Health, Bethesda, Maryland). Images were examined using Zen imaging software (v 8.1, Carl Zeiss Microscopy, Oberkocken, Germany), and figures were prepared on CorelDraw software (v 12, Corel Corporation, Ottawa, Canada). Adjustments to the brightness and contrast were made to individual images. No other manipulations were made.

Fluorescence-activated cell sorting

FACS was performed on bones from C57BL/6 mice or *Wnt1*-cre; *ChR2-EYFP* mice to examine the different populations of cells that expressed GFRα3 or Wnt1-EYFP in the bone marrow. Long bones were dissected and gently crushed with a mortar and pestle in sterile PBS serum (PBS supplemented with 2% fetal bovine serum and 3 mM EDTA) to remove the bone marrow. Bone fragments were washed twice in PBS and digested in collagenase type I (3 mg/ml; Worthington, NJ) in PBS twice for 25 min at 37 °C. The released digested bone suspensions were washed, centrifuged at 400×*g* for 5 min and resuspended in PBS serum. Cells were then incubated for 30 min on ice with an antibody cocktail comprised of allophycocyanin (APC)-eFluor780 conjugated haematopoietic lineage markers (CD3, CD4, CD8α, CD11b, CD45, Gr-1, Ter119 and B220), CD31-peridinin chlorophyll protein (PerCP)-eFluor710, CD51-phycoerythrin (PE), Sca1-Pacific blue and PDGFRα-PE-CF594, together with the GFRα3 antibody (see Table 2). Cells were then washed twice in PBS serum and incubated with the secondary anti-goat AF488 antibody for 30 min on ice. Analysis of the background fluorescence of the GFRα3 antibody was conducted using the corresponding isotype-matched control primary antibody (normal goat IgG). Single stain controls were also prepared using BD CompBeads (BD Biosciences) to set fluorescence compensation. The expression of Sca-1 and CD51 in $EYFP^+$ bone marrow cells from *Wnt1*-cre; *ChR2-EYFP* mice was assessed in separate experiments. All FACS data were acquired using a FACSAria Cell Sorter (BD Biosciences, San Jose, CA) and analysed with FlowJo software (v. 8.8.7, Treestar, Ashland, OR, USA).

Results

Morphology and distribution of GFRα3-expressing cells in the bone marrow

We used immunohistochemistry to identify GFRα3 expression in the femoral bone marrow of C57BL/6 mice. Longitudinal sections through the whole femur showed nucleated GFRα3-expressing cells distributed along the length of the marrow cavity ($n = 17$, Fig. 1a–e). These cells were found in greater numbers at the central diaphysis (Fig. 1a). Double

Table 2 Antibodies used for flow cytometric analysis

Antigen	Conjugate	Manufacturing details	Dilution
CD3	APCeFluor780	Jomar Biosciences; #47-0031-82	1:200
CD4	APCeFluor780	Jomar Biosciences; #47-0041-82	1:200
CD8α	APCeFluor780	Jomar Biosciences; #47-0081-82	1:200
CD11b	APCeFluor780	Jomar Biosciences; #47-0112-82	1:200
CD45	APCeFluor780	Jomar Biosciences; #47-0451-82	1:200
Gr-1	APCeFluor780	Jomar Biosciences; #47-5931-82	1:200
Ter119	APCeFluor780	Jomar Biosciences; #47-5921-82	1:200
B220	APCeFluor780	Jomar Biosciences; #47-0452-82	1:200
Sca-1	Pacific Blue	BioLegends; #108120	1:250
CD51	PE	BioLegends; #551187	1:250
CD31	PerCPeFluor 710	Jomar Biosciences; #46-0311-82	1:500
PDGFRα	PE-CF9594	BioLegends; #562775	1:500
GFRα3		R&D Systems; #AF2645	1:100
Isotype control		R&D Systems; #AB108C	1:100
Donkey α Goat Alexa Flour488		Molecular Probes; #A11055	1:100

labelling with an antibody directed against α-smooth muscle actin (α-SMA) revealed that the majority of these cells were found within the vicinity of blood vessels covered by α-SMA-positive cells (Fig. 1f, g) and particularly around the nutrient foramen. The GFRα3-expressing cells associated with larger vessels were often elongated, spindle-structured cells with extensive processes that wrapped around the length of the vessels (Fig. 1f). A number of single, thin-shaped GFRα3-expressing cells were also noted along smaller vessels near the endosteum or appeared to be distributed away from blood vessels within the bone marrow (Fig. 1g). Staining of these elongated GFRα3-expressing cells was absent when the bone marrow sections were incubated with a GFRα3 isotype-matched control primary antibody.

GFRα3-expressing cells are distinct from nerve terminals in the bone marrow, but some bone marrow nerve terminals also express GFRα3

To determine if the GFRα3 expression was on cells distinct from peripheral nerve terminals in the bone marrow, we used double labelling with antibodies directed against PGP9.5. This revealed a relatively rich distribution of nerve fibres and terminals in the bone marrow, particularly around the nutrient foramen and branching arterioles ($n = 8$, Fig. 2). We noted on higher-power imaging and in images of single optical planes captured by confocal microscopy, that GFRα3-expressing cells were often closely associated with, but clearly distinct from the PGP9.5 immuno-labelled nerve terminals in the marrow cavity (Fig. 2d–g and h–k). The PGP9.5 immuno-labelled nerve terminals had a fine varicose appearance that appeared to make contact with the cell body of GFRα3-expressing cells (Fig. 2d–g) or the elongated processes of GFRα3-expressing cells (Fig. 2h–k). We also observed GFRα3 expression in some of the PGP9.5 immuno-labelled nerve terminals (Fig. 2l–o). We used an antibody directed against TH to determine if some of these nerve terminal endings were sympathetic ($n = 11$, Fig. 3). TH immuno-labelled nerve terminals were also in close contact with, but clearly distinct from, the soma (Fig. 3d–g) and elongated processes (Fig. 3h–k) of GFRα3-expressing cells, and GFRα3 expression was observed in some of the TH immuno-labelled nerve terminals (Fig. 3l–o).

We also used anterograde tracing ($n = 4$, Fig. 4) and an antibody directed against CGRP ($n = 3$, Fig. 5) to determine if some of the nerve terminal endings were of sensory origin. Anterograde-labelled, sensory nerve terminal endings had a similar relationship with the soma (Fig. 4d–g) and the elongated processes (Fig. 4h–k) of GFRα3-expressing cells to TH immuno-labelled nerve terminals, and some also expressed GFRα3 (Fig. 4l–o). CGRP immuno-labelled nerve terminal endings were characteristically varicose and also observed in close contact with, but distinct from, the soma of GFRα3-expressing cells (Fig. 5d–g) and their elongated processes (Fig. 5h–k), and some CGRP immuno-labelled nerve terminals expressed GFRα3 (Fig. 5l–o). Taken together, these findings show that GFRα3 is expressed in some sensory and sympathetic nerve terminal endings in the bone marrow, but that there are also many GFRα3-expressing cells that are closely apposed to, but distinct from both sensory and sympathetic nerve terminal endings in the bone marrow.

GFRα3-expressing cells are coexpressed with markers of nonmyelinating Schwann cells

We used the bone marrow obtained from *Nes*-cre; *EYFP* mice to further characterise the GFRα3-expressing cells. Sections

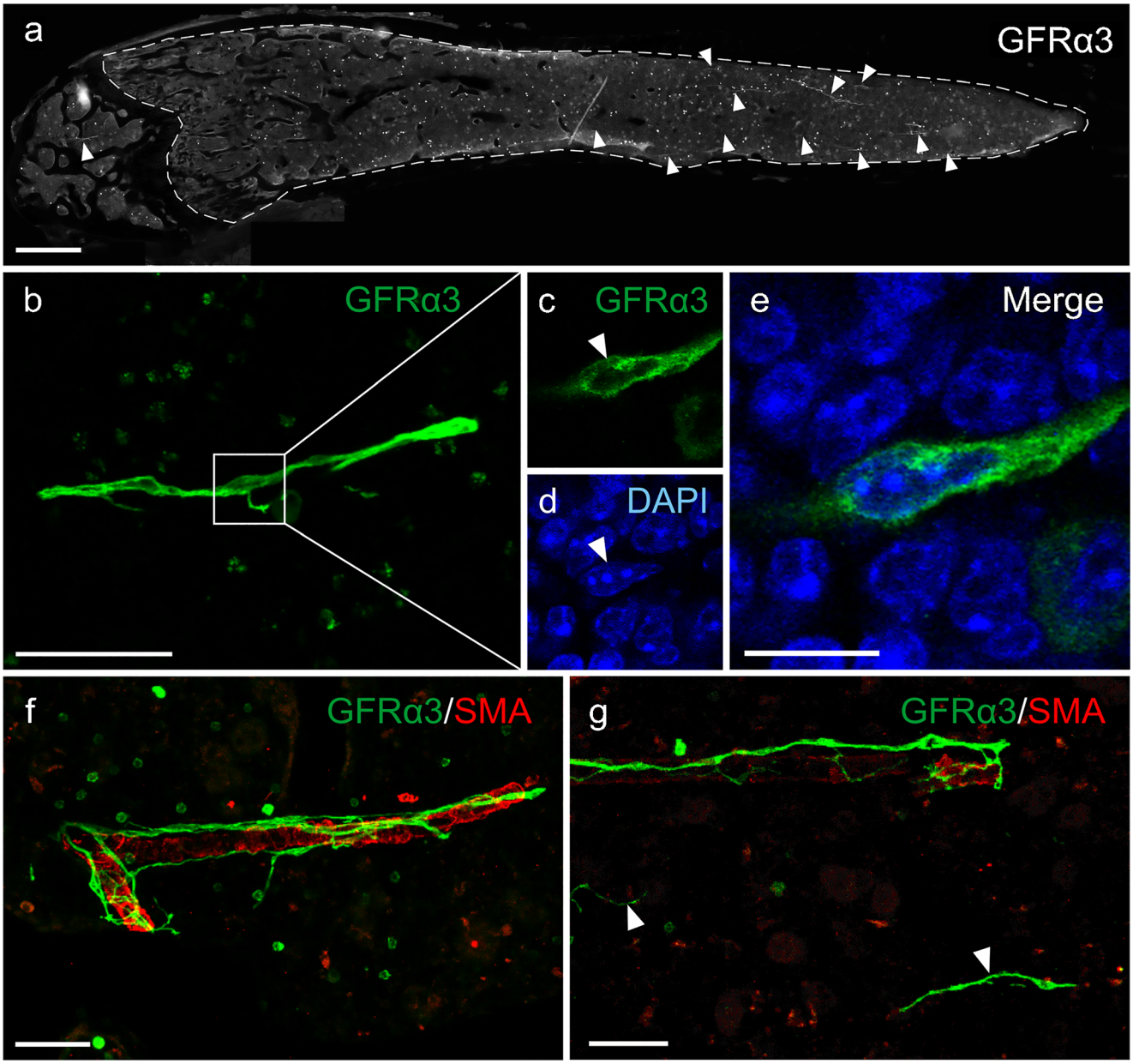

Fig. 1 Immunostaining for GFRα3 in the femoral bone marrow of C57BL/6 mice ($n = 17$). **a** Low-magnification image of the whole bone marrow shows the distribution of GFRα3-labelled cells in the medullary cavity (arrowheads). Dashed lines indicate marrow cavity. **b–e** High-magnification confocal image of a GFRα3-expressing cell. **b** Z-projection of cell through full thickness of the section. Insets (**c–e**) show single optical sections of **c** GFRα3, **d** DAPI and **e** a merged image of the same cell. **f–g** Representative Z-projection images showing a close association of the GFRα3-expressing cells with blood vessels. Sections were double-labelled with an antibody against α-SMA to identify arterioles. Arrowheads in **g** indicate single GFRα3-expressing cells along smaller vessels. Scale bars = 500 μm (**a**), 50 μm (**b**, **f** and **g**) and 10 μm (**e**)

were immuno-labelled with antibodies directed against EYFP (to detect nestin-targeted cells), p75NTR and GFAP, all known markers of nonmyelinating Schawnn cells (Jessen and Mirsky 1999; Mendez-Ferrer et al. 2010; Yamazaki et al. 2011).

The intermediate filament protein nestin is expressed in dividing cells during early development and has previously been used to identify neural crest-derived MSCs and nonmyelinating Schwann cells in the bone marrow (Isern et al. 2014; Yamazaki et al. 2011). Immuno-labelling of $EYFP^+$ cells in bone marrow sections obtained from *Nes*-cre; *EYFP* mice revealed that the elongated GFRα3-expressing cells in the bone marrow were targeted by nestin ($n = 5$, Fig. 6a–c). However, the majority of the nestin-targeted EYFP cells had a satellite-shaped appearance and did not express GFRα3 (Fig. 6a). We further characterised the GFRα3-expressing cells by double labelling with an antibody directed against p75NTR ($n = 6$, Fig. 6d–f and g–j), a receptor that is

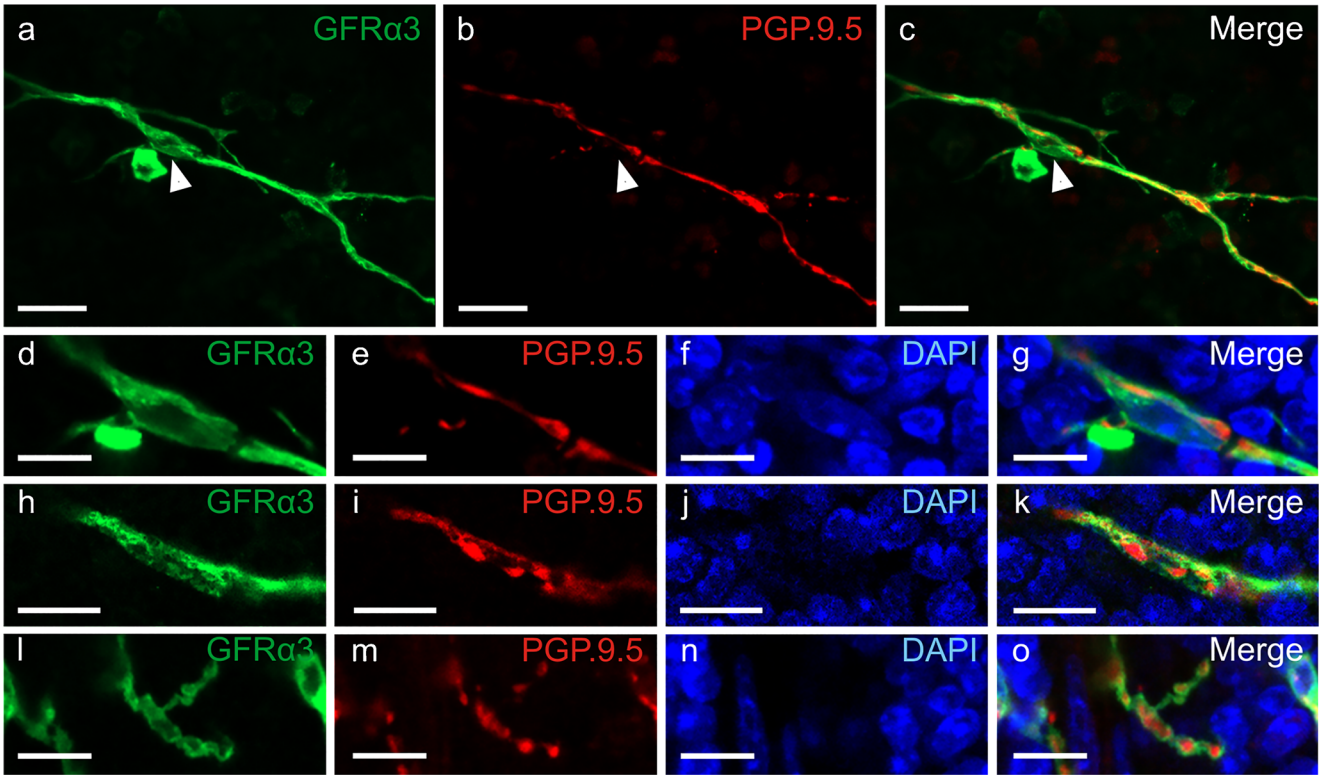

Fig. 2 GFRα3 and PGP9.5 immunostaining in the bone marrow of C57BL/6 mice ($n = 8$). **a–c** Confocal images of a Z-projection through the full thickness of GFRα3-expressing cells showing a close association with PGP9.5-labelled nerve fibres in the mouse femur. **d–g** High-magnification confocal images of single optical sections through the cell indicated by the arrowhead in **a–c**. This shows an example of a nucleated (DAPI stained) GFRα3-expressing cell with PGP9.5-labelled nerve terminal endings closely apposed to the surface of the cell. **h–k** High-magnification confocal images of single optical sections through the elongated process of a GFRα3-expressing cell. This shows an example of PGP9.5-labelled nerve terminals closely apposed to, but clearly distinct from, the GFRα3-expressing cell process. **l–o** High-magnification confocal images of single optical sections showing that some PGP9.5-labelled nerve terminals expressed GFRα3. Scale bars = 20 μm (**a–c**) and 10 μm (**d–o**)

involved in cell survival and/or apoptosis and axonal growth (Dechant and Barde 2002; Underwood and Coulson 2008; Yamashita et al. 1999), or GFAP ($n = 11$, Fig. 6k–m and n–q), a major component of immature and nonmyelinating Schwann cells (Jessen and Mirsky 2008). We observed that all of the GFRα3-expressing cells were positive for p75NTR and GFAP and vice versa.

The neural crest is the source of the GFRα3-expressing nonmyelinating Schwann cells in the bone marrow

The neural crest can give rise to diverse cell types in the peripheral nervous system, including both glial cells and peripheral neurons (Le Douarin and Dupin 2003; Stemple and Anderson 1992). Wnt1 signalling is important for maintaining proliferation of neural crest progenitors during development, and its expression on cells indicates a neural crest developmental origin (Ikeya et al. 1997). To determine whether bone marrow GFRα3-expressing cells were derived from the neural crest, we performed immunohistochemical analysis of *Wnt1*-cre; *ChR2-EYFP* transgenic mice ($n = 8$, Fig. 7). Double labelling of GFRα3-expressing cells with antibodies directed against EYFP revealed that all of the GFRα3 immunolabelled cells and/or nerve terminals in the bone marrow were positive for Wnt1 (Fig. 7f–h and i–l). This indicates that both GFRα3-expressing cells and nerve terminals in the bone marrow are derived from the neural crest.

Flow cytometric analysis identified GFRα3-expressing cells within the CD51$^+$Sca-1$^-$PDGFRα$^-$ population

To further characterise the GFRα3-expressing cells in the bone marrow, the expression of GFRα3 was examined in collagenase-digested bone marrow microenvironment cells ($n = 3$). Potential haematopoietic lineage (CD2, CD3, CD4, CD5, CD8, CD11b, Gr-1, CD45, B220, Ter119) and vascular endothelial (CD31) cells were initially gated out of bone marrow suspensions (Fig. 8a, b; see Table 2 for antibodies used). The resulting lin$^-$CD45$^-$CD31$^-$ cells were further subdivided using antibodies directed against integrin αV (CD51) and Sca-1 to resolve for four cell populations within the bone marrow (Fig. 8c–e): the mesenchymal stem/progenitor cells (CD51$^+$Sca-1$^+$), osteoblast-lineage cells (CD51$^+$Sca-1$^-$),

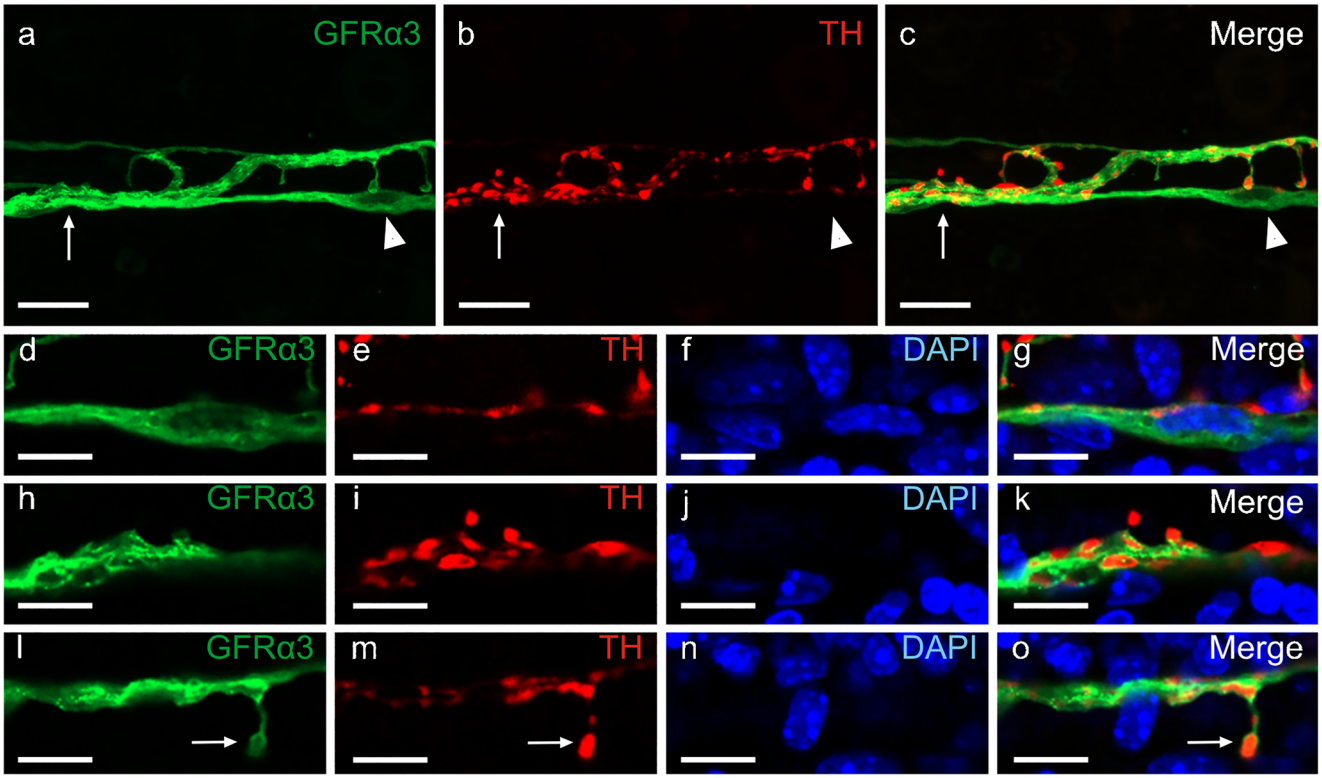

Fig. 3 GFRα3 and TH immunostaining in the bone marrow of C57BL/6 mice ($n = 11$). **a–c** Confocal images of a Z-projection through the full thickness of GFRα3-expressing cells showing that GFRα3-expressing cells are in intimate contact with TH-labelled sympathetic nerve terminals. **d–g** High-magnification confocal images of single optical sections through the cell indicated by the arrowhead in **a–c**. This shows an example of a nucleated (DAPI stained) GFRα3-expressing cell with TH-labelled nerve terminal endings closely apposed to the surface of the cell, but clearly distinct from GFRα3 labelling. **h–k** High-magnification confocal images of single optical sections through the elongated process of a GFRα3-expressing cell indicated by the arrow in **a–c**. This shows an example of TH-labelled nerve terminals closely apposed to, but clearly distinct from, the GFRα3-expressing cell process. **l–o** High-magnification confocal images of single optical sections showing that some TH-labelled nerve terminals expressed GFRα3 (arrow). Scale bars = 20 μm (**a–c**) and 10 μm (**d–o**)

$CD51^{-}Sca\text{-}1^{+}$ and $CD51^{-}Sca\text{-}1^{-}$ populations (Green et al. 2017).

We then examined the proportion of cells that expressed GFRα3 and PDGFRα within each subpopulation. PDGFRα is an important marker of MSCs and is required for mesodermal development (Pinho et al. 2013). Flow cytometric data revealed that the $CD51^{+}Sca\text{-}1^{-}$ population of cells expressed GFRα3 above the IgG isotype control, with 18.95 ± 4.26% (mean ± SD) of cells expressing GFRα3 ($n = 3$, Fig. 8c, d). Furthermore, the GFRα3-expressing cells in this population showed negligible PDGFRα expression. A second GFRa3-expressing population was identified in the $CD51^{+}Sca\text{-}1^{+}$ cells (Fig. 8d‘). These cells did express PDGFRα (mean ± SD 11.73 ± 1.86) ($n = 3$, Fig. 8d‘). No GFRα3 expression was detected in the $CD51^{-}Sca\text{-}1^{-}$ or $CD51^{-}Sca\text{-}1^{+}$ populations ($n = 3$, Fig. 8d“ and d“‘).

In a separate method of analysis, we examined the expression of $EYFP^{+}$ cells in different populations of collagenase-digested bone marrow microenvironment cells using the *Wnt1*-cre; *ChR2-EYFP* mice ($n = 3$, Fig. 8e, e‘, e“, e“‘). This revealed that 55.8 ± 13.02% of the Wnt1-EYFP cells are $lin^{-}CD31^{-}CD51^{+}Sca\text{-}1^{-}PDGFR\alpha^{-}$ cells. There was also another EYFP-positive population observed in the $lin^{-}CD31^{-}CD51^{-}Sca\text{-}1^{-}PDGFR\alpha^{-}$ population (39.83 ± 11.83% of Wnt1-EYFP+ cells). The $CD51^{+}Sca\text{-}1^{+}$ cells did not express EYFP.

Immuno-labelling of sections with GFAP and PDGFRα, or GFRα3 and Sca-1, confirmed that the elongated cells in the bone marrow do not express PDGFRα (Fig. 9a–c) or Sca-1 (Fig. 9d–f). It also revealed that $Sca\text{-}1^{+}$ and $GFR\alpha3^{+}$ cells were found along the endosteal surface of the cortical bone (data not shown). However, this labelling was not abolished by omission of the primary antibody and so was likely nonspecific staining.

Discussion

In the present study, we have identified and characterised a population of GFRα3-expressing nonneuronal cells in the bone marrow. We described

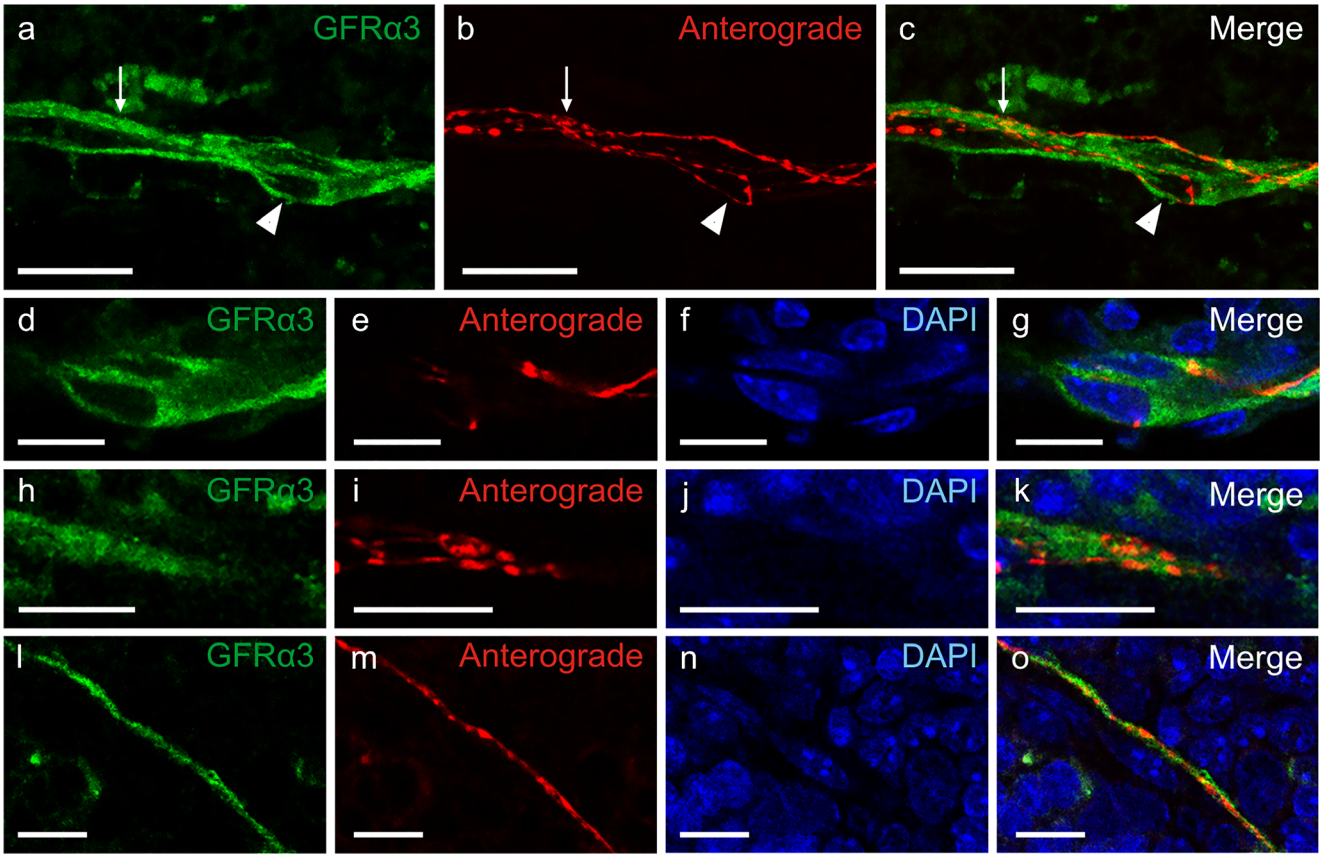

Fig. 4 Anterogradely labelled bone afferent neurons following injection of dextran biotin into lumbosacral DRG of C57BL/6 mice ($n = 4$). **a–c** Confocal images of a Z-projection through the full thickness of GFRα3-expressing cells showing that GFRα3-expressing cells are in intimate contact with anterograde-labelled sensory nerve terminals. **d–g** High-magnification confocal images of single optical sections through the cells indicated by the arrowhead in **a–c**. This shows an example of nucleated (DAPI stained) GFRα3-expressing cells with anterograde-labelled nerve terminal endings closely apposed to the surface of the cell, but clearly distinct from GFRα3 labelling. **h–k** High-magnification confocal images of single optical sections through the elongated process of a GFRα3-expressing cell indicated by the arrow in **a–c**. This shows an example of anterograde-labelled nerve terminals closely apposed to, but clearly distinct from, GFRα3-expressing cell processes. **l–o** High-magnification confocal images of single optical sections showing that some anterograde-labelled nerve terminals expressed GFRα3. Scale bars = 20 μm (**a–c**) and 10 μm (**d–o**)

the elongated, spindle-shaped morphology of the GFRα3-expressing cells and noted their close distribution around blood vessels. Anterograde tracing and immuno-labelling with neuronal markers (PGP9.5, TH and CGRP) revealed that GFRα3-expressing cells were very closely associated with, but clearly distinct from, both sensory and sympathetic nerve terminals in the bone marrow. In addition, it also revealed that GFRα3 was expressed in some sensory and sympathetic nerve terminal endings. We also found that markers of glial cells (e.g. GFAP and p75NTR) were expressed on the elongated GFRα3-labelled cells in the marrow cavity. By using Wnt1-reporter mice, we further demonstrated that the GFRα3-expressing cells are of a neural crest origin. Finally, we revealed with flow cytometry that a population of the GFRα3-expressing cells have a glial cell phenotype ($CD51^+$, Sca-1^- and PDGFRα^-). Taken together, the findings indicate that the elongated GFRα3-expressing cells are bone marrow glial cells, more specifically neural crest-derived, nonmyelinating Schwann cells. Schwann cells have well-established roles in the development of the peripheral nervous system, regeneration of injured peripheral nerves and regulation of haematopoiesis. Our findings suggest that artemin/GFRα3 may be relevant to these functions of nonmyelinating Schwann cells in the bone marrow.

The GFRα3 receptor is expressed in developing sympathetic postganglionic neurons (Baloh et al. 1998) and a subpopulation of small-diameter peptidergic sensory neurons (Ernsberger 2008; Orozco et al. 2001). It has also been shown to be expressed in the soma of at least one subpopulation of small-diameter peripheral sensory neurons retrogradely labelled with injections from the rat tibia (Nencini et al. 2018). Whilst some of the GFRα3 immuno-labelling we report in the bone marrow in the present study is of peripheral nerve terminal

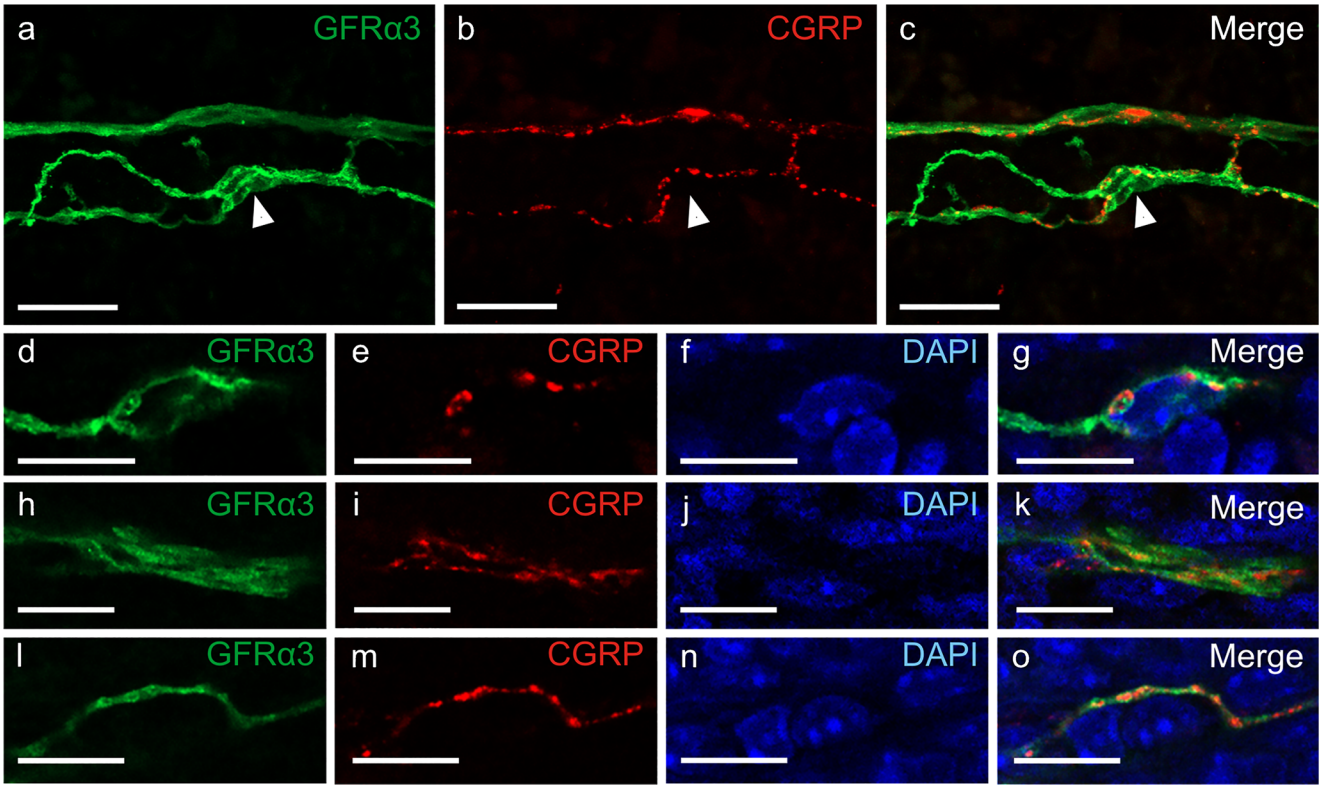

Fig. 5 GFRα3 and CGRP immunostaining in the bone marrow of C57BL/6 mice ($n = 3$). **a–c** Confocal images of a Z-projection through the full thickness of GFRα3-expressing cells showing that GFRα3-expressing cells are closely associated with CGRP-labelled peptidergic sensory nerve terminals. **d–g** High-magnification confocal images of single optical sections through the cell indicated by the arrowhead in **a–c**. This shows an example of a nucleated (DAPI stained) GFRα3-expressing cell with CGRP-labelled nerve terminal endings closely apposed to the surface of the cell, but clearly distinct from GFRα3 labelling. **h–k** High-magnification confocal images of single optical sections through the elongated process of a GFRα3-expressing cell. This shows an example of CGRP-labelled nerve terminals closely apposed to, but clearly distinct from GFRα3-expressing cell processes. **l–o** High-magnification confocal images of single optical sections showing that some CGRP-labelled nerve terminals express GFRα3. Scale bars = 20 μm (**a–c**) and 10 μm (**d–o**)

endings, we have clearly shown that GFRα3 is also expressed in nonmyelinating Schwann cells that have a close association with sensory and sympathetic nerve terminals in the bone marrow.

Signalling by artemin requires the presence of both the ligand specific GFRα3 receptor and the tyrosine kinase coreceptor RET. However, a number of studies have reported a distinct lack of RET expression on Schwann cells of the sciatic nerve (Trupp et al. 1997, 1999), or that artemin could even interact with Schwann cells in the absence of RET (Iwase et al. 2005; Paratcha et al. 2001). This suggests that artemin might interact with GFRα3 in a RET-independent manner to regulate Schwann cell function. Some studies have indeed described a role for a non-RET signalling receptors for artemin, including NCAM and Syndecan-3 (Bespalov et al. 2011; Schmutzler et al. 2011). How this is relevant to the function of the nonmyelinating Schwann cells we report in the bone marrow remains to be determined.

Schwann cells have a well-established role in the development of both sensory and sympathetic components of the peripheral nervous system (Corfas et al. 2004; Monk et al. 2015). Our findings of GFRα3 expression on nonmyelinating Schwann cells in the bone marrow of both adult and postnatal day 4 mice suggest that artemin may contribute to the development of neurons that innervate the bone marrow. There is indeed evidence by in situ hybridization that GFRα3 is expressed on neural crest-derived cells, including Schwann cell precursors, during development (Widenfalk et al. 1998). Furthermore, immature Schwann cells are known to express high levels of artemin which can function in a paracrine or autocrine fashion (Baloh et al. 1998; Fontana et al. 2012). Thus, artemin/GFRα3 may be involved in the dynamic interactions between migrating glial cells and developing axons during early innervation of the bone marrow. In the vasculature, artemin is also known to be expressed in smooth muscle cells and acts as a survival and guidance factor for neurons to follow blood vessels as they project towards their target tissue (Honma et al. 2002). This is likely to be relevant to the innervation of the bone marrow which clearly follows a vascular route into

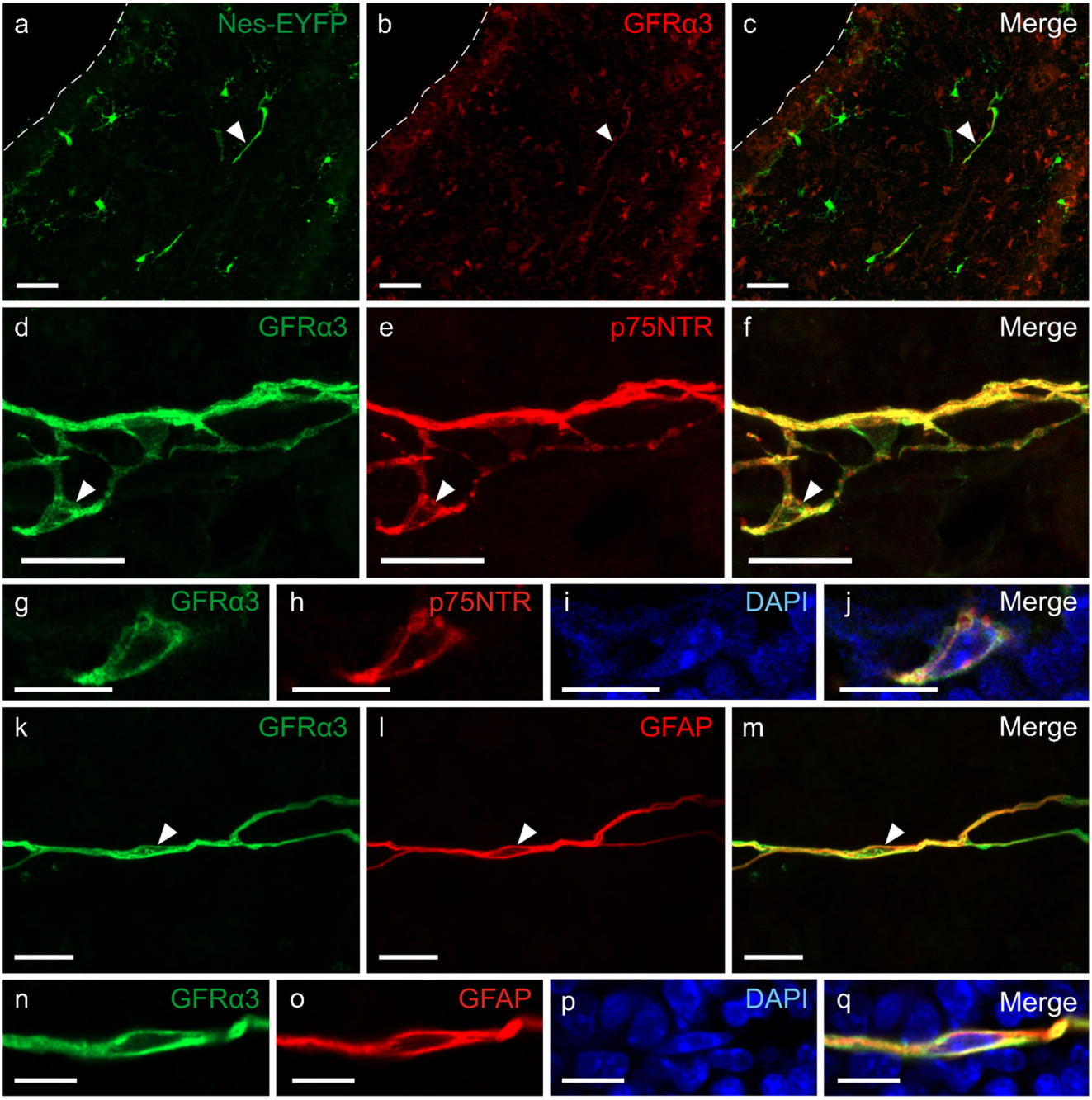

Fig. 6 Coexpression of GFRα3 with cell markers of glial cells. **a–c** Nestin-targeted EYFP$^+$ cells in tibial bone marrow sections of *Nes*-cre; *EYFP* mice ($n = 5$). Note that GFRα3 is expressed on only a small subset of the Nes-EYFP$^+$ cells that have an elongated, spindle-shaped morphology (arrowhead). Dashed lines indicate marrow cavity. **d–f** Double labelling shows colocalisation of GFRα3 with p75NTR in both cells and nerve terminals ($n = 6$). **g–j** High-magnification confocal images of single optical sections through the cell indicated by the arrowhead in **d–f**. This shows an example of nucleated (DAPI stained) GFRα3-expressing cells that also expresses p75NTR. **k–m** Double labelling shows colocalisation of with GFRα3 with GFAP ($n = 11$). **n–q** High-magnification confocal images of single optical sections through the cell indicated by the arrowhead in **k–m**. This shows an example of nucleated (DAPI stained) GFRα3-expressing cells that also expresses GFAP. Scale bars = 50 μm (**a–c**), 20 μm (**d–f**, **k–m**) and 10 μm (**g–j**, **n–q**)

the bone. Glial cell migration is an important process during peripheral nerve development, and further experiments are required to clarify the role of artemin/GFRα3 towards this in the bone marrow.

Schwann cells are also known to support the regeneration of injured peripheral nerve fibres. They can migrate, express growth promoting factors and transform their morphological and molecular properties to ensure appropriate repair (Jessen et al. 2015). Baloh et al.

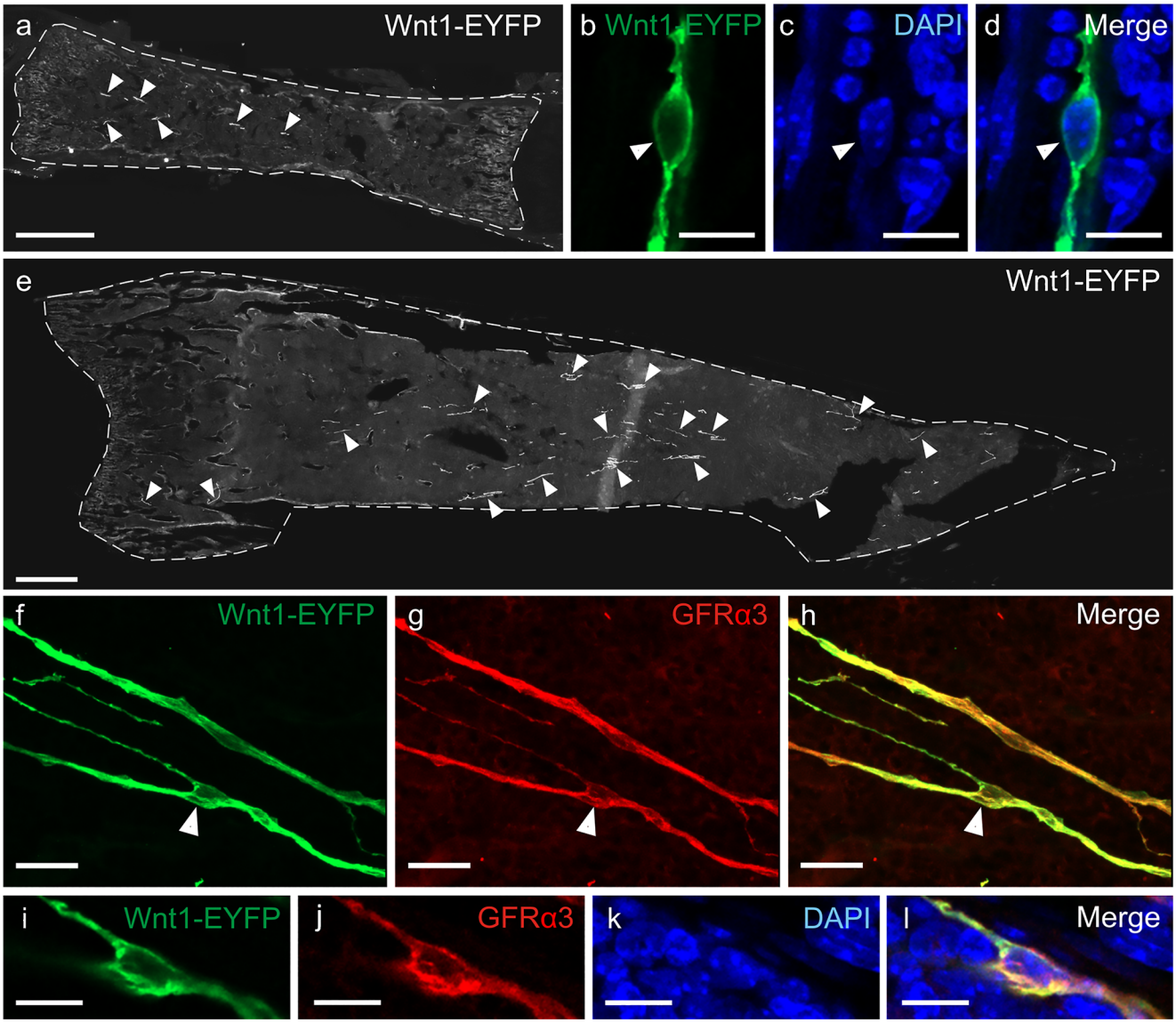

Fig. 7 GFRα3-expressing cells in bone marrow sections of *Wnt1*-cre; *ChR2-EYFP* mice. Cells positive for Wnt1-EYFP are derived from the neural crest. **a**, **e** Low-magnification images of a whole bone marrow section of postnatal P4 pup (**a**, $n = 5$) and 7-week-old mice (**e**, $n = 3$) show the distribution of Wnt1-EYFP$^+$ cells (arrowheads). Dashed lines indicate marrow cavity. **b–d** High-magnification confocal images of single optical sections through a Wnt1-EYFP$^+$ cell counterstained with DAPI. **f–h** Confocal images of a Z-projection through the full thickness of a section showing colocalisation of GFRα3 with Wnt1-EYFP. **i–l** High-magnification confocal images of single optical sections through the cell indicated by the arrowhead in **f–h**. All GFRα3-expressing cells are Wnt1-EYFP$^+$ and are therefore neural crest-derived cells. Scale bars = 500 μm (**a**, **e**), 10 μm (**b–d**, **i–l**) and 20 μm (**f–h**)

(1998) found that following sciatic nerve transection, adult Schwann cells reacquire transcription factors associated with an immature state in a process of dedifferentiation and subsequently upregulate the expression of artemin. Fontana et al. (2012) further showed that artemin released by Schwann cells supports target reinnervation and axonal outgrowth after nerve axotomies. Given that our current study showed GFRα3 expression on nonmyelinating Schwann cells in the bone marrow, and that there is such a close association between these cells and both sensory and sympathetic nerve terminal endings, it is possible that axonal regeneration to the bone marrow may result from effects of artemin through GFRα3 on nonmyelinating Schwann cells. However, the molecular mechanisms involved in nerve regeneration are highly complex, and there have been limited studies that have examined the role of GFRα3.

Recent studies have further suggested that nonmyelinating Schwann cells can act as functional components of the HSC niche that are important for regulating HSC quiescence (Yamazaki et al. 2011).

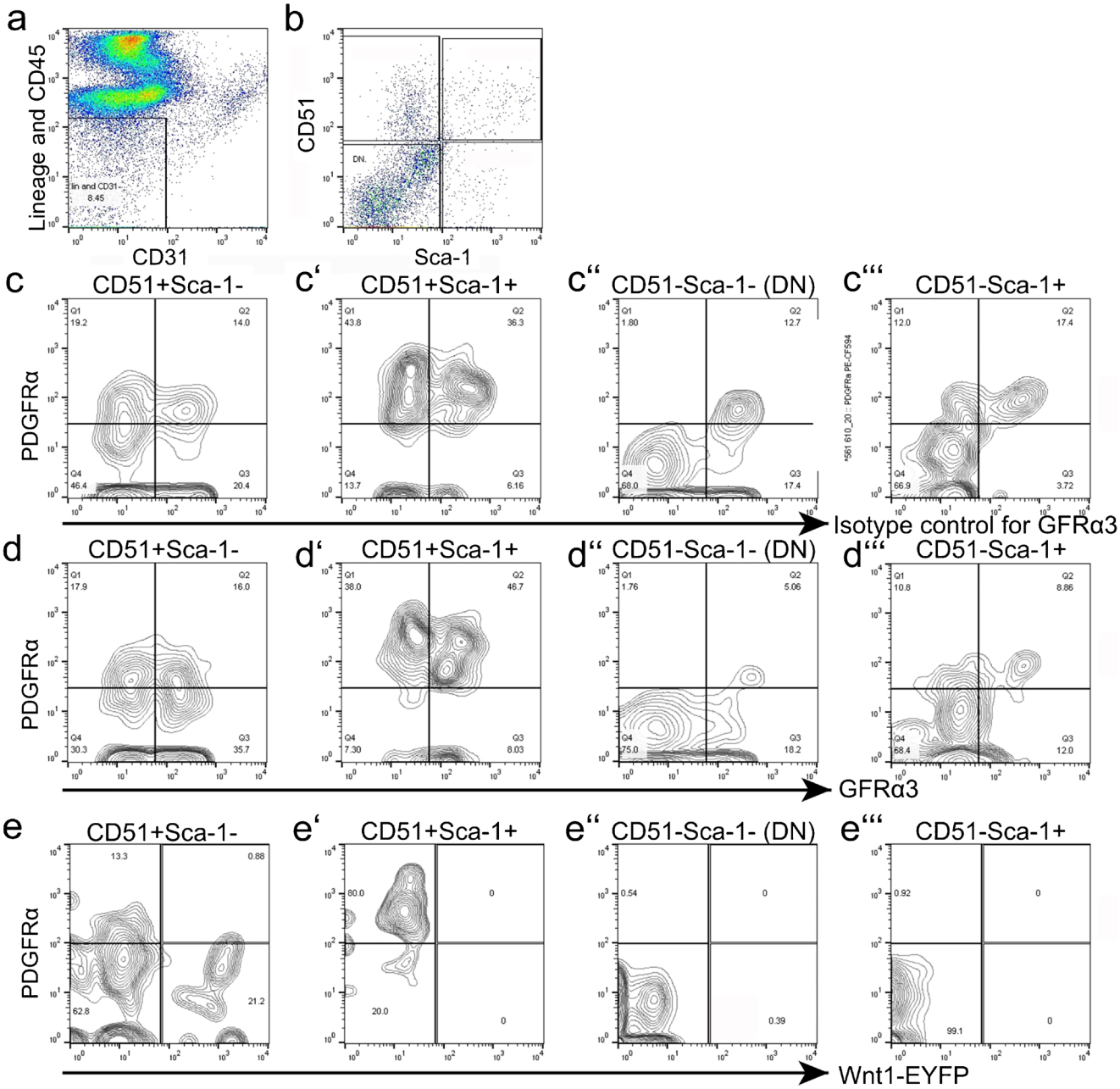

Fig. 8 Representative flow cytometric analysis of GFRα3$^+$ cells in the C57BL/6 murine bone marrow ($n = 3$). **a**–**b** Gating strategy for the identification of the GFRα3$^+$ population by flow cytometry. **a** The collagenase-digested bone and bone marrow cells were first gated to exclude mature haematopoietic lineage cells (Lin and CD45) and endothelial cells (CD31). **b** The stromal cells were further subdivided with Sca-1 and CD51 to resolve for four populations: the lin$^-$CD45$^-$CD31$^-$CD51$^+$Sca-1$^-$ osteoblasts-lineage (**c**, **d** and **e**), the lin$^-$CD45$^-$CD31$^-$CD51$^+$Sca-1$^+$ mesenchymal progenitor cells (**c‘**, **d‘** and **e‘**), the lin$^-$CD45$^-$CD31$^-$CD51$^-$Sca-1$^-$ populations (**c“**, **d“** and **e“**) and the lin$^-$CD45$^-$CD31$^-$CD51$^-$Sca-1$^+$ (**c“‘**, **d“‘** and **e“‘**). **c**, **c‘**, **c“**, **c“‘** and **d**, **d‘**, **d“**, **d“‘** The populations were analysed for their expression of PDGFRα and GFRα3. **c**, **c‘**, **c“**, **c“‘** Isotype controls for GFRα3 antibody are shown. **d** GFRα3 signal above isotype control levels could be seen in the CD51$^+$Sca-1$^-$ population of cells, and they have negligible expression of PDGFRα. A significant proportion of CD51$^+$Sca-1$^+$ PDGFRα$^+$ cells also expressed GFRα3 (**d‘**). No GFRα3 signals could be detected in the CD51$^-$Sca-1$^+$ and CD51$^-$Sca-1$^-$ populations (**d“** and **d“‘**). **e**, **e‘**, **e“**, **e“‘** Expression of EYFP was examined using *Wnt1*-cre; *ChR2-EYFP* mice ($n = 3$). A significant proportion of Wnt1-EYFP$^+$ cells were observed in two populations: CD51$^+$Sca-1$^-$PDGFRα$^-$ and CD51$^-$Sca-1$^-$PDGFRα$^-$ (**e** and **e“**). The CD51$^+$Sca-1$^+$ cells did not express EYFP (**e‘**)

The authors identified GFAP-positive, nonmyelinating Schwann cells as the source of active TGFβ/SMAD signalling in the bone marrow which induces HSC dormancy by inhibiting lipid raft clustering and activating maintenance pathways on HSCs (Yamazaki et al. 2009, 2011). Furthermore, Isern et al. (2014) found that nestin-positive cells in the bone marrow, including MSCs and Schwann cell precursors, were involved in

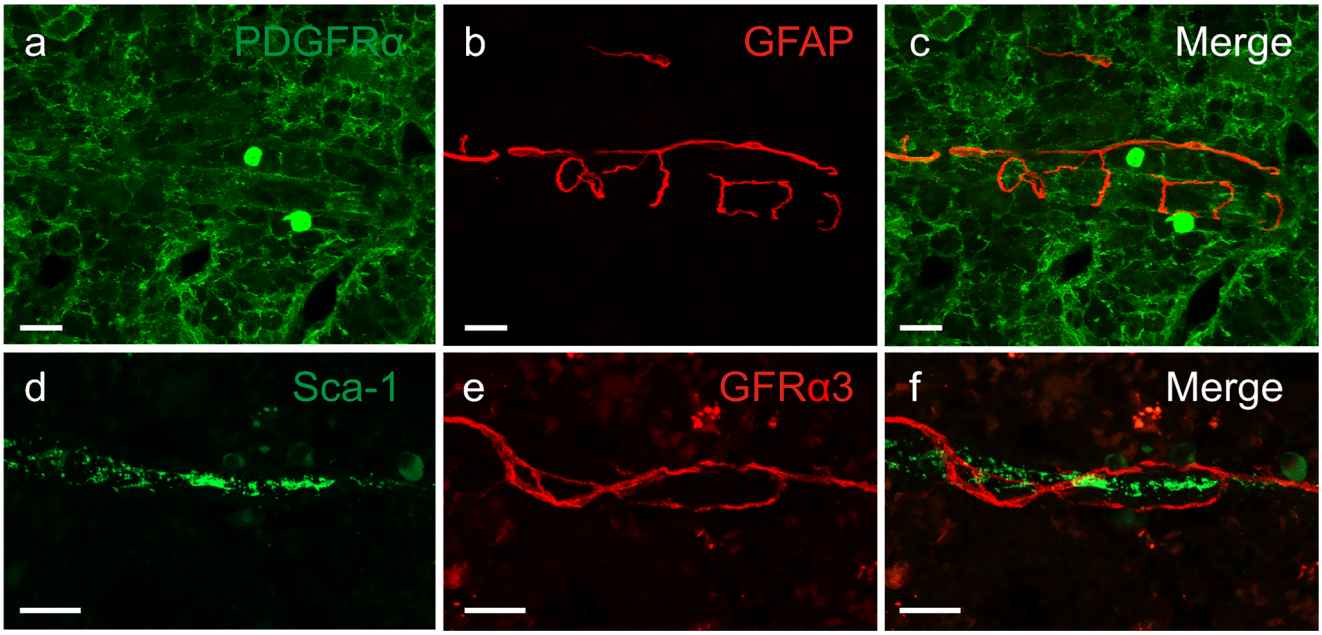

Fig. 9 Immunohistochemical staining for PDGFRα and Sca-1 in the bone marrow of C57BL/6 mice. High-magnification confocal images of GFAP and PDGFRα (**a–c**), or GFRα3 and Sca-1 (**d–f**), confirmed lack of coexpression in the bone marrow. Scale bars = 20 μm

migration of blood progenitor cells to the bone marrow for maturation. These findings provide support for the role of nonmyelinating Schwann cells in maintaining the haematopoietic stem cell niche. Given the close association between the GFRα3-expressing nonmyelinating Schwann cells and nerve terminals we have reported in the bone marrow, it is also possible that Schwann cell activity is regulated by the nerve endings that they are wrapped around and vice versa (Bruckner 2011). This is consistent with the findings of some other studies that have revealed a functional role for the peripheral nervous system in controlling HSC egression into the vasculature system (Afan et al. 1997; Garcia-Garcia et al. 2018; Katayama et al. 2006; Mendez-Ferrer et al. 2008). Schwann cells, MSCs and peripheral nerve fibres form important components of the HSC niche. Whether functional artemin/GFRα3 interactions are involved in any of these processes remains to be determined.

An additional population of GFRα3-expressing cells were identified by FACS that expressed CD51, Sca-1 and PDGFRα, cell surface markers that are associated with MSCs (Morikawa et al. 2009; Nakamura et al. 2010). These cells were not targeted by Wnt1-reporter mice, hence are not neural crest-derived MSCs. A number of studies have confirmed that Sca-1+ CD51+ MSCs can also express p75/CD271 (Alvarez-Viejo et al. 2015; Barilani et al. 2018; Li et al. 2016; Lopes et al. 2016). We were unable to unequivocally identify CD271+ MSCs using immunohistochemistry in our study and so do not comment on them further here.

Conclusion

We have demonstrated that GFRα3 is expressed in both nerve terminal endings and a population of nonneuronal cells in the bone marrow that we have identified as nonmyelinating Schwann cells. Our findings provide for a better understanding of the expression pattern of GFRα3 in the bone marrow microenvironment and have revealed that GFRα3 expression may be a novel marker for the identification of nonmyelinating Schwann cells in the bone marrow.

Acknowledgements The authors acknowledge Professor Roberto Cappai and Phan Truong for generously providing us with tissue from C57BL/6 mice. Louise E. Purton was an NHMRC Senior Research Fellow.

Funding information This work was supported by funding from the National Health and Medical Research Council and the Operational Infrastructure Support Program from the Victorian Government (to St. Vincent's Institute).

Compliance with ethical standards

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors. All experiments performed were in accordance with ethical standards of the University of Melbourne Animal Experimentation Ethics Committee, St. Vincent's Health Animal Ethics Committee and the Animal Welfare Committee of Flinders University of South Australia.

Conflict of interest The authors declare that they have no conflict of interest.

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