

Characterisation of a mural cell network in the murine pituitary gland

Laura O'Hara^{1,2}  | Helen C. Christian³  | Nathan Jeffery² | Paul Le Tissier¹  | Lee B. Smith^{2,4} 

¹Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, UK

²MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, UK

³Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK

⁴School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW, Australia

Correspondence

Laura O'Hara, Centre for Discovery Brain Sciences, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK.
Email: l.o'hara@ed.ac.uk

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Abstract

The anterior and intermediate lobes of the pituitary are composed of endocrine cells, as well as vasculature and supporting cells, such as folliculostellate cells. Folliculostellate cells form a network with several postulated roles in the pituitary, including production of paracrine signalling molecules and cytokines, coordination of endocrine cell hormone release, phagocytosis, and structural support. Folliculostellate cells in rats are characterised by expression of S100B protein, and in humans by glial fibrillary acid protein. However, there is evidence for another network of supporting cells in the anterior pituitary that has properties of mural cells, such as vascular smooth muscle cells and pericytes. The present study aims to characterise the distribution of cells that express the mural cell marker platelet derived growth factor receptor beta (PDGFR β) in the mouse pituitary and establish whether these cells are folliculostellate. By immunohistochemical localisation, we determine that approximately 80% of PDGFR β + cells in the mouse pituitary have a non-perivascular location and 20% are pericytes. Investigation of gene expression in a magnetic cell sorted population of PDGFR β + cells shows that, despite a mostly non-perivascular location, this population is enriched for mural cell markers but not enriched for rat or human folliculostellate cell markers. This is confirmed by immunohistochemistry. The present study concludes that a mural cell network is present throughout the anterior pituitary of the mouse and that this population does not express well-characterised human or rat folliculostellate cell markers.

KEYWORDS

folliculostellate, folliculostellate, mural, pituitary, SOX2

1 | INTRODUCTION

The pituitary is an endocrine gland located underneath the brain that produces and releases circulating hormones in response to signals from the hypothalamus. It is formed of three lobes. Neurones

in the posterior lobe (pars nervosa) connect to the hypothalamus and store oxytocin and vasopressin. The intermediate lobe (pars intermedia) is formed of melanotrophs that produce melanocyte-stimulating hormone and the anterior lobe (pars distalis) is formed of heterogeneous specialised endocrine cells that each make a

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particular hormone type: adrenocorticotrophic hormone is made by corticotrophs, growth hormone by somatotrophs, prolactin by lactotrophs, thyroid-stimulating hormone by thyrotrophs, and the gonadotrophs follicle-stimulating hormone and luteinising hormone by gonadotrophs. The folliculostellate cell is a further non-endocrine cell type in the anterior pituitary. Folliculostellate cells are named after their appearance in electron micrographs: they have a distinctive stellate shape and surround follicular structures.¹ Their long cytoplasmic processes connect to form a three-dimensional network² that is considered to perform multiple roles in the pituitary, including production of paracrine signalling molecules and cytokines,¹ coordination of endocrine cell hormone release,³ phagocytosis,⁴ and structural support.⁵ There is evidence that folliculostellate cells in rats are composed of multiple subpopulations that may arise from different precursors.⁶

Mural cells such as vascular smooth muscle cells and pericytes are required for the formation and stability of the vasculature and the support of endothelial cells. They are characterised by the expression of the markers chondroitin sulfate proteoglycan 4 (more commonly known as nerve/glial antigen 2 or NG2), alpha smooth muscle actin (SMA) and platelet derived growth factor receptor beta (PDGFR β).^{7,8} In rat anterior pituitaries, they have also been shown to express desmin.⁹ A recent study reported that mouse folliculostellate cells express NG2 and that inactivation of retinoblastoma protein in NG2+ cells resulted in adeno-hypophysial tumours with immunohistochemical and ultrastructural features resembling those of aggressive Pit1-lineage tumours in humans.¹⁰ Furthermore, PDGFR β has been shown to be expressed in the mouse TtT/GF folliculostellate cell line.¹¹ Taken together, this evidence suggests that either mouse pituitary folliculostellate cells may have mural cell-like properties, or mural cells form a network similar to that of folliculostellate cells in the anterior pituitary. The present study aims to characterise the distribution of PDGFR β + cells in the mouse pituitary and establish whether these cells have folliculostellate cells and/or mural-like properties.

2 | MATERIALS AND METHODS

2.1 | Breeding and maintenance of mice

Mice were fed a soya-free diet. Experimental procedures and animal breeding and maintenance were approved by University of Edinburgh Animal Welfare and Ethical Review Body and were carried out with licenced permission under project licence 70/8804 held by Professor Lee B. Smith in line with the UK Home Office Animals (Scientific Procedures) Act, 1986.

2.2 | Immunohistochemistry

NG2 staining was only possible with cryopreserved sections. To co-stain for NG2 and PDGFR β , Pituitaries were frozen on dry ice

and transferred to a -80°C freezer. Prior to sectioning, pituitaries were encompassed in Tissue-Tek OCT compound (Sakura, Osaka, Japan), mounted on a cryostat chuck, sectioned at $5\text{ }\mu\text{m}$ and mounted on poly lysine coated slides (PO425-72EA; Sigma-Aldrich, St Louis, MO, USA). Sections were dried at room temperature for 12 hours, then sections fixed under a droplet of 4% buffered formaldehyde for 5 minutes. Slides were then stained with a double fluorescent tyramide method as described previously^{12,13} using antibodies to NG2 (dilution 1:8000; catalogue no. AB5320; Millipore, Billerica, MA, USA; RRID:AB_91789) and PDGFR β (dilution 1:8000; catalogue no. ab32570; Abcam, Cambridge, MA, USA; RRID:AB_777165). A non-pressurised heating by microwave in pH6 0.01 mol L⁻¹ sodium citrate buffer was performed after the first tyramide reaction to denature remaining antibodies from the first set and avoid non-specific reactions with the second set of antibodies.¹⁴ Double staining of either NG2 first or PDGFR β first, both with and without primary antibodies, was performed to confirm this (see Supporting information, Data S1).

All other antibodies could be used on formaldehyde-fixed paraffin-embedded sections. Pituitaries were fixed for 24 hours in 4% buffered formaldehyde. Fixed tissues were processed and embedded in paraffin wax and sections ($3\text{ }\mu\text{m}$) were used for immunohistochemical analysis.

Fluorescence immunostaining was performed either by a single or double antibody tyramide fluorescence immunostaining method, as described previously.^{12,13} Antibodies used were PDGFR β (dilution 1:1000; catalogue no. ab32570; Abcam; RRID:AB_777165), S100 (dilution 1:2000; catalogue no. Z0311, lot number 20038616; Agilent, Santa Clara, CA, USA; RRID:AB_10013383) and sex determining region Y-box 2 (SOX2) (dilution 1:500; catalogue no. ab92494; Abcam; RRID:AB_10585428) optimised for the method and conditions used. A non-pressurised heating by microwave in 0.01 mol L⁻¹ sodium citrate buffer (pH 6) was performed after the first tyramide reaction to denature remaining antibodies from the first set and avoid non-specific reactions with the second set of antibodies.¹⁴

On resin-perfused sections, staining was performed using a colorimetric method. Briefly, sections were deparaffinised and rehydrated, and high-pressure antigen retrieval was performed in citrate buffer. Slides were incubated with normal goat serum/Tris-buffered saline/bovine serum albumin (NGS/TBS/BSA) to block non-specific antibody binding, before being incubated overnight with rabbit anti-PDGFR β (dilution 1:400; catalogue no. ab32570; Abcam; RRID:AB_777165) in NGS/TBS/BSA. The next day, slides were incubated with biotinylated goat anti-rabbit (dilution 1:500; catalogue no. BA-1000; Vector Laboratories; RRID:AB_2313606), then streptavidin-conjugated alkaline phosphatase (dilution 1:200; catalogue no. SA-5100; Vector Laboratories; RRID:AB_2336093), then PermaBlue Plus/AP (K058; Diagnostic BioSystems, Pleasanton, CA, USA; diluted in accordance with the manufacturer's instructions) to form a blue precipitate localised to PDGFR β protein. Slides were then mounted using an aqueous mounting medium and visualised with a Provis light microscope and camera (Olympus, Tokyo, Japan).

2.3 | Magnetic activated cell sorting (MACS)

Pituitaries were removed during dissection and the anterior lobes were dissected and used for MACS. Anterior pituitaries from approximately 40 mice on a mixed C57Bl/6 background were used in each MACS experiment, and MACS was repeated three times. The pool of anterior pituitaries was digested into a single cell suspension in accordance with a previously published protocol.¹⁵ MACS was performed using protocols and reagents from Miltenyi Biotec (Bergisch Gladbach, Germany). Briefly, the single cell suspension was incubated first with 1:500 PDGFR β antibody (catalogue no. ab32570; Abcam; RRID:AB_777165), then with anti-rabbit IgG magnetic microbeads (130-048-602; Miltenyi) before passing through a Miltenyi MS column (130-042-201; Miltenyi) in a magnetic holder. Both the column-bound cell fraction (MACS+) and the flow-through cell fraction were kept, washed and pelleted.

2.4 | Preparation of cDNA

Recovered whole pituitaries, dissected pituitaries and MACS cell pellets were frozen on dry ice before being transferred to a -80°C freezer for storage. RNA was isolated using the RNeasy Mini extraction kit (Qiagen, Crawley, UK) in accordance with the manufacturer's instructions. RNA was quantified and assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All RNA samples had a A_{260}/A_{280} ratio of above 1.9 and a A_{260}/A_{230} ratio of above 1.5. Random hexamer primed cDNA was prepared using the SuperScript VILO cDNA synthesis kit (Life Technologies, Grand Island, NY, USA) in accordance with the manufacturer's instructions.

2.5 | Quantitative reverse transcriptase-polymerase chain reaction (qPCR)

Multiplex qPCR was performed on cDNA for the genes listed in Table 1 using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the Roche Universal Probe library (Roche, Welwyn, UK). The expression of all genes was related to an internal housekeeping gene assay for *Actb* (Roche) as described previously.¹³ *Actb* Ct values were similar between samples

when normalised to the number of cells that had been RNA extracted. The resulting data were analysed using the $\Delta\Delta\text{Ct}$ method.

2.6 | Resin perfusion

Resin perfused pituitaries were obtained from Dr Diane Rebourcet (University of Newcastle, Callaghan, NSW, Australia), prepared in accordance with previously published protocols.¹⁶

2.7 | Immunogold electron microscopy

Pituitaries were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol L⁻¹ phosphate buffer (pH 7.2) at room temperature for 3 hours, then transferred to 0.25% glutaraldehyde and 0.2% paraformaldehyde in 0.1 mol L⁻¹ phosphate buffer (pH 7.2) and then kept at 4°C. Pituitaries were prepared for immunogold electron microscopy as described previously.¹⁷ Briefly, cells were post-fixed in osmium tetroxide (1% w/v in 0.1 mol L⁻¹ sodium phosphate buffer), contrasted with uranyl acetate (2% w/v in distilled water), dehydrated through increasing concentrations of ethanol (70%-100%) and embedded in Spurr's resin. Ultra-thin sections (50-80 nm) cut and stained with antibody to PDGFR β (catalogue no. ab32570; Abcam; RRID:AB_777165). Sections were viewed with a JEM-1010 transmission electron microscope (JEOL USA Inc, Peabody, MA, USA).

3 | RESULTS

3.1 | PDGFR β cells were found in the anterior, intermediate and posterior lobes of the mouse pituitary

Stellate PDGFR β cells were widely distributed in the anterior and posterior lobes of the adult pituitary, with fewer seen in the intermediate lobe (Figure 1A). PDGFR β cells were also present in the day (d)1 neonatal anterior pituitary as stellate-shaped cells and cell clusters and in a layer of spindle-shaped cells around the outside of the anterior pituitary (Figure 1B). Mural pericytes have a characteristic location and morphology, defined as being adjacent to endothelial

TABLE 1 Primers and Roche UPL probes for the quantitative reverse transcriptase-polymerase chain reaction assays used in the present study

Gene	Forward primer	Reverse primer	Probe
<i>Cspg4</i>	cttgcccttggtgcat	cacctccagggtgtctcc	16
<i>Des</i>	gccacctaccggaagctact	gcagagaaggtctggataggaa	15
<i>Acta2</i>	gacaccaccaccagagt	acatagctggagcagctct	20
<i>Pdgfra</i>	ggtcctcagctgtctctca	cgtttgggaggatagagggtta	51
<i>Pdgfrb</i>	gctgatgaaggtctccaga	ggagctccaggggactgt	69
<i>Gfap</i>	acatcgagatcgccactac	ggatctggagggttgagaaag	9

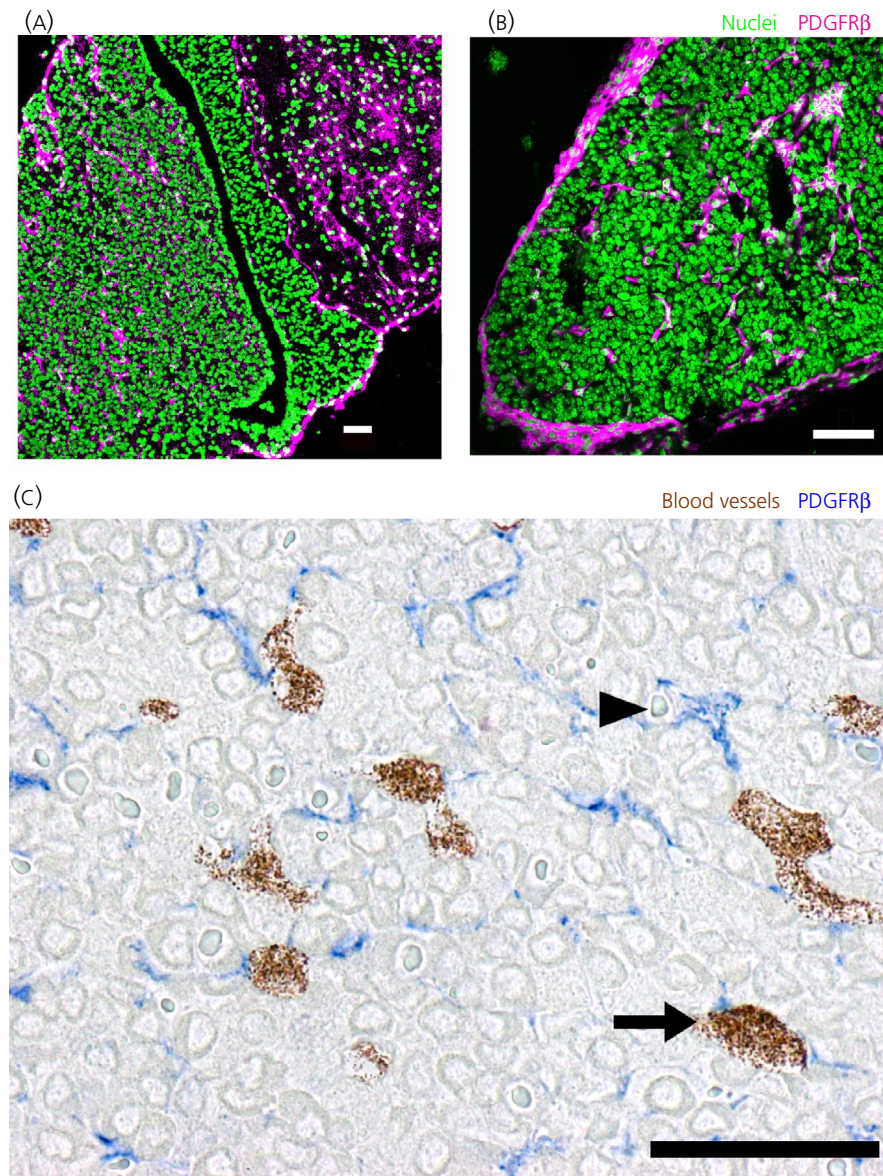


FIGURE 1 Platelet derived growth factor receptor beta (PDGFR β) cells are found in the anterior and posterior lobes of the pituitary. A, Fluorescence immunohistochemical staining of PDGFR β (magenta) in formaldehyde-fixed paraffin-embedded thin sections of wild-type mouse pituitary with green counterstain, showing anterior (left), intermediate and posterior lobes. B, Anterior lobe of postnatal day (pnd) 1 mouse pituitary showing both clusters of stellate-shaped cells and cells around the periphery of the lobe staining for PDGFR β . All scale bars = 50 μ m. C, Perma-blue staining of PDGFR β (blue) in thin sections of pituitary from mice intravascularly perfused with an opaque resin at termination, marking larger vessels in brown (arrow) and smaller vessels in iridescent green (arrowhead), showing that PDGFR β -positive cells are present both adjacent to pituitary blood vessels and in the parenchyma. Scale bar = 50 μ m

cells and wrapping around the vasculature.¹⁸ In the rat anterior pituitary, desmin-positive perivascular cells can be found detached from capillaries, although with cytoplasmic processes still in contact with the capillary wall.¹⁹ To visualise whether PDGFR β + cells are found only in a perivascular location in the anterior pituitary, we immunostained for PDGFR β in sections of pituitary from adult mice that had been intravascularly perfused with an opaque resin at termination.¹⁶ Both large and small blood vessels can be visualised by this technique. The percentage of PDGFR β + cells in contact with and not in contact with blood vessels was then quantified. Although 20% of PDGFR β + cells were seen to be either contacting or surrounding anterior pituitary blood vessels in a location characteristic of pericytes, 80% were located in the pituitary parenchyma in a location and distribution characteristic of folliculostellate cells (Figure 1C). To identify PDGFR β cells by ultrastructure, immunogold electron microscopy was performed with a PDGFR β antibody. Folliculostellate cells and pericytes were identified by location and ultrastructure

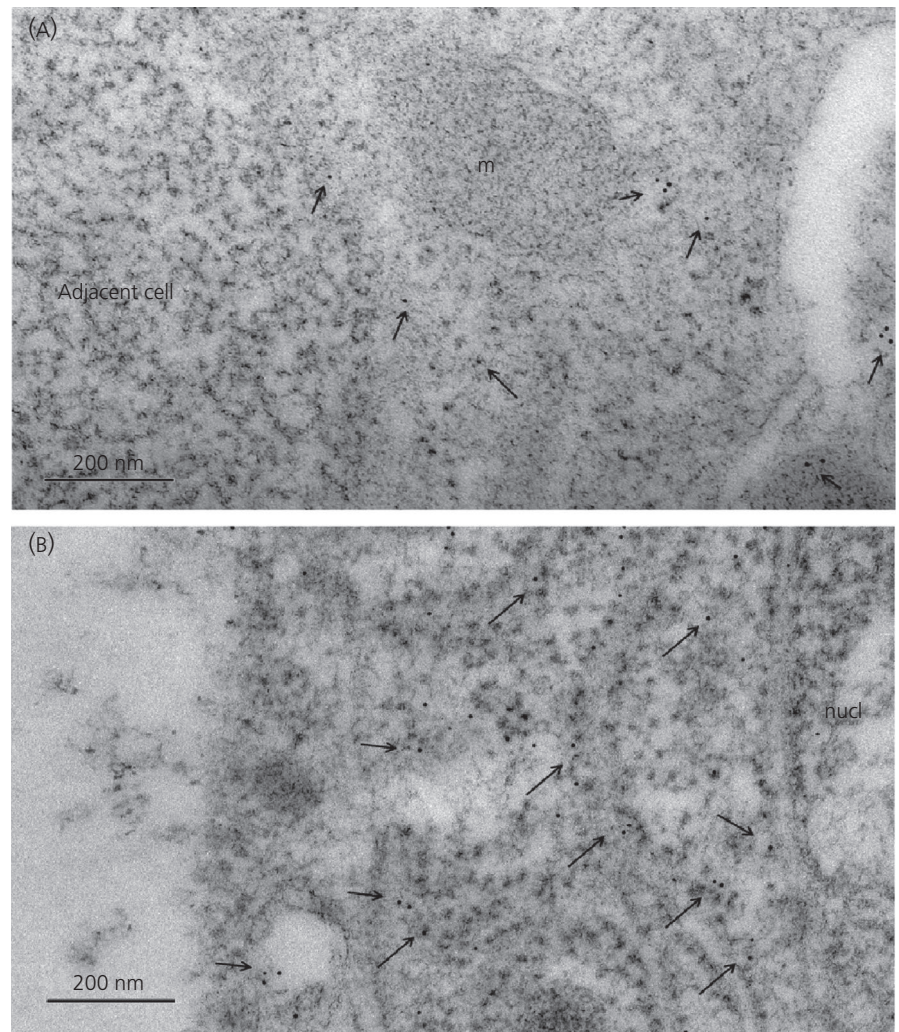
(see Supporting information, Supplementary Data S2). Both folliculostellate cells (Figure 2A) and pericytes (Figure 2B) were found to localise PDGFR β .

3.2 | PDGFR β cells of the anterior pituitary were found to express other mural cell markers

To investigate whether pituitary PDGFR β + cells express other mural cell markers, a population of cells enriched for PDGFR β was generated by MACS from micro-dissected anterior pituitaries using the same PDGFR β antibody employed for immunohistochemistry (Figure 3A). These markers were examined by qPCR in whole pituitaries and dissected anterior pituitaries, as well as PDGFR β positive and negative purified MACS populations.

Cspg4 (NG2 transcript) was enriched in a PDGFR β MACS+ population at levels 10-fold higher compared to the MACS- population,

FIGURE 2 Platelet derived growth factor receptor beta (PDGFR β) is localised to cells with folliculostellate and pericyte morphology. Immunogold electron microscopy was performed with a PDGFR β antibody. A, Localisation in folliculostellate cells identified from location and ultrastructure (m= folliculostellate cell). B, Localisation in pericytes identified from location and ultrastructure (nucl= nucleus). Scale bars = 200 nm



seven-fold higher compared to the whole anterior pituitary and seven-fold higher compared to the whole intact pituitary (Figure 3B). This was confirmed by immunohistochemistry for NG2 protein in cryosections of whole mouse pituitary. Staining of NG2 colocalised with PDGFR β in the anterior lobe in a stellate-shaped cell pattern (Figure 3C). Other mural cell markers were enriched in the PDGFR β cell population of the mouse pituitary. *Acta2* (SMA transcript) was enriched in the MACS+ population at levels five-fold higher compared to the MACS- population, nine-fold higher compared to the whole anterior pituitary and two-fold higher compared to the whole intact pituitary PDGFR α transcript (Figure 3D). Desmin transcript *Des* was enriched in the MACS+ population at levels 11-fold higher compared to the MACS- population, three-fold higher compared to the whole anterior pituitary and 3.5-fold higher compared to the whole intact pituitary (Figure 3E). Because PDGFR β is also considered a marker of mesenchymal stem cells (MSCs),²⁰ the PDGFR β enriched cell population was also tested for *Pdgfra*, a MSC marker. *Pdgfra*²¹ was enriched in the MACS PDGFR β + population at levels 12.5-fold higher compared to the MACS- population, 2.5-fold higher compared to the whole anterior pituitary and 1.4-fold higher compared to the whole intact pituitary (Figure 3F). These results indicate that the PDGFR β + cell population in the anterior pituitary is enriched for mural cell and perivascular MSC markers.

3.3 | PDGFR β cells of the mouse anterior pituitary did not express rat or human folliculostellate cell markers

We then investigated whether pituitary PDGFR β cells express the same markers as rat and human folliculostellate cells. These markers were examined by qPCR in whole pituitaries and dissected anterior pituitaries, as well as positive and negative purified MACS populations. Sections of whole pituitary were also stained for the markers where an appropriate antibody was available.

Localisation of S100 calcium-binding protein B (S100B) is a characteristic of folliculostellate cells in rats.²² *S100b* transcript was not enriched in the MACS+ population compared to the MACS- population and was present at levels of 1.6-fold lower compared to the whole anterior pituitary and four-fold lower compared to the whole intact pituitary. (Figure 4A). Because it was found to be enriched in whole pituitaries compared to dissected anterior pituitaries, it is likely that it is enriched in intermediate or posterior lobe cells. This was confirmed by immunohistochemistry for pan-S100 protein in whole mouse pituitary sections. S100 staining was not seen in the parenchyma of the anterior lobe (Figure 4B) but was seen in

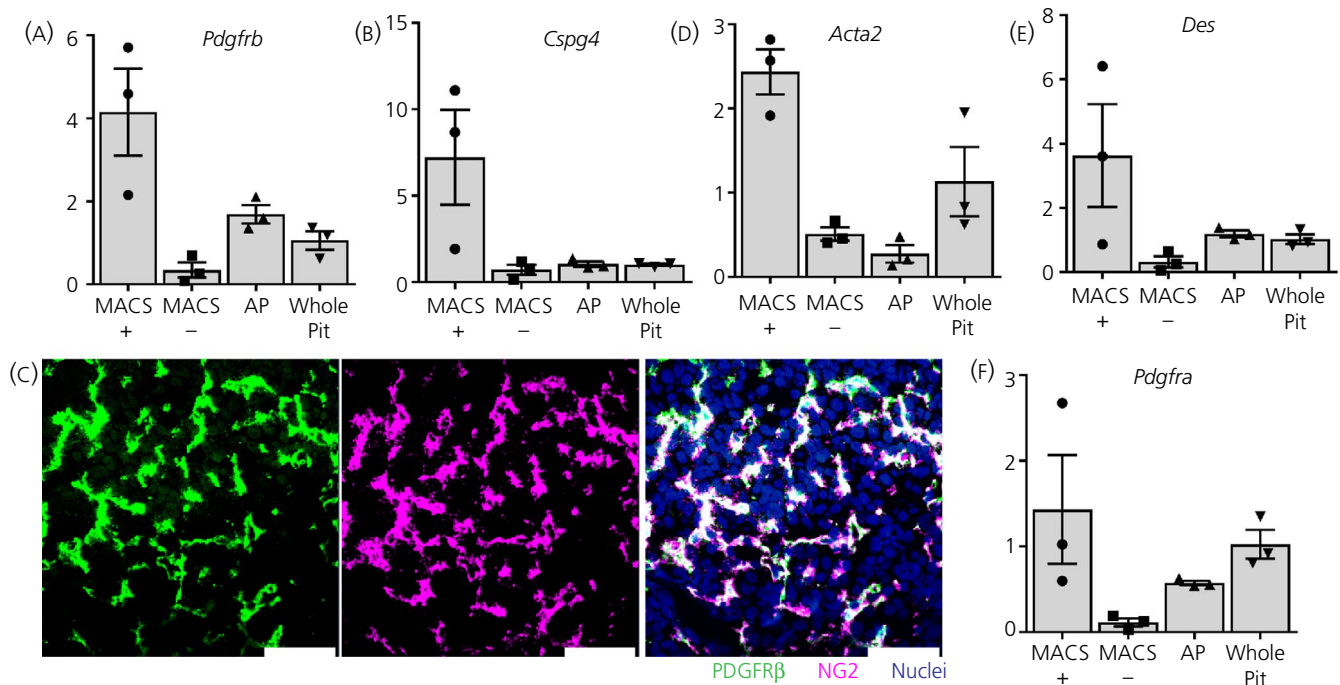


FIGURE 3 Platelet derived growth factor receptor beta (PDGFR β) cells of the anterior pituitary (AP) are enriched for mural cell markers. A, *Pdgfrb* is enriched in the MACS PDGFR β + population at levels 12.1-fold higher compared to the MACS- population, 2.5-fold higher compared to the whole anterior pituitary and 3.9-fold higher compared to the whole intact pituitary. B, *Cspg4* (NG2 transcript) is enriched in a PDGFR β MACS+ population at levels 10-fold higher compared to the MACS- population, seven-fold higher compared to the whole anterior pituitary and seven-fold higher compared to the whole intact pituitary. C, Fluorescence immunohistochemistry for NG2 protein (magenta) in cryosections of whole wild-type mouse pituitary colocalises with PDGFR β (green) in the anterior lobe in a stellate-shaped cell pattern. Blue counterstain, scale bar = 50 μ m. D, *Acta2* (SMA transcript) is enriched in the MACS+ population at levels five-fold higher compared to the MACS- population, nine-fold higher compared to the whole anterior pituitary and two-fold higher compared to the whole intact pituitary. E, *Des* is enriched in the MACS+ population at levels 11-fold higher compared to the MACS- population, three-fold higher compared to the whole anterior pituitary and 3.5-fold higher compared to the whole intact pituitary. F, *Pdgfra* is enriched in the MACS PDGFR β + population at levels 12.5-fold higher compared to the MACS- population, 2.5-fold higher compared to the whole anterior pituitary and 1.4-fold higher compared to the whole intact pituitary. For graphical figures, individual data points are indicated by circles (MACS+), squares (MACS-), point-up triangles (AP) and point-down triangles (whole pituitary).

a population of cells lining the cleft on both the anterior lobe and intermediate lobe sides (Figure 4C). As positive controls to ensure that the antibody is functional, staining could be seen both in astrocyte and tanyocyte cells in the mouse hypothalamus (Figure 4D) and in sections of rat pituitary (Figure 4E), as expected. Glial fibrillary acid protein (GFAP) is a folliculostellate cell marker in humans.²³ *Gfap* was not enriched in the MACS+ population compared to the MACS- population, nor compared to whole anterior pituitary, although it was present in whole pituitaries at a level of 14.5-fold compared to the MACS+ cell population (Figure 4F). Because it was highly enriched in whole pituitaries compared to dissected anterior pituitary samples, it is likely that it is enriched in intermediate or posterior lobe cells. This was confirmed by immunohistochemistry for GFAP protein in whole mouse pituitary sections. Staining was not seen in the parenchyma of the anterior lobe (Figure 4G). As a positive control to ensure that the antibody is functional, staining could be seen in astrocytes in the mouse brain (Figure 4H). These results indicate that the PDGFR β + cell population in the anterior pituitary is not enriched for rat or human folliculostellate cell markers.

3.4 | SOX2+ cells in the adult pituitary gland did not express PDGFR β

To clarify whether PDGFR β + cells in the pituitary express the previously characterised pituitary stem cell marker SOX2, we performed double immunohistochemistry for these proteins. PDGFR β + cells did not co-stain with SOX2 in adult pituitaries (Figure 5A) or in neonatal pituitaries (see Supporting information, Supplementary Data S3).

4 | DISCUSSION

4.1 | A network of mural cells in the mouse pituitary has a spatial distribution similar to folliculostellate cells

In the present study, we have shown that staining for PDGFR β is present throughout the mouse pituitary in a network of cells with a stellate shape. PDGFR β is a marker of mural cells such as

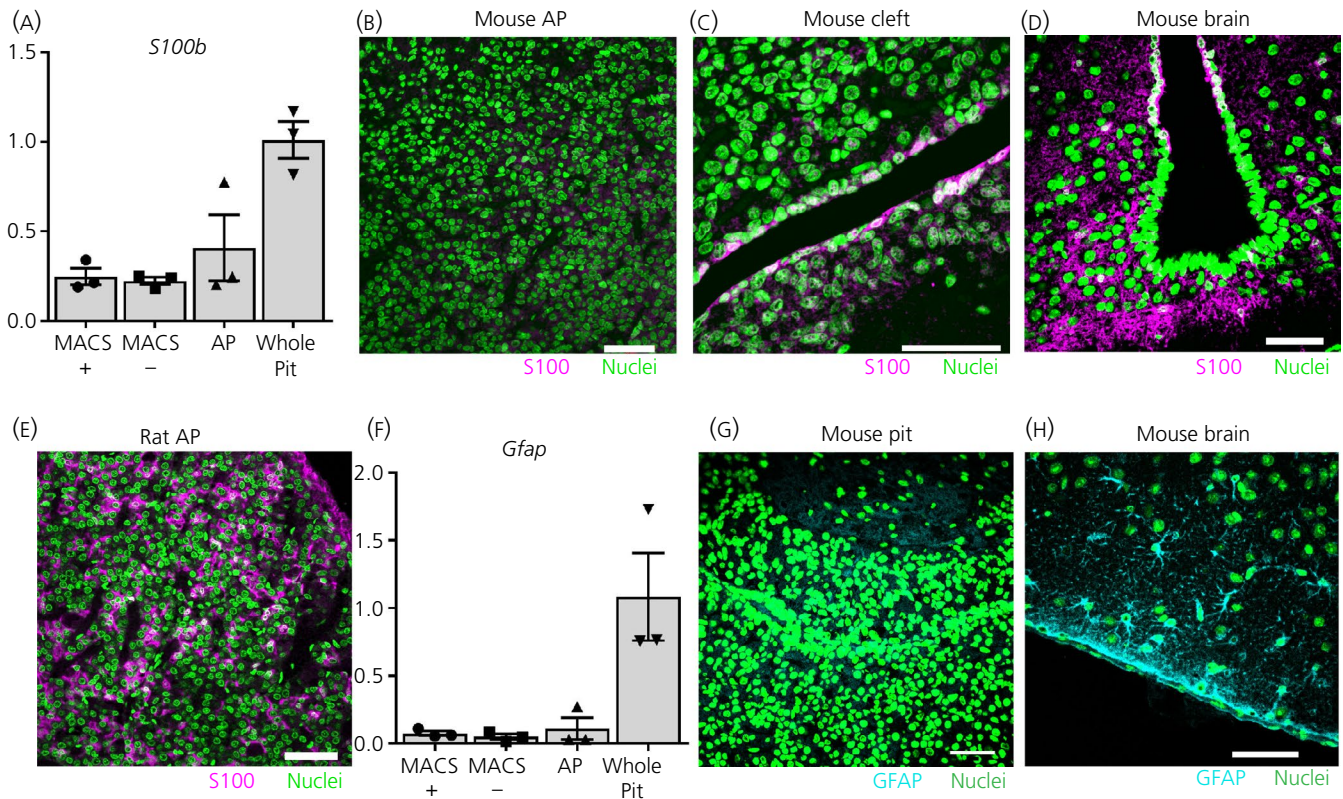


FIGURE 4 Platelet derived growth factor receptor beta (PDGFR β) cells of the mouse anterior pituitary (AP) are not enriched for rat folliculostellate cell markers. A, *S100b* is not enriched in the MACS+ population compared to the MACS- population and is present at levels 1.6-fold compared to the whole anterior pituitary and four-fold lower compared to the whole intact pituitary. B-E, Fluorescence immunohistochemical staining of pan-S100 (magenta) in formaldehyde-fixed paraffin-embedded thin sections of wild-type mouse pituitary and brain with green counterstain, scale bars = 50 μ m. B, Staining is not seen in the parenchyma of the anterior lobe of the pituitary. C, Staining is seen in a population of cells lining the cleft on both anterior and intermediate sides. D, As positive controls to ensure that the antibody is functional, S100 staining can be seen both in astrocyte and tanycyte cells in the mouse hypothalamus. E, S100 staining is seen in folliculostellate cells in sections of rat pituitary. F, *Gfap* is not enriched in the MACS+ population compared to the MACS- population, or compared to whole anterior pituitary, but is present in whole pituitaries at a level of 14.5-fold compared to the MACS+ cell population. G,H, Fluorescence immunohistochemical staining of glial fibrillary acid protein (GFAP) (cyan) in formaldehyde-fixed paraffin-embedded thin sections with green counterstain, scale bars = 50 μ m. G, GFAP staining is not seen in the parenchyma of the pituitary anterior lobe. H, In mouse brain positive controls, GFAP staining can be seen in astrocytes in the mouse brain. For graphical figures, individual data points are indicated by circles (MACS+), squares (MACS-), point-up triangles (AP) and point-down triangles (whole pituitary).

pericytes and vascular smooth muscle cells, and also of MSCs²⁰ and fibroblasts²⁴ of some tissues. Although 20% of the cells seen to stain for PDGFR β in the anterior pituitary interact with blood vessels, 80% did not make contact. The expression pattern of PDGFR β in the pituitary parenchyma was similar to the previous staining of folliculostellate cells in the mouse pituitary using an antibody to ER-BMDM1.²⁵ Because other cells such as MSCs and fibroblasts express PDGFR β , we aimed to characterise whether mouse anterior pituitary parenchymal PDGFR β + cells expressed any other mural cell markers. In the present study, we have shown that anterior pituitary PDGFR β + cells co-stain with NG2, which is also a marker of mural cells, but not MSCs²⁰ or fibroblasts.²⁶ This expression pattern is consistent with a previous study that has also localised NG2 to a network of cells with a stellate shape, although that study designated the cells as mouse pituitary folliculostellate cells.¹⁰ A few cells appear to stain for NG2 or PDGFR β only. This could be an artefact of the staining, or it could be that there are

some cells in the anterior pituitary that are NG2+/PDGFR β - and NG2-/PDGFR β +. Without further investigation, it is difficult to conclude which possibility is true. Our evidence suggests that a population of mural cells in the mouse anterior pituitary has a network structure very similar to that of folliculostellate cells, and that this network extends away from the vasculature.

4.2 | The novel mural cell network does not express folliculostellate cell markers

The folliculostellate cells of the pituitary also form a network that is commonly characterised by expression of S100B or GFAP markers in immunohistochemistry. In total, 473 papers published between 1972 and November 2019 mentioning the words "folliculostellate" or "folliculostellate" in their abstract have been indexed on PubMed (Supplementary Data S4). Of these, 426 are primary research papers

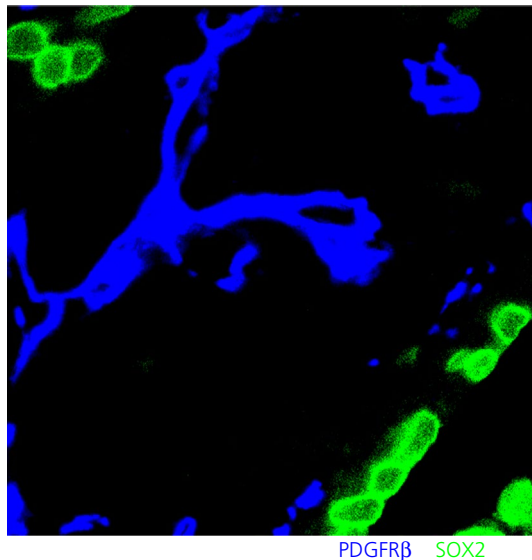


FIGURE 5 No colocalisation of sex determining region Y-box 2 (SOX2) and platelet derived growth factor receptor beta (PDGFR β) in anterior pituitary. Fluorescence immunohistochemical staining of PDGFR β (blue) and SOX2 (green) in formaldehyde-fixed paraffin-embedded thin sections of wild-type mouse pituitaries

and 47 are reviews. Of the 426 primary research papers, 202 have used rats as their animal model to investigate pituitary folliculostellate cells, either in whole pituitaries, primary cell culture or derived cell lines. Folliculostellate cells in rats are defined by their expression of the calcium binding protein S100B but are considered functionally heterogeneous, arising from three different developmental origins.⁶ Astrocyte-like folliculostellate cells express GFAP as a marker, dendritic cell-like folliculostellate cells are thought to arise from MAC-1+ early macrophages and express MH-class II as a marker, and epithelial-like folliculostellate cells express neither of these. The use of S100B as a marker and the availability of S100-GFP reporter rats²² have resulted in the rat becoming the most widely used model organism for folliculostellate cell research, and most folliculostellate cell characterisation is based on their phenotype in rats. Pericytes and folliculostellate cells are distinct populations in rats,²⁷ although there is also evidence in rats that pericyte-like cells may be found in contact with (but not enveloping) capillaries.¹⁹

Mouse folliculostellate cells are not well-investigated compared to rats and only 25 of these 423 primary research papers use mice as a model. To determine whether the network of PDGFR β cells that we characterised in the mouse anterior pituitary expressed the rat folliculostellate cell marker S100B or the human folliculostellate cell marker GFAP, we attempted to detect these proteins by immunohistochemistry on whole anterior pituitaries and transcripts by qPCR on a MACS purified population of PDGFR β cells. Our results showed that GFAP is not expressed in the anterior pituitary and that S100 is only expressed in cells of the anterior pituitary lining the pituitary cleft, and not in the body of the anterior pituitary. Neither GFAP, nor S100 are enriched in mouse anterior pituitary PDGFR β cells. However, immunogold electron microscopy for PDGFR β shows that it is localised to cells with ultrastructural characteristics

of both folliculostellate cells and pericytes. Previous studies (including those using the same antibody as that employed in the present study) have shown S100 staining in the pituitary,^{28–34} although these S100-positive cleft cells are often defined as ‘folliculostellate cells’ but do not show staining throughout the parenchyma. Interestingly, Allaerts et al³⁵ note that they do not observe S100 staining in the mouse pituitary. The S100+ cells found in the cleft are also likely to be SOX2+ as a result of their location and previous reports of co-staining of SOX2 and S100 in the rat.³⁶

A further 54 papers have been published using the TtT/GF cell line, which is an immortal cell line generated from a mouse thyrotrophic pituitary tumour,³⁷ classified as ‘folliculostellate-like’ based on strong immuno-positive staining for GFAP and weak staining for S100 protein.³⁷ The TtT/GF cell line was isolated from a mouse thyrotrophic tumour that had been induced by radiothyroidectomy and so it is possible that the transformation of these cells and their culture *ex vivo* has changed the characteristics of the cell line from that of the originating cell *in vivo*. However, it is also interesting to note a recent study reporting that the TtT/GF cell line has ‘pericyte characteristics’ including the expression of NG2.³⁸ It is likely that this is a folliculostellate cell line but has either switched on or increased expression of S100 as well as mural cell markers, or that it has been derived from a sub-population of folliculostellate cells,⁶ potentially the from the SOX2+ S100+ population of cleft cells. Our evidence suggests that the mural cell network in the mouse anterior pituitary does not comprise folliculostellate cells, although we could not establish a folliculostellate marker to unambiguously determine this, and so there is still the possibility that these two networks overlap.

4.3 | SOX2+ cells of the anterior pituitary are not PDGFR β +

The source of dividing cells in the anterior pituitary is assumed to change throughout development. Previous studies show that the very earliest progenitor cells in the developing pituitary come from SOX2+ cells lining Rathke's pouch, which, at embryonic day (e)12.5, ubiquitously express the transcription factor SOX2 and are highly proliferative.³⁹ From e12.5 to e17.5, between 20% and 30% of SOX2+ cells are dividing at any given point.⁴⁰ By e14.5, SOX2 is confined to the intermediate lobe and periluminal cells in the anterior lobe and, by e16.5, dividing cells begin to switch off SOX2 and switch on the lineage-specific transcription factors TPIT (also known as TBX19: corticotrophs and melanotrophs), SF1 (gonadotrophs) and PIT1 (also known as POU1F1: thyrotrophs, somatotrophs and lactotrophs).³⁰ Lineage tracing studies that use a tamoxifen-inducible Sox2-Cre to permanently express fluorescent marker proteins in these cells and their progeny have shown that all types of pituitary anterior lobe endocrine cells can develop from early SOX2+ cells, although not all cells are marked by the lineage tracer.^{28,29,39} A small number of SOX2+ cells persist in the postnatal pituitary, found lining the anterior-pituitary slide of the

cleft and in small groups through the parenchyma of the anterior pituitary. When isolated and cultured, these cells form pituitary spheres that can differentiate into all types of pituitary endocrine cells, which has led to the suggestion that they might be pituitary stem cells.³⁰

In the first 3 weeks of postnatal life, the pituitary undergrows rapid growth in which differentiated or differentiating endocrine cells increase their contribution to cell division, and SOX2+ cells decrease their contribution.⁴¹ SOX2+ cells contribute approximately 64% of dividing cells at d1, reducing to 19% at d10, and are mostly quiescent at d20. By contrast, the percentage of dividing cells that are Pit1-positive (a marker of differentiating lactotrophs, somatotrophs and thyrotrophs) increases from 16% to 87% over this time.³⁹ Two lineage tracing studies for SOX2+ cells have investigated the early postnatal period and concluded that only a small percentage of SOX2+ cells present in the neonatal pituitary develop into pituitary endocrine cells^{29,39} and that most of the SOX2+ dividing cells develop into gonadotrophs.

In adulthood, it is thought that the adult pituitary gland is maintained by stochastic cellular self-replication rather than stem cell replenishment, although stressing the pituitary by ablating a cell population or removing a feedback gland can prompt the mobilisation of SOX2+ stem cells. Lineage tracing experiments show that, when SOX2-Cre is induced in adulthood, labelled cells rarely differentiate into different pituitary lineages and mostly remain as SOX2+ stem cells.^{28,29,39} Taken together, this evidence shows that SOX2+ stem cells have a large commitment to differentiating cells in the embryonic period but a diminishing contribution after birth and little during adulthood. The present study shows that the SOX2+ cells lining the anterior epithelium of the pituitary cleft do not express PDGFR β in adult or neonatal pituitaries.

5 | CONCLUSIONS

The anterior pituitary is composed of well-characterised networks of endocrine and folliculostellate cells.⁴² Here, we present evidence indicating that there is a population of mural cells in the anterior pituitary of the mouse extending away from the vasculature to form a further network. The role of this mural cell network requires further characterisation: because the pituitary vasculature is important for the coordination of hormone release,⁴³ it is possible that it is involved in coordinating the communication between the vasculature and endocrine cells.

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PEER REVIEW

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Laura O'Hara  <https://orcid.org/0000-0001-7790-9261>

Helen C. Christian  <https://orcid.org/0000-0002-4263-5499>

Paul Le Tissier  <https://orcid.org/0000-0002-7220-5705>

Lee B. Smith  <https://orcid.org/0000-0002-4103-3074>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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