Identification and characterisation of a novel SOX2+ stem cell population in the adrenal medulla

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Declaration

I, Alice Santambrogio, confirm that the work presented in this thesis, unless otherwise acknowledged, is my own original work. The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.

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Abstract

The adrenal glands are two major endocrine organs responsible for multiple physiological processes, including the stress response, modulating the immune system and metabolism. The adrenal is composed of an outer cortex and an inner medulla with distinct developmental origin and function. While tissue-specific stem/progenitor populations of the adrenal cortex have been widely identified and characterised, the presence of a functional stem/progenitor population in the medulla is unclear. Establishing cell hierarchy of the adrenal medulla essential to understand normal homeostasis, disease pathogenesis and establishing regenerative medicine strategies, therefore the identification of a stem/progenitor population would provide an important starting point for further basic and translational studies. Cell composition of the adrenal medulla includes three main cell types: chromaffin cells, which secrete catecholamines, neurons, which stimulate catecholamine production, and a third cell type with an unspecified "support" function called sustentacular cells.

Using transcriptomics and genetic approaches in mouse, I established that a population of sustentacular cells express the stem/progenitor marker SOX2. These cells are present throughout life and have a developmental origin congruent with the rest of the gland. Through genetic lineage-tracing using the *Sox2*^{CreERT2} strain, I demonstrate that SOX2+ cells are an expanding population, capable of giving rise to the catecholamine-producing chromaffin cells, consistent with a stem cell role *in vivo*. I further demonstrate the self-renewal potential of SOX2+ cells through *in vitro* isolation and expansion, using a *Sox2*^{eGFP} mouse line. Analysis of SOX2+ cells in physiological organ challenge suggests potential involvement of these cells in the response to perturbation of normal homeostasis. Through

analysis of FFPE sections of human adrenals, I confirm the presence of SOX2+ cells in the normal adult organ, as well as in pheochromocytomas.

Taken together, these data support the identification of a previously undescribed stem cell population in the mammalian adrenal medulla and confirm its functional relevance.

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List of Abbreviations

AADC Aromatic Amino-acid Decarboxylase

AR Adrenergic Receptor

BMP Bone Morphogenetic Protein

BSA Bovine Serum Albumin

CAH Congenital Adrenal Hyperplasia

CART Cocaine and Amphetamine-Regulated Transcript

CHGA Chromogranin A

CRH Corticotrophin-Releasing Hormone

CVS chronic variable stress

CYP11B1 11β-hydroxylase

CYP11B2 Aldosterone Synthetase

DAPI 4' 6-diamidino-2' -deoxyuridine

DBH Dopamine beta-hydroxylase

DHEA Dehydroepiandrostenedione

DMEM Dulbecco's Modified Eagle Medium

DNAse Deoxyribonuclease

DOPA Dihydroxyphenylalanine

DTA Diphtheria Toxin Subunit A

EDTA Ethylenediaminetetraacetic Acid

EdU 5- ethynyl-2' -deoxyuridine

EGF Epidermal Growth Factor

EGFP Enhanced Green Fluorescent Protein

FACS Fluorescent-Activated Cell Sorting

FBS Fetal Bovine Serum

FGF Fibroblast growth factor

GFAP glial fibrillary acidic protein

GFP Green Fluorescent Protein

HBSS Hank's Balanced Salt Solution

HPA Hypothalamus-Pituitary-Adrenal

HRP Horseradish Peroxidase

NBF Neutral Buffered Formalin

NGF Nerve Growth Gactor

OCT Optical Cutting Temperature

PBS Phosphate Buffered Saline

PCC Pheochromocytoma

PCR polymerase chain reaction

PENK Proenkephalin

PGL paraganglioma

PLP1 Proteolipid Protein 1

PNMT Phenylethanolamine N-methyltransferase

PPGL Pheochromocytoma and Paraganglioma

RET Rearranged During Transfection

S100B S100 Calcium Binding Protein B

SCP Schwann Cell Precursor

SF1 Steroidogenic Factor 1 NR5A1

SHH Sonic Hedgehog

SOX10 Sry-related box 10

SOX2 Sry-related box 2

SOX9 Sry-related box 2

TGF-β (transforming growth factor-beta)

TH Tyrosine Hydroxylase

TUJ1 Beta Tubulin 3

WT Wild Type

1 Introduction

1.1 The adrenal glands

The adrenal glands are essential endocrine organs involved in several key physiological processes including the regulation of the immune system, production of sex hormones and "fight or flight" response. They are a main component of the hypothalamus-pituitary-adrenal (HPA) stress axis. Adrenal glands are located above the kidneys and composed of an outer cortex and an inner medulla with distinct developmental origin and function.

The adrenal cortex derives from the mesoderm and shares its embryonic origin with the gonads. In mice, the adrenogonadal primordium is first observed at embryonic day 9.0 (E9.0), defined by the expression of the transcription factor NR5A1 (hereby referred to as SF1, steroidogenic factor 1), and a definitive adrenal cortex is formed by E13.5. The postnatal adrenal cortex is composed of three histologically distinct concentric zones of cells with specialised functions, and an outer capsule (Figure 1.1). The main function of the zona glomerulosa is to produce mineralocorticoid aldosterone, which regulates blood pressure and electrocyte balance. Corticosterone is converted to aldosterone by aldosterone synthetase (CYP11B2), which is selectively expressed by cells of the zona glomerulosa. The zona fasciculata produces the glucocorticoids cortisol and corticosterone, involved in immune and metabolic responses. Cells of the zona fasciculata express the 11β-hydroxylase (CYP11B1) which coverts 11-deoxycortisol to cortisol and 11-deoxycorticosterone to corticosterone. The zona reticularis produces dehydroepiandrostenedione (DHEA) which can act as an androgen, estrogen or neurosteroid. In mice, the zona reticularis is missing and it is substituted by cells with a similar downstream function forming the X zone. This zone is present only at some

developmental stages and produces pregnenolone. The adrenal cortex is a highly dynamic and proliferative organ which in normal homeostasis has a complete turnover every 3 months. Stem cells and progenitors of the adrenal cortex are located in the outer capsule and subcapsular area: GLI1+ SF1- non-steroidogenic capsular cells are capable to generate steroidogenic descendants of all inner zones of the adrenal cortex both in embryonic development and postnatally; SHH+ SF1+ non-steroidogenic cells of the zona glomerulosa situated in the sub-capsular region can also proliferate and differentiate to all steroidogenic cell types of the adrenal cortex. (Berger et al., 2019; Lyraki & Schedl, 2021; Oikonomakos et al., 2021)

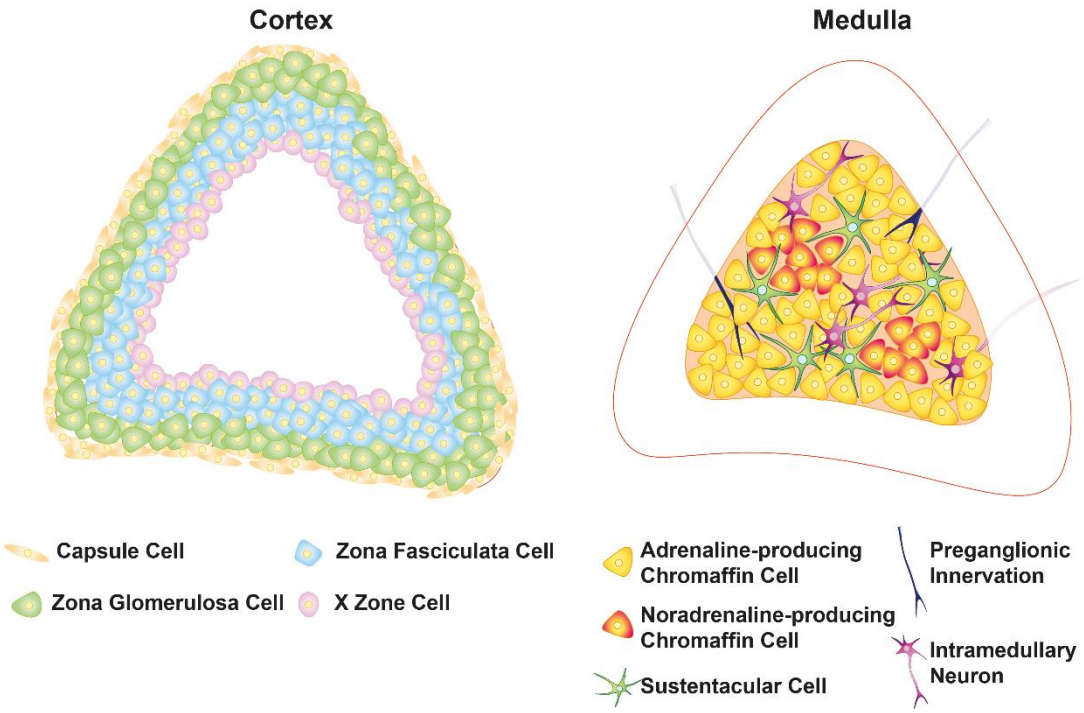

Figure 1.1 Cellular composition of adrenal glands.
The adrenal cortex is formed of concentric zones, from outer to inner: capsule, zona glomerulosa, zona fasciculata, X zone (zona reticularis in human). The adrenal medulla is composed of adrenaline- and noradrenaline- producing chromaffin cells, efferent and afferent nerve fibres, intramedullary neurons and sustentacular cells.

The adrenal medulla is derived from the neural crest, and its main function is to regulate the sensory-metabolic cascades generated by the "fight or flight" response through the production of catecholamines. The adrenal medulla includes three main cell types:

chromaffin cells, the functional unit of the tissue, which produce catecholamines, neurons and neuronal termination, which stimulate the catecholamine production, and sustentacular cells of glial origin that are thought to have a support function (Figure 1.1). Chromaffin cells are further subdivided in to two types, ones that produce noradrenaline and ones that express Phenylethanolamine N-methyltransferase (PNMT), which converts noradrenaline to adrenaline. Adrenal medulla development, function and cell composition will be discussed further in the next chapters. (reviewed in Bechmann, Berger, Bornstein, & Steenblock, 2021; Kastriti, Kameneva, & Adameyko, 2020)

1.2 Adrenal medulla physiology

Physiological functions of the adrenal medulla are mainly mediated by the release of the catecholamines adrenaline and noradrenaline. At resting state, low levels of these catecholamines maintain normal blood pressure and sympathetic tone. A 2- to 10- fold increase of circulating levels of adrenaline and noradrenaline is observed upon activation of the sympathetic system given by physical, psychological or environmental acute stress, exposure to cold, lack of oxygen or fear and alarm (Tank & Wong, 2015). This is referred to as the "fight or flight" response, which indicates the activation of physiological processes necessary to overcome acute stress (Cannon & de la Paz, 1911). The surge of catecholamine production initiated by the "fight or flight" response prepares the animal for immediate fight or to run from danger. This means catecholamines orchestrate a response with the involvement of multiple systems and affect organs both at a macro- and cellular level. Adrenaline and noradrenaline bind to G-protein-coupled adrenergic receptors (AR) on target organs to initiate a series of responses, αAR have a higher affinity for noradrenaline, whereas βAR show higher affinity for adrenaline. Both adrenaline and noradrenaline increase heart rate and the force of myocardial contraction; they initiate radial muscle contraction in the eye to widen the pupil; adrenaline relaxes bronchial smooth muscle in the lungs as well as smooth muscle of the gastrointestinal tract. In the kidneys, catecholamines compensate the normal sodium waste given by the increase of blood pressure with enhancement of sodium reuptake and activation the renin-angiotensin-aldosterone system. The "fight or flight" response leads to a major redistribution of the blood flow from the kidneys, gastrointestinal tract and skin to skeletal muscle. To facilitate the activity of skeletal muscle, heart and brain, adrenaline affects the metabolic activity of multiple target tissues to increase circulating levels of glucose and free fatty acids. Adrenaline inhibits the secretion of insulin and stimulates

glucagon secretion in the pancreas; catecholamines activate triglyceride lipase to release free fatty acids and glycerol (reviewed in Tank and Wong 2015).

The adrenal medulla is also involved in mediating an adaptive response to long-term sustained stress through its role as a component of the hypothalamus-pituitary-adrenal (HPA) axis. The hypothalamus controls the release of adrenocorticotrophic hormone produced by the pituitary gland, which leads to an increase in glucocorticoids levels secreted by the adrenal cortex. Amongst the multiple functions of glucocorticoids, these stimulate the production of adrenaline in the adrenal medulla (reviewed in Tank and Wong 2015; Tsigos and Chrousos 2002).

Beside its links to the sympathetic nervous system, the adrenal medulla also works as a sensory organ itself: chromaffin cells directly monitor the levels of plasma glucose, pH and oxygenation and release catecholamines in response to hypoxia and hypoglycaemia (Fujiwara et al., 1994; SC et al., 1999; Schwartz et al., 1987).

1.3 Embryonic Development of the Adrenal Medulla

The two main compartments of the adrenal gland have distinct developmental origins. While the adrenal cortex derives from the intermediate mesoderm, the adrenal medulla develops from the neural crest. Despite its distinct origin and function, the adrenal medulla requires the presence of an adrenal cortex to develop correctly. In cortex-impaired SF1 null mutant mice, it has been shown that the adrenal medulla is still formed, however chromaffin cells cannot express the enzyme PNMT, necessary for adrenaline production (Gut et al., 2005).

Early studies in chick identified a sympathoadrenal domain at the level of somites 18-24, which gives rise both to sympathetic neurons and the adrenal medulla (Le Douarin & Teillet, 1974). The presence of bipotent sympathoadrenal progenitors is also observed in mammals (Anderson & Axel, 1986). In mouse, at E10.5, the first migrating neural crest cells expressing SOX10 locate near the dorsal aorta, which produces BMP4/7 to drive lineage commitment of the migrating cells (Saito et al., 2012). At E11.5 the first adrenomedullary and sympathetic ganglion progenitors appear near the dorsal aorta. These populations are indistinguishable and express both chromaffin cell marker TH and sympathetic progenitor marker 'Cocaine and Amphetamine-Regulated Transcript' (CART). At E12.5 the populations start becoming distinct, with only 38% of TH+ cells being also CART+. At E13.4 the sympathetic ganglia primordium and the adrenal anlagen are anatomically defined, with CART being a marker of sympathetic progenitors (sympathoblasts) expressed by 91% of this population. However, the adrenal medulla primordium includes few cells with CART immunoreactivity. At E14.5, chromaffin cells of the adrenal medulla start expressing PNMT, an enzyme necessary for adrenaline production and by E16.5, 60% of all chromaffin cells express PNMT (Hei Chan et al., 2016). However, this model of bipotent neural crest progenitors could not explain observations of heterogeneity of neuronal markers arising prior to the cells arrival at the dorsal aorta in

chick (Ernsberger et al., 2004). Furthermore, mouse sympathoadrenal progenitors deficient of *Phox2b*, a transcription factor necessary for chromaffin cell differentiation, are still able to colonise the adrenal anlage (Pattyn et al., 1999), suggesting bifurcation of the sympathoadrenal lineage does not necessarily need to occur to form the adrenal medulla primordium.

A recent study of adrenal development in mouse (Furlan et al., 2017) used single cell transcriptomic techniques combined with *in vivo* validation to provide new insights. These observations confirm that migratory neural crest cells and derivatives form the suprarenal ganglion near the dorsal aorta, subsequently giving rise to sympathoadrenal progenitors. However, this study indicates that the segregation between sympathoblasts and adrenomedullary chromaffin cells occurs before a putative sympathoadrenal common progenitor reaches the dorsal aorta. Labelling migrating neural crest cells with a *Plp1*-driven linage tracing system in mouse from E10.5, this shows these cells give rise to about 50% of adrenomedullary cells and less than 10% sympathetic ganglia by E17.5. A *Ret*-driven lineage tracing system induced at E10.5 shows traces specific to sympathetic ganglia by E17.5 with only minor tracing in the adrenomedullary primordium, indicating that while some common sympathoadrenal progenitors might still be present at this point, segregation has already occurred. Interestingly, when investigating Schwann Cell Precursors (SCPs) using *Plp1*- and *Sox10*-driven lineage tracing systems induced at E11.5 (when neural crest migration is complete and all SOX10+FOXD3+ cells are associated with innervation), it was shown that SCPs give rise almost exclusively to adrenomedullary cells by E17.5, indicating that SCPs give rise preferentially to adrenomedullary cells. Diphtheria Toxin Subunit A (DTA)-based genetic ablation of *Sox10*+ SCPs at E11.5 shows a reduction of intra- and extra-medullary SOX10+ cells and TH+ chromaffin cell depletion in the adrenomedullary primordium, while sympathoblast numbers were unaffected. Unlike neural crest and common sympathoadrenal progenitors, which are able to migrate freely, SCPs use nerves to reach their final location. To confirm the importance of SCPs in forming

the adrenomedullary primordium, genetic ablation of nerves required for SCPs migration was achieved using a *Hb9*$^{Cre/+}$;*Isl2*$^{DTA/+}$ mouse line. Nerve ablation resulted in a loss of 78% of adrenomedullary cells by E14.5, confirming SCPs have a major role in the formation of the adrenal medulla. Furthermore, transcriptional investigation with single cell RNA sequencing revealed the presence of an intermediate SCP/chromaffin cell state, coined "bridge cell", which expresses *Htr3a*. Another study (Lumb et al., 2018) confirmed that nerve-associated SOX10+ progenitors are required to give rise to chromaffin cells, ablation

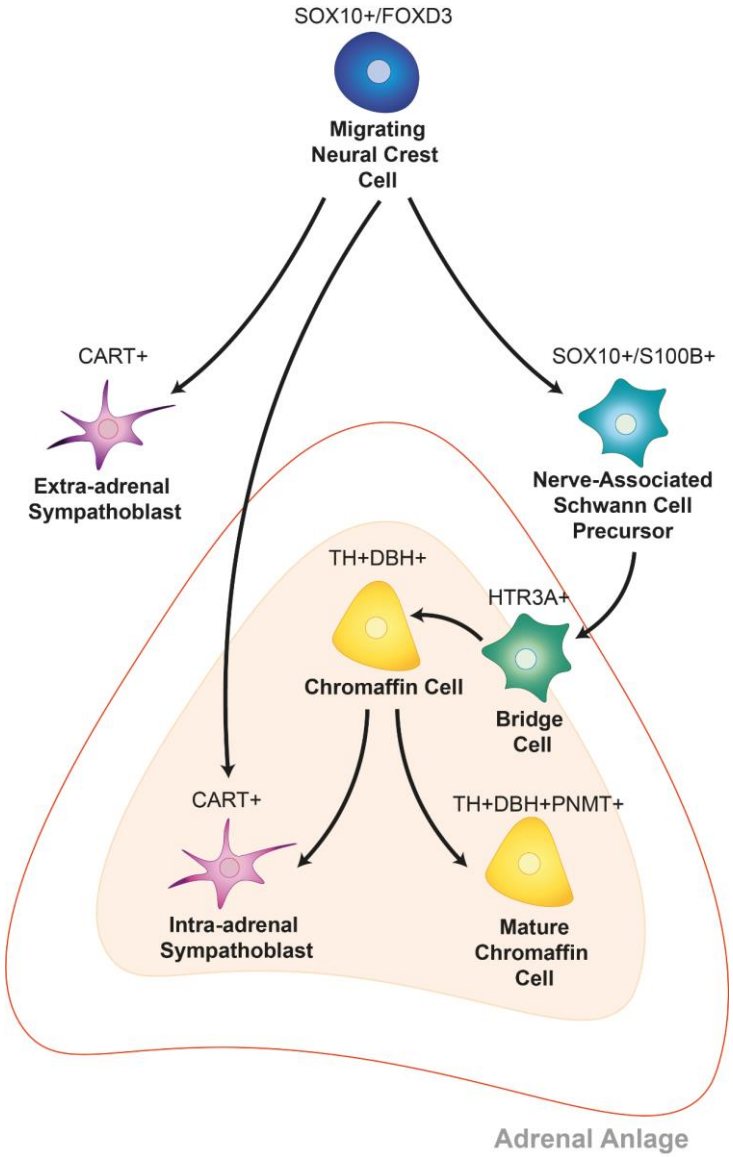

Figure 1.2 Simplified model of murine adrenal medulla development.
Neural Crest Cells give rise to SCPs as well as extra-adrenal and intra-adrenal sympathoblasts. SCPs give rise to chromaffin cells, which can also give rise to intra-adrenal sympathoblasts before reaching full maturation.

of preganglionic neurons via *Olig2*$^{Cre/+}$;*Rosa*$^{DTA/+}$ mouse line at E12.5 results in a 69% reduction of chromaffin cells, which is maintained at E13.5.

The latest study in human and mouse adrenomedullary development (Kameneva, Artemov, et al., 2021) used bioinformatic and *in vivo* observations to provide a refinement of the role of SCPs during adrenomedullary embryonic development. In mouse, as previously described, it is confirmed that extra-adrenal sympathoblasts derive from migrating neural crest cells traced by a *Ret*-driven lineage tracing system induced at E10.5. While part of the intramedullary sympathoblasts (10%) is also derived by this population, further analysis of a *Plp1*-driven lineage tracing system induced at E11.5 revealed that several CART+ intramedullary sympathoblasts are derived from embryonic SCPs. *In silico* analyses, together with temporal observations (chromaffin cells appear at 11.5 while CART+ intramedullary sympathoblasts at E13.5), indicate that intramedullary sympathoblasts are derived from immature SCP-derived chromaffin cells (Figure 1.2). Bioinformatic analyses of single cell RNA sequencing of human developing adrenal medulla reveal that SCPs are able to transition both into chromaffin cells and intramedullary sympathoblasts and that sympathoblasts can turn into chromaffin cells (Kameneva, Artemov, et al., 2021).

1.3.1 Molecular mechanisms of medulla development

The development of the adrenal medulla is composed of two migratory waves of cells originating in the neural crest (reviewed in Bechmann et al. 2021; Kastriti, Kameneva, and Adameyko 2020). The first cells (SOX10+, FOXD3+, ERBB2/3+, NRP1/2+) delaminate from the dorsal neural tube and migrate ventrally towards the dorsal aorta, which secretes signals necessary for sympathoadrenal lineage maturation, as well as chemotactic signals. Bone morphogenetic proteins 4 and 7 (BMP4 and BMP7) ligands produced by the dorsal aorta initiate the expression of chemoattractant stromal cell-derived factor-1 (SDF1, also

called CXCL12) and Neuregulin 1 (NRG1). SDF1 and NRG1 act on delaminating neural crest sympathetic progenitors expressing their respective receptors CXCR4 and EGF family-ERBBs, to initiate accumulation of sympathoadrenal progenitors near the dorsal aorta. Once reaching the dorsal aorta, BMP4 and BMP7 signals act directly on the sympathoadrenal progenitors where they are necessary for special segregation of adrenomedullary/sympathetic progenitors and they upregulate a network of transcription factors involved in early sympathoadrenal lineage differentiation and maturation including PHOX2B, MASH1, INSM1, HAND2 and GATA2/3. Furthermore, NRG1/ERBB signalling drives adrenomedullary progenitors to their final destination. (Britsch et al., 1998; Huber et al., 2009; Reissmann et al., 1996; Saito et al., 2012; Shah et al., 1996)

The second migratory wave originates from nerve-associated SCPs (SOX10+, S100B+, NRP1/2+), which along preganglionic axons, are guided by SEMA3 signalling through neuropilins (NRP1/2) to reach the sympathoadrenal primordium (Lumb et al., 2018) There they will give rise to adrenomedullary cells via an intermediate population of *Htr3a+* 'bridge' cells (Furlan et al., 2017).

The homeodomain transcription factor PHOX2B has a pivotal role in the development of both chromaffin cells and sympathetic neurons *in vivo*. PHOX2B-deficient sympathoadrenal progenitors do not lose their ability to colonise the adrenal anlage but they fail to form a centrally localised medulla. In addition, developing chromaffin cells fail to express enzymes necessary for catecholamine production Dopamine beta-hydroxylase (DBH) and TH (Huber et al., 2005; Pattyn et al., 1999). The lack of production of these enzymes is also found in adrenomedullary cells of mouse embryos lacking helix-loop-helix transcription factor MASH1 (ASCL1), where the chromaffin cells present an immature phenotype and persistence of neuronal markers. However, a small fraction of MASH1-deficient chromaffin cells were found to differentiate normally at birth (Huber et al., 2002). This could be consistent with a role in facilitating differentiation as it has been

shown in sympathetic neurons (Pattyn et al., 2006) or MASH1 could be differentially expressed in subsets of chromaffin cells (reviewed in Huber, Kalcheim, and Unsicker 2009). Zinc finger transcription factor INSM1 is another important component of the transcription factor network regulating the development of chromaffin cells and sympathetic neurons. Loss-of-function mice show delayed differentiation and smaller sympathetic ganglia, while chromaffin progenitors fail to differentiate and, despite showing initial normal numbers, cell death is later enhanced. Furthermore, INSM1 deficient chromaffin cells show a reduced expression of TH, DBH and PNMT, enzymes involved in catecholamine synthesis as well as chromogranin A and B, necessary components of secretory granules (Wildner et al., 2008). Basic helix-loop-helix transcription factor HAND2 determines the noradrenergic phenotype in the sympathetic ganglia (Morikawa et al., 2007), *Wnt1*-driven *Hand2* deletion, affecting specifying and migrating neural crest cells leads to a reduced number of adrenomedullary progenitors, together with a reduction in number of cells expressing PHOX2B and GATA3 (Hendershot et al., 2008). Zinc finger transcription factors GATA2 and GATA3 are both expressed by the sympathoadrenal lineage. In chick, GATA2 expression starts after MASH1, PHOX2a and HAND2 expression, but before TH and DBH (Tsarovina et al., 2004). *Gata2/3* expression is missing in $Phox2b^{-/-}$ mice and $Gata3^{-/-}$ mice show severely compromised differentiation and cell number both in sympathetic ganglia and chromaffin cells at later stages. Nonetheless, differentiation up to E10.5 appears almost normal except for reduced TH expression, indicating this transcription factor has a role in later stages. $Gata3^{-/-}$ mutant chromaffin cells also show reduced levels of *Mash1*, *Hand2* and *Phox2b*, suggesting cell differentiation is controlled by a feedback of transcriptional interactions between GATA3, MASH1, HAND2 and PHOX2B (Moriguchi et al., 2006). The LIM-Homeodomain transcription factor Islet-1 (ISL1) acts downstream of PHOX2B in the development of neurons and chromaffin cells. Conditional deletion of *Isl1* driven by $Wnt1^{Cre}$ shows ISL1 is not essential for the initial differentiation of sympathoadrenal cells, but it is necessary for

sympathetic neuron survival and for initiation of the adrenaline synthetising enzyme PNMT. Developing chromaffin cells of *Isl1* deficient mice display normal expression levels of TH, DBH and the transcription factors *Phox2b*, *Mash1*, *Hand2*, *Gata3* and *Insm1*, but the expression levels of the transcription factors *Gata2* and *Hand1*, and *AP-2b* were significantly reduced (Huber et al., 2013). The transcription factor AP-2β, member of the AP2 family, is expressed and has a crucial role in sympathetic ganglia of the developing mouse embryo. It is also expressed in developing chromaffin cells and AP-2β deficient mice show significantly reduced expression levels of catecholamine biosynthesising enzymes DBH and PNMT, as well as transcription factor PHOX2B, indicating AP-2β is necessary for correct adrenaline-producing chromaffin cell maturation. Chromaffin cell content of adrenaline is diminished and AP-2β binds upstream of the promoter of PNMT, therefore directly regulating the production of adrenaline (Hong et al., 2008).

Studies in chick, quail and mouse embryos indicate that BMP/TGF-β (transforming growth factor-beta) signalling has a role in the developing chromaffin cells beyond the secretion of BMPs from the dorsal aorta during early sympathoadrenal development. In chick, it has been shown that BMP4 is expressed by SF1+ adrenocortical cells while BMP receptors are expressed by TH+ chromaffin cells and upon BMP signalling inhibition with Noggin in explants, the number of TH+ cells decreases while the number of SOX10+ cells increases, indicating BMP4 has a role in chromaffin cell differentiation (Huber et al., 2008). Further evidence of the role of BMP/TGF-β signalling has been reported in quail: TGF-β1,2,3 inhibition with monoclonal antibodies induces an increase in number of proliferating TH+ cells, indicating TGF-β has a role in the control of proliferation of chromaffin cells during embryonic development (Combs et al., 2000). This role was confirmed using *Tgf-b2*$^{-/-}$ and *Tgf-b3*$^{-/-}$ mouse lines, with a specific role for the isoform TGF-β2 (Rahhal et al., 2004). Together with BMP/ TGF-β signalling, other pathways of possible relevance during embryonic adrenomedullary development are the canonical WNT pathway, NOTCH and

Hedgehog, which were bioinformatically identified as statistically significant in single cell RNA sequencing at E12.5 and E13.5 (Furlan et al., 2017), however investigation on the role of these signalling pathways has not been carried out yet.

1.4 The postnatal adrenal medulla

The main functional cell components of the postnatal adrenal medulla are chromaffin cells. Chromaffin cells produce catecholamines, which mediate adrenomedullary functions such as the "fight or flight" response. Axons from preganglionic and sensory neurons of the sympathetic nervous system innervate the adrenal medulla to direct catecholamine secretion and postganglionic intramedullary neurons regulate feedback mechanisms. A third cell type of glial nature, termed "sustentacular" provides unspecified support for chromaffin cells.

The adrenal medulla was historically considered a post-mitotic organ with no regenerative capacities until it was identified that a small number of chromaffin cells are able to proliferate in rodents (Tischler et al., 1989). Experiments based on BrdU incorporation showed the adrenal medulla has a calculated turnover of 1% per day. A single BrdU injection in adult rats shows an uptake of 0.46%, which increased to 0.77% after 12 hours with no further increase for 7-8 days, while continuous infusion of BrdU showed 40% of the adrenal medulla cells were labelled after 73 days (A. A. Verhofstad, 1993). These studies suggest chromaffin cells are capable of proliferation, given that they are the most abundant cell type in the postnatal adrenal medulla. However, these cannot exclude that sustentacular cells or even postganglionic neuronal bodies proliferate in the postnatal medulla. Nonetheless, these studies demonstrate that the adrenal medulla is a dynamic organ at a cellular level. Observations in the postnatal adrenal from weaning to adulthood indicate a considerable growth in volume and sexual dimorphism. At 3 weeks of age, the volume of adrenal medulla is significantly larger in females compared to male mice and this is maintained at least until 11 weeks of age; between 3 and 7 weeks of age the medulla volume in both males and females increases. Between 9 and 11 weeks of age, the volume of the medulla in females further increases by 60% whereas it slightly decreases in males (Bielohuby et al., 2007). This suggests that analysis of the adrenal

medulla at early postnatal timepoints might reveal meaningful insights on adrenal cell hierarchy, and that differences between males and females are expected. Adrenomedullary sexual dimorphism has not been fully characterised at a cellular or tissue homeostasis level, but adrenomedullary function has shown to be regulated by estrogen in a sex-specific manner (Khasar et al., 2005).

1.4.1 Chromaffin cells

Chromaffin cells are the functional unit of the adrenal medulla. The term "chromaffin" was associated to these cells by Alfred Kohn in the 19th century, after observing that an unidentified substance in the adrenal medulla (adrenaline) reacts with ferric chloride to produce a green colour and with chromium salts to produce a yellow-brown colour. Chromaffin cells are modified post-ganglionic sympathetic neurons that secrete the content of their vesicles in the bloodstream instead of innervating target organs. Adrenomedullary chromaffin cells synthesise, store, transport and release adrenaline, noradrenaline, and dopamine. Chromaffin cells of the adrenal medulla are mainly described as two separate sub-populations of adrenaline-producing and noradrenaline-producing chromaffin cells. Chromaffin cells appear arranged in clusters and have a polygonal or columnar shape in the tissue and a spheroidal shape in culture. Occasionally, they show thin processes of variable length (Díaz-Flores et al., 2008). Catecholamine-containing chromaffin granules are localised in the cytoplasm of chromaffin cells, appear electron-dense and they vary in size and shape according to the type of secretory content (Coupland, 1965). Besides catecholamines, these granules contain multiple proteins necessary for catecholamine production, stimulation and secretion, such as chromogranin A and B, enkephalins, calcium ions, dopamine β-hydroxylase (DBH) and tyrosine hydroxylase (TH) (Kataoka et al., 1985; Winkler & Fischer-Colbrie, 1992). The mechanism of catecholamine biosynthesis is shown in Figure 1.3 In brief, TH catalyses the

hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA), which is decarboxylated by aromatic amino-acid decarboxylase (AADC) to produce dopamine. Dopamine is hydroxylated by DBH to produce noradrenaline. Only in adrenaline-producing chromaffin cells, noradrenaline is methylated by PNMT to form adrenaline (A. A. J. Verhofstad et al., 1985).

In rodents, about 80% of the total chromaffin cells are adrenaline-producing, while 20% are noradrenaline-producing (A. A. J. Verhofstad et al., 1985). Adrenaline- and noradrenaline-producing chromaffin cells of the adrenal medulla have been identifiable as two separate populations since the 1950s using classical histochemistry techniques (Coupland, 1965; Hillarp & Hoekfelt, 1954), and are regulated by distinct neural efferent pathways to the adrenal medulla (reviewed in De Diego, Gandía, and García 2008).

Given their developmental origin, chromaffin cells share the same neurotransmitters, neuropeptides and transduction mechanisms as the brain, skin and gut. Because of the accessibility and abundance of adrenomedullary chromaffin cells and the feasibility to culture these as primary cells *in vitro*, chromaffin cells have been largely used as a model to investigate basic concepts of neurochemical transmission, in particular neurostransmitter-containing vesicle exocytosis (Bornstein et al., 2012). Upon administration of nerve growth factor (NGF) during gestation, chromaffin cells are able to differentiate into sympathetic neurons (Aloe & Levi-Montalcini, 1979) and NGF administration *in vitro* leads to neurite outgrowth, which is impaired by glucocorticoids (K. Unsicker et al., 1978). The plasticity shown by chromaffin cells together with their secretory profile and abundance, have rendered them useful to tackle neurodegenerative disease. Transplants of chromaffin cells have been used in clinical trials to treat Parkinson's disease and chronic pain, bringing significant improvements but of limited duration, possibly because of the post-mitotic nature of most of the chromaffin cells

transplanted. Pre-clinical experiments have shown potential for chromaffin cell transplantations to treat Alzheimer's disease and depression (Ambriz-Tututi et al., 2012).

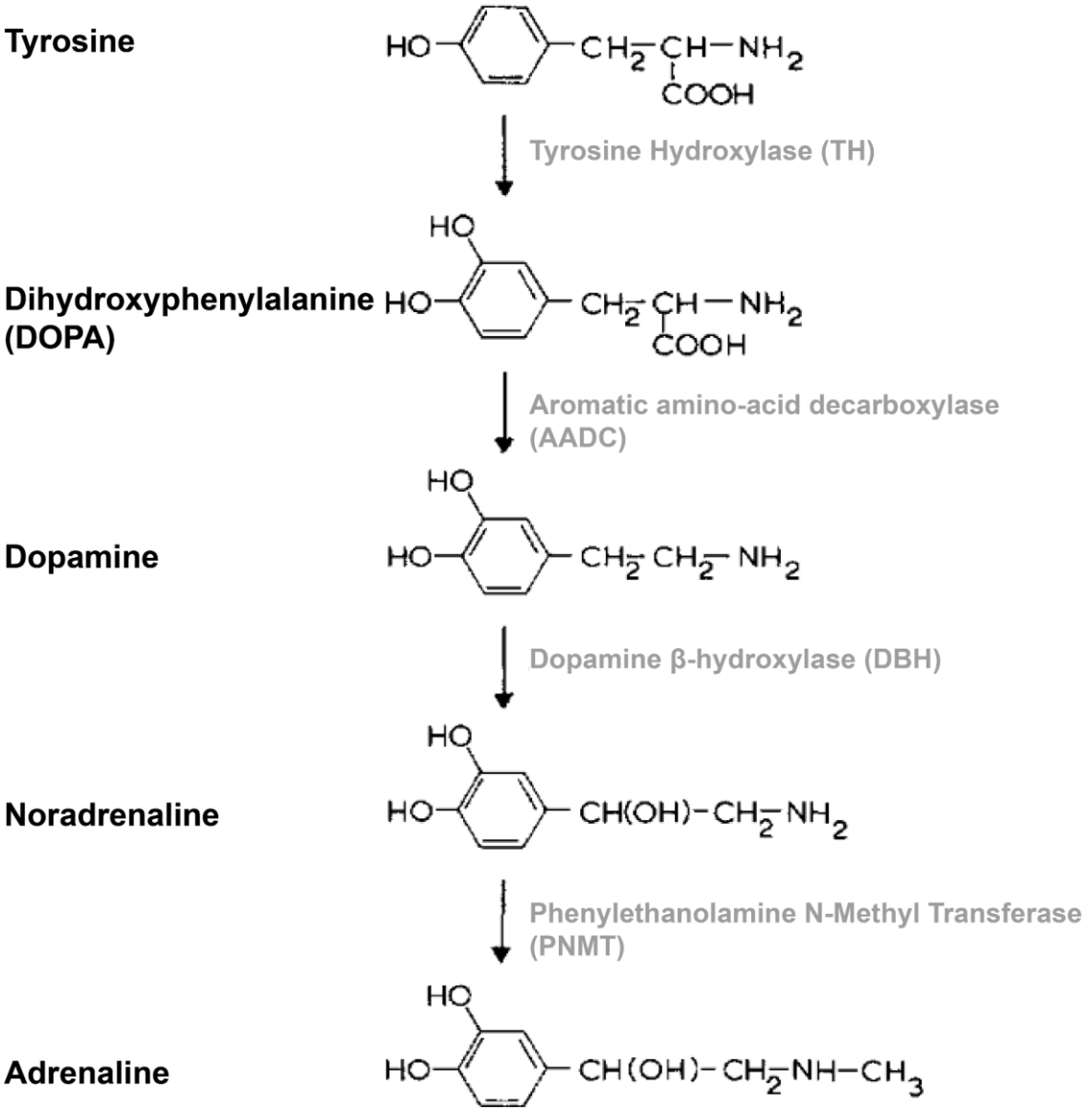

Figure 1.3 Adrenaline biochemistry.
Modified from Verhofstad 1985. Tyrosine is converted to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH). Amino-acid decarboxylase (AADC) decarboxylate DOPA to form dopamine. Dopamine is converted to noradrenaline by dopamine β-hydroxylase (DBH). Phenylethanolamine N-methyl transferase (PNMT) methylates noradrenaline to produce adrenaline.

1.4.2 Sustentacular cells

Besides chromaffin cells and neurons, a third main cell type is found in the adrenal medulla: sustentacular cells. These cells have been described both in rodent and human as "satellite" or "support" cells and are immunoreactive for glial markers S100B, GFAP and Vimentin (Cocchia & Michetti, 1981; Magro & Grasso, 1997; Suzuki & Kachi, 1995). Sustentacular cells present a small and spindle-shaped cytoplasm together with processes of variable length, show a filamentous cytoskeleton and occasional small lipid droplets, and are located in proximity to chromaffin cells, their bodies usually localised at the periphery of the chromaffin cells clusters (Díaz-Flores et al., 2008). *In vitro* experiments show that bovine Nestin+;Vimentin+;SOX9+ sustentacular cells cultured in non-adherent primary cultures called chromospheres, are capable of forming new chromospheres once dissociated (Chung et al., 2009). Rodent Nestin+;SOX10+;S100B+;GFAP+ sustentacular cells are able to form self-renewing spheres and differentiate into chromaffin cells and neurons *in vitro*. Furthermore, lineage tracing using a $Nestin^{CreERT2}$–driven system, show the derivation of CHGA+ chromaffin cells and TUJ1+ neurons *in vivo* in acute stress conditions (Rubin De Celis et al., 2015). Strong evidence for a role of sustentacular cells *in vivo* and in normal homeostasis has not yet been provided.

While there is little experimental *in vivo* evidence that sustentacular cells could act as stem/progenitor cells of the postnatal adrenal medulla, numerous observations indicate this population as the most likely candidate. Sustentacular cells have been identified as a very distinct population from chromaffin cells and intramedullary neurons, with which they do not show any marker overlap. Interestingly, reported markers of sustentacular cells resemble the ones identified in embryonic SCPs, leading to the speculation that this cell population might be a postnatal source of progenitor cells. Single cell transcriptomics experiments in mouse show a continuous population of *S100b*+ *Erbb3*+ SCPs at 7 combined timepoints from E13.5 to P5, which are predicted to give rise to differentiated

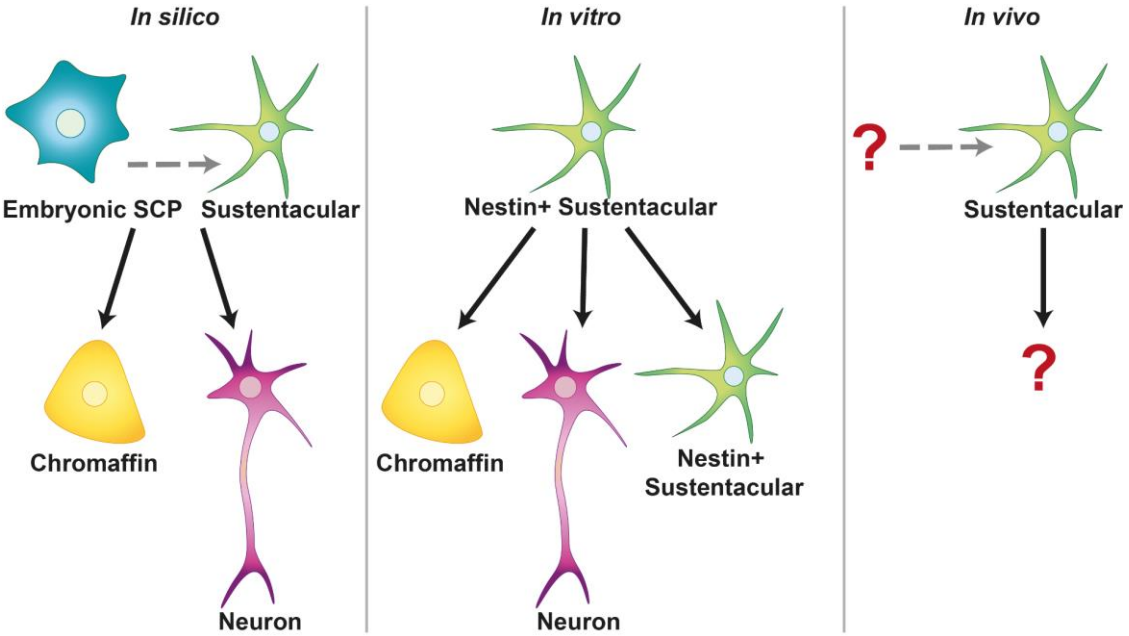

Figure 1.4 Role of sustentacular cells.
***In silico*, combined embryonic and early postnatal datasets indicate SCPs/postnatal cells with markers compatible with sustentacular cell nature can give rise to chromaffin cells and intramedullary neurons. This suggests postnatal sustentacular cells might be derived from embryonic SCPs. *In vitro*, cultured as non-adherent spheres, Nestin+ sustentacular cells have shown to be able to self-renew as well as differentiate towards chromaffin and neuronal fate. *In vivo*, there is no evidence of the developmental origin of sustentacular cells or their differentiation potential under normal physiological conditions.**

cells bioinformatically based on pseudotime (Hanemaaijer et al., 2021). It has been shown in other non-canonical SCP-derived tissues that SCPs persist postnatally where they contribute to tissue homeostasis. In colon, postnatal SCPs differentiate into neurons to contribute to the enteric nervous system (Uesaka et al., 2015); in tooth, postnatal SOX10+ PLP1+ nerve-associated cells of glial nature generate mesenchymal stem cells that produce pulp cells and odontoblasts during homeostasis (Kaukua et al., 2014). The carotid body, a catecholaminergic organ mainly composed of glomus cells that are very similar to chromaffin cells, also presents a SOX10+ S100B+ GFAP+ sustentacular cell population. Sustentacular cells of the carotid body have been defined as stem cells as they can differentiated towards glomus cells going through an intermediate Nestin+ state (Pardal et al., 2007). These parallel observations suggest that sustentacular cells of the adrenal

medulla might act as a progenitor/stem cell population *in vivo* to maintain normal organ homeostasis, but this has yet to be demonstrated (Figure 1.4).

1.4.3 Innervation and blood supply

Innervation

The adrenal medulla is innervated by axons of sympathetic preganglionic neurons originating in the spinal cord, via the splanchnic nerve. Bundles of neurons pierce the adrenal cortex and divide into thinner fibres when reaching the medulla (Strack et al., 1988). Synapses of sympathetic preganglionic neurons terminate directly on either adrenaline or noradrenaline-producing chromaffin cells (Morrison & Cao, 2000). Sensory neurons with cell bodies located in the dorsal root ganglia also innervate the adrenal medulla with endings associated with chromaffin cells and vasculature (Mohamed et al., 1988). Intra-adrenal ganglionic neurons can be divided into two subtypes: type I neurons express enzymes for catecholamine synthesis except PNMT, while type II neurons express vasoactive intestinal polypeptide (VIP) (A. Dagerlind et al., 1990; Oomori et al., 1994). Intramedullary neurons have been shown to function as postganglionic neurons, innervating both chromaffin cells and the adrenal cortex (Maubert et al., 1990), and retrograde tract-tracing experiments showed they project to the splanchnic nerve, where it is thought they contribute to a feedback loop modulating the preganglionic innervation of the adrenal gland (Å. Dagerlind et al., 1995).

Blood supply

The adrenal glands are one of the tissues with the highest blood supply rate as its catecholaminergic activities are mediated by the release of adrenaline and noradrenaline

in the bloodstream (Ehrhart-Bornstein et al., 1998). The adrenal arteries derive from the abdominal aorta, the renal artery, and the phrenic artery. Most of the adrenal arteries perfuse the adrenal capsule and cortex, whereas the medulla arteries reach the adrenal medulla directly (Vinson et al., 1985). Once in the medulla, the arteries divide into smaller branches to form a capillary network amongst chromaffin cells, which is necessary for efficient release of catecholamines throughout the body (Coupland & Selby, 1976; Díaz-Flores et al., 2008).

1.5 Pathologies of the adrenal medulla

Given its important role in homeostasis and regulation of metabolism, pathologies of the adrenal medulla can have a significant impact on physiological functions.

1.5.1 Congenital adrenal hyperplasia

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder of cortisol biosynthesis, with severe forms occurring in one in 15,000 births worldwide. 95% of CAH cases are caused by a defect of 21-hydroxylase, leading to cortisol deficiency, with or without aldosterone deficiency, and androgen excess. The lack of circulating cortisol leads to a disrupted negative feedback mechanism to the hypothalamus, which overproduces corticotrophin-releasing hormone (CRH), leading to overactivation of the intact functions of the adrenal cortex. Despite this being an adrenal cortex defect, it has an impact on the adrenal medulla and CAH patients present with adrenaline deficiency, which leads to hyperinsulinism. (Merke & Bornstein, 2005). The adrenal medulla is defective due to the lack of cortisol during development, which is necessary for chromaffin cell precursors to express PNMT and therefore adrenaline production (Bohn, 1983; Gut et al., 2005).

1.5.2 Dopamine beta-hydroxylase deficiency

Dopamine beta-hydroxylase (DBH) deficiency is an extremely rare congenital disease caused by mutations in the *DBH* gene, which is essential in noradrenaline synthesis, therefore leading to absence of production of both noradrenaline and adrenaline. DBH deficiency is characterised by cardiovascular disorders such as hypotension, together with muscle hypotonia, hypothermia and hypoglycaemia. (Senard & Rouet, 2006)

The incidence of DBH deficiency not defined, with only 25 patients from 20 families identified started from the 1980s (Wassenberg et al., 2021). The real prevalence of DBH deficiency is unknown and might be higher than observed, given that studies in *Dbh*$^{-/-}$ mice have shown that only 5% of DHB deficient mice reach adulthood. Most *Dbh*$^{-/-}$ mice die embryonically or perinatally, with *in utero* survival dependant on catecholamine transfer across the placenta (Thomas et al., 1995).

1.5.3 Stress-induced medullary dysfunction

The hypothalamic-pituitary-adrenal (HPA) axis and the sympathoadrenal axis are the main systems responsible for the maintenance of homeostasis during stress. In case of acute stress, the adrenal medulla plays a crucial role in orchestrating a fast "fight or flight" response, as well as in promoting finely tuned feedback mechanisms that prompt a fast return to homeostasis. However, frequent or prolonged activation of both the HPA axis and the sympathoadrenal system can lead to changes on the functional tone of the organs involved, including the adrenal medulla. Multiple studies in rats showed that chronic stress paradigms such as repeated cold exposure, mild stress or long-term repeated immobilisation stress, lead to a permanent increase of TH activity and levels, both of mRNA and protein (Bhatnagar et al., 1995; Duncko et al., 2001; Kvetnansky et al., 2003). Interestingly, it has been shown that after chronic stress induction with 41 consecutive immobilisations, rats exposed to novel stressors such as cold, insulin or 2-deoxyglucose showed no increase of TH levels whatsoever (Kvetnansky et al., 2003), confirming that chronic stress impairs the normal function of the adrenal medulla, which would normally increase its catecholamine production to restore homeostasis after acute stress. Experiments on rats, with the chronic variable stress (CVS) paradigm for 14 days, show that chromaffin cells of the adrenal medulla present cellular hyperplasia, while differences

in proliferation were not observed compared to non-stressed controls (Ulrich-Lai et al., 2006).

Obesity, another type of metabolic stress, has an opposed effect on adrenomedullary function. While the size of the adrenal medulla remains unchanged in obese mice that were fed with a high fat diet (Swierczynska et al., 2015), clinical studies on obese patients revealed that obesity leads to adrenomedullary dysfunction, specifically to a reduction of adrenaline secretion both in basal conditions and in response to carbohydrate ingestion (Del Rio, 2000; Reimann et al., 2017).

1.5.4 Neuroblastoma

Neuroblastoma is a heterogeneous malignancy arising during development from the neural crest cells that give rise to the sympathoadrenal lineages. It is the most common extra-cranial malignant tumour reported in children, accounting for 7% of all childhood cancers and 10% of cancer deaths (Park et al., 2013). Neuroblastoma normally occurs along the chain of sympathetic neural tissue along the spinal cord and in the adrenals, with the adrenal medulla being the most common site for tumour initiation, accounting for 47% of cases (Vo et al., 2014). About 1% of neuroblastoma patients have a familial predisposition with specific mutations in *PHOX2B* and *ALK* (Mosse et al., 2004, 2008). Common germline variants predisposing to sporadic neuroblastoma have also been identified including *LINC00340*, *BARD1*, *LMO1*, *DUSP12*, *DDX4/IL31RA*, *HSD17B12*, *LIN28B*. 90% of high-risk neuroblastoma present fragmental chromosomal alterations resulting in *MYCN* amplification, 17q gain or 11q loss (Pugh et al., 2013); 25% present somatic mutations of single genes such as *ALK*, *PTPN11*, *ARTX*; 20-30% show upregulation of telomerase reverse transcriptase (*TERT*) (Valentijn et al., 2015). Neuroblastomas present high inter- and intra- heterogeneity, with at least two types of cells observed in the same

tumours: undifferentiated mesenchymal cells and committed adrenergic cells. This intra-heterogeneity is representative of the multipotency of neural crest cells, whereas the inter-heterogeneity can be explained by the fact that somatic mutations can arise at different timepoints during development, therefore at different differentiation stages. While it is known that neuroblastoma specifically arises in neural crest derivatives of the sympathoadrenal lineage, the exact origin of this tumour is still unclear. Recent studies in SCP biology are elucidating developmental processes that might be linked to neuroblastoma pathogenesis (Hanemaaijer et al., 2021; Kameneva, Artemov, et al., 2021).

1.5.5 Pheochromocytoma and paraganglioma

Pheochromocytomas (PCCs) are rare neuroendocrine tumours affecting adrenomedullary chromaffin cells. These often arise in conjunction with paragangliomas (PGLs), which affect extra-adrenal sympathetic ganglia. About 25% of pheochromocytomas and paragangliomas (PPGLs) are malignant and able to metastasise in non-chromaffin tissues including bone, liver, lungs and lymph nodes, with scarce therapeutic approaches available and a survival rate of less than 50% after 5 years; PPGLs classified as benign are still associated with high morbidity and mortality due to their phenotype of excessive catecholamine production, which leads to hypertension, arrhythmia and stroke (Ayala-Ramirez et al., 2011; Hescot et al., 2013). Strikingly, PPGLs are associated with the highest degree of heritability in human neoplasms, with about 40% of PPGLs carrying a germline mutation in at least one of the following described genes: *VHL*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *RET*, *NF1*, *TMEM127*, *MAX*, *HIF2A* and *HRAS*. PPGLs are classified in three subgroups: “kinase signalling”, “pseudohypoxia” and “WNT-altered”. The kinase signalling subtype shows the highest expression of PNMT and is caused by somatic and germline mutations in *NF1*, *RET*, *TMEM127* and *HRAS* as well as increased expression of the RAS-MAPK signalling pathway and reduced response to DNA damage. The pseudohypoxia subtype is

caused by germline mutations in genes encoding subunits of SDH (succinate dehydrogenase), and somatic mutations in *VHL* and *EPAS1*. This is the subtype with the most genome duplications, and these are found across most chromosomes. The WNT-altered cluster consists of PCCs/PGLs with somatic mutations in genes including *WNT4*, *WNT7B*, *WNT5B*, *FZD7*, *FZD8*, *FZD3*, *GSK3* and *CTNNB1*, that all lead to an elevated WNT response. This subtype has the highest overexpression of chromogranin A, relevant to chromaffin cell function. (Alrezk et al. 2018; Fishbein et al. 2017; Lenders and Eisenhofer 2014)

The cellular origin of PPGLs is currently unknown, however a number of stem cell markers including OCT4, SOX2, LIN28, NGFR and THY1 are expressed in multiple types of PPGLs as reviewed in (Scriba et al., 2020). The lack of a pin-pointed cell of origin of these tumours together with their heterogeneity, severely limits the generation of *in vivo* experimental models of PPGLs, however *in vitro* models are widely used. The most well-known cell line used as a model of PCC is the PC12 rat pheochromocytoma cell line, which was originally isolated from a PCC developed in an irradiated rat in 1976 by (Greene & Tischler, 1976). Although this has been widely used for basic studies of chromaffin cell biology as well as investigation of PCCs, these cells are only noradrenergic and do not express PNMT. Other *in vitro* systems include mouse pheochromocytoma cell lines (MPCs) and mouse tumour tissue cells (MTTs), derived from different types of PCCs arising from mice with specific mutations, which express PNMT and produce adrenaline; hPheo1, which are progenitor cells derived from a human pheochromocytoma; immortalised chromaffin cells (imCCs) which derive from a *Sdhb* mutant mouse and RS0 cells, which are derived from an *Sdhb* mutant rat (all reviewed in Martinelli et al., 2020). However, *in vitro* models are restrictive as they do not recapitulate the complexity of *in vivo* PPGLs, limiting observations to one or few cell types at the same time. Multiple attempts have been made in the production of *in vivo* mouse models, especially ones carrying germline hereditable mutations, however

these have generally failed to produce PPGLs *in vivo* (reviewed in Martinelli et al., 2020). A deeper understanding development and postnatal cell hierarchy of the adrenal medulla is imperative to be able to determine the origin of PPGLs, not only with the goal to produce animal models or more complex *in vitro* models such as organoids, but also to lead to new effective therapeutic approaches.

1.6 Putative cell hierarchy of the postnatal medulla

Based on observations reported in literature, it is possible that the postnatal adrenal medulla contains a stem cell compartment. Sustentacular cells are the population of choice, and based on *in silico* and *in vitro* studies, as well as developmental parallels, it is expected of these cells to give rise to at least the two adrenaline- and noradrenaline-producing chromaffin cell populations, and possibly to intramedullary neurons. It has been shown that chromaffin cells containing adrenaline and noradrenaline can proliferate (Tischler et al., 1989), indicating this is a dynamic population that might need replenishment from the differentiation of a putative stem/progenitor population, whereas information on the dynamics of intramedullary postganglionic neuronal bodies is scarce. Interestingly, the latest study in adrenomedullary development indicates that chromaffin cells give rise to intramedullary neurons during development. Numerous *in vitro* studies reported the capabilities of chromaffin cells to differentiate into neurons, in response to NGF induction (K. Unsicker et al., 1978), confirming postnatal chromaffin cells retain this potential, therefore it cannot be excluded that even postnatally, chromaffin cells could give rise to intramedullary neurons. Adrenaline- and noradrenaline-producing chromaffin cells differ for their catecholamine secretion, which is mainly dictated by the presence of the enzyme PNMT. Transcription factors involved in chromaffin cell maturation are specific for promoting the expression of PNMT such as ISL1 (Huber et al., 2013), indicating a transcriptional heterogeneity beyond the functional enzymatic profile that has been scarcely investigated. During embryonic development, it has been shown that all differentiated TH+ chromaffin cells derive from the same origin, however the possibility that SCPs could directly give rise to chromaffin cells expressing PNMT, without passing through an intermediate DBH+PNMT- “immature” noradrenergic state, has not been explored.

Adult stem cells have been described in multiple organs and tissues and have been classically defined as a rare population which is capable of self-renewal and differentiation into more specialised cell types. Often, stem cells differentiate into an intermediate state termed "progenitor" cells, which maintain their differentiation ability but not self-renewal. For example, LGR5+ small intestinal crypt stem cells give rise to two types of progenitors: cycling absorptive, which differentiate into enterocytes; noncycling secretory, which will then give rise to goblet cells, Paneth cells and enteroendocrine cells. Interestingly, the two types of progenitors derived from LGR5+ stem cells are able to revert back to LGR5+ stem cells in case of extensive loss of the latter (Buczacki et al., 2013; Tetteh et al., 2016). In the airway epithelium in the lung, basal cells are the dominant stem cell, capable of giving rise to ciliated cells, secretory cells and neuroendocrine cells. However, secretory cells are also capable of self-renewal and differentiation to ciliated cells (Rao Tata & Rajagopal, 2017). In the mammary gland, stem cells give rise to a luminal progenitor and a myoepithelial progenitor, which are both capable of self-renewal and independently maintain specific differentiated cell types: the luminal progenitors will give rise to ductal luminal cells and alveolar cells (going through a ductal and alveolar progenitor respectively), while the myoepithelial progenitor gives rise to myoepithelial cells (Van Keymeulen et al., 2011). In the pituitary gland, SOX2+ stem cells give rise to three different defined progenitors, each capable of giving rise to specific hormone producing cells (Cynthia Lilian Andoniadou et al., 2013). The nature of adult stem cells is often associated with their low proliferative activity, meaning many adult stem cell populations are quiescent or label-retaining. However, with continuous new knowledge about adult stem cells, it has become extremely difficult to have a broad definition of adult stem cells that is relatable to every organ and tissue. For example, the association of a quiescent nature has been widely challenged by the proliferative nature of adult stem cells in highly dynamic tissues such as the intestinal crypt (Clevers, 2013). Given the heterogeneity of the stem cell phenotype throughout different adult tissues, it has been suggested that the definition of stem cell is being

increasingly linked to their function in homeostasis and in organ challenge (Clevers & Watt, 2018).

Regarding the adrenal medulla, putative Nestin+ sustentacular stem cells have been described to be differentiating in the context of the stress response, and show differentiation and self-renewal capabilities *in vitro* (Rubin De Celis et al., 2015). However, there have been no *in vivo* reports of newly formed differentiated cells arising from a putative stem/progenitor cell in normal homeostasis in the postnatal adrenal medulla.

1.7 SOX2 as a stem cell marker

SOX2 (Sry-related box 2) is part of the high-mobility group (HMG) box transcription factors family. SOX2 has been widely associated to pluripotency and in mammals it is expressed from early embryogenesis to adulthood, predominantly in stem cells. In the early embryo, SOX2 is expressed in the inner cell mass, where it is responsible for the maintenance of pluripotency (Masui et al. 2007). During development, SOX2 is necessary for the formation of several endodermal and ectodermal tissues, including the nervous system (Bylund et al. 2003). Postnatally, SOX2 expression is retained in multiple adult tissues, where SOX2 is a marker multiple stem/progenitor cell lineages, including the brain (Ellis et al. 2004), salivary gland (Emmerson et al. 2017), the trachea (Que et al. 2009) and the pituitary gland (Andoniadou et al. 2013).

SOX2 is a known regulator of neural crest development and it is expressed by SCPs migrating along peripheral nerves (Furlan et al., 2017; Wakamatsu et al., 2004). Experiments of SOX2 overexpression *in vitro* in avian trunk neural crest cells indicate SOX2 inhibits neuronal differentiation (Wakamatsu et al., 2004), suggesting a similar role to the one observed in the developing central nervous system, where postnatal cells retaining SOX2 expression act as stem cells (Ellis et al., 2004). These observations, together with preliminary data obtained from our group which indicate SOX2 expression in the postnatal adrenal medulla, suggest SOX2 expression could be pursued as a possible marker of stem/progenitor cells in the adrenal medulla.

1.8 Thesis Aims

The postnatal adrenal medulla is a poorly studied organ, with very little known about its cell types, cell hierarchy and its ability to maintain tissue homeostasis in normal conditions. It is not known whether newly formed chromaffin cells arise during the postnatal life of the organ and furthermore, a stem/progenitor cell population with a function in normal homeostasis has not been identified.

This thesis strives to provide a detailed picture of the postnatal adrenal medulla at a cellular level, a necessary step to further establish cell hierarchy. Literature reports and preliminary data from our group have indicated the presence of SOX2+ adrenomedullary cells. SOX2 is a known pluripotency marker, which is expressed by somatic stem and progenitor populations. The overarching aim of this thesis is to determine whether SOX2+ adrenomedullary cells are a progenitor/stem cell population of the adrenal medulla. The specific aims are:

1. To define the transcriptional profiles of adrenomedullary cell types.

Given the poor understanding of cell hierarchy of the adrenal medulla, I aim to create the first postnatal mouse adrenal medulla cellular atlas using single cell RNA sequencing and identify the transcriptomic signatures for functional adrenomedullary populations.

2. To characterise the function of SOX2+ adrenomedullary cells.

Combining *in vivo*, *in vitro* and *in silico* techniques, I aim to study the SOX2+ adrenomedullary cell population to characterise their cellular attributes, document population dynamics during postnatal physiology and establish their developmental origin.

3. To determine if SOX2+ adrenomedullary cells are stem cells.

In order to determine whether SOX2+ cells are a stem/progenitor cell population of the postnatal adrenal medulla, I will assess whether these cells are capable of self-renewal and the generation of new functional endocrine cells through *in vitro* and *in vivo* assays, supported by i*n silico* analyses.

2 Materials and Methods

2.1 Animals

2.1.1 Mouse Strains

Procedures were carried out in compliance with the Animals (Scientific Procedures) Act 1986, Home Office license and King's College London (KCL) ethical review approval. All mouse colonies were maintained under 12:12 hours light/dark cycle and fed *ad libitum*. I used the *Sox2*$^{eGFP/+}$ mouse line where one allele of *Sox2* was replaced with *eGFP* (Ellis et al., 2004), and *Sox2*$^{CreERT2/+}$ inducible Cre system where also one allele was replaced with *Cre* recombinase linked to estrogen receptor T2 (Cynthia Lilian Andoniadou et al., 2013). I used the *Wnt1*$^{Cre/+}$ strain where Cre expression is under the control of the *Wnt1* enhancer (Danielian et al., 1998) and a *Sox10*$^{iCreERT2/+}$ inducible Cre, where one allele of *Sox10* was replaced with *iCre* recombinase linked to estrogen receptor T2 (Laranjeira et al., 2011). The following reporter strains were used: *R26*$^{mTmG/+}$ where all cells express membrane Tomato until, upon Cre activity, targeted cells excise Tomato and, together with their progeny, express membrane GFP (Muzumdar et al. 2007); *R26*$^{tdTomato/+}$ (Jax #155793) where upon Cre recombination, Tomato is expressed by successfully recombined cells and their progeny. As a model of metabolic disease, I used 10-month-old *Ob/ob* mice which are homozygous null for the gene *Lep*. *Lep* codes for leptin, a hormone that inhibits hunger. *Ob/ob* mice are fed *ad libitum* and therefore show obesity, with associated hyperphagia, hyperglycaemia, glucose intolerance, elevated plasma insulin and increased hormone production from pituitary and adrenal glands (Garris & Garris, 2004; Lindström, 2007).

All mice were bred and maintained on mixed backgrounds and consistently backcrossed on CD1. To generate *Sox2*$^{CreERT2/+}$;*R26*$^{mTmG/+}$ mice, *Sox2*$^{CreERT2/+}$ males were crossed with *R26*$^{mTmG/mTmG}$ females or *Sox2*$^{CreERT2/+}$;*R26*$^{mTmG/mTmG}$ males were crossed with WT females. To generate *Wnt1*$^{Cre/+}$;*R26*$^{mTmG/+}$ mice, *Wnt1*$^{Cre/+}$ males were crossed with *R26*$^{mTmG/mTmG}$ females. To generate *Sox10*$^{iCre/+}$;*R26*$^{mTmG/+}$ mice, *Sox10*$^{iCre/+}$ males were crossed with *R26*$^{mTmG/mTmG}$ females. To generate *Sox2*$^{CreERT2/+}$;*R26*$^{tomato/+}$ mice, *Sox2*$^{CreERT2/+}$ males were crossed with *R26*$^{tdTomato/tdTomato}$ females.

2.1.2 Genotyping and phenotyping

Ear biopsies were obtained from pups after postnatal day 10 and lysed in DNAreleasy (Anachem, 95016898) following manufacturer's recommendations. PCRs were performed using Red Taq (PCR Biosystems, PB10.13) following manufacturer's instructions, using the primers listed in Table 2.1. *Sox2*$^{eGFP/+}$ pups up to postnatal day 4 were phenotyped using a Dual Fluorescent Protein Flashlight (Nightsea) to detect GFP expression in the brain. Cre recombination for lineage tracing was confirmed by observing positive fluorescence of relevant organs with a MZ10 F Stereomicroscope (Leica Microsystems).

Generic Cre (*Sox2*^{CreERT2}, *Wnt1*^{Cre})	Primer 1	GAT GCA ACG AGT GAT GAG GTT CGC
	Primer 2	ACC CTG ATC CTG GCA ATT TCG GC
R26^{mTmG}	Primer 1	CTC TGC TGC CTC CTG GCT TCT
	Primer 2	CGA GGC GGA TCA CAA GCA ATA
	Primer 3	TCA ATG GGC GGG GGT CGT T
Sox10^{iCreERT2}	Primer 1	TGC CCA GAG TCA TCC TTG GC
	Primer 2	GAG GGA CTA CCT CCT GTA CC
Sox2^{eGFP}	Primer 1	CGC TTC CTC GTG CTT TAC G
	Primer 2	GGC TTC TCC TTT TTT TGC AGT
R26^{tdTomato}	Primer 1	CTG TTC CTG TAC GGC ATG G
	Primer 2	GGC ATT AAA GCA GCG TAT CC
	Primer 3	AAG GGA GCT GCA GTG GAG T
	Primer 4	CCG AAA ATC TGT GGG AAG TC

Table 2.1 Primers used to genotype relevant strains.

2.1.3 Tamoxifen administration

Tamoxifen (Sigma, T5648) was injected intraperitoneally with a single dose of 0.15mg/g body weight in postnatal mice. Pregnant females were injected by a single intraperitoneal injection of tamoxifen, capped at 1.5mg and one dose of Progesterone (Sigma P0130) at 0.75mg. Pregnant females were injected by Dr Emily Lodge.

2.1.4 Physiological challenge experiments

Experiments of chronic social defeat stress were performed by Dr Juan Pablo Lopez at the Max Plank Institute of Psychiatry in Munich (Germany), as described in (Lopez, Brivio, Santambrogio, De Donno, et al., 2021) on 7 weeks old C57BL/6N males. Restraint acute stress experiments were performed by Ilona Berger at the Technische Universität Dresden (Germany) on 8 week-old C57BL/6N males, as reported in (Steenblock et al., 2018).

2.2 Tissue processing

2.2.1 Paraffin-embedded tissue

Adrenal glands were dissected using one Dumont Curved-Tip Tweezer #7 and one Dumont Straight-Tip Dumostar Tweezer #5 in 1X PBS, surrounding fat was removed using Wescott Scissors and samples were fixed in 10% neutral buffered formalin (NBF) (Sigma, HT501128) overnight at room temperature (RT, 20-25 °C) whilst rolling. Tissue was embedded manually over three days following the protocol in Table 2.2. Samples were sectioned at 5 µm using a Leica Microtome and mounted on SuperFrost Plus slides (Thermo Fisher, J1830AMNZ) and dried overnight at 40°C.

Day 1		
PBS I	1 hour	RT
PBS II	1 hour	RT
PBS III	1 hour	RT
25% EtOH	1 hour	RT
50% EtOH	1 hour	RT
70% EtOH	1 hour	RT
70% EtOH	Overnight	4 °C
Day 2		
80% EtOH	1 hour	RT
90% EtOH	1 hour	RT
95% EtOH	1 hour	RT
100% EtOH	Overnight	4 °C
Day 3		
100% EtOH	1 hour	RT
Neoclear I	10 minutes	RT
Neoclear II	10 minutes	60 °C
Neoclear : Paraffin 1:1	15 minutes	60 °C
Paraffin I	1 hour	60 °C
Paraffin II	1 hour	60 °C
Paraffin III	1 hour	60 °C

Table 2.2 Paraffin embedding steps.

2.2.2 Cryopreserved tissue

Adrenal glands were dissected in 1X PBS, surrounding fat was removed and samples fixed in 4% PFA at 4°C for 4 hours. Adrenals were washed in 1X PBS for 30 minutes, followed by immersion in 30% Sucrose in 1X PBS overnight at 4°C. Adrenals were embedded in Optical Cutting Temperature (OCT) compound (VWR, 361603E) and flash-frozen in dry ice with 100% Ethanol. Cryopreserved tissues were stored at -80°C. Samples were sectioned at 8-12 µm thickness using a Bright OTF5000 cryostat, mounted on SuperFrost Plus slides and stored at -20°C.

2.3 RNAscope mRNA *in situ* hybridisation

RNAscope was carried out on paraffin-embedded sections with the RNAscope 2.5 HD Duplex Kit (ACD Bio, 322430) following the manufacturer's protocol, with optimised retrieval time of 12 minutes and optimised protease time of 30 minutes. Probes used are listed in Table 2.3. Tissue was counterstained with Hematoxylin QS (Vector Laboratories, H-3404-100) and slides were mounted in VectaMount Permanent Mounting Medium (Vector Laboratories, H-5000-60).

Target	Cat. No
Ctgf	314541
Cyr61	429001
Gfap-C2	313211-C2
Lats2	420271
Plp1	428181
S100b-C2	431731-C2
Sox10	435931
Sox2	401041
Sox2-C2	401041-C2
Wls	405011
Ppib +ve CTRL	313911
DapB -ve CTRL	310043

Table 2.3 RNAscope probes used.
C2 denotes Channel 2 probe (all ACD Bio).

2.4 Immunostaining

2.4.1 Immunohistochemistry

Mounted paraffin sections were deparaffinised with Neoclear (Merk, 109843), 3 times for 10 minutes and rehydrated in a descending ethanol series for 5 minutes per wash as follows: 3 times 100%, 95%, 90%, 80%, 70%, 50%, 25%, distilled water; then washed under running water for 10 minutes. Antigen retrieval was performed with 1X Declere, pH 6 (Cell Marque, 921P-04) in a Decloaking Chamber NXGEN (Menarini Diagnostics, DC2012-220V) at 110°C for 3 minutes. Slides were allowed to cool down to room temperature and then washed twice for 2 minutes in 1X PBS 0.1% Triton X-100 (Sigma, X100) (PBST) with agitation. ImmPRESS Excel Amplified HRP Polymer Staining Kit Anti-Rabbit IgG (Vector Laboratories, MP-7602-50) was used following the manufacturer's instructions. Primary antibodies were used at the concentrations listed in Table 2.4. Nuclei were stained with Vector Hematoxylin QS (Vector Laboratories, H-3404-100) and slides were mounted in VectaMount Permanent Mounting Medium (Vector Laboratories, H-5000-60).

2.4.2 Immunofluorescence on paraffin-embedded sections

Mounted paraffin sections were deparaffinised with and rehydrated in a descending ethanol series for 5 minutes per wash as follows: 3 times 100%, 95%, 90%, 80%, 70%, 50%, 25%, distilled water; then washed under running water for 10 minutes. Antigen retrieval was performed with 1X Dako Target Retrieval Solution, pH 9 (Agilent, S236784-2) in a Decloaking Chamber NXGEN (Menarini Diagnostics, DC2012-220V) at 110°C for 3 minutes. Slides were allowed to cool down to room temperature and then washed twice

for 2 minutes in PBST with agitation. Sections were blocked for 1 hour at room temperature in blocking buffer (0.15% glycine, 2mg/ml BSA, 0.1% Triton X-100 in PBS) with 10% sheep serum. Primary antibodies were diluted in blocking buffer with 1% sheep serum at the concentrations described in Table 2.4 were incubated overnight at 4°C. After washing 3 times for 5 minutes in PBST with agitation, samples were incubated for 1 hour at room temperature in secondary fluorophore-conjugated antibodies (dilution 1:500, listed in Table 2.5) and Hoechst (Life Technologies, H3570) (dilution 1:10,000) in blocking buffer with 1% serum. Tyrosine Hydroxylase and PNMT antibodies were amplified with biotin-streptavidin by incubating at room temperature for 1 hour with anti-mouse biotinylated secondary antibody (dilution 1:300, listed in Table 2.5) and Hoechst (Life Technologies, H3570) (dilution 1:10,000), washed 3 times for 5 minutes in PBST with agitation and incubated at room temperature for 1 hour with fluorescent-labelled streptavidin (dilution 1:500, listed in Table 2.5). Before mounting, slides were washed 3 times for 5 minutes with agitation in PBST. Coverslips were mounted with Vectashield Antifade Mounting Medium (Vector Laboratories, H-1000-10).

2.4.3 Immunofluorescence on cryosections

Frozen pre-fixed sections in OCT were air-dried for 10 minutes and washed for twice for 2 minutes in PBST with agitation at room temperature. Sections were blocked for 1 hour at room temperature in blocking buffer (1% BSA, 0.1% Triton X-100, 5% goat serum). Primary antibodies were diluted in blocking buffer at the concentrations shown in Table 2.4 and incubated overnight at 4°C. After washing 3 times for 5 minutes in PBS with agitation, secondary fluorophore-conjugated antibodies (dilution 1:500, listed in Table 2.5) and Hoechst (Life Technologies, H3570) (dilution 1:10,000) were diluted in in blocking buffer were incubated for 1 hour at room temperature. Before mounting, slides

were washed 3 times for 5 minutes in PBS with agitation. Coverslips were mounted with Vectashield Antifade Mounting Medium (Vector Laboratories, H-1000-10).

2.4.4 Immunofluorescence on cells

For immunofluorescence on cells, coverslips were washed twice in PBS and fixed with 4% PFA on ice for 10 minutes. After washing 3 times for 5 minutes in PBST, cells were blocked for 1 hour at room temperature in blocking buffer (0.15% glycine, 2mg/ml BSA, 0.1% Triton X-100 in PBS) with 10% sheep serum. Primary antibodies were incubated overnight at 4°C in blocking buffer with 1% sheep serum at the concentrations shown in Table 2.4. After washing 3 times with PBST, sections were incubated for 1 hour at room temperature in secondary fluorophore-conjugated antibodies, diluted 1:500 (listed in Table 2.5) in blocking buffer with 1% serum. After washing 3 times in PBST, coverslips were mounted with Vectashield HardSet Antifade Mounting Medium with DAPI (Vector Laboratories, H-1500-10).

Antibody	Species	Supplier	Protocol/ Concentration
Beta Tubulin III	Mouse monoclonal	Abcam ab78078	IF-C/1:300
Endomucin	Rat monoclonal	Abcam ab106100	IF-F/1:300
GFAP	Rabbit polyclonal	Dako Z0334	IF-F/1:500
GFAP	Chicken polyclonal	Antibodies.com A85307	IF-F/1:1000
GFP	Chicken polyclonal	Abcam ab13970	IF-P/1:300 IF-F/1:300 IF-C 1:100
Ki67	Rabbit polyclonal	Abcam ab15580	IHC/1:500
Ki67 (SolA15)	Rat monoclonal	eBioscience 14-5698-82	IF-F/1:300
Nestin	Chicken polyclonal	Novus NB100-1604	IF-F/1:1000
PENK	Rabbit polyclonal	ABclonal Technology A6302	IF-P/1:300
PNMT	Mouse monoclonal	Santa Cruz sc-393995	IF-P/1:300
S100b (EP1576Y)	Rabbit monoclonal	Abcam ab52642	IF-F/1:300
SOX10 (SD204-04)	Rabbit monoclonal	Novus NBP2-67812	IF-F/1:300
SOX2 (EPR3131)	Rabbit monoclonal	Abcam ab92492	IHC/1:800 IF-F/1:300 IF-C 1:100
Tyrosine Hydroxylase	Mouse monoclonal	BD Biosciences 612300	IF-P/1:300
Tyrosine Hydroxylase	Rabbit polyclonal	Abcam ab6211	IF-F/1:300 IF-C/1:300

Table 2.5 Primary antibodies list.
IHC = immunohistochemistry, IF-P = Immunofluorescence on paraffin embedded sections, IF-F = immunofluorescence on frozen sections, IF-C = immunofluorescence on cells.

Antibody	Species	Suppplier	Concentration
Anti-Chicken Alexa Fluor 488	Goat polyclonal	Invitrogen A-11039	1:500
Anti-Chicken Alexa Fluor 633	Goat polyclonal	Invitrogen A-21103	1:500
Anti-Mouse Alexa Fluor 488	Goat polyclonal	Abcam ab150113	1:500
Anti-Mouse Biotinylated	Goat polyclonal	Abcam ab6788	1:300
Anti-Rabbit Alexa Fluor 488	Goat polyclonal	Invitrogen A-11008	1:500
Anti-Rabbit Alexa Fluor 594	Goat polyclonal	Abcam ab150080	1:500
Anti-Rabbit Alexa Fluor 633	Goat polyclonal	Invitrogen A-21070	1:500
Anti-Rat Alexa Fluor 647	Goat polyclonal	Invitrogen A-21247	1:500
Streptavidin Alexa Fluor 555	Goat polyclonal	Invitrogen S32355	1:500

Table 2.4 Secondary antibodies list

2.5 Tissue culture techniques and assays

2.5.1 Primary cell culture

Adrenal glands were dissected as described in section 2.2.1, and the adrenal medulla isolated manually using two Dumont Straight-Tip Dumostar Tweezer #5. Medullae were placed in an enzymatic digestion mix containing 50 µg/ml DNAse I (Sigma, D5025), 10 mg/ml Collagenase II (Worthington, LS004177), 2.5µg/ml Fungizone (Gibco, 15290026), 0.1X Trypsin-EDTA (Sigma, 59418C) in 1X Hank's Balanced Salt Solution (HBSS) (Gibco, 14025050). The enzyme mix was activated in a water bath at 37°C and after 10 minutes adrenals were triturated by pipetting up and down 100 times with a P1000 pipette, then put back in the water bath. After 5 minutes, medullas were triturated again as above to ensure a single-cell suspension, and enzymes inactivated by addition of 10 times volume of serum-containing Base Media: DMEM/F-12 (Gibco, 31330-038) + 5% FBS (Merk, F0804) + 50U/ml Penicillin-Streptomycin (Gibco, 15070063). The cell suspension was centrifuged at 281g for 5 minutes at room temperature and resuspended in PBS, centrifuged again and washed in PBS, before being resuspended in Complete Media: Base Media + 20ng/ml bFGF (R&D Systems, 234-FSE) + 50µg/ml cholera toxin (Sigma, C8052). Two days after isolation, an equal volume of fresh media was added to each plate. Media were fully changed every 2-3 days. For immunostaining, cells were plated on glass coverslips coated with 0.1% gelatine diluted in PBS.

2.5.2 Passaging primary adherent adrenal medulla cell cultures

Cells were washed with PBS once and treated with pre-warmed 0.25% Trypsin-EDTA (Sigma, 25200-056) for 3 minutes at 37°C. Trypsin was inactivated with the addition of

Base Media, cells were centrifuged at 281g and resuspended in Complete Media for plating.

2.5.3 Flow Sorting

Adrenal glands from *Sox2*$^{eGFP/+}$ mice were dissected and tissue was dissociated as described for primary cell culture. At the last step, cells were resuspended in FACS buffer (2.5% HEPES solution 1M (Sigma), 1% FBS in PBS), passed through a 40µm cell strainer (Corning, CLS431750) and stained with DAPI 0.05µg/ml (Biolegend, 422801), before being flow sorted by a FACSAria Cell Sorter (BD Biosciences) with gating strategy as per Figure 2.1. Wild Type littermates were used as a negative control. Flow sorting was performed by staff of the BRC flow core at KCL. Cells were collected in 1.5ml tubes containing 50µl of Complete Media. After sorting, cells were directly plated in Complete

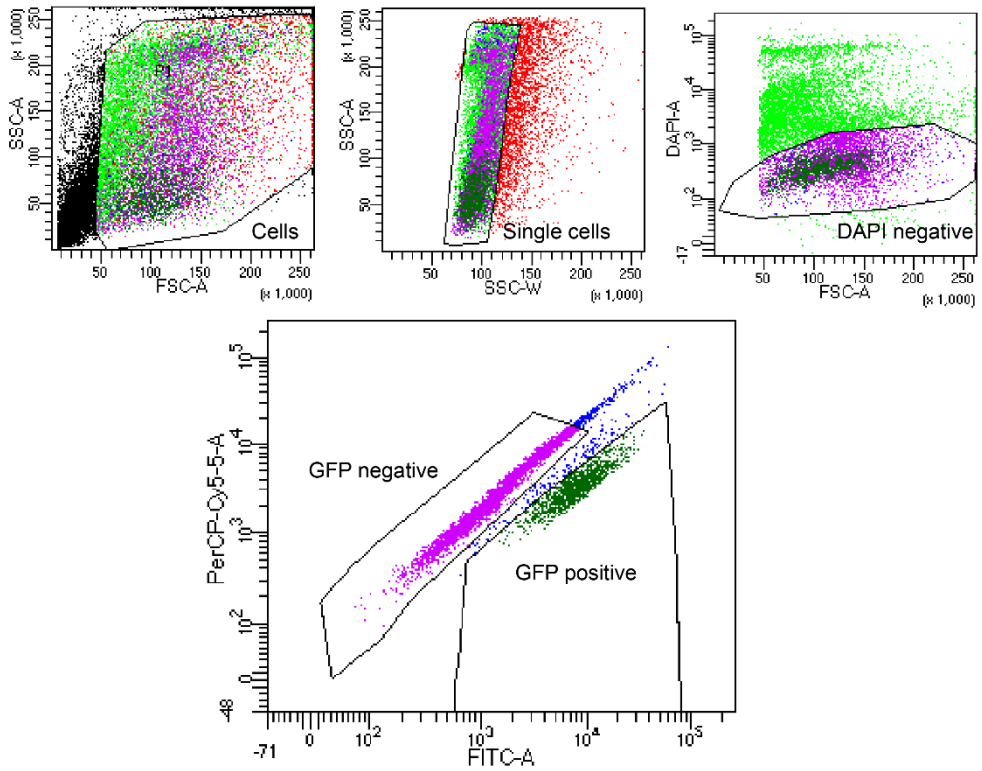

Figure 2.1 FACS gating strategy for *Sox2*$^{eGFP/+}$ adrenal medulla primary cells.

Media.

2.5.4 Colony Forming Assay

For colony forming assays, adrenals from *Sox2*$^{eGFP/+}$ mice were dissected and tissue was dissociated as described for primary cell culture. GFP+ and GFP- cells were separated by flow sorting. After sorting, GFP+ and GFP- cells were plated at clonal density of 500 cells/well in a 12-well plate. Two days after isolation, an equal volume of medium to the one present in the plate was added. After that, media were changed every 2-3 days.

After 14 days of culture, cells were washed 3 times in PBS and fixed with 10% NBF for 10 minutes at room temperature. After washing 3 times with PBS, cells were stained for 10 minutes with Crystal Violet Solution: 0.5% Crystal Violet powder (Sigma, C0775), 20% methanol in distilled water. Excess crystal violet was washed with running tap water and plates dried before colony observation and imaging.

2.6 Imaging and Analysis

2.6.1 Image acquisition and processing

Images of immunofluorescence staining were taken with a Leica TCS SP5 confocal microscope, using an HCX Plan-Apochromat CS 20x/0.7 dry objective and an HCX Plan-Apochromat CS 63x/1.3 Glycine objective (both Leica Microsystems). Stacks of 0.5µm were taken through the entire section thickness. Immunohistochemistry and RNAscope stained sections were scanned with a Nanozoomer-XR Digital slide scanner (Hamamatsu), close-up images were taken with an Olympus BX34F Brightfield microscope using a 40X objective. Cell culture images were taken with an Olympus Phase Contrast microscope using a 4X or 20X objective.

Images were processed with Fiji (Schindelin et al., 2012) and with Nanozoomer Digital Pathology View. Final figures were created in Adobe Illustrator.

2.6.2 Analysis

Cell counting was performed manually with Fiji's "Cell Counter" plugin. For mouse samples, a minimum of three sections per sample were counted. For human samples, 5 representative fields were selected at 20X magnification for each sample and counted. Statistical analysis and graphs (except for single cell RNA sequencing analysis) were produced using GraphPad Prism.

2.7 Human samples

Pheochromocytoma samples were obtained with full informed consent and KCL ethical approval LRS-19/20-20118, from patients at St Bartholomew's Hospital, University College London Hospital (London, UK), National Hospital for Neurology and Neurosurgery (London, UK). Normal adrenal tissue samples were obtained from the Uniklinikum Würzburg (Germany). Normal adrenal glands removed as part of tumour nephrectomy and proven to be histologically normal, showing no neoplastic tissue. Samples were provided by Prof Paul Chapple at Queen Mary University London, Dr Nicole Bechmann at Uniklinikum Carl Gustav Carus Dresden and Prof Martin Fassnacht at the University of Würzburg.

2.8 2.8. Single Cell RNA Sequencing

2.8.1 Tissue Preparation for Whole Medulla RNA-Seq Dataset

10 adrenals from 5 mixed sex P15 *Sox2*$^{eGFP/+}$ mice were dissected on ice and dissociated as previously described. Pellets were resuspended in a solution of HBSS 2.5% FBS, an aliquot of 10,000 viable cells were used for the experiment.

2.8.2 Tissue Preparation for Sox2-enriched Dataset

30 adrenals from 15 mixed sex P15 *Sox2*$^{eGFP/+}$ mice were dissected on ice, dissociated, and GFP+ cells were isolated via FACS as described, centrifuged at 300 g for 5 minutes and resuspended in a solution of HBSS 2.5% FBS. 2,000 viable cells were used for the experiment.

2.8.3 Library Preparation and Sequencing

Library preparation and sequencing were performed by the BRC Genomics Core at KCL. Cells from experiments described in 8.1 and 8.2 were processed singularly at different times. Library preparation was done using the Chromium 10X Single Cell 3' Reagent Kit v3 (10X Genomics) and a Chromium Controller (10X Genomics) following the manufacturer's protocol. Once obtained, barcoded transcripts from single cells were sequenced with an Illumina HiSeq 2500.

2.8.4 Computational Analysis

Pre-processing of the sequencing datasets was performed by the BRC Genomics Core at KCL using Cell Ranger. Once raw matrices were obtained, I performed the analysis of both datasets in RStudio with the Seurat package, v3 and 4 (Hao et al., 2021; Stuart et al., 2019), following tutorial instructions.

For the "Whole Medulla", the dataset was subset to exclude cells with <500 or >5000 genes or with >20% mitochondrial genes. After normalisation, the 2000 most variable features were identified, dataset was scaled and PCA was estimated based on the previously identified variable features. A UMAP was generated using the top 10 PCs and a resolution of 0.4. Clusters were identified based on literature (described in Chapter 3), raw counts from clusters showing medulla-specific markers were extracted and the matrix re-analysed with the same parameters. This allowed to resolve clustering further and select the final "Medulla Only" dataset, which was once again re-analysed with the same parameters. Cell cycle analysis was performed using the CellCycleScoring function in Seurat, following tutorial instructions.

For the "Sox2-enriched" dataset, cells with <1000 or >5000 genes or with >10% mitochondrial genes were excluded. After normalisation, the 2000 most variable features were identified, the dataset was scaled and PCA was estimated based on the previously identified variable features. A UMAP was generated using the top 10 PCs and a resolution of 0.4. To produce the "Sox2 only" sub-set, I used the WhichCell function to select only cells with *Sox2* normalised expression >1. Once extracting the raw counts from these cells, I re-analysed the dataset with the same parameters. Cell communication analysis was performed using the CellChat package (Jin et al., 2021), following tutorial instructions.

For Velocity analysis (La Manno et al., 2018), output BAM files were processed using the Python implementation of the RNA Velocity package (velocyto.py). In brief, sorted BAM files obtained as standard output from the cell ranger pipeline were counted using the "run10x" function of velocyto.py. The resulting loom files were loaded into RStudio and further processed using the Seurat pipeline. Seurat was also used to cluster the cells and assign cell type identities. Using the SeuratWrappers package, the analysed datasets were exported as loom files and imported into python. Using scVelo (La Manno et al., 2018), velocities were projected onto the Seurat UMAP embedding and further analysed to corroborate the results obtained from Seurat.

3 *In silico* study of the postnatal adrenal medulla cell composition

3.1 Introduction

Cell composition and cell hierarchy of the adrenal medulla have been widely characterised during embryonic development, but little is known about the postnatal medulla. The main cell types identified in the mouse postnatal adrenal medulla are adrenaline or noradrenaline producing chromaffin cells and sustentacular cells. Postganglionic neuronal cell bodies were identified in the rat adrenal medulla. All chromaffin cells express specific enzymes necessary for their function: tyrosine hydroxylase (TH), aromatic amino-acid decarboxylase (DDC), dopamine β-hydroxylase (DBH), adrenaline producing chromaffin cells also express phenylethanolamine N-methyl transferase (PNMT), which is responsible for the conversion of noradrenaline to adrenaline (A. A. J. Verhofstad et al., 1985). Most of postnatal adrenal medulla studies focus on the physiological function of chromaffin cells and mechanisms of catecholamine biosynthesis and secretion. Investigation of the postnatal molecular profile, signalling and transcriptional heterogeneity of chromaffin cells could provide significant insights on any hierarchy, cell communication, signalling and function. Intramedullary neurons of the postnatal adrenal have been observed in rat *in vivo* and described based on their morphological aspects. Sustentacular cells express are supporting cells of glial nature that express S100B, GFAP, SOX10, Nestin and Vimentin. Sustentacular cells present an expression profile similar to that of embryonic SCPs, and stress-responsive progenitors have been identified as Nestin-expressing cells (Rubin De Celis et al., 2015), pointing towards the hypothesis that these cells might also play a progenitor role in normal homeostasis (Kastriti et al., 2020). Primary chromaffin cells are

capable to differentiate into neurons when given specific signals *in* vitro (Vukicevic, Schmid, et al., 2012), indicating a certain plasticity within the adrenal medulla. During embryonic development, it has been shown that SCPs, which share markers with sustentacular cells, differentiate towards a chromaffin cell fate passing through a transitionary state, and that chromaffin cells can then give rise to postganglionic neurons in the developing medulla (Furlan et al., 2017; Kameneva, Artemov, et al., 2021). The nature of these postganglionic neurons is not well-defined and it is extremely difficult to discriminate between these and noradrenaline producing chromaffin cells, given that intramedullary neurons express all key enzymes necessary for catecholamine production except for PNMT and have a noradrenergic phenotype (A. Dagerlind et al., 1990), leaving an open question whether these are two different populations. In order to proceed with a further understanding of cell hierarchy and cell composition of the adrenal medulla, it is important to determine the cell populations that make up the gland. Single cell RNA sequencing techniques have been pivotal in producing atlases of multiple organs and tissues (Schaum et al., 2018), however no publications have successfully performed single cell RNA sequencing of the postnatal adrenal medulla with a big enough number of cells to be able to analyse adrenomedullary populations. In the following chapter I will produce the first in-depth atlas of the mouse adrenal medulla.

3.2 Results

3.2.1 Elucidating cell hierarchy of the postnatal medulla using single cell RNA sequencing

To gain insights on the cell composition of the adrenal medulla, I performed 10X single cell RNA sequencing on 10 adrenal glands from 5 mice at P15, which coincides with a period of rapid postnatal growth of the medulla. In brief, adrenals were dissected, excess cortex manually removed, and organs dissociated to a single cell suspension. Library preparation for 10X Genomics single cell RNA sequencing and Cell Ranger analysis were performed by the KCL Genomics facility. Using the Seurat package in RStudio, after excluding low quality cells as described in Materials and Methods, 9961 cells remained, which were included in the analysis (Figure 3.1A). Unsupervised clustering revealed 18 clusters representative of 18 different transcriptional signatures (Figure 3.1B, C). Top differentially expressed genes for each cluster are shown in Table 3.1. To determine cluster identity, I used known markers of cell types composing the adrenal gland: *Cxcl2*, *C1qc* for immune cells, *Emcn*, *Cd34* for endothelial cells, *Star*, *Nr5a1* for cortex cells, *Chga*, *Th*, *Pnmt* for chromaffin cells, *Sox10*, *S100b*, *Gfap* for sustentacular cells *Mki67*, *Top2a* for cycling cells. Expression of these markers (Figure 3.1D) allowed identification of the clusters and exclusion of cortex, endothelial and immune cells. Endothelial and immune cells were not considered in the analysis of adrenal medulla because impossible to discriminate whether these cells belong to cortex or medullary tissue.

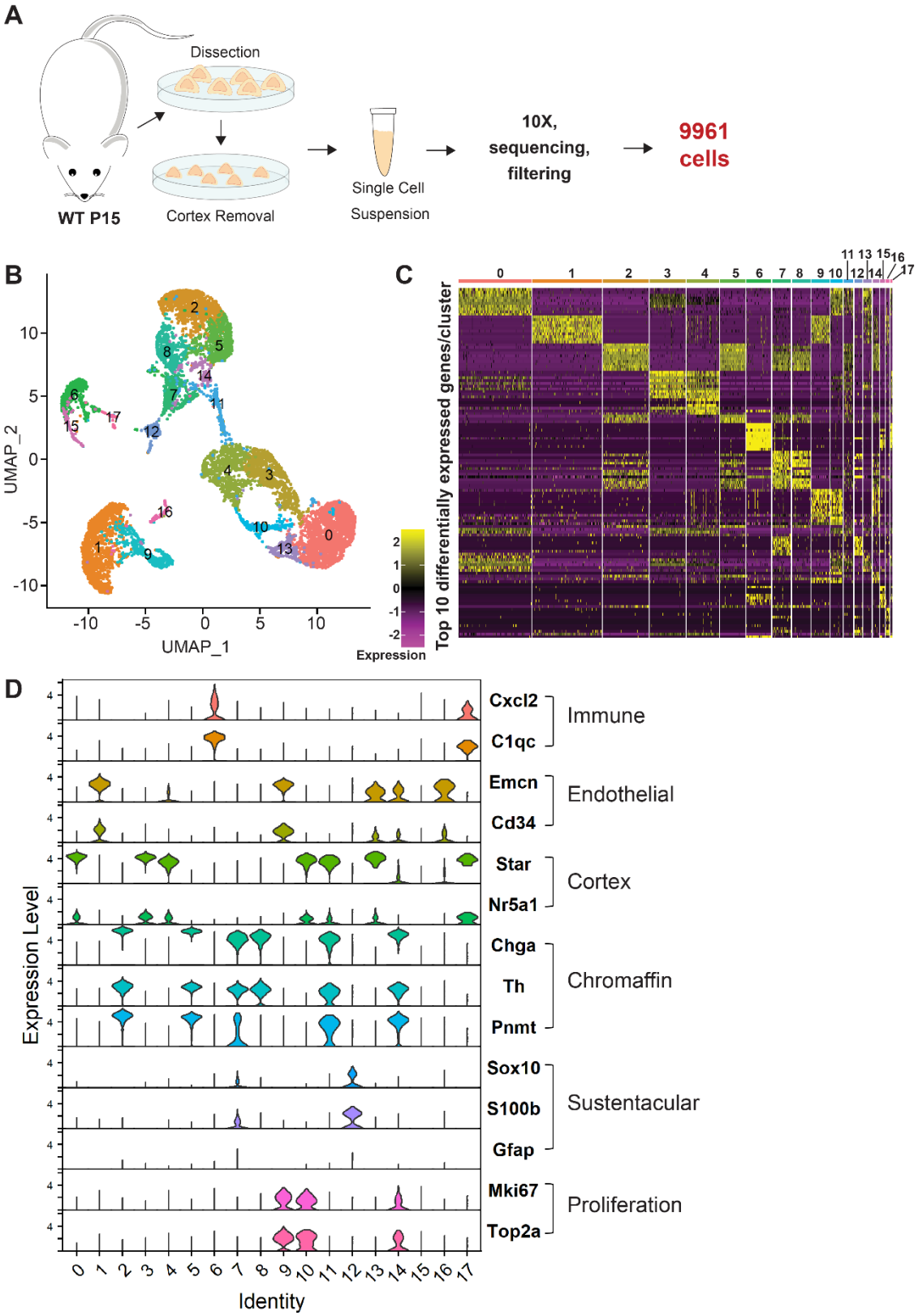

Figure 3.1 Single cell RNA sequencing of the postnatal medulla – "whole" dataset. A) Experimental strategy; B) UMAP of the entire dataset obtained (9961 cells); C) Heatmap showing the transcriptional signatures of 18 clusters; Top 10 genes of each cluster are listed in Table 3.1. C) Violin plots indicating expression of different markers to identify each cluster.

Cluster	Gene	Cluster	Gene	Cluster	Gene	Cluster	Gene	Cluster	Gene	Cluster	Gene
0	*Apoc1*	3	*Cyp11b1*	6	*C1qa*	9	*Hmgb2*	12	*Cryab*	15	*Ccl5*
0	*Akr1c18*	3	*Cdkn1c*	6	*C1qb*	9	*Top2a*	12	*Scn7a*	15	*Cd52*
0	*Abcb1b*	3	*Cyp21a1*	6	*Cd74*	9	*Mki67*	12	*Plp1*	15	*Plac8*
0	*Me1*	3	*Hao2*	6	*C1qc*	9	*Prc1*	12	*Fbln5*	15	*Nkg7*
0	*Mgst1*	3	*Hsd3b1*	6	*Lyz2*	9	*Pclaf*	12	*Plce1*	15	*Ptprc*
0	*Akr1cl*	3	*Akr1b7*	6	*Apoe*	9	*Hist1h1b*	12	*Apod*	15	*Coro1a*
0	*Cyp2f2*	3	*Cyp11a1*	6	*Tyrobp*	9	*Birc5*	12	*Vim*	15	*Arhgdib*
0	*Nrep*	3	*Mc2r*	6	*Ctss*	9	*Nusap1*	12	*Pmepa1*	15	*Ifi27l2a*
0	*Fdps*	3	*Aebp1*	6	*Fcer1g*	9	*Cenpf*	12	*Fxyd1*	15	*Lyz2*
0	*Ankrd55*	3	*Mgarp*	6	*Rgs1*	9	*Tubb5*	12	*Cst3*	15	*S100a6*
1	*Fabp4*	4	*Ly6d*	7	*Cox8b*	10	*Cenpm*	13	*Abcb1b*	16	*Mgp*
1	*Gpihbp1*	4	*Ecrg4*	7	*Ncam1*	10	*Birc5*	13	*Akr1c18*	16	*Tagln*
1	*Rgcc*	4	*Wnt4*	7	*Cryab*	10	*Mki67*	13	*Nrep*	16	*Dcn*
1	*Emcn*	4	*Kcnk3*	7	*Nnat*	10	*Pclaf*	13	*Pik3c2g*	16	*Col1a2*
1	*Fam167b*	4	*Dab2*	7	*Stmn2*	10	*Cenpf*	13	*Me1*	16	*Ogn*
1	*Egfl7*	4	*Aebp1*	7	*Epha5*	10	*Top2a*	13	*Abhd2*	16	*Ace2*
1	*Plvap*	4	*Cyp11a1*	7	*Pmepa1*	10	*Prc1*	13	*Idh1*	16	*Cfh*
1	*Ramp2*	4	*Cdkn1c*	7	*Gal*	10	*Ccdc34*	13	*Star*	16	*Col1a1*
1	*Ednrb*	4	*Cyp11b1*	7	*Dbh*	10	*H2afz*	13	*Idi1*	16	*Bgn*
1	*Ctla2a*	4	*Cpe*	7	*Cst3*	10	*Akr1c18*	13	*Emb*	16	*Igfbp7*
2	*Pnmt*	5	*Chga*	8	*Cox8b*	11	*Th*	14	*Spc25*	17	*Ms4a6c*
2	*Chga*	5	*Scg2*	8	*Nnat*	11	*Akr1cl*	14	*Nusap1*	17	*C1qc*
2	*Cfap61*	5	*Pnmt*	8	*Ndufa4l2*	11	*Dbh*	14	*Cenpf*	17	*Ctss*
2	*Dlk1*	5	*Meg3*	8	*Epha5*	11	*Rian*	14	*Prc1*	17	*C1qb*
2	*Chgb*	5	*Npy*	8	*Ddc*	11	*Adh1*	14	*Mt2*	17	*C1qa*
2	*Scg2*	5	*Rab3c*	8	*Stmn2*	11	*Mgst1*	14	*Npy*	17	*Tyrobp*
2	*Npy*	5	*Th*	8	*Gpr22*	11	*Clu*	14	*Top2a*	17	*Lyz2*
2	*Th*	5	*Dlk1*	8	*Gal*	11	*Ly6d*	14	*Stmn1*	17	*Fcer1g*
2	*Pcsk1n*	5	*Peg3*	8	*C1ql1*	11	*Cyp2f2*	14	*Hmgb2*	17	*Cd74*
2	*Resp18*	5	*Chgb*	8	*Chgb*	11	*mt-Nd6*	14	*Cks2*	17	*Apoe*

Table 3.1 Top 10 differentially expressed genes for the 18 identified clusters in "whole" dataset.

By subsetting and re-clustering, I obtained a "medulla only" dataset composed of 2708 cells including sustentacular and chromaffin cells, as well as proliferating cells within those populations (Figure 3.2A). Unsupervised clustering of these cells revealed 8 cell clusters determined by 8 transcriptional signatures (Figure 3.2B, C). Top differentially expressed genes for each cluster are shown in Table 3.2. Cluster identity was assigned using known markers of the different cell types. *Chga*, *Th*, *Ddc*, *Dbh* indicate all chromaffin cells, adrenaline-producing and noradrenaline-producing chromaffin cells were identified by the expression of *Pnmt*, which is necessary only for adrenaline production. *Sox10*, *S100b* and *Gfap* indicate sustentacular cells, while markers of proliferation *Mki67* and *Top2a* were used to identify proliferating cells. Expression of these markers (Figure 2D) allowed identification of 8 clusters as follows: 0 – adrenaline-producing chromaffin cells (AdrChr), 1 – adrenaline-producing chromaffin cells (AdrChr), 2 – noradrenaline-producing chromaffin cells (NorAdrChr), 3 – sustentacular cells transitioning to noradrenaline-producing chromaffin cells (T-NorAdrChr), 4 – sustentacular cells, 5 – adrenaline-producing chromaffin cells (AdrChr), 6 – sustentacular cells transitioning to adrenaline-producing chromaffin cells (T-AdrChr), 7 – cycling adrenaline-producing chromaffin cells (Cycling AdrChr). Both the transitioning clusters as chromaffin cells express known markers of sustentacular cells. The presence of these intermediate cell types or cell states was previously not described and is consistent with literature reports of sustentacular cells being able to differentiate towards chromaffin cells *in vitro.* These findings lead to a strong indication that sustentacular cells could have a stem/progenitor cell role. Interestingly, I was not able to identify a separate cluster of neurons, indicating that the transcriptional overlap within this population and the adrenaline-producing chromaffin cells is extended enough for these two putatively different cell types to be considered one. For the scope of this thesis, I will refer to these cells as noradrenaline-producing chromaffin cells.

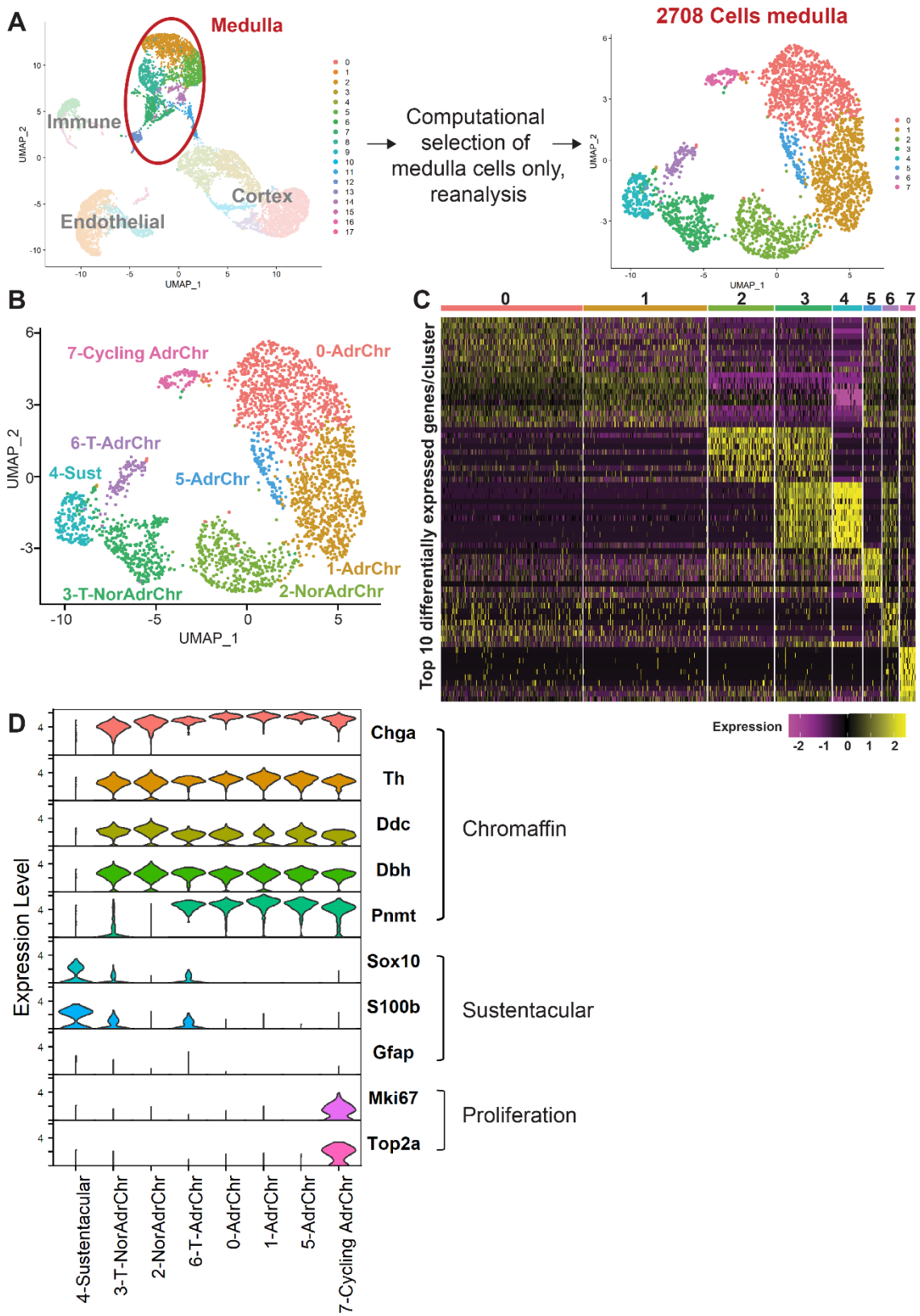

Figure 3.2 Single cell RNA sequencing of the postnatal medulla – "medulla only" dataset.
A) Subsetting strategy; B) UMAP of selected medulla cell types (2708 cells); B) Heatmap showing the transcriptional signatures of 8 clusters; Top 10 genes of each cluster are listed in Table 3.2. D) Violin plots indicating expression of different markers to identify each cluster.

Cluster	Gene	Cluster	Gene	Cluster	Gene	Cluster	Gene
0	*Cplx2*	2	*Cox8b*	4	*Timp3*	6	*Timp3*
0	*Gata2*	2	*Nnat*	4	*Igfbp7*	6	*Ly6h*
0	*Rab3c*	2	*Lix1*	4	*Fxyd1*	6	*Pmepa1*
0	*Tmem130*	2	*Penk*	4	*Pmepa1*	6	*Agrp*
0	*Pcdh10*	2	*Epha5*	4	*Sparc*	6	*Prph*
0	*Slc24a2*	2	*Myl1*	4	*Apoe*	6	*Hhip*
0	*Alcam*	2	*Aldh1a3*	4	*Col4a1*	6	*Ccnd1*
0	*Dock1*	2	*Zdbf2*	4	*Vim*	6	*Scgn*
0	*Pclo*	2	*Igfbp5*	4	*Dbi*	6	*Snhg11*
0	*Gm42418*	2	*Gal*	4	*Cst3*	6	*Klf9*
1	*Pnmt*	3	*Timp3*	5	*Atf3*	7	*Cenpf*
1	*Cfap61*	3	*Sparc*	5	*Fosb*	7	*Birc5*
1	*Tmsb10*	3	*Apoe*	5	*Jun*	7	*Top2a*
1	*Dlk1*	3	*Apod*	5	*Junb*	7	*Mki67*
1	*Resp18*	3	*Pmepa1*	5	*Ier2*	7	*Cks2*
1	*Pcsk1n*	3	*Ifitm3*	5	*Fos*	7	*Nusap1*
1	*Mt1*	3	*Cst3*	5	*Egr3*	7	*Prc1*
1	*Ramp1*	3	*Cox8b*	5	*Dusp1*	7	*Hmgb2*
1	*Mt2*	3	*Nnat*	5	*Btg2*	7	*Stmn1*
1	*C1qtnf4*	3	*Epha5*	5	*Dnajb1*	7	*H2afz*

Table 3.2 Top 10 differentially expressed genes for the 18 identified clusters in "medulla only" dataset.

Cell cycle regression analysis *in silico* (Figure 3.3A, B) reveal that 23,6% (641 cells) within the medullary cell types analysed are in phase G2/M of the cell cycle, suggesting the organ is active and proliferative at this age. *In vivo* validation with immunofluorescence staining on WT P15 samples shows expression of Ki67 by 8.87% of medullary cells (Figure 3.3C, D), indicating the adrenal medulla is an actively proliferating organ, and confirming this age is appropriate to investigate cell hierarchy.

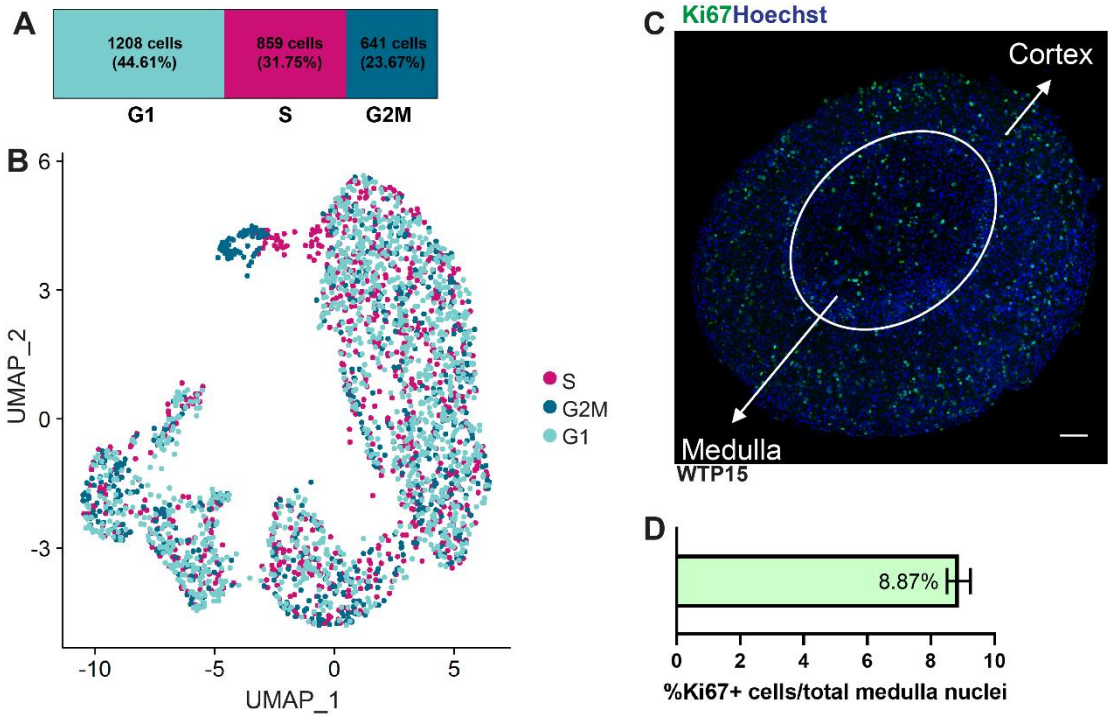

Figure 3.3 Cell Cycle analysis of "medulla only" dataset and validation.
A) Computationally identified percentages of medullary cells in different cell cycle phases: 44.61% (1208 cells) are in G1, 31.75% (859 cells) are in S, 23.67% (641 cells) in G2/M. B) UMAP showing the distribution of cell cycle states over the dataset. C) Immunofluorescence of WT P15 adrenals with antibodies anti-Ki67 (green), nuclei stained with Hoechst, scale bar 50µm. D) Quantification of %K67 cells in the adrenal medulla at P15: 8.87%, n = 4, plotted mean and SEM.

3.2.2 Identification of novel noradrenaline/adrenaline producing chromaffin cell markers

Using the "medulla only" single cell RNA sequencing dataset, I investigated the distribution of known markers of chromaffin cells and aimed to identify new markers. Feature plots in Figure 3.4A confirm chromaffin cell markers *Chga*, *Th*, *Ddc*, *Dbh* are expressed in all clusters except the sustentacular cluster and *Pnmt* expression is restricted to the adrenaline-producing chromaffin clusters (Figure 3.4B). *Pnmt* expression was used to discriminate between adrenaline- and noradrenaline-producing chromaffin cells, therefore I determined whether analysis of the dataset could reveal positive markers of noradrenaline-producing chromaffin cells. I analysed differentially expressed genes and identified the ones restricted to either adrenaline- or noradrenaline-producing chromaffin cells and selected genes expressed by >70% of the cells considered. Adrenaline-producing chromaffin cell markers include *Gata2*, *Tmeff2*, *Hand1*, *Pcdh10*, *Isl1*, *Scgn*, *Sema5a*, *Kcnip1*, *Lrp1b*, *Fxyd2* and *Pcnx* (Figure 3.4B), while noradrenaline-producing chromaffin cell markers include *Cox8b*, *Lix1* and *Penk* (Figure 3.4D). The adrenaline-producing chromaffin cells express the general neuroendocrine marker *Scgn* and they present a specific set of channels and receptors: *Tmeff2*, a transmembrane protein with EGF-like and follistatin-like domains involved in MAPK signalling and cancer in other endocrine organs (H. Han et al., 2019); *Pcdh10*, a cadherin-related neuronal receptor; *Kcnip1*, a potassium channel, *Lrp1b*, a member of the low density lipoprotein (LDL) receptor family; *Fxyd2*, an ion transport regulator. Adrenaline-producing chromaffin cell markers express transcription factors relevant in embryonic development of the adrenal medulla: *Gata2* (Tsarovina et al., 2004) and *Isl1* (Huber et al., 2013) are involved in chromaffin cell differentiation and *Isl1* was identified as a marker of sympathoblasts (Kameneva, Artemov, et al., 2021); *Hand1* is a transcription factor driving sympathetic neurogenesis (Vincentz et al., 2012) and expressed in committing and chromaffin cells in embryonic development (Furlan et al.,

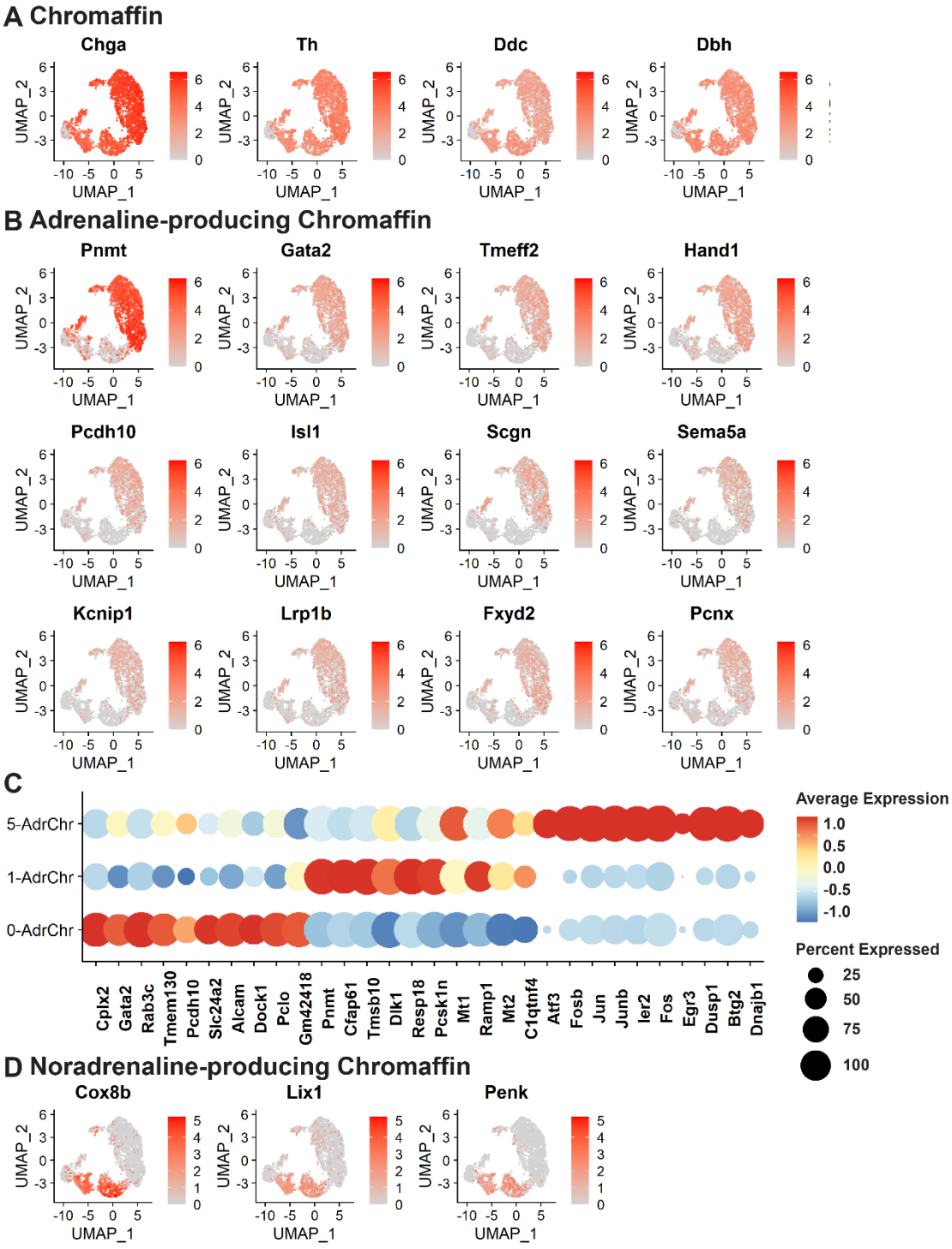

Figure 3.4 Chromaffin cell markers.
A) Feature plots of known chromaffin cell markers. B) Feature plots of newly identified markers of adrenaline-producing chromaffin cells. C) Dot plot showing differences amongst Cluster 0, 1, 5 of adrenaline-producing chromaffin cells. D) Feature plots of newly identified markers of noradrenaline-producing chromaffin cells.

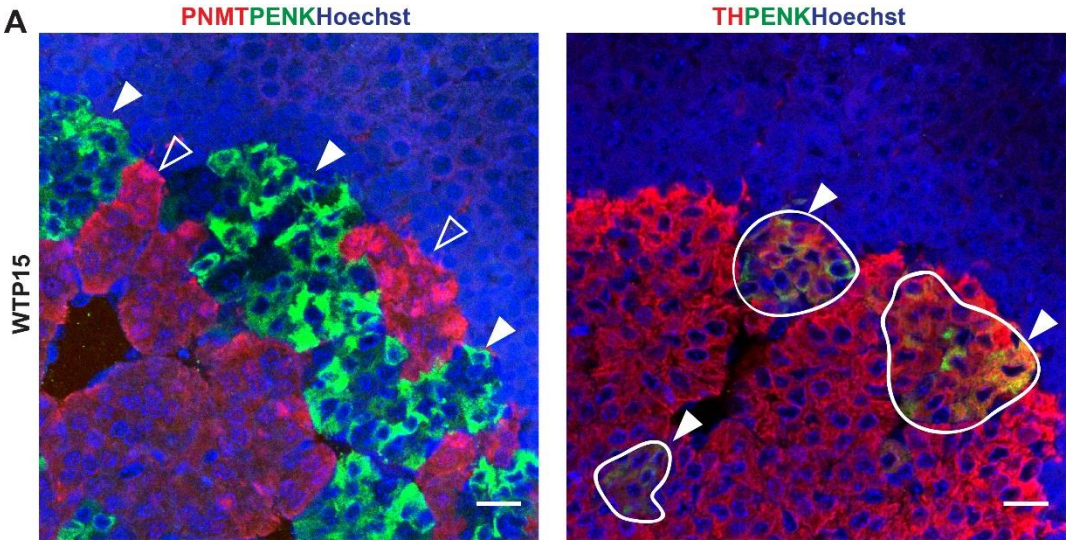

Figure 3.5 PENK is a marker of noradrenaline-producing chromaffin cells.
A) Immunofluorescence on WT P15 with antibodies anti-PNMT or TH (red) and anti-PENK (green). Nuclei stained with Hoechst, scale bar 20µm. White arrowheads indicate PENK staining, empty arrowheads indicate absence of PENK staining.

2017). Noradrenaline-producing chromaffin cell marker *Cox8b* is a component of the cytochrome c oxidase; *Lix1* is known to be expressed in the dorsal root ganglia during embryonic development (Moeller et al., 2002) and is a marker of sympathoblasts in embryonic development (Kameneva, Artemov, et al., 2021); *Penk* is expressed in committing and chromaffin cells during embryonic development (Kameneva, Artemov, et al., 2021). Using immunofluorescence I validated PENK to be a marker of noradrenaline-producing chromaffin cells, showing PENK+ cells are also TH+ but PNMT- (Figure 3.5A).

I was not able to identify markers associated exclusively with any of the different clusters of adrenaline-producing chromaffin cells (Clusters 0,1,5). Instead, all markers associated strongly with each cluster was also expressed at lower levels in the other clusters, indicating the differences among these clusters mainly reside in expression levels. I used the DotPlot function to plot the top 10 differentially expressed genes for each cluster indicated in the heatmap in Figure 2C (Figure 3D). Cluster 0 shows higher expression of *Gata2*, together with genes involved in synaptic vesicle trafficking (*Cplx2* and *Pclo*) and biogenesis (*Rab3c* (Schonn et al., 2010)); genes coding for transmembrane (*Tmem130*), cell adhesion (*Pcdh10*, *Alcam*) and ion transporter (*Slc24a2*) proteins; as well as *Dock1*,

which is a guanine nucleotide exchange factor for small Rho family G proteins and *Gm42418*, a non-coding RNA.

Cluster 1 shows higher expression of *Pnmt*, *Dlk1*, which is a Notch ligand previously reported to be expressed in chromaffin cells during embryonic development (El Faitwri & Huber, 2018); as well as cilia and cytoskeletal associated genes *Cfap61* and *Tmsb10*. *Resp18*, a protein involved in secretory pathway in multiple endocrine organs (Atari et al., 2019), *Pcsk1n*, involved neuropeptide signalling. *Mt1* and *Mt2*, metallothioneins and transcriptionally regulated both by heavy metals and glucocorticoids; *Ramp1* transport calcitonin-receptor-like receptor (CRLR) to the plasma membrane; and *C1qtnf4*, a secreted protein involved in inflammation.

Cluster 5 shows differential expression for components of the AP-1 signalling pathway: *Atf3*, *Fosb*, *Jun*, *Junb*, *Ier2*, *Fos*, *Egr3*, *Dusp1*, *Btg2*, *Dnajb1*. Components of the AP-1 pathway have shown to be involved in catecholamine biosynthesis (Shi et al., 2019).

3.2.3 Markers and transcriptional heterogeneity of sustentacular cells

Markers of sustentacular cells identified in literature include S100B, GFAP, SOX10, Vimentin, Nestin. S100B and GFAP are markers of cells of glial origin, while SOX10 of embryonic Schwann cell precursors, Vimentin is expressed both in the sustentacular cells and endothelial cells in rat (Suzuki & Kachi, 1995), Nestin+ cells give rise to chromaffin cells *in vitro* and have a role in stress *in vivo* (Rubin De Celis et al., 2015). All of these markers are restricted to the sustentacular and transitioning cell clusters in the medulla dataset generated (Figure 3.6A). Intrigued by the overlap between sustentacular cells and endothelial cells, I used the dataset before subsetting to retain only medulla cells, in order to investigate whether any of the other known sustentacular cell markers are expressed by endothelial cells (Figure 3.6B). As reported for rat (Suzuki & Kachi, 1995), *Vim* is expressed in both murine sustentacular and endothelial cells, as well as by immune cells. *Nes* expression is surprisingly predominant in endothelial cells, beside sustentacular cells (Figure 3.6B). Using immunofluorescence, I performed triple staining for Nestin, Endomucin and SOX10, which showed that Nestin+ cells can be either SOX10+ or Endomucin+, showing that Nestin expression marks subsets of both sustentacular cells and endothelial cells. *S100b*, *Gfap* and *Sox10* expression is mostly restricted to sustentacular cells, with *Sox10* and *S100b* being the most highly and widely expressed markers. These findings indicate that reliable "classical" markers of sustentacular are *S100b*, *Gfap* and *Sox10*.

To identify new markers of sustentacular cells, I analysed differentially expressed genes and selected the ones restricted to the sustentacular and transitioning cell clusters and not expressed by endothelial or immune cells. Candidate genes identified are *Scn7a*, *Plp1*, *Col28a1*, *Kcna1*, *Lgi4*, *Cdh19*, *Serpine2*, *Atp1a2*, *Apod*, *Lpar*,*1 Sostdc1*, *Fabp7*, *Gfra3*, *Scrg1*, *Col18a1*, *Sfrp1*, *Postn*, *Kcna2*, *Htra1* (Figure 3.7A). Among these genes I found several

reported to be expressed in embryonic SCPs: *Plp1*, *Lgi4*, *Fabp7*, *Sfrp1* (Furlan et al., 2017) and *Cdh19* (Takahashi & Osumi, 2005); generally expressed in the adrenal medulla: *Gfra3* (Widenfalk, 1998), *Postn* (VanDusen et al., 2014); and to be involved in neurodegenerative changes and neuroblastoma: *Scrg1*, *Lpar1* (Wei et al., 2013), *Htra1* (D'Angelo et al., 2014). Furthermore, expression of *Scn7a*, *Atp1a2 Kcna1* and *Kcna2* suggest that sustentacular cells express specific sodium and potassium channels, and *Col28a1*, *Col18a1* encode specific collagens. The remaining markers identified are *Serpine2*, a serine (or cysteine) peptidase inhibitor, *Apod*, a secreted glycoprotein and *Sostdc1*, a BMP antagonist.

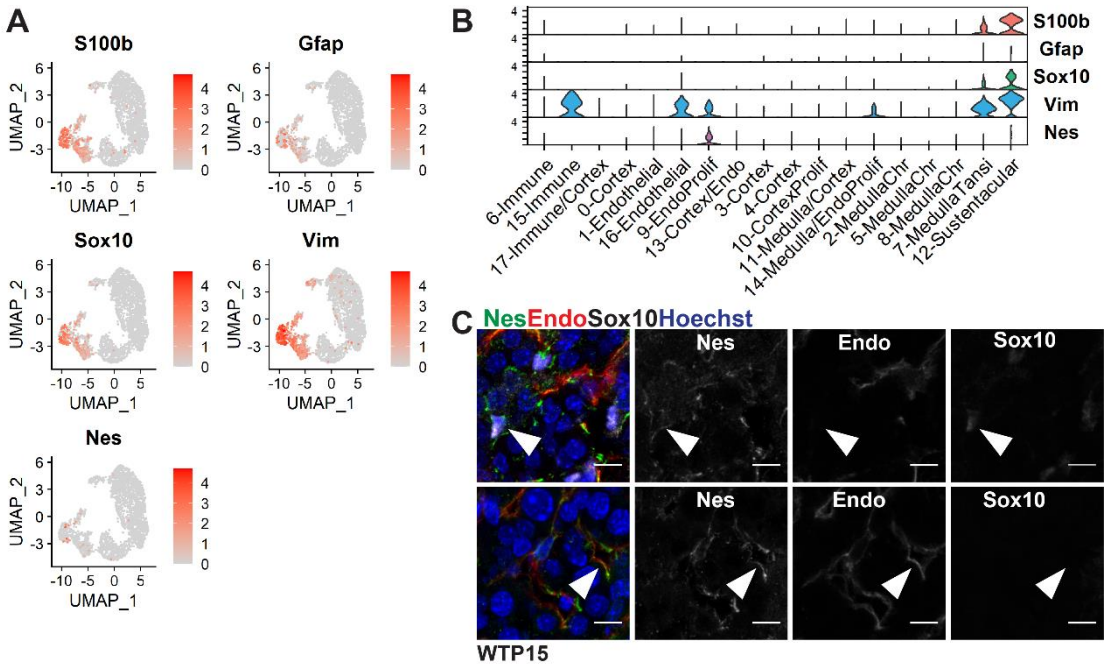

Figure 3.6 Known sustentacular cell markers.
A) Feature plots of known sustentacular cell markers. B) Violin plots of known sustentacular cell markers in the "whole" dataset. C) Immunofluorescence on WT P15 with antibodies anti-Nestin (green), anti-Endomucin (green) and anti-SOX10 (white). Double positive cells indicated by white arrowhead. Nuclei stained with Hoechst, scale bar 10µm. D) Feature plots showing newly identified sustentacular cell markers.

I decided to include *Plp1* in further analyses of sustentacular cells, as it is a consolidated marker of embryonic SCPs *in vivo* and is expressed by 80% of the cells in the Sustentacular cluster. To determine whether any of the sustentacular markers considered for further analysis identify predominantly with the Sustentacular or the two Transitioning clusters, I calculated the percentage of cells expressing *S100b*, *Gfap*, *Sox10* and *Plp1* in the Sustentacular cluster (Sust), each Transitioning cluster T-NorAdrChr or T-AdrChr, the two Transitioning clusters together or all of the sustentacular-containing clusters (All). As shown in Figure 3.8A, *Plp1* is expressed by the highest percentage of cells across all clusters (80% Sust, 62.5% T-NorAdrChr, 66.7% T-AdrChr, 63.4% TNAC+ T-AdrChr, 68% All), second is *S100b* (61.9% Sust, 45.1% T-NorAdrChr, 38.7% T-AdrChr, 43.7% T-NorAdrChr + T-AdrChr, 48% All), third *Sox10* (42.5% Sust, 29% T-NorAdrChr, 29% T-AdrChr, 29% T-NorAdrChr + T-AdrChr, 32.7% All) and last *Gfap* (16.2% Sust, 15.9% T-NorAdrChr, 12.9% T-AdrChr, 15.2% T-NorAdrChr + T-AdrChr, 15.5% All). These results show that all markers have the highest percentage of positive cells in the Sustentacular cluster, suggesting these are all expressed by a similar cell type/state. Furthermore, these percentages show that sustentacular cells must express these markers simultaneously. To investigate heterogeneity, I used the ggvenn function in RStudio and analysed the cells of the Sustentacular cluster only (Figure 3.8B). 8.7% of the cells expresses only *S100b*, 2.5% *Sox10*, 0.6% *Gfap*, 18.6% *Plp1*. Double positive *S100b*+*Sox10*+ cells are 5% of the total, *S100b*+*Gfap*+ 2.5%, *S100b*+*Plp1*+ 20.5%. *Sox10*+*Gfap*+ 0.6%, *Sox10*+*Plp1*+ 11.8%, *Gfap*-+*Plp1*+ 2.5%. Triple positive *S100b*+*Sox10*+*Gfap*+ 0.6%. *S100b*+*Sox10*+*Plp1*+ 16.8%, *S100b*+*Gfap*+*Plp1*+ 4.3%, *Sox10*+*Gfap*+*Plp1*+ 1.9%. 3.1% of the cells express all markers. These results indicate that *Plp1* is the marker expressed by the highest number of sustentacular cells, strengthening the connection between postnatal sustentacular cells and embryonic SCPs. Furthermore, combinations of *S100b*, *Gfap*, *Sox10* and *Plp1* are

expressed by different cells, confirming the transcriptional heterogeneity among sustentacular cells. I validated these findings *in vivo* using immunofluorescence with antibodies against GFAP and S100B (Figure 3.8C), and RNAscope with probes against *S100b*, *Gfap*, *Sox10* and *Plp1*, confirming overlap across all markers (Figure 3.8D).

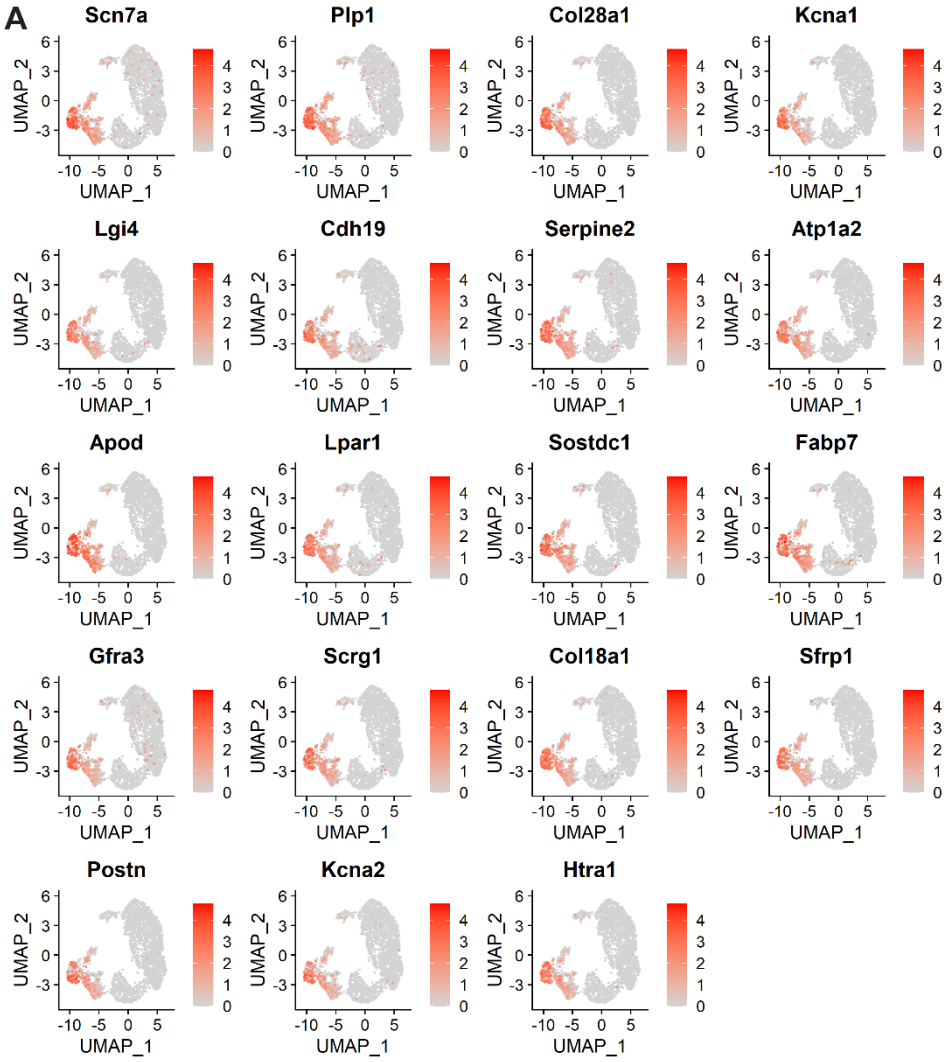

Figure 3.7 New sustentacular cell markers.
A) Feature plots showing newly identified sustentacular cell markers.

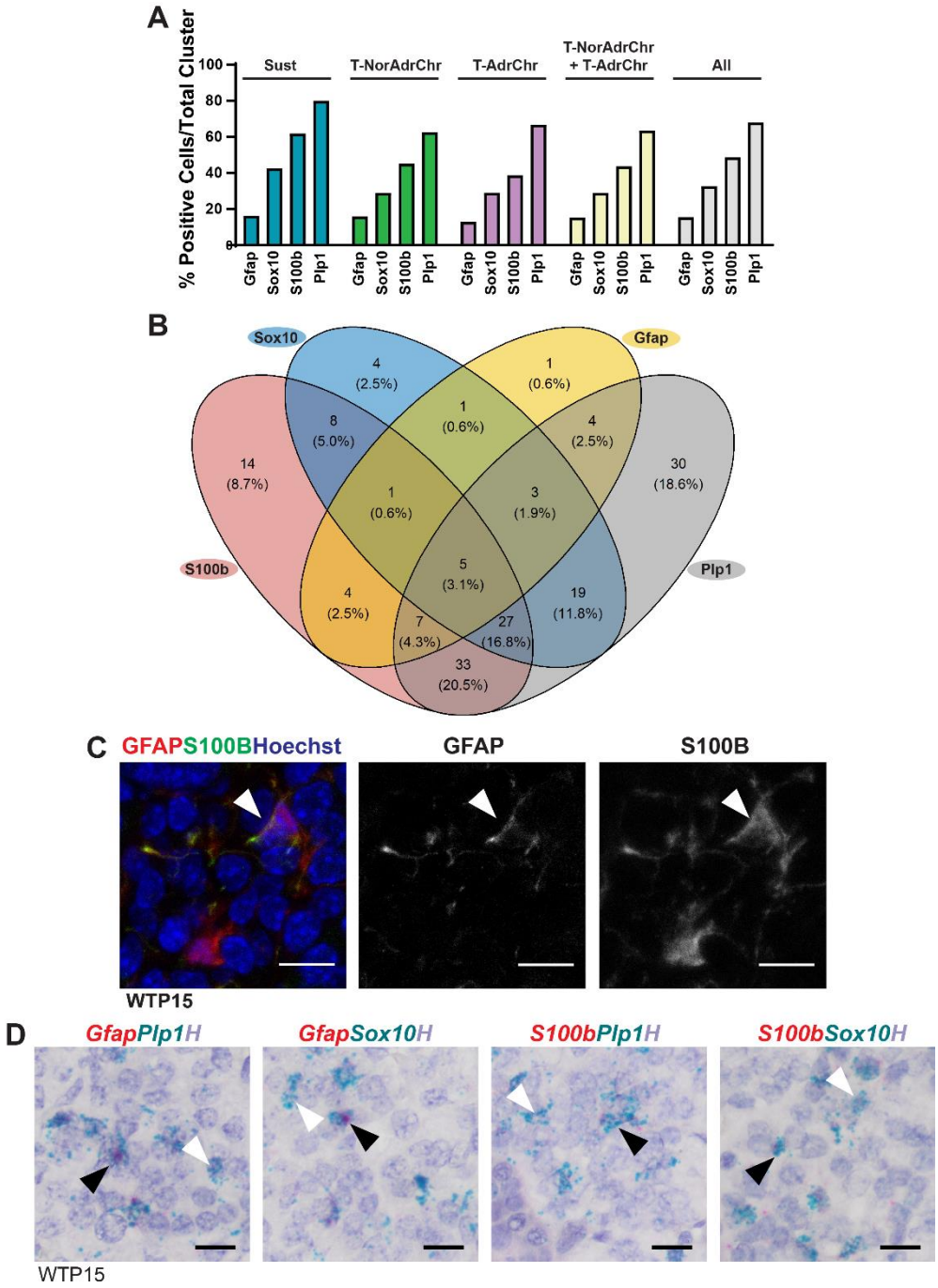

Figure 3.8 Heterogeneity of sustentacular cells.
A) Percentage of cells expressing each marker in each sustentacular/transitioning cluster. B) Venn diagram showing the number of cells expressing one of multiple markers of sustentacular cells. C) Immunofluorescence on WT P15 with antibodies anti-GFAP (red), anti-S100B (green). Double positive cells indicated by white arrowhead. Nuclei stained with Hoechst, scale bar 10µm. D) Double RNAscope on WT P15 samples shows double positive for *Gfap* (red) and *Plp1* (blue), *Gfap* (red) and *Sox10* (blue), *S100b* (red) and *Plp1* (blue), *S100b* (red) and *Sox10* (blue) black arrowheads, or single positive, white arrowheads. Nuclei counterstained with Hematoxylin, scale bar 10µm.

3.2.4 Postnatal cell composition of the medulla resembles embryonic development

Cell cluster arrangement in the postnatal medulla strongly resemble the embryonic adrenal medulla, as reported in literature (Furlan et al., 2017) (Kameneva, Artemov, et al., 2021) and newly identified markers have a close relationship to those identified in the developing adrenal medulla. To further investigate similarities and differences in cell arrangement and marker expression of the embryonic and postnatal medulla, I used published datasets from Furlan et al. at E12.5 and E13.5 (Smart-seq2), and Kameneva et al. at E13.5 (10x sequencing), which I re-analysed using the Seurat package in R following

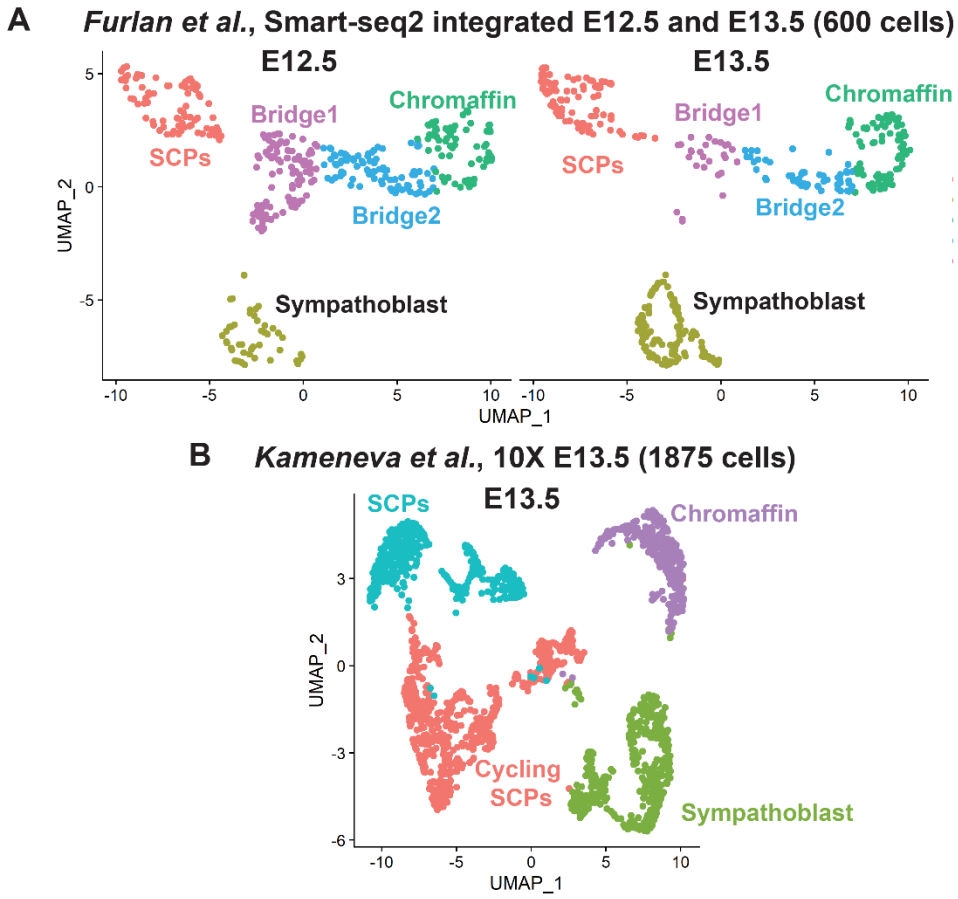

Figure 3.9 . Embryonic single cell datasets of the developing adrenal medulla.
A) Split UMAP of integrated datasets from E12.5 and E13.5 Smart-seq2 adrenal medulla from *Furlan et al., 2017*. B) UMAP of E13.5 10X dataset from *Kameneva et al., 2021*.

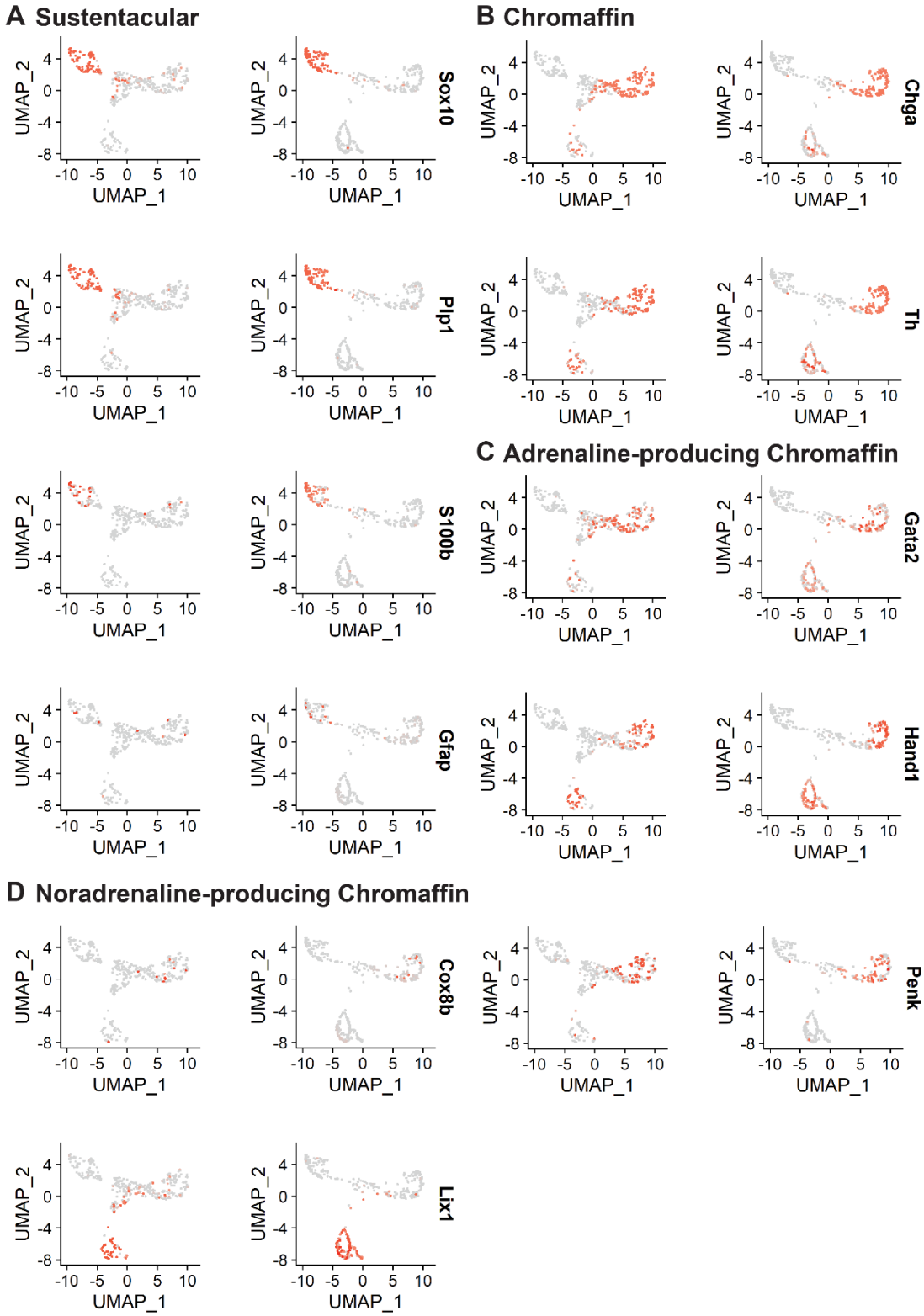

Figure 3.10 Embryonic (Furlan) expression of markers identified in postnatal dataset.
A) Feature plots showing the expression of postnatal sustentacular markers. B) Feature plots showing the expression of postnatal chromaffin cells. C) Feature plots showing the expression of postnatal adrenaline-producing chromaffin cells. D) Feature plots showing the expression of postnatal noradrenaline-producing chromaffin cells.

standard pipeline. Furlan E12.5 and E13.5 were integrated, and clustering of both datasets was performed to resemble the published findings. The Furlan dataset (Figure 3.9A) shows 5 clusters: one cluster of SCPs, two clusters of "bridge" cells thought to be transitioning towards chromaffin fate, one cluster of chromaffin cells and one cluster of sympathoblasts. The model proposed in the paper involves a single trajectory from SCPs to chromaffin cells, passing through two clusters of transitioning cells, both at E12.5 and E13.5. The Kameneva dataset (Figure 3.9B) shows 4 clusters: one SCP cluster, one cycling SCP cluster, one chromaffin cluster and one sympathoblast cluster. This new dataset provided an update on the previous model, and while the transitioning clusters are not identified based on clustering, Velocity analysis predicted a first transition from SCP to chromaffin, and a second transition from chromaffin to sympathoblast. The postnatal adrenal cell composition resembles the ones observed in the embryonic datasets, as I was able to identify two transitioning clusters which show expression of sustentacular cell-associated genes as well as chromaffin cell markers.

I investigated the expression of the previously and newly identified genes marking the different cell types by plotting the gene expression with the FeaturePlot function in RStudio. In the Furlan dataset, embryonic SCPs and sustentacular postnatal markers *Sox10*, *Plp1*, *S100b* and *Gfap* are expressed in the SCP cluster, with *Gfap*+ cells being very rare (Figure 3.10A). Markers of chromaffin lineage *Chga* and *Th* are expressed in the chromaffin cluster, as well as the second "bridge" cluster of transitioning cells and sympathoblasts (Figure 3.10B). Newly identified markers of postnatal adrenaline-producing chromaffin cells *Gata2* and *Hand1* follow the general chromaffin marker expression pattern (Figure 3.10C). Postnatal markers of noradrenaline-producing cells *Cox8b*, *Lix1* and *Penk* all show distinct patterns of expression, with *Cox8b* being expressed by few cells in the chromaffin cluster, *Penk* expression is restricted to the chromaffin

cluster, while *Lix1* to the sympathoblast cluster and partially in the transitioning "bridge" cell cluster 1 (Figure 3.10D).

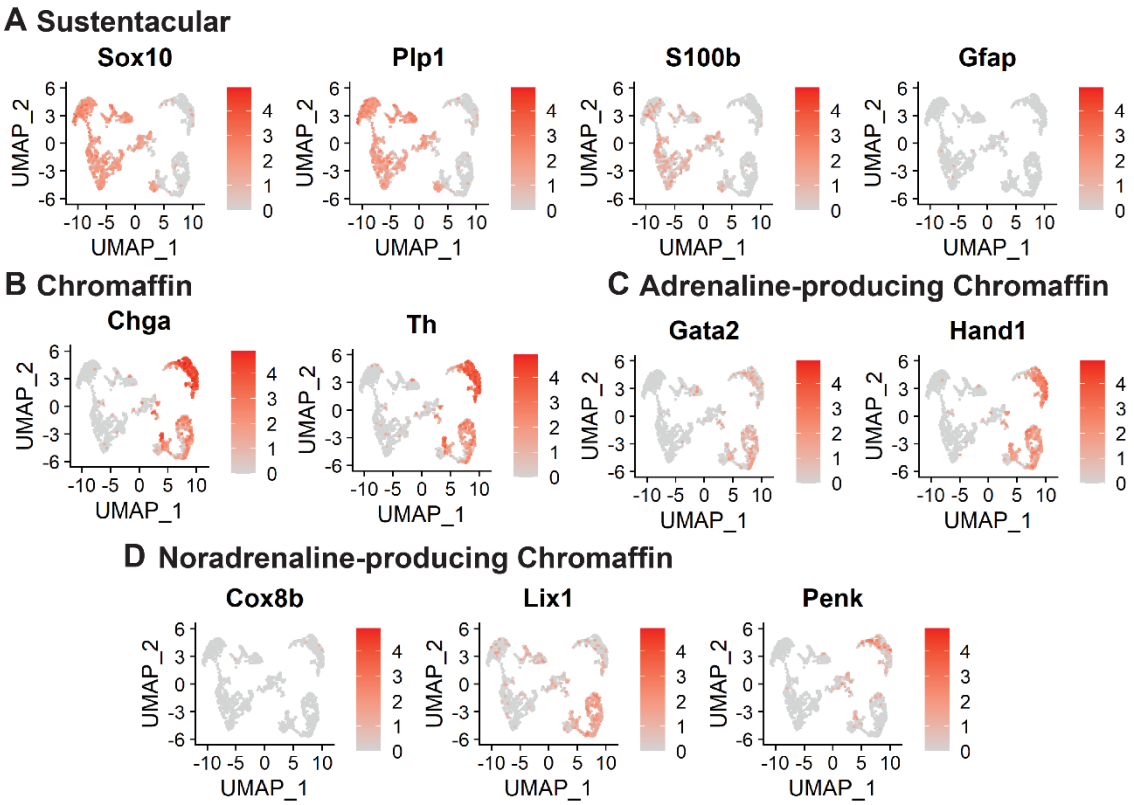

Figure 3.11 E13.5 (Kameneva) expression of markers identified in postnatal dataset.
A) Feature plots showing the expression of postnatal sustentacular markers. B) Feature plots showing the expression of postnatal chromaffin cells. C) Feature plots showing the expression of postnatal adrenaline-producing chromaffin cells. D) Feature plots showing the expression of postnatal noradrenaline-producing chromaffin cells.

For all genes analysed, the expression pattern remains similar between E12.5 and E13.5 in this dataset. In the E13.5 Kameneva dataset, the expression of all markers is very consistent with that observed in the Furlan dataset (Figure 3.11A, B, C, D). Looking into specific genes, the correlation between embryonic and postnatal cell arrangement appears even stronger, confirming sustentacular cells are close relatives of SCPs and might hold the potential to act as stem or progenitor cells *in vivo* in the postnatal adrenal medulla.

3.2.5 Signalling signatures of the postnatal medulla

To identify the possible implication of signalling pathways in the regulation of the different cell types of the adrenal medulla, I determined the expression of the following pathways known to be involved in stem cell regulation and differentiation: WNT, Notch, BMP/TGF, Hippo and MAPK.

The WNT pathway (Figure 3.12A) signals in neural crest cell lineages during medulla development (Becker & Wilting, 2018), plays a pivotal role in stem cell differentiation in multiple organs including the adrenal cortex (Wood & Hammer, 2011). Activating mutations in WNT pathway components lead to pheochromocytoma (Fishbein et al., 2017a) and WNT signalling also plays a role during neuroblastoma pathogenesis (Szemes et al., 2019). In the presented dataset, *Wnt6*, *Sfrp1* and *Sfrp5* are expressed in the sustentacular and transitioning cells, indicating the WNT pathway to be active in these cell types. The WNT target *Axin2* is predominantly expressed in the adrenaline-producing clusters, while transcriptional effector *Tc7l2* is expressed in the sustentacular and transitioning clusters, as well as the proliferating chromaffin cluster. Transcriptional effector *Lef1*, is expressed predominantly in the noradrenaline-producing cluster. *Lgr5*, which encodes a receptor involved in amplifying WNT-signals but also a target, is expressed throughout all the clusters except the sustentacular cells. *Wls*, which codes for a key protein responsible for WNT secretion, is expressed throughout the dataset; *Dkk3*, a WNT inhibitor, is expressed by all clusters except the sustentacular cells. These observations indicate that sustentacular cells might secrete WNT signals, while the differentiated cell types might respond to the WNT secretion by transcribing target genes.

Notch signalling (Figure 3.12B) was identified as a necessary signal to differentiate medulla progenitors *in vitro* (Vukicevic, De Celis, et al., 2012), I identified expression of *Notch1* and Notch-target genes *Hey2*, *Heyl* and *Hes1* in the sustentacular cells and the

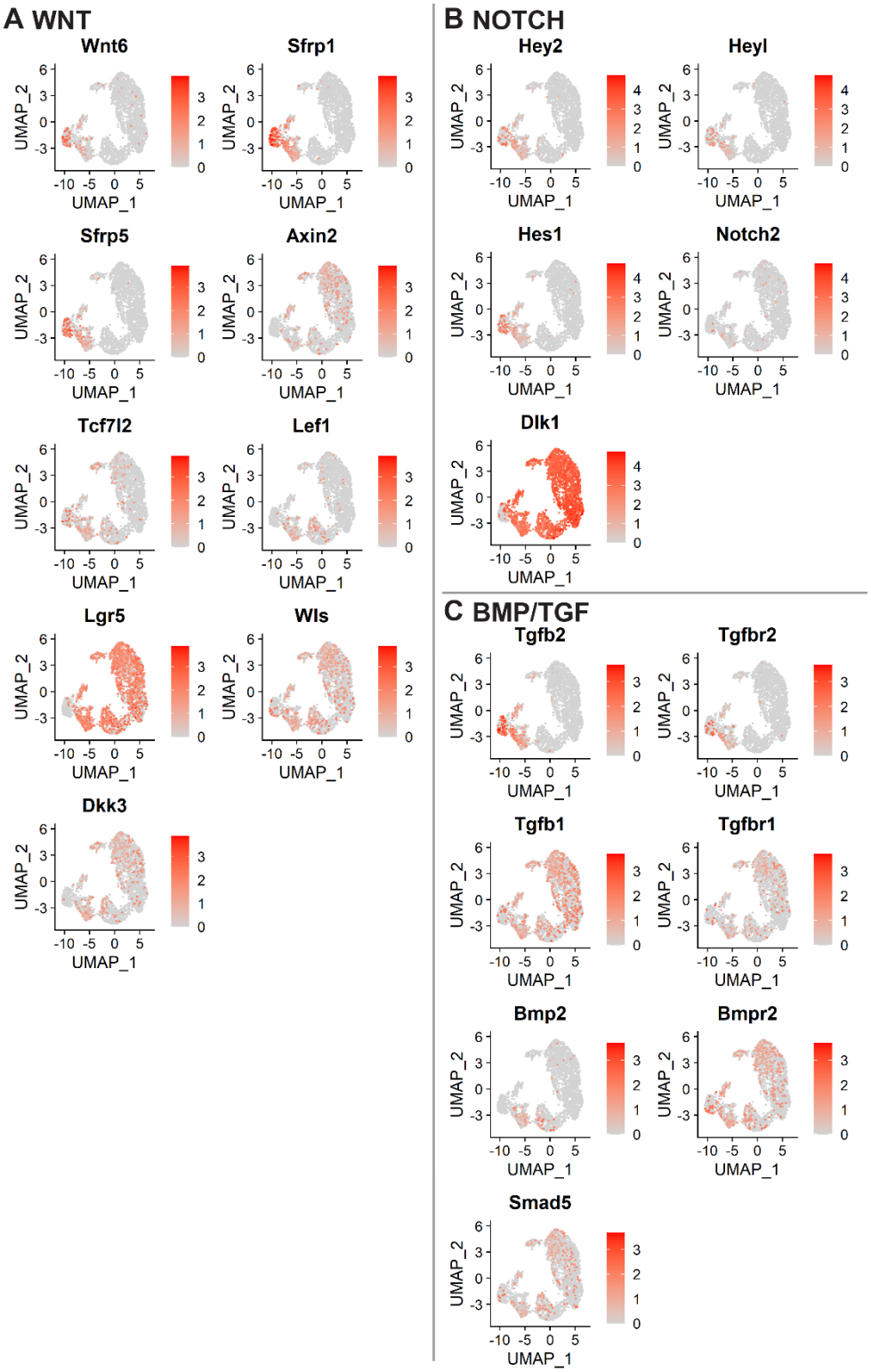

Figure 3.12 Postnatal expression of signalling pathways: WNT, Notch, BMP/TGF. A) Feature plots showing expression of WNT signalling pathway. B) Feature plots showing expression of Notch signalling pathway. C) Feature plots showing expression of BMP/TGF signalling pathway.

transitioning clusters, while expression of *Dlk1* was detected in all chromaffin cell clusters. DLK1 was previously reported to be expressed in chromaffin cells during embryonic development, and DLK1 expression is dependent on PHOX2B, a transcription factor involved in chromaffin cell maturation (El Faitwri & Huber, 2018). The expression of the Notch signalling pathway components observed is consistent with a Delta-Notch interaction within differentiated-sustentacular cells.

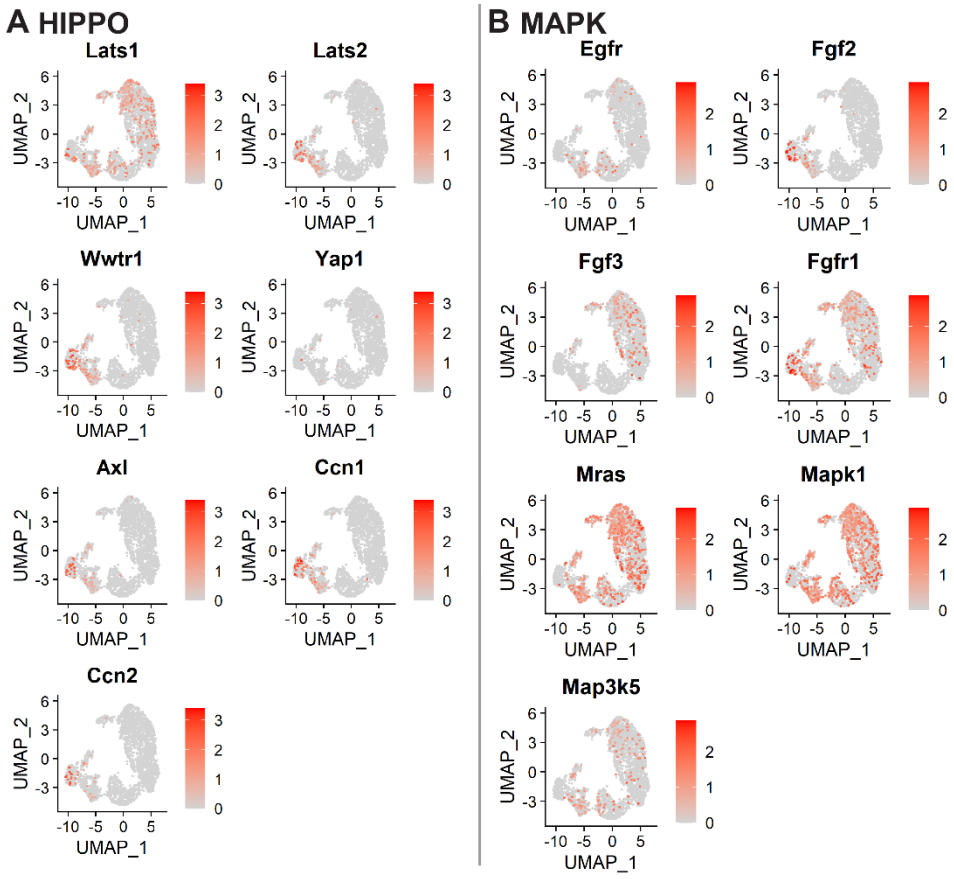

Figure 3.13 Postnatal expression of signalling pathways: YAP/TAZ and MAPK.
A) **Feature plots showing expression of YAP/TAZ pathway. E) Feature plots showing expression of MAPK pathway.**

TGFb signalling has shown to be involved in chromaffin cell differentiation and proliferation during medulla development (Rahhal et al., 2004). Therefore, I investigated components of the TGF/BMP signalling pathway (Figure 3.12C). In the postnatal dataset, I identify *Tgfb2* and *Tgfbr2* to be expressed in the sustentacular and transitioning clusters, while *Tfgb1* and *Tgfbr1* are expressed in the differentiated cell types. *Bmp2* is expressed

only in the noradrenaline-producing clusters, while its receptor *Bmpr2* is expressed ubiquitously. BMP/TGF signalling effector *Smad5* is expressed in all clusters. These expression patterns suggest that BMP/TGF signalling might have a role in all the different cell types of the postnatal medulla.

The YAP/TAZ signalling pathway (Figure 3.13A) is involved in the regulation of dorsal root ganglia and glial populations during embryonic development (Serinagaoglu et al., 2015). *Lats1* and *Lats2*, kinases involved in the phosphorylation and negative regulation of YAP/TAZ, are expressed in the adrenal medulla, with *Lats2* expression restricted to the sustentacular and transitioning clusters only. *Yap1* is expressed a low level by few cells throughout the dataset, while *Wwtr1 (Taz)*, together with Hippo pathway targets *Axl*, *Cyr61 (Ccn1)* and *Ctgf (Ccn2)* are found in the sustentacular and transitioning clusters. The expression of many YAP/TAZ signalling components seems to be predominant in sustentacular and transitioning cells, suggesting the Hippo pathway might have a role in the regulation of sustentacular cells.

Mutations in MAPK pathway components (Figure 3.13B) are found both in neuroblastoma (Eleveld et al., 2015) and pheochromocytoma (Fishbein et al., 2017a). *Egfr* is expressed in the noradrenaline-producing chromaffin cell cluster and its transitioning counterpart, *Fgf2* is only expressed in sustentacular and transitioning cells. *Fgf3* is only expressed in adrenaline-producing chromaffin cells. *Fgfr1* is expressed in all clusters except the noradrenaline producing chromaffin cluster. Expression of downstream components of the MAPK pathway such as *Mras*, *Mapk1* and *Map3k5* is enriched in the differentiated cell types of the medulla. This indicates that the MAPK pathway could be involved in regulation of both sustentacular cells and chromaffin cells.

3.3 Discussion

The results presented in this chapter provide the first atlas of the mouse postnatal adrenal medulla. I defined unique transcriptional and signalling attributes of the main cell types of the postnatal mouse adrenal medulla and identified a putative cell hierarchy. Further investigation of signalling pathways detected could help defining the mechanisms that maintain organ homeostasis in the postnatal adrenal medulla. Investigating published RNA sequencing datasets of embryonic development timepoints, I find comparable similarities with the transcriptional signatures identified in the postnatal medulla, indicating that the well-studied developmental mechanisms of differentiation and maturation of the adrenal medulla might be recapitulated during the postnatal development of the gland.

3.3.1 Single cell RNA sequencing

Single cell RNA sequencing datasets present in the literature fail to provide significant data for the postnatal adrenal medulla, which is always represented by very few cells, preventing a thorough analysis (X. Han et al., 2020; Hanemaaijer et al., 2021; Lopez, Brivio, Santambrogio, Donno, et al., 2021). I believe these results are due to the need/choice to process the entire adrenal gland using a digestion method optimised for the adrenal cortex, which proves to be too harsh for adrenomedullary cells, leading to cell death. The methodology I used, which consists of manually removing most of the cortex just based on morphological observations does not prove to be perfect, given that many cells of the cortex were still captured. However, I believe the significant improvement in the methodology I developed, lies in the use of an enzymatic digestion mix that is optimised for the adrenal medulla in terms of timings of incubation/manual dissociation. This

allowed me to produce a dataset with an adequate number of single cells to perform a thorough analysis. However, because so many cortex cells were included, it was impossible to determine whether the endothelial and immune cells present in the dataset belong to the adrenal medulla or to the adrenal cortex. Improvements on this could be done by optimising the manual dissection of the adrenals to be able to remove more cortex, for example using *Wnt1*^{Cre} driven lineage tracing to obtain all medulla cells to be fluorescent and manually remove or flow sort the non-fluorescent cortex. Alternatively, immunohistological and mRNA *in situ* hybridisation techniques could be used to investigate whether there are specific immune/endothelial markers that can allow to discriminate whether these cells belong to cortex or medulla. To investigate further both cortex and medulla together with single cell transcriptomics techniques, the two components of the adrenal will need a different preparation to guarantee the quality of the cells. This could be achieved by providing a mixture of non-separated adrenal glands dissociated according to cortex requirements and medulla-enriched adrenals dissociated according to medulla requirements. Alternatively, 10x Visium Spatial Gene Expression technology, or a future version with higher resolution, could be used, so that the special information can be combined with the single cell sequencing information. At the moment of writing, this technique is still quite limited.

3.3.2 Chromaffin cells

Single cell RNA sequencing revealed the presence of 5 chromaffin cell clusters. Only one of the clusters lacked the expression of *Pnmt* but maintained all other catecholamine synthesis enzymes, making it the only cluster of noradrenaline-producing cells.

Adrenaline-producing chromaffin cells are more transcriptionally heterogeneous and are represented by 4 different clusters: 0-adrenaline-producing chromaffin cells, 1-

adrenaline-producing chromaffin cells, 5-adrenaline-producing chromaffin cells, 7-cycling adrenaline-producing chromaffin cells. Cluster 6 contains proliferative cells and is more distinct, while the three remaining clusters show the expression of comparable markers, presenting different expression levels of specific genes. Cluster 0 and 1 share similar gene expression profiles, with differences in the levels of specific genes associated with secretion and cytoskeleton. Heterogeneity at this level is expected, since *in vitro* observation of bovine adrenomedullary chromaffin cells revealed at least two distinct types of adrenaline-producing chromaffin cells based on the density of their granules and cytoplasm (Koval et al., 2000). Further investigation could link the previously observed phenotypes with the transcriptional differences observed *in silico*. Cluster 5 shows upregulation of the AP1 pathway, which, in response to stress, regulates numerous cellular functions including proliferation, differentiation and apoptosis. Furthermore, the AP1 pathway was shown to be involved in catecholamine biosynthesis showing correlation to TH expression in response to EPHB6 (Ephrin type-B receptor 6) activation (Shi et al., 2019). This suggests that Cluster 5 could be a unique sub-population of chromaffin cells and should be investigated further.

One of the chromaffin cell clusters analysed is *Pnmt* negative, while expressing all the other enzymes necessary for catecholamine production. I therefore assigned this cluster a noradrenaline-producing chromaffin cell identity. Transcriptional differences between noradrenaline- and adrenaline-producing chromaffin cells are poorly studied, therefore I focused on identifying genes that are differentially expressed between the two cell types. This analysis revealed three main new markers of noradrenaline-producing chromaffin cells: *Penk*, *Cox8b* and *Lix1*. I validated PENK expression at the protein level and found PENK+ cells to be also TH+ but not by PNMT+, making this a good marker of noradrenaline-producing chromaffin cells. The specific expression of *Cox8b* only in noradrenaline-producing cells might suggest a difference in metabolism, which is expected

given the different requirements in production of adrenaline vs noradrenaline. *Lix1* is expressed in the dorsal root ganglia and sympathoblasts during development. It is not uncommon for chromaffin cells to share many markers with the sympathetic system, given their common origin, which leads to difficulties in observing a transcriptomic distinction between chromaffin cells and intra-adrenomedullary neurons.

3.3.3 Sustentacular cells

The identified sustentacular cell cluster expresses a range of expected markers, compatible with what is already shown in the literature. Markers of sustentacular cells are the most distinct from all chromaffin cell types, however there are two clusters that co-express these markers, indicating these might represent cells committing to a different fate.

Trying to elucidate the difference amongst the previously reported markers of sustentacular cells, I clarified the use of Nestin as a marker of sustentacular cells. Nestin+ cells were described as progenitors both in the adrenal cortex and medulla (Rubin de Celis et al., 2015; Steenblock et al., 2017, 2018) and able to give rise to differentiated progeny *in vitro* upon sphere formation, with scarce *in vivo* validation. Using *in silico* analyses and *in vivo* validation at protein level with immunofluorescence, I identified that Nestin+ cells are found in two different populations: sustentacular cells and endothelial cells. Therefore, while the studies published remain relevant, Nestin is not a good marker to indicate sustentacular cells alone, and future studies using this marker will require the introduction of staining for endothelial cells e.g., Endomucin, to ensure the population observed is the Nestin+; Endothelial-, or to use alternative markers such as SOX10 or S100B to indicate Nestin+ sustentacular cells. The overlap of other main markers described S100B, SOX10, PLP1, GFAP is heterogeneous both at the mRNA level *in silico* and

in vivo, as well as at protein level *in vivo*. While *in silico* differences could be explained by technical limitations such as possible dropouts in the reads or the variability of expression that might be lost due to normalisation and scaling, the *in vivo* data demonstrate that this heterogeneity exists and it is translated to differences in protein expression, suggesting there could also be functional heterogeneity. Intrigued by the hypothesis that different markers could indicate different cell hierarchy, using the single cell RNA sequencing dataset obtained, I calculated the number of positive cells for a certain marker in relation to the sustentacular cell cluster and the transitioning clusters and found no marker to be preferentially expressed in one rather than the other one. Possible future experiments to determine whether these markers all indicate cells with the same function could be carried out by using different lineage tracing systems *in vivo*.

Sustentacular cells are transcriptionally distinct from chromaffin cells. However, in the dataset analysed I identified two clusters that share markers of chromaffin cells and sustentacular cells: 3-sustentacular cells transitioning to noradrenaline-producing chromaffin cells and 6-sustentacular cells transitioning to adrenaline-producing chromaffin cells. No overlap of sustentacular cell markers and chromaffin cell markers was never reported in literature and not observed with double immunofluorescence staining a protein level (data not shown), therefore Cluster 3 and Cluster 7 could represent a transcriptional intermediate state, which suits the hypothesis that sustentacular cells are stem cells/progenitors of chromaffin cells. Given the presence of these intermediate committing states, it is also possible to hypothesise that sustentacular cells could be stem cells of the adrenal medulla, while the transitioning states identified might indicate progenitor cells. However, I was not able to identify a marker for these intermediate states *in silico*, making the identification of these cells *in vivo* challenging. Double immunofluorescence of TH and sustentacular cell markers does not reveal any overlap at the protein level (data not shown), suggesting that these populations might be a

transitioning committing state, rather than a distinct intermediate cell population. Interestingly, one population shows markers of adrenaline-producing chromaffin cells together with sustentacular cells, while the other one is PNMT negative. This leads to the hypothesis that sustentacular cells could directly give rise to adrenaline-producing and noradrenaline-producing chromaffin cells independently.

3.3.4 Neurons

UMAP and cluster assignment of the ''adrenal medulla only" dataset show that the adrenal medulla cell types are mainly divided into chromaffin cells and sustentacular cells. I was not able to identify a cluster of intramedullary neurons at this timepoint. Previously reported markers of intramedullary neurons such as *Vip* or *Nos1* were not detected in the dataset. Other neuronal markers like *Npy*, *Ache, Tubb3* and *Th* are expressed in all clusters except sustentacular cells, and it is known that TH is expressed by chromaffin cells. Pan-neuronal markers *Nefl* and *Rbfox3* are expressed by a subset of the cells clustering in what I have called noradrenaline-producing chromaffin cell cluster. RNAscope mRNA *in situ* hybridisation shows expression of *Nos1* in sporadic cells, while I was not able to identify any intramedullary cellular body using immunostaining for TUJ1 (data not shown). The identity of postnatal postganglionic neurons in the adrenal medulla has not been defined in mouse, and studies in rats report an overlap of cell markers and function with noradrenaline-producing cells (A. Dagerlind et al., 1990). The data gathered at a transcriptional level indicate that noradrenaline-producing chromaffin cells and postganglionic neurons might be variations of the same cell type. However, more investigation needs to be performed to establish this. Mindful that postganglionic neurons might have been lost during sample preparation for single cell RNA sequencing, it would be appropriate to use histological techniques with double/triple immunostaining or

multiplex RNAscope mRNA *in situ* hybridisation to investigate the overlap of multiple neuronal markers with multiple noradrenaline-producing cell markers.

4 Characterisation of SOX2+ cells of the adrenal medulla

4.1 Introduction

SOX2 is a transcription factor associated with pluripotency. SOX2+ stem and progenitor cells have been identified in multiple organs including the brain (Ellis et al., 2004) salivary gland (Emmerson et al., 2017), the trachea (Que et al., 2009) and the pituitary gland (Cynthia Lilian Andoniadou et al., 2013). SOX2 has been shown to be expressed in progenitors during embryonic development of adrenal medulla (Furlan et al., 2017) and in human pheochromocytoma (Oudijk et al., 2015). I decided to investigate whether SOX2 could indicate a population of stem cells or progenitors of the postnatal adrenal medulla. Before assessing the functional relevance of SOX2+ adrenomedullary cells, which will be addressed in Chapter 5, I decided to characterise adrenomedullary SOX2+ cells. In this chapter, I provide insights on SOX2+ cells in the adrenal medulla through *in vivo* and *in silico* investigation and I establish a method of culturing SOX2+ cells *in vitro* in attachment conditions.

4.2 Results

4.2.1 Stem cell marker SOX2 is expressed by sustentacular cells

Single cell RNA sequencing analysis shows known stem cell marker *Sox2* is expressed both in embryonic SCPs at E13.5 (Figure 4.1A) and in postnatal sustentacular cells at P15 (Figure 1B). Focussing the analysis at P15, I used immunofluorescence to show SOX2+ cells express sustentacular cell markers S100b, GFAP and SOX10 and (Figure 4.2A, B, C).

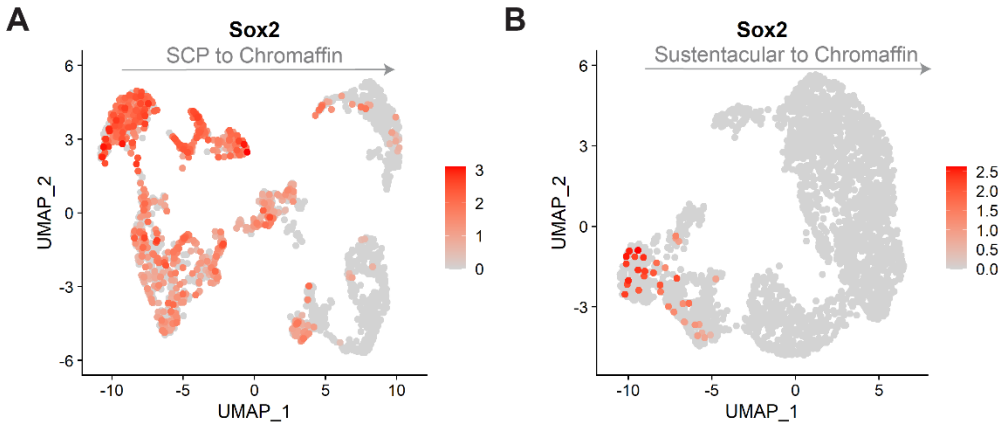

Figure 4.1 *Sox2* expression in single cell RNA sequencing datasets.
A) **FeaturePlot showing *Sox2* expression (in red) in E13.5 dataset from *Kameneva et al.*, 2021. B) FeaturePlot showing *Sox2* expression (in red) in P15 dataset. Scale shows relative expression in normalised units.**

To facilitate immunofluorescence investigation, I used the validated *Sox2*$^{eGFP/+}$ mouse line (Ellis et al., 2004), where GFP protein expression is consistent with SOX2 protein expression (Figure 4.2D). The same markers are expressed by *Sox2*+ cells at mRNA level, including SCP marker *Plp1*, as shown via RNAscope mRNA in situ hybridisation (Figure 4.3A, B, C, D). Quantification of immunofluorescence staining (Figure 4.2A, B, C) shows 100% of SOX2+ cells express S100B, whereas 58.72% of S100B+ cells express SOX2, indicating SOX2+ cells are a subpopulation of S100B+ sustentacular cells. 43.48% of SOX2+ cells express GFAP, whereas 89.55% of GFAP+ cells express SOX2, indicating GFAP+ cells are predominantly SOX2+, however they are not a complete subpopulation of SOX2+ cells. 91.49% of SOX2+ cells express SOX10, whereas 84.31% of SOX10+ cells

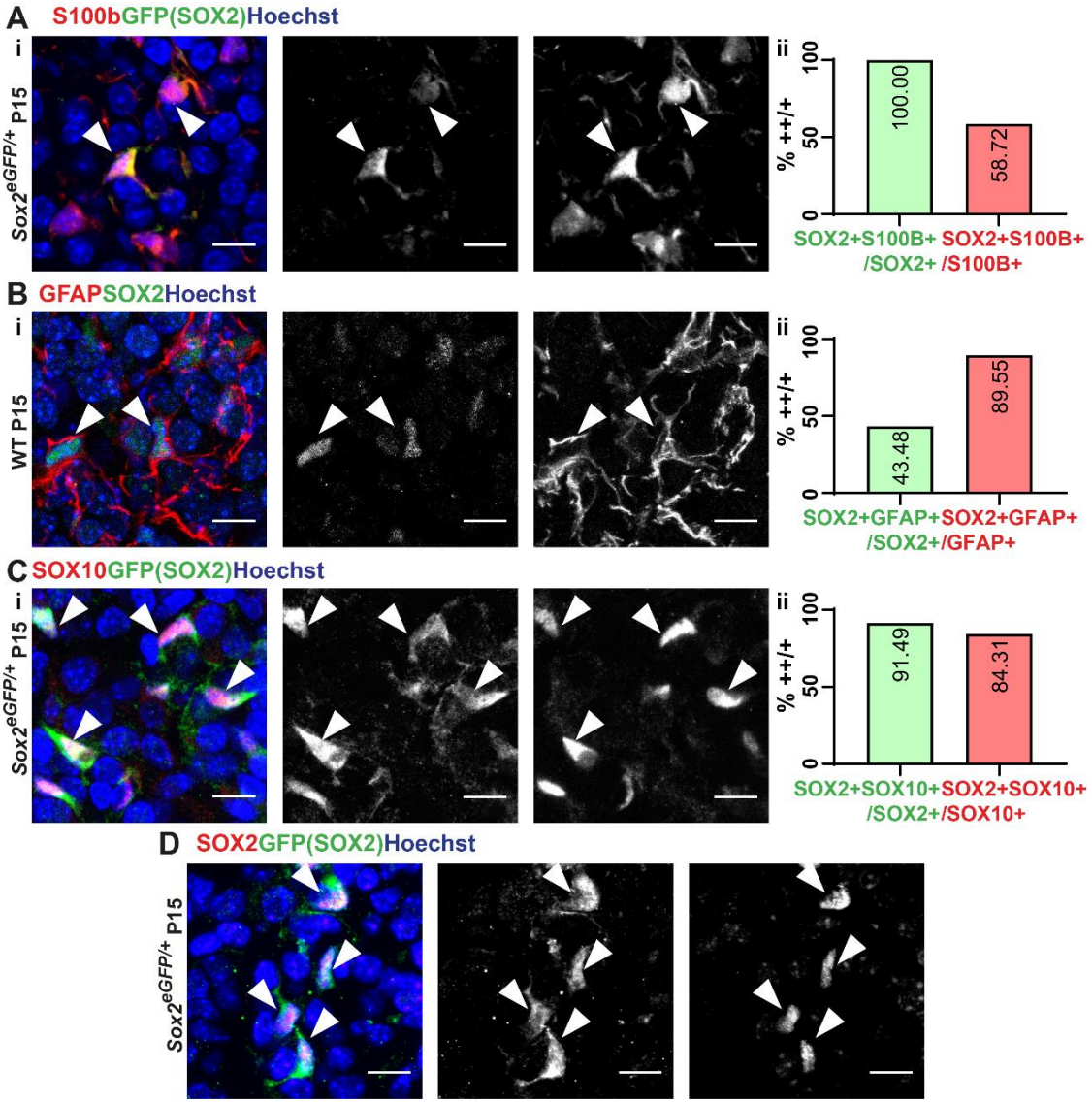

Figure 4.2 Protein expression of known sustentacular markers in SOX2+ cells.
A) i – Immunofluorescence staining of a P15 *Sox2*$^{eGFP/+}$ sample using antibodies against S100b (red) and GFP (green), shows double positive cells (arrowheads), nuclei counterstained with Hoechst, scale bars 10µm; ii – quantification of the percentage of SOX2+ cells expressing S100B (SOX2+S100B+/SOX2+) and of the percentage of S100B+ cells expressing SOX2 (SOX2+S100B+/S100B+). B) i – Immunofluorescence staining of a P15 WT sample using antibodies against GFAP (red) and SOX2 (green) shows double positive cells (arrowheads), nuclei counterstained with Hoechst, scale bars 10µm; ii – quantification of the percentage of SOX2+ cells expressing GFAP (SOX2+GFAP+/SOX2+) and of the percentage of GFAP+ cells expressing SOX2 (SOX2+GFAP+/GFAP+). C) i – Immunofluorescence staining of a P15 *Sox2*$^{eGFP/+}$ sample using antibodies against SOX10 (red) and GFP (green) sample shows double positive cells (arrowheads), nuclei counterstained with Hoechst, scale bars 10µm; ii – quantification of the percentage of SOX2+ cells expressing SOX10 (SOX2+SOX10+/SOX2+) and of the percentage of SOX10+ cells expressing SOX2 (SOX2+SOX10+/SOX10+). D) Immunofluorescence on *Sox2*$^{eGFP/+}$ P15 using antibodies against GFP (green) and SOX2 (red) shows all cells are double positive (arrowheads). Nuclei counterstained with Hoechst, scale bars 10µm.

express SOX2, indicating these two transcription factors are expressed together very frequently. These results show SOX2+ cells are a novel population of sustentacular cells that does not completely overlap with any of the previously identified populations indicated by the markers analysed.

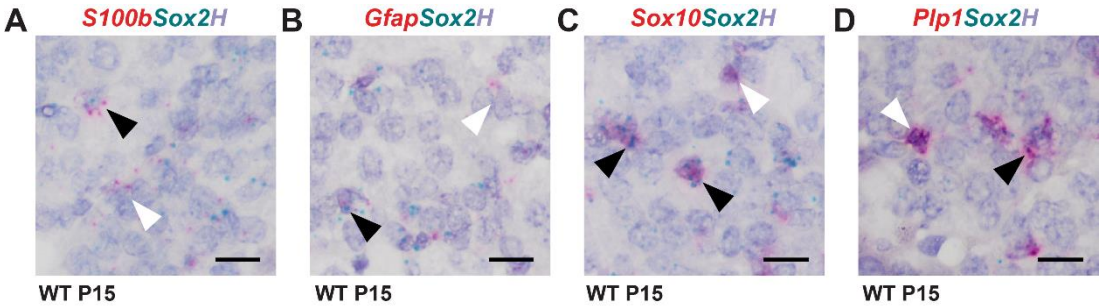

Figure 4.3 mRNA expression of known sustentacular markers in *Sox2*+ cells.
A) Double RNAscope mRNA in situ hybridisation on wild type P15 samples shows double positive cells for *S100b* (red) and *Sox2* (blue) (black arrowheads) or single positive (white arrowheads). B) Double RNAscope mRNA in situ hybridisation on wild type P15 samples shows double positive cells for *Gfap* (red) and *Sox2* (blue) (black arrowheads) or single positive (white arrowheads). C) Double RNAscope mRNA in situ hybridisation on wild type P15 samples shows double positive cells for *Sox10* (red) and *Sox2* (blue) (black arrowheads) or single positive (white arrowheads). D) Double RNAscope mRNA in situ hybridisation on wild type P15 samples shows double positive cells for *Plp1* (red) and *Sox2* (blue) (black arrowheads) or single positive (white arrowheads). All nuclei counterstained with Hematoxylin, scale bar 10µm.

4.2.2 SOX2+ cells persist throughout life in mouse and human adrenal medulla

To determine whether Sox2+ cells are transient population or if they are a long-lived population that is present throughout life, I investigated SOX2 expression at multiple postnatal timepoints in mouse and human adrenals. Using immunohistochemistry to identify SOX2+ cells, I counted the % of SOX2+ cells in the entire adrenal medulla at P15, P17, P21, P28, P84, P176, P365 in mouse and at 17, 29, 48, 55, 56, 71, 75 years of age in human. In mouse, the percentage initially increases from 4.95% at P15 to 7.04% at P17, decreases to 5.22% at P21 and decreases further at P28 (3.55%), to then reach a plateau at P84 (2.71%), P178 (2.82%) and P365 (2.99%) (Figure 4.4A, B). The trends observed might suggest that SOX2+ cells could be of relevance in the early postnatal timepoints. The increase in the percentage of SOX2+ cells from P15 to P17 might indicate that this population could have a driving role for the expansion of the medulla, and the decrease at later timepoints is consistent with a progenitor/stem cell role. Previous reports indicate a difference in volume of the medulla in female vs male adrenals during organ growth in mouse (Bielohuby et al., 2007). To determine whether this could be linked to a difference in the number of SOX2+ cells, I separated my analysis by sex but identified the percentages to be comparable (Figure 4.4A, C).

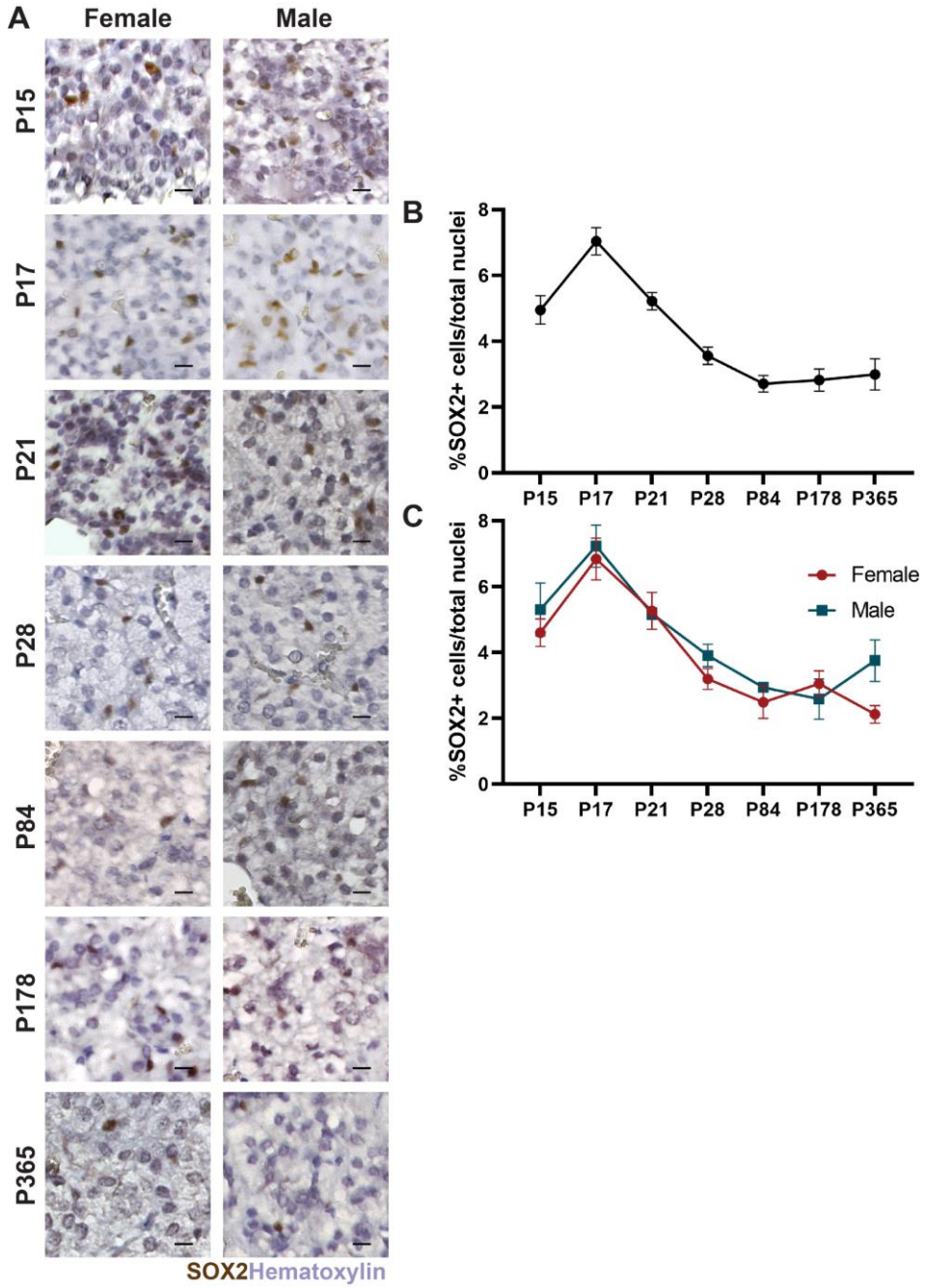

Figure 4.4 SOX2+ cells throughout life in mouse.
A) Immunohistochemistry with antibodies against SOX2 (brown) at P15, 17, 21, 28. 84, 178, 365, female and male. Nuclei counterstained with Hematoxylin, scale bar 20µm. B) Quantification of SOX2+ cells over the total nuclei of adrenal medulla. n = 6 for each timepoint, plotted mean and SEM. C) Quantification of SOX2+ cells over the total nuclei of adrenal medulla, split by sex. n = 3 per sex, each timepoint, plotted mean and SEM.

Investigation of SOX2+ cells in human adrenal medulla shows a similar trend to the one observed in mouse. I calculated the percentage of SOX2+ cells in 5 female samples (17, 48, 55, 71, 75 years of age) and 3 male samples (29, 56, 71 years of age). When grouping the samples in "<50 years of age", "50-70 years" of age and ">70 years of age", the percentage

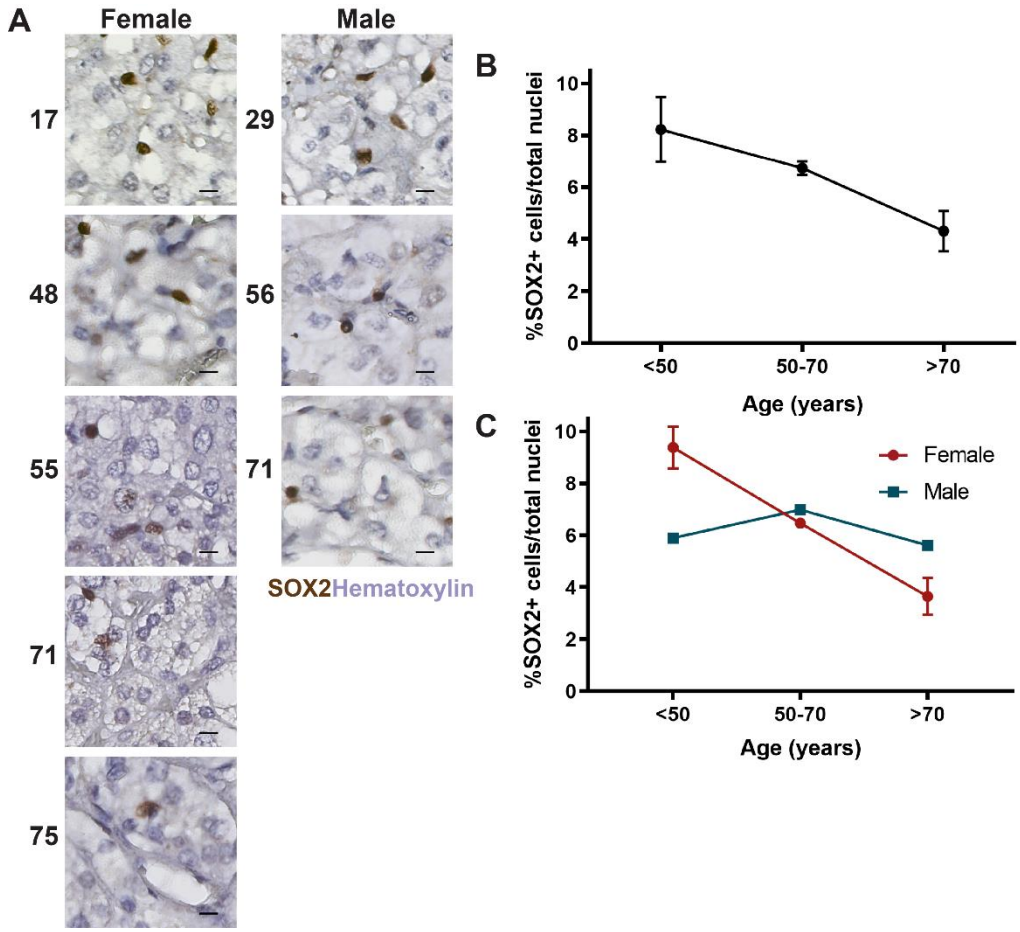

Figure 4.5 SOX2+ cells throughout life in human.
A) Immunohistochemistry with antibodies against SOX2 (brown) at 17, 48, 55, 71, 75 years of age in females and 29, 56, 71 years of age in male. Nuclei counterstained with Hematoxylin, scale bar 20µm. B) Quantification of SOX2+ cells over the total nuclei of adrenal medulla. n = 2-3 for each timepoint, plotted mean and SEM. C) Quantification of SOX2+ cells over the total nuclei of adrenal medulla, split by sex. n = 1-2 females, n = 1 males, each timepoint, plotted mean and SEM.

of SOX2+ cells among the total number of cells in the adrenal medulla decreases with age: the <50 group averages at 8.23% SOX2+ cells/total nuclei, the 50-70 group at 6.73% and the >70 group at 4.31% (Figure 4.5A, B). Given the small cohort analysed, it is impossible to consider differences between male and females (Figure 4.5C).

Given the lack of published information on proliferation of the mouse adrenal medulla during postnatal growth, I used immunohistochemistry to mark Ki-67+ cells in order to calculate the number of cycling cells in the whole adrenal medulla at P15, P17, P21, P28, P84, P176 and P365. Similarly to SOX2+ cells, the percentage initially increases from 8.87% at P15 to 10.34% at P17, confirming the organ is very dynamic at these timepoints. The percentage of Ki-67% cells then decrease to 5.02% at P21 and 4.87% at P28, it is further decreased at P84 (1.02%), P178 (0.56%) and P365 (0.66%) (Figure 4.6A, B). Analysis of male vs female samples at the same timepoints reveals no significant difference (Figure 4.6C). These findings indicate previous reports of the differences in volume between male and female mouse adrenal medulla is not dependant on proliferation. I used double immunofluorescence for SOX2 and Ki-67 to confirm SOX2+ cells are capable of proliferating and found that an average of 3.2% SOX2+ cells are Ki67+ and therefore proliferating at P15 (Figure 4.6D).

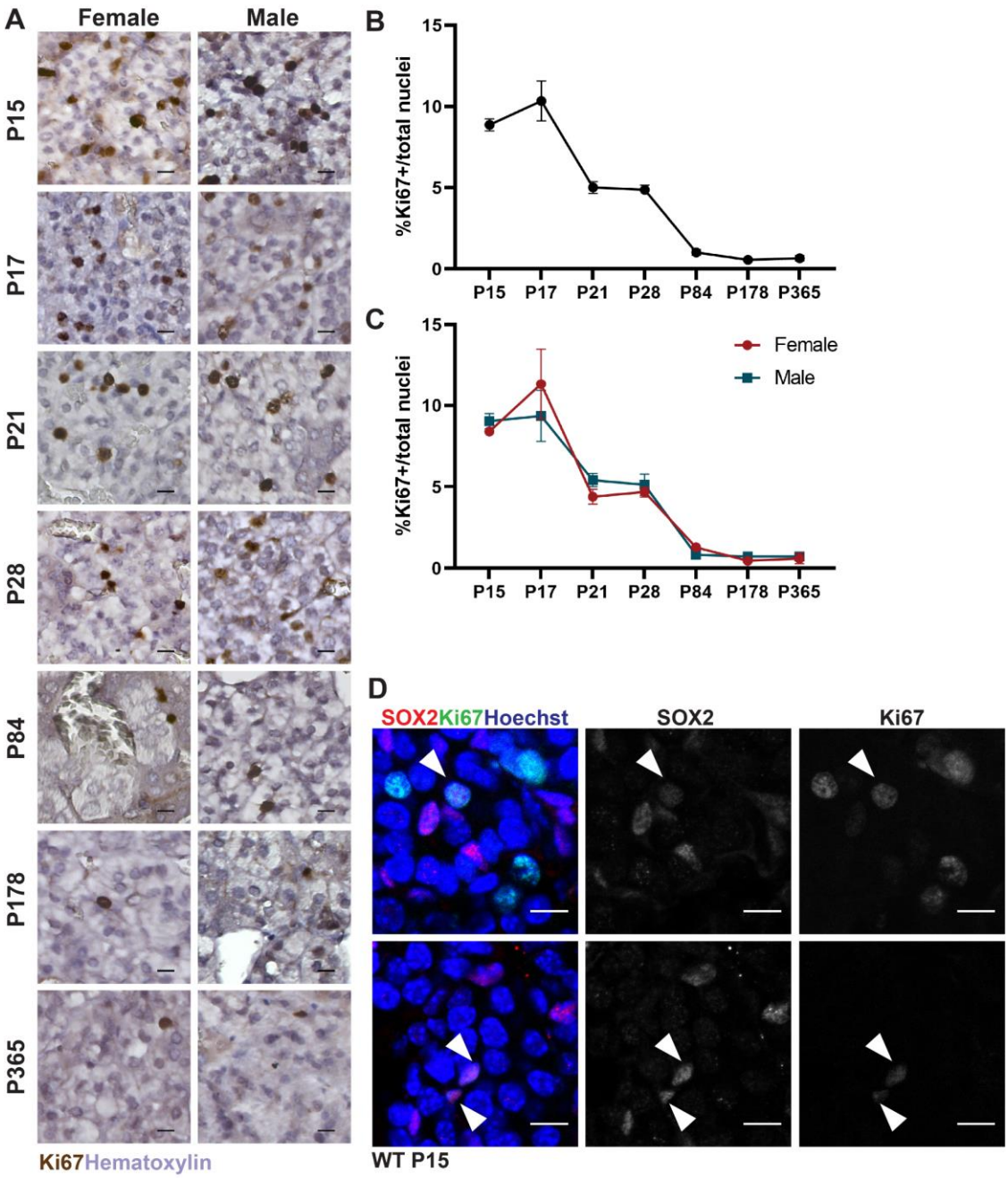

Figure 4.6 Proliferation in the postnatal adrenal medulla.
A) Immunohistochemistry with antibodies against Ki-67 (brown) at P15, 17, 21, 28. 84, 178, 365, female and male. Nuclei counterstained with Hematoxylin, scale bar 20µm. B) Quantification of Ki-67+ cells over the total nuclei of adrenal medulla. n = 4-6 for each timepoint, plotted mean and SEM. C) Quantification of Ki-67+ cells over the total nuclei of adrenal medulla, split by sex. n = 1-3 per sex, each timepoint, plotted mean and SEM. D) Antibody staining of a WT P15 sample shows double positive cells for SOX2 (red) and Ki67 (green) (arrowheads). Nuclei counterstained with Hoechst, scale bars 10µm.

4.2.3 SOX2+ cells derive from embryonic SCPs

To confirm SOX2+ cells have an origin consistent with the rest of the adrenal medulla and to investigate whether they derive from embryonic SCPs, I designed experiments using two lineage tracing systems: *Wnt1*$^{Cre/+}$;*R26*$^{mTmG/+}$ marking all neural crest derivatives and *Sox10*$^{CreERT2/+}$;*R26*$^{mTmG/+}$ marking all *Sox10* expressing cells and their derivatives upon tamoxifen induction. *Wnt1*$^{Cre/+}$;*R26*$^{mTmG/+}$ adrenals were harvested at P15 and double immunofluorescence for SOX2 and GFP shows double positive cells (Figure 4.7A, arrowheads), indicating SOX2+ cells are derived from the neural crest and therefore have an origin congruent with the rest of the gland. To determine whether SOX2+ cells are derived from embryonic SCPs, *Sox10*$^{CreERT2/+}$; *R26*$^{mTmG/+}$ were induced at E11.5 and analysed at P15, GFP+ traced cells make up for 85% of the medulla analysed at P15. Double immunofluorescence for GFP and SOX2 shows double positive cells (Figure 4.7B), confirming SOX2+ cells are derived from embryonic SCPs.

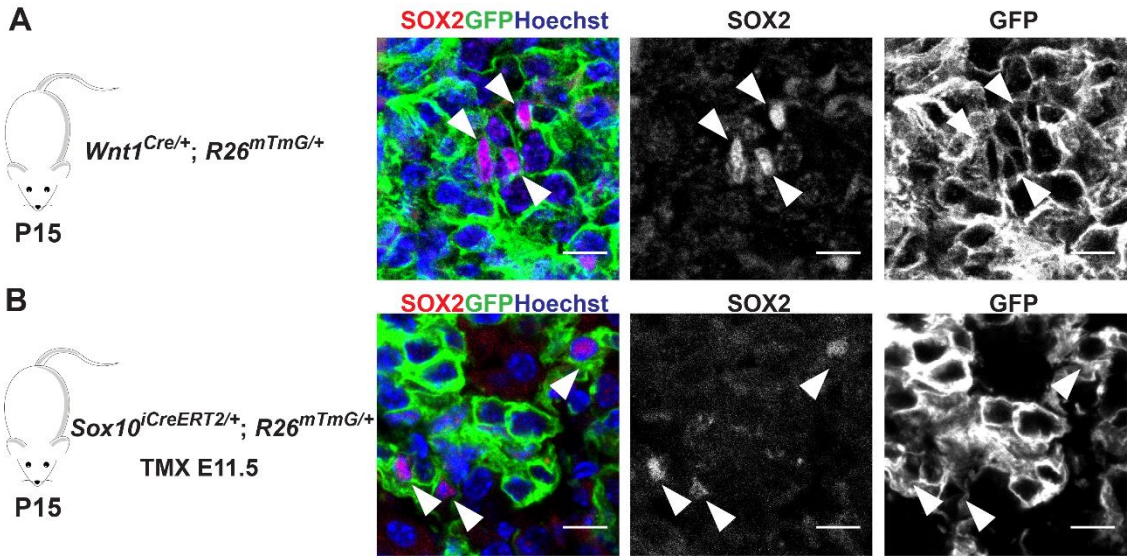

Figure 4.7 Origin of Sox2+ cells.
A) Immunofluorescence on P15 mouse adrenals from a *Wnt1*$^{Cre/+}$; *R26*$^{mTmG/+}$ line – immunostaining shows double positive cells with antibodies anti-SOX2 (red) and anti-GFP (green), arrowheads. B) Immunofluorescence on P15 mouse adrenals from a *Sox10*$^{CreERT2/+}$; *R26*$^{mTmG/+}$ line induced at E11.5 – immunostaining shows double positive cells with antibodies anti-SOX2 (red) and anti-GFP (green), arrowheads. Nuclei stained with Hoechst, scale bars 10µm.

4.2.4 SOX2+ cells can be isolated and cultured *in vitro*

To gain a tool for further investigation, I established an adherent culture system for SOX2+ cells of the adrenal medulla (Figure 4.8A). Using a *Sox2*$^{eGFP/+}$ mouse line, I was able to isolate the GFP+ cells with FACS (Figure 4.8B), which account for about 10% of the cells sorted. After FACS, GFP+ cells were cultured in stem cell promoting media, on tissue culture plastic. Media composition followed the one used to culture SOX2+ mouse pituitary gland stem cells (Gaston-Massuet et al., 2011): DMEM/F12, 5% FBS, cholera toxin, bFGF. Two days after isolation, the medium was topped up. After that, media were fully replaced every 2-3 days. The first colonies were observed after 3-5 days; these were expanded in culture (Figure 4.8C). GFP(SOX2)+ cells can be passaged and are able to form new colonies for at least two passages (Figure 4.8E, F), retaining their GFP expression (Figure 4.8G). This culture system allows for further characterisation of *in vitro* abilities of SOX2+ cells.

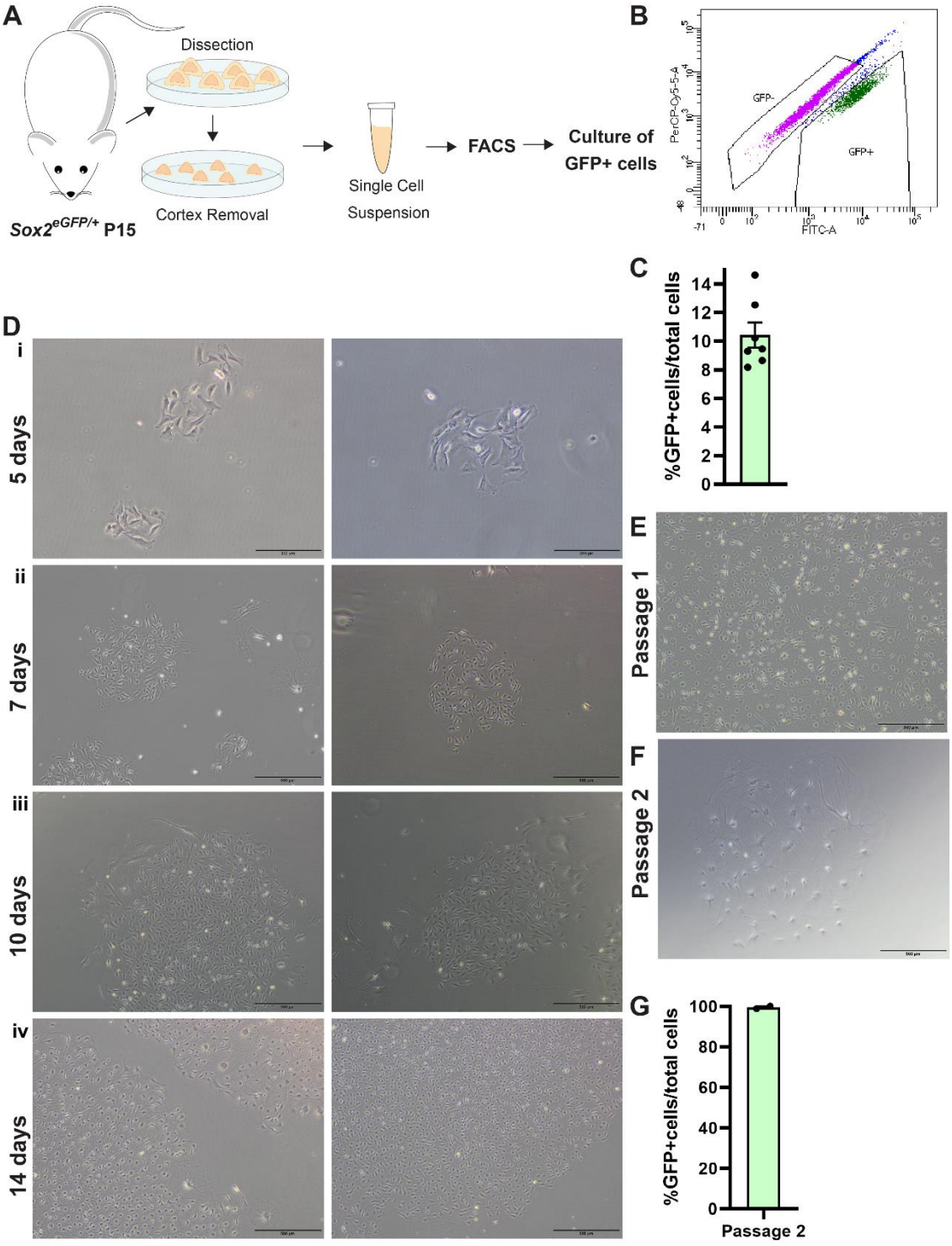

Figure 4.8 Sox2+ cell culture system.
A) Schematic of the experimental design. B) FACS plot showing the gating of GFP+ and GFP- cells. C) Quantification of the percentage of GFP+ cells/total cells isolated with FACS. n = 7, plotted mean and SEM. D) Brightfield images of cultured GFP+ cells: i – 5 days, ii – 7 days, iii – 10 days, iv – 14 days after isolation. Scale bars 200 µm (i) and 500 µm (ii-iv). E) Brightfield image of cultured GFP+ cells after 1 passage, scale bar 500 µm. F) Brightfield image of cultured GFP+ cells after 2 passages, 500 µm. G) Quantification of the percentage of GFP+ cells/total cells counted with flow cytometry after passage 2. n = 2, plotted mean and SEM.

4.2.5 Heterogeneity and signalling signature of SOX2+ cells

To gain insights on the nature and possible heterogeneity of SOX2+ cells I performed 10X single cell RNA sequencing on an enriched population of SOX2+ cells using the *Sox2*$^{eGFP/+}$ mouse line previously described. In brief, 30 adrenals were dissected from 15 *Sox2*$^{eGFP/+}$ P15 mice, cortex was manually removed and, after enzymatic digestion, GFP(SOX2)+ cells were isolated via FACS. Cell suspension was submitted to the KCL Genomics facility for library preparation for 10X Genomics single cell RNA sequencing and Cell Ranger analysis. Using the Seurat package in RStudio, I excluded low quality cells as described in Materials and Methods and obtained a dataset of 1563 cells. Of these, *Sox2* expression was detected in 493 cells, which were selected for final analysis (Figure 4.9A).

Unsupervised clustering shows two transcriptional signatures of *Sox2*+ cells (Figure 4.9B, C) indicating transcriptional heterogeneity within this population. Expression of sustentacular cell markers *S100b*, *Gfap*, *Sox10* and *Plp1* is confirmed in the whole dataset (Figure 4.9D). Top differentially expressed genes of Cluster 0 include *Kank1*, *Ebp41l2* and *Cntrf*, genes involved in cytoskeleton regulation; Notch targets *Heyl* and *Hey2* and insulin-like growth factor binding protein *Igfbp4*. Top differentially expressed genes of Cluster 1 include AP1 signalling components *Fosb* and *Btg2*, long non-coding RNA *Gm26825* and transcription factors *Klf2* and *Klf4*.

To investigate the regulation of *Sox2*+ cells, I used the package CellChat, which allows analysis of all the ligand-receptor interactions of a given dataset. 59.9% of the *Sox2*+ population is involved in secreted signalling, 21.4% in ECM-receptor, 18.7% in cell-cell contact (Figure 4.10A). Cluster 0 has both more interactions (Figure 4.10B) and a highest strength of interaction (Figure 9C), both within the same cluster and with Cluster 1. This,

together with the analysis of top differentially expressed genes, indicates Cluster 0 might have a more active role than Cluster 1.

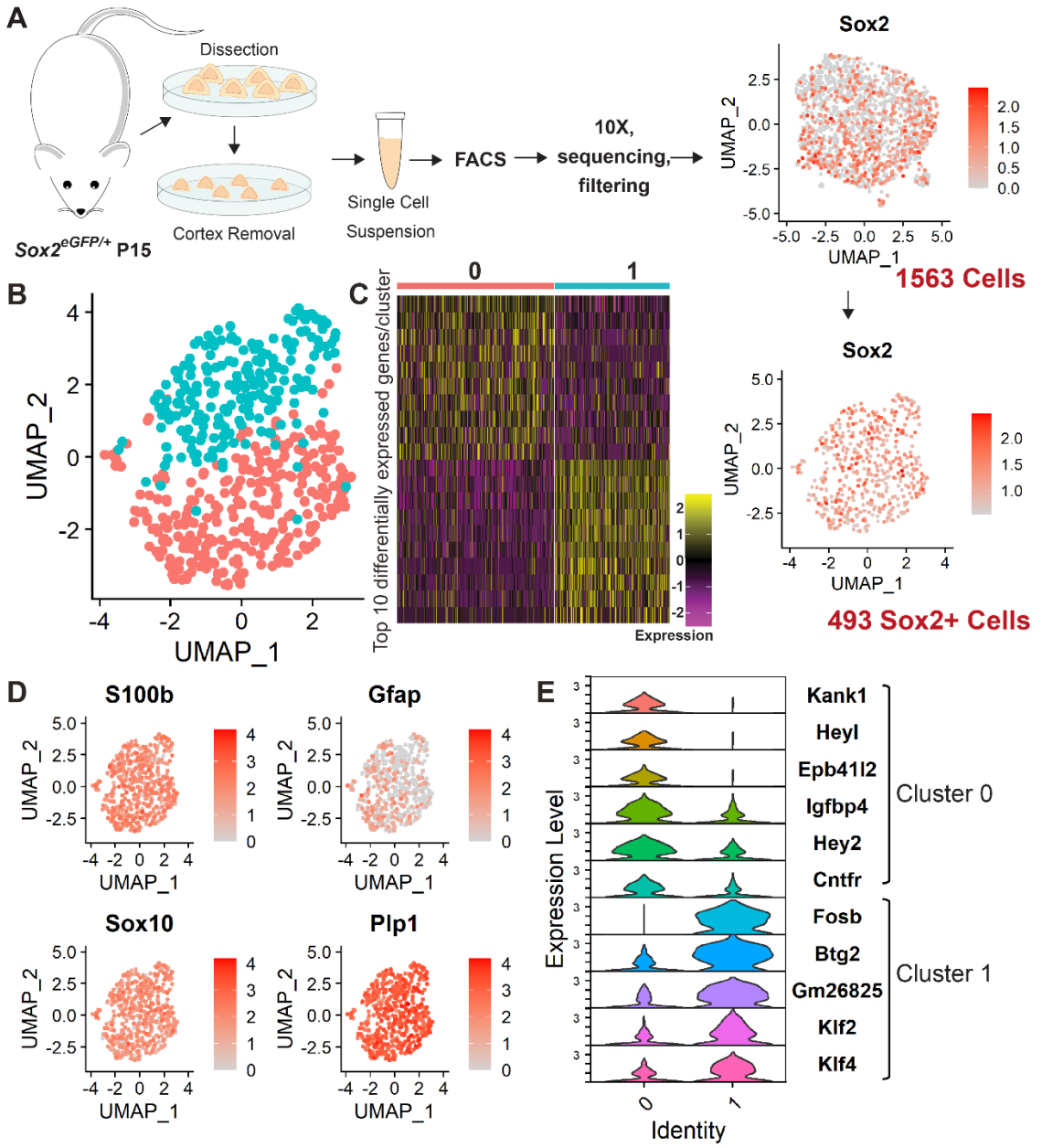

Figure 4.9 Single cell RNA sequencing enriched for Sox2+ cells.
A) Experimental design. B) UMAP showing two clusters. C) Heatmap showing the expression of the top 10 differentially expressed genes for each cluster. Genes shown in Appendix X. D) Feature plots showing the expression of *S100b*, *Gfap*, *Sox10*, *Plp1* in the dataset. E) Violin plots showing the expression of highly expressed genes in each cluster.

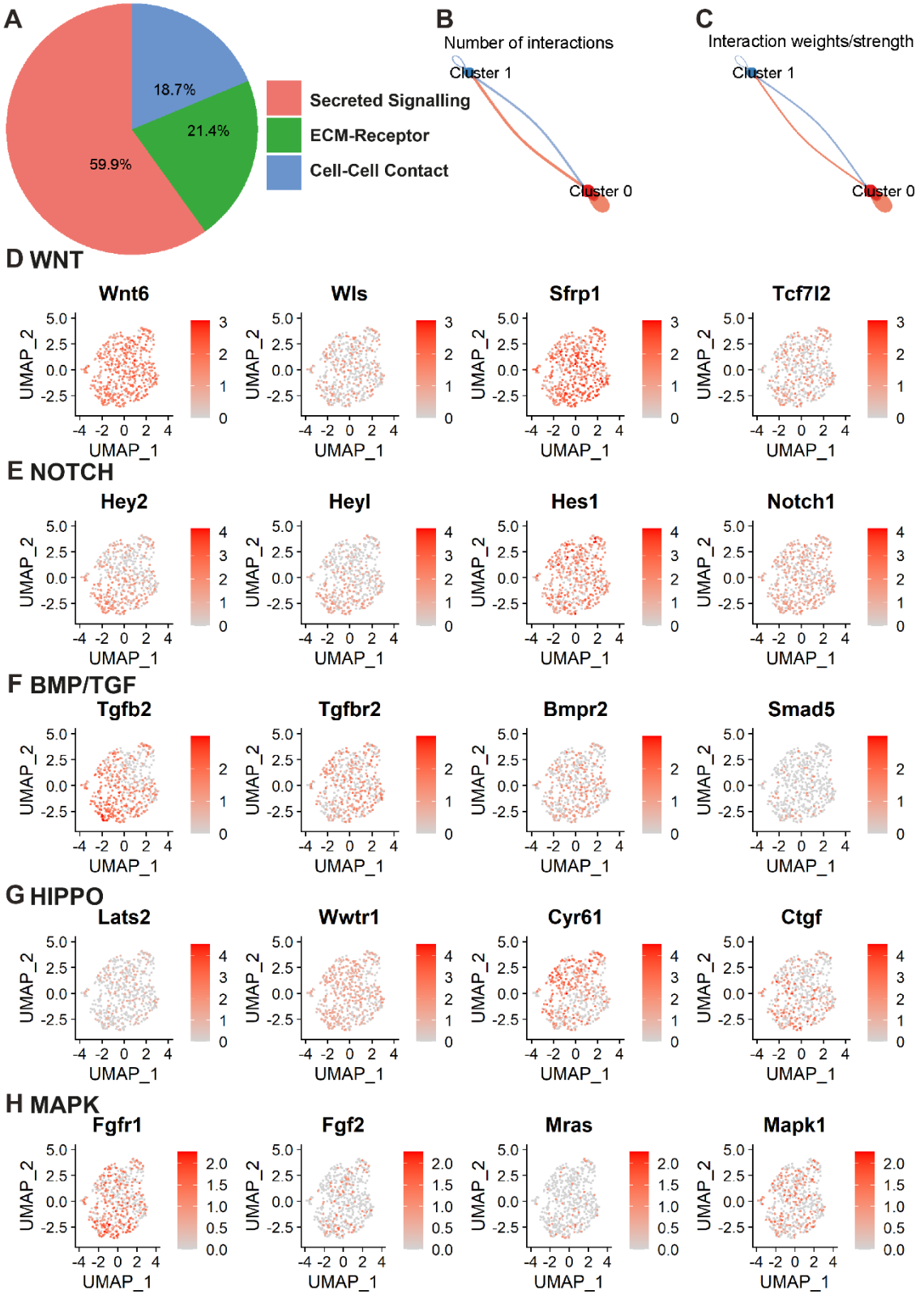

Figure 4.10 Signalling signature of Sox2+ cells.
A) Pie chart of types of ligand-receptor interaction in the dataset. B) Chord plots showing the number of ligand-receptor interactions and the weight/strength of interactions in the dataset. D) Feature plots showing components of the WNT pathway. E) Feature plots showing components of the NOTCH pathway. F) Feature plots showing components of the BMP/TGF pathway. G) Feature plots showing components of the HIPPO pathway. H) Feature plots showing components of the MAPK pathway.

I observed the distribution of components of stem cell-regulating signalling pathways already investigated in the whole medulla dataset in Chapter 1. I find WNT signalling (Figure 4.10D) to be relevant in *Sox2*+ cells, which express both *Wnt6* and *Wls*, indicating these cells are capable of WNT secretion, together with *Sfrp1* but also some of the *Sox2*+ express WNT target/effector *Tcf7l2*. Notch pathway (Figure 9E) targets *Hey2* and *Heyl* expression is higher in Cluster 0, while *Hes1* and *Notch1* are expressed by both clusters, indicating Cluster 0 might be particularly active in responding to Notch signalling. BMP/TGF signalling (Figure 4.10F) shows *Tgfb2* to be expressed more highly in Cluster 0, while other components *Tgfbr2* and *Bmpr2* are expressed ubiquitously, *Smad5* is lowly expressed. Components of the Hippo pathway cascade (Figure 4.10G) *Lats2* and *Wwtr1(Taz)* are expressed throughout the dataset, as well as target *Ctgf*. Hippo target *Cyr61* is differentially expressed in Cluster 1. Molecules associated with MAPK signalling are expressed scattered through the whole dataset including *Mapk1* and lowly *Fgf2* and *Mras*. *Fgfr1* is expressed predominantly in Cluster 0. These observations suggest that the two *Sox2*+ subgroups identified are active in multiple pathways associated with stem cell regulation. I validated some of these findings with RNAscope mRNA in situ hybridisation, where I confirm that *Sox2*+ cells express *Ctgf*, *Cyr61*, *Lats2* and *Wls* (Figure 4.11A, B).

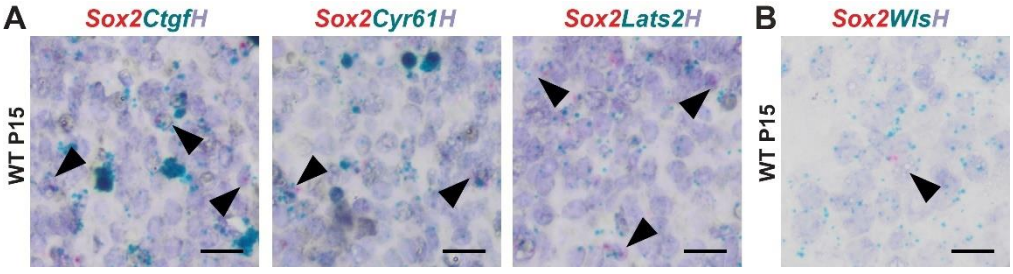

Figure 4.11 Validation of signalling in Sox2+ cells.
A) Double RNAscope on WT P15 samples shows double positive for either *Ctgf*, *Cyr61*, *Lats2* (blue) and *Sox2* (red), black arrowheads. B) Double RNAscope on WT P15 samples shows double positive cells for *Wls* (blue) and *Sox2* (red), black arrowheads. Nuclei counterstained with Hematoxylin, scale bar 10µm.

4.3 Discussion

In this chapter, I performed a characterisation of the SOX2+ cells of the adrenal medulla, established and *in vitro* tool and produced a dataset which will be pivotal for further studies of this population.

4.3.1 SOX2+ cells and sustentacular cells

SOX2+ cells are sustentacular cells of the adrenal medulla and present some overlap with markers of this cell population. As expected, these cells derive from the neural crest and from SOX10+ SCPs, which is consistent with the rest of the gland's origin. While there is clear heterogeneity in the expression of marker genes, it is not known whether subtypes of sustentacular cells have a different function/phenotype, or whether the differential gene expression represents alternative states of differentiation. Further investigation of the transcriptional heterogeneity of this population could be carried out using computational methods, such as looking into the possible intra-sustentacular cell hierarchy with the Velocity tool. An *in vivo* approach using a cell ablation system such as DTA (Diphtheria Toxin A), driven by a sustentacular-specific Cre, such as SOX2, S100B, SOX10 or PLP1 could provide information on cell hierarchy.

4.3.2 SOX2+ cells *in vivo* observations

The presence of SOX2+ cells at different timepoints during the postnatal growth period of the murine and human adrenal medulla follows a trend that is not unexpected for a stem/progenitor cell population. In fact, the highest percentage of SOX2+ cells is observed at the earliest postnatal timepoints analysed, when a most active role for this stem cell

type is to be expected (Cynthia L. Andoniadou et al., 2012). While more extensive analyses have been performed on mouse samples, human samples follow the same trend. Surprisingly, differences between male and female were not observed. From (Bielohuby et al., 2007) it is known that there is a difference in the growth rate of the volume of the adrenal medulla between males and females, but this was just measured on volume. Analysing cell proliferation at different cell types, I conclude that the difference in volume is not due to different proliferation kinetics. This leads to the speculation that the volume differences observed could be due to differences in cell size or an increase in extracellular matrix deposition.

4.3.3 Primary cell culture of SOX2+ cells

I established and *in vitro* system to culture SOX2+ cells in attachment conditions. SOX2+ cells can expand and can be passaged for at least 3 passages. However, despite extensive optimisation, the use of FACS to isolate SOX2+ cells leads to the loss of many cells, possibly due to the stress caused by the technique. Adjustments to the cell culture system to gain higher number of cells could include the use of a substrate to coat the plates for more successful attachment, or the introduction of specific growth factors that could promote cell survival. Preliminary experiments plating the entire medulla without sorting for GFP+ cells indicate this could provide a valid alternative, with 98% of all cells plated expressing GFP after 14 days in culture, but this will need to be assessed further.

4.3.4 Profile of SOX2+ cells

I performed single cell RNA sequencing of SOX2+ cells given the small number of *Sox2* expressing cells in the general dataset. *In vivo* calculations show that at P15 4.95% of all medulla nuclei are SOX2+ (Chapter 4.2.2), while in the "medulla only" dataset, the

percentage of *Sox2*+ cells is 1.3%. This could be given by a discrepancy between mRNA and protein levels, or *Sox2* could be accidentally lost in the bioinformatic analysis because it is a "short" monoexonic gene. To create the "Sox2 enriched" dataset, I used a *Sox2*^{eGFP} mouse line that I had previously validated for protein expression of SOX2 (Chapter 4.2.1). We (together with Thea Willis) mapped *Gfp* in the genome and analysed its expression in the dataset obtained. This was expressed only by some of the cells obtained, possibly indicating this gene could also be easily lost in the process or that the flow sorting protocol was not accurate. To make sure that only correct cells are analysed, I decided to select only for cells with confirmed expression of *Sox2* for further analyses. I find two transcriptional signatures of *Sox2*+ cells: Cluster 0 and Cluster 1. In both clusters, the top differentially expressed genes have a function in cytoskeleton regulation, cell signalling and gene transcription. This confirms the transcriptional heterogeneity previously characterised in sustentacular cells (Chapter 3). I could not find unique markers to separate these two populations *in vivo*, therefore it is more likely that these two transcriptional signatures might indicate two states of the same population. Because of this, further studies regarding cell signalling will be of particular importance. Further investigation using mouse models to modulate key signalling pathways identified, will provide information on the regulation of these cells.

5 SOX2+ cells potential in normal homeostasis

5.1 Introduction

The classical definition of "stem cell" is that of a cell with the ability of differentiation and self-renewal. The presence of specific cells with these abilities has been described from the early embryo throughout pre-natal development, and populations of postnatal stem cells have been described in most of all postnatal organs and tissues. One step below in terms of abilities and potential are populations coined as "progenitors" which share the ability to differentiate into one or multiple cell types but are not able to maintain a reserve of their own population through self-renewal. While stress-responsive progenitors of the adrenal medulla have been described to be able to differentiate to chromaffin cells and neurons *in vitro* (Rubin de Celis et al., 2015), the adrenal medulla is one of few organs with an unknown stem cell and progenitor component in normal homeostasis. There is no evidence showing the occurrence of newly formed chromaffin cells *in vivo* in the postnatal medulla, however the postnatal development of the mouse adrenal medulla shows a significant increase in size with time (Bielohuby et al., 2007), especially within the first 3 postnatal weeks. Results presented in section 4.2.2 show high proliferation between P15 and P21, with then a rapid descend. It has not been established whether this growth is given simply by chromaffin cell proliferating or whether there is a different stem/progenitor cell capable of giving rise to chromaffin cells *in vivo*.

In this chapter, I use *in silico*, *in vitro* and *in vivo* techniques to test whether postnatal SOX2+ cells (characterised in Chapter 4) could act as progenitor or stem cells of the adrenal medulla.

5.2 Results

5.2.1 SOX2+ sustentacular cells show predicted stem cell behaviour *in silico*

To assess the stem cell potential of *Sox2*+ cells *in silico*, we used the Velocity package, which assesses the differentiation state in a single cell sequencing dataset based on splicing. This analysis was performed with Dr Val Yianni. Single cell RNA sequencing original whole medulla dataset was re-analysed to accommodate further data interrogation (Figure 5.1A). Two terminal states were identified as sustentacular cells (*Plp1*+) and chromaffin cells (*Pnmt+ Penk+*) (Figure 5.1B). RNA velocity analysis suggests that sustentacular cells transition to chromaffin cells through multiple intermediate states (Figure 5.1C) and that they can give rise to both noradrenaline-producing (*Penk*+) and adrenaline-producing (*Pnmt*+) chromaffin cells. To investigate where *Sox2*+ cells fall in this transition, we integrated the whole medulla dataset with the *Sox2*-enriched dataset previously described (Figure 5.1D, E, F). RNA velocity analysis shows *Sox2* expressing cells incur in a similar transition to the one observed in sustentacular cells in the whole medulla dataset (Figure 1G), giving rise to multiple intermediate states, which then progress to terminally commit to both the adrenaline- and noradrenaline- producing chromaffin cell lineages.

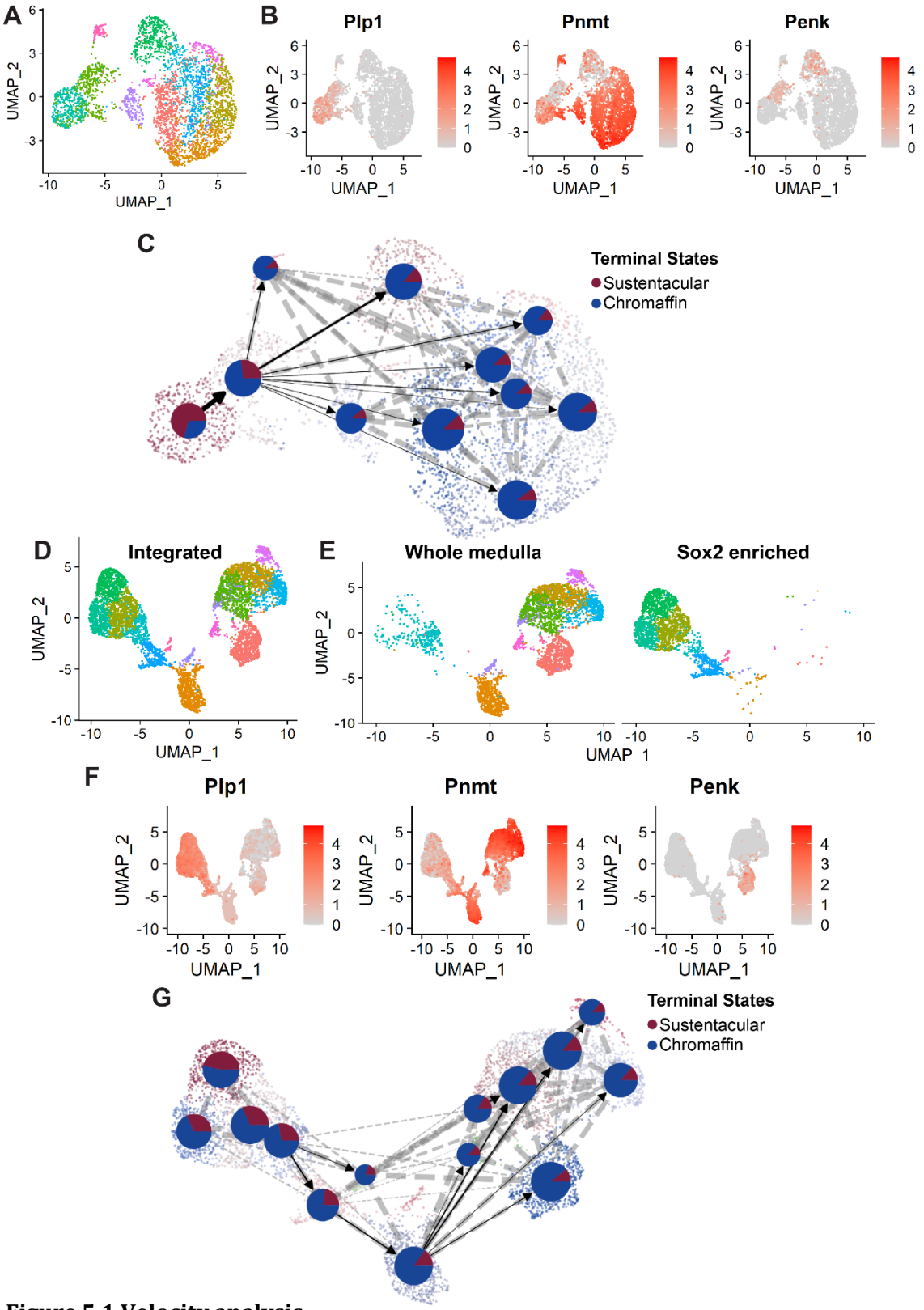

Figure 5.1 Velocity analysis.
A) UMAP of whole medulla dataset reanalysed for Velocity. B) Feature plots showing expression of *Plp1*, *Pnmt*, *Penk* in whole medulla dataset. C) Velocity analysis of whole medulla dataset – pie charts indicate probability that the cluster would progress towards each of the terminal states. D) UMAP of integrated whole medulla and Sox2 enriched dataset. E) Split UMAP of the integrated whole medulla and Sox2 enriched dataset. F) Feature plots of *Plp1*, *Pnmt*, *Penk* in the integrated dataset. C) Velocity analysis of the integrated dataset.

5.2.2 SOX2+ sustentacular cells can self-renew and differentiate *in vitro*

To validate the abilities of Sox2+ cells indicated *in silico*, I first designed *in vitro* experiments to test the self-renewal and differentiation potential of these cells. I used the previously described culture system with a *Sox2*$^{eGFP/+}$ mouse line and isolated GFP+ from GFP- cells. Frist, I plated both populations in clonal conditions and performed crystal violet assay to visualise the colonies formed after 14 days in culture (Figure 5.2A). No colonies were observed in the GFP-(SOX2-) wells, which include differentiated cell types such as chromaffin, neuronal cells, endothelial cells, as well as any SOX2- sustentacular cell population (such as S100B+SOX2-, GFAP+SOX2-, SOX10+SOX2- cells). The GFP+ (SOX2+) population were the only cells able to produce colonies, indicating these cells have a unique potential. I used immunofluorescence to determine that after 14 days the majority of cells forming the colonies are still GFP+ and SOX2+ (Figure 5.2B) and quantified with flow cytometry that an average of 98.94% of the expanded cells in the colonies remain GFP+ at 14 days (Figure 5.2C). These results show SOX2+ cells are capable of giving rise to new SOX2+ cells, indicating the ability to self-renew.

While GFP- populations are not capable of giving rise to colonies in these culture conditions, a small percentage of GFP- cells are found in the colonies formed by GFP+ cells. This suggests GFP+ (SOX2+) cells can give rise to a GFP- (SOX2-) population *in vitro*, consistent with the *in silico* analyses that suggest SOX2+ cells are upstream of chromaffin cells. To investigate this, I used a *Sox2*$^{CreERT2/+}$*;R26*$^{Tomato/+}$ lineage tracing system combined with immunofluorescence staining to identify SOX2-derived differentiated progeny *in vitro*. In brief, I induced mice at P13 with a single injection of TMX, harvested the adrenals at P15 and cultured primary medulla cells (Figure 5.2D). 7 days after culture I detected the presence of Tomato+TH+ chromaffin cells and Tomato+TuJ1+ neurons.

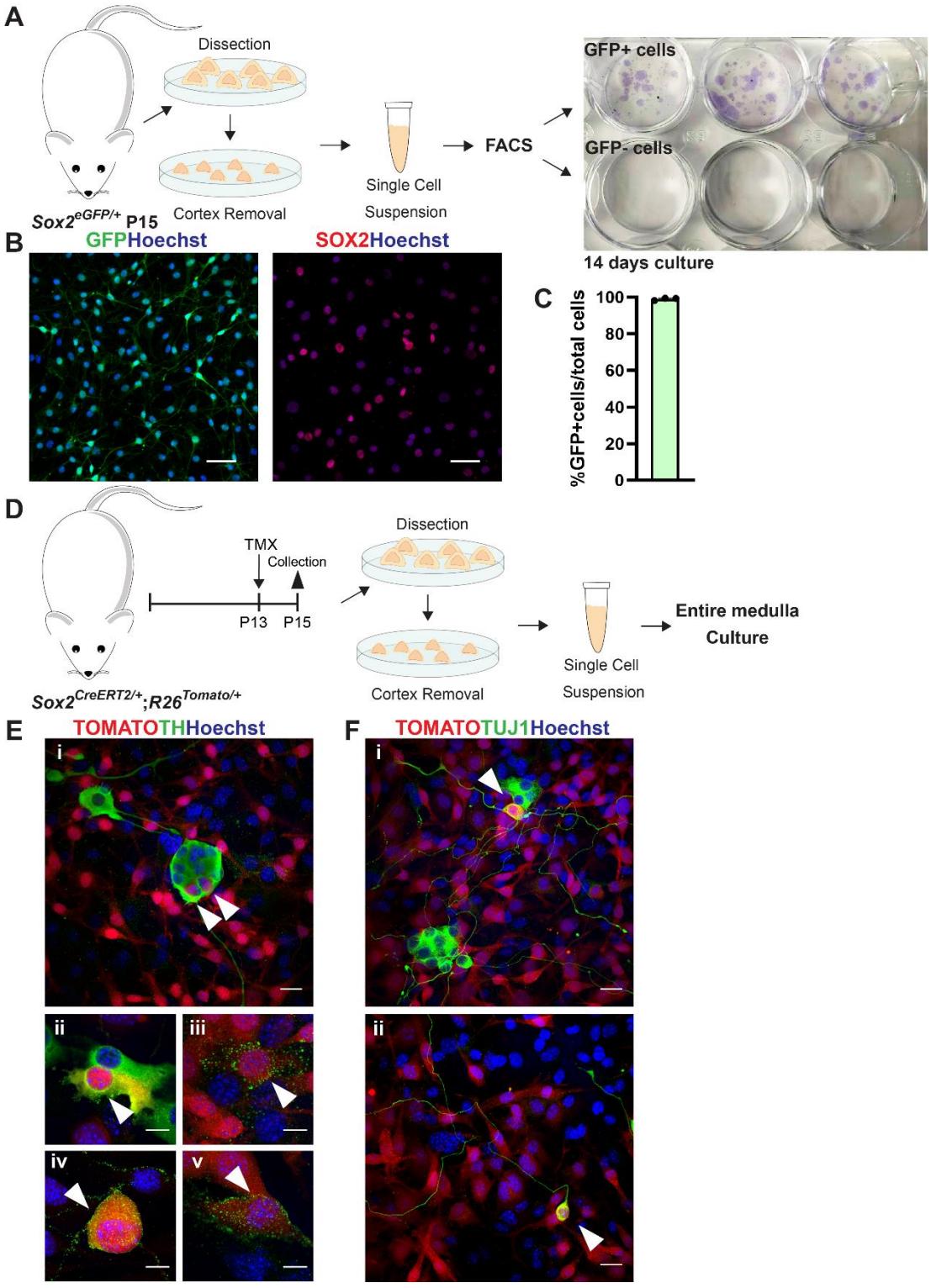

Figure 5.2 SOX2+ cells potential *in vitro*.
A) Experimental design and crystal violet assay. B) Immunofluorescence staining of GFP+ primary cells from *Sox2*$^{eGFP/+}$ medulla cultured for 14 days: GFP (green), SOX2 (red), nuclei stained with DAPI, scale bar 50µm. C) Quantification of GFP+ cells via flow cytometry after 14 days of culture, n = 2. D) Experimental design for lineage tracing. E) Immunofluorescence of primary cells from *Sox2*$^{CreERT2/+}$; *R26*$^{Tomato/+}$ medulla after 7 days of culture: Tomato is in red, TH antibody staining in green. Nuclei stained with DAPI, scale bar 20µm (i), 10µm (ii – v). F) Immunofluorescence of primary cells from *Sox2*$^{CreERT2/+}$; *R26*$^{Tomato/+}$ medulla. Tomato is in red, TuJ1 staining in green. Nuclei stained with DAPI, Scale bar 20µm.

This indicates that SOX2+ cells are capable of differentiation *in vitro*, maintaining the ability to give rise to both neurons and chromaffin cells, as do embryonic SCPs.

5.2.3 SOX2+ cells give rise to an expanding and differentiating population *in vivo*

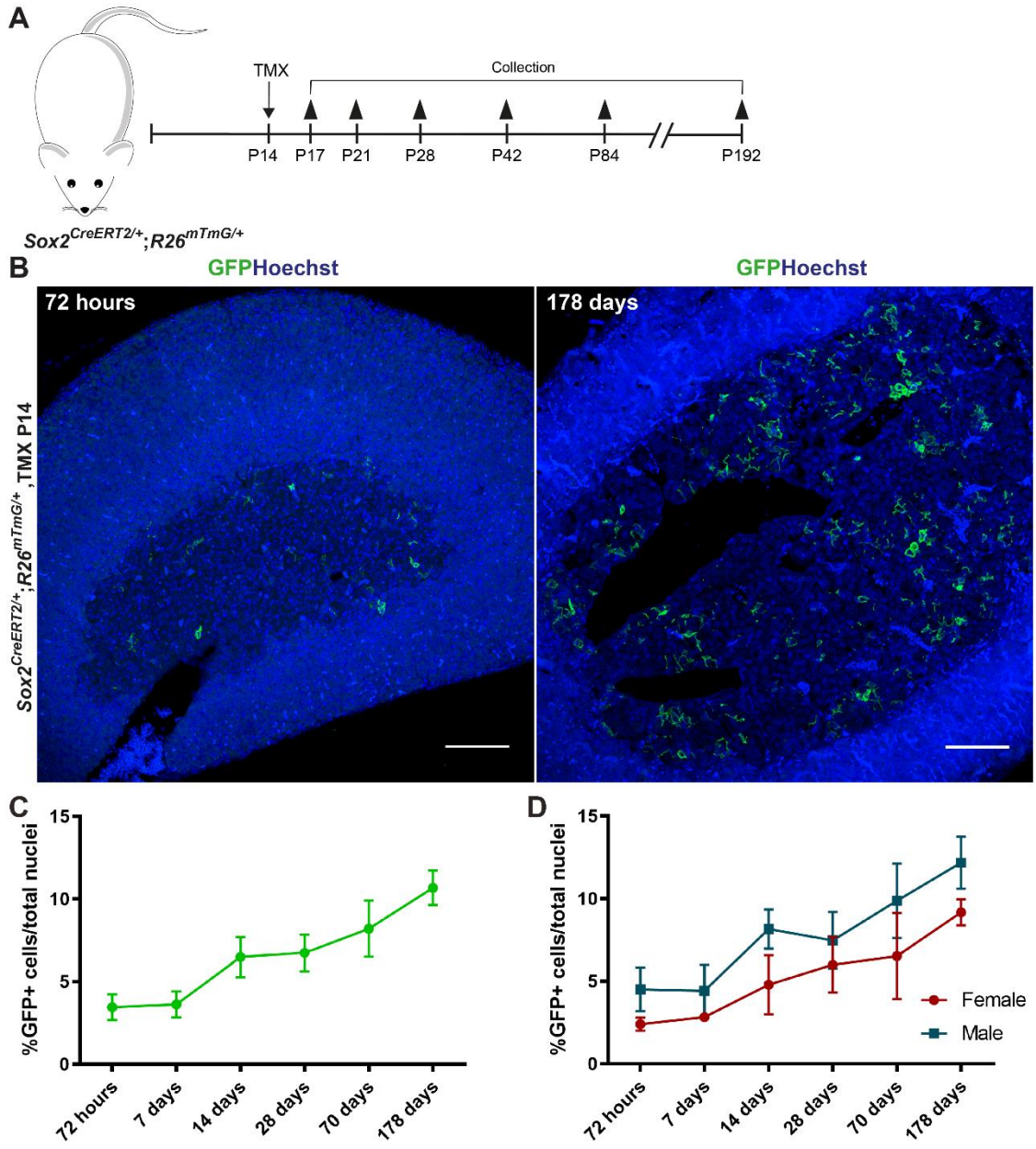

Figure 5.3 SOX2+ cells potential give rise to an expanding population *in vivo*.
A) Experimental design indicating tamoxifen (TMX) induction at P14 and all time points analysed. B) Immunofluorescence of *Sox2*$^{CreERT2/+}$;*R26*$^{Tomato/+}$ induced at P14, collected after 72 hours and 178 days. GFP in green, nuclei stained in Hoechst, scale bar 100µm. C) Quantification of GFP+ cells/total nuclei of adrenal medulla at different timepoints. n = 6 for each timepoint, plotted mean and SEM. D) Quantification of GFP+ cells/total nuclei of adrenal medulla at different timepoints, split by sex. n = 3 per sex, per timepoint, plotted mean and SEM.

To investigate whether the abilities of Sox2+ cells shown *in vitro* and *in silico* are conserved and relevant *in vivo*, I used lineage tracing with a $Sox2^{CreERT2/+};R26^{mTmG/+}$ to follow Sox2+ cells and their progeny in *vivo*. In brief, I induced the mice with a single Tamoxifen injection at P14, and collected samples after 72 hours, 7 days, 14 days, 28 days, 70 days and 178 days (Figure 5.3A). Using immunofluorescence with antibodies against GFP, I show that Sox2+ cells are giving rise to an expanding population *in vivo* (Figure 5.3B, 4A). I quantified the % of GFP+ cells in the whole medulla: 72 hours after induction GFP+ cells account for an average of 3.4% of the whole medulla; at 7 days there is a small increase to 3.72%, which increases further at 14 days to 6.47%; 28 days after induction to 6.73%, 70 days to 8.20%, 178 days to 10.67% (Figure 5.3B). I investigated the differences

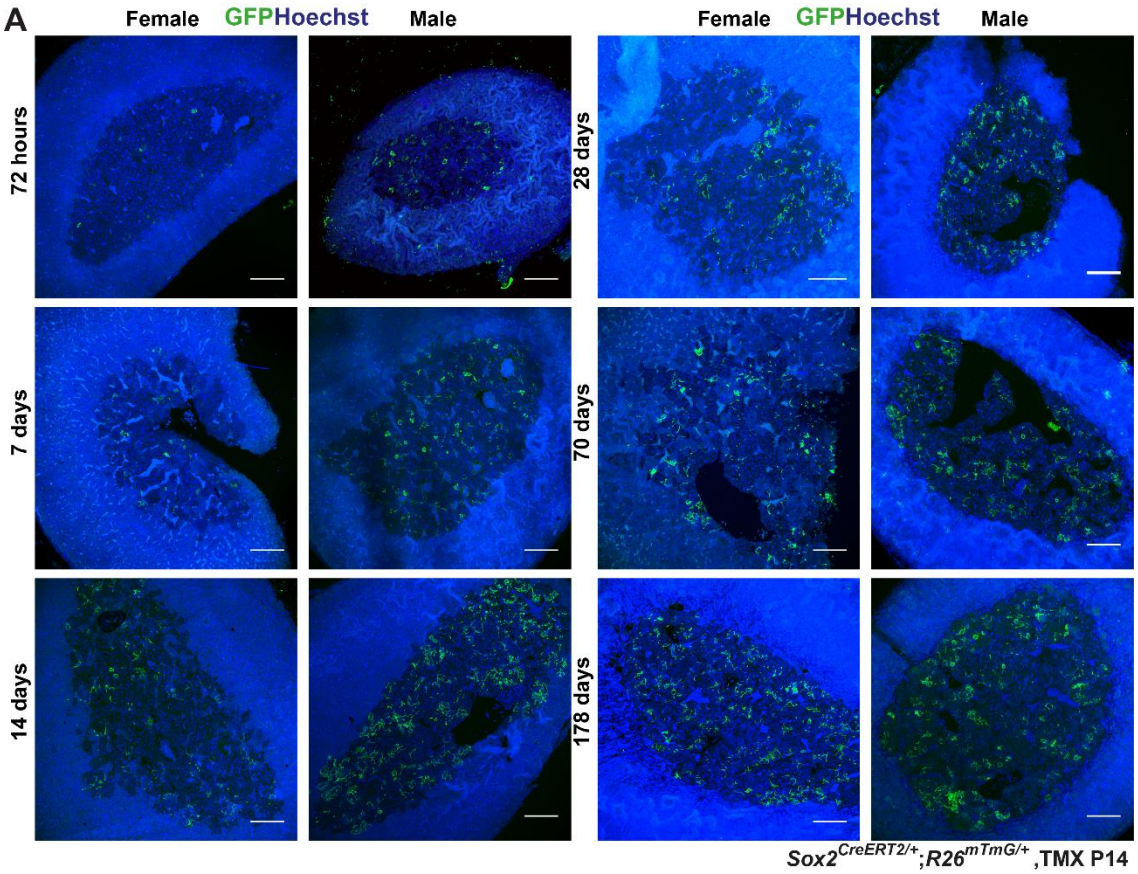

Figure 5.4 SOX2+ cells potential give rise to chromaffin cells *in vivo*.
A) Immunofluorescence of $Sox2^{CreERT2/+};R26^{Tomato/+}$ induced at P14, collected after 72 hours, 7 days, 14 days, 28 days, 70 days and 178 days. GFP in green, nuclei stained in Hoechst, scale bar 100µm.

between sexes and found no significant difference (Figure 5.3D and 5.4A). From my observations, female samples have an overall lower percentage of GFP+ cells but the trend among timepoints is comparable to males. Results presented in Chapter 4 (section 2.2) indicate that the proportion of SOX2+ cells decreases over time, while the absolute number of GFP+ cells traced from SOX2+ cells increases over time. This suggests that SOX2+ cells are giving rise to SOX2- cells. To prove this, I used immunofluorescence on 178 day-traces of *Sox2*$^{CreERT2/+}$;*R26*$^{mTmG/+}$ induced at P14 to show TH+, PENK+ and PNMT+ cells within the traced GFP+ population (Figure 5A, B, C). This confirms Sox2+ cells residing in the postnatal adrenal medulla give rise to both adrenaline- and noradrenaline-producing chromaffin cells *in vivo* during normal organ growth. 365 days after lineage tracing induction, SOX2+ cells were still present within the traced population (Figure 5.6A), confirming that this stem/progenitor population is long-lived, either due to persistence of positive cells from P14, or through self-renewal generating new SOX2+ cells over time.

To assess if the capacity of SOX2+ cells to generate chromaffin cells is a property of these cells only during the early postnatal period, I induced Sox2-expressing cells from *Sox2*$^{CreERT2/+;}$ *R26*$^{mTmG/+}$ mice with tamoxifen at P133 and analysed the GFP contribution after 28 days. Double immunofluorescence staining using antibodies against GFP and TH, reveals that *Sox2*+ cells are still capable of giving rise to TH+ chromaffin cells (Figure 5.6B).

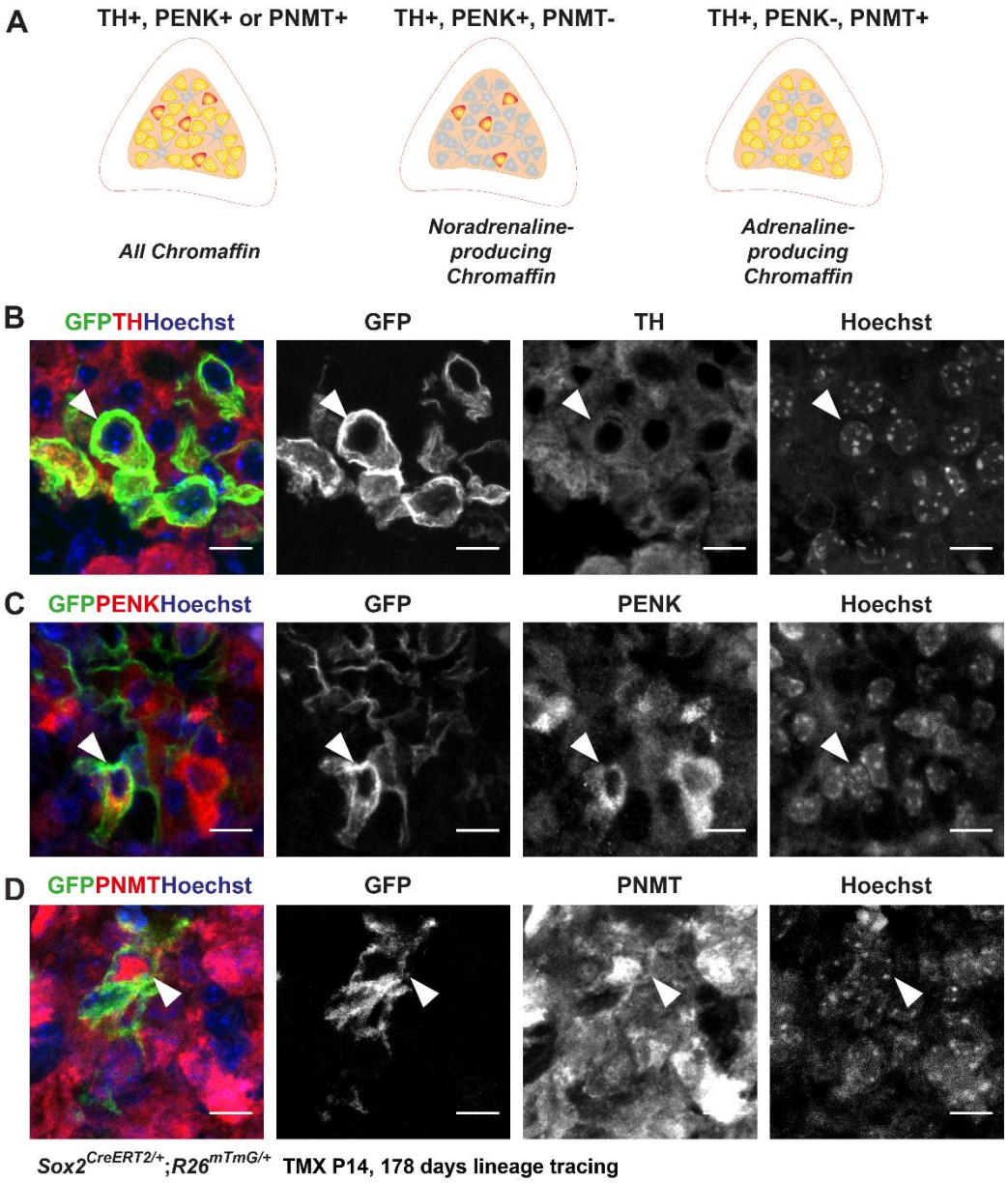

Figure 5.5 SOX2+ cells have the potential give rise to chromaffin cells *in vivo*.
A) Double immunofluorescence on *Sox2*$^{CreERT2/+}$;*R26*$^{Tomato/+}$ induced at P14, collected after 178 days. GFP shown in green, TH in red, nuclei counterstained with Hoechst (blue), scale bar 10µm. B) Double immunofluorescence on *Sox2*$^{CreERT2/+}$;*R26*$^{mTmG/+}$ induced at P14, collected after 178 days. GFP shown in green, PENK in red, nuclei counterstained with Hoechst, scale bar 10µm. C) Double Immunofluorescence of *Sox2*$^{CreERT2/+}$; *R26*$^{mTmG/+}$ induced at P14, collected after 178 days. GFP in green, PNMT in red, nuclei counterstained in Hoechst, scale bar 10µm.

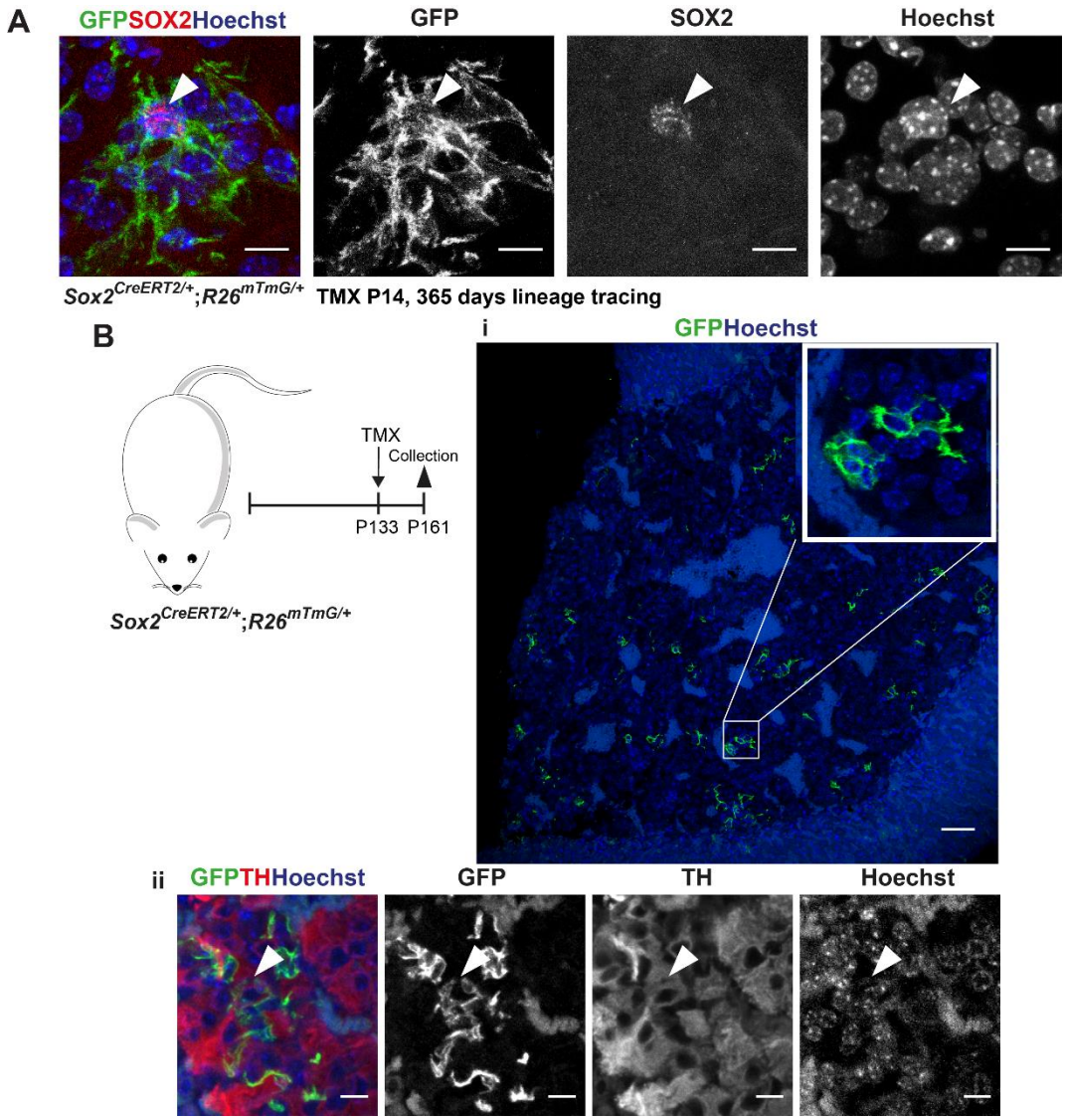

Figure 5.6 SOX2+ cells are a long-lived population capable of generating new cells. A) Double Immunofluorescence on *Sox2*$^{CreERT2/+}$;*R26*$^{mTmG/+}$ adrenals induced at P14 and collected after 365 days. GFP in green, SOX2 in red, nuclei counterstained in Hoechst, scale bar 10µm. B) Double Immunofluorescence on *Sox2*$^{CreERT2/+}$;*R26*$^{mTmG/+}$ adrenals induced at P133, collected after 28 days. i - GFP in green, nuclei counterstained in Hoechst, scale bar 100µm. ii – GFP in green, TH in red, nuclei counterstained in Hoechst, scale bar 10µm.

5.3 Discussion

The data presented in this chapter establishes that SOX2+ cells of the adrenal medulla show stem cell properties *in silico*, *in vitro* and *in vivo*.

5.3.1 In silico

In silico prediction with Velocity on the previously described "medulla only" dataset shows that sustentacular cells can give rise to the differentiated populations of the adrenal medulla. Merging this dataset with the ''SOX2 enriched'' dataset, shows that SOX2+ cells are also predicted to give rise to differentiated progeny, therefore indicating that SOX2+ cells could be a stem/progenitor population of the adrenal medulla in normal homeostasis. From this initial analysis, it is unclear whether sustentacular cells transition towards either to an adrenaline- or noradrenaline-producing fate and whether these two cell types can commit further into one another. From the "medulla only" analysis, this seems to be the case, but when this dataset is integrated with the "Sox2 enriched" dataset, the prediction changes to all sustentacular cells first transitioning towards a PNMT+ population. Further investigation on this is needed, and transcriptional comparisons between the transitionary populations identified by Velocity and determined as such following marker-based Seurat analysis need to be defined. An in-depth analysis using the Velocity tool could reveal significant insights on the transitory populations identified and possibly provide information regarding any hierarchy within the sustentacular cells. A limitation of this analysis is that *Sox2* is not identifiable because of its monoexonic nature and therefore it cannot be spliced, however, cells expressing *Sox2* could be mapped to the Velocity plots after extracting the cell name from the Seurat analysis.

5.3.2 In vitro

In vitro experiments confirmed the stem cell potential of SOX2+ cells. In stem cell promoting conditions, only SOX2+ cells are capable of giving rise to colonies. This result provides a strong indication that SOX2+ cells might be upstream or more relevant than SOX2 negative sustentacular cells, making the cell type marked by SOX2 a population with unique potential. This cell culture model was established based on anterior pituitary gland stem cell culture, therefore it is expected to promote the survival of cells of similar nature, however it cannot be excluded that different culture conditions could give different results. An encouraging observation is that within the SOX2 negative populations, some cells attach; however, they do not expand and die after a few days in culture, potentially indicating that the culture conditions might be permissive for other cell types, but these simply do not have the ability to proliferate in culture.

Expanding colonies of SOX2+ cells give rise to almost exclusively (98.94%) cells that retain SOX2 expression, indicating these cells can self-renew and therefore show a pivotal characteristic of stem cells. Investigation of further markers known to be expressed by SOX2+ cells *in vivo* could indicate whether the cells maintain a similar gene expression to the initial cells plated, confirming self-renewal as more than just the expression of SOX2 and its GFP reporter. This could be done with qPCR, analysing the expression of genes identified in the "SOX2 enriched" single cell RNA sequencing dataset. However, the sporadic nature of these cells paired with the loss of cells during flow sorting, does not allow for high numbers of cultured cells, and attempts at mRNA extraction resulted in modest yields. Comments on the improvements of the culture system were made in section 4.3.3. Given the observed transcriptional heterogeneity of SOX2+ cells described in Chapter 4, further investigation on whether all the SOX2+ cells have the same ability to form colonies and self-renew. This could be assessed by immunofluorescence, to

determine the expression pattern of the top differentially expressed genes of the SOX2+ clusters identified via single cell RNA sequencing.

When analysing SOX2+ cultures after 14 days, there is a small percentage of SOX2 negative cells emerging (1.06%), indicating differentiation might occur in culture. I used a lineage tracing system to investigate whether SOX2+ cells could give rise to differentiated derivatives and found TH+ chromaffin cells and TUJ1+ neurons within the traced cells, indicating SOX2+ cells can give rise to both these cell types *in vitro*. Further investigation is needed to assess whether SOX2+ cells can give rise to both adrenaline- and noradrenaline-producing chromaffin cells, which can be achieved with the same system, performing immunostaining for PNMT (adrenaline-producing chromaffin cells) and PENK (noradrenaline-producing chromaffin cells). The expression of differentiated cell markers is not enough to establish whether SOX2+ cells are giving rise to functional chromaffin cells and neurons *in vitro*. Measurements of secretion of catecholamines in the culture media could assess chromaffin cell maturation, while for neurons, the presence of an action potential could determine whether these are functional cells.

5.3.3 In vivo

Using an *in vivo* lineage tracing system, I established that SOX2+ cells give rise to an expanding and differentiated population, indicating these cells act as stem/progenitor cells *in vivo* in normal homeostasis. This finding provides the first evidence that new chromaffin cells arise postnatally, and that a stem/progenitor population in the adrenal medulla in normal homeostasis.

Lineage tracing of SOX2+ cells shows an expansion of derivatives over time, consistent with the postnatal growth of the adrenal. This indicates that SOX2+ cells actively

contribute to the postnatal growth of the gland by giving rise to chromaffin cells. I investigated the difference between male and female and found no significant distinction in the trend over time, however the number of cells traced in females is lower than in males. Knowing from the analysis in Chapter 4 that the number of SOX2+ cells is not sex dependant, I can assume the difference lies in the recombination efficiency. Administration of tamoxifen was performed based on the mouse weight consistently in males and females, therefore no experimental differences related to this were expected. This discrepancy could be explained by the interaction of some sex specific hormones or competition at receptor level with tamoxifen, or by a difference in blood supply. Anatomical variance of the arterial supply in rat adrenals has been identified (Kigata & Shibata, 2017), however, this is not sexually dimorphic. Further detailed morphological analyses of the same samples could be performed to clarify the differences observed.

I showed that SOX2+ cells can differentiate into adrenaline- and noradrenaline-producing chromaffin cells. I was not able to establish whether SOX2+ cells differentiate to neurons *in vivo* as I was unable to find any cell body in the adrenal medulla, as discussed in Chapter 3. Due to limitations with immunostaining, I was not able to determine whether SOX2+ cells can give rise SOX2-S100B+, SOX2-SOX10+ sustentacular cells. Such experiments would confirm the *in vitro* observation that SOX2+ cells might be upstream of SOX2-sustentacular cells in terms of potential.

I induced lineage tracing in adult mice and determined that SOX2+ cells maintain their potential to give rise to chromaffin cells after the growth of the adrenal has reached a plateau, consistent with a stem/progenitor role. Analysis of this potential at multiple timepoints could provide information regarding the role of SOX2+ cells over time.

6 SOX2+ adrenomedullary cells in challenge and disease

6.1 Introduction

Beyond the classical definition of "stem cell" based on differentiation and self-renewal potential, postnatal stem and progenitor cells normally have the function to protect the organ or tissue from disruption of homeostasis and disease. This is normally achieved by regulating the differentiation into specialised cell types based on organ hyperfunction or hypofunction (Bornstein et al., 2019; Rubin de Celis et al., 2015; Steenblock et al., 2018; Werdermann et al., 2021). In case of tumorigenesis, stem cells can be directly affected by a mutation and initiating cancer, or they could be indirectly affected by the environment (Batlle & Clevers, 2017). To investigate whether the SOX2+ component of the adrenal medulla shows a behaviour in line with a stem/progenitor cell nature, obtained mouse adrenals from multiple organ challenge systems, and human samples of adrenomedullary tumour and Cushing's disease. Studies in rats have shown that following chronic stress, chromaffin cells present cellular hyperplasia, while there is no difference in proliferation and TH levels (Ulrich-Lai et al., 2006). Metabolic stress such as obesity leads to a reduction of adrenaline secretion (Del Rio, 2000).

Pheochromocytomas (PCCs) are chromaffin cell tumours that arise within the adrenal medulla and are often found in conjunction with paragangliomas (PPGLs), which affect extra-adrenal paraganglia. These tumours are characterised by excessive catecholamine production, leading to hypertension, arrhythmia and stroke. About 25% of PCCs/PGLs are malignant and form metastases in non-chromaffin tissues, leading to limited treatment

options and poor prognosis (Ayala-Ramirez et al., 2011). PPGLs are classified in different subgroups based on their somatic or germline mutations: “pseudohypoxia”, including somatic and germline mutations in subunits of SDH (SDHx), VHL and EPAS1; “signalling”, including somatic and germline mutations in NF1, RET, TMEM127, HRAS and present increased expression of RAS-MAPK signalling pathway; and “WNT altered” which presents somatic mutations that lead to an elevated WNT pathway response (Fishbein et al., 2017a).

In this chapter, I investigate the expression of SOX2 in mouse models of obesity, acute and chronic stress and in human samples of different types of pheochromocytoma.

6.2 Results

6.2.1 Acute stress

To assess potential implications of SOX2+ cells during adaptation to stress, I analysed the adrenal medulla from mice that underwent acute stress. In brief, 8 weeks old males were restrained for 2 hours per day for 6 days, adrenals were collected on day 6 (Figure 6.1A). Ilona Berger designed and performed the stress experiments. Using immunohistochemistry, I labelled SOX2+ cells and counted these across 9 sections from 3

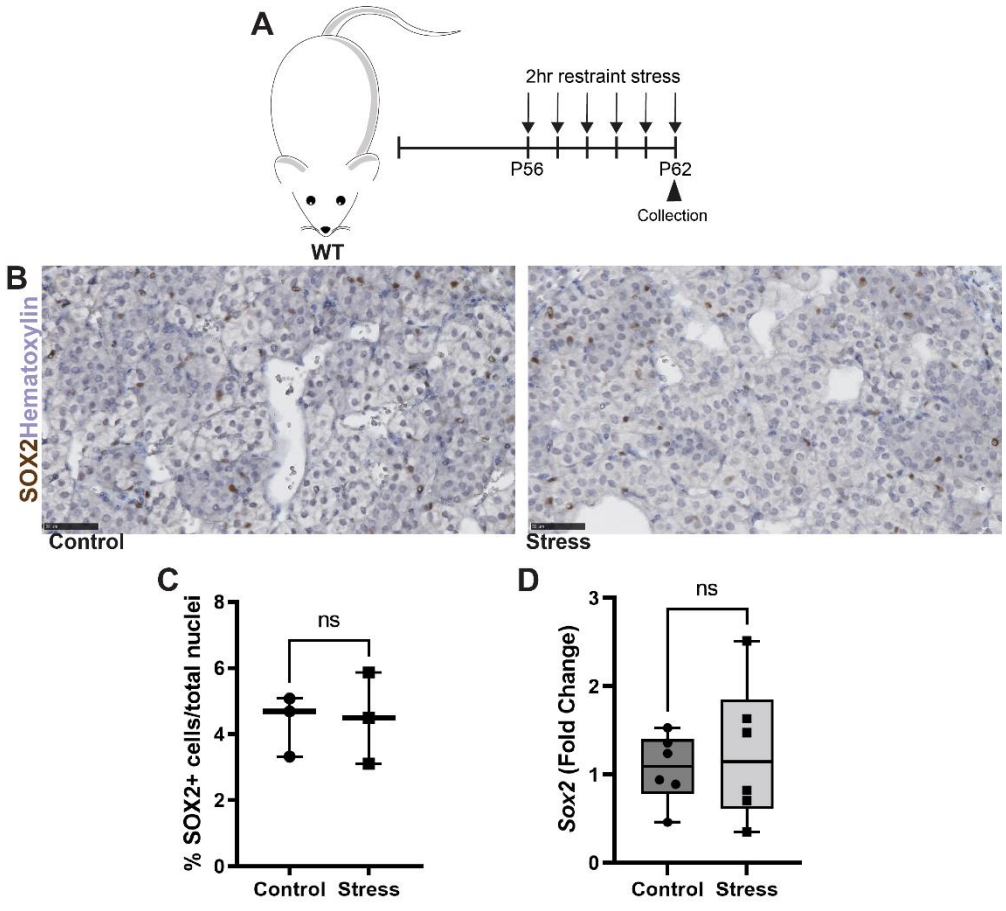

Figure 6.1 SOX2+ cells in acute stress.
A) Experimental design. B) Immunohistochemistry with antibodies against SOX2 (brown) in stress and control samples. Nuclei counterstained with Hematoxylin, scale bar 50µm. C) Quantification of SOX2+ cells over the total nuclei of adrenal medulla. n = 3 each, plotted mean and SEM, Unpaired t test with Welch's correction. P value = 0.9026. C) mRNA expression of *Sox2*. n = 3, 2 repeats each, plotted mean and SEM. Unpaired t test with Welch's correction. P value = 0.6311

adrenal medullae. SOX2+ cells were expressed as a percentage of the total nuclei, identified by counterstaining by Hematoxylin (Figure 6.1B, C). No significant difference in the percentage of SOX2+ cells was observed between stressed (4.49%) and control (4.36%) samples. Ilona Berger performed qPCR to investigate expression levels of *Sox2* (Figure 6.1D) and found no significant difference between stress (1.07 fold change) and control samples (1.25 fold change), n = 3 each.

6.2.2 Chronic stress

To investigate further the role of SOX2+ cells in stress, I analysed samples from animals that have undergone chronic stress, using the chronic social defeat paradigm. In brief, 7 week-old mice underwent social defeat stress as described in (Lopez, Brivio, Santambrogio, Donno, et al., 2021) for 21 days. After a recovery time of 48 hours, adrenals were collected (Figure 6.2A). Stress experiments were designed and performed by Dr Juan Pablo Lopez. After immunohistochemistry, I counted the number of SOX2+ cells across 3 comparable sections per sample and then calculated the percentage of SOX2+ cells over the total medulla and found the percentage of SOX2+ cells is significantly lower in stressed samples (3.70%), compared to control (5.53%) (Figure 6.2B, C).

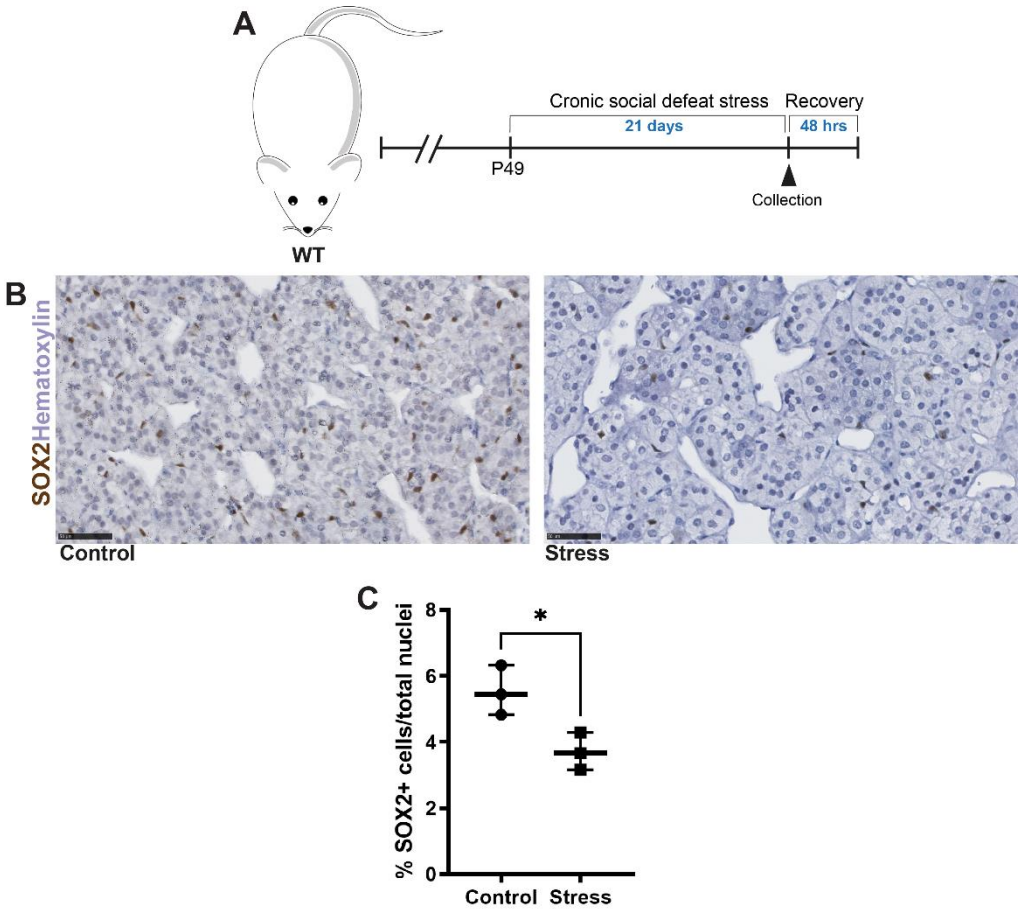

Figure 6.2 SOX2+ cells in chronic stress.
A) Experimental design. B) Immunohistochemistry with antibodies against SOX2 (brown) in stress and control samples. Nuclei counterstained with Hematoxylin, scale bar 50µm. C) Quantification of SOX2+ cells over the total nuclei of adrenal medulla. n = 3 each, plotted mean and SEM, Unpaired t test with Welch's correction. P value = 0.0318.

6.2.3 Obesity

I used *ob/ob* obese mice as another model of metabolic challenge. These mice present a spontaneous mutation which results in a homozygous null for the gene *Lep*, which codes for the protein Leptin, a hormone responsible for inhibiting hunger. *Ob/ob* mice present multiple metabolic dysfunctions, including obesity, hyperphagia, hyperglycaemia, glucose intolerance, elevated plasma insulin and increased hormone production from pituitary and adrenal glands (Garris & Garris, 2004; Lindström, 2007). Using lean wild type age- and background-matched controls, I analysed medulla from *ob*/*ob* 10 months old males and and with immunohistochemistry I counted the percentage of SOX2+ cells (Figure 6.3A, B) and found no significant difference between *ob*/*ob* (4.9%) and lean control (4.43%).

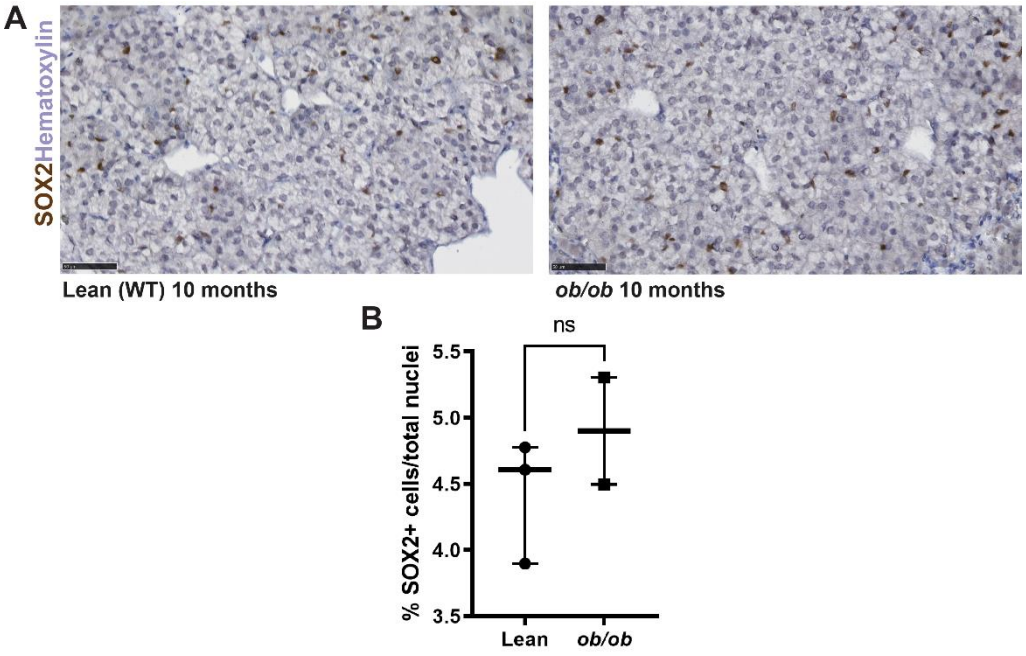

Figure 6.3 SOX2+ cells in obesity.
A) Immunohistochemistry with antibodies against SOX2 (brown) in *ob/ob* and control samples. Nuclei counterstained with Hematoxylin, scale bar 50µm. B) Quantification of SOX2+ cells over the total nuclei of adrenal medulla. n = 3 each, plotted mean and SEM, Unpaired t test with Welch's correction. P value = 0.4369.

6.2.4 Human Pheochromocytoma

As a first step to assess relevance of SOX2+ cells in human disease, I characterised the distribution of SOX2+ cells in human pheochromocytoma and paragangliomas and compared these to healthy control samples (previously described in Results 4.2.2). I obtained a cohort of paraffin-embedded sections from 16 samples of human pheochromocytoma (PCC), some of which arose in conjunction with paraganglioma (PPGL), with or without metastasis and/or germline or somatic mutations (Table 6.1).

Sample	Tumour location	Metastasis	Germiline mutations	Somatic mutations
1	PCC		NF1	
2	PCC		NF1	
3	PCC			VHL
4	PPGL			VHL
5	PCC		SDHC	
6	PCC		NF1	
7	PPGL	YES	SDHB	
8	PCC	YES		
9	PPGL		SDHB	
10	PCC	YES		
11	PCC			
12	PCC			
13	PPGL	YES	SDHB	
14	PPGL		SDHB	
15	PPGL		SDHB	
16	PPGL		SDHB	

Table 6.1 List of human pheochromocytoma samples.
PCC = pheochromocytoma, PPGL = pheochromocytoma and paraganglioma

Using immunohistochemistry, detected the expression of SOX2 and counted the percentage of SOX2+ cells over the total nuclei of 5 randomly selected comparable areas. The percentage of SOX2+ cells is not significantly different when comparing control normal adrenals (6.38%) with PCC (4.51%) or with PPGL (5.67%) and within PCC and PPGL (Figure 6.5A). Both PCC and PPGL can be subdivided into different clusters based on their genetic landscape. Some of the samples obtained have confirmed germline or somatic mutations which indicate their belonging to the "pseudohypoxia" (SDHx, VHL) and the "signalling" group (NF1). I found no significand differences in the percentage of SOX2+ cells of pseudohypoxia group tumours (5.11%) vs control normal adrenals (6.38%), signalling group (5.63%) vs control and between pseudohypoxia and signalling (Figure 6.5B). While no significant change was observed in non-metastatic tumours (5.47%) vs control normal adrenals (6.38%), the percentage of SOX2+ cells is significantly lower in metastatic tumours (3.66%) vs control, but it does not reach significance when compared with non-metastatic tumours (Figure 6.5B). I grouped tumours presenting SDHx mutations (SDHB or SDHC), given this mutation is the most common of germline mutations. The percentage of SOX2+ cells is lower in these tumours (4.12%) compared to control normal adrenals (6.38%) and to non-SDHx mutation tumours (5.56%), however this is not a significant change. Further investigation and a larger cohort of tumours are needed to confirm and explain these findings, however these results suggest that the expression of SOX2 might change in metastatic and SDHx mutated tumours when compared to normal adrenal control and tumours outside these cohorts.

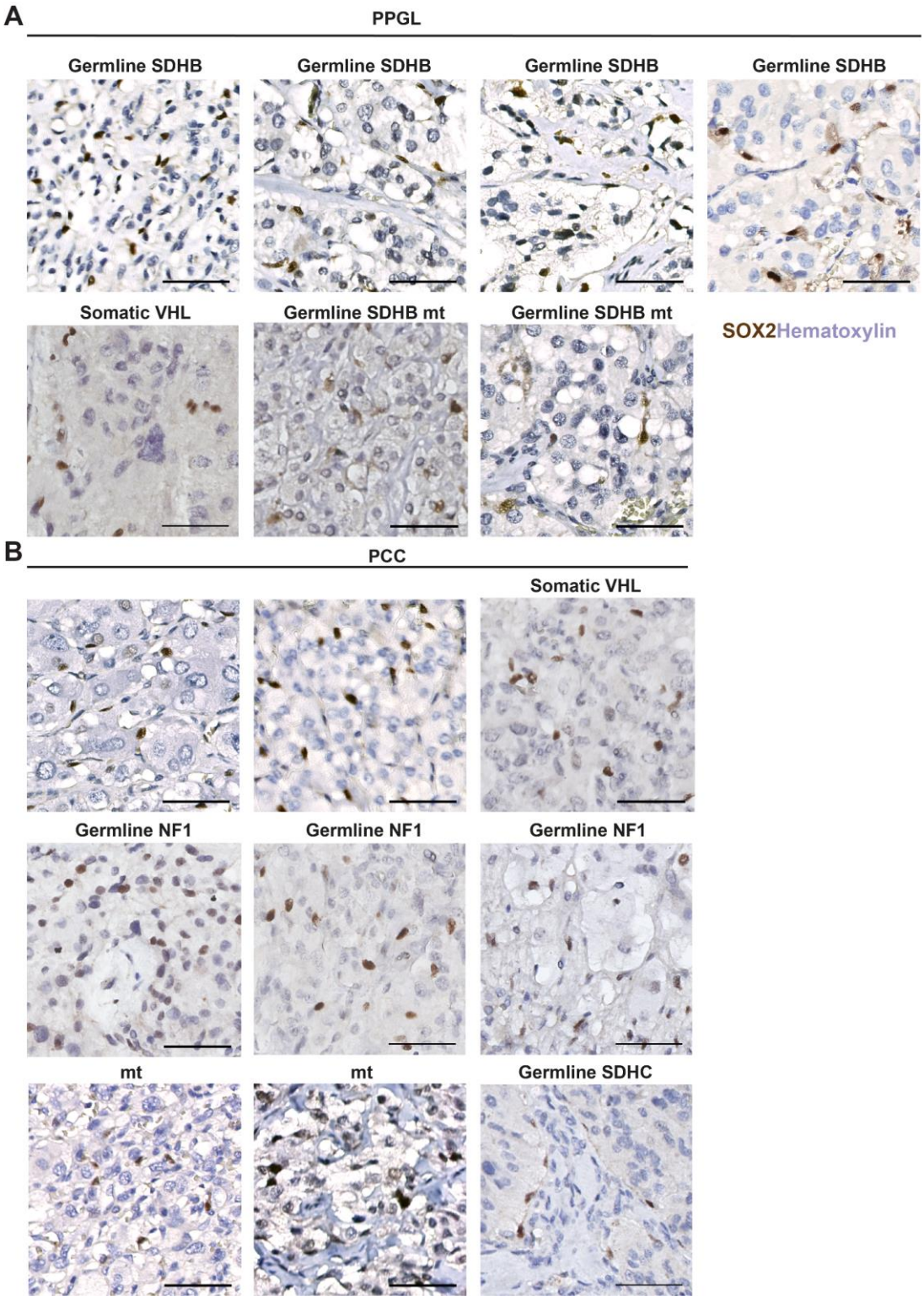

Figure 6.4 SOX2+ cells in adrenomedullary tumours.
A) Immunohistochemistry with antibodies against SOX2 (brown) pheochromocytoma that arise in conjunction with paragangliomas (PPGL), with defined mutations, with or without metastasis (mt). B) Immunohistochemistry with antibodies against SOX2 (brown) of pheochromocytomas (PCC) with defined mutations, with or without metastasis (mt). Nuclei counterstained with Hematoxylin, scale bar 50µm

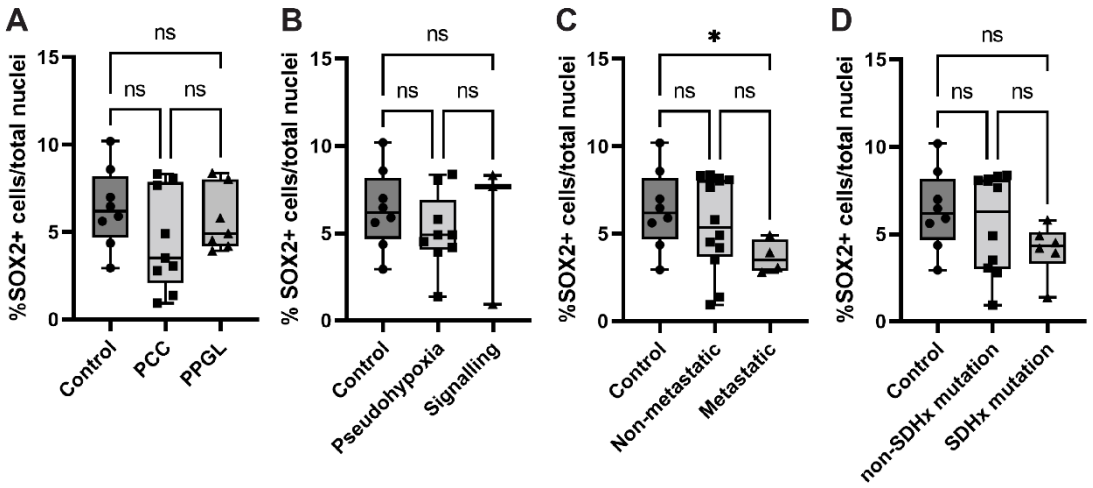

Figure 6.5 Quantification of SOX2+ cells in pheochromocytoma.
A) Comparison of the percentage of SOX2+ cells in PPCs vs PPGLs vs normal adrenal control. Plotted mean and SEM. Control n= 8, PCC n = 9, PPGL n = 7. Multiple comparisons Brown-Forsythe and Welch ANOVA tests: CTRL vs PCC P value = 0.3848, CTRL vs PPGL P value = 0.8754, PCC vs PPGL P value = 0.7004. B) Comparison of the percentage of SOX2+ cells in pseudohypoxia group vs signalling group vs normal adrenal control. Plotted mean and SEM. Control n = 8, Pseudohypoxia n = 9, Signalling n = 3. Multiple comparisons Brown-Forsythe and Welch ANOVA tests: CTRL vs Pseudohypoxia P value = 0.5695, CTRL vs Signalling P value = 0.9835, Pseudohypoxia vs Signalling P value = 0.9939. C) Plotted mean and SEM. Control n= 8, Non-metastatic n = 12, metastatic n = 4. Multiple comparisons Brown-Forsythe and Welch ANOVA tests: CTRL vs non-metastatic P value = 0.7993, CTRL vs metastatic P value = 0.0450, non-metastatic vs metastatic P value = 0.0450. D) Plotted mean and SEM. Control n= 8, SDHx mutation n = 7. Unaired t test with and Welch's correction, P value = 0.1666.

6.3 Discussion

A primary analysis of mouse models of organ challenge and of human disease samples indicates SOX2+ adrenomedullary cells might be involved in stress adaptation in mouse and in metastatic human tumours.

6.3.1 Mouse models

Analysis of acute stress and metabolic dysfunction samples did not indicate any change in the percentage of SOX2+ sustentacular cells when compared to their respective controls. This does not necessarily mean that SOX2+ adrenomedullary cells do not have a role in the organ's response to stimuli. To investigate this further, analysis of SOX2+ cell proliferation, general proliferation or lineage tracing experiments could indicate whether SOX2+ cells have an increased contribution to the formation of new chromaffin cells when compared to control, or whether this function is diminished.

Significant decrease in the percentage of SOX2+ cells in a chronic stress model might indicate that there is an expansion of the differentiated cell compartment. While chromaffin cell hyperplasia has been observed in similar chronic stress models, comments on an increase in the number of chromaffin cells has not been made. Using lineage tracing systems, this could be clarified. The reduction of the percentage of SOX2+ cells could also indicate that the SOX2+ compartment is being depleted following giving rise to new chromaffin cells to satisfy any physiological demand during chronic stress, which might indicate SOX2+ cells might not be able to self-renew, or that this ability is impaired due to a chronic disruption of homeostasis.

6.3.2 Human disease

I investigated the expression of SOX2 in human adrenomedullary tumours. PCCs and PPGLs consist of an excessive growth of the adrenal medulla with overproduction of catecholamines (Fishbein et al., 2017a). Given the impressive expansion of the chromaffin cell compartment, I hypothesise this could be sustained by a progenitor/stem cell population. If the stem cell role of SOX2+ cells was to be comparable to the one observed in mouse, the expansion of chromaffin cells observed could be the result of: 1) an initial proliferation of SOX2+ cells that could lead to more cells capable of differentiating to newly formed chromaffin cells; 2) proliferation of SOX2+ cells that directly give rise to differentiated progeny; 3) depletion of the SOX2+ cell compartment given by differentiation and absence of/defect in self-renewal. The data presented do not indicate a significant trend between tumours divided by PCC/PPGL nature or pseudohypoxia/signalling subgroups vs control. A significant decrease in the percentage of SOX2+ cells in metastatic tumours was observed when compared to healthy control. This is in line with previous reports that the mean count of S100B+ sustentacular cells is lower in malignant PCCs compared to benign PCCs (Białas et al., 2013). Even if non-significant, a similar trend was observed when grouping tumours presenting SDHx mutations, which are the most common germline mutations and often link to poor prognosis. This observation is compatible with all three hypotheses and transcriptomic analyses of these cells could help identify whether SOX2+ cells are directly affected and if they are, which mechanisms could be involved. Expanding the cohort of tumours analysed and obtaining more information about the samples (stages of disease, any other co-morbidity) would help define whether the differences observed in the SOX2+ population are consistent and only associated with tumour type.

7 Final Discussion

7.1 SOX2+ adrenomedullary sustentacular cells are stem cells in the normal postnatal adrenal medulla

The data presented in this thesis provide a first significant step towards the understanding of the postnatal adrenal medulla cell hierarchy. Given the association of SOX2 expression with pluripotency, I used SOX2 as a marker of the sustentacular cell population and found these cells to present stem cell attributes such as self-renewal and differentiation. I demonstrated that sustentacular cells act as a stem cell population and that these cells go through a transitory committing state to then differentiate into adrenaline- and noradrenaline-producing chromaffin cells.

The single cell RNA sequencing dataset generated (Chapter 3) identified a link between sustentacular cells and chromaffin cells through a transitioning committing cell state. Analysis of the differentiated cell compartments of the postnatal medulla shows transcriptional heterogeneity of adrenaline-producing chromaffin cells, which indicates possible separate functional roles for the populations identified. Additionally, I detected transcriptional homogeneity between a subset of chromaffin cells and postganglionic neurons, appearing transcriptionally indistinguishable from PNMT- noradrenaline-producing chromaffin cells, which suggests noradrenaline-producing chromaffin cells could have multiple roles in the postnatal adrenal medulla. These results challenge and bring definition to the classical view of cell types observed in the postnatal adrenal medulla.

In vitro analyses of SOX2+ adrenomedullary sustentacular cells showed that these cells have a unique potential to self-renew and form colonies, while having the capacity to commit towards differentiated cell types of the adrenal medulla. I confirmed that their ability to differentiate was recapitulated *in vivo* through lineage tracing experiments, which showed that SOX2+ cells give rise to adrenaline- and noradrenaline-producing chromaffin cells during normal homeostasis, contributing to the postnatal growth of the adrenal medulla (Chapter 5). This result shows for the first time that the postnatal adrenal medulla can generate new chromaffin cells *in vivo* and confirms the importance of sustentacular cells as more than "support cells", defining this cell population as stem cells of the adrenal medulla (Chapter 4,5).

Further analyses of mouse models of organ challenge and human disease provide evidence that SOX2+ adrenomedullary sustentacular cells might have an involvement in stress adaptation and in metastatic human adrenomedullary tumours (Chapter 6).

For the scope of the investigation presented in this thesis, I produced two tools that will be pivotal for further studies to understand the cellular composition, hierarchy and function of the postnatal adrenal medulla: single cell RNA sequencing datasets and a cell culture system.

The single cell RNA sequencing datasets obtained (Chapter 3) present the first complete atlas of the mouse postnatal adrenal medulla, with over 2700 single cell transcripts to analyse over all adrenomedullary cell types and an additional enriched dataset for SOX2+ sustentacular cells. Both sustentacular and chromaffin cells remain poorly studied cell types at both the transcriptional and cell signalling level. The information provided by these datasets could lead to a number of further studies investigating these populations at a molecular level, providing essential information regarding physiological processes,

differentiation mechanisms and cell communication. Comparing these datasets across different timings and species could provide further insights on developmental and evolutionary mechanisms. Comparing these datasets with human postnatal medulla data could help refine murine disease/challenge models to make these more relevant to human physiology.

The cell culture system I optimised (Chapter 4) is the first cell culture system that allows sustentacular cells to grow in attachment conditions. The only previously published culture system of postnatal adrenomedullary sustentacular cells (Rubin de Celis et al., 2015) is based on floating spheres. Cell attachment conditions provide a more controlled environment for morphological observations and a simpler manipulation of cells, and the reagents used are common and cost-effective, making this approach simple and reproducible. The system optimised allows SOX2+ cells to expand, and these can be easily passaged, maintaining their potential for at least 3 passages. Only SOX2+ cells attach and expand in these culture conditions and preliminary experiments show that plating the adrenal medulla directly without the need for flow sorting still results only in the culture of SOX2+ sustentacular cells. Therefore, high numbers of primary cells can be cultured and expanded, allowing for molecular biology and biochemical quantification experiments, drug treatment and possibly cell transfection for *in vitro* genetic manipulation, making this an excellent tool to further study the nature and function of sustentacular cells. Furthermore, this protocol has the potential to be used for isolation of the equivalent cell type from the human adrenal.

7.1.1 Cell types and cell hierarchy of the mouse postnatal adrenal medulla

1) Subtypes of adrenaline-producing chromaffin cells

The single cell RNA sequencing dataset obtained (Chapter 3) reveals heterogeneity in the adrenaline-producing chromaffin cell population of the postnatal medulla. Three transcriptional signatures indicate differential upregulation of specific genes: Cluster 0 and 1 differ for secretion- and cytoskeleton-associated genes, while Cluster 5 shows an upregulation of the AP1 pathway genes. Previous observations reported in the literature identified two types of adrenaline-producing chromaffin cells based on the density of their granules and cytoplasm (Koval et al., 2000). Using flow cytometry techniques, it might be possible to separate these cell types and relate their transcriptional profiles with their morphological features. This might give an indication on whether the phenotype observed is linked to any specific transcriptional program and whether it indicates a different function or state of differentiation or maturation. The AP1 pathway regulates multiple cellular mechanisms, and it has been shown to be involved in catecholamine biosynthesis (Shi et al., 2019). Cluster 5 could indicate a particularly active population of chromaffin cells which could be investigated further, for example in organ challenge models to determine whether these cells could have a unique function.

2) Are noradrenaline-producing chromaffin cells and postganglionic intramedullary neurons the same cell type?

Single cell RNA sequencing investigation (Chapter 3) indicates that neurons are not transcriptionally separate from chromaffin cells and do not form their own transcriptional cluster based on differentially expressed genes. This finding is in line with reports present

in literature indicating that postganglionic intramedullary neurons show an enzymatic profile equal to the one of noradrenaline-producing chromaffin cells and produce noradrenaline (A. Dagerlind et al., 1990; Holgert et al., 1996), with the identification of this population mostly based on their morphological, neuronal-like features. Given that all chromaffin cells show neuronal-like functions and characteristics, have the same developmental origin and they have been described to sometimes present axons (Díaz-Flores et al., 2008), it is extremely difficult to discriminate between the two cell types *in vivo*. Further investigation of markers, paired with morphological observations might help define this population.

3) Defining lineage commitment trajectories

In silico, *in vitro* and *in vivo* observations (Chapter 5) indicate that SOX2+ adrenomedullary sustentacular cells can differentiate towards a chromaffin cell fate. *In silico* analysis showed the presence of committing "transitioning" populations, which I was not able to validate *in vivo* for lack of unique markers. Of the populations observed, one shows markers of adrenaline-producing chromaffin cells together with sustentacular cells, while the second one is PNMT negative, therefore more similar to noradrenaline-producing cells. This leads to the question of whether sustentacular cells could commit towards a noradrenaline-producing cell fate, which can then mature towards an adrenaline-producing cell fate; alternatively, they may be able to directly differentiate towards an adrenaline-producing cell fate. *In vitro* analysis might help define these trajectories by culturing colonies at clonal density and doing an immunohistological analysis. If one SOX2+ cell is only able to produce one of the two cell types, single colonies will present either PENK+ noradrenaline-producing cells or PNMT+ adrenaline producing cells. If one SOX2+ cell is capable to give rise to both cell types, colonies showing both the populations will be observed. However, if both populations are observed in one colony, it cannot be

excluded that one multipotent SOX2+ cell might give rise to two SOX2+ unipotent cells, which is why it is necessary to investigate the intra-heterogeneity of SOX2+ cells (described in Chapter 4). Additional lineage tracing driven by the newly identified markers of noradrenaline-producing chromaffin cells (*Penk*, *Cox8b*, *Lix1*) would be able to define whether these can then differentiate/mature to adrenaline-producing chromaffin cells. Equally, lineage tracing systems for one of the identified markers of adrenaline-producing chromaffin cells (such as *Pnmt*, *Gata2*, *Tmeff*, *Hand1*, *Isl1*) could identify whether these can give rise to noradrenaline-producing cells. If noradrenaline-producing cells and neurons converge in the same cell type postnatally, it could be expected that adrenaline-producing chromaffin cells could give rise to this population as it has been identified during embryonic development (Kameneva, Artemov, et al., 2021).

4) Heterogeneity of sustentacular cells

The transcriptional profile of sustentacular cells is extremely different from the one of chromaffin cells, making them a separate cell type with the ability to differentiate (Chapters 3, 4, 5). However, the nature of sustentacular cells has been poorly studied and association of markers with this cell type has proven inaccurate (Chapter 3.2.3). The main markers used in this thesis are SOX2, S100B, SOX10, GFAP, PLP1, however expression of these markers is heterogeneous within all the cells expressing them. It is not clear whether any of these markers could have a higher stem cell potential than others, however *in vitro* experiments suggest that SOX2+ sustentacular cells might have a more prominent role, given they are the only ones capable of forming colonies. Therefore, this suggests that cells expressing the other markers but not SOX2 might indicate a less potent, possibly differentiating cell type. Further experiments are needed to prove which of the populations could be hierarchically superior to the others. This could be done by performing single cell RNA sequencing of just this cell type and simulating possible

differentiation trajectories based on splicing (Velocity tool). This could be achieved by separating sustentacular cells with FACS for the expression of PLP1, which according to *in silico* analysis is the marker expressed by most cells in the sustentacular cell cluster.

7.1.2 Molecular mechanisms of chromaffin cell differentiation in the postnatal adrenal medulla

1) Could the transcription factors involved in chromaffin cell differentiation during embryonic development have a similar function in the postnatal medulla?

I identified that sustentacular cells give rise to chromaffin cells in the postnatal adrenal medulla. Given that SOX2+ sustentacular cells originate from embryonic SCPs (Chapter 4.2.3), it is likely that the differentiation process of sustentacular to chromaffin cells could be controlled by the same molecular mechanisms as differentiation from embryonic SCPs to chromaffin cells. While a thorough characterisation of the molecular mechanisms regulating chromaffin cell development has not been carried out, several transcription factors are known to have an essential role for the differentiation and maturation of chromaffin cells: PHOX2B, MASH1, INSM1, HAND2, GATA 2/3 (Klaus Unsicker et al., 2013). All these transcription factors are expressed in chromaffin cells in the postnatal adrenal medulla and transcriptional signatures of the postnatal medulla are generally comparable to the ones of the embryonic adrenal medulla (Chapter 3.2.4), therefore these transcription factors might still be relevant during the postnatal development period and perhaps in the adult. Conditional deletion of these transcription factors in SOX2+ cells could be performed to identify whether these are essential for chromaffin cell differentiation in the postnatal adrenal and if their absence leads to differentiation defects.

2) Which signalling pathways are responsible for the maintenance of cell turnover during organ homeostasis?

To gain insights on how tissue homeostasis is maintained in the postnatal medulla, further study of major signalling pathways might help understand the molecular mechanisms orchestrating cellular maintenance and differentiation. Primary analyses shown in Chapter 3.2.5 indicate that multiple signalling pathways (WNT, Notch, BMP/TGF, Hippo and MAPK) are active in the postnatal adrenal medulla. Extensive *in vivo* validation of the obtained results could be coupled with mouse models to investigate any disruption of tissue homeostasis given by an overactivation or downregulation of these pathways. Alternatively, the expression of known signalling molecules could be investigated in models of organ challenge and disease. WNT and MAPK are pathways known to be disrupted in adrenomedullary tumours (Fishbein et al., 2017a), therefore a more thorough analysis of these might provide relevant information on tumour initiation and progression. Based on the *in silico* investigations on the expression of its components, the WNT pathway seems to be particularly relevant, with sustentacular cells secreting signals (*Wnt6*, *Sfrp5*, *Sfrp1*) and differentiated cells expressing target genes of the pathway (*Axin2*, *Lef1*), suggesting a possible paracrine interaction. A similar scenario takes place in the pituitary gland, where SOX2+ stem cells control the expansion of differentiated cells through paracrine WNT secretion (Russell et al., 2021). Expression of the components of the Notch signalling pathway suggests an interaction between sustentacular cells in a Delta-Notch manner, with sustentacular cells presenting activation of Notch with expression of its target genes (*Hey2*, *Heyl* and *Hes1*), while *Dlk1* is expressed in all differentiated cell types. DLK1 is known to be expressed in chromaffin cells during embryonic development of the adrenal medulla and its expression to be dependent on PHOX2B, a transcription factor necessary for chromaffin cell maturation (El Faitwri & Huber, 2018).

7.1.3 Further investigation of the role of SOX2+ cells

1) Do SOX2+ cells function as stem cells the postnatal mouse adrenal medulla?

In this thesis, I have shown that sustentacular cells expressing the transcription factor SOX2 are a long-lived population that gives rise to new endocrine cells *in vivo* and has the potential for self-renewal *in vitro* (Chapter 5), but this remains to be shown *in vivo*. *In vivo* experiments using clonal tracing with the Confetti tool can be used to define whether one single SOX2+ cell can give rise to another SOX2+ cell, to observe self-renewal *in vivo*. However, this view of self-renewal will still be limited to the expression of SOX2 and not the complete comparison of downstream potential and transcriptional profiles of the parent and daughter cell. To obtain *in vivo* functional proof that SOX2+ sustentacular adrenomedullary cells are stem cells of the adrenal medulla, I would propose extensive functional validation to prove that these cells are essential and necessary for the postnatal function the adrenal medulla. Ablating all SOX2+ cells using a DTA system driven by a specific Cre in the early postnatal stages would allow to determine whether the postnatal development and growth of the glands are dependent on SOX2+ cells. Alternatively, the stem cell potential of SOX2+ cells could be demonstrated with classical experiments such as injecting SOX2+ cells under the renal capsule to determine whether these are capable to singularly give rise to functional medulla tissue, or injection directly into the medulla to demonstrate tissue re-colonising and differentiation into the appropriate cell types.

2) Heterogeneity of this population

The data presented in Chapter 4.2.5 indicate that the adrenomedullary sustentacular SOX2+ cell population presents transcriptional heterogeneity, identifying two different

transcriptional signatures. Investigating this heterogeneity further could define a cell hierarchy internal to the SOX2+ cells, indicating one less- and one more-committed state, or a different function/level of activity. Understanding the intra-heterogeneity of SOX2+ cells will be pivotal to determine whether this entire population or just a portion of it could be used for regenerative medicine approaches or as a therapeutic target to restore homeostasis. Further investigation in the expression differences observed could indicate whether these are related to a difference in potency or signalling response. Using the adherent cell culture system presented in this thesis, it could be possible to investigate whether both or only one of these populations have colony-forming potential *in vitro* using immunostaining and comparing protein expression levels of the genes identified *in silico*. *In vivo* or *in vitro* manipulation of signalling pathways shown to be active in SOX2+ cells and thorough analysis of their differentiation and self-renewal potential could indicate whether any of these are essential for SOX2+ cells to exert their function.

3) Relevance in physiological organ challenge and disease pathogenesis

Preliminary analysis of organ challenge and disease (Chapter 6) found a decrease in the number of SOX2+ adrenomedullary cells in a chronic stress model in mouse as well as in metastatic human tumours. Both of these conditions involve an expansion of the adrenal medulla, which suggests it could be sustained by a stem/progenitor cell population. SOX2+ adrenomedullary cells could undergo proliferation and/or differentiation in response to homeostatic disruption; they could directly promote proliferation of chromaffin cells orchestrating a response based on signalling pathways (e.g. paracrine signalling); or they could regulate the amplification and proliferation of a putative intermediate committing population yet to be defined.

To further investigate the involvement of SOX2+ cells in stress adaptation, mouse models of chronic stress could be used in conjunction with *Sox2*-driven lineage tracing, to identify

whether SOX2+ cells might undergo an upregulation in their differentiation rate, which could explain the result in a lower percentage of SOX2+ cells.

Regarding the role of SOX2+ cells in human tumours, first it will be crucial to assess whether SOX2+ is conserved as a marker of cells with similar properties as the ones described in mouse, given that there is an already reported discrepancy amongst cellular markers between mouse and human (Kameneva, V.Artemov, et al., 2021). With the accessibility of new single cell RNA sequencing datasets (Bedoya-Reina et al., 2021), and in time, multi-ome analyses to incorporate chromatin accessibility and epigenetic status, a thorough comparison between human and mouse signatures might be able to clarify this. At the moment of writing, while bulk-RNA sequencing datasets are available (e.g (Fishbein et al., 2017)) there are no single cell RNA sequencing datasets published for pheochromocytoma. These would provide an extremely important tool to be able to determine the poorly understood mechanisms of tumour progression and cell of origin. The involvement of sustentacular cells in PPGLs is poorly understood, and the presence of a tumour-initiating cancer stem cell is just a hypothesis (Scriba et al., 2020). Given the fact that PCCs are often associated with paragangliomas that arise in extra-adrenal ganglia along the neural crest migration path, an origin from a common developmental progenitor could be suggested, indicating necessary further investigation in the role of sustentacular cells and their embryonic counterparts, SCPs. However, PPGLs often arise during adulthood, therefore it is possible that sustentacular cells might spontaneously accumulate mutations that drive tumour initiation or that defective signalling from the environment (e.g. stress) could lead to excessive differentiation; alternatively, sustentacular cells could simply sustain the expansion of the chromaffin cell compartment in response to signals coming from differentiated cells or altogether not have a role in PPGLs. Should human primary sustentacular cells behave similarly to mouse primary SOX2+ cells, then an *in vitro* culture system could be optimised based on the one presented

in this thesis. This could allow to either study the behaviour of primary PPGL sustentacular cells and determine whether these can differentiate *in vitro*, or to use primary normal sustentacular cells and express known tumorigenic mutations to assess whether and how these could drive differentiation/proliferation.

8 Summary

The data presented and discussed in this thesis bring definition to the understanding of the postnatal adrenal medulla at a cellular level. I identified a novel SOX2+ cell population with stem cell abilities documented with bioinformatic, primary cell culture and *in vivo* analyses (Figure 8.1), with potential involvement in stress adaptation and relevance to human adrenomedullary tumours. I created the first postnatal adrenal medulla cell atlas with the use of single cell RNA sequencing, and I optimised the first cell culture system that allows sustentacular cells to grow under attachment conditions. These tools, together with the foundation knowledge provided by the analyses performed, will allow for an exponential growth of this field, when so little is known about the postnatal adrenal medulla and the behaviour and hierarchy of its cell components. Furthermore, any confirmed relevance of the identified SOX2+ population in organ challenge and disease, would provide a prime target for regenerative medicine and therapeutic approaches.

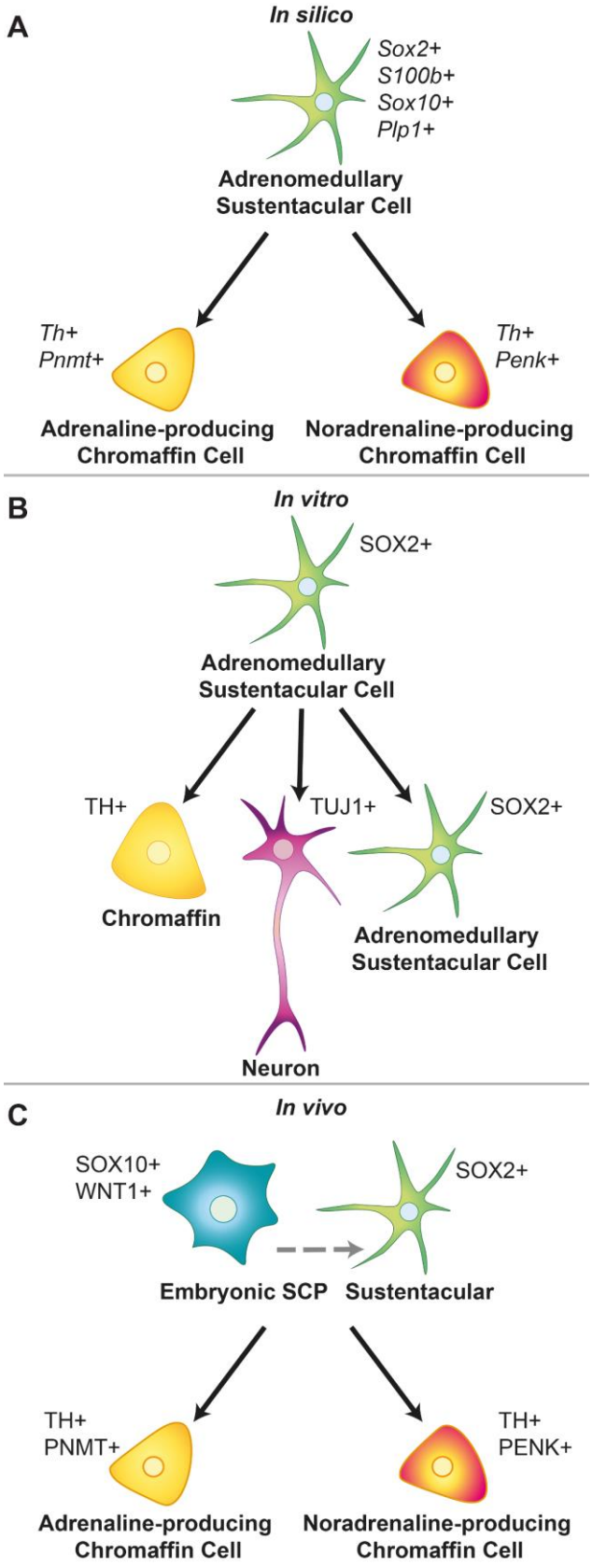

Figure 8.1 Thesis summary.
A) *In silico* prediction (Velocity) and observations indicate that *Sox2+* sustentacular cells can differentiate towards adrenaline- and noradrenaline-producing chromaffin cells. B) Using primary culture under attachment conditions, I determined that SOX2+ adrenomedullary cells can self-renew, producing new SOX2+ cells, and at the same time committing towards a TH+ chromaffin fate and a TUJ1+ neuronal fate. C) Using different lineage tracing systems *in vivo*, I determined that SOX2+ adrenomedullary cells are derived from embryonic SCPs, and that SOX2+ cells contribute to the postnatal growth of the adrenal medulla by giving rise to PNMT+ adrenaline-producing chromaffin cells and PENK+ noradrenaline-producing chromaffin cells.

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10 Appendix

10.1 Peer-reviewed original manuscripts published during this PhD