REVIEW

Cell population characterization and discovery using single-cell technologies in endocrine systems

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Abstract

In the last 15 years, single-cell technologies have become robust and indispensable tools to investigate cell heterogeneity. Beyond transcriptomic, genomic and epigenome analyses, technologies are constantly evolving, in particular toward multi-omics, where analyses of different source materials from a single cell are combined, and spatial transcriptomics, where resolution of cellular heterogeneity can be detected *in situ*. While some of these techniques are still being optimized, single-cell RNAseq has commonly been used because the examination of transcriptomes allows characterization of cell identity and, therefore, unravel previously uncharacterized diversity within cell populations. Most endocrine organs have now been investigated using this technique, and this has given new insights into organ embryonic development, characterization of rare cell types, and disease mechanisms. Here, we highlight recent studies, particularly on the hypothalamus and pituitary, and examine recent findings on the pancreas and reproductive organs where many single-cell experiments have been performed.

Key Words

- ▶ technology
- ▶ single cell
- ▶ microfluidics
- ▶ multi-omics
- ▶ transcriptome
- ▶ endocrine organs

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Introduction

Single-cell technologies have become an essential tool, offering an unprecedented glimpse into cellular heterogeneity. The decreasing costs of next generation sequencing, allowing the sequencing of millions or billions of DNA fragments in parallel, and the development of microfluidic techniques, easing the handling of a large number of single cells, both underlaid the emergence and success of relevant new technologies over the last 15 years. Among single-cell technologies, transcriptomic (scRNAseq) analyses are most frequently performed, followed by genome and epigenome sequencing. As new technologies are constantly emerging, and progressing very rapidly, the proteome and metabolome may also

soon be analyzed at the single-cell level (Pali et al. 2019) (for review see Duncan et al. 2019). In parallel, multi-omics technologies are utilized to profile simultaneously different material sources from one single cell, enabling, for example, correlations between genomic mutations and alteration of gene expression using G&T-seq (Genome and Transcriptome-seq; Macaulay et al. 2015; for review see Macaulay et al. 2017). Furthermore, combining scRNA-seq with genome-editing tools, genetic screens can now be conducted with an unprecedented level of characterization using tools such as PERTURB-seq (Dixit et al. 2016), CRISP-seq (Jaitin et al. 2016) and CROP-seq (Datlinger et al. 2017). While one obvious

caveat of most of these analyses is the loss of spatial information, because most techniques rely on the physical isolation of single cells or nuclei from their *in vivo* or *in vitro* context, progress has been made to solve this issue by performing *in situ* sequencing. Several different platforms, such as seqFISH (Eng *et al.* 2019), MERFISH (Chen *et al.* 2015), FISSEQ (Lee *et al.* 2015) and TIVA (Lovatt *et al.* 2014), have been developed, and this technology is fast becoming accessible to the wider community (<https://spatialtranscriptomics.com/>).

To handle the large quantity of sequencing data generated from thousands up to hundreds of thousands of cells, specialized bioinformatic tools are essential. They need to be more sophisticated to deal with the particularities of single-cell datasets compared to bulk analyses, such as the low starting material, leading to enhanced noise and batch effects. Similar to bulk analyses, these tools are first used to check the quality of the data, remove noise, and generate the results, by aligning it to the relevant genome. More advanced algorithms, with the frequent development of new ones (Amezquita *et al.* 2019), are then used to extract as much information as possible from the sequencing data, to visualize results and extrapolate biological meaning. Essentially, the first step in result analysis is dimensional reduction, where cells with similar gene expression profiles are grouped together, allowing separation and, therefore, identification of distinct cell populations. Principal component analysis (PCA), t-distributed stochastic neighbour embedding (t-SNE) and uniform manifold approximation and projection (UMAP) are commonly used algorithms to achieve dimensional reduction. The Seurat package from the Satija lab comprises quality check and dimensional reduction analyses and is very commonly used to analyze single-cell RNA-seq datasets (Stuart *et al.* 2019). Then, according to the biological question, ordering of cell populations may be performed, because cells have been captured as they transit between different states or stages and this information can be used to reconstruct cellular hierarchies and trajectories. A commonly utilized algorithm to allow distribution of cells along a pseudo-time trajectory is Monocle from the Trapnell lab (Trapnell *et al.* 2014). Other widely used tools comprise RNA velocity, where trajectories are reconstituted according to the balance between unspliced and spliced transcripts, reflecting how recently a gene has been up-regulated (La Manno *et al.* 2018), and many others have been developed and extensively used (for review see Todorov & Saeys 2019). Because the quantity of datasets generated is now exponential, bioinformatic tools

are currently developed to allow comparisons between different datasets and platforms (Adey 2019). In this constantly evolving technological landscape, the range of possibilities is truly amazing. However, it is biological validation that gives meaning to dataset mining, and single-cell datasets are rather the beginning than the end of the story. Here, we will mostly focus our attention on transcriptome and genome analyses.

Applications and remaining hurdles of single-cell genome and transcriptome analyses

While the focus of this review will be on endocrine systems, we will first briefly review more widely the advances brought up by single-cell genomic and transcriptomic analyses. We will focus on pioneer studies and illustrate the areas where these technologies have been particularly instrumental in opening new avenues for investigation. Broadly, the immense advantage of single-cell approaches is that they highlight and inform about cell heterogeneity, while previous bulk analyses wiped away all this precious information (for review see Trapnell 2015). However, there are still technical hurdles. In genome analyses, allelic or locus dropouts and incomplete coverage lead to failed detection of single-nucleotide variants, while in transcriptome analyses, not all transcripts are reverse transcribed, and therefore, absence of a certain gene in the dataset does not always mean that it is not expressed. This is more of a problem for lowly transcribed genes, such as transcription factors. It clearly implies that a substantial amount of information is simply missing from the datasets. Spike-ins (RNAs in known quantities) can be added as internal controls, but they may not always truly reflect the rate of capture of endogenous transcripts. Platforms, reagents and algorithms are constantly improved to respectively reduce and take into account these problems. For genome analysis, insertion of transposons for linear amplification (LIANTI) improves both coverage and fidelity compared with exponential amplification (Chen *et al.* 2017a). In addition, there are notable technological differences between the different platforms used for transcriptomic analyses (for review see Svensson *et al.* 2018). In term of results, the two main differences are sequencing depth (number of reads) and the number of cells processed, which are both inversely correlated (Ziegenhain *et al.* 2017). Maybe counterintuitively, it appears that sequencing more cells, at the detriment of the number of reads, is better for dimensional reduction. Furthermore, it is sometimes difficult to obtain a good quality single-cell

suspension or this is simply not an option when working with frozen clinical samples. An alternative is to use single nuclei where transcriptome is comparable to that found in the cytoplasm, albeit less abundant, enriched in nuclear and ncRNA, and intronic sequences (Bakken *et al.* 2018).

The success of these technologies is illustrated by the proposal for ambitious projects such as the Human Cell Atlas, an international collaborative initiative aiming to complete characterization of all of the human body cell types (Regev *et al.* 2017). But, single-cell approaches have already made a significant impact on description, characterization, and therefore understanding of biological systems, while considerably expanding investigative possibilities.

In cancer research, identification of a tumor genomic mutational landscape at single-cell resolution allows reconstitution of cellular hierarchies, unravels tumoral evolution, and potentially, identifies the mutation at the origin of tumorigenesis (Navin *et al.* 2011, Wang *et al.* 2014). In pioneer scRNA-seq studies, an unanticipated heterogeneity, recapitulating neural development, was revealed in human glioblastoma. This was shown to be clinically relevant because higher heterogeneity in individual tumors is associated with lower survival (Patel *et al.* 2014), maybe reflecting a more primitive, undifferentiated and proliferative phenotype of the cells fueling tumor initiation and growth. Furthermore, rare cell types can be identified, such as circulating (CTC) and disseminated (DTC) tumor cells (Ni *et al.* 2013, Lohr *et al.* 2014). In addition, single-cell analyses allow segregation of stromal niche cells away from tumor cells, providing clues to understand how the microenvironment interact with the tumor cells. For example, in lung tumors, stromal cell RNAseq analyses uncovered complexity of this compartment and characterized new subpopulations (Lambrechts *et al.* 2018). In healthy subjects, investigation of the immune system in an innovative study (Villani *et al.* 2017) revealed the existence of a new type of dendritic cells that robustly activate T-cells and could therefore be utilized to strengthen anti-cancer immunity. Therefore, in a relatively short time, these new technologies have led to important discoveries in tumor and immune cell biology which are expected to impact significantly both the diagnosis and development of treatments.

Cell heterogeneity and trajectories are also central questions in stem cell and developmental biology. Better characterization of differentiation pathways is useful *in vivo*, in embryos and stem cells (SC), and *in vitro*, in embryonic SC (ESC) and induced pluripotent SC (iPSC) to improve disease modelling, drug screening assays,

and ultimately for regenerative medicine. Single-cell approaches have thus been instrumental to resolve cell heterogeneity in different SC populations, such as neural stem cells (NSC) (Llorens-Bobadilla *et al.* 2015, Shin *et al.* 2015, Dulken *et al.* 2017, Kalamakis *et al.* 2019, Mizrak *et al.* 2019). They have given new insights into mechanisms of activation and metabolism in adult NSC (Llorens-Bobadilla *et al.* 2015, Shin *et al.* 2015, Dulken *et al.* 2017) and during aging (Kalamakis *et al.* 2019). These studies have also suggested that quiescent SC are poised for activation because some genes associated with commitment toward a certain cell fate are active in these, but only transcriptionally (for review see van Velthoven & Rando 2019). This changes our view of quiescence and thus strategies toward activation for regenerative purposes. In developing zebrafish (Wagner *et al.* 2018), frog (Briggs *et al.* 2018), mouse (Cao *et al.* 2019; Pijuan-Sala *et al.* 2019) and human embryos (Xue *et al.* 2013, Yan *et al.* 2013, Blakeley *et al.* 2015, Leng *et al.* 2019), single-cell RNAseq have provided a further level of description of cell lineages emergence and cell-fate acquisition mechanisms. In addition, sophisticated approaches combining genome editing and scRNAseq have allowed reconstitution of developmental trajectories where single-cell progenies can be followed after integration in their genome of a unique barcode (Wagner *et al.* 2018). As we will show in the following section, these approaches have similarly remodelled pre-existing knowledge in endocrine organs. They moreover provide powerful tools to investigate normal and perturbed systems.

Broadly, these studies have often revealed an unanticipated degree of cellular heterogeneity at the transcriptional level. It is now important to examine whether this transcriptional diversity is translated at the protein level and therefore has a functional relevance. It is possible that transcriptional heterogeneity within cell populations simply reflects the presence of functionally distinct, stable sub-populations. Alternatively, transcriptional heterogeneity may highlight an unanticipated plasticity between different cell states or phenotypes. However, even if cases where this transcriptional diversity is not translated, such as in stem cells, where the presence of untranslated transcripts suggests a primed state toward differentiation (for review see van Velthoven & Rando 2019), we still have much to learn from these data that reveal, in particular, the importance of post-transcriptional regulatory controls.

From the gonads to the pineal gland, most endocrine organs have now been sequenced at the single-cell level in different organisms and contexts (Table 1).

Table 1 Summary illustrating single studies in endocrine systems.

Organ / discovery	Origin of the cells	Platform/methodology	Number of cells sequenced
Endocrine pancreas			
Resource comparing <i>in vivo</i> progenitors and ES cell-derived ones (Krentz <i>et al.</i> 2018)	Mouse embryonic and ES cell-derived pancreatic progenitors	10X Genomics	17,993
Characterization of murine pancreatic lineages emergence (Bastidas-Ponce <i>et al.</i> 2019)	Mouse embryonic pancreatic cells	10X Genomics	36,351
Characterization of murine pancreatic lineages emergence (van Gurp <i>et al.</i> 2019)	Mouse embryonic pancreatic cells	(van Gurp <i>et al.</i> 2019)	4620
Differential emergence and maturation of α and β cells (Qiu <i>et al.</i> 2017)	Mouse embryonic and post-natal pancreatic cells	Smart-seq2	891
Characterization of β cells heterogeneity during post-natal expansion (Zeng <i>et al.</i> 2017)	Mouse post-natal pancreatic cells	FluidigmC1	387
Characterization and improvement of <i>in vitro</i> human β -cells differentiation (Veres <i>et al.</i> 2019)	Differentiating human iPS cells	inDrop	>100,000
Characterization of embryonic human pancreatic progenitors (Villani <i>et al.</i> 2019)	Second trimester human pancreas	10X Genomics	8000
Comparison of mouse and human β cells, characterization of β cells heterogeneity (Baron <i>et al.</i> 2016)	Adult human and mouse pancreatic cells	inDrop	12,000
Human adult pancreas atlas (Muraro <i>et al.</i> 2016)	Adult human pancreas	Cell-seq2	1500
Diabetic islet transcriptional profiles suggest dedifferentiation (Wang <i>et al.</i> 2016)	Pediatric and adult pancreas from healthy and diabetic donors	FluidigmC1	635
Transcriptomic changes associated with diabetes (Xin <i>et al.</i> 2016)	Adult pancreas from healthy and diabetic donors	FluidigmC1	1492
Transcriptomic changes associated with diabetes rare pancreatic cell types profile (Segerstolpe <i>et al.</i> 2016)	Adult pancreas from healthy and diabetic donors	Smart-seq2	2209
Increased transcriptional noise suggesting altered β cell identity during aging (Enge <i>et al.</i> 2017)	Aging human pancreas	(Enge <i>et al.</i> 2017)	2544
Male reproductive organs			
Transcriptional events surrounding male sex determination (Stevant <i>et al.</i> 2018)	Mouse embryonic gonadal somatic cells	Fluidigm C1	400
Definition of spermatogenesis stage-specific markers and characterization of azoospermia in one patient (Wang <i>et al.</i> 2018)	Testicular cells from normal and azoospermia patients	Smart-seq2	3028
Characterization of male meiosis and spermatogenesis (Hermann <i>et al.</i> 2018)	P6 and adult mice and adult human spermatogonial cells	Fluidigm C1	>62,000
Adult mouse testis atlas (Green <i>et al.</i> 2018)	Adult mouse testes	Drop-seq	35,000
Female reproductive organs and embryos			
Transcriptional heterogeneity of female germ cells (Vertesy <i>et al.</i> 2018)	Human fetal female gonadal cells	Smart-seq2	195
Transcriptional changes during germ cell migration and gonadal somatic cells transcriptome heterogeneity (Guo <i>et al.</i> 2015)	Human male and female gonadal cells	(Tang <i>et al.</i> 2010)	319
Characterization of different stages during germ cells development and interactions with somatic cells (Li <i>et al.</i> 2017)	Human male and female gonadal cells	Smart-seq2	2167
Effect of endometriosis on oocyte transcriptome (Ferrero <i>et al.</i> 2019)	Oocytes from healthy and endometriosis patients	Smart-seq V4	32
Transcriptomic differences associated with PCOS in oocytes and cumulus cells (Liu <i>et al.</i> 2016)	Oocytes and granulosa cells from healthy and PCOS patients	Smart-seq2	48
Transcriptional changes during folliculogenesis in oocytes and interaction with granulosa cells (Zhang <i>et al.</i> 2018)	Human oocytes and granulosa cells	(Wang <i>et al.</i> 2016)	151
Identification of organ cell types and changes associated with folliculogenesis (Fan <i>et al.</i> 2019)	Adult human ovaries	10X genomics	56,206

(Continued)

Table 1 Continued.

Organ / discovery	Origin of the cells	Platform/methodology	Number of cells sequenced
Transcriptional changes occurring during early development, determination of conserved and human specific events, characterization of parent-of-origin specific transcripts (Xue <i>et al.</i> 2013)	Human and murine oocyte to morula stages	(Tang <i>et al.</i> 2010)	251
Comparison mouse-human early development and validation at the protein levels (Blakeley <i>et al.</i> 2015)	Human and murine zygote to blastocyst stage	Smarter ultra low	Integration of datasets from several studies
Parent-specific transcripts, effects on genome activation and early differentiation (Leng <i>et al.</i> 2019)	Human biparental and uniparental zygote to morula stages	Smart-seq2	296
Differences between early embryonic stages progression and ES cells at different passages (Yan <i>et al.</i> 2013)	Human zygote to blastocyst stage and embryonic stem cells	(Tang <i>et al.</i> 2010)	124
Uterus cell type characterization and effect of <i>Hox</i> gene loss (Mucenski <i>et al.</i> 2019)	WT and <i>Hox9,10,11</i> mutant mouse uteri	Drop-seq	11,855
Characterization of a proliferative effect of estrogen on ovarian epithelial cells (Vuong <i>et al.</i> 2018)	Cultured mouse ovarian surface epithelial cells	Fluidigm C1	20
Characterization of endometrial progenitor cells and their potential role in uterine malformations and tumorigenesis (Saatcioglu <i>et al.</i> 2019)	Newborn rat uterine cells	(Hrvatin <i>et al.</i> 2018)	9801
Atlas of fetal-maternal interface during early gestation (Vento-Tormo <i>et al.</i> 2018)	Human first-trimester placenta	10X Genomics and smart-seq2	70,000
Cell-type characterization in the human normal and pre-eclampsia placenta (Tsang <i>et al.</i> 2017)	Human placenta from C-sections	10X Genomics	20,518
Cell-type characterization in the human normal placenta (Pavlicev <i>et al.</i> 2017)	Human placenta from C-sections	FluidigmC1	87
Adrenals			
Characterization of cellular trajectories during Schwann cell precursors differentiation in the adrenal medulla (Furlan <i>et al.</i> 2017)	Mouse neural crest derived embryonic adrenal medulla cells	Smart-seq2	768
Pituitary			
Gonadotroph cell line response to GnRH not linked to cell cycle (Ruf-Zamojski <i>et al.</i> 2018a)	GnRH- and control-treated L β T2 gonadotroph cell line	10X Genomics	3881
L β T2 cells respond to GnRH in bimodal all-or-nothing manner (Ruf-Zamojski <i>et al.</i> 2018b)	GnRH- and control-treated L β T2 gonadotroph cell line	Fluidigm C1 and 10X genomics	>1000 (C1) and 6332 (10X)
<i>FoxP2</i> expression in gonadotrophs, and possible sterol-producing somatotroph subpopulation (Cheung <i>et al.</i> 2018)	Seven-week-old C57BL/6J male whole pituitary	10X Genomics	13,663
Transcriptional similarity and diversity between related corticotrophs and melanotrophs (Mayran <i>et al.</i> 2019)	Four-month-old C57BL/6J male anterior and intermediate pituitary	10X Genomics	9269
Novel posterior pituitary pituicyte markers (Chen <i>et al.</i> 2020)	Three-month-old C57BL/6 male intermediate and posterior pituitary	10X Genomics	528
Compensatory pituitary stem cell gene expression in genetic model of retinoic acid signaling inhibition (Cheung & Camper 2020)	Whole pituitary from control and conditional knockout P4 female mice	10X Genomics	13,197
Transcriptional relationship between folliculostellate cells and hormone-producing cells (Fletcher <i>et al.</i> 2019)	Postpubertal Sprague–Dawley male and female whole pituitary	10X Genomics	6896
Detection of multihormonal cells; differential gene expression patterns between male and female mice, lactating and non-lactating females, and in an acromegalic mouse model (Ho <i>et al.</i> 2020)	Eight-week-old CD1 male and female mice whole pituitary; 8-week-old acromegalic female pituitary (<i>mt/Ghrh</i> transgenics); 13-week-old CD1 lactating and virgin female pituitary	10X Genomics and drop-seq	21,185

(Continued)

Table 1 Continued.

Organ / discovery	Origin of the cells	Platform/methodology	Number of cells sequenced
Hypothalamus			
Spatial markers and developmental trajectories of diencephalic progenitor cells (Guo & Li 2019)	EGFP-labeled diencephalon of 12.5dpc <i>Gbx2^{CreER-ires-eGfp}</i> embryos	10X Genomics	7365
Novel periventricular dopamine population that responds to circadian rhythm-regulated neuromedin S (Romanov <i>et al.</i> 2017)	Central column of hypothalamus in sections of P14–28 C57BL/6/N male and female mouse brain	Fluidigm C1	3131
Novel somatostatin population in lateral hypothalamic area regulating repetitive locomotor behavior (Mickelsen <i>et al.</i> 2019)	Lateral hypothalamic area of brain sections from P30 C57BL/6 male and female mice	10X Genomics	7218
Novel arcuate somatostatin population regulating feeding behavior; differential transcriptional response to dietary changes between AgRP and Pomc neurons (Campbell <i>et al.</i> 2017)	Microdissected arcuate nucleus and median eminence from 4–12-week-old male and female mice fed varying diets	Drop-seq	20,921
Markers of novel neuronal subtypes; differential transcriptional responses to dietary changes (Chen <i>et al.</i> 2017b)	Dissected hypothalamus from 8–10-week-old female B6D2F1 mice on normal or restricted diet	Drop-seq	3319
Identity and markers of novel neuronal populations activated by specific social behaviors (Moffitt <i>et al.</i> 2018)	Preoptic hypothalamic area from 78-week-old C57BL/6J male and female mice	10X Genomics	31,299
Novel vascular progenitor in embryonic hypothalamus (Hu <i>et al.</i> 2019)	Hypothalamus of 16.5dpc C57BL/6 embryos	10X Genomics	959
Pineal gland			
Identification of new pinealocyte subtypes and circadian transcriptional differences (Mays <i>et al.</i> 2018)	Rat whole pineal gland	10X Genomics	17,000

Most studies have been performed in mice, but reports of human tissues sequencing are emerging. Studies have described organ development, such as sex determination (Stevant *et al.* 2018), allowed better characterization of rare cell types, like β -cells in the endocrine pancreas (DiGrucchio *et al.* 2016) and approached disease mechanisms, by examining, for example, islets in diabetic patients (Baron *et al.* 2016, Muraro *et al.* 2016, Segerstolpe *et al.* 2016, Wang *et al.* 2016, Xin *et al.* 2016). Here, we will highlight recent studies, particularly those conducted on the hypothalamus and pituitary where the advances brought by these techniques have not been discussed yet, and examine recent advances in the pancreas and reproductive organs where most experiments have been performed.

Single-cell analyses in the hypothalamo-pituitary axis

The vast majority of cases of congenital hypopituitarism are of unknown origin. In these patients, hormonal deficiencies can result from either hypothalamic,

pituitary or combined defects. The hypothalamus and pituitary gland form indeed the top two levels of the neuroendocrine axes and hold, therefore, significant potential for discovery using single-cell technologies, which have already begun in earnest. Both organs are critical for homeostatic maintenance of a range of physiological processes. The neuroendocrine axes control growth, lactation, reproduction, stress response, metabolism, and water retention, while hypothalamic neurons additionally connect to the CNS to regulate sleep, appetite, and body temperature (for review see Kelberman *et al.* 2009, Xie & Dorsky 2017). The different cell types within both organs form functional networks controlling precise physiological functions. However, while a certain degree of organization underlines these networks, both in the hypothalamus and the pituitary, where cells are respectively organized in groups of neurons or nuclei, or interconnected in homotypic networks (Mollard *et al.* 2012), these are not morphologically apparent. Therefore single-cell approaches are particularly well-suited for identifying cell types and investigating the gene regulatory networks responsible for their fate and function.

Developmental origin of the hypothalamus

The hypothalamus is a conserved vertebrate forebrain structure that, unlike some brain regions such as the cortex where the different layers are morphologically distinct, contains structurally contiguous nuclei, without physical/morphological boundaries/demarcation. A combination of competing Wnt, fibroblast growth factor (FGF), bone morphogenetic protein (BMP) and sonic hedgehog (SHH) signals pattern the diencephalon into the thalamus and the hypothalamus (for review see [Xie & Dorsky 2017](#), [Blackshaw *et al.* 2010](#)). A caudal portion of the ventral diencephalon evaginates toward the oral ectoderm-derived pituitary anlagen or Rathke's pouch, to form the infundibulum, which will give rise to the median eminence, pituitary stalk and posterior pituitary, thereby connecting the hypothalamus and pituitary gland into an integrated neuroendocrine unit. Different neuroendocrine cells reside in hypothalamic nuclei and some project axons through the arcuate nucleus and median eminence into the posterior pituitary. However, most neuroendocrine secretions are conveyed to the anterior pituitary by the hypophyseal portal system at the median eminence. A recent study performed scRNAseq of the developing diencephalon at 12.5dpc in mice ([Guo & Li 2019](#)); however, the study focused on the molecular profiles of progenitors giving rise to the thalamus rather than the hypothalamus. Going forward, scRNAseq surveys of the ventral diencephalon throughout embryonic development will provide a deeper mechanistic understanding of the molecular pathways governing the patterning of the hypothalamus as well as the subsequent formation of neural circuits (for review see [Blackshaw *et al.* 2010](#)).

Single-cell studies of the hypothalamus

The adult hypothalamus contains a wide variety of neurons producing neurotransmitters and neuropeptide hormones, with some cell types grouped within distinct anatomical nuclei, while others are dispersed throughout large tracts of the hypothalamus. While this cellular diversity both among and within the different hypothalamic nuclei remained unclear, recent scRNAseq studies have focused on resolving the molecular landscape of each nuclei, leading to the discovery of novel neuronal subtypes. For example, dopamine (DA) neurons are located both in multiple dopaminergic cell groups (A11, A12, A14, A15) spread throughout the hypothalamus and also concentrated in the arcuate nucleus of the hypothalamus (for review see [Ang 2006](#)). SHH and BMP signaling pathways drive their

specification ([Ohyama *et al.* 2005](#)), but little is known about the molecular differences between DA neurons that reside in different sites. Romanov *et al.* were one of the first to use scRNAseq in the hypothalamus ([Romanov *et al.* 2017](#)), identifying 62 neuronal cell-types in a central column of the medial-ventral hypothalamus, including four subtypes of dopamine neurons, one of which may regulate diurnal dopamine repression of prolactin release based on connections with suprachiasmatic neurons expressing *Per* genes. Furthermore, the arcuate nucleus and median eminence contain a high concentration of other neuroendocrine neurons, acting as the major control centers for neuroendocrine axes. A recent molecular census of these two nuclei identified 26 distinct neuronal cell types ([Campbell *et al.* 2017](#)) and additionally identified novel populations such as rare cells co-expressing transcripts for the orexigenic hormone agouti-related protein (AgRP) and the growth hormone-inhibiting factor somatostatin (Sst), two neuropeptide hormones generally thought to be expressed in distinct cell types. Whole hypothalamus scRNAseq identified molecular markers of neuronal subtypes including α -tanyocytes ([Chen *et al.* 2017b](#)), specialized glial cells lining the third ventricle that form the hypothalamic stem cell niche ([Robins *et al.* 2013](#)). Chen *et al.* further observed that food deprivation induced differential transcriptional effects on specific neuronal populations, pinpointing populations mostly affected by diet variation. In a later study, a massive survey of 1 million cells from the mouse hypothalamic preoptic region, combining scRNAseq and spatial transcriptomics, identified 66 neuronal subtypes ([Moffitt *et al.* 2018](#)). Furthermore, Moffitt *et al.* were able to identify the cells that were specifically activated during sexually dimorphic behaviors and their spatial organization, allowing further investigation of underlying neuronal circuits ([Moffitt *et al.* 2018](#)). In a more recent study, 30 classes of glutaminergic and GABAergic neurons were found in the lateral hypothalamic area by scRNAseq ([Mickelsen *et al.* 2019](#)), including sst-expressing neurons that, when selectively activated by designer receptors exclusively activated by designer drugs (DREADDs), induced a reduction in resting and an increase in repetitive locomotor behavior in mice. So far, few studies have used single-cell RNAseq to study the developing hypothalamus. One study utilized a newly developed clustering tool (PanoView) in the 16.5dpc hypothalamus to identify many expected cell-types and a novel vascular progenitor population ([Hu *et al.* 2019](#)), while a currently unpublished study performed scRNAseq at 12 timepoints between 10dpc and P45 to elucidate regional subdivisions of the hypothalamus and

markers repressing and promoting hypothalamic fate (Kim 2019). These studies have provided rich resources for characterizing the cell populations existing within different regions of the hypothalamus, and future studies will likely turn toward understanding the biological role of these novel cell-types and subpopulations in health and disease.

Development and cell-turnover in the pituitary gland

The anterior and intermediate lobes of the pituitary gland are derived from the oral ectoderm as it invaginates upward toward the presumptive hypothalamus to form Rathke's pouch from about embryonic day 8.5dpc in response to BMP and FGF signaling from the ventral diencephalon (for review see Cheung *et al.* 2017). Over the course of development, pituitary progenitor/stem cells will diverge into distinct lineages to give rise to each of the six mature differentiated hormone cell-types (for review see Prince *et al.* 2011). The hypophyseal portal system arises from pituitary epithelial cells intercalating with endothelia cells from the surrounding mesenchyme to form the capillary network that, along the pituitary stalk, connect the hypothalamus to the pituitary (Hashimoto *et al.* 1998, Scully *et al.* 2016). The pituitary gland demonstrates remarkable plasticity during the lifetime of an animal, with cellular and physiological adaptations to adequately secrete the hormones required for specific life stages (for review see Florio 2011, Le Tissier *et al.* 2017). Modulation of hormone secretion is achieved at different levels, such as variation in hormone release, synthesis and storage, but also in endocrine cell numbers. While adult endocrine cells keep the ability to divide, their proliferative rate is low, but nonetheless sufficient, at least for corticotrophs, to ensure normal cell turnover (Langlais *et al.* 2013). However, upon physiological challenge (Rizzoti *et al.* 2013) or endocrine cell ablation (Fu *et al.* 2012), adult pituitary stem cells, normally quiescent in the adult, are able to give rise to new endocrine cells. Utilization of single-cell sequencing to study pituitary cells during all these different timepoints is currently in its infancy, but the increasing frequency of such studies over the past 2 years indicate it is likely to unveil significant novel discoveries in the years to come.

Single-cell studies of the pituitary

Single-cell RNA sequencing was first applied for pituitary studies using the L β T2 gonadotroph cell line, to dissect

mechanisms of activation by GnRH (Ruf-Zamojski *et al.* 2018a). Cells transcriptional responses to GnRH were found to be highly variable and this was not linked to cell cycle progression (Ruf-Zamojski *et al.* 2018a). In a follow-up study (Ruf-Zamojski *et al.* 2018b), the authors refined their approach and suggest that responding L β T2 cells do not linearly increase gene transcription, but respond bimodally to rising GnRH concentrations (Ruf-Zamojski *et al.* 2018a). However, it is unclear whether gonadotrophs *in vivo* exhibit this behavior as well and what its functional consequence may be. Single-cell RNAseq is increasingly being applied to study primary pituitary tissues. Cheung *et al.* (Cheung *et al.* 2018) first described the identification of known pituitary populations and novel biomarkers, with computational analysis to suggest a sterol-producing subpopulation of growth hormone-producing somatotrophs. In another study (Mayran *et al.* 2019), Mayran and colleagues utilized single-cell RNA sequencing as part of a wider study to understand transitional and epigenetic differences between melanotrophs and corticotrophs, both of which transcribe the prohormone pro-opiomelanocortin. Fletcher *et al.* microdissected anterior pituitaries from postpubertal male and female rats, finding a much larger proportion of S100 β -expressing folliculostellate cells (FSCs) than expected (Fletcher *et al.* 2019). Folliculostellate cells are a glial-cell like population forming a network throughout the gland. They have been shown to have an endocrine cell-supporting function and are hypothesized to optimize and harmonize endocrine secretion across the gland (Fauquier *et al.* 2002). In addition, they express the stem cell marker SOX2, but it is so far unclear whether they also have stem cell properties (Fauquier *et al.* 2008, Andoniadou *et al.* 2013). Fletcher *et al.* distinguished two subtypes of FSCs, although they do not yet provide a description of the transcriptomic differences between the two types. It is possible that the two FSC populations represent the differentially located S100b/SOX2-expressing cells in the marginal zone and the pituitary parenchyma, which as yet are not known to be functionally distinct *in vivo*. There is longstanding evidence for multihormonal cell types in the pituitary gland that produce more than one of the characteristic anterior pituitary hormones, such as mammosomatotrophs that express both growth hormone and prolactin (Frawley *et al.* 1985, Leong *et al.* 1985). However, there are virtually no known genetic markers to distinguish these populations from their single-hormone counterparts. Remarkably, while three pituitary scRNAseq studies did not find evidence of mammosomatotrophs (Cheung *et al.* 2018, Fletcher *et al.* 2019, Mayran *et al.* 2019),

one single-cell study using 8-week-old WT CD1 male and female mice detected that approximately 30% of cells expressing either *Gh* or *Prl* also expressed the other hormone in both sexes (Ho *et al.* 2020). Furthermore, this study also appeared to show that the majority of *Pomc*-expressing cells also express *Gh* and *Prl* and that the majority of *Tshb*-expressing cells also express *Gh*. It is unclear why multihormone pituitary cells are so variably detected in different studies. Further studies are required to confirm and characterize these plurihormonal cells, but single-cell approaches have the potential to identify markers and thus allow us to characterize and better understand the role of these cells. Ho *et al.* also explored differential gene expression patterns between males and females, lactating and virgin adult female mice, and in an acromegalic mouse model (Ho *et al.* 2020). Recently a study also utilized scRNAseq to characterize the post-natal consequences of retinoic acid inhibition in the pituitary. These analyses showed a compensatory expression of adhehyde dehydrogenase in the stem cell compartment, while there was no manifestation of pituitary disease in this genetic model of retinoic acid inhibition (Cheung & Camper 2020). Furthermore, the first scRNAseq study of the posterior pituitary identified novel markers of pituicytes, a specialized posterior pituitary astroglia cell-type (Chen *et al.* 2020).

In conclusion, due to the novelty of single-cell studies in pituitary tissue, the functional role of novel genes identified in individual cell types has yet to be investigated. Because most cases of hypopituitarism are of unknown aetiology, these studies have the potential to identify new regulators of endocrine cell fate and function. While a previous study has studied pituitary tumors on the single-cell level using single-cell calcium imaging and immunohistochemistry (Senovilla *et al.* 2015), scRNAseq is likely to prove powerful in understanding the molecular origins of different types of pituitary tumors (Muller 2014, Molitch 2017).

Single-cell studies in the pancreas

The endocrine pancreas has a central role in maintaining and adapting the blood glucose concentration in vertebrates. The islets of Langerhans that form the endocrine pancreas comprise five main different cell types: α cells producing glucagon, β cells secreting insulin, δ cells synthesizing sst, ϵ cells making ghrelin and γ cells, source of pancreatic polypeptide. Diabetes, resulting from a failure to control blood glucose concentration, results from β -cells deficiency and it afflicts more than

8% of the world population aged over 18 years (WHO). It is incurable, and while current treatments reduce symptoms, there are long-term complications. A major goal is to stop its progression and restore β cells (for review see Zhou & Melton 2018). It is therefore not surprising that, among endocrine organs, the pancreas has been by far the most investigated gland at the single-cell level. Single-cell technologies appear indeed particularly suited to understand the underlying mechanisms and functional significance of pancreatic β -cell heterogeneity, in both health and disease. With the ultimate goal to generate new β cells for diabetic patients, either *in vitro*, for transplantation, or *in vivo* by stimulating regeneration or trans-differentiation, single-cell studies have already provided valuable insights. We will present here a concise review of representative studies and refer readers to recent and more comprehensive reviews (Carrano *et al.* 2017, Tritschler *et al.* 2017).

Morphogenesis and cell turnover

Embryonically, the pancreas is a foregut endoderm derivative (Jennings *et al.* 2015, Larsen & Grapin-Botton 2017). Several single-cell RNAseq datasets have now been generated, using different strategies, to further unravel endocrine developmental pathways (Krentz *et al.* 2018, Bastidas-Ponce *et al.* 2019, van Gurp *et al.* 2019). At 9.5dpc in the mouse, signals from the mesoderm induce the formation of buds from the primitive gut endoderm. From these buds, an intensive branching develops, invading and interacting with the surrounding mesenchyme, ultimately giving rise to epithelial monolayers organized around a single lumen. At the tips of the branches, endocrine progenitors differentiate from neurogenin-3 unipotent progenitors. From 9.5dpc until birth, committed precursors delaminate from the epithelium and cluster to form the islets of Langerhans just before birth. Post-natally, there is proliferation, in particular of endocrine cells, to reach the mature organ size, but after this early phase, proliferation and cell turnover are low and there is clearly a limited regenerative potential in humans. β -cell turnover appears to rely on division of pre-existing β cells, but ablation studies in the mouse have highlighted the potential of α and δ to trans-differentiate into insulin-producing cells. In human, α and δ cells have recently been successfully reprogrammed *in vitro* into β cells, highlighting the conservation of this phenotypic plasticity and its potential use for therapeutic approaches (Furuyama *et al.* 2019). The presence of an adult population of islet stem cells has been suggested

based on histological observations; however, there is not, so far, any convincing experimental evidence of their existence (Zhou & Melton 2018). This is in contrast with the exocrine pancreas, where scRNAseq analyses have helped to uncover a small population with progenitor characteristics (Wollny *et al.* 2016).

Single-cell RNAseq studies

β cells are known to be a highly heterogeneous population, especially post-natally because there is substantial organ growth in rodents and cells are at different stages of maturation. Therefore, to characterize mechanisms underlying efficient β -cell generation at these early stages, single-cell analyses are useful to segregate the different cell states and arrange them along a differentiation trajectory. To unravel these early events, scRNAseq was performed at multiple timepoints post-natally in the mouse (Qiu *et al.* 2017, Zeng *et al.* 2017). Together, these two studies highlight new maturity markers and proliferation pathways that are active early on. The latter ones could be used and be re-activated in the adult to potentially stimulate endogenous regeneration. As mentioned earlier, δ cells have the potential to trans-differentiate into β cells both in mice and humans (Furuyama *et al.* 2019). However, because they are a rare cell type, many questions remain about their function. ScRNAseq helped to show that they are important regulators of β cells through their unique ability to respond to Ghrelin (DiGrucchio *et al.* 2016, Xin *et al.* 2016).

Most studies have been expectedly performed in mice, but it is particularly important to examine human pancreas because there are substantial known differences between both species. Single-cell analyses have thus been used to characterize human islets during development (Petersen *et al.* 2017, Ramond *et al.* 2018, Villani *et al.* 2019) and in both healthy and diabetic post-natal tissues (Baron *et al.* 2016, Muraro *et al.* 2016, Segerstolpe *et al.* 2016, Wang *et al.* 2016, Xin *et al.* 2016). These analyses have revealed an unanticipated complexity within the cell populations, particularly from the diseased samples. However, similar subpopulations were identified between the different datasets, along with characterization of transitional states, opening new avenues for potential treatments (for review see Dominguez-Gutierrez *et al.* 2019). Finally, aging in the healthy pancreas is associated with an accumulation of somatic mutations and an increased transcriptional noise which may reflect a compromised islet identity (Enge *et al.* 2017). It is not yet known how the diseased pancreas evolves with time.

Single-cell approaches have also been used to assess the protocol and quality of β cells generated *in vitro*. The comparison of datasets generated from fetal human samples with those generated *in vitro* highlighted differences and confirmed the inability to reach a fully mature state (Hrvatin *et al.* 2014, Petersen *et al.* 2017, Ramond *et al.* 2018), which remains a problem for most differentiation protocols. A similar conclusion was made comparing scRNAseq mouse embryonic datasets with ones obtained from β cells derived from human ES cells (Krentz *et al.* 2018). Comparisons between *in vivo* development and *in vitro* differentiation are useful to pinpoint steps where differences exist and where improvements are required. Finally, more than 100,000 human pluripotent stem cells were recently analyzed during 3D pancreatic endocrine differentiation (Veres *et al.* 2019). These extensive datasets reveal intermediate differentiation states, better markers and guide toward further improvements to obtain more mature β cells (Veres *et al.* 2019).

The exocrine pancreas secretes digestive enzymes through the pancreatic duct. The pancreatic duct is also the site of the most common pancreatic tumors. Pancreatic ductal carcinomas are often discovered late, and therefore, the prognosis is poor and these tumors represent the fourth highest cause of cancer-related deaths (Foucher *et al.* 2018). Single-cell RNAseq approaches have been used to resolve tumor heterogeneity, characterize new markers for prognosis, and pinpoint potential target and immune anti-tumor strategies (Peng *et al.* 2019). In addition, datasets have been generated from precursor lesions to describe events preceding tumorigenesis and characterize markers (Bernard *et al.* 2019).

In conclusion, most efforts have been focalized toward the generation or re-generation of β cells, and single-cell analyses have certainly provided clues on how to improve current protocols. Single-cell characterization of diabetes has highlighted an unexpected heterogeneity between patients, and more work is needed to reveal the biological significance of these results and pinpoint common features that could be used to improve current treatments.

Single-cell analyses in reproductive organs

About one in eight women and one in ten men have experienced infertility in the UK between 2010 and 2012 (Datta *et al.* 2016). In males, tests, diagnosis and treatments are not standardized, and in 2017, the WHO recommended that more research was needed to better characterize germ cells and the effect of lifestyle choices on their differentiation and functionality (Barratt *et al.* 2017).

In women, infertility causes are unknown in about 25% of the cases (Somigliana *et al.* 2016). Because infertility is associated in particular with implantation failure, a better characterization of the female reproductive organs and modalities of embryonic implantation are required. Single-cell technologies have moreover been used to investigate mechanisms of sex determination, and this subject has been recently reviewed (Stevant & Nef 2018). Here, we will instead focus on discoveries made using single-cell analyses of germ and supporting cells, the uterus and early stages of pregnancy.

Mechanisms of sex determination

In mammals, the undifferentiated gonad adopts a male differentiation pathway upon expression of the Y chromosome sex determination factor SRY, at 10.5dpc in the mouse. Its expression is restricted to the NR5A1 positive presumptive Sertoli cells, a spermatogenesis-supporting cell type. A recent single-cell analysis analyzed these early events and examined the transcriptome of NR5A1 positive cells from 10.5 to 16.5dpc in the mouse (Stevant *et al.* 2018). The authors show that these cells are multipotent and that, in the male, they give rise sequentially to Sertoli then Leydig cells, interstitial cells producing testosterone (Stevant *et al.* 2018). The mechanisms explaining this shift in cell fate are currently unknown, but because deficiency/malfunction in Sertoli, and more frequently Leydig cells underlie infertility, these results have important clinical implications. In XX embryos, the transcription factor Foxl2 and the Wnt signaling pathways are thought to be crucial determinants. At the time of sex determination, between 10 and 11dpc, primordial germ cells, originating from the post-implantation epiblast, migrate through the endoderm to the gonads. Male and female specific differentiation pathways subsequently antagonize each other to promote development of the gonad and associated organs and tissues characteristic of genetic sex of the individual and actively prevent the alternative process. For example, the Mullerian ducts, that will give rise to the uterus, develop along the Wolfian ducts, that will later degenerate in the female, while in the male, presumptive Sertoli cells secrete the anti-Mullerian hormone (AMH) causing Mullerian ducts regression, whereas the Wolfian ducts give rise to the vas deferens.

Single-cell RNAseq studies

Testicular cells have been analyzed both in mouse (Green *et al.* 2018) at different stages (Hermann *et al.* 2018)

and in humans, in both healthy (Hermann *et al.* 2018) and infertile subjects (Wang *et al.* 2018). In the mouse, scRNAseq analyses of testicular cells provide a thorough description of spermatogenesis (Green *et al.* 2018, Hermann *et al.* 2018) and of supporting cells, revealing new subtypes (Green *et al.* 2018). In addition, differences between juvenile and adult mouse spermatogenesis have been transcriptionally characterized (Hermann *et al.* 2018). These will be useful when trying to devise *in vitro* approaches for germ cell differentiation (Zhou *et al.* 2016). In human, these analyses allow better description of pathologies, as demonstrated by Wang *et al.* who characterized abnormal gene expression patterns in Sertoli cells when comparing expression profiles from testicular cells in normal and azoospermic samples (Wang *et al.* 2018). A similar approach was used to characterize the effects of endometriosis in females. Endometriosis causes infertility, because adhesions develop in the female reproductive tract. However, it has also been suggested to affect oocyte quality. Its impact on oocyte was thus evaluated using scRNAseq (Ferrero *et al.* 2019). These analyses revealed that the disease affects oocyte transcriptomes and that these changes suggested a reduced quality (Ferrero *et al.* 2019). Oocytes and granulosa cells were similarly investigated in normal and polycystic ovary syndrome (PCOS) samples and the positive impact of fertility treatments detected at the transcriptional level (Liu *et al.* 2016). Human folliculogenesis was moreover examined in several studies to better characterize oocyte activation and cell interactions in the ovary, in particular with the supporting granulosa cells and the steroidogenic theca cells (Zhang *et al.* 2018, Fan *et al.* 2019). These analyses provide a precise description of these processes. They also suggest new markers of ovarian reserve which could be used as fertility assessors (Zhang *et al.* 2018) and provide a thorough description of the different ovarian somatic cell types (Fan *et al.* 2019). Important epigenome changes, such as X-chromosome re-activation in the female, are observed during meiosis. Several studies have examined these changes in human male and female primordial germ cells at the single-cell level by performing exome, methylome and transcriptome analyses (Guo *et al.* 2015, Li *et al.* 2017, Vertesy *et al.* 2018) to understand mechanisms of reprogramming underlining germ cell development. Furthermore, preimplantation embryonic stages have also been examined in mouse and human, in several studies, characterizing mechanisms of genome re-activation, early cleavage processes and species-dependant differences (Xue *et al.* 2013, Yan *et al.* 2013, Blakeley *et al.* 2015, Leng *et al.* 2019). Finally, anomalies of the uterus are estimated to account for a fifth of female

infertility cases. ScRNAseq was used in mice to examine development of this organ in juvenile animals (Mucenski *et al.* 2019, Saatcioglu *et al.* 2019). In humans, the maternal-fetal interface has been characterized by performing single-cell sequencing of the placenta and decidua during the first trimester of pregnancy, focusing on characterization of cellular interactions to better understand mechanisms underlining development of this complex and transient environment (Vento-Tormo *et al.* 2018). Studies were also performed on placenta at term (Pavlicev *et al.* 2017), in particular to characterize anomalies characterizing pre-eclampsia (Tsang *et al.* 2017).

In conclusion, single-cell studies have so far been used to unravel sex determination mechanisms and characterize conditions underlying infertility. It is expected that these discoveries will lead to improved treatments and care.

Conclusion

The robustness of new single-cell technologies has enabled analyses of an unprecedented numbers of single-cells from heterogeneous tissue systems. While technical hurdles remain, the impact of these technologies is already obvious and it is expected to further increase, along with technological advances. Multi-omic approaches are expected to expand investigative potential, while spatial transcriptomic approaches will allow examination of cell heterogeneity *in situ*. In parallel, it will also be important to examine whether this transcriptional diversity within cell populations underlines functional differences or maybe simply highlight different cell states. Furthermore, the potential of these technologies for characterization of mutant phenotypes and disease mechanisms appear particularly interesting because both the cells that are directly affected and their potential effect on surrounding cells can be distinguished. In particular, single-cell analyses of tumor development in endocrine organs should help characterize these diseases and potentially point toward new therapeutic approaches. While many studies have so far characterized normal organ cell heterogeneity, these technologies hold great potential for further functional characterization in different contexts.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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