The role of Stem Cell Factor in recovery from neurological injury

By

Maureen Cassidy Turner

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Neuroscience

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor John Ngai, Chair
Professor Richard Harland
Professor Daniela Kaufer
Professor David Schaffer

Summer 2018

Abstract

The role of Stem Cell Factor in recovery from neurological injury

By

Maureen Cassidy Turner

Doctor of Philosophy in Neuroscience

University of California, Berkeley

Professor John Ngai, Chair

Stroke, Alzheimer's Disease, and other neurological pathologies are not only among the leading causes of death in the United States but also account for a huge portion of national healthcare expenditure (National Center for Health Statistics, 2016 report). Advancing our understanding of the bodily response to neural injury and the mechanisms that might be used to prevent or heal that injury is of paramount importance. This dissertation is a presentation of work aimed at understanding the molecular cascades that govern regeneration after neural injury using the olfactory neuroepithelium (OE) - one of the few locations in the adult where such regeneration is naturally able to occur - as a model.

Throughout the human lifespan, the OE is continually subject to chemotoxic and mechanical insult. Remarkably, the epithelium has the ability to fully regenerate all cell types, both neurons and supporting cells after minor or extensive injury. Understanding the biological regulation that allows for this regeneration serves two purposes: One, these mechanisms may be usefully employed in the development of translational methods for treating neurological diseases. Two, the cells and stem cells of the OE constitute a rich potential source of cells for possible transplantation in translational approaches to pathologies where neural death has occurred. In this dissertation, we evaluated the functional importance of the Stem Cell Factor growth factor (SCF) and C-Kit, the receptor tyrosine kinase SCF binds to, in neural regeneration.

The regenerative capability of the human epithelium is faithfully recapitulated by the murine OE, making it an ideal model of the human system for use in researching regenerative mechanisms. Using transgenic mouse models, we examined the expression of SCF and C-Kit in the OE via a combination of molecular imaging and single-cell transcriptional sequencing, and we discovered that SCF is expressed by all sustentacular (SUS) supporting cells and all horizontal basal stem cells, both in the uninjured and in the injured/regenerating OE. C-Kit is expressed by a large fraction of the globose basal cells, a population which is heterogeneous and consists both of stem and committed progenitor cells, and some microvillous cells, a population which is the subject of much current research. Interestingly, during regeneration after experimentally induced injury, the majority of cells express SCF or C-Kit. These striking expression patterns led us to look for deficits in SCF knock-out transgenic mice with the hope of better understanding the function of this growth factor. We found that SCF is critical to production of normal numbers of neurons and microvillous cells during regeneration. Extensive assessment of the expression patterns and impact of the ablation of SCF

function in the OE led us to a novel understanding of the role of this growth factor/receptor pair in neural recovery after injury: SCF is critical for OE stem cells to make neuronal fate decisions during regeneration from injury.

Acknowledgements

There are many people I'd like to thank for their help with this dissertation.
I couldn't have done it without any of you.

Particularly, I'd like to thank:
Russell and Dave for teaching me a whole lot of what I know about biology,
Mike and Todd for teaching me a whole lot of what I know about life,
and Sam and Ariane for keeping me (mostly) sane.

And to my family:
I love you all more than I have words to express. Thank you, thank you for supporting me in all my far-flung endeavors. I hope to do you proud.

Table of Contents

List of Figures

Abbreviations

OE	Olfactory epithelium/olfactory neuroepithelium
HBC	Horizontal Basal Cell
GBC	Globose Basal Cell
TA	Transit Amplifying Cell
INP	Immature Neural Precursor Cell
SUS	Sustentacular Cell
OSN	Olfactory Sensory Neuron
MV	Microvillous Cell
BG	Bowman's Gland
E	Embryonic day
P	Post-natal day
SS RNAseq	RNA-sequencing single cell transcriptional sequencing
dpi	Days post-injury/days post-methimazole administration
wpi	Weeks post-injury/weeks post-methimazole administration
SCF	Stem Cell Factor/Steel Factor/Kit Ligand

Chapter 1 | The Mammalian Olfactory Epithelium: Structure and Function

1.1 | Significance of the present work

The present work focuses on elucidating the biological mechanisms governing neural creation by stem cells in the olfactory neuroepithelium (OE) in health and during regenerative response to injury over the lifespan of the organism. This system is of paramount importance in organisms where olfactory cues convey virtually all social information, such as the mouse. Yet, it is also of importance to us. Though humans do not rely on olfactory cues to the same extent as some other species, any mechanisms that maintain neural systems in the face of decline are of potential use to us as we stave off the neurological decline inherent to our long lifespans.

The real importance of this work lies in the public health burden of degenerative and traumatic neural injury, which is poised only to increase in the coming years. Despite having two minor proliferative neural stem cell zones, the adult brain, largely, cannot heal itself. The regulatory networks governing recovery from neural injury are critical to understand. Here, we focus on just such a mechanism – the growth factor Stem Cell Factor (SCF) and its cognate receptor C-Kit.

1.2 | Mammalian olfactory epithelium

The olfactory epithelium is situated within the nasal cavity, where it lines the surface of the bony nasal turbinate folds (Farbman, 1992; Frisch, 1967; Morrison & Costanzo, 1992) (See Figure 1). Because this location is directly exposed to the external environment, olfactory neurons are able to access the odorants they detect, but they are also susceptible to exposure to mechanically harmful and chemotoxic environmental stimuli (DeMaria & Ngai, 2010; Dulac, et al., 1995). The OE, therefore, undergoes continual neural death and consequent neurogenesis throughout adult life (Brann, et al., 2014; Graziadei & Graziadei, 1979; Iwai, et al., 2008; Leung, et al., 2007; Mackay-Sim, et al., 1991; Murray, et al., 1999) . In the absence of injury, normal cell turnover in the OE is maintained by globose basal cells (GBCs) which proliferate to replenish lost neurons (Caggiano, et al., 1994; Schwob, et al., 1994) (Figure 1).

In addition to normal 'steady state' cell turnover, the OE is capable of regenerating after more extensive injury (Burd, 1993; Matulionis, 1975; Schultz, 1960; Schwob, et al., 1995). During recovery of this sort, the role of progenitor shifts from the GBCs to the horizontal basal cells (HBCs), which are situated just below the GBCs in the OE. The HBCs are thought to have a wider stem potential than the GBCs, allowing them to regenerate not just neurons, but all supporting cell types in the OE as well (Iwai, et al., 2008; Leung, et al., 2007). Understanding this ability is important, both to elucidate the underlying biology of the OE, but more so as a step towards understanding how we might one day harness this biology to regrow the human brain after traumatic and neurodegenerative injury.

Though the present work relies on the murine olfactory epithelium as a model, human and murine epithelia are, for the most part, incredibly similar (see Moran, et al., 1982 and Morrison & Costanzo, 1990 for evidence that the cell types and organization of those cells in the human OE is dominantly the same as in the mouse). The OE is an excellent model of neurogenesis in the adult organism. In addition to being the only readily accessible neural stem cell niche in mice and humans (Figure 1), the OE has been well characterized using molecular and genetic markers. A hierarchy of transcription factors identify distinct cell states as maturing cells move basally to apically through a progression from quiescent stem cells to transit-amplifying proliferation to terminal differentiation into neuronal and non-neuronal cells (reviewed in Zhang & Fedoroff, 1999). This simple and consistent progression represents a significant advantage in studying stem cell molecular dynamics, as it is easy to assess both

initial gene expression and also changes in expression during regeneration after neural injury, making the OE is a superb model of neurologic injury and recovery, and a rich source of translational inspiration.

1.3 | Ultrastructure and cell types in the OE

The OE is composed of a few core cell types, which are illustrated in a schematic in Figure 1. Similar to the human, the mouse OE houses four classes of cells: quiescent stem cells, active stem cells and neural progenitors (globose basal cells), immature and mature neurons, and supporting cells (sustentacular [SUS], Bowman's Gland [BG], microvillous) (Graziadei & Graziadei, 1979; Mackay-Sim & Kittel, 1991) (Figure 1). Starting with the most basal cell layer (those cells directly apposed to the basal lamina), the bottom layer of cells in the OE consists of the horizontal basal cells, so named in reference to their flattened, elongated shape. The HBCs sit in an adherent monolayer on the basal lamina. Directly above the HBCs sit the globose basal cells, which are also named for their morphology (Holbrook, et al., 1995; Fletcher, et al., 2011; Packard, et al., 2011). Both the HBCs and the GBCs are genetically homogeneous populations of cells and are the subject of ongoing study (Caggiano, et al., 1994; Cau, et al., 1997). Filling in the intervening layers between the GBCs and mature neurons are several layers of neuronal transit-amplifying cells and immature neurons that travel progressively apically as they mature. This structure can simplistically be characterized as having the most stem-like cells in the most basal layers, with cells of increasing 'maturity' in each layer moving basal to apical.

Though the HBC quiescent stem cell population holds translational promise, here we focus on the GBCs, owing to their critical role in SCF/C-Kit signaling in the OE. The regulatory and dynamic features of the HBCs have been well described (Caggiano, et al., 1994; Calof, et al., 2002; Cau, et al., 1997; Fletcher, et al., 2011; Goldstein & Schwob, 1996; Joiner, et al., 2015 Iwai, et al., 2008; Leung, et al., 2007; Schultz, 1941). Despite interest for therapeutic reasons, the active progenitors of the OE, the GBCs, have proven harder than HBCs to fully characterize owing, in part, to their genetic heterogeneity (Goldstein, et al., 1998; Leung, et al., 2007; Jang, et al., 2008). Under steady state conditions, GBCs divide roughly once a day – in contrast to HBCs, which, in the absence of injury, undergo cell division no more frequently than every 30-60 days (Caggiano, et al., 1994; Huard, et al., 1998; Leung, et al., 2007; Mackay-Sim & Kittel, 1991).

GBCs are a mixed class of cells, containing at least two distinct subclasses: unipotent neuronal precursors (Caggiano, et al., 1994; Cau, et al., 1997) and multipotent stem cells (Chen, et al., 2004; Gokoffski, et al., 2011; Huard, et al., 1998, Manglapus, et al., 2004). Though heterogeneous, the GBCs are mostly actively proliferative, even under steady-state conditions (Caggiano, et al., 1994; Graziadei & Graziadei, 1979; Huard & Schwob, 1995; Schwob, et al., 1994), and express a predictable series of transcription factors over the course of their tenure as GBCs before terminal differentiation which can be used as markers of maturity for experimental purposes.

Apoptosis is apparent throughout the entire extent of the neuroepithelium throughout the nasal cavity and turbinates in an ongoing way over the lifespan of the animal (Carr & Farbman, 1993; Holcomb, et al., 1995). As normal neural turnover occurs under steady-state conditions, the GBCs proliferate and differentiate to replace dead cells (Calof, et al., 2002; Fletcher, et al. 2011; Fletcher, et al., 2017; Goldstein & Schwob, 1996; Huard, et al., 1998; Smart, 1971). Lineage tracing experiments have shown that more profound injury results in the activation of HBCs, which proliferate and differentiate to rebuild the entire OE (Chen, et al., 2014; Fletcher, et al., 2017; Gayde et al., 2017; Gokoffski, et al., 2011; Goldstein, et al., 2015; Leung, et al., 2007). Studies have demonstrated that the newly rebuilt OE is functionally capable to an extent that is similar to the epithelium before injury (Graziadei, 1973).

Mature neurons rest just underneath the sustentacular (SUS) cell layer that makes up the most apical portion of the OE (Figure 1). These neurons each extend a short stalk capped with a knob of cilia above the SUS layer, which allows them to sample the odorants borne in on the lumenal air (Menco, et al., 1980). These apical dendrites are lined with G Protein Coupled Receptors (GPCRs) that bind odorants and effect signal transduction within the olfactory cell (Buck & Axel, 1991; Mombaerts, 2004). Each sensory cell expresses one olfactory receptor type from the over 913 total olfactory receptor genes (Godfrey, et al., 2004). Olfactory sensory neurons extend axons down through the layers of the OE. These axons fasciculate and pass through the basal lamina and out of the epithelium through the cribiform plate (DeMaria, et al., 2010). Once through the plate, bundles of axons converge on the olfactory bulb (OB), where axons derived from sensory neurons tuned to like odorants synapse in a common location called a glomerulus, of which the mouse has been estimated to have ~1800 (Royet, et al., 1988).

With cell bodies aligned neatly along the most apical portion of the OE, SUS cells are visible, extending their cilia into the lumen of the nasal epithelium. The SUS cells are the major glial constituent of the OE niche and serve a protective, secretory role ensheathing and supporting the local neurons. Consistent with their role in detoxification, SUS cells are known to express glutathione-S-transferases and cytochrome p450 (Beites, et al., 2005).

Several other supporting cell types are present in the OE, though they are secondary in numbers to neurons and SUS cells. These include microvillous cells and cells of the Bowman's Gland. Despite increasing interest in recent years, microvillous cells remain poorly understood (Elsaesser & Paysan, 2007). It is presently unclear the degree to which microvillous cells are a heterogeneous population. At least some microvillous cells express TRPM5 (Lin, et al., 2008). It has been suggested that these microvillous cells derive from Ascl1+ progenitors historically thought to be solely involved in the generation of typical neurons (Yamaguchi, et al., 2014), which has been supported by current research (Fletcher, et al., 2017; Gayde, et al., 2017). While distinct populations among the microvillous cells are known to be cholinergic (Ogura, et al., 2011). The functional role of these populations is, as yet, unknown.

1.4 | Modeling neural tissue regeneration

Human actively proliferating adult neural stem cells continuously rebuild the olfactory neuroepithelium as neurons are killed by exposure to environmental toxins and mechanical insult (Mackay-Sim & Kittel, 1991). Within the OE, both at homeostasis and following injury, there is a huge amount of regenerative capacity. Neural stem cells in the OE are capable of completely regenerating the architecture of the niche, including all cell types, neuronal and non-neuronal, after large-scale injury throughout the life of the organism (Leung, et al., 2007; Iwai, et al., 2008, Brann, et al., 2014), though with diminished capacity during aging (Child, et al., 2018). The ability of the OE to perform continuous neural regeneration makes the OE an ideal model system for the study of neurogenesis. Understanding this ability is important, both to elucidate the underlying biology of the OE, but more so as a step towards understanding how we might one day harness this biology to regrow the human brain after traumatic and neurodegenerative injury.

While many experimental methods (axotomy, bulbectomy, methyl bromide, zinc sulfate) cause sufficient cell death in the OE to stimulate GBC proliferation-based regeneration (Murray & Calof, 1999; Jang, et al., 2003; Schwob, 2005), the model of OE injury and regeneration our lab relies on is systemic administration of methimazole, which causes a more complete ablation of the cells in the

OE and more comprehensive activation of the niche's regenerative potential. Intraperitoneal or intranasal methimazole administration results in the death and subsequent sloughing off of all of the cell types layered on top of the HBCs in the OE. This cell death causes the mitotic activation of the HBCs which regenerate all of the cells (including neurons and gland cells) that were lost. Over the course of about two weeks, this regeneration paradigm allows for examination of the genetic framework supportive of the birth, in the adult animal, of a myriad of neural and supporting cell types (SUS cells, microvillous cells, cells of the Bowman's Glands) (Bergman, et al., 2002; Fletcher, et al., 2011; Jia & Hegg, 2012, Leung, et al., 2007; Packard, et al., 2011; Schwob, et al., 1995).

As much of the currently available body of research on neurogenesis is based on developmental and adult central nervous system stem cell niches, there is tremendous and growing interest in use of the OE as a model of neural regeneration and healing. Recent work in our lab has provided key insights about the intricate genetic mechanisms which control cell fate decisions in the regenerating OE (Fletcher, et al., 2017). Further, we've been able to parse the genetic controls mediating stem cell choices between self-renewal and differentiation (Gayde, et al., 2017). These works both succinctly answer long-standing mysteries in stem cell biology, and also serve to demonstrate the power of the OE niche as a model system in combination with cutting-edge genetic assays and analysis.

While the broad genetic mechanisms which regulate OE responses under homeostasis and during recovery from injury are becoming clearer, it is not yet entirely clear what each specific gene that is implicated in the process functions to do. Recent work has approached regulation of the commitment of HBCs to neuronal or non-neuronal fates during regeneration after injury (Packard, et al., 2016; Herrick, et al., 2018), and the signaling mechanisms by which these cells are induced to activate for regeneration (Herrick, et al., 2017).

The GBCs have proved to be a more heterogeneous and difficult to elucidate population, despite evidence that these cells are thymidine label-retaining (a hallmark of stem cells) (Jang, et al., 2013) and multipotent (Cau, et al., 1997; Cau, et al., 2002; Chen, et al., 2004; Chen, et al., 2014; Manglapus, et al., 2004). Past studies have focused on the role of basic helix-loop-helix transcription factors in the neuronal progression of GBCs (Cau, et al., 1997; Cau, et al., 2002; Manglapus, et al., 2004). More recently, several studies have examined the genetic regulation of these cells during neurogenic responses, including role of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, in GBC stem cell dynamics (Guo, et al., 2010; Jang, et al., 2013). Recent work has even demonstrated genetic control of the "dedifferentiation" of these cells during the OE response to injury (Lin, et al., 2017), a mechanism reminiscent of their putative role in the generation of HBCs during development (Packard, et al., 2011), though it remains unclear if this state is best characterzed as dedifferentiation or as a distinct activated state (Gayde, et al., 2017).

While many studies are currently approaching an understanding of stem cell regulation in the OE model system by looking 'top down', with a focus on large gene sets, in the present set of experiments, we take an opposite approach. We tackle one particularly evocative aspect of the complex genetic control of GBC stem cell identity, using the powerful methimazole model of OE neurogenesis to study changes in and the functional importance of C-Kit signaling cascade expression during neural recovery.

1.5 | Growth factor receptor C-Kit and its ligand Stem Cell Factor

Growth factor Stem Cell Factor (also called SCF, Steel factor, or Kit Ligand [KitL]) and its receptor tyrosine kinase, C-Kit, are pivotal in fundamental cellular processes including proliferation,

differentiation, survival and cell migration in diverse stem cell niches embryonically and in the adult organism. These proteins, encoded at the Steel and W loci respectively, are known to effect myriad changes in cells via interaction with a variety of downstream signaling pathways (PI3K, Src, MAPK, PLC, and others [reviewed in Lennartsson & Rönnstrand, 2012]).

The C-Kit receptor is a ~150 kDa protein (Ashman, et al., 1999) initially identified at the protein level as the target of monoclonal antibody YB5.B8 (Ashman, et al., 1991), that can be found in diverse cell types including hematopoietic cell types, endothelial cells, gut epithelial cells, brain astrocytes and neurons, renal tubules, and germ cells, among others (reviewed in Ashman, 1999). C-Kit initially drew attention as a trophic target in neural injury, when up-regulation of C-Kit and SCF mRNA production was observed in cells surrounding and infiltrating experimental cortical stab wounds (Orr-Urtreger, et al., 1990). Cells at the sites of cortical brain injury up-regulate expression of SCF, which causes the migration of C-Kit+ neural precursors from other zones in the brain to the location of the injury (Keshet, et al., 1991; Sun, et al., 2004). Further, it was found that adding SCF at the site of induced stroke in mice caused proliferation and aided functional recovery (Jin, et al., 2002; Zhao, et al., 2007). Together, these studies underscore the central importance of SCF signaling in neural precursor cell proliferation and in healing from neural injuries and pathologies. However, the mechanisms by which C-Kit signaling promotes recovery after neural injury remain obscure, as does the extent of the ubiquity of these mechanisms.

More is known about SCF's role in healing in biological niches outside of the OE. Notably, in the hematopoietic system, SCF expression by mesenchymal cells drives progenitor proliferation and differentiation in response to injury (Heissig, et al., 2007) - roles SCF mediates by promoting physical cellular adhesion between ligand and receptor expressing cells (Kimura, et al., 2011), an effect reliant on membrane-bound SCF being expressed (Driessen, et al., 2003; Toksoz, et al., 1992). However, SCF's role is not consistent throughout the body. In the colon, for instance, Lgr5+ stem cells express SCF, while supporting goblet cells express C-Kit (Chen, et al., 2015). Yet more variations on these themes can be seen in stem cell niches producing melanocytes, mast cells, in reproductive niches, and beyond.

Considering that C-Kit+ cells are competent to drive recovery in a wide variety of other, non-neural tissues (cardiac (Morigi, et al., 2004), hepatic (Rojas, et al., 2005), renal (Liang, et al., 2013), bronchial (Norlin, et al., 2015)), there has been interest in its expression and possible role in the OE. Several studies have reported active C-Kit/SCF transcription in the OE stem cell niche both during development (Krolewski, et al., 2012; Shetty, et al., 2005) and during adulthood (Ellison, et al., 2013). C-Kit transcription increases during recovery from injury in the OE (Kinnman, et al., 2001). The presence of a C-Kit+ contingent of cells in our stem cell niche of interest, the OE, has been noted (Bernex, et al., 1996 ; Matsui, et al., 1990; Orr-Urtreger, et al., 1990). C-Kit is expressed by a small complement of cells with GBC-morphology and bearing traditional GBC histological markers. The GBC population contains stem and progenitor cells – though potential appears to depend on experimental conditions: Transgenic lesion-based studies suggest that cells marked by C-Kit expression primarily produce neurons (Goldstein, et al., 2015), and lineage tracing of these cells has demonstrated that they are multipotent under injury, but not steady-state, conditions (Goss, et al., 2016). Discovery of C-Kit activity in the OE provides an opportunity to understand both the basic molecular mechanisms driving recovery in this niche, and also is a chance to learn more generalizable information about SCF signaling in stem cells and neurologic injury. Given the myriad roles of SCF in other niches and that it is poised to impact stem cells and neural progenitors in the OE, it is important to know how SCF signaling fits into the broader OE niche and what functional role it plays

in niche dynamics.

1.6 | Results

In pursuit of these aims we first sought to find the origin of the SCF signal in the OE. Using a combination of single-cell transcriptional sequencing methods and immunochemical analysis in transgenic animals, we examined expression profile of SCF and its receptor C-Kit. We show for the first time that SCF is expressed by all quiescent HBC stem cells and SUS supporting cells throughout the OE. Using the same methods, we transcriptionally characterized the identity of C-Kit+ GBCs confirming and expanding previous reports (Goldstein, et al., 2015; Goss, et al., 2016). Using a chemical lesion, we characterize the remarkable expression of this growth factor throughout recovery from injury – finding that, during the critical early days of recovery, expression of SCF or C-Kit marks nearly every cell in the recovering epithelium.

To uncover the functional necessity of SCF signaling under steady-state and injury conditions, we used transgenic models to selectively knock-out SCF expression in HBCs or all OE cells during both normal turnover and recovery from injury. Analysis of the knockout animals revealed that SCF signaling is required for the birth of normal numbers of neurons from stem cells during recovery from injury, but plays a less evident role in normal turnover during healthy maintenance. We provide the first account of the functional role of SCF signaling in adult neurogenesis in the OE niche. This result builds off of what has been seen in previous reports about the role of cells marked by C-Kit (Goldstein, et al., 2015; Goss, et al., 2016).

A major contribution of the current work was further examination of the nature and transcriptional identity of microvillous cells in the OE. Though this was not a principal aim of this project from the outset, our discovery that microvillous cells express C-Kit and further characterization of these cells presents an exciting advance beyond what was previously known.

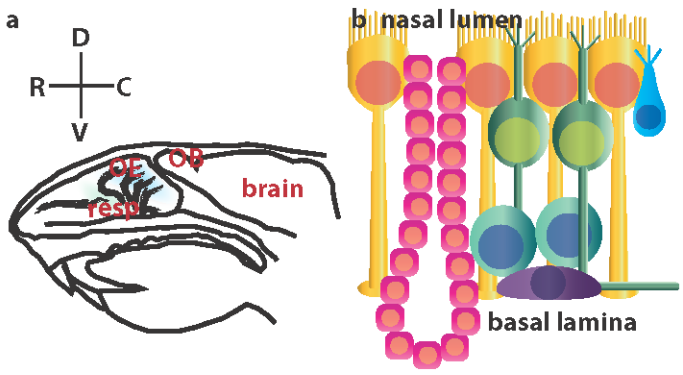


Figure 1 | Olfactory epithelium cellular architecture. **a,** Saggital section of the murine skull with the OE, respiratory epithelium, olfactory bulb, and brain indicated. The OE and respiratory epithelium line the nasal cavity just rostral, across the cribiform plate, from the OB, to which all OE neurons project axons. **b,** Cellular constituents of the OE. Sustentacular (yellow), Bowman's Gland (fuchsia), horizontal basal cells (purple), globose basal cells (teal), neurons (green), microvillus cells (blue). Immature neuronal-layer cells not shown.

References

Ashman, L.K. (1999). The biology of stem cell factory and its receptor C-kit. *International Journal of Biochemistry & Cell Biology, 31*(10): 1037-1051.

Ashman, L.K., Cambareri, A.C., Bik To, L., Levinski, R.J. & Juttner, C.A. Expression of the YB5.B8 antigen (C-kit proto-oncogene product) in normal human bone marrow. *Blood Journal, 78*(1): 30-37.

Beites, C.L., Kawauchi, S., Crocker, C.E. & Calof, A.L. (2005). Identification and molecular regulation of neural stem cells of the olfactory epithelium. *Experimental Cell Research, 306*: 309-316.

Bergman, U., Ostergren, A., Gustafson, A. & Brittebo, E. Differential effects of olfactory toxicants on olfactory regeneration. *Archives of Toxicology, 76*(2): 104-112.

Bernex, F., De Sepulveda, P., Kress, C., Elbaz, C., Delouis, C. & Panthier, J.J. (1996). Spatial and temporal patterns of c-kit expressing cells in WlacZ/+ and WlacZ/WlacZ mouse embryos. *Development, 122*(10): 3023-3033.

Brann, J.H. & Firestein, S.J. (2014). A lifetime of neurogenesis in the olfactory system. *Frontiers in Neuroscience, 8*(182): 1.

Buck, L. & Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell, 65*: 175-187.

Burd, G.A. (1993). Morphological study of the effects of intranasal zinc sulfate irrigation on the mouse olfactory epithelium and olfactory bulb. *Microscopy Research & Technique,* doi: https://doi.org/10.1002/jemt.1070240302.

Calof, A.L., Bonnin, A., Crocker, C., Kawauchi, S., Murray, R.C., Shou, J. & Wu, H. (2002). Progenitor cells of the olfactory receptor neuron lineage. *Microscopy Research and Technique, 58*: 176-188.

Caggiano, M., Kauer, J.S. & Hunter, D.D. Globose basal cells are neuronal progenitors in the olfactory epithelium: A lineage analysis using a replication-incompetent retrovirus. *Neuron, 13*: 339-352.

Carr, V.M. & Farbman, A.I. (1993). The dynamics of cell death in the olfactory epithelium. *Experimental Neurology, 124*: 308-314.

Cau, E., Casarosa, S. & Guillemot, F. (2002). Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. *Development, 129*: 1871-1880.

Cau, E., Gradwohl, G., Fode, C. & Guillemot, F. (1997). Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development, 124*: 1611-1621.

Chen, E.C., Karl, T.A., Kalisky, T., Gupta, S.K., O'Brien, C.A., Longacre, T.A., van de Rijn, M., Quake, S.R., Clarke, M.F. & Rothenberg, M.E., (2015). Kit signaling promotes growth of color

xenograft tumors in mice and is upregulated in a subset of human colon cancers. *Gastroenterology, 149*(3): 705-717.

Chen, M., Tian, S., Yang, X., Lane, A.P., Reed, R.R. & Liu, H. (2014). Wnt-responsive Lgr5+ globose basal cells function as multipotent olfactory epithelium progenitor cells. *Journal of Neuroscience, 34*: 8268-8276.

Chen, X., Fang, H. & Schwob, J.E. (2004). Multipotency of purified, transplanted globose basal cells in olfactory epithelium. *Journal of Comparative Neurology, 469*: 457-474.

Child, K.M., Herrick, D.B., Schwob, J.E., Holbrook, E.H. & Jang, W. (2018). The neuroregenerative capacity of olfactory stem cells is not limitless: implications for aging. *Journal of Neuroscience*, DOI: https://doi.org/10.1523/JNEUROSCI.3261-17.2018.

Dulac, C. and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. *Cell, 83*(2): 195-206.

DeMaria, S. and Ngai, J. (2010). The cell biology of smell. *Journal of Cell Biology, 191*: 443-452.

Driessen, R.L., Johnston, H.M. & Nilsson, S.K. (2003). Membrane-bound stem cell factory is a key regulator in the initial lodgment of stem cells within the endosteal marrow region. *Experimental Hematology, 31*(12): 1284-1291.

Ellison, G. M., Vicinanza, C., Smith, A. J., Aquila, I., Leone, A., Waring, C. D., Henning, B. J., Stirparo, G. G., Papait, R., Scarfo, M., Agosti, V., Vigiletto, G., Condorelli, G., Indolfi, C., Ottolenghi, S., Torella, D. & Nadal-Ginard, B. (2013). Adult c-kit(pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. *Cell, 154*(4): 827-842.

Elsaesser, R. & Paysan, J. (2007). The sense of smell, its signaling pathways, and the dichotomy of cilia and microvilli in olfactory sensory cells. BMC Neuroscience, 8, doi: doi:10.1186/1471-2202-8-S3-S1.

Farbman, A.I. (1992). Cell biology of olfaction. Cambridge: Cambridge University Press.

Fletcher, R.B., Das, D., Gadye, L., Street, K.N., Baudhuin, A., Wagner, A., Cole, M.B., Flores, Q., Choi, Y.G., Yosef, N., Purdom, E., Dudoit, S., Risso, D. & Ngai, J. (2017). Deconstructing olfactory stem cell trajectories at single-cell resolution. *Cell Stem Cell, 20*(6): 817-830.

Fletcher, R.B., Prasol, M.S., Estrada, J., Baudhuin, A., Vranizan, K., Choi, Y.G. & Ngai, J. (2011). p63 regulates olfactory stem cell self-renewal and differentiation. *Neuron, 72*: 748-759.

Frisch, D. (1967). Ultrastructure of mouse olfactory mucosa. *American Journal of Anatomy, 121*: 87-120.

Gayde, L., Das, D., Sanchez, M.A., Street, K., Baudhuin, A., Wagner, A., Cole, M.B., Choi, Y.G. Yosef, N., Purdom, E., Dudoit, S., Risso, D., Ngai, J. & Fletcher, R.B. (2017). Injury activates transient olfactory stem cell states with diverse lineage capacities. *Cell Stem Cell, 21*(6): 775-790.

Gesteland, R. C , Yancey, R. A. & Farbman, A. I. (1982). Development of olfactory neuron selectivity in the rat fetus. *Neuroscience, 7:* 3127-3136.

Godfrey, P.A., Malnic, B. & Buck, L.B. (2004). The mouse olfactory receptor gene family. *Proceedings of the National Academy of Sciences, 101*(7): 2156-2161.

Gokoffski, K.K., Wu, H.H., Beites, C.L., Kim, J., Kim, E.J., Matzuk, M.M., Johnson, J.E., Lander, A.D. & Calof, A.L. (2011). Activin and GDF11 collaborate in feedback control of neuroepithelial stem cell proliferation and fate. *Development, 138*: 4131-4142.

Goldstein, B.J., Fang, H., Youngentob, S.L. & Schwob, J.E. (1998). Transplantation of multipotent progenitors from the adult olfactory epithelium. *NeuroReport, 9*(7): 1611-1617.

Goldstein, B.J., Goss, G.M., Hatzistergos, K.E., Rangel, E.B., Seidler, B., Saur, D. & Hare, J.M. (2015). Adult c-kit+ progenitor cells are necessary for maintenance and regeneration of olfactory neurons. *Journal of Comparative Neurology,* doi: https://doi.org/10.1002/cne.23653.

Goldstein, B.J. & Schwob, J.E. (1996). Analysis of the Globose Basal Cell compartment in rat olfactory epithelium using GBC-1, a new monoclonal antibody against Globose Basal Cells. *Journal of Neuroscience, 16*(12): 4005-4016.

Goss, G.M., Chaudhari, N., Hare, J.M., Nwojo, R., Seidler, B., Saur, D. & Goldstein, B.J. (2016). Differentiation potential of individual olfactory c-kit+ progenitors determined via multicolor lineage tracing. *Developmental Neurobiology, 76*(3): 241-251.

Graziadei, P. (1973). Cell dynamics in the olfactory mucosa. *Tissue and Cell, 5*(1): 113-131.

Graziadei, P.P. & Graziadei, G.A. (1979). Neurogenesis and neuron regeneration in the olfactory system of mammals. *Journal of Neurocytology, 8*: 1-18.

Guo, Z., Packard, A., Krolewski, C., Harris, M.T., Manglapus, G.L. & Schwob, J.E. (2010). Expression of Pax6 and Sox2 in the adult olfactory epithelium. Journal of Comparative Neurology, 518: 4395-4418.

Heissig, B., Lund, L.R., Akiyama, H., Ohki, M., Morita, Y., Ramer, J., Nakauchi, H., Okumura, K., Ogawa, H., Werb, Z., Dano, K. & Hattori, K. (2007). The plasminogen fibrinolytic pathway is required for hematopoietic regeneration. *Cell Stem Cell, 1*(6): 658-670.

Herrick, D.B., Guo, Z., Jang, W., Schnittke, N. & Schwob, J.E. (2018). Canonical Notch signaling directs the fate of differentiating neurocompetent progenitors in the mammalian olfactory epithelium. *Journal of Neuroscience*, 0484-17; doi: 10.1523/JNEUROSCI.0484-17.2018 4-517.

Herrick, D.B., Lin, B., Peterson, J., Schnittke, N. & Schwob, J.E. (2017). Notch1 maintains dormancy of olfactory horizontal basal cells, a reserve neural stem cell. *Proceedings of the National Academy of Sciences, 114*(28): 5589-5598.

Holbrook, E.H., Szumowski, K.E. & Schwob, J.E. (1995). An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium. *Journal of*

Comparative Neurology, 363: 129-146.

Holcomb, J., Mumm, J. & Calof, A. (1995). Apoptosis in the neural lineage of the mouse olfactory epithelium: regulation in vivo and in vitro. *Developmental Biology, 172*: 307-323.

Huard, J.M., Youngentob, S.L., Goldstein, B.J., Luskin, M.B. & Schwob, J.E. (1998). Adult olfactory epithelium contains multipotent progenitors that give rise to neurons and non-neural cells. *Journal of Comparative Neurology, 400*: 469-486.

Iwai, N., Zhou, Z., Roop, D.R. & Behringer, R.R. (2008). Horizontal basal cells are multipotent progenitors in normal and injured adult olfactory epithelium. *Stem Cells, 26*: 1298-1306.

Jang, W., Lambropoulos, J., Woo, J.K., Peluso, C.E. & Schwob, J.E. (2008). Maintaining epitheliopoietic potency when culturing olfactory progenitors. *Experimental Neurology, 214*(1): 25-36.

Jang, W., Youngentob, S.L. & Schwob, J.E. (2003). Globose basal cells are required for reconstitution of olfactory epithelium after methyl bromide lesion. *Journal of Comparative Neurology, 460*: 123-140.

Jang, W., Chen, X., Flis, D., Harris, M. & Schwob, J.E. (2013). Label-retaining, quiescent globose basal cells are found in the olfactory epithelium. *Journal of Comparative Neurology, 522*(4): 731-749.

Jia, C. & Hegg, C.C. (2012). Neuropeptide Y and extracellular signal-regulated kinase mediate injury-induced neuroregeneration in mouse olfactory epithelium. *Molecular and Cellular Neuroscience, 49*(2): 158-170.

Jin, K., Mao, X. O., Sun, Y., Xie, L. & Greenberg, D. A. (2002). Stem cell factor stimulates neurogenesis in vitro and in vivo. *The Journal of Clinical Investigation, 110*(3): 311-319.

Joiner, A.M., Green, W.W., McIntyre, J.C., Allen, B.L., Schwob, J.E. & Martens, J.R. (2015). Primary cilia on horizontal basal cells regulate regeneration of the olfactory epithelium. *Journal of Neuroscience, 35*(40): 13761-13772.

Keshet, E., Lyman, S. D., Williams, D. E., Anderson, D. M., Jenkins, N. A., Copeland, N. G. & Parada, L. F. (1991). Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. *The EMBO Journal, 10*(9): 2425-2435.

Keshet, E., Lyman, S.D., Williams, D.E., Anderson, D.M., Jenkins, N.A., Copeland, N.G. & Parada L.F. (1991). Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. *EMBO Journal, 10*(9): 2425-2435.

Kimura, Y., Ding, B., Imai, N., Nolan, D.J. & Butler, J.M. (2011). C-Kit mediated functional positioning of stem cells to their niches is essential for maintenance and regeneration of adult hematopoiesis. *PLoS ONE, 6*(10): e26918.

Kinnman, N., Goria, O., Wendum, D., Gendron, M. C., Rey, C., Poupon & Housset, C. (2001). Hepatic stellate cell proliferation is an early platelet-derived growth factor mediated cellular event in rat cholestatic liver injury. *Laboratory Invesitgation, 81*(12): 1709-1716.

Krolewski, R. C., Packard, A., Jang, W., Wildner, H. & Schwob, J. E. (2012). Ascl1 knockout perturbs differentiation of nonneuronal cells in olfactory epithelium. *PLoS ONE, 7*(12): e51737. doi:10.1371/journal.pone.0051737.

Lennartsson, J. & Ronnstrand, L. (2012). Stem Cell Factor receptor/c-Kit: From basic science to clinical implications. *Physiological Reviews, 92*(4): 1619-1649.

Leung, C.T., Coulombe, P.A. & Reed, R.R. (2007). Contribution of olfactory neural stem cells to tissue maintenance and regeneration. *Nature Neuroscience, 10*(6), 720-726.

Liang, J., Wu, Y. L., Chen, B. J., Zhang, W., Tanaka, Y. & Sugiyama, H. (2013). The c-kit receptor-mediated signal transduction and tumor-related diseases. *International Journal of Biological Sciences, 9*(5): 435-443.

Lin, B., Coleman, J.H., Peterson, J.N., Zunitch, M.J., Jang, W., Herrick, D.B. & Schwob, J.E. (2017). Injury induces endogenous reprogramming and dedifferentiation of neuronal progenitors to multipotency. *Cell Stem Cell, 21*(6): 761-774.

Lin, W., Ezekwe, E.A.D., Zhao, Z., Liman, E.R. & Restrepo, D. (2008). TRPM5-expressing microvillous cells in the main olfactory epitheliem. *BMC Neuroscience, 9*: 114.

Mackay-Sim, A. & Kittel, P. (1991). Cell dynamics in the adult mouse olfactory epithelium: a quantitative autoradiographic study. *Journal of Neuroscience, 11*: 979-984.

Manglapus, G.L., Youngentob, S.L. & Schwob, J.E. (2004). Expression patterns of basic helix-loop-helix transcription factors define subsets of olfactory progenitor cells. *Journal of Comparative Neurology, 479*: 216-233.

Matsui, Y., Zsebo, K.M. & Hogan, B.L.M. (1990). Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. *Nature, 347*: 667-669.

Matulionis, D.H. (1975). Ultrastructural study of mouse olfactory epithelium following destruction by $ZnSO_4$ and its subsequent regeneration. American Journal of Anatomy: https://doi.org/10.1002/aja.1001420106.

Menco, B.P. (1980). Qualitative and quantitative freeze fracture studies on olfactory and nasal respiratory surfaces of frog, ox, rat, and dog. *Cell Tissue, 211*: 361-373.

Mombaerts, P. (2004). Genes and ligands for odorant, vomeronasal and taste receptors. *Nature Reviews Neuroscience, 5*: 263-278.

Moran, D.T., Rowley, J.C., Jafek, B.W. et al. (1982). *Journal of Neurocytology, 11*: 721. https://doi.org/10.1007/BF01153516.

Morigi, M., Imberti, B., Zoja, C., Corna, D., Tomasoni, S., Abbate, M., Rottoli, D., Angioletti, S., Benigni, A., Perico, N., Alison, M. & Remuzzi, G. (2004). Mesnchymal stem cells are renotopic, helping repair the kidney and improve function in acute renal failure. *Journal of the American Society of Nephrology, 15*: 1794-1804.

Morrison, E. E. & Costanzo, R. M. (1990). Morphology of the human olfactory epithelium. *Journal of Comparative Neurology, 297*: 1–13. doi:10.1002/cne.902970102

Morrison, E.E. & Costanzo, R.M. (1992). Morphology of olfactory epithelium in humans and other vertebrates. *Microscopy Resolution Technology, 23*: 49-61.

Murray, R.C. & Calof, A.L. (1999). Neuronal regeneration: lessons from the olfactory system. *Seminars in cell and developmental biology, 10*: 421-431.

Norlin, E. M., Vedin, V., Bohm, S. & Berghard, A. (2015). Odorant-dependent, spatially restricted induction of c-fos in the olfactory epithelium of mice. *Journal of Neurochemistry, 93*: 1594-1602.

Ogura, T., Szebenyi, S.A., Krosnowski, K., Sathyanesan, A. & Lin, W. (2011). Cholinergic microvillous cells in the mouse main olfactory epithelium and effect of acetylcholine on olfactory sensory neurons and supporting cells. *Journal of Neurophysiology, 106*(3): 1274-1287.

Orr-Urtreger, A., Avivi, A., Zimmer, Y., Givol, D., Yarden, Y. & Lonai, P. (1990). Developmental expression of c-kit, a proto-oncogene encoded by the W locus. *Development, 109*: 911-923.

Packard, A., Giel-Moloney, M., Leiter, A. & Schwob, J.E. (2011). The progenitor cell capacity of NeuroD1-expressing globose basal cells in the mouse olfactory epithelium. *Journal of Comparative Neurology, 519*: 17

Packard, A., Schnittke, N., Romano, R.A., Sinha, S. & Schwob, J.E. (2011). Delta-Np63 regulates stem cell dynamics in the mammalian olfactory epithelium. *Journal of Neuroscience, 31*(24): 8748-8759.

Packard, A.I., Lin, B. & Schwob, J.E. (2016). Sox2 and Pax6 play counteracting roles in regulating neurogenesis within the murine olfactory epithelium. *PLoSONE, 11*(5): e0155167. doi:10.1371/journal.pone.0155167.

Piao, C. S., Gonzalez-Toledo, M.E., Xue, Y. Q., Duan, W. M., Terao, S., Granger, D. N. Kelley, R. E. & Zhao, L. R. (2009). The role of stem cell factor and granulocyte-colony stimulating factor in brain repair during chronic stroke. *Journal of Cerebral Blood Flow and Metabolism, 29*: 759-70.

Rojas, M., Xu, J., Woods, C. R., Mora, A. L., Spears, W., Roman, J. & Brigham, K. L. (2005). Bone marrow-derived mesenchymal stem cells in repair of the injured lung. *American Journal of Respiratory Cell and Molecular Biology, 33*: 145 – 152.

Royet, J.P., Souchier, C., Jourdan, F. & Ploye, H. (1988). Morphometric study of the glomerular population in the mouse olfactory bulb: numerical density and size distribution along the rostrocaudal axis. *Journal of Comparative Neurology, 270*: 559-568.

Schultz, E.W. (1941). Regeneration of olfactory cells. *Experimental Biology and Medicine, 46*(1): 41-43.

Schultz, E.W. (1960). Repair of the olfactory mucosa with special reference to regeneration of olfactory cells. *American Journal of Pathology, 37*(1): 1-19.

Shetty, R. S., Bose, S. C., Nickell, M. D., McIntyre, J. C., Hardin, D. H., Harris, A. M. & McClintock, T. S. (2005). Transcriptional changes during neuronal death and replacement in the olfactory epithelium. *Molecular and Cellular Neuroscience, 30*: 90-107.

Schwob, J.E. (2005). Restoring olfaction: a view from the olfactory epithelium. *Chemical Senses, 30*: 131-132.

Schwob, J.E., Huard, J.M., Luskin, M.B. & Youngentob, S.L. (1994). Retroviral lineage studies of the rat olfactory epithelium. *Chemical Senses, 19*: 671-682.

Schwob, J.E., Youngentob, S.L. & Mezza, R.C. (1995). Reconstitution of the rat olfactory epithelium after methyl bromide-induced lesion. *Journal of Comparative Neurology, 359*: 15-37.

Smart, I.H.M. (1971). Location and orientation of mitotic figures in the developing mouse olfactory epithelium. *Journal of Anatomy, 109*: 243-251.

Sun, L., Lee, J. & Fine, H. A. (2004). Neuronally expressed stem cell factor induces neural stem cell migration to areas of brain injury. *The Journal of Clinical Investigation, 113*(9): 1364-1374.

Toksoz, D., Zsebo, K.M., Smith, K.A., Hu, S., Brankow, D., Suggs, S.V., Martin, F.H. & Williams, D.A. (1992). Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor. *Proceedings of the National Academy of Sciences, 89*(16): 7350-7354.

Yamaguchi, T., Yamashita, J., Ohmoto, M., Aoude, I., Ogura, T., Luo, W., Bachmanov, A.A., Lin, W., Matsumoto, I. & Hirota, J. (2014). Skn-1a/Pou2f3 is required for the generation of Trpm5-expressing microvillous cells in the mouse main olfactory epithelium. *BMC Neuroscience, 15*: 3.

Zhang, S.C. & Fedoroff, S. (1999). Expression of stem cell factor and c-kit receptor in neural cells after brain injury. *Acta Neuropathologica, 97*(4): 393-398.

Zhao, L. R., Singhal, S., Duan, W. M., Mehta, J. & Kessler, J. A. (2007). Brain repair by hematopoietic growth factors in a rat model of stroke. *Stroke, 38*(9): 2584-2591.

Chapter 2 | Stem Cell Factor and C-Kit are expressed by distinct subsets of cells

Background

Early experimentation indicated that C-Kit and SCF were expressed by mast cells (Majumder, et al., 1988; Mayrhofer, et al., 1987), melanocytes (Nocka, et al., 1989), germ cells (Majumder, et al., 1988), and in bone marrow (Wang, et al., 1989). Kit was later discovered to also mark a wide array of endothelial and epithelial cell types (Broudy, et al., 1994). In adult humans, C-kit protein is expressed in virtually all stem cell niches as well as in myriad body tissue cell types and germ cells (Lammie, et al., 1994).

Accordingly, we sought to discover first whether C-Kit/SCF were expressed in the OE, then to characterize the subsets of cells expressing the receptor and the growth factor. The nature of the isoforms expressed was of interest. With respect to SCF, two murine isoforms in particular were of interest, differing by the presence or absence of exon 6 of the gene, with exon 6 present in the soluble (potentially long-range) and exon 6 absent in the membrane-bound form (contact-mediated signaling) (Ashman, et al., 1999). The two isoforms have functionally distinct roles in mice. Previous work has shown that engineering mice to express only soluble SCF in the absence of its membrane-bound counterpart, results in lack of mast cells, anemia and pigmentation defects, among other defects (Russell, et al., 1979). The ratios of the two SCF isoforms varies substantially in different tissues (Huang, et al., 1992). This ratio was, therefore, of interest to us, though research to date has yet to fully reveal its functional importance.

Murine C-Kit is known to have two isoforms, that differ by the presence of a four amino acid GNNK sequence in the juxtamembrane region of the protein via the use of an alternative 5' splice acceptor site, a feature conserved between mice and humans (Crosier, et al., 1993; Motro, et al., 1991). Comparing the two isoforms, the GNNK- isoform was more able to promote cell growth, but required cell contact for that growth to occur, while the GNNK+ isoform was less able to promote growth, yet could do so in a contact-independent way (Caruana, et al., 1999). The two C-Kit isoforms are co-expressed in many tissues, though the GNNK- form is generally predominant (Crosier, et al., 1993) – the importance of this ratio to biological functioning remains unclear (Piao, et al., 1994).

Methods

Tissue collected from mice used in immunohistochemistry experiments was fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), decalcified for five days in 10% ethyleneaminetetracetic acid in PBS and equilibrated overnight in 30% sucrose. Frozen tissue was mounted in freezing medium (Triangle Biomedical Sciences), cryostat sectioned to 12um thick and slide mounted for further processing.

For immunohistochemistry sections were permeabilized in 0.1% Triton X-100 in PBS and stained with antibodies diluted in donkey serum overnight. Fluorophore-conjugated secondary antibodies were used and detected as described in Duggan et al., 2008. Secondary antibodies (Alexa 488, 594, 555, 568 and 647 (Invitrogen)) were used in conjunction with Hoechst 33342 nuclear stain for imaging. Slides were cover slipped with Vectashield (Hard Set, Vector Labs). Primary antibodies were used as follows:

chicken anti-GFP 1:500 (Abcam)
mouse anti-p63 1:100 (Santa Cruz Biotechnology)
goat anti-Sox2 1:100 (Santa Cruz Biotechnology)

goat anti-C-Kit 1:100 (R&D Systems)
rabbit anti-Ki67 1:250 (Abcam)
mouse anti-neuronal tubulin 1:250 (Tuj1; Abcam)
guinea pig anti-Ascl1 1:10,000 (gift from Jane Johnson)
rabbit anti-caspase3 1:250 (Cell Signaling Technology)

Images were acquired using epifluorescence and confocal microscopes. Image assembly and analysis was done in ImageJ (National Institutes of Health) and Photoshop (Adobe). Quantification was done using the Cell Counter ImageJ plugin (Kurt de Vos). Quantification of numbers of cells positive for various markers was done for approximately 5000um length of septal OE in each of 3 or 4 knockout and 3 or 4 control animals for each condition. Quantification was done in the olfactory epithelium only (not respiratory/non-sensory, which lines the ventral portion of the nasal cavity). Quantification was restricted to septal regions for consistency.

Strains of transgenic mice (Krt5-CrePR [Zhou et al, 02]), mice carrying cre-inducible SCF knock-out allele (SCF-lox-Exon1-lox-, Kitltm2.1Sjm, Jackson Laboratory) and mice carrying a cre-inducible YFP allele under the Rosa26 promoter (Srinivas et al., 2001; Rosa26-YFP) were bred on a C57/B6 background. Mice used in this study included the following:

Krt5-CrePR$^{Tg/+}$; Rosa26$^{YFP/+}$; SCF$^{lox/lox}$ (knock-out)
Krt5-CrePR$^{Tg/+}$; Rosa26$^{YFP/+}$; SCF$^{+/+}$ (wildtype)

Regeneration from neural injury was modeled by methimazole administration. Mice were co-housed with parents and littermates until until post-natal day 21, at which point methimazole was administered by intraperitoneal injection (0.01mg methimazole/g bodyweight). Methimazole causes the death of virtually all cells in the epithelium aside from the HBCs within 24 hours of drug administration (Leung, et al., 2007). Mice used for 'injured' tissue collection were each sacrificed at one of three experimental time points after administration of methimazole (4 days post-injury, 7 days post-injury, or 14 days post-injury). 'Healthy' tissue was collected at post-natal day 21 from mice that were not administered methimazole.

RNA-sequencing data analyzed here were from Fletcher, et al., 2017. Briefly: mouse OE was dissociated (detailed previously in Fletcher, et al., 2011), FACS sorted to enrich for cells of interest, captured (Fluidigm C1) from animals before injury (healthy) and at various time points following injury (24h, 48h, &ct), and sequencing libraries generated (Nextera [library synthesis], Beckman Coulter [Library size selection], Illumina [library sequencing]). All RNA-sequencing data used in this paper have been deposited and are freely available at the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/).

For expression analyses and comparisons, cells were aligned, filtered, normalized and clustered as described in Fletcher, et al., 2017. Biplots in Figure 3 were generated by comparing the expression of various genes in cells belonging to the GBC cluster in data from healthy OE cells.

For the isoform analysis, sequencing reads were aligned using Bowtie (1.2.0, Johns Hopkins University). Reads mapped to the chromosomes bearing C-Kit and SCF (Ch5 and Ch10 respectively) were isolated and used for further analysis. Stringtie (1.3.3, Johns Hopkins University) was used to assemble transcripts and quantify reads with the C-Kit and SCF chromosomal loci (Ch5: 75,574,916-75,656,722 and Ch10: 100,015,630-100,100,416 respectively in the GRCm38.p5 c57/Bl6 reference genome

[National Center Biotechnology Information]). Assemblies were visualized in Integrated Genomics Viewer (2.3.92, Broad Institute) aligned to the GRCm38.p5 c57/Bl6 reference genome, and splice variants were assessed using the Variant Call Format (VCF 4.0, European Bioinformatics Institute). Analysis pipelining and scripting was done in a Jupyter Notebook (IPython (Perez and Granger, 07)) running Python (3.6.1, Python Software Foundation, https://www.python.org/) and R (3.4.0, R Project for Statistical Computing; rpy2, 2.8.5, Laurent Gauthier).

Results

2.1 | C-Kit is expressed by 'late' GBC neural cycling progenitors

We started our investigation of the role of SCF in the OE by determining what cells this growth factor and its receptor are expressed by. At steady-state, we observed three morphologically distinct populations of cells in the OE that express the C-Kit receptor and are, accordingly, potential targets of SCF signal. Consistent with previous reports, the majority of cells that express the C-Kit receptor are morphologically and transcriptionally identifiable as GBCs, though there are also cells with cell bodies more apical than the SUS layer that morphologically appear to be microvillous cells. Additionally, there are a small number of immunochemically C-Kit+ cells midway between the stem/progenitor layers and the SUS cells, mixed in among the neuronal layers (Figure 2). These are elongated cells, and may be traveling apically upwards through the epithelium.

Because the GBCs are a heterogeneous population, we sought to correlate C-Kit expression with other known genetic markers for GBCs at varying stages of maturation. Conditional ablation of the p63 gene in transgenic mice (Krt5-CreER;Trp63 -/-; Rosa26YFP) provides an excellent model of differentiation in the uninjured OE (Fletcher, et al., 2011; Fletcher, et al., 2017; Gayde, et al., 2017). For other experiments in our lab, using single-cell transcriptional sequencing (Fluidigm C1 cell capture; Illumina sequencing), cells from adult (post-natal day 21) mice 72 and 96 hours after Cre activation by tamoxifen administration were profiled. 681 YFP+ cells (indicative that these cells were actively moving through the stages of differentiation as a result of the conditional knock out) were collected. Additionally, 169 Sox2-eGFP+;ICAM1-;SCARB/F3- cells were collected and sequenced in order to capture a population of cells representative of GBCs, microvillous cells and neuronal progenitors. After removal of doublets, sequencing data from 687 cells remained, which we used for the present analysis. To assess the identities of cells that express C-Kit, we compared the level of expression of C-kit mRNA transcripts with those of other widely used GBCs marker genes (Figure 3). Of the genes compared (C-Kit, Sox2, Lgr5, Ascl1, Ki67), C-Kit expression correlated the most strongly with Ascl1 ($r = 0.4916$, $n = 97$ cells, $p < 0.05$) and Ki67 ($r = 0.3263$, $n = 97$ cells, $p < 0.05$), but less strongly with Sox2 ($r = 0.0117$, $n = 97$ cells, $p > 0.10$).

To confirm what was apparent by single-cell sequencing, we quantified overlapping labeling of OE cells co-labeling fixed tissue sections from healthy adult mice with an antibody for C-Kit and antibodies for widely accepted transcriptional markers of GBCs (Figure 3). The greatest degree of overlapping expression was between C-kit and Ascl (59.1% C-Kit+/Ascl1+; 36.2% C-Kit+/Ascl1-; 4.7% C-Kit-/Ascl+). GBCs expressing Ascl1 are known to be neuronally committed transit amplifying progenitor cells in the healthy OE (Cau, et al., 1997; Cau, et al., 2002; Murray, et al., 2003). Consistent with this, a large percentage of cells expressing C-Kit were actively cycling, when stained with an antibody against the mitotic marker Ki67 (44.2% C-Kit+/Ki67+; 28.8% Ckit+/Ki67-; 27.0% C-Kit-/Ki67+). Interestingly, we found that there are also a large contingent of cycling cells are not C-kit+. This is suggestive that, either C-kit marks only 'earlier' or 'later' GBCs and that part of their time cycling, they don't express C-Kit, or that not all GBCs pass through a C-Kit+ state in the healthy OE. To clarify whether C-Kit+ cells might mark a class of early GBCs, we quantified co-expression with

Sox2, which is known to mediate stemness in many niches (Liu, et al., 2013) and is expressed by HBCs, some GBCs and SUS cells in the OE niche (Guo, et al., 2010). C-Kit had relatively little overlap with Sox2 (8.7% C-Kit+/Sox2+; 36.1% C-Kit+/Sox2-; and 55.2% C-Kit-/Sox2+; quantification included only the morphologically distinct cells of the GBC layer and not HBC or SUS cells because these cells were never observed to express C-Kit in the healthy OE). It therefore seemed most likely that C-Kit marks late GBCs or that not all GBCs pass through a C-Kit+ state.

2.2 | C-Kit is expressed by a number of apical cells with the characteristic 'teardrop' morphology of microvillous cells

Expression of C-kit by these cells has not been previously reported, and is particularly interesting in light of the role that microvillous cells have been proposed to play in regeneration and disease (Jia, et al., 2013). While a rigorous study of the subclasses of cells presenting microvillous morphology has not yet been done, both chemosensory, secretory and protective roles have been proposed for subsets of these cells (Lemons, et al., 2017).

We observed C-Kit expression in a large number of cells with teardrop morphology and cell bodies situated above or in line with the cell bodies of the SUS cell layer – likely microvillous cells (Figure 3). In addition to cells that are characteristic of GBCs and microvillous cells, C-Kit expression also appears in cells that are more apical in the epithelium than GBCs, but not so apical as to be in the microvillous cell layer (Figure 2). The identity of these cells is unclear. Our findings that C-kit is expressed by cells moving apically in the OE and also by at least some subset of microvillous cells is particularly evocative given recent work suggesting that differentiation of stem and progenitor cells into microvillous cells is accomplished as progenitors that were previously thought to be directed to strictly neural fates branch off to form microvillous cells (Fletcher, et al., 2017). This placement may be interpreted as C-Kit expression by GBCs migrating as they differentiate into neurons or microvillous cells.

2.3 | SCF is expressed by quiescent basal stem cells and sustentacular cells

We investigated the localization of SCF expression in the OE. Using transgenic SCF-eGFP mice (Kitltm2.1Sjm, Jackson Laboratory; in which one allele of SCF has been replaced by an eGFP transgene such that all cells which would normally express SCF express GFP protein and a decreased [but haplosufficient] amount of SCF protein) in combination with known immunochemical markers for various cell types in the OE, we determined the pattern of expression of SCF in the OE.

Previous studies have reported SCF expression by SUS cells in the healthy OE (Goldstein, et al., 2015). We confirmed the findings of others - SCF was expressed by SUS cells, which are morphologically distinct, goblet-shaped cells. Staining for Sox2 (Figure 2) confirmed the expression of SCF by SUS cells, which are, without exception, Sox2+ (Goldstein, et al., 2015). Sox2 marks two further populations of cells: quiescent HBC stem cells, which make up the most basal layer of the epithelium, and a subset of GBCs, just above the HBCs (Krolewski, et al., 2012). In addition to SUS cells, we observed that Sox2+ cells in the HBC layer expressed SCF. We sought to corroborate this finding, since no previous study had reported expression of SCF by HBCs, and because it can be difficult to differentiate morphologically between the end feet of the SUS cells, which terminate in and around the HBCs, and HBCs themselves. In the healthy OE, p63 marks all HBCs (Fletcher, et al., 11). We found that GFP co-localized with p63 by immunohistochemistry (Figure 2), and that the presence of SCF in HBCs was also confirmed by the sequencing data set we used. Fletcher, et al. (2017) used a set of marker genes for different types of cells in the OE. HBCs were best characterized by Trp63, Krt5 and Krt14. Examining correlations of expression among the cells in their dataset, showed that SCF

expression is significantly correlated with the expression of all three HBC marker genes (Trp63: $r = 0.457$, $p = 4.795 \times 10^{-33}$; Krt5: $r = 0.418$, $p = 1.963 \times 10^{-27}$; Krt14, $r = 0.415$, $p = 4.555 \times 10^{-27}$, $n = 625$ cells). Similarly, SCF expression was found to correlate with expression of a genetic hallmark of SUS cells, Cyp2g1 ($r = 0.148$, $p = 2.276 \times 10^{-4}$, $n = 625$ cells).

This finding is particularly interesting considering that HBCs are situated just beneath GBCs, which are responsible for ongoing regeneration of lost neurons at steady state. In the hematopoietic niche, SCF is known to act as both a soluble, long-range cue and also as a local, paracrine signaling factor, resulting in nuanced control of cells expressing the receptor (Li & Wu, 2011). GBCs that express the C-Kit receptor are in direct contact with two sources of SCF, while cells located more apically in the epithelium, including microvillous cells, that express the receptor are poised to receive paracrine SCF cues only from SUS cells.

2.4 | Multiple isoforms of SCF and C-Kit are expressed by different types of cells

In order to clarify which sources of SCF are driving the behavior which C-Kit+ cells, we examined the differential expression by HBCs and SUS cells of two murine isoforms of SCF, SCF 220 (-exon 6) and SCF 248 (+exon 6) that are known, to code for soluble - potentially long-range - and membrane-bound - contact-mediated, short range - signaling respectively. The two isoforms are functionally distinct (Ashman, et al., 1999) (Figure 4).

Soluble and membrane-bound forms of SCF are produced as a result of alternative splicing of mRNA which removes a main proteolytic cleavage site in the SCF 220 isoform, and consequent differing efficiencies of proteolysis of the SCF protein once anchored in the plasma membrane (Broudy, et al., 1997; Huang, et al., 1992). These two isoforms exist in variable ratios depending on the tissue and developmental stage of the organism (Huang, et al., 1992). Phenotypes associated with mutations at the Sl locus are known to manifest at variable distances from the source of SCF (Bennett, 1956; McCulloch, et al., 1965; Mayer and Green, 1968; Dexter and Moore, 1977; Russel, 1979; Silvers, 1979), which is consistent with the different functional relevance and biological activity of the membrane-bound and soluble isoforms. These unique ratios appear to be partially responsible for the pleiotropic effects of the SCF growth factor. Huang, et al. (1992) compared the relative RNA expression of the two isoforms of SCF in eleven different tissues and found that SCF 220 was not expressed exclusively in any tissue assayed, while SCF 248 was expressed essentially exclusively in bone marrow and brain. The two isoforms were co-expressed in the majority of tissues, with SCF 248 predominating in the spleen, kidney, lung, and the heart, and SCF 220 predominating in the testis. Relative ratios of isoforms were noted to differ, even between tissues with similar expression levels, though, because the analysis was done on whole tissue lysate, it is unclear whether this reflects real variance or differences in relative abundance of cell types in each tissue.

From the single-cell transcriptome sequencing data, we extracted the subset of cells harboring SCF mRNA and quantified the relative percentages of these cells that expressed SCF 220 mRNA, SCF 248 mRNA, or both isoforms of mRNA (Figure 4). Cells were characterized according to three patterns of SCF expression, with each cell classified as having predominance of one isoform or the other, or a similar preponderance of both isoforms.

Cells that expressed SCF tended to express either SCF 220 (42.92% of 229 total cells) or an approximately equal mix of SCF 220 and SCF 248 (47.03%). Relatively few cells expressed mainly SCF 248 (10.05%). This is in keeping with the findings of Huang, et al. (1992), who suggested that SCF 220 was not found alone in any tissue of the body, though it expands this line of experimentation

by showing that SCF 220 is found alone in individual cells. Our findings are also interesting considering Kapur, et al. (1998), who used transgenic mutants which expressed either membrane-bound or soluble SCF but not both to show that the membrane-bound isoform was responsible for anemia, loss of bone marrow cells and 'runt' body types. The soluble form of SCF was linked primarily to the presence of increased numbers of myeloid progenitors. Cells of the OE, we have found rely on a combination of both isoforms.

Importantly, the analysis presented in Figure 4 includes all cells expressing SCF, meaning that both HBCs and SUS cells are included together, though the two types of cells may in fact have different underlying patterns of SCF expression. Among the 229 cells with discernible expression of one or both isoforms, there was no observable difference between the distribution of isoforms in HBCs versus SUS cells. This may reflect limitations in the resolution of our genetic analysis (there were relatively few SUS cells with clear expression of one or both isoforms) or could mean that the two populations of cells express comparable ratios of isoforms. To clarify this, genetic data from more cells would be needed.

Murine C-Kit is known to have two isoforms, that differ by the presence of a four amino acid GNNK sequence in the juxtamembrane region of the protein. This sequence determines whether C-Kit acts in a cell-contact-dependent or a long-range soluble manner due to the presence or absence of a site where proteolytic cleavage can occur. Differential expression of these isoforms has been shown to lead to functional differences between cells. For example, only the GNNK- isoform, but not the GNNK+ was able to lead to high rates of tumorogenicitiy among NIH3T3 cultured fibroblasts (Caruana, et al., 1999).

Cells that expressed C-Kit tended to express both the GNNK+ and the GNNK- isoforms (54.00% of 47 total cells). Many expressed the GNNK+ isoform in isolation (31.33%), while few expressed only the GNNK- variant (14.67%). Essentially, the OE relies on a mix of C-Kit isoforms for signaling. As with SCF, different types of cells may be expressing different patterns of C-Kit isoforms, however the number of cells included in this analysis is too low to make generalizations based on type.

2.5 | Recovery after injury is marked by extensive expression of SCF and C-Kit.

SCF and C-Kit play an important role during development and healthy, 'steady state' maintenance of numerous tissues in the adult organism. It is also known that this growth factor is critically important in mediating the response to injury in many of these niches, including both immune reactions and attempted regeneration to heal injured tissues.

Administration of methimazole to the mouse results in the death of all cells in the OE except the HBCs which are situated in the most basal layer of the epithelium. Remarkably, the HBCs then go on to regenerate all cell types constituting the entire OE in a matter of days. This regeneration follows a predictable time course, with the HBCs first converting to an 'activated' transcriptional state where they are poised for rapid proliferation and differentiation into a number of fates (Gayde, et al., 2017).

During the first few days following injury, HBC proliferation generates predominantly precursor cells. In histological staining, at 24 hours post-methimazole administration, the previously thick epithelium is visible as tattered, dead tissue sloughed off into the nasal lumenal space, while, attached to the basal lamina, is a monolayer of activated HBCs which have begun to express an array of proliferative markers. At 48 hours post-methimazole administration, the monolayer has expanded to a bilayer. Thus early in regeneration, the majority of cells in both layers remain proliferative, and there is little

distinction between the two layers with respect to the transcriptional identities of the cells present. By five days post-methimazole administration, however, the newly re-grown epithelium has begun to resemble its steady state organization. On top of the layers of basal cells, which are still highly proliferative, rests a newborn complement of immature neuronal cells in their own layer. Above these neurons, is a layer of cells resembling SUS cells. As recovery progresses, more neurons are born, bringing the OE to full thickness. Other cell types continue to be born and progress to maturity until, at roughly fourteen days post-methimazole administration, regeneration is complete and the OE is indistinguishable from its steady state counterpart. Considering the marked inability of the central nervous system to recover and the limited ability of the peripheral nervous system to do so, this rapid and flawless regeneration of a complex neural tissue is truly incredible.

Having observed the striking pattern of SCF and C-Kit expression in the OE of healthy steady state mice, and considering the role that this growth factor plays both in health and during injury in other tissues and stem cell niches, we next aimed to determine if SCF was involved in the stem cell-mediated recovery from injury in the OE. To parallel the foregoing work described in this chapter, we approached the question of a role for SCF in regeneration by first conducting immunohistochemical studies assessing whether SCF and C-kit proteins were evident at different time points following methimazole ablation of the OE. Figure 5 shows representative images, giving an idea what the widespread expression of this growth factor looks like during regeneration after injury. HBCs, which we have previously shown to be SCF+ in steady state, uninjured tissue, predominantly appear to remain SCF+ early in recovery (Figure 5). Throughout the process there is a core contingent of cells apposed to the basal lamina which express SCF.

Of note, however, these images were collected in a mouse with an eGFP transgene knocked into the Sl locus (Kitltm1.1Sjm, Jackson Laboratory) and bred to carry the allele homozygously. Our laboratory has noticed that the eGFP produced in the cells of mice utilizing this transgenic strategy occasionally perdure for a brief time even after the cell has ceased to express the gene of interest. We have no reason to suspect this problem is occurring in the Kitltm1.1Sjm mice, however, it is worth bearing in mind now as the cells which express SCF in the steady state experiments that we have done (HBCs and SUS cells) are cells which rarely divide. As such, perdurance would be unlikely to significantly bias our interpretation of these images. However, in the present assessment of recovery after injury, there is a great deal of rapid proliferation and accompanying rapid changes in transcriptional states and protein expression profiles. If perdurance were occurring, it might substantially alter the interpretation of our images. Because the OE is almost entirely SCF+ at very early time points, but then, mere hours later there are a number of clearly SCF- progeny that cannot but be from these SCF+ progenitors, it is reasonable to assume that GFP perdurance is a minor problem if present at all in these mice.

At 48 hours post-methimazole injury, the expected bilayer of highly proliferative cells is visible (Figure 5). Within the bilayer, SCF+ cells are the majority, while C-Kit+ cells and cells not immunoreactive to either antibody make up the remainder. Interestingly, most C-Kit+ cells are proliferative at this time point, as indicated in Figure 5 by a Ki67 antibody co-stain. The SCF+ fraction is split between those positive and negative for Ki67. Very few cells co-express SCF and C-Kit.

As recovery progresses, SCF+ and C-Kit+ cells can be seen to take on a certain degree of macro organization. For example, at 72 hours post-methimazole, C-Kit+ cells have formed loose clusters about broad swathes of mixed SCF+ and SCF- cells (Figure 5). C-Kit+ cells remain almost universally proliferative at this point, while SCF+ cells retain a variety of proliferative states. It is tempting to surmise that the non-proliferative SCF+ cells at this stage might be early SUS cells, but little can be

said to that end from this experiment alone.

At five days post-methimazole, the OE structure has begun to resemble that of the steady state epithelium (Figure 5). C-Kit+ cells fill out much of the middle layers of the tissue, with not infrequent C-Kit+ cells rising above the middle layers with their cell bodies infiltrating the SUS apical layer. This evocative placement suggests the possibility that microvillous cells are being born concurrent with neuronal cells from a common, or at least similarly C-Kit+, progenitor.

Conclusions and Discussion

C-Kit and SCF are expressed in the OE stem cell niche. Previous work (Goldstein, et al., 2015) examining the expression of the receptor and its ligand found that, in the adult mouse, SCF is expressed exclusively by SUS cells and bone marrow stromal cells in vasculature beneath the OE. They further described C-Kit as sparsely expressed by cells in the GBC layer along with rare instances of C-Kit+ cells present in Bowman's Glands evident in the lamina propria. The authors included one picture pointing out a single cell which was described as "microvillar or sustentacular," though no further comment was made addressing its existence.

We found that, in the uninjured OE, SCF is expressed by both HBC stem cells and by SUS supporting secretory cells, while its receptor, C-Kit is expressed in a less clearly defined population of cells, inclusive of at least some GBCs and also a subset of microvillous cells. These results function as a significant extension upon the findings presented in Goldstein, et al. (2014). Excitingly, using the same antibodies and similar experimental and imaging methodologies, we were able to discern expression of SCF not just in SUS cells, but also in each and every HBC. This result was confirmed by our assessment of single cell sequencing data confirming that cells expressing HBC marker genes also express SCF. The presence, anatomically, of SCF both below GBCS in HBCS and on the scaffold of SUS cells that maturing cells may follow, points to roles for SCF expression across the maturation process of cells in the OE.

Our examination of C-Kit localization in the uninjured, adult OE also serves to expand on the work of Goldstein, et al. (2015). While they relegated C-Kit to expression by GBCS, we noted that there was also clear expression in cells above the GBC layer, with examples of cells with microvillous morphology staining just as markedly as their GBC counterparts. Though this class of C-Kit+ microvillous cells were evident in the post-injury images shown by Goldstein, et al., and the authors found that lineage tracing C-Kit+ progenitors led to the generation of both neurons and microvillous cells, the authors did not seem to notice these remarkable C-Kit+ microvillous cells. Of note, we saw no evidence of the C-Kit+ Bowman's Gland cells Goldstein et al. observed. Despite pursuing co-stains with typical Bowman's Gland marker cells, we were unable to locate these cells.

The pattern of expression we observed is particularly interesting considering the possible functional interplay of the ligand and receptor in this niche. As C-Kit expression co-localizes with Ascl1 expression and also with Ki67, C-Kit+ GBCs are likely to be actively proliferating neuronal progenitors. However, there are likely to be multiple distinct populations of cells among the GBCs. Given the existence of a label-retaining GBC (Jang, et al., 2013), it would be interesting to know if these cells are C-Kit+, or if C-Kit+ cells are more transient progenitors.

We also found C-Kit+ cells in the middle and top strata of the OE. A number of these cells had their cells bodies above the cell bodies of the SUS cells and displayed the teardrop cell body shape associated with microvillous cells. While this has not been reported previously in the literature,

considering the suggestion by Fletcher, et al. (2017) that microvillous lineages 'branch off' at the GBC-stage, it is perhaps unsurprising. Given that many GBCs strongly express C-Kit, it is plausible that their microvillous progeny might retain this expression. With only the anatomical datasets used in this chapter, we were unable to make any claims about the functional role played by C-Kit in the GBC-to-microvillous fate transition. Accordingly, the work in later chapters addresses this point.

The existence of C-Kit+ cells above the GBC layer, but below the SUS cell bodies provides a possible hint as to the stage of maturation at which C-Kit is expressed in GBCs. Based on the observations in this chapter, we wondered whether SCF and C-Kit might play a critical role as the final differentiated fate of the cell is being chosen. Subsequent experimentation was carried out to address whether C-Kit's role in the niche might relate to stem cell or progenitor fate, as opposed to proliferation, another key characteristic of regenerative cells.

During recovery from injury, SCF and C-Kit were expressed in striking patterns. While, under homeostatic conditions, the ligand and receptor were expressed in confined populations of cells, during early regeneration, the pair were expressed by nearly all cells in the recovering epithelium. As healing progressed, their expression came to resemble more closely their homeostatic placement, albeit with profound numbers of cells with immature neuronal morphology expressing C-Kit, a population not apparent at steady state, possibly due to the lower rate of neuronal turnover in the absence of injury. These findings are consistent with the findings after injury in Goldstein, et al. (2015), though these authors only observed expression at a single time point late in the recovery process (10 days post-injury). This pattern of expression bolstered our suspicion that SCF signaling might function to regulate the fate choices of GBCs as they transitioned to differentiation, since C-Kit expression appeared so profoundly linked to cells in the neuronal lineage after injury.

Two isoforms of SCF and two isoforms of C-Kit that are conserved between mice and humans were found to be expressed in OE cells, though our analysis did not reveal precisely which isoforms are expressed in which cell types. While most SCF+ cells express either the soluble, long-range-acting isoform exclusively or a combination of both the soluble and membrane-attached isoforms, few expressed the membrane-attached isoform in isolation. This finding is useful insofar as it suggests that there are multiple different profiles of SCF expression within the niche, though whether these profiles map in a straight-forward way onto the HBC and SUS cell types remains a question that we could not satisfactorily answer with the present dataset. Similarly, cells tended to express a combination of both isoforms of C-Kit, with smaller contingents of cells expressing either isoform alone. This finding is even harder to parse without knowing if and which distinct classes of GBCs and microvillous cells express C-Kit. The findings from this isoform analysis constitute the first step in the OE towards a more fine-grained, molecular understanding of SCF signaling's role in the niche. The experiments described in this manuscript, however, were designed to explore the role of SCF at a more 'macro' functional level.

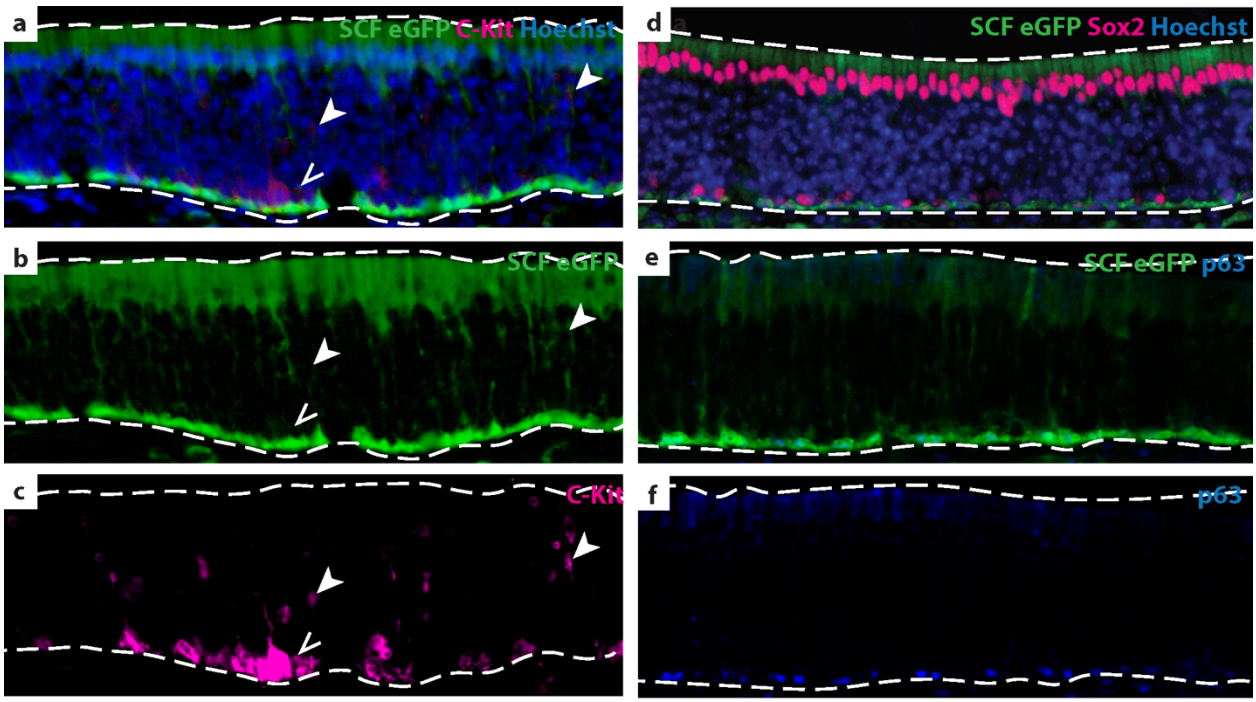


Figure 2 | C-Kit and SCF are expressed by predictable subgroups of cells in the olfactory epithelium. **a,b,c,** C-Kit antibody staining in a SCF eGFP transgenic background. C-Kit is expressed predominantly by cells with location and morphology typical of Globose Basal Cells (open arrowhead), and also by cells located more suprabasally (closed arrowheads). **d,** SCF eGFP co-localizes with Sox2 in Horizontal basal cells. **e, f,** SCF eGFP co-localizes with p63 in Horizontal Basal Cells.

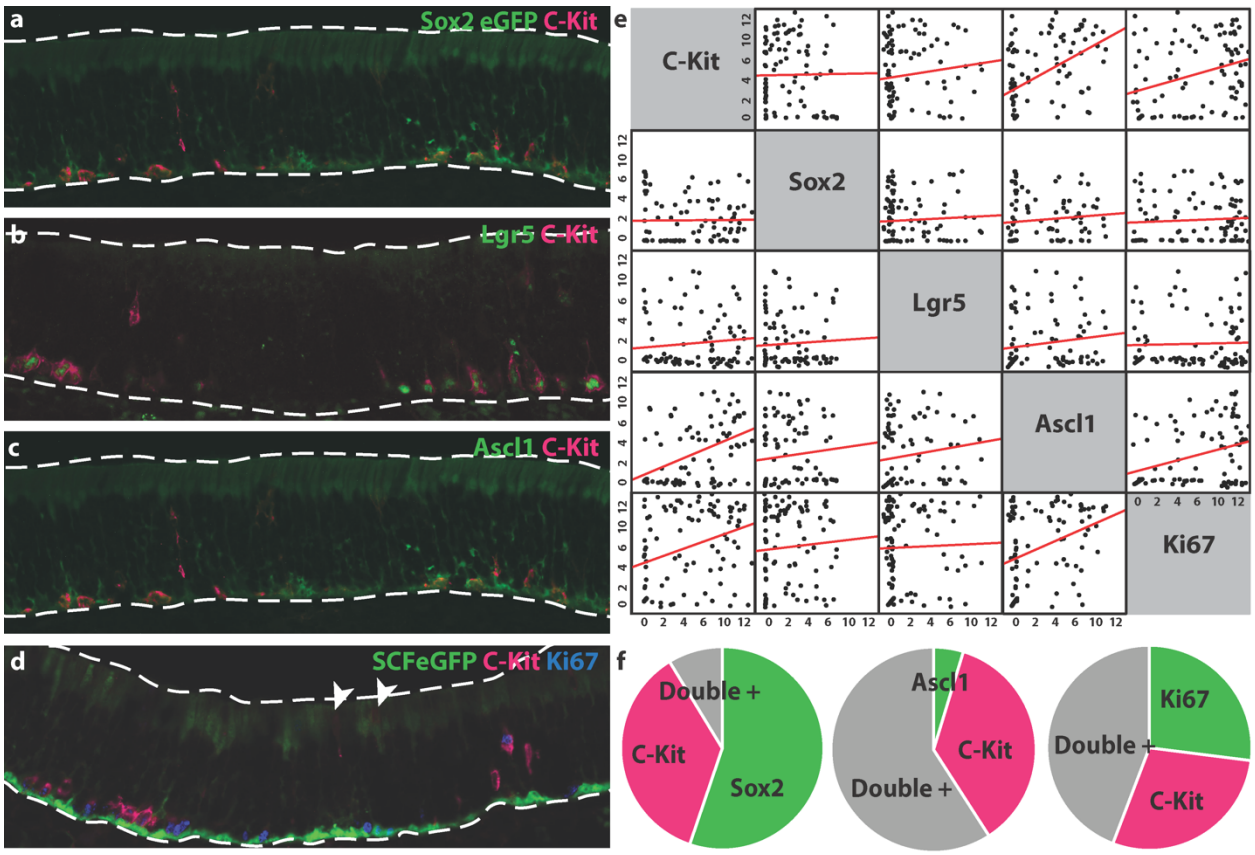


Figure 3 | C-Kit is expressed in proliferative Globose Basal Cells. **a, b, c, d,** C-Kit co-labels GBC-layer, morphologically characteristic cells, including cells which are actively moving through the cell cycle (see Ki67 in d). Cells with microvillous morphology indicated by arrowheads. **e,** Biplots show correlation in expression between gene pairs in individual OE cells. Red lines indicate degree of correlation (Pearson's Coeffcient) between genes, with steeper slope indicating a stronger correlation. Each dot represents correlation between two genes in a single cell. **f,** Degree of overlap between gene pairs as observed via in antibody staining. Percentages represent comprehensive counts done across four animals.

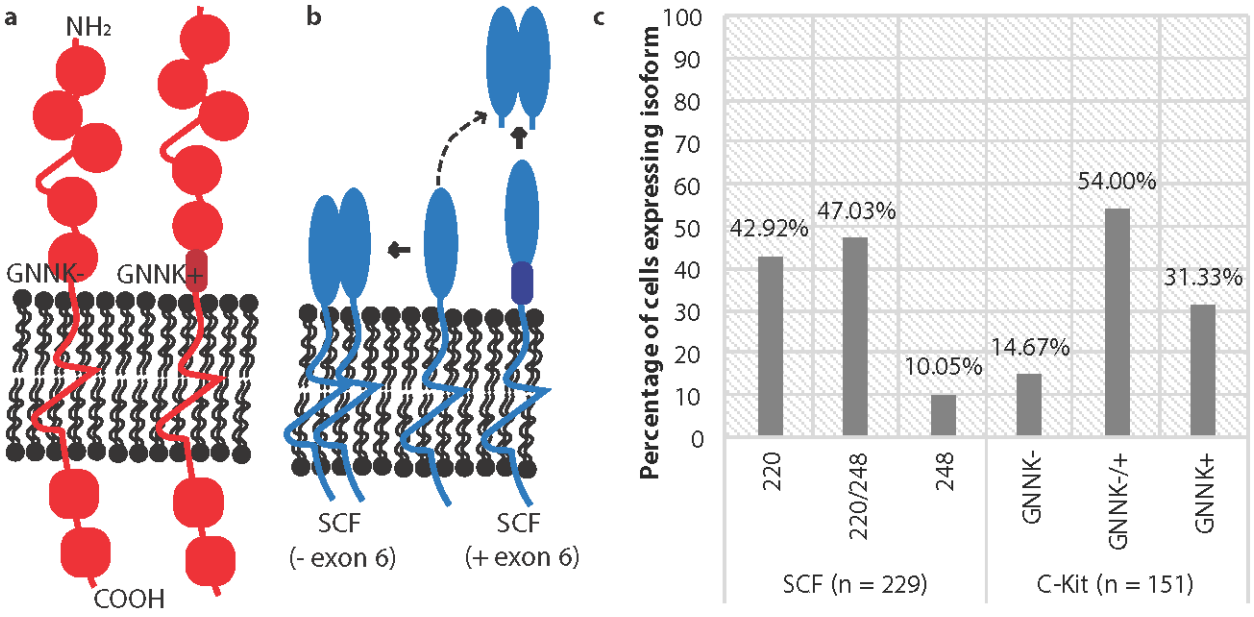


Figure 4 | Multiple isoforms of C-Kit and SCF are expressed in the OE. **a,** GNNK-, but not GNNK+, C-Kit is capable of leading to a high degree of transforming/tumorigenic activity. **b,** Two isoforms of SCF, plus and minus exon 6, lead to different amounts of solubilized SCF signal. **c,** Isoforms of C-Kit and SCF detected in OE cells.

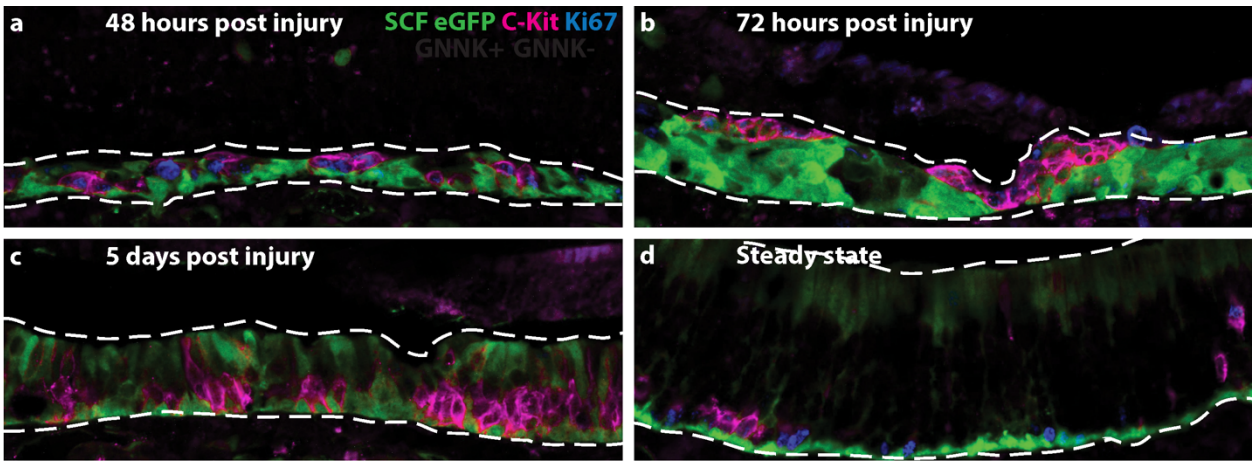


Figure 5 | C-Kit and SCF are expressed in the OE during recovery from chemically induced injury. **a , b ,** C-Kit and Ki67 antibody staining in SCF eGFP mouse. C-Kit and SCF are expressed in virtually all cells at early time points during recovery. **c,** By 5 days post-injury, the OE is stratified into neuronal, mostly C-Kit+, and SUS, all SCF+, layers. **d,** At steady state or after full recovery from injury, C-Kit is expressed only in small cell clusters, while SCF is in all HBCs and SUS cells. (Magenta tissue above the OE in b and c is dead, but still present, since it takes time to slough off after injury.)

References

Ashman, L.K. (1999). The biology of stem cell factory and its receptor C-kit. *International Journal of Biochemistry & Cell Biology, 31*(10): 1037-1051.

Baumann, U., Crosby, H.A., Ramani, P., Kelly, D.A. & Strain, A.J. (1999). Expression of the Stem Cell Factor Receptor c-kit in Normal and Diseased Pediatric Liver: Identification of a Human Hepatic Progenitor Cell. *Hepatology, 30*(1): p112-117.

Bennett, D. (1956). Developmental analysis of a mutant with pleiotropic effects in the mouse. *Journal of Morphology, 98*: 199-234.

Bocchi, R., Egervari, K., Carol-Perdiguer, L., Viale, B., Quairiaux, C., De Roo, M., Boitard, M., Oskouie, S., Salmon, P. & Kiss, J. K. (2017). Perturbed Wnt signaling leads to neuronal migration delay, altered interhemispheric connections and impaired social behavior. *Nature Communications, 1158*: doi:10.1038/s41467-017-01046-w.

Broudy, V.C. (1997). Stem Cell Factor and Hematopoiesis. Blood. 90. 1345-1364.
Chen, S.Q. & Xiong, A.Q. (2002). The progress and implication of stem cell factor. *Basic Medical Sciences and Clinics, 22*(5): 385-390.

Broudy, V.C., Kovach, N.L., Bennett, L.G., Lin, N., Jacobsen, F.W. & Kidd, P.G. (1994). Human umbilical vein endothelial cells display high-affinity c-kit receptors and produce a soluble form of the c-kit receptor. *Blood, 83*: 2145-2152.

Caruana, G., Cambareri, C. & Ashman, L.K. (1999). Isoforms of c-kit differ in activation of signaling pathways and transformation of NIH3T3 fibroblasts. *Oncogene, 18*: 5573-5581.

Cau, E., Casarosa, S. & Guillemot, F. (2002). Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. *Development, 129*: 1871-1880.

Cau, E., Gradwohl, G., Fode, C. & Guillemot, F. (1997). Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development, 124*: 1611-1621.

Christensen J. L, Wright D. E, Wagers A. J, Weissman I. L. (2004). Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biology, 2*: E75.

Crosier, P.S., Ricciardi, S.T., L.R. Hall, Vitas, M.R., Clark, S.C. & Crosier, K.E. (1993). Expression of isoforms of the human receptor tyrosine kinase c-kit in leukemic cell lines and acute myeloid leukemia. *Blood, 82*: 1151-1158.

Dexter, T.M. & Moore, M.A.S. (1977). In vitro duplication and cure of hematopoietic defects in genetically anemic mice. *Nature, 269*: 412-414.

Duggan, C.D., DeMaria, S., Baudhuin, A., Stafford, D. & Ngai, J.N. (2008). FoxG1 is required for development of the vertebrate olfactory system. *Journal of Neuroscience, 28*(20): 5229-5239.

Fletcher, R.B., Das, D., Gadye, L., Street, K.N., Baudhuin, A., Wagner, A., Cole, M.B., Flores, Q., Choi, Y.G., Yosef, N., Purdom, E., Dudoit, S., Risso, D. & Ngai, J. (2017). Deconstructing olfactory stem cell trajectories at single-cell resolution. *Cell Stem Cell, 20*(6): 817-830.

Fletcher, R.B., Prasol, M.S., Estrada, J., Baudhuin, A., Vranizan, K., Choi, Y.G. & Ngai, J. (2011). p63 regulates olfactory stem cell self-renewal and differentiation. *Neuron, 72*: 748-759.

Gayde, L., Das, D., Sanchez, M.A., Street, K., Baudhuin, A., Wagner, A., Cole, M.B., Choi, Y.G. Yosef, N., Purdom, E., Dudoit, S., Risso, D., Ngai, J. & Fletcher, R.B. (2017). Injury activates transient olfactory stem cell states with diverse lineage capacities. *Cell Stem Cell, 21*(6): 775-790.

Goldstein, B.J., Goss, G.M., Hatzistergos, K.E., Rangel, E.B., Seidler, B., Saur, D. & Hare, J.M. (2015). Adult c-kit+ progenitor cells are necessary for maintenance and regeneration of olfactory neurons. *Journal of Comparative Neurology,* doi: https://doi.org/10.1002/cne.23653.

Guo, Z., Packard, A., Krolewski, C., Harris, M.T., Manglapus, G.L. & Schwob, J.E. (2010). Expression of Pax6 and Sox2 in the adult olfactory epithelium. Journal of Comparative Neurology, 518: 4395-4418.

Hu, B. & Colletti, L. M. (2008). Stem cell factor and c-kit are involved in hepatic recovery after acetaminophen-induced liver injury in mice. *American Journal of Physiology, 295*(1): 45-53.

Huang, E.J., Nocka, K.H., Buck, J. & Besmer, P. (1992). Differential expression and processing of two cell associated forms of the Kit-ligand: KL-1 and KL-2. *Molecular Biology of the Cell, 3*: 349-362.

Ishizuka, K., Kamiya, A., Oh, E. C., Kanki, H., Seshadri, S., Robinson, J. F., Murdoch, H., Dunlop, A. J., Kubo, K., Furukori, K., Huang, B., Zeledon, M., Hayashi-Takagi, A., Okano, H., Nakajima, K., Houslay, M. D., Katsanis, N. & Sawa, A. (2011). DISC1-dependent switch from progenitor proliferation to migration in the developing cortex. *Nature, 473*: 92-96.

Jang, W., Chen, X., Flis, D., Harris, M. & Schwob, J.E. (2013). Label-retaining, quiescent globose basal cells are found in the olfactory epithelium. *Journal of Comparative Neurology, 522*(4): 731-749.

Jia, C. & Hegg, C.C. (2012). Neuropeptide Y and extracellular signal-regulated kinase mediate injury-induced neuroregeneration in mouse olfactory epithelium. *Molecular and Cellular Neuroscience, 49*(2): 158-170.

Kapur, R., Majumdar, M., Xiao, X.G., McAndrews-Hill, M., Schindler, K. & Williams, D.A. (1998). Signaling through the interaction of membrane-restricted stem cell factor and c-kit receptor tyrosine kinase: genetic evidence for a differential role in erythropoiesis. *Blood, 91*: 879-889.

Kim C. H. & Broxmeyer H. E. (1998). In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor, and the bone marrow environment. *Blood, 91*: 100-110.

Krams, M., Parwaresch, R., Sipos, B., Heidorn, K., Harms, D. & Rudolph, P. (2004). Expression of the C-Kit receptor characterizes a subset of neuroblastomas with favorable prognosis. *Oncogene, 23*: P588-595.

Krishnamoorthy, N., Oriss, T.B., Paglia, M., Fei, M., Yarlagadda, M., Vanhaesebroeck, B., Ray, A. & Ray, P. (2008). Activation of c-Kit in dendritic cells regulates T helper cell differentiation and allergic asthma. *Nature Medicine, 14*: 565-573.

Krolewski, R. C., Packard, A., Jang, W., Wildner, H. & Schwob, J. E. (2012). Ascl1 knockout perturbs differentiation of nonneuronal cells in olfactory epithelium. *PLoS ONE, 7*(12): e51737. doi:10.1371/journal.pone.0051737.

Lammie, A., Drobnjak, M., Gerald, W., Saad, A., Cote, R. & Cordon-Cardo, C. (1994). Expression of c-kit and kit ligand protein in normal human tissues. *Journal of Histochemistry and Cytochemistry, 42*(11): 1417-1425.

Lemons, K., Fu, Z., Aoudé, I., Ogura, T., Sun, J., Chang, J., Mbonu, K., Matsumoto, I., Arakawaand, H. & Lin, W. (2017). Lack of Trpm5-expressing microvillous cells in mouse main olfactory epithelium leads to impaired odor-evoked responses and olfactory-guided behavior in a challenging chemical environment. *Eneuro, 4*(3): doi.org/10.1523/ENEURO.0135-17.2017

Lev, S., Blechman, J.M., Givol, D. & Yarden, Y. (1994). Steel factor and c-kit protooncogene: genetic lessons in signal transduction. *Critical Reviews in Oncogenomics, 5*: 141-168.

Li, T. & Wu, Y. (2011). Paracrine molecules of mesenchymal stem cells for hematopoietic stem cell niche. *Bone Marrow Research*, Article ID 353878.

Liu, K., Lin, B., Zhao, M., Yang, X., Chen, M., Gao, A., Liu, F., Que, J. & Lan, X. (2013). The multiple roles for Sox2 in stem cell maintenance and tumorigenesis. *Cellular signaling, 25*(5): 1264-1271.

Majumder, S., Brown, K., Qiu, F.H. & Besmer, P. (1988). C-kit protein, a transmembrane kinase: identification in tissues and characterization. *Molecular Cell Biology, 8*(11): 4896-4903.

Mansuroglu, T., Baumhoer, D., Dudas, J., Haller, F., Cameron, S., Lorf, T., Fuzesi, L. & Ramadori, G. (2009). Expression of stem cell factor receptor c-kit in human nontumoral and tumoral hepatic cells. *European Journal of Gastroenterology and Hepatology, 21*(10):1206-1211.

Matsui, Y., Zsebo, K. M. & Hogan, B. L. (1990). Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. *Nature, 347*: 667-669.

Mayer, T.C. & Green, M.C. (1965). An experimental analysis of the pigment defect caused by mutations at the W and Sl loci in mice. *Developmental Biology, 18*: 62-75.

Mayrhofer, G., Gadd, S.J., Spargo, L.D.J. & Ashman, L.K. (1987). Specificity of a mouse monoclonal antibody raised against acute myeloid leukaemia cells for mast cells in human mucosal and connective tissue. *Immunology Cell Biology, 65*: 241.

McCulloch, E.A., Siminovitch, L., Till, J.E., Russell, E.S. & Bernstein, S.E. (1956). The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype Sl/Sl^d. *Blood, 26*: 399-410.

Ming, G. & Song, H. (2011). Adult neurogenesis in the mammalian brain: Significant answers to significant questions. *Neuron, 70*(4): p687-702.

Motro, B., van der Kooy, Rossant, J., Reith, A. & Bernstein, A. (1991). Contiguous patterns of c-kit and steel expression: analysis of mutations at the W and Sl loci. *Development, 133*: 1207-1221.

Murray, R.C., Navi, D., Fesenko, J., Lander, A.D. & Calof, A.L. (2003). Widespread defect in the primary olfactory pathway caused by loss of Mash1 function. *Journal of Neuroscience, 23*(5): 1769-1780.

Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A. & Besmer, P. (1989). Expression of c-kit gene products in known cellular targets of W mutations in normal and mutant mice – evidence for an impaired c-kit kinase in mutant mice. *Genes and Development, 3*: 816-826.

Oriss, T. B., Krishnamoorthy, N., Ray, P. & Ray, A. (2014). Dendritic cell c-kit signaling and adaptive immunity: implications for the upper airways. *Current Opinions in Allergy Clinical Immunology, 14*(1): 7-12.

Perez, F. & Granger, B.E. (2007). Python for scientific computing. *Computing in science and engineering*.

Piao, X., Curtis, J.E., Minkin, S., Minden, M.D. & Bernstein, A. (1994). Expression of the kit and kitA receptor isoforms in human acute myelogenous leukemia. *Blood, 83*: 476-481.

Ren, X., Hogaboam, C., Carpenter, A. & Colletti, L. (2003). Stem cell factor restores hepatocyte proliferation in IL-6 knockout mice following 70% hepatectomy. *Journal of Clinical Investigation, 112*(9): 1407-1418.

Russel, E.S. (1979). Hereditary anemias of the mouse: a review for geneticists. *Advances in Genetics, 20*: 357-459.

Silvers, W.K. (1979). White-spotting, patch and rump-white. The coat colors of mice: A model for gene action and interaction. New York: Springer-Verlag.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M. & Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Developmental Biology, 1*: 4

Wang, C., Curtis, J.E., Geissler, E.N., McCulloch, E.A. & Minden, M.D. (1989). The expression of the proto-oncogene c-kit in the blast cells of acute myeloblastic leukemia. *Leukemia, 3*(10): 699-702.

Zhou, Z., Wang, D., Wang, X.-J., and Roop, D.R. (2002). In utero activation of K5-CrePR1 induces gene deletion. *Genesis, 32*: 191–192.

Chapter 3 | Stem Cell Factor is required for neurogenesis following injury

Background

The functional role of C-Kit/SCF signaling was initially determined on the basis of their function in mast cells (Anderson, et al., 1990; Flanagan and Leder, 1990; Nocka, et al., 1990a,b; Williams, et al., 1990; Zsebo, et al., 1990). In both the bone marrow and connective tissue hematopoietic niches, SCF serves to stimulate proliferation of mast cells (Martin, et al., 1990). Further, SCF, in combination with other growth factors, promotes the proliferation of both erythroid cells and hematopoietic stem cells (Nocka, et al, 1990a).

The function of SCF/C-Kit goes well beyond mast cell proliferation however. Expression of SCF and C-Kit has been detected across a huge array of tissues in both development, the adult organism, and disease. Broadly, SCF signaling has been shown in multiple epithelia, the nervous system, the hematopoietic system and immune cells, and germ cells. Of all these locations, expression and function in the hematopoietic system has received by far and away the most research attention. C-Kit is expressed in many cell types, including bone marrow/hematopoietic stem cells, immune cells, germ cells (spermatogonia, oogonia) (Lammie, et al., 1994), mast cells, skin epithelium (melanocytes), lung epithelium, transitional epithelium of the bladder, corneal epithelium of the eye, Cajal cells in the intestinal epithelium, liver cells (Baumann, et al., 1999), breast glandular epithelium (Lammie, et al., 1994), CNS (particularly in cerebellum, hippocampus, and dorsal horn of the spinal cord (Lammie, et al., 1994)), cancer (neuroblastomas (Krams, et al., 2004)), development (neural crest cells), and other niches.

A number of studies have looked at the specific role of SCF/C-Kit signaling following injury. Hu & Colletti (2008) found that SCF knock-out mice display dysfunctional hepatocyte proliferation at 48 and 72 hours post-injury and also an increased number of apoptotic cells compared to wild-type controls. Normally, increased SCF production in response to injury is induced by IL-6, and causes hepatocyte proliferation via the JAK/STAT pathway (specifically, STAT3) (Ren, et al., 2003), a finding which echoes research showing that increased SCF and C-Kit expression and signaling in immune system dendritic cells results in increased IL-6 production by these cells mediating immune response to tissue damage (Krishnamoorthy, et al., 2008).

Notably, in the hematopoietic system, SCF expression by mesenchymal cells drives progenitor proliferation and differentiation in response to injury (Heissig, et al., 2007) - roles SCF mediates by promoting cellular adhesion between ligand and receptor expressing cells (Kimura, et al., 2011), an effect reliant on membrane-bound SCF being expressed (Driessen, et al., 2003; Toksoz, et al., 1992). SCF's role is not consistent throughout the body. In the colon, for instance, Lgr5+ stem cells express SCF, while supporting goblet cells express C-Kit (Rothenberg, et al., 2012). Yet more variations on these themes can be seen in stem cell niches producing melanocytes, mast cells, in reproductive niches, and beyond. In the rodent liver, oval cells, which are the liver stem cells responsible for response to tissue injury, are activated by SCF (Baumann, et al., 1999). C-Kit receptor mRNA is expressed in both normal and injured human liver tissue, with an increase in the number of C-Kit+ cells following a variety of types of injury (Baumann, et al, 1999; Hu & Colletti, 2008). Mansuroglu et al. (2009) have shown that the majority of human liver cells in health, injury, and hepatic cancer are C-Kit+, demonstrating a role for SCF signaling across an array of conditions.

In the hematopoietic system, SCF is responsible for regulating the survival, proliferation, differentiation, and migration of cell types in multiple lineages (Chen & Xiong, 2002). SCF has been

shown to have a role in adaptive immune responses such as allergic reaction, as T2/T17 immune-inducing stimuli cause an increase in SCF and C-Kit on dendritic cells (Oriss, et al., 2014). This C-Kit activation actually biases the immune response away from T1 and prevents the activation of NK cells (Krishnamoorthy, et al., 2008). Many liver cells also express C-Kit, including oval cells, a stem cell population capable of regenerating the live following cirrhotic and other types of injury (Mansuroglu, et al., 2009). Both SCF and its receptor C-Kit are subject to common mutations that, owing to germline expression of both, can result in pervasive and lethal effects. Since the pair are often present in stem in progenitor cells both during development and in the adult organism, mutations can result in a variety of cancers, and both are considered proto-oncogenes. With cancer, C-Kit expression in tumor cells sometimes correlates with decreased severity of disease prognosis because activation of the receptor often leads to differentiation of cells which would otherwise continue to proliferate unchecked contributing to pathology (Lev, et al., 1994). Notably, this is true in neuroblastomas (Krams, et al., 2004).

C-Kit initially drew attention as a trophic target in neural injury, when up-regulation of C-Kit and SCF mRNA production was observed in cells around experimental cortical stab wounds (Sun, et al., 2004). Cells at the sites of cortical brain injury up-regulate expression of SCF, which causes the migration of C-Kit+ neural precursors from other zones in the brain to the location of the injury (Sun, et al., 2004; Sun, et al., 2006). Further, it was found that adding SCF at the site of induced stroke in mice caused proliferation and aided functional recovery (Jin, et al., 2002; Zhao, et al., 2007). Together, these studies underscore the central importance of C-Kit signaling in neural precursor cell proliferation. However, the mechanisms by which C-Kit signaling promotes recovery after neural injury remain obscure.

Methods

Transgenic knock-out of genes of interest is a useful tool in determining the function of proteins in vivo. Our research group is particularly well equipped to do this in the OE using a variety of lines of transgenic mice harboring a Cre cassette under the transcriptional control of promoters in the loci of genes known to predictably mark particular cell types in the OE and with sufficient rates of transcription to consistently effect whatever Cre-inducible transgenes are bred in along with the drivers. In this work, we use two cell-type-specific Cre driver mice (Figures 6 & 7).

The first line we used had Cre-ERT2 placed under the control of the Sox2 promoter in an inbred Black 6 genetic background (B6;129S-Sox2tm1(cre/ERT2)Hoch, Jackson Laboratories). Cre-ERT2 codes for a fusion protein of Cre recombinase and a mutated version of the human estrogen receptor which makes the recombinase activatable in response to the presence of 4-hydroxytamoxifen ("tamoxifen"), rather than its native ligand, 17β-estradiol (Metzger, et al., 1995). Administration of tamoxifen to the mouse and subsequent binding to the estrogen receptor allows the normally cytoplasmically restricted fusion protein to enter the nucleus of the cell. Once inside the nucleus, Cre recombination acts at transgenically inserted loxP sites, allowing for deletion of genetic sequences flanked by two loxP sites ("floxing").

We bred mice expressing the Cre fusion protein in Sox2+ cells to mice with homozygously floxed Sl loci (Kitltm2.1Sjm, Jackson Laboratories, described in detail below). This combination results in mice which, upon the administration of tamoxifen (0.25mg/g body weight, intraperitoneal injection), lose functional production of SCF protein in all Sox2+ cells. At steady state in the OE, Sox2 is expressed by all HBCs and SUS cells in addition to a subset of GBCs, likely those with the most stem cell-like genetic character, given the role Sox2 plays in maintaining stemness (Arnold, et al., 2011). Based on

the antibody assays described in Chapter 2, we had little reason to suspect that SCF would be expressed in these GBCs, but it is worth noting this as an experimental imperfection. Effectively, this transgenic constitutes a tamoxifen-inducible knock-out of SCF in the two groups of cells which express the growth factor in the OE: HBCs and SUS cells.

Even with the standard administration in suspension of 0.25mg tamoxifen/g body weight of mouse via intraperitoneal injection, Cre recombinase induction and nuclear translocation does not occur with complete reliability in all Sox2+ cells. To control for this deficiency in experimental methods, we bred in a third transgenic allele, a Cre-inducible fluorescent reporter. This allowed us to visually determine which cells had undergone experienced effective recombinase activity.

Additionally, we used a second Cre driver line (K5.CrePR1, Laboratory of Dennis Roop (Zhou, et al., 2002)), differing in both promoter driving Cre expression and in the nature of the Cre recombinase protein itself. For this mouse, the promoter that harbored the Cre transgene was the cytokeratin 5 promoter, which is expressed in all HBCs (Leung, et al., 2007). The Cre allele in the K5 locus coded for a fusion protein of Cre recombinase and the human progesterone receptor, which is constitutively activated even in the absence of any kind of drug administration (Zhou, et al., 2002). This transgenic strategy is useful in two instances. First, if recombination is desired in HBCs only. Second, this is useful for looking at transgenic modifications in all cell types derived from HBCs in the OE during recovery from injury. Because the entire OE is regenerated from HBCs during regeneration after methimazole administration, in these mice, the OE is regenerated with the transgenic recombination accomplished in the HBCs present in every single cell of the re-build epithelium. This is a useful tool to target the OE as whole without there being any one promoter that would be on in all OE cell types and not so widespread in the organism as a whole as to cause profound developmental defects.

Several other mice were particularly useful to us in the present work. We bred in an allele from Cre recombinase reporter mice (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J, Jackson Laboratory), which produce yellow fluorescent protein (YFP) in cells in which Cre recombinase is active. In these mice, a floxed stop codon followed by sequence for YFP is in the Rosa 26 constitutively active locus. In the presence of Cre, the stop is floxed out and YFP begins to be produced. Additionally, all of our knock-out experiments relied on a line of mice with loxP sites situated on either side of exon one of the gene coding for SCF (Kitltm2.1Sjm/J, Jackson Laboratory). In the presence of Cre, SCF protein ceases to be produced in mice carrying this mutation homozygously.

Strains of transgenic mice (Krt5-CrePR [Zhou, et al., 2002]), mice carrying cre-inducible SCF knock-out allele (SCF-lox-Exon1-lox-, Kitltm2.1Sjm, Jackson Laboratory) and mice carrying a cre-inducible YFP allele under the Rosa26 promoter (Srinivas, et al., 2001; Rosa26YFP) were bred on a C57/B6 background. Mice used in this study included the following:

Krt5-CrePR$^{Tg/+}$; Rosa26$^{YFP/+}$; SCF$^{lox/lox}$ (knock-out)
Krt5-CrePR$^{Tg/+}$; Rosa26$^{YFP/+}$; SCF$^{+/+}$ (wildtype)

Regeneration from neural injury was modeled by methimazole administration. Mice were co-housed with parents and littermates until until post-natal day 21, at which point methimazole was administered by intraperitineal injection (0.01mg methimazole/g bodyweight). Methimazole causes the death of virtually all cells in the epithelium aside from the HBCs within 24 hours of drug administration (Leung, et al., 2007). Mice used for 'injured' tissue collection were each sacrificed at one of three experimental time points after administration of methimazole (4 days post-injury, 7 days post-injury, or 14 days

post-injury). 'Healthy' tissue was collected at post-natal day 21 from mice that were not administered methimazole.

Tissue collected from mice used in immunohistochemistry experiments was fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), decalcified for five days in 10% ethyleneaminetetracetic acid in PBS and equilibrated overnight in 30% sucrose. Frozen tissue was mounted in freezing medium (Triangle Biomedical Sciences), cryostat sectioned to 12um thick and slide mounted for microscopy.

For immunohistochemistry sections were permeabilized in 0.1% Triton X-100 in PBS and stained with antibodies diluted in donkey serum overnight. Fluorophore-conjugated secondary antibodies were used and detected as described in Duggan, et al., 2008. Secondary antibodies (Alexa 488, 594, 555, 568 and 647 (Invitrogen)) were used in conjunction with Hoechst 33342 nuclear stain for imaging. Slides were cover slipped with Vectashield (Hard Set, Vector Labs). Primary antibodies were used as follows:

chicken anti-GFP 1:500 (Abcam)
mouse anti-p63 1:100 (Santa Cruz Biotechnology)
goat anti-Sox2 1:100 (Santa Cruz Biotechnology)
goat anti-C-Kit 1:100 (R&D Systems)
rabbit anti-Ki67 1:250 (Abcam)
mouse anti-neuronal tubulin 1:250 (Tuj1; Abcam)
guinea pig anti-Ascl1 1:10,000 (gift from Jane Johnson)
rabbit anti-caspase3 1:250 (Cell Signaling Technology)

Images were acquired using epifluorescence and confocal microscopes. Image assembly and analysis was done in ImageJ (National Institutes of Health) and Photoshop (Adobe). Quantification was done using the Cell Counter ImageJ plugin (Kurt de Vos). Quantification of numbers of cells positive for various markers was done for epithelium in each of 3 or 4 knockout and 3 or 4 control animals for each condition with an average of 18000um OE scored per animal. Quantification was done in the olfactory epithelium only (not respiratory/non-sensory, which lines the ventral portion of the nasal cavity). Quantification was restricted to septal regions for consistency.

Results

3.1 | Neither SCF knock-out in SUS cells nor in HBCs has a substantial impact in steady state OE

We next aimed to examine the functional role of SCF in the OE. Using the $Sox2CreER^{T2/+};Sl^{fl/fl};Rosa26^{YFP/(+ \text{ or } YFP)}$ mice described in the Methods section of this chapter, we examined the effect of SCF knock-out in steady state OE tissue. Under homeostatic conditions, there is constant normal turnover of OE neurons, which are replaced by dividing GBCs (Caggiano, et al., 1994; Huard, et al., 1998; Leung, et al., 2007; Mackay-Sim and Kittel, 1991). We therefore conjectured that, if SCF is involved in the birth and migration of newborn neurons in the OE, we might expect to see signs of a defect manifest over the course of a month after tamoxifen administration in our transgenic mice. This was not the case, however. Ablation of local SCF signal in HBCs and SUS cells, did not lead to any apparent change in the morphology of the OE or relative cell types and localizations. We limited the assessment of this line of inquiry to qualitative impressions of imaging with immunohistochemistry for basic cell markers, because it was fairly clear, even in early trials, that any effect that was evident would not be sufficient to account for the striking degree of and pattern

of expression of this particular growth factor in the OE. We assessed different experimental time points, 30, 60 and 90 days post-tamoxifen administration, and no change was evident at any of these (data not shown). We further assessed the impact of SCF knock-out after methimazole injury (Figure 6). Considering the images from the Sox2CreER$^{T2/+}$;Sl$^{fl/fl}$;Rosa26$^{YFP/(+ or YFP)}$ mice, it became apparent that our transgenic Sox2 had not been sufficiently strongly induced to catalyze recombinase mediated changes in all of the cells we were targeting. Namely, the YFP reporter appeared bright in patches along the OE, while other sections remained dark.

We pursued two avenues in response to this realization. First, we trialed intranasal, rather than intraperitoneal administration of tamoxifen in the hope that this delivery route might result in more even and robust induction. Second, we repeated the experiment using a different Cre driver. This time we used a constitutive, rather than tamoxifen-inducible driver, so that we were assured reliable and comprehensive knock-out of SCF in cells expressing the driver gene - in this case Keratin 5. These mice were K5CrePR$^{T2/+}$;Sl$^{fl/fl}$;Rosa26$^{YFP/(+ or YFP)}$, providing a near parallel to our first attempt, but with a driver mouse that our lab has used successfully in many previous instances (Figure 7). One notable weakness arose with this change in experimental mouse, however. While the Sox2 driver meant that we could expect ablation of SCF in all SCF expressing cell types in the OE, the K5 driver only produces Cre recombinase in basal HBCs. This means that the SUS cells, a major source of SCF spanning the entire basal to apical breadth of the epithelium, were still able to produce SCF protein in our knock-out. Further, because we used the CrePR allele in order to allow for maximum effect, we also collaterally knocked-out SCF during development, which might have had impacts beyond the scope of the intended aims of our work in the present study.

The results of examining the epithelia of the K5CrePR$^{T2/+}$;Sl$^{fl/fl}$;Rosa26$^{YFP/(+ or YFP)}$ knock-out mice were disappointing (data not shown). Again, little, if any, observable change occurred in knock-out mice relative to controls

3.2 | Absence of SCF signal after injury leads to incomplete recovery and a thinner epithelium

At this point, we wondered if we might be looking in the wrong place for the function of SCF and C-Kit in the OE. In many other niches, neurogenic and non-neurogenic alike, SCF appears to be most important in mediating response to and recovery from injury. Using transgenic SCF knock out mice we examined the ultrastructural differences in the OE during regeneration from injury in animals selectively deficient in SCF in the OE.

We crossed mice harboring the CrePR transgene under the Keratin5 promoter (described in Methods) with lines carrying flowed alleles of the SCF gene and a Cre-dependent fluorescent transgene under the ubiquitous Rosa26 locus. Keratin5 is a consistent marker of all HBC cells (Leung, et al., 2007). In these mice, SCF knock-out is limited to HBCs only under healthy conditions. Methimazole-induced injury kills virtually all cell types apical to the HBCs, leaving the HBCs to divide and differentiate to restore the epithelium. Administration of methimazole in our mouse line therefore resulted in the ablation of all cells not harboring the complement of transgenes and the subsequent regeneration of the entire epithelium from SCF-deficient progenitor cells. The fluorescent YFP transgene remains expressed in all SCF knock-out progeny of transgenic HBC parent cells, which provided confirmation of SCF knock out in the epithelia we quantified. Importantly, in our regeneration model of SCF-deficiency, both sources of the growth factor in the epithelium are knocked-out, during and after regeneration. Though some SCF may still reach the OE via the bloodstream, ablation of systemic sources is beyond the scope of the present inquiry.

To begin to address the functional impact of SCF in the OE, we first measured the thickness of the OE in health and then during regeneration in knock-out and wild type mice (Figure 8). At steady state, no difference was observed in epithelial thickness at post-natal day 30, 60 or 90 (data not shown). This may reflect that the Krt5 driver in these healthy knock-out mice excises the SCF gene in HBCs, but not in SUS cells. Though SCF from each of these sources may differ in functional impact, both have direct contact with the largest population of receptor-expressing cells, the GBCs. It is conceivable that we did not detect any differences in healthy (un-ablated) mice because SCF from SUS cells is able to sufficiently compensate for loss of the HBCs as a source of the growth factor. It may also be that SCF plays a small or redundant role in the healthy epithelium.

Because SCF signaling is pivotal in other niches during injury response and developmental cell generation periods, we next examined the impact of SCF knock-out during recovery from methimazole injury in the OE. We administered tamoxifen to post-natal day 21 mice and measured epithelial thickness at various time points during recovery. Comparing wild-type and SCF knock-out mice, we found that the OE is thinner in knock-outs by 7 days-post-injury (Figure 8). We reasoned that number of explanations for this phenomenon were possible: The OE might simply regenerate more slowly in the absence of SCF, in which case, we would expect to see that the epithelia of knock-out and wild type mice were of equal thickness at experimental time points after the completion of the injury-induced regeneration of the OE. Alternately, the macroscopic changes we observed might be the result of thinning of the OE, resulting from failure to produce or untimely death of one or many types of cells. This could stem from changes in the identity of progeny produced by progenitors during regeneration, changes in the number of cells entering into the cell cycle, changes in the number of times cells underwent cycling, death of cells due to apoptotic processes during the cell cycle, or death of cells due to failure to thrive as differentiating precursors.

3.3 | Lack of SCF signal after injury leads to defects in neural birth

We reasoned that the thinning of the OE in knock-out animals likely stemmed from either a change in the morphology and cellular make up of the OE resulting in overall compaction or from a decrease in the number of one or multiple cell types produced during regeneration. The first hypothesis, general compaction of the OE morphology, seemed unlikely, as knockout OE at all time points closely resembled the organization of the OE in non-mutant animals. Accordingly, we pursued the second hypothesis, that there was a deficit in the number of certain cell types during recovery.

To determine the nature of underlying deficits in the K5CrePR SCF knock-out mouse that led to thinning of the epithelium during and after recovery from injury, we first used immunochemical stains to determine the relative abundance of various cell types present in knock-outs during recovery compared to wild-type mice during recovery. In keeping with previous studies, we looked for deficits in neuronal production. Tuj1 (also called Gap43) marks immature neurons. Cells that are Tuj1+ are committed to neuronal fate and their transcriptional profile indicates that they are well along toward being mature neurons. Tuj1+ cells never divide. Quantifying Tuj1 expression allows us to determine deficits in neuronal production earlier during recovery from injury than quantifying expression of a marker of more mature neurons, such as Olfactory Marker Protein (OMP), would.

Because of the association of SCF with neural birth and regeneration in other niches, it was plausible that the cellular defect accounting for the thinner OEs of SCF mutant animals was a decrease in the number of neurons produced during regeneration. Examining the epithelia of mice recovering from methimazole ablation of the OE using immunohistochemical staining for Tuj1, we observed that there

were less neurons produced in mice lacking SCF during healing after injury. At four days after injury, the OE lacks sufficient neurons to observe a deficit (Figure 9). A difference was first observed at the seven days post-methimazole administration time point (Figure 10), which is consistent with the timeframe in which maturing neurons begin to appear during regeneration. We wondered if the observed lack of neurons at seven days as a result of SCF knock-out would be made up for by other mechanisms in the cell as regeneration progressed. Accordingly, we quantified a further experimental time point, at fourteen days post-methimazole administration, to see if the effect was durable over the full course of regeneration (Figure 11). Starting at seven days post-methimazole administration, this deficiency was manifest at all time points included in the analysis. The function(s) mediated by SCF in the OE during regeneration are necessary for normal neural birth and not redundant with any other mechanism.

Though our results indicate that SCF signaling is functionally implicated in production of neurons during recovery from injury, it is interesting to note that the penetrance of the effect is less profound than that seen in a well-executed experimental paradigm that simply killed all C-Kit+ cells (thus using C-Kit as a marker of a class of cells, rather than examining its function) (Goldstein, et al., 2015). There are a number of reasons this might have occurred: Sources of SCF outside of the OE may be able to make up for the sources we eliminate in our knock-out model. Perhaps most interestingly, the intracellular components of SCF signaling (like many receptor tyrosine kinase cascades) overlap with a variety of other signaling pathways that phosphorylate similar substrates and rely on the same downstream molecular partners. Accordingly, in our SCF knock-out, incomplete blockade of the birth of new neurons may reflect the ability of parallel signaling pathways to make up for C-Kit. Similarly, there may be pathways along which cells born of HBCs can differentiate that do not involve passing through a C-Kit+, and thus SCF-dependent, state, though work of the Goldstein group strongly implies that this is not the case.

Conclusions and Discussion

SCF signaling is implicated in neurogenic birth after injury in the OE. While defects were not apparent in the epithelia of knock-out animals under steady state conditions, transgenic mice lacking SCF production in the OE presented with significant defects in neural birth during regeneration.

Essentially, four interpretations of these findings are possible: It is possible that SCF is involved in the specification of neural fates over supporting cell fates in multipotential progenitor cells, serving in tandem with other molecular cues to balance the relative size of each of these populations of cells. If this is the case, during regeneration, we would expect to see an increase in the number of supporting cells formed that corresponds to our observed decrease in number of neurons.

It is also possible that SCF is involved primarily in the proliferation of various different cell types among the GBCs: either multipotential progenitors, progenitor cells committed to neural fates, or both of these. In any of these three cases, we would expect to see a decrease in number of neurons such as that which we observed. However, a loss of progenitors would likely also lead to a decrease in the number of supporting cells as well as a decrease in neurons, delineating this from the previous possibility described.

Further, based on our findings, it might be the case that SCF participates in the cascade of signals that mediate the survival of either neurally committed progenitor cells, multipotential progenitor cells, supporting cells which facilitate the survival of any of the preceding cell, or some combination of these cell types. Maintaining newborn cells in the face of apoptosis during regeneration is a critical

mediator of recovery.

Another interesting possibility is that loss of SCF during regeneration impairs migration of newly born cells. Considering the importance of local paracrine signals to the fate specification of differentiating cells, loss of organizational cues in the OE might significantly impair neural production and/or maturation. Perhaps the most striking example of the delicate interplay between migration and fate specification is the intricate radial migratory dance done by newborn cortical cells as they differentiate into neurons during development (see Ming & Song, 2011 for a review).

A migratory role for SCF and C-Kit has already been suggested in both central and peripheral nervous system injury. In the central nervous system, SCF mRNA is strongly expressed in neurons at sites of acute tissue injury. C-Kit is expressed by neural stem and progenitor cells which migrate to these areas, while SCF induces the migration of these cells to the site of injury (Sun, et al., 2004; Sun, et al., 2006). Given the alteration in neuronal populations we observed in SCF knock-out mice, it is possible that SCF is playing a similar role in the OE neuroepithelium to that which it plays in the brain and broader central nervous system. As shown in Chapter 2, the placement of SCF expressing cells - basal HBCs and SUS cells, which extend their processes from the most basal to the most apical positions in the OE - leaves these cells poised to guide the migration during maturation of stem and progenitor cells, which initially rest in a more basal position, to more mature apical neurons. Though there have been other proposed roles for SCF signaling, a migratory function would be particularly well served by this expression placement. Additionally, recent work has shown that activation of the C-Kit receptor on maturing neurally committed progenitor cells is necessary for timely migration of these cells to appropriate cortical locations (Bocchi, et al., 2017).

This finding mirrors the function in the blood, and other niches, where SCF signaling is responsible for adhesion and migration of hematopoietic stem and progenitor cells to their bone marrow niche (Kim, et al., 1998; Matsui, et al., 1990). Hepatic stem cells display a similar reliance on SCF and C-Kit for migratory cues (Christensen, et al., 2004). Bocchi, et al. (2017) showed that SCF production and C-Kit expression are increased in response to Wnt3a (canonical Wnt signaling), which had previously been implicated in the transition of the same neuronal progenitors from a proliferative mode to a post-mitotic differentiation program (Ishizuka, et al., 2011). Migration defects were found to be a result of poor neuron-glia attachment in mutant mice. Interestingly, the authors found that the majority of cells in the mutants did eventually reach their laminar destination and displayed normal cell fate markers, but their migration was much slower than wild-type cells, raising the possibility of different types of migratory effects in our data. Localization and/or kinetic defects are possible. There is some evidence suggesting that the mechanisms regulating neural migration in development are still operational in the adult, though there has been relatively little work looking at the relevance of this signaling cascade in the few neural stem cell niches that remain operational in the adult (Bocchi, et al., 2017).

These findings create a problem in our interpretation of our own results. If SCF signal in the OE primarily functions to regulate the features and timing of progenitor migration, we would expect to see slowed establishment of the neural layer during regeneration after methimazole injury in the knockouts. However, our assay is not particularly well suited to differentiate this type of a defect from a defect in proliferation or differentiation. Since our method for determining the number of neurons produced in regeneration involved counting the Tuj1+ apical dendrites of neurons in the OE at various points in time following injury, no matter the nature of the defect, we would expect to see lower numbers of Tuj1+ cells. The ideal experiment to determine if the defect in SCF mutants was

due to migratory kinetics might have been to count neurons in mice long after the completion of regeneration, such that slowly migrating cells would have had enough time to reach their final destination, and mutant and wild-type numbers of neurons would have equalized in a migration/kinetic mutant, but not in a mouse with a problem with differentiation or proliferation.

In order to understand better what role SCF plays, we decided to look at the numbers of non-neuronal cells in our knockout mice, reasoning that, if SCF is involved in fate specification, then we might see changes in the relative numbers of non-neuronal cells (if progenitors were producing less neurons without changing their rate of mitotic activity). The work described in Chapter 4 describes these experiments.

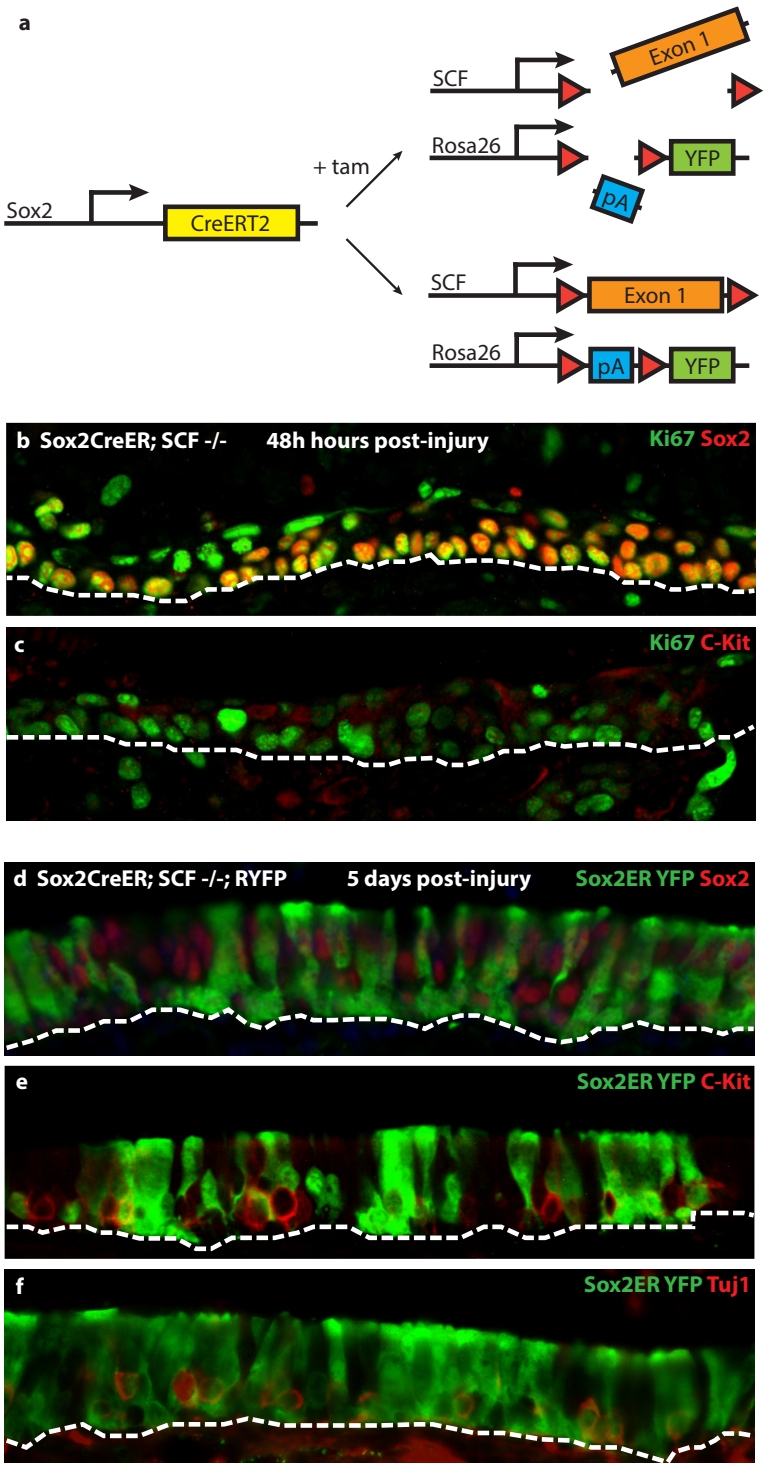


Figure 6 | Preliminary assay of SCF knock-out phenotype. **a,** Mouse design for knockout of SCF and lineage tracing. At injection of tamoxifen, Cre recombinase was produced in cells expressing Sox2, resulting in the excision by floxing of Exon1 of the SCF gene and YFP lineage tracing by floxing out a stop signal just upstream of a YFP sequence knocked into the ubiquitously expressed Rosa26 locus. b-f are all from SCF KO animals. Knockouts appear to have normal cell numbers at 48 hours post-injury of **b,** Ki67+ proliferative cells, Sox2+ activated stem cells and **c,** C-Kit+ cells. Knockouts also appear to have normal cell numbers at 5 days post-injury of **d,** Sox2+ stem cells and SUS cells (upper layer), **e,** C-Kit+ cells and **f,** Tuj1+ immature neurons.

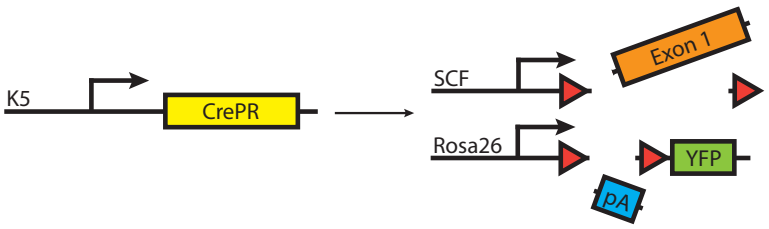


Fig 7 | Experimental procedure to target and lineage trace SCF knock out in K5+ cells Mouse design for knockout of SCF and lineage tracing in all cells derived from K5+ cells. Because the entire OE regenerates from HBCs after methimazole injury, this design results in nearly ubiquitous removal of SCF from OE cells during recovery and after injury.

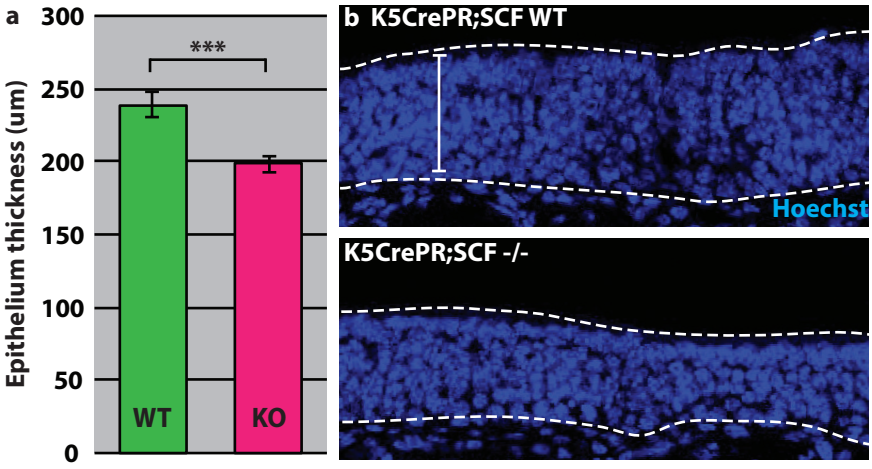


Figure 8 | SCF/C-Kit signaling knock-out results in a thinner olfactory epithelium. **a,** Transgenic mice with the K5-promoter driving the floxed excision of the SCF gene allowed for knock-down of Kit signaling upon regener-ation of the OE from K5+ HBC progenitors. In this condition, OE was much thinner than those of wild-type controls. **b,** sample wild-type control and **c,** knock-out epithelia.

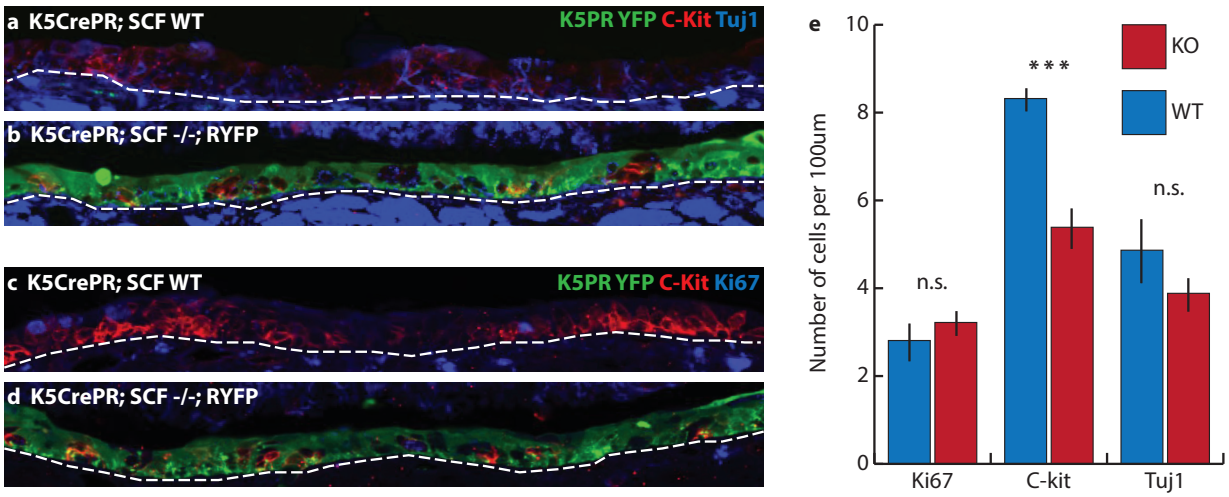
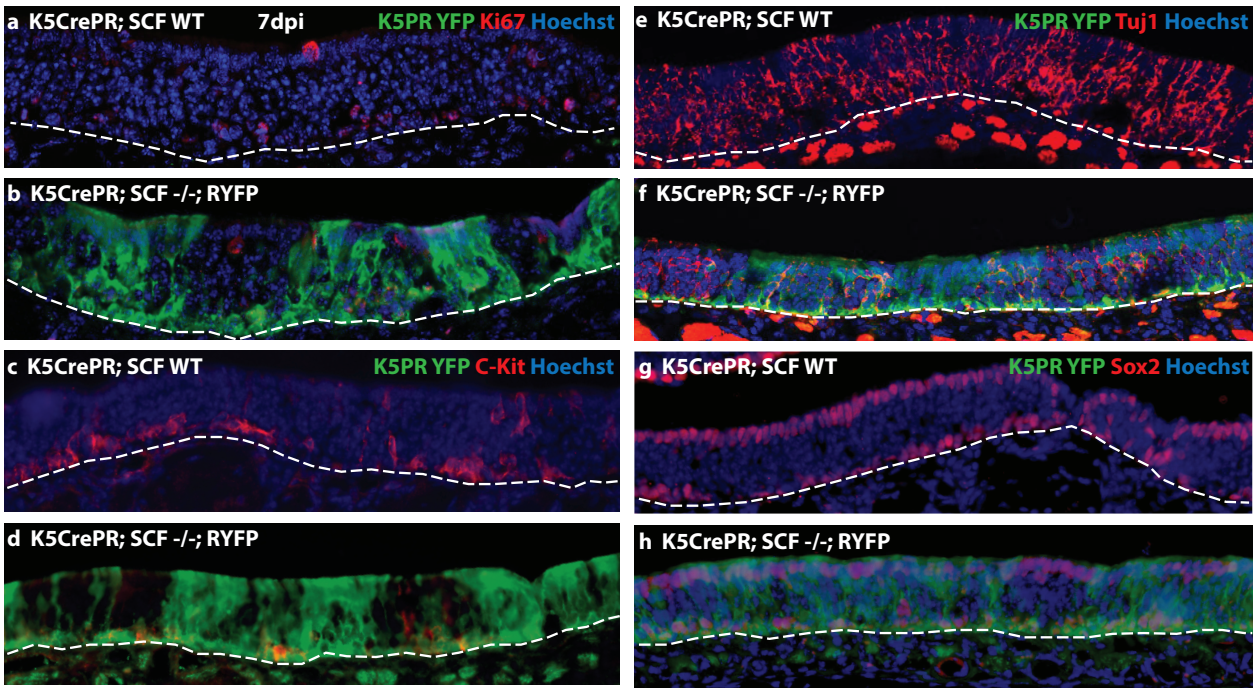


Figure 9 | Marker gene expression changes at four days post-injury in SCF knockout mice. **a,b,** Regenerating OE from SCF wild type (a) and knock out (b) tissue. Numbers of C-Kit+ cells are diminished. Numbers of Tuj1+ cells are similar, possibly because there are relatively few immature neurons this early in regeneration. Interestingly, C-Kit+ and Tuj1 cells infrequently co-localize with YFP tracing, suggesting that these cells have escaped SCF knock out. **c, d,** Numbers of Ki67 + cells are similar between wild type and control mice. **e,** Quantification of transcriptional differences. Error bars indicate s.e.m.; ***P<0.05.



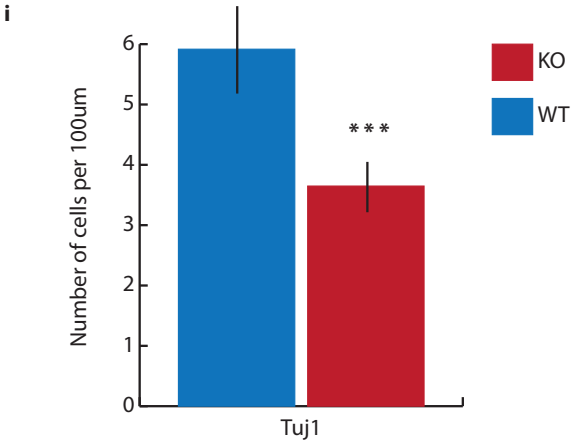


Figure 10 | Marker gene expression changes at seven days post-injury in SCF knockout mice. **a, b,** Regenerating OE tissue at seven days post-injury. Relative numbers of proliferative Ki67+ cells are similar between knockouts and wild type littermates. **c, d,** Numbers of C-Kit+ cells appear to be decreased in knockouts, but this data has not yet been quantified. **e, f,** Numbers of Tuj1+ cells are significantly decreased in knockout animals. **g, h,** It's not yet clear if there is a difference in number of Sox2+ basal or sustentacular cells. **i,** Quantification of transcriptional differences. Error bars indicate s.e.m.; ***P<0.05.

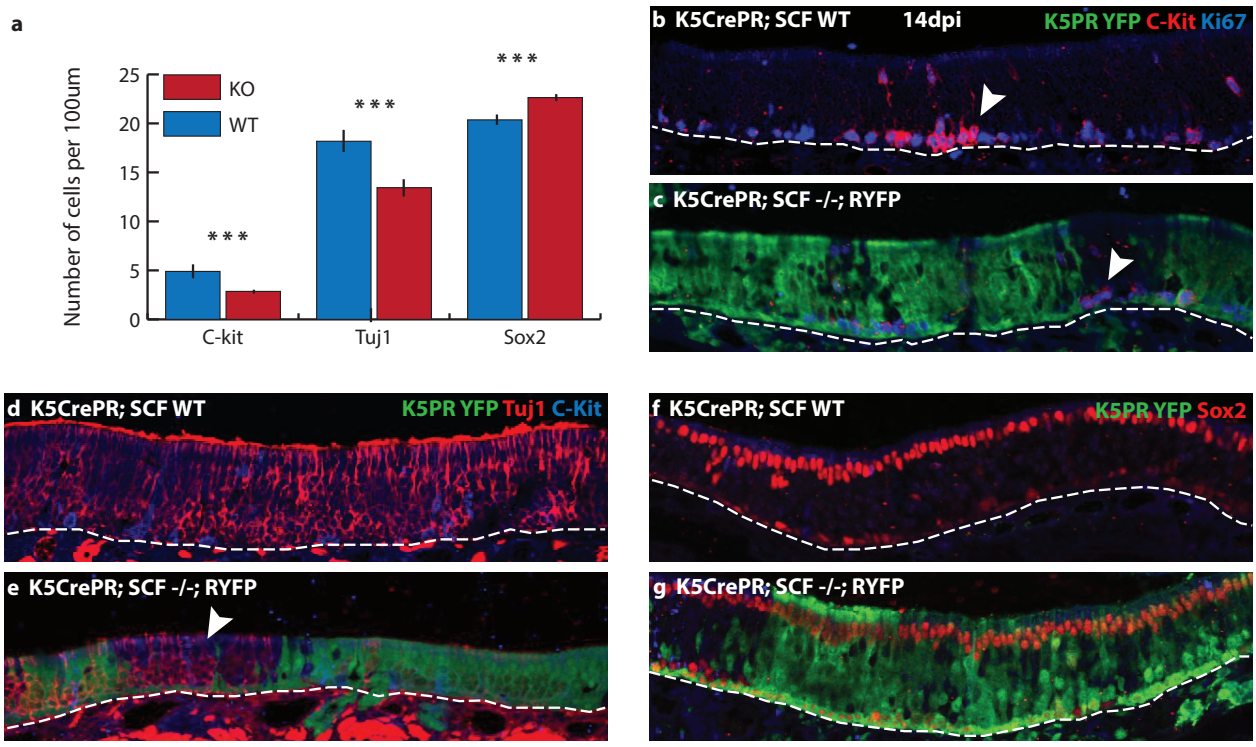


Figure 11 | Marker gene expression changes at fourteen days post-injury in SCF knockout mice. **a,** Quantification of transcriptional differences. Error bars indicate s.e.m.; ***P<0.05. **b, c,** Regenerating OE from wild type (b) and SCF knock out (c) tissue. At fourteen days post-injury, the deficit in C-Kit+ cell numbers remains. Clusters of C-Kit+ basal cells producing cells moving more apically (white arrowhead, b) are not evident in knock out animals (white arrowhead, c). No differences exist in numbers of Ki67+ proliferating cells. **d, e,** Decreased numbers of immature neurons have become pronounced by fourteen days post-injury. Of note, in areas where the knockout in incomplete - indicated by lack of YFP+ staining, many more Tuj1+ cells are present (white arrowhead). **f, g,** Interestingly, numbers of Sox2+ SUS-layer cells are increased in SCF knock outs, suggesting a possible fate-switch from neuronal (Tuj1+) to SUS (Sox2+) in knockouts.

References

Anderson, D.M., Lyman, S.D., Baird, A., Wignall, J.M., Eisenman, J., Rauch, C., March, C.J., Boswell, H.S., Gimpel, S.D., Cosman, D. & Williams, D.E. (1990). Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell, 63*: 235-243.

Arnold, K., Sarkar, A., Yram, M.A., Polo, J.M., Bronson, R., Sengupta, S., Seandel, M., Geijsen, N. & Hochedlinger, K. (2011). Sox2+ adult stem and progenitor cells are important for tissue regeneration and survival in mice. *Cell Stem Cell, 9*: 317-329.

Baumann, U., Crosby, H.A., Ramani, P., Kelly, D.A. & Strain, A.J. (1999). Expression of the Stem Cell Factor Receptor c-kit in Normal and Diseased Pediatric Liver: Identification of a Human Hepatic Progenitor Cell. *Hepatology, 30*(1): 112-117.

Bocchi, R., Egervari, K., Carol-Perdiguer, L., Viale, B., Quairiaux, C., De Roo, M., Boitard, M., Oskouie, S., Salmon, P. & Kiss, J. K. (2017). Perturbed Wnt signaling leads to neuronal migration delay, altered interhemispheric connections and impaired social behavior. *Nature Communications, 1158:* doi:10.1038/s41467-017-01046-w.

Caggiano, M., Kauer, J.S. & Hunter, D.D. (1994). Globose basal cells are neuronal progenitors in the olfactory epithelium: A lineage analysis using replication-incompetent retrovirus. *Neuron, 13*: 339-352.

Chen, S.Q. & Xiong, A.Q. (2002). The progress and implication of stem cell factor. *Basic Medical Sciences and Clinics, 22*(5): 385-390.

Christensen J. L, Wright D. E, Wagers A. J, Weissman I. L. (2004). Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biology, 2*: E75.

Driessen, R.L., Johnston, H.M. & Nilsson, S.K. (2003). Membrane-bound stem cell factor is a key regulator in the initial lodgment of stem cells within the endosteal marrow region. *Experimental Hematology, 31*(12): 1284-1291.

Duggan, C.D., DeMaria, S., Baudhuin, A., Stafford, D. & Ngai, J.N. (2008). FoxG1 is required for development of the vertebrate olfactory system. *Journal of Neuroscience, 28*(20): 5229-5239.

Flanagan, J.G. & Leder, P. (1990). The kit ligand: a cell surface molecule altered in Stell mutant fibroblasts. *Cell, 63*: 185-194.

Goldstein, B.J., Goss, G., Hatzistergos, K.E., Rangel, E.B., Seidler, A., Saur, D. & Hare, J.M. (2015). Adult c-Kit(+) progenitor cells are nexessary for maintenance and regeneration of olfactory neurons. *Journal of Comparative Neurology, 523*(1): 15-31.

Heissig, B., Lund, L.R., Akiyama, H., Ohki, M., Morita, Y., Romer, J., Nakauchi, H., Okumura, K., Ogawa, H., Werb, Z., Dano, K. & Hattori, K. (2007). The plasminogen fibrinolytic pathway is required for hematopoietic regeneration. *Cell Stem Cell, 1*(6): 658-670.

Hu, B. & Colletti, L. M. (2008). Stem cell factor and c-kit are involved in hepatic recovery after

acetaminophen-induced liver injury in mice. *American Journal of Physiology, 295*(1): 45-53.

Huard, J.M., Youngentob, S.L., Goldstein, B.J., Luskin, M.B. & Schwob, J.E. (1998). Adult olfactory epithelium contains multipotent progenitors that give rise to neurons and non-neural cells. *Journal of Comparative Neurology, 400*: 469-486.

Ishizuka, K., Kamiya, A., Oh, E. C., Kanki, H., Seshadri, S., Robinson, J. F., Murdoch, H., Dunlop, A. J., Kubo, K., Furukori, K., Huang, B., Zeledon, M., Hayashi-Takagi, A., Okano, H., Nakajima, K., Houslay, M. D., Katsanis, N. & Sawa, A. (2011). DISC1-dependent switch from progenitor proliferation to migration in the developing cortex. *Nature;473:* 92-96.

Jin, K., Mao, X.O., Sun, Y., Xie, L. & Greenberg, D.A. (2002). Stem cell factor stimulates neurogenesis in vitro and in vivo. *The Journal of Clinical Investigation, 110*(3): 311-319.

Kim, C. H. & Broxmeyer, H. E. (1998). In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor, and the bone marrow environment. *Blood, 91*: 100-110.

Kimura, Y., Ding, B., Imai, N., Nolan, D.J., Butler, J.M. & Rafii, S. (2011). C-Kit-Mediated Functional Positioning of Stem Cells to Their Niches Is Essential for Maintenance and Regeneration of Adult Hematopoiesis. *PLoS ONE 6*(10): doi.org/10.1371/journal.pone.0026918.

Krams, M., Parwaresch, R., Sipos, B., Heidorn, K., Harms, D. & Rudolph, P. (2004). Expression of the C-Kit receptor characterizes a subset of neuroblastomas with favorable prognosis. *Oncogene, 23*: 588-595.

Krishnamoorthy, N., Oriss, T.B., Paglia, M., Fei, M., Yarlagadda, M., Vanhaesebroeck, B., Ray, A. & Ray, P. (2008). Activation of c-Kit in dendritic cells regulates T helper cell differentiation and allergic asthma. *Nature Medicine, 14*: 565-573.

Lammie, A., Drobnjak, M., Gerald, W., Saad, A., Cote, R. & Cordon-Cardo, C. (1994). Expression of c-kit and kit ligand protein in normal human tissues. *Journal of Histochemistry and Cytochemistry, 42*(11): 1417-1425.

Leung, C.T., Coulombe, P.A. & Reed, R.R. (2007). Contribution of olfactory neural stem cells to tissue maintenance and regeneration. *Nature Neuroscience, 10*: 720-726.

Lev, S., Blechman, J.M., Givol, D. & Yarden, Y. (1994). Steel factor and c-kit protooncogene: genetic lessons in signal transduction. *Critical Reviews in Oncogenomics, 5*: 141-168.

Mackay-Sim, A. & Kittel, P. (1991). Cell dynamics in the adult mouse olfactory epithelium: a quantitative autoradiographic study. *Journal of Neuroscience, 11*: 979-984.

Mansuroglu, T., Baumhoer, D., Dudas, J., Haller, F., Cameron, S., Lorf, T., Fuzesi, L. & Ramadori, G. (2009). Expression of stem cell factor receptor c-kit in human nontumoral and tumoral hepatic cells. *European Journal of Gastroenterology and Hepatology, 21*(10): 1206-1211.

Martin, F.H., Suggs, S.V., Langley, K.E., Lu, H.S., Ting, J., Okino, K.H., Morris, C.F., McNiece, I.K.,

Jacobsen, F.W., Mendiaz, E.A., Birkett, N.C., Smith, K.A., Johnson, M.J., Parker, V.P., Flores, J.C., Patel, A.C., Fisher, E.F., Erjavec, H.O., Herrera, C.J. Wypych, J., Sachdev, R.K., Pope, J.A., Leslie, I., Wen, D., Lin, C.H., Cupples, R.L. & Zsebo, K.M. (1990). Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell, 63*: 203-211.

Matsui, Y., Zsebo, K. M, & Hogan, B. L. (1990). Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. *Nature, 347:* 667-669.

Metzger, D., Clifford, J., Chiba, H. & Chambon, P. (1995). Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proceedings of the National Academy of Sciences, 92*(15): 6991-6995.

Ming, G. & Song, H. (2011). Adult neurogenesis in the mammalian brain: Significant answers to significant questions. *Neuron, 70*(4): 687-702.

Nocka, K., Buck, J., Levi, E. & Besmer, P. (1990a). Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast-derived growth factor stimulates mast cells and erythroid progenitors. *European Molecular Biology Association Journal, 9*: 3287-3294.

Nocka, K., Huang, E., Beier, D.R., Chu, T.Y., Buck, J., Lahm, H.W., Wellner, D., Leder, P. & Besmer, P. (1990b). The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell, 63: 225-233.

Oriss, T. B., Krishnamoorthy, N., Ray, P. & Ray, A. (2014). Dendritic cell c-kit signaling and adaptive immunity: implications for the upper airways. *Current Opinion in Allergy Clinical Immunology, 14*(1): 7-12.

Ren, X., Hogaboam, C., Carpenter, A. & Colletti, L. (2003). Stem cell factor restores hepatocyte proliferation in IL-6 knockout mice following 70% hepatectomy. *Journal of Clinical Investigation, 112*(9): 1407-1418.

Rothenberg, M.E., Nusse, Y., Kalisky, T., Lee, J.J., Dalerba, P., Scheeren, F., Lobo, N., Kulkarni, S., Sim, S., Qian, D., Beachy, P.A. Pasricha, P.J., Quake, S.R. & Clarke, M.F. (2012). Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5(+) stem cells in mice. *Gastroenterology, 142*(5): 1195-1205.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M. & Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Developmental Biology, 1*: 4

Sun, L., Hui, A., Su, Q., Vortmeyer, A., Kotliarov, Y., Pastorino, S., Passaniti, S., Menon, J., Walling, J., Bailey, R., Rosenblum, M., Mikkelsen, T. & Fine, H. A. (2006). Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. *Cancer Cell, 9*(4): 287-300.

Sun, L., Lee, J. & Fine, H.A. (2004). Neuronally expressed stem cell factor induces neural stem cell migration to areas of brain injury. *Journal of Clinical Investigation, 113*(9): 1364-1374.

Toksoz, D., Zsebo, K.M., Smith, K.A., Hu, S., Brankow, D., Suggs, S.V., Martin, F.H. & Williams,

D.A. (1992). Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expression the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor. *PNAS, 89*(16): 7350-7354.

Williams, D.E., Eisenman, J., Baird, A., Rauch, C., Ness, K.V., March, C.J., Park, L.S., Martin, U., Mochizuki, D.Y., Boswell, H.S., Burgess, G.S., Cosman, D. & Stewart, D.L. (1990). Identification of a ligand for the c-kit proto-oncogne. Cell, 63: 167-174.

Zhao, L.R., Singhal, S., Duan, W.M., Mehta, J. & Kessler, J.A. (2007). Brain repair by hematopoeitic growth factors in a rat model of stroke. *Stroke, 38*(9): 2584-2591.

Zhou, Z., Wang, D., Wang, X.-J., and Roop, D.R. (2002). In utero activation of K5-CrePR1 induces gene deletion. *Genesis, 32*: 191–192.

Zsebo, K.M., Williams, D.A., Geissler, E.N., Broudy, V.C., Martin, F.H., Atkins, H.L., Hsu, R.Y., Birkett, N.C., Okino, K.H., Murdock, D.C., Jacobsen, F.W., Langley, K.E., Smith, K.A., Takeishi, T., Catanach, B.M., Galli, S.J. & Suggs, S.V. (1990). Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell, 63*: 213-224.

Chapter 4 | Stem Cell Factor is critical for cell fate choice in neurogenesis, but not for proliferation or cell survival

Background

Considering our findings that the presence of fully operational SCF signaling in the OE is critical for production of normal numbers of neurons during epithelial regeneration but not under healthy, non-injury conditions, we next sought to understand the precise nature of the deficit leading to reduced numbers of neurons in order to gain insight into the exact function of SCF and C-Kit expression. Essentially, SCF might be involved in proliferation, differentiation, survival, or migration. SCF might impact stem cells, committed progenitors, and/or supporting cell lineages. Given the large number of potential intracellular targets impacted by C-Kit as a receptor tyrosine kinase, some combination of these defects and cell types is not only possible but likely, making interpretation somewhat challenging. To assess the defect, we conducted experiments examining a number of different cell types and processes.

If the observed deficit in number of neurons regenerated during healing is due to defect in proliferation, we would expect to see changes in the numbers or kinetics of proliferative cells. Accordingly, we examined the number of proliferative cells at a time point just before neurons begin to be created in the regenerating epithelium. To address the possibility that the deficit stems from changes in cell survival in knock-out animals, we assayed the expression of a marker of cell death at an experimental time point preceding the emergence of the observed neuronal deficit.

Designing an experiment to definitively assess whether the $SCF^{-/-}$ defect might be best understood as a deficit in ability of cells to differentiate along the lineages they follow in wildtype animals is less straightforward than for proliferation or survival. We reasoned that, if SCF functions solely to impact fate differentiation, we would see differences in other cell fates that are quantitatively consistent with the changes in numbers of neurons presented in Chapter 3. If a cell that would normally divide to produce daughters in the neuronal lineage divides as normal, but does not produce neuronal or neural progenitor offspring, it must produce daughters from another lineage. In the OE, the possibilities for non-neuronal daughters include cells in the SUS lineage or cells in the microvillous lineage. In this chapter, we sought to detect and decipher changes in these other cell types that might shed light on the underlying function that was lost when SCF signaling was knocked-out.

The SUS and neuronal lineages have often been treated as a nearly binary choice for OE stem cells, so an examination of SUS fates in $SCF^{-/-}$ animals was an obvious choice. Microvillous cells factor into differentiation in the niche in a more complicated way. Microvillous cells are located in the most apical layer of the OE, with their cell bodies in line with or just above those of the SUS cells (blue colored cells in diagram in Figure 1). Using electron microscopy, Morrison & Costanzo (1990) identified two types of microvillous cells in the human based on morphology: flask and pear shaped. Both types of cells extend an apical tuft of cilia into the nasal lumen and a single long process towards the basal lamina, though it remained unclear where this process terminates and what sort of function it might serve. More recent studies have added a third morphological type to the mix (Hansen & Finger, 2008). Microvillous cells are generated by a population of Ascl1-positive basal cells (GBCs) (Yamaguchi, et al., 2014), a finding which was recently corroborated at the genetic level in Fletcher, et al. (2017). Given the findings presented in Chapter 2 which detailed our discovery that at least some, and possibly all, classes of microvillous cells express the C-Kit receptor, we included an analysis of microvillous cells in $SCF^{-/-}$ animals both to shed light on our neuronal defect, but also because the function of SCF signaling in this type of cell is interesting in its own right.

Methods

Mice used in experiments described in this chapter were the same lines as the knock-out and wild type control mice used in Chapter 3. Strains of transgenic mice (Krt5-CrePR [Zhou, et al., 02]), mice carrying cre-inducible SCF knock-out allele (SCF-lox-Exon1-lox-, Kitltm2.1Sjm, Jackson Laboratory) and mice carrying a cre-inducible YFP allele under the Rosa26 promoter (Srinivas, et al., 2001; Rosa26YFP) were bred on a C57/B6 background. Mice used in this chapter included the following:

Krt5-CrePR$^{Tg/+}$; Rosa26$^{YFP/+}$; SCF$^{lox/lox}$ (knock-out)
Krt5-CrePR$^{Tg/+}$; Rosa26$^{YFP/+}$; SCF$^{+/+}$ (wildtype)

Regeneration from neural injury was modeled by methimazole administration. Mice were co-housed with parents and littermates until until post-natal day 21, at which point methimazole was administered by intraperitineal injection (0.01mg methimazole/g bodyweight). Methimazole causes the death of virtually all cells in the epithelium aside from the HBCs within 24 hours of drug administration (Leung, et al., 2007). Mice used for 'injured' tissue collection were each sacrificed at one of three experimental time points after administration of methimazole (4 days post-injury, 7 days post-injury, or 14 days post-injury). 'Healthy' tissue was collected at post-natal day 21 from mice that were not administered methimazole.

Tissue collected from mice used in immunohistochemistry experiments was fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), decalcified for five days in 10% ethyleneaminetetracetic acid in PBS and equilibrated overnight in 30% sucrose. Frozen tissue was mounted in freezing medium (Triangle Biomedical Sciences), cryostat sectioned to 12um thick and slide mounted for microscopy.

For immunohistochemistry sections were permeabilized in 0.1% Triton X-100 in PBS and stained with antibodies diluted in donkey serum overnight. Fluorophore-conjugated secondary antibodies were used and detected as described in Duggan, et al., 2008. Secondary antibodies (Alexa 488, 594, 555, 568 and 647 (Invitrogen)) were used in conjunction with Hoechst 33342 nuclear stain for imaging. Slides were cover slipped with Vectashield (Hard Set, Vector Labs). Primary antibodies were used as follows:

chicken anti-GFP 1:500 (Abcam)
mouse anti-p63 1:100 (Santa Cruz Biotechnology)
goat anti-Sox2 1:100 (Santa Cruz Biotechnology)
goat anti-C-Kit 1:100 (R&D Systems)
rabbit anti-Ki67 1:250 (Abcam)
mouse anti-neuronal tubulin 1:250 (Tuj1; Abcam)
guinea pig anti-Ascl1 1:10,000 (gift from Jane Johnson)
rabbit anti-caspase3 1:250 (Cell Signaling Technology)

RNA in situ stains for Cochlin RNA labeling of microvillous cells were done using dioxygenin-conjugated probes and detected with a secondary antibody to dioxygenin and BCIP/NBT (as described in Duggan, et al., 2008). The Cochlin-sensitive probe was generated using primers (5'-CACCCACCTTCAGGTAAAAGAC -3' and 5'- CATTAGTGCCACTTTCCCAACA -3').

Images were acquired using epifluorescence and confocal microscopes. Image assembly and analysis

was done in ImageJ (National Institutes of Health) and Photoshop (Adobe). Quantification was done using the Cell Counter ImageJ plugin (Kurt de Vos). Quantification of numbers of cells positive for various markers was done for approximately equal lengths of epithelium in each of 3 or 4 knockout and 3 or 4 control animals for each condition. Quantification was done in the olfactory epithelium only (not respiratory/non-sensory, which lines the ventral portion of the nasal cavity). Quantification was restricted to septal regions for consistency.

All RNA-sequencing data analyzed here were from Fletcher, et al., 2017. Briefly: mouse OE was dissociated (detailed previously in Fletcher, et al., 2011), FACS sorted to enrich for cells of interest, captured (Fluidigm C1) from animals before injury (healthy) and at various time points following injury (24h, 48h, etc.), and sequencing libraries generated (Nextera [library synthesis], Beckman Coulter [Library size selection], Illumina [library sequencing]). All RNA-sequencing data used in this paper are freely available at the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). For expression analyses and comparisons, cells were aligned, filtered, normalized and clustered as described in Fletcher, et al., 2017.

For the isoform analysis, sequencing reads were aligned using Bowtie (1.2.0, Johns Hopkins University). Reads mapped to the chromosomes bearing C-Kit and SCF (Ch5 and Ch10 respectively) were isolated and used for further analysis. Stringtie (1.3.3, Johns Hopkins University) was used to assemble transcripts and quantify reads with the C-Kit and SCF chromosomal loci (Ch5: 75,574,916-75,656,722 and Ch10: 100,015,630-100,100,416 respectively in the GRCm38.p5 c57/Bl6 reference genome [National Center Biotechnology Information]). Assemblies were visualized in Integrated Genomics Viewer (2.3.92, Broad Institute) aligned to the GRCm38.p5 c57/Bl6 reference genome, and splice variants were assessed using the Variant Call Format (VCF 4.0, European Bioinformatics Institute). Analysis pipelining and scripting was done in a Jupyter Notebook (IPython (Perez and Granger, 07)) running Python (3.6.1, Python Software Foundation, https://www.python.org/) and R (3.4.0, R Project for Statistical Computing; rpy2, 2.8.5, Laurent Gauthier).

Results

4.1 | Ki67 and Cas3

A number of marker genes give us insight into the functional state of cells. Here we rely on two, Ki67, a nuclear protein which is expressed in actively proliferating cells, and Caspase 3, an enzyme which is expressed during the course of apoptosis, because C-Kit is known to be involved in both proliferation and apoptosis in other niches.

To determine whether differences in neural birth were due to a decrease in the number of cycling cells, we quantified the number of Ki67+ cells at an early time point (4dpi) that just precedes the birth of neurons (and the appearance of the observed defect in neural regeneration) during recovery from injury. We reasoned that, if SCF signaling serves to promote or permit progenitor cells entering the cell cycle, then we would see a decrease in the number of proliferative cells at just before the time that newborn immature neurons start to be born. We observed no such deficit in Ki67+ cells that could account for the overall decrease in neuronal regeneration in these transgenic mice. $SCF^{-/-}$ mice had an average of 3.220 Ki67+ cells per 100um (S.E. = 0.269), while wild-type control mice had an average of 2.809 Ki67+ cells per 100um (S.E. = 0.424) ($p = 0.281$, n.s.) (Figure 9), suggesting that the deficiency in neuronal production during recovery after injury observed in $SCF^{-/-}$ mice is unlikely to be best explained as a deficit of proliferation.

We next turned to whether this deficit might be accounted for by an increase in cell death. Assessing

Cas3 expression in the recovering OE, revealed that little Cas3 was expressed in either wildtype or $SCF^{-/-}$ animals (data not shown). Insufficient numbers of cells marked by our antibody made it difficult to achieve statistics with a satisfactory level of statistical error, however, it is worth noting that there was no appreciable cell death occurring in either our knockouts or controls, making it somewhat improbable that the neuronal deficit is due to cell death. This analysis would require more extensive future investigation before a role for C-Kit in cell death in the OE could be ruled out entirely.

4.2 | Lack of SCF signal after injury leads to reduced numbers of C-Kit+ cells starting early in recovery.

Our finding that neither deficits in progenitor cycling nor increases in the rate of cell death in animals lacking SCF could account for the decrease in number of newborn neurons in the regenerating epithelium. We next asked whether there were simply fewer neural progenitors born of knock-out HBCs. We would likely have seen a decrease in the number of cycling (Ki67+) progenitors and no change in the relative amount of cell death. Because we observed no change in the number of cycling progenitors in our 4dpi ki67 quantification, we figured the observed decrease in numbers of neurons in the knock-out animals was not due to a decrease in the number of progenitors in the OE. To verify this assumption, we quantified the fraction of C-kit+ cells present at experimental time points throughout regeneration. In SCF deficient mice, there were less C-Kit-positive cells at 4 and 14 days of regeneration (Figures 9 and 11).

If the C-Kit receptor were expressed as cells moved from HBC-like states to GBC-like states during recovery from injury, and SCF provided a confirmation signal that the cell should continue down that neural progenitor fate pathway, rather than turning off C-Kit expression and producing supporting SUS cells, then we would expect to see the results we observed. While this finding does not delineate between our remaining two explanations (SCF mediates migration and/or differentiation), it does raise an interesting issue. In Chapter 1, we suggested that C-Kit is likely expressed by "late" GBCs due to the presence of a number of C-Kit+ cells located midway between the GBC and SUS layers of the epithelium. However, this is not consistent with the hypothesis that SCF mediates passage from the HBC to the progenitor stage – if this were true, we would expect C-Kit expression in "early" GBCs. There are several possible explanations for this apparent inconsistency. Chief among them is that C-Kit might mediate multiple processes and stages in the OE.

4.3 | Lack of SCF signal after injury leads to increased numbers of SUS cells and decreased numbers of microvillous cells

Broadly speaking the processes governing stem cells can be divided into three categories: molecules involved in proliferation (that is, the entrance of cells into and progression of cells through the cell cycle), self-renewal (where a cell divides to create at least one cell similar to itself) and differentiation (where a cell divides to create at least one daughter cell of more limited fate potential than the parent cell). Given that SCF signaling did not appear to be involved primarily in processes subserving proliferation during the regeneration of neurons, we wondered if the key role of the pathway might be in determining cell fate decisions. In the OE, during regeneration, there are multiple differentiated cell types that must be regenerated, including neurons, SUS cells, microvillous cells and cells of the Bowman's glands. Lineage tracing of C-Kit cells following methimazole injury shows that these cells produce primarily neurons, but are truly multipotent, in that they are also competent to produce SUS, microvillous and BG cells (Goss, et al., 2016). We reasoned that, if the functional role of SCF is to govern the fate decisions made by C-Kit+ progenitors during recovery from injury, then we might see a corresponding increase in the number of one or a few other cell types in our knock-out model.

For confirmation that SCF is critical to maintaining the fate of early neuronal precursors during recovery from injury, we examined whether other cell types linked to this process were affected in ways consistent with this hypothesis. Specifically, if SCF functions to maintain neural progenitor identity, we expected to see that, in the absence of SCF, the production of SUS cells would be favored secondary to deficits in neural birth. Secondly, based on recent work suggesting the neurons and microvillous cells share a common progenitor during recovery from injury (Fletcher, et al., 2017), we expected that we might see a decrease in the number of microvillous cells in knock-out mice accompanying defects in neural birth, and, assuming that microvillous-fated progenitors and progenitors fated to produce neurons are fundamentally similar, at least through a SUS versus neuron or microvillous fate decision.

We quantified the number of SUS and microvillous cells in SCF knock-out mice at a late experimental time point during regeneration, using apical Sox2+ cells to count SUS and Cochlin+ cells to count microvillous cells (Figures 13 and 14). Looking at 14dpi rather than earlier, allows us to ensure that we are quantifying all cells of these types. It is possible that Cochlin, despite being a very clear marker, and excellent for quantification does not mark all microvillous cells. We predicted that, if SCF governs fate, there might be a decrease in the number of microvillous cells proportional to the decrease in neurons, since many microvillous cells express C-kit in the steady-state epithelium and after recovery from injury. Our quantification showed that there was a decrease in the number of microvillous cells following regeneration from injury. These cells likely stem from C-Kit+ progenitors during recovery from injury much as neurons do and share a common progenitor with newborn neurons (Fletcher, et al., 2017). A deficit in regulation of the fate of this common progenitor might account for this phenotype (reduction in birth of both neurons and microvillous cells).

On the contrary, we observed an increase in the number of SUS cells regenerated in the SCF knock-out epithelium (Figure 14). This difference was small, but extremely reliable. We wondered if the number of neurons lost would be matched one-for-one by the number of SUS cells gained per unit length of tissue. However, this was not the case. While interpretation of this result remains speculative, it does give us some insight into the point in the progression from stem cell (HBC) to fully differentiated progeny that SCF signaling is involved in regulating. During recovery from injury, some HBCs are thought to differentiate, essentially, directly into SUS cells, while others produce intermediate progenitors which proliferate and form neurons (Fletcher, et al., 2017). If SCF signaling is pivotal in supporting/allowing the birth of intermediate progenitors, we would expect to see HBCs which might have produced a progenitor that would have proliferated to create multiple neurons, instead divide to create a smaller number of SUS cells, since no amplifying progenitor stage is involved in this lineage pathway. Indeed, we find that the increase in SUS cells per distance quantified is commensurate with the decrease in number of C-Kit+ progenitor cells, rather than with the number of their neuronal offspring. This suggests that SCF is critical to the conversion of stem cell GBCs into transit amplifying neuronal progenitors, and that, in the absence of SCF, these early GBCs may differentiate by default to sustentacular fates.

Conclusions and Discussion

The function of SCF signaling in the OE does not appear to relate straight-forwardly to either cell proliferation or to cell death, as we saw no decrease in the number of Ki67+ cells and no decrease in the number of Cas3+ cells in $SCF^{-/-}$ animals. Interestingly, in light of these findings, we observed a decrease in the number of C-Kit+ cells in the same knockouts, suggesting that receiving SCF signal perpetuates the expression of C-Kit in C-Kit+ cells.

We also observed that knock out of SCF led to the presence of a decreased number of microvillous cells, with a concurrent increase in the number of SUS cells. These changes, together with the observations presented in earlier chapters, are consistent with the interpretation that SCF/C-Kit signaling in the OE plays a role in cell fate choices during regeneration from injury. Considering the cell lineages which are affected are neuronal, microvillous and SUS, dovetails with our earliest findings that C-Kit is expressed in GBCs, while SCF is expressed in cells spatially poised to drive GBC fate decisions.

One of the most interesting questions we can hope to answer with our data set is what subset of GBCs C-Kit is expressed in, since knowing this would allow us to better understand the functional import of SCF in terms of the general programs that GBCs are running at that stage in the OE lineage. In Chapter 2, we presented single-cell sequencing data which showed that, among common GBC marker genes, C-Kit expression correlated most strongly with expression of Ki67 (a marker of proliferation) and Ascl1 (a marker associated with neuronal commitment among GBCs) – two markers that suggest C-Kit expression is strongest in "late" GBCs, a group of cells which are predominantly a transit amplifying progenitor pool for neuronal fates in the OE.

Yet, here we see that a reduction in SCF function leads to changes in more than just neuronal fates. It is not surprising that our main effect, a reduction in the number of neurons in the regenerating OE is accompanied by a reduction in the number of microvillous cells, given the recent work of Fletcher, et al. (2017) demonstrating that the microvillous lineage branches off from neuronally committed GBCs. Since we see the same effect in these cells types, C-Kit likely has its effects before this branch point. The really interesting finding, then, is that we observe the opposite effect among SUS cells, which is suggestive that the C-Kit mediated effects we see here are occurring in even "earlier" GBCs – in cells which are still truly stem cells and have the potential to commit to either neuronal or supporting cell lineages.

These anatomical and functional findings are not necessarily at odds, as C-Kit need not have its maximal expression levels at the time that it exerts its most profound functional impact. SCF signaling is implicated in a huge number of different cellular functions in the various tissues where it has been studied. In the OE it seems likely that SCF is pivotal for stem cell GBCs in making the fate choice between a neuronal or a supporting lineage.

When injury occurs in the OE, neurons are not produced immediately. First an initial flurry of relatively undifferentiated proliferation and production of supporting cells occurs. Then, once a sufficient foundation is built, neurons are produced. A common-sense model of the role of C-Kit emerges where, upon injury, stem GBCs are re-established in an environment where there is a relatively low concentration of SCF, since there are not yet a layer of SUS cells above them. In this milieu, stem GBCs differentiate toward SUS fates. As a sufficient number of SUS cells are formed, the amount of SCF reaching the stem GBCs is increased, which will start to drive these cells, via the C-Kit receptor, toward neuronal/microvillous fates.

One finding that is not well explained by this model is the expression of SCF by HBCs, though the finding is not in consistent with the model. It may be that SCF is also performing its well-known role in recruiting immune response to tissue injury, and that HBCs express SCF for this reason, however this is not within the scope of our present work and has already been well documented in many tissues.

Taken together, the results presented in this manuscript represent an advance on what was previously

known both about recovery from injury in the OE and about the presence and functional role of SCF signaling in this niche.

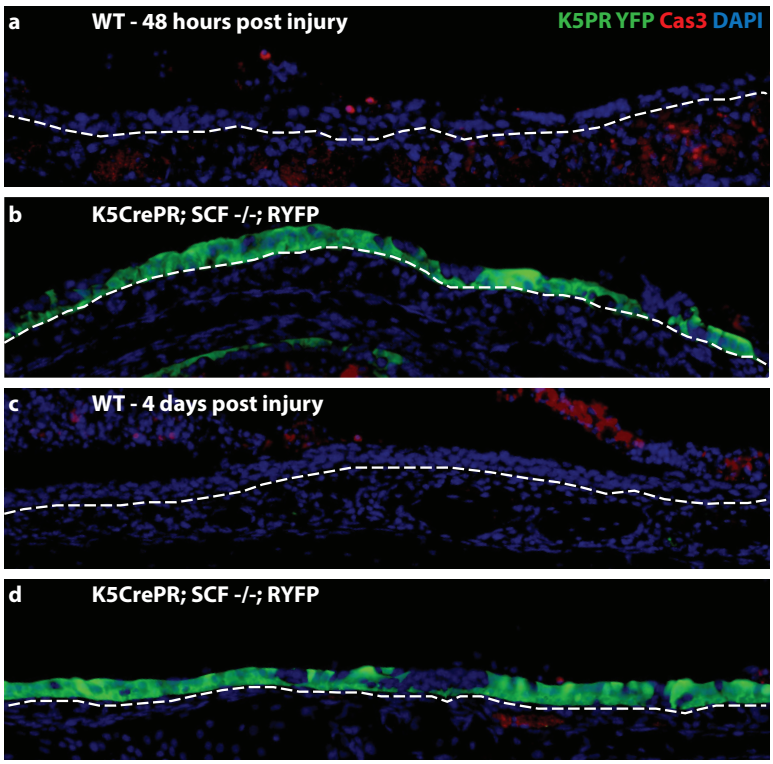


Figure 12 | Decreased numbers of C-Kit+ cells in SCF knockouts cannot be accounted for by increased cell death during regeneration in the absence of SCF. **a, b,** At 48 hours post-injury, there are very few Caspase 3+ (a marker for cell death) cells present in the WT or knockout epithelia. **c, d,** Similarly, there is very little apparent cell death in the recovering epithelium at 4 days post-injury, indicating that the decrease in numbers of C-Kit+ cells at this time point cannot be accounted for by the death of C-Kit+ cells in the absence of SCF ligand.

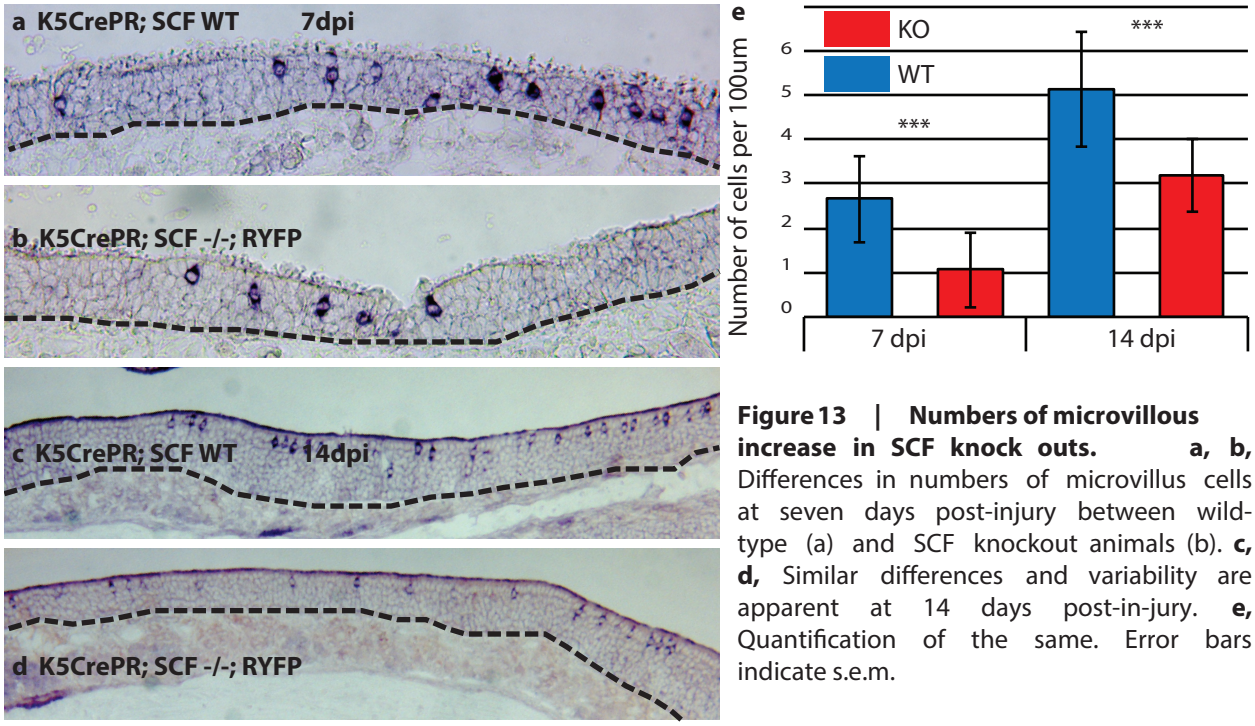
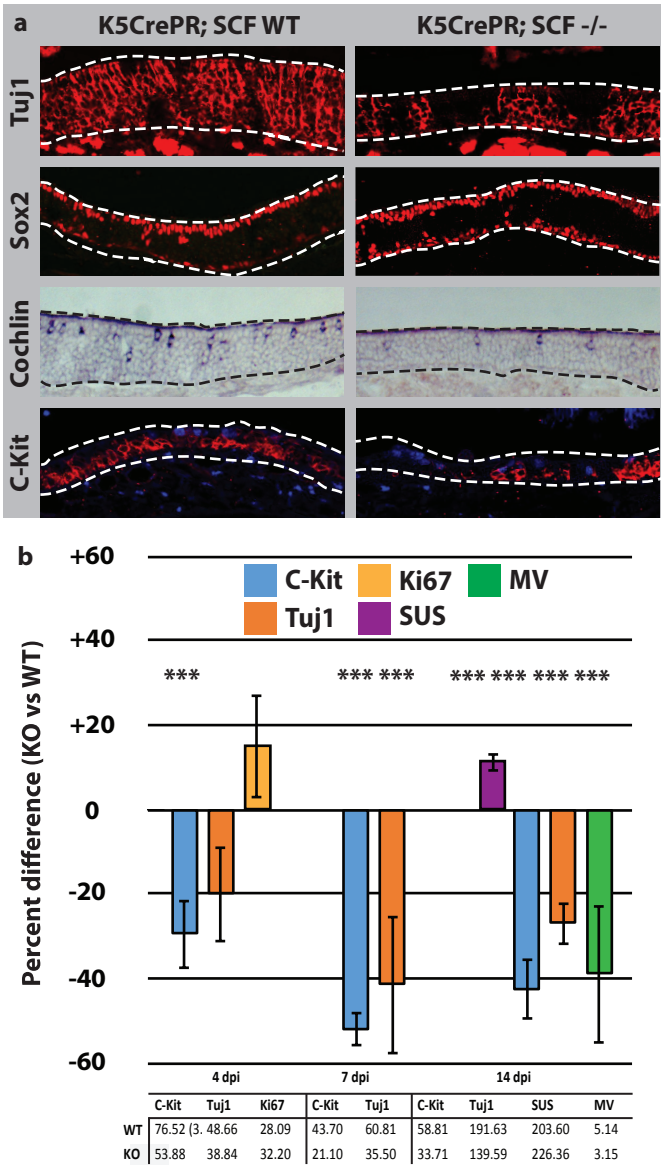


Figure 13 | Numbers of microvillous increase in SCF knock outs. **a, b,** Differences in numbers of microvillus cells at seven days post-injury between wild-type (a) and SCF knockout animals (b). **c, d,** Similar differences and variability are apparent at 14 days post-in-jury. **e,** Quantification of the same. Error bars indicate s.e.m.



	C-Kit	Tuj1	Ki67	C-Kit	Tuj1	C-Kit	Tuj1	SUS	MV
WT	76.52 (3.	48.66	28.09	43.70	60.81	58.81	191.63	203.60	5.14
KO	53.88	38.84	32.20	21.10	35.50	33.71	139.59	226.36	3.15

Figure 14 | Proportions of neural and non-neuronal cell types shift in SCF knock-out animals during recovery from neurotoxic injury.

a, Example images showing OE from wild-type (left) compared to SCF knock-out (right) animals. Tuj1, shown at 14 days post-injury, stains show a decreased number of neurons. Sox2, shown at 14 days post-injury, stains show an increased number of sustentacular cells. Cochlin in situs, shown at 14 days post-injury, show a decreased number of microvillus cells. C-Kit stains, shown at 7 days post-injury, show a decreased number of GBCs. **b,** Quantification of percent change in cell number at 4, 7, and 14 days post-injury. Numbers represent the percent increase or decrease in observed number of cells in knockout animals, compared to wildtype controls.

References

Baumann, U., Crosby, H.A., Ramani, P., Kelly, D.A. & Strain, A.J. (1999). Expression of the Stem Cell Factor Receptor c-kit in Normal and Diseased Pediatric Liver: Identification of a Human Hepatic Progenitor Cell. *Hepatology, 30*(1): 112-117.

Bocchi, R., Egervari, K., Carol-Perdiguer, L., Viale, B., Quairiaux, C., De Roo, M., Boitard, M., Oskouie, S., Salmon, P. & Kiss, J. K. (2017). Perturbed Wnt signaling leads to neuronal migration delay, altered interhemispheric connections and impaired social behavior. *Nature Communications, 1158*: doi:10.1038/s41467-017-01046-w.

Carr, V.M., Farbman, A.I., Colletti, L.M. & Morgan, J.I. (1991). Identification of a new non-neuronal cell type in rat olfactory epithelium. *Neuroscience, 45*: 433-449.

Chandrakesan, P., May, R., Weygant, N., Qu, D., Berry, W.L., Sureban, S.M., Ali, N., Rao, C., Huycke, M., Bronze, M.S. & Houchen, C.M. (2016). Intestinal Tuft Cells Regulate the ATM Mediated DNA Damage Response Via Dclk1 Dependent Mechanism for Crypt Restitution Following Radiation Injury. *Scientific Reports, 6*: 37667.

Chen, S.Q. & Xiong, A.Q. (2002). The progress and implication of stem cell factor. *Basic Medical Sciences and Clinics, 22*(5): 385-390.

Christensen J. L, Wright D. E, Wagers A. J & Weissman I. L. (2004). Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biology, 2*: E75.

Duggan, C.D., DeMaria, S., Baudhuin, A., Stafford, D. & Ngai, J.N. (2008). FoxG1 is required for development of the vertebrate olfactory system. *Journal of Neuroscience, 28*(20): 5229-5239.

Elsaesser, R. & Paysan, J. (2007). The sense of smell, its signaling pathways, and the dichotomy of cilia and microvilli in olfactory sensory cells. *BioMed Central Neuroscience, 8*: doi:10.1186/1471-2202-8-S3-S1.

Fletcher, R.B., Das, D., Gadye, L., Street, K.N., Baudhuin, A., Wagner, A., Cole, M.B., Flores, Q., Choi, Y.G., Yosef, N., Purdom, E., Dudoit, S., Risso, D. & Ngai, J. (2017). Deconstructing olfactory stem cell trajectories at single-cell resolution. *Cell Stem Cell, 20*(6): 817-830.

Fletcher, R.B., Prasol, M.S., Estrada, J., Baudhuin, A., Vranizan, K., Choi, Y.G. & Ngai, J. (2011). p63 regulates olfactory stem cell self-renewal and differentiation. *Neuron, 72*: 748-759.

Gayde, L., Das, D., Sanchez, M.A., Street, K., Baudhuin, A., Wagner, A., Cole, M.B., Choi, Y.G. Yosef, N., Purdom, E., Dudoit, S., Risso, D., Ngai, J. & Fletcher, R.B. (2017). Injury activates transient olfactory stem cell states with diverse lineage capacities. *Cell Stem Cell, 21*(6): 775-790.

Goldstein, B.J., Goss, G.M., Hatzistergos, K.E., Rangel, E.B., Seidler, B., Saur, D. & Hare, J.M. (2015). Adult c-kit+ progenitor cells are necessary for maintenance and regeneration of olfactory neurons. *Journal of Comparative Neurology,* doi: https://doi.org/10.1002/cne.23653.

Goss, G.M., Chaudhari, N., Hare, J.M., Nwojo, R., Seidler, B., Saur, D. & Goldstein, B.J. (2016).

Differentiation potential of individual olfactory c-kit+ progenitors determined via multicolor lineage tracing. Developmental Neurobiology, 76(3): 241-251.

Hansen, A. & Finger, T. (2008). Is TrpM5 a reliable marker for chemosensory cells? Multiple types of microvillous cells in the main olfactory epithelium of mice. *BioMed Central Neuroscience, 9*(115): doi.org/10.1186/1471-2202-9-115.

Hegg, C.C., Jia, C., Chick, W.S., Restrepo, D. & Hansen, A. (2010). Microvillous cells expressing IP3 receptor type 3 in the olfactory epithelium of mice. *European Journal of Neuroscience, 32*: 1632–1645.

Hu, B. & Colletti, L.M. (2008). Stem cell factor and c-kit are involved in hepatic recovery after acetaminophen-induced liver injury in mice. *American Journal of Physiology, 295*(1): 45-53.

Ishizuka, K., Kamiya, A., Oh, E. C., Kanki, H., Seshadri, S., Robinson, J. F., Murdoch, H., Dunlop, A. J., Kubo, K., Furukori, K., Huang, B., Zeledon, M., Hayashi-Takagi, A., Okano, H., Nakajima, K., Houslay, M. D., Katsanis, N. & Sawa, A. (2011). DISC1-dependent switch from progenitor proliferation to migration in the developing cortex. *Nature, 473*: 92-96.

Jia, C., Hayoz, S., Hutch, C.R., Iqbal, T.R., Pooley, A.E., Hegg, C.C. (2013). An IP3R3- and NPY-expressing microvillous cell mediates tissue homeostasis and regeneration in the mouse olfactory epithelium. *PLoS One,8*: doi:10.1371/journal.pone.0058668pmid:23516531

Kim, C. H. & Broxmeyer, H. E. (1998). In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor, and the bone marrow environment. *Blood, 91*: 100-110.

Krams, M., Parwaresch, R., Sipos, B., Heidorn, K., Harms, D. & Rudolph, P. (2004). Expression of the C-Kit receptor characterizes a subset of neuroblastomas with favorable prognosis. *Oncogene, 23:* P588-595.

Krishnamoorthy, N., Oriss, T.B., Paglia, M., Fei, M., Yarlagadda, M., Vanhaesebroeck, B., Ray, A. & Ray, P. (2008). Activation of c-Kit in dendritic cells regulates T helper cell differentiation and allergic asthma. *Nature Medicine, 14*: 565-573.

Lammie, A., Drobnjak, M., Gerald, W., Saad, A., Cote, R. & Cordon-Cardo, C. (1994). Expression of c-kit and kit ligand protein in normal human tissues. *Journal of Histochemistry and Cytochemistry, 42*(11): 1417-1425.

Lemons, K., Fu, Z., Aoudé, I., Ogura, T., Sun, J., Chang, J., Mbonu, K., Matsumoto, I., Arakawaand, H. & Lin, W. (2017). Lack of Trpm5-expressing microvillous cells in mouse main olfactory epithelium leads to impaired odor-evoked responses and olfactory-guided behavior in a challenging chemical environment. *Eneuro, 4*(3): doi.org/10.1523/ENEURO.0135-17.2017

Leung, C.T., Coulombe, P.A. & Reed, R.R. (2007). Contribution of olfactory neural stem cells to tissue maintenance and regeneration. *Nature Neuroscience, 10*: 720-726.

Lev, S., Blechman, J.M., Givol, D. & Yarden, Y. (1994). Steel factor and c-kit protooncogene: genetic lessons in signal transduction. *Critical Reviews in Oncogenomics, 5*: 141-168.

Liman E.R. (2007) TRPM5 and Taste Transduction. In: Flockerzi V., Nilius B. (eds) Transient Receptor Potential (TRP) Channels. *Handbook of Experimental Pharmacology, vol 179*. Springer, Berlin, Heidelberg.

Lin, W., Margolskee, R., Donnert, G., Hell, S.W., Restrepo, D. (2007). Olfactory neurons expressing transient receptor potential channel M5 (TRPM5) are involved in sensing semiochemicals. *Proceedings of the National Academy of the Sciences, 104*: 2471–2476.

Mansuroglu, T., Baumhoer, D., Dudas, J., Haller, F., Cameron, S., Lorf, T., Fuzesi, L. & Ramadori, G. (2009). Expression of stem cell factor receptor c-kit in human nontumoral and tumoral hepatic cells. *European Journal of Gastroenterology and Hepatology, 21*(10): 1206-1211.

Matsui, Y., Zsebo, K. M. & Hogan, B. L. (1990). Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. *Nature, 347*: 667-669.

Ming, G. & Song, H. (2011). Adult neurogenesis in the mammalian brain: Significant answers to significant questions. *Neuron, 70*(4): 687-702.

Morrison, E. E. & Costanzo, R. M. (1990). Morphology of the human olfactory epithelium. *Journal of Comparative Neurology, 297*: 1–13.

Oriss, T. B., Krishnamoorthy, N., Ray, P. & Ray, A. (2014). Dendritic cell c-kit signaling and adaptive immunity: implications for the upper airways. *Current Opinion in Allergy Clinical Immunology, 14*(1): 7-12.

Perez, F. & Granger, B.E. (2007). Python for scientific computing. *Computing in science and engineering*.

Ren, X., Hogaboam, C., Carpenter, A. & Colletti, L. (2003). Stem cell factor restores hepatocyte proliferation in IL-6 knockout mice following 70% hepatectomy. *Journal of Clinical Investigation, 112*(9): 1407-1418.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M. & Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Developmental Biology, 1*: 4.

Yamaguchi, T., Yamashita, J., Ohmoto, M., Aoude, I., Ogura, T., Luo, W., Bachmanov, A.A., Lin, W., Matsumoto, I. & Hirota, J. (2014). Skn-1a/Pou2f3 is required for the generation of Trpm5-expressing microvillous cells in the mouse main olfactory epithelium. *BioMed Central Neuroscience, 15*(13): doi:10.1186/1471-2202-15-13pmid:24428937.

Yamashita, J., Ohmoto, M., Yamaguchi, T., Matsumoto, I. & Hirota, J. (2017). Skn-1a/Pou2f3 functions as a master regulator to generate Trpm5-expressing chemosensory cells in mice. *PLoS One, 12*(12): doi.org/10.1371/journal.pone.0189340.

Zhou, Z., Wang, D., Wang, X.-J., and Roop, D.R. (2002). In utero activation of K5-CrePR1 induces gene deletion. *Genesis, 32*: 191–192.

Chapter 5 | Current and future directions

5.1 | Creation of an in vitro model of the OE, optimization of assay tools for use in this system and assessment of degree to which this system recapitulates the OE in vivo.

Independent of the C-Kit project, as a collaborative effort, our lab has developed an in vitro model of the OE. We had two separate aims. First, to select for a 'self-sustaining' cell in the OE that we could plate and then allow to propagate indefinitely, allowing for passaging, controlled generation of different OE cell types and more high-throughput experiments. We were not successful in this aim. These efforts were detailed in the thesis work of my labmate Michael Sanchez, so I will not discuss them further here. Second, we hoped to design a protocol for growing primary cultures of OE cells - essentially, take cells straight from the mouse and plate them for use in pharmacological manipulations that would be more involved to do in vivo. We've had some success with this, and refinement is ongoing. I've been more involved and more interested in the latter pursuit, since I like the idea of recapitulating in vivo niche cell types and environment as much as possible for mechanistic experiments, something primary culture seems ideally suited for.

Building off the protocols I developed early in my degree while attempting to culture quiescent HBC stem cells for viral gene editing conversion to induced pluripotent stem cells, Michael Sanchez, Russell Fletcher and I have dedicated significant time to figuring out culture conditions, media and substrate recipes, trying to culture different subsets of cells and testing and optimizing the use of a large number of antibodies on these cultured cells. Currently, our standard experimental setup is as follows:

1. Injure the OE, ablating all cells except HBCs, which become activated to proliferate: Intraperitoneal injection of postnatal day 21 mice with 50ug methimazole/g body weight. 24 waiting period for cell death to take place.
2. Isolate 'activated' HBCs: FACS for HBCs using ICAM-PE antibody.
3. Plate HBCs and allow to proliferate: Plate FACS-isolated cells at 50K cells per cm2 on fibronectin coated slides in stem cell media (NeuroCult system, plus EGF, FGF, Heparin, L-Glutamine, Penicillin/Streptomycin).
4. Fix cells and use for immunochemistry: I've collected timepoints ranging from 48 hours to two weeks.

Using HBCs that have been activated by injury is critical. We previously attempted to plate quiescent HBCs and Sox2+ cells and neither population proliferated in vitro. Subjecting the HBCs to injury prior to collecting and plating them, guarantees that we are plating a proliferative population. When plated, these cells proliferate until quite confluent, at which point contact death starts to occur. The choice of media and additives was somewhat arbitrary and based solely on culture work that M. Sanchez and I had done in other labs that we've worked in. Since HBCs are K5+, R. Fletcher and I have conducted several runs comparing a Keratinocyte-specific media to the neural stem cell cocktail that we normally use, and, while it does seem to shift the balance of cell types in the culture (based on morphology alone), it's not clear that either more faithfully recapitulates the niche environment in vivo. Importantly, at this point, results vary widely from run to run, even when conditions are ostensibly held constant.

C-Kit/SCF signaling provides a good test case for our new in vitro system. Using SCFeGFP knock-in reporter mice it is clear that there are a large number of SCF+ cells in our primary cultures (Figure 15). It's unclear if these cells are more like HBCs (the cells we plate initially) or like SUS cells. We do not yet have a reliable antibody marker for SUS cells in vitro, and both cell types express Sox2, which

would be the next most logical marker. However, we know from optimizations runs of the p63 antibody, that there are relatively fewer p63+ cells in our typical cultures than there are SCF eGFP+ cells, so the green clusters seen in Figure 10 are likely to contain at least some SUS-like cells.

At this point, we think that C-Kit+ cells are likely to be neuronally committed progenitors, at least in the healthy OE and during late regeneration of the injured OE. Knowing that C-Kit and SCF are expressed very early during recovery from injury, we were interested to know about the potency of these cells at this stage. Figure 16 shows a close-up image of a culture that was seeded with cells FAC sorted using a C-Kit-APC+ antibody from 24 hour post-injury animals. C-Kit+ cells from early points in injury appear to be multipotent, at least in vitro. However, given that it is often the case that cells express different genes or are differentially potent in vitro than they are in vivo, this qualitative data is merely suggestive. Future work in this vein might include, with a C-Kit CreER driver mouse, lineage tracing C-Kit+ cells during recovery from injury in vivo and establishing a more physiologically relevant hierarchy.

Attempts to pharmacologically rescue cultured SCF knockout cells by the addition of SCF protein have yielded mixed results. In an effort to establish whether the minimal phenotype seen in vivo might be worth pursuing, we cultured injury activated HBCs from SCF homozygous knockout animals according to the protocol described above. Wells of these knock out cultures were examined and compared with wells where SCF protein had been added to the media.

These experiments have yielded mixed, mostly disappointing results. At this point, we have done seven repetitions of this experiment, and have only seen a visually striking phenotype one time (examples shown in Figure 17). Limited quantification of Tuj1, Ki67 and Sox2 antibodies suggests that there is little to no difference between the knockout cells with and without added protein to 'rescue' them. While the conditions have varied between runs – in an effort to find conditions which work – and this variation might have led us to overlook a phenotype, it is clear by now that this line of experimentation is not going to give results that are strong enough to bolster what we've seen in vivo by any significant margin.

At this point, our goal is to uncover any effects that are profound enough to build a scientific story around. Given that these culture experiments require genetically rare transgenic animals and have shown variable results, we have decided to set them aside in favor of an experimental line using pharmacological tests in cells from wild type animals to assay C-Kit and SCF in vitro.

5.2 | Pharmacological inhibition of C-Kit receptor or over-expression of SCF protein in vitro have also yielded mixed results but constitute a promising future direction. These experiments are ongoing.

Currently, we're using wild type mouse activated HBCs to seed plates (Figure 18). One third of plates have added SCF protein, essentially an over-expression condition, because these cells have SCF functioning perfectly intact. One third of wells have added ACK2 antibody, which is a function-blocking antibody that binds to the C-Kit receptor, creating a knockdown condition. The last third of wells receive no treatment.

The benefit to this design is that availability of animals will not be rate limiting, so this will be a much more high throughput way of looking at C-Kit signalling in the OE. This will allow us to test out a greater variety of experimental manipulations, as well as have the appropriate number of replicates to draw quantitative conclusions from. Of note, the over-expression condition is of limited relevance.

It really just serves as a foil to any effect that we find in the knockdown – we would hope to find the opposite effect in the over-expression wells.

So far, we've tried this experimental design twice, without any profound changes. Before continuing, we will do a run using various concentrations of SCF and ACK2 to try to see if we are using too much or too little to see an effect. While previous experiments have been done using concentrations in keeping with literature in a variety of tissues, we are open to raising the concentrations, so long as we stay within physiologically relevant bounds.

5.3 | Generation, via CRISPR/Cas9 gene editing, of a 'better' knockout mouse, designed to knock out function of the C-Kit receptor

Our current knockout mouse arrests production of the ligand, SCF. This is not ideal. Even if our Cre driver mouse were 100% effective in knocking out SCF in all HBCs, there are other sources of SCF in the body, and the protein is known to act at very long distances. Also, our knockout relies on injury to recover the epithelium from HBCs which have SCF knocked out in them. But we know that our injury model is also imperfect. There are other cells than HBCs that are occasionally spared during injury and which contribute to the recovery of the epithelium. Even if there are very few cells that do not have the knockout allele, they may be able to produce sufficient quantities of SCF to drastically attenuate any phenotype we may see.

Our lab in involved in the development of a 'pipeline' for the creation of transgenic mice using the CRISPR/Cas9 system to genetically alter embryonic stem cells that are then used for the creation of mice (Figure 19). At present, this pipeline has been carried through, in our lab, to completion (birth of chimeric mice) in at least one instance, so it is feasible for us to simply create our own C-Kit inducible knockout mouse (since such a mouse is not available commercially) and redo the bulk of this project with that mouse.

There are a variety of different Cre driver mice that we might use to induce knockout of the C-Kit gene in our floxed mouse. Continuing to use the K5PR driver and the injury-recovery based model of establishing a knockout epithelium is non-optimal for reasons similar to our current mouse. Our lab also has a Sox2CreER driver mouse. There are two problems with this mouse. One, C-Kit expression does not co-express with Sox2, so we would still be reliant on the injury-recovery style of accomplishing a total knockout. Two, this mouse requires heavy induction with tamoxifen to get a reliably high level of Cre induction, which results in very sick mice. Since I expect the C-Kit knockout under the rather broad Sox2 driver may take a rather large toll on the health of the animal on its own, the necessity of multiply dosing with tamoxifen will likely result in large number of unhealthy or dead mice.

We are going through the process of making the C-Kit floxed mouse, and so it may be the most wise option, experimentally, for us to also make a C-Kit CreER driver mouse by the same process and at the same time. This mouse would inducibly knock out C-Kit receptor in C-Kit expressing cells. Clearly, this is the cleanest choice of driver experimentally and the most likely to get us the results we want, but making a mouse represents a significant commitment of resources and time, so we've not yet determined if this is the best path, overall, to take.

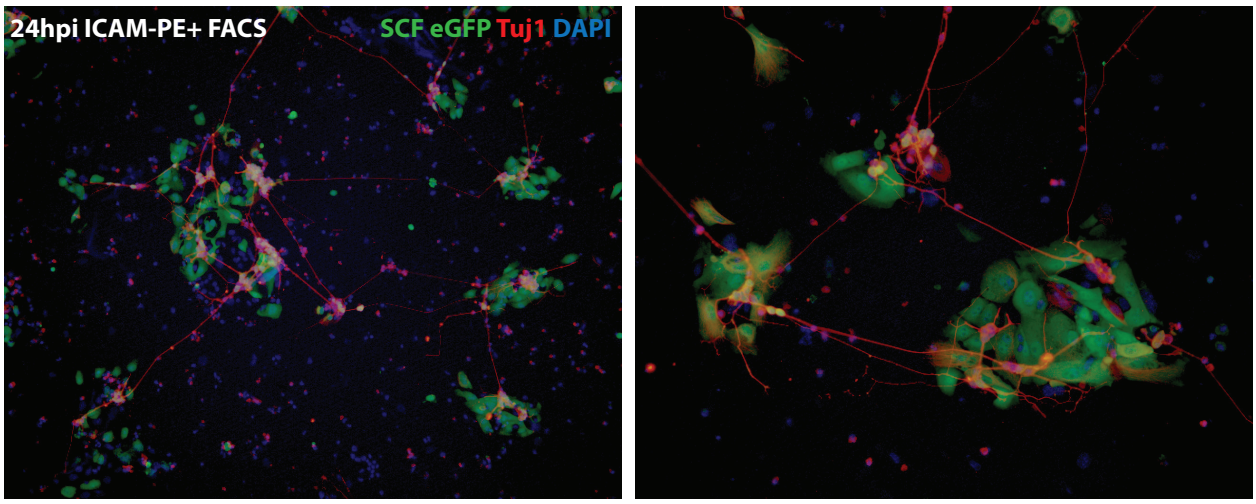


Figure 15 | Development of an in vitro model of the OE. ICAM-PE+ cells are isolated by FACS 24 hours post injury in WT mice. Cells are plated at a density of 50K cells/cm^2 on glass 8 well chamber slides, coated with fibronectin. **a, b,** Example plating where cell types appear morphologically and transcriptionally to mirror the in vivo niche, at least on a superficial level. Cell types and transcriptional identities of cells are highly variable between experimental runs at this point. Factors accounting for this variation are, as yet, unknown.

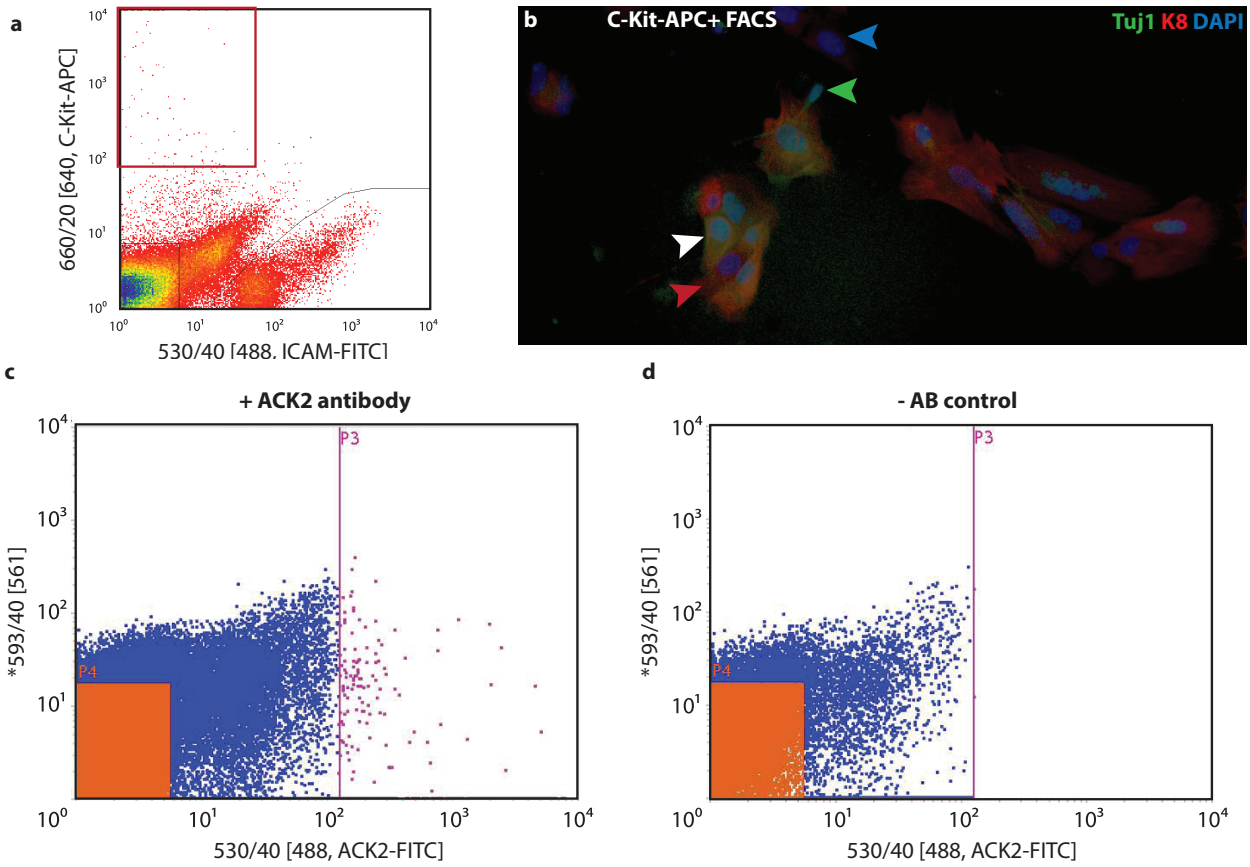


Figure 16 | Identity of progeny of C-Kit+ progenitor cells in vitro. In an attempt to determine what sort of cells arise from C-Kit+ GBCs, C-Kit-APC+ cells were isolated by FACS from 24 hours post-injury OE (a) and plated at a density of 50K cells/cm^2. Allowed to grow for 5 days in vitro, C-kit+ cells produced progeny that were Tuj1+ (green arrowhead), K8+ (red arrowhead), Tuj1+/K8+ (white arrowhead) and Tuj1-/K8- (blue arrowhead), suggesting that C-Kit cells are multipotent under in vitro conditions (b). **c,** Cells in region P3 were sorted as C-Kit+ by FACs with ACK2-FITC conjugated antibody. **d,** Each run was gated based on a negative control from the same sample.

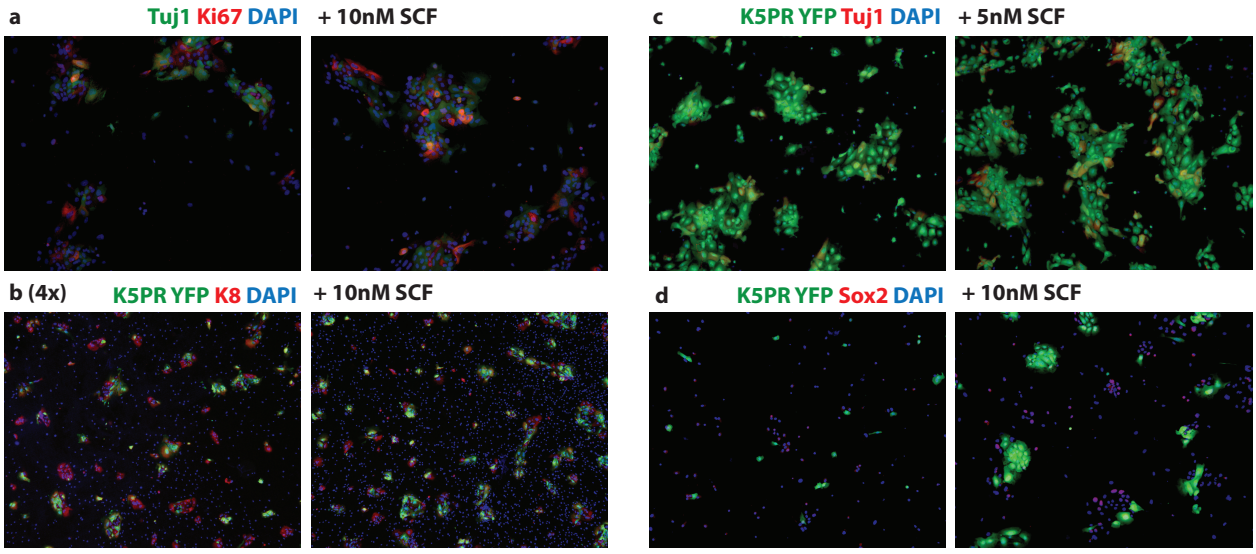


Figure 17 | Attempts to rescue SCF f/f cells in vitro with SCF protein result in subtle, if any, change. 24 hour post injury ICAM-PE+ cells from K5CrePR; SCF f/f; Rosa26YFP +/+, +/- or -/-. Example images from four (of 7 total) experimental runs. Results vary widely, with generally small qualitative increases in the number of cells when SCF protein is added. Runs lack sufficient replications for statistical power. All cells plated at an initial density of 50k cells/cm^2. Time at fixation varied by run. **a, b,** Cells fixed at five days post-plating. Images on the left are knockout cells with no added protein. The images on the right show wells with cells from the same mice that have been cultured in the same media, but with 10nM SCF protein added. Differences between conditions in (a) are not apparent, while there does appear to be an increase in the total number of cells in the SCF rescue condition in (b). **c, d,** Same experimental design as (a) and (b), but cells were fixed at three days post-plating. (d) displays prominent differences. Reasons for variability in results are not clear at this point.

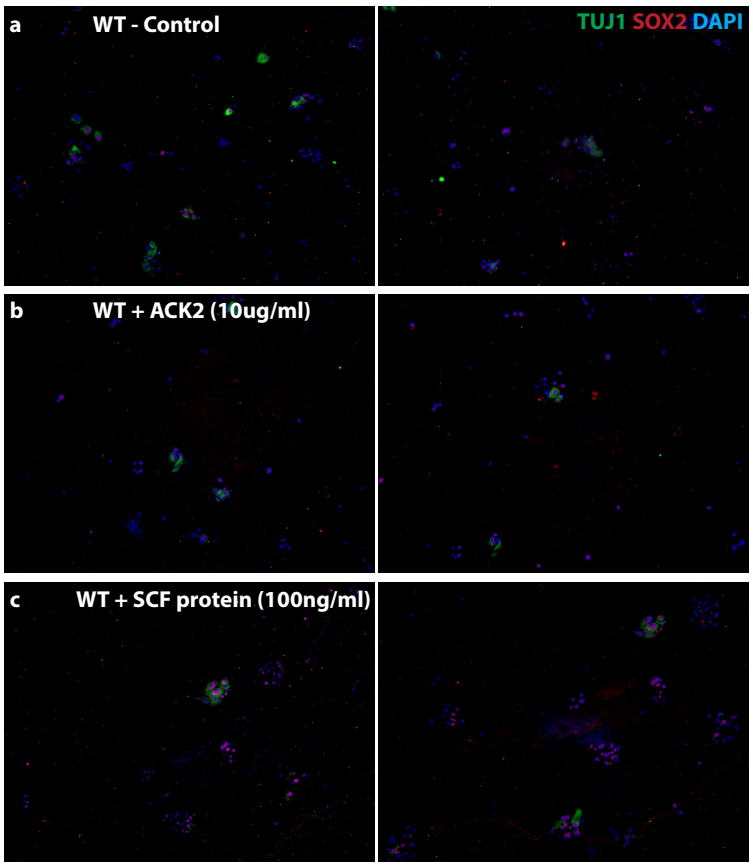


Figure 18 | Pharmacological knock-down and overexpression of C-Kit signaling in wild type mice. Initial trial run of pharmacological manipulations in wild type animals. Having only done one replicate of this design, there is no quantitative aspect at this point. **a,** Wild type cells (24 hours post-injury selected via FACS for ICAM-PE+ cells). Staining is for Tuj1 and Sox2, which, in vivo, would indicate immature neurons or HBC/GBC/SUS. Its not clear what cell 'types' exist in the in vitro system. **b,** Addition of C-Kit receptor function blocking antibody qualitatively results in a small decrease in total cell number, **c,** while addition of SCF protein (effectively over-expession, since SCF producing cells are already in the culture) results in a slight qualitative increase in the total cell number and cluster size. Further replication is required to determine if these effects, which mirror those found in vivo, will be borne out statistically.

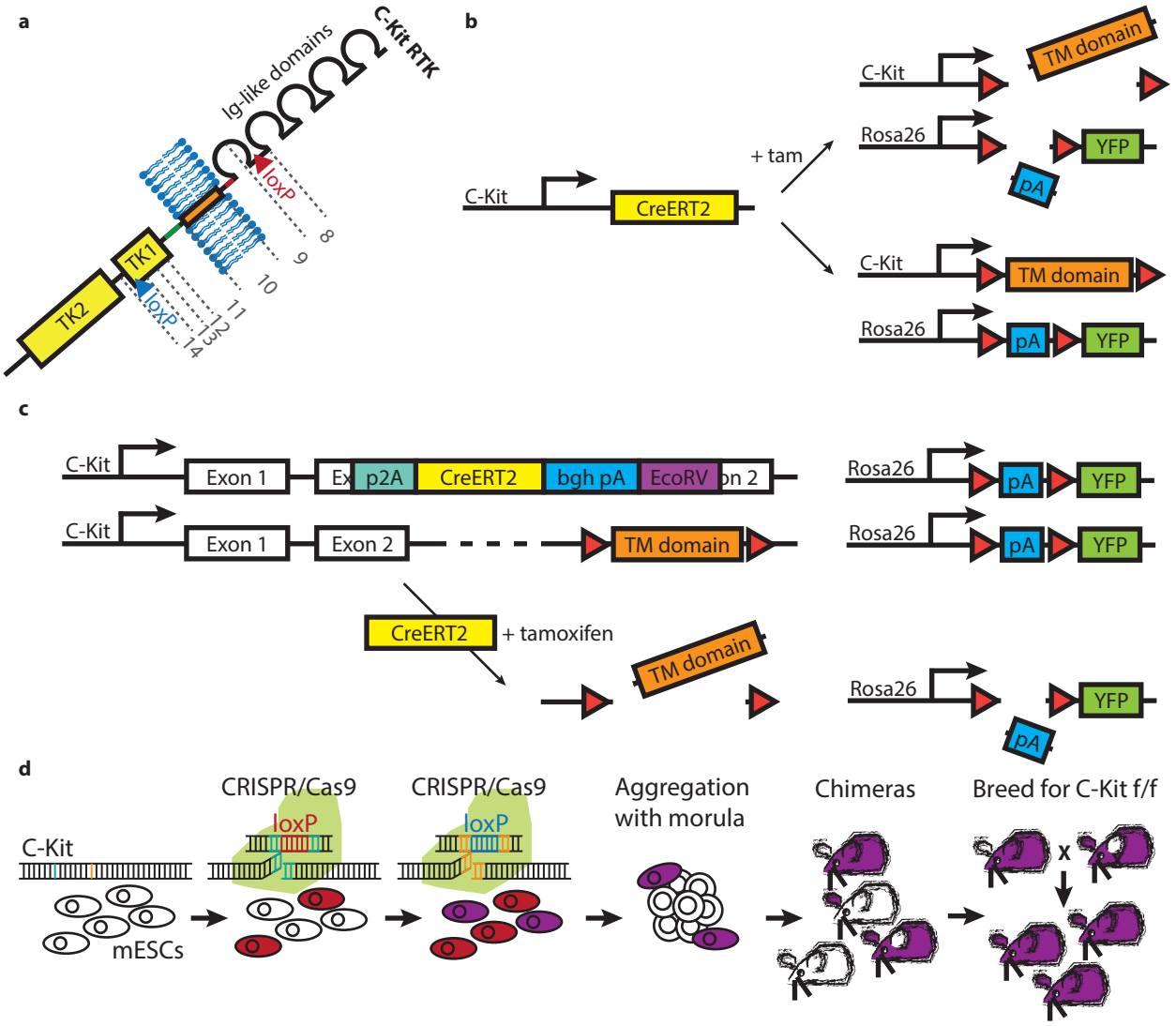


Figure 19 | Strategy for creation of a better knock-out mouse. **a,** C-Kit receptor tyrosine kinase showing the targeting of two loxP sites to introns 8 and 13, flanking the transmembrane region of the receptor. **b,** Genetic strategy for using the floxed C-Kit inducible knock-out mouse. Crossing the floxed mouse to a C-Kit CreER driver mouse and a mouse carrying an inducible YFP lineage tracing cassette in the Rosa26 locus results in a mouse where the addition of tamoxifen knocks out the C-Kit receptor and induces fluorescent lineage tracing in C-Kit+ cells. **c,** Strategy for making the floxed C-Kit mouse using the CRISPR/Cas9 gene editing system. LoxP sites will be inserted individually into mESCs from agouti mice during two rounds of CRISPR transfection. After each round, cells will be screened using PCR and Southern blot to assure homozygous integration of the transgene. Once colonies are established that are homozygous for both LoxP sites, these mESCs will be integrated into albino morulae, resulting in chimeric mice. Mice that appear to be primarily derived from floxed cells (as evidenced by primarily agouti coat color) will be bred to each other and, if there is germline expression, will produce C-Kit f/f offspring. These mice will then be bred to C-Kit CreER driver mice to produce experimentally useful offspring.