Dissecting adult epithelial cell plasticity using a model of oesophageal-to-skin lineage conversion

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A thesis submitted for the degree of
Doctor of Philosophy

June 2022
A mis padres y a mi hermana.
To my loving parents and sister.
Declaration

This thesis describes work done between October 2018 and June 2022 at the Cambridge Stem Cell Institute under the supervision of Dr Maria P Alcolea and the co-supervision of Prof. Benjamin D Simons.

The work presented in this dissertation is my own work and I confirm that where information has been derived from other sources or in collaboration, this has been specifically indicated in the text. Some of the data included in Chapter 3, 5 and 6 has been deposited in BioRxiv and is currently being considered for publication.

This work has not been previously submitted, in part or as a whole, to any university or institution for any degree, diploma, or other qualification.

In accordance with the guidelines of the Degree Committee of the School of Clinical Medicine, this thesis does not exceed 60,000 words in length including all figures, tables and references.

Paula Jiménez Gómez
June 2022
Abstract

Paula Jiménez Gómez: Dissecting adult epithelial cell plasticity using a model of oesophageal-to-skin lineage conversion

Epithelial cells possess a remarkable capacity to rapidly adapt their cell fate programme in response to changing tissue demands. Upon tissue injury or environmental perturbations, adult committed cells can reacquire stem cell properties, thereby expanding the pool of cells that contribute to tissue regeneration. Notably, this ability to rewire the cell fate programme – known as cell fate plasticity – extends beyond physiological constraints. When exposed to ectopic cues, epithelial cells can alter their identity as directed by the surrounding microenvironment. A deeper understanding of the mechanisms that govern these changes in cell identity holds great promise for regenerative medicine. However, our current knowledge of these processes is very limited.

Here, I adapted an ex vivo regenerative model to investigate adult oesophageal cell fate in response to the ectopic microenvironment of the skin. For this, I exposed the appendage-free mouse oesophageal epithelium to the mouse skin dermis, bearing empty niches for hair follicles. Whole-mount techniques together with immunofluorescence analysis revealed that oesophageal cells re-epithelialized the skin dermis and underlying niches, forming a new epithelium with associated appendages structurally similar to hair follicles.

By looking into surrogate markers of oesophageal and skin lineages, I found that oesophageal cells were instructed to change towards the skin lineage. Further investigation confirmed that the cues dictating lineage conversion emerged from the skin dermis. Yet, histological characterization and transcriptomic analysis unveiled high heterogeneity in response to dermal signals, denoting the inefficiency of the lineage conversion process.

To investigate the mechanisms promoting/preventing oesophageal-to-skin lineage conversion, I made use of an in-depth single cell RNA sequencing
dataset. Interestingly, cells transitioning towards skin identity showed a regenerative profile defined by a marked hypoxic signature. To further study the relevance of this signature for lineage conversion, I used gain and loss of function experiments targeting the hypoxia-inducible factor-1α (HIF1α) and its downstream target SRY-box transcription factor 9 (SOX9). These results unveiled that the HIF1α-SOX9 axis poses a barrier to oesophageal-to-skin lineage conversion. In turn, when this barrier is lifted cells respond better to the dermal signals instructing alternative fate choices.

Finally, I explored the contribution of oesophageal cells to hair follicle formation following transplantation into the skin. These in vivo experiments confirmed that oesophageal cells have the ability to reconstitute functional hair follicles giving rise to hair.

Taken together, the results of my PhD project reveal the existence of barrier mechanisms to cell fate plasticity, whereby the same cues that promote tissue regeneration prevent free-access to alternate fates. Future studies will be needed to investigate the physiological relevance of these mechanisms in the context of wound-healing and cancer, where plasticity is known to operate.
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<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>D</td>
<td>Day</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEG</td>
<td>Differentially Expressed Genes</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMOG</td>
<td>Dymethyloxalylglycine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Dermal papilla</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EdU</td>
<td>5-Ethynyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial Growth Factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FB</td>
<td>FACS Buffer</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HF</td>
<td>Hair Follicle</td>
</tr>
<tr>
<td>HFSC</td>
<td>Hair Follicle Stem Cell</td>
</tr>
<tr>
<td>HIF1a</td>
<td>Hypoxia-inducible factor 1-alpha</td>
</tr>
<tr>
<td>IFE</td>
<td>Interfollicular Epidermis</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>KRT</td>
<td>Keratin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MMP</td>
<td>Metalloproteinases</td>
</tr>
<tr>
<td>OE</td>
<td>Oesophageal Epithelium</td>
</tr>
<tr>
<td>oHF</td>
<td>in vitro oesophageal-derived hair follicle</td>
</tr>
<tr>
<td>oIFE</td>
<td>in vitro oesophageal-derived interfollicular epidermis</td>
</tr>
<tr>
<td>oOE</td>
<td>in vitro oesophageal-epithelium</td>
</tr>
<tr>
<td>oSKIN</td>
<td>in vitro oesophageal-derived skin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PB</td>
<td>Permeabilization Buffer</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>REAC</td>
<td>Reactome</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SC</td>
<td>Stem Cell</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SOX9</td>
<td>SRY-box transcription factor 9</td>
</tr>
<tr>
<td>sSKIN</td>
<td>in vitro skin-derived skin</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TC</td>
<td>Transitioning Cells</td>
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<tr>
<td>TEC</td>
<td>Thymic Epithelial Cells</td>
</tr>
<tr>
<td>TET</td>
<td>Ten-eleven translocation</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>UMAP</td>
<td>Uniform Manifold Approximation and Projection</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
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Chapter 1. Introduction

In this chapter, I describe the background of my project. I begin setting out a historical perspective of the plasticity field, as well as describing some of the tissues that have contributed to our knowledge on cell fate plasticity. Next, I analyse the aspects thought to regulate cell fate changes and present the oesophageal epithelium as a valuable system to investigate them. Following this, I compare and contrast the characteristics of the two main tissues investigated in my thesis: the skin and the oesophageal epithelia. Finally, I introduce an ex vivo 3D organotypic culture as a platform to investigate epithelial cell fate plasticity and summarize the aims of my PhD project.

1.1 Cell plasticity

For many years, it was accepted that tissue maintenance was sustained by a designated pool of tissue stem cells (Alonso & Fuchs, 2003; D. L. Jones & Wagers, 2008; Kalabis et al., 2008; Mascré et al., 2012). Stem cells were thought to have the unique ability to self-renew over the long term, generating a range of specialised cell types through the production of progenitor daughter cells (Smith, 2006). Progenitor cells were seen as having a more limited range of cell fate options, committed to becoming specific cell types and unable to revert back to a stem cell state (Braun & Watt, 2004; Croagh, Cheng, et al., 2008; Seery & Watt, 2000).

However, recent research has challenged this traditional view of tissue maintenance. Studies in various tissues – particularly in an epithelial context – have shown that cell fate is more adaptable than previously thought; in turn, tissue maintenance is not reliant on traditional stem cells. Instead, committed and differentiated cells can change their identity and acquire new properties through a process called cell fate plasticity (Ito et al., 2005; Levy et al., 2007; Tata et al., 2013; Tian et al., 2011; van Es et al., 2012). This process enables cells to broaden their potency (i.e. the range of cell fate options available to a cell) and adapt to extreme conditions like wounding, chemical stress, or niche vacancy (Blanpain & Fuchs, 2014; Gonzales & Fuchs, 2017; Jonathan M.W. Slack, 2016; Smith, 2006). This new understanding has led to a redefinition of the roles of stem and progenitor cells, which are now described as
those cells contributing to tissue maintenance and repair long-term (Doupe et al., 2012; P. Jones & Simons, 2008; Piedrafita et al., 2020a; Visvader & Clevers, 2016).

1.1.1 Plasticity evolution

Decades of research have addressed how embryonic stem cells give rise to all specialised cell types of an organism. During embryonic development stem cells progressively restrict their potency in a process known as cell fate specification, ultimately committing to a specific lineage (J M W Slack, 1991). This long-standing notion is illustrated by the famous Waddington’s landscape model, which represents embryonic stem cells as a ball at the top of a mountain. In this model stem cells progressively limit their potency as the ball rolls down the valleys, making cell identity choices at every bifurcation and eventually reaching a mature state at the bottom of the mountain (Waddington, 1961). This model postulated that during cell fate specification, cells permanently inactivated genes associated with other lineages; hence, making cell fate irreversible.

Seminal tissue recombination studies – combining epithelium and mesenchyme from different developing tissue origins – established the ability of embryonic cells to change their fate and form appendages of a different nature, a process known as cell fate re-specification (Dhouailly et al., 2004; Fliniaux et al., 2004; Mauger, 1972). Investigations using the chick embryo as a model unveiled the capacity of epidermal cells to develop into either feather or scales according to the transplanted mesenchyme grafted underneath (Dhouailly & Hardy, 1978; Mauger, 1972). Similarly, recombination of the mammary epithelium and salivary mesenchyme resulted in the development of salivary-like glands (Sakakura et al., 1976). These studies highlighted the cell fate plasticity of embryonic epithelia as instructed by the relevant mesenchyme. Of note, this ability was long thought to be restricted to a narrow temporal window during embryonic development (Kratochwil, 1969; Lu et al., 2016; Jonathan M.W. Slack, 2016).

Revolutionary work by Gurdon proved for the first time that mature cells can return to an undifferentiated state by exposure to the developing cues of an embryo, challenging the notion that adult cell fate is irreversible (Fischberg et al., 1958).
Particularly, this study employed transplantation of *Xenopus Laevis* somatic cell nuclei into enucleated eggs. Although highly inefficient, nuclear transplantation led to a proportion of viable embryos that developed into sexually-mature frogs. These results suggested that unused genes may be silenced, rather than permanently inactivated, during cell fate specification allowing their reactivation upon exposure to adequate stimuli. This pioneering work revealed that mature nuclei can be reverted to a totipotent state – process known as cell reprogramming – and opened up exciting questions on the mechanisms controlling it.

In the coming years it became clear that adult cells have the ability to change their identity also under physiological conditions, particularly in response to regenerative stimuli (e.g. upon wounding or stress). Early experiments in various mammals showed that following retinal excision, pigmented epithelial cells in the eye lost melanin and acquired retinal traits consistent with an identity switch or lineage transition (Coulombre & Coulombre, 1965; S. Zhao et al., 1997). More recently, the use of genetic labelling of one or more cells in their physiological context using transgenic animals has allowed researchers to trace the subsequent cell progeny, which forms clones carrying the genetic label. This method – known as lineage tracing –, together with clonal analysis, has proven to be powerful to evaluate the contribution of different cell types to tissue homeostasis and repair *in vivo* (Alcolea & Jones, 2013) greatly aiding to our understanding of cell fate plasticity in diverse tissues including the skin, airways, intestine and liver among others (Buczacki et al., 2013; Clayton et al., 2007; Piedrafita et al., 2020a; Tata & Rajagopal, 2017; Tetteh et al., 2016).

1.1.2 Physiological relevance of epithelial plasticity

Epithelial tissues provide a vital barrier that protects the organism from external aggressions. Indeed, barrier disruption may compromise survival due to dehydration or microbial infection (Gonzales & Fuchs, 2017). In this context, it is logical that epithelial cells have evolutionarily adapted to maintain the integrity of the epithelial barriers at all costs, even if that involves rewiring their cell fate programme (Tetteh et al., 2015). The physiological relevance of plasticity during tissue regeneration has been elegantly demonstrated by multiple lineage tracing studies (Ge et al., 2017a;
Chapter 1. Introduction

Hoeck et al., 2017; Levy et al., 2005; Tata et al., 2013). Several investigations in the skin revealed that following wounding labelled hair follicle and sweat gland cells switched fates towards epidermal lineages to contribute to wound-healing (Ito et al., 2005; Levy et al., 2005; Lu et al., 2012). Similarly, studies in the intestinal epithelium have shown that upon tissue damage lineage-committed cells undergo dedifferentiation, i.e. the re-acquisition of stem-like properties (Buczacki et al., 2013; Tetteh et al., 2015; van Es et al., 2012). Critically, blocking this process resulted in delayed tissue repair proving its functional relevance for an efficient regenerative response.

Yet, cell fate plasticity makes epithelial tissues highly susceptible to disordered cell growth and cancer. In fact, the emergence of cell identity changes in cancers has long been recognized. Histological characterization of squamous cell carcinomas (SCC) as well as colon and lung tumours have shown the expression of markers associated with different lineages, a process recently defined as ‘lineage infidelity’ (Curtis et al., 2010; Ge & Fuchs, 2018). Remarkably, a recent study employing lineage tracing together with gene editing techniques demonstrated that squamous cell carcinomas are dependent on this ‘lineage-infidelity’ to propagate (Ge et al., 2017a). Cell plasticity has also been proposed to play a role in tumour relapse in the context of colon cancer, where Lgr5-negative cells (non-stem cells) have been shown to dedifferentiate and drive tumour recurrence (de Sousa e Melo et al., 2017).

Altogether, these studies demonstrate the duplicity of plasticity in epithelial cells as it is required for tissue regeneration but, when abnormally regulated, can lead to disordered cellular response and cancer. Below, I describe some of the key tissues that have contributed to our understanding of cell fate plasticity, including the murine skin, airways, intestine and liver. Of note, their distinct specialized functions (thermal, absorptive, secretory, detoxifying etc.) and different embryonic origins support the idea that cell fate plasticity is a conserved mechanism among tissues/organs (Tetteh et al., 2015). Due to its relevance to my PhD work, a special focus is placed on the skin.
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1.1.3 Skin

The skin forms the first barrier that protects the body from external insults like extreme temperatures or microbial infection (Gonzales & Fuchs, 2017). The compact nature of the skin barrier leads to a mildly hypoxic environment that is key for skin physiology as well as repair (W. X. Hong et al., 2014). Structurally, it is formed by an outer epithelial layer (i.e. the epidermis) underlined by the tissue stroma (i.e. the dermis) (Figure 1; left). The murine skin is punctuated by various appendages including sweat glands and pilosebaceous units (hair follicles and sebaceous glands), which are spatially restricted to different body regions (Lu et al., 2016).

While the murine skin has greatly aided in our understanding of skin physiology, there are some interspecies differences that should be considered when translating murine studies into a human context. The human skin is significantly thicker, composed of 6-10 cell layers as opposed to the 2-3 layers found in the mouse skin (Pasparakis et al., 2014). Another important difference is that human skin contains undulations between the epidermis and the dermis, known as rete ridges, of which their function is not fully understood (Zomer & Trentin, 2018). Additionally, it should be noted that in the human skin, both hair follicles and sweat glands are co-localized within the same body regions, in contrast to the anatomical segregation of these appendages observed in the murine skin (Lu et al., 2016).

- Skin compartments: Interfollicular epidermis

The outermost layer of the skin consists of a stratified squamous epithelium termed the interfollicular epidermis (IFE), which excludes hair follicles and other appendages (Figure 1). It is a multi-layered epithelium maintained by proliferating cells that are confined to the basal layer; upon commitment to differentiation, cells detach from the basal membrane, leaving their niche to progressively migrate throughout the suprabasal layers (i.e. spinous, granular and cornified) (MESSIER & LEBLOND, 1960). As cells stratify they undergo profound morphological and biochemical changes. In fact, cells in either compartment can be accurately identified by the expression of different keratins: basal cells express KRT14 and KRT5, while differentiating cells in the suprabasal compartment switch on KRT10 and KRT1, among others (Figure 1; right) (Fuchs & Green, 1978; Fuchs & Hanukoglu, 1986).
Terminally differentiated cells in the uppermost layers lose their nuclei and synthesize filamentous proteins forming the cornified envelope, where cells are ultimately shed from.

**Figure 1. Structure of the murine tail skin.**

Schematic view of the murine skin (left) formed by the dermis (stromal compartment), epidermis, hair follicles, and sebaceous glands (red). Individual hair follicles are separated by the interfollicular epidermis (IFE) (dashed lines, left; inset, right). Proliferation is restricted to the basal layer. Upon commitment to differentiation, basal cells exit the cell cycle and migrate to the suprabasal compartment until they are shed from the tissue (right). Basal cells express keratin 14 (KRT14) and keratin 5 (KRT5). Suprabasal cells switch off expression of basal keratins and turn on KRT1 and KRT10. BM, basal membrane. Figure created with biorender.com

Understanding how epidermal cells contribute to tissue maintenance has been an area of intense research. Two distinct models have been proposed to explain basal cell behaviour: (I) the stem cell/transit amplifying model suggests that the epidermis is divided into proliferative units sustained by a slow-cycling stem cell population that divides asymmetrically to generate one stem cell (SC) and one transit amplifying (TA) cell, which only divides several times before undergoing differentiation (Potten & Booth, 2002); (II) the single progenitor model suggest that a single functionally equivalent cell population, termed progenitor population, sustains tissue homeostasis through balanced cell division and differentiation. The use of genetic lineage tracing techniques has enabled detailed investigation of these opposing models of cell behaviour. When single basal cells of the murine ear, hind paw and tail skin were randomly labelled and clones followed up long-term, several studies agreed that the single progenitor model applied to these skin body regions (Clayton...
et al., 2007; Doupé et al., 2010; Lim & Nusse, 2013). Later on, another report combined two labelling methods: an unbiased one, similar to the one used by Clayton et al., as well as a more targeted approach selectively labelling basal cells expressing the gene \textit{Involucrin} (Mascré et al., 2012). Clonal analysis of these two lineage tracing strategies originally pointed towards basal cell heterogeneity, with a long-lived stem cell pool and a committed progenitor population as proposed by the SC/TA model. Given that one major source of variability in the interpretation of these studies was the different modelling approaches, a recent quantitative study used a common approach to analyse the above-mentioned datasets. Notably, it concluded that all the presented lineage tracing data was consistent with the single progenitor model (Piedrafita et al., 2020a).

Adding to the ongoing debate about the model of murine epidermal maintenance, a single-case study in a human patient proposed that the human epidermis follows the SC/TA model (Hirsch et al., 2017). This impressive work used gene-editing techniques together with the ability of epidermal cells to regenerate the whole skin, to treat a patient displaying junctional epidermolysis bullosa; a genetic disease that leads to chronic wounds in the skin. Autologous cells were gene-edited using viral vectors, cells grown in vitro, and the resulting epidermal sheet successfully grafted onto the patient. Interestingly, 21 months later punch biopsies of the transgenic skin were taken allowing investigation the integrational profile of the grafted cells compared to the originally transplanted cells. The authors hypothesised that in case that an equivalent progenitor population maintained the skin, they would recover thousands of integrations; while if only a discrete stem cell population accounted for tissue turnover, the number of integrations would be proportional to the number of stem cells originally transplanted. Investigation of the integration profile together with clonal analysis supported the latter model. While it is possible that murine and human skin follow different models of tissue maintenance, the authors did not consider that the model used here is not homeostatic but rather regenerative; hence, it remains plausible that a stem cell population is activated during epidermal repair while deemed unnecessary for tissue maintenance, as previously suggested in animal models (P. Jones & Simons, 2008; Mascré et al., 2012). Together, these studies fuel the controversy with regards to the cellular heterogeneity of the skin basal cell layer.
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- **Skin compartments: Hair Follicles**

Hair follicles (HFs) are found throughout the vast surface of the murine skin including the tail and back skin. These appendages undergo constant cycles of regeneration in order to continuously produce hair, forming a coat that aids in thermal protection (Fuchs, 2016; Rompolas & Greco, 2014). Of note, HF regeneration is orchestrated by the dermal papillae found in close proximity to the lower HF (Jahoda et al., 1984; Rompolas et al., 2012).

![Hair follicle stem cell markers](image.png)

**Figure 2. Hair follicle stem cell markers.**
Schematic of hair follicle compartments illustrating the expression of proposed stem cell markers. Figure adapted from Alcolea et al. 2014 and created with biorender.com

In contrast to the interfollicular epidermis, it is widely accepted that HFs host several stem cell populations; each of which is restricted to discrete compartments including the bulge protrusion, the hair germ (forming the lower HF), the infundibulum – with associated sebaceous glands – and the isthmus (Figure 2). The bulge was the first HF stem cell compartment to be described (Cotsarelis et al., 1990). Bulge stem cells were identified by their slow-cycling nature, as determined by label retaining assays that measure cell division rate according to the dilution of a cell dye that is
progressively split among daughter cells. Further to this, bulge stem cells were found to express the stemness marker CD34 as well as KRT15, which allowed the isolation and investigation of this cell pool (R. J. Morris et al., 2004; Trempus et al., 2003). Serial transplantation experiments demonstrated that they contributed to hair regeneration long term, which was the ultimate evidence that bulge stem cells have long-term self-renewal capacity (Blanpain et al., 2004; Claudinot et al., 2005; Tumbar et al., 2004). During the growth phase of the hair follicle (known as anagen) bulge stem cells generate a pool of committed progenitors that form the hair germ, which produces all the differentiated cell types required for hair growth. The bulge and hair germ form the lower regenerating HF, undergoing cycles of growth and regression (Müller-Röver et al., 2001; Tumbar et al., 2004). While they both express Lgr5 and Sox9 stem cell markers, hair germ progenitor cells no longer express CD34 (Figure 2) (Ito et al., 2004; Tumbar et al., 2004). Of note, regional differences in self-renewal denote a gradient of SOX9 and Lgr5 expression that is highest towards the bottom markedly regenerative area (Rompolas et al., 2013; Rompolas & Greco, 2014).

The upper HF does not contribute to HF regeneration under homeostatic conditions and is maintained constant throughout the HF cycle (Müller-Röver et al., 2001). It is comprised of the infundibulum, where the sebaceous gland is adhered, and the isthmus. This region contains at least two additional stem cell populations: sebaceous glands are maintained through unipotent Lgr6+ Lrig1+ stem cells, whereas the infundibulum is sustained by a Lrig1+ population (Figure 2) (Alcolea & Jones, 2014). In addition to stem cell markers, HFs can also be identified by the expression of keratins different to those displayed by the epidermis, including KRT17 and KRT24 among others (Joost et al., 2018; K M McGowan & Coulombe, 1998).

Of note, under steady state conditions HFs are self-maintained and are independent from epidermal progenitors (Ito et al., 2005; Levy et al., 2005). Yet, numerous studies have shown that upon niche vacancy (following HF plucking, targeted cell ablation or wounding), HF and epidermal cells have the ability to change identity and account for the loss of neighbouring cells (Andrew et al., 2021). Following bulge stem cell depletion, upper HF stem cell populations repopulate the vacant niche gaining properties of bulge stem cells, including CD34 expression (Hoeck et al., 2017; Ito et al., 2004). Similarly, committed progenitor cells from the hair germ have been found
to dedifferentiate into multipotent bulge stem cells (Rompolas et al., 2013). Altogether, these studies demonstrate that upon niche vacancy stem cells, and even committed progenitors, can dedifferentiate to mediate niche replenishment. Therefore, while HF stem cell pools maintain discrete compartments under homeostatic conditions, wide plasticity is unleashed in response to tissue perturbations (Blanpain & Fuchs, 2014).

- **Epidermal fate and hair follicle morphogenesis**

During mouse embryonic development, the epidermis forms from the ectodermal layer soon after gastrulation at embryonic day (E) 8.5-18.5. During this process, Wnt and BMP signalling have been shown to be essential for the epidermal cell fate choice. Indeed, loss of function experiments targeting Wnt or BMP result in a blockage of epidermal fate and differentiation into neural lineages (Fuchs, 2007; Hardman et al., 1998; Okada, 1980; Wilson & Hemmati-Brivanlou, 1995).

Hair follicle bud formation starts at E14.5 (Figure 3) (S. Liu et al., 2013). The first stage of HF development is the formation of the epidermal placode, where Wnt expression is required (Stefanie et al., 2006). In fact, mutant mice lacking the Wnt signalling mediator *Lef1* don’t develop HF appendages (van Genderen et al., 1994). Likewise, Sox9 expression is necessary for subsequent HF stem cell specification as determined by loss of function experiments depleting Sox9 at E14.5, which resulted in alopecia (Vidal et al., 2005). Intriguingly, while BMP is a major inhibitor of HF formation, BMPs such as BMP2 and BMP4 are highly expressed in the nascent placode (S. Liu et al., 2013). It has been proposed that these signals may be diffused to the surrounding IFE to suppress follicular fate and ensure HF spacing, while short-range BMP inhibitors like Noggin counteract BMP signalling within the placode (Millar, 2002). In parallel, the nascent placode induces the aggregation of dermal fibroblasts that will form the dermal condensate, immediately below the HF bud (Figure 3). This will develop into the dermal papillae that is essential for HF morphogenesis as well as regeneration during adulthood (Higgins et al., 2013; Rompolas et al., 2013).
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Figure 3. Hair follicle morphogenesis.
Diagram summarizing the main stages during murine HF development. Key positive and negative regulators expressed by the developing HF are labelled in green and red, respectively. E, embryonic day; P, postnatal day. Figure adapted from Shuang et al., 2013 and created with biorender.com

Subsequently, secondary signals arising from the dermal papillae (DP) promote keratinocyte proliferation and downwards migration resulting in the hair germ (E15.5) (Jamora et al., 2005; Karlsson et al., 1999). From this stage, PDGFRa and Shh expression are essential for HF downgrowth (S. Liu et al., 2013; Lu et al., 2016). This is ultimately followed by a long phase of HF elongation and maturation (S. Liu et al., 2013). Collectively, the growth of the developing HF is known as the first anagen that spans from mid-gestation to approximately postnatal day (P) 12 (Müller-Röver et al., 2001; Oro & Higgins, 2003). The HF is considered mature from P4, when it has all the differentiated lineages including a hair shaft, and it will engage in the first postnatal cycle from P21 (S. Liu et al., 2013).

1.1.3.1 Wound-healing

The skin barrier is vital for survival. Disruption of this barrier triggers a wound-healing response that is designed to restore tissue integrity in a timely manner. It involves a highly coordinated process where epithelial cells, fibroblasts, endothelial cells and immune cells collaborate to restore the dermal niche as well as epidermal integrity (Arwert et al., 2012; Schäfer & Werner, 2008; Shaw & Martin, 2009). Briefly, the
typical response to wounding occurs in three partially overlapping stages: (I) Inflammation, mediated by TGFβ, PDGFα and VEGF signalling resulting in immune cell infiltration and activation of the coagulation cascade that prevent fluid loss and microbial infection (Gonzalez et al., 2016); (II) Tissue regeneration, including epithelial cell proliferation and migration as well as angiogenesis regulated by the hypoxia-inducible factor 1 (HIF1a) (W. X. Hong et al., 2014; Park et al., 2017); and (III) Tissue remodelling aimed at strengthening the newly formed tissue through the activity of metalloproteinases (MMPs) as well as deposition of ECM components such as collagen (Xue et al., 2006). Altogether, the coordinated activity of multiple cell types leads to wound-closure 7-10 days post-wounding under normal conditions (Arwert et al., 2012).

As a result of vascular disruption and high oxygen consumption by inflammatory cells, tissue injury is characterized by low oxygen availability (W. X. Hong et al., 2014; Rezvani, Ali, Nissen, Harfouche, de Verneuil, et al., 2011). This acute hypoxia, that has a beneficial role at the early stages of wound-closure, is progressively reversed by angiogenesis and re-epithelialization. The hypoxic response is regulated by the hypoxia-inducible factor 1alpha (HIF1a), which mediates some of its positive effects in wound-healing (Li et al., 2007). Besides promoting angiogenesis and vascular remodelling, HIF1a controls cell migration and proliferation playing a fundamental role in tissue re-epithelialization (Woodley et al., 2009). The role of HIF1a in migration has been shown to be mediated through regulation of ECM proteins and metalloproteinases (Lokmic et al., 2012; Ruthenborg et al., 2014). In support of its positive role during wound-healing, HIF1a-deficient keratinocytes lead to impaired wound-closure in vivo (Loh et al., 2009; Rezvani, Ali, Nissen, Harfouche, de Verneuil, et al., 2011). Conversely, sustained HIF1a expression upon wound closure results in excessive ECM deposition and fibroblast activation leading to fibrosis. Indeed, overexpression of HIF1a in fibroproliferative disorders correlates with increased fibrosis (W. X. Hong et al., 2014; Kimura et al., 2008). Therefore, tight regulation of HIF1a is critical for appropriate tissue regeneration.

Decades of research have investigated the contribution of epidermal cells to wound-healing. Nowadays, it is clear that epidermal progenitors are not the only cells contributing to tissue repair. In fact, wounding dissipates the sharp boundaries found
between HF and IFE compartments under normal homeostatic conditions; hence, allowing HF stem cells to exit their niche and participate in wound-closure (Ito & Cotsarelis, 2008). This has been elegantly demonstrated by lineage tracing studies targeting different HF stem cell populations including those expressing Shh and Sox9, as well as Krt15, Lgr5 and Krt19 that specifically target the lower HF stem cells including bulge stem cells (Andrew et al., 2021; Ito et al., 2005; Joost et al., 2018; Levy et al., 2005). Indeed, a comprehensive transcriptional study showed that after only one day post-wounding bulge stem cells rewire their cell fate programme to match that of the IFE (Joost et al., 2018). Critically, following wound-healing HF-derived cells have been reported to remain in the epidermis up to two-months post-wounding. Consistent with lineage re-specification, HF-derived epidermis is transcriptional and functionally indistinguishable from native epidermis (Andrew et al., 2021). Altogether, these studies highlighted the extraordinary plasticity displayed by epidermal and HF cells during tissue repair.

Further evidence of this is the emergence of de novo HF formation during wound-healing, a process known as wound-induced hair neogenesis. This observation was originally reported by histological analysis in rabbits and humans and later on confirmed by lineage tracing studies in the mouse skin (BILLINGHAM & RUSSELL, 1956; Ito et al., 2007; KLIGMAN & STRAUSS, 1956). Based on the knowledge that the Wnt pathway plays a major role in HF development, Ito used a mouse model that allows conditional Wnt inactivation to investigate its effect in wound-induced hair neogenesis. Interestingly, this resulted in a complete blockage of de novo HF demonstrating that developmental pathways are repurposed to mediate cell fate plasticity.

1.1.4 Airways

A pseudostratified columnar epithelium lines the internal cavities of the airways including the trachea, bronchi and bronchioles, which conduct gases to the alveolar spaces where gas exchange occurs (Tetteh, 2015). The airway epithelium is maintained by a basal stem cell pool (KRT5/KRT14+p63+) that generates the two main functional cell types of the airways: secretory Clara cells and ciliated cells (K. U. Hong et al., 2004; Rock et al., 2010). Further to this, the bronchioles harbour an
additional and independent progenitor cell population, neuroendocrine cells. Although largely quiescent under homeostatic conditions, airway epithelial cells displays robust regeneration following tissue perturbation (Lee & Rawlins, 2018).

While basal stem cells were originally regarded as the only population accounting for tissue repair, early studies hinted at the remarkable cell fate plasticity of the airway epithelium (Tata & Rajagopal, 2017). Pioneering work using lineage-specific markers and flow cytometry showed that non-stem cells regenerated the tracheal epithelium when grafted onto an epithelium-denuded trachea (J. Y. Liu et al., 1994). Within 4 days, cells were found to re-epithelialize the trachea, lose differentiation markers and acquire the basal stem cell marker KRT14 among others. In the following week a mucociliary epithelium formed expressing markers of Clara and ciliated cells. This study provided the first evidence that airway epithelial cells can dedifferentiate into functional stem cells that self-renew and give rise to differentiated lineages. This notion was later confirmed by genetic lineage tracing experiments demonstrating that Clara cells can revert into stem cells following basal stem cell ablation (Tata et al., 2013). The remarkable plasticity of the airway epithelium is further evidenced by cell transdifferentiation events, described as the conversion of one cell type to another (Tosh & Slack, 2002). Indeed, neuroendocrine cells have been shown to directly change identity into Clara and ciliated cells following naphthalene-induced injury (Yao et al., 2018). More recently, single cell RNA sequencing technologies have further dissected the plasticity of airway epithelial cells during tissue regeneration (Choi et al., 2021; Jeon et al., 2022).

1.1.5 Intestine

The intestine is lined by a simple columnar epithelium, a monolayer of column-shaped cells that form finger-like villi as well as epithelial pits known as crypts (van der Flier & Clevers, 2009). Together, these structures increase the surface area of the intestine aiding in nutrient digestion and absorption. The intestinal epithelium is maintained by an actively cycling Lgr5+ stem cell pool located at the base of the crypt. Crypt stem cells divide into lineage committed progenitors that progressively differentiate into four main lineages: enterocytes, Goblet cells, enteroendocrine cells and Paneth cells (Barker, 2014; Barker et al., 2007).
Despite the well-described hierarchy sustaining intestinal epithelium turnover, multiple lineage tracing studies have reported cell plasticity events under stress conditions. In response to niche vacancy, following ablation of Lgr5+ stem cells, the largely quiescent Bmi1+ committed progenitor cell pool can cross compartmental barriers and transition into actively cycling Lgr5+ stem cells, hence compensating for their loss (Tian et al. 2011). Similarly, numerous studies have reported dedifferentiation of committed progenitors as well as terminally differentiated cells to regenerate the damaged epithelium under physiological conditions (Buczacki et al., 2013; Tetteh et al., 2015; van Es et al., 2012).

Tumour cells have been proposed to hijack plasticity mechanisms to fuel malignancy (de Sousa e Melo et al., 2017; Ge et al., 2017a; Ge & Fuchs, 2018). Interestingly, the link between dedifferentiation and tumorigenesis has been well established in the intestine (de Sousa e Melo et al., 2017; Gidekel Friedlander et al., 2009). Indeed, a mouse model of intestinal cancer has shown that forced expression of Wnt leads to enterocyte dedifferentiation into Lgr5+ stem cells, acquiring properties of tumour stem cells and initiating tumour growth (Schwitalla et al., 2013). This work beautifully demonstrated that dedifferentiation can contribute to tumour initiation.

1.1.6 Liver

The liver is a largely quiescent organ (MACDONALD, 1961). It is composed mainly of hepatocytes and biliary-duct cells, which work together to pursue the liver metabolic and detoxification functions. During embryonic development, both cell types derive from a common progenitor cell (hepatoblasts), while after birth hepatocytes and duct cells self-duplicate to give rise to their respective lineages (Malato et al., 2011; Miyajima et al., 2014). Despite the absence of a dedicated stem cell pool, the liver displays remarkable regenerative capacity (Huch et al., 2013).

Following partial hepatectomy, proliferation of the hepatocyte compartment accounts for tissue restoration (Yanger et al., 2014; Yimlamai et al., 2014). When hepatocyte cell division is compromised, ductal cells have been shown to dedifferentiate into hepatoblasts that will later on mature into functional hepatocytes (Duncan et al.,
Probing deeper into liver regenerative capacity, another study combined bile duct ligation with toxin-mediated damage, impairing ductal dedifferentiation as well as hepatocyte proliferation. Remarkably, such challenges resulted in hepatocyte transdifferentiation into biliary duct epithelial cells that would engage in tissue repair (Michalopoulos et al., 2005; Tetteh et al., 2015; Yanger et al., 2013). Altogether, these studies highlight the extraordinary plasticity displayed by the liver to ensure organ function upon damage.

1.2 Plasticity regulators

1.2.1 Tissue microenvironment

The role of the niche in stem cell behaviour has long been recognized (D. L. Jones & Wagers, 2008; Scadden, 2006). The mesenchyme/stroma provides structural support as well as biochemical and physical cues that dictate cell function and behaviour. Indeed, the link between epithelial cell fate changes and the underlying stroma dates back to the 1960s. Early tissue recombination studies showed that embryonic epithelial cells have the ability to adapt their fate and develop appendages of a different nature, ranging from feathers to sweat glands, when exposed to relevant ectopic mesenchyme (Kratochwil, 1969; Mauger, 1972). These groundbreaking findings revealed that embryonic epithelial cell fate can be rewired by the underlying tissue mesenchyme.

In the last decades, several studies have revisited the role of the mesenchyme in eliciting cell fate changes (Bonfanti et al., 2010; Claudinot et al., 2020). Motivated by the expression of skin differentiation markers in clonogenic thymic epithelial cells (TEC), Bonfanti investigated the capacity of these cells to integrate into the skin using transplantation assays. Strikingly, they found that serially passaged embryonic, postnatal and adult TECs transplanted into the skin increased potency, acquiring both epidermal and hair follicle identity. Serial transplantation experiments, the hallmark functional assay for stem cells, proved their self-renewal capacity as they contributed to HF regeneration for more than 3 months. Consistent with an identity switch, transcriptional analysis revealed the downregulation of the TEC-associated signature and upregulation of typical epidermal markers. Altogether, the authors concluded that TECs rewired their fate due to the skin tissue microenvironment.
A follow up study by the same group, later reported that TECs were not the only cells that had the capacity to integrate into the skin (Claudinot et al., 2020). In fact, they showed that this capacity was shared between epithelial tissues expressing p63, a master transcription factor for the development of squamous tissues including the skin. Using serial transplantation experiments they demonstrated that epithelial cells from adult tissues including the oral mucosa, vagina and oesophagus can contribute to skin as well as hair follicle formation when exposed to the new-born skin microenvironment. Interestingly, some Tp63-expressing tissues, like the thymus, were only competent to change cell fate when previously passaged \textit{in vitro} suggesting a higher degree of lineage restriction. Conversely, Tp63-expressing epithelial cells from the trachea and ureter solely formed a squamous epithelia or cysts, respectively, but did not contribute to hair follicle formation even after \textit{in vitro} culture. Together these results highlighted the remarkable plasticity of embryonic and adult epithelial cells when exposed to the morphogenic signals of an ectopic niche, while raising questions about tissue-specific restrictions to those instructing cues.

Making use of a more physiological system, elegant work by Lu and colleagues demonstrated that manipulating mesenchymal signals in the developing embryo changed cell fate from sweat glands to hair follicles (Lu et al., 2016). Using genome-wide transcriptional analysis they first described that regional skin differences in mesenchymal expression of BMP directed sweat gland as opposed to hair follicle fate during morphogenesis. To investigate if changes in BMP activity could lead to different epithelial fates, they used transgenic mouse models inhibiting BMP signalling in the mesenchyme of the hairless foot pad skin. Remarkably, this resulted in hair follicle development as opposed to sweat glands demonstrating that epithelial-mesenchymal crosstalk can re-direct cell fate in embryonic epithelial cells.

In a physiological context, several studies have demonstrated the relevance of the niche for cell fate plasticity. A comprehensive lineage-tracing study using the murine cornea as a model showed that corneal-committed cells could only dedifferentiate to compensate for limbal stem cell loss if the underlying stroma was intact (Nasser et al., 2018). Indeed, chemical damage of the limbal epithelium and underlying stroma abolished dedifferentiation and recovery of the stem cell compartment leading to
aberrant repair. Similar conclusions have been drawn from work looking into the hair follicle, where the mesenchymal dermal papillae has long been known to be essential for regeneration (Blanpain et al., 2004; R. J. Morris et al., 2004; Tumbar et al., 2004). Recently, the link between both skin compartments was further investigated in real time in live mice showing that ablation of the mesenchymal dermal papilla impairs initiation of hair regeneration (Rompolas et al., 2012). Overall these studies emphasise the relevance of epithelial-mesenchymal interactions for cell fate changes during epithelial regeneration.

1.2.2 Transcription factors

Transcription factors (TF) shape cell fates by activating genes crucial for cell identity while inhibiting lineage-inappropriate genes (Cantor et al., 2008; Schaffer et al., 2010). While TFs generally work in concert with many others, master transcription regulators known as pioneering factors have the ability to impose their transcriptional programme by binding transcriptionally silent chromatin; hence, playing a dominant role on cell fate specification (S. A. Morris, 2016). This is the case for the Myogeneic determination gene (MyoD), which forced expression can convert fibroblasts into stable muscle cells (L. & Harold, 1992). Pioneering factors also include some of the Yamanaka reprogramming factors (Oct4, Sox2 and Klf4), whose ectopic expression drives somatic cell reprogramming (Takahashi & Yamanaka, 2006). These and many other studies highlight the key role of TFs establishing cellular states and raise questions about the transcriptional networks operating naturally in cell fate conversion events, for example during wound-healing and tumorigenesis.

Multiple studies have pointed towards Sox9 as a pioneering factor governing cell fate (Adam et al., 2015; Fuglerud et al., 2021). In fact, it has been shown to be involved in the formation of many organs including the hair follicles (HF), chondrocytes, testis, retina and sweat glands among others (Lefebvre et al., 2007; Nowak et al., 2008; Vidal et al., 2005). Specifically, its relevance for HF morphogenesis and skin physiology has been extensively studied (Adam & Fuchs, 2016; Ge & Fuchs, 2018; Kadaja et al., 2014). In the adult skin its expression is exclusively found in the lower HF (bulge and hair germ) as opposed to the upper hair follicle and the epidermis (Gonzales & Fuchs, 2017; Joost et al., 2018). However, Sox9 remains activated as
cells exit their niche to contribute to wound-healing (Adam et al., 2015). This observation was intriguing given that most HF identity genes are silenced as cells engage in epidermal repair (Ge et al., 2017a). Further investigation by Ge and colleagues demonstrated that activation of SOX9 is required for HF stem cells to progressively acquire an epidermal identity during wound repair, allowing them to exit their niche. In fact, transplantation assays modelling wound-healing revealed that Sox9-depleted cells did not contribute to tissue re-epithelialization. This study established the critical role of SOX9 in epidermal cell fate changes. Further to this, the role of Sox9 in epithelial homeostasis and regeneration in other tissues hint at its broader relevance in cell fate decisions (McConnell et al., 2011; Menzel-Severing et al., 2018; Nandan et al., 2014).

### 1.2.3 Epigenetics

Most of our knowledge about epigenetics and cell fate determination arises from *in vitro* studies in embryonic stem cells. Only recently, mouse genetic and functional studies have highlighted the physiological significance of global epigenetic landscapes in adult stem cells (Adam et al., 2015, 2020; Adam & Fuchs, 2016; Aloia, 2021; Gonzales & Fuchs, 2017).

Several studies have addressed the role of epigenetics in cell fate plasticity during regenerative processes. A comprehensive study investigating the mechanisms underlying ductal cell plasticity upon severe liver injury, described a transient genome-wide epigenomic remodelling mediated by Ten-eleven translocation (TET) 1 enzyme (Aloia et al., 2019). Interestingly, they found that this enzyme was a key epigenetic regulator required for the regenerative activation of ductal cells. In turn, TET-1-depleted ductal cells failed to compensate for the hepatocyte loss leading to fibrosis as a result of impaired regeneration. Building on the notion that epigenetic landscapes are remodelled during regeneration, work by the Fuchs laboratory has recently demonstrated that not all epigenetic changes are transient, indeed some chromatin domains remain in the ‘non-homeostatic’ configuration upon tissue repair (Andrew et al., 2021; Naik et al., 2017). The authors demonstrated that the maintenance of such epigenetic profiles endows epithelial cells with a memory of their previous experiences and identities i.e., inflammation, wounding or epidermal...
vs HF fate. For example, HF-derived epidermal cells several months following wounding retained epigenetic marks consistent with their HF origin. Functionally, they retained HF stem cell multipotency and had hair-forming ability, as determined by hair reconstitution assays, which represents the gold standard assay to assess long-term self-renewal and multipotency of HF stem cells. Similarly, they also retained a wounding memory resulting in a more efficient wound-healing response in the event of a secondary wound. Together these results highlight the role of epigenetics in cell fate plasticity, representing not only an acute mechanism in response to tissue perturbation but also a process that enhances epithelial adaptation to secondary microenvironmental challenges.

1.3 The murine oesophageal epithelium

The mouse oesophageal epithelium (OE) has recently been recognized as a valuable system to study squamous epithelial cell fate (Doupe et al., 2012; Fernandez-Antoran et al., 2019; Frede et al., 2016; Piedrafita et al., 2020a). Contrary to the skin, the murine OE lacks appendages such as hair follicles or sweat glands, making it a very uniform tissue particularly suited for lineage tracing studies, in which a heritable genetic label is induced in single dividing cells. As the labelled cell divides and differentiates, clusters of labelled daughter cells – termed clones – are formed. Monitoring clonal growth and stratification over time allows inference of cell fate of the originally labelled cell; hence shedding light on the oesophageal cell dynamics in various physiological or experimental conditions (Alcolea & Jones, 2014; Frede et al., 2016).

While representing an invaluable tool for in vivo studies, it is important to consider that the murine OE has some key differences when compared to the human tissue. Structurally, the latter is significantly thicker (40-60 cell layers compared to the 4-6 found in mice), lacks a cornified layer and has a more complex organisation, including papillae and submucosal glands. A further difference of the human OE lies in the presence of 5-6 proliferating cell layers as opposed to the single proliferating layer of the murine OE. Therefore, it is critical to consider these discrepancies when assessing the applicability of murine studies for human-related questions.
1.3.1 Tissue structure

The oesophagus is a tubular organ that transports food and liquids from the oral cavity to the stomach. Its piping function corresponds with its simple architecture, consisting of a squamous stratified epithelium covering the cavity of the organ, and underlined by the tissue stroma (submucosa) and associated muscle (Figure 4; left) (Doupe et al., 2012; McGinn et al., 2021). The mouse OE consists of a multilayer of keratinocytes, lacking typical structures found in the digestive tract such as papillae or secretory glands.

**Figure 4. Structure of the mouse oesophagus.**
Diagram depicting a cross section of the oesophageal tube (left), encompassing an outer muscle layer, the submucosa (stroma compartment) and a squamous epithelium (right). Proliferation is restricted to the basal cell layer. Upon commitment to differentiation cells exit the cell cycle and migrate towards the suprabasal compartment. Basal cells express keratin 14 (KRT14) and keratin 5 (KRT5), while suprabasal cells express KRT4 and KRT13. BM, basal membrane. Figure created with biorender.com

Constant wear and tear by the food bolus results in epithelial cells being shed from the lumen of the organ. To account for cell loss, cells in the basal layer undergo proliferation; hence, sustaining tissue turnover (MESSIER & LEBLOND, 1960). Upon commitment to differentiation, basal cells exit the cell cycle and migrate towards the tissue surface before being ultimately shed. Throughout the stratification process, cells undergo changes in morphology as well as keratin expression. The basal compartment consists of a layer of densely packed cuboidal cells – in contact with the basal membrane – that ubiquitously express KRT14 / KRT5 (Figure 4; right). Upon detachment from the basal membrane, cells become larger, flattened and start
to express the differentiation markers K4 / KRT13 (Frede et al., 2016a; Giroux et al., 2017; Rosekrans, 2015). During the final steps of differentiation, cells lose their nuclei and synthesize filamentous proteins such as filaggrin and loricrin among others that form the cornified envelope (Alcolea & Jones, 2013; Rosekrans, Baan, Muncan, & Van Den Brink, 2015).

1.3.2 Tissue maintenance

During the last decades numerous groups have addressed how OE cells sustain tissue turnover during homeostasis. Like in the epidermis, it was unclear whether all dividing cells are functionally equivalent or whether there is some degree of heterogeneity, as proposed by the SC/TA model (DeWard et al., 2014; Doupe et al., 2012; Giroux et al., 2017; Horsley, 2012; Kalabis et al., 2008). A variety of strategies have been used to address this question and dissect oesophageal epithelial cell behaviour.

Pioneering work by Leblond suggested that the OE is maintained by a single equipotent progenitor population that accounts for self-renewal and differentiation (MESSIER & LEBLOND, 1960). This study used radioactive nucleotide incorporation to label dividing cells in the rodent oesophagus. As cells undergo mitosis they incorporate the analogue nucleotide into their DNA, which is then passed on to daughter cells, allowing to trace their cell progeny. The authors observed that consecutive radioactive thymidine injections in vivo led to labelling of all the basal cells within 5 days, indicating that all cells were actively cycling. When tracing the behaviour of labelled pairs, they found three potential fate outcomes: a) both cells differentiating, b) both cells going on to divide (self-renewal), c) one dividing and one differentiating cell. Interestingly, the probability of each of these cell fates was comparable suggesting a model where progenitor cells with equal chances of undergoing self-renewal or differentiation divide stochastically to sustain OE tissue turnover.

In the coming years, several studies described a degree of cellular heterogeneity in the basal layer of the OE. One of such studies looked into cell kinetics and showed the presence of interspaced labelled basal cells with their progeny stacked above in
the suprabasal compartment (Croagh, Thomas, et al., 2008; Giroux et al., 2017). Despite the limited number of clones analysed, the authors hypothesised that this clonal distribution supported the SC/TA model, where slow-cycling stem cells divide asymmetrically to generate short-lived TA cells that ultimately differentiate after a few rounds of division. In line with this, work by Kalabis reported the presence of slow-cycling cells using label retaining assays (Kalabis et al., 2008). This assay measures cell division rate as determined by the dilution of a cell dye that is progressively split among daughter cells; hence quiescent or slow-cycling cells – so called label-retaining cells – retain the dye, while proliferating cells lose it. In addition, label retaining cells were reported to express the well-described stem cell marker CD34; this was interpreted by the authors as a further prove of their stemness. However, Kalabis and colleagues did not consider that the CD34+ slow-cycling population may correspond to tissue resident immune cells. In fact, a later study using a large battery of anti-CD34 antibodies together with label retaining assays found that CD34+ and label retaining cells present in the OE correspond to immune cells, as determined by expression of the pan-immune marker CD45 (Doupe et al., 2012).

Other studies used cell sorting and colony/organoid formation assays to assess the self-renewal capacity of different OE cell populations based on the expression of cell surface markers like integrin-α6, integrin-β1, CD71 and CD73 (Croagh, Thomas, et al., 2008; DeWard et al., 2014; Giroux et al., 2017; Kalabis et al., 2008). Although several independent studies described a degree of heterogeneity in cell self-renewal capacity, there is still no consensus regarding the putative OE stem cell markers. Of importance, the authors did not consider that the cell dissociation methods used prior to cell sorting (including long trypsinization incubations) may have altered the cell adhesion molecules leading to artefactual differences in their colony/organoid forming efficiencies.

In line with the early studies from Leblond, large-scale lineage tracing assays coupled with quantitative approaches are at odds with the SC/TA model of tissue maintenance in the OE (Doupe et al., 2012). Firstly, label retaining assays using the tetracycline-regulated expression of the histone H2B-GFP were used to estimate OE cell division rate. The authors observed that, excluding some immune cells, GFP protein expression was lost from the basal compartment within 4 weeks suggesting
the lack of a slow cycling stem cell pool. Next, to interrogate cell behaviour single cells were genetically labelled and clonal units traced for a year. Clonal analysis showed that the average size of persisting clones increased linearly with time and their size distribution acquired long term scaling behaviour. Such clonal behaviour is characteristic of an equipotent cell population like the one described in the epidermis (Clayton et al., 2007; Piedrafita et al., 2020a). Overall, these studies provide compelling evidence that the OE is maintained by a progenitor cell pool that divides stochastically to generate - on average - equal numbers of cycling and differentiating cells, hence achieving tissue homeostasis.

1.3.3 Oesophageal epithelial cell plasticity

Growing evidence demonstrate that OE progenitor cells can rapidly adapt their cell fate in conditions away from homeostasis like tissue repair. Using a transgenic mouse line, progenitor cells were conditionally labelled with a genetic yellow fluorescent protein (YFP) prior to oesophageal wounding and clonal behaviour analysed thereafter. Interestingly, the authors found that 1-day post wounding the YFP-labelled clones migrated towards the wounded area contributing to epithelial proliferation and migration (Doupe et al., 2012). This suggested that the same progenitor population that maintains the tissue under homeostatic conditions can mediate wound repair. To investigate whether all basal cells contributed equally to tissue repair, cell division rate was measured by label retaining assays revealing that indeed all cells increased their proliferation rate equivalently. Importantly, following wound resolution progenitor cells reverted back to a balanced production of proliferating and differentiating cells, i.e. homeostatic behaviour. Overall, this study demonstrated that same progenitor population that accounts for tissue maintenance can change behaviour to contribute to tissue repair as later demonstrated in the epidermis (Doupe et al., 2012; Piedrafita et al., 2020a).

Various studies have addressed the effect of tumorigenic mutations in OE cell dynamics (Alcolea et al., 2014; B Colom et al., 2021; Bartomeu Colom et al., 2020; Fernandez-Antoran et al., 2019; Frede et al., 2016). Several models of carcinogenesis in the mouse oesophagus, including consecutive treatment with tumour-promoting agents as well as the induction of an inactivating Notch mutation,
have shown that progenitor cells undergo a bias in the production of proliferating cells similar to that observed transiently in wounds (Alcolea et al., 2014; Frede et al., 2016). This cell fate bias resulted in cell expansion, leading to dysplasia in response to tumour-promoting agents and field cancerization by Notch mutant clones. Interestingly, once mutant cells expanded through the tissue, cells reacquired a more homeostatic behaviour; hence suggesting their resilience and capacity to adapt to ensure tissue integrity is sustained (Alcolea et al., 2014). To investigate whether similar cell fate changes were observed in advanced cancers, work by Frede used a chemical mutagen treatment together with expression of an oncogenic Ras allele (KrasG12D). This protocol resulted in oesophageal squamous cell carcinoma. Interestingly, they found a similar phenotype to that observed in earlier lesions with the only exception that cell behaviour was particularly tilted to produce and excess of progenitor cells (Frede et al., 2016).

Overall, these studies further support the maintenance of the OE through a single progenitor model, whereby an equivalent progenitor population balances the production of progenitor and differentiating cells to adapt to wounding and tumorigenic agents/mutations.

1.4 Epithelium of the murine skin and oesophagus: compare and contrast

Epithelial tissues are highly specialised according to their specific functions e.g. preventing water loss, absorption, thermal protection etc. Hence, a variety of cell morphologies, appendages, and differentiation programmes can be found in different epithelial organs (Ferraris et al., 2000; Wabik & Jones, 2015).

The surface of the mouse skin as well as the luminal side of the mouse oesophagus are lined by a stratified squamous epithelium (Figure 5). This type of epithelium consists of multiple layers of squamous cells, that provide a solid barrier against abrasion as well as dehydration (Gonzales & Fuchs, 2017). To sustain the multi-layered architecture of the tissue, an apical-lateral network of adherent junctions and desmosomes link epithelial cells together (Green et al., 2010). The uppermost layer
of the epithelium is highly keratinized, forming the cornified envelope, that aids in the impermeable epithelial barrier.

Figure 5. Histological comparison of skin and oesophageal epithelia. 
H&E of the murine adult skin (top) and oesophagus (bottom) showing the characteristic tissue structure. Insets show representative images of the squamous epithelia that line either organ. Characteristic basal and suprabasal markers are shown. KRT, keratin; scale, 100 µm (left) and 10 µm (right).

Although there is still some debate in the field (as described in Section 1.1.3 and 1.3.2), comprehensive lineage tracing studies in the skin and oesophageal epithelia have converged on a common paradigm of tissue maintenance, whereby a single progenitor population balances the production of progenitor and differentiating cells (Clayton et al., 2007; Doupe et al., 2012; Frede et al., 2016; Lim & Nusse, 2013; Park et al., 2017; Piedrafita et al., 2020a). This simple model sustains the turnover of the epithelium without the need of a dedicated stem cell population. Progenitor cells are located in the basal layer, the only proliferating compartment of both tissues. Interestingly, basal cells in the skin and oesophageal epithelium share expression of
KRT14 / KRT5, while they display different programmes of differentiation: epidermal cells turn on expression of KRT10 / KRT1 and oesophageal cells express KRT4 / KRT13, among others. Moreover, the epidermis contains appendages, including hair follicles and sebaceous glands, which express their own set of keratins. This is the main histological difference between the murine epidermis and the oesophagus, that lacks any secondary structures.

All in all, the murine skin and oesophageal epithelia are very similar. Of importance, the skin epithelium differs in its complexity having both hair follicles and sebaceous glands. As described in Section 1.1.3 and 1.3, this is not the case for the human tissues, where both oesophagus and skin contain appendages.

1.5 3D organotypic cultures to study cell behaviour ex vivo

Cell culture approaches provide a powerful tool to complement in vivo experiments. The development of self-assembling organ-like structures ex vivo, also known as 3D cultures, offer a controlled system that can be easily manipulated to study epithelial cell behaviour (Abaci et al., 2018; Chacón-Martínez et al., 2018; Rheinwald & Green, 1975; Sato & Clevers, 2013). A benefit of these models when compared to traditional 2D culture systems is that they recreate the 3D structural organisation and distinctive cell lineages found in the in vivo tissue. One limitation is that their maintenance depends on complex media formulations, supplemented with growth factors, that continuously boost cell expansion. Further to this, traditional organotypic cultures are typically established from single dissociated cells, hence requiring trypsinization treatments that are known to interfere with normal cell behaviour (Tsuji et al., 2017). A next generation of 3D culture systems, that retain the tissue structure with minimal interference from dissociation methods or growth factors, may serve as a model to study epithelial cell behaviour recapitulating a condition closer to that found in vivo.

In order to investigate oesophageal cell fate dynamics during epithelial regeneration, a novel ex vivo organotypic culture that encompasses intact oesophageal tissue was first described by Doupe et al. 2012. This system combines two oesophageal tissue strips grown on the live tissue submucosa, therefore reproducing the 3D organ structure while maintaining the unperturbed tissue (Figure 6a). At the onset of the
culture, epithelial basal proliferation and migration are found to mediate stromal re-
epithelialization, reproducing a wound-healing like response (Doupe et al., 2012). After 10 days in culture, the newly formed OE acquired the typical marker distribution found in the \textit{in vivo} tissue, with KRT14 expression restricted to the basal layer and KRT4 to the suprabasal compartment (Figure 6b). Importantly, cell division was consistent with ‘homeostatic-like’ behaviour as determined by EdU incorporation.

Adaptation of this recombination culture system - combining epithelium and stroma from different tissue-origins - represents the basis of my PhD work.

Figure 6. \textit{Oesophageal 3D ex vivo cultures.} 
(a) Schematic representation of the oesophageal 3D ex vivo culture strategy. Oesophageal epithelial strips were laid over denuded isolated submucosa and cultured for up to 10 days. During this time new epithelium forms between the strips (OE \textit{in vitro}), re-epithelializing the submucosa. Figure adapted from Doupe 2013 and created with biorender.com (b) Side view of the \textit{in vivo} OE (top panel) and \textit{in vitro} oesophageal wholemount 10 days upon culture (bottom panel). Blue, DAPI; red, KRT4; green, KRT14; white, EDU, scale, 20 μm.
1.6 Objectives of my PhD project

The ability of epithelial cells to redefine their cell fate programme in response to changing tissue demands has emerged as a new paradigm in stem cell biology. Following tissue perturbations, committed and differentiated cells have the capacity to reacquire stem cell features hence expanding the pool of cells contributing to tissue regeneration. Of importance, this plasticity extends beyond physiological constraints. Transplantation experiments have shown that cells can re-define their identity when exposed to an ectopic microenvironment instructing alternate fates (i.e. feathers, salivary glands or feathers). However, the mechanisms that control cell fate changes remain largely unknown. Understanding how epithelial cells modulate their fate was the overarching question of my PhD work.

The main objectives of my PhD project were:

i) To investigate cell fate changes in the adult oesophageal epithelium when exposed to the instructive signals of the skin stroma.

Based on previous recombination studies, I speculated that combining epithelium and stroma from different tissue origins would unleash cell plasticity changes. To this end, I decided to make use of an ex vivo regenerative system that allowed culturing of two structurally similar tissues: the skin and the oesophagus. Critically, this system was amenable for wholemount techniques allowing one to investigate cell fate changes at the whole tissue level. First, I characterized tissue re-epithelialization temporally. To investigate the emergence of epithelial cell identity changes, I used quantitative analysis of surrogate markers associated with oesophageal or skin identity. Following the identification of oesophageal-to-skin lineage conversion events, I evaluated the effect of the skin stromal cues on such cell fate changes. I then explored whether other epithelial tissues may be permissive for cell fate plasticity upon exposure to skin cues.
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ii) To define the molecular mechanisms regulating oesophageal-to-skin lineage conversion.

To this end, I first developed a microdissection-coupled RNA sequencing strategy that allowed molecular profiling of spatially-defined regions. Yet, given the low resolution of this bulk approach, I next turned to single cell RNA sequencing. In depth investigation of cells transitioning from oesophageal to skin lineage revealed a clear candidate regulator, the hypoxia-inducible factor 1 alpha (HIF1α). Thereafter, I combined transgenic mouse lines with in vitro drug treatments to investigate the functional relevance of this transcription factor in lineage conversion.

iii) To explore the oesophageal cell contribution to hair formation in vivo.

For this, I first used a full-thickness grafting approach that enabled transplantation of intact oesophageal-dermal composites equivalent to the recombinant cultures investigated in vitro. A second approach included the use of classical hair reconstitution assays i.e., transplantation of dissociated OE cells into the murine skin.

Note that a significant proportion of the data and some of the text presented in my thesis was previously deposited in BioRxiv and are currently being considered for publication. Unless otherwise specified, all the presented data is my own work. Collaborative work has been done in conjunction with Dr Maria T Bejar and Dr Maria P Alcolea. Computational work was performed by Ilias Mospolous, Dr Irina Mohorianu and Dr Seugmin Han. Critical advice in epithelial cell biology has been provided by Prof. Benjamin Simons.
Chapter 2. Materials & Methods

2.1 Animal work

Animal work was conducted in accordance to Home Office project licence PPL70/8866 “Epithelial stem cell plasticity; relevance to cancer” at the Wellcome – Medical Research Council Cambridge Stem Cell Institute biomedical facility and the Wellcome – Cancer Research UK Gurdon Institute. All experiments comprised a mixture of male and female mice, with no gender specific differences observed. For RNA sequencing experiments exclusively, males were used to avoid confounding effects due to the estrous cycle. Unless otherwise specified, all animal cohorts were adults between 8-25 weeks of age.

2.1.1 Mouse strains

To determine the tissue-of-origin in 3D organotypic cultures, fluorescent reporter mouse lines were used. Unless otherwise specified, oesophageal tissue was obtained from reporter mouse lines constitutively expressing tdTomato localized in the membrane (R26mTmG; stock #007676, Jackson Laboratory) or nuclei (R26nTnG; stock #023537, Jackson Laboratory). The tail skin was harvested from animals constitutively expressing EGFP in the nucleus (CAG::H2B-EGFP; kindly provided by J. Nichols).

Lgr5 expression was detected using the Lgr5-EGFP mouse line (Lgr5-EGFP-IRES-creERT2; stock #008875, Jackson Laboratory) (Barker et al., 2008) that incorporates an EGFP cassette targeted to the 3’ untranslated region of the Lgr5 gene.

To visualize cycling cells, Fucci2a animals (R26Fucci2a, kindly provided by Ian J. Jackson) that constitutively express cell cycle reporter proteins (G1 marked by mCherry-hCdt1, and S/G2/M marked by mVenus-hGem), were used.

For Sox9 conditional knock out experiments, Sox9^{flox/flox} mice (obtained from MRC-Harwell, which distributes these mice on behalf of the European Mouse Mutant Archive https://www.infrafrontier.eu) were crossed onto K14^{CreERT} mice (stock
#005107, Jackson laboratory) to generate the K14\textsuperscript{CreERT}/ Sox9\textsuperscript{flox/flox} enabling genetic inactivation of Sox9 expression in epithelial cells upon tamoxifen induction.

For transplantation experiments, C57BL/6J wild type mice (strain code, 632) and nude athymic mice (strain code, 490) were purchased from Charles River (UK).

### 2.1.2 Induction of Cre mediated recombinase

For recombination of K14\textsuperscript{CreERT}/ Sox9\textsuperscript{flox/flox}, 25 mg/ml Tamoxifen dissolved in sterile sunflower seed oil was used. Animals received two subcutaneous and one intraperitoneal tamoxifen injections (5 mg per 20 g of body weight) over 3 consecutive days. The esophagi of treated animals were collected 24h after the last TAM administration.

### 2.2 Tissue culture

In order to investigate adult cell fate plasticity, I adapted the \textit{ex vivo} organotypic system previously described to study oesophageal cell regeneration (Figure 6) (Doupe et al., 2012). This method enabled co-culturing of adult epithelium and stroma of different tissue origins (i.e. heterotypic culture) (Figure 7a). Equivalent culture systems combining epithelium and stroma from the same tissue of origin (i.e. isotypic cultures; like the one used by \textit{Doupe et al.}) were used as controls (Figure 7b).

#### 2.2.1 3D Oesophageal-Dermal Heterotypic cultures

3D heterotypic cultures combined epithelium and stroma from oesophageal and tail skin origin, respectively (unless otherwise stated in Section 3.4). This co-culture system allowed investigation of the emergence of cell fate changes during ectopic regeneration. To determine the tissue of origin, oesophageal tissue was taken from tdTomato+ mice (mTmG or nTnG mouse lines) and skin-derived tissue was taken from EGFP+ mice (H2B-EFGP mouse line).

The tail skin was cut into 7 x 9 mm pieces and incubated for 4 hours in 5mM EDTA (Ethylene-diamine-tetra-acetic acid; Life Technologies, 15575020) at 37°C. The skin
was then denuded of its epithelium and remaining tissue (dermis) pieces placed onto transparent ThinCert™ inserts (0.4 μm pore size; Greiner Bio-One Ltd; Cat#657641).

Figure 7. Ex vivo organotypic culture set up.
(a) Oesophageal-dermal 3D heterotypic cultures. Tail epidermis was peeled away from the underlying stroma (dermis), leaving it denuded of its epidermal compartments (i.e. hair follicles and sebaceous glands). Inset shows pilosebaceous units remaining in the peeled epidermis. Heterotypic cultures were established by placing two strips of oesophageal tissue (black dotted lines) on top of the denuded skin dermis. (b) Oesophageal isotypic cultures. Two strips of oesophageal tissue were overlaid on the peeled oesophageal stroma (submucosa). Scale 1mm.
Subsequently, the murine oesophagus was longitudinally opened with spring scissors and muscle removed using fine forceps. The remaining tissue – epithelium and underlying submucosa – was cut into strips of approximately 5 x 1 mm in size (Figure 7a). Immediately afterwards two oesophageal strips were laid on top of the denuded dermis.

Heterotypic composites were then allowed to settle for 5 minutes at 37ºC to ensure attachment, and 700 μl of minimal medium (mFAD; lacking growth factors) containing one-part DMEM (4.5 g/L D-Glucose, Pyruvate, L-Glutamine), one-part DMEM/F12, supplemented with 5% fetal calf serum, 5 μg/ml insulin, 0.18 mM adenine, 5-10 μg/ml transferrin and 5% Penicillin-Streptomycin added. Cultures were grown for up to 10 days at 37ºC with 5% CO2, replacing the medium on alternate days. The denuded/peeled dermis was progressively re-epithelialized by cells of oesophageal origin. The newly formed epithelium is referred to as oesophageal-derived skin (oSKIN), including oesophageal-derived interfollicular epidermis (oIFE) and hair follicles (oHF).

2.2.2 Oesophageal isotypic cultures

The oesophageal epithelium was grown on its native stroma – the submucosa – using the method described by Doupe et al. (Figure 7b) (Doupe et al., 2012). For this, the submucosa was denuded of its epithelium after 3 hours incubation in 5mM EDTA at 37ºC and cut into 5 x 10 mm pieces. Subsequently, oesophageal tissue strips (1 x 5 mm; processed as in 1.2.1) were laid on top. Isotypic cultures were maintained for up to 10 days in mFAD at 37ºC with 5% CO2 (as above). The submucosa was progressively re-epithelialized by cells of oesophageal origin, forming a new epithelial sheet referred to as oesophageal-derived oesophagus (oOE).

2.2.3 Skin isotypic cultures

The dermis was denuded of its epithelial compartment as above (section 2.2.1) and cultured in the absence of tissue strips. Cultures were maintained under the same regime described above. This system allowed the dermis to re-epithelialize itself by epidermal host cells that remained in the tissue. The resulting epidermal sheet is
referred to as skin-derived skin (sSKIN), which includes associated skin compartments (sHF, Hair Follicle; sIFE, Interfollicular Epidermis).

### 2.2.4 Heterotypic cultures: other epithelial tissues

Although the focus of this work was to investigate cell fate changes in the oesophageal epithelium, the response of other tissues to dermal cues was also explored. For this, tissues (trachea, forestomach, corpus stomach, small intestine and colon) were processed and grown on the skin dermis as described for oesophageal-dermal cultures (Section 2.2.1). Briefly, the lumen of the organs was flushed with PBS supplemented with 10% Penicillin-Streptomycin, the organs opened using spring scissors and tissue cut into 1 x 5 mm strips. Forestomach strips were peeled off their underlying muscle using fine forceps and a scalpel. Subsequently, tissue strips were laid on the dermis and grown for up to 10 days under the same conditions described above.

### 2.2.5 Limited re-epithelialization assay

To inhibit regenerative cues, the oesophageal epithelium was peeled off its underlying submucosa following a 3-hour incubation in 5mM EDTA at 37°C. The epithelial tissue (lacking the stroma) was then laid on the dermis as in section 2.2.1. Of note, in this assay the epithelial tissue was cut into bigger pieces of approximately 5 x 8 mm to account for the anticipated drop in epithelial expansion.

### 2.2.6 Explant cultures

To study SOX9 expression under non-homeostatic conditions, oesophageal explant cultures were used. Following opening of the oesophageal tube and removal of its underlying muscle, the remaining tissue (epithelium and submucosa) was cut into 5 x 5 mm pieces. Explants were then placed onto ThinCert™ inserts with the epithelium facing upwards and the submucosa on the membrane. To ensure attachment, explants were dried for 5 minutes at 37°C before adding the medium. Cultures were then grown for up to 10 days under mFAD media conditions (see section 2.2.1).
2.2.7 HIF1a inhibitor and stabilizer treatments

HIF1a translation inhibitor KC7F2 (10 μM; SML1043, Merck) and the HIF1a protein stabilizer DMOG (1 mM; 71210, Cayman) were used to dissect the role of HIF1a in heterotypic cultures. Control cultures were treated with DMSO vehicle alone. Cultures were grown in a normoxic humidified incubator at 37°C, 5% CO2 for a period of 8 days and medium changed on alternate days.

2.3 Histology

Hematoxylin and Eosin (H&E) staining was performed on 4.5 μm paraffin-embedded sections by the Histology Core Service at the Cambridge Stem Cell Institute and subsequently imaged by myself with an Apotome Imaging System (Zeiss) using 10x, 20x or 40x objectives.

2.4 Tissue preparation

2.4.1 Oesophageal wholemounts

The freshly dissected oesophageal tube was opened with spring scissors and flattened under a dissecting microscope. Muscle was peeled away and remaining tissue – consisting of epithelium and submucosa – incubated in 5 mM EDTA for 3 hours at 37°C on a rotor. Following incubation, the oesophageal epithelium was gently peeled from the submucosa using fine forceps and fixed in 4% paraformaldehyde (Alfa Aesar; 043368) in PBS (4% PFA) for 30 minutes at 37°C. Oesophageal wholemounts were then washed three times in PBS to remove any remaining fixative and stored in PBS at 4°C until further analysis.

2.4.2 Skin wholemounts

Skin wholemounts were prepared based on a previously described method (Braun et al., 2003). Briefly, the middle third of the tail was cut longitudinally with a scalpel and skin gently peeled from the tail bone with forceps. Skin was cut into pieces of 9 x 7 mm and incubated in 5 mM EDTA for 4 hours at 37°C on a rotor. The intact sheet of epidermis was then peeled from the dermis and incubated in 4% PFA for 30
minutes at 37°C. Storage conditions prior to downstream analysis were the same as described above.

2.4.3 Wholemounts

To separate the new epithelial sheet grown in vitro, cultures were incubated in 5 mM EDTA for 2 hours at 37 °C. The epithelium was then carefully dissected from the underlying stroma using fine forceps and fixed in 4% for 30 minutes at 37°C. This method was used to wholemount the epithelia derived from heterotypic, oesophageal, and skin cultures (as in section 2.1, 2.2 and 2.3, respectively).

Figure 8. Schematic illustrating epithelial whole-mounting.
Epithelia from the in vivo oesophagus (top) the in vivo skin (middle) and 3D oesophageal-dermal heterotypic cultures (bottom) were peeled off the underlying stroma (i.e. submucosa or dermis) prior to fixing for immunofluorescence analysis.
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2.4.4 Thick cryosections

When epithelial whole-mounting was not possible, full thickness tissues (including epithelial and stromal compartments) were fixed in 4% for 1 hour at 37°C and embedded in optimal cutting temperature compound (OCT; Fisher Scientific, 12678646). OCT-embedded tissues were then cryosectioned (150 μm sections), sections placed onto glass slides and immediately transferred into PBS. To permeabilize the tissue, sections were washed three times in 0.3% Triton-X100 PBS for 10 minutes and kept in PBS at 4°C until processed for immunostaining analysis.

2.5 Immunofluorescence

2.5.1 Immunostaining

Wholemount staining was performed based on the protocol described by Jensen et al. 1999. Samples were processed as described in section 2.4, then incubated for 15 minutes in permeabilization (PB) buffer – containing 0.5% bovine serum albumin (VWR International; 126575-10), 0.25% fish skin gelatin (Sigma, G7765), and 0.5% Triton X-100 (Fisher Scientific Ltd; 10102913) in PBS – and blocked for 1 hour in blocking buffer (PB buffer containing 10% donkey serum, according to the secondary antibody used). Primary antibodies were incubated at appropriate concentrations (see Table 1) in blocking buffer overnight at 4°C and were subsequently washed 4 times for 20 minutes in 0.2% Tween-20 in PBS at RT. Secondary antibodies were incubated for 3 hours in blocking buffer at RT and washed with 0.2% Tween in PBS twice. To counterstain cell nuclei samples were incubated overnight in 1 μg/ml of 4’,6-diamidino-2-phenylindole (DAPI). Samples were mounted in 1.52 RapiClear mounting media (SUNJin Lab; RC152001) with the basal epithelial layer facing the coverslip. To facilitate imaging from either side, cryosections were mounted between coverslips using a 50 μm iSpacer (SUNJin Lab; Cat #IS201). All incubation steps were done on an orbital shaker.
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2.5.2 Antibodies

Primary antibodies used in this project are listed in Table 1. Secondary antibodies used were Donkey anti Chicken 488 (Jackson Immuno Reserch) and Goat or Donkey Alexa Fluor 488/555/647 (Molecular Probes).

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<td>Millipore</td>
<td>AB5535</td>
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</tr>
</tbody>
</table>

Table 1. List of primary antibodies used for immunofluorescence staining of wholemounts and cryosections.

2.6 Microscopy and quantitative analysis

2.6.1 Confocal imaging and analysis

Confocal images were captured using an inverted Leica SP5 (Leica Microsystems) with standard laser configuration. Typical settings for z-stack image acquisition included optimal pinhole, line average 3, bi-directional scan with speed of 400 Hz and a resolution of 512 x 512 or 1024 x 1024 pixels using 10x, 20x or 40x objectives. Images were typically acquired with 40x objective and digital zoom 3x for oesophageal and interfollicular epithelium and 1x for hair follicles. Images were
analysed and processed using Volocity Analysis Software 5.3.3 (PerkinElmer) or Arivis 3.5.0A (Vision4D).

To calculate the percentage of HF structures expressing lineage markers (HF/oesophageal), a minimum of 150 HF units were quantified. Randomly selected HF triplets throughout the sample were examined to provide a representative estimate of the whole sample. A HF was considered to be positive when at least one cell expressing the relevant marker was found. Normally, 20 HFs were quantified per replicate, and at least two replicates per animal (n=3 animals).

To quantify the number of oHF cells expressing a marker, a different approach was used (Figure 9). Given that I found some expression variability between the area adjacent to the strip and the central area of the culture (which is re-epithelialized at later time points), I only considered the former to minimize the presence of confounding factors in the analysis. Specifically, only the two HF triplets adjacent to the OE strips were examined. A minimum of 9 fields of view i.e., 18 HF units (from at least 3 biological replicates) were imaged by confocal microscopy. Rendered confocal images were then analysed using their xyz views to quantify the number of cells expressing the relevant markers as well as total number of cells, as determined by DAPI nuclear staining. Representative images are shown in figures.

Figure 9. Schematic illustrating the method used to quantify the percentage of cells expressing HF markers in oesophageal-derived hair follicles.
Chapter 2. Materials and Methods

Epithelial wholemounts were immunostained for the relevant markers and imaged by confocal microscopy. Briefly, 9 fields of view – each including a HF triplet – adjacent to the OE strip were acquired and rendered images examined in xyz views. Each HF was then quantified independently and the number of total cells, as determined by DAPI counterstained nuclei, as well as the number of positive cells quantified to calculate the percentage of cells expressing the relevant HF marker. A minimum of 18 HF units were quantified (i.e., ~2000 cells; n=3 biological replicates).

2.6.2 Oesophageal-derived HF formation efficiency

To quantify the efficiency of HF re-epithelialization, 7 x 9 mm tail skin pieces from Lgr5-EGFP reporter mice (labelling bulge HF stem cells) were incubated for 30 minutes in 4 μg/ml DAPI in PBS. Samples were then tile-scanned on a confocal microscope and the number of HFs present in the tissue quantified. Immediately after imaging, the quantified tail pieces were used to perform heterotypic cultures (as described in section 2.2.1). In short, skin pieces were washed in PBS and incubated in 5mM EDTA for 4 hours at 37ºC. The epidermis was dissected away from the dermis and oesophageal tissue from mTmG mice laid on top. Heterotypic cultures were grown for 10 days and full thickness tissues fixed in 4% PFA for 1 hour. Wholemounts were then washed three times in PBS and mounted in 1.52 RapiClear mounting media for imaging. The number of oesophageal-derived HF structures (tdTomato+) were quantified using xyz views of rendered confocal tiled images. The efficiency of oHF formation was calculated as the percentage of oHF formed in vitro relative to the number of original HFs in the in vivo skin piece. A total of 3 independent samples were quantified (n=3 animals).

2.6.3 Quantification of re-epithelialized area

To quantify dermis re-epithelialization, full thickness tissues were imaged using an Apotome Imaging system (Zeiss). Tile scan images were taken using the 2.5x objective and individual images stitched automatically by the software. Tissue re-epithelialization was then estimated as the percentage of the area covered by the newly-formed epithelium (tdTomato+) compared to the total dermis area. At least 3 independent samples were analyzed per experiment (n=3).
2.6.4 Fluorescence intensity analysis

Fluorescence intensity analysis of individual cells was performed using Arivis 3.5.0A (Vision4D). Nuclei segmentation was performed through DAPI counterstaining and nuclear staining intensity measured within the identified nuclei. A minimum of 18 HF units (from a minimum of n=3 animals) were analysed.

2.7 Dermal decellularization assay

The decellularization protocol was based on previously described methods (Kristofik et al., 2017; Simsa et al., 2018). Freshly isolated dermal pieces, processed as described in section 2.2.1, were incubated for 4 hours in a hypertonic solution (0.1M NaOH, 1M NaCl and 25 mM EDTA in DI water) at 37ºC to lyse cells, followed by an overnight incubation in 100mM EDTA at 4 ºC. Enzymatic digestion of DNA was achieved by a 6 hours incubation in a solution containing 2 U/ml Benzonase, 47 mM Tris, 1.4 mM MgCl², 19 mM NaCl in deionized water at 37ºC. The dermis was then washed overnight in 100 mM EDTA at 4ºC, followed by a 2-hour incubation in a solution containing 8 mM CHAPS, 1 M NaCl and 25 mM EDTA in PBS at 37ºC and finally washed in 100 mM EDTA solution overnight. The decellularized dermis was then thoroughly washed with PBS for 30 minutes. All incubation steps were done in an orbital shaker. Between different solutions the dermis was washed in PBS (3 washes of 10 minutes on each occasion).

2.8 In vivo transplantation experiments

2.8.1 Heterotypic grafting assay

Heterotypic oesophageal-dermal grafts were prepared as described for the in vitro assay (see Method 2.2.1). Briefly, a dermis piece was cut with a 6-8 mm diameter puncher and overlaid with a 4 x 6 or 4 x 8 mm strip of oesophageal tissue. Typically, dermis was obtained from H2B-EGFP animals and oesophageal tissue from mTmG or nTnG mice. To reduce tissue dehydration prior grafting, composites were kept in a humidified environment until transplantation.

The grafting technique was adapted from previously described methods (Ge et al., 2017b; Plikus et al., 2008). Inbred C57BL/6J wild type animals as well as nude
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Athymic mice were used as recipients. In a typical experiment, recipient mice were anesthetized by isoflurane inhalation and underwent an excision on the dorsal shoulder using sterile 6-8 mm diameter biopsy punch. Immediately afterwards, a graft of equivalent dimensions was fitted and adhered to the surrounding tissue with Vetbond tissue adhesive (3M). The area was further sutured using steri-strip wound closure strips (3M) and covered with Tegaderm dressing film (3M) to protect the tissue implant. Animals were monitored thereafter, and skin tissue harvested up to 4 weeks post-transplantation.

2.8.2 Hair reconstitution assay

Hair reconstitution studies were adapted from previously described protocols (Jensen et al., 2010). In short, back skin was obtained from postnatal day 2 (P2) H2B-EGFP mice and placed epidermal side up, floating in 10ml 0.25% trypsin overnight at 4°C. After washing with PBS, the epithelium was peeled off using fine forceps and thoroughly minced with a scalpel. The epidermal cell suspension was repeatedly pipetted to disaggregate clumps, filtered through a 30μm strainer and spun down at 500 g for 8min at RT. Epidermal cells were resuspended in cFAD medium, containing one-part DMEM (4.5 g/L D-Glucose, Pyruvate, L-Glutamine), one-part DMEM/F12, supplemented with 5% fetal calf serum, 5–10 μg/ml insulin, 5–10 μg/ml transferrin, 0.5 μg/ml hydrocortisone, 1nM cholera enterotoxin, 10 ng/ml EGF and 5% Penicillin-Streptomycin. The neonatal dermis was separately minced and incubated in 0.25% collagenase for 25min at 37°C. The dermal cell suspension was then filtered through a 100μm strainer and the HF buds were pelleted by centrifugation at 100 g for 5min at 4°C. The supernatant was transferred to a new tube, centrifuged at 300 g for 5min at 4°C and resuspended in cFAD medium.

Oesophageal epithelial cells were isolated from adult nTnG mice. Longitudinally opened oesophagi were cut in 4 pieces and incubated in 0.5mg/ml Dispase in PBS for 15min at 37°C. The epithelium was peeled, minced with a scalpel and transferred to a new tube with fresh Dispase 1 solution. After a 5 min incubation at RT, EDTA was added at a final concentration of 5 mM to inhibit Dispase activity. Cells were mixed by pipetting and filtered through 30 μm strainers. Oesophageal cell
suspensions were centrifuged at 300 g for 5 min at 4°C, and resuspended in cFAD medium. All cell suspensions were kept on ice until transplantation.

A cell suspension typically containing $5 \times 10^5$ oesophageal epithelial cells, $5 \times 10^6$ neonatal dermal cells and $10^6$ neonatal epidermal cells was prepared and surgically implanted onto a PDMS silicon chamber secured on the dorsal fascia of recipient nude mice, following a full-thickness punch biopsy (8 mm diameter). Grafted mice were monitored and tissue harvested for immunofluorescence analysis 6 weeks post-grafting.

### 2.9 Oesophageal biopsy wounding

Oesophageal wounding was performed by controlled micro-endoscopic biopsying of the mouse oesophagus as previously described (Doupe et al., 2012). Briefly, C57BL/6J mice undergoing endoscopy were anesthetized using a combination of 100 mg/kg Ketamine (Pfizer Animal Health) and 10 mg/kg Xylazine administered intraperitoneally. Animals without a complete loss of righting reflex after anaesthetic induction were topped up with inhaled isoflurane prior to intervention. A 9.5 Fr diagnostic miniature endoscope with a 3 Fr instrument channel was used in conjunction with an AIDA COM II image capture system for visualization (Karl Storz GmBH). 3 Fr diameter biopsy forceps with double action jaws (Karl Storz GmBH) were used to create one superficial wound in the middle third of the mouse oesophagus of between 0.4 – 0.9 mm diameter. Anaesthesia was reversed using Atipamezole (Pfizer Animal Health) given at 1 mg/kg subcutaneously at least 20 minutes after induction. Animals were euthanized 24 h after wounding.

### 2.10 RNA sequencing

#### 2.10.1 Bulk RNA sequencing

**2.10.1.1 Sample processing and microdissection**

Heterotypic and oesophageal cultures were prepared as indicated above (see Method 2.2.1 and 2.2.2, respectively). Oesophageal tissue was obtained from nTnG mice and denuded tail skin dermis from H2B-EGFP animals. The resulting epithelia (oesophageal control, oOE; and oesophageal-derived skin, oSKIN) were harvested
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10 days post-culture (D10). Adult oesophagus, adult skin and postnatal day 10 (P10) skin were included as reference controls of tissue identity.

To separate the epithelium from the underlying stroma, samples were cut into 5 x 5 mm pieces and incubated in 50 mM EDTA for varying timepoints (12-55 min) according to tissue requirements. The peeled epithelium was then processed under a Leica DMI6000 B microscope (Leica Microsystems): single HF's were harvested using fine forceps and 0.5 x 0.5 mm pieces of epithelial tissue micro-dissected using a sterile microsurgical knife (Surgical Specialties Corporation; 74-1000). Freshly micro-dissected samples were collected in 5 μl of RLT lysis buffer (Qiagen) containing 1% b-Mercaptoethanol to inhibit RNAases. Total RNA was then extracted using a RNeasy Micro Extraction Kit (Qiagen), including DNase I treatment to degrade any genomic DNA carry-over. RNase-free material was used throughout the process. Following reverse transcription-mediated amplification, samples displaying good cDNA yields were sent for library preparation.

2.10.1.2 Library preparation and sequencing

Libraries were generated using the NovaSeq kit (Illumina) at the Cambridge Stem Cell Institute Genomics Facility. The libraries were multiplexed and single-end 50bp sequenced on a single lane Illumina HiSeq 4000 at the CRUK - CI Genomics Core Facility of the CRUK Cambridge Institute.

2.10.1.3 Analysis

Pre-processing quality checks were performed using FastQC (v0.11.2) and summarised with MultiQC (v1.8). Adapter trimming was implemented using TrimGalore (v0.6.4_dev) and standard alignment to the mouse reference genome (genome assembly GRChm38.97 of *M musculus*) performed using STAR (STAR_2.5.2a) with default parameters. Density plots, violin plots, MA plots, Jaccard similarity index, dendrograms and PCA were performed as further quality checks. The data was normalised using quantile normalisation and noise was removed using a fixed threshold of 20 (only genes entirely under the threshold were discarded and abundances <20 were set to 20). Differential expression analysis was performed using edgeR (v3.28.1) and enrichment analysis on the set of differentially expressed
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genes against a background of all genes expressed in the dataset was performed using the g:profiler (https://biit.cs.ut.ee/gprofiler/gost) R package (v0.1.8).

2.10.2 Single cell RNA sequencing

2.10.2.1 Sample preparation and cell sorting strategy

Heterotypic 3D cultures were prepared as indicated above using oesophagus from nTnG mice and denuded tail skin dermis from H2B-EGFP mice. As controls samples, oesophagus from nTnG was grown over peeled oesophageal stroma from H2B-EGFP mice. The resulting epithelia (oesophageal control, oOE; and oesophageal-derived skin, oSKIN) were harvested 3 and 10 days post-culture (D3 and D10, respectively), at which point the epithelia were carefully peeled from their underlying stroma following a 50mM EDTA incubation for 15 min at 37°C. A single-cell suspension was obtained by rinsing the peeled epithelia with PBS, and incubating it with 0.5 mg/ml Dispase (Sigma) for 5 min. EDTA was then added to the samples at a final concentration of 5 mM, and the suspension diluted 1/5 by adding FACS Buffer (FB; 2% heat-inactivated Fetal bovine serum (Life Technologies; 26140079), 25mM HEPES (Life Technologies; 15630056)) in order to inhibit Dispase activity. The cell suspension was filtered through a 30μm cell strainer, and centrifuged at 300 g for 10 minutes at 4°C. Cells were finally resuspended in FB containing 1 U/μl RNAse Inhibitor. Single viable epithelial cells were sorted (using a BD FACSARia™ III cell sorter) by tissue of origin, tdTomato (oesophageal origin) and EGFP (host tissue stroma). Sorted tdTomato cells from Batch 1 were spiked in with EGFP host cells as an internal control to define epidermal skin identity. Batch 2 was kept free of EGFP host cells to avoid confounding effects (see details in Table 2).
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2.10.2.2 Library preparation and sequencing

scRNA-seq libraries were generated and sequenced using 10X Genomics kits (Single Cell 3’ v3) at the CRUK-CI Genomics Core Facility of the CRUK Cambridge Institute. Libraries were processed in two different batches/dates. Batch 1 included 3 biological replicates for eEE at D3, eSKIN at D3, eEE at D10, and eSKIN at D10, rendering 12 libraries; Batch 2 included 2 biological replicates for eSKIN at D10, rendering 2 libraries. Each biological replicate consisted of pooled cultures from 4-5 wells with a total of 9,000 sorted cells per sample. The libraries were multiplexed and sequenced on 4 lanes Illumina NovaSeq6000 S2 flow cells.

2.10.2.3 Analysis

Pre-processing quality checks were performed on the R2 reads using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). The data was then

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Table 2. Sample collection details for single cell RNA-sequencing experiment.

*Percentage of EGFP+ cells spiked in each sample at the cell sorting stage. Please note replicates eSKIN-D10 4 and 5 did not include EGFP spiked-in cells as a control to avoid confounding factors.
processed using the CellRanger software (v3.1.0) with standard alignment (genome assembly GRCm38.97 of *M musculus*) and default filtering parameters. Upper and lower bounds on the distributions of counts, features, mitochondrial and ribosomal RNA were used to remove outlier cells. Cells were included in the analysis if their sequencing depth was over 8750, the number of expressed genes was between 2000 and 8000, and the percentage of mitochondrial and ribosomal DNA was lower than 15% and 45%, respectively. Mitochondrial and ribosomal genes were subsequently removed from the matrix. As a result, a total of 107,445 cells, discarding doublets, were selected for further analysis. The raw expression matrix was normalized using sctransform; and the downstream analysis was performed using the Seurat R package (v3.2.2). To filter out non-epithelial cells, cells expressing fibroblast or immune marker genes including *Col1a2*, *Pdgfra* or *Ptprc* were excluded from the analysis.

Dimensionality reductions (PCA and UMAP), as well as clustering were performed using Seurat. The biological replicates for each time point overlapped well with each other, confirming negligible batch effects between samples and conditions, therefore no batch effect correction was necessary. Based on the characteristics of the dataset (stability of partitioning assessed in an incremental manner), we focused on the 1000 most abundant genes to identify the main transcriptional features for each cluster. After the gene expression analysis (identified by testing for differential gene expression using the Wilcoxon Rank Sum test on the Bonferroni adjusted p-value, with a natural log-fold change threshold of 0.25 and a p-value threshold of 0.01), we defined 19 relevant clusters in the present dataset. Clustering stability was evaluated using a PAC analysis.

Pseudotime analysis was performed using the monocle3 R package (v0.2.3.0)\textsuperscript{145}. Since C18 Host cells served as a reference for the skin identity switch, these were, therefore, fixed as the endpoint of the pseudotime trajectory. To focus on the exclusive features that drive the TC away from their original identity (Oesophagus), we selected the genes that had > 0.2 logFC between the Oesophagus and the TC (357 genes). We then scaled the expression value for each gene in the [0,100] range using an affine transformation and summarized the scaled expression levels in a clustered heatmap using the Pearson correlation for the clustering to reveal patterns
Chapter 2. Materials and Methods

of expression. The nine unsupervised clusters were then grouped in four distinctive patterns of expression. Here, we focused on Pattern 3 (162 genes). We ran gene enrichment analysis for each of the observed patterns using g:Profiler, against the standard GO terms, the KEGG142 and Reactome143 pathway databases, and TRANSFAC146 as transcription-factor-binding motifs prediction database. The background set was comprised of all genes expressed in clusters 8 and 18. Terms and genes were manually curated in accordance to the biological context of this work. We then displayed relevant DEGs resulting from the analysis along the pseudotime trajectory; for each gene, auto-scaled expression was plotted using a generalized additive model for smoothing.

2.11 Figure preparation

Figures were prepared using Adobe Illustrator. Images were generated with Biorender.com as indicated in the relevant legends.

2.12 Statistics

The number of animals used for each experiment are indicated in the figure legends. A minimum of 3 biological replicates were used, unless otherwise specified in the text (“n” in legends refers to number of animals, independent replicates per time point, and/or condition). For image analysis, a minimum of 3 replicates were taken for each sample and representative images are shown in figures. Experimental data are expressed as mean values ± SD, unless otherwise indicated.

Differences between groups/conditions were assessed by using a two-tailed unpaired t-test, one-way, and two-way analysis of variance (ANOVA) as indicated in figure legends. ANOVA based analysis was followed by Tukey’s test for multiple comparisons. All graphs with average fields measurements were corrected by biological replicate, while individual cell measurements have been kept separate. Details are given in relevant Figure legends and exact p values are indicated in up to ten decimal places. Statistical differences between groups were assessed using GraphPad Prism software.
Chapter 3. Results 1 - Oesophageal-to-skin lineage conversion in 3D heterotypic cultures

3.1 Overview

Traditionally, epithelial tissues were thought to be maintained and repaired solely by a designated pool of stem cells (Braun et al., 2003; Croagh, Cheng, et al., 2008; Kalabis et al., 2008; Mascré et al., 2012). More recently, it has become widely accepted that epithelial cell fate is more dynamic than originally thought and cells other than stem cells can contribute to tissue maintenance and repair. In fact, even differentiated cells can re-wire their fate to resume proliferation and contribute to tissue restoration (Blanpain & Fuchs, 2014; Doupe et al., 2012; Ge et al., 2017a; Tata et al., 2013; van Es et al., 2012). This mechanism ensures the readiness of the tissue to respond to external challenges, hence preserving tissue integrity and survival.

Further to cell fate changes in response to stress, epithelial cells are also able to change their identity towards different lineages when exposed to relevant instructive signals (Bonfanti et al., 2010; Claudinot et al., n.d.; Ferrari et al., 1998; Mauger, 1972). Early recombination experiments (i.e. combining epithelium and stroma from different tissue origins) have shown the ability of epithelial cells to adapt their fate, developing into the typical appendages (e.g. salivary glands, hair follicles or feathers) normally found in the host tissue. This capacity is particularly attributed to developing tissues, which are thought to be more permissive to cell fate changes; however, the response of adult tissues has been studied to a lesser extent (Kratochwil, 1969; Lu et al., 2016).

Here, I aimed to shed light onto adult epithelial cell plasticity by combining two architecturally similar tissues: the murine oesophagus and the skin. The main architectural difference between them is that the oesophagus lacks any of the appendages typically found in the skin, including hair follicles (HFs) and sebaceous glands (Giroux et al., 2017). Based on previous recombination studies, I hypothesised that the inductive signals of the skin microenvironment may push
oesophageal cells towards skin lineages. In this Chapter, I investigated this hypothesis by adapting an ex vivo assay that allows co-culture of the oesophageal epithelium and denuded skin stroma (Doupe et al., 2012). Next, I used immunofluorescence and quantitative analysis to evaluate the emergence of cell identity changes. Altogether, I concluded that this system represents a valuable model to capture early plasticity events.

3.2 Adult oesophageal cells re-epithelialize the skin dermis

In order to expose the oesophageal epithelium to the inductive signals of the skin stroma (the dermis), I first sought to denude it from its native epithelium (the epidermis). For this, the epidermal layer – and associated appendages – were carefully dissected away as described in Method 2.2.1. The resulting dermal wholemounts were stained for the cell adhesion receptor integrin-α6 (ITGA6), which labels cells in the basal epithelial layer. The staining revealed that the dermis was largely denuded from any epidermal structures (Figure 10a). Interestingly, dermal niches (i.e. HFs and sebaceous glands) partially retained their architecture even when denuded of their epithelium. Sporadically, some epithelial left overs were found within HFs (see top area in Figure 10a). Altogether, these results confirmed that our technique enabled the dermis to be largely denuded from its overlying epidermal compartment, leaving the skin niches accessible to be re-epithelialized.

Having validated the epidermis-dermis separation, I used the dermis as a substrate to grow a new epithelium of oesophageal origin. Briefly, I cultured pieces of intact oesophageal tissue — including epithelium and stroma — over a larger piece of the dermis (Figure 10b) (see Method 2.2.1). To identify the origin of cells from the two different tissues, samples were harvested from different constitutive reporter mouse lines: the oesophagus was obtained from mTmG animals (R26mTmG), while skin derivatives came from H2B-EGFP mice (CAG::H2B-EGFP), unless otherwise stated (Hadjantonakis & Papaioannou, 2004; Muzumdar et al., 2007). These lines express fluorescence reporters under the control of ubiquitous gene promoters; hence, labelling all oesophageal-derived cells with tdTomato (red) and all skin-derived cells
Figure 10. Oesophageal-dermal 3D heterotypic cultures set up.

(a) Dermis denuded of epidermal compartments. Basal projected view of typical wild-type dermis wholemount following peeling of the epidermis. Insets (dotted lines) show sporadic instances of hair follicles (HFs) remaining in the dermis (right top panels) and a representative socket devoid of HFs (right bottom panels). Remaining basement membrane immunostained for integrin \( \alpha_6 \) (ITGA6) is shown. Blue, DAPI; greyscale, ITGA6; scale 1 mm; inset 50 \( \mu \)m. (b) Schematic representation of the 3D heterotypic culture strategy. Oesophageal epithelial strips obtained from adult tdTomato reporter mice were laid over denuded isolated dermis from H2B-EGFP mice and cultured for up to 10 days. During this time new epithelium forms between the strips, re-epithelializing the dermis. Scale 1 mm.
with EGFP (green). This approach allowed us to follow oesophageal cell growth while visualizing epidermal host cells that may potentially remain. To minimize variation in the epithelial regenerative capacity as well as the dermal inductive signals, all samples were harvested from adult animals between 8-25 weeks of age (exact age is specified in figure legends).

Figure 11. Oesophageal-dermal 3D heterotypic culture as a model of ectopic niche regeneration.
(a) Representative images of wholemounts from 3D heterotypic cultures at day 3 (D3, top) and day 10 (D10, bottom) as in Fig.7a. Donor EGFP mouse dermis, and oesophagus from tdTomato mouse line. (b) Confocal images showing sporadic chimeric (oesophageal-tdTomato/host-EGFP) skin in vitro. Tissues for cultures were harvested from adult animals between 18-22 weeks of age. Green, EGFP; Red, tdTomato; scale 50 µm.
Subsequently, oesophageal-dermal composites — from now onwards referred to as 3D heterotypic cultures — were maintained in vitro under minimal culture conditions (i.e. without added growth factors, other than those intrinsically present in serum; see Methods 2.2.1). Full-thickness tissue wholemounts were then inspected at several time points to monitor epithelial cell growth. Oesophageal cells (tdTomato) were found to migrate out of the tissue strips and progressively re-epithelialize the denuded dermis (EGFP) (Figure 11a). This process was reminiscent of tissue re-epithelialization during wound-healing; here, the denuded dermis represents a shallow wound, where the dermis is intact, and the oesophageal explant model the damaged epithelium contributing to tissue repair (Aragona et al., 2017; Doupe et al., 2012; Park et al., 2017). Over a period of 10 days (D10) in culture, a new epithelial sheet was formed (oesophageal-derived SKIN; oSKIN) (Figure 11a). Interestingly, I noticed that host epidermal cells (EGFP) occasionally contributed to tissue re-epithelialization forming EGFP+ patches in the otherwise tdTomato+ oSKIN (Figure 11b). This observation indicated that host epidermal cells retained — at least partially — their expansion potential in 3D heterotypic cultures, providing a valuable internal control of epidermal identity (skin-derived SKIN; sSKIN).

To investigate the architecture of the newly formed epithelium at temporal resolution, heterotypic cultures were sectioned at different time points. Confocal imaging showed that the dermis was typically covered with the new oSKIN by D5; however, a more mature tissue structure — denoted by epithelial stratification and thickening — was reached at later time points (D8-10) (Figure 12a). Of key importance, oesophageal cells also repopulated the dermal sockets left by the original epidermal HFs and corresponding sebaceous glands. To distinguish between oesophageal-derived compartments, from now on I will refer to the HF-like structures as oesophageal-derived HFs (oHF) and to the overlying epithelium as oesophageal-derived epidermis (oIFE), which together comprise the oSKIN (oHF + oIFE = oSKIN).

Importantly, the newly formed oSKIN epithelium was amenable for whole-mounting techniques allowing its 3D visualization by confocal microscopy (Figure 12b). Closer inspection of oHF appendages unveiled their strikingly similar morphology when compared to native skin HFs. In line with this, oHF histological analysis revealed the formation of a squamous stratified epithelium (Figure 12c); with a highly compact
basal cell layer and considerably larger and flattened cells in the suprabasal compartment. This, together with the formation of a highly-keratinized cylinder at the centre of the oHF, suggested that cell differentiation occurred concentrically as in native HFs. Altogether, these observations prompted me to investigate oHF structures further.
Figure 12. Adult oesophageal cells re-epithelialize the denuded dermis forming typical skin appendages.

(a) Cryosections of heterotypic cultures at the indicated time points throughout the 10-day culture.Insets show higher magnification images, illustrating dermis re-epithelialization by the oesophageal-derived cells (tdTomato, red). n=3 animals. Scale 1 mm; inset 50 μm. (b) Confocal images showing epithelial wholemounts of skin (top) from H2B-EGFP mice and oesophageal-derived skin from heterotypic cultures (bottom) at 10 days (D10) as in Fig. 7a. Insets depict typical hair follicles (HF; top) and oesophageal-derived HFs (bottom). Scale 1 mm. (c) H&E sections showing formation of oesophageal-derived HFs in heterotypic cultures over a 10-day period. Dashed grey lines indicate basal membrane. Right panel is a representative image of basal and suprabasal cell layers in oesophageal-derived HFs at D8-10. Tissues used for cultures were from adult animals between 18-22 weeks of age. Green, EGFP; red, tdTomato; scale 50 μm.
Figure 13. Oesophageal-derived HF quantification denotes high re-epithelialization efficiency.
(a) Schematic exemplifying the method to quantify the re-epithelialization efficiency of the heterotypic culture. Donor green Lgr5-EGFP mouse dermis, and oesophageal epithelium from tdTomato mouse line. (b) 3D images of in vivo skin wholemount (left image, inset shows in vivo Lgr-5GFP HFs, delineated by white dashed lines) and oesophageal-derived skin from heterotypic culture at 10 days (D10; right image, white dashed lines delineate oesophageal strips). The number of individual HF units (white crosses) were measured before (green) and after (red) peeling to measure the efficiency of oesophageal-derived HF formation. n=3 animals. Animals were adults between 18-22 weeks of age. Green, EGFP; Red, tdTomato; scale 1 mm.

To measure the efficiency of HF re-epithelialization, the original number of HFs in the skin as well as the number of oHF formed upon culture were quantified (Figure 13). For this, I made use of the Lgr5-CreGFP mouse line that constitutively expresses EGFP (green) under the control of Lgr5 promoter; hence, labelling HF stem cells

Efficiency = 91.4 ± 1.8%
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(Barker et al., 2008). This allowed me to accurately quantify skin HFs despite the high hair density. Subsequently, the quantified skin samples were denuded of their epidermal layer and combined with tdTomato oesophageal tissue as previously described (see Methods 2.2.1). Upon 10 days, the number of \textit{de novo} oHFs (red) was quantified. These results estimated that 91.4 ± 1.8\% of the original HFs had been replaced by \textit{de novo} oHFs. From this data it was possible to conclude that HF re-epithelialization in 3D heterotypic cultures is a very efficient and highly reproducible event.

Finally, I sought to define tissue integrity in the newly formed epithelium. To this end, cell proliferation and cell death were investigated in oSKIN — both oIFE and oHF — and compared to oesophageal controls generated as isotypic cultures (oesophageal epithelium reconstituted over its own native stroma; oOE) (Figure 14) (Doupe et al., 2012). To visualize actively cycling cells, oesophageal tissue was obtained from R26Fucci2a reporter mouse line that incorporates fluorescent reporters associated with cell cycle stage-specific proteins (Figure 14a) (Miyawaki et al., 2014). In line with the \textit{in vivo} oesophageal epithelium, oOE proliferation was restricted to the basal cell layer (Doupe et al., 2012; MESSIER & LEBLOND, 1960; Piedrafita et al., 2020b). This was also the case for oSKIN, suggesting that independently of the tissue substrate cell proliferation remains compartmentalized. To further assess epithelial tissue integrity, I investigated the presence of apoptotic cells. Caspase-3 staining showed sporadic cell death in the basal layer of oOE and oSKIN wholemounts (Figure 14b and c). However, opposite to the UV-irradiated control, these numbers were negligible and comparable to those found in the \textit{in vivo} tissues (OE, HF and IFE). Altogether, these results suggest that oSKIN retains \textit{in vivo} features, being sustained by basal cell proliferation and retaining low Caspase 3 levels.

Overall, these results showed that the adult oesophageal epithelium is competent to re-epithelialize the skin dermis \textit{in vitro}, reconstituting a stratified squamous epithelium and forming HF-like structures. Of key note, these samples were amenable for whole-mounting techniques and 3D imaging; hence, representing a comprehensive model to investigate cell fate plasticity.
Figure 14. Oesophageal-derived skin is sustained by basal cell proliferation and retains low Caspase 3 activity.

(a) Top: images illustrating oesophageal (control) and heterotypic cultures. Oesophagi from Fucci2a mice were grown for 10 days as isotypic cultures (oesophagus on submucosa; top) and heterotypic (oesophagus on dermis; bottom). Bottom: side view of oesophageal-derived oesophagus (oOE) and oesophageal-derived skin (oSKIN), including oesophageal-derived interfollicular epidermis (oIFE; left panel) and oesophageal-derived HF (oHF; right panel). Dashed white lines indicate basal membrane. Schematic at the bottom illustrates the fluorescent expression pattern of the cell cycle reporter mouse line Fucci2a (mCherry, G1 cells; mVenus, S/G2/M cells).
Blue, DAPI; red, mCherry; Green, EGFP; scale 50 μm. (b) Representative single-plane confocal images showing expression of active Caspase3 (aCaspase3+). Ultraviolet (UV)-exposed oesophageal epithelium was used as a positive staining control (UV). Oesophageal epithelium (OE) in vivo and in vitro as tissue of origin controls. Hair follicles (HF) in vivo and in vitro (from heterotypic cultures). Interfollicular epidermis in vivo and in vitro (from heterotypic cultures). Blue, DAPI; red, tdTomato; green, EGFP; scale 50 μm (c) Quantification from (b) of active Caspase3+ cells in vitro and in vivo tissues as a percentage of the total number of cells (DAPI+). Data presented as mean ± SEM and analysed using a two-tailed unpaired t-test. Points in the graph show individual measurements and statistics were performed on biological replicates (grey and blue scale indicate biological replicates, n=3). The UV control was used as a reference qualitative control. Animals were adults between 15-18 weeks of age.

3.3 Expression of tissue-associated markers

Having developed a model of ectopic niche regeneration, I decided to investigate whether this system enabled oesophageal cells to transition towards skin identity as recently described in a transplantation model system (Claudinot et al., 2020). To this end, oSKIN wholemounts were immunostained for typical HF and oesophageal markers, and analysed by confocal imaging. The marker expression pattern was then compared to that of: (i) in vivo oesophageal and skin wholemounts (OE and SKIN), and (ii) the individual tissues cultured as isotypic cultures (oesophagus and skin epithelium grown over their native stroma; oOE and sSKIN;) (Figure 15).
Figure 15. Schematic illustrating in vivo and in vitro samples analysed for expression of tissue-associated markers.

The oesophageal epithelium (OE) and the skin, including interfollicular epidermis (IFE) and hair follicles (HF), were harvested from tdTomato and H2B-EGFP mice, respectively. Oesophageal-derived oesophagus (oesophagus on submucosa; oOE) and skin-derived skin (epithelium derived from host epidermal cells remaining in the dermis; sSKIN) - grown in culture for 10 days - were used as controls. Likewise, oesophageal-derived skin (oesophagus on dermis; oSKIN) was harvested following 8/10 days in culture.

3.3.1 Hair follicle-associated markers

First, I investigated expression of typical HF keratins, Keratin 17 (KRT17) and Keratin 24 (KRT24), as well as the well-known HF stem cell marker CD34 (Hsu & Fuchs, 2021; Kevin M McGowan et al., 2002; Trempus et al., 2003). For this, oSKIN wholemounts and relevant controls were immunostained with appropriate antibodies. Confocal analysis revealed that oesophageal cells regenerating oHF structures often
showed positive staining for these markers (Figure 16). Of note, oesophageal cells grown on their native stroma (oOE) showed no signal for any of them, consistent with the in vivo oesophageal epithelium. This result ruled out artefactual effects derived from in vitro conditions and suggested that HF marker expression was induced in response to the HF niche. Further evidence of this was the observation that oIFE cells did not express HF markers; therefore, recapitulating the marker distribution found in the in vivo skin (Figure 17) (Rompolas & Greco, 2014; Trempus et al., 2003). Overall, these results suggest that cells exposed to HF inductive cues re-specify their fate and acquire HF typical markers.

As described in Chapter 1, the adult skin HFs are highly compartmentalized organs. Typically, markers are confined to particular regions: KRT17 and KRT24 are found throughout the follicular compartment – but not in the sebaceous glands –, while CD34 is restricted to the bulge region (Figure 16) (Ito et al., 2004; Joost et al., 2018; Trempus et al., 2003). In contrast, oHF lacked clear morphological compartments and HF keratins and CD34 expression was found throughout the oHF (including the regions corresponding to the sebaceous glands). It is important to note that the in vitro skin (sSKIN) expression did not recapitulate the in vivo pattern either. This result suggests that the in vitro conditions may alter the typical HF marker compartmentalization.
Figure 16. Oesophageal-derived hair follicles switch on expression of hair follicle markers.

(a) Representative 3D rendered confocal z-stacks showing expression of the typical hair follicle markers CD34 (top panel), KRT17 (middle panel) and KRT24 (bottom panel).
panel). Oesophageal epithelium in vivo (OE) and in vitro (oOE) from tdTomato mice as tissue of origin controls (left columns). Oesophageal-derived skin (oSKIN) from heterotypic cultures (middle column). In vitro (sSKIN) and in vivo skin (SKIN) from EGFP mice. All in vitro samples were cultured for 8/10 days. Images were acquired with equivalent confocal settings. For each marker, n=3 animals. (b) Illustration indicating the percentage of oHF units expressing KRT17, KRT24 and CD34 relative to total oHF, quantified from images in (a). Presented as mean ± SEM. n=3 animals. Tissues were harvested from adult animals between 9-13 weeks of age. Green, EGFP; red, tdTomato; white, HF markers CD34 (top panel), KRT17 (middle panel), KRT24 (bottom panel); scale 50 μm.

Figure 17. Oesophageal-derived interfollicular epidermis does not acquire expression of typical HF markers.
Representative confocal z-stacks showing a basal view of oesophageal-derived IFE (oIFE) upon 8/10 days in culture. Images were acquired with equivalent confocal settings as in Fig.16. n=3 animals. Tissues were harvested from adult animals between 9-13 weeks of age. Red, tdTomato; white, HF markers KRT17 (left column), KRT24 (middle column) and CD34 (right column); scale 50 μm.
To gain a quantitative insight into cell fate conversion, I assessed the proportion of oHF units expressing HF markers. For this, a minimum of 150 oHF units (n=3 animals) were examined by confocal microscopy (see Method 2.6.1). Interestingly, although most oHF exhibited marked levels of KRT17 and KRT24 (~78% and ~91%, respectively), only a subset of them expressed the HF stem cell marker CD34 (~28%) (Figure 16b). This was in direct contrast to the well-studied skin HFs, that are known to be sustained by a CD34+ stem cell population that is found in all adult HFs independently of their cycle phase (Alcolea & Jones, 2014; Rompolas & Greco, 2014; Trempus et al., 2003; Vidal et al., 2005). This discrepancy prompted me to investigate the number of CD34+ cells per HF unit in oSKIN as well in vivo skin. For this, a minimum of 18 HF units (n=3 animals) were quantified (see Method 2.6.1). This analysis revealed a remarkably low proportion of CD34+ cells (8.8 ± 1.34 cells per oHF on average) when compared to in vivo skin wholemounts (177.9 ± 15.7 cells per HF on average) (Figure 18a). Not only was CD34 expression found to be a rare event but also widely variable between oHF structures, ranging from 1 to 59 CD34+ cells per oHF (Figure 18a; inset). I reasoned that the observed disparity in marker expression may be due to an inefficient cell identity transition, where most oHF cells start a lineage conversion process — as indicated by KRT17 and KRT24 expression — but only a subset executes the transition, acquiring the CD34 HF stem cell marker.
Figure 18. The limited CD34 expression in oesophageal-derived hair follicles is not a result of cell fusion nor contribution of residual mesenchymal cells.

(a) Violin plots showing the distribution of CD34 positive cells per HF in skin and oesophageal-derived skin (oSKIN). Quantification represented a total of 4091 CD34+ cells out of 23 HF (n=8 animals) and 678 CD34+ cells out of 77 oHF (n=14 animals).

(b) Schematic illustrating the strategy to identify cell fusion events. Nuclear labelling of oesophageal and epidermal cells (with tdTomato and EGFP, respectively) allows visualization of double-positive nuclei as well as binucleated cells. (c) Schematic illustrating a representative chimeric oHF, where tdTomato (oesophageal origin) and
EGFP (skin origin) cells co-exist but do not fuse. No cell fusion events were found in over 60 oHF (n=3). (d) CD34+ cells have an epithelial origin. Top: Single plane confocal images showing CD34+ cells express the epithelial marker KRT14 and are negative for the fibroblast marker PDGFRα. Bottom: stromal fibroblasts showing CD34 and PDGFRα staining. Tissues were harvested from adult animals between 9-16 weeks of age. Magenta, KRT14; cyan, PDGFRα; white, CD34; scale 50 μm.

Subsequently, I aimed to rule out alternative sources of variability that may have explained the sparse CD34 expression: sporadic cell fusion events and the intermittent contribution of mesenchymal cells (CD34+). Transplantation studies have shown the emergence of cell fusion events between cells of different tissue origins (Alvarez-Dolado et al., 2003; Balsam et al., 2004; Xin Wang et al., 2003). This event may result in one nucleus imposing its transcriptional programme on the other (Bonfanti et al., 2012; Terada et al., 2002). To explore this possibility, I cultured nuclear tdTomato oesophageal explants on nuclear EGFP recipient dermis. I speculated that if cell fusion events were occurring, this approach together with E-cadherin cell membrane staining would unveil cells containing double positive nuclei or, alternatively, binucleated cells expressing either reporter protein (Figure 18b). While I found some chimeric tdTomato/EGFP oHFs (Figure 18c), confocal imaging of 60 oHF units (approximately 18,000 cells) revealed one single binucleated cell (<0.0001%) (in collaboration with Maria T Bejar). This rate was significantly lower than that of CD34+ cells (8.8 ± 1.34% CD34+ cells per oHF); therefore, ruling out the likelihood that cell fusion events accounted for the CD34+ population. Instead, I considered whether CD34+ cells may correspond to fibroblast cells, known to express this marker (Kacar et al., 2012). To this end, oSKIN wholemounts were immunostained for CD34 together with epithelial and mesenchymal markers; KRT14 and PDGFRα, respectively (Figure 18d). Immunofluorescence analysis showed negative PDGFRα staining in CD34+ cells, ruling out their mesenchymal origin. In line with this, co-expression of CD34 and the epithelial basal marker KRT14 confirmed the epithelial nature of this population.

Overall, these results support the notion of an inefficient oesophageal-to-skin lineage conversion process, where most oesophageal cells acquire typical HF keratins but only a portion express the CD34 HF stem cell marker. In light of these results, and
given the well described role of CD34 as a HF marker (R. J. Morris et al., 2004), here CD34 was used as a proxy of oesophageal-to-skin lineage conversion.

### 3.3.2 Oesophageal-associated markers

Next, I interrogated the expression of the oesophageal differentiation marker keratin 4 (KRT4). Wholemount analysis of *in vivo* oesophageal tissue showed KRT4 labelling throughout the suprabasal layers, as previously described (Figure 19) (Frede et al., 2016; Giroux et al., 2017; Rosekrans, Baan, Muncan, & van den Brink, 2015). Such a pattern was maintained *in vitro* (oOE) (Doupe et al., 2012). Conversely, oIFE cells only retained KRT4 expression in the upper-most suprabasal layers suggesting a progressive loss of this oesophageal differentiation marker. In support of this, 29% of oHF units were negative for KRT4.

Altogether, the loss of an oesophageal marker together with the acquisition of HF-associated markers support the emergence of oesophageal-to-skin lineage conversion.

*Figure 19. Oesophageal-derived skin downregulates expression of the oesophageal marker KRT4.*
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(a) Side view images of in vivo and in vitro oesophagus (OE and oOE). Of note, the KRT4 restriction to the first suprabasal layer is a wholemount artefact as it is expressed throughout the suprabasal compartment. (b) Side view and single plane images for oesophageal-derived skin (oIFE and oHF, respectively) and single plane images of in vivo and in vitro skin hair follicles (HF and sHF, respectively). Images were acquired with equivalent confocal settings. Dashed lines indicate basement membrane. Animals were adults between 18-22 weeks of age. Blue, DAPI; red, tdTomato; green, EGFP; white, KRT4; scale 50 μm.

3.4 Relevance for other epithelial tissues

Motivated by the previous findings, I decided to evaluate if cell fate changes towards skin lineage were restricted to oesophageal epithelial cells or, alternatively, other epithelial tissues retained skin competency in our 3D heterotypic culture system. To this end, tissue explants from the trachea (pseudostratified epithelium), forestomach (squamous epithelium), corpus stomach (columnar epithelium), and intestine (columnar epithelium) were exposed to the skin dermis in an equivalent manner to oesophageal-skin cultures (see method 2.2.4; Figure 20a). Briefly, tissue explants from tdTomato donor animals were grown on EGFP recipient dermis and maintained in vitro under the same culture conditions indicated above. In parallel, oesophageal-skin cultures were used as controls.

Initially, I addressed the capacity of the different tissues to reconstitute the skin epithelium upon 10 days in culture. To this end, heterotypic wholemounts were imaged and the re-epithelialized area calculated as the percentage of dermis covered by donor epithelium (red). Interestingly, marked variation was observed among different tissues (Figure 20b). The corpus stomach and intestinal epithelia did not grow. Similar to what happens in the oesophagus, the tracheal-derived epithelium re-epithelialized most of the dermis (98.36 ± 0.3%) forming HF-like structures (Figure 20b and c). Finally, forestomach-derived epithelium showed partial re-epithelialization (55.4 ± 8.9% on average), including formation of HF-like structures (Figure 20b and c).
Figure 20. **Tracheal and forestomach cells re-epithelialize the skin dermis in 3D heterotypic cultures.**

(a) Schematic representation of the 3D heterotypic culture strategy using different tissues as epithelial donors. Tracheal, forestomach, corpus stomach and intestine obtained from adult tdTomato reporter mice were laid over denuded dermis from H2B-EGFP mice as in Fig. 7a. Cultures were grown for 8 days. (b) Quantification of the area covered by the newly formed epithelium (tdTomato+) expressed as a percentage of the total dermis area. The oesophagus was used as a positive control. The points show biological replicates, mean ± SEM (n=3 animals). Statistics was performed on the biological replicates using a one-way ANOVA test. Not significant p-values not shown (p-value>0.05). (c) Representative images of wholemounts from tracheal-dermal (top) and forestomach-dermal (bottom) 3D heterotypic cultures at day 8 (D8). Insets depict typical tracheal-derived (top) and forestomach-derived (bottom) HF
structures. Animals were adults between 18-22 weeks of age. Red, tdTomato; scale 1mm; inset 50 µm.

In order to investigate cell re-specification events, I sought to evaluate the acquisition of the HF marker CD34 in tracheal and forestomach-derived skin. One limitation of these tissues is that they were not amenable for epithelial whole-mounting. Hence, CD34 expression had to be assessed in thick sections. Similar to observations in the oesophagus, the tracheal-derived SKIN showed CD34 staining in the lower areas of sporadic HF structures, but not in the overlying epithelium (Figure 21a). Reassuringly, in vivo tracheal controls didn’t show any labelling for CD34 suggesting that its expression was acquired following heterotypic culture (Figure 21b). Conversely, no staining was found in forestomach-derived HF structures (Figure 21c). However, it is worth noting that CD34 expression in tracheal and forestomach-derived HFs was only evaluated once (1 independent experiment). Further experiments and analysis would be required to fully confirm these observations.

This data suggests that the cell fate plasticity in 3D heterotypic cultures goes beyond oesophageal-to-skin lineage conversion, and supports recent findings emphasising that epithelial tissues have variable permissibility to skin cues (Claudinot et al., 2020).
Figure 21. Tracheal cells grown in 3D heterotypic cultures form a squamous stratified epithelium and HF-like structures expressing the CD34 hair follicle stem cell marker.

(a) Tissue cryosections of tracheal-dermal cultures at day 8 encompassing tdTomato tracheal tissue and wild type dermis. Inset depicts single plane images of tracheal-derived IFE (top) and tracheal-derived HFs (bottom). n=3. (b) Tracheal tissue cryosections from tdTomato mice. The white dashed line delineates the basal membrane. (c) Tissue cryosections of forestomach-dermal cultures at day 8. n=1. Animals were adults between 10-15 weeks of age. Blue, DAPI; Red, tdTomato; white, CD34; scale 50 µm (insets 20 µm).
3.5 Importance of the dermis signals

Hair follicle development and regeneration are controlled by soluble signals arising from the dermal papillae (DP), which is located in close proximity to the lower HF (Jahoda et al., 1984; Rompolas et al., 2012). Of note, dermal tissue used in 3D heterotypic cultures retained the DP as indicated by Sox2 staining (Figure 22a). To investigate if the DP may play a role in oesophageal-to-skin lineage conversion, CD34 distribution along the length of the HF was investigated in oSKIN wholemounts. I reasoned that if CD34 was randomly distributed throughout the oHF structure then the specific location and/or the proximity to the DP niche may not account for lineage conversion. In collaboration with Dr Maria T Bejar, a semi-automated segmentation pipeline was employed to determine the cellular CD34 expression intensity along the oHF longitudinal axis (Y) (Figure 22b). This data revealed preferential CD34 expression in the lower oHF, suggesting that signals from this region – possibly arising from the DP – instruct cell fate re-specification. These results support the notion that the specific position of a cell within its niche determines its cell fate, as shown by live-imaging in the HF (Matos et al., 2020; Rompolas et al., 2013).
Figure 22. CD34 expression is highest in the lower oHF in close proximity to the DP.

(a) Tail skin tissue cryosections from H2B-EGFP (green) adult mice before (top panels) and after (bottom panels) peeling off the epidermis. The epidermis (positive for KRT14, cyan) was removed in the peeling process, whereas dermal papillae (positive for SOX2, magenta) remained in the HF socket. (b) CD34 intensity quantification along the oHF longitudinal axis (Y) shows increased expression towards the lower oHF. Y axis position was normalised to the lowest point for each individual oHF. Animals were adults between 10-15 weeks of age. Blue, DAPI; White, CD34; Scale 50 μm (a), 20 μm (b).
Next, I sought to test whether the biochemical/mechanical cues arising from the dermal cells were indeed required for oesophageal cell identity changes or, alternatively, whether the dermis 3D architecture was sufficient. To discern between these possibilities, a dermis scaffold devoid of its cellular component was used for cultures. The skin dermis was decellularized using a combination of chemical and enzymatic methods (as described in Method 2.7). This approach has been shown to efficiently remove the tissue cellular component with minimal ECM damage (Kristofik et al., 2017; Simsa et al., 2018). Indeed, histological analysis confirmed that this decellularization method led to complete cell removal while maintaining the dermis architecture (Figure 23a). Subsequently, tdTomato oesophageal tissue was grown on the decellularized dermis. While oesophageal cells were able to expand and colonize the decellularized dermal HF 'sockets', their morphology was impaired when compared to non-decellularized cultures (Figures 23b). In line with this, cell re-epithelialization was also compromised; with re-epithelialization being 3-fold lower when compared to controls (Figure 23c and d). These results indicated that biochemical/mechanical signals from the dermal cells are required for appropriate oHF development. To confirm that cells were not undergoing cell fate conversion, I evaluated CD34 expression in epithelial wholemounts grown in combination with control or decellularized dermis. Remarkably, no CD34 staining was found in oesophageal-derived structures grown on decellularized dermis (Figure 23e). These results indicate that the dermis scaffold is not sufficient for lineage conversion in our system and emphasize the need of the dermal cellular component.
Figure 23. Dermis scaffold as a substrate for 3D heterotypic cultures.
(a) H&E sections of control and decellularized dermis showing efficient removal of the cellular component and maintenance of skin appendage structures following decellularization. Dashed lines indicate basement membrane. (b) oHF grown on decellularized dermis show impaired morphology. Confocal side view showing oSKIN (tdTomato) cultured on top of denuded live dermis under control conditions (as per Figure 7a) and over decellularized dermis (from panel a; bottom) for 8 days. Red, tdTomato. (c) Representative basal views of typical heterotypic culture wholemounts showing impaired re-epithelialization of decellularized dermis compared to controls. Red, tdTomato. (d) Quantification of re-epithelialization (tdTomato) in control and decellularized dermis (from c), expressed as percentage of re-epithelialized area relative to total dermis area. Data expressed as mean ± SEM. Points in the graph show individual measurements; grey and blue scale indicate biological replicates (n=3). Statistics was performed on biological replicates using a two-tailed unpaired t-test. (e) Decellularized dermis fails to induce CD34 expression in oesophageal-derived HF...
(oHF). Representative 3D rendered side views showing CD34 expression in oHF grown over control and decellularized dermis (as in b). Out of a total of 82 oHFs, no CD34+ cells were found when exposed to decellularized dermis (n=3 animals). Animals were adults between 10-15 weeks of age. Greyscale, CD34; scale 50 µm (a-b, d-e), 1 mm (c).
3.6 Discussion

In this chapter I described the development of a 3D heterotypic culture system that combines adult oesophageal tissue and the adult skin dermis constituting a model of ectopic niche regeneration. Temporal inspection of this model revealed dermis re-epithelialization as well as the formation of hair follicle-like structures by oesophageal cells (oHFs). Here, I used immunofluorescence together with quantitative analysis to investigate the potential of this ex vivo system as a model to study epithelial cell fate plasticity.

Initially, I interrogated the emergence of cell fate changes using surrogate markers of skin identity; a commonly used approach in the plasticity literature (Bonfanti et al., 2010; Claudinot et al., 2020; Ferrari et al., 1998; Lu et al., 2016). Interestingly, this revealed once the expression of HF keratins (KRT17, KRT24) and the HF stem cell marker CD34. In this regard, it is important to highlight that although CD34 expression was once reported in the oesophageal epithelium (OE), a later study using an extensive battery of CD34 antibodies confirmed that only resident immune cells (CD45+) sporadically found in the OE expressed this marker. Consistent with this, in our hands OE controls were negative for CD34; hence, validating this marker as a surrogate of lineage conversion towards HF lineage.

The selective acquisition of HF markers in oHF structures but not in the overlying oesophageal-derived epidermis (oIFE) was reminiscent of the compartmentalized nature of the skin and supported the theory that niche location imposes distinct stem cell fates (Matos et al., 2020; Rompolas et al., 2013; Y. V Zhang et al., 2009). The differences in response to either dermal niche (that of the HF or IFE) could be explained by the embedding of oesophageal cells within the HF region – therefore, increasing the 3D exposure to the localized dermal signals – as well as their proximity to the dermal papillae. Interestingly, despite the fact that oHFs showed expression of HF markers, it is worth noting that their expression did not recapitulate the distribution found in the in vivo adult HFs (Joost et al., 2018; Rompolas & Greco, 2014; Trempus et al., 2003; Tumbar et al., 2004). In fact, it is arguable how much the newly formed oHF mirror the different and distinctive niches found in vivo. The lack of well-defined HF compartments was reminiscent of the developing HFs that don’t
acquire their characteristic morphology – including the bulge protrusion – until the first postnatal hair cycle (~p20-21) when mice have a full hair coat (Nowak et al., 2008; Schmidt-Ullrich & Paus, 2005).

The acquisition of HF markers convinced us that lineage conversion was happening. Interestingly, quantitative analysis of HF markers revealed that while most oHFs expressed HF keratins (>75%) only a small subset of them acquired the HF stem cell marker CD34 (~28%). Further to this, a remarkably low number of cells expressed this marker. Following this, I explored potential sources of variability that could account for the low number of CD34+ cells; including the sporadic emergence of oesophageal-epidermal cell fusion events, as previously identified in other systems (Alvarez-Dolado et al., 2003; Balsam et al., 2004; Xin Wang et al., 2003), or the arbitrary contribution of mesenchymal cells (CD34+) to oHF formation. However, cell fusion and mesenchymal cells did not account for CD34 expression. Hence, given the well-documented role of CD34 in HF stem cell identity I hypothesized that such discrepancy in marker expression may be due to a halted lineage conversion process; in which most oHF cells commence a lineage transition – acquiring HF keratins – but only a limited number of those execute the transition and acquire CD34. This hypothesis is further reinforced by previous studies describing cell reprogramming and transdifferentiation events as highly inefficient processes (Bonfanti et al., 2010; Fischberg et al., 1958; Kratochwil, 1969; Saito et al., 2015; Takayama et al., 2010).

Consistent with limited lineage conversion, investigation of the oesophageal marker KRT4 revealed that only a proportion of oHF (~29%) lost its expression. Given the similar numbers when compared to CD34+ oHF (~28%), it is tempting to speculate that the loss of KRT4 and the acquisition of CD34 are connected. It would be of interest to look into the potential correlation between these throughout the lineage conversion process, which may give a temporal insight into the loss of oesophageal traits and the acquisition of skin identity. This data would help to determine whether oesophageal-to-skin lineage conversion is a binary process or, alternatively, as described in other transdifferentiation processes such as EMT, it occurs in a step-wise manner through different intermediate stages (Pastushenko et al., 2018; Jingyu Zhang et al., 2014).
To evaluate if lineage conversion was specific for oesophageal cells, I investigated the phenotype of other adult epithelial tissues when exposed to the skin dermis. I found that the competency to form a new epithelium with HF-like structures was not a distinct feature of the oesophageal epithelium, as later revealed by an in vivo recombinant transplantation model (Claudinot et al., 2020). Interestingly, despite its pseudostratified epithelial architecture, tracheal-derived cells formed a stratified squamous epithelium suggesting that dermal cues could change their morphogenic capacity. Like in the oesophagus, tracheal-derived HFs also expressed the HF stem cell marker CD34. This was in direct contrast with the work by Claudinot and colleagues, that grafted serially passaged tracheal cells into the newborn skin. Several months following transplant, tracheal cells had formed a skin-like epithelium but no tracheal-derived HFs were found. These conflicting results may be explained by the markedly different experimental designs used in the two studies. Here, I investigated tissue re-epithelialization in vitro, as opposed to de novo HF formation upon cell transplantation. Further to this, the considerably longer timepoint used by Claudinot– 122 days post-transplantation – raise the possibility that while HFs were formed, they were not maintained long term.

When other donor epithelial tissues were used in 3D heterotypic cultures I observed significant phenotypical differences. While the squamous epithelium of the forestomach was competent to form HF structures, it showed compromised dermal re-epithelialization. Nonetheless, given the wide variation observed between experiments and even repeats it is likely that these differences arise as a result of technical variation. In fact, in contrast to the oesophagus, the stomach tightly adhers to the underlying muscle – hampering its separation –; as a result, remaining muscle fibers may have led to impaired epithelial expansion. The lack of lineage conversion events in forestomach-derived HF structures – as determined by CD34 expression – was unexpected given that the transitional epithelium between the upper digestive tract (squamous) and the corpus stomach (columnar) is thought to be more susceptible to cell plasticity (Van Zanten et al., 1999). However, given the limited number of repeats (n=1 independent experiment), more data would be needed to draw any conclusion. Other tissues from the gastrointestinal tract (including corpus stomach, and intestine) failed to expand and re-epithelialize the skin dermis in 3D
Chapter 3. Results 1 - Lineage conversion in vitro

heterotypic cultures. This is most likely due to their dependency on growth factors such as Wnt agonist Rspondin, EGF and BMP inhibitor Noggin, as shown by intestinal and gastric organoids (Sato et al., 2011; Sato & Clevers, 2013; Seidlitz et al., 2021). Further to this, grafting experiments have shown that the intestinal epithelium is refractory to skin morphogenetic signals (Claudinot et al., 2020). Altogether, this data suggested that lineage conversion towards skin lineage was not specific for oesophageal cells, as previously reported by transplantation studies (Bonfanti et al., 2010; Claudinot et al., 2020). However, due to technical limitations I could not investigate the extent of this plasticity by quantitative analysis.

Finally, I described that the oesophageal-to-skin lineage conversion events were dependent on the dermal cellular component. Further evidence of this was the observation that cells at the lower oHF - in close proximity to the DP - expressed higher levels of the HF marker CD34 than those located in the upper oHF. Overall these results suggested that lineage-conversion may be regulated by the DP, the main orchestrating centre during HF growth (Abaci et al., 2018; Higgins et al., 2013; Jahoda et al., 1984). This hypothesis may be further investigated by laser-induced ablation of the DP (Rompolas et al., 2012), which would allow me to determine whether the DP plays a major role compared to other mesenchymal components.

The results summarised above cover the questions set out at the beginning of this chapter, suggesting that adult murine oesophageal cells are able to change identity when exposed to the ectopic cues of the skin stroma. Indeed, oesophageal cells were found to reconstitute the skin epidermis and acquire expression of typical skin markers, denoting a lineage conversion towards skin identity. Of particular interest, this process was very inefficient suggesting that there must be mechanisms that restrict cell fate plasticity at the cellular level. Together, this chapter described an in vitro model that unleashes cell plasticity events providing a valuable platform to investigate the regulatory processes that govern cell identity changes.
Chapter 4. Results 2 - Transcriptional analysis reveals a signature consistent with epithelial morphogenesis

4.1 Overview

Mounting evidence support the idea that epithelial cell fate is more dynamic than originally thought. Nowadays, it is well accepted that epithelial cells have the ability to rapidly adapt their fate in response to changing tissue needs (Doupe et al., 2012; Ito et al., 2004; Levy et al., 2007; Tata et al., 2013; van Es et al., 2012). This plasticity grants cells with great regenerative capacity, ensuring tissue integrity. Recombination studies have shown that epithelial cells can change their identity and form appendages from a different nature (e.g. feathers, scales, salivary glands) when exposed to ectopic cues from the relevant tissue (Bonfanti et al., 2010; Ferraris et al., 2000; Kratochwil, 1969; Mauger, 1972). Here, I described an *in vitro* regenerative system, where oesophageal epithelial cells are instructed towards the epidermal lineage. Interestingly, I found that a proportion of cells acquired the new identity as instructed by the ectopic niche (as described in Chapter 3). Yet, the lineage conversion efficiency was remarkably low raising questions about the processes regulating cell fate changes.

In this Chapter, I sought to investigate the mechanisms leading to cell fate conversion as well as those that may limit this process. This knowledge has traditionally been obscured due to the low resolution of available technologies, hence preventing the investigation of a scarce event such as cell fate conversion. Given the advent of RNA-sequencing technologies, here I chose to use low-input RNA sequencing of micro-dissected samples. This approach allowed me to molecularly profile specific tissue compartments (i.e. HF and IFE), hence aiding in spatial resolution. Altogether, I concluded that oesophageal cells re-epithelializing the skin dermis activate a developmental signature consistent with epithelial morphogenesis.
4.2 Low-input RNA sequencing method

Histological characterization of 3D heterotypic cultures (oesophageal epithelium grown over skin dermis; oSKIN) unveiled that oesophageal cells are directed towards epidermal identity when exposed to the instructive cues of the skin. Of particular importance, only a subset of cells was found to redefine their identity, acquiring traits associated with the epidermal lineage (as described in Chapter 3). Here, to investigate the processes modulating the cell identity switch while partially retaining sample heterogeneity, I chose to execute a low input RNA-sequencing experiment.

To this end, I developed a microdissection technique that allowed the manual isolation of micrometric tissue samples enabling the independent analysis of different tissue compartments i.e. oHF and oIFE (Figure 24a). These were analysed together with appropriate controls (as described in Chapter 3), which included: (i) isotypic cultures as an in vitro control (i.e. oesophageal epithelium on the oesophageal stroma; oOE); (ii) the adult skin as reference control of the host tissue, and (iii) the adult oesophagus as a tissue-of-origin control. Further to this, given the regenerative nature of my in vitro system, where cells are starting to respond to dermal signals, I speculated that the oSKIN may be more similar to neonatal skin than adult skin. To investigate this possibility, I included an additional skin control at the latest stages of the first anagen before the onset of the first postnatal HF cycle: (iii) neonatal skin at postnatal day 10 (p10) (Müller-Röver et al., 2001). All the dissected samples are summarized in Figure 24b.

Briefly, oesophageal tissue strips obtained from tdTomato mice (R26nTnG mouse line) were cultured onto denuded stroma (submucosa/dermis) constitutively expressing EGFP (CAG::H2B-EGFP). Cultures were maintained in vitro under minimal medium i.e. absence of added growth factors other than those inherent to serum (as described in Method 2.2.1). The newly-formed epithelial sheet was separated from the stroma at D10 and the regions of interest micro-dissected under the fluorescence microscope, together with in vivo controls (Method section 2.10.1). The dissected samples were then lysed in a single drop of lysis buffer prior to RNA extraction. Initial optimization of the technique included the assessment of RNA yields and quality check. Bioanalyzer analysis determined that single oHF units
Figure 24. Microdissection-coupled RNA sequencing experimental workflow. (a) Overview of the low-input RNAseq experimental workflow. Left: The epithelial sheet from 3D heterotypic cultures (oesophageal tissue grown on dermis; oSKIN) and isotypic cultures (oesophageal tissue grown on submucosa; oOE) were peeled from the underlying stroma at day 10 post-culture. The skin and oesophageal epithelium of adult tdTomato mice (mTmG mouse line), together with neonatal skin epithelium (mTmG mouse line) were harvested as in vivo controls for cell identity. Right: Epithelial samples were manually micro-dissected into single oesophageal-derived HF (oHF) or oesophageal-derived IFE (0.5 x 0.5 mm) together with controls of equivalent size. Samples were then lysed and sent for RNA sequencing (Nextera). Red, tdTomato; scale 1mm. (b) Sample collection details for RNA sequencing experiment. Tissues for cultures were harvested from adult animals of 8-12 weeks of age, while those for in vivo reference control purposes were collected from either postnatal mice of 10 days of age or adults between 8-9 weeks of age.
yielded ~50 pg/μg RNA with suitable quality for sequencing purposes. In contrast, skin HFs gave poor RNA integrity. Given that this was not the case for neonatal HFs (nHFs) or any other sample type, I reasoned that poor RNA yields may be due to the mature hair shaft interfering with the RNA isolation step and/or contributing to RNA degradation. To circumvent this, I repeated the isolation of adult HFs cutting off the hair prior to tissue lysis; however, similar outputs were achieved. For this reason, adult skin HFs were not included in the analysis.

Further to this, libraries were generated using Nextera Libraries at the Cambridge Stem Cell Institute Genomics facility. The resulting 57 libraries were sequenced using Illumina NovaSeq in the CRUK-CI Core Facility. Subsequent processing and analysis of the RNA-seq data was executed in collaboration with Ilias Moutsopoulos and Dr Irina Mohorianu (from the Cambridge Stem Cell Institute Bioinformatics facility).

4.3 Comparisons of in vitro and in vivo samples

First, I sought to define whether oHF and oIFE samples – exposed to the skin niches – showed a similar signature to that of in vivo skin samples. I reasoned that if oesophageal-to-skin lineage conversion events were occurring in these samples, their transcriptional profile would share traits with the skin. To investigate this, in vivo and in vitro samples were visualized by principal component analysis (PCA) (Figure 25). This showed a partial overlap between in vitro samples (oHF, oIFE and oOE), which were widely distributed throughout the PCA. In contrast, in vivo samples (nHF, nIFE, IFE, OE) formed compact clusters clearly segregated in the PCA space. Of note, in vitro samples were found to be transcriptionally different to in vivo samples. This data suggested that in vitro conditions impose a new signature. From this, I concluded that the experimental conditions (i.e. in vitro or in vivo) may account for most transcriptional differences observed between samples.
Figure 25. Principal component analysis (PCA) denotes segregation between in vitro and in vivo samples.

(a) Principal component analysis (PCA) for 50 most abundant genes from RNA sequencing experiment (see Figure 24). Green circle encompasses in vitro oesophageal-derived tissues including oesophageal epithelium (oOE), oesophageal-derived HFs (oHF) and oesophageal-derived IFE (oIFE). In vivo controls include adult oesophageal epithelium (OE), adult epidermis (IFE), neonatal HFs (nHF) and neonatal IFE (nIFE). Points represent individual samples (in vitro samples, n=3; in vivo samples, n=1).
To identify what transcriptional changes drive oesophageal-to-skin lineage conversion (oOE to oHF), I assessed common differentially expressed genes (DEG) in these conditions. To this end, I searched for genes upregulated in the nHF and oHF when compared to their respective oesophageal counterparts (OE and oOE) (Figure 26a). Interestingly, 35 genes were found to be upregulated in HF samples (nHF and oHF). Gene Set Enrichment Analysis (GSEA) revealed the upregulation of genes associated with tissue development and epithelial morphogenesis, consistent with immature cell states (Figure 26b). Of note, these denoted activation of the BMP signalling pathway, a key regulator of epidermal cell fate specification (Bmp1, Fst, and Id3) (Figure 26c). Further to this, I found an enrichment for genes involved in cell migration (Igfbp3, Arhgef1, Lamc2 and Nrp2), as well as hair cycle (Krt17, Krt16, and Msx2) (Figure 26d) (Chao et al., 2021; Kevin M McGowan et al., 2002; Nasarre et al., 2013; Rompolas et al., 2016; Rosekrans, Baan, Muncan, & van den Brink, 2015). Although the latter was of particular interest, the 3 enriched genes found in this gene ontology are involved in various regenerative processes not necessarily associated with HF biology. Hence, I could not conclude that oHF are actively cycling. Altogether, this data suggested that oHF cells activate developmental and regenerative features, and migratory genes.
Figure 26. Oesophageal-derived HFs (oHF) and neonatal HFs upregulate genes associated with tissue development and cell migration.

(a) Venn diagram showing the overlap of differentially expressed genes (DEGs) between oesophageal-derived hair follicles (oHF) and neonatal hair follicles (nHF) when compared to the in vitro oesophagus (oOE) and the in vivo OE, respectively. (b) Gene enrichment analysis for shared HF signatures (a), compiled from Gene Ontology Biological Process (GO:BP), Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Reactome (REAC) databases. (a) (d) Violin plots showing the expression of genes associated with tissue development (c) and cell migration (d) found to be upregulated in the HF-shared signature (a, b). Data expressed as arbitrary units (a.u.).
4.4 Comparison of *in vitro* transcriptional profiles

Next, I considered whether the differences between *in vivo* and *in vitro* conditions may have masked distinct signatures among *in vitro* samples. To investigate oesophageal-to-skin lineage conversion in more detail, *in vitro* samples were analysed separately. Differential expression analysis showed over 800 differentially expressed genes (DEG) between oSKIN (oHF and oIFE samples) and oOE (Figure 27a). Further to this, over 600 DEG were found between oHF and oIFE samples. These results supported the emergence of different cell profiles based on the stroma tissue-of-origin (i.e. oesophageal or skin), as well as the specific dermal compartment to which cells are exposed (i.e. HF or IFE). This data was consistent with the histological characterization shown in Chapter 3.

To investigate the molecular changes associated to ectopic regeneration, I performed GSEA of genes upregulated in oHF or oIFE relative to oOE. Interestingly, both samples showed enrichment for genes associated to tissue development (Figure 27b) denoting the activation of the Wnt (*Wnt4, Wnt10a, Ccn1* and *Stk4*) and the BMP signalling pathways (*Bmp1, Bmpr2, Id1* and *Id3*), important regulators of epidermal cell fate specification as well as HF development (Figure 27c) (Lim & Nusse, 2013; Rodriguez et al., 2010; Juan Zhang et al., 2012). Concomitantly with the activation of developmental pathways, oHF samples – but not oIFE - upregulated genes related to epithelial differentiation/keratinization (*Dsg1a, Lce1a2, Dsc3* and *KRT16*) as well as lipid metabolic processes (*Ajuba, Cidea* and *Sgpl1, Elovl1*). This signature could potentially reflect cell differentiation in response to the HF niche (Figure 27b and d) (Bach et al., 2017; Palmer et al., 2020; Rudan et al., 2020). Of note, wide gene expression variation was observed among oHF samples, raising the possibility that only a proportion of them acquire a developmental signature.

Altogether, these results suggested that cells exposed to the skin stroma present a signature consistent with epithelial morphogenesis. Interestingly, only cells exposed to the HF niche showed an epithelial differentiation profile, possibly as a result of the structural and biochemical cues associated to this particular niche (Abaci et al., 2018; Higgins et al., 2013; Watt, 2016).
Figure 27. Oesophageal-derived IFE and HF are enriched for developmental pathways, while only oHF upregulate differentiation genes.
(a) Graphical representation of differentially expressed genes (DEGs) between in vitro samples including the oesophageal-epithelium (oOE; from isotypic cultures) and oesophageal-derived HFs and IFE (oHFs and oIFE, respectively; from 3D heterotypic cultures). (b) Tables showing enriched terms upregulated in oIFE (top) and oHFs (bottom). Tables contain a selected compilation of representative terms compiled from Gene Ontology Biological Process (GO:BP), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome (REAC) databases, with their corresponding p-values. Closely related terms are grouped together. (c) Violin plots showing the expression of genes upregulated in oHF and oIFE corresponding to developmental genes as determined by gene enrichment analysis. Data expressed as arbitrary units (a.u.). (d) Violin plots showing the expression of genes upregulated exclusively in the oHF including genes associated to keratinocyte differentiation (top) and lipid metabolic processes (bottom). Data expressed as arbitrary units (a.u.).
4.5 Discussion

In this Chapter I investigated the molecular signature of oesophageal-to-skin lineage conversion. Here, I described a microdissection-coupled RNA sequencing technique to profile anatomically defined areas in oesophageal derived skin (oSKIN). This method enabled me to interrogate the pathways activated during ectopic regeneration as well as those specifically associated to the oHF, where cells undergo lineage conversion (as described in Chapter 3). Further to this, analysis of in vivo oesophageal epithelium and skin counterparts allowed me to compare their transcriptional signatures with that of the oHF.

Initially, I found similarities between in vivo and in vitro HF signatures (nHF and oHF). Indeed, some of the defining pathways of nHF (as opposed to OE) were switched on during oesophageal-to-skin lineage conversion. These included terms related to tissue development, epithelial morphogenesis, cell migration and hair cycle. Of note, genes known to be essential for HF formation and cycling such as \textit{Igfbp3}, \textit{Krt17}, \textit{Msx2} and \textit{Krt6} were identified (Ma et al., 2003; Kevin M McGowan et al., 2002; Tong & Coulombe, 2006; Weger & Schlake, 2005). The relevance of these genes in HF physiology has been demonstrated by numerous loss of function studies \textit{in vivo}. Knockout mouse models targeting Krt17 develop alopecia a few days after birth due to premature entry into catagen (Kevin M McGowan et al., 2002)(McGowan, 2002; Xuemei Tong, 2006). Similarly, \textit{Msx2}-deficient mice undergo hair defects ultimately leading to hair loss (Ma et al., 2003; Satokata et al., 2000). These results suggested that oHFs activate pathways associated with HF development and maintenance. It is tempting to speculate that oesophageal cells starting to respond to the dermis partly recapitulate the newly-formed neonatal HFs of the skin. Yet, to fully confirm these observations it would be of interest to include in the analysis adult HFs as well as neonatal oesophagus to further evaluate whether the observed signature emerges as a skin-specific profile or rather a developmental/regenerative one.

Interestingly, I observed that oSKIN samples (including oHF and oIFE) showed an upregulation of two key epidermal cell fate regulators: WNT and BMP (Botchkarev & Sharov, 2004; Lim & Nusse, 2013; Lu et al., 2016; Matos et al., 2020). The role of these pathways in epidermal development as well as hair follicle morphogenesis has
been extensively studied. Indeed, loss of function of either BMP or Wnt during embryogenesis blocks ectodermal differentiation into epidermal fate, giving rise to neural lineages (Hardman et al., 1998; Wilson & Hemmati-Brivanlou, 1995). Remarkably, WNT and BMP are also highly expressed during HF placode formation where WNT promotes HF development while diffusible BMPs expressed by the placode are thought to suppress follicular fate in the surrounding epithelium (S. Liu et al., 2013; Millar, 2002). In this regard, it is worth mentioning that given the particularities of my \textit{in vitro} system, where the dermal HF niches are already formed, molecules instructing appendage formation may not need to become patterned as it is occurs during HF morphogenesis (Sennett et al., 2015). Instead, I found that cells re-epithelializing the denuded IFE and HF niches turn on the Wnt and BMP pathways irrespectively of the skin compartment. Of note, the fact that such profile was not found in the \textit{in vitro} oesophagus (oOE) suggests that it emerged in response to the ectopic skin niches rather than the intrinsic \textit{in vitro} conditions.

Remarkably, the role of Wnt in epithelial cell fate plasticity has been reported in several tissues including the intestine and the skin (Baker et al., 2010; Ito et al., 2007; Schwitalla et al., 2013; Silva-Vargas et al., 2005). Using a model of intestinal carcinogenesis, Schwitalla described that Wnt upregulation mediates dedifferentiation of non-stem cells, highlighting its role in the acquisition of stem cell properties. In the skin, Wnt/\(\beta\)-catenin overexpression also results in cell fate changes leading to ectopic HF formation (Baker et al., 2010). Indeed, the use of a stabilised \(\beta\)-catenin transgene under the control of different lineage-specific promoters demonstrated that cells from the IFE and sebaceous gland are competent to form HFs. In support of these observations, wounding-associated \textit{de novo} HF formation has been shown to be mediated by Wnt signalling (Ito et al., 2007). The authors described that HF neogenesis molecularly and morphologically resembled embryonic HF development, suggesting that wounding induced an embryonic phenotype in the skin. It is likely that the re-acquisition of developmental features aids in cell permissibility to ectopic signals (Kratochwil, 1969; Lu et al., 2016). Given the observation that oesophageal-derived skin activates an epithelial morphogenetic signature, it would be of interest to investigate whether Wnt overexpression in oesophageal-derived skin would promote the lineage conversion phenotype.
The microdissection-coupled RNA sequencing technique described here was valuable to dissect the effect of specific niches on epithelial cell fate with spatial resolution. However, despite the low cell input, it was still a bulk analysis hence obscuring the differences of cells transitioning towards skin identity. Indeed, lineage conversion heterogeneity appeared to be a clear feature of this model as shown by immunohistochemical analysis (as described in Chapter 3). This cell heterogeneity together with the bulk effect of this approach may explain why when plotting in vivo and in vitro samples together, the experimental differences seem to be driving the transcriptional changes. In view of this data, a more sensitive approach allowing cell profiling at single cell resolution would be required to uncover the mechanisms dictating cell fate changes in oesophageal-to-skin lineage conversion.

Taken together, these results suggested that cells cultured on the skin stroma (oSKIN) acquire a signature consistent with epithelial morphogenesis. However, the bulk nature of this approach lacked the sufficient sequencing depth to interrogate the processes driving cell fate changes. As a result, I next decided to explore the molecular signature of lineage conversion in more detail using single-cell RNA-sequencing.
Chapter 5. Results 3 - Regenerative cues pose barriers to oesophageal-to-skin lineage conversion

5.1 Overview

Research dedicated to understanding epithelial cell fate choices has spanned decades but we still have very little knowledge about the processes governing cell fate changes (Adam et al., 2020; Bonfanti et al., 2010; Dhouailly & Hardy, 1978; Ge et al., 2017a; Ito et al., 2007; Kratochwil, 1969). Tissue recombination experiments dating back to the early 1960s established that during embryonic development epithelial cells can change their identity in response to an ectopic stroma. For example, recombination of feather-free epidermis and feather-forming dermis resulted in feather formation (Mauger, 1972). More recent transplantation experiments have demonstrated that cell plasticity extends beyond embryonic development, including adult cells. Indeed, epithelial cells from various adult tissue origins have been shown to respond to dermal cues forming skin-typical appendages (Claudinot et al., 2020; Ferraris et al., 2000).

Building on earlier recombination studies, I developed a regenerative ex vivo system where adult oesophageal cells re-epithelialize the adult dermis. I demonstrated that dermal re-epithelialization was accompanied by the formation of hair follicle-like structures (as described in Chapter 3). Yet, only a moderate proportion of cells acquired the typical hair follicle stem cell (HFSC) markers. In line with this, transcriptomic analysis indicated heterogeneity in response to ectopic cues (as described in Chapter 4). The variable response to ectopic cues in the oesophageal epithelium, thought to be maintained by an equivalent progenitor population (Doupe et al., 2012; Frede et al., 2016; Piedrafita et al., 2020a), suggested the existence of mechanisms that balance cell fate plasticity at the cellular level.

In this Chapter I aimed to investigate the mechanisms that govern adult cell plasticity in oesophageal-derived skin (oSKIN). To account for the heterogeneous response to ectopic cues, I made use of a single cell RNA-sequencing dataset in my laboratory. This single-cell approach allowed me to define the molecular signature of the cell
subpopulation transitioning towards skin lineage. Next, I used functional experiments to evaluate the contribution of candidate genes regulating the oesophageal-to-skin lineage conversion. Altogether, I concluded that regenerative cues, and specifically the HIF1a-SOX9 axis, restrict cell fate plasticity; hence, maintaining the original cell identity.

Note that the single cell RNA sequencing dataset was generated and analyzed by Dr Maria T Bejar, Ilias Moutsopoulos and Dr Irina Mohorianu.

5.2 Single cell RNA-sequencing

Here, I sought to define the processes associated with oesophageal cell fate changes. For this, I referred to an existing single cell RNA-sequencing dataset in my laboratory that investigated oesophageal controls (oOE) and oesophageal-derived skin (oSKIN). Analysis of these samples allowed me to discern between the processes occurring during normal re-epithelialization and those associated with lineage conversion, respectively. To gain a temporal overview of the lineage conversion process, the newly-formed epithelia were analyzed at two time points: early during re-epithelialization at day 3 (D3) and once the epithelium has been fully reconstituted at day 10 (D10) (Figure 28a). The full protocol is described in Methods 2.10.2.

Briefly, oesophageal tissue strips obtained from tdTomato mice (R26nTnG mouse line) were cultured onto denuded stroma (submucosa/dermis) constitutively expressing EGFP (CAG::H2B-EGFP). Cultures were maintained in vitro under minimal medium i.e. absence of added growth factors other than those inherent to the serum (as described in Method 2.2.1). The newly-formed epithelial sheet was separated from the stroma at D3 and D10 and cells dissociated. Subsequently, single viable epithelial cells were sorted based on tdTomato expression as well as cell size (Figure 28a and b). As shown by immunofluorescence (Chapter 1), host epidermal cells (EGFP+) sporadically remain in the denuded dermis forming a tdTomato/EGFP chimeric epithelium. In turn, EGFP+ cells were found in the cell suspension and a 5% of them added for analysis as a control of cell identity.
Chapter 5. Results 3 - Barriers to lineage conversion

Data processing and subsequent biological mining was carried out by Ilias Moutsopoulos and Dr Maria T Bejar. Briefly, raw data was aligned to the mouse reference genome and quality control metrics assessed (see methods 2.10.2). Poor quality cells were filtered out of the analysis when the sequencing depth was below 8750 reads, the number of expressed genes was below 2000 or over 8000, or the percentage of mitochondrial and ribosomal DNA was higher than 15% and 45%, respectively. Additionally, residual contaminating cells expressing fibroblast or...

Figure 28. Single cell RNA sequencing experimental workflow.
(a) Overview of the scRNA-seq experimental workflow (10x Genomics platform). Epithelial oOE and oSKIN viable cells (tdTomato+ and EGFP+, respectively) from organotypic cultures were sorted at day 3 and 10 post-culture (D3 and D10). Sorted samples were spiked with ~5% EGFP+ host-derived epidermal sSKIN cells as an internal control of host cell identity. Minimum of 3 libraries per sample and time point (x10, Chromium). Tissues were harvested from adult animals of 12 weeks of age. (b) Flow cytometry gating strategy for isolation of oesophageal (tdTomato+) and host (EGFP+) cells from 3D heterotypic cultures. In vitro cell suspensions were gated to sort the single viable oesophageal (tdTomato) or host skin (EGFP+) populations. Representative plots from oSKIN sample are shown.

Data processing and subsequent biological mining was carried out by Ilias Moutsopoulos and Dr Maria T Bejar. Briefly, raw data was aligned to the mouse reference genome and quality control metrics assessed (see methods 2.10.2). Poor quality cells were filtered out of the analysis when the sequencing depth was below 8750 reads, the number of expressed genes was below 2000 or over 8000, or the percentage of mitochondrial and ribosomal DNA was higher than 15% and 45%, respectively. Additionally, residual contaminating cells expressing fibroblast or...
immune marker genes including *Col1a2*, *Pdgfra* or *Ptprc* were excluded. As a result, a total of 107,445 cells were considered for downstream analysis.

In line with *in vivo* observations (Doupe et al., 2012; McGinn et al., 2021), cluster annotation identified three epithelial cell populations present in all sample types: i) basal cycling progenitors, identified by their strong proliferative signature and the lack of differentiation associated markers; ii) basal resting cells, discerned by a marked basal signature and a reduced level of cell cycle-related genes; and iii) differentiated cells, characterized by high expression of differentiation associated genes (Figure 29a).

Unsupervised clustering resulted in the identification of 19 clusters, as illustrated in the dimension reduction space UMAP (Figure 29b). In order to define the clusters containing oesophageal-to-skin lineage conversion events, it was critical to identify the epidermal spike-in cell population (EGFP+) as this would later serve as internal control for epidermal identity. To this end, we focused on genes known to discriminate cells of epidermal and oesophageal origin (EGFP+ and tdTomato+, respectively). Gene classifiers of epidermal identity included *Egfp* and *Gt(ROSA)26Sor* (Figure 29b). Note, *Gt(ROSA)26Sor* expression is blocked in nTnG oesophageal cells due to the targeting of the nTnG construct into the Rosa26 locus (Prigge et al., 2013). Further to this, the oesophageal basal marker Sox2 was used as a negative marker of epidermal identity (Figure 29c and d). Altogether, expression of epidermal gene classifiers and lack of the Sox2 oesophageal marker revealed that the host epidermal spike-in population corresponded to Cluster 18 (C18) in the UMAP.
Chapter 5. Results 3 - Barriers to lineage conversion

Figure 29. Cell clustering, annotation and identification of epidermal spike-in cells.

(a) UMAPs showing expression of representative markers for basal cells (top panels), cell cycle (middle panels) and differentiated cells (bottom panels). Color bars of UMAPs indicate log2-transformed normalized expression levels. (b) UMAP representing cell clusters based on louvain clustering (19 clusters in total labelled 0 to 18). (c) SOX2 remains as a distinctive oesophageal marker even when exposed to ectopic signals. 3D rendered confocal images of chimeric oSKIN wholemounts reveal the exclusive expression of SOX2 (white) in cells of oesophageal origin (tdTomato, red) and not in EGFP+ host skin cells (green). Scale, 50 μm. (d) UMAP showing Sox2 expression. Zoomed-in inset (dotted lines) in the upper right shows negligible Sox2 expression in the EGFP host cluster, C18. Colour bar indicates log2-transformed normalized expression levels.
5.3 Transitioning cells present a marked regenerative signature

In order to investigate molecular changes associated with lineage conversion, it was first necessary to identify the specific set of oesophageal cells undergoing a transition towards skin/HF identity. To this end, we looked for expression of epidermal-associated genes in the basal cell compartment, where progenitor cells reside (Doupe et al., 2012). As anticipated, we found that typical epidermal genes (CD34, Gata3 and Cd200, among others) were enriched in C18 host epidermal cells (Figure 30a). Of key interest, the adjacent Cluster 8 (C8) – containing cells of oesophageal origin, including oOE and oSKIN at day 3 and day 8 – was also enriched for these markers (Figure 30b). Further to this, these two clusters showed partial overlap denoting close transcriptional profiles. From this data we were able to conclude that lineage conversion events were mainly restricted to these two clusters.

Figure 30. Epidermal gene expression is restricted to host epidermal cells (C18) and a subset of oesophageal cells (C8).
(a) UMAPs showing the expression of classical epidermal genes (enriched in the reference cluster 18), and (b) hair follicle-related genes (enriched in reference cluster 18)
Chapter 5. Results 3 - Barriers to lineage conversion

18 and transitioning cluster 8; bottom panels). Colour bars of UMAPs indicate log2-transformed normalized expression levels. Insets in the lower left of each UMAP show a zoomed-in view of cells in C8 and C18.

Hence, to further define the transition from oesophageal-to-skin lineage we focused on C8 and C18. To this end, we re-clustered them separately (Figure 31a). In the new UMAP distribution cells segregated by tissue of origin, resulting in two main islands enriched for oesophageal and epidermal cells, respectively. While this indicated that the tissue of origin accounted for most transcriptional differences between samples, we noted that oSKIN cells clustered in-between oOE cells and host cells (Figure 31b). Such distribution was compatible with a shift in the transcriptional landscape of oSKIN cells, progressively moving away from oOE cells and approaching host epidermal cells (C18). It is worth noting that oSKIN and host epidermal cells partially overlapped at the cluster boundary, further reinforcing the notion of an identity switch (Figure 31b; inset). In line with my histological analysis, the number of cells showing signs of an identity transition only represented a small subset of the oSKIN cells, illustrating the inefficiency of the lineage conversion process also at the molecular level.
Figure 31. Oesophageal cells transitioning towards skin identity show a marked hypoxic signature.

(a-b) UMAP representation of C18 and C8 cells after re-clustering. Colours show the identity of the original clusters (a) or the sample origin (b). (c) Pseudotime trajectory for C18 and C8 cells on the UMAP representation. Colour bar denotes pseudotime. (d) Violin plots showing the distribution of cells along the pseudotime trajectory from oesophageal to skin identity for each sample type. Cells were split in three main states along the pseudotime trajectory: Oesophageal identity, Transitioning cells (TC; purple shade) and Skin Host identity. Expression-driven rug plots show the position of individual cells along the pseudotime trajectory. (e) Left: Pattern of genes upregulated in transitioning cells (TC). Grey, relative expression profile of individual genes; pink, averaged value. To calculate the relative expression profiles, log-transformed
To explore the trajectory of the identity switch, pseudotime analysis was used. This algorithm inferred the fate trajectory of oOE cells (C8) towards epidermal cell fate (C18) with transitioning oSKIN cells arranged between the two (Figure 31c and d). To focus on the defining aspects driving transitioning cells away from their original identity, three main areas were designated based on expression of oesophageal and epidermal genes: (i) Oesophageal, enriched for oOE and oSKIN cells at the onset of the trajectory, (ii) Transitioning, enriched for oSKIN cells moving along the trajectory, and (iii) Host, defined by epidermal cells representing the end of the trajectory (Figure 31d). Next, we focused on those genes specifically upregulated in transitioning cells (TC). GSE analysis of these genes revealed processes previously shown to be associated with cell fate plasticity (Shaw & Martin, 2016; Xi et al., 2017). This included terms defining an active tissue remodelling signature i.e. wound healing, invasion, EMT and tumour progression (Hbegf, Serpine1, Bsg, Plod2, Cited1) (Figure 31e) (Méniel et al., 2013; Nabeshima et al., 2006; Shirakata et al., 2005; Xu et al., 2017). Other genes denoting the active regenerative state of TCs, including those associated with ECM reorganization (Adam8, Itgb1, Pthlh) and glycolytic metabolism (Aldoc, Gapdh, Gpi1). Remarkably, the most recurrent process was hypoxia, showing an upregulation for HIF1a target genes (Adm, Bsg, Cited1, Ddit4, Er01l, Hbegf, Pgf, Bnip3, Cav1, Egln3, Ptgcs, Vegfa) (Figure 31f and g) (del Peso et al., 2003; Dengler et al., 2014; Keleg et al., 2007; Y. Wang et al., 2012). Importantly, HIF1a upregulation further supported an ongoing regenerative signature, as the acute hypoxia that follows tissue injury is known to activate the HIF1a pathway in an attempt to recover oxygen supply and aid in the healing response (W. X. Hong et al., 2014; Rezvani, Ali, Nissen, Harfouche, De Verneuil, et al., 2011; Ruthenborg et al., 2014).
Having identified hypoxia as the main defining trait of transitioning cells, I sought to validate this signature at the protein level. I reasoned that if transitioning cells were highly hypoxic, HIF1a – the functional hallmark of hypoxia – would be upregulated in oSKIN samples relative to oOE controls. Immunocytochemical analysis showed that HIF1a expression was indeed higher in oSKIN than oOE throughout tissue re-epithelialization (Figure 32). Interestingly, while high HIF1a expression was observed in oOE at day 3 (D3), it later dropped once tissue re-epithelialization was completed at day 8 (D8); hence, denoting a drop in tissue regeneration. In direct contrast, HIF1a
expression persisted in oSKIN at D8, further supporting the idea that transitioning oSKIN cells retain a highly regenerative profile (Shaw & Martin, 2016).

Altogether, I concluded that oesophageal cells transitioning towards skin identity present a marked regenerative state defined by a hypoxic profile. This motivated me to evaluate the functional link between regeneration/hypoxia and lineage conversion.

5.4 Regenerative cues may restrict lineage conversion events

Having described the regenerative profile of transitioning cells, and given the well-documented link between regeneration and plasticity, I aimed to investigate the role of regeneration in lineage conversion (Doupe et al., 2012; Ge et al., 2017a; Tata et al., 2013; van Es et al., 2012). Indeed, while epithelial regeneration is an intrinsic feature of my in vitro system, regenerative cues can be tweaked to limit re-epithelialization.

Here, I sought to avoid epithelial expansion and investigate the in situ oesophageal tissue grown in 3D heterotypic cultures rather than the newly-formed epithelium derived from it. I hypothesised that depriving the epithelium from its stroma would minimize regenerative cues and reduce re-epithelialization. To this end, oesophageal tissue of bigger dimensions (5 x 8 mm) was separated from its underlying stroma and overlaid on the skin dermis (Figure 33a; Method 2.2.5). As a control, the unpeeled oesophageal tissue was used as previously described (Method 2.2.1).

First, I evaluated the cell proliferative capacity in the stroma-deprived (peeled OE) and the control (unpeeled OE) culture systems. For this, tissue re-epithelialization – identified by tdTomato expression – was measured in tissue wholemounts after 8 days in culture (D8). As expected, epithelial cell expansion in the stroma-deprived (peeled OE) cultures was reduced when compared to controls (23.3% and 60.8%, respectively) (Figure 33a and b). Instead, given the absence of the OE stromal layer between the dermis and the oesophageal epithelium, epithelial cells were found to migrate downwards directly into the HF sockets. The in situ oesophageal tissue and
associated oHF were then compared to control cultures i.e. the newly formed epithelium derived from the oesophageal tissue. To investigate lineage conversion, the CD34 HF marker was used as a readout of skin identity (Figure 33c). Interestingly, immunofluorescence analysis revealed that the peeled OE cultures led to an increase in the percentage of CD34+ cells compared to controls (19.6±4.1 % and 3.6 ± 0.6 % of CD34+ cells per oHF, respectively). Even if this increase was not statistically significant, the upwards trend was clear. Although additional biological replicates would be needed to fully confirm this tendency, these results suggested a rise in lineage conversion efficiency (Figure 33c and d). The increasing trend in lineage conversion following regeneration impairment made me hypothesised that tissue regeneration may pose a constraint for cell re-specification. Importantly, alternative mechanisms may have influenced/led to this result. Firstly, it is possible that the absence of stroma in peeled OE cultures results in cellular stress, which is known to enable a permissive state to external signals, favouring cell plasticity (Andrew et al., 2021). Secondly, peeled OE cultures are uniquely exposed to dermal signals – as opposed to conflicting signals from two stromal compartments – potentially favouring the transition towards epidermal lineage.
Figure 33. Tissue regeneration may limit oesophageal-to-skin lineage conversion.

(a) Left: schematic depicting the strategy for the limited re-epithelialization assay. Controls were performed with unpeeled oesophageal tissue (top), while limited assay used only oesophageal epithelium to reconstitute the denuded dermis (bottom). Right: Representative tilescan confocal images of controls (top) and limited re-epithelialization assays (bottom) following 8 days in culture. Red; tdTomato; green; EGFP; scale 1mm. (b) Quantification of oesophageal re-epithelialization from (a), expressed as percentage of area re-epithelialized by oesophageal cells (tdTomato, red) relative to the dermis area. A minimum of 6 cultures were quantified from 3 animals. Data expressed as mean ± SEM and statistics performed on biological replicates using a two-tailed unpaired t-test. The points show individual
measurements, greyscale indicates biological replicates (n=3). (c) 3D confocal side views of oesophageal-derived skin wholemounts showing typical CD34 expression in oHFs derived from either unpeeled oesophagus (controls) or peeled oesophageal epithelium. Dashed lines delimit hair follicles borders. Blue, DAPI; white, CD34; scale 50 μm. (d) Quantification of CD34+ cells per oHF relative to the total number of DAPI+ cells (from C). A minimum of 1700 cells were quantified out of total 17 oHFs. Data expressed as mean ± SD and statistics performed on biological replicates using a two-tailed unpaired t-test. The points show individual measurements, greyscale indicates biological replicates (n=3). Tissues were harvested from adult animals between 9-15 weeks of age.

5.5 HIF1a-Sox9 axis poses barriers to lineage conversion

Having identified the upregulation of HIF1a-inducible genes in transitioning cells, I next sought to investigate the relevance of the HIF1a pathway for lineage conversion. To this end, I chose to modulate HIF1a using two different approaches: growing cultures in a hypoxic incubator and employing pharmacological treatments.

Firstly, 3D heterotypic cultures were exposed to hypoxic conditions (2% O2) throughout culture (Figure 34a). Yet, samples could not be analysed as the low oxygen levels proved to be too severe resulting in poor cell viability and minimal cell re-epithelialization (Figure 34b). Secondly, I used two well-described pharmacological modulators of HIF1a: KC7F2, that downregulates HIF1a protein synthesis; and DMOG, a HIF1a stabilizer (Chen et al., 2020; Masterson et al., 2019). As expected, KC7F2 treatment resulted in a significant drop in HIF1a basal intensity compared to the DMSO control (Figure 34a and b). The opposite effect was observed in response to DMOG. Importantly, regardless of the treatment, oesophageal cells expanded and formed oHF structures. Overall, these results validated the inhibitory and activating effect of KC7F2 and DMOG, respectively.
Figure 34. Hypoxic conditions lead to poor cell re-epithelialization in 3D heterotypic cultures.

(a) Experimental protocol. 3D heterotopic cultures were kept in vitro in a hypoxic incubator with 2% O2. 10 days post-culture samples were fixed and the re-epithelialized area analysed. (b) Quantification of re-epithelialization in control samples as well as those exposed to hypoxia, expressed as percentage of re-epithelialized area relative to total dermis area. Data expressed as mean ± SEM and statistics performed on biological replicates using a two-tailed unpaired t-test. The points show individual measurements (greyscale indicates values from each of 3 mice). Tissues were harvested from adult animals between 9-15 weeks of age.
Figure 35. HIF1α poses barriers to oesophageal-to-skin lineage conversion.
(a) Representative 3D confocal side views of oSKIN wholemounts showing HIF1α expression in oesophageal-derived HF (oHF) following treatment with HIF1α activator (DMOG; middle) or HIF1α inhibitor (KC7F2; right) for 8 days. Vehicle controls were DMSO treated. Blue, DAPI; white, HIF1α; scale 50 μm. (b) Quantification of HIF1α intensity, as measured by immunofluorescence (a.u.), in individual cells from oHF.
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following HIF1a pharmacological treatment (from a). A minimum of 2,000 cells were quantified out of 18 eHF and 3 animals. (c) 3D confocal side views of tissue wholemounts showing CD34 and SOX9 expression in oHF following treatment with HIF1a activator (DMOG) or HIF1a inhibitor (KC7F2). Vehicle controls were DMSO treated. Blue, DAPI; magenta, SOX9; white, HIF1a; scale 50 μm. (d) Quantification from (c) of CD34+ cells (d) and SOX9+ cells (e) per oHF, expressed as a percentage of the total number of cells (DAPI+). A minimum of 1,600 cells were quantified from out of 18 eHF and 3 mice. Data expressed as mean ± SD and statistics were performed on biological replicates using one-way ANOVA test followed by multiple comparisons. The points show individual measurements (greyscale indicates biological replicates, n=3). Tissues were harvested from adult animals between 9-15 weeks of age. (f) Plot showing correlation between SOX9 and HIF1a protein expression, measured as the intensity of individual oHF cells by immunostaining. n=3 mice; r, Pearson correlation coefficient. A total of 8,397 cells were quantified from out 27 oHF and 3 mice.

To further investigate whether SOX9 was operating downstream of HIF1a, the levels of SOX9 and HIF1a intensity were measured in untreated samples. Results showed that HIF1a+ cells were more prone to express high levels of SOX9, indicating a positive correlation between HIF1a and SOX9 (Figure 35f). Altogether, this data suggested that HIF1a may be acting through SOX9 to limit cell fate plasticity changes.

The role of SOX9 has been extensively studied in HF morphogenesis and cycling as well as skin regenerative processes such as wound-healing (Adam et al., 2015; Ge et al., 2017a; Nowak et al., 2008; Vidal et al., 2005). While it has been associated with regenerative processes in other tissues, its role in epithelial regeneration has been studied to a lesser extent (McConnell et al., 2011; Nandan et al., 2014). Here, I decided to investigate oesophageal SOX9 expression in non-homeostatic conditions including wound-healing, tissue expansion during neonatal development and in vitro cultures (heterotypic/isotypic cultures and explant cultures) (Figure 35) (in collaboration with Dr Maria T Bejar). Interestingly, while immunofluorescence analysis of the homeostatic oesophageal epithelium showed negative staining, all tested conditions of tissue perturbations showed marked SOX9 staining. These
results support previous findings establishing a link between SOX9 and epithelial regeneration/development beyond skin physiology (Lu et al., 2012; McGinn et al., 2021; Xiuyu Wang et al., 2019).

**Figure 36. SOX9 is expressed in oesophageal tissue under non-homeostatic conditions.**

Rendered confocal images showing SOX9 expression in basal cells in oesophageal epithelium under different experimental conditions. Left, in vivo oesophageal tissue denoting adult homeostasis. Right, non-homeostatic conditions including: in vivo neonatal tissue (harvested at postnatal day 7, P7), adult tissue following micro-endoscopic biopsy wounding in vivo, in vitro oesophageal epithelium (isotypic culture) and oesophageal explant culture. Diagrams at the bottom depict the different experimental conditions analysed for SOX9 expression. Tissues were harvested from adult animals between 9-15 weeks of age or postnatal animals 7 days post-birth. Blue, DAPI; magenta, SOX9; scale 50 μm.
Having defined a link between SOX9 and oesophageal regeneration, I sought to investigate whether SOX9 was acting as a further blockage to lineage conversion. For this, I made use of $Krt14^{CreERT}/Sox9^{fl/fl}$ transgenic animals that conditionally inactivate Sox9 upon tamoxifen-mediated recombination (Figure 37a) (in collaboration with Dr Maria T Bejar). As anticipated, oSKIN tissue derived from $Krt14^{CreERT}/Sox9^{fl/fl}$ showed reduced SOX9 intensity validating Sox9 inactivation (Figure 37b). Next, I evaluated its effect on lineage conversion by assessing CD34 expression. In line with its role in regeneration, Sox9 inactivation showed an increased percentage of CD34+ cells compared to controls (20.9±1.9 % and 2.8±0.7 %, respectively) suggesting that SOX9 limits lineage conversion (Figure 37c and d). Together these results support the notion that the HIF1α-SOX9 axis act as a barrier to lineage conversion.
Figure 37. Sox9 poses barriers for oesophageal-to-skin lineage conversion.
(a) Experimental protocol. K14CreERT/Sox9fl/fl mice were injected with tamoxifen (TAM) for three consecutive days to conditionally delete Sox9 in epithelial cells (Sox9 cKO). The oesophagi of treated animals were collected 24h after the last TAM administration and cultured over EGFP+ dermis as 3D heterotypic cultures for 8 days. (b) SOX9 intensity quantification, as measured by immunofluorescence (a.u.), in individual eHF cells derived from control and Sox9 cKO mice). A minimum of 8,000 cells were quantified out of 27 eHF (≈8,000 DAPI cells) and 3 animals. Individual cell measurements were analysed using two-tailed unpaired Mann-Whitney test. (c) 3D confocal side views of tissue wholemounts showing CD34 and SOX9 expression in oHF derived from uninduced (left panels) and induced (right panels) K14CreERT/Sox9fl/fl derived oHFs following 8 days of culture. Blue, DAPI; magenta, SOX9; white, CD34; scale 50 μm. (d) Quantification (from c) of CD34+ cells per oHF, expressed as a percentage of the total number of cells (DAPI+). A minimum of 6,400 cells were quantified out of 30 oHF and 3 mice. The points show individual measurements (greyscale indicates values from each of 3 mice). Data expressed as mean ± SD and statistics performed on biological replicates using a two-tailed unpaired t-test. Tissues were harvested from adult animals between 9-15 weeks of age.
5.6 Discussion

Here, I investigated the molecular mechanisms that regulate the oesophageal-to-skin lineage conversion. The experiments presented in this chapter were based on a single cell RNA-sequencing dataset that analysed oesophageal cells grown in heterotypic or isotypic culture (oSKIN and oOE, respectively) as well as host epidermal cells used as internal controls of epidermal identity. This single cell strategy allowed us to interrogate the molecular signature of the cell subset transitioning towards epidermal lineage, revealing a marked regenerative profile, defined by a hypoxic response. Functional validation assays unveiled that this signature arises naturally during regeneration, ultimately safeguarding cell identity and preventing cell fate plasticity.

Pseudo temporal ordering of cells from oesophageal to skin identity allowed us to investigate the molecular profile of transitioning cells, as previously done in other systems (Joost et al., 2018; Scialdone et al., 2016). Cells transitioning between oesophageal and epidermal lineages were defined by a prominent regenerative signature, including the upregulation of genes associated with the HIF1α pathway, ECM remodelling and glycolytic metabolism (W. X. Hong et al., 2014; Rezvani, Ali, Nissen, Harfouche, De Verneuil, et al., 2011; Shapira & Christofk, 2020). Among these processes, the HIF1α signalling pathway was the most recurrent term. Indeed, the high metabolic demands associated with tissue regeneration are known to result in a hypoxic microenvironment that turns on HIF1α activity (W. X. Hong et al., 2014; Xi et al., 2017). In line with its positive effect in tissue repair, we found activation of HIF1α-inducible genes involved in cell migration and EMT (Hbegf, Serpine1, Bsg, Plod2, Cited1) (Nabeshima et al., 2006; Shirakata et al., 2005; Xu et al., 2017). In this regard, an additional control would have helped to determine whether this phenotype was specifically associated with lineage conversion: skin-derived skin (sSKIN) – where skin explants re-epithelialize the whole denuded dermis, as opposed to the remaining host epidermal cells –. Analysis of these cells would unveil whether HIF1α upregulation was characteristic of epidermal cell regeneration more widely, or whether it was acquired as part of the lineage conversion process.
Chapter 5. Results 3 - Barriers to lineage conversion

Having identified regeneration as the main defining trait of transitioning cells, I sought to dissect its role in lineage conversion. Interestingly, I found a negative correlation between OE regenerative/re-epithelialization capacity—modulated by peeling the OE stroma—and lineage conversion as determined by CD34 expression. Although additional functional studies with more epidermal markers would be needed to fully confirm these conclusions, these results may suggest that the regenerative signature observed in transitioning cells may act as a regenerative blockage that prevents re-specification to epidermal lineage. In turn, reducing epithelial regeneration—as modelled when peeling the OE stroma—resulted in an increased trend in the oesophageal-to-skin identity switch.

Motivated by the above results, I speculated that the upregulation of HIF1a may represent a regenerative barrier to lineage conversion. In support of this notion, I found that HIF1a downregulation allowed cells to exit the transitioning state and acquire epidermal fate. Interestingly, studies in the skin have shown that the hypoxic state that promotes early wound closure must be resolved at later stages in order to guarantee an adequate healing process (W. X. Hong et al., 2014; Ruthenborg et al., 2014). Indeed, HIF1a is essential for the early adaptative response to hypoxia mediating re-epithelialization and rapid tissue repair (Li et al., 2007; Rezvani, Ali, Nissen, Harfouche, de Verneuil, et al., 2011). Yet, its aberrant expression following wound-closure results in excessive ECM deposition leading to fibrosis and scarring (Kimura et al., 2008; Lokmic et al., 2012). It would be of interest to investigate whether temporal modulation of HIF1a in 3D heterotypic cultures would aid in the lineage conversion process. Based on the wound-healing literature, it is possible that HIF1a is initially required for cells to acquire a regenerative signature needed for cell fate rewiring, while its enduring activation may halt cells in an unresolved regenerative state. This possibility could be tested using pharmacological HIF1a inhibitors for the last days of culture.

Further mechanistic insight suggested that HIF1a blockage operates through its downstream effector SOX9. The direct link between HIF1a and SOX9 has been established by studies in chondrogenesis, where both are required for differentiation during skeletogenesis (Amarilio et al., 2007; C. Zhang et al., 2011). In addition, SOX9 has recently suggested to be a pioneering factor driving cell transitional stages during
wound-healing, tumorigenesis and epithelial-mesenchymal transitions (EMT) (Adam et al., 2015; Adam & Fuchs, 2016; Fuglerud et al., 2021; Yang et al., 2017). Comprehensive epigenetic and lineage tracing studies in the skin have demonstrated that the cell fate plasticity observed in HF stem cells during wounding and tumorigenesis is sustained by SOX9 activity (Adam et al., 2015; Ge et al., 2017a). Further evidence of its role in cell identity changes comes from a recent investigation describing that the ectopic expression of SOX9 in endothelial cells is sufficient to drive reprogramming into a mesenchymal phenotype. Indeed, only 5-days post-transduction endothelial cells changed to a spindled-shaped morphology, gained migratory properties and acquired mesenchymal markers (Fuglerud et al., 2021).

In line with the role of SOX9 in cell fate choices, it has been shown to be essential for HF stem cell fate determination during embryogenesis (Nowak et al., 2008; Vidal et al., 2005). However, it is worth noting that SOX9 is not required for short-term CD34+ HF stem cell maintenance in an adult context. In fact, conditional targeting of Sox9 in the adult HF has been shown to be compatible with CD34 expression (Kadaja et al., 2014). Only after one full hair cycle (~3-6 weeks), the Sox9-depleted stem cell pool became exhausted failing to restart HF regeneration and undergoing epidermal differentiation (Song et al., 2014; L. Wang et al., 2019). Therefore, our results are not irreconcilable with the role of SOX9 in adult HF physiology. Indeed, the data presented here suggested that given the SOX9 regenerative properties, its inactivation promoted lineage conversion and acquisition of HF stem cell properties as dictated by the niche. Yet, it is possible that in light of the work by Kadaja Sox9-deficient oHF cells fail to maintain stemness long-term. This hypothesis may be tested using hair reconstitution assays (Jensen et al., 2010).

The work presented here points at SOX9 as a key player in regenerative processes well beyond skin physiology. In fact, we found that the oesophageal cells switch on SOX9 activity under a range of non-homeostatic conditions, including neonatal development, wound-healing and in vitro cell expansion. This was in line with a recent single cell transcriptional study revealing SOX9 activity in the neonatal oesophagus as opposed to the homeostatic adult tissue (McGinn et al., 2021). Further to this, SOX9 has been reported to drive stemness and malignant progression in oesophageal squamous cell carcinoma (Song et al., 2014; Xintao
Yang, 2019; Wang, 2019). Indeed, its involvement in tumorigenesis extends to multiple epithelial tissues including the stomach, prostate and bladder among others (Aguilar-Medina et al., 2019; Santos et al., 2016; Song et al., 2014; Wan et al., 2017).

Altogether, given the long-standing link between regeneration and plasticity, it is tempting to speculate that while an initial regenerative process with high HIF1a/SOX9 activity may be needed for cells to kick-start a plastic transitional state, it later acts as a barrier that prevents cell re-specification towards a different lineage. During wound-healing cells are known to exit their niche and migrate long distances to contribute to tissue repair. In this context, the existence of plasticity barriers intrinsic to regeneration may represent a temporally regulated mechanism to impede the premature acquisition of alternate fates. In turn, once cells reach their final destination downregulation of such a barrier allows them to respond to the surrounding signals dictating alternate fates. In line with this notion, a recent epigenomic study described NFIB and NFIX as stemness transcription factors that enable regeneration while repressing unwanted lineages (Adam et al., 2020).

The results summarised above have answered the aims set out at the beginning of this chapter, defining the molecular changes that characterize oesophageal-to-skin lineage conversion. Firstly, these results pointed at regeneration and specifically the HIF1a pathway as the defining trait of cells transitioning towards skin identity. Secondly, functional validation experiments revealed that HIF1a and its downstream effector SOX9 act as a barrier that prevent cell re-specification, therefore maintaining transitioning cells in an unresolved regenerative state. In turn, lifting this regenerative lock releases the fate barrier allowing cells to respond to instructive skin signals. Altogether, these results shed light onto the central role of HIF1a-SOX9 axis in modulating adult epithelial cell plasticity.
Chapter 6. Results 4 - *In vivo* oesophageal-to-skin lineage conversion

6.1 Overview

In the previous Chapters I described an *in vitro* model where the oesophageal epithelium is grown on the skin dermis (3D heterotypic cultures), eliciting oesophageal-to-skin lineage conversion (as described in Chapter 3). This system offered a valuable platform to interrogate the earliest stages of cell fate plasticity events (as described in Chapter 4 and 5). However, 3D heterotypic cultures could not be maintained *in vitro* long-term, which prevented investigation into the capacity of oesophageal cells to form functional hair shafts.

Here, I sought to investigate whether oesophageal cells have the ability to contribute to hair formation. To this end, two complimentary approaches were used. First, we developed a heterotypic grafting technique that enables transplantation of oesophageal-dermal tissue composites. Next, hair reconstitution assays were used (Jensen et al., 2010; Weinberg et al., 1993). Overall, these experiments supported the notion that oesophageal cells can reconstitute functional HFs *in vivo*.

The work presented in this chapter was done in collaboration with Dr Maria T Bejar and Dr Maria Pilar Alcolea, who performed the surgeries, while I processed and analysed the graft tissues.
6.2 Development of heterotypic grafting method

In order to investigate the capacity of oesophageal cells to form hair, we adapted a full skin thickness grafting technique that allowed the transplantation of heterotypic composites equivalent to those used in vitro (Ge et al., 2017a; Plikus et al., 2008). In short, the peeled dermis was cut into 6- or 8-mm diameter implants and oesophageal tissue (6 x 5 or 8 x 5 mm) laid on top (Figure 38a; see Method 2.8.1). In order to identify the tissue of origin, the dermis was obtained from H2B-EGFP animals and the oesophageal tissue from mice expressing tdTomato (mTmG or nTnG mice). Freshly-prepared heterotypic grafts were then transplanted onto recipient animals following a biopsy excision with a sterile biopsy punch (6-8 mm) (Figure 38b). To minimize animal use, 2 grafts were typically transplanted per animal.

Several pilot experiments were performed to optimize and validate the method. Initially, we assessed the optimal graft size for engraftment. For this, we transplanted 6- and 8-mm diameter grafts in immunocompetent animals (C57BL/6J). While the grafting efficiency 2-weeks post-transplant was shown to be limited (10%, n=20 grafts), the graft size did not have a major impact on engraftment. Hence, to increase the tissue availability for downstream analysis we chose to use 8 mm diameter grafts. One limitation of this strategy was the short-term maintenance of grafts, which precluded the assessment of hair growth. To circumvent graft rejection, we used two strategies: i) transplantation of dermal grafts into recipient animals from the same litter, hence minimizing the MHC mismatch; ii) using nude athymic recipient animals. Yet, both approaches resulted in the progressive loss of grafts indicating poor tissue engraftment rather than immune rejection. In turn, no graft could be recovered after more than 4 weeks post-transplant (n=20 grafts). We concluded that this grafting technique was not suitable to evaluate the ability of oesophageal cells to form hair.
Figure 38. Heterotypic grafting strategy.
(a) Oesophageal-dermal heterotypic tissue grafts were prepared overlaying a 4x8mm oesophageal tissue strip from tdTomato expressing mice on top of an 8mm diameter piece of peeled dermis from the tail skin of EGFP expressing mice. (b) The implants were grafted onto the back skin of either immunocompetent (C57BL/6J / H2B-EGFP) or nude animals following an excision with a biopsy punch. Scale, 4mm.
6.3 Oesophageal-derived hair follicle formation in vivo

Given that grafts could not be maintained long-term, they were collected 1-4 weeks post-transplant and full-thickness tissue analysed by confocal microscopy. This revealed the presence of HF-like structures (tdTomato; oesophageal origin) that protruded throughout the donor dermis (EGFP) (Figure 39a). Tissue cryosections further confirmed that oesophageal cells re-epithelialized the denuded dermis, reconstituting a new epithelium and associated HF structures (Figure 39b). Of note, this was not an isolated event; instead, over 60% of the engrafted implants unequivocally showed formation of these structures (n=14 total engrafted samples). Remarkably, we found that oesophageal-derived HFs contained a central fibre reminiscent of the hair shaft (Figure 39c). As a result, we chose to use heterotypic grafts to investigate the early stages of cell fate switching in vivo.

First, proliferation was assessed in the newly formed epithelium using the Fucci2a mouse line, which labels cells according to their cell cycle status – G1 marked by mCherry and S/G2/M marked by mVenus – (Miyawaki et al., 2014). Briefly, heterotypic transplants were set up with Fucci2a oesophageal tissue overlaid on wild type dermis. Confocal imaging revealed the presence of mVenus cells in vivo oHFs and oIFE, indicating that they were actively cycling up to 2 weeks post-transplant (Figure 39d). This result confirmed the cell viability of in vivo oesophageal-derived skin.
Figure 39. Oesophageal cells contribute to hair follicle formation in vivo.

(a) Representative 3D rendered basal view of a full thickness skin wholemount 1-2 weeks post-graft (as in Figure 38), showing the in vivo formation of oesophageal-derived skin (tdTomato). Grafted dermis was obtained from H2B-EGFP mouse tail skin. Blue, DAPI; Green, EGFP; Red, tdTomato; scale, 1 mm. (b, c) Side views showing oesophageal-derived HF structures from in vivo heterotypic skin grafts. Full-thickness skin graft (tissue cryosections, b), 3D rendered confocal inset (c). Asterisk in (c) denotes structure reminiscent of a hair shaft. Blue, DAPI; Red, tdTomato; scale, 50 μm. (d) Representative confocal side view showing cycling oesophageal-derived skin cells in vivo 2 weeks after heterotypic skin graft transplant (tissue cryosections). Schematic at the bottom illustrates the fluorescent expression pattern of cell cycle reporter mouse line Fucci2a (mCherry, G1 cells; mVenus, S/G2/M cells). n=2 animals. Recipient and donor animals were between 9-15 weeks of age. Blue, DAPI; Red, mCherry; Green, mVenus; scale 50 μm.

Having identified the formation of oHF structures in vivo, we aimed to investigate if they acquired hair follicle-associated markers as previously described in heterotypic cultures grown in vitro. One key difference was that, in contrast to the latter, heterotypic grafts were not amenable for epithelial whole-mounting. As a result,
immunofluorescence analysis of KRT17, KRT24 and CD34 HF markers was performed in tissue sections.

For this, heterotypic grafts as well as oesophageal and skin controls were sectioned and stained for hair follicle-associated markers. In vivo oesophageal-derived HFs showed acquisition of typical HF keratins, including KRT17 and KRT24 (Figure 40a). Regarding expression of the HF stem cell marker CD34, the use of tissue sections together with the high expression found in the stromal compartment precluded reaching a conclusion. To further investigate the expression of HF stem cell markers, we looked into Lgr5 expression. For this, the Lgr5-CreGFP mouse line, that constitutively expresses EGFP (green) under the control of Lgr5 promoter, was used (Barker et al., 2008). We speculated that if Lgr5 was activated in response to the skin signals, oesophageal cells would be labelled with EGFP. Heterotypic composites including Lgr5-CreGFP oesophageal tissue and wild type dermis were transplanted onto wild type animals (C57BL/6J). Animals were harvested two weeks upon transplant, and the EGFP reporter signal magnified with the appropriate antibody as previously described (Schuijers et al., 2014). However, no signal was detected in the in vivo oHFs, as opposed to control skin tissue harvested from the donor Lgr5-CreGFP animal (Figure 40b). Yet, given the limited number of recovered grafts we could not draw any conclusion about the expression of HF stem cell markers.

Altogether, the heterotypic grafting method confirmed that oesophageal cells have the ability to form HF-like structures in vivo expressing typical skin keratins. However, we did not detect expression of HFSC markers as previously observed in vitro (Chapter 3).
Figure 40. Expression of hair follicle markers in oesophageal-derived hair follicles in vivo.

(a) Confocal side views showing typical oesophageal-derived HF structures from in vivo heterotopic skin grafts 2 weeks after transplant, compared to oesophageal and skin controls. Full thickness skin graft/tissue cryosections reveal upregulation of the HF marker KRT24 in oesophageal-derived HFs (red, tdTomato) in vivo. EGFP (green) denotes skin origin. White, KRT24; scale 50 μm. (b) Confocal side views showing an oesophageal-derived HF, compared to skin control. Full thickness skin graft/ tissue cryosections show absent staining for the HF stem cell marker Lgr5 in oesophageal-derived HFs. Both recipient and donor animals were between 10-16 weeks of age. Blue, DAPI; Green, Lgr5; scale 50 μm.
6.4 Contribution of oesophageal cells to hair shaft formation

While the above heterotypic grafting approach served its purpose to investigate \textit{in vivo} ectopic regeneration, grafts were eventually lost preventing the long-term evaluation of oesophageal-derived hair formation. To address this, a well-described HF reconstitution assay was used (Jensen et al., 2010; Weinberg et al., 1993).

Briefly, dissociated oesophageal cells (tdTomato+) were combined with neonatal skin cells (EGFP+; including epidermal and dermal cells). The heterotypic cell suspension was then grafted onto the back skin of nude mice using a silicon chamber as previously described (Figure 41a) (U Lichti et al., 1993; Ulrike Lichti et al., 2008). This strategy resulted in the emergence of oesophageal/epidermal (tdTomato/EGFP) chimeric HFs that generated hair shafts 6 weeks post-grafting (Figure 41b and c). The oesophageal contribution to HF formation \textit{in vivo} was found to be limited (only 4 mosaic HFs out of 3x10^6 cells grafted, n=6). This was consistent with the inefficient oesophageal-to-skin lineage conversion rate found \textit{in vitro}, where only a proportion of oesophageal cells acquired HF stem cell markers (as described in Chapter 3). Nevertheless, the presence of hair shafts containing tdTomato+ cells clearly demonstrated that adult oesophageal cells retain the ability to contribute to functional hair follicle structures.
Figure 41. Contribution of oesophageal cells to hair formation in vivo.
(a) Schematic showing heterotypic cell grafting. Oesophageal epithelial cells (5 \( \times \) \( 10^5 \)) from adult tdTomato mice were isolated and mixed with epidermal (10^6) and dermal (5 \( \times \) \( 10^6 \)) single-cell suspensions from postnatal day 2 (P2) H2B-EGFP mice. Cells were injected into a silicon chamber on the back of a nude mouse, and allowed to reconstitute skin and grow hair for 6 weeks. Figure created with BioRender.com (b) Representative image showing hair outgrowth in the back skin of a nude mouse 6 weeks after grafting (as in a). Scale, 4mm. (c) 3D rendered side views of a full thickness skin wholemount 6 weeks after cell grafting (from b), showing oesophageal-skin chimeric hair follicles giving raise to oesophageal-derived hair shafts (tdTomato). Dotted line indicates graft-host skin interface. Both recipient and donor animals were between 9-15 weeks of age. Dashed lines indicate basement membrane. Blue, DAPI; Green, EGFP; Red, tdTomato; scale, 50 \( \mu \text{m} \).
6.5 Discussion

In this Chapter I described two complimentary transplantation strategies with the aim to investigate the capacity of adult oesophageal cells to contribute to functional hair follicles in vivo. First, we adapted a full-thickness grafting technique that allowed the transplantation of intact heterotypic grafts equivalent to those used in vitro. Although this strategy served its purpose to characterize the early stages of cell fate changes, grafts were ultimately lost. In turn, to evaluate the long-term formation of hair we used a well-established hair reconstitution assay. The use of these transplantation techniques together with immunofluorescence analysis demonstrated that oesophageal cells contribute to functional hairs and acquired the expression of keratins typically expressed in HFs.

Hair reconstitution assays have previously been used in the literature to assess the hair-forming capacity of non-skin epithelial cells (Bonfanti et al., 2010; Claudinot et al., 2020). Early work by Bonfanti demonstrated that clonogenic thymic epithelial cells (TEC) contributed to HF regeneration for months when transplanted into the skin of newborn mice (Bonfanti et al., 2010). Later on, adult epithelial cells from various tissue origins including the oral mucosa, the vagina, and the oesophagus were also found to contribute to hair formation when exposed to the morphogenic signals of the developing skin (Claudinot et al., 2020). These studies involved the transplantation of dissociated epithelial cells into the newborn mouse skin. Here, to investigate whether intact oesophageal tissue was competent to form hair, we initially used a heterotypic grafting assay (as those used in vitro). The value of this system is that the unperturbed tissue, as opposed to dissociated cells, is exposed to the skin dermis; therefore, maintaining the 3D structure of the oesophageal epithelium and denuded dermis (e.g. cell-cell interactions, ECM structure etc). A further novelty of this experiment was the use of adult recipient animals (at least 8-week old), hence evaluating the inductiveness of fully mature tissue.

Heterotypic grafting experiments revealed that adult oesophageal cells re-epithelialized the adult denuded dermis forming viable HF structures that were positive for typical HF keratins and contained a fibre highly reminiscent of a hair shaft. While encouraging, the heterotypic grafting technique posed some limitations that
hindered in-depth analysis of oesophageal-to-skin lineage conversion and hair growth. First, the engraftment efficiency was restricted and therefore the number of recovered grafts was reduced. While it remains plausible that a fully mature hair shaft was formed, it is not surprising that - due to graft scarring - hair outgrowth was not observed. Further to this, grafts were not amenable for epithelial whole-mounting and therefore had to be analysed either as full-thickness tissue or as tissue cryosections. As a result, the reduced number of recovered oHF and the limited spatial resolution hampered the identification of HF stem cell markers. Hence, more samples would be needed to fully confirm oesophageal-to-skin lineage conversion events in vivo.

Next, we turned to classical hair reconstitution assays (Jensen et al., 2010; Weinberg et al., 1993). These demonstrated that adult oesophageal cells form functional hair follicles as determined by the emergence of tdTomato+ HFs containing a red hair shaft 6-weeks post-grafting. These results indicated that adult oesophageal cells retain the competency to form hair in vivo, as previously demonstrated (Claudinot et al., 2020). In addition, the work by Claudinot provided evidence that oesophageal cells can contribute to all lineage of the pilosebaceous units as well as to HF regeneration for several months, even after serial transplantation.

Of note, only a reduced number of oHFs were found upon transplantation. In line with in vitro observations, these results supported the notion that oesophageal-to-skin lineage conversion is highly inefficient. Further to this, these results were consistent with the invariably low lineage conversion efficiency reported in the plasticity literature (Bonfanti et al., 2010, 2012; Kratochwil, 1969; Miyawaki et al., 2014). In the work by Bonfanti, transcriptional analysis comparing TEC-derived skin samples and skin HFs showed wide variability in the transcriptional signature of the first. Indeed, only a proportion of samples clustered closer to HFs than to TECs. Interestingly this pattern was maintained even after a second transplantation into the skin, suggesting that additional exposure to the ectopic skin niche did not aid in reprogramming. In support of the limited cell identity changes, they found that TEC-derived cells only contributed to long-term HF regeneration in 18% of the grafts.
Future experiments will investigate whether the plasticity barrier mechanisms observed in vitro, i.e. HIF1a-SOX9 axis (as described in Chapter 4), also operate in oesophageal cells upon transplantation. For this, the contribution of oesophageal cells obtained from HIF1a or SOX9-floxed animals to lineage conversion will be assessed in hair reconstitution assays. Alternatively, following transplantation animals may be treated with pharmacological treatments targeting the HIF1a pathway. The effect of either condition will be evaluated assessing the expression of HF markers as well as the contribution of oesophageal-derived cells to HF regeneration long-term.

Altogether, the results presented in this chapter confirmed that adult oesophageal cells retain skin competence upon transplantation contributing to hair follicle formation and giving rise to hair.
Chapter 7. Discussion

7.1 Summary

In the event of tissue perturbations, committed and differentiated cells have the ability to reacquire stem-like properties to contribute to tissue repair. This cell fate plasticity grants tissues with great regenerative capacity. However, remarkably little is known about the mechanisms that finely control cell fate changes to promote regeneration while preventing disorganized tissue growth.

Based on early recombination studies showing that epithelial cells can change their identity when exposed to ectopic stromal signals, here I sought to investigate the processes regulating lineage conversion beyond physiological constraints. For this, I made use of \textit{ex vivo} regenerative assays as well as grafting techniques that allowed me to combine epithelium and stroma from different tissue origins: the oesophagus and the skin, respectively. Characterization of oesophageal-dermal composites revealed that oesophageal epithelial cells have the ability to acquire skin identity when exposed to the instructive cues of the dermis \textit{ex vivo} and \textit{in vivo}. Remarkably, I found that oesophageal cells can contribute to the formation of functional hairs when transplanted into the skin.

The \textit{ex vivo} model used in this work was amenable for 3D imaging reconstruction allowing me to investigate cell fate changes in a spatial-temporal manner. Using immunofluorescence together with quantitative analysis, I demonstrated that only a small subset of cells executed the cell identity switch as instructed by the skin microenvironment. These results highlighted the inefficiency of the lineage conversion process.

Investigation into the defining signature of transitioning cells revealed a highly regenerative profile characterized by HIF1a activity. Finally, using gain and loss of function experiments I found that the regenerative HIF1a-SOX9 axis acts as a barrier to cell re-specification; hence, halting cells in an unresolved regenerative state. Indeed, lifting this regenerative lock releases the fate barrier, enabling cells to respond to signals instructing alternative fate choices (Proposed model Figure 42).
Taken together, my work sheds light onto the mechanisms that control the plasticity of adult epithelial cells.

**Figure 42. Suggested model.**

Oesophageal epithelium growing over the foreign stroma of the skin (the dermis) remains partially locked in a HIF1α-SOX9 driven regenerative state that restricts the ability of cells to change their identity towards skin. Lifting the regenerative barrier, by inhibiting the HIF1α-SOX9 axis, enhances the response of cells to niche signals instructing an alternative fate, and favours the lineage conversion process. Created with BioRender.com.
7.2 Contribution to the field

It has now become widely accepted that cell fate decisions are not as predetermined as originally thought. In response to injury or environmental perturbations, adult epithelial cells present a remarkable fate plasticity allowing them to respond to changes in tissue needs (Ito et al., 2005; Tata et al., 2013; van Es et al., 2012). Yet, if dysregulated, this adaptability can promote epithelial disease and cancer (Blanpain & Fuchs, 2014; Ge et al., 2017a; Schwitalla et al., 2013). Hence, understanding the largely unclear mechanisms balancing cell fate plasticity is critical to unveil its regenerative potential while preventing inherent tumorigenic risks (Ge et al., 2017a).

Under normal homeostatic conditions, progenitor and stem cells are known to differentiate into a limited range of fates, giving rise exclusively to the cell types needed to ensure adequate tissue function and integrity (Doupe et al., 2012; Piedrafita et al., 2020a). Traditionally, different models of maintenance across tissues converged on the notion that cell fate is unidirectional, with commitment towards differentiation being irreversible (Clevers & Watt, 2018; Waddington, 1961). And despite historical evidence arguing in favour of a less rigid cell fate programme, it has only recently become accepted that adult stem cells have the ability to alter their identity in response to extreme conditions, endowing them with the ability to dedifferentiate or transdifferentiate (Chacón-Martínez et al., 2018; Dhouailly & Hardy, 1978; Ferraris et al., 2000; Gola & Fuchs, 2021; Kratochwil, 1969).

Following tissue perturbations, cell fate plasticity extends the pool of cells able to contribute to tissue damage, guaranteeing an efficient repair process (Tata, 2013; van Es, 2012; Lu, 2012; Levy, 2007; Ito, 2005). Yet, reactivation of regenerative capacity can be aberrantly triggered by the tumour microenvironment to fuel tumour growth (Ge, 2018; Blanpain, 2014). This dual role of plasticity has been described in multiple tissues including the skin and the intestine, where the same cell fate changes transiently triggered in response to injury are reproduced by cancer cells (Ge, 2018). Indeed, squamous cell carcinomas (SCCs) simultaneously express genes associated with epidermal and HF lineages, similar to the transient process operating during wound repair (Ge, 2017). Similarly, intestinal differentiated cells acquire stem cell-like features to contribute to tumorigenesis; a process reminiscent
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of the controlled dedifferentiation that drives intestinal repair upon injury (Scwitalla, 2013; van es, 2012; Buczacki, 2013).

Over the years, efforts to understand the rules governing cell fate plasticity have unveiled the key role of the microenvironment in this process (Chacón-Martínez et al., 2018; Gola & Fuchs, 2021). Early tissue grafting studies in chicks uncovered that epidermal cells, regardless of their region of origin, are able to form feathers or scales if instructed to do so by dermis bearing those structures (Mauger, 1972). More recently, multiple epithelial tissues have been shown to bear the potential to switch towards HF identity when exposed to the signals of the developing skin stroma (Bonfanti et al., 2010; Claudinot et al., 2005; Ferraris et al., 2000; Lu et al., 2016). In line with these, my work supports the notion that the signals informing the oesophageal-to-skin identity switch emanate from the skin dermis. Interestingly, my results suggest that lineage conversion events are not restricted to developing tissues as originally thought, revealing that adult tissues retain both the permissibility and instructive capacity to execute changes in cell identity. This may explain clinical case reports describing the development of aberrant hairs and sebaceous glands in adult ectopic tissues, like the oral mucosa or the oesophagus (Baughman & Heidrich, 1980; Jhang et al., 2016; Miles, 1960).

An additional novelty of the regenerative in vitro system presented in my thesis is that it is amenable for epithelial whole-mounting techniques and 3D imaging allowing me to investigate cell fate plasticity at spatial-temporal resolution. This comprehensive model together with immunofluorescence analysis revealed that only a subset of oesophageal cells acquired HF stem cell markers, highlighting the inefficiency of oesophageal-to-skin lineage conversion. Interestingly, I found that oesophageal-derived HFs lacked the compartmentalized structure characteristic of the fully mature HFs (Gonzales & Fuchs, 2017; Rompolas & Greco, 2014). In this regard it is worth noting that the characteristic morphology and marker distribution of the adult HFs is not established until the first postnatal hair cycle (~p20-21) when the hair coat is fully formed (Nowak et al., 2008; Schmidt-Ullrich & Paus, 2005). This raises the possibility that oesophageal-derived HFs partially recapitulate HF morphogenesis.
Consistent with cell identity changes, I found that the oesophageal cell differentiation marker KRT4 was partially lost. Yet, transcriptional analysis did not support a full erasure of the oesophageal cell fate programme. Instead, cells maintained the expression of Sox2, a key transcription factor for oesophageal development that marks adult progenitor cells (DeWard et al., 2014). Of note, although Sox2 is known to be expressed in the skin in response to wounding and tumour formation, I did not find Sox2 expression in the in vitro skin controls (Siegle et al., 2014; Uchiyama et al., 2019). The maintenance of the tissue or origin developmental programme has been previously reported in other recombination studies (Claudinot et al., 2020). Indeed, Claudinot et al. described that epithelial cells contributing to hair follicles retained lineage genes defining their tissue of origin (e.g. thymus, bladder, oesophagus etc) even months after transplantation into the skin. My work further supports the idea that adult epithelial cells from various origins (e.g. oesophagus and trachea) retain the ability to form skin when instructed by relevant stromal signals. These results raise the possibility that modulating tissue-specific stromal cues may dictate adult epithelial cell fate as previously shown in developing tissues (Lu et al., 2016).

Recent research efforts have demonstrated that cells outside their niche remodel their epigenetic and transcriptional network acquiring a new transitional state before cell fate selection (Adam et al., 2015; Ge et al., 2017a; Joost et al., 2018). Such unresolved identity endows cells with fate flexibility, allowing them to rapidly adapt to changing microenvironments (Adam & Fuchs, 2016). However, other than the fact that tissue perturbations favour plasticity, the specific regulatory processes that dictate the ability of cells to respond to signals instructing alternative fates have remained obscure. As opposed to other studies, the early time points analysed in this work allowed me to interrogate the molecular signature of all cells exposed to an ectopic microenvironment, including those that failed to reach the alternate identity. This was of paramount importance to define the processes limiting cell fate plasticity. The findings of my thesis reveal that, during regeneration, epithelial cells exposed to ectopic niche signals, remain locked in a HIF1α-SOX9 dependent regenerative state that restricts their ability to change their identity. Once this regenerative blockage is resolved, re-specification is favoured and the number of lineage conversion events builds-up.
Interestingly, studies in the skin have shown that the hypoxic state that promotes the early wound response must be resolved in order to guarantee an adequate healing process (W. X. Hong et al., 2014; Ruthenborg et al., 2014). Indeed, while HIF1a deficiency leads to delayed wound-healing its aberrant expression following wound-closure is associated with fibrosis and scarring (Kimura et al., 2008; Lokmic et al., 2012; Rezvani, Ali, Nissen, Harfouche, De Verneuil, et al., 2011). In line with the notion that the HIF1a-SOX9 axis operate together during regeneration, SOX9 plays a key role during early re-epithelialization but its downregulation 5-days upon wounding has recently been shown to reduce scarring (Ge et al., 2017a; J. Zhao et al., 2021). Hence, the HIF1a-SOX9-dependent regenerative blockage points to a remarkably simple self-regulatory process where, in response to injury, plasticity would initially be kept at bay by the same hypoxic signals that promote tissue repair in the first place. This mechanism would ensure that the fate outcome of individual cells contributing to tissue repair is not allocated until later stages of the injury response, when hypoxic barriers are lifted and the final instructive niche is established. In a wound, often several tissue compartments are affected, and cells are known to migrate out of their niche repopulating adjacent structures (Andrew et al., 2021; Ge et al., 2017a; Ito et al., 2005; Joost et al., 2018; Levy et al., 2005; Lu et al., 2012; Page et al., 2013). Therefore, it is tempting to speculate that the existence of barriers to cell conversion during tissue repair may represent a temporal switch preventing cells from prematurely changing their identity in response to transient regenerative cues.

The present work uses a murine 3D organotypic culture system that unleashes oesophageal-to-skin lineage conversion as a model to demonstrate the existence of barriers to cell fate plasticity during tissue regeneration. Importantly, the conclusions derived from this model may not translate to humans; especially given the key architectural differences between mouse and human skin/oesophagus. A further limitation of this study is that the in vitro assay used could only be maintained for up to 10 days, hindering the long-term investigation of epithelial cell fate in oesophageal-derived hair follicles (e.g. hair follicle functionality and cycling). Investigating whether similar mechanisms operate in vivo remains an outstanding aspect in order to understand the full regenerative potential of epithelial tissues. However, to date, existing in vivo models of epithelial plasticity lack the spatio temporal resolution, and
present a very heterogeneous and inefficient response that limits \textit{in vivo} scalability. Indeed, attempts to overcome \textit{in vitro} limitations, using heterotypic transplantation assays, resulted in highly inefficient grafting preventing a comprehensive \textit{in vivo} characterization.

This study offers new insights into the mechanisms that enable adult epithelial cells to become competent to respond to signals instructing alternative fate choices upon tissue perturbations. Future work will be required to unveil the relevance of the HIF1a-SOX9 blockage, shown to limit cell fate re-specification during regeneration, for clinically relevant models such as wound healing and cancer, where plasticity is known to operate (Blanpain & Fuchs, 2014; Ge et al., 2017a; Schwitalla et al., 2013).


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