Homeostasis of the esophageal epithelium: A quest for the stem cell
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Chapter 7

Summary and Perspectives
SUMMARY

The esophageal epithelium differs from the rest of the gastrointestinal tract. The stomach and the intestine are lined by a single layer of columnar epithelium. In contrast, the esophagus is covered by a multilayered squamous epithelium. The deepest layer of the esophageal epithelium, underlined with the basement membrane, is called the basal layer. Basal cells are neatly organized with their nuclei perpendicular to the basement membrane. Proliferation exclusively is restricted to cells within this layer. In the layers above the basal layer, towards the lumen, cells are gradually differentiating. In the suprabasal layers cells become flattened and parallel to the basement membrane. These cells have large cytoplasm, causing the enlarged nuclei to be spread further apart compared to the basal layer. Approaching the lumen, nuclei are degraded. Eventually cells are shed off into the lumen. Unlike the human esophagus, the outer layer of the murine esophagus is keratinized, forming a strong protective layer against rough food. Under normal homeostasis there is a tight balance between proliferating cells in the basal layer and cell loss at the luminal surface. The esophageal epithelium is subjected to constant epithelial renewing. The capacity to regenerate cells in a tissue is generally assigned to stem cells residing within a tissue. Stem cells are characterized as undifferentiated cells with two main properties, the ability to give rise to other stem cells (self-renew) and the capacity to differentiate into all specialized cells in a tissue (potency).

In the last decade epithelial stem cells have been identified in various tissues. In the gastrointestinal tract stem cells have been identified in the intestine\(^1\) stomach, liver and pancreas.\(^2-4\) Surprisingly little research has been performed to address the identity of esophageal stem cells. It would be logical to assume that esophageal stem cells represent a subpopulation of basal cells. However, there has been a huge debate considering esophageal stem cell existence. To make the matter of esophageal stem cell more complicated, there have been reports stating that all basal cells are equal and no cell matches the criteria of a stem cell. In chapter 2 the current literature on esophageal development and epithelial homeostasis is reviewed. Evidently, we are just at the beginning of unraveling the complex mechanisms of proliferation, differentiation and stem cell biology in the esophageal epithelium. This thesis provides new findings that will help to better understand epithelial dynamics of the adult mouse esophagus.

In chapter 3 we focused on the role of Sonic Hedgehog (Shh) signaling in the adult mouse esophageal epithelium. Hh signaling plays an important role in the development of the esophagus, as is described in chapter 2. However, no functional experiments had been performed to address the potential role of Hh signaling in homeostasis of the adult esophagus in vivo. We used two different conditional mouse models to induce Hh signaling and described its function in the adult esophagus. Our data showed that the esophageal epithelium is a direct target of Hh signaling. Furthermore we demonstrated
for the first time that increased Hh signaling in the esophagus positively regulates precursor cell fate and impairs epithelial maturation and migration. Ptch1 mutant and Gli1 induced mice show features of esophageal dysplasia. Due to systemic induction of these mice, we did not have a chance to follow the mice in time. However, our data suggest that increased Hh signaling might be involved in the development of esophageal cancer. In accordance to this, in the skin Hh signaling is linked to the development of basal cell carcinoma.\(^5\) The Hedgehog signaling pathway plays an important role in the development of several other types of cancer as well, including medulloblastoma and rhabdomyosarcoma.\(^6\)

The role of Hedgehog signaling in the intestinal adenoma-to-carcinoma sequence is described in chapter 4. Shh is the main Hh expressed in the esophageal epithelium, on the other hand in epithelium of the small intestine and colon Indian Hedgehog (Ihh) is predominantly expressed.\(^7,8\) Hedgehog in the esophagus signals in an autocrine manner, whereas signaling in the intestine is directed from the epithelium to the mesenchyme.\(^9\) Loss of Ihh in a mouse intestinal adenoma model resulted in specific loss of different stromal cells suggesting that active Hh signaling from Apc mutant cells is essential to maintain the underlying stromal cells. In turn, these stromal cells are the likely supportive matrix adenomas need in order to progress.

Part two of this thesis focusses on the effect of ER stress on the adult murine esophageal epithelium. We have previously published that induction of ER stress in the intestinal epithelium causes specific loss of stem cells through a process of differentiation.\(^10\) XBP1, a key component of the ER stress signaling pathway has recently been linked to the risk of developing esophageal squamous cell carcinoma.\(^11\) However, the role of ER stress in esophageal homeostasis and the way it may protect against tumorigenesis remained completely uncharacterized. In chapter 5 we described a heterogeneous expression pattern of components of the ER stress pathway in progenitor cells in the basal layer of the esophagus and in differentiated cells. We use both a pharmacological and a genetic model to show that ER stress forces esophageal progenitor cells to differentiate. Our data suggest that sensitivity of esophageal progenitor cells to ER stress may protect against tumorigenesis by forcing progenitor cells with potentially deleterious changes to differentiate.

In chapter 6 we combined the fact that esophageal progenitor cells differentiate upon induction of ER stress and that intestinal stem cell markers are specifically lost upon induction of ER stress. We investigated expression of genes down-regulated upon induction of ER stress in esophageal squamous cell carcinoma cell lines. Nine genes were found to be expressed in a subpopulation of the proliferating basal cells, which would fit with the potential stem cell position in the esophagus. We further examined one of these genes, Id2. Id2 is described to be expressed in progenitor cells of the respiratory tract.\(^12\) By immunohistochemistry for Id2 and immunohistochemistry for GFP
in a Id2-GFP mouse we confirmed this heterogeneous expression within the basal layer. Using the lineage tracing experiments with Id2Cre-ZsGreen mouse we could establish long lived clones up to 6 months. Mathematical analysis confirmed our suspicion that we were in fact looking at cells with a long term progenitor capacity. The behavior of the Id2 derived clones seemed to conform neutral stochastic expansion opening a possibility that Id2 marks a cell with dynamics resembling a stem cell.

**PERSPECTIVES**

The esophagus, like the rest of the gastrointestinal tract, is derived from endoderm. However, it is a squamous epithelium, unlike the stomach and the intestine which are composed of columnar epithelium. In this aspect it resembles the ectoderm derived squamous epithelium of the skin. Well known markers of the basal layer of the esophagus are expressed as well in the basal layer of the skin, for example keratin 5, keratin 14 and p63. Moreover, loricrin, involucrin and keratin 13 mark differentiated cells in both tissues. Similarly to the mouse esophagus the skin forms a keratinized layer at the top. There are numerous publication on epithelial homeostasis in the skin and more specifically epidermal stem cells. The esophagus is devoid of a geographic niche for a potential stem cell, on the other hand the skin has the hair follicle. Different stem cell populations have been described in different regions of the hair follicle in the skin. Established stem cell markers in the hair follicle are CD34, Lgr5, Lgr6, keratin 15, Lrig1 and Blimp1. As there is a striking resemblance in epithelial structure, comparison between skin and esophagus can likely provide us with clues about esophageal stem cell. Several of the epidermal stem cell markers have been studied in the esophageal epithelium by us and by others. CD34 was tested in a label retaining model by Kalabis et al. They identified CD34 as a marker of label retaining cells and demonstrated that these cells are capable of forming 3D organotypic culture. Doupé et al. approach the quest for stem cell identification in a similar fashion. They investigate label retaining cells by using a doxycycline inducible Histone-2B-GFP mouse. Doxycycline withdrawal causes dilution of GFP. After 4 weeks only 0.4% of basal cells were GFP positive. Careful experiments proved these label-retaining cells to be of hematopoietic origin. Thus, the existence of quiescent stem cells in the esophageal epithelium is disputable as experiments of Doupé et al suggests that they might be hematopoietic cells. Furthermore, none of the tested stem cell markers, among others CD34, were observed in these label-retaining cells.

We have tested expression of other hair follicle stem cell markers Lgr5 and Lgr6 in the mouse esophagus by *in situ* hybridization. In the skin Lgr5 is expressed in the bulge region of the hair follicle, whereas Lgr6 expression is at the base of sebaceous gland. We confirmed this expression with our Lgr5 and Lgr6 *in situ* hybridization probes
(Figure 1). Surprisingly, no Lgr5 expression was seen in the esophageal epithelium. However, Lgr6 is expressed in a subpopulation of epithelial cells in the basal layer. As we believe the esophageal stem cell needs to be expressed in a heterogeneous manner in the basal layer, Lgr6 is a likely candidate for a stem cell marker. Unfortunately, we did not perform any functional experiments confirming this potential role of Lgr6 yet. The in situ hybridization screen described in chapter 6 was also performed on mouse skin samples. We found that most of the genes tested were expressed in the hair follicle, with the majority being restricted the bulge of the hair follicle, Id2 confirming this (figure 2).

**Figure 1** | *In situ* hybridization for Lgr5 and Lgr6 in the mouse esophagus and the mouse skin
Clearly, the identity of the esophageal stem cell has not been resolved yet. Further functional experiments have to be done in order to shed more light into the esophageal stem cell existence. Currently we are working on establishing an esophageal organotypic culture system using FACSorted cells that are grown under special conditions. We could use this system to provide additional evidence of Id2 being an esophageal stem cell marker. We would need to investigate whether Id2 positive basal cells have a growth advantage compared to Id2 negative basal cells. However, it is questionable if esophageal cells taken out of their natural environment and put in culture system will retain their stem cell properties. On the other hand, non-stem cell proliferating cells might act like stem cells \textit{in vitro}. Therefore, even better experiment would be to investigate the dynamic of clones derived from Id2 negative basal cells. As Id2 is downregulated upon induction of ER stress, components of the ER stress pathway would be likely candidate loci for such tracing experiment.
REFERENCES