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Epithelial progenitor cells from the human Fallopian tube

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Abstract

The human Fallopian tube (hFT) provides an ideal environment for oocyte fertilization and subsequent development of the resulting blastocyst. Since the inner epithelial mucosa is a dynamic tissue that underlies a constant turnover of cells, a long-term regeneration capacity is required.

Adult stem cells and their pluripotent progeny, the transit-amplifying cells, have been identified in various tissues. These cells have been found to be responsible for adult tissue repair and homeostasis. So far, the self-renewal mechanisms of the tubal epithelium are poorly understood.

In this study, we were able to detect undifferentiated epithelial progenitor cells in the tubal mucosa and found evidence for the presence of adult stem cells existing along with common terminally differentiated cells. Further, we established a novel three-dimensional ex vivo model of epithelial cells derived from the hFT, the “organoids”. By immunofluorescence staining, polarized epithelial cells expressing pluripotency markers were identified within the organoids. These cells are assumed to reside in the tubal mucosa as progenitor cells. Furthermore, we performed in situ hybridization for Lgr5, the most reliable marker for adult stem cells. Our preliminary results indicate the existence of Lgr5 positive cells in the organoids. To ultimately verify the presence of Lgr5 positive stem cells in the tube, the expression has to be demonstrated at protein level using our generated monoclonal antibodies.

To date, there was nothing known about progenitor cells in the hFT. Our results indicate that the mucosal epithelium of the hFT contains pluripotent progenitor cells and potential Lgr5 positive adult stem cells, providing a possible mechanism for tissue homeostasis. In addition, a 3D ex vivo model mimicking in vivo conditions is important to facilitate the study of bacterial infections. Of particular interest is *Chlamydia trachomatis*, which leads to tubal scarring and infertility.

Zusammenfassung

Der menschliche Eileiter stellt eine optimale Umgebung für die Befruchtung der Eizelle sowie die darauf folgende Entwicklung des Blastozysten dar. Die innere epitheliale Schleimhaut ist ein dynamisches Gewebe, das einer konstanten Zellerneuerung unterliegt und daher die Fähigkeit zur ständigen Regeneration benötigt.

Adulte Stammzellen, sowie deren pluripotente Nachkommen, die Vorläuferzellen, wurden in einer Vielzahl von Geweben identifiziert. Diese Zellen regulieren Wundheilung und ermöglichen Gewebshomöostase. Die Mechanismen, die der Selbsterneuerung des Schleimhautepithels im Eileiter zu Grunde liegen, waren bislang unbekannt.

In dieser Arbeit ist es uns gelungen, undifferenzierte Vorläuferzellen sowie mögliche adulte Stammzellen des Schleimhautepithels im menschlichen Eileiter zu identifizieren. Weiters konnten wir ein neues 3D ex vivo Modell epithelialer Eileiterzellen generieren, die sogenannten „Organoide“. Immunfluoreszenz-Analyse hat gezeigt, dass die Zellen in den Organoiden polarisiert sind und pluripotente Eigenschaften aufweisen. Mittels In situ Hybridisierung wurde die zelluläre Expression des adulten Stammzell-Markers Lgr5 bestimmt. Unsere vorläufigen Ergebnisse sprechen dafür, dass Lgr5 positive Zellen in den Organoiden und damit wahrscheinlich auch im Eileiter vorkommen. Durch weitere Tests mit den Seren der ebenfalls generierten monoklonalen Antikörper gegen Lgr5 gilt es diese Annahme zu überprüfen.

Zusammengefasst haben wir gezeigt, dass das Schleimhautepithel des menschlichen Eileiters pluripotente Vorläuferzellen enthält. Außerdem sprechen unsere Ergebnisse für die Anwesenheit von Lgr5 positiven adulten Stammzellen. Diese Resultate liefern einen möglichen Mechanismus für die Regeneration des Schleimhautepithels im Eileiter. Darüber hinaus kann unser 3D ex vivo Modell helfen, bakterielle Infektionen des Eileiters – insbesondere durch *Chlamydia trachomatis* – zu erforschen.

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1 Introduction

The human Fallopian tubes are two thin tubes in the female body that connect the ovaries with the uterus. These tubes, which are also known as uterine tubes, oviducts, and salpinges, enable the passage of the oocyte or zygote to the uterus (Figure 1). The tubes are structured into three different regions: the infundibulum with its associated fimbriae tubae uterinae; the ampullary part, where the fertilization takes place; and the tighter isthmic part near the uterus[1,2].

In the tube, two layers of muscles (myosalpinx) and a parenchyme layer (lamina propria) surround an inner mucosal layer (endosalpinx), which is folded out into the lumen to connect the ovum with the epithelial surface[3]. The mucosa consists of an columnar epithelial monolayer with two different types of cells: the non-ciliated peg cells and the ciliated cells. The peg cells produce cycle-dependent a mucoid fluid, while the ciliated cells establish a flow in the direction of the uterus. Taken together, the two cell types of the mucosal epithelium enable the transport of the gametes and subsequently of the fertilized egg cell to the uterus[2].

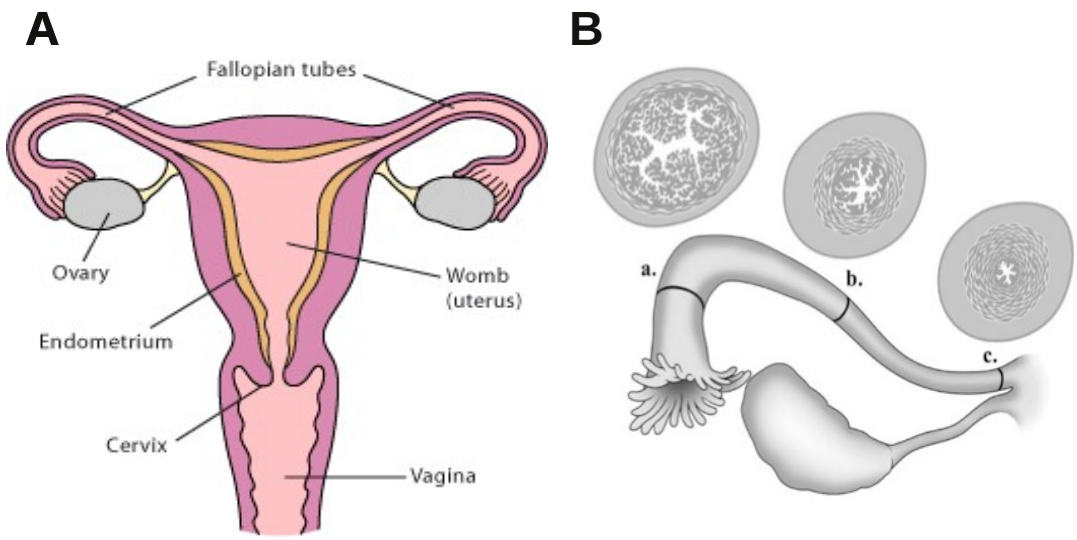

Fig. 1. The anatomy of the human Fallopian tube. (A) Schematic overview of the female reproductivity system[4]. (B)The inner mucosal epithelium (endosalpinx) is folded into large folds that connect with the ovum. The mucosa is surrounded by connective tissue (lamina propria) and two muscle layers (myosalpinx). (a) The infundibulum with its associated fimbriae tubae uterinae. (b) The ampulla, where fertilization takes place. (c) The isthmic part of the tube next the the uterus[1,5].

This characteristic tubal anatomy provides the microenvironment for the fertilization of the egg and the subsequent transport of the developing blastocyst. A patent tube is therefore essential for the female reproductive health.

Because every mucosa is a dynamic tissue, a constant turnover of cells occurs. It is known that the Fallopian tubes undergo endocrine-induced changes during the menstrual cycle and that they have a great regeneration capacity[6,7]. In order for the mucosal tissue to function and for the cells to stay alive, they have to regenerate and to replace cells lost through cycles of growth, differentiation, and shedding. This process is called homeostasis. It is known from other tissues how this self-renewal of cells during tissue

maintenance and response to injury takes place: pluripotent adult stem cells maintain the turnover of cells[8].

To date, the mechanisms of epithelial renewal in the human Fallopian tube are unknown. The question of interest is whether the tubal epithelium also contains adult stem cells that drive the long-term regeneration capacity of the Fallopian tube.

1.1 Adult stem cells – features and division strategies

In the last few years, adult stem cells have been identified in a wide variety of tissues and organs, such as the central nervous system, bone marrow, retina, endometrium and the gastrointestinal tract[9-11]. These undifferentiated cells were identified by the classical characteristics of a stem cell: the ability of unlimited self-renewal and multipotency, which means they can generate all differentiated cell types of a tissue and therefore are responsible for adult tissue homeostasis and repair.

Watt and Hogan[12] reported about two different strategies for stem cell-renewal and differentiation. First, single stem cell asymmetry, where two daughter cells with different fates are generated: through infrequent division of a stem cell remains one daughter cell in an undifferentiated state as a stem cell, while the other cell starts differentiation and is then called “transit-amplifying cell” (Figure 2). These more mature progenitor cells represent an intermediate cell population between the stem cells and the terminally differentiated effector cells; they are “transit”. A transit-amplifying cell is still multipotent and divides more frequently than a stem cell, but has only a limited number of cell divisions left[13].

The second strategy of self-renewal is population asymmetry: cell division does not have to be strictly asymmetric, but can also be symmetric. To maintain long-term homeostasis within the stem cell compartment, loss and gain of stem cells must occur with the same frequency. Some stem cells are lost due to differentiation or damage; subsequently other cells may also divide symmetrically into two stem cells. This mechanism therefore preserves a constant pool of stem cells.

Recent studies were able to confirm population asymmetry as the mode of self-renewal and show that the rate of stem cell regeneration is similar to the division rate. Therefore, loss of stem cells occurs in normal tissue[14].

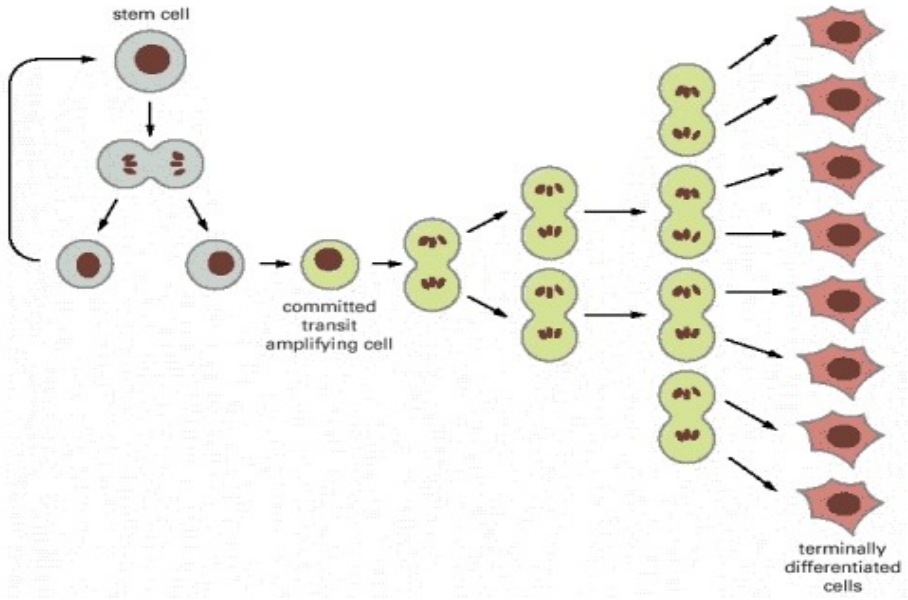

Fig. 2. From the slow-cycling stem cell to a rapidly-cycling terminally differentiated cell. A multipotent adult stem cell generates two daughter cells with different fates: one cell remains in the stem cell compartment, while the other cell is a transit-amplifying cell (TA cell). The more mature, multipotent TA cell passes a limited number of division cycles and finally generates the terminally differentiated effector cells of a tissue[13].

1.1.1 The stem cell niche

Stem cells require a specific environment that supports growth and regulates homeostasis – the stem cell niche. Stem cell niches are specific anatomic locations that regulate how stem cells participate in tissue generation, maintenance, and repair[15]. The stem cells get intrinsic and extrinsic cues from surrounding non-stem cells and interact with the extracellular matrix (ECM). These regulatory processes ensure the balance between the rate of retaining stem cells and cells that commit a terminally differentiation pathway.

1.1.2 Lgr5 as a marker of adult stem cells

Adult stem cells are difficult to isolate and characterize, because there are no specific markers that enable the distinction between stem cells and their multipotent progeny, the TA cells. However, in 2007, Barker et al.[16] were able to identify adult stem cells in the small intestine and colon by the marker gene Lgr5, which is a target of the Wnt pathway. These studies showed the exclusive expression of Lgr5 in the cycling columnar base cells at the bottom of the intestinal crypts. Rare cells also expressed Lgr5 in the eye, brain, hair follicle, mammary gland, and the reproductive organs.

Lgr5, also known as G-protein receptor 49 (Gpr49), is a leucine-rich repeat-containing G-protein coupled receptor (Figure 3 A). To date, the ligands of this orphan receptor are still unknown.

The 100 kDA-sized protein contains a large N-terminal extracellular (ecto-) domain with sixteen leucine-rich repeats (LRR) that mediate ligand interaction, followed by seven transmembrane domains[17] (Figure 3 B). The glycoprotein Lgr5 is structurally related to the hormone receptors thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH)[18].

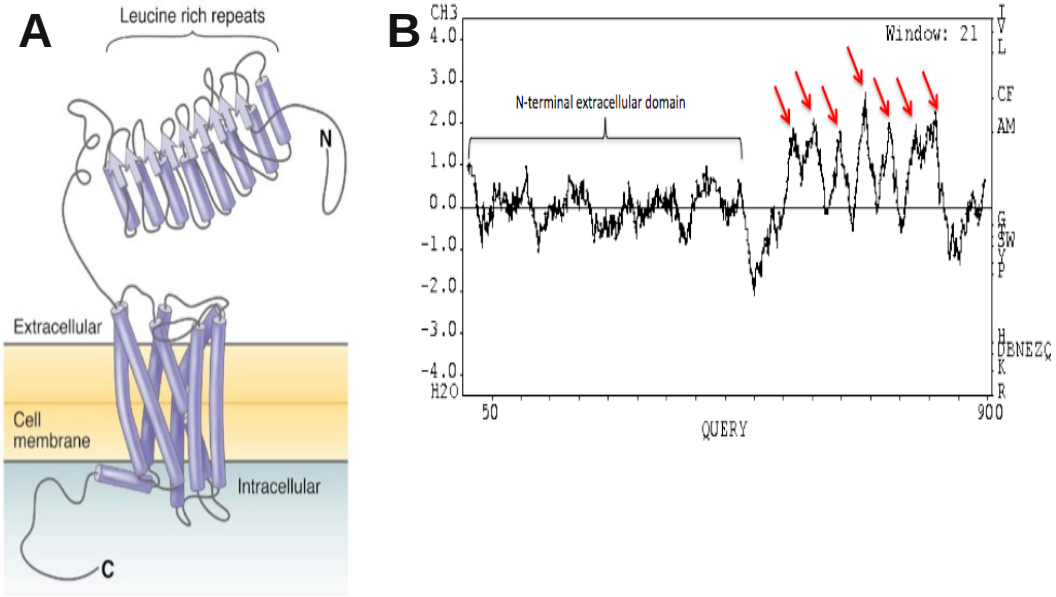

Fig. 3. Leucine-rich repeat-containing G-protein coupled receptor 5. (A) Predicted strucutre of Lgr5[19]. (B) Hydrophobicity plot for Lgr5 performed with window size 21. The large N-terminal extracellular (ecto-)domain (540 AA) with the 16 leucine-rich repeats (LRR) is followed by the seven transmembrane domains (marked with red arrows). The small cytoplasmic domain at the end consists of 84 AA[17,18,20].

Since cancers of the colon and the ovary have been found to show a high expression of Lgr5, it is suggested that these Lgr5 positive cells may have important roles in cellular transformation processes and oncogenesis in the tissues[21].

1.2 The cancer stem cell hypothesis

Within the last years growing evidence supports the theory of the “cancer stem cell” (CSC). As already mentioned before, the balance between stem cell regeneration and differentiation is important to prevent uncontrolled tissue growth or tissue damage. If a niche no longer controls a stem cell, it may convert into a cancer stem cell and give rise to aberrant-growing malignant cells. Lapidot et al.[22] first reported about the existence of CSC in acute myeloid leukemia and over the years, many studies are pointing to the existence of CSC in several solid tumors, including breast cancer, brain tumors and colorectal cancers[23].

Referring to the cancer stem cell hypothesis, a tumor consists of various cell types that are hierarchical organized within the tumor[23,24]. Rare, slowly dividing stem cells produce rapidly multiplying cells, the transit-amplifying cells that again differentiate to the terminally differentiated cells of the tumor[13]. Only a small population of cancer stem cells induces extensive tumor proliferation and subsequently drives tumorigenesis. Studies show that cancer stem cells display characteristics of adult stem cells, like self-renewal (by symmetric and asymmetric division) and production of rapidly amplifying cells[25]. Because this subpopulation of cancer cells is resistant to common cancer therapies such as radiation and chemotherapy, they are responsible for tumor return and progression and may further cause tumor spreading (metastasis) and tumor dormancy[26].

Beside the cancer stem cell hypothesis there is another theory of tumor propagation – the clonal evolution model[27]. It relies on the hypothesis that malignant cell growth arises from one mutated cell, which has a growth advantage over the other cells and is therefore selected and expanded. Tumor

growth can be driven by all differentiated cells, who have an equal potential to acquire further mutations. However, both models – the cancer stem cell hypothesis and the clonal evolution model are not mutually exclusive, since CSC also undergo clonal selection. Both models are assumed to exist in human cancer[23,27,28].

1.2.1 Different ways of CSC formation

Taken together, studies postulate three different hypotheses how cancer stem cells develop (Figure 4). Genetically altered CSC may arise from stem cells, their partly differentiated progeny – the progenitor cells – or the terminally differentiated cells. Mutations occurring in these cells may lead to the transformation of normal cells, onsetting of self-renewing genes and subsequently to aberrant cell growth resulting in neoplasms[29].

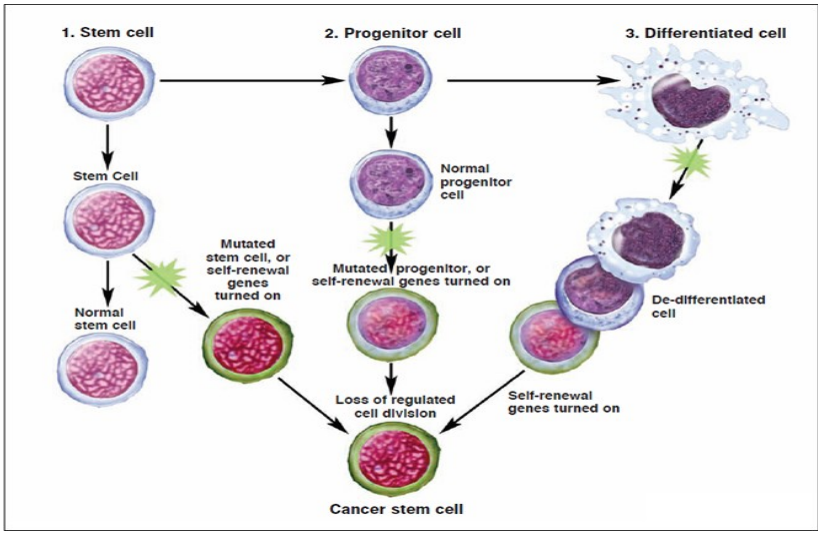

Fig. 4. Ways of cancer stem cell development. (1) A stem cell undergoes a mutation. (2) A progenitor cell undergoes two or more mutations. (3) A fully differentiated cell undergoes several mutations that reset it to a stem-like state. All three processes lead to a cancer stem cell, which has lost the ability to regulate its cell divisions and subsequently leads to tumor formation[29].

1.3 Side population cells

Side-population cells (SP cells) were first described in 1996 by Goodell et al.[30] as a subpopulation of hematopoietic stem cells in the bone marrow. To date, this subset of stem cells was isolated and characterized in various tissues, such as brain, liver, and kidney[31]. Furthermore, they seem to play an essential role in cancer genesis in solid tumors[32]. Various neoplasms have been shown to contain SP cells, including cancers of the breast, lung, prostate, and the ovary[33]. Thus, it is likely that SP cells represent a subset of solid tumor cells in human[33-36].

Based on fluorescence-activated flow cytometric analysis (FACS), SP cells have been identified by their ability to efflux the DNA-binding dye Hoechst 33342 compared to the main population[30]. SP cells express high levels of ATP-binding cassettes (ABC-transporter). The ABC-transporter is a transmembrane protein, which uses ATP hydrolysis to transport small molecules out of the cell[13]. It seems reasonable to presume that the expression of ABC-transporters on cancer SP cells contributes to the multi-drug resistance of some primary tumors[33].

In 2007, Kato et al.[37] morphologically characterized SP cells in the human endometrium and described them as small, round cells with a long-term proliferation capacity in vitro. Little is known about the significance of these cells, but results indicate that the SP phenotype might represent a common molecular feature for stem cells.

1.4 The Wnt signaling cascade and its importance

Wnt signaling is important during development and for the maintenance of stem cells within their niche[38]. Beside molecular events in individual cells,

cell-cell communication and paracrine signaling pathways in the tissue have equal importance for the development of cancerous lesions. A major factor that plays a role in tumor formation is the canonical Wnt pathway.

1.4.1 Canonical Wnt signaling – an overview

In the absence of a Wnt signal, the key molecule of the Wnt pathway, β-catenin, is marked for ubiquitination and subsequently degraded (Figure 5). A protein degradation complex binds the molecules while the two serine/threonine kinases CK1 (casein kinase 1) and GSK3 (glycogen-serine kinase 3) phosphorylate β-catenin. This phosphorylation marks the molecule for ubiquitination by the E3 ubiquitin ligase, which primes β-catenin for proteasomal degradation[13,39].

The scaffolding proteins Dishevelled, axin, APC (adenomatous polyposis coli) and the serine/threonine kinases CK1 and GSK3 regulate β-catenin labeling for degradation. While axin and APC associate with the proteasomal degradation complex, CK1 and GSK3 phosphorylate the bound β-catenin.

Binding of the Wnt ligand to the seven-transmembrane protein Frizzled (Fz) and its co-receptor LRP5 or LRP6 (low-density lipoprotein receptor-related protein 5 and 6) leads to the formation of a complex and the transduction of the signal into the cell – the intracellular signaling cascade begins. Wnt brings Fz and the co-receptor close together and in a poorly understood process, GSK3 and CK1 phosphorylate the cytoplasmic tail of LRP. As a consequence, LRP recruits axin and binds it, while Dishevelled is recruited by Fz. Bound to the membrane, the degradation complex is inactivated, β-catenin is no longer degraded, accumulates in the cell and translocates in the nucleus[13].

Without Wnt pathway stimulation, the translation of the Wnt target genes is inhibited by a silencing complex formed by members of the LEF/TCF family. This silencing complex is associated with Groucho, a co-repressor. In the presence of Wnt, β-catenin is translocated into the nucleus and binds LEF/TCF as a co-activator. Groucho is displaced and the transcription of the Wnt target genes can begin. Wnt target genes include – amongst others – Lgr5, the adult SC marker, and c-myc, a stimulator of cell growth and proliferation[13,40].

Frizzled binds Wnt proteins with high affinity through its cysteine-rich extracellular domain (CRD)[41]. Besides Frizzled, also other Wnt receptors are known. Ryk, a receptor tyrosine kinase, is an alternative co-receptor required for Wnt signaling mediated by Wnt3a for the stimulation of neurite outgrowth[42].

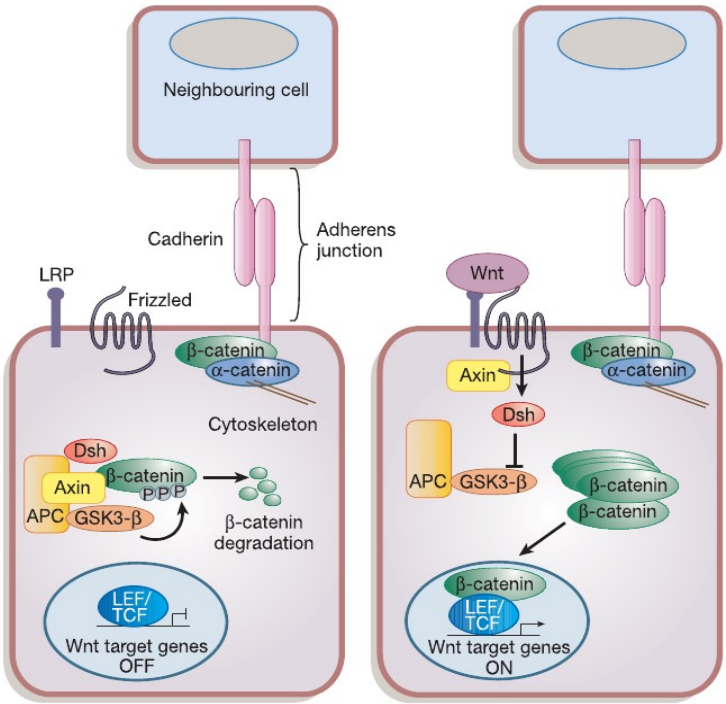

Fig. 5. Wnt signaling pathway. In the absence of Wnt, β-catenin is degraded by the destruction complex. Binding of Wnt leads to the inhibition of the degradation and β-catenin translocated in the nucleus, where it binds the LEF/TCF complex and activated the transcription of the Wnt target genes[39].

1.4.2 The Wnt3a protein

The term Wnt is composited from the Drosophila gene Wingless and its homolog in mice, the proto-oncogene Int-1[43]. In mammals, 19 different Wnt proteins are known[40]. Wnt proteins are signal molecules that act as local mediators and morphogens during development[13]. One of the best studied members of this highly conserved protein family is Wnt3a.

Wnt3a is a secreted protein with a signal domain. It consists of 352 amino acids (AA) and is post-translationally modified : positions 87 and 298 are glycosylated, while a conserved cysteine residue at position 77 is modified by a thioester-linked palmiate. Serine 209 shows acetylation with palmitoleic acid, an unsaturated fatty acid[44,45]. Palmitoylation is performed by the porcupine acyl-transferase[38]. Due to these particular lipid modifications, Wnt proteins are hydrophobic. However, the modifications seem to be important for the secretion of the protein into the extracellular space[44].

Wnt secretion from the producing cell is guided by the multipass-transmembrane protein Wntless (Wls)/Evenness interrupted (Evi), whereas lipoprotein particles are required for the extracellular transport[46,47].

1.4.3 β-catenin and its role in cell-cell adhesion

β-catenin is not only found in the nucleus, where it is a hallmark of active Wnt signaling, but also in adherent junctions (AJ). Adherent junctions are protein clusters at the lateral membrane of polarized epithelial cells, which are necessary for the formation of epithelial layers[48].

1.4.4 Wnt signaling – from stem cell maintenance to cancer

As already described, the cancer stem cell theory has increased our understanding in the formation of malignant neoplasms. It is known that various pathways which regulate self-renewal of stem cells are also activated in malignant cells. Besides Notch and Shh (sonic hedgehog) signaling, the Wnt pathway is one of them[39].

Lgr5, the main marker for adult stem cells, is a target of active Wnt signaling and was found to be up-regulated in colon cancer. The transformation process of cells in colorectal cancer was assumed to take advantage of the unique dependence of stem and progenitor cells on the Wnt pathway – this implication was confirmed with the discovery of Lgr5. Both, normal long-living stem cells at the base of intestinal crypts and malignant transformed colon cancer cells express the Wnt target gene Lgr5[39]. This indicates that dysregulation of the Wnt cascade leads to cell transformation.

Once the Wnt pathway is constitutively activated by mutation, the affected cells keep their capacity of self-renewal. Since Wnt acts as a morphogen, the induction of paracrine signaling will affect neighboring cells in the tissue and lead to aberrant cell growth, which can result in tumor formation.

Deregulated activation of the Wnt cascade occurs in different biological context; studies showed that mutations in β-catenin cause onset of the Wnt pathway in gastric cancer and mutational inactivation of APC leads as well to Wnt activation in colon cancer[49,50].

1.5 Chlamydia trachomatis

Chlamydia trachomatis (*Ctr*) is an obligatory intracellular human bacterium that infects the mucosal surface. Infection causes in both man and woman urethral inflammations, which at worst can lead to infertility in the female through Fallopian tube damage[51,52]. *Chlamydia trachomatis* can also infect the eye, with trachomas and possible blindness as a result. *Chlamydia* infections count to the leading sexually transmitted diseases – worldwide there are more than 600,000 reported cases every year[51].

1.5.1 The life cycle of Ctr

Chlamyida trachomatis has two distinct forms: the infectious, non-multiplying elementary body (EB), and the non-infectious reticulate body (RB) (Figure 6). The elementary body enters the host cell by binding to a yet unknown receptor and subsequent endocytosis. Inside the cell, the EB forms a cytoplasmic aggregate, the inclusion body. After few hours, the EB differentiates within the inclusion into its vegetative form, the RB. The RB is larger and divides by binary fission. During the second differentiation process, the multiplied RBs convert into EBs. Little is known about the liberation of the elementary bodies, but either by pathogen-induced host cell lysis or by mechanical bursting, they are released and able to infect new host cells[51].

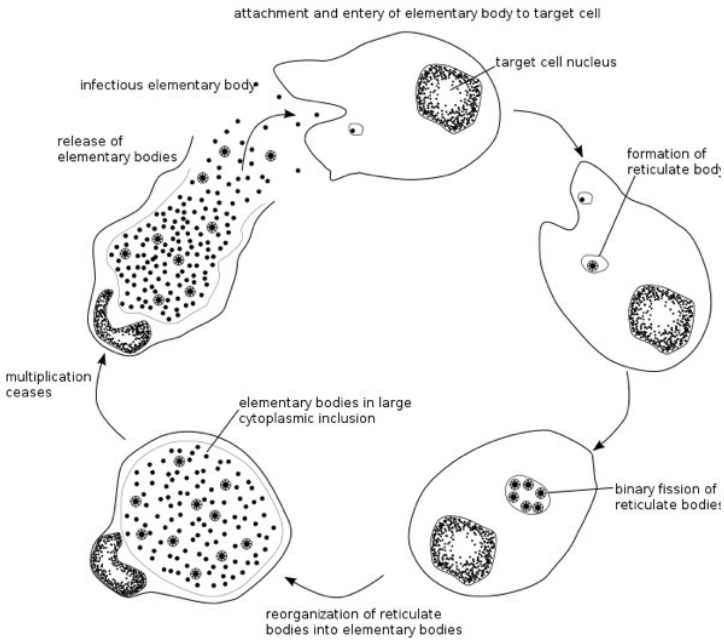

Fig. 6. Life cycle of *Chlamydia trachomatis*. After the infectious RB enters the host cell, it forms in an inclusion body. Within this inclusion, the RB differentiates into the non infectious EB, which multiplies via binary fission. The EBs differentiate back into the RB form and are released from the host cell[53].

2 Aim of the study

Accumulating clinical evidence suggests that the majority of ovarian tumors actually arise from the Fallopian tube epithelium rather than from the ovarian surface cells[54]. Studies showed that the complete removal of the tubes during procedures such as tubal ligation and hysterectomies strongly reduces the risk of developing serious ovarian carcinomas later in life[55].

The disruption of the Fallopain tube's mucosal epithelium by bacteria in the first line, including *Chlamydia trachomatis* and *Neisseria gonorrhoe*, is a serious hazard for the female reproductive capacity. Fallopian tube infection leads to salpingitis (tubal inflammation). Salpingitis causes increased tubal fluid production or even suppuration. Untreated, the infection leads to tubal damage and scarring, which emerges during the healing process. As a result, oocyte fusion with the sperm cell is prevented, leading to infertility[56].

Hence, a primary ex vivo model is required to enable a better understanding of the pathogen-host interaction in vivo and etiology of *Chlamydia* triggered pathology in the tube. *Chlamydia trachomatis* infection is the leading causative agent of sexually transmitted diseases, yet the entering process in the host cell is poorly understood. *Chlamydia trachomatis* infects a variety of cultured cells, but so far, there is nothing known about putative receptors. Another fact that contributes to the urgent requirement of an primary ex vivo model, is the absence of a suitable in vivo model. HeLa cells are also not suited for in vitro infection studies, because HeLa cells are tumor-derived and therefore genetically modified through countless mutations. This leads to non-significant conclusions about the infection process.

The aim of this study is to identify epithelial progenitor cells in the human Fallopian tube and to provide a long-term in vitro model to study bacterial, especially *Chlamydia* infection.

3 Material and Methods

3.1 Human Fallopian tubes

All Fallopian tube samples were obtained from the Klinik für Fauenheilkunde und Geburtshilfe der Charité, Campus Virchow-Klinkum Berlin. The tubes were transported on ice and prepared within one to two hours after dissection.

3.1.1 Treatment of the tubes and cell isolation

The tubes were washed in DPBS and the connective tissue was removed. By cutting the tube open, the inner mucous surface was unfold. The mucosa was treated with collagenase (0.5 mg/ml) (Biochrom) to disrupt the peptide bonds of the extracellular matrix and incubated at 37°C for one hour. After the enzymatic treatment, the epithelial cells in the lumen were scratched out with a scalpel. The primary cell suspension was transferred in a new falcon tube and subsequently centrifuged for 5 minutes at 400 rpm at 4°C. The supernatant and possibly existing blood cells were removed with a pipette and the cell pellet was resuspended in Advanced DMEM F12+ P/S + 10% FCS (complete medium) and EGF (10 ng/µl) as a growth supplement.

3.1.2 Cultivation

The primary epithelial cells were cultured in 25 cm^3 flasks at 37°C and 5% CO_2. Every two days, the cells were provided with fresh medium. When the cells were confluent – usually one week after dissection – they were passaged. For this purpose, the medium was removed, the cell layer was washed with DPBS, treated with trypsin/EDTA and incubated for 10 minutes at 37°C. The process was stopped by addition of complete medium. The cells were transferred into new flasks with fresh medium. The splitting process was repeated every three to four days up to passage seven, yielding usually more than five flasks with 1 x 10^7 cells each.

To remove fibroblasts, the cells were differentially trypsinated. This means that the culture was incubated with trypsin/EDTA only for two minutes and then the reaction was stopped by addition of complete medium. Fibroblasts are supposed to dissolve earlier than epithelial cells.

All steps were performed under germ-free conditions under a sterile hood.

3.1.3 Stock preparation of epithelial cells

The cultivated cells were expanded in 75 cm^3 flasks and stocked in an early passage (P), normally P2 or P3. In order to this, at least 1 x 10^6 cells were trypsinized, then centrifuged for 4 minutes at 800 rpm at 4°C, washed with complete medium and centrifuged again. The cell pellet was resuspended with precooled freezing medium (FCS + 10% DMSO). DMSO is an organic solvent and used as a cryoprotectant. The primary suspension was transferred in a 1 ml-cryotube (Nunc) and placed in a precooled cryobox

(Qualifreeze). The box was stored at -80°C overnight to guarantee a gentle freezing process. The cryotubes were transferred into liquid N_2 and kept for eventual use.

3.1.4 Thawing and re-cultivation of epithelial cells

The frozen cells were thawed at 37°C in a water bath. Subsequently, the cells were resuspended in complete medium, transferred into a 75 cm^3 flask and cultivated at 37°C and 5% CO_2. The medium was changed the next day.

3.2 Cultivation of three-dimensional organoids in Matrigel

The epithelial cells were seeded in Matrigel to test their behavior in 3D. The cells were trypsinized, pelleted and counted in a Neubauer chamber. A total of 20.000 cells were pipetted in 50 µl chilled Matrigel (BD Biosciences) in 24-well plates.

Matrigel is a serum derived from murine Engelbreth-Holm-Swarm (EHS) sarcoma cells that supports the three-dimensional cell growth in vitro. Matrigel is rich in extracellular matrix proteins and mimics an extracellular matrix (ECM). Main components of Matrigel are laminin, collagen IV, entactin, and various growth factors, such as EGF and TGF-β[57].

The plate was incubated at 37°C for 15 minutes until the gel drop with the cells was solidified. Subsequently, 500 µl organoid medium were added.

Composition of 1 ml organoid medium:

		Final concentration
Complete medium	220 µl	
Wnt3a (in-house production)	500 µl	
R-spondin (in-house production)	250 µl	
human B27 (Invitrogen)	20 µl	1x
Nicotinamide (Sigma)	10 µl	122 ng/µl
murine Noggin (PrepoTech)	1 µl	100 ng/µl
human EGF (10 ng/µl) (Invitrogen)	1 µl	0.01 ng/µl
human FGF10 (100 µg/ml)(PrepoTech)	1 µl	0.1 ng/µl

The Matrigel cultures were incubated at 37°C and 5% CO_2. The organoid medium was changed every two days.

3.2.1 Passaging of organoids

To passage the Matrigel-originated organoids, the medium was removed from the wells. 500 µl ice-cold complete medium were used to liquefy the Matrigel drop and the suspension was transferred in a new falcon tube. The organoids were mechanically disrupted with a fire-polished Pasteur pipette until all organoids were dissociated. The cells were pelleted by centrifugation for 5 minutes at 1200 rpm at 4°C. Two layers have been formed – one upper layer containing the Matrigel and one lower cell layer. The supernatant and the upper layer were removed and the cells were re-seeded in fresh Matrigel (see Matrigel seeding) with organoid medium. Passaging was performed every 8-9 days.

3.2.2 Coating of cover slips with Matrigel

In order to grow epithelial cells on a Matrigel basement, cover slips were coated with 30 µl 40% Matrigel and put in a 24-well plate. The Matrigel dilution was prepared with conventional double-distilled water (ddH_2O). Prior to cell seeding, the plate with the coated cover slips was dried for at least 30 minutes at 37°C.

3.2.3 Cell fixation with PFA

All cells and organoids were fixated with 3.7% PFA (para-formaldehyde). The medium was removed, the wells were washed with DPBS and fixated with 3.7% PFA at room temperature (RT). 2D cell cultures were treated for 20 minutes with PFA, whereas organoids in Matrigel were incubated overnight to ensure the penetration of PFA through the gel. Afterwards, the cells were washed twice with DPBS and the plates were stored at 4°C until usage.

3.2.4 Paraffin embedding and microtome sections

The fixed samples were placed in embedding cassettes and dehydrated in a series of alcohol dilutions in the Shandon Citadell 1000 rondell: 60% ethanol (1 h), 70% ethanol (1 h), 80% ethanol (1 h), 90% ethanol (1 h), 96% ethanol (2x 2 h), isopropanol (2x 2 h), acetone (2x 2 h), paraffin wax (2x 2 h).

With the Microm Paraffin Console, the dehydrated samples were embedded in paraffin blocks and cut in 5 µm thick slices using a Microm Paraffin Rotation Microtome. The paraffin sections were mounted on glass object slides and dried overnight at 37°C.

3.2.5 Immunofluorescence staining of fixed paraffinised organoids

Immunofluorescence staining is based on the specific binding of primary antibodies on the molecule of interest and their subsequent detection by secondary, labeled antibodies. These secondary antibodies are labeled with a fluorescence dye and can be detected by fluorescence microscopy.

Prior to the staining, the paraffin sections were put in a glass slide holder and dewaxed in a series of alcohol dilutions: xylene (2x 5-10 min), 100% ethanol (10-20 sec), 90% ethanol (10-20 sec), 70% ethanol (10-20 sec), and 50% ethanol (10-20 sec). The slides were washed for two times in water, transferred into preheated (95°C) 1x Dako target retrieval solution (ready to use) and incubated for 30 minutes at 95°C in the water bath, 20 minutes at RT, followed by 5 minutes under running water.

After dewaxing and antigen retrieval, the slides were dried by wiping around the sections with a tissue. The sections were surrounded with a DAKO fat pen and covered with PBS to save the sections from drying out.

To minimize unspecific bindings of the primary antibody, the sections were blocked with 200 µl IFF (immunofluorescence buffer) for 30 minutes.

IFF buffer
PBS + 1% BSA + 2% FCS

The following steps were performed at room temperature on a rocking platform. The primary antibody was diluted in IFF and incubated on the sections for 1 h. The slides were placed into a humidified chamber to prevent drying. After washing with PBS (3x 5 min), the secondary antibody (1:200) and Draq5 (1:1000, both diluted in IFF) were incubated for 1 h in the dark to

prevent bleaching of the fluorophores. Draq5 is a fluorescence dye that binds double-stranded DNA with high affinity and labels the cell nuclei. The slides were washed with PBS (3x 5 min) and rinsed in water. Finally, the sections were mounted in Mowiol, coated with coverslips and dried overnight. The dried samples were stored at 4°C in the dark.

3.2.6 Immunofluorescence staining of fixed cells

The same staining process was used for the fixed, non-paraffinized cell cultures, but the dewaxing and antigen retrieval steps were skipped. Further, instead of IFF buffer, 0.2% BSA in PBST was used.

1x PBST
1x PBS + 0.1% Triton-X

3.3 Microscopy

All confocal images were acquired with the Leica TCS SP-1 microscope using Leica Confocal Software.

Fluorophor	Absorption max (nm)	Emission max (nm)	Emission color
Cy2 (Alexa 488)	495	519	green
Cy3	550	570	red
Draq5	633	660	red

Table 1. Wavelength absorption and emission of various fluorescent dyes[58-60].

3.4 Antibodies

Primary antibodies	**name**	**origin**	**supplier**	**dilution**
	EpCAM	rabbit	Epitomics	1:100
	β-catenin	rabbit	abcam	1:100
	E-cadherin (Cdh1)	mouse	BD Transduction Laboratories	1:200
	Muc1	mouse	Cell Signaling	1:50
	CD133	rabbit	Cell signaling	1:50
	Olfactomedin4	rabbit	abcam	1:50
	Oct4	mouse	Sigma-Aldrich	1:50
	Nanog	rabbit	abcam	1:50
	Sox2	rabbit	Cell signaling	1:100
	acetylated Tubulin	mouse	Sigma-Aldrich	1:100
	Occludin	Mouse	Invitrogen	1:100
	Digoxigenin (Dig)	sheep	Roche	1:100

Secondary antibodies	**name**	**origin**	**supplier**	**dilution**
	Alexa 488 (Cy2) anti-mouse	donkey	Molecular Probes/MoBiTech	1:200
	Alexa 488 (Cy2) anti-rabbit	donkey	Molecular Probes/MoBiTech	1:200
	Cy3 anti-mouse	donkey	Dianova	1:200
	Cy3 anti-rabbit	donkey	Dianova	1:200
	Cy2 anti-sheep	donkey	Dianova	1:100

Table 2. Primary and secondary antibodies used for immunofluorescence analysis.

3.5 Transfection of primary cells with Wnt reporter construct

In order to generate a stably transfected primary cell line, epithelial cells from the human Fallopian tube were transfected with the Wnt reporter 7TGC (Addgene), which was inserted by a lentiviral vector and eventually stably integrated into the genome of the host cells. The lentiviruses were produced in 293T cells (human embryonic kidney cells) and the virus-containing supernatant was subsequently used to infect the target cells.

Lentiviruses belong to the family of retroviruses. Since lentiviruses replicate within non-dividing as well as dividing cells, they are used as gene delivery vectors to insert foreign DNA into eucaryotic cells.

3.5.1 Transformation of electro competent E.coli

To amplify the 7TGF construct and the packaging plasmids necessary for the transfection of the primary cells (pMD2.G and psPAX2), the plasmids were transformed in electro competent *E.coli*. The bacteria were thawed on ice and incubated with 1 µl (50 ng-500 ng) plasmid-DNA for 20 minutes on ice. The mix was transferred into a electroporation cuvette (Eppendorf) and electroporated at 2,4 kV. It was ensured that the time constant was between 4-5 ms. The transformated bacteria were resuspended in 1 ml prewarmed LB medium without antibiotics and incubated at 37°C and 250 rpm to recover. After one hour, the cells were transferred in an Erlenmeyer flask containing 30 ml LB medium and appropriate antibiotics and incubated over night at 37°C and 250 rpm. The next day, the in the bacteria amplified plasmids were purified.

3.5.2 Plasmid purification

The purification of the amplified plasmids was performed according to the manufacturer's protocol of the Qiagen Plasmid Midi Kit. The preparation efficiency was validated by measurement of the DNA concentration (NanoDrop) and the purified plasmids were stored at -20°C.

3.5.3 Production of the lentiviruses in 293 T cells

The principle of the Wnt reporter is based on a 7xTcf-eGFP reporter cassette and a SV40-mCherry selection cassette, which enable the differentiation between infected and non-infected cells as well as the detection of Wnt pathway activation in the transfected primary cells[61].

For the production of the lentiviruses in 293T cells, 20 µg 7TGC-DNA were pipetted together with 15 µg psPAX2 and pMD2.G (VSV.G). The mixture was filled up with 250 µl ddH_2O. 500 µl $CaCl_2$ were added to the DNA mix and the reaction mixture was pipetted in drops to 500 µl 2x HBS buffer under vortexing. The mix was incubated for 30 minutes on ice and subsequently transferred to the 293 T cells. The 293T cells were cultivated in DMEM + 10% FCS + 2mM L-glutamine and incubated at 37°C and 5% CO_2. The medium was changed 12 hours after lentiviral infection.

Plasmid	function	supplier	Publications
7TGC	Wnt reporter	Addgene	Fuerer, Nusse. PLoS One, 2010
PsPAX2	lentiviral packaging vector	Addgene/Tronolab	
PMD2.G (VSV.G)	lentiviral envelope vector	Addgene/Tronolab	

Table 3. Plasmids used for the production of the lentiviruses.

1 M $CaCl_2$
110.98 g in 1 liter ddH_2O

2x HBS (pH 7)
50 mM HEPES
280 mM NaCl
1.5 mM Na_2HPO_4
pH adjusted with NaOH and sterile filtrated (0.22 µm filter)

3.5.4 Transfection of the primary hFT cells

After two days, the virus-containing supernatant was removed from the 293T cells and filtrated through a 0.45 µm filter in a fresh tube. To receive a higher transfection efficiency, the supernatant was concentrated with LentiX Concentrator (Clontech) according to the manufacturer's protocol.

The target primary cells (~500.000 cells) were seeded in a 10 cm dish and cultivated for at least two days prior to the transfection in complete medium. The concentrated virus-containing supernatant was added to the target cells and incubated at 37°C and 5% CO_2. After 24 hours, the medium was changed.

3.5.5 Cultivation of organoids from transfected primary cells

After the transfection, the primary cells carrying the Wnt reporter were seeded in Matrigel as described before. The cells were not cultivated in 24-well plates, but in glass-bottom dishes (MaTek), which enable improved live cell-imaging.

3.5.6 Live cell fluorescence microscopy

Two days post seeding, the organoids were supplied with fresh organoid medium and put in a humidified incubation chamber at 37°C and 5% CO_2. Images were acquired at 10x magnification with an Olympus IX81 microscope equipped with a Hamamatsu C9100-02 CCD camera. The acquired images were processed by using the Metamorph software (Molecular Devices).

3.6 Monoclonal antibodies against Lgr5

To obtain monoclonal antibodies, which are produced by one single B-cell line and therefore recognize the same epitope of an antigen, mice have to be stimulated to make antibody-producing B cells. This is accomplished by injecting an cloned antigen construct – which corresponds the antibody of interest – in mice and gaining the resulting B cells. In further experiments, the antibody-containing serums are tested for their ability to recognize the target antigen and subsequently immortalized to produce an immortalized B-cell line that produces the monoclonal antibodies of interest. All steps performed in mice were accomplished by the Core facility for Proteomics of the Max Planck Institute for Infection biology, Berlin.

3.6.1 JumpStart RedTaq PCR

To amplify the Lgr5 gene, JumpSart RedTaq PCR was performed. PCR (polymerase chain reaction) is a method to amplify a gene sequence of interest. The concept of PCR is based on an enzyme, the polymerase, which catalyzes the polymerization of nucleotides complementary to a template DNA strand. Essential for the reaction to start is a free 3'OH, which is provided by short DNA oligonucleotides, the primers.

Lgr5 is a large transmembrane protein with a size of approximately 100 kDA. As membrane proteins contain large hydrophobic domains, they tend to insolubility by aggregation if overexpressed heterogeneously in a bacterial system. Therefore, we decided to use two small fragments of Lgr5 as an antigen – a large extracellular portion (540 AA) and a small cytoplasmic fragment (84 AA).

N-terminal extracellular segment (540 AA)	GSSPRSGVLLRGCPTHCHCEPDGRMLLRVDCSDLGLS ELPSNLSVFTSYLDLSMNNISQLLPNPLPSLRFLEELRLA GNALTYIPKGAFTGLYSLKVLMLQNNQLRHVPTEALQN LRSLQSLRLDANHISYVPPSCFSGLHSLRHLWLDDNAL TEIPVQAFRSLSALQAMTLALNKIHHIPDYAFGNLSSLV VLHLHNNRIHSLGKKCFDGLHSLETLDLNYNNLDEFPT AIRTLSNLKELGFHSNNIRSIPEKAFVGNPSLITIHFYDNP IQFVGRSAFQHLPELRTLTLNGASQITEFPDLTGTANLES LTLTGAQISSLPQTVCNQLPNLQVLDLSYNLLEDLPSFS VCQKLQKIDLRHNEIYEIKVDTFQQLLSLRSLNLAWNKI AIIHPNAFSTLPSLIKLDLSSNLLSSFPITGLHGLTHLKLT GNHALQSLISSENFPELKVIEMPYAYQCCAFGVCENAYK ISNQWNKGDNSSMDDLHKKDAGMFQAQDERDLEDF LLDFEEDLKALHSVQCSPSPGPFKPCEHLLDGWLIR
Cytoplasmic C-terminal domain (84 AA)	NPHFKEDLVSLRKQTYVWTRSKHPSLMSINSDDVEKQ SCDSTQALVTFTSSSITYDLPPSSVPSPAYPVTESCHLSS VAFVPCL

Table 4. Amino acid sequence of the selected fragments from Lgr5.

To minimize non-specific amplification, the JumpStart RedTaq PCR (Sigma-Aldrich) method was used. The JumpStart RedTaq Polymerase is inhibited by an antibody to prevent unspecific DNA amplification. In the first step of the amplification cycle, this antibody is denatured and the polymerase is released.

The following ingredients were mixed and incubated together in the PCR thermocycler:

JumpStart RedTaq PCR Mastermix	25 µl
Lgr5 pCR BluntII DNA (imaGenes)	0.5 µg
primer-pair mix (MWG-Biotech AG)	1 µl
ddH_2O	22 µl

The primer-pair mix for each PCR reaction was prepared by mixing 5 µl forward primer and 5 µl reverse primer in 40 µl ddH_2O.

Forward primer (Lgr5 extra)	5' GGGGACAAGTTTGTACAAAAAAGCAGGC TCCGGCAGCTCTCCCAGGTCTGGTGTGT 3'
Reverse primer (Lgr5 extra)	5' GGGGACCACTTTGTACAAGAAAGCTGGG TCTTCTGATCAGCCAGCCATCAAGCAG 3'
Forward primer (Lgr5 cyto)	5' GGGGACAAGTTTGTACAAAAAAGCAGG CTCCAATCCTCACTTTAAGGAGGATCTGG 3'
Reverse primer (Lgr5 cyto)	5' GGGGACCACTTTGTACAAGAAAGCTGGG TCTTAAACCGAGTTTCACCTCAGCTCT 3'

Table 5. Sequence of the used primers for the JumpStart RedTaq PCR.

Cycler program for the JumpStart RedTaq PCR:

15 min	95°C
40 cycles:	
1 min	95°C
1 min	51°C
2 min	72°C
10 min	72°C

3.6.2 DNA purification

After conventional agarose gel electrophoresis of the PCR products, the fragments were purified with the Qiagen QIAquick Gel extraction kit according to the manufacturer's protocol.

3.6.3 Midi prep of vectors for Gateway cloning

Transformed bacteria containing the pDONR 221 and pETG 30A were plated on LB agar plates with appropriate antibiotics and incubated at 37°C overnight. Single colonies were picked and transferred to 30 ml liquid LB medium containing ampicillin (1:1000) or kanamycin (1:1000). The cells were incubated overnight at 37°C and 250 rpm. Subsequent plasmid purification with the Qiagen Plasmid Midi Kit was performed according to the manufacturer's protocol.

3.6.4 Gateway cloning

The Gateway cloning system enables fast cloning of the target DNA sequence into an expression vector. The cloning concept relies on the recombination of specific attachment (att) sides by two enzymes derived from the bacteriophage lambda – the BP Clonase and the LR Clonase. This method allows cloning with a high efficiency, while retaining the reading frame[62,63].

The purified PCR products were eventually cloned into an expression vector to create a Lgr5-GST fusion protein. We amplified the Lgr5 fragments using primers that contained the attB1 or attB2 sequence at the end. The entry vector pDONR221 contains a kanamycin resistance that allows selection, as well as an attP1 and attP2 site. The BP Clonase recombines these matching

sites resulting in an entry plasmid containing the attL1 and attL2 side. The destination vector pETG-30A was utilized to N-terminally tag the Lgr5 fragments with glutathione S-transferase (GST) to produce a Lgr5-GST fusion protein. The GST tag facilitates subsequent affinity purification of the fusion protein. Besides an ampicillin selection cassette, pETG-30A contains the attR1 and attR2 sites, which enable the LR reaction by the LR Clonase. This recombination results in the expression plasmid containing the Lgr5-GST fusion protein flanked by the attB1 and attB2 sites.

All steps were performed according to the manufacturer's protocol of Gateway cloning (Invitrogen). Following each recombination reaction, the recombined plasmids were amplified in *E.coli*. For this purpose, the plasmids were transformed into electro competent *E.coli*, plated and incubated at 37°C over night. Subsequent plasmid purification was performed with the Qiagen Plasmid Mini Kit according to the manufacturer's protocol.

After BP and LR reaction, the cloning efficiency was validated by restriction enzyme digest and sequencing. All restriction enzyme digests were performed using FastDigest Restriction enzymes (Fermentas) in accordance to the manufacturer's protocol. The sequencing processes were done by MWG-Biotech AG.

3.6.5 Induction of heterogeneous overexpression

To produce a soluble Lgr5-GST fusion protein that can be injected into mice to stimulate antibody production, the protein was heterogeneously overexpressed in *E.coli*. Since we had problems with the overexpression of the extracellular fragment and the production of monoclonal antibodies is very time-consuming, we decided to produce only antibodies against the cytoplasmic fragment of Lgr5.

The expression vector pETG-30A containing the Lgr5-GST fusion protein was used for protein overexpression in *E.coli*. The expression of the fusion protein is controlled by the Lac operator and can be induced by addition of IPTG (Isopropyl-β-D-thiogalactopyranoside).

3.6.6 Chemical transformation

To amplify the destination vector pETG-30A with the inserted Lgr5-GST fusion protein, the plasmid was transferred in competent cells via chemical transformation. The process was performed in order to the manufacturer's protocol of the BL21 (DE3) chemically competent *E.coli* strain (Novagen). The transformed bacteria were incubated in 30 ml LB medium containing ampicillin (1:1000) overnight.

3.6.7 IPTG-induced heterogeneous overexpression

20 ml of the overnight-culture were diluted with 500 ml fresh LB medium containing ampicillin and incubated at 37°C and 220 rpm for 4 hours. After the incubation time, 5 ml of the non-induced sample were removed for later control. IPTG was added to receive a final concentration of 0.5 mM in the medium. The culture was incubated for another 3.5 hours at 30°C and 180 rpm. 5 ml of the induced culture were removed for later control. The bacteria from the culture as well as from the removed controls were harvested by centrifugation at 10.000 rpm for 15 minutes. The pellets were stored at -80°C until usage.

IPTG (100 mM)
23.83 mg in 1 ml ddH_2O

3.6.8 Solubilisation of the fusion protein

The removed non-induced and induced control were subsequently tested for the solubility of the amplified and overexpressed Lgr5-GST fusion protein. The bacteria pellets were resuspended in lysis buffer. In order to provide an extract containing the soluble fusion protein, the cells were disrupted by sonification for 30 seconds on ice and incubated for 30 minutes at RT. The suspension was centrifuged for 5 minutes at 4000 rpm.

Lysis buffer
200 µl 1x protease inhibitor cocktail complete (Roche)
40 µl Triton X-100
400 µl 1 M CHAPS
10 ml PBS

1 M CHAPS
614 mg in 1 ml ddH_2O

Protein purification was accomplished using glutathione sepharose beads (Sigma-Aldrich). Since the substrate of GST is glutathione, the Lgr5-GST fusion protein in the supernatant of the cell lysate binds the glutathione sepharose beads with high affinity and can be purified.

Prior to applying the supernatant, the glutathione sepharose beads were washed with PBS. The supernatant was applied on the washed beads and rotated over night at 4°C. Afterwards, the suspension was centrifuged for 1 minute and the beads with the bound fusion protein were analyzed by 10% denaturing SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

SDS-PAGE was used to separate the proteins according to their molecular weight. First, the 10% resolving gel was poured, covered with ddH_2O and incubated for 10 minutes at RT. After polymerization, the water was removed and the stacking gel was poured over the resolving gel. After polymerization, the gel was transferred in the gel apparatus (BioRad) and the gel chambers were filled with 1x SDS buffer.

The samples were boiled at 95°C for 5 minutes with 1x SDS sample buffer. 25 µl of each sample were pipetted in the gel slots. To detect the size of the proteins in the gel, a prestained protein ladder (Fermentas) was applied additionally. The gel was run at 90 V for 30 minutes, and after the samples reached the lower resolving gel, the potential was raised up to 150 V for another 45 minutes. Coomassie staining with PageBlue Protein Staining solution (Fermentas) was used to visualize the separated proteins.

Stacking gel (Upper gel)
2.5 ml upper gel buffer
1.5 ml acrylamide
6 ml ddH_20
50 µl 10% APS
7 µl TEMED

Resolving gel (Lower gel)
2.5 ml lower gel buffer
3 ml acrylamide
4.45 ml ddH_20
50 µl 10% APS
7 µl TEMED

Upper gel buffer (pH 6.8)
0.5 M Tris HCl
0.4% SDS

Lower gel buffer (pH 8.8)
1.5 M HCl

10x SDS buffer (pH 8.3)
144 g Glycine
30.2 g Tris-Base
ddH_2O ad 1 liter

The solubilised Lgr5-GST fusion protein was handed to the Core facility for Proteomics of the Max Planck Institute for Infection biology, who performed the immunization process of the mice and gained the antibody-containing serums.

3.6.9 Western blot

After immunization of the mice with the produced fusion protein, the murine serums were tested for the presence of Lgr5-detecting polyclonal antibodies by the Western blot method. Freshly dissected Lgr5 positive cell-containing crypts from the human stomach were used as a positive control. Furthermore, freshly dissected hFT mucosal tissue as well as cultivated epithelial hFT cells were tested. To obtain the proteins extracts from the tissue samples, the organ pieces were transferred in 400 µl ice-cold protein preparation buffer and homogenized with an ultraturrax (IKA) on ice (3x 20 sec). The lysates were centrifuged at 20.000xg for 10 minutes at 4°C. The remaining protein samples were boiled with 4x SDS sample buffer at 95°C for 5 minutes.

Protein preparation buffer (pH 7.4)
50 mM HEPES
330 mM sucrose
1x protease inhibitor cocktail complete
2 µM ortho-vanadate

The medium from the epithelial hFT cultures was removed and 4x SDS sample buffer was added to the cells. The cells were scraped out of the flask, transferred to a new cup and boiled at 95°C for 5 minutes.

All samples were analyzed with 10% denaturing SDS-PAGE. Afterwards, the separated proteins were transferred from the gel to a PVDF (polyvinylidene fluoride) membrane. For this purpose, the semi-dry electrophoretic transfer system (BioRad) was used according to the manufacturer's protocol.

After the transfer, the membrane was shortly rinsed in water and blocked for 1 h at RT in 3% skim milk in PBST. The serums gained from the immunized mice were used to detect Lgr5 on the membrane. The two serums were diluted (1:300 and 1:1000) and incubated with the membrane overnight at 4°C. Subsequently, the membrane was washed with PBST (4x 15 min) and incubated with the secondary antibody (1:3000, diluted in blocking solution), which is conjugated to horseradish peroxidase (HRP) for 1 h at RT. After washing with PBST (4x 15 min), the proteins were visualized by chemiluminescence.

3.7 In situ hybridization (ISH)

In situ hybridization is a technique that allows highly specific detection of mRNA coding for a certain protein. In this technique, an antisense RNA probe complementary to the mRNA of interest is produced by in vitro transcription with Digoxigenin-labeled nucleotides. This probe hybridizes under suitable conditions only with the target mRNA and can be subsequently detected by immunofluorescence staining.

3.7.1 In vitro transcription

All following steps were performed under RNase-free conditions.

In situ hybridization requires single stranded-RNA probes that detect their complement mRNA in situ with high sequence specificity. These probes were synthesized by in vitro transcription. For this purpose, the following reagents were mixed and incubated for two hours at 40°C:

EcoRV-linearized Lgr5 (SP6 promoter)	antisense RNA	2 µg
AgeI-linearized EpCam (T7 promoter)	positive control	2 µg
HindIII-linearized Lgr5 (T7 promoter)	negative control	2 µg
RNAse inhibitor (Ambion)		2 µl
SP6 RNA Polymerase (NEB)		4 µl
or		
T7 RNA Polymerase (Ambion)		4 µl

Subsequently, 4 µl DNaseI (Ambion) were added and incubated for 15 minutes at 37°C. Then, 5 µl 4 M LiCl and 160 µl precooled (-20°C) ethanol abs. were added and the mixture was incubated for 30 minutes at -70°C. After centrifugation for 15 minutes at 20.000xg at 4°C, the supernatant was discarded and 200 µl precooled (-20°C) 70% ethanol were added. The suspension was centrifuged for 5 minutes at 20.000xg at 4°C. The supernatant was discarded and the pellet was dried for 2 minutes under vacuum in a speed-vac concentrator system (Thermo Scientific). Finally, the dried RNA was dissolved in 50 µl nuclease-free H_2O and stored until usage at -70°C.

3.7.2 Fragmentation of the probes

After synthesis, 50 µl 2x carbonate buffer were added to the dissolved RNA in order to fragment the probes. The mixture was incubated at 65°C for 30 minutes. Subsequently, 100 µl stop solution were added to stop the fragmentation process. 600 µl ethanol abs. were added, mixed and incubated at -70°C for 30 minutes. After centrifugation for 15 minutes at 20.000xg at 4°C, the supernatant was discarded and 200 µl prechilled 70% ethanol were added to the pellet. The mixture was centrifuged for 5 minutes at 20.000xg at 4°C, the supernatant was discarded and the pellet was dried for 2 minutes under vacuum. The fragmented probes were dissolved in 50 µl nuclease-free H_2O and stored at -70°C. The concentration was measured via NanoDrop.

2x carbonate buffer (pH 10.2)
120 mM Na_2CO_3
80 mM $NaHCO_3$

Stop solution (pH 6)
200 mM NaAcetate

3.7.3 Preparation of the samples

All following incubation steps were performed in glass jars rinsed with ethanol abs. The glass object slides with the paraffinised organoid sections were dewaxed with xylene (2x 2 h) and in a series of alcohol dilutions: 100% ethanol (2x 3 min), 95% ethanol (1x 3 min), 70% ethanol (1x 3 min), and 50% ethanol (1x 3 min). The slides were rinsed in nuclease-free H_2O and fixed in prewarmed 3.7% PFA (37°C) for 20 minutes at RT. Subsequently, the slides were washed 5x with 1x TBS, treated with 0.2 M HCl for 10 minutes and rinsed 5x in 1x TBS.

Acetic anhydride solution was added to the samples and incubated for 10 minutes under a chemical hood. The slides were washed in 1x TBS (5x 1 min) and treated with proteinase K (30 µg/ml) in TBS + 2 mM $CaCl_2$ for 20 minutes at 37°C. After washing with 1x TBS (5x 1 min), cold 1x TBS was added and the slides were incubated for 5 minutes at 4°C. The sections were dehydrated in a series of ethanol solutions: 30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol (for 3 minutes each). After dehydration the samples were rinsed in chloroform.

10x TBS
80 g NaCl
2 g KCl
30 g Tris-Base
ddH_2O ad 1 liter

2 M HCl
193 ml HCl
add ddH_2O to 1 liter

0.2 M Hcl
1 ml 2 M HCl
9 ml ddH_2O

acetic anhydride solution (pH 8.0)
100 ml ddH_2O
1,34 ml triethanolamine
0,6 ml acetic anhydride just before use
0,4 ml concentrated HCl (to adjust pH)

3.7.4 Hybridization

The treated slides were placed in a chamber humidified with 2x SSC/50% formamide for 30 minutes at 50°C in a water bath. 5 µl of each RNA probe were diluted in 200 µl hybridization buffer and added directly on the slides. The slides were coated with cover slips and placed on a 94°C heat block for 4 minutes. The slides were subsequently transferred in the humidified chamber and incubated for 16 hours at 50°C in the water bath.

Hybridization buffer
500 mg dextran sulfate
1.9 ml ddH_2O
500 µl 20x SSC
50 µl 2% SDS
50 µl 1% herring sperm DNA (Promega)

20x SSC (pH 4.5)
175.3 g NaCl
88.2 g NaCitrate
10 g citric acid to adjust pH
ddH_2O ad 1 liter and autoclaved

3.7.5 Post hybridization washes

Post hybridization washes are necessary to remove unbound RNA probes that will complicate the analysis and falsify the results by producing a strong background signal. The slides were removed from the humidified chamber and washed twice in 2x SSC. After removing the cover slips, the samples were washed in 50% formamide/2xSSC (3x 20 min at 50°C), in 2x SSC (3x 5 min at RT), and in PBS (3x 5 min at RT).

3.7.6 Immunological Detection

The sections were surrounded with a DAKO fat pen and covered with PBS. Blocking solution was added on the samples and incubated for 1 h. The primary antibodies anti-Digoxigenin and anti-E-cadherin were diluted in the blocking solution (1:50), added and incubated for 90 minutes in a humidified chamber at RT. After washing with PBS (3x 5 min), the secondary antibodies (1:100) and Draq5 (1:1000, both diluted in the blocking solution) were incubated for 1 h in the dark to prevent bleaching of the fluorophores. The slides were washed with PBS (3x 5 min) and rinsed in water. Finally, the sections were mounted in Mowiol, coated with coverslips and dried overnight. The dried samples were stored at 4°C in the dark.

Blocking solution

0.5% BSA + 1% FCS in PBS

3.8 Chemicals

Agarose (gelelektrophoresis)	Biozym
BSA	PAA Laborotories GmbH
Hoechst 33342	Molecular probes
L-Glutamin	Serva
Paraformaldehyde	Fluka
RNase AWAY	Molecular BioProducts
Tris base	AppliChem

All other chemicals, which have been used, but are not listed here, were obtained from Roth, Roche, Sigma-Aldrich, or Merck.

3.9 Cell culture

Advanced DMEM F12	Invitrogen
DMEM	Invitrogen
DPBS	Invitrogen
DMSO	Sigma-Aldrich
FCS	Biochrom
Trypsin/EDTA	Invitrogen

4 Results

4.1 Cultivation of mucosal epithelial cells from the Fallopian tube

Epithelial cells from the mucosa were isolated by enzymatic and mechanical treatment of the Fallopian tubes. Prior to the cell isolation, the surrounding connective and vascular tissue was removed (Figure 7 A). In order to isolate the epithelial cells, the mucosal folds were exposed by longitudinal opening of the tube. Limited enzymatic digestion was followed by peeling off the epithelial layer. This process ensured minimal contamination with parenchymal cells and fibroblasts.

Co-isolation of fibroblasts occurred rarely, and they could be differentiated by their spindle-shaped morphology compared to the more round phenotype of the epithelial cells. Usually between 2-3 days in culture, the epithelial cells started to form characteristic epithelial islands (Figure 7 B). We have noticed unusually long life span and growth potential of the epithelial cultures. The cells of one tube can be expanded up to seven passages, yielding usually more than five flasks with 1 x 10^7 cells each. This was unusually long for terminally differentiated epithelial cells and therefore strongly indicated that the culture contains cells with a maintained pluripotency.

Dependent on the patient, the tubes behaved different regarding cell growth and morphology. This might be due to the tubal region that was dissected and the patient's age as well as its physical condition. In order to set up a library of cells from every patients' tube we worked with, the cells were routinely expanded and stocked in an early stage (passage 2-3).

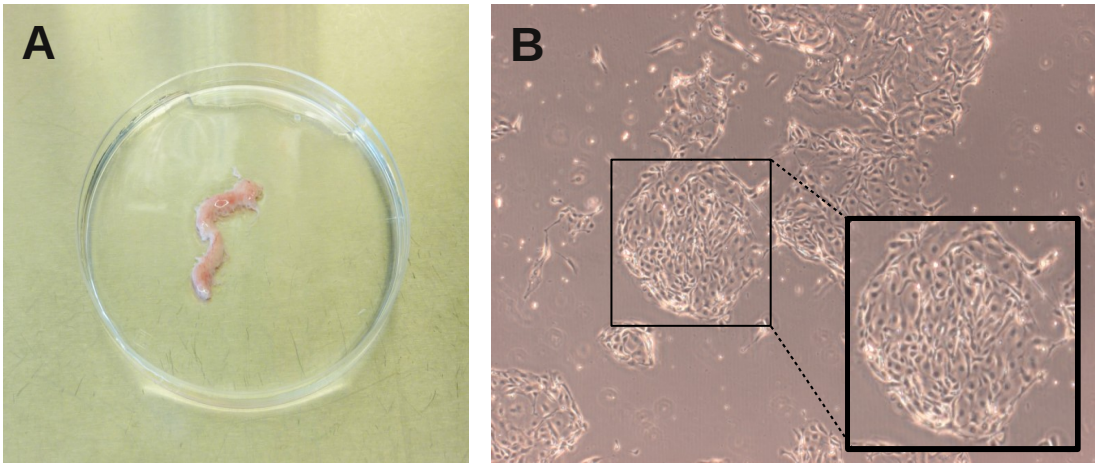

Fig. 7. Cultivation of epithelial cells from the human Fallopian tube. (A) The connective tissue of the Fallopian tube was removed and the cells from the inner mucosal epithelium were isolated. (B) After a few days in culture, the isolated cells started to form characteristic epithelial islands. The cultivated cells show the major morphologic features of epithelial cells and can be easily differentiated from the spindle-shaped fibroblasts, which are rarely co-isolated.

4.2 Establishment of a three-dimensional ex vivo model

To test the capacity of the epithelial cells to from three-dimensional structures in vitro, the cultivated cells were seeded in 3D using Matrigel to mimic the extracellular matrix.

The medium contained growth supplements which were compatible with other known adult stem cell systems in vitro[64]. These conditions imitate the tubal microenvironment and provide an optimal stem cell niche in vitro:

- Wnt3a as an activator of the Wnt signaling pathway
- R-spondin, a Wnt agonist
- Noggin, an inhibitor of bone morphologenetic proteins (BMP), which initiate cell differentiation[65]
- B27, which is known to maintain growth and viability of neurons[66]
- Nicotinamide, a substrate for the nicotinamide phosphoribosyl-transferase, which mediates cell survival by inhibiting apoptosis[67]
- EGF and FGF10, which both drive proliferation and differentiation and have been found to be essential for the formation of organoids

Small groups of cells aggregated in the Matrigel and formed complex patterns. The resulting three-dimensional structures, the so-called “organoids”, developed in general within 4-6 days post seeding, depending of the tube (Figure 8). Furthermore, we were able to passage the organoids and to keep them up to six weeks in culture.

After analysis with the light microscope, we strongly assumed that the cells form a hollowed ball, which contains apoptotic cells inside. Interfering with the growth, the color of the organoids changed and turned darker. This supported the hypothesis, that cells from the epithelium are shed and remain inside the organoid, visible as darker structures. However, the real organoid structure remained to be determined by immunofluorescence staining.

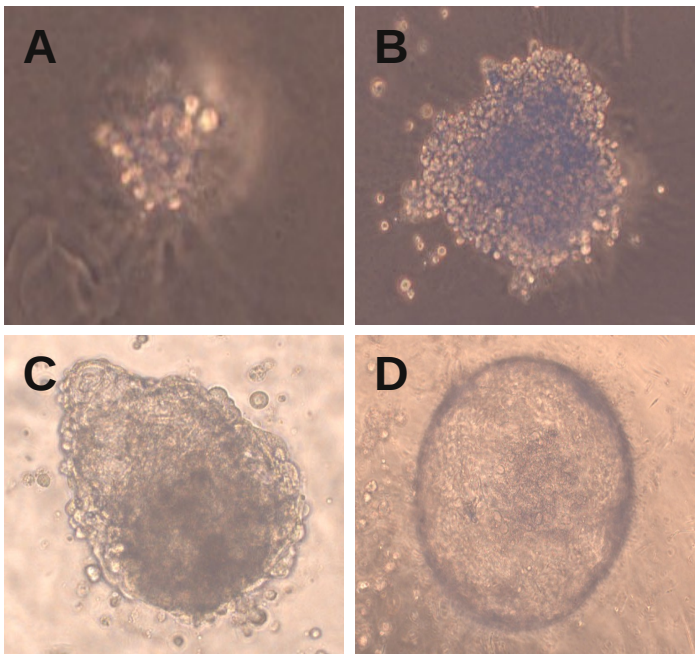

Fig. 8. The formation of organoid-like structures at different time stages after cell seeding in Matrigel as a support in vitro. (A) 24h (B) 36h (C) 72h (D) 120h. The seeded cells form small groups in the Matrigel and eventually build compact 3D structures, the so-called “organoids”.

4.2.1 Analysis of the organoid structure

Various organoid staining methods have been performed, but since the organoids reside within the Matrigel drop and cannot be retrieved without disruption, it was difficult to achieve penetration of the immunofluorescence antibodies. Finally, paraffinization and subsequent production of paraffin cuts conduced to clear results. We analyzed the morphological structure of these organoids using fluorescence immunostaining and visualization by confocal imaging. Notably, during the dehydration and paraffinization process prior the immunostaining, the organoid-shape was altered under these harsh conditions. Nevertheless, the cellular structure and organization were sufficiently preserved to enable characterization.

Detailed analysis of the structures revealed that – as expected – the organoid consists of a columnar epithelial monolayer of cells (Figure 9). The cells within this monolayer exhibit the characteristic “honeycomb”-pattern as well as apicobasal polarity. The apical side of the cell faces the lumen, which consists of potentially apoptotical cells that were shred during the process of growing. The lateral cell side makes cell-to-cell contacts with the adjacent cells (tight and adherent junctions), while the basal side adheres to the surrounding Matrigel by forming hemidesmosomes[68].

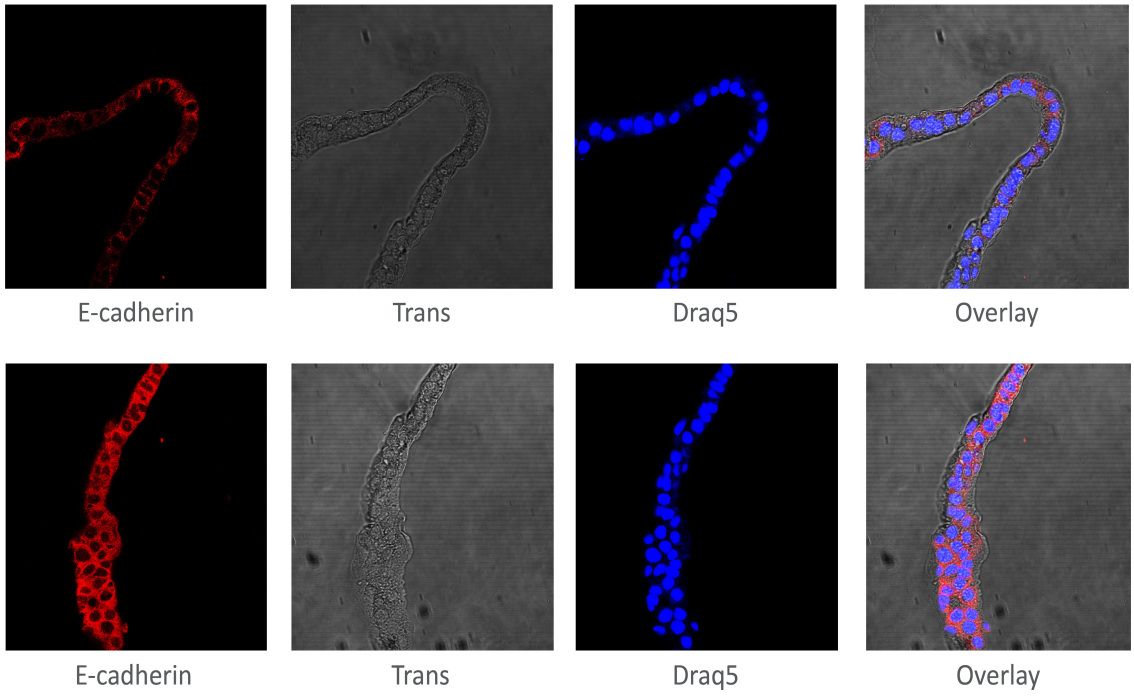

Fig. 9. Analysis of E-cadherin staining in organoids derived from the hFT and cultured in vitro for 8 days. The cells within the organoid are epithelial and display a well-organized and polarized phenotype. This distinct columnar cell layer shows apicobasal polarity: its apical side faces the lumen, which consists of apoptotical cells; the lateral side makes cell-to-cell contacts; the basal side adheres to the Matrigel via cell-to-extracellular matrix interactions.

4.2.2 The cells within the organoid are epithelial

To further characterize the organoid-forming cells, the cells were stained with specific epithelial markers, like E-cadherin, EpCAM, β-catenin, and Muc1. E-cadherin (Cdh1) is necessary for cell adhesion (adherens junctions) and found in all epithelial tissues at the basolateral side. EpCAM (epithelial cell adhesion molecule) is expressed on most epithelial cells, but is enriched in non differentiated and pluripotent cells. As already mentioned, β-catenin is necessary for cell-to-cell adhesion by interaction with E-cadherin and a component of the adherent junctions between cells. Mucin1 (Muc1) is another epithelial marker and is expressed only at the apical side of the cells.

All cells within the epithelial monolayer expressed at least one of these markers and the columnar epithelium displayed an arrangement of cells that is reminiscent of a bead chain (Figure 10). Furthermore, all tested samples showed no signs of contamination with other cell types, like fibroblasts.

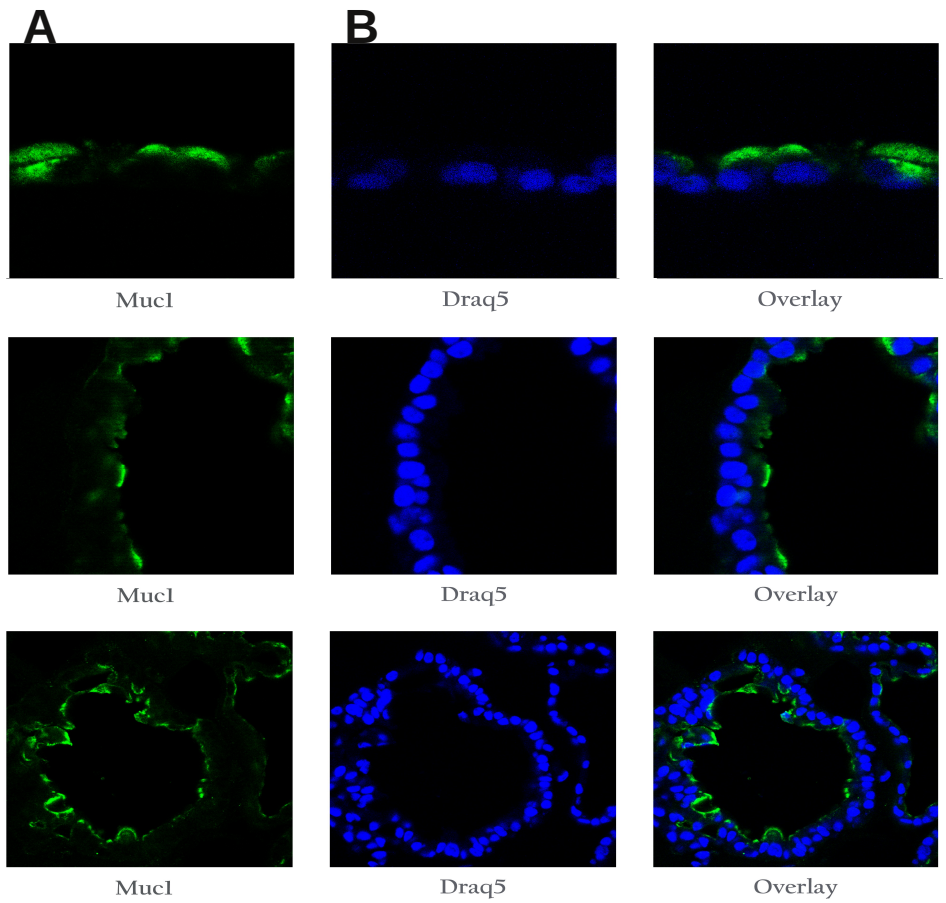

Fig. 10. Immunostaining of in vitro developed organoids cultured for 8 days. (A) Most cells within the organoid show positive staining for the epithelial marker Muc1 on the apical side of the epithelial monolayer. (B) Nuclear staining with Draq5 clearly displays the characteristic pattern of the polarized epithelium, comparable to a bead chain.

4.2.3 Organoids contain undifferentiated progenitor cells

To determine the differentiation state of the cells within the organoids, immunofluorescence staining with pluripotency markers was accomplished. Oct4 (POU5F1), CD133 (Prominin 1), and Olfm4 (Olfactomedin 4) are factors that have been found to be expressed in undifferentiated cells and stem cells. Oct4 is an active transcription factor in self-renewing cells and therefore only expressed in the nucleus. A major part of the nuclei in the organoid cells were

Oct4 positive. Comparison of these cells with one another revealed an differential expression level.

CD133 is a cell surface protein with five transmembrane domains. At present, its function is poorly understood, but it is supposed to be expressed in stem cells in normal and cancerous tissue[69]. Kyo et al.[11] described the increasing importance of CD133 as a marker for cancer stem cells. Olfm4 is an anti-apoptotic factor that promotes cell proliferation. Tissues from colon cancer and stomach cancer show expression of Olfm4 at high levels[70]. However, immunofluorescence staining with CD133 and Olfm4 antibodies showed a wide distribution throughout the cells in the whole organoid.

Interestingly, the majority of cells displayed an undifferentiated phenotype and are therefore suggested to be transit-amplifying cells, here called progenitor cells. As previously suggested, these cells represent an intermediate cell population between the stem cells and the terminally differentiated effector cells. Taken together, the cells within the organoid express at least three different pluripotency markers (Figure 11). Based on these findings, we suggest that the human Fallopian tube contains undifferentiated cells, the progenitor cells. Furthermore we assume that some of these undifferentiated cells are stem cells. Since there are no reliable markers for stem cells known, it remains difficult to distinguish between stem cells and their more mature progeny, the progenitor cells.

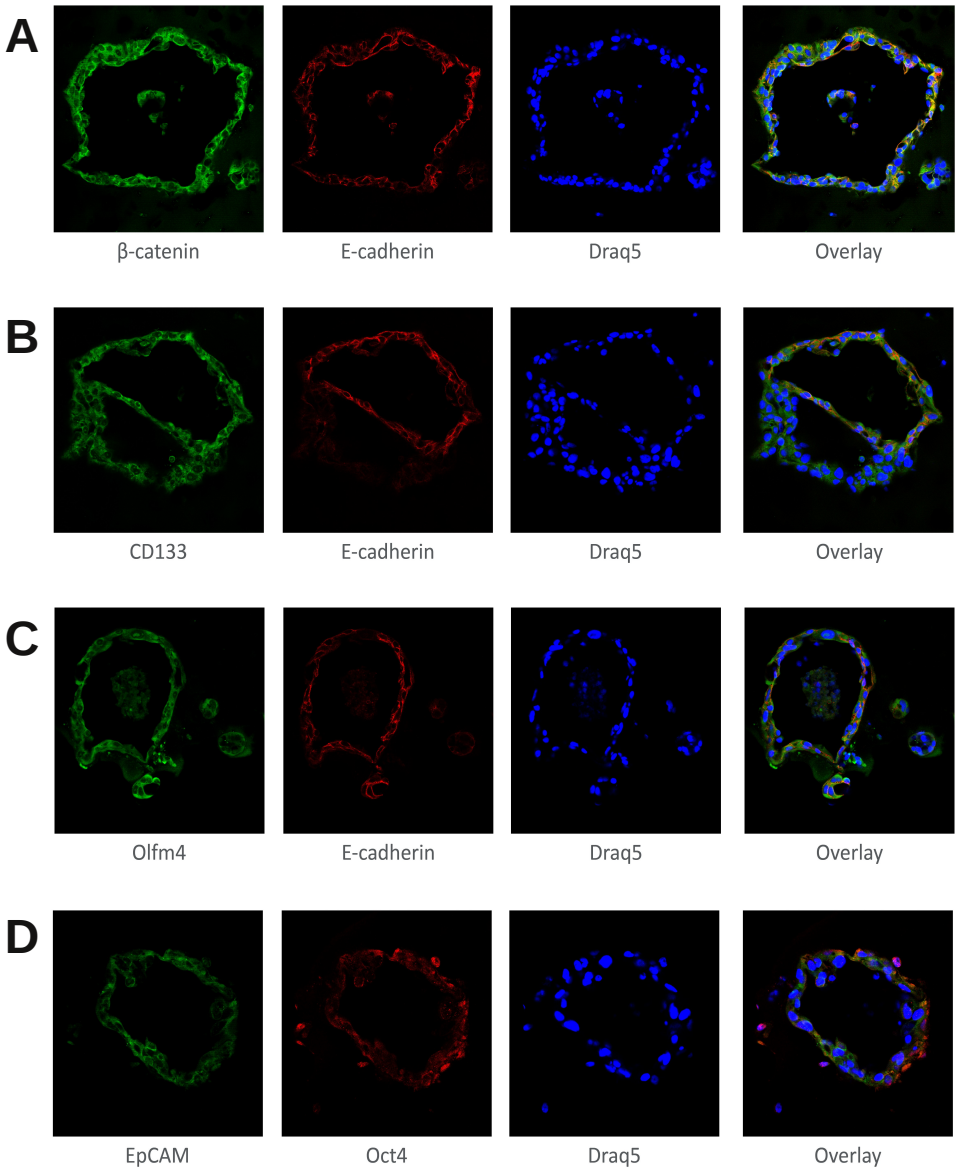

Fig. 11. The organoids contain pluripotent cells, the progenitor cells. The paraffin cuts were stained with various markers specific for epithelial cells and pluripotency. (A) E-cadherin is basolaterally distributed in all cells, whereas nuclear β-catenin indicates active Wnt signaling in the organoids. β-catenin is also involved in cell adhesion and ensures epithelial polarity. (B-D) Positive staining for CD133, Olfm4, and Oct4 reveals the pluripotent and undetermined state of the cells within the organoid.

4.2.4 Cells within the organoid differentiate to ciliated cells

The tubal mucosa is composed of two different cell types, which ensure its functional integrity – non-ciliated Peg cells and ciliated cells. The main component of the motile cilia is acetylated tubulin.

Immunofluorescence staining revealed that few single cells in the organoid have differentiated into ciliated cells and therefore expressed acetylated tubulin in the cilia (Figure 12). It is excluded that the ciliated cells were co-cultivated after cell isolation from the tube, because they are terminally differentiated and would not have persisted during the cultivation and expansion process. Moreover, ciliated cells would have been visible in the culture with light microscopy analysis.

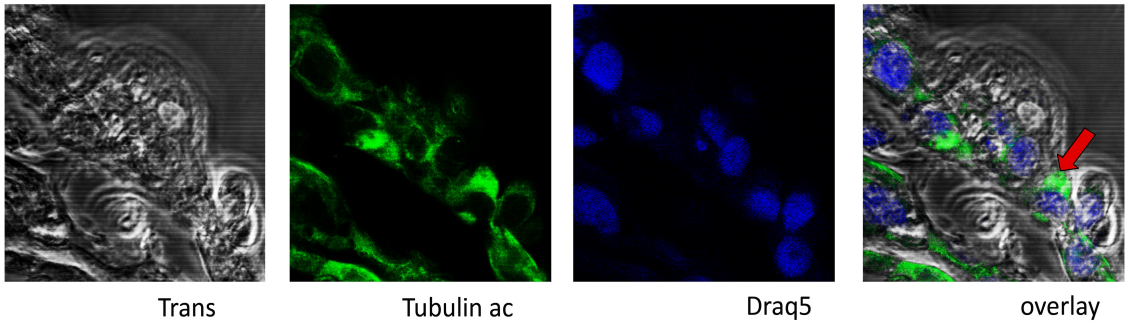

Fig. 12. Immunostaining of in vitro developed organoids for acetylated tubulin, the main component of the motile cilia. The two different cell types of the hFT are the non-ciliated Peg cells and the ciliated cells. Some cells within the epithelial monolayer of the cultivated organoids showed positive staining for acetylated tubulin (red arrow). These cells differentiate into ciliated cells.

These studies reveal that the majority of cells within the organoids are epithelial cells in an undifferentiated state – the epithelial progenitor cells of the human Fallopian tube. However, few cells are also differentiated into ciliated cells. Taken together, the phenotype of the cells within the organoid in vitro resembles the columnar epithelium in the female tube.

4.3 Epithelial 2D cultures contain undifferentiated progenitor cells

In order to reach definite results about the tubal progenitor cells, the features of the two-dimensional cell cultures were tested. The hypothesis was that terminally differentiated cells as well as undifferentiated progenitor cells and stem cells are cultivated from the tube after the isolation process. Due to passaging and cell expansion, only cells with the ability to self-renew will be able to remain in culture and eventually form organoids under suitable conditions.

Immunofluorescence staining of cells in 2D culture, which were cultured for more than two weeks, revealed that the cells are epithelial and display stem-cell like features (Figure 13). The majority expressed both Oct4 and Nanog. Oct4 (POU5F1) and Nanog are known to be involved in self-renewal and are expressed in undifferentiated cells. Since both are transcription factors, positive staining is only visible in the nuclei of the cells.

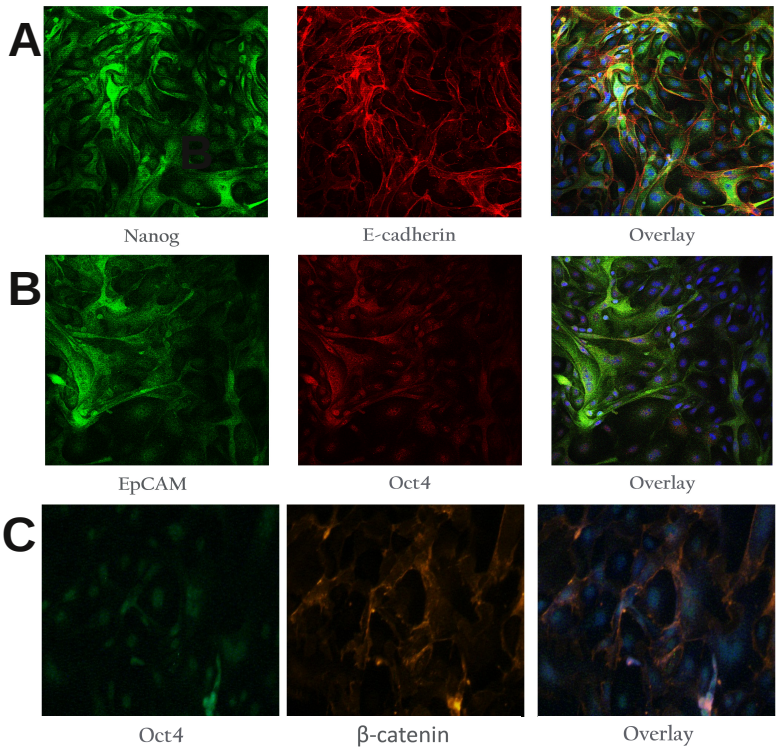

Fig. 13. Immunofluorescence staining of epithelial cells in 2D. The cells were cultivated on cover slips and stained when they were confluent. (A) All cells express E-cadherin as an epithelial marker and most cells show nuclear expression of the pluripotency marker Nanog. (B) EpCAM staining reveals the epithelial origin of the cells, whereas expression of oct4 in the nuclei indicated that the cells have pluripotent features. The red channel shows Cy2 (Alexa488) fluorescence bleeding and as a result false cytoplasmic staining. (C) Oct4 is clearly visible in the nuclei, while β-catenin is important for cell-to-cell adhesions.

Contrary to the cells within the organoid, light microscopy analysis showed that the cells cultivated in 2D are not polarized. To investigate whether these cells can be polarized under suitable conditions, we used coverslips that were coated with diluted Matrigel as a basal lamina that mediates the cell attachment in 2D.

The transmembrane protein occludin is together with the protein claudin the main component of tight junctions. Tight junctions connect adjacent cells and seal gaps in the epithelial cell sheet (Figure 14 D). The formation of tight junctions is associated with cellular apicobasal organization: the basal side that attaches to the basal lamina, and the lumen-facing apical side that is exposed to the medium[13].

Immunofluorescence staining revealed that the epithelial cells grown on the Matrigel basement exhibited distinct morphologies, reminiscent of their organization in vivo. The cells were arranged in a hexagonal pattern, the “honeycomb”-pattern, which is characteristic for polarized epithelial cell sheets (Figure 14 A-C). This arrangement is a result of the boundary contraction of the cells and strong contacts between the cells[71]. Occludin was expressed in the tight junctions of the epithelial cell layer. By using Matrigel coated cover slips, this method enabled the directed polarization of epithelial cells.

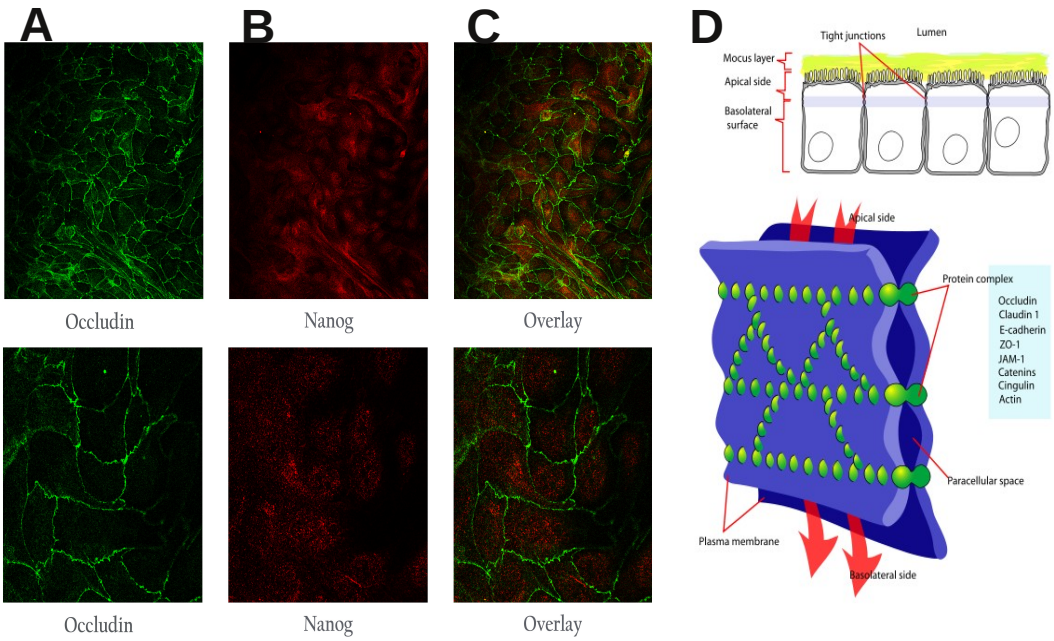

Fig. 14. Analysis of immunofluorescence staining of cultivated cells on a Matrigel-coated basement in 2D. The lower panel shows magnification of the upper panel. (A-C) The cells grow in a hexagonal “honeycomb” pattern and polarize. (A) The expression of occludin indicates that the cells are polarized and form tight junctions. (B) Nanog is expressed in the nuclei of most cells, which have pluripotent, stem-cell like features and are therefore called the progenitor cells. (D) Schematic overview of tight junctions in epithelial cells. Tight junctions connect adjacent cells, seal gaps in the epithelial cell sheet and are associated with apicobasal cell polarity; their main component is occludin[72].

4.3.1 Polarization of epithelial cells under suitable conditions

Once the organoid cells were identified as epithelial polarized progenitor cells, we wanted to answer the question which growth component from the organoid medium triggers the cell proliferation and which component leads to differentiation of the progenitor cells. For this purpose, the cells were seeded on Matrigel-coated cover slips and treated with medium (Advanced DMEM F12 + EGF) containing different growth supplements for 5-6 days. The

confluent cell layers were eventually analyzed by immunofluorescence staining for β-catenin.

Different cell phenotypes were detected, depending on the growth conditions in presence of the specific factors. Interestingly, the Noggin-treated cells showed a significant increased proliferation rate (Figure 15 A). Moreover, the morphology of the cells did not look totally polarized. Noggin is known to be an agonist of BMP signaling. The BMP ligands are members of the TGF-β superfamily and active signaling is involved in several developmental events[73]. These results suggest that Noggin is a factor that contributes to cellular expansion and can be used to prolong the undifferentiated state of the cells.

However, treatment of the cells with the organoid medium resulted in polarization of the cells (Figure 15 G-I). Strong expression of β-catenin in the adherent junctions as well as the sealed gaps in the cell layer indicated this cell polarization. Furthermore, nuclear expression of β-catenin represented active Wnt signaling in the cells. Since β-catenin gives information about adhering junctions and Wnt pathway activation, but is no polarity marker, the polarization of the cells should be confirmed in ongoing experiments, such as the determination of the subapical localization of occludin in the cell layer.

Nevertheless, the treatment of cells with other growth cocktails displayed no significant difference regarding the cell proliferation and the polarization (Figure 15 B-F).

Taken together, the cells treated with Noggin look less polarized than with the organoid medium and do not differentiate. These findings indicate that the process of polarization can be triggered in vitro with organoid medium, while Noggin prolongs the non-differentiated state of the progenitor cells.

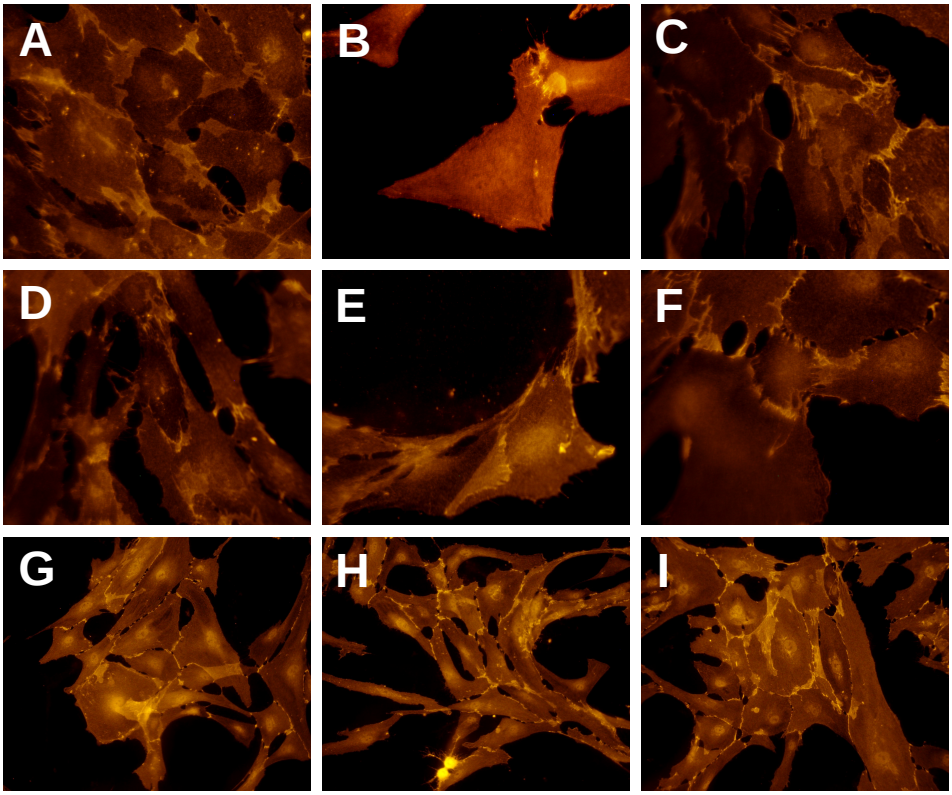

Fig. 15. Test for polarization of cultivated cells in 2D on Matrigel-coated coverslips under different growth conditions. All cells were stained with an antibody against β-catenin. (A) Medium containing EGF and Noggin. (B) Medium containing EGF, B27, and Nicotinamide. (C) Medium containing EGF and Wnt3a. (D) Medium containing EGF and R-spondin. (E) Medium containing EGF, Wnt3a and R-spondin. (F) Medium containing EGF, Wnt3a, R-spondin, and Noggin. (G) Organoid medium without FGF10. (H-I) Organoid medium. Noggin as a growth supplement increases the cell number during the cultivation. The cells propagate, but complete polarization does not occur. In contrast, cultivation of the cells in the organoid medium leads to polarization and nuclear β-catenin expression, a hallmark of active Wnt signaling. Figures G-I clearly illustrate the strong cell contacts and the sealed gaps in the cell layer. Additionally, the expression of β-catenin in the adherens junctions is obvious.

4.4 Potential side-population cells in epithelial cell cultures

We found cells exhibiting a phenotype distinct from the epithelial cells in a cell culture that was cultivated for more than seven weeks (Figure 16). It is excluded, that the cells were isolated and cultivated from the beginning, because the cell cultures were observed every day through light microscopy. The cells appeared after seven times of passaging and their phenotype resembles side-population cells (SP cells).

SP cells have been identified in various normal and cancerous tissues[31,32,38], including the human endometrium. They were described as small, round cells with a long-term proliferation capacity in vitro[37]. Interestingly, SP cells are supposed to represent a common molecular feature for stem cells and – since they have been detected in various solid tumors – they seem to be important for cancerogenesis[33-36].

The reported description of SP cells fits the phenotype of the cells we found. The cell's shape is round and reminiscent of a fried egg. However, the hypothesis that the differently looking cells emerging in hFT cultures after some weeks equate to SP cells has to be confirmed by various experiments, such as performance of an CFU (colony forming unit) assay to determine the proliferative potential and testing their ability to efflux Hoechst 33342.

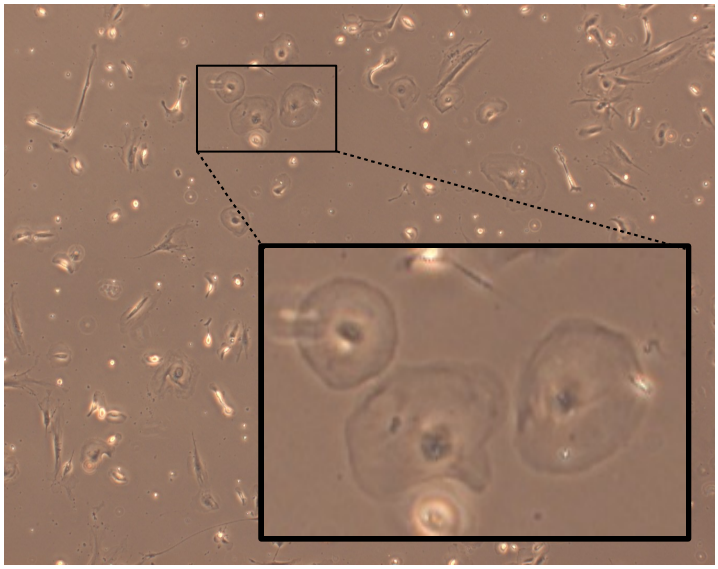

Fig. 16. Potential side-population cells from the tubal epithelial cells. The cells appeared in a cell culture after seven weeks. Their morphology resembles SP-cells, which were found in various healthy as well as cancer-derived tissues and described as small, round cells with a long-term proliferation capacity in vitro.

4.5 Transfection of primary cells with the Wnt reporter 7TGC

Since the Wnt pathway plays important roles in various developmental events and stem cell maintenance[38], it is interesting to understand the role of paracrine Wnt signaling during the organoid formation. As previously described, nuclear β-catenin, an indicator for active Wnt signaling, was observed in organoids and in hFT cells cultured with the organoid medium, which contains Wnt3a. With this in mind, we wanted to investigate the role of Wnt pathway activation during organoid formation.

This was achieved using the Wnt reporter 7TGC for transfection of the primary hFT cells (Figure 17 A). This construct contains a 7xTcf-eGFP reporter cassette as well as a SV40-mCherry selection cassette and enables the generation of an stably transfected primary cell line. The mCherry selection cassette, driven by the SV40 promoter, is constitutively expressed in all cells which have

integrated the plasmid in their genome. In the presence of a Wnt ligand, the Tcf promoter – a Wnt target – drives eGFP expression, which can be easily detected using fluorescence microscopy[61].

Subsequently to the transfection, the cells were seeded in Matrigel and cultivated in organoid medium. The majority of cells were red under the fluorescence microscope, thus inserted the reporter construct. The organoid formation process was analyzed by using a live-imaging system in combination with a humidified incubation chamber at 37 °C and CO_2 supply (Figure 17 B). This technique allows a constant cell cultivation and simultaneous time lapse imaging. All pictures were obtained at 10x magnification in the green channel. Since nearly all cells fluoresced red prior to the time lapse imaging, we only acquired images of the GFP channel to minimize the stress exposition of the cells.

Single cells showed Wnt signaling activation during the organoid formation (Figure 17 C). Interestingly, these cells displayed pathway activation at different time stages. These cells might be the stem cells that induce the formation of the organoid by paracrine signaling events. Our hypothesis is that besides terminally differentiated cells and undifferentiated progenitor cells, a small number of stem cells are isolated and co-cultivated from the tubal mucosa. As mentioned before, the terminally differentiated cells have a very limited division number and get lost during the cultivation. The remaining slow-cycling stem cells are supposed to give rise to their undifferentiated, but more mature daughter cells, the progenitor cells. Nevertheless, additional experiments will have to confirm this suggestion and to definitely identify and characterize the cells displaying active Wnt signaling in the organoid formation.

In addition, time lapse imaging gave information about the organoid formation process itself. As previously assumed, the seeded cells formed small groups within the Matrigel drop. By and by, this groups fused with others resulting in a complex three-dimensional sphere – the organoid.

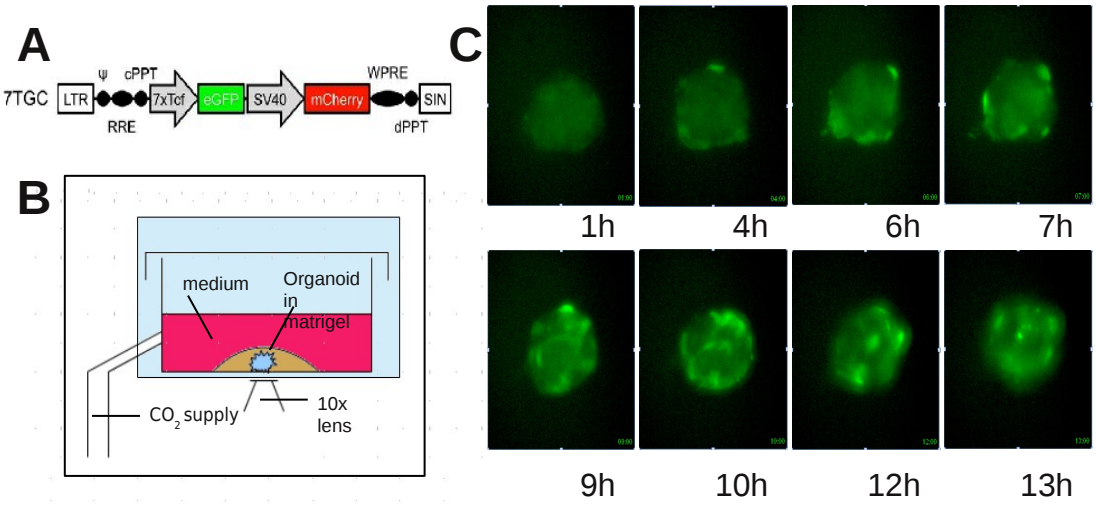

Fig. 17. Time lapse of organoid formation from primary cells transfected with the Wnt reporter 7TGC. (A) 7TGC virus with a SV40-mCherry selection cassette downstream of a 7xTcf eGFP reporter cassette. This construct can be transfected in the cells of interest and reports on active Wnt signaling in the cells[61]. (B) Schematic overview of the live cell imaging system. (C) Formation of an organoid from stably transfected primary cells at different time stages. The cultivated epithelial hFT cells were transfected with the 7TGC virus and eventually seeded in Matrigel. The subsequent formation of organoids was aquired with life-cell fluorescence microscopy. Infected cells fluorescence green in the presence of the Wnt signaling. At different times, single cells display active Wnt signaling. These cells might be the stem cells that induce organoid formation via paracrine signaling.

4.6 Production of monoclonal antibodies against Lgr5

So far, Lgr5 is the main and the only reliable marker for adult stem cells present in the mucosa of the gastrointestinal tract and hair follicle[16]. Lgr5 is

supposed to be expressed at a very small level, because only few cells within the tissues are assumed to be adult stem cells, which ensure tissue homeostasis and tissue repair after injury[11].

To date, there are no specific antibodies commercially available, which are able to to detect these small amounts of Lgr5 in the tissue. Because of that, we decided to produce monoclonal antibodies for the identification of possible Lgr5 positive cells in the hFT.

Monoclonal antibodies originate from one unique B-cell line and are therefore identical. Hence, they recognize only a single antigen (epitope). The general concept of producing monoclonal antibodies is to generate an antigen-construct, which eventually is injected into a mouse (Figure 18 B). After the immunization, the mouse develops various B cells, which each recognizes another epitope. At this stage, the antibodies contained in the serum are polyclonal. The antibody-producing cells – some are specific for the injected antigen – are gained through the removal of the spleen and subsequently fused with immortalized myeloma cells, resulting in hybridoma cells. Single hybridoma cells are cultivated and the secreted antibodies are tested for their ability to detect the epitope of interest[74]. This can be accomplished by using conventional Western blot analysis.

Since Lgr5 is a large protein with the size of approximately 100 kDA and a seven-transmembrane domain that complicates overexpression and solubility in a prokaryotic system, we decided to use only fragments of Lgr5 (Figure 18 A). The cytoplasmic part of the transmembrane protein was utilized to produce an antigen for heterogeneous overexpression in *E.coli* by cloning the target fragment with the Gateway cloning system (Figure 18 C-D).

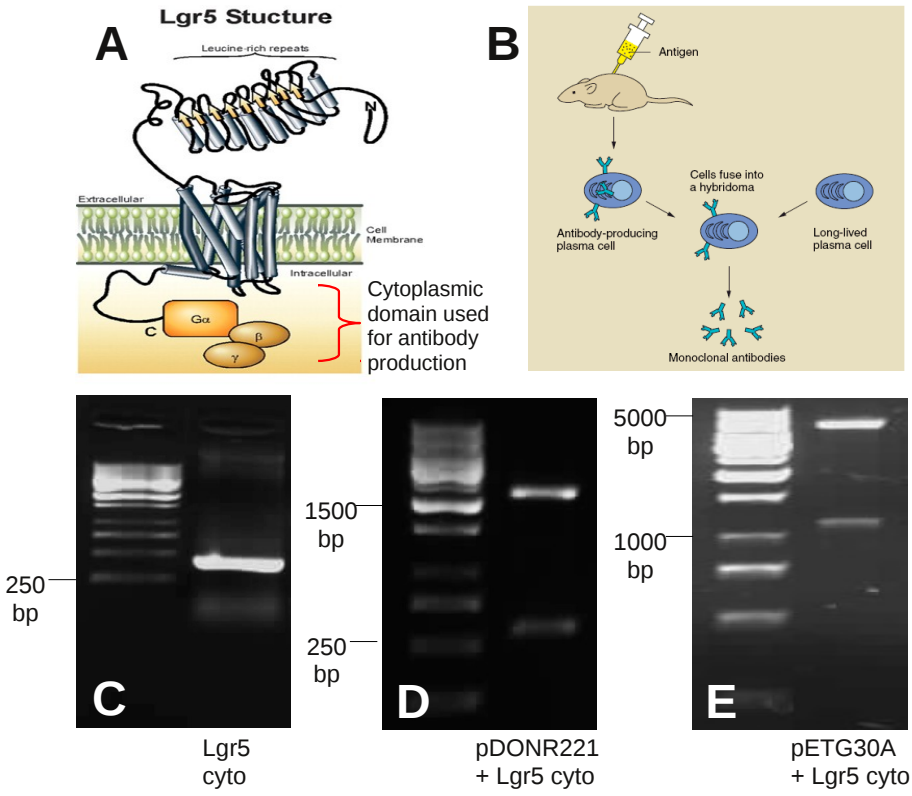

Fig. 18. Monoclonal antibodies against Lgr5. (A) Lgr5 is a leucine-rich repeat-containing G-protein coupled receptor with a large N-terminal extracellular domain and a smaller intracellular domain[10]. A fragment of the cytoplasmic domain was selected for antigen creation and subsequent antibody production in mice. (B) Schematic overview of the production of monoclonal antibodies[75]. (C-D) Production of an antigen-construct with the Gateway cloning system. (C) Purification of PCR products. PCR was performed for the Lgr5 cytoplasmic domain (252 bp). (D) BP reaction resulting in pDONR221 (2578 bp) containing the Lgr5 cytoplasmic domain (252 bp). (E) LR reaction resulting in pETG30A (6064 bp) containing the Lgr5-GST fusion protein (912 bp).

To amplify the cytoplasmic domain of Lgr5, conventional PCR was performed and the PCR products were cleaned up by gel extraction (Figure 18 C). The primers used in this PCR reaction contained 5' flanking attB sites that were recombined with the attP sites of the donor vector pDONR221 during the BP reaction.

Enzymatic digestion and Agarose gel electrophoresis confirmed the inserted cloning product (Figure 18 D). To produce the final Lgr5-GST fusion protein, the cytoplasmic domain of Lgr5 was transferred into the destination vector pETG30A by LR reaction (Figure 18 E). The vector pETG-30A was utilized to N-terminally tag the Lgr5 fragment with GST.

After the production of the cloned Lgr5-GST fusion protein, the construct was heterogeneously overexpressed in *E.coli* to obtain large amounts for the subsequent immunization of mice, performed by the Core facility for Proteomics of the Max Planck Institute for Infection biology. The overexpressed protein was purified and analyzed by SDS-PAGE (Figure 19).

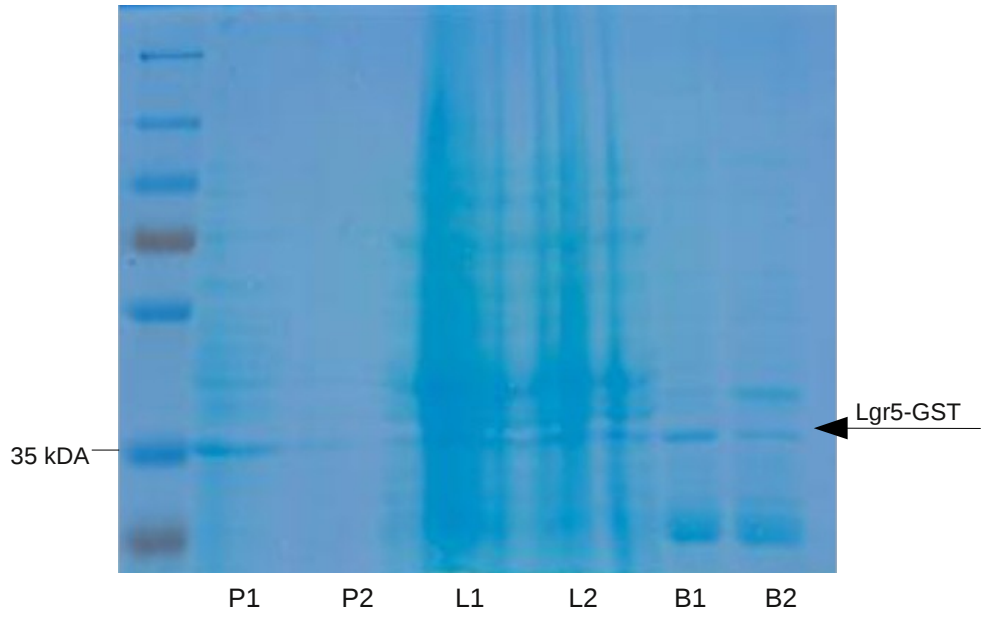

Fig. 19. IPTG-induced overexpression of the Lgr5-GST fusion protein in *E.coli*. The overexpression in *E.coli* was performed twice and the products were separated by SDS-PAGE: P – bacterial pellet, L – total lysate, B – Gluthatione bead purification. The Lgr5-GST fusion protein has a size of approximately 33.4 kDA. In the pellets and total lysates, the fusion protein is visible a prominent band besides other bacterial proteins appearing as a smear. After the purification with Glutathione beads, most of the bacterial proteins are removed. The other bands visible beside Lgr5-GST in B1 and B2 may be proteins that show a similar structure to GST and are therefore bound to the beads.

Prior to the hybridoma production, the serums from the immunized mice were tested for the presence of Lgr5-detecting polyclonal antibodies by the Western blot method.

The serum containing the polyclonal antibodies was tested at two different dilutions – 1:300 and 1:1000. Because it was shown that Lgr5 positive cells reside in the stomach[16], total protein extracts from freshly dissected human gastric crypts were used as a positive control. Besides, we tested epithelial cell cultures from the human Fallopian tube as well as total protein extracts from a freshly dissected hFT sample.

The positive control – the human gastric crypts – and the homogenized hFT show strong background. Since the serum was not affinity-purified yet, it contained the whole polyclonal immune response of the immunized mice against the antigen (Lgr5-GST fusion protein) besides the specific antibodies for Lgr5 and GST. This can lead to cross reactions with other components, such as GST-related proteins in the tested samples. However, the Western blot detected expression of an 100 kDA-sized protein (Figure 20). This would match the Lgr5 protein and show that the serum contains antibodies specific for Lgr5. Furthermore, this hypothesis would include that Lgr5 positive cells, which may act as adult stem cells, are expressed in the cell cultures at a low level.

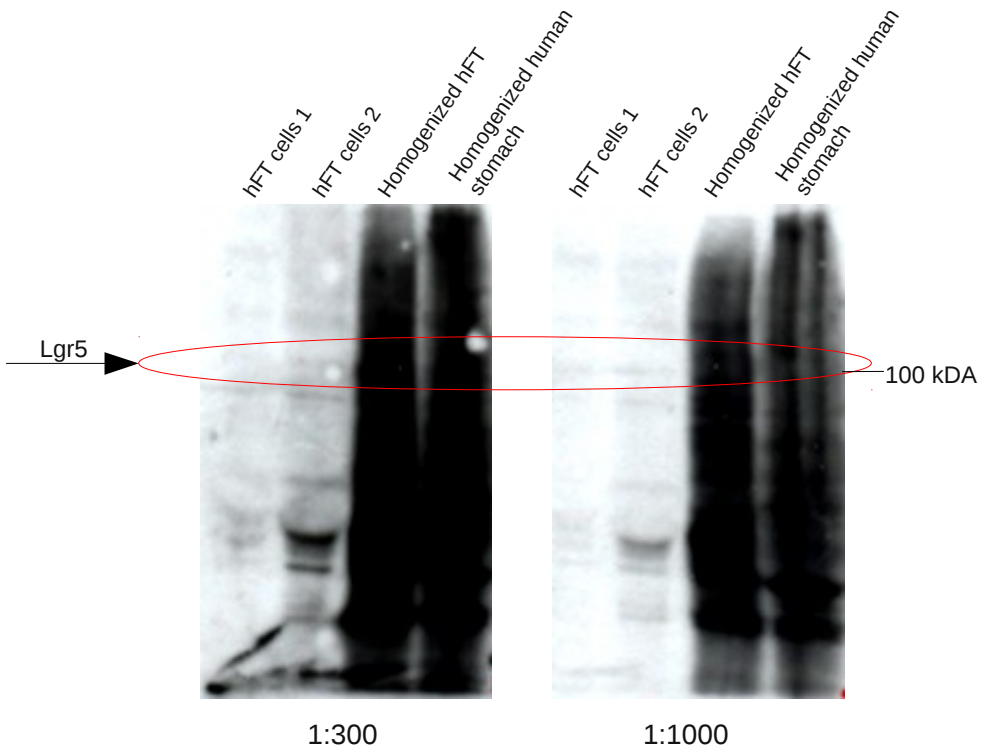

Fig. 20. Western blot to test the specificity of the mouse-derived serum. At both serum dilutions, a slight line with the size of 100 kDa is visible. This suits to the Lgr5 protein, which has an approximate size of 100 kDa. Therefore, it is assumed that the serum contains antibodies specific for Lgr5 and that Lgr5 positive cells might be expressed in the hFT epithelium at low levels.

4.7 In situ hybridization for the stem cell marker Lgr5

Besides the production of monoclonal antibodies against Lgr5, we wanted to detect the Lgr5 expression in the organoids also on the RNA level. Therefore, in situ hybridization (ISH) was performed using paraffin cuts obtained from cultivated organoids.

In addition to the antisense probe for the detection of the target mRNA, in situ hybridization requires a negative and a positve control. The epithelial cell marker EpCAM was selected as a positive control, because previous

immunofluorescence analysis revealed that all cells within the organoid are epithelial. The negative control was the sense probe for Lgr5.

If at all, we expected a rare expression of Lgr5 within the organoid. The subsequent immunostaining showed single positive cells within the spheroidal organoid (Figure 21 A). However, due to the strong background signal it remains to be determined, if these positive cells are indeed Lgr5 positive cells. This would strongly suggest that the hFT epithelium contains so far unidentified adult stem cells and needs to be further analyzed.

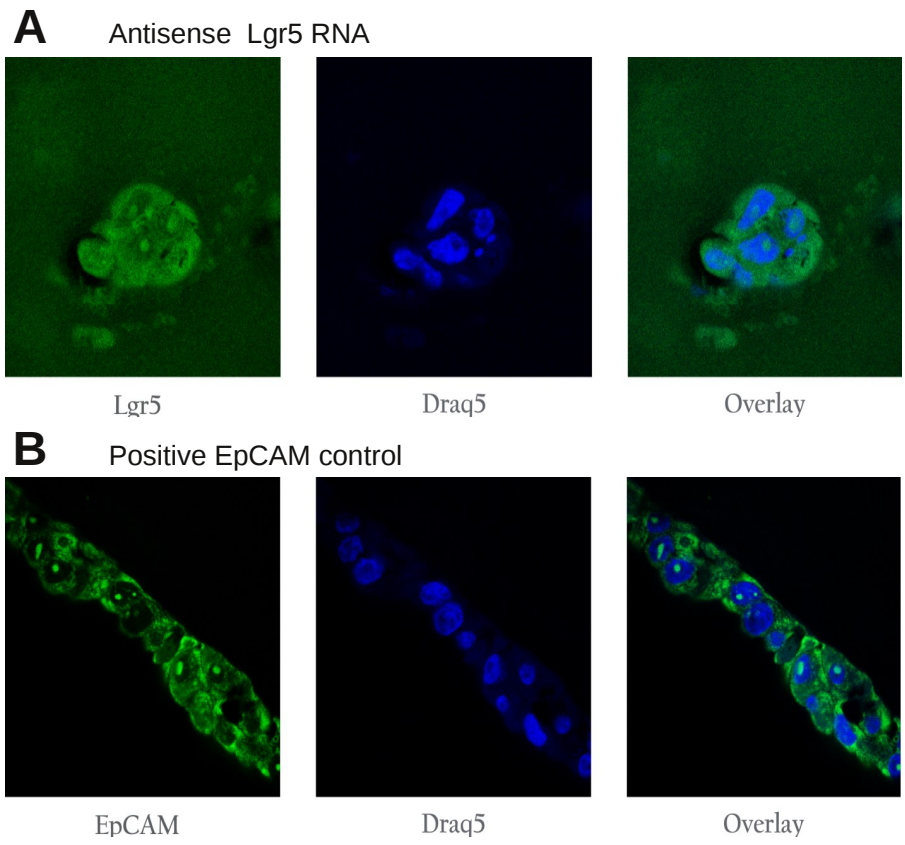

Fig. 21. In situ hybridization for the stem cell marker Lgr5. (A) Antisense Lgr5 RNA. Rare cells showed a positive signal for Lgr5 RNA in the nuclei. Because of the strong background signal, these results have to be confirmed by further experiments. (B) The positive control was performed with EpCAM, an epithelial marker. The positive signal is clearly visible in the cell nuclei.

5 Discussion

To date, there was nothing known about progenitor cells in the human Fallopian tube. In this study, we were able to isolate undifferentiated epithelial progenitor cells from the Fallopian tube and to establish a novel three-dimensional ex vivo model system. Furthermore, we attempted to detect adult stem cells in the hFT tissue by producing monoclonal antibodies and performing in situ hybridization against the adult stem cell marker Lgr5.

Epithelial cells from the tubal mucosa have been isolated and showed the capacity of forming compact three-dimensional structures in vitro. These so-called organoids could represent a valuable tool for investigating the mechanisms of bacterial infections in the hFT. The capacity to form organoids in Matrigel varied between the tubes from different patients. We suppose that the tubal region that was dissected, the patient's age as well as his physical conditions contribute to these observations. After thawing, the cells were able to grow in epithelial islands and to form three-dimensional structures in Matrigel, although less efficient than before the stocking and thawing.

In our study we established culturing conditions promoting the formation of organoids from hFT epithelial cells. These conditions can be presumably improved by the usage of other growth supplements to create a better microenvironment for the possibly existing stem cells. Since the mucosal epithelium in the tube is constantly exposed to hormonal changes due to the female menstrual cycle, it might be necessary to add certain hormones, such as oestradiol, to the culture. This could improve the efforts to maintain a mature organoid epithelium with fully differentiated cells.

We further demonstrate the presence of undifferentiated epithelial progenitor cells, which reside within the tubal mucosa. These cells are assumed to originate from adult stem cells. Progenitor cells have still pluripotent features, although they are more committed than adult stem cells.

The finding of these progenitor cells is important for a better understanding of pathology mechanisms concerning the integrity of the tubal mucosa and indicates the existence of adult stem cells in the tubal tissue. To investigate how the stem cells and their progeny contribute to tissue response following injury or inflammatory processes in the hFT will help understanding the etiology of tubal diseases caused by *Chlamydia trachomatis* and *Neisseria gonorrhoe*. Bacterial infection is a serious hazard for the female reproductive capacity, as the infection frequently leads to tubal damage and scarring, which often results in infertility. The existence of pluripotent progenitor cells in the hFT mucosal niche opens new questions about potential long-term impact of bacterial infections on epithelial cell homeostasis. In this model, the accumulation of mutations during tubal inflammation and injury response, as well as changes in epithelial homeostasis, could play an important role in tissue health beyond acute infection.

Our preliminary in situ hybridization data indicate the existence of Lgr5 positive cells in the organoids. However, the expression of Lgr5 needs to be confirmed on protein level using a specific Lgr5 antibody. For this purpose, immunostaining of hFT tissue, hFT cell cultures and organoids has to be accomplished. For a better understanding of the regulatory mechanisms of the tubal cell homeostasis, it would be necessary to clearly identify these Lgr5 positive cells as adult stem cells. This could be achieved by using the long term label retaining cells (LRC) approach[76]. In this pulse-chase approach, a labeled histone is tissue specifically expressed. As this tag is lost during cell division, only the slow cycling stem cells will retain it. This makes the stem cells readily identifiable.

In addition, observed side-population cells within the cultures have to be definitely characterized in ongoing studies including colony forming unit (CFU) assay or Hoechst 33342 exclusion. Potential Lgr5 expression of the SP cells in an immunostaining experiment remains to be examined.

Our findings, showing that single cells display Wnt signaling activation during the formation of organoids, provides a reasonable explanation for how the organoid formation process occurs. We hypothesize that these single positive cells act as adult stem cells residing in the tubal tissue and give rise to the pluripotent progenitor cells found within the organoids. Furthermore, they are supposed to be activated by Wnt ligands in the medium and to induce the grouping and subsequent formation of organoid-like structures by paracrine signaling events. It would be interesting to elucidate which factors, presumably secreted by the progenitor cells, induce this formation of 3D structures by epithelial cells. The data presented herein suggest an important role of Wnt signaling in the process of epithelial tissue renewal in the hFT. This prominent capacity of Wnt signaling in the tubal tissue renewal is emphasized by similar observations in the gastric and intestinal tissue by Barker et al[10].

In a clinical study[55], it could be shown that the frequency of ovarian cancer in females is significantly reduced if the Fallopian tubes are removed. This supports the suggestion that most ovarian neoplasms actually arise from the Fallopian tube epithelium rather than from the ovarian surface cells[54]. These findings led amongst others to the assumption that stem cells in the Fallopian tube could contribute to the onset of ovarian cancer. According to our results, at least undifferentiated epithelial progenitor cells and probably adult stem cells exist in the Fallopian tube. These stem cells could accumulate mutations, adopt a degenerated state and migrate into the ovary. As tumors in the Fallopian tube occur very seldom, there has to exist a mechanism, either preventing the growth of the proposed degenerated hFT stem cells in the Fallopian tube, or promoting the growth of these stem cells in the ovary. One

of these mechanisms could lead to ovarian cancer in cases, where the degenerated cells manage to migrate into the ovary.

Smith et al. provide some evidence that *Ctr* infection and cancer are linked[77]. Beside accumulation of mutations, another possible reason for the transformation of stem cells in the hFT could be *Chlamydial* infection. Recent studies by Kessler et al. (submitted manuscript, personal communication) show that acute *Chlamydia trachomatis* infection disrupts the cell homeostasis of the tubal epithelium, in infected cells as well as in neighboring uninfected cells. This *Ctr* induced transformation of hFT cells could promote the onset of ovarian cancer.

Taken together, this study provides insight into the possible process of tissue homeostasis in the human Fallopian tube. We were able to detect undifferentiated epithelial progenitor cells in the tubal mucosa and found evidence for the existence of adult stem cells. For the first time, other cell types than terminally differentiated cells have been verified in the human Fallopian tube and a possible mechanism for tissue homeostasis is provided.

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7 Reference of Figures

Fig. 1 A Fertility problems
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Fig. 1 B Lyons, R.A., Saridogan, E. & Djahanbakhch, O. The reproductive significance of human Fallopian tube cilia. Hum. Reprod. Update 12, 36-372 (2006).

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Fig. 3 A Barker, N. & Clevers, H. Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. Gastroenterology 138, 1681-1696 (2010).

Fig. 3 B Misc Protein Analysis
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Fig. 4 Are Stem Cells Involved in Cancer?
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Fig. 5 Reya, T. & Clevers, H. Wnt signalling in stem cells and cancer. Nature 434, 843-850 (2005).

Fig. 6 Chlamydia trachomatis – Wikipedia, the free encyclopedia
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Fig. 17 A Fuerer, C. & Nusse, R. Lentiviral vectors to probe and manipulate the Wnt signaling pathway. PLoS ONE 5, e9370 (2010).

Fig. 18 A Barker, N., Bartfeld, S. & Clevers, H. Tissue-resident adult stem cell populations of rapidly self-renewing organs. Cell Stem Cell 7, 656-670 (2010).

Fig. 18 B Monoclonal Antibodies http://www.web-books.com/eLibrary/Medicine/Physiology/Immune/Monoclonal.htm

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8 Abbrevations

AA	amino acid
ABC-transporter	ATP-binding cassette-transporter
AJ	adherent junction
APC	Adenomatous polyposis coli
APS	ammonium persulfate
BSA	bovine serum albumine
CBC cell	columnar crypt base cell
CFU	colony forming unit
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate
CK1	casein kinase 1
CRD	cysteine-rich extracellular domain
CSC	cancer stem cell
Ctr	*Chlamydia trachomatis*
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EB	elementary body
E.coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylenediaminetatraacetic acid
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
EHS	Engelbreth-Holm-Swarm sarcoma
EpCAM	epithelial cell adhesion molecule
FACS	fluorescence-activated flow cytometric analysis
FCS	fetal calf serum
FGF	fibroblast growth factor
Fz	Frizzled
GSK3	glycogen-serine kinase 3

GFP	green fluorescent protein
GST	glutathione S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IFF	immunofluorescence buffer
ISH	in situ hybridization
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton
kV	kilovolt
Lgr5	Leucine-rich repeat-containing, G-coupled receptor 5
LRP5/6	Low-density lipoprotein receptor-related protein 5 and 6
LRR	Leucine-rich repeats
Muc1	Mucin 1
M	molarity
ms	millisecond
P	passage
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline Tween 20
PVDF	polyvinylidene fluoride
P/S	penicillin and streptomycin
RB	reticulate body
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SC	stem cell
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shh	sonic hedgehog
SP cell	side-population cell
TA cell	transit-amplifying cell
TBS	Tris buffered saline
TEMED	N, N, N', N'-tetramethylethylenediamine
3D	three-dimensional

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