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ORAL MUCOSA KERATINOCYTES AND THEIR EXOSOMES FOR EPITHELIAL TISSUE REGENERATION

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Oral mucosa keratinocytes and their exosomes for epithelial tissue regeneration
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This thesis is dedicated to Gustav Sjöqvist.

Gustav was my dad and one of my closest friends. Sadly, he left us a few weeks ago.

He taught me never to be afraid to try something new.

"Det är skönare lyss till den sträng som brast än att aldrig spänna en båge."

- Verner von Heidenstam

"It is fairer to listen to the string that broke than to never strain a bow"

ABSTRACT

Early tumors, including high grade dysplasia and intramucosal invasive cancer, of the esophagus can today be removed using endoscopic resection, often using a technique called endoscopic submucosal dissection (ESD). This treatment is better tolerated and has considerably less mortality and morbidity compared to conventional, more invasive surgery, which usually entails esophagectomy. However, after larger endoscopic dissections, stricture formation is a common complication. Such strictures are usually treated with balloon dilatations, but the procedure often has to be repeated several times and is associated with risks such as perforations. In recent years, Japanese researchers developed a new method to reduce the risk of strictures. About two weeks before the treatment, an oral mucosa biopsy is taken from the patient from which epithelial cells are isolated and grown on special temperature-responsive polymer-coated surfaces. The polymer changes morphology and wettability properties depending on temperature, which enables non-enzymatic cell harvesting – the cells can be detached as contiguous sheets and with a large amount of extracellular matrix (ECM) maintained. After the ESD, cell sheets can be transplanted to the wound bed without the need for suture or other fixative, it is thought that the remaining ECM acts as a glue. Nine patients were treated in Tokyo and we subsequently transferred the technology to Stockholm where five additional patients were treated. Although one of nine patients in the Japanese cohort and three of five patients in the Swedish cohort still developed strictures, they appeared to be milder and easier to treat than expected. However, the aim of these projects was to evaluate safety and feasibility, further studies are needed to evaluate efficacy of the treatment.

Since some patients still developed strictures despite the cell sheet transplantation, we next aimed to evaluate if exosomes from the cell culture media could be used as a pro-regenerative agent, perhaps in combination with cell sheet therapy. Media was collected from clinical-grade production of cell sheets from eight healthy donors. The media was concentrated by ultra-filtration and exosomes were isolated by size-exclusion chromatography. The exosomes were characterized by western blot (CD9+, Flotillin-1+, GRP94-), electron microscopy and nanoparticle tracking analysis (~125 nm). They reduced the proliferation of skin fibroblasts and stimulated upregulation of gene expression of growth factors relevant for wound healing. We studied the exosomes' adhesion to esophageal wound bed by topical application to porcine esophageal wounds *ex vivo* and could detect signal after as little as one minute adhesion time. We also found that the exosomes stimulated wound healing of full-thickness skin wounds in immunocompetent rats, both at the 6^{th} day and 17^{th} day time point. In conclusion we found that exosomes could be isolated from cell sheet media and that they exhibited pro-regenerative properties even in a *xenogeneic* setting. Further studies are necessary to evaluate their potential to stimulate mucosal wound healing and reduce stricture formation of the esophagus.

LIST OF SCIENTIFIC PAPERS

I. Jonas E, **Sjöqvist S**, Elbe P, Kanai N, Enger J, Haas S L, Mohkles-Barakat A, Okano T, Takagi R, Ohki T, Yamamoto M, Kondo M, Markland K, Lim M L, Yamato M, Nilsson M, Permert J, Blomberg P, Löhr J M: **Transplantation of tissue-engineered cell sheets for stricture prevention after endoscopic submucosal dissection of the oesophagus**. *United European Gastroenterology Journal* 02/2016

II. **Sjöqvist S**, Ishikawa T, Shimura D, Kasai Y, Imafuku A, Bou-Ghannam S, Iwata T, Kanai N: **Exosomes derived from clinical-grade oral mucosal epithelial cell sheets promote wound healing**. *Journal of Extracellular Vesicles* 01/2019.

III. **Sjöqvist S**, Kasai Y, Shimura D, Ishikawa T, Iwata T, Kanai N: **Gingival keratinocyte-derived exosomes regulate proliferation of fibroblasts and epithelial cells**. *Unpublished manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CAR-T-Cells	Chimeric Antigen Receptor-T-Cell
CBB	Coomassie Brilliant Blue
CCK	Cell Counting Kit
CD9	Cluster of Differentiation 9
CFU	Colony Forming Unit
CTGF	Connective Tissue Growth Factor
DMEM	Dulbecco's Modified Eagle's medium
EBD	Endoscopic Balloon Dilatation
ECM	Extracellular Matrix
EGD	EsophagoGastroDuodenoscopy
EMR	Endoscopic Mucosal Resection
ESD	Endoscopic Submucosal Dissection
EV	Extracellular Vesicle
FBS	Fetal Bovine Serum
FEx	Fibroblast Derived Exosomes
FGF2	Fibroblast Growth Factor2
GERD	Gastro Esophageal Reflux Disease
GMP	Good Manufacturing Practice
GVHD	Graft-Versus-Host Disease
H&E	Hematoxylin and Eosin
HFFs	Human Foreskin Fibroblasts
HGD	High-Grade Dysplasia
HGF	Hepatocyte Growth Factor
HOK	Human Oral Keratinocyte
HSCT	Hemapoetic Stem Cell Transplantation
HSP70	Heat Shock Protein 70
IPCL	IntraPapillary Capillary Loops
IV	IntraVenous

KGM	Keratinocyte Growth Medium
MDT	Multi-Disciplinary Team
MSCs	Mesenchymal Stromal Cells
MVB	Multivesicular Body
NBI	Narrow Band Imaging
NHDF	Normal Human Dermal Fibroblasts
NK-Cells	Natural Killer Cells
NTA	Nanoparticle Tracking Analysis
OCT	Optimal Cutting Temperature
OKEx	Oral Keratinocyte Derived Exosome
OMEC	Oral Mucosal Epithelial Cell
PBS	Phosphate Buffered Saline
pCLE	Probe-base Confocal Laser Endomicroscopy
PCR	Polymerase Chain Reaction
PPI	Proton Pump Inhibitor
QOL	Quality of Life
RIPA	Radio Immuno Precipitation Assay
SM	Submucosa
TEM	Transmission Electron Microscopy
TRPS	Tunable Resisive Pulse Sensing
VEGF	Vascular Endothelial Growth Factor

1 INTRODUCTION

This thesis involves two very exciting, but also very complex research fields: regenerative medicine and extracellular vesicles (EVs). In this chapter, I will first briefly explain our clinical problem which is esophageal strictures developed after endoscopic submucosal dissections. I will then give an introduction to regenerative medicine and EVs.

1.1 ESOPHAGEAL STRICTURES AFTER ENDOSCOPIC SUBMUCOSAL DISSECTION

Today, early esophageal tumors or dysplasia can effectively be removed by endoscopic means, providing a much less invasive treatment compared to conventional surgery. The first technique was developed in the late 1980s, named endoscopic mucosal resection (EMR)[1,2]. In principle, a saline-based liquid is injected in the submucosa, to elevate the lesion. The lesion is then inserted into a snare which can be closed to resect the tissue. EMR has been shown to be safer than conventional surgery (esophagectomy), with considerably lower mortality and morbidity[1]. One of the limitations of EMR is that larger lesions cannot be removed *en bloc* (in one piece) which is more favorable for histopathological analysis to confirm radical resection. For this reason, endoscopic submucosal dissection (ESD) was developed. The lesion's margins are first marked, and a liquid is injected in the submucosa. A special electric knife is then used to dissect the lesion *en bloc* from the underlying muscular layer[3–5].

A common complication after ESD is stricture formation, resulting in a decrease of the esophageal lumen which leads to dysphagia. The risk of stricture formation is related to the size of the dissection, especially the percentage of esophageal circumference (**Fig 1**) – a dissection involving 75% - 99% of the circumference was found to have a stricture rate of around 36%. In the cases where the whole circumference was dissected, 83% of the patients developed strictures[4]. Strictures can be treated by endoscopic balloon dilatation (EBD), but this often has to be repeated which impacts the patients' quality-of-life (QOL) and there is a significant risk of esophageal perforation due to the dilatations[6,7].

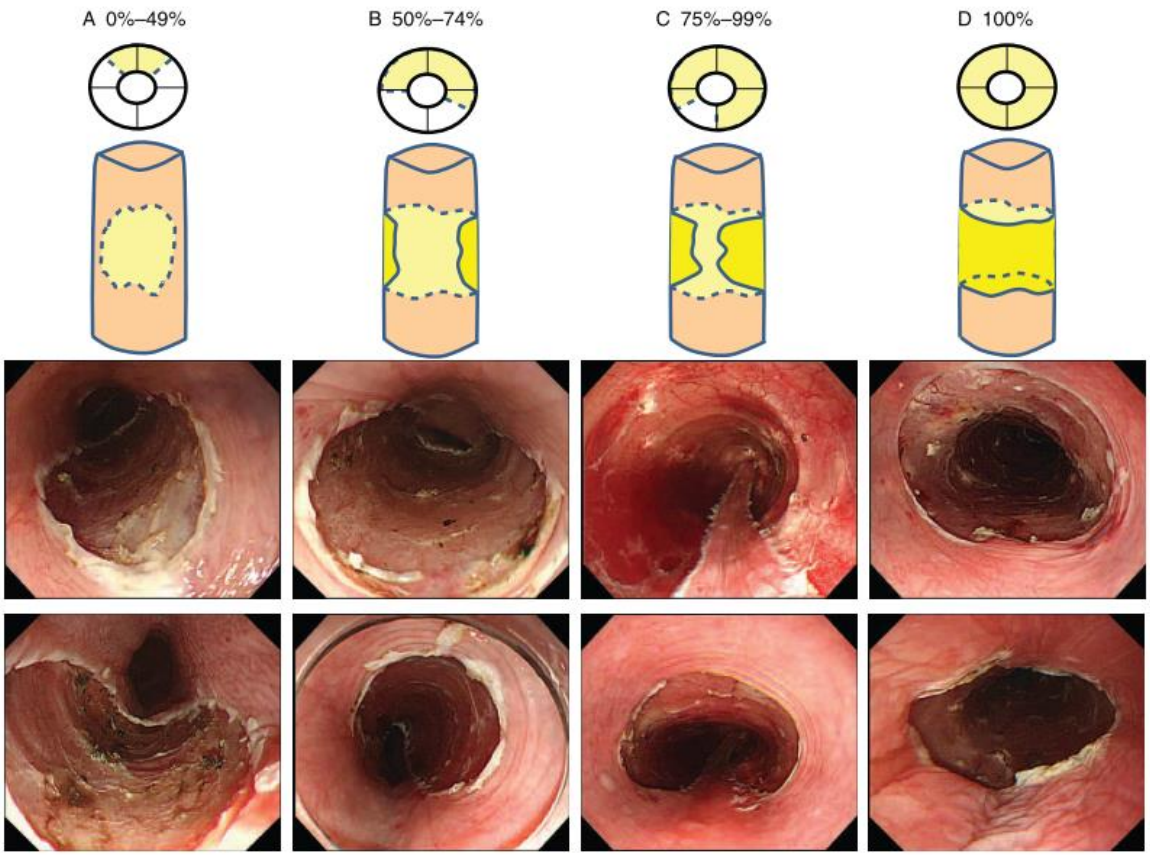

Figure 1- Grading of esophageal mucosal defects.
Reproduced with permission from: Funakawa K et al. Effect of Submucosal Dissection for Superficial Esophageal Neoplasms and Risk Factors for Postoperative Stricture. Medicine (Baltimore). 2015. 94(1):e373. https://insights.ovid.com/pubmed?pmid=25569662

Prevention of such post-ESD strictures remains a clinical challenge. Several methods have been reported but none has yet gained general acceptance[8]. In principle, the tried methods fall into four different categories: I) corticosteroids (local injection[9] or systemic administration[10]), II) stent[11] / prophylactic dilatations[12], III) tissue engineering solutions (cell based[13] or biomaterial[14,15] based), IV) other (for example small interfering RNA[16] or botulinum toxin[17] injections).

1.1.1 Steroid treatment

Corticosteroids are thought to prevent stricture formation by reducing collagen synthesis and stimulating collagen degradation. The treatment is cost-effective but steroid treatment, especially systemic, has adverse effects including risk of infections, diabetes, osteoporosis *etc*[8]. System treatment is generally administered for at least three weeks. Another method involves local injections into the wound bed, which will likely reduce the risk of adverse effects, but the treatments have not been found to reduce the frequency of strictures, although some researchers suggest that the severity of the developed strictures is reduced[8].

1.1.2 Prophylactic stenting

Stents can be placed after the ESD, in order to prevent the development of strictures. Studies have reported successful outcomes, but, the risk of stent migration is considerable. The stents

can be made from degradable or non-degradable material, and in some cases the stents are drug-eluting. However, in general, the sample-sizes in the published studies are too small to draw any strong conclusions[8].

1.2 REGENERATIVE MEDICINE

The fact that the parts of the human body can regenerate has been known for a long time. A good example is that of the Greek mythology titan Prometheus (**Fig 2**) who stole fire from the gods and gave it to the humanity. As a punishment, the gods sentenced him to eternal suffering, he was bound to a rock and an eagle would feed on his liver every day. By the following day, the liver had regenerated and the eagle could once again feed. Interestingly, despite the liver being one of the most regenerating organs in the human body, being able to regenerate after surgical removal of 2/3 of its volume, expanding hepatocytes *in vitro* is very challenging[18].

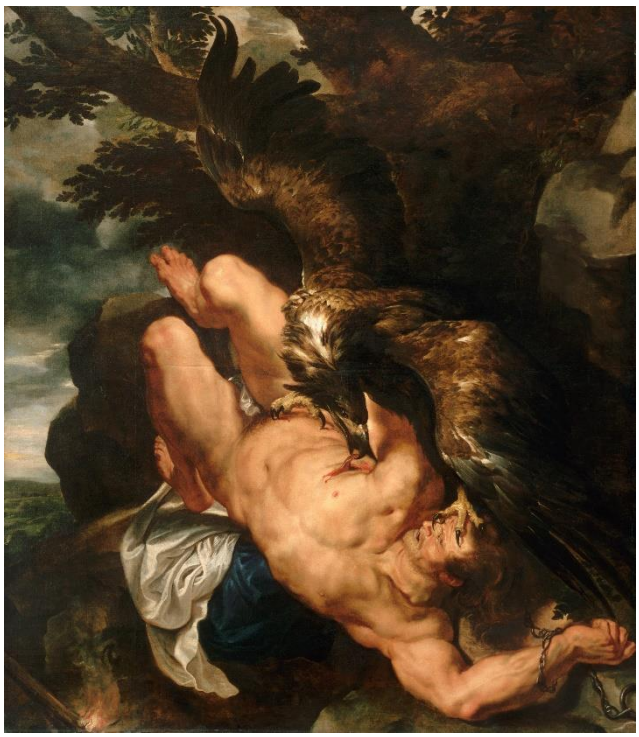

Figure 2 - "Prometheus Bound" by Peter Paul Rubens, 1618.

There is no globally accepted definition of regenerative medicine, but the aim is commonly stated to be to repair or replace damaged, injured or missing tissue with methods such as administering cells (cell therapy), applying biomaterials or a combination of the two (tissue engineering). Regenerative medicine is a relatively new research field, which is thought to have been used for the first time in 1992[19]. Since year 2000 there has been an enormous increase of interest in the field, with over 7000 articles published in 2018 (**Fig 3**).

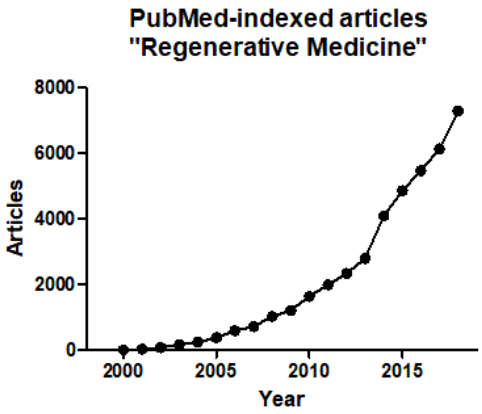

Figure 3 - Number of publications with key words "Regenerative Medicine". Data from https://www.ncbi.nlm.nih.gov/pubmed/

1.2.1 Cell therapy

1.2.1.1 Hematopoetic stem cell transplantation

Hematopoetic stem cell transplantation (HSCT) as a treatment for leukemia represent a success story which was first performed in patients by Dr Donall Thomas in 1957[20] and today is a clinical routine treatment. Dr Thomas shared the Nobel Prize in Physiology or Medicine with Dr Joseph Murray in the year 1990. During the years, the indications for the treatment has widened, and today include both hematopoetic and solid tumors[21]. The general principle of the treatment is to first reduce the diseased bone marrow using chemotherapy and / or irradiation treatments. Subsequently, donor (or in some cases the patients' own) cells are infused, with the hope that they will engraft and eliminate any residual malignant cells. In some cases, this does not happen, and remission is never achieved. A common complication to HSCT is graft-versus-host disease (GVHD), where the infused cells are reacting with the recipient tissue, typically causing symptoms in mucosa and skin[22]. Many of the GVHD cases can be managed by pharmacological treatment using corticosteroids, but steroid-refractory GVHD is a very severe condition with high mortality rates. The absence of treatments for these patients led to the development of a new cell therapy method using mesenchymal stromal cells (MSCs)[23].

1.2.1.2 Mesenchymal stromal cells for steroid-refractory GVHD

The interest for MSCs has been enormous and the cells have been isolated from a variety of tissues including bone marrow[24], adipose and skeletal muscle tissue[25]. These cells have been found to be pro-regenerative, immunomodulatory and well tolerated in *allogeneic* setting. Subsequently, researchers have tried to use them for many indications, for example, liver fibrosis[26], skeletal muscle regeneration[27], lung injury[28,29] and many others[30–35].

Due to the lack of treatment options for patients with steroid-refractory GVHD, Ringdén and colleagues investigated the use of MSCs for this indication. Bone marrow was harvested from donors, mononuclear cells were isolated through Redigrad gradient separation and the cells plated and maintained in incubators. Finally, the cells were resuspended in phosphate buffered saline (PBS) with 10% human plasma, and administered to the patients through infusion. In six of eight patients, the GVHD disappeared completely, and the survival rate was significantly better than controls[23]. Today there are several completed and ongoing clinical studies based on these findings. However, according to a recent Cochrane systematic review, the evidence today is not strong enough to support a conclusion that the treatment is effective[36]. Hopefully, larger trials with more participants will shed light on the efficacy of the treatment.

1.2.1.3 Chimeric Antigen Receptor-T-cells

This very exciting approach was developed in recent years. In brief, a cancer patient's T-cells are isolated and genetically engineered to target tumor antigen. This is achieved by introducing a synthetic antigen-recognizing receptor to the T-cells. The cells are then

expanded and infused to the patient[37]. This treatment was developed by Israeli researcher Zelig Eshhar in 1993 and by 2017, FDA approved the first CAR-T-based therapy, targeting acute lymphoblastic leukemia which was developed by Novartis[38]. The treatment is now under investigation for other indications, including diffuse large B-cell lymphoma, multiple myeloma and chronic lymphocytic leukemia[37,39].

1.2.2 Tissue engineering

While cell therapy generally consists of injection or infusion of single cell suspensions, tissue engineering adds another dimension by including some sort of structure, usually referred to as a scaffold. In principle, any adherent cell can be used, and the scaffold can be either of synthetic / artificial or biologic origin. Regardless of the origin of the scaffold, the aim is to have a structure that resembles the target tissue, can support and direct cell growth and permit (or even better stimulate) ingrowth of blood vessels. Of course, the scaffold should be non-immunogeneic, and where necessary, suturable to enable surgical implantation. Artificial scaffolds can be created in many different ways, for example electrospinning[40–44] or 3D-printing[45–50]. Biological scaffolds are often created by removing cells from donor tissue, so called decellularization[51,52]. Using different reagents, often detergents and enzymes, cells can be removed from the tissue without disrupting the underlying extracellular matrix (ECM). The ECM can often retain similar mechanical properties to the native tissue but at the same time be biocompatible as the immunogenic elements are removed. Cells can subsequently be seeded and cultured onto the ECM surface (**Fig 4**).

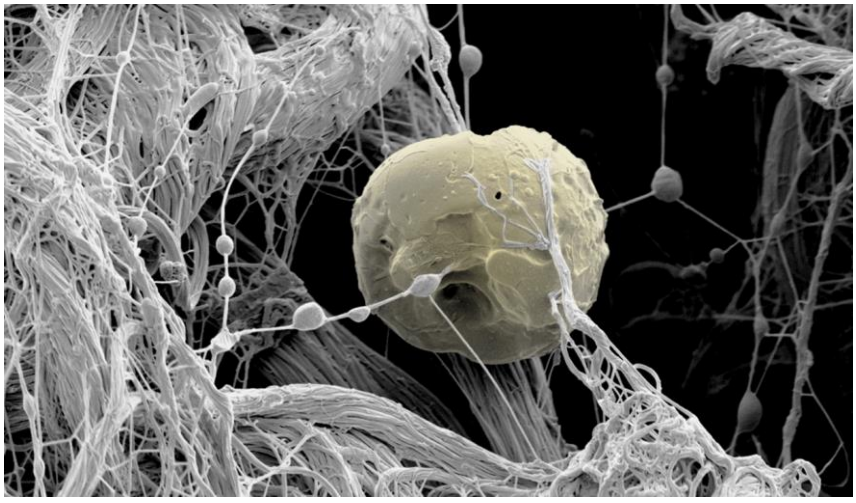

Figure 4 - Scanning electron microscopy of mesenchymal stromal cell growing on decellularized tissue. Source: private

One of the groundbreaking examples in this field was the *ex vivo* construction of a beating heart. Ott and colleagues decellularized rodent hearts and could observe macroscopical beating after reseeding the scaffolds with cells[53]. The same technique was later used to create bioartifical lungs which were capable of contributing to gas exchange[54]. Other researchers have used similar methodology to regenerate blood vessels[51,55,56], kidneys[57], esophagus[58,59], cornea[60] and aortic valves[61].

1.2.2.1 The "Vacanti mouse"

In 1997, one of the early examples of tissue engineering was conducted by the group of Dr Vacanti, in which they aimed to create a cartilage structure resembling an external ear. An artificial scaffold was used, consisting of polyglycolic acid-polylactic acid shaped to resemble an external ear of a 3-year-old child. The scaffolds were seeded with chondrocytes from bovine articular cartilage and were incubated for one week, after which they were implanted subcutaneously in athymic mice. Non-seeded scaffolds served as controls. They found that chondrocytes could adhere to and survive on the scaffold *in vitro*. In the seeded scaffolds, neo-cartilage observation could be observed after *in vivo* implantation, no neo-cartilage was found in the non-seeded scaffolds. They also found that an external stent was necessary to keep the three-dimensional structure. The image of the so called "ear-mouse" or "Vacanti-mouse" has become a symbol for tissue engineering (**Fig 5**). Dr Vacanti predicted that the image would lead to controversy and avoided to share images of the mouse with journalists[62]. However, the image still reached the media and allegedly sparked protests against genetic engineering – despite that no genetic engineering was involved in the research.

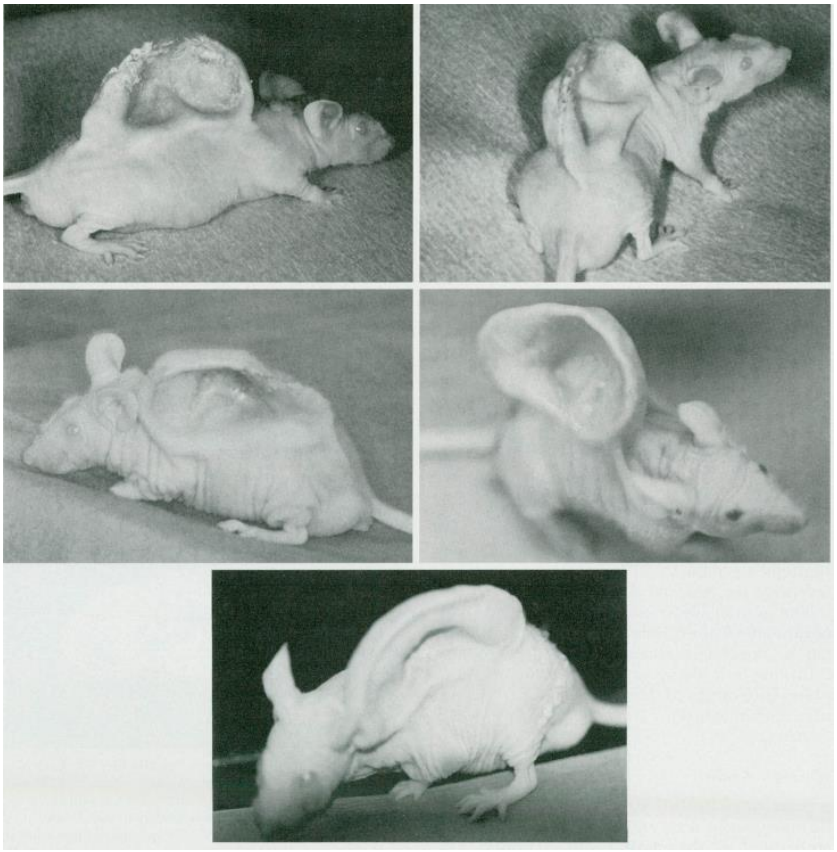

Figure 5 - "Vacanti mouse" – one of the early examples of tissue engineering. Reproduced with permission from: Cao, et al. Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear. Plast. Reconstr. Surg. 1997. 100(2): 297-302. https://insights.ovid.com/pubmed?pmid=9252594

1.2.2.2 Bladder and genital organs

Roughly a decade after the Vacanti mouse, urologist Anthony Atala made a breakthrough in urinary bladder tissue engineering. The team had constructed transplantable structures made from collagen or collagen-polyglycolic acid composite, seeded with *autologous* urothelial and muscle cells. The transplantations were done in seven patients and had positive functional outcomes (decreased mean bladder leak points, increase of volume and compliance *etc*)[63]. Eight years later, the same group published the use of a similar technology to create vaginal organs in patients with congenital aplasia. The follow-up period was up to eight years and there was functional restoration including gain of sexual function[64]. Professor Atala

could also successfully replace a large portion of rabbit penises by using *autologous* cells seeded on collagen scaffolds. The functional outcomes were remarkable: the animals receiving the grafts could impregnate female rabbits[65].

1.2.2.3 Esophageal mucosa regeneration

This example of tissue engineering has the same aim as our cell sheet project which will be mentioned in section 1.2.3.2, and in article two of this thesis. Early tumors or high-grade dysplasia of the esophagus can today be removed by endoscopic resection. However, a common complication after larger resections is stricture formation. Professor Badylak has developed a method of applying a tubularized sheet of *xenogeneic* ECM (derived from porcine small intestine). In this article, five patients underwent an endoscopic treatment whereby circumferential, long segment mucosa was removed. The biological scaffold was then held in place by a stent, which was removed after 9-18 days when the scaffold had integrated to the native tissue. Although all five patients developed strictures, they were limited to areas that were not covered by the scaffold and the authors conclude that the treatment had a beneficial effect and stimulated constructive remodeling[66].

1.2.3 Cell sheet engineering

In mid-1990s, Prof Okano described the novel use of a temperature-responsive polymer for use in cell cultures. The polymer Poly(N-isopropylacrylamide) changes conformation and wettability based on temperature: in 37 °C, the polymer, covalently attached to cell culture surface, is around 20 nm and hydrophobic. However, when the temperature is lowered below 32 °C, the polymer transforms to 60 nm and becomes hydrophilic. Cells can grow on the surface in a heated incubator, but once the temperature is lowered, the cells will detach[67]. Conventionally, cells are detached / harvested using enzymatic treatment, for example trypsin. However, using these enzymes commonly damages various proteins, including cell-to-cell connections and ECM. Harvesting the cells using temperature reduction results in contiguous cell sheets, with maintained cell-to-cell connections[68] and ECM[69]. For transplantations, the sheets readily adhere to the target tissue, without the need of sutures or tissue glue[70,71], which likely is due to the maintained ECM[69]. The cell sheet technology has been used for a variety of indications such as cardiac[68,72], osteoarthritis[73] and liver applications[74]. Following, I will describe three clinical applications.

1.2.3.1 Cell sheets for corneal replacement

There is a constant turnover of corneal epithelial cells, with a renewing of cells stemming from progenitor cells in the vascularized limbus area. Loss of these cells, so called limbal stem cell deficiency can lead to vascularization of the cornea and irregular epithelium, with subsequent discomfort and impaired vision. Common treatments are *autologous* transplantation of limbal cells from the opposite eye or transplantation of donated cornea. However, in some cases the injury is bilateral and there is a constant lack of available donors. This led Dr Nishida and colleagues to explore possible solution through cell sheet technology. They investigated the use of oral mucosal keratinocytes due to their similarities to corneal epithelium, the availability of these cells and the fact that buccal grafts had been used in ocular surgery before. Four patients with total limbal stem-cell deficiency were included. Biopsies were taken from the patients' oral mucosa, epithelial cells isolated and grown into cell sheets. Fourteen days after the biopsy, the cell sheets were ready to be transplanted. The corneal stroma was surgically re-exposed and the cell sheet was placed directly onto the stromal bed. The sheets attached without any suturing. All four patients had improved corneal transparency and visual acuity, and transparency remained during the mean follow-up of 14 months (**Fig 6**). No complications were found[70].

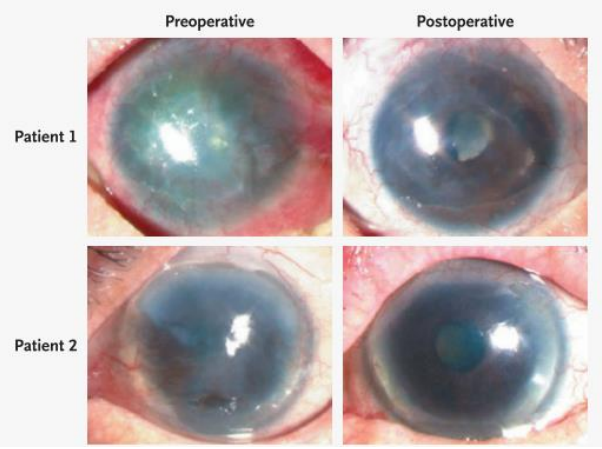

Figure 6 - Cell sheet transplantation for cornea regeneration. Reproduced with permission from (Nishida, K. et al. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium), Copyright Massachusetts Medical Society.

1.2.3.2 Cell sheets for esophageal stricture prevention

Researchers at Tokyo Women's Medical University aimed to reduce the risk of stricture formation of the esophagus after ESD. The technique was first evaluated in a canine model. Oral mucosal epithelial cells were isolated and grown into cell sheets. ESD wounds, 5 cm long and 180° of the esophageal circumference, were made and half of the animals received cell sheet treatment. The sheets adhered to the wound bed within ten minutes and without any sutures or clips. The transplanted cells could be detected on the eighth day. The wounds that were treated with cell sheets were completely healed at four weeks, in contrast to the control wounds that still were in an intermediate wound healing phase. Similarly, the treated wounds were completely re-epithelialized, while control wounds were not. Further the amount of inflammatory cells in the wounds were considerably higher in the controls[75].

After the promising results from the canine study, Dr Ohki and colleagues aimed to study safety and efficacy of the treatment by an open-label, single-arm and single-institution study[71]. Nine patients received the cell sheet treatment after ESD (**Fig 7**). Cell sheets could be manufactured from the biopsies from each patient. Two to eight cell sheets were transplanted and follow-up were performed by endoscopic evaluation and by dysphagia scoring (Mellow-Pinkas score). The dissected circumference ranged from ½ to "almost whole" (patient 4) (**Fig 8**), and dissected area from 231 mm^2 to 3850 mm^2. Patient 4 developed a stricture that was treated with 21 balloon dilatations, while the remaining patients had a favorable outcome without strictures or dysphagia. The authors conclude that the wound healing was faster than their previous experience (3.5 *vs* 4 weeks, except for patient 4 = 16 weeks). Transplanted cells could be detected one week after transplantation, but this was only evaluated in one patient.

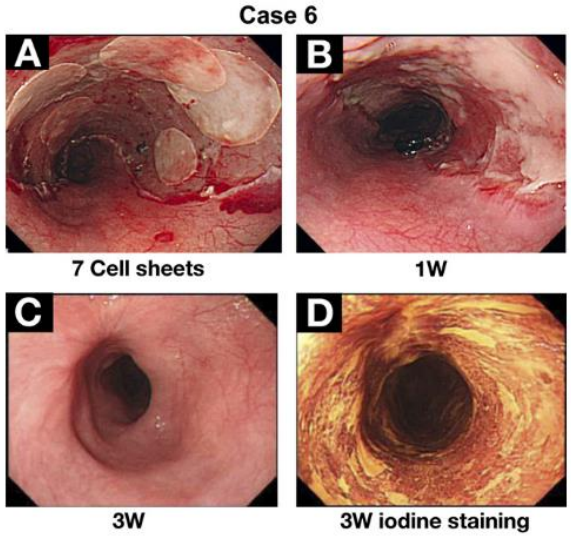

Figure 7 - Cell sheet transplantation, no stricture formation. Reprinted from Gastroenterology 2012. 143(3), Ohki, et al., Prevention of Esophageal Stricture After Endoscopic Submucosal Dissection Using Tissue-Engineered Cell Sheets, 582-588, Copyright (1012), with permission from Elsevier

I joined Tokyo Women's Medical University for a research exchange program in 2010 and was introduced to the esophagus project. In 2011, the transfer of this technology to Karolinska Institutet and Karolinska University Hospital was initiated. The report of the first five patients, described in 2016, is "article 1" of this thesis[76] and will be discussed in detail under chapter 4.

Later, a new collaborative project was started between Nagasaki University Hospital and Tokyo Women's Medical University[6]. The aim was to study the feasibility of air-transportation of biopsy and cell sheets between the two sites, a distance of 1200 kilometer. This technique could enable cell sheet transplantation almost all over Japan, regardless of whether the hospital has the technical capacity to manufacture cell sheets or not. Ten patients were included, cell sheets could successfully be manufactured in Tokyo and transported back to Nagasaki. The authors also found that the cell sheets produced vascular endothelial growth factor (VEGF) and interleukin-12.

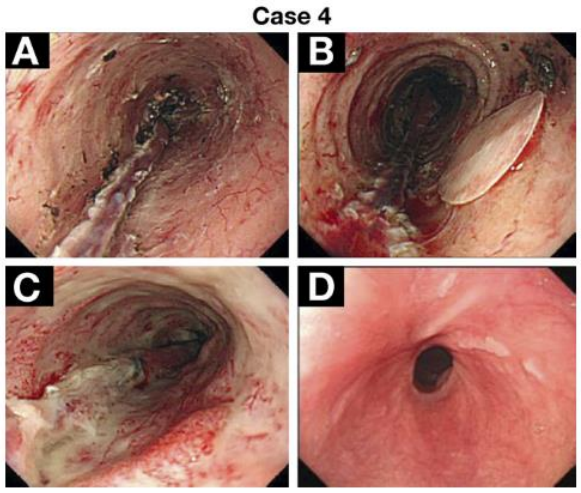

Figure 8 - Stricture formation despite cell sheet transplantation.
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The ESDs were in general larger than the patients transplanted in Tokyo. Circumference ranged from 5/6 of the circumference to complete circumference, and the area ranged from 1350 to 7985 mm^2. Three of the patients also received radiotherapy due to lymphovascular invasion and submucosal invasion (SM2). After one week, all cell sheets could be observed and were accompanied with vascularization. However, after two weeks, the sheets were not detectable. Four of the patients developed post-ESD strictures, requiring a maximum of seven balloon dilatations. Otherwise, no significant adverse effects were observed during the median follow-up time of 105.5 weeks. Although four patients developed strictures, the authors indicate that the risk was lower compared to similar subsets of patients where the incidence was 90%. However, the authors acknowledge that further controlled studies are necessary to verify the efficacy of the treatment compared with corticosteroids.

1.3 EXTRACELLULAR VESICLES

Originally, the main function of extracellular vesicles (EVs), was thought to be waste removal – basically a way for cells to get rid of unwanted cargo[77]. However, in recent years the vast functions of EVs have gained the interest of many researchers. EVs can transport protein, lipids and nucleic acids between cells and are important in both physiological and pathological states[78]. The vesicles are enclosed by a lipid bilayer and include transmembrane proteins[79]. EVs can generally be divided into three main categories, microvesicles, apoptotic bodies and exosomes[80]. Microvesicles are generated by an outward budding of the cell membrane and roughly have a diameter of 50 – 1,000 nm. Apoptotic bodies are larger (500 – 2,000 nm), are also produced by an outward budding of the cell membrane, but in this case from apoptotic cells[80]. Exosomes, on the other hand, are smaller (less than 150 nm), and produced in several steps. First, an inward budding of the cell membrane produces an endosome, and inward budding of that endosome produces multivesicular bodies (MVBs) filled with smaller vesicles (termed exosomes). Once the MVBs fuse with the cell membrane, the exosomes are released into the extracellular space (**Fig 9**).

EVs are highly complex and contain many component including membrane protein, lipids, adhesion molecules, enzymes and nucleic acids[78]. They can transmit signals to target cells through receptor-ligand binding or through absorption by phagocytosis, endocytosis or fusion with cell membrane (**Fig 9**)[79]. EVs can be used in many different fields of biomedical research, from diagnostics[81–85], investigating biological processes[86] and therapeutics[87–90].

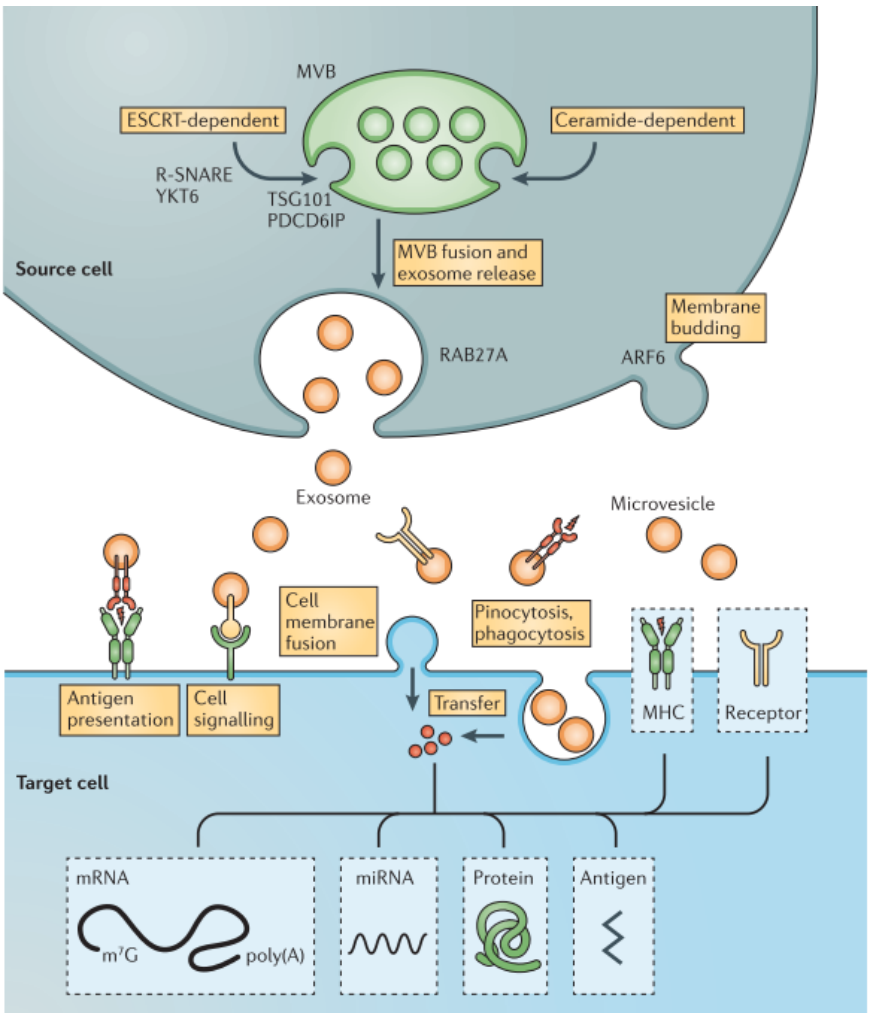

Figure 9 – Extracellular vesicle biogenesis and target cell interaction.
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1.3.1 Clinical applications of extracellular vesicles as therapeutics

The use of exosomes or EVs as therapeutics is still a new field and there are only few clinical examples. Besse and colleagues reported the use of small EVs derived from dendritic cells for end-stage cancer[91], with the aim to boost the patients' T-cell and natural killer cell (NK-cell) immune response. The indication was inoperable non-small cell lung cancer. The primary end-point, progression-free survival 4 months after chemotherapy cessation in 50% of patients, was not met, but the authors conclude that the exosomes could boost the patients' antitumor immunity.

Based on the rationale that MSCs can be used for severe graft-vs-host disease (GVHD), and that this effect likely could be driven by secreted factors, a German group treated one patient with MSC-exosomes[87]. The group isolated exosomes from four different donors and used *in vitro* assays to determine which of the donors' exosomes that were most suitable, based on immunomodulatory and anti-inflammatory properties. The exosomes were administered to the patient and the dose was increased every second day, in total seven administrations were

performed. No adverse effects were found. The patient's pro-inflammatory cytokine response reduced during the treatment course and his GVHD-symptoms improved considerably within two weeks. The patient was stable for several month, but unfortunately passed away seven months after the treatment. Although an interesting case, the results from one single patient have to be carefully interpreted.

2 THESIS AIMS

2.1 PAPER I

The aim of this paper was to evaluate the safety and feasibility of using oral mucosal epithelial cell sheet-transplantation after esophageal ESD in a Western population. The technology was transferred from Tokyo Women's Medical University to Karolinska Institutet and Karolinska University Hospital. Regarding my own contribution to the project, I was assisting in the technology transfer and preparation of regulatory documents. I also studied clinical-grade cell sheets that were not transplanted regarding histological architecture, ECM-composition, viability, cell proliferation *etc*, aiming to further characterize and assess the general health of the transplanted cell sheets.

2.2 PAPER II

The primary aim of this paper was to investigate if culture media from clinical-grade cell sheet production could be used as a source of therapeutic exosomes. Considering that some patients still develop stricture, despite the cell sheet transplantation, we wanted to investigate if these exosomes could have a regenerative effect, and possibly be used in combination with cell sheets. Additionally, we believed that this project could improve our understanding of the mode-of-action of these cell sheets. The isolated exosomes were analyzed *in vitro* and *in vivo*.

2.3 PAPER III

In Paper II, we found that exosomes from the cell sheet cultures had pro-regenerative effects *in vitro* and *in vivo*. However, these exosomes have two origins, they are both derived from the oral keratinocytes and from the *autologous* serum. In this paper we used commercially available cells (gingival epithelial cells) to prepare conditioned medium without serum, thus generating a purer exosome isolate. The aim was to understand more of the exosome-cell interactions.

3 METHODOLOGY

Detailed description of the methods used is available in each paper. Here, I will briefly and discuss key methods used in the papers.

3.1 CELL SHEET PRODUCTION AND QUALITY CONTROL

Oral mucosal biopsies were obtained from patients (paper I) and healthy donors (paper II). The samples were sent, together with *autologous* serum, to cell processing center (Vecura,

Stockholm for paper I, CellSeed Inc, Tokyo for paper II). Cell isolation and cultures were performed under strict, clinical grade conditions. For quality control, detachment tests were performed, and cell numbers, viability and purity were assessed. Endotoxin and microbiological testing was also performed.

3.2 HUMAN DONORS

All five patients in paper I and eight healthy donors in paper II were male, aged 56-70. A more diverse population would have been favorable, or even better, matched controls between male and females, so any gender differences could have been investigated. Hopefully, future studies can shed light on any gender-specific differences.

3.3 EXTRACELLULAR VESICLE ISOLATION

There are various ways to isolate EVs, each with pros and cons. We decided to use a combination of ultrafiltration and size exclusion chromatography. Cells and debris were removed from the conditioned media using centrifugation or 0.22 µm filtration. The media was then concentrated using ultrafiltration, in order to reach a volume that fit the size exclusion chromatography columns (500 µl). These columns are driven by gravity – the sample is loaded onto the columns and will pass through a gel. The gel has small pores (70 nm) wherein smaller molecules will enter, and thus take longer time to pass the gel. Vesicles, which do not enter the pores as easily, will pass through the gels quicker and be diluted in the first fractions. This is an easy and quick method to isolate vesicles from bulk protein. Of course, it is impossible to completely exclude all bulk protein, why a better term probably is "exosome / EV enrichment".

3.3.1 Isolation from cell sheet media

Optimally, serum free media is used to produce the conditioned media. The reason for this is that serum also contains vesicles, meaning that the final isolate will not be purely derived from the cultured cells. In the case of paper I, this was not possible as we had to follow the already established clinical-grade production protocols, which includes 5% *autologous* serum. The isolated exosomes in this project are therefore a mix of cell culture-derived exosomes and serum derived exosomes. This would have been suboptimal if the primary aim of the project was to understand the mode-of-action of the cells. However, our primary aim was to investigate whether "waste-products" from clinical grade cultures could be transformed into a therapeutic agent, so in this case the "less pure" exosomes are acceptable. We also included exosomes from non-conditioned media, entirely derived from the *autologous* serum, to act as a control

3.3.2 Isolation from gingival keratinocytes

For paper III, we performed the cell cultures in our own institute. Unfortunately, we couldn't get the same cells that were used in paper II (from healthy volunteers), as these were used in other experiments. Instead we used commercially available cells derived from gingiva. Although these cells also are derived from oral mucosa, there are differences, for example,

the buccal mucosa has a higher turnover time than other tissues in the oral cavity[92]. How this affects the exosomes is difficult to predict.

3.4 NOMENCLATURE – EXOSOMES OR SMALL EXTRACELLULAR VESICLES

In paper II and III, we refer to the isolated (or enriched) vesicles as exosomes. This was based on a paper in Cell from 2016, which describes exosomes as vesicles with a diameter less than 150 nm. Paper II and III were mainly written during 2018 and in November 2018, the International Society for Extracellular Vesicles published updated guidelines about EV research[93]. In principle, they state that since specific markers for each vesicle subtype does not (yet) exist, researchers should avoid using subtype terms, including "exosomes". In hindsight, it would have been more appropriate to name the vesicles described in paper II and III as "small extracellular vesicles" (sEVs). For consistency, I refer to the vesicles less than 150 nm diameter as exosomes in this thesis.

3.5 SIZE DISTRIBUTION OF EXOSOMES

According to a statement paper from the international society for extracellular vesicles[94], size distribution should be measured using suitable techniques. This hardware was not available at the institute where the research was performed, so we had to rely on collaborators for these analyses. This unfortunately led to the use of two different techniques for paper II and III, which makes comparing the results more complicated. In paper II we used nanoparticle tracking analysis (NTA) which briefly is performed by illuminating the particle suspension with laser and recording the light which is scattered by the particles. Video clips are recorded and it is then possible to calculate the size of the particles based on Brownian motion (the random motion of the particles is depending on the particle size). The NTA also calculates the concentration of particles in the solution.

For paper III we used a different technology, tunable resistive pulse sensing (TRPS). In this technique, the particle solution is passed through a nanoscale pore and the ionic current flow through the pore is recorded. When the particle is passing through the pore, there is a blockage of the ionic current flow, and from this signal, the size, charge and concentration of particles can be calculated.

3.6 ADHESION ASSAY TO ARTIFICIAL ESOPHAGEAL WOUND BED *EX VIVO*

This experiment was performed in order to evaluate the feasibility of the potential to use these exosomes after esophageal ESD. Perhaps the exosome solution could be sprayed onto the wound bed which was not covered by cell sheets. But considering the patients' swallowing of saliva, would the exosomes remain in the intended location? This was the background of this assay where we added labelled exosomes to artificial ulcers in healthy pig esophageal tissue. After pipetting the exosomes onto the wound bed, we let them adhere for 1, 2 or 5 minutes, after which it was completely submerged and washed in PBS, and visualized under a fluorescent dissection microscope.

3.7 SKIN WOUND MODEL

As mentioned previously, the aim of paper II was to investigate if waste product (used / excess culture media) could be transformed into a therapeutic agent to be used in conjunction with cell sheets to prevent esophageal strictures. Naturally, a model where these exosomes were used on actual esophageal wounds would have been logical. So, why did we choose to perform skin wounds instead? There were several reasons. First of all, we had no idea if the hypothesis would hold. Surely, exosomes have been used several times for wound healing[90] and other regenerative medicine applications[95]. But, to our knowledge, exosomes derived from oral mucosal epithelial cells had never been analyzed in similar settings. Secondly, skin wounds can be performed in a more control fashion compared to esophageal wounds. Previous esophageal wounds for cell sheet therapy used large animals, which also represent a greater cost, effort and animal suffering. Additionally, those wounds were much larger than the ones we created in the rats (thus requiring more exosomes), and our supply of exosomes was very limited, as they were derived from donors. For these reasons we decided to continue with a model that was easier to control, and where the wounds could be smaller.

RESULTS

In this chapter I will discuss the findings of each paper in a broader perspective than what's already described in the papers. Paper I describes the safety and feasibility of oral mucosal epithelial cell sheets for stricture prevention. In paper II, we confirmed that exosomes derived from conditioned media from such cell sheets stimulate wound healing, even in a *xenogeneic* setting. In paper III, we described the gingival keratinocyte-derived exosome isolation, uptake and regulation of cell proliferation.

3.8 PAPER I

The cell sheet technology for esophageal application could successfully be transferred to Sweden. From each of the included patients, oral mucosal epithelial cell sheets could be produced, all meeting the defined product specifications. The dissections were 75 – 100% and 5 – 10 cm long, three were due to adenocarcinoma (T1a and T1b) and two were due to Barrett's mucosa with high-grade dysplasia. Cell sheets could successfully be transplanted to all patients. Probe-based confocal laser endomicroscopy (pCLE) indicated that transplanted areas hosted epithelial cells, while non-transplanted areas mostly were covered by fibrin. pCLE also indicated that the cell sheets remained healthy on the transplant site, and that intrapapillary capillary loops (IPCLs) could not be detected in the regenerated mucosa, up to four weeks after ESD. Evaluation of IPCLs has been suggested to be a diagnostic tool for esophageal carcinoma[96], the relevance of their absence in this case, or if they regenerate at a later stage, remains unclear. The mucosal defects healed within a mean time of three weeks and the three patients with 100% circumferential dissections developed strictures. The aim of this project was not to evaluate the efficacy of the treatment, but the clinicians' opinions were that the strictures were easier to treat than what would've been expected.

The evaluations of excess cell sheets consisted of histology, immunohistochemistry, gene expression analysis, functional assays and transmission electron microscopy (TEM). The sheets were evaluated after that they had been transported to the operating theater and found to be excessive. This means a minimum of 30 minutes in room temperature and normal air (compared to 5% CO_2 atmosphere in the incubator). Hence, the status of the sheets better reflected the status of transplanted sheets, compared to sheets being analyzed straight in the production facility. We found that the cells were metabolically active and with few dead cells. The sheets were 3-5 cells thick and proliferative cells were found in the basal part of the sheets, resembling the structure of an epithelium. Compared to fibroblasts, the cells had an upregulation of epithelial marker keratin 18, and pluripotency markers OCT4 and SOX2, while NANOG could not be detected. Immunohistochemistry revealed retained ECM-proteins collagen I and IV, elastin and laminin. Laminin was more strongly expressed in the basal parts, also consistent with a normal epithelium. Connexin 43 and TEM-imaging suggest maintained gap junctions. It would have been interesting to compare the cell sheets to cells harvested though enzymatic treatment such as trypsin, in terms of gap junctions and ECM-proteins, to confirm that these properties are more highly maintained by cell sheet technology.

Pluripotency markers SSEA4, C-kit, Oct-4 and Sox-2 were also identified on protein level.

The biopsies used to isolate the cells are full-thickness mucosal samples, and thus include cells from the basal part of the epithelium. Researchers at TWMU were unsuccessful to produce cell sheets from "swabs" or brushings of the oral mucosa, possibly due to the absence of proliferative cells which are more basal. Other researchers have also reported that the lamina propria of the oral mucosa harbors a stem cell population[97].

3.9 PAPER II

Since some patients still developed stricture despite cell sheet transplantation, we here aimed to transform used or excessive culture media from the cell sheet production, into a therapeutic agent that possibly could be used in combination with cell sheets. We received both unused (non-conditioned) and conditioned media from healthy donors. The yield was approximately 2 µg exosomes / mL and 1 µg exosomes / mL for non-conditioned ("ncExo") and conditioned ("cExo") respectively. The yielded number of particles per milliliter showed the same pattern, but without significant difference, which is likely due to fewer data points (n=3 each). These results were surprising, we expected the opposite results – higher yield in the cExo, as the keratinocytes are releasing exosomes during the culture. It could be explained by that exosomes are internalized and degraded by the cells during the culture, to a higher extent than they produce.

Total protein measurements and gel electrophoresis showed that the majority of bulk-proteins diluted in "non-EV"-fractions from the size-exclusion chromatography. Albumin, the most abundant protein in serum, could effectively be separated from the exosome preparations. Using western blot, we detected two markers that are expected to be enriched in EV-preparations: CD9 and Flotillin-1 and negativity for GRP94, which is a marker for endoplasmic reticulum (common contaminant). We confirmed the morphology by TEM and size distribution by NTA. Taken together, we could effectively isolate exosomes with undetectable levels of contaminants.

We used fibroblasts in our *in vitro* studies because they are key cells in the formation of strictures[98] as well as scars[99]. We also included dexamethasone as a control substance as it is also used clinically to prevent stricture formation of the esophagus[8]. We first studied the proliferation of fibroblasts. Exosomes from both groups effectively reduced cell proliferation, but cExo was in general more effective. We used fraction 12 from the chromatography to test "non-EV-proteins" and found that their effect was considerably lower. Neither cExo nor ncExo showed any cytotoxicity towards the fibroblasts.

Exposure to exosomes from both groups led to a large increase of gene expression for several growth factors relevant for wound healing: hepatocyte growth factor (HGF), vascular endothelial growth factor A (VEGFA), fibroblast growthfactor-2 (FGF2) and connective tissue growth factor (CTGF). cExo gave a higher response in general, but only significantly for HGF. Dexamethasone, did only increase the expression of CTGF. HGF, VEGFA and FGF2 are in general thought of being "pro-regenerative" growth factors. CTGF, on the other

hand, is more complex. It is known to promote fibrosis and can have both pro- and anti-angiogenic effects[100]. Whether the increase of CTGF is an advantage or not in our case remains unknown. The increase of HGF was also confirmed on protein level.

Since cells from the oral mucosa has been shown to have antibacterial properties[101], we investigated if the exosomes could have similar effect. We could see a small, but significant, reduction of *S. aureus* at the 3h time point, and only with cExo, not ncExo.

Next we evaluated if exosomes could adhere to "ESD-ulcers" – we used biopsy punches to create small wounds in the mucosa of porcine esophagus. We found that the exosomes adhered to the tissue very quickly, we could detect a positive signal even after only one minute adhesion. For *in vivo* experiments, we used cutaneous, full-thickness wound healing in immunocompetent rats. Signal from the exosomes could be detected until the sixth day.

We applied two protocols, one where 7.6 µg of exosomes were applied, divided on day 0 and day 1, and a second protocol where 12.5 µg were delivered only on day 0. The animals were sacrificed on day 6. There was no difference between the groups when comparing macroscopic wound areas (measured from macroscopic digital photos). Images of histological sections were analyzed by two blinded, independent researchers, revealing that cExo accelerated wound healing (reduced wound width), especially from protocol 1. ncExo showed no effect using protocol 1, but some reduction from protocol 2. That protocol 1 was more effective, despite a lower total dose, suggest that repeated administration is of higher importance than dose. We proceeded with a 17-day time point for protocol 1 and found that cExo significantly reduced the wound width, while ncExo had no effect. These *in vivo* experiments were done in a *xenogeneic* setting: human-derived exosomes applied to immunocompetent, Sprague-Dawley rats.

In a hypothetic clinical scenario, the yield would not be enough to administer the same dose (7.6 µg / 0.1963 $cm^2 \approx 38.7$ µg / cm^2). Based on Ohki's paper[71], the average area was around 14 cm^2, which would require about 542 µg and based on the yield of approximately 0.96 µg / mL conditioned media, we would need 520 mL media. One cell sheet has 4 mL media, and conditioned media could be collected eight time → 520 / (4 x 8) = 16.25 sheets. That is more than the number of sheets usually produced per patient (which was as low as three sheets for one patient in paper I). However, this is just a hypothetical calculation and perhaps even lower doses could be effective.

3.10 PAPER III

After the promising results from paper II, we here wanted to understand more about the oral keratinocyte derived exosomes. It has been shown that exosomes from mesenchymal stromal cells can stimulate the growth of tumor cells[102,103]. Since we potentially would use the oral keratinocyte after ESD for esophageal cancer, it could be a safety concern, if the exosomes stimulated any residual malignant cells to grow. We also aimed to understand more of the mode-of-action of the cell sheets. In this study, we could get a more pure isolation of exosomes, as we could remove the serum from the media.

We compared exosomes from gingival oral keratinocytes and skin fibroblasts. Buccal keratinocytes (same origin as for paper II), would have been more suitable but we could not acquire such. Measured as µg vesicles produced per million cells per 24h, the yield was higher from keratinocytes compared to fibroblasts. However, when yield was calculated as number of particles released, there was an opposite trend. This suggest that the keratinocyte-derived exosomes (OKex) had more protein per particle compared to fibroblast-derived exosomes (FEx). Both groups showed similar morphology and size distributions. When studying exosome-uptake into fibroblasts or keratinocytes, we could detect signal after as little as 90 minutes, and all groups were positive at the 16 hour time point. The exosomes are small and are not detectable by themselves under light or fluorescent microscope. The dots that are seen in the figures likely represent aggregations of a large amount of exosomes. The uptake is probably even faster than the 90 minutes that we found, just that our methodology is not sensitive enough.

In order to understand more of the mode-of-action of the cell sheets, we used fibroblasts and keratinocytes as target cells – both which are abundant and important in healing of wounds. Both groups of exosomes reduced the proliferation of fibroblasts, and higher dose had a stronger effect. The effect at the highest tested dose was similar to that of dexamethasone. For keratinocytes, the effect was a little bit more complex. OKEx showed an "inversed dose-dependency", where a higher dose had smaller effect than lower doses. FEx suppressed the proliferation more strongly and at similar levels for the tested concentrations.

We also tested two tumor cell lines. For HaCaT-cells, OKEx suppressed the proliferation at doses over 150 ng / mL, while at the other doses there was no significant change compared to control (no exosomes). FEx reduced the proliferation at most tested concentrations (strangely not at 300 ng / mL). Dexamethasone effectively suppressed the proliferation as well. Next we examined TR146, a carcinoma cell line with its primary tumor in the buccal mucosa. OKex reduced the proliferation at 600 ng / mL, and did not affect the proliferation at lower doses. FEx suppressed the proliferation at 75 and 18.75 ng / mL, but had no effect at other doses. Interestingly, for these cells, dexamethasone did not affect the proliferation.

One question regarding this study might be why we didn't use the same concentration of exosomes in the *in vitro* assays, as we used in paper II. The reason for this is that since we anyway didn't know what proportion of the exosomes were derived from the cell sheet compared to the serum, it would anyway be impossible to compare the results.

We believe that the above results, where OKEx often showed similar results to dexamethasone, could indicate one part of the mode-of-action of the cell sheet therapy. The results between the two groups of exosomes were similar, so could we use fibroblast sheets instead? That would possibly not be a good idea, based on the fact that strictures (and scars) show an abundancy of fibroblasts, basically, we aim to prevent excessive amount of fibroblasts in the tissue – transplanting more could defeat the purpose. The findings that OKEx suppressed the proliferation of two tumor cell lines would add to the safety profile of the use of OKEx after ESD, although this should be studied in more tumor cell lines.

4 FUTURE PERSPECTIVES

In paper I, we described the use of *autologous* oral mucosal epithelial cell sheets to reduce the risk of esophageal strictures. The therapy is currently very costly, mainly due to the cell sheet production costs. Steroid treatment, on the other hand, represents a very low cost. Currently, it is not known which is the better therapy, but for cell sheets to become a standard treatment, my guess is that the production cost has to be greatly reduced. To my understanding, the big portion of the costs come from the large amount of required manual labor. Automatization by robotics is currently being developed in Japan and could potentially reduce the costs significantly. The costs can also likely be reduced if the production volume goes up. Another potential idea is to explore the use of *allogeneic* cells, which would reduce the number of necessary steps in the production.

We found in paper II that exosomes derived from conditioned media could stimulate wound healing to a larger extent than exosomes from non-conditioned media. Although we found some differences in our *in vitro* assays, the reason for the differences remain unclear. We are currently planning for proteomic and sequencing studies of these exosomes, which hopefully could shed light on the differences of these two samples.

Another interesting finding was that repeated administration of exosomes was more important than the dose. For future studies, it would be interesting to evaluate some sort of slow-releasing systems for *in vivo* applications, for example based on hydrogels. In our studies, we evaluated the wound healing histologically earliest on day 6. Re-epithalialization had already occurred in all groups. It would be interesting to try a shorter time point. Additionally, it would be interesting to study the histological sections more in detail, in order to evaluate differences in the regeneration of structures such as nerves, blood vessels, skin adnexa etc.

As mentioned under 4.3, several tumor cell lines should be studied, especially esophageal tumor cells. Finally, an esophagus injury model would be very interesting.

5 CONFLICT OF INTEREST

I am main inventor for a patent application submitted by Tokyo Women's Medical University, regarding the use of oral keratinocyte exosomes.

6 ACKNOWLEDGEMENT

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