Salivary gland stem cells:

 *A new therapy for hyposalivation*

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**Index**

**Introduction……………………………………………………………………………………………………………………….……......……2**

**Aim**……………………………………………….………….…………………………………………………………………..…………………..…..**.2**

**Head neck cancer treatment and side effects…………………………….………………….……………………….........…..3**

**Pathophysiology of hyposalivation…………...…………………………………………….....…………………………….……….3**

**Stem cells……………………………………………………………………………………………………………………....…………....……5**

*SGSCs transplantation in animal model…………………………………………………………………………….…………6*

*From animal model to a human model……………………………………………..……………..…………….………..…9*

**Discussion……………………………………………………………………………………………………………………….……….....….…9**

**Future perspectives…………………….…………………………………………………………………………………….………….….11**

**Conclusion…………………………………....…………………………………………………………….………………………….…….…11**

**References………………………………………………………………………………….…………………………………………..…..…..11**

**Introduction**

Cancers of the head and neck (HNC) include cancers of the oral cavity, larynx, pharynx, salivary glands and nasal passages. HNC accounts for about 3% of all cancers in the West. Approximately 500.000 new patients are registered with HNC worldwide every year [Jemal A, 2006]. Tobacco, alcohol, human papillomavirus (HBV) and Epstein Barr Virus (EBV) are the most important risk factors for developing HNC [Sankaranarayanan R, 1998]. When the HNC is found at an early stage, it can be well treated. There are several treatment options, which can be used in combination or as single treatment such as surgery, radiation therapy (RT) and chemotherapy [Dobbs, 1999]. Although eliminating the tumor is the primary goal of cancer treatment, preserving the function of the nearby nerves, organs, and tissues is also very important. Treating HNC is still complicated and side effects can have negative influences on the quality of life of the patients. For example, one of the major side effect of RT is the destruction of the salivary glands near the mouth region. The lining of the mouth is very sensitive to radiation, when treated with RT the salivary glands near the oral mucosa can get damaged, resulting in hyposalivation [Burlage FR, 2001]. Saliva is a clear, slightly acidic, mucoserous exocrine secretion and an important component of the mouth. It keeps the mouth moist and comfortable, helps with chewing, taste, swallowing and the immune components keep theoral gravity healthy and prevent bad breath. In addition, saliva also helps break down carbohydrates with the enzyme ptyalin and it lubricates the passage of food from the pharynx down to the esophagus and stomach [Dawes C et al., 2015]. Hyposalivation causes problems with speech, mastication and swallowing. Moreover, having less saliva also gives other pathological conditions, such as gingivitis or candidiasis. Patients with hyposalivation have a lower quality of life and suffer from their symptoms every day [Ronald L, 1996].

Aim

Currently there is no effective treatment for hyposalivation. Patients who suffer from hyposalivation are treated to relieve them from their symptoms, there is no cure for hyposalivation. The best treatment option would be to replace the destructed salivary glands with functional salivary glands. One potential way to make new salivary glands is by using stem cells. Stem cells can be differentiated in many different cells. If it is possible to differentiate stem cells into salivary glands, then these new salivary glands could be implanted back in the oral cavity and thereby hyposalivation can be cured.

Therefore, the research question of this thesis is: *how can stem cells be differentiated into salivary glands cells for the treatment of hyposalivation?*

In this thesis HNC will be discussed, the treatment for HNC using radiation therapy and the major side effect hyposalivation. The focus will be on how stem cells can be used to treat the hyposalivation. First we discuss the production of saliva, then we go into the pathophysiology of hyposalivation and how this can be treated with stem cell therapy.

**Head neck cancer treatment and side effects**

Treatment of HNCs depend on the location of the tumor, the stage of the cancer and the person's age and general health. The main treatment option is RT and surgery, whereby chemotherapy is often used as an additional treatment [National Cancer Institute].

 RT is particularly used when the tumor is confined to a specific area in the face region. RT uses particles or waves moving at a high frequency to target the DNA of cells in the tissue and change the way they can replicate. By manipulating the replicative ability of the cell, the growth is inhibited so that cells, among them cancer cells, cannot divide that fast anymore [American Cancer Association]. However, this means that RT will damaging *all* the cells that grow, and no distinction is made between cancer cells and healthy growing cells. Stem cells are one of the healthy growing cells and divide whenever new cells are needed in the organ. This means that during cancer treatment stem cells can be damaged as well.

Even the most advanced and highly focused radiation techniques can only spare one, but not all major salivary glands in the head and neck region because salivary glands are close to most head and neck tumors, and thus, the ionizing radiation has to pass through the salivary glands to effectively target the tumor(figure 1). Radiation allowed for exposure of parotid salivary glands must be below a cumulative dose of 24-26 Gy in 2 Gy fractions/day, so that there is space for recovery of salivary function [Yun Li, 2006]. Although there are limits in the radiation doses, and clinicians use try to use the minimal radiation dosage, there is not an ideal dose that would eliminate the cancer cells *and* at the same time prevent damage to the salivary glands.

Therefore, patients who are treated for their HNC suffer from hyposalivation, because one salivary glands are damaged and second the stem cells are lost and therefore no new salivary glands can be formed.

**Pathophysiology of hyposalivation**

The average daily flow of whole saliva varies in healthy persons between 1.0 and 1.5 L. When a person has 50% less saliva production than normal, a pathological condition, called hyposalivation occurs. Hyposalivation is a condition where patients suffer from dry mouth because of salivary gland dysfunction [Ronald L. Ettinger, 1995]. Hyposalivation affects millions patients throughout the world, its prevalence varies between 12% and 30% worldwide. The pathological condition affects mostly women around 65 years [Marta Tanasiewicz, 2016]. The condition has therefore long been considered a problem of aging.

Radiation-induced hyposalivation starts early during treatment: in the first week, a 50% to 60% decrease in salivary flow occurs and after 7 weeks of conventional RT, salivary flow diminishes to approximately 20% [Franzen L, 1992]. Tissues with a rapid turnover rate are more susceptible to RT than tissues with a slow turnover rate. Despite the fact that salivary gland cells turnover is slow, production and quality of saliva decreases after radiation [Lombaert et al., 2008].

The turnover capacity is a mechanism that is adapted for every organ specifically, so that tumor formation is prevented. However, this has consequences for patient who already have cancer and are treated with RT. When patients receive RT /chemotherapy for an abdominal cancer, the damaged cells are replaced because of the fast working stem cells in the gut. But when the patients receives RT in the head neck area, such as by HNC, then the slow turnover of the stem cells here has a negative effect for the patient, as the tissue cannot be replaced. Therefore, patients with excessive salivary gland damage in the head neck region suffer from hyposalivation whereas in some other organs the damaged tissues can be replaced by new tissue formed by stem cells [Pringle, 2016].

 Moreover, there are differences in radiosensitivity among the various types of salivary glands, for example, the submandibular gland is less radiosensitive than the parotid gland [Hall E, 2000]. As a consequence, the larger glands will be damaged first and patients suffer from hyposalivation at an early stage of RT. In addition, the damage depends on how long and what the doses of the radiation is. For example, a study showed that twenty-four hours after radiation exposure with a doses of 5-Gy, approximately 30% of mouse parotid glands went into apoptosis [Humphries MJ, 2006]. So the more patients are exposed to RT, the worse the damage will be.

There is not a right doses of RT to prevent the salivary gland damage. Therefore, as a consequence of HNC treatment, patient will suffer from hyposalivation as a major side effect.



**Figure 1. Anatomy of the salivary glands and the radiation zone.** Showing Sublingual, submaxillary and parotid gland.

**Stem cells**

RT-induced hyposalivation can be permanent [Grundmann et al. 2009]. To produce enough saliva again patients would need new salivary glands. One option would be the transplantation of salivary glands from healthy persons to patients with hyposalivation. However, allogeneic transplantation is not working well, because there is a high risk that the immune system of the host will reject the donor glands. The patients would need to take immunosuppressive medication to counteract the rejection. But, immunosuppressive medication has a lot of other complication such as weakening the immune system and making the patient more prone to infections. A better solution would be to use an autologous therapy. The benefits are that no rejection will occur and patients are not dependent on healthy donors. The most favorable autologous cellular therapy are the stem cells. Stem cells are continuously forming new tissues, while simultaneously renewing themselves through division.

The first stem cell therapy potential came from the embryonic stem cells (ESCs). Here the inner cell mass of an embryo is harvested and expanded in vitro and then differentiated into any cell of the body [Marting, 1981]. However, ESCs comes with a lot of ethical dilemmas, therefore research in this field is not progressing very well. The stem cell therapy made another breakthrough after a few decades, where the induced pluripotent stem cells (iPSCs) were introduced by the group of Thakashi and Yamanak in 2006. Here the researchers showed that mature cells can be rewind to pluripotent stem cells. Unfortunately also this technique has its limitations, safety issues related to teratoma formation still needs to be proven [Noir et al. 2015]. At the moment, adult stem cells are very promising for regenerative medicine. These cells are different from other cell types by two important characteristics. First, they are unspecialized cells and are capable of renewing themselves by each cell division. Secondly, they can be induced to become any tissues or organ, depending on the environment [Bray GA, 2004]. The reason why adult stem cells are preferred above iPSCs for salivary gland regeneration is that there is less chance that a tumor will develop. Also, tumors in salivary glands are uncommon. So therefore, the chance of salivary gland stem cells to become tumorigenic cells is very small.

The best approach to differentiate adult stem is to apply the knowledge about the embryonic development. During embryonic development, cells make decisions that result in specialized cells. These decisions are made by secreting chemicals coming from neighboring cells: direct communication with neighboring cells by cell-cell contact or mechanical strain. All of these signaling interactions lead to changes in gene activity, which determines the cell's differentiation. The same mechanism can be applied to the stem cells when they are harvested and put in a petri dish. To stimulate the embryonic salivary gland development in a petri dish, the following factors are important: growth factors, cell culture substrate, culture environment; inclusive 3D environment and signaling inhibition [U.S. Department of Health and Human Service]. These factors need to be in the right dosage and balance to successfully differentiate SGSCs into salivary glands. An example of one of the most successful stem cell application is the bone marrow transplantation of stem cells in leukemia patients.

The salivary glands stem cells are also adult stem cells. The human salivary gland stem cells (hSGSCs) are normally active when there is damage to the salivary glands and new salivary epithelia cells need to be formed. Patients who are treated with RT have damaged salivary glands and also a reduced amount of SGSCs. To be able to form new salivary epithelia cells, it is important to have abundant numbers of SGSCS who can differentiate into these cells.

Adult stem cells are currently the best cells to use for regenerative medicine. Especially the differentiation of SGSCs in salivary glands are promising in treating hyposalivation.

*SGSCs transplantation in animal models*

Several groups have tried to harvest and differentiate SGSCs into working salivary glands. At this moment the animal models are showing promising results. The first autologous SGSC transplants were performed in mouse and rat models. Table 1 gives an overview of the steps that need to be taken to transplant SGSCs in animal models. In mice SGSCs can be harvested by using specific markers. A few suggested markers are: CD49f, CD29 [Matsumoto et al., 2007] and c-Kit, all which are located in the ducts cells [Lombaert et al., 2008]. It is logical that the stem cell markers are located here, because the SGSCs are in the ductal compartment of the saliva gland. The group of Coppes showed that only the c-Kit marker have stem cell like characteristics. In their study [Lombaert et al., 2008] only the c-Kit positive stem cells were able to rescue salivary gland dysfunction in vivo and show that the c-Kit positive cells have self-renewal capacity. Moreover, next to c-Kit there are currently two other SGCSs markers, Sca-1+and Musashi-1 [Hisatomia, 2004]. But these two markers have not shown great results as c-Kit. Therefore, the best marker right now for finding SGSCs is c-Kit. However, it is not very convenient to use only one marker, because there is a large heterogeneity in stem cells. To exclude other lineages, more than one marker is needed. Therefore, c-Kit together with less optimum markers Sca-1 and Mushasi-1 are used to find the SGSCs. To improve the selective harvesting of SGSCs, better makers, next to c-Kit are needed.



**Table 1.** **Overview of SGSCs transplantation in mouse model.** SGSCs can be transplanted in a mouse model. This procedure contains harvesting, culturing, differentiating and transplanting the SGSCs.

The SGSCs can be isolated from submandibular glands. Submandibular glands are the best salivary gland to isolate SGSCs. These glands have the best anatomy for harvesting the SGSCs, they produce the most saliva during rest and the right saliva composition, making them the best gland for regeneration [Humphrey, 2001]. After harvesting SGSC, the cells are multiplied ass follow: First, the stem cells are harvested by surgical removal of the salivary glands. Than the SGCS are sorted by using the right markers. These cells are then put in a DMEM/Ham's F12 medium containing EGF, FGF-2, N2 and insulin, with hyaluronidase and collagenase. The content of the medium will provide what is needed for the SGSCs to survive and grow (Figure 2). Once the SGSCs multiply, they are called spheres. Also the spheres need to have the characteristics of SGSCs before transplantation, meaning that they must be c-Kit, Sca-1, and Musashi-1 positive.



**Figure 2: Culturing stem cells over a period of 10 days**. Submandibular gland stem cells dividing over a period of 10 days (D0 to D10). BrdU staining in brown, nuclei in blue. The SGSCs in the medium are BrdU positive, indicating that the cells are proliferating. Also notice that the cells are increasing in size. Scale bar = 20 micrometer [Lombaert et al, 2008].

After the spheres have formed, they can be differentiated into acinar and duct cells. The group of Coppes (Lombaert et al. 2008) showed with an immunohistochemical analyses that the spheres can indeed be differentiate into acinar and ductal cells (figure 3). At day one (D0) the acinar and ductal cells were seen separately, with time the acinar and duct cell grew into each other (figure 3, D10) and a morphology similar to salivary gland was seen (D10). Also noticeable is that the SGSCs markers were diminishing over time. This means that the SGSC are differentiating and therefore are losing their stem cell characteristics.

To test the functional of the differentiated spheres, the group focused on the key enzymes that should be secreted by salivary glands, such as amylase. They used immunohistochemistry and RT-PCR to see if key enzymes were secreted and indeed the results were positive for amylase. Showing that the spheres were indeed functional. However, the study did not look for other key factors of functional salivary glands also produce, such as antimicrobial agents or glycoproteins.

So far, the SGSCs can be harvested from mice and are showing stem cells properties. The SGSCs are able to differentiate into spheres with characteristics of salivary glands in vitro.



**Figure 3: c-Kit expression in SGSCs.** Submandibular salivary glands growing from stem cells are c-Kit positive (day 0). Over time the c-Kit marker decreases (less green, day 10). Green indicates ductal cells and blue acinar cells. D=ductal cells, AC=acinar cells. Scale bar = 20 micrometer [Lombaert et al, 2008].

The in vitro results were positive, next step was to see if they would differentiated into salivary glands in vivo. To do so, RT damage was mimicked in mice. First, the head neck part of the mice were irradiated by 15 Gy, damaging the salivary glands. Indeed, after 90 days there was an expected decline in acinar functioning. Then the pre-cultured spheres were injected via direct intraglandular injection in the salivary gland area of the irradiated mice. PAS staining was used to see if the acinar cells were formed and proliferating cell nuclear antigen (PCNA) is used to see if there is proliferation of the stem cells. The results showed that after transplantation, the PAS staining and PCNA were both positive. In control mice, who were irradiated but not treated with the stem cells, the positive staining was not seen. Therefore, the research group concluded that the transplanted spheres were differentiatedinto salivary glands.

The group showed that the salivary glands were differentiated, but the most important part is that the salivary glands work properly. Therefore, the group examined the function of the newly formed salivary glands by measuring the amount of saliva production in the mice. They compared the saliva production in irradiated mice with SGSC treatment, irradiated mice without treatment and healthy mice. The results showed that after stimulation of saliva with pilocarpine, the irradiated mice with treatment had 70% restored salivary production in comparison to control group. Also the morphology of salivary glands was improved in comparison to the irradiated mice without treatment [Lombaert et al, 2008, Nanduri LS, 2013].

 Factors that help SGSC to proliferate are still being searched for. Recently, the group of Coppes has shown that Wnt-signaling also plays a role in stem cells proliferation [Maimets et al. 2016]. Wnt-signaling regulates aspects of cell faith determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development [Logan and Nusse, 2004]. Looking back at the embryology, Wnt-signaling together with beta-catenin expression regulates the epithelial cell growth. Epithelial cells are epithelial cell adhesion molecule (EpCAM) positive, which means that EpCAM can be used as a marker to find epithelial cells. It is known that salivary glands are made of epithelial cells and EpCAM can be a marker to find salivary glands. Pingle et al. found a higherEpCAM expression in ductal cells and lower EpCAM expression in acinar cells. Therefore, a high EpCAM expression is correlated with ductal cells in salivary glands. The transcription of EpCAM is activated with the Wnt/beta-catenin signaling [Maimets, 2016]. The group of Coppes hypothesized correctly, that Wnt/ beta-catenin pathway plays a role in the development of salivary glands. The researchers used a Wnt-agonist and a Wnt-antagonist to observe the effect of Wnt-signaling on the isolated SGSCs. The results showed that the EpCAM high positive cells responded to the Wnt proteins and the cells started to self-renew and to expand. However, when a Wnt inhibitor was used, the expansion of the stem cells was not sufficient [Maimets, 2016, Pingle et al. 2013]. Meaning that Wnt-signaling is important for the salivary glands proliferation.

 The group also tested this in vivo. They pre-treated stem cells with Wnt agonist and transplanted them into the irradiated mice. The result was a restoration of saliva secretion and an increase in functional salivary glands. So the Wnt agonist is a growth factor that has a beneficial influence on the growth of the SGSC in mouse models. Therefore, the researchers conclude that the Wnt proteins are a requirement for SGSC self-renewal and long-term expansion in cultures [Maimets et al, 2016].

*From animal model to a human model*

Stem cell therapy can only be performed when a patients come to the physician *before* damage has occurred in the salivary glands. This means that the patients need to donate their SGSCs before they are treated for their HNC. After the patients have finished the HNC treatment, their SGSCs can be replaced back. The SGSCs will than differentiate into a new salivary gland.

To continue with hSGSCs, the group of Coppes focused on isolating human SGSC and differentiating them into salivary glands. First they isolated hSGSCs from healthy individuals and expanded and differentiated them in vitro. Secondly, they tested its function by transplanting the hSGSCs into a mouse model.

After harvesting the stem cells by biopsies from healthy persons and culturing the cells, the self-renewal and differentiation capacity of SGSCs were observed. The self-renewal capacity was seen after the primary culture was divided into single cells. The single cells were able to form secondary spheres. This could repeat itself more than 10 times, showing that the hSGSCs were able to self-renew. To see if the hSGSCs were also able to differentiate into salivary glands, specific markers for each cell type was used, such as cytokeratin as a marker for ductal cell, alpha-amylase as a marker for salivary enzyme production and aquaporins-5 as marker for water channel proteins. Also here the culture was stained positive for the markers, meaning that hSGSCs are showing salivary gland characteristics.

After showing that the human stem cells were able to self-renew and differentiate in vitro the next step was to test its functional. To test this hSCSCs were transplanted by xenotransplantation in mice. First the mice were irradiated with 15 Gy in the neck region, which damaged the salivary glands. One month post-irradiation, the differentiated human stem cells were transplanted in the mice. The results are promising, the treated mice had a better saliva production and showed a morphology similar to that of salivary glands. In addition, the stem cells markers could still be identified as well. The group showed that hSGSCs are able to self-renew and differentiate in vitro and in vivo.

Jeong et al. also worked with human SGSC, but this group transplanted the stem cells in a rat model. The results of this study was that the acinar and duct cell structure were restored, and a decrease in the amount of apoptotic cells was seen. It can therefore be stated that human salivary glands are capable of differentiation in both mice and rats models [Jeong et al., 2013].

**Discussion**

The main question of this thesis is: *How can stem cells be differentiated into salivary glands cells for the treatment of hyposalivation?* To differentiate SGSCs into salivary glands, markers such as c-Kit are needed to find the SGSCs. than the SGSCs are differentiated into salivary glands by using factors as Wnt-signaling. When the SGSCs can have multiplied in vitro they can be transplanted back to the patient. Great results are already shown with mice and rat models and xenotransplantation of human differentiated salivary glands in mice.

The main findings in the field were made by the group of Coppes. They showed that mouse salivary gland contains stem cells and that these stem cells were able to differentiate into salivary glands. The replacement of the damaged salivary glands with stem cells was successful and the differentiated salivary glands showed good functionality and morphology [Lombaert et al., 2008]. However, they measured the functionality by measuring the whole saliva production for *all* the salivary glands but the group only transplanted the submandibular glands. They treated the mice with RT, but that does not mean that all the salivary glands were damaged. It is therefore not clear if the measured saliva comes from the transplanted glands or from a combination of the other, still functioning, salivary glands. A correct way to test the saliva production of the transplanted salivary glands would be stopping the other working glands and then measuring the production of saliva.

The group of Coppes also used xenotransplantation as a method to test whether hSGSCs are capable to differentiate and function in vivo. So far good results have been achieved in the differentiation and functionality of hSGSCs in the mouse model. However, xenotransplantation has its own limitations, because the mouse used is not a t representative model for human use. The mouse used were immunosuppressed, while human have an intact immuunsystem, which can interfere with the transplantation of the differentiated salivary glands. Also other factors could play a role which are not seen in the mouse model. T. An Ideal way to test the function of the harvested hSGSCs would be to transplant it back to the patients. Because than the reaction to the hSGSCs can be observed directly. However, transplanting the hSGSCs directly back to humans is ethical not correct, because it is in a trial stage, meaning that potential risks are not foreseen and the health of the patient could be affected. This is one of the limitation that makes it difficult to transplant SGSCs directly back in patients.

Taking the results of the studies together, so far no group has performed a prospective study to determine how effective the salivary glands work [Lombaert et al, 2008, Jeong et al., 2013, Maimets et al, 2016, Nanduri LS, 2013]. The groups focused on how the SGSCs worked in the short term. However, it is possible that the differentiated glands work differently over a longer period. The studies would be more promising if the research groups performed a follow upto see if the differentiated salivary glands are still producing saliva and whether the saliva composition is the right quality.

Another point is that all studies used submandibular gland SGSC because these glands produce the right composition of saliva to counteract hyposalivation. However, during the RT all salivary glands can be damaged and every gland has its own specialty. It is therefore interesting to see whether the stem cells of different glands also can be differentiated into salivary glands. And whether hyposalivation could be treated more effectively when transplanted by a combination of SGSCs from different salivary glands.

A limitation in the studies is that not all factors that play a role in harvesting and differentiation process of salivary glands are found. Now Xiao et al showed that the glial cell line–derived neurotropic factor (GDNF) also plays a role in saliva production in vivo and spheres formation in vitro. This group suggest that next to the Wnt- pathway the GDNF pathway may have potential therapeutic benefit for management of RT-induced hyposalivation [[Xiao](https://www.ncbi.nlm.nih.gov/pubmed/?term=Xiao%20N%5BAuthor%5D&cauthor=true&cauthor_uid=25036711), 2014]. To specify and improve the differentiation of SGSCs into salivary glands all factors that play a role need to be found, which is not the case at the moment.

A down side of SGSC therapy is that therapy is based only for RT induced hyposalivation. Other patients who also suffer from hyposalivation such as Sjogren’s syndrome patients or the very elderly are not benefiting from this therapy. Because these big group of patients lack SGSC the cells cannot be harvested. To make this treatment also possible for these patient allogeneic transplantation is needed. But these options have other limitation, such as graft-versus-host disease.

**Future perspectives**

More research in finding the right signaling for the differentiation of SGSCs into salivary glands is needed. As mentioned before, Wnt-signaling and GDNF are important factors for the differentiation process into salivary glands. It would be interesting to also look at other growth factors as well, such as platelet derived growth factor or fibroblast growth factor and their influence on the salivary gland differentiation. These growth factors may also have an influences on the expansion or growth of SGSC [Maimets et al., 2016].

 Lastly, clinical trials are needed before this therapy can be introduced to patients. It is already known how to harvest hSGSCs and how to culture those [Feng et al. 2009]. And xenotransplantation is successful. The next logical step would be to transplant the previously harvested hSGSCs back to the patients after they success their HNC treatment. Promising result are underway, as the Netherlands is currently progressing towards a clinical phase 1 trial for transplanting autologous hSGSCs as a therapy.

**Conclusion**

Salivary gland stem cells are successfully differentiated into salivary glands in animal models. However, there are improvement points in harvesting the salivary glands stem cells and in the differentiation into salivary glands.

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