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Regeneration of irradiated salivary glands by stem cell therapy

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CHAPTER 1

General Introduction and Aim of the Thesis

INTRODUCTION

1. HEAD AND NECK CANCER

Three percent of all malignancies are head and neck cancer, with yearly more than ~40,000 new patients diagnosed in the United States and 500,000 world-wide [1]. Head and neck cancer comprise neoplasms (i.e. abnormal growth) that may occur in structures such as the lip, oral cavity, tongue, pharynx, larynx, nasal cavity, sinuses, ear, orbit, skull base, and salivary glands. The majority of the neoplasms are squamous cell carcinomas (80%), and less common adenocarcinomas, adenoid cystic carcinomas, melanomas, and lymphomas. As the primary factor for squamous cell carcinoma is tobacco use [2], there is strong concern that the incidence will increase, especially in the younger smoking population.

Curative treatment, directed towards elimination of the primary tumor and any neck nodal metastases, includes surgery and radiotherapy, often in combination. Chemotherapy is mainly used concomitant to radiotherapy for curative intents. Prognosis for head and neck cancers varies considerably according to the anatomical site and the stage of the tumor, with 5-year survival rates up to 40-50% [1].

2. TREATMENT-RELATED TOXICITIES

Two main types of injury, namely face malformation and organ dysfunction, may occur in head and neck cancer as a direct consequence of tumor growth or as a result from the treatment.

For radiotherapy, the dose-limiting factor is the sensitivity of normal tissues lying in the radiation field. These include salivary glands, spinal cord, skin, bone, and oral mucosa. To maximally spare the normal tissue, radiotherapy is conventionally given in daily fractions of 1.8 to 2 Gray (Gy), up to total doses of 66-70 Gy over 6-7 weeks. Still, acute and late side effects are caused in virtually all patients. Acute toxicity develops within the first weeks of radiotherapy treatment, and includes mucositis, dermatitis and xerostomia (= dry mouth syndrome). The latter can result from exposure of the salivary glands to radiation, which may result in progressive loss of saliva flow rate and alteration of saliva composition from thin (neutral pH) to a thick acidic secretion during and after the course of radiotherapy [3]. Xerostomia burdens the patient with oral dryness or pain, dental caries, reduced taste and smell, increased risk for oral infections, hampered speech, and problems with food mastication [3,4]. In 64% of all cases these symptoms persist during the life-time of the patient [5]. This defines xerostomia as one of the most serious radiation-induced side-effects.

Thus, although the damage to the salivary glands and subsequent consequences *per se* are not life-threatening, the undesirable side effects of head and neck cancer treatment may severely reduce the quality of life, and pose a new health problem for the patients. Therefore, both treatment and prevention of xerostomia are of major importance.

3. TREATMENT AND PREVENTION OF XEROSTOMIA

Current treatment of xerostomia include stringent oral hygiene with fluoride agents and antimicrobials to prevent dental caries and oral infections, saliva substitutes to relieve symptoms, and sialogogues to stimulate saliva production. Many salivary substitutes have been developed so far, containing ingredients that duplicate saliva properties, but do not entirely replace the complex substance of saliva. They may relieve symptoms to some extent [6], but in most cases are found unsatisfactory or just equally useful as water. Moreover, they do not exclude the need for antibacterial mouthwashes.

Post-irradiation administration of pilocarpine hydrochloride, a sialogogue, may reduce the effect of radiation-induced xerostomia in some patients [7]. This drug stimulates saliva secretion by acting on the muscarinic receptor (parasympathetic stimulation), but requires some residual function of the salivary gland in order to be working optimally. Although pilocarpine may produce some symptomatic improvement, it may not be adequate enough and can cause additional side-effects such as excessive sweating (diaphoresis), and in some cases pancreas secretion, urinary frequency problems, lacrimation and rhinitis [8].

Since clinical management of xerostomia is rarely effective, prevention is paramount. Several strategies have been developed to reduce the radiation-induced hyposalivation without compromising tumor treatment. These include sparing of tissue with state of the art techniques as IMRT (Intensity Modulated Radiation Therapy), surgical salivary gland transfer and the use of cytoprotectants.

As the extent of radiation-induced damage depends on the exposed volume of the tissue and the delivered radiation dose [9], IMRT has been rapidly implemented in head and neck cancer treatment. IMRT, when compared with conventional radiotherapy, delivers more high conformal dose to the tumor with minimum exposure of the normal tissue [10]. Despite promising outcomes, IMRT is not applicable for all patients, for example in those with tumors that originate from the midline or cross the midline, or in patients with contralateral neck lymph node metastasis [11]. Thus, IMRT can offer a solution for gland dysfunction to certain cases, but there is still need for other strategies.

Another approach to protect normal tissue against radiation/chemotherapy-induced damage is the agent Amifostine (WR-2721, Ethyol®) [12]. This potential radioprotector rapidly enters the bloodstream, and is converted to its active form (WR-1065) through hydrolyzation by the alkaline phosphatases of the endothelium. It then acts as a potent scavenger against free radicals, thereby reducing radiation-induced DNA damage. Protection of tumor tissue is considered to be avoided as its endothelium lacks expression of the alkaline phosphatase [13]. Studies have shown beneficial effects of Amifostine with a reduction of acute xerostomia from 78% to 51% [14] up to 2 years after treatment [15]. Important issues, however, are the toxicity of Amifostine with common side effects (nausea and emesis (i.e. vomiting)), and the high costs and intensive labor may suggest the relevance of other prevention treatments.

Prophylactic pilocarpine administration may also significantly protect against radiation-induced hyposalivation. However, in rats, its beneficial effect has been shown to diminish with dose and time after radiation, when salivary glands are completely in the radiation field [16]. However, both non-irradiated and irradiation-surviving cells are stimulated to proliferate after pilocarpine administration which may be clinically relevant, especially after unilateral gland irradiation. It was found that pilocarpine elicits a compensatory response in the non-irradiated part of the gland, resulting in a gain of saliva production that was related to the extent of damage inflicted to the irradiated gland [17,18]. Therefore, pilocarpine could be of clinical interest in a specific dose range and irradiation volume [19]. Further research will be directed to determine this exact range in humans.

It has been reported that tissue may be spared by surgical transfer of one submandibular gland to a position outside the radiation field [20,21]. This may only be practicable in patients who will receive post-operative radiotherapy, as the transfer is performed as part of the surgical intervention. Despite the success of the transfer (83% had no or minimal xerostomia) [22], it is not straightforward to predict in which patients this might be applicable in the future.

To date, the above described approaches are insufficient and not applicable to all patients. Therefore, other strategies are urgently needed. However, to optimally develop new therapies to counteract radiation-induced side-effects, biological insight of the salivary gland is needed.

4. PHYSIOLOGY OF THE SALIVARY GLAND

Saliva is produced in and secreted by salivary glands. Most saliva is produced by the three major glands, the glandulae submandibularis (~65%), glandulae parotidae (~23%) and the glandulae sublingualis (~4%); in humans situated in respectively, the cheek, ear, and tongue area (Fig. 1). Additionally, hundreds of minor salivary glands are randomly localized in the mucosa of the mouth and produce ~8% of the total saliva volume [23].

Saliva secretion is primarily controlled by the autonomic nervous system. Both (ortho)sympathetic and parasympathetic nerve systems regulate the secretion by the salivary gland. Parasympathetic stimulation (via the neurotransmitter acetylcholine that binds muscarinic receptors) is the main stimulus and may induce high volumes of aqueous saliva. The neurotransmitter noradrenaline (sympathetic nerve system) induces some saliva flow, but is mainly in control of protein secretion [24]. Both protein content of the saliva, size of acinar cells, gland weight and saliva production are decreased after denervation, underlining the role of nerve control in maintaining gland structure and function [25]. Further, physiological factors like age [26] and gender [27] affect salivary flow rate and composition. A fasting diet results in decreasing saliva flow [28], and excessive masticatory stimulation (like sugar-less gum chewing) not only increases the saliva output, but also the pH and buffer capacity of the saliva, which may help to reduce plaque acidogenicity [29].
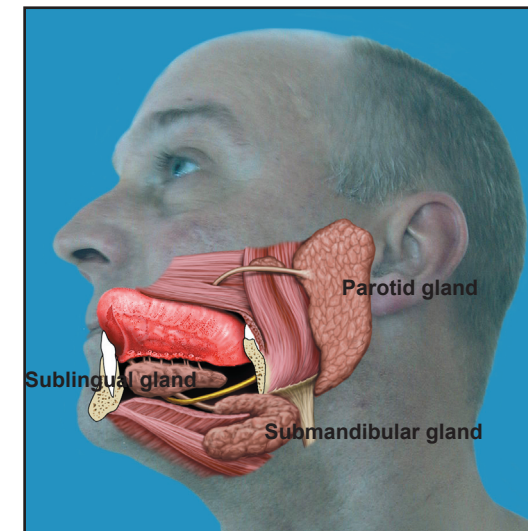

FIGURE 1. LOCALIZATION OF HUMAN SALIVARY GLANDS. Modified from: Stegenga, B., Vissink, A., de Bont, L. Mondziekten en Kaakchirurgie, Chapter 8. Van Gorcum, The Netherlands (2000).

Saliva, of which a healthy person produces about 1.5L per day, consists mainly of water, ions and proteins. It aids speech and swallowing (by continuous flow), and initiates the digestion process of food (by amylase). Protection of the oral mucosa is provided by components such as mucins (MUC5B and MUC7) and secretory IgA, as well as histatins and agglutinin. Other components protect the teeth (e.g. praline rich proteins, statherins, calcium, phosphate), have essential antibacterial (lysozyme, lactoferrin, IgA, lactoperoxidase-thiocyanate) or buffering functions (sodium, potassium, growth factors EGF and NGF) for protection and cleansing [30].

The basic secretory units of the salivary glands are pyramidal shaped acinar cells (Fig. 2). Each type of gland has specific acinar cells which are classified according to the proteins secreted. Human parotid glands almost entirely consist of serous acinar cells, secreting a serous watery fluid, while the sublingual gland mainly contains mucous acinar cells (mucus-rich saliva). The submandibular gland is a mixed gland with predominantly serous cells and some mucous cells. The primary saliva produced by acinar cells is secreted into a small lumen of a cluster of acinar cells, the acinus (Fig. 2). The saliva subsequently flows into a lumen surrounded by intercalated duct cells that coalesce into striated ducts and finally into columnar excretory (intralobular) ducts. These ducts further process the saliva and modify the electrolyte content by removal of sodium chloride and addition of potassium and biocarbonate [31]. Eventually, a single large (interlobular) excretory duct, referred to as Wharton's duct (submandibular gland), Stenson's duct (parotid gland), or Bartholin's duct (sublingual gland), transports the saliva into the oral cavity.

Apart from the above mentioned parenchymal cells, also myoepithelial cells comprise an important part of the gland (Fig. 2). Their long cytoplasmic extensions envelop both acinar cells (mostly in submandibularis) and intercalated duct cells (parotid and submandibularis) [32], and less extensively the striated duct cells. Consequently, nerve-mediated myoepithelial cell contractions increase pressure of the underlying cell, resulting in saliva release into the duct lumens [33]. Several of the grape-like acini and ducts are held together in lobules (secretory units), which are separated by connective tissue (interlobular stroma). This stroma is filled with capillaries, larger blood vessels, fibroblasts, small nerve fibres bundles, and lymphocytes.

As diets vary from one species to another, also the composition of acinar cells in the glands differs between rodents, which are mostly used in research, and mammals. Rodent submandibular and sublingual glands are exclusively composed of mucous cells, while the parotid gland contains exclusively acinar cells of the serous type. Furthermore, an extra ductal cell type, the granular convoluted tubule ductal cell (GCT), is positioned between intercalated and striated duct cells.

This cell type is under hormonal control and more abundantly present in males than in females. While most of the ductal cells secrete only small amounts of proteins, these GCTs are packed with additional kallikreins, serous granules and growth factors [34].
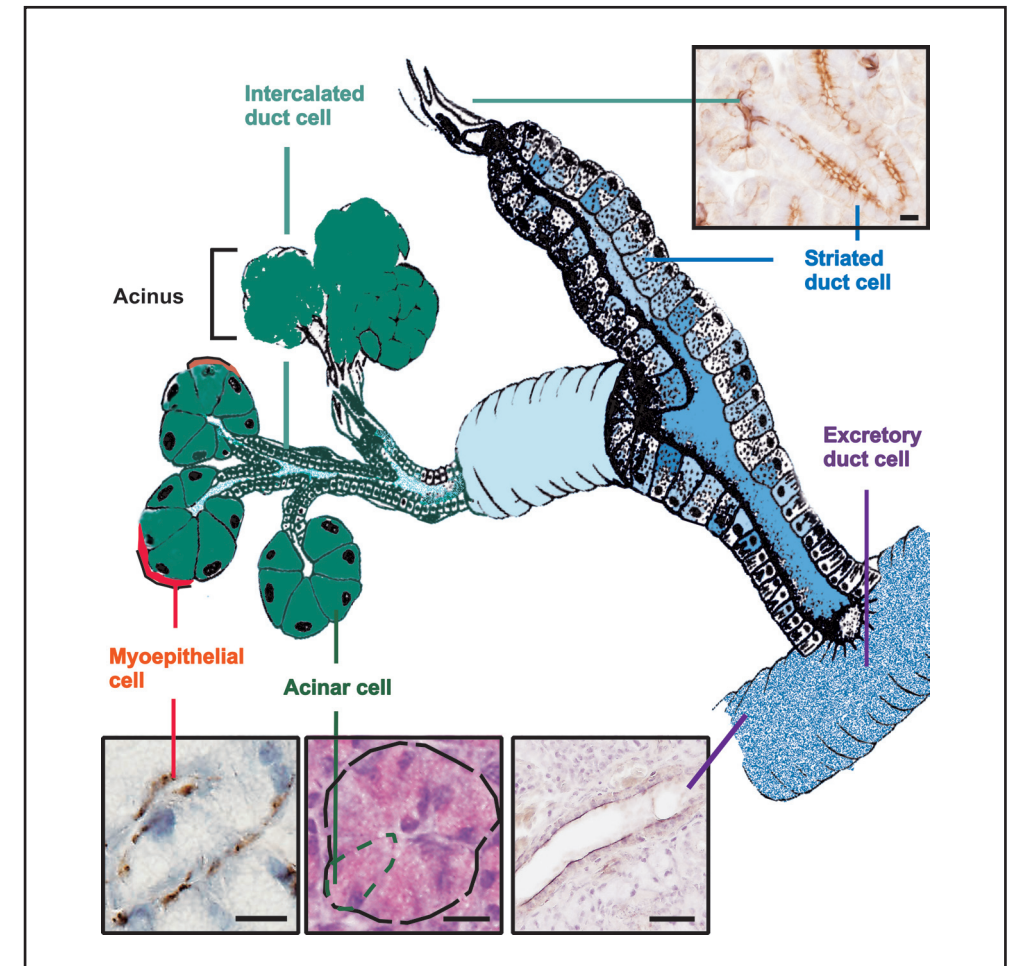
FIGURE 2. SCHEMATIC REPRESENTATION OF THE SALIVARY GLAND MORPHOLOGY. Primary saliva secreting acinar cells are clustered in an acinus, which transports the saliva towards intercalated ducts, subsequently to striated ducts and finally to excretory ducts. Contraction of myoepithelial cells, which surround acinar and duct cells, results in release of saliva into the oral cavity. Inserts represent morphological pictures stained with antibodies directed against alpha-smooth muscle actin (myoepithelial cell), c-Kit (excretory duct cell), CK 8 (duct cells), and a Periodic Acid Schiff's base staining (acinar cell). Scale bar, upper panel = 50 µm, lower panel = 20 µm. (Picture modified from Robert A. Freitas Jr., Nanomedicine, Volume I: Basic Capabilities, Landes Bioscience, Georgetown, TX, 1999).

5. PATHOPHYSIOLOGY OF THE IRRADIATED SALIVARY GLAND

The acute radiation response and high radiosensitivity of salivary glands is rather peculiar. Classically, tissues with slow tissue turnover are late responding [35,36]. Nonetheless, although the saliva secreting acinar cells are highly differentiated and have a slow turnover rate (~60 days) [37], they acutely respond to radiation as the saliva production is already reduced within the first 24 hours after irradiation [38].

Shortly after irradiation, several morphological changes have been described in the glands of rats [39-41], mice [42], primates [43], and humans [44], including cell death, acinar and GCT cell degranulation, cytoplasmic vacuolization of acinar cells and pyknotic nuclei. Serous cells (abundant in the parotid gland) even appeared to be more affected by radiation than mucous cells, while ductal cell types seemed to be relatively radioresistant [45].

Some twenty years ago, the degranulation hypothesis was presented by Abok et al. as an explanation for this acute response [39,46,47]. Membranes of cells, enclosing organelles rich in heavy metals (Zn, Mn, and Fe), could be subjected to radiation-induced lipid peroxidation, evoked by a metal-catalization process. As a consequence of this membrane damage, leakage of granules containing proteolytic enzymes would evoke immediate cell lysis, causing the acute response [39]. This explanation, however, cannot be the sole cause. Firstly since proteolytic enzymes are exclusively present in GCT cells, and secondly because no significant cell lysis (i.e. loss) was observed in rat glands after clinical relevant doses [48]. Another suggestion for the acute response proposed by Stephens et al. included cell apoptosis. Although significant apoptosis was described in rhesus monkey salivary glands [43], this could no be verified in rats [48].

The mechanism behind the acute response seemed to be more complicated, as further research revealed a radiation-induced hampered excretion function in the acinar cells [49]. The receptor-mediated signalling pathway regulating water excretion was abrogated by radiation-induced membrane damage. Both a defect in calcium mobilization and the translocation of Protein Kinase C to the membrane, two processes which are involved in water movement, lead to acute gland hyposalivation [49] and sticky saliva (i.e. initial loss of water content relative to mucins). Moreover, using scintigraphy in patients, it was observed that early after radiotherapy the trapping of technetium-pertechnetate was not affected, whereas the saliva excretion was severely reduced [50]. This finding indicated that the gland volume remained more or less intact, while the excretory function was impaired, confirming that a loss of secretory function of the salivary gland cell and not per se cell death may underlie the early radiation-damage to salivary glands.

Consequently, four phases of radiation damage have been proposed in rats; an acute phase (0-10 days post-irradiation) with no significant net loss in acinar cells, but rather a decrease in function of the acinar cells which leads to impaired water excretion [16,49,51], a second phase (10-60 days) with significant reduction in acinar cells and amylase secretion, which does correspond with the tissue turn-over time [16,42], a third phase (60-120 days) in which damage is stabilized (Fig. 3), followed by a fourth phase where both acinar cell number and gland function further deteriorate (120-240 days) [16]. In mice, this late phase already occurs at day 90-120 days post-irradiation and the third phase seems absent (Fig. 3), indicating that the time taken for each phase may be species dependent. For the late phases, the lack of replenishment of functional acinar cells is thought to be due to the radiation-induced sterilization of gland stem cells.

In conclusion, although saliva substitutes, sialogogues, and salivary gland-sparing techniques are important to reduce radiation-induced hyposalivation, the ultimate replacement of stem cells in the irradiated gland by stem cell therapy would be the only potential way to fully restore tissue homeostasis in the damaged gland. The potential of stem cell therapy to reduce radiation-induced damage to the salivary glands will be evaluated in this thesis.
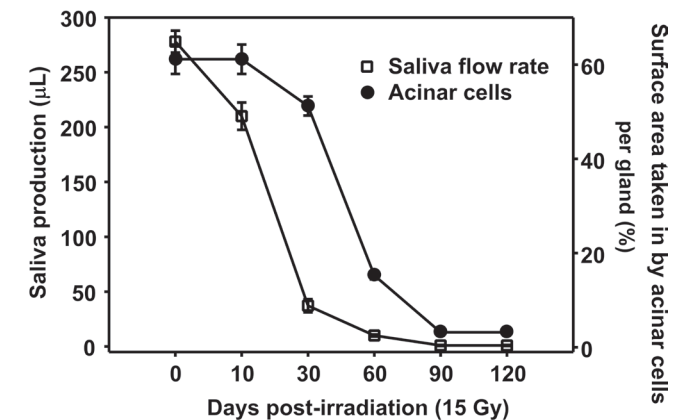

FIGURE 3. FUNCTION AND ACINAR MORPHOLOGY OF IRRADIATED MOUSE SALIVARY GLANDS. Radiation evokes acute salivary gland dysfunction within the first days post-radiation. Later in time, saliva flow rate progressively decreases. In contrast, acinar cells disappear well after flow rate has dropped (~30 days post-radiation).

6. THE POTENTIAL OF STEM CELL THERAPY

Stem cell therapies are currently investigated for their potential to treat a vast array of clinical disorders. Stem cells are considered to possess the capacity to self-renew and to produce more differentiated cells. To accomplish this, asymmetric cell division occurs, by which a stem cell divides to generate one daughter which remains a stem cell and one progenitor cell that will further differentiate (Fig. 4). Subsequent transition of these progenitor cells, also called transit-amplifying cells, towards mature cell lineages may involve amplification of progeny (restrictive division). This phenomenon is particularly observed during (post)-embryonic development [52], and during tissue regeneration after injury [53]. However, assymetric cell divisions do not allow stem cells to expand in number. To achieve expansion, stem cells can also divide symmetrically, i.e. a stem cell gives rise to two identical daughter cells, both with stem cell properties. The balance between symmetric and asymmetric divisions depends on the developmental stage of the tissue and on environmental signals [54]. To retain long-term organ repair both stem cell maintenance and the formation of differentiated cells is required. For clinical therapies, it is critical to transplant stem cells and not progenitor cells. Despite the latter being able to form differentiated cells, organ repair will only be temporarily restored as the progenitors are not able to self-renew.

Two stem cell types are currently investigated for their potential use in therapy: embryonic stem (ES) cells and adult stem cells. Embryonic stem cells, derived from the inner cell mass of the blastocyst, differentiate into all cell lineages of a living organism (i.e. are truly pluripotent) (Fig. 5). Potentially, they are a virtually unlimited source of cells for stem cell-based therapy, but in practice these cells have not been yet successfully used in clinical trials. Despite their pluripotency, it is still difficult to regulate their proliferation and differentiation *in vitro* or *in vivo*. As ES cell-based therapy will inevitably be in an allogenic setting, these cells face a substantial risk of immune rejection *in vivo*, which can potentially be circumvented by somatic cell nuclear transfer (i.e. therapeutic cloning or SCNT) [55]. By transfer of a nucleus from a somatic cell into an enucleated oocyte, the oocyte can form a blastocyst upon stimulation, from which embryonic stem cells can be isolated that are genetically matched to the donor. Although no such human embryonic stem cell lines have been obtained yet, SCNT may in the future be useful for research purposes to create disease-specific stem cell lines (e.g. SCNT from Parkinson's somatic cell). In any event, caution needs to be taken with respect to their tumorigenic potential, as they readily form teratomas. Additionally, ongoing debates relating to the ethical aspect of embryonic stem cell use are daily news. These ethical issues appear to have been circumvented in experiments where differentiated (fibroblast) cells were reprogrammed to pluripotent stem cells [56-58].

In contrast to ES cells, adult stem cells are generally organ restricted, and typically only form cell lineages of the organ from which they originate (unipotency) (Fig. 5).
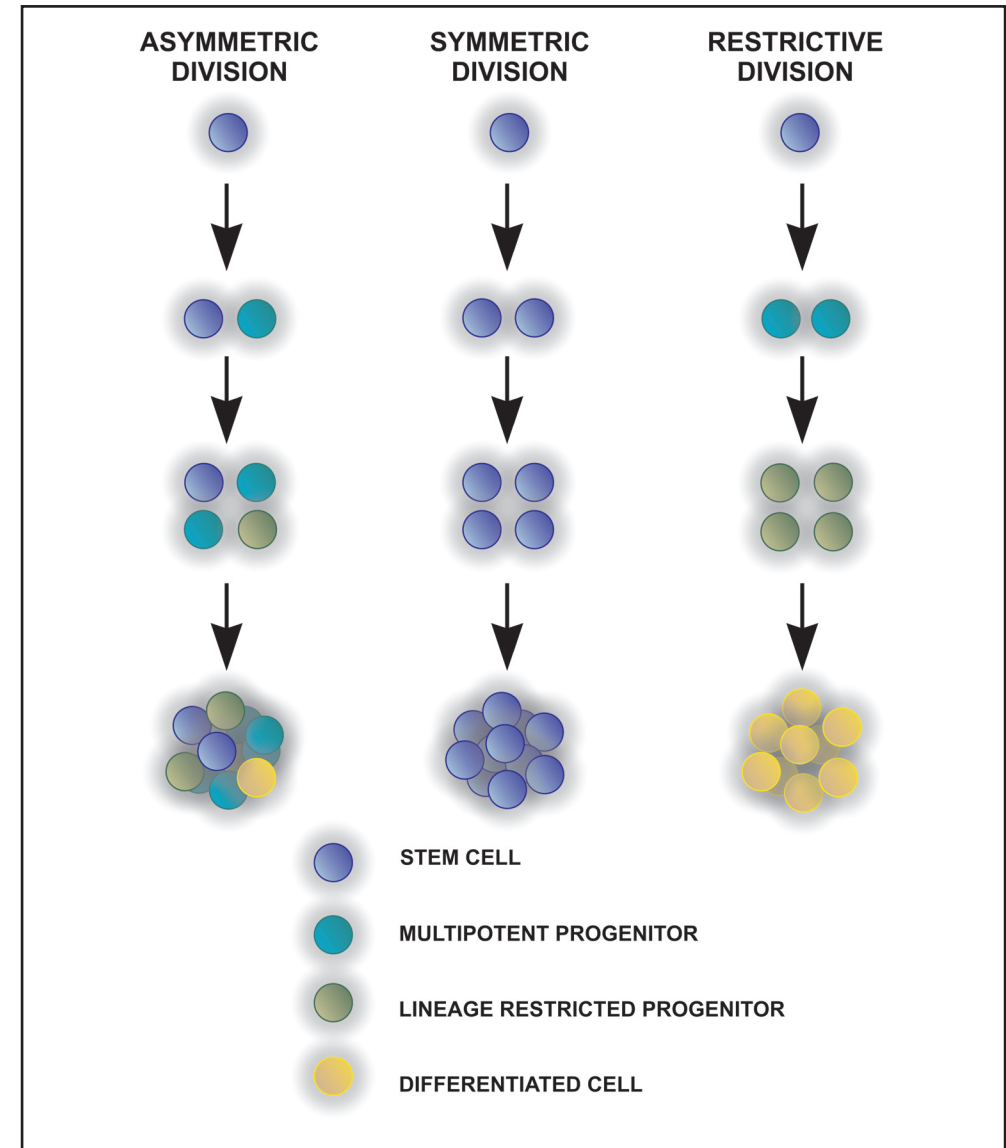
FIGURE 4. DIVISION PATTERN OF STEM CELLS. Asymmetric division of a stem cell involves the generation of one stem cell and a more differentiated progenitor cell. In contrast, via symmetric divisions stem cells are able to maintain and multiply their own number. When two differentiated daughter cells are produced, the process is called a restrictive division.

These cells are also referred to as somatic stem cells or tissue-derived stem cells. The most-intensively studied adult stem cells are hematopoietic stem cells that reside in the bone marrow. Since the 1960's, an exciting scientific field has opened with the demonstration of the rescue of mice from lethal irradiation with bone marrow stem cell transplantation [59,60]. The first human bone marrow transplant to treat leukemia followed in 1965 [61]. To date, adult stem cells have been identified in multiple organs. The general use of stem cells as medical treatment has been actively advocated and researchers now design stem cell-based therapies for a variety of diseases, such as myocardial infarction, Parkinson's and Alzheimer disease, diabetes type 1 and 2, chronic liver failures, muscular disorders, and skin, eye and kidney disorders.

In recent years, the consensus that each adult organ could only be rescued by its own tissue-specific stem cells was reconsidered after the observation that, under certain conditions, stem cells from one tissue can regenerate other, unrelated, tissue cell types (multipotency) [62]. Studies in which bone marrow-derived stem cells (BMCs) contributed to the regeneration of injured organs such as brain [63-65], liver [66-69], lung [70-72], vascular tissue [73], kidney [74-76], skin [77] and heart [78], opened the perspective of using multipotent BMCs to regenerate injured tissues. However, the potential of bone marrow-derived stem cells to regenerate non-hematopoietic tissue has been debated ever since. Some experimental evidence supports the hypothesis that BMCs can change their phenotype when present in an injured organ different from bone marrow (reviewed in Vieyra *et al*. [79]). Cell fusion with tissue-specific differentiated cells, however, has been proposed as an alternative mechanism [80] to explain observations of stem cell plasticity.

Separately, the potential of endothelial progenitor cells to treat vascular injuries has recently been discovered. These cells have been identified in bone marrow and peripheral blood, and may incorporate into injured vessels to participate in re-endothelialization and neo-vascularization [81]. Although the ultimate mechanism of endothelial progenitor cell-induced repair is not entirely elucidated, these cells may also stimulate local angiogenesis by secreting growth factors in a paracrine manner. This mechanism has also been proposed to explain organ repair by BMC therapy, whereby endogenous tissue (stem) cells are stimulated by the presence of BMCs [82].

Stem cell therapy treatment for radiation-induced hyposalivation may include all of the above mentioned possibilities. In this thesis, the application and potential outcome of BMC therapy to restore injured salivary glands will be explored. However, treatment of salivary gland dysfunction with stem cells derived from the salivary gland itself would be a preferred way of therapy. Unfortunately, data on salivary gland stem cells are scarce, and techniques to help define/isolate these cells need to be explored.
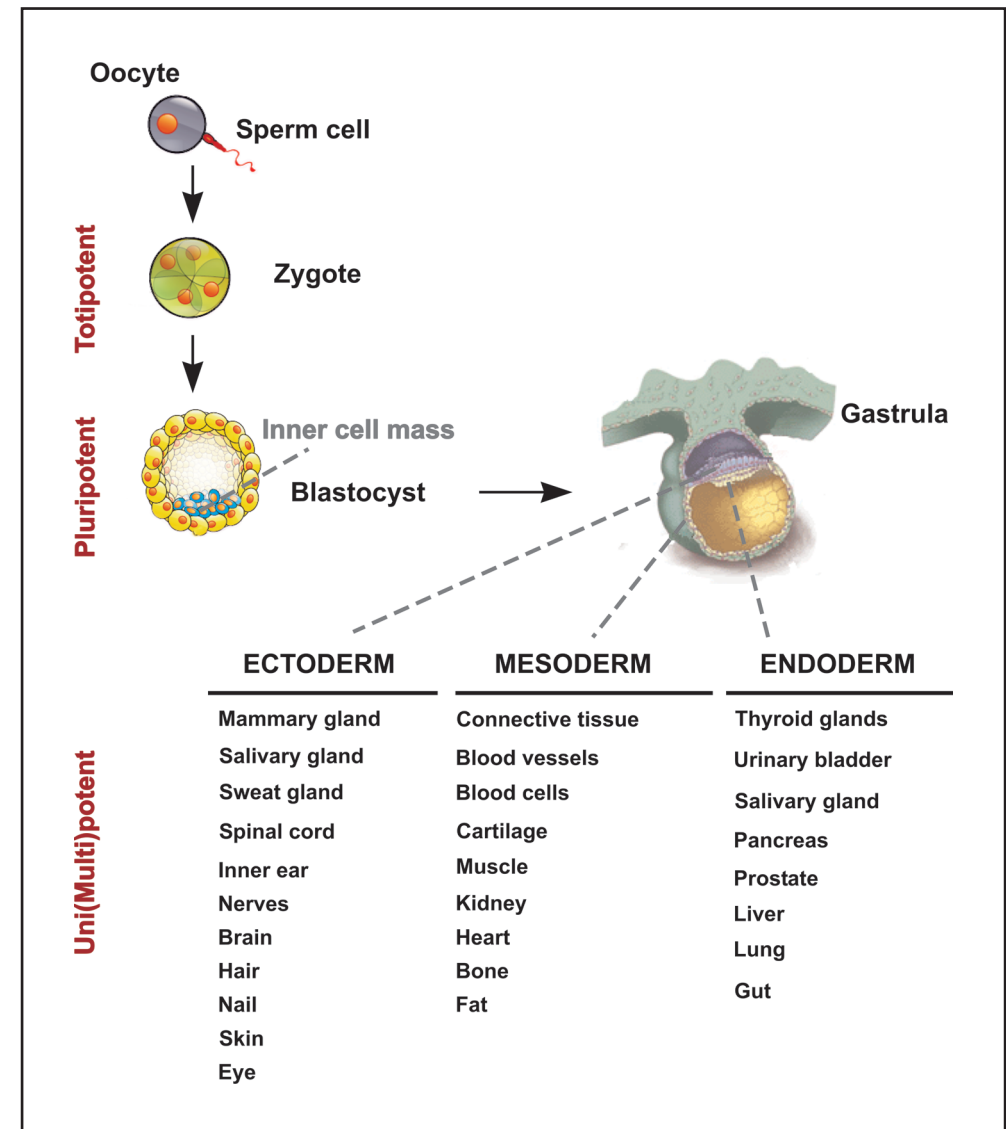
FIGURE 5. STEM CELL FATE. While cells of a zygote are still totipotent, embryonic stem cells, isolated from the inner cell mass of a blastocyst, are pluripotent. Eventually, during gastrulation, three different layers will be formed which are called the endoderm, mesoderm and ectoderm. Each layer gives rise to a variety of organs. In adults, each tissue may harbour tissue specific stem cells that are either unipotent or multipotent. (Picture modified from National Center for Biotechnology Information, Bethesda MD, revised November 2007).

7. THE TISSUE SPECIFIC STEM CELL

In order to efficiently perform stem cell therapy, certain general hurdles need to be taken. First of all, tissue-specific stem cells need to be isolated and characterized. Secondly, their potential to differentiate into tissue-specific lineages needs to be determined, as well as the ability to self-renew and their regenerative potential in pre-clinical animal models. Finally, if possible, expansion of their number *in vitro* may be necessary to obtain enough cells for clinical application.

For all of these purposes, precise knowledge of cellular behavior and the molecular mechanisms that lead to stem cell differentiation and self-renewal is required. The most commonly applied techniques will be addressed in the following paragraphs.

7.1 LOCALIZATION OF STEM CELLS

LABEL RETAINING CELLS

Although the hematopoietic cell system is most extensively studied, the exact location of hematopoietic stem cells (HSCs) in the bone marrow has not been resolved. In contrast, much knowledge has been obtained on the *in situ* stem cell location in certain other organs, including the intestinal crypts [83], the bulge of the hair follicle [84,85], bladder [86], the limbal region of the cornea [87], and the terminal end bud of mammary glands [88]. For all these tissues, one of the oldest techniques to define stem cell location was used: the Label-Retaining Cell assay. After a period of continuous administration of nucleotide analogues such as BrdU or ^{3}H-TdR, these are incorporated and label the DNA of all dividing cells. In a subsequent chase period, during which no nucleotide analogue is administered, the label will be diluted with every cell division. The less frequently dividing cells will retain the label, and are referred to as label-retaining cells (LRC) [89] and this population is considered to contain the stem cells. In these cells, the label is retained either due to quiescence or because of asymmetric segregation of the chromosomes, i.e. stem cells selectively retain a set of chromosomes that contain old template DNA [83,90]. However, recently, hematopoietic BrdU label retaining cells were shown to be less enriched for stem cells as first thought (only 0.5% were HSCs) due to random segregation of chromosomes [91]. As an alternative for the LRC assay, transgenic reporter mice have been developed expressing a stem cell specific protein fused to GFP or LacZ [92-95] for the imaging and the lineage tracking of stem cells in tissues like skin and intestine. One of these studies [92] clearly demonstrated that the most primitive intestinal stem cells are different from those observed by BrdU label tracking. Therefore, the LRC technique for stem cell detection is debated, and the obtained results need to be validated.

STEM CELL MARKERS

One of the most important obstacles in stem cell research is the absence of expression of a common and unique marker allowing identification of the stem cell. Such a marker would be of great help to unravel the stem cell niche. Consequently, the identification of novel stem cell-related markers is of major interest. Both histological and genetic analyses have revealed the existence of stem/progenitor cell-related markers, i.e. epitopes on the cell surface (e.g. Sca-1, CD24, CD133, CD49f, c-Kit) or intracellular proteins (e.g. Musashi-1). Some of these are cell-type and organ-specific, but others are expressed in several tissues. However, since a single marker that defines a stem cell has not been found yet, and may indeed not exist, several markers need to be combined. Nevertheless, in addition to *in vitro* and *in vivo* functional assays, morphologic observation of expression of potential markers might be a helpful tool in the identification of tissue stem cells and the niche in which it lives.

7.2 STEM CELL ISOLATION AND CHARACTERIZATION

Independent from information about their exact localization *in situ*, stem cells need to be physically isolated. Basically, three methods have been used so far: enrichment by selective cell culturing, sorting using FACS (Fluorescent Activated Cell Sorting) based on expression of cell surface markers or based on biochemical and physical properties (Side Populations, Rhodamine[dull] cells, cell size, cell density). However, in practice, a combination of these methods will be required to achieve the most optimal enrichment.

IN VITRO CULTURE OF STEM CELLS FROM SOLID TISSUES

Isolation of stem cells from solid tissues can be obtained by exposing the tissue to (several rounds of) digestion procedures with enzymes that disrupt the extracellular matrix (e.g. collagenase, hyaluronidase) and/or dissociation on cell-cell level (e.g. trypsin). The choice of one enzyme over the other is often arbitrary and more based on trial and error and past experience than on understanding why a specific method works, and differs between organs and even between laboratories. After enzymatic processing and seeding of cells *in vitro* in appropriate media conditions, often spherical, non-adherent cell clusters are formed. This selective culture system, often containing FGF and/or EGF as mitogenic growth factor, enriches for stem/progenitor cells. The most well-known are spheres obtained from mammary glands (mammospheres) [96] and brains (neurospheres) [97], both of which have been shown to contain progenitor and stem cells. For neurospheres, 4%-20% of the cells have stem cell potential, whereas the remaining population consist of progenitor cells in various stages of differentiation [98].

Subsequently, the self-renewal capability of stem cells can be demonstrated by formation of secondary and tertiary spheres from single cells originating from the original sphere [99]. Based on the frequency of cells that are capable of forming these secondary and tertiary spheres, the percentage of stem cells can be estimated (clonogenic assay).

Additional assays, including culture dish-adherent cells or cells growing in 3D culture systems, can subsequently be used to show differentiation of stem cells into all tissue lineages (e.g. oligodendrocytes, astrocytes, neurons for neurospheres or duct and alveolar cells for mammospheres).

Sorting of stem cells

Cell markers

Another possiblility to enrich and/or select for stem cells is by making use of cell type specific markers. Using FACS analysis, stem cells can be tagged with fluorescently labeled antibodies and may be ultimately sorted as single cells. Unfortunately, as mentioned above, no unique molecular marker has been found to be exclusively expressed on stem cells. For instance, a commonly used set of markers to enrich for HSC uses the lineage$^-$Sca-1$^+$c-Kit$^+$ protocol [100] (lineage includes antibodies raised against epitopes associated with terminal maturation of blood cell types, Sca-1 refers to Stem cell antigen-1 and c-Kit is the Stem Cell/Steel Factor receptor). Similarly, mammary gland stem cells have been isolated based on the expression of Lin$^-$CD24^{med}CD29^{high} markers [101,102]. Subsets of these cells can be further divided based on the expression of CD49f^{high}, Sca-1^{low}, CK14$^{+/-}$, SMA$^{+/-}$ or Hoechst 33342 [96,101].

A full description of all potential markers used to purify stem cells is beyond the scope of this thesis. It is clear, however, that the number of markers necessary to select putative stem cells is very high and differs between tissues. This also implies that none of these markers are associated with unique stem cell functions, that certain marker expressions may change over time (i.e. are not stable), and that presumably multiple types of stem cells exist even within a single organ.

Fluorescent tagging of genes

A second approach for direct isolation of stem cells is based on genetic manipulation of stem cells to express a fluorescence label. For example, skin stem cells expressing GFP from a stem cell specific promoter have been selected by FACS [94,95]. Many of the knock-in reporter models are currently being developed and will greatly improve our knowledge on adult tissue stem cells.

Dye exclusion

Stem cells are thought to efficiently express ABC transporters (i.e. membrane pumps able to exclude toxic substances), which enables them to be protected from potentially harmful chemicals. Cell suspensions can be enriched for stem cells using their ability to exclude fluorescent dyes such as Rhodamine 123 and Hoechst 33342. The latter is a DNA-binding dye which can easily enter living cells and which is actively exported from the cell by ABC-ATPase transporters present in stem cells. Rhodamine 123 stains mitochondria with increasing intensity as cells become proliferatively activated. The degree of efflux activity is related to the maturation state: the more primitive stem cells exhibit the highest efflux activity [103]. These transporters can be specifically inhibited by agents such as Verapamil, which serves as control for the specificity of the isolated cell population. Quiescent stem/progenitor cells, such as HSCs [103], can be highly enriched by selecting for reduced Rhodamine 123 or Hoechst 33342 accumulation, and are respectively referred to as Rhodamine$^{dull\ or\ low}$ cells and Side Population (SP) cells. SP cells have now also been described in many other organs, including skeletal muscle, lung, liver, heart, testis, skin, mammary gland, and cardiac muscle (reviewed in Challen *et al.* [104]). However, recently, it became clear that the mammary gland SP population is more enriched for progenitor cells than stem cells [105], and emphasizes once more that each technique by itself is not strict enough for isolation of a particular stem cell. However, in the absence of cell surface markers, SP selection has been proven highly useful as a primary purification step, but a combination with other assays is necessary to achieve purity. Additionally, one should keep in mind that small differences in isolation or staining protocols vary among laboratories, and will influence viability, homogeneity and yield of the obtained SP cells [106].

7.3 Transplantation of stem cells

Once stem cells are successfully isolated, their properties need to be demonstrated. It is important to stress that true stem activity can only be ascertained in functional assays. Showing clonality is one of the most powerful tools to prove stem cell identity, i.e. the capacity of a single cell to give rise to almost all cell type lineages of the appropriate tissue. *In vitro,* this is accomplished by the serial generation of clones from one single cell and their subsequent differentiation in different progeny cell types. However, recording the clonal ability of a single stem cell *in vivo* is technically very difficult. A solution for this is the demonstration that one single purified candidate stem cell is able to regenerate/repopulate a particular organ. In fact, this single stem cell should be able to integrate itself in the host environment, give rise to the appropriate mature cell types, and maintain itself in time. In the following paragraphs, promising stem cell transplantations are described which may be, or are already, clinically applicable.

Bone marrow

The only routinely clinically applied stem cell therapy to date is hematopoietic stem cell transplantation, which is used to restore blood production after myeloablation of patients suffering from leukemia, as well as various other types of (blood) disorders. The conditions for clinical hematopoietic stem cell transplantations have largely been determined in a very extensive series of pre-clinical (rodent) studies. In these experimental stem cell transplantations, HSCs are transplanted to recipients that received total body irradiation to destroy the endogenous bone marrow compartment. Co-transplantation of a defined population of helper cells ensures survival of all recipients. After reconstitution of the bone marrow (mostly after 3 months), blood/bone marrow chimerism is evaluated to assess stem cell quality and calculate the CRU (Competitive Repopulating Unit) of the transplanted cell pool. Serial *in vivo* transplantations are an extension to this assay, and are a useful tool to study the exhaustion of stem cells (i.e. their lifespan) and assess expansion limits.

Skin

Skin stem cell therapy is basically initiated to assist in skin and hair transplants for patients with burns or skin-related disorders. Clinically, human skin keratinocytes can be cultured to form epithelial sheets on fibrin matrices, which can be used to treat patients with burns [107]. In mouse studies, progress has been made in selecting and transplanting skin stem cells on the skin of recipient mice, and these cells have been shown to grow hair in nude mice [108]. Interestingly, the engrafted cells contributed to both the formation of hair follicles, epidermis and the sebaceous glands, indicating that these stem cells spontaneously differentiated into other tissue specific cells when placed in an appropriate environment.

Mammary gland

The best demonstration of the existence of mammary stem cells was made by Shackleton *et al.* [101,102] who showed that a whole organ could be formed from one single stem cell. The isolation of Lin$^-$CD29^{high}CD24$^+$ cells was followed by a transplantation of a single multipotent mammary gland cell into a cleared fat pad of virgin or pregnant mice. This transplantation resulted into a fully developed functional mammary gland with all lineages (alveolar, myoepithelial and luminal cells). Subsequent serial transplantations demonstrated self-renewal of the transplanted stem cell, and proved the principle that organ formation can occur from one single stem cell.

Eye

Stem cell therapy in the eye has been aimed at treating corneal Limbal Stem Cell Deficiency (LSCD) and retinal degenerations, and has become a major area of research in ophthalmology. The first *ex vivo* culture of human limbal stem cells was reported by Pellegrini *et al.* in 1997 [109]. Limbal stem cells were transplanted in the cornea of a patient with alkali burns [110]. Later in time, stromal scarring and neo-vascularisation produced a healthy organized cornea, allowing 100% recovery of visual acuity. Since then, additional reports have been published on the use of these stem cells to cure LSCD (reviewed in Shortt *et al.* [111]).

Kidney

In 2005, a study of Bussolati *et al.* [112] reported the successful transplantation of isolated human kidney CD133$^+$ stem cells in immuno-compromised mice. CD133$^+$ cells were subcutaneously placed into Matrigel, and ten days later, tubular structures were formed. The CD133$^+$ cells also survived in glycerol-induced tubulonecrosis kidneys of SCID mice when intravenously injected. Within the following three days, these human cells had integrated and were proliferating in the proximal and distal tubulus. In contrast, in normal undamaged kidneys, a minimal number of transplanted cells were found to home to the tissue. This study shows the capability of human renal (stem) cells to differentiate into renal cell lineages, and to take part in the regeneration of damaged tissue. Additionally, it demonstrates that the success of stem cell therapy is depending on the environment (uninjured/injured) in which the cells are transplanted.

Brain

For brain disorders such as Parkinson's disease (a degeneration of dopaminergic neurons) or Huntington disease (mutation in Huntingtin gene), most studies are focussed on the use of embryonic or fetal stem cells. However, also adult neuronal stem cells have been isolated from rats and grafted into the brain of a Parkinson disease rat model [113]. Although transplantation led to a high survival and migration rate of the donor cells, no efficient neuronal differentiation was observed. This is in contrast with a mouse study of Meissner *et al.* [114] where mouse neuronal stem cells from cultured neurospheres were injected in the subventricular zone of brains of Parkinson diseased mice. This study did show differentiation into astrocytes and neurons that reduced motor defects. These are the first studies showing the beneficial effects of adult stem cell therapy in brain diseases.

HEART

The heart has a very low regenerative capacity after injury. This is suggested to be due to the small number of endogenous cardiac stem cells. Nevertheless, adult cardiac stem cells might still be candidates for stem cell therapy. Research has focused on other cell types beside the adult cardiac stem cells such as skeletal myoblasts, bone marrow-derived cells, mesenchymal stem cells, and embryonic stem cells to use for transplantation purposes. Although different subsets of cardiac stem cells have been observed (lin-,c-Kit+ [115], Sca-1+ [116], SP cells [117], Isl-1 [118]), only the lin-c-Kit+ population has been documented to improve heart function up to 70% of its activity after injection into the border zone of ischemic-induced hearts [115] or led to a 29% decrease in infarct size after delivery into the coronary arteries [119].

In conclusion, the above described findings support the promising potential of stem cell therapy to restore injured and non-functioning organs with tissue specific stem cells. Transplantation of salivary gland stem cells into irradiated glands, however, has never been attempted and little information on putative salivary gland stem cells is available. A brief overview of existing data on salivary gland progenitor and stem cells will be outlined in the next paragraph.

8. STEM AND PROGENITOR CELLS IN THE SALIVARY GLAND

Multiple studies have attempted to determine the precise localization of the stem/progenitor cell in the salivary gland, but no adequate techniques have yet been developed to accurately isolate these cells for transplantation therapies. As salivary glands have the capacity to regenerate after partial extirpation [120] or duct obstruction [121], it seems likely that they contain stem/progenitor cells. Therefore, both of these techniques have been used to characterize and isolate stem/progenitor cells [122-125].

8.1 CHARACTERIZATION STUDIES

To identify the cells that are responsible for regeneration of salivary glands, duct ligation experiments have been the gold standard. Ligation of the excretory duct causes total atrophy of the gland, which is characterized by disorganization of the parenchyma, disappearance of acini, dilation of the interlobular ducts, infiltration of inflammatory cells and pronounced fibrosis of the glands. In the rat, after seven days of obstruction, this results in a 30-40% reduction of the total gland weight [123,126] and a loss of 85% in acinar cell mass [123]. Based on measurements of areas occupied by each epithelial cell type [122,123] or determination of proliferation [124,125,127], a decrease in (proliferating) acinar cells and an increase in intercalated duct cells was shown.

Following the removal of the obstruction, within 7 days an impressive proliferation rate and regeneration of acinar cells was observed with concomitant reduction in number of intercalated duct cells. These observations were the first indications that after acinar cell loss intercalated duct cells (ID) are able to extensively proliferate and differentiate in acinar cells.

Subsequently, LRC studies confirmed that intercalated duct cells exhibited high proliferation marker labeling, which are diluted in time at the acinar/ID and GCT(Granular Convoluted Tubule)/ID junctions [128,129].

Based on these studies, it was proposed that the intercalated duct cells contained the progenitors for acinar and GCT cells [128,129], and that striated duct cells were presumably replaced by more primitive excretory duct cells [129].

8.2 STEM CELL CULTURES

FORMATION OF ACINAR CELLS

Many efforts have been made to culture cells of salivary gland from both rodents and humans/primates, either to investigate acinar cell behavior or to assess ductal cell differentiation. First attempts date from 1959 [130] showing that when submandibular explants were cultured, acinar cells rapidly (24-48 hrs) degenerated *in vitro*, while most ductal epithelium became hyperplastic. Several years later [131-134], it was noticed that cells of the mouse submandibular gland responded *in vitro* to hydrocortisone and insulin, resulting in improved cell survival, although it should be noted that these cultures were severely contaminated with fibroblasts. Again years later, human salivary gland duct cells could be induced to form monolayers, which were passaged for several times in serum-free medium with a low concentration of calcium [135]. Apparently, the calcium concentration influenced the degree of desmosome formation and cell survival [136]. Based on this knowledge, human [137], macaque [137] and rat [138] submandibular/parotid (duct) gland cells were cultured on 3T3 feeder layers, resulting in formation of acinar-like cells, showing that gland cells could be harvested and cultured, and that ductal cell types were able to differentiate into acinar cells.

Human gland cells, isolated as either full tissue aggregates or as dissociated cells, can be cultured as undifferentiated parotid ducts (0.2 M Ca+2) [136], as acinar cells (0.2-1 mM Ca+2) [136,139], or as non-neoplastic cell lines [140]. Although adequate cell characterization was not performed in these studies, they suggest that ductal cells can differentiate into acini *in vitro*. Unfortunately, until now, none of these culture methods have been proven to be ideal for duct stem/progenitor cell selection.

Colony forming assay

Kishi T. *et al.* [141] were the first to succeed in developing a stem cell assay based on colony forming units (CFU). They showed colony formation of single cells of both neonatal and adult submandibular rat glands plated at low-density on coated dishes in serum-containing medium. All three cell lineages could be detected by the demonstration of cell type specific markers (AQP-5 for acini, SMA for myoepithelium, N^+K^+ATPase, S-100, CK19, c-met for ducts). Apparently, addition of EGF and HGF significantly increased colony formation. As a higher number of colonies could be obtained from neonatal tissue when compared to adult glands, they concluded that the latter contain less "stem" cells. Unfortunately, this study lacks important information as to what the origin is of the cell types at the beginning of seeding, how the difference between fibroblast and epithelium-containing colonies was determined, how the formation of a colony from one single cell was ascertained, and whether they were able to serially passage the stem cells. As *in vivo* functional characterization of these cells was not assessed, it appears premature to refer to these cells as salivary gland stem cells.

8.3 Transplantation studies

Atrophic glands

Transplantation of cultured salivary gland cells into atrophic glands was attempted by Sugito T. *et al.* [142]. Enzymatically dissociated rat submandibular gland cells, cultured in serum-rich medium on Mitomycin C-treated 3T3 cells [138], were injected into a gland seven days after duct ligation and subsequent deligation. Two weeks later, transplanted cells had migrated from the injection site, and remained adjacent to, or inside, the ductal system. No differentiation in any myoepithelial (SMA) or acinar (Mucin-1) cell type could be observed. This study was a first attempt to isolate and transplant gland stem/progenitor cells, but it failed to show formation of any acinar cell nor were functional measurements performed. Therefore, no proof that stem/progenitor cells were isolated has been obtained.

Regeneration of pancreatic/hepatic-diseased tissue using salivary gland (stem) cells

Other researchers have attempted to isolate salivary gland stem/progenitor cells from both mice [143] or rats [144,145] via cell sorting. They selected for Sca-1+c-Kit+ or α6+β1+-integrin cells from respectively mouse [143] and rat glands [145] after duct ligation. Ligation was necessary to enhance the number of these cells *in vivo*. In order to demonstrate the trans-differentiation capacity of the isolated gland stem/progenitor cells, cells were subjected to media used for the culture of pancreatic or hepatic cells.

After two weeks of culture, the cells were transplanted into the portal vein of partially hepatectomized livers, where after integration of albumin-expressing donor cells in the liver were observed. Subsequently, rodent cells were replaced with human and swine salivary gland material [146,147], and similar *in vitro* trans-differentiation results from CD49f+Thy-1+ selected cells were observed. Although induction of liver/pancreas specific genes in the cultured cells was observed [143,147], more selective and functional methods will be necessary to prove multipotency *in vitro* and *in vivo*.

8.4 Other regenerative approaches to restore function of salivary glands

The group of Baum *et al.* [148-150] reasoned that remaining duct cells in an irradiated gland might be stimulated to secrete water in order to prevent oral and food lubrication problems. However, the ductal cells in salivary glands are water-impermeable, in contrast to fluid-secreting acinar cell types. To solve this problem they transferred a human aquaporin-1 (hAQP1) cDNA sequence into duct cells with the use of a recombinant adenovirus. Their study in miniature pigs [150] showed a temporary restoration to 80% of the normal saliva flow after gene therapy, although their attempts in irradiated rhesus monkeys glands [149] yielded rather inconsistent outcomes. The exact mechanism by which the duct fluid secretion is established still needs to be elucidated, and detailed toxicological studies need to ensure the safety of patients if clinical trials are considered.

The same group has attempted to construct an artificial human gland. Hereto, three important substances are needed: a porous biodegradable substrate, a coated extracellular matrix on the surface, and a polarized epithelial cell layer to allow uni-directional fluid secretion. Although the first attempts appeared promising, the subsequent formation of a connection system between the different gland cells (duct, acinar, and nerve cells) still needs to be established and may be problematic [151-153].

GENERAL CONCLUSION

Malfunctioning of salivary glands and consequential xerostomia is a ubiquitous and long-term complication after radiotherapy in head and neck cancer patients. Significant progress has been achieved in the prevention and treatment of xerostomia. However, to date, many patients still continue to suffer from xerostomia. This implies that the above described prevention and treatment strategies are not sufficient. Although new efforts are directed towards creating artificial glands or gene therapy, these techniques are not fully standardized, nor yet clinically applicable. Therefore, further research and development of novel approaches including stem cell therapy are warranted to investigate.

AIM OF THE THESIS

This thesis aims to explore the potential of adult tissue stem/progenitor cell therapy to treat radiation-induced hyposalivation.

OUTLINE OF THE THESIS

CHAPTER 2: First attempts to prevent radiation-induced hyposalivation were performed using bone marrow-derived (stem) cells (BMCs). These cells have been suggested to be involved in the repair of a variety of injured tissues. BMCs were mobilized into the blood circulation using G-CSF (Granulocyte-Colony Stimulating Factor). Their homing and potential to trans-differentiate into salivary gland cells and to repair the injury were assessed.

CHAPTER 3: BMCs to some extent induced repair of radiation damage in salivary glands. In this chapter, it was questioned whether increasing the number of circulating BMCs, using a combined treatment with G-CSF, Flt-3L, and Stem Cell Factor, would lead to an increased repair of the irradiated submandibular glands.

CHAPTER 4: This chapter focuses on the isolation of submandibular gland stem/progenitor cells, their capability to differentiate into gland cells *in vitro,* and their *in vivo* capacity to regenerate irradiated submandibular glands after (serial) transplantation.

CHAPTER 5: This chapter explores which cells in mouse submandibular glands express (stem) cells markers, and how their expression is influenced by irradiation and subsequent transplantation of salivary gland stem cells.

CHAPTER 6: Keratinocyte Growth Factor (KGF/FGF-7) has been documented to prevent radiation-induced damage in many organs. This chapter investigates the role of stem cells in the amelioration of radiation-induced damage to the submandibular gland after ∆N23-KGF treatment.

CHAPTER 7: Finally, the results described in the different chapters are summarized, and their implication in future salivary gland stem cell research is discussed.