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Engineering a Human Embryonic Stem Cell Reporter Line for Salivary Lineage Screening

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by

Bahar Houshmand

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Abstract of the Thesis

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Head and neck cancer radiotherapy and Sjogren Syndrome are two major conditions associated with irreversible damage to salivary glands and, a considerable decrease in saliva secretion. Currently there is no treatment available for these patients other than those relieving the symptoms. Cell-based therapy holds a great promise for treatment of these conditions, however the source of salivary progenitor cells is limiting. Human embryonic stem cell (hESC) can be induced to differentiate in to all cells in the body and may provide a potential source of progenitor cells for regeneration of damaged salivary gland. To direct efficient differentiation of hESC to salivary gland progenitor cells however, well-defined culture conditions must be developed. One way to screen culture conditions that support differentiation of hESC to a specific lineage is to generate lineage-specific reporter lines. This is achieved through genetic modification of hESCs with a tissue-specific promoter driving a reporter gene. In addition, generation of reporter progenitor cells will facilitate the purification of specific cell populations from heterogeneous differentiated hESC progeny. Keratin (K) 19 is a marker of salivary gland progenitor cells. Here I describe the construction of a lentiviral vector in which EGFP expression is controlled by human K19 promoter. When compared to ubiquitously expressed EF1□-EGFP, the K19-EGFP reporter construct exhibited high levels of activity in prostate epithelial cells (PC3) which express high levels of K19, and low levels of activity in fibroblasts and keratinocytes which normally do not express K19. This demonstrated the fidelity and specificity of transgenic K19 promoter. Transgenesis of a hESC line with lentiviral vector encoding K19-

EGFP resulted in minimal or no EGFP expression while high levels of EGFP was detected in those transduced with EF1□-EGFP. This transgenic line could be useful in high through-put assays to define conditions that reproducibly and efficiently induce differentiation of salivary lineage.

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Abbreviations

AAV	Adeno-associated viral vectors
AQP	Aquaporin 1
Ascl3	Achaete-scute complex homolog 3
BAC	Bacterial artificial chromosomes
BMP	Bone morphogenic protein
BMDC	Bone marrow derived cells
BSA	Bovine serum albumin
CM	Conditioned medium
CMV	Cytomegalovirus
DMEM	Dulbecco`s minimal essential medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco`s Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
EF1-α	Elongation factor 1- α
EGF	Epidermal growth factor
EGFP	Enhanced Green fluorescent protein
FACS	Fluorescent- activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBS	Hank`s balanced solution
Hela-70	Cervical carcinoma line
HepG2	Hepatocellular carcinoma line
hESC	Human embryonic stem cell
hFB	Human fibroblasts
hKC	Human keratinocytes
IVF	In vitro fertilization
ICM	Inner cell mass
IGF	Insulin-like growth factor
K	Keratin
KGF	Keratinocyte growth factor
LRC	Label retaining cells
LVV	Lentiviral vector
MEF	Mouse embryonic fibroblast
MFI	Median fluorescent intensity
MOI	Multiplicity of infection
NGF	Nerve growth factor
P	Passage
PC3	Prostate adenocarcinoma line
PFA	Paraformaldehyde

Pgk	Phosphoglycerate kinase
RT	Room temp
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SS	Sjogren Syndrome
TA	Transit-amplifying cells
TGF	Transforming growth factor
VSV-G	Vesicular somatitis virus glycoprotein
γMEF	gamma-irradiated embryonic fibroblasts

Chapter I. Introduction

I.1. Importance of Salivary Glands in Human Health:

Saliva is secreted from acinar cells of salivary glands and plays a major role in protection and maintenance of upper gastrointestinal tract and initiation of food digestion (Amerongen et al. 2002). Patients without functional salivary acinar cells have dramatically reduced salivary secretion. These patients suffer from dry mouth, dysphagia, increased oral infections (e.g., dental caries, oropharyngeal infections), mucositis and generalized oral discomfort which affect their quality of life (Vissink et al. 2003).

I.2. Major Conditions Associated with Salivary Gland Dysfunction:

There are two major conditions which cause irreversible salivary gland hypofunction due to parenchymal injury: 1) Sjogren Syndrome (SS) and 2) head and neck radiotherapy.

I.2.1. Sjogren Syndrome:

Sjogren Syndrome is an autoimmune disease with unknown etiology which affects between 2 and 4 million people in the United States (Fox 2007). SS is characterized by generation of autoantibodies which bind to and inhibit muscarinic receptors, hence decreasing saliva production (Dawson et al., 2006). In addition, massive mononuclear cell infiltration into glandular tissues leads to acinar atrophy of salivary glands leading to hyposalivation (Pillemer et al. 2001; Lodd et al. 2006).

I.2.2. Head and Neck Radiotherapy:

Head and neck cancers comprise 2-3% of all cancers in the United States and are the cause of 2-3% of all cancer-related death. Head and neck cancers comprise neoplasms of various structures in the oral cavity (52.1%), larynx (25.5%) and pharynx (22.3%). Regional lymph node metastasis and distant metastasis are seen in most of the patients at the time of diagnosis. More than 90% of head and neck cancers are squamous cell carcinomas. Other less common types include mucoepidermoid carcinoma, adenoid cystic carcinoma and adenocarcinoma. About 40,000 new patients are affected every year in the United States (Jamal et al., 2009). Treatment is directed toward elimination of primary tumor and affected nodes in the neck and includes surgery and radiation therapy. One of the most serious side effects of radiotherapy is mucositis

and hyposalivation or dry mouth syndrome (Vissink et al., 2003). Exposure of salivary glands to radiation can lead to atrophy of salivary glands due to the loss of acinar cells and their progenitors resulting in progressive loss of saliva production and alteration in saliva composition (Coppes et al., 2001; Redman 2008).

Hyposalivation burdens patients with oral dryness, increased oral infection and dental caries, and leads to difficulty with swallowing and speech. Current treatments for hyposalivation provide symptomatic relief not a cure (Wang et al., 2010). Therefore, there is a great interest in development of novel therapeutic approaches to reconstruct salivary glands or to increase salivary flow for these patients. One potential approach for treatment of these patients is stem cell therapy. One of the challenges for patients with damaged salivary gland is the lack of sufficient stem cells for transplantation and tissue regeneration. Most of these patients are old and gland tissues are atrophic in older patients, therefore, isolating sufficient number of stem cells and expanding them may be difficult for auto-transplantation. Moreover, establishing optimized culture conditions for each patient may be difficult (Sumita et al., 2011). Therefore, development of salivary progenitor cells derived from embryonic stem cells capable of ultimately differentiating to acinar/ductal cells may overcome these problems. To develop such therapies, biological insights into gland development and function are needed.

I.3. Physiology of Salivary Gland:

In mammals, there are three major salivary glands known as the submandibular, sublingual, and parotid. In addition, there are numerous minor salivary glands. In humans the parotid glands are situated in the tissue anterior and inferior to the ears and superficial to the ramus and angle of the mandible while the sublingual and submandibular glands are located inferior to the mandible and the floor of the mouth (Fig.1). The human minor salivary glands are placed in the tissues immediately subjacent to the oral mucosa in the buccal mucosa, anterior floor of mouth, labial mucosa, posterior hard and soft palate, tonsillar pillars, and posterior dorsal and anterior ventral tongue (Reviewed by Redman 2008). The major salivary glands supply approximately 90% of saliva production.

Each gland's structure, cellular composition and secretory products is unique, but all are made up of two major cell types, the acinar cells and the duct cells. The acinar cells, or acini, are

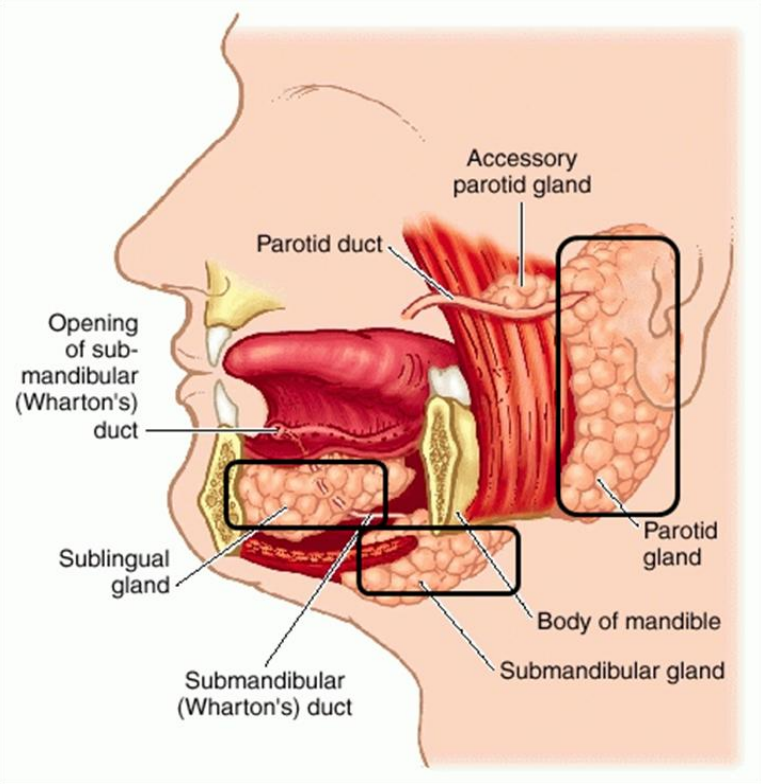


Figure 1. **Location of major salivary glands in humans**. Schematic presentation of the three main salivary glands and their ducts including parotid, submandibular and sublingual glands. Parotid glands are located anterior and inferior to the ears and superficial to the ramus and angle of the mandible. Sublingual and submandibular glands are located inferior to the mandible and the floor of the mouth (Adapted from Dorland's Medical Dictionary for Health Consumers. © 2007 by Saunders)

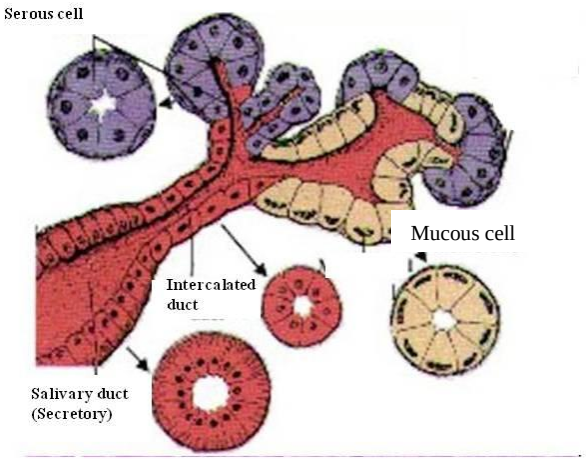
further subdivided into serous, mucous and seromucous cells (Young and van Lennep 1978, Hand 1987). Mucous cells secrete mucin protein but serous cells do not. Instead, serous cells secrete other proteins such as amylase. Seromucous cells produce both mucins and serous proteins. Mucin lubricates food, protects teeth against acid and helps protect against bacteria, viruses and fungi. Serous cells secret a group of digestive enzymes, anti-bacterial, anti-fungal, anti-viral agents and ions which help protect teeth and soft tissue against acidic conditions.

Saliva secretion is controlled by the autonomic nervous system (sympathetic and parasympathetic). It involves binding of acetylcholine and norepinephrine neurotransmitters to muscarinic and adrenergic receptors, respectively, signaling via guanine nucleotide-binding regulatory proteins (G-proteins) and activation of intracellular calcium signaling (Baum, 1993). Both parasympathetic and sympathetic nervous stimulation can cause myoepithelium contraction and expulsion of secretions from the secretory acinus into the ducts and finally to the oral cavity (Redman, 1994).

Human saliva is composed of 98% water and the remaining 2% consists of other compounds such as electrolytes (sodium, potassium, calcium, magnesium, chloride, bicarbonate , phosphate, iodine), lubricants (mucins, proline-rich glycoproteins), antimicrobial compounds (complement, defensins, lysozyme, salivary lactoperoxidase, cyatatins, histatins, statherin, thrombospondin, secretory leukocyte protease inhibitor and secretory immunoglobulin A), various enzymes (amylases, lipase, ribonuclease, protease) and growth factors (EGF, TGF-α, TGF-β, FGF, IGF-I, IGF-II and NGF) (Reviewed by Kaufman et al, 2002). The initial fluid secreted by acinar cells is isotonic but it is modified by ductal cells which absorb most of the Na^+ and Cl^- ions from the primary saliva and secrete K^+ and $HCO3^-$ and some proteins. Thus, the final saliva which enters the oral cavity is hypotonic to plasma (Baum 1993).

The secretory acinar cells, arranged in clusters, generate the initial saliva which is drained into the intercalated ducts that eventually lead to the excretory ducts. The ducts, which are composed of duct cells, are arranged in branching networks which modify and conduct the saliva to the oral cavity (Fig.2.A). The contraction of myoepithelial cells, which surround the acini and intercalated ducts, directs the flow of the saliva from acini through the intercalated ducts into the larger ducts (Reviewed by Redman 1994). In humans the submandibular gland is comprised of

A)



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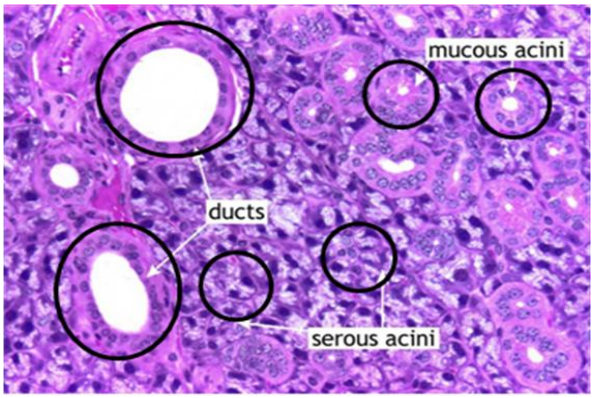

Figure 2.A) Schematic presentation of salivary gland compartments. Acinar cells producing saliva, composed of serous and mucous cells are shown. Saliva is conducted through the ducts into the oral cavity (adopted from www.capraispana.com/fisiologia). B) Cross section of human submandibular gland. Acini (Serous and mucous) and ducts are stained with H & E staining (Adapted from www.Deltagen.com).

both serous and mucous cells, the parotid gland is comprised of mainly serous cells and the sublingual gland consists mainly of mucous cells (Fig.2.B).

I.4. Salivary Gland Development and Growth

The general pattern of mammalian salivary gland development is similar between different species. Salivary gland development begins by thickening of the primitive oral epithelium that grows into the first branchial arch mesenchyme to form the solid epithelial placode. This placode grows inwardly into the mesenchyme forming a solid mass of epithelial cells (initial bud) which is connected to the oral epithelium by a stalk of immature duct epithelial cells. Epithelial-mesenchymal interaction leads to cleft formation which separates the primary bud into multiple buds (Jaskol and Melnick, 2000). This process repeats multiple times through pseudoglandular, canalicular and terminal bud stage. While the main duct undergoes lumenization, acini and lumen formation starts in end buds so that a continuous lumenized duct connecting the acini to the oral cavity forms. Nerve and blood vessels develop in association with the branching epithelium. Cellular differentiation and branching morphogenesis occur at the same time. The undifferentiated cells of the embryonic gland differentiate into acini, myoepithelium, intercalated, striated and excretory ducts (Tucker. 2007; Jaskol and Melnick., 2005; Patel et al., 2006).

Homeostasis of adult salivary glands is maintained by proliferation and differentiation of progenitor/stem cells and self-proliferation of existing differentiated cells (Denny et al., 1997; Kimoto et al., 2008), suggesting that normal salivary gland turnover is maintained by more than one cell population. In the adult salivary gland, radiolabeling and immunohistochemical studies have shown that intercalated ductal cells harbor a population of undifferentiated stem/progenitor cells which differentiate into acinar/ductal cells of the gland (Zajick et al., 1985; Man et al., 2001).

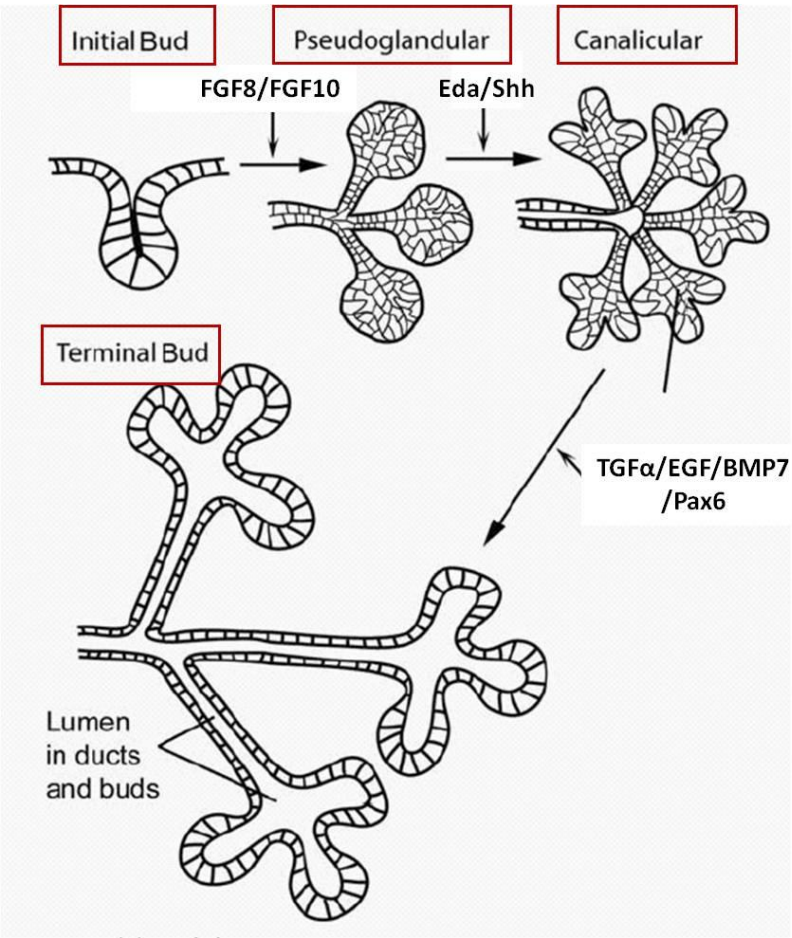

Figure 3. Schematic representation of salivary gland branching morphogenesis. Morphogenesis starts by initial bud stage, followed by pseudoglandular, canalicular and finally the terminal bud stage. Cellular differentiation and branching morphogenesis occur at the same time. (Adapted from Jaskoll and Melnick, Cells Tissues Organs 2000; 170: 83-98)

I.5. Salivary Gland Progenitor/Stem Cells:

The homeostasis of self-renewing tissues such as hematopoetic system or hair follicle is dependent on tissue specific stem cells. Tissue specific stem cells participate in organogenesis and persist throughout life in specialized microenvironments called niche (Spradling et al., 2008). Stem cells undergo divisions to generate daughter cells that either remain stem cells or differentiate to the more committed transit-amplifying cells (TA cells) that migrate from the stem cell niche and go through rapid proliferation and eventually differentiate (Fuchs, 2009; Morrison and Kimble, 2006).

The presence of stem cells in tissues with slow turn-over, such as the liver or the pancreas however, is more controversial. Renewal and regeneration of these tissues appear to be independent of stem cells. For instance, although during embryonic development of the pancreas, β cells are generated from endocrine progenitor cells (Gu et al., 2002; Murtaugh, 2007), during postnatal life, β cell expansion and maintenance relies on the proliferation of pre-existing differentiated β cells. (Dor et al. , 2004; Lee et al., 2006; Teta et al. , 2007; Brennand et al., 2007; Nir et al., 2007).

Salivary glands renew every 2-4 months (Zajicek et al., 1985) and are considered as "slowly renewing organs" (Schwartz-Arad et al 1988). Homeostasis of adult salivary gland in normal and regeneration states is derived from differentiation of progenitor/stem cells (Denny and Denny 1999; Man et al., 2001) and also is attributed to division of differentiated cells (Denny et al., 1993; Ihrler et al., 2002). Different studies have been done to identify the stem/progenitor cell population in salivary glands. Duct ligation is used as a model to study the injury-induced regeneration process in adult salivary glands. After 1 week of main excretory duct ligation, almost all of the acinar cells disappear and ductal cells proliferate extensively (Walker and Gobe 1987; Walker 1987). After re-opening of the obstructed duct, proliferation of residual acini (if any) is followed by proliferation and differentiation of intercalated ductal cells into acinar cells suggesting that ducts give rise to precursor cells that, through ongoing differentiation and proliferation, reconstitute the atrophic gland (Takahashi et al., 1998, 2004, 2005; Man et al., 2001).

Neonatal rat submandibular gland cells are capable of colony formation in culture. Although the exact characteristics of these cells have not been identified, they were able to form all cell types of submandibular gland and expressed specific submandibular gland markers (Kishi et al., 2006) suggesting the presence of stem cells in salivary gland.

Stem cells are slowly dividing cells which retain DNA labels long after exposure. Label retaining cells (LRCs) have been identified in different parts of the salivary gland including acinar cells, ductal cells, myoepithelium and connective tissue cells (Denny et al., 1999; Man et al., 2001; Kimoto et al., 2008) suggesting the presence of multiple stem/progenitor cells in the adult salivary gland. It has been shown that most of LRCs reside in intercalated ductal cells and can differentiate into both acinar/ductal cells suggesting that intercalated ductal cells could be a source of undifferentiated progenitor/stem cells in the adult salivary gland (Zajicek et al., 1985; Man et al., 2001; Kimoto et al., 2008).

To date, the characteristics of salivary gland stem cell have not been identified completely. In the following section, some identified markers of salivary gland stem/progenitor cells are discussed:

1.5.1. Potential Markers of Salivary Gland Stem Cells:

cKit:

In the adult salivary gland cKit is expressed in the excretory and striated duct cells. Transplantation of $cKit^+$ cells isolated from mouse submandibular glands into dysfunctional irradiated salivary gland has been shown to restore gland function with almost the normal number of healthy acinar cells and a nearly normal saliva production. It has been shown that ductal and acinar cells were originated from $cKit^+$ transplanted cells suggesting that $cKit^+$ cell populations contained true stem cells (Lombaert et al., 2008).

Ascl3:

Genetic lineage tracing demonstrated that Ascl3 positive cells isolated from the mouse salivary gland gave rise to ductal and acinar cells (Arany et al., 2011). Ascl3, also known as Sgn1, is a member of the mammalian achaete scute (Mash) gene family of transcription factors, which specifically localized to the duct cells of salivary glands (Yoshida et al., 2001). During

mouse salivary gland development, expression of Ascl3 is restricted to a subset of duct cells. Although Ascl3^{+} cells identifies a population of progenitor cells in salivary gland , only a subpopulation of the submandibular gland are derived from these cells (Arany et al., 2011), suggesting the presence of other stem cells.

K19:

During embryonic development of the submandibular gland, K5+ progenitor/ stem cells differentiate toward the developing salivary gland lumen and co-express K19. However, as differentiation continues K5 expression is lost (Knox et al., 2010). Sox2, an embryonic self-renewal marker, is expressed in K5+/K19+ and K5-/K19+ cells suggesting that K19-expressing cells have self-renewal potential (Lombaert et al.,2011)

K19 is a 40 kDa type I intermediate filament protein that is expressed in both stratified and simple epithelia, such as skin, esophagus, tongue, exocervix ,vagina, small intestine, colon, exocrine pancreas, bladder, gallbladder, and the ductal cells of the salivary gland and liver (Bosch et al., 1988; Omary et al., 2009). In developing salivary gland K5+ progenitor/ stem cells co-express K19 (Knox et al., 2010). In addition, in adult rodent salivary gland, intercalated ductal cells which give rise to ductal/acinar cells express K19. Since K19 positive cells also express the self-renewal marker Sox2, they are suspected to also have the self-renewal ability (Lombaert et al., 2011) and may serve as stem cells in mature salivary gland.

Although the relationship between Ascl3 cells, cKit+ cells and K5+/K19+ cells remain to be defined, currently these markers are considered to be salivary gland progenitor/stem cell markers. Any long-term therapeutic strategy to reverse gland function needs to be targeted to stem/progenitor cells.

I.6. Potential Approaches for Treatment of Salivary Gland Hypofunction:

I.6.1. Gene Therapy:

Several studies have focused on gene transfer of aquaporin 1 (AQP1) to human salivary gland cells in order to develop a method for restoring saliva production to clinically relevant levels (Aframian et al.2002; Tran et al.2005; Baum et al.2010). AQP1 is a member of a family of integral membrane proteins that function as water channels (Preston and Agre., 1991). Increasing

the water permeability of surviving salivary duct cells after radiation through transferring the human aquaporin-1 (hAQP1) cDNA adeno-associated viral vectors (AAV) was shown to be successful in increasing the saliva secretion in mice and minipigs (Delporte et al., 1997; Shan et al., 2005; Baum et al., 2006). This vector increased fluid secretion from surviving duct cells of minipig to 35% of pre-radiation levels. The rationale is that if an osmotic gradient is produced by the survived ductal cells, then AQP1 can increase fluid secretion from the irradiated gland (Gao et al., 2011). Currently there is a phase I/II clinical trial using AAV to transfer hAQP1 cDNA into the surviving ductal cells after radiation (http://www.clinicaltrials.gov).

In another study AAV encoding human keratinocyte growth factor (hKGF=BMP7), which is an epithelial cell–specific growth and differentiation factor, was administered to mouse submandibular gland before radiation (Zheng et al., 2011). Salivary flow rates of these mice were not significantly different from non-irradiated control mice. hKGF expressing vector prevented salivary hypofunction dramatically (Zheng et al., 2011).

Although these results seem to be promising, gland atrophy in these patients limits the number of cells that could be targeted by gene therapy. Therefore, these patients may not be appropriate candidates for this treatment approach.

I.6.2. Cell-based Therapy:

Tissue regeneration occurs through the activation of tissue specific somatic stem cells located in a niche or by transplantation of stem cell/ progenitor cells (Brockes et al., 2002). Unless the injury is too severe or chronic, the salivary gland responds to damage by its resident stem/progenitor cells serving as the source of new epithelial cell population. Long term effects of radiation therapy which leads to atrophy of salivary glands and lack of recovery after radiation is due to the loss of salivary gland progenitor/stem cells (Coppes et al., 2001). Thus, it is reasonable to propose that regeneration of the salivary gland could be greatly improved by transplantation of progenitor cells in the injured gland. Stem cell-based therapies using either adult or embryonic stem cells are currently under investigation for their potential to treat salivary gland hypofunction and will be discussed more in this section:

I.6.2.1. Multipotent Bone Marrow- Derived Stem Cells:

It has been reported that transplantation of bone marrow cells ameliorates radiation-damage in murine salivary glands by increasing the amount of saliva production (Lombaert et al., 2008). Sumita et al have shown that after transplantation of bone marrow derived cells (BMDCs) not only saliva secretion increased but also approximately 9% of salivary epithelial cells were derived from the donor BMDCs, (Sumita et al., 2011). The mechanism by which BMDCs can improve the function of damaged salivary glands is not yet clear but a combination of transdifferentiation, vasculogenesis and paracarine effect of BMDCs is suggested to play a role in the improvement of salivary gland function (Tran et al., 2011). Although cell therapy using BMDCs can regenerate damaged salivary gland tissue and improve its function, the therapeutic effects on salivary flow rate gradually diminish by time. To have a stable salivary flow rate further studies are required to optimize BMDC dosage, efficient injection time interval and local cell delivery methods to salivary glands.

I.6.2.2. Salivary Gland Progenitor Cells:

As discussed in section I.5, transplantation of salivary gland stem/progenitor cells have shown promising results in regeneration of damaged salivary glands. $cKit^+$ cells and $Ascl3^+$ cells isolated from mouse salivary glands, in two different studies, have been shown to differentiate into acinar/ductal cells and enhance saliva production in irradiated salivary glands (Lombaert et al,.2008; Arany et al., 2011). Although transplantation of tissue specific stem cells offers many advantages over other stem cells, the accessibility, stable phenotype and tissue type compatibility restricts their clinical use (Coppes and Stokman. 2010). In addition, many patients with head and neck cancer are old and gland tissues are atrophic in older patients, therefore isolating sufficient number of stem cells and expanding them for autologous grafting may be difficult. Moreover, establishing optimized culture conditions for each patient may be difficult (Sumita et al., 2011). Therefore, another source of stem cell is necessary for clinical use in future.

I.6.2.3. Embryonic Stem Cell as a Source of Tissue Stem Cells:

Embryonic stem cells have been used for regeneration of neural tissue, lung and skin in animal models (Placantonakis et al., 2009; Guenou et al., 2009; Wang et al., 2010). Alveolar epithelial lung progenitor cells derived from hESC have been reported to abrogate acute lung injury in mice (Wang et al., 2010). In addition neural cells derived from hESCs have shown

promising results in obtaining a homogeneous population of specialized cells (Placantonakis et al., 2009).

In October 2010 the first phase-I clinical trial using hESCs-derived oligodendrocyte progenitor cells was started as a multicenter trial. The objective of this study was to examine the safety and feasibility of these cells in patients with acute spinal cord injury. The second I/II phase trial was started in November 2010 for patients with Stargardt`s macular dystrophy. In this study retinal cells derived from hESC will be injected in the eyes of the patients (www.clinicaltrials.gov). In another effort French researchers are trying to coordinate a clinical trial by applying cardiac cells derived from hESCs (AFSSAPS). Therefore, salivary gland progenitor cells derived from hESCs cells may provide a transplantable cell source for regeneration of salivary glands.

I.7. Embryonic Stem Cells:

Development starts with totipotent zygote that gives rise to all body tissues. The zygote forms a blastocyst after 7-8 cell divisions. The outer wall of blastocyst, called trophoblast, adheres to the uterine wall and the inner cell mass generate the embryo. hESCs are derived from the inner cell mass when the fetus is at the blastocyst stage which is usually day 5 post-fertilization (Trounson, 2006) (Fig.4). Cultured hESCs have self-renewal potential and can be induced to differentiate into the three germ layers including ectoderm, mesoderm and endoderm (Thomson et al., 1998; Chen et al., 2009), and therefore hold great promise for regenerative medicine.

Three transcription factors, Oct-4, Sox-2 and Nanog, are core pluripotency and self renewal factors for hESCs (Orkin, 2005). Co-culturing of hESCs on fibroblast feeder cells or culturing them on Matrigel (an extracellular matrix derived from mouse sarcoma) or natural laminin maintains them in the undifferentiated state (Xu et al., 2001).

Differentiation of hESCs is induced when cells are cultured in suspension to form embryoid bodies which can be further differentiated into diverse cell lineages. This recapitulates many aspects of cell differentiation during early embryogenesis. The disadvantage of the embryoid body is that they inevitably contain multiple cell lineages and cell types. Therefore, new protocols are being developed to direct differentiation of hESCs in monolayer cultures toward a specific cell lineage (Karp et al., 2006).

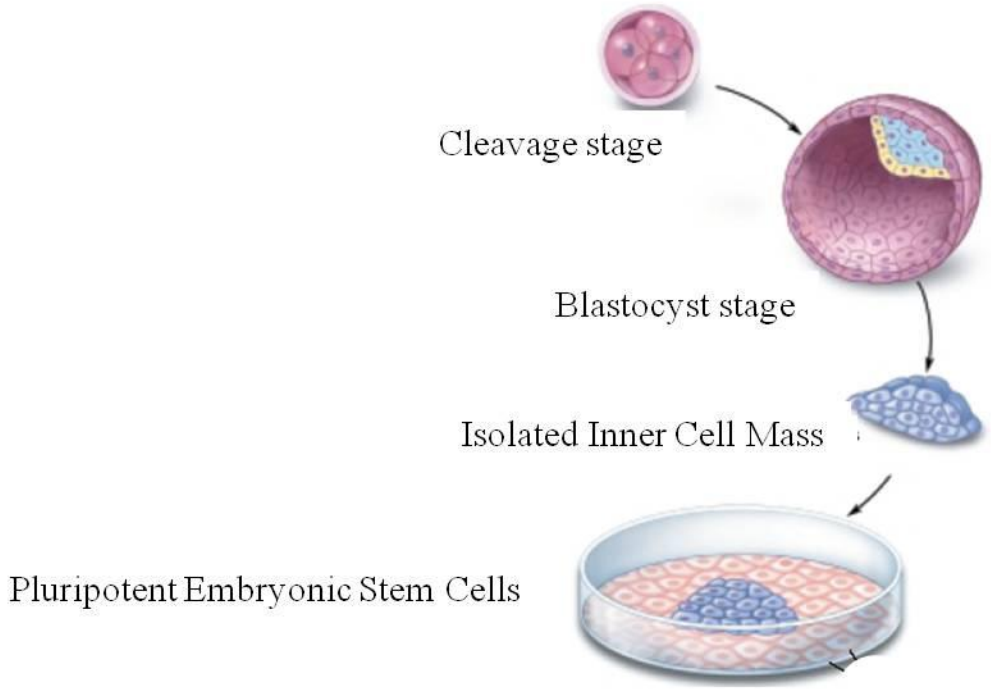

Figure 4. **Derivation of hESCs from early embryos**. Blastocyst-stage embryos are chosen for derivation of hESCs. The inner cell mass is isolated, mechanically or by immunosurgery, for further isolation of hESCs (Adapted fromhttp://we.vub.ac.be).

I.8. Directed Differentiation of hESCs:

Efficient and reproducible differentiation of hESCs to specific cell lineage offers an unlimited source of cells for transplantation and regeneration of defective tissues. The goal of *in vitro* differentiation protocols is to induce differentiation by mimicking embryonic developmental steps. Embryogenesis is a complex, orchestrated and stepwise process. Cells within the inner cell mass (ICM), where embryonic stem cells are extracted from, give rise to either primitive endoderm or epiblast cells. Primitive endoderm gives rise to parietal endoderm and visceral endoderm that together with the trophoblast form extra-embryonic tissues. Epiblast cells give rise to three germ layers of the embryo: endoderm, ectoderm and mesoderm (Beddington et al., 1999). Endoderm ultimately forms endodermal organs including the gut, pancreas, liver, lung and submandibular/sublingual salivary glands. Ectoderm gives rise to tissues such as skin, neurons and parotid salivary gland. All these complex processes is highly regulated and although many signals and cytokines that govern developmental steps have been identified in many organs based on the results from model organism studies. Still there is no culture system which can recreate all signals present *in vivo* (Roelandt et al., 2010).

While modeling the early steps of embryonic development *in vitro* may provide the best method for generating differentiated cells, providing protocols for differentiation of pluripotent stem cells is a very complicated, empirical task. Step-wise differentiation protocols for generation of endoderm and ectoderm derived tissues have been reported (Roelandt et al., 2010; Troy and Turksen. 2005; Guenou et al. 2009). hESCs treated with Activin A differentiate to endoderm lineage as Activin A activates Nodal pathway which plays a key role in endodermal differentiation (D'Amour et al., 2006; McLean et al., 2007; Kroon et al., 2008). hESCs treated with BMP4 and ascorbic acid have been shown to differentiate toward ectoderm (Guenou et al. 2009). During gastrulation, BMP-4 promotes epidermal commitment; therefore it is one of the key growth factors in epidermal induction (Munoz-Sanjuan and Brivanlou, 2002)

Salivary glands develop from epithelial-mesenchymal interactions. They develop initially as epithelial invaginations which form buds, and then undergo branching morphogenesis and differentiation. The salivary gland is a complex organ with epithelial, myoepithelial and neuronal compartments. Parotid gland is derived from ectoderm lineage while submandibular and sublingual glands develop from endodermal lineage (Redman 2008). Thus, to mimic the

developmental stages of epithelial cells of salivary glands during embryogenesis, the first step is to induce differentiation of hESCs to either ectoderm or endoderm and then providing the necessary growth factors normally provided by the surrounding mesenchymal tissue to induce differentiation toward the salivary lineage. Although the effects of several growth factors including FGFs, BMPs and TNFs during development, morphogenesis and differentiation of salivary glands have been studied (Harunaga et al., 2011). To date, no attempt has been made to direct differentiation of ES cells toward salivary gland cells

I.9. Transgenesis of ES cells:

Generation of hESC lines expressing fluorescent reporters from lineage-specific promoters is an important first step toward development of strategies for directed differentiation of hESCs to any specific lineage including the salivary lineage (Braam et al., 2008; James et al., 2010). Two gene transfer method are currently used to engineer transgenic ES cell lines. These include bacterial artificial chromosome (BAC) mediated and lentiviral vector (LVV)-mediated transgenesis.

In BAC-mediated transgenesis, the entire regulatory elements of the gene of interest are present which ensures that the expression of transgene matches closely to that of the endogenous gene. BAC-mediated transgenesis of hESC lines have been recently described (Placantonakis et al., 2009). Although this system allows more restrictive tissue-specific gene expression when compared to the promoter fragment approaches, chromosomal integration of the modified BAC is inefficient (Gray et al., 2011) and working with BACs is complex and difficult.

Recombinant lentiviral vectors are the most efficient method for stable gene transfer. These vectors can transduce both dividing and non-dividing cells and integrate into the host genome with very high efficiency (Chang et al., 2001). Unlike adenovirus, lentiviral vector-mediated gene transfer results in stable integration of new genetic material into host cell genome which is required in lineage tracing experiments. Lentiviral vectors have been used successfully for production of transgenic mice by targeting mouse embryonic stem cells. When transduced clones were injected into blastocyte stage embryo, chimeric offsprings were generated. Successful germ line transmission of the gene targeted embryonic stem cells has confirmed the permanent integration of the transgene of interest into embryonic stem cells (Michalkiewicz et al., 2007;

Park 2007; Okada et al., 2009). Compared to other methods, lentiviral-mediated transduction of the H1 and H9 hESC lines is highly efficient (Cao et al., 2010) and does not alter the pluripotency potential of hESCs (Gropp et al., 2003).

I.10. Goal of Thesis:

ESC-derived cells have been shown to improve the function of damaged tissues and their use has been studied in many animal models (Cui et al., 2011; Mignone et al., 2010). However, to our knowledge, there is no published study to examine the ability of ES-derived cells to improve functional outcome of damaged salivary gland. This study is aimed to engineer a hESC line expressing enhanced green fluorescence protein (EGFP) under the control of salivary-specific promoter as a first step toward development of strategies for directed differentiation of ES cells to salivary gland epithelial progenitor cells and eventually for regeneration of damaged salivary glands.

Chapter II. Materials and Methods

II.1. Cells:

II.1.1. Culture Conditions for Growth of Various Cell Types:

Human foreskin keratinocytes (hKC) were grown in cultures in the presence of irradiated 3T3 cells using keratinocyte medium (Wu et al., 1982) supplemented with 5% fetal bovine serum (FBS). Human foreskin dermal fibroblasts (hFBs), HepG2 (hepatocellular carcinoma line) and Hela-70 (cervical carcinoma line) cells were grown in Dulbecco's minimal essential medium (DMEM) (Cat# 11885, Invitrogen) supplemented with 10% FBS and antibiotics including penicillin (100 U/ml) and streptomycin (100 µg/ml). PC3 (Prostate Adenocarcinoma) were grown in F-12K media (Cat# 30-2004, ATCC) supplemented with 10% FBS and antibiotics. 293T cells were grown in DMEM (Cat# 21013, Invitrogen) supplemented with 10% FBS and antibiotics.

II.1.2. Human Embryonic Stem Cell (hESC) Culture:

Human ESC (WA07-FTDL-1) at passage 28 were purchased from National Stem Cell Bank, (NSCB, Madison, WI) and maintained on gamma-irradiated mouse embryonic fibroblasts (γMEF) according to NSCB protocols. Each well of a 6 well plate was coated with 2 mls of 0.1% gelatin (Sigma) in water and incubated at 37°C for at least 3 hours. Gelatin was removed and wells were washed once with DPBS. hESCs were cultured on 0.1% gelatin coated 6-well plates in DMEM-F12 (Cat# 11330, Invitrogen) supplemented with 20% Knockout Serum Replacement (Cat# 35050061, Invitrogen), 1 mM L-glutamin (Cat# 10828, Invitrogen), 0.1 mM β-mercapthoethanol, 1% nonessential amino acids (Cat# 11140-050, Invitrogen), 5 ng/ml basic fibroblast growth factor (bFGF; Cat# 100-18B, Peprotech) and antibiotics. hESCs were passaged following collagenase IV (Cat# 17104019, Invitrogen) treatment every 7-10 days , depending on the cell density. Cells were treated with 1 mg/ml collagenase IV in Knock-Out DMEM (Cat# 10829018, Invitrogen). When the edge of colonies were about to be lifted from the plate, cells were rinsed twice with Dulbeco`s Phosphate Buffered Saline (DPBS, Ca^{2+} and Mg^{2+} free), culture medium was added and colonies were mechanically dispersed into small clusters using a 1 ml micropipette and passaged at 1:3 split ratios.

II.1.3. Isolation of Mouse Embryonic Fibroblasts (MEF):

MEFs were isolated from CF-1 mice (code 023, Charles River, Wilmington, MA) at embryonic day 13 (E13) and cultured in DMEM (Cat# 21013, Invitrogen) supplemented with 10% FBS and penicillin (100 U/ml) and streptomycin (100 µg/ml). To isolate MEFs, a pregnant mouse at day 13 post-conception was sacrificed, rinsed with 70% ethanol and then allowed to dry. Using sterile techniques an incision was made across the abdomen and skin was grasped and drawn back to expose the viscera of the gut. The uterus was cut between each embryo and sections with a single embryo were placed into a beaker containing sterile DPBS on ice. The uterus pieces were squeezed with forceps to expel the embryo. Embryos were washed with DDPBS in a fresh beaker to remove any remaining maternal blood. Cleaned embryos were transferred into a 10 cm dish. The abdominal wall was incised vertically and pink hematopoetic tissue (liver and spleen) and tubular intestine was removed. The central nervous system was removed by dissecting head and neck away above the level of oral cavity. Embryos were then transferred to a beaker containing 0.5 ml 0.25% trypsin/embryo and minced by blunt-end scissors for 2-3 min to create bits smaller than 2 mm. Minced embryos were suspended in 10 ml of 0.25% trypsin/EDTA and transferred into 50 ml conical tube and incubated in 37°C water bath for 30 min. Cells were vigorously triturated several times with a wet 10 ml pipette to further dissociate tissue bits. Ten ml MEF media was added to inactivate the trypsin. Cells were centrifuged at 180×g for 5 min. Supernatant was aspirated and the cell pellet was resuspended in fresh media (2 ml MEF media for each embryo). Isolated cells were cultured at a density of 6×10^6 cells per each 10 cm dish. It took 3 days for the cultures to become confluent. Passage 0 (P0) cells were either cryopreserved or passaged at 1:4 ratios up to P4. Cells at the fourth passage were irradiated with 5000 R in a Cesium radiation source for 7 min. Gamma irradiated MEFs (γMEF) were cryopreserved in 90% MEF media and 10% DMSO.

II.1.4. Culturing Irradiated MEFs:

Irradiated MEFs were seeded at $5x10^5$ in a gelatin-coated well (10 cm^2) and incubated overnight in MEF media. Next day MEF media was removed, the plate was washed once with

DMEM-F12 to remove remnants of MEF media and replaced with hESC media. hESCs were seeded on these feeder cells.

II.1.5. Preparation of Matrigel Coated Plates:

To coat plates with Matrigel, a 1:100 dilution of growth factor reduced Matrigel (Cat# 356230, Becton Dickinson, Bedford, MA) in cold DMEM/F12 was made and 2 ml was added to each well of a 6-well plate and allowed to polymerize for 1 h at room temperature. Excess Matrigel was removed and wells were rinsed once with DPBS before seeding the cells.

II.1.6. Preparation of MEF-Conditioned Media:

hESCs were maintained in MEF-conditioned media on Matrigel. γMEFs were seeded at $5\times10^4/cm^2$ in MEF media. After 4 h, medium was replaced with hESC media. Conditioned medium was collected daily, stored at 4°C and supplemented with an additional 4ng/ml bFGF before feeding hESCs. γMEFs were used for 7-10 days for collection of conditioned medium.

II.2. Recombinant Lentiviruses

II.2.1. Construction of Lentiviral Vectors:

A lentiviral vector in which expression of enhanced green fluorescent protein (EGFP) is driven by human cytokeratin 19 (hK19) promoter was constructed. A 2952 bp upstream regulatory sequences of hK19 was excised from pACK19-EGFP (provided by Dr. Zheng, NIH,Bethesda, Maryland) using HindIII restriction enzyme and ligated to HindIII restriction site of pBluescript. Then it was excised using ClaI/BamHI restriction enzymes and inserted into Cla I/BamH I site of Lenti-CMV-EGFP-Pgk-Puro plasmid (658-5,Addgene) to replace the CMV promoter. EF1α promoter was excised using Cla I/BamH I restriction enzymes from Lenti-EF1α-IRES-EGFP-wsin18 and inserted to Cla I/BamH I site of Lenti CMV-EGFP-Pgk-Puro plasmid to be used as a control. Stbl2 bacteria (Invitrogen) were used for transformation. DNA constructs were verified with BamHI/CalI and EcoRI restriction enzymes, grown in Stbl 2 bacteria and plasmid was purified using a midi preparation kit (Zymo Research).

II.2.2. Generation of Recombinant Lentiviruses:

Lentiviral vectors were transfected into 293T cells, using calcium phosphate co-precipitation protocol to generate recombinant lentivirus. The day before transfection, $5x10^6$ 293T cells were plated in 10 cm dishes and the following morning 293T cells were triple co-transfected with the transfer vector, pMD.G and pCMV^ R8.91 as follows: To a 15 ml polystyrene tube 1.5 mls of 2X HBS (50 mM Hepes pH 7.1, 280 mM NaCl, 10 mM KCl, 12 mM Dextrose, and 1.5 mM Na2HPO4.2H2O) and 10 μg plasmid pMD.G, 20 μg plasmid pCMV^ R8.91 and 20 μg of either EF1α-EGFP-Pgk-Puro or hK19 -EGFP-Pgk-Puro were added. While vortexing the DNA/HBS solution, equal volume of 0.25M $CaCl_2$ was added drop-wise and allowed to precipitate at room temperature for 5 min. Precipitates were added drop-wise to culture media in presence of 25 μM chloroquine and incubated for 8-10 hrs. Medium was then removed and replaced with 6 ml of fresh media. Virus containing media was collected at 24 and 48 hrs post transfection, filtered through a 0.45 μM pore size filter and stored at -80 ºC until used.

II.2.3. Titration of Lentiviruses:

To determine viral titer, 5×10^4 293T cells were plated per well of a 6-well dish. The next day, 293Ts were transduced with 2, 10, or 50 μl of each virus plus 8 μg/ml polybrene in 500 ul of medium. Cells in a representative well were counted to determine the cell number at the time of transduction. Cells were incubated with virus for 3 hours, while rocking every 30 min. After 3 hrs, virus-containing medium was replaced with fresh medium. Two days later, transduced cells from each well were trypsinized and the percentage of EGFP-expressing cells were determined by flow cytometry. To determine the viral titer (transducing unit/ml) the percentage of EGFP positive cells was multiplied by the number of cells at the time of transduction and divided by the volume of viral supernatant.

II.2.4. Transduction of hKCs, hFBs, 293T, HepG2 and PC3 cells:

For transduction, cells were seeded at 5×10^4 cells/well in six-well plates and transduced the next day at a multiplicity of infection (MOI) of 1 or 2 as described in section 2-3. After 3 h, fresh medium was added and 2 days after transduction, cells were either passed or harvested for FACS analysis.

II.2.5. Transduction and Puromycin Selection of hESCs:

Two days before transduction, hESCs grown on Matrigel were dissociated with 0.05% trypsin/EDTA and 3×10^{5} hESCs were seeded in each well of 6-well Matrigel-coated plates in γMEF conditioned medium supplemented with 4 ng/ml bFGF. hESC were transduced with Lenti hK19 promoter-EGFP-Pgk-Puro and EF1α-EGFP-Pgk-Puro at multiplicity of infection (MOI) of 2 for 12 hrs in 2 ml of media in the presence of 8µg/ml polybrene. Medium was then replaced with 2 ml of γMEF conditioned media. Transduced ESCs on Matrigel were selected by 300 ng/ml puromycin starting at 48 h post-transduction for 2 days. Transduced colonies on Matrigel were dissociated with 0.05% trypsin/EDTA and transferred to the MEF coated wells. EGFP expression was analyzed using fluorescent microscopy.

II.3. Flow Cytometric Analysis of EGFP:

For analysis of EGFP-labeled cells, cells were trypsinized with 0.1% trypsin, washed twice with DPBS, resuspended in 500 µl of DPBS and analyzed by flow cytometry analysis. Cells were fixed in 2% PFA and stored at 4 ºC (in the dark) if they were not analyzed immediately. The median fluorescent intensity was used to compare EGFP expression in transduced cells.

II.4. mRNA Expression Analysis:

Total RNA was isolated by Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After quantification of RNA samples, 1µg of total RNA samples were analyzed on a 1% agarose gel to confirm RNA integrity. Keratin 19 transcript levels were analyzed in 0.125 µg of total RNA by semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using the one step RT-PCR kit (Qiagen, Valencia, CA) and a set of specific primers indicated below. GAPDH was used as the house keeping gene. Conditions for K19 and GAPDH are as follows respectively: 50 ºC 30 min, 95 ºC 15 min; variable cycles of 94 ºC 30 sec, 55 ºC 30 sec, 72 ºC 1 min; and final extension at 72 ºC 10 min; All samples were analyzed by 1 % agarose electrophoresis after staining with ethidium bromide.

Primers used are as follows:

GAPDH forward: 5'ccccttcattgacctcaactac 3'

GAPDH reverse: 5 gagtccttccacgataccaaag 3

hK19 promoter forward: 5 aagctaaccatgcagaacctcaacgaccgc 3

hK19 promoter reverse: 5 ttattggcaggtcaggagaagagcc 3

II.5. Alkaline Phosphatase Staining:

hESCs grown on either MEF or Matrigel were washed with PBS and fixed in 4% PFA for 10 min at room temperature. Fixed cells were rinsed with PBS-0.05% Tween-20 (Cat # 23336-2500) three times. Cells were incubated with X-P buffer (100 mM Tris-Hcl, 100 mM NaCl, 5mM $MgCl_2$) for 10 min at RT. Equal volumes of the two component solution of Alkaline Phosphatase Blue Membrane Substrate Solution (Cat # AB0300-1, Sigma) containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) were mixed. Cells were incubated in X-P buffer in dark at RT for one hour. EDTA (20 mM) was used to stop the reaction.

Chapter III. Results

III.1. Generation of Reporter DNA Constructs and Titration of Recombinant Lentiviruses.

K19 may serve as a marker for salivary gland progenitor cells. Human K19 upstream regulatory sequences have been characterized and the transcriptional activities of various fragments (0.7 Kb bps, 2.2 Kb bps and 3.0 Kb bps) have been tested in salivary gland cells *in vitro* and *in vivo* (Zheng et al., 2005). These studies demonstrated that the 3.0 kb fragment has the highest transcriptional activity. AAV-K19-EGFP has been demonstrated to be active in both acinar and ductal cells with higher levels of activity in ductal cells (Zheng et al., 2005).

To generate a reporter construct for salivary lineage differentiation, a lentiviral vector was constructed in which EGFP expression was driven by upstream 2.9 kb regulatory region of hK19 (K19-EGFP). This vector also contained a puromycine-resistance gene under the control of the Pgk promoter for drug selection. Human EF1-α promoter, a ubiquitously active promoter which has been shown to be active in both undifferentiated ES cells and their differentiated derivatives (Chung et al., 2002) was used as a control (EF1α-EGFP) (Fig.5). Vesicular somatitis virus glycoprotein (VSV-G)-pseudotyped second generation recombinant lentiviruses were generated by transient transfection of 293T cells as described in "Material and Methods". Virus-containing supernatant was collected 24h and 48h after transfection. To determine the lentiviruses titration, 293T cells were transduced with different concentrations of each virus as described in Materials and Methods section. The percentage of EGFP-expressing cells was determined by flow cytometry (Fig.6B-C). The viral titer was calculated by the percentage of EGFP positive cells multiplied by the number of cells at the time of transduction and divided by the volume of viral supernatant. The viral titer for both viruses was 6.5×10^6 U/ml.

Figure 5. Schematic representation of strategies used to construct lentiviral vectors in which EGFP expression is under the control of either the K19 (A) or the EF1α promoter (B).

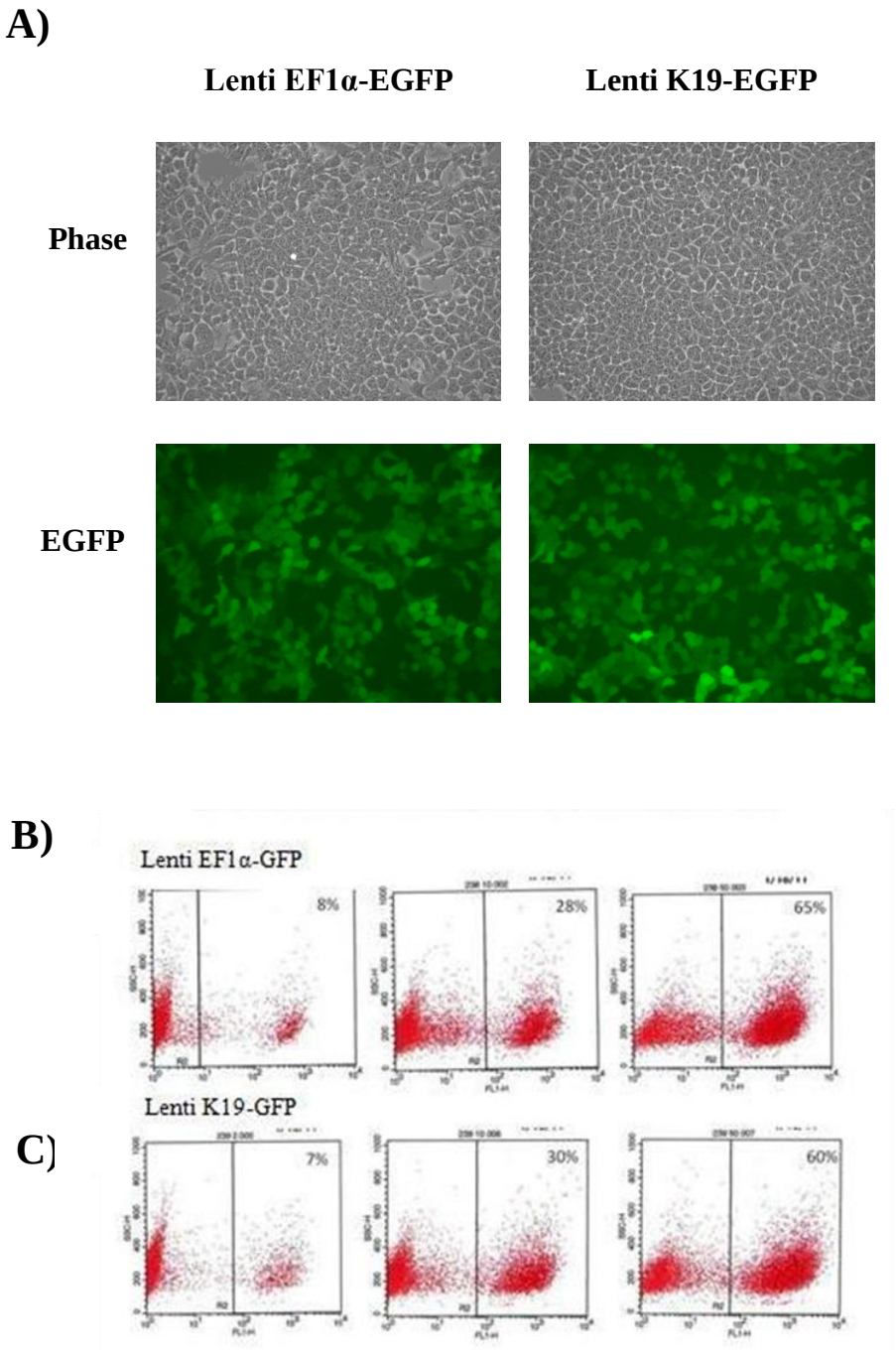

Figure 6. Analysis of viral titer and EGFP expression in 293T cells. A) Phase contrast and fluorescent microscope images of 293T cells transduced with 50 ul of LVV-EF1α-EGFP or LVV-K19-EGFP. Exposure time was the same for both images. B-C) Flow cytometry analysis 293T cells transduced with increasing volume of viral supernatant demonstrating the percentage of EGFP positive cells. These values were used for viral titer calculation. The viral titer for both viruses was 6.5×10^6 U/ml.

III.2. Comparison of hK19 Promoter and EF1α Promoter Activity:

Previous studies in our lab have shown that at transduction efficiencies below 30%, most cells that are transduced have one copy of the virus allowing a comparative analysis of promoter activity (Ghazizadeh, et al. 2004). To determine the level of activity of K19 promoter in the context of lentiviral vectors, 293T cells were transduced with the lentiviral vector LVV-EF1α -EGFP or with LVV-K19-EGFP at increasing MOIs and analyzed by FACS. EGFP expression level, as measured by median fluorescent intensity (MFI), was used to assess promoter activity. As shown in Figure 7 the activities of these two promoters in 293T cells were comparable indicating similar promoter activities for EF1α and K19 promoters in a permissive cell.

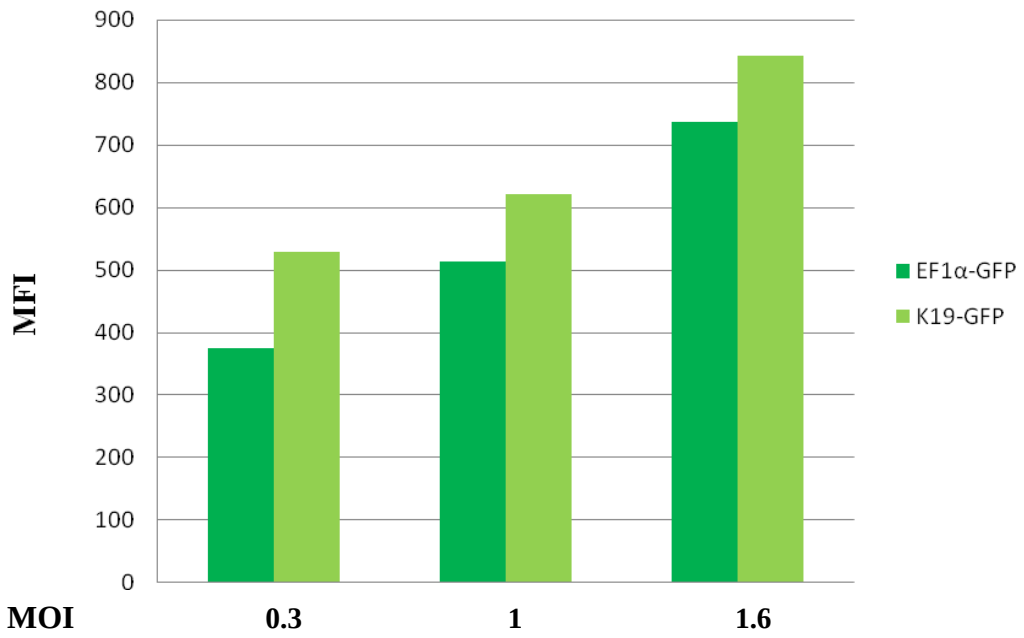

Figure 7. **Comparative analysis of EF1α and K19 promoter activity in 293T cells.** 293T cells were transduced with LVV EF1α or LVV-K19-EGFP at multiplicity of infections (MOIs) of 0.3, 1 and 1.6 and EGFP expression was analyzed by flow cytometry. Median fluorescent intensity (MFI) was used to compare EGFP expression levels. EF1α and K19 promoter transcriptional activities were comparable in a permissive cell line.

III.3. Identification of a Cell Line with High Levels of K19 Expression:

To determine the lineage specificity of K19 promoter in the context of lentiviral vectors, epithelial and non-epithelial cell lines were screened to identify a cell line with high expression of K19. It has been reported that Hela (Cervical carcinoma line), HepG2 (Hepatocellular carcinoma line) and PC3 (Prostate adenocarcinoma) cells express K19 at higher levels than non-carcinoma cells (Gorreta et al., 2005; Nagle et al., 1987). Therefore, K19 expression levels were assessed by RT-PCR in various cell lines including Hela, HepG2, PC3, hESC, hKC and hFB. K19 transcript was detected in PC3 cells and HepG2 cells but not in Hela, hESCs, hKCs and hFBs (Fig.8). The levels of K19 expression was the highest in PC3 cells followed by HepG2 cells.

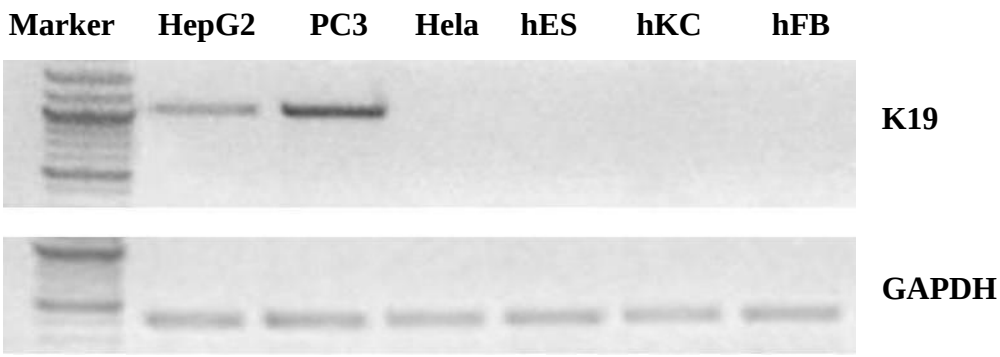

Figure 8. **Analysis of K19 transcript levels in various cell lines**. K19 transcript levels in hKC, hFB, hES, HepG2 and PC3 and Hela cells were determined by RT-PCR at 36 cycles. GAPDH was used a housekeeping gene. The highest level of K19 expression was detected in PC3 cells followed by HepG2. K19 transcript was not detectable in Hela, hESCs, hKCs and hFBs.

III.4. Comparative Analysis of Transgenic K19 Promoter Activity in Various Cell Lines.

A major criterion for a lineage-specific reporter construct is that the promoter activity is expected to increase upon up-regulation of endogenous gene during lineage commitment and differentiation. To determine if the transgenic K19 promoter activity was proportional to endogenous K19 gene expression, K19-low and high expressing cells were transduced with LVV-K19-EGFP or LLV-EF1□-EGFP and analyzed by FACS to determine EGFP expression levels. As the transduction efficiency and the levels of EF1α-driven EGFP were varied between different cell lines (Fig.9A), cultures were transduced at various MOIs to allow comparable transduction efficiencies, and the ratios of EGFP expression mediated by K19 promoter to that mediated by EF1α promoter in each cell line were determined. As shown in Figure 9B, the ratio of K19-EGFP to EF1□-EGFP was the highest in PC3 cells indicating higher K19 activity in these cells when compared to other cell types expressing low levels of endogenous K19. Both hKCs and hFBs showed similar low levels of K19 promoter activity and that of HepG2 cells was at intermediate levels. Overall, these data demonstrated that the transgenic hK19 promoter has higher activity in cells that express K19 indicating the specificity of transgenic K19 promoter activity.

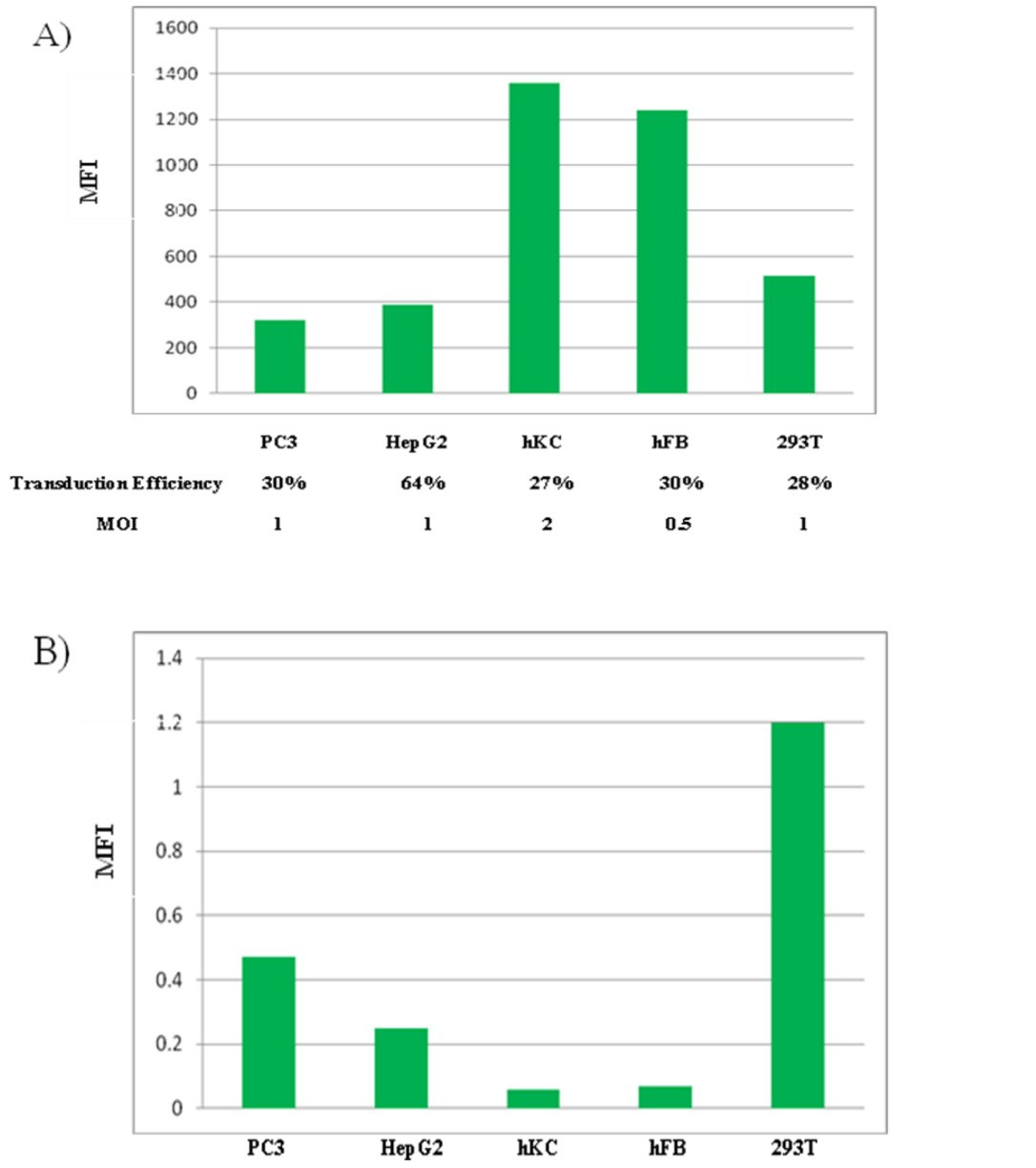

Figure 9. Comparative analysis of EF1α promoter and K19 promoter activity in hKC, hFB, HepG2, PC3 and 293T cells. A) Variable levels of EGFP driven by EF1α promoter was detected in various cell lines. Cells were transduced at MOIs of 1 or 2 to allow comparable transduction efficiencies and EGFP expression levels were analyzed by FACS. B) K19-driven EGFP expression is proportional to the levels of endogenous K19. The ratio of K19 to EF1α promoter activity was used to compare K19 promoter activity in various cell lines. The highest activity was observed in PC3 cells followed by HepG2, hFB and hKC.

III.5. Establishment of H-7 hESC in Culture:

Human embryonic stem cells (H-7: WA07-FTDL-1, NSCB, Madison, WI) were maintained either on γMEF feeder layer or on Matrigel and used at passage 30 to 36. hESCs grown on γMEF were passed following digestion with collagenase IV and those grown on Matrigel were passed following trypsinization as described in Materials and Methods. It has been reported that continuous culture of hESCs on Matrigel for over one year does not affect pluripotency, the expression of surface markers and transcription factors. However, subtle changes in morphology of the cells maintained in long term culture of hESCs in feeder free conditions has been reported (Rosler et al., 2004). In my hands cells seeded on Matrigel were less compact in comparison to the cells grown on feeders (Fig.10B). As shown in Figure 10C and D, colonies grown in both conditions were positive for alkalaine phosphatase (blue) staining confirming their undifferentiated state. Cells were passed approximately every 7-10 days, depending on cell condition and confluency.

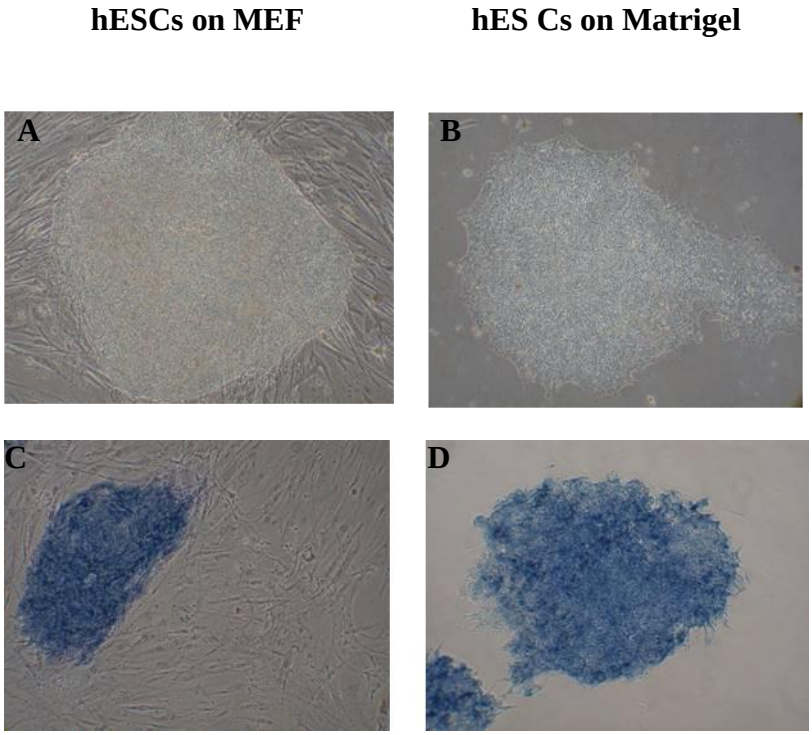

Figure 10. Morphology and alkaline phosphatase staining of hESC colonies grown on MEF or Matrigel. A-B) Morphology of hESC cultured on MEF feeder layer (A) or Matrigel (B) showing compact colonies with defined borders. C-D) Alkaline phosphatase staining of hESCs cultured on MEF feeder layer (C) or Matrigel (D). Undifferentiated colonies are positive for alkaline phosphatase.

III.6. Transduction of hES Cells with LVV- EF1α-EGFP and LVV-K19-EGFP

To determine the efficiency of lentivirus-mediated transduction of hESCs, cells were seeded on Matrigel at 3×10^4 cells/cm^2 and were transduced the next day with LVV--EF1α-EGFP. Transduction of hESCs with LVV-EF1α-EGFP at MOI of 2 resulted in transduction efficiency of 40%. Therefore, hESCs at passage 34 were transduced as described above with either LVV-K19-EGFP or LVV-EF1α-EGFP and EGFP expression was analyzed by Fluorescent Microscopy. As expected high levels of EGFP expression was observed in approximately 40% of cells transduced with LVV-EF1α-EGFP (Fig.11A), while no EGFP positive cells were observed in hESCs cultures transduced with LVV-K19-EGFP (Fig.12A).

Transduced cultures were selected in 300 ng/ml puromycin for 3 days and then transferred to MEF coated plates. As shown in Figure 11B almost all of the hESCs transduced with LVV-EF1α-EGFP were EGFP positive after puromycin selection. There were a few EGFP negative cells in culture transduced with LVV-EF1α-EGFP which was likely due to promoter interference and silencing of EF1□ promoter upon selection for puromycin-resistance which is controlled by Pgk promoter. All EF1α-EGFP- transduced colonies maintained EGFP expression after two passage (Fig.11C) indicating stable transgenesis of hESCs.

As shown in Figure 12, no EGFP was detected in the majority of cells in K19-EGFP-transduced cultures. There were a few EGFP positive cells in the first passage after transduction (Fig. 12 B). However, GFP expression was lost upon further passaging of these cultures (Fig. 12 C). Transient expression of K19-EGFP could be due to culture conditions which may have resulted in differentiation of a small number of hESCs and hence upregulation of K19 expression.

Overall, these data indicate that the transgenesis of a hESC line with lentiviral vector encoding K19-EGFP resulted in minimal or no EGFP expression while high levels of EGFP was detected in those transduced with EF1□-EGFP. This transgenic line will be useful in high through-put assays to define conditions that reproducibly and efficiently induce differentiation of salivary lineage

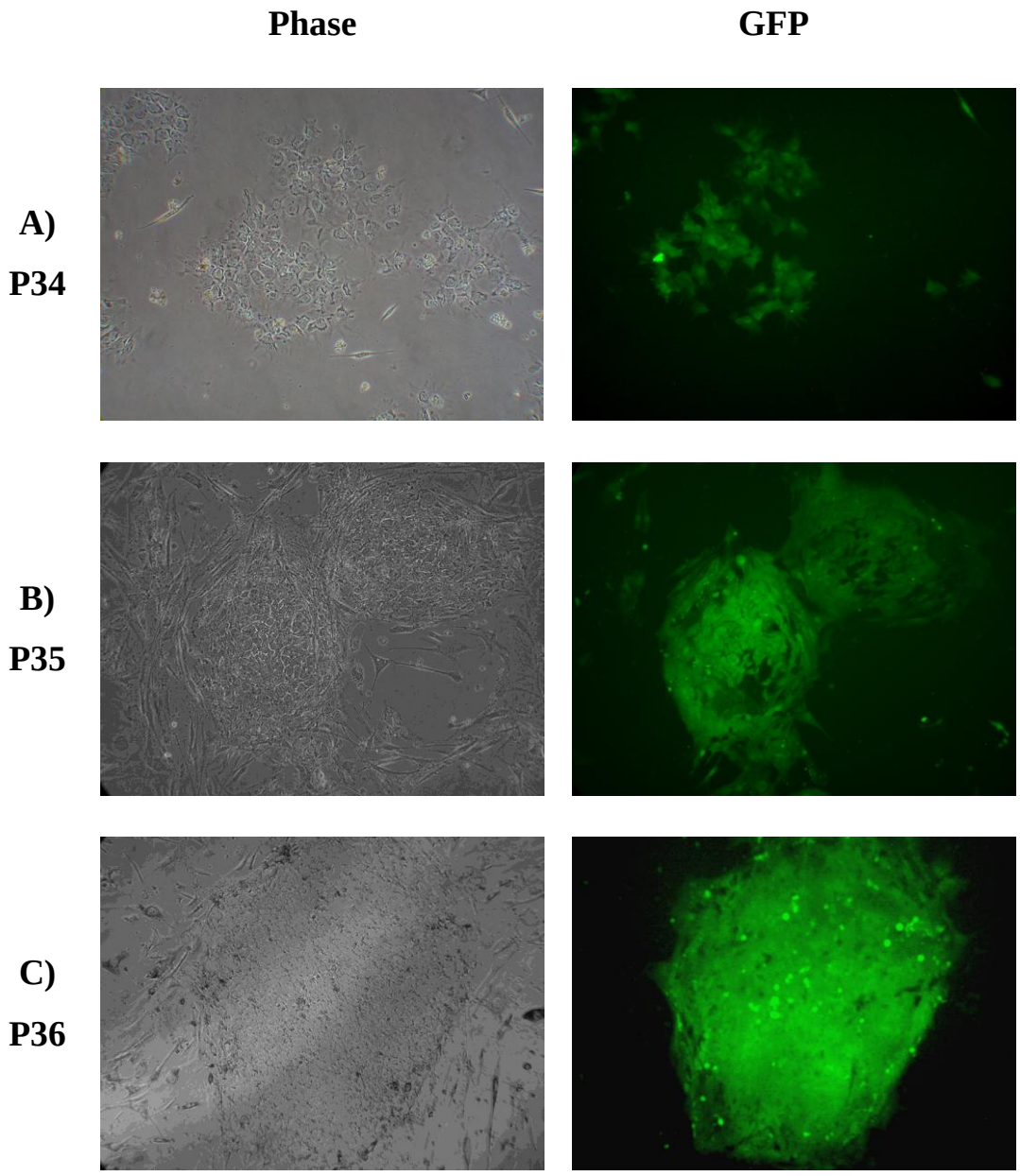

Figure 11. **EGFP expression of hESCs transduced with LVV-EF1α-EGFP at different passages**. Phase contrast and fluorescent microscope images of hESCs transduced with Lenti-EF1α-EGFP. A-B) hESCs grown on Matrigel (P34) express EGFP two days after transduction . C-F) Almost all of LVV-EF1α-EGFP transduced hESCs (P35-36) grown on MEF are expressing EGFP after puromycin selection.

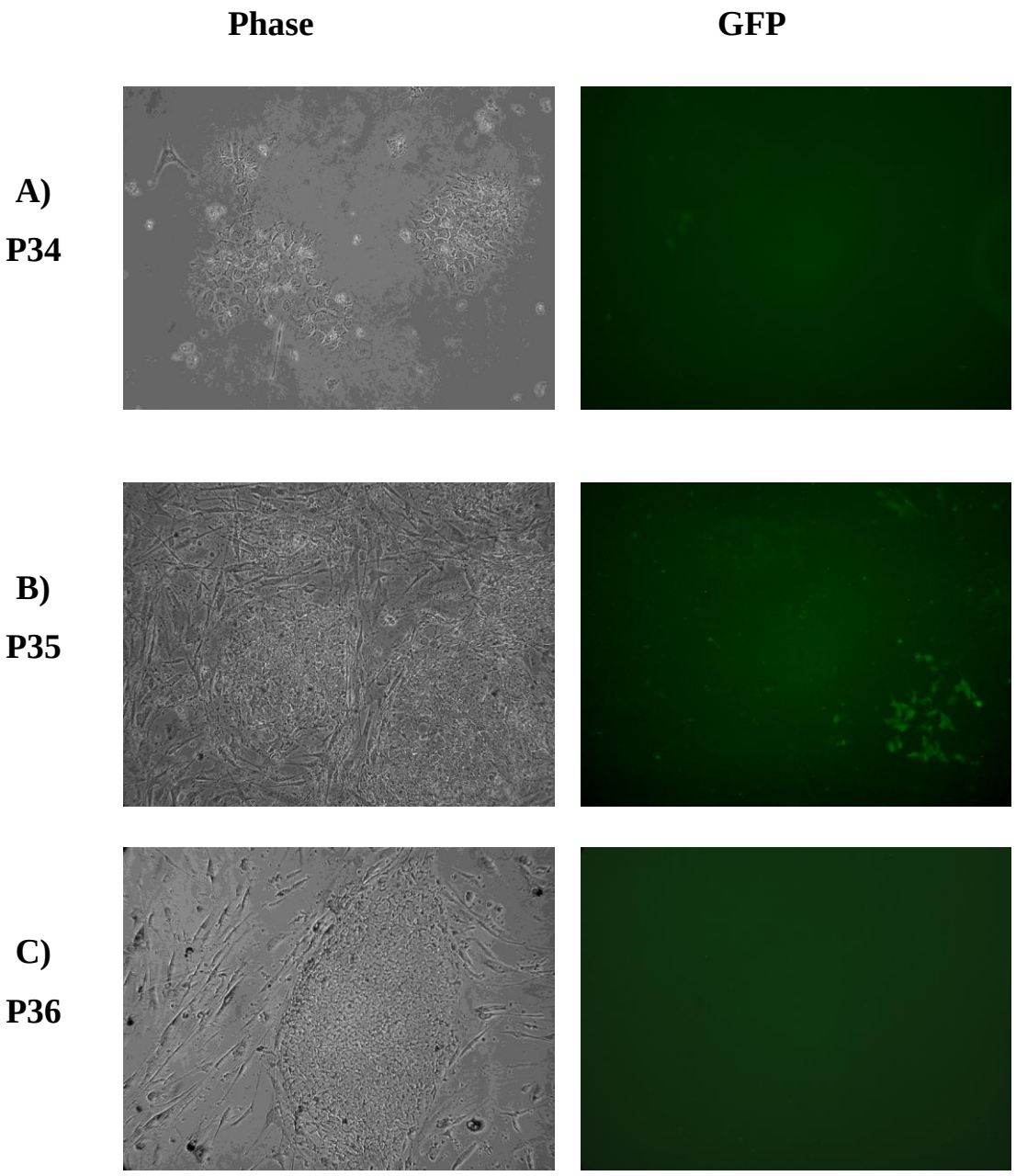

Figure 12. **Lack of EGFP expression in hESCs transduced with LVV-K19-EGFP**. Phase contrast and fluorescent microscope images of hESCs transduced with Lenti-K19-EGFP. A) hESCs grown on Matrigel (P34) do not express EGFP two days after transduction. B) hESCs grown on MEF (P35) showing EGFP expression in a few transduced cells (P35). C) hESCs grown on MEF (P36) lost EGFP expression.

Chapter IV. Discussion

In this study, a lentiviral vector harboring an internal K19 promoter to regulate EGFP expression was generated and successfully used to transduce a hESC line. This transgenic reporter line will be used for further screening of conditions resulting in differentiation of hESC cells toward a salivary lineage.

K19 is expressed in salivary gland progenitor cells (Knox et al., 2010). Furthermore, K19 promoter has been cloned and is available (Zheng et al., 2005). Thus, this promoter was used for this study. Other potential promoters are Ascl3 and cKit which are expressed in salivary gland progenitor cells as well. Ascl3 is only expressed in ductal cells during mouse salivary gland development. $Ascl3^{+}$ positive cells are progenitors of not all but a subset of acinar/ductal cells (Bullard et al., 2008). In addition, to our knowledge, Ascl3 promoter has not been cloned. On the other hand, although cKit is another marker of salivary gland progenitor cells, it is expressed on the surface of hematopoetic stem cells which are derived from mesoderm. cKit is not a suitable promoter candidate as for engineering salivary gland progenitor cells all effort is directed toward stepwise differentiation of hESCs toward endoderm/ectoderm and not mesoderm. Although K19 is a promising marker for screening epithelial salivary gland progenitor cells derived from hESCs, we should also bear in mind that K19 is expressed in tissues other than salivary gland such as liver and skin (Hu et al., 2007; Turksen et al., 2005). Therefore, to purify salivary gland epithelial progenitor cells from other hESC derivatives, putative salivary gland progenitor stem cell markers including cKit should be considered to eliminate possible interference from other cell lineages.

It has been shown that the lentiviral system is more favorable for stable integration of the transgene than other transgene delivery techniques in hESCs such as transfection or adenoviral transduction, which are more suitable for transient gene expression (St George et al., 2003; Siemen et al., 2005; Zwaka et al., 2003). In addition, HIV-1-derived lentiviral vectors are efficient tools for stable genetic modification of mammalian ES cells, since they are less susceptible to silencing than traditional retroviral vectors (Gropp et al., 2003; Hamaguchi et al., 2000; Jahner et al., 1982; Niwa et al., 1983; Pfeifer et al., 2002). Consistent with previous

reports, our experiments demonstrated the high efficiency of lentiviral vectors for stable gene transfer into hESCs.

Genetic modification of hESCs with a tissue specific gene promoter driving a reporter gene such as EGFP is an efficient approach to screen for growth factors/culture conditions promoting differentiation of ES cells toward a specific cell lineage (Braam et al., 2008; James et al., 2010). In addition detection of specific gene expression by reporter genes enables purification of desired cells in differentiating cultures and make it possible to track hESC derivatives in mixed cell cultures or when transplanted into animal models. Applications of EGFP-reporter cells derived from hESCs have been reported previously for analyzing stepwise differentiation of hESCs toward hepatic, pancreatic, neuronal and vascular lineages (Eiges et al., 2000; Lavon et al., 2006; James et al., 2010; Noisa et al., 2010). For instance, to define vasculogenic development of hESCs, endothelial cell specific VE-cadherin promoter driving EGFP has been used to screen for factors that promote vascular differentiation of hESCs (James et al., 2010). In order to have identifiable hESC-derived progenitor cells, we transduced hESC with hK19-EGFP lentivirus so that by application of stepwise endodermal/ectodermal differentiation protocols in future, population of hESC-derived cells which express K19 can be distinguished easily either by fluorescent microscope or FACS analysis. In all experiments EF1α-EGFP was used as a control for analyzing transduction efficiency and stability of gene transfer. Norrman et al. compared EF1α-EGFP versus CMV-EGFP long-term promoter activity in transduced hESCs and concluded that EF1α was more efficient than CMV in driving long-term expression of EGFP (Norrman et al., 2010). As shown in Figure 11, almost all undifferentiated hESC colonies were expressing EGFP after puromycin selection as expected, although there were a small population of selected cells that did not express EGFP. The lack of EGFP expression in some cells can be explained by the interference between EF1α and Pgk promoters. It has been shown that when a vector contains two genes under the control of two different promoters, the 3 gene could be suppressed when there is selection for expression of the 5' gene and the 5 gene could be suppressed when there is selection for expression of the 3 gene. The epigenetic reversible and cis-acting model has been proposed for this phenomenon in which transcription from one promoter leads to changes in the surrounding DNA structure, and therefore, transcription from a nearby promoter is inhibited. This inhibition is not absolute and transcription can occur from both promoters (Emerman and Temine., 1984).

Although we did not induce epithelial differentiation of K19-EGFP transduced hESCs, a few hESCs transduced with K19-EGFP were also EGFP positive in large colonies (Fig.12) which could be due to differentiation of a small number of cells in that colony (Schwartz et al., 2005). As hESCs are kept in culture for a prolonged period of time, it is possible that a certain percentage of cells may differentiate due to culture manipulations (Bhattacharya et al., 2004).

A reporter progenitor cell should have lineage specific activity. Thus, the potency of K19 promoter was tested in cell lines which express different levels of endogenous K19 (Fig.8). Analysis of EGFP levels in K19-EGFP transduced cell lines showed the highest K19 promoter activity in PC3 and HepG2 lines correlated with the level of K19 transcript detected in PC3 and HepG2 lines. K19 promoter displayed a 6.7 and 3.5 fold activity in PC3 and HepG2 cells respectively, when compared to hFBs. These data indicate that K19 promoter function more efficiently in cells expressing K19 and is likely to be controlled in a lineage specific manner during directed differentiation of hESC. It should be remembered that although generation of lineage reporter cell line by transgenesis is a fast and feasible method in comparison to other strategies such as knock-in or artificial chromosome approach, it has some limitations. Rearrangement or truncation of transgene could happen during integration of the transgene into the genome which may affect the pattern of gene expression. In addition, integration of a transgene into the host genome is random which also affect transgene expression (Alami et al., 2000; Noisa et al., 2010).

Although hESCs therapy is moving fast forward, three main obstacles are associated with transplantation of pluripotent stem cell derivatives: ethical issues, risks of tumorogenicity and immunogenicity. Although a teratoma is not a malignant lesion, its potential to grow makes it theoretically dangerous because it cannot be fully excluded if cells with pluripotent capacity lose control, or if the graft environment triggers dedifferentiation (Guenou et al., 2009). Immunological incompatibility between the cell donor and host is another issue to be considered. hESC-derived keratinocytes express low levels of MHC antigens suggesting low immunogenicity of the skin substitute (Guenou et al., 2009). Recent advances in reprogramming of somatic cells to produce induced pluripotent stem cells (iPS) which closely resemble embryonic stem cells may help resolve current problems related to the use of hESCs. However, the clinical use of iPS cells in regenerative medicine awaits their full characterization.

Furthermore, successful autologous stem cell therapy for autoimmune diseases such as Sjogren syndrome, requires an additional challenge to eliminate the antibodies which are responsible for the disease, or the stem cells should be modified to prevent the presentation of specific antigens that induce inflammation and tissue destruction (Coppes and Stokman., 2011).

The differentiation of hESCs into salivary gland progenitor cells is of great importance as a source of cell for treatment of salivary gland hypofunction. Although the differentiation of hESCs have been reported for several cell lines including nerve cells, cardiomyocytes, pancreatic and hepatic cells , the differentiation of hESCs to salivary gland cells has not been documented yet. In this study by using hESC genetic manipulation methodologies, we have taken the first step toward development of strategies to induce salivary-specific differentiation. By introducing EGFP under the control of K19 promoter, it may be possible to trace differentiation of hESC to salivary gland progenitors expected to upregulate K19 promoter and thus EGFP expression.

Chapter V. Future Directions

Salivary glands develop from ectodermal/endodermal embryonic layers (Redman 2008). Thus, the first step in stepwise differentiation of hESCs to salivary gland progenitors is to induce differentiation toward ectodermal/endodermal progenitor cells. Previous studies have shown that treatment of ESC with BMP4/Activin A for 6 days induce differentiation to ectoderm/endoderm lineage (Li et al., 2010; Harvey et al., 2010). Several growth factors, cytokines and extracellular matrix proteins are involved in the development of the salivary glands from embryonic endoderm/ectoderm. For instance, FGF7, FGF8, FGF10, TNFα, EGF, TGFβ, BMP7 are crucial for normal development of salivary gland. Therefore, according to diverse range of outcomes and the fact that K19 is expressed in multiple cell types, specific combinations of these factors involved in salivary gland development must be tested for their ability to induce differentiation of hESCs toward salivary gland progenitor cells.

First, differentiation capacity of human ES cells into salivary gland progenitor cells will be investigated and culture conditions will be defined for efficient induction toward salivary gland epithelial progenitor cells. Cultured ES cell-derived salivary gland progenitor cells will be examined for their ability to differentiate to acinar/ductal cells to demonstrate the potential of the progenitor cells *in vitro.* For *in vivo* analysis, $EGFP^+$ hESC derived salivary gland progenitor cells will be injected into irradiated salivary glands of immunodeficient mice to examine their ability to regenerate damaged tissue. The location and degree of differentiation of the transplanted cells could be traced based on EGFP expression. Furthermore, salivary flow rate and presence of acinar/ductal specific markers will be detected in glands to determine the effect of transplanted progenitor cells on saliva secretion and to analyze the ultimate morphology of the regenerated gland. Although, the progression of differentiation is expected to follow normal development, the inductive media may results in direct differentiation of hESCs to acinar cells which do not express K19, and therefore will not up-regulate EGFP expression. In this case, one needs to screen for expression of markers of acinar cells such as aquaporin-1.

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