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PERIPHERAL NERVE GLIA AS MULTIPOTENT PROGENITORS IN CRANIOFACIAL DEVELOPMENT

Maryam Khatibi Shahidi

Karolinska Institutet

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Cover illustration depicts a section from a PLPCreERT2/R26Confetti mouse at embryonic day 17.5. Lineage tracing was induced at embryonic day 8.5 to label migratory neural crest cells that contribute to craniofacial compartment. Clonal colour coding visualizes neural crest clones expressing cyan fluorescent protein (CFP), green fluorescent protein (GFP), red fluorescent protein (RFP), and yellow fluorescent protein (YFP). Note labelling in trigeminal ganglia and its nerve branches, the facial mesenchyme, tongue and skull bones. Traced neural crest-derived cells are also seen in molars in the maxilla and in incisor tooth structures in the mandibular jaw.

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Peripheral Nerve Glia as Multipotent Progenitors in Craniofacial Development

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“What you seek is seeking you”

Rumi

To My Family

ABSTRACT

Craniofacial development is complex. Numerous populations of progenitor cells coordinate activities to produce an array of highly integrated tissues inside the developing head. However, it is not clear how some key multipotent progenitors continue to exist in late developing head compartments. The general hypothesis of this thesis is focused around the idea of an embryonic infrastructure represented by peripheral nerves that serves as a niche for glial multipotent neural crest-like cells. The nerve-adjacent glial cells can change their fate and be recruited in a targeted way to produce tissues at remote destinations during fast growth, development and regeneration. Results presented in this thesis explain how the nerves contribute pulp cells and matrix-producing cells of odontoblast lineage to the developing and growing tooth. Glial cells as an unexpected progenitor source give rise to almost half of all pulp cells and odontoblasts in the growing incisor. Furthermore, lineage tracing with colour-coding of individual recombination events allowed us to discover new aspects of tooth development and coordination between pulp cell lineage and odontoblasts.

Another important component of the craniofacial compartment, the parasympathetic nervous system that targets glands in the head, is crucial for "rest-and-digest" or "feed and breed" activities especially during eating, salivation and lacrimation. Importantly, neurons of the autonomic parasympathetic nervous system are located very close to or inside the tissues they innervate and appear late in embryonic development. The discrepancy in developmental timing raised new questions: how do early neural crest-derived progenitors of parasympathetic neurons reach their destinations, and how do they acquire neuronal properties *in situ*? Furthermore, what is the nature of those progenitor cells? Our results clearly demonstrate that cells of glial origin located in the peripheral nervous system possess multipotency and gives rise to parasympathetic neurons during later developmental stages. Peripheral glial cells arrive to late-developing tissues on the pioneer presynaptic nerve fibres. Subsequently, some glial cells change fate, navigate for short distances and then convert into neurons and satellite cells of parasympathetic ganglia. Our conclusions redraw a fundamental principle on how the peripheral nervous system develops and provide a new type of logic, where both the cellular elements, as well as, the wiring are solved by a simple deposition of the postsynaptic elements from the presynaptic.

During our work we used a wide spectrum of approaches including advanced genetic tracing with multicolor reporters, analysis of numerous mouse mutants, *in vitro* cell cultures and 3D imaging of developing embryos. We have applied both genetic and surgical ablation techniques to the peripheral nerves and investigated targeted recruitment of glia from the nerves in each case.

Peripheral glia represents a novel amenable source of multipotent progenitor cells with putative regenerative potential that in the future might be applied for the treatment of congenital craniofacial pathologies, trauma cases or used for aesthetic body treatments.

LIST OF SCIENTIFIC PAPERS

I. Kaukua N*, **Shahidi MK***, Konstantinidou C, Dyachuk V, Kaucka M, Furlan A, An Z, Wang L, Hultman I, Ahrlund-Richter L, Blom H, Brismar H, Lopes NA, Pachnis V, Suter U, Clevers H, Thesleff I, Sharpe P, Ernfors P, Fried K, Adameyko I. *Glial origin of mesenchymal stem cells in a tooth model system*. Nature. 2014 Sep 25;513(7519):551-4. doi:10.1038/nature13536. Epub 2014 Jul 27.

II. **Shahidi MK***, Krivanek J*, Kaukua N, Ernfors P, Hampl A, Chubanov V, Romanov R, Gudermann, T, Harkany T, Adameyko I, Fried K. *Novel morphological features and tissue arrangements in the tooth revealed by 3D imaging of Odontoblasts and Pulp Cells*. Submitted.

III. Dyachuk V*, Furlan A*, **Shahidi MK**, Giovenco M, Kaukua N, Konstantinidou C, Pachnis V, Memic F, Marklund U, Müller T, Birchmeier C, Fried K, Ernfors P, Adameyko I. *Neurodevelopment. Parasympathetic neurons originate from nerve-associated peripheral glial progenitors*. Science. 2014 Jul 4;345(6192):82-7. doi: 10.1126/science.1253281. Epub 2014 Jun 12.

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CONTENTS

LIST OF ABBREVIATIONS

3D	Three-Dimensional
ANS	Autonomic Nervous System
bHLH	basic Helix-Loop-Helix
BMP	Bone morphogenetic protein
BMSCs	Bone Marrow Stem Cells
BrdU	5-Bromo-2-deoxyuridine
C.N.	Cranial Nerve
CFP	Cyan Fluorescent Protein
CL	Cervical Loop
CNCCs	Cranial Neural Crest Cells
CNS	Central Nervous System
CT	Chorda Tympani Nerve
DNA	Deoxyribonucleic acid
DPSCs	Dental Pulp Stem Cells
DRG	Dorsal Root Ganglia
E	Embryonic day
ENS	Enteric Nervous System
FACS	Fluorescence-Activated Cell Sorting
GFP	Green Fluorescent Protein
GSPN	Greater Superficial Petrosal Nerve
HSCR	Hirschsprung's disease
IEE	Inner Enamel Epithelium
JN	Jacobson Nerve
LRCs	Label-Retaining Cells
M	Musculus (Muscle *in latin*)
miRNA	microRNA
MSCs	Mesenchymal Stem Cells
N	Nervus (Nerve *in latin*)
NCCs	Neural Crest Cells
OEE	Outer Enamel Epithelium
P	Postnatal Day
PNS	Peripheral Nervous System
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
RT	Room Temperature
SCP	Schwann Cell Precursor
TA	Transit Amplifying
TACs	Transit Amplifying Cells
TG	Trigeminal Ganglia
TM	Tamoxifen
YFP	Yellow Fluorescent Protein

1 INTRODUCTION

1.1 CRANIOFACIAL DEVELOPMENT

Craniofacial development stems from interactions between between many different cell populations and a vast number of regulators spread over a relatively compact area. There is a multitude of cell types, tissues and organs that originate separately but develop in tight synchrony ensuring structural and functional unity. The complex integration yields difficulties in treatment of craniofacial disorders with reconstructive therapies. Many craniofacial tissues differ from their analogues elsewhere in the body due to their unique embryonic ancestry. Thus, much hope has been put in regenerative medicine approaches and advances within this field. Disruption in craniofacial development brings problems that can be physically, emotionally, and socially disabling. Even the most sophisticated reconstructive methods do not fully reproduce the fine complex function and shape that is the hallmark of craniofacial anatomy and physiology. Regenerative approaches might be expected to recapitulate the cellular interactions of the ectoderm, mesoderm and endoderm, as well as the generation of the important population of cranial neural crest cells (Figure 1ab) (Garland and Pomerantz, 2012).

1.1.1 Neural Crest Cells

Neural Crest Cells (NCCs) represents a unique embryonic cell type characterized by its ability to travel for long distances during early embryogenesis. The NCC can differentiate into a wide range of cell types, and is for this reason an attractive model within the field of stem cell research (Bronner-Fraser, 2004). NCCs are, nowadays, often referred to as the fourth germ layer (Noden and Schneider, 2006) due to their versatile capacities.

During early embryogenesis, at the end of the gastrulation stage, the three primary germ layers ectoderm, endoderm and mesoderm are formed. The ectoderm then becomes subdivided into two distinct domains: the non-neural or surface ectoderm and the neural ectoderm (Bhatt et al., 2013). As neurulation takes place, NCCs delaminate from the dorsal part of the neural tube through an epithelial-to-mesenchymal transition, migrate through the periphery in predefined routes and differentiate into a wide range of cells. These include neurons and glial cells, melanocytes, endocrine cells, mesenchymal cells and many others (Dupin and Sommer, 2012). The neural tube closes and the NCCs begin their journey through the mammalian body following distinct routes. NCCs are often considered as stem-like cells, due to their multipotency. Depending on the surrounding signals that influence them, they commit to different fates and differentiate at their target destinations. As an example, Wnt signalling influences NCCs to gain the properties of the sensory neuron lineage. In contrast, BMP (bone morphogenetic protein) signals bias these cells toward sympathetic neurons, while GGF (glial growth factor) commits them to a glial cell fate. Endothelin and later Wnt signals drive them toward a melanocyte fate (Bronner-Fraser, 2004).

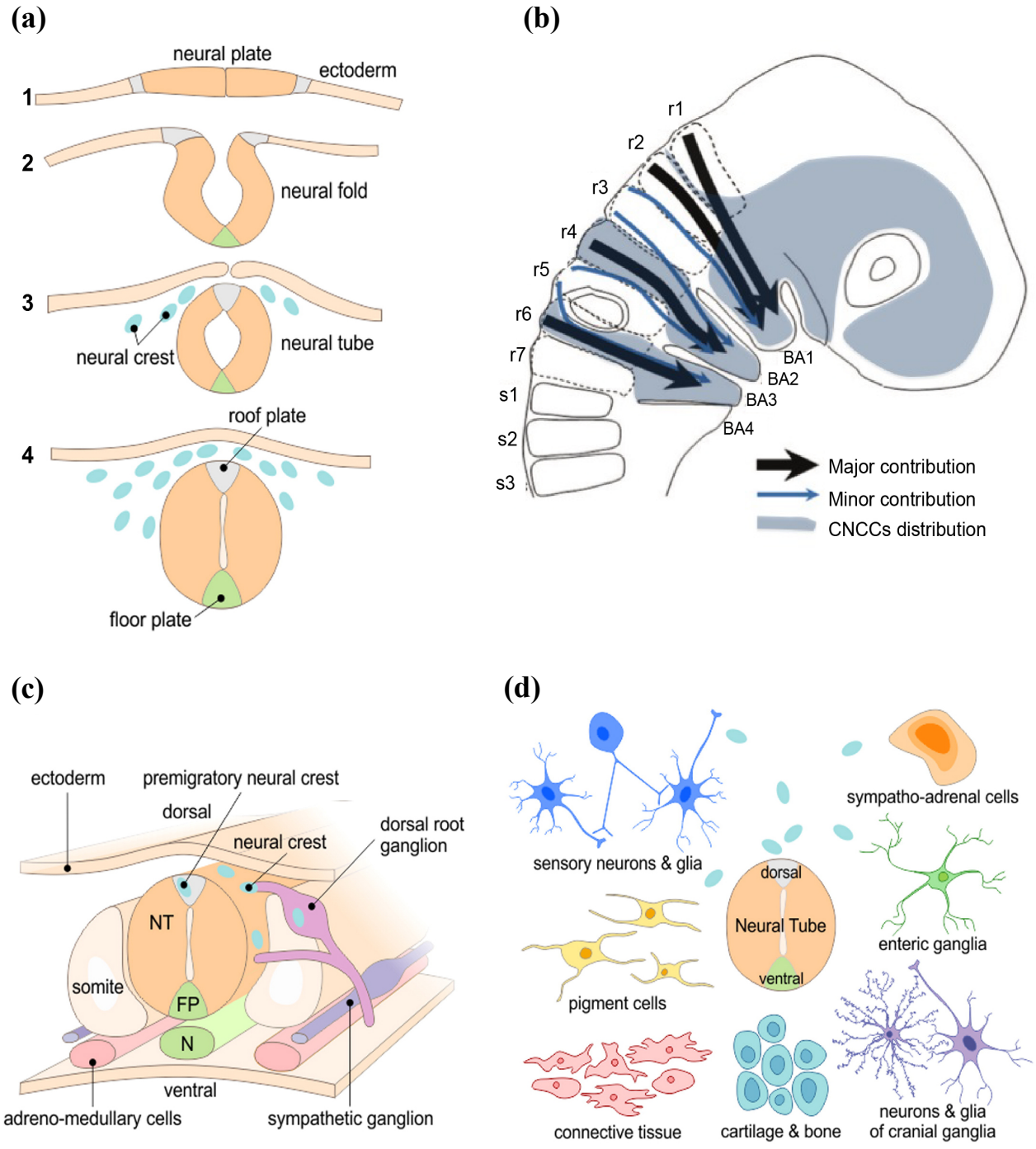

Figure 1.

(a) Schematic diagram illustrating the formation and migration of NCCs. (1) The neural plate part of the still flattened ectoderm thickens in comparison to the adjacent non-neural ectoderm tissue. (2) During neurulation the neural plate folds and begins to close. (Hazen et al.) At the time of neural tube closure, NCCs start their migration and subsequent journey from the dorsal part of the neural tube (4) throughout the tissue into the adjacent mesenchyme. **(b)** Cranial NCCs regional migration pattern is outlined with arrows with different thicknesses depending on the level of contribution to branchial arch development. BA – branchial arch, R – rhombomeres, S – somites. **(c)** The trunk NCCs aggregate to form multiple derivatives such as dorsal root ganglion, sympathetic ganglia and adrenomedullary cells. **(d)** Schematic diagram illustrating the wide range of cell types that arise from NCCs.

(a),**(c)**,**(d)** are reprinted from (Butler and Bronner, 2014), with permission from Elsevier, and **(b)** is modified from (Kuo and Erickson, 2010); Copyright © 2010 Landes Bioscience.

The neural tube gives rise to the central nervous system (CNS), whilst the NCCs that migrate into the periphery contribute to the formation of the peripheral nervous system (PNS). As the NCCs move closer to the dorsal aorta, they will give rise to sympatho-adrenal precursors while some of the NCCs migrate through the somites and aggregate to form sensory dorsal root ganglia (DRGs). Sensory cranial ganglia are also largely generated by the neural crest. In addition to this, the NCCs form the enteric neurons and glia of the gut, i.e. the PNS nerve cells that are in control of gut peristaltic and motility (Butler and Bronner, 2014).

The NCCs that arise from cranial levels are denoted cranial NCCs (CNCCs). In addition to the NCC-derived cell types and tissues mentioned above, CNCCs give rise to the majority of craniofacial bones, cartilages, adipose and other soft tissues (Achilleos and Trainor, 2012, Matsuoka et al., 2005). The migration patterns of the NCCs have been extensively examined using quail-chick transplantation studies (Le Lievre, 1978, Couly et al., 1993), and more recently with transgenic mice carrying reporter constructs that are activated in neural crest (Matsuoka et al., 2005). These studies have also has clarified the developmental homology between different species. The CNCCs migrate mostly in a dorsolateral pathway in-between the ectoderm and the underlying mesoderm to reach their future destinations. Figure 1C shows the migration patterns of the CNCCs and the extent of contribution to craniofacial development during early embryogenesis (Noden, 1975, Noden, 1978). As the cells migrate in streams into the first branchial arch (BA1, Figure 1B) they give rise to parts of the mandible, and the cells that originate from rhombomer 2 participate in forming the facial skeleton and the skull. Each branchial arch consists of a core of mesenchyme surrounded by neural crest that is externally covered by ectoderm and internally by endoderm (Richtsmeier and Flaherty, 2013). Most bones of the body are derived from the mesoderm and ossify by endochondral ossification, whilst the bones of the face and parts of the cranium that originate from CNCCs undergo predominantly intramembranous ossification during development (Jiang et al., 2002). The CNCCs that migrate more caudally form the hyoid arch and the necessary cartilages, bones and muscles for proper articulation and mastication. The hard-matrix producing cells of the teeth, the odontoblasts, are also derived from the neural crest (see further below, *section 1.1.7*), the neurons of sensory trigeminal mesencephalic (Mes V) nucleus, as well as the craniofacial parasympathetic ganglion neurons originate from CNCCs, and so do the telencephalic meninges and the connective tissue of the lacrimal glands (Baker et al., 1997a). Interestingly, craniofacial blood vessels ranging from the big arteries stemming from the heart to the very fine capillaries of the vascular tree in the face and the anterior brain are surrounded by cells of neural crest origin (Dupin et al., 2006). Neural crest has also been shown to provide the craniofacial and prosencephalic blood vessels with the right cues so cells can be locally induced to acquire pericyte and smooth muscle cell phenotypes (Etchevers et al., 2001) Finally, the CNCCs have been proven to be required for proper development of the mammalian forebrain (Baker et al., 1997b, Etchevers et al., 1999).

1.1.2 Placodes

The two main important embryonic cell sources that contribute to the vertebrate sensory systems are NCCs and cranial neural placodes. Both arise in close proximity to each other at the border of the neural plate, even though the placodal cells originate in a common preplacodal region slightly more lateral from where NCCs originate (i.e. more in the midline, at the closure point of the neural tube). A sequential activation of sets of transcription factors subdivides the thickened ectoderm (Figure 1a) over time into smaller domains of progenitors for the CNS, neural crest, epidermis and sensory placodes (Streit, 2004). The Six and Eya network determines sensory progenitor specification. In a positive feedback loop these factors preserve their own expression and stabilize pre-placodal fate, while simultaneously repressing neural and neural crest specific factors. Downstream of the Six and Eya cassette, Pax genes together with other factors start to convey local identity to placode progenitors (Grocott et al., 2012). Sensory placodes are formed by a unique columnar epithelium with neurogenic potential. They contribute to form the lens of the eye, the inner ear and the olfactory epithelium and, the anterior pituitary that is the major hormonal control organ in vertebrates. Together with NCCs they also contribute to the cranial sensory ganglia (Patthey et al., 2014). The trigeminal and ophthalmic placodes are established due to common activity of placodal epithelial cells and NCCs. The epibranchial placodes produce cells that directly delaminate to contribute sensory neurons to the distal parts of the VIIth, IXth and Xth cranial ganglia and nerves (geniculate, petrosal and nodose placodes, respectively) that innervate the taste buds in the oropharyngeal cavity as well as the heart, respiratory system, gastrointestinal tract and external ear (Steventon et al., 2014).

Non-neural placodes are induced in multiple and often stereotypical location throughout the epidermis of the head and contribute cells to the epithelial compartments of epidermal appendages, such as hairs, feathers, scales, horns and teeth (Biggs and Mikkola, 2014).

1.1.3 Endoderm

Endoderm interplay with CNCCs is of importance for proper facial bone and cartilage development (Couly et al., 2002). During the development of the first branchial arch, pharyngeal endoderm is thought to prepattern the orofacial epithelium, which in turn provides instructive signals to pattern the CNC-derived mesenchyme (Haworth et al., 2004). However, it has also been claimed that the ectoderm is critical for providing the instructive information for facial morphogenesis (Aoki et al., 2002). Endoderm contributes to the formation of minor mucous glands of the tongue (but no major salivary glands), along with the circumvallate papilla and foliate papillae (taste buds on the back of the tongue). The mucous minor salivary glands of the palate, however, are of mixed ectodermal and endodermal origin (Rothova et al., 2012). Furthermore, the endoderm derivates contribute to formation of the respiratory tract, the gastrointestinal tract, and follicular cells of the thyroid gland (Garland and Pomerantz, 2012).

1.1.4 Mesoderm

The cranial paraxial mesoderm has an organization that is different from that of the trunk paraxial mesoderm. The term paraxial mesoderm indicates that the mesoderm is located on either side of the midline embryonic notochord. The cranial paraxial mesoderm can be roughly divided into (1) preotic head mesoderm, which lacks any sign of segmentation and never forms somites; or (2) apparent occipital somites, which are caudal to the otic vesicle and give rise to muscles of the neck, the pharyngeal and laryngeal muscles that develop in the caudal branchial arches, as well as the tongue muscles (Noden, 1983, Couly et al., 1992, Huang et al., 1999). There are profound morphological and molecular differences between the cranial and trunk paraxial mesoderm, e.g. that genes that drive mesoderm segmentation in the trunk do not exist in the pre-otic mesoderm in the head. The cranial paraxial mesoderm provides a substratum for migrating CNC cells to populate the branchial arch. Studies in chick suggest that cranial paraxial mesoderm is able to direct CNC cell movement. To conclude, paraxial mesoderm forms the skeletal muscles, some skeletal elements and vascular tissues in the head (Trainor and Tam, 1995).

1.1.5 Tooth Development

Teeth have played an essential role in vertebrate evolution. The tooth anlagen develops during early embryogenesis, as a result of an intricate signalling between the epithelium of the oral cavity and the underlying neural crest-derived dental mesenchyme. Tooth morphogenesis is similar to morphogenesis of other partly ectoderm-derived organs that interact with cells of neural crest or mesodermal origin, to construct structures such as hair, feathers, salivary glands and mammary glands (Pispa and Thesleff, 2003). The functional elements of the tooth are the crown, root(s) and supportive tissue. The crown

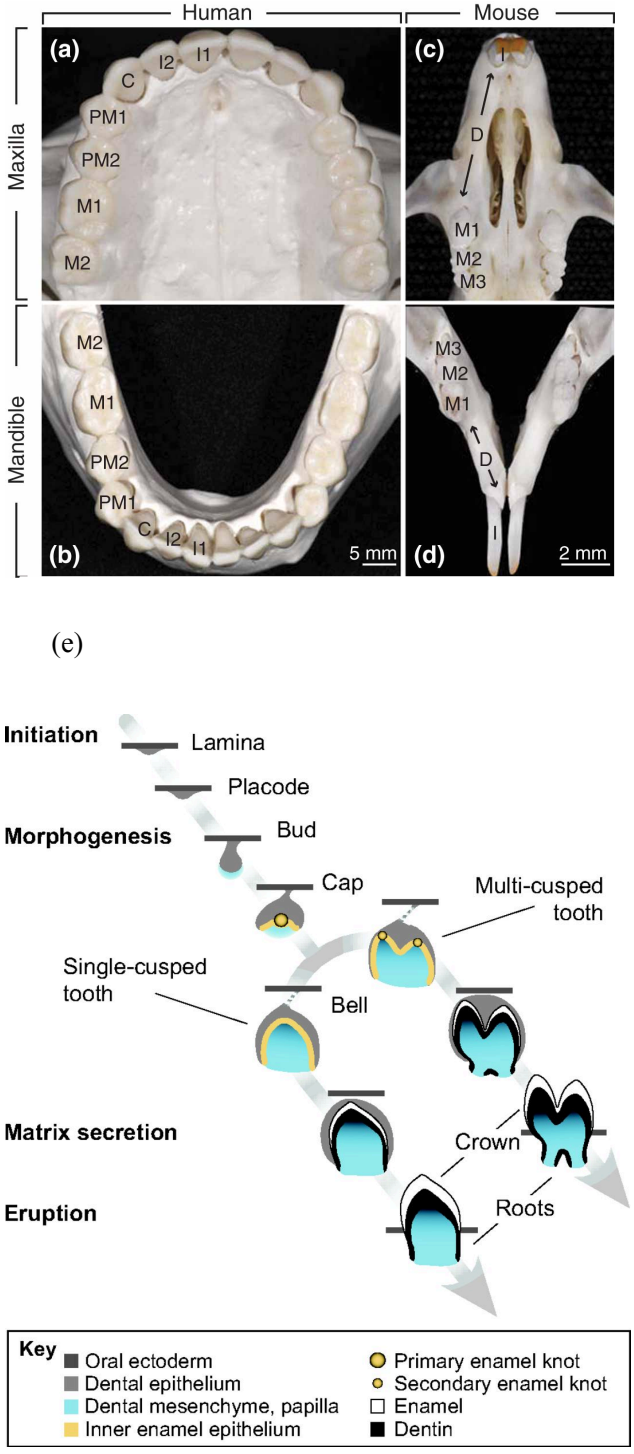

Figure 2.
Maxillary **(a, c)** and mandibular **(b, d)** dental arches show dentitions in adult human **(a, b)** and mouse **(c, d)**. The third molar or wisdom tooth (M3) is absent in the human specimen. I, incisor; I1, central incisor; I2, lateral incisor; C, canine; PM1, first premolar; PM2, second premolar; M1, first molar, M2, second molar; M3, third molar; D, diastema. Images are courtesy of Dr. Kyle Burke Jones (UCSF). **(e)** Displays the sequential growth route of uni- and multi-cusped teeth.

Figure 2 (a)-(d) are reproduced from Jheon et al, (2012) and **(e)** is reproduced from Jernvall and Thelseff (2012), with permission of Company of Biologists, via Copyright Clearance Center.

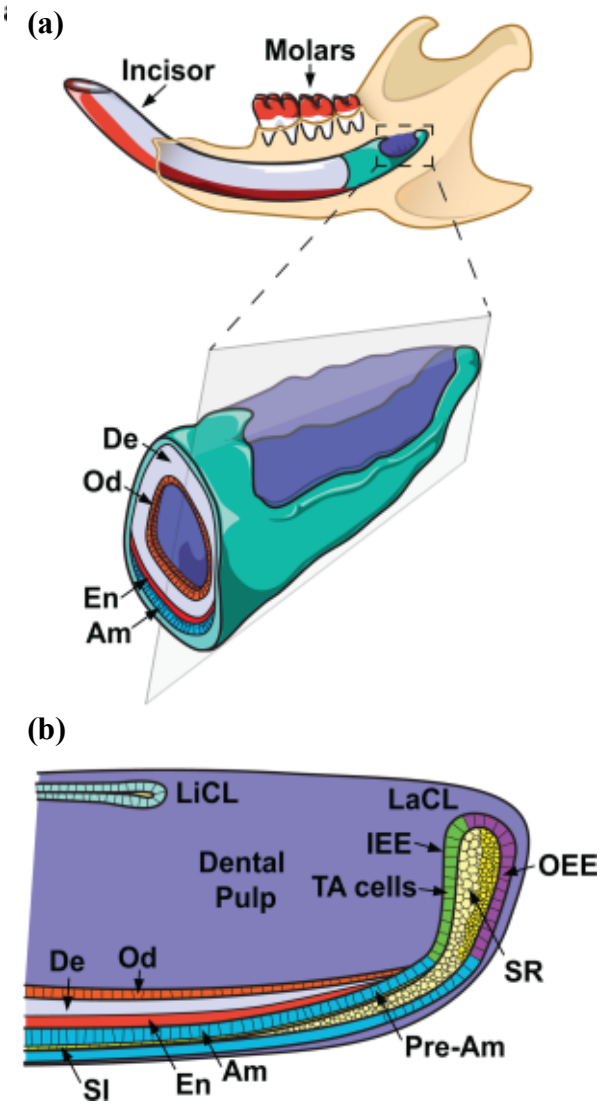

Figure 3.
Schematic illustrations of the rodent jaw and incisor **(a)** The incisor is to a great extent embedded in the jawbone, and the stem cell compartment is located at the proximal end of the tooth. Am – ameloblasts, Od - odontoblasts, En –enamel, De – dentine. **(b)** The incisor epithelial stem cells are located in the outer enamel epithelium (OEE) and stellate reticulum (Fried et al.) of the labial cervical loop (laCL). These cells are quiescent but give rise to transit-amplifying (TA) cells in the inner enamel epithelium (IEE) that undergo rapid proliferation. SI – stratum intermedium, liCL – lingual cervical loop.

is the part of the tooth that is visible in the oral cavity, whilst the root keeps the tooth anchored in the jawbone due to supportive tissue, i.e. root cement and fibres. Enamel, dentin and cement are the mineralized building blocks of the tooth. Enamel is the hardest material of the body and is produced by the ameloblasts, which are of epithelial origin. Below the enamel layer is the dentin layer that is generated by the odontoblasts, while cementoblasts create cementum that covers the root surface. Human primary dentition is composed of 20 teeth that are eventually replaced by the permanent dentition. The permanent teeth, in total 32, consist of 8 incisors, 4 canines, 8 premolars and 12 molars (Figure 2ab). In contrast, the mouse dentition has only 16 teeth, 4 continuously growing incisors and 12 molars that never are replaced (Figure 2cd, Figure 6a). Between the mouse incisor and molars in each jaw quadrant, there is a toothless spacing called a diastema (Kuang-Hsien Hu et al., 2014). Tooth formation is initiated at approximately embryonic day 11 - 12 (E11-12) during mouse embryogenesis, and at week 7 of gestation in human. The oral epithelium starts to thicken at specific sites. Subsequently the thickened epithelial placode forms a bud that grows into the underlying mesenchyme that in turn starts to condensate. This stage is termed the bud stage and is followed by the cap stage at E14 when the epithelial part begins to surround the condensed mesenchyme that will form the shape of the tooth. Now, the mesenchyme directs the folding of the epithelium, and tooth shape patterning takes place as cusps are formed via signalling centers, the enamel knots. The first mouse tooth to erupt is the mandibular incisor that becomes visible at postnatal day 9 (P9), shortly followed by the maxillary incisor. Eruption of molars takes place around P15 (Jheon et al., 2013).

The spatiotemporal expression of genes involved in tooth development is known to a large extent. However, the precise roles of each factor are not yet fully elucidated (Jheon et al., 2013, Michon et al., 2010, Thesleff and Tummers, 2008). The genetic mechanisms behind tooth initiation and formation are conserved between different species. This makes it possible to gain an understanding of human dental development by analysing animal models. Different animal models have different advantages. Mammals have in general the most complex set of teeth with restricted capacity for renewal. The relatively late and concise developmental time of the mouse dentition makes it useful for investigations at both early and later developmental stages (Jernvall and Thesleff, 2012). Furthermore, the mouse incisor provides an excellent model to analyse different stem cell niches. The mouse incisor, as in other rodents, grows continuously throughout life. The tip of the tooth is subjected to constant grinding during biting and chewing, and wears off, but is replaced by new material. Owing to mesenchymal stem cells (MSCs), which give rise to new mesenchymal derivatives, and epithelial stem cells, which produce epithelial derivatives, a growth-wear cycle of the incisor continues throughout the life span of the mouse. Dental epithelial stem cells are proposed to reside in the stellate reticulum and/or outer enamel epithelium (OEE) of the labial CL (Figure 3, 4) (Kuang-Hsien Hu et al., 2014, Cao et al., 2013). Both the labial and lingual cervical loop host epithelial stem cells, but only the ones of the labial CL produce ameloblasts, and enamel is uniquely deposited on the labial surface (Cao et al., 2013). The epithelial stem cells give rise to fast dividing transit-amplifying cells (TACs), which in turn differentiate into pre-ameloblasts and further into ameloblasts (Figure 3b) (Seidel et al., 2010). The MSC niche of the mouse incisor is known to reside in the apical part of the tooth, between the cervical loop regions. It has been proposed that incisor MSCs that can give rise to transit amplifying cells (TACs) are localized near the cervical loop region (Feng et al., 2011, Lapthanasupkul et al., 2012). TACs can be easily identified due to their active proliferation, and they give rise to committed preodontoblasts and then terminal differentiated odontoblasts. This rapid turnover makes the incisor mesenchyme an excellent model for studying MSCs. The growth rate of the mouse incisor is estimated to be 365 μm/day (Smith and Warshawsky, 1975). Hence, the time span for tooth turnover is 35 – 45 days in total (Zegarelli, 1944).

1.1.6 Signalling Mechanisms Governing Tooth Development

During development, teeth are formed through an intricate signalling between the ectoderm-derived epithelium and the underlying neural crest-derived ectomesenchyme. In addition, some species have an endodermal contribution to the epithelial compartment in this process (Soukup et al., 2008). Tooth development has to a large extent remained conserved throughout vertebrate evolution, both in terms of genetic basis and in the structure of teeth; however, vertebrates have much more diverse dentitions (Tucker and Fraser, 2014). Some species, e.g. reptiles, have continuous life-long tooth replacement, whilst mammals have a set number of teeth but some of these teeth may grow continuously, as seen in e.g. rodents. Continuous growth of teeth is executed through stem cells in a stem cell niche in the apical region. Understanding the mechanisms behind this activity may pave the way for bioengineered teeth in the future (Renvoise and Michon, 2014).

The most common molecular signalling pathways during odontogenesis include Eda (Ectodysplasin, a TNF, tumour necrosis factor, signal), FGF (fibroblast growth factor), Notch, Shh (sonic hedgehog), TGF-ß superfamily and Wnt (Thesleff and Tummers, 2008). Through complex interactions, they govern the different aspects of morphogenesis. Each pathway consists of ligands, cell surface receptors and intracellular signalling cascades. The different systems vary in their effector mechanisms. Some pathway ligands can act as morphogens that form gradients within tissues and affect function at a distance from the ligand-secreting cell, while others are dependent on proximity to their target to carry out their function(s). It has been shown by mathematical modelling that almost any tooth shape can be generated by changing the molecular parameters that influence position and function of secondary enamel knots (i.e. the signalling center that controls the developing tooth placode, see *Figure 2e*) (Salazar-Ciudad and Jernvall, 2002).

The tooth is an ectodermal organ, in common with scales in reptiles and fish, feathers in birds, and hair and exocrine glands in mammals. There is a striking developmental similarity between all ectodermal organs up to the bud stage (as the ectoderm is growing into the condensing mesenchyme underneath), and the signals that mediate these processes are highly conserved. After the bud stage, each organ has its own pattern of signalling to differentiate into the organ-specific cell types and tissues (Biggs and Mikkola, 2014). The expression patterns of over 200 genes that are developmentally regulated during tooth morphogenesis can be viewed in the graphical database at http://bite-it.helsinki.fi. Localized expression of Pitx2 and Shh genes has been reported to be of importance in the primary dental lamina (present in all species so far examined, such as fish, reptiles and several mammals) (Thesleff and Tummers, 2008). These genes are essential for tooth formation, and deletion of Pitx2 function results in tooth agenesis in mice and humans (Lin et al., 1999). Shh signalling is required for tooth initiation in mouse embryos, and subsequently for normal morphogenesis and differentiation of dental epithelium into ameloblasts (Hardcastle et al., 1998, Dassule et al., 2000, Gritli-Linde et al., 2002). There are many more genes of importance for tooth formation, and deletion of many of them leads to arrest of tooth formation or complete tooth agenesis (Thesleff, 2003)

The genetic regulation behind tooth replacement is not yet fully understood. Pitx2 and Bmp4 have been associated with tooth replacement in fish (Fraser et al., 2006). The ferret has been

used for analysis of tooth replacement in mammals, and the results showed that tooth replacement originated in the (secondary) dental lamina that develops as part of the primary tooth. Genes associated with this process include Runx2 and Il11rα (interleukin-11 receptor-alpha-α), which are expressed in the mesenchyme around the successional lamina in the ferret (Jussila et al., 2014). Furthermore, the expressions of some regulatory genes such as the BMP/Wnt inhibitor Ectodin (Sostdc1) were localized to the site of replacement tooth initiation. However, the actual stem cells responsible for replacement tooth formation have not yet been identified (Jarvinen et al., 2009). Even though mouse teeth are normally not replaced, *de novo* tooth formation could be induced in transgenic mice by stimulating the canonical Wnt pathway (Jarvinen et al., 2006). Studies *in vitro* in snake teeth have demonstrated that the epithelial compartments are critical for the arrangement of organs that develop in successional sequence, and the role of Wnt/β-catenin signalling in those processes seem to be crucial (Gaete and Tucker, 2013).

The environment regulates the tooth epithelial and mesenchymal stem cell niches. *Table 1* shows an overview of the factors involved and their roles (for an overview of signalling regulating epithelial-mesenchymal tooth morphogenesis see Thesleff, 2003). Transcription factor Sox2 is a specific marker for the epithelial stem cells in the tooth (Juuri et al., 2012). Tentative MSC markers in the tooth are (in human dental MSCs) STRO-1, CD90, CD146, CD34 and the recently suggested TfR (Park et al., 2013) Cells with MSC characteristics in the pulp neurovascular bundle have been shown to be Gli1+ (Zhao et al., 2014). It is likely that new markers for MSCs will emerge, depending on what subdivision that is investigated.

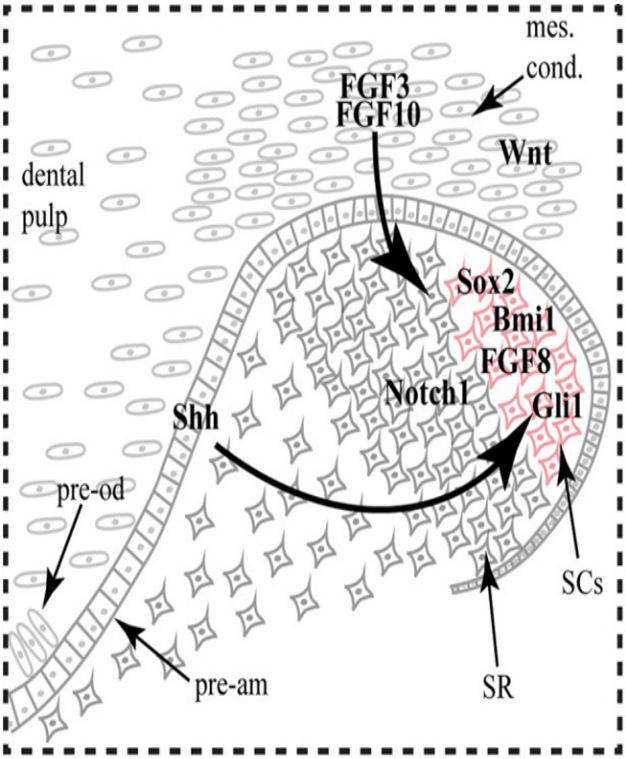

Figure 4. The mouse incisor stem cell niches.

In the laCL, the stem cells (SCs) are adjacent to the stellate reticulum cells. When the SCs divide and differentiate, they exit the niche to become integrated into the enamel epithelium and form pre-ameloblasts (pre-am). Condensated mesenchymal stem cells (mes. cond.) surround the epithelial niche. These cells give rise to the dental pulp and the pre-odontoblasts (pre-od). The mesenchyme expresses Fgf3 and Fgf10, which are important for the SC maintenance. While the SR cells express Notch1, the SCs express FGF8, Bmi1, Gli1 and Sox2. Gli1 expression is part of a feedback loop induced by Shh expression in the SC early progeny.

MOLECULAR REGULATION OF TOOTH STEM CELL NICHES

FACTOR:	ACTIONS:	REFERENCE:
BMP4 /Activin	• Expressed by dental mesenchyme. Modulates the amount of stem cells/ progenitors. BMPs have dual roles in regulating Wnt signalling activity (antagonize or induce). Non-canonical BMP signalling plays a major role in regulating dental epithelial cell proliferation.	(Wang et al., 2007) Yuan et al., 2015
Fgf	• Fgf-3 and Fgf-10 regulate the Notch pathway in dental epithelial stem cells in cervical loops. Crucial for survival, proliferation and fate decision of stem cells. Fgfr1b and Fgfr2b are enriched in the stem cell niche. Fgf8 induces Sox2 expression and therefore plays a direct role in stem cell regulation.	(Harada et al., 1999) (Harada et al., 2002) (Wang et al., 2007) (Juuri et al., 2012)
PCR1 complex	• Regulates TAC compartment of the dental MSC niche and cell differentiation, required for molar root formation.	(Lapthanasupkul et al., 2012)
Pitx2	• Pitx2 is induced by FGF8, and is essential for tooth development beyond the bud stage.	(Lin et al., 1999)
Follistatin	• TGFß inhibitor. Limits the amount of stem cells/progenitors on the lingual side by antagonizing the effect of Activin. Inhibits enamel formation in the differentiation zone by antagonizing BMP-induced ameloblast differentiation. Follistatin determines the asymmetry of enamel formation and the size difference between labial and lingual cervical loops.	(Wang et al., 2007)
Gli1	• Part of a feedback loop induced by the Shh expression by the SC early progeny.	(Seidel et al., 2010)
Notch	• Important regulators of stem cell function. Induce proliferation or differentiation. May be of importance for odontoblast differentiation.Suggested to be required for epithelial stem cell survival and enamel formation in the continuously growing mouse incisor.	(Mitsiadis et al., 2011) (Felszeghy et al., 2010)

Shh	• Signals back to progenitor stem cells through a positive feedback loop to produce more progeny. Supports the MSCs in the neurovascular bundle.	(Seidel et al., 2010) (Zhao et al., 2014)
Sprouty	• Negative regulators of FGF signalling. Involved in the maintenance of asymmetry of the stem cell niche in the incisor and seem to play a crucial role in the lingual inhibition of enamel-producing ameloblasts.	(Klein et al., 2006)
Wnt	• Wnt/β-catenin signalling controls the odontogenic fate and partly regulates cell proliferation in dental epithelium during early odontogenesis. Overexpression of Wnt3 in dental epithelium inhibits ameloblast differentiation.	(Suomalainen and Thesleff, 2010) (Juuri et al., 2012) (Millar et al., 2003) (Yuan et al., 2014)

Table 1. Molecular regulation of tooth stem cell niches.

1.1.7 Odontoblasts

Historically, the odontoblast has been difficult to study due to its shielded localization in the pulp periphery with its process surrounded by the hard-mineralized matrix that the cell has generated. This fact has hampered both microscopical and functional studies of these important cells. Odontoblasts are often compared to the osteoblasts since they share ancestry as well as functional similarities during their matrix-producing phase (Arana-Chavez and Massa, 2004). The odontoblast possesses an autophagic-lysosomal system to secure organelle and protein renewal. They are believed to remain functional, although at decreasing levels of efficiency, throughout the lifetime of the individual. (Couve et al., 2013). In cases when the odontoblasts are damaged or die because of trauma or caries lesions, progenitor cells from subodontoblastic cell layer or other unknown sources are thought to differentiate into second-generation odontoblasts that are odontoblast-like cells with capacity to generate hard mineral (Goldberg and Smith, 2004). The types of dentine that are produced are primary dentine during crown and root formation of the tooth, secondary and tertiary dentine. Secondary dentine is deposited throughout the life of the tooth, leading to a gradual narrowing of the pulp chamber, root canals and peritubular area. Primary and secondary dentine is similar in structure, whilst tertiary dentine, which is laid in response to damage or trauma, differs in histology (as it is more irregular). Primary dentine matrix is deposited at the rate of 4μm/day and secondary dentine at approximately 0.4μm/day (Simon et al., 2009). It is as yet not settled with certainty at what time point and under what circumstances developmental commitment and subsequent differentiation of the odontoblast occurs (Ruch, 1998). With

regard to odontoblast morphology, it depends on phase in development. However, most available descriptions focus on the adult mature cell. According to these, the functional mature odontoblast layer consists of postmitotic cells in the pulp periphery with tall (50 - 60 μm) columnar shapes and a polarized distribution of organelles. The main process of an odontoblast secretes the predentine that mineralizes into dentine (Ruch, 1998). , It extends into the predentine-dentine, and contains a cytoskeleton and exocytotic as well as endocytotic vesicles. With age the odontoblast shrinks due to autophagy, but remains functional (Couve et al., 2013). Odontoblasts are believed to have an advantageous localization to sense both external stimuli, because their processes extend into the fluid-filled dentinal tubules, and internal stimuli since their cell bodies are situated in the pulp periphery (Magloire et al., 2009). It is believed that hydrodynamic forces that are generated by dentinal fluid movement can affect nerve fibres (Hildebrand et al., 1995), with or without an involvement of the adjacent odontoblasts (Arana-Chavez and Massa, 2004). Odontoblasts possess membrane and cytoplasmic components, including TRP (Transient receptor potential) channels, voltage-gated sodium channels and ionotropic glutamate receptors (Allard et al., 2006, Magloire et al., 2009) that makes it reasonable to assume that they are involved in in stimulus transduction (Fried and Gibbs, 2014).

It is thought that the pulp cells in the subodontoblastic cell layer also denoted Höhl layer, (Ingle JI et al., 2008), originates, from the last pre-odontoblast cell division, where one cell assumes the odontoblast fate and the other one remains pulpal. One interesting hypothesis is that the sub-odontoblastic cells take part in replenishment of the odontoblast layer. If so, they could be activated to become terminally differentiated if needed (Goldberg, 2014). This hypothesis is supported by the fact that the subodontoblastic cells express high alkaline phosphatase (ALP) activity. Furthermore, when stimulated with BMP-2, the subodontoblastic cells exhibited accelerated ALP activity, forming Alizarin red-positive matrix (Hosoya et al., 2012).

1.2 CRANIOFACIAL PROGENITORS AND REGENERATIVE SOURCES

Stem cells can roughly be divided into 4 categories, depending on type: 1) embryonic and 2) adult stem cells are “physiological” categories that are existent at different stages of life. 3) cancer stem cells of tumours possess some stem cell properties (Alvarez et al., 2012), and 4) engineered or ‘induced’ pluripotent stem cells (iPS) have recently been introduced (Takahashi and Yamanaka, 2006). Stem cells can be defined as a population of undifferentiated cells characterized by the ability of massive proliferation (self-renewal) that usually arises from a single cell (clonal). They may differentiate into a wide range of different cells and tissues, thus being a highly potent source for tissue renewal. Pluripotent cells can differentiate into tissue from all three germ layers (endoderm, mesoderm, and ectoderm). Multipotent stem cells have the ability to differentiate into tissue derived from a single germ layer, i.e. the MSCs which form adipose tissue, bone, and cartilage (Kolios and Moodley, 2013). Stem cells are generally considered to be more “primitive” and are the precursors of progenitor cells, which in turn are more lineage-committed with less capacity to self-renew, and may be more organ-specific. Yet, recent reports challenge the view that organ stem cells

(also denoted tissue-resident stem cells) is a homogenous pool. It is rather believed that lineage-biased subtypes already exist within the stem cell population (Challen et al., 2010). Stem cells are tightly regulated by their microenvironment, the stem cell niche (Mitsiadis et al., 2007). Hence, it is of importance to understand the microenvironment that determines the maintenance and differentiation of the stem cells. The view on stem cell classification has changed dramatically over the past years. Label retention and quiescence are no longer seen as obligatory stem cell characteristics. A more explicit understanding of how organs maintain and repair themselves in adults is required in order to properly utilize the derivatives of pluripotent cells in therapy (Grompe, 2012).

If accepting the concept that massive self-renewal is needed, there are few adult tissues that qualify as "stem cell niche"-containing. There are many different kinds of progenitor sources hosted in craniofacial peripheral tissue, such examples are neural-, epithelial-, mesenchymal-, NC-like stem cells and pericytes. The list can however be made longer.

1.3 PULPAL NERVES AND VESSELS – TIGHTLY INTERCONNECTED STEM CELL NICHE

The vasculature of the pulp follows the same routes as the nerves, as elsewhere in the body. A layer of loose connective tissue surrounds many arteries and nerves, forming a neurovascular bundle. This bundle constitutes a niche for MSCs that participate in both homeostasis and injury repair *in vivo* (Zhao et al., 2014). It has been shown that Schwann cell precursors on the peripheral nerves secrete CXCL12 that attracts the endothelial cells to align adjacent to the nerves during development (Li et al., 2013). This demonstrates the presence of a sophisticated system where nerves direct the development of an accompanying primary vessel network. Hypothetically, a continued nerve-vessel crosstalk might also influence the neurovascular tissue homeostasis in the adult.

Furthermore, the vasculature contains pericytes - contractile multifunctional cells that wrap around the endothelial cells of capillaries and venules within the vascular basement membrane (Sims, 1986). No singular molecular marker can be used to unequivocally identify pericytes and distinguish them from vascular smooth muscle cells (vSMCs) or other mesenchymal cells. The multiple markers commonly applied are neither absolutely specific nor stable in their expression. The best markers so far include NG2 (Neuron-glia 2), PDGF (Platelet-derived growth factor) ß-receptor and α-SMA (α-smooth muscle actin) (Winkler et al., 2010, Murfee et al., 2005). Cephalic pericytes in all parts of the CNS seem to be neural crest derived, as demonstrated in chick-quail chimeras (Etchevers et al., 2001, Korn et al., 2002) and in mice (Heglind et al., 2005). Pericytes in the other parts of the body are believed to be of mesodermal origin (Mills et al., 2013). It has been suggested that pericytes (also called adventitial or Rouget cells) may represent mesenchymal stem or progenitor cells, since they can differentiate into a variety of MSC cell types, such as fibroblasts, chondroblasts, osteoblasts, odontoblasts, adipocytes, vascular smooth muscle cells and myointimal cells (Diaz-Flores et al., 2006, Feng et al., 2011, Armulik et al., 2011). Some pericytes express markers characteristic for stem cells, such as Sca1 (Brachvogel et al., 2005) and STRO-1. STRO-1 is an early common marker for MSCs (Yoshiba et al.,

2012). Furthermore, Gli1 marks a network of perivascular MSC-like cells found in many organs, as in lung, liver, kidney and heart. It has been demonstrated that upon tissue damage pericytes leave the perivascular space and generate myofibroblasts, thus playing a central role in organ fibrosis after injury. Ablating these cells ameliorates fibrosis and rescues organ function (Kramann et al., 2014). In conclusion, pericytes play an important role in vascular development and homeostasis, are sources of fibrogenic cells in pathological situations, and may also serve as a possible reservoir of stem or progenitor cells for adult tissue repair.

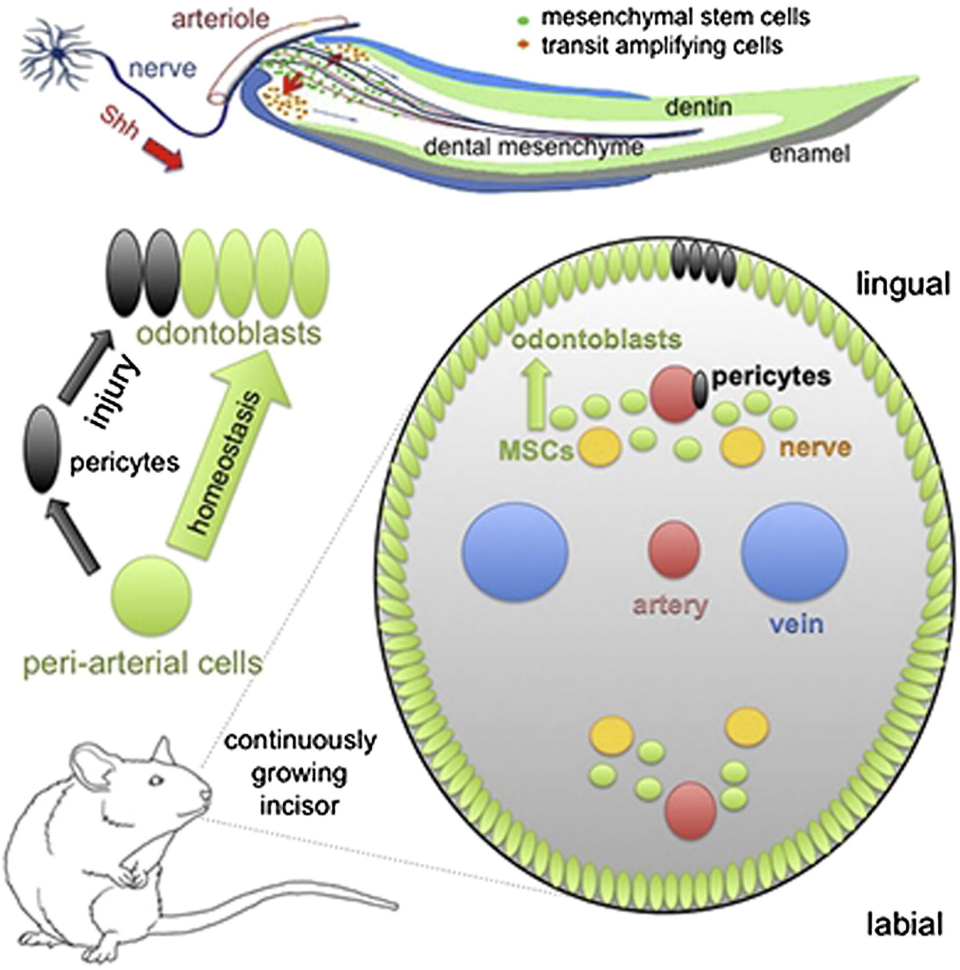

Figure 5. Sensory neurons in the trigeminal ganglion produce Shh, which is transported through axons and injected into the incisor mesenchyme. Shh activates Gli1 expression in the stem cells surrounding the arterioles near the cervical loop region and regulates the odontogenic differentiation process. The image shows the schematic cross-section of a continuously growing mouse incisor. The neurovascular bundle provides a niche to peri-arterial cells to support the continuous turnover of incisor mesenchyme. These quiescent stem cells continuously give rise to actively dividing TACs in homeostasis, which then differentiate into odontoblasts and other mesenchymal domains of the tooth. This microenvironment also provides pericytes that contribute mainly to injury repair but not during homeostasis.

1.4 PERIPHERAL INNERVATION

Peripheral Nervous System (PNS) is the link between the CNS and the peripheral structures in the body. It consists of different classes of neurons as well as an array of fibres, including afferent sensory fibres and efferent motor axons. PNS targets all parts of the body starting from early stages of embryonic development, and is to a large extent derived from multipotent migratory neural crest stem cells. PNS is often divided into the somatic nervous system (SNS), the autonomic nervous system (ANS, also known as the visceral nervous system), and the enteric nervous system (ENS) based on function. The SNS consists of the sensory spinal nerves with cell bodies in the dorsal root ganglia (DRG) and motor spinal nerves with cell bodies in the spinal cord anterior horn. The SNS is under voluntary control while the ANS is automated and not controlled by will (Catala and Kubis, 2013). The ANS plays an essential role in maintaining body homeostasis,, and is in close relation with the ENS that constitutes the essential innervation of the digestive tract. The ANS is subdivided into the parasympathetic and sympathetic nervous systems, which generally are each other's opposite in terms of effects. Neurons (both sensory and motor) of the ANS innervate a wide range of tissues and organs i.e. cardiac muscles, smooth muscles, and various glands and controls processes such as heart and respiratory rate, levels of O_2, CO_2, digestion, perspiration etc. Peripheral innervation is established early during embryonic development. The somatic peripheral nerves, for instance, reach into the limb buds already at E10.5 - E11.5 during mouse development and E4.5 – 5.5 in chick development (Kaucka and Adameyko, 2014).

1.4.1 Craniofacial Innervation

The orofacial region is densely innervated, which is reasonable, considering its involvement in a series of fundamental functions such as e.g. vision, olfaction, tactile perception, fending and feeding. During feeding, intake of food, mastication and swallowing are tightly regulated and fine-tuned by oral and pharyngeal sensory nerves in central coordination with motor axons (Dodds et al., 1990). This involves salivary glands that upon signalling from parasympathetic nerves produce saliva for lubrication as well as for initial enzymatic digestion of the food bolus. Saliva is also of utmost importance for proper homeostasis in the oral cavity, to maintain the function of teeth and mucosa.

The head is innervated in total by 12 pair of cranial cranial nerves (I–XII, see *Table 2*), most of which execute and coordinate the sensory and motor functions of this part of the body (Netter, 2006). Cranial nerves, except for I and II, originate in the brain stem (which includes midbrain, pons and medulla oblongata). The cranial nerves can be divided into sensory, motor or mixed, and in addition III, VII, IX and X carry parasympathetic nerve fibres. The nuclei in the brain stem that project to the craniofacial region are the *Edinger-Westphal nucleus* (EW) (also denoted accessory oculomotor nucleus), the *Superior salivatory and lacrimal nuclei*, and the *Inferior salivatory nucleus* (see *Figure 7*). Parasympathetic nerves signal to their target organs by releasing acetylcholine, and their target organs expressing the M1–M5 muscarinic receptors that induce specific intracellular responses to acetylcholine. EW is composed of an intermingled cell population of the midbrain that differ dramatically in connectivity and neurochemistry. The EW is generally seen as the part of the oculomotor complex that is the source of the parasympathetic preganglionic motoneuron input to the

ciliary ganglion (*Figure 9a*), through which it controls pupil constriction and lens accommodation. Albeit, the EW also includes a population of centrally projecting neurons involved in sympathetic consumptive and stress-related functions (Kozicz et al., 2011). The parasympathetic division of the EW and its necessity for proper ganglion formation in the periphery is expanded upon in Paper III.

The *Superior salivatory and lacrimal nucleus* of the facial nerve is a visceromotor cranial nerve nucleus located in pons (part of the brain stem). Many of the neurons send their (preganglionic parasympathetic) fibres to the submandibular ganglia and to the intra-lingual ganglia in the anterior part of the tongue, via the intermediate, chorda tympani and chorda-lingual nerves. A subdivision of parasympathetic neurons from this nucleus also innervates the extraorbital lacrimal gland (Matsuo and Kang, 1998, Toth et al., 1999). The *Inferior salivatory nucleus* is located in the medulla oblongata and its axons leave the brain with the glossopharyngeal nerve to govern secretion from the parotid gland after synaptic coupling in the otic ganglion (Rezek et al., 2008).

The sympathetic nerves of the head have their cell bodies in the *Superior cervical ganglion* (SCG). It is the largest and most rostral (superior) of the three cervical ganglia. The SCG provides sympathetic innervation to the pineal gland, oral-facial-cranial blood vessels, the choroid plexus, the eye, the carotid body and the salivary and thyroid glands (Cardinali et al., 1981).

Sensory innervation of the head and neck region is regionalized and maintained by several different cranial nerves. The trigeminal nerve (C.N. V) with its three different sub-branches provides the primary sensory innervation to the face. The ophthalmic nerve (V1) innervates the cornea, ciliary body, lachrymal glands, conjunctiva, nasal mucosa, and the skin of the nose, eyelid, and forehead. The maxillary nerve (V2) innervates the mid-portion of the face, the side of the nose, the lower eyelid, and upper teeth. Finally, the mandibular nerve (V3) innervates the lower third of the face, the anterior two-thirds of the tongue, the oral mucosa of the mouth, and the lower teeth (Sanders, 2010). Cutaneous and muscular sensory innervation of the neck and part of the ear, as well as posterior scalp is supplied by the upper cervical spinal nerves (C2, C3). Furthermore, the facial (C.N. VII), glossopharyngeal (C.N. IX) and vagus (C.N. X) nerves provide small portions of sensory innervation to e.g. the ear and pharynx (Baker and Schuenke, 2010). For a summary of cranial nerve pathways, see Table 2.

CRANIAL NERVES I – XII

C.N. I	**Olfactory Nerve** Governs the olfactory nerve fibres that register smell.	**C.N. VIII**	**Vestibulocochlear Nerve** Supplies sensory innervation to the inner ear.
C.N. II	**Optic Nerve** Governs the ocular and sensory functions.	**C.N. IX**	**Glossopharyngeal Nerve** Provides taste sensation for the posterior third of the tongue, sensation to the tonsils, pharynx and middle ear and motoric fibres to the stylopharyngeus muscle and the parotid gland.
C.N. III	**Oculomotor Nerve** Motorically innervates the previously mentioned eye muscles and allows the eye to move within the orbit.	**C.N. X**	**Vagus Nerve** Function is to provide sensation to the heart, lungs, trachea, bronchi, larynx, pharynx, gastrointestinal tract and the external ear.
C.N. IV	**Trochlear Nerve** Motorically governs the abduction, depression and internal rotation of the eye.	**C.N. XI**	**Accessory Nerve** Controls m. Trapezius and m. Sternocleidomastoideus as well as swallowing movements.
C.N. V	**Trigeminal Nerve** Branches into three: V/I ophthalmic V/II maxillary V/III mandibular nerves. Function is to govern the sensory innervation of the face, sinuses and teeth.	**C.N. XII**	**Hypoglossal Nerve** Controls tongue movements and innervates m. Infrahyoidei (that is important for speech) sensorically.
C.N. VI	**Abducent Nerve** Function is to retract the eye within the orbit.		
C.N. VII	**Facial Nerve** Governs facial motoric and also sensation to the anterior two thirds of the tongue.		

Table 2. Overview of cranial nerves (C.N.) and their functions in brief.
Reference: Frank H. Netter (Urban and Fischer), Atlas of Human Anatomy, 4th Edition, Saunders, 2006, Plates 12 and 13. Author: Dr Alexandra Sierosławska.

1.4.2 Tooth Innervation

The tooth is a highly vital organ that is densely vascularized and innervated. The tooth pulp receives its nerve supply very late (P3-4) in comparison to the surrounding mesenchymal tissue, which is densely innervated already early (E12.5) when tooth placode are formed (Fried et al., 2000). The ingrowth of Trigeminal Ganglion (TG) nerve fibres into the pulp does not occur until the crown shape is set and the mineralization of enamel and dentin has started (Figure 5). Prior to this, nerve fibres have formed basketlike structures just below the tooth anlagen (Fried et al., 2007, Moe et al., 2008). The delayed nerve penetration of the dental papilla/pulp tissue might be caused by neurite-inhibitory factors in the dental papilla. However, these seem to decrease in expression at later stages, when pulpal neurotrophic factors increase in concentration. These events coincide in time with initial innervation of the tooth pulp (Kettunen et al., 2005).

Approximately 70-90% of the axons in the tooth pulp are unmyelinated, while the rest are mainly A-delta fibres. However, most sensory pulpal axons seem to be end branches of larger or much larger parent axons that belong to A-beta neurons and have characteristics of mechanoreceptors (Fried and Devor, 2011). Pulpal axons travel through the root and crown, and then branch heavily in the odontoblast region of the crown. Terminal endings, all unmyelinated, are closely associated with odontoblasts, and many continue through the predentin and for some distance into the dentinal tubules (Hildebrand et al., 1995).

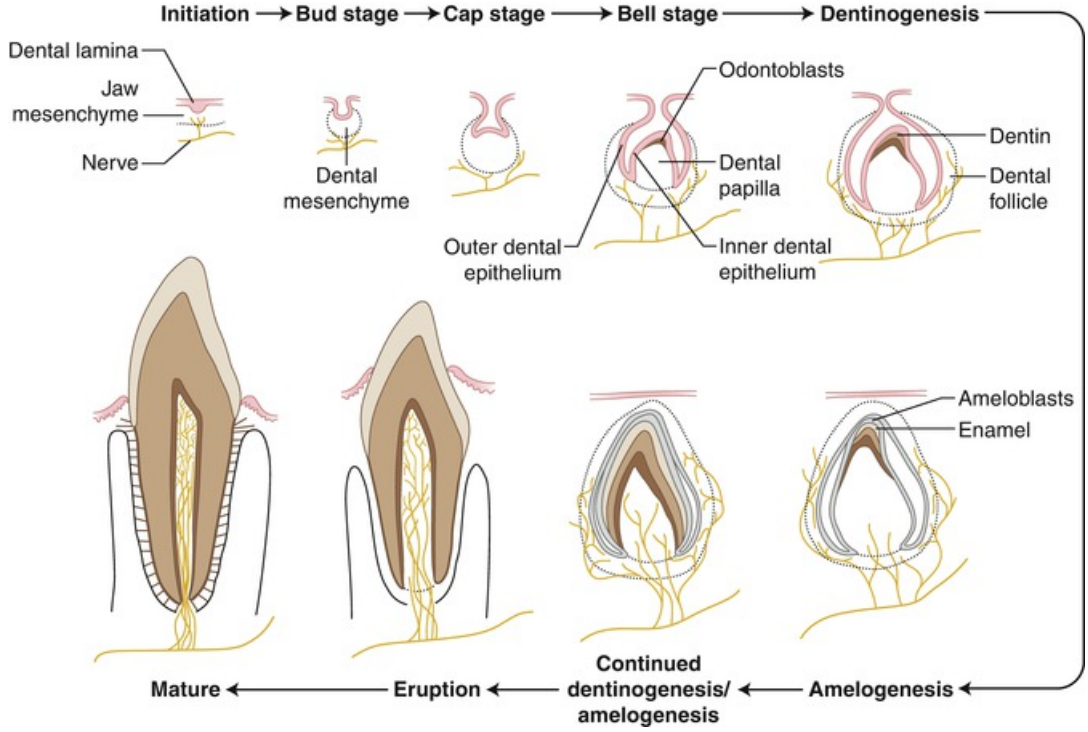

Figure 6. Modified from Fried et al., 2007. Illustration shows the relation between tooth development and innervation. Arrows indicates the sequential development order.

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1.4.3 Parasympathetic Ganglia

The autonomic neurons that belong to the ANS modulate the activity of internal organs such as the salivary- and tear glands, blood vessels, heart, lung, and gut, aiming at maintaining homeostasis of body functions in response to varying external circumstances. The ANS is, as already mentioned, subdivided into the parasympathetic system that is activated when the body is at rest whilst the sympathetic system is on when the body is subjected to fight-and-flight situations. There is a constant fine-tuning of the system and balance between the parasympathetic and sympathetic systems. All regulation is done by a set of reflex circuits passing through the hindbrain. Most autonomic neurons arise from neural crest (D'Amico-Martel and Noden, 1983, Lee et al., 2003). The parasympathetic neuronal bodies form ganglia in a close proximity to their target organs. There are four parasympathetic ganglia in the head: the ciliary, pterygopalatine (sphenopalatine), otic and submandibular ganglia (Figure 8).

1.4.3.1 Parasympathetic Lineage Differentiation

The efferent pathway consists of the preganglionic (or visceromotor) and postganglionic neurons of the parasympathetic and enteric nervous systems. All these neuronal types depend for their differentiation on a dedicated homeobox gene, Phox2b, which can be seen as a master gene of the ANS. In higher vertebrates, all sensory neurons derive either from precursors in the placodes of the head (for cranial sensory ganglia) or from the neural crest (for trunk sensory neurons). Neurogenin-1 and -2 (ngnl and ngn2) are related bHLH factors that are expressed in precursors of both placode- and neural crest-derived sensory neurons but not those of autonomic neurons. When either one is knock-outed, cranial sensory ganglia fail to form (Ma et al., 1998, Fode et al., 1998). Ascl1 (Achaete-scute homolog 1, also named Mash1 - a mammalian homologue of Ascl1 gene complex), however, promotes differentiation of committed neuronal precursors towards autonomic neurons (Guillemot et al., 1993).

The ontogeny of parasympathetic neurons is yet another interesting question to address. Current dogma states that neural crest cells give rise to parasympathetic neurons. The guidance cues that initially are found to promote migration of neural crest cells to the correct site to shape parasympathetic nervous system are indicated to be GDNF/GFRa1/Ret (Young et al., 2004). The cells differentiate into MASH1-expressing autonomic neuronal precursors under the influence of BMP-family. A gradient of BMP expression determines the fate switch, if subjected to high concentrations the precursors differentiate into sympathetic neurons meanwhile low concentrations permit parasympathetic neuron differentiation. Parasympathetic neurons are dependent on Phox2 expression in addition to Hand2 and Gata2 (also belonging to bHLH, basic helix-loop-helix, transcription factor). The coalescence of precursors in the ganglia requires GDNF and neurturin acting on GFRα1 or GFRα2 complexes with c-Ret (Lemke, 2010). GDNF and neurturin are suggested of importance to promote growth of the axons of the ciliary neurons towards their targets (Rossi et al., 2000). Analysis of knockout mice lacking molecules required for GDNF and neurturin signalling has uncovered that some parasympathetic ganglia are more

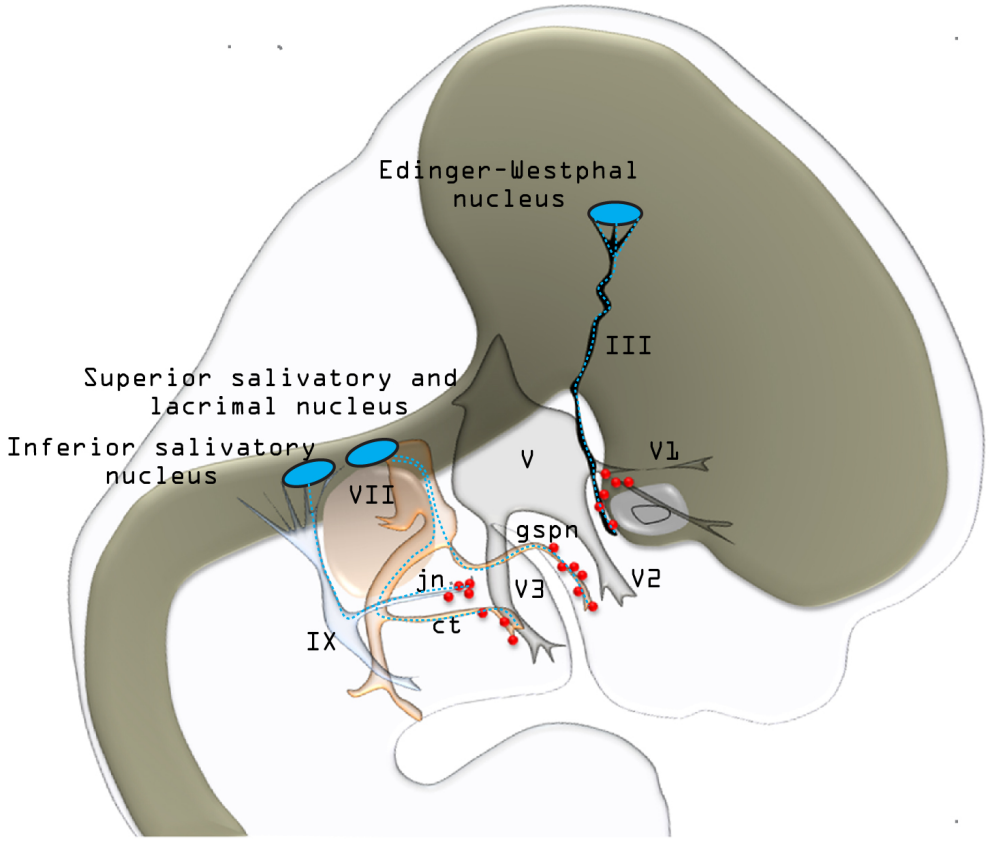

Figure 7. Schematic drawing illustrates some of the major parasympathetic nuclei; Edinger-Westphal nucleus, Superior salivatory and lacrimal nucleus, and Inferior salivatory nucleus. III – The Oculomotor Nerve, V – Trigeminal ganglia, with its three branches: V1 – Ophthalmic nerve, V2 maxillary nerve, V3 mandibular nerve, gspn –Jacobson nerve, ct – Chorda tympani, VII – The Facial nerve, and IX The Glossopharyngeal Nerve.

Illustration kindly provided by Dr Igor Adameyko.

dependent on their expression pattern. The pterygopalatine (sphenopalatine) ganglion seems to require GDNF and not neurturin whereas the otic ganglion requires both GDNF and neurturin to form (Enomoto et al., 2000).

There is certainly more to explore in regard of local cues at the site of parasympathetic ganglion formation and during development, as there are vast number of questions that are not answered concerning how the postsynaptic parasympathetic ganglion develops.

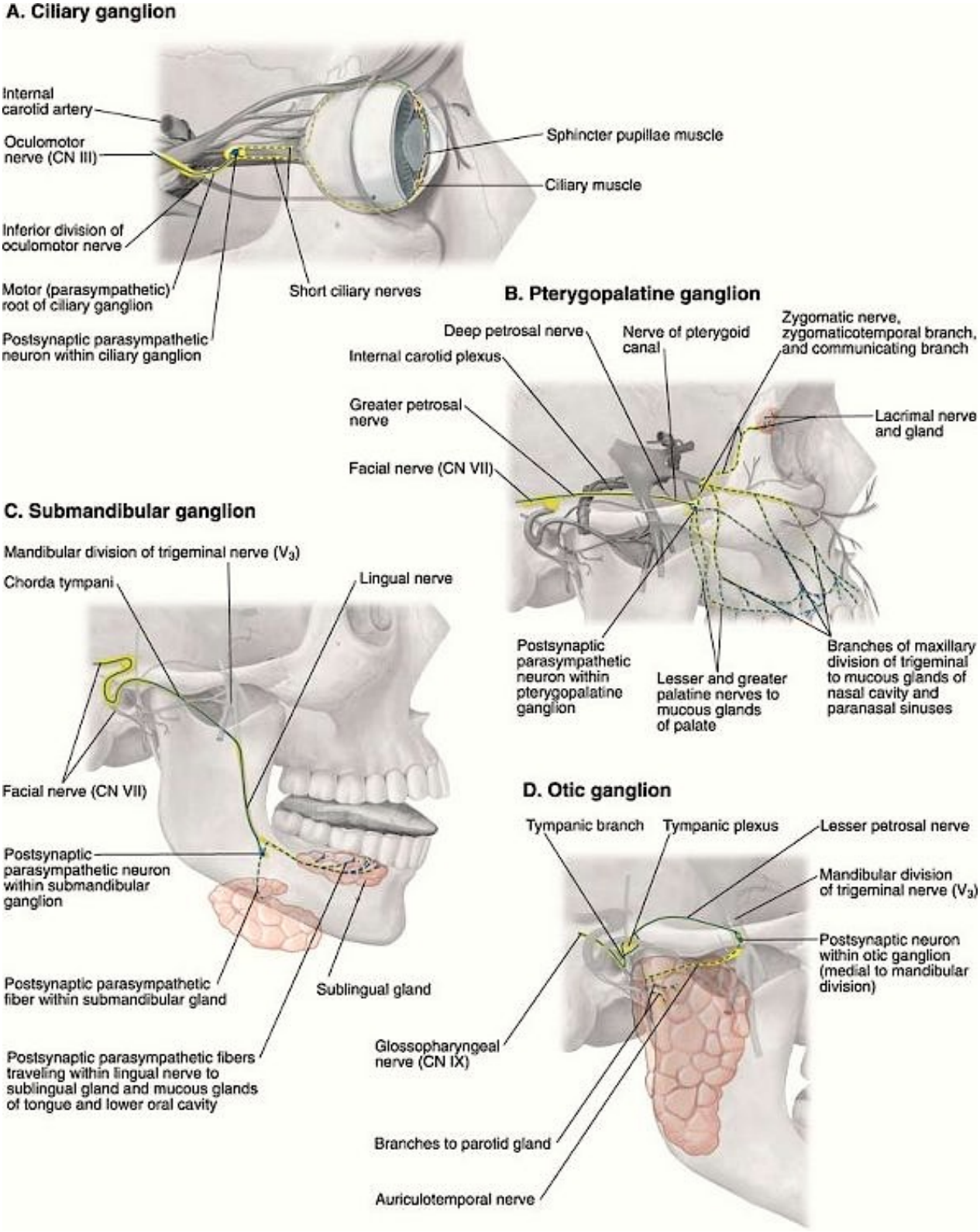

Figure 8. (A) Ciliary ganglion localization behind the orbita and the nerve passage to its targets. **(B)** Illustrates Pterygopalatine ganglion, **(C)** shows the innervation of submandibular gland through the submandibular ganglion, and **(D)** Branching of the parasympathetic nerves that shape the tympanic plexus and also branches that innervate the parotid ganglion.

Taken from: Lippincott Williams & Wilkins Atlas of Anatomy, Tank, Patrick W.; Gest, Thomas R., Chapter 8 - The Autonomic Nervous System.

1.5 GLIA

Glial cells are found both in CNS as well as PNS. Glial cells are known to have a crucial role in the maintenance of the neural system. CNS glia are referred to as "macroglial cells" or more properly "neuroglial cells". These are the *astrocytes*, *oligodendrocyte*s and *microglia*. Astrocytes participate in forming the blood-brain barrier, are of importance for neurotransmitter reuptake, metabolic coupling of synaptic activity and injury response. Oligodendrocytes are known for their role in myelination that enables efficient impulse propagation. The microglia stem from macrophages that invade the brain during early development and then remain throughout the CNS. The glial cells of the PNS are Schwann cells and the satellite cells of sensory, cranial and autonomic ganglia (Verkhratsky and Butt, 2007, Nave and Trapp, 2008). Many sensory nerve endings in the skin are closely associated with glial cells that form the innermost part of larger structures. The Pacinian corpuscle is one example, another is found at the axon terminals of the skeletal neuromuscular junction, which are tightly covered by terminal glia (teloglia). Satellite cells cover the neuronal cell bodies in DRG as well as in sympathetic and parasympathetic ganglia.

The Schwann cell lineage is initiated in NCCs by the generation of Schwann cell precursors (SCPs) that develop into immature Schwann cells around the time of birth. SCPs are dependent on nerves for their survival and provide the nerves with essential trophic support for their survival. They are also necessary for normal nerve fasciculation, and probably involved in the production of the endoneural fibroblasts in the peripheral nerves. Schwann cell precursors can be regarded as the transitional stage between the neural crest and the immature Schwann cell (Figure 9). Which type of nerves they connect with determines the future of the immature Schwann cells. If associated with a nerve with a diameter larger than 1µm, the cell will be myelinating. Myelinating Schwann cells wrap their plasma membrane around the nerve and produce a multilamellar compacted sheet of myelin for rapid transmission of saltatory impulses. In other cases, single non-myelinating Schwann cells will ensheath from one to multiple axons with diameters below 1µm, forming what is known as Remak bundles (Nave and Trapp, 2008). A major difference between Schwann cells and its earlier precursors is the ability of the Schwann cell to ensure its own survival through autocrine survival circuits (see *Figure 10*) (Meier et al., 1999). There has been considerable interest in the potential of SCPs to act as multipotent progenitor cells of the PNS. Thus, these cells can undergo fate switch to become melanocytes (pigment cells) of the skin (Adameyko et al., 2009). Peripheral nerves display a certain degree of plasticity. The myelinating

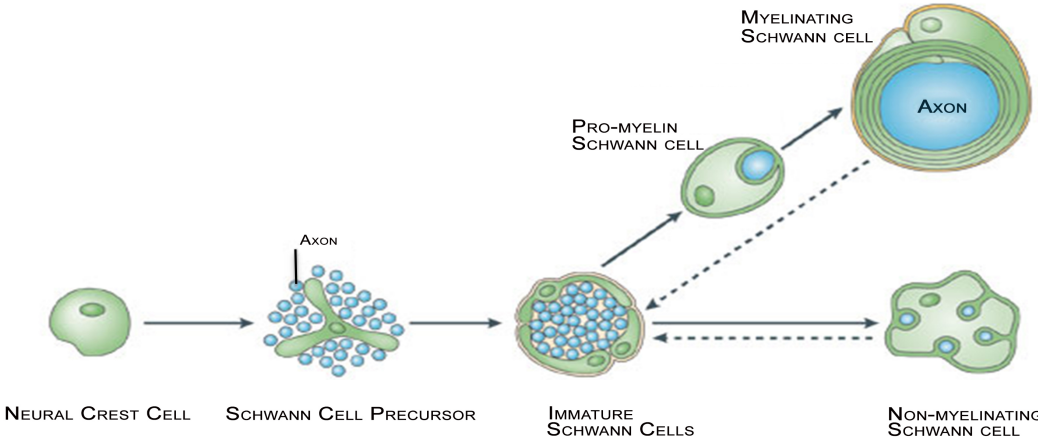

Figure 9. Reproduced from Jessen and Mirsky et al (2005).

Schwann Cells can for instance dedifferentiate back to their precursor state, SCPs, in case of a nerve damage (see *Figure 9*) (Jessen and Mirsky, 2008). The intrinsic negative regulators, including c-Jun, Notch, Sox2, Krox24, Pax3 and others drive the dedifferentiation process, promote axon regrowth and nerve repair following the trauma. Positive regulators including Krox20, Sox10 and Oct6 are pro-myelinating factors. Together they ensure that myelination of nerves occurs at the correct time during development and also reinforce reestablishment of the Schwann cell fate after injury. The capacity of regeneration in the PNS, in contrast to CNS was observed already very early. Although the microscopes of Santiago Ramón y Cajal's did not yet permit resolution of Schwann cells and axonal plasma membranes, he and others including Augustus Volney Waller (1816-1870) and Louis-Antoine Ranvier (1835-1922) demonstrated that axonal regrowth occurred from the proximal to distal stump (Ramon y Cajal, 1928). He concluded correctly that axons were extensions of the neuronal cell body and that axonal recovery in the PNS was due to "symbiotic" interactions between axons and Schwann cells (Kidd et al., 2013).

Wallerian degeneration is a term referring to the process of tissue cleaning/preparation at the distal stump of a damaged nerve were axonal regrowth is supposed to occur. Schwann cells and macrophages interact to remove debris, specifically myelin and the damaged axon, from the distal injury site (Carbonell et al., 1991, Guertin et al., 2005). As Wallerian degeneration proceeds, bands of Büngner form, comprising longitudinally aligned Schwann cell strands that guide selectively regrowing axons (Ribeiro-Resende et al., 2009). Similarities exist between Schwann cell-derived band of Büngner repair cells and immature Schwann cells. Yet, they are not identical, since Büngner band cells exhibit traits that are not strongly expressed in immature Schwann cells (Arthur-Farraj et al., 2012). Thus, cells that reside in bands of Büngner may represent a separate type in the Schwann cell lineage that participates in peripheral nerve regeneration (Kaucka and Adameyko, 2014).

Sox10, one of the most important peripheral glial genes, belongs to a family of high-mobility group-box transcription factors, namely the sex-determining region on Y chromosome-box (Sox) transcription factor family. This family can be further subdivided into classes, SoxA to SoxJ (Bowles et al., 2000). Sox10 gene expression is found in neural crest cells that delaminate from the neural tube and start their migration. However, the expression of this gene is quickly downregulated in neural crest-derived mesenchyme and neurons, and persists only in the glial lineage and in melanocytes. Later, the expression of Sox10 is reinitiated in developing cartilage and epithelial compartments of glands. Continued glial development is dependent on Sox10 as transcription factor (Jessen and Mirsky, 2005). It has a profound influence on the specialization of SCPs from NCCs. In addition to the terminal differentiation of NCCs into SCPs, Sox10 also regulates the maintenance and the myelin gene expression of Schwann cells (Weider et al., 2013).

Diseases that involve congenital pathology due to the inability of CNCCs to migrate or survive are termed neurocristopathies. A wide range of mutations in different genes can be involved in these kinds of developmental disruptions. The cranium and face are sites of a considerable amount of birth defects, almost one third of all such malformations. Theoretically, this might be due to the complexity of tissue interactions with a vast number of genes in the development of a relatively small area (Snider and Mishina, 2014). Mutation of

the Sox10 gene gives rise to neurocristopathies such as Waardenburg-Shah syndrome type IV (WS-IV) that has clinical combinational traits of Waardenburg syndrome, and Hirschsprung's disease (HSCR) characterized by cochlear deafness, pigmentary defects and enteric aganglionosis in distal parts of colon (Mollaaghababa and Pavan, 2003).

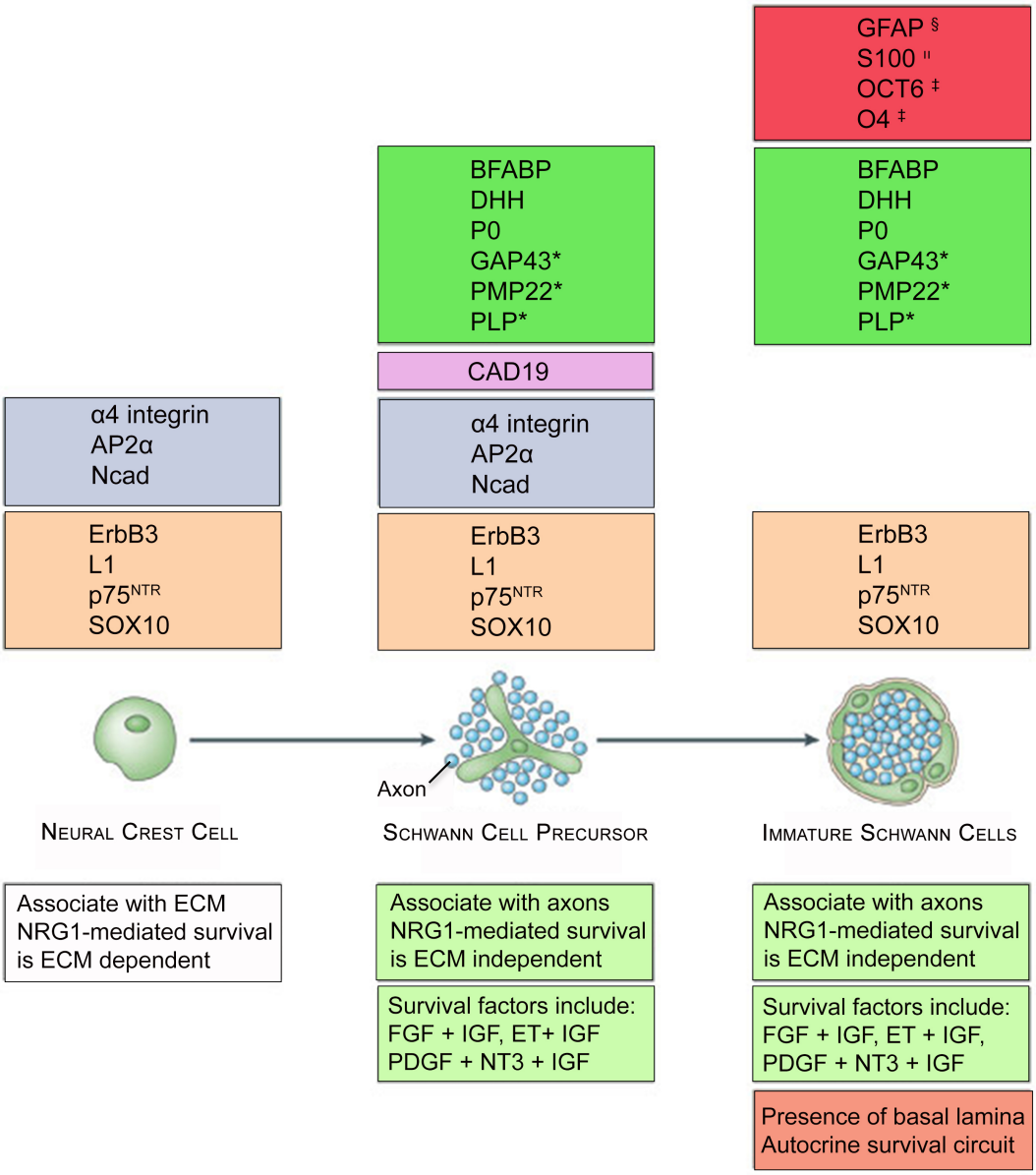

Figure 10. Overview of the expression profile and transitional characteristics for neural crest, Schwann cell precursor and the immature Schwann cell. Distinct colours indicate shared profiles. The boxes above the lineage drawing indicate changes in gene expression that take place during embryonic Schwann cell development. Gene expression shown here is based on observations of endogenous genes rather than on observations of reporter genes in transgenic animals. *Proteins that also appear on neuroblasts/early neurones. ‡Markers depends on axons for expression. §GFAP is a late marker of Schwann cell generation, as significant expression is not seen until about the time of birth, reversibly suppressed in myelinating cells. ||SCPs have been shown to be S100 calcium-binding protein (S100)-negative and Schwann cells S100-positive using routine immunohistochemical methods.

1.6 NERVE – ORGANOGENESIS INTERPLAY

Diseases causing abnormal cranial nerve development often lead to aberrant growth of target organs and tissues. One such example is Möbius syndrome that causes palsy and nerve weakness, which in turn gives facial asymmetry. This congenital disease is characterized by deficient innervation (abducens (VI) and facial (VII) nerves), and causes clinical symptoms, like deafness, tooth anomalies, and cleft palate (Rizos et al., 1998). It is evident that peripheral innervation in many cases is needed for proper development and function of orofacial structures. Etiologies behind congenital facial abnormalities vary. They can be caused by genetic or, most commonly, unknown factors. Deformations in facial growth can be environmentally influenced during embryogenesis and disruptions in the growth can also have metabolic, vascular and/or teratogenic causes. Even though both the severity and the etiologies behind facial anomalies differ widely, all cause some degree of functional impairment. If these embryonic defects are too extensive they can result in drastically reduced foetal survival rate (Sperber et al., 2010).

New findings suggest that there is a much more intrinsic interplay between peripheral nerves and their surroundings than initially thought. Craniofacial glands and their nerves are examples of such interesting cross-talk. In mice, parasympathetic axons that surround the salivary epithelial bud are first detected at E12. These axons follow the branching pattern of the developing salivary gland epithelium (Coughlin, 1975). Little has been known about the functional contribution of innervation to organogenesis until Knox and colleagues discovered that parasympathetic innervation is important for maintenance of the epithelial keratin 5–positive progenitor cells during salivary gland organogenesis. This maintenance is governed by the cholinergic signalling through muscarinic (M1) receptors and epidermal growth factor receptor that increases the epithelial morphogenesis and proliferation of the progenitor pool of cells (Knox et al., 2010). Parasympathetic nerves have been shown to be of importance for tubulogenesis and lumen expansion processes when the salivary gland ducts are formed (Nedvetsky et al., 2014), and also for salivary gland regeneration after damage (Proctor and Carpenter, 2007).

Sympathetic denervation in rodents affects the salivary gland target tissue. As an example, does ablation of sympathetic nerve cause hypotrophic alterations of the acinar cells and decreases their granule content (Henriksson et al., 1985).

Generally, it is known that regeneration in vertebrates is nerve-dependent (Brockes and Kumar, 2005). Yet another example where peripheral innervation seems indispensable for proper tissue or organ generation/regeneration is provided by fish teeth (Tuisku and Hildebrand, 1994). Cichlids were used to perform unilateral denervation studies of the lower jaw, followed by extraction of mandibular teeth. Corresponding teeth were extracted from both sides of the jaw, and the unoperated side served as control for the denervated side. Cichlids have continuous tooth replacement under normal circumstances. Interestingly, after a one-year follow-up, it was found that the teeth on the side where the nerve was lesioned did not regenerate, in contrast to the control side with the intact nerve. This is a strong indication that there is a link between peripheral innervation and organ formation. It can be speculated whether the nerve is providing the signal for tooth formation by secreting or presenting certain factors influencing the mesenchyme or epithelium. During what phases of

odontogenesis these hypothetical interactive signals would be transmitted is unknown. When embryonic mouse tooth germs are dissected and epithelial and mesenchymal compartments are cultivated separately they fail to undergo cytodifferentiation. It is generally accepted that a cross-talk between these two tissue compartments induces their normal differentiation and/or survival, governed by diffusible signaling mechanisms (Koch, 1967, Koch et al., 1970). It is also known that the odontogenic potential of the epithelium can partially be replaced by recombinant BMP4 protein in an organ culture of dental mesenchyme (Vainio et al., 1993, Chen et al., 1996). Organotypic *in vitro* and *ex vivo* cultures suggest that tooth initiation and development can proceed without neuronal contribution. Experiments have shown that entire teeth can be developed in *ex vivo* cultures regardless of the presence or absence of trigeminal ganglia (Lumsden and Buchanan, 1986). Many attempts have been made to develop different methodologies for tooth germ culture in *in vitro*/ *ex vivo* settings. Lately, refined methods such as slice culture techniques, biodegradable scaffolds and techniques to bioengineer teeth have been presented which provide tools to mimic *in vivo* settings better and allow more accessible experimental manipulations (Alfaqeeh and Tucker, 2013, Zhang et al., 2005, Oshima and Tsuji, 2014). These advances bring important information regarding basic mechanisms that regulate odontogenesis. Tooth germs can develop in standard tissue culture conditions for 7-8 days, but eventually become flattened and disorganized. When using 3D culture systems, this period can be slightly extended (Sun et al., 2014). Different transplantation methods have also been adopted to recapitulate the normal developmental conditions for the tooth germ where revitalisation/revascularisation can occur (Yokohama-Tamaki et al., 2006). However, many questions remain unanswered regarding the intra- and extracellular mechanisms that shape the tooth into its final form and what role the peripheral nerves may play.

Exactly what processes that might initiate and regulate a nerve–organogenesis interplay is not known. It has been indicated that sensory nerves that innervate hair follicles can regulate the repair process after injury (Brownell et al., 2011). Recently, it was proposed that stem cells are harboured by peripheral nerves also in the adult, and that they can be mobilized for repair as a response to tissue damage (Kaucka and Adameyko, 2014).

1.7 CONCLUDING REMARKS

CNCCs have long since been thought to be one the outermost important stem cell sources in the craniofacial region. Throughout this thesis work emphasis has been put on finding additional as yet unidentified sources. One such potentially interesting source may be tracked further down in the NCC developmental lineage tree, and of interest are the Schwann cell precursors (SCPs), the undifferentiated glial cells. Of interest is to investigate if tooth-adjacent peripheral nerves hold niches for multipotent progenitor cells that can be recruited in a targeted way and contribute to the mesenchymal derivates of developing teeth.

The extensive parasympathetic innervation of the head governs vital functions such as salivary, lacrimal and mucosal gland secretions. The developmental processes that lead to the formation of craniofacial parasympathetic ganglia are poorly understood. In line with our hypothesis that immature Schwann cells from peripheral nerves can be recruited at local sites and contribute to organ or tissue formation, we will here examine if the peripheral parasympathetic system of the head receives glial progenitor cells that contribute to gangliogenesis.

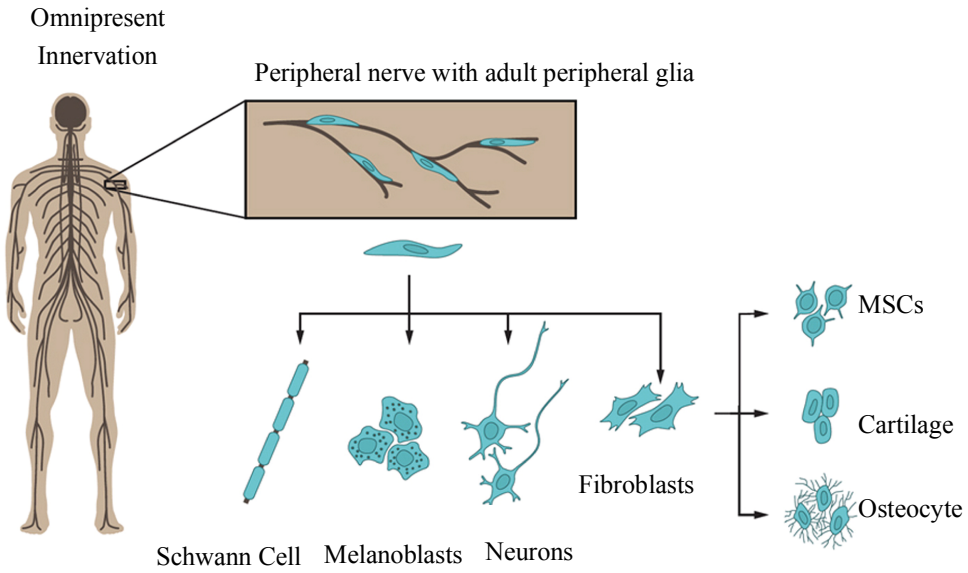

Figure 11. Peripheral glial cells are pervasive and represent easily accessible regenerative source for many neuro-glial and mesenchymal cell types.

2 AIMS OF THE THESIS

The general aim of this thesis work is to investigate if peripheral nerve glia is a source of multipotent progenitor or stem cells that contribute to the development and/or regeneration of craniofacial tissues and organs

Specific aims:

I) To investigate the potential role of peripheral glia in embryonic and adult tooth formation and regeneration.

II) To explore the fine morphology and potential functions of glia-derived odontoblasts and pulp cells using high-resolution 3D imaging.

III) To investigate if glial cells, as a multipotent stem cell reservoar, contribute to the formation of craniofacial parasympathetic ganglion nerve cells and, if so, to elucidate the steps in the the glia-to-neuron transition process.

3 MATERIAL AND METHODS

3.1 TRANSGENIC ANIMALS

All animal work was permitted by the Ethical Committee on Animal Experiments (Stockholm North Committee) and conducted according to The Swedish Animal Agency's Provisions and Guidelines for Animal Experimentation recommendations.

MOUSE STRAINS:	REFERENCE:	USED IN PUBLICATIONS:
Erbb3$^{-/-}$ (knock-out)	(Riethmacher et al., 1997)	I, III
Ascl1CreERT2/R26^{TOMATO}	Ascl1^{CreERT2} and R26^{TOMATO} purchased from The Jackson Laboratory were bred.	III
Ascl1$^{KINgn2/KINgn2}$	(Parras et al., 2002) Generated by Prof. F. Guillemont (NIMR, UK), received from U. Marklund Lab (KI, Sweden).	III
PLP^{CreERT2}/R26^{Confetti}	(Snippert et al., 2010)	I, II, III
PLP^{CreERT2}/R26^{YFP}	(Adameyko et al., 2009) (Leone et al., 2003)	I, II, III
Thy1^{CreERT2}/R26^{YFP}	(Dewachter et al., 2002)	I
Ret$^{CFP/CFP}$	Received from Prof. E Hideki (Riken, CDB, Japan).	III
Ascl1$^{CreERT2/CreERT2}$;Rosa26$^{Tomato/+}$	Purchased from The Jackson Laboratory.	III
Ascl1$^{CreERT2/+}$;Rosa26$^{Tomato/+}$	Purchased from The Jackson Laboratory.	III
SOX10$_{CreERT2}$/R26^{YFP}	(Laranjeira et al., 2011)	I, II, III

The confetti reporter is based on the Brainbow-2.1 cassette inserted into Rosa26 locus under control of the strong CAGG promoter and a LoxP-flanked NeoR-cassette providing a transcriptional roadblock in the absence of recombination. TM-induced recombination resulted in stochastic expression of one out of four fluorescent proteins (Snippert et al., 2010).

For tracing experiments, tamoxifen (4-HT, Sigma) was dissolved in corn oil (Sigma) and delivered via intra-peritoneal (i.p.) injection to pregnant dams. Concentration of Tamoxifen solution ranged from 1.0 - 5.0 mg/animal á 4mg/100µl injection volume, in order to achieve a range of recombination efficiencies. When administering sub-optimal concentrations we could obtain infrequent tracing that labeled less clones and gave valuable information regarding clonality properties of glial cells and their progeny. For all experiments, the day

when the plug was detected was considered as E0.5 and the day when the mice were born was considered to be P0.

3.2 IMMUNOHISTOCHEMISTRY

Whole-mount fluorescent immunostaining of mouse embryos, 3D imaging, visualisation and analysis were performed as previously described (Adameyko et al., 2012).

ANTIBODY:	SOURCE:	USED IN:
2H3 (NF200)	DSHB	I, III
CD13	BD Pharmingen, #558745	I
CD31 (PECAM-1)	BD Pharmingen, #553370	I, III
CD90 (THY1 [IBL-6/23])	Abcam, #AB3105	I, III
Collagen IV	AbD Serotec, #2150-1470	I
CRE	Novagen, #69050	I, III
DsRed	Clontech, # 632496	III
ERBB3	R&D Systems, #AF4518	I, III
GFAP	Abcam, #ab7260	I, III
GFP	Aves Labs Inc., #GFP-1020	III
GFP (FITC-conjugated)	Abcam, #ab6662	I, II, III
KI67 (SP6)	Thermo Scientific, #RM-9106-S1	I, III
Krox20	Nordic Biosite, not produced any longer.	I
MITF	R&D Systems, #AF5769	III
Myelin Basic Protein [12]	Abcam, #ab7349	I
NeuN	Millipore, #MAB377	III
NG2	Millipore, #AB5320	I, II
P0	Abcam, #134439	I
P75	Promega, #G323A	I
PGP9.5	Cederlane, #CL95101	I, III
PHOX2B	R&D Systems, #AF4940	I, III
Piezo2	Sigma, #HPA040616	II
S100b	DAKO A5110	I
Sox-10	Santa Cruz, #17342	I, III
Tuj1 (Anti-βIII Tubulin)	Promega, #G712A	I, III
VAChT	Phoenix Pharmaceuticals	III
Nuclear DAPI-staining (49,6-diamidino-2-phenylindole), Invitrogen #D1306, 300 nM, 5 min, RT.		

BFABP was a kind gift from Dr T. Müller (Paper I).
Rabbit anti-PHOX2B was a kind gift from Dr Jean-Françoie Brunet (Paper II).
Guinea pig anti-SOX10 was a kind gift from Dr Micheal Wegner (Paper II).
TrpM5 antibody was a kind gift from Dr Vladimir Chubanov (Paper III).

Standard immunohistochemical protocol has been used to stain the tissue, kindly see separate papers referred to for further details.

3.3 CONFOCAL IMAGING

Acquisition of images was done using Zeiss LSM510, LSM700, LSM 710 and Zeiss LSM780 confocal systems. IMARIS and Image J software were used for processing and analysis of all images. Bitplane IMARIS software was used also for 3D visualisation and analysis of confocal stacks. To avoid high levels of noise, stacks were rendered in 3D and non-specific fluorescent background was removed as described previously (Adameyko et al., 2012).

3.4 DATA ANALYSIS

Statistics and Quantification, Paper I: Statistical data are represented as mean s.e.m. Unpaired and paired versions of Student's t-test were used to calculate the statistics (P value). Pearson's product-moment correlation coefficient (r) was calculated to investigate the association of variables in Paper I, Fig. 3t–v (n529 for Fig. 3t–u and n527 for Fig. 3v). Every value corresponding to a dot refers to a single clone; in total, clones were analysed from11 different animals. Linear regression was used to build an approximation line in Fig. 3t. To analyse the position of dental MSCs in relation to the cervical loop (Fig. 3t–u), multiple sequential sections were analysed. Generally, we devoted several (always more than three) animals to every experiment to accomplish at least a biological triplicate. This was valid for all non-quantitative analyses including work done on sections or in a whole-mount. For the genetic tracing experiments reported as graphical panels, at least six embryos derived from at least two females were analysed; in most cases 15–20 embryos were used before conclusions and supporting graphics were generated. During our study more than 100 genetically traced animals of different strains were analysed before concluding final results. The animals were selected and distributed into groups in all experiments randomly. The control for the denervation experiment was an internal control coming from the same animal: the non-operated contralateral side (biological (number of individual animals) n=55, while technical (number of sections analysed) n=515). To quantify the contribution from Schwann cell-derived MSCs, we analysed three animals per condition counting three sections in every animal (technical n=59). YFP^+ cells and $DAPI^+$ cell nuclei inside the tooth were identified on confocal images segmented in IMARIS software and counted in a semi-automated way.

Statistics and Quantification, Paper II: Statistical data are represented as mean s.e.m. Rendered isosurfaces and 3D analysis in IMARIS. Graphs were obtained with Graph Pad Prism program. Figure 1 O-P, number of odontoblasts analysed were n=20. Graph 5 G data represents the morphological features of cells in the pulp at a distance from the Höhl layer, and cells within the Höhl layer in close proximity of odontoblasts. Number of branches measured were n=10 in each case.

Statistics and Quantification, Paper III: All quantification was conducted using the ImageJ software. Results were processed in Prism6 software and showed as mean+/- s.e.m. for each condition. At least three heads were analysed per quantification.

3.5 OTHER METHODS

For information regarding the techniques below, please see papers I,II and III, respectively.

- Sample preparations (Paper I, II, III)
- Immunohistochemistry details (Paper I, II, III)
- *In Situ* hybridization (Paper I)
- BrdU and EdU incorporation analysis (Paper I)
- Flow cytometry (Paper I)
- Tooth damage experiments (Paper I)
- Explant culture, cell sorting and osteogenic culture (Paper I)
- Denervation studies (Paper I)
- Genetic nerve ablation (Paper III)

4 SHORT SUMMARY AND DISCUSSION

Our work highlights a novel function of peripheral nerves as a niche for multipotent glial cells that can be recruited locally and transformed into a spectrum of differentiated cell types. By using state of the art techniques including clonal color-coding with genetic tracing we have labelled single peripheral glial cells *in vivo* and shown their diverse progeny.

4.1 PAPER I

Here we demonstrated a contribution from glial cells to the mesenchymal compartment of the tooth, and we also provided a general logic for how dental MSCs generate specialisedd tooth cells: odontoblasts and pulp cells are, organised clonally in space and are compartmentalized inside the tooth.

- We identified Schwann cell precursors and Schwann cells as novel sources that contribute to tooth formation during embryonic development and adult growth. During adult growth of mouse incisor the glial cells contribute almost half of odontoblasts and pulp cells after nine months of genetic tracing. These findings were obtained using two independent genetic tracing systems that recombine specifically in glial cells.

- We highlighted the importance of the nerve for the continuous tooth growth by surgical denervation in combination with genetic tracing. Upon denervation the progeny of glial cells ceased to appear in the tooth.

- We discovered that the progeny of glial cells produced reparative matrix after trauma *in vivo,* and *in vitro* in an osteogenic assay.

- We explored the dynamics of tooth development and growth by looking at the progeny arrangements that result from individual stem cells. We found that both odontoblast and pulp fates are generated from single dental MSC and that the fate of the clonal progeny depends on the proximity of dental MSC to the cervical loop. This leads to the formation of compact clonal sections inside of the mesenchymal compartment of the tooth. These clonal sections included both pulp cells arranged in proximity to the odontoblasts of the same clone. Despite a significant overlap between several clonal sections, each single dental MSCs contribute cells only to defined spatial domains inside of the tooth (Figure 12).

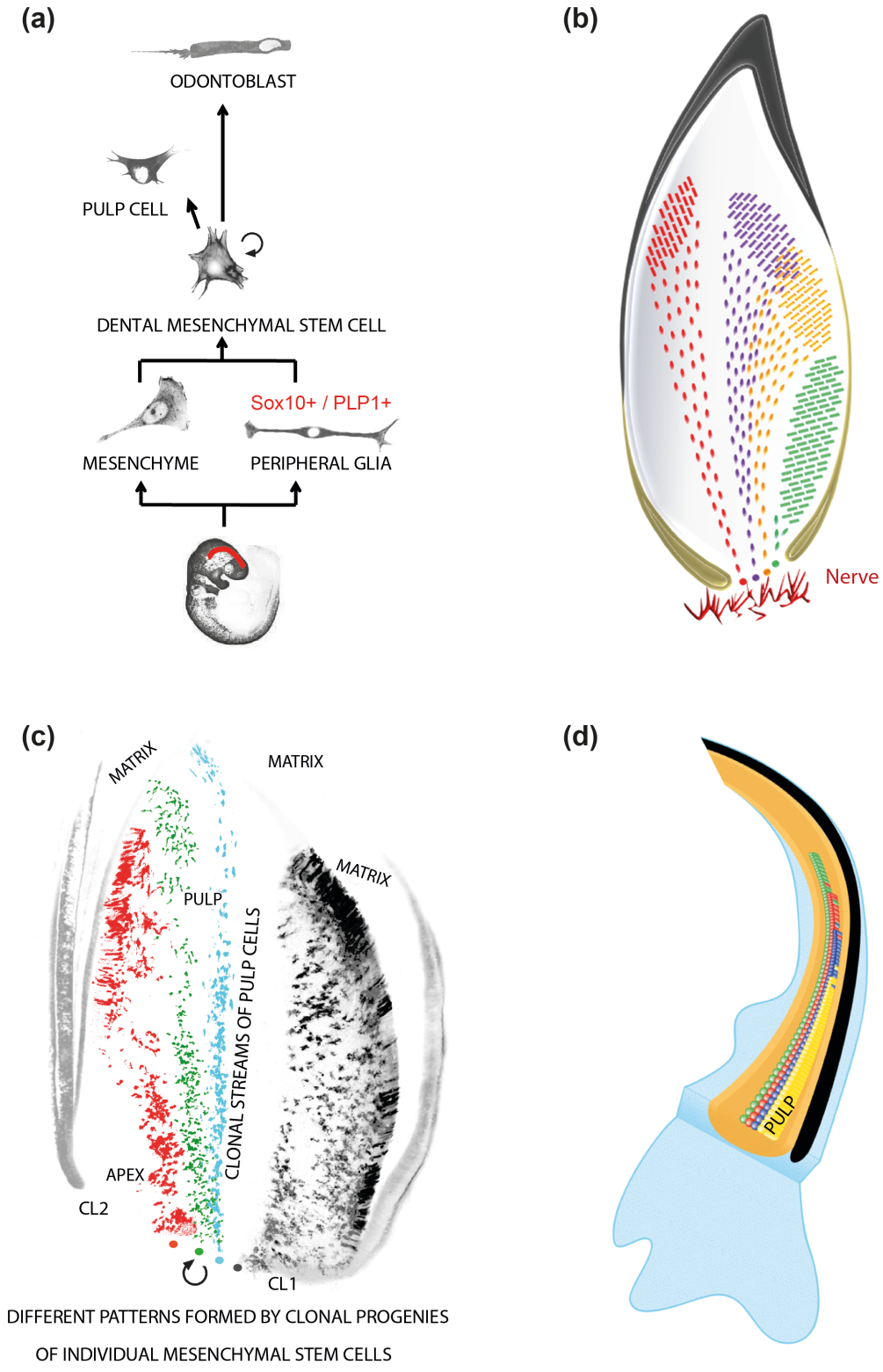

Figure 12. Conceptualisation of Paper I in brief: **(a)** Reconstructed lineage tree of the neural-crest-derived compartment in the tooth**. (b)** Illustration of clonally organised pulp and odontoblasts in embryonic incisor. **(c)**, **(d)** Clonal organisation of mesenchymal compartment in adult incisor and visualisation of how proximity of dental MSCs (dMSCs) to cervical loop (CL) correlates with clonal size and proportion of odontoblasts in a clone.

Illustrations are from Kaukua and Shahidi et al., 2014.

4.2 PAPER II

Gaining a better understanding of cell morphology and physical cell-cell interactions is of importance for future advances in clinical applications and for regenerative medicine purposes. Odontoblasts yield special interest, since in addition to their matrix-producing properties they have been proposed to participate in sensory functions in tooth. For this purpose, we took advantage of genetic tracing and the visualisation techniques developed in *Paper I*. The fact that odontoblast processes are embedded in the dentin limits their access to structural as well as functional studies. Consequently, many aspects of odontoblast activities remain obscure. To overcome this limitation, we used un-decalcified tooth sections from genetically traced animals. This provided a very useful method to obtain confocal microscope images of odontoblasts based on expression of fluorescent proteins, images that subsequently were assembled into 3D reconstructions. Simultaneous labelling of different glial clones with random fluorescent proteins allowed for visualisation of dental MSCs and their clonal progeny. In turn, it enabled examination of the fine morphology and spatial arrangements among odontoblasts that originated from different stem cells.

- We examined the different stages of the preodontoblasts during maturation into fully formed odontoblasts. This gave an understanding of how the main secretory pole, the odontoblast process, is initially formed as a brush-like structure which later becomes refined and streamlined as maturation proceeds.

- Using 3D reconstructions we identified a morphological compartment in the mature odontoblast that we named the “odontopode”, due to its foot-like configuration. The odontopode is located adjacent to the matrix, and the odontoblast process is emitted from here (Figure 13a).

- We demonstrated, for the first time, that odontoblasts express Piezo2 and TRPM5. This gives further indications that odontoblasts may have sensory functions, including mechanotransduction, since both these proteins are associated with action potential generation.

- We found that cells in the subodontoblast region emit long processes that extend far into the odontoblast cell layer. A large number of these processes appear to establish specialisedd contacts with odontoblasts. This strongly indicates that odontoblasts and subodontoblast pulp cells communicate and form a functional integrated cellular network with hitherto unknown properties.

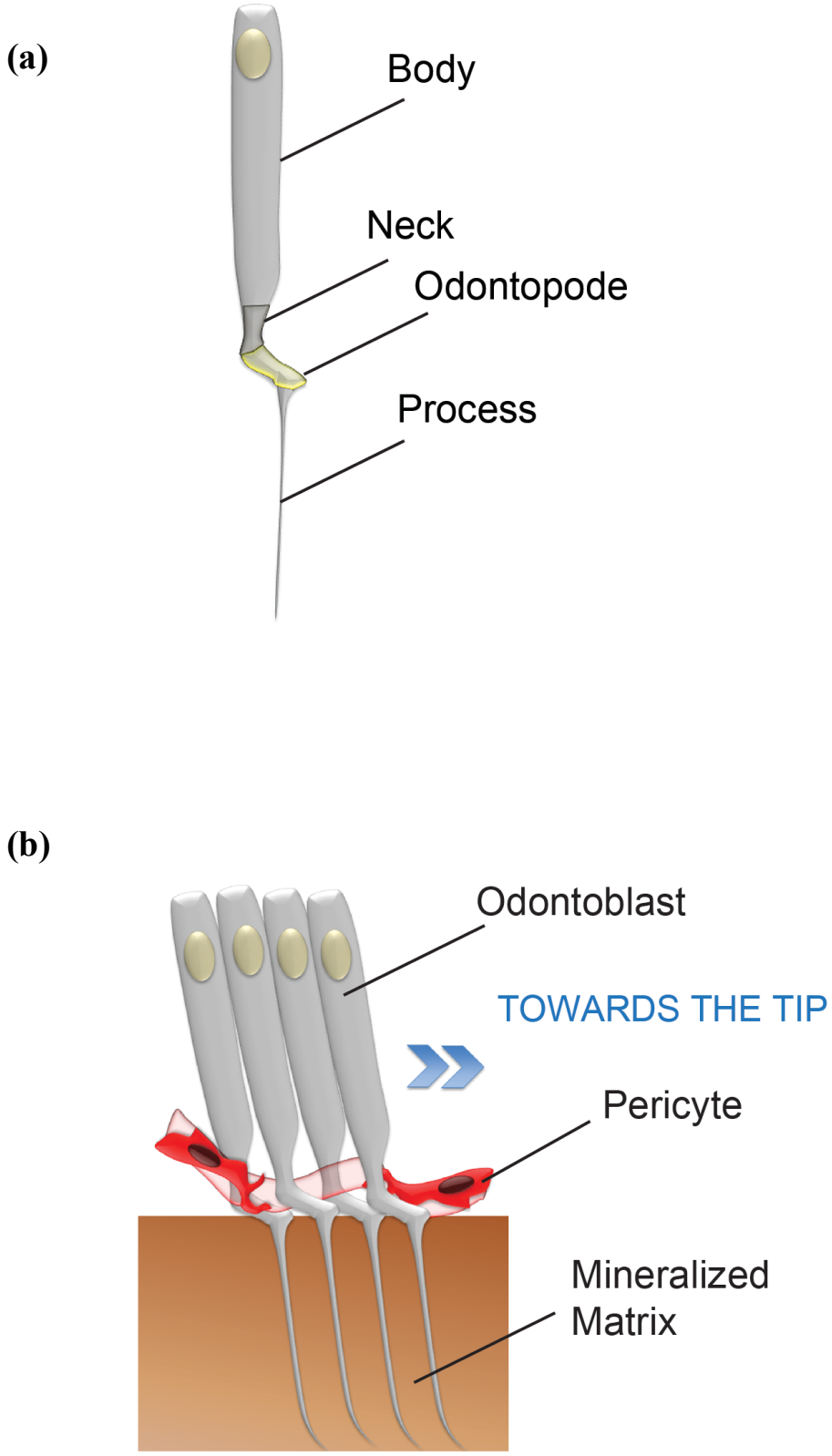

Figure 13. Conceptualization of Paper II. (a) Schematic illustration showing cellular compartments of odontoblasts. **(b)** Illustration depicts the angulated orientation of odontoblasts in relation to the dentinal mineralized matrix. Note pericytes on the vessels in the odontopode region of the odontoblast layer.

[Unpublished Data]

4.3 PAPER III

The influence of PNS on normal tissue and organ development is apparent but not yet fully understood and explored. Our findings that peripheral glia contribute to tooth formation sought us to expand upon the concept of immature PNS glia as a source of progenitors for autonomic parasympathetic neurons. Many studies have elaborated upon the development of sensory, sympathetic and enteric nervous system, but parasympathetic neuron development has been less explored. In this thesis work we show the following.

- Using genetic tracing with glia specific cre lines, we demonstrated that glial cells (Schwann cell precursors) give rise to all craniofacial parasympathetic neurons in all ganglia (ciliary, pterygopalatine, lingual, submandibular and otic).

- We showed that the first commited progenitors of parasympathetic neurons appear adjacent to specific nerve branches, namely preganglionic visceral motor nerves in the developing head.

- We examined if a transient progenitor cell stage exists between recruitment of glial cells and the emergence of parasympathetic neuron. Our results refute this concept and suggest an almost direct conversion of glia into neurons.

- We provided a detailed description of molecular markers that determine the transition between glial and parasympathetic phenotypes within crucial nerves at the sites of future parasympathetic ganglia.

- We discovered that when a neurogenic program was blocked in the glia-derived progenitors of parsympathetic neurons, the progenitors remained in the nerve and gave rise to peripheral glial cells instead. This was made through a knock-out of the critical neurogenic transcription factor Ascl1 combined with genetic tracing using CreERT2 inserted into the Ascl1 locus.

- We elucidated in what way parasympathetic ganglia formation was dependent of the presence of peripheral nerves by performing genetic ablation of selective visceral preganglionic motor nerves using Ret-mouse mutants. This resulted in a total absence of the parasympathetic neurons that had been expected to be formed from the now ablated nerves. This shows that preganglionic visceral motor fiber are necessary for formation of the parasympathetic neurons.

- We depleted all nerve-adjacent SCPs in early embryos using Erbb3 knock-out mice, and found that all parasympathetic neurons were lost. Taken together, our results show that the outgrowing preganglionic nerves serve as niches and transport systems for SCPs, which are the precursors of parasympathetic neuron.

The present work demonstrates that peripheral nerve glia represents a versatile source of multipotent progenitor cells with putative regenerative potential. However, much work remains in order to fully understand the microenvironment that regulates the development, regeneration, growth and homeostasis in this neural stem cell niche. Such stem cells might then in the future be employed for clinical use, in repair of congenital craniofacial pathologies, in trauma cases and in for aesthetic body treatments.

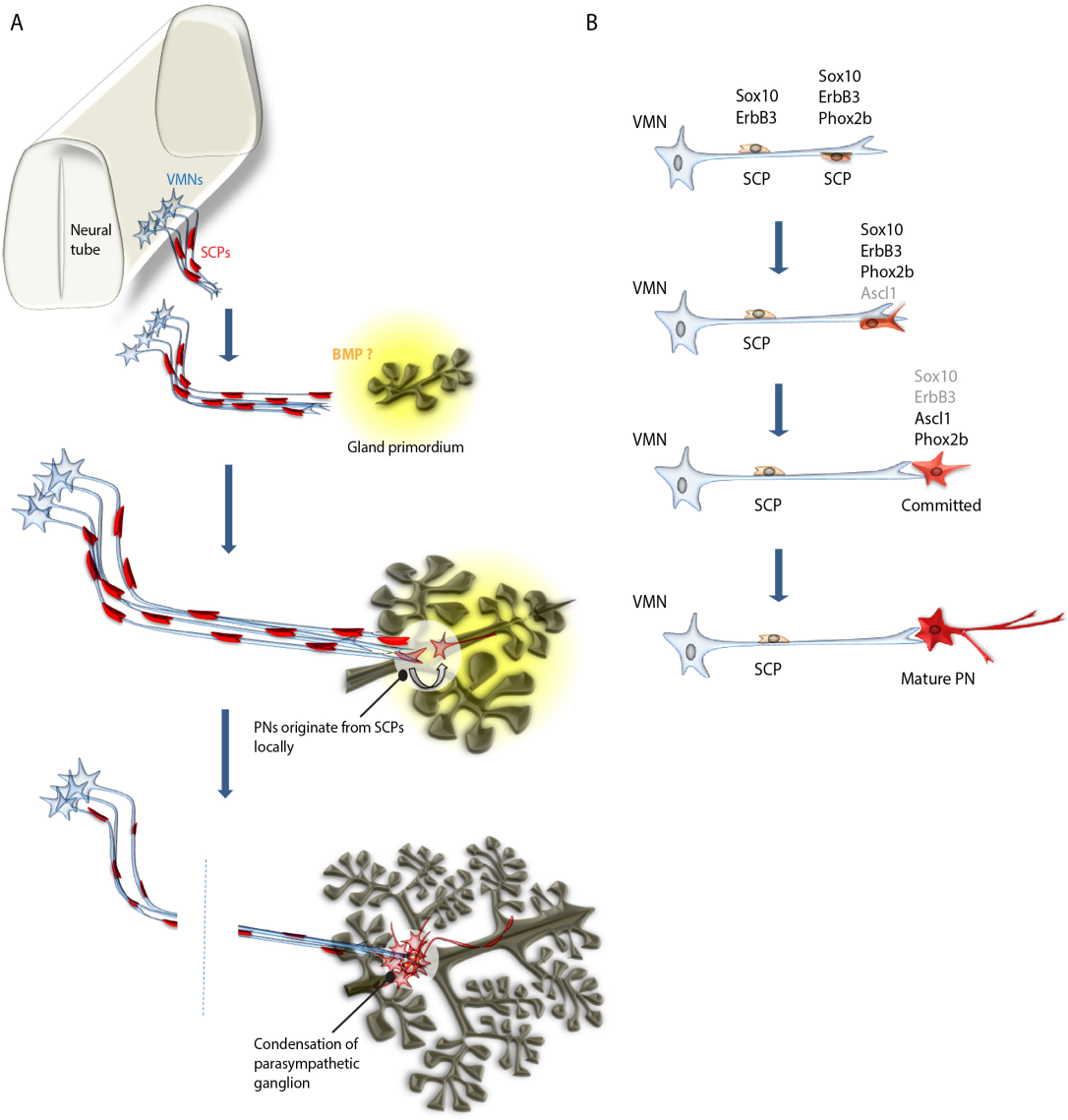

Figure 14. Conceptualization of Paper III in brief: (A) Nerves transport cellular sources of parasympathetic neurons to the locations of future ganglia. **(B)** A Schwann cell precursor (SCP) navigates along the visceral motor nerve (VMN) to the ganglion site where the SCP change fate to become a mature parasympathetic neuron (PN). Expression pattern during the different stages of the SCP differentiation process is indicated above the cel ls. The factors detailed in the present study are highlighted. VMN – visceral motor neuron, SCP – Schwann cell precursor, PN – parasympathetic neuron.

Illustration is kindly provided by Dr Igor Adameyko.

5 ACKNOWLEDGEMENTS

"It is Not the Destination, but the Journey that Matters Most"

I am thankful to all those who have been a part of my life over the past years, by brightening my days and in different ways helping me to lay the puzzle that in the end resulted in this thesis.

My journey into the world of science started during my high school years (in 2003-2004). I was intrigued by neurodevelopment and brain research and consequently my graduation project was performed on etiology and medical advances within the field of Alzheimer's disease (also involving my experiences as a nursery assistant at eldercare for dementia patients). I was fortunate enough to make the acquaintance of **Helene Hägglund** at the occasion of **Chengxuan Qiu**'s dissertation ("*The Relation of Blood Pressure to Dementia in the Elderly*"). Helene kindly introduced me to my future bosses Professors **Laura Fratiglioni** and **Bengt Winblad**. I was hired as a research assistant at the *Aging Research Center* (ARC) and gained experience within epidemiological research. Helene, thank you for taking a leap of faith and believing in me early on!

As a fresh dental student at Karolinska Institutet I was accepted onto the Summer Research School in 2005. As my interest was oriented towards craniofacial neurodevelopment, Professor **Kaj Fried** was my first choice of supervisor. Thank you Kaj for accepting me as a research student at that time, remaining as a supervisor during my PhD years, and helping me evolve my scientific writing skills. I would like to thank Associate Professor **Rachael Sugars** and Professor **Manuel Patarroyo** for their guidance throughout my initial PhD year. I am also very grateful for proof-reading help at times and our scientific chats Rachael.

Assistant Professor **Igor Adameyko**, you have been an inspirational source for me during the years we have done research together. Thank you for teaching me and guiding me as a supervisor but also doing research side-by-side with me. I have learnt a tremendous amount from you and look forward to remaining your collaborator.

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