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Comparing Stem cell Characteristics of Paired Stem cells from Apical Papillae and Dental Pulps before and after Cryopreservation

Dissertation

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Einleitung

Medicine as an initiative revolves around concepts such as healing, prevention, and naturally pain relief. Needless to say, the quest for pain relief must have been in pursuit so long as humans existed. Therefore, it is not surprising that papyrus scrolls dating back to 3,500 years ago reveal that ancient Egyptians used willow barks, also referred to as ‘natures aspirin‘ to alleviate pain.[2]. Within the next millennium, it was documented that the father of medicine, Hippocrates of Kos, advised towards the utilisation of willow barks to alleviate inevitable labour pains [2, 3]. The pursuit of pain relief was then introduced in the name of the present day pharmaceutical medicine by the German scientist, Friedrich Sertürner in 1804 when he lucratively brought to light the means to isolate Morphine, an indispensably crucial medicament until our present day [4]. About 90 years later, the earliest scientific explanation of why willow barks were effective for pain relief was established and consequently pure acetylsalicylic acid was formulated and developed as the Aspirin medicament[5, 2].

1.1 Regenerative Medicine

Medicine today is slowly shifting to encompass what is referred to as regenerative medicine, where rather than suppressing or alleviating pain, attention is redirected to treating the injury point in a regenerative manner. Generally, the typical route proceeding an injury involves several wound healing cascades that occur within the individual injured cells and amongst neighbouring and distant cells. These cascades have the goal of rehabilitating the injured site into a state of health. Nevertheless, it is well documented that wound healing more often results in repair, rather than regeneration [6, 7].

The disadvantage of repair is that it more often than not leads to tissue remodelling which entails haphazardly laid down extracellular matrix (ECM) together with a fibroblastic cellular component, also referred to as a fibrotic scarring. This repair scenario is less than ideal due to the consequence of a compromised tissue function, which can inevitably lead to additional impairments[7, 8]. On the other hand, regenerative medicine employs the notion of attempting to decelerate the accelerated fibrotic repair reaction, in order to enable inherently present stem/progenitor cells to take the lead and permit tissue regeneration[7]. Regenerative medicine can therefore ideally be described as utilizing and orchestrating the body's inherent self healing components and capabilities in order to fulfil the target of functional regeneration rather than repair[9].

1.2 Tissue Engineering Triad

The tissue engineering triad (TET) can be employed to explain the detailed orchestration required to reach the goal of regenerative medicine. As the name implies, the tissue engineering triad dictates the presence of the following three angles/components; stem/progenitor

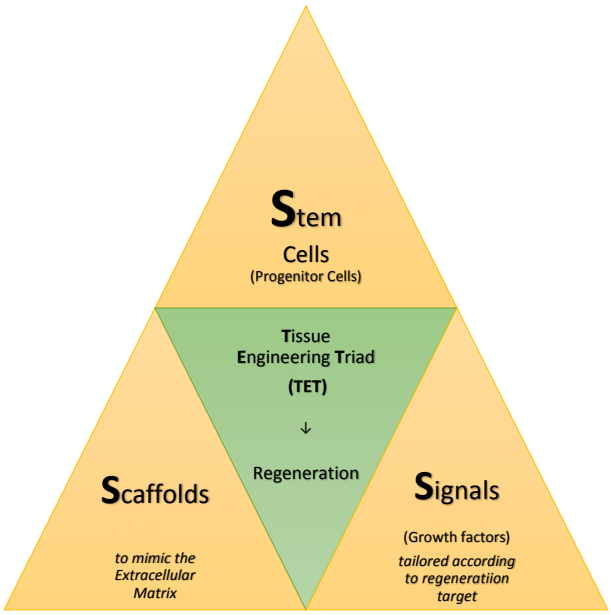


Figure 1.1: Tissue Engineering Triad

cells, growth factors/signals, and scaffolds mimicking the extracellular matrix (ECM)[10, 11] as illustrated in Figure 1.1.

1.3 Stem cells; a crucial element of the triad

The term stem cell that is in use nowadays was first introduced in 1868 by Ernst Haeckel, a german biologist who used the term to express how a mother cell, such as the fertilized egg can transform into a fully developed organism with differently specialized cells[12]. Twenty years later, William Sedgwick, a bacteriologist employed the stem cell word to delineate portions of plants that portrayed a regenerative capability. Further down the line, in 1957 E. Donnall Thomas, a physician striving to treat leukemia performed the earliest human bone marrow transplantation surgery based on the concept of blood regeneration by stem cells in

bone marrow[13, 12].

Similarly, the human body as a whole portrays stem cell characteristics in its ability to self heal or more appropriately speaking, in its ability to regenerate diseased tissues. Nevertheless, this regeneration is witnessed to vary greatly amongst tissues, for instance when comparing the turnover rate also known as the regeneration rate of skin fibroblasts versus for example, the very sought after neuroblasts. Mechanisms involved entailing factors such as timing and frequency of cellular division and hence turnover are noticeably different among stem cells residing in different tissues. Not only that, it could be the case that some cells require stimuli for turnover, where as others turnover in a more automatic or regulated manner [14, 15].

When examined more closely, a stem cell can be classified as an undifferentiated and thereby a non specialized type of cell. Furthermore, a stem cell is often informally referred to as the mother of all cells due to its inherent ability to give rise to various types of specifically differentiated and specialized cells, as illustrated in Figure 1.2. [12, 16]. This property further contrasts stem cells or non specialized cells against so to say non stem cells or specialized cells, where the latter divides to give rise to identical cells of the same specialty as illustrated in Figure 1.3, contrary to the former which divides to give rise to up to numerous types of differently specialized cells, thereby proving its multipotency and often times also its pluripotency[12].

Accordingly speaking, stem cells theoretically have the potential to shift the face of medicine from attempting to treat diseases through the usage of external pharmaceuticals to stimulating the body to treat itself by employing its inherent elements and capabilities. This can especially apply to diseases that have no adequate medicinal therapies to date, such as Parkinson's disease, Alzheimer's, and malignant tumors in general[12]. Therefore, it is of

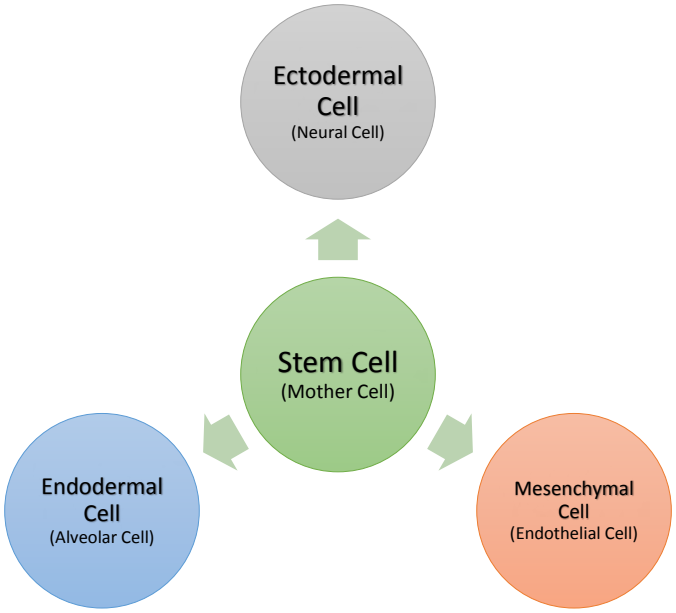


Figure 1.2: Potential Stem Cell derivatives

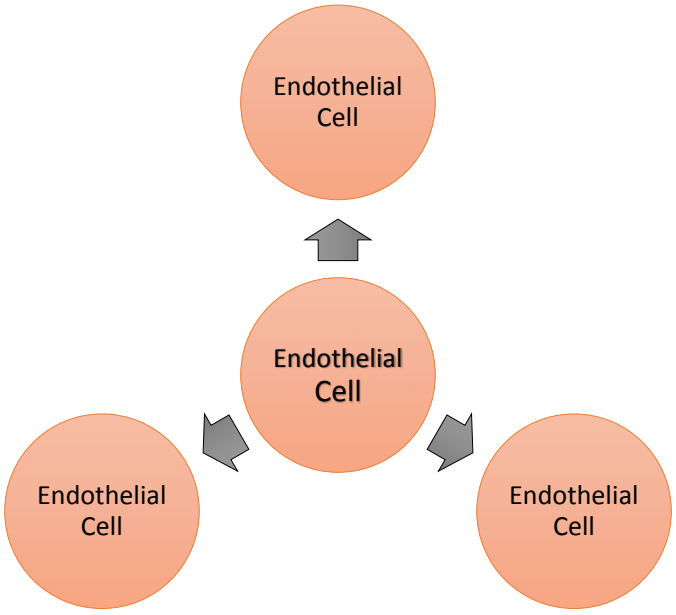


Figure 1.3: Specialized Cells derivatives (ex: Endothelial cell)

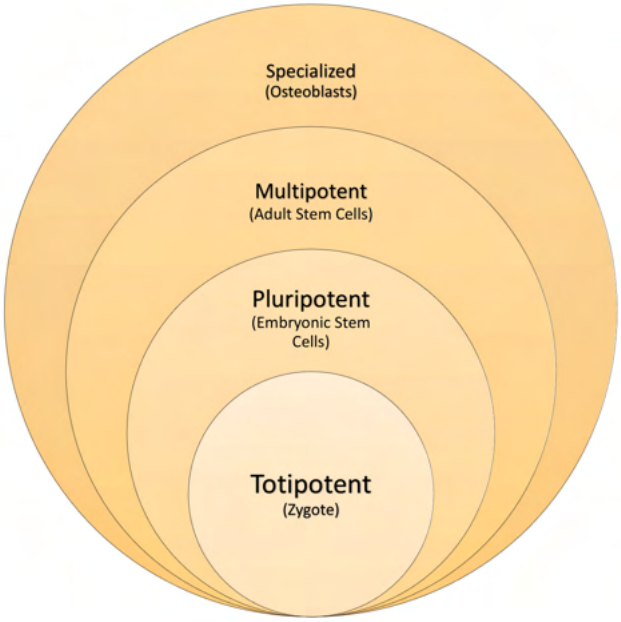


Figure 1.4: Evolution of Stem Cells; starting at the core with the most potent, non-specialized totipotent stem cells until reaching the least potent, specialized cells at the periphery.

vital importance to be able to pinpoint existing cell resources with stem cell characteristics for tissue engineering and eventually regenerative medicine applications[7].

1.4 Stem Cells based on Origin & Potential

It is definitely reassuring that one of the vital components of the tissue engineering triad, namely the stem cells can be obtained from several sources. As illustrated in Figure 1.4 , stem cells can be majorly categorized based on their origin, i.e tissues where they are retrieved, as well as based on their inherent potential to generate tissues from germ layers, whether they are restricted to one germ layer or are more expansive to include generating

tissues from more than one germ layer.

Accordingly if stem cells are categorized according to their potential to generate tissues from several germ layers to generating tissues from single germ layers, their potential rank would be dictated in the following respective order: Totipotent, pluripotent, and multi-potent stem cells, with totipotent stem cells taking the lead since they are those stem cells that lead to the formation of the embryo as a whole, i.e inclusive of all three germ layers, in addition to the extra embryonic placental tissues needed to sustain the growth of the 3 germ layers. The aforementioned potential renders the totipotent stem cell to be the most powerful. Second in line in terms of potential would be the pluripotent stem cell, which can also lead to the formation of the embryo as a whole, i.e inclusive of the 3 germ layers, only without the extra embryonic tissue formation. Last but not least, is the multi-potent stem cell which has the potential to generate several tissues restricted to one germ layer [17].

Moving on to categorization based on origin, stem cells can be derived from different origins during the human life span, starting all the way from the embryo state, progressing into the fetal state, where they can be derived from the associated umbilical cord and well throughout adult life, where they can be derived from adult tissues such as the most commonly known adult tissue for stem cell therapy, the bone marrow[17, 12, 18].

Based on the two aforementioned categorization, stem cells can be more comprehensively categorized as follows; with adult stem cells being most commonly multipotent stem cells, and embryonic stem cells being superiorly pluripotent stem cells as illustrated in Figure 1.4.

Due to the fact that pluripotent stem cells are bound to an early embryonic state of life, they are deemed superior to multipotent ones. However, they continue to raise ethical concerns since they are indeed isolated from the inner cell mass of the blastocyst. Nevertheless scientists have manoeuvred their way into reaching maximum benefit with

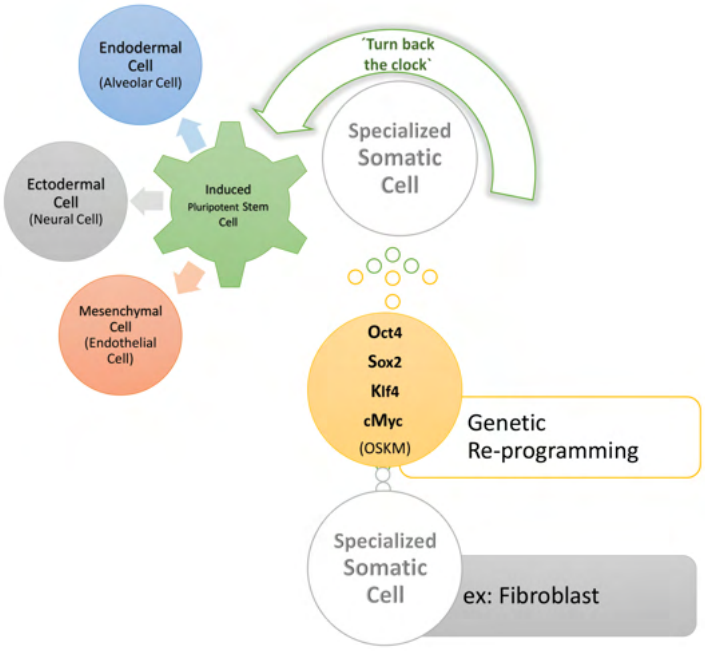


Figure 1.5: Genetic reprogramming of specialized somatic cells using Yamanaka's Factors to generate induced Pluripotent Stem Cells (iPSCs)[1]

minimal harm through the science of biomimetics [19].

Moreover and to not only lift the ethical controversy on the retrieval of pluripotent embryonic stem cells, but to also enable mass production, pluripotent stem cells have been proven in 2007 by Yamanaka et al to be genetically generated as induced pluripotent stem cells from basically any type of an already differentiated adult somatic cell[1] as illustrated in Figure 1.5. . This 'turning back the clock' notion on any type of adult somatic cell renders endless possibilities for exploration and implementation of stem cell therapies, with the only initial limitation being the access to a fully equipped laboratory necessary for genetic reprogramming.

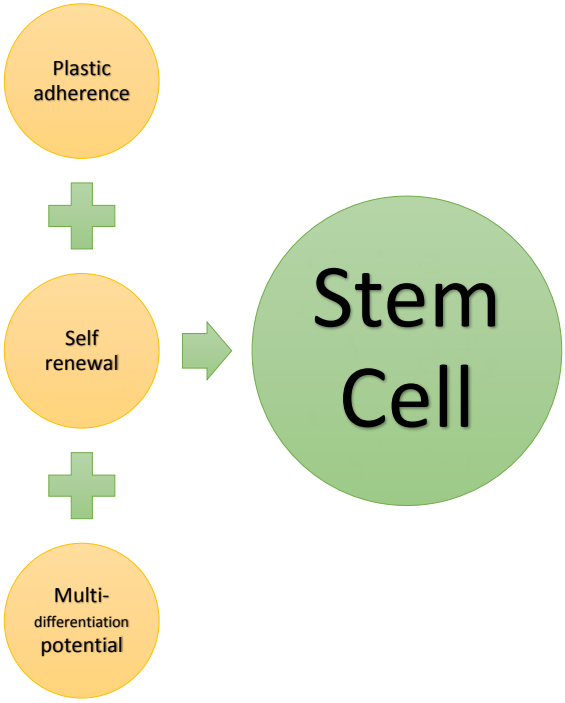


Figure 1.6: Stem Cell Criteria

1.5 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) were traditionally referred to as those stem cells isolated from mesenchymal bone marrow tissue. MSCs were considered a breakthrough in the field of medicine due to their striking proliferation and multi-differentiation potential (see illustration 1.6) which made them a valuable element in the tissue engineering triad. Nevertheless, despite their abundance in the human body[20], their isolation was not only inconvenient, it was also severely morbid to the donor through the painfully invasive retrieval procedure. Moreover, it has long been established that almost every tissue in the body harbours its own specific stem cells[21]. These tissue specific stem cells can be looked at

as inherent tools needed to maintain each individual tissue [22]. Nevertheless, these same tissue specific cells, based on their ease of retrieval can be utilised as external tools where they are most needed through regenerative medicine applications.

The most inviting source of stem cells are most likely those that are not limited to a specific time period such as embryonic stem cells (ESCs), but that are rather readily available postnatally and throughout life, more commonly referred to as adult or postnatal stem cells. Amongst adult or postnatal stem cells are the recently well known dental stem cells.

1.6 Dental Stem Cells

Dental stem cells are most infamously known for the dental pulp stem cells. However, there is more than the pulp when it comes to teeth. Teeth have been proven to harbour a wide variety of adult or post natal stem cells. In fact, these adult post natal stem cells are not only restricted to teeth, but they rather include the entirety of the oral cavity, rendering oral tissues in general a valuable source of adult postnatal stem cells.

Besides the discovery of dental pulp stem cells from the insides of developed permanent third molars in 2000 by Gronthos et al[23],stem cells were also isolated from the surrounding outer periodontium as periodontal ligament stem cells (PDLSCs)[24]. Moreover, stem cells were also proven to exist in the pulpal tissues or the remaining core of naturally exfoliated deciduous teeth, properly known as stem cells from exfoliated deciduous teeth (SHED)[25] as illustrated in Figure 1.7.

Moving away from fully developed and exfoliated teeth, teeth or teeth germs extracted during their developmental stages also have a lot to offer. Starting from tooth germ stem cells

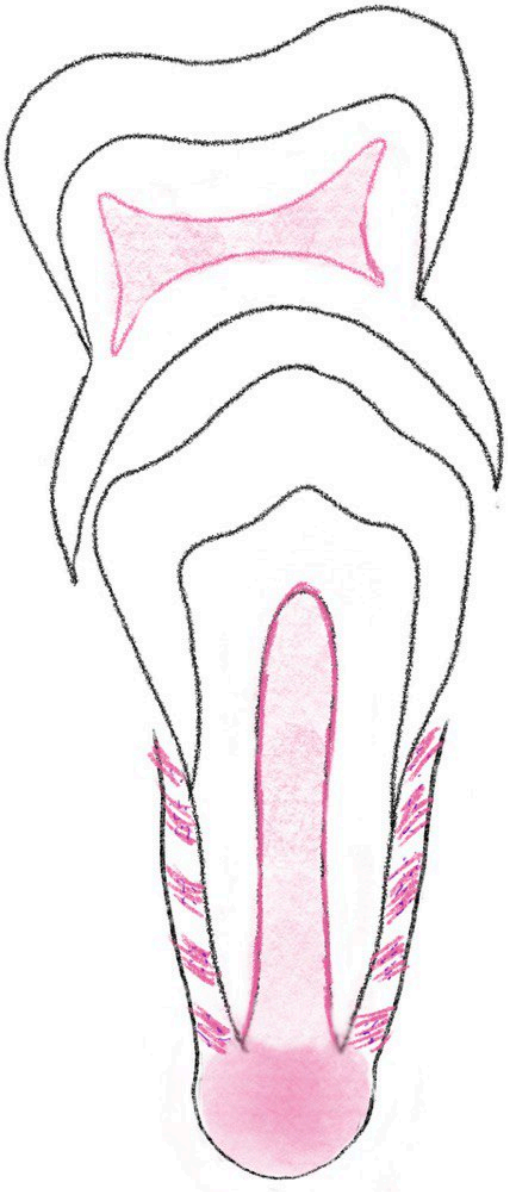


Figure 1.7: Illustration of anatomical locations of DPSCs, SCAP, PDLSCs, and SHED in a permanent tooth and its deciduous predecessor respectively

(TGSCs) in teeth germs during early teeth development onto the dental follicle progenitor cells (DFPCs) in so to say half way developed teeth (see Figure 1.8) , until finally reaching the final stages of teeth development where stem cells from apical papilla (SCAP) are found in association with developing roots and root tips[26, 16], as illustrated in Figure 1.9.

Besides the anatomical location of the cell groups, it is of prime importance to trace back how and from where did these dental stem cell niches come to existence. Studying teeth development reveals that the mesenchymal counterpart in the epithelial mesenchymal interaction involved in odontogenesis originated from migrating neural crest cells. This explains why recent in vitro research reported that DSCs and particularly DPSCs, SCAP, and SHED to be comitted to the neurogenic lineage through the secretion of various neurotrophic growth factors that stimulate neuronal guidance, outgrowth, and survival, amongst which are Vascular endothelial growth factors (VEGF), Brain derived neurotrophic factor (BDNF), Ciliary neurotrophic factor (CNTF), Glial cell-derived neurotrophic factor (GDNF), and Nerve growth factor (NGF) [27, 28, 29, 30]. Moreover, in vivo research has shown that DPSCs were even able to promote neurogenesis upon hippocampal transplantation [29, 31]. Similarly, SCAP were also reported to stimulate in vivo recovery when used to treat spinal cord injuries [32].

Therefore owing to their neural crest origin, DSCs are potentially reliable therapeutic starting points for neuro-degenerative disorders[33, 31, 34, 35, 36, 37].

It is worth highlighting that all the aforementioned cells can be isolated from regular medical waste, thereby inflicting minimal to non existent donor morbidity.

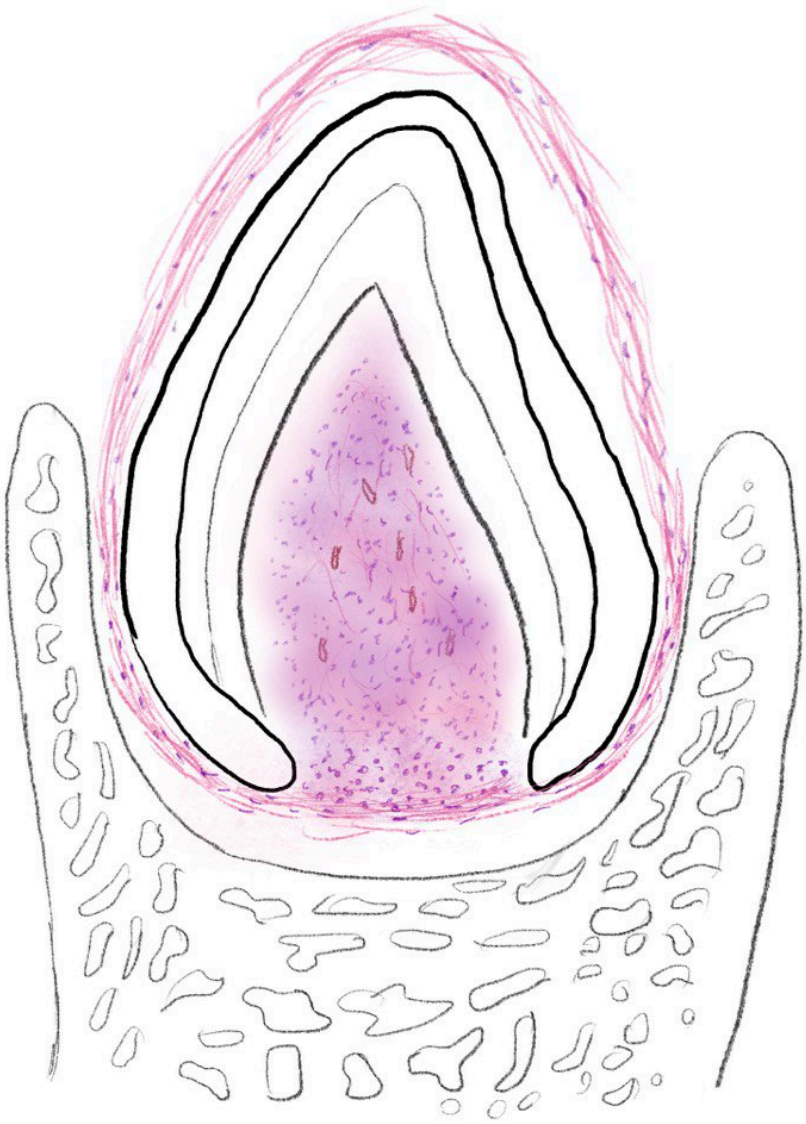


Figure 1.8: Illustration of anatomical locations of TGSCs and DFPCs within the core and surrounding the developing tooth germ respectively

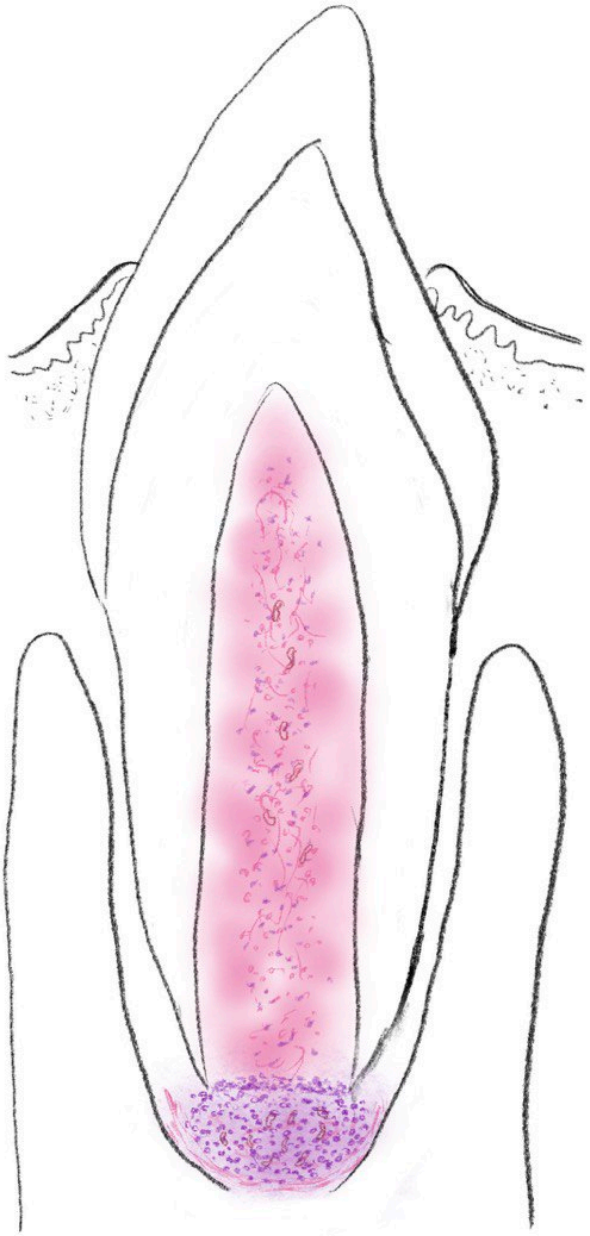


Figure 1.9: Illustration of anatomical location of SCAP in relation to a permanent tooth root tip with a 2/3 developed root

1.7 Dental Pulp Stem Cells

It is well known that dental pulp tissue which is found in the the core of the tooth is a neurovascular tissue of a soft nature that entails a large number of different type of cells, with the most common type of cell being the fibroblast. Besides fibroblasts, odontoblasts, and pericytes, dental pulp has been proved to also contain a sub-population of stem cells, descriptively named the dental pulp stem cells(DPSCs). Dental pulp stem cells were first reported nearly 20 years ago by Gronthos et al [23].

Dental pulp stem cells have been classified as mesenchymal stem cells due to the fact that their surfaces express specific MSC markers, such as CD146, CD105, CD90, CD73, CD59, CD44, CD29, CD13, and STRO-1, in addition to their fibroblast like spindle shaped morphology, their high proliferation ability and colony forming efficiency, as well as their inherent capability to adhere to plastic surfaces[38, 39, 40].

Not only that but previous research has shown that DPSCs are actually superior to so to say the original MSCs that are derived from the bone marrow, where DPSCs have shown to posses enhanced stem cell characteristics, in particular self renewal capacity, population doubling and strikingly the osteogenic differentiation potential[41, 42].

Moreover, Since DPSCs originate from migrating neural crest cells, with neurons being classified as ectoderm, DPSCs can thereby also be described as being neuro and ectodermaly derived stem cells that thereby owing back to their origin posses both neural and ectodermal characteristics [39, 43, 38]. Furthermore, harvesting of DPSCs is a rather simple procedure. Not only that, they are also the most protected and preserved dental stem cells, since they are enclosed within the core of the tooth. Rendering less chances for contamination that might occur post extraction as illustrated in Figure 1.10.

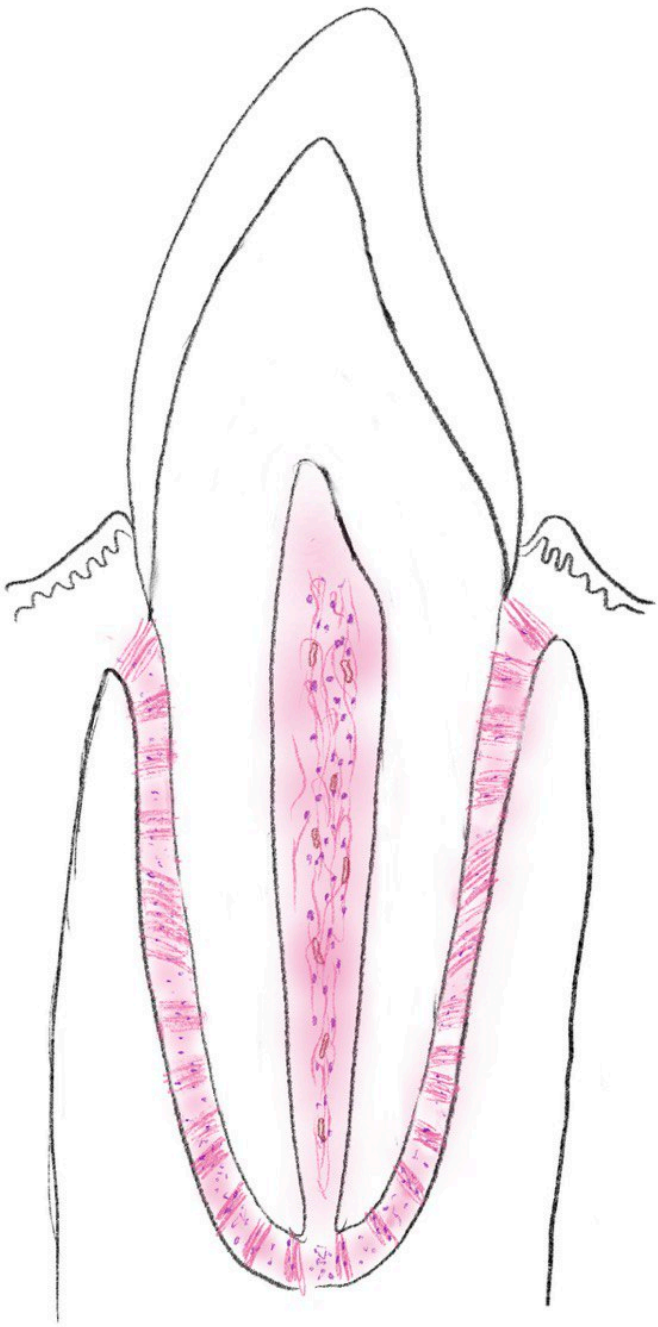


Figure 1.10: Illustration of a fully developed and erupted tooth with an enclosed pulp harboring DPSCs

Characteristically speaking, previous studies have all agreed on DPSCs possessing superior stem cell characteristics, such as proliferation and multi lineage differentiation abilities, where they were reported to successfully differentiate along osteogenic/odontogenic, angiogenic, adipogenic, myogenic and as justified earlier neurogenic lineages [44, 45, 46, 47, 48]. Not only that, but DPSCs were also reported to posses minimal immunogenicity, a significant prerequisite for in vivo applications[39].

1.8 Stem Cells from the Apical Papilla

As first discovered and described by Sonoyama et al, stem cells form the apical papilla (SCAP) are cells with stem cell characteristics that can be isolated from the papillary tissue at the apical most part of developing roots/root tips of human immature permanent teeth, hence the name apical papilla tissue [16, 49]. Moreover, the apical papilla tissue is a soft and richly vascular tissue due to its location in a perivascular niche[50, 51]. From a histological prespective and despite continuation of the pulp core with the apical papilla, since the former essentially developed from the latter, the apical papilla tissue can nevertheless be distinguished from the pulp tissue due to the fact that it harbours comparatively lower cellular and vascular elements[50, 16].

Furthermore, SCAP are also classified as MSCs due to their expression of MSC surface markers such as CD90, CD44, CD146, and STRO-1 [50, 52, 53]. In addition to the MSC surface markers, SCAP have been found to express a SCAP specific surface marker that has not been detected on neither the surface of bone marrow mesenchymal stem cells (BMMSCs) nor on that of DPSCs. That SCAP specific marker is actually a pluripotency marker referred to as CD24[54, 55]. As implied by the pluripotent classification of the

marker, CD24 can be regarded as a reference point for the undifferentiated state of the cell that expresses it, thereby reinforcing the stem cell characteristic of being non specialized/non differentiated [54, 56, 57].

Moving on to SCAPs differentiation potential, when it comes to SCAP leaving its state of undifferentiation to that of a differentiated nature, studies have proven that SCAP can differentiate along the osteogenic/odontogenic, adipogenic, angiogenic as well as strangely but not surprisingly along the neurogenic lineages due to their neural crest origin [50, 52, 53]. Supporting their neurogenic origin and potential, studies have shown that SCAP possess various neural markers such as neuroectodermal stem cell marker (Nestin), neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP), neurofilament M, Neuron-specific enolase (NSE), and Beta tubulin III [16].

Furthermore and in relevance to SCAPs eligibility for clinical use and in particular as an allogenic stem cell source, SCAP have not only been proven to posses immunosuppressive characteristics by interfering with the proliferation of defensive host T cells, but they have also been proven to elicit a rather low host immunogenicity [58, 59].

1.9 Factors modulating Stem Cell Characteristics; Second Triad element

The employment of the tissue engineering triad essentially attempts to replicate the natural processes of engineering occurring in the human body. If we look at the intricate engineering that takes place during tooth development as an example, the dynamic and reciprocal epithelial ectomesenchymal interaction is the most highlighted event for successful odontogenesis, where an abundant amount of signalling molecules and growth factors

such as epidermal and fibroblast growth factors (EGFs, FGFs), as well as various bone morphogenetic proteins (BMP) and insulin like growth factors (IGFs) posses a pivotal role in directing papillary cells towards odontogenic differentiation and eventually dentin formation [60, 61, 62, 63]

Generally speaking, mesenchymal stem cells themselves, being one of the crucial triad elements have been proven to have an innate paracrine secretory ability contributing to so to say self activation by producing/secreting various cytokines, chemokines, or growth factors[64]

1.10 Culturing of cells in an ‘in vitro´ environment

The in vitro environment normally dedicated for cell culture entails the use of growth media reinforced with varying percentages of fetal bovine serum (FBS) 2.1. FBS has been proven to be essential for cellular adhesion to the plastic cell culture surfaces, in addition to being essential for the prolongation of cellular survival until cells expand to the desired quantity needed to fulfil requirements of stem cell experimentation setups[37].

Despite the clear aforementioned benefits, there have been studies urging the minimal use of FBS in order to reduce the exposure of cells to FBS[65].This movement is especially essential when the transition from bench to bed side is in order, i.e from in vitro experimentation to in vivo implementation, seeing as though bovine proteins or peptodes within the FBS could themselves induce host immunogenic reactions[66]. Moreover and besides proteins, FBS consists further of a mixture of naturally occurring substances such as hormones, growth factors and nutrients that vary from one fetal bovine serum lot to another, rendering a less than perfect standardization of the FBS component of the in

vitro environment. Therefore, even though naturally occurring components are normally appealing, they are not in the case of research with the aim of reproducible and reliable results. Even more so when this research is going in the direction of human therapy[37, 67].

Due to the aforementioned reasoning, routine addition of FBS to cell culture media should be minimized and when possible even avoided. Multiple countries have in fact issued rulings regarding the use of FBS for clinical cell therapy procedures. Accordingly more and more studies have been directed towards minimizing the use of serum to as low as 1%, in addition to substituting FBS all together through the usage of serum free medium[37, 29, 68, 69]. Furthermore, Hirata et al. even reported that serum free medium not only boosts cells' proliferation and survival, but can also upregulate the expression of stem cell markers, thereby enhancing further the cultured cells' pluripotent qualities [69].

1.11 Mimicking in vivo by expanding from 2D to 3D cultures; third & final triad element

By transitioning to serum free medium, the in vitro microenviroment has the potential to direct the cultures away from a 2D monolayer setting onto a 3D one, all of which possibly brings us closer to mimicking the in vivo microenviroment, which is of prime importance when researching biological processes[42].

Despite the fact that cells lose readily available growth factors delivered in the serum when grown in serum free medium, previous studies described that in a serum free culture setting, cells naturally come to acquire greater contact with one another, leading to accentuated paracrine secretions of growth factors, such as transforming growth factor(TGFs), bone morphogenic proteins (BMPs), and fibroblast growth factors (FGFs), hence, contributing

to more potent stimulation of signaling cascade [8, 64]. Moreover, 3D cultures have been reported to contribute to a rise in the production of not only the extracellular matrix (ECM), but also in the secretion of adhesive proteins such as collagen, laminin, vitronectin, and fibronectin, together with the aforementioned growth factors have the ability to dictate the differentiation pathway taken by the stem cells involved, thereby potentially building up pluripotent stem cell qualities[64, 70].

1. Scaffold 3D cultures models

Scaffolds are regarded as an artificial alternative for the body's natural extracellular matrix. The use of scaffolds in tissue engineering is very common because they offer a wide range to fulfil the 3rd triad element, through the various customization of physical shapes that they can be made into in order to tailor cellular guidance. migration, and differentiation[42, 71]. Moreover, scaffolds themselves can be reinforced with growth factors such as BMPs to specifically guide cells in a desired direction. Despite the aforementioned advantages and possibilities to tailor the biological and mechanical properties of scaffolds, they remain to be a foreign construct that can potentially elicit an infection or an inflammatory reaction when introduced in vivo. Not only that, but scaffolds can also negatively interfere with the tissue being engineered through non anticipated scaffold degradation rates in vivo [61, 42].

2. Spheroid or scaffold free 3D culture models

Alternately, 3D culture models can be spontaneously generated without the use of external aid or supplements if cells are allowed to grow at a high seeding density. Therein cells acquire the ability to exogenously congregate and produce a desired extracellular microenvironment. This mechanism renders the scaffold free culture model as a true example of self sufficiency[42, 72].

Accordingly, studies have shown that spheroids generated in 3D cultures can be

classified as superior to conventional monolayers due to their enhanced ECM assembly and multilineage differentiation potential, thereby their regeneration and therapeutic potential [73, 42, 64]. More specifically, DPSCs when exposed to low serum medium have been shown to exhibit ESC markers, contributing further to the pluripotency notion of 3D cultures[29, 64].

1.12 Dental Stem Cells in ´in vivo´ regeneration

From a dental perspective, great progress has been made in the field of dentino-pulpal regeneration, better known as regenerative dentistry, where regeneration of dentinal tissues are becoming more and more attainable not only in vitro but also in vivo[11]. For instance, recent in vivo studies revealed that the injection of SCAP in the form of nanofibrous microspheres in the prescence of BMP-2 stimulates dentin regeneration[11].

Due to the natural location of SCAP, such findings are rather resourceful regenerative endodontics applications, where pre- existing/ remnant SCAP can be taken advantage of to serve the clinical dental need of dentinogenesis.

Furthermore, DSCs have also been recorded to have the potential for a greater medical benefit as a whole, where their employment can be utilized outside of their original anatomical location to expand further beyond regenerative dentistry and into the field of regenerative medicine.

Medically speaking, DPSCs have an existing potential to re-establish neurological function after neurological injuries such as strokes[39], in addition to having the potential to differentiate to cardiocytes to rehabilitate the myocardium in cases of myocardial infarction [47]. Moreover, in vivo studies have also proven that DPSCs can impede muscular dystrophy

and facilitate wound healing [8, 74].

1.13 Cryopreservation of SCAP & DPSCs for future applications

Previous studies in our group and elsewhere reported that DPSCs characteristics are reliably and conveniently maintained post cryopreservation and thawing[39, 40, 75]. Furthermore, SCAP cryopreservation was also proven to be non harmful according to reports of maintenance of SCAP´s stem cell characteristics as well as immunosuppresive traits post cryopreservation and thawing [76, 58, 59]. Accordingly, these reports reinforce the fine luxury of banking dental stem cell resources when the supply of extraction is high in dental surgery clinics and saving them for when demand is in order for clinical applications.

1.14 Aim of the study

The main objective of the current study was to explore the proposition that the two types of postnatal adult stem cells; SCAP and DPSCs acquired from extracted developing permanent wisdom and premolar teeth might possibly display dissimilar in vitro stem cell characteristics, differentiation potentials, and spheroid formation abilities. This study is particularly interesting since it tackles stem cells isolated from two structurally bound yet separated tissue entities, being the apical papilla tissues, from which SCAP is isolated and dental pulp tissues, from which DPSCs is isolated. All the above traits will also be investigated and contrasted after one year of cryopreservation to reinforce the extent of stability and convenience these cell lines have to offer in terms of bank today and use later.

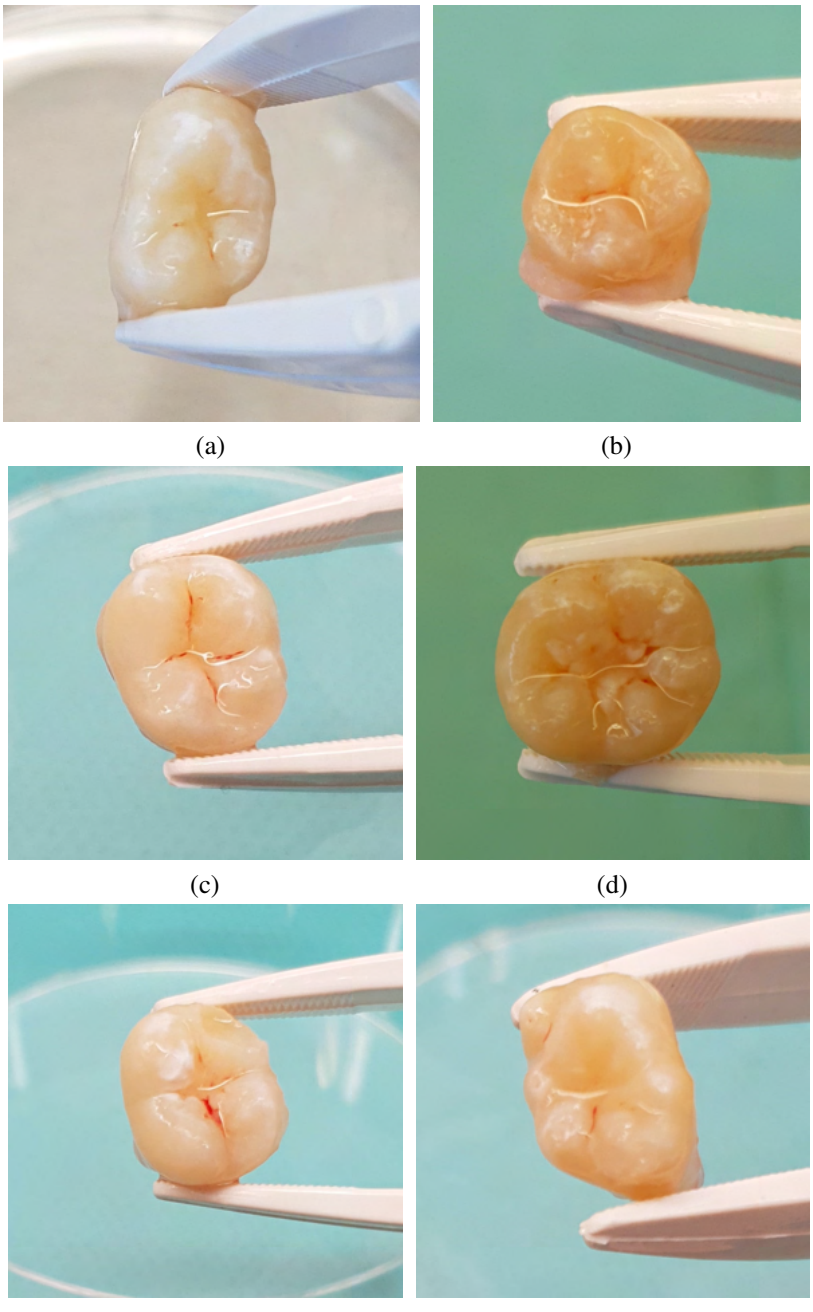
Additionally, light will be shed on whether or not SCAP are superior in expansion and differentiation potential to DPSCs since they are retrieved from developing tissues at a younger age in contrast to DPSCs which are retrieved from more developed tissues.

Chapter 2

Material und Methoden

2.1 Sample collection

Apical papilla and dental pulp tissues were harvested from sound developing human permanent teeth extracted from children and young adults with ages ranging from 12 to 23 years old (12 patients; 5 Females: 7 Males). All donors were healthy without any diagnosed diseases. Moreover, teeth involved in this study were either premolars or wisdom teeth, extracted under local anesthetic at the Oral and Maxillofacial Surgery clinics in the University medical center Hamburg Eppendorf with informed consent of the patients' guardians or patients respectively c.f. Figure 2.1. Furthermore, teeth extractions were performed for either prophylactic or orthodontic purposes and teeth were anonymously processed according to the local privacy protection regulation. After extractions, teeth were immersed in saline and transported to the laboratory immediately for cell culture processing. From each tooth, both apical papilla and dental pulp tissues could be retrieved.



(a) (b) (c) (d) (e) (f)

Figure 2.1: Sound extracted teeth; occlusal view.

2.2 Extraction of Apical Papilla tissue

Teeth were removed from the saline solution under the safety bench using sterile tweezers. Teeth were carefully grasped as far away from the root tip as possible in order to avoid touching the exposed apical papilla tissue. An ideal grasp was achieved at the tooth's cervix, c.f. Figure 2.2. Using another sterile tweezer, the apical papilla tissue was gently grasped and detached from the root tip as described by Sonoyama[16], see Figure 2.3.

In cases where resistance to pulling was sensed, the apical papilla tissue was gently separated from the developing root bodies using a sharp sterile scalpel blade as shown in Figure 2.4.

2.3 Retrieval of Dental Pulp tissue

Teeth were thoroughly disinfected with 70 % ethyl alcohol only after the removal of the exposed apical papilla tissues. Following disinfection, teeth were wrapped in sterile surgical drapes under the safety bench (Thermofisher Scientific). Drapes containing the teeth were further wrapped in heavy duty plastic bags. The plastic bag containing the wrapped tooth was then taken away from the safety bench and a strong hammer force was applied to the tooth with an aim on the cemento-enamel junction (CEJ) of the tooth in order to crack open the tooth at the weak cervical junction "decoronation" and thereby gain access to the pulp chamber and root canals, see Figure 2.5. After disinfection of the outer surface of the plastic bag, the cracked open tooth with the exposed pulp was unraveled carefully under the safety bench.

Finally, the teeth fragments were held by a sterile tweezer while the exposed dental pulp tissues were gently harvested from the inside of the tooth using dental files (Figure 2.6) or

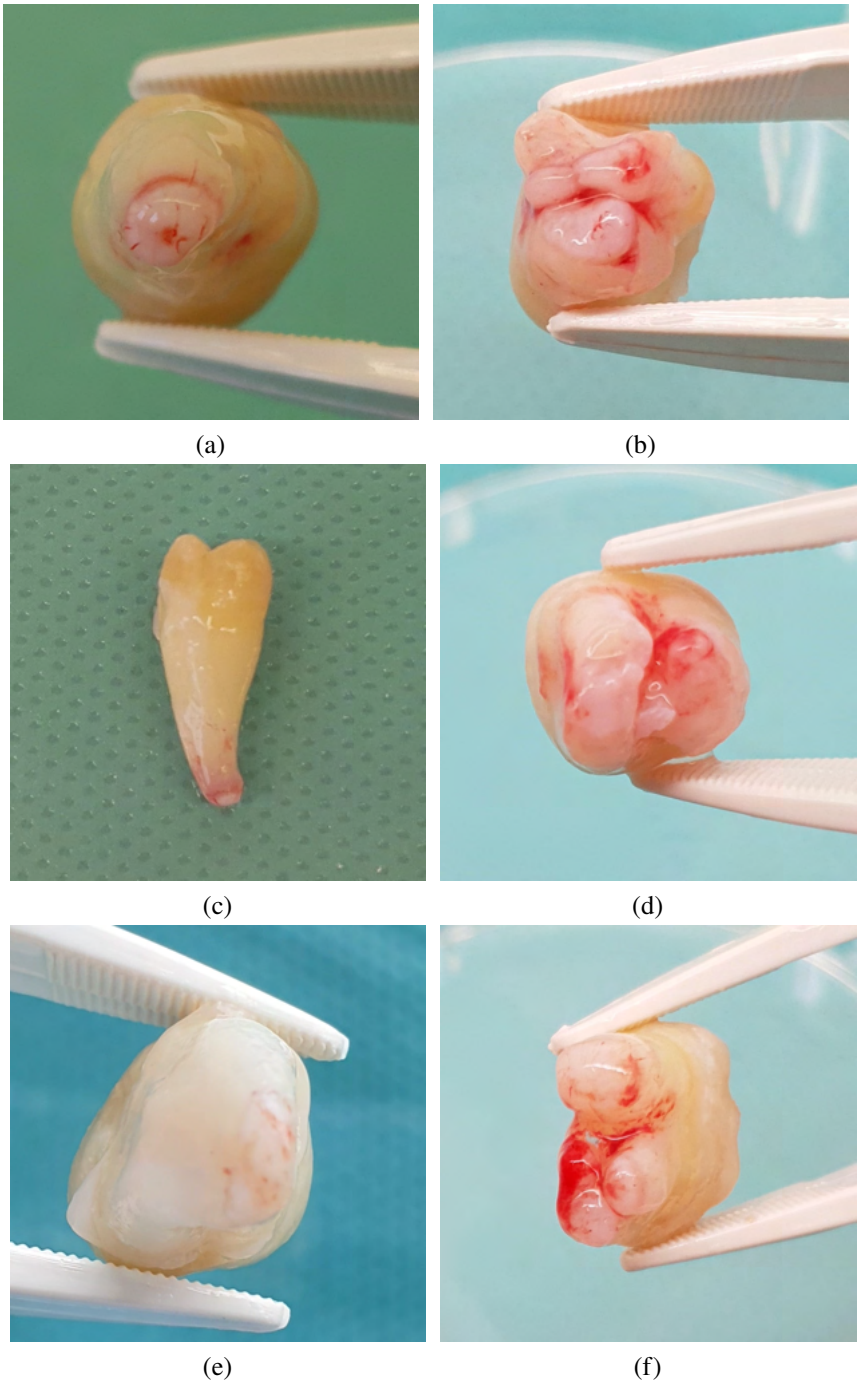
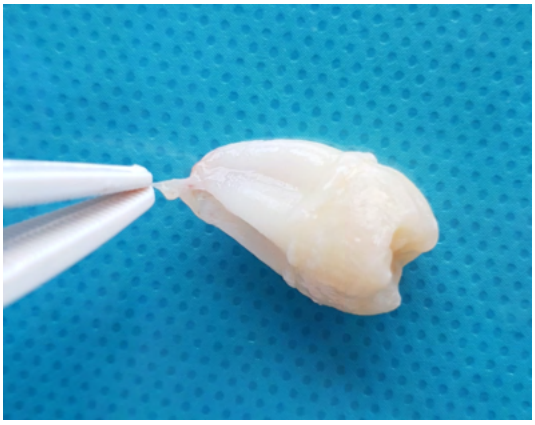
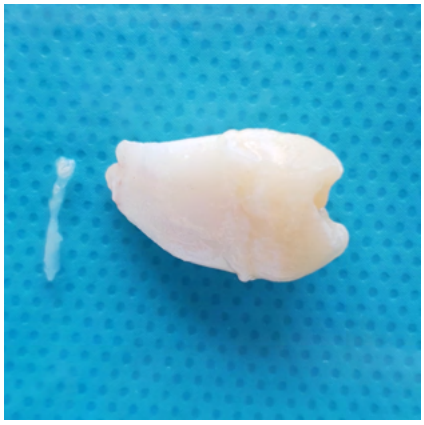


Figure 2.2: Freshly extracted teeth with evident and vascular apical papillae.

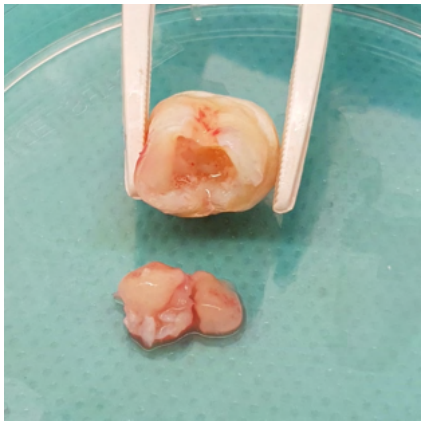


(a) Apical Papilla being detached.

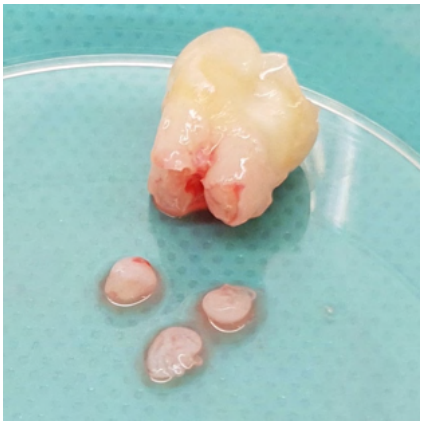


(b) Apical Papilla after being detached from molar with tweezers.

Figure 2.3: Apical Papilla

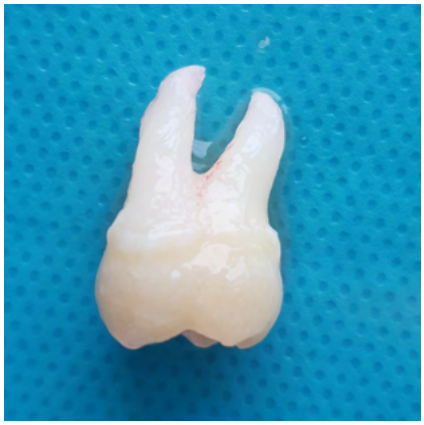


(a) Immature wisdom tooth with a large coherent apical papilla.

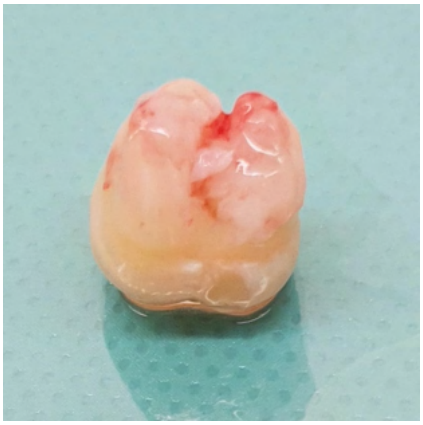


(b) Tooth with a 2/3 developed root - small distinct apical papillae tips.

Figure 2.4: Apical Papillae tissue dissected/detached from developing root trunk/tips using a scalpel blade.



(a) Developed molar with fully developed roots and an evident cementoenamel junction; patient age 23 years.



(b) Developing molar with a developed root trunk and bifurcation initiation; patient age 16 years.

Figure 2.5: Teeth at different developmental stages, with arrows pointing to the cementoenamel junctions.

tweezers with long slender tips.

2.4 Cell culture

The human cell cultures used in this study (n = 24) were derived from the apical papilla or the dental pulp of sound premolars and third molars with developing roots from 12 donors aged 12–23 years old (c.f. 3.1 and 3.2).

Apical papilla and dental pulp tissues were both washed separately with sterile phosphate-buffered saline (PBS; 0.01 M, pH = 7.4) for two times, see Figure2.7.

Washed tissues were then sectioned into 1- 2mm3 pieces using a sterile surgical blade.

Tissue fragments were thereafter plated onto cell culture dishes (Sarstedt) for cell culture using the explant method, where tissue fragments were then left to adhere for a few minutes, followed by the slow addition of medium composed of minimum essential medium (MEM)

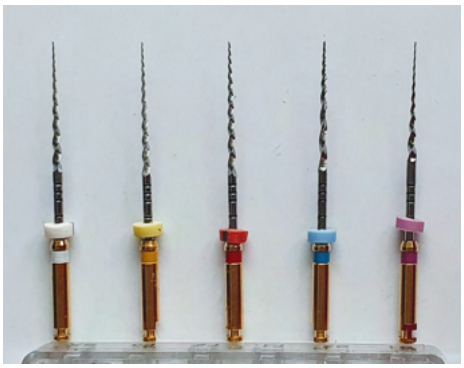
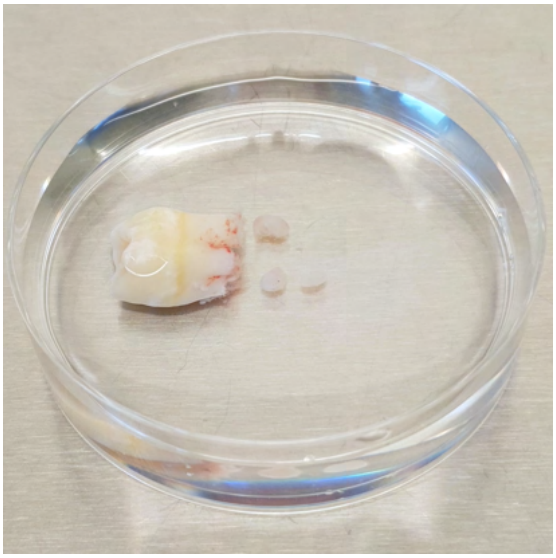
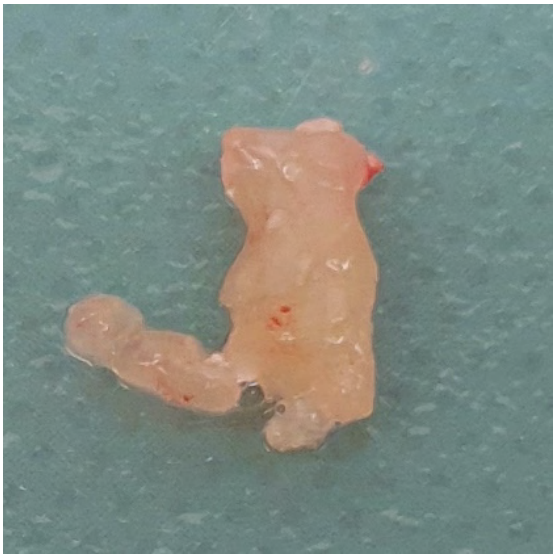


Figure 2.6: Sterile files in different diameters for pulp extirpation/retrieval purposes



(a) Apical papilla tissues



(b) Pulp tissue

Figure 2.7: Retrieved tissues in PBS ready for cutting and further processing

Table 2.1: Basic medium components

Reagent	Concentration/Quantity
MEM	1x
FBS	10%
L-Glutamine	2mM
Penicillin Streptomycin	100 U/ml
Amphotericin B	2.5µg/ml

Table 2.2: Digestion solution constituents

Reagent	Concentration/Quantity
Basal medium	1x
Collagenase	3 mg/ml
Dispase	4 mg/ml

supplemented with 10% fetal bovine serum (FBS)(Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mML-glutamine (Gibco) as presented below see Table 2.1

Tissue explants from the apical papilla were used to isolate stem cells from the apical papilla (SCAP) and those from the dental pulp tissue were respectively used to isolate dental pulp stem cells (DPSCs).

Alternatively, tissue fragments were also digested using Collagenase and Dispase (Sigma-Aldrich) as presented in Table 2.2.

Both growing cultures were maintained under these conditions until signs of cellular attachment was seen. Once several colonies were seen, the original media was changed twice a week until cellular confluence was reached. Upon confluence, cells were washed thoroughly with PBS, followed by trypsinisation using 0.25% trypsin solution (GIBCO). The yielded cell suspensions were then counted and and seeded at a density of 104 cells per 25 cm2 cell culture flask for further cellular expansion. All cultures were incubated at 37°C in a humidified 5% CO2 atmosphere. Cultures were observed daily under an inverted

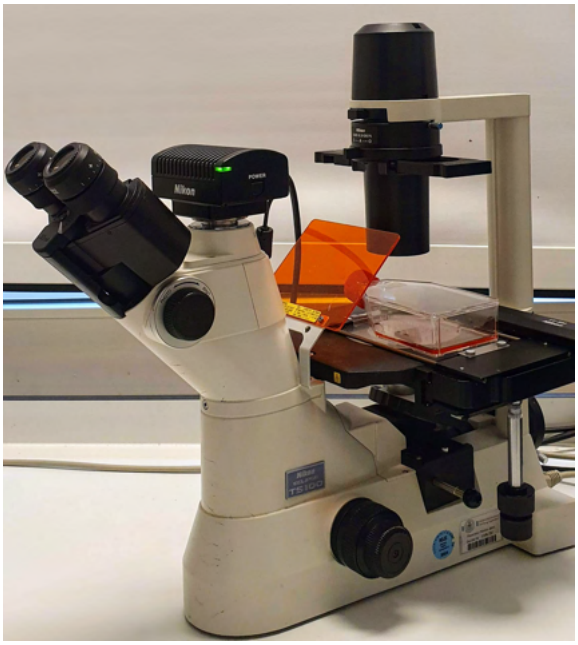


Figure 2.8: Inverted microscope used for all experiments, NIKON ECLIPSE TS100

microscope (Nikon Eclipse TS100, figure 2.8) to observe cell attachment and growth, in addition for an early detection of any signs of contamination. Microscopic pictures of the cells were captured using a microscope camera software (NIS-Elements BR software) at different magnifications.

2.5 Sub-culturing/passaging

When cell cultures reached 70 to 80% confluency (see Figure 2.9), cells were dissociated into single cells with 0.25% Trypsin-EDTA (GIBCO) in order to avoid spontaneous differentiation of the cells or cellular detachment.

To passage cells, cultures were first thoroughly washed with 1X PBS twice, followed by tryspinisation with 0.05% Trypsin-EDTA (GIBCO) for five minutes. The action of

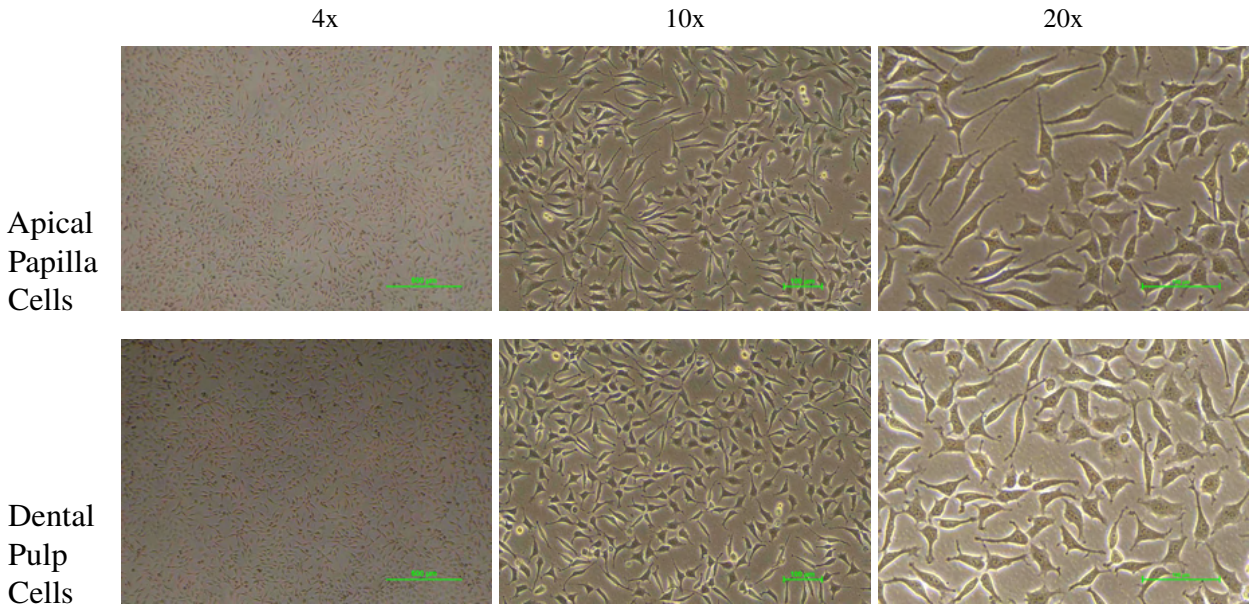


Figure 2.9: Cells reaching 80% confluency

trypsin was thereafter immediately neutralised by the addition of double the amount of cell culture medium containing 10% FBS. Detached cells were then transferred into a tube, centrifuged at 1500 rpm for ten minutes, followed by careful disposal of the supernatant while avoiding dislodging of the cell pellet. Yielded cells were then counted using a hemocytometer and thereafter either cryopreserved for later use as mentioned below or seeded at respective concentrations in diverse cell culture plasticware (see Figure2.10) in accordance to the following experimental plans: cellular growth curves, colony forming efficiency, multi-differentiation potential, and spheroid formation ability. Passages from 2 to 12 of SCAP and DPSCs were used for all the planned experiments. Furthermore, all experiments were performed in 3 replicates.

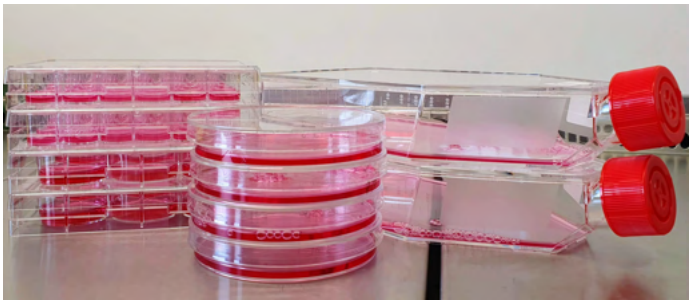


Figure 2.10: Cell culture plasticware (Sarstedt) used for seeding cells in accordance to experimental plans

2.6 Cryopreservation and thawing

For cryopreservtion, yielded cells were re-suspended in growth medium and dimethylsulfoxide (DMSO) (Sigma-Aldrich) at a ratio of 9:1. The cell suspension was then transferred to a cryovial and stored initially at –70°C, followed by storage in liquid nitrogen at -196°C after a few days for later use or long term storage.

When thawing of cells was desired, cryovials were placed in a 37°C water bath for 2 minutes or until no more ice crystals were seen. When the cryovial content was seen to be entirely in the liquid state, the cell suspension was transferred to a 15 ml tube and diluted with double the amount of fresh media, followed by a centrifuge cycle at 1500 rpm for 10 minutes. After the aspiration of the resultant supernatant, the cell pellet was re-suspended in fresh medium. Thereafter, cells were counted and seeded according to the experimental plans for cellular growth curves, colony forming efficiency, multi-differentiation potential, and spheroid formation ability. Cells were thawed after one year of cryopreservation.

2.7 Cell growth curve

Cells were seeded in triplicate at 2 × 105 cells/well in 6-well plates as described by Bakopoulou et al [77]. Cells were counted using a hemocytometer at the following day points: 2, 4, 6, 8, 10, and 12 days. The average value of each triplicate set was recorded for each time point.

2.8 Viability assay

As mentioned before, cells were cultured until around 70% confluency was reached. Following trypsinisation and resuspension in complete medium, 10 microliters(µL) of the cell suspension was mixed with 10 µL of trypan blue (Gibco) to yield a 20 µL cell:trypan blue suspension for the viability assay. Thereafter, 10µL of the prepared suspension was loaded to both chambers of a hemocytometer. Cells that were seen to be stained blue indicate a rupture of the cell membrane, and were therefore rendered as non viable. Accordingly, cells that were seen to remain unstained were rendered as viable, see Figure 2.11.

Both viable and non-viable cells were counted in duplicate under the inverted microscope (NIKON ECLIPSE TS100, Figure 2.8), followed by calculation of the viability percentage using the following formula:
Viable cells/ (Viable + Non-viable cells) x 100

2.9 Population doubling assay

Cells were seeded in T-75 culture flasks in basal medium at a recorded density. When the cells reached a state of 70 % confluency, cells were trypsinised and counted. Population

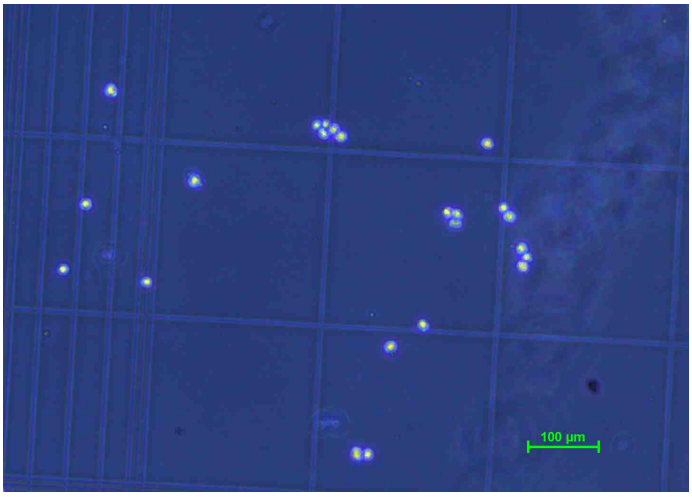


Figure 2.11: Microscopic view (10x) of trypan blue stained cell suspension (10µL) loaded onto a hemocytomer

doubling values were calculated at every passage using the following equation:

log2 (count of harvested cells at confluency/count of seeded cells).

The generated values dictated the population doubling ability of each cell type. Population doubling values were generated through three independent assays per cell sample. Moreover, these culture steps were repeated until the 12th passage.

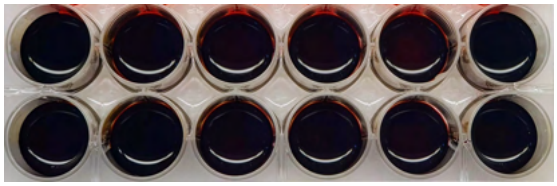
2.10 Colony forming efficiency

In order to evaluate the colony-forming efficiency of SCAP and DPSCs, 1000 cells from each cell type were seeded in duplicates into 60 mm petri dishes (Sarstedt). Cultures were maintained for 10 days followed by fixation and staining as described previously by Sonoyama et al [16]. All cellular monolayers were first washed with PBS to remove excess media followed by formalin fixation for 30 minutes. After fixation, traces of formalin were washed off with distilled water or PBS and the dishes were left to dry upside down on a

towel. Following excess liquid drainage, cells were stained with 0.1% Giemsa stain for one hour at room temperature. After removal of the stain solution, stain residues were rinsed off with distilled water until the water was seen to run clear of blue residues. In order to calculate the colony forming efficiency of cells, the following formula was used:
Average number of colonies formed/seeded cells x 100, where aggregations composed of 50 cells or more were regarded as colonies.

2.11 Osteogenic differentiation potential

In order to examine the odontogenic/osteogenic differentiation potential of SCAP and DPSCs, 20,000 cells were seeded per well in 24-well plates. After 3–4 days, when 70% confluency was observed, cell culture media was exchanged for odontogenic/osteogenic induction medium, where the induction medium was differently composed of alpha-MEM medium, supplemented with the following elements: 10 nM dexamethasone (DXM), 5 - 10mM beta-glycerophosphate (BGP), and 50 µg/ml ascorbic acid (Sigma–Aldrich). Simultaneously, control wells were exchanged for non inductive media, whereby the same media was used only lacking DXM, BGP, and ascorbic acid. A duplicate of the aforementioned experiment was set up in order to test the differentiation potential at 2 time intervals, being after 14 days, and 21 days respectively. During which, both inductive and non inductive media was continuously changed twice a week. As each time interval ended, both induced and non-induced wells were washed with PBS, followed by 10% formalin fixation and subsequent staining with Alizarin Red S (AR-S) in order to detect calcific deposits. At the 14 day and 21 day time interval, media was removed from all the wells and the cellular monolayers were washed thoroughly with PBS (without Ca2+ and Mg2+) (GIBCO) followed by fixation



(a) 24 well plate segment with Alizarin Red S stain solutions

(b) 96 well plate segment with solubilized alizarin red stain solutions ready for quantification

Figure 2.12: Alizarin Red S staining and subsequent quantification

for 1 hour with 10% neutral buffered formalin (Sigma–Aldrich) at room temperature. After fixation, cells were washed thoroughly with distilled water and left to dry with the wells turned upside down on a towel. After excess water was drained, cells were stained with 1% AR-S (Sigma–Aldrich) at a pH of 4.2 for 30 minutes at room temperature in the dark, see Figure 2.12a.

After removal of the stain, wells were rinsed up to 4 times with distilled water or until no red residue was detected in the distilled water. Adequate washing was necessary to exclude non-specific staining as much as possible. Calcified deposits were finally observed and photographed under an inverted light microscope (NIKON ECLIPSE TS100, Figure 2.8) that is equipped with a digital camera (NIS-Elements BR software).

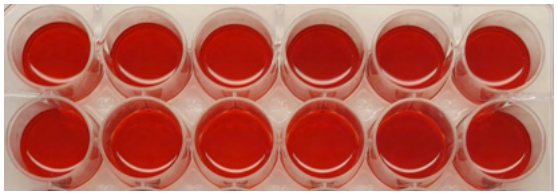
In order to obtain numerical values for the calcified deposits per well, the Alizarin Red S stain was solubilized in each well by adding 500 ul of the following formula 2 N NaOH:DMSO (1:1) as described before by Xiao et. al[78]. To solubilize the content of the wells, dishes were placed on a shaker for 10 minutes at room temperature. Following

Figure 2.13: Spectrophotometer used for quantification experiments (Biorad, Model 680)

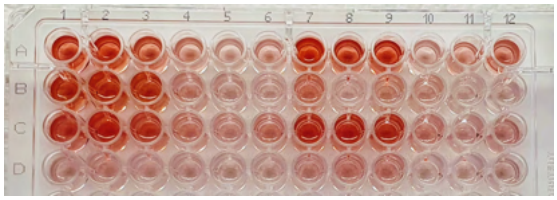
solubilization, a volume of 200 µl of the resultant purple solution was transferred from each well to a respective 96 well plate and absorbance values were measured using a spectrophotometer microplate reader (Biorad, Model 680, see Figure 2.13) at an absorbance wavelength of 542 nm. Quantification readings per original well were performed in triplicates and repeated three times, see Figure2.12b.

2.12 Adipogenic differentiation potential

In order to examine the adipogenic differentiation potential of SCAP and DPSCs, 20,000 cells were seeded per well in 24-well plates. After 3–4 days, when 70% confluency was observed, cell culture media was exchanged for adipogenic induction medium, where the induction medium was differently composed of alpha-MEM medium, supplemented with 0.01 µM DXM,1 µM indomethacin, 1 µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxantine (IBMX)and 5mM BGP(Sigma–Aldrich). Simultaneously, control wells were exchanged for non inductive media, whereby the same media was used only lacking DXM, indomethacin, insulin, IBMX and BGP. A duplicate of the aforementioned experiment was set up in order to test the differentiation potential at 2 time intervals, being after 14 days, and 21 days



(a) 24 well plate segment with Oil Red O stain solutions



(b) 96 well plate segment with solubilized oil red stain solutions ready for quantification

Figure 2.14: Oil Red O staining and subsequent quantification

respectively. During which, both inductive and non inductive media was continuously changed twice a week. As each time interval ended, both induced and non-induced wells were washed with PBS, followed by 10% formalin fixation for 20 minutes and subsequent staining with Oil Red O (OR-O) in order to detect oil droplets. After fixation, cells were first gently washed with PBS. Prior to staining, cells were immersed in 60% isopropanol for 5 minutes. Cells were then stained with freshly prepared 2% Oil Red O (Sigma-Aldrich) working solution for 15 minutes, see Figure 2.14a.

After stain removal, cells were washed profusely with distilled water until the water ran clear. Stained wells were finally viewed under the inverted microscope.

In order to obtain numerical values for the stained oil droplets per well, the oil red stain was solubilized in each well by adding 250 µL of 100% isopropanol. Following solubilization, a volume of 200 µl of the resultant colored solution was transferred from each well to a respective 96 well plate and absorbance values were measured using a

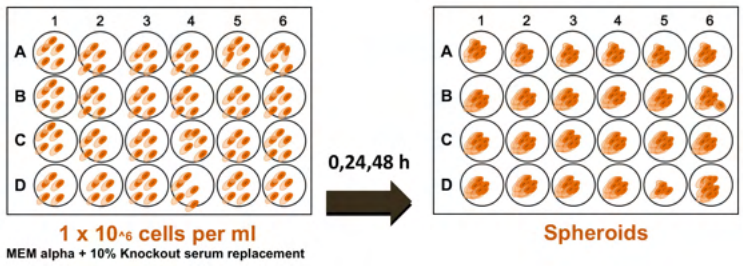


Figure 2.15: Experimental setup for spheroid formation assay

spectrophotometer microplate reader (see Figure 2.13) at an absorbance wavelength of 492 nm. Quantification readings per original well were performed in triplicates, see Figure 2.14b

2.13 Spheroid formation ability assay

Both SCAP and DPSCs were initially expanded as monocultures in basal medium until the required number of cells were attained in order to conduct the spheroid formation assay. Since high density seeding is required at a concentration of 1 million cells per ml, both cell types were mostly passaged until the 3rd passage until the desired cell count was reached. Using the aforementioned cell concentration, cells were subsequently seeded in 24 well suspension and attachement plates (Sarstedt). Two groups were allocated per experiment and per cell type. Moreover, sets of triplicates were implemented per group. Experimental setup of test and control groups was as illustrated in Figure 2.15, whereby the former group received knockout serum medium and the latter received basal fetal bovine serum medium.

Knockout serum medium (KSR-medium) was composed basally of minimum essential

medium Eagle alpha modification (MEM-α) with 10% knock out serum replacement (GIBCO) as described earlier [78]

Cellular monolayers (2D) or spheroids (3D) were photographed at a magnification of 10X while still in the 24 well plates. Photographs were taken using a digital camera that is connected to the inverted light microscope (NIKON ECLIPSE TS100, Figure 2.8) at the following time points: 0 hour, 24 hours, and 48 hours.

Images of spheroids were used to measure the sizes of the SCAP formed and DPSCS formed spheroids respectively. Both spheroid size measurements and analysis were performed using ImageJ software.

2.14 Statistical analysis

All experiments in this study were conducted in triplicate sets and the data sets were expressed as the mean ± standard deviation of the mean (SD), where both the mean and SDs were calculated using Microsoft Excel software. For qualitative results, descriptive analysis was implemented.

Paired student t test was employed to compare between SCAP and DPSCs in the following experimental results: Colony forming efficiencies, Growth curves, Viability percentages, Population doublings, quantified calcification at the different time intervals post odontogenic/osteogenic differentiation, quantified adipogenesis at the different time intervals post adipogenic differentiation, and Spheroid size analysis (significance assumed for $p < 0.05$).

Chapter 3

Ergebnisse

3.1 Cell culture success rate

In vitro explant outgrowth culture was positive for 50% (2/4) and 67% (4/6) of the premolar and wisdom teeth respectively (see figure 3.1), whereas the in vitro digestion cultures were positive for 100% (2/2) and 100% (4/4) of the premolar and wisdom teeth respectively as presented in Tables 3.1 and 3.2.

Four apical papilla tissues failed to completely show cellular attachment and thereby their dental pulp counterpart was also excluded from the study.

Cultures that did succeed in showing cellular adherence and eventually reached conflu-

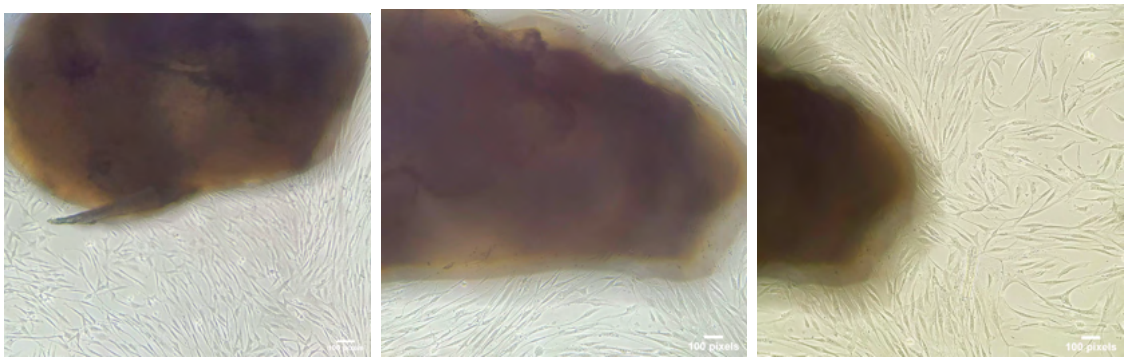
Table 3.1: Tabular results of success rates of SCAP

Tooth	Culture	Earliest cellular adhesion	Confluence	Success rate
Premolar (n=4)	Outgrowth	4 ± 2 days	10±6.2 days	50%
Wisdom (n=6)	Outgrowth	5 ± 2 days	10±6.2 days	67%
Premolar (n=2)	Digestion	3 ± 2 days	10±6.2 days	100%
Wisdom (n=4)	Digestion	3 ± 2 days	10±6.2 days	100%

Table 3.2: Tabular results of success rates of DPSCs derived from the same teeth

Tooth	Culture	Earliest cellular adhesion	Confluence	Success rate
Premolar (n=4)	Outgrowth	6 ± 2 days	14±3.4 days days	100%
Wisdom (n=6)	Outgrowth	5 ± 2 days	14±3.4 days	100%
Premolar (n=2)	Digestion	4 ± 2 days	10±6.2 days	100%
Wisdom (n=4)	Digestion	5 ± 2 days	10±6.2 days	100%

ence, demonstrated rather poor proliferation in the first days that extended up to possibly 1 week, where single cell colonies remained to be more isolated from one another. After one week, growth of colonies was notably accelerated until confluence was reached, where SCAP cultures on average reached confluency after 10±6.2 days whereas DPSCs took 14±3.4 days (see Figure 3.1).



(a) SCAP, 10x (b) SCAP, 10x (c) DPSCs, 10x

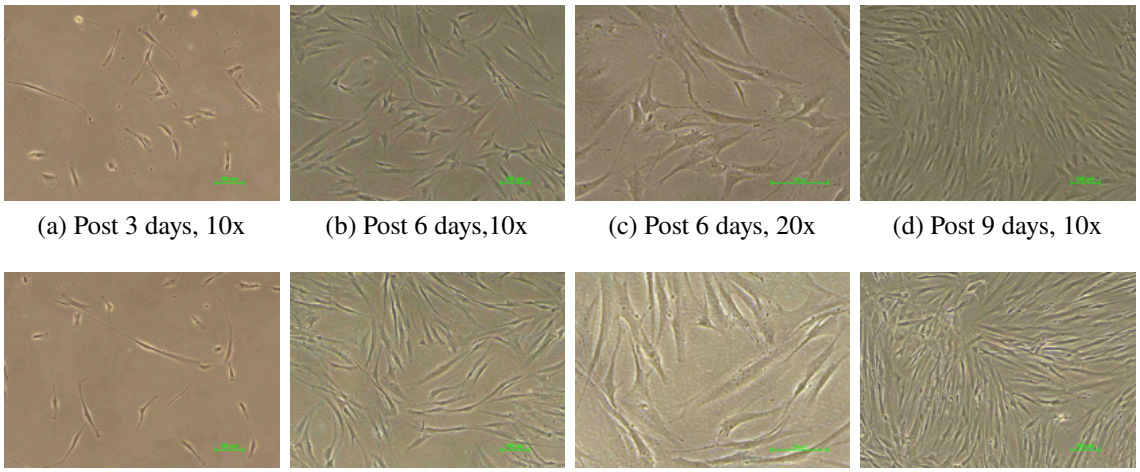
Figure 3.1: Explant outgrowth primary cultures at 10x magnification displaying various states of confluency

3.2 Apical Papilla and Dental Pulp cells' morphologies

Cells successfully cultured from the apical papilla tissues from developing root tips displayed a potent ability to form adherent cell colonies on plastic cell culture surfaces. Moreover,

cells were also successfully cultured from dental pulp tissues where they also displayed a potent ability to form adherent cell colonies on plastic cell culture surfaces.

Both SCAP and DPSCs grew out of the tissue explants or attached from the digested cell solution while protraying fibroblast-like spindle shaped morphology, as well as a polygonal shaped appearance, as displayed in Figure 3.1 and Figure 3.2 respectively. However,



(a) Post 3 days, 10x (b) Post 6 days,10x (c) Post 6 days, 20x (d) Post 9 days, 10x

(e) Post 6 days,10x (f) Post 9 days,10x (g) Post 9 days, 20x (h) Post 12 days, 10x

Figure 3.2: Primary culture of SCAP (a,b,c,d) and DPSCs (e,f,g,h) from first cell appearance and until confluency

as previously mentioned, the time required for primary cells to reach confluency varied considerably between SCAP and DPSCs from the same donor.

As cells reached confluency, the confluent monolayer was often seen to be comprised of tightly-packed cuboidal cells, as displayed previously in Figure 2.9. Finally, the morphological characteristics were more or less constant up until the 12th passage.

3.3 Cell growth curve

Cell growth was recorded every 48 hours by counting yielded cells at each time point. Initial seeding density was fixed at 20,000 cells per well. As displayed in Figure 3.3, both

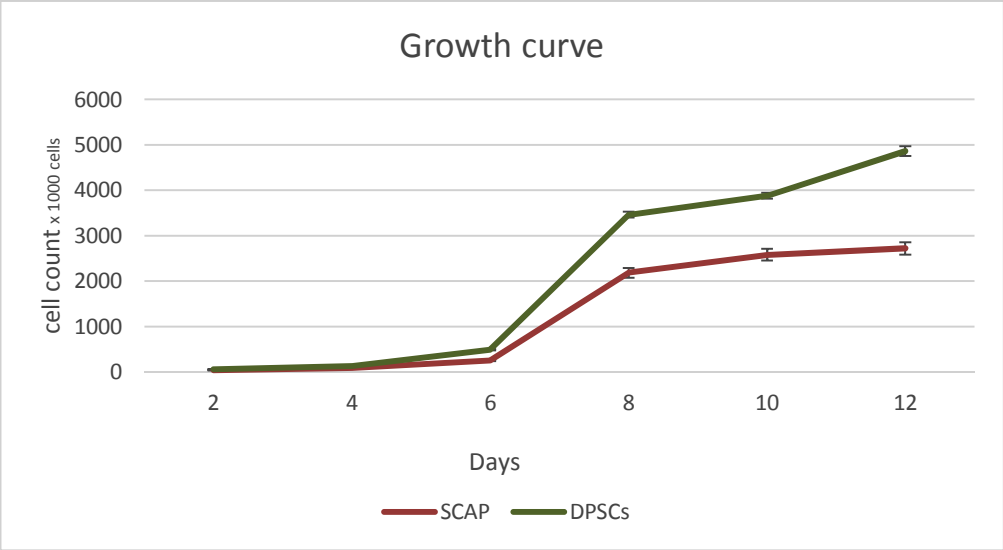


Figure 3.3: Growth curve of SCAP and DPSCs over the span of 12 days from 12 teeth samples

SCAP and DPSCs reached their peak of growth by the end of the 1st week, thereafter, their growth level was more or less maintained. Despite the fact that DPSCs yielded a slightly higher amount of cells, no significant difference was seen amongst their growth potential, see Figure 3.3.

3.4 Viability

When comparing the average difference in viability between the two groups of SCAP and DPSCs, DPSCs were found to score a slightly higher percentage on average, see Figure 3.4.

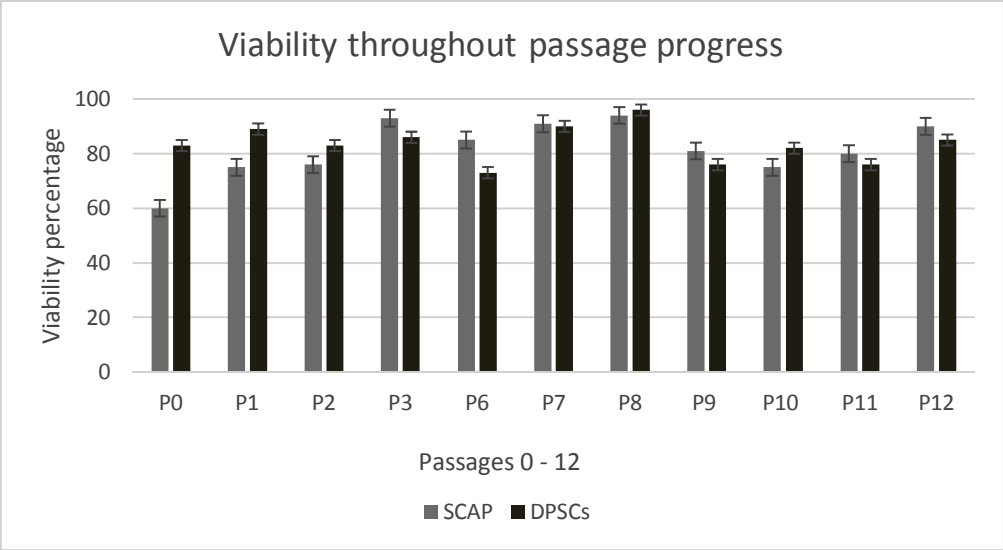


Figure 3.4: Viability of SCAP and DPSCs along twelve passages from 12 teeth samples

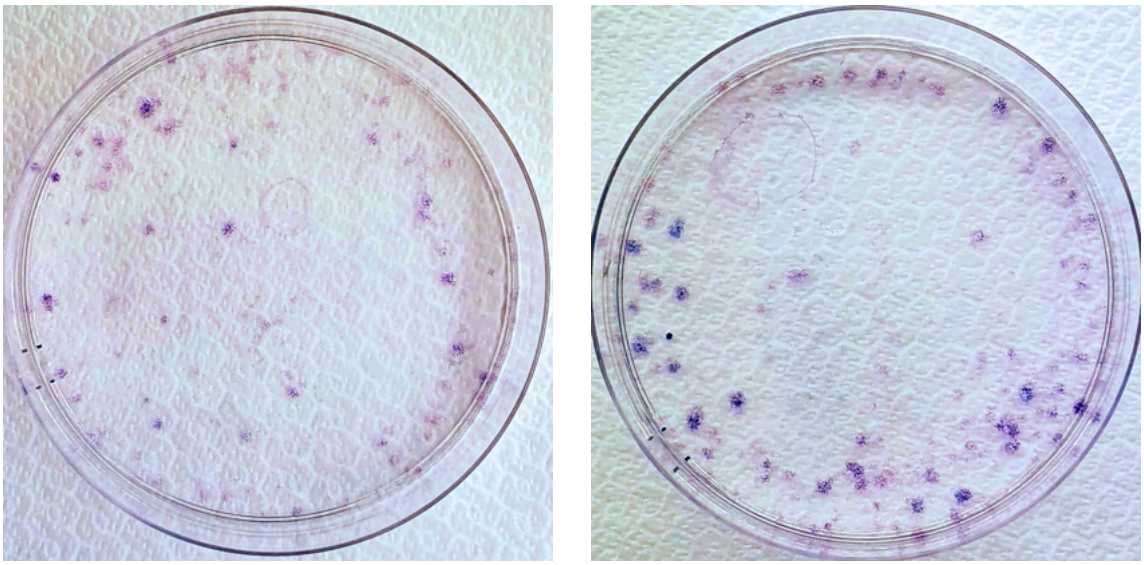
Nevertheless, the difference was not found to be statistically significant, denoting that DPSCs and SCAP have a rather similar viability rate.

3.5 Population doublings in various passages

SCAP and DPSCs displayed similar population doubling figures throughout the 12 passages, with DPSCs scoring 6.57±0.7 and SCAP scoring at 5.98±1. No statistical difference was observed amongst the two cell groups.

3.6 Colony forming efficiency

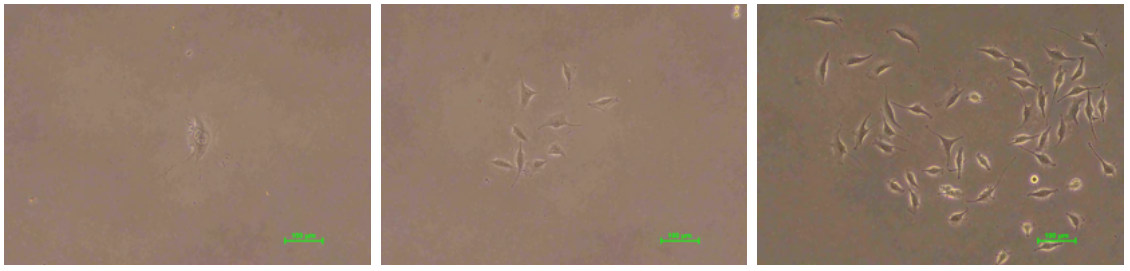
After seeding, single cells were observed microscopically for 10 days (see figure 3.6) until the resultant colonies were stained and counted as displayed in Figure 3.5. Throughout the



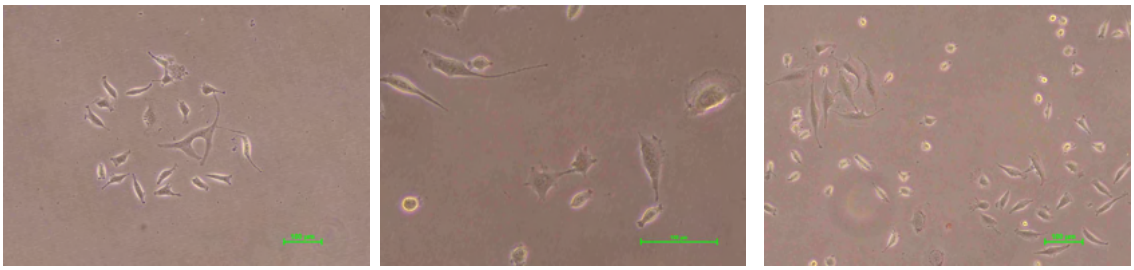
(a) SCAP (b) DPSCs

Figure 3.5: Giemsa staining after 10 days of culture showing the colony forming efficiency

10 day colony formation incubation period, cells were observed to progress from a single cell, through colony initiation and progression through cellular divisions (see figure 3.6). By the end of the 10 day incubation period, separate colonies were observed to come close



(a) Single cell, 10x (b) Colony initiation, 10x (c) Colony progression, 10x



(d) Cells dividing, 10x (e) Cells dividing, 20x (f) Two distinct colonies approaching each other, 10x

Figure 3.6: Microscopic observation of progressive colony formation

to one another, and often times they fused or coalesced. Fused colonies were excluded from the count after staining. On average SCAP scored a colony forming efficiency of 5.8%±1.9, whereas DPSCs yielded a slightly higher colony forming efficiency of 6.6%±1.2, see Figure 3.5. The difference was however not statistically significant.

3.7 Osteogenic differentiation

During the induction period and even before Alizarin red staining, a difference was clearly visualized between induced cells and non induced cells under the inverted light microscope. Within a week of induction, induced cells in both SCAP and DPSCs cultures were seen to aggregate greatly and form nodules of a different density, in contrast to non induced cells

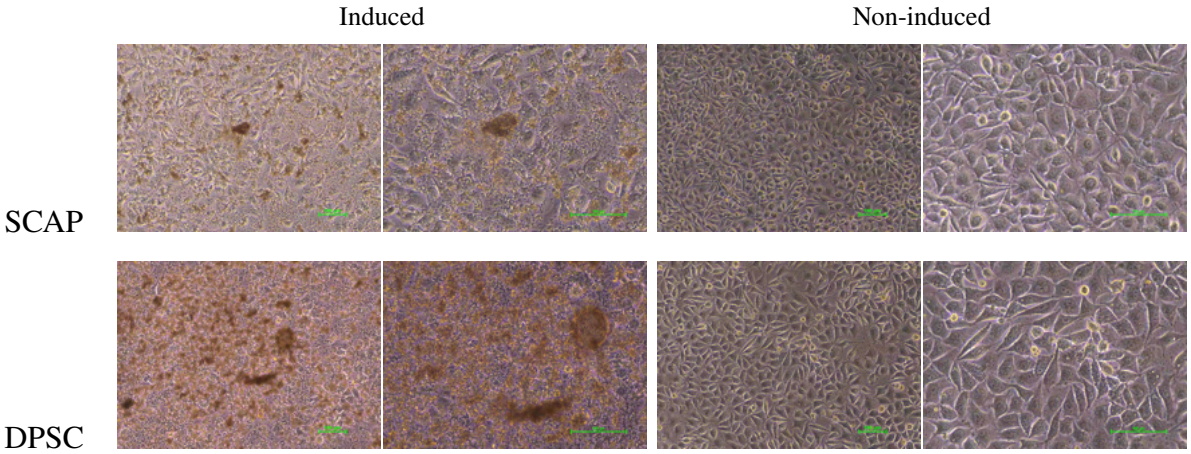


Figure 3.7: SCAP and DPSCs after one week of osteogenic induction, 10x & 20x

which portrayed overly confluent cultures but without local foci of variable densities, see Figure 3.7

Moving on to the 2nd week, both SCAP and DPSC cultures displayed an increase in potential calcific aggregations as seen in Figure 3.8, whereas non induced cultures continued to display overly confluent cultures with an increasing number of detached cells.

The observation of SCAP cultures revealed the following: As seen in Figure 3.7, after one week of induction SCAP cells generated discrete nodular deposits which later increased exponentially at the turn of the second week of induction as seen in Figure 3.8, where cultures revealed more generalized and diffuse nodular deposits covering a greater area. Higher magnification (20x) reveals that the distinct deposits remain to be of a nodular nature. Nevertheless, SCAP led to overall calcification in a diffuse manner.

Whereas, the observation of DPSCs cultures revealed the following: Induced monolayers were seen to develop deposits in a nodular and linear fashion as early as one week post induction as seen in Figure 3.7. This was clearly contrasted by the overly confluent non induced cultures lacking signs of deposition. Moving on to the second week (Figure 3.8),

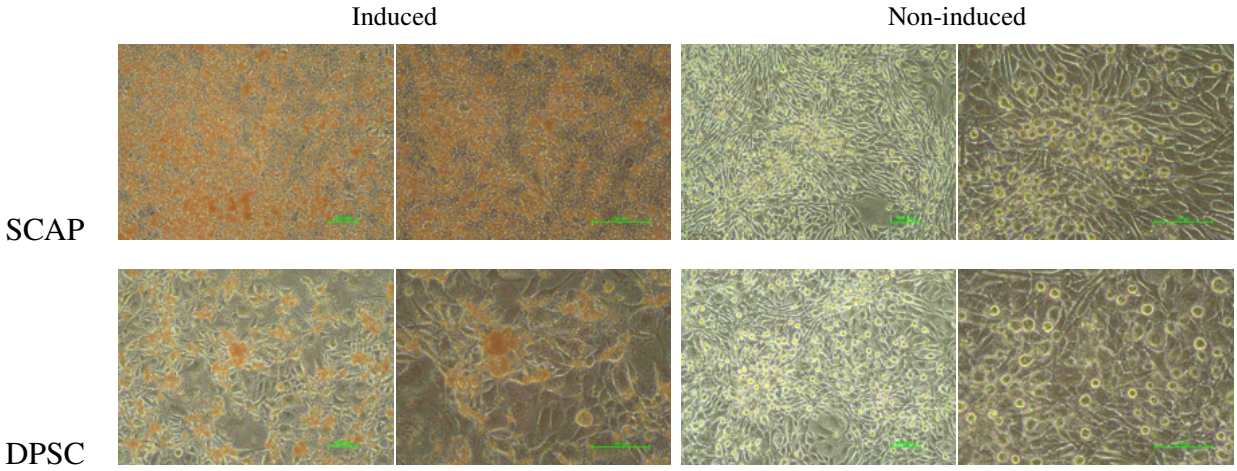


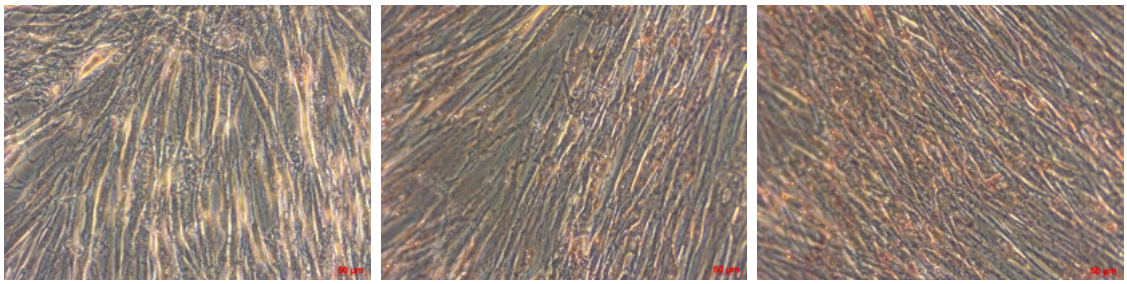
Figure 3.8: SCAP and DPSCs after two weeks of osteogenic induction, 10x & 20x

DPSCs revealed slight progress in calcific deposition, where deposits acquired colony like appearances. However neither as progressive as SCAP, nor was diffuse calcification observed, indicating a possible attenuation in DPSCs osteogenic differentiation potential when compared to SCAP. Moreover, upon consequential follow up of DPSC cultures throughout 14, 21, and 21 days of differentiation induction, gradual increase of focal calcification was clearly noted as displayed in figure3.9.

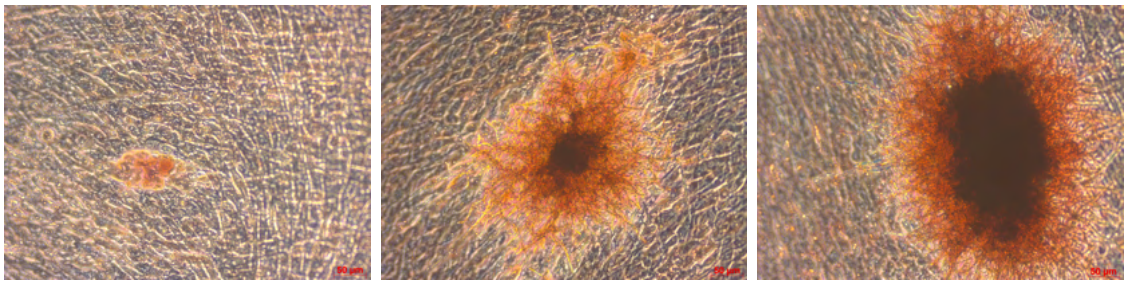
Quantification confirmed the qualitative observation of SCAP protraying a significantly greater osteogenic differentiation potential as dispalyed in Figure 3.10.

3.8 Adipogenic differentiation

Soon after the commencement of adipogenic induction and before oil red O staining, potential oil droplets were clearly visualized in induced wells under the inverted light microscope. Within a week of induction, induced cells in both SCAP and DPSCs cultures were seen to accumulate oil droplets within their cell bodies, in contrast to non induced



(a) Non-induced DPSCs, day 14 (b) Non-induced DPSCs, day 21 (c) Non-induced DPSCs, day 31



(d) Induced DPSCs, day 14 (e) Induced DPSCs, day 21 (f) Induced DPSCs, day 31

Figure 3.9: Alizarin Red S staining of DPSCs depicting progressive nodular calcification starting at day 14, through day 21 until reaching day 31 of osteogenic induction

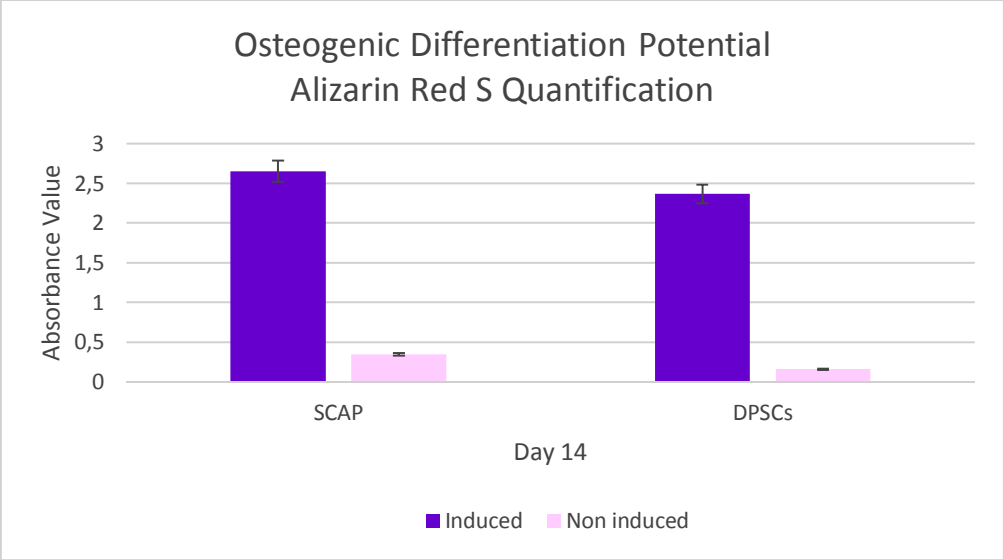


Figure 3.10: Quantification of Osteogenic Differentiation Potential after two weeks

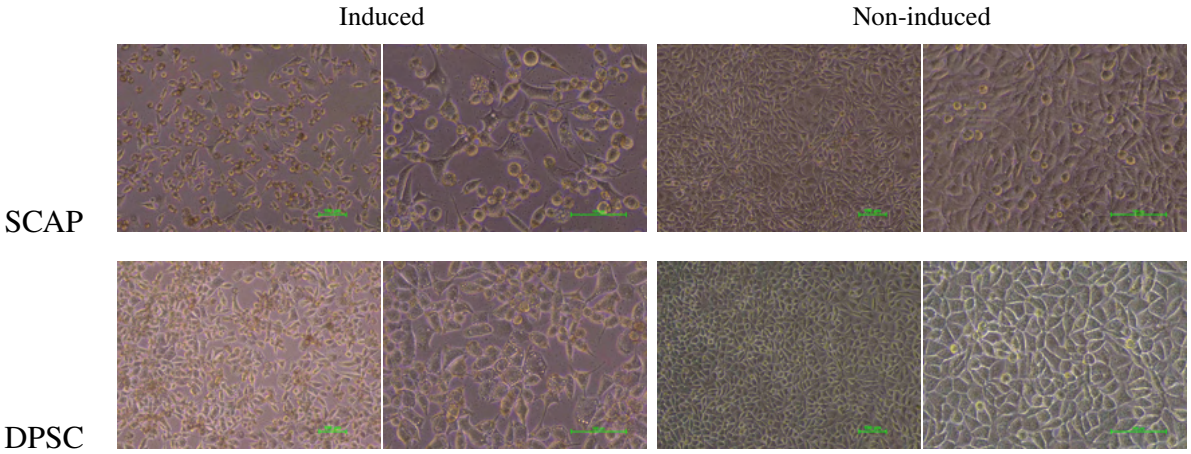


Figure 3.11: SCAP and DPSCs after one week of adipogenic induction, 10x & 20x

cells which portrayed overly confluent cultures that lacked oil like droplets, see Figure 3.11

Moving on to the 2nd week, both SCAP and DPSC cultures displayed an increase in potential oil droplet accumulations as seen in Figure 3.12. Oil Red O staining confirmed the presence of oil droplets in induced cultures of both SCAP and DPSCs, by retaining the red dye as displayed in Figure 3.13. Simultaneously, the non induced cultures failed to stain red, denoting the lack of formation of oil droplets.

Along passage progress, SCAP were observed to maintain their inherent adipogenic differentiation potential as represented through oil red O staining in Figure 3.14.

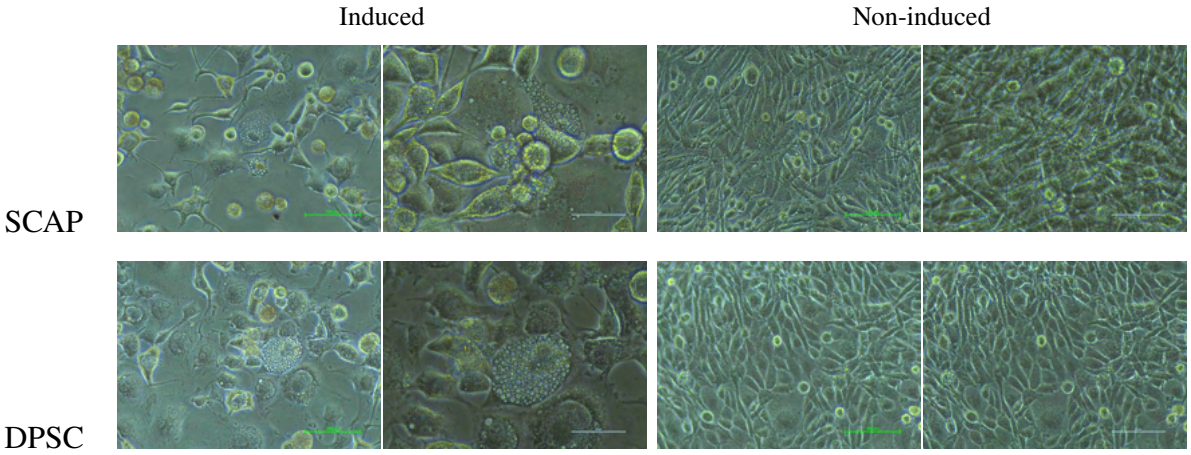


Figure 3.12: SCAP and DPSCs after two weeks of adipogenic induction, 10x & 20x

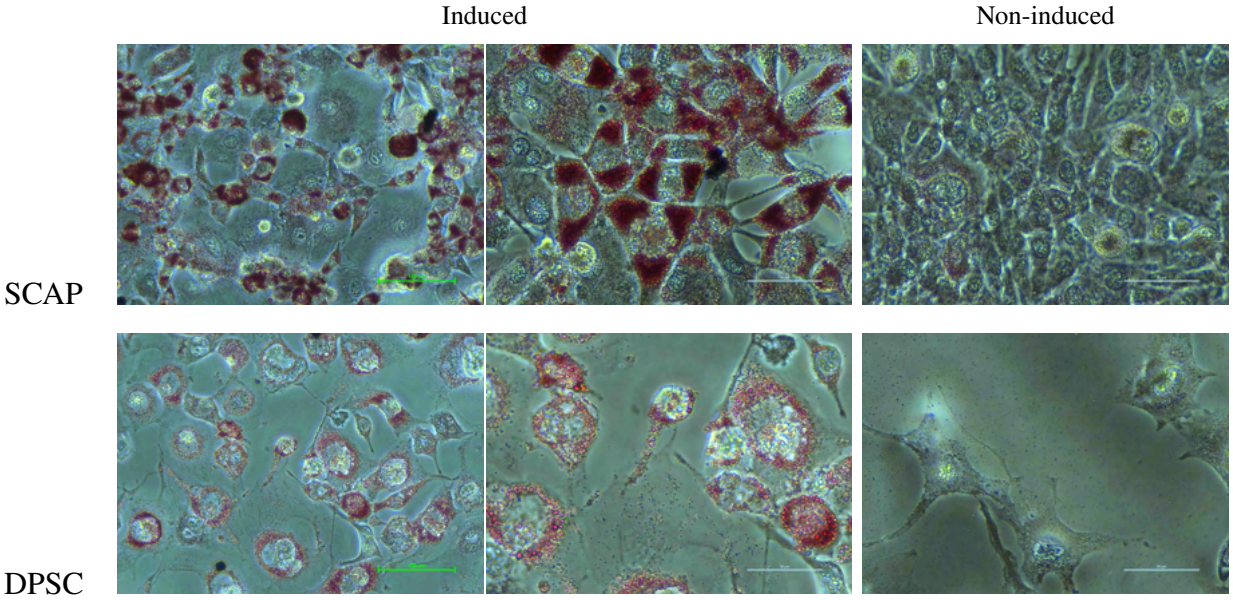
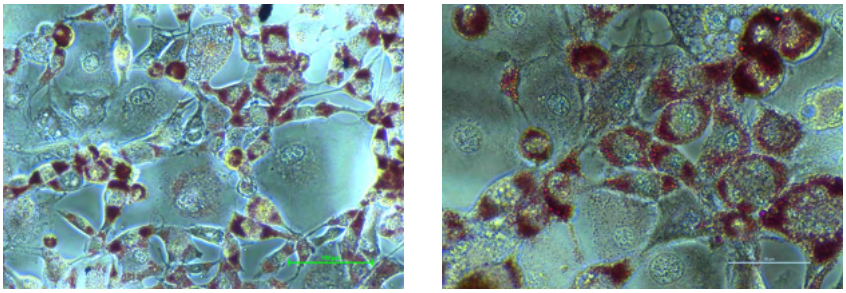
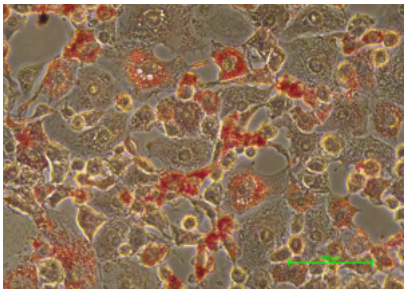


Figure 3.13: Oil Red O staining of SCAP and DPSCs after two weeks of adipogenic induction, 10x & 20x

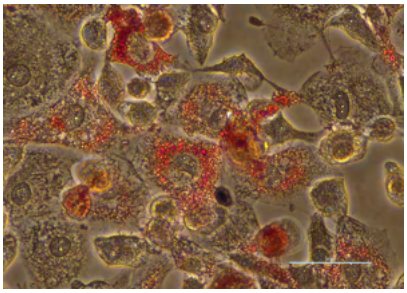


(a) Induced SCAP at P9, 20x

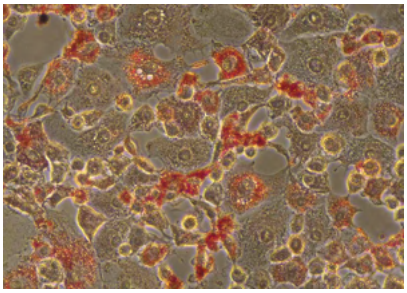
(b) Induced SCAP at P9, 40x



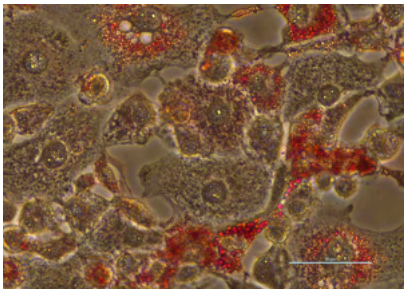
(c) Induced SCAP at P10, 20x



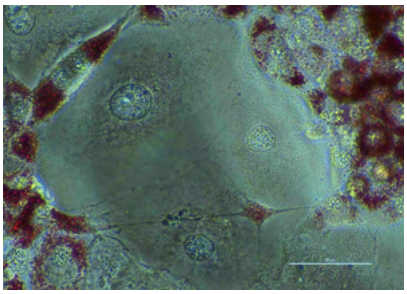
(d) Induced SCAP at P10, 40x



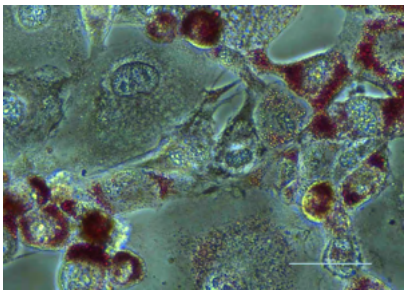
(e) Induced SCAP at P11, 20x



(f) Induced SCAP at P11, 40x



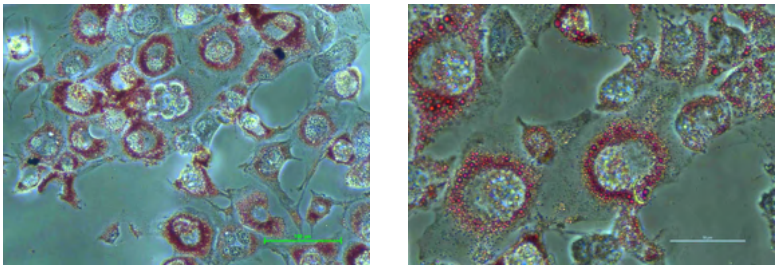
(g) Induced SCAP at P12, 40x



(h) Induced SCAP at P12, 40x

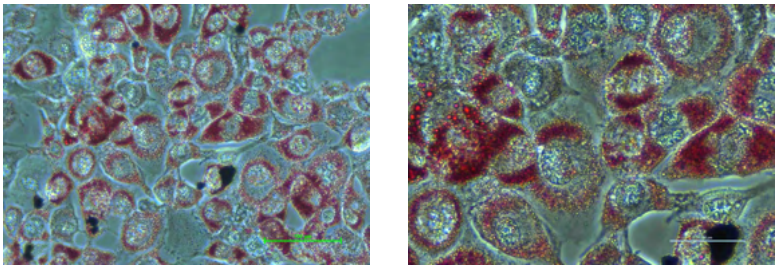
Figure 3.14: SCAP stained with Oil Red O after two weeks of adipogenic induction through passages 9-12 (P9-P12)

Along passage progress, DPSCs were also observed to maintain their inherent adipogenic differentiation potential as displayed through the oil red O staining in Figure 3.15. Moreover, DPSCs were observed to acquire a rounded morphology and detach from one another with passage progress, while still maintaining their adipogenic differentiation potential.



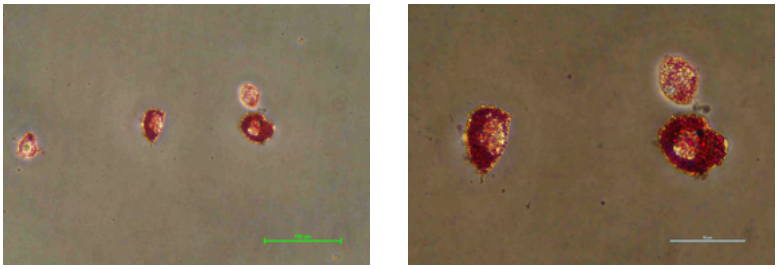
(a) Induced DPSCs at P9, 20x

(b) Induced DPSCs at P9, 40x



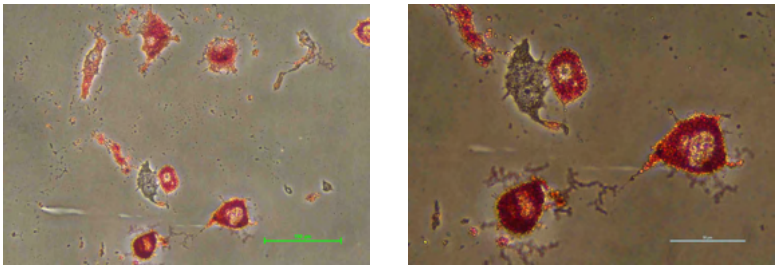
(c) Induced DPSCs at P10, 20x

(d) Induced DPSCs at P10, 40x



(e) Induced DPSCs at P11, 20x

(f) Induced DPSCs at P11, 40x



(g) Induced DPSCs at P12, 20x

(h) Induced DPSCs at P12, 40x

Figure 3.15: DPSCs stained with Oil Red O after two weeks of adipogenic induction through passages 9-12 (P9-P12)

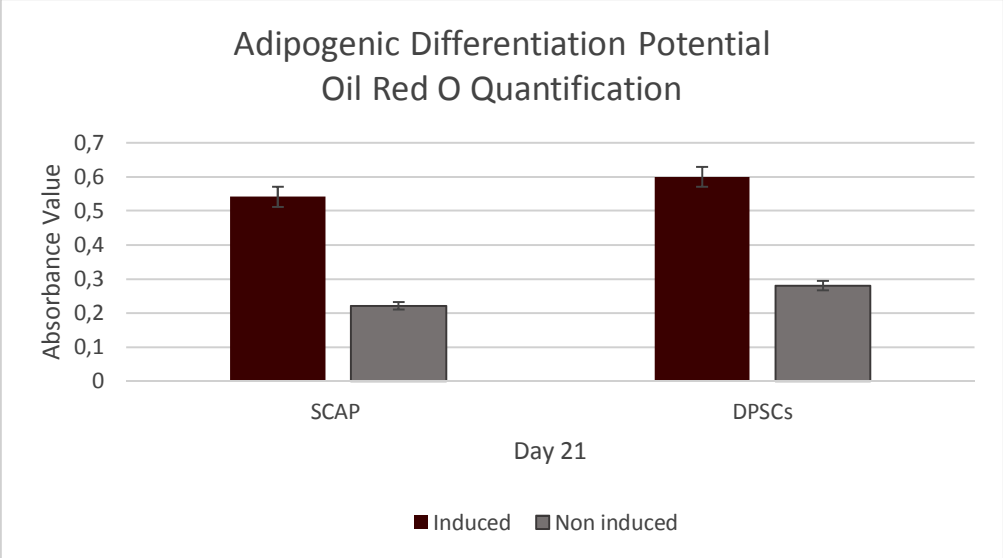


Figure 3.16: Quantification of Adipogenic differentiation potential

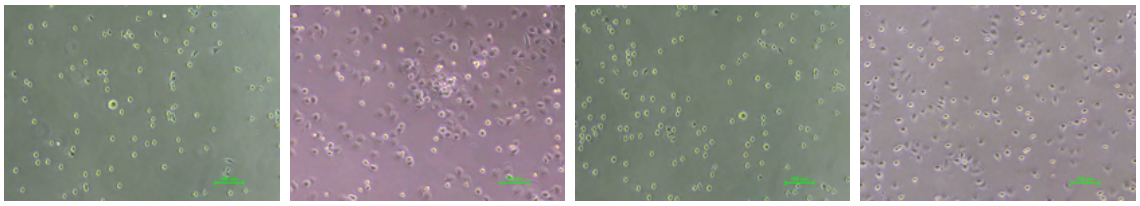
Adipogenesis quantification confirmed the qualitative observation of DPSCs displaying a slightly greater adipogenic differentiation potential, as displayed in Figure 3.16. However the slightly enhanced DPSCs adipogenic differentiation potential was not found to be statistically significant.

3.9 Spheroids formation

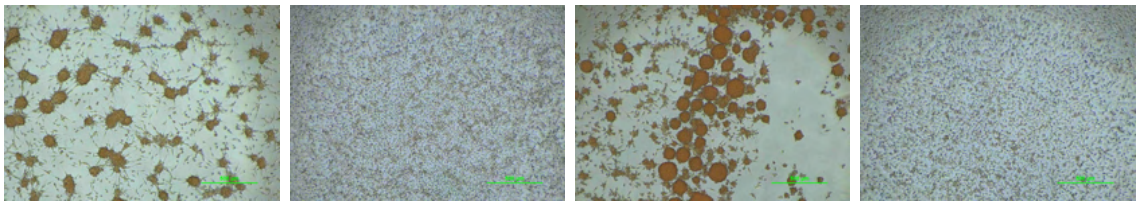
Spheroid formation was monitored under the inverted light microscope at the following time points: 0 hour, 24 hours, 48 hours, and 1 week. Microscopical examination revealed that

SCAP in knockout serum (KS) medium formed spheroids of similar size and morphology to DPSCs. On the other hand, SCAP and DPSCs in FBS medium showed attached cultures that were over-confluent as early as 24 hours, see Figure 3.17.

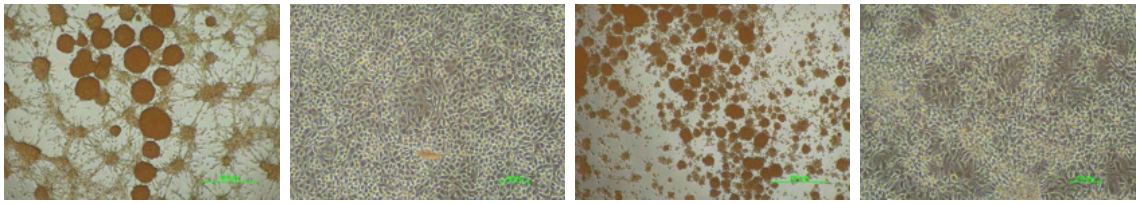
Within one day, SCAP and DPSCs cultured in a 24-well suspension plates formed small and loose spherical colonies. By the second day, multiple small sized spheroids were observed to come closer and often fuse or coalesce into larger and more dense spheroids. Moreover, with time, the larger coalesced spheroid masses were often observed to acquire an irregular outline rather than a spherical one as displayed in Figure 3.17. Furthermore, spheroids were observed to be maintained for a duration of 1 week.



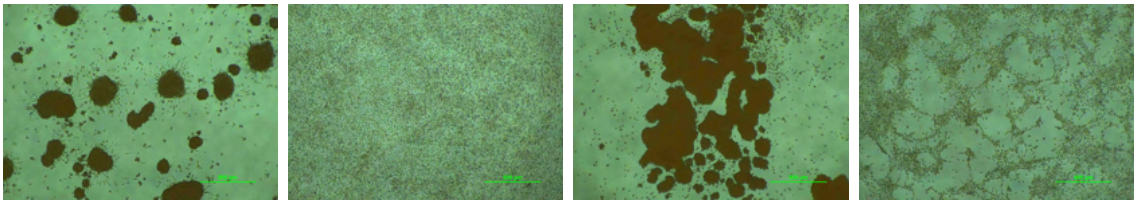
(a) SCAP in KS, 0 hour at 10x (b) SCAP in FBS, 0 hour at 10x (c) DPSCs in KS, 0 hour at 10x (d) DPSCs in FBS, 0 hour at 10x



(e) SCAP in KS, 24 h at 4x (f) SCAP in FBS, 24 h at 4x (g) DPSCs in KS, 24 h at 4x (h) DPSCs in FBS, 24 h at 4x

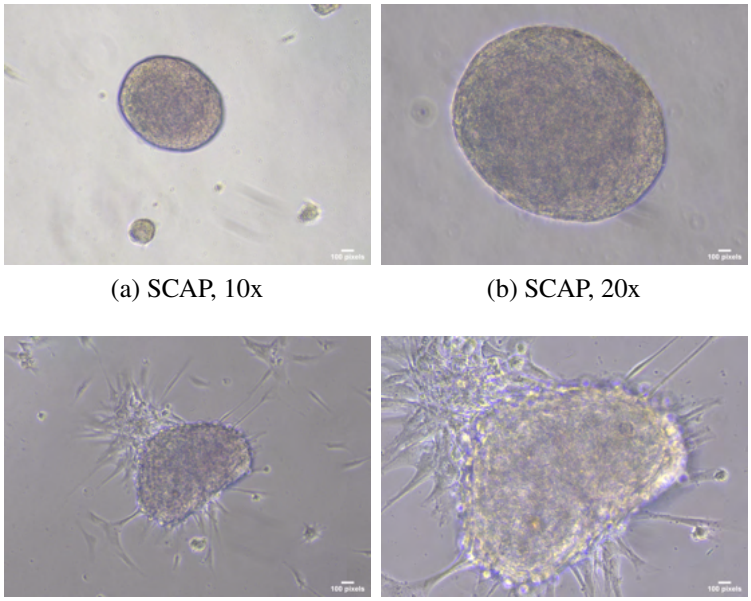


(i) SCAP in KS, 48 h at 4x (j) SCAP in FBS, 48 h at 4x (k) DPSCs in KS, 48 h at 4x (l) DPSCs in FBS, 48 h at 4x



(m) SCAP in KS, 1 week at 4x (n) SCAP in FBS, 1 week at 10x (o) DPSCs in KS, 1 week at 4x (p) DPSCs in FBS, 1 week at 10x

Figure 3.17: Spheroid formation ability of SCAP and DPSCs under the influence of KS and FBS medium



(a) SCAP, 10x (b) SCAP, 20x

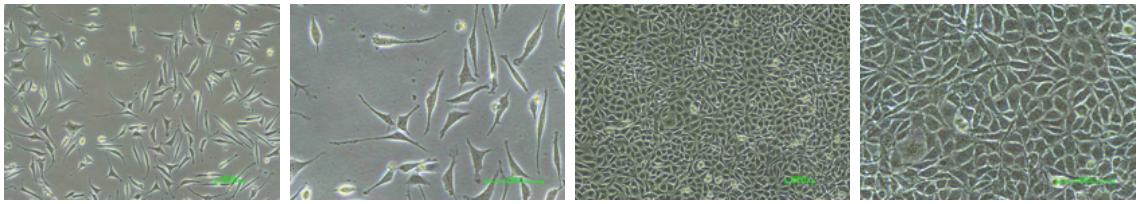
(c) DPSCs, 10x (d) DPSCs, 20x

Figure 3.18: Spheroid morphology at 10x and 20x displaying the cell dense core and a cellular peripheral zone

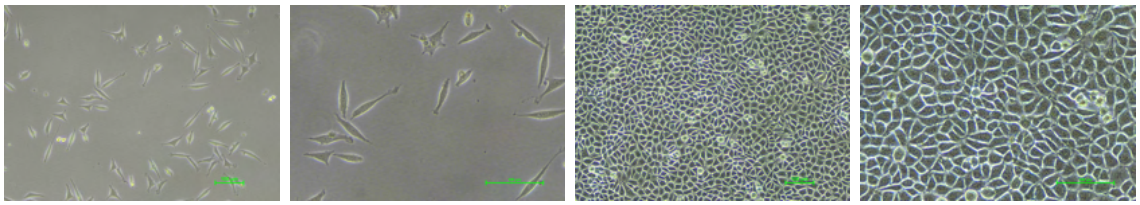
The 3D DPSC spheroids were observed to be comprised of two zones: a cell-dense core and an expanding cellular peripheral zone as displayed in Figure 3.18. However, 3D SCAP spheroids were observed to exhibit a cell dense core with a clean cut acellular peripheral zone in contrast to the ever expanding DPSC spheroid peripheral cellular zone as presented in figure 3.18.

SCAP and DPSCs could both generate multiple small-sized spheroids when cells were seeded in 24-well plates see Figure 3.17.

The average diameters of individual spheroid colonies formed by SCAP and DPSCs at day 1 were 250.8 ± 19 µm and 206.2 ± 22 µm respectively, growing to larger diameters by day 2, where SCAP and DPSCs spheroids measured at 319.5 ± 30 µm and 290.5 ± 21 µm respectively. The spheroid diameter difference between SCAP and DPSCs was found to be



(a) Post 3 days, 10x (b) Post 3 days,20x (c) Post 6 days, 10x (d) Post 6 days, 20x



(e) Post 3 days,10x (f) Post 3 days,20x (g) Post 6 days, 10x (h) Post 6 days, 20x

Figure 3.19: Thawed SCAP (a,b,c,d) and DPSCs (e,f,g,h) after one year of cryopreservation

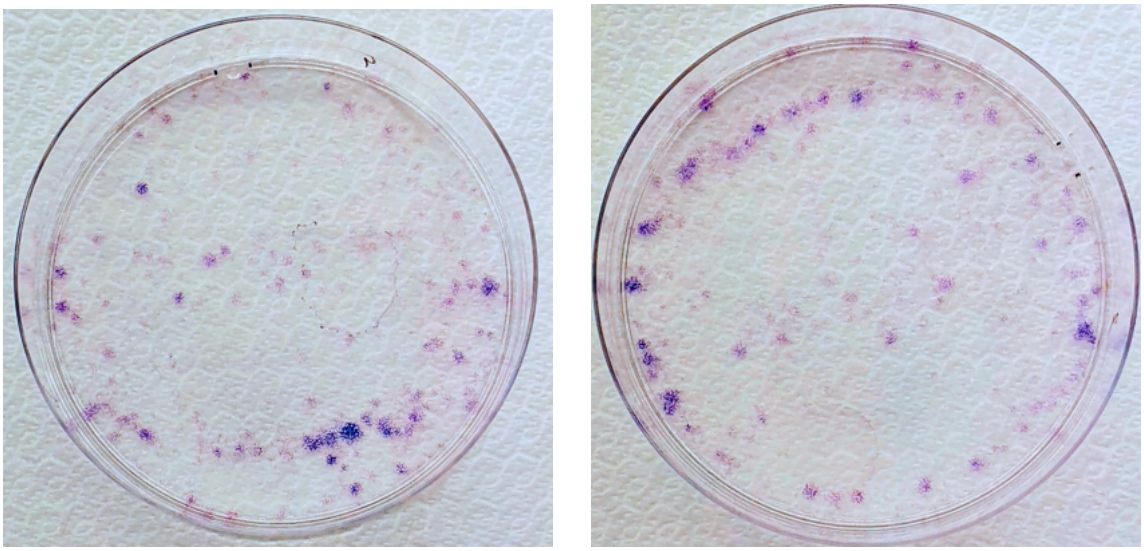
statistically significant with SCAP demonstrating a more potent spheroid formation ability.

3.10 Cryopreservation

Cell morphology of both SCAP and DPSCs cultures were observed to be unaltered post 1 year of cryopreservation. Moreover, cells retained their growth and proliferation rate, where they continued to reach confluent levels after 1 week of culturing, see Figure 3.19.

Moreover, viability assay results demonstrated that SCAP and DPSCs had a maintained level of viability before and after cryopreservation.

Viability assay results also demonstrated that SCAP and DPSCs had a maintained level of viability before and after cryopreservation. Viability percentages for SCAP was measured to be 89.4±5% before cryopreservation and 86±3% after cryopreservation. The same was true for DPSCs, scoring an average viability of 91±3% before cryopreservation and 90±5% after cryopreservation.



(a) SCAP (b) DPSCs

Figure 3.20: Colony forming efficiency of thawed SCAP and DPSCs following one year of cryopreservation

In terms of colony forming efficiency, as displayed in figure 3.20, SCAP maintained an efficiency of of 5.4%±1.9, with DPSCs scoring a slightly higher efficiency of 5.9%±1. with the difference once more being non statistically significant between the two cell groups.

Furthermore osteogenic and adipogenic differentiation potentials were also observed to be fully preserved post cryopreservation as displayed in figure 3.21 and figure 3.22 respectively, where figure 3.21 demonstrates the significantly greater osteogenic differentiation potential of SCAP compared to DPSCs and figure 3.22 demonstrates the advancement of DPSCs in adipogenic differentiation, with the difference in adipogenesis remaining to be non statistically significant.

Finally, cryopreservation also did not seem to have a negative influence on the spheroid formation ability.

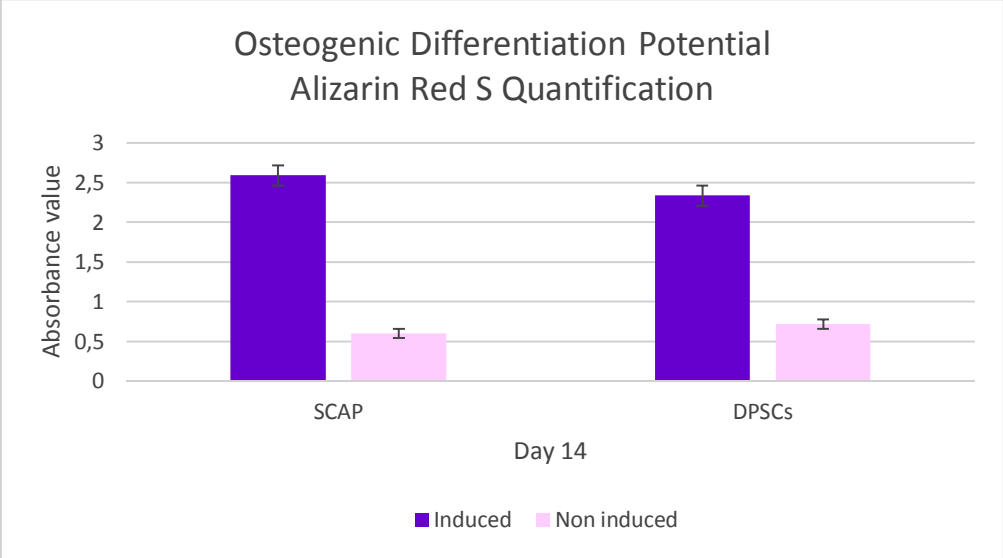


Figure 3.21: Quantification of Osteogenic Differentiation Potential following cryopreservation

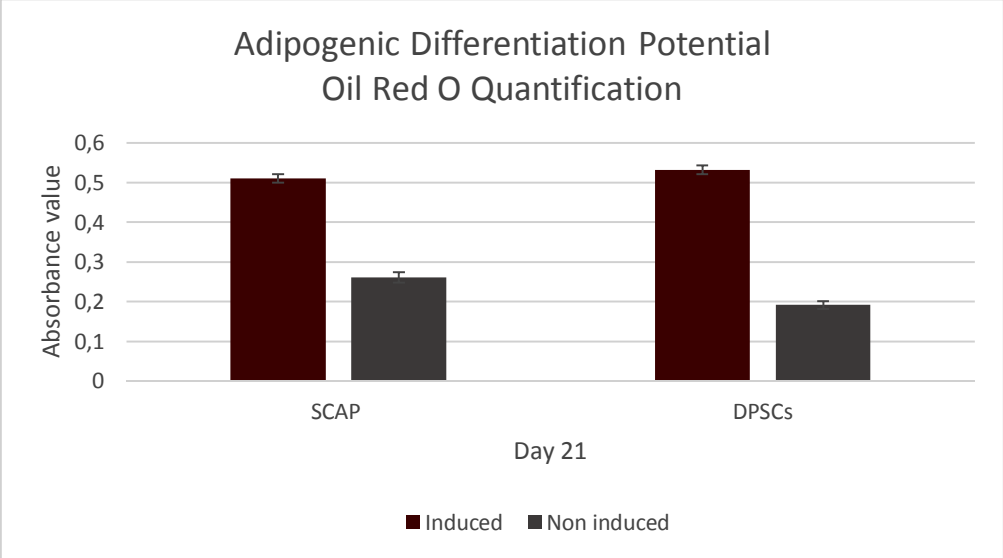


Figure 3.22: Quantification of Adipogenic Differentiation Potential following cryopreservation

Chapter 4

Diskussion

Tracing back intrauterine life revealed the intricate mechanisms involved in tooth development. While one might think that teeth can´t be of any use other than masticatory functions and aesthetics, learning how teeth were initially "generated" opens up endless possibilities in the field of regeneration. During tooth morphogenesis, the dental mesenchyme and its epithelial counterpart are the main active components, with the mesenchyme being composed of migrated neural crest derived cells[79].

The infamous sequence of reciprocal events that lead to tooth development involves the gradual engulfment of the dental papilla by the developing tooth germ where the segment that becomes partially enclosed becomes labelled as the developing dental pulp, while the non enclosed most apical part of the dental papilla at the tip of the ever progressing dental organ, becomes labelled as the future apical papilla tissue[79, 80, 81, 82].

Therefore, since cells located in the mesenchyme of the dental arches share a common origin with neural crest cells, tissues extracted from dental sources can very well be regarded as sources for neural crest stem cells (NCSCs) [78]. Hence, besides being employed for

mesenchymal regeneration, they can also be actively considered for neuronal regeneration in accordance with in vivo studies reporting that DSCs portray neuronal differentiation upon transplantation in various neuronal diseased tissues [80, 30].

This justifies the use of DSCs as a stem cell source against invasive sources like BMMSCs or even the readily available in large quantities, adipose stem cells (ASCs), since both BMMSCs and ASCs are restricted to mesenchymal regeneration[].Moreover, the ease and convenience of retrieving dental stem cells from dental waste is a highly adequate justification for their use as a stem cell source.

In the present study, two types of DSCs were addressed; SCAP and DPSCs were investigated after immediate isolation and after long term cryopreservation (for a duration of one year) throughout 12 consecutive passages. To ensure paired comparisons of SCAP and DPSCs from the same patients, apical papilla and dental pulp tissues were collected from extracted wisdom and premolar teeth (average ages of 19.5 and 11.5 years respectively). When one of the cell lines failed to be isolated, the entire pair was excluded from the study. Often times apical tissues failed to generate adherent cell cultures, wherein possible reasoning for this occasional failure could be the fact that the apical papilla tissue becomes directly exposed to the external environment post operative extraction. Thereby, subjecting the papilla tissue to the shock of being suddenly uncovered and compromised without its natural microenviroment of the corresponding developing bone socket, as well as making it more vulnerable to possible external contamination, both of which can lead to an incapacitated potential. This reasoning is in correspondence with Naz et al's observation of the failure of pulp tissues to generate adherent cells when the tissues were subjected to an osmotic shock because of inadequate media coverage during tissue processing[83].

To isolate the cells in culture, the two most commonly known techniques were employed,

namely being the digestion and explant outgrowth method, with the former technique displaying superior efficiency. Despite the convenience and cost efficiency of the latter outgrowth method where tissue extracts are simply cut into small pieces and incubated until cells naturally flow out of them, it could lead to sample loss when cells fail to attach. It is therefore our recommendation to favor the digestion method for a more guaranteed success rate and efficiency in isolation.

As reported by Karbanova et al., primary cultures started out slow with discrete and separated colonies for about 1 week until colonies started overlapping and eventually reached confluence within approximately 2 weeks time [29]. However, Bakopoulou et al. reported slightly shorter periods for primary cultures to reach confluency[77]. This could imply that teeth processing is technique sensitive and thereby differs from one operator to another. Moreover, it could also suggest that the greater the sample size, the higher the chances for variability since Bakopoulou et al had a sample size of 3 donors, whereas the present study tackled 10 donors.

Furthermore and in accordance with Gronthos et al. and Bakopoulou et al, cells cultured from apical papilla and dental pulp tissues displayed a potent ability to form adherent cell colonies on plastic cell culture surfaces, in addition to exhibiting fibroblast-like spindle shaped and polygonal shaped morphology[23, 77]

Moreover, previous studies fortified the basic media used to culture the isolated dental stem cells with ascorbic acid[16, 23, 52], whereas in the present study ascorbic acid supplementation in the basal media was entirely eliminated. Our data revealed that the lack of ascorbic acid supplementation did not negatively affect the isolation and growth of cultures and can thereby be regarded as a non essential addition to the dental stem cell basal medium. Further simplifying the process and reducing the overall cost of culturing dental

stem cells.

SCAP and DPSCs underwent experimentation to test their stem cell characteristics such as self renewal capacities, osteogenic/odontogenic and adipogenic differentiation potential as well as spheroid formation abilities. Even though prior research disclosed SCAP and DPSCs acquiring all the above stem cell characteristics, experimentation have always been limited to earlier passages (ranging from the 2nd passage till the 10th passage)[77, 16, 23, 50, 78], the current study explores beyond the 10th passage and up to the 12th passage.

The findings of present study revealed that SCAP and DPSCs exhibited growth patterns and population doublings that corresponded with previous studies [77, 84] and extended up until the 12th passage, concluding that SCAP and DPSCs are able to maintain potent growth abilities beyond the previously established 10 passages and well into 12 passages. However, growth curves in the present study opposed Bakopoulou et al, where DPSCs displayed a more advanced growth curve when compared to SCAP[77]. This could be attributed to the nature of the original tissues, where the dental pulp has a more fibrous nature when compared to the apical papilla, thereby possibly attributing the higher growth rate of DPSCs to their higher fibroblastic content.

Furthermore, in the light of our findings, the employment of simple experimentation setups such as the one introduced by [77] and adopted in the present study of seeding a certain concentration of cells and counting their cell count yield to denote growth is recommended to monitor and evaluate cell growth due to its simplicity and low cost.

As science dictates, stem cells are classified as being non specialized cells with a potential to differentiate along several lineages when subjected to certain conditions[59, 85]. This was in accordance to our findings where both SCAP and DPSCs transformed or differentiated to specialized cells of an osteogenic or adipogenic nature when exposed to

osteogenic and adipogenic environments respectively[16, 23, 86]. Further reinforcing the stem cell character of regenerating affected sites and reestablishing homeostasis or balance in the human body[59, 86].

Compared with DPSCs, SCAP showed a significant advancement in their osteogenic/odontogenic differentiation potential. This was not in accordance with results reported by Bakopoulou et al. where up until the second week, DPSCs were seen to protray a higher osteogenic potential , with SCAP only taking the lead at day 21[77]. Our results protrayed SCAP taking the lead from as early as day 14 with a significant difference when compared to its DPSC counterpart. Possible justification for this difference could be due to the slight change in the chemical cocktail used to induce differentiation where Bakopoulou et al. reported the use of monopotassium phosphate as a further supplement. Nevertheless, the pattern of calcification generated by each monoculture was seen to be in accordance with previous studies[77, 50].

Although additional research studies and in particular in vivo ones are required to acquire a more comprehensive and confirmed characterization of these cells, the results introduced from this research and previous ones can nevertheless serve as an adequate reference point that SCAP cultures derived from fresh and cryopreserved SCAP is a potential treasure for convenient and reliable allogenic osteogenic tissue regeneration[58, 76].

Furthermore on the choice of the confirmatory stain, we opted for Alizarin Red due to the simplicity and specificity of the stain to calcific deposits. Moreoever, the present study is directed towards simplifying and making stem cell experimentation attainable in the least equipped cell culture laboratory while fulfilling the purpose of stem cell characterization. Therein Alizarin Red Stain is considered to be a fairly simple stain to prepare with the only consideration going towards adjusting the pH level to 4.2 seeing as though its a critical key

point in the success of the staining.

Nevertheless, further genetic exploration of the generated calcific deposits is necessary, especially to understand which direction the differentiation is steered towards, whether it is of an osteogenic nature or an odontogenic nature as introduced previously by Wang et al. who previously reported IGF-1 to act as an osteogenic/odontogenic indicator, where it was found to be upregulated in cases of bone like calcification and down-regulated in dentin like calcification[63]. Further studies to understand and thereby control the osteogenic/odontogenic switch will further reinforce tailoring bone and dentin tissue regeneration applications.

Moreover on differentiation, DPSCs took the lead when compared to SCAP through displaying slightly elevated adipogenic differentiation potential. While the present findings might negate previous studies that suggested that SCAP has a generally more potent differentiation ability [52], the adipogenic enhancement portrayed by DPSCs were found to be non statistically significant. Thereby, rendering SCAP to remain to be superior due to not only SCAPs significant advancement in osteogenesis but also due to its relative ease of retrieval through simple detachment from the root tips, contrary to DPSCs which involves decoronating the tooth and digesting a rather large piece of tissue. SCAP on the other hand offers a simpler and faster isolation process, with comparable results and more superior osteogenesis. The only limitation that remains can be the availability of SCAP since it entails the presence of younger teeth with developing roots.

Furthermore, to test the effect of KSR-containing medium on the spheroid formation ability of SCAP and DPSCs, we performed a comparison of different culture media, including FBS containing medium and KSR containing medium. We demonstrated that under KSR-medium, both SCAP and DPSCs could readily and spontaneously form spheroids

without the use of complex scaffolds or sophisticated cell culture techniques, with SCAP taking the lead with a significantly superior ability to generate spheroids. Thereupon, in vivo conditions of establishing a 3D spheroid culture has been proved to be easily attainable up until the 12th passage. This enables and opens up more possibilities for future research that can test the potential of SCAP and DPSCs in a 3D culture environment rather than a 2D monolayer one, since the former is closer to the true representation in vivo. Reinforcing so, are previous reports that indicate that the altered cell shape in an in vivo like environment may contribute to 3D spheroids exhibiting superior differentiation qualities[42, 87, 88]. Furthermore, the 3D DPSC spheroids were observed in accordance to Friedrich et al[89], to be comprised of two zones: a cell-dense core and an expanding cellular peripheral zone as displayed in Figure 3.18. However, 3D SCAP spheroids were observed to exhibit a cell dense core with a clean cut acellular peripheral zone in contrast to the ever expanding DPSC spheroid peripheral cellular zone as presented in figure 3.18. Additionally, it has also been suggested that the hypoxic cores of spheroids may themselves be differentiation facilitators[42, 90].

Moreover and in correspondence with previous studies, spheroids were observed to be smaller and larger in quantity in the beginning, followed by progression in size due to their merging and coalescence[42]. From a spheroid perspective, the importance and effect of serum free media is highlighted where cultures are once more simplified and might possibly result in cells with more potent stem cell characteristics. Thereby, denoting the significance of shifting to serum free cultures and therefore developing 3D cultures.

Additionally in the present study, the effect of one year long cryopreservation periods on paired SCAP and DPSCs was also investigated and contrasted with the identical pairs that were left to expand without cryopreservation until the 12th passage. In the light of the

current knowledge, post cryopreservation qualities at later passages from paired cell sources have not been previously evaluated.

In accordance with previous studies tackling younger passages that reported that DPSCs can endure lengthy cryopreservation intervals with unaffected stem cell characteristics[39, 83, 59, 91, 92], our study also reports the same findings, where cellular viability was almost uncompromised before and after cryopreservation. The same was also observed to be true for SCAP well into the 12th passage.

The findings of present study also revealed that long-term cryopreserved SCAP and DPSCs possessed a marked maintenance in spheroid formation ability when compared to the formation ability of the same pairs that didn't undergo cryopreservation.

All the positive aforementioned findings in reference to cryopreservation are a great advantage especially when it comes to limitations that the present research faces of having an inconsistent patient flow; a non-deniable factor since the research study sample as a whole is based on dental waste composed of prophylactic teeth extractions. Therefore, positive cryopreservation findings such as those introduced in this study and recently in our group by Yan et al.[75] can shift the attention to stocking up on teeth samples/cultures through cryopreservation and benefiting from them on demand at a later time when the flow is constrained.

In conclusion, no comparative studies have yet been presented in such a comprehensive manner, therefore, the efforts invested in this thesis were aimed to bring the scientific community a step closer to eventually regenerate lost tissues and consequently restore function as William Haseltines described regenerative medicine as the field merging between stem cell biology and tissue engineering[93].

Appendix A

Abkuerzungsverzeichnis

Abbreviation	Meaning
2D	Two-dimensional
3D	Three-dimensional
°C	Celsius degrees
ASCs	Adipose stem cells
AR-S	Alizarin Red S
CO2	Carbon Dioxide
CNTF	Ciliary neurotrophic factor
BGP	Beta glycerolphosphate
BMMSCs	Bone marrow mesenchymal stem cells
BMP	Bone morphogenetic protein
BDNF	Brain derived neurotrophic factor
CEJ	Cemento-enamel junction
DXM	Dexamethasone

DFPCs	Dental Follicle Progenitor Cells
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPSCs	Dental pulp stem cells
DSCs	Dental Stem Cells
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ESCs	Embryonic Stem Cells
F12	DMEM Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
FBS	Fetal bovine serum
FGF	Fibroblast Growth Factor
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
g	Gram
IBMX	3-iso-butyl-L-methylxanthine
IGFs	Insulin like Growth Factors
KSR	Knockout Serum Replacement
MEM	Minimum Essential Medium
MEM-α	Minimum Essential Medium α
Mg	Milligram
Ml	Milliliter
mm3	Cubic millimeter
MSC	Mesenchymal Stem Cell
NCSCs	Neural Crest Stem Cells

NGF	Nerve growth factor
NSE	Neuron-specific enolase
NeuN	Neuronal nuclei
Nestin	Neuroectodermal stem cell marker
Nm	Nanometer
nM	Nanomole
OR-O	Oil Red O
PBS	Phosphate-buffered saline
PDLSCs	Periodontal ligament stem cells
SCAP	Stem cells from the apical papilla
SD	Standard deviation
SF	Serum free
SHED	Stem cells from Exfoliated Deciduous Teeth
TET	Tissue Engineering Triad
TGF	Transforming growth factor
TGSCs	Tooth Germ Stem Cells
U	Unit
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromole
VEGF	Vascular Endothelial Growth Factor

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Appendix B

Conclusion

In the light of the present study, the following can be concluded:

No significant differences are noted between SCAP and DPSCs in their viability, growth, population doublings, colony forming efficiencies, and adipogenic differentiation abilities.

Nevertheless, significant differences were credited to SCAP in being superior to DPSCs in reference to osteogenic differentiation and spheroid formation abilities.

Moreover, no significant differences were present when comparing fresh pairs of SCAP and DPSCs to their cryopreserved counterparts, deducing that cryopreservation can be a convenient option for storing SCAP and DPSCs until up to one year.

All of which contributes to future studies and applications of the two distinct, yet rather similar cell sources and types.

Further histological evaluation, multidifferentiation characterisation, and gene expression of the generated spheroids is particularly recommended in order to confirm their stemness.

Appendix C

Abstract

The purpose of the study is to compare stem cell characteristics between cells from the apical papilla and the dental pulp throughout 12 passages with and without being subjected to cryopreservation. The hypothesis is that with passage progress, cells are more likely to undergo senescence and lose their stem cell characteristics. The study also hypothesizes that cells from the apical papilla might display superior potency in stem cell characteristics owing to their origin from developing and therefore more immature and younger tissues.

Materials and Methods: Cell cultures from the apical papilla (n=12) and dental pulp (n=12) were retrieved from 12 prophylactically extracted premolars and wisdom teeth. Cultures were isolated using both explant outgrowth and enzymatic digestion. Stem cell characterisitics were examined and compared using growth curves, colony forming efficiency, population doublings, viability, adipogenic and osteogenic differentiaion potentials, and spheroid formation abilities. All experiments were repeated using the same pairs of apical papilla and dental pulp cells after a one year cryopreservation period.

Results: Cells from the apical papilla displayed a significant superiority in osteogenic differentiation and spheroid formation abilities. No significant differences were observed between cells from the apical papilla and the dental pulp in the remainder of all the stem cell characteristics. Finally, no significant differences were noted in stem cell charactersitics between the fresh and the cryopreserved cultures.

Conclusions: Apical papilla cells portray more potent osteogenic differentiation and spheroid formation abilities when compared to dental pulp cells. Both cell types displayed similar growth, colony forming efficiency, viability, population doublings, and adipogenic differentiation. Apical papilla and dental pulp cells can be cryopreserved for at least one year without substantial characteristic changes.

Keywords: SCAP, DPSCs, Adipogenic, Osteogenic, Spheroids, Cryopreservation

Appendix D

Zusammenfassung

Im Lichte der vorliegenden Studie kann folgendes gefolgert werden:

Es wurden keine signifikanten Unterschiede zwischen SCAP und DPSCs in Bezug auf Lebensfähigkeit, Wachstum, Populationsverdopplung, Koloniebildungseffizienz und adipogener Differenzierungsfähigkeit festgestellt.

Dennoch wurden signifikante Unterschiede zwischen SCAP und DPSCs in Bezug auf die osteogene Differenzierung und die Fähigkeit zur Sphäroidbildung festgestellt.

Darüber hinaus gab es keine signifikanten Unterschiede beim Vergleich von frischen SCAP- und DPSC-Paaren mit ihren kryokonservierten Gegenstücken, was darauf schließen lässt, dass die Kryokonservierung eine bequeme Option für die Lagerung von SCAP und DPSCs bis zu einem Jahr sein kann.

All dies trägt zu zukünftigen Studien und Anwendungen der beiden unterschiedlichen, aber doch recht ähnlichen Zellquellen und -typen bei.

Eine weitere histologische Auswertung, Charakterisierung der Multidifferenzierung und Genexpression der generierten Sphäroide wird besonders empfohlen, um deren Stammhaftigkeit zu bestätigen.

Appendix E

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Appendix F

Lebenslauf

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

Appendix G

Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe. Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe. Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

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