2015•2016
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef
The use of dental pulp stem cells as a cell-based therapy in head and neck squamous cell carcinoma

Promotor:
Dr. Annelies BRONCKAERS

Copromotor:
Prof. dr. Ivo LAMBRICHTS

Melissa Lo Monaco
Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen
Masterproef
The use of dental pulp stem cells as a cell-based therapy in head and neck squamous cell carcinoma

Promotor:
dr. Annelies BRONCKAERS

Copromotor:
Prof. dr. Ivo LAMBRICHTS

Melissa Lo Monaco
Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen
# Table of contents

Table of contents ........................................................................................................... I  
Acknowledgements ......................................................................................................... III  
List of abbreviations ........................................................................................................ V  
Abstract ........................................................................................................................... VII  
Samenvatting ..................................................................................................................... IX  
1. Introduction ................................................................................................................... 1  
  1.1. Head and neck squamous cell carcinoma (HNSCC) .................................................. 1  
  1.2. Angiogenesis .......................................................................................................... 2  
  1.3. Pathological angiogenesis and anti-angiogenic therapy in HNSCC ........................... 3  
  1.4. Mesenchymal stem cells (MSCs) and human dental pulp stem cells (hDPSCs) ....... 4  
  1.5. Mesenchymal stem cells and cell-based therapy in cancer ....................................... 5  
    1.5.1. Mesenchymal stem cells are able to migrate towards tumors ............................. 5  
    1.5.2. The controversial role of mesenchymal stem cells in tumor development .......... 6  
    1.5.3. Engineered mesenchymal stem cells in tumor treatment ................................... 7  
  1.6. Research aim and experimental set-up ..................................................................... 8  
  2. Materials and methods ............................................................................................. 9  
    2.1. Isolation and culturing of hDPSCs ....................................................................... 9  
    2.2. Culturing of tumor cells ...................................................................................... 9  
    2.3. Generation of conditioned medium (CM) of tumor cells ..................................... 9  
    2.4. Flow cytometry .................................................................................................. 9  
    2.5. Immunocytochemistry (ICC) ............................................................................. 10  
  2.6. Migration assays .................................................................................................... 11  
    2.6.1. Transwell migration assay .............................................................................. 11  
    2.6.2. Wound healing assay .................................................................................... 12  
  2.7. Cell proliferation assay ......................................................................................... 12  
  2.8. *In vivo* effect of hDPSCs on HNSCC growth ....................................................... 12  
  2.9. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) ........... 13  
  2.10. Immunohistochemistry (IHC) and histology ......................................................... 14  
  2.11. Statistical analysis .............................................................................................. 14  
  3. Results ....................................................................................................................... 15  
    3.1. Characterization of a human squamous cell carcinoma cell line called FaDu cells ... 15  
      3.1.1. Expression of various angiogenesis-related factors by FaDu cells .................... 15  
      3.1.2. FaDu cells antigen expression ...................................................................... 16  
    3.2. Migrating abilities of hDPSCs .............................................................................. 17  
      3.2.1. Evaluation of hDPSC migration towards tumor cells ...................................... 17  
      3.2.2. Evaluation of several chemotactic factors responsible for the migration of hDPSCs 18  
      3.2.3. The effect of chemotactic factors on hDPSC proliferation ............................... 19  
      3.2.4. Evaluation of the directional migration of hDPSCs in response to FaDu cells CM and SDF-1α ................................................................................................................. 20
3.3. The in vivo effect of hDPSCs on HNSCC growth ...............................................................21
3.4. Morphological and pathological properties of control and hDPSC-inoculated tumors ........22
3.5. The effect of hDPSC inoculation on HNSCC angiogenesis ............................................23
3.6. The effect of hDPSC inoculation on the aggressiveness of HNSCC .................................24
4. Discussion .........................................................................................................................25
5. Conclusion and synthesis .................................................................................................31
6. References .........................................................................................................................33
Supplemental information .....................................................................................................39
  S1 hDPSCs express the angiostatic chemokine, Platelet factor-4 variant (PF-4var), and a glycolytic regulator protein ..........................................................39
  S2 Migrating hDPSCs consist of a better migrating subpopulation compared to the non-migrating hDPSCs .............................................................................40
Acknowledgements

During the past eight months, I completed my senior internship at the department of Morphology at Hasselt University. This internship allowed me to experience that scientific research does not only involve experiments, criticism and scientific writing, but it also includes having fun with each other in the lab and helping each other. Therefore, I would like to express my gratitude to several people!

First of all, I would like to thank Prof. dr. Ivo Lambrichts for giving me the opportunity to be part of his team and research group during this internship period. A huge thank you goes to my promotor and daily supervisor dr. Annelies Bronckaers. She made me a real transwell assay expert! Annelies, thank you for your supervision, criticism and confidence. You gave me the responsibility and liberty to work in the lab, which allowed me to become independent in the lab. You were always there to help me when needed. Furthermore, your passion made me always smile, even in stressful periods!

Also, I would like to thank my second examiner dr. Myriam Gou Fabregas for her advice, criticism and suggestions during this project. This study would not have been imaginable without the provision of patients’ material by dr. Luc Vrielinck from the Ziekenhuis Oost-Limburg and Prof. dr. Constantinus Politis from Ziekenhuis Maas en Kempen.

Many thanks also to all members of the Morphology group: Jessica Ratajczak, dr. Petra Hilkens, Yorg Dillen, dr. Esther Wolfs, Pascal Gervois and Tim Vangansewinkel. Jessica, thank you for your help with the AxioVision software! Yorg, a big thank you for helping me with the focus of the Mirax slide scanner! Dr. Petra Hilkens, you were often there to help me in the lab when needed. Finally, dr. Esther Wolfs, thank you for the design of our vector! Furthermore, ‘thank you’ goes also out to Jeanine Santermans and Marc Jans for the sectioning of our tumor tissues and to Katrien Wauterickx, for all the help with the immunostainings during my junior internship. In addition, I would also like to express my gratitude to the group of Immunology, Marjan Vanheusden and Prof. dr. Niels Hellings, who allowed me to approach research during my junior internship. Thank you!

And of course, a big thank you goes out to all my fellow students for all the help, encouragements and advices. I really enjoyed the funny conversations and all the coffee breaks. Thank you not only for this year, but for all the years together! I wish you all the best of luck!

Last but not least, I would like to thank my parents, my brother and my boyfriend Federico for their support and for calming me down during stressful periods.

Melissa Lo Monaco; June 8th, 2016.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aMEM</td>
<td>Minimal Essential Medium, alpha modification</td>
</tr>
<tr>
<td>α-sma</td>
<td>Alpha smooth muscle actin</td>
</tr>
<tr>
<td>AAT</td>
<td>α1-antitrypsin</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxyethyl ester</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BD</td>
<td>Becton, Dickinson and Company</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence imaging</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CAM</td>
<td>Chick chorioallantoic membrane</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CXCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>Cyca</td>
<td>Cyclophilin A</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF(R)</td>
<td>Epidermal growth factor (receptor)</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated protein kinases 1 and 2</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FaDu</td>
<td>Human squamous cell carcinoma cell line</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fluc</td>
<td>Firefly luciferase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled chemokine receptor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>hDPSC</td>
<td>Human dental pulp stem cell</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia induced factor-1α</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>Human microvascular endothelial cells</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin growth factor-1</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>Insulin-like growth factor-binding protein 3</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PF-4(var)</td>
<td>Platelet factor-4 (variant)</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFKFB3</td>
<td>6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
</tr>
<tr>
<td>3PO</td>
<td>3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one</td>
</tr>
<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>RPL13a</td>
<td>Ribosomal protein L13 a</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcriptase quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal cell-derived factor 1α</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Neuroblastoma cell line</td>
</tr>
<tr>
<td>SIRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TAF</td>
<td>Tumor associated fibroblast</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Treg cell</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>VEGF(R)</td>
<td>Vascular endothelial growth factor (receptor)</td>
</tr>
<tr>
<td>Ywhaz</td>
<td>Yrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein zeta</td>
</tr>
</tbody>
</table>
Abstract

Introduction: The survival rate of patients with head and neck squamous cell carcinoma (HNSCC) is still dramatically low. Classic treatment options are hampered by the anatomic region of the tumor, often causing complications and a decreased quality of life. Histological data revealed that HNSCCs are characterized by an increased vascularization, suggesting that their progression depends on the formation of new blood vessels. Therefore, angiogenesis inhibition might be a promising contributory therapeutic approach. However, numerous limitations are reported to be associated with the systemic administration of anti-angiogenic agents. Consequently, the application of cells as delivery vehicles of anti-angiogenic proteins at the tumor site has been suggested. It has been proven that bone marrow mesenchymal stem cells (BM-MSCs) tend to specifically migrate towards tumors and may therefore function as on-site delivery vehicles of anti-angiogenic agents. Since the isolation of BM-MSCs involves several risks, we would like to use human dental pulp stem cells (hDPSCs), which are easy and safe to obtain.

We hypothesize that as a new treatment strategy for HNSCCs, hDPSCs can be applied as vehicles to deliver an angiostatic chemokine. Therefore, our objective is to study the effect of unmodified hDPSCs on tumor growth in vivo and whether hDPSCs are capable of migrating towards tumor cells in vitro.

Material & methods: The effect of hDPSCs on tumor growth in a HNSCC xenograft mouse model was investigated. 1x10^6 squamous carcinoma cells were subcutaneously injected into the dorsal flank of nude mice, two tumors per mouse. Ten days later, one of the tumors received intratumoral injection of 1x10^6 hDPSCs, while the other tumor received a control injection of medium. Tumor volume was measured two times a week (n=14). Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and histological analyses were performed on tumors to assess angiogenesis and aggressiveness of control and hDPSC-treated tumors (n=5).

To evaluate the migratory capacities of hDPSCs, a transwell migration assay was applied to evaluate migration towards tumor cells (n=5) and several factors (n=6).

Results: No significant difference in tumor volume between hDPSC-inoculated or control tumors could be observed during 24 days. RT-qPCR data revealed that the hDPSC-inoculated tumors had no increased expression of epithelial-mesenchymal transition (EMT)-specific markers, which are markers for tumor aggressiveness. In addition, both RT-qPCR as immunohistochemistry (IHC) revealed that blood vessel area was not increased in mice injected with hDPSCs. In the transwell system, hDPSCs were able to migrate towards tumor cells. In addition, high donor variability was observed.

Discussion & conclusions: The competence of hDPSCs to be used within the field of cell therapy combined with gene delivery has not been investigated before. Our data offer evidence for the application of hDPSCs for gene delivery in cancer as they do not promote tumor growth under the tested conditions in vivo and migrate towards tumor cells in vitro. Essentially, this study enhances the knowledge about the use of hDPSCs as cell therapy in cancer.
Samenvatting

**Introductie:** De overlevenkans van patiënten met hoofd en hals plaveiselcelcarcinoom is dramaticaal laag. Klassieke behandelingsstrategieën worden belemmerd door de anatomische ligging van de tumor, wat geassocieerd wordt met complicaties en een verminderde levenskwaliteit. Histologisch onderzoek onthulde dat deze kankers gekarakteriseerd worden door een verhoogde vascularisatie, wat suggereert dat de progressie afhankelijk is van de vorming van nieuwe bloedvaten, ook wel angiogenese genoemd. Omwille van deze redenen zou de inhibitie van de angiogenese een veelbelovende adjuvante therapeutische strategie zijn. Echter, systemische toediening van anti-angiogene middelen wordt geassocieerd met bijwerkingen en blijkt nauwelijks doeltreffend. Bijgevolg wordt het gebruik van stamcellen als producers van anti-angiogene eiwitten in de tumor als nieuwe therapeutische optie onderzocht. Het is reeds bewezen dat mesenchymale stamcellen (MSCs) uit het beenmerg specifiek migreren naar tumoren en daarom gebruikt kunnen worden als producers van anti-angiogene middelen. Omdat de isolatie van beenmerg MSCs invasief en pijnlijk is, gebruiken we als celtherapie in deze studie humane dentale pulp stamcellen (hDPSCs), die gemakkelijk en veilig te verkrijgen zijn. Deze studie veronderstelt dat als nieuwe behandelingsstrategie voor hoofd en hals plaveiselcelcarcinoom, hDPSCs toegepast kunnen worden om een angiostatisch chemokine te leveren aan de tumor. Daarom is onze doelstelling het effect van niet-gemodificeerde hDPSCs op tumorgroei na te gaan en om tevens te onderzoeken of hDPSCs kunnen migreren naar tumorcellen in vitro.

**Materiaal & methoden:** Het effect van hDPSCs op de tumorgroei werd geëvalueerd in een hoofd en hals plaveiselcelcarcinoom xenograft muis model. 1x10⁶ squameuze carcinoma cellen werden subcutaan geïnjecteerd in de dorsale flank van immunodeficiënte muizen, twee tumoren per muis. Tien dagen later, ontving een van de tumoren een intratumorale injectie van 1x10⁶ hDPSCs, terwijl de andere tumor een controle injectie van medium kreeg. Twee keer per week werd het tumorvolume gemeten (n=14). Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) en histologische analyses werden uitgevoerd om de angiogenese en agressiviteit van de controle and hDPSC-geïnjecteerde tumoren te evalueren (n=5). Om de migratie-eigenschappen van hDPSCs te onderzoeken, werd een transwell migratie assay gebruikt, waarbij migratie naar tumorcellen (n=5) en verschillende factoren werd getest (n=6).

**Resultaten:** Gedurende 24 dagen, kon er geen significant verschil in tumorvolume tussen de hDPSC-geïnjecteerde en controle tumoren waargenomen worden. RT-qPCR data onthulden dat de hDPSC injectie geen stijging veroorzaakte in het RNA niveau van EMT-specifieke genen, wat merkers zijn voor tumor agressiviteit. Tevens toonden zowel RT-qPCR als immunohistochemie (IHC) aan dat er geen verschil was in bloedvat-oppervlakte in de hDPSC-geïnjecteerde tumoren ten opzichte van controle tumoren. Tevens toonden wij aan dat in vitro, hDPSCs in staat zijn om te migreren naar tumorcellen.

**Discussie & conclusies:** De capaciteit van hDPSCs om gebruikt te worden binnen het gebied van celtherapie gecombineerd met gentherapie is nog niet eerder onderzocht. Onze data leveren het bewijs voor de toepassing van hDPSCs in cel- en gentherapie aangezien ze de tumorgroei in vivo niet bevorderen en dus veilig gebruikt kunnen worden. Daarnaast werd er voor het eerst bewezen dat hDPSCs in staat zijn om te migreren naar tumorcellen in vitro. Bijgevolg, bevordert dit onderzoek de kennis over het gebruik van hDPSCs als celtherapie bij kanker.
1. Introduction

1.1. Head and neck squamous cell carcinoma (HNSCC)

Ninety percent of cancers identified to be head and neck cancers mainly initiate from alterations in cell growth and replication of squamous cells. Therefore, these carcinomas are termed as **head and neck squamous cell carcinomas** (HNSCCs) (2, 3). Worldwide, HNSCC is the sixth most common cancer and among the eight most common causes of cancer mortality (4). It occurs mostly in patients over the age of 40 and among males (2, 5, 6). It is hypothesized that the main risk factors for evolving HNSCC are tobacco and alcohol use (2, 6). In addition, infection with certain viruses, especially with human papillomavirus (HPV), is a risk factor for developing HNSCC in the tonsils and the tongue. Clinically, these HPV-positive HNSCCs shape a separate cluster from the HPV-negative carcinomas. Furthermore, the expression of these HPV viral oncogenes seems to contribute to the progression of these cancers. Yet, besides altering the expression levels of several onco- and suppressor genes, the function of the virus assimilation needs still to be discovered (4). Other risk factors include the use of several nutrients (e.g. chewing betel), a poor oral health and job-related exposures (e.g. exposure to metal dust) (2, 6, 7).

![Figure 1: The head and neck cancer region (A) and clinical and histological progression of HNSCCs (B). HNSCCs originate from abnormal cell growth of squamous cells within the head and neck region, including the oral cavity, the (para)nasal cavity, the pharynx, the tongue and the larynx. Figure adapted from the National Cancer Institute (cancer.gov) and Pai et al. (2009) (1). HNSCC: Head and neck squamous cell carcinoma.](image)

HNSCCs can arise in different cavities of the head and neck region such as the oral cavity, the oropharynx, the (para)nasal cavity, nasopharynx, (hypo)pharynx and the larynx (Fig. 1). Consequently, these patients may present a variety of clinical manifestations. The most common symptoms are pain in the mouth, nasal obstruction, a persistent sore throat, painful swallowing and breathing, a hoarse voice, or swelling of lymph nodes (2, 6). HNSCCs are diagnosed with the use of histological analyses of taken biopsies. The degree of progression is further evaluated with the use of laboratory analyses and imaging, including computed tomography (CT), magnetic resonance imaging (MRI) and panendoscopy, to determine whether the cancer has spread,
providing information about the patient’s prognosis (2, 3, 6). HNSCC treatment strategies are comparable to other forms of cancer. Classic therapies often include surgery, radiotherapy, chemotherapy, or a combination (2, 8). However, their implementation is problematical because of the region of the tumor. For instance, radiation induces damage to neighboring tissues such as the aero-digestive tract (9). Furthermore, HNSCC patients experience a lot of tension and fear during treatment beyond their side effects caused by the treatment itself (10-12). The prognosis for patients with an early stage cancer is still the best. For these patients, a good quality of life can be well maintained (2, 13). In contrast, lots of patients are diagnosed in an advanced stage of cancer, which reduces the possibility of adequate and accessible treatment options (2). For these patients, treatment leads to a poor outcome and surgical resection is often associated with a lot of side effects, including loss of voice, problems with swallowing or eating, or aesthetic problems, which often needs reconstructive surgery (2, 13, 14). Therefore, despite advances in treatments and technologies, survival rate of patients with HNSCC nowadays has still remained low, increasing the need for a new therapy strategy.

1.2. Angiogenesis

Angiogenesis, which is the formation of new blood vessels from pre-existing blood vessels, is a well-orchestrated process regulated by several factors (15, 16). First, numerous angiogenic chemokines (e.g. vascular endothelial growth factor (VEGF), Angiopoietin (Ang)-2 and other chemokines) are produced by angiogenic signals. These factors are known to activate endothelial cells (ECs) in quiescent pre-existing blood vessels. Next, these cells start to detach in response to integrins and proteases, which degrade the basement membrane of the blood vessel allowing ECs to migrate. Subsequently, they move towards the angiogenic stimulus, which is coordinated by a ‘tip’ EC and supported by an extracellular matrix (ECM) scaffold. The adjacent cells, known as ‘stalk cells’, proliferate and mediate the elongation of the forming vessel. Next, myeloid bridge cells support the merging of two ‘tip’ ECs, forming a lumen in order to permit blood flow. Finally, newly formed vessels become supported and covered by pericytes (15, 16).

Normal angiogenesis is indispensable for the support of all organs (15, 16). However, cancer and several other diseases are able to induce an unbalanced and pathological blood vessel formation pattern (17). Angiogenesis is well-coordinated by an equilibrium of angiogenic activators and inhibitors. Generally, blood vessels are quiescent or dormant, however, when this balance is disturbed by an increased production of angiogenic actors, the balance is shifted towards blood vessel development (16). Tumor cells, for instance, are able to induce angiogenesis via the secretion of these angiogenic actors. In addition, angiogenesis is essential for tumor cells to migrate and to form metastases (15, 17). Therefore, targeting angiogenic processes is a promising tactic in order to slow tumor progression. Indeed, over the past decade, these angiogenic processes and anti-angiogenic therapies in cancer treatment have been extensively studied (17). Currently, numerous anti-angiogenic treatment strategies have been introduced into the clinic with moderate anti-tumor success. Several research groups address the blockage of the major angiogenic factor, which is VEGF. However, tumors are reported to adapt to these anti-angiogenic therapies by modifying towards a more aggressive status (15, 18). In addition, recent data indicate that angiogenesis is not only controlled by genetic and molecular signals, but also by
metabolic activities (19). Schoors et al. reported that ECs generate adenosine triphosphate (ATP) via the glycolysis and that the glycolytic regulator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) controls angiogenesis. Furthermore, blockage of PFKFB3 through 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) in vivo impaired pathological angiogenesis (20, 21). Therefore, the inhibition of blood vessel formation by blocking different metabolic or molecular pathways, besides the inhibition of VEGF, might thus be a promising therapeutic method (15, 18, 20). The ECM, for instance, plays an important role during angiogenic processes by providing a surface between vessel cells and adjacent tissues (15). During tumor growth and tumor invasion, the degradation of the ECM plays a crucial role in order to allow tumor cells to migrate and invade (17). Thrombospondin-1 (TSP-1), which is a glycoprotein, exerts its strong intrinsic anti-angiogenic features through different mechanisms (22-24). It is reported that TSP-1 influences the ECM function (22). Furthermore, it also has an effect EC functions and it deactivates VEGF (16, 22, 23). In addition, genetic alterations in tumor cells are often linked with a lower expression of TSP-1 (22). Other angiogenic regulating pathways could be achieved through chemokines. These molecules are generally recognized to contribute to leukocyte attraction. However, besides their immunological function, they seem to play a role in tumor development. They regulate angiogenesis, attract pro- or anti-angiogenic leukocytes and activate G-protein-coupled chemokine receptors (GPCRs) (25). Stromal cell-derived factor 1α (SDF-1α), for instance, is a pro-angiogenic chemokine (15, 25). Another chemokine regulating angiogenesis is the angiotropic chemokine platelet factor-4 variant (PF-4var). Vanderappellen et al. suggested that this factor might exert beneficial influence on cancer treatment. PF-4var is the non-allelic variant gene of platelet factor-4 (PF-4) and differs only in three amino acids from its antecedent. PF-4 is a chemokine, produced by platelets during platelet aggregation and belongs to the CXC chemokine family (25, 26). Struyf et al. established in vitro that PF-4var has stronger anti-angiogenic effects on ECs than PF-4. In vivo, the same chemokine seems to inhibit more substantially the angiogenesis in the rat cornea micropocket assay (27). The same research group described that these angiostatic activities are achieved by the binding of PF-4var on the CXC chemokine receptor CXCR3 (27). Recently, Prats et al. described that PF-4var and fibstatin can work synergistically in order to tackle the ability of a tumor to stimulate angiogenesis and to spread throughout the body (28). The delivery of combined anti-angiogenic molecules was selected because of the properties of the different molecules to address blood vessel formation through different molecular pathways (28). In conclusion, in order to target angiogenesis through different signaling pathways, TSP-1 or PF-4var seem to be very promising anti-angiogenic agents. Targeting the regulation of ECs and the ECM through TSP-1 could provide promising results within the field of cancer treatment, while PF-4var has shown to have auspicious anti-angiogenic features.

1.3. Pathological angiogenesis and anti-angiogenic therapy in HNSCC

For HNSCC, histological data propose a crucial role of angiogenesis in tumor progression (29). In these carcinomas, a high expression of numerous angiogenic actors, including VEGF, can be found. Furthermore, studies have proven that HNSCCs overexpressing VEGF are considered to be in a later and more aggressive stage of the disease (29-31). Hasina et al. proved the presence of two
separate pathways by which HNSCCs are able to promote angiogenesis. It was observed by gene expression and immunohistochemical analyses that normal tissue samples did not or little express the following factors VEGF, interleukin (IL)-8, fibroblast growth factor (FGF)-2, and hepatocyte growth factor (HGF), while these angiogenic actors were higher expressed in pathological HNSCC samples. Furthermore, it was observed that tumors with a high expression of VEGF and FGF-2 shaped a clinically distinct group of tumors with a higher microvessel mass compared to tumors expressing low levels of VEGF and FGF-2 and higher levels of IL-8 and HGF. (30). Furthermore, recently, it has been reported that the inhibition of epidermal growth factor (EGF) receptor (EGFR) reduces angiogenesis via hypoxia-inducible factor-1α (HIF-1α) and Notch1 in HNSCC mouse models. This suggests that the inhibition of the HNSCC angiogenesis through the blockage of HIF-1α and Notch1 might be favorable (32). Accordingly, these findings demonstrate the presence of various angiogenic actors in HNSCCs, stressing the need for targeting different molecular pathways in order to inhibit tumor growth and blood vessel formation.

1.4. Mesenchymal stem cells (MSCs) and human dental pulp stem cells (hDPSCs)

A stem cell is an undifferentiated cell that has the ability to differentiate into other cell types, is able to self-renew and to regenerate certain tissues in vivo. Various stem cell populations can be found in tissues of the human body at distinctive developing phases: totipotent, pluripotent, multipotent and unipotent stem cells. Totipotent stem cells originate from a fertilized egg and are able to differentiate into all tissues. Pluripotent stem cells are derived from the inner cell mass of a blastocyst and are characterized by a differentiation capacity towards all embryonic tissues. Multipotent stem cells are also labeled as adult stem cells and can be found in different adult organs. These stem cells are known to differentiate into organ-specific cells and are considered to be responsible for the natural turnover of organs. Finally, unipotent stem cells are considered to be able to differentiate into one particular cell type (33).

Mesenchymal stem cells (MSCs) are adult stromal stem cells that are characterized by a plastic-adherence, trilineage differentiation capacity and expression of certain markers such as CD105, CD90 and CD73 and the lack of CD45, CD14, CD34, CD11b CD79a or CD19 (34). Besides the trilineage differentiation potential towards chondrocytes, adipocytes and osteoblasts, MSCs are reported to be able to transdifferentiate into other atypical cell types, such as towards skeletal muscle cells or epithelial cells (33, 35, 36). Furthermore, they possess immune-suppressive characteristics making them attractive contenders for clinical applications within the field of regenerative medicine or inflammatory diseases (37). Gronthos et al. revealed for the first time the existence of human dental pulp stem cells (hDPSCs), located within the human tooth, specifically, within the dental pulp (38). These cells originate from the neural-crest derived mesenchyme and possess, besides their MSC-like characteristics, greater features than MSCs, including a better proliferation rate and differentiation capacity (39-41). hDPSCs are stated to be able to form a dentin-pulp-like complex containing odontoblast cells in vivo when preserved in a supporting scaffold. This suggests that these stem cells could have the potential to contribute within the field of engineered dental tissues (40, 41). In addition, our research group demonstrated strong pro-angiogenic effects of hDPSCs in vitro and in vivo, which insinuates that
hDPSCs could provide to the revascularization of regenerated dental tissues (40, 41). Furthermore, they are able to differentiate into several other cell types; hDPSCs differentiate into adipocytes, myocytes, osteocytes, chondrocytes and cells from the nervous system (39, 41, 42).

1.5. Mesenchymal stem cells and cell-based therapy in cancer

Nowadays, several anti-cancer and anti-angiogenic therapies have been approved for the clinic with moderate anti-tumor activities (17). Nonetheless, because of a short half-life of anti-angiogenic agents, a continued or frequent dosing is needed. Furthermore, a long-lasting administration of anti-angiogenic agents is reported to be toxic to patients (24, 43). Hence, directed and specific delivery of anti-cancer proteins is one of the most auspicious approaches in cancer therapy (44). One way to deliver a therapeutic gene to a tumor is by the use of gene therapy using viral vectors containing the therapeutic gene. Frequently, studies focusing on gene therapy use direct transfection of tumor cells with viral vectors in order to deliver the therapeutic gene (37, 45). However, several drawbacks are associated with this approach such as the possibility to elicit an immunological reaction. Furthermore, the use of these viral vectors for cancer gene therapy is hampered by a low tumor-specific homing ability, stressing the need for a direct intra-tumoral delivery (34, 37). Over the past decade, several types of stem cells such as neural stem cells (NSCs) and MSCs have been reported to be innately attracted by tumors (24, 46). This tumor-tropism has been exploited in order to carry and deliver a therapeutic gene over an ample distance to the tumor (37). Furthermore, stem cells have been extensively used in cell-based therapy because of several other benefits; they are easy to proliferate in vitro, possess ability to self-renew and can efficiently be transfected (37, 43). Therefore, the use of a stem cell-based therapy in which these cells function as vehicles to locally produce an anti-angiogenic protein has arisen (24, 44, 47-50). Several studies have been reported using NSCs as cell vehicles in order to treat glioblastomas because of their tumor-tropism (24, 46). However, NSCs originate from fetal tissues or the adult brain and their isolation is therefore associated with ethical concerns and uncertainty regarding their potential to become teratomas and elicit an immunologic reaction (33, 46, 51). This stresses the need for a different cell-based therapy using cells with a more available accessibility and with the possibility for an autologous use excluding an allogeneic immune reaction. MSCs, in contrast, are immune-privileged cells and a more accessible stem cell population, which offers more possibilities within the field of cell-based research and clinical applications (37, 46). However, their therapeutic use is troubled by the invasiveness of the isolation procedure (40, 41).

1.5.1. Mesenchymal stem cells are able to migrate towards tumors

There is increasing recognition that bone-marrow mesenchymal stem cells (BM-MSCs) possess an intrinsic feature to specifically migrate towards a tumor environment. This migrating capacity has been proved in numerous studies using xenograft models of cancers (34, 44, 52). Tumors can be considered as injuries causing constant inflammation and hypoxia (53). Since MSCs migrate towards injured and inflamed areas, it is assumed that these cells migrate towards tumors according to the same mechanisms (53). The list of molecules and signaling pathways managing
MSCs mobilization towards tumors is still expanding. MSCs have been reported to migrate through FGF-2 and VEGF in vitro. Furthermore, in a tumor environment, it is stated that MSCs migrate according to 1L-6, IL-8, EGF and monocyte chemoattractant protein-1 (MCP-1) (50, 54, 55). Previous studies have shown a role for SDF-1α in MSC homing (50, 53, 56). Finally, platelet-derived growth factor (PDGF) and matrix metalloproteinases (MMP)-8 also play a role in MSC migration towards glioblastoma in vitro and in vivo (47, 49, 50). Non-bone marrow derived MSCs have also been reported to migrate towards tumors such as umbilical cord-derived MSCs and adipose tissue derived MSCs (48, 51, 53).

1.5.2. The controversial role of mesenchymal stem cells in tumor development

Until now, the role of MSCs in tumors is rather uncertain and controversial. Some researchers describe that MSCs stimulate tumor growth, development and metastasis through different mechanisms (48, 57-59). On the other hand, others report that MSCs exert inhibitory effects on tumor growth (48, 53, 58, 60). However, these publications describing a tumor-suppressive role for MSCs are in the minority compared to articles describing a role of MSCs in tumor development. MSCs are suggested to increase tumor growth evidenced by several theories. (i) It is reported that MSCs interact with cancer cells by increasing their proliferation through the secretion of several molecules. It is postulated that these secreted factors have a paracrine effect by acting on cancer cells thus promoting tumor development (53, 61, 62). (ii) MSCs stimulate vascularization via different mechanisms. They secrete several angiogenic factors such as VEGF and EGF and can differentiate towards endothelial cells (53, 55, 57, 63). (iii) MSCs can differentiate into stromal cells, called the tumor-associated fibroblasts (TAF), which support the tumor stromal environment (64, 65). In fact, developing tumors are able to recruit numerous cells towards their environment, enlarging their stromal mass. This recruitment is organized via several secreted factors produced by the tumor itself. This stromal environment contains numerous cells originating from the mesenchymal germ layer such as fibroblasts and MSCs (53, 66, 67). (iv) Other research groups described that MSCs are able to induce chemoresistance by protecting the tumor environment (48). (v) MSCs are also known to be immune-modulatory by exerting different effects on immune cells. However, the mechanisms by which they are able to have an immune-protective role are partly described. First, they inhibit proliferation and maturation of several immune cells, and induce T cell differentiation towards regulatory T cells (Treg cells) (68, 69). In addition, MSCs are reported to secrete several immune-suppressive cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and nitric oxide (NO) (70). (vi) It is stated that MSCs stimulate the metastatic capacity of tumor cells via the inducement of epithelial-mesenchymal transition (EMT) (48, 53, 58, 59, 71, 72). On the other hand, some research groups described that MSCs have tumor-suppressive roles. It was demonstrated that when co-culturing human MSCs with breast cancer cells, a reduction in several proteins, which are important for the survival of cells, could be detected (73). Ohlsson et al. described a tumor-suppressive role of MSCs by observing pro-inflammatory effects caused by the MSCs (74). Finally, others reasoned that the tumor-suppressive effects are caused by the secretion of several other factors or an altered cell-cycle progression (75).
1.5.3. Engineered mesenchymal stem cells in tumor treatment

Recent studies have revealed that stem cell-based therapies assure great effects in the treatment of various human diseases. Because of the tumor-trophic properties of MSCs, numerous research groups have adopted MSCs to produce several anti-cancer drugs to inhibit tumor progression (34, 44, 52, 76). Ghaedi et al. transfected MSCs with α1-anti-trypsine (AAT) in order to locally deliver the anti-angiogenic protein. They showed an ample production of AAT with cytotoxic effects on human umbilical vein endothelial cells (HUVECs) in vitro (43). Pessina et al. modified MSCs to carry the chemotherapeutic drug Paclitaxel (PTX). The results showed that after co-injection of tumor cells and MSCs-PTX, the MSCs delivered PTX at the tumor site and postponed tumor development. In addition, tumor growth was reduced (77). Other research groups transduced MSCs to produce cytotoxic chemokines and showed prominent anti-tumor effects and increased survival in vivo compared to controls (Fig. 2) (24, 44, 46).

Figure 2: Mesenchymal stem cell-based therapies in order to treat tumors. Several studies have proven that stem cell-based therapies have great anti-tumor effects. Because of the tumor-homing ability of MSCs, numerous research groups have engineered MSCs to deliver numerous anti-angiogenic, anti-proliferative and cytotoxic genes to the tumor to inhibit tumor growth. aaTSP-1: Thrombospondin; MMP-2: Matrix metalloproteinase 2; MSCs: Mesenchymal stem cells; NK: Natural killer cell; TNF: Tumor necrosis factor. Figure adapted from Shah et al. (2012) (76).
However, even though MSCs have been widely used within the field of stem cell-based therapy, their therapeutic application is counterbalanced by the painful and invasive isolation method, which increases the need for a different adult stem cell source. Therefore, we would like to use hDPSCs as a cell-based therapy, which are easy and safe to obtain from extracted human third molars (40, 41).

1.6. Research aim and experimental set-up

As mentioned before, we would like to use hDPSCs as a stem cell-based therapy. During this project, we aim to apply engineered hDPSCs to reduce HNSCC growth by inhibiting angiogenesis via the on-site production of different angiostatic chemokines (Fig. 3). We first explore if hDPSCs are able to migrate towards the HNSCC by studying their migratory capacities in vitro. We also characterize the factors responsible for the migration towards the HNSCC. Secondly, we investigate the effect of unmodified hDPSCs on the HNSCC tumor growth. The present study will shed light on the development and possible use of a hDPSCs-based therapy in cancer.

**Figure 3: Global presentation of the study’s hypothesis.** We hypothesize that as a new treatment strategy for HNSCCs, which are often inoperable because of the anatomic region, hDPSCs can be applied as vehicles to deliver an angiostatic chemokine. Therefore, our first objective is to study the effect of unmodified hDPSCs on tumor growth in vivo and whether hDPSCs are capable of migrating towards tumor cells in vitro. BLI: Bioluminescence imaging; hDPSCs: Human dental pulp stem cells; HNSCC: Head and neck squamous cell carcinoma; PF-4var-Fluc-hDPSCs: Platelet factor-4 variant producing hDPSCs.
2. Materials and methods

2.1. Isolation and culturing of hDPSCs
Human third molars were obtained with written informed consent from patients (15–20 years of age) who were receiving extraction procedure for orthodontic or therapeutic reasons at Ziekenhuis Oost-Limburg, Genk, Belgium. Written informed consent of minor patients was acquired via their custodians. The study was accepted by the medical ethical committee of Hasselt University. After extraction of teeth, dental pulp was attained after mechanically fracturing every tooth. hDPSCs were isolated from the pulp tissue through the explant method described by Huang et al. (78). The explant method involves fragmentizing the dental pulp tissue into little pieces and culture them in a 6-well plate in standard hDPSC culture medium, and maintain them at 37°C, 5% CO₂. Every two days, the culture medium was replaced. hDPSC standard culture medium is Minimal Essential Medium, alpha modification (αMEM, Sigma Aldrich, St-Louis, United states of America) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Erembodegem, Belgium), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich). When cells were confluent, the cells were trypsinized with 0.05% trypsin/EDTA (Invitrogen life technologies, Thermo Fisher Scientific) and sub-cultured for additional experiments. hDPSCs were tested for several MSC markers such CD29, CD44, CD90, CD105 and CD146 by means of flow cytometry.

2.2. Culturing of tumor cells
The FaDu cell line, a human squamous cell carcinoma cell line, was purchased from the American Type Culture Collection (LGC Standards GmbH, Wesel, Germany). Tumor cells, the SH-SY5Y cells (neuroblastoma cell line, Sigma Aldrich) and the FaDu cells were both grown in Dulbecco’s Modified Eagle Medium (DMEM, Sigma Aldrich) supplemented with 10% FBS (Gibco) and 100 U/ml penicillin (Sigma Aldrich).

2.3. Generation of conditioned medium (CM) of tumor cells
Conditioned medium (CM) of tumor cells was prepared by seeding FaDu and SH-SY5Y cells at a density of 6 666.67 cells/cm² in 75 cm² cell culture flasks. After the cells were allowed to adhere during the night, cells were rinsed with phosphate buffered saline (PBS) (Lonza, Basel, Switzerland) and incubated with 10 ml of αMEM containing 0% FBS. After 48 hours, the medium was collected, centrifuged and stored at -80°C.

2.4. Flow cytometry
Flow cytometric analyses were performed on FaDu cells in order to characterize them and to determine surface markers to distinguish them from hDPSCs (Table 1). FaDu were trypsinized and 50 000 cells/well were seeded in a 96-well fluorescence-activated cell sorting (FACS) plate. Cells were washed twice in FACS buffer. Hereafter, cells were left for 30 minutes at room temperature in order to guarantee that the receptors on cells regain their extracellular expression. Next, cells were incubated with 1:100 primary antibodies for 45 minutes at room temperature. An unstained control sample and isotype controls for the labeled primary antibodies were also used (Table 1).
Subsequently, labeled cells were washed twice and analyzed with the flow cytometer FACSCalibur (Becton, Dickinson and Company (BD), Erembodegem, Belgium) for 5,000 events. Measurements were analyzed with BD CellQuest Pro™ Software (BD).

Table 1: Overview of the antibodies used for flow cytometry and immunocytochemical analyses with their corresponding isotype control, company and dilution.

<table>
<thead>
<tr>
<th>Antigens (Flow cytometry)</th>
<th>Isotype control</th>
<th>Label</th>
<th>Company</th>
<th>Dilution</th>
<th>Antigens (ICC)</th>
<th>Host</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24</td>
<td>IgG1</td>
<td>PE</td>
<td>eBioscience</td>
<td>1:100</td>
<td>IL-8</td>
<td>Mouse</td>
<td>R&amp;D Systems</td>
<td>1:100</td>
</tr>
<tr>
<td>CD29</td>
<td>IgG1</td>
<td>FITC</td>
<td>(12-0247-41)</td>
<td>1:100</td>
<td>VEGF</td>
<td>Mouse</td>
<td>R&amp;D Systems</td>
<td>1:100</td>
</tr>
<tr>
<td>CD31</td>
<td>IgG1</td>
<td>PE</td>
<td>Immunotools (21270314)</td>
<td>1:100</td>
<td>FGF-2</td>
<td>Mouse</td>
<td>Sigma-Aldrich</td>
<td>1:100</td>
</tr>
<tr>
<td>CD34</td>
<td>IgG1</td>
<td>PE</td>
<td>Immunotools (21270344)</td>
<td>1:100</td>
<td>MCP-1</td>
<td>Mouse</td>
<td>R&amp;D Systems</td>
<td>1:100</td>
</tr>
<tr>
<td>CD44</td>
<td>IgG2</td>
<td>PE</td>
<td>Immunotools (21270444)</td>
<td>1:100</td>
<td>IGFBP-3</td>
<td>Mouse</td>
<td>R&amp;D Systems</td>
<td>1:50</td>
</tr>
<tr>
<td>CD45</td>
<td>IgG1</td>
<td>PE</td>
<td>(12-0459-41)</td>
<td>1:100</td>
<td>PLGF</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td>CD54</td>
<td>IgG2</td>
<td>PE</td>
<td>Immunotools (21279544)</td>
<td>1:100</td>
<td>CXCR3</td>
<td>Mouse</td>
<td>R&amp;D Systems</td>
<td>1:50</td>
</tr>
<tr>
<td>CD73</td>
<td>IgG1</td>
<td>FITC</td>
<td>(11-0739-41)</td>
<td>1:100</td>
<td>PF-4var</td>
<td>Rabbit</td>
<td>Thermofisher</td>
<td>1:100</td>
</tr>
<tr>
<td>CD80</td>
<td>IgG1</td>
<td>PE</td>
<td>(12-0809-41)</td>
<td>1:100</td>
<td>PFKFB3</td>
<td>Rabbit</td>
<td>Thermofisher</td>
<td>1:100</td>
</tr>
<tr>
<td>CD86</td>
<td>IgG2</td>
<td>PE</td>
<td>(12-0869-41)</td>
<td>1:100</td>
<td>CXCR4</td>
<td>Mouse</td>
<td>R&amp;D Systems</td>
<td>1:100</td>
</tr>
<tr>
<td>CD90</td>
<td>IgG1</td>
<td>FITC</td>
<td>(11-0909-42)</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD104</td>
<td>IgG2</td>
<td>FITC</td>
<td>BioLegend</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD105</td>
<td>IgG1</td>
<td>PE</td>
<td>(12-1057-41)</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD117</td>
<td>IgG1</td>
<td>PE</td>
<td>(12-11178)</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD184</td>
<td>IgG2</td>
<td>PE</td>
<td>(12-9999-41)</td>
<td>1:50</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antigens (Immunocytochemistry)</th>
<th>Isotype control</th>
<th>Label</th>
<th>Company</th>
<th>Dilution</th>
<th>Antigens (ICC)</th>
<th>Host</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiopoietin 1</td>
<td>IgG2</td>
<td>FITC</td>
<td>BioLegend (327806)</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>(PA5 15456)</td>
<td></td>
<td>Thermofisher</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>(PA5 19472)</td>
<td></td>
<td>Thermofisher</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>(PA5 19476)</td>
<td></td>
<td>Thermofisher</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>(PA5 21944)</td>
<td></td>
<td>Thermofisher</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>(PA5 27984)</td>
<td></td>
<td>Thermofisher</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>(PA5 30546)</td>
<td></td>
<td>Thermofisher</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>(PA5 31266)</td>
<td></td>
<td>Thermofisher</td>
<td>1:100</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:1000</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>(PA5 359903)</td>
<td></td>
<td>BioLegend</td>
<td>1:20</td>
<td>TSP-1</td>
<td>Mouse</td>
<td>Novusbio</td>
<td>1:20</td>
</tr>
</tbody>
</table>

2.5. Immunocytochemistry (ICC)

Immunocytochemistry (ICC) on hDPSC was performed in order to investigate the expression of anti-angiogenic chemokines, PF-4var, and the expression of CXCR4 and PFKFB3 (Table 1). FaDu cells were stained for angiogenic factors and the PF-4var receptor CXCR3 (Table 1). The antibodies used for angiogenic factors were directed against FGF-2, IGFBP-3, IL-8, VEGF, MCP-1, Placental growth factor (PLGF) and TSP-1. Cells were seeded on a coverslip and fixed with 4% paraformaldehyde (PFA) for 20 minutes. Hereafter cells were washed with PBS. If necessary, cell permeabilization was performed with 0.05% Triton (Sigma Aldrich) (in PBS) during 30 minutes at 4°C. Next, the cells were blocked for 20 minutes at room temperature with serum-free protein block (Dako, Everlee, Belgium). Thereafter, primary antibodies were added in different dilutions (Table 1) for 1 hour at room temperature. Excess of antibodies was washed away three times for 5
minutes each, before incubation for 30 minutes at room temperature with the appropriate secondary antibody (donkey anti-mouse Alexa Fluor 555 (A21422), donkey anti-rabbit Alexa Fluor 488 (A21206) or donkey anti-mouse Alexa Fluor 488 (A21202) 1:500, Invitrogen life technologies). A blanco control staining for each antibody was performed by omitting the primary antibody. Then, the slides were incubated with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Thermo Fisher Scientific) for 10 minutes to stain the nuclei of cells. Finally, Dako fluorescent mounting medium (Dako) was used to mount cells. Cells were visualized with the Nikon Eclipse 80i fluorescent microscope (Nikon, Tokyo, Japan) connected to a PC computer. Images of cells were taken with a DS-2M-BW-C 2 megapixel camera and analyzed using NIS elements viewer (both Nikon).

2.6. Migration assays

2.6.1. Transwell migration assay

In vitro, the transwell migration assay is an appropriate method in order to evaluate the migration of cells from the upper compartment according to a chemotactic gradient towards a chemotactic factor in the lower compartment. Here, we performed this assay to define whether hDPSCs are able to migrate towards tumor cells and to elucidate which factors play a crucial role during the possible homing process. During this experiment, translucent ThinCert™ tissue culture inserts (Greiner bio-one, Vilvoorde, Belgium) with a pore size of 8 µm were used. First, wells and inserts were coated with Poly-L-Lysine (PLL) (Sigma Aldrich) at a concentration of 0.01 mg/ml for 1.5 hour at room temperature. hDPSCs of different patients with a cell density of 50 000 cells were applied on inserts, while the chemotactic agents were added in the lower compartment. The chemoattractant agents beneath the wells were: FaDu and SH-SY5Y CM (see section 2.3), FaDu and SH-SY5Y cells at a density of 13 157 cells/cm² and 26 315 cells/cm² in 500 µl αMEM 0% FBS in a 24 well plate (25 000 and 50 000 cells per well), VEGF, EGF, FGF-2, SDF-1α, IL-8, IL-6, Insulin growth factor-1 (IGF-1) and MCP-1 (100 ng/ml) (all ImmunoTools, Friesoythe, Germany). All factors were diluted in αMEM medium with 0%. Negative and positive controls were also included as a control, which were respectively αMEM medium with 0% and 10% FBS. Cells which were migrated towards the bottom side of the inserts were fixed with 4% PFA during 20 minutes and stained with 0.1% crystal violet for 10 minutes. Two representative light microscopic pictures of migrated hDPSCs were made with the Nikon eclipse TS100 inverted microscope (Nikon) with the camera Jenoptik ProgRes C3 (Jenoptik, Jena, Germany).

Otherwise, inserts were put in Non-enzymatic Cell Dissociation Solution 1x (Sigma Aldrich) in order to detach migrating cells. Next, viable migrating cells were labeled by calcein acetoxymethyl ester (AM) (BD) at a concentration of 1.67 mM. Living cells take up the non-fluorescent calcein AM and convert it towards green-fluorescent calcein. Fluorescent intensity of cells was measured with the FLUOstar Omega (BMG LABTECH, Ortenberg, Germany). Numbers of cells were calculated according to a calibration curve. This assay was performed on seven donors.
2.6.2. Wound healing assay
By a wound healing assay, also referred to as the scratch assay, the directional cell migration *in vitro* is assessed. During this experiment, the directional migration of hDPSCs in response to SDF-1α and CM of FaDu cells was verified. At starting point, the Ibidi culture inserts (Ibidi, Martinsried, Germany) were put in a 24-well plate. hDPSCs were seeded at a cell density of 40 000 cells at both sides of the culture insert in standard culture medium. Cells were allowed to adhere overnight. When cells were 90% confluent, the Ibidi culture inserts were removed and cells were washed with PBS. Next, αMEM containing 0% FBS (negative control situation), CM of FaDu cells and SDF-1α (100 ng/ml in αMEM containing 0% FBS) (ImmunoTools) were added for 24 hours to each well. At starting point, after 6 hours and after 24 hours, the migration of the cells was evaluated by taking three representative pictures with the Nikon eclipse TS100 inverted microscope (Nikon) with the camera Jenoptik ProgRes C3 (Jenoptik). Migration was quantified using Image J (The National Institute of Health, Maryland, USA) by measuring the width of the scratch between two monolayers of hDPSCs in each condition and at every time point. Finally, cells were fixed with 4% PFA for 20 minutes and stored at 4°C. This assay was performed on six donors.

2.7. Cell proliferation assay
The effect of the chemotactic factors mentioned in section 2.6 on hDPSC proliferation was measured using the 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. hDPSCs of different patients were seeded in a 96-well plate at a density of 294 cells/mm² (10 000 cells per well) in a 96 well plate in standard hDPSCs culture medium. After 24 hours, cells were washed twice with PBS and incubated with the appropriate conditions (see section 2.6). αMEM containing 10% FBS or 0% FBS was used as positive or negative control respectively. After 24 and 48 hours, the media from the cells were removed and 500 µg/ml MTT (Sigma Aldrich) was added to each well in standard hDPSC culture medium and incubated for 4 hours at 37°C. Then, the MTT solution was removed and a mixed solution of 0.01 M glycine and DMSO (Sigma Aldrich) was added to each well in order to induce reduction to formazan. The absorbance was measured at a wavelength of 570 nm with the iMark Microplate Reader (Bio-rad Laboratories, Temse, Belgium). This assay was performed on five donors.

2.8. *In vivo* effect of hDPSCs on HNSCC growth
To evaluate the effects of hDPSCs *in vivo* on tumor pathogenesis, direct intratumoral injection of hDPSCs was performed. FaDu cells (1.10⁶ cells in 50 µl DMEM 0% and 50 µl matrigel (BD)) were subcutaneously injected in each side of the back of nude mice. After 10 days, when tumors where well established, hDPSCs (1.10⁶ cells in 50 µl αMEM 0%) were injected directly into one of the tumors. Three donors were used for this experiment. For each mouse, a hDPSCs injected tumor was located on one side of the mouse’s back while the control tumor, receiving a control medium injection, was on the opposite side. Tumors were routinely measured two times a week with a caliper. The tumor volume was calculated using the following formula: 0.5 x (width (mm))^2 x length (mm). Finally, mice were sacrificed and tumors were dissected for *in vitro* analyses.
2.9. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Tumor tissues were frozen in liquid nitrogen and shredded. For the cell cultures, hDPSCs and FaDu cells were trypsinized, washed with PBS and a cell pellet was made of both cell types. Total RNA was extracted from the tumor tissues, hDPSC and FaDu pellet with the RNeasy Plus Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer’s guidelines. 500 ng of total RNA was used to synthesize cDNA in a total reaction mix of 20 µl using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, USA) following the manufacturer’s guidelines. Next, reverse transcription polymerase chain reaction (RT-PCR) was performed according to the reaction mixture and protocol listed in table 2 (Applied Biosystems, Carlsbad, CA, USA). All primers were supplied by Eurogentec S.A. (Seraing, Belgium) and are listed in table 3. For every gene, a non-template control was used. Data were analyzed with the StepOne Software (Applied Biosystems, version 2.3).

Table 2: Composition of qPCR reaction mix and applied program

<table>
<thead>
<tr>
<th>qPCR mix</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µl FAST Sybr Green Master Mix (Applied Biosystems)</td>
<td>Initial denaturation</td>
<td>95.0°C</td>
<td>20 sec</td>
<td>Step and hold</td>
</tr>
<tr>
<td>0.3 µl Forward primer (10 µM)</td>
<td>Denaturation</td>
<td>95.0°C</td>
<td>3 sec</td>
<td>40</td>
</tr>
<tr>
<td>0.3 µl Reverse primer (10 µM)</td>
<td>Annealing</td>
<td>60.0°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>2.4 µl MilliQ</td>
<td>Elongation</td>
<td>95.0°C</td>
<td>15 sec</td>
<td>1</td>
</tr>
<tr>
<td>2 µl cDNA (1/10 diluted in RNase-free water)</td>
<td>Final elongation</td>
<td>60.0°C</td>
<td>60 sec</td>
<td>1</td>
</tr>
<tr>
<td>Total 10µl reaction volume</td>
<td></td>
<td>95.0°C (+0.3°C)</td>
<td>15 sec</td>
<td>Step and hold</td>
</tr>
</tbody>
</table>

Table 3: Primer sequences for RT-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human TWIST Forward</td>
<td>CAT-CCT-CAC-ACC-TCT-GCA-TTC-T</td>
</tr>
<tr>
<td>Human TWIST Reverse</td>
<td>ACT-ATG-GTT-TTG-CAG-GCC-AGT-T</td>
</tr>
<tr>
<td>Human Snail 2 Forward</td>
<td>CTT-TTT-CTT-GCC-CTC-ACT-AC</td>
</tr>
<tr>
<td>Human Snail 2 Reverse</td>
<td>GCT-TCG-GAG-TGA-AGA-AAT-GC</td>
</tr>
<tr>
<td>Human α-sma Forward</td>
<td>GCA-CCC-CTG-AAC-CCC-AAG-GC</td>
</tr>
<tr>
<td>Human α-sma Reverse</td>
<td>GCA-CGA-TGC-CAG-TTG-TGC-GT</td>
</tr>
<tr>
<td>Mouse CD31 Forward</td>
<td>GAC-TCA-CCG-TGG-TGC-TCT-ATG-C</td>
</tr>
<tr>
<td>Mouse CD31 Reverse</td>
<td>TCA-GTT-GCT-GCC-CAT-TCA-TCA</td>
</tr>
<tr>
<td>Mouse VEGF Forward</td>
<td>CTC-CAG-GGC-TTC-ATC-GTA</td>
</tr>
<tr>
<td>Mouse VEGF Reverse</td>
<td>CAG-AAG-GAG-AGC-AGT-CC</td>
</tr>
<tr>
<td>Housekeeping genes</td>
<td>Sequence</td>
</tr>
<tr>
<td>Human RPL13a Forward</td>
<td>AAG-TTG-AAG-TAC-CTG-GCT-TTC</td>
</tr>
<tr>
<td>Human RPL13a Reverse</td>
<td>GCC-GTC-AAA-CAA-CCT-GAG-AC</td>
</tr>
<tr>
<td>Human Cyc A Forward</td>
<td>AGA-CTG-AGT-GGT-TGG-ATG-GC</td>
</tr>
<tr>
<td>Human Cyc A Reverse</td>
<td>TCG-AGT-TGT-CAC-GCA-TGA-GC</td>
</tr>
<tr>
<td>Mouse HPRT1 Forward</td>
<td>CTC-ATG-GAC-TGA-TTA-TGG-ACA-GGA-C</td>
</tr>
<tr>
<td>Mouse HPRT1 Reverse</td>
<td>GCA-GGT-CAG-CAA-AGA-ACT-TA-TAG-GC</td>
</tr>
<tr>
<td>Mouse Ywhaz Forward</td>
<td>GCA-ACG-ATG-TAC-TGT-CTC-TTT-TGG</td>
</tr>
<tr>
<td>Mouse Ywhaz reverse</td>
<td>GTC-CAC-AAT-TCC-CTT-CCT-GTC-ATC</td>
</tr>
</tbody>
</table>

A-sma: Alpha-smooth muscle actin; Cyc A: cyclophilin A; HPRT1: Hypoxanthine phosphoribosyltransferase 1; RPL13: Ribosomal protein L13 a; VEGF: Vascular endothelial growth factor; Ywhaz: Yrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein zeta.
2.10. **Immunohistochemistry (IHC) and histology**

Tumor tissues were fixed in 4% PFA. Afterwards, tumors were dehydrated in graded ethanol, embedded in paraffin and sectioned at 7 µm. Sections were first deparaffinized. This was performed by embedding slides for 2 minutes in following order of solutions: xylene, 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol.

3,3’-Diaminobenzidine (DAB) Envision™ Kit (Dako) was used to visualize CD146 positive cells. After rehydration, sections were incubated in 10% antigen retrieval solution (Dako) and heated three times with a microwave at 480W for 5 minutes with breaks of 2 minutes. Next, tissue sections were allowed to cool down for 30 minutes and were incubated with peroxidase block for 20 minutes at room temperature. The microscope slides were washed three times for 5 minutes each time in PBS. Then, they were blocked for 20 minutes at room temperature in serum-free protein block (Dako). Next, slides were washed again and were incubated for 1 hour at room temperature with the primary antibody rabbit anti-human CD146 (1:50) (Abcam ab75769, Cambridge, UK) after blocking. Excess of antibodies was washed away before incubation for 30 minutes at room temperature with the secondary antibody anti-rabbit labeled with horseradish peroxidase (HRP) (Dako). In blanco, the negative control, the primary antibody was omitted. Hereafter, DAB substrate buffer containing DAB chromogen (Dako) was added. This subsequently created a visible brown color. Counterstaining was performed by means of hematoxylin for 8 minutes. Finally, cells were washed with tap water twice for at least 10 minutes. Slides were mounted with Aquatex mounting medium (Merck, Overijse, Belgium).

For hematoxylin and eosin (H&E) staining, slides were counterstained by means of hematoxylin for 8 minutes and washed with tap water. Hereafter, slides were stained with eosin for 3 minutes. After rehydration, DPX mounting medium (Leica Biosystems, Diegem, Belgium) was used to mount tumor slices. Slides were visualized with the Mirax slide scanner (20×/0.8 NA objective) (Carl Zeiss NV-SA, Zaventem, Belgium) using the Mirax scan software. Photos of scanned slides were made with the Mirax viewer (Carl Zeiss NV-SA). Analyses and quantification was performed using the AxioVision software, edition 4.6.3 (Carl Zeiss NV-SA).

2.11. **Statistical analysis**

Statistical analysis was done using GraphPad Prism 5 Software (Graphpad, San Diego, CA). Normality was analyzed using the D’Agostino and Pearson omnibus normality test. Depending on the dataset, analyses were performed with a one way ANOVA followed by a Friedman test Dunn’s Multiple Comparison post-hoc test. This was the case for the transwell migration assay and the MTT assay. A two way repeated measures ANOVA was performed when analyzing the data from the in vivo tumor growth experiment and the wound healing assay. RT-qPCR data and the data of the quantification of the CD146 immunostained areas were analyzed by means of a two-tailed t-test followed by a Wilcoxon signed rank test. Data are represented as mean ± standard error of mean (SEM). A significant difference was specified when the p-value was smaller than 0.05 (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001).
3. Results

3.1. Characterization of a human squamous cell carcinoma cell line called FaDu cells

3.1.1. Expression of various angiogenesis-related factors by FaDu cells

Aggressive tumors are often associated with a high angiogenic potential (17). Therefore, in order to determine the angiogenic capability of FaDu cells, immunohistochemical analyses were performed to determine the expression of several angiogenic factors. In addition, these data could provide information about the production of chemokines or other factors that could imply recruitment of stem cells towards the tumor environment. IL-8, VEGF, FGF-2, MCP-1, IGFBP-3 and PLGF expression were detected on FaDu cells (Fig. 4 A-F). All factors are known to play a role in angiogenesis stimulating processes. On the other hand, FaDu cells also showed expression of TSP-1, which is an angiostatic chemokine (Data not shown).

Figure 4: FaDu cells express angiogenesis-related factors. FaDu cells were seeded at 10 000 cells per well on a coverslip and fixed with 4% PFA. All primary antibodies were diluted 1:100 (except for IGFBP-3 1:50). FaDu cells show expression of IL-8 (A), VEGF (B), FGF-2 (C), MCP-1 (D), IGFBP-3 (E) and PLGF (F). Scale bars = 50 µm. Images were taken at 20x magnification. FaDu: Human squamous cell carcinoma cell line; FGF-2: Fibroblast growth factor 2; IGFBP-3: Insulin-like growth factor-binding protein 3; IL-8: Interleukin 8, MCP-1: Monocyte chemoattractant protein-1; PLGF: Placental growth factor; VEGF: Vascular endothelial growth factor.
3.1.2. FaDu cells antigen expression

Flow cytometric analysis was performed in order to characterize surface antigens on FaDu cells. Results showed a homogenous population of cells according to size and granularity. Approximately 99.4% and 99.5% of FaDu cells proved positive surface expression of CD29 and CD44 respectively. Expression of CD31, CD34, CD45, CD80, CD86, CD90, CD104 and CD117 could not be detected on FaDu cells (Fig. 5).

![Flow cytometric analysis of antigen expression on FaDu cells.](image)

**Figure 5**: Flow cytometric analysis of antigen expression on FaDu cells. FaDu do not express CD31, CD34, CD45, CD80, CD86, CD90, CD104 and CD117. 50 000 cells per well were seeded in a 96-well FACS plate. Cells were incubated with 1:100 primary antibodies (1:50 CD184 and 1:20 CD309) for 45 minutes at room temperature. An unstained control sample and isotype controls for the labeled primary antibodies were also used. FACS: fluorescence-activated cell sorting; FaDu: Human squamous cell carcinoma cell line.
3.2. Migrating abilities of hDPSCs

3.2.1. Evaluation of hDPSC migration towards tumor cells

Previous studies confirmed the ability of MSCs to be able to migrate towards tumor cells by using *in vitro* and *in vivo* migration models. Therefore, we hypothesized that hDPSCs could also be able to migrate towards tumor cells, specifically towards human squamous cancer cells. The transwell migration assay was performed as described in section 2.6. First, the migration potential of cells towards 10% FBS (positive control) was monitored per donor. If hDPSCs of this donor were able to migrate, they were included in this study. In total ten patients were evaluated of which seven were included in this study. Consistent with previous data of MSCs, we found that hDPSCs could migrate towards tumor cells *in vitro* in the transwell migration assay. 5 097 ± 1 051 cells migrated towards the negative control condition, while 9 588 ± 1 429 hDPSCs migrated towards the positive control condition (Fig. 6 A). hDPSCs migrated significantly towards 25 000 and 50 000 FaDu cells (p<0.05), while they migrated not in the same amount towards of 50 000 SH-SY5Y cells. Interestingly, 12 853 ± 3 018 cells migrated towards CM of SH-SY5Y cells, while only 8 578 ± 1 009 hDPSCs migrated towards the CM of FaDu cells. Significant migration was only yielded when hDPSCs migrated towards 25 000 FaDu cells (11 767 ± 3 455) and 50 000 FaDu cells (11 600 ± 2 606). However, a positive trend of migration of hDPSCs towards FaDu CM and SH-SY5Y CM can also be observed (Fig. 6 A).

**Figure 6:** hDPSCs migrate towards 25 000 and 50 000 FaDu cells after 24h of incubation. hDPSC migration in culture inserts towards FaDu and SH-SY5Y cells and CM, standard hDPSC culture medium supplemented with 10% FBS (positive control) or 0% FBS (negative control) was assessed after 24h. A: Quantification of migrated hDPSCs per condition. B: Light microscopic pictures of migrated hDPSCs towards the negative control or the positive control situation (two donors). Data were analyzed with a one way ANOVA followed by a Friedman test Dunn’s Multiple Comparison post-hoc test. Data are represented as mean ± SEM and correspond to n = 5 assays. * = P-value < 0.05. Scale bars = 300 µm. Images were taken at 10x magnification. CM: Conditioned medium; FaDu: Human squamous cell carcinoma cell line; hDPSCs: Human dental pulp stem cells; SH-SY5Y: Neuroblastoma cell line.
3.2.2. Evaluation of several chemotactic factors responsible for the migration of hDPSCs

According to literature, several chemokines and factors, such as SDF-1α, MCP-1 and IL-8 play a role in the promotion of MSC migration towards tumors in vitro and in vivo (50, 54, 55). Furthermore SDF-1α has already been shown to play an important role in hDPSC migration (79, 80). Therefore, recombinant human VEGF, FGF, EGF, SDF-1α, IL-6, IL-8, IGF-1 and MCP-1 at a concentration of 100 ng/ml were added to the negative control medium in order to determine their influence on the migration of hDPSCs (as described in section 2.6). After 24 hours of incubation, there was a significant increase in the migration of hDPSCs towards the positive control condition (p<0.001) (Fig. 7 A). No statistically significant increased migration of hDPSCs towards other factors could be observed after 24 hours of incubation (Fig. 7 A and B). However, a positive trend of migration could be observed towards the SDF-1α condition, in which 6 856 ± 1 132 hDPSCs migrated (Fig. 7 A). Furthermore an increasing trend of migrating hDPSCs could also be observed towards EGF (6 535 ± 495) and FGF-2 (6 286 ± 1 023) (Fig. 7 A). Also a lot of donor variability was observed. In the positive control situation, migrated hDPSCs were separated from the non-migrating hDPSCs and were kept in culture. ICC showed that both populations, migrating and non-migrating hDPSCs, express CXCR4, which is the SDF-1α receptor (Data not shown). Next, a second transwell assay was performed. Quantification of the migration showed an increased homing capacity of the migrating hDPSC subpopulation towards the positive control condition compared to the non-migrating cells (Fig. S2 A and B).

![Figure 7: hDPSCs show a positive trend of migration towards SDF-1α after 24h of incubation. Inserts with hDPSCs were incubated for 24h with the negative control medium supplemented with VEGF, FGF, EGF, SDF-1α, IL-6, IL-8, IGF-1 or MCP-1 at a concentration of 100 ng/ml. A: Quantification of migrated hDPSCs towards VEGF, FGF, EGF, SDF-1α. B: Quantification of migrated hDPSCs towards IL-6, IL-8, IGF-1 and MCP-1. Data were analyzed with a one way ANOVA followed by a Friedman test Dunn’s Multiple Comparison post-hoc test. Data are represented as mean ± SEM and correspond to n = 6 assays (A) and n = 4 assays (B). *** = P-value < 0.001. EGF: Epidermal growth factor; FGF-2: Fibroblast growth factor 2; hDPSCs: Human dental pulp stem cells; IGF-1: Insulin growth factor-1; IL-8: Interleukin 8; IL-6: Interleukin 6; MCP-1: Monocyte chemoattractant protein-1; SDF-1α: Stromal cell-derived factor 1α; VEGF: Vascular endothelial growth factor.](image-url)
3.2.3. The effect of chemotactic factors on hDPSC proliferation

In order to assess whether the chemotactic factors used in the transwell migration assay had an influence on hDPSCs proliferation, an MTT assay was performed. This assay was performed in order to confirm that the seen effect in section 3.2.1 and 3.2.2 was due to migration and not to an induced proliferation. The positive control, which is αMEM containing 10% FBS, showed an increased cell viability compared to the negative control situation after 24 hours and 48 hours of incubation (Fig. 8 A and B). Compared to the negative control condition, the chemotactic factors did not significantly increase proliferation of hDPSCs after 24 hours (Fig. 8 A) and 48 hours (Fig. 8 B) of incubation. The measured absorbance values are comparable with the absorbance value of the negative control condition. After 24 hours, IGF-1 showed an increase in cell viability, however this effect was not statistically significant (Fig. 8 A). The FaDu cells CM, SH-SY5Y cells CM as well as SDF-1α show a trend in decreasing hDPSC viability after 24 and 48 hours of incubation (Fig. 8 A and B). This effect can possibly be explained by the production of several metabolites released by the tumor cells. Overall, the tested conditions did not induce hDPSC proliferation.

**Figure 8: hDPSC proliferation is not increased by the chemotactic factors used in the transwell assay after 24h and 48h of incubation.** hDPSCs were seeded at 10 000 cells per well in a 96 well plate and were incubated for 24h and 48h with VEGF, EGF, FGF-2, SDF-1α, IL-8, IL-6, IGF-1 and MCP-1 (all factors were diluted in αMEM medium with 0% FBS at a concentration of 100 ng/ml), FaDu and SH-SY5Y cells CM or standard hDPSC culture medium supplemented with 0% FBS (negative control) and 10% FBS (positive control). Afterwards an MTT assay was performed. Absorbance was measured at 570 nm. A: MTT assay after 24h of incubation. B: MTT assay after 48h of incubation. Data were analyzed with a one way ANOVA followed by a Friedman test Dunn’s Multiple Comparison post-hoc test. Data are represented as mean ± SEM and correspond to n = 5 assays. CM: Conditioned medium; EGF: Epidermal growth factor; FaDu cells: Human squamous cell carcinoma cell line; FGF-2: Fibroblast growth factor 2; hDPSCs: Human dental pulp stem cells; IGF-1: Insulin growth factor-1; IL-8: Interleukin 8; IL-6: Interleukin 6; MCP-1: Monocyte chemoattractant protein-1; MTT: 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; SDF-1α: Stromal cell-derived factor 1α; SH-SY5Y cells: Neuroblastoma cell line; VEGF: Vascular endothelial growth factor.
3.2.4. Evaluation of the directional migration of hDPSCs in response to FaDu cells CM and SDF-1α

Another way of examining migration is by assessing directional migration, with a wound healing assay. hDPSCs were seeded in two separate chambers, separated by a physical barrier (as described in section 2.6.2). When cells reached confluence, the insert was removed, thereby creating an empty space between two hDPSC monolayers. After 6 hours and 24 hours of incubation with SDF-1α at a concentration of 100 ng/ml or FaDu cells CM, the distance of migrating cells from the two hDPSCs monolayers was measured. There was an increased total directional migration in the negative control condition (172.45 ± 41.70 µm) and the FaDu cells CM situation (158.21 ± 34.03 µm) after 24 hours of incubation (Fig. 9 A). SDF-1α did not increase directional migration compared to the control situation.

Figure 9: The directional migration of hDPSCs is not altered by FaDu cells CM and SDF-1α after 6h and 24h of incubation. A scratch between two monolayers of hDPSCs was incubated with CM of FaDu cells, SDF-1α at 100 ng/ml and standard hDPSC culture medium with 0% FBS (negative control). A: Quantification of the total directional migration of hDPSCs. B: Light microscopic pictures of hDPSCs incubated with negative control, SDF-1α and CM of FaDu cells. Data were analyzed with a two-way repeated measures ANOVA followed by a Bonferroni posttest. Data are represented as mean ± SEM and correspond to n = 6 assays. Scale bars = 500 µm. Images were taken at 4x magnification. CM: Conditioned medium; hDPSCs: Human dental pulp stem cells; FaDu: Human squamous cell carcinoma cell line; SDF-1α: Stromal cell-derived factor 1α.
3.3. The *in vivo* effect of hDPSCs on HNSCC growth

To study the influence of hDPSCs on HNSCC growth *in vivo*, the effect of an intratumoral injection of hDPSCs on tumor growth was studied. First, mice received two injections of $1.10^6$ FaDu cells, giving rise to two tumors at each flank. When tumors were macroscopically detectable, 10 days later, hDPSCs were injected in one of the tumors, intratumorally at a ratio 1:1 in nude mice. Control tumors were injected with αMEM containing 0% FBS. Tumor volume was measured two times a week (Fig. 10 A). hDPSCs do not significantly influence tumor growth *in vivo* under the tested conditions (Fig. 10 B-E). However, a trend in increasing tumor growth can be observed after 24 days in the hDPSC-inoculated tumors. Control tumors were $279.72 \pm 59.23 \, \text{mm}^3$, while hDPSC-inoculated tumors had a volume of $436.11 \pm 72.22 \, \text{mm}^3$. In addition, three different hDPSC donors were used in this experiment. Analyses showed that this effect was not donor-dependent (Fig. 10 D and E).

**Figure 10:** hDPSCs do not influence tumor growth *in vivo* in a HNSCC xenograft mouse model. A: $1.10^6$ FaDu cells were subcutaneously injected in each side of the back of nude mice. After 10 days, $1.10^6$ hDPSCs were injected into one of the two tumors. Tumors were measured two times a week. B: Tumor volume of control tumors and hDPSC-injected tumors. Duration $t = 24$ days, $n = 14$. C: Tumor volume of control tumors and hDPSC-injected tumors. Duration $t = 33$ days, $n = 4$. D and E: Tumor volume of control tumors and hDPSC-injected tumors per donor. Duration $t = 24$ days, $n = 5$ (D) $n = 6$ (E). Data were analyzed with a two-way repeated measures ANOVA. Data are represented as mean $\pm$ SEM. FaDu: Human squamous cell carcinoma cell line; hDPSC: Human dental pulp stem cell; HNSCC: Head and neck squamous cell carcinoma; IHC: Immunohistochemistry; RT-qPCR: Reverse transcriptase quantitative polymerase chain reaction.
3.4. Morphological and pathological properties of control and hDPSC-inoculated tumors

We then evaluated the influence of the hDPSCs on tumor morphology and pathology with the use of histological examinations by H&E staining. The purpose was to visualize viable hDPSCs and morphological differences in tumor pathology. In both control and hDPSC-inoculated tumors, a large necrotic zone enclosed by a collar of viable cells could be observed (Fig. 11). However, on histological level, we were not able to distinguish hDPSCs from FaDu cells.

Figure 11: Influence of hDPSCs on pathological and morphological properties of tumors. Tumor tissues were fixed in 4% PFA and processed for histological analyses with a H&E staining. The necrotic area is indicated by a black arrow. Scale bars = 100 µm. hDPSC: Human dental pulp stem cell; H&E: Hematoxylin and eosin staining.
3.5. The effect of hDPSC inoculation on HNSCC angiogenesis

To determine whether hDPSCs have an effect on tumor angiogenesis, we evaluated the vascularization of the tumors after hDPSC inoculation.

We examined whether the blood vessel area was altered in control tumors and hDPSC-inoculated tumors. Sections of subcutaneous tumors were stained with an antibody directed against CD146 to visualize the blood vessels (Fig. 12 B and C). Total area of immunopositive cells was then quantified. The results of this analysis revealed that vessel area was not increased in tumors injected with hDPSCs (2.20 ± 0.32 %) compared to the control tumors (2.21 ± 0.69 %) (Fig. 12 A). This is in accordance with the RT-qPCR analyses, in which the mRNA expression levels of the endothelial cell marker CD31 and de angiogenic factor VEGF was analyzed. Both mouse CD31 as mouse VEGF mRNA levels in hDPSC-inoculated tumors were not statistically different compared to control tumors (Fig. 12 D and E). These data show that hDPSC inoculation did not alter the angiogenic potential of tumors under the tested circumstances.

**Figure 12:** hDPSCs do not influence the *in vivo* tumor angiogenesis in a HNSCC xenograft mouse model. A: Total area of CD146-positive cells was quantified in tumors. B and C: Representative images of blood vessels after staining with a CD146 antibody and hematoxylin. D and E: RT-qPCR analyses were performed on control and hDPSC-inoculated tumors for the assessment of mRNA expression levels of mouse CD31 (D), mouse VEGF (E). Data were analyzed with a two-tailed t-test followed by a Wilcoxon signed rank test. Data are represented as mean ± SEM and correspond to n = 5. Scale bars = 50 µm and 200 µm (images in black frame). Images were taken at 40x and 10x magnification. hDPSC: Human dental pulp stem cell; HNSCC: Head and neck squamous cell carcinoma; RT-qPCR: Reverse transcriptase quantitative polymerase chain reaction; VEGF: Vascular endothelial growth factor.
3.6. The effect of hDPSC inoculation on the aggressiveness of HNSCC

To determine whether hDPSCs could induce transcriptomic alterations associated with an improved metastatic ability, we evaluated the mRNA expression levels of several EMT-specific markers, which are markers for tumor aggressiveness, in control tumors or tumors after hDPSC inoculation. As determined by RT-qPCR analyses, we found that the mRNA expression levels of human TWIST, Snail 2 and alpha smooth muscle actin (α-SMA) were not statistically altered by the presence of hDPSCs (Fig. 13 A-C). In general, hDPSC inoculation did not increase the tumor’s transcriptomic phenotype of metastatic capacity under the tested conditions.

Figure 13: hDPSCs do not influence mRNA expression levels of tumor EMT-specific markers in vivo in a HNSCC xenograft mouse model. RT-qPCR analysis was performed on control and hDPSC-inoculated tumors for the assessment of mRNA expression levels of human TWIST (A), Snail 2 (B) and α-SMA (C). Data were analyzed with a two-tailed t-test followed by a Wilcoxon signed rank test. Data are represented as mean ± SEM and correspond to n = 5. α-SMA: Alpha smooth muscle actin; hDPS: Human dental pulp stem cell; HNSCC: Head and neck squamous cell carcinoma; RT-qPCR: Reverse transcriptase quantitative polymerase chain reaction.
4. Discussion

Despite improvements in cancer treatment, survival rate of patients with HNSCC has still remained incredibly low. This is often caused by the invasive character, making this type of cancer one of the leading causes of cancer death in the world (2-4). Therefore, there is a high need for new options to treat this type of carcinoma. Our own ICC data support the heterogeneity in expression of angiogenesis-related factors by FaDu cells. We showed expression of IL-8, FGF-2, VEGF, MCP-1 and IGFBP-3, all factors playing an important part within the angiogenesis process (15). Furthermore, according to literature, expression of VEGF, IL-8 and FGF-2 are associated with more pathological HNSCC samples (29, 30). Therefore, we used this squamous cell carcinoma cell line in order to define a new therapy strategy to target the HNSCC angiogenesis.

In the past few years, numerous researchers adopted BM-MSCs to function as vehicles for therapeutic proteins because of their capability of specifically migrating towards tumors (34, 44, 46-54, 56, 76). However, since the isolation of BM-MSCs involves several risks, we used an alternative stem cell source during the present study, namely hDPSCs (40, 41). We demonstrated the migratory behavior of hDPSCs towards FaDu cells. hDPSCs also showed a positive trend of migration towards SH-SY5Y CM and SH-SY5Y cells. Additionally, the migration towards FaDu cells was statistically significant in contrast to FaDu cells CM. This more marked influence of migration towards FaDu cells compared to the CM could possibly be explained by the presence of a medium containing more chemotactic factors. In addition, the CM of FaDu cells is stored at -80°C for an unknown extent of time, which may cause a decrease of chemotactic factors. In addition, 50 000 FaDu cells yielded the same amount of migrating hDPSCs as 25 000 FaDu cells. Possibly, the chemotactic factors produced by 25 000 FaDu cells resulted in saturation of receptors on hDPSCs. With the Boyden chamber assay, we also tried to evaluate the factors involved in the migration of hDPSCs in order to obtain an insight in which proteins secreted by FaDu are able to attract hDPSCs. We showed a positive trend of migration of hDPSCs towards SDF-1α, which is according to the literature (79, 80). The SDF-1α-CXCR4 axis has already been reported to play a role in migration of hematopoietic cells (81). Yang et al. already stated that hDPSCs are able to migrate towards SDF-1α (79). Di Scipio and colleagues saw migration of rat DPSCs towards injured cardiomyocytes according to SDF-1α (80). However, the involvement of SDF-1α in the migration of hDPSCs towards tumor cells needs still to be evidenced. Therefore, the blockage of CXCR4 by the use of antagonists, such as AMD3100, or the knockdown of the CXCR4 gene with the use of small interfering RNAs (siRNAs) could be performed in the future. Song et al. showed that CM of tumor cells could boost the expression of CXCR4 on MSCs. Yet, AMD3100 was not reported to be able to inhibit all MSC migration towards human prostate and breast cancer cells. This indicates the involvement of other factors besides SDF-1α in MSC migration (56). In addition, data from our own research group also indicated expression of CXCR4 on hDPSCs. Additional chemokines and receptors could also be involved in the migrating mechanisms of hDPSCs. Chemokines are known to play a role in migration of several cell types, such as leukocytes and hematopoietic stem cells (HSC) (81, 82). Several researchers already studied in a limited manner the expression of chemokines and chemokine receptors on DPSCs (83). However, analyses of a wide group of chemokines and receptors on hDPSCs should be performed in the first place (84). Though, the expression of these chemokines can vary due to practical lab conditions such as culturing
methods, isolation, and passage number (85). In addition to chemokines, growth factors and cytokines could also influence the migratory capacity of hDPSCs. Besides SDF-1α, we also demonstrate a positive trend of migration towards EGF and FGF-2, however, this migration was not statistically significant. A possible explanation can be found in the transwell assay methodology. Since the transwell assay system allows diffusion of fluids, the concentration of factors could be disturbed (86). IL-6, IGF-1, IL-8, PDGF-BB, FGF-2, EGF, VEGF and MCP-1 have been reported to play a role in migration of MSCs towards tumors (34, 44, 47-50, 53-55, 76, 87). In our case, migration towards EGF, FGF-2, VEGF, IGF-1, MCP-1, IL-8 and IL-6 was not statistically significant. No migration towards VEGF was detected, which is in accordance to findings of Di Scipio et al. (49). In contrast, Schichor et al. saw migration of MSCs through VEGF towards gliomas (54). Furthermore, Marchionni et al. showed expression of VEGF receptors (VEGFRs) on hDPSCs, which should indicate that hDPSCs should react in response to VEGF (88). In addition, migration of hDPSCs towards tumor cells should also involve invasion of tissues, thereby making MMPs possible interesting chemotactic factors. Indeed, Ho et al. showed the importance of MMP-1 in the migration of BM-MSCs towards human gliomas (50).

In the transwell system, we seeded 50 000 hDPSCs, from which a mean of 11 767 hDPSCs migrated across the membrane towards FaDu cells. This made us wonder whether there would be a superior migrating subpopulation within the hDPSCs. In order to target a tumor mass while reducing possible homing of hDPSCs towards other organs, the revelation of a better migrating subpopulation would serve in a favorable manner. Bolontrade et al. proved the presence of a population of MSCs with superior migration abilities towards CM of several tumor cell lines (89). This subpopulation of MSCs showed increased expression of integrins α2, α3 and α5 (89). Granulocyte-colony stimulating factor (G-CSF)-isolated hDPSC population also shows increased CXCR4 expression, CD105 and G-CSF receptor (90). Consequently, we performed a transwell assay and after 24 hours of incubation, we separated the migrating hDPSCs from the non-migrating cells. ICC analyses show that both populations express CXCR4. However quantification was not performed. In addition, the migrating sub-population showed an increased homing towards the positive control condition. However, this was only performed on one patient and should be repeated. Therefore, future experiments will be performed in which hDPSC subpopulations will be investigated with regard to an increased adhesiveness, characterized by altered integrin expression levels, and a superior migration towards CM of tumor cells. Furthermore, proteomic analyses or RNA sequencing can be performed on both subpopulations in order to reveal two distinct populations. The existence of a better migrating hDPSC subpopulation could additionally explain the donor-to-donor variability seen in the present study, explaining why several donors migrate better than others. Here, the screening of a varied set of chemokine receptors or integrins in migrating and non-migrating donors will be interesting as well. The identification of a possible subpopulation with superior migrating properties would first of all explain the donor variability and secondly would increase the migrating potential in vivo and the therapeutic efficacy as well.

Another question that arose was which signaling events are exploited by migrating hDPSCs. We show expression of PFKFB3 in hDPSCs. Carmeliet et al. showed that this glycolytic protein is involved in the energy fuel of endothelial cells (18, 20, 21). Therefore, one could consider whether
hDPSCs use the glycolysis during migration. In future, migration abilities after PFKFB3 gene knockdown or blockage can be performed to provide information about regulatory mechanisms in hDPSC migration. In addition, during the present study, we also evaluated whether migrating hDPSCs produced NO (data not shown). With the use of the Griess assay, we observed that hDPSCs do not produce NO when migrating or the NO levels lie under the detection limit. For MSCs, the regulatory pathways of migration are mainly undetermined. Accumulating evidences suggest that ATP influences MSC function. In addition, there are reports indicating that this ATP can induce a Ca²⁺ response in MSCs and that these Ca²⁺ channels play a role in MSC proliferation, differentiation and migration (91, 92). Tang et al. proved that Ca²⁺ channels, protein kinase C (PKC) and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) pathways are involved in MSC migration stimulated by acetylcholine (93). In addition, Ferrari et al. proved that MSC migration towards SDF-1α could be stimulated by ATP (94). Recently, Peng et al. showed that ATP concentrations influence Ca²⁺ signaling in hDPSCs migration through purinergic stimuli (95).

A second method of examining migration is by observing directional migration with the wound healing assay. In the wound healing assay a scratch is generated in a monolayer of cells, in which cells within this scratch are considered to be migrating cells. However, the thickness of the scratch can vary and be problematic when reproducibility is involved. Therefore we used a wound healing assay involving a physical barrier, in which cells grow around this physical barrier and can easily be repeated (96, 97). First, we tried to evaluate migration of hDPSCs towards a monolayer of FaDu cells. This experimental setting was hampered by an increased motility of FaDu cells compared to hDPSCs. This disallowed us to study migration of hDPSCs towards tumor cells. In a second experimental set-up, we used two monolayers of hDPSCs and evaluated directional migration of hDPSCs in response to SDF-1α or the CM of FaDu cells at different time points. Our data showed that the directional migration was not altered when cultured with the tested conditions compared to the negative control situation. A possible explanation could be that SDF-1α or the FaDu CM influences the expression of several integrins and receptors on hDPSCs, influencing the adhesiveness of cells. In addition, one could discuss whether the seen effect is caused by an increased proliferation, which should be remedied by the use of Mitomycin C. We also performed an MTT assay in order to evaluate cell proliferation when hDPSCs were cultured with the chemotactic factors used in the transwell assay, but no positive effect of SDF-1α of FaDu CM on proliferation could be observed. This shows that the increased number of migrating cells in the transwell assay can be completely attributed to chemotactic activities of SDF-1α or FaDu cells and not to any induction of cell proliferation.

The present study shows possible migrating capacities of hDPSCs in vitro. In the near future, in vivo migration experiments are planned. The hDPSCs that are injected are transduced with lentiviral particles containing the Firefly luciferase gene (Fluc). After the hDPSC injection, BLI is used to visualize the biodistribution of the cells. This allows us to visualize and follow independently the fate and location of the possible migrated hDPSCs and tumor in vivo. Another issue that needs to be solved is the way of hDPSC administration. Our first in vivo migration experiment is a pilot study with three experimental groups. The first group receives intravenous injection of hDPSCs. The second group receives subcutaneous injection and the last group receives
intraperitoneal injection of Fluc-hDPSCs. This permits us to select the way of administration for our next in vivo migration experiment.

Since MSCs are able to home towards carcinomas, many researchers proposed the application of stem cells for combining cell therapy with gene delivery (24, 43, 44, 46, 77). However, this led to an increased curiosity in order to speculate the contribution of unmodified stem cells on the tumor progression, especially their effect on angiogenesis and tumor aggressiveness, in order to evaluate the biosafety of these cells.

From our tumor growth experiment in vivo, we observed that hDPSC inoculation did not influence tumor growth. Though, several studies investigating the role of MSCs in tumor progression have led to a lot of incongruity on this specific topic as insinuated by Klopp et al (58). Numerous mismatches in terms of procedures, practice and theory can be stated among these studies. In several studies, a certain number of MSCs has been injected into a vast number of tumor cells. Yet, Studeny et al. and Keramidas et al. previously proved that MSCs only proliferate when cultured with tumor cells (98, 99). Therefore, the tumor-promoting roles of MSCs could be caused by an amplified amount of cells. During the present study, tumor volume was measured with a caliper. When implicating this tumor measurement method, one should interpreter the measured volume with caution, since the relative amount of tumor cells over hDPSCs is not known. In future, this can be excluded by various imaging methods. HDPSC survival, incorporation and fate can be visualized for instance by labeling them prior to transplantation with iron oxide nanoparticles or both, hDPSCs and FaDu cells, can be visualized by dual-luciferase reporter assay, where hDPSCs and FaDu cells are transfected with a different type of luciferase, recognizing different substrates and emitting light of a different wavelength (24, 98, 100). Other researchers proposed that the promoting roles of MSCs could be ascribed to their capacity to change into tumor cells (101). However, impurity of stem cells with tumor cell lines had been reported there (102). On the other hand, studies described that MSCs support tumor growth via their immune-modulatory actions (37, 48, 53, 70). Specifically, MSCs are reported to regulate differentiation of T-cells towards Treg cells, thereby contributing to the protection of the tumor (53, 68, 69). However, the influence of hDPSCs on the immune system and tumor immunology has not been investigated yet. In addition, the influence of hDPSCs on the tumor immunology could not be investigated in our model as we used athymic nude mice. In contrast, several other research groups proved that MSCs would be able to suppress tumor growth in vitro and in vivo. These observations were mainly attributed to the capacity of MSCs to alter the cell cycle progression of tumor cells (75).

Besides the observation that hDPSCs do not influence tumor growth in vivo, we also observed that there was no significant increased blood vessel formation as determined by a quantification of immunostained areas with the endothelial cell marker CD146 and by RT-qPCR for mouse CD31 and VEGF. In contrast to our findings, MSCs have been reported to support blood vessel formation in tumors by differentiating towards endothelial cells or secreting several angiogenic actors (55, 63, 103). In addition, our research group previously documented that hDPSCs have strong angiogenic properties by inducing migration of human microvascular endothelial cells (HMEC-1) and by the secretion of VEGF and MCP-1 (40, 41). In addition, in the chick chorioallantoic membrane (CAM) assay, hDPSCs were able to induce angiogenesis in a substantial manner (41). Furthermore, other
researchers also showed that hDPSCs have angiogenic effects in vivo mediated via paracrine actions (104). Therefore, one could expect an increase in blood vessel formation in the hDPSC-inoculated tumors. This was not the case in our model. One possible explanation might be the already profound induced angiogenesis of the FaDu cells, disallowing hDPSCs to exceed this blood vessel formation. Therefore, at the time point of the hDPSC injection, the tumor angiogenesis was already established and thus unchangeable. Another reason might be the paracrine actions of hDPSCs, thereby influencing the angiogenic effect of the tumor not receiving a hDPSC injection. In future, these drawbacks can be excluded by our tumor model, duration of inoculation or by the timing of the hDPSC injection. For MSCs, a decrease in blood vessels in MSC-inoculated tumors was shown by Keramidas et al. However, they showed an increase in vessels length (99). Lee et al. proved that MSCs secrete exosomes which reduced the angiogenesis in breast cancer by inhibiting the secretion of VEGF (105). However, in contrast to these data, Zhu et al. proved that the exosomes produced by MSCs enhance VEGF production (106). Furthermore, Otsu and colleagues proved that MSCs caused EC death when there is a 1:1 or 1:3 MSC to EC ratio (107). However, further experiments should be performed in order to evaluate the secretome of these hDPSCs co-cultured with FaDu cells.

Tumors are not only composed by abnormally growing cells, but also by TAFs, which are components of the stromal area of a tumor (53, 67). TAFs are often reported to have a mesenchymal origin (58). MSCs have been described to express TAF markers. This suggests that they could differentiate towards TAFs and stimulate tumor progression (58, 64, 65). However, we did not look for TAF differentiation of hDPSCs within tumors. RT-qPCR analyses showed no difference in mRNA expression levels of α-sma, which is a marker for TAFs, in hDPSC-inoculated tumors. Even so, as mentioned earlier, it is reported that these TAFs stimulate cancer progression. This seems to be managed by EMT (58). We observed that EMT-specific markers, TWIST and Snail 2 were not altered in tumors injected with hDPSCs. These findings are in contrast to numerous researchers who claim that MSCs allow an increase in expression of EMT-specific markers of tumors in vitro and in vivo (59, 71, 72). However, preliminary data from our research group showed that there was no difference in EMT markers when squamous cell carcinoma (SCC) cells were cultured for three weeks with hDPSC CM or in the same well as hDPSCs in an insert (data not shown). However, only secretion of paracrine factors was allowed to happen in those circumstances since hDPSCs and tumor cells were not in contact to each other. A lot of researchers describe increased tumor cells aggressiveness when co-cultured with MSCs (59, 71). Yet, these effects were all mediated by cell-cell contact. Lis et al. proved that when culturing MSCs with cancer cells there was a shift in increased gene expression of a metastatic tumor phenotype (71). Martin et al. also analyzed expression of numerous genes related to a more aggressive tumor phenotype. They showed increased levels of EMT-specific markers including TWIST and Snail after MSC inoculation in tumors (59). In addition, angiogenic factors, such as PDGF or EGF are reported to be EMT inducers (72). hDPSCs seem not to increase angiogenesis in hDPSC-inoculated tumors under the tested circumstances, which can explain the EMT data. Furthermore, as mentioned before, the increase in EMT induced by MSCs seems to be cell-contact regulated. In the present study, we dissolved our tumor cells in matrigel, which could play a role in decreasing contact between hDPSCs and FaDu cells. Nevertheless, understanding the conditions in which MSCs
promote or suppress tumor growth are important in order to use these cells for gene delivery in tumor treatment. Zhu and colleagues evaluated whether MSCs isolated from adults stimulated more tumor progression compared to fetal derived MSCs (108). In the present study, we used hDPSCs from a vast group of adolescents between the age of 15 and 20 years old. The conditions in which MSCs are cultured could also alter the behavior of MSCs (85). In addition, the amount of inoculated cells seems also to play an important role in this inconsistency of MSCs in tumor progression. We used a 1:1 proportion to reduce confusion regarding this topic (58). Besides the amount of cells, the way of inoculation could also participate in this inconsistency. Furthermore, a lot of studies co-inject tumor cells with MSCs, we, in contrast, chose to inject our hDPSCs after the tumor was established. Finally, donor variability in MSCs has also been reported to be important, because of genetic or epigenetic reasons (58). However, in the present study, we used three different donors and saw no donor-to-donor differences. Finally, the duration of the study and the fact that two tumors per mouse were used, each mouse being his own control, may have the potential to become a criticism. Therefore, future experiments are planned in which mice only receive one tumor and thus two separate experimental groups are used, namely a control group and a hDPSCs-inoculated group.

The next phase in order to prove our hypothesis would be to verify if hDPSCs could be engineered to have an anti-angiogenic effect. We aim to genetically modify hDPSCs to produce the angiostatic chemokine PF-4var and assess its anti-angiogenic effect in vitro and in ovo. We already investigated whether hDPSCs show expression of anti-angiogenic proteins. ICC data proved that hDPSCs already express angiostatic factors, such as PF-4var. However, overexpression of angiostatic proteins will shift the angiogenic balance towards an anti-angiogenic situation. Furthermore, we evaluated the presence of the PF-4var receptor on FaDu cells. We observed expression of CXCR3 on FaDu cells as determined by ICC (Data not shown). Consequently, we continue to verify the possible anti-angiogenic effects of PF-4var-producing hDPSCs in the HNSCC mouse model.
5. Conclusion and synthesis

HNSCC is one of the most common types of cancer worldwide and affects thousands of people every year. Nowadays, the survival rates for HNSCC patients are still incredibly low, with increasing health care costs. In general, cancer treatment aims for the elimination of all cancer cells within the body through surgery or the administration of chemotherapeutic agents. For HNSCCs, plastic reconstruction is frequently indispensable after tumor surgical resection. In addition, the administration of cytotoxic agents can often be unfavorable because of a reduced specificity to target the tumor. Therefore, there is an increasing need for targeted and effective cancer treatment. Cell-based therapy could admittedly be a promising adjuvant approach in order to do so. To date, several researchers are devoting their study to the use of different stem cell sources as delivery vehicles for anti-cancer agents. In the past few years, there are scientific evidences that MSCs are capable of specifically migrating towards tumors. However, since the isolation of BM-MSCs involves several risks, we used an alternative stem cell source during the present study, namely the hDPSCs. We are the first research group exploiting hDPSCs as on-site producers of a novel anti-cancer drug to inhibit the HNSCC angiogenesis. In this way, engineered hDPSCs could contribute to cancer therapy by obstructing tumor growth and by reducing the exposure to radiotherapy and chemotherapeutics. The present study focuses on two parts: we first aimed to evaluate the migratory capacities of hDPSCs in vitro. Secondly we evaluated the effect of unmodified hDPSCs on HNSCC in vivo.

For the first phase of the study, we proved the migratory behavior of hDPSCs towards tumor cells. We are the first research group to demonstrate that hDPSCs are able to migrate towards tumor cells, namely FaDu cells, in vitro. In the future, signaling pathways responsible for the migratory behavior towards a tumor environment need to be characterized, both in vitro and in vivo. In addition, hDPSCs also seem to migrate in a positive trend towards CM of SH-SY5Y cells, which is a neuroblastoma cell line, suggesting that further research within the area of using hDPSCs as vehicles for anti-cancer drugs towards other types of cancer should be taken into account. Our data suggest that hDPSCs might migrate towards tumor cells in vivo, which needs to be studied in future.

In a second phase, we observed that hDPSCs did not stimulate the in vivo tumor growth of HNSCCs under the tested conditions. In addition, this effect was not donor-dependent. Moreover, hDPSC treatment did not stimulate tumor angiogenesis or aggressiveness as determined by RT-qPCR analyses and immunohistochemistry. These findings suggest that hDPSCs can be safely used as a cell-based therapy in cancer treatment.

In conclusion, we show that hDPSCs are able to migrate towards cancer cells in vitro and that inoculation of hDPSCs in tumors did not have an effect on tumor growth, angiogenesis and EMT-specific markers expression. However, more profound research on the specific effects of hDPSCs on tumor growth needs still to be performed together with the in vivo evaluation of hDPSC migration with the use of BLI. Nevertheless, the present study can already form a good starting point for the strategy of a hDPSC-based therapy combined with gene delivery in cancer.
6. References


82. Ji JF, He BP, Dheen ST, Tay SS. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem Cells. 2004;22(3):415-27.


Supplemental information

S1 hDPSCs express the angiostatic chemokine, Platelet factor-4 variant (PF-4var), and a glycolytic regulator protein

Data from our own research group already proved the expression of pro-angiogenic factors by hDPSCs. We show the expression of an anti-angiogenic chemokine, PF-4var, as determined by ICC (Fig. S1 A). In addition, the expression of the glycolytic regulator, PFKFB3, on hDPSCs was also demonstrated (Fig. S1 B).

Figure S1: hDPSCs express anti-angiogenic factor PF-4var and the glycolytic regulator PFKFB3. hDPSCs were seeded on a coverslip and fixed with 4% PFA. Primary antibodies were diluted 1:100. hDPSCs show expression of PF-4var (A) and PFKFB3 (B) Scale bars = 50 µM. Images were taken at 20x magnification. hDPSCs: Human dental pulp stem cells; PF-4var: Platelet factor-4 variant; PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3.
S2 Migrating hDPSCs consist of a better migrating subpopulation compared to the non-migrating hDPSCs

50,000 hDPSCs were applied on inserts and migration towards the positive and negative control was tested. After 24 hours of incubation, the migrating hDPSCs were separated from the non-migrating cells with the use of trypsin/EDTA and were cultured. ICC demonstrated that both populations, migrating and non-migrating hDPSCs, express CXCR4 (Data not shown). A second transwell assay was incubated in which migration of the migrating hDPSCs and non-migrating hDPSCs was tested and quantified (Fig. S2 A and B). Quantification showed an increased migration of the migrating population towards the positive control situation (Fig. S2 B). These data could imply that the migrating cells consist of a better-migrating subpopulation. However, this experimental set-up should be repeated since this has only been performed on one patient.

Figure S2: Migrating hDPSCs could shape a subpopulation with superior migrating capacities within the hDPSC population. A: Light microscopic pictures of migrated hDPSCs towards the negative control or the positive control situation. B: Quantification of the migration of non-migrating and migrating hDPSCs. Data correspond to n = 1 assay. Scale bars = 500 µm. Images were taken at 4x magnification. hDPSCs: Human dental pulp stem cells.
Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:

The use of dental pulp stem cells as a cell-based therapy in head and neck squamous cell carcinoma

Richting: master in de biomedische wetenschappen-klinische moleculaire wetenschappen
Jaar: 2016

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identifieren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Lo Monaco, Melissa

Datum: 8/06/2016