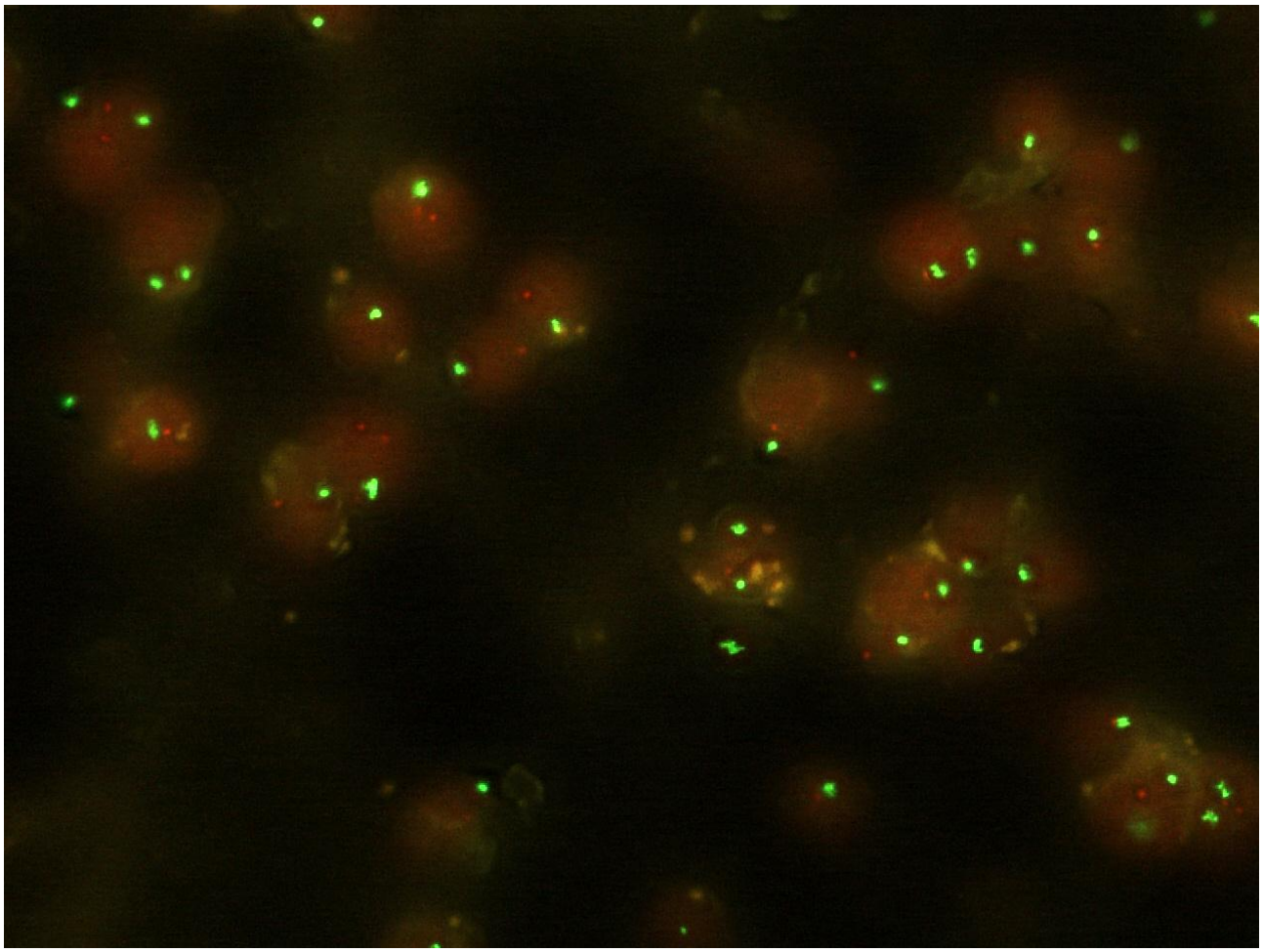
The Significance of Mesenchymal Stem cells for the Tumor Formation and Progression in Glioblastoma Multiforme



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Abstract:

Glioblastoma multiforme is the most aggressive type of all brain cancers. After diagnosis patients have a mean survival of 12-15 months. Mesenchymal stem cells are believed to play a role in the tumor development, among others by formation of tumor vessels, formation of cancer stem cell niches, promotion of invasive nature and fusion or transdifferentiation into malignant cells.

This project revolves around the pathology of glioblastoma multiforme, including cancer stem cells and the significance of mesenchymal stem cells in the tumor formation and progression. A protocol for in situ hybridization to a xenograft model of glioblastoma multiforme was set up and adipose derived stem cells were systemically injected into the model to determine the fate of the cells after systemic injection. Expression of CD105 was selected as a label for the adipose derived stem cells.

Results revealed successful hybridizations of human specific probes to tumor xenografts of the experimental mice. The adipose derived stem cells were not detectable upon immunohistochemical analysis for the selected marker, and it was concluded that labeling the cells before injection is more appropriate when tracing cells in vivo.

Preface

This master thesis is the result of the 9th and 10th semesters on the biomedicine track on Medicine with industrial specialization. The subjects on the semesters were optional, as to why the project was chosen to revolve around the significance of mesenchymal stem cells for the tumor formation and progression in glioblastoma multiforme tumor development.

The report is organized in three parts: A theoretical part that gives an insight into the present knowledge on glioblastoma multiforme pathology, including cancer stem cells in those tumors and the role of mesenchymal stem cells in this. Thereafter an experimental part, containing an experiment that served to clarify the fate of systemically injected mesenchymal stem cells in an in vivo model of glioblastoma multiforme on the basis of their expression of a certain marker. Furthermore, a protocol for in situ hybridization to the tumor xenograft was developed. Finally the report contains a discussion addressing the experimental findings together with conclusions and perspectives.

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Introduction

Glioblastoma multiforme is the most aggressive type of all brain cancers and accounts for 20 % of all intracranial tumors (Hou, et al., 2006). The tumor constitutes the worst stage of all astrocytomas and can arise in two ways; by transformation of astrocytes into glioblastoma multiforme cancer cells de novo or by upstaging a lower stage astrocytoma (Dimov, et al., 2011). Regardless of the development of the tumor, the prognosis of glioblastoma multiforme is very poor, after diagnosis patients have a mean survival of 12-15 months (Dimov, et al., 2011). This poor prognosis is due to a lack of curative treatments and a practical inevitable relapse of the disease (Hou, et al., 2006).

The treatment of brain cancers is complicated by poor accessibility to the tumor and risk of damaging the sensitive tissue irreversibly (McCance & Huether, 2005). If the tumor is accessible, surgery is used in attempt to cure, otherwise this method can be used to relieve the pressure created by the growth of the tumor (McCance & Huether, 2005). Additionally, standard treatment is chemo- and radiotherapy but often this only have palliative effects as glioblastoma multiforme is resistant in many cases (Hou, et al., 2006). Regrowth of the tumor occurs in more than 90 % of patients and the new tumor arises within only a few centimeters from the border of the old tumor (Hou, et al., 2006).

Currently, it is believed that the progression and regrowth of the glioblastoma multiforme tumors is caused by a rare population of highly malignant cells within the tumor.
The cells of glioblastoma multiforme is regarded a heterogeneous population that can be divided into groups with an internal hierarchy depending on the differentiation of the cells. It is believed that growth of the tumor is maintained by only a few highly malignant cells that make up the top of that hierarchy (Rahman, et al., 2011). This rare population of cancer cells has some stem cell like properties and the cells are therefore denoted cancer stem cells (Tan, et al., 2006). According to the cancer stem cell hypothesis the properties include ability of self-renewal and multi-lineage differentiation potential. Moreover, the cancer stem cells express markers similar to the markers expressed by normal stem cells (Altaner, 2008).

It therefore appears that it could be of great importance to identify the mechanisms of the development and maintenance of these very malignant cells. In the past few years, different studies have revealed an association between *mesenchymal stem cells* and these mechanisms. Furthermore, the mesenchymal stem cells have been suggested to play a role in a range of other tumor promoting mechanisms such as formation of blood vessels in the tumor, tumor invasiveness and others (Ramasamy, et al., 2007).

Overall Objectives and Aims

The purpose of this report is to gain an insight into some of the different theories about contributions of mesenchymal stem cells to the tumor formation in glioblastoma multiforme. This is done through a description of the pathologic features of glioblastoma multiforme, including cancer stem cells and a subsequent description of roles of mesenchymal stem cells in the tumor microenvironment. Furthermore an experiment was set up with the purpose to

- Evaluate to what extent adipose derived stem cells established by Laboratory of Stem Cell Research at Aalborg University were able to migrate to an in vivo model of glioblastoma multiforme.

- Set up a protocol for in situ hybridization to the in vivo model of glioblastoma multiforme.

Glioblastoma multiforme

In general, a brain tumor can be defined as a bulk of cells with neoplastic growth within the brain or spinal canal. The brain tumors can arise as either primary or metastatic tumors, however, while 25 % of patients with primary cancer outside the CNS develop secondary intracranial tumors, the primary brain tumors only rarely metastasize outside the CNS (al., 2009) (McCance & Huether, 2005). The different types of brain tumors can be categorized by the site or cell type of which they originate. For example, tumors arising in glial cells can be oligodendrogliomas or astrocytomas. Figure 1 shows some of the common sites and types of intracranial tumors (McCance & Huether, 2005).

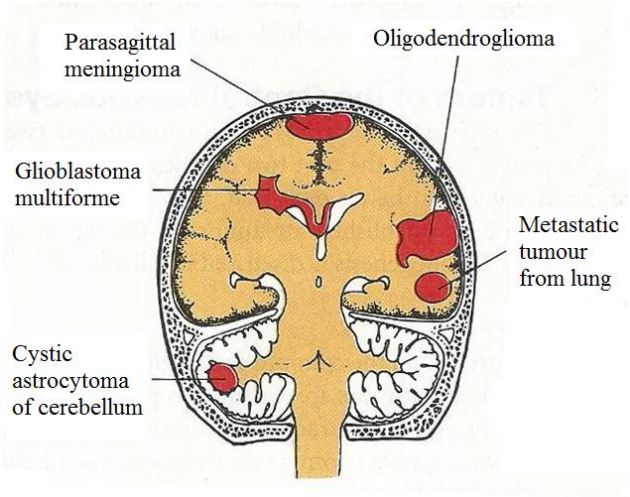

Figure 1. Common sites and types of some intracranial tumors. The examples are a parasagittal meningioma, an oligodendroglioma, a metastatic tumor from lung, a cystic astrocytoma of cerebellum and glioblastoma multiforme. Modified from (McCance & Huether, 2005).

Of all brain cancers one subtype of the astrocytomas, glioblastoma multiforme, denotes itself as the by far most malignant type of all brain cancers, with an extensive invasiveness of the tumor and a very high mortality (McCance & Huether, 2005). This tumor most often arises in the temporal lobe, the parietal lobe, the frontal lobe and the occipital lobe and often spreads into the ventricles (Ohgaki & Kleihues, 2005). Furthermore, the tumor often invades along tracts of the white matter and along the basal lamina of the blood vessels of the brain (Van Meir, et al., 2010). The mean survival after diagnosis is 12-15 months though 50 % of the patients have died already 6 months after diagnosis (al., 2009). Tumors of the central and peripheral nervous system only represent 1.3 % of all cancer diagnoses but 2.5 % of all cancer deaths are the result of these tumors. Thus the mortality is fairly high despite the relatively low incidence (Pecorino, 2008).
The World Health Organization, WHO, has set up a brain cancer staging system that is regarded the standard when staging newly diagnosed brain cancers (Louis, et al., 2007). The system has four grades, grade I being the most benign and grade IV the most malignant within each type of brain cancer. For the astrocytomas the following is characteristic in the different stages: Grade I and II astrocytomas show well differentiated astrocytes. They have a slow proliferation rate but are

infiltrative. Grade III and IV astrocytomas are large tumors that are well circumscribed and show poorly differentiated astrocytes, these tumors are highly infiltrative. Table 1 lists the characteristics of the different stages of the astrocytomas. Primary glioblastoma multiforme is classified as a WHO class IV astrocytoma while the initial diagnosis of secondary glioblastomas is made at a lower grade but changes into a class IV glioblastoma multiforme (Parsons, et al., 2008).

WHO classification	Name	Histologic features
I	Pilocytic astrocytoma	Well differentiated astrocytes Slow proliferation
II	Low-grade astrocytoma	Well differentiated astrocytes
III	Anaplastic astrocytoma	Poorly differentiated astrocytes
IV	Glioblastoma multiforme	Poorly differentiated astrocytes Highly infiltrative Necrosis Angiogenesis

Table 1. An overview of the four different classes of astrocytomas and their histologic features. WHO grade I is the most benign while grade IV is the most malignant. Modified from (al., 2009).

After the definitive diagnosis of glioblastoma multiforme has been made, the first line of standard treatment is surgery if the tumor is accessible for resection. The degree of resection is critical as it is related to survival, but it can be difficult to distinguish tumor tissue from normal brain tissue. An advance has been made with the exploit that fluorescent porphyrins accumulate within the glioblastoma multiforme tissue after peroral administration of 5 aminolevulinic-acid, 5-ALA (Sanai & Berger, 2008). The infiltrative nature of glioblastoma multiforme makes surgery alone inadequate (Villano, et al., 2009). Therefore the subsequent lines of standard treatment are chemotherapy and radiation. The alkylating agent temozolomide is approved by the US Food and Drug Administration, FDA, in the treatment of glioblastoma multiforme and a combination of this together with radiation significantly prolonges survival (Villano, et al., 2009).
A small fraction of glioblastoma patients demonstrate oligodendroglial-like differentiation in areas of the tumor and this indicates increased survival. The oligodendroglial component manifests itself by loss of chromosome arms 1p and 19q and is demonstrated by fluorescence in situ hybridization to tumor biopsies (Nagasaka, et al., 2007). Additional treatments aim at reducing the increased intracranial pressure and comprise among others the induction of hypothermia in the patient that reduces the cerebral blood flow and injections of the hypertonic solution mannitol (McCance & Huether, 2005).

In most cases the treatment only works palliative as resistance develops and the tumor relapses. Tumor regrowth is seen in 90 % of patients after a median time of 12 months and the regrowth is often seen within only a few centimeters of the original tumor (Hou, et al., 2006).

Pathogenesis of Glioblastoma multiforme

It is not known in which cell type glioblastoma multiforme arises. Generally, cancer is caused by accumulation of mutations in genes that control important functions of the cell. The mutations lead to activation of oncogenes or depression of tumor suppressor genes and result in cells exhibiting uncontrolled growth (Van Meir, et al., 2010).
Biopsies of glioblastoma multiforme consist of highly cellular tissue with polymorphous cells that have short and sometimes bipolar processes. The sizes of the nuclei are variable in between the cells but mitotically active cells can be seen. Two distinctive characteristics of the biopsies are areas of abnormal vasculature with increased diameter of the vessels and thick basement membranes, and areas of necrosis. Furthermore the necrotic areas exhibit adjacent tumor cells that form palisading structures (Hou, et al., 2006). A rare population of extremely malignant cells in the tumor bulk has been described and the earlier mentioned resistance and relapse of the tumor is thought to be caused by those cells. The cells have stem cell properties and this has caused scientists to designate the cells as cancer stem cells (Hou, et al., 2006). Mechanisms behind the tumor vessel formation and cancer stem cells are explained in the sections below. An example of a tumor biopsy with the traditional hallmarks can be seen on figure 2.

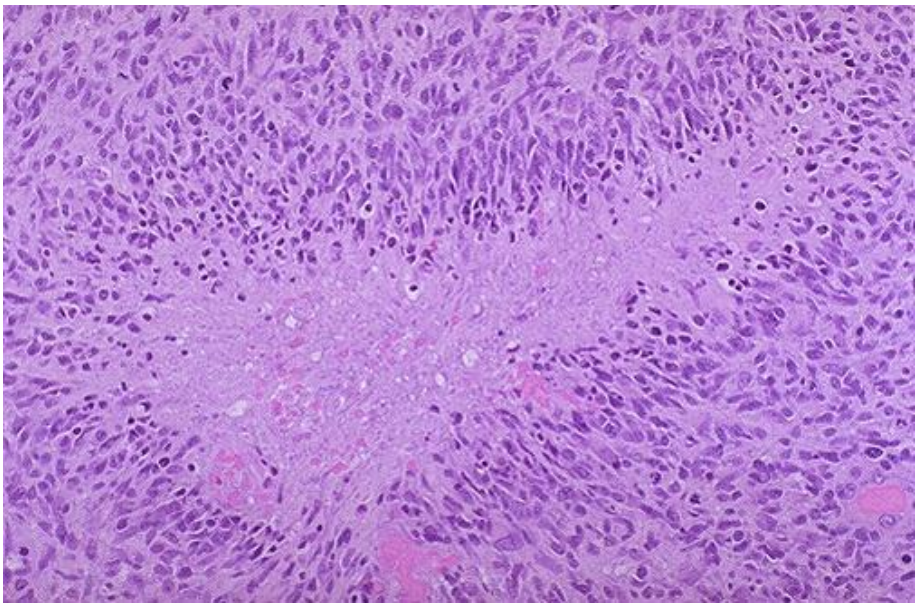

Figure 2: Biopsy of a glioblastoma multiforme tumor. The picture is from (Anon., u.d.)

Formation of tumor vasculature

As mentioned, a hallmark of glioblastoma multiforme is areas of abnormal vessel formation. Accumulating evidence suggest that the growth of the tumor relies on the formation on new blood vessels (Van Meir, et al., 2010). Tumors at a size above 2-3 mm^3 are penetrated by those blood vessels that transport nutrients and oxygen to the tumor. However, the newly formed blood vessels are abnormal and only produce low oxygen tension in the tumor which is though rendered a favorable milieu for the cancerous cells (Karamysheva, 2008).

The exact mechanism by which the vessels of the tumor develop is a subject of much discussion as the vessels are potential targets for new therapy. There is a distinction between tumor vessels developed by co-option of preexisting vessels, angiogenesis and vasculogenesis (Karamysheva, 2008) (Veeravagu, et al., 2008) (Hormigoa, et al., 2011) (Kioi, et al., 2010). Maybe the vessels in the tumor are a combination of all of the above mentioned.

Co-option of preexisting vessels

Preexisting vessels in the brain consist of endothelial cells, pericytes and astrocytes. These cells together form the blood brain barrier which is unique for the vessels in the brain. The blood brain barrier makes the brain tissue a privileged area as it protects against intrusion of potential harmful substances. This is among others managed by tight junctions between the endothelial cells and p-glycoprotein efflux transporters. The initial growth of the tumor is believed to occur along the preexisting vessels in the brain and thereby utilize the properties of those vessels. The BBB is disrupted when the tumor reaches a diameter of 1-2 mm and also the growth of the tumor causes compression of the vessels. All this ultimately leads to necrosis and hypoxia in the tumor cells and as a result of that secretion of growth factors related to angiogenesis (Jain, et al., 2007).

Angiogenesis

Some theories incline that the tumor vessel formation is a result of the sprouting of preexisting brain capillaries. In this theory, the pathological angiogenesis in tumors correspond to the normal physiological formation of new blood vessels upon formation of the primary vascular plexus during early embryogenesis (Karamysheva, 2008). The main factor involved in this process is VEGF-A, which has been shown to be produced by the glioblastoma multiforme cancer cells. Also other factors such as basic fibroblast growth factor, bFGF, angiopoietins, platelet derived growth factor, PDGF, and interleukin-8 are involved (Karamysheva, 2008) (Van Meir, et al., 2010).

The overall mechanisms of this theory are the following. A subpopulation of the endothelial cells in the preexisting vessel wall is known as the tip cells. These cells react to the gradient of VEGF-A created by the tumor by migrating against the gradient. Subsequently the remaining endothelial cells react to the VEGF-A by proliferation. Subsequently the new vessels mature by recruitment of pericytes and smooth muscle cells, derivatives of mesenchymal stem cells. This maturation is however distorted in tumor vessel formation resulting in leaky vessels (Karamysheva, 2008) (Hormigoa, et al., 2011).

Vasculogenesis

Another theory about the tumor vessel formation is the mechanism of vasculogenesis. Vasculogenesis has traditionally been connected to the formation of the primary vascular plexus from progenitors of hematopoietic and endothelial cells during early embryogenesis (Karamysheva, 2008) (Veeravagu, et al., 2008). Evidence suggests that endothelial progenitors and bone marrow derived cells play a role in the development of tumor vessels. Especially endothelial progenitor cells derived from the bone marrow are thought to play a role. It is unclear from which cell type the endothelial progenitor cells develop, but the theory assumes that it is the hemangioblast, that also give rise to hematopoietic stem cells, as they share some central markers.

The overall mechanisms of this theory are the following. VEGF is secreted by the tumor, enters the circulation and reaches the cells within the bone marrow. The hemangioblast responds by migrating towards the tumor where they incorporate into the wall of new vessels and differentiate to endothelial cells. This has been shown in many tumor types (Veeravagu, et al., 2008). In connection to the

recruitment of endothelial progenitor cells from the bone marrow, also mesenchymal stem cells respond at VEGF. After VEGF stimulation these cells are also recruited to sites with developing vessels where they differentiate to cell types of the vessel wall.

Cancer Stem Cells in Glioblastoma Multiforme

As mentioned, the relapse, resistance against chemotherapy and the ability to drive tumor growth is believed to be caused by a rare population of cells in the tumor bulk. These cells have characteristics that define normal stem cells and are therefore named cancer stem cells (Rahman, et al., 2011). Cancer stem cells were first identified in acute myeloid leukemia (Lapidot, et al., 1994) and have later been found in breast cancer, lung cancer and colon cancer. A special marker for cancer stem cells in glioblastoma muliforme has not yet been found but so far the population has been described by a selection of capabilities that are also present in normal stem cells; self-renewal, ability of multi-lineage differentiation and expression of stem cell markers. The characteristics of cancer stem cells are listed in table 2. The different abilities of the cancer stem cells are described in the sections below.

Characteristics of cancer stem cells
Self-renewal
Multi-lineage differentiation
Expression of stem cell markers

Table 2. An overview of the characteristics of cancer stem cells. The cancer stem cells have capability of self-renewal, ability of multi-lineage differentiation and express stem cell markers.

Self-renewal

Self-renewal is as indicated a process by which the stem cell renews itself and thereby maintain the number of stem cells in the body. It is a unique type of cell division that can be either symmetrical or asymmetrical. By symmetrical cell division both daughter cells are identical to the mother cell regarding functions and potential. In asymmetrical division the mother cell divides into one identical daughter cell and a daughter cell that is a more differentiated progenitor cell (Clarke, et al., 2006). In normal stem cells the capability of self-renewal is restricted by various signaling pathways that hinder constant divisions (Lobo , et al., 2007). Some of the signaling pathways that control self-renewal and proliferation are the Hedgehog and Notch pathways. Besides restriction of uncontrolled growth these pathways regulate apoptosis in the cell (Sagar, et al., 2007). Sonic Hedgehog and Notch have been found to be mutated in brain tumors and this has become an additional argument for the existence of cancer stem cells in the tumors as the pathways have only been found in stem cells (Altaba, et al., 2002) (Lobo , et al., 2007) (Pecorino, 2008).

Multi-lineage differentiation

Differentiation is a process where genetic mechanisms are activated in the progenitor cell, which define characteristics of the terminally differentiated cell. For example activation of the Bone morphogenetic protein (BMP) signaling cascade leads to differentiation of neural and intestinal progenitor cells. The differentiated cell can therefore become different from the progenitor cell in many ways hereunder shape, expression of surface markers and metabolism. By this process, the stem cell changes from an uncommitted to a committed state and this is also the state of most of the cells in the body (Anon., 2001).

Multi-lineage differentiation refers to the differentiation potential of the stem cells. A stem cell has the ability of multi-lineage differentiation when it is able to differentiate into cell types of different organ systems. For example have mesenchymal stem cells been shown to be able to differentiate into cells of ectodermal, mesodermal and endodermal germ layers.

The multi-lineage differentiation potential of cancer stem cells has been exploited in different cancer types. Cancer stem cells in glioblastoma multiforme have been shown to be able to differentiate to neurons, astrocytes and oligodendrocytes.

Recent studies have suggested differentiation therapy of brain cancers with BMP. Furthermore, differentiation therapy with the vitamin A derivative all-trans retinoic acid, ATRA, has made acute promyelocytic leukemia one of the most treatable cancers.

Expression of CD133

Today the most debated marker of the cancer stem cells is CD133, prominin-1. The marker is a glycoprotein with five transmembrane domains found in the cell membrane of hematopoietic stem cells in humans and neuroepithelial stem cells in mice. It has also been found in several cancers (Kania, et al., 2005). The function of the glycoprotein itself has not yet been clarified, but it is located in cytoplasmic protrusions. Therefore, it has been suggested that it takes part in the dynamic organization of these and participate in polarity, migration and interaction with adjacent cells and material (Dell'Albani, 2008).

A correlation has been found between expression of CD133 and poor prognosis, the higher expression the shorter survival. Furthermore $CD133^+$ cells from colon cancer have been shown to be resistant to therapy, thus further strengthening the hypothesis that $CD133^+$ cells are cancer stem cells. The use of CD133 as a marker for the cancer stem cell population as a hole is however doubtful. Studies on $CD133^+$ cells isolated from human glioblastoma multiforme biopsies have shown that only a few $CD133^+$ cells are needed to regenerate the original tumor when the cells are injected in vivo. Later it has been shown that $CD133^-$ cells have the same abilities, suggesting that more markers are needed to define the cancer stem cell population.

Origin of the Cancer stem cells

There are different hypotheses concerning the origin of cancer stem cells. Mainly, the discussed cells of origin are adult stem cells of the body, progenitor cells and differentiated cells that somehow gain stem cell properties (Sagar, et al., 2007) (Lobo , et al., 2007).

Adult stem cells of the body are a possible source of the cancer stem cells as the two cell types are very alike according to their abilities. Furthermore, the long life span and the numerous cell divisions of the adult stem cells provide sufficient time for the cells to accumulate the mutations necessary for a cell to be cancerous (Sagar, et al., 2007) (Lobo , et al., 2007). Regarding the cancer stem cells in glioblastoma multiforme, this theory therefore suggests that the stem cells of the brain, the neural stem cells, are the source of the cancer stem cells. In the brain, the neural stem cells are located close to blood vessels supplying the subventricular zone and an area extending from the subgranular zone of the hippocampus into the olfactory bulb (Veeravagu, et al., 2008). Furthermore, studies have indicated that CD133+ cancer stem cells of glioblastoma multiforme are solely located in areas with vasculature while $CD133^-$ cells doesn't have preferential areas of location, thus strengthening the hypothesis of relation between neural stem cells and the cancer stem cells of glioblastoma multiforme (Veeravagu, et al., 2008).

The progenitor cells are also relatively immature cells and therefore also shares some characteristics with the cancer stem cells. These cells are therefore also rendered suspects in providing a source of the cancer stem cells. Though the number of cell divisions in these cells is limited in compare to the adult stem cells, it is possible that they through mutations could reacquire capacity of self-renewal and thereby a longer life span in which they could accumulate the cancerous mutations (Sagar, et al., 2007) (Lobo , et al., 2007).

Recently it has been suggested that it might be the differentiated cells that give rise to the cancer stem cells, by somehow regaining stem cell like properties (Pecorino, 2008). This could possibly be through fusion between stem cells and non-stem cells. According to this theory, the cancer stem cells in glioblastoma multiforme tumors could develop as a result of fusion between cancer cells in the tumor and invading adult stem cells (Schichor, et al., 2012).

Contribution of stem cells to tissues of other germinal origin

During the last decade a series of controversial studies have reported an existence of genetic material from donor bone marrow cells in many tissues distant from the bone marrow, hereunder brain tissue (Horwitz, et al., 1999) (Theise, et al., 2000) (Okamoto, et al., 2002) (Körbling, et al., 2002) (Quaini, et al., 2002) (Krause, et al., 2001) (THEISE, et al., 2000) (Corbel , et al., 2003) (Weimann, et al., 2003) (Cogle, et al., 2004). It is not clear which subclass of bone marrow cells that have part in this phenomenon (Udani , et al., 2005) (Wagers & Weissman, 2004). Generally, the studies are based on female recipients that have been irradiated and subsequently receive sex mismatched bone marrow cells. Subsequently the tissues are analyzed for the presence of Y-chromosomes. Some studies have reported that the hematopoietic stem cells are able to contribute to tissues of non-hematopoietic lineage. However, later it has been suggested that also mesenchymal stem cells are able to contribute to tissues of other lineages an even xenograft models of breast, pancreatic, and ovarian adenocarcinomas (Spaeth, et al., 2009) (Sasaki, et al., 2008). A selection of studies demonstrating the phenomena is listed in table 3.

Tissue transplanted	Duration of experiment	Donor cells observed	Frequency	Reference	Specie
Bone marrow		Vessels of selected sarcomas, lymphomas and carcinomas	1 % - 12 %	(Peters, et al., 2005)	Human
Mesenchymal stem cells	91 days	Xenograft models of breast, pancreatic and ovarian adenocarcinomas		(Spaeth, et al., 2009)	
Bone marrow		Osteoblasts	1,5 % - 2 %	(Horwitz, et al., 1999)	Human
Bone marrow		Hepatocytes	2,2 %	(Theise, et al., 2000)	Human
Bone marrow		Gastrointestinal tract epithelium	0-4,6 %	(Okamoto, et al., 2002)	Human
Mobilized peripheral blood		Hepatocytes, GI tract and skin epithelium	0-7 %	(Körbling, et al., 2002)	Human
Heart		Cardiomyocytes	20 %	(Quaini, et al., 2002)	Human
Bone marrow	11 months	Skin, lung, GI-tract epithelial cells		(Krause, et al., 2001)	Mice
Bone marrow	1,3,5,7 days 2,4,6 months	Liver		(THEISE, et al., 2000)	Mice
Hematopoietic stem cells		Skeletal muscle		(Corbel , et al., 2003)	Mice with tissue damage
Bone marrow		Purkinje neurons		(Weimann, et al., 2003)	Mice
Bone marrow		Neurons Astrocytes Microglia		(Cogle, et al., 2004)	Human

Table 3. Studies revealing that transplanted bone marrow has contributed to tissues of other lineal origin. The donor cells have been observed as cells of tumor models, osteoblasts, hepatocytes, gastrointestinal tract epithelium, cardiomyocytes, skin, liver, skeletal muscles, purkinje neurons, neurons, astrocytes and microglia.

Role of Mesenchymal Stem Cells in Glioblastoma Multiforme

Mesenchymal stem cells are cells with a fibroblast like morphology. The cells belong to the group of adult stem cells and possess the stem cell abilities including self-renewal and differentiation. The cells have been shown to be able to differentiate into multiple lineages including adipocytes, chondrocytes, osteocytes and also neural cell types (Uccelli, et al., 2008). Besides their differentiation potential, the cells are defined by their expression of CD73, CD90 and CD105 together with their lack of expression of CD14, CD11b, CD34, CD45, CD19 and CD79 (Witkowska & Walenko, 2011). The protein CD105, endoglin, is a homodimeric transmembrane glycoprotein at a size of 180 kDa. The function of the protein is not very clear but it takes part in the TGF signaling cascade by interacting with the TGF-beta I and II receptors. Besides the mesenchymal stem cells the protein is expressed in hematopoietic stem cells, smooth muscle cells of vasculature, fibroblasts and macrophages. Furthermore it is associated with proliferating endothelial cells and has been found to be highly expressed in the vasculature of colon, breast, lung, prostate and cervical tumors (DUFF, et al., 2003).

Originally it was believed that the mesenchymal stem cells only existed in the bone marrow, however, this is under reconstruction as it has been shown that stem cells with mesenchymal potential are not solely located in the bone marrow but also in the periphery of vasculature in many tissues, including adipose tissue (Witkowska & Walenko, 2011).

As mentioned, glioblastoma multiforme tumors have been shown to secrete a range of factors that recruit the mesenchymal stem cells and derivatives to the site of tumor formation (Birnbaum, et al., 2007) (Schichor, et al., 2006) (Nakamizo, et al., 2005). These chemo attractants have been shown to be vascular endothelial growth factor-A, VEGF-A, interleukin-8, IL-8, transforming growth factor-ß1, TGF-ß1, and neurotrophin-3, NT-3 (Birnbaum, et al., 2007). Studies have revealed a positive contribution of the mesenchymal stem cells to the malignancy of tumors. Below is a collection of some of the contributions of the mesenchymal stem cells to glioblastoma multiforme tumor progression. As explained, mesenchymal stem cells have been suggested to play a role in the formation and survival of new vessels in glioblastoma multiforme. Furthermore, they have been suggested to take part in formation of cancer stem cell niches, to promote the invasive nature of the tumor, and lastly to fuse or transdifferentiate into cancerous cells of the tumor. The different contributions are explained below. An overview of the contributions of the mesenchymal stem cells to glioblastoma multiforme tumor formation is seen in figure 3.

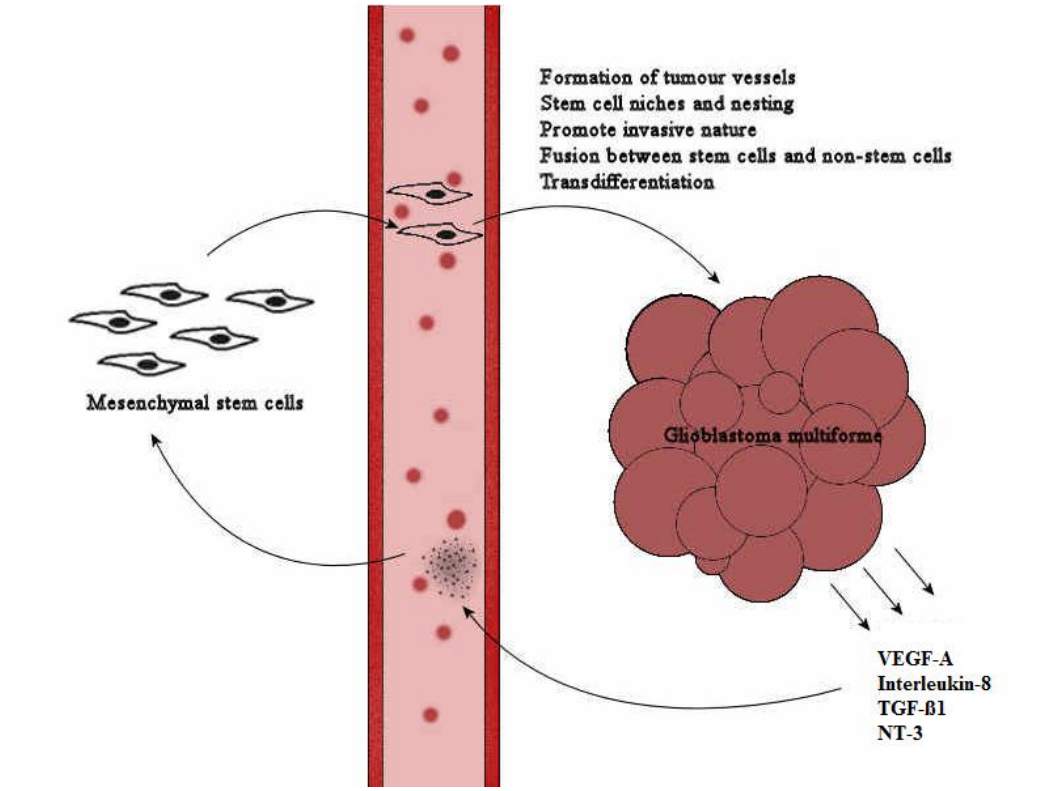

Figure 3. Role of mesenchymal stem cells in glioblastoma multiforme tumor formation and progression. The tumor secrete factors; vascular endothelial growth factor-A, VEGF-A, interleukin-8, IL-8, transforming growth factor-ß1, TGF-ß1 and neurotrophin-3, NT-3, into the blood stream that reaches mesenchymal stem cells in the body. These are then recruited to the site of the tumor where they contribute to formation of tumor vessels, cancer stem cell niches, promotion of invasive nature of the tumor and fuse or transdifferentiate into the glioblastoma multiforme tumor cells. Own creation based on (Roorda , et al., 2009)

Cancer stem cell niches

It has been suggested that the mesenchymal stem cells and their derivatives contribute to the cancer stem cell niches in glioblastoma multiforme in the same manner as they contribute to the hematopoietic stem cell niches and other stem cell niches of the body.
The hematopoietic stem cell niche is believed to have a critical role in the regulation of the hematopoietic stem cell pool and the trafficking of the hematopoietic cells. The cells of the niche secrete factors that control self-renewal potential of the stem cells, notch and hedgehog, to maintain the stem cell population. The niche shelters the stem cells from harmful substances. Furthermore, an important function of the niche is the physical anchoring of the hematopoietic stem cells to the cells of the niche. The mesenchymal stem cells have been shown to be effective as feeder layers when culturing hematopoietic stem cells (Prockop , et al., 2010). In connection to bone marrow transplantation, the mesenchymal stem cells have been shown to improve the regeneration of the hematopoietic system (Prockop , et al., 2010).

The cancer stem cell niches in glioblastoma multiforme are believed to be located in close proximity to vascular compartments of the brain as a study has revealed that $CD133^{+}$cancer stem cells are located in association with such vessels.

In line with the hypothesis that mesenchymal stem cells promote a cancer stem cell supporting milieu, in vitro studies on glioblastoma multiforme tumor cells cocultured with mesenchymal stem cells have revealed lower proliferation of the tumor cells. Also fewer cells in the experiment underwent apoptosis. However, contradictory studies have later been made as in vivo co-injection of tumor cells and mesenchymal stem cells promoted the number of cell divisions and thereby tumor growth (Ramasamy, et al., 2007) (Uccelli, et al., 2008).

Promotion of invasive nature

Generally, invasive nature of tumors and metastasis are two closely associated subjects. As malignancy increases, the tumor cells of the primary tumor get the ability to invade tissue adjacent to the primary tumor and sometimes even through tissue boundaries with resulting secondary tumor growth in other tissues of the body (Cairns, et al., 2003). The mechanisms of this process are not clarified but the first step is thought to be an epithelial to mesenchymal transition of selected cells that changes from an immobile phenotype to a mesenchymal like phenotype with ability of migration. Different kinds of invasion have been identified that distinguish between single migrating cells and collectively migrating cells (Yilmaz, et al., 2007). As mentioned, metastasis of glioblastoma multiforme to organs outside CNS is only a rare event though some sources report that the diagnoses of metastasized glioblastoma multiforme are increasing. However, the invasive nature of the tumor into adjacent brain parenchyma is evident. The phenotype of the invasive glioblastoma multiforme cells is not clarified but their development has been linked to actions taken by the cancer stem cell niche.

Invasive phenotype of glioblastoma multiforme seems to be linked to overexpression of CXCR4. The expression of the receptor has been found to be 25 to 89-fold higher than CXCR4 expression in non-invasive cancer cells and tumor cells expressing the receptor are prone to migrate against gradients of CXCL12, which is the ligand of the CXCR4 receptor. Furthermore, it has been reported that in many tumors mesenchymal stem cells and their derivatives secrete CXCL12, stromal-derived factor-1, thus suggesting the link between the mesenchymal stem cells and the invasive properties of the glioblastoma multiforme cells (Ehtesham, et al., 2006).

In connection to metastasis the mesenchymal stem cell and derivatives are also suggested to play a role. When tumors metastasize to or invade new areas a hypothesis of seed and soil has been suggested. In this hypothesis the microenvironments of the primary tumor and the target site of spread are prepared for invading tumor cells. This has been confirmed in studies where GFP expressing bone marrow cells where identified in organs of metastasis before the metastatic tumor cells had arrived. The cells in these premetastatic areas have been identified as hematopoietic progenitor cells but their adherence in the areas relies on newly produced ECM proteins by mesenchymal stem cell derivatives. The hematopoietic progenitor cells along with the adjacent stromal cells are then thought to change the local environment of the premetastatic areas among others by production of CXCL12 to which CXCR4 expressing tumor cells migrate (Kaplan, et al., 2006).
Accumulating evidence suggest that these premetastatic microenvironments are established as a secondary effect of the factors produced for new vessel formation, among others VEGF-A, thus making the primary tumor itself involved in the preparation of the premetastatic microenvironmets (Kaplan, et al., 2006).

The involvement of mesenchymal stem cells in metastasis of breast cancer has later been shown in a study on breast carcinomas which revealed that the metastatic potential of poorly metastasizing

breast carcinoma cells was increased when the cells were subcutaneously injected together with mesenchymal stem cells.

All of this together seems to describe important roles in the invasion-metastasis cascade of both mesenchymal stem cells in the tumor microenvironment and mesenchymal stem cells in the premetastatic niches (Karnoub, et al., 2007)

Transdifferentiation

The earlier mentioned studies on contribution of transplanted bone marrow cells to tissues of other origin has led to theories about ability of the cells to transdifferentiate or fuse into unexpected cells.
Transdifferentiation is a process by which a stem cell differentiates to a cell belonging to another germ-layer. In connection with mesenchymal stem cells this definition is though not appropriate as the mesenchymal stem cells not have one particular germlayer of origin but are described by their ability to differentiate into cells of the mesenchymal lineage which as mentioned is defined as adipocytes, chondrocytes and osteoblasts

In particular the contribution of bone marrow cells to cells of neuronal phenotype is controversial as neither hematopoietic cells nor mesenchymal cells of the bone marrow are related to the neuroectoderm. However, transdifferentiation of mesenchymal stem cells have been reported in several studies, especially studies focusing on tissue repair. In these, the cells have been shown to be able to trandifferentiate into airway epithelium, multiple cells of the skin and neural cells. In some of the experiments a proportion of the stem cells did not transdifferentiate but obtained the unexpected phenotype by fusion. (Spaeth, et al., 2009) (Spaeth, et al., 2009) (Hall, et al., 2007)

Fusion between stem cells and non-stem cells

Fusion between stem cells and non-stem cells is thought to be a mechanism that leads to the formation of hybrid cells. The process eventually leads to transfer of markers across cells from different germ layers.
The mechanisms behind fusion between stem cells and non-stem cells are completely unknown and the occurrence of the phenomenon is still controversial. This is partly due to the fact that the phospholipid bilayer of cell membranes is constructed particularly to hinder leakage of cellular compartments such as metabolites and genetic material. It therefore seems against inherent abilities of the cell if it undergoes cell-cell fusion (Chen & Olson, 2005). On the other hand, fusion between intracellular membranes is accepted as an important function of cellular activity. Of other well accepted cell-cell fusions are the fusion between egg and sperm cells during fertilization, the fusion between macrophages to osteoclasts and giant cells and the fusion of myoblasts in the development of multinucleated myofibers.

These purposes of the mesenchymal stem cells have never been explored in relation to glioblastoma multiforme tumor development (Schichor, et al., 2012).

The Experiment

The main objective of the study was to set up a protocol for in situ hybridization to X and Y chromosomes in an in vivo model of glioblastoma multiforme. Also, the fate of adipose derived stem cells after systemic injection into the experimental mice was investigated by immunohistochemical staining of CD105.

Study Design

Studies have shown that mesenchymal stem cells are able to migrate to glioblastoma multiforme tumors after injections contralaterally to the site of tumor formation (Birnbaum, et al., 2007) (Schichor, et al., 2006) (Nakamizo, et al., 2005). The migratory abilities have also been shown in transwell migration studies, but the fate of the stem cells has never been determined after systemic injection in an experimental mouse with a tumor xenograft in the brain. The roles of the mesenchymal stem cells in the tumor formation are manifold; contribution to formation of tumor blood vessels, contribution to formation of cancer stem cell niches, contribution to invasive phenotype of the tumor (Roorda , et al., 2009). Furthermore, controversial studies indicate that mesenchymal stem cells are able to fuse or transdifferentiate into tissues of other germinal origin in connection with tissue repair and also into a selection of tumors (Terada, et al., 2002).

Based on this the following in vivo model was set up. Nude mice were injected in the left striatum with cells of the female human glioblastoma-astrocytoma, epithelial like cell line U87-MG. The cells were genetically modified to express GFP. The injected dosages contained 50000 cells. After a period of 9 days the mice received tail vein injections of cells from a human adipose derived mesenchymal stem cell line with male karyotype, see figure 4. 24 hours after injection the mice were put down and the brains were conserved. Furthermore, an experimental mouse with two subcutaneous tumor xenografts was made, the right flank tumor was made of an injection of 500.000 U87 cells and the left was made of a co-injection of 250.000 U87 cells together with 250.000 adipose derived stem cells and 8 days later another injection of 500.000 adipose derived cells into the center of the tumor.

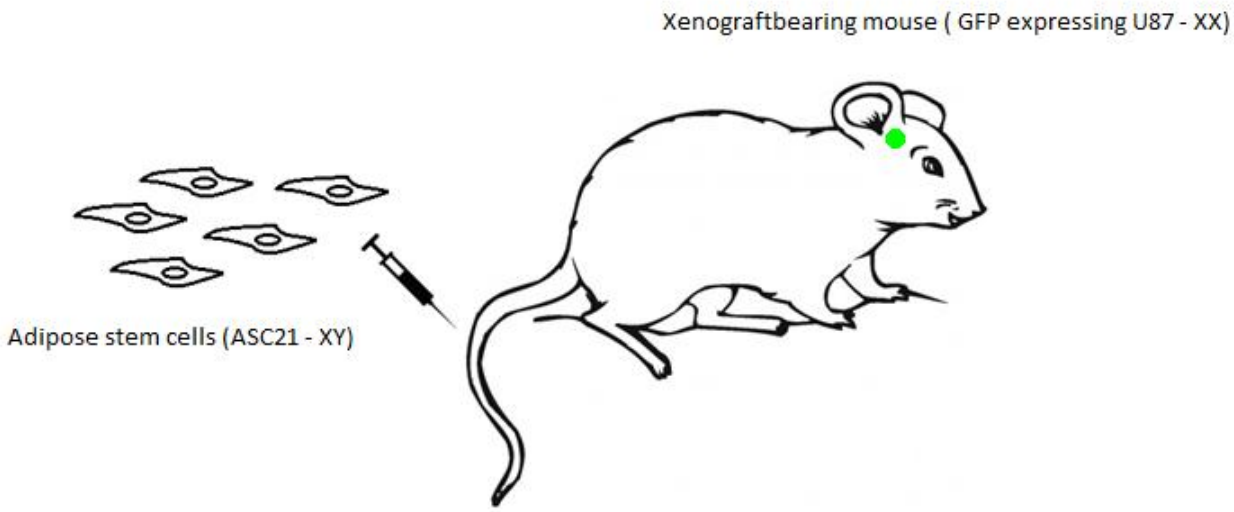

Figure 4. Overview of the study design. Nude mice were xenografted with cells of a GFP expressing U87 cell line. Subsequently the mice received tail vein injections of cells from an adipose derived mesenchymal stem cell line.

Materials and Methods

Cell Culture and Reagents

The human glioblastoma-astrocytoma, epithelial like cell line, U87-MG genetically modified to express GFP was used (the cells were kindly acquired from a Dutch study group). Also, human adipose derived stem cells isolated by the laboratory of stem cell research at Aalborg University were used. The cells were cultured in Dulbecco's modified Eagles Medium (Lonza, Cat. No. BE 12-614F) and Alfa-MEM (Gibco, Cat. No. 32561-029) respectively, supplemented with 10 % fetal calf serum (Gibco, Cat. NO. 10106-169) and 1 % Penicillin/Streptomycin solution (Gibco, Cat. NO. 15140-122) at 37 ^{o}C in a humidified atmosphere with 5 % CO_2. Medium was changed 1-2 times weekly. Cells were seeded at a seeding density of approximately 2800 cells/cm^2 in T175 flaks or 8-well chamber slides. The U87 cells were used at passages 6-8 and the adipose derived stem cells were used at passages 4-6.

Immunocytochemistry

Cells were fixed in 4 % buffered formaldehyde (Cas. NO. 82115-62-6) for 15 min, washed with PBS 2 x 5 minutes and blocked and permeabilized with 0,3 % triton X-100 (Sigma, Cas. No. 9002-93-1) in 1 % Bovine Serum Albumin (BSA) (Gibco, Cat. No.15561-020) for 1 hour. The primary antibodies mouse monoclonal antihuman CD105 (R&D systems, Cat. No. MAB10971) (Dako, Cat. No. M3527) (Abcam, Cat. No. ab114052) (AB serotec, Cat. No. MCA1557) were diluted 1:200 in blocking buffer and applied overnight at 4 ^{o}C. The cells were then washed 2 x 5 min. with PBS followed by addition of the secondary antibody. The secondary antibodies horse antimouse (Vector laboratories, Cat. No. TI-2000) and biotinylated goat antimouse (Dako, Cat. No. E0433) were diluted 1:200 in blocking buffer and applied for 1 hour at room temperature. Cells receiving biotinylated secondary antibodies were subsequently washed 2x5 min. with PBS and then incubated for 30 min. in streptavidin alexa 488 conjugate (Invitrogen, Cat. No. S-11223) diluted 1:200 in blocking buffer. As control for the secondary antibodies, cells without primary antibodies were made. Lastly, the cells were mounted with fluorescence mounting medium (Dako, Cat. No. S3023).

Implantation of brain Tumor Xenografts

Cells for the brain tumor xenograft were trypsinized, washed 2 times in sterile PBS and diluted in a total volume of 5 µl PBS pr. mouse. Male CD1 nude mice were used. The mice were anaesthetized by a subcutaneous injection of 0,235 ml/100 g body weight of Rompun cocktail (Ketalar 50 mg/ml, Xylazin 20 mg/ml, Plegicil 10 mg/ml) and fixated in a stereotactic instrument. The skin was incised and retracted and a small injection hole was drilled through the cranium above the injection site. The U87 cells were injected using a Hamilton syringe in the right striatum at coordinates related to bregma; 1.1 (medial-lateral), 1.4 (anterior-posterior) and 3.5 (superior-inferior). The skin edges were sutured and the mice were monitored during recovery from anesthesia and then daily.

Implantation of subcutaneous Tumor Xenografts

Cells for the subcutaneous tumor xenografts were trypsinized and washed 2 times in sterile PBS. The subcutaneous tumor xenografts were established by subcutaneous injection of 500.000 cells in a 0.5 ml volume of 50 % Matrigel (BD Biosciences, Cat. No. 356230) and 50 % sterile PBS.

Flourescence in situ hybridization

The mice were anaesthetized with the Rompun cocktail and perfusion fixed. Tissue for in situ hybridization was subsequently transferred to a 4 % buffered formaldehyde solution for 48 hours and embedded in paraffin. Sections of 5 micron meter or as thin as possible were cut and backed

overnight at 60 ^{o}C. The slides were then deparaffinized in xylene for 10 min followed by rehydration through ethanol series; 2 x 2 min in 99 % ethanol, 2 x 2 min in 96 % ethanol, and 2 min in 70 % ethanol. Dako FISH Accessory Kit (Dako, Cat. No. K5599) was used with the following modifications for the pretreatment of the slides. The slides were washed 2 x 3 min in wash buffer solution consisting of 20 ml wash buffer solution and 380 ml miliq water. Pretreatment solution consisting of 2.5 ml pretreatment solution and 48 ml miliq water was prewarmed in the microwave at highest power and the slides were then incubated in the solution in the microwave for 10 min at lowest power. The slides were afterwads kept in the cooling solution for 15 min and then washed 2x3 min in wash buffer solution. Pepsin was applied for 10 min and the slides were then washed 2x3 min in wash buffer solution. Before hybridization the slides were dehydrated through 70 %, 96 % and 99 % ethanol concentrations and air dried completely. The hybridizations were performed with Vysis SRY/CEP X FISH Probe Kit (Vysis, Cat. No. 6N29-20). The DNA probes were a mixture of the SRY probes specific for the human SRY gene region (Yp11.3) labeled with spectrum orange and the CEP X probes specific for human chromosome Xp11.1-q11.1 labeled with spectrum green. The probes were prepared according to the recommendations provided by Vysis. The hybridizer was programmed for co-denaturation at 73 ^{o}C for 5 min followed by hybridization at 37 ^{o}C for 20 hours. Posthybridization washes were performed as described. Stringency wash solution consisted of 5 ml 20X stringency wash buffer and 95 ml miliq water. A coplin jahr containing stringency wash buffer was placed in a waterbath at 65 ^{o}C until the temperature of the solution was stable. The fixogum and coverglasses were removed from the slides and the slides were then transferred to the solution and kept there for 10 min. The slides were then washed 2x3 min in wash buffer solution and dehydrated through 70 %, 96 % and 99 % ethanol series. Finally, the slides were mounted with DAPI antifade solution and coverglasses were sealed with nail polish. After 15 min the slides were ready for microscopy.

Immunohistochemistry

The mice were anaesthetized with the Rompun cocktail and subsequently perfusion fixed. The tissues were then transferred to 4 % paraformaldehyde and incubated at 4 ^{o}C for 24 hours after which they were transferred into potassium phosphate buffered saline (PPBS) (0,01M, pH 7.2-7.4) and incubated at 4 ^{o}C for additional 24-48 hours. The tissues were then transferred into 30 % sucrose solution and stored at 4 ^{o}C until they were sectioned in the cryostat. Tissue-tek® (Sakura) was used to attach the organs to cryostat plates. 30 μm slices were cut on the cryostat (Zeizz, MIC1ROM, serial number 3526) at -25 ^{o}C, and the sections were collected in PBS prior to immunohistochemical staining. Sections were blocked with M.O.M. blocking buffer (Vector laboratories, Cat. No. BMK-2202) for 1 hour. The primary antibodies mouse monoclonal antihuman CD105 (R&D systems, Cat. No. MAB10971) (Dako, Cat. No. M3527) (Abcam, Cat. No. ab114052) (AB serotec, Cat. No. MCA1557) were diluted 1:100 in M.O.M. protein concentrate solution (Vector laboratories, Cat. No. BMK-2202) and applied overnight at 4 ^{o}C. The slides were then washed 2 x 5 min. with PBS followed by addition of the secondary antibodies. The secondary antibodies horse antimouse (Vector laboratories, Cat. No. TI-2000) and biotinylated goat antimouse (Dako, Cat. No. E0433) were diluted 1:200 in M.O.M. protein concentrate solution (Vector laboratories, Cat. No. BMK-2202) and applied for 1 hour at room temperature. The sections were then washed in PBS 2x5 minutes and sections receiving biotinylated secondary antibodies subsequently received ABC solution (Vectastain, Cat. No. PK-6100) for 30 min, followed by 2x5 minutes of PBS wash, incubation in 10 min. with DAB substrate containing 0.033 % hydrogen peroxide and another 2x5 minutes of PBS wash. As control for the secondary antibodies, sections without primary antibodies were made. Finally, the sections were transferred to a 5 % gelatin solution and put on microscope slides.

Microscopy

For examination Zeizz Axioplan 2 imaging and Leica DMRXA microscopes were used. Images were captured with Zeizz AxioCam MRc and Nikon Digital sight DS-SMc cameras coupled with the microscopes and processed using AxioVision LE and NIS-Elements BR 3.0.

Results

Growth pattern of Adipose derived stem cells resembled growth pattern of fibroblasts

The adipose derived stem cells were plastic adherent in culture. The cells revealed branched cytoplasms with nucleoli in the middle, 48 hours after seeding. After 6 days of culture the cells exhibited confluent structures with more close lying cells, still branched cytoplasms see figure 5.

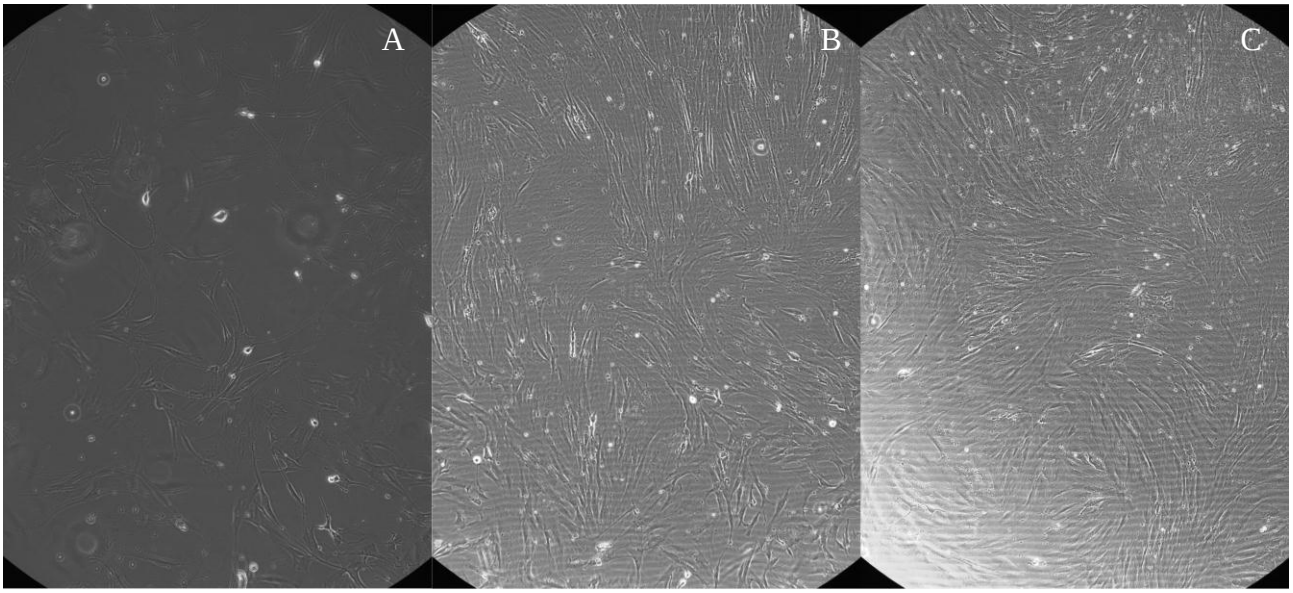

Figure 5. Growth pattern of adipose derived mesenchymal stem cells. A. 48 hours after seeding. B. 4 days after seeding. C. 6 days after seeding. Magnification X10.

Immunocytochemistry analysis of cultured adipose derived stem cells

Immunocytochemistry for human CD105 on the human adipose derived mesenchymal stem cells revealed equivocal results. Cells of passage 4-6 were stained and four different antibodies were used. Generally, control cells that did not receive primary antibodies also lighted up during microscopy.

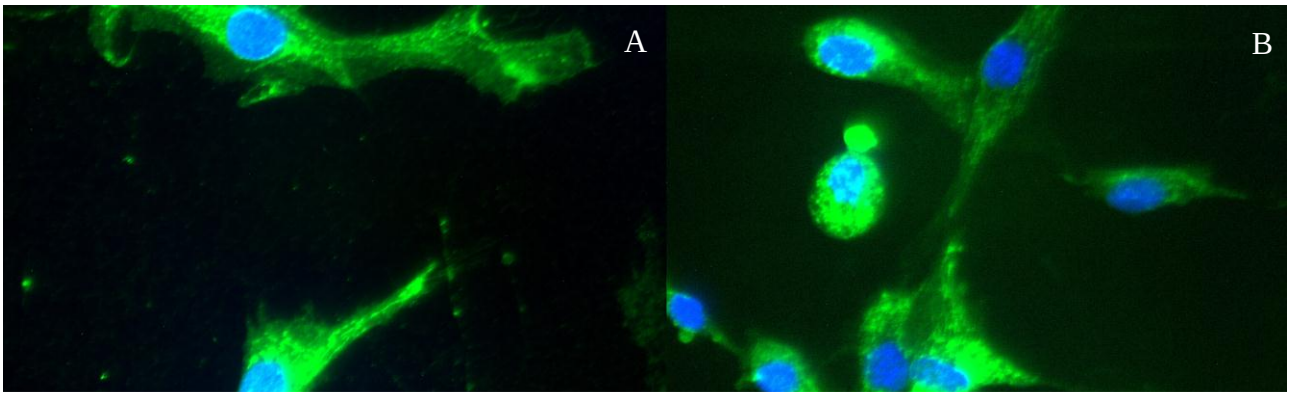

Figure 6. Immunocytochemistry analysis of cultured human adipose derived stem cells. A. The cells expressed CD105 and the protein was located in the cell membranes. B. Negative control without primary antibody. The negative controls were blank. Magnification X40.

Tumor formation in experimental mice after injection of GFP expressing U87

Injection of GFP expressing cells of the human glioblastoma cell line U87 resulted in tumor formation after a period of 19 days. The tumor exhibited a clear defined border. Nuclear staining of the cells reveals larger nuclei in the tumor than in the adjacent mouse cells, see figure 6.

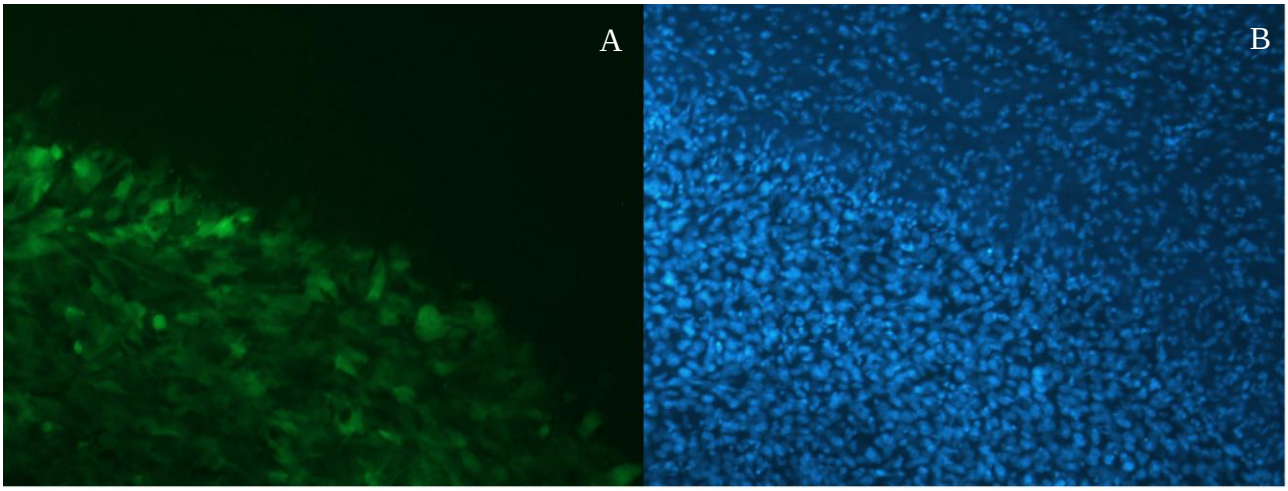

Figure 6. Tumor formation in brains of the experimental mice after injection of GFP expressing U87 cells. A. The glioblastoma cell line revealed sustained expression of GFP after in vivo injection. Also, a clear border of the tumor is visible. B. Nuclear staining of the area, nuclei in the tumor are different from the nuclei in the adjacent mouse tissue. Magnification X20.

Immunohistochemical analysis of brain sections of the experimental mice

Immunohistochemistry for human CD105 in sections of the tumor xenograft did not reveal any human CD105 in the tumor.

Unsuccessful detection of adipose derived stem cells after in vivo injection

Immunohistochemical analysis of brains, spleens and livers of the experimental mice did not reveal any human CD105. Thus the fate of the adipose derived stem cells after injection into the tail vein of the experimental mice remained unclear.

Fluorescence in situ hybridizations to biopsies of glioblastoma multiforme and the thyroid gland

To get an impression of what positive signals from the commercial probes would look like, in situ hybridizations to male human biopsies of glioblastoma multiforme was set up. As a negative control a human female biopsy from the thyroid gland was used. The male tissue revealed one red and one green signal in close to each cell nucleus, while the negative control revealed two green signals in close to all cells, thus suggesting that the Y-chromosomal specific probe did not bind in that tissue and that the stringency washes were appropriate.

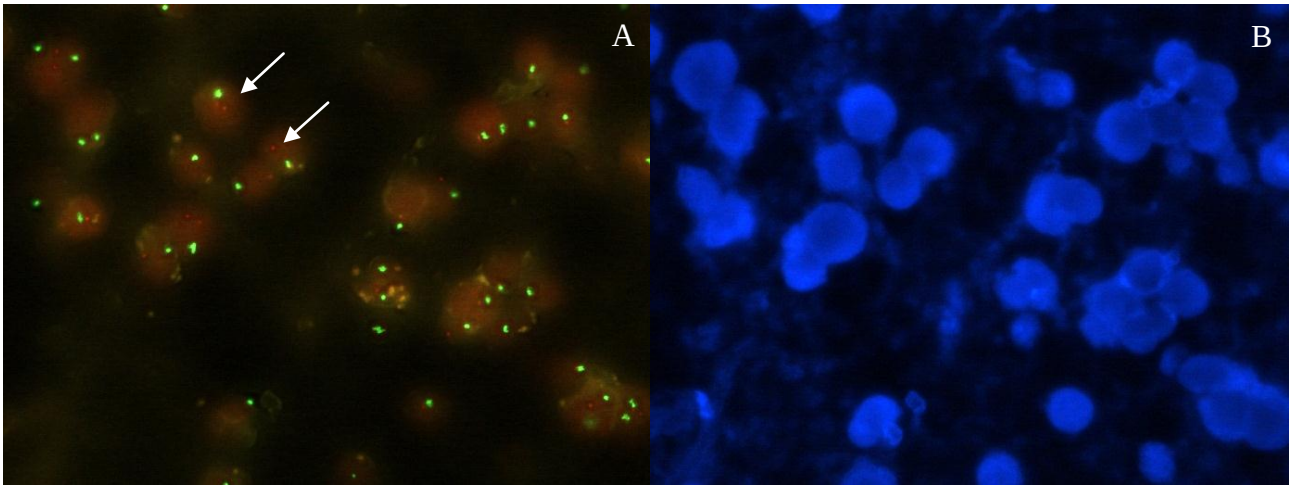

Figure 7. Fluorescence in situ hybridizations to a human biopsy of a male glioblastoma multiforme tumor. A. Signals from one X and one Y chromosome are present in almost every cell, arrows point at examples of the signals. B. Nuclear staining confirms that the signals seen in A are in fact signals from cell nuclei. Magnification X40.

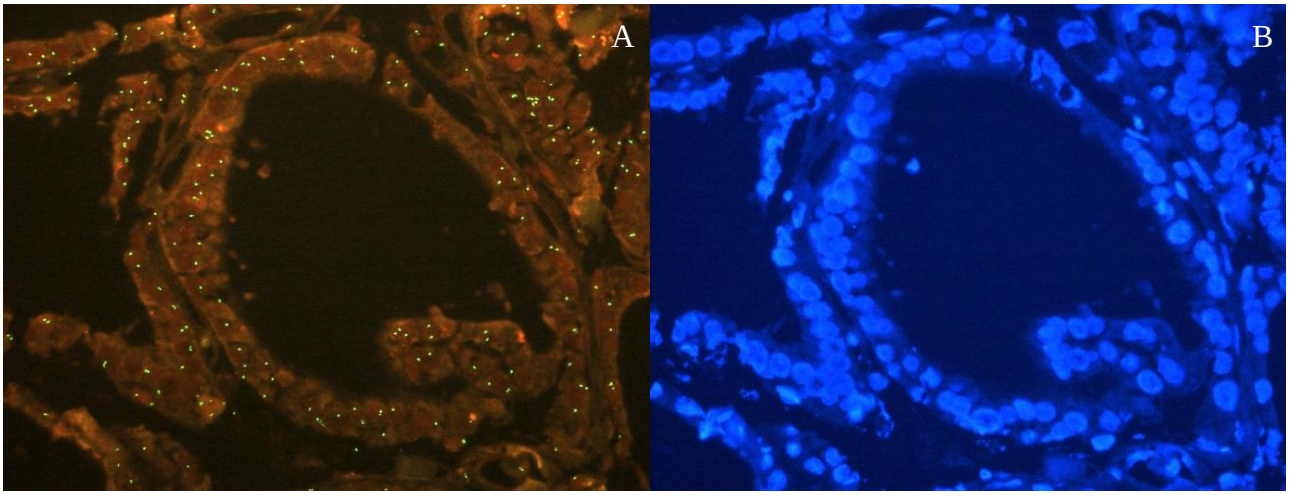

Figure 8. Fluorescence in situ hybridizations to a human tissue biopsy of a female thyroid gland. A. Signals from two X chromosomes are present in almost every cell. B. Nuclear staining confirms that the signals seen in A are in fact signals from cell nuclei. Magnification X40.

Fluorescence in situ hybridizations to brain sections of the experimental mice

Fluorescence in situ hybridizations to the brain sections of the experimental mice revealed binding of the human specific probes in a distinct bulk of cells close to the right ventricle. The cells in the bulk preferentially revealed x or xx karyotype and thorough examination of the bulk at high magnification revealed a very few cells that exhibited y signals. All nuclei of the bulk had a distinctive red appearance, see figure 9 and 10.

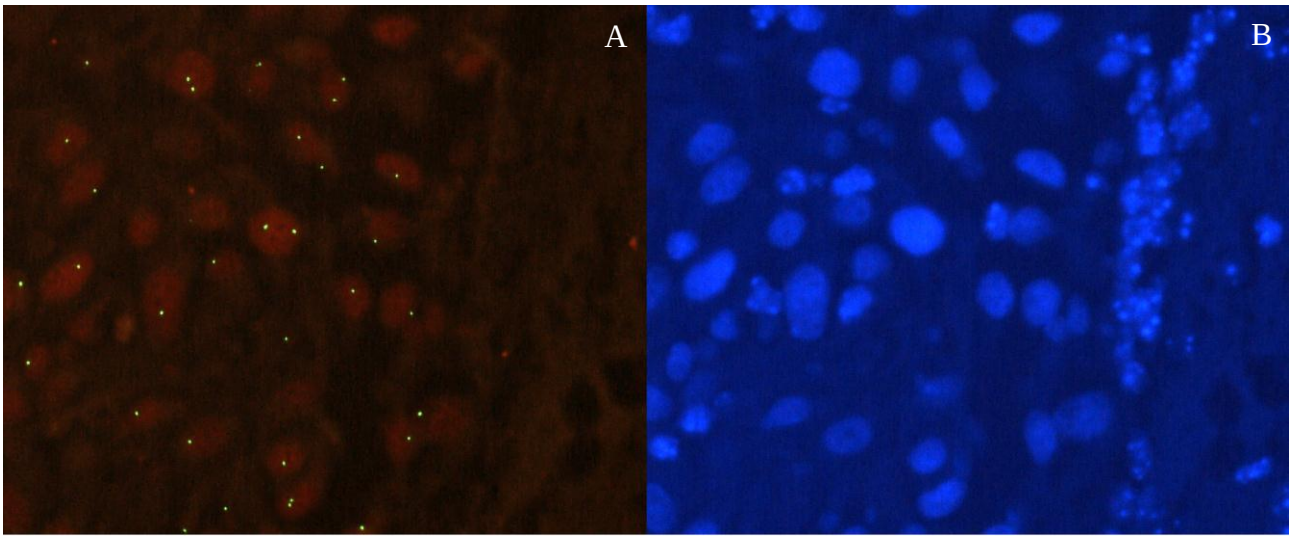

Figure 9. Fluorescence in situ hybridizations to brain sections of the experimental mice. A. Signals were present in a distinct bulk of cells in the brain. The cells revealed x or xx karyotype. B. Nuclear staining confirms that the signals seen in A are in fact signals from cell nuclei. Magnification X40.

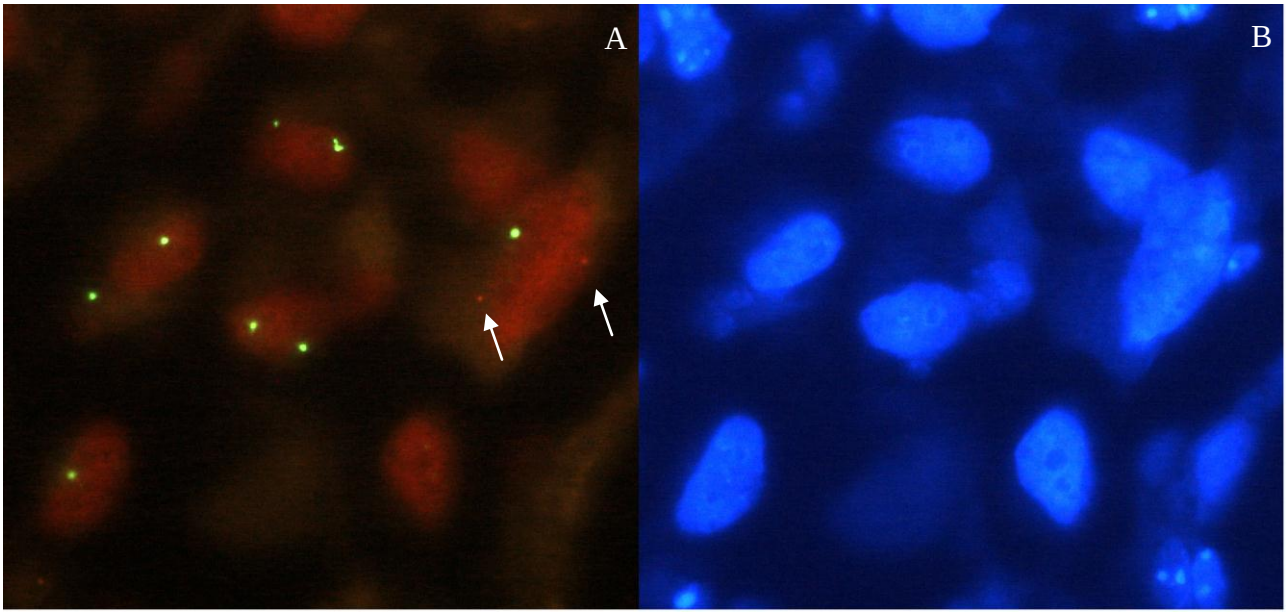

Figure 10. Fluorescence in situ hybridizations to brain sections of the experimental mice. A. Single cells exhibited Y chromosome signals. Arrows point at examples of the signals and a third signal is visible in the lower left corner of the picture. B. Nuclear staining confirms that the signals seen in A are in fact signals from cell nuclei. Magnification X100.

Fluorescence in situ hybridizations to flank tumors of the experimental mouse

Fluorescence in situ hybridizations to the flank tumors of the experimental mouse revealed x and xx karyotype of the cells of both the right and left flank tumor, see figure 11 and 12. Furthermore, the cells had a distinctive red appearance. Noticeably, thorough examination of the flank tumor slides at higher magnification did not reveal any cells with y chromosomes though the left flank tumor was made of a co-injection of 250.000 U87 cells together with 250.000 adipose derived stem cells and 8 days later another injection of 500.000 adipose derived stem cells into the center of the tumor.

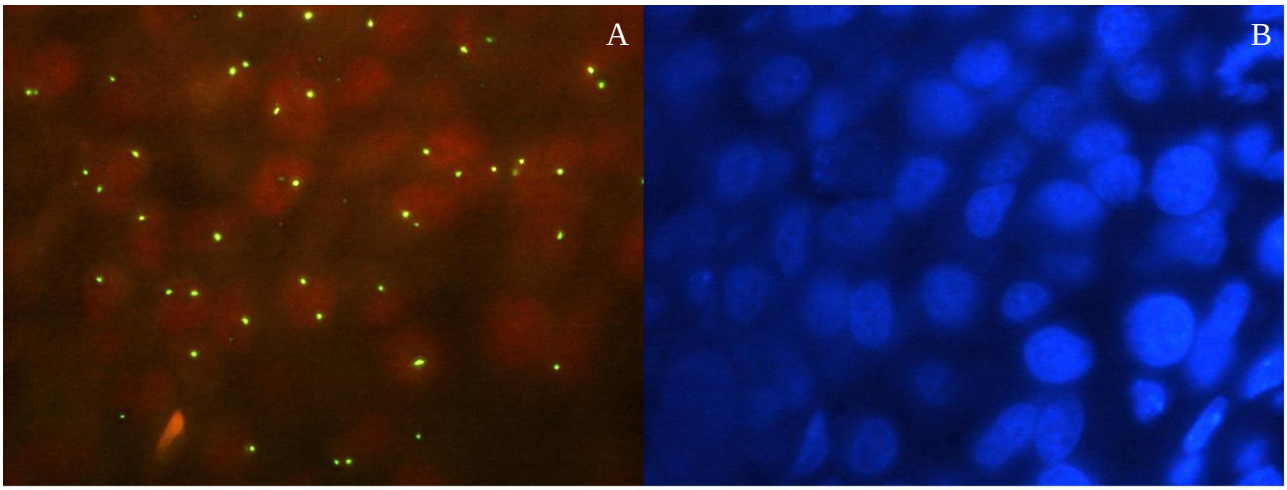

Figure 11. Fluorescence in situ hybridizations to sections of the right flank. A. the cells in the flank tumor revealed x or xx karyotype. B. Nuclear staining confirms that the signals seen in A are in fact signals from the cell nuclei. Magnification X40.

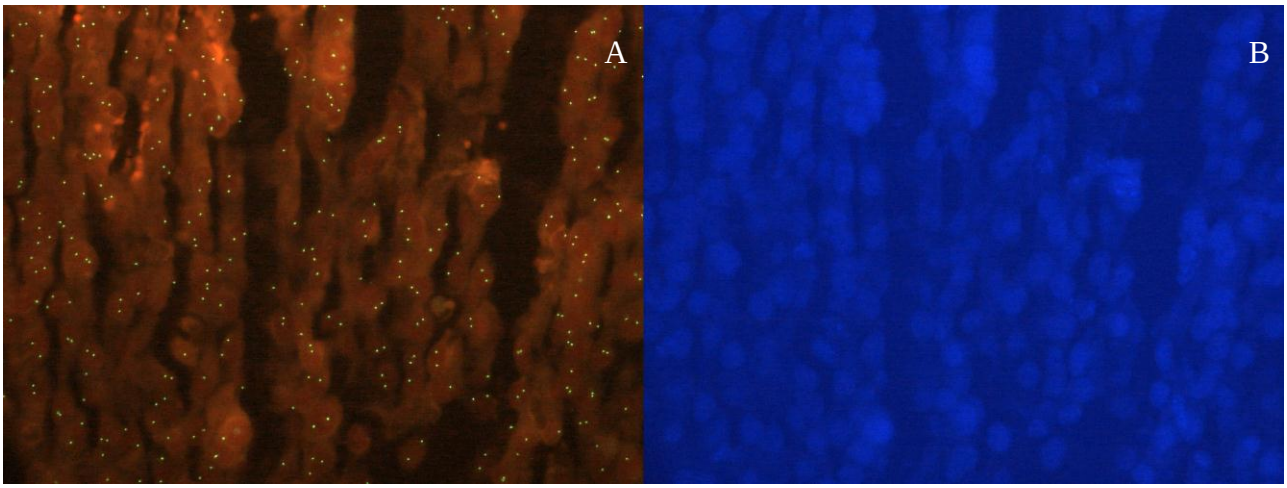

Figure 12. Fluorescence in situ hybridization to sections of the left flank. A. The cells in the flank tumor revealed x or xx karyotype. B. Nuclear staining confirms that the signals seen in A are in fact signals from the cell nuclei. Magnification X40.

Discussion

Expression of CD105 in cultures of adipose derived stem cells

Immunocytochemical stainings for CD105 in cultures of the adipose derived stem cells did not reveal any unequivocal expression of the protein. This could be connected with the number of passages that the cells had undergone. It is reported that early passages of cells obtained from the stromal vascular fraction (SVF) of liposuctions exhibit a heterogeneous population of cells that only express low levels of markers associated with stroma, including CD105. At passage 3 and 4 the cells are reported to become more homogenous and the expression of stromal markers is reported to increase. It is though unclear to which degree this involves CD105. In this experiment, cells of passage 4 to 6 were used, which, according to the reports, should be sufficient for detecting CD105. However, it is generally difficult to compare studies on adipose derived stem cells as there is no standardised way of isolating these, thus it is possible that the population of cells in this experiment is different concerning protein expression profile from the cell populations in the report (Phinney & Prockop, 2007) (Mitchell, et al., 2006). Another marker of adipose derived stem cells has been proposed to be CD90 and this protein could also have been tested as marker for the adipose derived stem cells in this experiment.

An alternative to the immunocytochemical stainings could have been to perform a reverse transcriptase PCR with primers specific for the complementary DNA of the markers.

Expression of human CD105 in tumor xenografts

Injection of cells from the GFP expressing human glioblastoma cell line, U87, into striatum of the experimental mice led to extensive tumor formation in the area of injection. Upon visual inspection, the tumors exhibited a clear defined border separating tumor cells from adjacent mouse cells. The U87 xenografts are reported to be highly vascularized tumors. (Gomez-Manzano, et al., 2008) Additionally, CD105 is reported to be expressed in association with vasculature of a range of tumors including colon, breast, brain, lung, prostate and cervical tumors. However, the immunohistochemical analysis of the U87 xenografts did not reveal any expression of human CD105 in the tumors. As the immunocytochemical stainings for CD105 in the cultures of the adipose stem cells did not succeed it is a possibility that the antibody procedures were inappropriate. Another explanation could be related to the origin of the vessels in the xenograft. By staining for anti-human CD105 it is assumed that the vessels develop from human cells.

Unsuccessful detection of adipose derived stem cells after in vivo injection

The perhaps most crucial question is why the detection of the in vivo injected adipose derived stem cells on the basis of immunostaining for CD105 proved unsuccessful. As already mentioned, it was doubtful if the cells in fact did express the protein. In connection with this it is questionable if immunhistochemical analysis is the best method when tracing in vivo injected adipose derived stem cells. Another way to trace the cells could be to label them before the systemic injection e.g. by GFP (Levi, et al., 2011). A stable labeling could be achieved through e.g. lentiviral tranfection of the cells with the gene encoding GFP. Alternatively, a transient label might be sufficient depending on the time frame of the experiment. This could be carboxyfluorescein succinimidyl ester, CFSE.

Fluorescence in situ hybridization to brain sections of the experimental mice

Fluorescence in situ hybridization to brain sections of the experimental mice revealed signals in a distinct bulk of cells close to the ventricular system, which could be the tumor xenograft. This could have been confirmed by the expression of GFP in the cells, but the protein was probably broken down by the pepsin treatments during the tissue preparation procedures for the hybridizations. However, the probes used for the hybridizations were human specific, suggesting that the cells of the bulk were in fact human cells. Also, the cells revealed female karyotype, which is also the karyotype of the U87 cell line. Noticeably, the number of hybridization signals varied between one and two X-chromosome signals within the cells. This is a well-known phenomenon when hybridizing to thin tissue sections. During tissue sectioning the nuclei of the cells can be cut through and therefore sometimes only the part of some of the nuclei without the chromosome to which the probe is targeted remains in the section. When diagnosing trisomy this problem is circumvented by a cell count, where above 50 % of the cells need to exhibit trisomy before a diagnosis can be made. Hybridizations to sections of flank tumors made of U87 cells also revealed a variation of one and two x chromosome signals within the cells, further strengthening the possibility that the bulk of cells with signals in the brain were in fact the cells of the tumor xenograft.

Y-chromosomes in the tumor xenograft

Two Y-chromosome signals were found in the tumor bulk, which unfortunately is too few to conclude anything. The signals could be probe leftover as a consequence of inappropriate stringency washes. However, according to the control tissue, which was male human biopsies of glioblastoma multiforme tumors, the level of stringency seemed to be adequate. One way to support the presence of human Y-chromosomes in the xenograft could have been to demonstrate Y-chromosomes in the adipose derived stem cells before they were injected e.g. by PCR or hybridization to cultures of the cells.

Fluorescence in situ Hybridizations to Sections of Flank Tumors

Fluorescence in situ hybridizations to sections of flank tumors revealed x and xx karyotype of the cells in the tumors. As for the brain sections, the explanation of the single x signals in some of the cells could be loss of chromosomes during sectioning of the flank tumor. Surprisingly, no Y-chromosome signals were seen in the left flank that was made of a co-injection of 250.000 U87 cells together with 250.000 adipose derived stem cells and 8 days later another injection of 500.000 adipose derived stem cells into the center of the tumor. The fate of the adipose derived stem cells in the left tumor is therefore unclear.

Mesenchymal Stem Cells and Cancer Stem Cells in Glioblastoma Multiforme

The significance of mesenchymal stem cells for glioblastoma multiforme tumor growth, progression and reoccurrence is unclear. It seems as though the cells take part in formation of new blood vessels, formation of cancer stem cell niches and promotion of the invasive nature of the tumor. Furthermore, it is possible that the mesenchymal stem cells directly contribute to the formation of cancer stem cells as a result of close interactions between the recruited stem cells and the glioblastoma mutiforme tumor cells. A new study from 2012 on the interactions between glioma cells and mesenchymal stem cells grown in co-culture reveal fusion between the two different cell types (Schichor, et al., 2012). The resulting cells reveal themselves as multinucleated cells but the degree to which the cancer cells are able to take advantage of the genes of the mesenchymal stem cells remains undiscovered. On the other hand, other studies have reported an inhibitory effect of the mesenchymal stem cells on the growth of the tumor (Schichor, et al., 2012). However, one role does not necessarily exclude another.

The interactions between the tumor and the mesenchymal stem cells of the body complicates the disease as it renders glioblastoma multiforme a disease affecting the body as a hole, not just the immediate infected part of the brain (Van Meir, et al., 2010).

Conclusions and Perspectives

Gliolastoma multiforme is the most lethal type of all brain cancers, after diagnosis the mean survival is 12-15 months. No curative treatment is available as the therapies of today allow the tumors to reoccur. The tumors are heterogenous bulks of cells and cancer stem cells are a highly malignant but relatively rare cell type within the bulks. These cells are thought to be responsible for the tumor regrowth. The cells share defining characteristics with adult stem cells of the body including ability to undergo self-renewal and multi-lineage differentiation. Furthermore they express markers similar to adult stem cells.

The Mesenchymal stem cells migrate to and exert significant responsibilities in the micro milieu of the tumor. Among others, they participate in the formation of new blood vessels in the tumor that prevents the tumor cells from undergoing necrosis, they contribute to the formation of cancer stem cell niches on which the survival of the cancer stem cells rely, they promote the invasive nature of the tumor, and they have been proposed to fuse or transdifferentiate into cancerous cells of glioblastoma multiforme.

In this study, fluorescence in situ hybridizations were successfully performed to glioblastoma brain tumor xenografts from mice that upon injections of female glioblastoma cells received tail vein injections of male adipose derived stem cells. Two single male chromosome signals were found in the tumor bulk, but this was too few to conclude the migration of the stem cells to the tumor. Furthermore, immunohistochemical analysis of CD105 expression in the tumor xenografts was performed, though this neither confirmed the prescence of the adipose derived stem cells in the experimental tumors.

Generally, the importance of mesenchymal stem cells in the micro milieu of the tumors suggests different perspectives. In the physiologic stem cell niches, a delicate balance of self-renewing and proliferating stem cells is maintained, but in the cancer stem cell niches this balance seems to be spoiled. The niches also have a protective role and loss of the cancer stem cell niches leads to loss of cancer stem cells. It is therefore tempting to consider future treatment strategies directed against the cancer stem cell niches as an important tool in the management of glioblastomas. However, it is still unknown if the growth of the tumors solely rely on the cancer stem cells.

Another perspective has emerged on the selective migration of the mesenchymal stem cells to the Glioblastoma multiforme tumors. These cells, derived from adipose tissue as well as bone marrow, have been proposed as cellular vehicles for targeted therapy against tumors. Studies on their effectiveness seem promising in both glioblastoma multiforme xenografts and other cancer models (Hall, et al., 2007) (Altanerova, et al., 2012). Theoretically, these cellular vehicles would also be targeted against infiltrative tumor cells, tumor metastases as well as the premetastatic niches. At lot of work still needs to be done on these cellular vehicles before they can be advantageous in the clinic. Unpredictable behavior of the cells should be taken into consideration. Also, it is unclear if the cellular vehicles will sustain their production of cytotoxic substances after the treatment of the tumor has finished.

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