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Construction of a lentiviral system for overexpression of hs-Sox5 in human glioma cells

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Summary

The most common primary brain tumors in adult humans are malignant gliomas (Y. Jiang and L. Uhrbom, 2012). Gliomas are classified by the World Health Organization (WHO) into astrocytomas, oligodendrogliomas, oligoastrocytomas and ependymomas based on which cell type is the most prominent in the tumor and into four different grades (I-IV) according to malignancy based on histopathology. Glioblastoma multiforme (GBM) is an astrocytoma of the highest grade (IV) is the most common and aggressive tumor of the central nervous system and is associated with a poor survival ranging between 12 and 15 month (Patrick Y. Wen and Santosh Kesari, 2008).

The formation of glioblastomas can occur either "de novo" or develop from a recurrent astrocytoma that progress to a higher grade of malignancy. The most common molecular alterations found in these tumor types are the loss of heterozygosity of chromosome 10 which harbors several tumor suppressor genes of which PTEN is the most important for GBM. The genetic pathways p53/MDM2 / $p14^{ARF}$ and CDK4/RB1/$P16^{INK4A}$ involved in cell cycle control are deregulated in most gliomas, including genes that promote proliferation, like EGFR. The variety and amount of these molecular alterations in glioblastomas have made it very difficult to find effective drugs to combat them.

The SOX family of proteins are transcription factors that contain a high-mobility group domain and have an important role in developmental processes. Genes in the *Sox* family play a critical role in the formation of tissues and organs during early development. These genes also maintain the normal function of certain cells after birth. There are 20 different *Sox* genes in mammals that are divided into eight subgroups (A-H) (V. Lefebvre 2010). Subgroup SoxD contains *Sox5, Sox6* and *Sox13* that have been found to contribute to gliogenesis. *Sox5* has also been identified as a brain tumor locus (Btl).

Sox5 has 15 exons and different splice variants give rise to different isoforms, such as long (*L-Sox5*) and short (*S-Sox5*). It has been shown that *Sox5* can suppress PDGFB-induced glioma development in Ink4a-deficient mice. The proliferation of human glioma cells (HGCCs) cultured under stem cell conditions is inhibited when *Sox5* is overexpressed (E. Tchougounova *et al* 2009). It has been also shown that there is a co-occurrence of *Sox5* overexpression and PDGFRA amplifications particularly in the proneural subtype of gliomas (M. Boije *et al* 2011).

List of Abbreviations

CDK4	Cyclin-dependent kinase 4
CDKN	Cyclin-dependent kinase inhibitor
EGFR	Epidermal growth factor receptor
GBM	Glioblastoma multiforme
GIC	Glioma initiating cell
HGCC	Human glioma cell culture
HMG	High mobility group
LOH	Loss of heterozygosity
MDM2	Murine double minute 2 homolog
NF1	Necrosis factor 1
NSC	Neural stem cell
PDGFR	Platelet derived growth factor receptor
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma
SOX	Sry-related HMG box
TGFb	Transforming growth factor b
TP53	Tumor supressor 53
TSG	Tumor supressor gene
WHO	World health organization

Introduction

Glioma

Gliomas are a heterogeneous group of primary tumors of the central nervous system (CNS), which are derived from glial cell. The disease affects men more often than women and it accounts for about 60 to 65% of all brain tumors. The incidence of brain tumors depends on the age of the patient and is between 6 and 7 cases per 100.000 pers/year (Deorah *et al* 2006). Some tumors do not cause symptoms until they are large enough and then can quickly damage the health of the person. Others cause symptoms that develop slowly. Symptoms depend on the size, location, degree of invasion and swelling. The most common symptoms are: changes in mental function of the person, headaches, seizures (especially in older adults), changes in hearing, etc (Buckner *et al* 2007).

Grading of gliomas
One can use tumor grades to indicate the difference between the types of slow-growing and rapid growing tumors. The tumor grade is based on how abnormal the cells look under a microscope and the likely speed in which the tumor will grow and spread.
Grade I includes tumors with low proliferative potential and possibility of cure with complete surgical excision. They mainly occur in children.
Grade II includes infiltrative tumors with capacity of recurrence and progression to greater malignancy, despite having a low level of proliferative activity (for example, low grade gliomas may progress to anaplastic gliomas and / or glioblastomas).
The grade III includes tumors with histological evidence of malignancy, like nuclear atypia and high mitotic activity.
Grade IV includes tumors with histological evidence of malignancy (nuclear atypia, high mitotic activity, necrosis, microvascular proliferation) and an aggressive clinical course with rapid progression and recurrence despite intensive treatment and high mortality
As a rule, high-grade gliomas almost always grow back even after complete surgical excision. On the other hand, low-grade gliomas grow slowly, often over many years, and can be followed without treatment unless they grow and cause symptoms (Wen and Kesari 2008; Louis *et al* 2007)

Classification of gliomas
Gliomas are classified by the World Health Organization (WHO) according to their histopathological appearance, the most common types are: astrocytoma, oligodendroglioma, oligoastrocytoma and ependynomas. Each type of glioma can be further subdivided into grades. Astroytic tumors can be divided into: Pilocytic astrocytoma is within the group of grade I gliomas (Louis *et al* 2007). A pilocytic astrocytoma is a rare tumor, usually well localized, which occurs in children and young adults. It grows slowly in the brain or spinal cord. Diffuse astrocytomas (AD) are low-grade gliomas grade II (Louis *et al* 2007). It affects mainly children and young adults between 20 and 40 years. A diffuse astrocytoma grows slowly, but often spreads into nearby tissues. Anaplastic astrocytoma (grade III): grows quickly and spreads into nearby tissues. They are characterized histologically by nuclear atypia, cellularity and a significant increase in proliferative activity. Anaplastic astrocytoma is also called malignant astrocytoma or high-grade astrocytoma. Glioblastoma (grade IV): grows and spreads very quickly. The tumor cells look very different from normal cells, also called glioblastoma multiforme (GBM). Oligodendroglial tumors can be divided into a number of sub-groups. Oligodendroglioma (OD), a glioma grade II according to the 2007 WHO classification (Louis *et al* 2007), is a rare tumor, infiltrating, diffuse, well-differentiated

location in the white matter of the cerebral hemispheres, and increased sensitivity to treatment and better prognosis than other gliomas. Anaplastic oligodendrogliomas (ODA) occur in adults with a peak incidence between 45-50 years (Louis *et al* 2007). They can arise from a low-grade oligodendroglioma. Oligoastrocytic tumours can be divided into the sub-groups as well. Oligoastrocytomas or mixed low-grade gliomas (OA) belong to the group of grade II gliomas according to WHO classification (Louis *et al* 2007), and are characterized by having a histopathological and molecular appereance like in diffuse astrocytomas and low-grade oligodendrogliomas. Anaplastic oligoastrocytoma (AOA) belongs to the group of grade III gliomas according to WHO classification 2007 (Louis *et al* 2007), and is characterized by having an histopathological and molecularappearence as anaplastic astrocytomas and oligodendrogliomas degree of III.
Ependymal tumours can be divided into the sub-groups: grade I ependymal tumors (ependymoma myxopapillary and subependymoma), grade II (ependymoma) and grade III (anaplastic ependymoma).
The diagnosis of gliomas is performed today through computed tomography, magnetic resonance imaging, and positron emission tomography. As a treatment, indicating the removal of the tumor, with microsurgery. It can also be treated with radiotherapy (a radiation beam to the tumor) and chemotherapy with drugs such as temozolomide.

GLIOBLASTOMA

Glioblastoma multiforme (GBM) is the most common tumor of the different types and unfortunately the most malignant of all gliomas (Kleihues *et al* 2002). Glioblastoma constitutes about 60-75% of all gliomas. GBM is associated with a poor survival ranging between 12 and 15 month. It is more common in adults, with an average age at diagnosis of 62 years. GBM is an astrocytoma of the highest grade (IV) (Louis *et al* 2007, Pollard *et al* 2009, Parsons *et al* 2008). The GBM is characterized histologically by presenting microvascular proliferation and / or necrosis, nuclear atypia and high mitotic activity.

The formation of glioblastomas is very diverse and can be presented in "de novo" or come from recurrent astrocytomas that progress to higher grades of malignancy. It is a diffuse tumor, infiltrative and poorly differentiated from glial cells. It usually originates in the white matter and the name multiforme is given by the heterogeneity that characterizes the images seen in microscopy (Pérez-Ortís *et al* 2000). GBM can develop from a low-grade astrocytoma that is undergoing anaplastic transformation and evolve to a secondary glioblastoma (GBM2) or be directly submitted as a primary glioblastoma or "de novo" (GBM1).

The biology of malignant gliomas is associated with a balance of the expression of the proteins that control the cell cycle positively or negatively, proliferation, motility, neovascularization and recognition of the immune system. These phenomena are the result of changes in the expression level of a normal gene or involve the loss of its expression. The signal for the proliferating normal cell commonly starts at the cell surface, where the growth factors are released to the extracellular matrix by the tumor itself or by interacting with specific receptors on the membrane, triggering various intracellular signaling mechanisms affecting gene expression which in turn promotes cell division. Alterations and mutations may exist in each one of these pathways that together or independently can lead to loss of cell cycle regulation, angiogenesis, proliferation and / or promote invasion (Demuth *et al* 2004). The frequencies of genetic alterations that are present in GBM1 and GBM2 are different depending on the age of patients in which they occur. GBM1 usually appears at later ages,

around 60-70 years and GBM2 usually occurs at 40-50 years. In the genesis of glioblastomas, there can be changes at the molecular level of tumor suppressor genes (TSG), oncogenes and genes involve in DNA repair. Figure 1 shows the progression of gliomas according to their molecular alterations that detailed below.

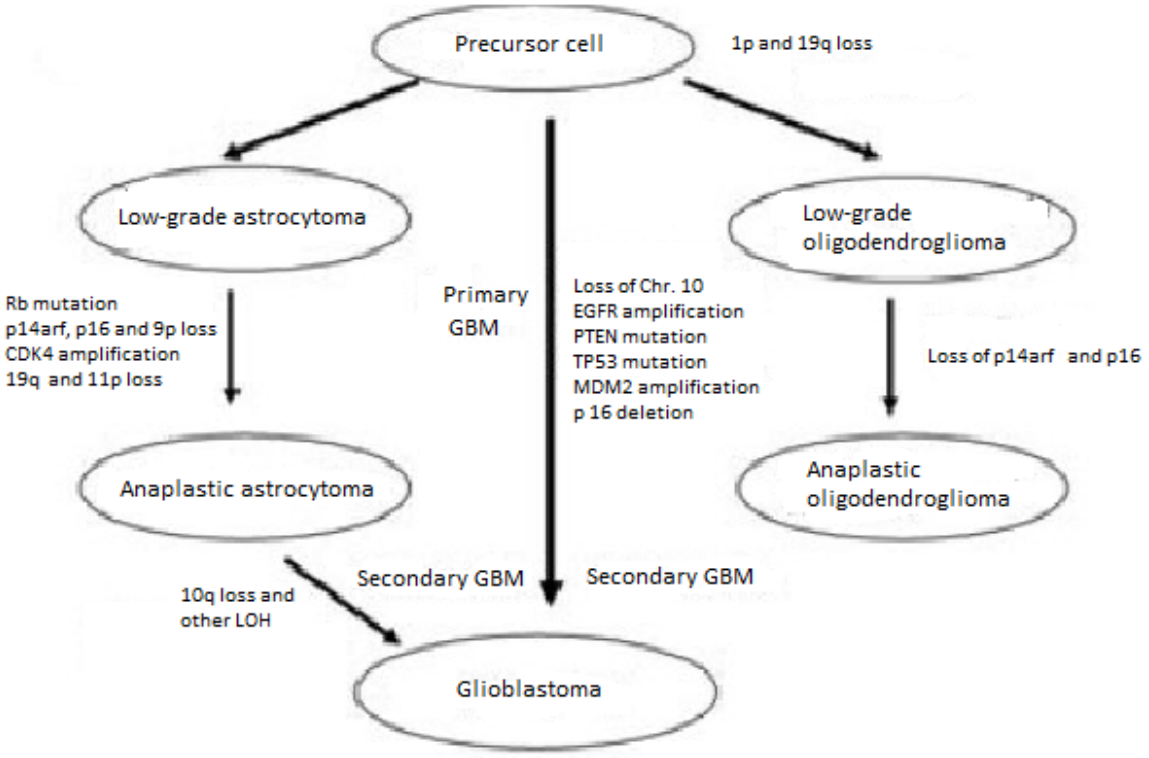


Figure1. Outline of the different mechanisms and molecular alterations that are frequently involved in the genesis of glioblastoma.

Glioblastoma multiforme presents four molecular subtypes, according to the data from The Cancer Genome Atlas (TCGA) Research Network. Different subtypes show different responses to chemotherapy and radiotherapy, with a difference of about 50% between subtypes. These findings could lead to more personalized approaches to therapy. The pathology of each subtype may start from different cell types and these differences might explain the variation in the response to treatment (Roel *et al* 2010, Verhaak *et al* 2010). The TCGA was based on 206 patient samples and sequence data from 601 genes in 91 patients. The genomic profiles allowed the classification into four GBM subtypes: Proneural, Neural, Mesenchymal and Classical. The reproducibility of this classification is shown in an independent validation set. It is unlikely that patients transition from one subtype to another during the course of the disease (Roel *et al* 2010).

GENETIC ALTERATIONS

Alterations of genes on chromosome 10

The most common abnormality identified in glioblastomas is loss of heterozygosity (LOH) at 10q (Ware *et al* 2003). The frequency of LOH on 10q is similar in GBM1 (70%) and GBM2 (63%) (Ohgaki *et al* 2006). On chromosome 10 have been identified different tumor suppressor gene (TSG). PTEN (phosphatase and tensin homolog) located at 10q23.3, DMBT1 (suppressor of malignant brain tumors) located at 10q25.3-q26.1, FGFR2 (fibroblast growth factor receptor 2) in the 10q26 region and MGMT (O6-methylguanine-DNA methyltransferase) that is located in 10q26. These TSG are involved in controlling the cell cycle and DNA repair. Inactivation of a TSG will produce a homozygous gene and therefore complete loss of their functionality (Mollenhauer *et al* 1997).

Alterations via TP53/MDM2/P14^{ARF}

The TP53/MDM2/P14^{ARF} genetic pathway is also subject to alterations in glioblastomas, (Koichi Ichimura *et al* 2000). This pathway is implicated in cellular response to stress, causing the activation of genes involved in cell cycle control, DNA repair and apoptosis (Amundson *et al* 1998). TP53 is a gene encoding the p53 protein which acts as a transcription factor (TF) binding to promoters of genes involved in DNA repair. This TF will promote the transcription of other genes involved in DNA repair and / or apoptosis. In normal cells the p53 protein is bound to its negative repressor MDM2.

In cells that do not have an altered pathway, the p53 binds to promoters that will activate genes involved in DNA repair. If the genetic material cannot be repaired the cell enters apoptosis before mitosis. Conversely, if the cell has the p53 mutated, cells that carry damage DNA will not be repaired resulting in increased cell division, decrease in apoptosis and in the repair of genetic material.

The p14^{ARF} may also intervene in this pathway; p14^{ARF} protein is a repressor of MDM2 when MDM2 is not bound to p53. When p14^{ARF} is not exerting a proper function, will trigger an excess of free MDM2, therefore high levels of MDM2 will bound more molecules of the p53 and as a result will occur a greater accumulation of DNA damage.

These genetic alterations in the cell may to changes triggering a tumor process. TP53 mutations, amplifications of MDM2 and / or deletions and methylation of p14^{ARF} are molecular alterations leading to an accumulation of other mutations in DNA, which may cause cell death or neoplastic transformation (Biernat *et al* 1997)

Alterations via p16I^{NK4}/RB1/CDK4

Another pathway that is involved in glioblastoma development is p16^{INK4} / RB1/CDK4. RB1 (retinoblastoma gene) is located on 13q, its protein is controlled by the G1-S transition in the cellular cycle (Sherr and Roberts 1999). When RB1 is not phosphorylated it binds to and inactivate E2F transcription factors that activate genes involved in the G1-S transition of the cell cycle when it is not attached to RB1. Phosphorylation of RB1 is produced by CDK4 (cyclin dependent kinase 4) and inhibit the protein responsible for this cyclin is p16^{INK4} (an inhibitor of cyclin dependent kinase 4). The homozygous loss of p16^{INK4}, and / or amplification of CDK4 cause continuous phosphorylation of RB1 phosphorylated thereby preventing it from binding to E2F, and as a result there is uncontrolled cell division (Burns *et al* 1998).

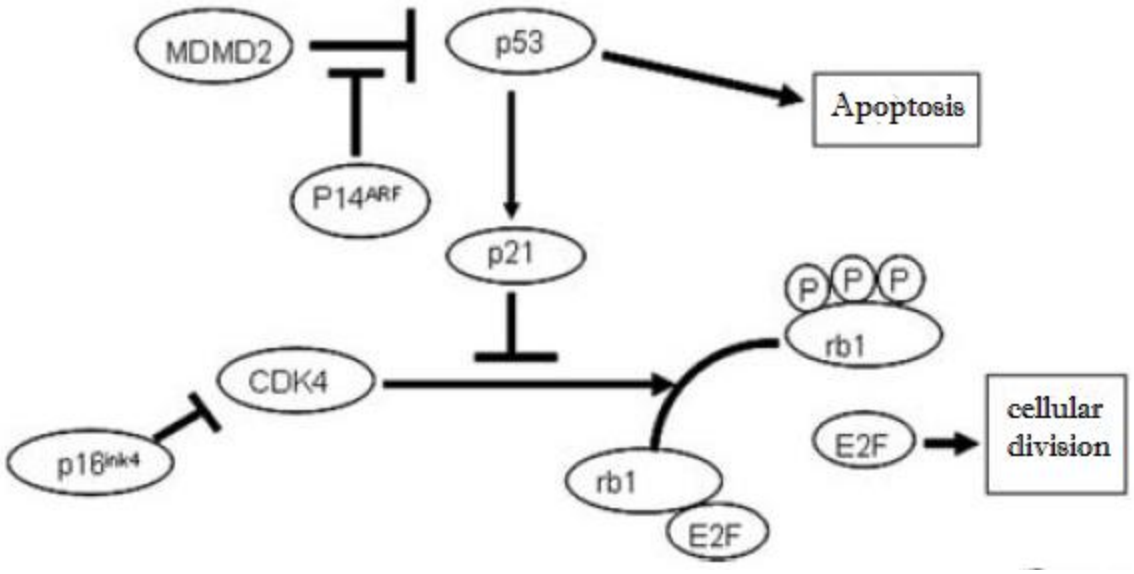


Figure 2 illustrates the connection of two pathways that control cell cycle TP53/MDM2/P14^{ARF} and CDK4 / P16^{INK4}/RB1. These two pathways are altered in glioblastomas. The pathway impairment associated TP53/MDM2/P14^{ARF} decreased however altering apoptosis pathway leads CDK4/P16^{INK4}/RB1 directly to an increase cell division (Hernandez and Martinez 2007).

EGFR gene alterations

Another genetic alteration of glioblastoma is present in the amplification of the gene encoding the growth factor receptor (EGFR) (Arjona *et al* 2006). EGFR is located on 7p12 and overexpression of its gene may be due, among other disturbances, to a tandem amplification giving rise to variants of the protein, the most common of which is the EGFRvIII13 variant. The ability of tumor cells to generate these functionally mutant forms of EGFR may contribute to the ability of gliomas to evade chemotherapy and, in addition, mutations present in the gene can also affect the prognosis or treatment (Layfild *et al* 2006).

PDGF/PDGFR alterations

It has been found that astrocytomas coexpress PDGF (Platelet derived growth factor) and its receptor, which does not occur in normal brain tissue. It has been suggested that the PDGFR-A isoform and its ligands play a role in the initial phases of gliogenesis because overexpression of this receptor and its ligands is often found in all grades of astrocytoma including in glioblastoma. Overexpression of PDGFR is usually associated with deletion of chromosome 17p (where the p53 gene locus is). It has been suggested that this chromosomal abnormality is unable, by itself, to trigger malignant transformation and therefore it requires the "collaboration" of PDGFR. There are many reports that show the importance of PDGF expression in glioma because it enhances the growth of glioma cell lines. For this reason there have been many attempts to identify different pathways that could suppress the expression of the protein and/or the receptor (Nazarenko *et al* 2012).

SOX PROTEINS

Transcription factors in the SOX superfamily of proteins contain a high mobility group domain (HMG) and play an important role in a wide range of developmental processes. These genes also maintain the normal function of certain cells after birth. To carry out these roles, genes in the SOX family provide instructions for making proteins that bind to specific areas of DNA. By binding to regions of DNA near genes, SOX proteins help control the activity of those genes. There are 20 different *Sox* genes in mammals that are divided into eight groups (A-H) (Wegner and Stolt 2005, Bowles *et al* 2000). Mutations in these genes may lead to disorders that involve the abnormal development of tissues in which a particular *Sox* gene is expressed. As a result, the genetic disorders caused by *Sox* mutations typically have a wide variety of signs and symptoms.

SOX5 AND GLIOMA

Subgroup SoxD contains *Sox5, Sox6* and *Sox13* that have been found to contribute to gliogenesis. *Sox5* has also been identified as a brain tumor locus (Btl) (Stolt *et al* 2006, Johansson *et al* 2004). *Sox5* has 15 exons and different splice variants give rise to different isoforms, such as long *(L-Sox5*) and short (*S-Sox5*) (Lefebvre et al. 1998, Tchougounova *et al*. 2009). It has also been shown that SOX5 can suppress PDGFB-induced glioma development in Ink4a-deficient mice (Tchougounova *et al* 2009). Proliferation of human glioma cells (HGCCs) cultured under stem cell conditions is inhibited when SOX5 is overexpressed, and there is a co-occurrence of SOX5 overexpression and PDGFRA amplifications particularly in the proneural subtype of gliomas.

AIM

The initial aim of this project was to study the effects of overexpression of human *hs-Sox5* in glioma using cell lines derived from human glioma patients (HGCCs); cultured in stem cell conditions for the enrichment of glioma initiating cells (GICs). But because of problems with different plasmids and lack of time the aim was changed in to designing and constructing a *hs-Sox5* lentiviral overexpression vector. The second aim of this project was to grow different glioma cell lines, some with low and some with high mRNA expression of *Sox5* and to perform western blot analysis, to check the protein concentration. The one with the lowest SOX5 protein concentration will then be used for future experiments.

Materials and methods

1 HUMAN GLIOMA CELL CULTURE (HGCC)

Five HGCC were grown in accordance with use for protein extraction. Cell expansion was carried out using complete GIC medium which contains: DMEM (dulbecco modified eagle medium)/F12 Glutamax (GIBCO) and neurobasal medium mixed 1:1 with addition of 1 % B27 (Invitrogen), 0,5 % N2 (Invitrogen), 1 % penicilin/streptomycin (Sigma) and 10 ng/ml of EGF (epidermal growth factor) and FGF2 (fibroblast growth factor 2) (Prepotech). Culture vessels were coated with laminin (Sigma) for 1 hour at 10 µg/ml prior to use. Cells were routinely grown to confluence, dissociated using accutase (Sigma) and then split 1:4. Medium was replaced every 3-4 days.

2 WESTERN BLOT

2.1 Protein extraction
The protein extraction was done by scraping the cells into PBS, after which the cells were collected in an Eppendorf tube. The cells were centrifuged down at 4ºC for 5 min at 3000 rpm before adding lysis buffer (138 mM NaCl, 2 mM EDTA , 10 % Triton X-100, 10 mM Tris HCl pH 8, 0.5 % NP40) with protein inhibitors (57.4 mM PMSF, 100 % trasylol, 200 mM Na3VO4, 1M NaF, 1M ZnCl2) and incubated for 30 min on ice. It was important to maintain cold temperature from this step. Lysates were centrifuged for 15 min at 13000 x g at 4°C. The supernatant was collected into a fresh tube and store at -20 until use.

2.2 Determination of protein concentration
Protein concentrations were determined using the Pierce BCA protein Assay Kit (Thermo scientific).The Roti-Quant Bradford reagent was used for the determination of protein concentration of the samples. BSA (25 to 2000 µg/ml) was used to obtain a standard curve for determination of the protein concentration of unknown samples. The cells lysates were diluted to 1:20 before determining the protein concentration. Quantification was performed by measuring the OD at 595 nm, using the standard curve feature of an Eppendorf BioPhotometer (Eppendorf, Germany).

2.3 SDS-PAGE and Western
Separation of proteins was performed by denaturing on a NuPage 3.8 Tris-acetate gel of 1.0 mm x 10 wells (Invitrogen). Samples were resuspended in running buffer (35ml MOPS buffer, 1.75 ml antioxidant in 665 ml purified water) and heated to 96°C for 5 min prior to loading. The gel was run in a SDS-PAGE running buffer for 1 hour at 200V. Gene Ruler pre-stained marker (Fermentas, Germany) was used as a protein molecular weight marker. For gel transfer, the iBlot Gel transfer system (Invitrogen) was used to place the gel onto a nitrocellulose membrane according to the manufacturer's protocol. Transfer was performed at 200 mA for 60 min. The presence of protein was checked with Ponceau staining (Sigma). Membranes were blocked in 5 % BSA in TBS-T 0.1 % for 1 hour on a shaker and then incubated with primary antibodies overnight at 4°C. Membranes were washed 4 times for 10 min in TBS-T 0.1 %. Then it was incubated with the secondary antibodies for 1 hour at RT and then it was washed 4 times with TBS-T 0.1 %. After brief incubation with ECL solution the luminescence was detected on X-ray films.

3 AMPLIFICACION OF HS-SOX5

In order to amplify the *hs-Sox5* gene, primers were designed to target the gene (see appendix).

Polymerase chain reaction cycle
Temperature settings; for initial denaturation were 5 min at 95°C; 25 cycles of denaturation (98°C for 10 min), annealing (60°C for 10 sec) and extension (68°C for 1 min 20sec), the final extension step was for 2 min at 72°C. The reaction mixture is shown in table 1.

Table 1 Polymerase chain reaction mixture

Reaction mixture (total 25 µl)	Volume µl
5x PrimeSTAR Buffer (Mg2+ plus)	5
dNTP mixture (2.5mM each)	2
Template (Plasmid)- 50ng	0.5
PrimeStar Hs DNA polymerase	0.4
Forward primer (10 µM)	0.75
Reverse primer (10 µM)	0.75
distilled H2O	10.6

Three µl were taken from the PCR product and visualised on a 1 % agarose gel using TAE buffer and diluted in 6X DNA loading dye (Fermentas) before being loaded onto the gel. A 1 Kb DNA ladder (Fermentas) was used for size determination of the fragments.

4 CLONING OF THE HS-SOX5 INTO PENTR4 VECTOR TO CREATE AN ENTRY CLONE

4.1 Restriction digestion
A digestion reaction was carried out for 15 min at 37°C. The reaction mixture is shown in table 2.

Table 2 Restriction digestion reaction mixture

Components	Pentry4	Hs-SOX5 pcr product
DNA	4 µl	10 µl
HFSalI	0.5 µl	0.5 µl
HFNotI	0.5 µl	0.5 µl
BSA	0.2 µl	0.2 µl
Buffer 4	2 µl	2 µl
ddH20	13 µl	7 µl

Note that all the components were from Biolab

4.2 Gel electrophoresis and gel extraction
The plasmid (pentr4 vector) and PCR products were visualized by 1 % agarose low melting temperature gel. The DNA samples were diluted in 6X DNA loading dye (Fermentas) before being loaded onto gel. A 1 kb DNA Ladder (Fermentas) was used for size determination of the fragments. After gel electrophoresis, the gel was purified using the Qiaquick gel extraction Kit (Qiagen) according to the manufacture's protocol.

4.3 Ligation reaction

The ligation reaction was carried out in order to create an entry clone which had the hs-SOX5 inserted in the pentr4 vector. The molar ratio of the vector and plasmid was 1:3. Thereafter the mixture of all reagents (Table 3), the sample and control mixture were incubated in a 16°C water bath overnight.

Table 3 ligation reaction mixture

Components	Sample	Control
Vector	2 µl	1 µl
Insert	8.6 µl	-
5X T4 DNA ligase buffer	1.5 µl	1.5 µl
T4 DNA ligase	0.5 µl	0.5 µl
Sterile water	3.4 µl	12 µl

4.4 Tranformation

The sample and control ligation from the reaction shown in 4.3 were transformed into E.coli sbtl3 by the One Shot method for chemically competent cells (Invitrogen). Five µl of each ligation reaction (control and sample) was added into one vial of One Shot Stbl3 chemically competent E. coli. This reaction was kept on ice for 30 min and then subjected to a heat shock for 30 seconds at 42°C. After this the suspension was immediately transferred on ice for 2 min. After the treatment bacteria cells were mixed with 250 µl of SOC medium (15544-034, Invitrogen) and incubated at 37°C in shaker for 1 hour. The transformation mixture was placed and streaked onto kanamycin 50 µg/ml containing agar plates, and incubated overnight at 37°C. Single colonies were observed next day and used for culture.

4.5 Plasmid preparation and restriction digestion

Single colonies were picked from the LB agar plate and used to inoculate two starter cultures of 5 ml LB medium containing 50 µg/ml kanamycin. Cultures were incubated overnight with gentle shaking at 37°C. Following incubation the bacteria cells were harvested by centrifugation at 4000 g for 15 min at 4°C. All further purification steps were performed using the Quiaprep Spin Miniprep Kit (Qiagen) according to the manufacturer's directions. DNA was dissolved in dH2O and the concentration was measured using NanoDrop® ND-1000 spectrophotometer (Thermo Scientific).

Five µl of the purified plasmid (pentr4-hsSox5) were taken and digested with 1 µl of XmnI enzyme and to the mixture we also added 2 µl of Buffer 4, 0.2 ul of BSA and 12 µl of dH2O. The reaction mixture was incubated overnight at 37ºC and run on a 1 % agarose gel.

4.6 Polymerase chain reaction of the entry clone

Temperature settings were carried out as described in 3, the components used in the mixture are shown in table 4. The PCR product was run in 1 % agarose gel.

Table 4 PCR mixture

Reaction mixture (total 50 µl)	Volume µl
5x PrimeSTAR Buffer (Mg2+ plus)	5
dNTP mixture (2.5mM each)	1.5
Template (Plasmid)- 50ng	1
PrimeStar Hs DNA polymerase	0.5
Forward primer (10 µM)	1.5
Reverse primer (10 µM)	1.5
distilled H2O	39.5

5 CREATION OF AN EXPRESSION CLONE USING THE LR RECOMBINATION REACTION

A LR recombination reaction was carried out using the pLenti 6.3/TO/V5/Dest as the destination vector and the pEntr-gus control vector.
After that the components were mixed (Table 5), we added 2 µl of Gateway LR Clonase II plus enzyme mix to each of the recombination reactions and incubated at 25°C overnight. Thereafter we added 1 µl of the Proteinase K solution to each recombination reaction and incubated for 10 min at 37°C, after the incubation we preceded to transformation in E. coli stbl3 competent cells. All following steps were performed as described in 4.4 but instead of kanamycin we added ampicilin 100 µg/ml.

Table 5 LR recombination reaction mixture

Component	Sample	Control
Entry clone (50 ng/reaction)	1 µl	-
Destination vector (150ng/reation)	1 µl	1 µl
pEntr- gus (50 ng/ µl)	-	1 µl
TE buffer, ph 8.0	4 µl	4 µl

5.1 Colony PCR
The colony PCR was performed on 7 colonies. Temperature settings were initial denaturation for 5 min at 95°C; 25 cycles of denaturation (98°C for 10 min), annealing (60°C for 10 sec) and extension (68°C for 1 min 20sec), final extension step was for 2 min at 72°C.

Table 6 PCR reaction mixture

Components	**Volume**
KAPA2G Fast Genotyping Mix	5 µl
Forward primer CMV	0.25 µl
Reverse primer V5	0.25 µl
dH2O	4.5 µl

The PCR product was run on 1 % agarose gel electrophoresis, following steps were performed as described in 5. We took three positive colonies and grown in 5 ml LB medium with 100 µg/ml ampicilin and incubated overnight. The next day the cultures were spin it down for 12 min at 4000g 4ºC. All further purification steps were performed using the Quiaprep Spin Miniprep Kit (Qiagen) according to the manufacturer's directions. DNA was dissolved in dH2O and the concentration was measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA).

6 GENEART SITE-DIRECTED MUTAGENESIS (SDM) (INVITROGEN)

The V5 epitope of our expression clone had a stop codon that did not permit the translation of the V5 tag. In accordance to express the V5 tag it was added an AC nucleotide by using the site directed mutagenesis method (SDM). With the SDM method it could be inserted the two nucleotides that it was needed to translate our entire sequence.

6.1 Methylation and mutagenesis reaction
The plasmid must first be methylated and amplify by PCR with primers containing the desired mutation (see appendix).

6.1.1 PCR
Temperature settings were methylation of the plasmid for 20 min at 37ºC, initial denaturation for 2 min at 94°C; 18 cycles of denaturation (94°C for 20 sec), annealing (57°C for 30 sec) and extension (68°C for 1 min 30 sec), final extension step was for 5 min at 68°C. The reaction mixture is shown in table 7. Five µl of the PCR product was analyse in 0.8 % agarose gel.

Table 7 Polymerase chain reaction mixture

Component	Volume µl
10X AccuPrime Pfx reaction mix	5.0
10X Enhancer	5.0
Primer mix (10 µM each)	1.5
Plasmid DNA (20 ng/µl)	1
DNA Methylase (4U/µl)	1.0
25X SAM	2.0
AccuPrime Pfx (2.5 U/µl)	0.4
PCRWater	35.1

A recombination reaction was performed to enhance the colony output.
After all components were mixed (Table 8), the recombination reaction was incubated at RT for 10 min. The reaction was stopped by adding 1 µl 0.5 M of EDTA and was continued immediately to transformation into E. coli stbl3 competent cells. All following steps were performed as described in 4.4. The E. coli sbtl3 cells have the MrBC endonuclease which digest the methylated plasmid so that the cells with the non-methylated plasmid which have the desired mutation are the ones that survived.

Table 8 Recombination reaction mixture

Component	Volume µl
5X reaction buffer	4
PCR water	10
PCR product	4
10X enzyme mix	2

RESULTS AND DISCUSSION

1 WESTERN BLOT

To performed western blot we used 5 different cell lines of which two had low mRNA expression levels and the other three had high mRNA expression (see appendix). We wanted to know which cell line had the lowest expression of the SOX5 to further use it for transfection with the sox5 gene and study overexpression of the gene in glioma cell line.

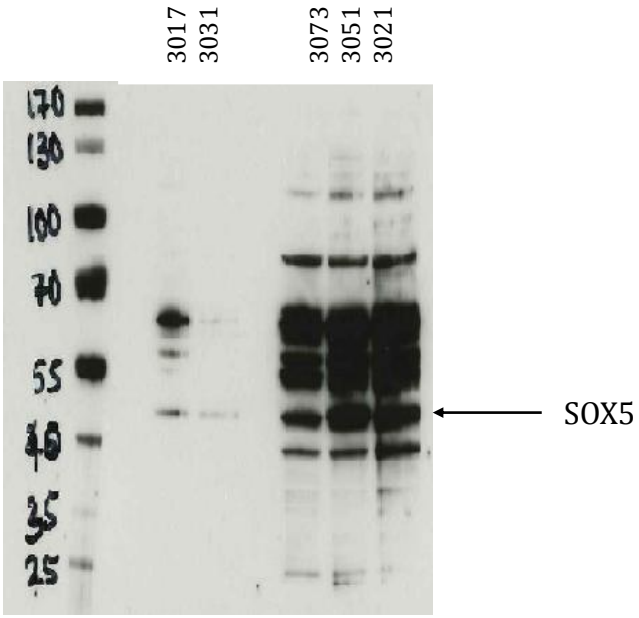
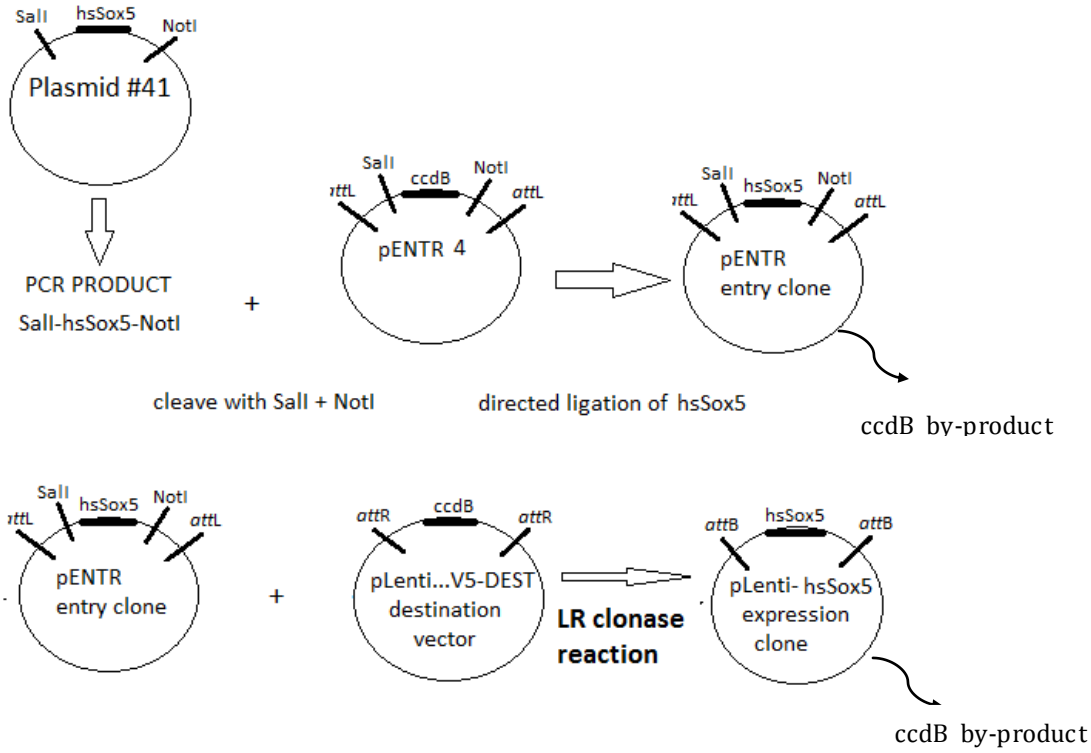


Figure 1 shows the Sox5 protein concentration of five different cell lines. The proteins were separated by SDS-PAGE and then they were transferred into a nitrocellulose membrane. The membrane was incubated with the first antibody ms-Sox5 (1:1000) then it was incubated with the second antibody □-ms-HRP (1:2000) and as a blocking solution 5% BSA. SOX5 protein has a molecular mass of 41.99 kDa

Western blot was used to confirm if the mRNA expression was in accordance with the protein concentration of SOX5 in the different cell lines According to the results, the protein concentrations agree with the mRNA expression of the cell lines used in this experiment. The cell line U3031 is the one that shows the lowest protein expression and therefore the one that is more susceptible to study the overexpression of SOX5. We used different dilutions because the concentration of the extracted lysate was not sufficient and it was not possible to extract more because of time issue. The western blot can be improve in the future by using another blocking solution like milk so that one does not get not specific bands and also tried different dilutions of the antibodies.

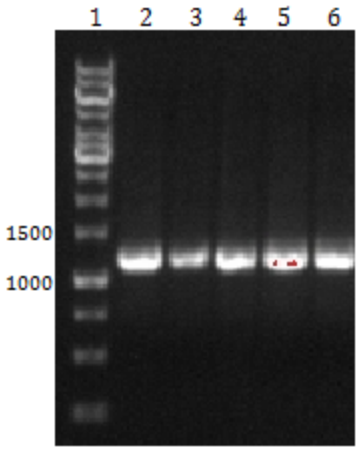
2 SCHEMATIC OVERVIEW OF THE LENTIVIRAL SYSTEM



Note: The *ccdB* gene gives negative selection of the plasmid.

3 VERIFICATION OF THE AMPLIFICATION OF HS-SOX5

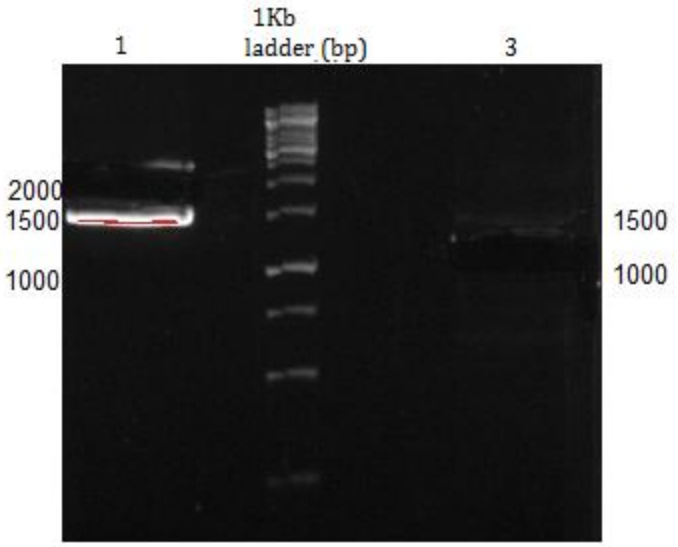
PCR was performed to amplify the *hs-Sox5* gene. The PCR was performed using gene specific primers (see appendix), and the PCR product was analyzed on a 1 % agarose gel together with a 1kb DNA ladder to see the fragment size. The results from the PCR showed the amplified *hs-Sox5* gene (Figure 2). According to the used DNA ladder, the *hs-Sox5* gene has the expected size of around 1131 base pairs.



Figur 2 PCR product use to amplifiy *hs-Sox5* gene using the Hu_s-Sox5_SalI forward primer and the Hu_s-Sox5_NotI reverse primer. Four ul of the PCR were run onto 1 % agarose gel Lane1: 1kb DNA ladder (bp) and Lane 2-6: amplified *hs-Sox5*.

4 CONSTRUCTION OF THE PENTRY VECTOR

The amplified PCR product and the pentr4 vector were digested with HFNotI and HFSalI restriction digestion enzymes. The reactions were loaded on 0.8 % low melting agarose gel (Figure 3). A positive digestion of the pEntry4 gives 1486 and 2271 bp meanwhile hsSox5 1131 bp.



Figur 3 The restriction digestion of the pEntry 4 vector and *hs-Sox5* gene using HFNotI and HFSalI enzyme. Lane 1 pentry4 vector, Lane 2 1Kb DNA ladder and Lane 3 *hs-Sox5* gene.

The PCR product and the pentr4 vector were extracted from the gel and ligated. Following the ligation, the vector was transformed into E. coli stbl3 competent cells. Selection of positive transformed cells was done making use of the kanamycin resistance. A restriction enzyme digestion was conducted using XmnI enzyme to verify the existence of the vector. The expected fragments sizes were 538 and 2871 bp

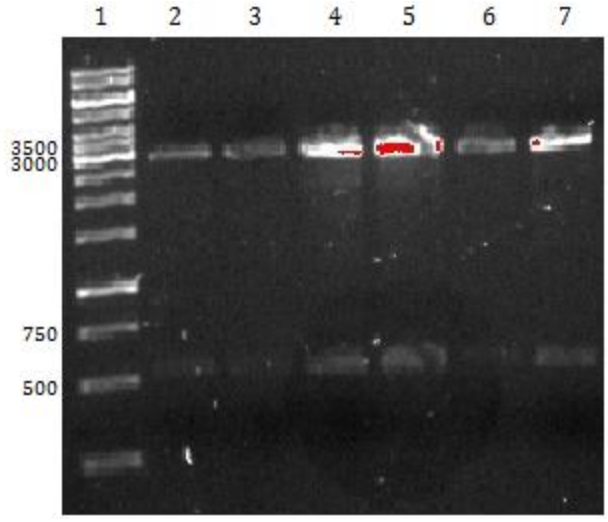


Figure 4 XmnI was used to digest the pentr4-hsSox5 plasmid to confirm that a positive ligation had occurred to yield a positive entry clone. A 1% agarose gel was used to detect DNA digestion products with the size of 538 and 2871 bp. Lane1: 1 kb DNA ladder (bp); Lane 2-7: digested entry clone.

Construction of the pEntry vector containing the *hs-Sox5* gene was further confirmed by PCR using specific primers (see appendix) that targeted the gene. According to the restriction digestion result (Figure 4), sample 3, 4 and 6 were chosen for the PCR reaction. All three samples gave a positive insertion of the *hs-Sox5* gene (Figure 5).

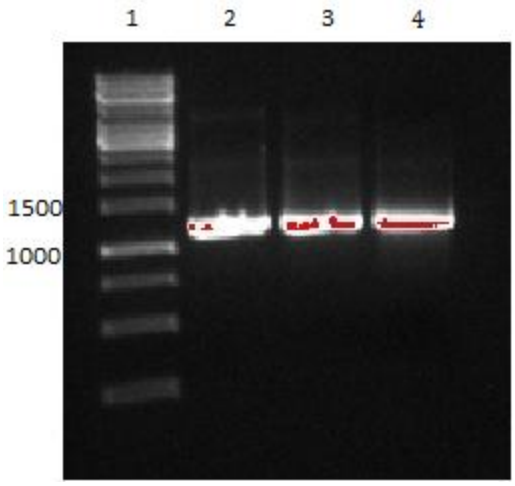


Figure 5 PCR product used to confirm the existence of the *hs-Sox5* gene into the pEntr4 vector using Hu_s-Sox5_SalI forward primer and the Hu_s-Sox5_NotI reverse primer. Four ul of the PCR were run onto 1 % agarose gel Lane1: 1Kb DNA ladder (bp) and Lane 2-4: amplified hs-Sox5. A positive amplified gene gives 1131 bp.

5 CONSTRUCTION OF THE EXPRESSION CLONE

The positive entry clone was use to create an expression clone in which the *hs-Sox5* was inserted in an appropriate destination vector in this case it was pLenti6.3/TO/V5-Dest. To confirm the cloning of the *hs-Sox5* into the destination vector a PCR was conducted to verify the integrity of the *hs-Sox5* gene into the vector and to confirm the orientation of ligation using one primer targeting the gene and the other primer targeting the vector (see appendix).

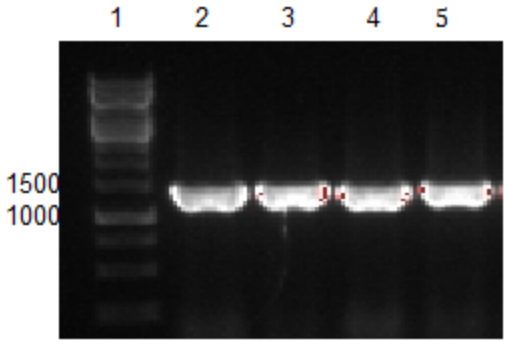


Figure 7 PCR product used to confirm the existence of the *hs-Sox5* gene in the expression clone using the CMV forward primer and the V5 reverse primer. Four ul of the PCR were run onto 1 % agarose gel to check the present of the gene. Lane 1: 1 kb DNA ladder (bp), Lane 2-5: positive amplified fragment (1314bp)

XmnI was used to digest the expression clone to confirm that right recombination had occurred between the pLenti6.3 vector and the *hs-Sox5* gene. A 1% agarose gel was used to detect DNA digestion products with the size of 538, 1950, 2518 and 3872 bp.

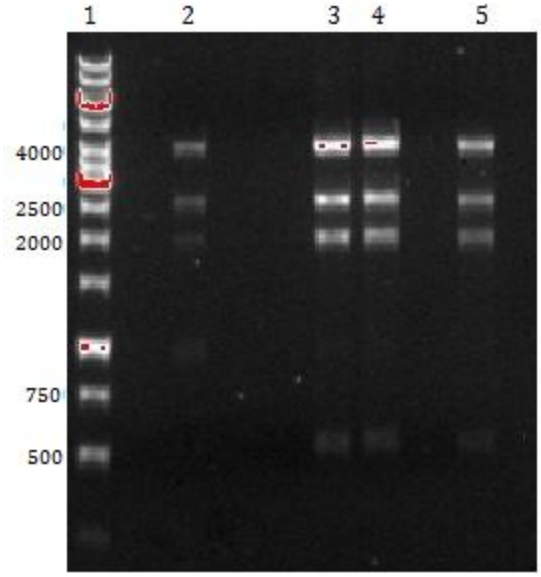


Figure 6 XmnI was used to digest the pLenti6.3-hsSox5 to confirm the creation of a positive expression vector. A 1 % agarose gel was used to detect the DNA digestion products with the size of 538, 1950, 2518 and 3872 bp. Lane 1: 1 kb DNA ladder (bp); Lane 2: negative expression clone; Lane 3-5: positive expression clone.

The *hs-Sox5* gene was successfully integrated into pEntr4 vector. However, there were some problems with the construction of the expression clone. After the transformation into E.coli sbtl3 competent cells, I got no growth in some plates and non-ampicilin bacterial grow in others. It could be possible that the transformation worked but the growth of ampicilin non-resistant bacteria might overgrow the real transformants. To improve transformation efficiency, the plates were let to growth in less time around 16 h. After this change it was possible to get positive clones but still with growth of ampicilin resistant colonies. Another way in which this could be avoided could be by using not only ampicilin but also chloramphenicol as an additional selection of the plasmid.

FUTURE PERSPECTIVES

The pLenti_ Hs_s-Sox5 will be co-transfected with the viral packaging mix into the 293F producer cell lines. The viral supernatant containing the pLenti_ Hs_s-SOX5 is then harvested and used to infect the HGCCs. The Lentiviral vector is then going to be used to infect the human glioma cell to further analyse the overexpression of the *Sox5* gene in cell proliferation, colony forming ability, self-renewal, apoptosis, senescence and in vivo tumorigenesis by comparing to control infected HGCCs.

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APPENDIX

Hu-man s-Sox5 protein sequence
377 a.a. MW=41.99 kDa

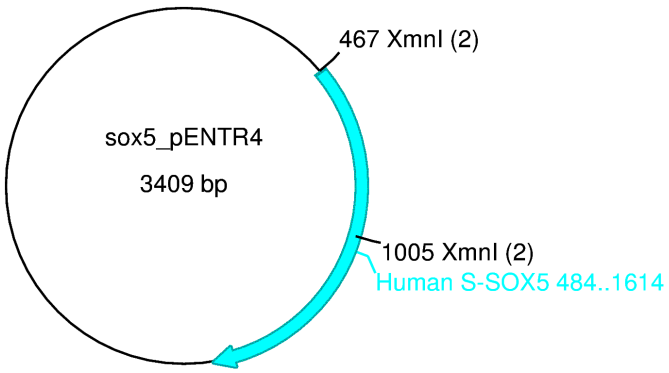
MHDEVAQPLNLSAKPKTSDGKSPTSPTSPHMPALRINSGAGPLKASVPAALASPSARV
STIGYLNDHDAVTKAIQEARQMKEQLRREQQVLDGKVAVVNSLGLNNCRTEKEKTT
LESLTQQLAVKQNEEGKFSHAMMDFNLSGDSDGSAGVSESRIYRESRGRGSNEPHIK
RPMNAFMVWAKDERRKILQAFPDMHNSNISKILGSRWKAMTNLEKQPYYEEQARLS
KQHLEKYPDYKYKPRPKRTCLVDGKKLRIGEYKAIMRNRRQEMRQYFNVGQQAQIP
IATAGVVYPGAIAMAGMPSPHLPSEHSSVSSSPEPGMPVIQSTYGVKGEEPHIKEEIQA
EDINGEIYDEYDEEEDDPDVDYGSDSENHIAGQAN

Primer name	Sequence 5' to 3'
Hu_s-Sox5_SalI.F	GCATGTCGACATGCATGATGAAGT GGCACAGCC
Hu_s-Sox5_NotI.R	GCATGCGGCCGCGTTGGCTTGTCC TGCAATATGG
CMV.F	CGCAAATGGGCGGTAGGCGTG
V5.R	ACCGAGGAGAGGGTTAGGGAT
SDM_F	AAGCCAACACGCGGCCGC
SDM_R	GCGGCCGCGTGTTGGCTT

From Invitrogen Custom Primers

Cell Line	S-Sox5 mRNA
U3017	771.577
U3031	76.462
U3073	116.7
U3051	758.322
U3021	1488.868

Map of the sox5-pEntr4 digested with XmnI



pLenti 6.3/TO/V5-Dest-hs-Sox5 Map