Using CRISPR/Cas9 to develop a serglycin-deficient canine mammary tumor cell line

Preparation for future exploration of the involvement of serglycin in carcinogenesis

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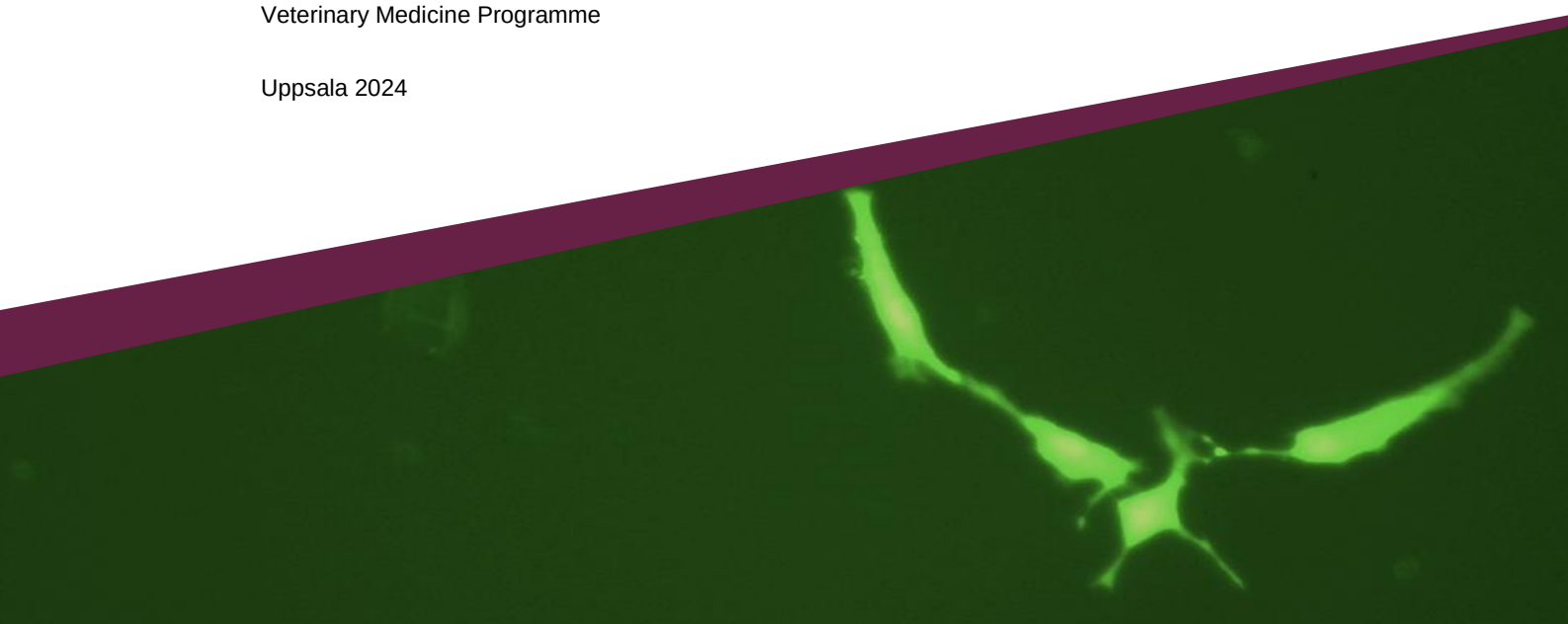

Using CRISPR/Cas9 to develop a serglycin-deficient canine mammary tumor cell line – Preparation for future exploration of the involvement of serglycin in carcinogenesis

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Abstract

Canine mammary tumor (CMT) is the most common form of cancer in bitches and is a common cause of death. Around a third to a half of the CMT are malignant, and carcinomas are a majority of those. In this study a cell line, called CMT-U27, derived from a canine mammary carcinoma was subjected to an attempt to knock out a gene by using the common gene editing method CRISPR/Cas9. With CRISPR/Cas9 it is possible to create a double-strand break in the DNA and insert a piece of donor DNA to give the cell certain desired abilities. In this study the donor DNA used contained sequences for fluorescent proteins and antibiotic resistances to enable the selection of the cells with a successful knock-out.

The goal was to knock out the gene for serglycin, a proteoglycan expressed by a wide variety of cells. Serglycin is strongly associated with inflammation and has normally many different functions. Among them, it has an important role in storage and secretion of bioactive substances in different immune cells and plays a part in regulation of inflammation. Changes in the expression of serglycin has been reported in multiple forms of cancers and serglycin has been linked to an increased malignancy. Establishing a serglycin-deficient cell line would facilitate further research about the involvement of serglycin in carcinogenesis.

The CMT-U27 cell line were transfected with the donor DNA by using the method electroporation. The cells then underwent an antibiotic selection to try to discern the transfected cells from the others. The cells showed promising results early after the transfection. Some of the cells emitted green and/or red fluorescence which is a sign that the donor DNA have been incorporated and expressed. A double fluorescence can indicate a bi-allelic knock-out which is the desired outcome. Unfortunately, the cells did not survive the antibiotic selection. A possible reason for their death could be an unsuccessful integration of the donor at the desired location or a too immediate start of the antibiotic selection after the transfection. No confirmed serglycin-deficient cell line was able to be established due to a lack of time but experiences from this experiment will be valuable for future trials.

Keywords: Serglycin, canine mammary tumor, CMT, CMT-U27, CRISPR, Cas9, homology-directed repair, HDR

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Abbreviations

Cas9	CRISPR associated protein 9
CMT	Canine mammary tumor
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DSB	Double-strand break
EMT	Epithelial-mesenchymal transition
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
HDR	Homology-directed repair
NHEJ	Nonhomologous end joining
PAM	Protospacer-motif
PBS	Phosphate-buffered saline
RFP	Red fluorescent protein
RPMI	Roswell Park Memorial Institute
sgRNA	Single guide RNA
SLU	Swedish University of Agricultural Sciences
tracrRNA	Trans-activating CRISPR RNA

1. Introduction

The purpose of this study was to develop a serglycin-deficient carcinoma cell line to be used as an in-vitro model for future exploration of functional aspects of the proteoglycan serglycin in canine mammary tumors. The objective was to achieve a knock-out of the serglycin gene by using CRISPR/Cas9, a commonly used system for gene editing.

Canine mammary tumor (CMT) is the most common form of cancer in bitches (Sleeckx *et al*. 2011) and is a common cause of death as well (Bonnett *et al*. 2005). Around a third (Rivera 2010) to a half of all CMT are malignant and carcinomas constitutes a majority of those (Sleeckx *et al*. 2011).

Serglycin is a proteoglycan expressed by many types of cells and is very common in different immune cells (Kolset & Pejler 2011). Serglycin has a wide variety of functions and is strongly associated with inflammation (Korpetinou *et al*. 2014). A lot of different forms of cancer have been shown to have an increased expression of serglycin (He *et al*. 2013; Korpetinou *et al*. 2015). There are correlations between elevated expression of serglycin and an increased malignancy in various forms of cancer (Li *et al*. 2011; He *et al*. 2013; Guo *et al.* 2017).

The studies in the previous paragraph are focused on human forms of cancer and very few studies have been performed on CMT and serglycin. However, there are multiple sets of genes and signal pathways that follow the same pattern when it comes to canine mammary tumors and human breast cancer (Dobson 2013), and therefore both species benefit from advances done in this field of research.

2. Literature review

2.1 Serglycin

2.1.1 Overview

Proteoglycans consists of a core protein and chains of glycosaminoglycans (GAG) connected to it. Serglycin is a proteoglycan with a long repetitive sequence of serine and glycine in the core protein and this repetitive sequence is the origin of its name (Kolset & Tveit 2008). Serglycin has different glycosaminoglycans, such as heparin, heparan sulfate or chondroitin sulfate, connected to the core protein depending on the cell type. Heparin and heparan sulfate are the main GAG in mast cells while lymphocytes mainly carry chondroitin 4-sulfate. The level of sulfation differs between the types of glycosaminoglycan and the glycosaminoglycans are a probable source of the different function serglycin has in the different cells that express it.

Serglycin is found mainly intracellularly (Kolset & Tveit 2008) but have also been shown to be attached to the cell surface and to be secreted (Purushothaman & Toole 2014). Monocytes and macrophages are cell types that secrete it constantly (Kolset & Tveit 2008). Many hematopoietic cells have a high expression of the proteoglycan but a lot of other types of cells such as endothelial cells, smooth muscle cells and chondrocytes also express serglycin (Kolset & Pejler 2011).

Serglycin have a wide variety of functions and is strongly associated with inflammation (Korpetinou *et al*. 2014). It has an important role in storage and secretion of bioactive substances such as proteases and granzymes in different kinds of immune cells. It interacts with mast cell proteases and regulates their activity, plays a part in apoptosis, and interacts with matrix metalloproteases. It has an immunomodulating effect *e.g.*, by inhibiting the complement system (Skliris *et al*. 2011).

2.1.2 Genetics

The gene for serglycin has three exons and is located on chromosome 4 in dogs (Sayers *et al.* 2022). The coding sequence for the repeat of serine and glycine in the core protein is found in exon 3 (Kolset & Tveit 2008). An alternative splicing of the serglycin gene which lacks exon 1 have been transcribed and this splicing results in a 146 amino acid long protein instead of the original one with 159 amino acids (Martin *et al.* 2023)

In humans, the serglycin gene is located in chromosome 10 and its three exons result in the core protein consisting of 158 amino acids (Manou *et al*. 2020b). An alternative splicing without exon 2 has been known for a long time in humans (Castronuevo *et al.* 2003).

2.2 Canine mammary tumour

Canine mammary tumour (CMT) is the most common form of cancer in unneutered bitches and a majority have multiple neoplasms (Sleeckx *et al*. 2011). CMT is one of the most common cancer related sources of death in dogs regardless of gender but especially in bitches (Bonnett *et al*. 2005). The risk of dying of cancer is almost twice as high for bitches than it is for male dogs, and it is most likely because of the high mortality rate of mammary tumors.

There are different types a CMT can be classified as depending on the tissue or tissues it originates from and the grade of malignancy. There are multiple forms of carcinomas, adenomas, sarcomas, and combinations of different types of tissue. Around half of all CMT have been reported to be malignant and carcinomas constitutes a majority of those (Sleeckx *et al*. 2011) but the frequency of malignant tumors may depend on the study population because a study on Swedish dogs showed that 32% of the examined CMT were malignant (Rivera 2010).

CMT triggers an inflammatory response with different inflammatory cells such as mast cells and lymphocytes but the connection between malignancy and the inflammatory response is yet to be determined (Sleeckx *et al.* 2011).

2.3 The role of serglycin in cancer

2.3.1 Canine mammary tumor

Very few studies have been conducted on the relationship between serglycin and canine cancer forms. However, in two recent projects done by veterinary students,

formalin-fixed and paraffin-embedded CMT samples were analyzed with qPCR and the results indicate that serglycin levels were increased in malignant forms of CMT but decreased in benign forms (Jönsson 2019; O'Shee I Sánchez 2021).

2.3.2 Human cancer forms

Cancer cells can express more serglycin than their original non-neoplastic cell type. Serglycin expression has been shown to be upregulated in patients with hepatocellular carcinoma and it was linked with a poor prognosis (He *et al*. 2013). The cancer cells can also express different amounts of serglycin depending on their grade of malignancy. A study showed that a highly aggressive breast cancer cell line expressed more serglycin than the medium aggressive cell line, and the least aggressive cell line expressed the least amount of serglycin (Korpetinou *et al.* 2013). The expression of serglycin was elevated in more aggressive forms of breast cancer, lung cancer and prostate cancer when compared to their less malignant forms, but the circumstances were reversed with colon cancer (Korpetinou *et al*. 2015). Not all cancer forms have an increased expression on serglycin. Decreased expression of serglycin in gastric cancer has been linked to bad prognosis (Wang *et al*. 2016b). The decreased expression was associated with for instance increased metastasis, increased depth of invasion and poor survival. In another study, the levels of serglycin in colon tumors were neither increased or decreased (Suhovskih *et al*. 2015).

Breast cancer cells have been shown to have an increased expression of serglycin, but it has also been propositioned that it may be the stromal cells that are the major source of elevated serglycin levels in breast tumor tissue (Tellez-Gabriel *et al*. 2022). It may be because of the invading immune cells such as mast cells and lymphocytes and not the cancer cells, at least in the examined aggressive breast cancer subtype.

Serglycin has also been linked to an increased malignancy. Serglycin has been shown to enhance the metastatic potential, *e.g.* through increased cellular migration and invasiveness, of nasopharyngeal carcinomas (Li *et al*. 2011). Suppression of the expression of serglycin in glioblastoma gives a decrease in aggressive behavior, pluripotency and tumor growth (Manou *et al*. 2020a). The upregulated serglycin expression in hepatocellular carcinoma was also correlated to changes in levels of biomarkers signalling epithelial-mesenchymal transition (EMT) (He *et al*. 2013). Neoplastic cells that are undergoing EMT is gaining more malignant properties such as increased metastatic abilities and resistance to chemotherapy (Tellez-Gabriel *et al.* 2022). Secreted serglycin promotes the aggressiveness of non-small cell lung cancer through interaction with the cell surface receptor CD44 which is linked to promoting EMT (Guo *et al*. 2017).

Serglycin from multiple myeloma cells inhibits activation of both the classical and the lectin pathway of the complement system (Skliris *et al*. 2011). This inhibits the activation of some proinflammatory processes and may obstruct the early defence against the tumorigenesis and it protects multiple myeloma cells from chemo-therapy induced complement attack. Serglycin from aggressive breast cancer cells have also been shown to inhibit the classical pathway (Korpetinou *et al*. 2013).

In a study with serglycin-deficient mice it was shown that serglycin is a necessity for metastasis to the lungs from mammary tumors (Roy *et al*. 2016). The cancer cells were able to undergo intravasation but were not able to extravasate.

2.4 CRISPR/Cas9

2.4.1 Overview

Clustered regularly interspaced short palindromic repeats (CRISPR) is originally an immunological defense system used by many bacteria and archaea to cleave invading genetic material (Wang *et al*. 2016a). The CRISPR-system uses many different CRISPR-associated proteins (Cas) for the targeted cleaving of incoming foreign DNA. Of those, Cas9 is the most frequently used for genome editing and the most common Cas9 originate from *Streptococcus pyogenes* and are therefore named Sp Cas9. Cas9 is an endonuclease that cleaves DNA, and it is guided to its target by a guide RNA (gRNA). A feature of Cas9 is that it requires a protospacer-motif (PAM) of DNA in proximity to the RNA-binding site to be fully functional. The PAM-site required for Sp Cas9 is NGG or NAG where N is any of the four nucleotides. The gRNA consists of one piece of RNA called CRISPR RNA (crRNA), that binds to the DNA and one piece, the trans-activating CRISPR RNA (tracrRNA), that binds to the crRNA and is a necessity for the binding of Cas9. When used as a toolkit in genomic editing the crRNA and the tracrRNA are often fused together to a single guide RNA (sgRNA). The mixing of sgRNA and Cas9 then forms a ribonucleotide complex (RNP) suitable for delivery into the cells.

Cas9 cleaves the DNA and creates a double-strand break (DSB) (Wang *et al.* 2016a). The cell repairs the DSB through one of two methods, nonhomologous end joining (NHEJ) or homology-directed repair (HDR). The result of NHEJ is mutations in the DNA caused by random deletion or insertion of nucleotides during the repair process. The insertions or deletions may cause a mutation in a vital part of the protein or a frameshift mutation and thereby cause a knock-out of the gene. HDR can be utilized to insert a piece of donor DNA in the DSB by homologous

recombination. This enables vast possibilities of specific genome editing e.g., gene knock-out, gene knock-in and mutagenesis.

2.4.2 Transfection

For Cas9 to be able to edit the genome it needs to gain access to the cells, and this can be done by a method called transfection. Transfection is a process of delivering foreign nucleic acid into eukaryotic cells which makes it possible to modify the host cells own genetic material (Chong *et al*. 2021). There are many different methods to transfect a cell and they can be physical, chemical, or viral based. Electroporation is a common physical method, and it creates temporary pores in the cellular membrane by subjecting the cells to a pulse of electrical voltage.

3. Material and methods

3.1 Preparations before knock-out

3.1.1 Cell culturing

The cell line used in this study was a cell line by the name CMT-U27 which is derived from canine mammary carcinoma. The cell line was provided by Eva Hellmén (SLU, Department of Anatomy, Physiology and Biochemistry). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium with GlutaMAX (Thermo Fischer Scientific) and an addition of 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (of the stock solution 10 000 U/ml penicillin, 10 mg/ml streptomycin) and 1% sodium pyruvate (of the stock solution 100 mM). The cells were grown in T75-cell culture flasks before transfection with Cas9. The medium was exchanged two times a week. The cells were incubated in a cell incubator with a temperature of 37°C and a CO_2-level of 5% during the whole study.

3.1.2 Design of gRNA and donor DNA

The design of the gRNA and donor DNA was done by assistant supervisor Sofia Tengstrand. The gRNA was designed using the program CRISPOR. The sequence of the gRNA used is: GCAGTGTACCCACTGGTACG. The gRNA is designed to cause a DSB in exon 2 in the serglycin gene since the alternative splicing without exon 1 have been reported.

In this study, the donor DNA chosen were of two different variants. Both contains three stop codons in different reading frames. The first variant is coding for green fluorescent protein (GFP) and resistance against puromycin. The other one is coding for red fluorescent protein (RFP) and resistance against blasticidin. The antibiotic resistance is driven by the endogenous promoter in the serglycin gene. The fluorescence is driven by an exogenous promoter in the donor DNA.

3.1.3 Amplification of donor DNA

The donor DNA had to first be amplified with PCR. The protocol used for the PCR-reaction are from the user guide for the TrueTag™ Donor DNA Kit (Thermo Fischer Scientific) and can be seen in appendix 1. The protocol was adjusted by raising the annealing temperature from 55°C to 70°C. The duration of the extension phase was set to 2 minutes and 30 seconds.

A gel electrophoresis with a 1% agarose gel were run to ensure that the PCR product were the correct length. The amplified donor DNA were then cleaned using the method described in the user guide for TrueTag™ Donor DNA Kit (Thermo Fischer Scientific). See appendix 2 for more details. The concentration of the cleaned donor DNA:s were measured using Nanodrop 8000 (Thermo Fischer Scientific) so the correct amount of donor DNA could be used for the transfection.

3.2 Knock-out of the serglycin gene

3.2.1 CRISPR/Cas9

The CMT-U27 cells were washed with phosphate-buffered saline (PBS) twice and then trypsinized 24 hours before the transfection. This was repeated on the day of the transfection to harvest them from the cell culture flask. The cells were then counted in a Fuchs-Rosenthal counting chamber. The transfection was done with approximately 0.5 million cells per reaction. The cells were washed with PBS and spun down to a cell pellet which was then dissolved in 500 µl electroporation buffer (Bio-Rad Laboratories Inc.). The cell suspension was put in electroporation cuvettes (Bio-Rad Laboratories Inc.). A total of two reactions were made, one with a mixture containing Cas9 and one negative control without the Cas9-mixture.

A mixture of TrueCut™ Hifi Cas9 Protein (Thermo Fischer Scientific), True-Guide™ sgRNA (Thermo Fischer Scientific) and the TrueTag™ donor DNA (Thermo Fischer Scientific), was created. See table 1 for the concentrations and volumes used. The donor DNA were coding for either GFP-Puromycin or RFP-blasticidin, a total of 1000 ng of each donor DNA were used.

The mixture was left to incubate for approximately 15 minutes for the RNPs to form. This mixture with Cas9 was then added to the electroporation cuvette containing the cells. The negative control received an addition of 50µl of electroporation buffer instead of the mixture with Cas9.

Table 1. Contents of the Cas9-mixture.

Reagent	Concentration	Volume (µl)
TrueCut™ Hifi Cas9 Protein	5 µg/ml	1
TrueGuide™ sgRNA	1 µM	37.5
Knockout Enrichment GFP-Puromycin	144.33 ng/µl	6.9
Knockout Enrichment RFP-Blasticidin	103.62 ng/µl	9.7
Total volume per reaction		**55.1**

3.2.2 Transfection

The method used for transfection was electroporation. The Gene Pulser® Apparatus (Bio-Rad Laboratories Inc.) was used to deliver the pulse to the cells. The settings used were a voltage of 250 V and a capacitance of 500 µF. Both the sample with Cas9 and the control without Cas9 were electroporated. The time constant for the cuvette with Cas9 was 49 ms and for the control 48 ms.

3.3 Verification of knock-out

3.3.1 Cell culturing and antibiotic selection

One way to confirm the integration of the donor DNA is by culturing the cells in medium with antibiotics in which the cells would normally not survive. The survival of the cells is made possible by the integrated donor DNA which makes the cells resistant against these antibiotics.

After the electroporation the cells were transferred from the electroporation cuvettes to a 6-well plate with RPMI medium. The cells were cultured in the regular RPMI medium for approximately one day and then the selection with antibiotics began. The transfected cells were divided into five wells, one containing RPMI medium without antibiotics and four containing RPMI medium with different concentration of the antibiotics puromycin and blasticidin. The concentrations are seen in table 2. After the transfected cells had been transferred to the different wells with antibiotics, the wells were examined with a fluorescence microscope to make sure all wells contained fluorescent cells. The selection with antibiotics were performed for 9 days with frequent renewal of the medium.

Table 2. Concentration of puromycin and blasticidin. Based on (Thermo Fischer Scientific Inc. n.d.)

Well	Concentration of puromycin	Concentration of blasticidin
A	0 µg/ml	0 µg/ml
B	0.5 µg/ml	1 µg/ml
C	1 µg/ml	2 µg/ml
D	2 µg/ml	5 µg/ml
E	5 µg/ml	10 µg/ml

3.3.2 Fluorescence

A fluorescence microscope was also used to evaluate the integration of the donor DNA. The cells were evaluated for both green and red fluorescence. Images were taken with the camera connected to the microscope.

3.3.3 Continued cell culturing

Based on the results of the antibiotic selection it was necessary to further examine the cells that had not undergone the antibiotic selection. The fluorescent cells from well A were transferred into new wells in a 96-well plate with one to a couple of fluorescent cells per well. A pipette was used to scrape off the cells from the bottom of the well, aspirate them up into the pipette tip, and transfer them to the new well. The wells were examined with fluorescence microscope to make sure fluorescent cells had been transferred to the new wells.

4. Results

4.1 Fluorescence

Fluorescent cells were visible already a couple of days after transfection. Some were only emitting green fluorescent light, few were only emitting red fluorescent light, and some were emitting both green and red fluorescent light. Below in figure 1 is an example of a cell emitting both green and red fluorescent light. The images were taken two days after the transfection.

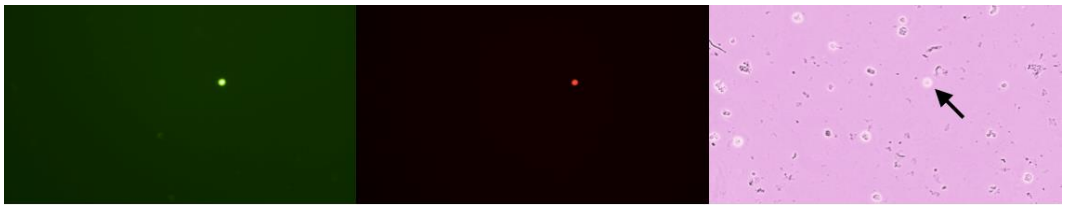

Figure 1. Images of the same cell emitting both green and red fluorescent light, and the same cell viewed under the optical microscope.

After additional days of culturing some of the cells had adhered to the bottom of the wells and started to form colonies. Figure 2 and figure 3 shows two different cell colonies emitting both green and red fluorescent light. The cell colonies were in well A which was not undergoing selection with antibiotics. The images were taken 13 days after transfection. Approximately 35 to 40 fluorescent cells were seen at that time.

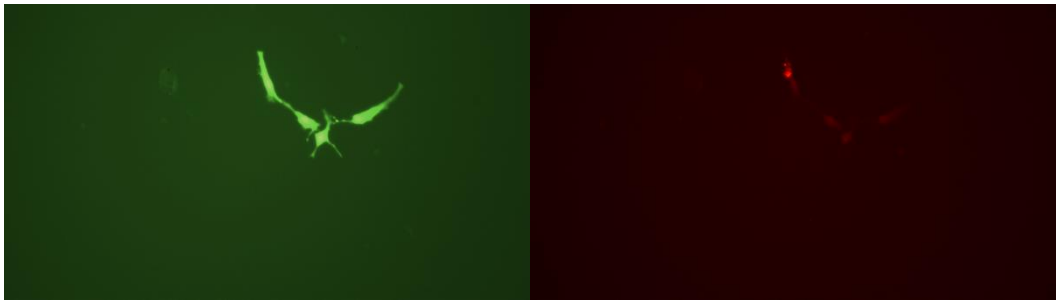

Figure 2. Images of a cell colony emitting both green and red fluorescent light.

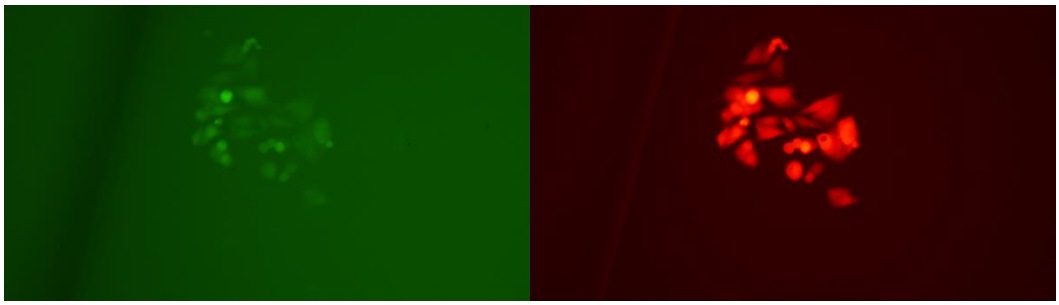

Figure 3. Images of a cell colony emitting both green and red fluorescent light.

4.2 Antibiotic selection

Culturing the cells in the medium with the antibiotics puromycin and blasticidin resulted in the death of both the non-fluorescent cells and the fluorescent cells. No fluorescent cells could be found after the selection.

4.3 Continued cell culturing

Fluorescent cells were seen directly after the transfer to the new wells, but no fluorescent cells could be found in the new wells five days after the transfer. They had not survived the process of being moved from one well to another with the pipette.

5. Discussion

The purpose of this study was to develop a serglycin-deficient carcinoma cell line to be used as an in-vitro model for future exploration of functional aspects of the proteoglycan serglycin in canine mammary tumors. The experiment could be seen as a form of pilot experiment for testing methods for the larger PhD project to be executed by assistant supervisor Sofia Tengstrand.

5.1 Fluorescence

The desirable outcome with the knock-out was to achieve a bi-allelic knockout. Since eukaryotic cells have a double set of chromosomes, they therefore have two copies of the serglycin gene and both of them needs to be knocked out to achieve a serglycin-deficient cell.

The idea behind using two different donor-DNA, one giving green fluorescence and one giving red fluorescence, is to visualize that both alleles have been knocked-out. If a cell is emitting both green and red fluorescent light, it should mean that the donor DNA with green fluorescence is integrated in one allele and the donor DNA with red fluorescence is integrated in the other allele. If only one type of fluorescent color is used for the donor DNA, then it is not possible, with a fluorescent microscope, to tell if one or both alleles have been knocked out. If a cell is only emitting green fluorescent light, then the cell could have the donor DNA for green fluorescence in both alleles or it could be that one of the alleles is still intact.

This experiment managed to create cells with double fluorescence according to the plan. Some of the cells had only one type of fluorescence, meaning that they could have had a bi-allelic knockout or only a mono-allelic knock-out.

However, a cell with a double fluorescence is not a certainty for a knock-out of the serglycin gene. The sequence for the fluorescence is driven by an exogenous promotor in the donor DNA which means that the cell still can express the fluorescence if the donor DNA is integrated in another locus than the serglycin gene. But nonetheless, the cells were managed to get transfected and continued to grow and divided themselves which can serve as a platform for future experiments. To ensure

that the double fluorescent cells had the integrated donor DNA on the correct locus, a series of genomic PCR were supposed to be conducted. Forward and reverse primer on different sites inside the donor DNA and in the serglycin gene were designed and should be used to show that the donor DNA was intact and had been integrated correctly. Another step to verify a successful knock-out is to do a sequencing of the genome with the serglycin gene. Unfortunately, based on the lack of time and viable cells these experiments were not able to be conducted.

5.2 Antibiotic selection

No fluorescent cells survived the antibiotic selection. They should in theory be resistant to the antibiotics puromycin and blasticidin if the donor DNA have been correctly integrated in the serglycin gene. Since the cells were fluorescent, they would hopefully survive the antibiotic selection. But opposite to the fluorescence, the antibiotic resistance is driven by an endogenous promotor which means that the donor DNA needs to be integrated in the assigned place in the reading frame for the cell to acquire the antibiotic resistance abilities. A possible explanation to the death of the cells could therefore be that the donor DNA had been incorporated incorrectly and did not have access to the promotor region.

However, another possible explanation was that the antibiotic selection began to rapidly after the transfection, so the cells had not been able to recover completely after the electroporation and therefore were not capable to survive the antibiotic selection even though the donor DNA may had been integrated correctly. For the next experiment it could be preferred to initiate the antibiotic selection later when the cells have had more time to adhere, recover and reach a higher confluency.

The concentration of puromycin and blasticidin for the antibiotic selection were based on concentration used on other cell types such as human breast cancer cell line MCF-7. No data for CMT-U27 where able to be found. Before future experiments, a kill curve specific for CMT-U27 and blasticidin and puromycin should be developed.

5.3 Conclusion

No confirmed serglycin-deficient cell line was able to be established according to the original plan. There may have been cells with a successful knock-out but due to a lack of time it was not possible to confirm it and establish the cell line. However, a lot of important information for future trials have been gathered. Hopefully, a serglycin-deficient CMT cell line is not too far from establishment and then the role of serglycin in carcinogenesis can be further researched and understood.

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Popular science summary

Mammary tumors

Mammary tumor is a very common form of cancer in dogs and is one of the most common causes of death, especially for bitches. Around half of the cases of mammary tumors are malignant, in other words, they spread in the surrounding tissues or to other places in the body. Different cell types can cause mammary tumors and one kind is called carcinoma. This study will be based on cells that come from a carcinoma form of mammary tumor in dogs.

Serglycin

Serglycin is a type of molecule called proteoglycan. Many different cells and especially different cells in the immune system use serglycin for their function. The immune cells use serglycin to store and release different forms of molecules active in the immune response. Serglycin has more functions and not all of them are known. In human medicine, it has been shown that there is a connection between serglycin and cancer. Some forms of cancer have increased levels of serglycin, and some forms of cancer have decreased levels. It has also been reported that serglycin affects the malignant properties of the cancer cells, such as their ability to spread thorough the body and their resistance to cancer treatments. The goal of this study was to create cells without serglycin that can be used in future studies to further understand the role of serglycin in the development of cancer.

CRISPR/Cas9

To achieve the goal of creating cells without serglycin this experiment aimed to destroy the gene for serglycin, to do a so-called knock-out of the gene. CRISPR/ Cas9 is a system for editing genes, recently awarded the Nobel Prize, and was the method used in this study. Cas9 is an enzyme that cuts DNA and can be directed to cut the DNA at a desired place, such as the serglycin gene. It is also possible to insert other predesigned pieces of DNA in the spot where the Cas9 cut the original DNA. In this study we chose to insert DNA that codes for proteins that makes the cell fluorescent and that makes them resistant to two types of antibiotics. There were two variants of the DNA that could be inserted, one that gave green fluorescence and one that gave red fluorescence. The fluorescence and antibiotic

resistance help to make it possible to determine which cells have had the serglycin gene knocked-out.

For the Cas9 to be able to cut the DNA it needs to enter the cell through the cellular membrane. This can be done using a method called electroporation. The cells receive a pulse of electricity which creates small holes in the cellular membrane which makes it possible for the Cas9-enzymes to enter the cells. When the Cas9 has entered the cells, it is called that the cells have been transfected.

Results

A couple of days after the cells were transfected it was possible to see cells with green and/or red fluorescence. The preferred result is if the cells have both green and red fluorescence. Every cell has two copies of the serglycin gene. If the cell has both green and red fluorescence it means that both genes have been knocked out and had the new DNA inserted. If the cell only has one color, it can be because one of the gene copies are intact.

The DNA inserted in the gene in this study was also supposed to make the cells resistant against two types of antibiotics. The theory behind this is that only the cells which have successfully been transfected is going to survive when the cells are exposed to these two antibiotics. However, all cells that received the antibiotics in this study died. It is not possible to tell if it was because they were not successfully transfected or if they just had not had time to recover after the electroporation.

No cell line without serglycin was able to be established according to the plan due to lack of time and viable cells, but a lot of lessons have been learned for future trials. Hopefully, it is not far from the establishment of a cell line without serglycin and then the role of serglycin in the development of cancer can be further researched and understood.

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Appendix 1 – PCR of donor DNA

Generating your TrueTag™ donor DNA

This protocol will generate >5 µg of linear dsDNA donor with the desired gene specific homology arms.

1. Set up two 50-µL PCR reactions:

Component	Amount
2X Phusion™ Flash High Fidelity PCR Master Mix	25 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
Donor template (20 ng/µL)	1 µL
Water	22 µL
Total	50 µL

2. PCR parameters:

Step	Cycles	Temperature	Time
Denature	1	98°C	10 seconds
Amplification	30	98°C	1 seconds
		55°C	5 seconds
		72°C	15–30 seconds/kb
Final extension	1	72°C	2 minutes
Final hold	1	4°C	HOLD

3. Confirm successful PCR reaction by running 2 µL of PCR on 1–2% agarose gel. A successful PCR will have the following band sizes:

TrueTag™ DNA donor type	Band Size (kb)
Fluorescent	1.3–1.5
Epitope	0.5–0.9
Stem	1.4–1.8
Knockout Enrichment	2.1–2.4

Appendix 2 – Clean-up of donor DNA

4. PCR Cleanup using the TrueTag™ PCR Purification module. All steps should be carried out at room temperature. All centrifugations should be carried out in a table-top microcentrifuge at >12,000 × *g* (10,000–14,000 rpm, depending on the rotor type).
   - Prior to the initial use of the purification module, dilute the Wash Buffer (concentrated) with 9 mL of ethanol (96–100%)
   - Examine the Binding Buffer for precipitate before each use. Re-dissolve any precipitate by warming the solution to 37°C and cooling to 25°C.

   a. Combine the two completed 50-µL PCR reactions for each donor into 1 tube

   b. Add a 100 µL of Binding Buffer to completed PCR mixture (1:1). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.

   c. Transfer the 200 µL of the solution from step b to the GeneJET™ purification column. Centrifuge for 30–60 seconds. Discard the flow-through.

   d. Add 700 µL of Wash Buffer (diluted with ethanol) to the GeneJET™ purification column. Centrifuge for 30–60 seconds. Discard the flow-through and place the purification column back into the collection tube.

   e. Centrifuge the empty GeneJET™ purification column for an additional 1 minute to completely remove any residual wash buffer.

   Note: This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.

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   f. Transfer the GeneJET™ purification column to a clean 1.5 mL microcentrifuge tube (not included). Add 50 µL of Elution Buffer (10 mM Tris-HCl, pH 8.5) or nuclease-free water to the center of the GeneJET™ purification column membrane and centrifuge for 1 minute.

   g. Discard the GeneJET™ purification column and store the purified DNA at –20°C.

   h. Concentration can be measured by A_{260}. If higher concentration is required a SpeedVac™ vacuum concentrator can be used.

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