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Faculty of Veterinary Medicine and Animal Science
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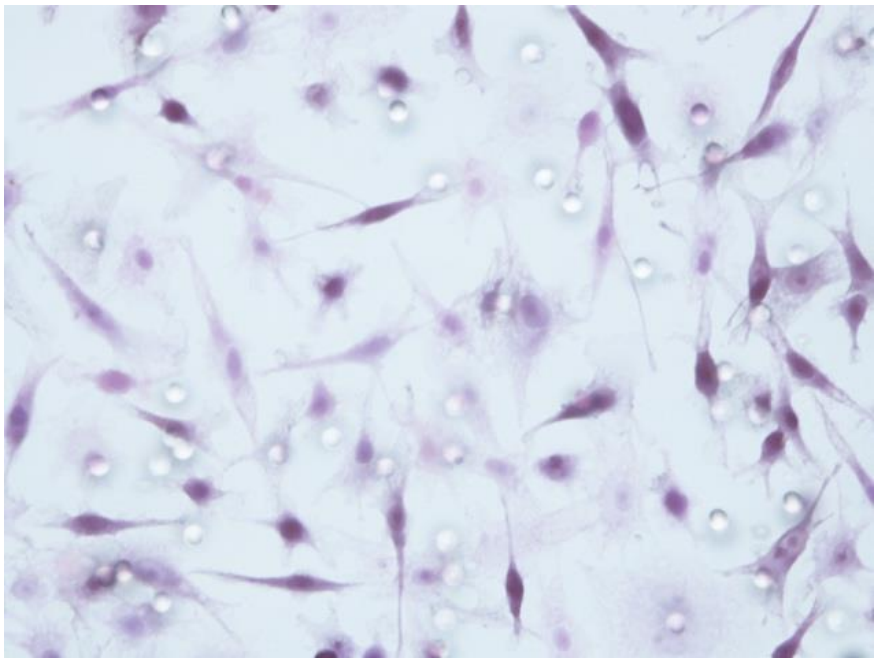

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The role of CSF-1R on migration and invasion of canine mammary tumors

CSF-1R och dess roll i migration och invasivitet vid juvertumörer hos hund

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SUMMARY

The colony stimulating factor-1 receptor (CSF-1R) is a tyrosine kinase receptor expressed in mammary tumors of canines and breast carcinomas of humans. The expression of this receptor has been associated with increased invasion and migration *in vitro.* In breast cancer patients, expression of CSF-1R in the tumor tissue is associated with increased levels of lymph node metastasis. Epithelial cancer cells can secrete the ligand, colony stimulating factor-1(CSF-1). CSF-1 may cause autocrine stimulation of the receptor and attract and educate macrophages towards a tumor associated macrophage (TAM) phenotype. The CSF-1R is also important in the regulation of macrophages in organogenesis including mammary development. In this study, the expression of CSF-1R in CMT-U229 avl 2 cell line was examined using flow cytometry and immunohistochemistry. During flow cytometry the cells were sorted in two groups of CSF-1R positive cells and CSF-1R negative cells. The migration and invasion of the CSF-1R positive and CSF-1R negative 2 was evaluated using a wound healing assay and Matrigel invasion assay. The CMT-U229 avl 2 cells expressed CSF-1R in 93 % of the cells according to the result of the immunohistochemistry and in 21.4 % of the cells according to the result of the flow cytometry. The CSF-1R positive cells showed a slight increase in migration rate and a larger increase in invasion compared to the CSF-1R negative cells. In conclusion the CSF-1R was expressed in the CMT-U229 avl 2 cell line and this expression could be associated with invasiveness in the CMT-U229 avl 2 cell line.

SAMMANFATTNING

Colony stimulating factor-1 receptor (CSF-1R) är en tyrosinkinasreceptor som uttrycks i juvertumörer hos hundar och bröstcarcinom hos människor. Uttryck av receptorn har kopplats till ökad invasivitet och migration *in vitro.* Hos bröstcancerpatienter är uttryck av CSF-1R i tumörvävnaden kopplat till ökade nivåer av metastasering till lymfknuta. Epitelial cancerceller kan även utsöndra liganden, colony stimulating factor (CSF-1). CSF-1 kan stimulera CSF-1R autokrint och attrahera makrofager samt förändra makrofager till en mer anti-inflammatorisk fenotyp. CSF-1R är även viktig för reglering av makrofager i organogenesen vilket inkluderar juverutveckling. I denna studie användes flödescytometri och immunohistokemi för att undersöka uttrycket av CSF-1R i cellinjen CMT-U229 avl 2. Cellerna sorterades även i en grupp av CSF-1R positiva och en grupp av CSF-1R negativa celler. Migration och invasivitet utvärderades hos de CSF-1R positiva och CSF-1R negativa genom en så kallad wound healing assay och Matrigel invasion assay. 93 % av cellerna uttryckte CSF-1R vid immunohistokemi och 21.4 % av cellerna uttryckte receptorn vid flödescytometri. CSF-1R positiva celler visade en något större förmåga att migrera och en större ökning i förmågan till invasivitet jämfört med de CSF-1R negativa cellerna. Sammanfattat uttrycks receptorn CSF-1R av cellinje CMT-U229 avl 2 och detta uttryck kan vara kopplat till ökad invasivitet.

CONTENT

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CSF-1R	Colony stimulating factor 1 receptor
CSF-1	Colony stimulating factor 1
CCL2	C-C motif chemokine ligand 2
CCL 8	C-C motif chemokine ligand 8
$Csf1r^{-}/Csf1r^{-}$ mice	Homozygous disruption of the *Csf-1r* gene in mice
$Csf1^{op}/Csf1^{op}$mice	Osteoporotic CSF-1-nullizygous mice.
DAB	3,3'-Diaminobenzidine
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
Erk/MAPK	Extracellular signal regulated kinases/Mitogen-activated protein kinases
Ets	E twenty-six
FBS	Fetal bovine serum
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GRB2	Growth factor receptor-bound protein 2
HBSS	Hanks balanced salt solution
HER2	Human epidermal growth factor receptor 2
IHC	Immunohistochemistry
KI	Kinase insert
LPS	Lipopolysaccharide
PE	Phycoerythrin
PBS	Phosphate buffer
PG	Progesterone receptor
PtdIns 3/PI3K	Phosphatadylinositol-4,5 biphosphate 3/Phosphoinositide 3-kinase
PTK	Protein tyrosine kinase
PyMT	Polyoma middle T oncoprotein
TAM	Tumor associated macrophage
TGF-β	Transforming growth factor β
Tyr	Tyrosine
uPA	Urokinase plasminogen activator

INTRODUCTION

Expression and function of the colony stimulating factor receptor (CSF-1R) has been studied in human and canine mammary tumor cell lines and murine models. Canine mammary tumors previously studied had a high expression of CSF-1R and this expression has been associated with increased proliferation and metastasis. Learning more about the CSF-1R receptor and its role in mammary cancer pathogenesis of canines could improve treatment and diagnosis of canine cancer patients. In this study the CMT-U229 avl 2 cell line was analyzed for expression of CSF-1R and association between CSF-1R, migration and invasion *in vitro*. This has previously not been studied in the CMT-U229 avl 2 cell line and the aim of the study was to learn more about the role of CSF-1R in this tumor cell line.

LITERATURE REVIEW

Molecular structure of the receptor

The colony stimulating factor receptor (CSF-1R) is a tyrosine kinase receptor with demonstrated ligand dependent protein tyrosine kinase (PTK) activity. There are six known tyrosine residues (Tyr) on the human CSF-1R receptor that become phosphorylated: Tyr 571, Tyr 561, Tyr 809, Tyr 723, Tyr 708 and Tyr 699 (Shurtleff *et al*., 1990; Roussel, 1997). These tyrosine domains of the CSF-1R receptor have been examined in murine fibroblasts with human CSF-1R transfected. Tyr 561 seems to be the primary binding site of the src family kinases. Tyr also 561 seems to be important for signal transduction in fibroblasts and is probably involved in changes of morphology and growth rate (Roche *et al.*, 1995). Tyr809 likely stimulates mitogenity by signaling to myc and cyclin D. Tyr 809 may also induce activation of src, Ras, Erk 1 and Erk 2 (Roussel, 1997). Tyrosine residues 699, 708 and 723 are part of the kinase insert domain (KI) (Shurtleff *et al.*, 1990). The KI stimulates PtdIns 3 kinase activity and mitogenesis but is not essential for either (Shurtleff *et al.*, 1990). Studies of murine fibroblast CSF-1R show a connection of GRB2 to the tyrosine residue 697. Tyr697 corresponds to the human 699 residue (van der Geer and Hunter, 1993).

The role of CSF-1R in physiology and reproduction

General physiology

The role of CSF-1R in the body has been studied using mice with targeted homozygous disruption of the Csf-1r gene ($Csf1r^{-}/Csf1r^{-}$) and CSF-1 nullizygous mice ($Csf1^{op}/Csf1^{op}$). $Csf1r^{-}/Csf1r^{-}$ mice and $Csf1^{op}/Csf1^{op}$ mice showed overall similar phenotypical changes but several of the phentypical characteristics of the $Csf1r^{-}/Csf1r^{-}$ mice were more severe. The fact that these phenotypical characteristics were more severe indicated that CSF-1 independent activation could have a subtle effect on CSF-1R. Both the $Csf1r^{-}/Csf1r^{-}$ mice and $csf1^{op}/csf1^{op}$ mice were severely osteoporotic and lacked osteoclastic development at the metaphysis resulting in increased bone density of the metaphysis and shortened long bones. The growth rate and survival was lower. Before 8 months of age they showed decreased volume of femoral bone marrow and femoral bone marrow

cellularity. Production of blood monocytes and lymphocytes was decreased but production of granulocytes was increased. Levels of mononuclear phagocytes in liver, kidney, skin and synovial membranes were decreased. CSF-1R also affected normal reproduction, in that diestrus of females increased. All of these phenotypical changes indicated CSF-1R is important for development and functions of many organs and tissues of the body (Dai *et al.*, 2002). Some macrophages expressing CSF-1R require CSF-1 and these macrophages seem to be important in tissue remodeling and organogenesis. (Cecchini *et al*., 1994) Levels of mononuclear phagocytes was decreased in several tissues like liver tissue, kidney tissue, skin and synovial in mice with homozygous disruption of CSF-1R (Dai *et al.*, 2002).

Mammary gland physiology

The phenotype of the mammary glands of pregnant mice with homozygous disruption of CSF-1R changes. The change of the mammary gland phenotype was likely caused by failure of branching morphogenesis of the ductal epithelium (Dai *et al.*, 2002). Transgenic overexpression of CSF-1 and/or CSF-1R in the mammary gland of murine breast cancer model Polyoma middle T oncoprotein (PyMT) mice was on the other hand associated with increased ductal branching and proliferation of the mammary epithelium (Kirma *et al.*, 2004). In the human breast tissue, CSF-1R and CSF-1 have been observed in epithelial cells that line lactating ducts and alveoli but CSF-1R has not been found in non-lactating epithelium (Sapi *et al.*, 1998a). Lactogenic hormones like insulin and prolactin can increase the expression of CSF-1 but not CSF-1R *in vitro.* Glucocorticoids can increase the CSF-1R expression *in vitro* but has no effect on CSF-1 expression (Kacinski, 1997).

Hormonal regulation of CSF-1R and CSF-1

Lactogenic hormones like insulin and prolactin can increase the expression of CSF-1 but not CSF-1R *in vitro.* Glucocorticoids can increase the CSF-1R expression *in vitro* but has no effect on CSF-1 expression (Kacinski, 1997).

Localization of the receptor

Expression of CSF-1R has been observed on both the cell surface and in the nucleus. In some breast carcinomas, CSF-1R can also localize within the nucleus associating with chromatin of carcinoma cells. The level of CSF-1R measured in western blot may vary depending on the type of buffer used, in some studies a buffer which mainly extracts plasma membrane proteins has been used (Barbetti *et al.*, 2014). A study of 40 human invasive breast cancer tissue samples (luminal A and B, human epidermal growth factor receptor 2 (HER2)+/- triple negative) expressing CSF-1R showed that 9 samples expressed CSF-1R in both nucleus and cytosol, three expressed CSF-1R in the nucleus only and 28 expressed CSF-1R in only the cytoplasm (Barbetti *et al.*, 2014).

CSF-1R found in mammary tumors and breast cancer

Spontaneous mammary tumors in the murine mammary cancer model Polyoma middle T oncoprotein (PyMT) mice do not express CSF-1R, only the tumor associated macrophages express the receptor (Strachan *et al.*, 2013). Presence of CSF-1R protein and mRNA expression of CSF-1R has been studied in various human mammary tumor cell lines. Many breast cancer cell lines in humans show some degree of CSF-1R surface protein, CSF-1 expression and CSF-1R mRNA expression respectively (Morandi *et al.*, 2011; Kacinski *et al.*, 1991). In a study where flow cytometry was used to study the presence of CSF-1R in 15 different breast cancer cell lines (luminal and basal carcinomas, 5 of them Human epidermal growth factor 2 (HER2)+) from humans, 14 expressed appreciable levels of CSF-1R. Two immortal breast epithelial cell lines (MCF-10A and MCF-12A) were analyzed in the same study, these cell lines had extremely low levels of CSF-1R (<4 %) protein but showed relatively high levels of CSF-1R mRNA expression. The percentage of cells positive for the CSF-1R protein in each cell line varied from 4 % - 25 %. (Morandi *et al.*, 2011). The low levels of CSF-1R surface proteins may however not be enough to stimulate mitogenic response to CSF-1 (Wrobel *et al.*, 2004). Presence of CSF-1R in carcinomas has also been analyzed immunohistochemically of slides from tissue samples, many of the breast carcinomas analyzed show presence of CSF-1R (Barbetti *et al.*, 2014; Kacinski *et al.*, 1991). In a study of 581 breast cancer tissue samples with varying status of HER2, estrogen receptor (ER) and progesterone receptor (PR), 500 had some level of CSF-1R staining (Kluger *et al.*, 2004). Secretion of CSF-1 also seems to be common in breast carcinomas. In the previously mentioned study of 14 breast carcinoma cell lines (Luminal, basal A and B) and two immortal epithelial cell lines using ELISA and western blot, all showed some level of CSF-1 secretion (Morandi *et al.*, 2011). In one immunohistochemical study of slides from breast carcinoma tissue samples the majority of cells were negative for CSF-1 (Kacinski *et al.*, 1991). From another study, 34 of 37 examined infiltrating ductal carcinomas and 12 of 13 lobular carcinomas were positive for CSF-1 expression. However, only 5/14 of preinvasive carcinomas were positive for CSF-1 expression (Tang *et al.*, 1992). Level of CSF-1R transcripts in epithelial cells correlated with histological grade and clinical stage in carcinomas (Kacinski, 1997) Król *et al.* published a study of CSF-1R expression in 5 canine mammary tumor cell lines (adenocarcinoma, anaplastic cancer, simple carcinoma and spindle cell mammary tumor). When flow cytometry was used all of these cell lines expressed CSF-1R in a higher degree than previously reported in human cell lines The percentage of cells expressing CSF-1R varied from 58.7 % to 88.7 % (Król *et al.*, 2013).

The role of CSF-1R in cancer

The exact role CSF-1R and CSF-1 seems to vary with different cell lines, species or if evaluated through *in vivo* or *in vitro* methods. CSF-1R has been demonstrated to increase tumor progression and invasion in two different ways (Patsialou *et al.*, 2009): (1)Autocrine stimulation in which the tumor cells express CSF-1R and excrete CSF-1. (2) Tumor cells secrete CSF-1, CSF-1 binds to the CSF-1R of macrophages or monocytes which attracts macrophages to the tumor and influences the phenotype of the macrophages. The macrophages then increase the invasiveness of the tumor cells, this may involve secretion of CSF-1 by the macrophages.

CSF-1 signaling to macrophages

Epithelial tumor cells can secrete CSF-1 which attracts tumor associated macrophages (TAMs), Epidermal growth factor (EGF) was secreted by the macrophage binding to EGFR of the tumor cells. (Goswami *et al.*, 2005; Wyckoff *et al.*, 2004; Patsialou *et al.*, 2009). This paracrine interaction with macrophages has been observed in both murine mammary carcinoma cells (PyMT) and human breast carcinoma cells. CSF-1 signaling increases motility of macrophages *in vivo*, which was regulated through the 721 phosphorylation site in association with PI3K (Sampaio *et al.*, 2011). Apart from secreting EGF, TAMs can also affect cancer cells (including carcinomas, spindle cell tumors, anaplastic cancer) in several different ways like upregulation of genes involving inflammation, apoptosis, increased angiogenesis, increased expression of C-C motif chemokine ligand 2 (CCL2) and increased expression of CSF-1R. (Rybicka *et al.*, 2016) (Król *et al.*, 2013). An *in vitro* study of 5 canine mammary tumor cell lines (including CMT-U27) co-cultured with macrophages showed an increased expression of CSF-1R and higher migration rate of the co-cultured tumor cells in all cell lines (Król *et al.*, 2013; Król *et al.*, 2012). TAMs found in tumor tissue has been correlated to metastasis in canine mammary carcinomas (Król *et al.*, 2011). When the same 5 cell lines were cultured without macrophages *in vitro* but supplemented with exogenous CSF-1 the expression of CSF-1R increased and the increased expression of CSF-1R was associated with increased migration rate in the wound healing assay (Król *et al.*, 2013). When the CMT-U27 cell line was co-cultured with macrophages in another study it affected carcinoma cells in several different ways like upregulation of genes involving inflammation, apoptosis, increased angiogenesis, increased expression of CCL2 . The effect on CSF-1R or CSF-1 expression was not mentioned in this study (Rybicka *et al.*, 2016).

The ratio of CSF-1/Granulocyte macrophage colony stimulating factor (GM-CSF) affected genes in the development from monocytes to macrophages (Brochériou *et al.*, 2011). CSF-1 increased the expression of CCL2 and C-C motif chemokine ligand 8 (CCL8) and increased the secretion of CCL2, CCL2 is important for macrophage recruitment and (Lipopolysaccharide) LPS-induced activation of macrophages. (Sierra-Filardi *et al.*, 2014; Beirão *et al.*, 2015).

CSF-1R inhibition could delay tumor growth in mammary carcinomas in PyMT mice and cervical carcinomas in mice, by decreasing the recruitment of TAMs and enhancing infiltration of CD8+ T-cells (Strachan *et al.*, 2013). Overexpression of CSF-1 in the mammary gland may on the other hand increase infiltration of tissue macrophages and cause preneoplastic changes in mammary tissue (Kirma *et al.*, 2004). PyMT mice lacking CSF-1 showed delayed lung metastases, delayed progression to a more aggressive carcinoma stage and decreased levels of infiltrating macrophages. PyMT mice expressing CSF-1 do on the other hand have increased levels of TAMs right before this progression and focal infiltrations of leukocytes were often seen (Lin *et al.*, 2001).

In another study on lung cancer, CSF-1R inhibition did however not affect pulmonary metastasis (Strachan *et al.*, 2013; Patsialou *et al.*, 2009). Anti CSF-1R treatment did also increase metastasis to the lungs and tumor draining lymph nodes in the murine 4T1 stage IV

breast cancer model (Hollmén *et al.*, 2016). This breast cancer model is known to excrete high levels of Granulocyte colony stimulating factor (G-CSF) (Waight *et al.*, 2011). When CSF-1R was inhibited in this model, G-CSF mediated a type of anti-inflammatory TAMs. G-CSF can also recruit anti-inflammatory monocytes to the tumor. Anti-CSF-1R treatment can lead to a decreased number of subcapsular sinus CD169+macrophages and, these macrophages are important for induction of anti-tumor immunity (Hollmén *et al.*, 2016) (Asano *et al.*, 2011).

Autocrine CSF-1 *signaling*

Human breast carcinoma cells may be less dependent on macrophages compared to carcinoma cells in murine breast cancer model PyMT (Wyckoff *et al.*, 2004; Patsialou *et al.*, 2009). The human MDA-MB-231(triple negative basal like) cell line was dependent on both paracrine signaling involving EGF and autocrine signaling. The autocrine regulation was amplified *in vivo* through a tumor environment induced upregulation of CSF-1R expression (Patsialou *et al.*, 2009). Transforming growth factor β (TGFβ) seems to be responsible for this upregulation of CSF-1R. Another *in vitro* wound healing assay of the human MCF-10A epithelial cell line (infected by retrovirus to express more CSF-1R) showed increased migration in cells co-expressing CSF-1R and CSF-1 compared to CSF-1R expressing cells treated with CSF-1 (Wrobel *et al.*, 2004). The coexpression of CSF-1R and CSF-1 of MCF-10A cells also induced hyperproliferation and a profound disruption of junctional integrity of acinar structures. Overexpression of either CSF-1R or CSF-1 was associated with preneoplastic changes in PyMT mice that could lead to tumor formation. (Kirma *et al.*, 2004).

MATERIALS AND METHODS

Cell lines

CMT-U229 avl2 is a cell line established from an atypical canine mammary complex adenoma (Hellmén, 1992).

Flow cytometry

The cells were harvested by trypsinization. The cell concentration was calculated using a Bürker chamber and 1×10^5 cells were added to each test tube. The cells were centrifuged 5 minutes at 1000 rpm. The supernate was removed and the primary antibody was added in 100 µl Hanks balanced salt solution (HBSS) with 2 % fetal bovine serum (FBS). The primary antibody used was anti-mouse CD-115 for CSF-1R (concentration 0.2 µg/µl eBioscience Catalog No:12-1152 (Thermo Fisher Scientific, Waltham, MA, USA) and rat IgG2a-PE (0,2 µg/µl eBioscience, BD Pharmingen Catalog: 553930 (Stockholm, Sweden) for the control. After incubation for 15 minutes on ice the solution was suspended in HBSS with 2 % FBS to a final volume of 250 µl. The cells were kept on ice until the flow cytometry analysis. The CSF-1R expression of the CMT-U27 cell line, which has previously been shown to expressed CSF-1R to a high degree, was also analyzed as a positive control. The following samples were analyzed.

- CMT-U229 avl CSF-1R-PE
- CMT-U229 avl Rat IgG2a-PE (isotype control)
- CMT-U229 avl unstained (control)
- CMT-U27 CSF-1R-PE
- CMT U27 Rat IgG2a-PE (isotype control)
- CMT U27 unstained (control)

The cells were analyzed using Bio-rad S3e cell sorter and FACSDiva 8.0.1 software (BD Biosciences). Gating was first made based on morphological criteria, then the CSF-1R positive cells were gated showing the amount of CSF-1R-positive cells in the cytoplasm. Cut off Value for the CSF-1R positive and CSF-1R negative cells was decided based on the level of Phycoerythrin (PE)-fluorescence.

Immunohistochemistry

All slides were deparaffinized in xylene and decreasing alcohol concentration 1 % H_2O_2 suspended in absolute alcohol. Then the slides were boiled in a Decloaking chamber (Biocare medical) with 500 ml of distilled water and unmasking solution (Vector 1:100) for 8 minutes. After boiling, the slides were left in the chamber for 60 minutes with the lid on to allow the pressure and temperature to decrease. Then the slides were washed with distilled water three times and then Tris buffer (0,05 M Tris-HCl pH 7,6) three times. All slides were incubated with normal goat serum for 60 minutes (1:50) to block non-specific-bindings. The primary antibody used for CSF-1R was a rabbit polyclonal (ABIN683788, antibodies online) diluted at 1:100 and 1:500. The slides were incubated with the primary antibody at +4ºC overnight. After incubation with the primary antibody the slides were washed with Tris buffer three times 5 minutes. Biotinylated anti-rabbit (1:200) was used as the secondary antibody, and the slides were incubated for 30 minutes, after this the slides were again washed in Tris three times. ABC-Elite was prepared suspending 20 µl of reagent A and 20 µl of reagent B in 1 ml of the Tris buffer and left for 30 minutes before use. The slides were washed again with Tris buffer and thereafter incubated with ABC-elite solution for 45 minutes. After ABC-Elite the slides were washed with Tris buffer as above. 3,3'-Diaminobenzidine (DAB) was used as substrate and consisted of a solution of 2,5 ml distilled water, 2 drops of buffer, 2 drops of DAB and 1 drop of H_2O_2 (Vector Burlingame, CA, USA). After DAB the slides were washed with distilled water three times. Mayer's hematoxylin was used to counterstain the nuclei. The slides were then washed in tap water and dehydrated two times in 95 % and absolute alcohol respectively. After this the slides were cleared in xylene and mounted in Pertex (Histolab Sweden). More than 100 cells from each slide were photographed using a Nikon microscope. ImageJ and the IHC toolbox plugin for ImageJ were used to process light micrographs to find DAB-staining. The level of staining was determined based on the processed light micrographs and the original light micrographs. Two slides of CSF-1R positive cells (1:100 and 1:500) and one slide of CSF-1R negative cells from each cell line were used. Canine lymph node tissue and the cell line CMT-U27 were used as positive controls. This experiment was conducted one time.

Wound healing assay

The sorted CSF-1R positive and CSF-1R negative cells were harvested by trypsinization. The concentration of cells was decided by cell counting using a Bürker chamber. Approximately $4x10^4$ cells were suspended in 20 ml Dulbecco's modified eagle medium (DMEM) cell culture medium supplemented with 5 % Fetal bovine serum (FBS), one batch of CSF-1R positive cells and one batch of CSF-1R negative cells. The cells were seeded from the test tubes into 6 different 60 mm dishes, three dishes of CSF-1R positive cells and three dishes of CSF-1R negative cells respectively. The dishes were then placed in a cell culture incubator at 37°C and 5 % CO_2 until the cells had grown into a confluent layer. The dishes of CSF-1R positive cells had grown into a confluent layer after 8 days and the CSF-1R negative cells took 9 days to grow into a confluent layer of cells. When the cells had grown into a confluent layer a scratch was made by drawing a line through the cell layer across the plate using a pipet tip. The scratch was made after removing the cell culture medium and adding three ml of phosphate buffer (PBS) to the plate. Markings were made at the bottom of the plate at different sites of the scratch to make sure that the same view was photographed at every time point. The cells were then washed two more times with PBS to smooth out the edges and remove debris from the medium. Five ml of fresh DMEM cell culture medium with 5 % FBS was added to the plates. The scratch was photographed using a Lumenera Infinity 1 camera at the marked sites after the scratch was made. The sites were then photographed three more times at intervals of two hours. After 6 hours, the gap was closed at some sites. The cells were kept in a cell culture incubator at 37 °C when they were not being photographed. The average gap closing speed per hour was calculated in every light micrograph using ImageJ and Microsoft Excel.

Invasion assay

The Corning BioCoat Growth Factor Reduced Matrigel Invasion Chamber, 24-well was used for the invasion assay and Corning BioCoat Control Inserts, 24-well was used for control (Corning, Bedford, MA, USA). The cells were grown in serum-free DMEM during 24 hours before the experiment started. The membrane of the inserts in the Matrigel invasion chamber was rehydrated by filling every insert with 0.5 ml serum-free DMEM and then placing the inserts in an incubator at 37 °C. The sorted CSF-1R positive and the CSF-1R negative cells were grown in different dishes and at about 80 % confluency the cells were harvested by trypsinization and suspended in two test tubes, respectively. The concentration of the harvested cell in each cell suspension was calculated using a Bürker chamber. After 2 hours of rehydration the Matrigel invasion chamber was taken out of the incubator. The six lateral wells to the left were filled with 0.75 ml DMEM with 5 % fetal bovine serum (FBS) and the six lateral wells to the right were filled with serum-free DMEM. The inserts were filled with the cell suspension, 0.5 ml DMEM and 0.1 % BSA with $2.5x10^4$ cells/well. Six of the inserts were filled with CSF-1R negative cells and six of the inserts were filled with CSF-1R positive cells. Of the six inserts containing CSF-1R positive cells, three were placed in wells with serum free DMEM and three were placed in wells with DMEM and 5 % FBS. The same thing was done to the control inserts. Both the Matrigel invasion chamber and the control plate were incubated in 37 °C and 5 % CO_2.

After 24 hours the Matrigel invasion chamber plate as well as the control plate were taken out of the incubator and the cells were fixated. One by one, the inserts were scrubbed with a cotton swab to remove free cells and then placed in formalin for fixation during 6 minutes. The membranes were then stained in hematoxylin for 6 minutes each and stained with eosin for 20 seconds. After being put in eosin each insert was washed with distilled water and then placed with the bottom up to dry for one hour. The same thing was done with the controls.

After drying off the membrane was cut out from the inserts using a scalpel and the membranes were assembled with the bottom up on a glass slide. The number of cells were counted manually in the center of every membrane using the 40 x objective. The invasion percentage was calculated according to the manufacturers manual. This means that the average number of invading cells counted in the membrane of the Matrigel coated inserts were divided with the corresponding membrane of the uncoated control inserts. For example, the membrane of the coated insert with CSF-1R positive cells incubated with FBS in the well were divided with the number of cells in the membrane of the uncoated control insert with FBS in the well. This way, the ability of the cells to migrate could be separated from the ability to invade through the Matrigel. The invasion percentage is the percentage of migrating cells which also had the ability to invade through the Matrigel coated membrane.

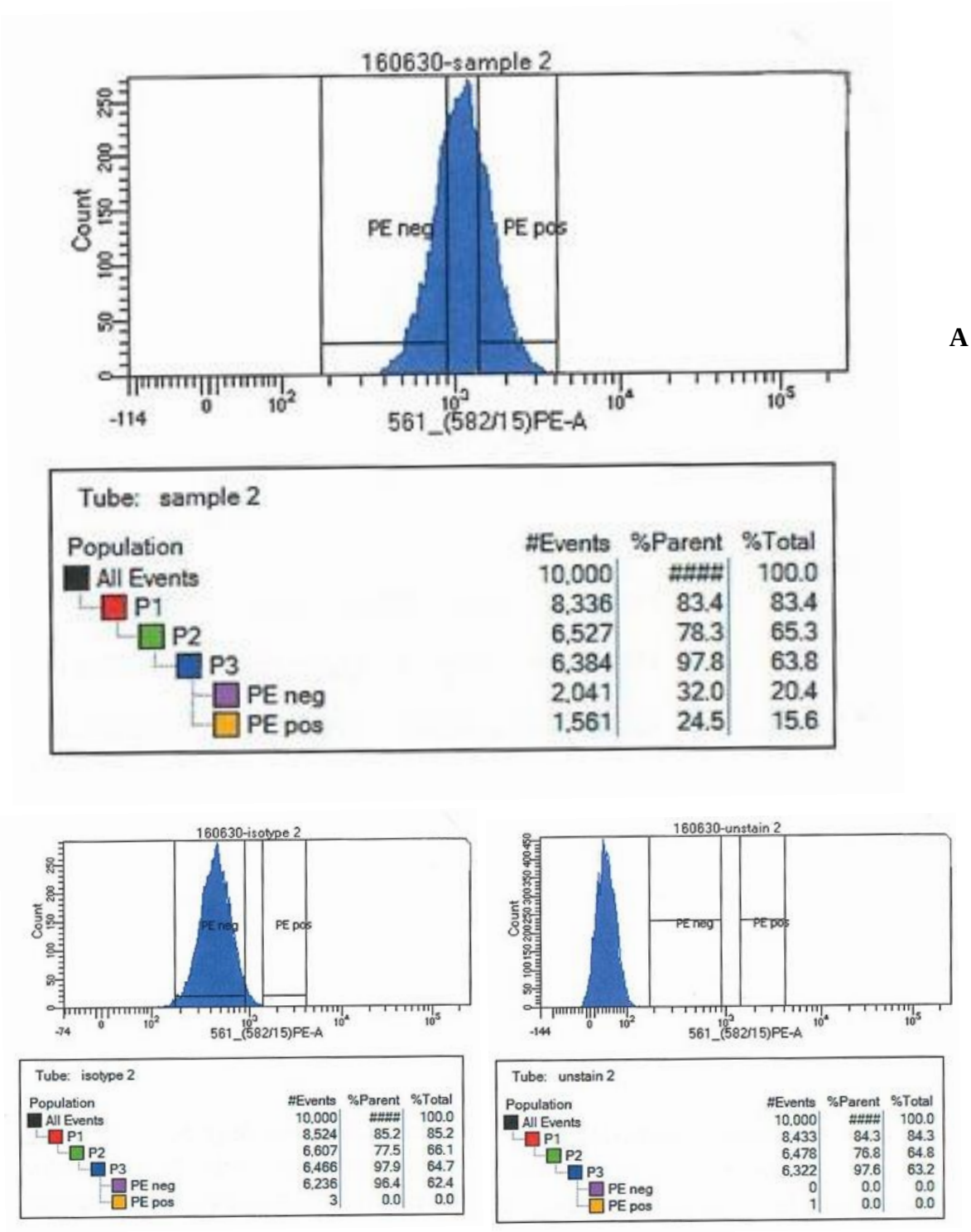
Statistical analysis

Statistical analysis of the wound healing assay was conducted using the student's t test in Microsoft Excel analyzing the average wound healing rate per hour. Average wound healing rate per hour was calculated based on the graphs of the wound healing made in Microsoft Excel, mean wound healing rate expressed in mean +/- S.D. P-value <0.05 was regarded as significant and p-value <0.001 was regarded as highly significant. Because the invasion assay, flow cytometry and immunohistochemistry was only conducted once as described in the results no statistical analysis was made of this data.

RESULTS

Flow cytometry

Flow cytometry was used to determine the level of CSF-1R expression and to sort the CSF-1R positive from the CSF-1R negative cells. The expression of CSF-1R was 24.5 %. Zero percent of the cells were positive for the CSF-1R receptor using isotype control and unstained control cells indicating absence of non-specific background signals. CMT-U27 cells were also analyzed by flow cytometry as a positive control and, the expression of CSF-1R in this cell line was 37.0 %. This experiment was conducted one time. (Figure 1).



A

Figure 1. *Expression of CSF-1R in the canine mammary tumor cell line CMT-U229 avl 2 unsorted cells passage 137. Histograms of* ***(A)****CSF-1R-PE-stained sample with 24.5 % CSF-1R-positive cells,* ***(B)*** *Isotype control with 0 % CSF-1R positive cells,* ***(C)*** *Unstained control with 0 % CSF-1R positive cells. Bio-rad S3e cell sorter and BD FACSDiva 8.0.1 software (BD Bioscience) was used to analyze the expression.*

Immunohistochemistry

The unsorted CMT-U229 avl 2 cells with the primary CSF-1R antibody diluted at 1:100 had 93 % positive cells and 7 % negative cells. Thirtytwo percentage of the cells had a strong DAB-staining. The macrophages of the positive control slides of canine lymph node were strongly positive with the CSF-1R antibody. CMT-U27 was used as a positive control and showed positive staining in 29 % of the cells. The cells from the cell lines were only counted as positive when the cytoplasm had a DAB staining. The negative control slide of the canine lymph node also had a brown coloring of the macrophages. This experiment was conducted one time (Figure 2)

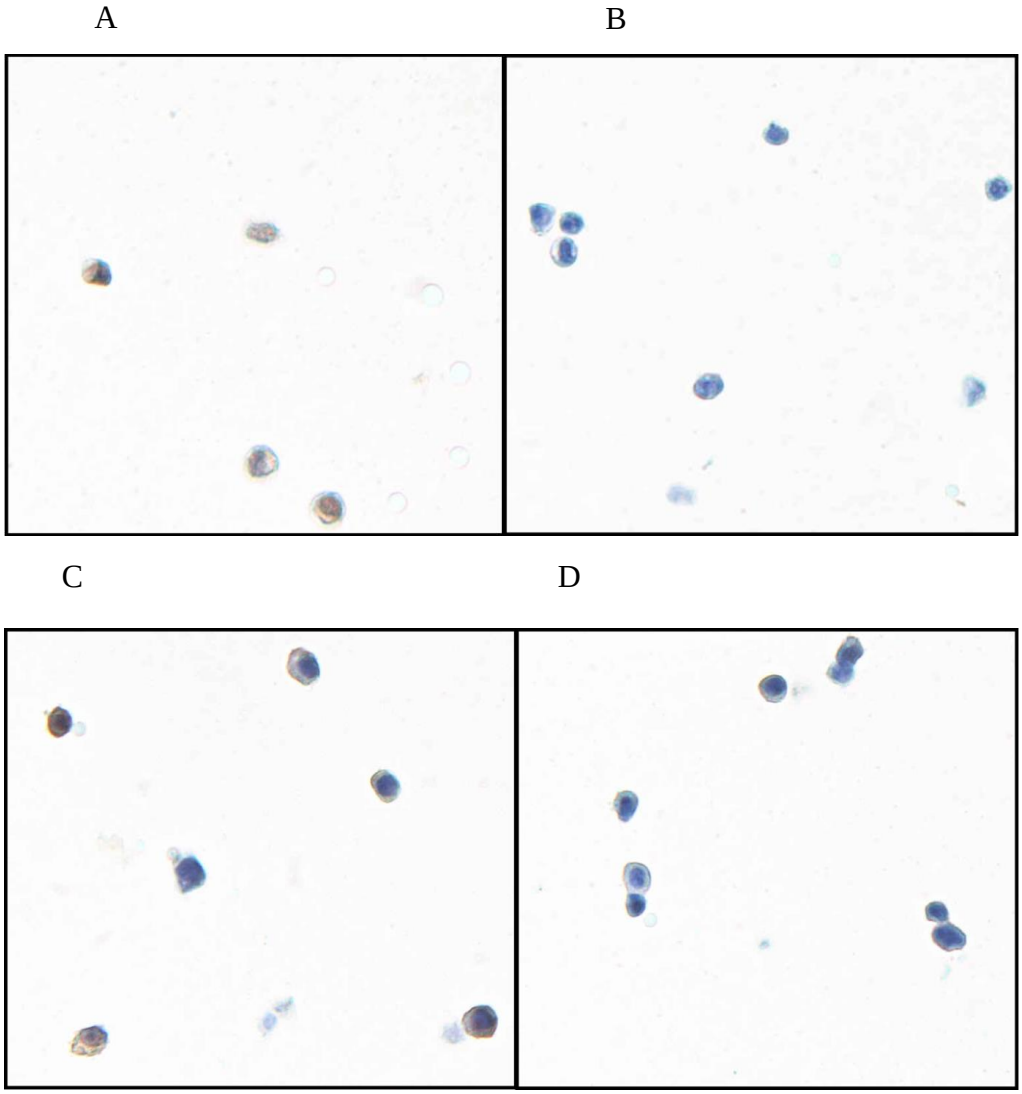
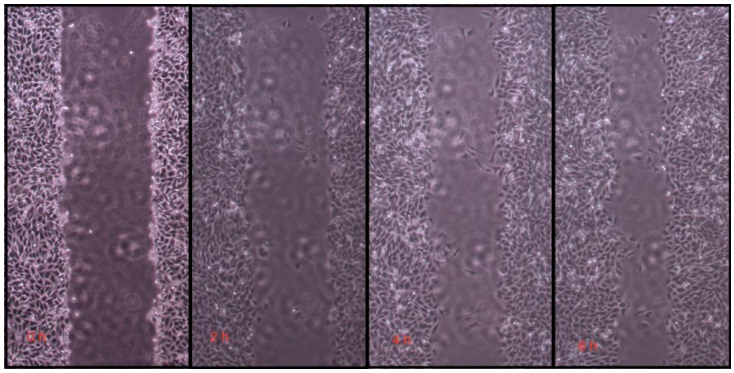


Figure 2. *Expression of CSF-1R in the canine mammary tumor cell lines CMT-U229 avl 2 unsorted and positive control CMT-U27 using immunohistochemistry. Representative light micrographs were taken at 20 x magnification of:* ***(A)*** *Stained CMT-U229 avl 2 cells, 93 % showed a positive DAB-stain and 29 % of them showed a strong positive staining.* ***(B)*** *Unstained negative control of the CMT-U229 avl 2 cell line. No cells in the negative unstained control had any brown coloring.* ***(C)*** *Positive control cells from the CMT-U27 cell line and, 29 % of the cells had a positive DAB-staining.* ***(D)*** *Unstained CMT-U27, no cells had any brown coloring. DAB was used as a substrate and Mayer's hematoxylin was used as a nuclear counterstain in all samples.*

Wound healing assay

The average wound healing rate of the CSF-1R positive cells was 17.5 % higher than the average wound healing rate of the CSF-1R negative cells. Statistical analysis showed a P value of 0.000037 which makes the result highly significant. These results indicate that expression of CSF-1R is correlated with a higher migratory ability for the CMT-U229 avl 2 canine mammary cell line. The average wound healing rate of the CSF-1R positive and CSF-1R negative cells was very similar for the first two hours. After two hours had passed the wound healing of the CSF-1R positive cells started to increase compared to the CSF-1R negative cells making the total wound healing during the six hours higher for the CSF-1R positive cells. The wound healing of the CSF-1R negative cells increased to a similar rate as the CSF-1R positive cells after four hours. The wound healing assay was terminated after six hours because some wounds were almost completely closed making analysis of the wound area difficult. After six hours, on average 38 % of the wound had closed in the CSF-1R positive dishes and 32 % of the wound in the CSF-1R negative dishes. Since the scratch was made using a pipette tip it was not possible to make the width of the wound the same in every dish, the average size of the wound was however very similar in the CSF-1R positive and the CSF-1R negative cells (Figure 3 and Figure 4).

A



B

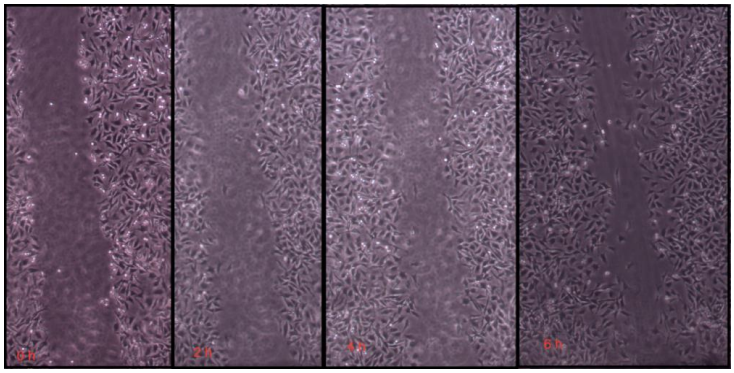


Figure 3. *Wound healing assay of the CMT-229 avl 2 cell line, comparing the migration rate of CSF-1R positive and CSF-1R negative cells. A wound was first made using a pipette tip, light micrographs were then taken at time intervals and the decreasing wound area was measured. The images show representative light micrograph of the* ***(A)*** *CSF-1R positive cells and* ***(B)*** *CSF-1R negative cells at time 0 hours (0 h), two hours (2 h), four hours (4 h) and six hours (6 h). The wound healing rate of the CSF-1R positive cells were 17.5 % higher than the CSF-1R negative cells and the difference was statistically significant (P<0.000037). This indicates that the CSF-1R positive cells have an increased ability to migrate compared to the CSF-1R negative cells.*

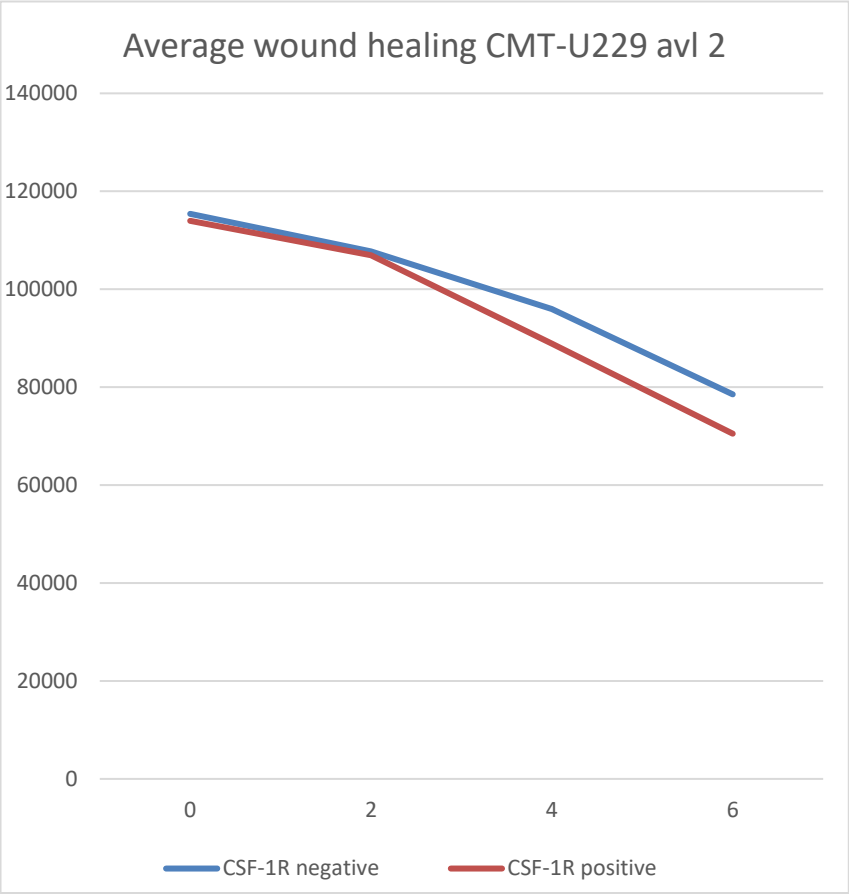


Figure 4. *Graph of the average decreasing wound area (y) per hour (x) in the dishes with CSF-1R positive cells and the dishes with CSF-1R negative cells. The decrease in wound area per hour corresponds to the average migration rate. The average migration rate of the CSF-1R positive and CSF-1R negative cells were similar for the first two hours. Then the average migration rate of the CSF-1R positive cells increased. After four hours the average migration rate of the CSF-1R negative cells increased to the same migration rate as the CSF-1R positive cells.*

Invasion assay

Differences between CSF-1R positive and CSF-1R negative cells when FBS was used as a chemoattractant

The invasion percentage of the CSF-1R positive cells was much higher compared to the CSF-1R negative cells (35.44 % compared to 4.77 %). The average number of cells counted were greater for the CSF-1R positive cells compared to the CSF-1R negative cells in both the Matrigel coated inserts and the control inserts. This indicates that while the migratory capacity of the CSF-1R positive cell was higher than the CSF-1R negative cells the difference in invasive capacity was even greater (Figure 5 and Table 1).

Differences between Matrigel coated inserts and uncoated control inserts when FBS was used as a chemoattractant

When the CSF-1R positive and CSF-1R negative cells were added together and divided with the control the invasion percentage was 23.6 %. The decreased level of cells in the Matrigel coated inserts indicates that the Matrigel coat works as a barrier inhibiting some cells to pass through the pores while other cells could invade through the Matrigel. The cells in the control inserts could move through the pores more easily because no Matrigel layer was there to stop them. The cells of the control inserts showed a different morphology with a more elongated shape than the cells from the Matrigel-coated membranes, this shape is similar to how the cells

look when they are attached to the bottom of a dish. The cells from Matrigel coated membranes had a rounder shape with protrusions pointing in different directions (Figure 5 and Table 1).

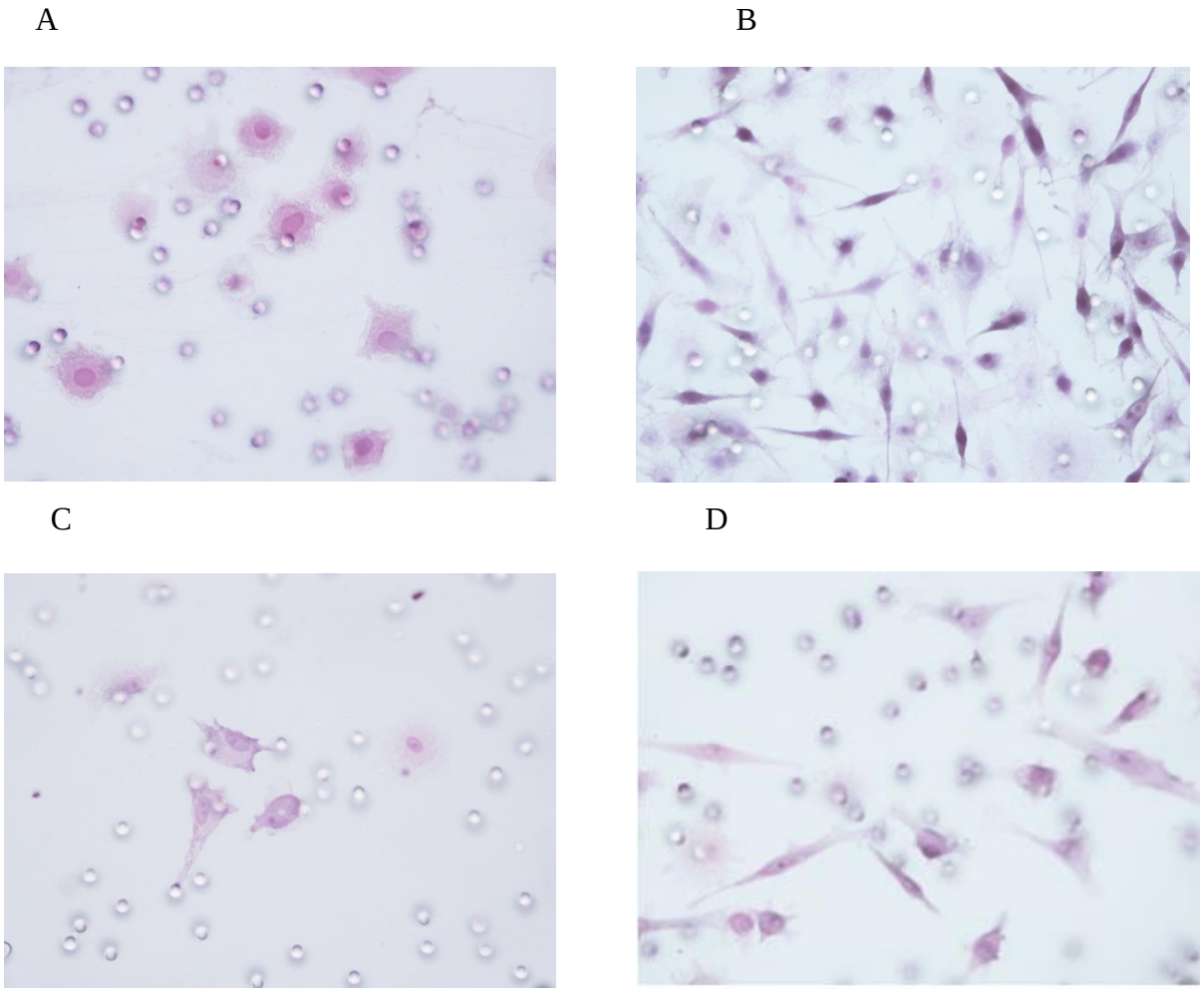


Figure 5**.** *Representative light micrographs of the cells in the membranes of the inserts used in the invasion assay. The light micrographs were taken at 40 x magnification in the center of the membrane. CSF-1R positive cells from* ***(A)*** *Matrigel coated inserts and* ***(B)*** *uncoated control inserts and CSF-1R negative cells from* ***(C)*** *Matrigel coated inserts and* ***(D)*** *uncoated control inserts. Morphological differences were seen between the cells in the Matrigel coated inserts and uncoated control inserts. The cells of uncoated inserts had an elongated shape while the cells of the Matrigel coated inserts had a rounder shape.*

Differences between inserts in wells containing cell culture medium with FBS or wells containing cell culture medium without FBS

FBS added to the well clearly increase the invasive behavior of the cells. On average only 0.67 cells were found in the center field of the coated inserts and 0 cells were found in the center field of the control membranes. The invasive percentage of the cells could not be evaluated when serum-free medium was used in the wells because the average number of cells counted in the membrane was very low (0-2 cells), (Table 1).

Table 1. *Table of the average number of cells found at the center of the membranes (CSF-1R positive/negative, Matrigel coated/uncoated control, FBS added/not added to the well). The number of cells was counted in the center of the membrane at 40 x magnification. The invasion percentage was calculated by dividing the average number of cells at the center of a Matrigel coated membrane with the average number of cells in the center of the corresponding uncoated control membrane. The invasion percentage of the CSF-1R positive cells was higher than the invasion percentage of the CSF-1R negative cells(35.44 % to 4.77 %). The number of cells in the inserts with no FBS added to the well was very low in both the inserts containing CSF-1R positive and inserts containing CSF-1R negative cells (not shown).*

	Matrigel insert	Control insert	% Invasion
CSF-1R positive with FBS	19.67	55.5	35.44
CSF-1R positive without FBS	0.67	0	-
CSF-1R negative with FBS	1.67	35	4.77
CSF-1R negative without FBS	0	0	-

Limitations of the first conduction of the experiment

This experiment was conducted two times but only the second time is described above. The first time the level of cells found in the membrane was very different in each individual group making it difficult to note a difference between the CSF-1R positive and CSF-1R negative. There were also a lot of cells in some of the membranes, sometimes overlapping each other making cell counting difficult. In this first experiment control inserts were not used making calculation of invasion index impossible. There was a clear lack of cells in the membranes of inserts placed in wells without FBS in both the first and second experiment confirming this result. Cells were also found in the membrane of inserts containing both the CSF-1R positive and CSF-1R negative cells confirming that both the CSF-1R positive and CSF-1R negative cells had the ability to invade the Matrigel.

DISCUSSION

Expression of CSF-1R in the CMT-U229 avl 2 cell line

This study confirms that expression of CSF-1R is common in canine mammary tumor cell lines. The expression seems to be more common in canine mammary tumor cell lines compared to human breast cancer cell lines and more cells in each cell line express the receptor *(Morandi et al.*, 2011; Król *et al.*, 2013) In a previous study of 14 human breast cancer cell lines no cell lines expressed CSF-1R in more than 25 % of the cells. (Morandi *et al.*, 2011). Canine mammary tumor cell lines studied by Król *et al.* on the other hand expressed CSF-1R in over 50 % of the cells in each cell line (Król *et al.*, 2013). So why is the expression of CSF-1R so much higher in canine mammary cancer? The CSF-1R receptor could play a more important

role in tumor development of canines compared to humans. The increased expression of CSF-1R in canine mammary tumor cell lines does not in itself indicate that CSF-1Rs play are more important role in cancer pathogenis in female dogs. The role of CSF-1R expression in the tumor cell is also affected by for example the levels of CSF-1 binding to the receptor, the signaling pathways activated by the receptor or overactivity of the receptor. To my knowledge the expression of CSF-1R has not been studied in normal mammary tissue of canines. Thus, it would be interesting to study the expression of CSF-1R in normal canine mammary tissue and compare it to the expression of CSF-1R in canine mammary tumor tissue. The receptor can be expressed in normal non-cancerous breast epithelium of humans during lactation (Sapi *et al.*, 1998a). Insulin and prolactin did not influence the expression of CSF-1R in human mammary epithelium *in vitro* but could increase the expression of CSF-1, especially when insulin and prolactin were added together (Kacinski, 1997). This increased CSF-1 expression could possibly attract macrophages, increase CSF-1R expression and bind to CSF-1R *in vivo* explaining the increased CSF-1R expression during lactation. The canine mammary gland goes through changes similar to lactation during diestrus including ductal arborization, lobuloalveolar differentiation and secretory capacity (Santos *et al.*, 2010). These differences in mammary structure means that it cannot be assumed that the receptor would only be expressed during lactation or in cancerous tissues of canines. The studies previously described about the role of CSF-1R in mammary gland development and cancer pathogenesis of mice does not describe any expression of CSF-1R in the murine mammary epithelium. This makes it difficult to know the role of CSF-1R in the canine mammary gland based on studies of CSF-1R and its role in the mammary gland in murine models.

The role of CSF-1R in CMT-U229 avl 2

Since the CMT-U229 avl2 CSF-1R positive cells migrated and invaded the membrane to a larger degree it is likely that CSF-1R expression is associated with increased migration and invasion. However the fact that the CSF-1R negative cells also migrated and invaded indicates that some other cell signaling pathways also drive the migration and invasion of cells in this cell line independent of the CSF-1R expression. It is also unclear if ligand binding by CSF-1 (possibly from fetal bovine serum) or autocrine stimulation is what increased invasion of the CSF-1R positive cells. While the group of CSF-1R positive cells did have better migratory and invasive abilities, the invasion was also not necessarily induced by stimulation of CSF-1R. Instead expression of CSF-1R could correlate with a population of CMT-U229 avl 2 cells which happens to be of a migratory and invasive phenotype. Macrophages can change the phenotype and gene expression of canine tumor cells in many ways making them more invasive *(*Król *et al*., 2012; Król *et al.*, 2014). This means that while the increased CSF-1R expression is indicative of exposure to CSF-1 secreted by TAMs or the tumor cells, some other factor involving TAMs could also increase invasiveness of the CSF-1R positive cells.

Incubation of canine mammary tumor cells with TAMs lead to a switch from canonical to a non-canonical Wnt pathway. (Król *et al.*, 2014). This lead to changes in the morphology of neoplastic cells with formation of branches. Phosphorylation of fascin and formation of complexes with protein kinase C was caused by activation of non-canonical Wnt-signaling and

fascine is essential for the formation of philopodia which is associated with increased net protrusion (Mejillano *et al.*, 2004). Since macrophages increase the expression of CSF-1R and cause this switch to the non-canonical Wnt-signaling the increased motility of the CSF-1R cells could also be explained by these cytoskeletal changes. Macrophages secreted epithelial mesenchymal transition (EMT)-activators and the secretion was further induced by neoplastic cells (Król *et al.*, 2014). Epithelial mesenchymal transition is a process in which epithelial cells lose epithelial characteristics and become more like mesenchymal cells. Conversion of breast epithelial cells to a mesenchymal phenotype has previously been shown to increase invasion, migration and create a claudin-low phenotype compared to epithelial cells (Asiedu *et al.*, 2011). Co-culture of canine mammary tumor cells with TAMs can decrease expression of the epithelial marker cytokeratin and increase expression of mesenchymal marker vimentin (Król *et al.*, 2014). Vimentin has been known to be required in a human breast carcinoma cell line (MDA-MB-231) for the function of a type of protrusions which are involved in invasion through the basement membrane (Schoumacher *et al.*, 2010). However expression of CSF-1R and increased invasion have previously been proven to increase after exposure to CSF-1 without co-culturing with macrophages (Król *et al.*, 2013). The only known ligands to the CSF-1R receptor is CSF-1 and IL-34. Since homozygous depletion of the CSF-1R leads to a similar phenotype as the $Csf1^{op}/Csf1^{op}$mice it is likely that CSF-1 is the most important ligand for CSF-1R (Dai et al., 2002). The ability of IL-34 to induce migration and invasion has to my knowledge not been examined.

Autocrine and exogenous CSF-1R signaling

Transfection of CSF-1R to a mammary epithelial cell line which expresses large quantities of CSF-1 can increase invasion without adding exogenous CSF-1, exogenous CSF-1 might not increase invasion more in a mammary epithelial cell line with high CSF-1 expression (Sapi *et al.*, 1996). Autocrine CSF-1R stimulation may have a larger effect on invasion compared to exogenous CSF-1 and this is regulated by TGF-β (Patsialou *et al.*, 2015). CSF-1R expression is regulated by TGF-β specifically in claudin-low breast carcinoma cell lines and CSF-1R expression is higher in claudin low cell lines. Autocrine CSF-1R signaling leads to increased invasion in claudin low cell lines and also maintains a claudin-low state and attenuates expression of tight junctions. Proliferation of the claudin-low cell lines was on the other hand decreased by TGF-β stimulation. TGF -β was expressed in the membranes of the inserts used in this invasion assay and this could possibly increase the autocrine CSF-1R stimulation leading to increased invasion (Wrobel *et al.*, 2004). Expression of CSF-1 has not been evaluated for the CMT-U229 avl 2 cell line, but since CSF-1 expression is common in breast cancer cell lines, autocrine CSF-1R stimulation or secretion of CSF-1 is a possibility (Morandi *et al.*, 2011). The level of claudin-expression in the CMT-U229 avl 2 cell line is however not known. It is also possible that the fetal bovine serum (FBS) contains some CSF-1, in addition to other growth factors, which could stimulate the receptors. Serum starvation can decrease the mean expression and phosphorylation of CSF-1R (Król *et al.*, 2013). A study of collected bovine serum from fetuses age 152-278 days of gestational age at slaughterhouses show that the serum contained bovine CSF-1 and the level differentiates among individuals. This study did however not mention if bovine CSF-1 would have any effect on human or canine cells. (Yoshihara *et al.*,

2003). No invasion or migration was seen in the inserts placed in FBS free medium in the invasion assay. This could be because the FBS-free medium lacked CSF-1 but other signal-substances in the FBS could also have influenced the cells to migrate into the membrane. Fetal bovine serum may also contain TGF-β among other growth factors (Zheng *et al.*, 2006). Of course it is possible that the CSF-1R receptor in this cell line could be stimulated by a combination of autocrine as well as exogenous CSF-1. When CSF-1R expression in canine mammary carcinomas was analyzed CSF-1R was found in both macrophages and carcinoma cells and this total CSF-1R expression was related to the ability to metastasize (Król *et al.*, 2011; Gonçalves *et al.*, 2016).

CSF-1R and cell migration

CSF-1R has previously been shown to disrupt cell adhesions by relocalization of E-cadherin from the membrane to intracellular vehicles in breast epithelium cells, this likely contributes to increased motility (Wrobel *et al.*, 2004). This CSF-1R signaling pathway is dependent on Src. The CMT-U229 avl 2 cell line expresses E-cadherin and, CSF-1R could have the same effect on E-cadherin in this cell line and thereby increase motility and migration of the cells. Usually a few cells would separate from the wound margin and move into the gap before the wound margins would start to move closer together. The disrupted adhesions might have made it easier for the individual cells of the CSF-1R positive group to migrate into the wound early. There is also the possibility that some of these cells seen on their own in the wound gap are single cells floating in the medium which attached to the bottom of the well in the middle of the gap. Free cells floating in the cell culture medium were sometimes observed in both the CSF-1R positive and CSF-1R negative group despite carefully and repeated washing the dish with PBS and changing cell culture medium right after the scratch was made. Ideally this experiment should have been conducted until the wound of both the CSF-1R positive cells and the CSF-1R negative cells had closed completely. Since the wound healing rate of the CSF-1R positive and CSF-1R negative cells was very similar for the last 2 hours it is possible that the wound healing rate would have continued to be similar in both cell lines until wound was completely closed, making the wound healing rate more similar if measured for a longer time.

CSF-1R and cell invasion

The increased invasion of the CSF-1R positive cells in this study is similar to the results of the invasion assay reported by Król *et al.* (2013). CSF-1 has also been shown to increase invasion in several human and murine cancer cell lines like ovarian carcinoma, lung carcinoma and mammary carcinoma (Chambers *et al.*, 1995; Filderman *et al.*, 1992; Patsialou *et al.*, 2009) Although it is established that CSF-1R can increase invasion in many cancer cell lines, adding CSF-1 will not necessarily increase invasion in an CSF-1R expressing breast epithelial cell lines (Gunawardane *et al.*, 2005) . To invade, the cell needs to go through morphological changes making invasion and migration possible like ability break down of the surrounding tissue to be able to move. Invasive behavior has been connected to cell-surface associated proteolytic activity stemming from interaction with urokinase plasminogen activator (uPA) and its surface

receptor (Ossowski, 1992; Ossowski, 1988). Expression of CSF-1R has been connected to increased secretion of uPA in murine mammary epithelial cell lines and human breast carcinomas (Sapi *et al.*, 1996; Kacinski, 1997). CSF-1 stimulation can also stimulate uPA-mediated invasion in ovarian and carcinomas and induce increased uPA mRNA in murine fibroblasts (Chambers *et al.*, 1995; Stacey *et al.*, 1995) Increasing CSF-1R using either dexamethasone or CSF-1 results in increased invasiveness in increased uPA production and invasion in mammary carcinoma cells (Kacinski, 1997). CSF-1 stimulated induction of uPA in murine fibroblasts was reported to involve activation of E twenty-six (Ets) transcription factors and Ras signaling (Stacey *et al.*, 1995). Mutation of Ets-2 can interrupt CSF-1 signaling pathways in the breast carcinoma cell line BT20 making CSF-1 unable to cause increased invasion. This happens by inhibition of CSF-1 induced c-myc, c-fos and c-jun (Sapi *et al.*, 1998b). Apart from uPA, other factors are involved in invasive activity of CSF-1R expressing cells. Inhibition of collagenases can decrease CSF-1 stimulated invasion (Chambers *et al.*, 1995). The fact that both the cells expressing CSF-1R and the CSF-1R negative cells could be found in the membrane of the Matrigel-coated inserts show that this cell line has some ability to invade the Matrigel, this could in part be caused by an increased level of uPA and collagenases in the CSF-1R positive cells. An increased ability of the cells to migrate toward the chemoattractant could also increase invasion. One invasive cell can break down the Matrigel and make path for less invasive cells to migrate and a cell with higher motility would move through the pores faster. Some cells were also found in the membrane of the CSF-1R negative Matrigel-coated inserts which indicate that this cell line is not completely dependent on CSF-1R stimulation for invasion.

Effect of the Matrigel on the morphology of the cells

The Matrigel used in this study did according to the manufacturer contain extracellular matrix (ECM) proteins (including laminin, collagen type IV, heparine sulfate proteoglycane and entactin), growth factors and other components. The levels of growth factors were reduced in this Matrigel, excluding TGF-β. According to the manufacturer, Corning Matrigel was extracted from the Engelbreth-Holm-Swarm mouse sarcoma. Matrigel might not just represent a barrier for the cells to invade, the component of the Matrigel may also have an effect on gene expression, growth characteristics and morphology of the cells. Components of the ECM can provide critical physical and biochemical cues to epithelial cells (Sisci *et al.*, 2004). Laminin-111 has previously been shown to affect breast carcinoma cells by making them grow in web-like cell organizations and reducing Estrogen receptor-β at mRNA-levels and laminin-1 can also change epithelial cell polarity (Neubauer *et al.*, 2009; Gudjonsson *et al.*, 2002). Co-injection fibroblasts and breast-carcinoma cell line (MCF-7) with Matrigel induced increased tumor growth. (Noël *et al.*, 1993) Adhesion to Collagen IV and fibronectin can however reduce cell invasion in breast carcinoma cell line MCF-7 (Sisci *et al.*, 2004).

Differences in the results of immunohistochemistry and flow cytometry

The level of CSF-1R expression using flow cytometry was very different from the level of CSF-1R using immunohistochemistry in the CMT-U229 avl 2 cell line. For the

immunohistochemical study, a different antibody was used from the flow cytometry. The same antibody could not be used because the antibody used in flow cytometry could not stain the paraffin embedded samples for immunohistochemistry. This could in part explain the differences of the CMT-U229 avl 2 in CSF-1R positive cells observed in immunohistochemistry compared to flow cytometry. The antibody used for immunohistochemistry could have a greater sensitivity for the receptor. The increased level of positive cells could also be a result of the selection which happens while culturing and passaging cells. Because of the generally weak staining using the 1:500 dilution this result was discounted from the immunohistochemistry. If the experiment was to be repeated an antibody dilution of 1:250 would probably be ideal for staining of the CSF-1R receptor in these cell lines.

Another explanation for the different percentages of CSF-1R expression in flow cytometry and immunohistochemistry could be the choice of cut-off. The areas gated in the flow cytometry histograms were the ones with strongest Phycoerythrin (PE)-signals i.e. PE positive and lowest PE-signals i.e. PE-negative respectively (Figure 1 A). To quantitatively decide the continuum of positivity by eye is almost impossible in immunohistochemical stainings. Thus, a software to measure this should have been beneficial and would most probable have given more similar results on the CSF-1R expression.

CONCLUSIONS

The colony stimulating factor-1 receptor is expressed in the CMT-U229 avl 2 cell line and the expression is associated with increased migration and invasion *in vitro.* Several signaling pathways involving E-cadherin, uPA and more have previously been shown to be involved in the CSF-1R signaling pathway. These signaling pathways could possibly be the cause of increased migration and invasion of the CSF-1R positive cells in the CMT-U229 avl 2 cell line. The results indicate that CSF-1R may play a role in the development of metastasis in canine mammary cancer.

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