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**Transcriptome and proteome profiling of
canine mammary tumors: dog as a genetic
model for unraveling mammary cancer
molecular signatures**

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Transcriptome and proteome profiling of canine mammary tumors: dog as a genetic model for unrevealing mammary cancer molecular signatures

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Para a Cati, o amor da minha vida

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Abstract

Within this work, two canine mammary tumors (CMT) derived cell lines named FR37-CMT and FR10-CMT were immortalized and characterized. These cell lines are good models for the development of new therapeutic compounds and novel strategies for the management of CMTs and for the study of CMT resistance to chemotherapy. FR37-CMT cell line is also a good model for the study of epithelial-to-mesenchymal transition (EMT) while the FR10-CMT cell line can be considered a model for the study of malignant CMTs with repression of EMT.

Two new organometallic compounds, TS262 and TS265, have been tested in FR37-CMT and FR10-CMT cell lines. In both cell lines, the IC_{50} for TS262 and TS265 were significantly lower than the displayed by cisplatin and doxorubicin ($1.05\mu\text{M}$ and $1.39\mu\text{M}$ respectively for FR37-CMT and $0.55\mu\text{M}$ and $0.80\mu\text{M}$ respectively for FR10-CMT) and are thus good therapeutic possibilities for the treatment of CMTs.

A novel therapeutic approach using nanotechnology has also been successfully used to enhance the effects of chemotherapeutic compounds. Indeed, functionalized gold nanoparticles (AuNPs) were used for the first time in veterinary medicine to transport TS262 (nanoTS262) and

TS265 (nanoTS265) into FR37-CMT and FR10-CMT cells, enhancing the cytotoxicity displayed by the free compounds.

A quantitative proteomic analysis was also performed in the FR37-CMT cells exposed to TS262 and TS265 in order to obtain more insights into the mechanisms of toxicity and the responses of the cells induced by these compounds.

Using principal component analysis (PCA) of gene expression, it was possible to distinguish metastatic from non-metastatic grade III CMTs using our set of 21 biomarkers (10 miRNAs and 11 mRNAs). The differential expression of miR-155, *DICER1* and *ESR1* between metastatic and non-metastatic grade III CMTs highlighted the importance of these genes in the metastatic transition of CMTs.

Keywords: Canine mammary tumors; Epithelial to mesenchymal transition; chemotherapeutic compounds and nanotechnology; Quantitative proteomics; FR37-CMT; FR10-CMT

Resumo

No trabalho desenvolvido para esta tese, foram imortalizadas e caracterizadas duas novas linhas celulares obtidas a partir de tumores mamários caninos (TMCs), FR37-CMT e FR10-CMT. Estas linhas são ótimos modelos para o desenvolvimento de novos compostos e estratégias terapêuticas para o tratamento de TMCs e para o estudo da resistência à quimioterapia exibida por estes tumores. A linha FR37-CMT é também um ótimo modelo para o estudo da transição epitelial-mesenquimatosa (TEM) enquanto que a linha FR10-CMT pode ser considerada um modelo para o estudo de TMCs malignos com repressão de TEM.

Dois novos compostos metálicos, T262 e TS265, foram testados nas linhas celulares FR37-CMT e FR10-CMT. Em ambas as linhas, o IC_{50} para TS262 e TS265 é significativamente mais baixo do que o exibido pela cisplatina e doxorubicina ($1.05\mu M$ e $1.39\mu M$, respectivamente, para FR37-CMT e $0.55\mu M$ e $0.80\mu M$, respectivamente, para FR10-CMT) e são assim boas possibilidades terapêuticas para o tratamento de TMCs.

Usando nanotecnologia, foi possível usar com sucesso uma nova abordagem terapêutica para aumentar os efeitos de compostos quimioterapêuticos. De facto, pela primeira vez em medicina veterinária, partículas de ouro (AuNPs) funcionalizadas foram usadas para transportar

TS262 (nanoTS262) e TS265 (nanoTS265) para o interior das células das linhas FR37-CMT e FR10-CMT, aumentando a citotoxicidade exibida pelos compostos livres.

Foi efectuada uma análise de proteómica quantitativa em células da linha FR37-CMT expostas a TS262 e TS265 de maneira a aprofundar o conhecimento dos mecanismos de toxicidade e resposta celular induzidas por estes compostos.

Foi possível distinguir TMC metastáticos de não metastáticos utilizando análise de componentes principais (ACP) da expressão genética de um conjunto de 21 biomarcadores (10 miRNAs e 10 mRNAs). A diferença de expressão de miR-155, *DICER1* e *ESR1* entre os TMCs metastáticos e não metastáticos sublinhou a importância destes genes na transição metastática dos TMCs.

Palavras-Chave: Tumores mamários caninos; transição epitelial mesenquimatosa; compostos quimioterapêuticos e nanoterapia; Proteómica quantitativa; FR37-CMT; FR10-CMT

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List of Abbreviations

- 2-DE – Two-dimensional electrophoresis
- 5-AzaC - 5-Azacytidine
- BCRP - breast cancer resistance protein
- BSA – Bovine serum albumin
- CAFs - carcinoma-associated fibroblasts
- CDKN1B - cyclin-dependent kinase inhibitor 1B
- CDKN2A - Cyclin Dependent Kinase Inhibitor 2A
- CMT – Canine mammary tumors
- CSC – Cancer stem cell
- DDAVP - 1-deamino-8-d-arginine vasopressin
- DION - 1,10-phenanthroline-5,6-dione
- DLS – Dynamic light scattering
- DFS – Disease free survival
- DNMT – DNA methyltransferase
- DMEM - Dulbecco's Modified Eagle's Medium
- DMEM-FBS-PenStrep – DMEM supplemented with 10% fetal bovine serum, and a mixture of penicillin (100 U/mL) and streptomycin (100 mg/mL)
- EGF – Epithelial growth factor
- EGFR – Epidermal growth factor receptor
- EMT – Epithelial to mesenchymal transition
- ESS – English Springer Spaniels
- ER α - Estrogen receptor α protein

FBS – Fetal Bovine Serum
 FGF - fibroblast growth factor
 GH – Growth hormone
 GHR – Growth hormone receptor
 GnRH – Gonadotropin releasing hormone superagonist
 GWAS – Genome wide association study
 HBC – Human breast cancer
 HE – Hematoxylin and eosin
 HER2 – Human epidermal growth factor receptor 2
 IBC – Inflammatory breast carcinoma
 ICMT – inflammatory canine mammary tumor
 ICP-MS - Inductively Coupled Plasma Mass Spectrometry
 IEF – Isoelectric electrophoresis
 IGF-I – Insulin-like growth factor I
 IHC - Immunohistochemistry
 IMC – Inflammatory mammary carcinoma
 INSR – Insulin receptor gene
 HIF I – Hypoxia induced factor I
 MDR – Multidrug resistance
 MES - 2-(N-morpholino)ethanesulfonic acid
 miRNAs – micro RNAs
 MMPs – matrix metalloproteinases
 MRP1 - multidrug resistance-associated protein 1
 MVD – Microvessel density
 NanoTS262 – gold nanoparticle system composed of polyethylene-glycol, bovine-serum-albumin and TS262
 NanoTS265 - gold nanoparticle system composed of polyethylene-glycol, bovine-serum-albumin and TS265

NSAID – Non-steroidal anti-inflammatory drugs

OS – Overall survival

PBS – Phosphate buffer saline

PCA – Principal component analysis

PEG - Polyethylene glycol

PGR – Progesterone receptor

PI3K/AKT - Phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT serine/threonine kinase 1

PTEN – Phosphatase and tensin homolog

RT – Room temperature

SDS-PAGE - Sodium dodecyl sulfate polyacrilamide gel electrophoresis

siRNA – Small interference RNA

SPR - surface plasmon resonance

TAM – Tumor-associated macrophages

TBST – Tris buffered saline with 0.1% (v/v) Tween 20

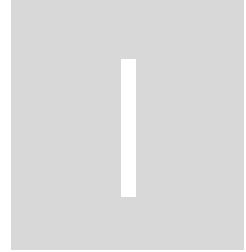
TEM – Transmission electron microscopy

TGF- β - tumor growth factor beta

VEGF - Vascular epithelial growth factor

WHO – World health organization

WNT - Wingless-type MMTV integration site family



I. Bibliographic review in Canine Mammary Tumors

I.1. Thesis Motivation

Canine mammary tumors (CMTs) are the most frequent neoplasms diagnosed in non-spayed female dogs. From these, 50% of all CMTs are malignant, and mastectomy (and ovariectomy in intact dogs) is the only broadly accepted treatment since chemotherapy with tamoxifen, doxorubicin and docetaxel, *e.g.*, has not been proven effective against CMTs.

In Figure I.1, the outline of the project is presented.

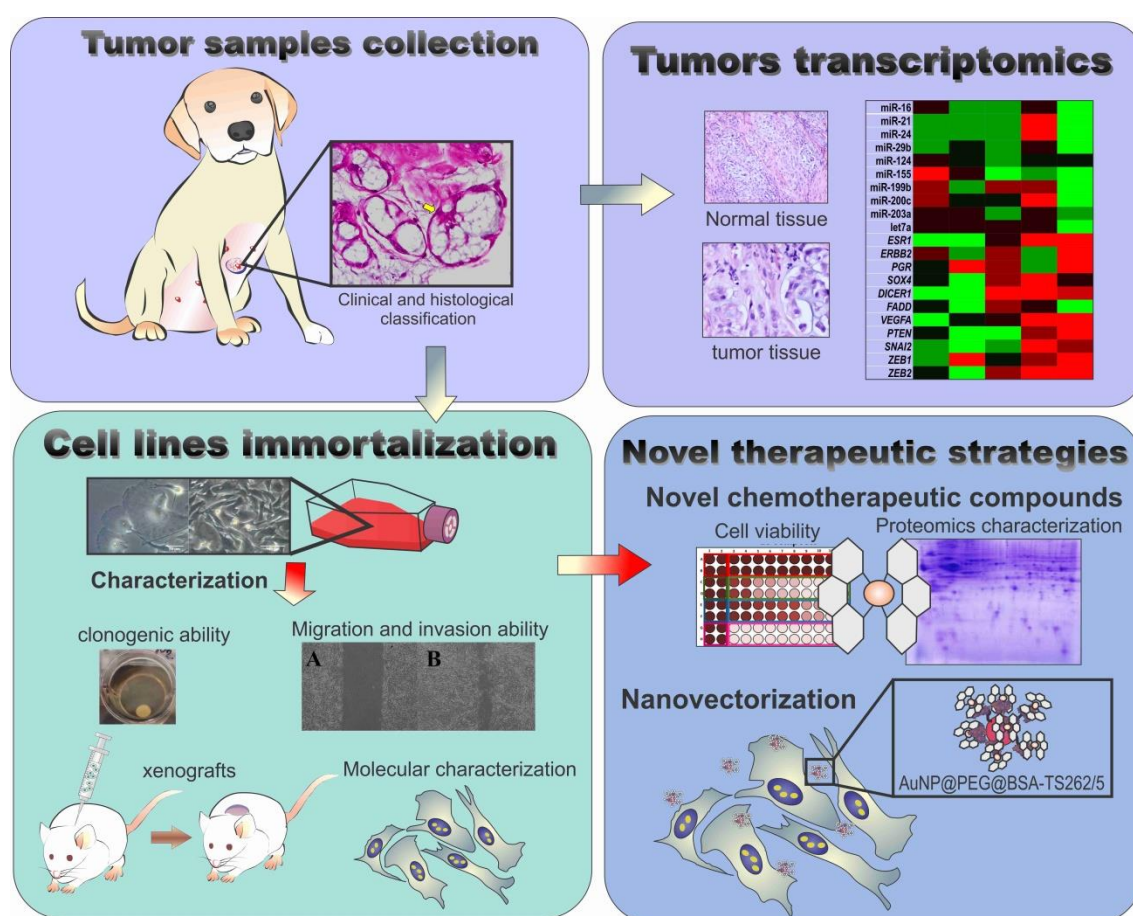


Figure I.1 – Work plan highlighting major scopes of this thesis. CMTs will be collected and processed for i) immunohistopathology characterization ii) spontaneous immortalization of CMT cell lines iii) CMT transcriptomics. After detailed characterization, the CMT cell lines obtained this way will also be used for the study of novel therapeutic compounds and the application of novel strategies, such as nanovectorization with gold nanoparticles, for the treatment of canine mammary cancer. They also will serve to explore the mechanisms of toxicity and resistance by quantitative proteomics, for instance. The analysis of RNA expression in CMTs will be compared with matched normal mammary tissue. The expression of 21 genes, related with mammary cancer and EMT, will be accessed for their potential use as biomarkers for the metastatic progression of CMT tumors.

Our aim was to establish new, well characterized CMT cell lines derived from the primary tumors by spontaneous immortalization. Relevant information on the CMTs collected during this work is described in table A.1 in the appendix. The molecular characterization of the cell lines will allow the identification molecular events and pathways responsible for their metastatic potential and cancer resistance.

The acquisition of metastatic potential has been linked to epithelial to mesenchymal transition (EMT) in human breast cancer (HBC). Based on the information available on HBC and in CMT models, we aimed to select panels of microRNAs (miRNAs) and mRNAs linked to this transition in order to identify molecular profiles linked to the metastatic transition in the original CMTs tissues and in the immortalized cell lines. At the same time, these new cell lines will serve as models for the identification of novel compounds with chemotherapeutic potential. For the most promising compounds their mechanism of action and molecular targets will also be explored using a quantitative proteomic analysis.

I.2. Incidence

Mammary cancer is the most common neoplasia of the intact female dog and the second most frequent when both sexes are considered. In the Alameda county, California, USA the incidence of mammary cancer, was 252 per 100 000 female intact dogs per year.¹ The reported incidence of CMTs in the United Kingdom is 205 per 100 000 dogs per year,² while in Sweden is 111 per 10 000 female dogs per year.³ The numbers described in the USA and the UK are similar to those reported for dogs in Genoa, Italy, where the incidence is 192 per 100 000 dogs per year being 70% of the all cancer cases diagnosed in female dogs, while only 25 per 100 000 male dogs were affected per year.⁴ In the Vicenza and Venice provinces of Italy, the local Animal

Tumor Registry reported that 56% of the diagnosed tumors were of the mammary gland while only 1.9% of male dogs were affected.⁵ These numbers are consistent with the findings in Switzerland, where the country's canine cancer registry revealed that 20.5% of all tumors in both sexes were mammary tumors.⁶ The differences of incidence between sexes are consistent with previous reports.⁷ Among the histopathological characterized CMTs, approximately 50% of the tumors are malignant.⁸⁻¹⁰

I.3. CMT Risk factors

I.3.1.Age

The majority of CMT cases are reported in dogs with 4 years of age or more.^{1-3, 8} The average age of onset for benign CMT was been reported to be 8.5 years while for malignant CMT it has been reported to be 9.5 years.¹¹ However, the average age of onset for overall CMTs has also been reported to be 7.3 and 8.0 years.^{3, 12} Despite the differences in age of onset reported, it is largely described that the incidence of CMT peaks at ages 9-12,^{1-4, 9} which is also the case when all types of canine cancer are considered.^{5, 6, 13}

I.3.2.Diet

Nutritional factors have been implicated in CMT onset. A study in pet dogs in the United States found that among spayed dogs, the risk of developing mammary cancer was reduced if the dogs were thin at 9 to 12 months of age.¹⁴ A study in Spain made a similar observation, that obesity at 1 year of age was a risk factor for the development of benign and malignant mammary tumors without consideration of ovariohysterectomy but not after the first year of life.¹⁵ It was also

observed that the consumption of homemade meals, when compared to the intake of commercial foods was also significantly associated with a higher incidence of CMTs.¹⁵ These authors also associated the consumption of beef and pork with increased risk of the dogs developing mammary tumors.¹⁵

I.3.3.Hormonal dependency

The data collected from different canine tumor registries point that CMTs are rare in male dogs and frequent in intact female dogs.^{1, 4, 6, 7, 9} Furthermore, the incidence of CMTs is greatly reduced in spayed female dogs when compared to intact female dogs.^{1, 9, 16} These observations confirm that the onset of CMTs is dependent of sexual hormones. This also may be the reason for the increased incidence of CMT in Sweden (5 times higher) when compared to USA and UK, since in Scandinavia pet dogs are not routinely spayed.³

The relative risk of acquiring malignant tumors in female dogs spayed before the first estrus is 0.5%.¹⁶ If the dogs are spayed after the first or second estrus the relative risk rises to 8% or 26%, respectively.¹⁶ The risk for benign tumor decreases even when neutering is performed later in the life of the dog but this does not reduce the risk of acquiring malignant tumors.¹⁷ A protective effect of spaying is also observed when the animals are sterilized within two years after the detection of the malignant tumors.^{18, 19} The protective effect of early pregnancy observed in women has not been demonstrated in dogs.¹⁶

The administration to dogs of progesterone or synthetic progestins to prevent estrus have been shown to induce full lobulo-alveolar development and hyperplasia of epithelial and myoepithelial cell elements in the canine mammary gland, alongside with hyperplasia of secretory (epithelial) and myoepithelial cell elements.²⁰ Studies with beagles revealed an earlier and more

frequent development of benign tumors, in dogs treated with progestins.²¹⁻²⁴ A report by Geil *et al.* 1977 reported that combinations that treatment of dogs with combinations of progestins with estrogens led to the development of malignant mammary tumors in the animals tested.²⁵

I.3.4.Breed dependency

Purebreds have been shown to be more predisposed to CMTs than crossbreds.¹ It was calculated a 7-fold higher risk for the purebred animals when compared with cross breed dogs.¹ In particular, several spaniel breeds, the poodle, the terrier and dachshund breeds seem to be more predisposed to the condition.^{7, 13, 26-28} The differences in risk between breeds and between purebreds and crossbreeds have been suggested to be due to genetic heritable components such as the ones observed in human breast cancer (see chapter I.3.5).²⁸ Two Japanese studies reported a correlation between the size of the dogs and mammary cancer: smaller dog breeds have a higher incidence of CMTs when compared with large dog breeds.^{13, 29}

I.3.5.Germline mutations that predispose for CMTs

The *BRCA1* and *BRCA2* genes are known to increase the risk of HBC.³⁰ Studying English Springer Spaniels (ESS) one of the breeds with the higher risk of developing CMTs, showed that this risk was also associated with germline mutations in the canine *BRCA1* and *BRCA2* genes.³¹ Until now, several polymorphisms and insertion/deletion mutations have been identified in canine *BRCA1* and *BRCA2* genes that may be implicated in CMT predisposition.³²⁻³⁵

Besides *BRCA1* and *BRCA2* genes, mutations in other genes have also been recently implicated in CMT predisposition.³⁶⁻³⁸ Mutations in the canine *ESR1* gene, which codes for the

estrogen receptor α protein, ER α , were also implicated in the increased risk of the ESS breed to develop CMTs.³⁶

A germline deletion in the canine p53 gene, *TP53*, (exons 3 to 7) was described as being associated with the development of CMT in a boxer female dog.³⁷

A genome wide association study (GWAS) performed in ESS dogs demonstrated an association between a deletion in chromosome 11, spanning the *CDK5RAP2* gene, with the predisposition of the breed to CMTs.³⁸

I.4. CMT classification

According to the World Health Organization (WHO) guidelines defined in 1999, CMTs, can be classified in three different types: i) carcinomas (previously adenocarcinomas) - malignant epithelial or myoepithelial CMTs, ii) sarcomas - malignant CMTs of mesenchymal origin and iii) carcinosarcomas – malignant mixed tumors, with malignant epithelial or myoepithelial and malignant mesenchymal components (Figure I.2).³⁹

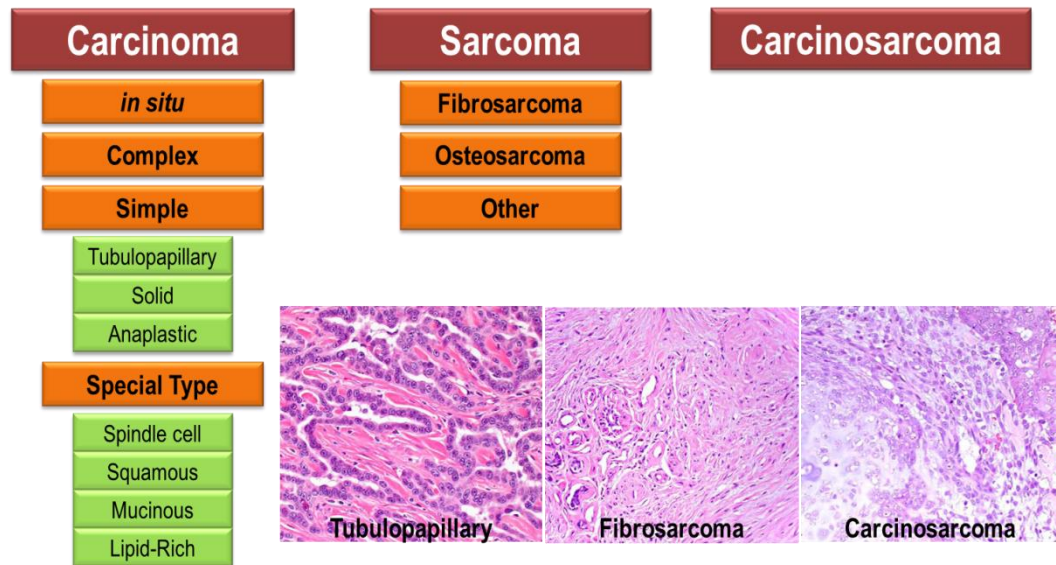


Figure I.2 – Classification of CMTs according to the 1999 WHO guidelines. Carcinomas are classified as *in situ* (non-infiltrating), complex (with involvement of myoepithelial cells), simple and mixed (with involvement of mesenchymal cells). Less frequent types of carcinoma include spindle-cell, squamous, mucinous or lipid-rich carcinomas. Canine mammary sarcomas are classified as fibrosarcoma, osteosarcomas or other types of rare mammary tumors such as chondrosarcomas or liposarcomas. Carcinosarcoma is a rare type of mammary mixed tumor in which both the epithelial and mesenchymal components of the tumor are malignant. Images exemplifying a carcinoma, a sarcoma and a carcinosarcoma are also depicted.

Epithelial carcinomas are the most common form of malignant CMTs.^{8, 39} The most rare types of malignant CMT are carcinosarcoma and carcinoma or sarcoma in benign tumor.^{39, 40} Sarcomas such as chondrosarcomas and liposarcomas can also found be found in the mammary gland of dogs but are uncommon.^{39, 40}

Besides man, dog is the unique animal species in which spontaneous inflammatory mammary carcinoma (IMC) has been reported.⁴¹ Both human inflammatory breast carcinoma (IBC) and canine IMC are considered the most malignant type of mammary cancer with an extremely poor prognosis and is still poorly characterized in dogs.⁴¹ IMC and HBC cannot be defined in a specific histologic subtype: infiltrating ductal carcinomas, other carcinomas and unspecified malignant tumors have been described as involved with IBC. Pena *et al.* 2003 found the same histologic indefiniton in 20 canine inflammatory carcinomas.⁴¹

I.5. CMT Etiology and clinical prognostic factors

CMTs progress from the benign to the malignant form.^{8, 11, 39} CMTs usually present themselves in older intact female dogs, with 1 or, in approx. 70% of the cases, multiple tumors in one or both mammary chains and are predominantly located in the caudal abdominal and inguinal mammary glands.^{8, 11} When metastases occur, the primary sites are the regional lymph nodes and the lungs due to the anatomy of the canine lymphatic system.⁴²⁻⁴⁴ The risk of dogs developing primary CMTs is 32% greater in patients with previous benign or malignant CMTs and 58% if the CMTs are located in the ipsilateral side of the mammary gland.^{11, 45}

The Elston and Ellis grading system is widely used in veterinary for the grading of CMTs, with slight adaptations (Table I.1).^{40, 46-49}

Table I.1 – The Elston and Ellis grading system evaluates three morphological features of mammary carcinomas observable in histopathological routine, each scored from 1 to 3. In tubule formation, the percentage of the tumor area displaying tubules is determined. If more than 75% of tumor area displays tubules, a score of 1 is given; a score of 2 is given to tumors with 10 to 75% of tubules in the area observed and the highest score of 3 is given to carcinomas with less than 10%. In nuclear pleomorphism, a score of 1 is given to tumors displaying regular uniform nuclei; scores of 2 and 3 are given accordingly to the degree of variation in the size and shape of the nuclei. In the mitotic counts there have been described two adaptations of the method to score canine mammary carcinomas from 1 to 3. In the method described in Clemente *et al.*, 2010 a score of 1 is given to tumors displaying less than 10 mitotic cells per 10 high power fields (hpf); a score of 2 is given to carcinomas with less than 20 mitotic figures in 10 hpf; a score of 3 is given to tumors with more than 20 mitotic figures per 10 hpf. In the method described by Misdorp, 2002 a score of 1 is given to mammary carcinomas with occasional hyperchromatic nuclei or mitotic figures per hpf; a score of 2 is given to tumors with 1 or 2 hyperchromatic nuclei or mitotic figures per hpf; a score of 3 is given to carcinomas with 2 to 3 or more hyperchromatic nuclei or mitotic figures per hpf. Canine mammary carcinomas are classified as Grade I (low malignancy) or well differentiated if the mammary carcinomas reach a score of 3 to 5; Grade II (intermediate malignancy) or moderately differentiated if the tumors are scored between 6 or 7; Grade III (high malignancy) or poorly differentiated if the tumors are scored with 8 or 9.^{46, 48, 49}

Tumor Feature	Grading
Tubule formation	1-3
Nuclear Pleomorphism	1-3
Mitotic counts	1-3

This method has been demonstrated as having prognostic value since the two year survival after surgery of dogs is higher in grade I CMTs, lesser in grade II and the smallest overall survival was found in the patients with grade III CMTs.⁴⁷ Also the TNM staging system, which uses information on tumor size (T), the presence of metastases at the local lymph nodes (N) and at other organs (M), and classifies CMTs in clinical stages I (initial stage) to V (final stage), has been shown to possess prognostic value (Table I.2).¹⁹ It was demonstrated that patients with tumors greater than 5 cm in diameter and with clinical stages IV or V are associated with the worse two year survival.¹⁹

Table I.2 – TNM (Tumor-node-metastasis) staging system as defined by the WHO for CMTs. The staging system considers the size of the primary tumor (T), the presence of metastasis at the regional lymph nodes (N) and the presence of metastasis in distant organs (M). Patients with stage I CMTs have higher overall survival time and on the contrary, patients with tumors larger than 5 cm or with metastasis at the lymph nodes (stage IV) or distant metastasis (stage V) have a poor overall survival time.

Stage	Tumor maximum diameter	Regional lymph node status	Distant Metastasis
I	< 3 cm	No metastasis	No
II	3 – 5 cm	No metastasis	No
III	> 5 cm	No metastasis	No
IV	Any size	Metastasis	No
V	Any size	With or without metastasis	Detected

HBC have been classified in 5 molecular subtypes associated with different clinical outcomes: luminal A, luminal B, HER2-overexpressed, basal-like and normal-like.⁵⁰⁻⁵³ Luminal A HBCs have the best prognosis and basal-like HBCs have the worst prognosis.⁵³ Luminal B HBC has a worse outcome than Luminal A and a better prognosis than HER2-enriched HBC.^{51, 52} This

classification has been increasingly adopted in HBC, due to their proved prognostic value and the discriminative value for the use of targeted therapy for ER and HER2 expressing tumors.⁵⁴

In CMT, identification of the molecular subtype of tumors has been also attempted by immunohistochemistry (IHC).⁵⁵⁻⁶¹ However, four independent transcriptomic studies performed did not confirm the existence of these (or other) molecular subtypes.⁶²⁻⁶⁵ Despite this limitation, several authors assigned molecular classifications to the various CMTs with different conclusions on the prognostic value of the identification probably due to the different histopathologic markers used in the studies.^{56-58, 61} Also, the assumption made in all works, that luminal B mammary tumors are HER2 positive and that luminal A CMTs are HER negative do not correlate with HBC, in which 14% of luminal A are HER2 positive and 24% of luminal B tumors are HER2 positive.⁵⁴ Interestingly, Gama *et al.* 2008 observed a significant statistical correlation between poor survival, grade III and CMTs assigned to the basal-like phenotype.⁵⁷ However, in a study by Sassi *et al.* 2010, the majority of grade III CMTs were luminal B while tumors classified as basal-like were grade I and no observable prognostic value was attributed to the molecular classification.⁵⁸ A third study by Im *et al.* 2014 confirmed a correlation between poor prognosis, grade III and basal-like CMTs.⁵⁶ Another IHC study used ER α , HER2 and CAV-1 staining for molecular phenotyping, and Shinoda *et al.* 2014 found a significant correlation between the level of positive staining, ER α localization, HER2 and CAV-1 staining and the behavior and prognosis of the tumor.⁶¹ However, the authors did not confirm a correlation between CMTs classified into the 5 molecular phenotypes and prognosis of the CMTs.⁶¹

I.6. Canine mammary carcinogenesis

At the moment, there is a small understanding on which specific genetic alterations contributes to the progression of CMTs. However, major changes in the cellular metabolism of the mammary gland may contribute to the formation and development of CMTs.

I.6.1. Role of steroid hormones and growth factors in the initiation and malignant progression in mammary carcinogenesis

As depicted in I.3.3, epidemiology of CMTs shows a clear connection between sexual steroid hormones and the initiation of mammary carcinogenesis.

Estrogens and progesterone levels also appear to be essential for the acquisition of malignant behavior in CMTs. Serum levels of progesterone, 17β -estradiol, androstenedione, dehydroepiandrosterone, testosterone, estrone sulfate, prolactin, growth hormone (GH) and of insulin-like growth factor I (IGF-I) were higher in the serum and in the tumor tissues of female dogs with malignant tumors compared with controls or with dogs with benign tumors.⁶⁶⁻⁷⁰ Higher levels of epithelial growth factor (EGF) were also found in the tissue of dogs with malignant CMTs.⁷¹ The levels of prolactin, GH, IGF-I and EGF were significantly correlated with the levels of 17β -estradiol and progesterone.^{66, 67, 71}

It has also been established that patient dogs with higher serum and mammary tissue concentrations of progesterone, 17β -estradiol, androstenedione, dehydroepiandrosterone and estrone sulfate, prolactin, GH and IGF-I were correlated with shorter disease free interval and shorter overall survival time.⁶⁸⁻⁷⁰

Administration of progestins to female dogs was shown to increase the secretion of GH in the mammary gland.⁷²⁻⁷⁴ Also, mammary cells producing GH were shown to be positive for the progesterone receptor (PGR) by IHC.⁷⁵ Parallel to the increase in GH production, a rise in the blood levels of IGF-I and IGF-II has been shown to occur, which stimulate mammary cell proliferation.^{66, 76} Likewise, a synergy has been established between 17 β -estradiol and IGF-I although the systemic or local origin of the IGF-1 has not been established by this study.⁶⁶ It is possible to speculate that GH/IGF-I stimulate the proliferation of mammary stem cells as a first step in the process of mammary carcinogenesis in progesterone dependent CMTs.^{66, 76} A recent study observed an association between increased levels of aromatase, poorly differentiated (grade III) CMTs and overweight and obese dogs.⁷⁷ The authors also found a correlation between the expression of aromatase, leptin and IGF-I and the presence of ER α and PGR in the CMTs studied.⁷⁷

These findings may justify that a faster growth of CMTs is probably due to the higher expression of aromatase and associated conversion of cholesterol into steroid hormones in the tumors or in the adjacent mammary tissue.⁷⁷ The autocrine/paracrine production at the mammary gland of GH, IGF-I, EGF and prolactin has been described.^{66, 70, 74-76, 78-80} It is possible that tumor formation and malignant transformation depend on cellular division and growth induced by the increased expression of steroid hormones and, consequently, of the above mentioned growth factors by CMTs and/or by the adjacent normal mammary cells. *In vitro*, it was shown that knock-down of the growth hormone receptor (GHR) in CMT-U27 cell line (derived from canine mammary carcinoma) reduced the percentage of cells dividing, by down regulating the ERK1/2 signaling pathway, and increased the percentage of cells in apoptosis.⁸¹

It has been documented by IHC that both ER α and PGR are more abundant in the normal and benign lesions of the mammary gland when compared with mammary carcinomas and their

expression is even more reduced in metastasis.⁸²⁻⁹⁰ Transcriptomic studies also corroborate these findings: mRNA levels of *ESR1* and *PGR* genes are decreased in metastatic CMTs.^{64, 65} The mRNA levels of Insulin receptor gene (*INSR*) were also found to be downregulated in metastatic CMTs.⁹¹

The ErbB protein family or epidermal growth factor receptor (EGFR) family is a family of four structurally related receptor tyrosine kinases, EGFR (ErbB1), HER-2 (ErbB2), ErbB3 and ErbB4.⁹² IHC has shown that EGFR is overexpressed in almost 50% (40.7% to 55.7%) of the malignant CMTs analyzed while it is overexpressed in approximately 18.5% (17.4 to 19.6%) of benign CMTs.⁹³⁻⁹⁶ This overexpression has been positively correlated with tumor necrosis, histological grade of malignancy (and in particular one of its features, mitotic index) and clinical stage (and with two of its features, tumor size and presence of metastasis at the regional lymph nodes).^{93, 95} Furthermore, RNA based studies show another aspect of the expression of *EGF* and *EGFR* genes in metastatic CMTs: a downregulation of both in metastatic CMTs when compared to normal mammary gland and non-metastatic CMTs.^{64, 97}

IHC of CMTs for canine HER-2 receptor revealed that approximately 16% (12.5% to 19.1%) of malignant CMTs are overexpressing HER-2 while the benign CMTs are reported to express from 0% to 8.6%.^{96, 98-100} These values are in the same range as seen for HBC.⁹⁸ Remarkably, HER-2 genomic amplification, which is present in 85% to 90% of the HBC HER-2 overexpressing tumors, was not found in the HER-2 positive CMTs.⁹⁸

Discrepancies between the above summarized values of HER-2 expression are possible to observe in three other papers. A paper by Rungtipat *et al.* 1999 reported that 50% of benign CMT overexpress HER-2 receptor as evaluated by IHC¹⁰⁰ which is very different from the described means in the other reports. A source of variability might reside in the treatment of the samples prior to staining: the authors used a hydrated autoclave treatment to enhance reactivity

of HER-2 which was not used in the other works.^{96, 98-100} Two other studies by Hsu *et al.* 2009 and Ressel *et al.* 2013, reported that 29.7% and 28.65% of malignant CMTs, respectively, had overexpression of HER-2.^{101, 102} The discrepancy between these studies and the previously reported values for malignant CMTs is due to the interpretation of the Herceptest results. The authors of both papers consider tumors HER-2 positive when graded with the score of 2+, though the samples should only be considered positive with a score of 3+ in order to avoid equivocal results.⁹⁸ This discrepancy in the analysis of IHC results is possible to observe even in works from the same research group, *e.g.* Dutra *et al.* 2004 and Bertagnolli *et al.* 2011. In Dutra *et al.* 2004 the authors also assumed that CMT samples were positive when scored 2+ and reported an average of 34.4% of HER-2 positive results.⁹⁹ For the purpose of this thesis, it was possible to review the results available within that paper allowing the calculation of the percentage of HER-2 positive samples scored 3+ (12.5%). This number is similar to the 14.8% of HER-2 positive CMTs reported made in Bertagnolli *et al.* 2011 (from the same group) in which it was considered that only CMTs scored 3+ would be considered positive.⁹⁶

I.6.2. Major genomic alterations involved in canine mammary carcinogenesis revealed by global expression studies

Genomic copy number alterations are a main genomic event present in both HBC and CMTs.^{103, 104} Flow cytometry studies have shown that genomic instability increases from benign to malignant CMTs as measured by DNA aneuploidy; 15% to 25% of benign CMTs display aneuploidy while 50% to 60% malignant tumors have shown to have either an increase or a decrease in total amount of DNA.¹⁰⁵⁻¹⁰⁷ Variations in gene copy number have been reported in CMTs: loss of copy number has been described for *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CDKN2A/B*,

CHEK2, *COL9A3*, *CYP2E1*, *PTEN*, *MAP2K4*, *RB1* and *TP53* genes, while gain in copy number has been described for *MYC*, *KIT* and *PFDN5* genes.^{103, 104}

The importance of several signaling pathways in both HBC and CMT was highlighted in a microarray analysis of mRNA expression in mammary tumors from humans and dogs by Uva *et al.* 2009.⁶³ Analysis of gene expression data revealed a great degree of similarity in the affected signaling networks in both types of cancer.⁶³ The acquisition of malignancy in CMTs was accompanied by molecular signatures compatible with loss of phosphatase and tensin homolog (PTEN) function, hypoxia induced factor I (HIF I) activation [both signatures compatible with activation of the Phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT serine/threonine kinase 1 (PI3K/AKT) signaling pathway], immune response expression signature, interferon signaling, activation of KRAS signaling pathway and expression loss of negative regulators of Wingless-type MMTV integration site family member (WNT)/ β -catenin and MAPK signaling pathways.⁶³ A recent work by Yu *et al.* (ahead of print) confirmed the importance of the role of WNT/ β -catenin signaling in CMTs.¹⁰⁸ In malignant CMTs it was found a significant upregulation of *DKK1*, *SFRP1*, *FZD3*, *CTNNB1*, and *LEF1* which correlated with higher levels of β -catenin and LEF1 protein in tumor cells.¹⁰⁸

Analyzing malignant CMTs with different malignancy grades, Pawlowski *et al.* 2013 were able to find a significant overlap between mRNA expression patterns and histological grade III.⁶² The grade III CMTs were shown to have an increased expression of genes involved in inflammation and cytokine signaling, which can indicate that these events occur later in the canine mammary carcinogenesis.¹⁰⁹

Analysis of the transcriptome of metastatic CMTs revealed up-regulation of genes involved in cell division, DNA damage repair and matrix invasion and downregulation of genes associated with epithelial differentiation, cell adhesion and angiogenesis.^{64, 65} It was possible to observe in

this work an overall decrease in the expression of membrane receptors, such as *ESR1*, *PGR*, *EGFR*, *FGFR1*, *GHR*, *PDGFR*, *TGFDR*, etc. which would indicate that metastatic CMTs are no longer dependent of steroid hormones or growth factors to proliferate.^{64, 65}

The expression changes of three genes (overexpression of *BMP2* and *DERL1* genes and downregulation of *LTBP4* gene) were proposed to be discriminative of malignancy in CMTs.⁹⁷

Proteomic studies were able to identify different expression patterns between benign, malignant and metastatic CMTs.^{110, 111} The benign expression profile consisted in the overexpression of β -actin, keratin 8, keratin 17, keratin 19 and phosphoglycerate mutase 1 proteins and downregulation of Calumenin, fibrinogen beta chain, fibrinogen gamma chain and siderophilin in non-malignant CMTs.¹¹⁰ The malignant expression profile involved the overexpression of eukaryotic translation initiation factor 4A3, creatine kinase B, tropomyosin 1 α and 14-3-3- ζ proteins and the downregulation of Gelsolin and peptidase D proteins in all malignant CMTs.¹¹⁰ In the metastatic expression profile it was possible to observe the overexpression of adenosine deaminase, bomapin, coronin 1A, ornithine aminotransferase, proliferating cell nuclear antigen, D-3-phosphoglycerate dehydrogenase, Ranspecific GTPase-activating protein, tropomyosin 3 and thioredoxin domain containing 5 proteins and the downregulation of Annexin A5, Rho GTPase activating protein 1, calretinin, fibrinogen β chain, isocitrate dehydrogenase 1, maspin, myosin light chain 2, peroxiredoxin 6 and tropomyosin 1 proteins in the metastatic CMTs.^{110, 111}

I.6.3. Oncogenes and tumor suppressor genes implicated in canine mammary carcinogenesis

As already stated in chapter I.3.5, germline mutations in the canine genes *BRCA1* and *BRCA2* genes are connected with the increased risk of CMTs onset and progression.³¹⁻³⁵ The loss of *BRCA1* nuclear localization was associated with ER α negative, PGR negative and triple negative CMTs, and with the increased abundance of a high proliferation marker, Ki67, in malignant CMTs when compared to normal mammary gland and with dysplasia lesions.^{112, 113} The loss of nuclear localization of *BRCA1* was accompanied by the ectopic localization of the protein in the cytoplasm of tumor cells.^{112, 113} The levels of *BRCA2* mRNA were found to be reduced in primary CMTs when compared to normal mammary tissue.¹¹⁴ In another study, conflicting results were reported.¹¹⁵ *BRCA2* gene was up-regulated in 50% of the malignant CMTs analyzed and in 50% of the lymph nodes metastases of CMTs.¹¹⁵ At the same time, the expression levels of *RAD51* mRNA were found to be up-regulated in 60% of the primary CMTs and in 80% of the lymph nodes CMTs present in the study.¹¹⁵

As mentioned in chapter I.3.5, a germline deletion in canine *TP53* gene was correlated with increased risk of dogs acquiring CMTs,³⁷ however, somatic mutations in this gene have been mainly encountered in malignant CMTs, suggesting that p53 has a similar role in CMTs and in HBC in the induction of malignancy in mammary cancer.^{37, 116-121}

The Cyclin Dependent Kinase Inhibitor 1A, *CDKN1A*, mRNA levels for p21^{CIP1} were found to be increased in all malignant CMTs tested, whereas only 40% of the benign CMTs and 40% of metastases CMTs tested displayed overexpression of *CDKN1A* mRNA.¹²² This may be an indication that up-regulation of *CDKN1A* may be necessary for the development of the malignant phenotype in CMTs.¹²²

The *CKN1B* cyclin-dependent kinase inhibitor 1B (*CDKN1B*) gene which codes for p27^{KIP1} was found to downregulated in 40% of the benign CMTs, 90% of the malignant CMTs and 80% of the metastatic CMTs metastases tested.¹²² By IHC, it was possible to identify nuclear p27^{KIP1} in 90% of benign of benign CMTs and in approximately 20% of malignant CMTs.¹²³ Taken together these observations seem to indicate that loss of p27^{KIP1} ability to inhibit cell cycle progression may be associated with malignant progression in CMTs.^{122, 123}

As previously mentioned in I.6.2, the loss of *PTEN* copy number and function are important events in canine mammary carcinogenesis.^{63, 103, 104} The levels of *PTEN* mRNA were shown to be 10 fold reduced in malignant CMTs and 100-fold reduced in metastatic CMTs when compared to normal mammary cells.¹²⁴ However, only 33% of malignant CMTs had loss of PTEN protein, as evaluated by IHC.¹²⁵

Genetic alterations in genes involved in apoptotic pathways have been demonstrated to be crucial for the development of cancer.¹²⁶ CMTs have been demonstrated to possess increased expression of anti-apoptotic proteins such as Bcl-2, Bcl-X and survivin and a significant decreased expression of pro-apoptotic proteins such as Bax, Caspase 8 and Caspase 3.^{127, 128}

I.6.4. Influence of tumor microenvironment in CMT growth, migration and invasion

As tumors grow, the masses of cells need more nutrients from the microenvironment.¹²⁹ Interaction of CMTs with the microenvironment will promote angiogenesis which will allow tumor growth.¹²⁶

Vascular epithelial growth factor (VEGF) is one of the most important factors that stimulates endothelial cell proliferation and has been found to be more expressed in malignant

CMTs.¹³⁰⁻¹³² Independent studies demonstrated positive correlations between VEGF increased expression and other factors including CMT grade, microvessel density (MVD), HIF-1 α , intra-tumor FoxP3 expression, COX-2, tumor-associated macrophages (TAM) and a shorter overall survival (OS) of the patient.^{130, 133-136} VEGF expression was also associated with the expression of the receptor VEGFR-2 in CMT cells and in endothelial cells.¹³⁷

Expression of COX-2 enzyme, an inducible enzyme important in the normal inflammatory response and angiogenesis through the production of prostaglandin H₂ (PGH₂), has been shown to be overexpressed in malignant CMTs.¹³⁸⁻¹⁴⁰ Expression of COX-2 has been significantly correlated with HER-2 overexpression, EGFR, VEGF, VEGFR-3, MVD, presence of regional metastasis, decreased OS and disease free survival (DFS).^{93, 135, 136, 141-143}

These observations reveal the importance for VEGF and COX-2 in the angiogenesis associated with CMTs and its importance for tumor growth, and as biomarkers of tumor dedifferentiation and poorer prognosis.

In CMTs, TAMs inhibit the canonical WNT signaling pathway and, at the same time, induce the non-canonical WNT pathway in the CMTs which induced epithelial to mesenchymal transition (EMT) in tumor cells.¹⁴⁴ *In vitro* it was also possible to observe the up-regulation of angiogenesis and WNT related genes in macrophages co-cultured with canine mammary cell lines.¹⁴⁵ In cancer cells it was possible to observe the up-regulation of genes involved in cytokine/chemokine pro-inflammatory signaling and myeloid specific antigen related genes.¹⁴⁵ Up-regulation of genes associated with invasion, angiogenesis and EMT were also found in CMT derived cancer cells co-cultured with carcinoma-associated fibroblasts (CAFs).¹⁴⁶

The EMT is a major molecular reprogramming event implicated in canine mammary cancer progression, invasion and tolerance to chemotherapeutic agents.^{147, 148} Hallmarks of EMT are the

gain of mesenchymal characteristics by epithelial cells, such as the expression of vimentin, increased cellular mobility and loss of epithelial markers, e.g. E-cadherin.^{147, 149} Three major signaling pathways have been described as implicated in the induction of EMT: WNT, tumor growth factor beta (TGF- β) and fibroblast growth factor (FGF).^{147, 149} Twist, Snail, Slug, ZEB1 and ZEB2, are known transcriptional factors that induce EMT in cancer cells.^{147, 149} In HBC models, EMT has been shown to generate cancer stem cell (CSC)-like cells expressing stem cell associated CD44⁺/CD24⁻ antigenic profile and with self-renewal capabilities.^{150, 151}

CSCs or tumor initiating cells are capable of self-renewal and are capable of generate a secondary tumor with the phenotypic heterogeneity of the primary tumor.^{147, 152} Thus, CSCs have been proposed to be very important in mammary tumor invasion, formation of metastasis and resistance to chemotherapeutic compounds.^{147, 152, 153} CSCs have been identified in CMTs and in tumor derived canine mammary cell lines.¹⁵⁴⁻¹⁵⁶

A class of small, 20 to 22 nucleotides, non-coding RNA, called miRNAs are negative post-transcriptional regulators of the gene expression, affecting the translation of more than 60% of genes by connecting.¹⁵⁷ Genetic silencing is accomplished by base-pairing between the seed region (nucleotides 2-8 in the 5' region) of the miRNA and the complementary region in the 3'-untranslated region of the target mRNAs in the RISC complex.^{158, 159} Therefore, miRNAs can act as key regulators of a particular pathway, regulating at the same time the expression of hundreds of genes while other miRNAs may target individual targets and some miRNAs have also been shown to regulate cooperatively the same mRNAs.^{157, 159} Differential expression of miRNAs has been linked to carcinogenesis as tumor suppressors or oncogenes in several human tumors and in CMTs (Figure I.3).¹⁵⁹⁻¹⁶¹

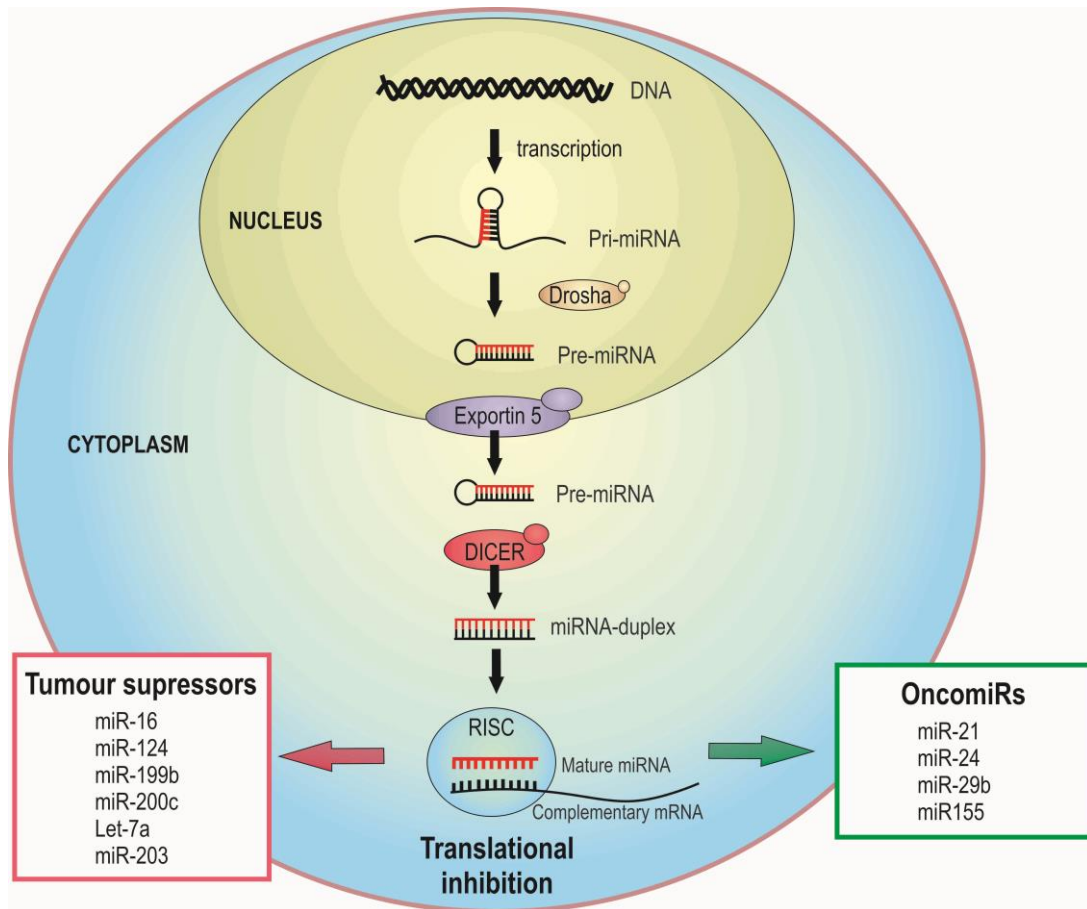


Figure I.3 – Biogenesis of miRNAs. The primary miRNAs, pri-miRNAs, are transcribed by RNA polymerase II and by RNA polymerase III.¹⁶²⁻¹⁶⁴ These pri-miRNAs are long molecules (more than 1 Kbp) with a terminal loop, a stem of approximately 33 base pairs (bp) and flanking segments of single stranded RNA.¹⁶⁵ In the nucleus, the Microprocessor complex cleaves the pri-miRNA. This complex has two major subunits, Drosha, a type III RNase and DiGeorge syndrome critical region gene 8, DGCR8, a double stranded RNA binding protein.¹⁶⁶⁻¹⁶⁸ DGCR8 interacts with the folded pri-miRNAs and assists Drosha to cut the stem of pri-miRNA 11 bp away from the junction of single stranded RNA with double stranded RNA.^{169, 170} The cleavage of the pri-miRNA by Drosha originates 60 to 70 nt precursor miRNAs, pre-miRNAs, maintaining the stem-loop conformation, with a two nucleotides (nt) 3' overhang characteristic of type III RNase activity.¹⁷¹ Pre-miRNAs are then exported to the cytoplasm by the Ran GTP-binding export receptor, exportin 5.¹⁷² The loop of the pre-miRNA is then cleaved in the cytoplasm by Dicer, another type III RNase thus forming the mature double stranded miRNA with 22 nt and two 3' overhangs.¹⁷³⁻¹⁷⁵ These miRNAs are incorporated as single stranded RNAs into the RNA-induced silencing complex, RISC.¹⁷¹ Although not necessary for its catalytic activity, Dicer has been shown to associate with two double strand RNA binding proteins, TRBP and PACT.^{176, 177} Dicer, TRBP, PACT and Ago2 the RISC Loading complex.^{178, 179} Ago2 is the effector unit of the human RISC complex.¹⁷⁹ Genetic silencing is accomplished by base-pairing between the seed region (nucleotides 2-8 in the 5' region) of the miRNA in the RISC complex and the complementary region in the 3'-untranslated region of the target mRNAs.¹⁵⁸ In boxes, the tumor suppressors and the oncogenic miRNAs (OncomiRs) used in this work.

Analysis of miRNA expression has shown an up-regulation of TGF- β signaling in CSCs obtained from canine mammary cell lines.¹⁸⁰ Since their discovery, miRNAs, have been increasingly recognized as an important class of regulatory small non-coding RNAs that function

as negative regulators of gene expression.^{181, 182} The differential expression of miRNAs has been associated with tumor suppressive and oncogenic patterns.^{160, 161, 183} The observed miRNAs expression variations in CMT are very similar to those described in HBC.^{160, 161, 183} Recently, a large miRNA expression study of CMT derived cell lines, described a miRNA expression profile that is associated with down-regulation of the Cyclin Dependent Kinase Inhibitor 2A (*CDKN2A*) family tumor suppressor genes.¹⁸³

I.7. CMT treatment

Mastectomy and, in intact animals, ovariectomy will be sufficient to heal dogs with benign and non-aggressive tumors.^{44, 184} Patients with aggressive or metastatic disease should benefit from adjuvant chemotherapy.^{44, 184} Due to high similarity between CMTs and HBC, the majority of treatment protocols used in patient dogs are adaptations of the from human medicine and the drugs used are off-label human medicines.¹⁸⁵

I.7.1. Cytotoxic chemotherapy

Several studies tested chemotherapeutic agents as co-adjuvant therapy in CMTs. Protocols combining cyclophosphamide with 5-fluorouracil and cyclophosphamide, mitoxantrone and vincristine have been shown to increase overall survival time (OS) in animals with inflammatory CMTs (ICMT) with minor side-effects.^{186, 187}

Treatment of dogs with doxorubicin alone or with combinations of doxorubicin with cyclophosphamide or doxorubicin with cyclophosphamide, 5-fluorouracil and prednisone had no significant effect on OS.¹⁸⁸⁻¹⁹⁰

Treatment protocols with the taxanes paclitaxel and docetaxel revealed partial response (20%) and high toxicity associated with the treatment protocol while the treatment protocol with docetaxel revealed minor toxicity in the subject animals despite no significant difference was observed in DFS and OS when compared to the control group patients.^{189, 191}

Animals treated with gemcitabine alone did not show any significant changes in DFS and OS.¹⁹² Dogs treated with a protocol combining gemcitabine with carboplatin showed only partial response (13%) and gastrointestinal toxicity in patients of the test group.¹⁹³ However, animals treated with carboplatin alone had longer OS than the animals in the control group with minor side-effects reported.¹⁹⁴

The lack of effectiveness of the chemotherapeutic drugs used in the treatment of CMTs may be due to the expression of efflux pumps such as multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) that belong to the ABC transporters super family.¹⁹⁵ It has been shown that malignant CMTs have an increased abundance of BCRP and MDR1 transporter.¹⁹⁶ Canine BCRP expression was associated, in cell line models, with increased tolerance of CMTs to doxorubicin and cyclophosphamide, MDR1 was associated with tolerance to vinblastine and cisplatin resistance was associated to the expression of MDR1, MDR3 and BCRP.^{195, 197}

I.7.2.Hormonal treatment

Due to the hormonal dependency of CMTs several hormonal-related therapies have been assayed.

Co-adjuvant therapy with tamoxifen, an estrogen receptor antagonist, has been attempted in a clinical trial but treated dogs displayed severe estrogen like side effects and no benefit to patients was proven.⁴⁴

Aglepristone, a PGR antagonist, was shown to reduce the expression of PGR and proliferation of tumors in dogs subject to treatment before surgery.¹⁹⁸ However, no clinical trial evaluating aglepristone treatment for CMT has been described so far.

Co-adjuvant treatment of dogs with goserelin, a gonadotropin releasing hormone superagonist (GnRH agonist), has shown to be promising.¹⁹⁹ The dogs in the treatment group (9 animals) presented reduced levels of 17 β estradiol and progesterone in blood and a reduction of tumor size.¹⁹⁹ An objective response was observed in 100% of the subjects and DF was increased.¹⁹⁹ However, these results have not been confirmed in a larger group of animals.

I.7.3.Non-steroidal anti-inflammatory drugs (NSAID)

The correlation of COX-2 expression and angiogenesis provides support for a potential role of COX-2 inhibitors for the prevention and the treatment CMTs.^{136, 143} Piroxicam, a non-selective COX inhibitor, has been tested in dogs with ICMT, with different results. De M Souza *et al.* 2009 observed an increase of the mean survival time of the dogs treated with piroxicam (183 days) compared with a control group treated with the anthracycline doxorubicin (7 days) while Clemente *et al.* 2009 reported a mean survival of 35 days of dogs treated with piroxicam.^{186, 188} The use of protocols with a combination of carboplatin and piroxicam or carboplatin with firocoxib, a selective COX-2 inhibitor, in the treatment of dogs with CMTs.¹⁹⁴ The treatment with the NSAID drugs showed an increase in OS when compared to the group of dogs treated with carboplatin.¹⁹⁴

I.7.4.Receptor Tyrosine Kinase (RTK) Inhibitor, SU11654 (Toceranib)

A Phase I trial has been performed with SU11654, a selective inhibitor of the split kinase members of the RTK family, which include Flk-1/KDR, PDGFR, and Kit.²⁰⁰ Objective responses were measure in subjects with CMTs, with 80% of CMT patients responding to treatment.²⁰⁰

I.7.5.Desmopressin and p62 vaccine

Desmopressin, 1-deamino-8-d-arginine vasopressin (DDAVP), is a synthetic derivative of antidiuretic hormone that has been proven effective as co-adjuvant treatment of surgery in CMTs.^{201, 202} Perioperative administration of DDAVP has shown to significantly increase DFS and OS in animals with stage III and IV CMTs and in dogs with grade 2 and 3 CMTs.^{201, 202}

The p62 vaccine is a DNA vaccine which consists in the pcDNA3.1 vector carrying the ubiquitin-binding protein p62 gene (*SQSTM1*).²⁰³ Dogs with advanced CMTs treated with the P62 DNA vaccine displayed tumor shrinkage or stabilization.²⁰³

I.7.6.Therapeutic compounds tested *in vitro* for the treatment of CMTs

Several promising compounds have been tested in CMT derived cell lines for the treatment of CMTs.

Three selenium compounds, sodium selenite, methylseleninic acid, and methylselenocysteine, have been shown to significantly decrease cell viability and growth in CTM1211 cell line while inducing apoptosis.²⁰⁴ These selenium compounds were also shown to downregulate VEGF, angiopoietin-2 (Ang-2), and HIF-1 α and overexpress PTEN.²⁰⁴

The DNA methyltransferase (DNMT) inhibitor 5-Azacytidine (5-AzaC) has been shown to be toxic to cells of a primary tumor culture obtained from a CMT and reduced *in vitro* tumorigenicity.²⁰⁵

The non-steroidal aromatase inhibitor, letrozole has shown to be effective in reducing proliferation and cell viability of an ICMT derived cell line, IPC-366.²⁰⁶

A combined treatment of doxorubicin and deracoxib, a NSAID selective COX-2 inhibitor, has been shown to reduce 3 times the IC₅₀ of doxorubicin in CMT-U27 cells which further supports the use of COX-2 inhibitors in a combined therapy strategy.²⁰⁷

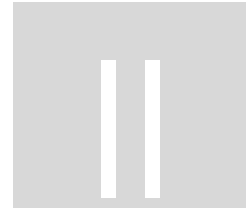
Masitinib, a selective inhibitor of the c-KIT, has been proven to sensitize CMT12 and CMT27 cell lines to the effects of gemcitabine.²⁰⁸

The use of small interference RNA (siRNA) to silence *EGFR* and *ERBB2* genes as an useful therapeutic tool has been proven since it has reduced cellular proliferation, colony formation and migration of cells from REM134 and LILLY cell lines.²⁰⁹ The use of Gefitinib, an inhibitor of EGFR, and GW583340, a dual inhibitor of EGFR and HER-2, have also been proven to reduce tumorigenicity of REM134 and LILLY cells.²⁰⁹

The anti-progestins mifepristone and onapristone have been shown to reduce cell viability in CMT-U27 cell line.²¹⁰

Two natural-derived compounds that were described as inhibitors of metastasis, migrastatin analogues, were tested in CMT-W1, CMT-W2, CMT-W1M and CMT-W2M.²¹¹ Two migrastatin analogues could inhibit cell viability cell migration and invasion capabilities in 3 of the cell lines.²¹¹

Although promising, these compounds have not yet been tested in patient dogs and thus their value as treatment for canine mammary cancer is still unknown.



II. Immortalization and characterization of a new canine mammary tumour cell line FR37-CMT

Disclaimer: Results and data presented in this chapter were published in:

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Raposo L.R., Santos S., Henriques, J., Alves, M., P Faísca, P., Beselga, A., J Correia, J., Fernandes, A. R.. "Immortalization of primary canine cell lines from mammary tumors: a protocol optimization." XVIII Meeting of the Portuguese Society of Animal Pathology. Évora. May 9-10, 2013.

II.1. Abstract

Here we describe the establishment of a new canine mammary tumour (CMT) cell line, FR37-CMT that does not show a dependence on female hormonal signalling to induce tumour xenografts in NOD-SCID mice. FR37-CMT cell line has a stellate or fusiform shape, displays the ability to reorganize the collagen matrix in the collagen colony assay, expresses vimentin, CD44 and shows the loss of epithelial markers, such as E-cadherin. Loss of E-cadherin is considered to be a fundamental event in epithelial to mesenchymal transition (EMT). The up-regulation of *ZEB1*, the detection of phosphorylated ERK1/2 and the downregulation of *DICER1* and miR-200c are also in accordance with the mesenchymal characteristics of FR37-CMT cell line. FR37-CMT shows a higher resistance to cisplatin ($IC_{50}>50\text{ }\mu\text{M}$) and to doxorubicin ($IC_{50}>5.3\text{ }\mu\text{M}$) compared to other CMT cell lines. These results support the use of FR37-CMT as a new CMT model that may assist the understanding of the molecular mechanisms underlying EMT, CMT drug resistance, fostering the development of novel therapies targeting CMT.

II.2. Keywords

Canine Mammary Tumours; cisplatin, doxorubicin, epithelial to mesenchymal transition, miRNAs

II.3. Introduction

Canine mammary tumours (CMTs) are the most common tumours diagnosed in intact female dogs.^{6, 9} Approximately 50% of all CMTs are malignant, for which mastectomy (and ovariectomy in intact dogs) is the only broadly accepted therapy since chemotherapy with agents, such as doxorubicin and docetaxel, does not radically increase overall survival time.¹⁸⁹ Despite the established dependence of CMT development on oestrogens and progesterone, tamoxifen [an oestrogen receptor alpha (ER α) antagonist] has not been proven effective against CMT.⁴⁴ Therefore, it is crucial to understand the mechanisms underlying the inefficacy of chemotherapy either by intrinsic or acquired drug resistance in dogs.

The epithelial to mesenchymal transition (EMT) is an important molecular reprogramming event that may allow canine mammary cancer cells to acquire mobility through changes in the expression of adhesion molecules (*e.g.* loss of E-cadherin expression and gain of N-cadherin) and reorganization of the cytoskeleton (*e.g.* gain of vimentin expression), for example, that result in the change of cellular polarity from top to bottom to back to front.¹⁴⁷ The shift in polarity may also be accompanied by the expression of other proteins such as metalloproteinases that allow extracellular matrix reorganization and therefore facilitate not only migration but also invasion.¹⁴⁷ These changes are induced by several transcription factors, such as Twist, Snail, Zeb1, Zeb2, that modulate expression of receptors involved in cell signalling, such as epithelial growth factor receptor (EGFR) and ER α , and alter the tolerance of cancer cells to chemotherapeutic agents.^{147, 148} Hallmarks of EMT are the gain of mesenchymal characteristics by epithelial cells, such as the expression of vimentin, increased cellular mobility and loss of epithelial markers, *e.g.* E-cadherin.¹⁴⁷ Consequently, it is of major importance to develop cellular models of CMT progression that are simultaneously suitable for drug screening. Only but a few CMT cell lines have been established that may assist elucidation of these matters. Here, we describe the

immortalization and characterization of a novel CMT cell line derived from a grade II complex canine mammary tumour that may serve as a model for the characterization and understanding of CMT progression, for development of novel therapies with new chemotherapeutic agents and also to improve the understanding of drug resistance mechanisms in CMT.

II.4. Materials and methods

II.4.1. Sample collection

Written informed consent was obtained from patient owners for all samples. The study was approved by the local ethical committee (Comissão de Ética da Faculdade Medicina Veterinária, Universidade Lusófona). Matched normal and CMT biopsies were collected by surgical excision following normal surgical procedures for neoplastic removal. Biopsies were divided into three fractions for further analysis: i) fixed in 10 % (v/v) neutral buffered formalin (Sigma) for histopathology; ii) conserved in RNAlater (Life Technologies) for posterior RNA extraction; and iii) washed three times in DMEM-5XPenStrep, Dulbecco's Modified Eagle's Medium, DMEM, (Life Technologies) supplemented with a mixture of 500 U/mL penicillin and 500 mg/mL streptomycin (Life Technologies) and kept on ice until further processing in the cell culture laboratory.

II.4.2. Establishment of immortalized CMT primary cell line

Biopsy pieces in DMEM-5XPenStrep were minced in TrypLE Express (Life Technologies) and incubated for one hour at 37 °C. The unattached cells were resuspended in DMEM (Life Technologies) supplemented with 10 % (v/v) Fetal Bovine Serum (FBS) (Life Technologies), 100 U/mL penicillin and 100 mg/mL streptomycin (Life Technologies), DMEM-FBS-PenStrep, seeded in a 25 cm² vented tissue culture flasks (VWR) and grown at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v)

relative humidity until the formation of a confluent primary cell monolayer. In each passage, the cell monolayer was treated with TrypLE Express (Gibco) and detached cells were centrifuged at 1500 $\times g$ for 5 minutes (min), resuspended in DMEM-FBS-PenStrep, counted and 5×10^5 cells seeded in 75 cm² vented tissue culture flasks (VWR). Cells were then grown at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity until reach a monolayer with 80 % confluence. The same passage procedure was performed consecutively and for passage 20 onwards, the number of cells seeded was serially lowered to ensure the establishment of a monoclonal cell culture.

II.4.3. Tumour sample preparation for histopathology and immunohistochemistry

The samples fixed in 10 % (v/v) neutral buffered formalin were processed routinely for paraffin embedding, sectioned at 5 μm and stained with hematoxylin and eosin (HE). Paraffin-embedded sections were placed on positively charged slides and submitted for immunohistochemistry using Cytokeratin Pan Ab-1 (Thermo Scientific) and Novocastra™ Liquid Mouse Monoclonal Antibody Vimentin (ref. NCL-L-VIM-V9 Leica biosystems). Immunohistochemistry staining was performed using the EnVision Detection Systems Peroxidase/DAB Rabbit/Mouse (Dako) according to the manufacturer's instructions. Slides were observed in a BX-51 microscope (Olympus) and images acquired using a DP50 camera (Olympus).

II.4.4. Chromosome preparations from FR37-CMT cell line

FR37-CMT cells were allowed to grow until 60% confluence in 25 cm² vented tissue culture flasks (VWR) in DMEM-FBS-PenStrep at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity. Colcemid™ (Sigma) was added to the cell media to a final concentration of 0.1 $\mu g/mL$ and cells

were incubated for 4 hours (h), treated with TrypLe Express (Gibco), pelleted and resuspended in a solution of 0.04M KCl and 0.025 sodium citrate. Cells then were incubated at room temperature for 30 min and fixed with an equal volume of methanol:acetic acid (3:1) solution. Following this, cells were pelleted, resuspended in methanol:acetic acid solution and incubated on ice for 10 min, and again pelleted and resuspended in methanol:acetic acid solution. Aliquots of the solution were dropped onto slides from a minimum height of 30 cm and air-dried. The slides were let to age by sitting at room temperature for at least one week before incubation with TrypLE express (Gibco) for 2 min and staining of the slides was made with Giemsa (Sigma) working solution (1:20) in distilled water. Slides were incubated for 15 min, air-dried before observation in BX-51 microscope (Olympus). Photographs were taken with a DP50 camera (Olympus).

II.4.5. Determination of the doubling time of FR37-CMT cell line

The doubling time of the FR37-CMT cell line was calculated by determination of the specific growth rate. For that purpose, 4500 cells were seeded in a 24-well tissue culture plates (VWR) and grown at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity. Daily cell counts were performed by treating the cells from 3 wells with TrypLE Express, pelleting by centrifugation at 1500 xg for 5 min and resuspending the pellet in 1 mL DMEM-FBS-PenStrep. Three independent experiments were performed to calculate the averaged specific growth rate and respective doubling time.

II.4.6. Clonogenic assays: Soft agar colony formation and collagen colony assays

For the soft agar assay, 10^5 CMT cells were resuspended in 0.5 % (v/v) agar (Oxoid) in DMEM-FBS-PenStrep and poured on top of 35 mm² tissue culture plates with 1 % (v/v) agar (Oxoid) in supplemented DMEM. After agar solidification, samples were covered with DMEM-FBS-PenStrep.

For the collagen colony assay, 10^5 cells were resuspended in rat tail collagen, type I (First Link, Ltd.) and poured into wells of a 24-well tissue culture plate (VWR). After the collagen hardened, it was covered with DMEM-FBS-PenStrep. Cell growth and formation of cell aggregates were monitored in a TMS inverted microscope (Nikon).

II.4.7. Growth of FR37-CMT cell line on the top of a Fibroblast cell monolayer

Human Fibroblasts (ATCC-PCS-201-010) were grown in a 25 cm² vented tissue culture flasks (VWR) in DMEM-FBS-PenStrep at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity. Upon confluent fibroblast monolayer, two concentrations of cells from the FR37-CMT cell line (2×10^3 and 4×10^3 cells/cm²) were added per flask. Co-cultures were then incubated at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity and monitored in a TMS inverted microscope (Nikon).

II.4.8. Wound healing assay

FR37-CMT cell line was seeded in 35 mm² tissue culture plates (VWR) and grown at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity until a confluent monolayer was obtained. With a sterile 100 µL micropipette tip, a scratch was made in the surface of the tissue culture plate. After

24 h of incubation in the same conditions, the cell culture was photographed, the scratches measured using ImageJ 1.49v software and the remission percentage calculated.

II.4.9. Tumorigenicity of FR37-CMT cell lines in NOD-SCID mice

All animal experiments were carried with the permission of the Portuguese Authority (Direcção Geral de Alimentação e Veterinária) and the study was approved by the Local Ethical Committees (Comissão de Ética Experimentação Animal da Faculdade de Farmácia, Universidade de Lisboa; Comissão de Ética da Faculdade Medicina Veterinária, universidade Lusófona), and in accordance with the Declaration of Helsinki, the EEC Directive (2010/63/UE) and Portuguese law (DL 113/2013, Despacho nº 2880/2015), and all following legislations for the humane care of animals in research. Animals were fed with sterile standard laboratory food and water ad libitum. Adequate measures were taken in order to minimize stress, pain or discomfort of the animals. A group of 6 non-obese severe combined immunodeficient, NOD/SCID, mice (Instituto Gulbenkian de Ciência), 3 female and 3 male, with 13 weeks old, were inoculated with 10^6 FR37-CMT cells in Phosphate buffered saline (PBS) into the scruff of the neck and xenografts allowed to grow until the appearance of tumors with a diameter of circa 1 cm. Mice were then anesthetized with Isoflurane (Isoflo, Esteve Farma), sacrificed by neck hyperextension and the tumours collected for histopathology and cell culture as described above.

II.4.10. DNA extraction from FR37-CMT cell line and from tumour xenografts cells monolayers

Cells from the tumour xenografts and from FR37-CMT were grown until 80 % confluence in 25 cm² vented tissue culture flasks (VWR) in DMEM-FBS-PenStrep at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity. DNA from cells was extracted using the High Pure PCR Template Preparation Kit (Roche).

II.4.11. PCR for *cOR9S13* and *PRCD* canine genes

PCR was performed to amplify two canine genes, the olfactory receptor family 9 subfamily S-like (*cOR9S13*) and the progressive rod-cone degeneration (*PRCD*).

The *PRCD* gene amplification was carried out in a 25 µL reaction containing MyTaq Reaction Buffer 1x (Bioline, Meridian Life Science, USA), 10 pmol of each primer (PRCD-Fw: 5'-GGTTGGCTGACCCCACTAAT-3', PRCD-Rev 5'-ACTGGAGGTCTCTCTCCGAC-3'), 1 U of MyTaq DNA Polymerase (Bioline, Meridian Life Science, USA) and approximately 100 ng of DNA. PCR was carried out in a Rotor-Gene Q thermal cycler (Qiagen, Valencia, CA), with the following conditions: 4 minutes at 95 °C, followed by 30 cycles of 30 seconds at 95 °C for denaturation, 30 seconds at 62 °C for primer annealing and 30 seconds at 72 °C for extension; a final extension of 7 minutes at 72 °C completed the reaction.

The *cOR9S13* gene amplification was carried out in a 25 µL reaction containing MyTaq Reaction Buffer 1x (Bioline, Meridian Life Science, USA), 20 pmol of each primer (*cOR9S13*-Fw: 5'-AATGCACTGGCCAACTTCTT-3', *cOR9S13*-Rev 5'-ATCCTCCATCAAGGTTGCAG-3'), 1 U of MyTaq DNA Polymerase (Bioline, Meridian Life Science, USA) and approximately 100 ng of DNA. PCR was carried out in a Rotor-Gene Q thermal cycler (Qiagen, Valencia, CA), with the following conditions: 10 minutes at 95 °C, followed by 35 cycles of 45 seconds at 95 °C for

denaturation, 30 seconds at 60 °C for primer annealing and 1 minute at 72 °C for extension; a final extension of 7 minutes at 72 °C completed the reaction.

For both *PCRD* and *cOR9S13* PCR reactions positive and negative controls were always included. The PCR amplification products were identified after electrophoresis in 1.5 % (w/v) agarose gels stained with ethidium bromide.

II.4.12. RNA extraction

Total RNA was extracted from RNAlater preserved biopsies of paired normal/ tumour tissue and from the FR37-CMT cell line using the SV total RNA Isolation System kit (Promega), accordingly to the manufacturer procedure.

II.4.13. Quantitative PCR (RT-qPCR)

For mRNA detection and quantification, cDNA was synthesized with the NZY M-MuLV First-Strand cDNA Synthesis Kit (NZYTech, Lda) accordingly to the manufacturer procedure. RT-qPCR for *18S*, *ESR1*, *ERBB2*, *PGR*, *DICER1*, *SOX4*, *FADD*, *VEGFA*, *PTEN*, *SNAI2*, *ZEB1* and *ZEB2* mRNA was performed in a Rotor-Gene 6000 (Corbett Research) using HOT FIREPol® Evagreen® qPCR Mix Plus (ROX) (Solis Biodyne). The primers used in RT-qPCR are described in Table II.1. Amplification of cDNA was performed using EvaGreen® HRM buffer 1X with 3 mM MgCl₂ and 0.2 µM of each primer. Conditions used for cDNA amplification are described in Table II.2. RNA 18S was used as endogenous control for RT-qPCR levels. Relative gene expression analysis was performed using the $2^{-\Delta\Delta C_t}$ method.^{212, 213}

Table II.1 - Sequences of the primers used for canine mRNA quantification.

Gene	Primers	Amplicon size (bp)
<i>18S</i>	Forward - 5'-GTAACCCGTTGAACCCATT-3' Reverse - 5'-CCATCCAATCGGTAGTAGCG-3'	151
<i>ESR1</i>	Forward - 5'-CCTTCAGTGAAGCTTCGATG-3' Reverse - 5'-AGAAGGTGGACCTGATCATG-3'	130
<i>ERBB2</i>	Forward - 5'-CAGCCCTGGTCACCTACAA-3' Reverse - 5'-CCACATCCGTAGACAGGTAG-3'	120
<i>PGR</i>	Forward - 5'-TGCAGGACATGACAACACCA-3' Reverse - 5'-CTGCCACATGGTGAGGCATA-3'	310
<i>DICER1</i>	Forward - 5'-CGAGGACTCTTGCCCAAAT-3' Reverse - 5'-GCCAATTCACAGGGGGATCA-3'	126
<i>SOX4</i>	Forward - 5'-ATGTCCTGGGCAGTTTCAG-3' Reverse - 5'-GATCATCTCGCTCACCTCGG-3'	282
<i>VEGFA</i>	Forward - 5'-CTTGCCTTGCTGCTCTACCT-3' Reverse - 5'-GTCCACCAGGTCTCAATGG-3'	144
<i>FADD</i>	Forward - 5'-TGGAGGAGACTGGCTCGTTA-3' Reverse - 5'-GCTCTTCCAGACTCTCAGCG-3'	117
<i>PTEN</i>	Forward - 5'-GCTATGGGGTTTCCTGCAG-3' Reverse - 5'-GCTGTGGTGGATTATGGTCTTC-3'	193
<i>SNAI2</i>	Forward - 5'-CACACTGGGGAGAAGCCTTT-3' Reverse - 5'-CACAGCAGCCAGATTCTCA-3'	178
<i>ZEB1</i>	Forward - 5'-ACAGTCCGGGGTAATCGTA-3' Reverse - 5'-TGAGTCCTGTTCTTGGTCGC-3'	224
<i>ZEB2</i>	Forward - 5'-ATATGGTGACGCACAAGCCA-3' Reverse - 5'-TTGCAGTTTGGGCACTCGTA-3'	172

Table II.2 - Amplification conditions used for canine mRNA quantification.

Cycle Steps	Temperature	Duration	N° Cycles
Initial denaturation	95 °C	15 min	1
Denaturation	95 °C	10 sec	45
Annealing	60 °C	10 sec	
Elongation	72 °C	10 sec	

For the detection of specific miRNA (miRNAs) expression levels, cDNA was synthesized using Exiqon's Universal cDNA Synthesis Kit II (Accordingly to the manufacturer procedure) and RT-qPCR was performed in a LightCycler 480 (Roche Diagnostics) through Real-Time PCR assay using Exiqon's LNA technology using ExiLENT SYBR® Green master mix (Exiqon) and individual microRNA LNA™ PCR primer sets for miR 16-5p, miR-21-5p, miR-24-3p, miR-29b-3p, miR-124-3p, miR-155-5p and miR-200c-3p (Exiqon). U6 snRNA was used as the endogenous control and amplified using U6 snRNA PCR primer set (Exiqon).

miRNAs and mRNA gene expression values were considered with a significant differential expression relative to normal tissue from values 2-fold higher ($2^{-\Delta\Delta Ct} > 2$) or 2-fold lower ($2^{-\Delta\Delta Ct} < 0.5$).

II.4.14. Total protein extraction

FR37-CMT cell line and MCF cell line (ATCC HTB-22) were grown in a 75 cm² vented tissue culture flasks (VWR) in DMEM-FBS-PenStrep at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity. At 80 % confluence, cells were scraped and centrifuged at 1500 xg for 5 minutes, washed three times with PBS and resuspended in lysis buffer (150 mM NaCl; 50 mM

Tris, pH 8.0; 5 mM EDTA, 2 % (v/v) NP-40, 1X phosphatase inhibitor (PhosStop, Roche), 1X protease inhibitor (cOmplete Mini, Roche), 1 mM PMSF and 0.1 % (w/v) DTT). After one hour incubation on ice, whole cell extracts were obtained by centrifugation at 14,000 xg for 30 min at 4 °C. Supernatant was collected and samples stored at -80 °C. Protein concentrations were determined using the Pierce 660 nm protein assay kit (Thermo Scientific) according to the manufacturer's specifications.

II.4.15. Western blot

For Western Blot analysis, 50 µg of total protein extracts were separated by sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) in a 10 % (37.5:1) acrylamide-bisacrylamide gel (Merck). Following electrophoretic transfer onto 0.45 µm nitrocellulose membranes (GE Healthcare) and blocking with 5 % (w/v) milk solution in TBST, Tris buffered saline with 0.1 % (v/v) Tween 20 (Sigma), blots were incubated according to the manufacturer's instructions for one hour at room temperature (RT) or overnight at 4 °C with primary antibodies against Estrogen Receptor alpha, ERα (ref. SAB4500810, Sigma), Human Epidermal Growth Factor Receptor 2 homolog, HER2 (ref. SAB4500789, Sigma), phosphorylated Extracellular Signal-Regulated Kinases 1 and 2, p-ERK1/2 (ref. sc-101761, Santa Cruz Biotechnology), E-cadherin (ref. WH0000999M, Sigma), Vimentin (ref. V6389, Sigma), Epithelial Cell Adhesion Molecule, EPCAM (ref. SAB4200473, Sigma), P53 (ref. SAB1404483, Sigma), CD44 (ref. SAB1402714, Sigma) and β-actin (ref. A5441, Sigma) (which was used as an endogenous control). Membranes were washed with TBST and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase, HRP (ref. 7074 and 7076, Cell Signaling Technology). WesternBright ECL (Advansta) was applied to the membranes and signal acquired with Hyperfilm ECL (GE Healthcare) and/or in GelDoc imager (Bio-Rad).

II.4.16. Chemotherapeutic agents

Cisplatin 1 mg/mL stock solution in 0.9 % (v/v) NaCl₂ (Teva Parenteral Medicines, Inc. Teva Pharmaceuticals) was kept at room temperature according to the manufacturer's instructions. Doxorubicin hydrochloride (Sigma) 5 mg/mL stock solution was made in DMSO (Sigma) and kept at 4 °C as recommended by the manufacturer.

II.4.17. Cell viability assays in presence of cisplatin and doxorubicin

FR37-CMT cell line was seeded in 96-well plates (VWR) at a concentration of 10,000 cells per well and incubated for 24h at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity. The media was then removed and replaced with fresh media supplemented with the appropriate dilutions of cisplatin, (Teva Parenteral Medicines, Inc. Teva Pharmaceuticals), doxorubicin (Sigma) or the respective vehicle control, 0.9 % (w/v) NaCl (Sigma) for cisplatin and DMSO (Sigma) for doxorubicin. After 48 h of incubation at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity, cell viability was evaluated with CellTiter 96[®] AQueous non-radioactive cell proliferation assay (MTS, Promega). Absorbance at 490 nm was measured in a Microplate reader Infinite M200 (Tecan) as previously described.²¹⁴

II.4.18. Statistical analysis

Statistical analysis was performed using GraphPad Prism v6.01. All data expressed as mean ± SD from at least three independent experiments. Statistical significance was considered when p-value < 0.05.

II.5. Results

II.5.1. Immortalization of FR37-CMT cell line

A total of 96 mammary tumours biopsies were cultured and putative immortalization assessed. One CMT cell line, FR37-CMT, was the first cell culture to reach 100 passages and capable to grow in a simple, broadly used DMEM medium.

Histopathology of the original tumour biopsy revealed a non-encapsulated mammary tumour with proliferation of both epithelial and myoepithelial cells (Figure II.1 A and B). The population of epithelial cells is arranged in irregular tubules lined by a single layer of cuboidal cells with eosinophilic cytoplasm with moderate anisokaryosis and anisocytosis (Figure II.1 B). There are occasional foci of squamous differentiation. The myoepithelial cells are spindle shaped and are arranged in irregular bundles within a myxoid matrix. The centre of the tumour is necrotic. The mitotic index is 10 mitosis per 10 high power fields.

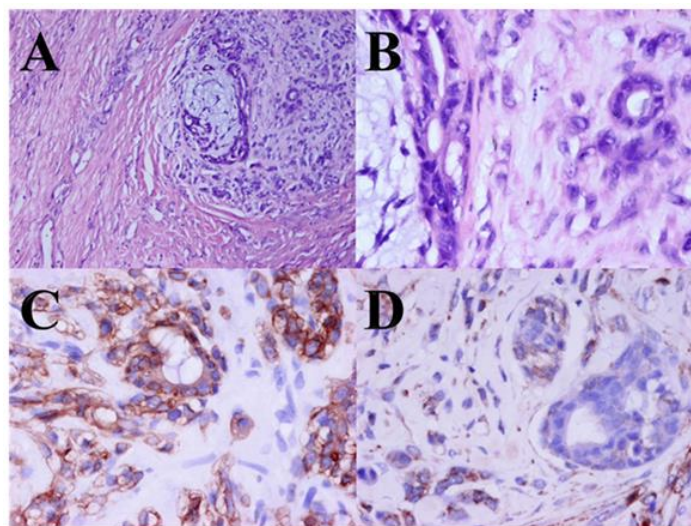


Figure II.1 - Representative images of the original tumour from which FR37-CMT cell line was originated, stained with HE (A, B), and the immunostaining for cytoplasm cytokeratins (C) and for vimentin (D). The non-encapsulated tumor has proliferation of epithelial and myoepithelial cells. Epithelial cells are arranged in irregular tubules lined in a single layer. The myoepithelial cells are arranged in irregular bundles within a myxoid matrix. Amplification: A, - 100X; B, C and D - 400X.

The immunohistochemistry study of the original tumour revealed strong cytoplasmatic positive staining of the epithelial and myoepithelial cells (Figure II.1 C), some of the myoepithelial cells also showed positive vimentin staining in the cytoplasm (Figure II.1 D).

The FR37-CMT cell line shows an adherent stellate/spindle shaped morphology (Figure II.2 A, B and C) and cells are able to grow on the top of each other after occupying all the surface of the tissue culture flask. This is a key characteristic of cancerous cells, which commonly lose contact inhibition and thus are able to grow in an uncontrolled manner even when in contact with neighbouring cells^{215, 216} (Figure II.2 D).^{194, 195}

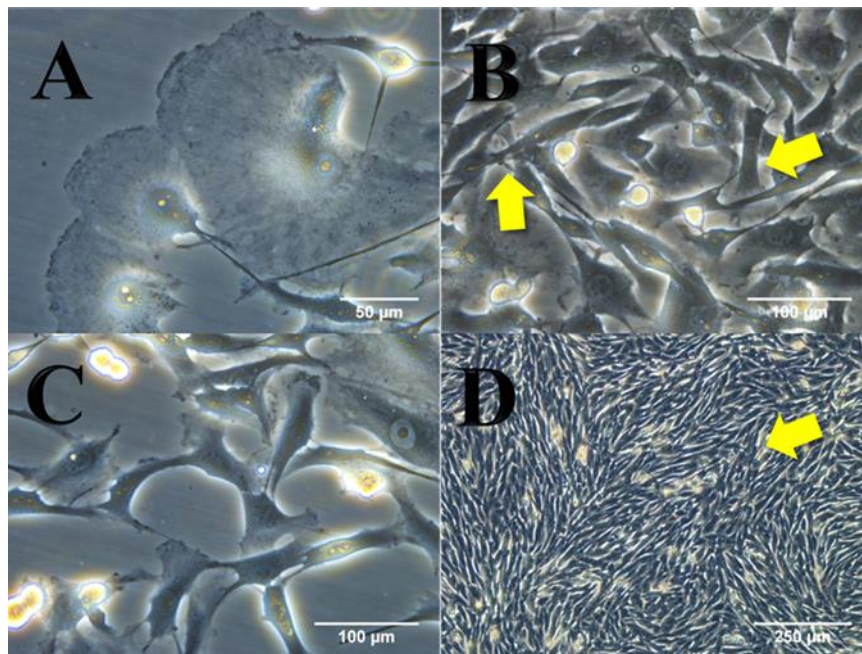


Figure II.2 - Representative images of adherent FR37-CMT cells. FR37-CMT cells are stellate/spindle shaped (A, C), capable of occupying 100 % of the surface of the tissue culture flask (B, D). It is possible to observe cells growing on top of other cells (yellow arrows). Amplification: A, 600X; B, C: 400X; D, 100X.

FR37-CMT shows a specific growth rate of 0.047 h^{-1} and a doubling time of 17 h, which is lower than the doubling time of the well-known human breast cancer derived cell line, MCF7 (29 h) according to the ATCC Website.²¹⁷

The canine metaphase karyotype consists of 78 chromosomes (Figure II.3). The presence of the *cOR9S13* and *PRCD* canine genes was evaluated by PCR in DNA extracted from FR37-CMT, confirming the canine origin of the cell line (Figure II.4).

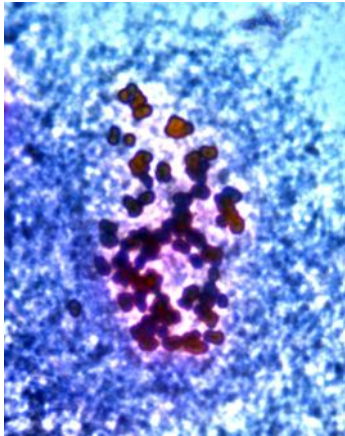


Figure II.3 - Chromosome preparations of the FR37-CMT cell line. Description of data: Representative image of chromosomes preparation of FR37-CMT cells with Giemsa staining. Amplification: 1000X.

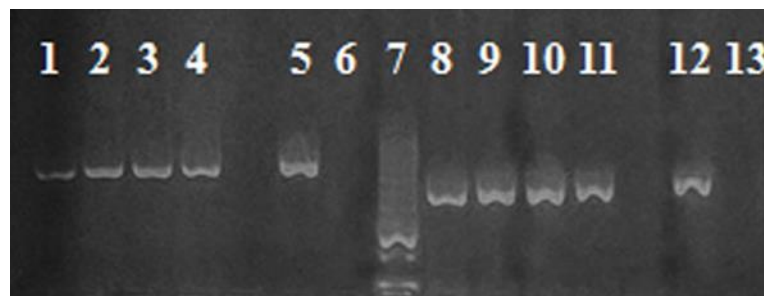


Figure II.4 - PCR results for *PRCD* and *cOR9S13* canine gene amplification. Lanes 1, 2 and 3 – xenograft tumour 1, 2 and 3 amplified with *PRCD* primers, Lane 4 – FR37-CMT amplified with *PRCD* primers; Lane 5 – Positive control for *PRCD* amplification, Lane 6 – Negative control for *PRCD* amplification, Lane 7 – Hyper ladder IV (Bioline), Lanes 8, 9 and 10 – xenograft tumour 1, 2 and 3 amplified with *cOR9S13* primers, Lane 11 – FR37-CMT amplified with *cOR9S13* primers; Lane 12 – Positive control for *cOR9S13* amplification Lane 13 – Negative control for *cOR9S13* amplification.

II.5.2. Loss of contact inhibition and invasion ability

To further assess the loss of contact inhibition in FR37-CMT cells as well as their invasion ability, clonogenic assays were performed, including the soft agar colony formation assay and collagen colony formation assay. FR37-CMT cells were able to form colonies in soft agar after 5

days of incubation and an overall increase in colony size was detected until the 14th day of the experiment (Figure II.5). After this period of time, no significant changes were observed.

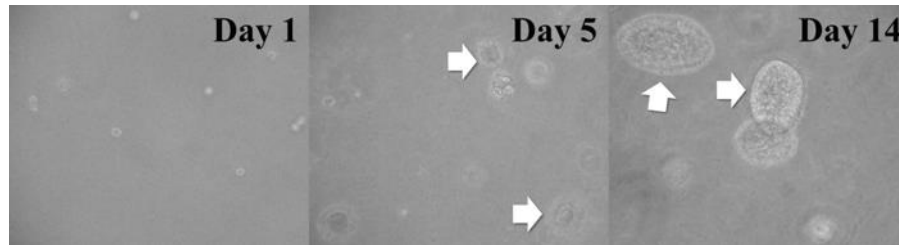


Figure II.5 - Representative images of the Soft agar assay. Amplification: 40X.

The growth on collagen revealed the ability of the FR37-CMT cells to reorganize the collagen matrix resulting in the formation of spherical aggregates of cells after 6 days of growth (Figure II.6). At the same time, the bottom surface of the wells was also completely occupied with cells.

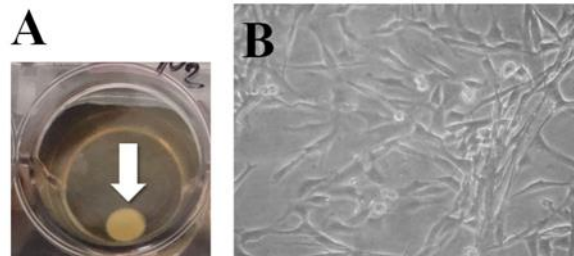


Figure II.6 - Representative images of the collagen assay. Amplification (B): 40X.

To evaluate FR37-CMT invasion and mobility capabilities FR37-CMT cells were allowed to grow on top of a fibroblast cell monolayer (Figure II.7) and a wound healing assay was performed. Results showed that FR37-CMT cells are able to grow on the top of a confluent monolayer of human fibroblasts (Figure II.7). Indeed, starting with 2×10^3 cells/cm² of FR37-CMT 100 % of confluence was observed after 5 days (Figure II.7). Interestingly, the number of fibroblasts in the co-culture seemed to be inversely proportional to the number of FR37-CMT

cells, and a total absence of fibroblasts was observed 5 days after the beginning of the co-culture (Figure II.7).

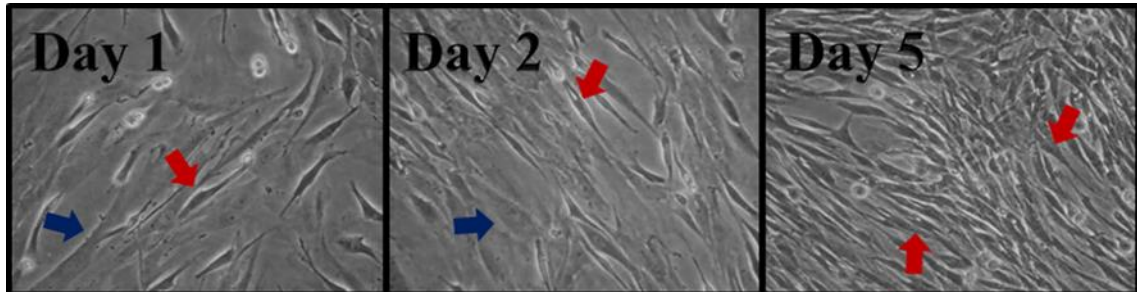


Figure II.7 - Representative images of FR37-CMT growth on the top of a human fibroblasts monolayer. Blue arrows indicate the fibroblast cells and the red arrows indicate the FR37-CMT cells. After 5 days, no fibroblasts were observed. Amplification: 40X.

FR37-CMT migration potential was evaluated via the wound healing assay. A wound remission percentage of $79.2\% \pm 5.03$ was observed at 24 h following the scratch (Figure II.8). For comparison, it should be noted that the remission percentage described for the human breast adenocarcinoma cell line MCF7 is approximately 60% after the same period of time (24 h) ²¹⁸.

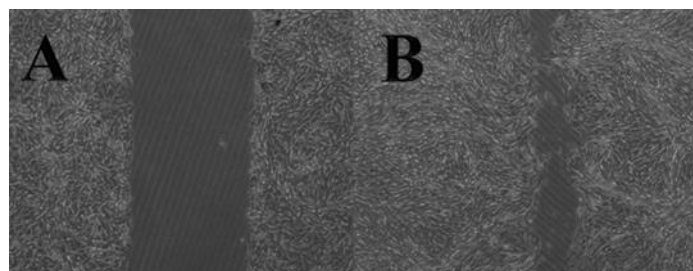


Figure II.8 - Representative images of the wound healing assay at 0 h (A); and 24 h (B). 24 h after the scratch there is a $79.2\% \pm 5.03$ (Mean $\pm$ SD) of wound remission percentage as observed in B).

II.5.3. Tumorigenicity of FR37-CMT in NOD-SCID mice

To assess FR37-CMT capability to form tumours, 6 NOD-SCID mice (3 males and 3 females) were subcutaneously inoculated at the scruff of the neck with 10^6 cells. All inoculated mice developed tumours at the injection site and 4 mice developed two separate tumours at the place of FR37-CMT cell inoculation. One male mouse was sacrificed 7 weeks post-injection, the other two male mice were sacrificed 8 weeks post-injection and the 3 female mice were sacrificed 9 weeks post-injection. Following histopathology, the ten tumour xenografts were anaplastic, composed mostly by spindle mesenchymal type cells disposed in multidirectional bundles in a phenotype compatible with a sarcoma. Some of the tumour xenografts present areas of myxoid stroma and two of them present foci of chondroid metaplasia. The tumour xenografts were also studied by immunohistochemistry for the presence of cytoplasmic cytokeratins and vimentin (Figure II.9). The neoplastic cells revealed strong positive cytoplasmic vimentin staining but no immunostaining was observed for cytoplasm cytokeratins.

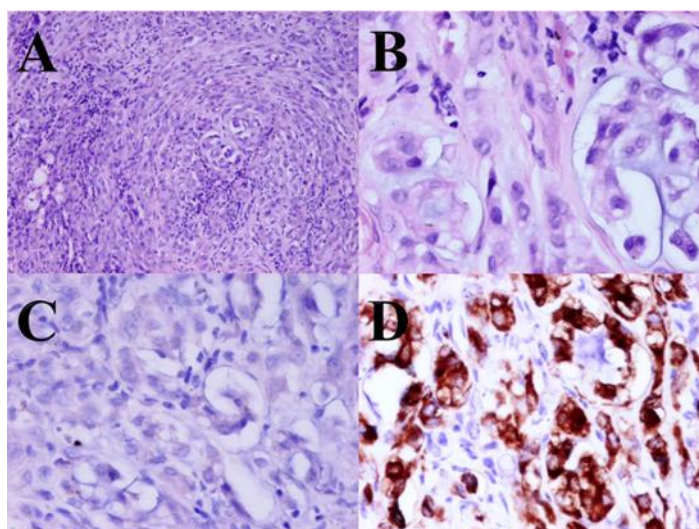


Figure II.9 - Representative images of a tumor xenograft stained with HE (A, B), and the immunostaining for cytoplasm cytokeratins (C) and for vimentin (D). The tumor is composed by non-encapsulated spindle cells arranged in multidirectional bundles (A). There are foci of epithelial/myoepithelial-like cells in mucinous stroma (B). The neoplastic cells revealed strong positive cytoplasmic vimentin staining, but no immunostaining was observed with cytokeratin AB-1. Amplification: A, 100X; B, C, D 400X.

DNA extracted from three of the xenografted tumours classified as sarcomas by PCR (Figure II.4) were positive for the cOR9S13 and PRCD canine genes, confirming that their canine origin was due to inoculation of FR37-CMT.

II.5.4. Molecular characterization of FR37-CMT cell line

In order to characterize the FR37-CMT cell line at the molecular level, we analysed the expression of 11 cancer related genes (Figure II.10): *ESR1*, *ERBB2* and *PGR*, the mostly commonly affected receptors in breast and in mammary cancers,^{50, 219} *DICER1* and its direct regulator *SOX4* have been implicated in the deregulation of miRNA expression in breast cancer; VEGF ligand gene, *VEGFA*, important for induction of angiogenesis in breast and mammary cancer,¹³¹ tumour suppressor genes *PTEN* and *FADD* in breast cancer,^{103, 220} and *SNAI2*, *ZEB1* and *ZEB2* genes that are involved in EMT.^{221, 222} The results attained for FR37-CMT were compared to the expression levels of all genes in RNA extracted from the original tumour biopsy (Figure II.10).

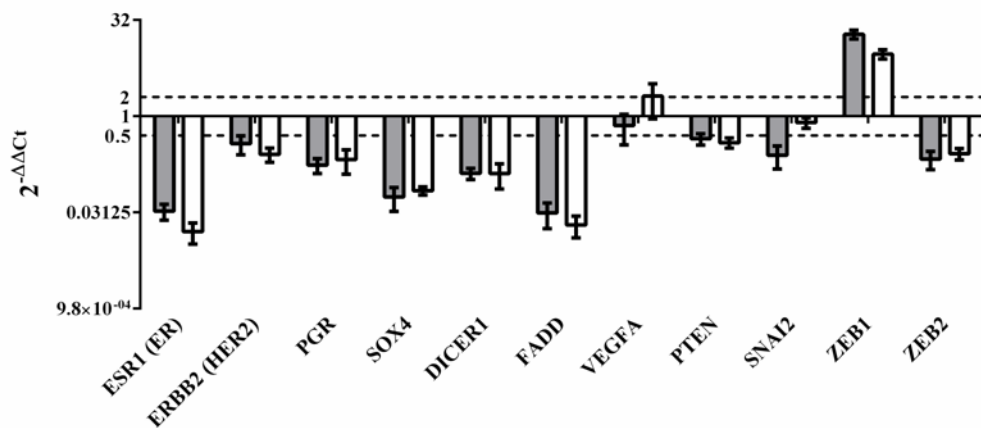


Figure II.10 - Relative expression of genes involved in breast and mammary tumorigenesis in the tumour that originated the cell line (grey bars) and the FR37-CMT cell line (white bars) normalized to the expression in matched normal mammary tissue. The dotted lines mark the threshold that considers altered expression: values 2-fold higher ($2^{-\Delta\Delta Ct} > 2$) or 2-fold lower ($2^{-\Delta\Delta Ct} < 0.5$). Described values are mean $\pm$ SD. *, ** significant expression differences, p-value < 0.05, between the FR37 line values relative to the original tumour.

There are no significant expression level alterations for 9 out of the 11 genes evaluated in the original tumour and in FR37-CMT when compared to the matched normal mammary tissue. The observed variations indicate an overall expression decrease of the selected mRNAs, 8 out of the 11 tested. The only expression increase relative to the normal mammary tissue was observed for *ZEB1* in both the original tumour and in FR37-CMT (Figure II.10). The overexpression of *ZEB1* is lower in the cell line when compared to the levels of the original tumour (Figure II.10). FR37-CMT *SNAI2* expression was lower in the original tumour than for FR37-CMT, where expression was almost at normal level.

The differential expression of miRNAs has been associated with breast cancer progression and also with canine mammary cancer.^{160, 161} The expression of 7 miRNAs, miR-16, miR-21, miR-24, miR-29b, miR-124, miR-155 and miR-200c was evaluated for the FR37-CMT cell line (Figure II.11~~Erro! A origem da referência não foi encontrada.~~). These miRNAs have already

been described as important for cancer progression and metastasis in breast and/or in canine mammary cancer.^{160, 223-226}

Figure II.11 shows that only three miRNAs are altered in the original tumour relative to the normal tissue: miR-16, miR-21, and miR-24. These miRNAs are also under-expressed in FR37-CMT but the levels of miR-21 are significantly lower in the cell line when compared to the original tumour. miR-29b and miR-200c are only under-expressed in the FR37-CMT. miR-124 and miR-155 expression levels are not significantly altered between the original tumour, the matched normal mammary tissue and FR37-CMT.

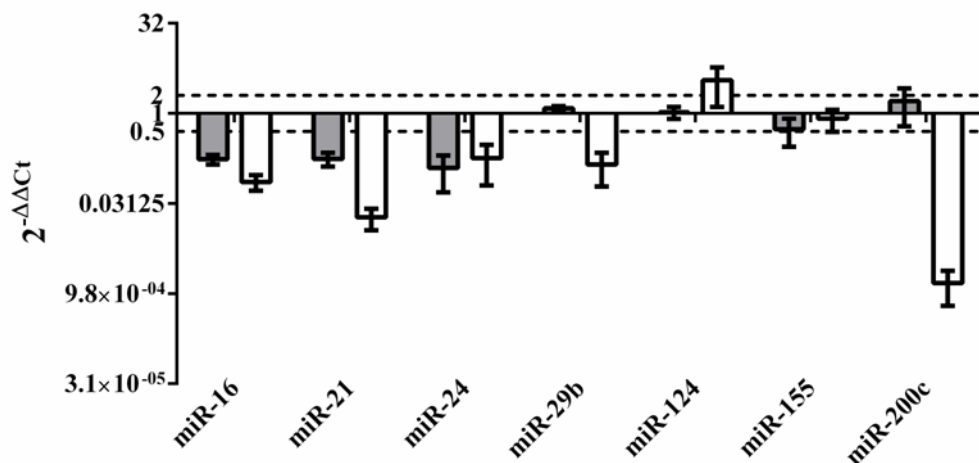


Figure II.11 - Relative expression of miRNAs involved in breast and mammary tumorigenesis in the tumor that originated the cell line (grey bars) and the FR37-CMT cell line (white bars) normalized to the expression in matched normal mammary tissue. The dotted lines mark the threshold that considers altered expression: values 2-fold higher ($2^{-\Delta\Delta Ct} > 2$) or 2-fold lower ($2^{-\Delta\Delta Ct} < 0.5$). Described values are mean $\pm$ SD. *, **, *** significant expression differences, p-value < 0.05, between the FR37 line values relative to the original tumor.

Figure II.12 shows a similar expression of p53 tumor suppressor protein, ER α and HER2 receptors, Epcam and Vimentin in FR37-CMT and MCF-7. When compared to MCF-7, FR37-CMT shows higher expression of CD44 but does not express E-cadherin. FR37-CMT exhibits the

phosphorylated forms of ERK1 and ERK2 MAPKinases (which were not detected in the MCF-7 cell line), which indicates activation of the ERK1/2 signalling pathway.

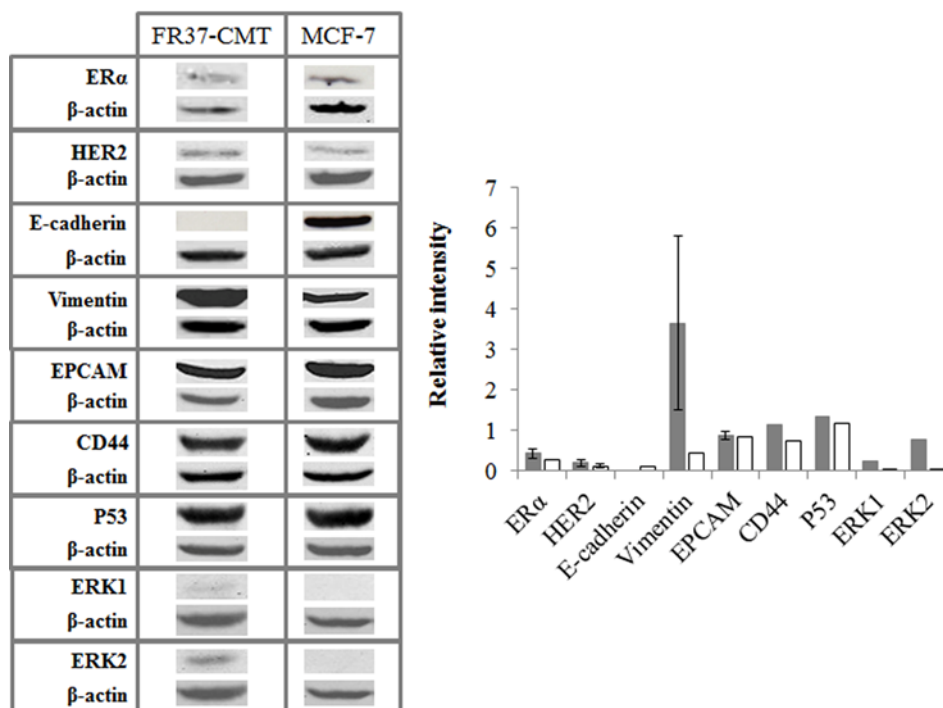


Figure II.12 - Proteins expressed in FR37-CMT and MCF-7 cell lines. A- Representative images of western blot results. β -actin was used as internal loading control. B. Relative intensity values (normalized to the endogenous control β -actin) of each protein in FR37-CMT (grey bars) and MCF-7 (white bars) cell lines. Results are the mean of at least three independent experiments.

II.5.5. Effect of cisplatin and doxorubicin in the cell viability of FR37-CMT cell line

The effect of the widely used chemotherapeutic agents, cisplatin and doxorubicin in FR37-CMT cell viability was evaluated using the MTS assay (Figure II.13). The IC_{50} for cisplatin is higher than 50 μ M, and for doxorubicin is 5.3 μ M. These IC_{50} values demonstrate a high level of resistance of this CMT cell line to cisplatin compared to doxorubicin.

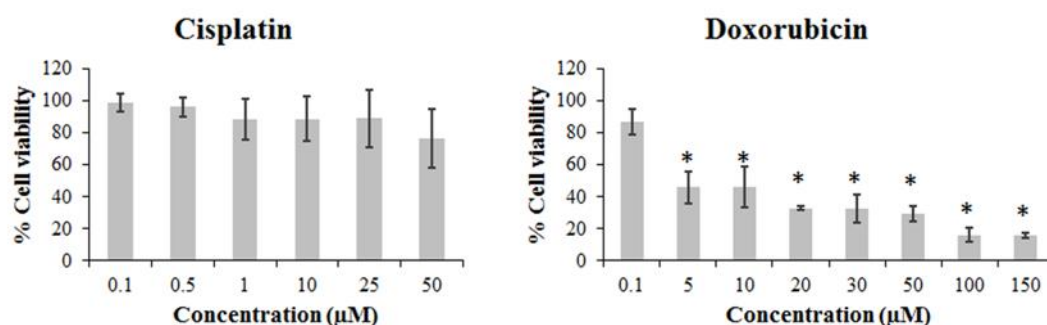


Figure II.13 - Cell viability of FR37-CMT cell line after 48 h of exposure to different concentrations of cisplatin and doxorubicin. The results are expressed as mean $\pm$ SD to controls from at least three independent experiments. *P-value < 0.05 relatively to the % viability of 0.1 μ M of doxorubicin.

II.6. Discussion

Here we report the establishment of the malignant canine mammary tumour cell line, FR37-CMT. The fact that FR37-CMT grows in a commonly used cell culture medium and has a smaller doubling time than the human breast adenocarcinoma cell line, MCF-7, 17 h vs. 29 h,²¹⁷ will facilitate its use as a model for the community and assist the study of canine mammary cancer biology. Karyotype analysis and the amplification of the canine specific genes *cOR9S13* and *PRCD* confirmed the canine origin of the presented cell line (Figure II.3 and Figure II.4). The malignant potential FR37-CMT was confirmed since all the NOD-SCID mice inoculated with 10^6 FR37-CMT cells developed tumours at the site of injection. Xenograft mice data also show that FR37-CMT tumorigenicity is independent of the female hormonal regulation, since all male NOD-SCID mice yielded tumour masses. The histopathology of the tumour xenografts revealed that the cells share the same spindle shape of the CMT-FR37 cells, which led to the histological classification of the xenograft tumours as sarcomas. However, some of these xenograft tumours had myxoid stroma and chondroid metaplasia which are characteristic of complex and mixed

mammary gland carcinomas.^{39, 56} The tumour xenografts analysed by immunohistochemistry revealed strong staining for vimentin and no staining for cytoplasm cytokeratins (Figure II.9).

The molecular characterization of FR37-CMT revealed a decreased expression of *ESR1*, *ERBB2* and *PGR* genes (Figure II.10) when compared to the matched normal mammary tissue. However, this decrease in the mRNA levels of *ESR1* and *ERBB2* did not correlated with an absence of the ER α and HER2 receptors from the cells (Figure II.12). Despite the presence of ER α in cells of the CMT-FR37 cell line there is a lack of hormonal dependency displayed by the growth of tumour xenografts in the male NOD-SCID mice.

Western blot (Figure II.12) demonstrated the expression of EPCAM, Vimentin and CD44 in FR37-CMT and the absence of E-cadherin. EPCAM is a marker of epithelial cells, whereas expression of vimentin and absence of E-cadherin receptor are characteristic of the myoepithelial or mesenchymal cells of the mammary gland.^{59, 227}

Together, the absence of the E-cadherin, presence of vimentin filaments and expression of the CD44 marker (Figure II.12) are also associated with the EMT and acquisition of stemness properties by cancer cells.¹⁵² This EMT phenomenon has been associated with the basal-like phenotype in breast cancer.²²⁸ The EMT phenotype in the FR37-CMT cell line is further supported by the activation of the ERK1/2 MAPK signalling pathway revealed by western-blot (Figure II.12), *ZEB1* overexpression (Figure II.10) and downregulation of miR-200c (Figure II.11). The ERK1/2 pathway has been shown to participate in TGF- β dependent EMT.^{149, 229-231} Overexpression of *ZEB1* and *ZEB2*, mediated by TGF- β signalling, has been described to lead to downregulation of miR-200 family (miR-200a, miR-200b and miR-429 cluster and the miR-200c, miR-141 cluster). A feed forward feedback loop downregulation of miR-200 is achieved by the binding of Zeb1 and Zeb2 transcriptional repressors to the miR-200 promoters, which in turn will result in higher expression of *ZEB1* and *ZEB2* (Figure II.14).^{221, 223, 232} Furthermore, a stable

prolonged TGF- β signalling induces reversible methylation of the miR-200 promoters that further reduce the levels of miR-200.²³³

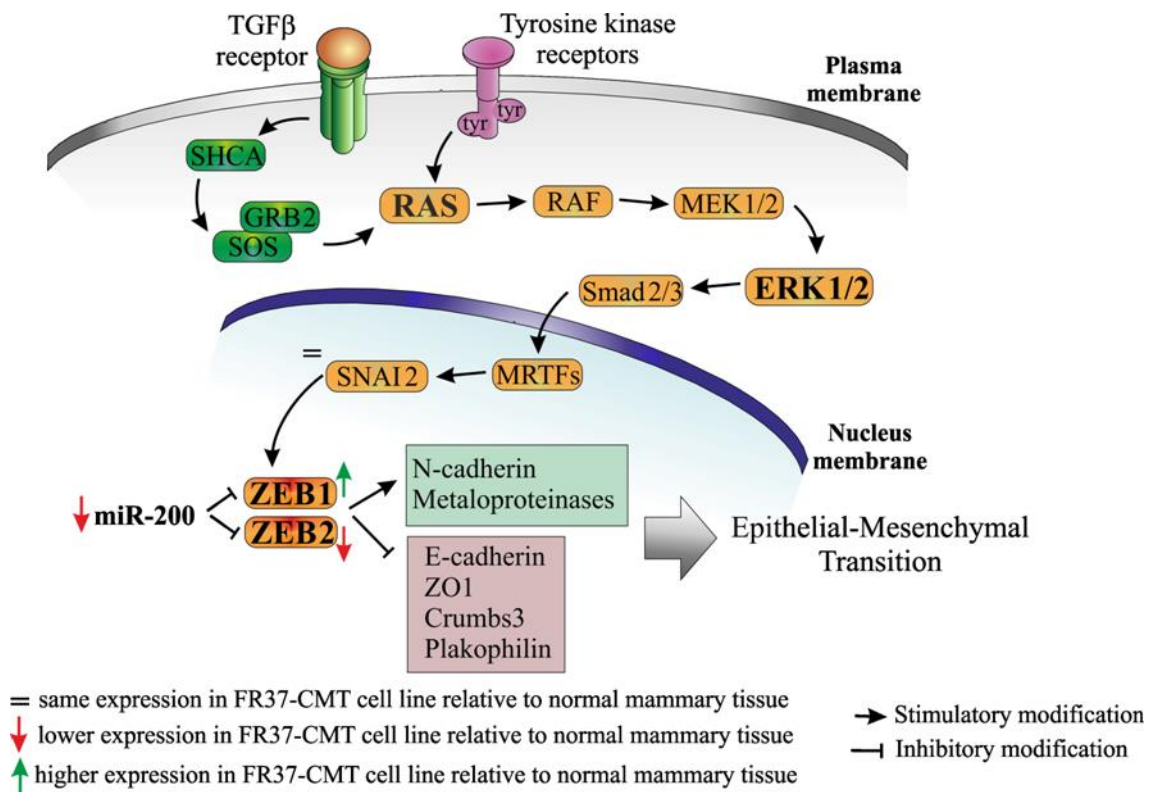


Figure II.14 - EMT mediated by the RAS-ERK1/2-SNAI2 signalling pathway. RAS is activated by tyrosine kinase receptors or by activation of Transforming growth factor- β receptor (TGF- β), which phosphorylates the adaptor protein SRC homology 2 domain-containing-transforming A (SHCA), and consequently generates a site for docking of growth factor receptor-bound protein 2 (GRB2) and son of sevenless (SOS), that in turn stimulates RAS protein. RAS activation is followed by the activation of RAF, MEK1 and MEK2, ERK1 and ERK2 protein kinases and SMAD2 and SMAD3 signalling effectors. Induction of SMAD3 activity results in the induction of the expression and nuclear import of myocardin-related transcription factors (MRTFs), leading to activation of SNAI2 (also named Slug) with consequent activation of the transcriptional factors Zinc-finger E-box binding 1 (ZEB1) and 2 (ZEB2), which regulate the expression of genes that ultimately will lead to EMT. It is also referred the gene expression tendency in FR37-CMT cell line relative to the matched normal mammary tissue.

The normal levels of *SNAI2* and, in particular, the downregulation of *ZEB2* observed in FR37-CMT when compared to normal mammary tissue are not in accordance with the commonly accepted model for EMT.²³³ Nevertheless, the over-expression of *ZEB1* alone has been shown to

be sufficient to induce EMT *in vitro*.^{232, 234} There are statistically significant differences in the expression levels of *ZEB1*, *SNAI2* and miR-200c between the FR37-CMT cell line and the original tumour (Figure II.10 and Figure II.11). There is a decrease in *ZEB1* expression accompanied by a slight increase of *SNAI2* and a decrease of miR-200c expression in FR37-CMT. These fluctuations could be seen as a homeostatic regulation in order to maintain EMT, since the *SNAI2* is a regulator of EMT and miR-200c is a negative regulator of *ZEB1*. Reorganization of the collagen matrix observed in the collagen colony assay (Figure II.6) by FR37-CMT cells is consistent with a previous report that correlated the activation of ERK1/2 MAPKinase pathway with a higher abundance of the Matrix Metalloprotease 9 (MMP-9) protein and a corresponding increase in the migration and invasion abilities of SCC-12F cells.²³⁵

Analysis of tumour suppressor genes revealed that *FADD* and *PTEN* were under-expressed in both the original tumour and in the FR37-CMT cell line (Figure II.10). Western blot identified the p53 protein without any detectable truncated form (Figure II.12).

A decreased expression level of *DICER1* has been associated with EMT, formation of metastasis and a poorer prognosis in breast cancer.^{236, 237} *SOX4* knockdown has been associated with major changes in the expression pattern of miRNAs in human melanoma cells, due to reduced expression of Dicer protein.²³⁸ Both in the original tumour and in FR37-CMT, *DICER1* and its transcriptional activator *SOX4* are equally downregulated when compared to the normal mammary tissue. Downregulation of *DICER1* is associated with a decrease in the overall expression of miRNAs²³⁹ that could explain the downregulation of miR-16, miR-21 and miR-24 in both the original tumour and in FR37-CMT. The under-expression of miR-16 present in the original tumour and in FR37-CMT, is a frequent event in breast cancer and canine mammary cancer.^{160, 240} One of the targets of miR-16, BMI1 proto-oncogene protein is essential to the EMT regulator coded by *TWIST1*.²⁴¹ The up-regulation of miR-21, miR-24, miR-29b and miR-155 are

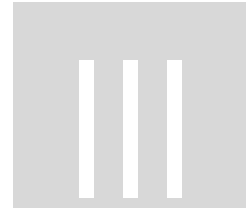
common in canine and breast cancer.^{160, 161, 222, 240} Under-regulation of miR-21 and miR-24, seen in FR37-CMT and in the original tumour, has been already described in a setting of induced tamoxifen resistance in MCF7 cells by over-expression of miR-221/222.²⁴² The under-regulation of *PTEN*, a miR-21 and miR-29b target, present in both the FR37-CMT cell line and in the original tumour could explain the absence of up-regulation of miR-21 and miR-29b. However, the miR-29b statistically significant downregulation in the cell line but not in the original tumour cannot be explained by decreased levels of *DICER1* alone. Up-regulation of miR-155 has been described as a marker of poor prognosis in breast cancer and in canine mammary cancer and thus be considered a later event in tumour progression.^{160, 240, 243} The absence of miR-124 down-regulation, a regulator of *SNAI2*,²²² in FR37-CMT cell line and the original tumour might be unnecessary for tumorigenesis since EMT may be mediated by the higher levels of *ZEB1*.

The IC_{50} for cisplatin in CMT-U27, CMT-U309, P114, CMT-W1 and CMT-W2 has been reported.¹⁹⁷ FR37-CMT shows an $IC_{50} > 50 \mu M$, higher than the reported in the literature, which indicates a higher level of tolerance to cisplatin by FR37-CMT (Figure II.13). The IC_{50} described for doxorubicin in DTK-E, DE-F and DE-SF CMT cell lines are lower than $1 \mu M$ and for DE-E and DTK-SME higher than $1 \mu M$.^{244, 245} The IC_{50} for doxorubicin in FR37-CMT is $5.3 \mu M$, higher than those for the other CMT cell lines (Figure II.13). The intrinsic resistance of FR37-CMT cell line to common antitumor drugs points out the interest of using this cell line in the screening for novel chemotherapeutic agents.

II.7. Conclusions

The newly established tumorigenic canine mammary cell line, FR37-CMT is not dependent on female hormonal signalling and may be used as a model for the study of the epithelial to mesenchymal transition in canine mammary gland tumours.

The cell line also displays characteristics of EMT mediated by TGF- β /ZEB1/miR-200 regulatory loop. When compared to other CMT cell lines in the literature, FR37-CMT revealed higher tolerance to cisplatin and doxorubicin, thus making this cell line a good model for the study of chemotherapy resistance in canine mammary tumours.



III. Targeting canine mammary tumors via gold nanoparticles functionalized with promising Co(II) and Zn(II) compounds

Disclaimer: Results and data presented in this chapter were published in:

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III.1. Abstract

Background: Despite continuous efforts, the treatment of canine cancer has still to deliver effective strategies. For example, traditional chemotherapy with doxorubicin and/or docetaxel does not significantly increase survival in dogs with canine mammary tumors (CMTs). Aims: Evaluate the efficiency of two metal compounds $[\text{Zn}(\text{DION})_2]\text{Cl}$ (TS262, DION = 1,10-phenanthroline-5,6-dione) and $[\text{CoCl}(\text{H}_2\text{O})(\text{DION})_2][\text{BF}_4]$ (TS265) and novel nanovectorizations designed to improve the anti-cancer efficacy of these compounds in a new CMT derived cell line (FR37-CMT). Materials and Methods: FR37-CMT cells were exposed to different concentrations of TS262 and TS265 and two new nanoparticle systems and cellular viability was determined. These nanosystems are composed of polyethylene-glycol, bovine-serum-albumin and TS262 or TS265 (NanoTS262 or NanoTS265, respectively). Results: In FR37-CMT, TS262 and TS265 displayed IC_{50} values well below those displayed by doxorubicin and cisplatin. The nanovectorizations further decreased the IC_{50} values. Discussion: TS262 and TS265 proved to be effective against FR37-CMT cells and more effective than of doxorubicin and cisplatin. The Nanosystems efficiently delivered the cytotoxic cargo inducing a significant reduction of cell viability in FR37-CMT cell line when compared to the free compounds. Conclusions: TS262 and TS265 are compounds with potential in the treatment of CMTs. NanoTS262 and NanoTS265 demonstrate that such simple nanovectorization via gold nanoparticles shows tremendous potential as anti-cancer formulations, which may easily be expanded to suit other cargo.

III.2. Keywords:

1,10-Phenanthroline-5,6-dione metal compounds; canine mammary tumors; cisplatin; doxorubicin; FR37-CMT; gold nanoparticles; nanotechnology

III.3. Introduction

The most common tumors in non-spayed female dogs are canine mammary tumors (CMTs),^{6, 9} whose standard treatment consists in mastectomy and ovariohysterectomy.⁴⁴ Approximately 50 % of these CMTs are malignant and these patients would further profit from adjuvant chemotherapy. However, the efficiency of common anticancer drugs, such as doxorubicin and docetaxel, used for the treatment of HBC, has not increased the disease free or overall survival time of tested animals.¹⁸⁹ For instance, a CMT model cell line, FR37-CMT, has been shown to be resistant to cisplatin up to 50 μ M, making it impossible to use as therapeutic agent in dogs.²⁴⁶ Despite the hormonal dependency of CMTs, the use of tamoxifen, an antagonist of the estrogen receptor alpha, is not proven to be an effective therapeutic adjuvant. Consequently, it is extremely important to test new therapeutic compounds or new strategies that can significantly reduce the growth of CMTs. Here, two metal compounds with 1,10-phenanthroline-5,6-dione (DION) ligands, $[\text{Zn}(\text{DION})_2]\text{Cl}$ (TS262) and $[\text{CoCl}(\text{H}_2\text{O})(\text{DION})_2][\text{BF}_4]$ (TS265) that have shown remarkable antiproliferative effect against human colorectal carcinoma cells (HCT116), human hepatocellular carcinoma cells (HepG2) and breast adenocarcinoma cells (MCF7),²⁴⁷⁻²⁴⁹ were used to evaluate their potential against CMT.

Gold nanoparticles (AuNPs) have been proposed as valuable platforms for the delivery of anti-cancer drugs in humans, showing enhanced selectivity and improved efficacy. Due to their high surface to volume ratio and high reactivity with biomolecules via simple water phase chemistry, these AuNPs may be easily modified with numerous functional moieties, such as polyethylene glycol (PEG), bovine serum albumin (BSA), oligonucleotides, peptides, *etc.*²⁵⁰ AuNPs modification may increase biocompatibility, assist transport chemotherapeutic compounds and allow for specific targeting without toxicity.²⁵⁰⁻²⁵² Here, the two aforementioned compounds were loaded onto 14 nm AuNPs functionalized with bifunctional PEG $[\text{SH-EG}(8)\text{-(CH}_2)_2\text{-COOH}]$

and BSA, and used as a nanovectorization platform against FR37-CMT cell line. We have previously showed the strong interaction between TS265 and BSA,²⁴⁹ which will allow loading both compounds (TS265 and TS262) onto the nanoconjugate. Nanovectorization of these compounds was assessed and the anti-cancer activity against FR37-CMT weighed to evaluate their potential in CMT therapy – Figure III.1.

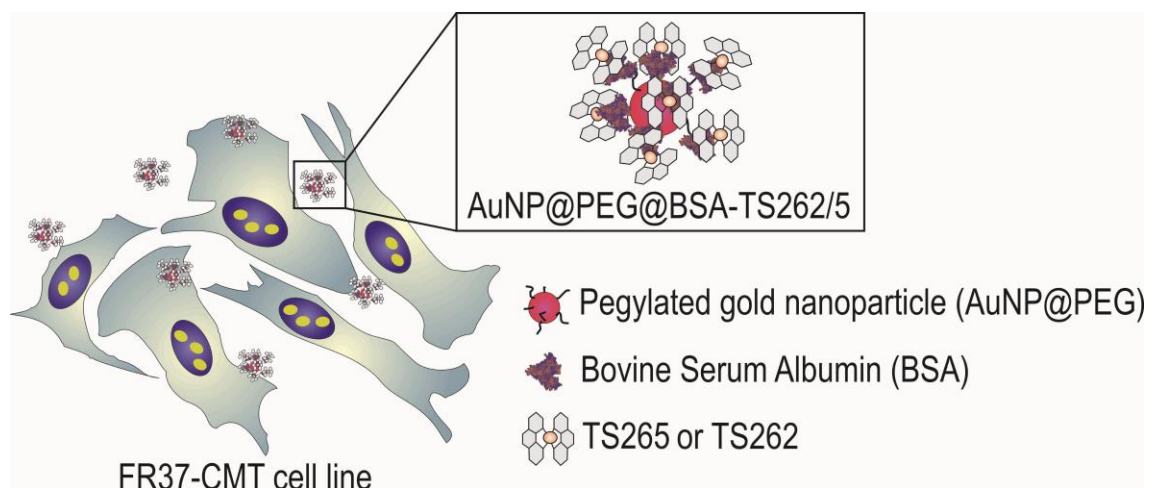


Figure III.1 - Gold nanoparticles as Nanovectorization systems for the delivery of TS262 and TS265 in FR37-CMT cell line.

III.4. Materials and methods

III.4.1. Compounds

TS262 and TS265 compounds were synthesized as previously described.²⁴⁸ Type I ultrapure water was used to prepare stock and working solutions. Stock solutions were kept at -20°C until used. Cisplatin 1 mg/mL stock solution in 0.9% (v/v) NaCl₂ (Teva Parenteral Medicines, Teva Pharmaceuticals, Petah Tikva, Israel) was kept at room temperature according to the manufacturer's instructions. Doxorubicin hydrochloride (Sigma) 5 mg/mL stock solution was made in DMSO (Sigma, Munich, Germany) and kept at 4 °C as recommended by the manufacturer.

III.4.2. Gold nanoparticles synthesis and assembly of Au-nanoconjugates

AuNPs with an average size of 14 nm were synthesized by the citrate reduction method described by Lee and Meisel²⁵³ and characterized by UV-Vis spectroscopy, transmission electron microscopy (TEM) and dynamic light scattering (DLS) using a Nanoparticle Analyzer SZ-100 (Horiba Scientific, Kyoto, Japan) at 25 °C, with a scattering angle of 90°. ^{254, 255} AuNPs functionalization with PEG was performed incubating a 10 nM AuNPs solution with 0.028 % (w/v) Sodium dodecyl sulfate (SDS, Sigma), and 3 µg/ mL SH-EG₍₈₎-(CH₂)₂-COOH (Iris-Biotech, Marktredwitz, Germany) for 16 h with agitation at room temperature (RT). Excess PEG chains were removed by centrifugation at 14 000 xg for 30 min at 4 °C and the degree of PEG coverage on the surface of PEGylated AuNPs (AuNPs@PEG) evaluated via Ellman's Assay.^{252, 254}

Functionalization of AuNPs@PEG with BSA (Sigma, MW 66,120 kDa) was performed by a process based on a N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide (EDC/NHS) reaction.²⁵⁶ A reaction mixture with 21 nM of the synthesized AuNPs@PEG, 1.25 mg/mL sulfo-NHS (Sigma, MW 217.13 Da) and 0.312 mg/mL EDC (Sigma, MW 191.70 Da) in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 6 (Sigma, MW 195.24 Da) was incubated for 30 min and centrifuged at 14 000 xg for 30 min at 4 °C. The supernatant was removed and replaced by 2.5 mM pH 6 MES buffer with 10 µg/mL of BSA. The reaction mixture was incubated for 16 h and then centrifuged three times at 14 000 xg for 30 min at 4 °C in order to separate the unbound BSA (in supernatant) from the AuNP@PEG with bound BSA (AuNP@PEG@BSA). BSA concentration was quantified from the supernatant recovered from the washes using Bradford Assay (Thermo Scientific, Waltham, Massachusetts, USA). The difference between the BSA added to the reaction mixture and BSA in the supernatants indicates the amount of BSA bound to the AuNP.

Finally, 6 nM of AuNPs@PEG@BSA, were mixed with either 50 µM of TS262 (AuNPs@PEG@BSA-TS262 - NanoTS262) or 50 µM of TS265 (AuNPs@PEG@BSA-TS265 - NanoTS265) and incubated for 1 h at 4 °C. After this period, solutions were centrifuged at 14 000 xg for 30 min at 4 °C, to remove unbound TS262 and TS265 (supernatants). Quantification of Zn for TS262 or Co for TS265 in the supernatants was performed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

All gold nanoconjugates were characterized by UV-Vis spectroscopy and DLS as above.

III.4.3. FR37-CMT cell culture

In all assays, FR37-CMT cells were grown at 37 °C, 5% (v/v) CO₂ and 99% (v/v) relative humidity in Dulbecco's Modified Eagle's Medium, DMEM, (Life Technologies, Waltham, Massachusetts, USA) supplemented with 10 % (v/v) Fetal Bovine Serum, FBS (Life Technologies), 100 U/mL penicillin and 100 mg/mL streptomycin (Life Technologies), DMEM-FBS-PenStrep, as previously described.²⁴⁶

III.4.4. Cell viability assays

Cell viability was evaluated by the production of formazan by viable cells from a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), the MTS assay.^{214, 246} Briefly, 1x10⁵ FR37-CMT cells per well were seeded in 96-well plates (VWR, Radnor, Pennsylvania, USA) and incubated for 24 h. Then, the media were refreshed and supplemented with the appropriate dilutions of TS262, TS265, AuNP@PEG@BSA, NanoTS262, NanoTS265 or Type 1 ultrapure water as a control. After an incubation of 48 h, cell viability was determined using the CellTiter 96® AQueous non-radioactive cell proliferation assay according to the manufacturer's instructions (Promega, Fitchburg, Wisconsin, USA). Absorbance at 490 nm was measured in a Microplate reader Infinite M200 (Tecan, Mannedorf, Switzerland).

III.4.5. Wound healing assay

Cells from FR37-CMT cell line were grown in 35 mm² tissue culture plates (VWR, Radnor, Pennsylvania, USA) until a confluent monolayer. A scratch was made with a sterile 100 µL micropipette tip on the surface of the plates. Cells were then exposed to 1.5x IC₅₀ of TS262 or TS265 or vehicle control (water) and incubated for 24 h in normal culture conditions. Cell cultures

were photographed and the remission percentage was calculated by measuring the width of scratches with the use of ImageJ 1.49v software (Wayne Rasband National Health Institutes, USA).²⁴⁶

III.4.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism v6.01 (GraphPad Software, Inc, La Jolla, CA, USA). The data is expressed as mean $\pm$ SEM from at least three independent experiments. Statistical significance was considered when p-value < 0.05.

III.5. Results

III.5.1. Synthesis of Gold nanoconjugates

Colloidal AuNPs with an average 14 nm diameter show maximum absorption at 518 nm that indicates the surface plasmon resonance (SPR) peak (Figure III.2A). Functionalization of AuNPs leads to a red-shift of the SPR, which may be used as indicator of effective binding of molecules to the AuNPs' surface. Following PEGylation, the SPR peak of AuNPs@PEG shifted from 518 nm to 520 nm confirming the covalent binding of PEG chains to the surface of the AuNPs (Figure III.2B). Further functionalization with BSA resulted in a shift to 523 nm (Figure III.2C). On average, the nanoconjugates have 2080 $\pm$ 150 PEG chains and 7 BSA molecules per nanoparticle.

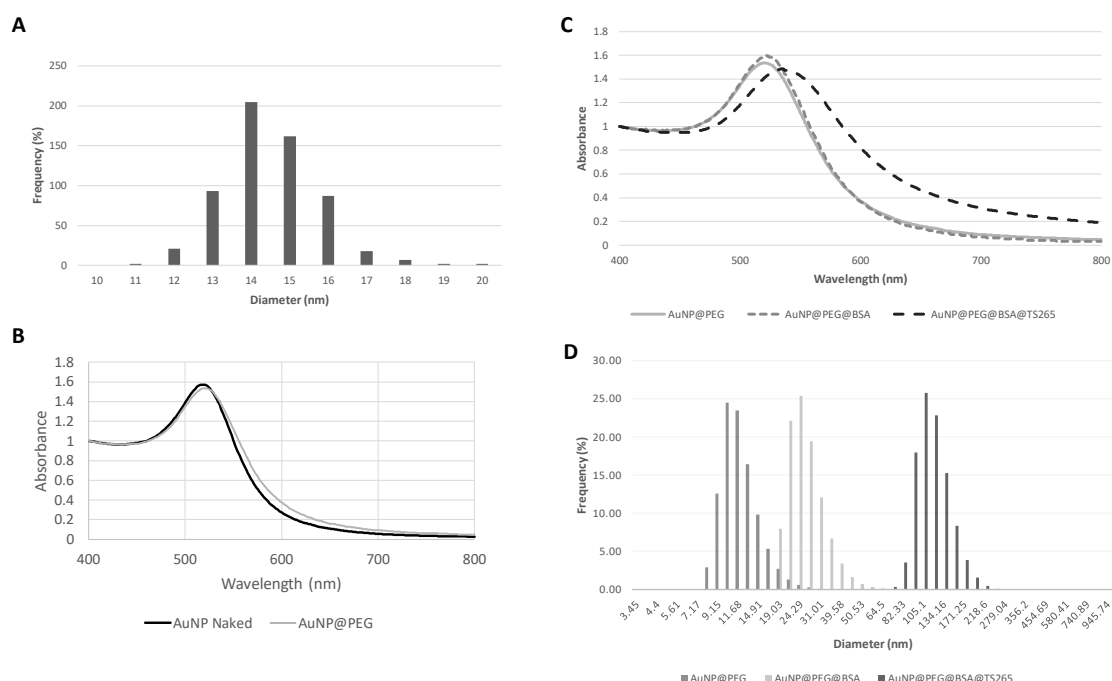


Figure III.2 - Physicochemical characterization of AuNP constructs. A. Transmission electron microscopy (TEM) analysis of core naked AuNPs evidencing an average diameter of 14 nm B. UV/Vis spectra of naked and PEGylated AuNP constructs; C. UV/Vis spectra of AuNPs@PEG, AuNPs@PEG@BSA and AuNPs@BSA-TS265 (NanoTS265); D. DLS analysis of AuNPs@PEG, AuNPs@PEG@BSA and AuNPs@BSA-TS265 (NanoTS265) confirming UV/Vis analysis.

Functionalization of AuNPs@PEG@BSA with each compound was confirmed by UV-spectroscopy and DLS (Figure III.2, C and D for TS265 and Figure III.3, A and B for TS262). The maximum absorption peak of AuNPs@PEG@BSA-TS265 (NanoTS265) is at 535 nm (Figure III.3, B) and for AuNPs@PEG@BSA-TS262 (NanoTS262) it is at 530 nm (Figure III.3, A).

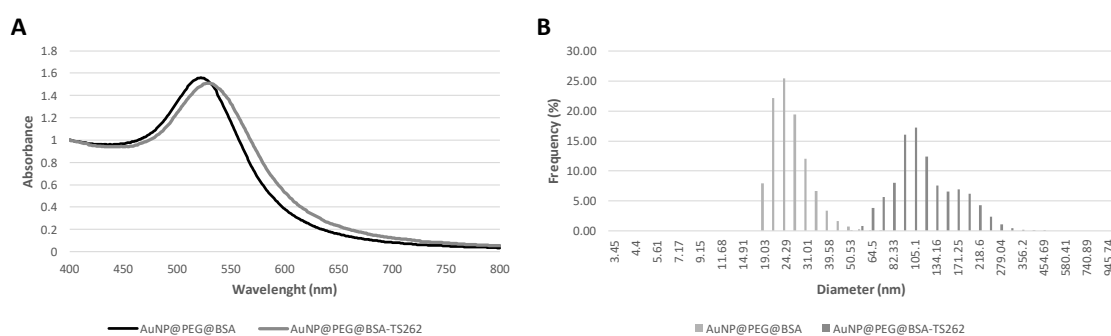


Figure III.3 - A. UV/Vis spectra of AuNPs@PEG@BSA and AuNPs@BSA-TS262 (NanoTS262). B. DLS analysis of AuNPs@PEG@BSA and AuNPs@BSA-TS262 (NanoTS262).

III.5.2. Effects of TS262 and TS265 on cell viability

The *in vitro* antiproliferative potential of the TS262 and TS265 compounds was evaluated in FR37-CMT cell line employing the spectrophotometrical quantification of formazan that resulted from the reduction of MTS by cells with active mitochondrial dehydrogenases, using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay. The *in vitro* cytotoxicity was expressed as the concentration of compound that inhibits proliferation of cells by 50% as compared to untreated cells (IC_{50} ; μM). A decrease of the cell viability, in a dose-dependent manner, was observed for both compounds (Figure III.4). The IC_{50} for TS262 is $1.05 \pm 0.15 \mu M$ and $1.39 \pm 0.23 \mu M$ for TS265. These IC_{50} are 50x lower than those described for cisplatin ($>50 \mu M$) and 5x lower than that described for doxorubicin ($5.3 \mu M$) on the FR37-CMT cell line (Figure II.13).²⁴⁶ This is the first report on the high antiproliferative activity of novel cobalt and zinc metal compounds for CMTs. The lack of good chemotherapeutic regimens for dogs with CMT highlights the relevant potential of these compounds against CMT.

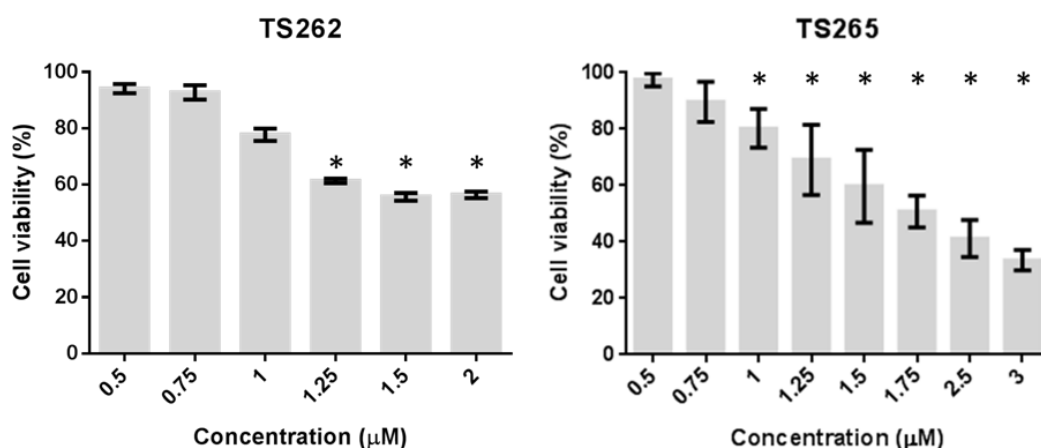


Figure III.4 - Viability of FR37-CMT cells after 48 h of exposure to different concentrations of TS262 and TS265. Results are expressed as mean $\pm$ SEM to controls from at least three independent experiments. * p-value <0.05 relative to the cell viability percentage of the $0.5 \mu M$ concentration.

III.5.3. Effect of TS262 and TS265 on the migration of FR37-CMT cells evaluated by wound healing assay

FR37-CMT cell line displays the ability to reorganize the collagen matrix, it also expresses vimentin, CD44 and shows the loss of E-cadherin that is considered a fundamental event in epithelial to mesenchymal transition (EMT) and has a very high migration potential (wound remission percentage of $79.2\% \pm 5.03$ at 24 h).²⁴⁶ The effect of Co(II) and Zn(II) compounds in the reduction of this migration potential was analyzed (Figure III.5). Cells were exposed to $1.6 \mu\text{M}$ of TS262 and $2.0 \mu\text{M}$ of TS265 ($1.5\times$ the respective IC_{50}) to ensure an observed effect. At these concentrations, there is a clear reduction of the wound remission rates in FR37-CMT cells exposed to both compounds, particularly for TS265 (by circa 20 %) (Figure III.5).

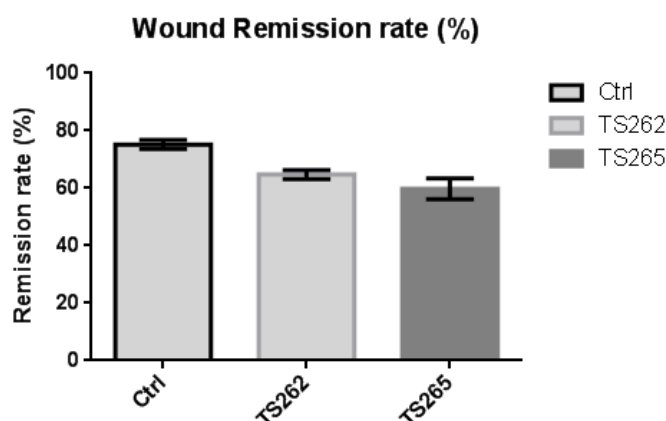


Figure III.5 - Wound healing assay of FR37-CMT cells exposed to $1.5\times \text{IC}_{50}$ concentrations of TS262 ($1.6 \mu\text{M}$) and TS265 ($2.0 \mu\text{M}$). The remission rates were calculated by measuring the scratches at time 0 h and after 24 h exposition to the compounds. Data is expressed as mean $\pm$ SEM of at least three independent experiments.

III.5.4. Effect of NanoTS262 and NanoTS265 on FR37-CMT cell line

Despite the high antiproliferative effect of free TS262 and TS265 in FR37-CMT cells, it has been demonstrated that gold nanoparticles are excellent carriers for drug delivery.^{257, 258} To allow the direct comparison between free and nanoconjugated compounds, the effect of the NanoTS262 and NanoTS265 on FR37-CMT cells viability was performed at IC₅₀ concentrations, both for the free compounds and the nanovectorized. The amount of TS262 and TS265 on the surface of respective functionalized nanoparticles was evaluated (402 ±32 molecules of TS262 and 438 ±19 molecules of TS265) (Figure III.6). As control, cells were exposed to AuNPs@PEG@BSA at a concentration in AuNPs equivalent to that of the nanoconjugates. Figure III.6 shows that AuNPs@PEG@BSA had no effect on the viability of FR37-CMT cells. However, nanovectorization of the compounds significantly diminished cell survival when compared to free TS262 and TS265; 21% and 16%, respectively.

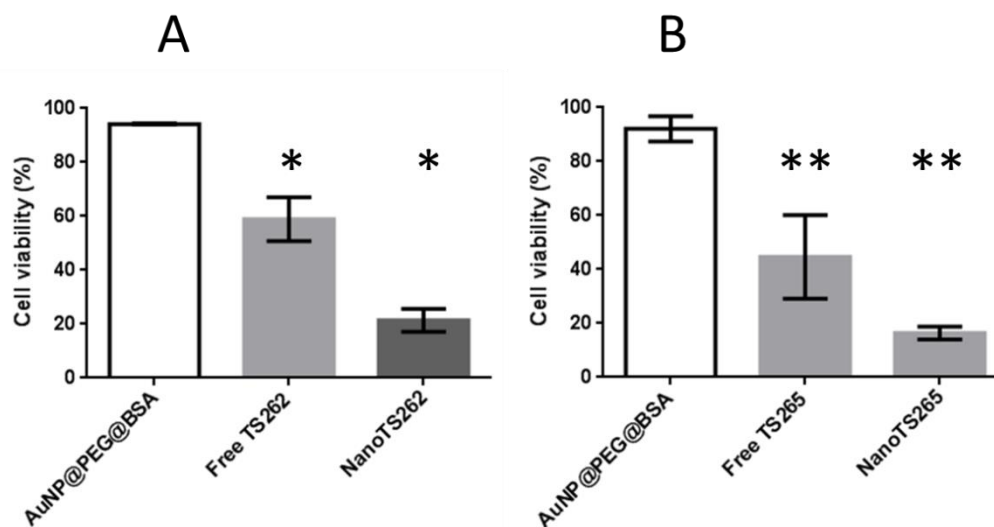


Figure III.6 – Cell viability of FR37-CMT cell line after 48 h exposure to A) 2.8 nM AuNPs@PEG@BSA, 1.05 μM free TS262 and equivalent concentration of AuNPs@PEG@BSA-TS262 (NanoTS262) to achieve 1.05 μM of TS262 (2.8 nM of particles), B) 4.8 nM AuNPs@PEG@BSA, 1.39 μM of free TS265 and equivalent concentration of AuNPs@PEG@BSA-TS265 (NanoTS265) to achieve 1.39 μM of TS265 (4.8 nM of particles). Results are expressed as mean ± SEM to controls from at least three independent experiments. * p-value<0.05 relative to the cell viability percentage of the 2.8 nM concentration and ** p-value<0.05 relative to the cell viability percentage of the 4.8 nM concentration.

III.6. Discussion

Unfortunately, there are no established chemotherapy treatment protocols for CMT and for most of the patients surgical mastectomy is the treatment of choice. However, it often fails in high-risk locally invasive mammary tumors.^{44, 197} Currently, the use of chemotherapeutic agents to combat the micro-metastatic disease is a reasonable consideration. Most of the chemotherapy protocols used in veterinary medicine have been translated from protocols used to treat breast cancer patients.¹⁸⁵ Among the chemotherapeutic agents, doxorubicin, cisplatin, vinblastine and cyclophosphamide either alone or in combination with other drugs, have been used for CMT therapy in veterinary practice.^{190, 197, 207}

Nevertheless, the failure of chemotherapy regimens with these agents due to dose-limiting toxicity and multidrug resistance is a major concern in the clinical management of CMTs.²⁰⁷ Therefore, there is an urgent need of novel compounds and strategies that increase the therapeutic efficacy and reduce the systemic toxicity in CMT.

The existence of CMT models that are not dependent on female hormonal signaling and may be used as a model for the study of the epithelial to mesenchymal transition (EMT) and drug resistance in canine mammary gland tumors is of extreme relevance. We have recently described a novel cell line, FR37-CMT that fulfils these characteristics.²⁴⁶

Here we report for the first time the effect of Co(II) and Zn(II) compounds bearing 1,10-phenantroline-5,6-dione ligands as antiproliferative agents for targeting CMT. Their solubility and stability in water encouraged their application as antiproliferative agents.²⁴⁸ Both compounds demonstrate a high cytotoxic activity in FR37-CMT cells with low IC₅₀ concentrations ($1.05 \pm 0.15 \mu\text{M}$ for TS262 and $1.39 \pm 0.23 \mu\text{M}$ for TS265) compared to doxorubicin (IC₅₀ = $5.3 \mu\text{M}$) and cisplatin (IC₅₀ > $50 \mu\text{M}$)²⁴⁶ thus making them excellent candidates for further research in CMTs treatment. The reduction of viability was accompanied with a slight impairment of cell mobility (Figure III.5) particularly relevant since this cell line has demonstrated high invasion and mobility capabilities.²⁴⁶

The high cytotoxic effect here demonstrated for the free TS262 and TS265 in the FR37-CMT cell line, highlights their further application using novel nano-delivery systems. Due to their size and ease of functionalization with different moieties (*e.g.* PEG for biocompatibility; BSA allowing the loading with the desired chemotherapeutics - here TS262 or TS265 as models) it has been demonstrated that gold nanoparticles can act as promising carriers for drug delivery further increasing the therapeutic index.^{257, 258} Despite the high number of applications of gold

nanoparticles as drug delivery agents in human cancer,²⁵⁸ as far as we are aware there is no description of their application in CMT.

Here we show for the first time the application of gold nanoparticles as drug delivery agents for delivering TS262 and TS265 compounds to CMT cells. TS262 and TS265 nanoconjugates (NanoTS262 and NanoTS265, respectively) significantly reduced the viability of FR37-CMT cells at previously determined IC_{50} concentrations of the free compounds (Figure III.6). Moreover, the control nanoconjugate - AuNP@PEG@BSA displayed no reduction of cell viability in FR37-CMT cells. The nanoconjugates – NanoTS262 and NanoTS265 increased cytotoxic activity can be due to a more efficient transport of the compounds into the cells as observed for tamoxifen.²⁵⁹ The conjugation of TS262 and TS265 compounds with gold nanoparticles functionalized with PEG and BSA, allowed the formulation of a promising new therapeutic approach for the treatment of CMTs.

III.7. Conclusions

The metal compounds, TS262 and TS265, displayed lower IC_{50} than cisplatin and doxorubicin against the FR37-CMT cell line. This indicates a potential therapeutic for these compounds. To further improve efficacy, we used AuNPs as vectorization platforms for these compounds, which led to a 20% increase in killing efficiency against this mammary tumor cell model. NanoTS262 and NanoTS265 are thus promising chemotherapeutic formulations for the treatment of CMTs and additional modification of these nanomedicines, e.g. with targeting moieties such as antibodies or peptides, may further improve efficacy.



IV. Proteomic study of FR37-CMT cell line exposed to Co(II) and Zn(II) compounds

Disclaimer: Results and data presented in this chapter are in preparation for publication in peer review journals.

IV.1. Abstract

Several chemotherapeutic agents have been tested for the treatment of CMTs but with little success. The FR37-CMT cell line has sensitivity to the organometallic compounds 1,10-phenanthroline-5,6-dione (DION) ligands, $[\text{Zn}(\text{DION})_2]\text{Cl}$ (TS262) and $[\text{CoCl}(\text{H}_2\text{O})(\text{DION})_2][\text{BF}_4]$ (TS265). Quantitative proteomics and two-dimensional electrophoresis were used to evaluate the response of FR37-CMT cell to exposure to these compounds. The proteome may give important answers towards the comprehension of the cellular mechanisms triggered with compounds' exposure and their major targets. It was possible to observe that FR37-CMT cells had a similar pattern of response towards TS262 and TS265. From the protein spots detected, 361 were common to all Control, TS262-treated and TS265-treated conditions. Fifteen protein spots were only present in TS262 treated conditions and 9 protein spots were only present in TS265 treated samples. Since the identification process is still being performed it is not possible, at the present time, to further discuss these modifications with more detail.

IV.2. Keywords:

1,10-Phenanthroline-5,6-dione metal compounds; 2-Dimensional Electrophoresis; Canine mammary tumors; FR37-CMT; Quantative Proteomics.

IV.3. Introduction

Canine mammary tumors (CMT) are the most common neoplasm in intact female dogs, whose gold standard treatment is surgery, effective in 50% of the malignant CMT cases that do not display (micro)metastases.⁴⁴ Although there are no established guidelines for treatment beyond surgery, dogs with localized advanced disease with metastatic CMT or with a biologically aggressive histological type of CMT may benefit from adjuvant treatment such as radiotherapy, chemotherapy or anti-angiogenic therapy.⁴⁴ Several chemotherapeutic agents have been tested in dogs; for the majority no positive effects due to development of multidrug-resistance (MDR) or high systemic toxicity.^{44, 260} Recently, the FR37-CMT cell line was described being a good model for chemotherapeutic development.²⁴⁶ This cell line is tolerant to high concentrations of cisplatin ($IC_{50} > 50 \mu M$) and doxorubicin ($IC_{50} = 5.3 \mu M$) making this cell also a good model for the study of resistance to chemotherapy in CMTs.²⁴⁶ As described in chapter III.5.2 the organometallic compounds 1,10-phenanthroline-5,6-dione (DION) ligands, $[Zn(DION)_2]Cl$ (TS262) and $[CoCl(H_2O)(DION)_2][BF_4]$ (TS265) are promising therapeutic agents in the treatment of CMT. FR37-CMT cell showed an IC_{50} of 1.05 μM and 1.39 μM for TS262 and TS265, respectively.

Quantitative proteomics has been commonly used to evaluate the effect of chemotherapeutic compounds at the cell level.²⁶¹ Indeed, modifications occurring at the proteome level after cell treatment with the compounds give important clues toward the understanding of chemotherapeutic agent major protein targets and/or alterations occurring in the cell metabolism. This work took advantage of the resolution of two-dimensional electrophoresis to deepen the knowledge on the FR37-CMT cells response to exposure to TS262 and TS265.

IV.4. Materials and methods

IV.4.1. Cell culture and samples preparation

The protocol used for cell culture and sample preparation for proteomic analysis was described elsewhere with few modifications.^{246, 252} Briefly, 40×10^5 FR-37 CMT cells were seeded in flasks with an area of 75 cm^2 and left at 37°C , 5 % (v/v) CO_2 and 99 % (v/v) relative humidity for 24 h to assure the complete adherence of cells to the flask. Culture medium was then removed and replaced with fresh medium supplemented with the correspondent concentration of the IC_{50} for TS262 and TS265, $1.05 \mu\text{M}$ and $1.39 \mu\text{M}$, respectively as described in III.5.2. After 48 h the supernatant was removed, cells were washed three times with Phosphate Buffered Saline (PBS) and removed from the flask. Cells were then pelleted by a centrifugation of $500 \times g$ for 5 min, resuspended in lysis buffer (150 mM NaCl; 50 mM Tris, pH = 8.0; 5 mM EDTA, 2% (w/v) NP-40) and stored at -80°C until use.²⁵² Biological duplicates of three samples were prepared: i) untreated FR37-CMT cells (control samples); ii) FR37-CMT cells treated with TS262 (TS262 samples); ii) FR37-CMT treated with TS265 (TS265 samples).

IV.4.2. Two-Dimensional Electrophoresis (2-DE)

Before 2-DE, cells were lysed by sonication, centrifuged at $5000 \times g$ for 10 minutes and proteins from the supernatant were precipitated with 2-DE cleanup kit (GE Healthcare, Little Chalfont, UK) for isoelectric electrophoresis (IEF) contaminants removal. Pelleted proteins were resuspended at RT for 24 h in IEF buffer [8 M urea, 2 % (w/v) CHAPS, 0.5 % (v/v) IPG buffer (pH 3-10NL), DTT 0.1 % (w/v)], quantified using 2-D Quant kit (GE Healthcare) and $200 \mu\text{g}$ protein were used to rehydrate and isoelectric focused in 7 cm long Immobiline DryStrip 3-10 NL (GE

Healthcare) in an Ettan IPGphor 3 focusing unit (GE Healthcare) as previously described.^{247, 252,}
²⁶¹ The second dimension was consisted in a molecular weight separation in a SDS Polyacrylamide gel electrophoresis (SDS-PAGE). All 2-DE gel images were digitalized using Image Scanner II (GE Healthcare) and analyzed with the Melanie 7.0 Software (GeneBio, Geneva, Switzerland). Each condition studied was evaluated in triplicate. The analysis was performed semi-automatically by the software, adhering to the following procedure: (1) spot detection; (2) spot matching from different gels; (3) assessment of the normalized percentage of volume of each spot. Normalization was performed as the ratio of the spot percentage of volume with the total volume percentage of all spots in the same gel. Variation of each protein expression level was calculated as the ratio of the normalized intensity of each protein spot in gels corresponding to each condition compared to those corresponding to control samples. The approximate molecular mass for each identified protein was determined by comparison with the relative positions of the proteins included (Color protein marker II, NZYTech, Lisbon, Portugal), which was run in the second dimension simultaneously with the samples under study. Spots with significantly altered intensities between conditions (up or down-regulated peptides in comparison to control samples) were selected.

IV.4.3. In-gel digestion and MALDI-TOF mass spectrometry analysis

Selected protein spots were manually excised from 2-DE gels and sent for Peptide Mass Fingerprinting analysis at Mass Spectrometry Laboratory in ITQB/iBET (paid service, Oeiras, Portugal).

IV.5. Results and Discussion

Chemotherapy of canine mammary tumors is being highly limited by the acquired resistance to the chemotherapeutic drugs.⁴⁴ Identification of cellular response to chemotherapeutic agents may provide important clues to predict drug resistance and toxicity. In this work, a quantitative proteomic analysis was performed in order to comprehend the mechanisms involved in toxicity of two promising chemotherapeutic compounds: TS262 and TS265. With that purpose, FR37-CMT cells were treated with the concentration of TS262 and TS265 equivalent to their IC₅₀: 1.05 μ M and 1.39 μ M, respectively. Figure 1 shows the representative 2-DE protein patterns obtained for control (untreated cells), TS262 and TS265 samples and more detailed results are presented in Table A.2 of the appendix.

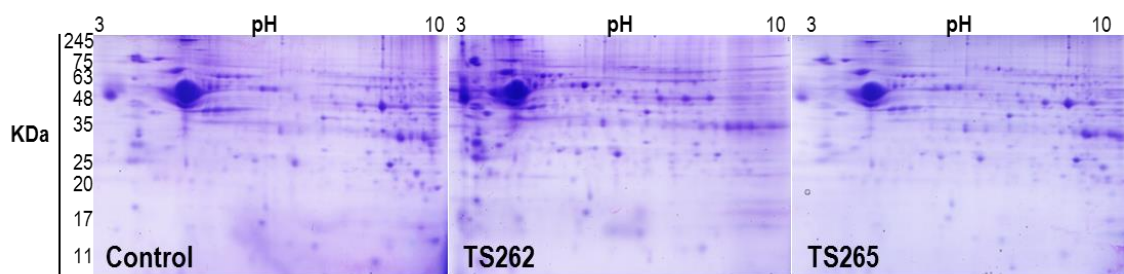


Figure IV.1 – Representative protein patterns of FR37-CMT cells untreated (control), treated with 1.05 μ M TS262 and with 1.39 μ M TS265. Proteins were separated by 2-DE (IEF/SDS-PAGE) and visualized by staining with Coomassie brilliant blue R-350.

Around 373, 384 and 383 protein spots were detected within pH range 3 to 10 in Control, TS262 and TS265 gels, respectively. Among these proteins, 361 were common to all gels, being 15 and 9 protein spots present only in TS262 and TS265 treated samples, respectively (Figure IV.2, A).

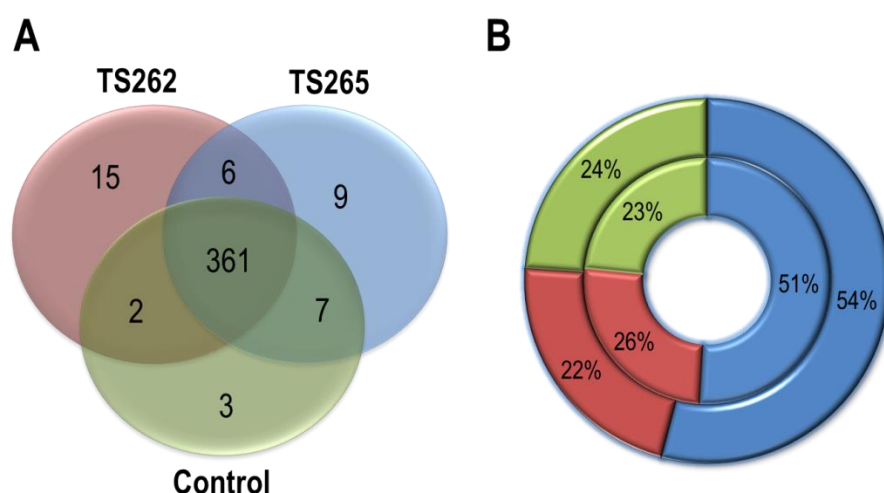


Figure IV.2 - A) Venn diagram demonstrating the number of spots that are in common among the 3 analyzed samples: untreated FR37-CMT cells (control), FR37-CMT cells treated with 1.05 μ M TS262 and FR37-CMT cells treated with 1.39 μ M TS265 cells. B) Doughnut diagram resuming the fold variations between TS262 (inner ring) and TS265 (outer ring) relative to the control sample. It is represented the percentage of protein spots that decreased (fold < 0.7; red section), increased (fold > 1.5, green section) or maintained (blue section) the abundance between the samples.

A similar percentage of altered proteins in TS262 and TS265 relative to the control sample were observed (Figure IV.2, B). This might reflect a similar cellular effect induced by both compounds in FR37-CMT cells. Indeed, a Principal Component Analysis (PCA) only discriminates both samples in the second principal component, which only reflects 32% of the samples variance (Figure IV.3). Among proteins that showed altered abundance, 6 proteins showed the highest alterations being represented as outliers in PCA (Figure IV.3). As an example, protein spot 203 showed an increased 4.9 fold and 2.8 fold in TS262 and TS265 samples, respectively, relative to the control sample. While protein spot 149 showed a decreased abundance in TS262 and TS265 of 0.48 and 0.64, respectively. On the other side, abundance of protein 101 was only decreased 0.11 times in TS262 condition. Due to their altered abundance, these proteins have high interest for identification. However, mass spectrometry identification is still being performed and it is not yet possible to discuss with more detail these modifications.

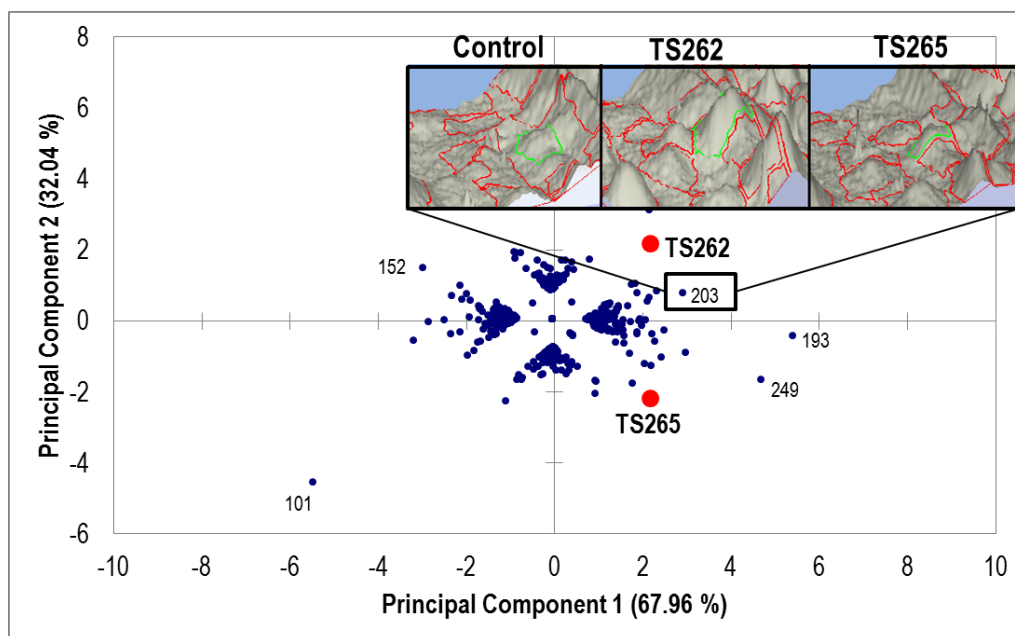


Figure IV.3 -: Principal component analysis of the protein fold obtained in FR37-CMT cells treated with TS262 and TS265 relative to untreated FR37-CMT cells (control). Red spots represent each sample prepared for the indicated condition. Blue spots represent the clustering of proteins detected in the corresponding 2-DE pattern. Outlier protein spots are numbered, being represented the 3D view of the protein spot 203 in the 3 conditions.

IV.6. Conclusions

A previous proteomic analysis of the effect of TS265 in human colorectal carcinoma cells (HCT116) revealed an increased abundance of proteins involved in oxidative stress regulation, such as Superoxide Dismutase (SODC) and peroxiredoxine 2 (PRDX2) and in the regulation of growth, such as PA2G4 (proliferation associated protein 2G4) and 14-3-3 σ (stratifylin/14-3-3 protein σ).^{247, 249} A decreased abundance of proteins involved in tumorigenesis, such as translationally controlled tumor protein (TCTP), heat shock protein beta 1 (HSP90B1) was also observed in the presence of TS265.²⁴⁷ All these observations were correlated with the antiproliferative activity of this compound mediating cell cycle arrest in the S-phase and the subsequent induction of apoptosis.^{247, 249} On the other hand, cytoskeleton-associated proteins TPM3 (tropomyosin 3) and EZRI (ezrin), where also found to be altered, suggesting some kind of

interactions of the compound with the cellular structural organization.²⁴⁷ It is expected that identification of the most altered proteins of FR37-CMT treated with TS262 and TS265 in comparison to control cells may enlighten the molecular targets and pathways associated with the cytotoxicity of these compounds, providing novel therapeutic targets in CMT.



V. Immortalization, characterization and tolerance to promising gold nanoparticles functionalized with Co(II) and Zn(II) compounds of a novel canine mammary tumor cell line FR10-CMT

Disclaimer: Results and data presented in this chapter are in preparation for publication in peer review journals and were partially published in:

Raposo L.R., Santos S., Henriques, J., Alves, M., P Faísca, P., Beselga, A., J Correia, J., Fernandes, A. R.. "Immortalization of primary canine cell lines from mammary tumors: a protocol optimization." XVIII Meeting of the Portuguese Society of Animal Pathology. Évora. May 9-10, 2013.

V.1. Abstract

In this work we describe the immortalization and characterization of a new cell line, FR10-CMT. FR10-CMT cells are cuboid shaped and are capable to form xenograft tumors in NOD-SCID female mice. The cell line shows some stemness characteristics such as expression of CD44 and vimentin and a downregulation of E-cadherin. However, the epithelial to mesenchymal transition (EMT) is apparently repressed by the overexpression of miR-200c. The cell line presents a down-regulation of *PTEN* and *SNAI2* that may be an indication that the Akt/mTOR and the basal WNT signaling pathways may be responsible for the proliferative phenotype of FR10-CMT cells. This cell line shows high tolerance to cisplatin ($IC_{50} > 50 \mu M$) but sensitive to doxorubicin ($IC_{50} = 3.96 \mu M$). The two metal compounds $[Zn(DION)_2]Cl$ (TS262, DION = 1,10-phenanthroline-5,6-dione) and $[CoCl(H_2O)(DION)_2][BF_4]$ (TS265), previously shown to be effective in reducing the viability of the FR37-CMT cell line were also effective in inhibiting the new FR10-CMT cell line, with IC_{50} values significantly lower than those of doxorubicin and cisplatin ($IC_{50} = 0.55 \mu M$ for TS262 and $IC_{50} = 0.80 \mu M$ for TS265). The novel nanovectorizations proved to efficient for chemotherapeutic compound delivery by significantly reducing cell viability in FR10-CMT cell line when compared to the free TS262 and free TS265. These results support the use of FR10-CMT cell as an important model for the understanding of CMT progression, to understand the mechanisms underlying tolerance to common chemotherapeutic agents such as cisplatin and for novel drug discovery fostering CMT treatment.

V.2. Keywords

1,10-Phenanthroline-5,6-dione metal compounds; cancer stem cells; Canine Mammary Tumors; cisplatin; doxorubicin; epithelial to mesenchymal transition; FR10-CMT; Gold nanoparticles; miRNAs; Nanotechnology.

V.3. Introduction

Canine mammary tumors (CMTs) are the most frequent neoplasms diagnosed in intact female dogs.^{6, 9} About 50% of all CMTs are malignant, and the patients with more aggressive tumors would benefit from co-adjuvant chemotherapy.^{44, 184} However, treatment with commonly used medicines used in HBC such as doxorubicin, docetaxel, tamoxifen for instance been proven effective.^{44, 189} Despite the established dependence of CMT development on estrogens and progesterone, tamoxifen (an estrogen receptor alpha antagonist) has not been proven effective against CMT.⁴⁴ Therefore, it is essential to test novel therapeutic options that can prove to be more effective in treatment of CMTs than the available options.

The epithelial to mesenchymal transition (EMT) is an important event associated malignancy, the formation of metastasis and resistance to chemotherapeutic agents.¹⁴⁷ For instance, cells will lose expression of E-cadherin and express N-cadherin and will express vimentin. EMT is also believed to have a role in the acquisition of stemness characteristics such as those exhibited by sub-populations of tumor cells called cancer stem-like cells which express the CD44⁺/CD24^{-/low} phenotype.^{147, 153, 262}

Recently, a new CMT cell line, FR37-CMT, has been described as a model for the understanding of EMT and the mechanisms implicated in the tolerance of CMTs to conventional chemotherapy.²⁴⁶ This cell line is tolerant to cisplatin ($IC_{50}>50\text{ }\mu\text{M}$) and is relatively resistant to doxorubicin ($IC_{50}=5.3\text{ }\mu\text{M}$).²⁴⁶ Also, two novel metal compounds with 1,10-phenanthroline-5,6-dione (DION) ligands, $[Zn(DION)_2]Cl$ (TS262) and $[CoCl(H_2O)(DION)_2][BF_4]$ (TS265) and two new nanovectorizations, nanoTS262 and nanoTS265 have been shown to more effective than doxorubicin.

In this work, a new immortalized canine mammary tumor derived cell line, FR10-CMT, is characterized. This cell line may be useful as new model for the comprehension of CMT development and for the development of new chemotherapeutic agents. Also, the antiproliferative activity of TS262, TS265, nanoTS262 and nanoTS265 will be evaluated in this novel cell line.

V.4. Materials and Methods

V.4.1. Sample collection

The sample collection consisted in surgical excision of the neoplasm following normal surgical procedures. This study was performed after approval by the local ethical committee (Comissão de Ética da Faculdade Medicina Veterinária, Universidade Lusófona) and written informed consent of the patient owners. Matched normal and tumor biopsies were immediately divided in three portions that were used for the following purposes: i) histopathology, by fixation in 10% (v/v) neutral buffered formalin (Sigma); ii) RNA extraction, by conservation in RNAlater (LifeTechnologies); and iii) establishment of cell line, by washing three times with DMEM-5XPenStrep, Dulbecco's Modified Eagle's Medium, DMEM, (Life Technologies) supplemented with a mixture of 500 U/mL penicillin and 500 mg/mL streptomycin (Life Technologies) and kept on ice until use.

V.4.2. Establishment of CMT primary cell line

For establishment of FR10-CMT cell line, biopsies were treated with the same procedure as previously described in Raposo *et al.* 2016 and in chapter II.4.2.²⁴⁶ Briefly, tumor pieces were immersed in TrypLE Express (LifeTechnologies), minced and incubated for 1 hour at 37 °C. After a centrifugation of 500 xg, the pelleted cells were resuspended in DMEM-FBS-PenStrep, DMEM

supplemented with 10% (v/v) Fetal Bovine Serum (LifeTechnologies), and a mixture of 100 U/mL penicillin and 100 mg/mL Streptomycin (LifeTechnologies), and seeded in a tissue culture flask that was maintained at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity until a confluent cell monolayer was observed. The following 20 passages consisted in the detachment of the cell monolayer with Tryple Express, centrifugation at 1500 *xg* for 5 minutes, resuspension of the pellet in 1 mL DMEM-FBS-PenStrep and seeding of 5x10⁵ cells to a new 75 cm² vented tissue culture flask. After 20 passages, the same procedure was performed, but the number of cells was serially lowered to ensure a monoclonal cell line. After the passage 50, it was considered that the cell culture was established and from this point on, 3x10⁵ cells were seeded in each new passage. These conditions were used along all the procedures used in this study involving cell culture maintenance and growth.

V.4.3. Chromosome preparations from FR10-CMT cell line

For chromosome visualization, a cell monolayer with 60% confluence was incubated with 0.1 µg/mL of Colcemid™ (Sigma) for 4 hours at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity. After this period, adherent cells were detached with TrypLE express, centrifuged at 1500 *xg*, the pellet resuspended in a solution of 0.04 M KCl and 0.025 M Sodium Citrate and incubated at RT for 30 min. Cells were then fixed in a methanol:acetic acid (3:1) mixture by incubation on ice for 10 minutes followed by centrifugation. This procedure was repeated one more time and pelleted cells were finally resuspended in the methanol:acetic acid solution. The prepared cell suspension was placed on top of slides by dropping the solution from a minimum height of 30 cm and let to dry and age at RT for at least 1 week. Preparations were incubated with TrypLE express for 2 min and stained with Giemsa (Sigma) using a 1:20 dilution in distilled water. After incubation for 15 min, slides were air-dried and chromosomes preparations were

visualized in a BX-51 microscope (Olympus). Images were obtained using a DP50 camera (Olympus).

V.4.4. Determination of the doubling time of FR10-CMT cell line

To calculate the specific growth rate of the cell line, 4500 FR10-CMT cells were seeded in a 24-well plate. After incubation for 24 h to allow cells to adhere, the number of cells were counted daily by unstacking the cells with TrypLE express, centrifugation at 1500 xg for 5 min, resuspension of the pellet in 1 mL DMEM-FBS-PenStrep and the number of cells determined using an hemocytometer. Three wells in three independent experiments were used to calculate the averaged specific growth and subsequently the doubling time.

V.4.5. Clonogenic assays

Soft agar colony formation

To infer if FR10-CMT cells are able to form colonies in soft agar, a 35 mm² tissue culture plate with 1 % (v/v) agar (Oxoid) in DMEM- FBS-PenStrep was first prepared. Then, 10⁵ cells in DMEM-FBS-PenStrep were mixed with a 0.5 % (v/v) agar solution, poured on top of the prepared plate, and after agar solidification covered with a layer of DMEM- FBS-PenStrep.

The wells of each experiment were monitored daily using a TMS inverted microscope (Nikon). Each experiment was repeated three times.

Collagen colony assays

The formation of colonies in collagen was also inferred by resuspending 10^5 cells in rat tail collagen, type I (First Link, LTd), let collagen to harden in a 24-well tissue culture plate and cover with DMEM- FBS-PenStrep.

The wells of each experiment were monitored daily using a TMS inverted microscope (Nikon). Each experiment was repeated three times.

V.4.6. Growth of FR10-CMT cell line on top of a Fibroblast cell monolayer

After obtaining a confluent monolayer of primary human fibroblasts (ATCC-PCS-201-010) in 25 cm² vented tissue culture flasks, there were added 2×10^3 and 4×10^3 FR10-CMT cells to 2 different flasks. The co-cultures were incubated at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity and monitored in a TMS inverted microscope. Experiments were repeated three times.

V.4.7. Tumorigenicity of FR10-CMT cell lines in NOD-SCID mice and tumor sample preparation for histopathology/immunohistochemistry

This animal experiment was carried out with permission of the Portuguese Authority (Direcção Geral de Alimentação e Veterinária), approved by the Local Ethical Committees (Comissão de Ética Experimentação Animal da Faculdade de Farmácia, Universidade de Lisboa; Comissão de Ética da Faculdade Medicina Veterinária, universidade Lusófona), and is in accordance with the Declaration of Helsinki, the EEC Directive (2010/63/UE) and Portuguese law (DL 113/2013, Despacho nº 2880/2015), and followed all legislations for the humane care of

animals in research. Animals were fed with sterile standard laboratory food and water *ad libitum*. A group of 3 non-obese severe combined immunodeficient, NOD/SCID, female mice (Instituto Gulbenkian de Ciência), with 13 weeks old, were inoculated with 10^6 FR10-CMT cells in Phosphate Buffer saline (PBS) into the scruff of the neck. When xenograft tumors reached a diameter of 1 cm, mice were anesthetized with Isoflurane (Isoflo, Esteve farma), sacrificed by neck hyperextension and tumors collected for cell culture, as described above, and histopathology. Briefly, tumor samples were fixed in 10 % (v/v) neutral buffered formalin, processed for paraffin embedding, sectioned with 5 μ m slices and stained with hematoxylin and eosin (HE). Paraffin-embedded sections were then placed on positively charged slides and submitted for immunohistochemistry using the EnVision Detection Systems Peroxidase/DAB Rabbit/Mouse (Dako) according to the manufacturer's instructions. Immunohistochemistry staining was performed using Cytokeratin Pan Ab-1 (Thermo Scientific) and Novocastra™ Liquid Mouse Monoclonal Antibody Vimentin (ref. NCL-L-VIM-V9 Leica biosystems). Slides were observed in a BX-51 microscope (Olympus) and images acquired using a DP50 camera (Olympus).

V.4.8. RNA extraction

The SV total RNA Isolation System kit (Promega) was used according to the manufacturer instructions to extract the total RNA from biopsies of paired normal and tumor tissue preserved in RNAlater and cells from FR10-CMT cell line.

V.4.9. Quantitative PCR (RT-qPCR).

As previously described in in chapter II.4.2 and Raposo *et al.* 2016,²⁴⁶ the analysis of relative expression of *18S*, *ESR1*, *ERBB2*, *PGR*, *DICER1*, *SOX4*, *FADD*, *VEGFA*, *PTEN*, *SNAI2*, *ZEB1* and *ZEB2* mRNA, cDNA was synthesized according the manufacturer instructions of the NZY M-MuLV First-Strand cDNA Synthesis Kit (NZYTech) and RT-qPCR performed in a Rotor.Gene 6000 (Corbett Research) using the mixture of Hot FirePol® Evagreen® qPCR MixPlus (ROX) (Solis Biodyne) with 3mM MgCl₂, 0.2 µM of forward and reverse primers and 2.5 ng cDNA. The primers and reaction conditions used for RT-qPCR are described in Table II.1 and Table II.2 from chapter II and in Raposo *et al* 2016. (Tables S1 and S2, respectively).²⁴⁶ Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method,^{212, 213} using RNA 18S and relative expression of the same gene in the normal tissue as controls.

For analysis of the miRNA expression, cDNA was first synthesized with Exiqon's Universal cDNA synthesis kit II, according to the manufacturer procedure, and a RT-qPCR mixture was prepared according to instructions of the ExiLent SYBR® Green Master Mix (Exiqon) using individual microRNA LNA™ PCR primer sets for miR 16-5p, miR-21-5p, miR-24-3p, miR-29b-3p, miR-124-3p, miR-155-5p, miR-200c-3p and U6 snRNA (Exiqon). RT-qPCR was performed in a LightCycler 480 (Roche Diagnostics) and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method,^{212, 213} and U6 snRNA and relative expression of the same miRNA in the normal tissue as controls.

A significant differential expression was considered when $2^{-\Delta\Delta Ct}$ values were 2-fold higher ($2^{-\Delta\Delta Ct} > 2$) or 2-fold lower ($2^{-\Delta\Delta Ct} < 0.5$).

V.4.10. Total protein extraction

After FR10-CMT and MCF7 (ATCC HTB-22) cells lines reached 80 % confluence in a 75 cm² vented tissue flask, cells were scrapped in PBS, centrifuges at 1500 xg for 5 min, washed 3 times with PBS and finally resuspended in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 8, 0, 5 mM EDTA, 2 % (v/v) NP-40, 1x phosphatase inhibitor (PhoStop, Roche), 1x protease inhibitor (cOmplete Mini, Roche), 1 mM PMSF and 0.1 % (w/v) DTT. Total cell proteins were obtained after 1h incubation on ice and centrifugation for 15 min at 5.000 xg to remove cell debris. Protein concentrations were determined using Pierce 660 nm protein assay kit (Thermo Scientific) according to manufacturer instructions.

V.4.11. Western blot

An amount of 50 µg of protein was first separated in a 10 % polyacrylamide gel by Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a 0.45 µm nitrocellulose membrane (GE Healthcare). After blocking with 5 % (w/v) milk solution in TBST (Tris buffered saline with 0.1 % (w/v) Tween-20 (Sigma)), the blots were incubated according to the manufacturer instructions with the following primary antibodies: anti-ERα (Estrogen Receptor alpha, ref. SAB4500810, Sigma), anti-HER2 (Human Epidermal Growth Factor Receptor 2 homolog, ref. SAB4500789, Sigma), anti-p-ERK1/2 (phosphorylated Extracellular Signal-Regulated Kinases 1 and 2, ref. sc-101761, Santa Cruz Biotechnology), anti-E-cadherin (ref. WH0000999M, Sigma), anti-Vimentin (ref. V6389, Sigma), anti- EPCAM (Epithelial Cell Adhesion Molecule, ref. SAB4200473, Sigma), anti-P53 (ref. SAB1404483, Sigma), anti-CD44 (ref. SAB1402714, Sigma) and anti-β-actin (ref. A5441, Sigma), which was used as an endogenous control. Membranes were washed 3 times with TBST for 5 min and incubated with appropriate secondary antibody conjugated with horseradish peroxidase, HRP (ref. 7074 and 7076, Cell

Signaling Technology). Blots were then treated with WesternBright ECL (Advansta) according to the manufacturer procedure and the signal was visualized in a GelDoc imager (Bio-Rad) or with Hyperfilm ECL (GE Healthcare).

V.4.12. Chemotherapeutic compounds

Cisplatin 1 mg/mL stock solution in 0.9 % (v/v) NaCl (Teva Parenteral Medicines, Inc. Teva Pharmaceuticals) was kept at room temperature according to the manufacturer's instructions. Doxorubicin hydrochloride (Sigma) 5 mg/mL stock solution was made in DMSO (Sigma) and kept at 4 °C as recommended by the manufacturer.

TS262 and TS265 compounds and NanoTS262 and TS265 vectorizations were synthesized as previously described in Silva *et al.* 2013 and in chapter III.4 of this thesis.²⁴⁸

V.4.13. Cell viability assays in presence of cisplatin and doxorubicin

The half maximum inhibitory concentration (IC₅₀) of cisplatin and doxorubicin in FR10-CMT cells was calculated as previously described for FR37-CMT.²⁴⁶ Briefly, FR10-CMT cells were let to adhere for 24h in 96 well tissue plate (VWR) in a concentration of 10.000 cells per well. The medium was then replaced by fresh medium supplemented with serial dilutions of doxorubicin and cisplatin. The respective solvent, DMSO and 0.9 % (w/v) NaCl, was also applied for control purposes. After 48 h incubation at 37 °C, 5 % (v/v) CO₂ and 99 % (v/v) relative humidity, the cell viability was measured using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer procedure. The percent of cell viability was calculated

relatively to the control samples and the IC₅₀ determined using GraphPad Prism vs 6.01 software (GraphPad software Inc.). These assays were repeated at least 3 times.

V.4.14. Statistical analysis

All data was expressed as mean $\pm$ SD from at least three independent experiments. Statistical analysis was performed using the GraphPad Prism v6.01 software. Data was considered with statistical significance when p-value < 0.05.

V.5. Results and discussion

V.5.1. Establishment of FR10-CMT cell line

In this work a novel CMT cell line able to grow in DMEM-FBS-PenStrep, one of the most common cell culture media was established. Together with FR37-CMT, this cell line may contribute to the understanding of the cellular and molecular mechanisms involved in canine mammary cancer progression, and for the development of new therapies that are still scarce for CMTs.

The novel FR10-CMT cell line is derived from a primary CMT mammary tumor. However, there is not a histopathological study of the CMT since it was lost during processing.

FR10-CMT is a monoclonal cell culture with a doubling time of 21 hours. At the morphological point of view, it is characterized as adherent cells with a cuboid shape (Figure V.1). When confluence is reached they occupy about 80-90% of the surface of the tissue culture flask, forming gaps with around 450 μ m diameter (Figure V.1, C). From that point onward, the

cells continue to grow on top of each other never fully occupying the total surface of the tissue culture flask.

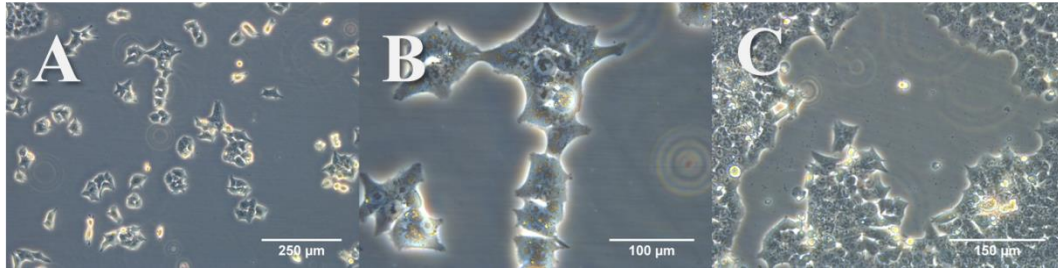


Figure V.1 - Representative images of adherent FR10-CMT cells. Adherent cells have a cuboid shape (A, B). When reach a higher confluence, they never occupy the total surface of the tissue culture flask (C). Amplification: A, 100X; B, 600X; C, 400X.

FR10-CMT cells are morphologically different from the FR37-CMT cell line described earlier in chapter II.5.1 and in Raposo *et al.* 2016.²⁴⁶ The FR10-CMT cells are cuboid while FR37-CMT cells are stellate/spindle shaped and FR10-CMT cells cannot occupy the whole surface of tissue culture flask while FR37-CMT cells can. FR10-CMT also has a slight higher doubling time (21 h vs 17 h) when compared to FR37-CMT cell line.

Chromosome counts made from the preparations of chromosomes revealed that the nodal number of chromosomes was 78 (Figure V.2), confirming the canine origin of the cell line.

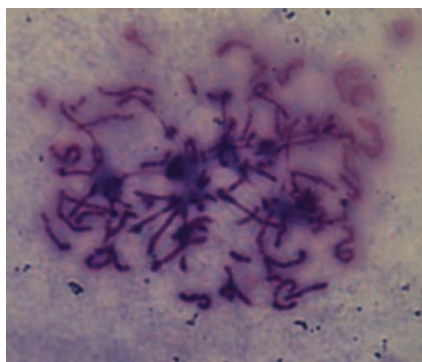


Figure V.2 - Representative image of chromosomes preparation of FR10-CMT cells with Giemsa staining. Amplification: 1000X.

V.5.2. Loss of contact inhibition and invasion ability of FR10-CMT

The assessment of the loss of inhibition of growth by contact as well as their invasion ability was performed by clonogenic assays, including the soft agar colony formation assay and collagen colony formation assay. While, after 5 days of incubation it was possible the visualisation of spherical colonies in soft agar (Figure V.3), that increased size until the 14th day of the experiment (Figure V.3). In collagen it was only possible the visualization of microscopic colonies only after 14 days of incubation (Figure V.3). Despite showing polarized growth, it was not observed any relevant tri-dimensional structures formed in collagen by FR10-CMT cell line.

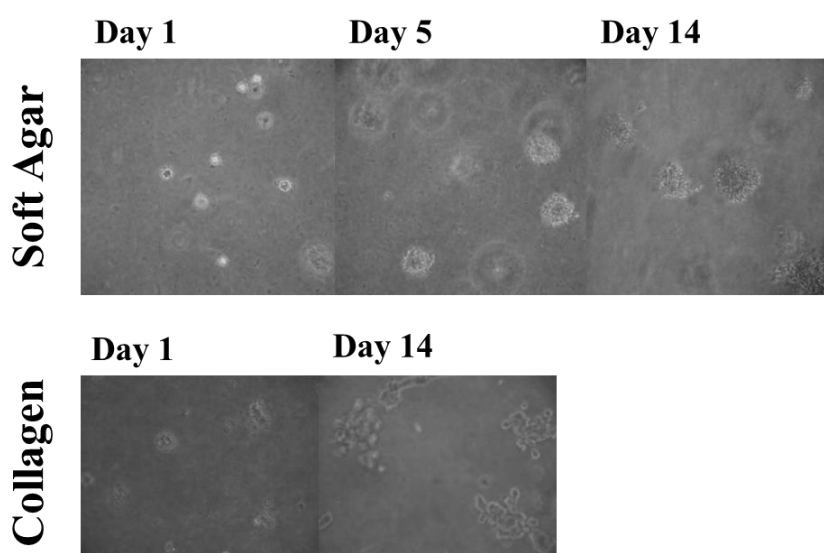


Figure V.3 - Representative images of the time course of clonogenic assays for FR10-CMT cell culture. Soft agar colony formation assay and collagen colony forming assay. FR10-CMT cells were able to grow in both soft agar and in collagen. Despite some cellular polarization, the cells growing in collagen did not form any recognizable tri-dimensional structures. Amplification 40x.

FR10-CMT cell growth in collagen displayed some polarity, a feature not exhibited by FR37-CMT cell line described in chapter II.5.2 and in Raposo *et al.* 2016 can (Figure II.6 and Figure V.3).²⁴⁶ However, FR10-CMT cells are unable to remodel the collagen matrix as FR37-CMT cells can.

Following an incubation of 5 days, FR10-CMT cell culture was able to grow on top of a fibroblast monolayer and reach 80 % confluence after an initial inoculation of 2×10^3 cells cm^{-2} . Cells formed clusters upon of fibroblasts (Figure V.4), suggesting that these malignant cells have lost contact inhibition.

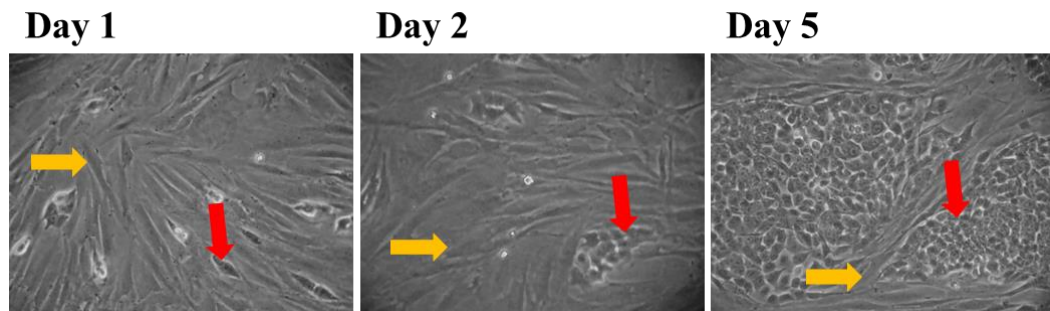


Figure V.4 - Representative images of FR10-CMT cell line growth on top of a human fibroblast monolayer. Yellow arrows indicate fibroblast cells and red arrows indicate FR10-CMT cells. Amplification: 40X.

At the end of the co-culture of FR10-CMT and fibroblast experiment, it was possible to observe fibroblasts surrounding the clusters of FR10-CMT cells. This is quite different from the observed in the co-culture experiment with FR37-CMT cells where fibroblasts were no longer observed at day 5 (Figure II.7 and Figure V.4).

The FR10-CMT migration ability was also evaluated by using the wound healing assay, which revealed that, after 24h, a wound remission percentage of $31.9\% \pm 7.2$ was attained (Figure V.5).

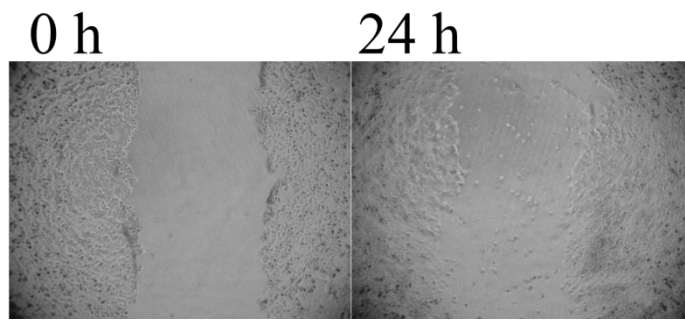


Figure V.5 - Representative images of wound healing assay after 0 h and 24 h of scratch. After 24 h, a remission percentage of $31.9\% \pm 7.23$ (mean $\pm$ SD) was observed Amplification: 40X.

FR10-CMT cells possess a lower remission rate compared to the FR37-CMT cell line (31.9% vs 79.2%) which indicates a lower migration potential for FR10-CMT (Figure II.8 and Figure V.5).²⁴⁶

V.5.3. Tumorigenicity of FR-10 Cells in Nod/SCID mice

To evaluate the FR10-CMT tumorigenic capabilities, 10^6 cells were subcutaneously inoculated at the scruff of the neck of 3 NOD-SCID female mice. The mice developed tumors at the site of injection, and 2 out three developed 2 separate masses at the place of injection. All mice were euthanized 9 weeks after injection.

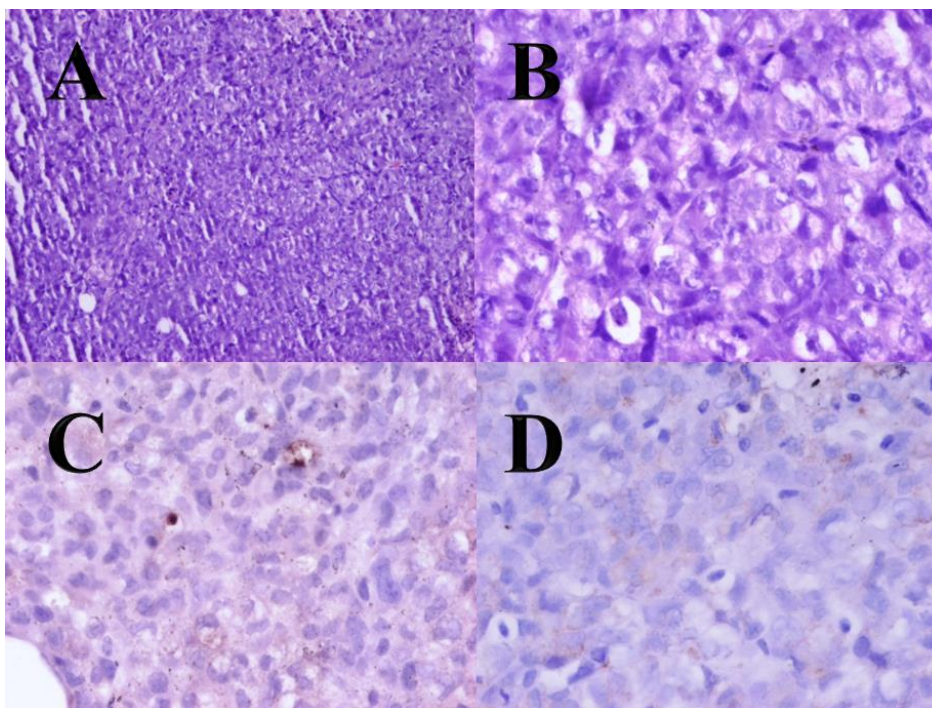


Figure V.6 – Representative images of a mouse tumor xenograft stained with HE (A, B). and immunostained for vimentin (C) and cytoplasm cytokeratins (D). Tumor is a solid mass of mass polygonal and ovoid shaped cells with frequent mitotic figures. No immunostaining was observed for for vimentin or for cytoplasm cytokeratins. Amplification of A) 100XB); C) and D) 400X.

The 5 tumor xenografts were histological classified as anaplastic, i.e. without a defined tridimensional shape, composed of ovoid and polygonal shaped cells growing on solid sheets. Cells displayed big nuclei and frequent mitotic figures. The shape of the tumor xenografts is consistent with the observed morphology of FR10-CMT cells cultured *in vitro* (Figure V.1 and Figure V.6). This has also been observed with the tumor xenografts derived from inoculation of NOD-SCID mice with FR37-CMT cells (chapter II.5.3).²⁴⁶

The immunohistochemistry study of a representative tumor xenograft revealed no staining for vimentin or cytoplasm cytokeratins (Figure V.6).

V.5.4. Molecular characterization of FR10-CMT cell line

The expression of 11 relevant mammary cancer-related genes, 7 microRNAs (miRNAs) and the presence of 9 proteins was evaluated in order to characterize FR10-CMT cell line as already described in chapter II.5.4 and in Raposo *et al.* 2016.²⁴⁶ The gene expression was evaluated for both the cell line and for the original tumor, using the normal paired tissue as control.

Analyzing the expression changes displayed in Figure V.7, it is possible to observe two different tendencies. The FR10-CMT tumor has an overall increase in the expression of the analyzed mRNAs (8 out of 11 up-regulated) and the FR10-CMT cell line has general decrease in the expression of the same mRNAs (6 out 11). Indeed, the *ERBB2*, *VEGFA*, *PTEN*, *SNAI2*, *ZEB1* and *ZEB2* mRNA are overexpressed in the FR10-CMT tumor while are downregulated in the FR10-CMT cell line. Noteworthy, the mRNAs for two of the three main receptors implicated in carcinogenesis of mammary gland, *ERBB2* and *PGR*, were overexpressed in the original tumor while in the FR10-CMT cell line, *ERBB2* was down-regulated and *PGR* was expressed at the same levels of the normal mammary gland. The only gene up-regulated in the cell line was *SOX4* which was statistically significantly higher than in the original tumor.

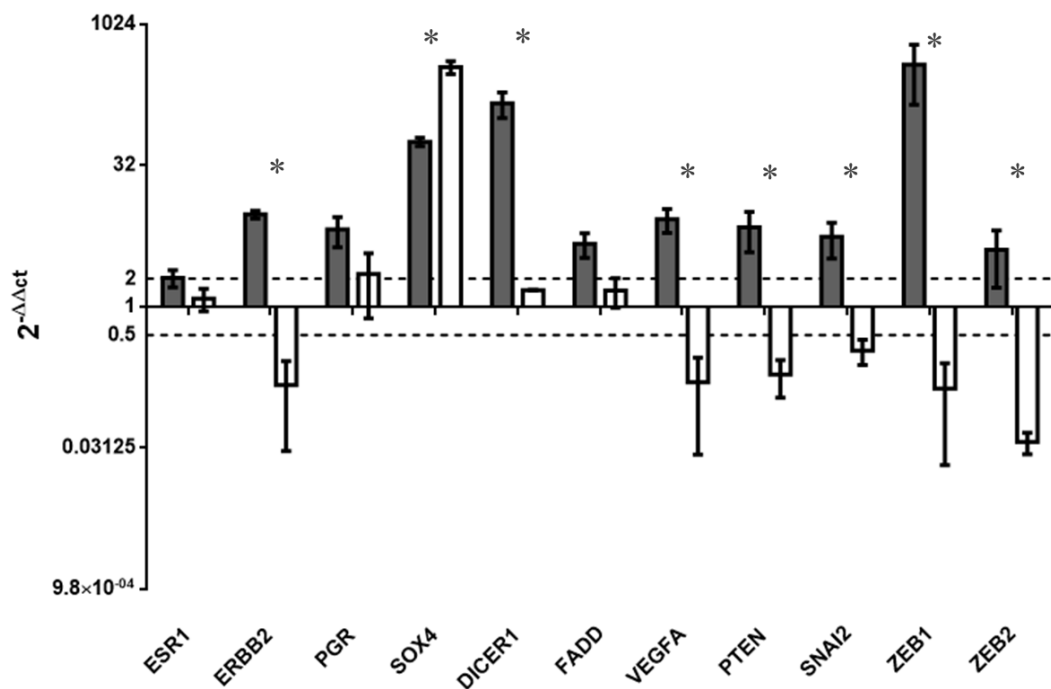


Figure V.7 - Relative expression of genes involved in breast and mammary tumorigenesis in the tumor that originated the cell line (grey bars) and the FR10-CMT cell line (white bars) normalized to the expression in matched normal mammary tissue. The dotted lines mark the threshold that considers altered expression: values 2 fold higher ($2^{-\Delta\Delta C_t} > 2$) or 2-fold lower ($2^{-\Delta\Delta C_t} < 0.5$). Described values are the mean of at least 3 independent experiments with standard deviation. * significant expression changes, $p\text{-value} < 0.05$, between FR10-CMT cell line values relative to the original tumor.

Analysis of the miRNA expression (Figure V.8) shows that the original tumor has a general decrease in 6 out of 7 miRNA while the FR10-CMT cell line only 3 miRNA were under-regulated. The FR10-CMT also showed an up-regulation of miR-24 and miR-200c. However, only the expression levels of miR-24, miR-155 and miR-200c were statistically different from the original tumor and the FR10-CMT cell line.

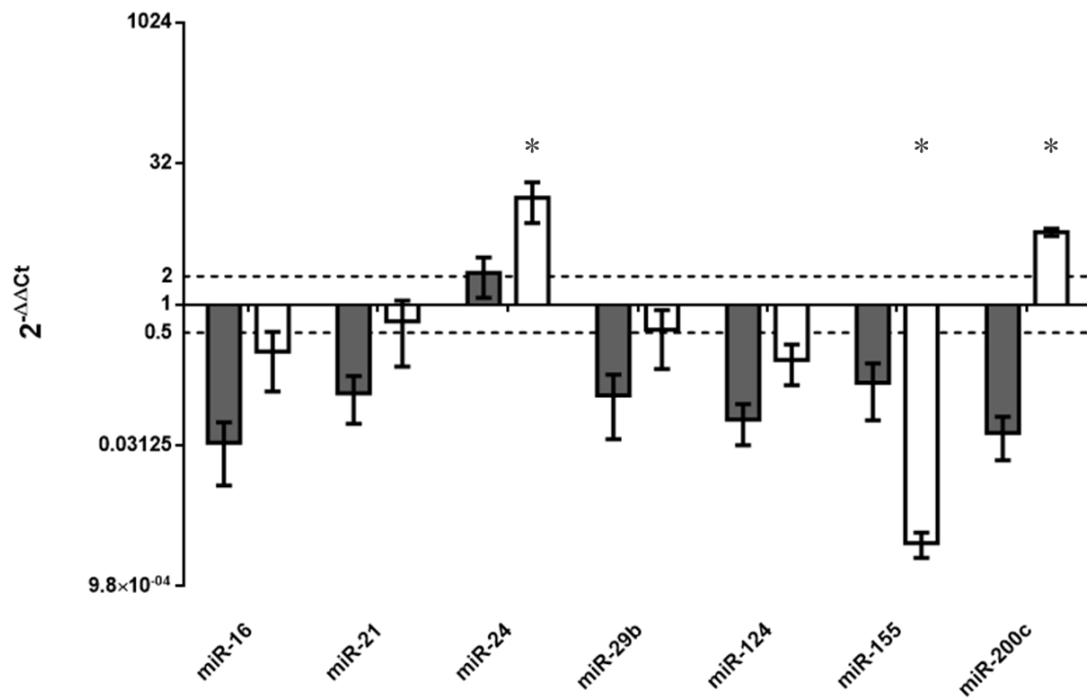


Figure V.8 - Relative expression of miRNAs involved in breast and mammary tumorigenesis in the tumor that originated the cell line (grey bars) and the FR10-CMT cell line (white bars) normalized to the expression in matched normal mammary tissue. The dotted lines mark the threshold that considers altered expression: values 2-fold higher ($2^{-\Delta\Delta Ct} > 2$) or 2-fold lower ($2^{-\Delta\Delta Ct} < 0.5$). Described values are the mean of at least 3 independent experiments with standard deviation. * significant expression changes, p -value <0.05 , between FR10-CMT cell line values relative to the original tumor

Results suggest that at the molecular level, the FR10-CMT cell line is very different from the tumor from which the line was derived (Figure V.7 and Figure V.8). Statistically significant differences were found in 11 out of the 18 mRNAs and miRNAs analyzed between the FR10-CMT cell and its original tumor. These overall differences were not observed between the FR37-CMT line and the respective original tumor as it can be seen in chapter II.5.4 and in Raposo *et al.* 2016 although minor differences were observed.²⁴⁶

This may be due to tumor heterogeneity and clonal selection during the immortalization process. Either the cell line is derived from a small sub-population of cells, within the tumor, that are different from the majority of cells within the tumor or the immortalization process selected a clone of cells that spontaneously mutated *in vitro* with proliferative advantage.

In order to obtain a more detailed molecular characterization of the FR10-CMT cell line, the expression levels of relevant protein biomarkers, such as ER α , HER-2, PGR, *etc*, were analyzed by Western-Blot. In Figure V.9 it is possible to observe that FR10-CMT cells did not express detectable levels of estrogen receptor alpha (ER α), phosphorylated ERK1 and phosphorylated ERK2 MAPKinases.

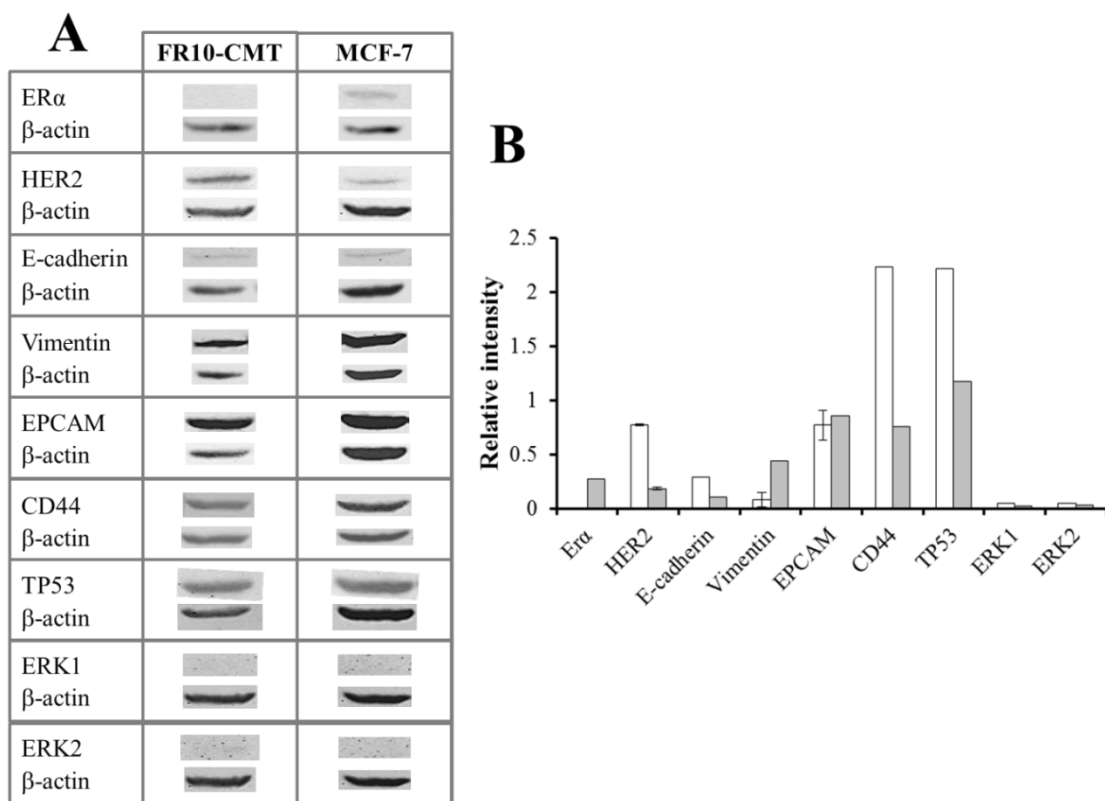


Figure V.9 - Proteins expressed in FR10-CMT and MCF-7 cell lines. A- Representative images of western blot results. β -actin was used as internal loading control. B. Relative intensity values (normalized to the endogenous control β -actin) of each protein in FR10-CMT (white bars) and MCF-7 (grey bars) cell lines.

Furthermore, when compared to MCF7, FR10-CMT cells express similar levels of EPCAM, have higher abundance in HER-2, E-cadherin, CD44 and p53 proteins and lower abundance of vimentin. The levels of phosphorylated ERK1 and ERK2 kinases expressed in FR10-CMT could indicate that there is not an activation of the ERK1/2 signaling pathway in FR10-CMT cell line.

Comparing FR10-CMT and FR37-CMT cell line, described in II.5.4 and in Raposo *et al.* 2016,²⁴⁶ it is possible to observe that, FR10-CMT expresses less ER α and does not have an activation of the ERK1/2 MAPKinase signaling pathway. This is an indication that proliferation of FR10-CMT cells is dependent of other signaling pathways, such as the PI3K/AKT/mTOR, KRAS or WNT pathways implicated in CMT proliferation. Both cell lines express similar levels of HER-2 and EPCAM. FR37-CMT expresses higher levels of vimentin than FR10-CMT and a lower abundance of CD44 and p53 proteins.

V.5.5. Effect of cisplatin and doxorubicin in the cell viability of FR10-CMT cell line

The effects of two widely used chemotherapeutic compounds on the FR10-CMT cell line was evaluated by MTS, as previously described in II.4.17 and in Raposo *et al.* 2016.²⁴⁶

As it can be seen in Figure III.4, the IC₅₀ for cisplatin is >50 μ M and for doxorubicin is 3.96 μ M.

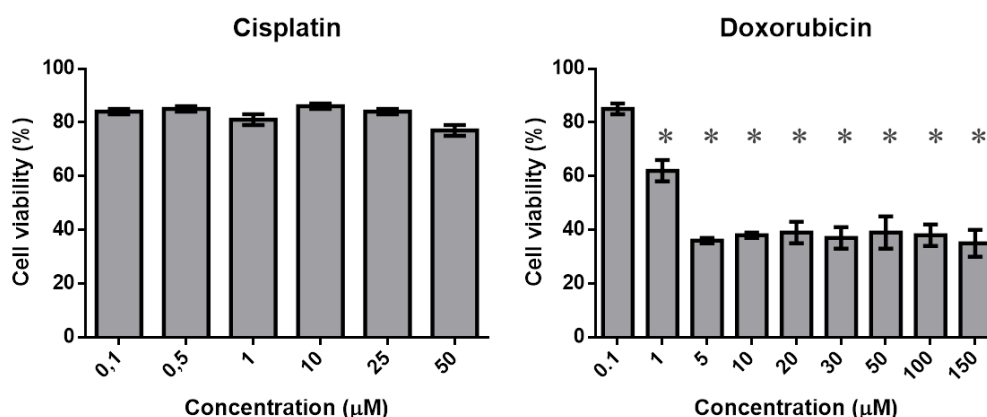


Figure V.10 - Cell viability of FR10-CMT cell line after 48 h of exposure to different concentrations of cisplatin and doxorubicin. The results are expressed as mean $\pm$ SD to controls from at least three independent experiments. *P-value < 0.05 relatively to the % viability of 0.1 μ M of doxorubicin. * p-value<0.05 relative to the cell viability percentage of the 0.1 μ M concentration.

As it can be seen in II.5.5 and in Raposo *et al.* 2016,²⁴⁶ the FR10-CMT and FR37-CMT cell lines display the same tolerance to cisplatin (IC_{50} >50 μ M) and similar sensitivities to doxorubicin (IC_{50} 3.96 μ M and 5.3 μ M, respectively).

V.5.6. Effect of TS262 and TS265 in the cell viability of FR10-CMT cell line

Two novel Co(II) and Zn(II) compounds bearing 1,10-phenantroline-5,6-dione ligands, TS262 and TS265 respectively, have exhibited high cytotoxic activity in FR37-CMT cell line with lower IC_{50} concentrations (1.05 μ M for TS262 and 1.39 μ M for TS265) when compared to doxorubicin (IC_{50} =5.3 μ M) and cisplatin (IC_{50} >50 μ M), as seen in chapters II.5.5 and III.5.2 and Raposo *et al.* 2016.²⁴⁶ For this reason, the cytotoxic effect of both compounds on FR10-CMT cell line were also evaluated (Figure V.11).

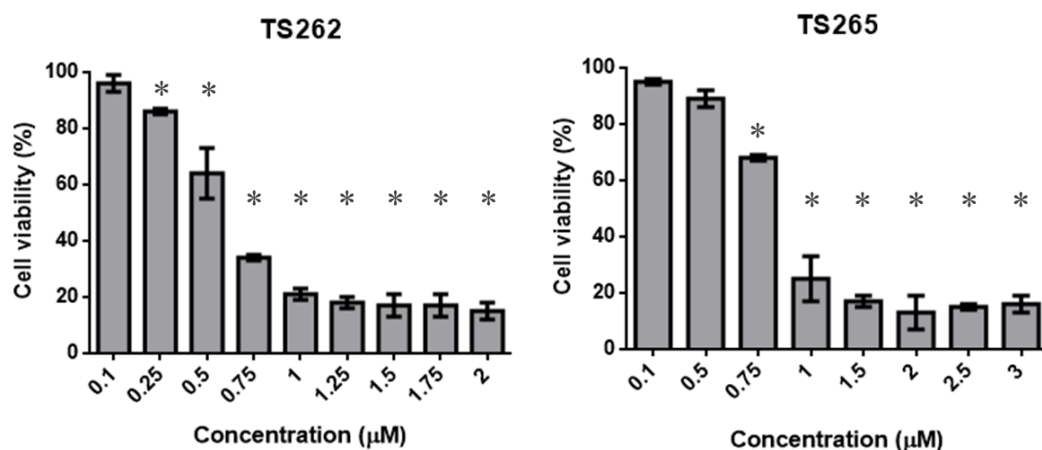


Figure V.11 - Cell viability of FR10-CMT cell line after 48 h of exposure to different concentrations of TS262 and TS265. The results are expressed as mean $\pm$ SD to controls from at least three independent experiments. *P-value < 0.05 relatively to the % viability of 0.1 μ M of doxorubicin. * p-value<0.05 relative to the cell viability percentage of the 0.1 μ M concentration

The IC_{50} in the FR10-CMT cell line for TS262 is 0.55 μ M and for TS265 the IC_{50} is 0.8 μ M. Comparing these results with the IC_{50} obtained for FR37-CMT, it is possible to observe that, with exception of cisplatin, the FR10-CMT cell line is more sensitive to doxorubicin than FR37-CMT (3.96 μ M and 5.3 μ M) and to both metal compounds tested, as seen in chapters II.5.5 and III.5.3, Figure V.10 and in Raposo *et al.* 2016.²⁴⁶

V.5.7. Effect of NanoTS262 and NanoTS265 in the cell viability of FR10-CMT cell line

The nanovectorization of TS262 (nanoTS262) and TS265 (nanoTS265) in functionalized gold nanoparticles with BSA (Au@PEG@BSA) were characterized in chapter III.5.1. They were demonstrated to greatly reduce FR37-CMT cell viability when exposed to IC_{50} equivalent concentrations of TS262 and TS265 (chapter III.5.4). The effect of these vectorizations on the FR10-CMT was also evaluated, using Au@PEG@BSA nanoparticles as controls (Figure V.12).

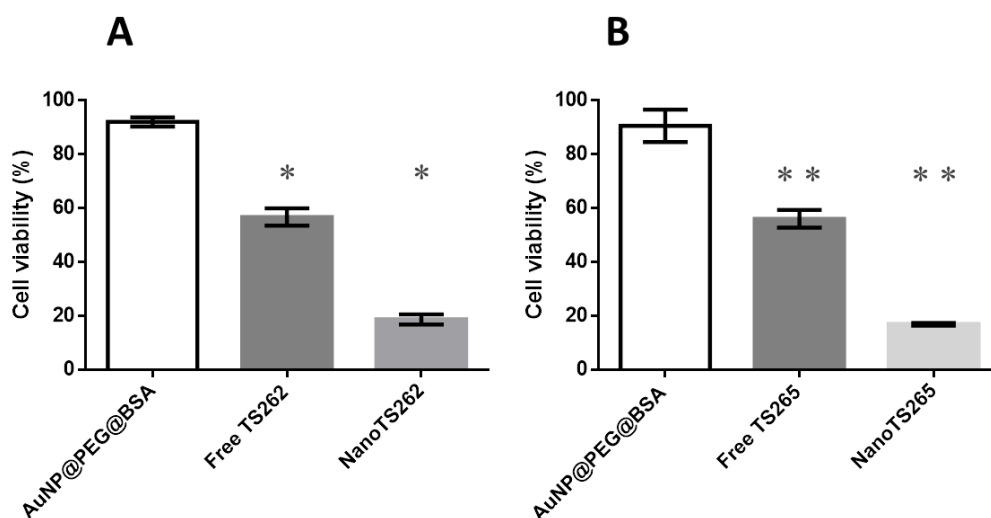


Figure V.12 – Cell viability of FR10-CMT cell line after 48 h exposure to A) 2.8 nM AuNPs@PEG@BSA, 0.55 μ M free TS262 and equivalent concentration of AuNPs@PEG@BSA-TS262 (NanoTS262) to achieve 0.55 μ M of TS262 (1.5 nM of particles), B) 2.8 nM AuNPs@PEG@BSA, 0.80 μ M of free TS265 and equivalent concentration of AuNPs@PEG@BSA-TS265 (NanoTS265) to achieve 0.80 μ M of TS265 (2.8 nM of particles). Results are expressed as mean $\pm$ SD to controls from at least three independent experiments. * p-value<0.05 relative to the cell viability percentage of the 1.5 nM concentration and ** p-value<0.05 relative to the cell viability percentage of the 2.8 nM concentration.

It is possible to observe in Figure V.12 a significant decrease in the viability of FR10-CMT exposed to IC₅₀ equivalent concentrations of nanoTS262 and nanoTS265 (18.8 % and 17.0 %, respectively) when compared to FR10-CMT cells exposed to free TS262 and TS265. The AuNPs@PEG@BSA nanoparticles had no significant effect on the cell viability. This effect has already been seen in the FR37-CMT cell line (chapter III.5.4) and further supports a role for these novel nanoconjugations for the treatment of mammary cancer in dogs.

V.6. Discussion

FR10-CMT is a new cell line derived from a primary CMT that grows in a widely used and simple culture medium, has a higher doubling time than FR37-CMT cell line (21h vs 17h).²⁴⁶ The chromosome counts confirmed the canine origin of the cell line (Figure V.2). FR10-CMT cells

originated xenograft tumors in NOD-SCID female mice. By histopathology analysis the xenograft tumors were classified as solid anaplastic tumors, which is in accordance with the shape of FR10-CMT cells and the characteristic growth in clusters displayed by the cells *in vitro* (Figure V.1 and Figure V.4). The tumor xenograft analyzed by immunohistochemistry did not stained positive for vimentin or cytoplasm cytokeratins (Figure V.6). These observations can be correlated with the levels of E-cadherin and vimentin detected by western-blot (Figure V.9). Despite these observations, FR10-CMT cells were shown to express vimentin (Figure V.9). Comparing these results with those obtained for FR37-CMT cells, which had detectable vimentin expression in the tumor xenografts, chapter II.5 and Raposo *et al.* 2016,²⁴⁶ the relative levels of vimentin were much higher in the FR37-CMT cell line. The lack of vimentin staining in the FR10-CMT tumor xenografts may be due to the low levels of vimentin present in cells.

The molecular characterization of the FR10-CMT cell line revealed that *ESR1* mRNA levels were the same as in normal matched mammary tissue, however, western-blot failed to reveal the presence of ER α receptor (Figure V.7 and Figure V.9). These results are intriguing and may be due to a post-translation regulation of *ESR1* or to an alteration of the tri-dimensional structure of epitope recognized by the antibody used for Western-blot. The receptor HER-2 was found in FR10-CMT cells although the expression analysis revealed a down-regulation of *ERBB2*.

The gain of expression of vimentin and CD44 marker and the downregulation of E-cadherin have been associated with the acquisition of stemness properties and EMT.⁵⁹ However, the FR10-CMT cell line does not display the molecular features associated with EMT. In fact, the overexpression of miR-200c associate with the downregulation of its direct targets *ZEB1* and *ZEB2* (Figure V.7 and Figure V.8) is associated with the repression of EMT.^{221, 233} Supporting this hypothesis, the EMT associated transcriptional factor *SNAI2* is also downregulated and there is no evidence of the activation of the ERK1/2 MAPKinase signaling pathway (Figure V.7).^{147, 149, 222}

The decrease expression of *PTEN*, a phosphatase that negatively regulates the Akt/mTOR signaling pathway, is an indicator that the proliferation potential of FR10-CMT cells may be due to the activation of Akt kinase.^{63, 103} The downregulation of *SNAI2* and *PTEN* has also been seen as part of the molecular alterations in canine mammary derived cell lines exhibiting high basal Wnt signaling.²⁶³ The downregulation of these two genes were not correlated with an increased expression of the repressors, miR-124 and miR-21 respectively (Figure V.8).^{222, 264} The downregulation of these miRNAs may be explained by the already decreased levels of their target genes.

The inhibition of mir-16 and the up-regulation of miR-24 are a common event in CMT and HBC and are also observed in FR10-CMT cell line.^{160, 240} The downregulation of miR-155, however, in FR10-CMT cells is not in accordance with what it is described in the literature.^{160, 240}

SOX4 is overexpressed in FR10-CMT cell but *DICER1*, one of its direct transcriptional targets remains constant in FR10-CMT cell line (Figure V.7). It has been shown that the metastatic behavior of cancer is connected with *DICER1* up-regulation mediated by *SOX4* transcriptional activator.²³⁸ The up-regulation of *SOX4* has also been shown to be dependent of the TGF- β , WNT and Notch signaling pathways.^{265, 266} However, since neither *DICER1* is overexpressed nor EMT is induced, it is not possible to infer the role of the *SOX4* in the molecular mechanisms inducing proliferation in FR10-CMT cells.

Comparing FR10-CMT and FR37-CMT, described in II.5.4 and in Raposo *et al.* 2016,²⁴⁶ cell lines it is possible to observe that both have some stemness characteristics in their cells but FR10-CMT lack the EMT induction seen in the FR37-CMT cell line. It is also corroborated with by the general morphology of the cells; while FR10-CMT cells are cuboid shaped, the characteristic shape of epithelial cells, the FR37-CMT cells have a fusiform shape, a form more common to mesenchymal cells.^{147, 149}

The FR10-CMT cell line is highly tolerant to cisplatin ($IC_{50} > 50 \mu M$) but much more sensitive to doxorubicin ($IC_{50}=3.96 \mu M$). Despite similar, the FR10-CMT cell line is slightly more sensitive to doxorubicin than FR37-CMT line ($IC_{50}=5.3 \mu M$) as it can be seen in chapter II.5.5 and in Raposo *et al.* 2016.²⁴⁶

Two Co(II) and Zn(II) compounds bearing 1,10-phenantroline-5,6-dione ligands, TS262 and TS265 respectively, and two novel vectorizations of these compounds in functionalized gold nanoparticles, nanoTS262 and nanoTS265, shown to be promising in the treatment of CMT, as seen in chapter III.5 were tested in FR10-CMT cells. The IC_{50} for TS262 is $0.55 \mu M$ and for TS265 the IC_{50} is $0.80 \mu M$. This cell line has lower IC_{50} for both compounds than FR37-CMT ($IC_{50}=1.05$ and $IC_{50}=1.39$ for TS262 and TS265 respectively). Also, the treatment of FR10-CMT cells with IC_{50} equivalent concentrations of nanoTS262 and nanoTS265 significantly reduced cellular viability demonstrating the interesting potential of these nanoformulations for future *in vivo* studies aiming to improve CMT treatment.

V.7. Conclusions

FR10-CMT cell line is a new model for the study of canine mammary tumorigenesis that displays some stemness characteristics (expression of CD44 marker, vimentin and reduced levels of e-cadherin) that are not correlated with canonical induction of EMT as does the recently described FR37-CMT cell line in chapter II and in Raposo *et al.* 2016.²⁴⁶ Also, it displays high tolerance to cisplatin and doxorubicin, being a good model for the study of the resistance to chemotherapy exhibit by CMTs.

FR10-CMT cells were also more sensitive to treatment with TS262 and TS265 than to cisplatin and doxorubicin, as already revealed in chapter III.5.2 for FR37-CMT. FR10-CMT cell

line is more sensitive than FR37-CMTs exhibiting lower IC_{50} for both compounds. Also the nanovectorization systems, nanoTS262 and nanoTS265, confirmed their huge potential as drug delivery agents in the treatment of CMTs by significantly reducing the viability of FR10-CMT as already shown for FR37-CMT (III.5.4).



VI. Molecular typing of grade III Canine Mammary Tumors can distinguish metastatic from non-metastatic tumors

Disclaimer: Results and data presented in this chapter are in preparation for publication in peer review journals and were partially published in:

LR Raposo, J. Henriques, P Faísca, M Alves, J Correia, AR Fernandes. “Molecular characterization of canine mammary tumours: the role of miRs and mRNAs as biomarkers in the metastatic transition.” European Society of Veterinary Oncology Annual Congress, ESVONC 2014, 22nd - 24th May 2014 Vienna, Austria.

LR Raposo, S Santos, J Henriques, M Alves, P Faísca, A Beselga, J Correia, AR Fernandes. “Insights into the molecular basis of canine mammary cancer: the use of miRs in the characterization of canine mammary tumors.” European Society of Veterinary Oncology Annual Congress, ESVONC 2013, 30th May – 1st June 2013 Lisbon, Portugal.

VI.1. Abstract

The expression of miRNAs has been increasingly used in human cancer medicine, but their value as biomarkers in canine mammary tumors, CMTs, is yet to be established. Analyzing the expression levels of 10 miRNAs and 11 mRNAs by Principal Component Analysis, it was possible to distinguish metastatic from non-metastatic grade III CMTs and at the same time group specific expression patterns. This clustering of grade III CMTs allowed to observe particular sets of expression in non-metastatic CMTs, including increased expression of *DICER1*, *VEGFA* and *ZEB1* and in metastatic CMTs, the over-expression of miR-155. The common expression levels of *DICER1* and *ESR1* mRNA and miR-155 in tubulopapillary and in metastatic CMTs, suggested a correlation between these tumors. Despite the low number of samples used in this work, this study opens a new avenue for CMT classification based on a molecular markers profiling.

VI.2. Keywords

Grade III Canine Mammary Tumors; Metastatic; mRNA; miRNAs; Principal component analysis.

VI.3. Introduction

Canine mammary tumors, CMTs, are the most common type of tumors found in female dogs.⁴⁴ The differential expression of miRNAs has been associated with tumor suppressive and oncogenic patterns in what concerns CMT phenotype.^{160, 161}. However, the clinical value of these miRNAs, for instance as cancer biomarkers in dog has not been completely proved. Although little information is available in CMT oncogenesis, it is possible that alterations in expression of miRNAs may allow their use as biomarkers of CMT progression to metastasis. Since there is not a clear sequence of molecular events in the progression of mammary tumors towards malignancy and metastatic behavior, this study was performed in five CMTs with high malignancy grade, grade III, with (2) and without (3) local metastasis in the lymph nodes.

VI.4. Materials and Methods

VI.4.1. Sample collection

The tumors in this study are identified by their histopathological classification and the presence of metastasis at the local lymph nodes. More information about the CMTs is available in Table VI.1. The patient owners gave written informed consent before surgery samples were taken. From each female dog both mammary tumors and normal adjacent healthy tissue were obtained for comparison. Tissue samples were preserved at -80°C until used for RNA extraction. The study was approved by the local ethical committee (Comissão Ética Faculdade Medicina Veterinária, Universidade Lusófona).

Table VI.1 - Summary of canine mammary tumors information, considering the age and breed of the animal, the location in the mammary chain where the biopsy was removed, the grade of the tumor, histological classification and the presence of metastasis in the lymph node.

Sample	Age	Breed	Location in mammary chain	Grade	Histopathologic classification	Metastasis at the lymph node
S1	12	Estrela Mountain	Left 4	III	Tubular	No
S2	6	Undifferentiated	Left 2	III	Simple Anaplastic	No
S3	8	Boxer and Irish Setter mixture	Left 4	III	Tubular	Yes
S4	13	Undifferentiated	Right 3-4	III	Adenosquamous	Yes
S5	11	Samoyed	Right 1	III	Tubulopapillary	No

Matched normal mammary tissue and CMT biopsies were collected by surgical excision following normal surgical procedures for neoplastic removal. Samples were divided in two pieces, one for histopathological characterization and the other for RNA isolation. For histopathology, the samples were fixed in 10 % (v/v) neutral buffered formalin and processed routinely for paraffin embedding, sectioned at 5 µm and stained with hematoxylin and eosin (HE). Slides were observed in a BX-51 microscope (Olympus) and images acquired using a DP50 camera (Olympus).

VI.4.2. Analysis of mRNA and miRNA expression

Total RNA was extracted from samples using the SV total RNA Isolation System (Promega). RNA integrity was evaluated through RIN parameter by using the Bioanalyzer platform. High quality total RNA (RIN values between 7.6 and 9.4) was used for cDNA synthesis with the Exiqon's Universal cDNA Synthesis Kit II (following the manufacturer instructions) and RT-qPCR was performed using Exiqon's LNA technology with ExiLent SYBR® Green master mix (Exiqon) and individual microRNA LNA™ PCR primer sets for miR 16-5p, miR-21-5p, miR-24-

3p, miR-29b-3p, miR-124-3p, miR-155-5p, miR-199b-5p, miR-200c-3p, miR-203a, let-7a-5p and U6 snRNA PCR primer set (Exiqon) in a LightCycler 480 (Roche Diagnostics). U6 snRNA was used as the endogenous control. The expression levels of 11 mRNAs *ESR1*, *ERBB2*, *PGR*, *SOX4*, *DICER1*, *PTEN*, *FADD*, *VEGFA*, *ZEB1* and *ZEB2* were further investigated in the CMTs and matched normal mammary tissue using expression of *18S* as the endogenous control. For this purpose, cDNA was synthesized with the NZY M-MuLV First-Strand cDNA Synthesis Kit (NZYTech, Lda) accordingly to the manufacturer's instructions and RT-qPCR was performed using HOT FIREPol® Evagreen® qPCR Mix Plus (ROX) (Solis Biodyne) in a Rotor-Gene 6000 (Corbett Research). Primer information and PCR conditions are available in Table VI.2 and Table VI.3, respectively.

Table VI.2 - Primer sequences and amplicon sizes used for canine mRNA quantification by RT-PCR.

Gene	Primer sequences	Size of amplicon (bp)
<i>18S</i>	Forward - 5'- GTAACCCGTTGAACCCATT-3' Reverse - 5'- CCATCCAATCGGTAGTAGCG-3'	151
<i>ESR1</i>	Forward - 5'-CCTTCAGTGAAGCTTCGATG-3' Reverse - 5'-AGAAGGTGGACCTGATCATG-3'	130
<i>ERBB2</i>	Forward - 5'- CAGCCCTGGTCACCTACAA-3' Reverse - 5'- CCACATCCGTAGACAGGTAG-3'	120
<i>PGR</i>	Forward - 5'-TGCAGGACATGACAACACCA-3' Reverse - 5'-CTGCCACATGGTGAGGCATA-3'	310
<i>DICER1</i>	Forward - 5'-CGAGGACTCTTGGCCCAAAT-3' Reverse - 5'-GCCAATTCACAGGGGGATCA-3'	126
<i>SOX4</i>	Forward - 5'-ATGTCCCTGGGCAGTTTCAG-3' Reverse - 5'-GATCATCTCGCTCACCTCGG-3'	282
<i>VEGFA</i>	Forward - 5'-CTTGCCTTGCTGCTCTACCT-3' Reverse - 5'-GTCCACCAGGGTCTCAATGG-3'	144
<i>FADD</i>	Forward - 5'-TGGAGGAGACTGGCTCGTTA-3' Reverse - 5'-GCTCTTCCAGACTCTCAGCG-3'	117
<i>PTEN</i>	Forward - 5'-GCTATGGGGTTTCCTGCAG-3' Reverse - 5'-GCTGTGGTGGATTATGGTCTTC-3'	193
<i>SNAI2</i>	Forward - 5'-CACACTGGGGAGAAGCCTTT-3' Reverse - 5'-CACAGCAGCCAGATTCCTCA-3'	178
<i>ZEB1</i>	Forward - 5'-ACAGTCCGGGGTAATCGTA-3' Reverse - 5'-TGAGTCCTGTTCTTGGTCGC-3'	224
<i>ZEB2</i>	Forward - 5'-ATATGGTGACGCACAAGCCA-3' Reverse - 5'-TTGCAGTTTGGGCACTCGTA-3'	172

Table VI.3 - Amplification conditions used for canine mRNA quantification.

Cycle Steps	Temperature	Duration	N° Cycles
Activation step	95 °C	15 min	1
Denaturation	95 °C	10 sec	45
Annealing	60 °C	10 sec	
Elongation	72 °C	10 sec	

Relative gene expression analysis was performed using the $2^{-\Delta\Delta C_t}$ method,^{212, 213} comparing to the corresponding miRNAs and mRNAs expression levels in healthy mammary tissue from the same animals, and U6 and 18S genes as internal controls for miRNAs and mRNAs expression, respectively. Every experiment was repeated at least 3 times. A Principal Component Analysis, PCA, an unsupervised analysis of variances, was performed using XLSTAT vs2014.5.03 software (Addinsoft), in order to understand if there can be found patterns of variance expression between the data of the grade III CMTs.

VI.5. Results and Discussion

In order to gain maximum information on molecular events mainly implicated in the acquisition of metastasis by CMTs, the expression levels of ten miRNAs, miR-16-5p, miR-21-5p, miR-24-3p, miR-29b-3p, miR-124-3p, miR-155-5p, miR-199b-5p, miR-200c-3p, miR-203a, let-7a-5p) and 11 mRNAs (*ESR1*, *ERBB2*, *PGR*, *SOX4*, *DICER1*, *PTEN*, *FADD*, *VEGFA*, *ZEB1* and *ZEB2*) involved in breast and CMT progression and in the EMT,^{44, 103, 160, 220, 238, 239, 267} were quantified by Real-Time PCR, qRT-PCR from cDNA synthesized from total RNA extracted from tumor and normal tissues (Table VI.4).

Table VI.4 - Expression levels of miRNAs and mRNA for each Grade III canine mammary tumor. Represented values are the mean and standard deviation of at least 3 independent experiments.

	Tubular	Anaplastic	Metastatic Tubular	Metastatic Adenosquamous	Tubulopapillary
miR-16	0.8 ± 0.18	2.4 ± 0.72	5.0 ± 1.00	0.7 ± 0.01	31.2 ± 2.60
miR-21	3.5 ± 0.75	10.0 ± 1.60	4.3 ± 1.6	0.3 ± 0.07	32.6 ± 2.99
miR-24	5.9 ± 0.31	4.7 ± 0.27	8.0 ± 1.20	0.4 ± 0.07	174.8 ± 13.35
miR-29b	3.2 ± 0.76	1.7 ± 0.53	3.1 ± 0.60	0.9 ± 0.07	14.8 ± 2.48
miR-124	0.9 ± 0.08	1.3 ± 0.21	2.4 ± 0.85	1.1 ± 0.28	1.5 ± 0.21
miR-155	0.1 ± 0.07	0.6 ± 0.12	36.9 ± 2.99	6.5 ± 0.81	14.1 ± 4.74
miR-199b	0.3 ± 0.07	2.5 ± 0.38	0.4 ± 0.21	0.4 ± 0.01	13.3 ± 7.74
miR-200c	0.3 ± 0.03	1.6 ± 0.38	1.6 ± 0.20	0.01 ± 0.001	223.6 ± 34.86
miR-203a	0.8 ± 0.20	0.9 ± 0.49	2.7 ± 1.21	1.0 ± 0.37	5.4 ± 0.62
let7a	1.0 ± 0.30	0.7 ± 0.07	0.8 ± 0.19	0.7 ± 0.25	231.1 ± 105.00
<i>ESR1</i>	21.4 ± 11.90	16.3 ± 4.48	0.7 ± 0.11	0.1 ± 0.01	0.02 ± 0.01
<i>ERBB2</i>	1.0 ± 0.43	4.1 ± 2.24	0.2 ± 0.1	3.9 ± 2.39	0.04 ± 0.03
<i>PGR</i>	1.9 ± 1.0	0.06 ± 0.03	0.2 ± 0.15	4.6 ± 1.08	0.005 ± 0.001
<i>SOX4</i>	1.1 ± 0.41	82.4 ± 0.86	0.2 ± 0.12	0.07 ± 0.03	0.55 ± 0.23
<i>DICER1</i>	13.6 ± 6.67	254.0 ± 164	0.08 ± 0.06	0.01 ± 0.008	0.004 ± 0.003
<i>FADD</i>	1.3 ± 1.17	318.6 ± 138.2	0.6 ± 0.47	0.9 ± 0.24	43.2 ± 15.3
<i>VEGFA</i>	10.5 ± 2.71	1.7 ± 0.37	0.7 ± 0.31	0.08 ± 0.03	0.008 ± 0.006
<i>PTEN</i>	1.1 ± 0.46	77.4 ± 70.45	65.2 ± 42.5	0.3 ± 0.12	0.05 ± 0.04
<i>SNAI2</i>	6.1 ± 1.42	2.3x10 ⁵ ± 2.05x10 ⁴	5.7 ± 1.40	0.03 ± 0.02	0.11 ± 0.07
<i>ZEB1</i>	9.6 ± 4.61	0.14 ± 0.04	1.1 ± 0.54	0.2 ± 0.07	1.25x10 ⁻⁶ ± 3.2x10 ⁻⁷
<i>ZEB2</i>	1.7 ± 0.75	3288.4 ± 1786.09	0.2 ± 0.16	0.1 ± 0.03	0.07 ± 0.11

Analysis of expression profile of miRNAs and mRNAs (Figure VI.1, A) allowed the identification of characteristic miR and mRNA expression patterns for each group. In the tubulopapillary CMT is possible to observe that over-expression of miR-24, miR-200c and let-7a are representative of this tumor (Figure VI.1, A). Remarkably, nine out of ten miRNAs analyzed were over-expressed in this tumor sample (Figure VI.1, A), while 9 of the 11 mRNAs evaluated, including *DICER1* mRNA, were under-expressed. This observation is intriguing, because reduced expression of Dicer is usually associated with decreased levels of mature miRNAs.²³⁹ In the same way, it is possible to observe that the increased levels of *DICER1* mRNA were only found

in the non-metastatic tumors and that the tubular and anaplastic tumors can be differentiated by different expression levels of *VEGFA*, *ZEB1* (overexpressed in the tubular tumor) and *SNAI2* (overexpressed in the anaplastic tumor).

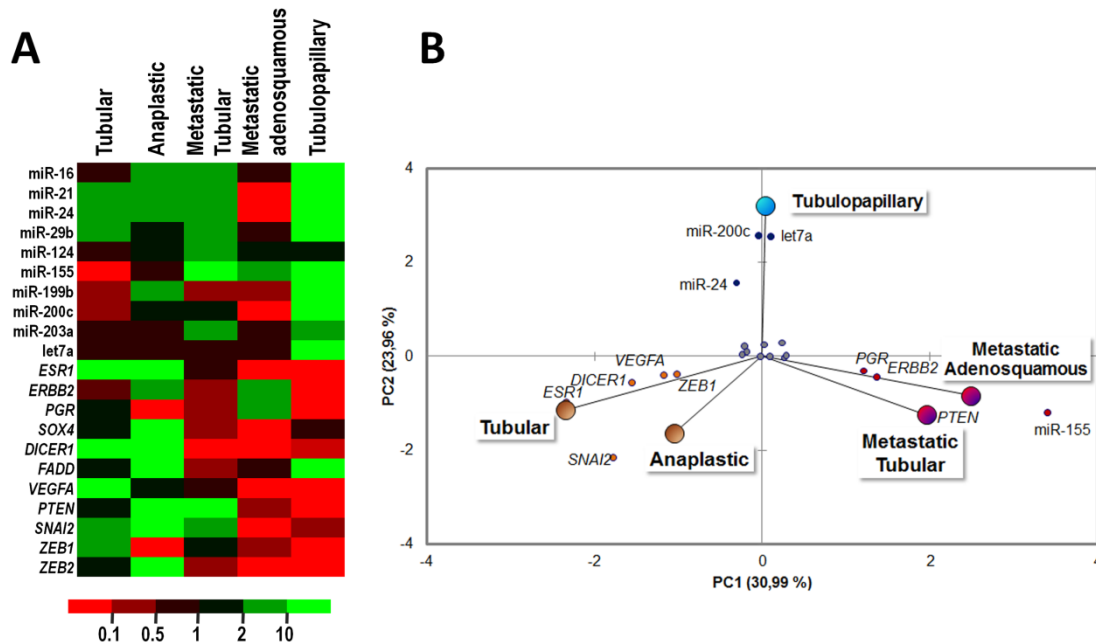


Figure VI.1 - Expression of miRNAs and mRNAs in grade III canine mammary tumors when compared to matched normal mammary tissue. A) Heat map of expression variations of miRNAs and mRNA in Grade III canine mammary tumors relative to the corresponding healthy mammary tissue. B) Principal component analysis of miRNA and mRNA expression variations.

Interestingly, the PCA (Figure VI.1, B) clearly separated the two metastatic CMTs from the other three non-metastatic CMTs in the first component. The second principal component, which represents 24% of the total variance, distinguished between the tubulopapillary from the other grade III CMTs. However, the similar expression behavior between *DICER1* and *ESR1* mRNA and miR-155 in both metastatic and tubulopapillary CMTs (Figure VI.1, A) might suggest a correlation between these CMTs, possibly a transition from a non-metastatic to a metastatic condition, since expression alterations in these genes have been implicated with poor prognosis and with breast/CMT progression.^{160, 238, 239}

VI.6. Conclusions

In conclusion, the simple molecular characterization of grade III CMT performed in this study allowed to discriminate metastatic tumors, giving a valuable tool to facilitate the identification of molecular events in the progression of tumorigenesis of the mammary gland of dogs. Nevertheless, we are aware that a low number of samples have been used and we are working to increase the number of CMT grade III samples.



VII. Concluding Remarks and Perspectives

For the purpose of this work, 96 mammary tumors from 78 female dogs were collected (see table A.1 in the appendix). From these, only two samples originated cell lines with at least 100 passages that were characterized in this thesis. Currently in our laboratory there is another sample with 30 passages so far and another that reached only 10 passages. The success of spontaneous immortalizations of CMTs was very low, approximately 2%. Adding hormones or growth factors to the culture media could improve the immortalization rate. The use of lentiviral, retroviral or adenoviral vectors or active viruses such as Epstein-Barr virus have also been used for the immortalization of different types of cells with higher efficiencies than those described in this thesis. However, the FR37-CMT or FR10-CMT lines established within this work do not require any supplementation of media with growth factors or hormones minimizing the occurrence of genomic alterations that might be induced by the transformation of cells with viral vectors.

The novel cell lines, FR37-CMT and FR10-CMT are capable of inducing the formation of xenograft tumors in NOD-SCID female mice. FR37-CMT cells were also able to xenograft tumors in male NOD-SCID mice, which is an indication that FR37-CMT cell tumorigenicity is not dependent on female hormonal signaling. Despite this observation and the observed

downregulation of the *ESR1* and *ERBB2* genes, western-blot showed that FR37-CMT cells expressed the ER α and HER-2 receptors. Surprisingly, western-blot in FR10-CMT cells did not detect the ER α receptor line, although *ESR1* expression was unaltered.

With the work developed for this thesis, the two cells lines described FR37-CMT and FR10-CMT were characterized at the transcriptome and at the protein expression level in order to gain insights in the oncogenic changes that occur in CMTs. The cell lines were also compared with the original tumors. From the 18 markers evaluated only 6 were differentially expressed between the FR37-CMT cell line and the original tumor, which makes this cell representative of the original tumor. In FR10-CMT cell line, 11 markers were differently expressed between the cell line and the original tumor. These observations can be explained in two different ways: FR10-CMT cell line could be originated from a small sub-population within the tumor or during immortalization, cells spontaneously mutated *in vitro* with proliferative advantages.

The molecular study of those cells revealed that both cell lines shared some characteristics, such as the downregulation of E-cadherin and expression of vimentin and CD44 marker, with cancer stem cells (CSC) which have a characteristic CD44⁺/CD24^{-low} phenotype. These particular cells are considered sub-clones present in the tumors that possess increased mobility and the ability to migrate and, thus, establish metastasis at distant sites. The CSCs have also been implicated in chemotherapy resistance. Recently it has also been proved the role of the epithelial to mesenchymal transition (EMT) in acquisition of malignancy and the formation of metastasis in CMTs and Human breast cancer (HBC). Epithelial cells undergoing EMT display alterations at the level of the adhesion molecules, usually loss of E-cadherin and gain of N-cadherin expression and reorganization of the cytoskeleton, with expression of the intermediate filament vimentin. The alterations in the cytoskeleton will ultimately affect cellular polarity and morphology. Also, commonly matrix metalloproteinases (MMPs) are expressed, enabling these

tumor cells to remodel the extracellular matrix increasing mobility. EMT has also been linked with acquisition of stemness related characteristics, as e.g. the CD44⁺/CD24^{-low} phenotype, by tumor cells.

FR37-CMT cells have molecular characteristics that are consistent with an induction of EMT: the up-regulation of *ZEB1* and the downregulation of its direct regulator miR-200c and *DICER1*. The activation of ERK1/2 MAPKinase signaling pathway, revealed by the presence of phosphorylated ERK1 and ERK2 proteins, is also an indication of EMT mediated by TGF- β signaling. These alterations are also reflected in the phenotype of the FR37-CMT cells which are fusiform instead of the more common cuboid morphology displayed by epithelial cells. Also, the ability of FR37-CMT cells to reorganize the collagen matrix is an indicator of the expression of MMPs, another characteristic of EMT. Thus, FR37-CMT cell line is a good model for the study of EMT in CMTs.

Although FR10-CMT cells have stemness related characteristics, the molecular characterization shows that EMT is repressed in this cell line, as it is possible to see by the up-regulation of miR-200c and the down-regulation of *ZEB1*, *ZEB2* and *SNAI2*, transcription factors implicated in the activation of EMT. This makes FR10-CMT a model for the study of CMT progression without the stimulation of EMT.

Although different in terms of EMT induction, FR37-CMT and FR10-CMT cell lines share other common features: the downregulation of miR-16 and *PTEN*, common features in HBC and CMTs, and high tolerance to chemotherapeutic compounds such as cisplatin and doxorubicin. Both cell lines have IC₅₀>50 μ M for cisplatin and for doxorubicin IC₅₀ were 5.3 μ M and 3.96 μ M for FR37-CMT and FR10-CMT, respectively. These values are higher than those described in the literature for other CMT derived cell lines which make these cells lines good models for the study

of CMT resistance to chemotherapeutic compounds and also for the development of novel therapeutic compounds and formulations.

The potential of two metal compounds with 1,10-phenanthroline-5,6-dione (DION) ligands, $[\text{Zn}(\text{DION})_2]\text{Cl}$ (TS262) and $[\text{CoCl}(\text{H}_2\text{O})(\text{DION})_2][\text{BF}_4]$ (TS265), as therapeutic agents against CMTs was also evaluated in FR37-CMT and FR10-CMT cell lines. These compounds had already shown to be effective against human cancer cell lines derived from colon (HCT116), liver (HepG2) and breast (MCF7) cancers. Noteworthy, unlike most organometallic compounds, these compounds are soluble in water, which makes them even more amenable for future use in medicine.

In both cell lines, the IC_{50} for TS262 and TS265 were significantly lower than the displayed by cisplatin and doxorubicin ($1.05\ \mu\text{M}$ and $1.39\ \mu\text{M}$ respectively for FR37-CMT and $0.55\ \mu\text{M}$ and $0.80\ \mu\text{M}$ respectively for FR10-CMT). These observations indicate a potential therapeutic in the treatment of CMTs.

Nanotechnology has been used for to bind or encapsulate therapeutic compounds enhancing transport efficiency and target selectivity. Due to the high surface to volume ratio, and its reactivity with several biomolecules, gold nanoparticles (AuNPs) can be easily modified with polyethylene glycol (PEG), bovine serum albumin (BSA), oligonucleotides, peptides, *etc.* that increase biocompatibility, facilitate the transport of therapeutic compounds and allow for specific targeting without toxicity.

AuNPs functionalized with PEG and BSA were used to vectorize TS262 (nanoTS262) and TS265 (nanoTS265) and used for the first time in veterinary medicine using FR37-CMT and FR10-CMT as new models of canine mammary tumors. Cells exposed nanoTS262 or nanoTS265 in equivalent concentrations of free TS262 and TS265 showed a significant decrease

in viability while the control nanoconjugate (AuNP@PEG@BSA) displayed no toxicity in FR37-CMT and FR10-CMT cells. These nanovectorization systems are thus promising new therapeutic strategies for the treatment of CMTs.

Quantitative proteomic analysis was performed in FR37-CMT cell line exposed to TS262 and TS265 in order to gain insights into the cytotoxicity mechanisms of these two compounds and the cellular responses triggered by their exposure. It was possible to identify protein spots characteristic of each of the responses. As soon as identification of the relevant proteins spots is completed, a deeper analysis will be conducted.

The development of biomarkers that could easily and accurately identify metastasizing tumors would be important for the correct diagnose and earlier treatment of patients. As discussed above, EMT transition is an important event implicated in the acquisition of metastatic phenotype. Differential expression of miRNAs have been increasingly linked to tumor suppressor and oncogenic function in several tumors, including CMTs. miRNAs are negative regulators of gene expression that have been implicated in the regulation of over 60% of protein coding genes. Some miRNAs function as key regulators while others have very specific targets. Some miRNAs are suggested to cooperate to regulate specific targets forming regulatory networks important for the regulation of the cellular metabolism. Therefore, alterations of the expression of miRNAs can be indicative of metabolic changes, including the metastatic progression of tumors.

With that in mind a set of 10 miRNAs and 11 mRNAs described to be involved in EMT or as markers of poor prognostic in HBC and in CMTs were selected. Tumor progression has been proposed to be the accumulation of genomic errors that will ultimately translate in an aggressive metastatic phenotype. For this reason, only highly malignant, grade III CMTs were included in this study, in order to identify expression changes mainly associated to appearance of metastasis in CMTs.

Using principal component analysis (PCA), an unsupervised statistical analysis of variance that will analyze the variability in data, it was possible to observe expression patterns in the grade III CMTs and it was possible to segregate metastatic CMTs from the non-metastatic CMTs. The PCA analysis also revealed the similar expression profile between miR-155 and *DICER1* and *ESR1* mRNA and in both metastatic and tubulopapillary CMTs which might suggest a correlation between expression of the genes and a transition from a non-metastatic to a metastatic condition. However, the number of grade III tumors available for this analysis is very small, not enabling us to confirm these results.

The new models FR37-CMT and FR10-CMT are good models for the study of sensibility of CMTs to novel chemotherapeutic compounds and new therapeutic approaches. Future work with these cell lines will also allow a better understanding of the importance of EMT in the carcinogenesis of CMTs. Since FR37-CMT has activation of EMT while FR10-CMT has inhibition of EMT, it is possible to compare responses of both cell lines to chemotherapy, for instance. A comparative proteomic analysis of FR10-CMT cells exposed to TS262 and TS265 would allow the evaluation the cellular responses of both cell lines to the metal compounds. The comparison of both responses would allow the identification of resistance mechanisms common to both cell lines and individual responses to chemotherapy. In this way, the effect of the EMT induced resistance could also be evaluated.

The use of FR37-CMT and FR10-CMT for the study modulation of the tumor microenvironment would also be interesting. A co-culture of cells from FR37—CMT and FR10-CMT with monocytes or with fibroblasts should give us information on the molecular reprogramming induced by the tumors and *vice-versa*.

The novel metal compounds TS262 and TS265 and their nanovectorizations nanoTS262 and TS265 are promising new chemotherapeutic agents that need further research. Their

potential for treatment should be tested in a murine model in order to determine the dose necessary to observe xenograft tumor regression and possible side effects before passing to a test with canine patients.

The effect of gold nanoparticles vectorizations with chemotherapeutics agents already available in the market, such as doxorubicin or paclitaxel for example, should also be tested in the FR37-CMT and FR10-CMT cell lines. The reduction of the IC_{50} and, consequently, the dose of doxorubicin or paclitaxel necessary to obtain an objective response would also reduce the side effects observed in canine patients.

The use of expression changes in 21 genes were demonstrated to be promising as biomarkers capable to distinguish metastatic from non-metastatic tumors using PCA. However, due to low number of samples analyzed, it was possible to reach a more reliable conclusion. The number of samples tested should be increased. Further testing should provide us with a smaller set of genes capable of detect CMTs with early metastasis. These biomarkers could be used in order to develop a clinical test, such as a microchip for instance, for the routine characterization of CMTs.

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A.Appendix

Table A.1 – List of CMTs collect during this study with respective relevant information

Lab ID	Age (years)	Breed	Histological classification of CMT	Malignancy Grade	Lymph node metastasis	Observation
73/11	9	Pointer	Simple Tubulopapillary Carcinoma	I	No	Passage 0
74/11	7	Undifferentiated	Hyperplasia	-	No	Passage 0
100/11	8	Undifferentiated	Hyperplasia Nodule	-	No	
			Benign Mixed tumor	-		Passage 4
122/11	10	Cocker	Benign Mixed tumor	-	No	Passage 0
134/11		Undifferentiated	Complex adenoma	-	No	Passage 0
139/11	6	Boxer	Hyperplasia	-	No	Passage 4
140/11	Unknown	Undifferentiated	Complex adenoma	-	No	Passage 5
			Simple Adenoma	-		
149/11	6	Poodle	Complex adenoma	-	No	Passage 0
160/11	18	Chihuahua	Simple Tubulopapillary Carcinoma	III	No	Passage 4
175/11	8	Undifferentiated	Complex adenoma	-	No	Passage 0
205/11	12	German Sheperd	Mammary carcinoma	III	No	Passage 1
210/11	13	Poodle	Benign Mixed tumor	-	No	Passage 1
			Complex adenoma	-		Passage 1
221/11	11	Undifferentiated	Complex adenoma	-	No	Passage 0
228/11	6	Undifferentiated	Mammary gland sclerosis	-	No	Passage 0
239/11	12	Undifferentiated	Anaplastic Carcinoma	III	No	Passage 1
51/12	12	Poodle	Complex adenoma	-	No	Passage 0
52/12	7	Undifferentiated	Hyperplasia	-	No	Passage 0
93/12	10	Undifferentiated	Benign Mixed tumor	-	No	Passage 1
-	10	Pitbull	Without histological characterization of the tumor		No	FR10-CMT cell line
148/12	10	Undifferentiated	Carcinoma and myoepithelioma (Malignant)	I	No	Passage 1

Table A.1 (continued)

Lab ID	Age (years)	Breed	Histological classification of CMT	Malignancy Grade	Lymph node metastasis	Observation
149/12	9	Cocker	Hyperplasia	-	No	Passage 1
155/12	5	Yorshire (half-breed)	Hyperplasia	-	No	Passage 2
156/12	Unknown	Yorshire Terrier	Benign Mixed tumor	-	No	Passage 3
157/12	8	Shi-tzu	Ductal adenoma	-	No	Passage 1
168/12	10	Undifferentiated	Benign Mixed tumor	-	No	Passage 0
204/12	9	Rottweiler	Neoplastic relapse of a mammary tumor	-	No	Passage 2
205/12	8	Poodle	Benign Mixed tumor	-	No	Passage 0
1896/12	9	Shi-tzu	Simple Tubulopapillary Carcinoma	II	No	Passage 1
			Complex mucinous cystic carcinoma (D4)	II		Passage 1
1925/12	12	Serra da Estrela	Complex cystic Tubulopapillary carcinoma (E2)	II	No	Passage 0
			Simple Tubular Carcinoma (E4)	III		Passage 1
226/12	8	Boxer	Carcinoma and myoepithelioma	I	No	Passage 1
1939/12	8	Rottweiler	Simple Tubulopapillary Carcinoma (E5)	II	Yes	Passage 1
227/12	11	Undifferentiated	Myoepithelioma (Malignant)	I	No	Passage 1
228/12	Unknown	Undifferentiated	Mixed mammary carcinoma	II	Yes	Passage 2
229/12	Unknown	Undifferentiated	Benign Mixed tumor	-	No	Passage 1
1949/12	6	Undifferentiated	Simple Anaplastic carcinoma	III	No	Passage 2
231/12	10	Undifferentiated	Simple Tubulopapillary Carcinoma (E2)	I	No	Passage 0
			Simple solid carcinoma (E5)	II		Passage 0
234/12	9	Boxer	Ductal adenoma	-	No	
2060/12	13	Undifferentiated	Adenosquamous Carcinoma	III	Yes	Passage 0
247/12	Unknown	Poodle	Benign Mixed tumor	-	No	Passage 0

Table A.1 (continued)

Lab ID	Age (years)	Breed	Histological classification of CMT	Malignancy Grade	Lymph node metastasis	Observation
2055/12	11	Undifferentiated	Cystic Tubulopapillary carcinoma (D2)	II	No	Passage 1
2056/12	9.5	Shi-tzu	Simple Mucinous carcinoma	II	No	Passage 30
004/13	12	Cocker	Ductal adenoma	-	No	Passage 4
005/13	Unknown	Labrador (half-breed)	Ductal carcinoma	I	No	Passage 0
2089/12	12	Undifferentiated	Tubular carcinoma (E4)	III	No	Passage 3
			Complex Tubulopapillary carcinoma (E5)	II		Passage 0
2090/12	8	Crossbreed (Boxer and Setter)	Simple Tubular carcinoma (E4)	III	Yes	Passage 2
			Complex Tubular carcinoma (D2)	I		Passage 0
0009/13	11	Undifferentiated	Simple tubulopapillary carcinoma	II	No	Passage 1
21/13	17	Yorkshire Terrier	Cystic Tubulopapillary carcinoma (E2)	II	No	Passage 1
			Complex Tubular carcinoma (E3)	II		Passage 0
41/13	14	Undifferentiated	Simple tubulopapillary carcinoma	II	No	Passage 1
105/13	10	Poodle	Complex Tubular carcinoma	I	No	Passage 1
81/13	11	Undifferentiated	Complex Carcinoma	II	No	Passage 5
			Benign mixed tumor	-		Passage 0
107/13	8	Undifferentiated	Simple cystic Tubulopapillary carcinoma	II	No	Passage 3
130/13	7	Epaniol breton	Simple Adenoma	-	No	Passage 0
166/13	5	Undifferentiated	Complex adenoma	-	No	Passage 0
167/13	13	Undifferentiated	Complex carcinoma (gl.2)	II	No	FR37-CMT Cell line
			Benign Mixed Tumor (gl.4)	-		Passage 1

Table A.1 (continued)

Lab ID	Age (years)	Breed	Histological classification of CMT	Malignancy Grade	Lymph node metastasis	Observation
314/13	10	Cocker	Simple Tubulopapillary carcinoma (E3)	II	No	Passage 1
			Complex tubulopapillary carcinoma (D4)	II		Passage 1
386/13	Unknown	Undifferentiated	Simple Tubular carcinoma (E1)	II	No	
			Complex Tubular carcinoma (E2)	I		Passage 2
			Complex Tubular carcinoma (E3)	II		
			Simple tubular carcinoma (E4)	II		
			Complex Tubular carcinoma (E4 mass 9 mm)	II		Passage 3
			Squamous carcinoma (E4 mass 7 mm)	II		
435/13	11	Samoyed	Simple tubulopapillary carcinoma (D1)	III	N.D.	Passage 5
472/13	10	Undifferentiated	Complex Cystic Tubulopapillary carcinoma (E4)	II	No	Passage 5
524/13	Unknown	Undifferentiated	Simple Tubular carcinoma (E2)	I	No	Passage 0
			Complex tubular carcinoma	I		
			Complex Cystic tubular carcinoma	II		Passage 12
602/13	15	Undifferentiated	Mammary Condro Osteosarcoma	-	Yes	Passage 2
			Squamous carcinoma (E4)	III		Passage 4
			Complex Cystic Tubular carcinoma (E2)	III		Passage 1
603/13	11	Golden Retriever	Complex Cystic Tubular carcinoma (E4)	III	No	Passage 2
			Complex tubulopapillary carcinoma (D3)	II		Passage 28

Table A.1 (continued)

Lab ID	Age (years)	Breed	Histological classification of CMT	Malignancy Grade	Lymph node metastasis	Observation
650/13	11	Undifferentiated	Squamous Carcinoma (E4)	III	No	Passage 4
779/13	9	German Sheperd	Complex Tubular carcinoma (E4)	III	No	Passage 2
H895/13	13	Poodle	Complex tubulopapillary carcinoma (E1)	I	No	
			Complex tubulopapillary carcinoma (E2)	II		Passage 0
			Tubular carcinoma (E3)	II		Passage 3
			Tubular carcinoma (E4)	I		Passage 4
			Complex tubulopapillary carcinoma (D2)	II		
			Complex tubulopapillary carcinoma (D3)	II		
			Complex Tubulopapillary Carcinoma (D4)	II		Passage 11
H927/13	8	Undifferentiated	Anaplastic Carcinoma (E5)	III	No	Passage 1
H928/13	12	Undifferentiated	Complex Tubulopapillary carcinoma (E5)	II	No	Passage 12
			Complex Cystic Tubulopapillary carcinoma (E5)	III		Passage 0
			Simple Cystic Tubular carcinoma (D3)	II		Passage 0
			Complex Tubulopapillary carcinoma (D5)	III		Passage 10
H930/13	10	Poodle	Complex Tubular carcinoma	I	No	Passage 5
H951/13	13	Undifferentiated	Squamous Carcinoma	III	No	Passage 9
H970/13	nd	Cocker	Complex Cystic Tubulopapillary carcinoma (E3)	I	No	Passage 8
H983/13	12	Undifferentiated	Complex tubular Carcinoma (E4)	I	No	Passage 11
			Complex Cystic Tbullopapillary carcinoma (D4)	I		

Table A.1 (continued)

Lab ID	Age (years)	Breed	Histological classification of CMT	Malignancy Grade	Lymph node metastasis	Observation
H1058/13	7	Podengo	Complex Cystic tubulopapillary carcinoma (D4)	II	No	Passage 6
H1091/13	13	Golden Retriever	Complex Cystic Tubular Carcinoma (D2)	II	No	Passage 7
H28/14	12	Undifferentiated	Lipoma (D3)	-	No	
			Hemangiosarcoma (E4)	-		
H42/14	10	Undifferentiated	Tubular carcinoma E4)	I	No	Passage 1
H156/14	11	Undifferentiated	Solid carcinoma (D2)	III	No	Passage 3

Table A.2 - Relative alteration (above 1.5-fold, green cells, or below 0.7-fold, red cells) of protein content in FR37-CMT cells treated for 48h with 1.05 μ M TS262 and 1.39 μ M TS265. The approximate isoelectric point and molecular mass of the protein in the gels is also referred.

Spot number	TS262 vs ctrl	TS265 vs ctrl	pI	MM (KDa)
5	0.65	0.87	5.0	60
10	0.68	0.89	5.5	50
12	0.30	1.16	5.0	55
13	1.07	0.66	5.0	50
14	1.10	0.59	5.0	55
15	0.60	0.30	5.0	50
17	0.61	1.16	4.5	100
19	1.13	0.64	4.0	80
20	1.01	1.64	4.0	100
21	0.83	0.61	4.0	100
22	2.02	0.84	4.0	100
23	2.79	2.73	3.5	75
24	0.55	0.34	4.0	80
25	1.77	0.72	3.5	63
26	1.77	0.40	3.5	65
27	a)	a)	3.5	60
28	a)	a)	3.5	60
29	2.13	1.02	3.5	70
30	a)	a)	4.0	63
32	1.38	0.29	4.0	50
34	1.02	1.51	4.0	65
35	2.96	3.64	4.0	65
36	0.71	2.33	4.0	60
37	1.67	1.66	4.0	60
38	1.82	0.52	4.0	60
39	3.55	2.13	4.0	60
40	1.83	1.84	4.0	60
41	2.24	1.14	4.0	50
42	b)	1.22	4.0	50
43	3.55	1.55	3.5	180
44	2.73	0.71	4.0	140
45	a)		4.0	130
46	a)		4.0	130
48	0.59	0.72	5.0	200
49	1.17	0.56	5.0	150

Spot number	TS262 vs ctrl	TS265 vs ctrl	pI	MM (KDa)
50	2.21	1.61	5.0	140
51	0.71	0.50	5.0	140
52	2.10	2.91	5.0	140
53	0.30	0.33	5.0	140
54	1.52	1.18	5.0	100
55	1.94	1.45	5.0	100
56	2.10	0.85	5.0	100
57	0.77	1.63	5.0	100
58	1.29	1.82	5.0	100
59	1.14	1.82	5.0	100
60	0.54	1.97	5.0	75
61	0.76	0.60	5.5	70
62	0.71	0.68	6.0	70
63	0.91	0.46	6.0	70
65	2.42	1.53	6.0	75
66	0.53	0.75	6.5	70
67	0.44	0.95	5.0	63
68	1.73	1.48	6.0	75
70	0.61	0.54	7.0	60
71	1.90	1.46	5.5	75
74		a)	4.5	240
75	1.34	2.24	6.0	140
76	2.06	1.56	5.5	75
77	2.24	1.06	5.0	75
78	2.38	0.76	5.0	75
79	7.04	0.92	5.0	75
80	1.96	1.53	5.0	50
81	1.87	1.30	5.0	48
84	1.65	1.66	5.0	50
85	1.69	1.22	5.0	50
86	b)	b)	5.0	50
87	0.96	2.42	5.5	60
88	0.53	0.99	6.0	48
89	0.78	0.60	6.0	50
91	0.22	1.60	6.0	40

Table A.2(continued)

Spot number	TS262 vs ctrl	TS265 vs ctrl	pI	MM (KDa)
92	2.12	2.07	6.0	60
94	0.38	0.89	6.5	75
95	b)	b)	6.5	75
96	1.47	2.35	6.5	65
97	1.41	0.69	6.5	65
98	0.42	1.72	6.0	63
99	0.65	1.22	7.0	48
100	0.28	0.92	6.0	35
101	0.07	0.97	6.0	35
106	0.58	0.91	6.0	40
108	0.65	1.53	7.0	70
110	0.99	2.10	7.0	70
111	1.99	1.73	7.0	60
112	1.71	2.04	7.5	70
113	2.49	1.47	7.5	65
114	2.01	2.99	7.5	65
115	1.37	0.30	7.0	70
116	1.24	2.05	7.5	60
118	1.91	1.32	8.0	60
119	0.92	0.70	7.0	75
122	3.75	2.05	8.0	60
123	0.69	0.90	8.0	70
124	0.59	0.82	8.0	70
125	0.32	1.28	7.5	48
126	0.84	0.61	7.5	50
128	0.44	1.30	8.0	50
129	0.67	0.66	8.0	50
131	0.55	0.66	8.0	50
132	0.43	0.94	8.0	50
133		a)	9.0	63
134	0.56	1.75	9.0	50
135	0.90	0.65	8.0	50
136	0.66	1.07	9.0	50
137	1.25	1.99	9.0	63
138	1.73	0.81	9.0	50
139	1.05	3.45	9.0	48
140	2.53	0.89	9.0	45
141	0.88	2.21	9.0	48

Spot number	TS262 vs ctrl	TS265 vs ctrl	pI	MM (KDa)
142	0.40	1.26	9.0	50
143	1.24	2.94	9.0	50
144	1.21	4.51	9.0	50
148	0.82	1.83	9.0	50
149	0.48	0.64	8.5	50
150	1.89	1.08	8.5	63
151	1.52	0.31	8.0	48
152	0.49	0.18	8.5	50
153	0.86	0.58	8.0	40
154	0.69	1.34	8.0	40
156	1.89	1.62	8.0	48
159	2.37	1.11	7.5	48
162	1.87	1.52	7.5	48
163	1.33	2.02	8.0	48
164	1.19	0.53	7.0	48
167	2.54	2.56	7.5	65
168	1.10	0.62	7.0	50
170	0.73	0.50	7.0	45
174	a)	a)	7.0	63
175		a)	7.0	63
176	0.82	2.07	7.5	50
177	1.53	1.19	7.0	45
178	0.74	1.71	8.0	50
180	2.78	1.70	7.0	50
181	2.34	1.52	7.0	55
183	1.82	2.01	7.0	50
184	0.56	1.45	7.0	30
185	0.56	1.26	7.0	30
187	0.75	0.64	7.0	35
188	0.54	1.03	7.0	30
189	1.90	2.22	7.0	55
191	1.73	0.83	7.0	48
192	0.60	1.62	7.0	35
193	6.66	7.50	7.0	48
194	0.40	0.41	6.5	48
196	2.77	5.04	7.0	50
197	1.53	1.47	7.0	40
199	3.38	0.96	7.0	30

Table A.2 (continued)

Spot number	TS262 vs ctrl	TS265 vs ctrl	pI	MM (KDa)	Spot number	TS262 vs ctrl	TS265 vs ctrl	pI	MM (KDa)
200	0.98	0.65	7.0	35	249	4.04	8.15	4.0	30
201	1.16	0.31	7.0	40	251	0.58	1.03	5.0	25
202	0.43	0.77	6.0	40	253	1.11	1.57	5.0	25
203	4.95	2.85	7.0	35	255	1.23	0.50	4.0	25
204	1.47	1.53	7.0	30	258	1.06	2.65	4.0	17
206	0.31	1.29	5.0	30	259	0.88	1.72	4.0	11
207	1.64	0.92	6.5	35	262	0.64	0.75	4.0	11
208	0.71	0.51	6.0	35	263	1.52	2.68	3.5	5
209		a)	5.0	35	264	0.45	0.58	7.0	20
210		a)	5.0	35	265	0.64	0.60	4.0	18
212	1.86	1.62	7.0	35	266	0.52	1.30	5.0	11
213	2.45	2.83	7.0	35	267	b)	b)	4.0	11
214	0.39	1.00	6.5	30	268	1.78	0.47	3.5	10
215	1.31	0.65	6.0	35	269	a)		4.0	5
216	0.97	0.64	6.5	40	271	a)		4.0	11
219	3.70	1.07	7.0	30	273		a)	6.0	20
220	0.66	1.53	6.5	25	274	0.49	0.60	6.0	35
221	0.83	0.49	6.0	45	278	0.73	0.53	6.5	25
222	0.85	0.52	6.0	45	279	1.09	1.68	6.5	20
223	2.60	0.59	5.0	63	280	0.51	0.69	7.0	35
225	1.09	0.40	5.0	45	281	1.67	0.72	6.5	20
226	1.30	0.51	5.0	40	282	2.48	3.41	7.0	25
228	0.46	0.83	4.5	40	284	0.60	0.57	6.5	30
229	0.33	0.82	5.0	30	285	0.59	0.70	7.0	25
230	2.65	2.52	4.5	35	286	1.55	0.64	7.0	18
233	a)		4.0	48	288	b)	1.08	7.0	18
234	1.86	0.55	4.0	45	289	1.56	0.92	7.5	15
236	1.83	4.51	4.0	35	290	0.40	0.63	7.0	15
237	2.01	1.13	4.0	35	291	0.27	0.42	7.5	17
238	a)	a)	4.5	40	292	0.57	0.39	8.0	5
239	a)	a)	5.0	40	293	0.62	0.75	8.5	10
242	1.53	1.06	3.5	30	294	0.30	0.47	9.0	15
243	0.44	0.49	4.0	30	295	0.65	0.26	7.5	17
244	1.53	1.08	3.5	30	296	0.56	0.41	8.0	17
245	3.85	1.14	3.5	35	297	0.26	0.29	9.0	5
246	2.50	0.56	3.5	30	299	0.20	0.32	6.0	11
247	0.76	0.52	3.5	30	300	0.54	0.45	7.0	11
248	0.65	0.92	4.0	30	301	0.55	0.42	7.0	20

Table A.2 (continued)

Spot number	TS262 vs ctrl	TS265 vs ctrl	pI	MM (KDa)
302	0.52	0.85	9.0	11
306	0.45	1.31	9.0	35
307	0.65	3.89	9.0	30
308	0.61	2.11	9.0	30
309	0.25	0.92	9.0	30
310	0.46	0.27	9.0	30
311	0.99	3.43	9.0	25
312	0.60	2.39	9.0	25
313	0.66	0.74	8.0	25
314	2.28	3.74	9.5	20
315	0.63	1.05	9.0	17
316		a)	9.0	17
317		a)	9.0	20
318	a)		9.0	25
320	1.09	4.25	9.0	20
322	0.49	0.31	8.0	20
323	0.95	1.78	8.0	20
326		a)	8.0	25
328	1.05	1.75	8.0	25
330	1.09	1.83	7.5	25
332	1.63	1.36	8.0	25
333	a)		7.0	25
334	a)		6.0	35
335	a)		6.0	75
336	a)		6.0	75
337	a)		5.0	75
338	a)		6.0	50
339	a)		7.0	75
340	1.38	2.22	7.5	30
342	a)		8.0	50
343	a)		9.0	63
344	1.08	1.61	9.0	30
345	0.57	0.85	8.5	35
351	0.46	1.01	6.0	100
352	0.45	0.62	6.0	100
353	0.65	0.93	5.0	100

Spot number	TS262 vs ctrl	TS265 vs ctrl	pI	MM (KDa)
355	0.41	1.69	5.0	100
356	0.32	1.06	6.0	100
357	0.97	3.51	5.0	100
358	0.73	1.69	5.0	100
359	0.59	0.74	6.0	100
360	0.66	1.00	6.0	100
361	0.32	0.79	6.5	100
362	1.10	0.66	6.5	100
364	b)	1.17	6.5	75
365	1.66	1.27	7.0	100
366	4.23	2.03	7.0	100
367	1.59	2.24	7.0	120
369	1.83	0.77	7.5	120
371	2.26	2.35	7.5	120
373	0.67	0.70	7.5	100
374	1.73	2.44	7.5	135
375	1.10	0.49	7.5	100
376	1.30	1.79	8.0	100
378	1.27	0.59	8.0	100
379	1.01	0.70	8.0	100
380	1.57	2.37	8.0	120
381	0.92	0.66	8.0	110
382	1.81	0.95	8.0	120
386	0.64	0.78	8.5	75
387	0.93	0.58	9.0	110
389	0.55	0.51	8.5	70
393	b)	0.91	8.5	75
394	b)	1.05	8.5	75
395	0.55	0.68	9.0	80
396	0.46	0.64	9.0	100
397	b)	1.08	8.5	75
398	b)	4.61	9.0	60
399	0.67	0.88	8.5	100
400	1.20	1.70	8.0	120
401	0.61	0.81	8.0	100
402	0.74	0.59	8.5	130

a) Spots not found in control condition and found in the referred condition

b) Spot not found in the referred condition

Immortalization and characterization of a new canine mammary tumour cell line FR37-CMT

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Abstract

Here we describe the establishment of a new canine mammary tumour (CMT) cell line, FR37-CMT that does not show dependence on female hormonal signaling to induce tumour xenografts in NOD-SCID mice. FR37-CMT cell line has a stellate or fusiform shape, displays the ability to reorganize the collagen matrix, expresses vimentin, CD44 and shows the loss of E-cadherin which is considered a fundamental event in epithelial to mesenchymal transition (EMT). The up-regulation of ZEB1, the detection of phosphorylated ERK1/2 and the downregulation of *DICER1* and miR-200c are also in accordance with the mesenchymal characteristics of FR37-CMT cell line. FR37-CMT shows a higher resistance to cisplatin ($IC_{50} > 50 \mu M$) and to doxorubicin ($IC_{50} > 5.3 \mu M$) compared with other CMT cell lines. These results support the use of FR37-CMT as a new CMT model that may assist the understanding of the molecular mechanisms underlying EMT, CMT drug resistance, fostering the development of novel therapies targeting CMT.

Keywords

canine mammary tumour, cisplatin, doxorubicin, epithelial to mesenchymal transition, miRNAs

Introduction

Canine mammary tumours (CMTs) are the most common tumours diagnosed in intact female dogs.^{1,2} Approximately 50% of all CMTs are malignant, for which mastectomy (and ovario-hysterectomy in intact dogs) is the only broadly accepted treatment since chemotherapy with agents, such as doxorubicin and docetaxel, does not radically increase overall survival time.³ Despite the established dependence of CMT development on oestrogens and progesterone, tamoxifen [an oestrogen receptor alpha (ER α) antagonist] has not

been proven effective against CMT.⁴ Therefore, it is crucial to understand the mechanisms underlying the inefficacy of chemotherapy either by intrinsic or acquired drug resistance in dogs.

The epithelial to mesenchymal transition (EMT) is an important molecular reprogramming event that may allow canine mammary cancer cells to acquire mobility through changes in the expression of adhesion molecules (e.g. loss of E-cadherin expression and gain of N-cadherin) and reorganization of the cytoskeleton (e.g. gain of vimentin expression), for example, that result in the change of cellular polarity from top to bottom to back to

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ORIGINAL ARTICLE

Targeting canine mammary tumours via gold nanoparticles functionalized with promising Co(II) and Zn(II) compounds

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Despite continuous efforts, the treatment of canine cancer has still to deliver effective strategies. For example, traditional chemotherapy with doxorubicin and/or docetaxel does not significantly increase survival in dogs with canine mammary tumours (CMTs). Using CMT-derived cell line (FR37-CMT) as model, we showed the efficacy of 2 metal compounds [Zn(DION)₂]Cl (TS262, DION = 1,10-phenanthroline-5,6-dione) and [CoCl(H₂O)(DION)₂][BF₄] (TS265), with IC₅₀ values well below that of doxorubicin and cisplatin. Here, we have developed a gold nanoparticle system composed of polyethylene glycol, bovine serum albumin and TS262 or TS265 (NanoTS262 or NanoTS265, respectively) to improve the anti-cancer efficacy of these compounds against CMT. The nanosystems efficiently delivered the cytotoxic cargo inducing a significant reduction of cell viability in FR37-CMT cell line when compared with the free compounds. NanoTS262 and NanoTS265 demonstrate that such simple nanovectorization via gold nanoparticles shows tremendous potential as anti-cancer formulations, which may easily be expanded to suit other cargo.

KEYWORDS

1,10-phenanthroline-5,6-dione metal compounds, canine mammary tumours, cisplatin, doxorubicin, FR37-CMT, gold nanoparticles, nanotechnology

1 | INTRODUCTION

The most common tumours in non-spayed female dogs are canine mammary tumours (CMTs)^{1,2} whose standard treatment consists of mastectomy and ovariectomy/hysterectomy.³ Approximately, 50% of these CMTs are malignant and these patients would further profit from adjuvant chemotherapy. However, the efficiency of common anti-cancer drugs, such as doxorubicin and docetaxel, used for the treatment of human breast cancer, have not increased the disease-free or overall survival time of tested animals.⁴ For example, a CMT model cell line, FR37-CMT, has been shown to be resistant to cisplatin up to 50 µM, making it impossible to use as therapeutic agent in dogs.⁵ Despite the hormonal dependency of CMTs, the use of tamoxifen, an antagonist of the oestrogen receptor alpha, is not proven to be an effective therapeutic adjuvant.² Consequently, it is extremely important to test new therapeutic compounds or new strategies that can significantly reduce the growth of CMTs. Here, 2 metal compounds

with 1,10-phenanthroline-5,6-dione (DION) ligands, [Zn(DION)₂]Cl (TS262) and [CoCl(H₂O)(DION)₂][BF₄] (TS265) that have shown remarkable anti-proliferative effect against human colorectal carcinoma cells (HCT116), human hepatocellular carcinoma cells (HepG2) and breast adenocarcinoma cells (MCF7),⁶⁻⁸ were used to evaluate their potential against CMT.

Gold nanoparticles (AuNPs) have been proposed as valuable platforms for the delivery of anti-cancer drugs in humans, showing enhanced selectivity and improved efficacy. Because of their high surface-to-volume ratio and high reactivity with biomolecules via simple water phase chemistry, these AuNPs may be easily modified with numerous functional moieties, such as polyethylene glycol (PEG), bovine serum albumin (BSA), oligonucleotides, peptides and so on.⁹ AuNP modification may increase biocompatibility, assist transport chemotherapeutic compounds and allow for specific targeting without toxicity.⁹⁻¹¹ Here, the 2 aforementioned compounds were loaded onto 14 nm AuNPs functionalized with bi-functional