Aus dem Institut für Veterinär-Biochemie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Cellular crosstalk between canine fibroblasts and a mast cell tumour cell line and its significance in fibroblast activation

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Matías Ignacio Aguilera Rojas
Tierarzt aus Licantén, Chile

Berlin 2020
Journal-Nr.: 4215

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To my family and loved ones.

“There is only one way to happiness and that is to cease worrying about things which are beyond the power of our will.”

Epictetus

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List of abbreviations

ACTA2	Alpha-smooth muscle actin
CAF	Cancer-associated fibroblast
CXCL12	CXC-chemokine ligand
ECM	Extracellular matrix
EV	Extracellular vesicle
FAP	Fibroblast activating protein
FGF	Fibroblast growth factor
IL	Interleukin
ILV	Intraluminal vesicles
JAK	Janus tyrosine kinase
Mb	Mega base
miRNA	MicroRNA
mRNA	Messenger RNA
MVB	Multivesicular bodies
ncRNA	Non-coding RNA
NTA	Nanoparticle tracking analysis
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
pri-miRNA	Primary miRNA transcript
RNAi	RNA interference
S100A4	Fibroblast specific protein 1
STAT	Signal transducer and activator of transcription
STC1	Stanniocalcin 1
TAM	Tumour-associated macrophage
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor-β
TME	Tumour microenvironment
UTR	Untranslated regions
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

1. Introduction

Dogs have been described as the first human companion species and the only large carnivore to ever be domesticated. Although still not clear, it is believed that the domestication of the dog took place between 15,000 and 33,000 years ago and since then dogs have played a significant role in the history and development of human civilisation (Ovodov et al., 2011; Freedman et al., 2014; Larson and Bradley, 2014). More so than other wolf-like canids, many extensive phylogenetic studies have concluded that the grey wolf is the direct ancestor of the domestic dog (Leonard et al., 2002; Lindblad-Toh et al., 2005; Parker et al., 2010). Dogs evolved in a parallel manner to humans likely because of the mutually beneficial relationships between both species, sharing the same living environment and food sources. Over the last centuries, humans have actively bred and selected certain attributes and features in dogs, resulting in the creation of several breeds that both mimic human behaviours and assist human needs (Lindblad-Toh et al., 2005). Genes involved in digestion and metabolism, neurological processes and cancer have been found to be evolving in parallel in humans and dogs for thousands of years. Natural selection might have therefore acted on these group of genes, in the two different genomes, as a result of convergent environmental pressure (Wang et al., 2013). Furthermore, many other positively selected genes seem to be involved in similar diseases in both species. As a matter of fact, the domestic dog carries numerous disease syndromes that are common to humans, more than any other laboratory or domestic species, and clinical manifestations in the two species are often similar (Ostrander and Wayne, 2005; Wayne and Ostrander, 2007; Shearin and Ostrander, 2010; Wang et al., 2013).

Over the past years, as a result of an improved and more accessible veterinary medical care, life expectancy of pet dogs has increased significantly. At the same time, the incidence of various diseases has also risen, particularly the frequency of cancer occurrences and/or its diagnosis (Davis and Ostrander, 2014; Komazawa et al., 2016). Some purebred dogs may indeed be predisposed to a higher risk of developing specific types of cancers or cancers in general (Schiffman and Breen, 2015). Broadly speaking, cancer is characterised by a continuous and uncontrolled cell proliferation; signals that regulate the normal cell behaviour are evaded and cancer cells grow freely, invading local tissue and organs, and eventually developing metastases (Sarkar et al., 2013). Interestingly, cumulative data show that the tumour microenvironment (TME) also plays a pivotal role in the progression of cancer. As cancer evolves, tumour cells and resident cells of the TME start a reciprocal crosstalk by means of releasing growth factors, cytokines, proteases, microRNAs (miRNAs) and other bioactive molecules. These interactions result in an immunosuppressed TME that protects and nourishes the developing tumour (Ariztia et al., 2006; Ganguli and Sarkar, 2018). Indeed, the fact that fibroblasts represent a large cellular population within the tumour stroma and often

command the tumour outcome, makes them attractive targets for studying cancer progression and interactions between cancer cells and TME components (Monteran and Erez, 2019).

Carcinogenesis in dogs is in many aspects very similar to humans, which is why translational and comparative studies become especially valuable and beneficial for both species (Gardner et al., 2016). In this respect, further research into the complex molecular relationships between cancer cells and TME components, such as fibroblasts, may lead to the discovery of new strategies, for both the diagnosis and treatment of cancer. Overall, the present study aims to expand the understanding of intercellular communications between non-cancer and cancer cells. Cell culture experiments involving normal canine dermal fibroblasts and a mast cell tumour cell line allowed the assessment of cellular crosstalk. The employed *in vitro* system was able to detect molecular changes in fibroblasts that were induced by cancer cells and may have identified a potential mechanism for fibroblast activation. In this context, miRNA, mRNA and protein expression were analysed in order to evaluate such evoked cellular responses.

2. Literature overview

2.1. Cancer in dogs

Cancer is the most common cause of death in dogs, as it is for humans. Incidence reports of tumours naturally occurring in dogs estimate that approximately 50% of dogs over 10 years and about 25% of dogs of all ages die from this disease (Bronson, 1982; Adams et al., 2010; Dobson, 2013; Davis and Ostrander, 2014). Pet dogs are diagnosed with several of the same cancers as humans, displaying complex interactions between the immune system and tumour cells, extensive intra-tumour and inter-tumour heterogeneity, similar treatment response, development of chemotherapy resistance, and metastasis, mirroring the biology and diversity of the human neoplasms (Khanna et al., 2006; Cadieu and Ostrander, 2007; Davis and Ostrander, 2014). Furthermore, specific biochemical pathways known to be involved in carcinogenesis in humans are also frequently deregulated in canine cancers, which at the same time suggests similar genetic mechanisms for tumorigenesis in both species. As both dogs and humans spontaneously develop cancers and share many characteristics of the disease, studying neoplasms in dogs may deliver more valuable information than research on induced tumours in other animal models (Gardner et al., 2016).

Studies in cancer incidence in pet dogs performed in Europe show that the occurrence of tumours in females is higher, with mammary tumours being the most frequently diagnosed neoplasm. Further common malignancies found in male and female dogs are mast cell tumours, lymphomas and melanomas (Merlo et al., 2008; Brønden et al., 2010; Grüntzig et al., 2016). Various canine breed predispositions have been established for certain cancers in veterinary medicine, reinforcing the concept of a shared genetic predisposition and therefore a common ancestor during breed development (Gardner et al., 2016).

When considered as a species, dogs bear wide genetic variations across all dog breeds, likely being as extensive as for human populations. Conversely, if looking at single breeds, genetic diversity is limited (Lindblad-Toh et al., 2005; Vonholdt et al., 2010). Reproductive programmes to develop breeds with particular phenotypic traits have widely extended the practice of dog breeding, resulting in high levels of consanguinity and inbreeding, alongside various health concerns, including cancer. However, such restricted genetic variation and increased risk of cancer in many purebred dogs allow for an easier identification of the genetic basis of cancer from a comparative point of view (Dobson, 2013; Schiffman and Breen, 2015). This is why canine cancer systems have gained great significance for studies of basic biology and treatment assessment. They allow researchers to study specific untraceable genes of certain human populations that are potentially easier to detect in particular dog breeds (Lindblad-Toh et al., 2005; Cadieu and Ostrander, 2007).

2.2. The dog as a model for human cancer

When in 2005 the canine genome was sequenced and made available worldwide for the research community (Lindblad-Toh et al., 2005), it revealed that dogs share around 650 Mb of ancestral sequence with humans that is absent in mice. At the same time, it showed that canine DNA and protein sequences are more similar to humans than to mice. It makes sense therefore to assume that many aspects of human biology are more significant in dogs than in mice (Rowell et al., 2011). Indeed, many inherited diseases occurring in humans have also been characterised in dogs; complex disorders such as heart disease, neurological disorders and numerous types of cancers are included in this group (Parker et al., 2010; Rowell et al., 2011; Shaffer, 2019).

Clinical presentation and histological characteristics of various canine neoplasms are frequently very similar to their human counterparts. The life expectancy of pet dogs represents a five- to eight-fold times faster aging process compared to humans. This makes possible long-term studies of cancer development, progression and treatment, positioning dogs in a very exclusive status for comparative research studies (Rowell et al., 2011; Schiffman and Breen, 2015). An additional highly important and unique advantage of naturally occurring cancer in dogs is that these tumours have evolved a wide variety of immune evasion strategies, much like human cancers have (Thamm, 2019). Such strategies to evade immune responses include the expression of immune regulatory molecules such as PD-L1 protein (Maekawa et al., 2017) or non-coding RNAs (ncRNAs) like miRNAs (Gioia et al., 2011; Eichmüller et al., 2017), increased levels of circulating myeloid derived suppressor cells (Goulart et al., 2012; Sherger et al., 2012) and secretion of numerous immunosuppressive cytokines (Itoh et al., 2009; Troyer et al., 2017), as well as infiltration of tumour-associated macrophages (TAMs) (Monteiro et al., 2018; Seung et al., 2018) and activation of cancer-associated fibroblasts (CAFs) in tumour tissues (Król et al., 2012; Wang et al., 2018).

Table 1 shows particular canine malignancies that have been proposed as reliable comparative models for human neoplasms. While some of these cancers have allowed significant advances in understanding the genetics of susceptibility, others have identified factors that contribute to tumour growth and development (Cadieu and Ostrander, 2007; Ostrander et al., 2019). Mast cell tumours are the most common skin malignancy in dogs, whereas in humans its presentation is rare. However, both species display activating mutations of the c-KIT tyrosine kinase receptor, which are considered to contribute to disease evolution (Ranieri et al., 2013). Tyrosine kinase inhibitors against c-KIT have been developed but clinical responses are unpredictable and often transitory in both species. Therefore, based on the higher incidence of mast cell tumours in dogs, further efforts to develop new and improved treatment strategies could be focused on canine studies. Future investigations will

Table 1. Canine malignancies proposed as reliable comparative models for human neoplasms.

Canine cancer model for humans	References
Colorectal cancer	Tang et al. (2010); Wang et al. (2018)
Cortisol-secreting adrenocortical tumour	Kool et al. (2015); Galac (2016)
Histiocytic sarcoma	Hedan et al. (2011); Shearin et al. (2012)
Leukaemia	Breen and Modiano (2008); Richards et al. (2013)
Mammary tumours	Lutful Kabir et al. (2016); Kabir et al. (2017); Lee et al. (2019); Zizzo et al. (2019)
Melanoma (especially oral melanoma)	Murakami et al. (2011); Gillard et al. (2014); Simpson et al. (2014); Prouteau and André (2019)
Non-Hodgkin's lymphoma	Breen and Modiano (2008); Ito et al. (2014); Panjwani et al. (2016)
Osteosarcoma	LaRue et al. (1989); Mueller et al. (2007); Kirpensteijn et al. (2008); Angstadt et al. (2012); Sakthikumar et al. (2018)
Prostatic carcinoma	Elshafae et al. (2017); Sun et al. (2017)
Transitional cell carcinoma of the bladder	Decker et al. (2015); Maeda et al. (2018)

then elucidate whether canine mast cell tumour models for humans may indeed help enhance the therapy and prognostic in both species (Willmann et al., 2019).

2.3. The tumour microenvironment

Tumorigenesis is a complex and dynamic process that involves cellular genotypic and phenotypic changes, which alter the normal balance between cell proliferation and cell death. It consists of three stages: initiation, progression, and metastasis (Wang et al., 2017a). Currently, ten hallmarks of cancer have been recognised, these comprise: sustained multiplication, evasion from growth suppressors, evasion of immune checkpoints, acquisition of unlimited replicative potential, tumour-associated inflammation, invasion and metastasis, induction of angiogenesis, genome instability and mutation, bypassing of cellular death, and deregulation of cellular energetics (Hanahan and Weinberg, 2011).

Despite the increased understanding of cancer cell biology, current therapies are often transient or only partially effective for most cancers. In this regard, while genetic alterations in tumour cells are essential for tumour development, they are not sufficient to establish the malignant status of cancer. As such, tumours are not composed of cancer cells alone, instead they are highly heterogeneous and complex “ecosystems” that combine different cell types and non-cellular elements. Hence, cancer cells recruit and reprogramme surrounding non-cancer cells to serve as contributors to tumour progression. All these components of the tumoral niche

and their dynamic interactions comprise the TME (Sounni and Noel, 2013; Valkenburg et al., 2018).

The structural and functional elements in the stroma of the TME include the extracellular matrix (ECM) and its associated soluble factors, as well as non-cancer cells, such as fibroblasts, neuroendocrine cells, adipose cells, immune cells, inflammatory cells, and blood and lymphatic vascular networks (Figure 1) (Chen et al., 2015; Alkasalias et al., 2018). Inside the TME, a dynamic network of cytokines, chemokines, growth factors, and inflammatory and matrix remodelling enzymes drive complex intercellular communication processes in order to modify the physical and chemical properties of the local tissue (Balkwill et al., 2012). The TME has been increasingly recognised in the research community as playing a crucial role in multiple stages of tumorigenesis, particularly in therapy resistance, immune-escaping, and metastasis (Chen et al., 2015; Valkenburg et al., 2018). Interestingly, studies have reported that when the TME is in a healthy state, it can exert a protective profile against tumour development during early stages of cancer. As tumours grow, however, the TME shifts its function into a tumour promoter, since cancer cells utilise the TME-derived growth factors to facilitate their own survival and proliferation (Wang et al., 2017a; LeBleu and Kalluri, 2018).

The ECM constitutes the non-cellular milieu of the TME, along with extracellular vesicles (EVs), as well as physical and chemical parameters including pH, oxygen tension and interstitial pressure. More than purely a static structure that only maintains the tissue morphology, the ECM represents a dynamic element of the TME. Chemical signals arising from ECM elements and ECM organisation processes mediate the biological crosstalk between stromal and tumour cells. Thereby, the non-cellular component of the TME is being slowly and constantly degraded by enzymes, such as collagenases and matrix metalloproteases, and replaced by fibroblast secretions. This represents a local interaction of collagens, proteoglycans, glycoproteins, glycosaminoglycans, and other macromolecules, all contributing to the intercellular signalling inside the TME. Therefore, any modification of the ECM synthesis, degradation, density, or rigidity can considerably influence the capacity of the TME to promote cancer cell proliferation, migration and invasion (Sounni and Noel, 2013; Valkenburg et al., 2018; Wei et al., 2020). Moreover, it has been shown that ECM stiffness and density can promote the growth and the invasive phenotype of mammary gland tumours in humans (Levental et al., 2009) and in dogs (Case et al., 2017).

Stromal cells within the TME provide cancer cells with growth-promoting signals via autocrine and paracrine mechanisms, including growth factors, cytokines and chemokines (Yuan et al., 2016). In this regard, to cover the increasing proliferative demand, growth factor receptors are overexpressed on either cancer cells or neighbouring cells (Hanahan and Weinberg, 2011). The higher need for oxygen in the cells triggers angiogenesis, which is induced by numerous growth factors such as vascular endothelial growth factor (VEGF) and

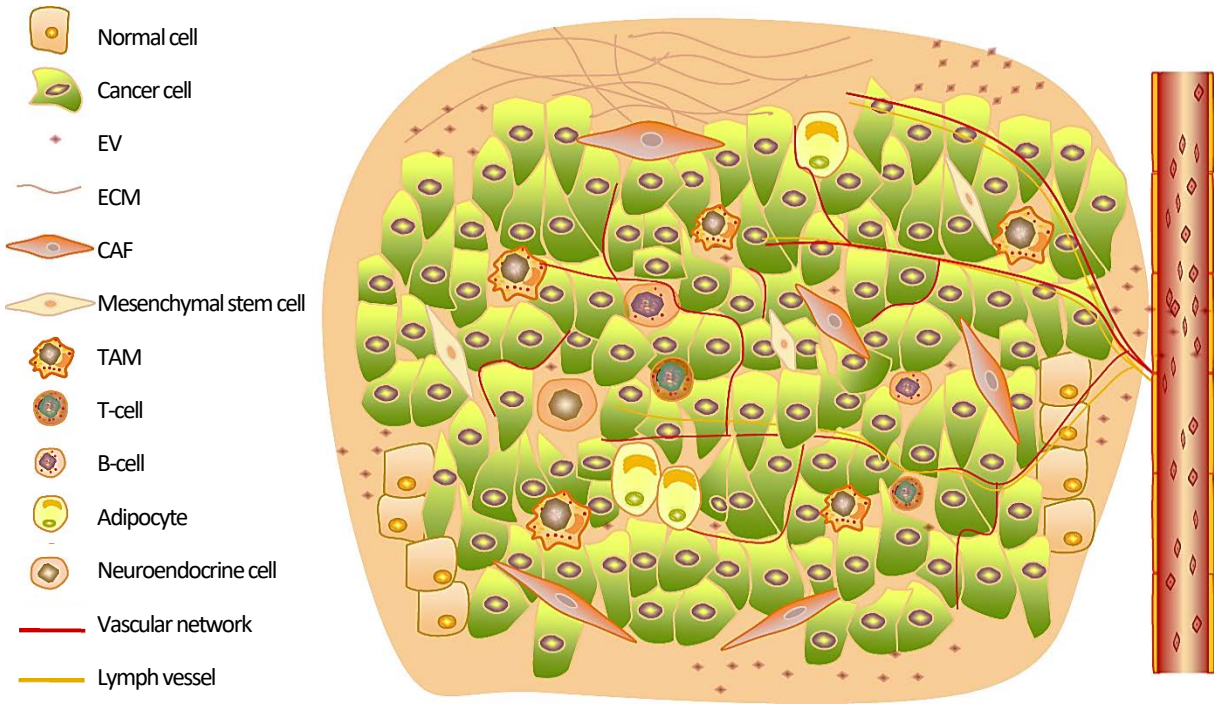

Figure 1. Schematic representation of the heterogeneous and complex tumour microenvironment, showing the relationship between its cellular and non-cellular constitutive elements. Taken from Wei et al. (2020) (open access article distributed under the Creative Commons Attribution License).

different fibroblast growth factors (FGFs) (Carmeliet and Jain, 2000; Thakkar et al., 2019). TAMs represent the major percentage of the leucocytic infiltrate in the TME and secrete many of the growth factors and cytokines that induce tumour-associated angiogenesis (Ireland and Mielgo, 2018). Indeed, TAMs' infiltration has been correlated with higher presence of vascular invasion in canine mammary tumours (Monteiro et al., 2018). In general, over-expression of certain FGFs is associated with an increased tumour growth rate in humans (Kwabi-Addo et al., 2004) and canine malignancies (Palmieri, 2015). Likewise, CAFs-derived ECM proteins and cytokines are essential for endothelial cell lumen formation (Newman et al., 2011) and it has been reported in dogs that CAFs can promote the expression of angiogenesis-related genes in mammary cancer cells (Król et al., 2012). Chemokines also contribute significantly to cancer progression. They represent a family of secreted and structurally related chemotactic cytokines and play important roles in cellular transformation and cancer-related inflammation (Ariztia et al., 2006; Mantovani et al., 2010). CXC and CC chemokines are currently the most studied families of cancer-associated chemokines, since they are responsible for the recruitment and activation of TAMs, angiogenesis, cancer cell proliferation, and metastasis (Bottazzi et al., 1983; Mollica et al., 2019). Additionally, miRNAs are also capable of controlling the cellular metabolism, tumorigenesis and tumour progression within the TME via post-

transcriptional modifications of gene expression (Calin and Croce, 2006). Effective delivery of miRNAs via exosomes has been confirmed to occur between cancer cells and stromal cells in a reciprocal way, which categorises miRNAs also as paracrine signalling molecules (Kosaka et al., 2010; Suetsugu et al., 2013).

Hypoxia and an acidic environment are known to be present in solid tumours as a result of the increased metabolic status and nutritional needs of the cells in the TME (Rotin et al., 1986). Locally, cancer cells grow in a manner such that available oxygen becomes insufficient, a situation that leads to a hypoxic condition. In order to continue developing under limited amounts of oxygen, cancer cells enhance their glucose uptake and aerobic glycolysis to gain energy, which generates large amounts of lactic acid, a phenomenon named Warburg effect. At the same time, increased fermentation of lactic acid produces free protons that reduce the pH and acidify the environment (Yuan et al., 2016; Thakkar et al., 2019). Both hypoxia and acid environment favour processes such as metastasis, immunosuppression and resistance to various treatment strategies, which is associated with poor prognosis (de la Cruz-López et al., 2019).

Since TME actively contributes to tumour progression and metastasis, approaches focusing on the TME may represent a great therapeutic potential. Moreover, because non-tumour cells recruited into the TME are presumably genetically more stable than cancer cells, therapies also targeting the TME components rather than cancer cells alone are less likely to cause adaptive mutations and/or rapid growth and metastasis formation (Auboeuf, 2016; Yuan et al., 2016).

2.4. Cancer-associated fibroblasts

Among the cellular components in the TME of solid tumours, the mesenchymal or fibroblastic cell type, also referred to as cancer-associated fibroblasts (CAFs), can be found neighbouring or in direct contact with neoplastic cells. CAFs represent not only one of the most important members but also the dominant cell type within the tumour stroma and bear high proliferative, migratory, contractile, and secretory properties (LeBleu and Kalluri, 2018; Wei et al., 2020). The mechanisms through which CAFs significantly impact cancer progression include a direct promotion of cancer cell proliferation, induction of angiogenesis, mediation of tumour-associated inflammation, and promotion of tumour cell invasion/metastasis by remodelling the ECM. CAFs manage all these functions via secretion of various growth factors, cytokines, chemokines, enzymes, ECM, and miRNAs (Kalluri and Zeisberg, 2006; Wang et al., 2017b; Monteran and Erez, 2019). On the other hand, fibroblasts in non-malignant tissues are generally present in small numbers as supportive elements residing in a given organ. These are quiescent cells that are capable of responding to extrinsic signals to become activated (Kalluri, 2016; LeBleu and Kalluri, 2018).

CAFs comprise a heterogeneous subpopulation that derives from diverse origins. It has been proposed that CAFs are mainly recruited and reprogrammed from resident fibroblasts, whose quiescent state was activated. Nevertheless, trans-differentiation of bone marrow-derived mesenchymal cells, adipocytes, and epithelial and endothelial cells undergoing mesenchymal transition can also induce CAFs' formation (Madar et al., 2013; Tommelein et al., 2015; Kalluri, 2016; Monteran and Erez, 2019). Similarly, during the wound healing process resident fibroblasts also become activated to enable the tissue remodelling cascade. This phenomenon endows fibroblasts with ECM synthesising and remodelling properties, as well as with immune cells recruitment abilities to further restore the tissue homeostasis. However, upon completion of wound healing, activated fibroblasts undergo apoptosis. In this manner, cancers are considered as "wounds that never heal" because CAFs remain permanently activated and share many similarities with wound-activated fibroblasts (Öhlund et al., 2014; LeBleu and Kalluri, 2018).

Cancer cells are directly involved in the activation of CAFs via aberrant secretion of fibroblast-activating molecules. Various chemical mediators including growth factors, cytokines, miRNAs and metabolites produced not only by the malignant cells, but also by the recruited immune cells have been reported to induce CAF activation (Kalluri, 2016; Wang et al., 2017a; Yang et al., 2017a). Accordingly, such key regulators leading to CAF's differentiation in humans are, for example, transforming growth factor-β (TGFβ), platelet-derived growth factor (PDGF), FGFs, and interleukins (IL) (Kalluri and Zeisberg, 2006; Tommelein et al., 2015; Liu et al., 2019). Thus, changes leading to the irreversible transformation of fibroblasts into CAFs have been mostly linked to epigenetic alterations, although some authors suggest that genetic modifications could also be involved in CAFs' reprogramming. Both DNA methylation and miRNA-mediated epigenetic changes have been identified as responsible for CAFs' activation (Hu et al., 2005; Albrengues et al., 2015; Du and Che, 2017).

During tumour progression, CAFs are the main players in the dysregulation of collagen turnover that leads to tumour fibrosis (known as desmoplasia), which is characterised by an excessive collagen accumulation in the TME (Pankova et al., 2016; Nissen et al., 2019). At the same time, CAFs influence the tumour growth and progression via paracrine signalling through the secretion of multiple soluble factors. CAFs' secretome is very broad and variable between CAFs subpopulations; TGFβ, VEGFA, IL6, FGFs, CXC-chemokine ligand (CXCL12), and stromal cell-derived factor-1, among others, have been reported to play significant roles in modulating cancer cell migration, invasion and angiogenesis. Interestingly, CAFs' secreted pro-oncogenic factors are often the same molecules that induce their activation, which remarks the reciprocal intercellular communications occurring inside the TME (Öhlund et al., 2014; Tommelein et al., 2015; Liu et al., 2019). Thus, the close interactions of CAFs with immune

cells and endothelial cells facilitate the activation and/or migration of resident immune cells, such as macrophages into TAMs, and the formation of new blood vessels into poor oxygenated tissues regions (Kendall and Feghali-Bostwick, 2014). Furthermore, CAFs-derived exosomes carrying miRNAs have also emerged as positive mediators of cancer progression, stroma remodelling, and chemotherapy resistance (Kalluri, 2016; Richards et al., 2017; Hu et al., 2019).

Several studies have attempted to identify activated CAFs by characterising specific CAF markers, although their heterogeneity has hampered a precise and exclusive identification of CAFs and their distinction from quiescent fibroblasts and other cell types (Marsh et al., 2013; Alkasalias et al., 2018). The CAF phenotype involves changes in the cellular morphology and enhanced migratory and contractile properties, similar to myofibroblasts. Markers such as alpha-smooth muscle actin (ACTA2), fibroblast activating protein (FAP), fibroblast specific protein 1 (S100A4) and stanniocalcin 1 (STC1) have been widely used to identify CAFs (Peña et al., 2013; Liu et al., 2019; Nissen et al., 2019). In this manner, canine CAFs have been shown to express higher levels of ACTA2 in various epithelial tumours compared with normal fibroblasts (Yoshimoto et al., 2017).

CAFs are one of the most studied cells within the TME in humans, studies performed in canine CAFs, however, are still scarce. Despite CAFs' heterogeneity and plasticity being until now poorly understood, targeting CAFs and/or their secretome may provide effective ways to overcome cancers by reducing the local immunosuppression and the remodelling of the TME (Liu et al., 2019).

2.5. Exosomes

As aforementioned, stromal cell- and cancer cell-derived EVs, in particular exosomes, play essential roles in mediating the intercellular crosstalk within the TME. Exosomes are extracellular nanosized vesicles with an approximate diameter of 30 to 150 nm and are secreted from all types of cells. Exosomal vesicles are originated from the inward budding of the plasma membrane to form intracellular endosomes. As early endosomes mature, intraluminal vesicles (ILVs) are formed as a result of the invagination of the endosomal membranes. Late endosomes containing ILVs are now named multivesicular bodies (MVBs). The MVBs can undergo lysosomal degradation or fuse with the plasma membrane, in which case they release their ILVs through exocytosis to the extracellular environment. These vesicles are then referred to as exosomes (Beach et al., 2014; Colombo et al., 2014; Doyle and Wang, 2019). Thus, the luminal content of exosomes is likely to be comparable to that of the cytoplasm of the cell of origin (Raposo and Stoorvogel, 2013). Exosomes are released *in vivo* into virtually all types of biological fluids of humans and dogs, while *in vitro* all tested cell

cultures have been found to shed exosomes to different extents (Raposo and Stoorvogel, 2013; Ichii et al., 2017; Fish et al., 2018).

Exosomes transport an assorted biological cargo which not only replicates the donor`s cell composition, but also reflects the enrichment of specific molecules selectively incorporated into their lumen (Valadi et al., 2007; Raposo and Stoorvogel, 2013). Various proteins such as transcription factors, enzymes, soluble factors, as well as DNA, RNAs (mRNA and miRNA), lipids and metabolites comprise the exosomal content (Mashouri et al., 2019). In this way, through this variable cargo, exosomes mediate the cellular crosstalk and modulate the downstream signalling pathways in their receiver cells (Anand et al., 2019). Because of their origin, a variety of endosome-associated proteins are found in exosomes, including alix, annexins, tumour susceptibility gen 101 (TSG101), and Rab proteins. Additionally, membrane proteins associated with microdomains in the plasma membrane or endosomes are often also increased in exosomes. Some of these comprise tetraspanins CD9, CD63, and CD81 (Raposo and Stoorvogel, 2013). Many of these proteins are frequently used as exosome markers. However, despite being commonly found in exosomes, endosome-associated proteins do not represent specific markers but rather enriched exosomal proteins. Interestingly, the variable relative proportion of different proteins may allow to distinguish diverse subsets of exosomes expressing common markers (Lötvall et al., 2014).

Exosomes bound to their recipient cells can either fuse with the cellular membrane and directly release their content into the cytoplasm, or get internalised via endocytosis. In addition, exosomes can also induce the activation of juxtacrine signalling, without delivery of their content. Cellular responses induced by RNA species rely on the cytoplasmatic incorporation of the exosomal cargo. After exosome uptake, a wide range of stimulatory or inhibitory functional outcomes may be induced in the recipient cell. Cellular proliferation, angiogenesis, apoptosis, cytokine production, immune modulation, and metastasis have been described following cellular interaction with exosomes (McKelvey et al., 2015; Mathieu et al., 2019).

The bidirectional communication between cancer cells and stromal cells is based on the transference of soluble factors or exosomes, which act as biological messengers (Ruivo et al., 2017). TME-derived exosomes are significantly involved in the modulation of the biological behaviour of their recipient cells via the transfer of their pro-oncogenic content. Cancer cells and immune cells, for example, contact each other through exosome-derived miRNAs to induce immune suppression (Zhou et al., 2018; Othman et al., 2019), or to promote angiogenesis (Ying et al., 2016). Likewise, exosome-dependent crosstalk between CAFs and cancer cells contributes to cancer progression and metastasis (Yang et al., 2019). Numerous miRNAs have been found enriched in CAF-derived exosomes in different cancer types. For instance, exosomal transference of miR-21 can suppress cancer cell apoptosis and increase resistance to chemotherapy (Yeung et al., 2016), as well as induce metastasis (Bhome et al.,

2017; Li et al., 2018). Cancer cell-secreted exosomes can, at the same time, induce the upregulation of matrix metalloproteinases in recipient cells (Rana et al., 2013), trigger fibroblast differentiation into CAFs (Goulet et al., 2018), and in general stimulate a pro-tumoral environment that supports cancer progression and survival (Melo et al., 2014; Othman et al., 2019).

The cellular status influences the composition and production of exosomes. Cellular stress in tumours increases the exosome release and also alters the composition of the messenger cargo (King et al., 2012). Hypoxia and acidic conditions are common stress characteristics within the TME. Other exogenous stimulus including chemotherapy and irradiation therapy can also induce changes in exosomal RNA and protein composition. Thus, exosomal-mediated crosstalk may influence the response of distant cells to stress by providing protective signals (Villarroya-Beltri et al., 2014). Remarkably, it has been shown that cancer exosomes are specifically enriched in miRNAs (Melo et al., 2014). Exosomal biomarkers have been in consequence proposed for various cancers, as exosomes contain a comparable molecular signature to a particular cancer type or to the cells they are released from (Othman et al., 2019).

2.6. MicroRNAs

Complex regulatory networks and interactions, which occur within the stroma and involve cancer cells, CAFs, immune cells, and non-cellular elements, dictate the tumour outcome. Thus, the cellular crosstalk has a major role modulating the immune response and has emerged as an important regulator of the tissue's healthy and cancerous status. In this context, it has been widely demonstrated that miRNAs mediate such crosstalk between the TME components and are recognised as crucial molecular players capable of regulating cancer progression (Paladini et al., 2016; Yang et al., 2018).

Mature miRNAs are single-stranded small ncRNA molecules containing in average 22 nucleotides in length. They can negatively regulate gene expression at a post-transcriptional level via imperfect base-pairing with complementary sequences within mRNA molecules. In most cases, miRNAs bind to the 3′ untranslated regions (UTR) of target mRNAs, although interactions with additional regions, including the 5′ UTR, coding sequences, and gene promoters have also been described for miRNAs. Accordingly, binding of a specific miRNA to its target mRNA typically leads to the translational repression of a protein-coding message or to the exonucleolytic decay of the mRNA (Bartel, 2009; Friedman et al., 2009; Ha and Kim, 2014; O'Brien et al., 2018).

It has become evident that miRNAs are involved in a wide variety of fundamental biological processes. They target genes controlling cellular processes such as cell cycle regulation, differentiation, apoptosis, migration, stress response, and inflammation, among

others. Thus, miRNA's aberrant expression has been associated with numerous diseases in humans and dogs, and not surprisingly also with cancers (Wagner et al., 2013; Di Leva et al., 2014; Ivey and Srivastava, 2015). Moreover, exosome-derived miRNAs are secreted into extracellular fluids and act as signalling molecules to mediate cellular crosstalk. For example, the characterisation of plasma-derived exosomal RNAs in humans detected miRNAs as the most common fraction (~42%) (Huang et al., 2013). Many researchers have taken advantage of this and investigated extracellular miRNAs as potential biomarkers for several diseases (Mizuno et al., 2011; Wagner et al., 2013; O'Brien et al., 2018).

The evidence shows that in tumours miRNAs act either as suppressors or promoters of the cancer phenotype, by inhibiting the expression of oncogenes or by repressing tumour suppressor genes, respectively (Medina et al., 2010). Therefore, the expression of tumour-suppressive miRNAs (also named anti-oncomiRs) is decreased in cancers, while oncogenic miRNAs (oncomiRs) are overexpressed. Moreover, researchers have identified that cancers may become dependent on specific miRNAs. This is why targeting single miRNAs could significantly reduce cancer cell proliferation, metastasis, and/or survival, despite the extensive complexion of tumorigenesis (Medina et al., 2010; Svoronos et al., 2016).

Approximately one-third of the known mammalian miRNAs are encoded within the introns of protein-coding genes, whereas most other miRNAs derive from miRNA gene loci. miRNA biogenesis begins with the transcription of a primary miRNA transcript (pri-miRNA). Stress signals in cancer cells alter the functionality of certain transcription factors that work as activators or repressors of pri-miRNAs, which in turn leads to a dysregulated miRNA expression. Additionally, genomic variations occurring in cancer cells also alter pri-miRNA transcription (Lin and Gregory, 2015). miRNA's aberrant expression is currently considered a common characteristic of all tumours and associated with nearly every, if not all, stages of cancer initiation and progression, highlighting the importance of miRNAs within the TME (Pan et al., 2020). In this context, miRNAs stabilise cancer hallmark features in neighbouring cells through direct manipulation of tumour angiogenesis, immune invasion and tumour-stromal interactions (Suzuki et al., 2015). Such pro-tumorigenic effects of miRNAs are based on the induction of diverse soluble factors (including TGFβ, PDGF, VEGFA, IL6, FGFs, CXCL12) and the activation of specific signalling pathways (Lei et al., 2009; Li et al., 2012; Givel et al., 2018).

Studies based mostly on human carcinomas have recognised the abnormal expression of specific miRNAs as one of the major mechanisms for differentiation and activation of CAFs (Mansoori et al., 2017). The variety of miRNAs involved in CAF activation among tissues and tumour types may be directly correlated with the heterogeneity of CAFs' origins and functions (Wang et al., 2017b). Therefore, particular dysregulation of miR-15a and miR-16 (Musumeci et al., 2011), miR-21 (Chen et al., 2018), miR-1, miR-206 and miR-31 (Shen et al., 2016), miR-31, miR-155 and miR-214 (Mitra et al., 2012), and miR-222 (Chatterjee et al., 2019) has been

shown to contribute to or induce the reprogramming of CAFs in different cancer types. Interestingly, data suggest that CAF-derived miRNAs can also mediate a paracrine activation of non-activated fibroblasts, thus expanding the reactive phenotype to adjacent fibroblasts and supporting cancer progression (Doldi et al., 2015). So far, however, there are no studies in canine cancers evaluating interactions between miRNAs and CAFs.

miRNAs released by CAFs can in turn also influence various characteristics of cancer cells. Available data indicate that in order to preserve the pro-tumorigenic environment, CAFs and cancer cells communicate with each other via miRNAs contained in EVs (Kogure et al., 2019). As such, exosome-derived miRNAs from CAFs have been identified as responsible for inducing epithelial-mesenchymal transition and downregulating the expression of tumour suppressor genes (Josson et al., 2015), for stimulating stemness and an aggressive cancer phenotype (Donnarumma et al., 2017), as well as for promoting chemoresistance (Fang et al., 2019) and metastasis (Sun et al., 2019).

Cumulative data are revealing that the dysregulation of miRNAs may be in fact an emerging hallmark of cancer, both in the tumour itself and in the TME (Price and Chen, 2014). Remarkably, numerous miRNAs are evolutionary conserved between humans and dogs, highlighting the importance of the domestic dog as model organism for miRNAs within the field of cancer research. It is also very likely that in canine malignancies these molecules are expressed in a similar manner and exert analogous functions. Thus, the identification of dysregulated miRNAs and their expression patterns in specific types of cancers may lead to the discovery of novel biomarkers and/or new miRNA-based therapies for both species (Wagner et al., 2013).

3. Aim of the study

CAFs play significant roles within the TME by promoting and supporting the tumour growth. However, despite CAFs being one of the most investigated cell types within the TME in humans, only few studies have been performed on canine CAFs. From a cell-to-cell communication approach, investigating how normal fibroblasts are transformed into CAFs may deliver valuable information to understand such interactions. Cellular crosstalk is a key inducer of fibroblast activation and exosome-derived miRNA signalling seems to be a major effector of such cellular reprogramming. Furthermore, numerous studies have shown the similarities between dog and human malignancies, as well as how pet dogs are unique as animal models in cancer genetics.

To improve the understanding of how normal fibroblasts can be activated by cancer cells, the effects of intercellular communication between normal canine dermal fibroblasts and a mast cell tumour cell line were investigated. It was hypothesised that the co-culture of canine primary fibroblasts with the mast cell tumour cell line C2 induces the activation of fibroblasts via miRNA dysregulation, which is mediated by intercellular crosstalk. Therefore, the specific aims of this project were as follows:

1. Identification and characterisation of intact cell culture-derived exosomes in dogs, which evaluates the potential for cellular crosstalk
2. Assessment of C2-induced miRNA dysregulations in canine fibroblasts and their potential to induce fibroblast activation
3. Identification of potential interactions between miRNAs and their cancer-associated target genes, via mRNA and protein expression profiling
4. Support the role of the dog as an animal model in the field of cancer research

4. Subsuming the published work

4.1. Publication 1: Exploration of serum- and cell culture-derived exosomes from dogs

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Exploration of serum- and cell culture-derived exosomes from dogs

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Abstract

Background: Exosomes are defined as extracellular membrane vesicles, 30–150 nm in diameter, derived from all types of cells. They originate via endocytosis and then they are released through exocytosis to the extracellular space, being found in various biological fluids as well as in cell culture medium. In the last few years, exosomes have gained considerable scientific interest due to their potential use as biomarkers, especially in the field of cancer research. This report describes a method to isolate, quantify and identify serum- and cell culture-derived exosomes from dog samples, using small volumes (100 μL and 1 mL, respectively).

Results: Quantification and sizing of exosomes contained in serum and cell culture samples were assessed by utilizing nanoparticle tracking analysis, transmission electron microscopy and immunoelectron microscopy. Detected particles showed the normal size (30–150 nm) and morphology described for exosomes, as well as presence of the transmembrane protein CD63 known as exosomal marker.

Conclusions: Based on a validated rapid isolation procedure of nanoparticles from small volumes of different types of dog samples, a characterization and exploration of intact exosomes, as well as facilitation for their analysis in downstream applications was introduced.

Keywords: Exosomes, Serum, Cell culture medium, Dog, Transmission electron microscopy, Nanoparticle tracking analysis, Biomarkers

Background

Exosomes are extracellular nano-sized membrane vesicles, reported as 30–150 nm in diameter, derived from all types of cells and released into practically all biological fluids such as blood, urine, cerebrospinal fluid, milk, sputum, saliva, seminal fluid, as well as into cell culture medium [1, 2]. These vesicles originate via endocytosis, initially forming endosomes and followed by invagination of the endosomal membrane to create multivesicular bodies (MVBs). Afterwards through exocytosis, the content of the MVBs is released as exosomes to the extracellular space once merging with the plasma membrane [3, 4].

The exosomal membrane consists mostly of lipids and proteins, while the luminal cargo is mainly represented by proteins and nucleic acids, including mRNAs, microRNAs, other non-coding RNAs and DNA [5–7]. Exosomes have been proven to possess several functions, for instance, intercellular communication, genetic exchange and antigen presentation, allowing cells to transport their cargo in a short and long distance manner and subsequently having a significant effect at a cellular and biological level [6, 7]. Since exosomes are of endosomal origin, they contain a distinct set of proteins involved in membrane transport and fusion (e.g. Rab GTPases, annexins, flotillin), biogenesis of MVBs (Alix, TSG101), major histocompatibility complex class I and II, in processes requiring heat shock proteins (hsc70 and 90), integrins and tetraspanins (e.g. CD63, CD9, CD81 and CD82) [6, 8, 9]. Even though some of these proteins are used as exosome markers, exosomal protein composition might differ based on the origin of the cells or tissue [7, 10].

Analyses of cargo proteins and nucleic acids present in exosomes show significant potential to be employed as exosomal biomarkers. Taking this into consideration, together with the ability to easily isolate exosomes from body fluids (liquid biopsy), these vesicles may deliver an additional valuable non-invasive biomarker for predisposition, prognosis and treatment monitoring in the cancer research field

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[7, 11]. Furthermore, when understanding endogenous transmission of distinct macromolecules between tissues via exosomes, a (dys) functional cell-cell communication could be focused (diagnostic tool) and subsequently modified (therapeutic tool).

In this report we describe a method to isolate and identify serum- and cell culture-derived exosomes from dog samples. This study provides comprehensive techniques such as transmission electron microscopy, nanoparticle tracking analysis and immunodetection to identify and characterize exosomes, allowing them to be quantified and sized, as well as characterized through specific morphology and a distinct protein expression.

Methods

Blood serum

Samples ($n = 10$) were gathered from 5 female and 5 male dogs of different ages (between 1 and 7 years old), non-cancer ($n = 6$) and cancer patients ($n = 4$), presented at the Small Animal Clinic, Department of Veterinary Medicine at the Freie Universität Berlin. Blood samples were collected in tubes without anticoagulant and left at room temperature to allow clotting for 30 min to 2 h. The main portion of the serum was used for the original diagnostic laboratory analyses, while the remaining amount was employed for this study. The protocol to separate and store serum was based on a published technical note from QIAGEN (miRNeasy Serum/Plasma Handbook 02/2012). Briefly, tubes were first centrifuged at 2000 x g for 10 min at 4 °C to separate residual cellular components of the blood. The supernatant was then placed in another tube and centrifuged at 16,000 x g for 10 min at 4 °C to separate any left cellular debris. Afterwards, the purified serum was taken and stored in − 80 °C until exosome isolation.

Cell cultures

C2 cell line

C2 cells, a canine mast cell tumour cell line, were kindly provided in August 2016 by Dr. Patrice Dubreuil (Centre de Recherche en Cancérologie de Marseille, Inserm U1068, Marseille, France), after previous consent of the cell line originator, Dr. Warren Gold (University of California San Francisco, School of Medicine, California, USA) [12]. Cells were cultured in RPMI 1640 medium, supplemented with 10% foetal bovine serum (FBS) superior, 100 U/mL penicillin/streptomycin (all from Biochrom, Berlin, Germany), 1 mM/mL sodium pyruvate and 2 mM/mL glutamine (both from Sigma, MO, USA), and incubated in a 5% CO_2 atmosphere at 37 °C. Special culture conditions were applied before exosomes were harvested (see exosome isolation paragraph).

Primary canine fibroblasts culture

Fibroblasts (FBs) were obtained from a portion of healthy skin of a female Golden Retriever, within 20 min after the animal was euthanized at the Small Animal Clinic, Department of Veterinary Medicine at the Freie Universität Berlin. The skin was collected in sterile Dulbecco's phosphate buffer saline (DPBS) (Sigma, MO, USA) and then placed in a Petri dish. Dermis was separated from epidermis using sterile forceps and scalpels. The dermis was cut in small pieces (1 × 3 mm approx.) and washed in DPBS, supplemented with 100 U/mL penicillin/streptomycin and 250 μg/mL amphotericin B (Biochrom, Berlin, Germany). Then, a 5 min centrifugation at 300 x g was performed and the supernatant was discarded. The sediment, representing the FBs, was resuspended in an enzymatic digestion medium containing 0.15% collagenase I (Biochrom, Berlin, Germany), RPMI 1640 medium, supplemented with antibiotic and fungicide as described above, and 1% 70 mM $CaCl_2$ (Merck, Darmstadt, Germany). The sample was transferred into a Petri dish and incubated at 37 °C for 2 h under constant agitation, then placed into a sterile 50 mL tube and centrifuged 5 min at 300 x g, the supernatant was discarded. The pellet was washed twice in warm (37 °C) RPMI 1640 medium, supplemented with 20% FBS, 100 U/mL penicillin/streptomycin, 250 μg/mL amphotericin B, 1 mM/mL sodium pyruvate and 2 mM/mL glutamine, and centrifuged 5 min at 300 x g. Lastly, the resulting pellet was seeded in a T25 flask in 7 ml of the same medium used for the last two washing steps and incubated in a 5% CO_2 atmosphere at 37 °C. The first passage was performed 10 days after seeding and passage number 5 was used for the exosome isolation. Special culture conditions were applied before exosomes were harvested (see exosome isolation paragraph).

Exosome isolation

Serum samples

Exosome isolation from serum samples was accomplished using a commercial kit (Total Exosome Isolation Reagent – from serum; Invitrogen, Vilnius, Lithuania) following the manufacturer's protocol. Briefly, purified serum was passed through a 0.22 μm pore PVDF filter (Rotilabo, Karlsruhe, Germany). After that, 100 μL of filtered serum was mixed with 20 μL of reagent and incubated at 4 °C for 30 min. Then, samples were centrifuged at 16,000 x g for 10 min at room temperature and the supernatant was discarded. The pellet containing exosomes was resuspended in 20 to 50 μL of DPBS, depending on the downstream applications.

Cell culture samples

In cell culture medium from the C2 cell line and from the primary FBs culture, exosome isolation was performed utilizing a commercial kit (Total Exosome

Isolation Reagent – from cell culture media; Invitrogen, Vilnius, Lithuania), although some modifications to the manufacturer's protocol were applied. For this purpose, prior to culturing cells for exosome isolation, 50–80% confluent C2 cells and primary FBs were washed twice in DPBS and further cultured in an exosome-free medium as described above, except for using exosome-depleted FBS (Gibco, USA). Briefly, cell culture medium was harvested after 48 and 72 h of incubation with exosome-depleted medium and centrifuged at room temperature; first, 5 min at 300 x g to remove floating cells and a subsequent 30 min 3000 x g centrifugation to eliminate cellular debris. Afterwards, the purified medium was passed through a 0.22 μm pore PVDF filter and then 1 mL of filtered medium was mixed with the volume of reagent indicated by the manufacturer. The mixture was incubated at 4 °C overnight and finally centrifuged at 4 °C at 11,000 x g for 60 min. The pellet containing exosomes was re-suspended in 20 to 50 μL of DPBS, depending on the downstream applications.

Transmission electron microscopy (TEM)

To identify exosomes and investigate their ultrastructural morphology, a Zeiss EM 109 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) operating at 80 kV was utilized, following the protocol developed by Théry et al. (2006) [13], with some modifications.

Native exosomes

For analysis of native exosomes, PBS-suspensions containing unfixed exosomes were differentially diluted in filtered PBS (0.22 μm pore PVDF filter). For serum-derived exosomes; undiluted suspension and 1:100, 1:1000 and 1:2000 dilutions were used, while for culture-derived exosomes; undiluted suspension and 1:50, 1:100 and 1:200 dilutions were applied. Formvar-carbon-coated 100 mesh nickel grids (Plano, Wetzlar, Germany) were laid on a 5 μL drop of the exosome-suspension and incubated 20 min at room temperature for adhesion (coated side of the grid facing the suspension), then washed 3 times for 3 min in filtered PBS. Next, grids were placed 2 times on drops of 50 mM glycine/PBS for 3 min and then transferred to a drop of 0.5% bovine serum albumin (BSA)/PBS blocking solution for 10 min. For contrasting the exosomes, grids were laid on 2% uranyl acetate drops for 6 min, followed by 2 washes with distilled water. Grids were allowed to dry overnight.

Immuno-gold labelled exosomes

For examination of immuno-gold labelled unfixed exosomes, anti-CD63 (ABIN1440014, antibodies-online), a goat polyclonal multi-species primary antibody, and a secondary antibody anti-goat IgG (whole molecule) labelled with 10 nm gold (Sigma, MO, USA) were used. The first part of the protocol was identical to the procedure for analysis of native exosomes up to placing the grids on drops of 0.5% BSA/PBS blocking solution for 10 min. This was followed by an incubation step for 2 h at room temperature with the primary antibody anti-CD63 (dilution 1:50 in 0.5% BSA/PBS). Afterwards, grids were washed 5 times for 3 min in drops of 0.5% BSA/PBS and an additional incubation with the secondary antibody anti-goat IgG-10 nm gold (dilution 1:50 in 0.5% BSA/PBS) was performed for 1 h at room temperature. Grids were washed again 5 times for 3 min in 0.5% BSA/PBS drops, and then laid on 2% uranyl acetate drops for 6 min for contrasting, followed by 2 washes in distilled water. Grids were allowed to dry overnight.

Nanoparticle tracking analysis (NTA)

Quantification and size determination of dog exosomes purified from serum and cell culture medium was assessed by using the NanoSight NS500 instrument (Malvern, Worcestershire, UK). The NTA 3.0 (build 0064) software visualizes and analyses nanoparticles in real time by associating Brownian motion with particle size. Fresh serum- and cell culture-derived exosomes samples were processed in duplicate and diluted in filtered PBS (0.22 μm pore PVDF filter) until reaching a concentration between 10 and 100 particles per image (optimal ~ 50 particles per image) before examination with the NTA system [14]. The instrument was set up to operate at 25 °C, three videos, 30 s each, were recorded for each specimen and outcomes were analysed with the NTA software.

Statistical analysis

The data analysis was performed using the software Microsoft Excel 2010 (Microsoft, Redmond, WA, USA), through one-way analysis of variance (ANOVA) and Bonferroni corrected post-hoc Student's t-tests. P value < 0.05 was considered as significant.

Results

A rapid protocol was validated to isolate nanoparticles from dog samples suitable to further detect size, quantity and evaluate selected protein expression.

Size and quantification of exosomes by NTA

A suitable real-time visualization and analysis of exosomes present in fluid samples could be easily performed by the NTA system, both in blood serum (Table 1, Fig. 1a) and in culture media (Table 2, Fig. 1b and c).

Serum samples

100 μL of canine serum was employed to isolate serum-derived exosomes from 6 non-cancer and 4 cancer dog patients. Most of the observed nanoparticles

Table 1 Exosome concentration and size distribution

Sample ID	Exosome concentration (xE10/mL)	Particle size mean (nm)	Type of sample
S1	403.2 +/− 25.8	71.3 +/− 3.1	Non-cancer
S2	107.4 +/− 6.8	90.5 +/− 14.5	Non-cancer
S3	322.8 +/− 24.0	89.5 +/− 1.4	Non-cancer
S10	198.0 +/− 19.9	89.9 +/− 13.2	Non-cancer
SHB	374.4 +/− 21.8	112.5 +/− 12.8	Non-cancer
SNT	219.6 +/− 17.3	111.3 +/− 10.7	Non-cancer
S8	397.2 +/− 18.6	113.9 +/− 9.0	Splenic mast cell tumour
SP	225.6 +/− 10.4	99.0 +/− 8.4	Prostatic carcinoma
S15	500.4 +/− 76.4	84.7 +/− 1.5	Perianal adenoma
SVT	277.2 +/− 11.3	84.5 +/− 0.6	Vaginal leiomyosarcoma

Serum-derived exosomes from non-cancer and cancer dog patients (mean +/− standard error)

were found to be 30 to 150 nm in diameter (Table 1, Fig. 1a), i.e. the normal size described for exosomes [1, 2], however few particles showed a larger size. The mean size range for serum-derived exosomes observed was between 71.3 +/− 3.1 and 113.9 +/− 9.0 nm. In terms of exosome concentration, in non-cancer patients nanoparticle concentration (xE10/mL) was between 107.4 +/− 6.8 and 403.2 +/− 25.8, while in cancer patients the lowest and the highest concentrations (xE10/mL) were 225.6 +/− 10.4 and 500.4 +/− 76.4, respectively. Nevertheless, no significant difference ($P > 0.05$) between non-cancer and cancer samples could be calculated, although a large variation within individual samples was detected.

Cell culture medium samples

Exosomes could be isolated from 1 mL of culture medium obtained from cultured C2 cells and primary FBs, after 48 and 72 h of incubation under exosome-depleted media conditions. Likewise in the analysis of dog serum samples, the commercial kit was found suitable for isolating exosomes derived from dog cell cultures. The majority of the nanoparticles exhibited the normal size described for exosomes, 30 to 150 nm in diameter (Table 2, Fig. 1b and c) [1, 2], while a small number was found to be in the 150–300 nm range. The mean size range for culture-derived exosomes observed in both types of cultures and both time-points was between 110.3 +/− 5.0 and 129.0 +/− 7.4 nm. In terms of nanoparticle quantification, after 48 and 72 h C2 cells cultures showed a significant ($P < 0.05$) 2–3-fold higher exosome concentration compared to primary FBs cultures but no difference ($P > 0.05$) between incubation times (48 vs. 72 h) was observed in either group.

Negative controls

To screen for potentially contaminating particles, samples from all solutions used (PBS, RPMI 1640 medium, RPMI 1640 + exosome isolation kit, and complete culture medium + exosome isolation kit) were analysed as negative controls. The number of particles detected in these fluids was low and did not affect the total concentration of exosomes per mL counted by the NTA system. Therefore, exogenous contamination interacting with a

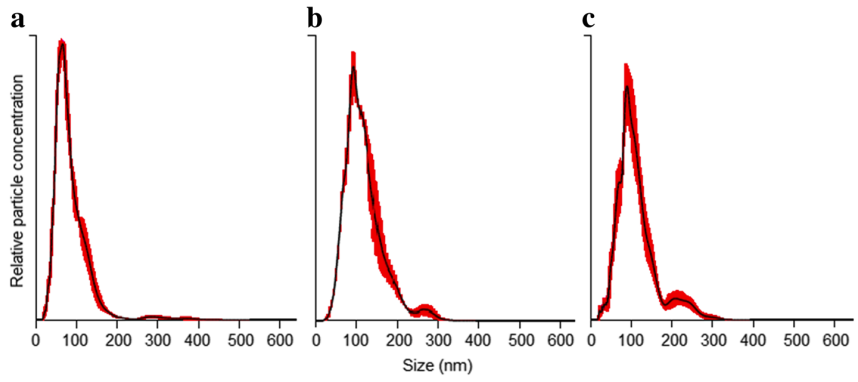

Fig. 1 Nano track analysis size distribution of exosomes isolated from samples of canine origin. **a** blood serum-derived exosomes, **b** C2 cell line culture-derived exosomes and (**c**) primary fibroblasts culture-derived exosomes. Red error bars indicate +/− standard error of the mean

Table 2 Concentration and size distribution of exosomes

Sample ID	Exosome concentration (xE10/mL)	Particle size mean (nm)	Type of sample
C248	17.5 +/− 0.9	120.9 +/− 2.1	C2 cells culture medium, 48 h incubation
C272	12.8 +/− 1.7	118.0 +/− 4.5	C2 cells culture medium, 72 h incubation
FB48	6.4 +/− 0.7	110.3 +/− 5.0	Primary FBs culture medium, 48 h incubation
FB72	7.3 +/− 1.5	129.0 +/− 7.4	Primary FBs culture medium, 72 h incubation

Culture medium-derived exosomes from C2 cell line and primary fibroblasts, after 48 and 72 h of incubation under exosome-free media conditions (mean +/− standard error)

valid characterization of canine exosomes can be excluded as a factor in our system (Table 3).

Exosome morphology by TEM

General morphology and ultrastructure of serum- and culture-derived exosomes of canine origin was assessed by using TEM technology, allowing visualization of the characteristic central depression or "cup shape" of exosomes [15, 16], either single (Fig. 2a and b) or aggregated (Fig. 2c and d). All samples revealed single and aggregated nanoparticles; non-diluted samples showed a higher number of exosome aggregates, whereas samples diluted 1:2000 displayed more individual exosomes, yet it was more difficult to localize them on the grids. Morphology and size of the depicted nanoparticles correspond to their exosomal origin, as described in several studies performed in samples of human fluids and cell culture origin [1, 13–15].

Protein expression by immunoelectron microscopy

Results presented in Fig. 3 revealed the presence of the transmembrane protein CD63 in all samples investigated in this study. It is important to note that not every single exosome observed by TEM expressed this protein. Indeed, the number of exosomes negative for CD63 was slightly greater to the number of exosomes expressing the protein.

Discussion

Size and quantification

The commercial kit used states a simple and quick precipitation method for isolation of intact exosomes, allowing them to be collected by a short, low-speed centrifugation easily applicable in most clinical laboratories [2]. The introduced NTA technology overcomes some limitations inherent to TEM-based methods, such as lack of absolute quantification and quick size determination of exosomes, as well as time-consuming protocols for sample preparation. For that reason, NTA-based procedures appear highly suitable to rapidly characterize size distribution and number of exosomes. However, the NTA system is not able to distinguish between extracellular vesicles (EVs) and other similar sized particles, such as clusters of exosomes, cellular debris or protein aggregates. Moreover, especially when working with precipitation methods, co-isolation of non-exosomal particles, for instance larger serum/plasma protein aggregates or lipoproteins, cannot be excluded [1, 17, 18]. These data might explain why we were able to also observe minor signals showing particles between 150 and 400 nm in the size distribution graph (Fig. 1) in addition to the major peak around 100 nm.

In our approach, culture-derived exosomes were found to have a significantly larger average size than serum-derived specimens, but an obvious size variation between both types of cell cultures was not found. Different studies have provided evidence that EVs vary in size depending on their cells of origin and there are even data published showing variation based on the method of visualization [19–21].

The exosome quantification variability between both cell culture types under the same culture conditions might be explained by the fact that C2 cells represent a cancer cell line. Since exosome secretion is normally increased in cancer [22], a higher exosome concentration in C2 cell medium was expected. Compared to primary FBs, the faster growth rate of C2 cells determines the number of cells contained in each culture flask, an aspect that certainly influences the exosome production. Moreover, it has been well documented that some types of cancer cell lines shed higher amounts of exosomes

Table 3 Negative controls

Sample ID	Exosome concentration (xE7/mL)	Particle size mean (nm)	Type of sample
CN1	0.88 +/− 0.36	188.2 +/− 62.1	PBS
CN2	1.33 +/− 0.75	138.9 +/− 69.6	RPMI 1640
CN3	1.40 +/− 0.34	130.4 +/− 11.5	RPMI 1640 + Exosome isolation kit
CN4	1.26 +/− 0.61	147.6 +/− 22.6	Complete culture medium + Exosome isolation kit

Solutions employed during harvest and dilution processing of exosomes (mean +/− standard error)

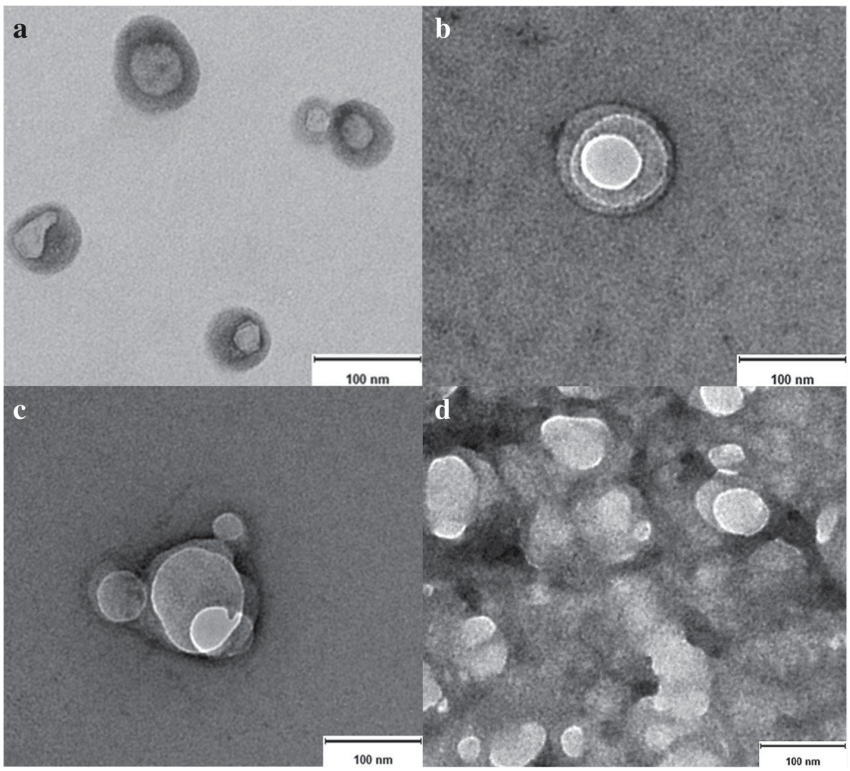

Fig. 2 Transmission electron microscopy of native exosomes isolated from samples of canine origin. **a** blood serum-derived exosomes, **b** C2 cell line culture-derived exosomes and (**c** and **d**) primary fibroblasts culture-derived exosomes. Size bar = 100 nm

than others and conditions like hypoxia may increase exosome production up to 90% [23, 24]. Many other elements can also affect exosome shedding in normal and diseased cells, including chemical factors like, calcium, calcium ionophores, phosphatidylinositol 3-kinase, and pH, as well as physical factors such as heat, ischemia, cellular stresses, and loss of cellular attachment [25].

Compared to serum samples, the number of exosomes found in cell culture media was significantly lower. It is known that in vivo exosomes are shed by all types of cells, in normal and diseased conditions [1, 2]. Taking that into consideration, along with the intercellular cross-talks occurring in complex organisms, the total number of cells in a living organism (a dog in this case) releasing exosomes into all body-fluid compartments, is in fact not comparable to the limited number of cells ($2–3 \times 10^6$) contained in our in vitro culture system.

It is worth to mention that although an aim of this report was to isolate and identify exosomes from different dog serum samples, no differences in size distribution and quantification between non-cancer and cancer dog patients were noticed. Hence, further investigations exploring potential variations between healthy and diseased groups including a larger number of individuals shall follow, since some reports have already shown that cancer cells secrete more exosomes than non-cancer cells [22].

Morphology and protein expression

Electron microscopy allowed the assessment of morphology and protein expression. Since most optical methods using light scattering to analyse substances or matter, such as flow cytometry and optical microscopy, are hardly able to detect particles smaller than 200 nm, TEM is essential to study the morphology of exosomes and is considered the standard method in this regard [15, 26]. When referring to morphology of nanoparticles, it involves their overall shape, while TEM detects also ultrastructural differences in their shape, contrast and surface patterns [3]. Although we and other researchers described the morphology of exosomes as cup shaped when observed by TEM, it seems to be an artefact generated by fixation and/or contrasting steps [13, 15], that is also associated with shrinking of vesicles [27, 28]. Studies employing scanner electron microscopy and cryo-electron microscopy revealed that exosomes have indeed a round/spherical shape [15, 20, 29, 30].

Immunoelectron microscopy allowed the detection and direct imaging of the transmembrane protein CD63,

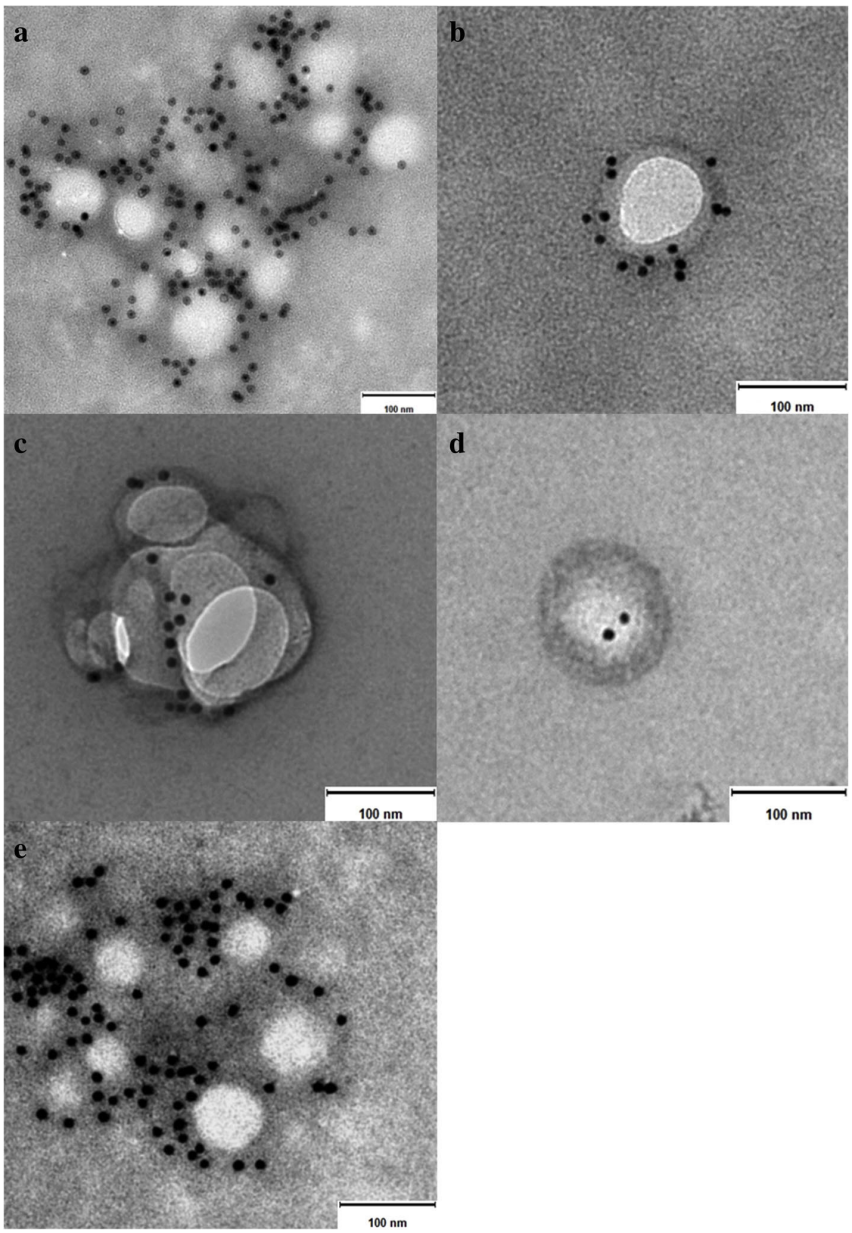

Fig. 3 Immunoelectron microscopy images of exosomes isolated from samples of canine origin. **a** and **b** serum-derived exosomes, **c** C2 cell line culture-derived exosomes, **d** and **e** primary fibroblasts culture-derived exosomes. Note the gold particles bound to the exosome membrane indicating presence of the tetraspanin CD63. Size bar = 100 nm

which bound to a selective secondary antibody labelled with gold particles [31] (Fig. 3). Exosomes represent a heterogeneous population of EVs expressing diverse patterns of molecules. Numerous studies have shown that some of these molecules are found frequently in exosomes, and therefore, they have gained support to be used as exosomal markers, e.g. proteins [13, 15, 31]. Since they all bear an endosomal origin, it is expected that exosomes contain different cargos of tetraspanin proteins, a family of membrane proteins. The tetraspanin CD63 is currently being used widely as a molecular exosome marker by diverse research studies in this field [7, 10]. The tetraspanin family includes a large amount of transmembrane proteins and only the most common members are made available as molecular exosomal markers, including CD63, CD9, CD81 and CD82 [5, 7,

10]. Several investigations have already demonstrated that the molecular characteristics vary broadly among exosomes from different sources, even across exosomes secreted by the same type of cells [10, 22, 25]. Accordingly, the fact that not all of the observed exosomes expressed CD63 was indeed contemplated.

Conclusion

Our results evidence the feasibility to easily and rapidly isolate intact exosomes from small volumes of serum, as well as from a tumour cell line and a primary fibroblast culture, all from dog origin, allowing nanoparticles to be analysed in downstream applications. The NTA system provides a quick and easy way to size and quantify exosomes, while TEM facilitates the morphology assessment and distinct immunodetection. The exosome research field has in the past years become an emerging area among researchers of all biological sciences. However, in veterinary medicine it is not yet a well-developed matter. Hence, by demonstrating techniques of isolation, characterization and exploration, this report supports the data until now available in the veterinary diagnostic field, encouraging scientists and clinicians to further explore exosomes of canine origin.

Abbreviations

ANOVA: Analysis of variance; BSA: Bovine serum albumin; DPBS: Dulbecco's phosphate buffer saline; EVs: Extracellular vesicles; FBs: Fibroblasts; FBS: Foetal bovine serum; MVBs: Multivesicular bodies; NTA: Nanoparticle tracking analysis; PBS: Phosphate buffer saline; PVDF: Polyvinylidene fluoride; TEM: Transmission electron microscopy

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Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MAR, prepared the manuscript. MAR, BWR and RE, were responsible for the conception and design of the study. BWR and RE supervised the activity planning and execution. RE, JP and BK revised the manuscript critically. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The ethics committee from the Governmental Office for Health and Social Affairs Berlin (LAGeSo Berlin), file number StN 0005/17, do not classify the use of residual blood from diagnostic samples from dogs or cats and the use of euthanized animals as animal experimentation.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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References

1. Helwa I, Cai J, Drewry MD, Zimmerman A, Dinkins MB, Khaled ML, et al. A comparative study of serum exosome isolation using differential ultracentrifugation and three commercial reagents. PLoS One. 2017;12(1):e0170628.
2. Li M, Rai AJ, DeCastro GJ, Zeringer E, Barta T, Magdaleno S, et al. An optimized procedure for exosome isolation and analysis using serum samples: application to cancer biomarker discovery. Methods. 2015;87:26–30.
3. Urbanelli L, Magini A, Buratta S, Brozzi A, Sagini K, Polchi A, et al. Signaling pathways in exosomes biogenesis, secretion and fate. Genes (Basel). 2013;4(2):152–70.
4. Zhang J, Li S, Li L, Li M, Guo C, Yao J, et al. Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics Proteomics Bioinformatics. 2015;13(1):17–24.
5. Kumar D, Gupta D, Shankar S, Srivastava RK. Biomolecular characterization of exosomes released from cancer stem cells: possible implications for biomarker and treatment of cancer. Oncotarget. 2015;6(5):3280–91.
6. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. J Proteome. 2010;73(10):1907–20.
7. Vlassov AV, Magdaleno S, Setterquist R, Conrad R. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochim Biophys Acta. 2012;1820(7):940–8.
8. Simons M, Raposo G. Exosomes–vesicular carriers for intercellular communication. Curr Opin Cell Biol. 2009;21(4):575–81.
9. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomics. 2009;6(3):267–83.
10. Lötvall J, Hill AF, Hochberg F, Buzás EI, di Vizio D, Gardiner C, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles. 2014;3:26913.
11. Tickner JA, Urquhart AJ, Stephenson S-A, Richard DJ, O'Byrne KJ. Functions and therapeutic roles of exosomes in cancer. Front Oncol. 2014;4:127.
12. DeVinney R, Gold WM. Establishment of two dog mastocytoma cell lines in continuous culture. Am J Respir Cell Mol Biol. 1990;3(5):413–20.
13. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. 2006;Chapter 3(Unit 3):22. https://doi.org/10.1002/0471143030.cb0322s30.
14. Salomon C, Yee S, Scholz-Romero K, Kobayashi M, Vaswani K, Kvaskoff D, et al. Extravillous trophoblast cells-derived exosomes promote vascular smooth muscle cell migration. Front Pharmacol. 2014;5:175.
15. Wu Y, Deng W, Klinke DJ. Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers. Analyst. 2015;140(19):6631–42.
16. Zhang X, Yuan X, Shi H, Wu L, Qian H, Xu W. Exosomes in cancer: small particle, big player. J Hematol Oncol. 2015;8:83.
17. Mørk M, Pedersen S, Botha J, Lund SM, Kristensen SR. Preanalytical, analytical, and biological variation of blood plasma submicron particle levels measured with nanoparticle tracking analysis and tunable resistive pulse sensing. Scand J Clin Lab Invest. 2016;76(5):349–60.
18. Tang Y-T, Huang Y-Y, Zheng L, Qin S-H, Xu X-P, An T-X, et al. Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum. Int J Mol Med. 2017;40(3):834–44.
19. Gheldof D, Hardij J, Cecchet F, Chatelain B, Dogné J, Mullier F. Thrombin generation assay and transmission electron microscopy: a useful

combination to study tissue factor-bearing microvesicles. J Extracell Vesicles. 2013;2(1):19728.
20. Green TM, Alpaugh ML, Barsky SH, Rappa G, Lorico A. Breast cancer-derived extracellular vesicles: characterization and contribution to the metastatic phenotype. Biomed Res Int. 2015;2015:634865.
21. Singh R, Pochampally R, Watabe K, Lu Z, Mo Y. Exosome-mediated transfer of miR-10b promotes cell invasion in breast cancer. Mol Cancer. 2014;13:256.
22. Whiteside TL. Tumor-derived exosomes and their role in cancer progression. Adv Clin Chem. 2016;74:103–41.
23. King HW, Michael MZ, Gleadle JM. Hypoxic enhancement of exosome release by breast cancer cells. BMC Cancer. 2012;12:421.
24. Shedden K, Xie XT, Chandaroy P, Chang YT, Rosania GR. Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles. Cancer Res. 2003;63(15):4331–7.
25. Zhang HG, Grizzle WE. Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. Am J Pathol. 2014;184(1):28–41.
26. van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R. Optical and non-optical methods for detection and characterization of microparticles and exosomes. J Thromb Haemost. 2010;8(12):2596–607.
27. van der Pol E, Coumans FA, Grootemaat AE, Gardiner C, Sargent IL, Harrison P, et al. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. J Thromb Haemost. 2014;12(7):1182–92.
28. Yang VK, Loughran KA, Meola DM, Juhr CM, Thane KE, Davis AM, et al. Circulating exosome microRNA associated with heart failure secondary to myxomatous mitral valve disease in a naturally occurring canine model. J Extracell Vesicles. 2017;6(1):1350088.
29. Ichii O, Ohta H, Horino T, Nakamura T, Hosotani M, Mizoguchi T, et al. Urinary exosome-derived microRNAs reflecting the changes of renal function and histopathology in dogs. Sci Rep. 2017;7:40340.
30. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. 2013;200(4):373–83.
31. Viens A, Harper F, Pichard E, Comisso M, Pierron G, Ogryzko V. Use of protein biotinylation in vivo for immunoelectron microscopic localization of a specific protein isoform. J Histochem Cytochem. 2008;56(10):911–9.

4.2. Publication 2: Deregulation of miR-27a may contribute to canine fibroblast activation after coculture with a mast cell tumour cell line

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Deregulation of miR-27a may contribute to canine fibroblast activation after coculture with a mast cell tumour cell line

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Keywords
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The tumour microenvironment comprises a diverse range of cells, including fibroblasts, immune cells and endothelial cells, along with extracellular matrix. In particular, fibroblasts are of significant interest as these cells are reprogrammed during tumorigenesis to become cancer-associated fibroblasts (CAFs), which in turn support cancer cell growth. MicroRNAs (miRNAs) have been shown to be involved in this intercellular crosstalk in humans. To assess whether miRNAs are also involved in the activation of fibroblasts in dogs, we cocultured primary canine skin fibroblasts with the canine mast cell tumour cell line C2 directly or with C2-derived exosomes, and measured differential abundance of selected miRNAs. Expression of the CAF markers alpha-smooth muscle actin (ACTA2) and stanniocalcin 1 confirmed the activation of our fibroblasts after coculture. We show that fibroblasts displayed significant downregulation of miR-27a and let-7 family members. These changes correlated with significant upregulation of predicted target mRNAs. Furthermore, RNA interference knockdown of miR-27a revealed that cyclin G1 (CCNG1) exhibited negative correlation at the mRNA and protein level, suggesting that CCNG1 is a target of miR-27a in canine fibroblasts and involved in their activation. Importantly, miR-27a knockdown itself resulted in fibroblast activation, as demonstrated by the formation of ACTA2 filaments. In addition, interleukin-6 (IL-6) was strongly induced in our fibroblasts when cocultured, indicating potential reciprocal signalling. Taken together, our findings are consistent with canine fibroblasts being reprogrammed into CAFs to further support cancer development and that downregulation of miR-27a may play an important role in the tumour–microenvironment crosstalk.

Similar to humans, cancer is the leading cause of death in dogs with skin cancer being one of the most common types [1,2]. Cancer occurs naturally in humans and dogs in similar incidence rates, and since they are companion animals, dogs share the same environment and are exposed to similar risk factors as humans. Moreover, the molecular and clinical resemblances between human and dog cancers in terms of tumour genetics, molecular targets, histological features, response to conventional therapies and age of onset are significant [3,4]. Hence, the study of oncogenesis in dogs not only is important to further our understanding of the disease within this species, but also is likely to deliver additional valuable information that could be applied in the management of human cancer.

Abbreviations
CAFs, Cancer-associated fibroblasts; CT, Threshold cycle; ECM, Extracellular matrix; IF, Immunofluorescence; miRNA, MicroRNA; PF, Primary fibroblast; RNAi, RNA interference; RT-qPCR, Reverse transcription–quantitative PCR.

Cancer development implicates complex interactions between genetic and epigenetic modifications, resulting in the ability of cancer cells to escape programmed cell death and grow out of control [5]. As tumour cells proliferate, the surrounding stromal cells, as well as the extracellular matrix (ECM), start to play a dynamic role in the progression of cancer. ECM goes through mechanical and biochemical changes, leading to the remodelling of the local environment that promotes cancer progression. These biological adaptations illustrate the strong and constant interaction between cellular and noncellular components of the tumour microenvironment [6,7]. The major modulatory role of the ECM in tumour growth has stimulated a significant research interest in fibroblasts, the predominant stromal cell type. Fibroblasts are the cells primarily responsible for the synthesis of ECM, secreting various soluble growth factors and structural proteins, as well as remodelling proteases [8]. Additional factors, including hypoxia, as well as tumour- and non-tumour-derived cytokines and other signalling molecules, also influence the local tumour microenvironment [9,10].

Tumour and wound stroma share many similarities such as fibroblast activation, increased production of ECM proteins and enhanced tissue remodelling [11]. When the wound healing process is completed, all these modifications return to their homeostatic state. In contrast, during tumorigenesis, these changes are perpetuated and cancer-associated fibroblasts (CAFs) remain activated, supporting cancer development [10,12]. Despite some studies suggesting that most CAFs originate from quiescent local fibroblasts, further investigations have shown that they may also arise from the bone marrow, adipocytes and endothelial or epithelial cells going through mesenchymal transition [8,13].

Since genetic alterations in CAFs appear only at very low frequency, activation of CAFs seems to be led by epigenetic modifications instead [14]. MicroRNAs (miRNAs) are recognised as one of the major epigenetic gene regulators capable of influencing wide networks of genes at a post-transcriptional level [15,16]. miRNAs are short noncoding RNA molecules whose dysregulation has been observed in all types of cancer. They function either as oncomiRs by blocking the expression of tumour suppressors or as anti-oncomiRs through repression of oncogenes, by means of complementary base pairing within the 3′-UTRs of their mRNA targets, inhibiting their translation or triggering their decay [17,18]. Research has shown that pathologic expression of miRNAs favours the development of tumour microenvironment by directly or indirectly influencing interactions between cancer cells and CAFs [9,19].

Recent investigations focussed on the interaction between tumour and stroma have revealed that cancer cells can reprogramme fibroblasts to become CAFs via miRNAs. In return, CAFs support tumour cell growth, invasion and metastasis, and again, miRNAs appear to be involved in this process [6,12,19]. For example, dysregulation of let-7 family members and miR-27a, along with several of their target mRNAs, has been found to be involved in regulatory mechanisms of ECM and CAF metabolism [6,9,20]. Some of this intercellular crosstalk occurring within the tumour microenvironment is also attributed to exosomes [21]. Exosomes are small membrane vesicles secreted by all types of cells capable of mediating cell-to-cell communication by means of exchanging DNA, proteins, mRNAs and miRNAs [22,23]. For instance, *in vitro* and *in vivo* models for the human disease have shown that exosome-derived miR-27a may regulate the transformation of normal fibroblasts into CAFs and also modulate the growth and metastasis of tumour cells [21].

Given the important role that miRNAs play in cancer tumorigenesis, modification of the expression of particular cancer-associated miRNAs could therefore represent a valuable tool for therapeutics and management of cancer. In this study, we aimed to identify miRNA-derived mediators of fibroblast activation in dogs by studying differential expression of miRNAs and their predicted mRNA targets in canine fibroblasts after coculture with cancer cells or after exposure to cancer cell-derived exosomes. We focussed our research on canine orthologues of miRNAs with known deregulation in human and/or canine cancer. Analysis of our data identifies potential regulatory miRNA-mediated mechanisms and further implies that the canine disease could be a suitable model for human cancer developmental studies.

Materials and methods

Cell culture and total RNA isolation

Primary fibroblasts (PFs) were isolated and cultured as previously described [22], and passage number 5 was used for all experiments. C2 cells, a canine mast cell tumour cell line, were kindly provided by P. Dubreuil (Centre de Recherche en Cancérologie de Marseille, Inserm U1068, Marseille, France), after previous consent of the cell line originator, W. Gold (University of California, San Francisco, School of Medicine, CA, USA), and were cultured as described earlier [22].

All cell culture experiments were carried out using three biological replicates and evaluated after 24, 48, 72 and 96 h. Three different groups were examined: PF control group (PF group), coculture of PF and C2 cell group (CC-PF group) and C2 exosome-derived group (Exo-PF). In all three groups, 24 h before the experiments started, 2.2×10^5 PFs were seeded per well in a standard 6-well culture plate (Sarstedt, Nümbrecht, Germany). At time zero, culture medium was replaced with exosome-depleted medium, containing RPMI 1640 medium (Biochrom, Berlin, Germany), supplemented with 10% exosome-depleted FBS (Gibco, Gaithersburg, MD, USA), 100 $U \cdot mL^{-1}$ penicillin/streptomycin (Biochrom), 1 $mM \cdot mL^{-1}$ sodium pyruvate and 2 $mM \cdot mL^{-1}$ glutamine (both from Sigma, St. Louis, MO, USA). In the CC-PF group, C2 cells were cocultured with PF in a two-compartment cell culture system, by means of utilising hanging cell culture inserts (0.4 μm PET) (Merck Millicell, Darmstadt, Germany) that created a lower and an upper compartment. PFs were first seeded in the lower compartment, and then, 2.2×10^5 C2 cells were seeded into the insert (upper compartment). For the Exo-PF group, exosomes were isolated from C2 cell culture media as described earlier [22], using the commercial kit Total Exosome Isolation Reagent – from cell culture media (Invitrogen, Vilnius, Lithuania), after a previous 48-h incubation period in exosome-depleted medium. Exosomes from 2.5 mL medium were pelleted (10 000 ***g***, 60 min, 4 °C), resuspended in PBS and added to the culture medium of all wells of the Exo-PF group.

RNA isolation in PF was carried out using the mirVana miRNA Isolation Kit (Ambion, Darmstadt, Germany) according to the manufacturer's protocol. RNA quality and quantity was validated as described previously [24].

Selection of miRNAs and mRNAs, primer design and quantification by RT-qPCR

A total of 20 known *Canis familiaris* miRNAs were selected on the basis that they had previously been reported to play a role in cancer initiation and/or progression in dogs and humans (Table S1). Corresponding mRNA targets were predicted using the online resources RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) [25], TargetScan (v7.2; http://www.targetscan.org) [26] and miRmap (https://mirmap.ezlab.org/app/) [27]. A list of identified targets was loaded on the database for annotation, visualisation and integrated discovery (https://david.ncifcrf.gov/) [28]. Target genes were selected based on their involvement in signalling pathways related to cancer from Kyoto Encyclopedia of Genes and Genomes pathway classification [29] in annotation summary result.

The quantification of miRNAs and mRNAs through Reverse transcription–quantitative PCR (RT-qPCR) was performed as previously described [24,30], using measurements in triplicate (miRNA) or duplicate (mRNA) of three biological samples, based on the 2-ΔΔCT method [31] and following protocols detailed before [32]. miRNA and mRNA primers were designed as reported earlier [30] or using the tool Primer-BLAST (National Center for Biotechnology Information). miRNA expression was normalised using RNU6-2 and miR-326 as reference genes, while HPRT1 and RPS19 were employed for mRNA normalisation. All four genes exhibited stable expression in our system. The entire set of oligonucleotides used in this study is provided in Tables S2 and S3. All oligonucleotides were synthesised by Sigma-Aldrich (Darmstadt, Germany).

Transfection of miRNAs

Primary fibroblasts were seeded and cultured until reaching ≥80% confluency in standard 6-well culture plates. Cells were cultured as described above and transfected with let-7a and miR-27a inhibitors (mirVana® miRNA inhibitor, MH10050 and MH11579; Ambion, Austin, TX, USA) and mimics (mirVana® miRNA mimic, MC10050 and MC11579; Ambion), as well as a nontarget (NT) siRNA (D-001810-02-05; Dharmacon, Lafayette, CO, USA) as control and a fluorescent TAMRA-labelled siRNA (Sigma-Aldrich) as transfection efficiency control, using a final concentration of 32 nM. TransIT-TKO (Mirus, Madison, WI, USA) was employed as transfection reagent, following the manufacturer's protocol. Six different groups were assessed: NT (siRNA), 7aIH (let-7a inhibitor), 27aIH (miR-27a inhibitor), 7a/27aIH (let-7a plus miR-27a inhibitors), 7a/27aMM (let-7a plus miR-27a mimics) and IF (fluorescent siRNA), along with evaluation of three time points: 24, 48 and 72 h. After 24 h of incubation, the medium was removed from all wells, cells were washed with PBS, and fresh medium was added for further culturing. Cell viability was assessed by immunofluorescence (IF) as described below. RT-qPCR was used to estimate inhibitor and mimic effects post-transfection.

Immunofluorescence

Detection of IF in our experiments was conducted as outlined previously [33], with some modifications described below. Sterile 13-mm diameter coverslips (Sarstedt) were placed inside of a standard 24-well culture plate (Sarstedt), and then, 0.5×10^5 PFs were seeded per well and cultured in a two-compartment cell culture system for 96 h. Cells were washed with cold PBS and fixed with 4% Histofix (Carl Roth, Karlsruhe, Germany) at room temperature for 15 min, then washed twice with PBS for 5 min and permeabilised using 0.25% (v/v) Triton X-100/PBS for 5 or 10 min at room temperature. A blocking step with 1% BSA/PBST [0.1% (v/v) Tween-100 and 1% (w/v) BSA in PBS] was performed for 1 h at room temperature. Coverslips were taken from the wells and laid on a 40-μL drop of primary antibody dilution in 1% BSA/PBST for 1 h at room temperature. After washing twice with PBS for

5 min, the secondary antibody was incubated for 1 h at room temperature (40-μL drop). Cells were washed twice with PBS, and nuclei were counterstained with 1 $\mu g \cdot mL^{-1}$ 4′,6-diamidin-2-phenylindol (Sigma-Aldrich) in PBS for 3 min at room temperature. Coverslips were mounted in 50% glycerol in PBS on a glass slide.

To detect IF in transfected groups, 0.25×10^5 PFs were seeded per well in an 8-well cell culture chamber slide (Sarstedt), cultured and transfected as described above. The IF staining was carried out as in coculture experiments, except from the primary antibody incubation step, which was performed afterwards on the chamber slides.

Primary antibodies were as follows: Anti-vimentin (1 : 50, #5741; CST, Danvers, MA, USA) rabbit antibody was used as fibroblast marker (Fig. S1); anti-ACTA2 (1 : 300, AJ1028a; Abgent, San Diego, CA, USA), and anti-CCNG1 and anti-JAK2 (both at 1 : 200; orb213680 and orb318917, both from Biorbyt, St. Louis, MO, USA) rabbit antibodies were used for IF detection in PF. The secondary antibody was goat anti-rabbit IgG DyLight 488 (1 : 400, #35553; Thermo Fisher Scientific, Waltham, MA, USA). Cell viability after transfection was assessed using Calcein acetoxymethyl/Hoechst (Calcein-AM, Biotium, Hamburg, Germany/Hoechst 33342; Thermo Fisher Scientific, Paisley, UK): PFs were incubated with 0.4 μM Calcein-AM at 37 °C (5% CO_2, 30 min) and washed twice with PBS. Then, 5 $\mu g \cdot mL^{-1}$ Hoechst was added to each well, incubated for 5 min at room temperature and washed twice with PBS.

Images were acquired using a Leica DMI6000B inverted microscope and the Leica LAS-X software (Leica, Wetzlar, Germany). For direct comparison, IF images were taken under identical microscope and camera settings.

Western blot

Protein isolation and detection were performed as described earlier [34] using three biological replicates. Briefly, proteins were separated by SDS/PAGE using a Tris/glycine buffer system and transferred onto a polyvinylidene fluoride membrane via semidry blot. Primary antibodies were as follows: anti-CCNG1 (1 : 1000, orb213680; Biorbyt) rabbit antibody and anti-ACTB (1 : 1000, sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA) mouse antibody. Secondary antibodies were as follows: donkey anti-rabbit and sheep anti-mouse horseradish peroxidase-conjugated (1 : 30 000 and 1 : 40 000; NA934 and NA931, both from GE Healthcare, Buckinghamshire, UK) antibodies. Protein quantification was performed by densitometry using the software BIO-1D (Vilber Lourmat, Marne-la-Vallée, France).

Data analysis

A two-tailed Student's *t*-test was used for comparison between the control group and treated groups (two-group analyses). Results are expressed as means of triplicate or duplicate measurements (technical replicates) ±SD. A *P*-value < 0.05 was considered statistically significant.

Results

Differential expression of miRNAs and their mRNA targets in activated PF

We hypothesised that coculture of canine PFs with the canine mast cell tumour cell line C2 (CC-PF group) would induce an activation of normal fibroblasts and that this activation would be mediated by miRNAs. We also evaluated whether C2-derived exosomes (Exo-PF group) could trigger similar effects. We initially assessed this by measuring the expression of the protein alpha-smooth muscle actin (ACTA2) by IF, the marker most widely used for identifying CAFs [10,12]. While control PF and Exo-PF groups showed only weakly positive signals of a diffuse net of filaments, fibroblasts in coculture for 96 h exhibited strong ACTA2 fluorescent signals as well-defined and organised long intracytoplasmic filaments as would be expected for activated fibroblasts (Fig. 1). Corresponding changes were also observed at mRNA level for ACTA2 and for the CAF-associated marker stanniocalcin 1 (STC1) [35] (Fig. S2), while asporin (ASPN) and calcium binding protein A4 (S100A4) were not detectable (data not shown). These data confirmed that coculture of the PF with C2 cells did activate our canine fibroblasts.

The expression of 20 miRNAs with known dysregulation in human and/or canine cancer (Table S1) was then investigated in C2-PF coculture experiments to test whether activation of our fibroblasts correlated with a differential regulation of one or more of these miRNAs. While none of these miRNAs were significantly changed during the first 48 h of coculture (Fig. S3), let-7a, let-7b and miR-27a were significantly ($P < 0.05$) reduced more than 1.5-fold after 72 h (Fig. 2A,E) when compared to the PF control group. This downregulation continued after 96 h of coculture, with additional significant reduction in miR-16 (Fig. 2B,F).

To test whether similar changes could also be induced by exosome-derived signals, PFs were cultured in the presence of purified C2-derived exosomes (Exo-PF group). During the initial 48 h, no significant changes were observed in the miRNA expression of PF (Fig. S3). However, after 72 h and 96 h let-7a was again significantly downregulated in the Exo-PF group (Fig. 2C-F) when compared to the PF control group, while no changes in miR-27a expression were observed.

To evaluate whether the downregulation of the identified miRNAs also led to the upregulation of their predicted common mRNA targets, RT-qPCRs were

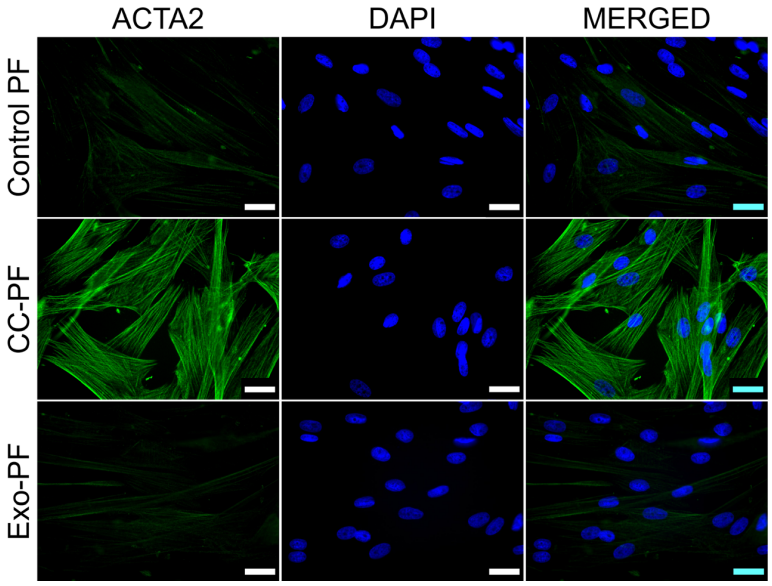

Fig. 1. Immunostaining in canine PFs for ACTA2 protein. Images show well-defined and organised long intracytoplasmic ACTA2 filaments in CC-PF 96 h after coculture compared with control PF and Exo-PF groups. IF representative images using at least two biological replicates were taken under identical microscope and camera settings. Scale bars represent 25 μm

performed on PF from both coculture and C2-derived exosome groups. Again, no significant changes were observed during the first 48 h in either CC-PF or Exo-PF groups (Fig. S4). However, after 72 and 96 h the CC-PF group showed a significant differential regulation of several predicted mRNA targets of more than 1.5-fold (Fig. 3A-D) when compared to the PF control group. After 72 h, mRNAs encoding Cbl proto-oncogene B (CBLB) and vascular cell adhesion molecule 1 (VCAM1) were upregulated, while cyclin D2 (CCND2) and sestrin 2 (SESN2) were downregulated (Fig. 3E). While CBLB and VCAM1 mRNAs were still upregulated after 96 h, significantly increased levels of cyclin G1 (CCNG1), Egl-9 family hypoxia-inducible factor 1 (EGLN1), fibroblast-associated protein (FAP), fibroblast growth factor 11 (FGF11), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Janus kinase 2 (JAK2) were now additionally observed, while CCND2, cyclin-dependent kinase 6 (CDK6) and forkhead box O1 (FOXO1) were downregulated (Fig. 3F). mRNA levels of the predicted target interleukin-6 (IL-6) were below the detection limit in all time points of the control PF group, but its expression was induced after 72 and 96 h in the CC-PF group. In contrast, the Exo-PF group showed very few changes with only CBLB mRNA exhibiting significant upregulation after 72 h (Fig. 3E), while CCND2 was the only downregulated target after 96 h (Fig. 3F). This showed that although coculture with C2-derived exosomes did lead to a significant reduction in let-7a in our PF, the exosome-containing medium was not sufficient to fully replicate the coculture experiment results.

Selective targeting of mRNAs using miRNA inhibitors and mimics

To test whether the observed reduction in let-7a and miR-27a was sufficient to induce activation of PF and to generate the observed changes in mRNA abundance of the predicted target genes, we tried to emulate the effects induced by C2 coculture on PF by means of RNA interference (RNAi)-mediated knockdown. PFs were transfected either with let-7a- or miR-27a-specific inhibitors (7aIH and 27aIH groups), or with their miRNA mimics (7a/27aMM group) as control, along with a NT control siRNA. Parallel transfection with fluorescently labelled control siRNAs indicated that the transfection efficiency was above 90% after 24 h, while cell viability was not visibly affected (Figs S5 and S6).

RT-qPCRs revealed that cells treated with either miR-27a inhibitor or mimic showed the expected significant down- or upregulation of miR-27a compared with the NT control. However, while PF transfected with let-7a mimic showed significantly increased levels of let-7a, cells transfected with let-7a inhibitor did not show decreased concentrations of this miRNA (Fig. S7), so that we did not further evaluate these cells. Therefore, predicted mRNA targets, whose expression was upregulated in the CC-PF group, were only further evaluated after RNAi knockdown of miR-27a.

ACTA2 protein expression was again assessed by IF to measure changes in the activation status of the transfected PF. Forty-eight hours after transfection

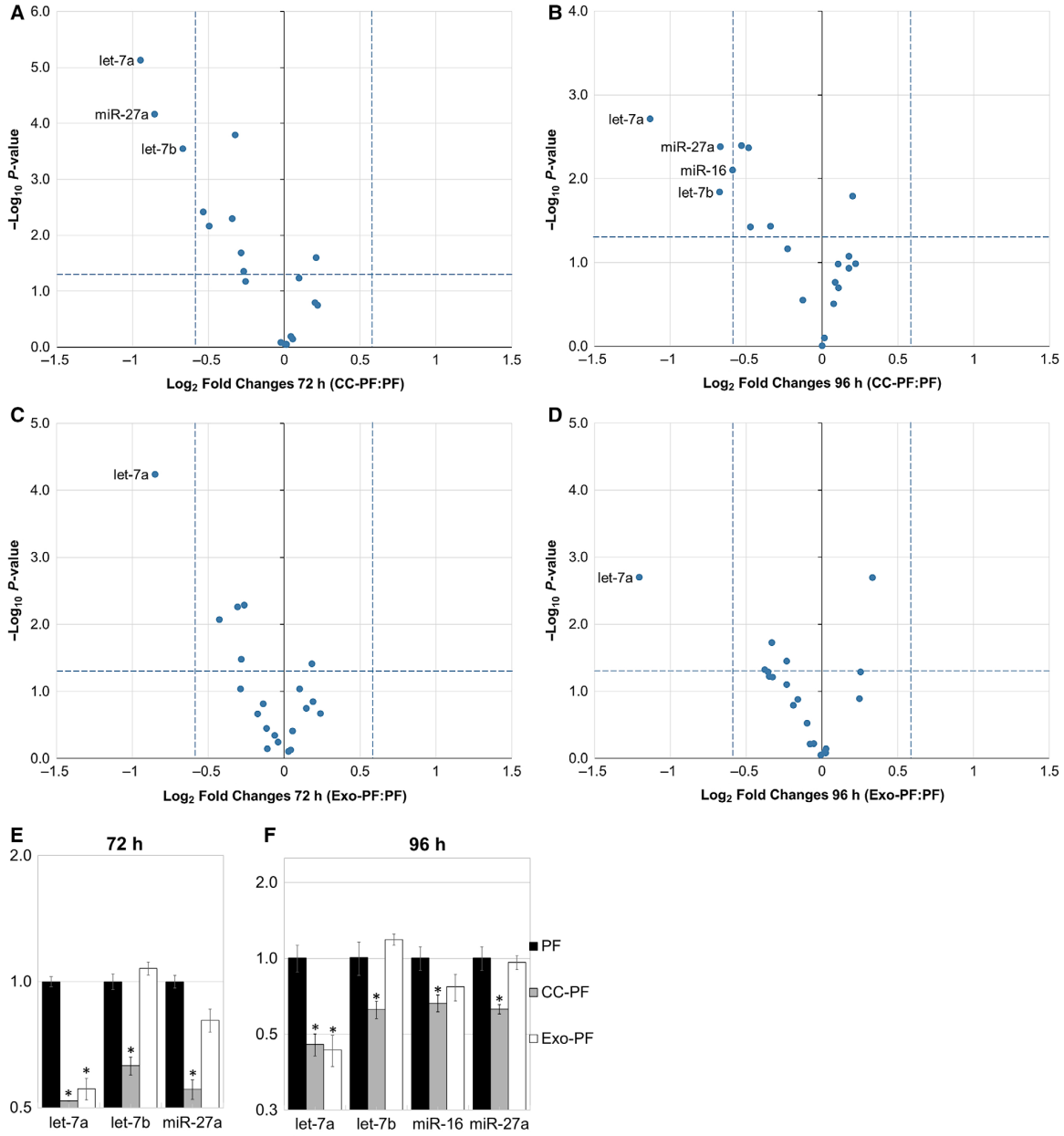

Fig. 2. Relative expression of miRNAs in canine PFs compared with PF control group. (A) 72 h and (B) 96 h after coculture with C2 cells: CC-PF group. (C) 72 h and (D) 96 h after culture with C2-derived exosomes: Exo-PF group. Regulated miRNAs in each group and time point (A-D) are indicated with their names in the plot. Bar charts show only regulated miRNAs after (E) 72 h and (F) 96 h in CC-PF and Exo-PF groups. Results were normalised to RNU6-2 and miR-326 and analysed using the 2-ΔΔCT method. Datasets are expressed as means of three biological samples and triplicate measurements ± SD, analysed with a two-tailed Student's *t*-test and transformed into $\log_2$ vs. $-\log_{10}$ *P*-value for volcano plots (A-D). Asterisks represent a statistical significance compared with the control group FB (*$P < 0.05$)

with miR-27a inhibitor, ACTA2 protein was observed as well-defined and organised long intracytoplasmic filaments consistent with fibroblast activation, while NT and 7a/27aMM groups exhibited only diffuse and weakly positive signals (Fig. 4). These results show that RNAi knockdown of miR-27a could emulate our coculture results by inducing the expression of ACTA2 at protein level in canine PF. Consistent with our IF

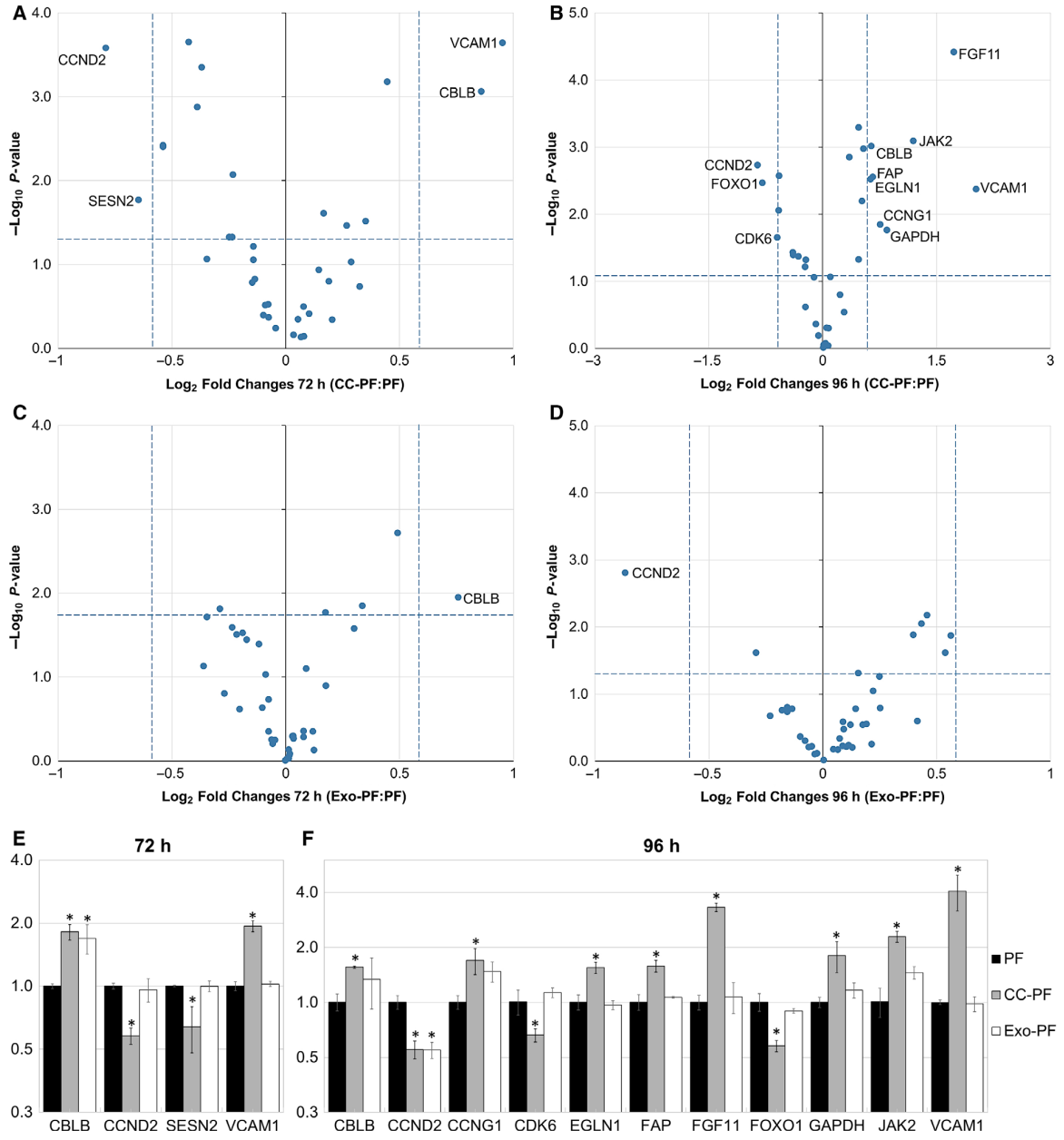

Fig. 3. Relative expression of predicted mRNA targets in canine PFs compared with PF control group. (A) 72 h and (B) 96 h after coculture with C2 cells: CC-PF group. (C) 72 h and (D) 96 h after culture with C2-derived exosomes: Exo-PF group. Regulated mRNAs in each group and time point (A–D) are indicated with their names in the plot. Bar charts show only regulated mRNA targets after (E) 72 h and (F) 96 h in CC-PF and Exo-PF groups. Results were normalised to HPRT1 and RPS19 and analysed using the 2-ΔΔCT method. Datasets are expressed as means of three biological samples and duplicate measurements ± SD, analysed with a two-tailed Student's *t*-test and transformed into $\log_2$ vs. $-\log_{10}$ *P*-value for volcano plots (A-D). Asterisks represent a statistical significance compared with the control group FB (*$P < 0.05$)

results, ACTA2 mRNA levels were significantly increased at 24, 48 and 72 h, and FAP mRNA, at 24 and 48 h (Fig. 5A,B), further confirming the activation of our fibroblasts. CCNG1 also exhibited significant and anticorrelative expression in PF in response to transfection with the miR-27a inhibitor after 24, 48

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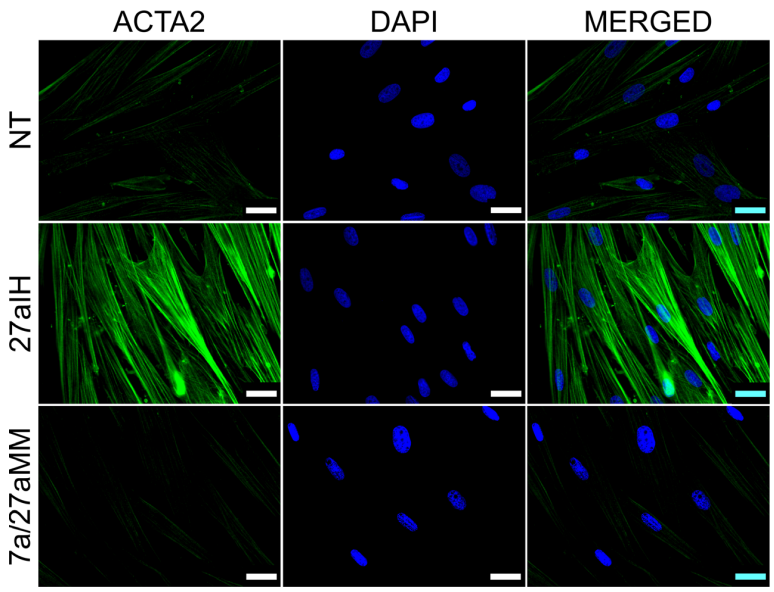

Fig. 4. Immunostaining in canine PFs for ACTA2 protein in transfection experiments. Images show the expression of intracytoplasmic ACTA2 filaments 48 h after transfection with miR-27a inhibitor compared with NT and 7a/27aMM groups. IF representative images using at least two biological replicates were taken under identical microscope and camera settings. Scale bars represent 25 μm

and 72 h (Fig. 5C), while CBLB was only significantly upregulated in PF transfected with miR-27a inhibitor after 24 h (Fig. 5D).

Transfection with the miRNA mimics gave a mixed response, with consistent significant decrease in CCNG1 mRNA (Fig. 5C), while FAP mRNA levels were significantly increased at 48 h (Fig. 5D) and FGF11 displayed reduced mRNA levels 72 h after transfection with inhibitor and mimics (Fig. 5E). Therefore, the inhibitor results indicate that CCNG1, CBLB and FAP, whose predicted miR-27a binding sites are conserved between dogs and humans (Fig. S8) along with ACTA2, may be the main targets for miR-27a in our system, while the mimic results were inconclusive.

Detection of target proteins in PF

Since CCNG1 was the only miR-27a target exhibiting a consistent and anticorrelative expression in transfected PF in our experiments, CCNG1 protein expression and localisation were further measured in PF by IF and western blot. As transfection of the let-7a inhibitor did not display significant changes, the 7aIH group was not tested for protein expression.

Figure 6A shows that PF cocultured for 96 h with C2 cells showed visibly increased expression of CCNG1 protein when compared to the control group. CC-PF cells showed clearly detectable signals for CCNG1 in the nucleus, while the fluorescence

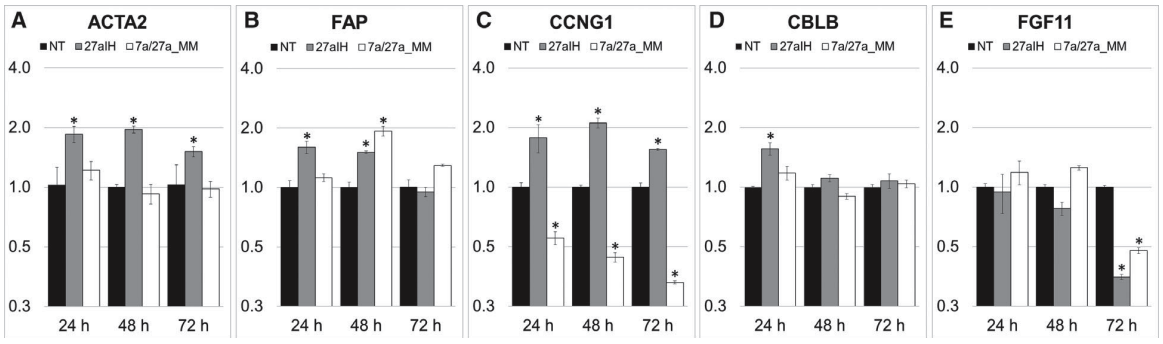

Fig. 5. Relative expression of predicted and regulated target mRNAs in canine PFs compared with the NT group. Twenty-four hours, 48 and 72 h after transfection in 27aIH and 7a/27aMM groups. (A) ACTA2, (B) FAP, (C) CCNG1, (D) CBLB and (E) FGF11. Results were normalised to HPRT1 and RPS19 and analysed using the 2-ΔΔCT method. Datasets are expressed as means of three biological samples and duplicate measurements ± SD, and analysed with a two-tailed Student's *t*-test. Asterisks represent a statistical significance compared with the control group NT (*$P < 0.05$)

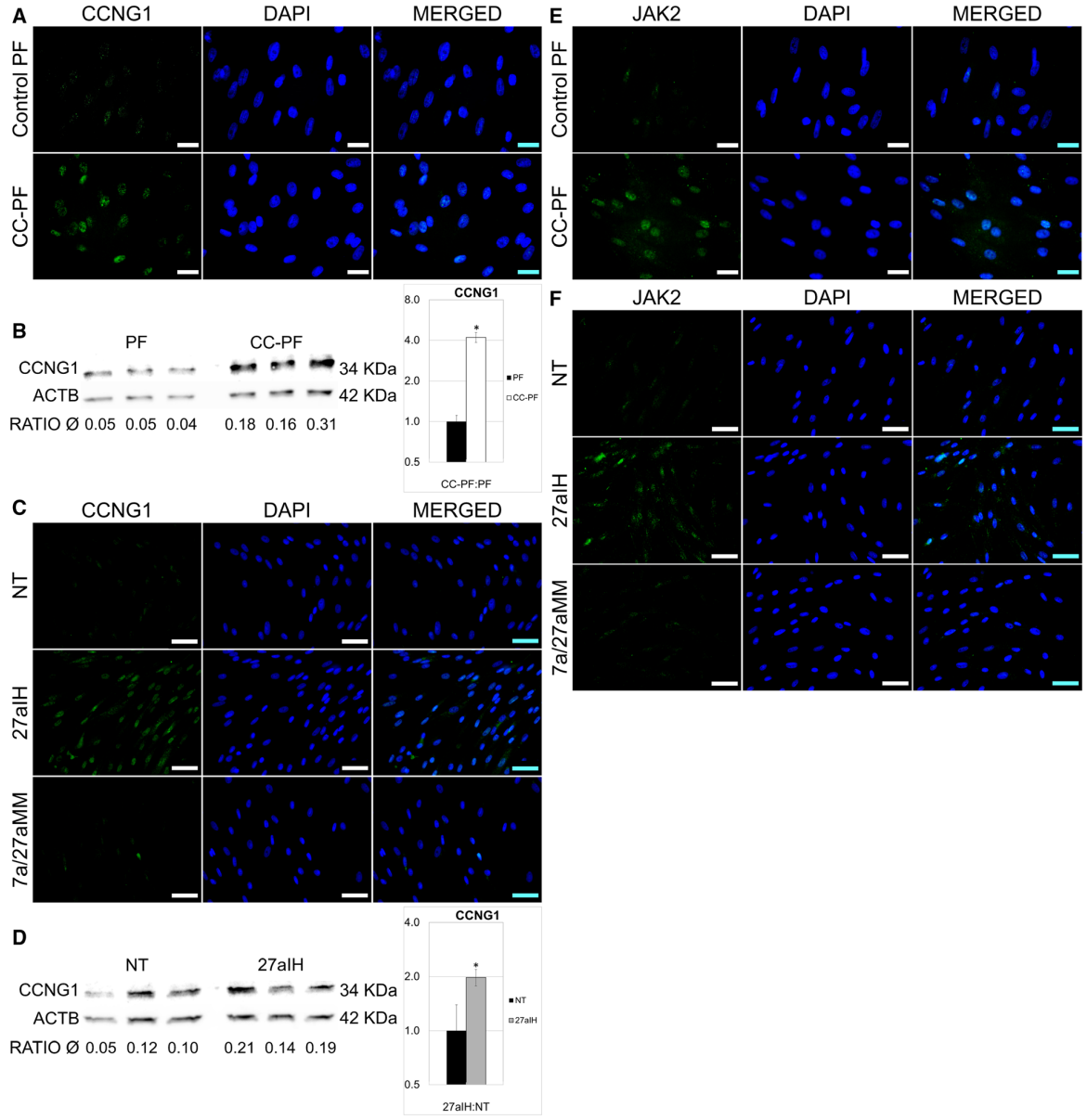

Fig. 6. Detection of CCNG1 and JAK2 proteins of in canine PFs. (A) IF staining of CCNG1 in CC-PF 96 h after coculture compared with control PF. (B) Western blot for CCNG1 in CC-PF 96 h after coculture compared with the PF control group. (C) IF staining of CCNG1 72 h after transfection with 27aIH (miR-27a inhibitor) compared with NT (siRNA) and 7a/27aMM (let-7a plus miR-27a mimics) groups. (D) Western blot for CCNG1 in PF 72 h after transfection with miR-27a inhibitor compared with NT. CCNG1 protein bands were quantified in three biological samples by densitometry relative to the respective beta-actin (ACTB) signals for both blots (B and D). Charts represent means of the biological triplicates ± SD and analysed with a two-tailed Student's *t*-test. Asterisks represent a statistical significance compared with the control group PF (*$P < 0.05$). (E) IF staining of JAK2 CC-PF after 96 h compared with control PF. (F) IF staining of JAK2 72 h after transfection with 27aIH compared with NT and 7a/27aMM groups. IF representative images were taken using at least two biological replicates, under identical microscope and camera settings. Scale bars represent 25 μm (A and E) and 50 μm (C and F).

observed in control PF under the same conditions was substantially lower. Similar results were observed when PFs were evaluated 72 h after transfection with miR-27a inhibitor. Again, nuclear fluorescence for CCNG1 was higher in the 27aIH group when compared to the staining of the NT and 7a/27aMM

groups (Fig. 6C). Western blots confirmed a significant increase in CCNG1 protein expression in CC-PF and 27aIH groups when compared to their respective controls (Fig. 6B,D). These observations confirmed the upregulation of CCNG1 protein in our fibroblasts through C2 coculture and transfection with miR-27a inhibitor, and are consistent with our findings at mRNA level (Figs 3F and 5C).

As JAK2 mRNA expression was also upregulated after 96 h in the CC-PF group (Fig. 3F), we assessed its protein expression by IF as well. Consistent with our RT-qPCR results, JAK2-specific nuclear fluorescence was enhanced in C2 coculture PF compared with the control PF group (Fig. 6E). Similar results were observed in PF transfected with the miR-27a inhibitor. Seventy-two hours after transfection, PF showed clearly enhanced nuclear fluorescence for JAK2 when compared to the NT and 7a/27aMM groups (Fig. 6F). Our JAK2 protein expression data therefore correlate with JAK2 mRNA levels observed in CC-PF (Fig. 3F). Interestingly, while transfection with the miR-27a inhibitor did not affect JAK2 mRNA expression, IF results showed a visible upregulation at protein level in PF, suggesting a post-transcriptional regulation of JAK2 protein.

Discussion

The tumour microenvironment is composed of a heterogeneous stroma including immune cells, endothelial cells and fibroblasts, along with the ECM. CAFs are the most abundant cell type in the stroma of solid tumours and the main producers of ECM [8]. Many of the pro-oncogenic phenomena and interactions taking place in the tumour microenvironment are driven via miRNAs [17,19]; hence, there is great interest in understanding how the malignant tumour cell–fibroblast crosstalk affects miRNA abundance in the surrounding environment. However, most of these studies have only been performed in human or rodent systems. To test whether the same crosstalk occurs in dogs, we have for the first time used a systematic approach to study miRNA expression in canine PF after coculture with the mast cell tumour cell line C2. For this purpose, we first assessed changes in abundance of selected canine orthologues of miRNAs in canine PF after coculture with C2 cells and with C2-derived exosomes, respectively. Secondly, we tried to evaluate computationally predicted mRNA targets *in vitro* via RNAi knockdown to indicate potential mechanisms of fibroblast activation.

Our results show that coculture with C2 cells induced downregulation of let-7 family members and miR-27a in canine PF. Furthermore, our data indicate that the growth-promoting cell cycle regulator CCNG1 is a target of miR-27a in dogs. PF cultured with C2-derived exosomes also displayed a significant downregulation of let-7a, though its predicted targets were not affected. These results indicate that exosomes do not appear to play a major role in modifying gene expression of the selected miRNAs in our PF-C2 cell system.

let-7a has been mostly described in humans as a tumour suppressor by targeting cancer-promoting genes in colon [36], prostate [37] and breast cancer [38]. In fibroblasts, downregulation of let-7a has been found to promote type I collagen expression, while its overexpression reduced fibrosis [20]. In our system, let-7a was downregulated in PF after coculture with C2 cells, as well as in PF cultured with C2-derived exosomes. Unfortunately, transfection with a let-7a inhibitor did not change the abundance of let-7a nor of its proposed mRNA targets. Thus, potential targets of let-7a could not be further assessed in this study.

In humans, miR-27a expression differs from one cancer type to another, acting either as oncomiR or as anti-oncomiR. It has been reported that in its oncogenic role, miR-27a modulates the malignant behaviour in osteosarcoma cells [39], promotes proliferation and invasion in lung cancer cells [40], and supports cell survival and angiogenesis in breast cancer [41]. On the other hand, Zhao and colleagues [42] showed that when functioning as anti-oncomiR, the downregulation of miR-27a contributed to metastasis in hepatocellular carcinoma. Likewise, it has been found that increased levels of miR-27a inhibited cell proliferation and enhanced apoptosis in colorectal [43] and lung cancer [44]. Research into miR-27a in fibroblasts has also yielded contradictory results. Experimental data demonstrated that exosome-derived miR-27a produced oxidative stress in human skin fibroblasts and inhibited their migration [23] and that miR-27a overexpression hindered lung fibrosis [45]. In contrast, one report indicated that miR-27a induced the reprogramming of fibroblasts into CAFs and also promoted proliferation, motility and metastasis of gastric cancer cells [21]. Our data have now shown that C2 cells can induce the expression of ACTA2 in PF and that this effect is likely to be mediated by means of miR-27a downregulation. It is known that normal fibroblasts can adapt to *in vitro* culture systems by promoting markers typically associated with fibroblast activation such as ACTA2 [46], which could explain the weak positive signal for ACTA2 in our control groups. Nevertheless, our results showed a marked increase in ACTA2 fluorescent signals and mRNA upregulation in coculture and transfection experiments. Our data therefore support recently published

observations that knockdown of miR-27a induces the expression of ACTA2 and enhances the differentiation of lung fibroblasts into myofibroblasts, and further support fibrosis [45].

Due to CAF heterogeneity, ACTA2 needs to be evaluated together with other CAF markers, including FAP, an important ECM-modifying enzyme that plays a significant role in matrix remodelling [7,10], and STC1, a glycoprotein secreted by activated CAFs with a protumorigenic role [35,47]. Both FAP and STC1 mRNA levels were increased in C2-cocultured PF, while FAP mRNA upregulation was also detected in miR-27a knockdown fibroblasts, confirming their activation and further supporting our hypothesis of tumour cell-derived activation of fibroblasts. However, other proposed CAF markers including ASPN and S100A4 [47] were not induced, confirming the variability between CAFs.

Analysis of the potential miR-27a targets further identified possible mechanisms of the fibroblast activation in our system. Expression of CCNG1 was enhanced in CC-PF after 96 h both at mRNA and protein levels, as well as in PF 24, 48 and 72 h after transfection with miR-27a inhibitor. CCNG1 expression has been associated with growth promotion and cell cycle progression [48]. The oncogenic behaviour of CCNG1 has been well documented, and its overexpression has been detected in several types of cancers [49,50], yet a cell growth-inhibitory function of CCNG1 has also been suggested [51]. However, in a study of normal human fibroblasts, transfection with a CCNG1 expression vector induced clonal expansion [52]. Furthermore, luciferase reporter assays confirmed that CCNG1 is a direct target of miR-27a in human osteosarcoma cells [51]. Our data also exhibited significantly upregulated CBLB mRNA levels in CC-PF after 72 and 96 h, as well as 24 h after transfection with miR-27a inhibitor. CBLB is a well-described oncogene, and its inhibition enhances anticancer immunity [53]. Our data are therefore consistent with a role of miR-27a as tumour suppressor and that its downregulation leads to an upregulation of the pro-oncogenic CBLB and CCNG1. Interestingly, a report indicated that miR-27b, another member of the miR-27 family, directly targets CBLB [54], though it is not clear whether miR-27a and miR-27b can interact with the same mRNA targets or are cell type-specific.

In addition to these possible fibroblast activation models by C2 cells, our data further revealed a potential mechanism for reciprocal fibroblast–tumour cell communication. Studies have shown a proproliferative effect of IL-6 on CAFs and have correlated its expression with increased levels of the CAF marker ACTA2 [55,56]. However, IL-6 is also an important mediator of a dynamic tumour cell–CAF crosstalk by not only promoting fibroblast activation, but also supporting tumour cell growth in humans [57]. IL-6 was induced in our PF cocultured with C2 cells, since it was not detectable in control PF or in Exo-PF cultures. Our data therefore are consistent with results described by Karakasheva and colleagues [57], making it likely that the enhanced IL-6 expression in our fibroblasts is not only part of a fibroblast activation programme but also a reciprocal signal to the tumour cells to enhance their growth. Interestingly, IL-6 has also been identified as direct target of let-7a and other members of the let-7 family [58], suggesting that downregulation of let-7a and let-7b observed in CC-PF could have influenced the expression of IL-6. In addition, a positive correlation between IL-6 and both JAK2 [59] and VCAM1 [60] has been previously shown, thereby supporting the results observed in our canine coculture approach.

Three additional upregulated genes in our coculture system further indicated a possible cellular response of PF to an enhanced metabolic status. Canine PF expressed increased mRNA levels of FGF11 and EGLN1 after 96 h. Both genes have been associated with adaptations to metabolic changes that improve cancer cell survival [61]. In contrast to other FGF family members, FGF11 is an intracellular nonsecreted growth factor and shows promitogenic and procell survival activities [62] and is therefore likely to be involved in the fibroblast activation itself. In addition, GAPDH expression was also increased in our cocultured PF, and although it is frequently employed as a housekeeping gene, GAPDH overexpression in fibroblasts has been shown to correlate with fibrosis and with an altered metabolism adapted to support a rapid cell growth [63]. Despite these genes being significantly upregulated at mRNA level after coculture, our transfection results indicated that they do not appear to be direct targets of miR-27a in our system. Similarly, CCND2, CDK6 and FOXO1 showed decreased mRNA levels in PF after C2 coculture and are consequently unlikely to be direct targets.

Experiments performed in mice showed that normal mast cells can affect fibroblast growth in coculture and enhance their growth rate [64]. We cannot therefore rule out that the observed gene expression changes are an effect of the mast cell origin of C2 cells and not just of their tumour nature. Unfortunately, no suitable normal canine mast cell line that would have allowed us to distinguish between these two possibilities was available to us. Nevertheless, our results have identified potential mechanisms for how C2 cells may induce

activation of primary dermal fibroblast that can now be further evaluated.

In conclusion, our data present the first systematic analysis of cellular crosstalk between a canine mast cell tumour and fibroblasts on a miRNA basis, providing essential data for further functional analyses. Our targeted approach identified distinct miRNAs significantly regulated in canine PF after coculture with the mast cell tumour cell line C2 and suggested potential mechanisms for their activation and reciprocal cell crosstalk. Results generated from our coculture system and other *in vitro* experiments have provided evidence that miR-27a is able to influence fibroblast protein expression associated with their activation. Therefore, our findings in this canine model are consistent with known human cancer cell interactions, reinforcing the idea that canine PFs are also reprogrammed into CAFs and may support cancer development. Downregulation of miR-27a could therefore play an important role in shaping the cancer microenvironment by further promoting the expression of its cancer-related targets in dogs. Our data thus further strengthen the concept of the dog as a suitable cancer model for humans.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MAR wrote the original manuscript, performed experiments and analysed data. SS revised and edited the manuscript, designed study, analysed data and supervised planning and execution. TS revised and edited the manuscript, provided experimental advice and analysed data. RE revised and edited the manuscript, provided advice in study design and supervised planning and execution. All authors have read and approved the final version of the manuscript.

References

1 Adams V, Evans K, Sampson J and Wood J (2010) Methods and mortality results of a health survey of purebred dogs in the UK. *J Small Anim Pract* **51**, 512–524.

2 Baioni E, Scanziani E, Vincenti MC, Leschiera M, Bozzetta E, Pezzolato M, Desiato R, Bertolini S, Maurella C and Ru G (2017) Estimating canine cancer incidence: findings from a population-based tumour registry in northwestern Italy. *BMC Vet Res* **13**, 203.

3 Paoloni M and Khanna C (2008) Translation of new cancer treatments from pet dogs to humans. *Nat Rev Cancer* **8**, 147.

4 Wagner S, Willenbrock S, Nolte I and Murua Escobar H (2013) Comparison of non-coding RNAs in human and canine cancer. *Front Genet* **4**, 46.

5 Herceg Z and Hainaut P (2007) Genetic and epigenetic alterations as biomarkers for cancer detection, diagnosis and prognosis. *Mol Oncol* **1**, 26–41.

6 Schoepp M, Ströse AJ and Haier J (2017) Dysregulation of miRNA expression in cancer associated fibroblasts (CAFs) and its consequences on the tumor microenvironment. *Cancers* **9**, 54.

7 Malik R, Lelkes PI and Cukierman E (2015) Biomechanical and biochemical remodeling of stromal extracellular matrix in cancer. *Trends Biotechnol* **33**, 230–236.

8 Gascard P and Tlsty TD (2016) Carcinoma-associated fibroblasts: orchestrating the composition of malignancy. *Genes Dev* **30**, 1002–1019.

9 Rupaimoole R, Calin GA, Lopez-Berestein G and Sood AK (2016) miRNA deregulation in cancer cells and the tumor microenvironment. *Cancer Discov* **6**, 235–246.

10 Räsänen K and Vaheri A (2010) Activation of fibroblasts in cancer stroma. *Exp Cell Res* **316**, 2713–2722.

11 Foster DS, Jones RE, Ransom RC, Longaker MT and Norton JA (2018) The evolving relationship of wound healing and tumor stroma. *JCI Insight* **3**, e99911.

12 Shiga K, Hara M, Nagasaki T, Sato T, Takahashi H and Takeyama H (2015) Cancer-associated fibroblasts: their characteristics and their roles in tumor growth. *Cancers* **7**, 2443–2458.

13 LeBleu VS and Kalluri R (2018) A peek into cancer-associated fibroblasts: origins, functions and translational impact. *Dis Models Mech* **11**, dmm029447.

14 Qiu W, Hu M, Sridhar A, Opeskin K, Fox S, Shipitsin M, Trivett M, Thompson ER, Ramakrishna M and Gorringe KL (2008) No evidence of clonal somatic genetic alterations in cancer-associated fibroblasts from human breast and ovarian carcinomas. *Nat Genet* **40**, 650.

15 Yao Q, Chen Y and Zhou X (2019) The roles of microRNAs in epigenetic regulation. *Curr Opin Chem Biol* **51**, 11–17.

16 Bronisz A, Godlewski J, Wallace J, Merchant AS, Nowicki MO, Mathsyaraja H, Srinivasan R, Trimboli AJ, Martin CK and Li F (2012) Reprogramming of the tumour microenvironment by stromal PTEN-regulated miR-320. *Nat Cell Biol* **14**, 159.

17 Kuninty PR, Schnittert J, Storm G and Prakash J (2016) MicroRNA targeting to modulate tumor microenvironment. *Front Oncol* **6**, 3.
18 Svoronos AA, Engelman DM and Slack FJ (2016) OncomiR or tumor suppressor? The duplicity of microRNAs in cancer. *Cancer Res* **76**, 3666–3670.
19 Kogure A, Kosaka N and Ochiya T (2019) Cross-talk between cancer cells and their neighbors via miRNA in extracellular vesicles: an emerging player in cancer metastasis. *J Biomed Sci* **26**, 7.
20 Makino K, Jinnin M, Hirano A, Yamane K, Eto M, Kusano T, Honda N, Kajihara I, Makino T and Sakai K (2013) The downregulation of microRNA let-7a contributes to the excessive expression of type I collagen in systemic and localized scleroderma. *J Immunol* **190**, 3905–3915.
21 Wang J, Guan X, Zhang Y, Ge S, Zhang L, Li H, Wang X, Liu R, Ning T and Deng T (2018) Exosomal miR-27a derived from gastric cancer cells regulates the transformation of fibroblasts into cancer-associated fibroblasts. *Cell Physiol Biochem* **49**, 869–883.
22 Aguilera-Rojas M, Badewien-Rentzsch B, Plendl J, Kohn B and Einspanier R (2018) Exploration of serum- and cell culture-derived exosomes from dogs. *BMC Vet Res* **14**, 179.
23 Tan W, Zhang Y, Li M, Zhu X, Yang X, Wang J, Zhang S, Zhu W, Cao J and Yang H (2019) miR-27a-containing exosomes secreted by irradiated skin keratinocytes delayed the migration of unirradiated skin fibroblasts. *Int J Biol Sci* **15**, 2240–2255.
24 Sharbati J, Lewin A, Kutz-Lohroff B, Kamal E, Einspanier R and Sharbati S (2011) Integrated microRNA-mRNA-analysis of human monocyte derived macrophages upon Mycobacterium avium subsp. hominissuis infection. *PLoS ONE* **6**, e20258.
25 Krüger J and Rehmsmeier M (2006) RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Res* **34**, W451–W454.
26 Agarwal V, Bell GW, Nam J-W and Bartel DP (2015) Predicting effective microRNA target sites in mammalian mRNAs. *elife* **4**, e05005.
27 Vejnar CE and Zdobnov EM (2012) MiRmap: comprehensive prediction of microRNA target repression strength. *Nucleic Acids Res* **40**, 11673–11683.
28 Dennis G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC and Lempicki RA (2003) DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol* **4**, R60.
29 Ogata H, Goto S, Sato K, Fujibuchi W, Bono H and Kanehisa M (1999) KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* **27**, 29–34.
30 Sharbati-Tehrani S, Kutz-Lohroff B, Bergbauer R, Scholven J and Einspanier R (2008) miR-Q: a novel quantitative RT-PCR approach for the expression profiling of small RNA molecules such as miRNAs in a complex sample. *BMC Mol Biol* **9**, 34.
31 Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods* **25**, 402–408.
32 Sharbati S, Sharbati J, Hoeke L, Bohmer M and Einspanier R (2012) Quantification and accurate normalisation of small RNAs through new custom RT-qPCR arrays demonstrates Salmonella-induced microRNAs in human monocytes. *BMC Genom* **13**, 23.
33 Sharbati J, Hanisch C, Pieper R, Einspanier R and Sharbati S (2015) Small molecule and RNAi induced phenotype transition of expanded and primary colonic epithelial cells. *Sci Rep* **5**, 12681.
34 Pawar K, Hanisch C, Vera SEP, Einspanier R and Sharbati S (2016) Down regulated lncRNA MEG3 eliminates mycobacteria in macrophages via autophagy. *Sci Rep* **6**, 19416.
35 Peña C, Céspedes MV, Lindh MB, Kiflemariam S, Mezheyeuski A, Edqvist P-H, Hägglöf C, Birgisson H, Bojmar L and Jirström K (2013) STC1 expression by cancer-associated fibroblasts drives metastasis of colorectal cancer. *Cancer Res* **73**, 1287–1297.
36 Li B, Chen P, Chang Y, Qi J, Fu H and Guo H (2016) Let-7a inhibits tumor cell growth and metastasis by directly targeting RTKN in human colon cancer. *Biochem Biophys Res Commun* **478**, 739–745.
37 Dong Q, Meng P, Wang T, Qin W, Qin W, Wang F, Yuan J, Chen Z, Yang A and Wang H (2010) MicroRNA let-7a inhibits proliferation of human prostate cancer cells *in vitro* and *in vivo* by targeting E2F2 and CCND2. *PLoS ONE* **5**, e10147.
38 Kim S-J, Shin J-Y, Lee K-D, Bae Y-K, Sung KW, Nam SJ and Chun K-H (2012) MicroRNA let-7a suppresses breast cancer cell migration and invasion through downregulation of CC chemokine receptor type 7. *Breast Cancer Res* **14**, R14.
39 Salah Z, Arafeh R, Maximov V, Galasso M, Khawaled S, Abou-Sharieha S, Volinia S, Jones KB, Croce CM and Aqeilan RI (2015) miR-27a and miR-27a* contribute to metastatic properties of osteosarcoma cells. *Oncotarget* **6**, 4920.
40 Chae DK, Ban E, Yoo YS, Kim EE, Baik JH and Song EJ (2017) MIR-27a regulates the TGF-β signaling pathway by targeting SMAD2 and SMAD4 in lung cancer. *Mol Carcinog* **56**, 1992–1998.
41 Mertens-Talcott SU, Chintharlapalli S, Li X and Safe S (2007) The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. *Cancer Res* **67**, 11001–11011.
42 Zhao N, Sun H, Sun B, Zhu D, Zhao X, Wang Y, Gu Q, Dong X, Liu F and Zhang Y (2016) miR-27a-3p suppresses tumor metastasis and VM by down-

regulating VE-cadherin expression and inhibiting EMT: an essential role for Twist-1 in HCC. *Sci Rep* **6**, 23091.

43 Bao Y, Chen Z, Guo Y, Feng Y, Li Z, Han W, Wang J, Zhao W, Jiao Y and Li K (2014) Tumor suppressor microRNA-27a in colorectal carcinogenesis and progression by targeting SGPP1 and Smad2. *PLoS ONE* **9**, e105991.

44 Yan X, Yu H, Liu Y, Hou J, Yang Q and Zhao Y (2019) miR-27a-3p Functions as a tumor suppressor and regulates non-small cell lung cancer cell proliferation via targeting HOXB8. *Tech Cancer Res Treat* **18**, 1533033819861971.

45 Cui H, Banerjee S, Xie N, Ge J, Liu R-M, Matalon S, Thannickal VJ and Liu G (2016) MicroRNA-27a-3p is a negative regulator of lung fibrosis by targeting myofibroblast differentiation. *Am J Respir Cell Mol Biol* **54**, 843–852.

46 Rodriguez LR, Emblom-Callahan M, Chhina M, Bui S, Aljeburry B, Tran LH, Novak R, Lemma M, Nathan SD and Grant GM (2018) Global gene expression analysis in an *in vitro* fibroblast model of idiopathic pulmonary fibrosis reveals potential role for CXCL14/CXCR4. *Sci Rep* **8**, 3983.

47 Orr B, Riddick A, Stewart G, Anderson R, Franco O, Hayward S and Thomson A (2012) Identification of stromally expressed molecules in the prostate by tag-profiling of cancer-associated fibroblasts, normal fibroblasts and fetal prostate. *Oncogene* **31**, 1130–1142.

48 Gordon EM, Ravicz JR, Liu S, Chawla SP and Hall FL (2018) Cell cycle checkpoint control: the cyclin G1/Mdm2/p53 axis emerges as a strategic target for broad-spectrum cancer gene therapy-A review of molecular mechanisms for oncologists. *Mol Clin Oncol* **9**, 115–134.

49 Reimer CL, Borras AM, Kurdistani SK, Garreau JR, Chung M, Aaronson SA and Lee SW (1999) Altered regulation of cyclin G in human breast cancer and its specific localization at replication foci in response to DNA damage in p53+/+ cells. *J Biol Chem* **274**, 11022–11029.

50 Skotzko M, Wu L, Anderson WF, Gordon EM and Hall FL (1995) Retroviral vector-mediated gene transfer of antisense cyclin G1 (CYCG1) inhibits proliferation of human osteogenic sarcoma cells. *Cancer Res* **55**, 5493–5498.

51 Lin T, Ma Q, Zhang Y, Zhang H, Yan J and Gao C (2018) MicroRNA-27a functions as an oncogene in human osteosarcoma by targeting CCNG1. *Oncol Lett* **15**, 1067–1071.

52 Smith ML, Kontny HU, Bortnick R and Fornace AJ Jr (1997) The p53-regulated cyclin G gene promotes cell growth: p53 downstream effectors cyclin G and Gadd45 exert different effects on cisplatin chemosensitivity. *Exp Cell Res* **230**, 61–68.

53 Liyasova MS, Ma K and Lipkowitz S (2015) Molecular pathways: Cbl proteins in tumorigenesis and antitumor immunity—opportunities for cancer treatment. *Clin Cancer Res* **21**, 1789–1794.

54 Chen D, Si W, Shen J, Du C, Lou W, Bao C, Zheng H, Pan J, Zhong G and Xu L (2018) miR-27b-3p inhibits proliferation and potentially reverses multi-chemoresistance by targeting CBLB/GRB2 in breast cancer cells. *Cell Death Dis* **9**, 188.

55 Goulet CR, Champagne A, Bernard G, Vandal D, Chabaud S, Pouliot F and Bolduc S (2019) Cancer-associated fibroblasts induce epithelial–mesenchymal transition of bladder cancer cells through paracrine IL-6 signalling. *BMC Cancer* **19**, 137.

56 Qin X, Yan M, Wang X, Xu Q, Wang X, Zhu X, Shi J, Li Z, Zhang J and Chen W (2018) Cancer-associated fibroblast-derived IL-6 promotes head and neck cancer progression via the osteopontin-NF-kappa B signaling pathway. *Theranostics* **8**, 921.

57 Karakasheva TA, Lin EW, Tang Q, Qiao E, Waldron TJ, Soni M, Klein-Szanto AJ, Sahu V, Basu D and Ohashi S (2018) IL-6 mediates cross-talk between tumor cells and activated fibroblasts in the tumor microenvironment. *Cancer Res* **78**, 4957–4970.

58 Iliopoulos D, Hirsch HA and Struhl K (2009) An epigenetic switch involving NF-κB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell* **139**, 693–706.

59 Yadav A, Kumar B, Datta J, Teknos TN and Kumar P (2011) IL-6 promotes head and neck tumor metastasis by inducing epithelial–mesenchymal transition via the JAK-STAT3-SNAIL signaling pathway. *Mol Cancer Res* **9**, 1658–1667.

60 Barnes TC, Anderson ME and Moots RJ (2011) The many faces of interleukin-6: the role of IL-6 in inflammation, vasculopathy, and fibrosis in systemic sclerosis. *Int J Rheumatol* **2011**, 721608.

61 Ye IC, Fertig EJ, DiGiacomo JW, Considine M, Godet I and Gilkes DM (2018) Molecular portrait of hypoxia in breast cancer: a prognostic signature and novel HIF-regulated genes. *Mol Cancer Res* **16**, 1889–1901.

62 Yang J, Kim WJ, Jun HO, Lee EJ, Lee KW, Jeong J-Y and Lee S-W (2015) Hypoxia-induced fibroblast growth factor 11 stimulates capillary-like endothelial tube formation. *Oncol Rep* **34**, 2745–2751.

63 Cruz-Bermúdez A, Laza-Briviesca R, Vicente-Blanco RJ, García-Grande A, Coronado MJ, Laine-Menéndez S, Alfaro C, Sanchez JC, Franco F and Calvo V (2019) Cancer-associated fibroblasts modify lung cancer metabolism involving ROS and TGF-β signaling. *Free Rad Biol Med* **130**, 163–173.

64 Dayton ET, Caulfield J, Hein A, Austen K and Stevens R (1989) Regulation of the growth rate of mouse fibroblasts by IL-3-activated mouse bone marrow-derived mast cells. *J Immunol* **142**, 4307–4313.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Literature overview of the selected 20 miRNAs. Expression patterns and their association with cancer in dogs and humans.

Table S2. Primer sequences of selected miRNAs. Genes for normalisation are presented in Italic.

Table S3. Primer sequences of selected mRNAs. Genes for normalisation are presented in Italic.

Fig. S1. Immunofluorescence staining of vimentin in canine primary fibroblasts. IF representative images represent at least two biological replicates. Scale bars represent 50 μm.

Fig. S2. Relative expression of mRNA levels for CAF markers ACTA2 and STC1 in canine primary fibroblasts compared with PF control group. Expression was evaluated in PF, CC-PF and Exo-PF groups after (A) 72 h and (B) 96 h. Results were normalised to HPRT1 and RPS19 and analysed using of the 2-ΔΔCT method. Datasets are expressed as means of three biological samples and duplicate measurements ± SD, analysed with a two-tailed Student's *t*-test. Asterisks represent a statistical significance compared with the control group FB (*$P = < 0.05$).

Fig. S3. Relative expression of miRNAs in canine primary fibroblasts compared with PF control group. (A) 24 h and (B) 48 h after coculture with C2 cells: CC-PF group. (C) 24 h and (D) 48 h after culture with C2-derived exosomes: Exo-PF group. No significantly regulated miRNAs were observed. Results were normalised to RNU6-2 and miR-326 and analysed using the 2-ΔΔCT method. Datasets are expressed as means of three biological samples and triplicate measurements ± SD, analysed with a two-tailed Student's *t*-test and transformed into $\log_2$ vs. $-\log_{10}$ *P*-value. No statistical significance was found compared with the control group FB ($P < 0.05$).

Fig. S4. Relative expression of predicted target mRNAs in canine primary fibroblasts compared with PF control group. (A) 24 h and (B) 48 h after coculture with C2 cells: CC-PF group. (C) 24 h and (D) 48 h after culture with C2-derived exosomes: Exo-PF group. No significantly regulated mRNAs were observed. Results were normalised to HPRT1 and RPS19 and analysed using of the 2^{-}ΔΔCT method. Datasets are expressed as means of three biological samples and duplicate measurements ± SD, analysed with a two-tailed Student's *t*-test and transformed into $\log_2$ vs. $-\log_{10}$ *P*-value. No statistical significance was found compared with the control group FB ($P < 0.05$).

Fig. S5. Representative images of transfection efficiency evaluated using a TAMRA-labelled siRNA. At the same time, viability of primary fibroblasts transfected with fluorescent siRNA control was also evaluated through Calcein and Hoechst fluorescent staining. IF representative images using at least two biological replicates were taken under identical microscope and camera settings. Scale bars represent 100 μm.

Fig. S6. Representative images of cell viability in primary fibroblasts evaluated by using Calcein and Hoechst fluorescent staining. (A) Cellular viability in NT group, (B) cellular viability in 27aIH group and (C) cellular viability in 7a/27aMM group. IF representative images using at least two biological replicates were taken under identical microscope and camera settings. Scale bars represent 100 μm.

Fig. S7. Relative expression of transfected miRNAs in primary fibroblasts compared with NT group. (A) 7aIH, (B) 27aIH, (C) 7a/27aIH and (D) 7a/27aMM groups. Results were normalised to RNU6-2 and miR-326 and analysed using the 2^{-}ΔΔCT method. Datasets are expressed as means of three biological samples and triplicate measurements ± SD, and analysed with a two-tailed Student's *t*-test. Asterisks represent a statistical significance compared with the control group NT (*$= P < 0.05$).

Fig. S8. Canine predicted binding sites in the 3' UTR region of regulated genes after miR-27a transfection, compared to human. Predicted interaction of each gene's target region (top) with miR-27a (bottom). (A) CCNG1 sequence has in both species 2 miR-27a binding sites in different positions due to nucleotide repetition. (B) CBLB and (C) FAP sequences have in both species a single miR-27a binding site. Pairing between genes and miR-27a was performed using the online resources TargetScan and RNAhybrid.

5. Summarising discussion

As tumours develop, cancer cells and non-cancer cells residing within the surrounding TME undergo numerous molecular changes. Cellular crosstalk is responsible for mediating such biological responses (Tommelein et al., 2015). The present study has shown that canine dermal primary fibroblasts and the mast cell tumour cell line C2 are each capable of shedding exosomes into the culture medium under normal culture conditions. Therefore, both cell types have the necessary machinery to transport biologically active cargo and potentially exchange it with each other. Furthermore, the activation of canine fibroblasts was observed after they were co-cultured with C2 cells, with miR-27a deregulation seemingly having contributed to this outcome. Additional cellular responses at miRNA, mRNA and protein levels were also detected.

5.1. Exosomes as important mediators of intercellular communication

Exosomes were first detected in reticulocytes as extracellular vesicles shed into the extracellular space with the capacity of transporting biomolecules (Harding and Stahl, 1983; Pan and Johnstone, 1983). A few years later, these vesicles were for the first time referred to as exosomes (Johnstone et al., 1987). Although once considered garbage bins for discarding unwanted material or simply seen as cell debris (Johnstone et al., 1987; Nawaz et al., 2019), empirical evidence has shown that exosomes are indeed messengers of biological information. Exosome-based intercellular communication regulates physiological and pathological cellular status. As such, in cancer disease they can manipulate both the local and the systemic environment to support tumour cell growth (Isola and Chen, 2017).

The first part of this study aimed to isolate and characterise canine exosomes from serum and cell culture media samples. Due to the great clinical potential for using exosomes as biomarkers for the diagnosis and treatment of cancers, it has become essential to optimise methods for exosome isolation and maximise their yield, purity and assay reproducibility (Patel et al., 2019). Numerous techniques based on size, density, precipitation, and surface proteins have been developed for exosome isolation/enrichment, each with a unique set of advantages and disadvantages (Li et al., 2017). Considered by some researchers as the gold standard method, differential ultracentrifugation at ≥100,000 *g* is the most used technique to obtain exosomes from biological fluids and cell culture supernatants (Bu et al., 2019). Although this procedure prevents contamination with cellular debris, it results in mixed fractions of exosomes, protein aggregates, and other vesicular structures (Jan et al., 2019). Additionally, it requires an expensive equipment, long run times, and large sample volumes (Jeppesen et al., 2014). Other isolation methods include serial filtration based on exosome size or molecular weight, immunoaffinity capture-based techniques against surface proteins, and commercial

kits using polymer precipitation (Jan et al., 2019). Studies analysing diverse types of biological fluids from canine origin have effectively isolated exosomes using ultracentrifugation (Fish et al., 2018), immunoaffinity-based methods (Yang et al., 2017b), and polymer precipitation techniques (Ichii et al., 2017; Loria et al., 2020). In this report, exosomes derived from canine fibroblasts and C2 cells were isolated using the precipitation method, which represents a quick and simple approach to obtain intact exosomes. Samples undergoing exosome isolation were first filtered (0.22 µm) to reduce co-precipitation of other EVs subpopulations and incubated with a polymer precipitation solution containing polyethylene glycol (PEG). A further short, low-speed centrifugation allowed the collection of exosomes from serum and culture media (Aguilera-Rojas et al., 2018). Polymers such as PEG possess a high water-binding capacity, which in biological fluids induces the precipitation of less soluble components, in this case exosomes. The exosome precipitation method is easy to use and no specialised equipment is required, which facilitates its clinical usage if required (Li et al., 2017).

Exosomes can be characterised based on their size, protein and even lipid content (Batrakova and Kim, 2015). In this study, the morphology and size of exosomes contained in the explored samples were analysed via negative-staining transmission electron microscopy (TEM). Besides, exosome quantification and size distribution were effectively assessed by nanoparticle tracking analysis (NTA). It should be noted, however, that exosome isolation via polymer precipitation may also co-precipitate non-exosomal particles such as proteins and polymeric material, which cannot be differentiated by the NTA system, especially in serum samples (Caradec et al., 2014; Batrakova and Kim, 2015). Both TEM and NTA techniques have often been used and widely accepted to confirm that isolated particles are indeed exosomes (Li et al., 2017). Furthermore, proteins from the tetraspanin family are considered as ubiquitously expressed on the exosomal surface (Lötvall et al., 2014). In particular, the expression of the tetraspanin CD63 was detected via immunogold labelling using TEM in canine serum- and culture media-derived exosomes (Aguilera-Rojas et al., 2018). These data align with findings that indicate a strongly enriched presence of CD63 in late endosomes (Ostrowski et al., 2010) and also resemble the results obtained by Kowal et al. (2016), who proposed CD63 as the preferred approach to identify exosomes. However, it has been shown that certain exosomes do not express CD63 and instead CD9 or CD81 should be evaluated (Kowal et al., 2016). This is why a single exosome marker should not be the exclusive confirmation method for exosome isolation. Unfortunately, the limited availability of specific canine antibodies in the market makes the detection of exosomal marker proteins in dogs a more difficult task than in, for example, humans or mice.

Cell-to-cell communication commonly involves soluble factors such as cytokines, chemokines, growth factors and neurotransmitters, and their binding to specific cell-surface receptors (Mittelbrunn and Sánchez-Madrid, 2012). Studies performed about two decades ago

revealed that exosomes also participate in intercellular communication, especially in immune responses and cancer (Raposo et al., 1996; Wolfers et al., 2001). However, it was not until 2007 when Valadi et al. showed for the first time that cells can communicate via transference of exosome-derived RNA species. The researchers identified that exosomes carry both mRNA and miRNA molecules, which can be delivered into different cells and induce functional modifications (Valadi et al., 2007). This discovery marked the beginning of the concept of exosomes as mediators of intercellular exchange of genetic material. To date, numerous studies have shown that exosomes can modify gene expression in their recipient cells and therefore alter their biological behaviour. As such, genetic information transported in tumour-derived exosomes can influence or even direct the fate of their target cells by inducing cellular activation, differentiation or de-differentiation, as well as promoting cellular migration, apoptosis or necrosis (Mittelbrunn and Sánchez-Madrid, 2012; Mathieu et al., 2019).

Autocrine and paracrine exosomal-mediated signalling have been widely shown to induce diverse cellular responses (Zhang and Grizzle, 2014; Asare-Werehene et al., 2020). It was observed that after co-culture of canine primary fibroblasts with the mast cell tumour cell line C2, the latter induced several changes in the gene expression of fibroblasts. Both cell types were never in direct contact, but cultured in different compartments of the same well (Aguilera-Rojas et al., 2020). Furthermore, it was also proved that both C2 cells and primary canine fibroblasts are each constantly secreting exosomes into the cell culture medium, which represents a potential source of interchangeable genetic material (Aguilera-Rojas et al., 2018). Based on the current evidence, it is likely that the observed gene regulation was led by exosome-related crosstalk, after fibroblasts incorporated genetic material from C2- or even fibroblast-derived exosomes into their cytoplasm (paracrine and autocrine signalling, respectively). Nonetheless, soluble factors may have also played a role in C2-fibroblast communication. Conversely, when purified C2-derived exosomes were added to canine primary fibroblasts cultures, only discrete variations in the gene expression of fibroblasts were observed. This could be explained by the number of exosomes mediating the crosstalk. In co-culture experiments, fibroblasts were in continuous interaction with exosomes (and soluble factors), which were being constantly produced by C2 cells. Unlike in experiments involving purified C2-derived exosomes, a single isolation procedure was performed from 2.5 ml of culture medium. This may have represented an insufficient amount of exosomes, not enough to induce more pronounced molecular changes in fibroblasts.

Microvesicles or ectosomes are a subpopulation of EVs, which are formed by the process of plasma membrane shedding and whose diameter size (100-1000 nm) is normally bigger than exosomes. They have been shown to transport functional RNA species and proteins between cells (Ratajczak et al., 2006; Stępień et al., 2018). This type of EVs partially overlap with the size range of exosomes (~30-150 nm) and share similar biophysical

characteristics in terms of density and membrane composition (Mathieu et al., 2019). Similarly, exomeres, a recently discovered type of extracellular non-membranous nanoparticles (<50 nm in diameter), have also been proven to contain and transfer functional cargo (Zhang et al., 2018; Zhang et al., 2019). Unfortunately, existing isolation methods cannot efficiently separate all types of subpopulations of EVs and most protocols may often contain a mixture of them (Mathieu et al., 2019). Based on these data, it cannot be excluded that the molecular changes observed in the canine fibroblasts after co-culture with C2 cells are solely induced by exosomes. However, the initial filtering step performed in this study in all samples eliminated all EVs with a diameter greater than 220 nm, as observed after analyses with the NTA and TEM techniques (Aguilera-Rojas et al., 2018).

5.2. C2 cells as inductors of fibroblast activation

The term “activated fibroblasts” was first coined in the wound healing context to describe cells that exhibit contractile properties. Initially, these cells were identified because of the expression of ACTA2 microfilament bundles (Hinz, 2010; Micallef et al., 2012). Quiescent fibroblasts serve as precursors for activated fibroblasts. Thus, resting fibroblasts undergoing this differentiation process are granted a significant proliferative activity and synthesising capacity. This means that activated fibroblasts are characterised by an increased production of growth factors, cytokines, and ECM. At the same time, they possess an enhanced migratory ability and are more vulnerable to epigenetic modifications. As such, activated fibroblasts can directly influence cancer metabolism, recruit immune cells and regulate tumour immunity, modulate chemoresistance, and stimulate angiogenesis (Kalluri, 2016; Alkasalias et al., 2018). In this context, activated fibroblasts associated with cancer have been termed CAFs (Liu et al., 2019).

The role of CAFs in canine tumours has not yet been widely investigated. Giuliano et al. (2017) found that the expression of FAP was increased in canine fibroblasts surrounding mast cell tumours. Their results showed that FAP expression has a positive correlation with histological grade, thus having the potential to be used as a negative prognostic factor in canine mast cell tumours. Data presented in this study show that after being co-cultured with C2 cells, canine fibroblasts increased the expression of ACTA2, at mRNA and protein levels, which indicates that C2 cells induced the activation of fibroblasts. These results are in accordance with the expression levels of FAP and STC1, two additional CAF markers that were also detected as increased. Further changes in gene expression were observed through the analysis of miRNA, their target mRNAs and protein expression (Aguilera-Rojas et al., 2020).

Exosomes shed by tumour cells can mediate local and systemic cell communication by delivering active growth factors and cytokines, miRNAs, proteins, mRNAs, and other

substances. These cargos are delivered to resting fibroblasts to induce their activation and differentiation. At the same time, exosomes released from CAFs are internalised by cancer cells and are able to favour cancer progression by transferring their content (Chen and Song, 2019; Yang et al., 2019). *In vitro* experiments have shown that miRNA-enriched exosomes derived from cancer cells induced the activation of quiescent fibroblasts into CAFs (Baroni et al., 2016; Fang et al., 2018). In the here presented co-culture study, the employed targeted miRNA approach did not find any upregulation in the evaluated candidate miRNAs, which made it challenging to look for miRNAs enriched in C2-derived exosomes. Interestingly, exosome-derived proteins have been also found to initiate the transition into CAFs (Yang et al., 2019). TGFβ transported in cancer-derived exosomes was shown to promote the differentiation process of fibroblasts into myofibroblasts (Webber et al., 2010). Similar results were observed by Goulet et al. (2018), who also detected TGFβ inside exosomes, representing up to ~85% of the total TGFβ present in the cancer cell supernatant. Furthermore, many ILs and other cytokines have been reported to be associated with EVs, either bound to their surface or encapsulated in the EVs (Fitzgerald et al., 2018).

The ability of miRNAs to recognise the complementary sites in the 3' UTR region of their target mRNAs directs the post-transcriptional gene repression (Bartel, 2009). Interactions between miRNAs and their target mRNAs are complex; multiple miRNAs can target a single gene and multiple genes can be regulated by a single miRNA, and therefore, the manifestation of such relationships is what determines the fine tuning of critical genes in a given biological setting (Peter, 2010). However, diverse mechanisms can also repress the expression of miRNAs in cancer. Genomic alterations may induce deletion or translocation of unstable regions of miRNA gene loci, altering pri-miRNA transcription and therefore miRNA expression. Transcription factors and epigenetic modifications such as hypermethylation of miRNA gene promoter regions and histone modifications are also known to suppress pri-miRNA transcription. Additionally, defects in core components of the miRNA biogenesis and processing machinery may contribute to the global repression of miRNAs in cancer (Lin and Gregory, 2015). As miRNAs take part in numerous regulatory networks/biological pathways, miRNA dysregulations can easily affect various cellular processes during cancer development and progression (Seo et al., 2017). In this study, the assessment of C2-induced miRNA dysregulations was approached in a targeted manner, as a pre-defined number of 20 canine miRNAs was evaluated. Each of these miRNAs has been previously reported in the literature to show an altered expression pattern in different human cancers. Some of them also play important roles in fibroblast activation, while others have been found to be dysregulated in canine cancers. For experimental validation within the co-culture system, cancer-related targets of the dysregulated miRNAs detected in fibroblasts were further computationally predicted (Aguilera-Rojas et al., 2020). Computational prediction of miRNAs target genes is a

valuable and powerful tool to identify putative miRNA-mRNA interactions. mRNA expression profiles alone are, however, not useful to predict protein translation processes since translation is a multistep regulatory procedure. mRNA and protein expression levels of the same gene may differ, depending on different regulatory processes such as formation of RNA secondary structures, alterations in translation initiation or elongation, and changes in protein stability. The assessment of protein expression profiles is therefore of significant relevance in gene analyses. Accordingly, in order to investigate relationships between miRNAs and the regulatory networks in cancer, the integration of miRNA, mRNA and protein expression datasets is required (Schwanhäusser et al., 2011; Fortelny et al., 2017; Seo et al., 2017).

The data presented here reveal that purified C2 cells-derived exosomes induced a significant downregulation in the expression of let-7a in fibroblasts. The same results were observed after 72 and 96 h of co-culture with C2 cells, although an additional downregulation in the expression levels of let-7b and miR-27a in fibroblasts was also induced (Aguilera-Rojas et al., 2020). let-7 family members are generally described as tumour suppressor miRNAs, since many of their targets are oncogenes (Gurtan et al., 2013). Downregulation of let-7 is frequently observed in various cancer types. Moreover, let-7 overexpression has been found to hinder the growth of cancer cells, either by inhibiting protein translation or via degradation of various cell cycle regulators (Boyerinas et al., 2010). Similarly, overexpression of let-7 in human primary fibroblasts was shown to induce cell cycle arrest (Legesse-Miller et al., 2009). miR-27a, in turn, has been identified as both a tumour suppressor and an oncogene, depending on the studied cell types and organs. Tanaka et al. (2015) found that human oesophageal fibroblasts transfected with miR-27a were activated and expressed ACTA2 filaments. On the contrary, it has also been shown that in human lung fibroblasts, the overexpression of miR-27a inhibited the differentiation of fibroblasts into myofibroblasts, whereas miR-27a RNA interference (RNAi)-mediated knockdown stimulated this process. Besides, ACTA2 was found to be a direct target of miR-27a in humans (Cui et al., 2016). In this canine system, miR-27a RNAi-mediated knockdown induced the activation of dermal primary fibroblasts, as confirmed by the increased expression levels of ACTA2 and FAP. Furthermore, miRNA, mRNA and protein integrated data analyses suggested that cyclin G1 (CCNG1) is a target gene of miR-27a in dogs, and may also be associated with the activation of fibroblasts (Aguilera-Rojas et al., 2020).

A tumour cell-induced switch from normal fibroblasts to CAFs unleashes a wide range of pro-tumorigenic cascade signals, which, together with a loss of the normal tissue architecture, creates an optimal niche for cancer cells growth. However, a surveillance mechanism against development and progression of cancer has been found to be driven by resting fibroblasts and the tumour-suppressive ECM produced by them (Alkasalias et al., 2018). Fibroblasts can inhibit the tumour cell proliferation and motility *in vitro* both in a cell-to-

cell contact and in a soluble factor dependent manner (Alkasalias et al., 2014), as well as suppress mammary epithelial tumours *in vivo* (Trimboli et al., 2009). Cancer cells therefore need to reprogramme resting fibroblasts into pro-tumorigenic CAFs to overcome the cellular surveillance (Chen and Song, 2019). Signals mediating the transition of quiescent fibroblasts into CAFs are complex. Eventually, the structure and function of the TME will change, gradually acquiring potent pro-oncogenic roles and simultaneously losing its tumour suppressive functions (Alkasalias et al., 2018). As such, it has been well documented that TGFβ plays a significant part in providing activating signals to fibroblasts. TGFβ promotes the activity of its main signal transducers, the Smad transcription factors, which in turn command the expression of the activated fibroblast marker ACTA2 and increase the activity of the contractile cytoskeleton (Sahai et al., 2020). Furthermore, numerous inflammatory modulators can also promote CAF activation. IL-6, for example, can activate the Janus tyrosine kinase (JAK) family members and leads to the activation of transcription factors of the signal transducer and activator of transcription (STAT) family (Čokić et al., 2015). Likewise, JAK-STAT signalling is recognised to maintain the contractile and pro-invasive fibroblasts capacities (Albrengues et al., 2015). Interestingly, an upregulation of the expression of vascular cell adhesion molecule 1 (VCAM1) has also been described to originate from the activation of the JAK/STAT signalling pathway (Shen et al., 2020). In addition, recent investigations have indicated that ACTA2 and FAP positive CAFs secrete a broad variety of cytokines, including IL-6 (Higashino et al., 2019; Liu et al., 2019). These data support the results obtained from the co-culture system presented in this study. C2 cells induced the secretion of IL-6 and increased both, the expression of JAK2 and VCAM1, as well as the expression of ACTA2 and FAP in canine fibroblasts. Unexpectedly, miR-27a knockdown fibroblasts, which were also ACTA2 and FAP positive, did not express IL-6 (Aguilera-Rojas et al., 2020). IL-6 expression, therefore, may have represented a phenomenon induced by C2 cells, which was not related to miR-27a RNAi-mediated downregulation. A possible explanation for this observation may be that activated mast cells can secrete soluble factors which benefit the tumour growth and can indirectly induce the production of the same molecules in fibroblasts (Theoharides and Conti, 2004; Maltby et al., 2009). Such interactions denote the complex multicomponent signal transduction pathways involved in cancer-related cellular responses.

5.3. Dogs are useful animal models in cancer research

Biomedical research has often relied heavily on animal models to make progress. In this way, canine models have long been employed in numerous diseases and their treatments (Parker et al., 2010). Dogs share exceptional similarities and unique features with humans. Their phenotypic diversity and the similarity with spontaneous developing human conditions make them an ideal animal model for complex human diseases (Rowell et al., 2011). In this

regard, naturally occurring cancers in dogs have the potential to be useful models in biomarker research. The biological and clinical behaviour of canine tumours, as well as their TME, are similar to those for human malignancies (Heishima et al., 2017). Particularly, research into miRNA in tumours has revealed that the expression profile of various canine malignancies is consistent with numerous reports on human cancers (Wagner et al., 2013; Heishima et al., 2017; Sahabi et al., 2018).

Most of the studies investigating cellular crosstalk between cancer cells and resting fibroblasts or CAFs, and their induced molecular responses, are based both on human or mouse research systems. The data presented here show for the first time, from a miRNA dysregulation approach, that canine resting fibroblasts are also reprogrammed into CAFs by C2 cells. These results also suggest that such cellular transition is mediated by intercellular crosstalk, since both cell types were never in direct contact with each other. The C2-induced miRNA dysregulation observed in canine fibroblasts resembles the dynamics of miRNA expression, as well as the molecular aspects of cellular responses in human tumours. Therefore, this study further strengthens the concept of the dog as a suitable cancer model.

6. Conclusion

The findings of the present study allow to conclude, firstly, that canine primary dermal fibroblasts and C2 cells each produce exosomes under standard cell culture conditions. And secondly, that canine dermal fibroblasts can be activated by C2 cells via intercellular crosstalk involving miRNA dysregulation, which may, in turn, foster the growth and proliferation of cancer cells. These results are comparable to data from the human system, which also describe interactions inside the tumour microenvironment, as well as more specifically between resting fibroblasts, CAFs and cancer cells. These observations further support the role of the domestic dog as a suitable animal model within the field of miRNA crosstalk and cancer research.

This targeted approach detected the dysregulation of specific miRNAs in canine fibroblasts within the co-culture system with the mast cell tumour cell line C2. Purified C2 exosomes also induced miRNA dysregulation, but at a lower scale. Additional RNA interference experiments provided evidence that miR-27a alone can influence the expression of proteins associated with fibroblast activation. Consequently, downregulation of miR-27a in dogs may contribute to the remodelling of the tumour milieu by controlling the expression of its cancer-associated target genes.

To complement these results, further functional analyses could describe in more detail the functions and gene interactions of miR-27a, or even find additional dysregulated miRNAs, and their related targets. This may also reveal other biomolecules associated with fibroblast activation, which could then be used as diagnostic or therapeutic tools for dogs. Future adaptations of those tools for humans may be possible as well.

7. Summary

Cellular crosstalk between canine fibroblasts and a mast cell tumour cell line and its significance in fibroblast activation

Naturally occurring cancers are the most common cause of death in dogs. Tumours represent complex interactive systems that combine different cell types and non-cellular elements. All components of the tumoral niche and their dynamic interactions comprise the tumour microenvironment (TME). Within the TME, fibroblasts are of significant interest as they are reprogrammed by cancer cells into cancer-associated fibroblasts (CAFs), which then further promote the abnormal growth and division of cancer cells. Various cancer-derived biomolecules including growth factors, cytokines, and microRNAs (miRNAs) have been reported to induce CAF activation. Exosomes transport an assorted biological cargo that plays an essential role in mediating the intercellular crosstalk within the TME. In this context, cancer cell-derived exosomes are specially enriched in miRNAs, which can trigger CAF differentiation and regulate cancer progression by targeting tumour suppressors or oncogenes. Moreover, dysregulation of miRNAs has been proposed as an emerging hallmark of cancer, both in the tumour itself and within the TME. Interestingly, known biochemical pathways involved in carcinogenesis in humans are also frequently deregulated in canine neoplasms. The essential similarities between canine and human genetic mechanisms for carcinogenesis make translational and comparative studies especially valuable and beneficial for both species.

This study initially detected that canine primary dermal fibroblasts and the mast cell tumour cell line C2 are each capable of releasing exosomes into the cell culture media under standard culture conditions. Then, from a targeted miRNA approach, the aim was to better understand how resting fibroblasts can be activated by cancer cells. To do so, the effects of intercellular communication between canine fibroblasts and C2 cells were investigated via co-culture of both types of cells and by culturing fibroblasts with purified C2 exosomes.

The results suggest that canine dermal fibroblasts are activated by C2 cells via intercellular crosstalk involving miRNA dysregulation, which may further support tumour cell proliferation. Additional RNA interference-mediated knockdown experiments provide evidence that miR-27a alone can influence the expression of proteins associated with fibroblast activation. Namely, downregulation of miR-27a in canine tumours may take part in the remodelling process of the TME by controlling the expression of its cancer-associated target genes.

These findings are comparable to data obtained from studies already performed on the human system, which further supports the notion of the domestic dog as a suitable animal model within the field of miRNA crosstalk and cancer research.

8. Zusammenfassung

Interzelluläre Kommunikation zwischen caninen Fibroblasten und einer Mastzell-Tumor-Zelllinie und ihre Bedeutung bei der Aktivierung von Fibroblasten

Natürlich vorkommender Krebs ist die häufigste Todesursache bei Hunden. Tumore stellen komplexe Wechselwirkungssysteme dar, die verschiedene Zelltypen und nicht-zelluläre Elemente kombinieren. Alle Komponenten der Tumornische sowie ihre dynamischen Interaktionen formen zusammen die Tumormikroumgebung. Innerhalb der Tumormikroumgebung sind die Fibroblasten von besonderem Interesse, da sie von Krebszellen zu Krebs-assoziierten Fibroblasten umprogrammiert werden, die dann das Wachstum und die Teilung der Krebszellen weiter begünstigen. Verschiedene vom Tumor erzeugte Biomoleküle, wie Wachstumsfaktoren, Zytokine und microRNAs (miRNAs), können Studien zufolge Krebs-assoziierten Fibroblasten aktivieren. Exosomen transportieren eine Mischung von diversen Biomolekülen, die eine essenzielle Rolle bei der Vermittlung der interzellulären Kommunikation innerhalb der Tumormikroumgebung spielen. In diesem Zusammenhang sind die aus Krebszellen hervorgegangen Exosomen besonders mit miRNAs angereichert, welche eine Differenzierung der Krebs-assoziierten Fibroblasten hervorrufen und die Tumorprogression regulieren können, indem sie auf Tumorsuppressoren oder Onkogene abzielen. Darüber hinaus wurde die Dysregulation von miRNAs, sowohl im Tumor selbst als auch innerhalb der Tumormikroumgebung, als neuartige Charakterisierung von Krebszellen vorgeschlagen. Interessanterweise sind die bekannten biochemischen Signalwege, die an der Karzinogenese beim Menschen beteiligt sind, häufig auch bei caninen Neoplasmen dereguliert. Die wesentlichen Ähnlichkeiten zwischen den genetischen Mechanismen der Karzinogenese bei Hunden und Menschen machen Translations- und Vergleichsstudien besonders wertvoll und vorteilhaft für beide Arten.

Im Rahmen der vorliegenden Studie wird zum einen gezeigt, dass primäre dermale Fibroblasten von Hunden und die Mastzelle-Tumor-Zelllinie C2 unter standardisierten Kulturbedingungen jeweils in der Lage sind, Exosomen in das Zellkulturmedium abzugeben. Anschließend soll mittels gezielter Charakterisierung ausgesuchter miRNAs besser verstanden werden, wie ruhende Fibroblasten durch Krebszellen aktiviert werden können. Zu diesem Zweck wurden die Effekte interzellulärer Kommunikation zwischen caninen Fibroblasten und C2-Zellen sowohl durch Ko-Kultur beider Zelltypen als auch durch die Kultivierung von Fibroblasten mit gereinigten C2-Exosomen untersucht.

Die Daten lassen darauf schließen, dass dermale Fibroblasten von Hunden durch C2-Zellen aktiviert sind, und zwar mittels interzellulärer Kommunikation auf Basis von miRNA-Dysregulation, was die weitere Proliferation der Krebszellen unterstützen könnte. Zusätzliche

RNA Interferenz-vermittelte Knockdown-Experimente geben Hinweise darauf, dass miR-27a auch allein die Expression von Proteinen, die mit der Aktivierung von Fibroblasten assoziiert sind, beeinflussen kann. Die Herabregulierung von miR-27a bei caniner Karzinogenese könnte an der Restrukturierung der Tumormikroumgebung beteiligt sein, wodurch die Expression Krebs-assoziierter Zielgene kontrolliert wird.

Diese Ergebnisse decken sich mit vergleichbaren Daten, die im Rahmen von Studien zum menschlichen System schon erhoben wurden, was wiederum die Annahme unterstützt, dass Haushunde ein gutes Tiermodell für Forschungen im Themenfeld der miRNA Kommunikation und Krebs darstellen.

9. References

Adams, V., Evans, K., Sampson, J., and Wood, J. (2010). Methods and mortality results of a health survey of purebred dogs in the UK. *Journal of Small Animal Practice* **51**: 512-524.

Aguilera-Rojas, M., Badewien-Rentzsch, B., Plendl, J., Kohn, B., and Einspanier, R. (2018). Exploration of serum-and cell culture-derived exosomes from dogs. *BMC Veterinary Research* **14**: 179.

Aguilera-Rojas, M., Sharbati, S., Stein, T., and Einspanier, R. (2020). Deregulation of miR-27a may contribute to canine fibroblast activation after coculture with a mast cell tumour cell line. *FEBS Open Bio* **10**: 802-816.

Albrengues, J., Bertero, T., Grasset, E., Bonan, S., Maiel, M., Bourget, I., Philippe, C., Serrano, C.H., Benamar, S., and Croce, O. (2015). Epigenetic switch drives the conversion of fibroblasts into proinvasive cancer-associated fibroblasts. *Nature Communications* **6**: 10204.

Alkasalias, T., Flaberg, E., Kashuba, V., Alexeyenko, A., Pavlova, T., Savchenko, A., Szekely, L., Klein, G., and Guven, H. (2014). Inhibition of tumor cell proliferation and motility by fibroblasts is both contact and soluble factor dependent. *Proceedings of the National Academy of Sciences* **111**: 17188-17193.

Alkasalias, T., Moyano-Galceran, L., Arsenian-Henriksson, M., and Lehti, K. (2018). Fibroblasts in the tumor microenvironment: shield or spear? *International Journal of Molecular Sciences* **19**: 1532.

Anand, S., Samuel, M., Kumar, S., and Mathivanan, S. (2019). Ticket to a bubble ride: cargo sorting into exosomes and extracellular vesicles. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **1867**: 140203.

Angstadt, A.Y., Thayanithy, V., Subramanian, S., Modiano, J.F., and Breen, M. (2012). A genome-wide approach to comparative oncology: high-resolution oligonucleotide aCGH of canine and human osteosarcoma pinpoints shared microaberrations. *Cancer Genetics* **205**: 572-587.

Ariztia, E.V., Lee, C.J., Gogoi, R., and Fishman, D.A. (2006). The tumor microenvironment: key to early detection. *Critical Reviews in Clinical Laboratory Sciences* **43**: 393-425.

Asare-Werehene, M., Nakka, K., Reunov, A., Chiu, C.-T., Lee, W.-T., Abedini, M.R., Wang, P.-W., Shieh, D.-B., Dilworth, F.J., and Carmona, E. (2020). The exosome-mediated

autocrine and paracrine actions of plasma gelsolin in ovarian cancer chemoresistance. *Oncogene* **39**: 1600-1616.

Auboeuf, D. (2016). Putative RNA-directed adaptive mutations in cancer evolution. *Transcription* **7**: 164-187.

Balkwill, F.R., Capasso, M., and Hagemann, T. (2012). The tumor microenvironment at a glance. *Journal of Cell Science* **125**: 5591-5596.

Baroni, S., Romero-Cordoba, S., Plantamura, I., Dugo, M., D'Ippolito, E., Cataldo, A., Cosentino, G., Angeloni, V., Rossini, A., and Daidone, M. (2016). Exosome-mediated delivery of miR-9 induces cancer-associated fibroblast-like properties in human breast fibroblasts. *Cell Death & Disease* **7**: e2312-e2312.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* **136**: 215-233.

Batrakova, E.V., and Kim, M.S. (2015). Using exosomes, naturally-equipped nanocarriers, for drug delivery. *Journal of Controlled Release* **219**: 396-405.

Beach, A., Zhang, H.-G., Ratajczak, M.Z., and Kakar, S.S. (2014). Exosomes: an overview of biogenesis, composition and role in ovarian cancer. *Journal of Ovarian Research* **7**: 14.

Bhome, R., Goh, R.W., Bullock, M.D., Pillar, N., Thirdborough, S.M., Mellone, M., Mirnezami, R., Galea, D., Veselkov, K., and Gu, Q. (2017). Exosomal microRNAs derived from colorectal cancer-associated fibroblasts: role in driving cancer progression. *Aging (Albany NY)* **9**: 2666.

Bottazzi, B., Polentarutti, N., Acero, R., Balsari, A., Boraschi, D., Ghezzi, P., Salmona, M., and Mantovani, A. (1983). Regulation of the macrophage content of neoplasms by chemoattractants. *Science* **220**: 210-212.

Boyerinas, B., Park, S.-M., Hau, A., Murmann, A.E., and Peter, M.E. (2010). The role of let-7 in cell differentiation and cancer. *Endocrine-Related Cancer* **17**: F19-F36.

Breen, M., and Modiano, J.F. (2008). Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans–man and his best friend share more than companionship. *Chromosome Research* **16**: 145-154.

Brønden, L.B., Nielsen, S.S., Toft, N., and Kristensen, A.T. (2010). Data from the Danish Veterinary Cancer Registry on the occurrence and distribution of neoplasms in dogs in Denmark. *Veterinary Record* **166**: 586-590.

Bronson, R. (1982). Variation in age at death of dogs of different sexes and breeds. *American Journal of Veterinary Research* **43**: 2057-2059.

Bu, H., He, D., He, X., and Wang, K. (2019). Exosomes: isolation, analysis, and applications in cancer detection and therapy. *Chembiochem* **20**: 451-461.

Cadieu, E., and Ostrander, E.A. (2007). Canine genetics offers new mechanisms for the study of human cancer. *Cancer Epidemiol Biomarkers Prev* **16**: 2181-2183.

Calin, G.A., and Croce, C.M. (2006). MicroRNA signatures in human cancers. *Nature Reviews Cancer* **6**: 857-866.

Caradec, J., Kharmate, G., Hosseini-Beheshti, E., Adomat, H., Gleave, M., and Guns, E. (2014). Reproducibility and efficiency of serum-derived exosome extraction methods. *Clinical Biochemistry* **47**: 1286-1292.

Carmeliet, P., and Jain, R.K. (2000). Angiogenesis in cancer and other diseases. *Nature* **407**: 249-257.

Case, A., Brisson, B.K., Durham, A.C., Rosen, S., Monslow, J., Buza, E., Salah, P., Gillem, J., Ruthel, G., and Veluvolu, S. (2017). Identification of prognostic collagen signatures and potential therapeutic stromal targets in canine mammary gland carcinoma. *PLoS One* **12**: e0180448.

Chatterjee, A., Jana, S., Chatterjee, S., Wastall, L.M., Mandal, G., Nargis, N., Roy, H., Hughes, T.A., and Bhattacharyya, A. (2019). MicroRNA-222 reprogrammed cancer-associated fibroblasts enhance growth and metastasis of breast cancer. *British Journal of Cancer* **121**: 679-689.

Chen, F., Zhuang, X., Lin, L., Yu, P., Wang, Y., Shi, Y., Hu, G., and Sun, Y. (2015). New horizons in tumor microenvironment biology: challenges and opportunities. *BMC Medicine* **13**: 45.

Chen, S., Chen, X., Shan, T., Ma, J., Lin, W., Li, W., and Kang, Y.A. (2018). MiR-21-mediated metabolic alteration of cancer-associated fibroblasts and its effect on pancreatic cancer cell behavior. *International Journal of Biological Sciences* **14**: 100.

Chen, X., and Song, E. (2019). Turning foes to friends: targeting cancer-associated fibroblasts. *Nature Reviews Drug Discovery* **18**: 99-115.

Čokić, V.P., Mitrović-Ajtić, O., Beleslin-Čokić, B.B., Marković, D., Buač, M., Diklić, M., Kraguljac-Kurtović, N., Damjanović, S., Milenković, P., and Gotić, M. (2015). Proinflammatory cytokine IL-6 and JAK-STAT signaling pathway in myeloproliferative neoplasms. *Mediators of Inflammation* **2015**: 453020.

Colombo, M., Raposo, G., and Théry, C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annual Review of Cell and Developmental Biology* **30**: 255-289.

Cui, H., Banerjee, S., Xie, N., Ge, J., Liu, R.-M., Matalon, S., Thannickal, V.J., and Liu, G. (2016). MicroRNA-27a-3p is a negative regulator of lung fibrosis by targeting myofibroblast differentiation. *American Journal of Respiratory Cell and Molecular Biology* **54**: 843-852.

Davis, B.W., and Ostrander, E.A. (2014). Domestic dogs and cancer research: a breed-based genomics approach. *ILAR Journal* **55**: 59-68.

de La Cruz-López, G.K., Castro-Muñoz, L.J., Reyes-Hernández, D.O., García-Carrancá, A., and Manzo Merino, J. (2019). Lactate in the regulation of tumor microenvironment and therapeutic approaches. *Frontiers in Oncology* **9**: 1143.

Decker, B., Parker, H.G., Dhawan, D., Kwon, E.M., Karlins, E., Davis, B.W., Ramos-Vara, J.A., Bonney, P.L., Mcniel, E.A., and Knapp, D.W. (2015). Homologous mutation to human BRAF V600E is common in naturally occurring canine bladder cancer—Evidence for a relevant model system and urine-based diagnostic test. *Molecular Cancer Research* **13**: 993-1002.

Di Leva, G., Garofalo, M., and Croce, C.M. (2014). MicroRNAs in cancer. *Annual Review of Pathology: Mechanisms of Disease* **9**: 287-314.

Dobson, J.M. (2013). Breed-predispositions to cancer in pedigree dogs. *ISRN Veterinary Science* **2013**: 941275.

Doldi, V., Callari, M., Giannoni, E., D'Aiuto, F., Maffezzini, M., Valdagni, R., Chiarugi, P., Gandellini, P., and Zaffaroni, N. (2015). Integrated gene and miRNA expression analysis of prostate cancer associated fibroblasts supports a prominent role for interleukin-6 in fibroblast activation. *Oncotarget* **6**: 31441.

Donnarumma, E., Fiore, D., Nappa, M., Roscigno, G., Adamo, A., Iaboni, M., Russo, V., Affinito, A., Puoti, I., and Quintavalle, C. (2017). Cancer-associated fibroblasts release exosomal microRNAs that dictate an aggressive phenotype in breast cancer. *Oncotarget* **8**: 19592.

Doyle, L.M., and Wang, M.Z. (2019). Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells* **8**: 727.

Du, H., and Che, G. (2017). Genetic alterations and epigenetic alterations of cancer-associated fibroblasts. *Oncology Letters* **13**: 3-12.

Eichmüller, S.B., Osen, W., Mandelboim, O., and Seliger, B. (2017). Immune modulatory microRNAs involved in tumor attack and tumor immune escape. *JNCI: Journal of the National Cancer Institute* **109**: djx034.

Elshafae, S.M., Kohart, N.A., Altstadt, L.A., Dirksen, W.P., and Rosol, T.J. (2017). The effect of a histone deacetylase inhibitor (AR-42) on canine prostate cancer growth and metastasis. *The Prostate* **77**: 776-793.

Fang, T., Lv, H., Lv, G., Li, T., Wang, C., Han, Q., Yu, L., Su, B., Guo, L., and Huang, S. (2018). Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. *Nature Communications* **9**: 1-13.

Fang, Y., Zhou, W., Rong, Y., Kuang, T., Xu, X., Wu, W., Wang, D., and Lou, W. (2019). Exosomal miRNA-106b from cancer-associated fibroblast promotes gemcitabine resistance in pancreatic cancer. *Experimental Cell Research* **383**: 111543.

Fish, E.J., Irizarry, K.J., Deinnocentes, P., Ellis, C.J., Prasad, N., Moss, A.G., and Bird, R.C. (2018). Malignant canine mammary epithelial cells shed exosomes containing differentially expressed microRNA that regulate oncogenic networks. *BMC Cancer* **18**: 832.

Fitzgerald, W., Freeman, M.L., Lederman, M.M., Vasilieva, E., Romero, R., and Margolis, L. (2018). A system of cytokines encapsulated in extracellular vesicles. *Scientific Reports* **8**: 1-11.

Fortelny, N., Overall, C.M., Pavlidis, P., and Freue, G.V.C. (2017). Can we predict protein from mRNA levels? *Nature* **547**: E19.

Freedman, A.H., Gronau, I., Schweizer, R.M., Ortega-Del Vecchyo, D., Han, E., Silva, P.M., Galaverni, M., Fan, Z., Marx, P., and Lorente-Galdos, B. (2014). Genome sequencing highlights the dynamic early history of dogs. *PLoS Genetics* **10**: e1004016.

Friedman, R.C., Farh, K.K.-H., Burge, C.B., and Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research* **19**: 92-105.

Galac, S. (2016). Cortisol-secreting adrenocortical tumours in dogs and their relevance for human medicine. *Molecular and Cellular Endocrinology* **421**: 34-39.

Ganguli, P., and Sarkar, R.R. (2018). Exploring immuno-regulatory mechanisms in the tumor microenvironment: Model and design of protocols for cancer remission. *PloS One* **13**: e0203030.

Gardner, H.L., Fenger, J.M., and London, C.A. (2016). Dogs as a model for cancer. *Annual Review of Animal Biosciences* **4**: 199-222.

Gillard, M., Cadieu, E., De Brito, C., Abadie, J., Vergier, B., Devauchelle, P., Degorce, F., Dréano, S., Primot, A., and Dorso, L. (2014). Naturally occurring melanomas in dogs as models for non-UV pathways of human melanomas. *Pigment Cell & Melanoma Research* **27**: 90-102.

Gioia, G., Mortarino, M., Gelain, M., Albonico, F., Ciusani, E., Forno, I., Marconato, L., Martini, V., and Comazzi, S. (2011). Immunophenotype-related microRNA expression in canine chronic lymphocytic leukemia. *Veterinary Immunology and Immunopathology* **142**: 228-235.

Giuliano, A., Dos Santos Horta, R., Constantino-Casas, F., Hoather, T., and Dobson, J. (2017). Expression of fibroblast activating protein and correlation with histological grade, mitotic index and Ki67 expression in canine mast cell tumours. *Journal of Comparative Pathology* **156**: 14-20.

Givel, A.-M., Kieffer, Y., Scholer-Dahirel, A., Sirven, P., Cardon, M., Pelon, F., Magagna, I., Gentric, G., Costa, A., and Bonneau, C. (2018). miR200-regulated CXCL12β promotes fibroblast heterogeneity and immunosuppression in ovarian cancers. *Nature Communications* **9**: 1-20.

Goulart, M.R., Pluhar, G.E., and Ohlfest, J.R. (2012). Identification of myeloid derived suppressor cells in dogs with naturally occurring cancer. *PloS One* **7**: e33274.

Goulet, C.R., Bernard, G., Tremblay, S., Chabaud, S., Bolduc, S., and Pouliot, F. (2018). Exosomes induce fibroblast differentiation into cancer-associated fibroblasts through TGFβ signaling. *Molecular Cancer Research* **16**: 1196-1204.

Grüntzig, K., Graf, R., Boo, G., Guscetti, F., Hässig, M., Axhausen, K.W., Fabrikant, S., Welle, M., Meier, D., and Folkers, G. (2016). Swiss canine cancer registry 1955–2008: occurrence of the most common tumour diagnoses and influence of age, breed, body size, sex and neutering status on tumour development. *Journal of Comparative Pathology* **155**: 156-170.

Gurtan, A.M., Ravi, A., Rahl, P.B., Bosson, A.D., Jnbaptiste, C.K., Bhutkar, A., Whittaker, C.A., Young, R.A., and Sharp, P.A. (2013). Let-7 represses Nr6a1 and a mid-gestation developmental program in adult fibroblasts. *Genes & Development* **27**: 941-954.

Ha, M., and Kim, V.N. (2014). Regulation of microRNA biogenesis. *Nature Reviews Molecular Cell Biology* **15**: 509-524.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. *Cell* **144**: 646-674.

Harding, C., and Stahl, P. (1983). Transferrin recycling in reticulocytes: pH and iron are important determinants of ligand binding and processing. *Biochemical and Biophysical Research Communications* **113**: 650-658.

Hedan, B., Thomas, R., Motsinger-Reif, A., Abadie, J., Andre, C., Cullen, J., and Breen, M. (2011). Molecular cytogenetic characterization of canine histiocytic sarcoma: a spontaneous model for human histiocytic cancer identifies deletion of tumor suppressor genes and highlights influence of genetic background on tumor behavior. *BMC Cancer* **11**: 201.

Heishima, K., Ichikawa, Y., Yoshida, K., Iwasaki, R., Sakai, H., Nakagawa, T., Tanaka, Y., Hoshino, Y., Okamura, Y., and Murakami, M. (2017). Circulating microRNA-214 and-126 as potential biomarkers for canine neoplastic disease. *Scientific Reports* **7**: 1-14.

Higashino, N., Koma, Y.-I., Hosono, M., Takase, N., Okamoto, M., Kodaira, H., Nishio, M., Shigeoka, M., Kakeji, Y., and Yokozaki, H. (2019). Fibroblast activation protein-positive fibroblasts promote tumor progression through secretion of CCL2 and interleukin-6 in esophageal squamous cell carcinoma. *Laboratory Investigation* **99**: 777-792.

Hinz, B. (2010). The myofibroblast: paradigm for a mechanically active cell. *Journal of Biomechanics* **43**: 146-155.

Hu, J., Wang, W., Lan, X., Zeng, Z., Liang, Y., Yan, Y., Song, F., Wang, F., Zhu, X., and Liao, W. (2019). CAFs secreted exosomes promote metastasis and chemotherapy resistance by enhancing cell stemness and epithelial-mesenchymal transition in colorectal cancer. *Molecular Cancer* **18**: 91.

Hu, M., Yao, J., Cai, L., Bachman, K.E., Van Den Brûle, F., Velculescu, V., and Polyak, K. (2005). Distinct epigenetic changes in the stromal cells of breast cancers. *Nature Genetics* **37**: 899-905.

Huang, X., Yuan, T., Tschannen, M., Sun, Z., Jacob, H., Du, M., Liang, M., Dittmar, R.L., Liu, Y., and Liang, M. (2013). Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics* **14**: 319.

Ichii, O., Ohta, H., Horino, T., Nakamura, T., Hosotani, M., Mizoguchi, T., Morishita, K., Nakamura, K., Hoshino, Y., and Takagi, S. (2017). Urinary exosome-derived microRNAs reflecting the changes of renal function and histopathology in dogs. *Scientific Reports* **7**: 1-11.

Ireland, L.V., and Mielgo, A. (2018). Macrophages and fibroblasts, key players in cancer chemoresistance. *Frontiers in Cell and Developmental Biology* **6**: 131.

Isola, A.L., and Chen, S. (2017). Exosomes: the messengers of health and disease. *Current Neuropharmacology* **15**: 157-165.

Ito, D., Frantz, A.M., and Modiano, J.F. (2014). Canine lymphoma as a comparative model for human non-Hodgkin lymphoma: recent progress and applications. *Veterinary Immunology and Immunopathology* **159**: 192-201.

Itoh, H., Horiuchi, Y., Nagasaki, T., Sakonju, I., Kakuta, T., Fukushima, U., Uchide, T., Yamashita, M., Kuwabara, M., and Yusa, S.-I. (2009). Evaluation of immunological status in tumor-bearing dogs. *Veterinary Immunology and Immunopathology* **132**: 85-90.

Ivey, K.N., and Srivastava, D. (2015). microRNAs as developmental regulators. *Cold Spring Harbor Perspectives in Biology* **7**: a008144.

Jan, A.T., Rahman, S., Khan, S., Tasduq, S.A., and Choi, I. (2019). Biology, pathophysiological role, and clinical implications of exosomes: a critical appraisal. *Cells* **8**: 99.

Jeppesen, D.K., Hvam, M.L., Primdahl-Bengtson, B., Boysen, A.T., Whitehead, B., Dyrskjøt, L., Ørntoft, T.F., Howard, K.A., and Ostenfeld, M.S. (2014). Comparative analysis of discrete exosome fractions obtained by differential centrifugation. *Journal of Extracellular Vesicles* **3**: 25011.

Johnstone, R.M., Adam, M., Hammond, J., Orr, L., and Turbide, C. (1987). Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *Journal of Biological Chemistry* **262**: 9412-9420.

Josson, S., Gururajan, M., Sung, S.-Y., Hu, P., Shao, C., Zhau, H., Liu, C., Lichterman, J., Duan, P., and Li, Q. (2015). Stromal fibroblast-derived miR-409 promotes epithelial-to-mesenchymal transition and prostate tumorigenesis. *Oncogene* **34**: 2690-2699.

Kabir, F.M.L., Deinnocentes, P., Agarwal, P., Mill, C.P., Riese, D.J., and Bird, R.C. (2017). Estrogen receptor-α, progesterone receptor, and c-erbB/HER-family receptor mRNA detection and phenotype analysis in spontaneous canine models of breast cancer. *Journal of Veterinary Science* **18**: 149-158.

Kalluri, R. (2016). The biology and function of fibroblasts in cancer. *Nature Reviews Cancer* **16**: 582.

Kalluri, R., and Zeisberg, M. (2006). Fibroblasts in cancer. *Nature Reviews Cancer* **6**: 392-401.

Kendall, R.T., and Feghali-Bostwick, C.A. (2014). Fibroblasts in fibrosis: novel roles and mediators. *Frontiers in Pharmacology* **5**: 123.

Khanna, C., Lindblad-Toh, K., Vail, D., London, C., Bergman, P., Barber, L., Breen, M., Kitchell, B., Mcneil, E., and Modiano, J.F. (2006). The dog as a cancer model. *Nature Biotechnology* **24**: 1065.

King, H.W., Michael, M.Z., and Gleadle, J.M. (2012). Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer* **12**: 421.

Kirpensteijn, J., Kik, M., Teske, E., and Rutteman, G.R. (2008). TP53 gene mutations in canine osteosarcoma. *Veterinary Surgery* **37**: 454-460.

Kogure, A., Kosaka, N., and Ochiya, T. (2019). Cross-talk between cancer cells and their neighbors via miRNA in extracellular vesicles: an emerging player in cancer metastasis. *Journal of Biomedical Science* **26**: 1-8.

Komazawa, S., Sakai, H., Itoh, Y., Kawabe, M., Murakami, M., Mori, T., and Maruo, K. (2016). Canine tumor development and crude incidence of tumors by breed based on domestic dogs in Gifu prefecture. *Journal of Veterinary Medical Science* **78**: 1269-1275.

Kool, M., Galac, S., Van Der Helm, N., Corradini, S., Kooistra, H., and Mol, J. (2015). Insulin-like growth factor--phosphatidylinositol 3 kinase signaling in canine cortisol-secreting adrenocortical tumors. *Journal of Veterinary Internal Medicine* **29**: 214-224.

Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y., and Ochiya, T. (2010). Secretory mechanisms and intercellular transfer of microRNAs in living cells. *Journal of Biological Chemistry* **285**: 17442-17452.

Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J.P., Primdal-Bengtson, B., Dingli, F., Loew, D., Tkach, M., and Théry, C. (2016). Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proceedings of the National Academy of Sciences* **113**: E968-E977.

Król, M., Pawłowski, K.M., Szyszko, K., Maciejewski, H., Dolka, I., Manuali, E., Jank, M., and Motyl, T. (2012). The gene expression profiles of canine mammary cancer cells grown with carcinoma-associated fibroblasts (CAFs) as a co-culture in vitro. *BMC Veterinary Research* **8**: 35.

Kwabi-Addo, B., Ozen, M., and Ittmann, M. (2004). The role of fibroblast growth factors and their receptors in prostate cancer. *Endocrine-Related Cancer* **11**: 709-724.

Larson, G., and Bradley, D.G. (2014). How much is that in dog years? The advent of canine population genomics. *PLoS Genetics* **10**: e1004093.

Larue, S., Withrow, S., Powers, B., Wrigley, R., Gillette, E., Schwarz, P., Straw, R., and Richter, S. (1989). Limb-sparing treatment for osteosarcoma in dogs. *Journal of the American Veterinary Medical Association* **195**: 1734-1744.

Lebleu, V.S., and Kalluri, R. (2018). A peek into cancer-associated fibroblasts: origins, functions and translational impact. *Disease Models & Mechanisms* **11**: dmm029447.

Lee, K.-H., Shin, T.-J., Kim, W.-H., Lee, S.-Y., and Cho, J.-Y. (2019). Methylation of LINE-1 in cell-free DNA serves as a liquid biopsy biomarker for human breast cancers and dog mammary tumors. *Scientific Reports* **9**: 1-10.

Legesse-Miller, A., Elemento, O., Pfau, S.J., Forman, J.J., Tavazoie, S., and Coller, H.A. (2009). let-7 overexpression leads to an increased fraction of cells in G2/M, direct down-regulation of Cdc34, and stabilization of Wee1 kinase in primary fibroblasts. *Journal of Biological Chemistry* **284**: 6605-6609.

Lei, Z., Li, B., Yang, Z., Fang, H., Zhang, G.-M., Feng, Z.-H., and Huang, B. (2009). Regulation of HIF-1α and VEGF by miR-20b tunes tumor cells to adapt to the alteration of oxygen concentration. *PloS One* **4**: e7629.

Leonard, J.A., Wayne, R.K., Wheeler, J., Valadez, R., Guillén, S., and Vila, C. (2002). Ancient DNA evidence for Old World origin of New World dogs. *Science* **298**: 1613-1616.

Levental, K.R., Yu, H., Kass, L., Lakins, J.N., Egeblad, M., Erler, J.T., Fong, S.F., Csiszar, K., Giaccia, A., and Weninger, W. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* **139**: 891-906.

Li, P., Kaslan, M., Lee, S.H., Yao, J., and Gao, Z. (2017). Progress in exosome isolation techniques. *Theranostics* **7**: 789.

Li, Q., Li, B., Li, Q., Wei, S., He, Z., Huang, X., Wang, L., Xia, Y., Xu, Z., and Li, Z. (2018). Exosomal miR-21-5p derived from gastric cancer promotes peritoneal metastasis via mesothelial-to-mesenchymal transition. *Cell Death & Disease* **9**: 1-17.

Li, X., Wu, Z., Fu, X., and Han, W. (2012). A microRNA component of the neoplastic microenvironment: microregulators with far-reaching impact. *BioMed Research International* **2013**: 762183.

Lin, S., and Gregory, R.I. (2015). MicroRNA biogenesis pathways in cancer. *Nature Reviews Cancer* **15**: 321-333.

Lindblad-Toh, K., Wade, C.M., Mikkelsen, T.S., Karlsson, E.K., Jaffe, D.B., Kamal, M., Clamp, M., Chang, J.L., Kulbokas Iii, E.J., and Zody, M.C. (2005). Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* **438**: 803.

Liu, T., Han, C., Wang, S., Fang, P., Ma, Z., Xu, L., and Yin, R. (2019). Cancer-associated fibroblasts: an emerging target of anti-cancer immunotherapy. *Journal of Hematology & Oncology* **12**: 1-15.

Loria, A.D., Dattilo, V., Santoro, D., Guccione, J., De Luca, A., Ciaramella, P., Pirozzi, M., and Iaccino, E. (2020). Expression of serum exosomal miRNA 122 and lipoprotein levels in dogs naturally infected by Leishmania infantum: a preliminary study. *Animals* **10**: 100.

Lötvall, J., Hill, A.F., Hochberg, F., Buzás, E.I., Di Vizio, D., Gardiner, C., Gho, Y.S., Kurochkin, I.V., Mathivanan, S., and Quesenberry, P. (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *Journal of Extracellular Vesicles* **3**: 26913

Lutful Kabir, F.M., Alvarez, C.E., and Bird, R.C. (2016). Canine mammary carcinomas: a comparative analysis of altered gene expression. *Veterinary Sciences* **3**: 1.

Madar, S., Goldstein, I., and Rotter, V. (2013). 'Cancer associated fibroblasts'–more than meets the eye. *Trends in Molecular Medicine* **19**: 447-453.

Maeda, S., Tomiyasu, H., Tsuboi, M., Inoue, A., Ishihara, G., Uchikai, T., Chambers, J.K., Uchida, K., Yonezawa, T., and Matsuki, N. (2018). Comprehensive gene expression analysis of canine invasive urothelial bladder carcinoma by RNA-seq. *BMC Cancer* **18**: 472.

Maekawa, N., Konnai, S., Takagi, S., Kagawa, Y., Okagawa, T., Nishimori, A., Ikebuchi, R., Izumi, Y., Deguchi, T., and Nakajima, C. (2017). A canine chimeric monoclonal antibody targeting PD-L1 and its clinical efficacy in canine oral malignant melanoma or undifferentiated sarcoma. *Scientific Reports* **7**: 1-12.

Maltby, S., Khazaie, K., and Mcnagny, K.M. (2009). Mast cells in tumor growth: angiogenesis, tissue remodelling and immune-modulation. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* **1796**: 19-26.

Mansoori, B., Mohammadi, A., Shirjang, S., and Baradaran, B. (2017). MicroRNAs in the diagnosis and treatment of cancer. *Immunological Investigations* **46**: 880-897.

Mantovani, A., Savino, B., Locati, M., Zammataro, L., Allavena, P., and Bonecchi, R. (2010). The chemokine system in cancer biology and therapy. *Cytokine & Growth Factor Reviews* **21**: 27-39.

Marsh, T., Pietras, K., and Mcallister, S.S. (2013). Fibroblasts as architects of cancer pathogenesis. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* **1832**: 1070-1078.

Mashouri, L., Yousefi, H., Aref, A.R., Mohammad Ahadi, A., Molaei, F., and Alahari, S.K. (2019). Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. *Molecular Cancer* **18**: 75.

Mathieu, M., Martin-Jaular, L., Lavieu, G., and Thery, C. (2019). Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nature Cell Biology* **21**: 9-17.

Mckelvey, K.J., Powell, K.L., Ashton, A.W., Morris, J.M., and Mccracken, S.A. (2015). Exosomes: mechanisms of uptake. *Journal of Circulating Biomarkers* **4**: 7.

Medina, P.P., Nolde, M., and Slack, F.J. (2010). OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature* **467**: 86-90.

Melo, S.A., Sugimoto, H., O'Connell, J.T., Kato, N., Villanueva, A., Vidal, A., Qiu, L., Vitkin, E., Perelman, L.T., and Melo, C.A. (2014). Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. *Cancer Cell* **26**: 707-721.

Merlo, D.F., Rossi, L., Pellegrino, C., Ceppi, M., Cardellino, U., Capurro, C., Ratto, A., Sambucco, P., Sestito, V., and Tanara, G. (2008). Cancer incidence in pet dogs: findings of the Animal Tumor Registry of Genoa, Italy. *Journal of Veterinary Internal Medicine* **22**: 976-984.

Micallef, L., Vedrenne, N., Billet, F., Coulomb, B., Darby, I.A., and Desmoulière, A. (2012). The myofibroblast, multiple origins for major roles in normal and pathological tissue repair. *Fibrogenesis & Tissue Repair* **5**: S5.

Mitra, A.K., Zillhardt, M., Hua, Y., Tiwari, P., Murmann, A.E., Peter, M.E., and Lengyel, E. (2012). MicroRNAs reprogram normal fibroblasts into cancer-associated fibroblasts in ovarian cancer. *Cancer Discovery* **2**: 1100-1108.

Mittelbrunn, M., and Sánchez-Madrid, F. (2012). Intercellular communication: diverse structures for exchange of genetic information. *Nature Reviews Molecular Cell Biology* **13**: 328-335.

Mizuno, H., Nakamura, A., Aoki, Y., Ito, N., Kishi, S., Yamamoto, K., Sekiguchi, M., Takeda, S.I., and Hashido, K. (2011). Identification of muscle-specific microRNAs in serum of muscular dystrophy animal models: promising novel blood-based markers for muscular dystrophy. *PloS One* **6**: e18388.

Mollica, P.V., Massara, M., Capucetti, A., and Bonecchi, R. (2019). Chemokines and chemokine receptors: new targets for cancer immunotherapy. *Frontiers in Immunology* **10**: 379.

Monteiro, L., Rodrigues, M., Gomes, D., Salgado, B., and Cassali, G. (2018). Tumour-associated macrophages: relation with progression and invasiveness, and assessment of M1/M2 macrophages in canine mammary tumours. *The Veterinary Journal* **234**: 119-125.

Monteran, L., and Erez, N. (2019). The dark side of fibroblasts: cancer-associated fibroblasts as mediators of immunosuppression in the tumor microenvironment. *Frontiers in Immunology* **10**: 1835.

Mueller, F., Fuchs, B., and Kaser-Hotz, B. (2007). Comparative biology of human and canine osteosarcoma. *Anticancer Research* **27**: 155-164.

Murakami, A., Mori, T., Sakai, H., Murakami, M., Yanai, T., Hoshino, Y., and Maruo, K. (2011). Analysis of KIT expression and KIT exon 11 mutations in canine oral malignant melanomas. *Veterinary and Comparative Oncology* **9**: 219-224.

Musumeci, M., Coppola, V., Addario, A., Patrizii, M., Maugeri-Sacca, M., Memeo, L., Colarossi, C., Francescangeli, F., Biffoni, M., and Collura, D. (2011). Control of tumor and microenvironment cross-talk by miR-15a and miR-16 in prostate cancer. *Oncogene* **30**: 4231-4242.

Nawaz, M., Malik, M.I., Hameed, M., and Zhou, J. (2019). Research progress on the composition and function of parasite-derived exosomes. *Acta Tropica* **196**: 30-36.

Newman, A.C., Nakatsu, M.N., Chou, W., Gershon, P.D., and Hughes, C.C. (2011). The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Molecular Biology of the Cell* **22**: 3791-3800.

Nissen, N.I., Karsdal, M., and Willumsen, N. (2019). Collagens and cancer associated fibroblasts in the reactive stroma and its relation to cancer biology. *Journal of Experimental & Clinical Cancer Research* **38**: 115.

O'Brien, J., Hayder, H., Zayed, Y., and Peng, C. (2018). Overview of microRNA biogenesis, mechanisms of actions, and circulation. *Frontiers in Endocrinology* **9**: 402.

Öhlund, D., Elyada, E., and Tuveson, D. (2014). Fibroblast heterogeneity in the cancer wound. *Journal of Experimental Medicine* **211**: 1503-1523.

Ostrander, E.A., Dreger, D.L., and Evans, J.M. (2019). Canine cancer genomics: lessons for canine and human health. *Annual Review of Animal Biosciences* **7**: 449-472.

Ostrander, E.A., and Wayne, R.K. (2005). The canine genome. *Genome Research* **15**: 1706-1716.

Ostrowski, M., Carmo, N.B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., Moita, C.F., Schauer, K., Hume, A.N., and Freitas, R.P. (2010). Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nature Cell Biology* **12**: 19-30.

Othman, N., Jamal, R., and Abu, N. (2019). Cancer-derived exosomes as effectors of key inflammation-related players. *Frontiers in Immunology* **10**: 2103.

Ovodov, N.D., Crockford, S.J., Kuzmin, Y.V., Higham, T.F., Hodgins, G.W., and Van Der Plicht, J. (2011). A 33,000-year-old incipient dog from the Altai Mountains of Siberia: evidence of the earliest domestication disrupted by the Last Glacial Maximum. *PloS One* **6**: e22821.

Paladini, L., Fabris, L., Bottai, G., Raschioni, C., Calin, G.A., and Santarpia, L. (2016). Targeting microRNAs as key modulators of tumor immune response. *Journal of Experimental & Clinical Cancer Research* **35**: 103.

Palmieri, C. (2015). Immunohistochemical expression of angiogenic factors by neoplastic epithelial cells is associated with canine prostatic carcinogenesis. *Veterinary Pathology* **52**: 607-613.

Pan, B.-T., and Johnstone, R.M. (1983). Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* **33**: 967-978.

Pan, Z., Tian, Y., Niu, G., and Cao, C. (2020). Role of microRNAs in remodeling the tumor microenvironment. *International Journal of Oncology* **56**: 407-416.

Panjwani, M.K., Smith, J.B., Schutsky, K., Gnanandarajah, J., O'Connor, C.M., Powell Jr, D.J., and Mason, N.J. (2016). Feasibility and safety of RNA-transfected CD20-specific chimeric antigen receptor T cells in dogs with spontaneous B cell lymphoma. *Molecular Therapy* **24**: 1602-1614.

Pankova, D., Chen, Y., Terajima, M., Schliekelman, M.J., Baird, B.N., Fahrenholtz, M., Sun, L., Gill, B.J., Vadakkan, T.J., and Kim, M.P. (2016). Cancer-associated fibroblasts induce a collagen cross-link switch in tumor stroma. *Molecular Cancer Research* **14**: 287-295.

Parker, H.G., Shearin, A.L., and Ostrander, E.A. (2010). Man's best friend becomes biology's best in show: genome analyses in the domestic dog. *Annual Review of Genetics* **44**: 309-336.

Patel, G.K., Khan, M.A., Zubair, H., Srivastava, S.K., Singh, S., and Singh, A.P. (2019). Comparative analysis of exosome isolation methods using culture supernatant for optimum yield, purity and downstream applications. *Scientific Reports* **9**: 1-10.

Peña, C., Céspedes, M.V., Lindh, M.B., Kiflemariam, S., Mezheyeuski, A., Edqvist, P.-H., Hägglöf, C., Birgisson, H., Bojmar, L., and Jirström, K. (2013). STC1 expression by cancer-associated fibroblasts drives metastasis of colorectal cancer. *Cancer Research* **73**: 1287-1297.

Peter, M. (2010). Targeting of mRNAs by multiple miRNAs: the next step. *Oncogene* **29**: 2161-2164.

Price, C., and Chen, J. (2014). MicroRNAs in cancer biology and therapy: current status and perspectives. *Genes & Diseases* **1**: 53-63.

Prouteau, A., and André, C. (2019). Canine melanomas as models for human melanomas: clinical, histological, and genetic comparison. *Genes* **10**: 501.

Rana, S., Malinowska, K., and Zöller, M. (2013). Exosomal tumor microRNA modulates premetastatic organ cells. *Neoplasia (New York, NY)* **15**: 281.

Ranieri, G., Gadaleta, C., Patruno, R., Zizzo, N., Daidone, M., Hansson, M.G., Paradiso, A., and Ribatti, D. (2013). A model of study for human cancer: spontaneous occurring tumors in dogs. Biological features and translation for new anticancer therapies. *Critical Reviews in Oncology/Hematology* **88**: 187-197.

Raposo, G., Nijman, H.W., Stoorvogel, W., Liejendekker, R., Harding, C.V., Melief, C., and Geuze, H.J. (1996). B lymphocytes secrete antigen-presenting vesicles. *The Journal of Experimental Medicine* **183**: 1161-1172.

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *Journal of Cell Biology* **200**: 373-383.

Ratajczak, J., Miekus, K., Kucia, M., Zhang, J., Reca, R., Dvorak, P., and Ratajczak, M. (2006). Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* **20**: 847-856.

Richards, K.E., Zeleniak, A.E., Fishel, M.L., Wu, J., Littlepage, L.E., and Hill, R. (2017). Cancer-associated fibroblast exosomes regulate survival and proliferation of pancreatic cancer cells. *Oncogene* **36**: 1770-1778.

Richards, K.L., Motsinger-Reif, A.A., Chen, H.-W., Fedoriw, Y., Fan, C., Nielsen, D.M., Small, G.W., Thomas, R., Smith, C., and Dave, S.S. (2013). Gene profiling of canine B-cell lymphoma reveals germinal center and postgerminal center subtypes with different survival times, modeling human DLBCL. *Cancer Research* **73**: 5029-5039.

Rotin, D., Robinson, B., and Tannock, I.F. (1986). Influence of hypoxia and an acidic environment on the metabolism and viability of cultured cells: potential implications for cell death in tumors. *Cancer Research* **46**: 2821-2826.

Rowell, J.L., Mccarthy, D.O., and Alvarez, C.E. (2011). Dog models of naturally occurring cancer. *Trends in Molecular Medicine* **17**: 380-388.

Ruivo, C.F., Adem, B., Silva, M., and Melo, S.A. (2017). The biology of cancer exosomes: insights and new perspectives. *Cancer Research* **77**: 6480-6488.

Sahabi, K., Selvarajah, G.T., Abdullah, R., Cheah, Y.K., and Tan, G.C. (2018). Comparative aspects of microRNA expression in canine and human cancers. *Journal of Veterinary Science* **19**: 162-171.

Sahai, E., Astsaturov, I., Cukierman, E., Denardo, D.G., Egeblad, M., Evans, R.M., Fearon, D., Greten, F.R., Hingorani, S.R., and Hunter, T. (2020). A framework for advancing our understanding of cancer-associated fibroblasts. *Nature Reviews Cancer* **20**: 174-186.

Sakthikumar, S., Elvers, I., Kim, J., Arendt, M.L., Thomas, R., Turner-Maier, J., Swofford, R., Johnson, J., Schumacher, S.E., and Alföldi, J. (2018). SETD2 is recurrently mutated in whole-exome sequenced canine osteosarcoma. *Cancer Research* **78**: 3421-3431.

Sarkar, S., Horn, G., Moulton, K., Oza, A., Byler, S., Kokolus, S., and Longacre, M. (2013). Cancer development, progression, and therapy: an epigenetic overview. *International Journal of Molecular Sciences* **14**: 21087-21113.

Schiffman, J.D., and Breen, M. (2015). Comparative oncology: what dogs and other species can teach us about humans with cancer. *Philosophical Transactions of the Royal Society B: Biological Sciences* **370**: 20140231.

Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., and Selbach, M. (2011). Global quantification of mammalian gene expression control. *Nature* **473**: 337.

Seo, J., Jin, D., Choi, C.-H., and Lee, H. (2017). Integration of microRNA, mRNA, and protein expression data for the identification of cancer-related microRNAs. *PloS One* **12**: e0168412.

Seung, B.-J., Lim, H.-Y., Shin, J.-I., Kim, H.-W., Cho, S.-H., Kim, S.-H., and Sur, J.-H. (2018). CD204-expressing tumor-associated macrophages are associated with malignant, high-grade, and hormone receptor–negative canine mammary gland tumors. *Veterinary Pathology* **55**: 417-424.

Shaffer, L. (2019). Special issue on canine genetics: animal models for human disease and gene therapies, new discoveries for canine inherited diseases, and standards and guidelines for clinical genetic testing for domestic dogs. *Human Genetics* **138**: 437.

Shearin, A.L., Hedan, B., Cadieu, E., Erich, S.A., Schmidt, E.V., Faden, D.L., Cullen, J., Abadie, J., Kwon, E.M., and Gröne, A. (2012). The MTAP-CDKN2A locus confers susceptibility to a naturally occurring canine cancer. *Cancer Epidemiology and Prevention Biomarkers* **21**: 1019-1027.

Shearin, A.L., and Ostrander, E.A. (2010). Leading the way: canine models of genomics and disease. *Disease Models & Mechanisms* **3**: 27-34.

Shen, H., Yu, X., Yang, F., Zhang, Z., Shen, J., Sun, J., Choksi, S., Jitkaew, S., and Shu, Y. (2016). Reprogramming of normal fibroblasts into cancer-associated fibroblasts by miRNAs-mediated CCL2/VEGFA signaling. *PLoS Genetics* **12**: e1006244.

Shen, J., Zhai, J., You, Q., Zhang, G., He, M., Yao, X., and Shen, L. (2020). Cancer-associated fibroblasts-derived VCAM1 induced by H. pylori infection facilitates tumor invasion in gastric cancer. *Oncogene* **39**: 2961-2974.

Sherger, M., Kisseberth, W., London, C., Olivo-Marston, S., and Papenfuss, T.L. (2012). Identification of myeloid derived suppressor cells in the peripheral blood of tumor bearing dogs. *BMC Veterinary Research* **8**: 209.

Simpson, R.M., Bastian, B.C., Michael, H.T., Webster, J.D., Prasad, M.L., Conway, C.M., Prieto, V.M., Gary, J.M., Goldschmidt, M.H., and Esplin, D.G. (2014). Sporadic naturally occurring melanoma in dogs as a preclinical model for human melanoma. *Pigment Cell & Melanoma Research* **27**: 37-47.

Sounni, N.E., and Noel, A. (2013). Targeting the tumor microenvironment for cancer therapy. *Clinical Chemistry* **59**: 85-93.

Stępień, E.Ł., Durak-Kozica, M., Kamińska, A., Targosz-Korecka, M., Libera, M., Tylko, G., Opalińska, A., Kapusta, M., Solnica, B., and Georgescu, A. (2018). Circulating ectosomes: determination of angiogenic microRNAs in type 2 diabetes. *Theranostics* **8**: 3874.

Suetsugu, A., Honma, K., Saji, S., Moriwaki, H., Ochiya, T., and Hoffman, R.M. (2013). Imaging exosome transfer from breast cancer cells to stroma at metastatic sites in orthotopic nude-mouse models. *Advanced Drug Delivery Reviews* **65**: 383-390.

Sun, F., Báez-Díaz, C., and Sánchez-Margallo, F.M. (2017). Canine prostate models in preclinical studies of minimally invasive interventions: part I, canine prostate anatomy and prostate cancer models. *Translational Andrology and Urology* **6**: 538.

Sun, L.P., Xu, K., Cui, J., Yuan, D.Y., Zou, B., Li, J., Liu, J.L., Li, K.Y., Meng, Z., and Zhang, B. (2019). Cancer-associated fibroblast-derived exosomal miR-382-5p promotes the migration and invasion of oral squamous cell carcinoma. *Oncology Reports* **42**: 1319-1328.

Suzuki, H., Katsura, A., Matsuyama, H., and Miyazono, K. (2015). MicroRNA regulons in tumor microenvironment. *Oncogene* **34**: 3085-3094.

Svoronos, A.A., Engelman, D.M., and Slack, F.J. (2016). OncomiR or tumor suppressor? The duplicity of microRNAs in cancer. *Cancer Research* **76**: 3666-3670.

Tanaka, K., Miyata, H., Sugimura, K., Fukuda, S., Kanemura, T., Yamashita, K., Miyazaki, Y., Takahashi, T., Kurokawa, Y., and Yamasaki, M. (2015). miR-27 is associated with chemoresistance in esophageal cancer through transformation of normal fibroblasts to cancer-associated fibroblasts. *Carcinogenesis* **36**: 894-903.

Tang, J., Le, S., Sun, L., Yan, X., Zhang, M., Macleod, J., Leroy, B., Northrup, N., Ellis, A., and Yeatman, T.J. (2010). Copy number abnormalities in sporadic canine colorectal cancers. *Genome Research* **20**: 341-350.

Thakkar, S., Sharma, D., Kalia, K., and Tekade, R.K. (2019). Tumor microenvironment targeted nanotherapeutics for cancer therapy and diagnosis: a review. *Acta Biomaterialia* **101**: 43-68.

Thamm, D.H. (2019). Canine cancer: strategies in experimental therapeutics. *Frontiers in Oncology* **9**: 1257.

Theoharides, T.C., and Conti, P. (2004). Mast cells: the Jekyll and Hyde of tumor growth. *Trends in Immunology* **25**: 235-241.

Tommelein, J., Verset, L., Boterberg, T., Demetter, P., Bracke, M., and De Wever, O. (2015). Cancer-associated fibroblasts connect metastasis-promoting communication in colorectal cancer. *Frontiers in Oncology* **5**: 63.

Trimboli, A.J., Cantemir-Stone, C.Z., Li, F., Wallace, J.A., Merchant, A., Creasap, N., Thompson, J.C., Caserta, E., Wang, H., and Chong, J.-L. (2009). Pten in stromal fibroblasts suppresses mammary epithelial tumours. *Nature* **461**: 1084-1091.

Troyer, R.M., Ruby, C.E., Goodall, C.P., Yang, L., Maier, C.S., Albarqi, H.A., Brady, J.V., Bathke, K., Taratula, O., and Mourich, D. (2017). Exosomes from osteosarcoma and normal osteoblast differ in proteomic cargo and immunomodulatory effects on T cells. *Experimental Cell Research* **358**: 369-376.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., and Lötvall, J.O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology* **9**: 654-659.

Valkenburg, K.C., De Groot, A.E., and Pienta, K.J. (2018). Targeting the tumour stroma to improve cancer therapy. *Nature Reviews Clinical Oncology* **15**: 366-381.

Villarroya-Beltri, C., Baixauli, F., Gutiérrez-Vázquez, C., Sánchez-Madrid, F., and Mittelbrunn, M. (2014). Sorting it out: regulation of exosome loading. *Seminars in Cancer Biology* **28**: 3-13.

Vonholdt, B.M., Pollinger, J.P., Lohmueller, K.E., Han, E., Parker, H.G., Quignon, P., Degenhardt, J.D., Boyko, A.R., Earl, D.A., and Auton, A. (2010). Genome-wide SNP and haplotype analyses reveal a rich history underlying dog domestication. *Nature* **464**: 898-902.

Wagner, S., Willenbrock, S., Nolte, I., and Murua Escobar, H. (2013). Comparison of non-coding RNAs in human and canine cancer. *Frontiers in Genetics* **4**: 46.

Wang, G.-D., Zhai, W., Yang, H.-C., Fan, R.-X., Cao, X., Zhong, L., Wang, L., Liu, F., Wu, H., and Cheng, L.-G. (2013). The genomics of selection in dogs and the parallel evolution between dogs and humans. *Nature Communications* **4**: 1860.

Wang, J., Wang, T., Sun, Y., Feng, Y., Kisseberth, W.C., Henry, C.J., Mok, I., Lana, S.E., Dobbin, K., and Northrup, N. (2018). Proliferative and invasive colorectal tumors in pet dogs provide unique insights into human colorectal cancer. *Cancers* **10**: 330.

Wang, M., Zhao, J., Zhang, L., Wei, F., Lian, Y., Wu, Y., Gong, Z., Zhang, S., Zhou, J., and Cao, K. (2017a). Role of tumor microenvironment in tumorigenesis. *Journal of Cancer* **8**: 761.

Wang, Z., Tan, Y., Yu, W., Zheng, S., Zhang, S., Sun, L., and Ding, K. (2017b). Small role with big impact: miRNAs as communicators in the cross-talk between cancer-associated fibroblasts and cancer cells. *International Journal of Biological Sciences* **13**: 339.

Wayne, R.K., and Ostrander, E.A. (2007). Lessons learned from the dog genome. *Trends in Genetics* **23**: 557-567.

Webber, J., Steadman, R., Mason, M.D., Tabi, Z., and Clayton, A. (2010). Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer Research* **70**: 9621-9630.

Wei, R., Liu, S., Zhang, S., Min, L., and Zhu, S. (2020). Cellular and extracellular components in tumor microenvironment and their application in early diagnosis of cancers. *Analytical Cellular Pathology* **2020**: 6283796.

Willmann, M., Hadzijusufovic, E., Hermine, O., Dacasto, M., Marconato, L., Bauer, K., Peter, B., Gamperl, S., Eisenwort, G., and Jensen-Jarolim, E. (2019). Comparative oncology: the paradigmatic example of canine and human mast cell neoplasms. *Veterinary and Comparative Oncology* **17**: 1-10.

Wolfers, J., Lozier, A., Raposo, G., Regnault, A., Théry, C., Masurier, C., Flament, C., Pouzieux, S., Faure, F., and Tursz, T. (2001). Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nature Medicine* **7**: 297-303.

Yang, F., Ning, Z., Ma, L., Liu, W., Shao, C., Shu, Y., and Shen, H. (2017a). Exosomal miRNAs and miRNA dysregulation in cancer-associated fibroblasts. *Molecular Cancer* **16**: 148.

Yang, N., Zhu, S., Lv, X., Qiao, Y., Liu, Y.-J., and Chen, J. (2018). MicroRNAs: pleiotropic regulators in the tumor microenvironment. *Frontiers in Immunology* **9**: 2491.

Yang, V.K., Loughran, K.A., Meola, D.M., Juhr, C.M., Thane, K.E., Davis, A.M., and Hoffman, A.M. (2017b). Circulating exosome microRNA associated with heart failure secondary to myxomatous mitral valve disease in a naturally occurring canine model. *Journal of Extracellular Vesicles* **6**: 1350088.

Yang, X., Li, Y., Zou, L., and Zhu, Z. (2019). Role of exosomes in crosstalk between cancer-associated fibroblasts and cancer cells. *Frontiers in Oncology* **9**: 356.

Yeung, C.L.A., Co, N.-N., Tsuruga, T., Yeung, T.-L., Kwan, S.-Y., Leung, C.S., Li, Y., Lu, E.S., Kwan, K., and Wong, K.-K. (2016). Exosomal transfer of stroma-derived miR21 confers paclitaxel resistance in ovarian cancer cells through targeting APAF1. *Nature Communications* **7**: 11150.

Ying, X., Wu, Q., Wu, X., Zhu, Q., Wang, X., Jiang, L., Chen, X., and Wang, X. (2016). Epithelial ovarian cancer-secreted exosomal miR-222-3p induces polarization of tumor-associated macrophages. *Oncotarget* **7**: 43076.

Yoshimoto, S., Hoshino, Y., Izumi, Y., and Takagi, S. (2017). α-Smooth muscle actin expression in cancerassociated fibroblasts in canine epithelial tumors. *Japanese Journal of Veterinary Research* **65**: 135-144.

Yuan, Y., Jiang, Y.-C., Sun, C.-K., and Chen, Q.-M. (2016). Role of the tumor microenvironment in tumor progression and the clinical applications. *Oncology Reports* **35**: 2499-2515.

Zhang, H.-G., and Grizzle, W.E. (2014). Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. *The American Journal of Pathology* **184**: 28-41.

Zhang, H., Freitas, D., Kim, H.S., Fabijanic, K., Li, Z., Chen, H., Mark, M.T., Molina, H., Martin, A.B., and Bojmar, L. (2018). Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nature Cell Biology* **20**: 332-343.

Zhang, Q., Higginbotham, J.N., Jeppesen, D.K., Yang, Y.-P., Li, W., Mckinley, E.T., Graves-Deal, R., Ping, J., Britain, C.M., and Dorsett, K.A. (2019). Transfer of functional cargo in exomeres. *Cell Reports* **27**: 940-954.e946.

Zhou, J., Li, X., Wu, X., Zhang, T., Zhu, Q., Wang, X., Wang, H., Wang, K., Lin, Y., and Wang, X. (2018). Exosomes released from tumor-associated macrophages transfer miRNAs that induce a Treg/Th17 cell imbalance in epithelial ovarian cancer. *Cancer Immunology Research* **6**: 1578-1592.

Zizzo, N., Passantino, G., D'Alessio, R.M., Tinelli, A., Lopresti, G., Patruno, R., Tricarico, D., Maqoud, F., Scala, R., and Zito, F.A. (2019). Thymidine phosphorylase expression and microvascular density correlation analysis in canine mammary tumor: possible prognostic factor in breast cancer. *Frontiers in Veterinary Science* **6**: 368.

10. Publications and scientific activity

10.1. Publications

- **Aguilera-Rojas, M.**, Sharbati, S., Stein, T., and Einspanier, R. (2020). Deregulation of miR-27a may contribute to canine fibroblast activation after coculture with a mast cell tumour cell line. *FEBS Open Bio* **10:** 802-816.
  DOI: https://doi.org/10.1002/2211-5463.12831
- **Aguilera-Rojas, M.**, Badewien-Rentzsch, B., Plendl, J., Kohn, B., and Einspanier, R. (2018). Exploration of serum- and cell culture-derived exosomes from dogs. *BMC Veterinary Research* **14**:179.
  DOI: https://doi.org/10.1186/s12917-018-1509-x
- Peñailillo, A.K., Sepulveda, M.A., Palma, C.J., Espinoza, A., **Aguilera, M.**, Burgos, R.A., Carretta, D., Islas, A., and Pérez, R. (2016). Haematological and blood biochemical changes induced by the administration of low doses of Escherichia coli lipopolysaccharide in rabbits. *Archivos de Medicina Veterinaria* **48:**315-320.
  DOI: http://dx.doi.org/10.4067/S0301-732X2016000300012

10.2. Conference and seminar presentations

- **M. Aguilera-Rojas,** S. Sharbati, T. Stein and R. Einspanier. Effects of mir-27a on reprogramming canine fibroblasts after co-culture with a mast-cell tumour cell line. Poster at the Tagung der DVG-Fachgruppe “Physiologie und Biochemie”. Leipzig, Germany. March 18th – 20th, 2020. **Conference was cancelled due to COVID-19*
- **Matias Aguilera-Rojas,** Brit Badewien-Rentzsch, Johanna Plendl, Barbara Kohn and Ralf Einspanier. Isolation and characterization of exosomes from serum and cell culture supernatants in dog samples. Poster at the 11. Doktorandensymposium & DRS Präsentationsseminar “Biomedical Sciences”. Berlin, Germany. September 21st, 2018
- **Matias Aguilera-Rojas,** Barbara Kohn, Johanna Plendl, Ralf Einspanier. Isolierung von Exosomen aus Blutserum und Zellkulturüberständen zu Diagnostikzwecken beim Hund - Isolation of exosomes from blood serum and cell culture supernatants for diagnostic purposes in dogs. Poster and oral presentation (plenary poster) at the Tagung der DVG-Fachgruppe “Physiologie und Biochemie”. Vienna, Austria. February 21st – 23rd, 2018
- **M. Aguilera,** B. Badewien-Rentzsch, K. Pawar, R. Einspanier. First identification of long non-coding RNA H19 expression in different canine cancer tissues. Poster presentation at the Tagung der DVG-Fachgruppe “Physiologie und Biochemie”. Berlin, Germany. March 30th – April 01st, 2016

- miRNA and gene expression in canine fibroblasts exposed to cancer cells. Oral presentation in the Tierbiochemisches Seminar/Animal Biochemistry Seminar at the Institut für Veterinär-Biochemie, Freie Universität Berlin. June 21st, 2019
- miRNAs in canine cancer. Oral presentation in the Tierbiochemisches Seminar/Animal Biochemistry Seminar at the Institut für Veterinär-Biochemie, Freie Universität Berlin. November 23rd, 2018
- Protein tumour markers in canines. Oral presentation in the Tierbiochemisches Seminar/Animal Biochemistry Seminar at the Institut für Veterinär-Biochemie, Freie Universität Berlin. June 16th, 2017
- Proteins as biomarkers in canine cancer. Oral presentation in the Tierbiochemisches Seminar/Animal Biochemistry Seminar at the Institut für Veterinär-Biochemie, Freie Universität Berlin. January 13th, 2017
- Identification of canine tumour markers. Oral presentation in the Tierbiochemisches Seminar/Animal Biochemistry Seminar at the Institut für Veterinär-Biochemie, Freie Universität Berlin. July 1st, 2016
- Project presentation (DAAD-CONICYT grant): From Chile to Berlin. Oral presentation in the Tierbiochemisches Seminar/Animal Biochemistry Seminar at the Institut für Veterinär-Biochemie, Freie Universität Berlin. June 5th, 2015

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12. Statement of contributions

The contributions of **Matías Ignacio Aguilera Rojas** to the published research articles presented in this cumulative doctoral thesis are detailed in the following table:

Contribution	Publication 1	Publication 2
Study design	++	+
Execution of experiments	+++	+++
Data analysis	+++	+++
Manuscript writing	+++	+++
Manuscript editing	++	++

Score: + = < 50%; ++ = 50 to 75%; +++ = > 75%

13. Selbstständigkeitserklärung

Hiermit bestätige ich, **Matías Ignacio Aguilera Rojas,** dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den **18.08.2020** **Matías Ignacio Aguilera Rojas**

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