ELUCIDATION OF GENOMIC STRUCTURE, BIOLOGICAL PROPERTIES, AND FUNCTIONAL STATUS OF CANINE MDA-7 AND ITS RECEPTORS

by

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ABSTRACT

Human melanoma differentiation associated gene-7/interleukin-24 (*mda-7*/IL-24) is a tumor suppressor gene with interleukin properties. Ectopic expression of MDA-7 protein causes growth suppression and induces apoptosis in a wide variety of cancer cells. In this study, we identified a canine ortholog of the human *mda-7* gene, and elucidated its genomic structue and biological properties. Canine *mda-7* was mapped to chromosome 7, located in a cluster of IL-10 family members. Canine MDA-7 was found to be endogenously expressed in cultured normal canine epidermal keratinocytes (NCEKs). When compared to human *mda-7* mRNA, canine *mda-7* mRNA had a very short 3' untranslated region. Pre-mRNA transcribed from the canine MDA-7 locus was alternatively spliced (Exon skipping and use of alternative 5' donor sites) to yield five splice variants in cultured NCEKs. Canine mda-7 splice variant1 (sv1) was the predominant splice variant expressed in NCEKs, while sv2 and sv5 were expressed at intermediate levels and sv3 and sv4 at the lowest level. These splice variants encode four isoforms of canine MDA-7 protein, which have similar amino acid sequences at their amino terminus. Canine MDA-7 is constitutively expressed in NCEKs and its expression was induced in PBMCs after lipopolysaccharide (LPS) stimulation. Similarly, expression of canine *mda-7* mRNA was increased in NCEKs after LPS stimulation. Canine MDA-7 mRNA was not expressed in most canine tumor cells and tumor samples. However, one cancer cell line, canine mammary tumor-12 (CMT12) expressed canine MDA-7 at very

high levels. When ectopically expressed from a plasmid vector, it suppressed the growth of canine and human tumor cells. Canine MDA-7 treated cancer cells accumulated in G2/M phase and underwent apoptosis. Canine MDA-7 has cytotoxic effects on cancer cells but not on normal canine fibroblasts (NCFs). In addition, canine MDA-7 protein isoforms also have growth inhibitory effects on cancer cells. Canine MDA-7 has a 28 amino acid long signal peptide sequence, and a possible cleavage site between the 28th and 29th amino acid. Canine MDA-7 was determined to be actively secreted and could bind to and signal through human MDA-7 receptors (IL-20R1/IL-20R2 and IL-22R1/IL-20R2). Secreted canine MDA-7 showed bystander antitumor activity against human tumor cells. In this study, we identified and showed that canine IL-20R1 and IL-22R1 subunits were expressed in NCEKs, canine tissues and tumor cells. A truncated mRNA sequence encoding for the extracellular domain of canine IL-20R2 subunit was also identified. The truncated canine IL-20R2 subunit does not have a transmembrane or intracellular domain and thus, it cannot translocate into the cell membrane to make functional canine MDA-7 receptor. Canine MDA-7 did not show bystander antitumor activity against canine tumor cells. This is either due to a lack of expression of functional MDA-7 receptors or due to expession of a truncated canine IL-20R2 subunit, which can block the activity of canine IL-24. In summary, we have shown that canine MDA-7 is indeed an ortholog of MDA-7. It has strikingly similarities in amino acid sequence, genomic and protein structure to human MDA-7. However, the canine MDA-7 receptor system appears to be defective, due to the absence of a full-length IL-20R2 subunit, leading to a failure of the mechanism responsible for the bystander antitumor effect.

Although the canine MDA-7 has the potential to be used for cancer gene therapy to treat canine cancers, however, the lack of a bystander effect in dogs may limit its usefulness.

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DEDICATED TO

MY FATHER, MOTHER

AND BROTHER

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LIST OF ABBREVIATIONS

AKT	Protein Kinase-B
AP-1	Activator Protein-1
ARE	AU Rich Elements
ATF6	Activating Transcription Factor 6
ATG5	Autophagy Protein 5
BAK	Bcl-2 Homologous Antagonist/Killer
Bcl-2	B Cell Lymphoma 2
Bcl-xL	B- cell Lymphoma Extra Large
bFGF	basic Fibroblast Growth Factor
C/EBP	CAAT/enhancer-Binding Protein
CD3	Cluster of Differentation 3
cDNA	Complementery DNA
CMT	Canine Mammary Tumor
CMV	Cytomegalo Virus
ConA	Concanavalin A
DD-PCR	Differential Display-Polymerase Chain Reaction
DISH	Differentiation Induction Subtraction Hybridization genes
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribose Nucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPXs	Endoperoxides
ERK1/2	Extracellular signal-regulated kinase 1/3
FADD	Fas-Associated protein with Death Domain (FADD)
FAK	Focal Adhesion Kinase
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FGFs	Fibroblast Growth Factors
FISP	Interleukin-4 Induced Secreted Protein
FNIII	Fibronectin Type III Domain
GADD	Growth Arrest and DNA Damage Inducible genes
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GST	Glutathione S – transferase
HaCaT	Human Keratinocyte Cell Line
HSPGs	Heparan Sulfate Proteoglycans
IFN-α	Interferon - α
IFN-β	Interferon-β
IL-10	Interleukin-10
IL-20R1	Interleukin-20 Receptor 1
IL-20R2	Interleukin-20 Receptor 2
IL-22R1	Interleukin-22 Receptor 1
IL-24	Interleukin-24

IFN-γ	Interferon - γ
JAK	Janus Kinase
LPS	Lipopolysaccharide
MDA-7	Melanoma Differentiation Associated Gene-7
MEZ	Mezerein
MMP	Matrix Metalloproteinase
mRNA	Messenger RiboNucleic Acid
NaCl	Sodium Chloride
NCF	Normal Canine Fibroblast
p38MAPK	p38 Mitogen Activated Protein Kinase
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly ADP Ribosyl Polymerase
PBMC	Peripheral Blood Mononuclear cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PERK	Protein kinase R – like Endoplasmic Reticulum Kinase
PHA	Phytohemagglutinin
PI3K	Phosphoinositide 3-kinase
PKR	Double Stranded RNA – dependent protein Kinase
PMSF	Phenylmethanesulfonylfluoride
PP2A	Protein Phosphatase 2A
qPCR	Quantitative Polmerase Chain Reaction
RNA	Ribonucleic acid

ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute-1640
SDS	Sodium Dodecyl Sulfate
STAT	Signal Transducer and Activator of Transcription
TBS	Tris-Buffered Saline
TGF-β	Transforming Growth Factor - β
Th2	Helper T cells type 2
TK	Tyrosin Kinase
TNF-α	Tissue Necrosis Factor - α
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor

I. REVIEW OF LITERATURE

1. Cancer pathogenesis

Cancer is the result of genetic and epigenetic changes that cause neoplastic transformation of normal cells. Genetic and epigenetic changes occur in the cell proliferation machinery that regulates the cell cycle (Weinstein 1988; Bishop 1991). These mutations occur either spontaneously or are acquired suddenly due to mutagens. These mutations vary widely, ranging from single base substitutions to chromosomal translocation (large fragments of DNA moving from one chromosome to another). Spontaneous mutations can occur due to gradual loss of an amine group on nucleotide bases, errors during replication, or failure of repair mechanisms. Rates of spontaneously occurring mutations are usually very low. However, the rate of mutation increases by several-fold when cells are exposed to various kinds of carcinogens (chemical compounds, ultraviolet and X-ray radiation etc). In normal wild type individual, most of these mutations are efficiently detected and repaired by DNA repair mechanisms. If the DNA lesions cannot be repaired, cells are induced to undergo apoptosis. In these cases mutations are resolved and do not get passed to progeny cells. However, no system of damage detection and repair is perfect. Consequently, during the lifetime of an individual, mutations accumulate in the genome of somatic cells. These mutations can occur both in non-coding and coding regions. Mutations that occur in genes encoding proteins disrupt important cellular pathways. Tumor suppressor genes, proto-oncogenes

and DNA repair genes are the most important genes that control cell growth, and mutations and play an important role in the development of various kinds of cancers (Weinstein 1988; Bishop 1991). Proteins encoded by tumor suppressor genes and proto-oncogenes control cell division. Under normal conditions most proto-oncogenes in the presence of appropriate growth and survival stimuli cause the cell to reenter cell division, while tumor suppressor genes inhibit cell division causing cell cycle exit, when the growth and survival stimuli are insufficient. Conversion of a proto-oncogene to an oncogene by mutation can stimulate the cells to undergo uncontrolled cell division even in the absence of mitogenic stimulation. Most of the mutations in proto-oncogenes to oncogenes are gain of function.

Similarly, a loss of function mutation in a tumor suppressor gene can also lead to uncontrolled cell growth (Weinstein 1988; Bishop 1991). Tumor suppressor genes code for proteins that function to restrict cell growth. These proteins can also function to aid in the repair or auto-destruction of cells with damaged DNA. When these proteins are damaged, the cell has a reduced ability to control its growth. This leads to uncontrolled cell growth, and is one of the factors in the development of cancer. However, cancer is not the result of mutations in a single gene because most cellular pathways are regulated by multiple mechanisms. Thus, multiple genes are involved in the progression of cancer. Ultimately, defects in a variety of these genes accumulate, and the cell loses its ability to control its replication. These mutations can provide a cell with selective advantages, allowing it to survive, grow and divide more vigorously than its neighbors. Over time repeated rounds of mutations, competition, and natural selection operating within the population of somatic cells, cause individual clones of mutant cells to proliferate at the

expense of their neighbors, leading to the development of cancer. Neoplastic cells also achieve cellular immortality helping them to evade cellular senescence. Thus, we can conclude that most cancers result from mutations that afttects the proper functioning of cell cycle or DNA repair machinery and result in unchecked cell growth (Weinstein 1988; Bishop 1991).

2. Discovery of human MDA-7

The unregulated growth characteristics of tumors provide both the target by which current chemotherapies work in rapidly dividing cells, and the means by which cells evade that therapy, namely mutations. Novel therapies, such as gene therapy are being developed to compensate for the defective genes with healthy ones or to introduce suicide genes (such as thymidine kinase) into cancer cells. A third approach is to transfer genes with antitumor properties into cancer cells. To develop an effective cancer treatment, we need to identify and characterize genes that have antitumor properties against a wide range of cancer cells. Differentiation therapy is an important experimental technique to identify novel genes with anticancer properties (Sachs 1978; Fisher and Grant 1985; Rowley 1991). This approach involves treatment of cancer cells with various substances that induce them to overcome their differentiation block. These substances include phorbal esters (Huberman and Callaham 1979; Huberman, Heckman et al. 1979), vitamin D metabolites (Wang, Elson et al. 1997), dimethylsulfoxide (DMSO) (Arcangeli, Carla et al. 1993; Yamada, Kondoh et al. 1997) and retinoic acid (Drach, McQueen et al. 1994; Gianni, Koken et al. 1998; Gianni, Ponzanelli et al. 2000). Once terminally differentiated cancer cells lose their proliferative capacity and tumorigenic potential.

Jiang and coworkers (1995) treated human melanoma cells (HO-1) with recombinant human fibroblast interferon–beta (IFN-β) and the protein kinase C activator mezerein (MEZ), which induced the HO-1 cells to terminally differentiate (Jiang, Lin et al. 1995; Jiang, Su et al. 1996). They hypothesized that the terminally differentiated phenotype of HO-1 cells was the result of altered gene expression (both qualitatively and quantitatively). Subtraction hybridization, a powerful technique to identify mRNAs that differ in abundance in time (undifferentiated vs. terminally differentiated cancer cells) and space (different tissues) was then used to identify various genes that were differentially expressed in terminally differentiated HO-1 cells. These genes were named melanoma differentiation associated (*mda*) and differentiation induction subtraction hybridization (*DISH*) genes. Melanoma differentiation associated gene-7 (MDA-7) was identified to be one of the genes with elevated expression levels in terminally differentiated HO-1 cells (Bonaldo, Lennon et al. 1996). Following its discovery in terminally differentiated HO-1 cells, MDA-7 was shown to be constitutively expressed by melanocytes, and its expression is inversely related to the level of melanocytic transformation (Su, Madireddi et al. 1998; Ekmekcioglu, Ellerhorst et al. 2001; Huang, Madireddi et al. 2001; Ellerhorst, Prieto et al. 2002).

3. Genomic structure of human *mda-7* gene

The human *mda-7* gene is located on chromosome 1, and this genomic locus is conserved in dog, rat, yeast and mouse (Jiang, Lin et al. 1995). Human *mda-7* has considerable structure and sequence similarity to IL-10, and is present in a cluster of IL-10 related cytokines (Kotenko 2002; Pestka, Krause et al. 2004). This cluster contains *mda-7*, IL-10, IL-19 and IL-20 and is spread over 195 kbps. Human *mda-7* is a single

copy gene and is mapped to the chromosome 1q31-1q32 locus. The coding sequences of human *mda-7* (transcription unit) are spread over a 5.33 kbp of the genomic region while its promotor occupies another 2.2 kbps (Huang, Madireddi et al. 2001). The *mda-7* locus is made up of seven exons and six introns. The human *mda-7* mRNA is 1718 bps long and encodes a 206 amino acid protein with a molecular weight of 23.8 kDa (Jiang, Lin et al. 1995).

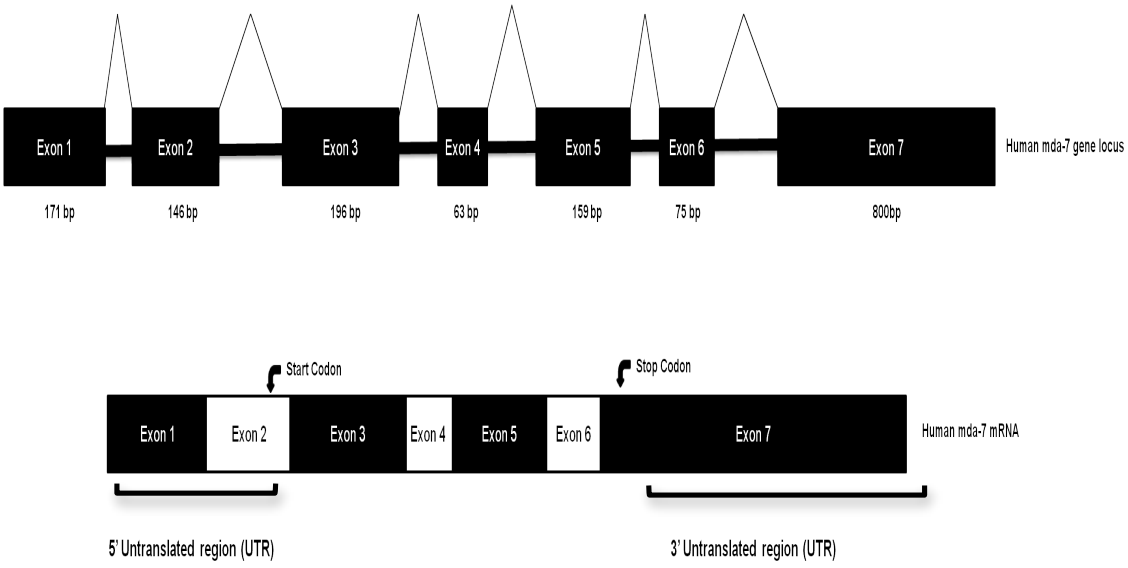


Fig 1.1: Structure of human *mda-7* gene locus and its mRNA. Human *mda-7* locus is spread over 5.5 kbps of genomic DNA. It contains seven exons (numbered 1 to 7) and six introns. After splicing of introns from the pre-mRNA, a 1.718 kbp long mRNA is produced which encodes a protein of 206 amino acids. Location of start codon (Exon 2) and stop codon (Exon 7) is shown. Human *mda-7* mRNA also contains a very long 5' and 3' untranslated region (UTR).

4. Structure of human MDA-7 protein

Human MDA-7 is also known as interleukin-24 (IL-24) because of its translational regulation, protein structure and chromosomal location (Huang, Madireddi et al. 2001; Pestka, Krause et al. 2004; Zdanov 2006). However, I will use mda-7 to represent both mda-7 and IL-24 in this manuscript. It is a member of the IL-19 subfamily of IL-10 related cytokines and has 19% amino acid identity to IL-10. Human MDA-7 protein is a member of the four-helix bundle family of cytokines (Chaiken and Williams 1996). Human MDA-7 has six alpha helicies named A through F. The antitumor activity of human MDA-7 resides in alpha helicies C to F. Gupta and coworkers made multiple mutants of mda-7, removing one alpha helix at a time. Deletions of alpha helices, A and B (1 to 103 amino acids) did not alter the biological properties of MDA-7. However, deletion of subsequent alpha helices did affect the antitumor activity of MDA-7 (Gupta, Walter et al. 2006). MDA-7 also contains an IL-10 signature motif, which is a conserved amino acid sequence present in all members of the IL-10 family (Gopalkrishnan, Sauane et al. 2004; Lebedeva, Sauane et al. 2005).

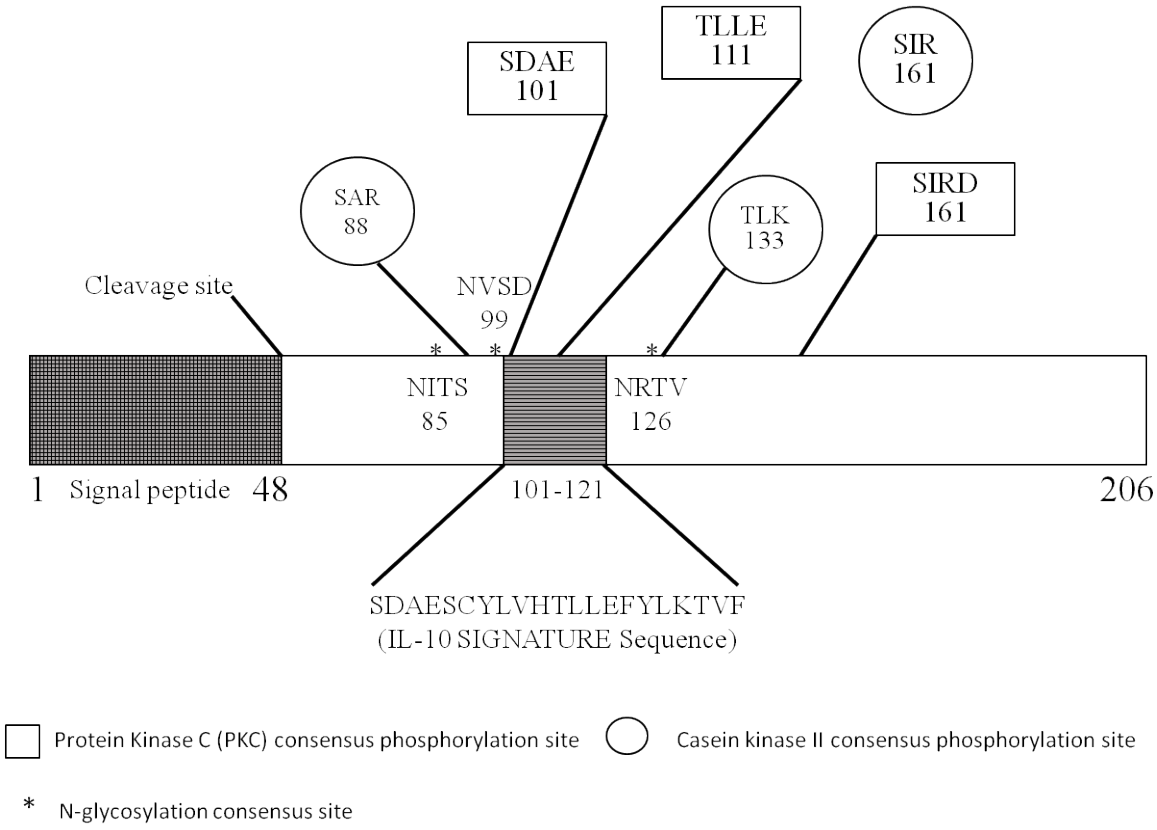


Figure 1.2: Protein structure of human MDA-7. Consensus amino acid sequences for phosphorylation (serine (S) in SDAE, SAR, SIR and SIRD) and (threonine (T) in TLLE and TLK) and N-glycosylation sites (Asparagine (N) in NVSD, NITS and NRTV) are shown in boxes and circles.

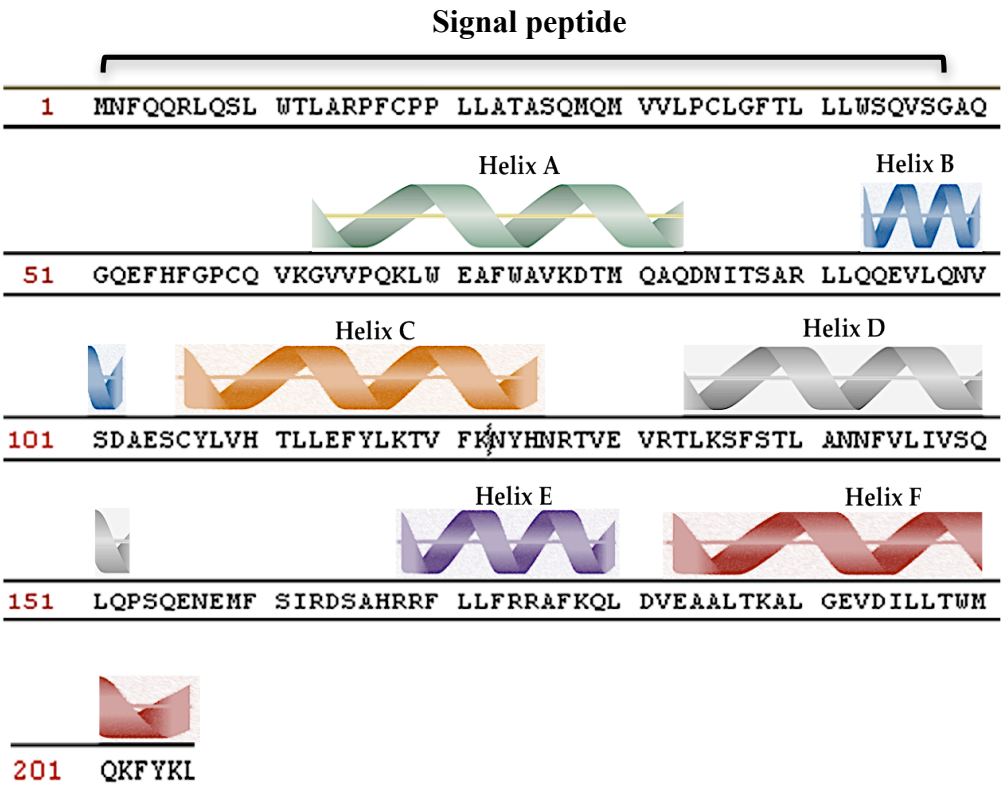


Fig 1.3: Protein structure of Human MDA-7. Human MDA-7 consists of 206 amino acids. The position of helices from A to F is shown. It has a 48 amino acid long signal peptide sequence at the N-terminus.

MDA-7 has an 48 amino acid long hydrophobic signal sequence, which is cleaved off during secretion (Nielsen, Kaestel et al. 1999; Nakai 2000; Pestka, Krause et al. 2004). It also has three potential N-linked glycosylation sites (residues 95, 109 and 126) that are distinctly glycosylated depending upon the species (Bairoch 1991; Attwood, Beck et al. 1997). Secreted MDA-7 protein is variably glycosylated, which causes it to migrate as multiple bands with different apparent molecular weights from 18 KDa to 35 KDa on Sodium Dodecyl Sulfate - Polyacrylamide Gel electrophoresis (SDS-PAGE) (Fuson, Zheng et al. 2009). Previously, it has been suggested that human MDA-7 might

form covalently linked homodimers (Cys59 – Cys59) and would not exist in monomeric form (Chang et al. 1999). However, Chada and coworkers (2004) predicted that a disulfide bond can be formed between Cys59 and Cys107 based on their homology model, thus suggesting a monomeric form of human MDA-7. Proper glycosylation and disulfide bond formation is thought to be necessary for the solubility, secretion and activity of MDA-7 (Fuson, Zheng et al. 2009).

5. Expression profile of human MDA-7

Human *mda-7* expression is highly restricted to certain cell types, including cells from the immune system. Its expression is detected in normal unstimulated or lipopolysaccharides (LPS) stimulated monocytes, and in LPS, phytohaemagglutinin (PHA) and concanavalin A (ConA) stimulated T cells (Huang, Madireddi et al. 2001; Caudell, Mumm et al. 2002; Garn, Schmidt et al. 2002; Wolk, Kunz et al. 2002; Poindexter, Walch et al. 2005; Ablin, Kynaston et al. 2011). Anti-CD3 stimulated T cells also express MDA-7. Human MDA-7 is expressed by cultured normal human epidermal keratinocytes (NHEKs) and skin keratinocytes. During wound healing, its expression peaks between the 2^{nd} to 10^{th} day in keratinocytes after injury. It functions to control the proliferation and migration of keratinocytes (Poindexter, Williams et al. 2010). Human MDA-7 also inhibits the TGF-α induced migration of keratinocytes. Furthermore, it has been proposed that MDA-7 inhibits the proliferation of keratinocytes during the resolution phase of wound repair. In addition, its expression is elevated in psoriatic skin. Physiological levels of MDA-7 are also detected in melanocytes, where its expression is inversely related to melanocytic transformation (Ekmekcioglu, Ellerhorst et al. 2001; Allen, Pratscher et al. 2004).

Expression levels of human MDA-7 are not controlled by transcription up-regulation, since *mda-7* gene is transcribed at the same levels in uninduced and IFN-beta and MEZ treated HO-1 human melanoma cells (Madireddi, Su et al. 2000). MDA-7 expression is normally regulated by transcription factor AP-1 and C/EBP at the level of transcription (Madireddi, Su et al. 2000). However, human *mda-7* mRNA encodes very long 5' and 3' untranslated regions (5' and 3' UTR). The 3' untranslated region of human *mda-7* mRNA contains three destabilization domains, i.e AU - rich elements (ARE, 3'-UTR-AUUUA) that are responsible for its rapid degradation. ARE-binding proteins bind to these AU rich elements that lead to *mda-7* mRNA decay. Activity of ARE-binding proteins is regulated by the p38 mitogen activated protein kinase (p38 MAPK) pathway. Activation of the p38 MAPK pathway in LPS stimulated PBMCs leads to mda-7 mRNA stabilization through altered binding properties of ARE-binding proteins (Madireddi, Su et al. 2000). The 3' UTR of mda-7 also contains three polyadenylation sequences (AAUAAA).

6. MDA-7 orthologs in other species

Orthologous sequences of the human *mda-7* gene have been identified in murine, simian, bovine, canine, feline and yeast genomes by southern blot analysis (Jiang, Lin et al. 1995). The first ortholog (c49a) was identified by differential display-polymerase chain reaction (DD-PCR) during wound healing in the rat. This gene has a 71.7 % nucleotide similarity in the coding region to human *mda-7*, however, it has only 58.7% amino acid similarity to human MDA-7. Its expression is increased in fibroblast-like cells during the inflammatory and granulation phase of wound healing (12 hr - 5 days).

However, the role that rat c49a plays during the wound healing process is still not known (Soo, Shaw et al. 1999).

In 2001, Schaefer and coworkers identified a murine ortholog of human mda-7, which was named Interleukin-4 - induced secreted protein (FISP). FISP has 93% and 69% amino acid similarity to rat c49a and human MDA-7, respectively. It is expressed in Th2 cells during their differentiation**.**

7. Alternative splicing at the *mda-7* gene locus

Alternative splicing is an important mechanism to increase the functional diversity of the eukaryotic transcriptome as it results in the expression of new protein isoforms (Sorek and Amitai 2001). The *mda-7* locus undergoes differential splicing to yield four different protein isoforms. The first splice variant (*mda-7s*) was detected in normal human melanocytes and is produced by skipping exons 3 and 5 (Allen, Pratscher et al. 2004; Allen, Pratscher et al. 2005). When translated, *mda-7s* encodes a protein with a molecular weight of 12KDa that has only 12 amino acids similar to wild type MDA-7. However, this protein can still heterodimerize with the full-length MDA-7, and inhibits its secretion. Subcutaneous and metastatic melanomas do not express *mda-7s,* thus suggesting a possible inverse relationship between expression levels of *mda-7s* and development of metastatic disease (Allen, Pratscher et al. 2004).

Two more splice variants lacking either the third (MDA-7del3) or the fifth exons (MDA-7del5) are also produced in human melanocytes. Skipping of exon 3 results in a similar splice variant to *mda-7s*. However, skipping the fifth exon (MDA-7del5) leads to deletion of the IL-10 signature sequence. This produces a protein that is more similar to wild type MDA-7 than the one produced from the previously discussed splice variant,

mda-7s (Allen, Pratscher et al. 2004). Recently, it has been shown that the expression of *mda-7* and its splice variant MDA-7del5 are necessary for the differentiation of monocytes (Yang, Duan et al. 2011). One more splice variant has been recognized that uses an alternate in-frame splice site in the 5' coding region and encodes a protein isoform that is one amino acid longer than the wild type MDA-7.

Alternative splicing also occurs at the locus of the murine homolog of *mda-7*, FISP. This splice variant of FISP, FISP-sp lacks 29 nucleotides from the 5'end of the fourth exon. FISP-sp lacks growth suppressing and antitumor activity, however, it can heterodimerize with FISP and prevents its secretion and apoptotic effect (Sahoo, Jung et al. 2008; Sahoo, Lee et al. 2011).

8. MDA-7 receptors

MDA-7 receptor complexes are members of the class II cytokine receptor family (Bazan 1990). Class II cytokine receptor family proteins have high amino acid similarity in their extracellular domains; however, the amino acid sequences in the transmembrane and intracellular domains are not conserved (Thoreau, Petridou et al. 1991; Moore, de Waal Malefyt et al. 2001). Extracellular domains of class II cytokine receptors contain 210 amino acids in two tandemly placed fibronectin type III (FNIII) domains (Thoreau, Petridou et al. 1991; Moore, de Waal Malefyt et al. 2001). These fibronectin type III domains have a characteristic pattern of proline and cysteine residues (Langer, Cutrone et al. 2004). Class II cytokine receptors are heterodimeric complexes that contains one alpha and one beta subunit. Alpha chains are longer, and are responsible for the affinity of receptor complexes to their respective cytokines (Roberts, Liu et al. 1998). Binding of ligand to an alpha subunit results in its oligomerization with the beta subunit. Beta

subunits of these receptor complexes do not bind to ligands directly (LaFleur, Nardelli et al. 2001). However, the beta subunit is bound to a janus kinase (JAK), which is responsible for cross activation of receptor subunits (Langer, Cutrone et al. 2004). Similarly, the alpha chain is associated with Jak1 tyrosine kinase, which is activated after ligand binding and causes phosphorylation of tyrosine residues. After phosphorylation, alpha chains recruit SH2 domain-containing proteins like STAT (signal transducer and activator of transcription). Phosphorylated STATs form homo or hetero-dimers and then translocate to the nucleus where they control the expression of interleukin responsive genes (Chai, Nichols et al. 1997; Nielsen, Kaestel et al. 1999).

After secretion, MDA-7 can bind to two different receptor complexes (Fig. 2). These receptor complexes are heterodimeric, and assemble from three different chains. One of the two different alpha chain (IL-20R alpha or IL-20R1, IL-22R alpha or IL-22R1) heterodimerizes with a common beta chain (IL-20R beta or IL-20R2) to form a functional receptor complex (Josephson, Logsdon et al. 2001). Binding of MDA-7 to its receptors leads to activation of JAK - associated with the alpha chains (Chai, Nichols et al. 1997; Nielsen, Kaestel et al. 1999). Activation of JAK causes phosphorylation of STAT3 and to a lesser extent STAT1, which then translocates to the nucleus, and causes up-regulation of BAX (pro-apoptotic) expression (Parrish-Novak, Xu et al. 2002). Receptors from the class II cytokine receptor family are often used by more than one cytokine. Other members (IL-10, IL-19 and IL-22) of the IL-10 cytokine family also use the above receptor complexes, however none of them induce apoptosis in cancer cells. Binding of these cytokines to MDA-7 receptors also results in activation of STAT3 with no cytotoxic effects (Dumoutier, Leemans et al. 2001; Parrish-Novak, Xu et al. 2002;

Wang, Tan et al. 2002; Ablin, Kynaston et al. 2011). Moreover, activation of MDA-7 receptors in normal cells also results in phosphorylation of STAT3 with no cell killing. However, the exact mechanisms by which these cytokines induce different responses through same receptor complexes are still not known

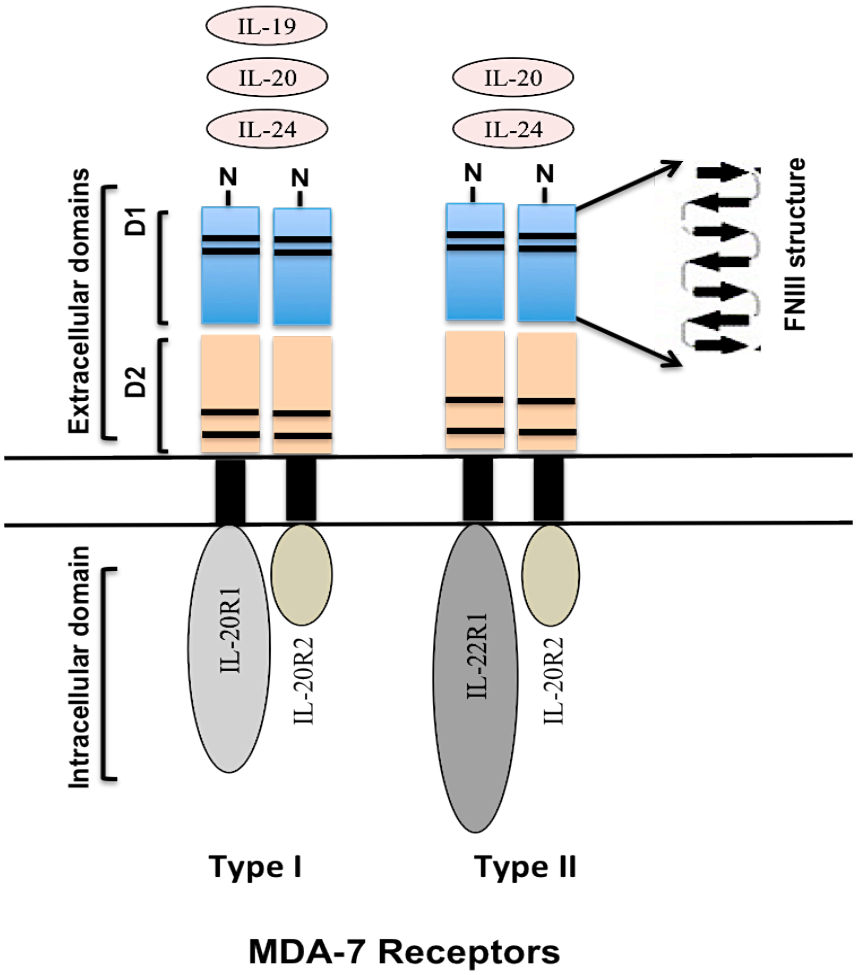


Fig 2: MDA-7 receptor structures. MDA-7 can bind and signal through two different types (Type I and Type II) of receptors. Type I receptor is composed of IL-20R1 and IL-20R2 chains, while type II receptors contain IL-22R1 and IL-20R2 chains. Each receptor chain contains two (DI and DII) fibronectin III (FNIII) domains in their extracellular domain. Fibronectin domain has conserved cysteine residues (—, represents cysteine residues) that are characterstics of type II receptor family.

9. Expression patterns and the role of MDA-7 receptors

Although activated monocytes or Th2 cells express MDA-7, expression of its receptor subunits (IL-20R1/IL-22R1) has not been detected in haematopoietic cells suggesting that cells of the immune system do not respond to secreted MDA-7 (Parrish-Novak, Xu et al. 2002; Ablin, Kynaston et al. 2011). Several studies have shown that MDA-7 receptor subunits are widely expressed in skin, thus making skin a major target organ for secreted MDA-7 (Sarkar, Lebedeva et al. 2007; He and Liang 2010). MDA-7 receptors are constitutively expressed on normal human epidermal keratinocytes as well as on melanocytes (Poindexter, Williams et al. 2010). The IL-20R1 subunit is expressed in lung, testis and ovary. IL-22R1 expression is detected in keratinocytes, hepatocytes, pancreatic acinar cells, colon and the small intestine. However, the activity of receptor complexes is controlled by restricted expression of IL-20R beta subunits in different tissues (Sarkar, Lebedeva et al. 2007).

IL-19, IL-20 and MDA-7 play an important role in cutaneous innate immunity, skin repair, and remodeling (Sarkar, Lebedeva et al. 2007). However, the exact role of each cytokine is still not clear. Skin samples from human psoriasis patients show high levels of MDA-7 expression. Infiltrating monocytes and macrophages are the major source of MDA-7 molecules in these psoriatic skin samples (He and Liang 2010). Similarly, MDA-7 transgenic mice also exhibit characteristic lesions of human psoriasis including a blockade in differentiation of keratinocytes, epidermal hyperplasia and neonatal lethality (He and Liang 2010). MDA-7 transgenic mouse was generated by cloning its cDNA under the bovine keratin-5 promoter control. These studies suggest a possible redundant role for MDA-7, IL-19 and IL-20 in the pathogenesis of psoriasis,

because overexpression of any one of these cytokines can induce epidermal hyperplasia. Furthermore, Susan and coworkers (2007) showed that IL-19, IL-20, IL-22, and MDA-7 regulate many of these same genes (S-100 family proteins, β-defensin) in reconstituted human epidermis. These genes are involved in molecular pathways that regulate inflammation, wound healing, re-epithelialization and differentiation.

This function of MDA-7 appears to be mediated by the IL-22R1 subunit because IL-22R1 transgenic mice showed similar symptoms to those shown by MDA-7 transgenic mice. However, a dimeric mouse sIL-20R2-Fc fusion protein can act as a potent MDA-7 antagonist (He and Liang 2010; (Wolk, Haugen et al. 2009)). This was further confirmed by Susan and coworkers (2007), when they showed that the biological effects of human MDA-7 can also be blocked using anti-IL-20R2 antibody but not by anti-IL-20R1 or IL-22R1 antibodies. This effect seems to be due to the high binding affinity of MDA-7 to IL-20R2 subunits, because anti-IL-20R2 antibody does not block IL-20 or IL-22 activity (Sarkar, Lebedeva et al. 2007).

10. Biological properties of MDA-7

The biological properties of MDA-7 vary according to its expression level. Its functions can be categorized at either physiological levels or at supra-physiological levels (Fisher, Gopalkrishnan et al. 2003; Aggarwal, Takada et al. 2004; Gopalkrishnan, Sauane et al. 2004; Dent, Yacoub et al. 2005; Fisher 2005; Emdad, Lebedeva et al. 2007; Emdad, Lebedeva et al. 2009; Dash, Bhutia et al. 2010; Dent, Yacoub et al. 2010; Dent, Yacoub et al. 2010). As discussed above, MDA-7 is expressed at physiological levels in melanocytes and keratinocytes (Ekmekcioglu, Ellerhorst et al. 2001; Aggarwal, Takada et al. 2004; Poindexter, Williams et al. 2010). Supra-physiological levels of MDA-7 can

only be achieved when it is ectopically expressed from plasmids or replication incompetent adenoviral vectors. Most of these vectors have a cytomegalovirus (CMV) promotor that drives expression of MDA-7 (Fisher, Gopalkrishnan et al. 2003; Aggarwal, Takada et al. 2004; Gopalkrishnan, Sauane et al. 2004; Dent, Yacoub et al. 2005; Fisher 2005; Emdad, Lebedeva et al. 2007; Emdad, Lebedeva et al. 2009; Dash, Bhutia et al. 2010; Dent, Yacoub et al. 2010; Dent, Yacoub et al. 2010).

10.1 Biological functions MDA-7 at supra-physiological levels

When MDA-7 is expressed at supra - physiological levels from either plasmid or replication incompetent adenoviral vectors, it exhibits potent growth suppressing and tumor killing properties. These biological functions of MDA-7 are not observed at physiological levels (Fisher, Gopalkrishnan et al. 2003; Aggarwal, Takada et al. 2004; Gopalkrishnan, Sauane et al. 2004; Dent, Yacoub et al. 2005; Fisher 2005; Emdad, Lebedeva et al. 2007; Emdad, Lebedeva et al. 2009; Dash, Bhutia et al. 2010; Dent, Yacoub et al. 2010; Dent, Yacoub et al. 2010). Antitumor properties of MDA-7 can be further categorized into the effects that are seen in either *in-vitro* or *in-vivo* studies.

10.1.1 Antitumor properties of MDA-7 in *in-vitro* models

Jiang and coworkers (1995, 1996) were the first to document that ectopic expression of MDA-7 results in growth arrest, and induces apoptosis in various cancer cells *in-vitro*. These workers transfected HeLa (human cervical carcinoma), MCF-7 (human breast adenocarcinoma), LS174T (colorectal carcinoma), HONE-1 (nasopharyngeal carcinoma), DU-145 (prostate carcinoma), T98G (glioblastoma) and Saos-2 (Osteosarcoma) cancer cells with a plasmid vector expressing MDA-7 protein. Transfected cancer cells were then used for monolayer colony formation assays. MDA-7

expression suppressed the growth and inhibited monolayer formation when compared to cancer cells transfected with either vector backbone or vector with a cloned MDA-7 gene in the reverse orientation (Jiang, Lin et al. 1995; Jiang, Su et al. 1996). Cells expressing high levels of MDA-7 also lost the property of anchorage independence. Antitumor effects of MDA-7 protein can be reversed with infection of a recombinant type-5 adenovirus expressing antisense *mda-7*. These cancer cells have a diverse genetic makeup. Some of them have mutated p53 or retinoblastoma (RB) genes. However, MDA-7 was still able to inhibit the growth of cancer cells having mutations in these genes, thus suggesting that they do not play an important role in MDA-7 mediated cancer cell killing (Su, Lebedeva et al. 2003). These results were further supported when malignant glioma cells expressing either wild-type or mutated p53 genes were infected with a replication incompetent adenoviral vector expressing MDA-7 or wild type p53 (Su, Lebedeva et al. 2003). Overexpression of p53 only suppressed the growth of malignant glioma cancer cells that have a mutated p53 gene. However, it failed to suppress the growth of cancer cells expressing wild type p53. MDA-7 treatment killed malignant glioma cells with either mutated or wild type p53 (Su, Lebedeva et al. 2003). Jiang and coworkers (1996) also transfected normal cells like human mammary epithelium, human skin fibroblasts and rat embryo fibroblasts, and did not observe any growth suppressing effects on these cells.

Replication deficient adenoviral vectors have been widely used to express MDA-7 in a variety of cancer cells including breast carcinoma (Su, Madireddi et al. 1998; Bocangel, Zheng et al. 2006; Chada, Mhashilkar et al. 2006; Zheng, Bocangel et al. 2007), prostate carcinoma (Emdad, Sarkar et al. 2006; Sauane, Su et al. 2008; Sauane, Su

et al. 2010), melanoma (Lebedeva, Su et al. 2002; Aggarwal, Takada et al. 2004; Chada, Mhashilkar et al. 2004), lung cancers (Saeki, Mhashilkar et al. 2000; Saeki, Mhashilkar et al. 2002; Aggarwal, Takada et al. 2004; Nishikawa, Ramesh et al. 2004; Ramesh, Ito et al. 2004; Ramesh, Ito et al. 2004; Oida, Gopalan et al. 2005; Inoue, Hartman et al. 2007), glioblastoma multiforme (Su, Lebedeva et al. 2003; Yacoub, Mitchell et al. 2003; Yacoub, Mitchell et al. 2003; Yacoub, Gupta et al. 2008), osteosarcoma (Huang, Madireddi et al. 2001), colon carcinoma (Emdad, Lebedeva et al. 2007), cervical carcinoma (Huang, Madireddi et al. 2001), ovarian carcinoma (Gopalan, Litvak et al. 2005; Emdad, Sarkar et al. 2006; Gopalan, Shanker et al. 2007; Shanker, Gopalan et al. 2007; Yacoub, Liu et al. 2010), renal carcinoma (Yacoub, Mitchell et al. 2003; Park, Hamed et al. 2011), gastric carcinoma (Wei, Fan et al. 2010; Yan, Zhang et al. 2010), hepatocellular carcinoma (Wang, Xue et al. 2006; Hu, Xue et al. 2008; Xue, Chen et al. 2008; Xue, Xiao et al. 2010) and all of these tumor cells undergo MDA-7 induced cell death except pancreatic carcinoma cells. Pancreatic carcinoma cells express a mutated form of the *k-ras* gene that blocks translation of *mda-7* mRNA. However, inhibition of the mutant *k-ras* gene makes these cells susceptible to MDA-7 induced apoptosis (Su, Lebedeva et al. 2001; Lebedeva, Sarkar et al. 2006).

MDA-7 protein does not have significant homology to other tumor suppressing proteins and it shows differential growth suppressing effects on cancer cells and, may represent a new class of cancer growth suppressing genes. This suppression of growth and induction of cancer-specific cell death by MDA-7 in cancer cells is mediated by modulating apoptotic and other signaling pathways.

10.1.1.1 MDA-7 uses various molecular mechanisms to induce apoptosis in cancer cells

MDA-7 overexpression leads to the down-regulation of anti-apoptotic genes such as Bcl-2 and Bcl-xL while causing up-regulation of pro-apoptotic genes such as Bax, Bad and Bak in breast cancer cells (Yacoub, Mitchell et al. 2003; Chada, Mhashilkar et al. 2006). This alteration in expression levels of pro-apoptotic genes and anti-apoptotic genes tilts the balance toward cell death. Lebedeva et al (2003) expressed MDA-7 from a replication incompetent adenoviral vector (*Ad.mda-7*). Infection of LNCap, DU-145 and PC-3 (prostate carcinoma) cells with *Ad.mda-7* decreased expression of Bcl-x_L (B-cell lymphoma-extra large) and bcl-2 (B-cell lymphoma 2) family protein members (Anti-apoptotic genes) while increasing expression levels of BAX (Bcl-2–associated X protein) and Bak (Bcl-2 homologous antagonist/killer) genes (pro-apoptotic genes). Several other studies have verified these results in various other cancer cell lines (Su, Madireddi et al. 1998; Su, Lebedeva et al. 2001; Lebedeva, Su et al. 2002; Su, Lebedeva et al. 2003).

The pro-apoptotic effects of MDA-7 are independent of the status of the p53, pRB and p21 genes (as shown before) and are mediated by activation of the p38 MAPK signaling pathway which, in turn, induces expression of growth arrest and DNA damage-inducible genes (GADD) (Sarkar, Su et al. 2002). Activation of the p38 MAPK pathway is necessary for MDA-7 induced apoptosis. When melanoma (FO-1) cells are treated with SB203580 (a selective inhibitor of the p38-MAPK pathway), it inhibits *Ad.mda-7*-induced apoptosis (Sarkar, Su et al. 2002). Similarly, when MDA-7 treated FO-1 cells are infected with an adenoviral vector expressing dominant negative mutant p38-MAPK, no pro-apoptotic effects are observed in these cells. MDA-7 mediated activation of p38-

MAPK culminates in increased expression of GADD153, GADD45α, and GADD34 genes in melanoma cells. Knocking down the expression of GADD family members in melanoma by antisense RNA protects FO-1 cells from MDA-7 induced apoptosis. (Sarkar, Su et al. 2002).

Treatment of prostate cancer cells (DU-145) with MDA-7 also causes cancer-specific endoplasmic stress which, in-turn, culminates in the induction of the unfolded protein response (UPR) (Sieger, Mhashilkar et al. 2004; Gupta, Walter et al. 2006). When expressed, MDA-7 localizes to endoplasmic reticulum (both in normal and cancer cells) and interacts through its C & F alpha helixes with the Bip/GRP78 (78-kDa glucose-regulated protein), a chaperone and an important intracellular target for MDA-7 (Gupta, Walter et al. 2006). Short hairpin RNA (shRNA) mediated knockdown of Bip/GRP78 protects cancer cells from MDA-7 induced apoptosis (Rahmani, Mayo et al. 2010).

Bip/GRP78 is an important chaperone that is required for the proper folding of several secreted proteins including members of IL-10 family (IL-19, IL-20, IL-22/IL-TIF, and IL-26/AK155) (Vandenbroeck, Alloza et al. 2002). Cancer cells have high levels of Bip/GRP78 protein compared to normal cells (Gupta, Walter et al. 2006). In normal conditions, Bip/GRP78 binds to activating transcription factor 6 (ATF6, endoplasmic stress response initiator) and inhibits its activity (Shen, Snapp et al. 2005). However, when MDA-7 is ectopically expressed, it binds to Bip/GRP78 and inhibits its interaction with ATF6, which then initiates the endoplasmic stress response that, in turn, leads to the unfolded protein response (UPR) (Gupta, Walter et al. 2006). The unfolded protein response then activates p38 MAPK activity which, in turn, leads to increased expression

of growth arrest and DNA damage inducible (GADD) genes and results in apoptosis of cancer cells (Sarkar, Su et al. 2002; Gupta, Walter et al. 2006).

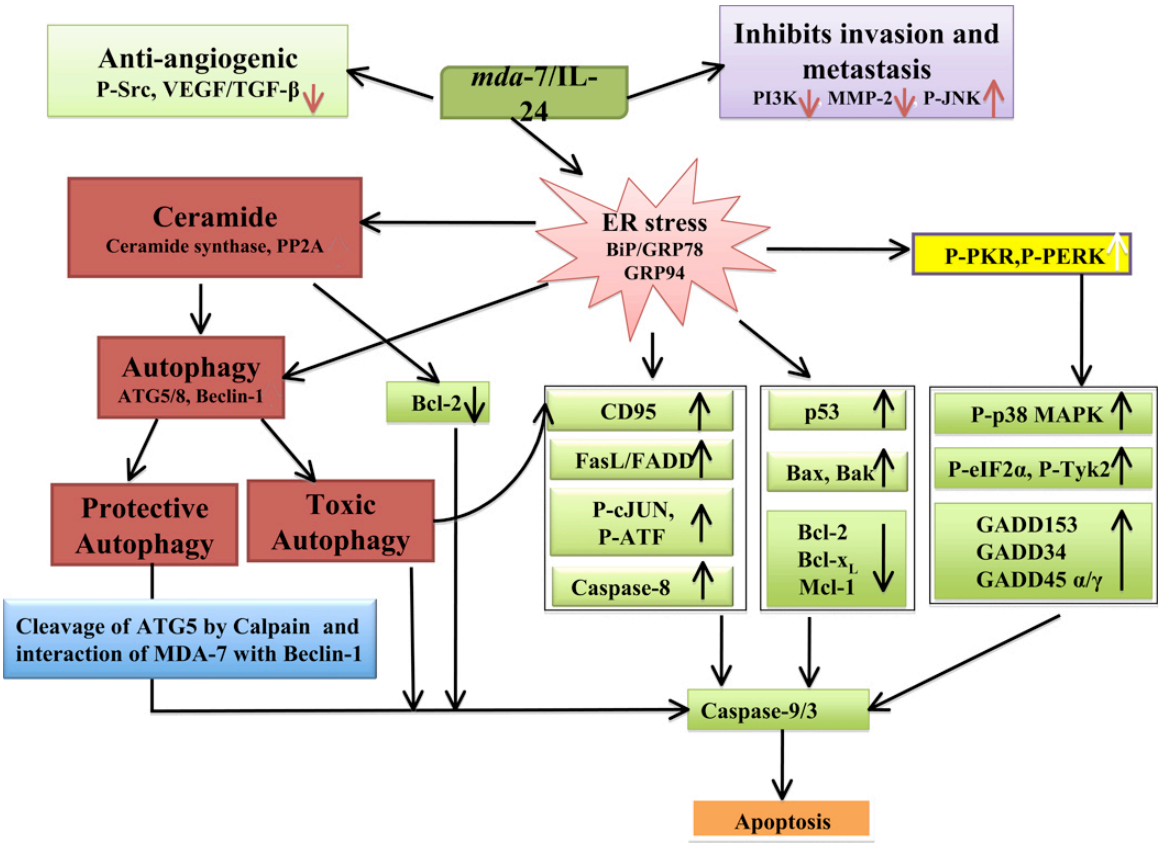


Figure 3: Molecular pathways modulated by ectopically expressed MDA-7 to induce cancer specific cell killing.

Ectopic expression of MDA-7 in lung cancer cells also activates double-stranded RNA-dependent protein kinase (PKR) (Pataer, Vorburger et al. 2005). Activation of PKR leads to the phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2α) resulting in inhibition of protein synthesis (Pataer, Vorburger et al. 2005). p38 MAPK is also one of the downstream targets of PKR whose role in MDA-7 induced

cancer cell death has already been discussed (Sarkar, Su et al. 2002; Gupta, Walter et al. 2006).

10.1.2 Antitumor properties of MDA-7 in *in-vivo* cancer models

The antitumor properties of MDA-7 against various cancer cell lines have been successfully replicated in *in-vivo* cancer models (Su, Madireddi et al. 1998; Su, Emdad et al. 2005). Two different approaches were used to demonstrate the antitumor activity of human MDA-7 in initial studies. In the first approach, tumor cells were first infected with *Ad.mda-7* and then injected subcutaneously into nude mice. In the second approach, tumor cells were first allowed to grow and establish a tumor mass (in-vivo therapy model) followed by injection of *Ad.mda-7* (Su, Madireddi et al. 1998; Madireddi, Su et al. 2000). Treatments of MCF-7 cells (ex-vivo) with *Ad.mda-7* prior to subcutaneous injection prevented formation of tumor masses in nude mice (Su, Madireddi et al. 1998). For in-vivo therapy models, HeLa cells were injected into nude mice and were allowed to develop into a tumor mass with an average volume of 150 -200 mm^3 (Madireddi, Su et al. 2000). After successful establishment, tumor masses were injected intratumorally with either *Ad.mda-7* or Ad.vec (vector control without mda-7) three times per week. Injection of tumor masses with *Ad.mda-7* inhibited their growth and disease progression and apoptotic changes were detected in tumor cells biopsied from tumors in *Ad.mda-7* injected mice. Injection of Ad.vec did not inhibit the growth of the tumor mass (Madireddi, Su et al. 2000). Similar results were also observed in non-small cell lung carcinoma (NSCLC) and human breast cancer xenograft models (Saeki, Mhashilkar et al. 2002). Furthermore, Sarkar and coworkers (2005, 2008) showed that injection of an adenoviral vector expressing MDA-7 eradicated not only primary injected tumor, but

distant tumor as well, due to what was termed the "bystander cancer-specific killing effect"which, in the case of MDA-7, was mediated through its two heterodimeric receptors IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (Sauane, Gopalkrishnan et al. 2003; Chada, Mhashilkar et al. 2004; Su, Emdad et al. 2005; Sauane, Gupta et al. 2006).

Based upon its properties in *in-vivo* and *in-vitro* models, MDA-7 antitumor therapy entered a phase I clinical trial (Fisher, Gopalkrishnan et al. 2003; Cunningham, Chada et al. 2005; Fisher 2005; Tong, Nemunaitis et al. 2005; Emdad, Lebedeva et al. 2007; Eager, Harle et al. 2008). In this trial, 28 human patients were injected intratumorally with replication incompetent adenoviral vector expressing MDA-7 (*Ad.mda-7*). One group of patients with advanced carcinoma were injected only once with 2 X 10^{10} virus particles (Tong, Nemunaitis et al. 2005; Cunningham, Chada et al. 2005). After 4 days of injection, the tumor mass was resected and evaluated for expression and biological effects of MDA-7. In another group of patients, multiple injections (12 doses) of *Ad.mda-7* (INGN241) were given intratumorally with a steady increase in the dose amount (2 X 10^{10} virus particle (vp) to 2 X 10^{12} vp). Intratumoral injection of *Ad.mda-7* resulted in transduction of 10-30% of the tumor cells. However, after injection, 70% of the tumor cells showed an apoptotic phenotype, substantiating its potent "bystander cancer-specific killing effect" (Tong, Nemunaitis et al. 2005; Cunningham, Chada et al. 2005). Multiple injections of *Ad.mda-7* were well tolerated by human patients with the exception of a few self-limiting mild toxicities (Dash, Bhutia et al. 2010). Moreover, these antitumor effects were clinically significant. Injection of *Ad.mda-7* led to increased levels of IL-6, IL-10, IFN-γ and TNF-α. However, levels of

these cytokines reverted to normal 15-30 days after injection (Fisher, Gopalkrishnan et al. 2003)

10.2 Biological functions of MDA-7 at physiological levels

As noted, human MDA-7 is expressed in Th2 cells and monocytes (Garn, Schmidt et al. 2002). Secreted MDA-7 binds to MDA-7 receptors activating the JAK/STAT signaling pathway (Huang, Madireddi et al. 2001; Caudell, Mumm et al. 2002; Wolk, Kunz et al. 2002). These receptors are widely expressed in lung, testis, ovary, keratinocytes and skin. Skin is the major target organ for MDA-7. In human skin samples, maximum expression levels of MDA-7 are observed between the 2^{nd} and 6^{th} days during the wound healing process. Cytokines involved in wound repair, such as IFN-α, IFN-β, TGF-α and TGF-β, have been shown to upregulate the expression of MDA-7 in normal human epidermal keratinocytes (NHEKs). During the wound healing process, MDA-7 inhibits the proliferation and migration of keratinocytes (Poindexter, Williams et al. 2010). Similarly, c49a, the rat ortholog of mda-7 is also expressed in fibroblast like cells during wound repair (Soo, Shaw et al. 1999).

The antitumor properties of MDA-7, suggesting that MDA-7 not only inhibits the growth of tumors but also kills various types of human tumor cells has already been discussed. However, cancer therapy can only be successful if the treatment not only kills cancer cells, but also induces antitumor immune responses that will enforce a check on the recurrence of the primary tumors as well as any metastases. Phytohaemagglutinin (PHA) - activated $CD56^{+}$ and $CD19^{+}$ subsets of peripheral blood mononuclear cells (PBMCs) have been shown to produce MDA-7 (Caudell, Mumm et al. 2002). These subsets include T cells, B cells, natural killer cells and macrophages. When HEK-293

cells produced MDA-7 was applied to human PBMCs, it induced secretion of Th1 cytokines including IL-6, IFN-γ, TNF-α (Caudell, Mumm et al. 2002).

Miyahara and coworkers (2006) evaluated the immune stimulatory properties of MDA-7 in a murine cancer vaccine model using an adenoviral vector to express MDA-7 (Miyahara, Banerjee et al. 2006). When Ad-mda-7 transfected UV2237m cells were injected into C3H mice, there was no tumor growth compared to control mice injected with Ad-luc transfected UV2237m cells. Similar effects were seen in nude mice. However, inhibition of tumor growth was not as pronounced in nude mice as in C3H mice suggesting a possible role for the immune response in controlling tumor growth. Tumor free C3H mice previously injected with *Ad.mda-7* transfected UV2237m cells were challenged with UV2237m cells. No tumor growth was observed in these mice either as opposed to control mice. This data clearly showed that MDA-7 has immunomodulatory activities and induced protective antitumor immunity. Moreover, splenocytes prepared from mice injected with *Ad.mda-7* transfected UV2237m cells showed high proliferation *in-vitro* when compared to splenocytes from control mice. There was also an increase in the population of $CD3^+$ $CD8^+$ T cells, and these splenocytes secreted high level of Th1 cytokines when compared to control mice (Miyahara, Banerjee et al. 2006).

Similarly, when *Ad.mda-7* (Replication incompetent adenoviral vector expressing MDA-7) was injected intratumorally into cancer patients in a phase I clinical trial, it induced an increase in the population of peripheral $CD3^+$ $CD8^+$ T cells in a cohort of patients 15 days after injection. These cells expressed very high levels of IL-6, IFN-γ and TNF-α (Tong, Nemunaitis et al. 2005).

11. Bystander antitumor effect of human MDA-7 protein

As noted, MDA-7 is a secreted protein with a long hydrophobic leader peptide of 48 amino acids which is cleaved during secretion (Nakai 2000). Secreted MDA-7 protein is heavily glycosylated with a variable molecular weight from 18 to 35 kDa and exhibits a potent bystander antitumor effect (Pestka, Krause et al. 2004; Fuson, Zheng et al. 2009). For MDA-7, the "bystander antitumor effect" can be defined as a paracrine effect that kills more than the expected number of tumor cells because of the secretion of MDA-7 from the *Ad.mda-7* transduced tumor cells.

The bystander antitumor effect of MDA-7 was first observed in pancreatic carcinoma cells (Su, Lebedeva et al. 2001). Pancreatic carcinoma cells are resistant to MDA-7 induced apoptosis due to mutations in the k-ras gene that are found in 85% of tumors of this type. Mutations in the k-ras gene inhibit translation of *mda-7* mRNA leading to reduced or no MDA-7 production in these cells. However, transfection of pancreatic carcinoma cells with a K-ras anti-sense construct not only blocked K-ras expression but also resulted in successful production of MDA-7 protein which then led to induction of apoptosis in all tumor cells. When it was observed that only ~8% of the cells received both treatments (k-ras anti-sense and *Ad.mda-7*), it was postulated that expression and secretion of MDA-7 proteins from the successfully transfected cells has potent bystander antitumor effect on neighboring cells. MDA-7 treated pancreatic cells not only showed reduced potential in cloning ability *in-vitro* but also did not have tumorigenic potential *in-vivo* (Su, Lebedeva et al. 2001). This bystander antitumor effect was further demonstrated when extracellular application of bacterially expressed and purified GST-MDA-7 protein caused growth suppression and apoptosis induction in

pancreatic cancer cells *in-vitro* (Sauane, Lebedeva et al. 2004). Additionally, intratumoral injection of *Ad.mda-7* results in transduction of only - 30% of tumor mass. However, later on 70% of the tumor cells were either apoptotic or dead reinforcing the likelihood that a bystander effect is occuring (Fisher, Gopalkrishnan et al. 2003; Cunningham, Chada et al. 2005; Lebedeva, Su et al. 2005).

Sauane et al. (2008) showed that application of recombinant MDA-7 protein up-regulated expression of endogenous *mda-7* in cancer cells (DU-145, A549, PC-3, HO-1, and HeLa cell lines). This up-regulation of MDA-7 protein levels is not a result of increased promotor activity of *mda-7* but is achieved through stabilization of *mda-7* mRNA by a receptor-dependent pathway. Recombinant MDA-7 protein acts through an autocrine loop to stabilize its own mRNA in cancer cells. After this, endogenous MDA-7 then increases expression of its target genes such as BiP/GRP78, GADD153, and GRP94 resulting in an unfolded protein response and tilting the balance toward expression of pro-apoptotic genes. Thus, the above data support the important role of the MDA-7 receptor in a bystander antitumor effect of MDA-7. However, activation of these receptors is not needed for initiating antitumor effects of intracellular MDA-7 (Sauane, Gopalkrishnan et al. 2003).

12. Expression of MDA-7 radiosensitizes cancer cells

Induction of apoptosis by generating reactive oxygen species in cancer cells is one of the important mechanisms targeted by various cancer therapeutic modalities. Treatment of cancer cells with ionizing radiation (radiotherapy) generates reactive oxygen species like hydroxyl radicals which can cause mitochondrial dysfunction (Pelicano, Carney et al. 2004). Treatment of cancer cells with MDA-7 also causes

oxidative stress resulting from increased levels of reactive oxygen species (ROS). Oxidative stress is cancer-specific because this effect is not seen in normal cells (Lebedeva, Sarkar et al. 2003; Lebedeva, Su et al. 2005; Sarkar, Lebedeva et al. 2007). Reactive oxygen species (ROS) such as hydrogen peroxide, singlet oxygen, superoxide anion and hydroxyl radical levels are increased after MDA-7 treatment of prostate cancer cells while there was no such effect in normal immortal P69 prostate epithelial cells (Lebedeva, Sarkar et al. 2003; Lebedeva, Su et al. 2005; Sarkar, Lebedeva et al. 2007). Moreover, as in radiotherapy MDA-7 treatment also raises ceramide levels and activates poly ADP-ribose polymerase in cancer cells (Mesicek, Lee et al. 2010; Yacoub, Hamed et al. 2010). Activation of poly ADP ribosyl polymerase leads to NADPH depletion that alters the redox potential of the cells which is sensed by mitochondria. Thus, it was hypothesized that MDA-7 radiosensitizes cancer cells by elevating levels of ceramide and ROS and may have an additive effect with this treatment by increasing the ROS levels. This hypothesis was validated by treating cancer cells with antioxidants, such as N-acetyl-L-cysteine (NAC) and Tiron, which prevented *Ad.mda-7*-induced apoptosis. A combinatorial treatment involving *Ad.mda-7* with various ROS-generating agents (arsenic trioxide, dithiophene, or 4–HPR (N-(4-Hydroxyphenyl)retinamide)), induced apoptosis not only in wild-type K-ras pancreatic carcinoma cells but also in pancreatic carcinoma cells with mutant k-ras by abolishing the *mda-7* mRNA translational block (Sarkar, Lebedeva et al. 2007). Lebedeva and coworkers (2007) evaluated the effects of combinatorial therapy involving MDA-7 and endoperoxides (EPXs) in mda-7 sensitive and resistant pancreatic cancer cells. EPXs can store and transport singlet oxygen and are a potent ROS inducer. Combined therapy with EPXs and MDA-7 not only killed the

chemotherapy resistant cancer cells, but also successfully induced apoptosis in MDA-7 resistant pancreatic carcinoma cells.

Simultaneous treatment with MDA-7 and radiotherapy has a synergistic effect on cancer cell killing. Yacoub and coworkers (2003) treated the glioblastoma multiforme (GBM) cells, with *Ad.mda-7* and ionizing radiation, and observed a synergistic effect with two treatments. *Ad.mda-7* treatment of GBM cells enhanced the activity of p38 and ERK1/2, however, it did not affect the activity of JNK and Akt. When MDA-7 expressing GBM cells were irradiated, there was suppression of ERK1/2 activity plus enhancement of JNK1/2 activity. However, irradiation did not alter the activity of either Akt or p38 MAPK. Inhibition of JNK1/2, but not p38, signaling abolished the radiosensitizing properties of MDA-7. Inhibition of ERK1/2 or PI3K signaling does not enhance the anti-proliferative effects of *Ad.mda-7*, whereas, combined inhibition of both pathways enhanced cell killing suggesting that ERK and PI3K signaling can be protective against MDA-7 lethality (Yacoub, Mitchell et al. 2003; Sarkar, Lebedeva et al. 2007; Lebedeva, Su et al. 2008).

13. Autophagy induction by MDA-7 in cancer cell toxicity

Autophagy is one of the most important cellular mechanisms that is responsible for breakdown of cellular components by lysosomal degradation. It regulates both survival and apoptosis of cancer cells (Kondo, Kanzawa et al. 2005; Eisenberg-Lerner, Bialik et al. 2009). MDA-7 can alter the expression levels of various mediators (ceramide, protein kinase R–like endoplasmic reticulum kinase (PERK)) involved in the autophagy pathway (Park, Yacoub et al. 2008; Yacoub, Gupta et al. 2008; Yacoub, Park

et al. 2008; Yacoub, Hamed et al. 2010). However, the role of MDA-7-induced autophagy in cancer cell death is still controversial.

Treatment of glioblastoma multiforme (GBM) cells with glutathione S-transferase tagged MDA-7 (GST-MDA-7, a bacterially expressed fusion protein) resulted in induction of type II programmed cell death, i.e. autophagy ((Yacoub, Gupta et al. 2008)). GST-MDA-7-induced endoplasmic reticulum stress and unfolded protein responses (UPR) (Yacoub, Gupta et al. 2008). High concentrations of GST-MDA-7 (300nM/L) activated protein kinase R–like endoplasmic reticulum kinase (PERK). PERK is an important target for MDA-7 because, it not only senses unfolded protein in endoplasmic reticulum (UPR induced by MDA-7), but it also direct the cell to undergo autophagy in response to UPR. PERK induces autophagy by activating c-Jun NH(2)-terminal kinase 1-3 (JNK1-3) while inactivating extracellular signal-regulated kinase 1/2 (ERK1/2) that leads to up-regulation of autophagy regulatory proteins like atg5 and Beclin-1 (Park, Yacoub et al. 2008; Yacoub, Gupta et al. 2008; Yacoub, Park et al. 2008; Yacoub, Hamed et al. 2010). Induction of autophagy is followed by a switch to the apoptotic pathway by cancer cells in an MDA-7 - dependent manner. MDA-7 physically interacts with beclin-1 and inhibits its activity and induces calpin-mediated degradation of atg5 directing cancer cells toward the apoptotic pathway (Bhutia, Dash et al. 2010; Bhutia, Das et al. 2011).

Ceramide is a tumor-suppressor lipid that regulates many diverse cellular processes, including cell cycle arrest and apoptosis (Koybasi, Senkal et al. 2004; Ogretmen and Hannun 2004; Sauane, Su et al. 2010). Increased ceramide levels induce the activity of protein phosphatase 2A (PP2A) which regulates important cell processes

including apoptosis. MDA-7 elevates ceramide levels in cancer cells by increasing *de-novo* synthesis. Increased ceramide levels also activate PERK-dependent autophagy in human renal carcinoma cells. Inhibiting the *de-novo* synthesis of ceramide or PP2A activity blocked MDA-7-induced apoptosis in renal carcinoma cells (Park, Walker et al. 2009). This data suggests that MDA-7-induced autophagy plays an important role in cancer cell death.

When expressed in chronic lymphocytic leukemia B-cells, MDA-7 also induces autophagy as it does in GBM cells and renal carcinoma cells, but this induction of autophagy promotes the survival of chronic lymphocytic leukemia B-cells rather than causing cell death (Yang, Tong et al. 2010). However, when MDA-7 - induced autophagy was blocked by a phosphatidylinositol 3-kinase inhibitor, wortmannin, it enhanced cell death in chronic lymphocytic leukemia B-cells. It is still not clear how MDA-7 induced autophagy provides a survival advantage to leukemia cells but a combinatorial therapy involving MDA-7 and an autophagy inhibitor (wortmannin) may provide a new strategy to treat leukemia (Yang, Tong et al. 2010).

14. Anti-angiogenic properties

An actively growing tumor mass requires a large supply of nutrients and oxygen which is achieved by formation of new blood vessels (angiogenesis). One of the approaches used in cancer treatment involves gene therapy to prevent formation of new blood vessels (Fidler and Ellis 1994). Antiangiogenic properties of MDA-7 were tested by an endothelial tube formation assay using human umbilical vein endothelial cells (HUVEC), an *in-vitro* assay that asssesses formation of new capillaries (Saeki, Mhashilkar et al. 2002). Treatment of HUVEC cells with *Ad.mda-7* prevented endothelial

tube formation. Moreover, these workers showed that the inhibition of tube formation was not a result of the cytotoxic effects of MDA-7 as determined by an alamar blue assay for cell viability. This effect of MDA-7 on HUVEC cells was similar to suramin, an inhibitor of tube formation. Suramin acts by preventing the interaction of FGFs and VEGF with heparan sulfate proteoglycans (HSPGs) and tyrosine kinase (TK) receptors on endothelial cells (Saeki, Mhashilkar et al. 2002). These workers also treated A549 and H1299 (lung cancer) subcutaneous tumor xenograft models with *Ad.mda-7* and showed that *Ad.mda-7*- treated tumors were smaller in size and less vascularized compared to control tumors groups (Ad.luc, adenoviral vector expressing luciferase gene) (Saeki, Mhashilkar et al. 2002). The tumor masses were stained for CD31, a marker of neoangiogenesis. *Ad.mda-7*- treated tumors had a significantly lower number of CD31-staining blood vessels compared to the Ad.luc treated tumors (Saeki, Mhashilkar et al. 2002).

The antiangiogenic effects of MDA-7 on endothelial cells were found to be greater than that of endostatin, a potent anti-angiogenic agent (Ramesh, Mhashilkar et al. 2003). Human MDA-7 exerts its anti-angiogenic effect by down-regulating the expression levels of vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) (Saeki, Mhashilkar et al. 2002). Both, VEGF and TGF-β are known modulators of angiogenesis (Kayton, Rowe et al. 1999). Similarly, it is also known that a wide variety of tumors express and secrete factors like VEGF, basic fibroblast growth factor (bFGF) and interleukin-8 (IL-8) that stimulate the formation of blood vessels (Fidler and Ellis 1994). Overexpression of MDA-7 reduces expression of VEGF and bFGF in A549 tumor cells (Nishikawa, Ramesh et al. 2004). In addition, MDA-7 had

additive negative effects on microvessel formation when combined with radiation therapy. This combined effect of MDA-7 and radiation therapy was different and statistically significanty different from the control treatment (PBS) (Nishikawa, Ramesh et al. 2004).

15. Anti-migration and anti-invasive properties

During metastasis, cancer cells use a variety of mechanisms to invade and disseminate to different parts of the body. Therefore, blocking tumor metastasis is an important target for development of cancer therapy. Tumor cells utilize various molecular pathways involving a variety of kinases such as PI3K, p38MAPK, p44/42MAPK, pJNK, and focal adhesion kinase (FAK) for invasion and migration. Ramesh and coworkers (2004) treated A549 (human NSCLC cell line) or H1299 (human large cell lung carcinoma cells) with *Ad.mda-7* and assessed the anti-migration effects of MDA-7 using a cell migration assay (measuring the movement of cancer cells across polycarbonate filters with 8 μm pores). MDA-7 treated A549 and H1299 cancer cells had significantly lower abilities to move across the polycarbonate filters (Ramesh, Mhashilkar et al. 2003; Ramesh, Ito et al. 2004). Overexpression of MDA-7 down-regulated the expression of p85, PI3K and FAK in human lung cancer cells (A549 and H1299 cell lines). MDA-7 treatment also decreased the expression of beta-catenin and PI3K in lung and breast cancer cells (Mhashilkar, Stewart et al. 2003). MDA-7 also induced apoptosis and inhibited migration of PAC1 cells, a smooth muscle cell line (Chen, Chada et al. 2003). Anti-migration effects of MDA-7 were also studied in the C8161 cell line (metastatic human melanoma cells). These cells showed reproducible invasive capacity *in-vitro*, and tumorigenesis in nude mouse models. Treatment with MDA-7 lowers the invasiveness of

C8161 cells through Matrigel-coated membrane inserts (Sauane, Gopalkrishnan et al. 2004). In contrast, secreted MDA-7 also acted through its receptors (IL-20R1/IL-20R2 and IL-22R1/IL-20R2) to prevent the invasion of cancer cells (DU-145). Su et al. (2005) expressed MDA-7 in normal cells (P69 cells) and co-cultured these MDA-7 expressing P69 cells with cancer cells. Cocultivation with *Ad.mda-7* infected P69 cells lowered the invasive ability of DU-145 or BxPC-3 (expresses MDA-7 receptors) while had no affect on A549 or NSCLC cells (which do not express the MDA-7 receptors) (Su, Emdad et al. 2005), suggesting the role of MDA-7 receptors in anti-migration properties of MDA-7.

During metastasis, tumor cells need to invade and migrate through the extracellular tissue matrix to successfully establish metastases at distant sites. During invasion, proteolytic enzymes produced by cancer cells degrade the extracellular matrix. Matrix metalloproteinases (MMPs) play an important role promoting tissue destruction during this process. Matrix metalloproteinases are expressed by a wide variety of tumor cells. Expression levels of MMPs are directly correlated with disease prognosis of various cancers including breast, lung, and colon. MDA-7 treatment down regulated expression of MMP-2 and MMP-9 in A549 cells. Similarly, it decreased the levels of MMP-2 in H1299 cells (Su, Emdad et al. 2005).

These investigators also studied the anti-migration and anti-invasive properties of MDA-7 in a lung metastasis model in nude mouse. They treated the A549 cells with *Ad.mda-7*, Ad.luc or PBS injected into the lung metastasis mouse models. *Ad.mda-7* treated A549 cells formed significantly lower numbers of lung tumor nodules compared to controls. The MDA-7 migration inhibiting activity was comparable to LY294002 and LY294002 also inhibited the expression of p85 PI3K and pFAK like MDA-7. Thus,

treatment of cancer cells with MDA-7 activated a signaling cascade that down regulated expression of MMPs, although the exact mechanism by which MDA-7 inhibited MMP-2 and -9 was not clear (Su, Emdad et al. 2005).

Thus, all of the above data suggested that human MDA-7 possesed a wide variety of antitumor properties that can kill various kinds of human cancer cells. Moreover, antitumor effects of human MDA-7 are cancer-specific, as it does not affect the growth of normal cells. It has also proven its use an as anticancer agent in phase-I clinical trial conducted on human patients (Fisher, Gopalkrishnan et al. 2003; Cunningham, Chada et al. 2005; Fisher 2005; Tong, Nemunaitis et al. 2005; Emdad, Lebedeva et al. 2007; Eager, Harle et al. 2008).

Currently, mice are used as the animal model for different human cancers, however murine cancer models differ from human cancer in several characteristics (mouse tumors are usually transplanted, the mouse population is homogenous, histopathological differences are seen between murine and human, and viral particles are sometimes present in the murine cancers). However, the dog provides several advantages as an intermediate animal model of human cancer. Similar to human beings, the risk of cancer increases in geriatric dogs. Cancer in dogs develops spontaneously in the presence of an active immune response due to exposure to the same environmental factors involved in human cancers. Moreover, canine cancers share many characteristics with human cancers (natural occurrence, histopathological appearance, biological behavior and response to different therapy, inter-individual and inter-tumoral heterogeneity etc (Khana *et a*l., 2006).

Thus, the aim of this study is to identify canine ortholog of human *mda-7* gene and its receptors (IL-20R1/IL-20R2 and IL-22R1 and IL-20R2) and characterize their biological properties.

II. Characterization of the canine *mda-7* gene, transcripts and expression patterns.

Introduction:

Cancer cells can be reprogrammed to undergo irreversible growth arrest and terminal differentiation using gene therapy. Melanoma differentiation associated gene-7 (*mda-7*) has such activity (Jiang, Lin et al. 1995; Jiang, Su et al. 1996). Human *mda*-7 is also known as interleukin-24 because it encodes a secreted protein with possible interleukin function, and belongs to the IL-10 family (Huang, Madireddi et al. 2001; Pestka, Krause et al. 2004). Human *mda*-7 is located in a cluster of IL-10 family members on chromosome 1q31-1q32. A 2.0 kbps long mRNA is transcribed from the *mda*-7 locus and encodes an evolutionarily conserved protein of 206 amino acids that belongs to the four-helix bundle family of cytokines (Huang, Madireddi et al. 2001; Pestka, Krause et al. 2004; Zdanov 2006). Human MDA-7 protein has a 48 amino-acid long signal peptide which is cleaved off when it is secreted (Nielsen, Engelbrecht et al. 1997; Nakai 2000). Normally, human MDA-7 has a highly restricted expression pattern. It is expressed at physiological levels by cells with immunological functions; including unstimulated or lipopolysaccharide stimulated monocytes and anti-CD3- stimulated Th2 cells. Its expression can also be detected in ConA or LPS stimulated human PBMCs (Huang, Madireddi et al. 2001; Caudell, Mumm et al. 2002; Garn, Schmidt et al. 2002; Wolk, Kunz et al. 2002; Poindexter, Walch et al. 2005). Expression of MDA-7 from human monocytes is also detected during influenza A virus infection (Garn, Schmidt et al. 2002).

Human MDA-7 is constitutively expressed at very high levels in normal human melanocytes (Ekmekcioglu, Ellerhorst et al. 2001; Huang, Madireddi et al. 2001), and its expression decreases progressively during melanocytic transformation (Ekmekcioglu, Ellerhorst et al. 2001; Ekmekcioglu, Ellerhorst et al. 2003; Allen, Pratscher et al. 2004). Similarly, human MDA-7 is not expressed by a wide variety of tumor cells with the exception of one human colon cancer specimen (Emdad, Lebedeva et al. 2007). However, its expression is induced in a wide variety of tumor cells after treatment with IFN-β and mezerein (Jiang, Lin et al. 1995; Jiang, Su et al. 1996). Despite much effort, little is known about the biological functions of MDA-7. It may act as a Th1- inducing cytokine because it induces expression of Th1 cytokines - TNF-alpha and IFN-γ in human PBMCs (Poindexter, Walch et al. 2005; Miyahara, Banerjee et al. 2006). Human MDA-7 may act as an immunostimulatory cytokine with respect to melanomas as it stimulates an immune response against melanoma-associated antigens. The primary target tissue of MDA-7, and its related cytokines, appears to be skin where it controls the proliferation of dermal cells (Poindexter, Williams et al. 2010). However, over the last decade, attention has been focused on its antitumor properties which are observed when human MDA-7 is expressed at high levels using either plasmid or replication-deficient adenoviral vectors (Jiang, Lin et al. 1995; Jiang, Su et al. 1996; Zhao, Dong et al. 2006; Dent, Yacoub et al. 2010; Dent, Yacoub et al. 2010; Zhang, Liu et al. 2011).

Overexpression of MDA-7 causes growth arrest and induces apoptosis in a variety of tumor cells including malignant glioma (Yacoub, Park et al. 2008; Yacoub, Hamed et al. 2010), melanoma (Chada, Mhashilkar et al. 2004), carcinoma of the breast (Su, Madireddi et al. 1998; Chada, Mhashilkar et al. 2006), lung (Kawabe, Nishikawa et al.

2002; Nishikawa, Ramesh et al. 2004; Inoue, Hartman et al. 2007), ovary (Leath, Kataram et al. 2004; Gopalan, Litvak et al. 2005; Emdad, Sarkar et al. 2006; Mahasreshti, Kataram et al. 2006) and prostate (Bhutia, Das et al. 2011; Dash, Azab et al. 2011). Importantly, normal cells when tested did not undergo apoptosis when exposed to MDA-7 indicating that its effects are cancer cell-specific (Dent, Yacoub et al. 2010; Dent, Yacoub et al. 2010). These growth suppressive and antitumor effects result from induction of apoptotic pathways and various other signaling pathways. MDA-7 overexpression leads to the down-regulation of anti-apoptotic genes (Bcl-2 and Bcl-xL) while causing up-regulation of pro-apoptotic genes (Bax, Bad and Bak) (Sarkar, Su et al. 2002). It also inhibits angiogenesis, invasion and migration of tumor cells, thus making it a potent candidate for cancer treatment (Ramesh, Mhashilkar et al. 2003; Ishikawa, Nakagawa et al. 2005; Xie, Sheng et al. 2008; Wang, Zhang et al. 2010).

Recently, orthologs of human *mda*-7 have been identified in rats and mice (Soo, Shaw et al. 1999; Schaefer, Venkataraman et al. 2001). Murine ortholog of *mda-7* is known as FISP (Interleukin 4-induced secreted protein) and is selectively expressed by Th2 cells. Murine MDA-7 also has antitumor properties. The rat ortholog of *mda-7* is known as c49a/Mob-5. Its expression is increased during wound healing, primarily in fibroblast-like cells on wound edges. The genomic structure, including exon/intron boundaries is conserved between these species and human MDA-7. Given that dogs are considered useful intermediate animal models of many human cancers (Hansen and Khanna 2004; Paoloni and Khanna 2007; Paoloni and Khanna 2008), knowledge of the canine *mda-7* gene and its functions would be useful. However, no studies to either

elucidate the biological properties or genomic structure of canine *mda-7* have been published to date.

In the present study, we sought to identify the canine ortholog of *mda-7* and examine its genomic structure and expression pattern. Our results demonstrate that the canine *mda-7* locus has a similar genomic structure when compared to human *mda-7*. However, canine *mda-7* has unique genomic elements, including novel exon sequences not identified in the human or murine genes, is restricted in its expression pattern and appears to undergo extensive splicing to generate five splice variants that encode four different protein isoforms with novel motifs, which could potentially have diverse biological properties.

Results:

1. Genomic structure of canine mda-7

To identify the canine *mda*-7 locus, we retrieved a 552 bp long predicted mRNA sequence (XM_846427) from the NCBI database and analyzed the sequence by BLAST against the canine genome. The putative canine *mda*-7 gene was localized to chromosome 7, which also contains the IL-10- related gene cluster of canine *IL-10*, *IL-19* and *IL-20*. To further characterize the canine *mda*-7 locus exonic/intronic structures as well as promoter sequences were examined. The *in-silico* analysis indicated that predicted mRNA sequence from the canine genomic *mda-7* locus was composed of five exons. A TATA element was identified by FPROM software analysis that was located 1389 bp upstream from the first nucleotide of the predicted canine *mda*-7 mRNA sequence. The sequences in and around the promoter were highly conserved when compared to the human *mda*-7 locus. A transcription start site was predicted 1359 bps upstream of the first nucleotide of predicted mRNA sequence. No sequences were identified in the 1389 base pair gap with similarity to human *mda-7*. In order to determine if the predicted promoter and transcription start site were used by canine *mda-7*, the full length mRNA sequence was obtained using rapid amplification of cDNA ends (5' and 3' RACE) from total RNA isolated from cultured normal canine epidermal keratinocytes (NECKs). Six hundred twenty seven additional bases of the mRNA were identified by this approach (Table 1). When the new 3' sequence was compared to human *mda-7* there was a high degree of similarity and the exon structure was identical. When the new 5' sequence was compared to human *mda-7*, a novel sequence was identified and the predicted transcriptional start site was confirmed. When aligned with

the canine genome, the full-length canine cDNA sequence indicated that the canine *mda-7* gene has eight exons, which vary in size from 62 to 216 bps, with introns ranging in size from 47 to 1159 bps. Six of the seven intron-exon boundaries have consensus-splicing signals (GT/AG)(Fig.4). However, the seventh intron uses GC as the splice donor and TG as the splice acceptor. The open reading frame starts in the second exon and terminates in exon 7 (Fig.4).

Primer Name	Primer Sequences
5' RACE adapter	5' – GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA - 3'
5' RACE outer primer	5'- GCTGATGGCGATGAATGAACACTG - 3'
5' mda-7 specific outer antisense primer	5'- AAAGTTGTTGGCCAGAGTAGAGAAT – 3'
5' RACE inner primer	5'- CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG - 3'
5' mda-7 specific inner antisense primer	5'- AGGATCAGACTCAGGCCGAGG – 3'
3' RACE adapter	5'- GCGAGCACAGAATTAATACGACTCACTATAGGT12VN - 3'
3' RACE outer primer	5'- GCGAGCACAGAATTAATACGACT - 3'
3' mda-7 specific outer sense primer	5'- GACACTGTGCAAGCTAAGGATAACATC – 3'
3' RACE inner primer	5'- CGCGGATCCGAATTAATACGACTCACTATAGG - 3'
3' mda-7 specific inner sense primer	5'- CAAAGACAATCACGGAAGGTT – 3'

Table 1: Sequences of primers used for 5' and 3' RACE of canine *mda-7*

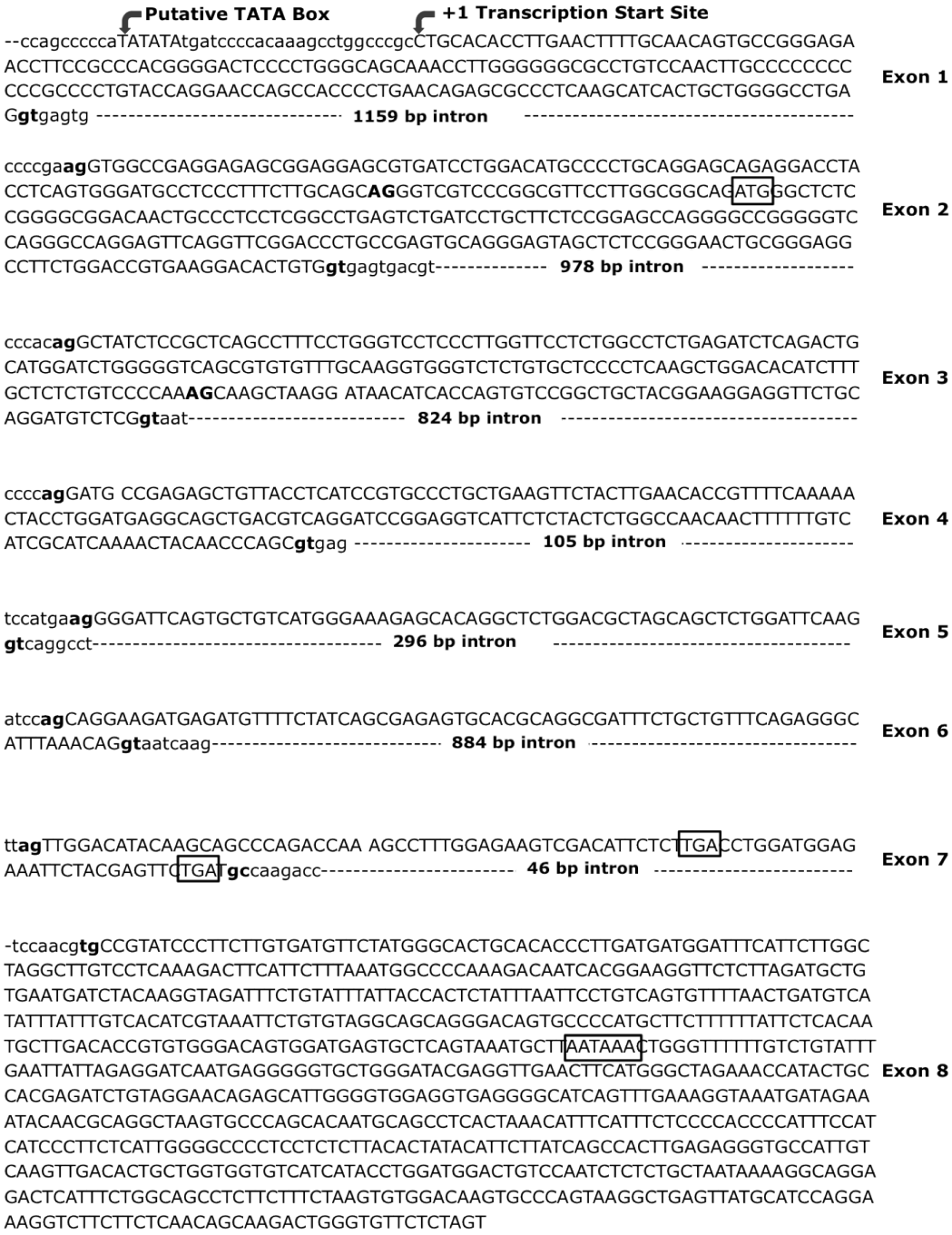


Fig. 4: Genomic structure of canine *mda*-7. The canine *mda*-7 locus is composed of eight exons and seven introns. Splice donor and acceptor sites are shown in bold. Alternative splice donor sites are located within exons 2 and 3. The start codon, stop codons and Poly (A) signal are shown in boxes. The size of each intron is noted in base pairs. Exon numbers are based on relative position and not on the corresponding human exon numbering.

A PCR primer set was designed to amplify the entire 6.0 kbps containing the canine *mda*-7 locus (promotor and gene). Genomic DNA isolated from canine (Beagle) muscle samples was used as template for PCR. In addition, genomic DNA from lymphocytes of an American Grey Wolf (*Canis lupus*) was also used as template for this PCR. A 6.0 kbp product from both templates was identified, gel-purified, cloned into a plasmid vector (pGEMT-easy) and sequenced. These sequences have 100% similarity to each other as well as to the published canine genome (Boxer), thus confirming that this locus is conserved in different dog breeds as well as in American Grey Wolf (data not shown).

2. Canine mda-7 pre-mRNA uses alternative splicing to generate five splice variants

Alternative splicing is an important mechanism to increase the functional diversity of the eukaryotic transcriptome as it results in the expression of new protein isoforms. Splice variants have been characterized in both human *mda*-7 as well as it murine ortholog FISP. While initially amplifying the open reading frame of canine *mda*-7, we identified a second transcript that appeared to be produced by alternative splicing. The second transcript was due to the use of an alternate 5' acceptor site within the second exon (Figs. 4 & 5B). This results in the deletion of 99 bp from the second exon of the transcript. We designated these splice variants canine *mda-7*sv1 (with the short version of the second exon) and canine *mda-7*sv2 (with the longer version of the second exon). However, canine *mda-7*sv1 and *mda-7*sv2 encode the same ORF. To determine if there were any other splice variants derived from the canine *mda-7* gene, two PCR primer pairs were designed and used sequentially for nested PCR. The outer primer pair was

complimentary to the sequences of the first and last exons. The inner primer pair was designed to amplify the open reading frame of the canine *mda-7* mRNA and was internal to the outer primer pair. These primer pairs were used to perform a nested PCR protocol on total RNA isolated from normal canine epidermal keratinocytes (NCEKs). PCR products were cloned into a plasmid vector (pCDNA3.1+/Hygro) and sequenced which resulted in the identification of the original 2 splice variants and three additional splice variants (Fig. 5A). The third splice variant, canine *mda*-7sv3, results from skipping the fourth exon (63 bps) (Fig 5B). However, deletion of this exon does not cause a frameshift in the reading frame. The fourth and fifth splice variants, canine *mda*-7sv4 and *mda-7*sv5 contain a novel fifth exon that is only present in these two variants (Fig. 5B). Addition of the fifth exon (62 nucleotides) causes a frameshift, so the protein isoforms encoded by canine *mda*-7sv4 and *mda*-7sv5 are dissimilar at the C-terminus from the protein isoforms encoded by the other three splice variants. The *mda-7*sv4 and -7sv5 splice variant's reading frames use a stop codon that is 29 bases upstream of the stop codon for sv1-3 in exon 7 (Fig 4). The fifth exon was not recognized by gene prediction software and has not been reported for *mda-7* in any other species. Lastly, the third exon has two 5' acceptor sites. All of the above splice variants (sv1, sv2, sv3 and sv4) except the fifth use a shorter version of the third exon (Fig. 5B). However, mda-7sv5 utilizes a 5' upstream alternate acceptor site for the third exon that adds 153 bp to this splice variant (Figs. 4 & 5B). Addition of 153 nucleotides does not cause a frameshift in the reading frame, however it results in the addition of 51 amino acids to the reading frame of canine *mda*-7sv5.

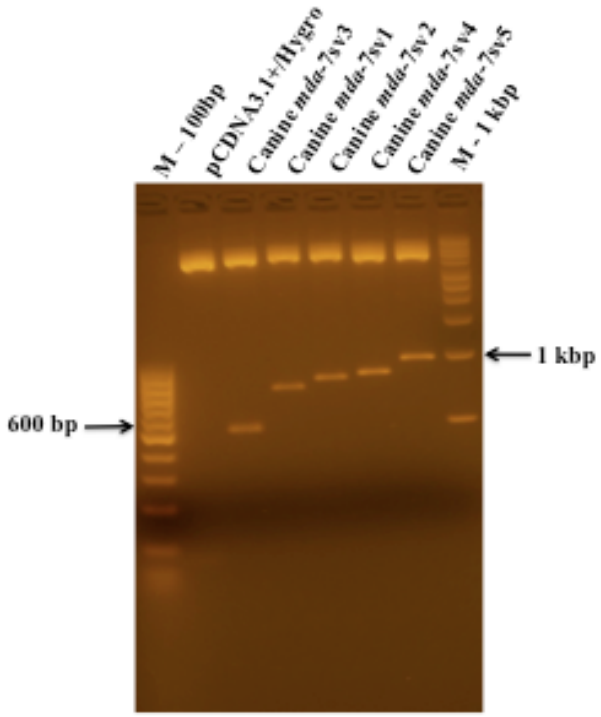


Fig. 5A: Identification of splice variants of canine *mda*-7. Canine MDA-7 splice variants were amplified and cloned into the pCDNA3.1+/Hygro vector. Recombinant clones were digested with *HindIII* and *XhoII* restriction enzymes to release the canine *mda*-7 inserts. Canine mda-7sv1- Splice variant 1, Canine mda-7sv2 - Splice variant 2, Canine mda-7sv3 - Splice variant 3, Canine mda-7sv4- Splice variant 4, Canine mda-7sv5- Splice variant 5 (M- 100bp Marker).

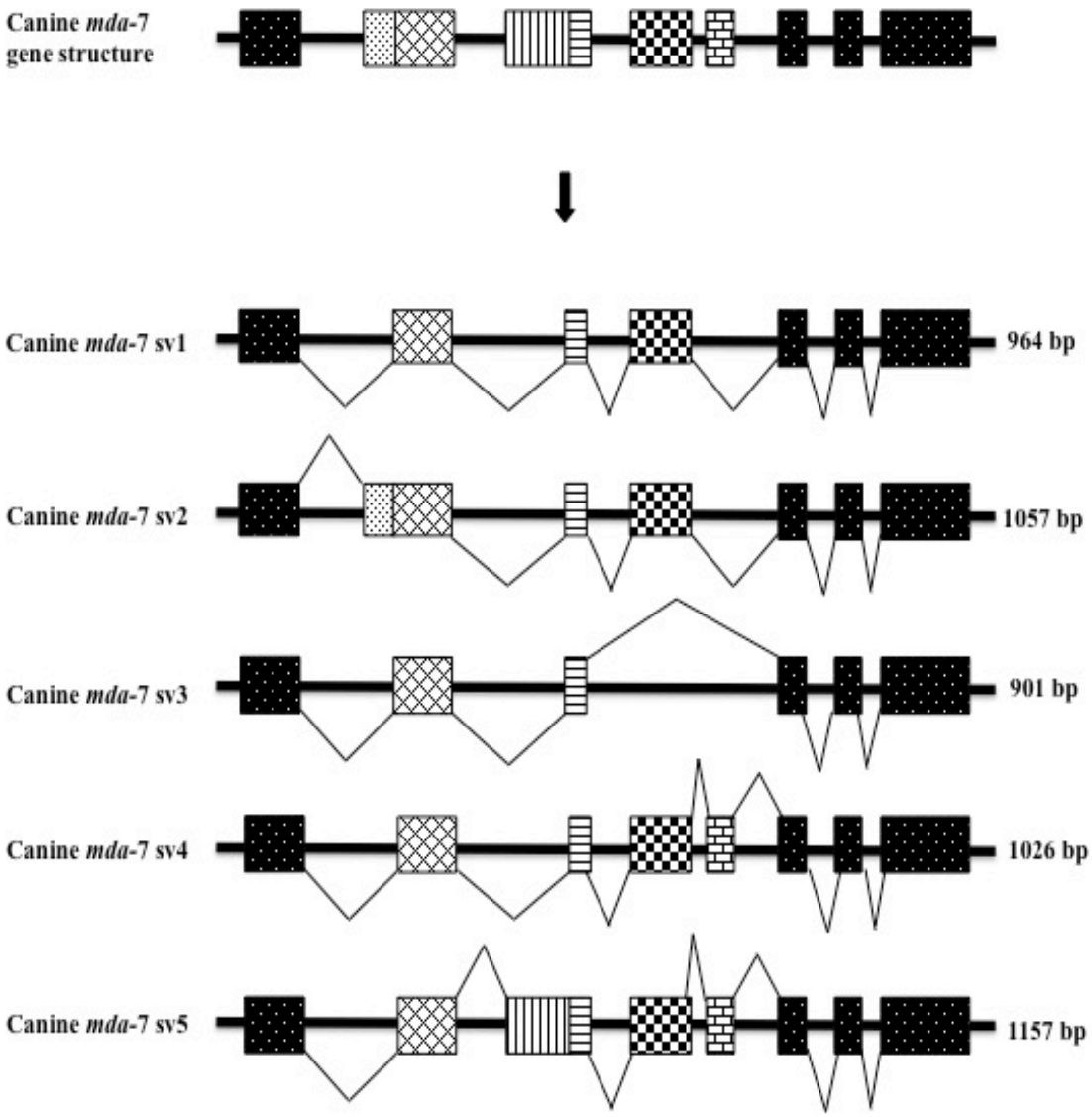


Fig. 5B: Alternative splicing at the canine MDA-7 locus results in production of five splice variants. Splice variants range in size from 901 to 1179 bps. Three of the splice variants use alternate acceptor sites for either the second or third exons; while exon skipping is used in four of the five splice variants. Commonly used splices are denoted by lines below the introns, while unique splices are denoted by lines above the introns.

3. Canine *mda-7* expression is restricted to canine keratinocytes and lipopolysaccharide stimulated canine PBMCs

Human *mda*-7 expression was first detected in IFN-β and mezerein-induced human melanoma (HO-1) cells and its expression is inversely correlated to progression of the melanoma. Subsequently, it was shown that human *mda-7* was expressed in cells and tissues related to the immune system such as monocytes, B cells, thymus and spleen. Furthermore, human *mda*-7 was not expressed in any of the human tumors examined. To determine the expression pattern of canine *mda-7*, a quantitative PCR assay was designed to detect canine *mda-7* mRNA. A canine *mda*-7 specific primer pair and TaqMan probe were designed to amplify the 3' region of mRNA to detect all of the splice variants (Table 2). Canine *mda*-7 was amplified by reverse transcriptase - PCR using these primers and was cloned into a plasmid vector (pGEMT-easy) to provide a standard reference. Recombinant plasmid containing canine *mda-7* was serially diluted to obtain copy numbers ranging from 300,000 to 30 per reaction. Following amplification, a standard curve was constructed using these dilutions with a correlation coefficient of 99.9 and a PCR efficiency of 100% (Fig. 6). The assay successfully detected as few as 30 copies of recombinant plasmid. This assay was then used to evaluate canine MDA-7 expression from different dog tissues. No canine *mda-7* mRNA was identified in unstimulated canine PBMCs, PHA, ConA or anti-CD3-stimulated canine PBMCs, thymus, lymphnode or spleen (Table 3). However, when dog PBMCs were stimulated with lipopolysaccharides (LPS) for 24 hours, canine *mda*-7 was expressed at very high levels (436.8 ± 21.33 copies/ng) (Table 3). Cultured primary canine keratinocytes also expressed canine *mda-7* at very high levels, and this expression increased significantly after stimulation with LPS (225.72±16 copies/ng to 329.54±31.36 copies/ng of total

RNA). In addition, primary canine tumor samples, including hepatic and splenic hemangiosarcomas, were assayed. None of the canine tumor samples expressed canine *mda-7* (Table 3). Cancer cell lines derived from various dog tumors were also evaluated. These cell lines included canine mammary tumors (CMT-28, CMT-27 and CMT-12), canine melanoma (CML-10) and lymphoma (OSW and 17-71). Five of the six cell lines, namely CMT-28, CMT-27, CML-10, OSW and 17-71 did not express canine *mda-7*. However, CMT-12 expressed canine *mda*-7 at very high levels (378.23±11.23 copies/ng of total RNA) (Table 3).

Primer Name	Primer and Probe Sequences
Canine mda-7 All transcripts Probe	[6-FAM]CAAGCAGCCCAGACCAAAGCCTTTGGA[TAMRA-6-FAM]
Canine mda-7 All transcripts CS	AGGCGATTTCTGCTGTTTCAGAGG
Canine mda-7 All transcripts NCS	AAGAAGGGATACGGCATTCAGAACTC
Canine mda-7 sv2 probe	[6-FAM]ATGCCTCCCTTTCTTGCAGC[TAMRA-6-FAM]
Canine mda-7 sv2 CS	AGAGGACCTACCTCAGTG
Canine mda-7 sv2 NCS	GAAGCAGGATCAGACTCA
Canine mda-7 sv3 Probe	[6-FAM]TCGGCATCCACAGTGTCCTT[TAMRA-6-FAM]
Canine mda-7 sv3 CS	GTCTGATCCTGCTTCTCC
Canine mda-7 sv3 NCS	GCACGAATGAGGTAACAG
Canine mda-7 sv4 Probe	[6-FAM]CTAGCGTCCAGAGCCTGTGC[TAMRA-6-FAM]
Canine mda-7 sv4 CS	AGGTCATTCTCTACTCTGG
Canine mda-7 sv4 NCS	CTTCCTGCTTGAATCCAG
Canine mda-7 sv5 probe	6-FAM]CTCCCTTGGTTCCTCTGGCA[TAMRA-6-FAM]
Canine mda-7 sv5 CS	GACACTGTGGCTATCTCC
Canine mda-7 sv5 NCS	CTGGTGATGTTATCCTTAGC

Table 2: Primer and probe sequences used for quantifying splice variants by TaqMan Assays (CS – coding strand, NCS – Non coding strand)

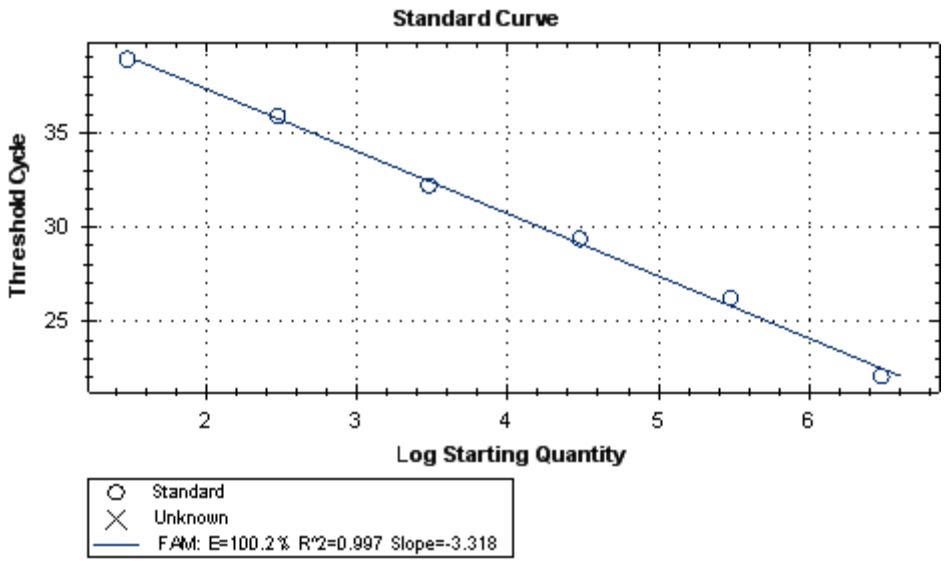


Fig. 6: Standard curve for the all Transcript TaqMan PCR. All transcript primers and probe were used to amplify log diluted (3×10^5 to 3×10^1 copy number) recombinant plasmid containing *mda-7* transcript. Copynumber was plotted on the X-axis while their respective C_T values were plotted on Y-axis. A standard curve was plotted and used for quantifying the copy number of canine mda-7 mRNA in different canine tissues.

Canine Tissue type	Canine *mda*-7 expression profile Copies/ng of Total RNA
Normal tissue	
Canine PBMCs Unstimulated PBMCs ConA stimulated PBMCs PHA stimulated PBMCs LPS stimulated PBMCs (100ng/ml of media) Anti-CD3 stimulated PBMCs	 Undetectable Undetectable Undetectable 436.8 ± 21.33 Undetectable
Spleen	Undetectable
Lymphnode	Undetectable
Thymus	Undetectable
Cultured normal canine epidermal keratinocytes	225.72 ± 5.58
Tumor sample and cancer cell lines	
Canine mammary tumor cell lines CMT-28 CMT-27 CMT-12	 Undetectable Undetectable 378.33 ± 9.12
Canine lymphoma cell lines OSW 17-71	 Undetectable Undetectable
Primary canine tumor samples Spleen hemangiosarcoma Liver hemangiosarcoma 5 more tumor samples	 Undetectable Undetectable Undetectable

Table 3: Quantification of mRNA levels of canine *mda-7* by TaqMan assay in various dog tissues.

4. Canine *mda*-7sv1 is the predominant splice variant expressed by canine keratinocytes.

The relative contribution of each of the five splice variants to the total amount of *mda-7* mRNA was evaluated with TaqMan PCR assays that were specific for each transcript. TaqMan probes were designed complementary to the sequences (canine *mda-*

7sv2, sv4 and sv5) that were either present only in specific transcripts or to the exonic junction (canine *mda*-7sv3) that was not present in other transcripts (Fig. 7). Copy number of canine *mda*-7sv1 was obtained by subtracting the cumulative means for the other transcripts from the copy number obtained from PCR for the total *mda-7* mRNA, as a unique probe set could not be identified for this variant. For absolute quantification, each transcript was cloned into a plasmid vector (pGEMT easy). This recombinant plasmid was used to develop a standard curve for each TaqMan probe. All of the TaqMan assays had a high PCR efficiency in addition to having a correlation coefficient in the range of 0.965 to 0.999 (Table 4). Canine *mda*-7sv1 was the predominant splice variant expressed (128.74/ng of RNA), sv2 and sv5 were expressed at an intermediate level (48.248 and 45.092 copies/ng respectively) and sv3 and sv4 were expressed at the lowest levels (1.104 and 2.528 copies/ng respectively) (Table 4). Relative expression of the splice variants was similar in both the unstimulated primary canine keratinocytes and stimulated canine PBMCs. However, when primary canine keratinocytes were stimulated with LPS, it resulted in a 50% increase in the copy number of canine *mda-7*sv1 and a two-fold increase in the copy number of canine *mda*-7sv2 with no alteration in the levels of the other splice variants ($p<0.0001$).

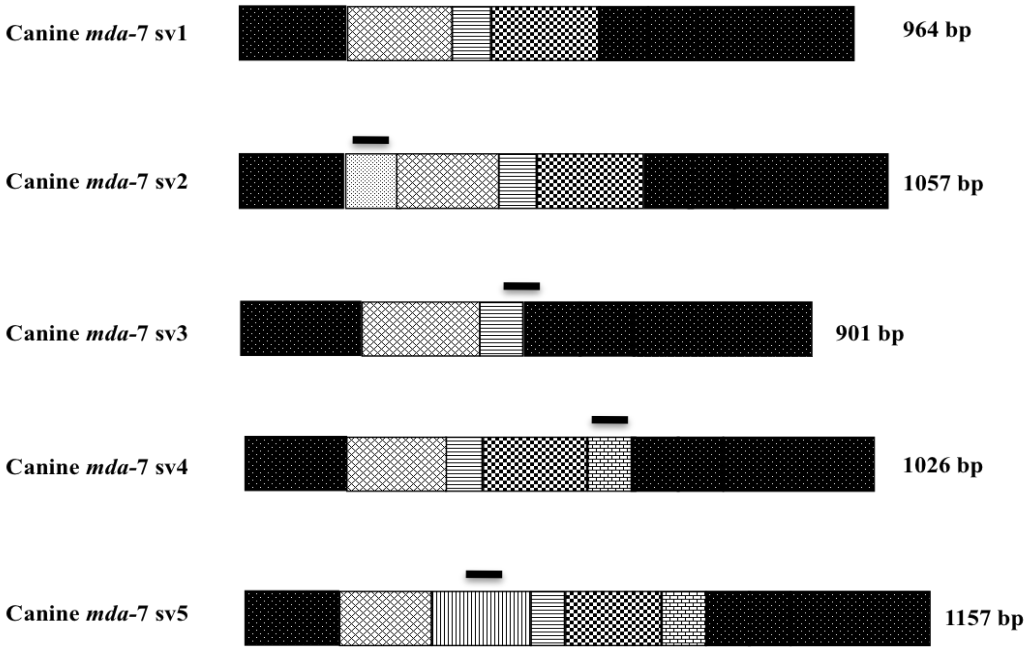


Fig. 7: Quantitative analysis of *mda-7* expression by TaqMan probe - based quantitative RT-PCR. One of the PCR primer pairs and probe were designed to amplify all of the different transcripts derived from the canine *mda-7* locus. The remaining TaqMan probes were designed against transcript specific sequences to detect sv2, sv3, sv4 and sv4. The copy number of canine *mda-7*sv1 was deduced by subtracting the sum of the copy number of sv2, sv3, sv4 and sv4 from the total copy number obtained from all transcript PCR. A) Diagram representing the position of splice variant TaqMan probes (bars).

Transcript Name	Protein Length	TaqMan Assay Efficiency/R^2 Value	Copy Number/ng of Total RNA	Copy Number/ng of total RNA After LPS Stimulation
Canine mda-7sv1	183	NA	128.74 ± 10.309	189.202 ± 9.08
Canine mda-7sv2	183	100.0/0.998	48.248 ± 9.489	94.668 ± 6.39
Canine mda-7sv3	162	96.2/0.967	1.104	Undetectable
Canine mda-7sv4	194	89.0/0.965	2.528	Undetectable
Canine mda-7sv5	245	96.5/0.982	45.092 ± 0.82	45.13 ± 2.69

Table 4: Quantitative analysis of relative expression levels of different splice variants in normal canine epidermal keratinocytes. Normal canine epidermal keratinocytes were cultured with or without lipopolysaccharides (LPS) for 24 hours (100ng/ml)

5. Predicted Canine MDA-7 protein

Human MDA-7 is a member of four-helix bundle family of cytokines. It is predicted to have 206 amino acids with a 49 amino acid signal peptide which is cleaved when human MDA-7 is secreted. The five splice variants of canine *mda*-7 that we identified, encode four different protein isoforms. These protein isoforms differ in length as well as in amino acid sequences. All of the canine MDA-7 protein isoforms use the same initiation codon, however, the isoforms derived from splice variants sv4 and sv5 have a frameshifting insertion and use a different stop codon than is used by the other splice variants making these two isoforms different from other isoforms at the C-terminus

(Fig. 8B). Canine *mda*-7sv1 and sv2 encode the same protein isoform, which is a 183 amino acid long protein with a predicted molecular weight of 18 kDa, and with the highest similarity to human MDA-7 protein. We amplified and expressed the open reading frame encoded by splice variants sv1 and sv2 into a plasmid vector to generate a fusion protein with the FLAG epitope (pCMV3TAG3). Expression from this vector resulted in tagging of canine MDA-7 with three copies of the FLAG epitope at its C-terminus which made it possible to detect the fused protein with an anti-FLAG antibody. Recombinant plasmid expressing FLAG tagged canine MDA-7 was transfected into HEK-293 cells. Expression of fused protein was detected by Western Blot analysis (Fig.8C) from the cell lysate of stable cell lines with a molecular weight of approximately 23 kDa, thus confirming that sv1 and sv2 encode the same ORF. A 28 amino acid long signal peptide was also predicted for canine MDA-7 protein using SignalP3.0 NN software (Fig. 8A). The predicted cleavage site lies between the 28^{th} and 29^{th} amino acid and is not conserved when compared to the cleavage site of human MDA-7 (Fig. 8A and 8B).

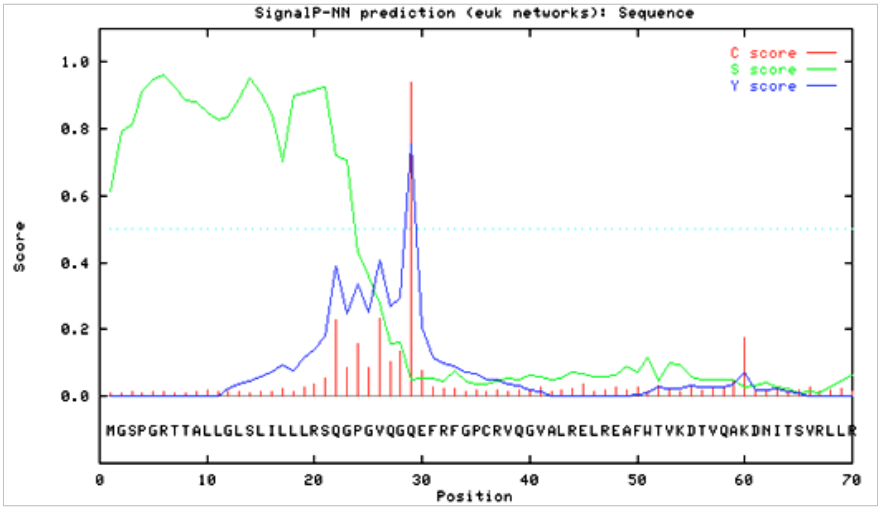


Fig. 8: Evidence that canine MDA-7 is a secreted protein. 8A) Prediction of signal peptide sequence for canine MDA-7 protein by signalP3.0 NN software. Canine mda-7 mRNA sequence was translated *in-silico* and analyzed by signalP3.0 NN software. It predicted a signal peptide sequence of 28 amino acids with a cleavage site between 28th and 29th amino acids.

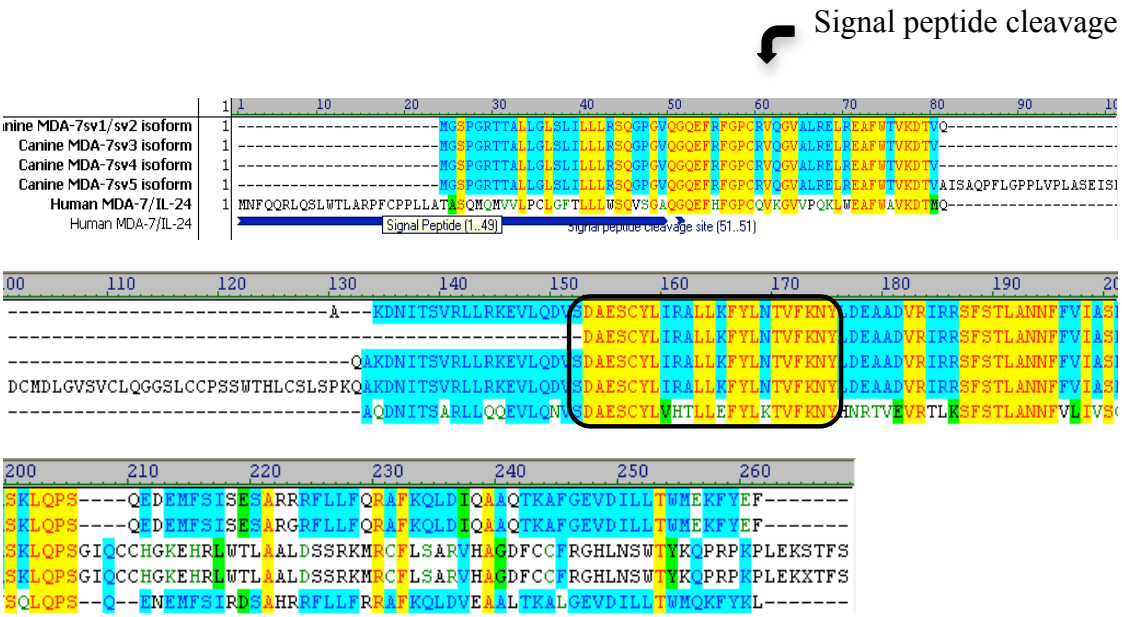


Fig. 8B: Alignment of canine MDA-7 protein isoforms with the Human MDA-7. Canine MDA-7 isoforms have a 28 amino-acid long predicted signal peptide sequence. The interleukin-10 signature motif (101-121) is shown within the box. Arrow - Signal peptide cleavage site.

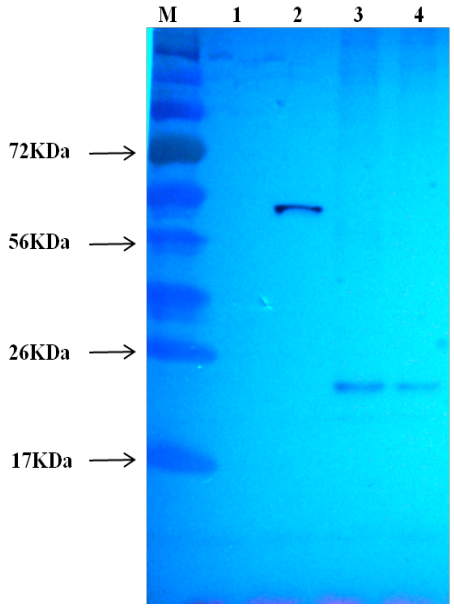


Fig. 8C: Western Blot showing canine MDA-7 fusion protein expressed from canine mda-7sv1 and sv2. Lane 1- HEK-293 lysate, 2- FLAG - bacterial alkaline phosphatase protein, 3 - HEK-293 transfected with pCMV 3TAG3 canine mda-7sv1 and 4 - HEK-293 transfected with pCMV 3TAG3 canine mda-7sv2. Estimated molecular weight of FLAG tagged canine MDA-7 is 23kDa.

Discussion:

Melanoma differentiation associated gene-7 *(mda-7)* is an important tumor suppressor gene that belongs to the IL-10 family of cytokines due to its chromosomal localization, sequence orthology and functional properties (Huang, Madireddi et al. 2001). MDA-7 shows antitumor properties in a wide variety of cancer cell lines derived from human tumors as well as in mouse tumor xenograft models and human solid tumors *in-vivo* (Dent, Yacoub et al. 2010; Dent, Yacoub et al. 2010). In the present study, we elucidated the genomic structure and expression profile of canine *mda*-7. As expected, much of the structure and sequence is conserved, however several novel features were identified in canine *mda-7*. The canine *mda*-7 gene locus contains eight exons (human *mda*-7 has seven exons) and consists of approximately 6.0 kbp, located on canine chromosome 7, within a cluster of IL-10 family members. The *mda*-7 locus is conserved between different breeds of dogs as well as in their close relative, the founding population of American Grey Wolf (*Canis lupus*). The canine *mda-7* promoter also contains a TATA element that has conserved sequences when compared to the promoter sequence of human *mda*-7. Human *mda-7* has a very long 3' untranslated region that has three poly(A) signal sequences that can be used for alternative polyadenylation. Human *mda-7* uses the third poly(A) signal to add a poly(A) tail. Canine *mda-7* mRNA does not have a long 3' untranslated region (3' UTR) like human *mda-7*. However, analysis of the genomic sequence of the canine MDA-7 locus identified all three polyadenyaltion signals. This suggests that the first poly(A) signal sequence is used to add the poly(A) tail. The 3' UTR of the human *mda-7* mRNA also has three destabilization domains or AU-rich elements (ARE) (3'-UTR-AUUUA). AU-rich elements interact with ARE binding proteins and target human *mda-7* mRNA for degradation. This is one of the

mechanisms used to regulate the expression levels of human *mda-7* (Otkjaer, Holtmann et al. 2010). The canine *mda-7* mRNA appears not to have these AU-rich elements because of the absence of the long 3' untranslated region. Thus, it seems that expression levels of canine *mda-7* are most likely primarly regulated through its promoter activity.

Canine *mda*-7 is constitutively expressed by cultured normal canine epidermal keratinocytes (NCEKs) although while it was not detectable in RNA isolated from whole dog skin samples, mimicking the expression profile seen with human MDA-7. The c49a, rat ortholog of *mda-7* is expressed in fibroblast-like cells during wound repair and human *mda-7* is expressed by epidermal keratinocytes during wound healing as well (He and Liang 2010; Poindexter, Williams et al. 2010). NCEKs also showed elevated expression of canine *mda-7* upon LPS stimulation. These results suggest that the culturing of epidermal keratinocytes (both NHEKs and NCEKs) stimulates them to express *mda-7*. Human MDA-7 is hypothesized to play an important role in maintaining the normal architecture of skin. During wound healing, MDA-7 inhibits the proliferation of epidermal keratinocytes, and induces them to return to their differentiated, non-proliferating state. Thus, our studies suggest the possibility of a similar role for canine MDA-7 in wound repair in dog skin as has been reported for human MDA-7.

Human MDA-7 is expressed by monocytes, subsets of T cells, and in LPS and PHA-stimulated PBMCs. Canine *mda*-7 mRNA was only detected in LPS-stimulated canine PBMCs. However, its expression was undetectable in unstimulated PBMCs as well as in PHA, ConA or Anti-CD3-stimulated PBMCs. The lack of expression in these canine cells is a significant difference between canine and human *mda-7* and may have broad implications with respect to the impact, or lack thereof, of canine *mda-7* on

conditions such as malignant cell growth where the human ortholog has a well defined activity.

Canine *mda*-7 mRNA was not detected in primary canine tumor samples and in most of tumor-derived canine cancer cell lines evaluated (OSW, 17-71, CML10, CMT28 and CMT27). However, one cell line, CMT12 expressed canine *mda*-7 mRNA at very high level. Expression of canine *mda*-7 did not seem to affect the growth of CMT12 cells. However, we do not know the mechanism that results in expression of canine MDA-7 in these cells.

Alternative splicing of pre-mRNA is an important mechanism to increase protein diversity in eukaryotes. We detected five different splice variants that encode four protein isoforms of canine MDA-7. These splice variants result from either exon skipping (exon 4 and 5) or from the use of alternate 5' acceptor sites (exon 2 and 3). Splice variants resulting from the use of alternate 5' splice sites are the predominant forms (canine *mda-7*sv1 (57.03%), canine *mda-7*sv2 (21.37%) and canine *mda-7*sv5 (19.97%). All of the splice variants were constitutively expressed in cultured normal canine epidermal keratinocytes (NCEKs). However, canine *mda*-7sv1 and sv2 were the only splice variants whose relative expression was elevated after LPS stimulation. This may suggest that these two splice variants are the primary transcripts. Similarly, human *mda*-7 pre-mRNA has been reported to undergo alternative splicing to yield at least three splice variants (Allen, Pratscher et al. 2004; Allen, Pratscher et al. 2005; Yang, Duan et al. 2011). These splice variants either lack the third exon or both exons 3 and 5 (Allen, Pratscher et al. 2005). The splice variant lacking the third and fifth exon was renamed *mda-7s*, and its expression was found to be inversely correlated to the stage of melanoma (expression

decreased as the melanoma progressed). This splice variant encodes a truncated protein that has only 14 amino acids in common with normal human MDA-7. However, this truncated protein can be coprecipitated with human MDA-7 protein, and prevents its secretion (Allen, Pratscher et al. 2004). Similarly, splice variants (FISP-sp) were also found for the murine ortholog of MDA-7, FISP. FISP-sp heterodimerizes with FISP and blocks its secretion, inhibiting the apoptotic effects of FISP (Sahoo, Jung et al. 2008). Thus, it is possible that the protein isoforms encoded by different splice variants of canine *mda*-7 may interact with each other to control the expression level, secretion and activity of the primary splice variant, canine *mda-7*sv1.

Protein isoforms encoded by splice variants sv1/2 and sv3 exhibit a high level of sequence identity (75%) to human MDA-7 at the amino acid level. All of the canine MDA-7 protein isoforms also have a conserved IL-10 signature sequence (Fig. 5b). Human MDA-7 is a heavily glycosylated protein with three consensus N-linked glycosylation sites (Fuson, Zheng et al. 2009). Glycosylation at these sites is necessary for human MDA-7 solubility and bioavailability. Canine MDA-7 isoforms have only one of these consensus N-linked glycosylation sites (Asn-85). The two other N-linked glycosylation sites (Asn-99 and Asn-126) are not present in canine MDA-7 protein isoforms. Human MDA-7 also posseses a disulfide bond between 59^{th} and 106^{th} cysteine. This disulfide bond is required for secretion and activity (Fuson, Zheng et al. 2009). These two cysteine residues are also conserved among different isosforms of the canine MDA-7. As human MDA-7 is a secreted protein, it has a very long signal peptide of 49 amino acids. Canine MDA-7 also has a predicted signal peptide, but it is a more typical 28 amino acids long.

Thus, we can conclude that the *mda-7* locus is present and expressed in dogs but that it has a more limited range of tissue expression than its human counterpart. Additionally, canine *mda*-7 undergoes extensive alternative splicing that results in generation of five splice variants which encode four protein isoforms of canine MDA-7. Canine MDA-7 has a potential signal peptide and conserved IL-10 signature sequence, thus confirming it to be a member of the IL-10 family of cytokines. Due to its high level of amino acid sequence similarity with human MDA-7, we predict that one or more of the splice variants of canine MDA-7 may also have antitumor properties which may have potential for designing new cancer therapeutics based on canine MDA-7 for canine cancer patients.

Material and methods:

1. **Isolation and culturing of normal canine epidermal keratinocytes (NCEKs) using CellnTec protocol:**

Canine skin samples ($1.5inch^2$) were collected in CnT-09 media (CELLnTEC advance cell system) supplemented with 10% supplement A (CELLnTEC), 400nM L-glutamine, penicillin (500I.U/ml) and streptomycin (500µg/ml) (complete CnT-09). Excess dermal tissue from the skin samples was removed under sterile conditions. The skin was cut into 0.5 cm square pieces. Cut skin samples were kept at 4^0C in 20 ml of complete CnT-09 media supplemented with 10 mg/ml dispase II (Roche Applied Science) for 21 hours. After that, the epidermis was removed from the dermis, washed in PBS (Mediatech, Inc.) and placed in TrypLE express (Invitrogen) for 30 minutes, at room temperature. The epidermis was rubbed with forceps to release the cells. After

incubation, the cell suspension was filtered through a 0.70 µM filter (Beckson Dickinson Labware, France). An equal amount of complete CnT-09 media was added to the cell suspension. Cells were centrifuged at 300 x g for 5 min. The cell pellet was resuspended in complete CnT-09 media and the cells were seeded (1.5 X 10^6 cells/flask) into a 75 cm^2 flask and maintained at 37^0C and 5% CO_2. Where noted, NCEKs were stimulated with lipopolysaccharides (100ng/ml) for 24 hours.

2. Isolation of canine PBMCs

Canine PBMCs were purified from healthy dogs by gradient density centrifugation using Histopaque™ (1.077 g/mL (Sigma-Aldrich, Inc.)) from blood collected in EDTA coated blood collection tubes. Isolated canine PBMCs (1 X 10^6 cells/ml) were cultured in Roswell Park Memorial Institute medium-1640 (RPMI-1640, Mediatech, Inc.) supplemented with 10% FBS, penicillin (100 I.U/ml) and streptomycin (100µg/ml) and maintained at 37^0C and 5% CO_2. Canine PBMCs were stimulated *in-vitro* with 25 ng, 100 ng, or 200 ng LPS/ml. Canine PBMCs were also stimulated *in-vitro* with phytohemagglutinin (5 µg/ml) and Concanavalin A (25 µg/ml) (Sigma-Aldrich, Inc).

3. Culture of canine cancer cell lines

Canine cancer cell lines namely CMT12, CMT27, CMT28, CML10, OSW and 17-71 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, penicillin (100 I.U/ml) and streptomycin (100µg/ml) and maintained at 37^0C and 5% CO_2. When cancer cells reached 75% confluency, they were used to isolate total RNA using Tri-reagent kit (Ambion, Inc.)

4. Rapid amplification of cDNA ends

5’ RNA ligase mediated RACE (RLM-RACE) and 3’ RACE were performed according to the manufacturer’s directions (First choice® RLM-RACE, Ambion, Inc.). Briefly, total RNA was isolated from normal canine epidermal keratinocytes (NCEKs) with TRI Reagent (MRC, Inc.). 1μg of this RNA was treated with Calf Intestine Alkaline Phosphatase (CIP) to remove the 5'-phosphate group from rRNAs, tRNAs and broken mRNAs. CIP-treated RNA was purified and precipitated by phenol: chloroform extraction and isopropanol precipitation. The RNA pellet was then dissolved in nuclease free water. After this, RNA was treated with Tobacco Acid Pyrophosphatase (TAP) to remove the 5’ cap from full-length mRNAs, leaving a 5'-monophosphate. CIP/TAP treated RNA was then ligated to a 45bp long RNA 5’ adapter using T4 RNA ligase. Adapter-ligated RNA was reverse transcribed into first-strand cDNA using random-primers for 5’ RACE. An outer 5' RLM-RACE PCR reaction was carried out under standard procedures using 5’ RACE outer and outer mda-7 specific antisense primers (Table 1). 2μl of the outer PCR reaction was used as template for an inner PCR reactions using 5’ RACE inner and inner mda-7-specific antisense primers. For 3’ RACE, RNA was reverse transcribed with the 3’ RACE adapter acting as a primer. An outer 3' RACE PCR reaction was run using the 3’ RACE outer and outer canine mda-7 specific sense primers. 2 μl of outer PCR reaction was used as template for an inner PCR reaction using the 3’ RACE inner and inner mda-7 specific sense primers. Amplified products were cloned into the pGEMT-easy vector and sequenced using T7 and SP6 primers at DNA Core Facility at Massachusetts General Hospital.

5. Quantitative PCR

Different splice variants were amplified using nested PCR and cloned into pGEMT easy (Promega) and pCDNA3.1+/Hygro vectors (Invitrogen, Inc). Absolute copy numbers of all the splice variants were calculated using quantitative polymerase chain reactions employing TaqMan® Probes. Primers and TaqMan® probes (Table 2) were designed to amplify and differentiate among splice variants respectively. Recombinant clones containing splice variants were diluted according to the copy number to generate a standard curve. Total RNA was isolated from NCEKs, canine PBMCs, LPS stimulated canine PMBCs and NECKs. One microgram of total RNA was reverse transcribed (qScript cDNA synthesis kit, Quanta Biosciences). 4µl of this reaction was used to quantify the copy number of splice variants by quantitative PCR (TaqMan Fast Universal PCR Master Mix, Applied Biosystem) under optimal conditions that included preheating to 95°C for 5 min followed by 40 cycles of 95°C for 10 seconds and 62°C for 20 seconds.

6. Amplification, cloning and expression of canine MDA-7

The open reading frame encoded by canine mda-7sv1 and sv2 were amplified and cloned into pGEMT-easy vector (Promega Inc.). Recombinant clones were confirmed by sequencing using T7 and SP6 primers. This ORF was further sub-cloned into the mammalian expression vector (pCMT-3TAG vector, Agilent technologies) for high-level expression. Expression from this vector also results in tagging of canine MDA-7 protein with three copies of the FLAG epitope at the C-terminus. For subcloning, pCMV-3TAG vectors were double-digested with *HindIII* and *XhoI* restriction enzymes and dephosphorylated with Calf Alkaline Phosphatase (CIP, New England biolab Inc.).

Canine *mda*-7 inserts were released from the pGEMT easy vector by double digestion with *HindIII* and *XhoI,* purified (GeneJET^{TM} gel extraction kit, Fermentas International Inc.), according to the manufacturer's instructions, and ligated with digested and dephosphorylated pCMV-3TAG vector using T4 DNA ligase. JM109 competent cells were transformed with the ligation reaction. Recombinant clones were confirmed by sequencing with T3 and T7 primers. Expression of canine MDA-7 from recombinant clones was confirmed both at RNA and protein levels after transfection into HEK-293 cells (Lipofectamine LTX, Invitrogen Inc) by PCR and western blot analysis. For western blot analysis, 30 μg of cell lysate was fractionated on 10% SDS-PAGE gels. Proteins were then electroblotted to nitrocellulose membrane. The membrane was then blocked with 5% non-fat dry milk. The blocked membrane was incubated with anti-flag antibody at a 1 to 1500-fold dilution (Clonetech Inc.). The membrane was washed three times with phosphate buffered saline (10 mM sodium phosphate, 0.15 M NaCl) containing 0.05% tween-20, incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse (Thermo Fisher Scientific Inc.) IgG at 1 to 200,000-fold dilution, and was washed again three times with PBS containing 0.05% tween-20 and then incubated with ECL substrate (Thermo Fisher Scientific Inc.) for five minutes. The membrane was dried and signal was detected by exposing the membrane to X-ray film for 3 min.

7. *In-silico* analysis

The predicted canine MDA-7 gene sequence was obtained from the National Center for Biotechnology IFNormation (NCBI) sequence database (Accession: XM_846427). This predicted sequence was analyzed by BLAST against the canine genome and the genomic sequence representing the canine MDA-7 locus

(NW_876323.1) retrieved. The canine MDA-7 gene locus was analyzed *in-silico* with different software packages including Vector NTI Advance 10 (Invitrogen), Spidey (NCBI), Genescan (MIT) and FGANESH (Softberry, Inc.). FPROM (Softberry, Inc.) was used to predict the promoter for the canine mda-7 gene. SignalP (3.0) was used to predict the signal peptide in canine MDA-7 protein (NCBI ; Softberry ; Spidey ; Burge 1997; Jannick Dyrløv Bendtsen 2004).

8. Statistical analysis:

Equal amounts of cDNA were used to compare the expression levels of splice variants. All the TaqMan PCRs were run in triplicate. The two sample t-test was used to compare the expression levels of canine mda-7 between stimulated and unstimulated NCEKs at the 95% confidence level and a p-value of less than 0.05 was considered statistically significant.

III. Canine melanoma differentiation associated gene-7/interleukin-24 (*mda-7*/IL-24) and its protein isoforms exhibit antitumor properties against canine and human tumor cells

Introduction

In recent years the incidence of cancer has been on the rise in the canine population due to advances in pet nutrition, vaccination and improved chemotherapy against infectious diseases (Hansen and Khanna 2004; Paoloni and Khanna 2007; Paoloni and Khanna 2008). Canine cancers have significant similarity to human cancers in etiology, pathogenesis, histological similarities as well as in biological complexity (Misdorp and Hart 1979; Patronek, Waters et al. 1997; Modiano, Ritt et al. 1999; Olson 2007). Canine cancers are spontaneously induced in the same environment as human cancers, and develop in the presence of an intact immune response. Moreover, mutations in the same set of oncogenes and tumor suppressor genes are involved in the pathogenesis of dog and human tumors (Haga, Nakayama et al. 2001; Koenig, Bianco et al. 2002; DeInnocentes, Agarwal et al. 2009). Thus, this large pool of canine cancer patients can be used to better understand tumor pathogenesis as well as to develop and evaluate new cancer treatments.

One important therapeutic approach to treat cancer is the replacement of mutated genes with healthy ones or the introduction of suicide genes (such as thymidine kinase) into cancer cells by cancer gene therapy. Cancer cells may also be modified with genes

that have growth suppressing and cell killing effects on these cells, specifically. Human melanoma differentiation associated gene-7 (*mda-7*) was identified by subtraction hybridization from cDNA libraries prepared from IFN-β and mezerein treated human melanoma cells (HO-1) (Jiang, Lin et al. 1995; Jiang, Su et al. 1996). Human MDA-7 is a single copy gene, and is located on 1q31-1q32 locus (Huang, Madireddi et al. 2001). The human *mda*-7 gene is composed of seven exons and six introns, and when transcribed, it produces an mRNA of 1718 nucleotides that encodes a protein of 206 amino acids (Jiang, Lin et al. 1995; Jiang, Su et al. 1996; Huang, Madireddi et al. 2001). Human *mda-7* is also known as interleukin-24 (IL-24) because of its translational regulation, protein structure and chromosomal location. It is a member of the IL-19 subfamily of IL-10 related cytokines, and has 19% amino acid similarity to interleukin-10 (Pestka, Krause et al. 2004; Zdanov 2006). It has an unusual 49 amino acid long hydrophobic signal sequence, which is cleaved off during secretion (Nielsen, Engelbrecht et al. 1997; Nakai 2000; Pestka, Krause et al. 2004).

Jiang and coworkers (1995, 1996) first showed the growth suppressing effects of human MDA-7 on various cancer cells lines. Overexpression of MDA-7 protein inhibits the growth and induces apoptosis in a wide variety of cancer cells including malignant glioma, melanoma and carcinoma of breast, lung, ovary and prostate *in-vitro* (Sarkar, Su et al. 2002; Fisher, Gopalkrishnan et al. 2003; Emdad, Lebedeva et al. 2007; Sarkar, Lebedeva et al. 2007). The antitumor properties of MDA-7 were further confirmed in *in-vivo* studies involving xenograft nude mouse models of human cancers (Saeki, Mhashilkar et al. 2002; Sarkar, Su et al. 2005; Sarkar, Su et al. 2008). MDA-7 not only inhibited their growth but also induced apoptosis in tumor cells *in-vivo*. Furthermore,

Sarkar *et al* (2005) showed that intratumoral injection of *Ad.mda-7* not only kills the primary tumors, but also shows a bystander antitumor effect on distant tumor masses. Bystander antitumor activity of MDA-7 is exerted through its two heterodimeric receptor complexes, IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (Chai, Nichols et al. 1997; Nielsen, Engelbrecht et al. 1997; Catlett-Falcone, Landowski et al. 1999; Dumoutier, Leemans et al. 2001; Wang, Tan et al. 2002; Wang, Tan et al. 2004). Binding of MDA-7 to its receptors leads to activation of Janus Kinase and phosphorylation of STAT3 (to a lesser extent of STAT1), which then translocate to nucleus (Parrish-Novak, Xu et al. 2002).

The antitumor effects of MDA-7 are mediated through a variety of molecular pathways. MDA-7 activates the p38 MAPK signaling pathway, which results in increased expression of growth arrest and DNA damage inducible genes (GADD) in a variety of cancer cells (Sarkar, Su et al. 2002; Sainz-Perez, Gary-Gouy et al. 2006). MDA-7 also causes endoplasmic stress in prostate cancer cells, which in-turn induces the unfolded protein response (UPR) that causes death of cancer cells via a Bip/GRP78 dependent pathway (Sieger, Mhashilkar et al. 2004; Gupta, Walter et al. 2006). MDA-7 also increases the oxidative stress in cancer cells by increasing the generation of reactive oxygen species (ROS) (Lebedeva, Sarkar et al. 2003; Lebedeva, Su et al. 2005; Sarkar, Lebedeva et al. 2007). Furthermore, MDA-7 exhibits anti-invasive, anti-migration and anti-angiogenic properties (Saeki, Mhashilkar et al. 2002; Nishikawa, Ramesh et al. 2004).

Fisher and coworkers confirmed all of the above properties of MDA-7 in a phase I trial conducted on solid tumors using *Ad.mda-7* (replication deficient adenoviral vector expressing MDA-7). Intratumoral injection of *Ad.mda-7* resulted in transduction of 10-

30% tumor cells. However, after injection, 70% of the tumor cells showed an apoptotic phenotype, demonstrating a potent "bystander cancer specific killing effect". Multiple injections of *Ad.mda-7* were well tolerated by human patients with the exception of few self-limiting mild toxicities (Fisher, Gopalkrishnan et al. 2003; Cunningham, Chada et al. 2005; Tong, Nemunaitis et al. 2005; Emdad, Lebedeva et al. 2007; Dent, Yacoub et al. 2010). Thus, all of the above data confirm that human MDA-7 has the properties required for development of an effective anticancer treatment.

Previously, we have identified a canine ortholog of the human *mda-7* gene. Canine *mda-7* is constitutively expressed in cultured normal canine epidermal keratinocytes (NECKs). The predicted canine MDA-7 protein is 183 amino-acid long, and has significant amino acid (75%) similarity to human MDA-7 protein. We also predicted a 28 amino-acid long signal peptide sequence that can direct canine MDA-7 to be secreted. Furthermore, alternative splicing of canine mda-7 pre-mRNA generates five splice variants that encode four isoforms of canine MDA-7 protein.

In this study, we investigated the biological properties of canine MDA-7 and its splice variants.

Results:

1. Canine MDA-7 is a secreted protein

In-silico analysis of the canine MDA-7 protein sequence by SignaIP 3.0 revealed a 28 amino acid long signal peptide that can direct its secretion. A cleavage site between the 28^{th} and 29^{th} amino acid was predicted. To confirm this, a eukaryotic expression vector expressing canine MDA-7 protein tagged with three copies of the Flag epitope was contructed. Human embryonic kidney-293 (HEK-293) cells were transiently transfected with the pCMV 3TAG3 canine MDA-7 expression plasmid. As a negative control, HEK-293 cells were transfected with an empty expression vector. 48 hours after transfection, cell cultured medium was collected and probed with anti-FLAG antibody to detect the secreted canine MDA-7 protein by western blot analysis (Fig. 9). We successfully detected the FLAG-tagged canine MDA-7 protein band in the supernatant as well as in the cell culture lysate of pCMV 3TAG3 canine MDA-7 transfected HEK-293 cells (Fig. 9). However, no protein band was detected in cell lysate and supernatant of HEK-293 cells transfected with empty vector. Thus, our data confirm that canine MDA-7 is a secreted protein.

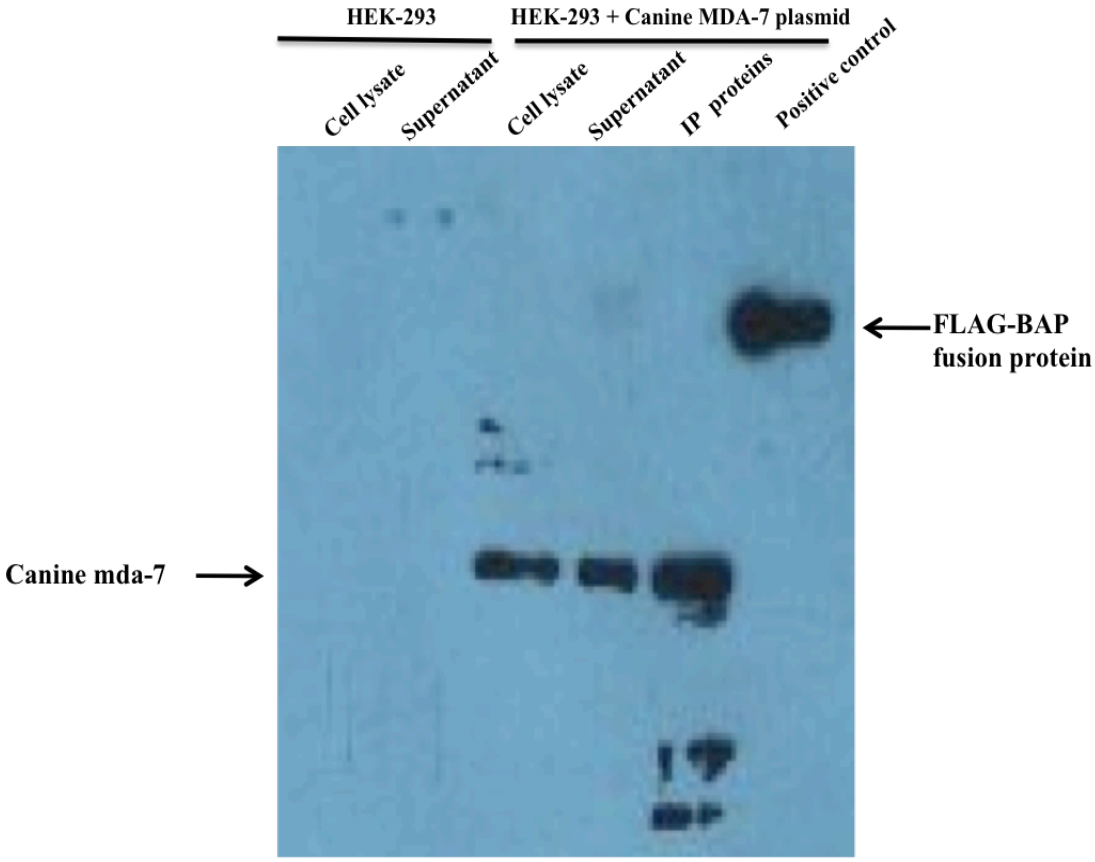
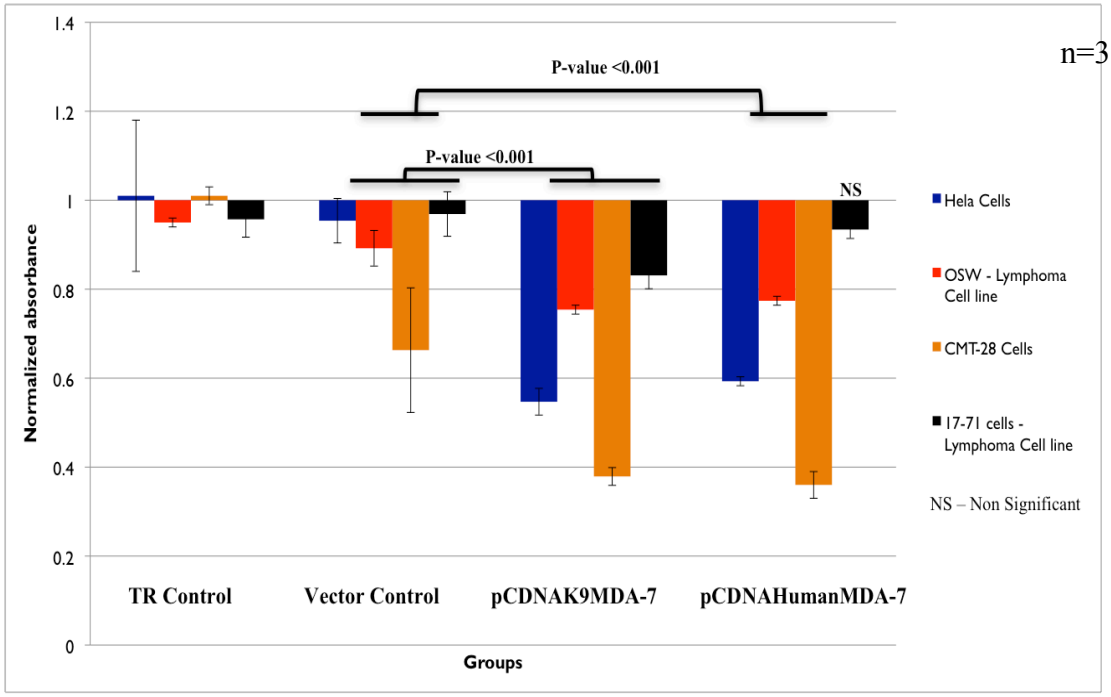


Fig. 9: Canine MDA-7 protein is a secreted protein. HEK-293 cells were transfected with pCMV 3TAG3 plasmid expressing canine MDA-7. After 72 hours, cells and cell culture media were harvested and probed for canine MDA-7 protein by Western Blot analysis using anti FLAG antibody.

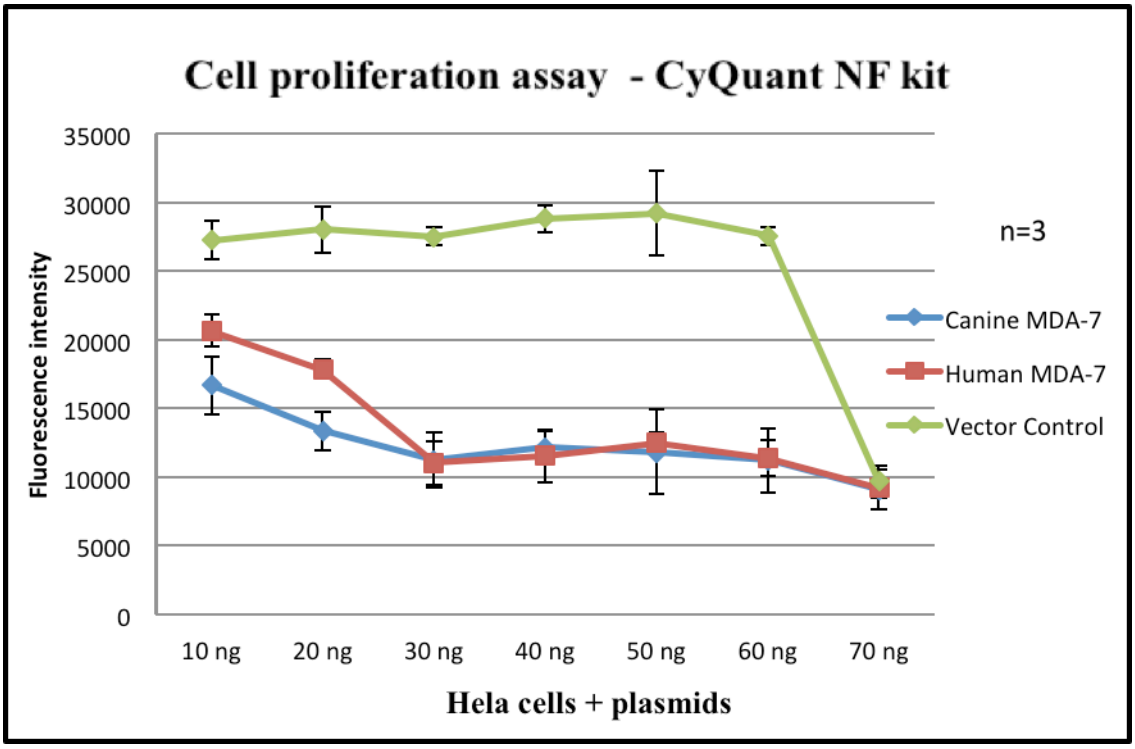
2. Ectopic expression of canonical canine MDA-7 inhibits the growth of tumor cells

Human MDA-7 shows potent growth-suppressing effects on a wide variety of cancer cells. We decided to evaluate the effects of canine MDA-7 expression on both human and canine cancer cells. Canonical canine MDA-7 was cloned and ectopically expressed from pCDNA3.1+/Hygro, an eukaryotic mammalian expression vector. Plasmid expressing human MDA-7 was used as positive control, and empty vector was used as a negative control. HeLa cells were cultured in 96-well plates (triplicate) and transfected with the expression plasmids described above. After 72 hours, the proliferation and viability of HeLa cells was determined by MTT assay. When transfected with canine MDA-7 plasmid, the proliferation of HeLa cells was inhibited (Fig. 10B), while no such effect was seen when HeLa cells were transfected with empty vector. Similarly, human MDA-7 protein also inhibited the growth of HeLa cells. Moreover, the cytotoxic effects of canine and human MDA-7 protein increased with increasing the amounts of (ng/well) of plasmid before reaching a point when there was no difference from the vector group (Fig. 10B). We also transiently transfected canine and human MDA-7 constructs into various canine cancer cells including canine mammary tumor (CMT-28), canine melanoma cells (CML-10) and lymphoma cell lines (OSW and 17-71). Expression of canine and human MDA-7 proteins showed cytotoxic effects on these dog tumor cells. Following transfection, there were fewer viable cells in groups treated with canine or human MDA-7 expression plasmids than in vector treated cells (Fig. 10A). Growth inhibitory effects of canine and human MDA-7 proteins were statistically significant from the control group ($p<0.01$). However, no growth inhibiting effects (Fig. 10C) were seen on normal canine fibroblasts (NCF).

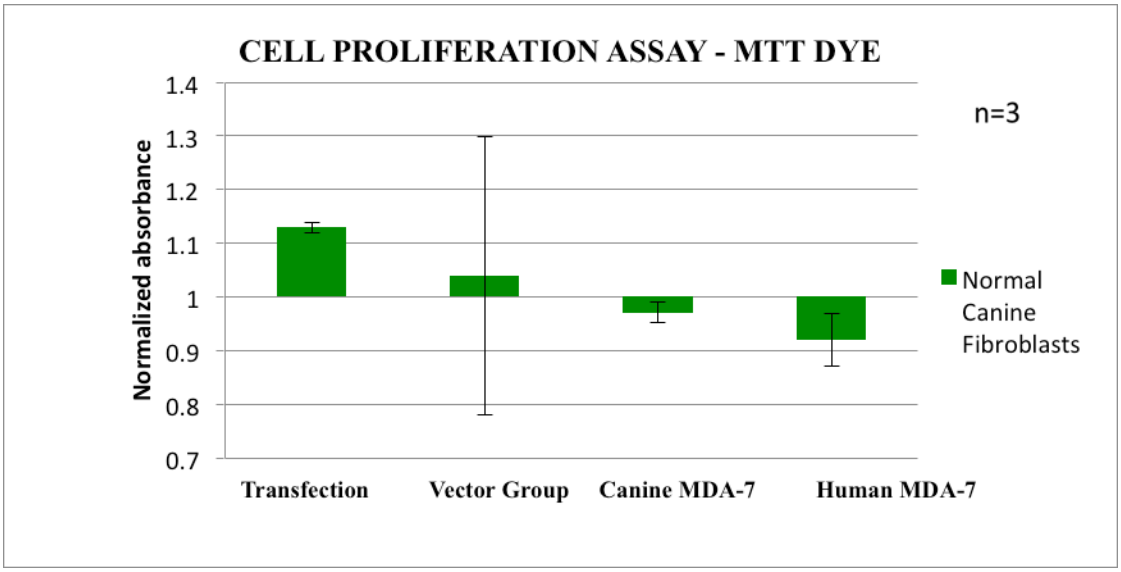
Fig. 10: Canine MDA-7 protein exhibits cytotoxic effect on cancer cells



10A. Canine and human tumor cells were trypsinized and plated in 96 well plates 24 hours before transfection with expression plasmids. 72 hours after transfection, cell viability and proliferation were measured by MTT assay. Data was normalized to the control group. The effect of different treatments was compared by one-way ANOVA, followed by the Scheffe test at a 95% level of confidence.



10B. HeLa cells were trypsinized and plated in 96 well plates 24 hours before transfection. On the next day, HeLa cells were transfected with increasing amounts of various expression plasmids. 72 hours after transfection, cells were quantified by using the CyQUANT˙NF assay kit. Effects of different treatments were compared by one-way ANOVA, followed by Scheffe test at 95% level of confidence.



10C. Normal canine fibroblasts were transfected with canine and human MDA-7 expression plasmids. Cell proliferation was measured by MTT assay and compared to the vector control group.

3. Canine MDA-7 induces apoptosis in cancer cells

HeLa cells transfected with canine MDA-7 were analyzed for the proportion of apoptotic and necrotic cells by flow cytometry. Cells were stained with annexin-V FITC and propidium iodide to detect early apoptotic and necrotic cells respectively. Transient transfection of HeLa cells with the human MDA-7 construct increased the population of apoptotic (26.9%) as well as necrotic cells (39.5%) as was seen in earlier studies (Su Z., 2005, Gupta P., 2006). Similarly, canine MDA-7 induced apoptosis and increased the proportion of apoptotic (35.4%) and necrotic cells (47.7%) compared to the percentage of apoptotic (21.4%) and necrotic cells (22.5%) seen when HeLa cells were transfected with empty vector (Fig. 11 B and A). Similar apoptosis inducing effects were observed with

canine and human MDA-7 on canine cancer cell lines. Furthermore, canine or human MDA-7 does not induce apoptosis in NCF cells (Fig. 11C).

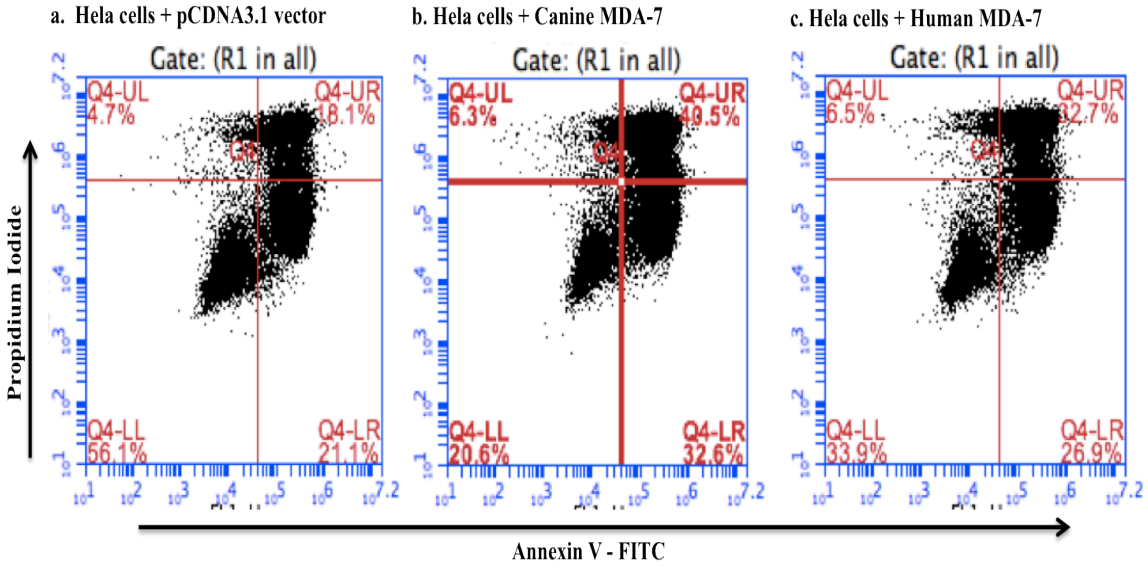


Fig. 11A: Canine MDA-7 induces apoptosis in cancer cells. HeLa cells (3 X10^5 cells/well) were plated in 6 well plates. 24 hours later the cells were transfected with 1.25 µg of expression plasmids (a, b, c). 72 hours after transfection, the cells were stained with annexin V-FITC and propidium iodide and analyzed by flow cytometry. Cells above the horizontal lines are necrotic and cells to the right of the vertical lines are apoptotic. (X-axis – apoptotic cells (Annexin-V FITC), Y-axis – necrotic cells (Propidium Iodide staining)).

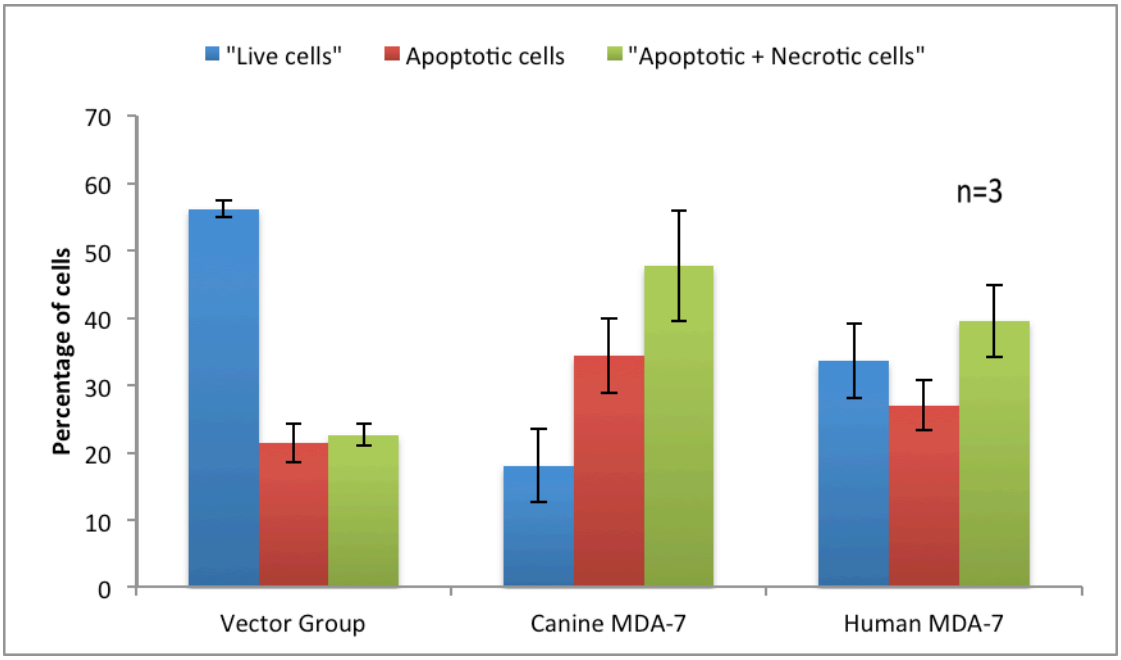
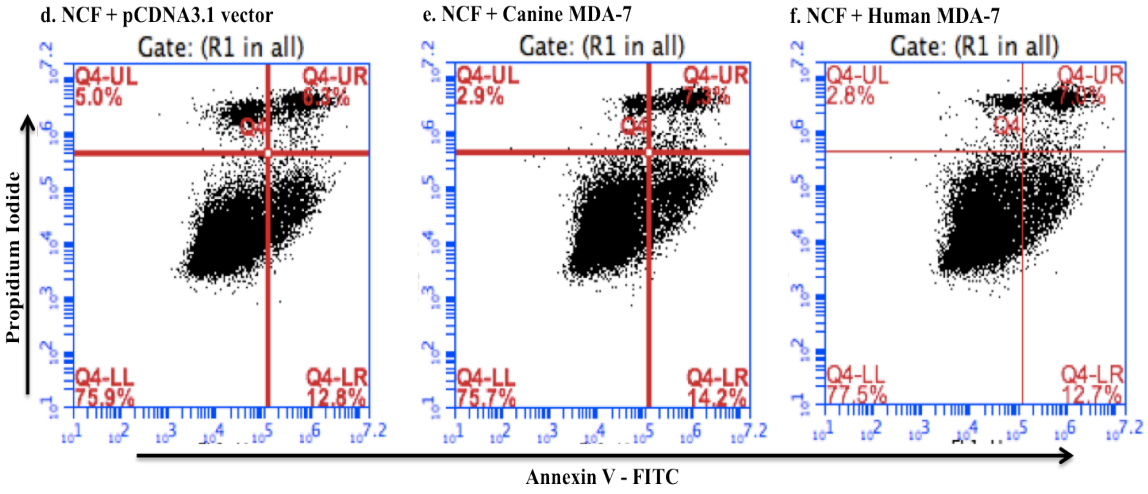


Fig. 11B: The above experiment (11A) was repeated three times and the percentage of live, apoptotic and necrotic cells in various treatment groups is depicted graphically.

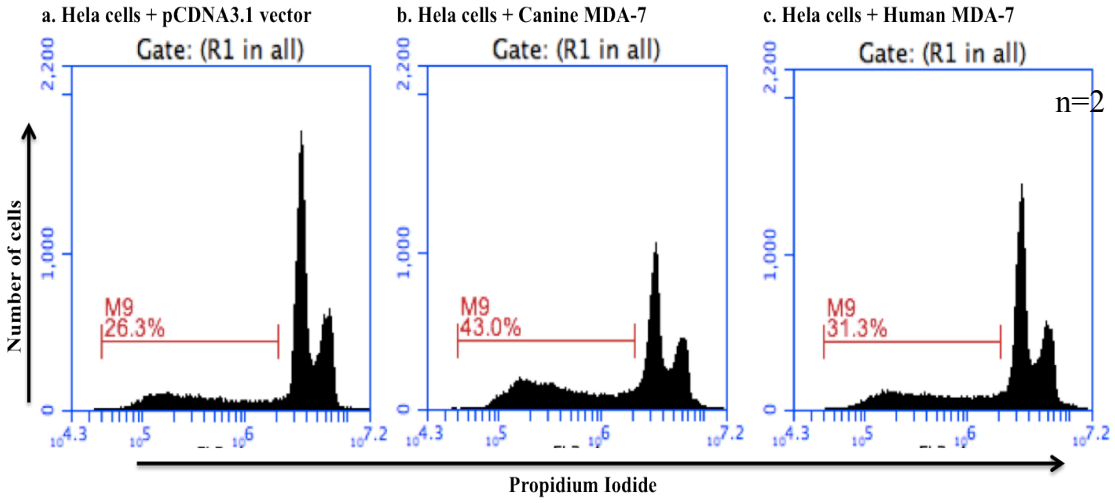


11C. Canine MDA-7 does not show any cytotoxic effects on normal cells. Normal canine fibroblasts were cultured, transfected with various expression plasmids (d, e, f) and apoptotic and necrotic cells were quantified by flow cytometry (n=2). Cells above the horizontal lines are necrotic and cells to the right of the vertical lines are apoptotic. (X-axis – apoptotic cells (Annexin-V FITC), Y-axis – necrotic cells (Propidium Iodide staining)).

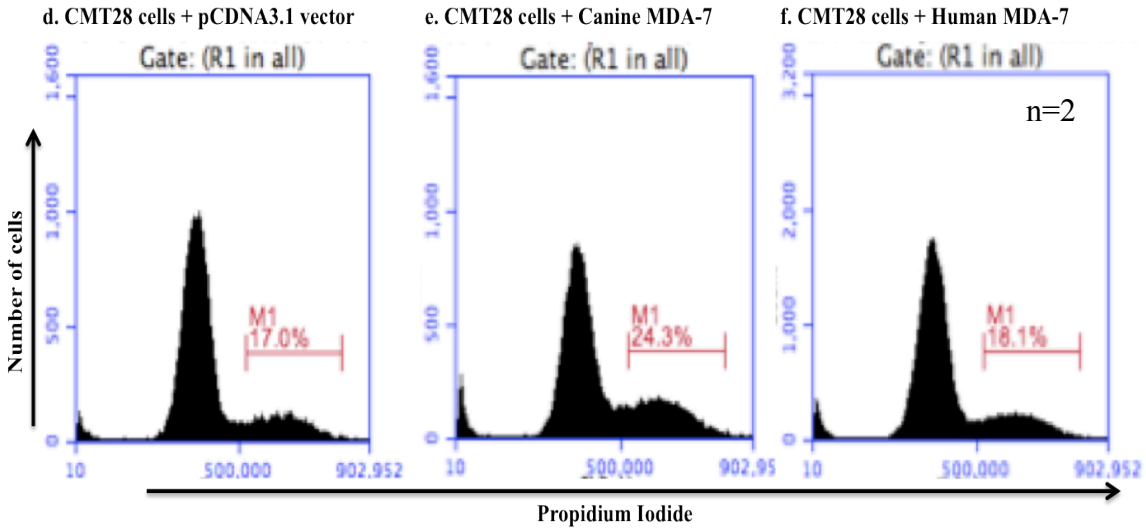
4. Canine MDA-7 causes cancer cells to accumulate in the G2/M phase

From the preceding experiments, we confirmed that canine MDA-7 protein, like human MDA-7, inhibited proliferation of cancer cells. We then sought to determine whether canine MDA-7 caused accumulation of cancer cells in the G2/M phase of the cell cycle. Cancer cells were cultured in six well plates and transiently transfected with canine and human MDA-7 protein expressing plasmids. After 72 hours, the cells were fixed and stained with propidium iodide solution for cell cycle analysis. Human MDA-7 protein increased the number of HeLa cells (31.3%) in A0, which represent the apoptotic cell population. Similarly, when HeLa cells were treated with canine MDA-7 protein, there was an increase (43.0%) in cell numbers in the A0 population when compared to vector treated HeLa cells (26.3%) (Fig.12A). However, canine and human MDA-7 do not increase the number of HeLa cells in G2/M phase. This may be due to the fact that most canine and human MDA-7 transfected HeLa cells were apoptotic (A0). When CMT-28 cells were transfected with the canine MDA-7 expression plasmid, it increased the population of CMT-28 cells in the G2/M phase (Fig. 12B). However, human MDA-7 did not increase the number of CMT-28 cells in G2/M (Fig. 12B).

Fig. 12: Canine MDA-7 causes G2/M arrest in cancer cells.



12A. HeLa cells were cultured, transfected with various expression plasmids (a, b, c). After 72 hours, cells were harvested, fixed and stained with propidium iodide for cell cycle analysis. HeLa cells accumulated in A0 (Gate M9) after transfection with canine (b) and human MDA-7 (c) expression plasmid compared to vector control group (a).



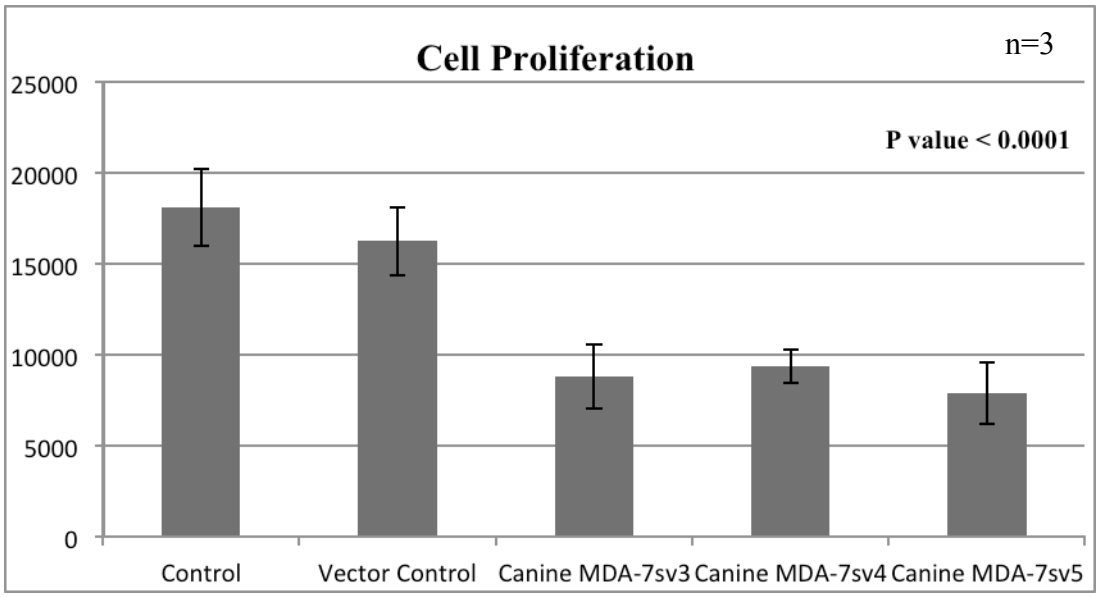
12B. Transfection with canine MDA-7 expression plasmid cause G2/M arrest in CMT28 cells. CMT28 cells were cultured, transfected with various expression plasmids (d, e, f). After 72 hours, cells were harvested, fixed and stained with propidium iodide for cell cycle analysis. CMT28 cells accumulated in G2/M phase after transfection with canine MDA-7 expression plasmid (e) compared to vector control group (d).

5. Canine MDA-7 splice variants also exhibit growth-suppressing effects on cancer cells

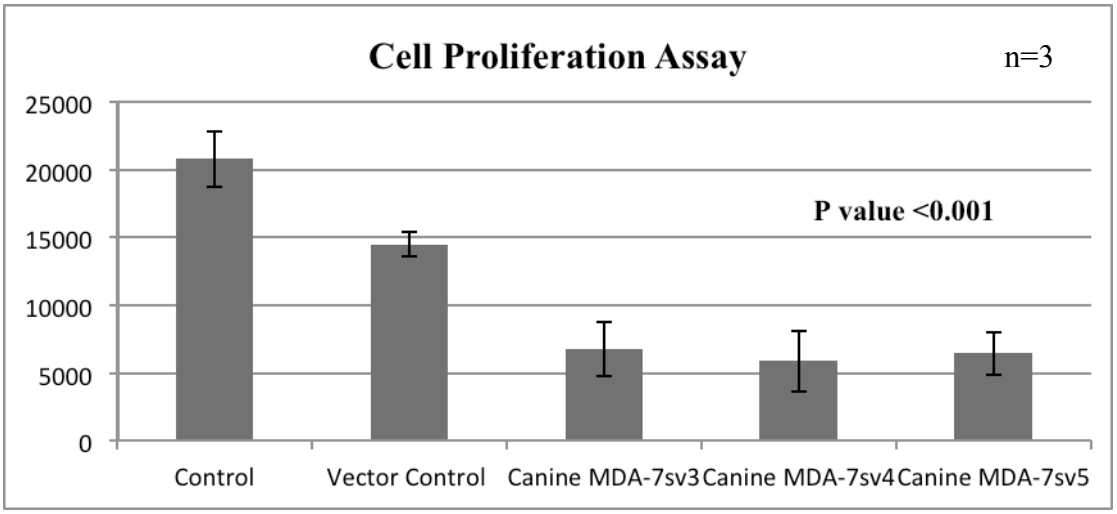
We have previously shown that five splice variants of canine mda-7 are expressed in normal canine epidermal keratinocytes (NCEKs). These splice variants encode four different isoforms of canine MDA-7 protein. Isoforms encoded by canine MDA-7sv4 and sv5 have a dissimilar C-terminus when compared to isoforms encoded by canine MDA-7sv1-3. The isoform encoded by canine MDA-7sv3 lacks 21 amino acids in the middle of protein. However, these canine MDA-7 protein isoforms have high amino-acid similarity with each other in their shared domains. In order to determine the contribution of each isoform to the overall activity of MDA-7, the open reading frames encoded by splice variants 3-5 were amplified and cloned into the pCDNA3.1+/Hygro vector. HeLa and MCF-7 cells were cultured in 96 well plates, and transiently transfected with these different constructs. After 72 hours, the proliferation of HeLa and MCF-7 cells were measured by using the CyQuant NF cell proliferation kit. Transient expression of canine MDA-7 protein isoforms suppressed the proliferation of HeLa and MCF-7 cells, and this cytotoxic effect of canine MDA-7 protein isoforms on HeLa and MCF-7 cells was statistically (<0.0001) different from effects shown by empty vector on these cells (Fig. 13A and 13B).

Fig. 13) Canine MDA-7 protein isoforms also show cytotoxic effects on human cancer cells. HeLa and MCF-7 cells were cultured in 96-well plate (triplicates) and transfected with various expression plasmids. After 72 hours, cell proliferation was quantified using CyQUANT• NF cell proliferation assay kit.

A) HeLa cells



B) MCF-7 cells



Discussion:

The development of new therapies for cancer is a long and costly process. Before an anticancer drug is approved for human use, its efficacy must first be validated in animal models. Murine models of human cancer are the most commonly used animal models, however, new drugs that show promising results in these models can fail to replicate their efficacy against human cancers (Hansen and Khanna 2004). Companion animals, especially the dog, develop a diverse range of tumors including melanoma, non-Hodgkin lymphoma (NHL), leukemia, osteosarcoma and carcinoma of prostate, mammary, lung, head and neck and bladder (Hansen and Khanna 2004; Mukaratirwa 2005; Olson 2007; Paoloni and Khanna 2007; Paoloni and Khanna 2008). These tumors are spontaneously occurring, develop in the presence of an intact immune response and share some common risk factors including age, sex, reproductive status and environment, with human cancer. Canine cancers have high histopathological similarity to human cancer (Porrello, Cardelli et al. 2006). Moreover, genetic alterations that are responsible for human cancer have also been characterized for canine cancer (Haga, Nakayama et al. 2001; Koenig, Bianco et al. 2002; DeInnocentes, Agarwal et al. 2009). In this study, we investigated the possibility of using cancer gene therapy based on canine melanoma differentiation associated gene-7/IL-24 to treat canine cancers.

Human *mda-7* is a tumor suppressor gene that has been shown to induce cell death in diverse array of human tumor cells. Previously, we identified the canine ortholog of human *mda-7* from cultured normal canine epidermal keratinocytes (NCEKs). Canine *mda-7* has a similar genomic structure, located in cluster of IL-10 cytokines, and has high amino acid similarity with human MDA-7. *In-silico* analysis of the amino acid sequence

of canine MDA-7 revealed the presence of a 28 amino-acid long signal peptide and a possible cleavage site between 28^{th} and 29^{th} amino acids. Like human MDA-7, canine MDA-7 is actively secreted from HEK-293 cells transfected with canine MDA-7 expression plasmid. Similarly, human MDA-7 has a 48 amino acid long signal peptide sequence and is actively secreted. In *in-vitro* studies, secreted human MDA-7 protein exhibits a potent bystander antitumor activity against cancer cells expressing MDA-7 receptors (Su, Lebedeva et al. 2001; Chada, Mhashilkar et al. 2004). These results are also confirmed in renal and prostate xenograft tumor models. Intratumoral injection of *Ad.mda-7* into established tumor mass on one flank, not only kills tumor cells primary injected tumor mass, but also causes growth arrest and apoptosis in tumor mass on the opposite flank (Sauane, Lebedeva et al. 2004; Su, Emdad et al. 2005; Emdad, Sarkar et al. 2006). Thus the current study demonstrates that canine MDA-7 is also a secreted protein like human MDA-7, and has the potential to show bystander antitumor activity in *in-vivo* cancer models.

Supra-physiological expression of canine MDA-7 not only causes growth suppression but also induces apoptosis in HeLa cells. Cytotoxic effects of canine MDA-7 on human tumors cells were found to be comparable to human MDA-7. Similarly, canine MDA-7 also has growth inhibitory effects on various dog tumor cells (CMT-28, CML-10, OSW and 17-71). Human MDA-7 protein also exhibits antitumor activity against canine tumor cells. These cell lines were derived from spontaneously induced canine cancers including mammary carcinoma (CMT-28), leukemia (OSW and 17-71) and melanoma (CML-10). Genetic defects in these cancer cell lines have been well characterized (Haga, Nakayama et al. 2001; Koenig, Bianco et al. 2002; DeInnocentes,

Agarwal et al. 2009). Furthermore, overexpression of canine or human MDA-7 does not suppress the growth of normal canine fibroblasts (NCF). Thus, these data suggest that canine *mda-7* is a tumor suppressor gene, and could potentially be used for development of cancer gene therapy to treat canine cancers.

We have previously shown that five spice variants of canine *mda-7* are expressed in cultured normal canine epidermal canine keratinocytes and LPS stimulated PBMCs. Human and murine MDA-7 isoforms (*mda-7*s and FISP-sp (Interleukin 4 induced secreted protein) to interact with wild type protein and inhibit its secretion and apoptotic effects (Schaefer, Venkataraman et al. 2001; Allen, Pratscher et al. 2004; Allen, Pratscher et al. 2005; Sahoo, Jung et al. 2008; Yang, Duan et al. 2011). However, unlike human and murine MDA-7 isoforms, canine MDA-7 protein isoforms have significant amino acid similarity to the wild type protein and all of them show growth inhibitory effects on tumor cells.

In summary, this study shows that the canine MDA-7 has similar biological properties to human MDA-7. Canine MDA-7 has tumor specific killing effects against a variety of canine and human tumor cells. Moreover, canine MDA-7 is actively secreted from pCMV3TAG3 canine mda-7 transfected HEK-293 cells. Thus, this data confirm the similarity between human and dog tumors especially in their susceptibility to MDA-7 induced apoptosis. Thus, we suggests that canine MDA-7 is an important candidate for cancer gene therapy to treat canine cancer, as well as a model system to better understand the molecular aspects of MDA-7 mediated cell killing.

Material and Methods:

1. Cloning and expression of canine *mda*-7sv1 splice variant

The open reading frame (ORF) encoded by canine MDA-7sv1 was amplified and cloned into the pCMT-3TAG vector (Agilent technologies) as previously described. Canine MDA-7 protein expressed from the pCMT-3TAG vector is tagged with three copies of the FLAG epitope at its C-terminus. This recombinant plasmid was transfected into HEK-293 cells (lipofectamine LTX, Invitrogen Inc). After 48 hours, 2 ml of cell culture media was collected, centrifuged at 2000g for 5 min and the resulting supernatant was used for western blot analysis. Companion total cell lysate was prepared by lysing transfected cells in RIPA buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific Inc.). 20 μg of cell extracts and 40 μl of supernatant were fractionated on 10% SDS-PAGE gels. Proteins were electroblotted to a nitrocellulose membrane. The blotted membrane was blocked with 5% non-fat dry milk for 2 hours, and then incubated with anti-flag antibody (1:1500 dilutions, Clonetech Inc.). After this, the membrane was washed three times with PBS/0.05% tween-20 and incubated with goat anti-mouse horseradish peroxidase (HRP) antibody (Thermo Fisher Scientific, Inc.) The membrane was washed again with PBS/ 0.05% tween-20 three times and then incubated with super signal west pico chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) for five minutes, dried and exposed to an X-ray film.

2. Cell transfection with canine and human MDA-7 expression plasmid

Cancer and normal cells were plated in triplicates in 96-well (8000 cells/well, MTT/viability) or 6-well plates (300,000 cells/well apoptosis/cell cycle), and after 24 hours, transfected with either plasmid expressing human MDA-7 or canine MDA-7 (lipofectamine LTX, Invitrogen, Inc). One group of cells was transfected with plasmid backbone (pCDNA3.1+/Hygro). Cells were grown for 72 hours in DMEM media at 37^0C in 5% CO_2.

3. Cell cytotoxicity assay using MTT dye

72 hours after transfection, cell proliferation and viability was determined by a cell proliferation kit (Roche Applied Science). Specifically, 10 µl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye was added to each well, and incubated at 37^0C for 4 hours. After 4 hours, the supernatant was removed and 100 µl of solubilization solution was added to each well and mixed thoroughly and incubated overnight at 37^0C. After 12 hours, absorbance was measure at 595 nm with a reference wavelength of 650 nm with a spectophotometer.

4. Cell viability assay with CyQUANT® NF Cell Proliferation Assay Kit

Cancer cells were plated and treated as above. 72 hours after transfection, the effects of canine and human MDA-7 on cancer cell viability were determined by CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen, Inc.). Specifically, media was removed after 72 hours, replaced with 100 µl of 1X dye binding solution and incubated for 45 minutes at 37^0C. 1X dye binding solultion contains a green fluorescent dye, CyQUANT® GR dye, which specifically binds to cellular DNA. A microplate

fluorescence reader was used to measure the fluorescence intensity (excitation-485 nm and emission-530 nm).

5. Apoptosis Assay

Cells were harvested from 6-well plates after 72 hours of transfection, and assayed using Apoalert® annexin-V kit (Clonetech) according to the manufacturer's instruction. Briefly, cells were resuspended in 500 µl of binding buffer, and stained with 5 µl of fluorescein isothiocyanate (FITC)-labeled annexin V and 10 µl of propidium iodide (PI) for 15 minutes. After 15 minutes, stained cells were assayed by flow cytometer (Accuri C6).

6. Cell cycle analysis

Cells were harvested from 6 well plates after 72 hours of transfection and washed once with PBS and resuspended in 1ml of PBS. Cells were then fixed by adding 3ml of 70% ice-cold ethanol. Fixed cells were incubated on ice for 30 min. Cells were centrifuged at 3000g for 2 min and resuspended in 100 µl of flow buffer (1% BSA in PBS). After this, 900 µl of stain solution (33 µl of propidium iodide (4.5mg/ml), 400 µl of RNAse A (4.5mg/ml), and 9.567 ml of nuclease free water) were added to 100 µl of cells and incubated for 30 min at room temperature. Stained cells were then analyzed by flow cytometer (Accuri C6).

7. Statistical analysis

All the experiments were done in triplicates and repeated three times. Data were represented as mean ± SD. Statistical comparisons between groups were performed

using one-way ANOVA, which was further analyzed using Post-Hoc analysis tests (Scheffe and Tukey test). All of the data is compared at 95% confidence levels of significance (p-value, 0.05).

IV. A truncated canine IL-20R2 protein inhibits the bystander antitumor activity of canine melanoma differentiation associated gene-7/IL-24 in canine cancer cells

Introduction

Human melanoma differentiation associated gene-7 (*mda-7*) is an important candidate for cancer gene therapy because of its wide range of antitumor properties, which include growth suppression, apoptosis induction, inhibition of invasiveness, migration and angiogenesis in various types of tumor cells *in-vitro,* as well as in *in-vivo* (Jiang, Lin et al. 1995; Jiang, Su et al. 1996; Su, Madireddi et al. 1998; Madireddi, Su et al. 2000; Huang, Madireddi et al. 2001; Fisher, Gopalkrishnan et al. 2003; Emdad, Lebedeva et al. 2007). Human MDA-7 belongs to the four-helix bundle family of cytokines, has 19% amino acid identity to interleukin-10 (IL-10) and has a conserved IL-10 signature motif (Chaiken and Williams 1996; Huang, Madireddi et al. 2001; Pestka, Krause et al. 2004; Zdanov 2006). The human *mda-7* gene is found within a cluster of IL-10 family cytokines (IL-19, IL-10 and IL-20) on chromosome 1. It has a 48 amino acid long signal peptide that directs secretion of the MDA-7 protein (Nielsen, Kaestel et al. 1999; Nakai 2000). MDA-7 has a highly restricted expression profile and is expressed in cells and tissues related to the immune system (Huang, Madireddi et al. 2001; Caudell, Mumm et al. 2002; Garn, Schmidt et al. 2002; Wolk, Kunz et al. 2002; Poindexter, Walch et al. 2005). Based on these observations (protein structure, expression profile, chromosomal location and translational regulation), the Human Gene Organization

(HUGO) renamed human *mda-7* as interleukin-24 (IL-24) (Huang, Madireddi et al. 2001; Pestka, Krause et al. 2004).

Jiang and coworkers (1995, 1996) first discovered the *mda-7* gene by subtraction hybridization in cDNA libraries of interferon-β (IFN-β) and mezerein treated human melanoma cells (HO-1). Expression of human MDA-7 at supra-physiological levels from either plasmid or replication-deficient adenoviral vectors inhibits tumor growth and induces apoptosis in a wide range of cancer cells *in-vitro* (Jiang, Lin et al. 1995; Jiang, Su et al. 1996; Su, Madireddi et al. 1998; Saeki, Mhashilkar et al. 2000; Huang, Madireddi et al. 2001; Su, Lebedeva et al. 2001; Aggarwal, Takada et al. 2004; Wang, Peng et al. 2008). Tumor cells with diverse genetic make ups (mutations in different molecular pathways like p53 or p21) are susceptible to MDA-7 induced apoptosis (Su, Lebedeva et al. 2003), as are tumor cells derived from various cancer types including malignant gliomas, melanomas, carcinomas of breast, lung, ovary and prostate (Su, Madireddi et al. 1998; Huang, Madireddi et al. 2001; Saeki, Mhashilkar et al. 2002; Chada, Sutton et al. 2004; Emdad, Lebedeva et al. 2007; Gopalan, Shanker et al. 2007; Sauane, Su et al. 2008; Yacoub, Park et al. 2008; Yan, Zhang et al. 2010). Several studies have confirmed the anticancer properties of human MDA-7 in xenograft nude mouse models of human cancers. In these studies, human MDA-7 not only inhibits tumor growth but also shows potent anti-invasive, anti-migration and anti-angiogenic effects (Madireddi, Su et al. 2000; Saeki, Mhashilkar et al. 2002; Su, Emdad et al. 2005).

Once expressed in tumor cells, MDA-7 regulates and alters the activity of different molecular pathways. MDA-7 activates p38 mitogen-activated protein kinase (MAPK), which then increases the expression of pro-apoptotic and growth arrest and

DNA damage (GADD) inducible genes (Sarkar, Su et al. 2002; Gupta, Walter et al. 2006). MDA-7 also causes endoplasmic stress in cancer cells, which in turn results in the unfolded protein response (UPR) and causes cancer cell death via a Bip/GRP78 dependent pathway (Sieger, Mhashilkar et al. 2004; Gupta, Walter et al. 2006).

Several studies have shown that human MDA-7 not only kills primarily transduced cancer cells, but also exhibits a potent "bystander antitumor activity" on cancer cells that are distant from the site of injection (Madireddi, Su et al. 2000; Saeki, Mhashilkar et al. 2002; Su, Emdad et al. 2005). Intratumoral injection of an adenovirus expressing MDA-7, in murine xenograft models, killed the primary (injected) tumors on one flank and inhibited the growth of tumor masses on the other flank (Sarkar, Su et al. 2005). A similar effect was observed by Fisher and coworkers when they evaluated the antitumor properties of human MDA-7 in a phase I trial using *Ad.mda-7* on solid tumors (Fisher, Gopalkrishnan et al. 2003; Cunningham, Chada et al. 2005; Tong, Nemunaitis et al. 2005; Emdad, Lebedeva et al. 2007). Intratumoral injection of *Ad.mda-7* lead to transduction of only 10-30% of the tumor cells, however, upon analysis 70% of the tumor cells in the tumor mass showed an apoptotic phenotype. The bystander antitumor activity of MDA-7 is due to the activation of its receptors, and cancer cells expressing the MDA-7 receptors are susceptible to exogenous MDA-7 protein (Su, Lebedeva et al. 2001).

After secretion, MDA-7 binds to two different heterodimeric receptor complexes, which are assembled from three different subunits, namely IL-20R alpha (or IL-20R1), IL-22R alpha (or IL-22R1) and IL-20R beta (or IL-20R2) (Dumoutier, Leemans et al. 2001; Wang, Tan et al. 2002). One of the two alpha subunits binds to the beta subunit to form a functional receptor complex (IL-20R1/IL-20R2 and IL-22R1/IL-20R2) (Chai,

Nichols et al. 1997; Catlett-Falcone, Landowski et al. 1999; Nielsen, Kaestel et al. 1999). Binding of MDA-7 to its receptors activates Janus Kinase (JAK) which in-turn phosphorylates Signal Transducers and Activators of Transcription protein (STAT3), which then translocate to the nucleus and causes up-regulation of BAX (pro-apoptotic) expression (Chada, Mhashilkar et al. 2004).

We previously identified a canine ortholog of human *mda-7* and showed that its mRNA was expressed in lipopolysaacharide stimulated PBMCs and cultured normal canine epidermal keratinocytes (NCEKs). In NCEKs, canine *mda-7* pre-mRNA undergoes differential splicing to produce five splice variants, which encode four isoforms of canine MDA-7 protein. Expression of all 4 isoforms from plasmid vectors caused growth suppression and apoptosis induction in canine as well as in human cancer cells. Like human MDA-7, canine MDA-7 is also a secreted protein and has a 28 amino-acid signal peptide.

In this study, we investigated the bystander antitumor activity of canine MDA-7 against canine and human tumor cells and characterized the expression profiles of the canine IL-20R1, IL-22R1 and IL-20R2 subunits.

Results:

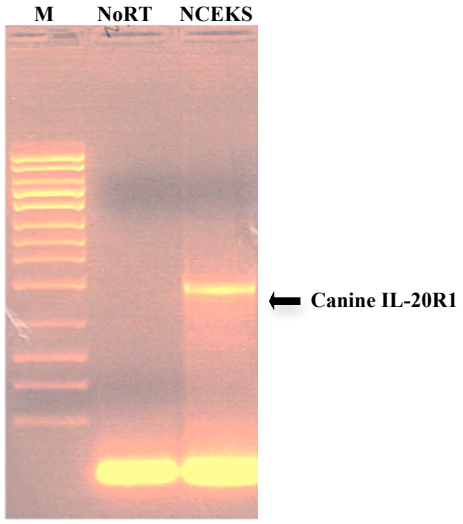
1. Alpha subunits of canine MDA-7 receptors are expressed in normal canine epidermal keratinocytes (NCEKs)

We have previously identified the canine ortholog of the human *mda-7* gene, and named it canine *mda-7* based on its high nucleotide and amino-acid identity to human *mda-7*. In the current study, the nature and role of canine MDA-7 receptors in the bystander antitumor activity of canine MDA-7 were investigated. Predicted mRNA sequences for the canine ortholog of human IL-20R1 (XM_541119) and IL-22R1 subunits (XM_850020) were retrieved from the NCBI database. Human keratinocytes have been shown to express both MDA-7 and its receptor subunits (Poindexter, Williams et al. 2010). In order to identify the canine transcripts, RNA from cultured NCEKs was amplified by RT-PCR. The mRNA sequences for the canine IL-20R1 (Fig. 14A) and IL-22R1 (Fig. 14B) subunits were successfully amplified cloned and sequenced. The open reading frame (ORF) encoded by these sequenced mRNAs were identified, and translated into amino acid sequences *in-silico*. The canine IL-20R1 and IL-22R1 proteins contain 562 and 579 amino acids respectively (Fig. 14C and 14D). Canine IL-20R1 and IL-22R1 proteins were then aligned and compared to the amino acid sequences of human IL-20R1 and IL-22R1 subunits respectively (Fig.14C and 14D). The canine IL-20R1 and IL-22R1 subunits have high amino acids similarity, 73% and 78% respectively, to their respective human orthologs (Fig.14C and 14D).

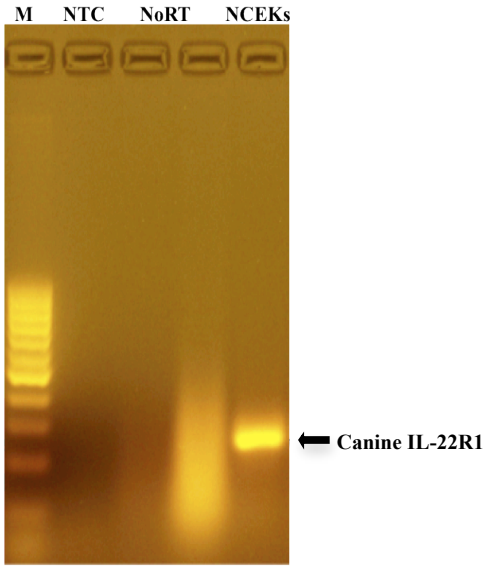
Based on the high degree of protein similarity, an anti-human IL-20R1 antibody was used to detect the expression of canine IL-20R1 protein. This antibody recognizes a

conserved epitope (human 159-175, EKSISIVLTAPEKWKRS), and can detect both canine and human IL-20R1 protein. MCF-7 cell lysate was used as a positive control. Protein bands representing the canine and human IL-20R1 subunit were successfully detected in cell lysates of MCF7 and NCEKs (Fig. 14E). LPS stimulation (100ng/ml) of cultured NCEKs increased the expression of canine IL-20R1 protein approximately 1.15 fold (Fig. 14E). Density of protein bands was determined using ImageJ software (National Institute of Health). A canine IL-20R1 protein band was also detected in cell lysate prepared from different dog tissues (kidney, ovary and uterus) (data not shown). These data suggest that canine IL-20R1 and IL-22R1 subunits are expressed in different dog tissues, and show considerable amino acid similarity with human IL-20R1 and IL-22R1 subunits.

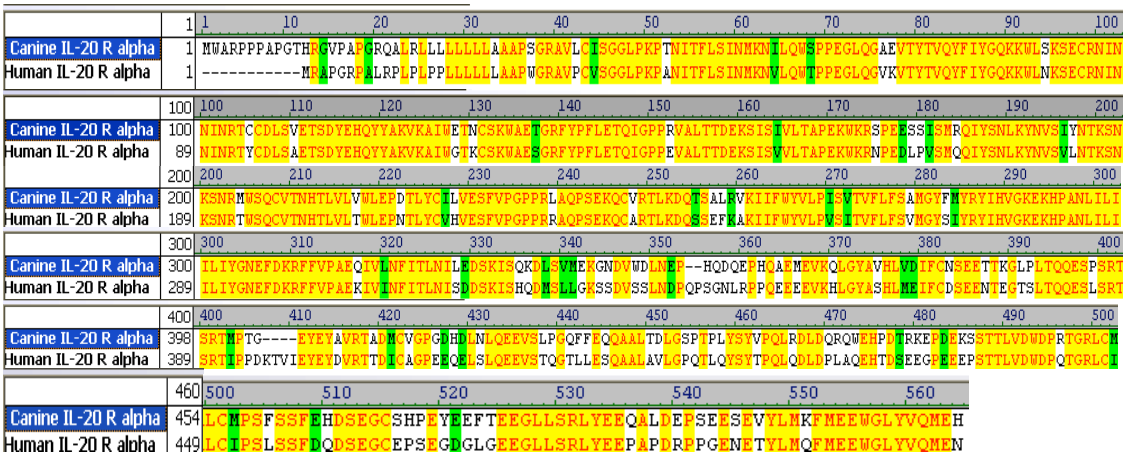
Fig 14: Amplification of canine IL-20R1 (A) and IL-22R1 (B) subunits. RNA was isolated from NCEKs and canine IL-20R1 and IL-22R1 mRNAs were amplified by reverse transcription – polymerase chain reaction (RT-PCR).



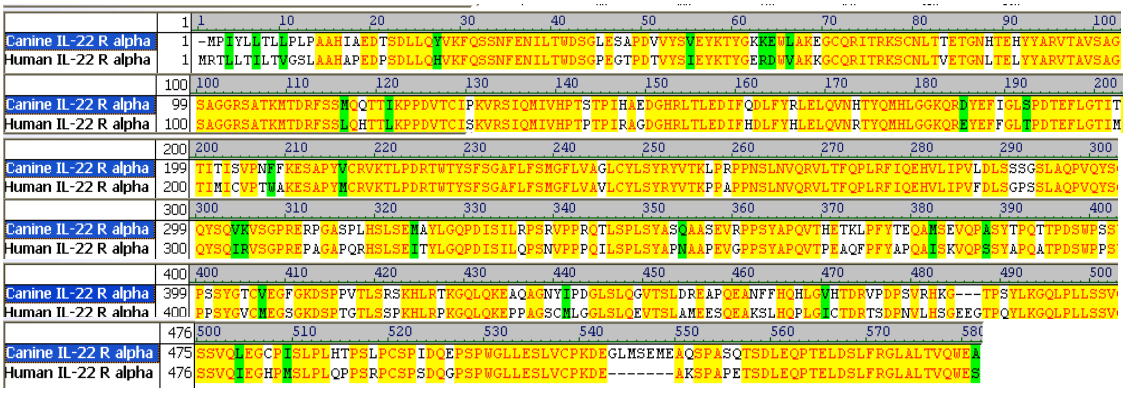
14A. Canine IL-20R1 subunit



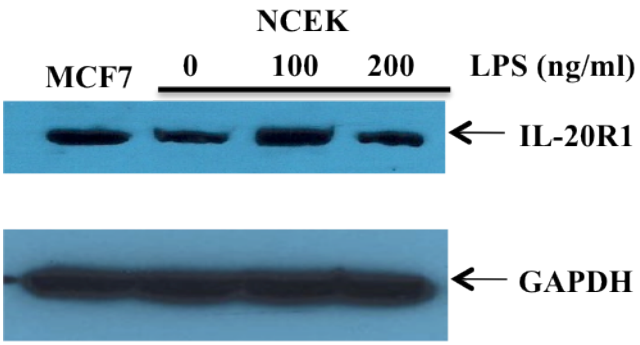
14B. Canine IL-22R1 subunit



14C. Sequence alignment of canine and human IL-20R1 subunits. Canine IL-20R1 mRNA sequence was translated in-silico using vector NTI 10.0 software and aligned with amino acid sequence of human IL-20R1 subunit. Human and canine IL-20R1 subunits have 73% amino acid similarity.



14D. Sequence alignment of canine and human IL-22R1 subunits. Sequence alignment of canine and human IL-22R1 subunit. Canine IL-22R1 mRNA sequence was translated in-silico using vector NTI 10.0 software and aligned with amino acid sequence of human IL-22R1 subunit. Human and canine IL-22R1 subunits have 78% amino acid similarity.



14E. Western Blot analysis of canine IL-20R1 subunit expression. Cell lysates were prepared from NCEKs and MCF-7 cells, and probed with anti IL-20R1 to detect canine IL-20R1 proteins. After successful detection of canine and human IL-20R1 subunits, anti IL-20R1 antibody is striped from the nitrocellulose membrane and reprobed with anti-GAPDH antibodies. The intensity of protein bands were determined using ImageJ software (NIH) and normalized to GAPDH.

2. Canine tumor cells express IL-20R1 and IL-22R1 subunit

After identifying canine MDA-7 receptor subunits, the expression profile of these receptor subunits was evaluated in various canine tumor cells. Two primer sets were designed to amplify the canine IL-20R1 and IL-22R1 subunit mRNAs. The specificity of the primer sets was confirmed by sequencing amplified products. RNA was isolated from canine tumor cell lines (CMT-28, CMT-27, CMT-12, OSW, 17-71 and CML-10) and was analyzed for expression of canine IL-20R1 and IL-22R1 subunits. Two dog tumor cell lines (CMT-12 and 17-71) expressed both canine IL-20R1 and IL-22R1 subunits. Two other tumor cell lines (CML-10 and OSW) only expressed the canine IL-20R1 mRNA, while CMT-28 cells expressed only the canine IL-22R1 subunit. However, the expression of 20R1 subunits could not be detected at all in CMT-27 cells (Fig. 15). Thus, this data suggests that if a functional R2 subunit is expressed in most canine cancer cells, these

receptors might then mediate bystander antitumor activity of canine or human MDA-7. CMT27 however, is unlikely to show the bystander effect due to the lack of any expression of either of the R1 subunits.

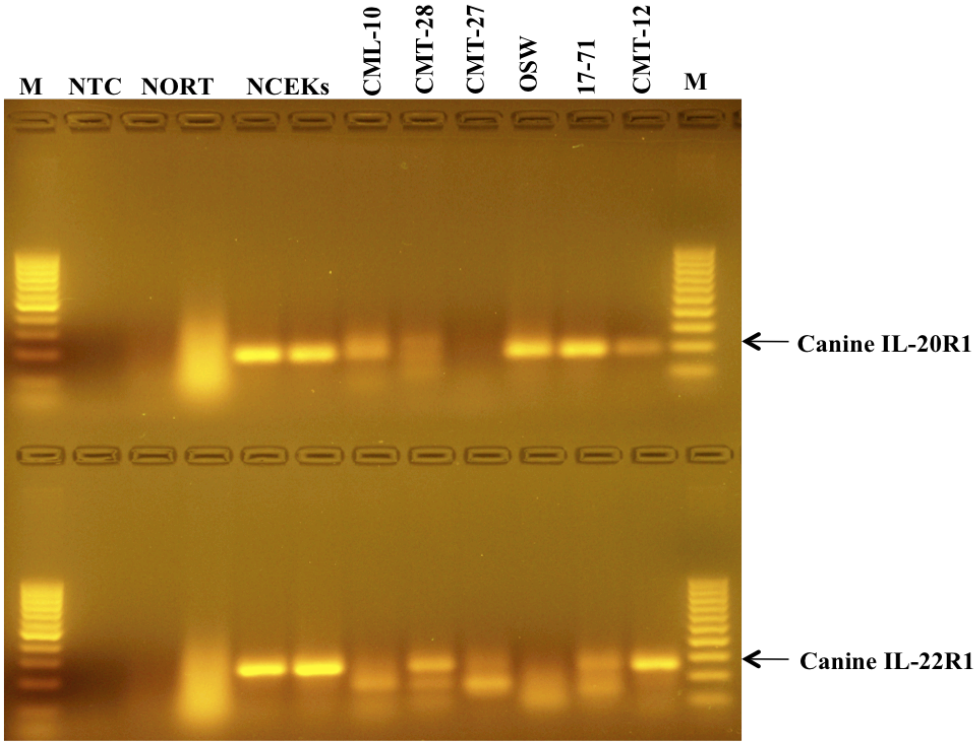


Fig. 15: Canine IL-20R1 and IL-22R1 subunits are expressed in canine cancer cells. RNA was isolated from different canine cancer cell lines, and canine IL-20R1 and canine IL-22R1 subunits were amplified by RT-PCR.

3. NCEKs express a truncated canine IL-20R2 subunit

IL-20R2 is the common beta subunit between the two receptor complexes and is required for formation of functional MDA-7 receptors. Therefore, we attempted to identify the mRNA sequence of the canine IL-20R2 subunit. No annotated or predicted mRNA sequence was available for the canine IL-20R2 subunit in the NCBI database. In order to identify the canine ortholog of IL-20R2, the human IL-20R2 subunit sequence was BLASTed against the dog genome. A genomic locus representing the canine IL-20R2 gene was identified, and nucleotide sequence for this locus was retrieved. The retrieved genomic sequence was analyzed by gene predicting software (GENESCAN and FGANESH), to identify any exonic sequences. A short mRNA sequence of 600 nucleotides was identified, which, when aligned to human IL-20R2 sequence had considerable nucleotide similarity. The central region of this transcript was amplified by RT-PCR from NCEK cells, demonstrating that the canine IL-20R2 gene was being transcribed. In order to determine the complete mRNA sequence of the canine IL-20R2 subunit the 5' end was amplified by RNA ligase mediated-Rapid amplification of cDNA ends (RLM-RACE) and the 3' end was amplified by classic RACE, based on the predicted mRNA sequence (Fig. 16 A, B, C). By combining the data from these approaches an mRNA of 829 base pairs was identified that had a stop codon and poly-A tail (Fig. 16D). *In-silico* comparison to human IL-20R2 revealed that this mRNA encodes a truncated canine IL-20R2 protein (Fig. 16D and E) that represents the extracellular domain of canine IL-20R2 subunit (Fig. 16 E). The truncated canine IL-20R2 protein has high amino acid (78%) similarity to human IL-20R2 in the portion that is present. Representative sequences encoding for the transmembrane and intracellular domains

were not present in this predicted protein (Fig. 16E). No alternative mRNA that might encode a longer version of canine IL-20R2 mRNA was identified.

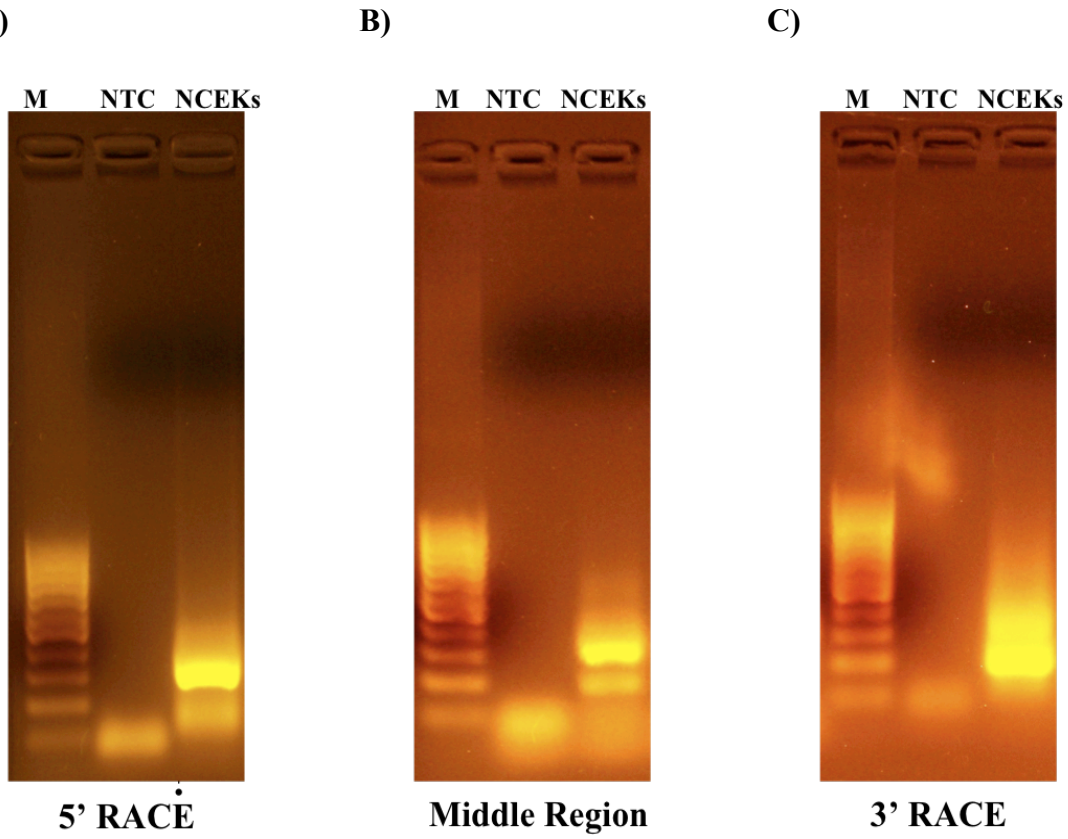
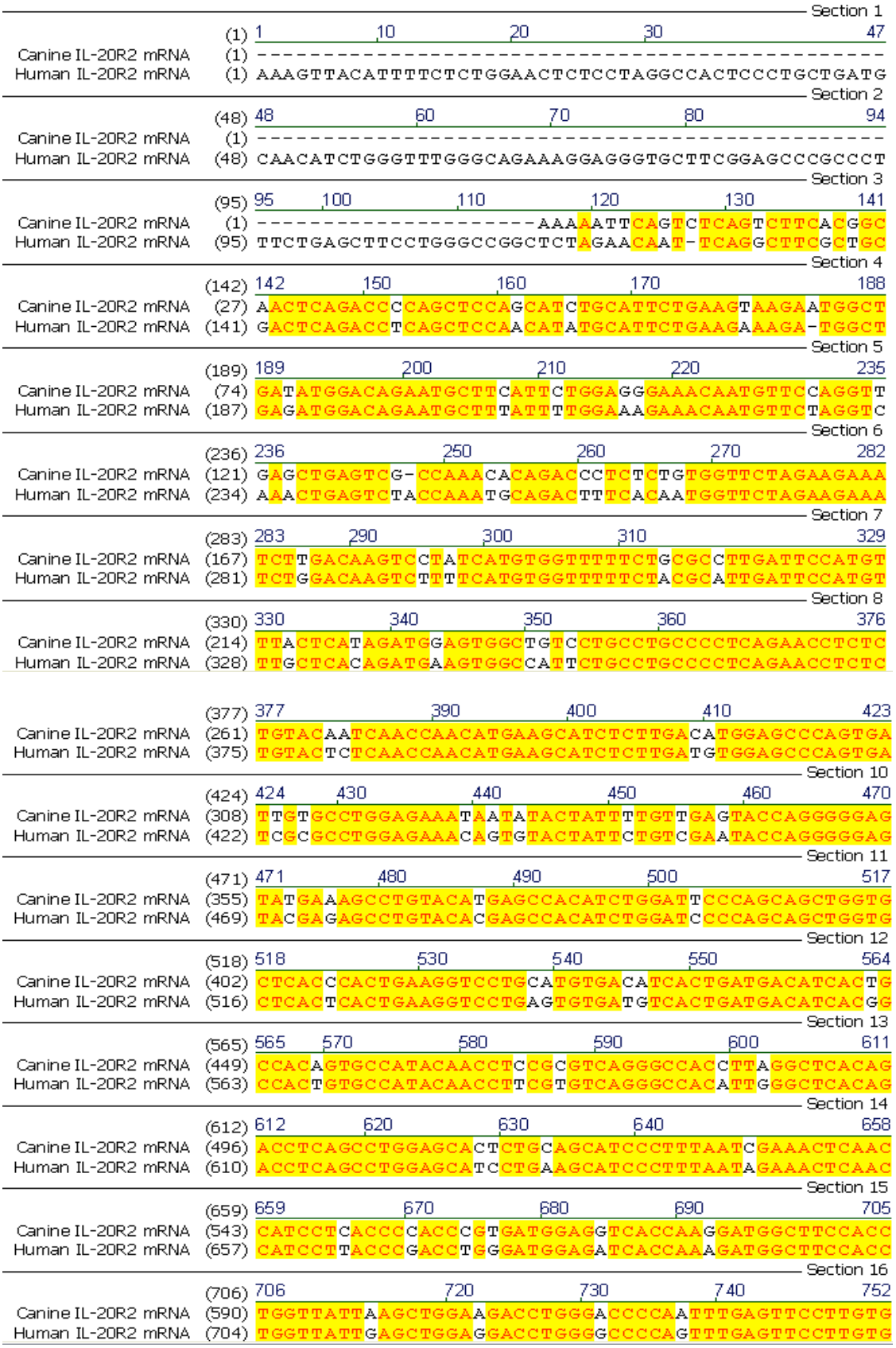
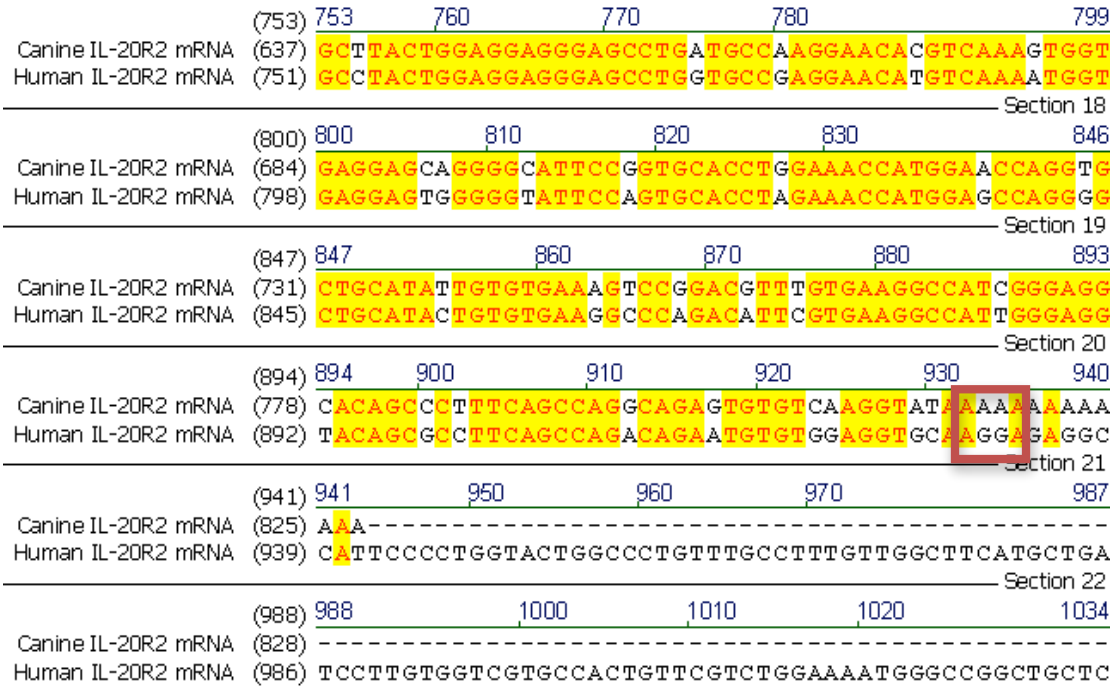


Fig. 16: RT-PCR amplification of the canine IL-20R2 subunit transcript. Canine IL-20R2 mRNA sequence was predicted using GENESCAN and FGANESH. Multiple primer sets were designed to amplify and sequence the canine IL-20R2 mRNA sequence from NCEKs. RLM-RACE was used to identify the 5' end (A), and 3' end (C) while standard RT-PCR was used to amplify the central portion of the transcript (B). (M – Marker, NTC - No template control, NCEKs - Normal canine epidermal keratinocytes.


Section 1
(1) 1 10 20 30 47
Canine IL-20R2 mRNA (1) ----------
Human IL-20R2 mRNA (1) AAAGTTACATTTTCTCTGGAACTCTCCTAGGCCACTCCCTGCTGATG
Section 2
(48) 48 60 70 80 94
Canine IL-20R2 mRNA (1) ----------
Human IL-20R2 mRNA (48) CAACATCTGGGTTTGGGCAGAAAGGAGGGTGCTTCGGAGCCCGCCCT
Section 3
(95) 95 100 110 120 130 141
Canine IL-20R2 mRNA (1) ----------AAAAATTCAGTCTCAGTCTTCACGGC
Human IL-20R2 mRNA (95) TTCTGAGCTTCCTGGGCCGGCTCTAGAACAAT-TCAGGCTTCGCTGC
Section 4
(142) 142 150 160 170 188
Canine IL-20R2 mRNA (27) AACTCAGACCCCAGCTCCAGCATCTGCATTCTGAAGTAAGAATGGCT
Human IL-20R2 mRNA (141) GACTCAGACCTCAGCTCCAACATATGCATTCTGAAGAAAGA-TGGCT
Section 5
(189) 189 200 210 220 235
Canine IL-20R2 mRNA (74) GATATGGACAGAATGCTTCATTCTGGAGGGAAACAATGTTCCAGGTT
Human IL-20R2 mRNA (187) GAGATGGACAGAATGCTTTATTTTGGAAAGAAACAATGTTCTAGGTC
Section 6
(236) 236 250 260 270 282
Canine IL-20R2 mRNA (121) GAGCTGAGTCG-CCAAACACAGACCCTCTCTGTGGTTCTAGAAGAAA
Human IL-20R2 mRNA (234) AAACTGAGTCTACCAAATGCAGACTTTCACAATGGTTCTAGAAGAAA
Section 7
(283) 283 290 300 310 329
Canine IL-20R2 mRNA (167) TCTTGACAAGTCCTATCATGTGGTTTTTCTGCGCCTTGATTCCATGT
Human IL-20R2 mRNA (281) TCTGGACAAGTCTTTTCATGTGGTTTTTCTACGCATTGATTCCATGT
Section 8
(330) 330 340 350 360 376
Canine IL-20R2 mRNA (214) TTACTCATAGATGGAGTGGCTGTCCTGCCTGCCCCTCAGAACCTCTC
Human IL-20R2 mRNA (328) TTGCTCACAGATGAAGTGGCCATTCTGCCTGCCCCTCAGAACCTCTC
(377) 377 390 400 410 423
Canine IL-20R2 mRNA (261) TGTACAATCAACCAACATGAAGCATCTCTTGACATGGAGCCCAGTGA
Human IL-20R2 mRNA (375) TGTACTCTCAACCAACATGAAGCATCTCTTGATGTGGAGCCCAGTGA
Section 10
(424) 424 430 440 450 460 470
Canine IL-20R2 mRNA (308) TTGTGCCTGGAGAAATAATATACTATTTTGTTGAGTACCAGGGGGAG
Human IL-20R2 mRNA (422) TCGCGCCTGGAGAAACAGTGTACTATTCTGTCGAATACCAGGGGGAG
Section 11
(471) 471 480 490 500 517
Canine IL-20R2 mRNA (355) TATGAAAGCCTGTACATGAGCCACATCTGGATTCCCAGCAGCTGGTG
Human IL-20R2 mRNA (469) TACGAGAGCCTGTACACGAGCCACATCTGGATCCCCAGCAGCTGGTG
Section 12
(518) 518 530 540 550 564
Canine IL-20R2 mRNA (402) CTCACCCACTGAAGGTCCTGCATGTGACATCACTGATGACATCACTG
Human IL-20R2 mRNA (516) CTCACTCACTGAAGGTCCTGAGTGTGATGTCACTGATGACATCACGG
Section 13
(565) 565 570 580 590 600 611
Canine IL-20R2 mRNA (449) CCACAGTGCCATACAACCTCCGCGTCAGGGCCACCTTAGGCTCACAG
Human IL-20R2 mRNA (563) CCACTGTGCCATACAACCTTCGTGTCAGGGCCACATTGGGCTCACAG
Section 14
(612) 612 620 630 640 658
Canine IL-20R2 mRNA (496) ACCTCAGCCTGGAGCACTCTGCAGCATCCCTTTAATCGAAACTCAAC
Human IL-20R2 mRNA (610) ACCTCAGCCTGGAGCATCCTGAAGCATCCCTTTAATAGAAACTCAAC
Section 15
(659) 659 670 680 690 705
Canine IL-20R2 mRNA (543) CATCCTCACCCCACCCGTGATGGAGGTCACCAAGGATGGCTTCCACC
Human IL-20R2 mRNA (657) CATCCTTACCCGACCTGGGATGGAGATCACCAAAGATGGCTTCCACC
Section 16
(706) 706 720 730 740 752
Canine IL-20R2 mRNA (590) TGGTTATTAAGCTGGAAGACCTGGGACCCCAATTTGAGTTCCTTGTG
Human IL-20R2 mRNA (704) TGGTTATTGAGCTGGAGGACCTGGGGCCCCAGTTTGAGTTCCTTGTG



16D. Sequence comparison of canine and human IL-20R2 subunit transcripts. Sequenced canine IL-20R2 was aligned using Vector NTI10.0 against human IL-20R2 sequence. The premature termination codon in the canine sequence is identified by a box.

16E. Sequence comparison of the truncated canine and full-length human IL-20R2 subunits. Protein domains, based on the human IL-20R2, are shown below the amino acide sequence. Truncated IL-20R2 protein sequence have high amino acid similarity (78%) to human IL-20R2 mRNA.

4. Secreted canine MDA-7 protein can bind to human MDA-7 receptors and inhibits proliferation of Hela cells

We have previously shown that over-expression of canine MDA-7 protein from a plasmid vector induces apoptosis and cell cycle arrest in both human and canine cancer cells. Canine MDA-7 is also actively secreted and has a 28 amino-acid amino terminal signal peptide sequence and a possible cleavage site between 28th and 29th amino acid. In order to evaluate the bystander antitumor activity of canine MDA-7, stable cell clones based on HEK-293 cells that constitutively produced either canine or human MDA-7 protein at high-levels were established. Expression of canine MDA-7 protein was

confirmed by western blot analysis using anti-FLAG antibody from these stable cell clones. Canine MDA-7 protein was detected in both cell lysates and cell culture media (Fig. 17A).

Secreted human MDA-7 protein binds to its receptors and inhibits the growth of tumor cells. HeLa cells have been shown to be susceptible to receptor mediated human MDA-7 induced growth suppression and apoptosis (Su, Emdad et al. 2005). This "bystander antitumor activity" was next assessed for canine MDA-7. HeLa cells were stained with carboxyfluorescein diacetate, succinimidyl ester (CFSE) dye to monitor their cell division. After 24 hours, CFSE stained HeLa cells were treated with HEK-293 conditioned media containing either canine or human MDA-7 for 72 hours. HEK-293 conditioned media containing no canine or human MDA-7 protein was used as a negative control. After 72 hours, CFSE stained hela cells were analyzed by flow cytometry. Treatment of HeLa cells with canine or human MDA-7 proteins inhibited their proliferation as indicated by higher CFSE concentrations (Fig. 17B). Fluorescence of CFSE stained cells is reduced to half after each cell division. The number of cell divisions required to reach the final mean fluorescence intensity were determined. When CFSE stained hela cells were treated with control media, they underwent approximately 7 cell divisions. Cells treated with conditioned media containing canine or human MDA-7 had slower growth rates and underwent only 4 cell divisions during the same time interval. Thus, this data suggest that canine MDA-7 protein is successfully secreted, and can also interact with human MDA-7 receptors and cause growth suppression of human cancer cells.

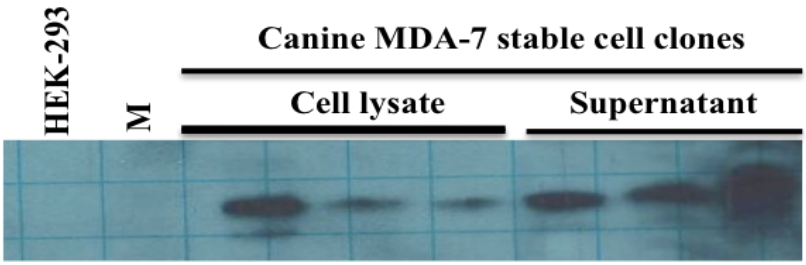
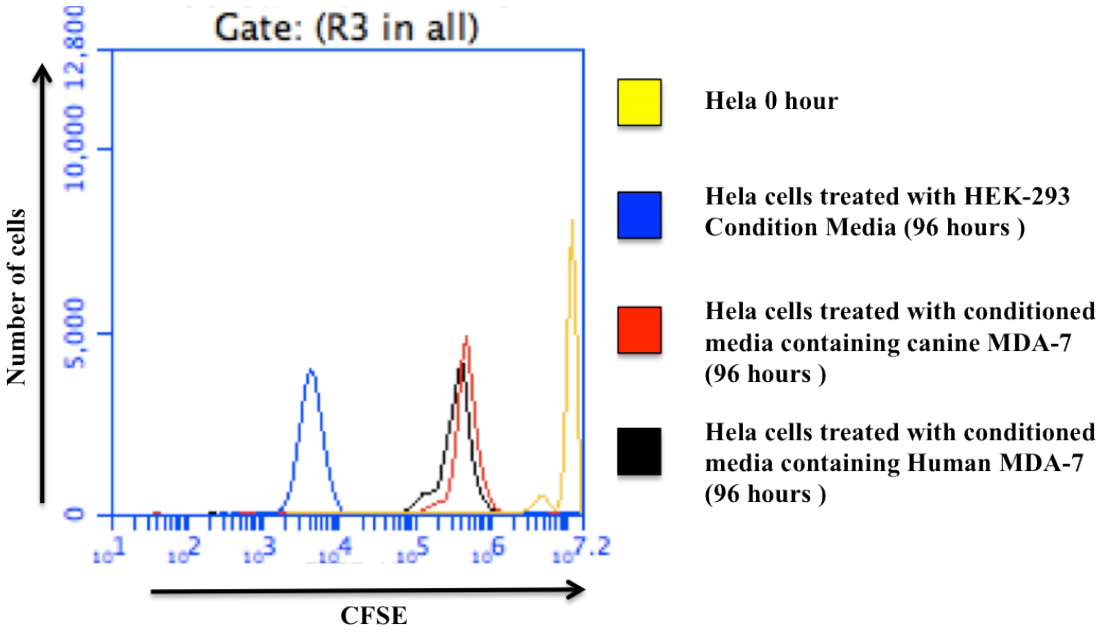


Fig. 17A. Stable cell clones expressing canine MDA-7 protein were established. Cell lysate and companion supernatant was then probed for canine MDA-7 expression by Western Blot analysis using anti-FLAG antibody.

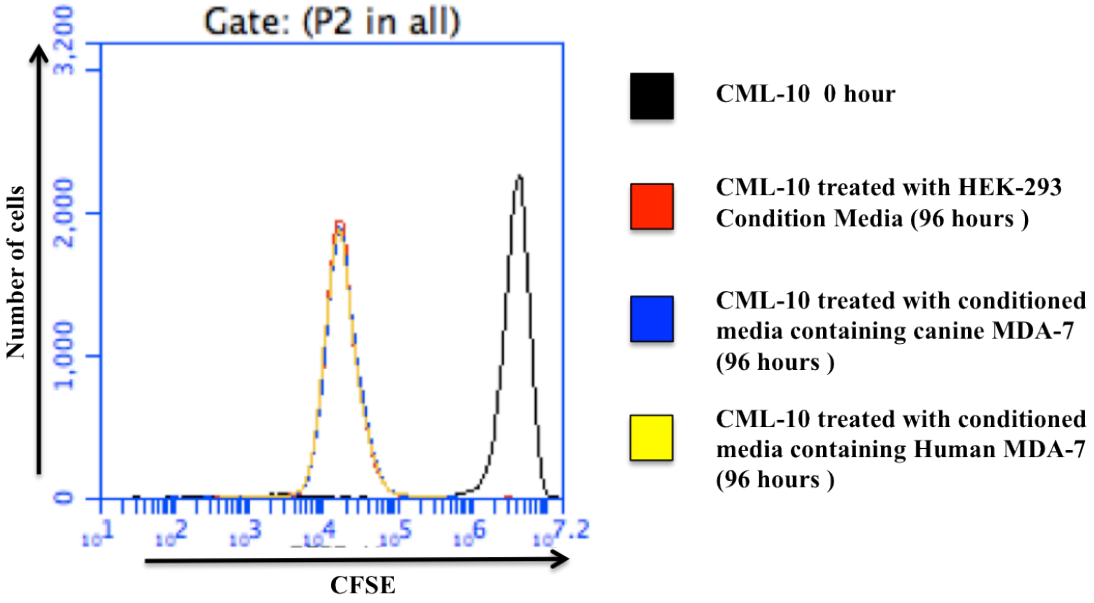


17B. Canine MDA-7 can interacts with human MDA-7 receptors and causes growth suppression of HeLa cells. CFSE stained HeLa cells were treated with culture media containing canine and human MDA-7 protein for 96 hours and were analyzed by flow cytometry. Treatment of HeLa cells with media containing either canine or human MDA-7 suppressed the growth of HeLa cells when compared to HeLa cells treated with control media.

5. Canine MDA-7 does not show bystander antitumor activity against canine cancer cells

After confirming bystander antitumor activity of canine MDA-7 against human cancer cells, its bystander antitumor activity against canine cancer cells was examined. Most of the human tumor cells that express MDA-7 receptors are susceptible to MDA-7 induced apoptosis. However, tumor cells (NSCLC) lacking MDA-7 receptors are resistance to MDA-7 induced growth suppression and apoptosis. We stained CMT-28 (canine breast carcinoma cells) and CML-10 (canine melanoma) cells with CFSE dye and performed a CFSE cell proliferation assay as described above (Fig. 18A and 18B). Unlike human tumor cells, canine MDA-7 protein did not inhibit proliferation of canine cancer cells (CMT28 or CML10). Similarly, human MDA-7 protein also did not suppress the growth of these canine cancer cells. In both cases, the MDA-7 treated canine cancer cells showed a similar proliferation profile to control treated canine cancer cells. Thus, this data suggest that neither canine nor human MDA-7 proteins exhibit bystander antitumor activity against canine tumor cells.

A) CML-10 cells



B) CMT-28 cells

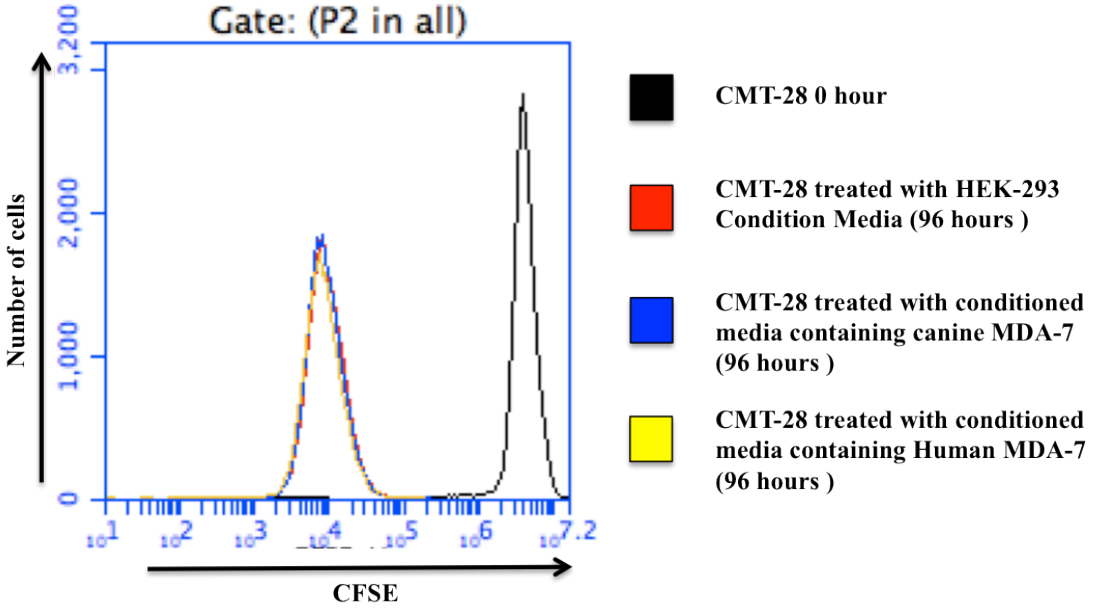


Fig. 18. Canine MDA-7 do not show bystander antitumor activity against dog tumor cells. Canine tumor cells were stained with CFSE, treated with canine or human MDA-7 protein for 96 hours and were analyzed by flow cytometry. Treatment of CML10 or CMT28 cells with media containing either canine or human MDA-7 did not suppress their growth.

Discussion:

Melanoma differentiation associated gene-7 (also known as Interleukin-24 (*mda-7*)) is a tumor suppressor gene with interleukin properties (Jiang, Lin et al. 1995; Jiang, Su et al. 1996; Huang, Madireddi et al. 2001; Pestka, Krause et al. 2004). Ectopic expression of MDA-7 from plasmid or adenoviral vectors causes growth suppression and apoptosis induction in cancer cells (Jiang, Lin et al. 1995; Jiang, Su et al. 1996). Human MDA-7 has a 48 amino-acid signal peptide sequence, and is actively secreted from *Ad.mda-7* infected normal and cancer cells (Nielsen, Kaestel et al. 1999; Nakai 2000). Secreted human MDA-7 protein then interacts with its two cell surface receptors, and initiates the JAK/STAT signaling pathway. MDA-7 receptors are heterodimers, and are derived from three different subunits, IL-20R1, IL-22R1 and IL-20R2 (Dumoutier, Leemans et al. 2001; Wang, Tan et al. 2002).

We have previously identified a canine ortholog of human *mda-7* and showed that it has genomic arrangement and sequence similarities to human MDA-7. Canine MDA-7 protein has amino-acid similarity to rat (66%) and human orthologs (65%). Canine MDA-7 is endogenously expressed in cultured normal canine epidermal keratinocytes (NCEKs) and can be actively secreted. Like human MDA-7, it causes growth suppression and cell death in canine and human tumor cells when ectopically expressed from a plasmid vector. Thus, the goal of the present study is to identify canine MDA-7 receptor subunits, elucidate their expression profile and investigate the bystander antitumor activity of canine MDA-7 protein.

In this study, we show that canine IL-20R1 and IL-22R1 mRNAs were shown to be endogenously expressed in cultured NCEKs. Canine IL-20R1 and IL-22R1 subunits have high amino acid similarity (73% and 78% respectively) to their respective human orthologs. NCEKs also expressed canine IL-20R1 protein with a molecular weight of ~ 73 KDa. Furthermore, expression of canine IL-20R1 protein increased (1.15) in NCEKs with LPS stimulation. An mRNA sequence for the canine IL-20R2 subunit was also identified from NCEKs. *In silico* analysis revealed that this mRNA sequence encodes the extracellular domain of the canine IL-20R2 subunit and has significant amino acid similarity (75%) to extracellular domain of human IL-20R2 subunit. This mRNA of canine IL-20R2 does not have representative nucleotide sequence for the intracellular and transmembrane domains. As the nucleotide sequence encoding for the transmembrane and intracellular domain are missing from this mRNA, we hypothesized that this truncated canine IL-20R2 protein cannot be expressed on the cell surface to form functional receptor complexes.

Human MDA-7 receptors are highly expressed on skin keratinocytes and are responsive to IL-20, IL-22 and MDA-7 stimulation (He and Liang 2010; Poindexter, Williams et al. 2010). Furthermore, secreted MDA-7 also plays an important role in skin biology where it is known to regulate keratinocytes proliferation and differentiation (Kunz, Wolk et al. 2006). MDA-7 transgenic mice show epidermal hyperplasia and defects in keratinocytes differentiation (He and Liang 2010). Thus, this data suggest that canine MDA-7 receptors might play a similar role, if a functional canine IL-20R2 subunit is expressed.

Expression of canine MDA-7 receptor alpha subunits was also confirmed on various canine tumor cells (Fisher, Gopalkrishnan et al. 2003; Emdad, Lebedeva et al. 2007). Most canine tumor cells endogenously expressed at least one R1 subunit. Similarly, human MDA-7 receptor subunits are expressed on a majority of tumor cells, and their expression is required for the bystander antitumor activity of MDA-7. Previously, it was been shown that HeLa cells were susceptible to MDA-7 induced apoptosis and were a model target cell line to study the bystander antitumor activity of MDA-7. In this study, secreted canine MDA-7 was shown to interact with the human MDA-7 receptor, suppress the growth of HeLa cells, and these growth-suppressing effects of canine MDA-7 were comparable to human MDA-7. Similarly, Wang and coworkers (2004) have shown that the rat ortholog of MDA-7, MOB-5, can interacts with human MDA-7 receptors and activate JAK/STAT signaling (Wang, Tan et al. 2004).

Bystander antitumor activity of canine and human MDA-7 was also evaluated against canine tumor cells. CMT-28 and CML-10 cells expressed the canine IL-22R1 and IL-20R1 subunit respectively. However, neither canine nor human MDA-7 protein inhibited the proliferation of canine cancer cells, thus failing to show bystander antitumor activity. This appears to be due to the lack of expression of a full length mRNA for canine IL-20R2 in canine tumor cells or possibly due to expression of truncated canine IL-20R2 protein.

Human MDA-7 has high binding affinity to the IL-20R2 subunit, and anti-IL-20R2 antibodies can block the activity of human MDA-7 (Wang, Tan et al. 2002). Lack of the IL-20R2 subunit expression can make tumor cells resistant to MDA-7 induced apoptosis. Although, a full-length mRNA of the canine IL-20R2 subunit was not

detected, the possibility of expression of full length mRNA for canine IL-20R2 subunit cannot be ruled out.

Interestingly, a recent study has shown that a homodimer of the human IL-20R2 subunit has high binding affinity for human MDA-7 (He and Liang 2010). These workers fused the extracellular domain of the IL-20R2 subunit with IgG1 Fc and expressed the fusion protein in CHO cells. This homodimer also lacks transmembrane and intracellular domains like the truncated canine IL-20R2 protein and can block the interaction of human MDA-7 protein with its receptors. Thus, we suggest that truncated canine IL-20R2 protein may play a similar role in canine tumor cells, and prevent MDA-7 induced bystander antitumor effects on dog tumor cells.

In summary, the current study showed that canine MDA-7 receptors subunits were highly expressed in cultured NCEKs and various other canine tissues. Secreted canine MDA-7 protein interacts with human MDA-7 receptors and initiates JAK-STAT signaling. Furthermore, canine MDA-7 shows a potent antitumor activity against HeLa cells. However, canine as well as human MDA-7 protein did not show bystander antitumor activity against canine tumor cells. This may be either due to a lack of expression of full-length canine IL-20R2 protein or due to expression of a truncated canine IL-20R2 protein, which may block the interaction of canine and human MDA-7 with functional canine MDA-7 receptors.

Material and methods:

1. Isolation and culturing of normal canine epidermal keratinocytes (NCEKs):

Normal canine epidermal keratinocytes were isolated as previously described. Briefly, dog skin samples were collected in CnT-09 media (CELLnTEC advance cell system) supplemented with 10% supplement A (CELLnTEC), 400nM L-glutamine, penicillin (500 IU/ml) and streptomycin (500µg/ml). Excess dermal tissue from the skin samples was removed under sterile condition. The skin was cut into 0.5cm square pieces. Cut skin samples were kept at 4^0C in complete CnT-09 media supplemented with 10mg/ml dispase II (Roche applied science) for 21 hours. The epidermal layer was collected by peeling it from dermis, washed in PBS (Mediatech, Inc.) and placed in TrypLE express (Invitrogen) for 30 minutes, at room temperature. The epidermis was rubbed with forceps to release the cells. Following incubation, the cell suspension was filtered through a 0.70µM filter (Beckson Dickinson Labware, France) and TrypLE express was neutralized with an equal amount of complete CnT-09 media. Cells were centrifuged at 300g for 5 min. The cell pellet was resuspended in complete CnT-09 media and seeded at 1.5 X 10^6 cells into a 75 cm2 flask and maintained at 37^0C and 5% CO_2.

2. Cells lines and reagents:

The human keratinocyte cell line (HACAT) and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100µg/ml). Canine cancer cells including canine mammary tumor lines (CMT-12, CMT-27, CMT-28), lymphoma cell lines (OSW and 17-71) and canine melanoma cells (CML-10) were also maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine

serum, penicillin (100 IU/ml) and streptomycin (100µg/ml). These cells were always maintained in log phase and were free of any contamination.

3. Cloning and expression of canine MDA-7

The open reading frame encoded by canine MDA-7sv1 was amplified and cloned into the pCMV 3TAG vector. This vector contains the cytomegalovirus (CMV) immediate early promotor and a FLAG epitope tag that is fused to the carboxyl terminus of the canine MDA-7 protein. A human MDA-7 expressing plasmid vector (pCDNA3.1+/Neo) was generously provided by Dr. Paul B. Fisher. The human mda-7 gene was released from this plasmid and cloned into the pCDNA3.1+/Hygro vector. These plasmids were then transfected into HEK-293 cells (lipofectamine LTX, Invitrogen Inc) and stable cell clones were isolated using geneticin (500µg/ml) or hygromycin (250µg/ml) selection for canine and human MDA-7 respectively. Cell cultures containing their respective MDA-7 proteins were collected and supplemented with Halt protease inhibitor cocktail (Thermo Scientific Inc.) and 0.05% sodium azide.

4. Western blot analysis:

Western blot analysis was performed on cell lysates and supernatants collected from MCF-7 and NCEK cells, and from various stable cell clones expressing MDA-7 protein. Protein samples were also prepared from different canine tissues including kidney, ovary and uterus. Briefly, 30 µg of cell extract was fractionated on a 10% SDS-PAGE gel. Proteins were then electroblotted to a nitrocellulose membrane and blocked with 5% non-fat dry milk. The blocked membrane was incubated overnight at 4^0C with primary antibody (anti-IL-20R1 (Polyclonal), anti-GAPDH, Thermo Scientific, Inc). The

membrane was then washed three times with phosphate buffer saline (PBS) containing 0.05% Tween-20. After washing, the membrane was incubated with a secondary antibody conjugated (anti-Rabit IgG, Thermo Scientific) to horseradish peroxidase (HRP). The membrane was washed again three times with PBS/0.05% Tween-20. The membrane was then incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Inc) for five minute. The membrane was dried and signal was detected by exposing X-ray film to the membrane for three minutes.

5. **Rapid amplification of cDNA ends:**

5’ RNA ligase mediated RACE (RLM-RACE) and 3’ RACE were performed according to the manufacturer’s directions (First choice® RLM-RACE, Ambion, Inc.). Briefly, total RNA was isolated from normal canine epidermal keratinocytes (NCEKs) with TRI Reagent (MRC, Inc.). 1μg of this RNA was treated with Calf Intestine Alkaline Phosphatase (CIP) to remove the 5'-phosphate group from the broken mRNAs. CIP treated RNA was purified by phenol: chloroform extraction and dissolved in nuclease free water. After this, the RNA was treated with Tobacco Acid Pyrophosphatase (TAP) to remove the 5’ cap from full-length mRNAs. CIP/TAP treated RNA was then ligated to a 45bp long RNA 5’ adapter using T4 RNA ligase. Adapter-ligated RNA was then reverse transcribed into cDNA, which was then ready for PCR amplification using the supplied outer RACE primers, and subsequently, with the inner RACE primers. For 3’ RACE, the RNA was reverse transcribed with the 3’ RACE adapter and subsequently amplified using 3’ RACE outer and inner primers. Amplified products were cloned into pGEMT-easy vector and sequenced using T7 and SP6 primers.

6. CellTrace CFSE cell proliferation assay

HeLa, CMT-28 and CML-10 cells were maintained in log phase in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100I.U/ml) and streptomycin (100µg/ml). Cells were trypsinized, centrifuged and resuspended as a single-cell suspension in PBS/0.1% BSA at a final concentration of 1 X 10^6 cells/ml. 2µl of CFSE dye solution was added to 1ml of cell suspension for a final concentration of 10µM. Cells were incubated at 37^0C for 10 minutes, and then the staining reaction was quenched with the addition of 5 ml of ice-cold DMEM media. Cells were then incubated on ice for 5 minutes, and pelleted by centrifuging at 150g for 5 minutes. Cells were washed three times, resuspended in DMEM media, and plated in a 6-well plate (2X 10^5 cells/well). After 24 hours, the media was exchanged for supernatant collected from stable cell clones expressing canine or human MDA-7 protein, and incubated at 37^0C for 72 hours. After 72 hours, cells were harvested and analyzed by flow cytometery (Accuri C6).

7. ***In-silico*** **analysis**

The predicted canine IL-20R1 and IL-22R1 gene sequences were obtained from National Center for Biotechnology IFNormation (NCBI) sequence database and analyzed *in-silico* with Vector NTI advance 10 (Invitrogen). The human IL-20R2 sequence was obtained from the NCBI database and compared to the canine genome using BLAST®. Canine genomic sequences representing the IL-20R2 gene locus were retrieved and analyzed by Genescan (MIT) and FGANESH (Softberry, Inc.) to predict the mRNA sequence of canine IL-20R2.

V. CONCLUSIONS

Cancer is a multi-step disease that requires multiple genetic and epigenetic alterations in a wide variety of genes that regulate important cellular pathways including the cell cycle, apoptosis and DNA repair. Mutations in cell cycle machinery and apoptotic pathways result in unchecked cell growth and these cells then develop into a tumor mass and may affect the functions of nearby organs and tissues (Weinstein 1988; Bishop 1991). Over the years, surgery, radiotherapy and conventional chemotherapy have remained the mainstay for treatment of a wide variety of cancers. Recent advancements in molecular biology have increased our understanding about tumor development and progression. Although, tumors are pleiotropic in nature, they share many biological characteristics, such as, self-sufficiency in growth signals, tissue invasion and migration, resistance to apoptosis, limitless replication potential, neo-angiogenesis and evasion from immune surveillance (Pavet, Portal et al. 2011). Thus, the common pathways that regulate these shared biological characteristics are important targets for development of cancer therapeutics. One such approach is based on cancer gene therapy using melanoma differentiation associated gene-7/Interleukin-24 (MDA-7) (Jiang, Lin et al. 1995; Jiang, Su et al. 1996; Fisher, Gopalkrishnan et al. 2003; Emdad, Lebedeva et al. 2007).

Human *mda-7* gene was first discovered in terminally differentiated human melanoma cells (HO-1) (Jiang, Lin et al. 1995; Jiang, Su et al. 1996). Subsequently, several studies have shown that expression of human MDA-7 protein at supra-

physiological levels from either plasmid or replication deficient adenoviral vectors suppresses growth and induces apoptosis in tumor cells (Cao, Mohuiddin et al. 2002; Leath, Kataram et al. 2004; Chen, Cheng et al. 2005; Chada, Mhashilkar et al. 2006; Gopalan, Shanker et al. 2007; Dong, Zhang et al. 2008; Hu, Xue et al. 2008; Kreis, Philippidou et al. 2008; Bao, Miao et al. 2009; Dash, Dmitriev et al. 2010; Chang, Yang et al. 2011). Furthermore, MDA-7 has potent anti-migration, anti-invasive and anti-angiogenic properties (Chen, Chada et al. 2003; Ramesh, Mhashilkar et al. 2003; Ramesh, Ito et al. 2004; Inoue, Branch et al. 2005; Inoue, Hartman et al. 2007; Wang, Zhang et al. 2010). The antitumor properties of MDA-7 have also been validated in a phase-I trial (Cunningham, Chada et al. 2005; Tong, Nemunaitis et al. 2005; Emdad, Lebedeva et al. 2007). In this study, we investigated the potential of developing a cancer gene therapeutic based on the canine ortholog of *mda-7*.

Development of an effective cancer therapeutic for human cancer requires its preclinical evaluations in animal models for safety and efficacy. Two kinds of animal models have been used for this purpose. The first are based on the mouse, which are either transplanted with human cancers or are genetically engineered to develop spontaneous cancers. Murine models have been very helpful in understanding the immunobiology of the cancer, however they are of limited use for the evaluation of new cancer treatment (Hansen and Khanna 2004).

The second type of animal model is based on companion animals, especially the dog. Dogs develop a diverse range of tumors including melanoma, non-Hodgkin lymphoma (NHL), leukemia, osteosarcoma and carcinoma of prostate, mammary, lung, head and neck and bladder (Hansen and Khanna 2004; Olson 2007; Paoloni and Khanna

2007; Paoloni and Khanna 2008). Canine tumors have strong similarities to human cancer including histological appearance, tumor genetics, biological behavior and response to conventional therapies. These tumors are spontaneously occurring, develop in the presence of an intact immune response and share some common risk factors including age, sex, reproductive status and environment, with human cancer. Moreover, genetic alterations that are responsible for human cancer have also been characterized for canine cancer. Thus, development of new therapeutics for canine cancer will not only help to treat canine cancer, but can be translated for human use (Haga, Nakayama et al. 2001; Koenig, Bianco et al. 2002; DeInnocentes, Agarwal et al. 2009).

In this study, we identified the canine ortholog of melanoma differentiation associated gene-7/Interleukin-24 (*mda-7*) and named it canine *mda-7* because of its sequence similarity (66%) to human MDA-7. The canine MDA-7 gene was identified on dog chromosome seven and found to be located in a cluster of interleukin-10 cytokines (canine IL-10, IL-19, IL-20 and *mda-7*) like the human and rat (c49a) orthologs ((Soo, Shaw et al. 1999)). In this study, we also showed that this locus is not only conserved in different breeds of dogs, but also in their close relative, the American Grey wolf (*Canis lupus*). The canine *mda-7* gene locus is spread over 6000 base pairs, and contains eight exons and seven introns. The promotor is located 29 bases upstream of the first exon and contains a conserved TATA element like the human *mda-7* gene (Huang, Madireddi et al. 2001). Thus, the canine *mda-7* locus maintains the overall structure of the human *mda-7* gene. However, we identified an extra exon (exon-5) and extra sequences for the 2nd and 3rd exon for canine *mda-7*, which are not present in the genomic structure of human *mda-7* (Huang, Madireddi et al. 2001).

The canine *mda-7* mRNA is only 964 nucleotides long, which is much shorter than the human *mda-7* mRNA (~2kb). The canine *mda-7* genomic locus contains three poly-A signals and AU-rich element (ARE) sequences. Use of alternative polyadenylation sites can produce mRNA of varying length. However, canine *mda-7* exclusively uses first polyadenylation site to add the poly-A tail. Similarly, the 3' UTR of human *mda-7* has three different poly-A signal sequences that can be used for alternative polyadenylation (Huang, Madireddi et al. 2001). However, no such alternative polyadenylation is observed for human *mda-7* mRNA, and the third polyadenylation signal sequence (most downstream) is exclusively used for addition of the poly-A tail. Use of this site results in a very long 3' untranslated region for human *mda-7* mRNA when compared to canine *mda-7* (Huang, Madireddi et al. 2001; Otkjaer, Holtmann et al. 2010). Furthermore, the 3' UTR of human mRNA also has three destabilization domains i.e. AU rich elements (ARE) (3'-UTR-AUUUA). These sequences, which are present in the canine gene, are absent in the mRNA of canine *mda-7* because of the use of the first polyadenylation site. ARE sequences are also present in mRNA of other cytokines (GM-CSF, TNF-α and IFN-γ). AU rich elements interact with ARE binding proteins and targets mRNA for degradation (Otkjaer, Holtmann et al. 2010). Activation of MDA-7 receptors activate p38MAPK, which then alters the binding affinity of ARE binding protein directly or by acting through different kinases (MK2, MK3 and MAPK-interacting kinases), and prevent degradation of *mda-7* mRNA (Otkjaer, Holtmann et al. 2010). The lack of AREs in the canine *mda-7* mRNA suggests that canine *mda-7* expression levels are not regulated by the same mechanisms (mRNA stabilization) that are used to control human

mda-7 expression. Thus, it seems possible that expression of canine *mda-7* is solely regulated through its promotor activity.

Canine *mda-7* is constitutively expressed in cultured normal canine epidermal keratinocytes (NCEKs) and its expression is increased after lipopolysaacharide (LPS) stimulation. Similarly, human *mda-7* is also expressed at high levels in cultured normal human epidermal keratinocytes (NHEKs). Cultured NHEKs secrete transforming growth factor-alpha (TGF-α), which then induces the expression human *mda-7* (Poindexter, Williams et al. 2010). Therefore, it seems likely that canine *mda-7* expression is also induced by canine transforming growth factor-alpha (TGF-α) in cultured NCEKs. Human *mda-7* expression is not detected in normal human skin samples, however during the wound healing process, its expression can be detected in keratinocytes, which peaks between the 2nd and 6th day. Human MDA-7 also alters the expression of psoriasis-related genes (S-100 family proteins, β-defensin etc) and affects differentiation and proliferation of human keratinocytes (Romer, Hasselager et al. 2003; Kunz, Wolk et al. 2006; Sarkar, Lebedeva et al. 2007; He and Liang 2010; Margue and Kreis 2010; Poindexter, Williams et al. 2010). Human *mda-7* mRNA and protein is also endogenously expressed in normal human skin melanocytes (Ekmekcioglu, Ellerhorst et al. 2001; Huang, Madireddi et al. 2001). Similarly, a rat ortholog of human *mda-7*, c49a is expressed in fibroblast like cells during wound repair. During wound healing, c49a inhibits the proliferation of epidermal keratinocytes, induces them to return their differentiated, non-proliferating state (Soo, Shaw et al. 1999). Moreover, *mda-7* transgenic mice show neonatal lethality, epidermal hyperplasia and defects in keratinocytes differentiation. *mda-7* transgenic mice showed increased expression of monocyte chemotactic protein-1 (MCP-1) protein from

keratinocytes, which then recruits macrophages into the dermal layer of skin (He and Liang 2010). Thus, this data suggest an important role for MDA-7 in skin biology, and we predict a similar role for canine MDA-7. However, further studies are needed to localize its expression in dog skin samples and roles it play in wound healing process in dogs.

Alternative splicing of pre-mRNA is an important mechanism to increase protein diversity in eukaryotes. Canine *mda-7* pre-mRNA is differentially spliced to produce five splice variants. These splice variants were named canine *mda-7*sv1, canine *mda-7*sv2, canine *mda-7*sv3, canine *mda-7*sv4 and canine *mda-7*sv5. Canine *mda-7*sv3 has the shortest length (901bp) while sv5 is the longest (1179bp). These splice variants encode four different protein isoforms that have a conserved amino terminus, but are dissimilar at the C-terminus. Canine *mda-7*sv1 and sv2 encode a protein of 183 amino acids, which has the highest amino acid similarity (66%) to human MDA-7 protein. Canine *mda-7*sv3 lacks the fourth exon, which is excluded from the final sv3 sequence by exon skipping. The second and third exons of the canine MDA-7 gene have 5' alternate acceptor sites. Use of these alternate acceptor sites can produce either a shorter or a longer version of the second and third exons, and most of the splice variants use the shorter version of both exons. However, canine *mda-7*sv2 has a longer version of the second exon, which increases the length of 5' untranslated region (UTR), but encodes the same protein as encoded by sv1. Similarly, splice variant 5 contains a longer version of the third exon that adds 99bp in the middle of the open reading frame with no effects on protein reading frame. Splice variants 4 and 5 also have an extra exon, which is not present in other splice variants. Representative sequences of the fifth exon are not present in human *mda-*

7 mRNA. The fifth exon has 62 bp and causes a frameshift in the open reading frame, which results in a change in the amino acid sequence at the C-terminus. In cultured NCEKs, canine *mda-7*sv1 wass expressed at the highest level (57.03%), sv2 and sv5 at intermediate levels (21.3% and 19.97% respectively) and sv3, sv4 were expressed at lowest levels. LPS stimulation of NCEKs caused increased expression of sv1 ad sv2. However, it did not alter the expression level of canine *mda-7*sv3, sv4 and sv5. This data suggest that canine *mda-7*sv1 is the primary transcript that encodes wild type canine MDA-7 protein, while the other splice variants may regulate the expression of canine *mda-7*sv1 by post-translational mechanisms. Splice variants have been reported for both human *mda-7* and its murine ortholog, FISP (Allen, Pratscher et al. 2004; Allen, Pratscher et al. 2005; Sahoo, Jung et al. 2008; Whitaker, Filippov et al. 2011; Yang, Duan et al. 2011). Human *mda-7* splice variants lack either the third exon alone or both the third and fifth exons. The splice variant lacking the third and fifth exon (*mda-7s)* encodes a truncated protein, which has 14 amino acids identical to wild type human MDA-7. However, expression of *mda-7s* is inversely related to the stage of melanoma (expression decrease as the melanoma progress). Furthermore, the truncated protein encoded by human *mda-7s* can heterodimerize and it coprecipitates with wild type human MDA-7, and blocks its secretion (Allen, Pratscher et al. 2004; Whitaker, Filippov et al. 2011; Yang, Duan et al. 2011). Similarly, a murine splice variant (FISP-sp) heterodimerizes with FISP, blocks its secretion and inhibits the apoptotic activity of FISP (Schaefer, Venkataraman et al. 2001). Thus, it is possible that the protein isoforms encoded by different splice variants of canine *mda*-7 may interact with each other to regulate the expression and activity of wild type canine MDA-7 protein (Sahoo, Jung et al. 2008).

Human MDA-7 has a highly restricted expression pattern and is expressed in cells and tissues related to the immune system. These cells include monocytes, subsets of T cells and lipopolysaccharide (LPS) and phytohaemagglutinin (PHA) stimulated PBMCs (Garn, Schmidt et al. 2002; Poindexter, Walch et al. 2005). When human melanocytes undergo transformation, they concurrently lose the expression of human MDA-7 expression. Thus, MDA-7 expression is inversely related to melanocytic transformation (Ekmekcioglu, Ellerhorst et al. 2001; Huang, Madireddi et al. 2001). Moreover, human MDA-7 is not expressed in most tumor cells (Fisher, Gopalkrishnan et al. 2003; Emdad, Lebedeva et al. 2007). However, its expression can be induced in different tumor cells with IFN-β and mezerein treatment. In this study, we showed for the first time that canine MDA-7 is expressed in LPS stimulated canine PBMCs. However, its expression is not detected in unstimulated or PHA, ConA or Anti-CD3 stimulated PBMCs. Like human MDA-7, canine MDA-7 is also not expressed by most of canine tumor cells (CML-10, CMT-28, CMT-27, OSW and 17-71). Thus, canine *mda-7* has a more limited expression profile than the human *mda-7*. However, CMT-12 (canine breast carcinoma) cells expressed canine *mda-7* at very high levels (378.23 ± 11.23 copies/ng of total RNA). We still do not know the mechanisms that induce expression of canine *mda-7* in CMT-12 cells. It will be interesting to understand the genetic and epigenetic changes that cause constitutive expression of canine *mda-7* in these cells.

Sequence analysis of canine *mda-7* revealed that it is a member of IL-10 family of cytokines. It has a conserved IL-10 signature sequence (Huang, Madireddi et al. 2001; Pestka, Krause et al. 2004; Zdanov 2006). Human MDA-7 has a 48 amino acid signal peptide sequence that allows its secretion, and is cleaved during secretion (Chaiken and

Williams 1996). Although the canine MDA-7 signal peptide sequence is very short (28 amino acids), it can still direct secretion of canine MDA-7. In this study, we showed that canine MDA-7 was actively secreted from pCMV 3TAG3 canine MDA-7 transfected HEK-293 cells. During secretion, human MDA-7 is heavily glycosylated at three consensus N-linked glycosylation sites. Glycosylation at these sites is necessary for human MDA-7 solubility and bioavailability (Mumm, Ekmekcioglu et al. 2006; Sauane, Gupta et al. 2006; Fuson, Zheng et al. 2009). Sequence analysis shows that canine MDA-7 protein has only one of the three consensus N-linked glycosylation sites (Asn-85). The two other N-linked glycosylation sites (Asn-99 and Asn-126) are not present in the canine MDA-7 protein. Furthermore, human MDA-7 has a disulfide bonds between the 59^{th} and 106^{th} resides (cysteine). This disulfide bond is required for its secretion and biological activity (Fuson, Zheng et al. 2009)). In this study, we showed that these two cysteine residues are conserved among different isoforms of the canine MDA-7 protein. Thus, our data suggest that canine MDA-7 has high protein structure similarity with human MDA-7 protein, and thus may play similar biological roles.

Ectopic expression of human MDA-7 protein from plasmid or adenoviral vectors causes growth suppression and induces apoptosis in a wide variety of tumor cells (Jiang, Lin et al. 1995; Jiang, Su et al. 1996; Dent, Yacoub et al. 2005; Emdad, Lebedeva et al. 2007; Altomare, Rybak et al. 2010; Dent, Yacoub et al. 2010). MDA-7 treated tumor cells lose invasion and migration abilities and are more susceptible to radiotherapy (Yacoub, Mitchell et al. 2003; Yacoub, Mitchell et al. 2003; Lebedeva, Su et al. 2007). Ectopic expression of human MDA-7 does not affect the growth of normal cells (Jiang, Lin et al. 1995; Jiang, Su et al. 1996; Emdad, Lebedeva et al. 2007). Human MDA-7

modulates multiple apoptotic pathways and causes cell death in tumor cells with diverse genetic make ups (Emdad, Lebedeva et al. 2007). In this study, we showed for the first time that over-expression of canine MDA-7 protein also inhibited the growth of human tumor cells. Furthermore, ectopic expression of canine MDA-7 protein induced the tumor cells to undergo apoptosis. Antitumor effects of canine MDA-7 were found to be comparable to human MDA-7. Overexpression of human MDA-7 protein also causes growth suppression and apoptosis induction in dog tumor cells. Likewise, canine MDA-7 has growth inhibitory effects on various canine tumor cells. These cell lines were derived from spontaneously occurring canine cancers including mammary carcinoma (CMT-28), leukemia (OSW and 17-71) and melanoma (CML-10). Genetic defects in these cancer cell lines have been well characterized. Moreover, overexpression of canine or human MDA-7 does not have any cytotoxic effects on normal canine fibroblasts (NCF). Thus, the above data suggests that canine and human tumor cells are susceptible to MDA-7 induced apoptosis, and canine MDA-7 is indeed an ortholog of *mda-7* and has very similar protein structure and biological properties.

Human MDA-7 protein expressed from normal and cancer cells is actively secreted (Chada, Sutton et al. 2004; Fuson, Zheng et al. 2009). Secreted MDA-7 binds to its two cognate receptors (IL-20R1/IL-20R2 and IL-22R1/IL-20R2) and activates the JAK/STAT signaling pathway. MDA-7 receptors are members of the class II cytokine receptor family. These receptor complexes are composed from three-receptor subunits-IL-20R1, IL-22R1 and IL-20R2 (Chai, Nichols et al. 1997; Catlett-Falcone, Landowski et al. 1999; Nielsen, Kaestel et al. 1999). MDA-7 receptors mediate the bystander antitumor activity of MDA-7 proteins, and their lack of their expression makes tumor cells resistance to

MDA-7 induced cell death (Arcangeli, Carla et al. 1993; Dent, Yacoub et al. 2010; Dent, Yacoub et al. 2010). Furthermore, these receptors are widely expressed in skin and other epithelial tissues (uterus, intestine, liver and lung) (Parrish-Novak, Xu et al. 2002). These receptors are hypothesized to play an important role in skin biology. In this study, we showed that cultured NCEKs express canine orthologs of the IL-20R1 and IL-22R1 subunits. These receptors are endogenously expressed in NCEKs, and expression of the canine IL-20R1 subunit is increased (1.15 fold) in NCEKs with LPS stimulation. Canine IL-20R1 is also expressed in other canine tissues including kidney, ovary and uterus. These two receptor subunits (IL-20R1 and IL-22R1) are widely expressed in various canine tumor cells.

In spite of the presence of IL-20R1 and IL-22R1, full-length mRNA encoding for the canine IL-20R2 subunit could not be amplified. A truncated mRNA was amplified and sequenced that encoded the extracellular domain of the canine IL-20R2 subunit. Sequences encoding the transmembrane and intracellular domains of canine IL-20R2 were not present in this truncated mRNA.

In this study, we showed that secreted MDA-7 was not only secreted but could also interact and signal through human MDA-7 receptors. Activation of MDA-7 receptors via canine MDA-7 protein suppressed the growth of HeLa cells. However, canine MDA-7 did not show bystander antitumor activity against canine tumor cells. Similar results were also seen with human MDA-7 protein against canine cells. We have shown that dog tumor cells express canine IL-20R1 and IL-22R1 receptor subunits, but lack expression of a complete canine IL-20R2 subunit. Absence of this subunit can make the canine MDA-7 receptor non-functional. Interaction with the IL-20R2 subunit is necessary for the

receptor mediated bystander antitumor activity of human MDA-7. Blocking MDA-7 interaction with IL-20R2 subunits using anti-IL-20R2 antibodies can inhibit the activation of MDA-7 receptors (Wang, Tan et al. 2002). Similarly, a homodimeric protein made from the extracellular domain of the IL-20R2 subunit can block the activity of human MDA-7 (He and Liang 2010). Based on these observations, we believe that truncated canine IL-20R2 protein may form a homodimer, and block the interaction of human and canine MDA-7 proteins if any functional canine MDA-7 receptors are expressed in canine tumor cells. Since these receptors are also used by other cytokines (IL-20 and IL-22), secretion of a homodimeric IL-20R2 might provide an additional mechanism to control receptor availability for different cytokines. Further studies are needed to explore the possibility of expression of full-length canine IL-20R2 receptor subunit.

In summary, we have shown that canine *mda-7* is indeed an ortholog of *mda-7*. It has strikingly similarities in amino acid sequence, genomic and protein structure to human MDA-7. Furthermore, canine MDA-7 is a secreted protein and possesses antitumor activity against human and canine tumor cells. However, the canine MDA-7 receptor system appears to be defective, due to the absence of a full-length IL-20R2 subunit, leading to a failure of the mechanism responsible for the bystander effect. Thus, we suggest that canine MDA-7 has the potential to be used for development of cancer gene therapy to treat canine cancers, however the lack of a bystander effect in the dog may limit its utility.

VI. BIBLIOGRAPHY

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