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Short communication

Targeted knockdown of canine KIT (stem cell factor receptor) using RNA interference

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1. Introduction

Mast cell tumours (MCT) are the most common skin malignancy in dogs and are thought to arise from neoplastic transformation of tissue mast cells (reviewed by Dobson and Scase, 2007; Welle et al., 2008). In order to become malignant, mast cells must be able to resist those mechanisms associated with programmed cell death, to allow survival and proliferation. Developing a greater understanding of how such cells prevent apoptosis might enable us to design more rational treatment strategies for this type of cancer.

Abbreviations: CHO, Chinese hamster ovary; siRNA, small inhibitory RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; B2M, beta- 2 microglobulin; TRAIL, TNF related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis protein.

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and is thought to contribute to oncogenesis (Cerveró et al., 1999; Hartmann et al., 2003). Mutations in the KIT gene are found in 15–50% of canine MCT, which typically consist of in-tandem duplications, located in or around exon 11 (London et al., 1999; Ma et al., 1999; Downing et al., 2002; Webster et al., 2006). When expressed, such mutations often result in autophosphorylation of the intracellular kinase domain of the receptor protein, leading to constitutive activation, even in the absence of SCF (London et al., 1999; Ma et al., 1999; Pryer et al., 2003). Thus, canine mast cell tumors are capable of developing independence from growth/survival signals, one of the key features of malignancy (Hanahan and Weinberg, 2000). The C2 mastocytoma cell line, which is commonly used to study canine malignant mast cell biology in vitro, expresses a 48-base-pair in-tandem duplication (London et al., 1999; Ma et al., 1999), although the precise location, nature and size of mutations can vary between different MCTs (Downing et al., 2002; Webster et al., 2006; Letard et al., 2008).

KIT activating mutations have been shown to be associated with MCT of a more aggressive phenotype, which is likely due to increased proliferation and resistance to apoptosis (Gleixner et al., 2007; Letard et al., 2008). This has led to several investigations into the therapeutic potential of receptor tyrosine kinase inhibitors (RTKi), designed to target this molecule. There are currently two drugs (masitinib, Masivet ®, AB Science; toceranib, Palat sine ®, Pfizer) that have recently been licensed for canine MCT. However, these compounds are not specific for KIT and also inhibit other receptor tyrosine kinases, such as platelet-derived growth factor receptor (PDGFR) and vascular growth factor receptor (VEGFR) (Rubin et al., 2001; Hayes and Thor, 2002; Humbert et al., 2009; London et al., 2009). Although the lack of specificity might enhance the anti-cancer efficacy of these drugs, this could also increase the risk of adverse effects in the patient. In addition, these RTKi will not have specific effects on malignant mast cells, as they will also inhibit wild-type KIT, expressed by tissue mast cells and other cell types including haematopoietic stem cells and melanocytes (Galli et al., 1994; Linnekin et al., 1997) Molecular techniques, such as gene silencing by RNA interference, offer an alternative, potentially more specific method for targeting mutant KIT that might be applicable for canine MCT, as well as for human gastrointestinal stromal cell tumours that commonly express similar KIT mutations (Lasota et al., 2003; Steigen et al., 2007; Du et al., 2008).

RNA interference can be used to target specific mRNA for degradation (Spee et al., 2006; Tsuchiya et al., 2006; Watanabe et al., 2009). Thus, gene expression can be selectively silenced, preventing production of the encoded protein, which allows experiments to be designed to determine the role of that specific gene product on cellular function. The aim of the current study was to determine whether small inhibitory RNA (siRNA) molecules could be designed to knock-down canine KIT expression. In particular we aimed to design novel siRNA molecules that specifically targeted the mutant KIT expressed by C2 cells that would spare expression of the wild-type receptor. Chemical transfection of C2 cells proved to be problematic, despite repeated attempts using various transfection reagents and protocols. Therefore, to allow investigation of the activity of the siRNA molecules to specifically interfere with their target canine mRNA, we investigated use of a reporter gene assay to assess the ability of canine KIT-specific SiRNA molecules to knock-down their target.

2. Materials and methods

2.1. Plasmid DNA constructs and siRNA molecules

Partial coding regions for selected canine genes were amplified by PCR from cDNA prepared from the C2 canine mastocytoma cell line (a generous gift from Dr B. Helm, University of Sheffield; originally generated by Prof. W. Gold, University of California) (Lazarus et al., 1986), which expresses both wild-type KIT as well as a KIT exon 11 mutation (Fig. 1). PCR products representing canine glyceraldehyde-3-phosphate dehydrogenase; GAPDH (sense primer: ACCACGTCCATGGCATCAC; antisense primer: TCCACCACGGTGTGCTGTA; 452 bp amplicon), beta-2 microglobulin; B2M (sense primer: TCTCTATCCCTGCTCT; antisense primer: GTCAGTTGTCTCGGTCCCAC; 333 bp amplicon), KIT126–407 (sense primer: ATTITTCCTCGTCTCTGCTC; antisense primer: ACCAGGTATCATGGCCCT; 382 bp amplicon), KIT1569–1920 (sense primer: CCTCTACACTTTTTGGA; antisense primer: TAGGGCTTCTCTGTGCTGA; 352 bp amplicon for wild-type, 400 bp amplicon for mutant) were cloned into the pSC-A vector (Stratagene, La Jolla, CA). Plasmid DNA from recombinant clones was sequenced to confirm the integrity of the inserts, which were then sub-cloned into the psiCHECK™-2 vector (Promega, Southampton, UK). This vector contains both firefly and renilla luciferase reporter genes under the control of separate promoters. Each canine gene-specific sequence was inserted downstream of the renilla luciferase element using NotI and Xhol restriction sites, which will result in expression of the target sequence in the 3′UTR of the renilla luciferase mRNA. Following transfection, any changes in renilla luciferase activity, which might result from siRNA binding and knockdown of the fusion mRNA, can thus be normalized against firefly luciferase activity. Plasmid DNA was then extracted from recombinant E.coli using the GenElute™ Endotoxin-free Miniprep Kit (Sigma, Poole, UK) in preparation for transfection studies. The SiRNA molecules used in the study are shown in Table 1. In addition, a scrambled SiRNA molecule (AllStars Negative Control SiRNA, Qiagen, Crawley, UK) was used as a negative control.

2.2. Cell culture and transfections

Chinese hamster ovary (CHO) cells were maintained in 75 cm² flasks of culture medium consisting of Minimal Essential Medium (Serotec, Kidlington, UK) supplemented with 10 mM Glutamax-1 (Invitrogen) 10% foetal bovine serum (Serotec, Kidlington, UK) and 25 μg/ml gentamicin (Sigma). Cells for transfection studies were plated out in triplicate wells in Corning 96 well clear bottom, white microtitre plates (Sigma) at 1 x 10⁴ cells in a 100 μl volume of culture medium lacking serum and antibiotics. Cells in each
Fig. 1. The C2 cell line expresses both wild-type KIT and a KIT exon 11 mutant. (a) cDNA was prepared from C2 cells and used as the template for PCR using primers spanning KIT exon 11. PCR products were separated by 2% agarose gel electrophoresis. Anticipated amplicon size = 352 bp for wild-type KIT and 400 bp for the mutant version, which contains a 48 bp in-tandem duplication. MWt = 200 bp molecular weight marker. (b) Sequence of KIT mutation in C2 cells. Partial genomic DNA sequence is shown with exon 11 highlighted. The 48 bp mutation is shown in bold and the region targeted by the SiRNA molecule is underlined.

well were transfected with 200 ng plasmid DNA and 5 pmol SiRNA using Lipofectamine 2000™ (Invitrogen) according to the manufacturer's instructions. Plates were incubated for 4 h then the complex-containing medium was replaced with culture medium lacking antibiotics.

2.3. Luciferase assay

Twenty four hours after transfection, cells were assayed for both firefly and renilla luciferase activity using the Dual-GLO® Luciferase Assay System (Promega). Briefly, cells were lysed and the firefly luciferase substrate added (50 μl per well Dual-GLO® Substrate/Buffer). After 15 min, luciferase activity was measured using a luminometer (Spectramax M2, Molecular Devices). Next the renilla luciferase substrate was added (50 μl per well Stop & GLO® Substrate/Buffer) and the luminescence measured after a further 15 min incubation. The renilla/firefly luciferase ratio was calculated from the mean luminescence values of triplicate wells, after blanking against values from untransfected cells. The percentage knockdown using test SiRNA molecules was calculated compared to the control scrambled SiRNA and data are shown as the mean of three experimental replicates.

3. Results & discussion

3.1. Knock-down in expression of canine housekeeping genes

Few studies have been performed using SiRNA to specifically knock-down canine gene expression. In terms of studying the biology of canine malignant cells, siRNA targeting apoptosis-modulator molecules including Bcl-2 (Watanabe et al., 2009), Bcl-xL (Tsuchiya et al., 2006) and XIAP (Spee et al., 2006) have previously been undertaken in

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Plasmid DNA construct</th>
<th>SiRNA molecule</th>
<th>SiRNA target sequence</th>
<th>SiRNA source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renilla luciferase</td>
<td>psiCHECK-2</td>
<td>Renilla</td>
<td>tgccttactctttcctcag</td>
<td>Custom synthesized (Ambion)</td>
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<td>cactttggaagctcatt</td>
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<td>Silencer® SiRNA 35 (Ambion)</td>
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<td>KIT#2</td>
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canine cancer cell lines. In the current study, several siRNA molecules were initially assessed that were designed to target canine housekeeping genes. Using a renilla luciferase siRNA molecule as a positive control, efficient knockdown of canine GAPDH was demonstrated, although siRNA molecules targeting beta-2 microglobulin were less effective (Table 2). The increase in firefly luciferase activity seen in CHO cells co-transfected with plasmid DNA with scrambled siRNA, compared to those cells transfected with plasmid DNA alone, might be explained by the greater amount of transfection reagent used, leading to increased transfection efficiency. A further increase in firefly luminescence was seen in CHO cells co-transfected with targeted siRNA compared to scrambled siRNA. This pattern of reactivity might be explained if firefly and renilla luciferase mRNAs compete for translation. Thus, following successful targeting of the renilla-fusion mRNA for destruction, this could lead to enhanced firefly luciferase protein expression.

### 3.2. Knock-down in expression of canine KIT

To investigate the biological effect of KIT RNA interference, the C2 canine mastocytoma cell line was chosen for in vitro studies. The mutation present in the KIT gene expressed by C2 cells consists of a 48 bp in-tandem duplication in exon 11. The C2 cells used in the current study expressed both wild-type and mutant KIT mRNA (Fig. 1), with the latter seeming to dominate. Since C2 cells are heterozygous for the mutation, it is possible that the mutant allele is transcribed at a higher level, or that the mutant transcript is more stable than the wild-type transcript. Expression of wild-type KIT seems to be variable in C2 cells, with expression reportedly absent in cells used in some (Ma et al., 1999) but not all previous studies (London et al., 1999).

Since no KIT siRNA molecules had been validated for the dog, several human KIT siRNAs that had been shown to be effective (Lefevre et al., 2004; Li et al., 2007; Sikarwar and Reddy, 2008) were assessed for sequence identity with the canine KIT sequence. A commercially available and validated KIT siRNA molecule (Silencer® KIT SiRNA, Ambion), which demonstrated complete sequence identity between human and canine target sequences, was selected which demonstrated >90% knock-down efficiency (Table 2). The KITexon2 siRNA molecule is not specific to the mutant KIT sequence and would be expected to knock-down both wild-type and mutant KIT mRNAs. The presence of the mutation generates a unique sequence at the 5′ junction of the insertion (Fig. 1). Therefore, a novel canine KIT siRNA molecule was designed, that targeted this region to determine whether it might be possible to specifically knock-down the mutant, while sparing expression of wild-type KIT. When tested against constructs containing either the wild-type sequence or the mutant version, this siRNA molecule demonstrated efficient knock-down when using the KIT2mutant construct (mean 93.1% knock-down) but had little effect on the KIT2wild-type construct (mean −2.98% knock-down) (Fig. 2).

Due to the relatively large size of the KIT coding sequence (almost 3kB) and the problems associated with cloning this in entirety, we opted to clone only the specific

<table>
<thead>
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<th>Table 2: Knockdown efficiency of canine housekeeping gene and KIT siRNA molecules.</th>
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<td>Transfection conditions</td>
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<tr>
<td>Firefly (LU)</td>
</tr>
<tr>
<td>psiCHECK™-2 + scrambled siRNA</td>
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<tr>
<td>psiCHECK™-2/B2M + scrambled siRNA</td>
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<tr>
<td>psiCHECK™-2/GAPDH + scrambled siRNA</td>
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<td>psiCHECK™-2/KIT1 + scrambled siRNA</td>
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<tr>
<td>psiCHECK™-2/KIT1 + KITexon2 siRNA</td>
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Triplicate wells of CHO cells were transfected with native or recombinant psiCHECK-2 plasmid DNA and co-transfected with either scrambled siRNA, siRNA targeted to renilla luciferase, or SiRNA targeting the inserted canine sequence. Both firefly and renilla luciferase activity were measured after 24 h incubation. Mean luminescence values are shown, following subtraction of the luminescence values of untransfected CHO cells. Knockdown efficiency of targeted siRNA was calculated compared to scrambled siRNA, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, B2M = beta-2 microglobulin. Ex-perimental units, KD (%) = percentage knockdown efficiency.
regions of the KIT coding sequence targeted by the SiRNA molecules selected/designed. This strategy has its disadvantages, since the secondary structure of the native KIT mRNA is lost, which might influence the efficiency of SiRNA silencing. This also precludes assessment of the effect of SiRNA on KIT protein expression, although the reporter gene assay is based on evaluation of luciferase activity, rather than mRNA expression. Both firefly and renilla luciferase activity were consistently lower when using the psiCHECK-2 construct containing mutant KIT, compared to the native vector or vector containing wild-type KIT sequences. Endotoxin contamination of the plasmid DNA was considered, but a new batch of plasmid DNA demonstrated the same response. The explanation for this reduced efficiency of luciferase activity with this particular construct remains unclear. Despite this, the KITexon11mut SiRNA molecule demonstrated specificity of activity against the mutant KIT construct, without affecting expression from the construct containing the equivalent wild-type KIT sequence.

In the current study, we have attempted to validate several canine gene-specific SiRNA molecules. These can now be assessed in canine cell lines and it is anticipated that alternative delivery systems (e.g. electroporation, viral vectors) will allow evaluation of these molecules in the C2 cell line. We have shown as “proof-of-concept” that SiRNA molecules can be designed to specifically target KIT mutations, although the heterogeneity of mutations seen in canine MCT means that it is unlikely that a single SiRNA molecule could be designed that would be effective in all cases.

Conflict of interest statement
No conflict of interest.

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