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1 **Original Article**

2  
3 **Development of recombinant canine single-chain insulin analogues, evaluation of their**  
4 **insulin receptor binding capacity and ability to stimulate glucose uptake**

5  
6  
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19

20 **Abstract**

21 Virtually all diabetic dogs require exogenous insulin therapy to control their  
22 hyperglycaemia. In the UK, the only licensed insulin product currently available is a purified  
23 porcine insulin preparation. Recombinant insulin is somewhat problematic in terms of its  
24 manufacture, as the gene product (preproinsulin) undergoes substantial post-translational  
25 modification in pancreatic  $\beta$  cells before it becomes biologically active. The aim of the present  
26 study was to develop recombinant canine single-chain insulin (SCI) analogues that could be  
27 produced in a prokaryotic expression system and which would require minimal processing.  
28 Three recombinant SCI constructs were developed in a prokaryotic expression vector, by  
29 replacing the insulin C-peptide sequence with one encoding a synthetic peptide (GGGPGKR)  
30 or with one of two insulin-like growth factor (IGF)-2 C-peptide coding sequences (human:  
31 SRVSRRSR; canine: SRVTRRSSR). Recombinant proteins were expressed in the periplasmic  
32 fraction of *E. coli* and assessed for their ability to bind to the insulin and IGF-1 receptors and  
33 to stimulate glucose uptake in 3T3-L1 adipocytes.

34

35 All three recombinant SCI analogues demonstrated preferential binding to the insulin  
36 receptor, compared to the IGF-1 receptor, with increased binding compared to recombinant  
37 canine proinsulin. The recombinant SCI analogues stimulated glucose uptake in 3T3-L1  
38 adipocytes compared to negligible uptake using recombinant canine proinsulin, with the canine  
39 insulin/cIGF-2 chimaeric SCI analogue demonstrating the greatest effect. Thus, biologically-  
40 active recombinant canine SCI analogues can be produced relatively easily in bacteria, which  
41 could potentially be used for treatment of diabetic dogs.

42

43 *Keywords:* Insulin; Insulin receptor; Canine diabetes; Glucose uptake

44

45 **Introduction**

46 Diabetes mellitus in dogs is characterised by the presence of hyperglycaemia caused by  
47 an absolute or relative deficiency in the pancreatic  $\beta$  cell hormone, insulin (Catchpole et al.,  
48 2008). Virtually all diabetic dogs require administration of exogenous insulin to control their  
49 blood glucose concentration. Whereas recombinant insulin is used to treat human diabetic  
50 patients, in the UK there is currently only one licensed insulin product available for treatment  
51 of diabetes in companion animals, which consists of purified porcine insulin (Caninsulin, MSD  
52 Animal Health)<sup>1</sup>. Recombinant human insulin has been used for many years in North America  
53 for treatment of canine diabetes, where until recently there was no FDA-approved insulin for  
54 companion animals (Rucinsky et al., 2010).

55  
56 Production of purified beef and pork insulin has been scaled down, with the  
57 introduction of recombinant techniques for production of human insulin. Since the supply of  
58 bovine and porcine insulin for veterinary use generally relies on human market availability,  
59 insulin for diabetic dogs is likely to become increasingly limited. Indeed, in recent years,  
60 bovine insulin products (formerly Insuvet soluble, lente and protamine zinc insulin, Zoetis)  
61 have been withdrawn from the veterinary market. Thus, there is an anticipated need for  
62 development of recombinant canine insulin preparations.

63  
64 Biologically active insulin is synthesised in pancreatic  $\beta$  cells by extensive post-  
65 translational modification of preproinsulin. After folding and disulphide bond formation  
66 between insulin A and B chains, cleavage of the connecting C-peptide is required for biological  
67 activity (Fig. 1A). Proinsulin demonstrates a somewhat modest 1-2% affinity for binding to the  
68 insulin receptor (INSR) compared to insulin and it is thought that there are two main reasons

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<sup>1</sup> See: [http://www.vmd.defra.gov.uk/productinformationdatabase/SPC\\_Documents/SPC\\_124274.doc](http://www.vmd.defra.gov.uk/productinformationdatabase/SPC_Documents/SPC_124274.doc).

69 for this (Peavy et al., 1985); insulin C-peptide does not seem to allow enough molecular  
70 flexibility to facilitate interaction with INSR binding sites and it interferes with important  
71 receptor-binding residues such as glycine at position A1. This presents a challenge for  
72 commercial production of recombinant insulin, since most methods are based on use of  
73 prokaryotic expression systems, with bacteria and yeast lacking the necessary cellular  
74 machinery and enzymes required for correct folding and processing of proinsulin to insulin.

75

76         The first recombinant insulin to become commercially available (Humulin, Eli Lilly)  
77 was based on a process whereby insulin A and B chains were produced separately in bacteria,  
78 then combined to form the biologically active molecule (Riggs and Itakura, 1979). However  
79 this process is somewhat inefficient and subsequently different techniques have been employed  
80 for commercial production of recombinant human insulin, which usually involves synthesis of  
81 a precursor molecule that is subjected to chemical and/or enzymatic modification (Christensen  
82 et al., 1991).

83

84         An alternative approach to synthesis of recombinant insulin is to produce single chain  
85 insulin (SCI) analogues, which do not require post-translational modification to exert their  
86 biological activity (Kristensen et al., 1995). In SCI analogues, the proinsulin C-peptide is  
87 substituted with alternative linking peptide sequences that allow folding and disulphide bond  
88 formation between A and B chains, but which do not require cleavage and interfere with  
89 binding to the INSR much less than the native C-peptide. One such construct, developed for  
90 gene therapy of diabetes, involved substituting the insulin C-peptide with a synthetic peptide  
91 linker (Lee et al., 2000).

92

93         There are other members of the insulin superfamily, with the most important being the

94 insulin-like growth factors IGF-1 and IGF-2 (Chan and Steiner, 2000). Unlike insulin, IGF-1  
95 and IGF-2 do not require cleavage of their C-peptide to bind to their cognate receptors. IGF-2  
96 has been implicated in the syndrome of non-islet cell tumour hypoglycaemia, which involves  
97 production of an IGF-2 related peptide by tumour cells that acts on insulin receptors to cause  
98 hypoglycaemia (Boari et al., 1995; LeRoith, 2004; Zini et al., 2007). Thus, insulin and IGF-2  
99 both have glucose-lowering properties that might be exploited for developing a novel  
100 therapeutic for canine diabetes.

101

102         The aim of the present study was to develop canine SCI analogues, whereby the insulin  
103 C-peptide was substituted with either a synthetic peptide linker or alternatively the human or  
104 canine IGF-2 C-peptide sequence, to express these recombinant SCI analogues in bacteria and  
105 to assess their ability to bind to the INSR and stimulate glucose uptake in cultured cells. The  
106 hypothesis was that such SCI analogues would be biologically active, without any requirement  
107 for post-translational modification.

108

## 109 **Materials and methods**

### 110 *Generation of canine single-chain insulin analogue constructs*

111         The coding sequence for recombinant canine proinsulin (rcPROINS) was amplified by  
112 PCR with a high-fidelity proof-reading polymerase (Easy-A High-Fidelity PCR cloning kit,  
113 Stratagene) using canine insulinoma cDNA as the template. Restriction sites for *EcoRI* and  
114 *SphI* were incorporated into the forward and reverse primers respectively (see Appendix:  
115 Supplementary material) to allow for subsequent directional subcloning of rcPROINS from the  
116 pSC-A cloning vector (Stratagene) to the pTAC-MAT-Tag-2 expression vector (Sigma-  
117 Aldrich).

118

119 Three SCI analogues were designed, each substituting the proinsulin C-peptide with a  
120 different peptide linker sequence (Fig. 1B). The pSC-A/rcPROINS plasmid DNA was used as  
121 the template for further PCR to generate the necessary coding sequences for each of the  
122 constructs. Initially, primers designed to anneal to insulin A chain or B chain were modified by  
123 adding specific synthetic oligonucleotide sequences encoding the linking GGGPGKR peptide  
124 as well as restriction sites to facilitate ligation of the two elements into a single construct. The  
125 pTAC-MAT-Tag-2/rcINS[GGGPGKR] construct was completed by digestion of PCR  
126 products, followed by ligation into the pTAC-MAT-Tag-2 vector (see Appendix:  
127 Supplementary material for more information on cloning strategy).

128

129 A synthetic oligonucleotide dimer encoding the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-  
130 Asp-Asp-Lys) was inserted into these two expression constructs immediately upstream of and  
131 in frame with the proinsulin coding sequence. Subsequently, pTAC-MAT-Tag-2/rcPROINS  
132 plasmid DNA was used as the template to generate amplicons required to create the canine  
133 insulin/IGF-2 C-peptide chimeric constructs, rcINS[cIGF-2C] and rcINS[hIGF-2C] (see  
134 Appendix: Supplementary material for more information on cloning strategy). Constructs were  
135 subcloned into the pFLAG-ATS (Sigma-Aldrich) periplasmic expression vector that had been  
136 modified to incorporate a 3' metal affinity tag (pFLAG-MAT-Tag-ATS). Using the same  
137 methodology, the MAT sequence was also inserted into the pFLAG-ATS+BAP (bacterial  
138 alkaline phosphatase) vector (Sigma-Aldrich) to create pFLAG-MAT-Tag-ATS+BAP, used as  
139 a positive control for expression studies. Plasmid DNA was transformed into BL-21(T1) *E.*  
140 *coli* (Sigma-Aldrich) for expression of recombinant proteins.

141

#### 142 *Expression of recombinant SCI analogues*

143 Expression of recombinant protein was performed in *E. coli*, transformed with plasmid

144 DNA encoding rcPROINS or the various SCI analogues. An individual colony was inoculated  
145 into 10 mL LB broth containing 100 µg/mL ampicillin (LB Liquid Amp, Fermentas) and  
146 incubated in an orbital shaker at 145 rpm, 37 °C overnight. Bacterial cultures were diluted by  
147 adding 1 mL to 50 mL pre-warmed LB/Amp in a 250 mL baffled shaker flask (Erlenmeyer  
148 Culture Flask Baffled Base, BD Falcon) and incubated at 145 rpm, 37 °C until they reached  
149 the desired turbidity (optical density at 600 nm; OD<sub>600</sub> = 2.0), as measured with an Eppendorf  
150 BioPhotometer. Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich) was added to  
151 cultures at 1 mM for 4 h to stimulate recombinant protein expression. Bacterial pellets were  
152 obtained by centrifuging samples at 2500 g for 15 min. Bacteria were lysed in 10 M guanidium  
153 hydrochloride (Sigma-Aldrich) in PBS pH 7.2, centrifuged at 13,000 g for 2 min and  
154 supernatants used for analysis. Alternatively bacterial pellets from 20 mL culture were  
155 resuspended in 40 mL/g osmotic shock buffer (500 mM sucrose, 30 mM Tris-HCl, 1 mM  
156 EDTA, pH 8.0; all Sigma-Aldrich) and centrifuged at 3500 g at 10 °C for 10 min. Pellets were  
157 then resuspended in 25 mL/g ice-cold distilled water, incubated for 5 min, then centrifuged at  
158 3500 g for 10 min at 4 °C to obtain the periplasmic fraction.

159

#### 160 *Analysis of recombinant SCI analogues by ELISA and Western blotting*

161 Fifty microlitres of bacterial lysate were added to nickel-coated ELISA wells (Ni-NTA  
162 HisSorb strips, Qiagen) and incubated at room temperature for 2 h. After three washes with  
163 200 µL PBS supplemented with 0.1% Tween-20 (PBST), 50 µL per well of anti-FLAG M2  
164 HRP antibody conjugate (Sigma-Aldrich) diluted 1:10,000 in PBST was added and plates  
165 incubated for 1 h. After a further six washes with PBST, binding of anti-FLAG was detected  
166 by adding 50 µL per well of 3,3',5,5'-Tetramethylbenzidine (TMB liquid substrate system for  
167 ELISA, Sigma-Aldrich) and the reaction stopped by adding 100 µL 2M sulphuric acid (SLS).  
168 The optical density at 450 nm (OD<sub>450</sub>) of each well was then measured using a SpectraMax M2



169 microplate reader. Results are shown as the mean OD of triplicate wells, following subtraction  
170 of values for background wells containing lysis buffer only. Estimation of FLAG-tagged  
171 recombinant protein concentration in bacterial periplasmic samples was performed by  
172 reference to a standard curve, constructed using a dilution series of FLAG-BAP control protein  
173 (Sigma-Aldrich) in an anti-FLAG ELISA.

174

175 Bacterial periplasmic fractions were reduced using 10%  $\beta$ -mercaptoethanol (Sigma-  
176 Aldrich) in sodium dodecyl sulphate (SDS) buffer (RunBlue LDS sample buffer, Expedeon)  
177 for 15 min at 95 °C. Denatured proteins were separated under reducing conditions using 16%  
178 PAGE gels (RunBlue Gel, Expedeon) in an X-cell SureLock Mini-cell (Invitrogen) at constant  
179 180V for 60 min. Transfer of the separated proteins to nitrocellulose membranes was performed  
180 in the X-cell II Blot module (Invitrogen) using transfer buffer (PAGEgel.com) under reducing  
181 conditions at constant 30 V for 60 min. Membranes were rinsed with PBST, blocked in PBST  
182 supplemented with 5% dried skimmed milk (Marvel, Premier International Food) overnight,  
183 then incubated for 60 min in 15 mL of anti-FLAG HRP antibody diluted 1:5000 in PBST/5%  
184 milk. Membranes were subsequently washed four times with PBST and antibody binding  
185 detected by enhanced chemiluminescence (Amersham ECL Western Blotting Detection  
186 Reagents, GE Healthcare) using autoradiography film (Amersham Hyperfilm ECL, GE  
187 Healthcare).

188

#### 189 *Receptor-binding assays*

190 Flat bottomed MaxiSorp microplates (Nunc) were coated with 50  $\mu$ L per well  
191 recombinant human insulin receptor (rhINSR) or IGF-1 receptor (rhIGF-1R) (R&D Systems)  
192 at 4  $\mu$ g/mL and incubated overnight at 4 °C. Negative control wells contained diluent (0.15 M  
193 PBS) only. After three washes with PBST, plates were blocked with 100  $\mu$ L per well PBST

194 supplemented with 5% bovine serum albumin (BSA), incubated for 3 h and washed a further  
195 three times. Recombinant FLAG-tagged SCI analogues from bacterial periplasmic fractions  
196 were added at 50  $\mu$ L per well at the indicated concentrations. Biotinylated bovine insulin  
197 (Sigma-Aldrich) or biotinylated recombinant human IGF-1 (bIGF-1; IBT Systems) were used  
198 as positive controls. Plates were incubated for 1 h prior to washing three times with PBST, then  
199 100  $\mu$ L per well of anti-FLAG HRP at 1:10,000 dilution or streptavidin HRP (Sigma-Aldrich)  
200 at 1:200 dilution (both in PBST/0.1% BSA) added. Plates were incubated for 1 h, washed six  
201 times with PBST then 50  $\mu$ L per well of supersensitive TMB (Sigma-Aldrich) added. The  
202 reaction was stopped by adding 100  $\mu$ L 2M sulphuric acid and the absorbance at 450 nm  
203 ( $OD_{450}$ ) measured using a SpectraMax M2 microplate reader. Results were calculated as the  
204 mean  $OD_{450}$  of triplicate wells following subtraction of background values in the absence of  
205 receptor; binding curves were constructed and analysed according to a four-parameter logistic  
206 equation using GraphPad Prism version 5.0 for Windows. Relative binding activities were  
207 compared using one-way ANOVA, followed by the Student's *t*-test with the Bonferroni post-  
208 hoc correction applied (PASW Statistics for Windows). Adjusted values were considered  
209 significant at  $P < 0.05$ .

210

### 211 *Glucose uptake assay*

212 The ability of recombinant SCI analogues to induce a biological effect was assessed by  
213 measuring insulin-stimulated glucose uptake in cultured adipocytes. This was achieved using  
214 a mouse fibroblast cell line (3T3-L1, ATCC) which were differentiated into mature adipocytes  
215 (see Appendix: Supplementary material), then cultured with a fluorescent glucose analogue, 6-  
216 (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG), according to a  
217 previously described protocol (Jung et al., 2011). Briefly, triplicate wells of 3T3-L1 cells  
218 cultured in 24-well plates were used for experiments on days 8-15 post-induction of

219 differentiation. Cells were cultured in serum-free, low glucose (1.5 g/L) Dulbecco's Modified  
220 Eagles Medium (Sigma-Aldrich) for 2 h at 37 °C, 5% CO<sub>2</sub>. Following aspiration of culture  
221 medium, cells were then incubated for 1 h at 37 °C, 5% CO<sub>2</sub>, in 250 μL PBS containing 100  
222 μM 6-NBDG in the presence or absence of 5 nM porcine insulin (Sigma-Aldrich), which is  
223 identical to canine insulin, or the different recombinant SCI analogues, isolated from bacterial  
224 periplasmic fractions. Cells were washed three times with 500 μL PBS and lysed using 650 μL  
225 per well 90% dimethyl-sulfoxide. Two hundred microlitres was transferred in triplicate to black  
226 96-well plates and fluorescence measured using a SpectraMax M2 microplate reader ( $\lambda_{\text{ex}} = 466$   
227 nm,  $\lambda_{\text{em}} = 540$  nm, cut-off = 530 nm). Fluorescence was compared between stimulated and  
228 unstimulated cells and between the different SCI analogues using one-way ANOVA, followed  
229 by the Student's *t*-test with the Bonferroni post-hoc correction applied (PASW Statistics for  
230 Windows). Adjusted values were considered significant at  $P < 0.05$ .

231

## 232 **Results**

### 233 *Characterisation of recombinant single-chain insulin analogues*

234 All constructs (rcPROINS, rcINS[GGGPGKR], rcINS[cIGF2C] and rcINS[hIGF2C])  
235 were confirmed by sequencing in both pTAC-MAT-Tag-2 and pFLAG-MAT-Tag-ATS  
236 expression vectors. All constructs were induced to express recombinant protein simultaneously  
237 for 4 h and ELISA of bacterial whole cell lysates demonstrated the presence of recombinant  
238 protein expressing both MAT and FLAG epitopes (Fig. 2). Since the SCI analogues were  
239 designed flanked by FLAG at the 5' end and MAT at the 3' end, this was evidence that  
240 recombinant protein was being expressed as anticipated, with expression from the pFLAG-  
241 MAT-Tag-ATS vector greater than from the pTAC-MAT-Tag-2 vector. Western blotting was  
242 performed on the periplasmic fractions of bacteria transformed with the pFLAG-MAT-Tag-  
243 ATS constructs, confirming that the recombinant proteins were of the anticipated sizes (Fig.

244 3).

245

### 246 *Binding of recombinant SCI analogues to insulin and IGF-1 receptors*

247 To assess differential binding of the SCI analogues to the INSR and IGF-1R, a direct  
248 ELISA was initially performed. This demonstrated that biotinylated insulin and IGF-1  
249 preferentially bound their cognate receptors (Fig. 4A). Although rcPROINS showed a degree  
250 of binding to the INSR and IGF-1R, binding to the INSR was greater and more selective,  
251 compared to binding to IGF-1R for the three SCI analogues assessed (Fig. 4A). Comparing the  
252 relative binding for ligands to rhINSR and rhIGF-1R, revealed ratios of 1.6:1 for rcPROINS,  
253 4.9:1 for rcINS[GGGPGKR], 3.1:1 for rcINS[cIGF-2C] and 4.9:1 for rcINS[hIGF-2C],  
254 compared to 5.5:1 for insulin and 0.17:1 for IGF-1.

255

256 Dose-response curves for binding of each recombinant protein to rhINSR demonstrated  
257 that saturation was reached at approximately 5 nM (Fig. 4B). The dose-response curve for  
258 rcPROINS was shifted to the right, whilst the curves for all SCI analogues were similar. A  
259 competitive-inhibition ELISA against biotinylated insulin was performed to obtain  
260 comparative IC<sub>50</sub> values for the binding of each rcINS to the insulin receptor (Fig. 4C). It was  
261 not possible to obtain an IC<sub>50</sub> value for rcPROINS, since sufficient inhibition of the signal  
262 could not be achieved with the maximum concentration of recombinant protein available. The  
263 relative IC<sub>50</sub> values for rcINS[cIGF-2C] and for rcINS[hIGF-2C] were similar, at 593 pM and  
264 613 pM respectively. The lowest IC<sub>50</sub> was demonstrated by rcINS[GGGPGKR], at 187 pM,  
265 suggesting a higher affinity for the insulin receptor compared to the rcINS[IGF-2C] constructs.

266

### 267 *Stimulation of glucose uptake by recombinant SCI analogues*

268 Fluorescent glucose uptake was not significantly different comparing unstimulated

269 cells to cells incubated with rcPROINS (Fig. 5). In contrast, fluorescent glucose uptake by cells  
270 incubated with porcine insulin, rcINS[GGGPGKR], rcINS[cIGF-2C] and rcINS[hIGF-2C] was  
271 significantly greater than in unstimulated cells ( $P < 0.05$ ). While cells cultured with each SCI  
272 analogue demonstrated significantly greater fluorescence than with rcPROINS ( $P < 0.05$ ), there  
273 was no significant difference comparing the three different recombinant SCI analogues.

274

## 275 **Discussion**

276 The present study developed a number of plasmid DNA constructs that could be used  
277 for prokaryotic expression of recombinant SCI analogues, where the insulin C-peptide was  
278 replaced with either a synthetic linker, canine IGF-2 C-peptide or human IGF2-C peptide.  
279 These SCI analogues were shown to be capable of binding to the INSR and stimulating glucose  
280 uptake in vitro.

281

282 Recombinant proteins produced in this study were expressed as epitope-tagged fusion  
283 proteins. Prokaryotic vectors were selected that produced recombinant proteins with a C-  
284 terminal metal-affinity tag. Such epitope-tagged recombinant proteins can be immobilised  
285 using metals such as nickel, which is useful for both detection and purification. A FLAG  
286 epitope was inserted upstream of the B-chain coding sequence, which also possessed an  
287 enterokinase cleavage site immediately before the start of the B-chain, so that this epitope could  
288 potentially be removed, if the presence of the FLAG peptide inhibited receptor binding.

289

290 Expression of each construct was assessed by both ELISA and Western blotting,  
291 demonstrating that recombinant proteins of the appropriate sizes were produced from both  
292 pTAC-MAT-Tag-2 and pFLAG-MAT-Tag-ATS vectors in *E. coli*. Expression from pTAC-  
293 MAT-Tag-2 resulted in production of recombinant protein for each rcINS construct, although

294 subsequent work demonstrated that this was primarily in the insoluble fraction (data not  
295 shown). Expression of recombinant protein in *E. coli* commonly results in aggregation and  
296 formation of inclusion bodies (Thomas and Baneyx, 1996). The reducing environment of the  
297 bacterial cytosol does not favour folding of eukaryotic protein, especially those reliant on  
298 disulphide bond formation (Mergulhao et al., 2004), such as insulin (Williams et al., 1982).

299

300 Unlike the bacterial cytosol, the periplasm (the space between the inner cytoplasmic  
301 membrane and external outer membrane of Gram-negative bacteria) provides an oxidising  
302 environment which is more favourable for protein folding and disulphide bond formation  
303 (Baneyx and Mujacic, 2004). Therefore, the pFLAG-MAT-Tag-ATS vector was employed in  
304 an attempt to increase the yield of soluble protein. The pFLAG-MAT-Tag-ATS vector encodes  
305 the outer membrane protein A signal peptide (OmpA) and incorporation of this sequence at the  
306 N-terminus targets recombinant protein to the periplasm, where the OmpA is cleaved by signal  
307 peptidase as the recombinant protein crosses the bacterial inner cytoplasmic membrane (Freudl  
308 et al., 1987). Western blot analysis of periplasmic preparations, isolated for use in downstream  
309 assays demonstrated the molecular weight differences between the different rcINS proteins  
310 (Fig. 3).

311

312 Receptor-binding assays were developed to assess the different SCI analogues against  
313 rhINSR and rhIGF-1R, with biotinylated insulin and IGF-1 used as control ligands. Although  
314 human recombinant receptors were used, this was considered appropriate since canine IGF-1<sup>2</sup>  
315 and human IGF-1<sup>3</sup> share an identical sequence and canine insulin<sup>4</sup> and human insulin<sup>5</sup> differ  
316 by only one amino acid in the B chain (B30A/T). However, it should be noted that these studies

---

<sup>2</sup> See: <http://www.uniprot.org/uniprot/P33712>

<sup>3</sup> See: <http://www.uniprot.org/uniprot/Q9NP10>

<sup>4</sup> See: <http://www.uniprot.org/uniprot/P01321>

<sup>5</sup> See: <http://www.uniprot.org/uniprot/P01308>

317 were undertaken with heterologous receptors, which might not represent the situation with  
318 autologous canine receptors. Initial studies established the working range of the assays with a  
319 receptor concentration of 2–4  $\mu\text{g/mL}$  found to be optimal, resulting in sensitivity for ligand  
320 binding down to approximately 0.1 nM. It was important to assess binding to rhIGF-1R as well  
321 as to rhINSR, since an insulin analogue might have the potential for increased affinity, resulting  
322 from changes to the peptide sequence, with subsequent mitogenic activity and increased  
323 oncogenic potential (Vajo et al., 2001).

324

325         Detection using anti-FLAG antibody confirmed that all SCI analogues were able to  
326 bind to both rhINSR and rhIGF-1R. Binding of rcPROINS to rhINSR resulted in a significantly  
327 weaker signal compared to each of the SCI analogues, suggesting lower affinity for the  
328 receptor. This is consistent with other studies, which demonstrate that proinsulin has relative  
329 affinity of 1–2% for INSR compared to insulin (Lee et al., 2000). There was no significant  
330 difference in signal for rhINSR binding comparing the different SCI analogues. Incubation  
331 with rhIGF-1R gave significantly reduced signals for rcPROINS and for each SCI analogue,  
332 compared to binding to rhINSR, and there was no significant difference in rhIGF-1R signal  
333 within the rcPROINS/rcINS group.

334

335         The results of the receptor-binding assays suggest that all SCI analogues demonstrate  
336 greater affinity for the rhINSR than for the rhIGF-1R, with rcINS[GGGPGKR] and  
337 rcINS[hIGF-2C] showing relative binding similar to that demonstrated by insulin. Using a  
338 competitive-inhibition ELISA against INSR, the rcINS[GGGPGKR] analogue demonstrated  
339 the highest relative affinity ( $\text{IC}_{50} = 187 \text{ pM}$ ), followed by rcINS[cIGF-2C] ( $\text{IC}_{50} = 593 \text{ pM}$ ) and  
340 rcINS[cIGF-2C] ( $\text{IC}_{50} = 613 \text{ pM}$ ). It was not possible to calculate  $\text{IC}_{50}$  for rcPROINS since  
341 sufficient inhibition could not be achieved, even using a concentration of 20 nM. This indicates

342 that each of the SCI analogues showed much greater affinity than rcPROINS. The two  
343 insulin/IGF-2 C-peptide chimaeric proteins demonstrated similar relative affinity for rhINSR,  
344 which is unsurprising since they differ by only two residues (Fig. 1B).

345

346 There are several other studies describing binding characteristics of SCI analogues. Lee  
347 et al. (2000), who developed a human SCI analogue containing the GGGPGKR linker,  
348 compared its receptor binding with both insulin and proinsulin, reporting an affinity for the  
349 INSR of 28% compared to native insulin, and 1350% that of proinsulin. In a more recent study  
350 investigating over 30 different SCI analogues, it was concluded that intermediate linking  
351 peptides of 7-10 residues, composed primarily of glycine residues (to promote molecular  
352 flexibility) along with a C-terminal arginine, demonstrate the greatest INSR affinities and  
353 bioactivity (Rajpal et al., 2009). Indeed, a SCI analogue with a hexapeptide linking sequence  
354 (GGGPRR) has demonstrated enhanced INSR affinity of 130% compared to native insulin,  
355 although in that particular molecule the A8:threonine residue was also replaced with histidine  
356 (Hua et al., 2008).

357

358 We are not aware of any studies that have investigated SCI analogues based on the  
359 concept of an insulin/IGF-2 C-peptide chimaera, although there has been a report of an  
360 insulin/IGF-1 C-peptide chimaera (Kristensen et al, 1995). The binding affinity for that  
361 molecule compared favourably with insulin, and was reported to be between 55-94%. This was  
362 surprising since the presence of the linking peptide was predicted to limit the flexibility of the  
363 insulin domains necessary for receptor binding, and it was also expected to impair the  
364 important A1:glycine residue essential for receptor binding (Pullen et al., 1976). It was  
365 proposed that the C-peptide of IGF-1 interacted directly with a conserved domain in the INSR  
366 since this receptor and the IGF-1R are similar. This is unlikely to be the case with an



367 insulin/IGF-2 C-peptide chimaera since the IGF-2R belongs to a functionally different receptor  
368 class.

369

370 In glucose uptake assays, adipocytes derived from 3T3-L1 cells incubated with porcine  
371 insulin, rcINS[GGGPGKR], rcINS[cIGF-2C] or rcINS[hIGF-2C] all resulted in significantly  
372 higher fluorescence (suggesting GLUT4 mediated 6-NBDG uptake) compared to unstimulated  
373 cells. All SCI analogues resulted in comparable 6-NBDG uptake, which was significantly  
374 greater than that seen with rcPROINS. This indicates that substitution of the proinsulin C-  
375 peptide for the various linker peptides resulted in biologically-active molecules.

376

### 377 **Conclusions**

378 The present study has demonstrated that it is possible to produce recombinant canine  
379 SCI analogues that are capable of INSR binding and stimulating glucose uptake in cells,  
380 without the need for post-translational processing. This work paves the way for production of  
381 recombinant canine insulin analogues that could be scaled up, with a view to developing novel  
382 therapeutics for canine diabetes.

383

### 384 **Conflict of interest statement**

385 None of the authors of this paper has any financial or personal relationship with other  
386 people or organizations that could inappropriately influence or bias the content of the paper.  
387 This work was undertaken as part of a BBSRC CASE studentship with MSD Animal Health  
388 as the industrial sponsor. MSD Animal Health had no involvement in the study design,  
389 collection, analysis and interpretation of data, writing of this manuscript or the decision to  
390 submit the article for publication.

391

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395

396 **Appendix: Supplementary material**

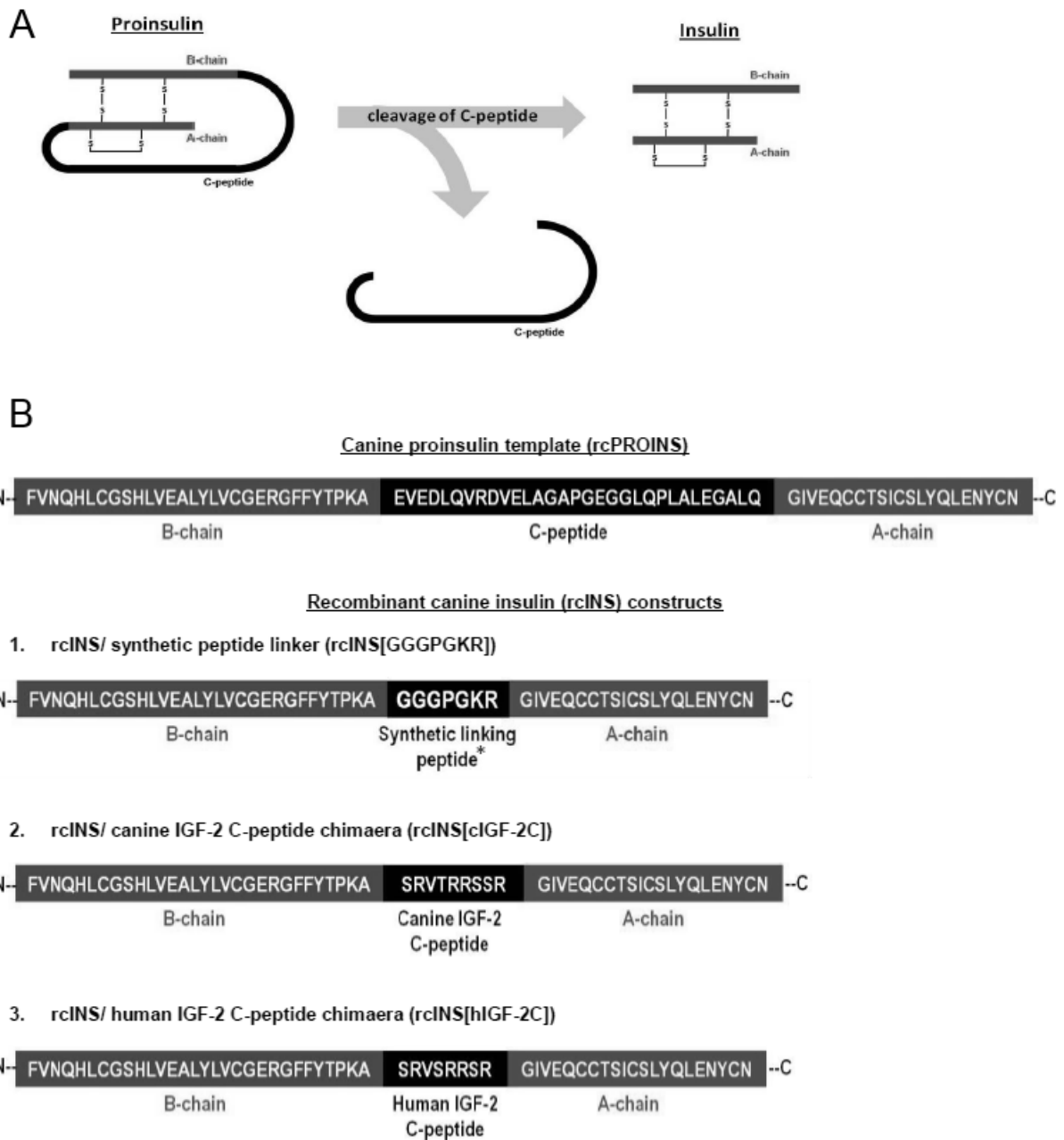
397 Supplementary data to this article can be found online at doi:xxx

398

399 **References**

- 400 Baneyx, F., Mujacic, M., 2004. Recombinant protein folding and misfolding in *Escherichia*  
401 *coli*. *Nature Biotechnology* 22, 1399-1408.  
402
- 403 Boari, A., Barreca, A., Bestetti, G.E., Minuto, F., Venturoli, M., 1995. Hypoglycemia in a dog  
404 with a leiomyoma of the gastric wall producing an insulin-like growth factor II-like  
405 peptide. *European Journal of Endocrinology* 132, 744-750.  
406
- 407 Borgono, C.A., Zinman, B., 2012. Insulins: Past, present, and future. *Endocrinology and*  
408 *Metabolism Clinics of North America* 41, 1-24.  
409
- 410 Catchpole, B., Kennedy, L.J., Davison, L.J., Ollier, W.E., 2008. Canine diabetes mellitus: from  
411 phenotype to genotype. *Journal of Small Animal Practice* 49, 4-10.  
412
- 413 Chan, S.J., Steiner, D.F., 2000. Insulin through the ages: Phylogeny of a growth promoting and  
414 metabolic regulatory hormone. *American Zoologist* 40, 213-222.  
415
- 416 Christensen, T., Dalboge, H., Snel, L., 1991. Postbiosynthesis modification: Human growth  
417 hormone and insulin precursors. *Bioprocess Technology* 13, 206-221.  
418
- 419 DeChiara, T.M., Efstratiadis, A., Robertson, E.J., 1990. A growth-deficiency phenotype in  
420 heterozygous mice carrying an insulin-like growth factor II gene disrupted by  
421 targeting. *Nature* 345, 78-80.  
422
- 423 Freudl, R., Schwarz, H., Degen, M., Henning, U., 1987. The signal sequence suffices to direct  
424 export of outer membrane protein OmpA of *Escherichia coli* K-12. *Journal of*  
425 *Bacteriology* 169, 66-71.  
426
- 427 Hua, Q.X., Nakagawa, S.H., Jia, W., Huang, K., Phillips, N.B., Hu, S.Q., Weiss, M.A., 2008.  
428 Design of an active ultrastable single-chain insulin analog: Synthesis, structure, and  
429 therapeutic implications. *Journal of Biological Chemistry* 283, 14703-14716.  
430
- 431 Jung, D.W., Ha, H.H., Zheng, X., Chang, Y.T., Williams, D.R., 2011. Novel use of fluorescent  
432 glucose analogues to identify a new class of triazine-based insulin mimetics  
433 possessing useful secondary effects. *Molecular Biosystems* 7, 346-358

434  
435 Kristensen, C., Andersen, A.S., Hach, M., Wiberg, F.C., Schaffer, L., Kjeldsen, T., 1995. A  
436 single-chain insulin-like growth factor I/insulin hybrid binds with high affinity to the  
437 insulin receptor. *Biochemical Journal* 305, 981-986.  
438  
439 Lee, H.C., Kim, S.J., Kim, K.S., Shin, H.C., Yoon, J.W., 2000. Remission in models of type 1  
440 diabetes by gene therapy using a single-chain insulin analogue. *Nature* 408, 483-488.  
441  
442 LeRoith, D., 2004. Non-islet cell hypoglycemia. *Annals of Endocrinology* 65, 99-103.  
443  
444 Liu, J.P., Baker, J., Perkins, A.S., Robertson, E.J., Efstratiadis, A., 1993. Mice carrying null  
445 mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF  
446 receptor (Igf1r). *Cell* 75, 59-72.  
447  
448 Mergulhao, F.J., Taipa, M.A., Cabral, J.M., Monteiro, G.A., 2004. Evaluation of bottlenecks in  
449 proinsulin secretion by *Escherichia coli*. *Journal of Biotechnology* 109, 31-43.  
450  
451 Peavy, D.E., Brunner, M.R., Duckworth, W.C., Hooker, C.S., Frank, B.H., 1985. Receptor  
452 binding and biological potency of several split forms (conversion intermediates) of  
453 human proinsulin. Studies in cultured IM-9 lymphocytes and in vivo and in vitro in  
454 rats. *Journal of Biological Chemistry* 260, 13989-13994.  
455  
456 Pullen, R.A., Lindsay, D.G., Wood, S.P., Tickle, I.J., Blundell, T.L., Wollmer, A., Krail, G.,  
457 Brandenburg, D., Zahn, H., Gliemann, J., Gammeltoft, S., 1976. Receptor-binding  
458 region of insulin. *Nature* 259, 369-373.  
459  
460 Rajpal, G., Liu, M., Zhang, Y., Arvan, P., 2009. Single-chain insulins as receptor agonists.  
461 *Molecular Endocrinology* 23, 679-688.  
462  
463 Riggs, A.D., Itakura, K., 1979. Synthetic DNA and medicine. *American Journal of Human*  
464 *Genetics* 31, 531-538.  
465  
466 Rucinsky, R., Cook, A., Haley, S., Nelson, R., Zoran, D.L., Poundstone, M., 2010. AAHA  
467 diabetes management guidelines for dogs and cats. *Journal of the American Animal*  
468 *Hospital Association* 46, 215-224.  
469  
470 Thomas, J.G., Baneyx, F., 1996. Protein misfolding and inclusion body formation in  
471 recombinant *Escherichia coli* cells overexpressing heat-shock proteins. *Journal of*  
472 *Biological Chemistry* 271, 11141-11147.  
473  
474 Vajo, Z., Fawcett, J., Duckworth, W.C., 2001. Recombinant DNA technology in the treatment  
475 of diabetes: Insulin analogs. *Endocrinology Reviews* 22, 706-717.  
476  
477 Williams, D.C., Van Frank, R.M., Muth, W.L., Burnett, J.P., 1982. Cytoplasmic inclusion  
478 bodies in *Escherichia coli* producing biosynthetic human insulin proteins. *Science*  
479 215, 687-689.  
480  
481 Zini, E., Glaus, T.M., Minuto, F., Arvigo, M., Hauser, B., Reusch, C.E., 2007. Paraneoplastic  
482 hypoglycemia due to an insulin-like growth factor type-II secreting hepatocellular  
483 carcinoma in a dog. *Journal of Veterinary Internal Medicine* 21, 193-195.  
484



486

487 Fig. 1. (A) Post-translational modification of proinsulin to yield the biologically-active insulin

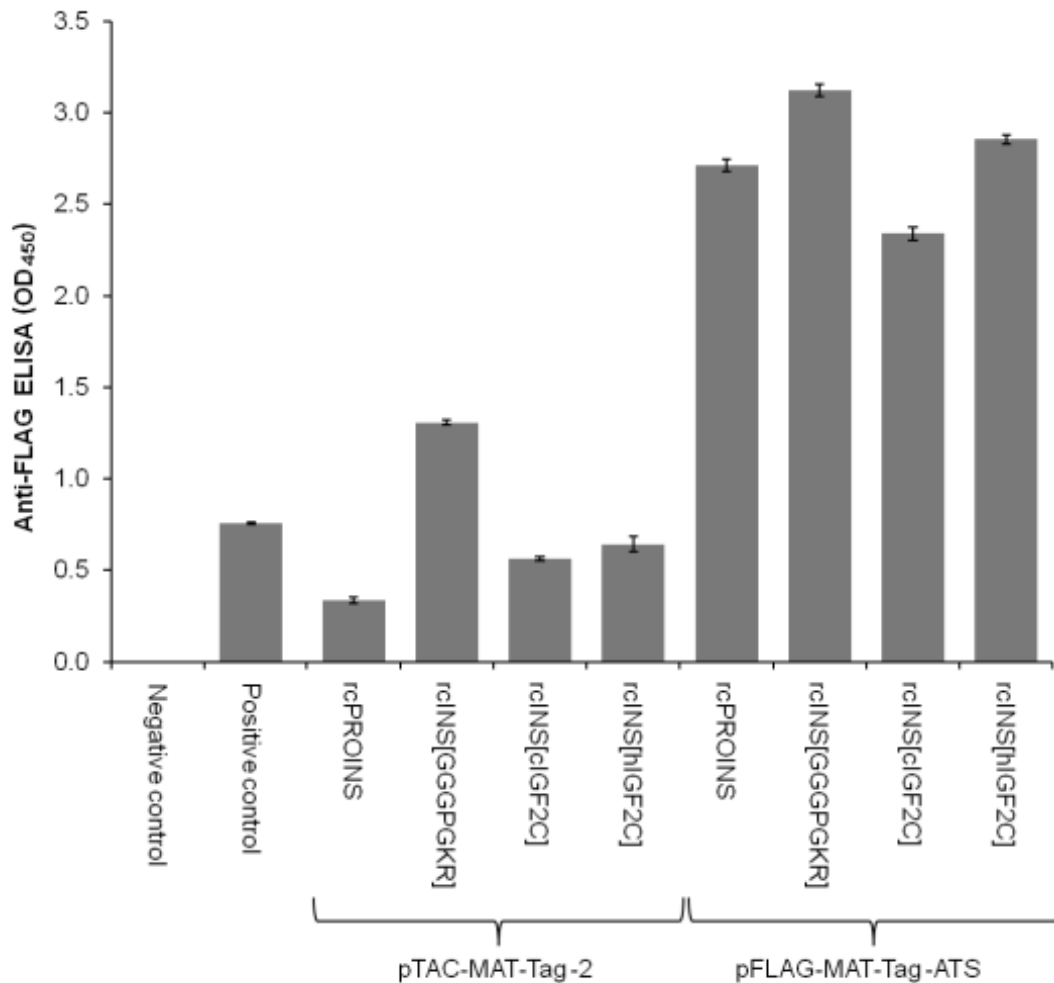
488 and C-peptide. (B) Details of single chain insulin analogues adapted from recombinant canine

489 proinsulin (rcPROINS) for the present study. Three different recombinant canine insulin

490 analogues (rcINS) were generated, where the insulin C-peptide was substituted with different

491 linking peptide sequences.

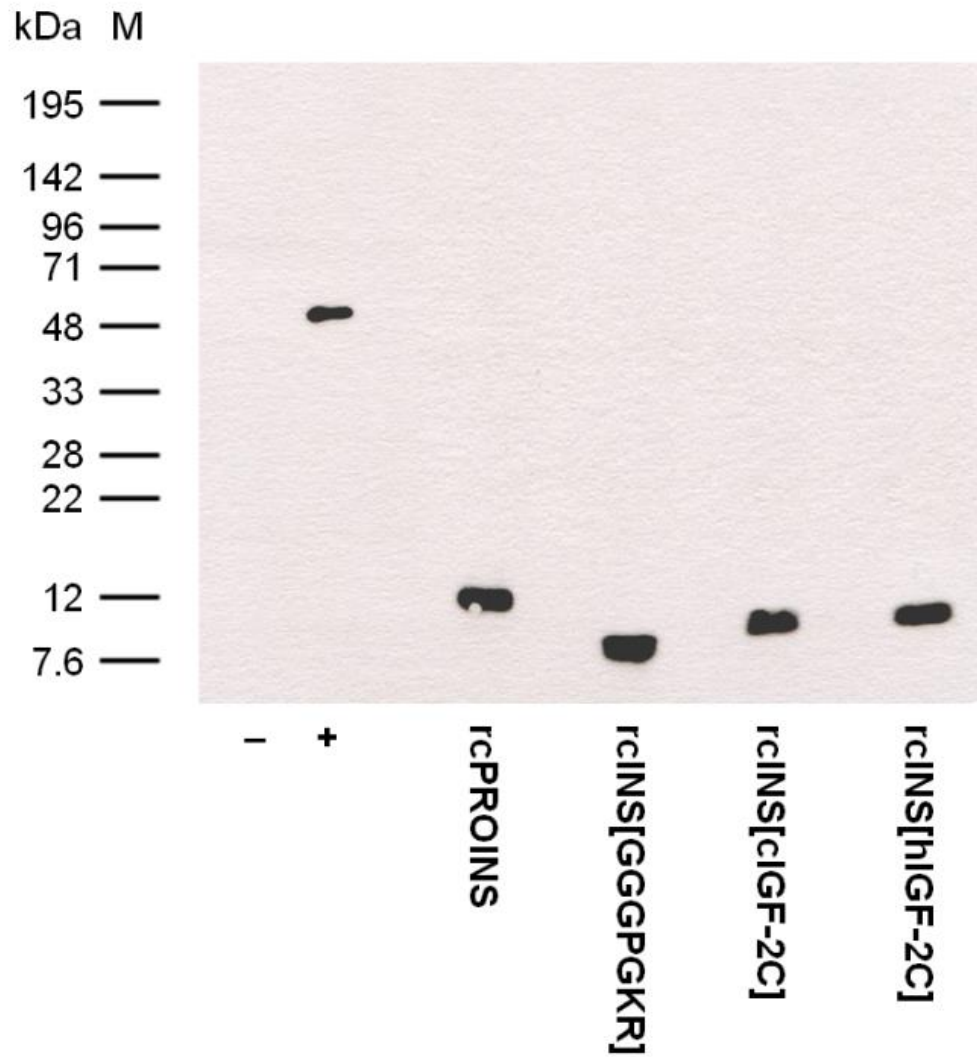
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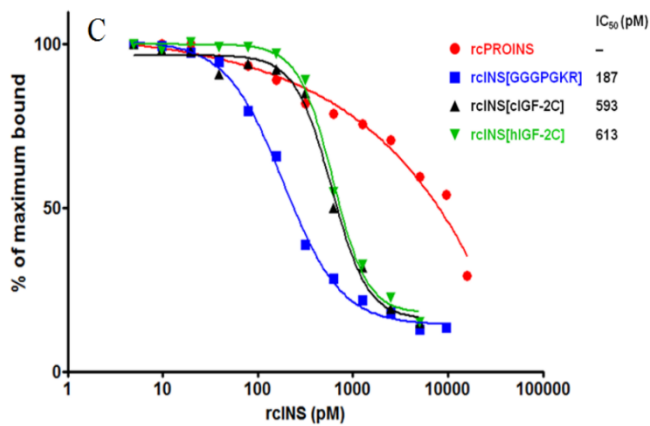
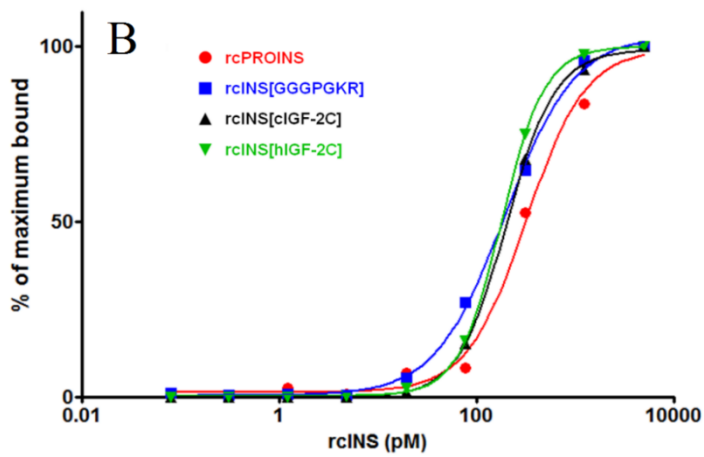
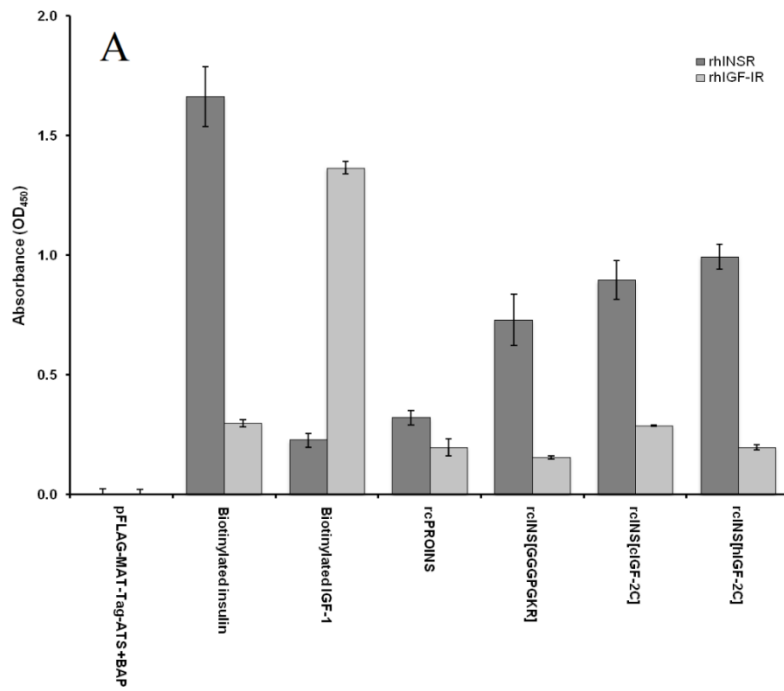
494 Fig. 2. Detection of FLAG-tagged recombinant protein. BL-21(T1) *Escherichia coli* were  
 495 transformed with each rcINS construct in one of two expression vectors. After induction of  
 496 expression, bacterial lysates were prepared and analysed by ELISA, using nickel-coated plates  
 497 to capture proteins expressing a metal-affinity tag and detected using an anti-FLAG antibody.  
 498 Absorbance is shown as the mean  $\pm$  standard error of the mean (SEM) of triplicate wells  
 499 following subtraction of background (lysis buffer only). Negative control is lysate from  
 500 bacteria transformed with wild-type pTAC-MAT-Tag-2 plasmid DNA. Positive control is lysate  
 501 from bacteria transformed with pFLAG-MAT-Tag-ATS+BAP. The experiment was repeated  
 502 with similar results.

503



504

505 Fig. 3. Western blotting of FLAG-tagged rcINS isolated from bacterial periplasmic fractions.  
 506 BL-21 (T1) *Escherichia coli* were transformed with pFLAG-MAT-Tag-ATS plasmid DNA  
 507 containing the indicated constructs. After induction of expression, bacteria were subjected to  
 508 osmotic lysis and periplasmic samples analysed by Western blotting, using an anti-FLAG  
 509 antibody, detected by enhanced chemiluminescence. The negative control (-) is from bacteria  
 510 transformed with wild-type pTAC-MAT-Tag-2 vector and the positive control (+) is from  
 511 bacteria transformed with pFLAG-MAT-Tag-ATS+BAP (anticipated size of bacterial alkaline  
 512 phosphatase ~50kDa). M, molecular weight marker.



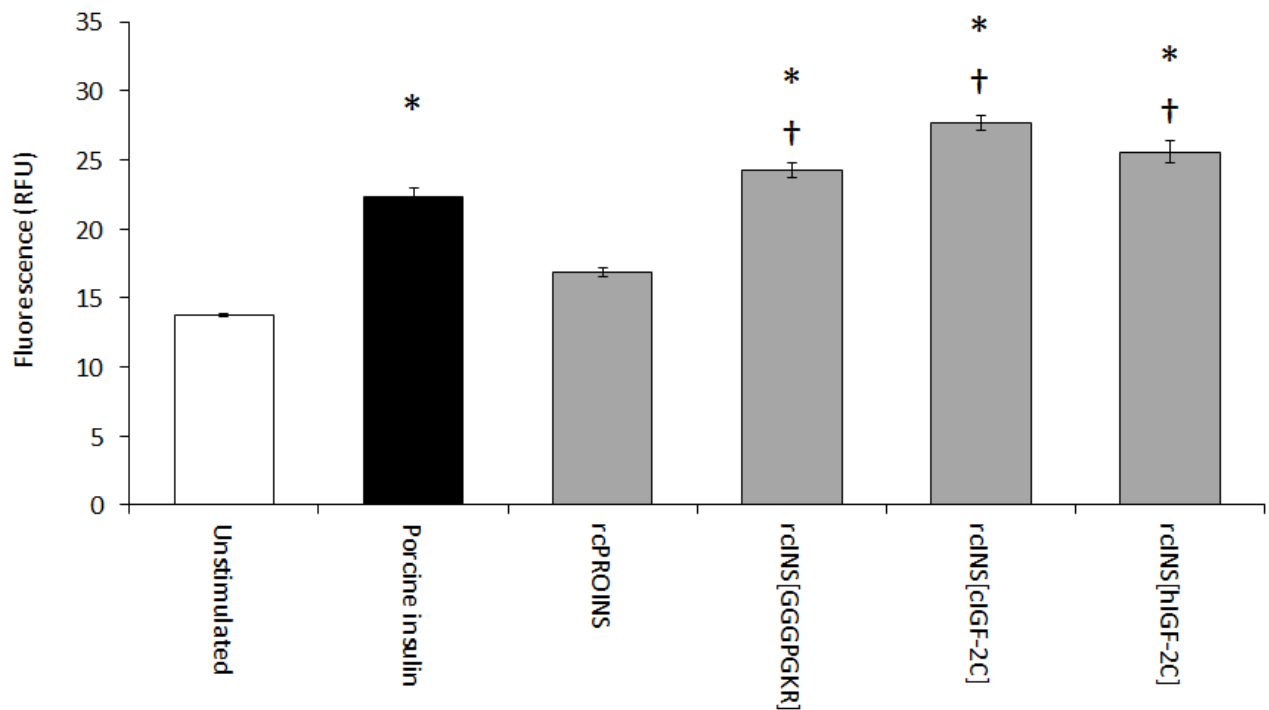
513

514 Fig. 4. (A) Binding of rcINS constructs to INSR and IGF-1R. ELISA wells were coated with 4

515  $\mu\text{g/mL}$  rhINSR or rhIGF-1R. Biotinylated bovine insulin, biotinylated human IGF-1 or

516 periplasmic fractions of the indicated constructs (all 5 nM) were added and binding detected  
517 using either streptavidin-HRP or anti-FLAG HRP antibody. Absorbance is shown as the mean  
518  $\pm$  standard error of the mean (SEM) of triplicate wells. (B) Dose-response curve for binding of  
519 rcINS constructs to INSR. (C) Competition-ELISA for binding of rcINS constructs to INSR.  
520 Increasing concentrations of rcINS protein were co-cultured in rhINSR coated plates in the  
521 presence of 3 nM biotinylated bovine insulin, with binding detected using streptavidin-HRP.  
522





523

524 Fig. 5. Assessment of insulin-stimulated glucose uptake in 3T3-L1 adipocytes. 3T3-L1  
 525 adipocytes were serum-starved in low-glucose DMEM for 2 h prior to incubation with  
 526 fluorescent glucose analogue 6-NBDG for 1 h in the presence of 5 nM porcine insulin,  
 527 recombinant canine proinsulin (rcPROINS) or the indicated recombinant canine insulin  
 528 analogues. Cells were lysed and fluorescence measured ( $\lambda_{\text{ex}} = 466 \text{ nm}$ ,  $\lambda_{\text{em}} = 540 \text{ nm}$ , cut-off  
 529 = 530 nm). Results represent the mean of triplicate samples  $\pm$  SEM. The Student's *t* test was  
 530 used to compare fluorescence of stimulated cells to unstimulated cells (\*  $P < 0.05$ ) and to  
 531 compare recombinant insulin analogues to rcPROINS ( $\dagger P < 0.05$ ).

532

533  
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537

## Appendix: Supplementary Table 1

Restriction endonuclease sites shown underlined; coding sequences in bold refer to explanation in target column.

Primer name	Target	Primer sequence	Amplicon length (bp)
PROINS pTACMAT <i>EcoRI/SphI</i>	Canine proinsulin coding sequence with additional restriction site <i>EcoRI</i> for cloning	For: <u>GAATTC</u> GTTAACCAGCACCTG	268
	Canine proinsulin coding sequence with additional restriction site <i>SphI</i> for cloning	Rev: <u>GCA</u> TGCCGTTGCAGTAATTCCTCCAG	
INS A CORE	Amplification of canine insulin A chain. Used with PROINS pTACMAT <i>SphI</i> REV.	For: GGCA <b>TCGTGGAGCAGTGC</b>	70
INS B CORE	Amplification of canine insulin B chain. Used with PROINS pTACMAT <i>EcoRI</i> FOR	Rev: GGCCTTAGGCGTGTAGAAG	93
INS A LINK	Modified INS A CORE primer to generate synthetic linker sequence (in bold). Contains <i>XbaI</i> restriction site. Used with PROINS pTACMAT <i>SphI</i> REV	For: <u>CCC</u> GGGTAAGAGAGGCATCGTGGAGCAGTGC	83
INS B LINK	Modified INS B CORE primer to generate synthetic linker sequence (in bold). Contains <i>XbaI</i> restriction site. Used with PROINS pTACMAT <i>EcoRI</i> FOR	Rev: ATAC <u>CCGGG</u> CCACCACCTGCCTTAGGCGGTGTAG	110
INSA cIGF2C	Amplification of canine insulin A chain and part of canine IGF-2 C peptide (in bold). Contains <i>BglII</i> site. Used with PROINS pTACMAT <i>SphI</i> REV	For: <u>AGATC</u> TAGCCGTGGCATCGTGGAGCAGTGC	82
INSB cIGF2C	Amplification of canine insulin B chain and part of canine IGF-2 C peptide (in bold). Contains <i>BglII</i> site. Used with PROINS pTACMAT <i>EcoRI</i> FOR	Rev: <u>GATC</u> TGCGAGTTACGCGAGAGGCCTTAGGCGGTGTAGAAG	113
INSA hIGF2C	Amplification of canine insulin A chain and part of human IGF-2 C peptide (in bold). Contains <i>XbaI</i> site. Used with PROINS pTACMAT <i>SphI</i> REV	For: <u>TCTAG</u> ACGCAGCCGTGGCATCGTGGAGCAGTGC	85
INSB hIGF2C	Amplification of canine insulin B chain and part of human IGF-2 C peptide (in bold). Contains <i>XbaI</i> site. Used with PROINS pTACMAT <i>EcoRI</i> FOR	Rev: <u>GATC</u> TAGACACGCGGCTGGCCTTAGGCGGTGTAGAAG	110

538

For, forward; Rev, reverse; bp, base pairs.

Separate coding sequences for canine insulin A-chain and B-chain were generated by PCR, using pSC-A/rcPROINS pDNA as template. The reverse primer for the insulin B chain, and the forward primer for the insulin A-chain incorporated the additional coding sequence for the synthetic peptide linker (GGGPGKR) as well as for *Xma*I, to facilitate ligation.

PCR product for **insulin B-chain + synthetic peptide linker** (INSB/GGG) coding sequence (110 bp). Primer pair: PROINS pTACMAT *Eco*RI For/ INS B LINK Rev  
 Incorporated 5' *Eco*RI site and 3' *Xma*I site:

*Eco*RI *Xma*I

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GAATTCGTTAACCAGCACCTGTGTGGCTCCCACCTGGTAGAGGCTCTGTACCTGGTGTGCGGGGAGCGGGCTTCTTCTACACGCCTAAGGCAGGTGGTGGCCGGGTAT
CTTAAGCAATTGGTCGTGGACACACCGAGGGTGGACCATCTCCGAGACATGGACCACACGCCCTCGCGCCGAAGAAGATGTGCGGATTCCGTCCACCACCAGGGCCATA
    
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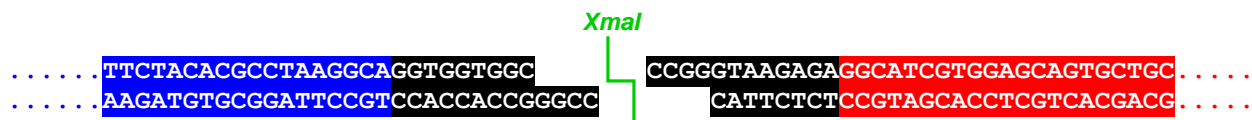
PCR product for **synthetic peptide linker + insulin A-chain** (PKGR/INSA) coding sequence (83 bp). Primer pair: INS A LINK For/ PROINS pTACMAT *Sph*I Rev  
 Incorporated 5' *Xma*I site and 3' *Sph*I site:

*Xma*I *Sph*I

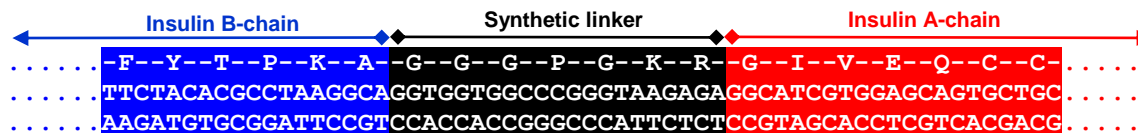
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CCCGGCTAAGAGAGGCATCGTGGAGCAGTGTGCTGCACCAGCATCTGCTCCCTCTACCAGCTGGAGAAATTACTGCAACGGCATGC
GGGCCATTCTCTCCGTAGCACCTCGTCACGACGTGGTTCGTAGACGAGGGAGATGGTCGACCTCTTAATGACGTTGCCGTACG
    
```

1. The complete coding sequence for rcINS[GGGPGKR] was constructed via ligation of sticky ends generated following digestion of coding sequences with *Xma*I.



2. rcINS[GGGPGKR] following ligation:



**Suppl Fig. 1. Generation of rcINS/ synthetic peptide linker (rcINS[GGGPGKR])**

Coding sequences for insulin B-chain highlighted in **blue**, for linking peptide sequence in **black**, and for insulin A-chain highlighted in **red**. Non-coding sequences are not highlighted. Primer binding sites are shown underlined. Restriction sites shown in **green**. Primer sequences are given in Supplementary Table 1.

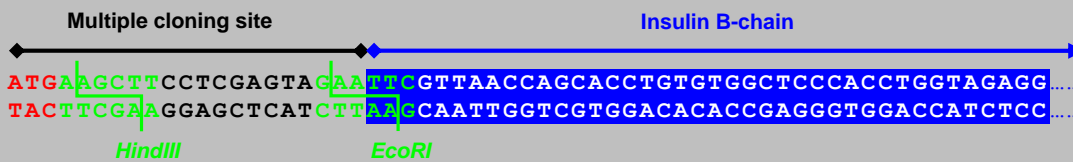
Oligonucleotides coding for FLAG® epitope with added bases for *HindIII* / *EcoRI* restriction sites when dimerised:

FLAG® oligo For : 5' -AGCTAGACTACAAGGACGACGATGACA -3'

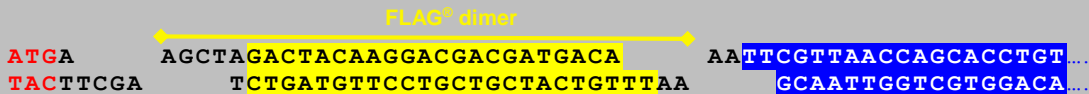
FLAG® oligo Rev: 5' -AATTTGTCATCGTTCGTCCTTGTAGTCT -3'



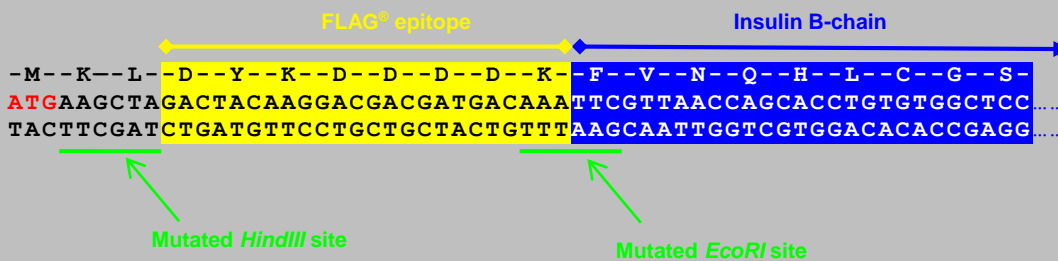
2. Canine insulin B-chain sequence cloned into pTAC-MAT-Tag®-2:



3. Insertion of FLAG oligonucleotide dimer following digestion of vector with *HindIII* and *EcoRI*:



4. Completed sequence:



Suppl. Fig. 2. Schematic illustrating insertion of FLAG® coding sequence into pTAC-MAT-Tag®-2/rcPROINS and pTAC-MAT-Tag®-2/rcINS[GGGPGKR]

FLAG oligonucleotides were annealed to form a dimer and then ligated into the pTAC-MAT-Tag®-2 vector containing rcPROINS or rcINS[GGGPGKR] which had been digested with *HindIII* and *EcoRI*. Recognition sites shown in green, start codon in red.

Separate coding sequences for canine insulin A-chain and B-chain were generated by PCR, using pSC-A/rcPROINS or pTAC-MAT-Tag<sup>®</sup>-2/FLAG<sup>®</sup>-rcPROINS pDNA respectively as template. The reverse primer for the insulin B chain, and the forward primer for the insulin A-chain incorporated the additional coding sequence for the canine IGF-2 C-peptide (cIGF-2C) as well as for *Bgl*III, to facilitate ligation.

PCR product for **insulin B-chain** + **canine IGF-2 C-peptide** coding sequence incorporating the FLAG<sup>®</sup> epitope (141 bp). Primer pair: FLAG<sup>®</sup> For/ INS B cIGF2C Rev  
 Incorporated 5' *Hind*III site and 3' *Bgl*III site (3' terminal T/A added by *Taq* DNA polymerase)

*Hind*III *Bgl*III

```

AAGCTTGACTACAAGGACGACGAAATGACAAATTCGTTAACCAGCACCT . . . . . GGAGCGGGCTTCTTCTACACGCCTAAGGCCTCTCGCGTAACTCGCAGATCT
TTCGAACTGATGTTCC TGCTGCCTTTACTGTTT TTGGTCGTGGACACACC . . . . . CCTCGCGCCGAAGAAGATGTGCGGATTCCGGAGAGCGCATTGAGCGTCTAGA
    
```

PCR product for **canine IGF-2 C-peptide** + **insulin A-chain** coding sequence (82 bp). Primer pair: INS A cIGF2C For/ PROINS pTACMAT *Sph*I Rev  
 Incorporated 5' *Bgl*III site and 3' *Sph*I site:

*Bgl*III *Sph*I

```

AGATCTAGCCGTGGCATCGTGGAGCAGTGTCTGCACCAGCATCTGCTCCCTCTACCAGCTGGAGAATTACTGCAACGGCATGC
TCTAGATCGGCACCGTAGCACCTCGTCACGACGTGGTTCGTAGACGAGGGAGATGGTTCGACCTCTTAATGACGTTGCCGTACG
    
```

1. The complete coding sequence for rcINS[cIGF-2C] was constructed via ligation of sticky ends generated following digestion of both coding sequences with *Bgl*III.



2. rcINS[cIGF-2C] following ligation:



Suppl. Fig. 3. Generation of rcINS/ canine IGF-2 C-peptide chimaera (rcINS[cIGF2-C])

Coding sequences for insulin B-chain highlighted in blue, for linking peptide sequence in black, and for insulin A-chain highlighted in red. FLAG<sup>®</sup> epitope coding sequence highlighted in yellow. Non-coding sequences are not highlighted. Primer binding sites are shown underlined. Restriction sites shown in green. Primer sequences are given in Supplementary Table 1.

Separate coding sequences for canine insulin A-chain and B-chain were generated by PCR, using pSC-A/rcPROINS or pTAC-MAT-Tag<sup>®</sup>-2/FLAG<sup>®</sup>-rcPROINS pDNA respectively as template. The reverse primer for the insulin B chain, and the forward primer for the insulin A-chain incorporated the additional coding sequence for the human IGF-2 C-peptide (hIGF-2C) as well as for *Xba*I, to facilitate ligation.

PCR product for **insulin B-chain + human IGF-2 C-peptide** coding sequence incorporating the FLAG<sup>®</sup> epitope (135 bp). Primer pair: FLAG<sup>®</sup> For/ INS B hIGF2C Rev  
 Incorporated 5' *Eco*RI site and 3' *Xba*I site (3' terminal T/A added by *Taq* DNA polymerase)

*Hind*III *Xba*I

```

AAGCTT GACTACAAGGACGACGAAATGACAAA TTCGTTAACCAGCACCT . . . . . GGAGCGCGGCTTCTTCTACACGCCTAAGGCCAGCCGCGTGTCTAGA
TTCGAACTGATGTTCCCTGCTGCCTTTACTGTTT TGGTTCGTGGACACACC . . . . . CCTCGCGCCGAGAAGATGTGCGGATTCGGTTCGGCGCACAGATCT
    
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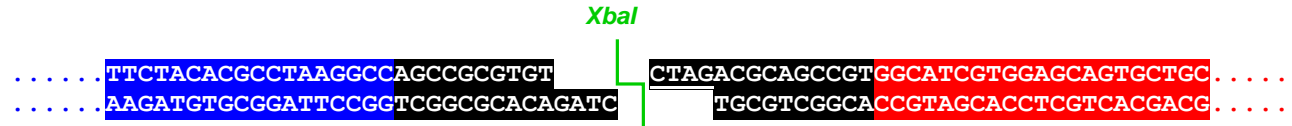
PCR product for **human IGF-2 C-peptide + insulin A-chain** coding sequence (85 bp). Primer pair: INS A hIGF2C/ PROINS pTACMAT *Sph*I Rev  
 Incorporated 5' *Xba*I site and 3' *Sph*I site:

*Xba*I *Sph*I

```

TCTAGACGCAGCCGTGGCATCGTGGAGCAGTGTGTCACCAGCATCTGCTCCCTCTACCAGCTGGAGAAATTAAGTCAACGGCATGC
AGATCTGCGTCGGCACCGTAGCACCTCGTTCACGACGTGGTTCGTAGACGAGGGAGATGGTTCGACCTCTTAATGACGTTCCGTACG
    
```

- The complete coding sequence for rcINS[hIGF-2C] was constructed via ligation of sticky ends generated by digestion of both coding sequences with *Xba*I.



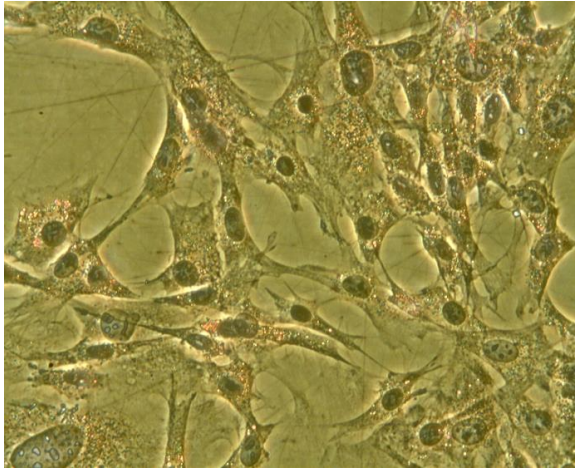
- rcINS/hIGF-2C following ligation:



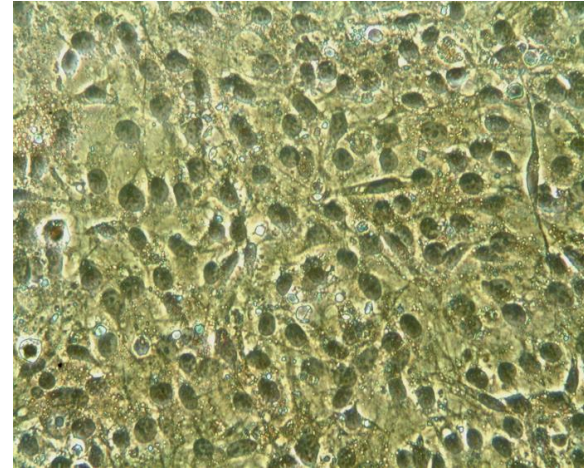
**Suppl. Fig. 4. Generation of rcINS/ human IGF-2 C-peptide chimaera (rcINS[hIGF2-C])**

Coding sequences for insulin B-chain highlighted in **blue**, for linking peptide sequence in **black**, and for insulin A-chain highlighted in **red**. FLAG<sup>®</sup> epitope coding sequence highlighted in **yellow**. Non-coding sequences are not highlighted. Primer binding sites are shown underlined. Restriction sites shown in **green**. Primer sequences are given in Supplementary Table 1.

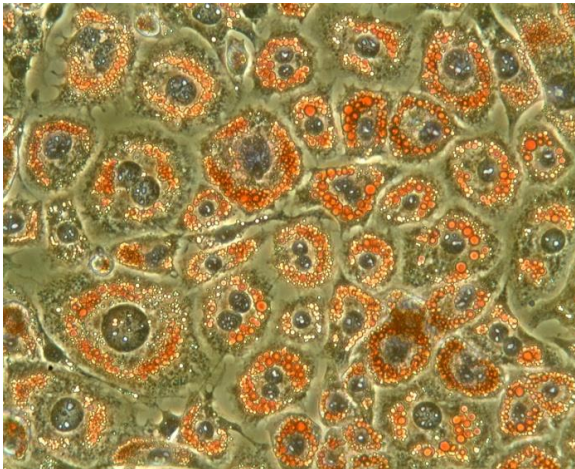
(a)



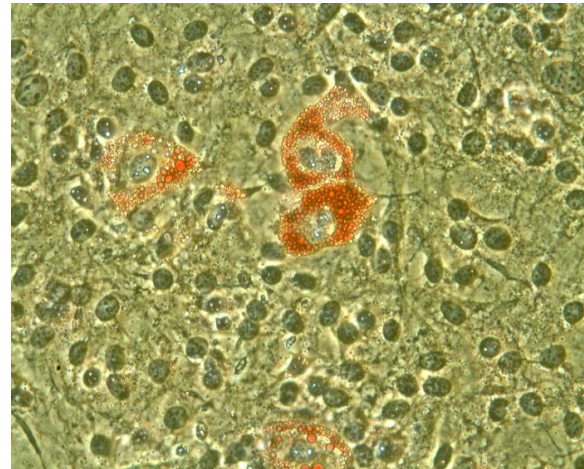
(b)



(c)



(d)



**Suppl Fig. 5 Murine fibroblasts were differentiated into adipocytes**

Murine fibroblasts (3T3-L1) were seeded at a density of  $1 \times 10^5$  cells per well in 24-well tissue culture plates. After growing to confluency in DMEM/ 10% bovine calf serum, cells were cultured for another 48 h before induction of adipogenesis was induced with insulin, IBMX and dexamethasone in DMEM/ 10% FBS (day 0). Adipocyte differentiation was usually complete by day 8. Oil Red O/ haematoxylin staining, x200.

(a) 3T3-L1 preadipocytes demonstrated a fibroblast-like morphology 2 d after seeding.

(b) Day 0: 48 h after cells became 100% confluent, usually 5-6 days after seeding.

(c) Day 8: 3T3-L1 cells had an adipocyte like morphology with much more rounded cytoplasm full of lipid droplets stained red with Oil Red O

(d) Day 8: 3T3-L1 cells which were not stimulated with insulin, IBMX and dexamethasone. There was approximately 1 differentiated cell/ hpf.