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1 **Notch pathway inhibition targets chemoresistant insulinoma cancer stem**
2 **cells**

3 Capodanno Y^{1*}, Buishand FO^{2,*}, Pang LY¹, Kirpensteijn J³, Mol JA², Argyle DJ¹

4 ¹ Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, EH25
5 9RG, United Kingdom.

6 ² Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht
7 University, Utrecht, The Netherlands.

8 ³ Hill's Pet Nutrition, 400 W 8th Ave, Topeka, KS 66047, USA

9

10 * These authors contributed equally

11 **Corresponding author:** Ylenia Capodanno, Royal (Dick) School of Veterinary Studies and
12 The Roslin Institute, University of Edinburgh, Easter Bush, EH25 9RG, United Kingdom,
13 ylenia.capodanno@roslin.ed.ac.uk

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16 tumours

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18

19

20 **Abstract**

21

22 Insulinomas (INS) are the most common neuroendocrine pancreatic tumours in humans and
23 dogs. The long-term prognosis for malignant INS is still poor due to a low success rate of the
24 current treatment modalities, particularly chemotherapy. A better understanding of the
25 molecular processes underlying the development and progression of INS is required to
26 develop novel targeted therapies. Cancer stem cells (CSCs) are thought to be critical for the
27 engraftment and chemoresistance of many tumours, including INS. This study was aimed to
28 characterise and target INS CSCs in order to develop novel targeted therapies.

29 Highly invasive and tumourigenic human and canine INS CSC-like cells were successfully
30 isolated. These cells expressed stem cell markers (*OCT4*, *SOX9*, *SOX2*, *CD133* and *CD34*),
31 exhibited greater resistance to 5-fluorouracil (5-FU), and demonstrated a more invasive and
32 tumourigenic phenotype *in vivo* compared to bulk INS cells. Here, we demonstrated that
33 Notch-signalling-related genes (*NOTCH2* and *HES1*) were overexpressed in INS CSC-like
34 cells. Protein analysis showed an active NOTCH2-HES1 signalling in INS cell lines, especially
35 in cells resistant to 5-FU. Inhibition of the Notch pathway, using a gamma secretase inhibitor
36 (GSI), enhanced the sensitivity of INS CSC-like cells to 5-FU. When used in combination GSI
37 and 5-FU, the clonogenicity *in vitro* and the tumourigenicity *in vivo* of INS CSC-like cells were
38 significantly reduced. These findings suggested that the combined strategy of Notch
39 signalling inhibition and 5-FU synergistically attenuated enriched INS CSC populations,
40 providing a rationale for future therapeutic exploitation.

41

42

43 **Introduction**

44

45 Insulinomas (INS) are the most common functioning neuroendocrine pancreatic tumours
46 (PancNETs) in humans and dogs. INS are insulin-producing tumours that arise from beta-
47 cells (Wang *et al.* 2004; Bailey *et al.* 2007; Polton *et al.* 2007; Athanasopoulos *et al.* 2011;
48 Baudin *et al.* 2014; Buishand *et al.* 2014). The treatment of choice for localised benign INS is
49 surgical resection (Bailey *et al.* 2007; Buishand *et al.* 2014). However, for advanced stage
50 disease medical treatment options for adjuvant therapy are limited. Combinations of
51 chemotherapies such as streptozocin plus 5-fluorouracil (5-FU) or doxorubicin have been
52 used in these cases, but response rates, are variable and generally disappointing (Corroller
53 *et al.* 2008; Mathur *et al.* 2012). Thus, effective new treatment strategies are required.

54 We hypothesise that the malignant behaviour and recurrence of INS is driven by a
55 subpopulation of cancer stem cells (CSCs). CSCs are unique subpopulations of the
56 heterogeneous cell population of a tumour, which are considered to be responsible for
57 tumour initiation, metastasis, and recurrence (Mitra *et al.* 2015). CSCs have been described
58 to be able to resist systemic anti-cancer treatment by several mechanisms including entering
59 into a quiescence state; up-regulation of expression of xenobiotic efflux pumps; and
60 enhancing anti-apoptotic and DNA repair pathways to allow cell survival (Bomken *et al.*
61 2010). Therefore, CSCs are able to survive and initiate tumour relapse after systemic
62 treatment, making them an essential target for novel anti-cancer drugs.

63 Despite the growing evidence to support the existence of CSCs in a wide array of solid
64 tumours, a comprehensive characterisation of INS CSCs has not yet been reported (Grande
65 *et al.* 2011). Previous studies have already identified pancreatic cells with a stem cell
66 phenotype in human and canine INS (Ordonez, 2001; Buishand *et al.* 2013). These so-called
67 amphicrine cells co-express both endocrine and exocrine markers (Ordonez, 2001).

68 Furthermore, recent studies have identified CD90 as a potential marker for CSCs in a human
69 INS cell line (Buishand *et al.* 2016). However, there are no consensus markers available to
70 identify INS CSC-like cells and additionally, recent studies show that several CSC
71 populations may reside within one tumour (Hou *et al.* 2014; Krampitz *et al.* 2016).

72 The lack of knowledge regarding CSCs in INS can be partly attributed to the low incidence of
73 human INS. With only four cases per million population per year, the availability of research
74 material is limited, especially for malignant subtypes (Callacondo *et al.* 2013). Previously,
75 investigators have analysed changes in gene expression of malignant INS mainly as part of
76 broad studies on PancNETs (Speel *et al.* 1999; Zhao *et al.* 2001). However, PancNETs
77 represent a heterogeneous group of tumours and therefore, the specific tumourigenesis of
78 INS is still poorly understood. The incidence of canine INS has not been specified yet but it is
79 higher compared to humans. Data collected at the Department of Clinical Sciences of
80 Companion Animals of Utrecht University have recorded 10 referral cases of malignant
81 canine INS on a yearly basis, out of a total of two million dogs in The Netherlands (FO
82 Buishand, unpublished observations). This provides readily available canine INS samples for
83 molecular studies.

84 Canine INS are classified as malignant tumours in 95% of the cases as they often
85 metastasise to abdominal lymph nodes and liver (Buishand *et al.* 2010). As in humans,
86 canine patients diagnosed with malignant INS are often presented with relapse of
87 hyperinsulinaemia due to the outgrowth of micrometastases that were not detected at the
88 time of initial surgery (Jonkers *et al.* 2007; Goutal *et al.* 2012). From a comparative oncology
89 perspective, which aims to utilise spontaneous tumours in pet animals as natural models for
90 the study of human cancer biology and therapy (Gordon *et al.* 2009), the close resemblance
91 of canine INS to human malignant INS, makes canine INS an interesting study model for
92 human malignant INS. The major benefit of comparing human INS cells to canine INS cells

93 instead of murine cells from genetically-induced INS mouse models (Schiffman *et al.* 2015) is
94 that spontaneous canine tumour cells are more representative of the complex heterogeneity
95 of INS, as they are not induced by a set of specific mutations, but arise spontaneously in a
96 dog. Therefore, the translational gap between pre-clinical *in vitro* studies and the application
97 of novel drugs in a clinical setting can be overcome by using naturally occurring canine INS
98 as model for human INS (Gordon *et al.* 2009).

99 Using a comparative oncology approach, the first goal of this study was to isolate and
100 characterise human and canine enriched INS CSC populations. As CSCs are known to often
101 co-opt stem and progenitor cell properties, we have used the potential functional
102 conservation of stem cell-surface and intrinsic enzymatic markers found on self-renewing
103 cells to identify and characterise tumourigenic cells. We then set out to identify therapeutic
104 targets in signalling pathways in INS, performing gene expression profiling of adherent INS
105 cells and CSC-enriched tumourspheres. We showed that the Notch pathway is a critical
106 pathway involved in INS CSC viability. Using both *in vitro* and *in vivo* models, we have
107 demonstrated the efficacy of targeting the Notch pathway in decreasing INS CSC survival
108 and resistance to 5-FU, thereby providing preclinical evidence that adjuvant anti-Notch
109 therapy may improve outcomes for patients with malignant INS.

110

111 **Materials and Methods**

112

113 ***Cell culture***

114 The human INS cell line CM (Baroni *et al.* 1999) was cultured in RPMI-1640 (Roswell Park
115 Memorial Institute Media, Invitrogen, Life Technologies, Paisley, UK) supplemented with 10%
116 foetal bovine serum (FBS) (Invitrogen) and 1% penicillin-streptomycin and plasmocin
117 (Invitrogen). The canine INS cell line canINS was derived from a primary canine INS, TNM
118 stage II (Buishand *et al.* 2010), resected from a 6-year old male Flatcoated Retriever at the
119 Faculty of Veterinary Medicine, Utrecht University. Using an insulin radioimmunoassay
120 (Cisbio, Codolet, France), it was determined that the first passage of canINS produced 305
121 $\mu\text{U/L}$ insulin, however insulin secretion was lost after the fourth passage, like in the CM cell
122 line. Further details on the characterisation of canINS can be found in the Supplementary
123 data 1 (Fig. S1-2). canINS was cultured in RPMI-1640 supplemented with 10% FBS, 1%
124 penicillin-streptomycin, 200ng/mL growth hormone (GH) (Source Biosciences, Nottingham,
125 UK). Both lines were cultured at 37°C with 5% CO₂ and cells were passaged on reaching 70-
126 80% confluence. Cell lines were authenticated using Short tandem repeat analysis (Cell
127 Check Human 9 and Cell Check Canine; IDEXX Bioresearch, Windsor, UK). All experiments
128 were conducted with cells from passage numbers 5-25.

129

130 ***Tumoursphere culture***

131 Spheres were grown in serum-free medium at a density of 60,000 cells/well (2 mL volume) in
132 6-well low adherence plates (Corning, New York, USA). The medium consisted of
133 DMEM/F12 (Invitrogen) supplemented with progesterone (20 nM), putrescine (100 μM),
134 sodium selenite (30 nM), transferrin (25 $\mu\text{g/mL}$), insulin (20 $\mu\text{g/mL}$) (Sigma-Aldrich, Dorset,
135 UK). Every two days, human recombinant EGF (10 ng/mL) and human recombinant basic

136 fibroblast growth factor (bFGF) (10 ng/mL) (Peprotech, London, UK) were added. Spheres
137 were passaged every week up until 15 passages. All experiments were conducted in
138 triplicate.

139

140 ***RNA extraction and quantitative real time PCR***

141 Total cellular RNA was extracted using RNeasy[®] kit (Qiagen, Redwood City, CA, USA) and
142 was reverse transcribed using the Omniscript[™] RT Kit (Qiagen) according to the
143 manufacturer's instructions. Quantitative real time PCR (qRT-PCR) was performed for genes
144 of interest by using the Stratagene M63000p qPCR system (Agilent, Santa Clara, CA, USA),
145 and the PlatinumH SYBRH Green qPCR SuperMix-UDG (Invitrogen) according to
146 manufacturer's instructions (primers are listed in Supplementary Tables 1 and 2). Relative
147 gene expression levels were obtained by normalisation to the expression levels of
148 housekeeping gene *GADPH*. Calculations were made using the Delta Delta Ct Method.

149

150 ***Protein extraction and western blotting***

151 Cells were lysed in urea lysis buffer (7 M urea, 0.1 M DTT, 0.05% Triton X-100, 25 mM NaCl,
152 20 mM Hepes pH 7.5). Then cells were transferred to 0.1 mL Bioruptor[®] Microtubes
153 (Diagenode, Seraing, Belgium) and sonicated using pre-chilled Bioruptor[®] Pico sonicator
154 (Diagenode) following the manufacturer's instructions. Equal amounts of protein were
155 separated by SDS polyacrylamide gel electrophoresis (SDS PAGE), transferred to Hybond-C
156 nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and
157 hybridised to the appropriate primary antibody and HRP-conjugated secondary antibody for
158 subsequent detection by ECL. Antibodies used against HES1 (EPR4226) (1:600), Beta actin
159 (AC-15) (1:5000), SOX9 (ab26414) (1:500) and OCT4 (ab18976) (1:1000) were purchased
160 from Abcam (UK). Secondary antibodies were obtained from Dako (Glostrup, Denmark)

161 (Goat anti-Rabbit-HRP; Rabbit anti-Mouse-HRP). The appropriate secondary antibody was
162 diluted 1:1000 (Rabbit anti-Mouse-HRP) or 1:2000 (Goat anti-Rabbit-HRP).

163

164 ***Choriollantoic membrane assay***

165 Fertilised ISA Brown layer strain chicken eggs (Roslin Institute Poultry Unit, UK) were
166 incubated in a humidified rotary incubator (Brinsea Octagon 40 OX incubator) at 37°C. As
167 chick embryo chorioallantoic membrane experimental protocols were conducted and
168 concluded during the first two-thirds of the incubation of the embryonated eggs, according to
169 the UK Animals (Scientific Procedures) Act 1986 regulated by the Home Office, we did not
170 require a licence (Home Office 2014).

171 On day 7, single cell suspensions of trypsinised adherent CM and canINS cells or spheres
172 were fluorescently labelled with PKH26 (Sigma-Aldrich, Dorset, UK) according to
173 manufacturers' instructions. Cells (1×10^4 for each condition) were suspended in a 1:1 mixture
174 of serum-free media and Matrigel Phenol Red Free (Corning) and 25 μ L was pipette-
175 inoculated directly onto the CAM. The shell windows were resealed and incubated without
176 turning. At day 11, pictures were taken using Axio ZoomV16 coupled with AxioCAM HRM
177 camera (Zeiss, Cambridge, UK). Images were processed using Zeiss pro image software and
178 then the fluorescence was calculated using ImageJ 1.46 software (open source). All data
179 were subtracted of background fluorescence and then averaged.

180 The embryos were decapitated and the area of the CAM inoculated with the fluorescent cells
181 was harvested and stored in 10% neutral buffered formalin solution (Sigma-Aldrich) and
182 embedded in an agarose block for cutting and staining. The staining was performed with anti-
183 cytokeratin (MNF116; Dako) as primary antibody at 1:50 dilution for 30 min followed by
184 staining with secondary antibody Envision anti-Mouse HRP (Dako). Images were taken using
185 a Nikon Eclipse Ni Brightfield Microscope and thereafter processed with Zeiss pro image
186 software (Zeiss).

187

188 ***Invasion assay***

189 The invasive ability of cells was determined using the QCM™ collagen-based cell invasion
190 assay kit (Millipore, Billerica, MA, USA) according to manufacturer's instructions. Briefly, cells
191 were seeded into the upper inserts at 1×10^5 cells per insert in serum-free RPMI. Cells were
192 incubated at 37 °C with 5% CO₂ for 48 hours. Non-invading cells were removed. Cells that
193 migrated through the gel insert to the lower surface were stained and quantified by
194 colorimetric measurement at 560 nm. Images were taken using an Eclipse Ni Brightfield
195 Microscope (Nikon UK Ltd., Surrey, UK) and thereafter processed with Zeiss pro image
196 software (Zeiss).

197

198 ***Flow cytometry***

199 CM and canINS were detached by trypsinisation, washed with PBS and stained with the
200 Zombie Violet Fixable Viability Kit (BioLegend Inc., San Diego, CA, USA) to detect dead
201 cells. Subsequently, cells were washed again with PBS and fixed in paraformaldehyde at 1%
202 for 10 min at 37°C and then chilled for one minute on ice. A batch of cells was also
203 permeabilised by adding ice-cold 90% methanol slowly to pre-chilled cells under gentle
204 vortexing. Cells were incubated for 30 min on ice, washed in incubation buffer (PBS 0.5%
205 BSA) twice and resuspended in 100 µL of the diluted primary antibody at 1:800 dilution. After
206 incubation with the primary antibody, cells were washed and incubated with a fluorochrome-
207 conjugated secondary antibody for 30 min. After washing with incubation buffer, cells were
208 resuspended in PBS and analysed using BD Fortessa (BD Biosciences, Oxford, UK). The
209 primary antibody used was monoclonal anti-rabbit Notch2 (D76A6) XP® with anti-rabbit IgG
210 (H+L) F(ab')₂ Fragment Alexa Fluor® 647 Conjugate (NewEnglandBio, Ipswich, MA, USA) as
211 a secondary antibody. Rabbit (DA1E) mAb IgG XP® Isotype control Alexa Fluor® 647
212 Conjugate (NewEnglandBio) was used as negative control.

213

214 Growth inhibition assays

215 CM and canINS adherent cells and spheres were trypsinised into single cell suspensions and
216 aliquots of 500 cells/well were seeded in triplicates in opaque 96-well plates (Corning) in 50
217 μ L medium and incubated overnight at 37°C with 5% CO₂. After 24 hours serial dilutions of 5-
218 FU (Tocris, R&D System, Minneapolis, Canada), or gamma-secretase inhibitor (GSI) N-[N-
219 (3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Sigma-Aldrich) were
220 added to the appropriate wells. Equal volumes of vehicles were used as controls. After
221 incubation for 48 hours, cell viability was measured using the CellTiter-Glo[®] Luminescent
222 Assay (Promega, Madison, WA, USA). Data of triplicate wells were averaged and normalised
223 against the average signal of control treated samples, and dose-response curves were
224 generated.

225

226 Colony formation assays

227 CM and canINS 2D and 3D cultures were trypsinised into single cell suspensions and seeded
228 at 500 cells per 10 cm plate (Corning). Cells were treated with 5-FU and DAPT whilst in
229 suspension. Plates were incubated at 37°C with 5% CO₂ until colonies were visible. Growth
230 media were changed once a week. The colonies were fixed by incubating in ice-cold
231 methanol for 5 min at room temperature. Colonies were stained with Giemsa (Invitrogen)
232 according to the manufacturer's instructions.

233

234 Statistical analysis

235 All experiments were repeated at least on two separate occasions. Quantitative analysis was
236 based on a minimum of three replicates. Data were analysed using Minitab[®] 17 Statistical
237 Software (Minitab Ltd., Coventry, UK) and all graphs and diagrams were generated using
238 Microsoft Office 2011 software (Microsoft Corporation, Redmond, WA, USA). *P*-values <0.05

239 were considered statistically significant. When data followed a normal distribution, two
240 sample *t*-tests were used to compare differences between two samples, or one-sample *t*-
241 tests to determine whether the sample mean was statistically different from a known or
242 hypothesised mean. IC₅₀ values were calculated using GraphPadPrism 6 (GraphPad
243 Software, La Jolla, CA, USA). To assess combined treatment effects on the canINS and CM
244 cell lines, the Bliss additivism model was used (Buck *et al.* 2006).

245

246 **Results**

247

248 ***CSC-like cells are enriched in human and canine INS spheres***

249 Human CM adherent cells (Fig. 1 A) gave rise to small and irregularly shaped spheres (Fig. 1
250 B), whereas canine canINS adherent cells (Fig. 1 C) gave rise to well-rounded large spheres
251 (Fig. 1 D). These cells repeatedly formed tumourspheres for up to 15 subsequent passages
252 when plated in low-adherent conditions. To further characterise tumourspheres we examined
253 the expression of embryonic stem cell markers OCT4 and SOX9. Both markers were
254 expressed at a higher level in human (Fig. 1 E) and canine (Fig. 1 F) tumourspheres
255 compared to parental adherent cells.

256 We investigated the gene expression levels of a number of CSC-associated genes including
257 stemness markers, stem cell surface related markers, epithelial-mesenchymal transition
258 markers, growth factor receptors, Notch signalling pathway receptors and target genes, and
259 pancreatic neuroendocrine and exocrine markers. *CD34*, *CD133*, *OCT4*, *SOX2*, *SOX9*,
260 *NOTCH2*, *HES1* and *HEY1* were all upregulated in both human and canine INS
261 tumourspheres compared with the adherent population (Fig. 1 G). There was no significant
262 difference in the expression of *NOTCH1*, *NOTCH3* and *NOTCH 4* in both human and canine
263 INS spheres, although these receptors demonstrated a trend to be downregulated in
264 tumourspheres.

265

266 ***INS CSC-enriched tumourspheres are highly invasive in vitro***

267 The invasive capacity of cells was tested *in vitro* using a collagen-based invasion assay.
268 CSC-like cells displayed a greater invasive potential compared to the non-enriched CSCs
269 (Fig. 2 A). When quantified, a statistically significant increased invasive potential was
270 recorded for both human and canine INS CSC-like cells compared with non-enriched CSCs
271 (Fig. 2 B-C).

272

273 ***INS CSC-enriched tumourspheres are more tumourigenic and invasive in vivo than***
274 ***adherent cells***

275 We developed a CAM assay protocol to monitor the tumourigenic and metastatic properties
276 of INS cancer cells. We recorded the amount of fluorescence in triplicate CAMs for both the
277 adherent cells and the CSC-enriched spheres and showed that the adherent INS cells did not
278 form tumours and did not proliferate in the CAM model. However, the CSC-like populations
279 proliferated on the CAM and gave rise to substantial tumours (Fig. 3 A-B). We quantified the
280 red fluorescence recorded in the CAM assay and obtained a statistical significant difference
281 for the amount of cells between the canINS adherent and CSC-like cells (Fig. 3 C). No
282 statistical difference was recorded between both cell populations of the human INS cell line
283 (Fig. 3 D).

284 We then tested whether the cells were able to migrate through the deep layers of the CAM.
285 Human and canine bulk INS cells (Fig. 4 A-B) were less invasive *in vivo* compared to human
286 and canine INS CSC-like cells (Fig. 4 C-D). INS CSC-like cells demonstrated invasive
287 behaviour moving from the outer ectoderm CAM layer through the mesoderm towards the
288 endoderm (Fig. 4 E-F). These findings were consistent with our *in vitro* invasion data.

289

290 ***INS CSC-enriched tumourspheres exhibit greater resistance to 5-FU compared with***
291 ***adherent cells***

292 After testing a set of chemotherapeutics commonly used in the treatment of human INS we
293 identified 5-FU as the most suitable drug to evaluate the INS cancer cells' chemoresistance.
294 The relative IC₅₀ values for 5-FU of adherent CM and canINS cells were 5 µM and 0.5 µM,
295 respectively, which reside within, or are lower than the therapeutic plasma dose range of 5-
296 FU (800 ng/mL-2000 ng/mL, 5 µM-15 µM) (Danquechin-dorval et al., 1996; Yamada, 2003;

297 Blaschke et al., 2012). Both CM and canINS CSC-enriched tumourspheres proved to be
298 more resistant to 5-FU treatment compared to adherent cells in cell viability (Fig.5 A-B) and
299 clonogenicity assays (Fig. 5 C-D).

300

301 ***The Notch pathway is overexpressed and active in 5-FU resistant INS cells***

302 Analysis of gene expression had revealed that Notch pathway related receptor, NOTCH2,
303 and its target gene, HES1 were upregulated in both CM and canINS CSC-enriched spheres
304 (Fig. 1 G). Using flow cytometry, we provided evidence that the NOTCH2 receptor is
305 constitutively activated as it is present both in its inactive form (extracellular level) and active
306 form (intracellular level) in adherent and CSC-enriched sphere populations (Fig. 6 A-B).
307 Using western blot analysis, we showed that both CM and canINS CSC-enriched spheres
308 demonstrated an intrinsic higher expression of NOTCH2 and HES1 compared to the
309 adherent INS cells. Furthermore, treatment of cells with 5-FU resulted in an increased
310 expression of both the inactive and active form of the NOTCH2 receptor in CM (Fig. 6 C) and
311 canINS cells (Fig. 6 D). In response to an increase in NOTCH2 expression, also its
312 downstream target gene HES1 demonstrated an increased expression in cells that were
313 resistant to 5-FU (Fig. 6 C-D).

314

315 ***Inhibition of Notch signalling decreases viability and 5-FU resistance in INS CSC-*** 316 ***enriched tumourspheres***

317 Since CSC-enriched INS spheres were more resistant to 5-FU treatment compared to
318 adherent cells and 5-FU resistant INS cells demonstrated an overexpression of active
319 NOTCH2, we evaluated the effect of Notch pathway inhibition on INS cells. Notch
320 inhibition using DAPT, preferentially decreased the viability of CM and canINS CSC-
321 enriched spheres (Fig. 7 A-B). CSC-enriched canINS spheres demonstrated increased

322 sensitivity to treatment with DAPT compared with CSC-enriched CM spheres. To confirm
323 whether the DAPT is able to specifically inhibit the Notch pathway, we treated the human
324 and canine cells with increasing doses of DAPT and observed, through western blot
325 analysis, a reduced expression of the intracellular form of NOTCH2 (NOTCH2-IC) and its
326 downstream target HES1 in both human and canine INS cell lines_(Fig. 7 C-D). We
327 demonstrated that a blockade of the Notch signalling occurs in CSC-enriched canINS
328 spheres at a lower dose of DAPT compared to CSC-enriched CM spheres (Fig. 7 C-D).
329 Finally, when DAPT was used in combination with 5-FU, we demonstrated that the
330 clonogenicity of CSC-enriched CM and canINS spheres was significantly reduced. This
331 effect was superior to use of either drug alone (Fig. 7 E-F). The synergistic effect of the
332 combination of 5-FU and DAPT was confirmed using the Bliss independence model (Fig. 7
333 G-H).

334

335 ***Notch inhibition enhances chemosensitivity to 5-FU treatment of INS CSC-enriched***
336 ***tumourspheres in vivo***

337 In order to validate the results obtained *in vitro*, we tested this approach in the *in vivo* CAM
338 model. Treatment with either 5-FU, DAPT, or their combination, in the CAM model
339 demonstrated that the human and canine INS CSC populations were not able to proliferate
340 when treated with a combination of 5-FU and DAPT (Fig. 8 A-B). We recorded the amount of
341 fluorescence in the triplicate CAMs for the different conditions and demonstrated that the
342 combination of 5-FU and DAPT significantly decreased the proliferation of INS CSC-like cells,
343 while neither treatment with DAPT, or 5-FU alone led to a significant reduction in cell
344 proliferation (Fig. 8 C-D).

345

346

347 **Discussion**

348

349 In the current study, we demonstrated that human and canine INS cell lines could be
350 enriched in CSCs by tumoursphere culturing. CSCs have been previously isolated from a
351 variety of human (Zhu *et al.* 2011; Mao *et al.* 2014; Paschall *et al.* 2016; Zhao *et al.* 2016;
352 Sakai *et al.* 2017) and canine cancer types (Wilson *et al.* 2008; Stoica *et al.* 2009; Pang *et al.*
353 2011, 2012, 2017; Rybicka & Król 2016). However, to our knowledge, we are the first to
354 report the isolation of CSC-like cells from a canine INS cell line and the use of this cell line as
355 comparative model for human INS.

356 CSC-enriched tumourspheres from both species demonstrated a common upregulation of
357 stem cell-associated markers *CD133*, *CD34*, *OCT4*, *SOX9*, and *SOX2*. Previously, *OCT4*,
358 *SOX2*, *SOX9* and *CD133* have been identified as stem cell markers of pancreatic endocrine
359 progenitor cells (Seymour *et al.* 2007; Koblas *et al.* 2008; Wang *et al.* 2009; Venkatesan *et al.*
360 2011). Of these markers, *CD133* expression was demonstrated to be a negative
361 prognosticator in PancNETs (Sakai *et al.* 2017).

362 Human and canine CSC-like INS cells were highly invasive *in vitro*, similar to CSCs isolated
363 in previous studies (Gaur *et al.* 2011; Pang *et al.* 2011; Gao *et al.* 2014). CSC-like INS cells
364 displayed a greater invasive potential compared to the bulk INS cells in both *in vitro* invasion
365 assays and *in vivo* CAM models. Previously, the CAM model has been used to model
366 metastatic behaviour in other cancer types such as breast, bladder, prostate, ovarian cancer
367 and head and neck cancers in humans (Deryugina *et al.* 2009; Lokman *et al.* 2012) and
368 mammary carcinoma and osteosarcoma in companion animals (Pang *et al.* 2013, 2014). In
369 our CAM assays, CSCs from INS tumourspheres developed visible tumours within 4 days,
370 and escaped the primary inoculation site and migrated to the inner layers of the CAM. The
371 invasive behaviour of INS CSCs in the CAM model with its highly vascularised structure,
372 closely mimics the mode of INS metastasis which involves INS cancer cell invasion and

373 spread through the abdominal lymphatic system to reach the site of metastases in either
374 lymph nodes or liver. Overall, these findings suggest CSCs may play a role in INS
375 carcinogenesis.

376 According to our results, INS CSC-like cells are more resistant to 5-FU compared to the
377 adherent cancer cells. This is consistent with the CSC model stating that despite the
378 sensitivity of bulk tumour cells to chemotherapy, CSCs are resistant and lead ultimately to
379 the failure of cytotoxic chemotherapy, increasing the need for new CSC-targeted therapies
380 (Guo *et al.* 2006). After isolating INS CSCs, we have identified the Notch pathway as a
381 potential target for INS CSC targeted therapy. Notch signalling pathway activation occurs
382 when a Notch receptor (NOTCH 1–4) binds to one of the five known Notch ligands (Delta-
383 like-1, -3, and -4 and Jagged-1 and -2). After receptor–ligand binding, there is a two-step
384 proteolytic cleavage, first by ADAM10, then by gamma-secretase of the intracellular
385 domain of the Notch receptor (NICD). NICD translocates to the nucleus, interacts with CSL
386 transcription factors (CBF1/RBP-J, Su(H), Lag-1) which activate and promote transcription
387 of downstream genes such as HES1, involved in various differentiation programmes
388 (Grande *et al.* 2011; Abel *et al.* 2014). For instance, Notch signalling has a major role in
389 pancreatic embryogenesis, influencing the balance between pancreatic endocrine
390 progenitors, exocrine cells and differentiated beta-cells (Angelis *et al.* 1999; Andersson *et*
391 *al.* 2011). The current study demonstrates that NOTCH2 is constitutively active in CM and
392 canINS cells. Furthermore, *NOTCH2* and *HES1* are overexpressed in human and canine
393 CSC-like cells, compared to the bulk INS cells. *NOTCH2* is the only Notch receptor that
394 have demonstrated overexpression in both human and canine INS suggesting that
395 *NOTCH2* is the most relevant Notch receptor through which signalling in INS CSCs is
396 mediated. The role of the Notch pathway has been previously described in various types
397 of NETs (Grande *et al.* 2011; Carter *et al.* 2013; Crabtree *et al.* 2016) but to our knowledge
398 this is the first study to evaluate the role of the Notch pathway in INS tumourigenicity and

399 in particular its role in maintaining the INS CSC population. Previous studies have
400 identified NOTCH2 as an oncogene in NETs (Carter *et al.* 2013; Crabtree *et al.* 2016): in
401 small cell lung carcinoma (SCLC) Notch2 signalling has shown a prominent role in tumour
402 promotion in SCLC xenografts in mice (Crabtree *et al.* 2016). Recently NOTCH2
403 overexpression has been related to increased tumourigenicity of cancer cells, and an
404 increased resistance to 5-FU in hepatocellular carcinoma (Rui *et al.* 2016). In our study,
405 we have demonstrated an increased activation of the Notch pathway in INS cells, after
406 treatment with 5-FU. The observed enhancement in Notch signalling may be explained by
407 a selective enrichment of the INS 5-FU resistant cells that display an active Notch
408 signalling. In accordance with this hypothesis, previous studies have demonstrated that
409 overexpression of HES1 has been related to an increased resistance to 5-FU in colon
410 cancer (Candy *et al.* 2013) and oesophageal squamous cell carcinoma (Liu *et al.* 2013).

411 Notch signalling in CM and canINS may contribute to carcinogenesis by inhibiting
412 differentiation, promoting cellular proliferation, and/or inhibiting apoptosis, yet no studies
413 have examined these endpoints in INS. Our results showed that NOTCH2 is constitutively
414 activated in both CSC-like cells and bulk INS cells, although the bulk cancer cell
415 population demonstrated a lower expression of HES1. Interestingly, Notch inhibition using
416 DAPT preferentially decreased the viability of the CSC-like population. Considering that
417 NOTCH2 was the only overexpressed Notch receptor in human and canine INS CSCs,
418 these data suggest that the Notch2-Hes1 signalling cascade plays an important role in
419 CSCs' survival and resistance to chemotherapy. Next, we have tested whether a
420 combined regimen of DAPT and 5-FU can reverse the 5-FU resistance of INS CSC-like
421 cells. Treatment *in vitro* with DAPT alone did not inhibit INS CSC-like cells clonogenicity,
422 however, the combination of DAPT and 5-FU significantly inhibited colony-forming ability of
423 INS CSC-like cells to a greater degree than either therapy alone. We have then used the

424 CAM model to study the effect of this combined treatment *in vivo*. The results from the
425 CAM assay were consistent with the *in vitro* findings, as tumour proliferation *in vivo* was
426 significantly decreased when the drugs were used in combination compared to their use
427 as single agents. Previous studies have already shown that Notch inhibition increased the
428 cytotoxic effects of chemotherapy in various types of cancer (Meng *et al.* 2009; Lee *et al.*
429 2015; Li *et al.* 2015): for example oxaliplatin-induced activation of Notch1 signalling in
430 metastatic colon cancer was reduced by simultaneous GSI treatment, resulting in
431 enhanced tumour sensitivity to oxaliplatin (Meng *et al.* 2009); in breast cancer, combined
432 inhibition of Notch with doxorubicin treatment resulted in decreased tumourigenicity in
433 mouse xenograft models (Li *et al.* 2015); and in gastric cancer, targeting the Notch
434 pathway significantly increased the cytotoxicity of 5-FU (Lee *et al.* 2015). Demonstrating
435 that inhibition of the Notch pathway has functional consequences provides further
436 evidence that this pathway is not only differentially expressed but plays a causative role in
437 INS carcinogenesis.

438 In summary, in the current study, we have isolated INS CSC-like cells from human and
439 canine INS cell lines and have demonstrated that both subpopulations of INS CSC-like
440 cells seem to be dependent on the Notch pathway for their survival. Furthermore, targeting
441 the Notch pathway led to a significant increase in cytotoxicity of 5-FU in the INS CSC-like
442 population, demonstrating a correlation between Notch activation and 5-FU resistance.
443 The increased expression of Notch in 5-FU resistant INS cells may be clinically significant,
444 as it provides a valuable rationale that INS patients whom developed chemoresistance
445 might benefit from a treatment with Notch small molecule inhibitors, such as GSIs. GSI
446 treatment has previously been used in a clinical setting to sensitise cancer cells to
447 chemotherapy in advanced stages of solid tumours (Richter *et al.* 2014). Since GSIs
448 including DAPT, inhibit cleavage of all Notch receptor families, our results may not be

449 exclusively due to Notch2 signalling effects. Therefore, future preclinical studies on INS
450 will focus on the use of specific inhibitors of either NOTCH2 or HES1, and further,
451 elucidate their potential in clinical settings.

452

453 **Declaration of interest**

454 The authors declare that there is no conflict of interest that could be perceived as prejudicing
455 the impartiality of the research reported.

456

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459

460 **Authors contributions**

461 J.K., J.A.M., F.O.B., L.Y.P. and D.J.A conceived the study; Y.C., F.O.B., L.Y.P., J.A.M.
462 and D.J.A designed the experiments. Y.C. performed the experiments and analysed the
463 data, interpreted the results and drafted the manuscript. Y.C. and F.O.B. wrote the final
464 version of the manuscript. L.Y.P., J.K., J.A.M. and D.J.A. revised and reviewed the
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466

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665

666

1 **Figure legends**

2

3 **Figure 1** Isolation and characterisation of CM and canINS cancer stem cells (CSC). **A-B:**
4 CM in adherent (A) and in tumoursphere (B) culturing conditions (scale bar: 100 μ m). **C-D:**
5 canINS in adherent (C) and tumoursphere (D) culturing conditions (scale bar: 100 μ m). **E-F:**
6 Western blot analysis of CM (E) and canINS (F) stem cell markers OCT4 and SOX9 and beta
7 actin as loading control. **G:** qRT-PCR of stem cell and self-renewal pathway related genes
8 comparing CM and canINS in both adherent and sphere culturing conditions. The mRNA
9 expression of embryonic stem cell genes (SOX9, OCT4, SOX2) and stem cell-associated
10 surface markers (CD133, CD34) were upregulated in sphere culturing conditions. The
11 expression of NOTCH receptor (NOTCH2) and downstream target genes (HES1, HEY1) was
12 upregulated, whereas no significant differences were recorded in NOTCH1, NOTCH3 and
13 NOTCH4 expression in human and canine INS spheres. Values are mean of triplicates \pm SD.
14 The P-values represent the comparison with a stated hypothesis (values >1) using one
15 samples t-test. *P-values <0.05 were considered statistically significant.

16

17 **Figure 2** Invasive properties of INS CSCs in vitro. **A:** Representative images of invasive
18 capacity of human (top row) and canine (bottom row) CSC-enriched spheres and adherent
19 cells using a collagen-based cell invasion assay kit (scale bar: 20 μ m) **B-C:** Invading cells
20 were stained and quantified by colourimetric measurement at 560 nm. Values are mean of 3
21 \pm SEM. *P-value < 0.05 .

22

23 **Figure 3** Putative canine and human INS CSCs show an increased in vivo tumourigenic
24 potential **A:** Representative photographs of the chorioallantoic membrane (CAM) 11 days
25 after inoculation with either canINS adherent cells or CSC-enriched spheres following red
26 fluorescent membrane labelling. Pictures on the top row show the merging of the brightfield
27 channel; pictures on the bottom row show the red channel. A3 represents a magnified picture

28 of the circles shown in A2. Magnification is specified on top of each picture. **B:**
29 Representative photographs of the chorioallantoic membrane (CAM) 11 days after
30 inoculation with either CM adherent cells or CSC-enriched spheres following red membrane
31 labelling. C3 represents magnified pictures of the circles shown in C2. **C-D:** Graphs show the
32 differences in fluorescence between the two populations after quantification using ImageJ.
33 Values are mean of $3 \pm \text{SEM}$. *P-value < 0.05.

34

35 **Figure 4** Invasive properties of INS CSCs in vivo. **A-F:** Representative images of
36 immunohistochemistry of CAM sections embedded in agar and stained with anti-cytokeratin
37 that stains only human and canine cells (brown). The structure of CAM layers is comprised
38 by ectoderm (ET), mesoderm (M) and endoderm (ED). Cancer cell matrigel grafts (CG) were
39 seeded on the CAM. Pictures show the migration of CM adherent (A) and canINS adherent
40 (B) and CM CSC-enriched sphere cells (C) and canINS CSC-enriched sphere cells (D) in the
41 inner part of the CAM 11 days after being seeded. Results show that the CM adherent (A)
42 and the canINS adherent (B) migrate less through the different layers of the CAM compared
43 with the CM CSC-enriched sphere cells (C) and the canINS CSC-enriched sphere cells (D).
44 High magnifications (20x and 60x) shows in details how the CM (E) and canINS (F) CSC-
45 enriched sphere cells disrupt the CAM membrane and invade through the CAM layers.
46 Magnification is specified on top of each picture (scale bar: 200 μm).

47

48 **Figure 5** Chemosensitivity and colony formation assays of CM and canINS. **A-B:**
49 Chemosensitivity assay in CM (A) and canINS (B): cells were treated with increasing
50 concentrations of 5-FU (from 0.5 to 5 μM) comparing the adherent population (dashed line)
51 and the CSC-enriched sphere population (continuous line). **C-D:** Colony formation assay CM
52 (C) and canINS (D): Human and canine cells were treated with increasing concentrations of

53 5-FU (from 0.5 to 5 μ M) comparing the adherent population (dashed) and the CSC-enriched
54 sphere population (solid). Values represent mean of triplicates \pm SD. The P-values represent
55 the comparison using 2 sample t-test within the adherent and the CSC-enriched spheres. *P-
56 value < 0.05 was considered statistically significant.

57

58 **Figure 6** Analysis of Notch pathway protein expression and activation in human and canine
59 insulinoma (INS) cells. **A-B:** Graph showing the percentage of cells positive to NOTCH2
60 antibody using flow cytometry in human (A) and canine (B) INS cell lines. **C-D:** Western blot
61 analysis of NOTCH2 in its inactive transmembrane form (NOTCH2-TM) and its active
62 intracellular form (NOTCH2-IC), and HES1 with beta actin as a loading control in human (C)
63 and canine (D) INS cell lines, treated with increasing doses of 5-Fluorouracil (5-FU).

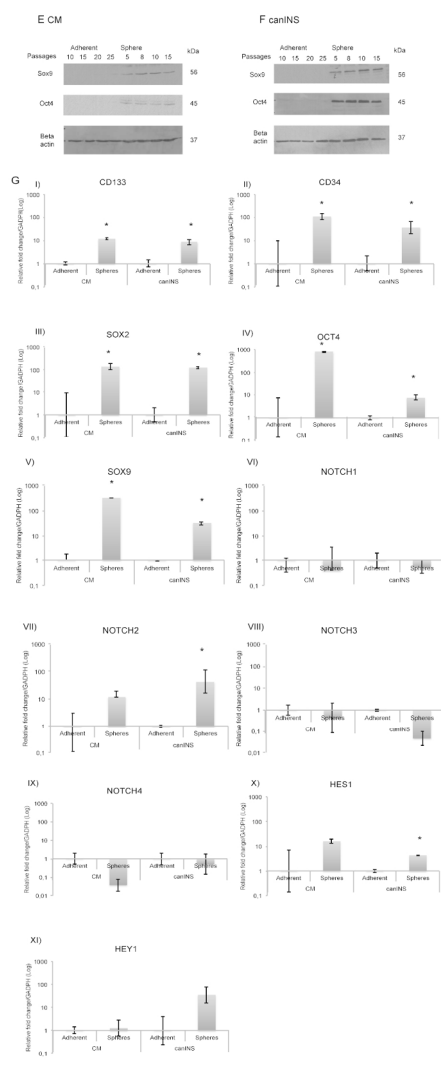
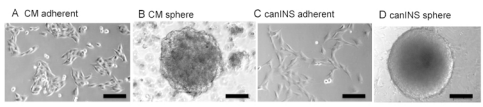
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65 **Figure 7** Function of the Notch pathway in canine and human insulinoma (INS) cancer stem
66 cells (CSC). **A-B:** Cell viability assay of human (A) and canine (B) INS cell lines using
67 increasing concentrations of DAPT comparing adherent cells (dashed line) against CSC-
68 enriched spheres (solid line). **C-D:** Western blot analysis of NOTCH2 in its inactive
69 transmembrane form (NOTCH2-TM) and in its active intracellular form (NOTCH2-IC), and
70 HES1, with beta actin as a loading control in human (C) and canine (D) INS cell lines treated
71 with increasing doses of DAPT. **E-F:** Colony formation assay of human (E) and canine (F)
72 INS cell lines using a combination of DAPT and 5-fluorouracil (5-FU). Values represent mean
73 of triplicates \pm SD. The P-values represent the comparison using 2 sample t-tests within the
74 adherent and the CSC-enriched spheres. *P-value < 0.05. **G-H:** Calculation of the synergistic
75 effect of the DAPT and 5-FU using e-bliss calculation in CM (G) and canINS (H). The method
76 compares the observed combined response with the predicted combined response. The
77 combined effect is synergistic as it is greater than the predicted one.

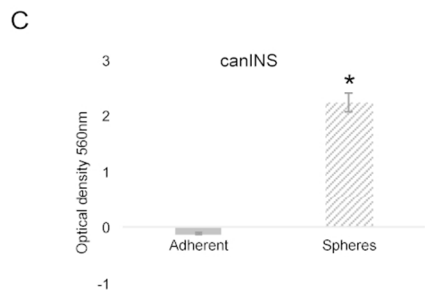
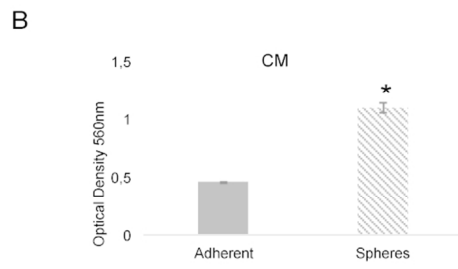
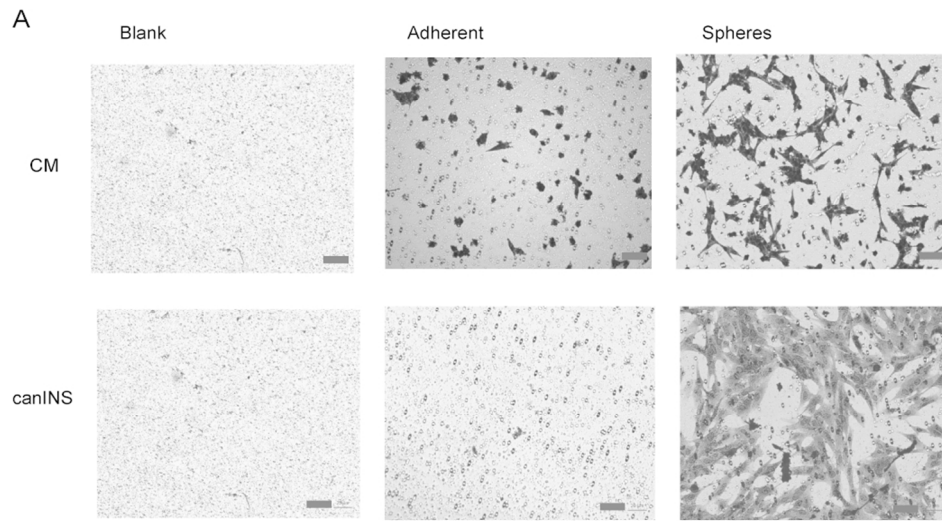
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79 **Figure 8** Combined 5-FU and DAPT treatment decreases human and canine INS CSC-like
80 cells tumourigenic potential in the in vivo chorioallantoic membrane (CAM) model. **A:**
81 Representative photographs of the CAM 11 days after inoculation with CSC-enriched CM
82 spheres following red membrane labelling. Cells have been treated with 5-FU (5 μ M) and
83 DAPT (20 μ g/mL). Pictures on the top row show the merging of the brightfield channel;
84 pictures on the bottom row show the red channel (scale bar: 100 μ m). **B:** Representative
85 photographs of the CAM 11 days after inoculation with CSC-enriched canINS spheres
86 following red membrane labelling. Cells have been treated with 5-FU (0.5 μ M) and DAPT (20
87 μ g/ml). Pictures on the top row show the merging of the brightfield channel; pictures on the
88 bottom row show the red channel (scale bar: 100 μ m). **C-D:** Graphs show the differences in
89 fluorescence between the different conditions after quantification using ImageJ. Values are
90 the mean of 3 \pm SEM. *P-value < 0.05.

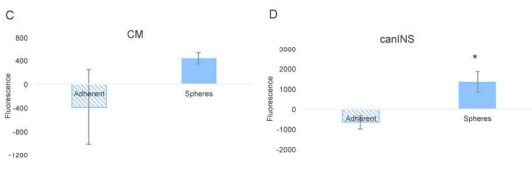
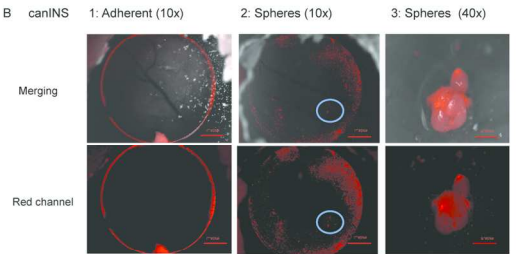
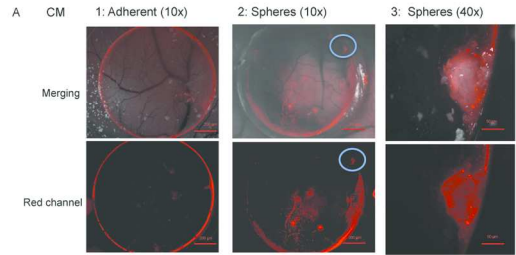
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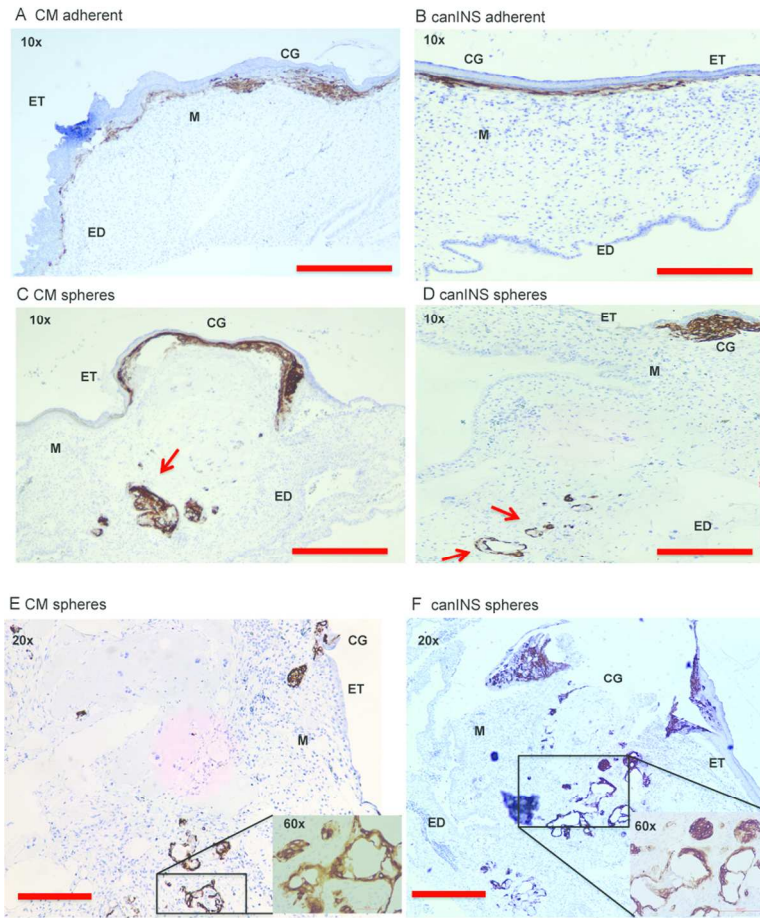
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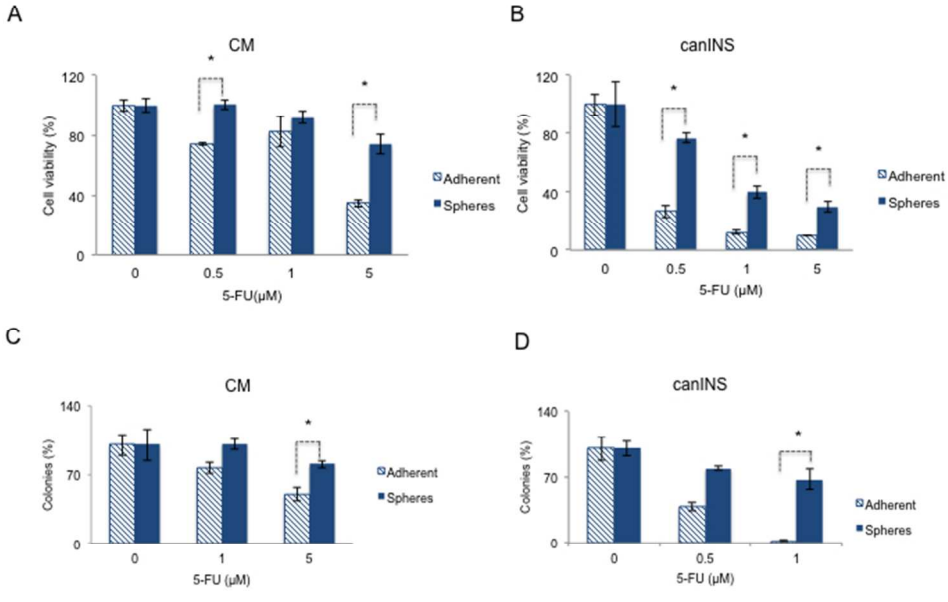
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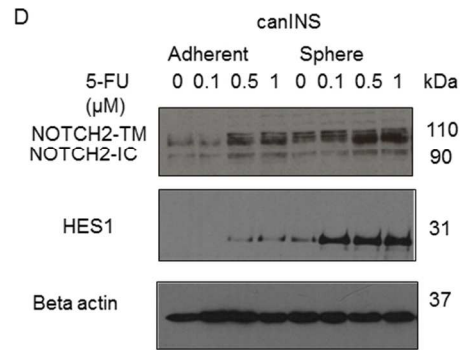
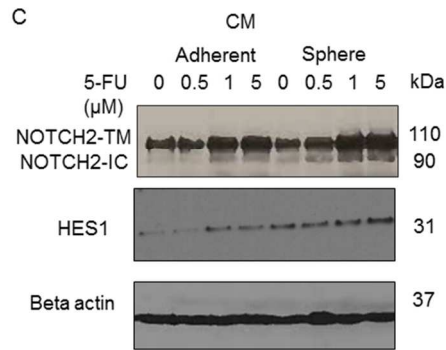
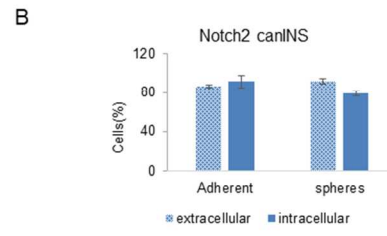
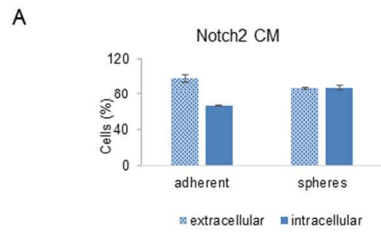
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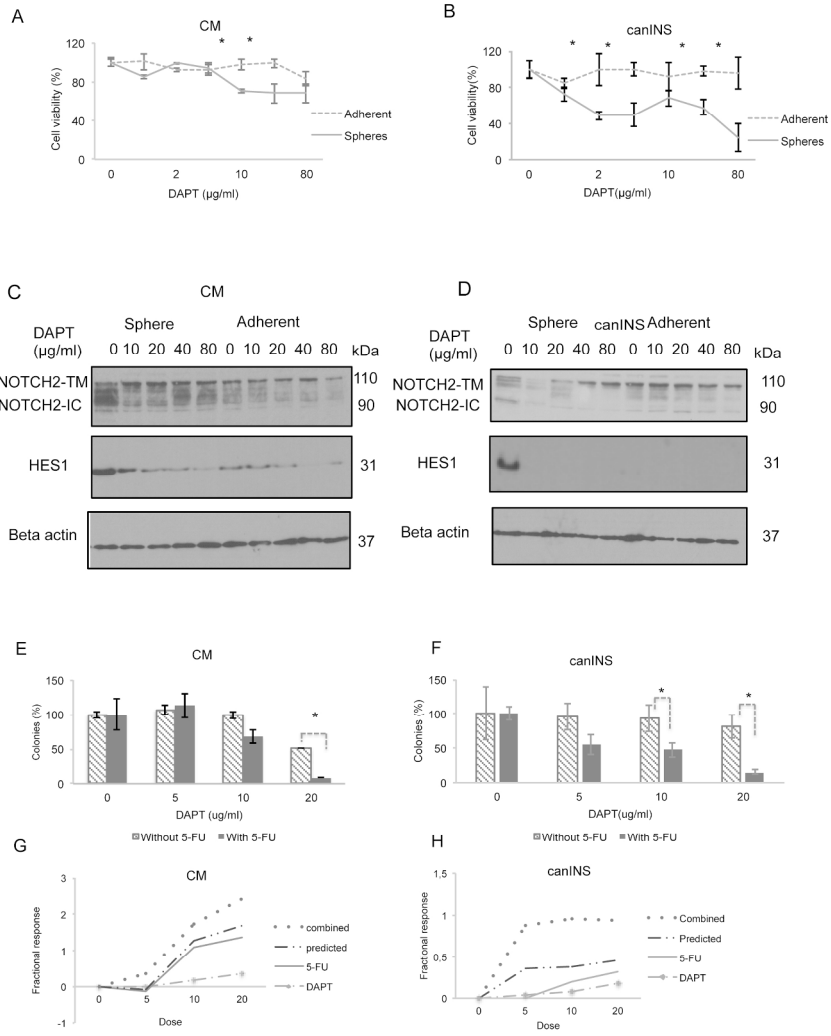
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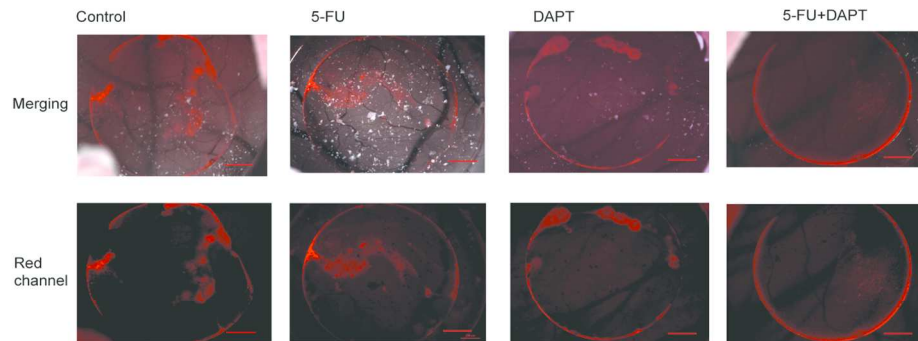


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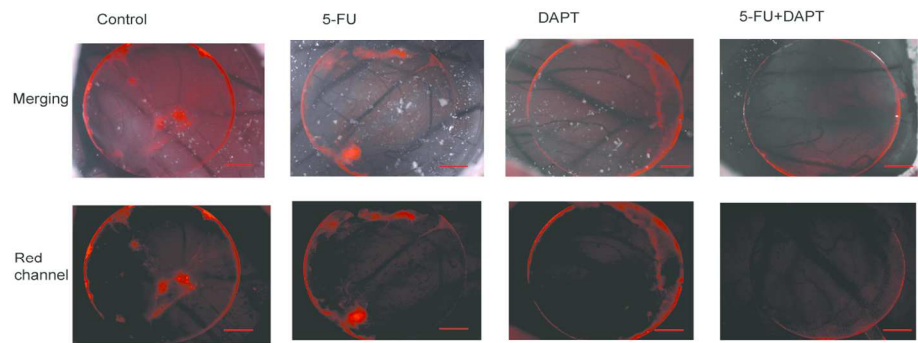


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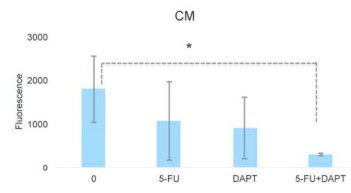
A: CM sphere



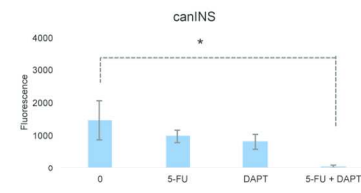
B: canINS sphere



C



D



239x303mm (150 x 150 DPI)