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A comparative study on the in vitro effects of the DNA methyltransferase inhibitor 5-Azacytidine (5-AzaC) in breast/mammary cancer of different mammalian species

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27

28   ## Abstract

29   Murine models are indispensible for the study of human breast cancer, but they have

30   limitations: tumors arising spontaneously in humans must be induced in mice, and long-

31   term follow up is limited by the short life span of rodents. In contrast, dogs and cats

32   develop mammary tumors spontaneously and are relatively long-lived. This study

33   examines the effects of the DNA methyltransferase (DNMT) inhibitor 5-Azacytidine (5-

34   AzaC) on normal and tumoral mammary cell lines derived from dogs, cats and humans,

35   as proof of concept that small companion animals are useful models of human breast

36   cancer. Our findings show that treatment with 5-AzaC reduces *in vitro* tumorigenicity in

37   all three species based on growth and invasion assays, mitochondrial activity and

38   susceptibility to apoptosis. Interestingly, we found that the effects of 5-AzaC on gene

39   expression varied not only between the different species but also between different

40   tumoral cell lines within the same species, and confirmed the correlation between loss of

41   methylation in a specific gene promotor region and increased expression of the associated

42   gene using bisulfite sequencing. In addition, treatment with a high dose of 5-AzaC was

43   toxic to tumoral, but not healthy, mammary cell lines from all species, indicating this

44   drug has therapeutic potential. Importantly, we confirmed these results in primary

45   malignant cells isolated from canine and feline adenocarcinomas. The similarities

46   observed between the three species suggest dogs and cats can be useful models for the

47   study of human breast cancer and the pre-clinical evaluation of novel therapeutics.

Background

Murine cancer models such as genetically engineered mice and human-mouse xenograft models have been extremely useful in studies on the complexity of human breast cancer, and these models are generally accepted to be the most effective means for studying and understanding breast cancer development [1]. However, and despite the unquestionable importance of these murine models in human cancer research, they have some limitations. The most significant is that tumors arise spontaneously in humans, but have to be induced in most mouse models. Furthermore, biological differences between naturally occurring cancers in humans and transplanted cancers in mice can lead to divergences in carcinogenesis including differences in telomerase activity, variation in activated gene sets and pathways, and changes in tolerance to certain drugs and proteins [2]. In addition, tumor development and responses observed in mouse models are not always predictive of human tumors of similar histology, and long-term follow up studies on tumor growth are limited due to the relatively short life span of mice. Therefore, more appropriate, spontaneous animal models that fully recapitulate the complex biology of breast cancer in human patients are needed. Spontaneous tumors in dogs and cats share many features with their human counterparts and offer valuable supplementary model systems for research aimed at elucidating important molecular mechanisms and gene signaling pathways that have a role in mammary tumorigenesis [3-5]. In addition,

71   mammary cancer in dogs and cats is an underused, but unique resource for preclinical,

72   translational research on cancer therapeutics because the hepatic enzyme homology of

73   these animals is much more similar to humans than that of rodents, allowing for a more

74   accurate extrapolation of pharmacokinetics, toxicity and dosing of anti-cancer drugs for

75   both human as well as animal use [3, 6, 7].

76   Breast cancer is induced by the accumulation of altered gene regulation. Besides

77   abnormalities in the DNA sequence (genetic mutations), changes in gene expression

78   profiles (epigenetic dysregulation) also have an important role in breast cancer

79   tumorigenesis [8, 9]. Because of the reversible nature of epigenetic alterations, the

80   potential of epigenetic modifiers in breast cancer drug development has gained

81   significant interest [10]. Several drugs that target epigenetic alterations, including

82   inhibitors of histone deacetylase (HDAC) and DNA methyltransferase (DNMT), are

83   currently approved for treatment of hematological malignancies and are being explored

84   for clinical investigation in solid tumors, like breast cancer [8, 10-12]. For example, the

85   DNA methyltransferase inhibitor 5-AzaC is a Food and Drug Administration (FDA)

86   approved drug used clinically to treat acute myelodysplastic leukemia [13] and has been

87   shown to inhibit cell invasiveness and growth of several human breast cancer cell lines

88   [14,15].

89   In contrast, much less is known about gene signatures in canine, and especially feline,

90   mammary cancer although this is imperative information to further strengthen the value

91   of naturally occurring mammary cancer in these animals as a model for human breast

92   cancer. Moreover, the potential of epigenetic modifiers, like 5-AzaC, as anti-cancer drugs

93   to treat mammary cancer in these species has only minimimally been explored.

94 Therefore, the aims of the present study were to evaluate the effects of 5-AzaC on

95 mammary tumorigenicity of canine and feline mammary cancer cells *in vitro*, and to

96 compare these results with results obtained in human breast cancer cell lines, in order to

97 further emphasize the importance of dogs and cats as powerful models in which to study

98 human breast cancer as well as explore new treatment options for all three species.

99

100 **Methods**

101 *Established mammary cell lines*

102 The human normal breast epithelial cell line MCF10A and its derivative

103 MCF10CA1a, a poorly differentiated invasive malignant carcinoma cell line, have been

104 intensively characterized [16-19]. MCF10A cells were cultured in Dulbecco's modified

105 Eagle medium (DMEM)/F12 (Corning, Acton, MA) supplemented with 5% horse serum

106 (Gibco), 1% penicillin/streptomycin (Invitrogen), 10 μg/ml human insulin 20 ng/ml

107 epidermal growth factor (EGF) and 0.5 μg/ml hydrocortisone (all from Sigma, St Louis,

108 MO) (Medium A); MCF10CA1a were cultured in DMEM/F12 supplemented with 5%

109 horse serum and 1% penicillin/streptomycin (Medium B). The non-invasive, oestrogen-

110 receptor (ER) positive MCF7 cell line [20,21] was cultured in DMEM supplemented with

111 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1%

112 penicillin/streptomycin (Medium C).

113 The feline normal mammary epithelial cell line (FMEC), a kind gift from Dr. John

114 Parker (Baker Institute for Animal Health, Cornell University, Ithaca, NY) [22], the

115 mammary adenocarcinoma cell line K12-72.1, a kind gift from William Hardey Jr.

116 (School of Veterinary Medicine, Cornell University, Ithaca, NY) [23] and the mammary

117 carcinoma cell line CAT-MT [24] were cultured in Medium C. The canine normal

118   mammary epithelial cell line (CMEC), which was established in-house using mammary

119   gland tissue from a healthy 2-year old female research Beagle and used at passages 40-

120   45, and the mammary carcinoma cell lines REM134 and CMT12 [25, 26] were also

121   cultured in Medium C.

122   All cell lines were maintained at 37°C in a humidified environment with 5% CO_2.

123   *Primary mammary tumor cell cultures*

124   Tissue samples were obtained from a dog and cat with owner consent for tissue

125   donation. The dog was an 11-year old spayed mixed breed with mammary

126   adenocarcinomas in two glands, but no observed metastasis based on chest radiographs.

127   The cat was a 10-year old unspayed female with palpable mammary adenocarcinomas in

128   three glands, one of which was accompanied by skin ulceration. Tissue samples were

129   collected during surgery (dog sample) or after euthanasia (cat sample), placed in PBS and

130   shipped to the laboratory overnight at 4°C. Tissues were minced into 1- 2-mm^3 pieces

131   and digested with 0.1% collagenase type III (Worthington Biochemical, Lakewood, NJ)

132   for 60 min at 37°C. Cell suspensions were subsequently filtered through a sterile 100 and

133   40 μm filter to obtain a single cell suspension, and centrifuged at 400 g for 10 min at

134   room temperature (RT). Cells were incubated in Medium C at 37°C in a humidified

135   environment with 5% CO_2. In addition, part of the tissue was fixed in 10% neutral

136   buffered formalin and histology sections were send to Cornell Anatomic Pathology for

137   tumor grading.

138   *Anchorage-independent growth assays*

139   Cells for soft agar assays were pretreated for 2 days with 5 μM 5-Azacytidine (5-

140   AzaC), or cultured under standard conditions, after which cells were detached using

141   (0.25%) Trypsin-EDTA (Corning Life Sciences, Manassas, VA) and counted. To set up

142   soft agar assays, 2 mL of 0.6% 2-hydroxyethylagarose melted in appropriate culture

143   medium were pipetted into wells of 6-well culture plates and plates were held at 4°C for

144   15 min until the agarose solidified. Ten thousand cells per well were gently resuspended

145   in 1.5 mL 0.3% 2-hydroxyethylagarose melted in appropriate culture medium, and

146   layered over the base agarose. Cells were cultured in soft agar for 7 to14 days at 37°C

147   with 5% CO_2. Every 3 days, cultures were fed with 1 mL 0.3% 2-hydroxyethylagarose

148   melted in appropriate culture medium. Cultures were photographed at 10x using a Nikon

149   Diaphot-TMD inverted light microscope with an attached Cohu CCD camera (Nikon,

150   Melville, NY). The number of spheres, defined as clusters of cells increasing in size due

151   to cell division, a universal feature of tumoral cell lines [31-34], was counted and average

152   sphere areas were determined using *Image J* software (http://rsb.info.nih.gov/ij/).

153   *Electric Cell-substrate Impedance Sensing*

154   Cell proliferation rates were measured by Electric Cell-substrate Impedance Sensing

155   (ECIS) using the ECIS Model Z instrument with 96W array station (ECIS, Applied

156   BioPhysics Inc., Troy, NY). To this end, cells were pre-treated for 48 h with 5 μM 5-

157   AzaC or left untreated, and were seeded at a density of 1.0 x 10^4 cells per well in a

158   96W1E$^+$ PET array chip (Applied BioPhysics Inc.) in appropriate culture medium with or

159   without 5 μM 5-AzaC. An alternating current (~1 μA, 32 kHz) was applied to the

160   electrodes to measure impedance (Ohms) and monitor proliferation in real-time for 20 h

161   post-plating. Impedance in wells containing cells treated with 5-AzaC was compared to

162   impedance in wells containing untreated control cells. A decrease in impedance was

163   indicative of a decrease in cell proliferation.

164   For invasion assays, 96W1E$^+$ PET array chips were coated with 25μg/ml bovine

165   plasma fibronectin (Life Technologies) for one hour, rinsed and seeded with bovine lung

166   microvessel endothelial cells (BLMVEC;VEC Technologies, Rensselaer, NY) at a

167   density of 1.0 x 10^5 cells per well. Upon confluency, an equal number of tumoral cells

168   were gently added to each well. An alternating current (~1 μA, 4 kHz) was applied to the

169   electrodes to measure impedance (Ohms) and monitor extravasation of the tumoral cells

170   through the endothelial cell monolayer in real-time for 20 h post-plating. Impedance in

171   wells containing tumoral cells was compared to impedance in wells containing healthy,

172   non-tumoral control cells, or impedance in wells containing tumoral cells pre-treated with

173   5 μM 5-AzaC. Decreased impedance signified disruption of the endothelial cell

174   monolayer by invasive cancer cells.

175   *Quantitative reverse-transcription (RT)-PCR*

176   RNA was extracted from cells using an RNeasy Mini Plus kit (Qiagen, Valencia, CA)

177   and cDNA was synthesized using M-MLV Reverse Transcriptase (USB, Cleveland, OH),

178   according to the manufacturer's protocol. All primers were designed using Primer3

179   software, based on sequences found in the National Center of Biotechnology Information

180   (NCBI) GenBank. Primers each spanned an intronic region to prevent amplification of

181   genomic DNA.

182   Quantitative RT-PCR (qRT-PCR) assays, using SYBR green technology, was carried

183   out on an Applied Biosytems 7500 Fast Real Time PCR instrument (Applied Biosystems,

184   Carlsbad, CA). Primers for 11 genes were designed to study their relative expression

185   levels after 5-AzaC (5 μM) treament. Reference genes were selected from panels

186   validated by other groups for dog [27], cat [28], and human mammary samples [29].

187 Four dog reference genes, four cat reference genes and 13 human reference genes were

188 tested on healthy and tumoral cells from each species. Only genes with Ct values < 28

189 and with a difference < 2 between healthy and tumoral cells, were included as reference

190 genes. An overview of all qRT-PCR primers can be found in Table 1. All samples (n=3)

191 were run in triplicate and the comparative C_t method ($2^{-\Delta\Delta Ct}$) was used to quantify gene

192 expression levels where $\Delta\Delta C_t = \Delta C_t$(sample) $- \Delta C_t$(references). Calculations were

193 preformed using the 7500 Fast software associated with the real time PCR thermal cycler.

194 *Bisulfite sequencing*

195 Genomic DNA was extracted from cells using the DNeasy Blood and Tissue Kit

196 (Qiagen, Valencia, CA). Bisulfite treatment of DNA was carried out with the

197 MethylCode Bisulfite Conversion Kit, according to manufacturer's instructions

198 (Invitrogen Life Technologies, Grand Island, NY). CpG islands upstream of the *PGP9.5*

199 gene were identified based on sequence information in the UCSC genome browser

200 (https://genome.ucsc.edu). Primers to amplify an upstream CpG island were designed

201 with MethPrimer software (http://www.urogene.org/methprimer) which amplified a 212

202 base pair region containing 16 CpGs. Traditional PCR using Taq DNA Polymerase

203 (Invitrogen Life Technologies) was performed to amplify the region of interest and a

204 portion of the PCR products were run on a 1.5% agarose gel containing GelRed

205 intercolating dye (Thermo Fisher Scientific, Waltham, MA) at 97 V for 1 h. Remaining

206 PCR products were purified using the PCR Purification Kit (Qiagen, Valencia, CA) and

207 cloned into DH5α competent *E.Coli* using the pGEM T-easy vector (Promega, Madison,

208 WI). Transformed *E.Coli* were plated on Luria-Bertani (LB) agar (Thermo Fisher Life

209 Technologies, Grand Island, NY) containing 100 µg/mL ampicillin and incubated for 16

210   h at 37°C. White colonies were picked and used to inoculate 4 mL LB broth with

211   ampicillin. After 16 h incubation at 37°C, cultures were centrifuged at 400 x g for 10 min

212   and 4°C to pellet bacteria. Plasmid DNA was collected using the QIAprep Spin Mini

213   Prep Kit (Qiagen, Valencia, CA) and a portion was digested with EcoR1 (NE Biolabs,

214   Ipswich, MA) and run on a 1.5% agarose gel containing GelRed intercolating dye to

215   assess insert length. From each treatment, five plasmid DNA samples containing inserts

216   of the predicted length were sequenced at the Cornell University Bioresource Center.

217   Sequences were aligned using Genious software and methylation status of the 16 CpGs

218   was assessed.

219   *Cell viability assays*

220   To evaluate the cytotoxicity of 5-AzaC on both normal and tumoral mammary cell

221   lines, cells were seeded at 10,000 per well in 96 well microplates. At 90% confluency,

222   low (5 µM) or high (50 µM) doses of 5-AzaC were added to triplicate wells. After 48 h of

223   culture, an MTT *in vitro* toxicology assay (Sigma Aldrich, Saint Louis, MO) was carried

224   out, according to manufacturer's instructions, and absorbance was measured at 570 nm

225   on a Multiskan EX plate reader (Thermo Fisher Scientific, Waltham MA). Optical

226   densities of wells treated with 5-AzaC were compared to those of untreated wells to

227   determine cell viability.

228   *Mitotracker C staining*

229   Cells for Mitotracker C staining were plated in 24 well culture dishes fitted with 35

230   mm coverslips. After one day of culture, 50 µM 5-AzaC was added to appropriate wells.

231   After 48 h of treatment, cells were washed twice with PBS and incubated with the

232   mitochondria-specific red fluorescent probe MitoTracker Red CMXRos (Cell Signaling

233 Technology, Danvers, MA) at a final concentration of 100 nM in serum free-culture

234 medium for 45 min at 37°C. Cells were then washed 3 times with PBS and fixed with ice

235 cold 90% ethanol for 10 min at -20°C. Cells were washed 3 times with PBS and

236 incubated with 0.5 μg/mL DAPI (BioLegend, San Diego, CA) for 5 min in PBS. After 3

237 washes with PBS, coverslips were carefully removed from culture wells and mounted on

238 glass slides using aqueous mounted medium (Dako, Carpenteria, CA). Samples were

239 examined with a Zeiss LSM confocal microscope (Oberkochen, Germany) and images

240 were captured with an attached camera controlled by ZEN imaging software.

241 *Immunocytochemistry (ICC) analyses*

242   Cells for ICC were grown in 24 well culture dishes fitted with 35 mm coverslips. After

243 one day of culture, 50 μM 5-AzaC was added to appropriate wells. After 48 h of

244 treatment, cells were rinsed with PBS and fixed in 4% PFA for 10 min. Following 3

245 rinses with PBS, cells were permeabilized using PBS + 1% Triton-X 100 + 1% BSA for

246 30 min at RT. Primary rabbit anti-active caspase-3 antibody (Abcam, Cambridge, MA) or

247 rabbit IgG, each diluted 1:00 in PBS, was added to the wells and incubated overnight at

248 4°C. Wells were then rinsed 3 times with PBS, and HRP-conjugated goat anti-rabbit

249 secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:100 in

250 PBS was added. After 30 min at RT, cells were washed 3 times with PBS and AEC

251 solution (Invitrogen Life Technologies, Grand Island, NY) was added for 15 min. Cells

252 were rinsed with PBS, counterstained with Gill's Hematoxylin (Thermo Fisher Scientific,

253 Waltham, MA) and washed with tap water. Coverslips were gently removed from wells

254 and mounted on slides using aqueous mounted medium (Dako, Carpenteria, CA). Images

255 were captured with a digital camera mounted on an Olympus BX51 light microscope

256   (Center Valley, PA). To determine percentage of cells positive for active caspase-3, cells

257   in three fields each containing at least 300 cells, were counted and classified as either

258   positive or negative based on presence or absence of red staining.

259   *Statistical analyses*

260   All experiments were repeated at least three times. Results are expressed as the mean

261   ± STDEV from three independent experiments, with the exception of the (ECIS) data,

262   which show the mean ± STDEV of endpoint impedance from 3 replicate wells of one

263   experiment, representing statistical trends calculated from 3 independent assays. Data

264   were analyzed by the Student's T-test and p values < 0.05 were considered statistically

265   significant. Single and double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively.

266

267   **Results**

268   *Characterization of in vitro tumorigenicity of canine and feline mammary carcinoma cell*

269   *lines and primary patient-derived mammary tumor cells.*

270   To characterize the *in vitro* tumorigenicity of the canine and feline tumoral cell lines

271   and primary tumor cells used in this study, soft agar and invasion/extravasation Electric

272   Cell-substrate Impedance Sensing (ECIS) assays were performed. The soft agar assays,

273   used to assess anchorage-independent growth, showed that significantly more spheres

274   were formed by the REM134 and K12-72.1 cell lines than by the CMT12 and Cat-MT

275   cell lines, respectively, and that these spheres had a tendency to a larger size (Figure 1A).

276   The ECIS assays, used to model tumor extravasation and assess the metastatic potential

277   of these cell lines did not show a statistically significant decrease in impedance from

278   confluent monolayers of bovine lung microvessel endothelial cells (BLMVEC) co-

279 cultured with any of the tumoral cell lines, when compared to BLMVEC co-cultured with

280 healthy control cell lines (Figure 1B).

281   The tissue samples that were used to isolate primary canine and feline mammary

282 adenocarcinoma-derived cells (CMADC and FMADC, respectively) were evaluated on

283 histopathology based on cell type involved, tubule formation, mitotic index and nuclear

284 variation, and scored as a grade 2 complex mammary carcinoma (canine tissue) and a

285 grade 2 simple mammary carcinoma (feline tissue) (Figure 2A). When using CMADC

286 and FMADC in soft agar assays to assess their anchorage-independent growth capacities,

287 however, no clear spheres were formed, not even after 40 days of culture (data not

288 shown). In contrast, CMAD and FMAD did show invasive potential using ECIS, since

289 co-culturing BLMVEC with these tumor cells resulted in a statistically significant

290 decreased impedance compared to co-culturing with healthy control cell lines (Figure

291 2B).

292 *Treatment of breast/mammary cancer cell lines and primary tumor cells with a low dose*

293 *of 5-AzaC suppresses both anchorage-dependent and -independent tumoral growth, as*

294 *well as tumor invasion activity.*

295   The effects of the DNA methylation inhibitor 5-azacytidine (5-AzaC) on tumor

296 proliferation (anchorage-dependent growth), *in vitro* tumorigenicity (anchorage-

297 independent growth) and tumor invasion potential of the canine, feline and human

298 tumoral cell lines were evaluated using a low dose of 5 μM 5-AzaC. Briefly, although a

299 significant reduction in viability of the cell lines MCF10CA1a, CMEC and CMT12 was

300 observed after treatment with this low dose of 5 μM 5-AzaC, the overall tendency was

301 this low dose did not negatively affect viability of the tumoral cells as determined by

302 MTT assays (Suppl. Figure 1). Indeed, since the absolute variation between the three

303 replicates of MCF10CA1a, CMEC and CMT12 was extremely small (Suppl. Figure 1),

304 the biological relevance of the statistically significant difference in these cell lines could

305 be questioned. To evaluate tumor proliferation, cells were treated with 5-AzaC or left

306 untreated (control) for 2 days, after which each cell line was added to wells of an ECIS

307 array slide at low density. Increases in impedance (a direct measure of the surface area of

308 the well covered by adherent cells) were recorded. As seen in Figure 3A, a significantly

309 lower impedance was observed in most of the tumoral cells lines treated with 5-AzaC

310 when compared to untreated controls, indicating that 5-AzaC treatment of tumoral cells

311 from all three species correlated with a decrease in proliferation. The exceptions were the

312 CAT-MT cell line, which showed a decrease in impedance when treated with 5-AzaC

313 that did not reach significance, the MCF7 cell line, which showed no difference in

314 impedance upon treatment, and the CMADC cells, which showed a significantly higher

315 impedance upon treatment (Figure 3A). When looking at the effects of 5-AzaC treatment

316 in soft agar assays, a reduced anchorage-independent growth capacity of all tumoral cell

317 lines was found as shown by a significantly reduced number of spheres, as well as a

318 reduced size of these spheres that reached significance for MCF10CA1a, MCF7 and

319 CMT12 (Figure 3B). The effects of 5-AzaC treatment on the anchorage-independent

320 growth of the primary tumor cells CMAD and FMADC was not assessed since these cells

321 do not form spheres in soft agar, as previously determined (data not shown).

322   Finally, the effects of 5-AzaC treatment on tumor invasiveness was evaluated using

323 the ECIS system. For these assays, only those cell lines/primary cells were used that

324 showed invasive potential based on our experiments using ECIS (Figure 2B) or previous

325 literature (Table 2). When MCF10CA1a, CMADC and FMADC or were added to a

326 confluent BLMVEC monolayer, they disrupted (invaded) the endothelial cells as

327 visualized by a rapid and sustained decrease in impedance as the endothelial cells were

328 displaced from the surface of culture wells (Figure 4). In contrast, when these cells were

329 treated with 5-AzaC they no longer induced a decrease in impedance as compared to the

330 untreated cells, indicating that 5-AzaC treatment of these human tumoral cells

331 significantly suppressed their metastatic potential (Figure 4).

332 *Treatment with a low dose of 5-AzaC affects gene expression with both inter- and intra-*

333 *species variability and the modified gene expression is caused by DNA methylation.*

334   To evaluate the effects of 5-AzaC treatment on gene expression, 11 genes were

335 selected that were shown previously to be upregulated upon 5-AzaC treatment in human

336 breast cancer cell lines [30-32]. An initial screen revealed that some genes were

337 downregulated whereas others were upregulated after 5-AzaC treatment (Table 3).

338 Interestingly, no consistent pattern was observed in gene expression between tumoral

339 cells from the 3 different species, and more importantly, not even between different

340 tumoral cell lines from the same species. For example, the gene *DKK3* was upregulated

341 in the human cell line MCF10CAa1 and the feline cell lines K12-72.1 and primary feline

342 FMADC, but not in MCF7 or any of the canine cell lines/primary cells (Table 3).

343 Another example is the gene *PGP9.5*, which was upregulated > 4-fold in CMADC, the

344 canine primary tumor cells, but not in the canine cell lines CMT12 and REM134 (Table

345 3). Next, the increase in expression of those genes that were upregulated > 4-fold after 5-

346 AzaC treatment was confirmed by repeating the qRT-PCR assays. The genes *FKBP6*,

347 *SYK* and *PGP9.5* were significantly upregulated in all cell lines/primary cells tested

348   (Figure 5A). The gene *NTN4* was significantly upregulated in FMAD but did not reach

349   significance in MCF10CA1a, and the gene *SFRP1* was upregulated in FMADC albeit

350   also without significance (Figure 5A). Since 5-AzaC is known to function as a DNA

351   methyltransferase (DNMT) inhibitor at low doses [33], bisulfite sequencing was used to

352   confirm the DNA methylation-dependent action of this drug in our current study. To this

353   end, the K12-72.1 cell line was used to evaluate the methylation status of CpG sites in the

354   promotor region of the gene *PGP9.5*. This gene was chosen based on the fact that 5-

355   AzAC treatment, at both 5 and 10 μM, induced a significant and robust upregulation in

356   this cell line (Suppl. Figure 1B). Sixteen CpG sites were examined and all 16 sites were

357   found to be methylated in untreated K12-72.1 (Figure 5B). In contrast, cells treated with

358   5 or 10 μM 5-AzaC only had between 12-15 and 1-14 CpG sites, respectively, methylated

359   (Figure 5B). These results showed that at low concentrations 5-AzaC indeed causes

360   demethylation of CpG sites in the promoter region of *PGP9.5* (Figure 5B) and that this is

361   associated with increased expression of this gene (Suppl. Figure 1B).

362   *Treatment with a high dose of 5-AzaC has a direct toxic effect on breast/mammary*

363   *cancer cell lines and primary tumor cells, without affecting healthy mammary cells.*

364   Aside from demethylating cellular DNA, 5-AzaC has been shown to cause DNA

365   damage by inducing double-strand breaks and to induce apoptosis by mitochondrial

366   membrane permeabilization and caspase activation in cancer cells like myeloma cells [34,

367   35]. To begin exploring these cytotoxic effects of 5-AzaC in breast/mammary cancer

368   cells, we treated the canine, feline and human cancer cells with a high dose of 5-AzaC

369   (50 μM) and evaluated viability using MTT assays. At this concentration, a significant

370   reduction in cell viability was observed in treated tumor cells as compared to untreated,

control cells (Figure 6A). Importantly, such an effect was not observed when normal

health mammary cell lines were treated with the same dose of 5-AzaC, with the exception

of a small, but significant, decrease in viability in the healthy canine mammary cell line

CMEC (Figure 6A). These data show that this drug is selectively toxic towards tumor

cells as compared to normal cells. To study the underlying mechanisms of this increased

toxicity in more detail, we performed a staining with the MitoTracker Red CMXRos on

untreated and 50 μM 5-AzaC-treated tumor cells and observed a loss of dye accumulation

in the treated tumoral cells, indicative of mitochondrial membrane permeabilization,

when compared to control tumoral cells (Figure 6B). Moreover, 5-AzaC treatment

activated caspases in mammary tumoral cells, as shown by an increased expression of

active caspase-3 in treated versus untreated tumoral cells (Figure 7). In contrast, no such

effects were observed when healthy mammary cells from all three species were treated

with 50 μM of 5-AzaC (Figure 6B and Figure 7), re-emphasizing the selective toxic

effects of 5-AzaC on tumoral cells, at least *in vitro*.

Discussion

The present comparative study was initiated to evaluate the effects of the epigenetic

modifier 5-AzaC on mammary tumorigenicity of canine and feline mammary cancer cells

in vitro, and to compare these results with results obtained in human breast cancer cell

lines. Our salient findings were that 5-AzaC at a low concentration (5 μM) could reduce

in vitro tumorigenicity and at a high dose (50 μM) had a direct toxic effect for tumoral,

but not healthy, mammary cells in all three species. The consistency of our results across

the three species supports the value of naturally occurring mammary cancer in dogs and

394   cats as a valuable translational model for human breast cancer, and provides the *in vitro*

395   rationale for using DNA methyltransferase (DNMT) inhibitors, like 5-Aza, as a potential

396   treatment option in veterinary oncology.

397   For our *in vitro* tumorigenicity studies, we used a combination of assays to evaluate

398   the anchorage-dependent and -independent growth, and invasive potential, of tumoral

399   cells in the presence and absence of 5 μM 5-AzaC. For the anchorage-dependent assays,

400   tumor cells were plated in 96W1E$^+$ PET plates and cell growth, also defined as

401   proliferation, was measured over time using Electric Cell-substrate Impedance Sensing

402   (ECIS). Anchorage dependence is a phenomenon that has been defined as an increase in

403   proliferation when cells are allowed to attach to a solid surface and is therefore relevant

404   to malignant transformation and tumorigenicity [36,37]. All tumoral cell lines showed a

405   significant reduced proliferation upon treatment with 5-AzaC with exception of the feline

406   CAT-MT cell line (reduction but not significant), the human MCF7 cell line (no effect)

407   and the canine primary tumor cell CMADC (significantly increased expression). Despite

408   this unexpected increase in proliferation of CMAD after 5-AzaC treatment, 5-AzaC could

409   still inhibit the invasiveness of these cells as shown by the tumor invasion/extravasation

410   ECIS assay [39, 40]. Interestingly, a study evaluating the effects of 5-AzaC on

411   proliferation and *in vitro* invasion of pancreatic adenocarcinomas also found

412   contradictory and adverse effects of 5-AzaC in some, but not all, pancreatic cell lines

413   [41]. More specifically, they found that whereas 5-AzaC could inhibit the proliferation of

414   all five pancreatic cancer cell lines, a significant increase, instead of the expected

415   decrease, in *in vitro* invasive potential was noted in four out of the five cell lines after

416   treatment with 5Aza [41]. Those data combined with our results indicate that the use of

417 methylation inhibitors to reduce tumorigenicity should be carefully evaluated, ideally

418 using the primary tumor cells isolated from the patient's tumor to ensure that these drugs

419 will not results in adverse effects when used clinically in that patient.

420 To study anchorage-independent growth, soft agar assays were used. These functional

421 *in vitro* assays are standard for modeling *in vitro* tumorigenicity, at least for human

422 cancer cells, and correlate fairly well with *in vivo* carcinogenesis [38]. Since soft agar

423 assays are not routinely used to evaluate anchorage-independent growth of feline and

424 canine cancer cells, we decided to first characterize the sphere formation of the tumoral

425 cell lines and primary cells used in this study. All cell lines (CMT12, REM134, CAT-MT

426 and K12-72.1) were capable of forming tumor spheres, however, and to our surprise, the

427 primary cells CMAD and FMAD were unable to form spheres in soft agar. A potential

428 explanation for this lack of sphere formation could be the specific requirements of these

429 patient-derived primary tumor cells and consequently, optimization of the soft agar

430 concentrations, seeding density, culture media (increasing serum, adding hormones like

431 EGF or IGF-1) and environmental conditions (oxygen, humidity) might be necessary for

432 these primary cells to be able to form spheres in the soft agar assay.

433 In addition to studying the effects of 5-AzaC on *in vitro* tumorigenicity of

434 breast/mammary cancer cells in all three species, we evaluated the expression profiles of

435 genes that were shown previously to be upregulated upon 5-AzaC treatment in human

436 breast cancer cell lines. Ten genes were shown to be upregulated in MCF7 cells [30] and

437 one gene, *SYK*, was shown to be upregulated in six *SYK*-negative breast cancer cell lines

438 [31-32]. When we evaluated the expression of those ten genes in 5-AzaC-treated MCF7

439 cells as a positive control, we only observed an upregulation in mRNA expression of

440   *SFRP1* and *NTN4*. This discrepancy might be explained by differences in the qRT-PCR

441   assay used to assess mRNA expression. The authors of the previous study used a TaqMan

442   approach and included only one reference gene, *GAPDH*, whereas we used SYBR green

443   and two reference genes, namely *GAPDH* and *HSPBC*. Another explanation could be the

444   treatment regiment: MCF7 cells were treated with an undisclosed amount of 5-AzaC for

445   3 days in the referenced study, instead of 5 μM 5-AzaC for 2 days that was used in our

446   present study. Still, when looking at the effects of 5AzaC on expression of the same

447   genes but in the 3 different species, not only considerable inter-species variability was

448   observed but also substantial intra-species variability, at least in this limited set of 11

449   genes. The latter is in line with what has been reported in the referenced study related to

450   the 5-AzaC-treated MCF7 [30], where they found that the genes that were upregulated in

451   5-AzaC-treated MCF7 showed variable levels of gene expression in other breast cancer

452   cell lines, indicating that a variable level of DNA methylation of the same genes exists in

453   different cancer cell lines. Although not the focus of our present study, this variability

454   between different tumors from the same tissue, like the mammary gland, could

455   potentially reduce the usefulness of DNA methylation of specific genes as biomarkers.

456   Finally, we also used 5-AzaC at a high dose to evaluate its direct cytotoxic effects on

457   cancer cells, as previously described [34, 35]. Treatment of these tumor cells with 50 μM

458   5-AzaC could significantly reduce viability of these cells, an effect caused by apoptosis

459   as determined by mitochondrial permeabilization and caspase-3 activation. Importantly,

460   treating healthy mammary cell lines with a high dose of 5-AzaC did not negatively affect

461   viability, indicating the selective toxicity of this drug towards tumoral cells, at least *in*

462   *vitro*. Confirming that apoptosis is the process by which a high dose of 5-AzaC reduces

463   viability in canine and feline mammary cancer cell lines (i) suggests that these cells

464   behave like human mammary cancer cell lines, supporting the use of dogs and cats as

465   models for human cancer and (ii) directs future drug studies toward exploring the power

466   of combination (epigenetic) therapies to induce potent and directed killing of dog and cat

467   tumor cells. Indeed, work with human breast cancer cell lines has shown that treatment

468   with 5-AzaC in combination with overexpression of the tumor suppressor gene Inhibitor

469   of growth family member 1 (*ING1*), a critical epigenetic regulator of cellular senescence,

470   had a synergistic cytotoxic effect [42]. Although each of these compounds causes

471   dysregulation of a distinct epigenetic pathway, they were shown to complement each

472   other by ultimately directing target cells towards apoptosis.

473   Based on the *in vitro* findings in our current study, the next step will be to evaluate the

474   effects of 5-AzaC in a mouse xenograft model of canine and feline mammary cancer. For

475   human breast cancer cell lines, the anti-cancer effects of 5-AzaC, alone or in combination

476   with other epigenetic drugs, have been evaluated in mouse xenografts [15,32]. In

477   contrast, and to our knowledge, 5-AzaC has not been evaluated in canine and feline

478   xenograft models of mammary cancer to date. Therefore, future experiments are planned

479   to first establish and characterize canine and feline mammary tumor xenograft models

480   with the primary CMADC and FMADC used in the present study, and then use these

481   patient-derived xenograft (PDX) models to evaluate the efficacy and safety of 5-AzaC, a

482   drug for which we could show promising anti-cancer effects *in vitro*.

483

484   **Conclusions**

485   Taken together, we believe the results of the present study verify the unique

486   comparative value of dogs and cats as models for breast cancer research in humans. More

487   specifically, we propose that evaluating anti-cancer drugs in these animals will not only

488   yield benefits for humans, but may improve treatments for veterinary species as well.

489

490   **List of Abbreviations**

491   DNMT: DNA methyltransferase; 5-AzaC: 5-Azacytidine; HDAC: histone deacetylase;

492   FDA: Food and Drug administration; MCF10A: human normal breast epithelial cell line;

493   MCF10CA1a: human malignant breast carcinoma cell line; DMEM: Dulbecco's

494   modified Eagle medium; EGF: epidermal growth factor; ER: oestrogen receptor; MCF7:

495   human breast adenocarcinoma cell line; FMEC: feline normal mammary epithelial cell

496   line; K12-72.1: feline mammary adenocarcinoma cell line; CAT-MT: feline mammary

497   carcinoma cell line; FBS: fetal bovine serum; CMEC: canine normal mammary epithelial

498   cell line; REM134: canine mammary carcinoma cell line; CMT12: canine mammary

499   carcinoma cell line; ECIS: Electric Cell-substrate Impedance Sensing; BLMVEC: bovine

500   microvessel lung endothelial cells; qRT-PCR: quantitative reverse-transcription PCR;

501   *DFNA5*: non-syndromic hearing impairment protein 5; *SFRP1*: secreted frizzled-related

502   protein 1; *NTN4*: netrin 4; SYK: spleen tyrosine kinase; *FKBP6*: FK506 binding protein

503   6; *LOXL4*: Lysyl oxidase-like 4; *PON1*: paraoxonase 1; *TRIM50*: tripartite motif-

504   containing 50; *OSPBL3*: oxysterol-binding protein 3; *DKK3*: dikkopf-related protein 3;

505   *PGP9.5*: ubiquitin carboxy-terminal hydrolase L1; *HSPBC*: heat shock protein 1;

506   *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *HPRT*: hypoxanthine guaine

507   phosphoribosyl transferase; *UBI*: polyubiquitin; *RPL30*: Ribosomal Protein L30;

508 *YWHAZ:* 14-3-3 protein zeta*; ICC:* immunocytochemistry; *HRP*: horseradish peroxidase;

509 PBS: phosphate buffered saline; BSA: bovine serum albumin; TBS: Tris buffered saline;

510 PFA: 4% paraformaldehyde; *AEC*: 3-amino-9-ethylcarbazole; CMADC: canine

511 mammary adenocarcinoma-derived cells; FMADC: feline mammary adenocarcinoma-

512 derived cells; ECM: extracellular matrix; NIH-3T3: murine fibroblast cell line; EC:

513 endothelial cells; ING1: inhibitor of growth family member 1; PDX: patient-derived

514 xenograft.

515

Competing interests

517 The authors declare they have no competing interests.

518

Authors' Contributions

520   RH carried out all laboratory procedures, was involved in conception and design, and

521 manuscript writing; TC provided expertise and technical assistance with the Electric Cell-

522 substrate Impedance (ECIS) assays; DA provided canine and feline mammary cancer cell

523 lines and SC provided the human cell lines; DA, SC and GVdW were involved in

524 conception and design; GVdW was involved in data analyses and manuscript writing. All

525 authors read and approved the final manuscript.

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657

658

Figure Captions

660 **Fig 1 Characterization of *in vitro* tumorigenicity of canine and feline mammary cell**

661 **lines. (A)** Representative phase contrast images of tumorspheres formed by canine and

662 feline mammary cell lines in anchorage-independence (soft agar) assays, and

663 quantification of sphere number and size determined from soft agar assays. n=3, *: P <

664 0.05, scale bars = 50 μm **(B)** Assessment of invasive potential of canine and feline

665 mammary cell lines measured by invasion/extravasation Electric Cell-substrate

666 Impedance Sensing (ECIS) assays. Representative phase contrast images of healthy

667 canine and feline mammary cell lines, and non-invasive canine and feline mammary cell

668 lines on top of endothelial cell monolayer in ECIS assay plate are shown. n = 3, scale

669 bars = 50 μm

670 **Fig 2 Characterization of primary mammary tumor cells cultured from canine and**

671 **feline mammary adenocarcenomas. (A**) Images of formalin-fixed, paraffin-embedded

672 tumor tissues stained with hematoxylin and eosin. scale bars = 50 μm **(B)** Assessment of

673 invasive potential of canine and feline adenocarcinoma-derived cells (CMADC and

674 FMADC, respectively) measured by invasion/extravasation Electric Cell-substrate

675 Impedance Sensing (ECIS) assays. Representative phase contrast images of healthy

676 canine and feline mammary cell lines on top of endothelial cell monolayer in ECIS assay

677 plates (upper images), and invasive CMADC ad FMADC incorporated into endothelial

678 cell monolayer (lower images) are shown. n = 3, scale bars = 50 μm

679 **Fig 3 Effects of low dose 5-AzaC on anchorage-dependent and –independent growth**

680 **of breast/mammary cancer cell lines and primary tumor cells. (A)** Anchorage-

681 dependent growth as measured by proliferation using Electric Cell-substrate Impedance

682   Sensing (ECIS) of canine, feline and human tumoral mammary cells lines/primary cells

683   treated with 5 μM 5-AzaC or left untreated. **(B)** Anchorage-independent growth as

684   measured by soft agar assays of canine, feline and human tumoral mammary cells lines

685   treated with 5 μM 5-AzaC or left untreated. Number and size of spheres were determined.

686   n=3, *: P < 0.05, **: P < 0.01

687   **Fig 4 Effects of low dose 5-AzaC on invasive potential of breast/mammary cancer**

688   **cell lines and primary tumor cells.** Invasive potential of MCF10CA1a, CMADC and

689   FMADC treated with 5 μM 5-AzaC or left untreated was measured by

690   invasion/extravasation Electric Cell-substrate Impedance Sensing (ECIS) assays.

691   Representative phase contrast images of untreated (invading) and 5-AzaC treated (non-

692   invading) cells on endothelial cell monolayer in ECIS assay plates are shown. n= 3, scale

693   bars = 50 μm

694   **Fig 5 Effects of low dose 5-AzaC on gene expression and methylation status in**

695   **breast/mammary cancer cell lines and primary tumor cells. (A)** Expression levels of

696   the genes *PGP9.5*, *SFRP1*, *NTN4*, *FKBP6* and *SYK* in canine, feline and human tumoral

697   mammary cells lines/primary cells treated with 5 μM 5-AzaC as determined by qRT-

698   PCR. Fold change from non-treated cells is shown. n=3, *: P < 0.05, **: P < 0.01. **(B)**

699   Graphic representation of the methylation status of CpG islands in the upstream promoter

700   region of the gene *PGP9.5* in untreated K12-72.1 cells, and K12-72.1 cells treated with 2

701   concentrations (5 and 10 μM) of 5-AzaC using bisulfite sequencing. Each column

702   represents a CpG site (16 total) and each row represents a genomic DNA clone (n=5).

703   Blue blocks indicate a methylated CpG site, red blocks an unmethylated CpG site. A

704   representative trace file showing a methylated cytosine (top panel), unaffected by

705 bisulfite treatment, and an unmethylated cytosine (bottom panel), converted into a uracil

706 by bisulfite treatment and amplified as a thymine during PCR, at position 139 (black

707 arrow) is shown.

708 **Fig 6 Effects of high dose 5-AzaC on viability and mitochondrial membrane**

709 **permeabilization in breast/mammary cancer cell lines and primary tumor cells. (A)**

710 Viability of canine, feline and human tumoral mammary cells lines/primary cells treated

711 with 50 μM 5-AzaC as determined by MTT assays. Percent viable cells, compared to

712 non-treated cells, set at 100%, are shown. n=3, *: $P < 0.05$, **: $P < 0.01$. **(B)**

713 Representative confocal images of canine, feline and human tumoral mammary cells

714 lines/primary cells treated with 50 μM 5-AzaC, or left untreated, and stained with

715 MitoTracker Red are shown. Scale bars = 10 μm.

716 **Fig 7 Effects of high dose 5-AzaC on caspase activation in breast/mammary cancer**

717 **cell lines and primary tumor cells.** Representative bright field images of canine, feline

718 and human tumoral mammary cells lines/primary cells treated with 50 μM 5-AzaC, or

719 left untreated, and stained with anti-active caspase-3 antibodies are shown. Numbers

720 show percentage of cells positive for anti-active caspase-3. Scale bars = 20 μm.

721

722 **Supplementary Figure 1.**

723 **(A).** Viability of canine, feline and human tumoral mammary cells lines/primary cells

724 treated with 5 μM 5-AzaC as determined by MTT assays. Percent viable cells, compared

725 to non-treated cells, set at 100%, are shown. n=3, *: $P < 0.05$. **(B).** Expression levels of

726 the gene *PGP9.5* in the feline cell line K12-72.1 treated with 5 and 10 μM 5-AzaC as

727 determined by qRT-PCR. Fold changes from non-treated cells is shown. n=3, *: $P < 0.05$.

728

Table 1. Primers used for qRT-PCR

A. Primers for genes reported to be affected by 5-AzaC*

Gene	Abbreviation	Species	Forward Primer (5'-3')	Reverse Primer (5'-3')
Non-syndromic hearing impairment protein 5	DFNA5	human, canine, feline	AGCCACAACAGACAGCTTTG	ACTGGTTCCAGGACCATGAG
Secreted frizzled-related protein 1	SFRP1	human, canine, feline	TGTCCCCAAGAAGAAGAAGC	AAGTGGTGGCTGAGGTTGTC
Netrin 4	NTN4	human, canine, feline	AAACTCTGGGCAGACACCAC	TAGGCAGCATTGCACTTGTC
Spleen tyrosine kinase	SYK	human, canine, feline	AAACTACTACAAGGCCCAGACC	TCCAGACGTCACTCTTGCTG
FK506 binding protein 6	FKBP6	human	TGACTTCCTGGACTGTGCTG	GTTCCGTAGCTGCCACTTTC
		canine	GAATGCTAAGGCCCTCTTCC	TGAAGGGTTGTTCCTTCTGG
		feline	TTGAGCTGCTTGACTTCCTG	TAGTTGCCAAACTCCCGTTC
Lysyl oxidase-like 4	LOXL4	human	CCAGCTTCTGTCTGGAGGAC	ATATCCACCCACTGGCAATC
		canine	CGCTTCTCAGCTGGAGTTTC	CAGACTGGGAGAGGCAGTTC
		feline	GCTTTGAAACAGCCTTGACC	ATACAGCGCACATTGTCCAG
Paraoxonase 1	PON1	human	AACCATCCAGATGCCAAGTC	AAAGTGCTCAGGTCCCACAG
		canine	CAGAGGTGATCCGAATCCAG	ACAGAGGCCACGGTACTTCC
		feline	TATTGTTGCTGTGGGACCTG	CCATCTGCCATCACTTGAAC
Tripartite motif-containing 50	TRIM50	human	AACAGTTCGGCAATGAGGAC	GCTTGATGTCAGCCTGGTG
		canine	GCTAGCGGCTCTCATCTCTG	CGGGTCCTATTGTTCACCAG
		feline	CAGGCTGACATCAAGCTGAC	TTGGAGAGTTCCAGGAGTGG
Oxysterol-binding protein 3	OSBPL3	human	CTATGCCGAAAGGCTACGAG	CTGGTCTGGCCTAAATCGAG
		canine	TGCAGAATCCATGCTGAGTC	ACCAGAGCTCGGTTGTCATC
		feline	GGAGTACAGCGAGCTTCTGG	GAACAGGATTGAAGGGCTTG
Dikkopf-related protein 3	DKK3	human	TGTGACAACCAGAGGGACTG	CTAGCTCCCAGGTGATGAGG
		canine	CTGTGCCTTCCAGAGAGGTC	CAGGCTCTAACTCCCAGGTG

		feline	CTGTGCCTTCCAGAGAGGTC	CAGGCTCTAACTCCCAGGTG
Ubiquitin carboxy-terminal hydrolase L1	*PGP9.5*	human	GAGATGCTGAACAAAGTGC	AGCCCAGAGACTCCTCTTCC
		canine	GTGGTACCATCGGGCTTATC	TTCAGGACTGACCCATCCTC
		feline	CAGTGGCCAATAACCAGGAC	GGTGACAGCTTCTCCGTTTC

B. Primers for reference genes

Gene	Abbreviation	Species	Forward Primer (5'-3')	Reverse Primer (5'-3')
Heat shock protein family B member 1	*HSPB1*	human	GGCATGACCAAAGCTGATCTC	ACCAAACTGCCCAATCATGG
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	human	GACAGTCAGCCGCATCTTCT	TTAAAAGCAGCCCTGGTGAC
Hypoxanthine phosphoribosyl transferase 1	*HPRT*	canine	TGCTCGAGATGTGATGAAGG	TCCCCTGTTGACTGGTCATT
Polybiquitin	*UBI*	canine	TCTTCGTGAAAACCCTGACC	CCTTCACATTCTCGATGGTG
Ribosomal protein L30	*RPL30*	feline	CCTCGGCAGATAAATTGGACTGC	TGATGGCCCTCTGGAATTTGAC
Tyrosine 3-mono oxygenase/tryptophan 5-monooxygenase activation protein, zeta	*YWHAZ*	feline	GAAGAGTCCTACAAAGACAGCAC	AATTTTCCCCTCCTTCTCCTGC

* These genes have been previously shown to be upregulated upon 5-AzaC treatment in human breast cancer cell lines [30-32].

Table 2. The invasive potential of the cell lines/primary tumor cells used in the present study.

Species	Normal mammary/breast epithelial cell lines	Mammary/breast adenocarcinoma cell lines and cells	
		Non-invasive	**Invasive**
Canine	CMEC	REM134 [25][*] CMT12 [26]	CMADC
Feline	FMEC [22]	K12-72.1 [23] CAT-MT [24]	FMADC
Human	MCF10A [16]	MCF-7 [20]	MCF10CA1a [17,18]

[*] References for previously published cell lines are included in between brackets.

Table 3. Changes in gene expression after 5-AzaC treatment.

Gene	Human cell lines		Canine cell lines			Feline cell lines		
	MCF7	MCF10CA1a	CMT12	REM134	CMADC	CAT-MT	K12-72.1	FMADC
DFNA5	-	ND	ND	ND	-	ND	ND	ND
SFRP1	+	+	-	+	+	-	+	++
NTN4	+	++	-	+	-	ND	ND	++
FKBP6	-	++	+	+	++	ND	+	+
LOXL4	-	ND	-	-	+	-	-	+
PON1	-	-	-	-	+	-	+	+
TRIM50	-	+	-	+	+	-	+	+
OSPBL3	ND	ND	-	-	-	-	-	+
DKK3	-	+	-	-	-	-	+	+
PGP9.5	-	ND	-	-	++	ND	++	+
SYK	-	+	+	+	-	-	-	+

ND: not determined; -: no upregulation; +: < 4-fold upregulation; ++: > 4-fold upregulation

A.

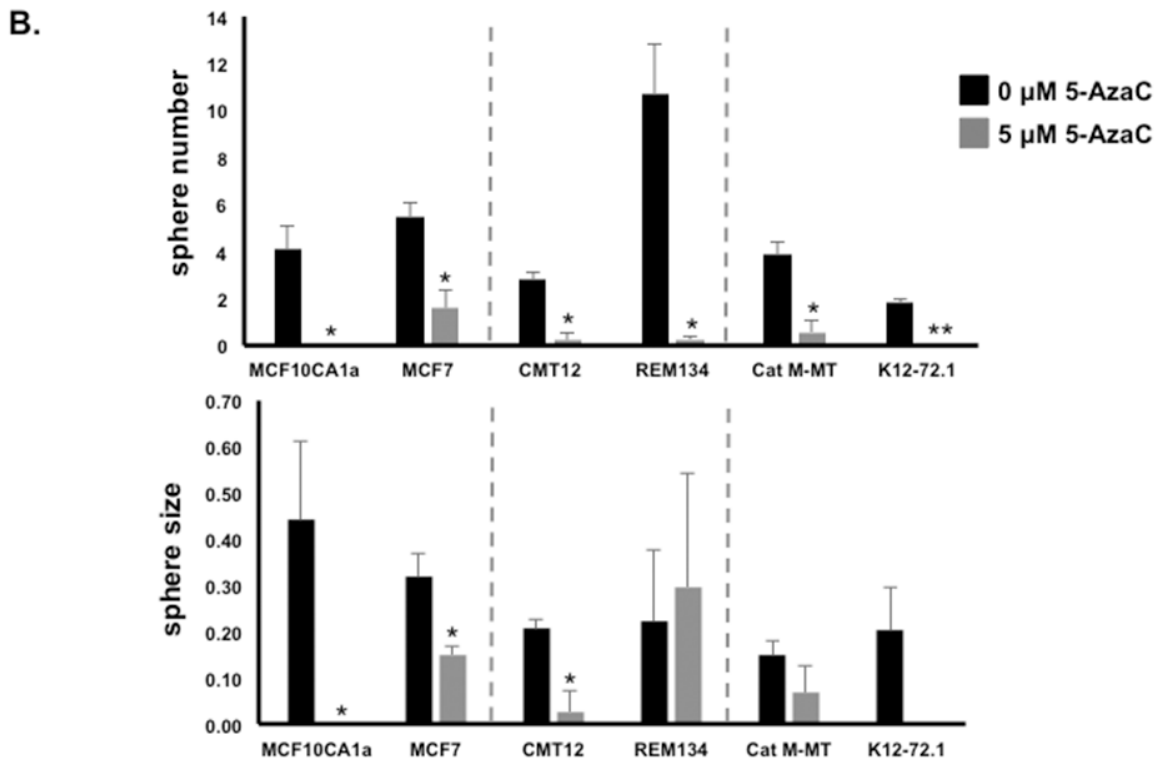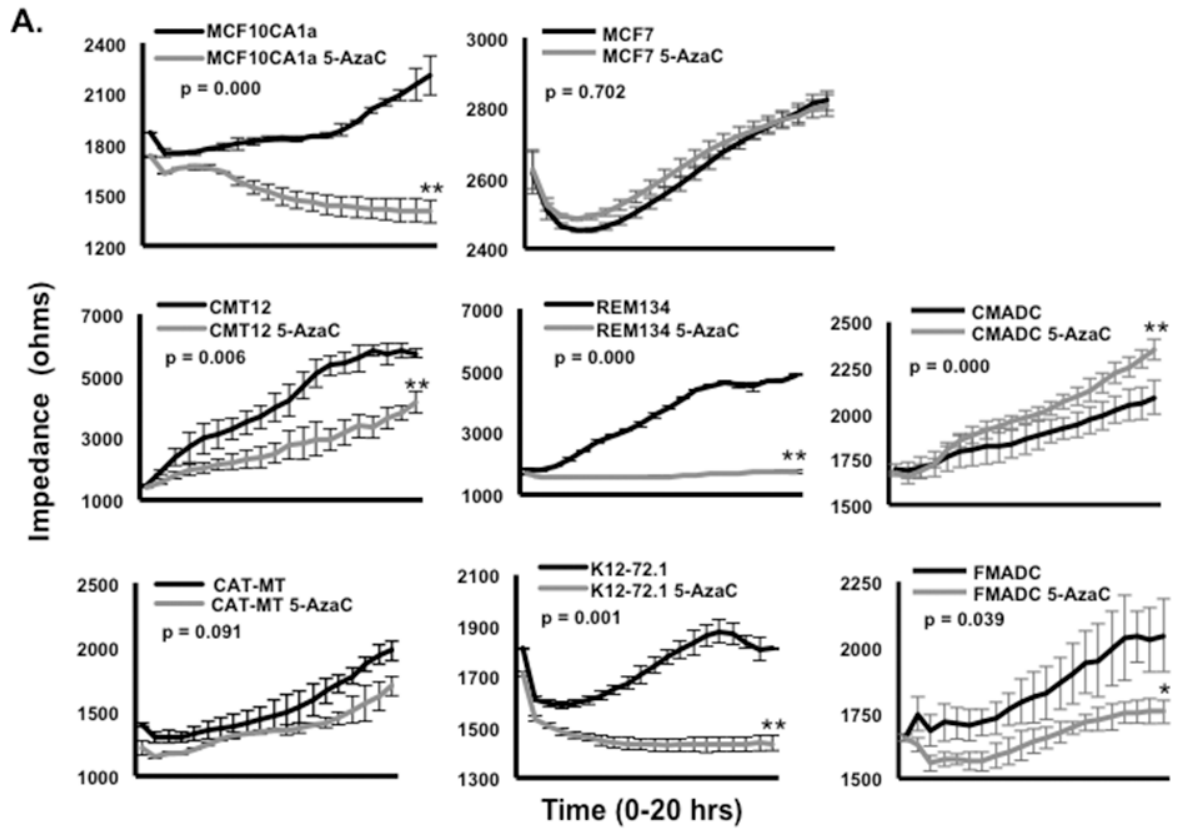CMT12 REM134 CAT-MT K12-72.1
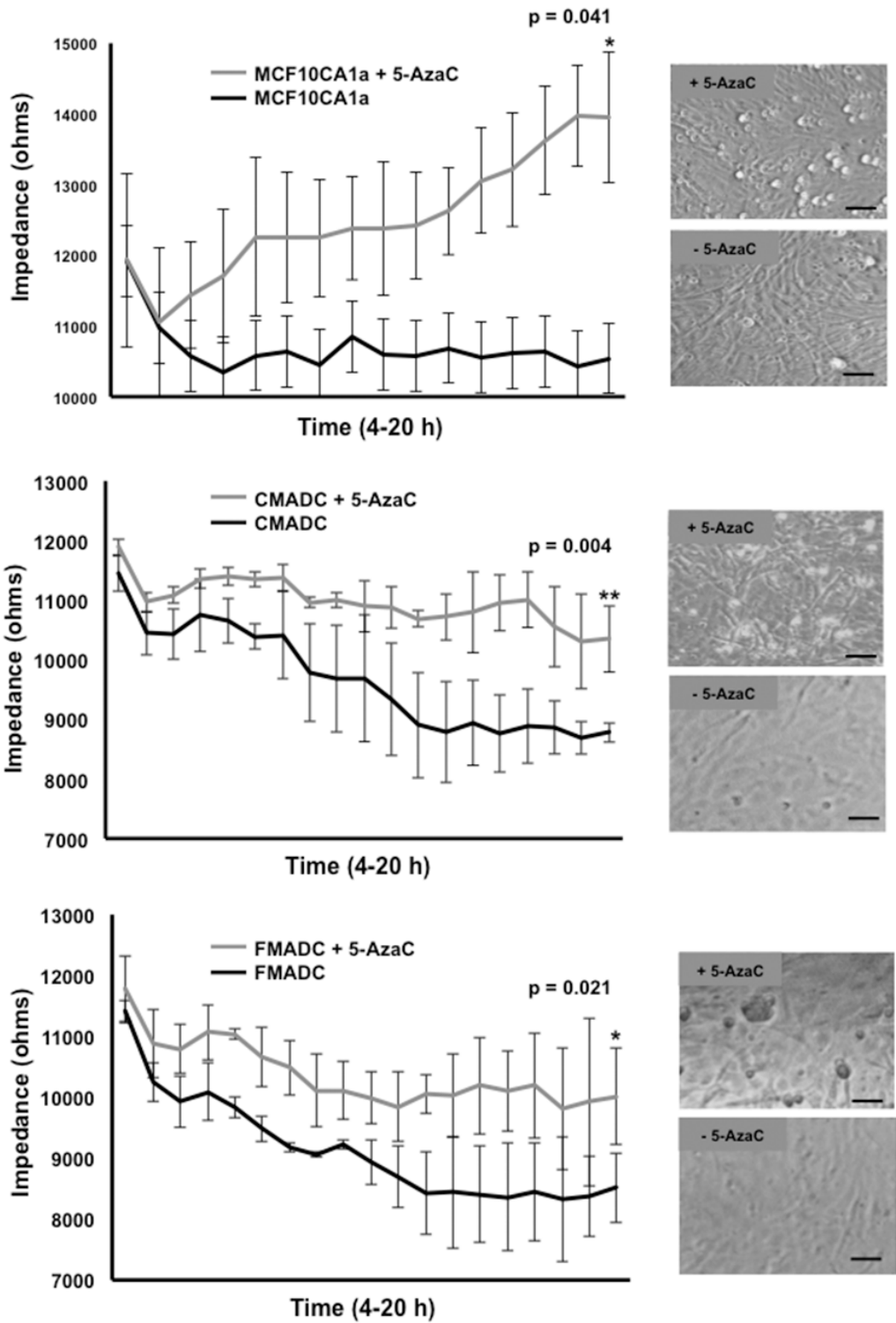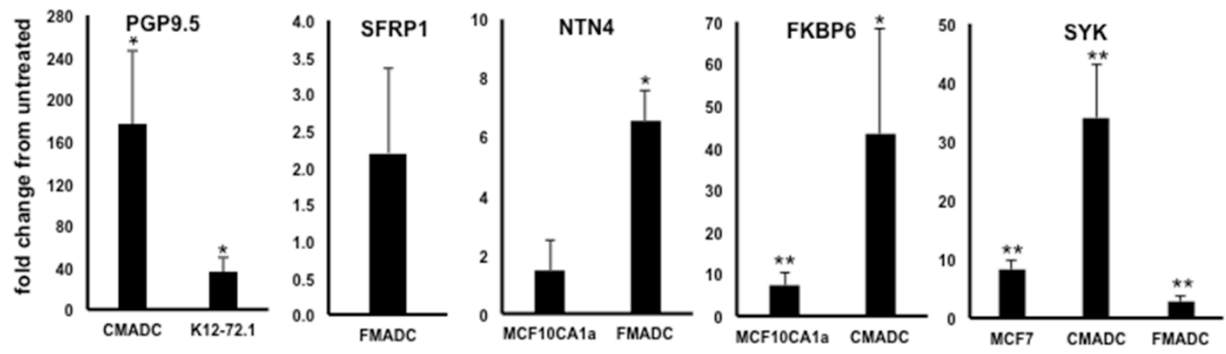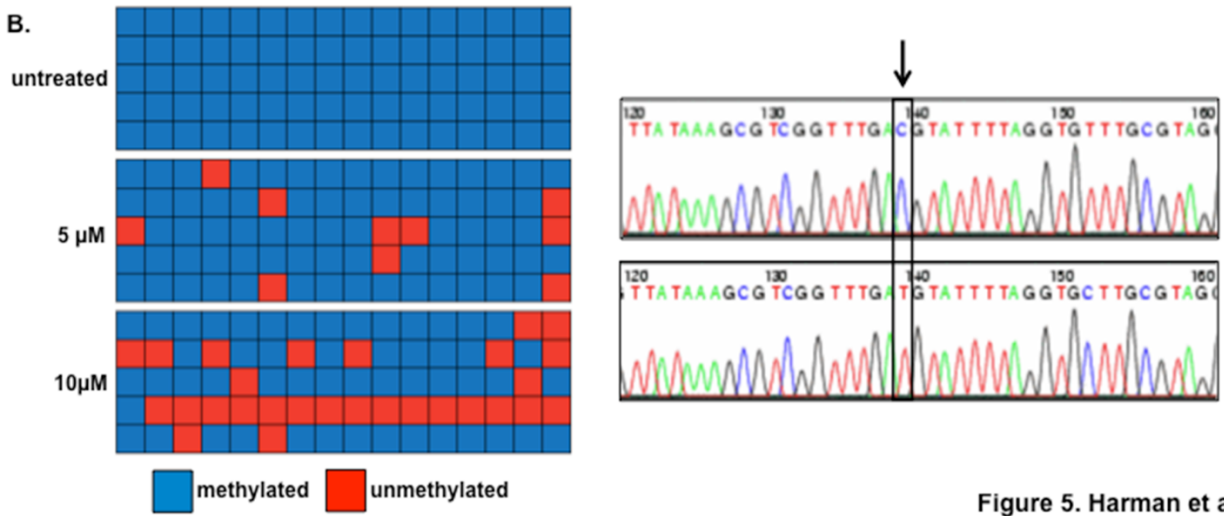
B.
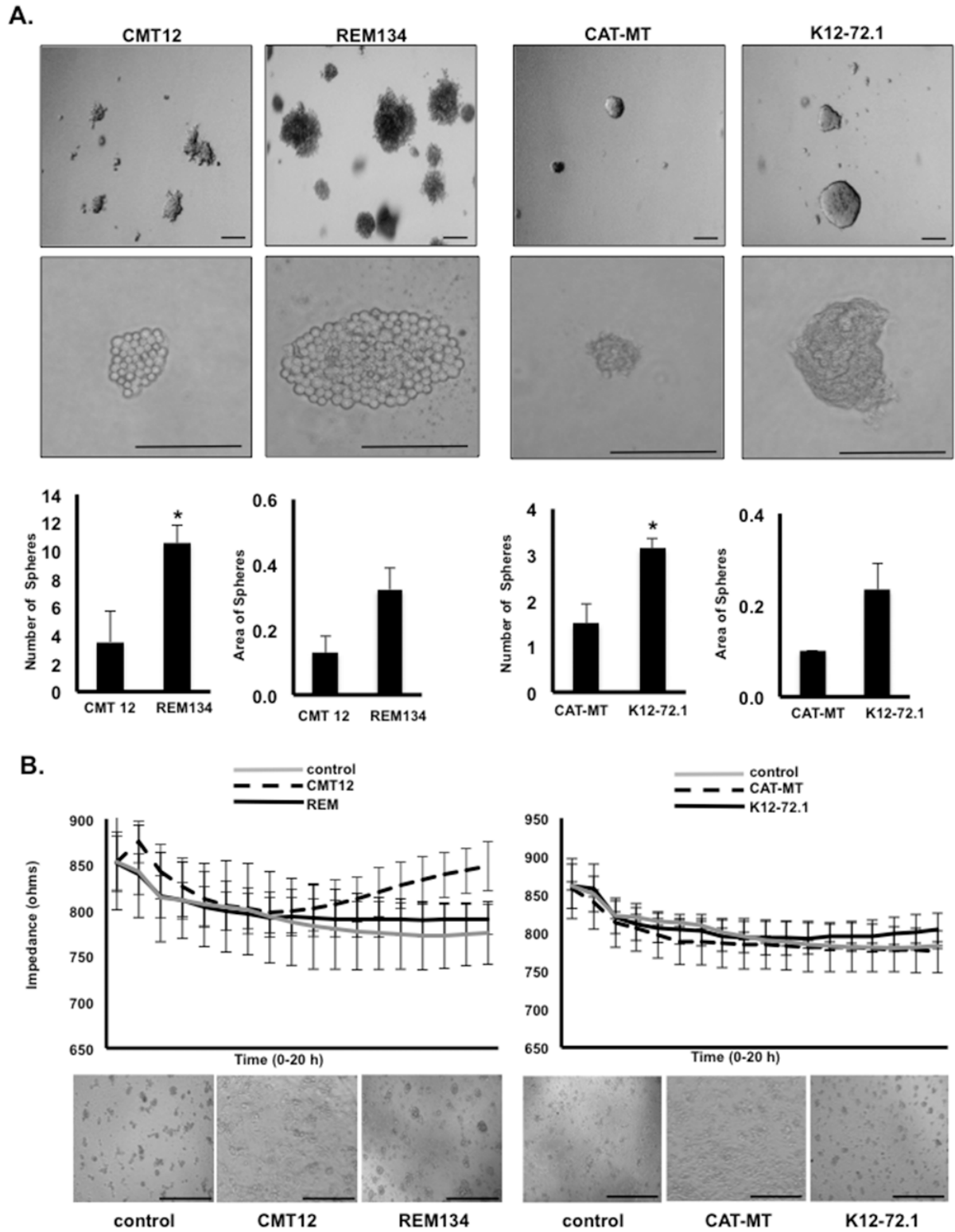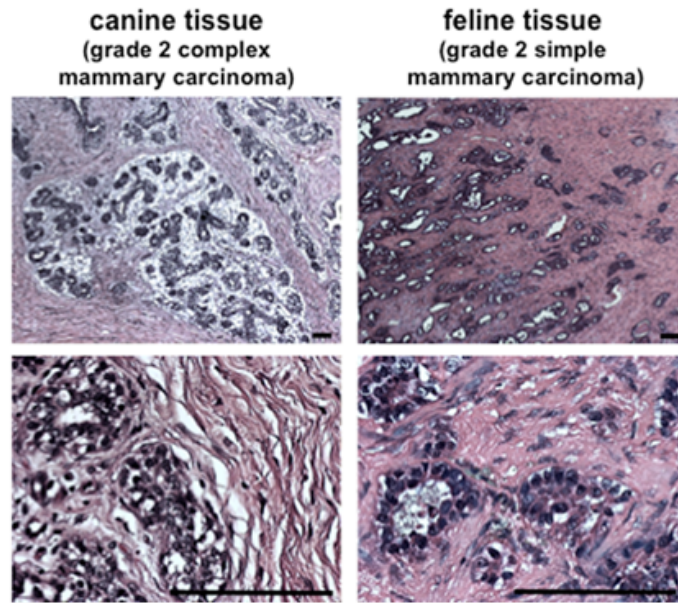
Figure 1. Harman et al.
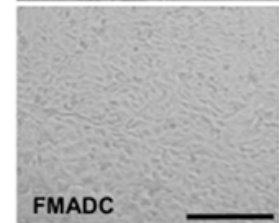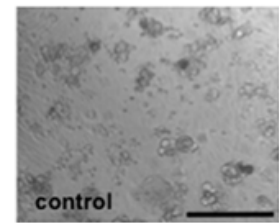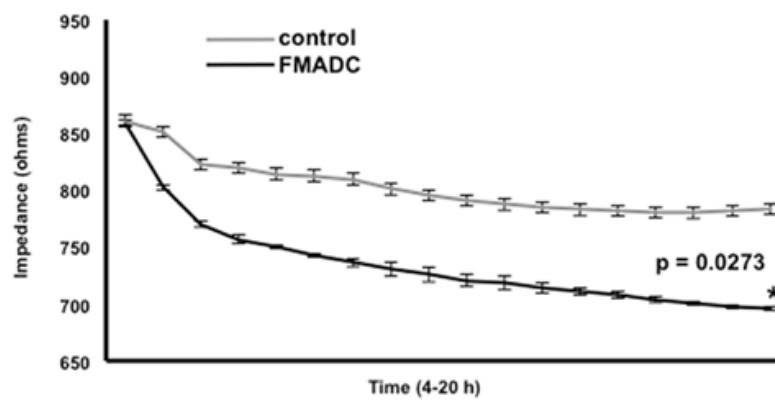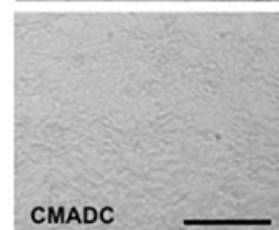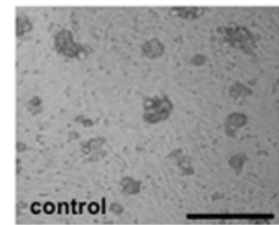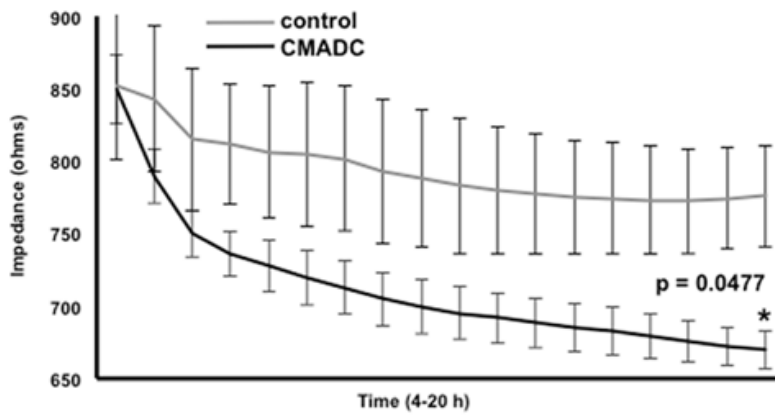
Figure 2. Harman et al.

Figure 3. Harman et al.

Figure 4. Harman et al.

Figure 5. Harman et al.

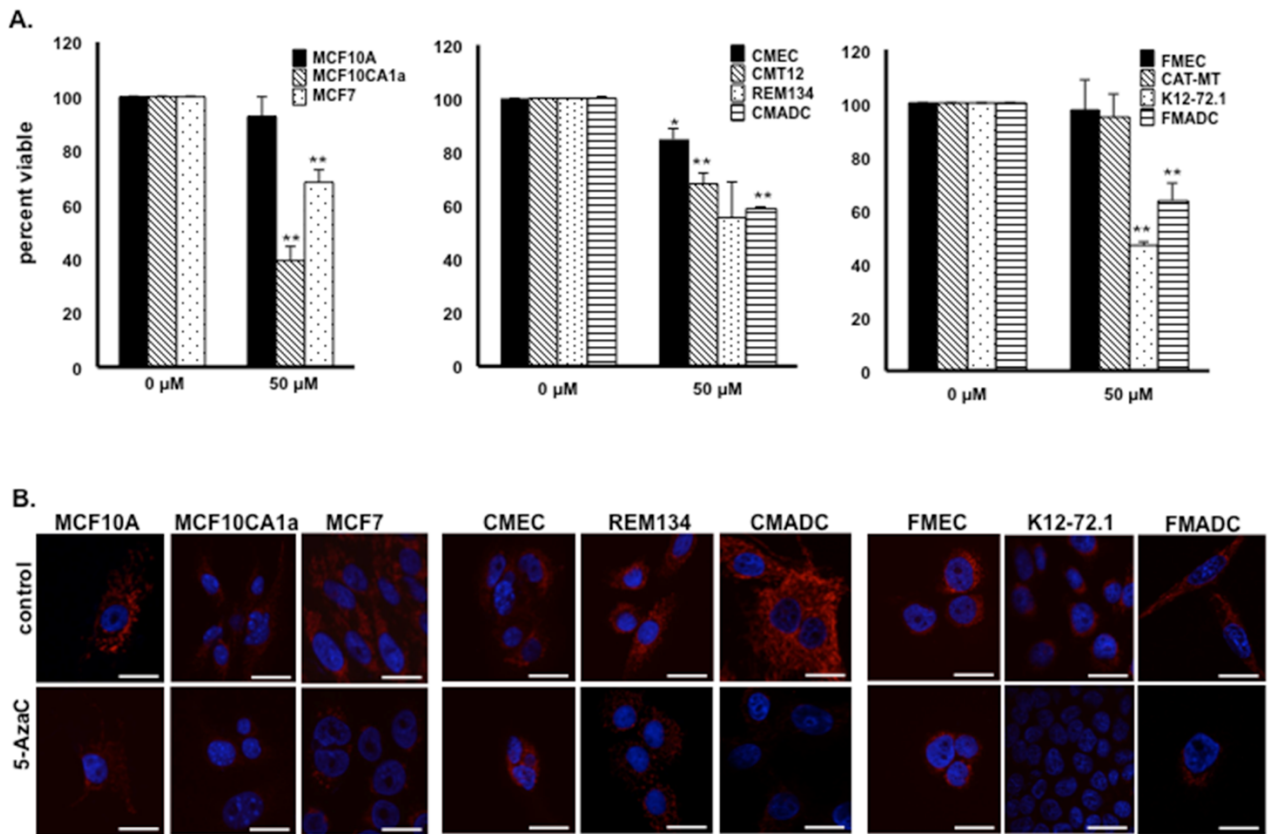**A.**

B.

Figure 6. Harman et al.

	MCF10A	MCF10CA1a	MCF7
control	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 1.2
5-AzaC	0.0 ± 0.0	45.7 ± 11.6	100.0 ± 0.0

	CMEC	REM134	CMADC
control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5-AzaC	0.0 ± 0.0	59.9 ± 5.9	90.3 ± 9.4

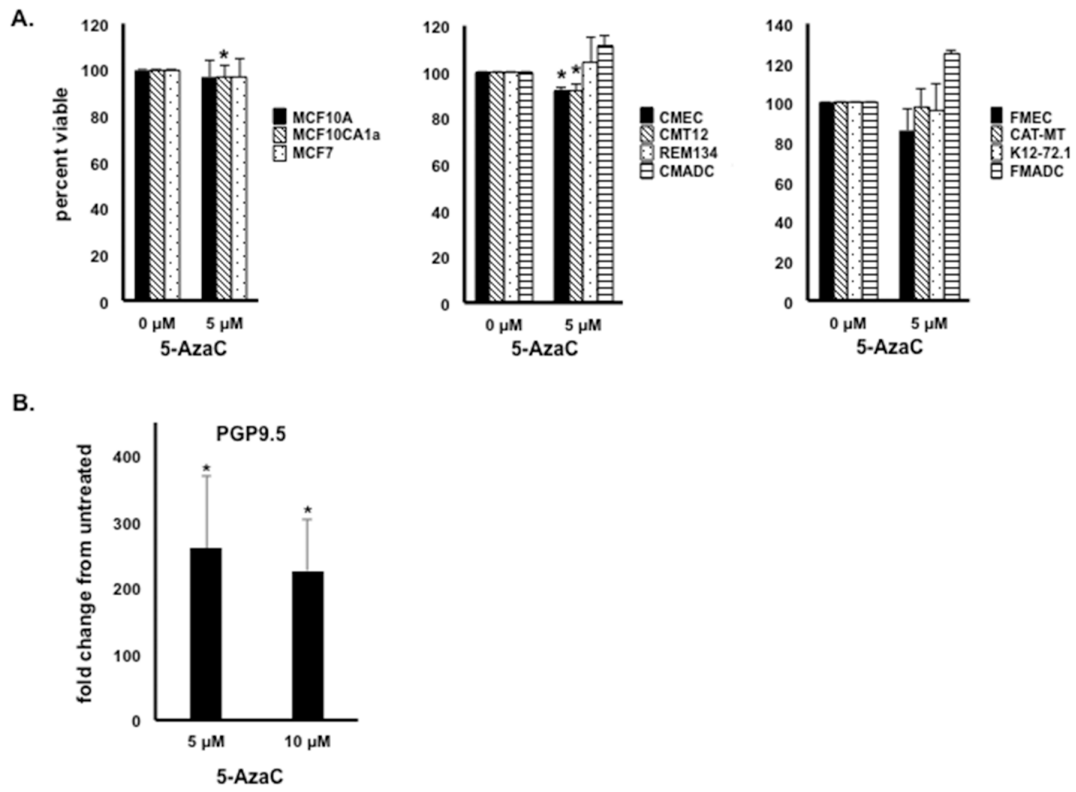|	FMEC	K12-72.1	FMADC
control	2.3 ± 3.2	0.0 ± 0.0	0.0 ± 0.0
5-AzaC	0.6 ± 0.6	56.3 ± 5.2	93.1 ± 7.2
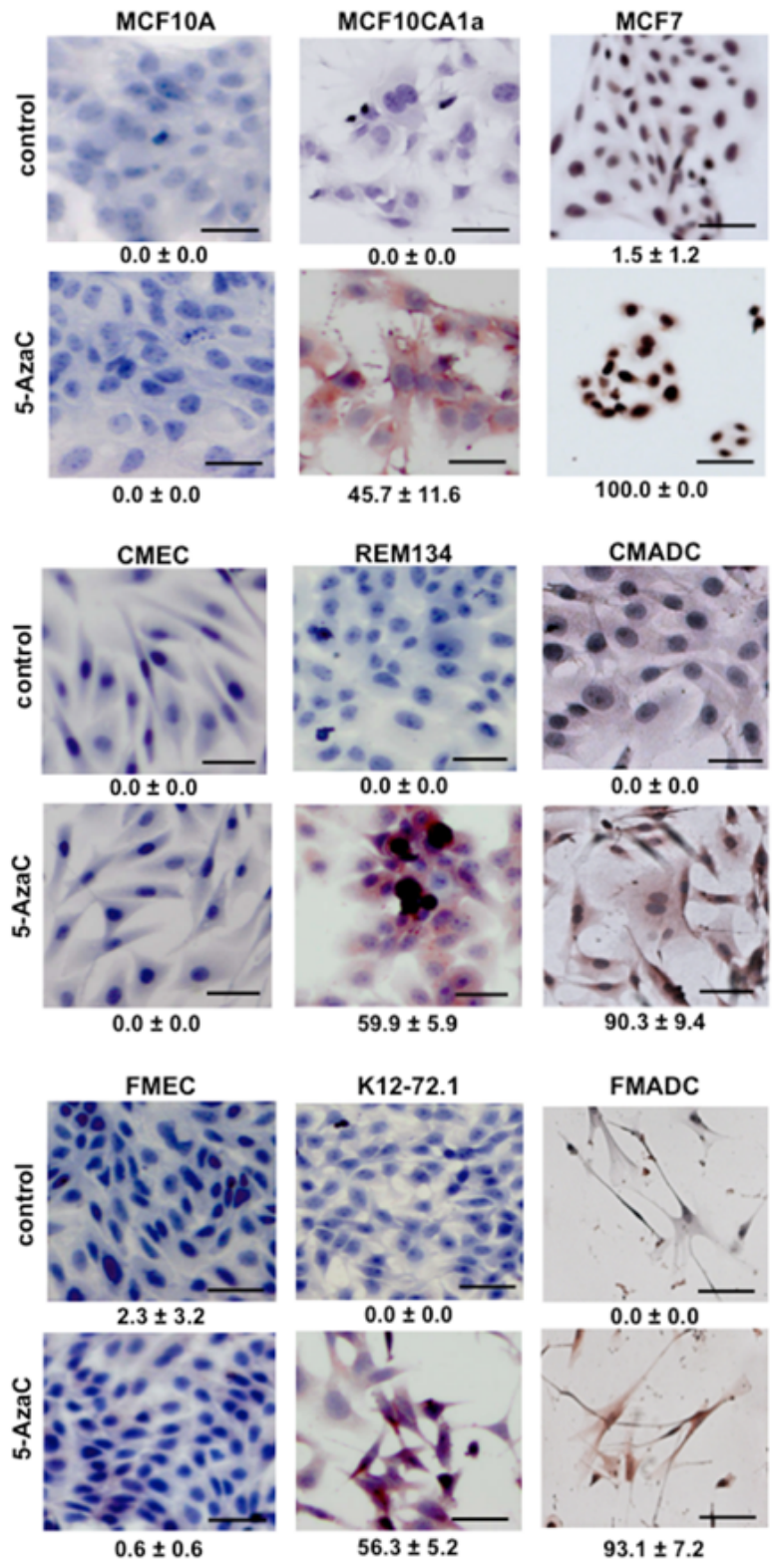
Figure 7. Harman et al.

A.

B.

PGP9.5

Supplemental Figure 1. Harman et al.