

1 **Comparing the Fate of Brain Metastatic Breast Cancer Cells in Different Immune Compromised Mice with**
2 **Cellular Magnetic Resonance Imaging**

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23 models

24

25 **Abstract**

26 Metastasis is the leading cause of mortality in breast cancer patients, with brain metastases becoming increasingly
27 prevalent. Studying this disease is challenging due to the limited experimental models and methods available. Here,
28 we used iron-based cellular MRI to track the fate of a mammary carcinoma cell line (MDA-MB-231-BR) *in vivo* to
29 characterize the growth of brain metastases in the nude and severely immune-compromised NOD/SCID/IL2Rg^{-/-}
30 (NSG) mouse.

31 Nude and NSG mice received injections of iron-labeled MDA-MB-231-BR cells. Images were acquired with a 3T
32 MR system and assessed for signal voids and metastases. The percentage of signal voids and the number and volume
33 of metastases were quantified. *Ex vivo* imaging of the liver, histology, and immunofluorescence labeling was
34 performed.

35 On day 0, iron-labeled cells were visualized as signal voids throughout the brain. The percentage of voids decreased
36 significantly between day 0 and endpoint. At endpoint, there was no difference in the number of brain metastases or
37 tumour burden in NSG mice compared to nudes. Tumour volumes in nude mice were significantly larger than in
38 NSG mice. Body images indicated that the NSG mice had metastases in the liver, lungs, and lymph nodes.

39 Characterization of the NSG and nude mouse is necessary to study breast cancer brain metastasis *in vivo*. Here, we
40 show that the 231BR cell line grew differently in NSG mice compared to nude mice. This work demonstrates the
41 role that imaging can play toward credentialing these models that cannot be done with other *in vitro* or
42 histopathologic methods alone.

43

44 **Background**

45 Breast cancer is a leading cause of death in women mainly due to the propensity of breast tumors to metastasize to
46 regional and distant sites, such as the lymph node, lung, liver, bone and brain [1]. The incidence of brain metastases
47 is increasing due to the introduction of more sensitive diagnostic methods and improved systemic therapies leading
48 to improvements in extra-cranial control and survival [2-4]. Breast cancer is a disease with a number of subtypes and
49 patients with metastatic ‘triple-negative’ breast cancer tend to develop brain metastasis at a high rate [5, 6]. For the
50 HER2 amplified subtype, the frequency of brain metastasis has been reported to be as high as 50% [7].

51
52 Once a metastatic cancer cell arrives in the brain one of three things can happen: (1) it may die, (2) it may proliferate
53 to form micrometastases, or (3) it may remain viable but dormant (‘non-proliferative’) [8, 9] If the solitary cells
54 proliferate to form micrometastases, they may again experience one of three fates: (1) they may die, (2) they may
55 continue to proliferate and form macrometastases, or (3) they may persist as “dormant” micrometastases, where
56 dormancy is defined as a balance between proliferation and apoptosis within the cell population such that there is no
57 net growth [9, 10]. The factors that tip the balance between dormancy and proliferation are poorly understood. Both
58 dormant single cells and dormant micrometastases are believed to be sources of cells that contribute to tumor
59 recurrence [11]. Dormant cancer cells also present a substantial therapeutic problem; since they are quiescent, they
60 are non-responsive to current therapies which target proliferating cells [8].

61
62 Currently, only a handful of models specific to breast cancer brain metastasis have been described and even fewer
63 allow for *in vivo* investigation of cancer cell dormancy. Both murine and human cancer cell lines have been
64 developed to mimic as many steps in the metastatic cascade as possible. A well characterized murine breast cancer
65 cell line is the 4T1-BR5 cell line, a highly tumorigenic and invasive cell line that has undergone multiple rounds of
66 selection to preferentially grow in the mouse brain [12]. There are several advantages to using a murine breast
67 cancer cell line to study brain metastasis, as growth and maintenance is easy and relatively inexpensive, and it can
68 be grown in immune-competent mice, which is of particular interest for studying this disease in a way that
69 recapitulates the tumor microenvironment [13].

70

71 A small number of human breast cancer brain metastatic cell lines have also been developed, including the
72 commonly used MDA-MB-231BR (231BR), MDA-MB-231BR-HER2, MA11, JIMT1-BR3, SUM190-BR3 cell
73 lines. The MDA-MB-231BR cell line has been particularly well characterized for studying the progression of brain
74 metastases in nude mice. Nude mice are the most commonly used immune-deficient strain for models which use
75 human cell lines. They have a genetic mutation that causes a deteriorated or absent thymus, resulting in a lack of T
76 cells [14]. The 231BR cell line grows selectively in the brain of nude mice, without metastatic growth seen in other
77 distant organs [15]. Human breast cancer cell lines are one of the most widely used models to study the metastatic
78 growth of cancer *in vitro* and *in vivo*, as they have been used to provide extensive insight into the characteristics of
79 human cells and can be used for high throughput screening of various drugs [16]. There are, however, significant
80 limitations to using human and murine breast cancer cell lines, as the quick progression *in vivo* can limit
81 opportunities for adequate therapeutic testing, and the growth of these cells *in vitro* prior to establishment in a mouse
82 can cause changes in the genetic composition due to clonal selection [17]. Many groups have shown that these
83 genetic changes result in these models failing to maintain tumor heterogeneity, which is now recognized as a critical
84 element for developing personalized treatments [18-20]. Studying breast cancer with immunocompetent mice also
85 has limitations, as tumor latency and growth can be variable and slow [21]. There is also increased difficulty in
86 establishing human tumors successfully in these mice. Immune-compromised mice lack a comparable tumor
87 microenvironment to clinical tumors and fail to provide a realistic result of interactions with the natural immune
88 response, particularly in the case of studying anticancer therapeutics [22].
89
90 In more recent years, researchers have moved towards studying breast cancer and its subtypes with patient-derived
91 xenografts (PDX), which allow for the growth of human primary breast cancer tissue that has been recently resected
92 from consenting patients into immune-compromised rodents [23]. NOD/SCID/IL2Rγ^{-/-} (NSG) mice are the preferred
93 strain of mice to engraft a PDX, as they are highly permissive to growing breast cancer metastasis, and resembles
94 the metastatic pattern seen in human patients. NSG mice are the most immunodeficient mouse strains to date,
95 lacking T cells, B cells, NK cells, and have defective macrophages and dendritic cells [24-26]. Even fewer PDX
96 models have been developed specific to breast cancer brain metastasis, including the novel F2-7 and E22-1 PDX cell
97 lines [27], as well as others who have established low passage PDX models of breast cancer brain metastasis and
98 those that implant fresh tumor tissue directly into the rodent brain [28].

99 The D2.0R and related D2A1 mouse mammary tumor cells have been well characterized as model systems for
100 tumor cell dormancy. In mice, D2.0R/R cells invade distant metastatic sites, remain as single quiescent cells for
101 prolonged periods of time, and occasionally proliferate into metastatic tumors. D2A1/R cells, have a much shorter
102 dormancy period before forming metastases in the lung, liver, and other organs [29]. A human breast cancer cell
103 line, MCF-7 has also been studied to examine the mechanisms of dormancy, however it is poorly metastatic [30].

104 For all of these brain metastasis models – cancer cell growth, metastasis and dormancy are typically studied using
105 methods such as histology, flow cytometry, immunohistochemistry, and fluorescent microscopy [31]. While these
106 techniques provide useful information about molecular and cellular markers and morphology, they are limited to
107 studying this disease after endpoint has been reached. There is a clear need to characterize brain metastatic models
108 of breast cancer *in vivo*, which can be accomplished through use of imaging modalities and cell tracking techniques.

109
110 Cellular magnetic resonance imaging (MRI) combines the ability to obtain high resolution MRI data with the use of
111 iron oxide nanoparticles for labeling specific cells, thereby enhancing their detectability [32, 33]. The presence of
112 the iron in cells causes a distortion in the magnetic field and leads to abnormal signal hypo-intensities in iron-
113 sensitive images (T2*-weighted images are most often used). Areas containing iron labeled cells therefore appear as
114 regions of low signal intensity on MRI images, creating negative contrast [34]. We have previously shown that it is
115 possible to use cellular MRI to track iron-labeled 231BR cancer cells in the nude mouse brain [35-37]. Proliferative
116 cancer cells lose the iron label over time; as the cells divide the particles are apportioned to daughter cells and
117 eventually some cells contain too little iron to be detectable by MRI. As brain metastases form, changes to the tissue
118 result in the tumor appearing brighter than the surrounding brain in MRI. Nonproliferative cancer cells retain the
119 iron particles and can be detected over long periods of time [37].

120
121 In this study, we used these cellular MRI techniques to characterize and compare the growth of 231BR brain
122 metastases and the persistence of iron-retaining cancer cells in nude and NSG mice. Our goal was to evaluate the
123 NSG mouse as a model for breast cancer brain metastasis and dormancy.

124 125 **Methods**

126 **Cell Culture and MPIO Labeling Procedure**

127 Brain trophic human breast cancer cells (MDA-MB-231BR) expressing green fluorescent protein (GFP) were
128 maintained with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (complete DMEM) at
129 37°C and 5% CO₂. 2x10⁶ of these cells were seeded and allowed to adhere for 24 hours. To iron label these cells,
130 cells were supplemented with 25 µg Fe/mL of micron-sized iron oxide particles (MPIO) (0.9 µm diameter, ~63%
131 magnetite, labeled with Flash Red; Bangs Laboratory, Fishers, IN, USA) in 10mL of complete DMEM in a T75-cm²
132 flask. Following this, cells were washed once with phosphate buffered solution (PBS) within the flask and
133 trypsinized with 0.25% Trypsin-EDTA. After, cells were harvested and washed three additional times with PBS in
134 the flask to thoroughly remove unincorporated MPIO prior to cell injections. Cell viability was assessed and
135 calculated using the Trypan blue exclusion assay. To visualize MPIO labelling, labeled cells were affixed to a glass
136 slide with a ThermoFisher Cytospin 4 cytocentrifuge and fixed with a Methanol/Acetic acid solution. Slides were
137 then stained with a Perl's Prussian Blue (PPB) solution and counterstained with Nuclear Fast Red. Slides were
138 dehydrated with increasing concentrations of ethanol, cleared with xylene, and coverslipped with a xylene-based
139 mounting medium. These PPB-stained slides were examined to assess the localization of MPIO within the cell and
140 to determine the labeling efficiency using a Zeiss AXIO Imager A1 Microscope (Zeiss Canada, Toronto, ON,
141 Canada). Iron oxide nanoparticles appear dark blue, and cells appear light pink in colour.

142

143 **Animal Model and Work Flow**

144 All animals were cared for in accordance with the standards of the Canadian Council on Animal Care, under an
145 approved protocol of the University of Western Ontario's Council on Animal Care and housed in a pathogen-free
146 barrier facility. Female nude mice (nu/nu Foxn1, aged 6-8 weeks, from Charles River Laboratories, Wilmington,
147 MA) and female NSG mice (NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ*, 6-8 weeks, Humanized Mouse and
148 Xenotransplantation Facility, Robarts Research Institute, University of Western Ontario, London, ON) were first
149 anesthetized with isoflurane (2% in 100% oxygen). Nude (n=10) and NSG mice (n=10) were then injected with a
150 suspension of 1.5x10⁵ MPIO-labeled MDA-MB-231BR/GFP+ cells in 100µL of sterile saline and 15% Vevo
151 MicroMarker microbubble solution (FUJIFILM, VisualSonics Inc., Toronto, ON, Canada). Cell suspension was
152 loaded into a 100µL Hamilton syringe with a 30G needle. Cells were administered by slow intracardiac injection
153 into the beating left ventricle of the heart with ultrasound imaging guidance on a Vevo 2100 ultrasound system
154 (FUJIFILM, VisualSonics Inc., Toronto, ON, Canada). Cancer cell arrest was evaluated with MRI on the day of the

155 injection (Day 0) and used to determine whether mice had a successful intracardiac injection. Absence of signal
156 voids in the brain indicated an unsuccessful injection, resulting in exclusion from the remainder of the study. Nude
157 mice with successful injections had MRI performed at day 21 and day 32 post-injection. NSG mice with successful
158 injections had MRI performed at day 21 post-injection (Figure 1).
159 Additionally, 1 nude mouse and 3 NSG mice received intracardiac injections with 1.5×10^5 MPIO labeled MD-MB-
160 231BR/GFP+ cells and intraperitoneal injections of 200 μ L of gadopentetate dimeglumine (Magnevist, Schering,
161 US; 0.5 mmol/mL) on Day 0 to assess cancer cell arrest in the liver. These mice were sacrificed on Day 0 by
162 isoflurane overdose approximately 40 minutes after intraperitoneal administration. These mice were then
163 exsanguinated, and brains and livers were removed for *ex vivo* imaging.

164

165 **Magnetic Resonance Imaging**

166 All brain and body MRI examinations were acquired on a 3.0 Tesla GE MR750 clinical MR scanner (General
167 Electric, Mississauga, ON, Canada) using a custom-built gradient coil (inner diameter = 17.5 cm, gradient strength =
168 500 mT/m, and peak slew rate = 3000 T/m/s). Brain and *ex vivo* liver images used a custom-built solenoidal mouse
169 brain radiofrequency (RF) coil. Body images were acquired using a 4.3 x 4.3 cm dual tuned $^1\text{H}/^{19}\text{F}$ surface coil
170 (Clinical MR Solutions, WI, USA), originally built for imaging small ROIs in humans. Mice were anesthetized with
171 2% isoflurane in 100% oxygen administered through a nose cone. *In vivo* brain and body images were acquired
172 using a 3D balanced steady-state free precession (bSSFP) sequence [Fast Imaging Employing Steady State
173 Acquisition (FIESTA) on a GE system] that has been optimized for simultaneous detection of signal voids produced
174 by iron-loaded cells and hyperintense metastases. This permitted the assessment of both cell arrest and retention as
175 well as the number and volume of metastases throughout the mouse brain. Brain images for nude mice were
176 acquired on day 0, day 21, and day 32. Brain imaging for NSG mice occurred on day 0 and day 21. Scanning
177 parameters were as follows: resolution = 100 x 100 x 200 μ m, repetition time (TR) = 7 ms, echo time (TE) = 3.5 ms,
178 bandwidth (BW) = 20.83kHz, flip angle (FA) = 35 degrees, signal averages = 2, phase cycles = 8, matrix = 150 x
179 150, scan time = approximately 33 minutes per mouse. Body images were acquired on Day 20. Body imaging
180 parameters were as follows: resolution = 200 x 200 x 200 μ m, repetition time (TR) = 4.7 ms, echo time (TE) = 2.3,
181 bandwidth (BW) = +/-31.25 kHz, flip angle (FA) = 35 degrees, signal averages = 2, phase cycles = 8, matrix = 250
182 x 250, scan time = approximately 20 minutes. *Ex vivo* liver imaging parameters were acquired using a spoiled

183 gradient echo sequence that also allows for the detection of signal voids throughout the liver. *Ex vivo* liver samples
184 were immersed in tubes containing Fluorinert™ FC-40 Electronic Liquid (3M, St. Paul's, MN, USA), a
185 fluorocarbon liquid which produces a black background in proton images. Images were taken of *ex vivo* livers
186 acquired on day 0 for both strains, day 21 for the NSG mouse, and day 32 for the nude mouse. The scanning
187 parameters for *ex vivo* liver images were as follows: resolution = 100 x 100 x 200 μ m, repetition time (TR) = 43 ms,
188 echo time (TE) 4.844 ms, bandwidth (BW) = 31.25kHz, flip angle (FA) = 60 degrees, signal averages = 1, phase
189 cycles = 8, matrix = 250 x 250, scan time = approximately 23 minutes.

190

191 **Image Analysis**

192 Images were analyzed using open-source OsiriX image software (Pixmeo, SARL, Bernex, Switzerland), version
193 10.0.4 and Horos image software, version 3.3.5. Brain images were evaluated for successful cell delivery by
194 assessment of signal voids on day 0. To quantify cancer cell arrest throughout the brain, the number of black pixels
195 within the total brain volume was determined from day 0 MRI images. The brain was manually segmented as a
196 region of interest and then a threshold value was determined based on the mean pixel intensity value of a
197 representative signal void within the brain region \pm 2 standard deviations. The total number of black pixels under
198 this threshold value within the entire brain volume signal was obtained from a pixel intensity histogram. To quantify
199 brain metastases at day 21 and day 32, tumors were counted in all image slices by a single observer. Each tumor was
200 manually segmented, and the tumor volumes were reconstructed using the OsiriX and Horos volume algorithm. To
201 calculate brain tumor burden at day 21 and day 32, an ROI was drawn around the outline of the brain in each slice,
202 and a 3D reconstruction using the OsiriX and Horos volume algorithm provided a quantification of the total brain
203 volume. The total volume of all segmented tumors was then determined by adding each individual tumor volume
204 measurement and calculating the percentage of the total brain volume occupied by tumors. Body images were
205 qualitatively assessed for the presence of metastases in the liver, lung, and lymph nodes. All quantitative values
206 were presented as the mean \pm standard error. Statistical analysis was performed using Welch's *t* tests on GraphPad
207 Prism version 8 software (GraphPad, San Diego, CA).

208

209 **Histology and Immunohistochemistry**

210 At each strain's respective endpoints, mice were euthanized by isofluorane overdose and perfused with 4%
211 paraformaldehyde. Brains were removed and placed into paraformaldehyde for another 24 hours. Fixed brains were
212 processed, paraffin embedded and then cut into 6 or 8 um sections. Sliced sections were deparaffinized and stained
213 with either hematoxylin and eosin (H&E) or immunofluorescently labelled for Ki67.

214

215 **Hematoxylin and eosin (H&E) staining**

216 Sections were washed briefly in distilled water, stained in Harris hematoxylin solution for 5 minutes and
217 differentiated in 1% acid alcohol for 30 seconds. After washing in 0.2% ammonia for 5 minutes, sections were
218 counterstained in eosin-phloxine solution for 30 seconds; dehydrated through 95% alcohol, 2 changes of absolute
219 alcohol, and 5 minutes each. Sections were then dehydrated and cleared through 95% ethyl alcohol, absolute alcohol
220 and xylene, and mounted with resinous medium.

221

222 **Immunofluorescent labelling**

223 Ki-67 immunostaining was performed using a rat anti-Ki-67 antibody (1:400 dilution; Catalog #14-5698-82,
224 Invitrogen). All sections were permeabilized with 0.2% Triton X-100 in PBS for 15 minutes and non-specific
225 protein binding was then blocked by incubation in a commercial blocking agent (ab156024, Abcam) for 1 hour at
226 room temperature. Sections were then incubated with the Ki-67 primary antibody in commercial antibody dilutant
227 (ab64211, Abcam) at room temperature for 1 hour. Negative controls (without addition of primary antibody) were
228 performed on adjacent sections. Unbound primary antibody was washed away through three 5-minute exchanges of
229 1xPBS. Sections were then incubated with an anti-rat Alexa Fluor-488 secondary antibody (1:300 dilution; Catalog
230 #A-11006, Invitrogen) for 1 hour. Unbound secondary antibody was washed away through three 5-minute
231 exchanges of 1xPBS. Finally, nuclei were counterstained with Hoechst 33258 for 5 minutes and rinsed sections
232 cover slipped for microscopic examination.

233

234 **Results**

235 ***In Vitro* Studies**

236 ***Cell Labelling***

237 MDA-MB-231BR cells were efficiently labeled with MPIO, as demonstrated by the Perl's Prussian blue staining of
238 cells shown in Figure 2a; cancer cells appear pink, and the intracellular iron is blue. Labelling efficiency of
239 $96.33 \pm 1.20\%$ was achieved with $97.33 \pm 0.88\%$ cell viability.

240

241 ***In Vivo Studies***

242 ***Experimental Endpoint***

243 In our experience, intracardiac injection of 1.5×10^5 231BR cells in nude mice results in brain tumor burden,
244 significant weight loss and neurological impairments which leads to a requirement for euthanasia at 28-34 days post
245 injection. In this study the nude mice reached experimental endpoint on day 32. In contrast, NSG mice could only
246 be studied until day 21, at which time they had reached a weight loss greater than 15% of their body weight,
247 resulting in extreme cachexia and anorexia. Additionally, these mice showed severe signs of neurological
248 impairment, resulting in the inability to perform normal functions and exhibiting paralysis and circling. Post-mortem
249 examination also revealed significant liver tumor burden, which also contributed to the early, unexpected endpoint.

250

251 ***Imaging Cell Arrest and Retention***

252 On day 0, bSSFP brain images confirmed the successful intracardiac injection of MPIO-labeled cancer cells in all
253 mice; iron-labeled cancer cells appeared as distinct regions of signal void throughout the brain due to their initial
254 arrest in brain vasculature (Figure 2b). Figure 3a shows representative day 0 images for each mouse strain. When
255 quantifying the percentage of the brain consisting of black signal voids, approximately $4.69\% \pm 1.71$ and $6.26\% \pm 1.86$
256 of the brains of NSG and nude mice contained arrested cancer cells, respectively. At each strain's endpoint, the
257 number of black pixels was again determined, with percentages significantly reduced to approximately $1.89\% \pm 0.57$
258 in NSG mice (day 21) and $2.45\% \pm 0.48$ in nude mice (day 32). While the number of arrested cancer cells
259 significantly differs from day 0 to endpoint for both NSG ($p = 0.03$) and nude mice ($p = 0.02$), there was no
260 significant differences between strains at each time point (Figure 3b).

261

262 ***Day 21 MRI***

263 On day 21, metastases were detectable in bSSFP brain images as regions of signal hyperintensity in both strains of
264 mice (Figure 4a). On day 21, the mean number of MRI-detectable metastases was significantly higher in NSG mice

265 (63.70±5.37) compared to nude mice (15.33±4.29) (Figure 4b). While the nude mice had fewer tumors, they were
266 significantly larger. The mean volume of tumors in nude mice was 0.06 mm³±0.01, compared to 0.04 mm³±0.003
267 for NSG mice (Figure 4c). This mean volume was based on a total number of 637 tumors in all NSG mice and 72
268 tumors counted in all nude mice. At day 21, the mean tumor burden was significantly higher for NSG mice (2.39
269 mm³±0.18) compared to nude mice (0.82 mm³±0.35) (Figure 4D).

270

271 ***Day 32 MRI***

272 Nude mice were followed out their endpoint of day 32 and imaging analysis was compared to the endpoint data for
273 the NSG mice (Figure 5). The mean number and volume of brain metastases in nude mice increased between days
274 21 and 32. On day 32 the mean number of brain metastases in nude mice was 39.60±10.99. When comparing the
275 mean number of metastases for NSG and nude mice at their respective endpoints there was no longer a significant
276 difference (p = 0.07) (Figure 5b).

277 On day 32 the mean volume of brain metastases in nude mice was 0.32±0.23 mm³, compared to 0.04±0.03 mm³ on
278 day 21. This was significantly higher (p = <0.0001) than the mean volume of brain metastases at the NSG endpoint
279 (Figure 5c). Accordingly, nude mice had significantly more tumor burden than NSG mice (Figure 4d), which was a
280 reversal from the previous timepoint at day 21. Due to the earlier endpoint of NSG mice but lesser brain tumor
281 burden, body MRI was acquired for an NSG mouse at day 20. Notably, there was significant tumor burden detected
282 by MRI within the liver, lungs (Figure 6A), and lymph nodes (Figure 6b) of the NSG mice. Nude mice had no MRI-
283 detectable tumors within the body outside the brain (Figure 6c).

284

285 ***Ex Vivo Liver MRI***

286 Discrete signal voids were visible in the day 0 images of *ex vivo* livers from each strain of mouse, suggesting the
287 arrest of iron labeled 231BR cells throughout the liver (Figure 6d,e). *Ex vivo* liver mages obtained from mice at
288 endpoint showed numerous regions of abnormal high signal intensity associated with liver metastases, confirming
289 the *in vivo* image results (Figure 6f). A photo of a representative liver sample clearly shows the liver metastases on
290 the surface (Figure 6h). No regions of abnormal signal hyperintensity were observed in *ex vivo* images of the nude
291 mouse liver (6g). H&E stained sections of the liver tissue from NSG mice confirmed the presence of metastases (6i)
292 and ki67 staining showed that they were highly proliferative (6j).

293 **Discussion**

294 This work demonstrates for the first time the use of *in vivo* longitudinal MRI based cell tracking to compare murine
295 models of brain metastatic breast cancer. We show that there are significant differences in tumor progression for
296 231BR cells in NSG versus nude mice. While the initial arrest, clearance, and retention of iron-labeled cells was
297 similar, brain metastases developed more quickly in NSG mice and NSG mice developed substantial body tumor
298 burden, particularly in the liver, leading to an earlier endpoint.

299
300 The 231BR cell line was developed by Yoneda et al [15]. and is a brain-colonizing subline of the metastatic triple-
301 negative MDA-MB-231 human breast cancer cell line, which was isolated by six repeated cycles of intra-cardiac
302 injection and harvesting from brain metastases grown in nude mice. In our lab, the intra-cardiac injections are
303 performed using ultrasound (US) guidance. Of note, was our observation by US of a thicker heart muscle
304 surrounding the left ventricle of the NSG mouse. This anatomical observation made intracardiac injections more
305 difficult, as the ventricular space appeared smaller with US. The observation of signal voids in brain images
306 acquired on day 0 was used to identify successful delivery of cells to the brain. In this study, all mice imaged had
307 successful injections and were included in the study. Delivery of cells to a specific organ is related to the cardiac
308 output that is delivered to that organ. The cardiac output to the brain of a mouse is ~9.5% [38]. Here, we injected
309 150,000 cells into the left ventricle of the heart, and therefore we can expect ~14,250 cells to be successfully
310 delivered to the brain. Only a percentage of these cells will arrest; previous studies, however, have shown that less
311 than 1% of cells are retained in the microcirculation of the brain after 2 hours post-injection [38]. In previous
312 studies, we have used PPB staining and fluorescence microscopy to demonstrate that these signal voids correspond
313 to the presence of iron-labeled cells in the brain [35, 36].

314
315 The size of the signal void created by iron-labeled cells is much larger than the actual cell size, due to what is known
316 as the blooming effect. The blooming effect is a susceptibility artifact that occurs as a result of the iron oxide
317 nanoparticle, which causes a local magnetic field inhomogeneity [34]. We have previously shown that we can detect
318 single iron-labeled cells arrested in the mouse brain using cellular MRI [35, 39, 40]. Our image showed that the
319 number of signal voids in the brain was similar for nude and NSG mice on day 0 and at endpoint. While we
320 acknowledge that there is potential for immune cell uptake of the iron oxide nanoparticles that may be released by

321 dead cancer cells, we believe that the majority of the signal voids that remain present are live cancer cells retaining
322 iron. For example, in Parkins et al. [39] we investigated this mouse model using luciferase-positive 231BR cells and
323 measured a strong correlation on day 0 between the number of signal voids detected in day 0 brain MR images and
324 the brain signal measured in bioluminescence images, which is only detected from viable cells, providing evidence
325 that signal voids represent live iron-labeled cells. In Hamilton et al. [41] we used fluorescence activated cell sorting
326 (FACS) to successfully isolate live 231BR cells that were GFP-positive and labeled with red fluorescent (Flash Red)
327 MPIO from the brains of mice. The GFP+ Flash Red+ cells were collected and expanded *in vitro*. The majority
328 (~90%) of cells adhered to tissue culture plastic and successfully expanded, displaying the same cell morphology as
329 the original cultured 231BR cells. This provide additional support for our claim that signal voids in MRI represent
330 viable iron-positive cancer cells.

331 Our previous work has shown that the number of signal voids detected in MRI of the brain decreases over the course
332 of the experiment, from day 0 to day 8, which is expected as the large majority of cancer cells that arrest in the brain
333 do not survive and are cleared with time [39]. The signal voids which do persist in the brain over time are thought to
334 represent iron-retaining, non-proliferative or dormant cancer cells. These cells have been shown to contribute to
335 tumor recurrence [41]. It appears that both nude and NSG mice are capable of clearing dead cells from the brain.

336
337 3D MRI of the entire mouse brain at high resolution allowed us to view brain metastases in all 3 orientations and to
338 digitally re-slice images to carefully interrogate image data. We counted and measured the volume of all MRI-
339 detectable brain metastases. Brain metastases developed more quickly in NSG mice. At day 21 post cell injection
340 NSG mice had more than 3 times the number of brain metastases. The NK cells and the remaining innate immune
341 cells in nude mice likely contribute to the reduced tumor growth at this timepoint. This finding is in agreement with
342 the results of other groups that show that more immune compromised mouse models are more permissive for tumor
343 growth and metastasis [42-45]. Puchalapalli et al. [45] have previously reported an increase in the metastatic burden
344 (in liver, lungs, brain and bones) in NSG compared to nude mice that were injected with the parental 231 breast
345 cancer cell line in an intracardiac experimental metastasis model. In this study, metastases in each organ were
346 enumerated from *ex vivo* fluorescence microscopy images. Our work demonstrates the advantages of using *in vivo*
347 and longitudinal 3D MRI for the accounting of metastases in the whole brain.

348

349 NSG mice had to be euthanized at day 21/22. This was unexpected, as volumetric analyses of the tumor burden in
350 the brain at this timepoint compared to the tumor burden of the nude mice at their endpoint of day 32 indicated that
351 the NSG mice had lesser tumor burden in the brain. This suggested that there was a possibility of metastases to have
352 occurred elsewhere in the body that could be contributing to a greater disease burden. Mouse body MRI confirmed
353 tumors in the liver, lungs, and lymph nodes.

354
355 Nude mice were imaged at a third timepoint. On day 32 the number of brain metastases in nude mice was similar to
356 the number of brain metastases in NSG mice on day 21. The mean volume of the brain metastases in the nude mice
357 on day 32 was more than 3 times greater than those in NSG mice at day 21, having had more time to develop.
358 Overall, the brain tumor burden at necessary endpoint was significantly greater in nude mice.

359
360 *Ex vivo* images of livers removed on the day of the intracardiac injection of iron labeled 231BR cells revealed
361 numerous signal voids in all lobes of the liver in both strains of mice indicating that there was similar cancer cell
362 arrest for both strains at this timepoint. We did not do a quantitative analysis of liver signal voids, however, in a
363 previous study where we injected MPIO-labeled melanoma cells via the mesenteric vein, mouse livers were imaged
364 *ex vivo* by MRI and the signal void area was shown to correlate with the number of cells injected [46]. *Ex vivo*
365 images of livers removed at endpoint showed that numerous metastases formed in NSG mice while no liver
366 metastases could be detected in nude mice.

367 The 231BR cell line was developed to selectively grow distant metastases in the brains of nude mice, and so the
368 proliferation of metastases of this cell line in the liver demonstrates the loss of selectivity to the brain in the NSG
369 mouse.

370

371 **Conclusion**

372 In summary, high resolution cellular MRI allowed us to characterize the 231BR cell line in both the NSG and nude
373 mouse models. We found marked differences in tumor incidence, volumes, and body tumor burden between strains.
374 Our *in vivo* comprehensive analysis of cancer cell arrest, clearance, and tumor progression is important for
375 understanding the metastatic cascade of a model of breast cancer brain metastasis that can be challenging to obtain
376 with *in vitro* or *ex vivo* methods alone.

377

378 **List of Abbreviations**

379	Abbreviations	Full Form
380	bSSFP	balanced steady-state free precession
381	BW	bandwidth
382	DMEM	Dulbecco's modified Eagle's medium
383	FA	flip angle
384	FACS	fluorescence activated cell sorting
385	FIESTA	Fast Imaging Employing Steady State Acquisition
386	GFP	green fluorescent protein
387	H&E	hematoxylin and eosin
388	MPIO	micron-sized iron oxide particles
389	MRI	magnetic resonance imaging
390	NSG	NOD/SCID/IL2rg ^{-/-}
391	PBS	Perl's Prussian Blue
392	PDX	patient-derived xenograft
393	RF	radiofrequency
394	TE	echo time
395	TR	repetition time
396	US	ultrasound
397	231BR	MDA-MB-231BR
398		

399 **Declarations**

400 **Funding**

401 This study was supported by the Canadian Institute for Health Research (P.J. Foster) and the Breast Cancer Society
402 of Canada (N.N. Knier).

403

404 **Conflicts of interest/Competing interests**

405 The authors declare that they have no competing interests.

406

407 **Ethics approval**

408 Animals were cared for in accordance with the standards of the Canadian Council on Animal

409 Care, and under an approved protocol of the Western University's Council on Animal Care (2018-135).

410

411 **Consent to participate**

412 Not applicable

413

414 **Consent for publication**

415 Not applicable

416

417 **Availability of data and materials**

418 The datasets used and/or analysed during the current study are available from the corresponding author on

419 reasonable request.

420

421 **Code availability**

422 Not applicable

423

424 **Author's Contributions**

425 P.J.F. designed the experiments. N.N.K, P.J.F, and A.M.H conducted the experiments. N.N.K. analyzed the data.

426 N.N.K. and P.J.F. wrote the main manuscript text. All authors reviewed the manuscript.

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430

431 **References**

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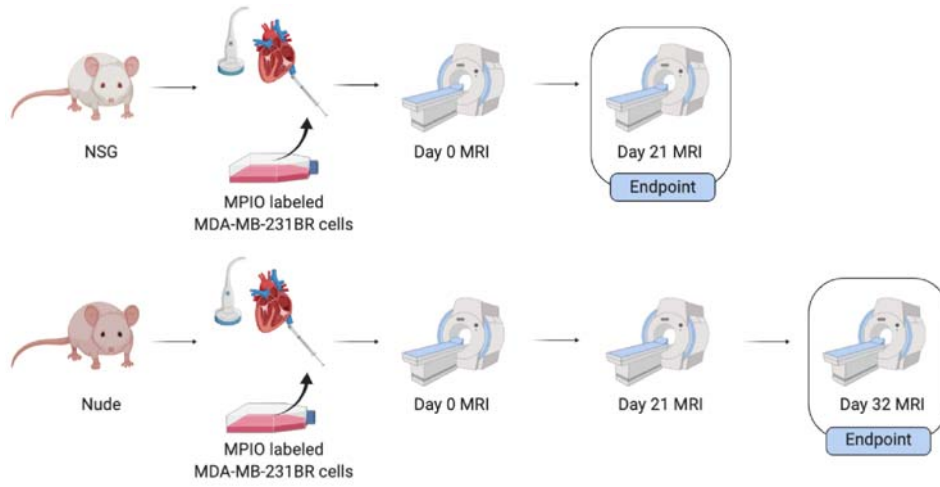
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Figure 1

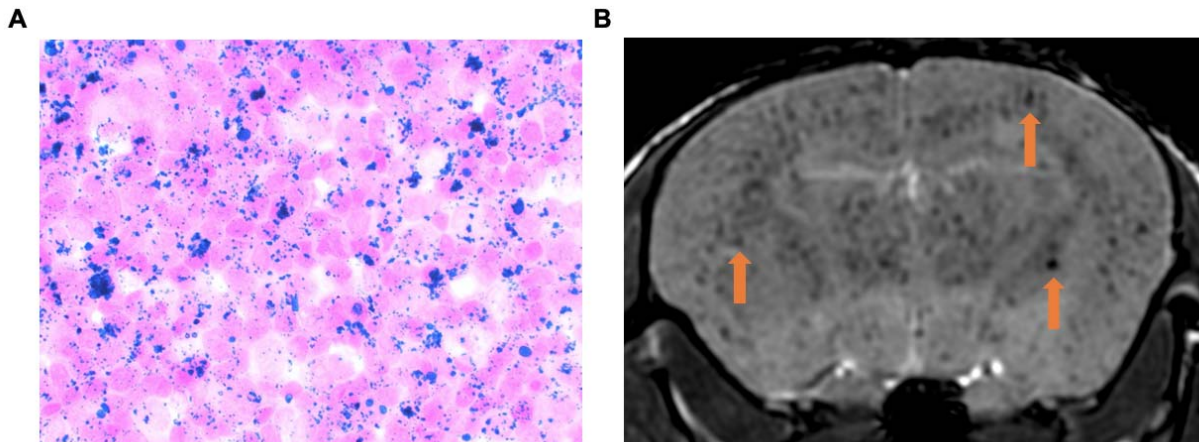
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591 **Figure. 1 Experimental design for the NSG and nude mouse model**

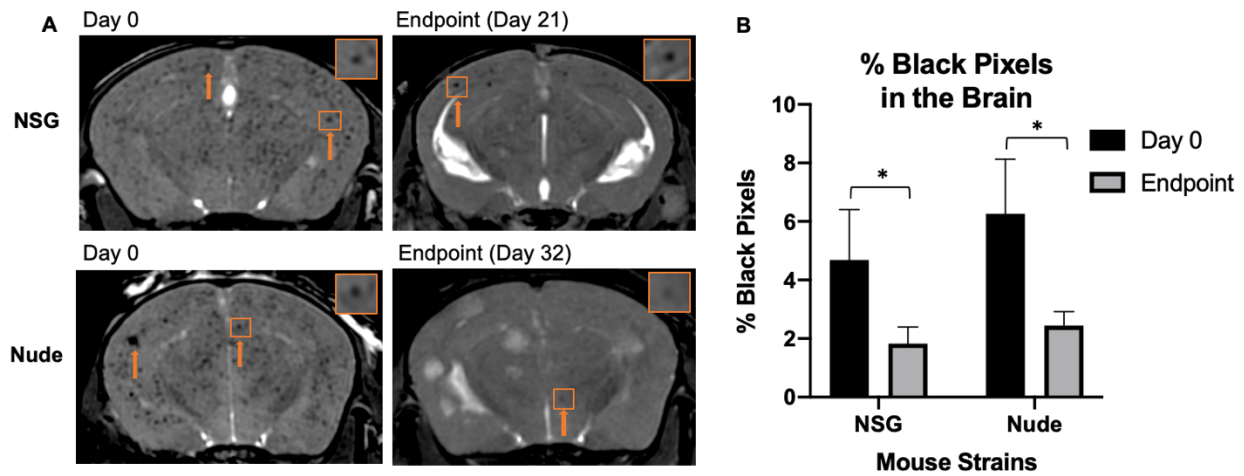
Figure 2



592

593 **Figure. 2 Iron labeling and injection of 231BR cells** (A) Perl's Prussian Blue stain showing intracellular iron
594 (blue, PPB) detected within MDA-MB-231BR cells (pink, Nuclear fast red) *in vitro*. (B) Representative day 0 image
595 showing visualization of successful iron-labeled cancer cell delivery by intracardiac injection as regions of signal
596 void (orange arrows)

Figure 3



597

598 **Figure. 3 Cancer cell arrest and clearance** (A) Balanced steady-state free precession images showing initial

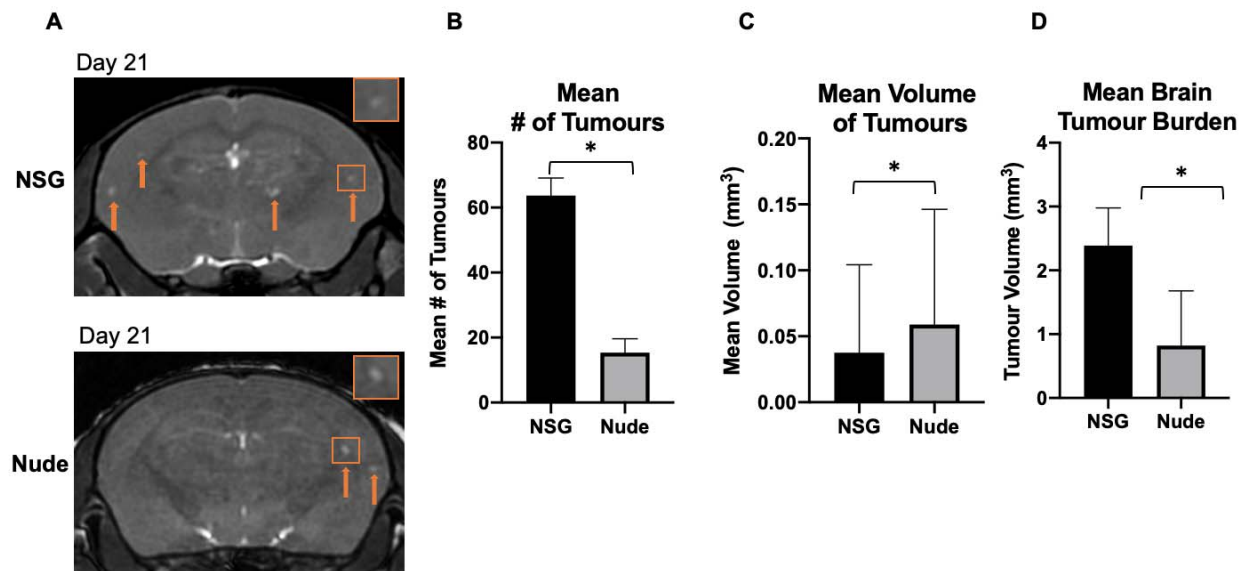
599 cancer cell arrest at day 0 and clearance and retention at endpoint. The orange arrows indicate regions of signal void.

600 (B) The percentage of black pixels, representing regions of signal void was not significantly different between

601 strains, however there was a significant decrease within strains from day 0 to endpoint, suggesting similar cancer

602 cell arrest and clearance between nude and NOD/SCID/IL2rg^{-/-} mice

Figure 4



603

604 **Figure. 4 Day 21 visualization and quantification of brain metastases** (A) Balanced steady-state free precession

605 images showing brain metastases in both strains of mice as regions of signal hyperintensity (orange arrows) at day

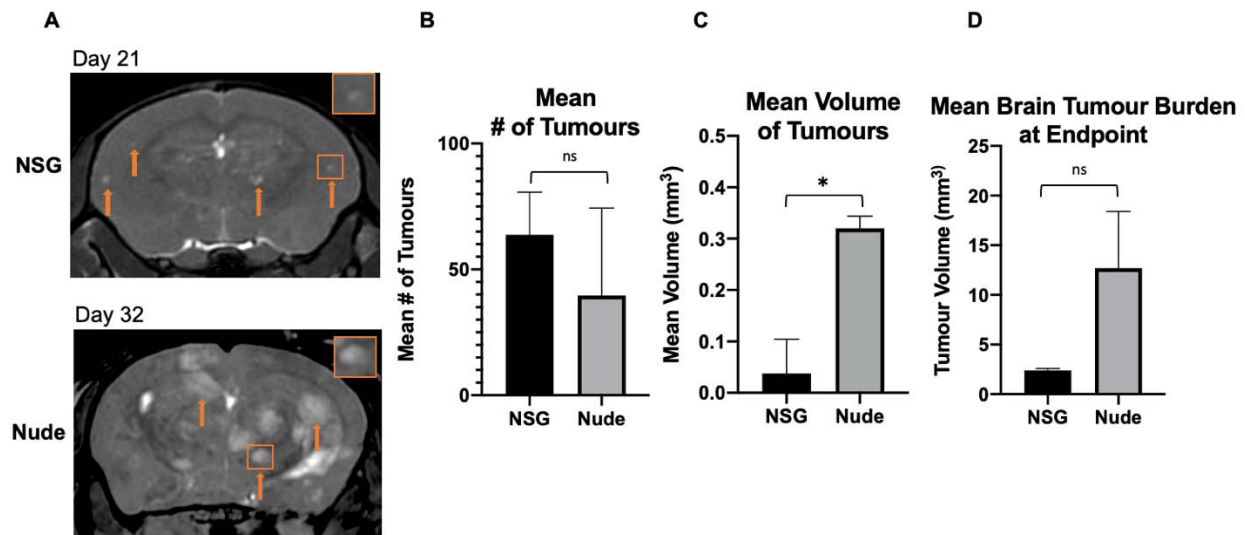
606 21. (B) There were significantly more brain metastases on average detected with MRI in NSG mice than nude mice

607 at day 21. (C) The mean volume of tumors present in the brain was significantly greater in nude mice than NSGs.

608 (D) The brain tumor burden of NOD/SCID/IL2rg^{-/-} mice was significantly higher than in nude mice at day 21. Data

609 is presented as mean +/- SEM. *indicates p < 0.05

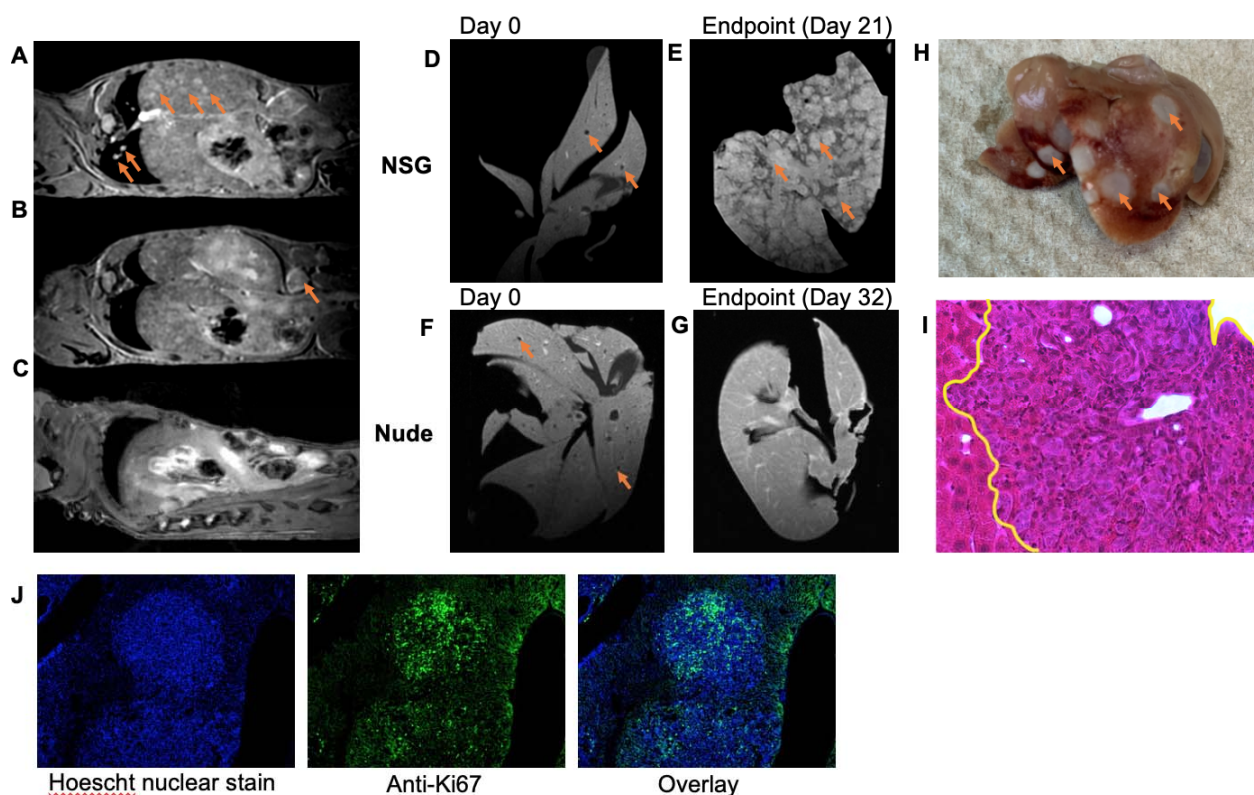
Figure 5



610

611 **Figure. 5 Endpoint visualization and quantification of brain metastases** (A) Magnetic resonance images of both
612 NOD/SCID/IL2rg^{-/-} and nude mice at each strain's respective endpoints (Day 21 for NOD/SCID/IL2rg^{-/-}, Day 32
613 for nude) showing regions of signal hyperintensity where brain metastases have developed (orange arrows). (B)
614 There was no significant difference between the number of brain metastases detected with magnetic resonance
615 imaging between strains at endpoint. (C) The tumors in the nude mice were significantly larger in volume than those
616 in the NOD/SCID/IL2rg^{-/-} mice. (D) The total tumor burden within the brain at endpoint in both nude and mice was
617 not significantly different. Data is presented as mean +/- SEM. *indicates p < 0.05

Figure 6



618
619 **Figure. 6 Body and liver imaging with correlative histology and immunofluorescence** (A) Body imaging of
620 NOD/SCID/IL1Irg^{-/-} mouse allowed for detection of significant tumor burden in the liver, lungs. (B) Tumors were
621 detected in the lymph nodes of NOD/SCID/IL1Irg^{-/-} mice with magnetic resonance imaging. (C) No tumors were
622 detected with magnetic resonance imaging in the nude mouse body. (D) Spoiled gradient echo sequence image
623 showing initial arrest of cancer cells in *ex vivo* liver at Day 0 for NOD/SCID/IL1Irg^{-/-} mouse (E) Spoiled gradient
624 echo sequence image of *ex vivo* liver showing regions of hyperintensity where liver metastases have developed in
625 the NOD/SCID/IL1Irg^{-/-} mouse. (F) Day 0 spoiled gradient echo sequence image of *ex vivo* liver of from a nude
626 mouse showing signal voids, representing arrested cancer cells. (G) Spoiled gradient echo sequence image of Day
627 32 (endpoint) *ex vivo* liver of a nude mouse with no magnetic resonance detectable metastases (H) Photo of a
628 representative *ex vivo* liver from an NOD/SCID/IL1Irg^{-/-} mouse showing tumors on the exterior of the organ. (I)
629 H&E-stained section showing liver metastases that had developed in the NOD/SCID/IL1Irg^{-/-} mouse (outlined in
630 yellow). (J) Ki67 staining showing a highly proliferative tumor within the NOD/SCID/IL1Irg^{-/-} liver