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1 **Prostate cancer-derived exosomes promote osteoblast differentiation and activity**
2 **through phospholipase D2**

3
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18 Running title:

19 Exosomes, osteoblasts and phospholipase D

20

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22

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24

25 **ABSTRACT**

26 Prostate cancer (PCa) is the most frequent cancer in men aged 65 and over. PCa mainly metastasizes
27 in the bone, forming osteosclerotic lesions, inducing pain, fractures, and nerve compression. Cancer
28 cell-derived exosomes participate in the metastatic spread, ranging from oncogenic reprogramming
29 to the formation of pre-metastatic niches. Moreover, exosomes were recently involved in the dialog
30 between PCa cells and the bone metastasis microenvironment. Phospholipase D (PLD) isoforms
31 PLD1/2 catalyze the hydrolysis of phosphatidylcholine to yield phosphatidic acid (PA), regulating
32 tumor progression and metastasis. PLD is suspected to play a role in exosomes biogenesis. We aimed
33 to determine whether PCa-derived exosomes, through PLD, interact with the bone
34 microenvironment, especially osteoblasts, during the metastatic process. Here we demonstrate for the
35 first time that PLD2 is present in exosomes of C4-2B and PC-3 cells. C4-2B-derived exosomes
36 activate proliferation and differentiation of osteoblasts models, by stimulating ERK 1/2
37 phosphorylation, by increasing the tissue-nonspecific alkaline phosphatase activity and the
38 expression of osteogenic differentiation markers. Contrariwise, when C4-2B exosomes are generated
39 in the presence of halopemide, a PLD pan-inhibitor, they lose their ability to stimulate osteoblasts.
40 Furthermore, the number of released exosomes diminishes significantly (-40%). When the PLD
41 product PA is combined with halopemide, exosome secretion is fully restored. Taken together, our
42 results indicate that PLD2 stimulates exosome secretion in PCa cell models as well as their ability to
43 increase osteoblast activity. Thus, PLD2 could be considered as a potent player in the establishment
44 of PCa bone metastasis acting through tumor cell derived-exosomes.

45

46 **Keywords**

47 Phospholipase D, exosomes, prostate cancer, osteoblast, mineralization

48

49 **Abbreviations**

50 AP: Alkaline Phosphatase

51 DLS: Dynamic Light Scattering

52 ILV: Intra-Luminal Vesicles

53 mOB: Mouse primary Osteoblasts

54 MVB: Multivesicular Bodies

55 PA: Phosphatidic Acid

56 PCa: Prostate Cancer

57 PLD: Phospholipase D

58 TNAP: Tissue-Nonspecific Alkaline Phosphatase

59

60 1. INTRODUCTION

61

62 Prostate cancer (PCa) remains an acute public health issue, with 1,276,106 new reported cases in
63 2018 [1]. PCa has a strong tropism for bone, with 90% of the metastases targeting the bone [2, 3].
64 Bone metastases are of bad prognosis, with a 5-year survival rate plummeting from 90-100% to 25-
65 30% [4]. Bone metastases induce severe comorbidities called skeletal-related events, such as pain,
66 fractures or nerve compression among others [5]. Those bone metastases mainly present mixed or
67 osteosclerotic behaviors [6], contrary to those occurring in breast cancer, which are pro-osteolytic.
68 This particular feature of PCa is still poorly understood. The available treatments include
69 bisphosphonates to prevent the metastatic-induced bone remodeling. More recently, the use of ²²³Ra
70 has emerged as a promising alternative [7, 8]. Established bone metastases are incurable, thus
71 preventing or retarding their onset seems to be the most effective strategy to adopt to prevent their
72 dreadful consequences.

73

74 It is now widely admitted that cancer cells strongly interact with their surrounding cells, and
75 exosomes emerge as new players in the regulation of the tumor microenvironment [9, 10]. Exosomes
76 are small (30-150 nm in diameter) extracellular nanovesicles containing miRNAs, mRNAs, DNAs,
77 proteins [11]. Exosomes affect PCa progression [12], mesenchymal stem cell differentiation into pro-
78 angiogenic and pro-invasive myofibroblasts [13] drug resistance [14], and bone metastasis [15]. It
79 has already been demonstrated that PCa-derived exosomes can modulate osteoblast activity in the
80 bone metastatic niche, mainly due to their cargo content in miRNAs [16-18]. Concomitantly, they
81 also inhibit osteoclast differentiation [19, 20]. Moreover, exosomes secreted by PCa cells
82 preferentially accumulate in the bone marrow when they are intravenously injected in mice [21].
83 Nevertheless, the available literature is still lacunar to this day and further work needs to be done to

84 assess other potential mechanisms of action of exosomes in the onset, establishment, and
85 maintenance of PCa bone metastasis.

86

87 Phospholipase D (PLD) has been proposed as a potential actor in exosome secretion [22] even
88 though the precise mechanism through which it acts remains to be fully elucidated. In humans, there
89 are 6 members of the PLD family, ranging from PLD1 to PLD6. Until now only PLD1 and PLD2
90 have been solidly described as having a role in cancer progression; they are also the sole isoforms
91 that are catalytically active [23, 24]. PLD catalyzes the hydrolysis of membrane phospholipids,
92 mainly phosphatidylcholine, which yields to the production of phosphatidic acid (PA) and choline.
93 PA is a potent regulator of mTOR [25], actin cytoskeleton organization [26, 27], and it also interacts
94 with a vast array of proteins [28, 29]. Above all, PA is negatively charged and has a “cone” like
95 shape which is thought to be helping with vesicles formation and stability [30], facilitating exosome
96 biogenesis. When overexpressed, PLD2 is transferred to exosomes produced by RBL-2H3 rat cells,
97 and PLD activity is detected in those exosomes [31]. PLD2 is also found in colon cancer-secreted
98 exosomes where it favors cancer-associated fibroblast senescence [32]. Hence, the role of PLD might
99 not be limited to the biogenesis of exosomes, but it might induce their secretion and their
100 internalization in the target cells. PA, the product of PLD activity, is a so-called “fusogenic” lipid,
101 that by its properties favors membranes fusion [33]. Thus, it is not unreasonable to hypothesize that
102 PLD might be involved in exosome fusion at the target cell plasma membranes. Therefore, PLD
103 could be a potent actor in three stages of exosome live: biogenesis, secretion, and internalization.

104 It was reported that PLD is overexpressed in PCa tissue samples and cell models [34-37]. A recent
105 analysis by mass spectrometry indicated that PA is more abundant in PCa samples of higher Gleason
106 scores (4+3 vs 3+4). Lipidomic analysis of patients’ sera lead to the conclusion that several lipid
107 classes are altered in PCa patients, except for PA concentration which were lower in PCa patients’
108 sera [38, 39]. Since PLD is linked to exosome biogenesis [22]; we sought to establish the link

109 between PLD, PCa, and exosomes. More precisely we posit that PLD overexpression may trigger the
110 formation of exosomes from PCa cells, enhancing cell to cell communications and affecting the
111 functions of osteoblasts to induce bone metastasis.

112 In this study, we focused on PLD, PCa-derived exosomes and their action on osteoblast activity.
113 First, we analyzed the presence of PLD on PCa-derived exosomes. Then, we investigated their
114 activity by using a pharmacological pan-inhibitor (halopemide) in PCa cells. Under these conditions we
115 analyzed exosome secretion and their distant action on proliferation, tissue-nonspecific phosphatase
116 (TNAP) activity, and osteogenic markers in osteoblasts.

117

118 **2. MATERIAL AND METHODS**

119

120 **2.1 Chemicals and reagents**

121 Cell culture medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), antibiotics, G418,
122 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Alizarin red, p-
123 nitrophenylphosphate, phosphatidic acid (PA), secondary antibodies (anti-mouse IgG and anti-rabbit
124 IgG) and Lactate dehydrogenase (LDH) cytotoxicity detection kit were from Sigma-Aldrich (Lyon,
125 France). Halopemide (Halo) was obtained from Cayman Chemicals (Montluçon, France).

126

127 **2.2 Cell culture**

128 Non-tumour human prostatic cell line WPMY-1 and PCa-derived cell line 22Rv1 were purchased
129 from ATCC (Manassas, USA). The 22Rv1 cell line was obtained from a xenograft serially
130 propagated in mice after castration-induced regression and relapse of the parental, androgen-
131 dependent CWR22 xenograft. Human PCa bone metastasis-derived cell lines C4-2B and PC-3 were
132 obtained from Dr O. Cuvillier's lab (Toulouse, France) and were purchased from Viromed
133 (Minnetonka, USA) and DSMZ (Braunschweig, Germany) respectively. C4-2B is a castration-
134 resistant PCa cell line isolated from the bone metastasis of a mouse xenograft inoculated with C4-2
135 cells, a subline of LNCaP cells. Murine pre-osteoblastic cell line MC3T3-E1 (subclone 4) was
136 obtained from ATCC.

137

138 22Rv1, C4-2B and PC-3 cell lines were cultured in Roswell Park Memorial Institute (RPMI) medium
139 supplemented with 10 % (v:v) FBS, penicillin (100 U/mL), and streptomycin (0.1 mg/mL). WPMY-
140 1 and MC3T3-E1 were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented
141 with 10 % (v:v) FBS, penicillin (100 U/mL), and streptomycin (0.1 mg/mL). All cells were
142 maintained in a humidified atmosphere consisting of 95% air and 5% CO₂ at 37 °C and passaged

143 twice a week. They were regularly monitored by the following tests: morphology check by visual
144 inspection, growth curve analysis and mycoplasma detection (Mycoplasma detection kit; InvivoGen,
145 California, USA). All experiments were started with low-passaged cells (<20 times).

146

147 **2.3 Generation of stable PLD2-overexpressing PCa cell lines**

148 C4-2B and PC-3 cells were transfected with custom plasmids carrying either the eGFP gene or an
149 eGFP-PLD2 fusion gene [27]. One μg of each plasmid was transfected using Lipofectamin®
150 (Invitrogen, California, USA) as explained by the manufacturer. A day later, cells stably expressing
151 the target genes were selected with G418 at 0.8 g/L for a week. Next, clones were isolated and
152 screened by Western blot for the expression of target proteins (eGFP or eGFP-PLD2).

153

154 **2.4 Osteoblast culture models**

155 For the MC3T3-E1 differentiation experiments, cells were seeded at 25,000 cells/cm². Four days
156 later, the culture medium was enriched with 50 $\mu\text{g}/\text{mL}$ of L-ascorbate and 10 mM of β -
157 glycerophosphate. Cells were grown in this media for 7 days, and up to 17 days for differentiation
158 experiments, with a medium change every two days.

159

160 Primary mouse osteoblasts (mOB) were isolated from 8-day-old mice calvaria as described [40].
161 Three days after seeding 50 $\mu\text{g}/\text{mL}$ of L-ascorbate were added to the media, and seven days later, 10
162 mM of β -glycerophosphate were added to induce osteoblastic differentiation, for 3 more days.

163

164 Whole calvaria were aseptically dissected and cultured as described [41, 42]. After dissection,
165 calvaria were cut in half and cultured in growth DMEM medium supplemented with 10 % (v:v) FBS,
166 2 mM L-Gln, 20 mM HEPES, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and amphotericin
167 B (2,5 $\mu\text{g}/\text{mL}$). Calvaria were cultured for 3 days and the culture dish was changed every other day.

168

169 All experiments were performed under the authorization n°69-266-0501 and were in agreement with
170 the guidelines laid down by the French Ministry of Agriculture (n° 2013-118) and the European
171 Union Council Directive for the protection of animals used for scientific purposes of September 22nd,
172 2010 (2010/63UE). The protocol was approved by the local ethical committee (Comité Ethique de
173 l'INSA-Lyon - CETIL, CRNEEA n°102) under the reference APAFIS#4601-2016032110173355.

174

175 **2.5 Exosome isolation and treatment in osteoblast cells and in calvaria**

176 Exosomes were isolated following the method reported by Théry and colleagues [43]. Briefly, non-
177 tumour human prostatic cell line WPMY-1, 22Rv1, C4-2B and PC-3 cells at 70% confluence were
178 incubated with culture media for two days. The media were harvested and centrifuged at 2,000 g for
179 5 min to remove dead cells. The supernatants were then ultracentrifuged using a Ti 50.2 rotor
180 (Beckman Coulter, Ireland) at 4°C. A first centrifugation was performed at 20,000 g for 30 min to
181 remove small debris, the supernatants were then centrifuged at 100,000 g for 2 h. The pellets
182 obtained at this stage were resuspended in ice-cold PBS, and centrifuged one last time at 100,000 g
183 for 1 h. The supernatants were then discarded and pellets containing exosomes were resuspended in
184 ice-cold PBS. The exosomal protein content was assayed by a BCA assay (Thermo Scientific,
185 Massachusetts, USA). Exosomes were immediately used to treat osteoblastic cell models; they were
186 diluted in 500 µL of culture media and incubated with MC3T3-E1 at 6 days of differentiation during
187 24 h or with mOB at 10 days of differentiation during 2 h respectively. For calvaria experiments, one
188 half of calvaria was used as a control, the other one was treated with 50 µg/mL of C4-2B-derived
189 exosomes for 24 h.

190 For the rescue experiments, C4-2B cells at 70% confluence were incubated with either Halopemide
191 at 1 µM, or PA at 100 µM, or a combination of both for 48 h. The media were then harvested and

192 processed as mentioned above to recover exosomes, which were used for the treatments of MC3T3-
193 E1 and mOB cells as well as calvaria.

194

195 **2.6 Western blot**

196 Cells were lysed on ice for 30 min with radio immunoprecipitation assay buffer (RIPA; 25 mM Tris-
197 HCl, 150 mM NaCl, 1% (w:v) NP-40, 1% (v:v) sodium deoxycholate, 0.1% (w:v) SDS, at pH 7.4).

198 The protein content of cell lysates and exosomes was measured with the BCA assay, and the same
199 amounts of proteins were boiled in reducing Laemmli buffer (Bio-Rad, California, USA) for 5 min,

200 except for the PLD immunodetection, for which where they were boiled for only 1 min. Samples

201 were then loaded on top of 12% acrylamide gels; the gels contained 4 M urea when performing PLD

202 immunodetection. Proteins were then transferred on nitrocellulose membranes (GE Healthcare,

203 France). After the transfer, membranes were blocked in 5% (w:v) BSA for 2 h at room temperature.

204 All Primary antibodies (see **Table 1** for detail) were incubated overnight at 4°C. Blots were then

205 probed with goat anti-mouse or goat anti-rabbit, HRP-coupled antibodies (Sigma Aldrich) for 1 h.

206 Immunoblots were revealed with the enhanced chemiluminescence (ECL) detection system (Bio-

207 Rad), images were captured with the XRS+ Chemidoc (Bio-Rad) camera. Band intensities were

208 quantified with Image J [44].

209

Antibody	Supplier – reference	Working concentration
Rabbit anti-ERK	Cell Signaling - #9102	1 µg/mL
Mouse anti-p-ERK	Cell Signaling - #9106	500 ng/mL
Rabbit anti-ALIX	Abcam – ab117600	500 ng/mL
Mouse anti-Claudin3	Abcam – ab214487	500 ng/mL
Mouse anti-Calnexin	Abcam – ab22595	100 ng/mL

Mouse anti-CD9	Abcam – ab92726	500 ng/mL
Rabbit anti-PLD1	Gift from Pr. Bourgoin Univ-Montréal	200 ng/mL
Rabbit anti-PLD2	Gift from Pr. Bourgoin Univ-Montréal	100 ng/mL

210

211 **Table 1:** List of antibodies used for Western blot

212

213 **2.7 Cryogenic-transmission electron microscopy**

214 Exosome morphology was analyzed by cryogenic-transmission electron microscopy (Cryo-TEM) at
 215 the “Centre Technologique des Microstructures” (CT μ) facility of the University of Lyon. For Cryo-
 216 TEM analysis diluted samples of exosomes were dropped onto 300 mesh holey carbon films
 217 (Quantifoil R2/1) and quench-frozen in liquid ethane using a cryoplunge workstation (made at
 218 Laboratoire de Physique des Solides-LPS, Orsay, France). The specimens were then mounted on a
 219 precooled Gatan 626 sample holder, transferred into the microscope (Phillips CM120) and observed
 220 at an accelerating voltage of 120 kV.

221

222 **2.8 Physico-chemical characterization of exosomes**

223 Exosomes size distribution was determined using Malvern Zetasizer® NanoZS (Malvern
 224 Instruments S.A., Worcestershire, UK). Exosomes extracted from 240 mL of conditioned culture
 225 media were brought to a volume of 1 mL in sterile PBS, in these conditions the protein concentration
 226 was around 0.25 μ g/mL Particle size and polydispersity index (PDI) were determined by Dynamic
 227 Light Scattering (DLS) diluting all samples with Milli-Q water to ensure correct calculation of size
 228 distribution by the method of cumulants. Analyses were carried out at 25 °C with an angle of
 229 detection of 173°. Measurements were performed in triplicate. Results were presented as mean
 230 diameter \pm standard deviation (SD) or PDI respectively.

231

232 2.9 Fluorescence microscopy

233 Cells stably expressing eGFP or eGFP-PLD2 were seeded at 10,000 cells/cm². The day after they
234 were fixed in a 3.7% (v:v) formalin solution for 20 min, and then stained with 1 µg/mL of DAPI
235 (Sigma Aldrich) for 2 min at room temperature. Images were then acquired with a fluorescence
236 microscope (Axiovert 40 CFL – Zeiss, Switzerland), and reconstructed with Image J.

237

238 2.10 Real time PCR analysis

239 The total RNA content from samples was isolated with the Nucleospin kit (Macherey Nagel,
240 France), following the manufacturer's instructions. One µg of RNA was then subjected to a reverse
241 transcription, using Super Script II reverse transcriptase (Invitrogen). The reaction was performed at
242 42°C for 30 min, and the enzyme was then heat inactivated for 5 min at 99°C. Real-time quantitative
243 PCR was completed using iTaq Universal SYBR green supermix on the CFX-96 Touch™ Real-
244 Time PCR system (Bio-Rad), with an annealing temperature of 60°C. At the end of the amplification
245 process, melt curves were generated to ensure that the amplified product was pure. The primers used
246 for the PCR amplification are listed on **Table 2**.

247

Gene	Forward (5'-3')	Reverse (5'-3')	Ta °C
<i>Gapdh</i>	GGCATTGCTCTCAATGACAA	TGTGAGGGAGATGCTCAGTG	60
<i>Osx</i>	AGGCACAAAGAAGCCATACG	GCCCAGGAAATGAGTGAGG	60
<i>Opn</i>	CTTTCACTCCAATCGTCCCTA	GCTCTCTTTGGAATGCTCAAGT	60
<i>Ocn</i>	AAGCAGGAGGGCAATAAGGT	CGTTTGTAGGCGGTCTTCA	60
<i>Dmpl</i>	GGCTGTCCTGTGCTCTCCCAG	GGTCACTATTTGCCTGT	60
<i>Alpl</i>	CAGGCCGCCTTCATAAGCA	GGGGCAGCGTCAGATGTTAAT	60

248

249 **Table 2:** List of primers used for the quantitative real-time PCR experiments.

250

251 **2.11 Alkaline phosphatase activity and mineralization assessment**

252 Alkaline phosphatase (AP) activity was measured using the same protocol as reported [45]. Briefly,
253 samples were collected in 0.2 % (v:v) NP-40 (polyethylene glycol nonylphenyl ether), and disrupted
254 by a mild sonication. AP activity was then assayed by the measure of the hydrolysis of p-nitrophenyl
255 phosphate into p-nitrophenolate (yellow compound with an absorbance at 405 nm) in alkaline
256 conditions (pH=10.3). For standardization, the protein content was determined using the BCA
257 protein assay kit. Matrix mineralization was quantified by Alizarin red (Sigma-Aldrich) staining
258 [46]. Matrix calcium content was determined by o-cresolphthalein complexone assay as described
259 [47].

260

261 **2.12 Viability and toxicity assays**

262 Viability was assayed through the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
263 bromide (MTT; Sigma-Aldrich, France) to formazan by metabolically active cells. MTT assay were
264 performed as described [34].

265

266 Toxicity was assayed by measuring the lactate dehydrogenase (LDH) released by dead cells in the
267 culture medium. For this purpose, we used the LDH detection kit (Roche Diagnostics, Germany),
268 according to the manufacturer's protocol.

269

270 **2.13 ³H-thymidine incorporation assay**

271 MC3T3-E1 cells were plated at 2,500 cells/cm², and cultured for 4 days in normal conditions, then
272 for 6 more days in mineralizing conditions. Cells were then treated with exosomes (1-50 µg/µL) for
273 24 h at 37°C. ³H-thymidine was added to the culture medium (0.5 µCi/mL) 6 h before the end of the

274 experiment. Cells were then washed once in ice cold PBS, and radioactivity measured as described
275 previously [48].

276

277 **2.14 Data analysis**

278 All data reported here are represented as the mean \pm SEM. To establish significance of our results,
279 data were analyzed by the use of two-sided Mann-Whitney U test. The level of significance was set
280 at $p < 0.05$. Graphs and calculations were done using Prism and InStat 3, respectively (GraphPad
281 software, California, USA). P values (p) and the number of experiments (n) are displayed in the
282 figure legends.

283

284 **3. RESULTS**

285 **3.1 Characterization of exosomes secreted by several prostate cell lines**

286 Exosomes were recovered from multiple prostate cell lines and were characterized. After purification
287 of exosomes and extraction of proteins from cell lysates, 20 µg of proteins were separated by SDS-
288 PAGE, transferred onto Hybond-P membranes (GE Healthcare Life Sciences), and subjected to
289 Western blot analysis. In this type of experiments, it is not possible to have a loading control such as
290 actin or tubulin because exosomes have different amounts of these proteins compared to cell lysate
291 content. To further validate our protocol and demonstrate the separation of exosomes, we performed
292 Western blot analysis of CD9, ALIX and CLN3, three exosome markers, compared to Calnexin, a
293 cellular marker. Each antibody was very specific and we obtained a single band of expression for
294 each one.

295 Exosomes from 22Rv1, C4-2B and PC-3 cell lines all expressed ALIX and CD9 but they did not
296 express the specific endoplasmic reticulum marker Calnexin, thus excluding a potential
297 contamination by cell debris (**Figure 1A**). Moreover, all PCa-derived cell lines also expressed
298 claudin-3 (CLN3), a major protein component of membranes of tight junctions that has been
299 significantly related to apparition of distant metastasis in different cancers [49, 50] (**Figure 1A**).
300 Non-tumor prostate cancer cell line WPMY-1 which was used as a control, only expressed faintly
301 ALIX and CD9 but not CLN3, indicating a decrease number of secreted exosomes.

302
303 DLS analysis revealed that C4-2B and PC-3 exosomes size was around 100-120 nm (**Figure 1B**).
304 Finally, exosomes harvested from C4-2B and PC-3 cells were observed by Cryo-TEM (**Figure 1C**)
305 to characterize their morphology. Exosomes derived from C4-2B and PC-3 cells were round-shaped
306 with a bilayer membrane (**Figure 1C**)

307

308 **3.2 PCa-derived exosomes only express PLD2**

309 We analyzed the expression levels of PLD1 and PLD2 in WPMY-1, 22Rv1, C4-2B and PC-3 cells
310 and in their respective exosomes. Both PLD1 and PLD2 were detected in ~~the~~ all cell lysates whereas
311 only PLD2 was present, at a lesser extent, in exosomes from C4-2B and PC-3 cells (**Figure 2A, 2B**).
312 To confirm these results, the localization of an eGFP-tagged PLD2 was examined in cells and
313 exosomes. We generated eGFP or PLD2-eGFP overexpressing cell lines (C4-2B or PC-3). PCa cell
314 lines stably overexpressing eGFP or PLD2-eGFP were generated with at least 50% of transfection
315 rate. PLD2-eGFP localized to the plasma membrane in C4-2B cells compared to eGFP, which was
316 diffuse through the cell (**Figure 2C**). Next, exosomes from C4-2B overexpressing eGFP or PLD2-
317 eGFP were analyzed by Western blot. Exosomes derived from C4-2B/PLD2-eGFP cells were more
318 enriched in ALIX, CD9 and CLN3 compared to C4-2B/eGFP-derived exosomes (**Figure 2D**).
319 Similar results were obtained for PC-3/PLD2-eGFP cells (data not shown). As expected, in C4-2B
320 and PC-3 cell lines PLD2-eGFP was efficiently exported in their respective exosomes (**Figure 2E,**
321 **2F**).

322

323 **3.3 Characterization of MC3T3-E1 osteoblastic cell mineralization**

324 Due to the osteoblastic nature of bone metastasis in PCa, we determined the effects of PCa-derived
325 exosomes on the bone microenvironment, and more specifically their interactions with osteoblasts.
326 Therefore, we selected exosomes from C4-2B cells, a PCa bone metastasis-derived cell line with a
327 pro-osteoblastic phenotype [51]. The MC3T3-E1 murine pre-osteoblast cell line was monitored
328 during its differentiation to find the optimal conditions for a treatment with C4-2B exosomes.
329 MC3T3-E1 cells were cultured in mineralizing conditions (50 µg/mL ascorbic acid and 10 mM β-
330 glycerophosphate) for 7 to 17 days. Calcium deposition and AP specific activity were measured in
331 osteoblasts, because they are major markers of the osteoblastic activity [52]. Osteoblasts were
332 stimulated by osteogenic factors to produce extracellular calcium deposits which are an indication of
333 successful in vitro mineralization. During this process, AP activity increase. The objective was to see

334 the effect of exosomes derived from C4-2B and WPMY-1 cells on AP activity in osteoblast and
335 whether they stimulate mineralization.

336 As expected, *Alpl* expression was significantly increased from day 7 of treatment (**Figure 3A**)
337 concomitantly with AP specific activity (**Figure 3B**). In parallel, extracellular matrix mineralization
338 was also significantly increased after 7 days of treatment either evidenced by Alizarin red staining
339 (**Figure 3C**) or calcium deposition quantification (**Figure 3D**). Early bone matrix mineralization
340 markers *Osx* and *Opn* expression (coding for osterix and osteopontin respectively) were significantly
341 enhanced after 7 days of culture in mineralizing conditions (**Figure 3E, 3F**). Finally, late bone
342 mineralization markers *Ocn* and *Dmp-1* (coding for osteocalcin and dentin matrix acidic
343 phosphoprotein 1 respectively) were significantly augmented after 10 days of treatment (**Figure 3G,**
344 **3H**). MC3T3-E1 cell cultures were strongly mineralized after 10 days of treatment. Therefore, we
345 selected the treatment of C4-2B-derived exosomes in MC3T3-E1 during early stages of
346 differentiation after 7 days of treatment in mineralizing conditions.

347 **3.4 C4-2B-derived exosomes increased proliferation and AP specific activity in MC3T3-E1** 348 **osteoblastic cells**

349 MC3T3-E1 cells were treated with different concentrations of C4-2B-derived exosomes during the
350 last 24 h of the 7 days of culture with osteogenic factors (L-ascorbate and β -glycerophosphate).
351 Exosomes did not affect MC3T3-E1 viability (measured with MTT assay) at 1 nor 10 $\mu\text{g}/\text{mL}$ but
352 they slightly diminished viability ($-11.3 \pm 3.8\%$) when used at 50 $\mu\text{g}/\text{mL}$ (**Figure 4A**). At this
353 concentration, exosomes induced around 5% of toxicity (measured with LDH assay) in MC3T3-E1
354 cells (**Figure 4B**). We then chose the concentration of 10 $\mu\text{g}/\text{mL}$ of C4-2B exosomes where the cells
355 were viable, to conduct experiments on the effect of exosomes released from PCa on osteoblasts.
356 Next, we determined if C4-2B-derived exosomes were able to stimulate MC3T3-E1 proliferation
357 after 24 h of treatment. Exosomes slightly induced proliferation at 10 $\mu\text{g}/\text{mL}$ ($+16 \pm 13.4\%$) as
358 demonstrated by the ^3H -thymidine incorporation assay (**Figure 4C**). At this concentration, they

359 significantly stimulated ERK 1/2 phosphorylation indicating the activation of several mitogenic
360 pathways (**Figure 4D**). Noteworthy, as for proliferation, exosomes significantly inhibited ERK1/2
361 activation when used at 50 µg/mL (**Figure 4D**), in accordance with the toxicity observed at this
362 concentration (**Figure 4B**). The relative AP specific activity, was significantly increased ($+22.8 \pm$
363 10.5%) in MC3T3-E1 cells when treated with exosomes at 10 µg/mL during 24 h (**Figure 4E**).

364

365 **3.5 PLD invalidation counteracts the effect of C4-2B-derived exosomes on osteoblast** 366 **proliferation and mineralization**

367 We aimed to understand if the localization of PLD2 in C4-2B-derived exosomes was directly related
368 to their capacity to stimulate the proliferation and differentiation of MC3T3-E1 cells. Therefore, we
369 treated C4-2B cells during 48 h with 1 µM of Halopemide (Halo), a pan-inhibitor of PLD activity,
370 before recovering exosomes. Exosomes from Halo-treated C4-2B cells displayed exactly the same
371 protein markers (ALIX, CD9, and CLN3) as exosomes from non-treated C4-2B cells (**Figure 5A**).
372 Moreover, their average size, determined by DLS, was the same (126 ± 3 nm for C4-2B cells
373 compared to 128 ± 17 nm for Halo pretreated C4-2B cells). Exosomes from Halo pre-treated cells
374 were unable to stimulate neither proliferation nor the ERK1/2 phosphorylation of MCT3-E1 cells
375 when used at the same concentration (10 µg/mL) (**Figure 5B, 5C**). Moreover, exosomes from Halo-
376 treated C4-2B cells lost their capacity to activate mineralization. Contrary to untreated C4-2B-
377 derived exosomes, they failed to increase mRNA levels of *Alpl*, *Osx*, and *Opn* as well as *Dmp1*
378 (**Figure 5D**). Moreover, they were not able to stimulate AP specific activity (**Figure 5E**). To exclude
379 a potential cell-type dependent artefact, we confirmed this result by using mOB, in which C4-2B-
380 derived exosomes significantly increased AP specific activity, contrary to exosomes produced in the
381 presence of Halo (**Figure 5E**). To strengthen these findings, we treated calvaria explants with 10
382 µg/mL exosomes for 24 h. Untreated C4-2B-derived exosomes were able to significantly increase
383 AP specific activity ($+29.7 \pm 3.6\%$). On the opposite, Halo pre-treated C4-2B-derived exosomes did

384 not substantially change AP specific activity of calvaria explants (**Figure 5E**). Finally, exosomes
385 derived from non-tumour prostatic cell line WPMY-1, were incapable of increasing mRNA levels of
386 *Alpl* and *Ocn* (**Figure 5F**) or AP specific activity of MC3T3-E1 osteoblastic cells (**Figure 5G**).

387

388 **3.6 PLD-derived PA is a requisite for PCa-derived exosomes generation**

389 Next, we aimed to determine if the product of PLD, PA, was directly involved in C4-2B-derived
390 exosome generation and if it was able to rescue the impact of Halo in exosome secretion. Pre-
391 treatment of C4-2B cells with 100 μ M PA alone or combined with Halo treatment during 48 h before
392 the recovery of exosomes, did not modify exosome features compared to the non-treated condition
393 (**Figure 6A**). Pre-treatment of C4-2B cells with Halo significantly reduced exosomal production (-
394 $39\pm 11.5\%$). The treatment with PA alone did not modify the quantity of exosomes produced, but it
395 was able to restore exosome secretion when combined with Halo (**Figure 6B**). This strongly suggests
396 that PLD acts through its product PA. Finally, the biological function of exosomes was ascertained
397 by treating MC3T3-E1 osteoblastic cells either with C4-2B/eGFP or C4-2B/PLD2-eGFP-derived
398 exosomes. We observed a more important increase in ERK 1/2 phosphorylation in MC3T3-E1
399 osteoblastic cells treated with exosomes delivered from C4-2B/PLD2-eGFP cells (Figure 6C). AP
400 specific activity of MC3T3-E1 cells was slightly increased (+14%) as compared with C4-2B/eGFP-
401 derived exosomes (Figure 6D). These results partially confirmed the role of exosomal PLD2 in
402 activating metabolism of osteoblastic cells.

403 **4. DISCUSSION**

404 In the present work, we have demonstrated for the first time the presence of PLD2 in exosomes
405 derived from PCa metastatic cells and its involvement in osteoblast activation by exosomes.
406 Moreover, we showed that PLD is a regulator of exosome secretion through its product, PA.
407 We successfully isolated extracellular vesicles secreted by diverse PCa cell lines, 22Rv1, C4-2B and
408 PC-3. Their identity as exosomes was confirmed by protein marker analysis using Western blot, by
409 morphology analysis using cryo-TEM and by particle size analysis using DLS. We confirmed the
410 expression of 3 exosomes markers (CD9, ALIX and CLN3) in 22Rv1, C4-2B and PC-3-derived
411 exosomes. DLS analysis revealed that C4-2B and PC-3 exosomes size was around 100-120 nm
412 (126 ± 3 nm for C4-2B cells, 110 ± 14 nm for PC-3 cells respectively) with a relatively small PDI value
413 indicating a monodispersed population (<0.2 nm for C4-2B cells, <0.3 nm for PC-3 cells
414 respectively). Cryo-TEM observations were performed to study exosomes morphology. This
415 technique allows a finer discrimination between smaller and bigger vesicles, which cannot be
416 evidenced by DLS measurement. PC-3 cells formed a quite monodispersed population constituted of
417 empty round shaped vesicles. In addition, multilayered vesicles were also detected. In the case of
418 exosomes derived from C4-2B cells, a more important contribution of smaller particles was noticed
419 which was not detected by DLS. These results suggest that our purified extracellular vesicles are
420 highly enriched in exosomes (30 – 150 nm). These measurements were in accordance with previous
421 reports [14, 20, 21].

422

423 PLD2 was found to be present in exosomes from C4-2B and PC-3 cells, even though to a lesser
424 extent than in cell lysates, which is consistent with previous observations in RBL-2H3 [31], HCT116
425 and LS180 colon cells [32]. On the contrary, the inability to detect PLD1, in our work and in
426 previous studies, strongly suggests that PLD1 is absent from tumor cell-derived exosomes. The
427 transfer of PLD2 to exosomes was further confirmed when it was overexpressed in C4-2B and PC-3

428 metastatic cell lines. PLD2 is mainly localized at the plasma membrane [23], whereas PLD1 is
429 mostly localized on the membranes of the perinuclear organelles. The first step of exosome
430 biogenesis being the budding and internalization of early endosomes [53], we might speculate that
431 the plasma membrane-associated PLD2 colocalizes with endosomes [54], explaining its presence
432 later in exosomes.

433 PCa-derived exosomes could affect function of osteoblasts to trigger the process of cancer
434 metastasis, by different ways. Exosomes from PCa cells target the bone [21] being internalized by
435 osteoblasts [16, 55]. 1) miRNAs in exosomes may regulate the activity of osteoblasts as evidenced
436 by other reports. Indeed, exosomal miR-141-3p derived from MDA-PCa-2b cells regulated
437 osteoblast activity and increased osteoprotegerin expression [16] whereas exosomal hsa-miR-940
438 derived from C4-2B cells stimulated the osteogenic differentiation of human mesenchymal stem
439 cells [17]. 2) The increase in expression of CXCL12 in osteoblasts may facilitate the interactions
440 between osteoblasts and PCa cells. In this respect, exosomes derived from C4-2B cells were able to
441 induce the expression of the chemokine CXCL12 in bone stromal cells, through the transfer of
442 pyruvate kinase M2-mediated HIF1 α -dependent pathway [21]. 3) Moreover, the delivery of-V-ets
443 erythroblastosis virus E26 oncogene homolog 1(Ets1) by exosomes from PC-3 and DU145 cells
444 induced osteoblast differentiation [56].

445 So far, no protein with catalytic activity has been linked to the promotion of bone metastasis by PCa
446 cells *via* exosomes. Here, we selected exosomes derived from the C4-2B cell line which induced
447 marked osteoblastic lesions in mice [51]. To investigate the effect of exosomes on the bone
448 microenvironment, and particularly osteoblasts, we used several osteoblast models: MC3T3-E1
449 osteoblastic cell line, primary culture of murine osteoblasts (mOB), and whole calvaria explants.

450

451 MC3T3-E1 cells treated with 10 μ g/mL C4-2B-derived exosomes increased phosphorylation of ERK
452 1/2 and proliferated slightly more. It has been demonstrated that TRAMP-C1 PCa cell line-derived

453 exosomes impaired RAW 264.7 pre-osteoclast proliferation [19]. Thus, exosomes may shift the bone
454 balance in favor of osteoblasts, stimulating bone formation. AP specific activity was also increased
455 under those conditions, for MC3T3-E1, mOB, and calvaria explants consistent with an increase in
456 TNAP gene expression and activity [16-18]. AP velocity (ie. The increase of AP in patients' sera
457 over time) is an excellent predictor of cancer progression and metastasis [57, 58], consistent with
458 higher AP expression in metastatic PCa cells than in healthy cells which is linked to the EMT and
459 migration. We could only find this osteogenic capacity in exosomes derived from C4-2B bone
460 metastatic cell line, since the non-tumor cell line WPMY-1 could not induce any osteoblastic
461 response in MC3T3-E1.

462 The modulation of proliferation and ERK phosphorylation are new mechanisms by which PCa-
463 derived exosomes could participate in the establishment of the bone metastatic niche. PLD is able to
464 activate both the MAPK and the PI3K/Akt pathways in different type of cells, such as metastatic
465 cancer cells [23]. Both pathways are then responsible for an increased cell proliferation, via the
466 activation of mTOR for instance [59]. Gastric cancer cell lines-derived exosomes can stimulate pAkt
467 pathway and proliferation of the recipient cells [60]. We may speculate that exosomes derived from
468 PCa, eventually through the transfer of the tumor PLD2 to osteoblasts, could mediate PI3K/Akt
469 signaling and could stimulate their proliferation and differentiation. We demonstrated that the
470 treatment with C4-2B-derived exosomes increased ERK1/2 phosphorylation in MC3T3-E1
471 osteoblastic cells. Indeed, the MAPK/ERK1/2 signaling pathway is an activator for osteoblast
472 differentiation and bone formation [61, 62].

473 Exosomes generated in the presence of halopemide lost their ability to stimulate expression of
474 osteoblast markers, even though they were used at the same concentration as exosomes from non-
475 treated cells. As a precaution, we checked that their structure and identity were unchanged. Both
476 their size by DLS and by immunoblotting were similar from control exosomes. This led us to
477 speculate that exosomes secreted in the presence of halopemide had an altered content, especially in

478 miRNA, since miRNA are the sole demonstrated exosome effectors in PCa bone metastasis. PLD2
479 was suggested to be an effector of the ARF6 – Synthenin – ALIX pathway in the budding process of
480 multiple vesicle bodies, in MCF-7 breast cancer cells [63]. The content of the resulting intraluminal
481 vesicles (ILVs) is also changed by the inhibition of PLD2, which is backed by the fact that
482 synthenins are also dependent on PA [63]. Investigating the miRNA content of exosomes produced
483 when PLD2 is inhibited could perhaps show a difference.

484

485 When we inhibited PLD with halopemide, exosomes were secreted by C4-2B cells at a lower rate
486 indicating that PLD affected exosomes release. PA rescued the loss of exosome secretion when
487 combined with halopemide. This strongly suggests that PLD acts on exosome secretion through its
488 product PA. PA is associated with membrane dynamics and fusion [64], which are required upon
489 exosome biogenesis, from the internalization of endosomes to the fusion of MVBs with the plasma
490 membrane. PA is present in exosomes, and some PLD activity could be detected in RBL-2H3
491 exosomes [65, 66].

492

493 The minor effect of PA in exosome production could be due to a saturation of the exosome
494 biogenesis pathway. PA might directly act on the synthenin-ALIX pathway, by binding to synthenin
495 as in MCF-7 breast cancer cells [63], triggering exosome release, which could be rapidly saturated.
496 The minor effect of PA can also be explained by the presence of the active phosphatidate
497 phosphatase 1 (PAP1) in exosomes, which could degrade PA rapidly into diglycerides [31].

498 Thus, the role of PLD2 once exosomes are secreted is probably less important than its role upon
499 ILVs formation. Alternatively, PA as a lipid mediator, through its action on phosphorylation of ERK
500 1/2, may trigger other cellular responses including production of miRNAs.

501

502 Our work opens alternate perspectives about the role of PLD in PCa, completing the landscape of a
503 multipotent enzyme in cancer progression. Its action in exosome secretion, by stimulating osteoblast
504 in metastatic processes [67], indicate that inhibition of PLD might be a target to prevent bone
505 metastasis in the case of PCa.

506

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514

515 **6. AUTHOR CONTRIBUTIONS**

516 M. Borel conducted experiments, interpreted the data and wrote the manuscript. G. Lollo realized the
517 physico-chemical characterization of exosomes and proofread the manuscript. D. Magne and R.
518 Buchet proofread the manuscript. S. Mebarek and L. Brizuela conceived the project, planned the
519 experimental design, participated in experiments, interpreted the data and wrote the manuscript.

520

521 **7. LEGENDS**

522 **Figure 1. Characterization of exosomes secreted by several prostate cell lines.** Prostatic cell line
523 WPMY-1, and tumoral prostatic cell lines 22rv1, C4-2B and PC-3 were cultured for 48 h, culture
524 media were harvested for exosome purification. The exosomes (E) and the corresponding cell lysates
525 (C) were analyzed by Western blot (A). The morphology of exosomes of the bone metastatic cell
526 lines C4-2B and PC-3 was analysed by cryoTEM (B) and size distribution was measured by DLS
527 (C). At least three independent measurements were performed

528

529 **Figure 2. PCa-derived exosomes only express PLD2.** Exosomes from WPMY-1, 22Rv1, C4-2B
530 and PC-3 cells were extracted and PLD1/PLD2 were analyzed by Western blot (A, B). C4-2B and
531 PC-3 were transfected with plasmids carrying eGFP or PLD2-eGFP constructs, after selection, we
532 checked the expression of the constructs by fluorescence imaging of the eGFP (green). Nuclei were
533 counter stained with DAPI and appeared blue. C4-2B eGFP and C4-2B/PLD2-eGFP cells (CB). The
534 exosomes secreted by C4-2B/eGFP and C4-2B/PLD2-eGFP were analyzed by Western blot for the
535 expression of calnexin, ALIX, CD9 and CLN3 (D). The exosomes secreted by C4-2B/PLD2-eGFP
536 and PC-3/PLD2-eGFP cells were ~~analyzed~~ checked by Western blot for the expression of PLD2 (E,
537 FC). * indicates the presence of a non-specific band.

538

539 **Figure 3. Characterization of MC3T3-E1 osteoblastic cell mineralization.** MC3T3-E1 were
540 cultured for up to 17 days in L-ascorbate and β -glycerophosphate containing medium. Mineralization
541 was assessed by measuring the *Alpl* gene expression (A) and AP specific activity (B), as well as
542 calcium deposition in the extracellular matrix using Alizarin red staining (C) and using o-
543 cresolphthaleine-complexone in a colorimetric assay (D). Gene expression of several osteoblastic
544 differentiation markers (*Osx*, *Opn*, *Ocn*, *Dmp1*) were measured (E to H). All the data are the means \pm
545 SEM, * p < 0.05, ** p < 0.01.

546

547 **Figure 4. C4-2B-derived exosomes increase proliferation and AP specific activity of MC3T3-E1**
548 **osteoblastic cells.** MC3T3-E1 cells were cultured in mineralizing media for 6 days and then treated
549 with C4-2B exosomes in concentrations ranging from 1 µg/mL to 50 µg/mL for 24 h. Their effects
550 on cellular viability (A) and toxicity (B) were assessed. Proliferation was determined by ³H-
551 thymidine incorporation for 6 h (C). Expression of ERK 1/2 phosphorylation was examined by
552 Western blot (D). The effects of exosomes on osteoblastic activity was assessed by measuring AP
553 specific activity (E). Experiments were conducted at least 4 times, and data are reported as the mean
554 ± SEM, * p < 0.05, ** p < 0.01.

555

556 **Figure 5. PLD invalidation counteracts the effect of PCa-derived exosomes in osteoblast**
557 **mineralization.** C4-2B were treated with Halo 1 µM for 48 h, exosomes were then extracted and
558 exosomes (E) markers were analyzed compared to whole cell (C) lysates by Western blot (A). Those
559 exosomes (10 µg/mL) were then used to treat MC3T3-E1 for 24 h. Proliferation was determined by
560 ³H-thymidine incorporation for 6 h (B). Expression of ERK 1/2 phosphorylation was examined by
561 Western blot (C). Osteoblastic differentiation was determined by the analysis of gene expression of
562 osteoblastic markers (*Alpl*, *Osx*, *Opn*, *Ocn*, *Dmp1*) after 24 h of treatment (D). C4-2B-derived
563 exosomes were also used to treat MC3T3-E1 for 24 h, mOB for 2 h or calvaria explants for 24 h and
564 subsequently AP specific activity was measured (E). MC3T3-E1 cells were treated with WPMY-1-
565 derived exosomes and gene expression of osteoblastic markers (*Alpl*, *Ocn*) was analyzed (F) and AP
566 specific activity was determined (G). Each experiment was repeated at least 4 times, and data are
567 reported as the mean ± SEM, * p < 0.05, ** p < 0.01. # denotes a significant difference between
568 normal exosomes and exosomes generated in the presence of Halo.

569

570 **Figure 6. PLD-derived PA is a requisite for PCa-derived exosomes generation.** C4-2B were
571 treated for 48 h with 1 μ M Halo, 100 μ M PA, or a combination of both, exosomes were then
572 purified. We analyzed exosomes (**E**) and the corresponding cell lysates (**C**) by Western blot (**A**). *
573 indicates the presence of a non-specific band. In parallel exosomes were quantified by the measure
574 of protein concentration, which was normalized by the number of cells (**B**). Next effects of C4-
575 2B/eGFP or C4-2B-PLD2-eGFP-derived exosomes in ERK 1/2 phosphorylation (**C**) and AP specific
576 activity (**D**) of MC3T3-E1 osteoblastic cells. Each experiment was repeated at least 4 times (n=2 for
577 panel C and D), and data are reported as the mean \pm SEM, * p < 0.05, *** p < 0.001.

578

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