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Article

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Abstract: Cancer stem cells remain a challenge to isolate and characterize because of their plastic phenotype. Using a microfluidic lab-on-a-chip based on ultra-high frequency dielectophoresis, we measured the electromagnetic signature of colorectal cancer cells and demonstrated that cancer stem cells show a distinct and lower electromagnetic signature than differentiated cells. The release of extracellular vesicles from tumor cells can drive tumor progression and metastasis development. As extracellular vesicles from cancer stem cells carry more aggressive content, we treated colorectal cancer cells with these vesicles to test whether the lab-on-a-chip can detect a change in phenotype. The electromagnetic signature of treated cells is modified in comparison to untreated cells and sometimes even when no biological change is observed. The lab-on-a-chip provides rapid and relevant result without prior labeling compared to conventional biological approaches. It could be useful in the clinic for early detection of cancer stem cells in the tumor mass and for monitoring the aggressive potential of extracellular vesicles in the bloodstream in order to adapt therapeutic management and prevent relapse.

Keywords: colorectal cancer; cancer stem cells; extracellular vesicles; high frequency dielectrophoresis; microfluidic lab-on-a-chip

1. Introduction

Colorectal cancer (CRC) is the second most diagnosed cancer in women and third most in men yet mortality in women is 25% lower than in men. Industrialized countries have the highest rates of CRC but they appear to be stabilizing or even decreasing due to screening campaigns and increased use of colonoscopy. Despite this, CRC is the fourth most deadly cancer in the world with nearly 900 000 deaths per year [1]. Furthermore, almost 25% of newly diagnosed CRC show distant metastasis [2] 50% of patients will develop metastasis during their lifetime [3,4]. Over the last decades, the diagnosis and care of CRC patients have improved considerably but relapses still occur and may be related to therapeutic resistance and/or the presence of a minimal residual disease (MRD).

A particular subpopulation within the heterogeneous tumor mass, known as cancer stem cells (CSCs) or tumor-initiating cells, is incriminated for both phenomena [5,6]. These are highly plastic undifferentiated cells, resistant to conventional therapies due to their

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stemness and self-renewal capabilities. In CRC, it has been reported a direct correlation between the number of undifferentiated cells and high risks of relapse [4,7].

Inside the stem cell niche, CSCs communicate with their microenvironment and this crosstalk relies on the secretion of extracellular vesicles (EVs). These are nanosized membrane vesicles ranging in size from 50 to 150 nm released by exocytosis of multivesicular bodies (MVB) from both tumor and healthy cells. They are found in all body fluids but usually, tumor cells release more EVs than their healthy counterparts [15,16].

The function of EVs in tumor development [2-8], drug resistance [2,8] and the transfer of aggressiveness [2,9-14] in solid cancers is widely documented. EVs act as a way of communication between cells by the exchange of genetic material [15]. We previously demonstrated that EVs can induce an aggressive phenotype by transferring neutrotrophin receptors (TrkB) to YKL-40-inactivated glioblastoma cells that have lost their aggressive potential [9]. Similarly, in glioblastoma (GBM), EVs can transfer the EGFRvIII, the truncated EGFR receptor associated with tumor progression [17]. In CRC, many studies have highlighted the role of EVs on the transfer of aggressiveness. EVs derived from CRC cell lines can deliver mRNAs, microRNAs and natural antisense RNAs to other solid cancer cell lines to control their gene expression [10] or to promote the cell migration of a hepatocellular cancer cell line [11]. Colon cancer-derived EVs may stimulate the tumor angiogenesis by activating endothelial cells [13], promote acquired resistance to 5-FluoroUracile (5-FU) [18] and facilitate the development and metastasis of CRC [14]. EVs can also affect the antitumor immune response. Indeed, they facilitate immune escape by inhibiting macrophage activation [19], by blocking T cell proliferation and promoting regulator T cell expansion [20]. In summary, EVs are considered an important source of circulating tumor biomarkers. Therefore, they could represent an efficient strategy to remotely monitor exchanges between CSCs and their microenvironment and thus predict drug resistance, tumor progression or the risk of relapse.

Isolation and characterization of CSCs are difficult because they represent less than 10% of the tumor mass [24] and the biological markers commonly used are shared with normal stem cells. Moreover, the level of expression of biological markers is not sufficient to characterize these cells and functional properties need to be explored. All these techniques are time consuming and quite expansive. Thus, the last few years, we developed a new label-free approach based on the detection of biophysical cell properties. It consists in measuring the electromagnetic (EM) signature of individual cells by dieletrophoresis (DEP) technique in the ultra-high frequency (UHF) range. A lab-on-a-chip implemented in a microfluidic channel allows the manipulation of individual cells to determine their EM signature called “crossover frequency”. This precise frequency is related to the intracellular content of the characterized cell. CSCs and differentiated cells from brain tumors have already been characterized with this device [21,22] making it an interesting tool for CSCs diagnosis in clinical routine.

In this work, we used this lab-on-a-chip on colorectal cancer-derived cells to investigate whether CSCs exhibit a different EM signature than differentiated cells, as previously observed in brain tumors [21,22]. In addition, EVs derived from CSCs carry more aggressive content than differentiated cells. We therefore performed aggressiveness transfer to demonstrate that the lab-on-a-chip can monitor phenotypic transformation. Here, we reported that CSCs show a lower EM signature than their differentiated counterparts, in both cell lines and primary CRC cultures. Moreover, this lab-on-a-chip can detect cellular changes caused by EVs intake that may eventually be missed in biology. This label-free technique allows biophysical characterization of cells and could be useful for early detection of CSCs in the tumor mass to improve the management of CRC patients.

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2. Materials and Methods

2.1 Cell culture

2.1.1 Cell lines and primary cultures of patient

SW480 and SW620 cell lines were purchased from the American Type Culture Collection (ATCC/LGC Promochem, Molsheim, France) and were grown in RPMI 1640 GlutaMAX™ (Gibco™, Thermo Fisher, MA, USA) medium supplemented with 10% FBS, 1% sodium pyruvate and 1% of penicillin / streptomycin (Thermo Fisher).

Two primary cultures from patients (CPP) were gently provided by Julie Pannequin- from the Institute of Fonctional Genomics (Univ. Montpellier, France) after informed consent of patients (Material Transfer Agreement CNRS 190287). CPP were grown in DMEM GlutaMAX™ medium supplemented with 10% FBS and 1% penicillin / streptomycin (Thermo Fisher).

Culture under FBS will be referenced as "Normal Medium" (NM).

2.1.2 Cancer stem cells (CSCs) enrichment

To enrich the cell population in CSCs, cell lines or CPP were cultured for at least one week in a "Define Medium" (DM): DMEM/F-12 (Thermo Fisher) medium without FBS but supplemented with 5 µg/mL of insulin, N-2 supplement (1X), 1% of penicillin / streptomycin (Thermo Fisher), 20 ng/mL of EGF and 20 ng/mL of bFGF (MACS Miltenyi Biotec, Bergisch Gladbach, Germany).

All cell types were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

2.1.3 Cell treatment with extracellular vesicles (EVs)

To undergo EVs transfer aggressiveness, cells were seeded in a 6-wells plate at $0{,}25.10^6$ cells and treated with EVs from 1.10^6 cells (NM or DM cultured cells) resuspended in a "exo-free" culture medium. Exo-free culture medium is a conventional culture medium with EVs-deprived FBS by ultracentrifugation at 120 000*g* for 16h. Cells were treated for 24h or 72h, once or twice with the same amount of EVs each time.

2.2 Extracellular vesicles

2.2.1 Isolation

Cells were seeded in a T75 flask at 1.10^6 cells for 10 mL of medium and cultured for 48h before collection of culture supernatant. To collect EVs, the culture supernatant was ultracentrifugated at 120 000*g* for 90 min twice to pellet and wash vesicles. Then, EVs were resuspended in phosphate-buffered saline (PBS, Thermo Fisher) for nanoparticle tracking analysis (NTA) or in culture medium for cell treatment or in RIPA buffer for western blot analysis.

2.2.2 Nanoparticle tracking analysis (NTA)

NTA was performed using NanoSight NS300 (Malvern Panalytical Ltd.) with specific parameters according to the manufacturer's user manual (NanoSight NS300 User Manual, MAN0541-01-EN-00, 2017). Captures and analysis were achieved by using the built-in NanoSight Software NTA3.3.301 (Malvern Panalytical Ltd.). The camera level was set at 14 and the detection threshold was fixed at 5. Samples were diluted in PBS (Thermo Fisher) and their concentration was adjusted by observing a particles/frame rate of around 50 (30–100 particles/frame). For each measurement, five consecutive 60-s videos were recorded under the following conditions: cell temperature—25 °C, syringe speed—22 µL/s (100 a.u.). Particles (EVs) were detected using a 488 nm laser (blue), and a scientific CMOS camera. Among the information given by the software, the following were studied: mode (i.e., the most represented EVs size population), the percentage of EVs between 50-150 nm

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and particles/mL. Using the last one and the number of cells collected, we calculated an EVs/cell ratio for each condition.

2.3 RNA extraction and RT-qPCR

Total RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and quantified by NanoDrop 2000 (Thermo Fischer). For RT-qPCR analyses, 2 µg of RNA was reverse-transcribed into cDNA using the cDNA Archive kit (Applied Biosystems, MA, USA). Quantitative gene expression was performed using SensiFAST Probe Hi-ROX kit (Bioline, London, UK) on QuantStudio 5 (Thermo Fischer) and stemness related genes (*PROM1, NANOG, SOX2, POU5F1, BMI1, LGR5*) were analyzed. Results were normalized to *GADPH* and *HPRT1* expressions and analyzed using the ΔΔCt method (control condition defined as 1).

2.4 Western blot

After collection, EVs were resuspended and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) supplemented extemporaneously with a cocktail of protease inhibitors (Roche, Bâle, Switzerland). Samples were separated on a 12% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membrane, blocked for 1 h in 5% (w/v) bovine serum albumin (BSA) in PBS and probed overnight at 4 °C with primary antibodies specific of EVs (Alix, Hsc70 and CD9). Membranes were then incubated with the proper secondary antibodies for 1 h and proteins revealed by Immobilon ECL Ultra (Merck Millipore, MA, USA) using a G-box imager with GeneSnap software (Syngene).

2.5 Flow cytometry

2.5.1 Stemness markers

Differentiated cells (NM) or CSCs (DM) were rinsed in PBS and 1.10^6 cells were labelled with extracellular anti-CD133, anti-Lgr5 and viability marker antibodies. Cells were incubated 30 min at RT in the dark, washed in PBS and then fixed 10 min in 4% PFA. After a wash, cells were permeabilized 30 min at 4°C in Perm buffer III (BD Biosciences, Le Pont de Claix, France). Finally, cells were labelled with intracellular antibodies of anti-Bmi1, anti-Oct4 and anti-Nanog for 30 min at RT in the dark. Samples were analyzed with a CytoFLEX flow cytometer (Beckman Coulter, CA, USA). Data analysis was performed using Kaluza software (v2.1, Beckman Coulter).

2.5.2 Cell cycle analysis

Cells were washed with ice-cold PBS, resuspended in ice-cold 70% ethanol and placed at -20°C overnight. The next day, after a wash, cells were incubated 20 min with RNase A (Sigma Aldrich, MO, USA) and Propidium Iodide (PI) before analysis on a CytoFLEX flow cytometer (Beckman Coulter). Results were analyzed using ModFit LT™ software (v5.0.9, Verity Software House, ME, USA).

2.6 Proliferation Assay

Five thousand cells were seeded in a 96-well plate and proliferation was measured using BrdU Cell Proliferation Assay Kit (Cell Signaling, MA, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm with a Multiskan FC Thermo Scientific microplate photometer (Thermo Fisher).

2.7 Metabolic activity assay

Five thousand cells were seeded in a 96-well plate and treated or not by EVs from NM or DM cultured cells for 24 or 72h at the same ratio as. After 24 or 72h of treatment,

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cells were treated with decreasing doses of 5-FU for 48h (500 µM to 1 µM). Cell toxicity was measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, WI, UA) according to the manufacturer's instructions and IC_{50} was determined graphically (GraphPad Prism 7.04).

2.8 Migration and invasion assays

Fifty thousand cells were seeded in an Incucyte® Imagelock 96-well Plate (Sartorius, Goettingen, Germany) and scratch migration and invasion assays were performed according to the manufacturer's instructions. Briefly, cells were seeded and allowed to adhere overnight. The next day, wounds were created with the 96-well WoundMaker™ (Sartorius), cells were washed twice and 100 µL of culture medium containing or not EVs was added to the wells.

For invasion assay, after wound creation, cells were overlay with 50 µL of Matrigel® (Corning, NY, USA) solution. After 30 min at 37°C for Matrigel® polymerization, 100 µL of culture medium containing or not EVs was added to the wells. The plate was placed in the Incucyte® system (Sartorius) and wound closure was analyzed every 2h for 2 days using IncuCyte 2021A software (Sartorius).

2.9 Self-renewal capacity

Five hundred of NM or DM cultured cells were seeded in a 96-well plate in define medium for 7 days and analyzed every 24h with the Incucyte® system (Sartorius) and analyzed using IncuCyte 2021A software (Sartorius).

2.10 UHF-DEP Crossover frequency measurement

With UHF-DEP, we are able to electromanipulate particles, such as biological cells. In a microfluidic channel is implemented a quadrupole electrode in which a suspension of individual cells is injected. The microfluidic flow allows to stop a single cell at the center of the microelectrode for its individual DEP characterization (Figure 1H). Signals in the Ultra-High Frequency (UHF) range of 30-450 MHz are used to probe the intracellular content of the cell. The movement of the cells from a "repulsive" state (nDEP) at the center of the quadrupole to an "attractive" state (pDEP) at one edge of the electrodes is done at a specific frequency called "crossover frequency" which is the cell's electromagnetic signature. The measurement of this crossover frequency allows to discrimintate cells by measuring the dielectric properties of their cytoplasmic content without any labeling. For further information, please see our precedent publication [21].

2.11 Statistical analysis

Values are represented by mean ± SEM obtained from at least three independent experiments except values from DEP analyses, represented as mean ± SD. Statistical analysis was performed using PAST software (version 2.17c) by one-way ANOVA (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

3. Results

3.1. Cancer stem cells from colorectal cancer cell lines show a lower EM signature than their differentiated counterparts

To test the lab-on-a-chip on CRC, two cell lines from the same CRC patient: SW480 as a stage II and SW620 as a stage III (tumor-invaded lymph nodes) were chosen. Each cell line was cultured under two distinct conditions: Normal Medium (NM) to enrich cell population in differentiated cells or Define Medium (DM) to enrich in CSCs. Define medium

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is a medium without FBS but supplemented with growth factors to generate stress on cells and force them to acquire stem-like cells properties. As shown in Figure 1A, cells cultured in DM are no longer adherent but grow as colonospheres, resulting in the acquisition of "stemness" properties. To confirm that these conditions induce a phenotypic shift to CSCs, we assessed their phenotypical and functional properties. Both cell lines cultured under define medium overexpress almost all stemness tested genes (*CD133*, *Sox2*, *Oct4*). Interestingly for both cell lines, stem-like cells underexpress *Lgr5* which is related to the level of cell activation, and SW620-DM overexpress *Bmi1* which is correlated to a quiescence state (Figure 1B). The same markers were investigated by flow cytometry to analyze their protein counterparts. No significant differences were observed with SW480 cell line whereas all markers were overexpressed by SW620 cells cultured in DM compared to SW620-NM, except from CD133 (Figure 1C). The SW480 cell line appears to require a longer time in define medium to overexpress these markers than the SW620 cell line, suggesting that stage III-derived cells have a more pronounced "cancer stem cells" burden. Indeed, SW620 cells always overexpress stemness related proteins compared to SW480 cells, regardless of culture conditions (Figure S1B). Since an overexpression of stemness related markers is not sufficient to consider a cell as a CSC, we explored its functional properties as well. For both cell lines, stem-like cells show a lower proliferation rate than differentiated cells (Figure 1D). Furthermore, the proportion of SW480-DM cells in the G2/M phase of the cell cycle is higher than that of SW480-NM cells, while the rate of cells in the G0/G1 phase is higher for SW620-DM cells than for SW620-NM cells (Figure 1G), consistent with the aforementioned overexpression of *Bmi1*. In a similar way, cellular response to 5-FU chemotherapy treatment (chemoresistance) was assessed by measuring metabolic activity and stem-like cells show a 5-fold higher IC50 than the differentiated ones (Figure 1E). Finally, we explored the ability of cells to self-renew by culturing them in define medium for seven days. For both cell lines, stem-like cells form more and larger colonospheres (Figure 1F). Altogether, these results allow us to consider cells cultured under define medium as cancer stem cells. In a previous work, we biophysically characterized brain tumor cells and the U87-MG glioblastoma cell line with the lab-on-a-chip and established a first EM signature related to it. Our results demonstrated that CSCs display a lower EM signature in the fx02 range whereas no difference was observed in the fx01 range [21]. On this basis, the fx02 range was selected to test CRC cells in our experiments. Similar results were observed for both CRC cell lines, as previously in the brain model, the EM signature is lower in CSCs than in differentiated cells (Figure 1I). Furthermore, we observe a complete change in electromagnetic signatures between CSCs and differentiated cells. For both cell lines, almost 100% of the differentiated cells show a crossover frequency between 200 and 400 MHz (88% for SW480 and 100% for SW620) while both CSCs show 100% of the cells between 0 and 100 MHz (Table 1, Figure S1I).

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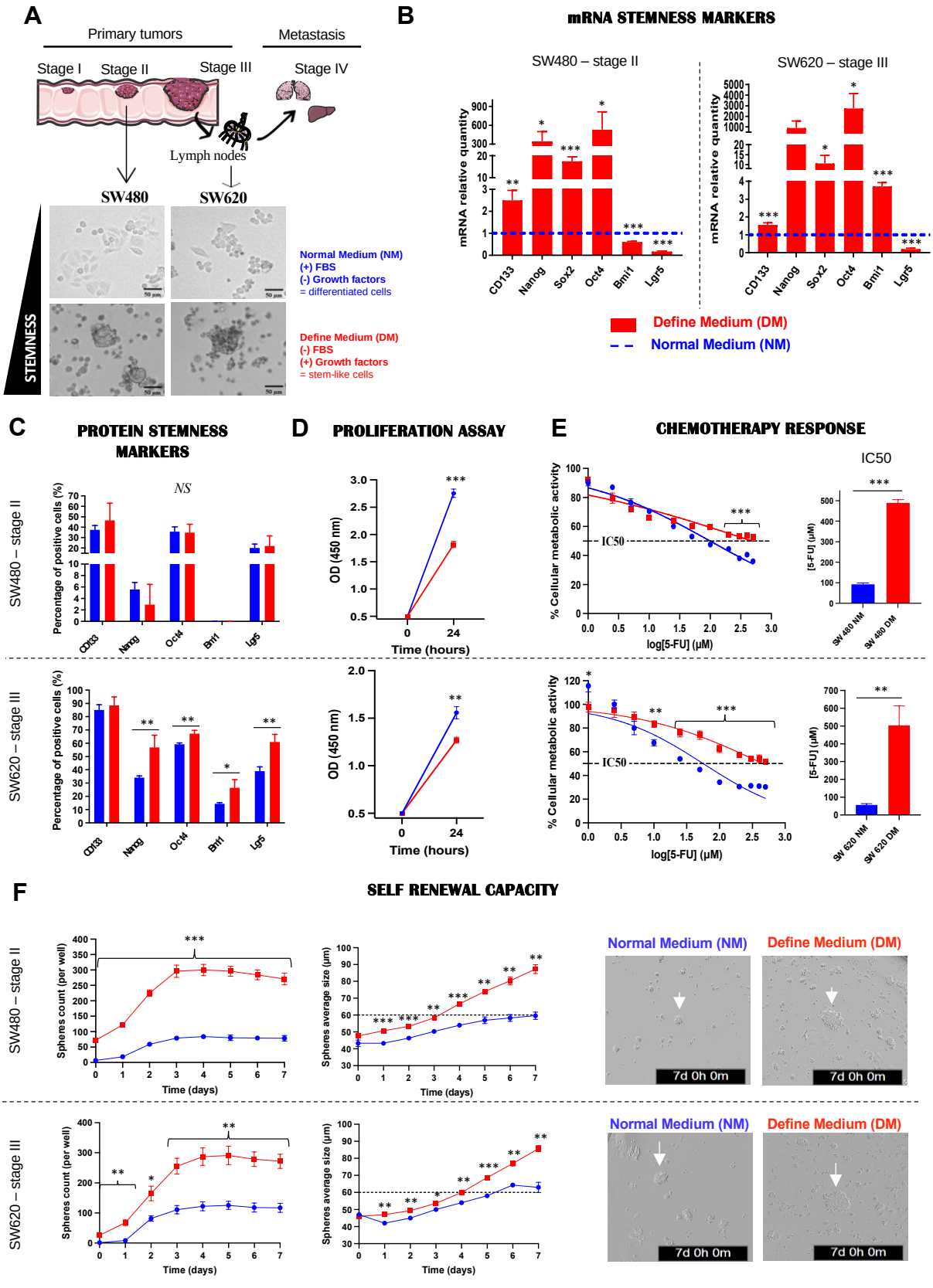

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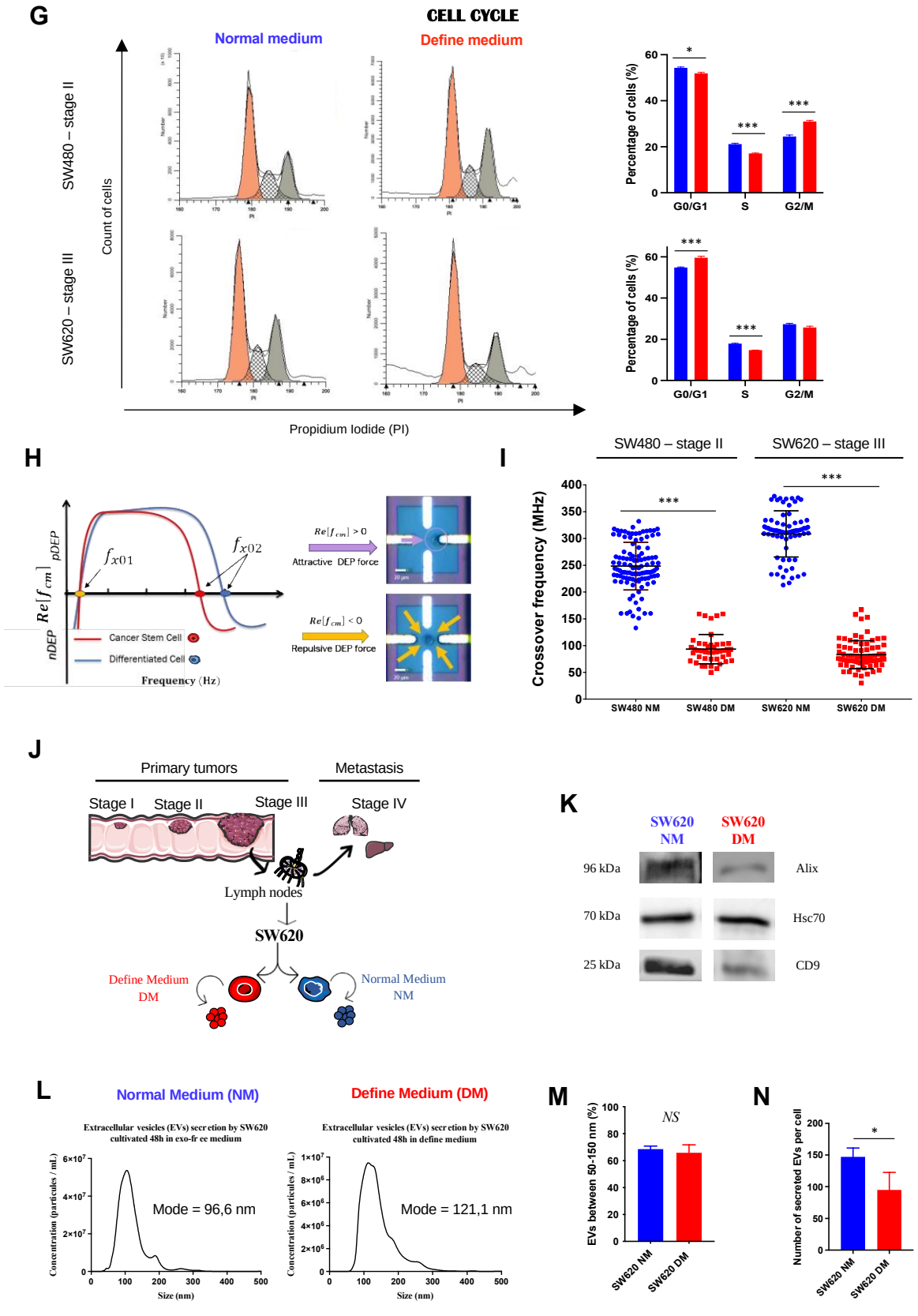

Figure 1. Characterization of cell lines and their extracellular vesicles according to culture conditions. (A) Cells were cultured under Normal Medium (NM) or Define Medium (DM) to enrich cell population in differentiated cells or CSCs. To validate culture conditions, stemness related genes (B)

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and stemness markers (C) were analyzed. Functional properties were explored with proliferation (D), response to chemotherapy (E), self-renewal capacity (F) and cell cycle analysis (G). The EM signature of both cell types was measured at UHF (fx02) with a lab-on-a-chip (H-I). EVs from SW620 cells (differentiated cells or CSCs) were collected by ultracentrifugation (J) and expression of EVs markers was assessed by western blot (K). NTA was performed on EVs regarding size distribution (L), percentage of EVs between 50 and 150 nm (M) and number of secreted EVs per cell (N). All results are represented as mean ± SEM except EM signatures represented as mean ± SD, NS *p*-value indicate not significant result, * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 using one-way ANOVA test.

Table 1. Cell proportions per frequency range for colorectal cancer cell lines

	SW480		SW620	
	Normal medium	**Define medium**	**Normal medium**	**Define medium**
0-100 MHz	0%	70%	0%	78%
100-200 MHz	12%	30%	0%	22%
200-300 MHz	68%	0%	24%	0%
300-400 MHz	20%	0%	76%	0%

Although these results confirm that CSCs are enriched in both CRC cell lines cultured in define medium, SW480-DM and SW620-DM are not dependent of the same step of cell cycle. Thus, SW480-DM appear to be a proliferative type of CSCs while SW620-DM appear to be a quiescent one. This EM signature is consistent with those previously obtained for glioblastoma and medulloblastoma tumors [21,22].

3.2. Extracellular vesicles transfer some aggressive properties and this change in state can be detected by biophysics in colorectal cancer cell lines

As EVs transfer genetic and protein materials derived from tumors, CSCs-derived EVs might transfer additional tumor aggressiveness compared to differentiated cells due to their more aggressive content. Indeed, our previous results put on evidence that EVs from undifferentiated cells related to CSCs transferred tumor aggressiveness to less aggressive tumor cells. In this context, SW480-NM (early-stage) was treated with EVs from the SW620 cell lines (late-stage) to assess a putative aggressiveness transfer. After culturing the SW620 cell line under both culture conditions, supernatant were ultracentrifugated to collect EVs (Figure 1J). NTA and western blot analyses confirmed their characteristics and CSCs secrete less EVs than differentiated cells (Figure 1K-N). The SW480 cell line, cultured in NM, was treated once for 24h with EVs derived from SW620 cell line (cultured in NM or DM) (Figure 2A). Analysis of stemness-related gene expression demonstrated that cells treated with EVs from SW620-DM overexpress *CD133*, *Sox2* and *Lgr5* whereas no difference was observed with EVs from SW620-NM (Figure 2B). Conversely, cells treated with EVs from SW620-NM show a higher proliferation rate that increases overtime, although no change was observed with EVs from SW620-DM (Figure 2C). Strikingly, this lack of change when cells are treated with EVs derived from CSCs suggests that these cells may be blocked in G0/G1 phase like SW620-DM cells, but the distribution in the cell cycle remains unchanged (Figure 2D). Regarding the response to chemotherapy, both types of EVs result to a 3-fold higher IC50 of treated cells (Figure 2E). Migration and invasion can lead to metastasis, a main hallmark of cancer [24]. These abilities were investigated upon EVs treatment, and no difference is observed on migration (Figure 2F) but both types of EVs improve the invasion of SW480-NM. Nevertheless, EVs derived from SW620-DM significantly enhance invasion properties that are triggered only 6 hours after treatment whereas the effect of EVs from SW620-NM is delayed to 48 hours (Figure 2G). Since the phenotype of targeted cells could be influenced by EVs derived from CSCs, the EM signatures of SW480-NM treated with EVs from the SW620 cell line

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were measured to demonstrate that the effects of EVs are not restricted to biological modifications. Cells treated with EVs from CSCs show a lower EM signature than untreated cells (Figure 2I), whereas the EM signature remains the same with EVs from differentiated cells (Figure 2H). To determine if the amount of EVs influences the treated cells in a dose-dependent manner, cells were treated twice for 72h. No dose effect is observed for cells treated with EVs from SW620-NM while the EM signature is even lower for cells treated with EVs from CSCs (Figure 2H,I). However, we observe a slight redistribution of the EM signatures with the appearance of cells with an EM below 100 MHz and 10% of additional cells with an EM higher than 300 MHz for cells treated once with EVs from SW620-NM, and 17% when treated twice (Table 2 and Figure S2B).

Table 2. Cell proportions per frequency range for SW480 treated with EVs

	SW480	**+ EVs SW620 NM**		**+ EVs SW620 DM**	
	Normal medium	**24h**	**72h**	**24h**	**72h**
0-100 MHz	0%	3%	2%	12%	15%
100-200 MHz	12%	19%	19%	31%	51%
200-300 MHz	68%	48%	42%	45%	27%
300-400 MHz	20%	30%	37%	11%	8%

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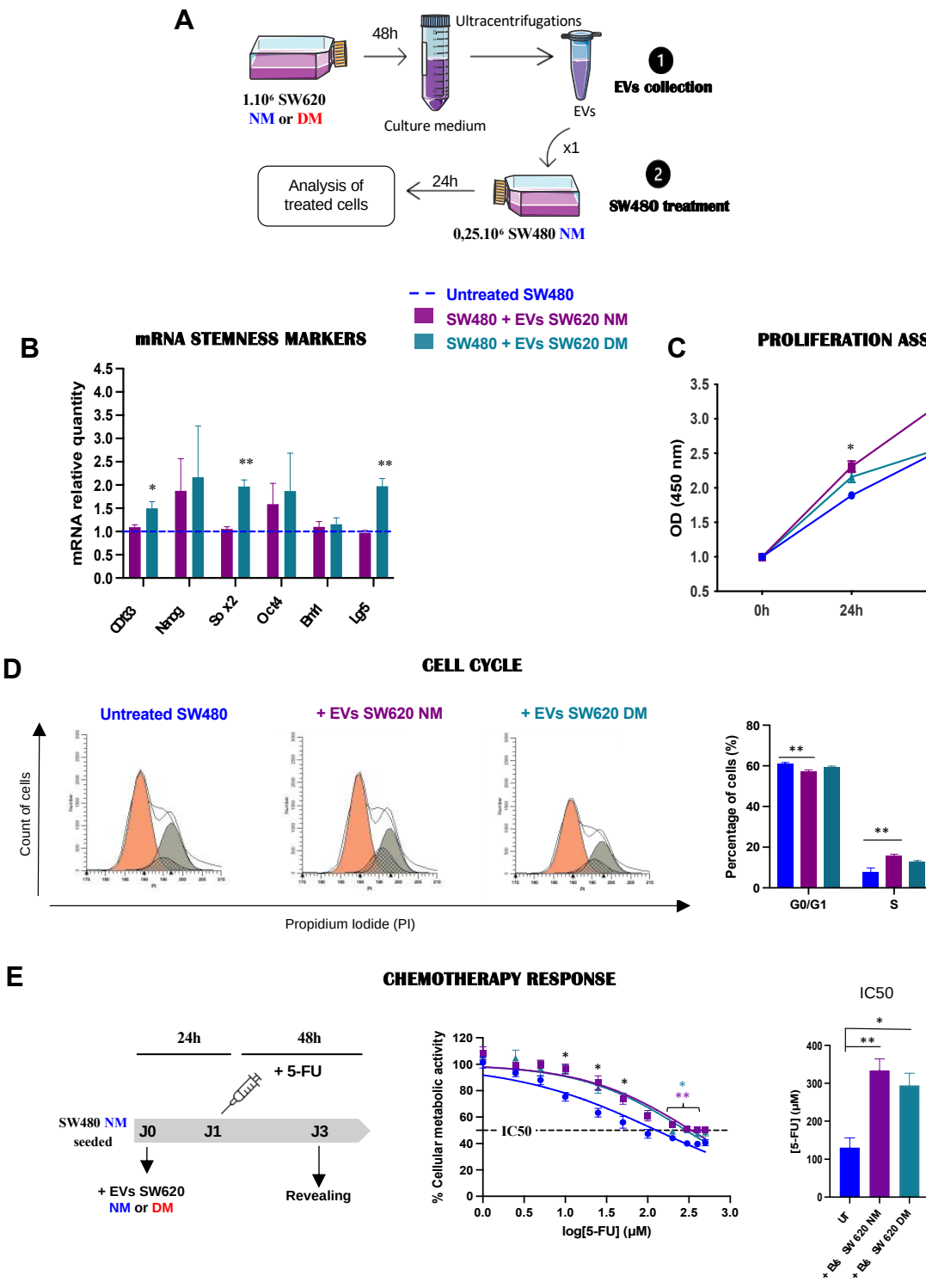

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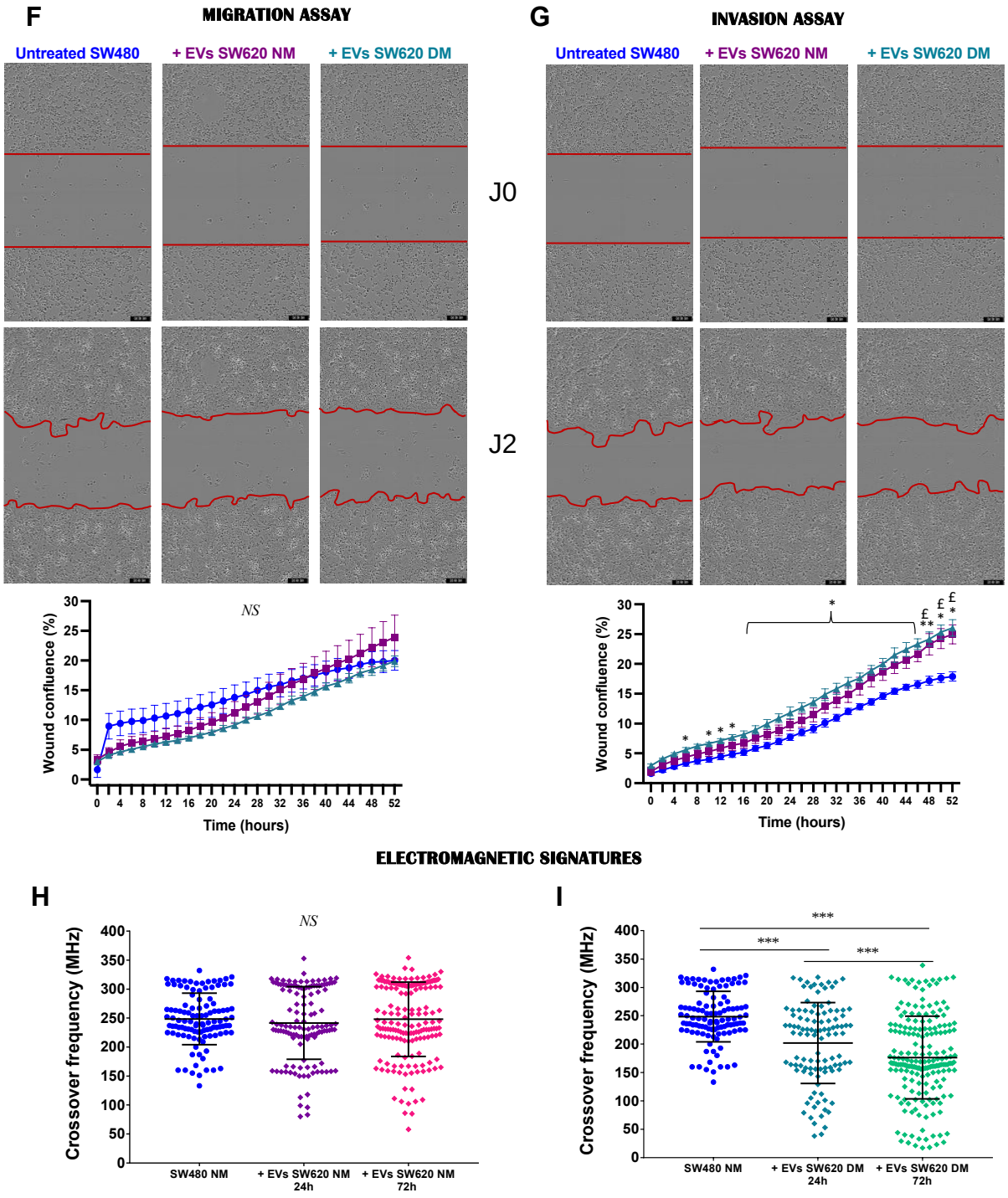

Figure 2. Impact of EVs derived from SW620 on the SW480 cell line. (A) SW480-NM cells were treated once for 24 h with EVs derived from the SW620 cell line (NM or DM cultured cells) to test an aggressiveness transfer. To assess the biological effects of this treatment, stemness related genes (B) were analyzed, as well as the proliferation rate (C), cell cycle (D) and chemotherapy response (E). The ability of cells to migrate (F) or invade (G) has also been explored (*: +EVs SW620 versus untreated SW480, £: +EVs SW620 NM versus untreated SW480) and EM signatures measured (H-I). All results are represented as mean ± SEM except EM signatures represented as mean ± SD, NS *p*-value indicate not significant result, * and £ *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 using one-way ANOVA test.

Both types of EVs influence treated cells but not at the same level. EVs from CSCs lead to the overexpression of some stemness-related genes, EVs from differentiated cells

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promote proliferation while both types enhance invasion and metabolic activity. Regarding the EM signatures, the lab-on-a-chip appears to detect a difference only for cells treated with EVs from CSCs. Since part of the biological changes triggered by CSCs derived-EVs are correlated with the change in EM signature, EM signature might be of primary importance to monitor the phenotypic transformation induced by the transfer of aggressive content from EVs.

3.3. Cancer stem cells from primary cultures of patient show a lower EM signature than their differentiated counterparts

To achieve a proof of concept in clinical conditions, our first results were transposed to tumor cells from CRC patients. Two primary cultures of patient (CPP) from primary tumors were used: CPP14 from a stage I and CPP6 from a stage IV (metastasis stage). As above, cells were cultured in the same two culture conditions and in define medium, they grow as colonospheres (Figure 3A) and overexpress all stemness related genes except *Bmi1* and *Lgr5* (Figure 3B). As for cell lines, CSCs show a lower EM signature compared to differentiated cells (Figure 3C). Compared to cell lines and CPP6, EM signature of CSCs from CPP14 is not opposite to that of differentiated CPP14, there is just a loss of cells between 200 and 300 MHz and an enrichment of cells between 0 and 100 MHz. For CPP6, 100% of differentiated cells are between 100 and 400 MHz while only 15% of CSCs are (Table 3 and Figure S3B).

Table 3. Cell proportions per frequency range for primary cultures of patient

	CPP14		**CPP6**	
	Normal medium	**Define medium**	**Normal medium**	**Define medium**
0-100 MHz	29%	55%	0%	85%
100-200 MHz	53%	45%	35%	15%
200-300 MHz	18%	0%	49%	0%
300-400 MHz	0%	0%	16%	0%

Like CRC cell lines, both CPP show the same behavior with a distinct EM signature for CSCs and always lower than cells cultured in normal medium.

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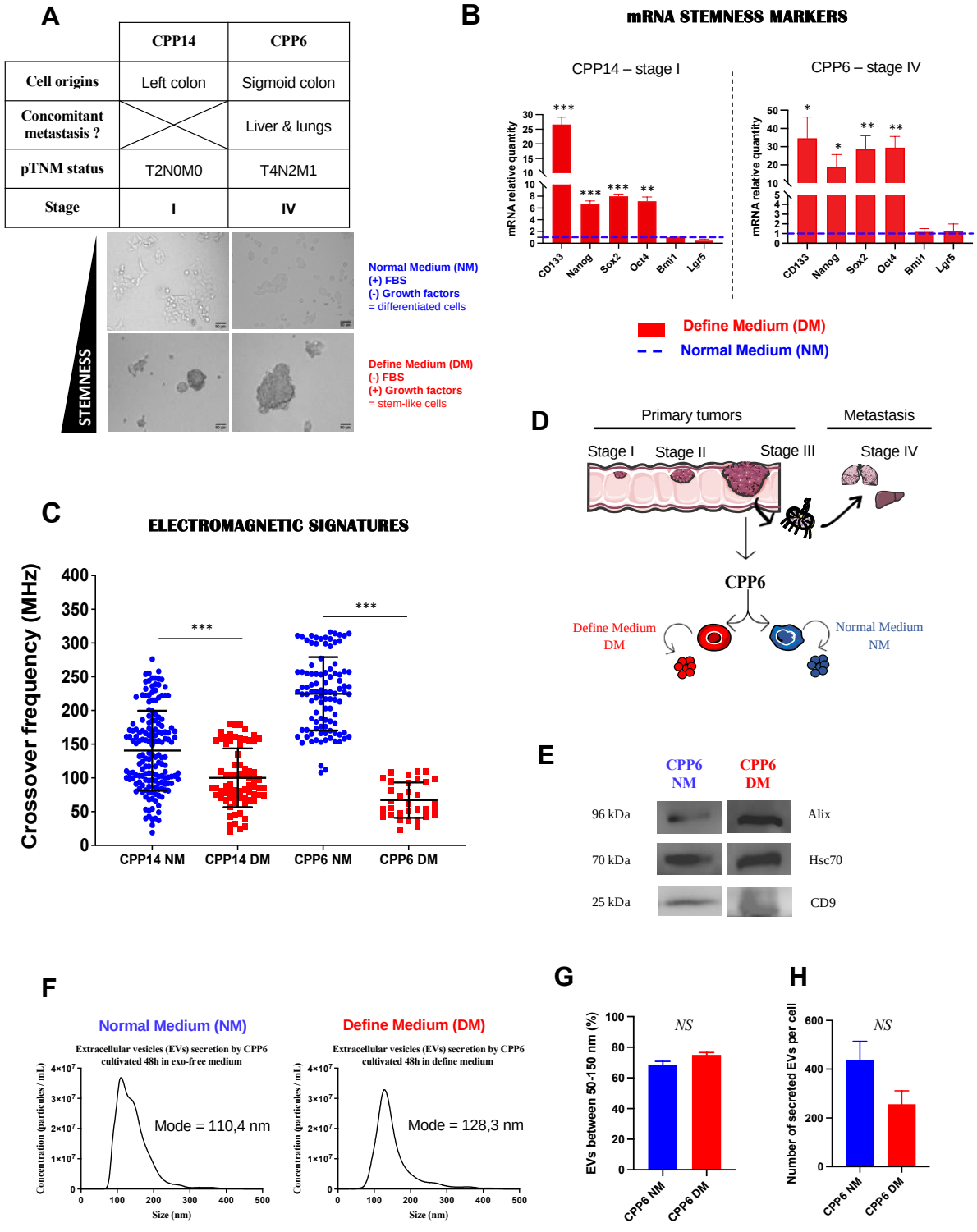

Figure 3. Characterization of primary cultures of patient and their extracellular vesicles according to culture conditions. (A) Two primary cultures of patients at different stages of CRC were cultured under NM or DM to enrich cell population in differentiated cells or in CSCs. The expression of stemness-related genes (B) was explored and EM signatures (C) measured. EVs from CPP6 (NM or DM cultured cells) were collected by ultracentrifugation (D) and expression of EVs markers was assessed by western blot (E). NTA was performed on EVs regarding size distribution (F), percentage of EVs between 50 and 150 nm (G) and number of secreted EVs per cell (H). All results are represented as mean ± SEM except EM signatures represented as mean ± SD, NS *p*-value indicate not significant result, * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 using one-way ANOVA test.

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3.4. Extracellular vesicles from primary cultures of patient can transfer aggressive properties and induce EM signature changes detected by biophysics

To demonstrate the transfer of aggressiveness due to EVs derived from aggressive cells, CPP14-NM cells were treated with EVs from CPP6 derived from a metastatic tumor (stage IV). CPP6 was cultured under normal or define medium to collect EVs by ultracentrifugation (Figure 3D). Both types of vesicles express specific EVs markers such as Alix, Hsc70 and CD9 (Figure 3E) and are between 50 and 150 nm in size (Figure 3F,G). Nevertheless, as previously described, CSCs tend to secrete less EVs than differentiated cells (Figure 3H). As with the cell lines, CPP14-NM were treated with EVs from CPP6-NM or CPP6-DM. Since the EM signature result for SW480-NM treated with EVs from SW620-DM was more pronounced with a two-step treatment for 72h, we chose to keep only this treatment (Figure 4A). When CPP14-NM are treated with EVs from CPP6-DM, they overexpress *Nanog*, *Sox2* and *Oct4*, whereas with EVs from CPP6-NM, cells significantly underexpress *CD133*, *Nanog* and *Oct4* (Figure 4B). In contrast, no significant effect of EVs on cell cycle (Figure 4C) or 5-FU response is obtained, although there is a trend toward increased metabolic activity, especially for cells treated with EVs from CSCs (Figure 4D). Strikingly, regardless of the origin of EVs used to treat CPP14-NM cells, the EM signatures increase significantly upon treatment suggesting that the biological changes (increase of CSCs-related genes) that were previously observed are not related to the change in EM signatures (Figure 4E,F, Table 4).

Table 4. Cell proportions per frequency range for CPP14 treated with EVs

	CPP14	**+ EVs CPP6 NM**	**+ EVs CPP6 DM**
	Normal medium	**72h**	**72h**
0-100 MHz	0%	2%	2%
100-200 MHz	12%	34%	37%
200-300 MHz	68%	60%	60%
300-400 MHz	20%	5%	1%

Both EVs alter the state of treated cells with a distinct higher EM signature. Biologically, we only see an overexpression of some stemness related genes for cells treated with EVs from CSCs and a trend toward increased metabolic activity.

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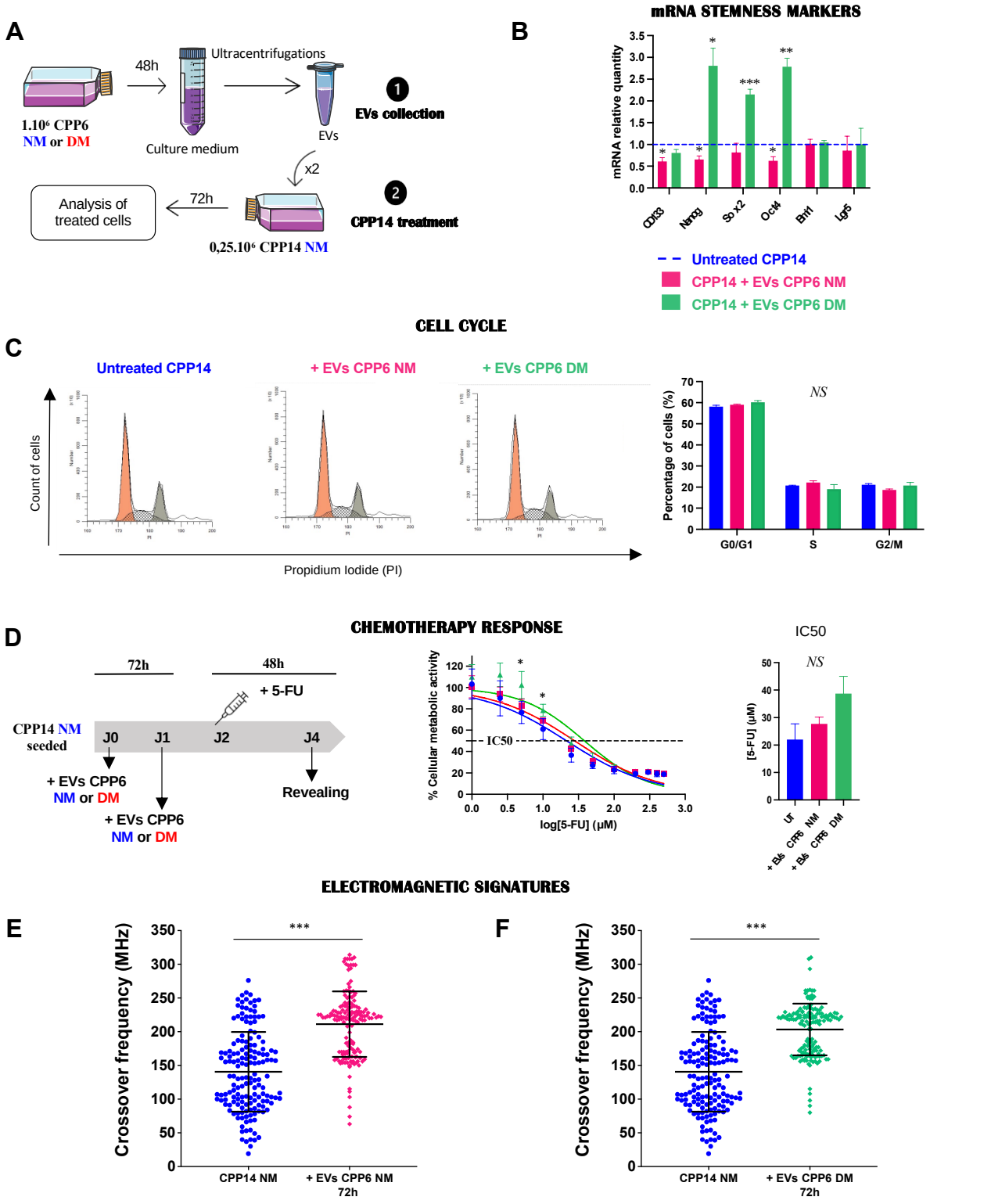

Figure 4. Impact of extracellular vesicles from CPP6 on CPP14 cells. (A) EVs from CPP6 cells (NM or DM cultured cells) were used to treat CPP14-NM cells twice and for 72h. To assess the biological effect of EVs, expression of stemness related genes (B), cell cycle (C), chemotherapy response (D) and EM signatures (E-F) were analyzed. All results are represented as mean ± SEM except EM signatures represented as mean ± SD, NS *p*-value indicate not significant result, * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 using one-way ANOVA test.

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3.5. Extracellular vesicles from the same primary culture of patient have an impact on their EM signature

Because CPP14 and CPP6 come from two CRC patients, they are genetically different and come from a different microenvironment, so we treated CPP6-NM with EVs from the same CPP but cultured in define medium, twice for 72h (Figure 5A). Unfortunately, we do not see any difference in stemness markers expression, cell cycle distribution or response to 5-FU upon treatment (Figure 5B-D). Interestingly, we observe a distinct and lower EM signature when cells are treated with EVs from CSCs (Figure 5E).

Table 5. Cell proportions per frequency range for CPP6 treated with EVs

	CPP6 Normal medium	**+ EVs CPP6 DM 72h**
0-100 MHz	0%	7%
100-200 MHz	35%	46%
200-300 MHz	49%	45%
300-400 MHz	16%	2%

No biological effects are observed when CPP6-NM are treated with EVs from CPP6-DM but the EM signature is lower. This does not mean that no biological change has occur but that those testes have not evolved with EVs intake. Moreover, this result highlights the relevance of the EM signature which is complementary to biological analysis and can reveal changes that may be missed in biology.

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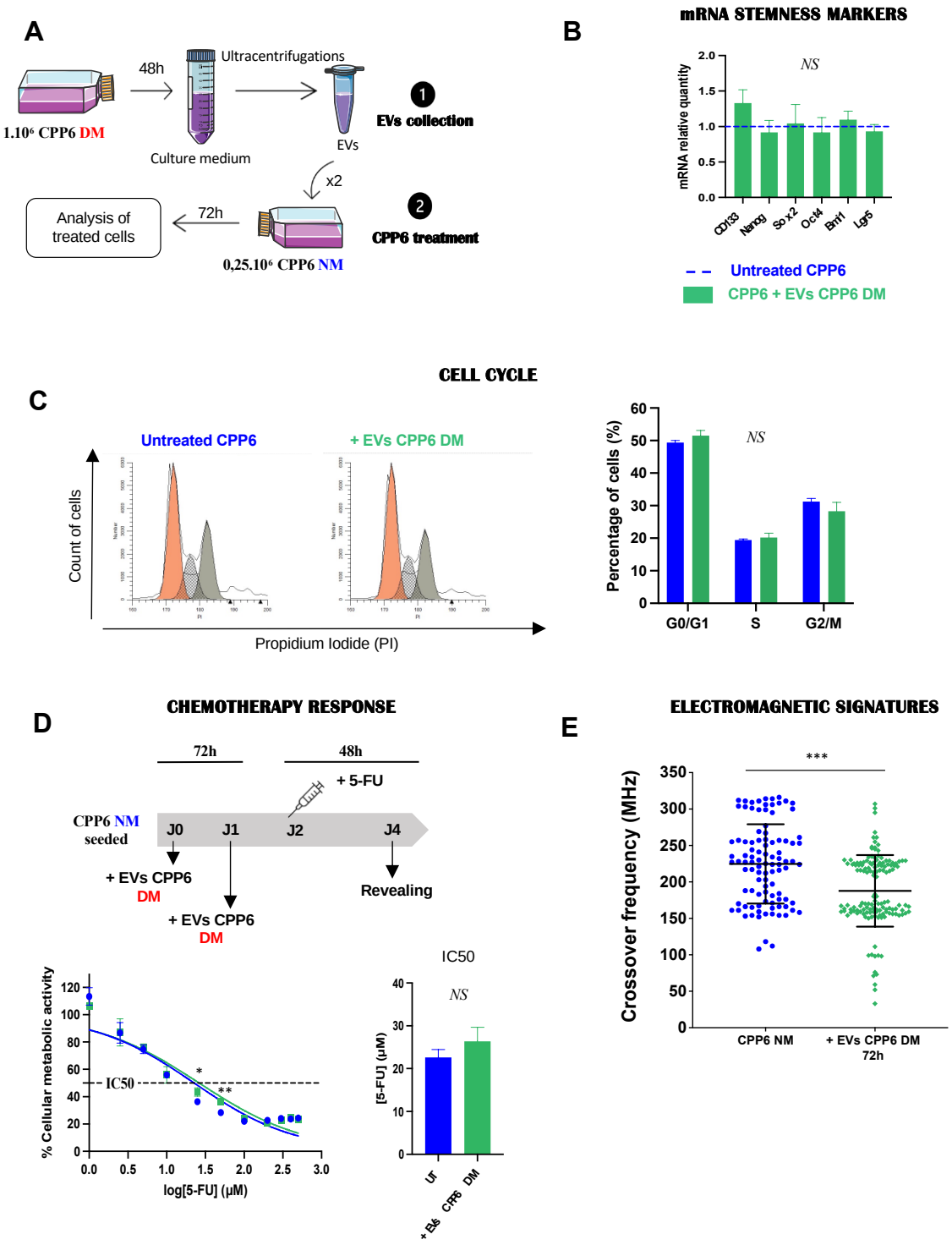

Figure 5. Impact of extracellular vesicles from CPP6 on the same type of cells. (A) EVs from CPP6 cultured under define medium were used to treat CPP6-NM cells twice and for 72h. To undergo the biological effect, stemness related genes (B), cell cycle (C), chemotherapy response (D) and EM signatures (E) were analyzed. All results are represented as mean ± SEM except EM signatures represented as mean ± SD, NS *p*-value indicate not significant result, * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001 using one-way ANOVA test.

4. Discussion

Our work highlights the relevance of our lab-on-a-chip using UHF-DEP analysis to discriminate and track cells status. Previously, the lab-on-a-chip allowed us to distinguish differentiated cells and CSCs in two types of brain tumors. In both cases, the study revealed that CSCs show a distinct and consistently lower EM signature than differentiated

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cells [21,22]. Our first goal was to transfer this technology to CRC to determine if the changes in EM signatures are solely a signature of brain tumors or if they can be applied to other tumors. Our main objective is to demonstrate that EM signatures can be modified when tumor cells are treated with EVs derived from aggressive tumor cells related to CSCs. By culturing cells in a define medium to enrich in CSCs, we observed in cell lines and in primary cultures of CRC patient that CSCs show a distinct and consistently lower EM signature than differentiated cells.

The characterization and discrimination of CSCs in a heterogeneous tumor mass remains a challenge since their discovery two decades ago. Initially, the scientific community thought that only stemness markers were needed to label a cell as a CSC. For many years, a lot of scientists have tried to find the best markers and characterization techniques to identify CSCs [5]. Since these markers are also expressed by normal stem cells, only their expression level could be useful to distinguish normal from cancer stem cells. Next, functional properties were assessed to complete the phenotypic characterization and to overcome the lack of universal CSC markers. Unfortunately, new evidence of massive cellular plasticity has emerged in recent years and has been adopted by the scientific community as a new "Hallmark of Cancer" in 2022 [24]. Due to this dynamic plasticity, it is more complicated to qualify a cell as a CSC based on phenotypic and functional characterizations. Moreover, these techniques are expensive, time consuming and require special equipment not still available in laboratories. Although these approaches are time consuming, they were necessary to ensure that cells cultured in define medium were enriched in CSCs. In contrast, UHF-DEP analysis allows us to characterize and discriminate CSCs in a short time of about one hour without prior labeling.

Second, as EVs have received increasing interest in recent years due to their ability to transfer aggressive properties to other cells or cells in the tumor microenvironment [2,9-14] (tumor cells, immune cells, stromal cells …), we wanted to test whether the lab-on-a-chip could detect a phenotypic change induced by EVs intake. We started with two cell lines from the same CRC patient. Based on EVs cargo secreted by CSCs carry more aggressive properties than those secreted by differentiated cells, SW480 cell line cultured under normal medium (SW480-NM) was treated with EVs from the SW620 cell line cultured under normal or define medium (SW620-NM or SW620-DM). Biological results confirm that stemness related genes (*CD133*, *Sox2*, *Lgr5*) were overexpressed by cells treated with EVs from CSCs. In contrast to the proliferation rate which was mainly increased in cells treated with EVs from differentiated cells. Regardless of the EVs treatment, resistance to chemotherapy increased threefold. No EM signature changes were detected between native SW480 and SW480 treated with EVs derived from SW620-NM suggesting that the EV content of the latter is unlikely to increase SW480 aggressiveness and induce the acquisition of stemness properties. On the contrary, when SW480 were treated with SW620-DM, the EM signature was significantly reduced compared to untreated SW480. Similar experiments were performed with primary CRC cells derived from two patients. However, treatment of early-stage primary cells (CPP14) with EVs from primary cells derived from a late-stage tumor (CPP6) was achieved to determine if EVs are able to transfer tumor aggressiveness as previously demonstrated in glioblastoma [9]. As expected, gene expression analysis confirms that only CPP14 treated with EVs from CPP6-DM overexpress genes related to CSCs (*Nanog, Sox2, Oct4*), suggesting that the ability of EVs to transfer aggressiveness depends on the nature of the cells that released them. The results also show that the cell cycle distribution was not altered by EVs intake compared to untreated cells and that the metabolic activity tended to increase by the supply of EVs from CSCs. Strikingly, the EM signatures increased with both EVs indicating a phenotypic change even for cells treated with EVs from differentiated cells while no biological changes were detected. This experiment suggests that the lab-on-a-chip might help to detect changes that are difficult to demonstrate by conventional biological approaches. In contrast, the last experiment showed that when CPP6-NM cells were treated with EVs from CPP6-DM, they retained similar phenotypic characteristics. However, the EM signature is significantly

reduced upon treatment, confirming that biophysical properties are relevant for detecting changes that can potentially be missed in biology. The lab-on-a-chip provides relevant results without pre-labeling and is less expensive to use than most biological techniques. It allows to distinguish CSCs from differentiated cells in several types of solid tumors but also to follow the cells to see their evolution [21, 22]. We used it to track cells treated with EVs, but it could be used on liquid biopsies to detect EVs in peripheral blood and monitor their aggressive potential even before analyzing their contents. The analysis of the EM signature of EVs would complement their biological analysis and allow assessment of tumor heterogeneity and the risk of relapse induced by the transfer of genetic and protein materials from therapy resistant cells. In addition, the development of a sorting device based on this technology could be used to isolate CSCs from the tumor mass. The isolation of CSCs will be faster and less expensive than conventional techniques based on phenotypic and functional properties of the cells without altering their properties by prior labelling. As a high number of undifferentiated cells is known to correlate with a high risk of relapse in CRC [4,7], this tool would allow potential early detection of the CSCs subpopulation in the tumor and therefore adapt the care of CRC patients.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Characterization of cell lines according to culture conditions; Figure S2: Impact of EVs derived from SW620 on the SW480 cell line; Figure S3: Characterization of primary cultures of patient according to culture conditions; Figure S4: Impact of EVs derived from CPP6 on cells.

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