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Characterization of the cell-free DNA released by cultured cancer cells

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ABSTRACT

The most prominent factor that delays the translation of cell-free DNA (cfDNA) analyses to clinical practice is the lack of knowledge regarding its origin and composition. The elucidation of the former is complicated by the seemingly random fluctuation of quantitative and qualitative characteristics of cfDNA in the blood of healthy and diseased individuals. Besides methodological discrepancies, this could be ascribed to a web of cellular responses to various environmental cues and stressors. Since all cells release cfDNA, it follows that the cfDNA in the blood of cancer patients is not only representative of tumor derived DNA, but also of DNA released by healthy cells under different conditions. Additionally, cfDNA released by malignant cells is not necessarily just aberrant, but likely includes non-mutated chromosomal DNA fragments. This may cause false positive/negative results. Although many have acknowledged that this is a major problem, few have addressed it. We propose that many of the current stumbling blocks encountered in *in vivo* cfDNA studies can be partially circumvented by *in vitro* models. Accordingly, the purpose of this work was to evaluate the release of cfDNA from cultured cells and to gauge its potential use for elucidating the nature of cfDNA. Results suggest that the occurrence of cfDNA is not a consequence of apoptosis or necrosis, but primarily a result of actively secreted DNA, perhaps in association with a protein complex. This study demonstrates the potential of *in vitro* cell culture models to obtain useful information about the phenomenon of cfDNA.

1. Introduction

Understanding the processes involved in the generation of cell-free DNA (cfDNA) is critical for inferring its role in biology and pathology, and expediting the translation of analyses to clinical practice. However, despite the apparent ubiquity of cfDNA in biofluids, the elucidation of its origin has only been a partial victory. Excluding exogenous DNA (bacterial, viral and parasitic, for instance), several possible sources and cognate mechanisms have been advocated. Firstly, it was presumed that cfDNA enters the blood following the lysis of cells on the interface between a tumor and circulation. Since it was shown that the concentration of cfDNA in the blood of cancer patients is greater than could be accounted for by the mass of cells present [1], this notion has been abandoned. Secondly, it was proposed that cfDNA may originate from the destruction of tumor micrometastases and circulating cancer cells. However, as regards the mutated cfDNA in the plasma of colorectal cancer patients, no mutated counterpart has been found in the cells of the Ficoll layer where micrometastatic cells should be present [2]. In addition, the fact that this layer is used as a normal control in microsatellite analysis indicates the lack of cancer cells in this material. On the basis of this information, the hypothesis was rendered erroneous [3]. There remain three more possible sources that may account for the occurrence of cfDNA, namely: apoptosis, necrosis, and active cellular secretion [4].

In most cases where plasma or serum DNA is subject to electrophoresis, a ladder pattern reminiscent to that obtained by apoptotic cells is seen [5]. Typically, these fragment sizes relate to multiples of nucleosomal DNA stretches, ranging from 150 to 1000 bp. Jiang et al. have reported a distinct size of 166 bp for the cfDNA of hepatocellular carcinoma (HCC) patients. This observation is consistent with their previous analyses on maternal blood [6], and similar results have been obtained by other independent groups [7–9]. Suzuki and colleagues characterized the cfDNA of healthy humans and demonstrated a size distribution between 61 and 567 bp, with a prominent peak between 161 and 170 bp. Since DNA fragments derived from necrotic cells are normally larger than 10,000 bp [10], these findings suggest an apoptotic origin for the bulk of cfDNA in diseased as well as healthy individuals [11–13]. In contrast, others have observed larger fragments of cfDNA in the blood of cancer patients, which suggests an origin from necrosis [14–18]. An argument against necrosis as the primary pathway for cfDNA release comes from observations that cfDNA levels decrease by approximately 90% following radiation therapy. If necrosis ensued, one would not expect a decline but a surge in cfDNA concentration [19,20]. However, it can also be argued that the reduction of cfDNA

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levels may be due to the inhibition of the cfDNA release pathways of healthy cells by the radiation [18].

On the other hand, many studies indicate that a significant fraction of cfDNA is derived from active cellular secretions, in which newly synthesized DNA in association with a lipid-protein complex is released in a homeostatic manner [21–25]. In 2010, Gahan termed this protein complex "the virtosome" [26]. Since then, other mobile protein complexes such as Argonaute2 [27] and high density lipoprotein [28] have been shown to be associated with nucleic acids and occur in the extracellular environment. Furthermore, cells have been shown to release extracellular vesicles, such as exosomes and prostasomes, which contain highly specific nucleic acids that often relate to the cell from which it originated [29–31].

It is evident that the origin of cfDNA is still elusive. Although most evidence suggests that the release of cfDNA is mainly a consequence of apoptosis, it is becoming increasingly clear that cfDNA may be released by more than one mechanism. It may also be of particular interest that Jiang and co-workers have detected a small amount of mitochondrial DNA and not only demonstrated that the fragments are shorter than that of nuclear DNA but also more abundant in HCC patients than in healthy subjects [32]. Since many of the confounding factors that affect the release of cfDNA *in vivo* are absent in cell cultures, we propose that many of the stumbling blocks encountered in *in vivo* cfDNA studies can be partially circumvented by *in vitro* models. Unfortunately, *in vitro* analyses of cfDNA are currently lacking. For this reason, the aim of this work was to assess the release and composition of cfDNA from cultured human osteosarcoma cells (143B) and to gauge its potential use for elucidating the nature of cfDNA. The release pattern of cfDNA was characterized over time, and the sizes of the cfDNA fragments at each of these intervals were evaluated. Apoptosis, necrosis, and the cell cycle profiles were also investigated at different time intervals using flow cytometry.

2. Materials and methods

2.1. Cell culturing and growth medium processing

The human bone cancer (osteosarcoma) cell line 143B was obtained from the American Type Culture Collection (ATCC® CRL-8303™). Cells were grown in Dulbecco's modified Eagle's medium (Hyclone DMEM/high glucose) (Thermo Scientific; #SH30243.01) containing 4 mM L-glutamine, 4500 mg/L glucose, and sodium pyruvate. It was further fortified with 10% fetal bovine serum (FBS) (Biochrom; #S0615) and 1% penicillin/streptomycin (Lonza; #DE17-602E). Cells were incubated in humidified atmosphere containing 5% CO_2 at 37 °C. Cells were seeded and cultured in bulk in 175 cm^2 cell culture flasks (Corning; #431080) (36 mL growth medium) and grown to confluency. To detach the cells from the flask surface, they were washed with phosphate-buffered saline (PBS) (Sigma; #SLBJ5110V) and then incubated for 5–10 min at 37 °C with 0.25% trypsin (Lonza; #2 MB258). After trypsinization, the cells were redistributed equally into twelve 75 cm^2 flasks, each containing 12 mL growth medium. The cells were then grown for 12 h, after which the growth medium was renewed. After this time, pairs of flasks were incubated for 4, 8, 12, 16, 20 and 24 h, respectively. At the end of incubation, the growth medium was collected in 15 mL nuclease-free tubes (Ambion; #3108090) and then centrifuged at 10,000 ×*g* for 10 min and transferred to fresh 15 mL tubes. The samples were then stored at −80 °C until extraction. In concurrence with growth medium collection, the cells were collected by trypsinization, which was then used for extraction and determination of total cellular protein content. This experiment was then also repeated for 28, 32, 36 and 40 h, respectively.

2.2. Extraction and quantification of cellular protein

Total cellular protein was liberated by sonication with the Bioruptor UCD-200 (Diagenode). Before usage, the Bioruptor was cooled to 4 °C using distilled water and ice. Samples were then vortexed and loaded into the Bioruptor wells. Sonication settings were: power, H-position (high); sonication cycle, 30 s on/30 s off; total sonication time, 5–10 cycles. Halfway through sonication, samples were removed and vortexed.

Proteins were quantified with the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies). This method relies on the design of a standard curve with proteins included in the Qubit® Protein Assay kit (0, 200 and 400 ng/μL). Briefly, working solution was prepared by adding a volume of 1 μL of Qubit™ reagent to 199 μL of Qubit™ buffer. For the standards, 10 μL was mixed with 190 μL of working solution in 0.5 mL Qubit® assay tubes. For the cellular protein samples, 2 μL was added to 198 μL of working solution. The tubes were vortexed for 3 s and then incubated for 15 min at room temperature. After incubation, the tubes were inserted into the Qubit® 2.0 Fluorometer and the stock concentration readings were noted. First, to evaluate the precision of the Qubit® 2.0 Fluorometer a stock solution of bovine serum albumin (BSA) (2 mg/mL) was diluted to 0.15, 0.20, 0.25, and 0.30 mg/mL and then quantified. It was then calculated that the Qubit is 93% accurate, which was sufficient for the purposes of subsequent experiments. This way, more tedious analyses like the BCA assay could be circumvented.

2.3. Extraction of cell-free DNA

CfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; #1502/001), according to the manufacturer's PCR clean-up instructions. Briefly, samples were removed from the −80 °C freezer and thawed at 37 °C in a temperature controlled water bath. After incubation, the samples were vortexed and centrifuged briefly. For each biological replicate, cfDNA was extracted in triplicate. For every sample, 600 μL of growth medium was mixed with 1200 μL of binding buffer. Samples were then vortexed, the entire volume of growth media was added to the spin column in small regiments, and centrifuged at 11,000 ×*g* for 1 min at room temperature. The columns were then washed twice, followed by the elution of cfDNA into 20 μL of elution buffer.

2.4. Quantification of cell-free DNA

PCR amplification of cfDNA was measured using a real-time quantitative assay for the β-globin gene. All assays were performed on a Rotor-Gene Q detection system (Qiagen) using a 72 well ring-setup. The reaction mixture consisted of 2 μL DNA and 23 μL master mix, which was composed of 8.1 μL H_2O, 12.5 μL TaqMan Universal MasterMix (Life Technologies; #1502032), 0.4 μL of 10 μM dual fluorescent probe 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3′, and 1 μL of 10 μM forward and reverse primers, respectively. The primers used were: F1, 5′-GTG CAC CTG ACT CCT GAG GAG A-3′, and R1, 5′-CCT TGA TAC CAA CCT GCC CAG-3′. These probe and primers were synthesized by Integrated DNA Technologies (IDT, Whitehead Scientific). PCR conditions were set to: 95 °C for 10 min, followed by 45 cycles of 15 s denaturation at 95 °C, 1 min annealing at 60 °C, followed by 30 s extension at 72 °C. Sequence data of β-globin is attainable from GenBank (accession number: U01317). The absolute concentration of the target gene was calculated by using a standard curve. In this study, a standard curve was generated using five-fold serial dilutions of genomic DNA (50,000, 5000, 500, 50 and 5 pg/μL). Each biological replicate was quantified in duplicate, and triplicates of the standard curve were included in each run (only assays with R^2 values >0.99 for the standard curve were used).

2.5. Fragment size evaluation of cell-free DNA

The size of cfDNA extracted at the different time intervals were analyzed by capillary electrophoresis (CE). This was done on a microchip using the High Sensitivity DNA kit and an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) equipped with Expert 2100 software. The assay was performed according to the instructions

provided by the manufacturer. After the nucleic acids are separated analogously to CE, they are normalized to a ladder and two DNA markers, and are represented as a virtual band. The software then automatically calculates the size of each band, and is represented as an electropherogram.

2.6. Flow cytometric detection of apoptosis and necrosis using the FITC annexin V assay

The BD Annexin V FITC assay (BD Biosciences) was utilized to determine whether the apoptotic profile of 143B cells differ at specific time intervals. 143B cells were washed twice with PBS and resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL at different time intervals (4, 12, 24 and 40 h). 100 μL of this solution was then transferred to 12 × 75 mm round bottom (conical) tubes (BD Biosciences) and mixed with 5 μL of FITC Annexin V and 5 μL of propidium iodide (PI). Samples were then vortexed and incubated in the dark at room temperature for 20 min. After incubation, 100 μL of 1X binding buffer was added to each tube and then analyzed using the BD FACSVerse System and BD FACSuite Software. For a positive apoptosis control, 143B cells were treated with 650 μM hydrogen peroxide for 4 h. Unstained cells were included for settings optimisation of the flow cytometer.

2.7. Flow cytometric detection of apoptosis with the TUNEL assay

An APO-BrdU™ TUNEL assay kit (Molecular Probes, Invitrogen) was used for the detection of DNA fragments as recommended by the manufacturer, with slight modifications. Briefly, an amount of 2×10^6 cells were suspended in 0.5 mL PBS. Cells were counted using the scepter (Merck, Millipore) and fixed by adding the cell suspension to 5 mL of 1% (*w*/*v*) paraformaldehyde (Fluka) on ice for 15 min. After fixation, the cells were centrifuged for 5 min at 300 ×*g*, and the supernatant was discarded. The cells were washed twice with 5 mL of PBS, and the pellet was resuspended in 0.5 mL of PBS followed by 5 mL of ice-cold 70% (*v*/*v*) ethanol. The cell suspensions were stored in a −20 °C freezer until analyzed for fragmented DNA. On the day of analysis, the samples were resuspended in PBS and 1 mL aliquots (approximately 1×10^6 cells) were removed and placed in 12 × 75 mm flow cytometry tubes (BD Biosciences). The cell suspensions were then centrifuged for 5 min followed by the removal of the remaining ethanol by aspiration. The cell suspensions were washed twice with 1 mL of wash buffer (Apo- BrdU™ TUNEL assay kit), and the supernatants were removed by aspiration. Then, the pellet was resuspended in 50 μL of freshly prepared DNA-labeling solution (Apo-BrdU kit) containing 10 μL reaction buffer, 0.75 μL of TdT enzyme, 8.0 μL of BrdUTP and 31.25 μL dH_2O. The cells were incubated in the DNA-labeling solution for 60 min at 37 °C. The cells were gently shaken every 15 min to keep it in suspension. At the end of the incubation time, the cells were washed twice in 1 mL of rinse buffer (Apo-BrdU kit) by centrifugation at 300 ×*g* for 5 min at room temperature.

The supernatants were removed by aspiration, and the pellet was resuspended in 100 μL of freshly prepared antibody staining solution containing 5 μL Alexa Fluor® 488 dye-labeled anti-BrdU antibody and 95 μL rinse buffer. Cells were incubated in this solution for 30 min at room temperature in a darkened room. After incubation, 0.5 mL of propidium iodide/RNase A staining buffer (Apo- BrdU™ TUNEL assay kit) was added. Cells were analyzed for DNA fragmentation using the BD FACSVerse System and BD FACSuite Software.

2.8. Flow cytometric cell cycle analysis

Cells were cultured as described in section 2.1. After 12 h of incubation, the point where medium was renewed, 5-ethynyl-2′-deoxyuridine (EdU) was added to a final concentration of 10 μM to each flask and

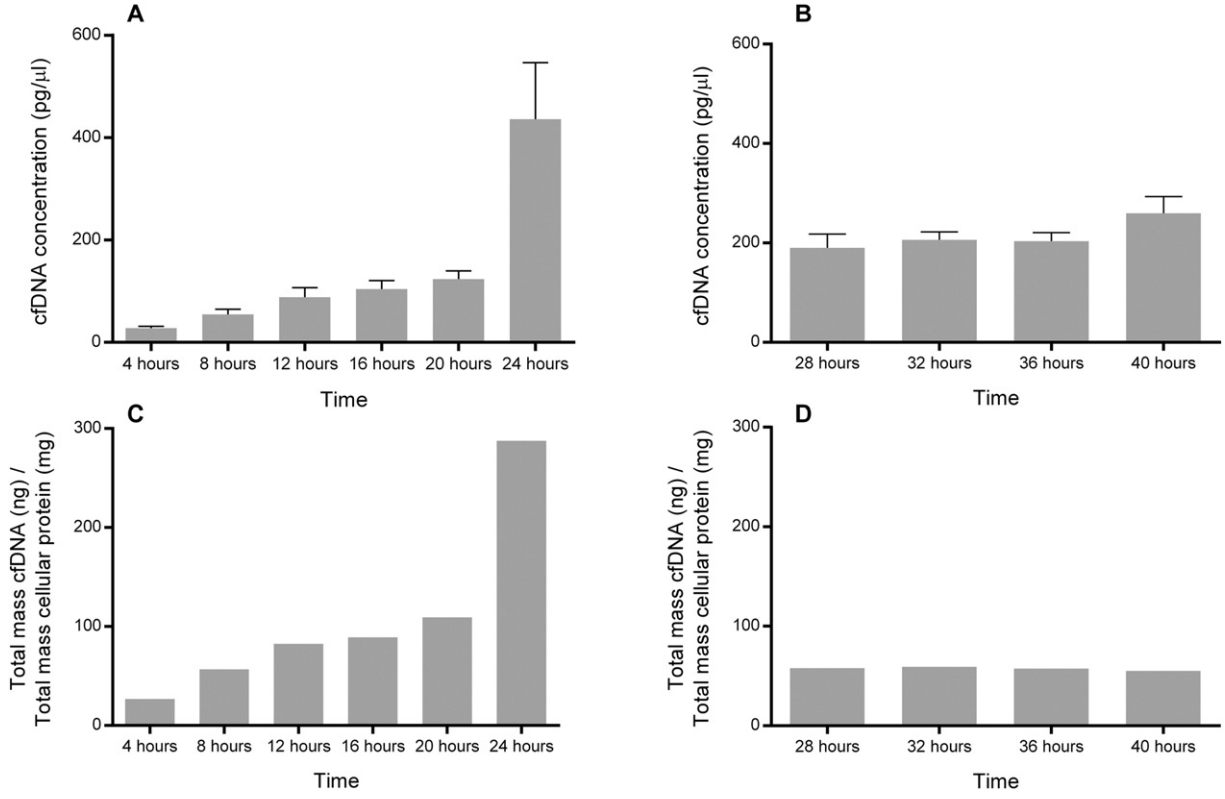

Fig. 1. Time-course characteristics of cfDNA released from 143 B cells. A. Bar graph showing the amount of cfDNA released by 143 B cells after 4–24 h of incubation following medium renewal. B. Bar graph showing the amount of cfDNA released by 143B cells after 28–40 h of incubation following medium renewal. The value of each bar represents the amount of cfDNA in 1 μL of a total of 20 μL of elution buffer obtained from 600 μL of growth medium. C & D Represents the amount of cfDNA released at each time-point normalized in terms of total cellular protein.

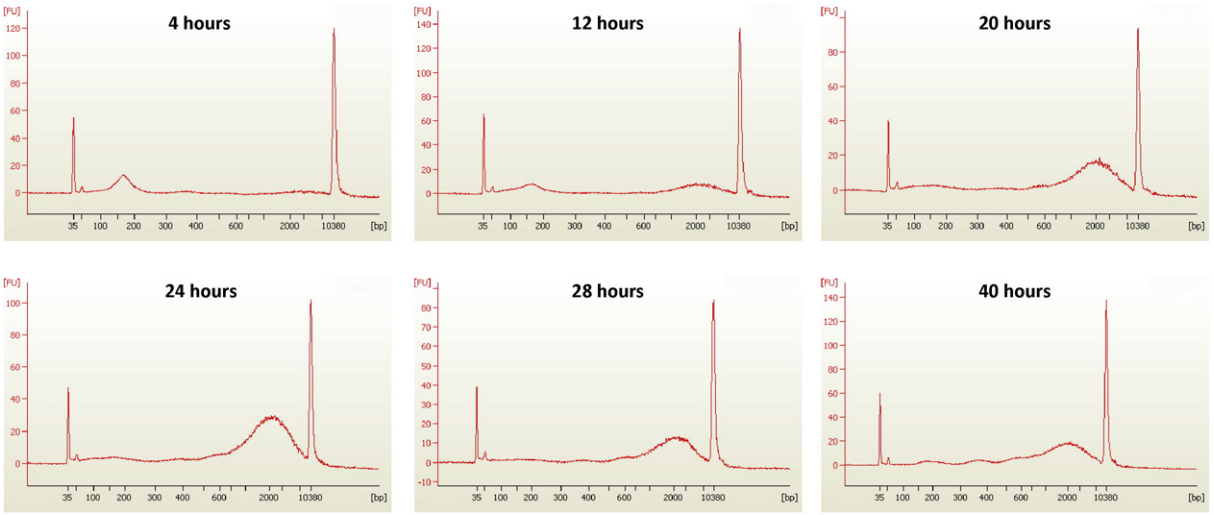

Fig. 2. Capillary electropherograms showing the size of cfDNA isolated after incubation at various times following medium renewal. In each electropherogram two major peaks can be seen, one at 35 bp and one at approximately 10,000 bp. These peaks correspond to the two size markers. The relative fluorescence of these markers is then used to calculate the size of the unknown samples. Thus, any deviation from the baseline, excluding the markers, indicates the size of cfDNA.

mixed well. Detection of EdU incorporation into DNA was performed with the Click-iT® Edu Alexa Fluor® 488 Cell Proliferation Kit (Molecular Probes, Invitrogen) according to the instructions of the manufacturer. Briefly, harvested cells were washed once with 1% BSA in PBS and pelleted by centrifugation at 500 $\times g$ for 5 min at room temperature. The supernatant was removed and the pellet resuspended at a density of 1×10^7 cells/mL in PBS. To fixate cells, 100 μL of the cell suspension was mixed with 100 μL of Click-iT® fixative agent in a 12 × 75 mm flow tube and incubated in a dark room for 15 min. After incubation, cells were washed with 3 mL of 1% BSA in PBS, pelleted, and the supernatant was discarded. The pellet was then dislodged, resuspended rigorously, and mixed with 100 μL Triton® X-100-based permeabilization reagent and then incubated for 30 min at room temperature in a dark room. After incubation, cells were washed with 3 mL of 1% BSA in PBS, pelleted, and the supernatant was discarded. The Click-iT® EdU reaction cocktail was prepared as recommended by the manufacturer and 5 mL was added to each cell pellet. Samples were incubated for 30 min at room temperature in a dark room and then washed with 3 mL of 1% BSA in PBS and pelleted. After the supernatant was discarded 0.5 mL of 1% BSA in PBS was added to the pellet. For staining of cellular DNA, 5 μL of Ribonuclease (RNAse A) was first added to each sample and mixed, after which 2 μL of the Click-iT® EdU CellCycle 488-red (7-AAD) and 2 μL of Propidium iodide was added to each sample and mixed again and then incubated at room temperature for 30 min. After incubation, cells were transferred to ice until analysis with a BD FACSVerse System and BD FACSuite Software.

2.9. Flow cytometry instrumentation and data analysis

Fluorescence of single cells was measured by a FACSVerse™ bench top flow cytometer equipped with blue (488 nm) and red (640 nm) lasers. Events were acquired on BD FACSuiteTM software, version 1 (Becton & Dickson, Mountain View, CA, USA). Amplification of signals were carried out at logarithmic scale and measurement of events plotted on forward light scatter (FSC), side light scatter (SSC), green fluorescent (FL1) and red fluorescent (FL2). A gating strategy was used to distinguish the fluorescently labeled cell population from unstained populations. A total of 10 000 events as defined by gates, were counted. Positive as well as stained and unstained negative controls were included in each analysis. The FACSVerse were calibrated using FACSuite™ CS&T research beads. Data was processed with FCS Express V4 software (De Novo Software, CA, USA). Gates were set on the dot plot FSC and SSC during analysis. The geometric means of fluorescence for all the parameters were calculated from the respective histograms or two parameter fluorescence dotplots.

Fluorescence results were expressed in arbitrary units as mean fluorescence intensity (MFI) and cell counts were expressed as percentage. Flow cytometry samples were analyzed blind, and the researcher responsible for analyzing flow cytometry data was blinded to the objectives and outcome of the experiments.

2.10. Treating growth medium with denaturing agents

cfDNA was isolated as described in section 2.3, except prior to extraction the growth medium was incubated with SDS (0.05%), proteinase K (1.5 mg/mL), and a combination of the two for 30 min, respectively. In the cases where SDS was used, buffer NTB was used instead of buffer NTI. As the kit makes no suggestions regarding the use of proteinase K, buffer NTI was used in this case. Since the medium of all the samples in the previous experiment was snap-frozen, an experiment was done to determine whether the high yields of cfDNA are wholly or only partly due to the addition of proteinase K, and not partly due to snap-freezing. To do this, four different scenarios were compared: (1) cfDNA was extracted from growth medium directly after collection, (2) growth medium was treated with proteinase K immediately after collection, followed by cfDNA extraction, (3) growth medium was snap frozen

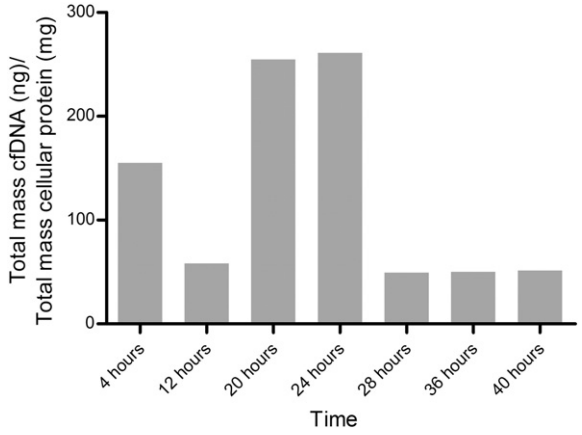

Fig. 3. Bar graph showing the amount of cfDNA released by 143B cells after 4–40 h of incubation following medium renewal. CfDNA was quantified by capillary electrophoresis using an Agilent 2100 Bioanalyzer.

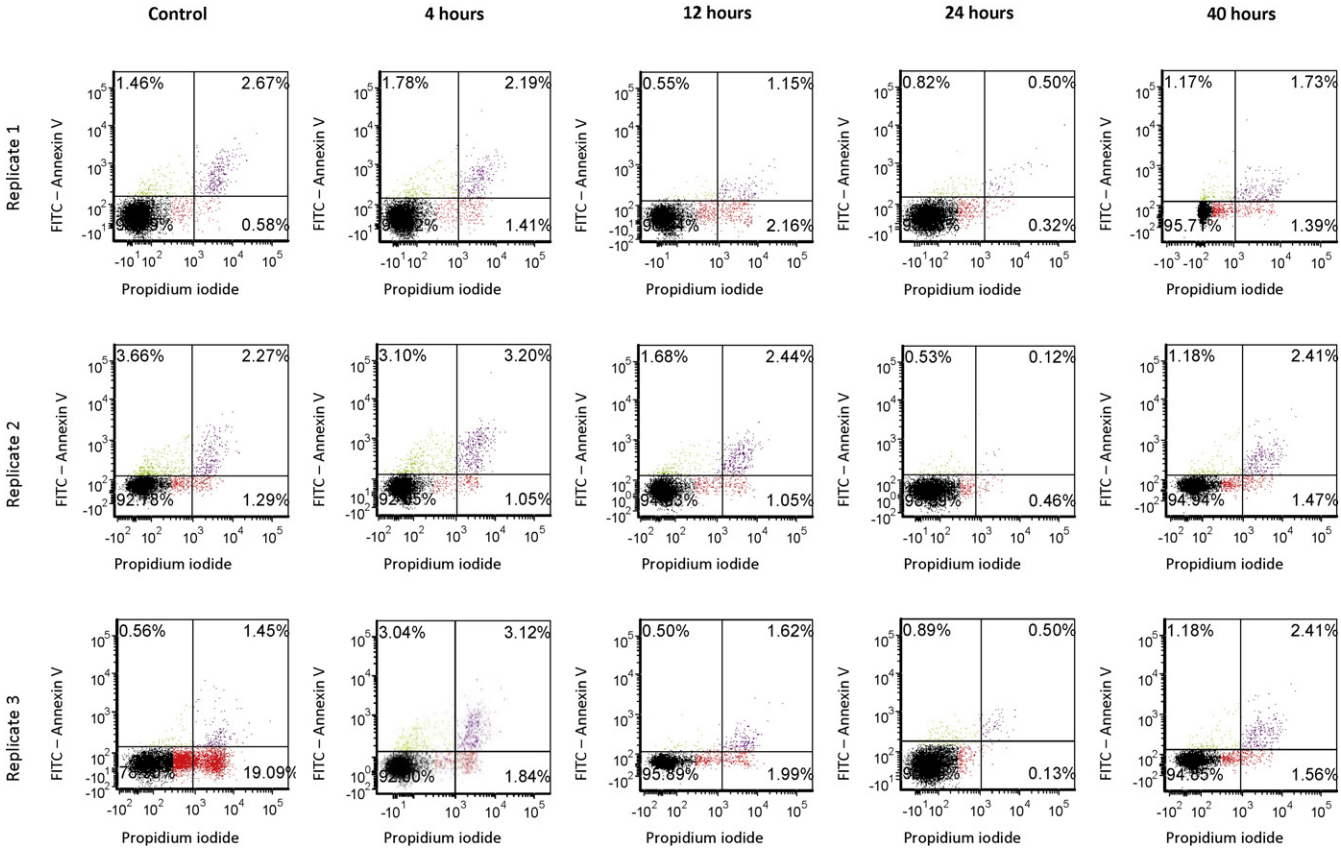

Fig. 4. Dot plots illustrating the amount of apoptotic and necrotic 143 B cells after different times of incubation. Flow cytometric analysis was performed on 143 B cells staining simultaneously with annexin-V-FITC and propidium iodide (PI). For each graph, the lower left quadrant represents viable cells, the upper left quadrant represents apoptotic cells, the upper right quadrant represents cells in late stage apoptosis, and the lower right quadrant represents necrotic cells. Every dot corresponds to a single cell. Replicates 1, 2 and 3 of the control column represent 143 B cells treated with 150, 300 and 600 mM of hydrogen peroxide, respectively.

before cfDNA extraction, and (4) growth medium was snap frozen and then thawed and treated with proteinase K prior to extraction.

3. Results and discussion

The origin of cfDNA is still elusive. There are several possible sources that may account for the occurrence of cfDNA, including apoptosis, necrosis, and active cellular release. In this study, the release of cfDNA from cultured cells was evaluated in order to gauge its potential use for elucidating the nature of cfDNA.

3.1. Kinetics of cfDNA release

Release of cfDNA was characterized over time (Fig. 1 A & B). After growth medium renewal, the amount of cfDNA increased incrementally, notably peaked after 24 h, and plateaued at a much lower level thereafter. Since the amount of cells increase over time, this is not surprising. However, when the values were normalized in terms of total cellular protein the tendency did not change, suggesting that more cfDNA is released per cell (Fig. 1 D & C). In the case where capillary electrophoresis was used to quantify cfDNA, the same pattern was obtained (Fig. 3). This phenomenon can be seen in at least three other reports in the literature [33–35]. Morozkin et al. assessed the kinetics and composition of cfDNA released by A431 (epidermoid carcinoma) and HeLa (cervical cancer) cells over 48 h. It was demonstrated that cfDNA concentration increases over time, and electrophoretic analysis indicated a molecular weight between 0.4 and 10 kilobase pairs. This suggested that the cfDNA is neither from apoptotic nor necrotic origin [34]. A few years later, they repeated the experiment but also assessed the DNA released by primary HUVEC (human umbilical vein endothelial) cells and mycoplasma infected

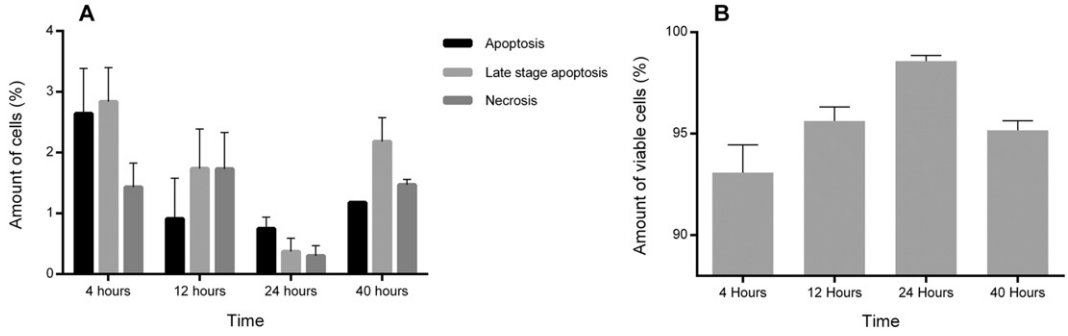

Fig. 5. Summary of the processed data obtained by the FITC Annexin V flow cytometric assay. A illustrates the percentage of apoptotic, necrotic, and late-stage apoptotic 143 B cells at the different time-points. B illustrates the percentage of viable 143 B cells at each time-point. These values represent the averages of the replicates showed in Fig. 3. Error bars indicate standard deviation.

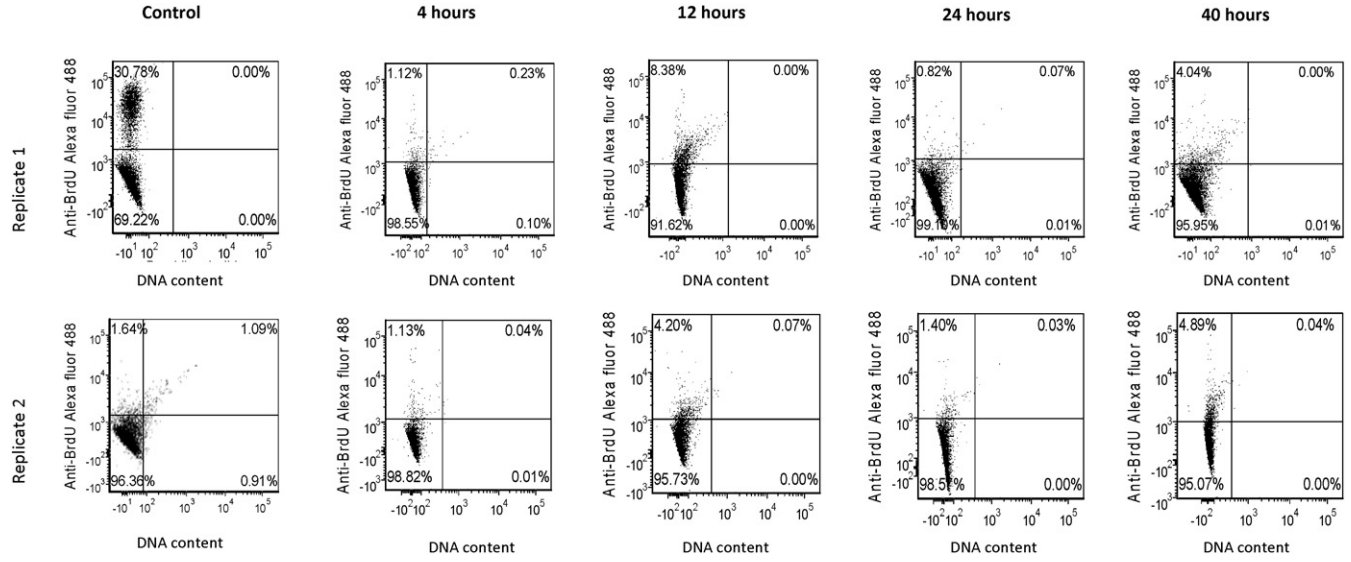

Fig. 6. Dot plots illustrating the detection of DNA fragmentation in 143B cells at different times as analyzed with the TUNEL assay by flow cytometry. The fluorescence of DNA content is plotted against Anti-BrdU alexa fluor 488 incorporation. Replicate 1 shows the positive control and replicate 2 shows the negative control. For each graph, the lower left quadrant represents viable cells, while the upper left quadrant represents apoptotic cells.

HeLa cells, showing that cfDNA concentration increased during the lag and beginning of the exponential growth phase. They considered this to be evidence for the active release of cfDNA [33]. Choi and coworkers designed a similar experiment for Jurkat (human T lymphocyte) and U937 (pleural effusion) cells. However, in addition to normal conditions, apoptosis and necrosis were induced in both cell lines. In the case of apoptosis, cfDNA concentration increased over time and also peaked rather dramatically after 24 to 48 h. CfDNA from untreated cells increased only slightly at these times. On the other hand, necrotic cells showed a decline of cfDNA over time. These results suggest that the release of cfDNA is mainly a consequence of apoptosis, but also suggests that cfDNA may be released by more than one mechanism [35].

3.2. Size of cfDNA

To examine the size of cfDNA isolated at the different time points, samples were subject to capillary electrophoresis (Fig. 2). After 4 h of incubation a prominent peak at 166 bp was observed, which is consistent with the literature and indicates an origin from apoptosis [32]. However, this peak diminished incrementally and disappeared after 24 h when a new peak of approximately 2000 bp dominated the scene. After 40 h of incubation, the cfDNA resembles multiples of nucleosomal repeats, with peaks forming at approximately 160, 340 and 540 bp, also suggesting an origin from apoptosis. These observations demonstrate a clear correlation between an increase in the release of cfDNA and the occurrence of higher molecular weight DNA. As far as we know, this distinct size of ~2000 bp has not yet been reported. This is peculiar, because its size suggests that it is neither from apoptotic nor necrotic origin.

3.3. Measurement of apoptosis and necrosis

To corroborate the observations made by electrophoretic analysis, and to help elucidate the origin of the cfDNA occurring at the different time points, the cells at each of the times correlating with the time-course study was analyzed for apoptosis, necrosis and cell cycle phase using three different flow cytometric assays. The FITC Annexin V assay

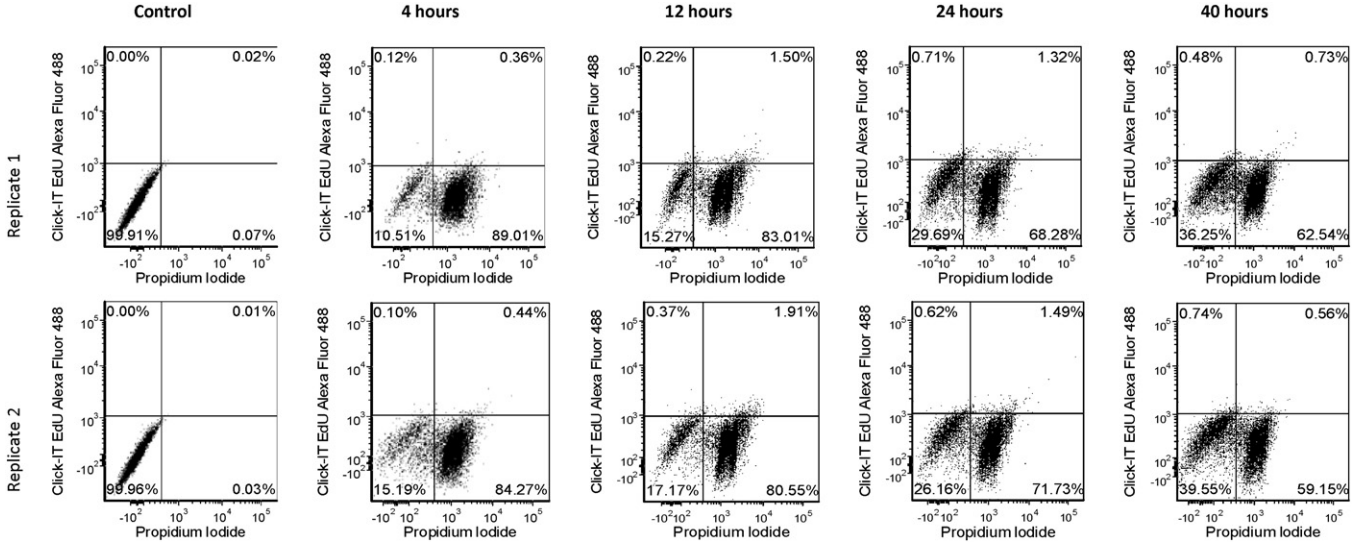

Fig. 7. Dot plots illustrating the proliferation of 143B cells at different times as analyzed with the Click-it EdU kit by flow cytometry. The DNA content (position in the cell cycle) is plotted against Edu alexa fluor 488 staining.

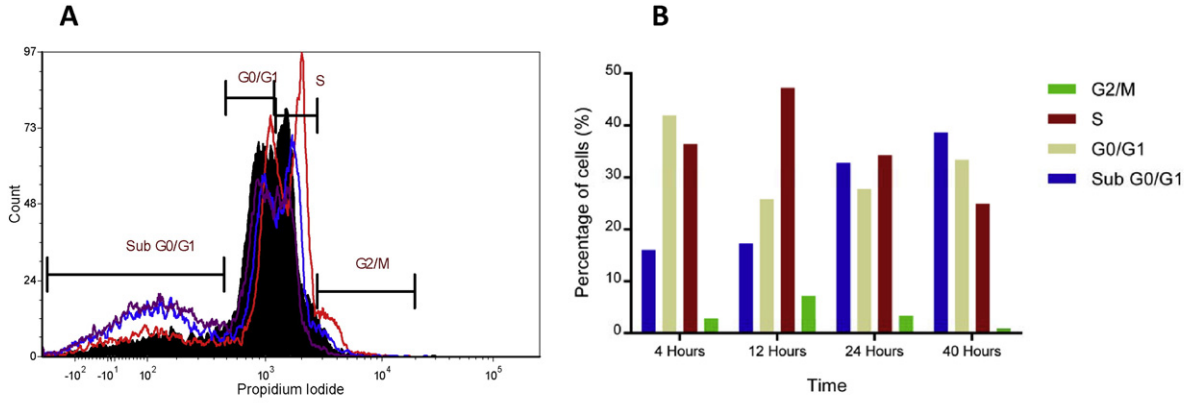

Fig. 8. Histogram and bar graphs showing the cell cycle distribution of 143 B cells at different points in time. In A, 4, 12, 24 and 40 h are represented by black, red, blue and purple, respectively. B shows the percentage cells in each phase as analyzed by FCS Express Multicycle. The high percentages of cells in the G0/G1 and S-phases are indicative of a fast growing cell population.

revealed that a fraction of cells are apoptotic after 4 h, whereas 24 h showed almost no apoptosis or necrosis (Figs. 4 & 5). Barring the time point of 4 h, these results were corroborated by the TUNEL assay (Fig. 6).

3.4. Cell cycle assessment

The assay used to measure cell proliferation reveals that, after 24 h of growth following medium renewal, there is a decline of cells in the S phase and significant increase of cells in the sub G0/G1 phase (Figs. 7 & 8). This suggests that the increase of cfDNA after 24 h of incubation is not associated with the process of DNA replication. The possible origin of cfDNA, in association with lipid-protein complexes (virtosomes), has been reviewed by Gahan where he reported literature showing that cfDNA in this form is released from both dividing and differentiated cells [26]. Since differentiated cells tend to be held in either the G0 or G1 phases of the cell cycle, Gahan suggested that this could be the realm in which cfDNA is synthesized, and that it is not likely related to mitotic DNA synthesis [26]. Our results support this notion.

3.5. Further characterization of cfDNA

To determine whether the cfDNA released from 143 B cells after 24 h of incubation could be similar to the virtosomes described by Gahan, growth medium was treated with denaturing agents prior to cfDNA extraction, and were compared to untreated samples (Fig. 9). In all cases, the yield of cfDNA was increased considerably by the addition of denaturing agents. Since it has been previously described that cfDNA is dissociated from the lipid-protein complex by freezing and thawing or by treatment with deoxycholate [36], the results of this experiment suggest that cfDNA is associated with proteins. Whether these proteins are simply nucleosomes, virtosomes, or extracellular vesicles remains unclear, and requires further experimentation.

4. Conclusion

Most *in vivo* studies report that the occurrence of cfDNA is associated with apoptosis or necrosis. However, the results obtained by this study suggest that the release of cfDNA from cultured 143 B cells after 24 h of incubation is not a consequence of apoptosis, necrosis, or a product of DNA replication, but primarily a result of actively released DNA, perhaps in association with a protein complex. Although other research groups have arrived at the same conclusion, very little attention has been drawn to this discovery.

The literature suggests that the purpose of actively released DNA is for the DNA to act as an intercellular messenger of sorts [37,38]. This is achieved by entering target cells and either integrating into the host genome, or to transiently elicit a biological effect [26]. Thus far, to mention the most prominent, the transfer of cfDNA between different cells has been implicated in the induction of tolerance against detrimental substances [40], immunomodulation [41], and the development of metastasis [42,43]. The latter has been demonstrated to occur *in vitro* [44] and *in vivo* [43]. In addition, results by Bergsmedh et al. suggest that the lateral transfer of cfDNA can not only mediate metastasis, but also generate the genetic instability necessary for malignancy [45]. On the other hand, Garcia-olmo and colleagues have recently shown that cfDNA derived from non-dividing healthy cells can halt tumor growth [46]. A recent review lends support to these findings and further illustrates the functional diversity of cfDNA [47]. It was found that apoptotic

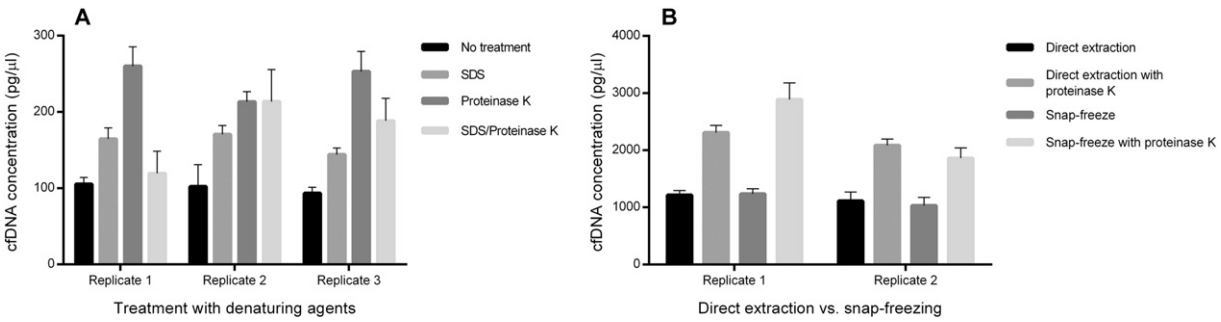

Fig. 9. The effect of treating growth medium with denaturing agents prior to extraction of cfDNA. A. The effect of the addition of denaturing agents. The use of SDS (0.05%), proteinase K (1.5 mg/mL), and a combination of the two were evaluated. In the cases where SDS was used, buffer NTB was used instead of buffer NTI. B. A comparison of cfDNA yield between direct extraction and snap-freezing, with and without the use of proteinase K. It is clear that, when the averages are calculated, snap-freezing does not increase the amount of cfDNA extracted and that the high yields can be ascribed solely to the addition of proteinase K.

death of stressed (irradiated) cells result in the release of oxidized cfDNA into circulation. This may serve as a biomarker of stress. In addition, it was demonstrated that these oxidized cfDNA molecules may confer survivability to other malignant cells by augmenting their resistance to radiation therapy. This is known as the bystander effect [39].

We further suggest, keeping in mind that cfDNA is readily transferred between cells, the possibility that cfDNA may play a role in the regulated generation of somatic genome variation. In addition, DNA has been detected in extracellular vesicles released by prostate cells that have been shown to interact with sperm cells [48,49]. This suggests that cfDNA may also be a potential bridge for information transfer between the soma and the germ line, which is still considered to be an unbridgeable chasm.

Although many aspects of the phenomenon of cfDNA messaging remain obscure, it is clear that the possibilities are intriguing. The observed messaging functions of cfDNA defy our traditional views of intercellular communication, especially relating to pathology. Furthermore, if cfDNA plays a role in somatic genome variation and transgenerational inheritance, it would strongly challenge the neo-Darwinian synthesis of evolution. Since cells in culture are isolated from the confusing variables encountered *in vivo*, *in vitro* characterization of the mechanisms that mediate the reactions of cfDNA should provide useful insight into its origin. This information is crucial for expediting the translation of *in vivo* cfDNA analyses to clinical practice, since it will provide novel markers for the diagnosis and therapy monitoring of several diseases, while ultimately leading to the treatment of certain diseases.

There are many ways in which cfDNA can be further characterized *in vitro*. For instance, the effect of cfDNA transfection on recipient cells can be evaluated by measuring cell viability, followed by metabolic and gene expression profiling. In addition, intracellular trafficking of these molecules can be traced by radioactive labeling, or alternatively by non-radioactive methods as described by Mansfield et al. [50]. Since cfDNA is often associated with extracellular vesicles such as exosomes, its cellular release and uptake could be visualized by live-cell microscopy using lipophilic dye and amino-reactive fluorophore labeling. This technique has been described by Tian et al. [51]. Arguably the best way to understand the molecular production of cfDNA is to examine its composition, in other words its sequence information. In the last couple of years, only a small number of papers have been published on the large scale sequencing of cfDNA. However, the objective of these studies was not to fully characterize cfDNA, but rather the sequence amplification of products to identify a small number of genes, while others have focused on methylation specific sequencing, or on the determination of molecular size [32,52–54]. Moreover, these studies were based on blood samples. The results of this study suggest that sequencing of cfDNA released by cultured cells may prove to be a useful way to circumvent the variables encountered in *in vivo* studies that may produce confusing sequencing results. In conclusion, the results of the current study demonstrate the potential of *in vitro* cfDNA analysis to aid in the elucidation of the nature of cfDNA.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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