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Reports

BioTechniques

Methods for simultaneous and quantitative isolation of mitochondrial DNA, nuclear DNA and RNA from mammalian cells

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ABSTRACT

The aim of this study was to assess two protocols for their capacities to simultaneously isolate RNA, mtDNA and ncDNA from mammalian cells. We compared the Invitrogen TRIzol-based method and Qiagen DNeasy columns, using the HepG2 cell line and human primary glioblastoma stem cells. Both methods allowed the isolation of all three types of nucleic acids and provided similar yields in mtDNA. However, the yield in ncDNA was more than tenfold higher on columns, as observed for both cell types. Conversely, the TRIzol method proved more reproducible and was the method of choice for isolating RNA from glioblastoma cells, as demonstrated for the housekeeping genes *RPLP0* and *RPS9*.

METHOD SUMMARY

Here we compare two methods – Invitrogen TRIzol reagent and Qiagen DNeasy columns – for simultaneously extracting RNA, mtDNA and ncDNA from mammalian cells.

KEYWORDS:

mammalian cells • mitochondria • mitochondrial DNA (mtDNA) • nuclear DNA (ncDNA) • RNA

Mitochondria are endowed with essential cellular functions. Mitochondrial dysfunctions, which are caused by mutations in both ncDNA and mtDNA, are associated with a number of severe metabolic diseases [1–3]. Changes in mtDNA copy number have also been directly associated with aging and disease [4–9]. As demonstrated recently, mitochondria and their intrinsic mtDNA are also exchangeable between cells, resulting in the metabolic reprogramming of the recipient cells, in tissue repair and cancer progression [1,10–13]. This metabolic and functional reprogramming is also associated with modifications of the cellular gene expression pattern. In metabolic diseases originating from mtDNA mutations, the degree of heteroplasmy – the percentage of mutated mtDNA – is an essential criterion for the declaration of the pathology. For intercellular mitochondrial exchange, the concentration of exogenous mitochondria also determines the phenotype of the recipient cells. Therefore, in both cases, gene expression as measured by mRNA levels needs to be assessed as a function of the mtDNA concentrations in order to establish mechanistic links. This prompted us to identify robust methods to concurrently isolate the mtDNA and RNA in these eukaryotic cells. In addition, we were looking for a protocol that allowed the simultaneous isolation of RNA and DNA in order to save both the starting biological material and isolation time. A number of protocols have already been proposed to isolate RNA [14–16], mtDNA [15,17,18] or ncDNA [14,15,19] and most commercial kits are recommended for the exclusive isolation of either RNA or DNA. However, this is more costly and requires more biological material.

We assessed two experimental methods for nucleic acid isolation and tested their yields in mRNA, mtDNA and ncDNA. One of these methods was based on the Invitrogen TRIzol™ reagent, designed primarily for RNA isolation from the aqueous extraction phase [20]. The second method used the Qiagen DNeasy Blood & Tissue Kit, based on the known silica-adsorption properties of DNA [21] and designed for the rapid purification of total DNA (i.e., ncDNA and mtDNA). We tested whether the aqueous phase recovered in the TRIzol-based protocol also contained ncDNA and mtDNA and allowed their quantitative recovery. Conversely, we tested whether the DNeasy Blood & Tissue Kit also allowed the quantitative recovery of RNA. We performed these tests on the HepG2 adherent human hepatocyte cell line and on nonadherent human primary glioblastoma stem cells (GSCs) (clone Gli4) [22].

Materials & methods

Cell culture

The human hepatocyte cell line HepG2 was grown in Minimum Essential Medium Eagle, alpha modification (Lonza Bioscience BE12-169F), supplemented with 10% fetal bovine serum (Sigma-Aldrich F7524) and 2 mM L-glutamine (Gibco 25030024).

Human primary GSCs (clone Gli4 [22]) were grown as spheroids in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Gibco 21331046), supplemented with 2 mM L-glutamine (Gibco 25030024), 0.3% D-glucose (Sigma-Aldrich G8769), 0.002% bovine insulin (Sigma-Aldrich I1882), N-2 supplement (Gibco 17502048), B-27 supplement (Gibco 12587010), 10 ng/ml human EGF (Miltenyi Biotec 130-097-750) and 10 ng/ml human FGF-2 (Miltenyi Biotec 130-104-924).

Human mesenchymal stem cells (MSCs) were isolated from the bone marrow of healthy donors at the authorized cell therapy unit (Biotherapy Team of General Clinic Research Center, French health minister agreement TCG/04/0/008/AA) at the Grenoble University Hospital. MSCs were cultured in Minimum Essential Medium Eagle, alpha modification (Lonza Bioscience), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Gibco) and 1 ng/ml FGF-2 (Miltenyi Biotec). All cell types were cultured at 37°C in 5% CO_2.

Nucleic acid isolation by the TRIzol method & on column

Nucleic acids were isolated from 5×10^5 cells. The TRIzol reagent (Invitrogen 15596026) procedure was performed according to the manufacturer's instructions. Briefly, cells were homogenized in 700 μl TRIzol reagent. The solution was separated into the two phases, aqueous and organic, by adding 200 μl chloroform. The upper aqueous phase was gently recovered, without touching or aspirating the interphase, and transferred to a microtube containing 1.5 μl glycogen (Invitrogen 10814010). The nucleic acids contained in this phase were precipitated with 500 μl isopropyl alcohol, washed twice with 900 μl ice-cold 70% ethanol, air-dried and resuspended in RNase-free water. The centrifugations for collecting and rinsing the nucleic acid pellets were all performed at 12,000×*g*, 4°C, for 15 min. Nucleic acid isolation with the Qiagen DNeasy Blood and Tissue kit (Qiagen 69506) was also performed according to the manufacturer's instructions. Briefly, cells were resuspended in 200 μl phosphate-buffered saline to which 20 μl proteinase K was then added. After the addition of 200 μl buffer AL, the samples were vortexed. Ethanol (100%, 200 μl) was then added and the samples vortexed again. The mixture was pipetted into a spin column which was then centrifuged at 6000×*g* for 1 min on top of a collecting tube. The column was then rinsed twice, first with 500 μl buffer AW1 (centrifugation at 6000×*g* for 1 min) and second with 500 μl buffer AW2 (centrifugation at 20,000×*g* for 3 min). Nucleic acids were eluted with 200 μl buffer AE (centrifugation at 6000×*g* for 1 min). All centrifugations were performed at room temperature. Nucleic acid concentrations and purity were assessed with a NanoDrop spectrophotometer.

Transfer of MSC mitochondria to GSCs by MitoCeption

The transfer of MSC mitochondria to GSCs was performed as previously described [23,24]. Briefly, GSC spheroids were dissociated 24 h prior to the mitochondria transfer and seeded as single cells immediately before the mitochondria transfer. On the day of mitochondria transfer, MSCs (5×10^5) were trypsinized in the absence of EDTA (Gibco 15090046). MSCs were mechanically lysed using a syringe with 25- and 27-gauge needles. MSC mitochondria were isolated by two differential centrifugations of 10 min each, at 800×*g* and 8000×*g* respectively, using a buffer containing 200 mM mannitol (Sigma-Aldrich M1902), 70 mM saccharose (Sigma-Aldrich S0389), 1 mM EDTA (Sigma-Aldrich E9884), 10 mM HEPES (pH 7.4) (Sigma-Aldrich H3375) and 1× protease and phosphatase inhibitor cocktail (Roche, Meylan, France; 04693159001). MSC mitochondria were then added to the previously-seeded single-cell GSCs and centrifuged at 3000 rpm for 15 min. GSCs were incubated for 24 h at 37°C in 5% CO_2.

qPCR (mtDNA & ncDNA)

Real-time quantitative PCR to quantify mtDNA and ncDNA was performed on 30 ng nucleic acids, directly after the nucleic acid isolation step, by using the SYBR Green Master PLUS Mix (Roche 03515885001) and the LightCycler 480 instrument (Roche), with the following program: 10 min at 95°C, 50 cycles of 10 s at 95°C, 15 s at 67°C and 15 s at 72°C. ncDNA was quantified by using the following primers: βglobin-F: 5′-ACACAACTGTGTTCACTAGC-3′; βglobin-R: 5′-CCAACTTCATCCACGTTCA-3′, targeting the nuclear β-hemoglobin gene. Total mtDNA was quantified by amplifying a DNA domain within the D-loop of mtDNA by using the following primers: Universal-F: 5′-TTAACTCCACCATTAGCACC-3′; Universal-R: 5′-GAGGATGGTGGTCAAGGGA-3′ [25]. To specifically amplify mtDNA from MSCs (donor MSC119), the following set of primers was used: MSC-F: 5′-AAGCAAGTACAGCAATCAACCCC-3′; MSC-R: 5′-TTAAGGGTGGGTAGGTTTGTAGC-3′. The amplification efficacies with the different sets of primers were verified with serial DNA dilutions.

mRNA quantification

RNAs were reverse transcribed using the M-MLV RT kit (Invitrogen 28025013) and random hexamers (Invitrogen N8080127). Real-time quantitative PCR was performed using the SYBR Green Master Mix (Roche 04887352001), with 250 nM primer concentrations and the LightCycler 480 instrument (Roche), with the following program: 10 min at 95°C, 50 cycles of 10 s at 95°C, 15 s at 65°C and 15 s at 72°C. The following primer sets were used for *RPLP0* and *RPS9*: RPLP0-F 5′-TCGACAATGGCAGCATCTAC-3′, RPLP0-R 5′-GCCTTGACCTTTTCAGCAAG-3′, RPS9-F 5′-ATGAAGGACGGGATGTTCAC-3′, RPS9-R 5′-GATTACATCCTGGGCCTGAA-3′.

Statistical analysis

Data were analyzed using GraphPad Prism 7 (GraphPad Software Inc., CA, USA). All data are presented as mean values with standard deviations. Unpaired Student *t*-tests with Welch's correction were performed to compare the concentrations of mtDNA, ncDNA and mRNA. Differences were considered statistically significant for $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

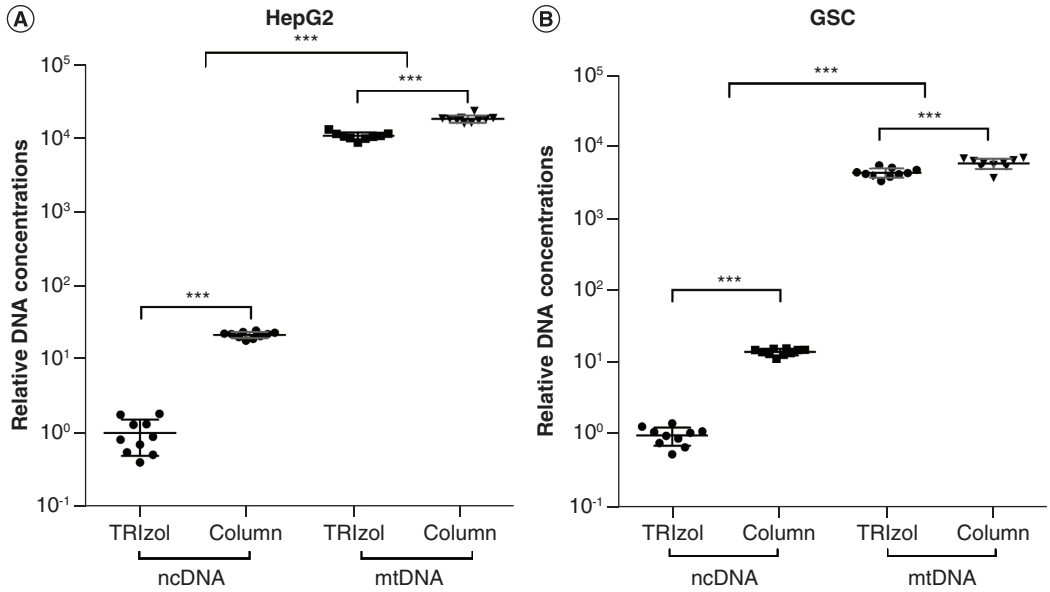

Figure 1. Comparison of the extraction yields by TRIzol reagent and Qiagen DNeasy Blood and Tissue kit of nuclear and mitochondrial DNA. Samples of (A) hepatocellular carcinoma cells (HepG2) and (B) human primary glioblastoma stem cells (Gli4) were used to extract nucleic acids, by using either the Invitrogen TRIzol-based method (n = 10) or the Qiagen DNeasy columns (n = 10). The relative concentrations of ncDNA and mtDNA were determined by qPCR using specific primers. Each dot represents an independent extraction. Represented are the means ± standard deviation. Unpaired Student *t* tests with Welch's correction were performed.
GSC: Glioblastoma stem cell.

Results & discussion

Isolation of mtDNA & ncDNA

We first showed that the TRIzol-based method allowed the isolation of both nuclear and mitochondrial DNA from the HepG2 cell line (Figure 1A). Both ncDNA and mtDNA were isolated from the aqueous phase also containing RNA, which contributes to a simplified isolation procedure. Of note, we were unsuccessful in isolating DNA from the TRIzol organic phase as we encountered both low isolation yields and reproducibility with the two human cell lines we tested. Isolation of mtDNA from the TRIzol aqueous phase showed high reproducibility and the yield with the column method was only 1.7-fold higher than with the TRIzol method. However, the column appeared superior for ncDNA isolation, providing a 21.3-fold higher yield than the TRIzol method. Similar results were obtained when DNA was isolated from human primary GSCs (Gli4 clone [22]). While the mtDNA yields with the column were only slightly higher than with the TRIzol method (1.3-fold), ncDNA isolation using the column was 14.5-times more efficient than with the TRIzol method (Figure 1B). Overall, these data show that both methods are highly reliable for quantitatively isolating mtDNA, while the column proved more efficient than the TRIzol-based method for ncDNA isolation.

Isolation of mRNA

We next checked whether the Qiagen DNeasy column procedure also allowed the isolation of mRNA. For this purpose, we prepared cDNA from the Qiagen DNeasy column samples, for both the HepG2 cells and the GSCs, and measured the expression of the two housekeeping genes *RPLP0* and *RPS9*. The same was done with the samples prepared by the TRIzol method. Interestingly, the HepG2 samples prepared on the Qiagen DNeasy columns yielded mRNAs in amounts close to those obtained with the TRIzol-based isolation method, albeit with yield differences depending on the analyzed genes (Figure 2A). For GSCs, the mRNA recovery appeared higher with the Qiagen DNeasy columns (Figure 2B). However, the isolation efficiency appeared less reproducible than with the TRIzol procedure, with more widespread mRNA concentrations, as indicated by standard deviations that were on average twice as big for the column samples than for the TRIzol-derived samples. These measures indicated that both the TRIzol-based and column methods allow the concurrent isolation of mRNA, mtDNA and ncDNA. However, the TRIzol-based method is to be preferred whenever experimental priority needs to be given to mRNA and mtDNA.

Application of the TRIzol-based method to quantify exogenous mtDNA following mitochondrial intercellular transfer

One of our research goals is to determine changes of gene expression in GSCs following the acquisition of exogenous mitochondria from MSCs. On the basis of the above study, we opted for the TRIzol-based method because it provides efficient and reproducible

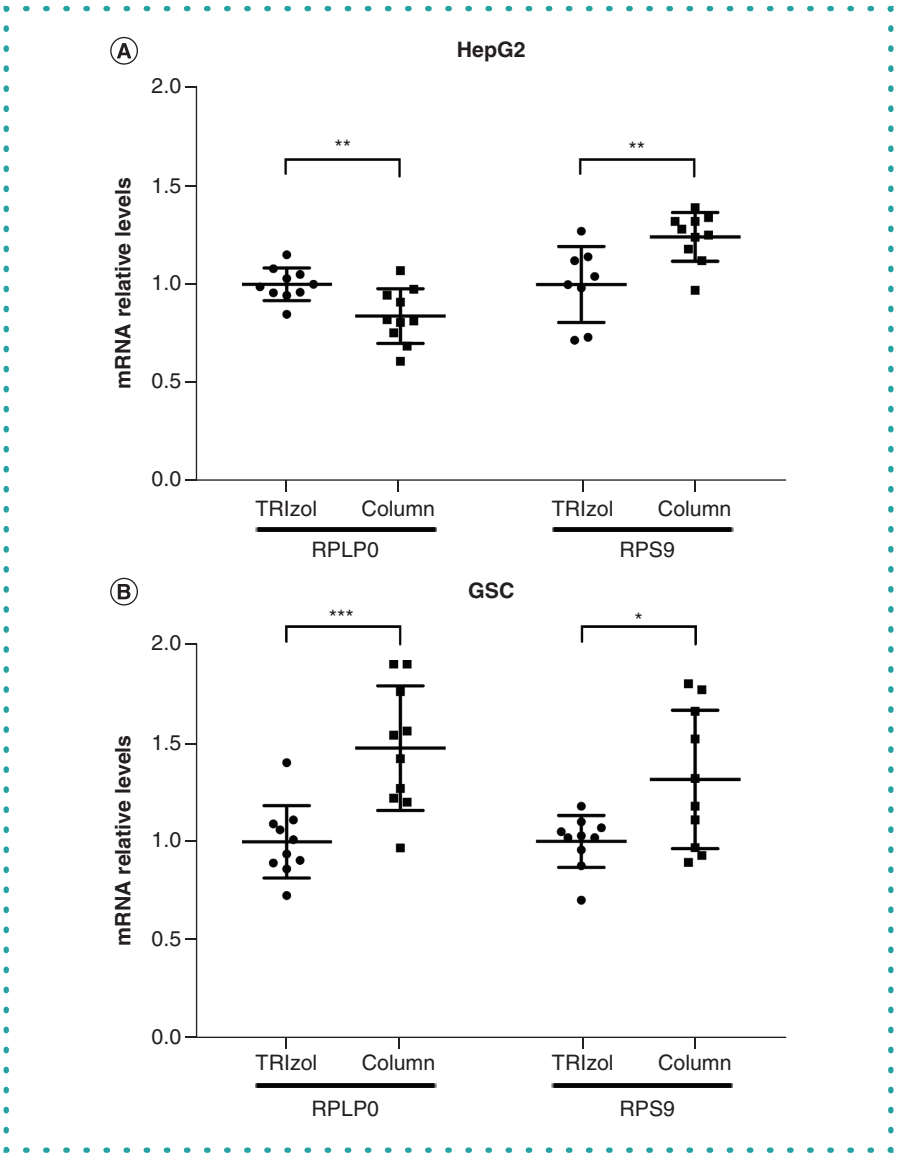

Figure 2. Comparison of the mRNA extraction yields by TRIzol reagent and Qiagen DNeasy Blood and Tissue kit. Samples of **(A)** hepatocellular carcinoma cells (HepG2) and **(B)** human primary glioblastoma cells (Gli4) were used to extract nucleic acids, by using either the Invitrogen TRIzol-based method (n = 10) or the Qiagen DNeasy columns (n = 10). cDNA was prepared and the relative concentrations of mRNA for the housekeeping genes *RPLP0* and *RPS9* were determined by qPCR. Each dot represents an independent extraction. Represented are the means ± standard deviation. Unpaired Student *t* tests with Welch's correction were performed.
GSC: Glioblastoma stem cell.

isolation of mRNA as well as mtDNA (required to quantify the amounts of acquired exogenous mitochondria). qPCR quantification of the exogenous mtDNA is based on the recognition of single nucleotide polymorphisms specific to the cell donor [23,24]. We first validated the qPCR conditions for specifically detecting MSC mtDNA (and not GSC mtDNA). For this, we isolated DNA from both the GSCs (Gli4) and the MSCs (MSC119) using the TRIzol-based method. We then performed serial dilutions of the MSC DNA (30 to 6×10^{-2} ng) in a solution containing the GSC DNA (total DNA amount 30 ng) and PCR-amplified the MSC mtDNA. As shown by the linear regression, this provided the range of quantitative detection for MSC119 mtDNA in GSC-Gli4 by qPCR (Figure 3A). We then transferred mitochondria, isolated beforehand from MSCs (MSC119), to GSCs (clone Gli4) using the MitoCeption protocol [23,24]. Following the transfer, we used the TRIzol-based method to isolate DNA from GSCs and determined the concentrations of MSC mtDNA by qPCR. This allowed us to demonstrate the dose–response acquisition of MSC mitochondria by the GSCs (Figure 3B). Additionally, the functional effects of MSC mitochondria acquisition were determined by quantifying mRNA concentrations and gene expression in these samples (data not shown). Overall, these data demonstrate that the TRIzol-based method allows the simultaneous and quantitative isolation of both mtDNA and mRNA from mammalian cells, and from GSCs in particular, and can be exploited in the context of intercellular mitochondrial transfers.

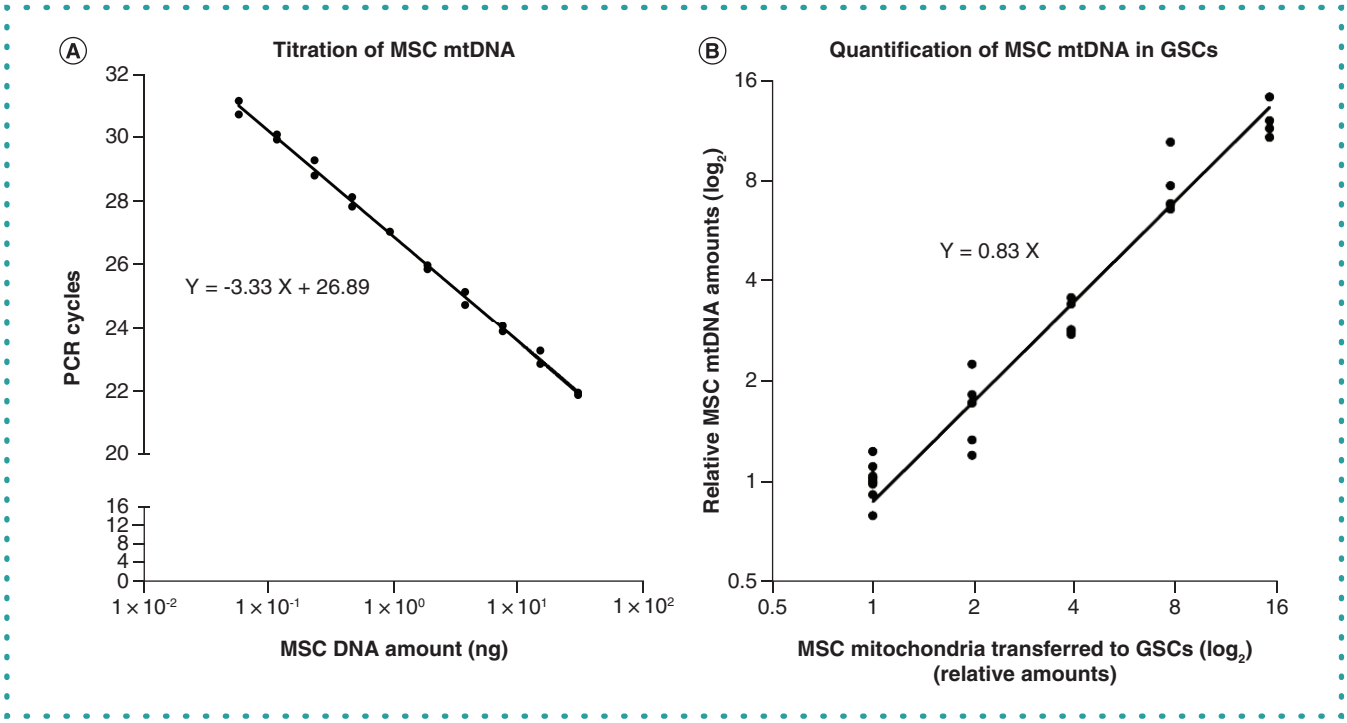

Figure 3. Quantification of mesenchymal stem cell mtDNA following the transfer of mitochondria to glioblastoma stem cells. (A) The range of detection of MSC (donor MSC119) mtDNA in GSCs (Gli4 clone) was determined by qPCR quantification of MSC mtDNA, following serial dilutions of MSC DNA with GSC DNA, both isolated by the TRIzol method. Two independent experiments were performed. Data are presented on a semi-log scale and shown are the line and equation of interpolation best-fit. **(B)** MSC mitochondria were transferred in increasing amounts to GSCs by MitoCeption. The following day, total DNA was isolated by the TRIzol-based method and the relative concentrations of MSC mtDNA were determined by qPCR and by using the titration curve shown in **(A)**. Shown are the measured concentrations of MSC mtDNA as a function of transferred MSC mitochondria, expressed as relative values. Each dot corresponds to an individual TRIzol extraction; data were obtained from four independent MitoCeption experiments. Data are presented on logarithmic scales (Log2) and shown are the line and equation of best-fit.
GSC: Glioblastoma stem cell; MSC: Mesenchymal stem cell.

As already observed [26–28], the respective yields in mitochondrial and nuclear DNAs are different depending on the DNA isolation method used. As the concentrations of mtDNA and ncDNA are often used to determine the number of mitochondria per cell, these method-related differences underline the caution required when drawing this type of conclusion.

Future perspective

The role played by mtDNA is beginning to emerge, in the fields of both mitochondrial intercellular exchanges and mitochondrial diseases (originating either from mutations in the mitochondrial DNA or from changes in mitochondrial DNA copy number). Studies in these fields need precise quantification of, first, the mtDNA concentrations of exogenous versus endogenous mitochondria and second, mutant versus wild-type mtDNA concentrations; therefore protocols enabling quantitative isolation are valuable. This further supports the need for robust and rapid mtDNA and mRNA isolation methods, as the characterization of the mtDNA sequence and copy number along with the gene expression pattern will be valuable for more accurate clinical evaluation. Studies on mtDNA characterization constitute an active and rapidly evolving field, as illustrated by novel technologies aiming at, for instance, single-cell mtDNA PCR detection [29,30]. In addition to qPCR, other technical approaches to determine mitochondrial DNA copy number have recently been proposed – for example, whole genome sequencing [31] – opening further possibilities for characterizing mitochondrial DNA sequences and copy number and, in the longer term, therapeutic manipulation of heteroplasmy [32–34].

Author contributions

The experiment was designed by J Nakhle, T Özkan and M Vignais and was undertaken by J Nakhle, T Özkan, K Lněničková and P Briolotti. The manuscript was prepared by J Nakhle, T Özkan and M Vignais. All authors had the opportunity to read and approve the manuscript.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Human MSCs were isolated from bone marrow from healthy donors at the authorized cell therapy unit (Biotherapy Team of General Clinic Research Center, French health minister agreement TCG/04/0/008/AA) at the Grenoble University Hospital. The line of GSCs that was used was previously published.

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