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## RESEARCH ARTICLE

# Understanding natural isotopic variations in cultured cancer cells

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**Rationale:** Natural variations in the abundance of the stable isotopes of nitrogen ( $\delta^{15}\text{N}$ ) and carbon ( $\delta^{13}\text{C}$ ) offer valuable insights into metabolic fluxes. In the wake of strong interest in cancer metabolism, recent research has revealed  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  variations in cancerous compared to non-cancerous tissues and cell lines. However, our understanding of natural isotopic variations in cultured mammalian cells, particularly in relation to metabolism, remains limited. This study aims to start addressing this gap using metabolic modulations in cells cultured under controlled conditions.

**Methods:** Prostate cancer cells (PC3) were cultured in different conditions and their  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were measured using isotope ratio mass spectrometry. Isotopic variations during successive cell culture passages were assessed and two widely used cell culture media (RPMI and DMEM) were compared. Metabolism was modulated through glutamine deprivation and hypoxia.

**Results:** Successive cell culture passages generally resulted in reproducible  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values. The impact of culture medium composition on  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of the cells highlights the importance of maintaining a consistent medium composition across conditions whenever possible. Glutamine deprivation and hypoxia induced a lower  $\delta^{13}\text{C}$  in bulk cell samples, with only the former affecting  $\delta^{15}\text{N}$ . Gaps between theory and experiments were bridged and the lessons learned throughout the process are provided.

**Conclusions:** Exposing cultured cancer cells to hypoxia allowed us to further investigate the relation between metabolic modulations and natural isotopic variations, while mitigating the confounding impact of changing culture medium composition. This study highlights the potential of natural  $\delta^{13}\text{C}$  variations for studying substrate fluxes and nutrient allocation in reproducible culture conditions. Considering cell yield and culture medium composition is pivotal to the success of this approach.

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## 1 | INTRODUCTION

Natural variations in isotopic abundances can offer insights into metabolic changes. For instance, the natural abundance of the heavy stable isotope of nitrogen  $^{15}\text{N}$  relative to its lighter counterpart  $^{14}\text{N}$  (expressed as  $\delta^{15}\text{N}$ ) has been shown to vary with changes in tissue amino acid catabolic relative to anabolic fluxes.<sup>1–4</sup> As for carbon, variations in the natural abundance of the heavy stable isotope  $^{13}\text{C}$  relative to  $^{12}\text{C}$  (expressed as  $\delta^{13}\text{C}$ ) have been shown to be informative on the relative fluxes of substrates like glucose and fatty acids towards biosynthetic pathways through the tricarboxylic acid (TCA) cycle.<sup>1,3,5</sup> Hence, natural  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  variations have the potential to help us track and better understand metabolic fluxes.

Tracking and understanding metabolism have been in the spotlight for many years in fields like cancer research, with malignant tissues undergoing metabolic rewiring to support tumor growth, spread, and resistance to treatment. It has been shown that probing metabolism can help assess tumoral tissue status.<sup>6–8</sup> In this context, recent research has shown natural  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  variations in cancerous tissues compared to non-malignant tissues.<sup>9–17</sup> Natural isotopic variations were also observed in the plasma of metastatic breast cancer patients supplemented with n-3 fatty acids that lowered systemic inflammation.<sup>18</sup> While clinical trials and the study of cancerous human tissues are critical to translate findings to the clinic, model organisms and immortalized cell lines allow one to dig deeper into the underlying mechanisms and are highly used in preclinical research. Some advantages of cell culture reside in the ability to manipulate genes and molecular pathways, as well as greater clonal and environmental homogeneity.<sup>19</sup> So far, one study in *Drosophila* exploited natural differences in  $\delta^{13}\text{C}$  to study tumor growth<sup>20</sup> and only two studies measured natural  $\delta^{15}\text{N}$  variations in cancer cell lines.<sup>11,21</sup> Both Tea et al<sup>11</sup> and Krishnamurthy et al<sup>21</sup> reported a global  $^{15}\text{N}$  depletion in cancerous compared to non-cancerous bulk cell line samples. Using more granular approaches, one group emphasized a role of glutamic acid metabolism in  $^{15}\text{N}$  depletion,<sup>21</sup> while another group also highlighted the involvement of arginine metabolism.<sup>11</sup> A natural  $^{13}\text{C}$  enrichment was also observed in cancerous compared to non-cancerous cell lines and related to pyruvate carboxylase, carbamoyl-phosphate synthase, and lactate metabolism.<sup>11,17</sup> Yet, we still have little understanding of natural  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  variations in cultured mammalian cells and of their relation to metabolism. Better knowledge about isotopic variations in cultured cells deserves attention if we hope to make more of their use in *in vitro* models and thereby better complement *in vivo* findings.

In this paper, we share the results obtained so far in an attempt to understand natural  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  variations in prostate cancer cells. Reproducibility over cell culture passages, and the effects of cell culture medium composition and of metabolic changes induced by glutamine deprivation and hypoxia were studied in order to bridge the gap between theory and experiments.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines, culture media, and cell culture protocol

Prostate cancer cell line PC3 (CRL-1435) was purchased from the American Type Culture Collection (ATCC). Cells were cultured in Roswell Park Memorial Institute (RPMI) media and in Dulbecco's modified Eagle medium (DMEM), with 5% fetal bovine serum (FBS). Commercially available RPMI media were purchased for the control (RPMI-1640, GlutaMAX™) and glutamine deprivation conditions (RPMI-1640, HEPES, no glutamine). A DMEM culture medium without glucose, glutamine, and phenol was purchased and supplemented with glucose (2000 mg/L) and glutamine (600 mg/L) for the control condition and glucose alone for the glutamine deprivation condition. Detailed culture media compositions are presented in Tables S1–S3 (supporting information). Cells ( $8 \times 10^5$ ) were seeded in T175 flasks with 25 mL of culture medium and cultured for 7 days without medium renewal. Cells were kept at 5%  $\text{CO}_2$  in a humidified incubator (37°C). For the hypoxia condition, cells in the control medium were subjected to the same culture conditions except that  $\text{O}_2$  level was set at 1% using a hypoxia chamber (InviO<sub>2</sub>, Baker Ruskinn, UK). Cells were put in hypoxia 24 h after seeding for the 6 remaining days. The cells were collected for isotopic analyses and the experiment was repeated on a weekly basis by using part of the cells from the control condition to seed flasks containing the control and glutamine deprived culture media.

### 2.2 | Cell extraction

Adherent cells were washed once with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS), detached using trypsin-EDTA (Eurobio Scientific; 2 mL, 5 min, 37°C), and, for all conditions, cells were resuspended in fresh control medium (8 mL), and pooled with floating cells extracted by centrifugation ( $190 \times g$ , 4 min) of the culture medium to maximize cell yield. Cells were counted (Cellometer Auto T4, Nexcelom Bioscience, USA) and pelleted by centrifugation ( $190 \times g$ , 4 min). The culture medium was removed and the cell pellet was resuspended in PBS (10 mL) by gently pipetting up and down to wash the cells of any remaining culture medium, and centrifugated again ( $190 \times g$ , 4 min). The PBS was removed, and cell pellets were frozen at  $-80^\circ\text{C}$ , then freeze-dried overnight.

### 2.3 | Isotope ratio mass spectrometry

Freeze-dried cells were pulverized, and the resulting powder was weighed into thin capsules (0.7 mg per capsule) that were introduced into an isotope ratio mass spectrometer (Inegra2, Sercon, UK). Glutamic acid was used for calibration and drift correction. Samples were analyzed in triplicate, except for the glutamine deprivation and

hypoxia conditions, where reduced cell proliferation often prevented triplicate analysis (1–2 capsules per sample were analyzed depending on cell yield). The natural abundance of carbon and nitrogen stable isotopes was expressed using the delta notation:

$$\delta (\text{‰}) = 1000 \times (R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}} \quad (1)$$

where  $\delta$  is the per mille difference between the heavy to light isotope ratios ( $^{15}\text{N}/^{14}\text{N}$  and  $^{13}\text{C}/^{12}\text{C}$ ) of a sample ( $R_{\text{sample}}$ ) and of a standard ( $R_{\text{standard}}$ , atmospheric  $\text{N}_2$  for  $^{15}\text{N}/^{14}\text{N}$  and Vienna Pee Dee Belemnite for  $^{13}\text{C}/^{12}\text{C}$ ).

## 2.4 | Statistical analyses

Statistical analyses were performed with jamovi (version 2.3). Differences between cells cultured in different media and the effect of glutamine deprivation in the RPMI culture medium were assessed using the Mann–Witney U test. The effect of glutamine deprivation and hypoxia in the DMEM culture medium was assessed using the Kruskal–Wallis test followed up by the Dwass–Steel–Crichtlow–Fligner test. Data are presented as mean  $\pm$  standard deviation.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Stability of isotopic composition over cell culture passages

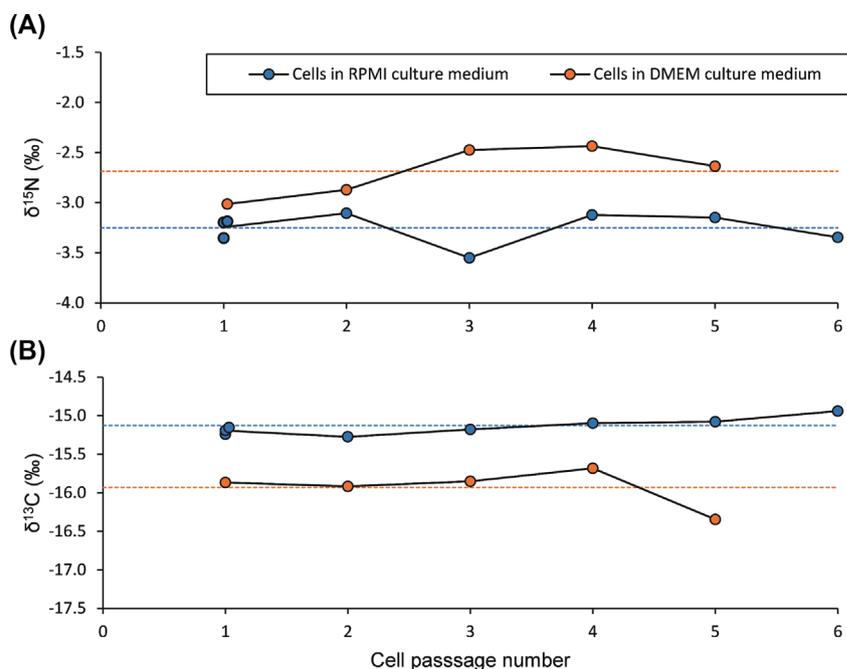
First, the stability of cell  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  over successive cell culture passages was evaluated. Cultured cells are usually subcultured or passed into a new culture vessel as cells replicate and approach confluency, around  $\sim 80\%$  confluency. This involved the removal of

the culture medium, the detachment of adherent cells, and the transfer of a fraction of the cells into a new growth vessel with fresh medium. In general, there were little variations of both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  over successive cell passages (Figure 1), demonstrating that reproducibility is achievable. Still, higher  $\delta^{15}\text{N}$  variations at passage number 3 and higher  $\delta^{13}\text{C}$  variations at passage number 5 in one of the two culture media used (Figure 1) show that greater isotopic variability can occur. Therefore, care must be taken to ensure consistency of culture conditions and in sample processing, minimizing variability between different cell culture passages. Although no *isotopic memory* was observed in this study after transferring the cells from the RPMI to the DMEM culture medium, it is advisable from a theoretical standpoint to wait at least one cell passage after their transfer to a different culture medium before analyzing their isotopic composition. The idea is to ensure a complete turnover of the cells with the nutrients in the new medium, as cell growth is supported by the incorporation of nutrients from the culture medium into cell mass, influencing the isotopic composition of the cells. The turnover time of the cells is affected by the duration of the cell cycle, i.e., the time required for a cell to divide into two daughter cells. This factor varies between cell lines and with culture conditions. Culturing cells in triplicate with three flasks for the first passage in the RPMI medium resulted in standard deviations of 0.09‰ for  $\delta^{15}\text{N}$  and 0.04‰ for  $\delta^{13}\text{C}$ .

### 3.2 | Effect of culture medium composition

Cell culture media come in a wide variety of formulations and can be supplemented with various additives, such as glutamine, glucose, buffers, etc., to support cell growth. There are therefore many potential sources of isotopic variability related to culture medium composition and the origin of the nutrients. We consequently

**FIGURE 1** Natural (A)  $\delta^{15}\text{N}$  and (B)  $\delta^{13}\text{C}$  values over successive passages of PC3 cells cultured in RPMI medium (blue circles) and DMEM (orange circles). Dashed lines represent the average of all cell culture passages. Each point represents the value obtained for one cell culture flask. For the first experimental cell passage in the RPMI culture medium, three different cultures were performed to study the variability of replicated experiments (SD: 0.09‰ for  $\delta^{15}\text{N}$  and 0.04‰ for  $\delta^{13}\text{C}$ ) [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



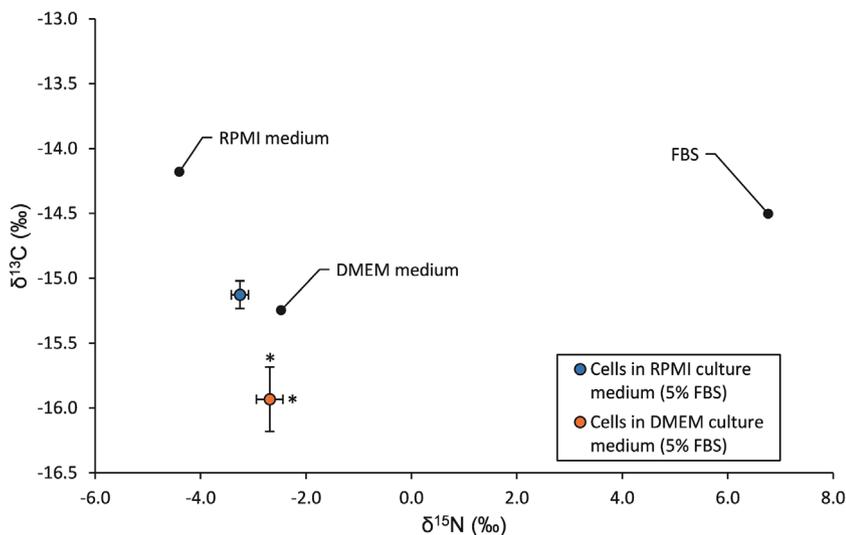
evaluated the effects of cell culture medium composition on  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of the cells. Figure 2 shows the isotopic differences between cells cultured in two different commercially available culture media (RPMI and DMEM). These isotopic differences are attributable, at least partly, to differences in the isotopic compositions between the two culture media (Figure 2), with nutrients that likely differ in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ . Compound-specific isotopic analyses would be especially useful to study this point further and to evaluate the relation between the isotopic composition of the different nutrients in the media and that of the cells, while considering the possibility of different metabolic impacts.

In addition, cell lines are often cultured in media supplemented with FBS that provides growth factors and nutrients like fatty acids. FBS is added in proportions that represent around 5–20% of the volume of the culture medium. Figure 2 shows the differences in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  between the two commercially available cell culture media used and the FBS used to supplement these media (5%). Negative  $\delta^{15}\text{N}$  values were observed in the studied culture media (analyzed before FBS addition), whereas  $\delta^{15}\text{N}$  was positive in the FBS that was added to these media (Figure 2). These bulk  $\delta$  values of the media analyzed without FBS and of the FBS are the sum of the  $\delta$  values of media and FBS individual components multiplied by their contribution to total carbon and nitrogen. The  $\delta$  values of the individual components in the media and FBS were not measured, but the compositions of the RPMI and DMEM (Tables S1–S3) show that even though there were vitamins and phenol red contributing to the  $\delta^{15}\text{N}$  values measured, amino acids represent most of the nitrogen in the culture media so that the negative  $\delta^{15}\text{N}$  values of the media can be attributed to amino acids. This is further confirmed by isotopic analysis of the commercially available glutamine that was added to the DMEM and had a  $\delta^{15}\text{N}$  of  $-2.51\%$ . Amino acids and glucose represented most of the carbon in the culture media (Tables S1–S3). The composition of FBS can vary between batch and providers, and while FBS is a lesser source of amino acids and glucose than the culture media, it is the sole source of fatty acids. The high  $\delta^{15}\text{N}$  of FBS is related to its animal origin, as animals are enriched in  $^{15}\text{N}$  compared to their diet, resulting in an increase in  $\delta^{15}\text{N}$  along food

chains.<sup>22</sup> As opposed to the culture media, FBS contains proteins that, due to their mass and concentrations, have a significant impact on the FBS  $\delta$  values that were measured (Figure 2). The relation between a  $\delta$  value measured in a matrix composed of many components like a cell culture medium depends on the  $\delta$  values of individual components and on their contributions to total carbon and nitrogen ( $p$ ) as represented below:

$$\delta_{\text{medium}} = \delta_{\text{component-1}} \times p_1 + \delta_{\text{component-2}} \times p_2 + \delta_{\text{component-3}} \times p_3 + \dots \quad (2)$$

Equation (2) reinforces the notion that the isotopic composition of individual components like amino acids and fatty acids in the culture media need to be analyzed after addition of all sources of such components, and therefore after addition of all supplements like FBS. In addition, it must be considered that the proteins in FBS are likely not a significant source of amino acids because amino acids in proteins are only bioavailable to cultured cells after protein degradation. We, therefore, present the  $\delta$  values of the media without FBS in Figure 2. Determining the  $\delta$  values of the nutrients that are bioavailable to cultured cells needs extraction and separation of fractions and compounds in cell culture media before measuring isotope ratios by isotope ratio mass spectrometry (IRMS). Altogether, these analyses illustrate that the best strategy to study isotopic variations related to metabolism in cultured cells is to keep the medium composition fixed in different experimental conditions where metabolism differs. This circumvents, at least to some extent, the need to analyze the isotopic composition of individual sources of carbon and nitrogen in the culture media. For instance, cancerous and non-cancerous cells must be cultured in the same medium to be compared, a practice not necessarily common for prostate cancer cells. The non-cancerous RWPE-1 cell line is typically cultured in keratinocyte SFM medium supplemented with human recombinant epidermal growth factor and bovine pituitary extract, but without FBS. Here, metabolic modulations were induced using both glutamine deprivation, inducing a change in culture medium composition, and hypoxia that did not.



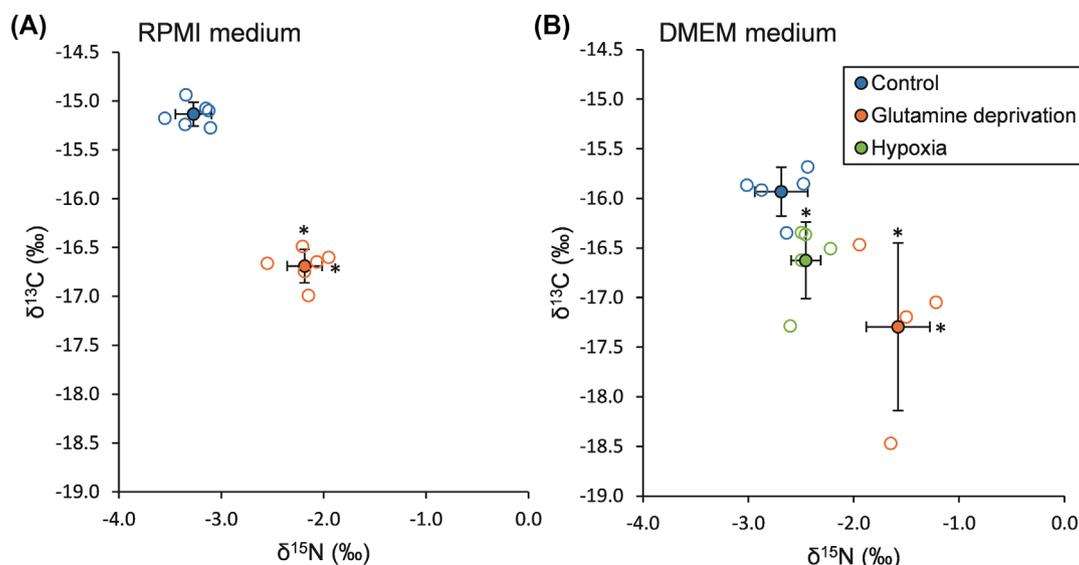
**FIGURE 2** Natural  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of cells cultured in RPMI medium (blue circle) and DMEM (orange circle) with 5% FBS. The cells were extracted from their media before the analysis of their isotopic compositions. The isotopic compositions of the fresh media without FBS and of the FBS are presented as well. The RPMI medium contained stabilized glutamine (alanyl-glutamine) (GlutaMAX™), while DMEM was purchased without glucose and glutamine and analyzed without these nutrients, which were later added to the medium. \*Significant difference between cells cultured in the different media ( $p < 0.01$ ) [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

### 3.3 | Effect of nutrient deprivation and hypoxia

In order to study how the metabolism of cultured mammalian cells influences their isotopic composition, metabolic modulations were induced using glutamine deprivation and hypoxia and the  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of PC3 cells were measured. Glutamine deprivation impacts the proliferation of PC3 cells and was expected to rewire their amino acid metabolism. For both RPMI and DMEM, glutamine deprivation resulted in higher  $\delta^{15}\text{N}$  values and in lower  $\delta^{13}\text{C}$  values of bulk cell samples (Figure 3). This was accompanied by a reduced cell proliferation as already documented<sup>23</sup> and as indicated by a cell count at the end of the culture (12.7% and 12.4% of the number of control cells were obtained with the glutamine deprived RPMI and DMEM, respectively). For instance, *in vivo*, glutamine was shown to affect whole-body amino acid metabolism by stimulating protein synthesis.<sup>24</sup> Cell yield was lowered by glutamine deprivation and was also consistently lower in DMEM than in RPMI medium (with 22.4% of the number of control cells obtained on average, suggesting different metabolic responses). As has been done in animal models,<sup>1,3,4</sup> it is tempting to relate the change in  $\delta^{15}\text{N}$  during glutamine deprivation to the induced rewiring of amino acid metabolism and the change in  $\delta^{13}\text{C}$  to changes in substrate fluxes to the TCA cycle. However, it cannot be ruled out at this point that the isotopic variations induced are not the sole reflection of the isotopic composition of the glutamine no longer incorporated into cell mass. The glutamine-free RPMI medium (Table S2), in contrast to the RPMI medium used for the control condition (Table S1), contained a HEPES buffer at a non-trivial concentration of 5958 mg/L, which significantly influenced the bulk isotopic composition of the medium (+3.87‰ for  $\delta^{15}\text{N}$  and -9.26‰ for  $\delta^{13}\text{C}$  compared with the control medium). Therefore, it is difficult to deduce the isotopic composition of the

glutamine in the RPMI medium. DMEM without glutamine was used so that the isotopic composition of the glutamine added to the medium for the control condition could be easily analyzed ( $\delta^{15}\text{N}$ : -2.51‰;  $\delta^{13}\text{C}$ : -13.37‰), confirming an impact of glutamine incorporation on the isotopic composition of the cells.

The issue of changes in the composition of the culture medium during glutamine deprivation was addressed by using hypoxia to induce metabolic changes without altering the culture medium composition (Figure 3B). Hypoxia is common in tumors, including in prostate tumors.<sup>25</sup> Unfortunately, one week of hypoxia did not significantly alter the  $\delta^{15}\text{N}$  of PC3 cells. Therefore, it was challenging to connect natural variations in  $\delta^{15}\text{N}$  to changes in amino acid metabolism beyond the differences observed between different cell lines grown in the same medium.<sup>11,21</sup> To determine whether changes in glucose incorporation into cell mass could be observed during hypoxia, glucose from maize with a  $\delta^{13}\text{C}$  (-11.38‰) slightly higher than that of DMEM (Figure 2) was added to glucose-free DMEM. Hypoxia lowered the  $\delta^{13}\text{C}$  of PC3 cells (Figure 3B) as expected. Hypoxia, via hypoxia-inducible factor 1, has been shown to increase glycolysis, reduce oxidative phosphorylation, and limit glucose carbon entry into the TCA cycle.<sup>26,27</sup> Confirming that PC3 cells respond this way to hypoxia despite an already glycolytic phenotype in normoxia,<sup>28</sup> the lower  $\delta^{13}\text{C}$  observed during hypoxia (Figure 3B) indeed suggests reduced glucose incorporation into cell mass, consistent with known effects of hypoxia. This result is promising for using natural  $\delta^{13}\text{C}$  variations to study nutrient routing to cultured biomass as done in animal models.<sup>1,3</sup> This technique has the advantage over enriched isotope tracers that it allows one to simultaneously study the fate of many metabolic precursors like glucose, fatty acids, and amino acids. Natural  $\delta^{13}\text{C}$  variations could serve as a screening tool for metabolic reorientations in cultured cells.



**FIGURE 3** (A)  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of PC3 cells cultured in control (RPMI 1640 GlutaMAX™, 5% FBS) and glutamine deprivation (RPMI 1640, HEPES, no glutamine, 5% FBS) conditions. (B)  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of PC3 cells cultured in control (DMEM, 5% FBS), hypoxia (DMEM, 5% FBS), and glutamine deprivation (DMEM, 5% FBS) conditions. DMEM without glucose, glutamine, and phenol was supplemented with glucose ( $\delta^{13}\text{C}$ : -11.38‰) and glutamine ( $\delta^{15}\text{N}$ : -2.51‰;  $\delta^{13}\text{C}$ : -13.37‰) or only with glucose for glutamine deprivation. \*Significant difference from the control condition ( $p < 0.05$ ) [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Although the lower  $\delta^{13}\text{C}$  during hypoxia might also reflect reduced incorporation of glutamine carbon into biomass, research has shown that glutamine replenishes TCA cycle intermediates during hypoxia.<sup>27,29</sup> Furthermore, the lower  $\delta^{13}\text{C}$  during hypoxia was accompanied by a reduced cell proliferation (44.1% of the number of control cells). While the lack of an effect on  $\delta^{15}\text{N}$  is reassuring, it remains to be fully determined whether the apparent metabolic shift induced by hypoxia and the associated  $\delta^{13}\text{C}$  variations are related to reduced cell density. The same applies to isotopic differences observed between different cell lines<sup>11,21</sup> that can grow at different rates. Altogether, the results presented here suggest a potential of using natural isotopic variations for studying cultured cell metabolism and help identify pitfalls in this application and research questions that remain to be answered.

### 3.4 | Limitations and lessons learned

Several factors need to be considered when using natural isotopic variations in cultured cells. In this study, we identified the amount of material required for isotopic analyses and culture medium composition as important factors to consider. Obtaining enough cells for isotopic analyses can be challenging, especially under conditions that limit cell growth, such as glutamine deprivation and hypoxia. This consideration is particularly crucial when isolating fractions like proteins and lipids and performing compound-specific isotope analyses, which require more material (~10 mg of dry mass) than the bulk analyses performed in this study. The use of high-yield culture vessels and pooling multiple vessels may be necessary. A tradeoff between extensively washing the cell pellet in PBS and cell yield must not be neglected, as cells can be lost during washing steps.

In this study, we pooled the floating cells with adherent cells to maximize the yield, which was particularly needed for the glutamine deprivation and hypoxia conditions but could have increased the number of non-viable cells in the analyzed pellets. While traditionally regarded as dead or dying, recent research has shown that most floating cancer cells are viable, and that this detachment share similarities with the metastatic process.<sup>30</sup> The number of non-viable cells, as assessed using a cell autounter, represented on average 1.5% and 2.3% of the cells collected for the control and glutamine deprivation conditions in the RPMI medium and 7.9%, 8.0%, and 16.5% of the cells collected for the control, glutamine deprivation, and hypoxia conditions in DMEM. Cell sorting techniques could be useful for IRMS applications and help assess whether dead cells introduce a bias. An initial screen to determine if experimental conditions significantly impact cell yield is recommended. Cell viability and cell cycle duration are important factors affecting yield. Assessing these parameters will help ensure complete cell turnover and a sound interpretation of natural isotopic variations.

We also identified differences in culture medium composition as problematic for interpreting natural isotopic variations in cultured

cells. While analyzing nutrients in commercially available culture media by gas chromatography–combustion–IRMS (GC-C/IRMS) would provide useful information, several challenges remain. It would be extremely difficult to analyze all nutrients in commercially available media using a conventional GC-C/IRMS approach. For instance, some amino acids, like arginine, are poorly detected using commonly employed derivatization methods for GC-C/IRMS analyses.<sup>31</sup> Additionally, glutamine and asparagine are deaminated. Using a homemade culture medium and analyzing the isotopic composition of the ingredients by IRMS prior to their incorporation into the media would provide nearly complete knowledge of the isotopic input in the system. Furthermore, analyzing the medium components both before and after the culture could provide information on the metabolic reactions that occurred.

## 4 | CONCLUSION

This study was performed to better understand natural variations in the abundance of the stable isotopes of nitrogen and carbon in cultured cancer cells. Evidence is provided that good reproducibility is achievable with stable  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values over cell culture passages. An effect on  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of cultured cells attributable to culture medium composition stresses the need for a stable medium composition and compound-specific isotope analyses, especially when medium composition varies across conditions. Manipulating cultured cell metabolism with hypoxia revealed that natural variations in  $\delta^{13}\text{C}$  show promise for studying substrate fluxes and nutrient allocation. More research and the use of compound-specific isotope analysis are required, especially with regards to nitrogen stable isotopes that remained unaffected by one week of hypoxia in this study.

### AUTHOR CONTRIBUTIONS

**Olivier L. Mantha:** Conceptualization; formal analysis; writing—review and editing; writing—original draft; methodology; investigation; project administration; supervision; visualization. **Marie Mahé:** Investigation; visualization; formal analysis; writing—review and editing. **Karine Mahéo:** Resources; writing—review and editing. **Gaëlle Fromont:** Resources; writing—review and editing. **Maxime Guéguinou:** Investigation; methodology; writing—review and editing. **Illa Tea:** Resources; writing—review and editing. **Régis Hankard:** Conceptualization; writing—review and editing. **Arnaud De Luca:** Conceptualization; project administration; supervision; writing—review and editing.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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