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(Citation)
Cancer Genomics & Proteomics,20(5):412-416

(Issue Date)
2023-09

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100483290

CANCER GENOMICS & PROTEOMICS *20*: 412-416 (2023)
doi:10.21873/cgp.20393

Methionine Restriction Increases Exosome Production and Secretion in Breast Cancer Cells

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Abstract. *Background/Aim: Methionine addiction is the elevated requirement for exogenous methionine for growth and survival of cancer cells, termed the Hoffman effect. Methionine-addicted cancer cells synthesize normal or excess amounts of methionine but still need an external source of methionine. Methionine restriction (MR) by either a methionine-free medium or in vivo by a low-methionine diet or by methioninase, selectively arrests cancer cells in the late S/G_2 cell cycle phase, but not normal cells. The present study focuses on the comparison of production and secretion of exosomes by cancer cells under MR and normal conditions. Materials and Methods: MDA-MB-231 cells (triple-negative breast cancer), containing exosomes labeled with CD63-GFP (CD63-GFP exosomes), were visualized by fluorescence microscopy. MDA-MB-231 cells expressing exosome-specific CD63-GFP were cultured in methionine-containing (MET+) or in methionine-free (MET−) DMEM conditions. Exosomes were isolated from conditioned medium of cultured MDA-MD-231 cells by ultracentrifugation and characterized by nanoparticle tracking analysis (NTA) and Western blotting. Results: When MDA-MB-231-CD63-GFP cells were cultured under MR conditions, they arrested their growth and CD63-GFP-expressing exosomes were strongly increased in the cells. MR resulted in approximately a 2-fold increase in exosome production and secretion per cell, even though cell growth was arrested. Methionine restriction thus resulted in elevated exosome production and secretion per surviving cell. Conclusion: Exosome production and secretion in the cancer cells increased under MR, suggesting a relation between MR and exosome production and secretion.*

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Key Words: Exosomes, cancer cells, methionine addiction, Hoffman effect, breast cancer, CD63, GFP, methionine restriction.

Exosomes are vesicles that vary in size between 30-150 nm. Exosomes originate from endosomal membranes (1) and are produced and secreted by cancer and other cells for intercellular communication and other purposes. Methionine addiction (2-12) is a universal and fundamental hallmark of cancer termed the Hoffman effect (2-18).

Methionine addiction is due to excess transmethylation reactions in cancer cells that results in much higher requirement for methionine for growth and survival than normal cells (3, 4, 6, 7, 14, 15). Cancer cells selectively arrest in the S/G_2 phases of the cell cycle after methionine restriction (16, 17).

In the present study we aimed to determine the effect of methionine restriction (MR) on exosome production and secretion in cancer cells. For imaging of exosome production in cancer cells under normal and MR conditions we previously introduced the exosome marker CD63 linked to green fluorescent protein (GFP) (CD63-GFP) to the human triple-negative breast cancer cell line MDA-MB-231, with a lentivirus vector (19). In MDA-MB-231-CD63-GFP cells, exosomes are brightly seen with GFP fluorescence. Surprising and paradoxical results of the present study show MR of MDA-MB-231-CD63-GFP cells resulted in increased production and secretion of exosomes, compared to normal-methionine conditions.

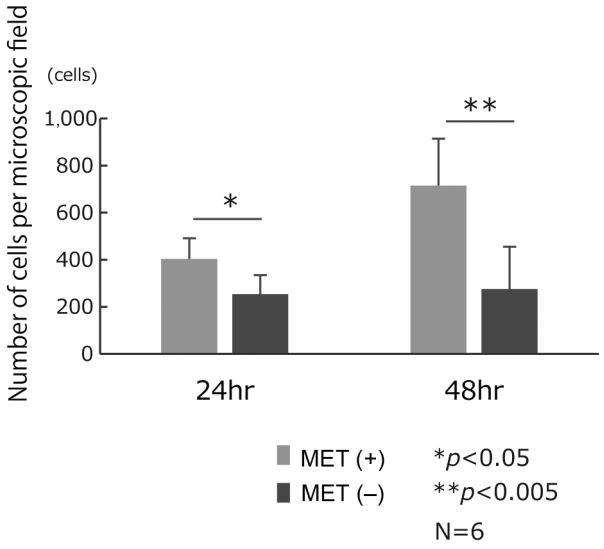


Figure 1. *Methionine addiction of MDA-MB-231-CD63-GFP cells. Bar graphs show the number of cells per microscopic field for MDA-MB-231-CD63-GFP cells at 24 and 48 h under methionine restriction and normal-methionine conditions.*

Materials and Methods

Cell line. The human breast cancer cell line, MDA-MB-231, was previously transformed to express the exosome-specific protein CD63 labeled with green fluorescent protein (CD63-GFP) (19).

The MDA-MB231-CD63-GFP cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin and incubated at 37˚C in an atmosphere of 5% CO_2 95% air. The cells were washed with phosphate-buffered saline (PBS), and the culture medium was replaced with DMEM with methionine (MET+) or without methionine (MET-) (4).

Preparation of conditioned medium and secreted exosomes. After incubation of MDA-MB-231-CD63-GFP cells for 24 h in MET+ or MET- medium, the conditioned medium (CM) was collected. Exosomes from the CM were isolated by an ultracentrifugation protocol, as reported previously (20). Briefly, the CM was centrifuged at 2,000 × *g* for 10 min to remove contaminating cells. The resulting supernatants were then transferred to fresh tubes and passed through a 0.22-μm filter (Millipore). The filtered CM was centrifuged for 90 min at 110,000 × *g* to pellet the enriched exosomes (Beckman Coulter, Brea, CA, USA). The pellets were washed with PBS and ultracentrifuged at 110,000 × *g* for another 90 min.

Preparation of cell lysates. Whole-cell lysates were prepared for western blotting with RIPA buffer (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) (21). After collection of the CM, the cells in culture dishes were washed with PBS and RIPA buffer. The cell lysates were measured for protein content using a Takara BCA protein assay kit (Takara, Bio Inc., Shiga, Japan).

Western blotting. Cell lysates were prepared for Western blotting as described above (21). An anti-CD63 antibody was used to quantify intracellular exosomes (BD Biosciences, Franklin Lakes, NJ, USA).

Exosome particle-size analysis. Exosome size, concentration and distribution were analyzed by nanoparticle-tracking-analysis (NTA) software using a NanoSight NS500 particle counter (Malvern Panalytical Ltd., Malvern, UK). The software was optimized to identify and track each exosome particle on a frame-by-frame basis and Brownian movement was tracked and measured from frame to frame (20).

Results

Growth of MDA-MB-231-CD63-GFP cells under methionine restriction. MDA-MB-231-CD63-GFP cells were seeded in normal methionine-containing medium, then the medium was replaced with normal or methionine-restricted (MR) medium. After 24 and 48 hours the number of cells in 6 fields of view under a fluorescence microscope was counted. MDA-MB-231-CD63-GFP cells significantly arrested their growth by 24 hours ($p<0.05$) under methionine restriction (MR) and by 48 hours under MR there was a very large difference in cell number between the 2 groups ($p<0.0005$) (Figure 1).

Exosome production by MDA-MB-231-CD63-GFP cells under methionine restriction. MDA-MB-231-CD63 cells were cultured in normal or MR media and then observed for CD63-GFP fluorescence in the cells. MDA-MB-231-CD63-GFP showed more fluorescence under MR than under normal conditions, suggesting greater production of exosomes under MR (Figure 2). Six fields of view were analyzed for the degree of GFP brightness using a Keyence BZ-X800 fluorescence analyzer. CD63-GFP fluorescence per cell was significantly higher under MR than normal conditions and at 48 hours there was a very large increase of fluorescence under MR ($p<0.005$) (Figure 3).

Production of CD63 by MDA-MB-231-CD63-GFP cells under methionine restriction. Western blotting of cell lysates showed CD63 production increased in MDA-MB-231-CD63-GFP cells under MR (Figure 4). Thus, the production of exosomes was strongly elevated in MDA-MB-231-CD63-GFP cells under MR.

Secretion of exosomes by MDA-MB-231 cells under methionine restriction. In order to count the number of exosomes excreted in the medium of MDA-MB-231 cells under normal and MR conditions, exosomes were isolated by ultracentrifugation using conditioned medium collected 24 h after exposure to MR or control conditions. The ultracentrifuged exosomes were quantified by NTA counting of secreted exosome particles which showed that the exosomes particle number per cell increased under MR for 24 hours ($p<0.05$) (Figure 5). The exosome size was similar under MR and normal conditions (Figure 6).

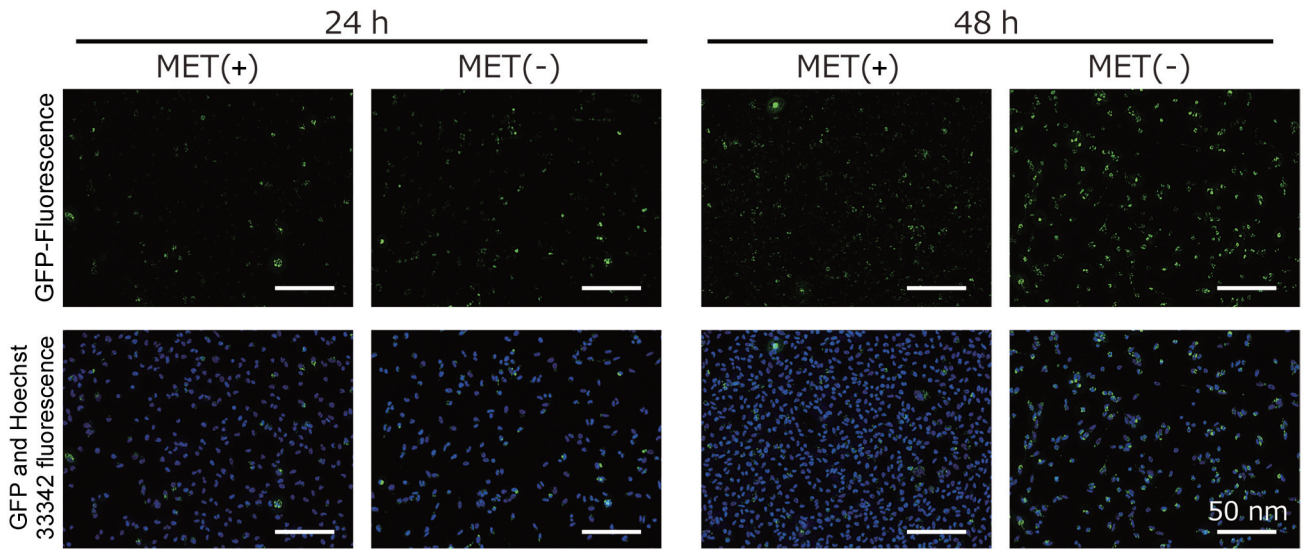


Figure 2. *Fluorescence imaging of CD63-GFP exosomes in MDA-MB-231-CD63-GFP cells under methionine restriction and normal-methionine conditions. The cell nuclei are stained with Hoechst 33342 (blue fluorescence).*

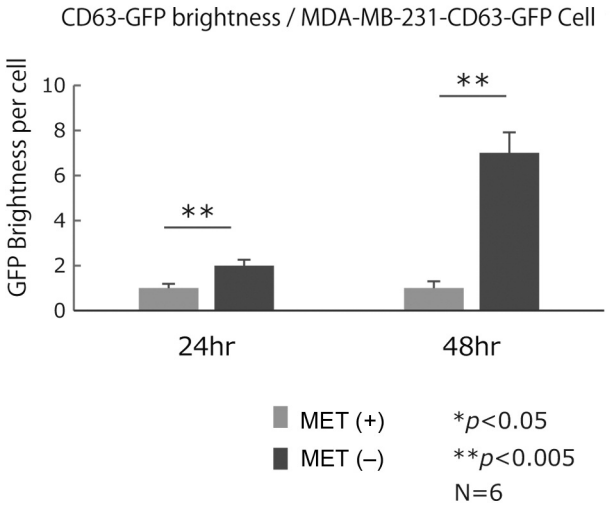


Figure 3. *CD63-GFP exosome fluorescence per MDA-MB-231-CD63-GFP cell under methionine restriction and normal conditions.*

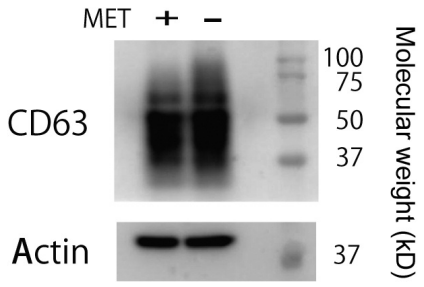
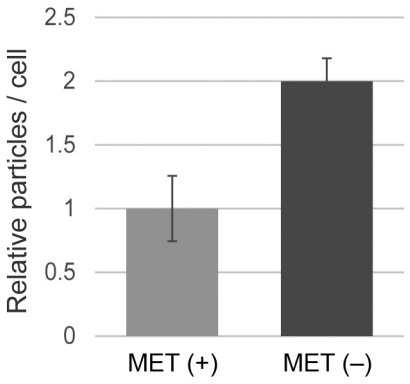


Figure 4. *Western blotting of CD63-GFP expression in MDA-MB-231-CD63-GFP cells under methionine restriction and normal conditions.*

Discussion

Methionine addiction of cancer cells was discovered by one of our team (RMH) (2) and is now termed the Hoffman effect (22). Cancer cells were previously found to be methionine dependent, unlike normal cells which could grow when methionine was replaced by homocysteine (23, 24). It was initially thought that cancer cells could not make methionine from homocysteine (24, 25) but later it was found that cancer cells make large amount of methionine from homocysteine (2, 3, 26). It was thus paradoxical when we showed cancer cells made more methionine from homocysteine than normal cells, but still needed external methionine for growth and survival, with the external methionine used by cancer cells for excess transmethylation reactions (3-7, 14, 15, 27). These results demonstrated methionine addiction in cancer cells (2, 3).

The present results were also surprising and paradoxical that under MR the MDA-MB231-CD63-GFP cells arrested their growth but produced and secreted much more exosomes. Why the cancer cell should expend resources on exosome production and secretion under MR where they are not able to grow and are dying is an important question and suggests a relationship between exosome production, secretion, and MR. Our current hypothesis is that the increase in exosomes production by cancer



	Particles/cell (× 10^2)
MET (+)	1.58 ± 0.41
MET (–)	3.16 ± 0.29

Figure 5. *Exosome secretion by MDA-MB-231 cells under methionine restriction [MET(-)] and normal conditions [MET(+)], as measured by a NanoSight NS500 particle counter.*

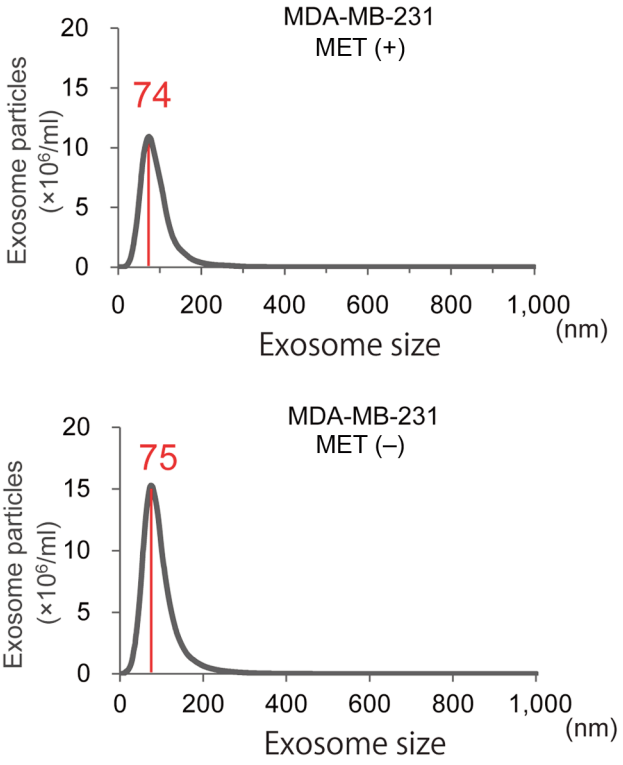


Figure 6. *Size of exosome particles secreted by MDA-MB-231 cells under methionine restriction [MET(-)] and normal conditions [MET(+)], as measured by a NanoSight NS500 particle counter.*

cells under MR is a stress response, Increased production of exosomes may serve as a biomarker of methionine starvation by cancer cells which may have clinical application for MR therapy (28) since exosomes can be measured in the body fluids such as tears (21). Future pre-clinical and clinical research is necessary.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conception and design: SI, and RMH. Performed the experiments: SI. Interpretation: TK, SM, SIn, MM, AS and HT. Manuscript writing: SI and RMH. Approval of manuscript: All Authors.

Acknowledgements

This work was supported by PI Training Program for International Collaborative Research, Kobe University, 2021 and JSPS KAKENHI.

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Received May 25, 2023
Revised July 3, 2023
Accepted July 18, 2023