PHARMACIA

Pharmacia 72: 1–5
DOI 10.3897/pharmacia.72.e141843

Research Article

Differences in phenotypes of normal and malignant cells concerning chromosomal fragility and ganglioside expression

Iskra Sainova[1], Vera Kolyovska[1], Desislava Drenska[2], Dimitar Maslarov[2,3], Dimitrina Dimitrova-Dikanarova[4], Tzvetanka Markova[5]

1 *Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*
2 *First MHAT "St. John Krastitel", Sofia, Bulgaria*
3 *Department of Neurology, Medical University of Sofia, Sofia, Bulgaria*
4 *Department of Biology, Medical University of Sofia, Sofia, Bulgaria*
5 *Department of Pharmacology and Toxicology, Medical University of Sofia, Sofia, Bulgaria*

Corresponding author: Iskra Sainova (iskrasainova@gmail.com)

Received 14 November 2024 ♦ **Accepted** 25 December 2024 ♦ **Published** 5 February 2025

Citation: Sainova I, Kolyovska V, Drenska D, Maslarov D, Dimitrova-Dikanarova D, Markova T (2025) Differences in phenotypes of normal and malignant cells concerning chromosomal fragility and ganglioside expression. Pharmacia 72: 1–5. https://doi.org/10.3897/pharmacia.72.e141843

Abstract

The spontaneous chromosomal fragility was tested by light microscopy observation of metaphases from peripheral blood lymphocytes of 8 patients with malignancies and of 8 healthy controls. In the tested patients, a significantly higher frequency of the spontaneous chromosomal fragility was observed compared to the controls, especially in the centromere chromosomal regions. Of particular interest were interactions involving gangliosides, the reduced form of tri-peptide glutathione (GSH) and/or of tumor-suppressor protein HACE1. The average titers of gangliosides and of anti-ganglioside antibodies in extracts from experimental *in vitro* models of laboratory-incubated cultures of mouse embryonic 3T3 fibroblasts, of mouse malignant myeloma cells, as well as of mixed cultures of both cellular types, were determined after previous passing of each one through GSH-agarose columns about the "selection" of the molecules in each one of the described samples, possessing affinity to GSH. Additionally, the presence and expression of the tumor-suppressor gene *HACE-1* in the genome of mouse embryonic stem cells (mESCs) and malignant human cervical carcinoma HeLa cells, both containing an additional copy of this gene, inserted by transfection with appropriate recombinant DNA vectors containing a copy of the tumor-suppressor gene, were tested. The developed experimental *in vitro* models show specific intermolecular interactions, which could prevent the disease development. Furthermore, a possibility about the production of antibodies/immunoglobulins by non-lymphoid cellular types was shown. Because the antibodies produced in this manner are outside the germinal centers in the specialized lymphoid tissues and organs, the control of their functions by small ions and molecules, such as gangliosides, is important.

Keywords

spontaneous chromosomal fragility, centromere chromosome regions, experimental *in vitro* models, gangliosides, tumor-suppressor proteins and peptides

Introduction

Chromosomes contain fragile sites serving as key breakpoints in the neoplastic transformations and chromosomal rearrangements. They are subdivided into common, or constitutive, fragile sites, which are present in the majority of individuals but may become manifest in cancer, and rare, or heritable, fragile sites (Daniel 1986). The latter fragile sites are highly unstable in mitosis and meiosis and have been found to correspond to tandemly repeated triplet sequences in disease-related genes (Zheng et al. 1993). In this relation, the intermolecular interactions in different cells, which could prevent the appearance of malignant changes, should be taken into consideration.

The reduced form of the tripeptide glutathione –GSH – is known to be an important immunomodulator, antioxidant, anti-neoplastic, and anti-aging substance (Jahngen-Hodje et al. 1997). This tripeptide exercises its diverse functions by participating in various intermolecular interactions and regulatory pathways. A still unresolved question is whether GSH influences the expression of the tumor-suppressor gene *HACE1*. In the human, this gene is located in the long arm of chromosome #6 (Zhang et al. 2007). Its protein product is an E3 ubiquitin ligase, which has been shown to participate in processes of degradation and inactivation of key proteins responsible for malignancy and metastasis processes (Gao et al. 2016).

For this goal, appropriate experimental *in vitro* models were developed and investigated. The attention in the concrete case was directed in particular to intermolecular interactions with participations of gangliosides, tripeptide GSH, and/or tumor-suppressor protein HACE1.

Materials and methods

Peripheral blood samples from eight patients with malignancies, as well as from eight healthy controls, were taken. They were incubated *ex vivo* in 4 ml RPMI 1640 (Sigma-Aldrich) medium with previously added 0.8–1 ml FBS and 0.2 ml phyto-hemagglutinin at 37 °C in an incubator with 5% CO_2 and 95% air humidification for 68–70 hours. Subsequently, further cell growth and proliferation were blocked by the addition of 0.2 ml colchicine, and the samples were placed back at 37 °C for 50 minutes. After careful shaking, they were centrifuged for 10 minutes at 1000 rpm. The supernatants were removed, and 0.3 ml from each pellet was resuspended in 10 ml of warm hypotonic 0.555% KCl solution. The samples were incubated at 37 °C for 10 minutes. After centrifugation at 1000 rpm for 10 minutes, the supernatants were removed, and 0.3 ml of each pellet was resuspended in 10 ml of a fixative mixture of methanol and glacial acetic acid (3:1). After centrifugation at 1000 rpm for 10 minutes, this step was repeated 3–4 times with fresh fixative mixture. Slides for light microscopy were prepared, stained by the G-banding technique, and observed by the microscope Amplival with immersion in order to determine the frequency of fragility in the respective chromosomes from both patients and healthy controls.

Total extracts from cultures of mouse embryonic fibroblasts of the 3T3 line, mouse malignant myeloma cells, as well as a mixed culture of co-incubated cells from the two types, were prepared by treatment with 10% Cl_3CCOOH and 0.48 M solution of K_3PO_4. Each of the extracts was subsequently passed through a GSH-agarose column to "select" molecules possessing affinity to GSH tripeptide (Cuattrecasas 1970). All prepared samples from the extracts of the three types of cellular *in vitro* cultures were subjected to enzyme-linked immunosorbent assay (ELISA) to determine the average titers of the anti-ganglioside IgG antibodies and of gangliosides in each one of them. The obtained titers were expressed as mean ± standard deviation (SD). The differences were considered statistically significant at $p < 0.05$ and $p < 0.01$.

Sub-populations of mouse embryonic stem cells (mESCs) and malignant human cervical carcinoma cells HeLa, both containing an additionally-inserted copy of the tumor-suppressor gene *HACE1* by transfection with appropriate recombinant DNA-vectors, based on Adeno-Associate Virus (AAV) DNA-genome, were used. Each one of the applied recombinant DNA-constructs contained inserted copy of the tumor-suppressor gene HACE-1, was selected by incubation in medium, supplemented with the synthetic analogue of neomycin – G418 - by taking into consideration the near location of the gene, responsible for neomycin resistance, to the inserted copy of the *HACE1* gene in the applied recombinant DNA vectors. The copy of mouse tumor-suppressor gene *HACE1* was isolated from 3T3 mouse embryonic fibroblasts, the marker gene, determining resistance to neomycin - from a plasmid of *E. coli* bacteria, and promoter of the Eukaryotic Elongation Factor-1 alpha (eEF-1α) was inserted (Chen et al. 2003)." Because of the murine origin of the inserted copy of gene *HACE1*, in the recombinant DNA vector applied for transfection of the human malignant cells HeLa, the tag gene *FLAG*, encoding the human protein FLAG was additionally inserted (Slootstra et al. 1997; Valdez-Sinon et al. 2020). Total genomic DNA material was isolated from transfected cells of both types, containing an additionally inserted copy of the tumor-suppressor gene *HACE1*, as described above. Standard polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) were performed, followed by electrophoresis in 1% agarose gel, to prove the presence and expression of the inserted copy of the tested gene of interest, respectively. Single-strand conformational polymorphism (SSCP) assay was applied, following the protocol of Dong and Zhu (2005). Appropriate primers with sizes of 750 to 2500 base pairs (bp), complementary on the based on the DNA genome of Adeno-Associated Virus (AAV) recombinant DNA constructs were applied.

Results

The highest frequency of spontaneous fragility in the tested patients was assessed in the centromere chromosomal regions (Fig. 1).

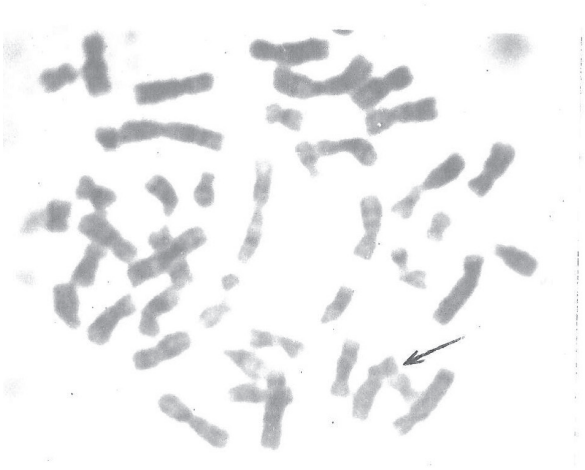

Figure 1. Spontaneous chromosomal fragility in the centromere region of human chromosome 6 (arrow) on metaphase chromosomes from human peripheral blood lymphocytes.

The average titers of anti-ganglioside antibodies (Fig. 2A) and of gangliosides (Fig. 2B) were determined in extracts of *in vitro* incubated cultures of mouse embryonic 3T3 fibroblasts, of mouse malignant myeloma cells, and also of mixed cultures of co-incubated cells from the two types. At dilutions of 1:40 and 1:100, the average titers of the anti-ganglioside antibodies in the samples from the culture of the mouse embryonic fibroblast 3T3 cell line were significantly higher (Fig. 2A) compared with the average titers of the gangliosides in the samples from the same line (Fig. 2B). Additionally, at dilutions of 1:40 and 1:100, the average titers of the anti-ganglioside antibodies in the extract from the culture of embryonic 3T3 cells were significantly higher compared with the average titers of the anti-ganglioside antibodies in the extracts from the mixed *in vitro* cell culture and the culture of mouse malignant myeloma cells at the same dilutions (Fig. 2A). At dilution 1:200, approximately equal average titers of the anti-ganglioside antibodies were found in the extract of the 3T3 cell culture and of the mouse malignant myeloma cell culture, and at dilution 1:400, approximately equal titers of the anti-ganglioside antibodies in the 3T3 culture and in the mixed culture (Fig. 2A). At dilutions of 1:40 and 1:200, the highest average titers of the gangliosides in the sample from the mixed culture were registered (Fig. 2B). At dilution 1:100, approximately equal average titers of gangliosides in the samples of the mixed culture and of the culture of mouse malignant myeloma cells were measured. At dilution 1:400, approximately equal average titers of the gangliosides in the samples from the three types of *in vitro* cell cultures were found (Fig. 2B). Only at dilution 1:100 of the sample from the extract of the embryonic 3T3 fibroblasts was a statistically significant difference in the significantly higher average titers of the anti-ganglioside antibodies compared to the other dilutions of the same sample, as well as to the same dilution of the extracts from the other cell cultures (Fig. 2A). At dilutions of 1:40 and 1:200, significantly higher average titers of the gangliosides were assessed in the extract of the mixed culture compared to the dilutions of 1:100 and 1:400 of the same extract, but also in comparison with the dilutions of 1:40 and 1:200 in the extracts from the *in vitro* cultures of the mouse embryonic 3T3 fibroblasts and of the mouse malignant myeloma cells (Fig. 2B).

The presence and expression of an additional copy of the tumor-suppressor gene *HACE1* in transfected mESCs and malignant human cervical carcinoma HeLa cells by appropriate recombinant DNA vectors were proven by standard PCR and RT-PCR, respectively (Fig. 2C). The assay was made on the total DNA material, isolated from the two types of cells, both subjected to transfection by recombinant DNA constructs, containing inserted (each one of them) copies of this tumor-suppressor gene.

Discussion

Our study found elevated levels of chromosomal fragility in patients with malignancies, which could possibly be used as a diagnostic or even a prognostic trait. It is not known, however, how the pathological process could unfold regarding other processes inside their cells. To elucidate this, we developed experimental *in vitro* models based on cultured mouse cell lines to study the levels and interactions of key molecules participating in the control of cell growth, proliferation, and differentiation. These molecules included lipids of the ganglioside group.

The present data confirm the role of the gangliosides in important signaling pathways, known from other published studies that have suggested these molecules as appropriate markers of cancer subgroups and potential targets for anti-neoplastic therapeutic approaches (Ustjanzew et al. 2024). The important role of gangliosides in the protection against immune attacks has also been discussed. In this way, the role of gangliosides as targets for autoimmunity and as receptors for viruses, bacteria, and/or toxins has been suggested. The expression patterns, amounts, and types of gangliosides, as well as of the specific antibodies to them, have been found to vary in the different cells. On the other hand, the key role of the tripeptide GSH (Jahngen-Hodje et al. 1997) and of the tumor-suppressor protein HACE1 (Zhang et al. 2007; Gao et al. 2016) in these intermolecular interactions is also taken into consideration. Additionally, the current results were in agreement with the literature data about the possibility of expression of genes coding immunoglobulins and/or their components/domains by non-lymphoid cells (Bebbington 1991; Deyev et al. 1993; Cho et al. 1999). Production of immunoglobulins by neoplastic cells has also been reported (Chen et al. 2009). The ability of non-immune cells to produce immune molecules directs attention to the development of novel diagnostic, prophylactic, and therapeutic approaches. A possible explanation is the possibility that subpopulations of immature stem-like cells can acquire basic lymphoid and myeloid properties. According to another hypothesis, separate immunoglobulin domains or chains could perform different functions (for instance, as enzymes). Because the immune molecules

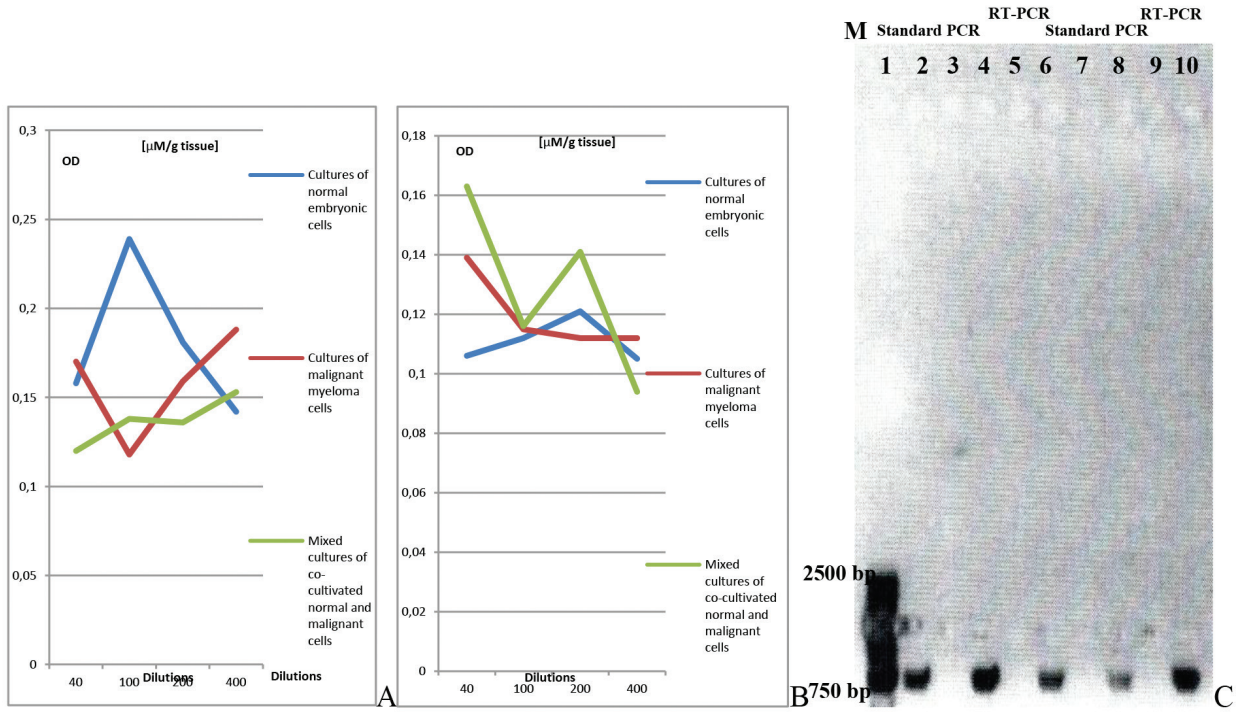

Figure 2. Samples from *in vitro* models of laboratory-incubated cells: **A, B**. Average titers in samples from lysates of *in vitro* cultures of non-malignant murine embryonic 3T3 fibroblasts, mouse malignant myeloma cells, and a mix of both cell types after passing through GSH-agarose columns: **A.** of anti-ganglioside antibodies; **B.** of gangliosides, on the horizontal axis are shown the dilutions of the respective biological samples in µM/g tissue/cell culture material, and on the vertical axis, the values of the OD; **C.** agarose gel electrophoresis of the presence (lanes 2 and 6) and expression (lanes 4, 8, and 10) of additionally inserted copy of tumor-suppressor gene *HACE1* in non-malignant mESCs (lanes 2 and 4) and malignant human cervical carcinoma HeLa cells (lanes 6, 8, and 10), both transfected by appropriate recombinant DNA constructs, containing previously inserted copies of this gene, by standard PCR (lanes 2 and 6) and RT-PCR (lanes 4, 8, and 10), respectively. Specific standards (M) with size 750–2500 bp were applied.

and/or their components produced in this manner are outside the germinal centers in the specialized lymphoid tissues and organs, the control of their production and functions by appropriate inter-molecular interactions is of key importance. In this respect, the influence of small ions and molecules, such as gangliosides, is very important. The registered higher average titers of the gangliosides in the extract from the mixed culture compared with the samples of the extracts from the other two cultures was in accordance with the literature data about the importance of stroma-derived gangliosides in the process of cellular differentiation to myeloid direction (Ziulkoski et al. 2009). In addition to gangliosides, our study also took into consideration the impact of the tri-peptide GSH and/or of the tumor-suppressor protein HACE1.

Conclusion

In the current research paper, the assessed differences in the frequency of the spontaneous chromosomal fragility in patients with malignancies and healthy controls were taken into consideration on the one hand, but also the availability of spontaneous chromosomal fragility in the controls (besides in the patients). In this relation, experimental *in vitro* models were developed from cultures of non-malignant cells, of malignant cells, and also from co-cultivated non-malignant and malignant cells. The main goal was directed to understanding specific intermolecular interactions, which could lead to the development of a pathologic process or, in the opposite case, to prevent it, with the participation of key molecules, in particular gangliosides, GSH, and the tumor-suppressor protein HACE1. The tested normal and malignant cell cultures, as well as their mix, showed differences in the average titers of the gangliosides and of the anti-ganglioside antibodies, which can be attributed to the participation of gangliosides in various intra- and extra-cellular inter-molecular interactions. Additionally, a possibility about the production of antibodies/immunoglobulins by non-lymphoid cellular types in appropriate conditions was proposed, including by malignant cells. Because the antibodies produced in this manner are outside the germinal centers in the specialized lymphoid tissues and organs, the control of their functions by small ions and molecules, such as gangliosides, is of key importance.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

Clinical trials: First MHAT "St. John Krastitel"
The authors declared that no experiments on humans or human tissues were performed for the present study.
The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.
The authors declared that no experiments on animals were performed for the present study.
The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

Funding

This work is supported by a grant on the research project D-137/14.06.2022 of the Bulgarian Ministry of Education and Science.

Author contributions

I.S. – the main ideas, experimental in vitro-models and research; V.K. – statistical assay; D.D. – biological samples from patients; D.M. – approach to the tested patients, from which were taken the most of the applied biological samples; D.D. – materials about laboratory work; Tz. Markova – materials about laboratory work on the put tasks, final reading and edition.

Author ORCIDs

Dimitrina Dimitrova-Dikanarova https://orcid.org/0000-0001-5399-6954

Data availability

All of the data that support the findings of this study are available in the main text.

References

Bebbington C (1991) Expression of antibody genes in nonlymphoid mammalian cells. Methods 2(2): 136–145. https://doi.org/10.1016/S1046-2023(05)80214-2

Chen S, Agarwal A, Glushakova OY, Jorgensen MS, Salgar SK, Poirier A, Flotte TR, Croker BP, Madsen KM, Atkinson MA, Hauswirth WW, Berns KI, Tisher CC (2003) Gene delivery in renal tubular epithelial cells using recombinant adeno-associated viral vectors. Journal of the American Society of Nephrology 14(4): 947–958. https://doi.org/10.1097/01.ASN.0000057858.45649.F7

Chen Z, Qiu X, Gu J (2009) Immunoglobulin expression in non-lymphoid lineage and neoplastic cells. The American Journal of Pathology 174(4): 1139–1148. https://doi.org/10.2353/ajpath.2009.080879

Cho SK, Webber TD, Carlile JR, Nakano T, Lewis SM, Zuniga-Pflucker JC (1999) Functional characterization of B lymphocytes generated *in vitro* from embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America 96(17): 9797–9802. https://doi.org/10.1073/pnas.96.17.9797

Cuattrecasas P (1970) Protein purification by affinity chromatography: derivatizations of agarose and polyacrylamide beads. Journal of Biological Chemistry 245(12): 3059–3065. https://doi.org/10.1016/S0021-9258(18)63022-4

Daniel A (1986) Clinical implications and classification of the constitutive fragile sites. American Journal of Medical Genetics 23(1–2): 419–427. https://doi.org/10.1002/ajmg.1320230134

Deyev SM, Lieber A, Radko BV, Polanovsky OL (1993) Production of recombinant antibodies in lymphoid and non-lymphoid cells. FEBS Letters 330(2): 111–113. https://doi.org/10.1016/0014-5793(93)80253-Q

Dong Y, Zhu H (2000) Single-strand conformational polymorphism analysis: basic principle and routine practice. Methods in Molecular Medicine 108: 149–157. https://doi.org/10.1385/1-59259-850-1:149

Gao Z-F, Wu Y-N, Bai Z-T, Zhang L, Zhou Q, Li X (2016) Tumor-suppressive role of HACE1 in hepatocellular carcinoma and its clinical significance. Oncology Reports 36(6): 3427–3435. https://doi.org/10.3892/or.2016.5205

Jahngen-Hodje J, Obin MS, Gong X, Shang S, Nowel TR, Gong J, Abasi H, Blumberg J, Taylor A (1997) Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. Journal of Biological Chemistry 272(45): 28218–18226. https://doi.org/10.1074/jbc.272.45.28218

Slootstra JW, Kuperus D, Plütckthun A, Melon RH (1997) Identification of new tag sequences with differential and selective recognition properties for the anti-FLAG monoclonal antibodies M1, M2 and M5. Molecular Diversity 2(3): 156–164. https://doi.org/10.1007/BF01682203

Ustjanzew A, Nedwed AS, Sandhoff R, Faber J, Marini F, Paret C (2024) Unraveling the glycosphingolipid metabolism by leveraging transcriptome-weighted network analysis on neuroblastic tumors. Cancer Metabolism 12(1): 29. https://doi.org/10.1186/s40170-024-00358-y

Valdez-Sinon AN, Gokhale A, Faundez V, Bassell GJ (2020) Protocol of immune-enrichment of FLAG-tagged protein complexes. STAR Protocols 1(2): 100083. https://doi.org/10.1016/j.xpro.2020.100083

Zhang L, Anglesio M, O'sullivan M, Zhang F, Yang G, Sarao R, Mai PN, Cronin S, Hara H, Melnyk N, Li L, Wada T, Liu PP, Farrar J, Arceci RJ, Sorensen PH, Penninger JM (2007) The E3 ligase HACE1 is a critical chromosome *6q21* tumor suppressor involved in multiple cancers. Nature Medicine 13(9): 1060–1069. https://doi.org/10.1038/nm1621

Zheng CJ, Byers B, Moolgavkar SH (1993) Allelic instability in mitosis: a unified model for dominant disorders. Proceedings of the National Academy of Sciences of the United States of America 90(21):10178–10182. https://doi.org/10.1073/pnas.90.21.10178

Ziulkoski AL, dos Santos AX, Andrade CM, Trindade VM, Daniotti JL, Borojevic R, Guma FC (2009) Anchored and soluble gangliosides contribute to myelosupportivity of stromal cells. Biochemical and Biophysical Research Communications 388(1):17–20. https://doi.org/10.1016/j.bbrc.2009.07.092