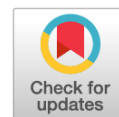


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NEURONAL PROTEIN GAP-43 IN EARLY MOUSE EMBRYOS

Faina M. Zakharova^{1, 2}, Nadezhda A. Yagovkina², Vladislav V. Zakharov³¹ Institute of Experimental Medicine, Saint Petersburg, Russia;² Saint Petersburg State University, Saint Petersburg, Russia;³ Institute of Macromolecular Compounds of the Russian Academy of Sciences, Saint Petersburg, Russia

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BACKGROUND: GAP-43 (growth-associated protein 43) is a specific neuronal protein of vertebrates, which is predominantly localized at the plasma membrane of axon terminals. GAP-43 plays an important role in axon growth cone guidance, neuroregeneration and synaptic plasticity. We have recently shown that GAP-43 is also present in mouse oocytes and zygotes, where the protein exhibits cytoplasmic localization, which presumably results from peculiar GAP-43 expression and modifications in these cells.

AIM: The aim of the research was to study GAP-43 localization in early (preimplantation) mouse embryos, from zygote to blastocyst stage.

MATERIALS AND METHODS: C57BL/CBA F1 hybrid mice were used in the work. Oocytes and zygotes were obtained by hormonal stimulation of female mice. For immunocytochemical staining of oocytes and early embryos, primary polyclonal antibodies to GAP-43 and Ser41-phosphorylated GAP-43 were used.

RESULTS: The intracellular distribution of GAP-43 protein in mouse oocytes (at the metaphase II stage) and early embryos — from the unicellular stage (zygote) to the blastocyst stage — was studied by immunocytochemical assay. In oocytes, there is a uniform distribution of protein throughout the cytoplasm with the highest intensity of staining in the meiotic spindle region. In early embryos, GAP-43 is present in the nuclei and cytoplasm. The relative amount of GAP-43 in the nucleus and cytoplasm varies depending on the stage of embryo development and the cell cycle phase of blastomeres. The phosphorylation of GAP-43 at Ser41 residue, which is characteristic of neurons, is also observed in the nuclei and cytoplasm of early embryo cells. At blastocyst stage, the high expression of GAP-43 is preserved only in the pluripotent cells of the inner cell mass.

CONCLUSIONS: For the first time, we have demonstrated the presence of GAP-43 protein in early mouse embryos. The significant difference between GAP-43 localization in neurons (plasma membrane) and early embryo cells (cytoplasm and nucleus) was revealed. The results suggest a specific role of GAP-43 in toti- and pluripotent cells of early embryos.

Keywords: mouse oocytes; mouse zygotes; early embryogenesis; GAP-43.

НЕЙРОНАЛЬНЫЙ БЕЛОК GAP-43 В РАННИХ ЭМБРИОНАХ МЫШИ

Ф.М. Захарова^{1, 2}, Н.А. Яговкина², В.В. Захаров³¹ Институт экспериментальной медицины, Санкт-Петербург, Россия;² Санкт-Петербургский государственный университет, Санкт-Петербург, Россия;³ Институт высокомолекулярных соединений Российской академии наук, Санкт-Петербург, Россия

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Обоснование. GAP-43 (growth-associated protein 43) — специфический нейрональный белок позвоночных животных, локализованный преимущественно на плазматической мембране аксонных окончаний. GAP-43 играет важную роль в ориентации конусов роста аксонов, в регенеративных процессах в нервной системе и в синаптической пластичности. Недавно нами было показано, что GAP-43 присутствует в ооцитах и зиготах мыши, где он локализован в цитоплазме, что, предположительно, связано с особенностями его экспрессии и модификаций в этих клетках.

Цель — исследование особенностей локализации GAP-43 в клетках ранних (предимплантационных) эмбрионов мыши — от стадии зиготы до стадии бластоцисты.

Материалы и методы. В работе были использованы мыши гибриды F1 C57BL/CBA. Ооциты и зиготы получали с помощью гормональной стимуляции самок. Для иммуноцитохимического окрашивания ооцитов и ранних эмбрионов использовали первичные поликлональные антитела к белку GAP-43 и к его фосфорилированной (по остатку Ser41) форме.

Результаты. С помощью иммуноцитохимического окрашивания было исследовано внутриклеточное распределение белка GAP-43 в ооцитах (на стадии метафазы II) и в ранних эмбрионах мыши — от одноклеточной стадии (зиготы) до стадии бластоцисты. В ооцитах наблюдается равномерное распределение белка по цитоплазме с наибольшей интенсивностью окрашивания в области веретена деления. В ранних эмбрионах GAP-43 присутствует в ядрах и цитоплазме. Соотношение количества GAP-43 в ядре и цитоплазме меняется в зависимости от стадии

развития эмбриона и фазы клеточного цикла бластомеров. Характерное для нейронов фосфорилирование GAP-43 по остатку Ser41 также наблюдается в ядрах и цитоплазме клеток ранних эмбрионов. В бластоцистах высокое содержание белка GAP-43 сохраняется только в плюрипотентных клетках внутренней клеточной массы.

Заключение. В настоящей работе мы впервые продемонстрировали присутствие белка GAP-43 в клетках ранних эмбрионов мыши и выявили существенные особенности локализации белка GAP-43 в них — локализацию в цитоплазме и ядре клеток вместо локализации на плазматической мембране, характерной для нейронов. Полученные данные позволяют сделать заключение об особой роли GAP-43 в toti- и плюрипотентных клетках ранних эмбрионов.

Ключевые слова: ооциты мыши; зиготы мыши; ранний эмбриогенез; GAP-43.

Background

Growth-associated protein 43 (GAP-43) is a specific neuronal protein found in vertebrate species. In neurons, this protein is predominantly associated with the plasma membrane of axon terminals [1–3]. GAP-43 plays an important role in the embryonic development of the nervous system, participating in axonal growth cone guidance. In adult organisms, GAP-43 supports synaptic plasticity and regenerative processes in the nervous system [4–9]. At the molecular level, GAP-43 clusters acidic phosphoinositides in the plasma membrane via its polycationic effector domain under the control of calmodulin and protein kinase C, thereby participating in the regulation of the actin cytoskeleton dynamics [10, 11]. GAP-43 function is presumably mediated by the formation of large oligomer complexes in the presence of acidic phospholipids [12, 13]. GAP-43 belongs to a group of natively disordered proteins, which are characterized by a variety of protein–protein interactions and multiple functions [13]. For a long time, GAP-43 was considered neuron-specific [1, 7]. However, there is currently evidence of GAP-43 presence in glial cells, chromaffin cells [1], podocytes [14], muscle [15], and cancer cells [16]. We have recently shown that GAP-43 is also present in mouse oocytes and zygotes. In metaphase II oocytes, GAP-43 is localized in tubulin-based structures — the meiotic spindle and microtubule-organizing centers — and phosphorylated by protein kinase C [17]. Notably, the expression, modifications and functions of GAP-43 vary significantly across different cell types, whereas its interactions with the main intracellular partners — protein kinase C, calmodulin, actin, and acid phospholipids — are preserved [17]. GAP-43 has also been detected in proliferating pluripotent cells (mouse embryonic carcinoma cells) [18] and multipotent cells (neuronal precursors), where it is localized in the cytoplasm, on centrosomes, and in the chromatin region [19].

The aim of the study was to investigate the localization of GAP-43 in toti- and pluripotent cells of early mouse embryos.

Materials and Methods

C57BL/CBA F1 hybrid mice were used in the study. To obtain oocytes and zygotes, female

mice were hormonally stimulated by administering 10 IU of pregnant mare serum gonadotropin (Mosagrogen, Russia) followed by 10 IU of human chorionic gonadotropin (Intervet International B.V., Netherlands) after 42–46 hours. The animals were euthanized by cervical dislocation 13–20 hours after the administration of human chorionic gonadotropin. The oocyte/zygote–cumulus cell complexes were then collected from the mouse oviduct. The mouse embryos were cultured up to the blastocyst stage in a CO₂ incubator (5% CO₂, 37 °C) in G-1 Plus medium (Vitrolife, Sweden).

Immunocytochemical staining of oocytes and early embryos was performed using a standard protocol [17]. The anti-GAP-43 primary antibodies AB5312 and AB5401 (Millipore, USA) were used at a 200-fold dilution. The secondary antibody, A-11034, conjugated with the Alexa Fluor 488 fluorescent dye (Invitrogen, USA) was used at a 500-fold dilution. Chromosomes were stained with DAPI. The immunostained oocytes and embryos were mounted on glass slides using the Vectashield mounting medium (Vector Laboratories, USA) and analyzed using an LSM-510 Meta confocal microscope (Carl Zeiss, Germany) at the Resource Center for the Development of Molecular and Cellular Technologies (St. Petersburg State University, Russia).

Results and Discussion

Previously, to study GAP-43 in mouse oocytes and zygotes, we used specific polyclonal antibody against rat GAP-43, AB5220, and AB5401 against the phosphorylated (at Ser41) form of GAP-43 (Millipore, USA) [17]. In this study, the distribution of GAP-43 in metaphase II oocytes and early mouse embryos, from the single-cell stage (zygote) to the blastocyst stage, was studied using immunocytochemical staining with polyclonal antibody against cat GAP-43, AB5312 (Millipore, USA).

In oocytes, a uniform distribution of GAP-43 is observed throughout the cytoplasm, with the highest intensity of staining in the meiotic spindle region (see Fig. 1, *a*). In zygotes, GAP-43 is present in the pronuclei (except for the nucleoli) and cytoplasm. Notably, the pronuclei generally exhibit a higher intensity of staining compared to the cy-

toplasm (see Fig. 1, *b*). In 2-cell and 4-cell stage embryos, a similar pattern is observed: GAP-43 is evenly distributed throughout the blastomere cytoplasm and is present in the nuclei, but not in the nucleoli (see Fig. 1, *c*, *d*).

At the morula stage, GAP-43 is also localized in the cytoplasm and nuclei (see Fig. 2, *a*, *b*). In some blastomeres, GAP-43 staining intensity is higher than in other blastomeres of the same embryo. Fig. 2a shows a morula at the beginning of compaction, with two of eight blastomeres having more intense GAP-43 staining. This probably indi-

cates that GAP-43 expression in early embryos is dynamical and depends on the phase of the cell cycle. Consequently, due to the asynchronous division of blastomeres, the amount of GAP-43 in different blastomeres may vary.

At the next stage, the blastocyst, the embryo cells differentiate into the trophoblast (the outer layer of cells of the blastocyst) and the inner cell mass, which later forms a pluripotent epiblast. Fig. 2, *c* shows an early blastocyst with a forming cavity. It is notable that the intensity of GAP-43 staining in the nuclei is higher than that in the cytoplasm.

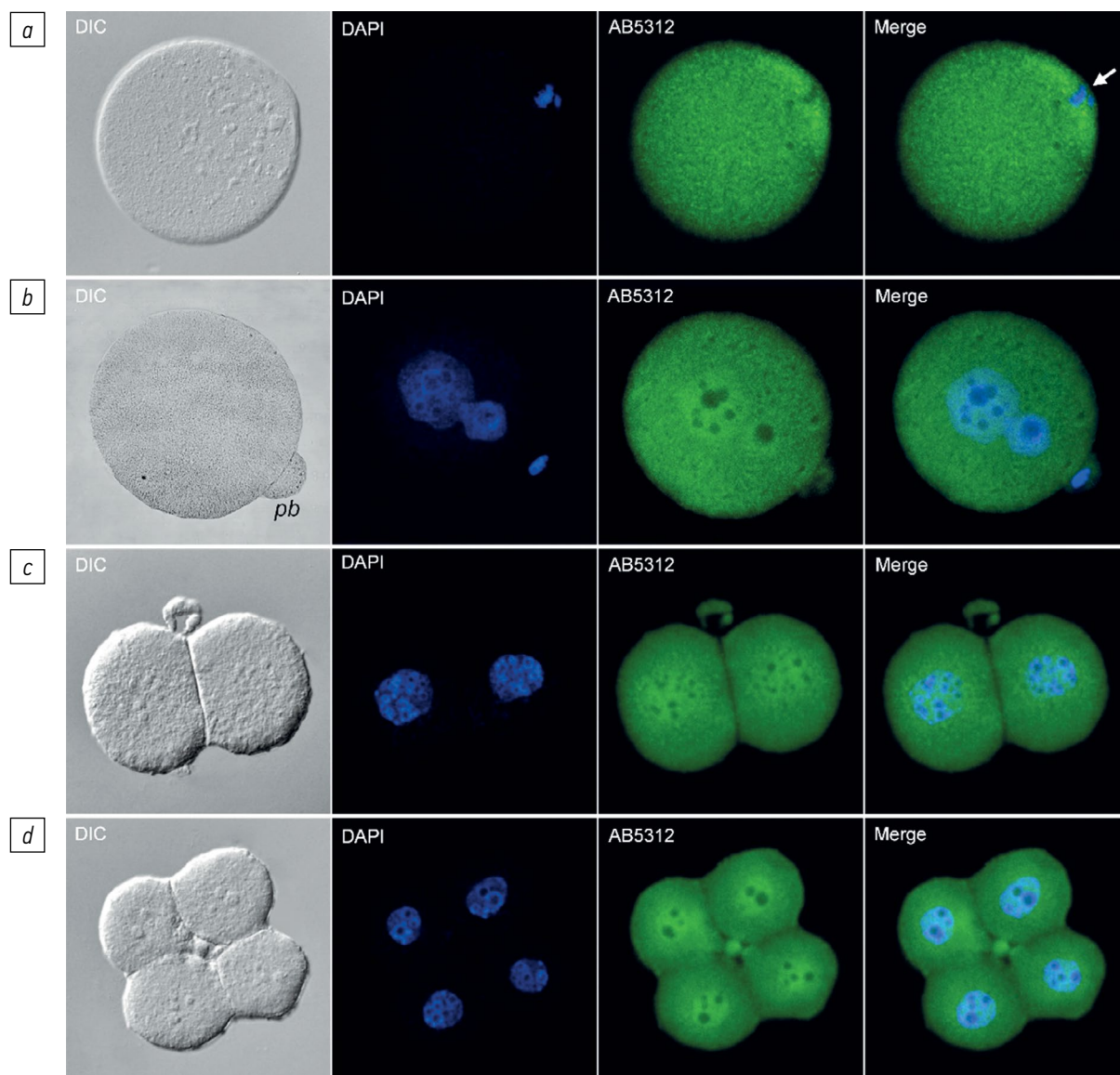


Fig. 1. Localization of GAP-43 in oocytes and early mouse embryos: *a*, metaphase II oocyte (arrow indicates the position of the meiotic spindle); *b*, interphase stage zygote (*pb*, the second polar body); *c*, 2-cell stage embryo; *d*, 4-cell stage embryo. DIC, differential interference contrast; DAPI, DNA staining (blue); AB5312, GAP-43 staining with AB5312 antibodies (green); Merge, DAPI and AB5312 combined

Рис. 1. Локализация белка GAP-43 в ооците и ранних эмбрионах мыши: *a* — ооцит на стадии метафазы II (стрелкой указано положение веретена с хромосомами); *b* — зигота на стадии интерфазы (*pb* — второе полярное тельце); *c* — эмбрион на стадии двух бластомеров; *d* — эмбрион на стадии четырех бластомеров. DIC — дифференциально-интерференционный контраст, DAPI — окрашивание ДНК, AB5312 — окрашивание GAP-43 антителами AB5312, Merge — совмещение каналов DAPI и AB5312

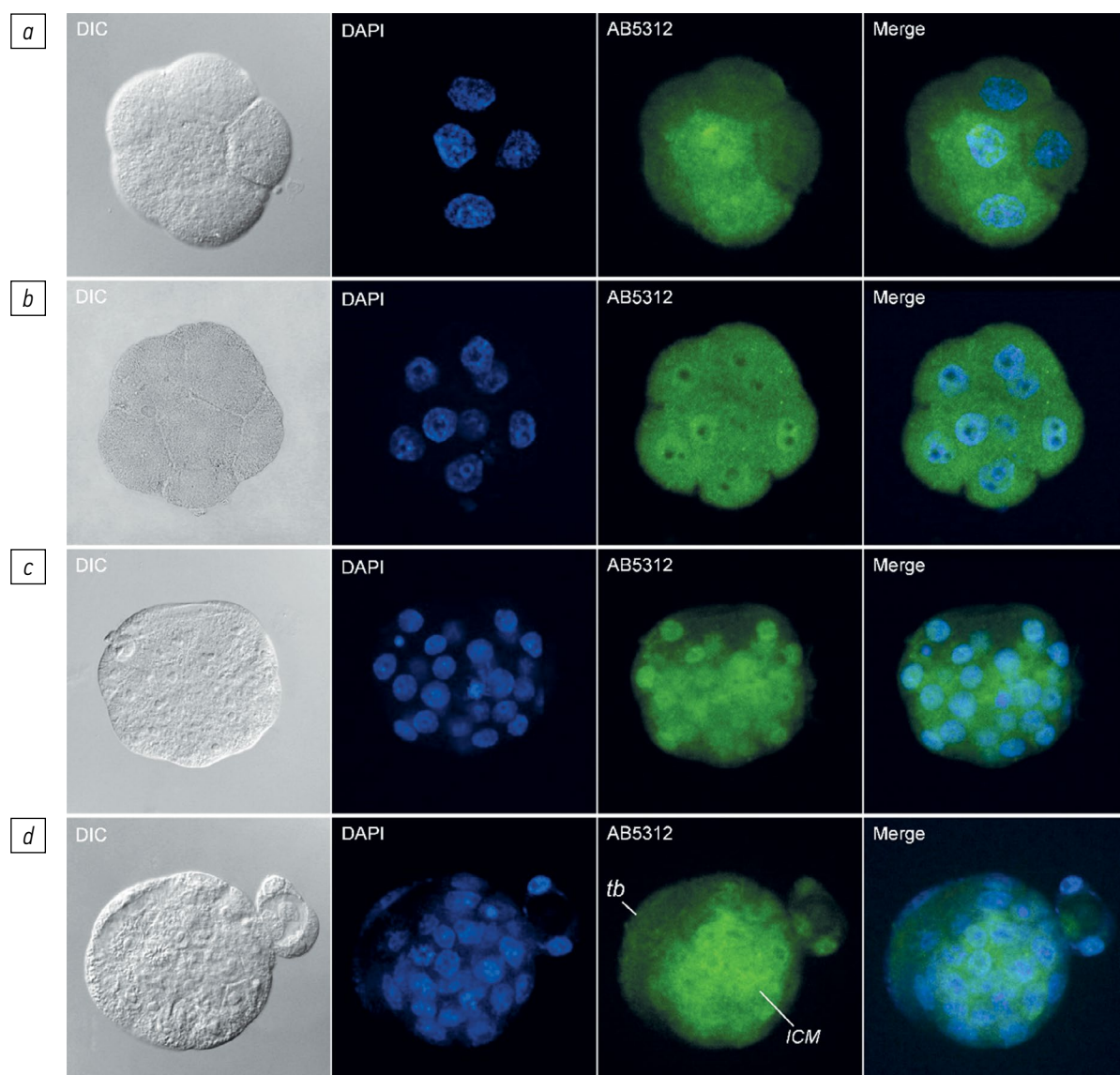


Fig. 2. Localization of GAP-43 in early mouse embryos: *a*, 8-cell stage morula; *b*, late morula; *c*, early blastocyst; *d*, hatching blastocyst (*ICM*, inner cell mass, *tb*, trophoblast cells). DIC, differential interference contrast; DAPI, DNA staining (blue); AB5312, GAP-43 staining with AB5312 antibodies (green); Merge, DAPI and AB5312 combined

Рис. 2. Локализация белка GAP-43 в ранних эмбрионах мыши: *a* — морула на стадии восьми бластомеров; *b* — поздняя морула; *c* — ранняя бластоциста; *d* — бластоциста на стадии выплывания (*ICM* — клетки внутренней клеточной массы, *tb* — клетки трофобласта). DIC — дифференциально-интерференционный контраст, DAPI — окрашивание ДНК, AB5312 — окрашивание GAP-43 антителами AB5312, Merge — совмещение каналов DAPI и AB5312

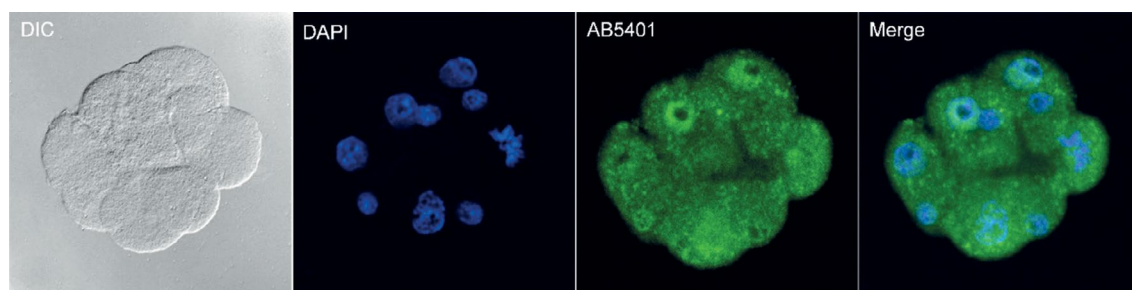


Fig. 3. Localization of Ser41-phosphorylated GAP-43 (pSer41-GAP-43) in morula. DIC, differential interference contrast; DAPI, DNA staining (blue); AB5401, pSer41-GAP-43 staining with AB5401 antibodies (green); Merge, DAPI and AB5401 combined

Рис. 3. Локализация белка GAP-43, фосфорилированного по остатку Ser41, в моруле. DIC — дифференциально-интерференционный контраст, DAPI — окрашивание ДНК, AB5401 — окрашивание pSer41-GAP-43 антителами AB5401, Merge — совмещение каналов DAPI и AB5401

In the blastocyst at a later stage (during hatching from the *zona pellucida*), a more intense staining of the inner cell mass is clearly visible compared to the trophoblast cells (see Fig. 2, *d*).

In this study, we described for the first time the expression of GAP-43 in early mouse embryo cells, from the zygote to the blastocyst stage. In early embryos, GAP-43 exhibits cytoplasmic and nuclear localization, which differs significantly from the predominantly membrane localization of GAP-43 in neurons. Intense staining of the cytoplasm with anti-GAP-43 antibodies is observed in embryos up to the 4–8-cell stage. GAP-43 is also localized in the zygote pronuclei and early embryo nuclei. Thus, the nuclear localization of GAP-43 was demonstrated for the first time. The presence of GAP-43 in the nucleus was also confirmed by antibody AB5401 against the Ser41-phosphorylated form of GAP-43 (Millipore, USA) and anti-rat brain GAP-43 monoclonal antibody G9264 (Sigma-Aldrich, USA). Both antibodies also stain cell nuclei in early mouse embryos (see Fig. 3; for G9264, data are not provided). Staining of the nuclei and cytoplasm with AB5401 indicates that in early embryos GAP-43 is phosphorylated, presumably by protein kinase C as in neurons.

It is well known that the membrane localization of GAP-43 in neurons is associated with palmitoylation of its two residues, Cys3 and Cys4 [20, 21]. Therefore, it can be concluded that GAP-43 is not palmitoylated in early mouse embryos. As we suggested earlier, the absence of palmitoylation may be explained by the expression of the short isoform of GAP-43 (GAP-43-2), lacking residues 1–4 and truncated at the N-terminus due to the alternative translation initiation from the Met5 codon [17]. We analyzed the amino acid sequence of GAP-43 using computer algorithms designed to predict the subcellular localization of the protein. The PSORT II [22], SCLpred [23], ESLPred2 [24], and Cello v.2.5 [25] programs predict nuclear localization of GAP-43. The MultiLoc2 [26] and BUSCA [27] programs predict nuclear/cytoplasmic localization of GAP-43. The cNLS Mapper [28] and NLStradamus [29] programs predict that the polycationic effector domain of GAP-43 contains a monopartite nuclear localization signal (residues 43–57). It is interesting to note that the two GAP-43-related proteins, MARCKS and BASP1, have also been shown to contain a nuclear localization signal in their effector domains [30, 31]. Thus, the prediction algorithms confirm the nuclear and cytoplasmic localization of unpalmitoylated GAP-43 revealed in the present study.

Comparing the data obtained using two polyclonal antibodies against GAP-43, AB5312 (this study) and AB5220 (previous study [17]), two differences can be noted. (1) AB5312 produce more

intense staining of the cytoplasm than AB5220. Consequently, staining of tubulin-based structures (the meiotic spindle and microtubule-organizing centers) with AB5312 is not as pronounced, although staining of the spindle region is still more intense than staining of the cytoplasm (see Fig. 1, *a*). (2) Unlike AB5220, AB5312 stains cell nuclei and zygote pronuclei. Thus, AB5220 and AB5312 have different specificities to the nuclear and cytoplasmic pools of GAP-43. Both AB5220 and AB5312 are rabbit polyclonal antibodies, but their immunogens are different: they were raised against rat and cat GAP-43, respectively. Approximately 20% of amino acid residues in the rat and cat GAP-43 sequences are different, which may result in different epitopes for these antibodies.

Based on the results of this and previous [17] studies, three different intracellular pools of GAP-43 can be distinguished in mouse oocytes and early embryos: (a) cytoplasmic, (b) nuclear, and (c) associated with tubulin-based structures (the meiotic spindle and microtubule-organizing centers). Moreover, our preliminary data show that these pools are selectively stained with different anti-GAP-43 monoclonal antibodies (the manuscript is being prepared for publication). This is probably due to various modifications of GAP-43 in these pools, which will be investigated in future work.

Early (preimplantation) embryos attract special interest due to the unique properties of their cells — toti- and pluripotency. In placental mammals, totipotency (the potential to develop into a complete organism from a single cell) includes the ability to generate both the embryo and the extraembryonic tissues. For mouse embryos, it has been shown that only the zygote and a single blastomer of the 2-cell and 4-cell embryos are totipotent [32]. Blastomeres of mouse embryos at later stages of development no longer possess this property. At the late blastocyst stage (before implantation into the uterus), a subset of cells within the inner cell mass forms the pluripotent epiblast. Epiblast cells are capable of differentiating into cells of all three embryonic germ layers; however, they are not capable of forming extraembryonic tissues. The epiblast is a source for obtaining pluripotent embryonic stem cells *in vitro*. In this study, GAP-43 was detected in mouse totipotent and pluripotent embryonic cells. In this regard, it should be noted that there are several reports on the expression of GAP-43 in pluripotent cells of mouse embryonic carcinoma. In these cells, *GAP43* gene transcription was found to be initiated from a promoter different from the one responsible for *GAP43* transcription in neurons [18, 33]. GAP-43 has also been detected in human embryonic stem cells [34] and cancer cells [16]. This suggests that GAP-43 plays a special role in undifferentiated cells.

Conclusion

In this study, we demonstrated for the first time the presence of GAP-43 in early mouse embryo cells, from the zygote to the blastocyst stage. In early embryo cells, GAP-43 is localized in the cytoplasm and nucleus, unlike in neurons, where it is predominantly associated with the plasma membrane. Since the putative role of GAP-43 in neurons is related to the regulation of the dynamics of the plasma membrane and actin cytoskeleton, our findings suggest specific functions of GAP-43 in toti- and pluripotent cells of early embryos. Our findings are also important for studying the role of GAP-43 in other undifferentiated (stem and cancer) cells.

Additional information

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Ethics approval. The protocol of the study was approved by the local Ethics Committee of the Institute of Experimental Medicine № 2/22 dated 2022 April 6.

Competing interests. The authors declare that they have no competing interests.

Author contribution. All authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study.

Personal contribution of each author: *F.M. Zakharova, V.V. Zakharov* — concept and design of the study, analytical processing of the results, writing the text of the article; *F.M. Zakharova, N.A. Yagovkina* — working with animals, immunocytochemical experiments, data analysis; *F.M. Zakharova* — working on a confocal microscope; *V.V. Zakharov* — working with databases and programs.

Дополнительная информация

Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

Этический комитет. Выполнение исследования одобрено протоколом этического комитета Института экспериментальной медицины № 2/24 от 25.04.2024.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Наибольший вклад распределен следующим образом: *Ф.М. Захарова, В.В. Захаров* — концепция и дизайн исследования, аналитическая обработка результатов, написание текста статьи; *Ф.М. Захарова, Н.А. Яговкина* — работа с животными, проведение иммуноцитохимических экспериментов, обзор результатов; *Ф.М. Захарова* — работа на конфокальном микроскопе; *В.В. Захаров* — работа с базами данных и программами.

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Information about the authors / Информация об авторах

Institute of Experimental Medicine, Saint Petersburg, Russia
 ФГБНУ «Институт экспериментальной медицины», Санкт-Петербург, Россия

Saint Petersburg State University, Saint Petersburg, Russia
 ФГБОУ ВО «Санкт-Петербургский государственный университет», Санкт-Петербург, Россия

Faina M. Zakharova — Cand. Sci. (Biology),
 Senior Research Associate
 at the Department of Molecular Genetics;
 Senior Lecturer at the Department of Embryology.
 ORCID: 0000-0002-9558-3979;
 eLibrary SPIN: 9699-5744;
 e-mail: fzakharova@mail.ru

Фаина Михайловна Захарова — канд. биол. наук,
 старший научный сотрудник отдела
 молекулярной генетики;
 старший преподаватель кафедры эмбриологии.
 ORCID: 0000-0002-9558-3979;
 eLibrary SPIN: 9699-5744;
 e-mail: fzakharova@mail.ru

Saint Petersburg State University, Saint Petersburg, Russia

ФГБОУ ВО «Санкт-Петербургский государственный университет», Санкт-Петербург, Россия

Nadezhda A. Yagovkina — 2nd year graduate student
of Faculty of Biology,
Departments of Embryology.
ORCID: 0009-0002-3090-9621;
e-mail: st110082@student.spbu.ru

Надежда Андреевна Яговкина — студентка 2-го курса
магистратуры биологического факультета,
кафедра эмбриологии.
ORCID: 0009-0002-3090-9621;
e-mail: st110082@student.spbu.ru

Institute of Macromolecular Compounds of the Russian Academy of Sciences, Saint Petersburg, Russia

ФГБУН «Институт высокомолекулярных соединений Российской академии наук», Санкт-Петербург, Россия

Vladislav V. Zakharov — Cand. Sci. (Biology),
Research Associate at the Laboratory No. 5
(natural polymers).
ORCID: 0000-0002-7871-632X;
eLibrary SPIN: 1203-0639;
e-mail: vlad.v.zakharov@mail.ru

Владислав Викторович Захаров — канд. биол. наук,
научный сотрудник лаборатории № 5
(природных полимеров).
ORCID: 0000-0002-7871-632X;
eLibrary SPIN: 1203-0639;
e-mail: vlad.v.zakharov@mail.ru

✉ **Corresponding author / Контактное лицо**

Faina M. Zakharova / Фаина Михайловна Захарова

Address: 12 Academician Pavlov St., Saint Petersburg, 197022, Russia

Адрес: Россия, 197022, Санкт-Петербург, ул. Академика Павлова, д. 12

E-mail: fzakharova@mail.ru