# Localization of Green Fluorescent Protein in Mouse Preimplantation Embryos

**A. A. Smirnov, N. V. Shishova*, N. Yu. Sakharova, A. M. Malashenko**, I. I. Selezneva, and B. K. Gavrilyuk**

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The localization of enhanced green fluorescent protein (EGFP) was studied in preimplantation embryos obtained from reciprocal mating of hemizygous C57Bl/6-Tgn (ACTbEGFP)1Osb/J mice with C57Bl/6 mice. Specific fluorescence of EGFP was observed in all oocytes and embryos obtained from transgenic females during all preimplantation stages and in embryos inheriting the EGFP gene from transgenic males starting from the 8 blastomere stage during the compactization period. EGFP mRNA or EGFP synthesized during oogenesis can be retained in embryos during the entire preimplantation period, while expression of EGFP gene transferred from the father coincides with the onset of compactization. The possibility of using these embryos in experimental mammalian embryology is discussed.

**Key Words:** *EGFP; mouse embryos; preimplantation development*

Green fluorescent protein (GFP) attracts the attention of specialists as a vital cell marker. GFP gene is widely used in biological studies as an extremely convenient reporter gene allowing visualization of GFP-containing cells under a fluorescent microscope. Using this method, the fate of certain cell populations (proliferation, differentiation, and migration of "labeled cells" in the body and during *in vitro* culturing) [5,6,9] can be traced.

The use of GFP as a vital marker is promising for modern biotechnological studies on early embryonic cells of mammals or embryonic stem cells [12]. For example, using this marker, it is possibly to trace the fate of early blastomeres during the preimplantation development and detect them during the postimplantation period [13].

The fact that GFP does not disturb normal development [8,11,13] opens new vistas for experimental embryology. The potentialities provided by GFP make it particularly promising for studies of early mammalian embryogenesis, for example, for clearing out the intricate cell-cell interactions essential for the fate of individual blastomeres and their descendants [10]. Injection of GFP reporter gene or the corresponding mRNA into the pronuclei or nuclei of early blastomeres often disturbs early development [4]. The use of transgenic animals seems to be a more safe and reliable method for evaluation of the GFP status in a developing embryo. In this case, it is important to know the parameters of GFP manifestation in embryonic cells depending on which of the parents was the carrier of the "green" protein gene.

We investigated the manifestation of green protein gene in early embryos obtained from hemizygous C57Bl/6-Tgn(ACTbEGFP)1Osb/J (B6-GFP) females or males mated with C57Bl/6 mice (B6).

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino; *Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino; **Research Center of Biomedical Technologies, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** nsakcharova@rambler.ru. N. Yu. Sakharova

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## MATERIALS AND METHODS

Mature oocytes were isolated from the ovaries treated with 1% collagenase, 48 h after injection of Follimag (gonadotropic preparation; Mosagrogen). The embryos at the zygote stage were isolated from the oviduct ampoule on day 1 of gestation (on the day of detection of the copulation plug in females).

Embryos at the stages of 2, 3, and 8 blastomeres, morula, and blastocyst were obtained by washing out from the oviducts and upper compartments of the uterine horns on days 2, 3, and 4 of gestation, respectively. Reciprocal mating of B6-GFP and B6 mice was used.

The resultant embryonic material in a drop of Witten's medium was transferred onto a slide with a well, covered with a coverslip, and examined under an Axioscop 40FL fluorescent microscope with a set of 09 filters (BP 450-490 nm, FT 510 nm, LP 515 nm; Zeiss) at λ=440-450 nm. The images were analyzed on a computer. A total of 82 embryos were used.

Transgenic mice with the reporter gene encoding enhanced green fluorescent protein (EGFP) synthesis under chicken β-actin promotor were obtained from Jackson Laboratory due to kind permission of A. V. Chervonsky. The location of EGFP was studied in mature oocytes and preimplantation embryos.

## RESULTS

Study of oocytes from hemizygous females carrying EGFP gene detected even intensive fluorescence of the oocyte cytoplasm and the first reduction body in all cells (Fig. 1). The cumulus cells surrounding the oocyte exhibited dull background fluorescence, which attested to its inspecific nature. The absence of GFP fluorescence in follicular cells was presumably due to epigenetic changes determined by chromatin methylation or acetylation in differentiated cells.

All two-blastomere embryos obtained from hemizygous females exhibited bright green fluorescence of the cytoplasm. At this stage, the reduction body exhibits specific EGFP fluorescence (Fig. 2, *a*, *b*) or does not exhibit it (Fig. 2, *c*, *d*). The absence of specific fluorescence can be explained by denaturation of this protein [5], which can be an initial sign of destruction of the reduction body. Under fluorescent microscope we can see a cytoplasmic bridge in the furrow dividing the first two blastomeres (Fig. 2, *c*, *d*).

All examined four-blastomere embryos exhibited specific fluorescence of the cytoplasm. Stained regions between the blastomeres indicate presumably retained cytoplasmic bridges between divided cells (Fig. 3, *a*, *b*). Cytoplasmic bridges between the blastomeres were of different width; the number of bridges connecting blastomeres also varied. Our morphological findings attest to intricate cell-cell relationships during the four-blastomere stage, which determine the subsequent fate of their descendants [2,10].

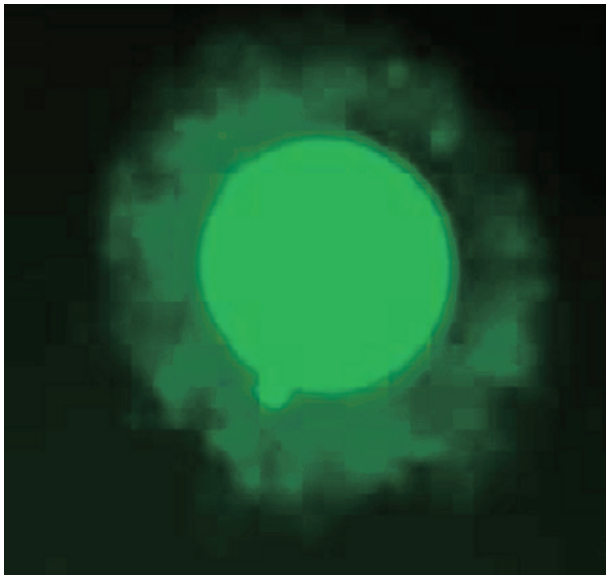

**Fig. 1.** Specific fluorescence of EGFP in oocyte from the ovary of B6-GFP female.

At the stage of eight blastomeres, all embryos also exhibited bright fluorescence of the cytoplasm, which persisted during the next stage (compact multicellular morule) (Fig. 4, *a*). Intensive specific fluorescence of the inner cell mass (ICM) during the blastocyst formation was noted in all embryos, but in the forming trophoblast (TB) the fluorescence intensity decreased and only some brightly fluorescing sites were detected (Fig. 4, *b*).

Thus, analysis of embryonic material from homizygous females carrying EGFP gene showed that specific fluorescence of this protein manifested in all studied objects from mature oocyte to embryos at the last preimplantation stage (blastocyst). After mating of B6-GFP females with B6 males, only some embryos carry EGFP gene, but specific fluorescence was detected in all embryos. Hence, EGFP and/or its mRNA synthesized in the oocytes of heterozygous females persisted in all embryos irrespective of their own genome. This is in line with the data indicating that mRNA is actively synthesized during the early oogenesis and the greater part of this mRNA is utilized in subsequent embryogenesis. It is known that up to 30% maternal mRNA is retained for more than 2 days and is

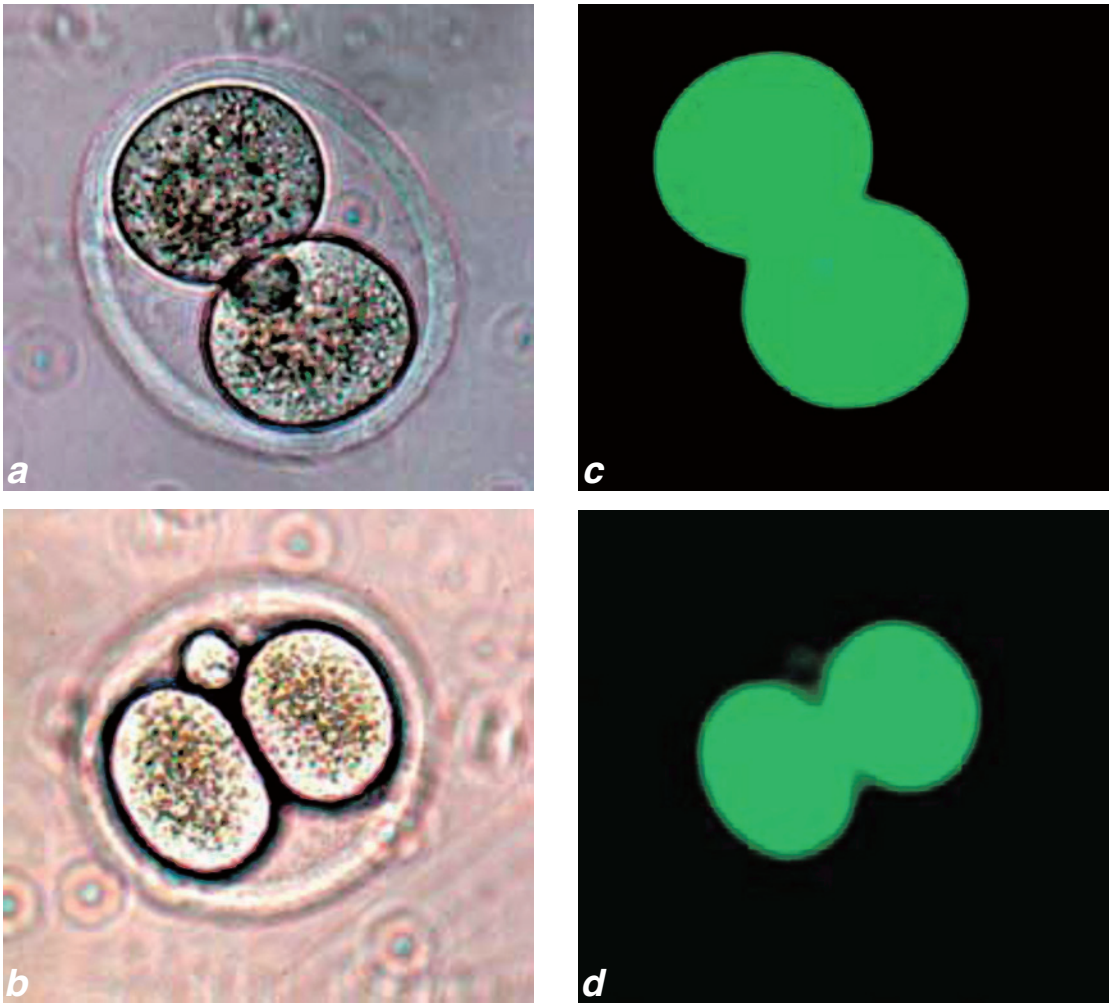


**Fig. 2.** Two-cell embryo of a B6-GFP female with different status of the reduction body. *a*, *b*) embryo with a reduction body, containing EGFP, in transmitting light (*a*) and in fluorescent light (*b*); *c*, *d*) embryo with cytoplasmic bridges between blastomeres and reduction body without EGFP fluorescence: *c*) in transmitting light; (*d*) in fluorescent light.

active even during the blastocyst formation [2]. On the other hand, proteins, synthesized during the oogenesis, are spent for the construction of the outer plasma membrane and membranes of the endoplasmic reticulum, *i.e.* are also utilized by the embryo [1]. The majority of proteins synthesized on maternal matrices are highly stable and detected at the early stages of division and in the blastocyst. It can be hypothesized that the difference in the fluorescence of ICM and TB is determined by the presence of maternal mRNA and EGFP in undifferentiated ICM cells and decreased expression and/or increased degradation of EGFP in differentiating TB cells.

In embryos from B6 females mated with B6-GFP males no fluorescence at the zygote stage or during subsequent stages of early division were noted. It appeared only at the stage of eight blastomeres and coincided with initial compactization (early morula) in only some embryos (Fig. 5). In some embryos, specific fluorescence appeared at all stages of the blastocyst formation until its release from *Z. pellucida* (Fig. 6). These data suggest that expression of EGFP gene inherited by the embryos from the male is first detected at the 8-cell stage, when compactization starts. Specific EGFP fluorescence was detected in half of embryos analyzed during this period (in 7 of 13). This proportion of fluorescent to nonfluorescent embryos (1:1) coincides with expected theoretical Mendelian ratio for the mating pattern used in this study. It seems that the detected fluorescence was really associated with the start of expression of EGFP gene inherited from the farther. This expression is delayed in comparison with the start of the main genome activity, which, as we know, is observed at the stage of two blastomeres. Our data are in line with the results of evaluation of the start of EGFP gene expression in

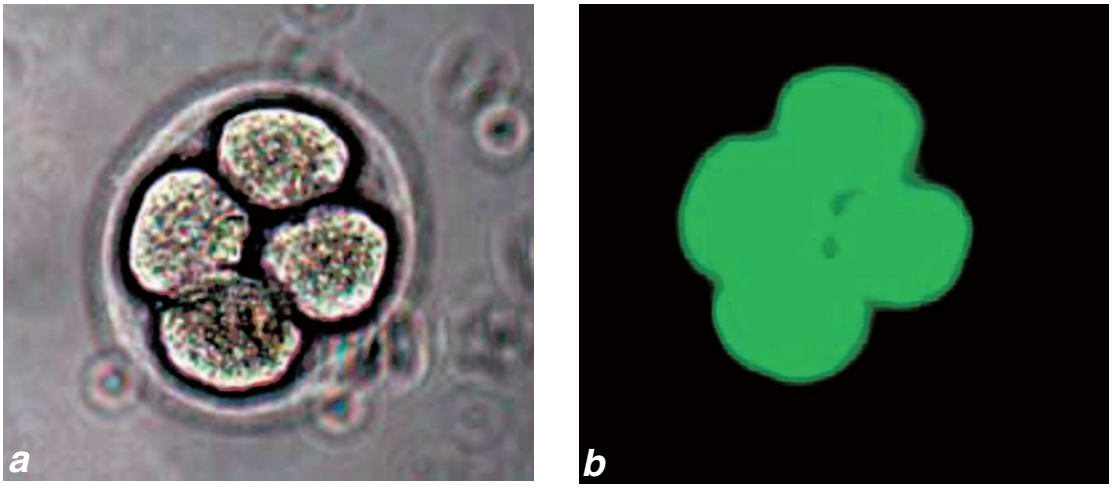


**Fig. 3.** Four-cell embryo from a B6-GFP female. *a*) in transmitting light; (*b*) in fluorescent light.

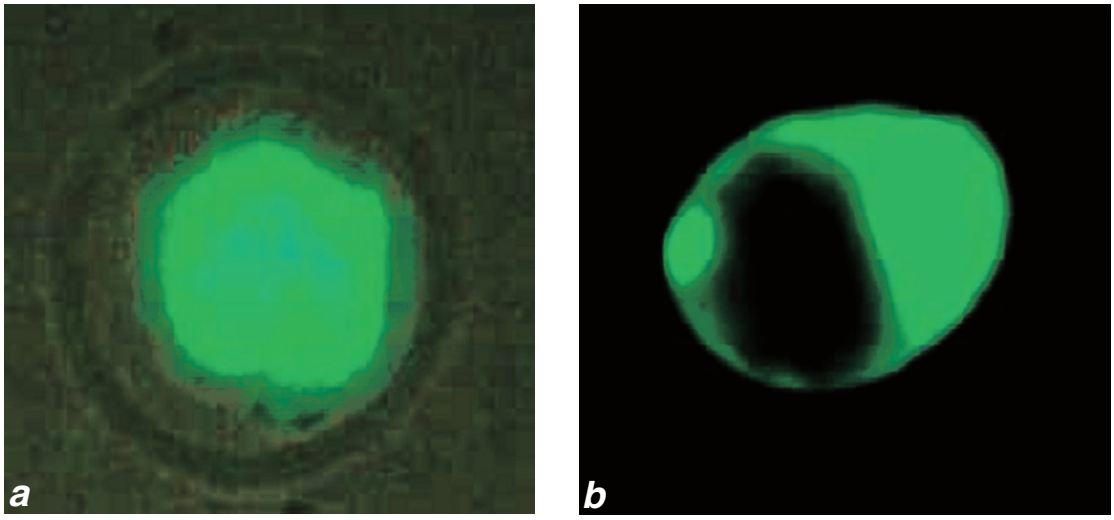


**Fig. 4.** Specific fluorescence of EGFP in embryos from B6-GFP female: morula (*a*) and blastocyst (*b*) stages.

another recombinant DNA (R26-EGFP-SV40polyA) [4] and showing that expression of this protein in mouse embryos first appears during transition to the 8-cell stage.

Our observations indicate that EGFP mRNA and, presumably, this protein synthesized in oocytes during the previtellogenesis in hemizygous B6-GFP females are retained in embryos irrespective of their own genotype during the entire preimplantation period. The location of the green protein at the early division stages in the division furrows can be helpful in studies of cell-cell relations in live embryos presumably determining the differences in the fate of descendants from the first blastomeres [10]. Blastomeres from early embryos obtained from heterozygotic B6-GFP females can be used in experiments on creation of chimeric embryos. Manipulations with this material require no preliminary control under a fluorescent microscope (exposure to its radiation can reduce embryo viability). Our observations showed that the fluorescent label was retained in cells from these embryos during the entire preimplantation period. However, in order to trace the changes in the cells at later developmental stages, for example, after the blastocyst release from *Z. pellucida* and under conditions of *in vitro* culturing of ICM cells, the embryos receiving the EGFP gene from the male should be used. In this case we can be sure that cytoplasm fluorescence is caused by expression of the EGFP gene. Embryos at the early morula stage for this analysis should be selected under a fluorescent microscope. It is noteworthy that the resistance to a harmful external exposure increases significantly in embryos during this stage in comparison with earlier stages [3]. One more reason why the embryos carrying the parental EGFP gene are preferable for long experiments is that the negative effect of high dose of

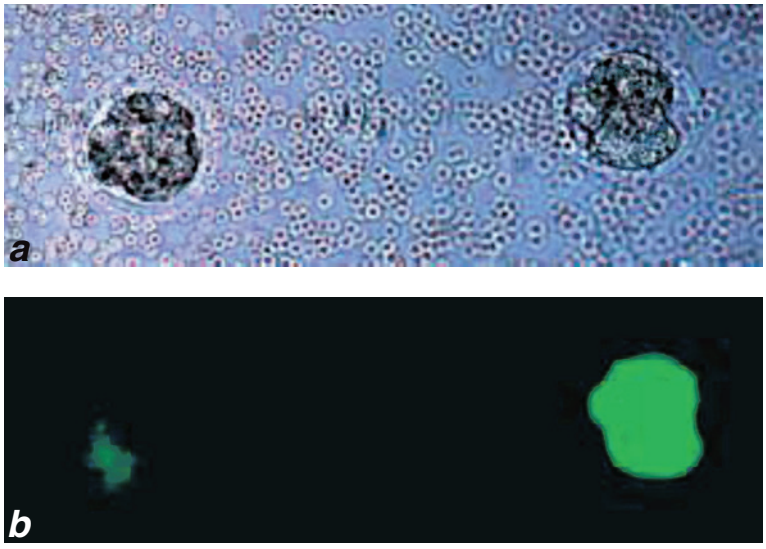


**Fig. 5.** Embryos from B6 female mated with B6-GFP male at the early morula stage. *a*) in transmitting light: two embryos and numerous blood cells occurring in culture medium; (*b*) in fluorescent light: only one embryo exhibits specific fluorescence.

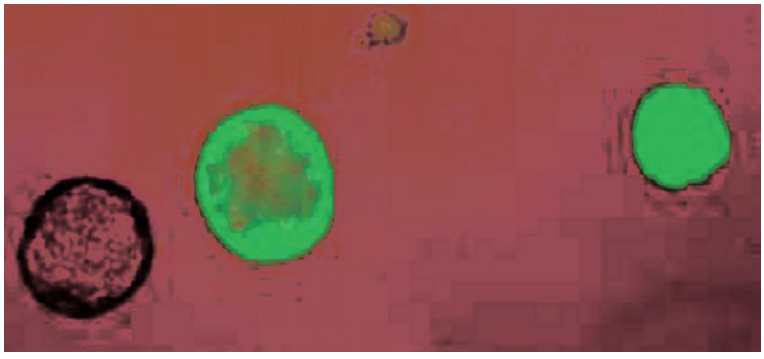

**Fig. 6.** Embryos from B6 female mated with B6-GFP male at late stages of preimplantation development. The picture made at combined illumination: fluorescent (green spectrum) and transmitting light. Left: two late (dilated) blastocysts, one carrying the EGFP gene; right: blastocyst released from *Z. pellucida* also carrying the EGFP gene.

green protein does not manifest in them [7], while in embryos from hemizygous females this effect is possible because EGFP synthesis is realized simultaneously on the embryonic and maternal matrices.

Hence, we detected the location of the green fluorescent protein in mouse early embryonic cells, depending on the source of EGFP gene in them. These data should be taken into consideration when using such embryos in experimental studies.

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## REFERENCES

1. L. V. Belousov, *Fundamentals of General Embryology* [in Russian], Moscow (2005).
2. S. Gilbert, *Developmental Biology* [in Russian], Moscow (1994).
3. L. M. Mezhevikina, N. Yu. Sakharova, and B. N. Veprintsev, *Problems in Preservation and Maintenance of Genetic Collections of Laboratory Animals* [in Russian], Pushchino (1991), pp. 102-112.
4. A. V. Sorokin, E. M. Noniashvili, L. K. Sasina, *et al.*, *Tsitologiya*, **46**, No. 10, 942-943 (2004).
5. M. Chalfic, J. Tu, G. Euskirchen, *et al.*, *Science*, **263**, 802-805 (1994).
6. A. B. Cubitt, R. Heim, S. R. Adams, *et al.*, *Trends Biochem. Sci.*, **20**, No. 11, 448-455 (1995).
7. V. Devgan, M. R. Rao, and P. B. Seshagiri, *Biochem. Biophys. Res. Commun.*, **313**, No. 4, 1030-1036 (2004).
8. M. Hara, X. Wang, T. Kawamura, *et al.*, *Am. J. Physiol. Endocrin. Metab.*, **284**, No. 1, E177-E183 (2003).
9. K. A. Lukyanov, D. M. Chudakov, S. Lukyanov, and V. V. Verkhusha, *Nat. Rev. Mol. Cell. Biol.*, **6**, No. 11, 885-891 (2005).
10. A. K. Tarkowski, W. Ozdenski, and R. Czolowska, *Int. J. Dev. Biol.*, **45**, No. 7, 811-816 (2001).
11. M. Xian, N. Hondo, J. Zhang, *et al.*, *J. Mol. Cell. Cardiol.*, **31**, No. 12, 2155-2165 (1999).
12. Xu Xin, J. Sh. Yu, H. C. Tsung, *et al.*, *Cell Res.*, **9**, No. 3, 201-208 (1999).
13. M. Zernica-Goetz, J. Pines, S. McLean Hunter, *et al.*, *Development*, **124**, No. 6, 1133-1137 (1997).