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Basic Neuroscience

# Pitfalls and fallacies interfering with correct identification of embryonic stem cells implanted into the brain after experimental traumatic injury

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## HIGHLIGHTS

- Identification of GFP-transfected cells transplanted into injured rat brains by intrinsic fluorescence proved difficult.
- At early time points transfected cells were identified by immunostaining.
- At later stages autofluorescent cerebral cells impeded ES cell identification.
- Staining procedure were implemented to circumvent these difficulties.
- Improved labeling protocols allowed for the correct identification of ES cells and the detection of stem cell phagocytosis.

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## ABSTRACT

Cell-therapy was proposed to be a promising tool in case of death or impairment of specific cell types. Correct identification of implanted cells became crucial when evaluating the success of transplantation therapy. Various methods of cell labeling have been employed in previously published studies. The use of intrinsic signaling of green fluorescent protein (GFP) has led to a well known controversy in the field of cardiovascular research. We encountered similar methodological pitfalls after transplantation of GFP-transfected embryonic stem cells into rat brains following traumatic brain injury (TBI). As the identification of implanted graft by intrinsic autofluorescence failed, anti-GFP labeling coupled to fluorescent and conventional antibodies was needed to visualize the implanted cells. Furthermore, different cell types with strong intrinsic autofluorescence were found at the sites of injury and transplantation, thus mimicking the implanted stem cells. GFP-positive stem cells were correctly localized, using advanced histological techniques. The activation of microglia/macrophages, accompanying the transplantation post TBI, was shown to be a significant source of artefacts, interfering with correct identification of implanted stem cells. Dependent on the strategy of stem cell tracking, the phagocytosis of implanted cells as observed in this study, might also impede the interpretation of results. Critical appraisal of previously published data as well as a review of different histological techniques provide tools for a more accurate identification of transplanted stem cells.

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*Abbreviations:* CsA, cyclosporin A; Cy3, fluorochrome cyanine 3; DAB, 3,3-diaminobenzidine; EScell, embryonic stem cell; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; NBT/BCIP, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; TBI, traumatic brain injury; USPIO, ultrasmall superparamagnetic iron oxide nanoparticle.

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## 1. Introduction

No effective therapy is currently available to promote the recovery from various diseases of central nervous system or cardiovascular system associated with an irreversible cell loss such as Parkinson disease or myocardial infarction. Cell replacement strategies have been proposed to be promising therapeutic alternative in such case (Bjorklund et al., 2002; Richardson et al., 2010; Strauer et al., 2002). In addition to functional and behavioral testing the correct identification and localization of implanted cells are crucial for the evaluation of the transplantation strategies. Various methods of cell labeling and trafficking have been employed in previously published studies. One of the widely used technologies implemented the intrinsic autofluorescence of green fluorescent protein (GFP), introduced into the stem cells prior to implantation (Burns et al., 2009; Cao et al., 2002; Richardson et al., 2010).

This strategy lead to a well-known controversy in the field of cardiovascular research. Promising results of Anversa et al., claiming the GFP-labeled bone marrow stem cells to be repopulating the zone of myocardial infarction and thus healing a damaged heart, were critically questioned by Murry (Murry et al., 2004; Orlic et al., 2001, 2003; Pearson, 2004). Based on Anversa's experimental data, first clinical trials on human subjects were initiated, only to be stopped soon after several other research groups failed to reproduce Anversa's initial experiment (Strauer et al., 2002). GFP-positive fluorescent cells that Anversa identified inside the myocardial infarction were later suspected to be autofluorescent artefacts (Pearson, 2004).

We report here similar methodological problems following the implantation of GFP-fluorescent stem cells into the injured brain. Survival, migration and differentiation of GFP-transfected pluripotent embryonic stem cells was reported following transplantation into rat brains in an experimental stroke model (Erdo et al., 2003, 2004; Hoehn et al., 2002). Based on these results we have implanted the same cells (using identical cell passage/clone) into the cortex of rat brains after the induction of a moderate traumatic brain injury (TBI), as previously published (Molcanyi et al., 2007; Riess et al., 2007).

The encouraging data of the above mentioned stroke studies was not reproduced in the model of TBI. We hypothesize that the conflicting results might partly be due to different methodological strategies used for cell detection and cell tracking. The identification of implanted cells by intrinsic GFP-fluorescence turned out to be challenging. The activation of microglia/macrophages, accompanying the implantation of stem cells post TBI was demonstrated to be a significant source of fallacies interfering with the correct identification of implanted stem cells. Migration and integration of embryonic stem cells as reported by Erdo et al. was not observed in the rat brain (Erdo et al., 2003, 2004).

In this study we encountered various methodological pitfalls including primary false-positive identification of implanted stem cells. Critical appraisal of different histological techniques helped us to acquire correct results. The impact of these findings on previously published data and current technologies implementing both in vitro localization and in vivo cell trafficking is discussed in detail.

## 2. Material and methods

### 2.1. Animal model

All experiments were performed in accordance with animal protection guidelines, and were approved by the local government authorities of North-Rhine Westphalia. Adult male Sprague-Dawley rats (weighting 250–300 g, age 12–14 weeks, supplied by Harlan-Winkelmann) were given food and water ad libitum. The animals were anesthetised with 60 mg/kg body weight pentobarbital intraperitoneally (i.p.) and then surgically prepared for lateral fluid-percussion brain injury or sham operation as originally described (McIntosh et al., 1989; Thompson et al., 2005). In brief, a 5-mm craniectomy was performed over the left parietal cortex, between lambda and bregma, leaving the dura mater intact. A hollow female Luer-Lock fitting was positioned over the craniectomy and held in place with dental cement. Animals were attached to the fluid-percussion device (containing a saline-filled cylinder) via the female Luer-Lock. Moderate/severe brain injury was then induced by a rapid injection of a pressure pulse of saline into the closed cranial cavity. Sham-operated animals were anesthezised and surgically prepared as described, but were not subjected to brain injury. After brain injury or sham-operation, the Luer-lock and the dental cement were removed, craniectomy left open and the skin was sutured. Animals recovered after injury or sham- operation on heating pads to maintain normothermia. All surgical procedures were performed on spontaneously breathing animals. Depth of anesthesia was monitored by breathing excursions of the thorax wall.

A group of $n = 36$ animals received a brain injury of moderate severity (exerted pressure = 2.3 atm). Another group $n = 13$ underwent only the initial surgical procedure without any injury and served as sham-operated (ShamOP) controls.

Twenty-four hours prior to implantation, animals received an intraperitoneal (i.p) injection of cyclosporin A (CsA, 10 mg/kg body

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