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Time-dependent segmentation of BrdU-signal leads to late detection problems in studies using BrdU as cell label or proliferation marker

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ABSTRACT

Bromodeoxyuridine incorporates into DNA during mitosis. A long-term stability of the incorporated BrdU is important for the recovery of BrdU-labeled cells. For testing the stability of BrdU incorporation into DNA we pulse-labeled mesenchymal stem cells with BrdU and observed these cells *in vitro* over 4 weeks. During this time the BrdU-signal was permanently decreasing. Starting with cells containing evenly stained BrdU-nuclei, so-called *filled* cells, already 3 days after BrdU removal we detected cells containing so-called *segmented* and *punctated* BrdU-signals. The number of those labeled cells continuously increased over time. Interestingly, the loss of BrdU in the nucleus was accompanied by an increasing labeling of the cytosol. Further, we injected BrdU intraperitoneally into rats after ischemia and detected BrdU-positive cells in the hippocampus 3 and 23 days after the last BrdU injection. While after 3 days most of the BrdU-positive cells in the hippocampus displayed a *filled* BrdU-signal, 23 days after BrdU removal an increased number of *segmented* and *punctated* BrdU-positive nuclei was detected. The gradual degradation of the BrdU-signal was not caused by cell death. The consequence of this BrdU degradation would be an underestimation of cell proliferation and an overestimation of cell death of newly generated cells.

1. Introduction

Already two decades ago, Miller and Nowakowski (1988) introduced the thymidine analog 5-bromo-2-deoxyuridine (BrdU) to examine the proliferation and migration of cells in the central nervous system. In comparison to [^{3}H]-thymidine, BrdU allows faster studies, the labeled cells are detectable in relatively thick tissues and, more interestingly, additional labeling can be performed with markers specific for different cell types. With the advance of confocal microscopy, BrdU-labeling allows the phenotypic identification of proliferated cells and their stereological quantification. BrdU has been proven to be a suitable marker for proliferating cells in numerous *in vitro* studies as well as *in vivo* studies (Thored et al., 2006; Lichtenwalner and Parent, 2006; Leuner et al., 2006; Taupin, 2006). Those *in vivo* studies include the identification of newly generated endogenous cells in the intact or damaged brain and in functional contexts such as learning and memory (Shors, 2006; Leuner et al., 2006). However, BrdU can also induce or enhance cell proliferation and alter the phenotype of cells. These activities mediated at the transcriptional level have profound consequences on cell proliferation and fate determination when using BrdU-labeling for studying adult neurogenesis (Taupin, 2007). Ongoing stem cell research, including research on neural stem cells, investigates two fundamental properties of stem cells: the seemingly unlimited capacity for self-renewal and the ability to generate multiple mature cell types, i.e. multipotentiality (Seaberg and van der Kooy, 2003). BrdU provides a methodological tool for *in vivo* investigations for both of these fundamental properties inherent to stem cells.

BrdU has also been used for *in vitro* pre-labeling of transplanted cells in order to track them in the recipient organism. Mesenchymal stem cells (MSC) belong to those stem cells which are intensively explored for transplantation purposes in regenerative medicine. MSC are among the most promising adult stem cells suitable for treatment of neurologic disorders like stroke (Woodbury et al., 2000; Sanchez-Ramos et al., 2000; Hermann et al., 2004; Wislet-Gendebien et al., 2005; Blondheim et al., 2006).

To clarify the fate of MSC in a neural environment, investigations explore both transplantation into animals and co-cultures *in vitro* (Sanchez-Ramos et al., 2000; Wislet-Gendebien et al., 2004; Bossolasco et al., 2005). Besides the detection of Y-chromosomes (Eglitis and Mezey, 1997; Mezey et al., 2000; Mahmood et al., 2001) or using transgenic GFP-animals (Brazelton et al., 2000; Priller et

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; ET-1, endothelin-1; eMCAO, endothelin-induced middle cerebral artery occlusion; MSC, mesenchymal stem cell; PBS, phosphate buffered saline.

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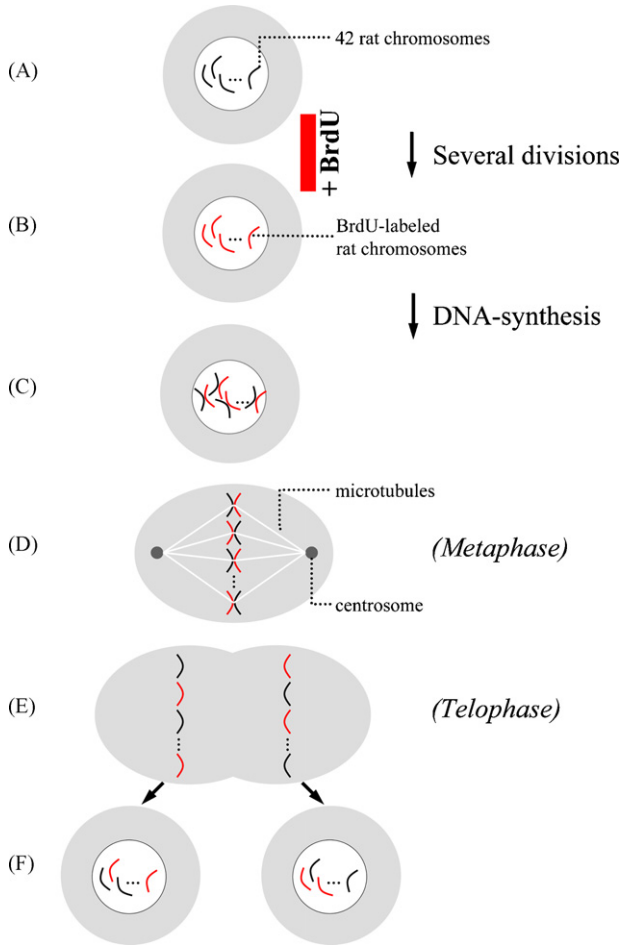

Fig. 1. Decrease of BrdU-signal in the nuclei by distribution of BrdU due to cell proliferation. (A) A non-labeled cell is exposed to BrdU. After multiple cell divisions (B) almost all chromosomes have been incorporated BrdU. (C) Subsequent cell divisions without exposure to BrdU result in newly synthesized chromatides without BrdU. (D) During the metaphase of the cell cycle the chromatides of the equatorial arranged chromosomes are randomly positioned to the centrioles. (E) During telophase the chromatides are randomly distributed to the future daughter cells. (F) As a result daughter cells contain slightly different numbers of BrdU-positive and BrdU-negative chromosomes. Additional cell cycles lead to a decreasing number of BrdU-positive chromosomes and the BrdU-signal is expected to resemble a mosaic pattern. After a sufficient great number of mitotic divisions cells without any BrdU-positive chromosomes will be generated.

al., 2001a,b; Nakano et al., 2001), labeling with BrdU (Kopen et al., 1999; Li et al., 2000, 2001) has been established primarily to identify and track these cells. Such labeled cells can be detected by immunohistochemistry and followed up in their differentiation-fate. Interestingly, the majority of transplanted BrdU-labeled cells depicted in publications possessed nuclei stained in unusual patterns. The nuclei are frequently stained not only inhomogeneously but are also segmented or punctated (Chen et al., 2001; Reubinoff et al., 2001; Munoz-Elias et al., 2004). Coyne et al. (2006) mentioned in their study the occurrence of nuclei with a fragmented BrdU pattern, however they did not comment on this phenomenon. Valero et al. (2005) speculated that the fragmentation of the BrdU-signal is a consequence of a punctated incorporation of BrdU during early, middle or late S-phase. However, this obvious fact was, for the most part, neither further addressed nor systematically investigated.

Fig. 1 demonstrates in a simplified illustration, how the proliferation of cells has influence to the BrdU-signal.

Divisions of once BrdU-labeled cells result in a distribution of the thymidine analogue to the progenies. The result is an increasing fragmentation of the immunocytological detectable BrdU-signal and in consequence a decrease in the ability to detect these cells.

An additional pitfall of BrdU is its apparent transfer from BrdU-labeled graft cells to the genome of adult neuronal cells in the recipient, leading to false positive results (Taupin, 2007). This also includes the transfer from BrdU-labeled neighbouring dividing cells (Burns et al., 2006). A report by Qu et al. (2004) suggests that BrdU, after long-term treatment of 3 weeks, may affect the mitotic activity and phenotype of those cells. The aforementioned observation regarding previously unrecognized stem cell plasticity had a great impact on research wherein tissue-specific stem cells have been described to cross lineage boundaries and generate tissues derived from multiple primary germ layers. These unexpected effects may also be enhanced or induced by BrdU, since it may act as a strong mutagen (Taupin, 2007).

In this study we systematically investigated, to our knowledge for the first time, the fate of the BrdU-signal over time by performing *in vitro* time series experiments. By examining the expected mosaic changes of the BrdU-signal, we analyzed pulse-labeled MSC immunocytologically up to 4 weeks at defined time points. The outcome is of huge impact for transplantation studies employing MSC, because it allows a conclusion regarding to the long-term traceability of these cells.

Furthermore, by studying the proliferation and neurogenesis in the rat dentate gyrus (DG) in previous studies (Baldauf and Reymann, 2005), we found varying distributions of the BrdU-signal in the nuclei of BrdU-positive cells. To further evaluate this phenomenon, in the present study we additionally performed focal ischemia in rats to increase neurogenesis in the DG and followed up the development of BrdU-positive cells for 7 or 28 days.

In both *in vitro* and *in vivo* experiments we detected a decreasing BrdU-signal in the nuclei of pulse-labeled cells over time and classified the cells into *filled*, *segmented*, and *punctated* cells. Further we describe for the first time the phenomenon that BrdU is obviously translocated from the nucleus to the cytosol *in vitro*.

2. Material and methods

2.1. Isolation and cultivation of rat MSC

Whole bone marrow was collected from 50 Sprague–Dawley rats (Harlan-Winkelmann, Germany). The juvenile rats (5 weeks of age) were euthanized by an overdose of the anesthetic gas halothane (Sigma–Aldrich, Germany) and both femurs were aseptically removed and placed in alpha-MEM (Biochrom, Germany) supplemented with 20% fetal bovine serum (FBS, Biochrom, Germany) and 1% penicillin/streptomycin (Biochrom, Germany), to which we refer as standard medium. The ends of the bones were cut and the bone marrow was flushed out with 5 ml standard medium using a 10-ml syringe and a 23-gauge needle. The pooled cell suspension was centrifuged at $500 \times g$ for 4 min. The pellet was resuspended in standard medium and gently triturated several times. Further cell separation was achieved by passaging the cells through a 23-gauge needle several times. The cells were counted using a Neubauer-hemocytometer and cultured with a density of 2×10^6 whole marrow cells/cm^2 in standard medium with 37 °C, 5% CO_2 and 92% relative humidity. After 3 days, non-adherent cells were removed by flushing with 10 ml PBS 0.1 M and the standard medium was replaced. The attached MSC were cultured until 80% of confluence was reached.

Then the cells were detached by incubation with trypsin/EDTA (0.25%, v/v/0.02%, v/v; Biochrom, Germany) dissolved in standard medium supplemented with 10% DMSO (Sigma–Aldrich, Germany) and apportioned for cryo-preservation.