Reports

A Cre-based double fluorescence indicator system for monitoring cell fusion events and selection of fused cells

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We have established an in vitro Cre/loxP-based assay for monitoring cell fusion events that specifically traces the transport of cytoplasm from one cell to its fusion partner. Cells with a double fluorescence vector indicate fusion with cells expressing Cre recombinase by switching expression from red to green fluorescent protein through a Cre-mediated recombination event that simultaneously activates puromycin-acetyltransferase expression. This strategy allows for both the observation and puromycin selection of indicator cells that have undergone fusion with a Cre recombinase-expressing partner. A fusion protein of Cre with estrogen receptor (ER) can be used to control Cre recombinase activity through the tamoxifen-induced translocation of the Cre-ER fusion protein to the nucleus. Here we have established a new methodology that not only allows the monitoring of the transport of cellular contents, but also enables the purification of fused cells using puromycin.

Introduction

Cell fusion has become of major scientific interest with the establishment of monoclonal antibodies, derived from fused antibody-producing B-lymphocytes with myeloma cells (1). Apart from the derivation of clonal antibodies, cell fusion has been utilized in a variety of applications, including transfer of cellular contents from one cell to its fusion partner, as recently demonstrated by the fusion of cardiomyocytes with CHO cells (2). Interestingly, fusion was also identified as a physiological process that occurs under different circumstances in vivo. It has been observed that bone marrow-derived cells are capable of fusing with a variety of cell types, including cardiomyocytes, hepatocytes, and Purkinje neurons (3–5). Detailed analysis of these processes revealed that the fusion of bone marrow cells with the different cell types is enhanced by tissue damage or inflammation (5–7). Formation of heterokaryons by in vivo fusion of bone marrow-derived cells with Purkinje neurons was followed by a reprogramming of the bone marrow-

derived nucleus into a state that corresponds to that of Purkinje neurons in terms of chromatin structure and gene expression profile (8).

In the last 10 years, the fusion of embryonic stem (ES) cells with somatic cells in vitro has been intensively studied owing to the ability of ES cells to reprogram somatic nuclei to an ES cell-like state (9–12). In 2005, Melton and colleagues demonstrated that fusion of human ES cells with human fibroblasts results in the reprogramming of the somatic nucleus, recovering a gene expression profile similar to that found in human ES cells alone (13).

The selection of fused cells requires marker genes to detect which cells have undergone fusion as well as to isolate these cells from non-fused cells. Usually this is accomplished by introducing two different selectable markers in each of the fusion partners (e.g., neomycin and hygromycin resistance) (13). After cell fusion, application of both selection agents enables purification of fused cells. This technique allows selection for tetraploid cells as a result of the fusion event. Here we describe

a novel selection methodology that not only allows tracking of fusion events between cells, but also enables selection of fused cells independently from the formation of polyploid derivatives.

Material and methods

RFPpig indicator plasmid construction

HcRed-pA was isolated from pHcRed1.1 (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) by *Bam*HI and *Afl*II digest (New England BioLabs, Frankfurt, Germany). The HcRed fragment was inserted between the first loxP site and the Westphal stop of pRFPGFP (Thomas Wunderlich, Institute for Genetics, University of Cologne; unpublished data) replacing DsRed. The GFP was excised by *Eco*RV digestion and puromycin-acetyltransferase-IRES-GFP was inserted as described (14).

Cell lines, culture conditions, and transfection

HEK 293 cells (provided by Toni Schneider, University of Cologne,

Cologne, Germany) were cultured using Iscove's Medium (IMDM) containing 100 μ M β -mercaptoethanol (Sigma-Aldrich, Munich, Germany), 1% non-essential amino acids (Invitrogen Life Technologies, Karlsruhe, Germany), 1% penicillin/streptomycin solution (Invitrogen), and 17% FCS (Invitrogen). We generated stably transfected cell lines by transfection with linearized RFPpig vector and linearized Cre-ER (ER, estrogen receptor) expression vector. Transfections were performed by electroporation using a Gene Pulser (Bio-Rad, Munich, Germany) (260 V; 500 μ F; 0.4-cm gap). Cell selection was started 48 h after transfection with 500 μ g/mL geneticin (PAA, Pasching, Austria) for RFPpig or 200 μ g/mL hygromycin (Invitrogen) for Cre-ER. Clones were picked after 2 weeks of selection. Cre-ER transgenic clones were assessed for Cre expression by staining with anti-Cre antibody.

Immunocytochemistry and cytosol staining by CMAC

For Cre staining, cells were fixed in ice-cold 99% methanol for 15 min. Blocking was performed with 5% BSA in PBS pH 7.4 for 1 h. Anti-Cre (polyclonal, Novagen, distributed by Merck KGaA, Darmstadt, Germany) was diluted 1:500 in 1% BSA and incubation was done overnight at 4°C. Secondary detection was performed with anti-rabbit Alexa Fluor 647 (Invitrogen) for 90 min at room temperature.

For 7-amino-4-chloromethylcoumarin (CMAC, Molecular Probes) labeling, cells were washed twice with PBS and incubated with 10 μ M CMAC in cell culture medium without FCS for 60 min. Cells were washed and incubated with IMDM supplemented with 17% FCS for 45 min to bind residual dye. Thereafter, cells were trypsinized and used for fusion immediately. Pictures were taken using a Zeiss Axiovert 200 equipped with an Apotom (Zeiss, Jena, Germany) device to facilitate optical sectioning. Images were processed with Axiovision 4.5 (Zeiss) and Corel Graphic Suite 11.

Cell fusion and FACS analysis

The 50% (w/w) PEG solution was produced by autoclaving 20 g PEG 6000 (Merck KGaA) and dissolving it in 20 mL serum-free cell culture medium. HEK 293 cells were trypsinized and $0.5\text{--}1.0 \times 10^6$ were resuspended with equal amounts of Cre transgenic cells. After spin-down of the cells ($800 \times g$) and aspiration of the supernatant, the pellet was loosened by gently flicking the tube. One milliliter of a 50% PEG solution (pre-warmed at 37°C) was added to the cell pellet and gently

Figure 1. Generation of the reporter cell lines. (A) To detect activity of Cre recombinase, the Cre indicator vector RFPpig was designed. The ubiquitously active CAGGS promoter drives expression of the red fluorescent HcRed protein in the non-recombined state of the reporter. The coding sequence of HcRed and a translational isolator (Westphal stop, WSS) are flanked by loxP sites. Activity of Cre recombinase results in the deletion of the loxP-flanked region and expression of the downstream puromycin-acetyltransferase (PAC) coding gene that is coupled to the expression cassette of enhanced green fluorescent protein (GFP) by an internal ribosome entry site. (B) Morphology of HEK 293 cells stably transfected with the Cre indicator RFPpig. (C) HEK 293 Cre indicator cell line expressing HcRed. (D,E) HEK 293 cells stably transfected with CreER were stained with antibodies against Cre (yellow) in the absence (D) and presence (E) of 4-OH-tamoxifen. Nuclei were counterstained with Hoechst dye (blue). Scale bars, 100 μ m (B,C) and 20 μ m (D,E).

Figure 2. Fusion of RFPpig transgenic Cre indicator HEK 293 cells with B-lymphocytes from a CD19-Cre transgenic mouse. (A) GFP expression in a colony formed after puromycin selection of fused cells. (B) Single GFP-positive cell that did not form a colony during a two-week selection period. Scale bar, 200 μ m. (C) PCR assay for the GFP and Cre transgenes on genomic DNA of puromycin-selected fusion colonies after clonal expansion. Clone 3 exhibits the presence of both transgenes.

Figure 3. Fusion of RFPpig transgenic Cre indicator HEK 293 cells with CreER transgenic HEK 293 cells and subsequent addition of 4-OH-tamoxifen. Fused cells displaying green fluorescence 48 h after fusion (A) were analyzed for GFP+ cells by flow cytometry (B). Flow cytometric analysis of non-fused control HEK 293 cells (C) and GFP+ RFPpig indicator cells after fusion and 2 weeks of subsequent puromycin selection (D). (E) Relative cell size as estimated by forward scatter of: unfused HEK 293 cells; GFP+ cells after 2 weeks of puromycin selection following fusion; total cells 48 h after fusion; and GFP+ cells 48 h after fusion. Scale bar, 100 μ m.

stirred with the pipet tip. Thereafter, serum-free cell culture medium was added dropwise while stirring with the pipet, according to the following scheme: 1 mL for 2 min, 2 mL for 2 min, 3 mL for 2 min, 5 mL for 2 min, 10 mL for 2 min, and 10 mL for 2 min. Cells were collected by centrifugation and plated on cell culture dishes. For splenocyte fusions, cell culture dishes were coated with 2.5 μ g/mL bovine fibronectin overnight at 4°C. When fusion was carried out with Cre-ER-expressing cells, 4-OH-tamoxifen was added to a final concentration of 0.5 μ M at the time of cell plating to enable Cre-ER to enter the nucleus. For fusion of adherent HEK 293 cells, the indicator and Cre-ER cells were plated in equal amounts on tissue culture dishes and cultured for 2 days.

PEG solution was added to cover the cells completely and incubation with PEG was performed for 5 min. The PEG solution was removed, then cells were rinsed with PBS carefully and overlaid with IMDM supplemented with 17% FCS and 0.5 μ M 4-OH-tamoxifen. Puromycin treatment of fused cells was initiated 48 h after fusion by addition of fresh medium containing 5 μ g/mL puromycin (PAA). For flow cytometry (FC), cells were trypsinized to achieve a single cell suspension and analyzed by a Becton Dickinson FACScan (Becton Dickinson GmbH, Heidelberg, Germany).

Preparation of splenocytes

A CD19-Cre mouse (CD19-Cre mice were contributed by F.T.W. and are maintained

at the Institute for Genetics, University of Cologne, Cologne, Germany) was killed by cervical dislocation. The spleen was isolated under sterile conditions and dissected into small pieces. Tissue was minced through a 100- μ m cell sieve and cells were washed out by addition of medium on the sieve. Total splenocytes were counted and collected by centrifugation.

Results and discussion

Validation of the Cre/loxP double fluorescence indicator construct

To monitor the presence of Cre recombinase following fusion with Cre transgenic cells, we designed an indicator construct, called RFPpig (Figure 1A), which allows detection of Cre activity, as well as purification of cells that have undergone Cre-mediated recombination. The red-fluorescent protein HcRed, driven by the ubiquitous active CAG promoter, was used to label non-recombined cells. Downstream of the HcRed, a transcriptional isolator (Westphal stop) (15) sequence prevents transcriptional read-through, while loxP sites flank the HcRed-Westphal stop cassette. The loxP-flanked HcRed-stop is followed by puromycin-acetyltransferase (PAC) and enhanced green fluorescent protein (GFP) coding sequences. The latter two expression cassettes are coupled via an internal ribosome entry site (IRES) that promotes formation of a polycistronic mRNA encoding for PAC and GFP. In the non-recombined state, without Cre, HcRed is constitutively expressed. Upon Cre-mediated recombination, the HcRed-Westphal stop cassette is deleted from the indicator and thereby induces expression of both PAC and GFP.

After transient transfection with the HcRed indicator vector, all transfected cells expressed the red-fluorescent HcRed. When co-transfected with a Cre-encoding plasmid, transfected cells switched from red to green fluorescence and expressed GFP within 24 h (data not shown).

Generation of reporter cell lines

We performed stable transfections of HEK 293 cells with the RFPpig indicator vector (Figure 1A). Depending on the red fluorescence intensity, six RFP-expressing clones were expanded (Figure 1, B and C). Clone 29 was used for all further experiments. To study the fusion process, a second stably transfected HEK 293 line with a plasmid coding for Cre-ER was generated. After colony picking, cells were assayed for Cre expression by immunocytochemistry using anti-Cre antibodies. A

Figure 4. Fusion of CMAC-labeled, Cre-ER transgenic HEK 293 cells with RFPpig transgenic HEK 293 Cre indicator cells. Red, green, and blue indicate RFP, GFP, and CMAC, respectively. (A–D) Four examples of fused cells 30 min after fusion. The left panel is the overlay of the middle and right panel showing CMAC and RFP, respectively. Binucleated cells with distinct colored nuclei could be observed. (E,F) Fused cells 7 h after fusion. (G,H) Fused cells 24 h after fusion. (I,J) Fused cells after 52 h of fusion. Panel G and I show the overlay of RFP, CMAC and GFP fluorescence. Panels H and J illustrate the distribution of CMAC solely, with GFP-expressing cells indicated by the dotted outlines. Scale bars, 50 μ m.

single clone, named Cre-ER1, was chosen for further experiments. Translocation of Cre from the cytoplasm to the nucleus was demonstrated by immunocytochemical staining against Cre following incubation with 0.5 μ M 4-OH-tamoxifen (Figure 1, D and E).

Fusion of RFPpig indicator HEK 293 cells with CD19-Cre B-lymphocytes

The spleen of a CD19-Cre mouse, where Cre is expressed under the control of the CD19 promoter exclusively in B-lymphocytes, was isolated and total splenocytes were prepared. Splenocytes (10^7) were mixed with 5×10^6 HEK 293 RFPpig indicator cells and collected by centrifugation. After fusion in the presence of PEG, cells were plated on fibronectin-coated tissue culture dishes. After 24 h of fusion, green fluorescence was detectable. Beginning on day 2 post-fusion, puromycin was added and selection was performed for 2 weeks. Thereafter, all cells expressed GFP and therefore appeared green (Figure 2A). Some cells did not proliferate during the selection period (Figure 2B), while others had formed colonies (Figure

2A). Ten colonies were picked and expanded on multi-well dishes. After one additional passage, DNA was extracted from each clone and PCR analysis was performed using GFP- and Cre-specific primers. While the coding sequence for GFP could be identified in all samples analyzed, we found only one clone that was clearly positive for the Cre transgene (Figure 2C). Repetition of the experimental procedure resulted in similar findings.

Fusion of RFPpig indicator HEK 293 cells with Cre-ER-expressing HEK 293 cells

Indicator HEK 293 cells were mixed with Cre-ER1 HEK 293 cells 1:1 (5×10^5 of each cell type). Cells were plated on 6-cm dishes (Cat. no. 734-0961; Falcon BD, distributed by VWR International GmbH, Darmstadt, Germany) and fusion was carried out by adding PEG 2 days after plating. The Cre-ER was activated by addition of 4-OH-tamoxifen. GFP-positive (GFP+) cells were identified 48 h after fusion (Figure 3A) and were collected by trypsin digestion for analysis by flow cytometry. The percentage of green cells at

this time point was calculated to be $3.96 \pm 0.59\%$ ($n = 8$, Figure 3B). In parallel, non-fused Cre-ER1 HEK 293 cells (Figure 3C), as well as GFP+ fused cells that had been selected with puromycin for 2 weeks (Figure 3D), were included as controls. The relative size of the unmodified HEK 293 control cells (Figure 3E, first bar) and of the GFP+ cells after 2 weeks of puromycin selection (Figure 3E, second bar) was similar. Forty-eight hours after fusion the relative size of the total population (Figure 3E, third bar) was similar to the size of the control cells. In contrast, gating for the GFP+ fraction 48 h after fusion revealed that the GFP+ cells were 70% larger relative to the control cells. (Figure 3E, fourth bar).

To measure the background of GFP+ cells in the absence of PEG-mediated fusion Cre-ER1, HEK 293 cells were plated together with RFPpig indicator cells at equal ratios. Induction with 4-OH-tamoxifen was started 2, 5, or 7 days following plating. In addition, co-culturing without induction was performed. GFP+ cells were quantified at day 8. Without application of PEG, only several single events of GFP+ cells could be observed pointing to a low incidence of spontaneous fusion (Supplementary Table S1 and Supplementary Figure S1).

The fusion of Cre-ER1 HEK 293 cells and RFPpig indicator cells resulted in no GFP+ cells (maximum number of GFP+ cells was 2 out of 11,665 cells analyzed) when no 4-OH-tamoxifen was added (Supplementary Table S2). This finding demonstrates a tight regulation of the Cre activity in the ER fusion protein Cre-ER.

Tracing the HEK 293 Cre-ER cells after fusion

To provide further evidence that substantial parts of the cytoplasm are transferred and induction of the green fluorescence is correlated with the cytoplasm transfer, an assay to trace the HEK 293 Cre-ER1 cellular proteins following fusion was developed. HEK 293 Cre-ER1 cells were stained blue by incubation with CMAC dye, which is metabolized inside the cell into a thiol-reactive product that covalently binds to cytosolic proteins. After staining, CMAC-labeled Cre-ER1 cells were mixed with an equal number of the red fluorescent RFPpig indicator cells. Fusion was carried out by PEG treatment of the cell pellet and Cre-ER nuclear translocation was induced by addition of 4-OH-tamoxifen to the plated cells.

After fusion, cells were monitored by fluorescence microscopy at 0.5, 7, 24, and 52 h. Approximately 30 min after PEG

Figure 5. Illustration of the fusion of Cre-ER transgenic cells with RFPpig Cre indicator cells. Upon fusion, cytoplasm is transferred from Cre-ER-expressing cells into the indicator cells. Some indicator cells potentially internalize almost the entire cytoplasm including the nucleus of the Cre-ER cell (left pathway) while others may retain only their individual nucleus (right pathway), although this is speculative. At this stage, 4-OH-tamoxifen is added to allow Cre-ER to enter the cell nucleus and perform recombination resulting in activation of the GFP expression from the indicator vector independent of the presence of the nucleus from the fusion partner.

treatment, we observed a population of cells containing two or more nuclei that appeared bi-colored (Figure 4, A–D). While the RFP protein was spread out more or less equally throughout the cell, the CMAC blue fluorescence was found in one nucleus and in the cytoplasm.

This can be explained by the hypothesis that the RFP, which derives from the indicator cell, is free to diffuse through all structures of the fused cell and stains the cytoplasm as well as all nuclei inside the cell. In comparison, most of the CMAC is probably bound to structural proteins and therefore limited in its ability to diffuse through the nuclear membrane of the indicator cell nucleus.

Seven hours after fusion, GFP fluorescence could be clearly identified (Figure 4, E and F). The GFP-labeled cells that had undergone recombination by Cre recombinase exhibited a clear blue fluorescence indicating the presence of CMAC-labeled protein originating from HEK 293 Cre-ER1 cells. The same results were found after 24 h of fusion (Figure 4, G and H). Although even after 52 h, the majority of the green fluorescent cells contained CMAC-labeled proteins (Figure 4, I and J), several cells were identified that had apparently lost the HEK 293 Cre-ER1-derived proteins since blue fluorescence was absent in these cells. Apparently, the reporter vector detects cell fusion events since the CMAC-labeled proteins can be observed in any green cell directly after fusion.

The Cre/loxP-based reporter system (Figure 5) described herein, which allows monitoring of fusion processes, has several advantages: (i) it can provide information about the efficiency of fusion procedures, even 6 h after the onset of the experiment by expression of GFP that can be observed under sterile conditions without harming cells, (ii) it allows an antibiotic-based purification of cells that have undergone fusion, and (iii) most important, the novel reporter system can indicate fusion processes independently of whether tetraploid cells are formed or only cytoplasm is transferred.

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Competing interests

The authors declare no competing interests.

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