EXPERIMENTAL CELL RESEARCH 320 (2014) 33–45

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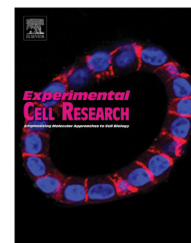

Research Article

Green fluorescent protein expression triggers proteome changes in breast cancer cells

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ARTICLE INFORMATION

Article Chronology:
Received 28 May 2013
Received in revised form
15 July 2013
Accepted 19 July 2013
Available online 27 July 2013

Keywords:
GFP expression
Proteomic change
Unfolded protein response
Cytoskeleton modifications
Cellular immune response

ABSTRACT

Green fluorescent protein (GFP) is the most commonly used reporter of expression in cell biology despite evidence that it affects the cell physiology. The molecular mechanism of GFP-associated modifications has been largely unexplored. In this paper we investigated the proteome modifications following stable expression of GFP in breast cancer cells (MDA-MB-231). A combination of three different proteome analysis methods (2-DE, iTRAQ, label-free) was used to maximise proteome coverage. We found that GFP expression induces changes in expression of proteins that are associated with protein folding, cytoskeletal organisation and cellular immune response. In view of these findings, the use of GFP as a cell reporter should be carefully monitored.

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Introduction

Green fluorescent protein (GFP) is responsible for the green bioluminescence of the jellyfish *Aequorea victoria*. This single-chain 238 amino acid polypeptide emits green fluorescence under 488 nm light. Because of this property GFP has been increasingly used to facilitate experiments in cell biology. It is commonly used as a reporter gene or a fusion tag with a particular protein (for review, see [1]). However, a number of cellular side effects resulting from GFP expression have been reported. For example, apoptosis has been reported in several cell lines when stable expression of GFP was attempted [2]. In muscle cells, overexpression of GFP has been linked to impairment of contraction in experimental models both *in vitro* and *in vivo* [3–6]. In neurons, expression of fluorescent proteins (eGFP and YFP) appears to alter cellular mechanisms and molecular characteristics [7,8].

In light of these findings, it is surprising that relatively few studies report on the molecular consequences of GFP expression. In endothelial cells, GFP has been shown to induce a marked increase of HSP70 at both mRNA and protein levels in a dose

Abbreviations: GFP, green fluorescence protein; 2-DE, two-dimensional electrophoresis; iTRAQ, isobaric tags for relative and absolute quantitation

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http://dx.doi.org/10.1016/j.yexcr.2013.07.019

dependent manner. This upregulation leads to an increased expression of cyclooxygenase-2 (COX-2) followed by increased prostaglandin E_2 (PGE_2) production [9]. In 2006, Baens et al. [10] reported a defect in polyubiquitination, a post-translational modification that affects a wide range of cellular processes. Finally, in an attempt to provide a more comprehensive view of the influence of GFP expression in cardiac myocytes, Badrian and Bogoyevitch [11], conducted a DNA microarray analysis which revealed changes in the expression of 212 genes and therefore suggested caution in the interpretation of experimental results where GFP-expressing cells are used as controls. In this study, we used a breast cancer cell line as a model system to analyse proteome changes associated with stable GFP expression.

Materials and methods

Cell culture

Parental MDA-MB-231 breast cancer cells (referred to as wild-type (WT) cells form hereon) were cultured in DMEM supplemented with 10% fetal bovine serum, sodium pyruvate and antibiotics at 37 °C and 5% CO_2. Stable MDA-MB-231 clones expressing GFP were generated using the plasmid pEGFP-C3 and the X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Australia) according to the manufacturer's instructions. Selection of the stable clones (expression of GFP was confirmed by fluorescence microscopy) and subsequent subcultures of one stable clone (used in the following experiments) were performed in the above conditions and with regular growth media supplemented with 1 mg mL^{-1} G418.

Protein extraction and quantification

For protein extraction, cells grown to 90% confluence in 175 cm^2 culture flasks were washed twice with PBS and detached from the flask by 5 min incubation at 37 °C in citric saline solution (1.35 M KCl, 0.15 M sodium citrate).

For protein analysis by two-dimensional electrophoresis (2-DE), cells were pelleted by centrifugation, resuspended in IEF buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% IPG buffer (pH 4–7) (GE Healthcare), 1% DTT, 0.001% bromophenol blue) and proteins were extracted with glass beads using a mini bead beater (4×20 s interspersed with 5 min cooling on ice). Samples were then centrifuged at 15,000*g* (15 min, 20 °C).

For the iTRAQ and MS-label free experiments, a total cell lysate was obtained by extracting the cell pellet in 10 volumes of ice-cold RIPA Buffer (Pierce, Sydney, Australia) using the mini bead beater as described above. Samples were then centrifuged at 15,000*g* (1 h, 4 °C).

Total protein concentration in the supernatant was determined using the 2-D Quant kit (GE Healthcare), using BSA as the reference standard.

Two-dimensional electrophoresis, protein visualization and image analysis

2-DE was performed essentially as previously described by Coumans et al. [12]. In summary, rehydrated 18 cm IPG strips pH 4–7 (GE Healthcare) were loaded with protein (300 μg for analytical gels or 400 μg for preparative gels) and IEF was carried out on the IPGphor II (GE Healthcare Life Science) at 20 °C with a current limit of 50 μA/strip to a total volt–hour-product of 30 kVh (analytical gels) or 45 kVh (preparative gels). Second dimension separation was achieved on Protean II XL Cell (Bio-Rad) with home cast 1.5 mm SDS polyacrylamide gels (12%) at 8 mA/gel until the bromophenol blue dye front reached the anodic end of the SDS-gel. Staining of the gels was performed using colloidal Coomassie Brilliant Blue G-250 [13] for analytical study and by Coomassie blue staining (50% methanol, 0.15% Coomassie blue R-250, 0.75% acetic acid) for preparative 2-DE. Image capture was performed as previously described by Coumans et al. [14]. Three biological samples and two technical replicates were grouped and analysed with PDQuest advanced 2-D analysis software (Bio-Rad). For comparison of expression data, the Student's *t*-test function in the PDQuest software was used and a $p\text{-value} \leq 0.05$ was considered to be statistically significant.

Sample preparation and iTRAQ labeling

100 μg of whole cell protein extract from either WT or GFP-expressing MDA-MB-231 cells was precipitated with 9 volumes of ice-cold acetone for 1 h at −20 °C. The protein precipitate was recovered by centrifugation, air dried (*ca.* 2–3 min) and dissolved in 50 mM sodium bicarbonate containing 0.1% SDS. Probe sonication was used to assist dissolution. Protein samples were then reduced using tris(2-carboxyethyl)phosphine (2.5 mM, 60 °C, 60 min) and alkylated with iodoacetamide (2 mM, 20 °C, 10 min), digested with trypsin (0.5 μg/50 μg protein) (Promega, Madison, WI) for 16 h at 37 °C and then labeled with the iTRAQ reagents according to the manufacturer's instructions [15].

Excess unbound iTRAQ labels, trypsin, SDS and solvents were removed by strong cation exchange (ICAT SCX cartridge, ABSciex, MA), solid phase extraction (SPE) using an Applied Biosystems Opti-Lynx cartridge holder and a syringe pump (KD Scientific, Holliston, MA) at a flow rate of 9.5 mL/h. The eluted peptide solutions were vacuum-dried, and resuspended in 500 μL of 0.2% heptafluorobutyric acid (HFBA) prior to a further SPE step using reverse phase (RP) chromatography on a C18 RP peptide macrotrap cartridge (Microm Bioresources, Auburn, CA). Briefly, the macrotrap was primed with 1 mL CH_3CN, then 1 mL 50% CH_3CN/0.1% formic acid, and equilibrated using 1 mL of 0.2% HFBA. The resuspended peptides were loaded, the macrotrap washed with 1 mL of 0.2% HFBA and the peptides eluted with 500 μL 50% CH_3CN/0.1% formic acid, followed by 200 μL neat CH_3CN. The eluants were then pooled and vacuum-dried. The pellet was dissolved in 25 μL 1% formic acid, 0.05% HFBA and analysed using 2D-LC–MS/MS. All labeling experiments were run twice (2 separate injections), to provide the two technical replicates and statistical significance was determined using the Student's *t*-test (Microsoft Exel).

Sample preparation for label-free LC–MS quantification

Whole cell protein extracts (100 μg) from WT and GFP-expressing MDA-MB-231 cells were treated and trypsin digested as described in the sample preparation for iTRAQ labeling.

Following trypsin digestion, the peptide samples were purified using SCX StageTips and C18 StageTips (Thermo Scientific) following the manufacturer's instructions.

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