Application of Fluorescent GPCR Ligands for Fluorescence Activated Cell Sorting (FACS) to Select Receptor Expressing Cell Sub-Populations

Introduction

G-protein coupled receptors (GPCRs) are routinely transfected into host cell lines, such as Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells, to generate recombinant cell lines for receptor research, assay development and drug discovery.

For stable cell transfection, the DNA construct encoding the recombinant GPCR is often coupled to a selectable marker (eg. G418 / Geneticin ® resistance) on the same DNA construct to allow only the transfected cells expressing the construct to be selected and propagated.

Having established a cell population that expresses the recombinant GPCR at various levels, individual cells are then cloned from the population, to propagate clones with low, medium or high levels of receptor expression dependent on how the construct has stably integrated into the host cell DNA. The cloned cell line(s) with the appropriate level of receptor expression are then selected as required for the particular application.

Although cloned cell lines expressing recombinant GPCRs can be maintained under selection to ensure continued expression at the same level over many passages, it is common for the selection agent to be omitted for economy in long-term or large-scale cell culture. As a consequence, selectable marker and GPCR expression can be lost in some cells of the clonally-derived population, generating variable and inconsistent receptor-dependent binding and functional data.

Radioligand saturation binding studies can be used to determine GPCR protein expression level (and any decline in expression that occurs with increasing passage), but this only indicates the average expression level of the cell population.

Hello Bio's range of CellAura fluorescent ligands provide an alternative to radioligands as a means to estimate GPCR expression level in the cell population. Furthermore, the CellAura fluorescent GPCR ligands can be used to visualise individual cells within a heterogeneous population to determine the proportion of the population that continue to express the receptor. Hence, by using flow cytometry / fluorescence activated cell sorting (FACS) of the live cell population, the sub-population of cells labelled by the fluorescent ligand (because they are still expressing the recombinant GPCR) can be selected and propagated to maintain and extend the useful lifespan of the cell line.

This Application Note provides an example of the use of the fluorescent dopamine D1 antagonist [SKF83566], as the fluorescent label in FACS analysis to select a population of cells expressing the human dopamine D1 receptor from a cell line showing heterogeneous receptor expression within the (unsorted) cell population.

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Materials and Methods

The CHO cell line heterogeneously expressing human dopamine D1 receptors was provided by AstraZeneca R&D, Charnwood. Cells were cultured in Dulbecco's minimal essential medium (D-MEM) / Ham's F12 medium supplemented with 10% foetal calf serum and 2mM glutamine at 37°C in 5% CO_2/humidified air. The cell line had been maintained in cell culture for a number of passages and had been cryopreserved at regular intervals, allowing different passages to be compared simultaneously.

The fluorescent D1 antagonist [SKF83566], was prepared by dissolving 0.2mg in 209µL of dimethyl-sulphoxide (DMSO) to give a 1mM stock solution in DMSO. This stock solution was stored as small aliquots at -20°C. An aliquot was thawed immediately before use, and briefly sonicated using an ultrasonic bath to ensure it was fully dissolved and the solution was homogenous.

To visualise cells expressing the dopamine D1 receptor with the fluorescent D1 antagonist [SKF83566], cells were harvested, resuspended in culture medium, seeded into clear-bottomed black 96-well plates (Perkin Elmer) at 5 x 10^4 cells/ well in 100µl medium, and grown to confluence over 48 hours. On the day of experimentation, culture medium was removed and the cells were stained with 1µM Hoechst stain in HEPES buffered saline (HBS) for 60 minutes at room temperature. Cells were then washed once with HBS before the addition of 50µl HBS to each well of the 96-well plate. Where appropriate, cells were pre-incubated with the unlabelled D1 competitor, SCH23390, at 1µM for 30 minutes at room temperature prior to the addition of the fluorescent ligand. The fluorescent D1 antagonist [SKF83566], was diluted from the 1mM stock solution in DMSO, to a 2x final concentration of 60nM in HBS; 50µl of this solution was added to each well of the 96-well plate, to give a final concentration of 30nM fluorescent ligand in HBS (+ 0.03% DMSO).

Fluorescent ligand binding was detected after a 10 minute binding period on the InCell 3000 (GE Healthcare). Fluorescent ligands were excited by a 647nm laser and imaged using a 695nm bandpass filter. The Hoechst stain was excited by a 365nm laser and imaged using a 450nm bandpass filter.

To label the cell population expressing the dopamine D1 receptor for selection using FACS, cells were grown to 70-80% confluency in T75 flasks. Medium was removed and cells were rinsed with HBS 3x to remove all traces of phenol red indicator. The cell monolayer was then incubated with 5ml HBS containing 500nM of the fluorescent D1 antagonist [SKF83566] for 20 minutes at room temperature before harvesting by scraping. The cell suspension was prefiltered to remove large cell clumps before analysis on a FACSAria flow cytometer (Becton Dickinson), excitation at 633nm, emission at 660nm. Gating was applied to sort cells into three separate populations (high, medium and low expressers) into tubes. Cells were subsequently cultured in T25 flasks prior to experiments.

Results

The human dopamine D1 expressing cell line was tested for receptor expression by fluorescence imaging at an early passage (P2) and a late passage (P22) before proceeding to FACS selection (Figure 1A and 1B). Both passages show a low number of cells labelled with the fluorescent ligand, indicating that receptor expression is not ubiquitous. Indeed, at P2, only approximately 10% of the cells appear to be expressing the D1 receptor (Figure 1A), while at P22 only individual cells appear to be expressing the receptor (Figure 1B).

Following FACS selection of three sub-populations of cells (high, medium and low expressing), selected from the cell cultures at P2 and subsequently cultured for a further 10 passages post-FACS selection, the cells were labelled with the fluorescent D1 antagonist to reveal that receptor expression now occurs in > 90% of the 'mid-expressing' cell population (Figure 1C). This binding can be displaced by the unlabelled, selective D1 competitor, SCH23390 (Figure 1D).

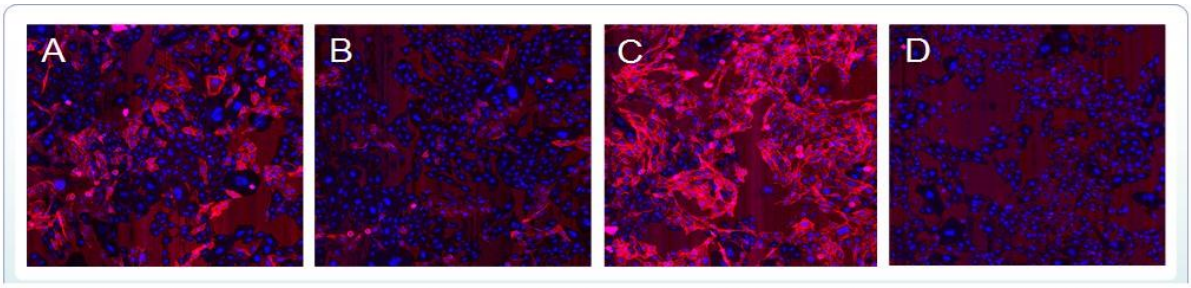

Figure 1: Expression, loss of expression and enhancement of expression of human dopamine D1 receptors in a CHO cell line, detected using the fluorescent D1 antagonist [SKF83566]

A: human D1 cells at P2, showing binding of the fluorescent D1 antagonist [SKF83566] (red) at 30nM to ~10% of the cell population.
B: human D1 cells at P22, showing very few cells labelled with the fluorescent D1 antagonist [SKF83566].
C: Mid-level human D1 expression cells at P10 after FACS selection, showing >90% of the cells labelled with the fluorescent D1 antagonist [SKF83566] at 30nM.
D: Mid-level human D1 expression cells at P10 after selection, pre-incubated with SCH23390 at 1 µM to block binding of the fluorescent D1 antagonist [SKF83566] at 30 nM
In all images, nuclei have been counter-stained with Hoechst (blue) to reveal cell location and density.

Conclusion

Expression of the human D1 receptor in a recombinant CHO cell line, labelled with the fluorescent D1 antagonist [SKF83566], was shown to be non-uniform, with a significant proportion of the cell population apparently not expressing the receptor.

Following FACS selection of live cells labelled with the fluorescent D1 antagonist, cells could be cultured for a further 10 passages with a substantially increased proportion of the cell population expressing the D1 receptor.

This indicates the utility of the fluorescent D1 antagonist to indicate the proportion of cells expressing the D1 receptor in the cell population, to then act as a fluorescent label for FACS selecting the D1 expressing cells, and then to confirm the enhancement of the proportion of the population expressing the D1 receptor post-selection.

It is expected that fluorescent ligands selective for other GPCRs will have similar utility to reveal receptor expression in individual cells, and to allow FACS selection of those cells expressing the appropriate GPCR.

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