Construction of a recombinant mu opioid receptor tagged with FLAG epitope and yellow fluorescent protein to generate a FLIP-In HEK293 stable cell line

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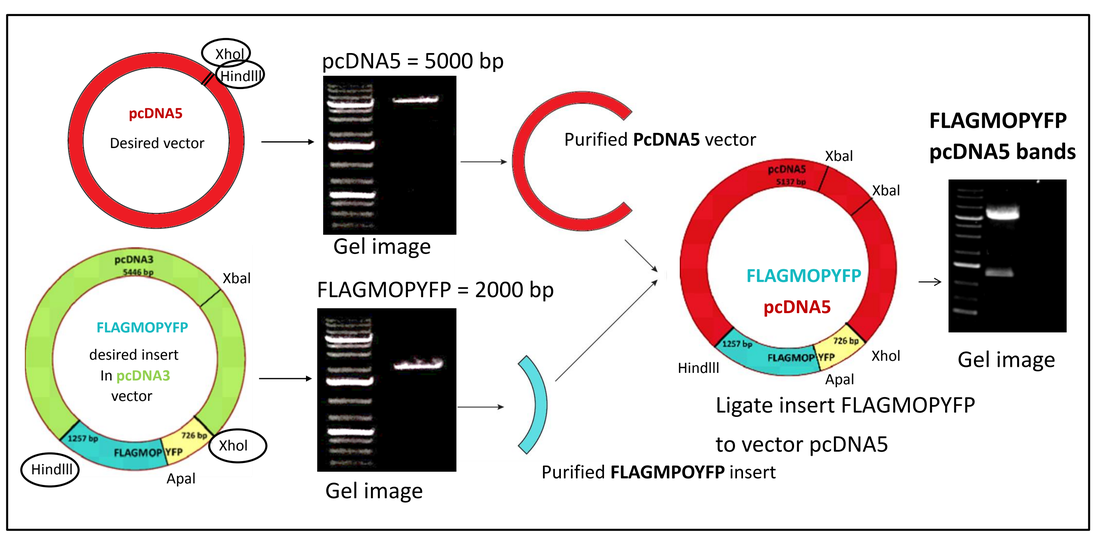
Summary :

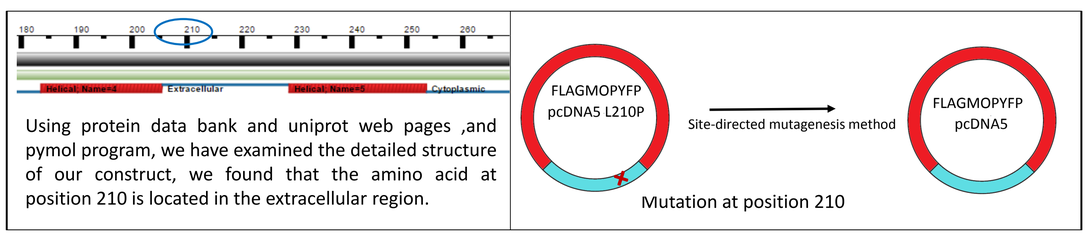
The opioid receptors belong to the G-protein-coupled receptor (GPCR) superfamily. The mu opioid receptor (MOP) is a member of the G protein-coupled receptor family and constitutes the main target of endogenous opioid neuropeptides and morphine. MOP activation by agonist compounds has a wide variety of pharmacological and physiological effects involved in addiction, pain treatment and others. Therefore our understanding of MOP receptor function is central to the development of different therapies. We have constructed a recombinant MOP receptor to be expressed in human embryonic kidney 293 (HEK) cells, by tagging a FLAG epitope at amino terminus and a yellow fluorescent protein (YFP) at carboxyl terminus. Subsequently FLAGMOPYFP receptor was subcloned into pcDNA5/FRT/TO vector to generate a future inducible FLIP-In HEK 293 stable cell line. In order to observe the cellular expression of FLAGMOPYFP in fluorescence microscopy, HEK 293 cells were transiently transfected with this chimeric MOP receptor subcloned in different expression plasmids, i.e. pcDNA3 and pcDNA5 vectors. Additionally, an amino terminus untagged form of MOPYFP receptor was also visualized to compare with the FLAGMOPYFP version. Finally, we concluded that the presence of FLAG epitope in amino terminus of MOPYFP receptor might be responsible of the different patterns of cellular distribution observed.

Subcloning of FLAGMOPYFP insert into pcDNA5/FRT/TO vector :



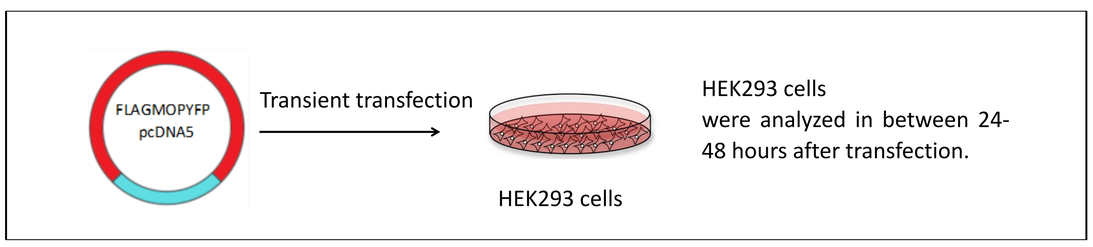
Subcloning of FLAGMOPYFP into pcDNA5 is a basic step in our project required to move the FLAGMOPYFP insert from vector pcDNA3 to vector pcDNA5 to gain the desired functionality to study our insert. We have released and purified our insert from the parent vector pcDNA3 by digestion of FLAGMOPYFPpcDNA3 with HindIII and Xho I. In parallel, the vector pcDNA5 was also digested with HindIII and XhoI , and subsequently the insert FLAGMOPYFP and the pcDNA5 vector were ligated. The ligation reaction was transformed into competent bacterial cells. Then the transformant cells underwent miniprep and maxiprep techniques to isolate the cDNA at the purity and scale we need. Afterwards, we prepared FLAGMOPYFPpcDNA5 samples for sequencing. The sequencing reactions resulted in one mutation in our construct at position 210, the original proline (CCT) was substituted by leucine (CTT), so our construct was not totally correct (FLAGMOPYFP L210P). Consequently, the next step was to resolve this unexpected problem.

DNA Mutation Repair in the sequence of mu opioid receptor :



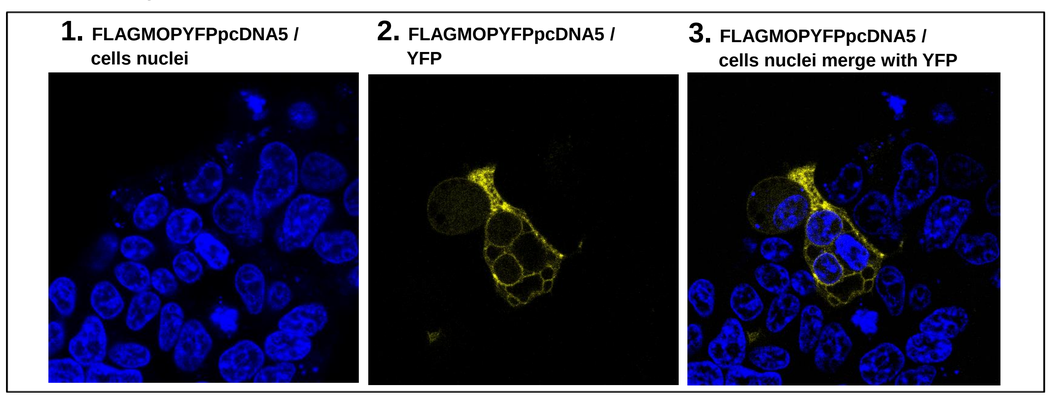
A close physical relationship between extracellular loop (EL) and the ligand binding pocket of MOP explains why mutating specific residues in EL can alter subtype selectivity of ligands. So we performed site-directed mutagenesis method to correct leucine mutation at position 210 in our sequence. By using two synthetic oligonucleotide primers containing the desired change, i.e. substitution of CTT by CCT, we made PCR reaction under a specific program for FLAGMOPYFP L210P that contain the undesired mutation with the designed primers that contain the desired change. The PCR reaction product was digested with DpnI to cut the methylated, parental DNA template, then we transformed the digested product into competent cells to get the correct construct of FLAGMOPYFP. After that we prepared sample of our new construct with a specific primer that covers the sequence at the mutation position 210, then we sent the sample for sequencing reactions service, to check mutation, and we found that the mutation has been corrected.

Transient expression of mu opioid receptor in HEK 293 cells:



Before transfection, a sterile high-quality FLAGMOPYFP pcDNA5 DNA was prepared. We used polyethylenimine (PEI) method to transfect our recombinant plasmid into HEK 293 cells. The DNA-PEI transfection complexes were performed in fresh medium and added to the cells. Transiently transfected cells express the foreign gene but do not integrate it into their genome. As a consequence the genetic material is not passed from generation to generation during cell division, so the transfected gene will not be replicated and therefore the transiently transfected cells will express the gene for a finite period of time, usually several days.

Visualization of expression of FLAGMOPYFP receptors in HEK293 living cells by yellow fluorescent protein :



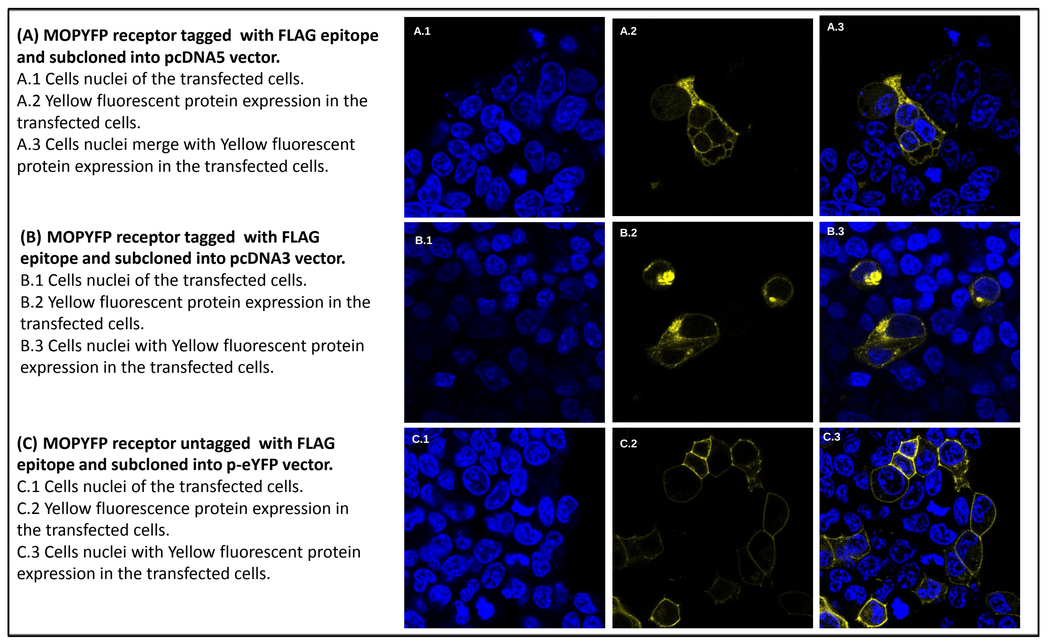
YFP was tagged with the construct of MOP for visualization in live cells to identify the positive expression of MOP recombinant in HEK 293 cells. We have assayed the cells about two days after the transient transfection and cells nuclei were stained with Hoechst 33342 reagent in order to visualize them by fluorescence microscopy. In this way, yellow fluorescent protein was detected in some cells indicating the presence of our gene of interest, FLAGMOPYFP, within that cells. We can notice in the images above that not all the cells were expressing FLAGMOPYFP receptors. This is due to the transiently nature of the heterologous expression in this case.

Intracellular distribution of mu opioid receptor might be caused by FLAG tag epitope :

(A) MOPYFP receptor tagged with FLAG epitope and subcloned into pcDNA5 vector.
A.1 Cells nuclei of the transfected cells.
A.2 Yellow fluorescent protein expression in the transfected cells.
A.3 Cells nuclei merge with Yellow fluorescent protein expression in the transfected cells.

(B) MOPYFP receptor tagged with FLAG epitope and subcloned into pcDNA3 vector.
B.1 Cells nuclei of the transfected cells.
B.2 Yellow fluorescent protein expression in the transfected cells.
B.3 Cells nuclei with Yellow fluorescent protein expression in the transfected cells.

(C) MOPYFP receptor untagged with FLAG epitope and subcloned into p-eYFP vector.
C.1 Cells nuclei of the transfected cells.
C.2 Yellow fluorescence protein expression in the transfected cells.
C.3 Cells nuclei with Yellow fluorescent protein expression in the transfected cells.



HEK 293 cells were transiently transfected with different versions of MOPYFP receptor in different expression plasmids. FLAG epitope was tagged with MOPYFP receptor, a short, hydrophilic protein tag, commonly used in conjunction with antibodies in protein to study protein-protein interactions. The FLAG tag was inserted at the N-terminus, because of its hydrophilic nature, the FLAG tag is commonly found on the surface of a fusion protein, which makes the protein more available to binding antibodies. We have observed that FLAGMOPYFP receptors are predominantly displaying an intracellular distribution in (A) and (B), while the distribution of MOPYFP receptor without FLAG tag (C), was essentially in the plasma membrane region. Therefore, we conclude that the presence of FLAG epitope in amino terminus of MOPYFP receptor might be responsible of the different patterns of distribution observed.

Concluding remarks :

There is a clear interest for heterologous expression systems that can provide the possibility to express genetically modified receptors to conduct molecular pharmacology investigations. To meet this interest, the main purpose of our research was the construction of a recombinant mu opioid receptor tagged with FLAG epitope and fused to YFP to generate an inducible FLIP-In HEK 293 stable cell line to conduct further investigations on its molecular pharmacology. Repair of DNA mutation in FLAGMOPYFP has been an essential process in this project, because the DNA is repository of genetic material in each living cell and its integrity is essential for viability of our construct. An intriguing result of our investigations was the different pattern of distribution observed when comparing the cellular expression of FLAGMOPYFP and MOPYFP that opens new avenues of research to further explore the reason behind this result.

Aknowledgements :

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