Human Melanocortin 4 receptor (MC4R) cell line

Technical Manual No. **Version 20080606**

I. Introduction

Adrenocorticotropic hormone (ACTH) and α-, β-, and γ-melanocyte-stimulating (α-, β-, γ-MSH) hormones derive from post-translational processing of the precursor molecule proopiomelanocortin (POMC) (Hadley and Haskell-Luevano, 1999). These POMC products are collectively called melanocortin peptides or melanocortins. Melanocortin peptides have multiple effects on the host. These effects are disparate and range from modulation of fever and inflammation to control of food intake, autonomic functions, and exocrine secretions (Goodfellow and Saunders, 2003).

The five MCRs cloned so far belong to the class A of guanine nucleotide-binding protein (G protein)-coupled, seven transmembrane receptors. They are the product of small genes, many of which are polymorphic. MCRs are the smallest G protein-coupled receptors known. They have short amino- and carboxyl-terminal ends and a very small second extracellular loop. All are functionally coupled to adenylyl cyclase and mediate their effects primarily by activating a cAMP-dependent signaling pathway (Chen *et al*., 2000; Goodfellow and Saunders, 2003).

Using HDB's assay development technologies, a cell line and assay protocol was established and pharmacologically validated for MC4R responsiveness to the MC4 receptor agonists. The MC4R receptor assay is ready for use in the identification of agonists and antagonists.

II. Cell Line Information

- Product Number: CH2098-4
- Description:

  The HUMAN MC4R is amplified by PCR using a high-fidelity enzyme and subcloned into the pcDNA3.1/G418(+) mammalian expression vector. The full-length ORF has been confirmed by sequencing. The MC4R cell line was created by cotransfection of pcDNA3.1/MC4R/G418(+) with pcDNA3.1/hygromycin(+) in an HDB parental cell line, HEK293/CRE/d2EGFP. The transfected cells are stably selected by 100μg/ml hygromycin. Single cell clones with high MC4R receptor inducibility and low GFP background are isolated using ring cloning. The clones with the largest dynamic ranges in GFP are chosen for pharmacological and stability studies.
- Cell Line Name: HEK/MC4R/CRE-d2EGFP
- Date Created: Nov, 2002
- Function: Cell-based, functional assay for MC4R
- Quantity: 2 vial (2 x 10^6/vial) frozen cells
- Host Cell: HEK293
- Cell phenotype: Adherent/epithelial
- Antibiotics Selection: G418 for parental cells and hygromycin for MC4R cells
- Freeze Medium: 90% FBS and 10% DMSO

- Plasmid: hu MC4R-pCDNA3.1/G418(+)
- Transfection: Full-length human MC4R cDNA (Genebank Accession Number: NM_005912.2)
- Recommended Storage Conditions: Liquid nitrogen upon delivery
- Propagation Medium: DMEM, 10% FBS

III. Cell Culture Conditions

Note: This clone often grows as clumps. It is necessary to separate cells into single cell suspensions whenever the cells are passaged or plated. MATRIGEL Matrix-coated flasks or plates are recommended for healthier cells.

Complete Culture Medium:

DMEM: 90%

FBS: 10%

Supplements:

L-glutamine 2.0 mM

Amp 100 μg/ml

Strep 100 μg/ml

Medium for Stable Line Propagation:

Add 3 μg/ml puromycin in complete culture medium.

Freezing Medium:

20-90% of fetal bovine serum and 10% dimethyl sulphoxide (DMSO)

Thawing Cells:

1. Quickly thaw frozen cells in a 37°C water bath, agitating continuously.
2. Using a 1 ml pipette, slowly pipet the cells up and down five times and add, drop by drop, to a 15 ml centrifuge tube containing 5 ml of fresh prewarmed complete DMEM medium. Then centrifuge at 1,000 rpm for five minutes.
3. Discard the supernatant medium and resuspend the cell pellet in 5 ml of fresh prewarmed complete DMEM medium. Transfer cells to a T25 flask and incubate at 37°C with 5% CO_2 until the cells reach >90% confluence. The recovery rate for frozen cells is usually 90% or above.

Subculturing:

When the cells reach confluence, they need split. This cell line is normally split twice weekly at 1:4 to 1:8 dilutions.

1. Carefully aspirate all the media. Gently rinse the cell layer with appropriate amount of 0.2% trypsin-EDTA, and aspirate it off.
2. Wait for about 1-3 minutes. Then dislodge the cells by gently tapping the sides of flask or dish.
3. Resuspend cells with appropriate amount of complete DMEM medium, and split cells as desired.

Changing Medium:

This is normally done every other day.

1. Gently aspirate off medium.
2. Transfer fresh warm complete DMEM medium (37°C) into a flask (5 ml for T25 and 10 ml for T75).

Freezing Cells:

1. Repeat the steps 1-3 of subculturing section.
2. Centrifuge down the cells at 1,000 rpm for five minutes.
3. Aspirate off the supernatant and resuspend the cells in fresh freezing medium at a density of 2-3 $\times$ 10^6 cells/ml. Add 1 ml cells per cryogenic vial.
4. Put the cryogenic vial of cells into cryo freezing container. Then transfer the container to a -80°C environment and leave it there overnight.
5. Transfer cryogenic vial into liquid nitrogen (-196°C).

IV. Assay Procedure

Transfection:

1) Plate cells in 6 cm dish at 50-70% density for stable transfection.
2) Cells are ready for transfection at 24 hours after plating: >90%.
3) Dilute 2 μg DNA in 250 μl OptiMEM I Reduced Serum Medium without serum (or other medium without serum). Mix gently.
4) Mix LipofectAmine™2000 (Invitrogen) gently before use, then dilute 5 μl in 250 μl OptiMEM I Reduced Serum Medium without serum (or other medium without serum). Mix gently and incubate for five minutes at room temperature.
5) After the five-minute incubation, combine the diluted DNA with the diluted LipofectAmine™2000 (total volume is 500 μl). Mix gently and incubate for 20 minutes at room temperature to allow the DNA-LipofectAmine™2000 complexes to form.
6) Remove the cells from the incubator, and gently aspirate off the medium. Then transfer 2 ml fresh warm complete DMEM medium (37°C) to the dish. Add the 500 μl DNA- LipofectAmine™2000 complexes to the 6cm dish. Mix gently by rocking the dish back and forth.
7) Incubate the cells at 37°C / 5% CO_2 for 24-48 hours until they are ready to assay for transgene expression or antibiotics selection.
8) For MC4R cell line development, MC4R cDNA is transfected into HEK/CRE-EGFP cell lines. Two rounds of transfection are performed, about one week was allowed to pass between the first and second rounds.

Cell colony selection:

1) For the development of stable cell lines:

   Passage the cells at a 1:10, 1:20 and 1:30 dilution into fresh medium 24 hours after transfection. Add selective medium (hygro, final concentration 100 μg/ml) the following day.
2) Keep exchanging the medium every other day for about two weeks' selection. This is the time needed for the antibiotics to act on the non-transfected cells, which then detach and are washed away during the medium exchange. Once all cells have died in the dish of the negative control (non-transfected), you can proceed with the cloning.
3) For MC4R cell line development, a total of 76 single clones are picked by ring cloning and transferred to 96-well plate. After the clones are developed, a calcium assay is used to screen positive clones, and α-MSH is used as an agonist. Cell clones of MC4R with significantα-MSH induction are picked and used for further characterization and validation.

Screening of cell clones:

1) Seed cells at a density of $2X10^4$ cells/well into the 96-well black wall and clear bottom plate (assay plate), and incubate in CO_2 incubator overnight.
2) Discard the growth media. Add 100μl/well of phenol red free media with 0.1% BSA and incubate for eight hours at 37°C in CO_2 incubator.
3) Add 10 μl/wellα-MSH (final concentration, 1 μM) into wells.
4) Incubate the plate at 37°C for 8-24 hours.
5) Capture the fluorescence pictures with Olympus Microscope (C5060). Quantify the fluorescence intensity using the Image-Pro Plus analysis software.

Filter parameter	
Excitation wavelength (nm)	488
Emission wavelength (nm)	510

Compound Preparation

The agonist/antagonist compound should be diluted with phenol red free media with 0.1% BSA for fluorescence induction assay. To a 96-well, add 10X working concentration of agonist/antagonist compound in phenol red-free media (e.g.: add 10 μl/well if the buffer volume is 100 μl).

Fluorescence Induction Assay of HEK293/MC4R/CRE-EGFP Cells

Standard protocol for fluorescence induction assay:

PART 1: Fluorescence induction assay of HEK293/MC4R/CRE-EGFP cells withα-MSH in 96-well plate

1) Cell plating: Cells are seeded at a density of $2x10^4$/well in a 96-well black wall, clean bottom plate and incubated in CO_2 incubator.
2) 12-24 hours later, discard the growth media. Add 100 μl/well of phenol red free medium (0.1% BSA) and incubate overnight (8~12 hrs) at 37°C/5% CO_2.
3) Prepare agonists addition plates in advance of assay. Add 10X work concentration of agonist compound in phenol free media
4) Then, add 10 μl 10X agonist solution into wells and incubate 8-24 hours at 37°C/5% CO_2.
5) Capture the fluorescence pictures with Olympus Microscope (C5060). Semi-quantify the fluorescence intensity using the Image-Pro Plus analysis software.

PART 2: Fluorescence induction assay of HEK293/MC4R/CRE-EGFP cells withβ-MSH in 96-well plate

1) Cell plating: Cells are seeded at a density of $2x10^4$/well in a 96-well black wall, clean bottom plate and incubated in CO_2 incubator.
2) 12-24 hours later, discard the growth media. Add 100 μl/well of phenol red free medium (0.1% BSA) and incubate overnight 8-12 hours at 37°C/5% CO_2.
3) Prepare agonists addition plates in advance of assay. Add 10X working concentration of agonist compound in phenol free media
4) Then, add 10 μl 10X agonist solution into wells and incubate 8-24 hours at 37°C/5% CO_2.
5) Capture the fluorescence pictures with Olympus Microscope (C5060). Semi-quantify the fluorescence intensity using the Image-Pro Plus analysis software.

PART 3: Fluorescence induction assay of HEK293/MC4R/CRE-EGFP cells with foskolin in 96-well plate

1) Cell plating: Seed cells at a density of $2x10^4$/well in a 96-well black wall, clean bottom plate and incubate them in CO_2 incubator.
2) 12-24 hours later, discard the growth media. Add 100 µl/well of phenol red free medium (0.1% BSA) and incubate overnight (8~12 hrs) at 37°C/5% CO_2.
3) Prepare agonists addition plates in advance of assay. Add 10X working concentration of foskolin in phenol-free media.
4) Then, add 10 µl 10X agonist solution into wells and incubate (8~24 hrs) at 37°C/5% CO_2.
5) Capture the fluorescence pictures with Olympus Microscope (C5060). Semi-quantify the fluorescence intensity using the Image-Pro Plus analysis software.

Note: The fluorescence induction assay can not be performed with DMEM with phenol red media, which shows strong background for fluorescence observation

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Mycoplasma Detection Protocol (Using Venor@GeM Mycoplasma Detection kit)

1) Allow cells to reach 90-100% confluence.
2) Transfer 100 µl of supernatant from the test culture to a sterile micro-centrifuge tube. The lid should be tightly sealed to prevent opening during heating.
3) Boil or incubate the sample at 95°C for five minutes.
4) Briefly centrifuge (five seconds) the sample supernatant to pellet cellular debris before adding to the PCR mixture.
5) Rehydration of the reagents
   A. Centrifuge tubes with lyophilized components (five seconds at maximum speed).
   B. Add appropriate amount of deionized, DNA-free water:
   Primer/Nucleotide Mix (per portion of 25 reactions) 130 µl
   Positive Control DNA 100 µl
   Internal Control DNA 220 µl
   C. Incubate for five minutes at room temperature.
   D. Votex and centrifuge again.
6) The PCR Mastermix

PCR components (µl)	Negative control	Positive control	Cell sample
dH_2O	26.8	24.8	24.8
10X PCR Buffer	5	5	5
$MgCl_2$（25 mM）	6	6	6
Primer/nucleotide Mix	10	10	10
Internal control	2	2	2
Positive control DNA	—	2	—
Cell sample	—	—	2
Taq polymerase (5U/µl)	0.2	0.2	0.2
Total volume	50	50	50

7) Thermal profile
   1 cycle 94°C for two minutes

35 cycles 94°C for 30 seconds
55°C for 30 seconds
72°C for 30 seconds
Agarose gel run and evaluation

Reverse Transcriptase PCR Process

RNA preparation:

1) Lyse cells directly in a culture dish by adding 1 ml of TRIZOL reagent to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette.
2) PHASE SEPARATION
Incubate the homogenized samples for five minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at 15°C to 30°C for two to three minutes. Centrifuge the samples at no more than 12,000×g for 15 minutes at 2°C to 8°C.
3) RNA PRECIPITATION
Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA or protein is desired. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL reagent used for the initial homogenization. Incubate samples at 15°C to 30°C for 10 minutes and centrifuge at no more than 12,000×g for 10 minutes at 2°C to 8°C.
4) RNA WASH
Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7,500×g for five minutes at 2°C to 8°C.
5) REDISSOLVING THE RNA
At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Partially dissolved RNA samples have an A260/280 ratio < 1.6. Dissolve RNA in RNase-free water by passing the solution a few times through a pipette tip and incubating for 10 minutes at 55 to 60°C. RNA can also be redissolved in 100% formamide (deionized) and stored at -70°C.

Reverse transcription

Reverse transcription is carried out with the SuperScript First-Strand Synthesis System for RT-PCR. The following procedure is based on Invitrogen's protocol.

1) Prepare the following RNA/primer mixture in each tube:
Total RNA 1 µg
Random hexamers (50 ng/µl) 1 µl
10 mM dNTP mix 1 µl
DEPC H_2O to 10 µl
2) Incubate the samples at 65°C for five minutes and then on ice for at least one minute.
3) Prepare reaction master mixture. Proportions follow:
10x RT buffer 2 µl
25 mM $MgCl_2$ 4 µl
0.1 M DTT 2 µl
RNAaseOUT 1 µl
4) Add the reaction mixture to the RNA/primer mixture, mix briefly, and then place at room temperature for two minutes.
5) Add 1 µl (50 units) of SuperScript II RT to each tube. Mix.
6) Incubate the tubes at 50°C for 50 minutes. Heat inactivate at 85°C for five minutes, and then chill on ice.
7) Add 1 µl RNase H and incubate at 37°C for 20 minutes.

8) Store the 1st strand cDNA at -20°C until use for PCR.

PCR:

1) Prepare the following mixture in each tube:

1st strand cDNA	1 µl
10X Taq buffer	2 µl
sense primer (5 µM)	1 µl
antisense primer (5 µM)	1 µl
2.5 mM dNTP mix	2 µl
H_2O	to 20 µl

2) PCR reaction:

95°C two minutes
95°C 20 seconds
55°C 30 seconds } 25 cycles
72°C 45 seconds
72°C 10 minutes
4°C ∞

V. Results

PART 1: Inducibility of HEK293/MC4R/CRE-EGFP Cell Line

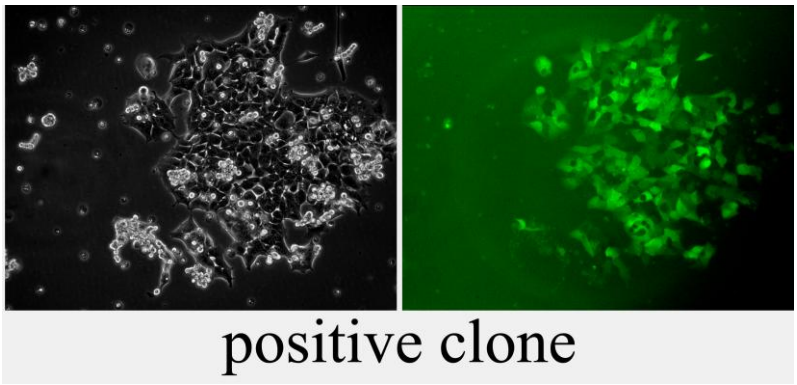

Figure 1. Development of 293-CRE-d2EGFP cells. Left: Formation of antibiotic cell clone. Right: Induction of green fluorescence in selected clone. The cells were incubated for 24 hours with 1 µM α-MSH. Cells were examined by fluorescence microscopy. Magnification: 400×

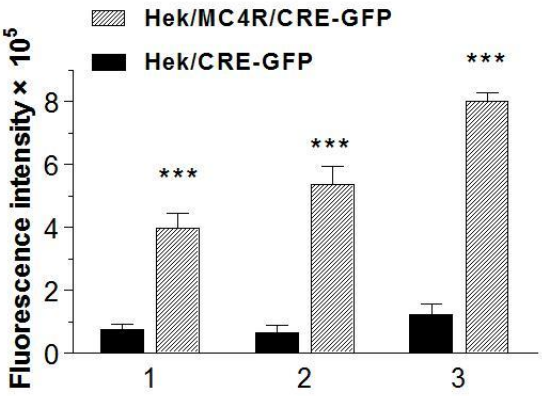

Figure 2. α-MSH -induced GFP response in HEK/MC4R/CRE-GFP and HEK/CRE-GFP parental cells. The cells were incubated with (1) 0.1 μMα-MSH , (2) 0.3 μMα-MSH , (3) 1 μMα-MSH in 96 well plates for 24 hours. Then the d2EGFP expression was measured as described under materials and methods. Means and standard deviations are from three independent experiments.*** indicate significant difference. (*P*<0.001)

Good clones of drug-resistant cell colonies were obtained from the co-transfection of MC4R and CRE-GFP plasmids into the HEK293 and CRE-GFP plasmid into HEK293 only as parental cells. The MC4R cell line showed good responses to the stimulation of α-MSH. However, Hek/CRE-GFP cells showed less response to the stimulation (Fig. 2).

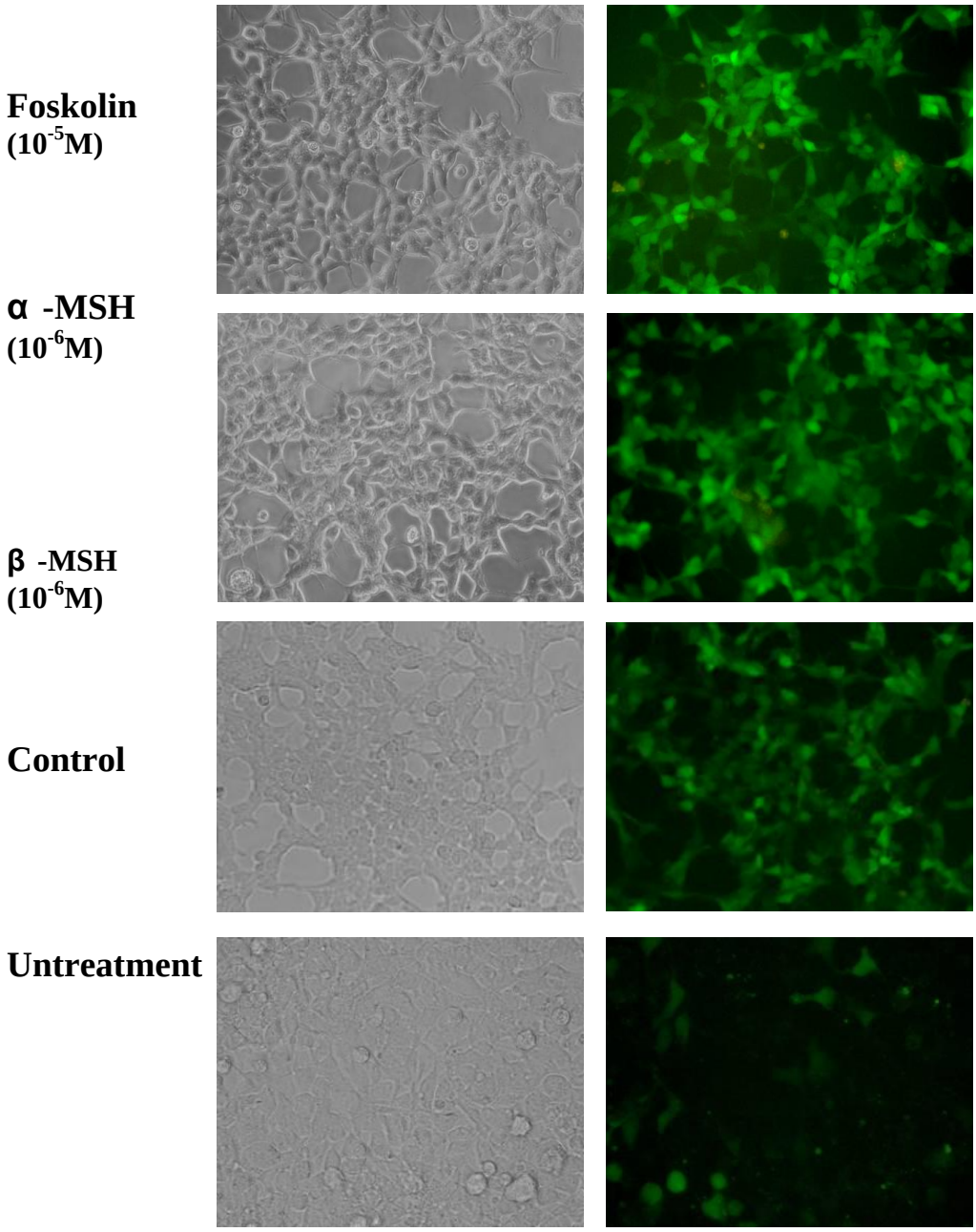

Figure 3. Fluorescence images of d2EGFP expression in MC4R cells following treatment with the foskolin and α/β–MSH and foskolin. The cells were incubated with 10 μM foskolin, 1 μM α–MSH and β–MSH in 96 well plates for 24 h. Then the fluorescence images were taken using an Olympus digital camera and show random fields of representative wells at 200×magnification.

We confirmed that HEK/MC4R/CRE-EGFP showed a good respond to the stimulation of α-MSH or β-MSH. The fluorescence intensity is close to that with foskolin stimulation (Fig.3).

PART 2: Assay development

Responses of HEK/MC4R/CRE-EGFP cell line to α-MSH and β-MSH

HEK/ MC4R/CRE-EGFP cells were validated as described above. Dose responses withα-MSH and β-MSH and foskolin were performed. α-MSH is the most potent one and its EC50 is 40.8 nM (Fig.B); and forβ-MSH, EC50 is around 140 nM (Fig.A); EC50 of foskolin is around 0.719 μM (Fig. C). Their rank order of potency was consistent with published data.

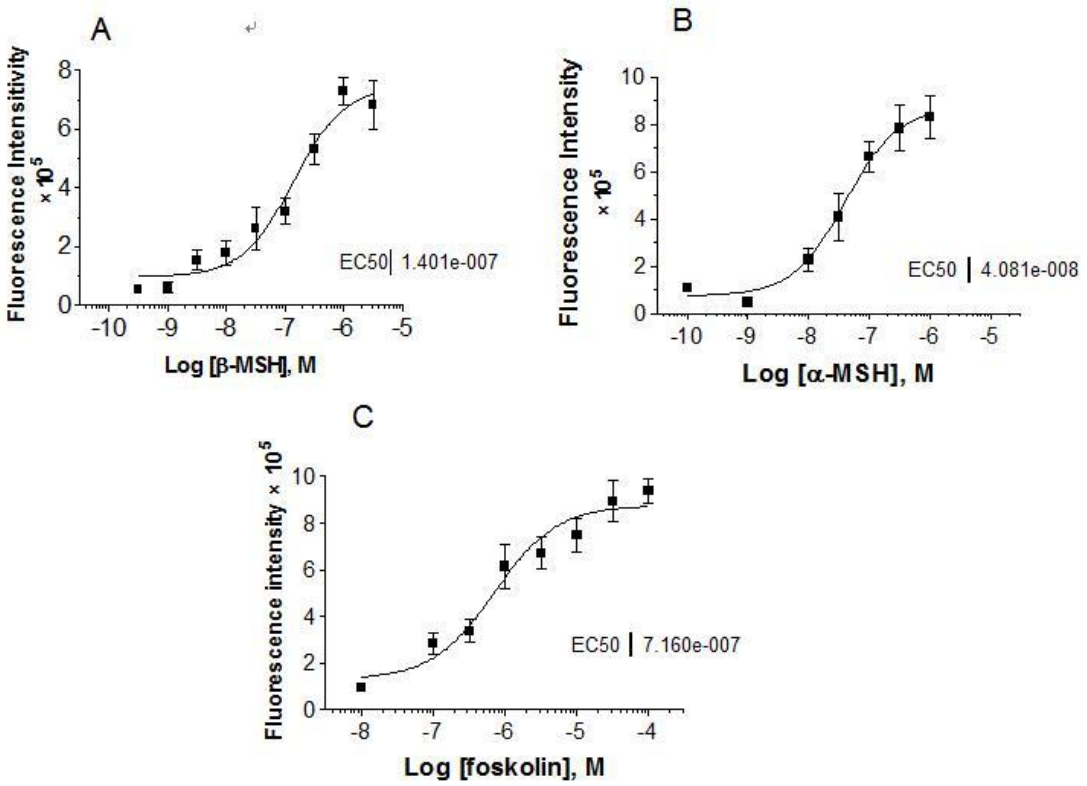

Figure 4. α-MSH, β-MSH and foskolin agonist dose response curves of MC4R cell line. (A–C) Dose–response ofα-MSH,β-MSH and foskolin induced GFP response in MC4R cell lines. The cell seeding, foskolin and MSH addition, cell incubation and fluorescence measurement were described in detail in the method. The data presented are the average standard deviation of three independent experiments performed with three replicates. Data represent means ± SEM for duplicate samples. EC50 value was determined using GraphPad Prism 5 software.

PART 3: Assay Validation

Reverse Transcriptase PCR (RT-PCR) confirmation of the MC4R cell lines

After HEK/MC4R/CRE-EGFP cell clones was selected, a RT-PCR experiment was preformed to confirm the expression of MC4R mRNA in the cells. As shown in the lane 1 of Fig. 5, the expression of MC4R cDNA (a band of 999 base pairs in lane 1) were positive in the MC4R cell line and negative in wt HEK293 cell line. The results of RT-PCR suggest that the HEK/MC4R cell line indeed contained human MC4R cDNA.

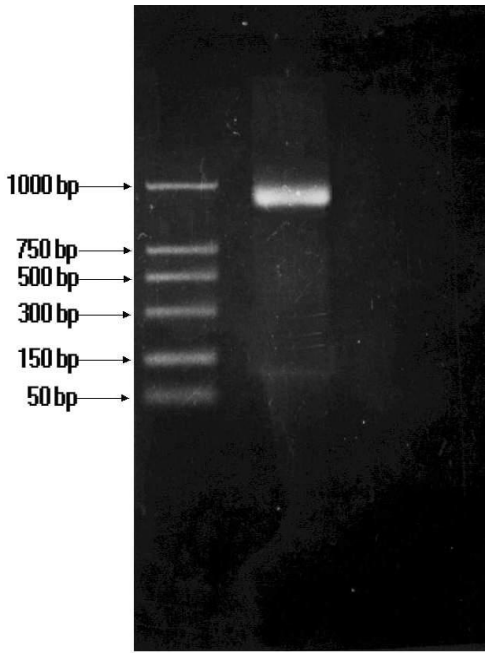

Figure 5. RT-PCR analysis of HEK/ MC4R/CRE-EGFP cell lines. RT-PCR was performed using total RNA isolated from the cells according to the procedures described above.

PART 4: Cell line stability test

α-MSH agonist dose responses were tested at five passages and 15 passage, respectively, in independent experiments. The signal has been stable and the calculated EC50 is comparable among them (Fig. 6, ranged from 48.99 nM to 58.58 nM), which is close to published data. No drop in activity was observed for the cell line.

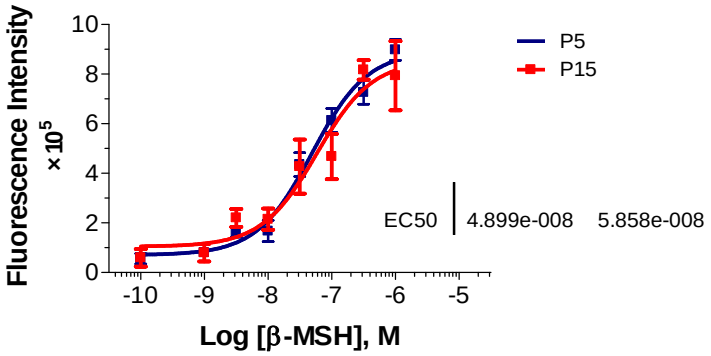

Figure 6. α-MSH agonist dose response curves of MC4R recombinant cell line at every five passages up to passage 15. Data represents means ± SEM for duplicate samples. EC50 value ofα-MSH dose response was determined using GraphPad Prism 5 software.

PART 5. Mycoplasma Detection

Mycoplasma detection using PCR method has been done routinely for MC4R cell line. The result demonstrates that cell line is mycoplasma-free (Fig.).

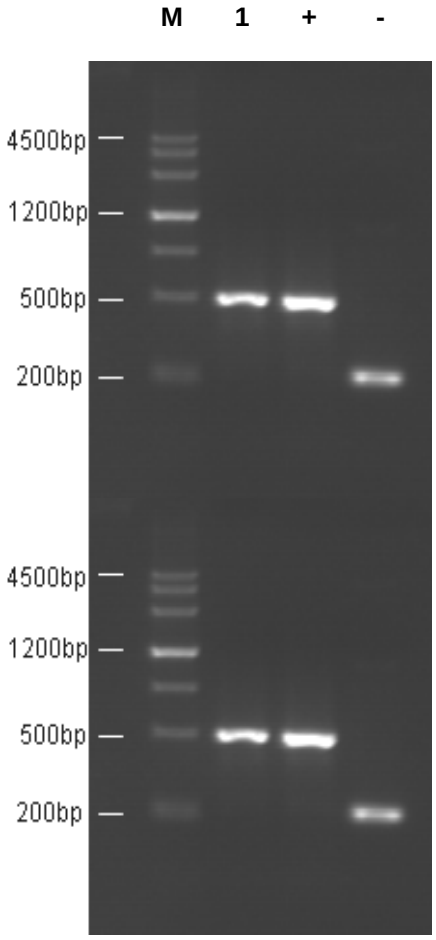

Figure7 . Mycoplasma detection of MC4R cell line. Assay was done using Venor@GeM Mycoplasma Detection kit. **M,** DNA marker; **1:** HEK293/MC4R/CRE-EGFP cell line; **+:** Positive Control; **-:** Negative control.

Conclusion

A cell-based functional assay was developed and validated for HEK293/MC4R /CRE-EGFP cell line through fluorescence induction observation. Our data show that the cell line is stable and the assay is specific for MC4R. It is also useful for lead compound validation and characterization.

VI. References

1. Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L, Metzger JM, Strack AM, Camacho RE, Mellin TN, Nunes CN, Min W, Fisher J, Gopal-Truter S, MacIntyre DE, Chen HY and Van der Ploeg LH (2000) Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nature genetics* **26**(1):97-102.

2. Goodfellow VS and Saunders J (2003) The melanocortin system and its role in obesity and cachexia. *Current topics in medicinal chemistry* **3**(8):855-883.

3. Hadley ME and Haskell-Luevano C (1999) The proopiomelanocortin system. *Annals of the New York Academy of Sciences* **885**:1-21.

VII. Appendix

Reagents & Consumables:

1. PfuUltra™ High-Fidelity DNA Polymerase, Stratagene, Cat # 600380.
2. QIAprep Spin Miniprep Kit, Qiagen, Cat # 27104.
3. QIAquick Gel Extraction Kit, Qiagen, Cat # 28706.
4. QIAquick PCR Purification Kit, Qiagen, Cat # 28106.
5. QuikChange Site-Directed Mutagenesis Kit, Stratagene, Cat # 200518.
6. TRIZOL: Invitrogen, Cat # 15596-026
7. DEPC: diethypyrocarbonate, Sigma, Cat #D5758
8. SuperScript III First-Strand Synthesis System for RT-PCR: Invitrogen, Cat #18080-051
9. DMEM: Dulbecco's Modified Eagle Medium powder, high glucose, Gibco BRL, Cat #12100-046.
10. DMEM: Dulbecco's Modified Eagle Medium liquid, high glucose, Gibco BRL, Cat #21063-029. Phenol red free
11. FBS: Fetal Bovine Serum, Hyclone, Cat #CH30160.03.
12. dFBS: Dialyzed Fetal Bovine Serum**,** Hyclone, Cat #SH30079.
13. L-Glutamine: 200 mM, Gibco BRL, Cat # 25030-081
14. Zeocin: Invitrogen, Cat # R25001
15. Trypsin: 1:250 rom Bovine Pancreas, Gibco BRL, Cat # 27250016
16. Trypsin-EDTA: prepared in HD Biosciences
17. PBS: prepared in HD Biosciences (see Media & Solution)
18. MATRIGEL: BD Bioscience, Cat# 354230
19. LipofectamineTM 2000: Invitrogen Cat #11668-019
20. DMSO: Dimethyl sulphoxide, Sigma, Cat #D8418
21. Hepes: Sigma Cat #H-3375
22. α-MSH: HDB
23. β-MSH: HDB
24. Venor@GeM Mycoplasma Detection kit: Minerva Biolabs Cat #11-1050
25. T25 flask: 25 cm^2 cell culture flask, Corning, Cat #430639
26. 6 cm dish: Orange Scientific, Cat # 2050200
27. 10 cm dish: Corning, Cat # 430167
28. 6-well plate: Corning, Cat #3516
29. Cryogenic Vial: Corning, Cat #430289
30. 96 Well Plate: Costar, Cat# 3603, BlackWall/Clear Bottom, Polystyrene, sterilized.

Media and Solutions:

1. PBS (for preparation of 500 ml)

1)	KCl:	0.1 g
2)	KH_2PO_4:	0.1 g
3)	NaCl:	4.0 g
4)	$Na_2HPO_4.12H_2O$:	1.4425 g

Dissolve the above components in double-distilled water (ddH_2O) and adjust pH to 7.4 with 0.1 N NaOH. Add ddH_2O to the final volume of 500 ml. Autoclave and store at 4°C.

2. Trypsin-EDTA (for preparation of 100 ml)

1)	Trypsin:	0.25 g
2)	2%EDTA:	2 ml
3)	PBS:	98 ml

Dissolve trypsin in 2%EDTA and PBS completely. Sterilize the solution by passing through a 0.20 μm membrane filter. Store at 4°C.

3. Culture medium (for preparation of 1 L)

1) Measure out 950 ml distilled water to dissolve the media components, stirring gently until the solution becomes clear.
2) Add $NaHCO_3$ 3.7 g for high glucose DMEM.
3) Adjust pH of medium to 0.2-0.3 below the desired final working pH (using 1 N NaOH or 1 N HCL is recommended). Add slowly while stirring.
4) Dilute to 1 liter with ddH_2O.
5) Sterilize the medium immediately using the method of membrane filtration. Store at 4°C.

4. Complete DMEM medium

1) DMEM: 90%, FBS: 10%
2) Supplements: L-glutamine 2.0 mM

5. Freezing Medium

1) DMEM: 70%, FBS: 20%
2) Supplements: dimethyl sulphoxide (DMSO) 10%

6. Serum-free DMEM medium
1) Phenol red free - DMEM
2) 0.1% BSA

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