目录

生化实验

分子实验

Molecular cloning

Point mutation

Design primers according to QuikChange® Site-Directed Mutagenesis protocol (*stratagene*) .

100ng template plasmid,

PCR 10 to 15 cycles. Purify the product.

Digest with DpnI. Purify again.

Transfer to DH5a or other appropriate competence cell.

RNA manipulation

Purification, follow 天根 #DP421 protocol.

Reverse transcription, follow 天根 #KR106 protocol.

Realtime PCR

Western blot

试剂配方：

Lysis buffer

PBS + 1% Triton X-100 + 1X complete protease inhibitor (Roche).

Running Buffer.

转膜 buffer.

To 10X stock buffer, Tris 40 g, Glycine 144 g, add Millipore water to 1L.

Protein purification

For Western-blots, cells were lifted, collected by spinning, and then washed by phosphate buffered saline (PBS). The cell pellets are then subsequently lysed on ice for 30 minutes with the following lysis buffer. The lysates were then sonicated for 10 seconds and spun at >20000 g at 4 ℃ for 10 minutes. The supernatants were then collected and the total protein concentration of each sample was measured by the BCA assay kit (Pierce). Lysates of same amount of total protein for each sample were then added with LDS loading buffer and Nupage reducing reagent (Invitrogen), and heated at 70 ℃ for 10 minutes.

Gel running

For full length HTT, 6% PAGE or 3-8% Tris-Acetate gels (Invitrogen) in the Tris-Acetate gel running buffer were used (Invitrogen).

For proteins ranged from 70-150KD, 8% PAGE.

For proteins ranged from 30- 70 KD, 10-12% PAGE.

For proteins smaller then 30KD, 15% PAGE

转膜

30-70KD

100ml, 10X stock buffer, add Millipore water to 800ml, add 甲醇 to 1000ml 20 度预冷, 200mA 恒流 1 小时。

Htt

100ml, 10X stock buffer, add Millipore water to 900ml, add 甲醇 to 1000ml 20 度预冷, 400mA 恒流 2 小时。

细胞实验

细胞培养

血清灭活

将血清放于 4 度冰箱中过夜融化，水浴锅 60 度 1 小时。

STHdh 细胞

STHdh cells are immortalized cell line generated from striatal neuronal progenitor cells in knock-in mice, and cultured in DMEM (Gibco, #11965) with 10% FBS (Gibco, # 10082-147).

Human fibroblast

Human HD fibroblasts were obtained from Coriell Cell Repositories (GMXXXX) and immortalized by SV40 large T-antigen. The cell culture medium used was MEM (Gibco, #10370) with 15% FBS (Gibco, # 10082-147) and 1X Glutamax (Gibco, # 35050079).

细胞转染

For siRNA in 6-well plate using lipo2000 (or lipo3000 in brackets)

1. prepare mixtures: A: siRNA with opti-MEM to 400nM for 250μl/well (125ul/well)

For example: if the concentration of simMAPK11 is 20nmol/ml, then add 5ul siRNA and 245ul OPTI-MEM (120ul/well)

B: 10ul (7.5ul) in opti-MEM at 250μl/well (125ul/well)

Mix A with B for 30 minutes.

The cell was suspended in complete medium (DMEM with 10% FBS)

2.count cell to get the concentration of the cell suspension, then dilute with complete medium to 50000 cells/ml

3.add 2ml of the cell suspension to each well on top of the transfection mixture (A/B)

4.shake the plates to mix the cells with the transfection mixture, then cultured in 33℃,5% CO2 incubator

For cDNA in 6-well plate using lipo2000 (or lipo3000 in brackets)

1. Plate cells one day before transfection at 200000/well (roughly 20% confluency)

2. Prepare mixtures:

A: cDNA with opti-MEM: 4ug cDNA in 250ul OPTI-MEM for lipo2000, or 2.5ug cDNA in 125ul OPTI-MEM for lipo3000. If the cDNA volume is large (>5ul), adjust the OPTI-MEM volume so that the total volume is close to 250ul(125ul)

B: 10ul (7.5ul) in opti-MEM at 250μl/well (125ul/well)

Mix A with B for 20 minutes or more.

3.add the mixture prepared in step 1 on top of the cells.

4.shake the plates to mix the cells with the transfection mixture, then cultured in 33℃,5% CO2 incubator

慢病毒包装

Calcium Phosphate Cell Transfection

Reagents:

2 x HBS (for 500ml): NaCl - 8g, KCl - 0.38g, Na2HPO4 - 0.1g, Hepes - 5g, Glucose - 1g, Bring pH to 7.05. PH 值非常重要，一般要求 PH6.90-7.15 做若干组，然后选择效率最高的一组。

2.5M CaCl2,

bi-distilled water

Method

Day 1 Plating (9-10am), Plate 2-2.5x106 of 293T cells per 10cm plate.

Day 2 Transfection (9-10am), Prepare calcium-phosphate precipitate (1ml/10cm plate). Transfer vector - 20μg, Packaging plasmid - 15μg (3rd generation: pMDL g/p RRE - 10μg + pRSV-Rev - 5μg), Envelope plasmid - 6μg. Add water to 0.5ml, add 0.5ml 2xHBS and mix well. Add 50μgl 2.5M CaCl2 and shake briefly, keep in RT for 20-25min, add dropwise on a plate and mix gently with a medium.

Change medium (6-8hrs later); remove medium with precipitate and add 6ml/plate of fresh medium.

Day 4 Collection (9-10am), Collect medium. Spin 3000rpm/5min/RT, Filter through 0.45 μm.

At this point virus can be used for transduction, frozen at -70° C for future use, or concentrated.

Lipofectamine

Plate of $9x10^6$ 293 FT cells per 15 cm dish in 25 ml medium (DMEM high glucose + 10 per cent FBS)at D_0

Transfect the cells at D_{+1} using the following transfection method:

Tube A: Packaging mix plasmids 6.24 l in 3 ml of OptiMEM + gene of interest 11.91 g mix well with pipetting up and down and incubate at RT for 5 min.

Tube B: 90 l of Lipofectamine in 3 ml of OptiMEM mix well pipetting up and down and incubate at RT for 5 min.

Mix Tubes A and B with pipetting up and down and incubate at RT for 20 min.

Add the transfection (6 ml) mix to 293 FT cells plate.

Concentration

Transfer 30ml of virus to 33ml Beckman conical tubes spin at 26.000rpm/2hrs/4° C in Beckman SW28 swingle bucket rotor. After spin discard supernatant and resuspend the virus in a desired volume of serum-free medium (e.g. Cellgro or Episerf) or PBS/1% BSA, aliquot and store at -70° C. For transduction of very delicate cells the virus can be concentrated on sucrose cushion, just put 4ml of 20% sucrose on the bottom of the tube and overlay with 26ml of viral supernatant.

细胞收集及样品处理

细胞染色

免疫荧光实验

电镜样品制备

电镜常规制样（单层细胞）

将细胞贴壁培养于六孔板或培养皿中（细胞密度尽量大）

吸去上层培养基，用 PBS 洗一遍。（注：加液体时沿培养皿壁小心加入，避免将贴壁细胞吹掉）
戊二醛、锇酸、epon812 从生化细胞所电镜平台领取。
加入 2.5%戊二醛，室温固定 1 小时后 4 度固定过夜
吸掉戊二醛，PBS 洗 4 遍，10min/次
加入 1%锇酸，后固定 1-2 个小时（时间不可过长）
PBS 洗 5 遍，3min/次
酒精梯度脱水：30% 10min 50% 10min 70% 10min 85% 15min 95% 15min 100% 2 次(无水乙醇用之前用无水硫酸钠过滤一下或者用新开的)，每次 30min
渗透：无水乙醇：epon812 2:1 1h, 无水乙醇：epon812 1:1 1h. 无水乙醇：epon812 1:2 1h.
纯 epon812 过夜。
包埋，吸掉过夜的 epon812,铺一层新鲜的树脂，将装满新鲜树脂的胶囊倒扣至细胞处，65 度烘箱聚合两天。

Pulse Chase

功能性实验

STHdh 细胞 stress
血清饥饿
6 孔板每孔接种 $1X10^5$ 细胞，48 小时后对照细胞换完全培养基，stress 细胞用 1ml 含有双抗的不加血清的培养基洗一次，吸干培养基后加入 1ml 含有双抗的不加血清的培养基，48 小时后固定染色
Heat shock
6 孔板每孔接种 $1X10^5$ 细胞，48 小时后置于 37OC, 42OC 培养箱中处理 2-4 小时后固定染色。

Patient fibroblast transfection and HTRF detection for mHTT:

A. Cell culture immortalized human HD fibroblasts

Culture medium: MEM (Gibco, #10370-021) + 15% Heat-inactivated FBS (Gibco, #10082-147) + 1X Glutamax (Gibco, #35050-061). Cells are normally cultured in T150 with 20 ml complete culture medium, in humidified 37°C + 5% CO_2 incubator. They normally double everyday and form monolayer attached cultures. Cells are normally subcultured twice a week at 1:10.

B. siRNA transfection (384 well format)

1. Dilute the siRNA stock with OPTI-MEM (Gibco, #31985-070) to the concentration of 0.2 µM. The plates of diluted siRNAs could be stored at -20 °C for months.
2. Make the transfection mix by mixing (3.9 µl OPTI-MEM + 0.1 µl Dharmafect1) and 4 µl diluted siRNA from step1. Incubate at room temperature for 1 hour.

3. During the incubation time, lift the cells by trypsinizing and collect the cells by centrifuging at 1300 rpm for 3 minutes.
4. Aspirate the medium and resuspend the cells with complete culture medium with 18% FBS. Determine the cell number/ml by Vi-Cell. Dilute the cells to the density at 1.71 X 10^5 cells/ml (for the Q45 cell line) or 1.14 X 10^5 cells/ml (for the Q68 cell line). The Q45 cell density is higher because they proliferate a little slower than the Q68 cells. The serum concentration is a little higher because the transfection mix does not have serum.
5. Plate 35 µl cells into each well and incubate for 72 hours in humidified 37°C + 5% CO_2 incubator before lysis and HTT detection. The final siRNA concentration is ~20 nM, whereas up to 40 nM worked as good in knock-down of HTT and showed better knock-down to some of the target genes

C. cDNA transfection (AMAXA, 96 well format)

The Amaxa Basic 96-well Nucleofector Kit1 Primary Fibroblasts (Lonza, # VHPI-1012) was used. The protocol is very similar to the one provided by the manufacturer:

1. Prepare the cDNA plate (2 µl of 0.5 µg/µl cDNA per well) in the V-shape bottom 96-well plate.
2. Lift the cells by trypsinizing and collect the cells by centrifuging at 1300 rpm for 3 minutes.
3. Resuspend the cells in complete medium and determine the cell density by Vi-Cell. Calculate the volume for the required number of cells (2 X 10^5 cells/well for Q45 and 1X 10^5 cells/well for Q68), take the volume and spin to collect the cells at 1300 rpm for 3 minutes.
4. Aspirate the medium and resuspend the cells with Solution1 supplemented with the Supplement Buffer (20 µl per well), both of which provided by the kit.
5. Plate the cells onto the cDNA plate (20 µl per well), mix by pipetting and transfer then transfer the mix to the 96-well shuttle plate.
6. Transfect with program 96-CA137
7. Add 180 µl complete medium into each well, and transfer them to the culture plates.
8. Incubate for 48 hours in humidified 37°C + 5% CO_2 incubator before lysis and HTT detection.

D. cDNA infection for InCell experiments (Lenti-virus, 96 well format)

1. Cells were plated in 96 well plates at 6000 cells/well in 180 µl complete culture medium on Day -1.
2. On Day 0, the culture medium was removed for each well and added with the virus of the indicated MOI in 30 µl serum-free medium (just the MEM) for each well. After 1 hour incubation, 150 µl of medium with 18% serum was added into each well.
3. Incubate for 48 or 72 hours in humidified 37°C + 5% CO_2 before InCell experiments. The staining condition for InCell is still being optimized.

E. HTRF detection of endogenous mutant HTT in human HD fibroblasts (384 well format; Directly lysis in well)

1. Aspirate the medium with plate washer so that ~6 µl medium remains in each well of the 384-well plates.
2. Prepare the antibody mix in lysis buffer: 1X HTRF buffer (50 mM NaH_2PO_4, 400 mM NaF, 0.1% BSA, 0.05% Tween 20) + 1% Triton X-100 + 2.5% EDTA-free protease inhibitor (Calbiochem, #535140) + 2B7-Tb (0.023 µg/ml) + MW1-D2 (1.4 µg/ml).
3. Add 5 µl of the solution in step2 into each well of the 384-well plates. Spin briefly, seal the plates and shake the plates at 700 rpm for 2 minutes.
4. Spin the plates at 1800 rpm for 1 minute and incubate the plates at 4 ^{o}C overnight.
5. The next day, warm up the plates to room temperature and spin the plates at 1800 rpm for 1 minute.
6. Remove the seal and read the HTRF by measuring the time-resolved fluorescence at 615 nM and 665 nM. The parameters of the Envision protocol needs to be optimized by the optimization function of the device.
7. Mutant HTT level correlates with the HTRF signal linearly within the range between 0 to ~8 µg total protein/well, whereas the direct lysis of the cells plated in well is normally 2~4 µg estimated by cell number.

Fractionation：P1，P2，S2：

The samples were lysed according to the following protocol to separate the membrane, nuclei and cytosolic fractions.

http://www.jbc.org/content/280/43/36464.full
http://www.jbc.org/content/280/43/36464/F2.large.jpg

Subcellular Fractionation and Western Blot Analysis—Subcellular fractionations, SDS-PAGE, and Western blot analysis were performed as described previously (5). Briefly, cell homogenates made in 10 mm HEPES, pH 7.4, 250 mm sucrose, 1 mm EDTA plus protease inhibitors were centrifuged at 2000 × g to obtain the crude nuclear pellet (P1) and the postnuclear supernatant (S1). S1 was again centrifuged at 100,000 × g to obtain the membrane pellet (P2) and the cytosolic fraction (S2). Lanes were loaded with 10 µg of protein from each fraction. Distribution of the transmembrane protein calnexin (an endoplasmic reticulum marker) was used to assess the purity of fractions. For Western blotting, the following concentrations of antibodies were used: anti-htt Ab 1 (0.5 µg/ml), anti-htt mAb 2166 (1:1000; Chemicon), and anti-calnexin (1:1000; Stressgen). Peroxidase-labeled secondary antibodies (Jackson ImmunoResearch) were diluted 1:5000. Blots were developed using ECL (Amersham Biosciences). Densitometry data from at least three separate experiments were analyzed using SigmaScan (Jandel); where a doublet was observed at the appropriate relative molecular mass, both

bands were measured. Statistical significance was determined using an ANOVA and post hoc Bonferroni tests (Graphpad Instat).

FREEZING/THAWING hESCs and hiPSCs BY VITRIFICATION IN CRYOVIALS

To improve recovery, we have optimized a protocol based on vitrification. This protocol uses cryovials. We typically observe 40-50% recovery using this protocol. This protocol is also significantly better for the maintenance of a normal karyotype and ES characteristics in both hESCs and hiPSCs.

Materials and reagents needed:

1. Ethylene glycol [Sigma cat E9129]
2. DMSO [Sigma cat. D2650]
3. Human ESC medium (hESM) see **section 1.2**
4. Sucrose [Fisher cat S5-500]
5. 1M HEPES solution [Invitrogen, cat 15630-080]
6. Cryovials
7. liquid nitrogen in an ice bucket
8. Square floating microtube rack [Nalgene 5974-0404]

Prepare Media:

HM (Growth Medium with 20mM HEPES):

80% hESM

20mM HEPES

HM+Sucrose:

3.42g Sucrose in 10ml HM

VS2:

30% HM

30% HM+Sucrose

20% ethylene glycol

20% DMSO

VS3:

40% HM+Sucrose

30% ethylene glycol

30% DMSO

WS3:

hESM +1M Sucrose

Caution: Prepare all reagents fresh, filter sterilize and maintain on ice while working.

Freezing protocol:

Caution: Work quickly. The HESC cannot be exposed to the cryoprotectants for very long or they will differentiate upon thawing.

Viability may also be reduced if timing is not closely controlled. Steps 4-11 must be timed accurately.

1. Harvest hESCs in clumps by manual dissection or collagenase/dispase treatment. This protocol can also be used on collagenase/dispase-harvested hESCs grown on Matrigel or MEFs.
2. Wash clumps well to remove collagenase/dispase if necessary.

3. Resuspend clumps into HM in about 0.5ml (depending on number of clumps). They can be kept at room temp in HM for up to 20min. Prolonged incubation will result in clumping and reduced attachment after thawing.
4. Transfer 40ul of the clumps into a sterile cryovial on ice in the microtube rack. Process no more than 5 vials at a time.
5. Add 40ul of VS2 and mix by gentle pipetting let sit a 10-20 seconds
6. Add 160ul of VS3 and mix by gentle pipetting. Steps 5-6 should be completed in no more than 1 min. Handle only as many tubes as can be processed in this amount of time. Remember that handling and capping the tubes will take time.
7. Submerge the tubes quickly in liquid nitrogen and swirl while freezing. The frozen solution should have **a pink glass-like appearance**, while a thin layer at the top might be opaque. Be sure the caps are tightened and transfer vials to liquid nitrogen storage boxes. It is important to do this quickly to prevent the small solution volume from thawing. We usually place a storage box in 1-2 inches of LN2 in a large rectangular ice bucket while processing and transferring the tubes.

Thawing protocol:

Thawing is performed in the tubes and all solutions must be prepared in advance. Steps 1-3 must be performed quickly so that the cells are not exposed to the high concentration of cryoprotectants for too long. The incubation times in steps 4-7 remove the sucrose slowly and prevent osmotic shock and lysis of the cells.
1. Remove a tube from liquid nitrogen storage and quickly submerge bottom of tube in warm sterile water in a beaker.
2. quickly wipe with 70%ETOH.
3. Immediately add 800ul of cold WS3 (growth medium + sucrose), mix gently by stirring with the pipette tip and let sit for 30 seconds.
4. Add 1ml of growth medium, mix as above, let sit for 2min.
5. transfer to 15ml conical tube.
6. Rinse cryotube 2 times with 1ml each of growth medium and add to 15ml tube, mixing gently, let sit for 1min.
7. Add 6ml growth medium slowly dropwise to cells over about 2 min.
8. Spin 1000 rpm / 4 min
9. Resuspend gently in 1ml of growth medium with p1000.
10. Using a p1000 to transfer to a well of a 6-well plate with MEFs in hESM. The colonies should recover and show signs of growth within a week. Change medium daily.

FREEZING/THAWING hESCs and hiPSCs Derived Neurons

Cells were exposed to 500 nM of the general caspase inhibitor benzyloxycarbonyl-V-A-D-O-methyl fluoromethyl ketone (z-VAD-FMK, R&D Systems) and incubated for 30 minutes before freezing. The cell suspension was frozen in 1.5 ml freezing media containing 10% DMSO (Hybri-Max; Sigma-Aldrich), 20% 500 mM myo-inositol (Sigma-Aldrich), 0.25 V% polyvinyl alcohol stock solution (Merck & Co., Whitehouse Station, NY, http://www.merck.com), and 65% GIBCO Knockout Serum Replacement (Invitrogen). Cells were directly transferred into a Nalgene Cryo freezing container (Nalge Nunc International, Rochester, NY, http://www.nalgenunc.com) and placed at −80°C to achieve a −1°C per minute rate of cooling. Final temperature was reached after freezing in liquid nitrogen. Thawing was carried out by dropping a frozen sample into a 37°C water bath for 2 minutes.

Reference:

Stem Cells. 2008 Jul;26(7):1705-12. Epub 2008 Apr 17.

Lineage selection of functional and cryopreservable human embryonic stem cell-derived neurons.

Ladewig J, Koch P, Endl E, Meiners B, Opitz T, Couillard-Despres S, Aigner L, Brüstle O.