Construct preparation of *hBRCA1* variants

Design oligos using The QuickChange Primer Design Program (http://www.stratagene.com/qcprimerdesign).

(Day1)
Dissolve the designed primers in $H_2O$ with a final concentration of 1µg/µl and prepare a working stock of 100 ng/µl by diluting them 10 times with $H_2O$.
Perform a PCR using the QuickChange Lightning Kit (Stratagene 210518).

10x Stratagene Reaction buffer 5.00 µl
*hBRCA1* Plasmid DNA (100 ng/µl) 1.00 µl
Forward primer (100 ng/µl) 1.25 µl
Reverse primer (100 ng/µl) 1.25 µl
Stratagene dNTPmix 1.00 µl
Stratagene Quick solution reagent 1.50 µl
$H_2O$ 39.00 µl
Stratagene Quickchange Lightning Enzyme 1.00 µl

PCR program: 2' 95°C; 18 x (20'' 95°C; 10'' 60°C; 10' 68°C); 10' 68°C.

Add 2 µl of DpnI restriction enzyme (Stratagene) directly after completion of the PCR and mix by thoroughly pipetting up and down. Incubate this mixture 5-10' at 37°C. Thaw XL-10 Gold ultracompetent cells (Stratagene) on ice and distribute 45 µl aliquots in prechilled 14 ml BD Falcon tubes. Add 2 µl of β-mercaptoethanol to the competent cells, swirl and leave them on ice for 2 minutes. Add 2 µl of the DpnI treated PCR reaction mixture to the competent cells, swirl and incubate on ice for exactly 30 minutes. Heat-pulse the samples at 42°C for exactly 30 seconds and subsequently put them on ice for 2 minutes. 0.5 ml of preheated NZY+ medium is added, after which the cells are incubated for 1 hr at 37°C in a shaking incubator at 225 rpm. Spin down the cells for 15 seconds at 14000 rpm, remove the excess of medium, approximately 400 µl, and plate the left over cell mixture on LB plates containing ampicillin. Incubate the plates overnight at 37°C.

(Day2)
Pick 8 colonies per construct and inoculate 10 ml LB cultures + ampicillin. Grow overnight at 37°C in the shaking incubator at 225 rpm.

(Day3)
Isolate DNA from the overnight cultures using Qiagen miniprep columns (include the PB-buffer wash step, it increases the yield of DNA; leave the elution buffer on the column for 15 minutes to increase the yield).

Sequence analysis of the *hBRCA1* variants

The full cDNA sequence of *hBRCA1* variants is analyzed to confirm the presence of the intended mutations and to exclude additional mutations. For this purpose, the following 10 sequencing primers are used (equally distributed over the *hBRCA1* cDNA sequence and flanking regions):

| | |
|---|---|
| Primer 1 | 5'-GCCTCAGACAGTGGTTC-3' |
| Primer 2 | 5'-GTCTCAGTGTCCAACTCTC-3' |
| Primer 3 | 5'-AATAAGCAGAAACTGCC-3' |
| Primer 4 | 5'-TACTAATAGTGGTCATG-3' |
| Primer 5 | 5'-AAGGGTTTTGCAAACTG-3' |
| Primer 6 | 5'-ATGCCAAATGTAGTATC-3' |
| Primer 7 | 5'-GTTTTGCAACCTGAGGTC-3' |
| Primer 8 | 5'-AACCAGGTAATATTGGC-3' |
| Primer 9 | 5'-AAGTTTGAGGTGTCTG-3' |
| Primer 10 | 5'-GGAAAGGACAGTGGGAG-3' |

| | | |
|---|---|---|
| Big Dye Terminator mix (Invitrogen) | 4.00 | µl |
| Sequencing primer (10 µM) | 0.75 | µl |
| DNA (200 ng/µl) | x | µl |
| $H_2O$ | 15.25 - x | µl |

Prepare the sequencing reaction and use the following sequencing program:
1' 96°C; 30x (30'' 96°C; 15'' 50°C; 4' 60°C).

Transfection and culture of *R26$^{CreERT2/RMCE}$;Brca1$^{SCo/\Delta}$* ES cells

ES cells are cultured on gelatin coated plates in 60% Buffalo Rat Liver cell (BRL) conditioned medium + 0.1 mM β-mercaptoethanol (Merck) and $10^3$ U/ml ESGRO LIF (Millipore) as described (1). It is recommended to use one batch of Brca1 SCo/RMCE ES cells and one batch of BRL conditioned medium for each series of experiments to ensure reproducibility of the assays.

*Media and solutions*

CM (complete medium)

| | |
|---|---|
| 1 x GMEM (Invitrogen 21710-025) | 500 ml |
| Sodium Pyruvate 100 mM (Invitrogen 11360-039) | 5 ml |
| Non-essential amino acids 100 x (Invitrogen 11140-035) | 5 ml |
| Fetal Calf Serum (Hyclone SV30160.03) | 50 ml |
| (FCS is tested for optimal growth of ES cells) | |

100% BRL medium

CM conditioned for one week on a monolayer of BRL cells

60% BRL + β-mercaptoethanol+ LIF (60% medium)

| | |
|---|---|
| 100% BRL medium | 150 ml |
| CM | 100 ml |
| L-Glutamin 200 mM (Invitrogen 25030-024) | 1.5 ml |
| β-Mercaptoethanol (1000x; Sigma M3148) | 0.25 ml |
| ESGRO LIF (100x; Millipore ESG1107) | 2.5 ml |

Gelatin

1 % gelatin (Sigma G1890) in PBS, dissolve 5 gram in 500 ml heat in microwave but do not boil, filtrate trough 0.22 µm filter while still warm.
Gelatinize tissue culture plates 20 minutes before cells are seeded using 0.1% gelatin in PBS.

PBS

Invitrogen 14190-169

β-Mercaptoethanol (1000x) *

0.1 M : 0.1 ml 2-mercaptoethanol,14.2 M (Merck 15433) + 14.1 ml water
Working conc.: 0.1 mM

*: do not use stock solution longer than one month

Trypsin-EDTA 0.05%

Invitrogen 25300-054

TVP (1x) †

| | | |
|---|---|---|
| PBS | | 477.5 ml |
| EDTA 40 mM | | 12,5 ml |
| Chicken serum | (Invitrogen 16110-033) | 5 ml |
| Trypsin 2.5% | (Invitrogen 25090-028) | 5 ml |

TVP (2x)

9.9 ml TVP (1x) plus 0.1 ml Trypsin 2.5%

LIF (100x)

$10^5$ Units/ml complete medium of ESGRO-LIF (Gibco 3275SB)
= 1 ml in 99 ml CM. Working concentration: $10^3$ U/ml

G418 (100x) †

20 mg G418 (Invitrogen 11811-031) per ml 60% medium, store at 4°C
Working concentration: 200-400 µg/ml

Hygromycin B (100x) †

15 mg Hygromycin B (Merck 400051) per ml 60% medium, 4 °C. Working concentration: 150 µg/ml

Puromycin (1000x)

18 mg Puromycin (Sigma P7255) per 10 ml PBS, -20 °C
Working concentration.: 1.8 µg/ml

†: solutions are filter sterilized

*Constructs*

Human BRCA1 VUS RMCE exchange vector (hBRCA1 VUS)
pFlpe (2)
pFlpo (3,4)
pCMV-EGFP (EGFP from Clontech 6077-1)

(Day1)
Thaw a frozen vial of mouse *R26*$^{CreERT2/RMCE}$*;Brca1*$^{SCo/\Delta}$ (Brca1 SCo/RMCE) ES cells, dilute 10-fold and pellet for 5' at 1200 rpm to remove DMSO. Plate in 3 ml ES cell medium on one well of a gelatinized 6-well plate.

(Day2)
Refresh medium.

(Day3)
Trypsinize the Brca1 SCo/RMCE cells with 0.5 ml 2x TVP, neutralize with 1.5 ml of medium and passage them 1:8 to a gelatinized 10cm dish in 15 ml medium.

(Day6)
Trypsinize the Brca1 SCo/RMCE cells in the 10cm dish with 2 ml 2x TVP and neutralize with 3 ml medium. Count the cells and seed for each VUS transfection 200.000 cells per well on a gelatinized 24-well plate in 0.5 ml medium.

(Day7)
Refresh the Brca1 SCo/RMCE cells the next day with 0.5 ml medium.

(Day8)
Refresh the approximately 80-90% confluent Brca1 SCo/RMCE cells with 0.5 ml medium 3 hours before transfection.
Prepare the transfection reagents as follows:

Mix A (per reaction):
OptiMEM (Invitrogen) 10.0 µl
Lipofectamine 2000 (Invitrogen) 1.2 µl
Incubate mix A 5 minutes at room temperature before use

Mix B (per reaction):
OptiMEM 10 µl
hBRCA1 VUS (400 ng) x µl
pFlpe (400 ng) 1.0 µl
pCMV-EGFP (20 ng) 1.0 µl (optional transfection control)
12.0 µl / rxn + 400 ng hBRCA1 VUS DNA

Add 10 µl of mix A to mix B
Mix by pipetting 5 times and incubate 20-40 minutes at room temperature
Add the mixture to a 24-well containing Brca1 SCo/RMCE cells, mix and incubate in the $CO_2$ incubator.
* Use 1 well per hBRCA1 VUS and take along negative controls to check for selection efficiency later (cells transfected with only GFP/Flpe and untransfected cells)

(Day9)
Check GFP expression in the transfected cells using a fluorescence microscope. GFP should be detectable in at least 20-30% of the cells. Trypsinize the transfected cells in 100 µl 2x TVP and neutralize with 1 ml medium. Resuspend them with a Gilson P1000 pipette and split the cells by transferring 2x 550 µl cell suspension to 2x 6-well containing 2 ml medium.

(Day10)
Refresh the transfected cells which are seeded in duplicate in 6-well plates with 2.5 ml medium containing 200 µg/ml G418, to start the selection.

(Day13)
Refresh the transfected cells with 2.5 ml medium containing 200 µg/ml G418, cell death should be clearly visible now.

(Day14)
Colonies of transfected cells should be visible now, check all wells for the presence of colonies.

(Day15)
Trypsinize the colonies in 0.5 ml 2x TVP and neutralize with 0.5 ml medium. Pool de duplo wells for each VUS in a 15 ml tube and spin 5 min at 1200 rpm. Resuspend the cells in 2.5 ml medium containing 200 µg/ml G418 and seed on a gelatinized 6-well.
When the wells of the negative controls are almost empty only refresh medium with 2.5 ml medium containing 200 µg/ml G418.

(Day19)
Trypsinize the transfected cells in 0.5 ml 2x TVP and neutralize with 0.5 ml medium. Add 1 ml freezing medium (20% DMSO / 80% ES cell medium) and divide over two vials, collect samples on ice and freeze down for storage in $N_2$ or at -150 °C.

- At this point cells have been selected on G418 10/11 days

<u>Western blots to check hBRCA1 protein expression</u>

After 14 days of G418 selection the cells are seeded in a 6-well plate in ES cell medium. When the wells are nearly confluent protein lysates are prepared.
The 6-well plates are placed on ice, washed 2x with PBS and lysed in 100 µl lysisbuffer (20mM Tris-HCl pH 8.0, 300mM NaCl, 2% NP40, 20% glycerol, 10mM EDTA, including strong protease inhibitors (Complete, Roche 11697498001, and Pefabloc, Roche 11429868001). After 20 minutes incubation on ice the cells are scraped and the lysate is collected. After a short spin the protein concentration of the supernatant is determined with the BCA assay (Pierce 23225). Protein samples of each VUS variant are prepared with Nupage 4x LDS sample buffer (Invitrogen NP0007) and Nupage 10x reducing agent (Invitrogen NP0009). The samples, containing 40 µg protein, are heated 10 minutes at 70°C, loaded on to a NuPage 3-8% Tris Acetate gradient gel (Invitrogen EA03752BOX) and run at 100V for 2-3 hours in Novex Tris-acetate running buffer (Invitrogen LA0041) supplied with Nupage antioxidant (NP0005).
The protein is transferred on a PVDF membrane (Millipore IPVH0001030; 30 seconds prewetted in methanol) in 50mM Tris, 36mM glycine containing 0.01% SDS, during 16 hours at 100mA.
The membranes are washed with $H_2O$ and stained with Ponceau S (Sigma P7170) for 5 minutes at room temperature to check the protein transfer. Aspecific binding is blocked by incubating the membranes in 5% ELK non-fat dry milk powder (Campina)/ TBS-T (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 60 minutes.
Subsequently the membranes are incubated overnight at 4°C with primary antibody raised against hBRCA1 (Cell Signalling, 1/1000 in 1% ELK/TBST), washed for three times with TBS-T and incubated with secondary antibody 1 hour at room temperature (DAKO, Goat-a-Rabbit-HRP 1/2000 in 1% ELK/TBST-T). After several washing steps with TBS-T and a final wash with TBS the signal is detected by

electrochemiluminescence (ECL)-using ECL Plus (Amersham RPN2132/ Pierce PX0080196).
As a loading control the membranes are blocked for 60 minutes in 5% ELK/TBS-T and reprobed for 60 minutes with a Pol.II primary antibody (Santa Cruz, 1/400 in 1% ELK/TBS-T). After washing three times with TBS-T the membranes are incubated with secondary antibody for 1 hour at room temperature (DAKO, Rabbit-a-Goat-HRP 1/1000 in 1% ELK/TBS-T).After several washing steps with TBS-T and a final wash with TBS the signal was detected by ECL (100mM Tris-HCL pH8.5, 2.5 mM luminol, 0.4mM p-coumaric acid mixed 1:1 with 100mM Tris-HCl pH8.5, 0.02% $H_2O_2$).

Cisplatin sensitivity assay

After G418 selection, seed the transfected Brca1 SCo/RMCE cells on a 12-well plate containing ES cell medium and culture for about 3 more days in the presence of 200 µg/ml G418 until confluent (14 days of G418 selection in total).

(Day 1)

Trypsinize the transfected Brca1 SCo/RMCE cells in 12-well plates with 0.25 ml 2xTVP, neutralize with 0.5 ml 60% BCM +/+ and seed 150 µl cell suspension (1:5 dilution) in a gelatinized 12-well in 1.5 ml 60% BCM +/+ containing 0.5 µM 4OHT (10 µl of 2.5 mM 4OHT stock in 50 ml medium) to switch the cells.
Also keep unswitched transfected Brca1 SCo/RMCE cells in culture by seeding 150 µl cell suspension in a gelatinized 12-well containing 1.5 ml 60% BCM +/+ without 4OHT.

(Day 2)

Refresh the switched cells with 1.5 ml 60% BCM +/+ to remove the 4OHT

(Day 4)

Store cell pellets for the unswitched cells by trypsinizing them with 0.25 ml 2x TVP, neutralize with 0.5 ml of medium and transfer the cell suspension to an eppendorf tube. Spin the cells for 5'at 3000 rpm, remove supernatant and store the cell pellets at -20°C.
Trypsinize the switched cells in 0.25 ml 2xTVP, neutralize them with 0.5 ml of medium and passage them 1:3 / 1:4 / 1:5 to a new gelatinized 12-well plate containing 1.5 ml ES cell medium + 1.8 µg/ml puromycin.
(some switched cells grow slower, dilution needs to be adapted depending on growth speed)

(Day 8)

Gelatinize black 96-well plates with µclear flat bottom (Greiner 655090). Trypsinize the transfected cells in 0.25 ml 2x TVP and neutralize with 1 ml of medium. Count the cells (e.g using a Casy Cell Counter) and prepare for each transfected VUS a cell

suspension of 60.000 cells in 6 ml. For cisplatin sensitivity assays each transfected variant is seeded on 3 rows at 1000 cells per well in volume of 100 µl, using columns 3-11 and omitting the first and the last rows. The wells of column 2 are filled with 100 µl medium as a medium control. The outer wells are filled with 200 µl PBS.

(Day 9)

Prepare a 1:3 serial dilution of cisplatin (1 mg/ml in NaCl, Mayne Pharma) in ES cell medium starting with 20 µM as highest concentration (= 10 µM final concentration on the cells). Add 100 µl of the drug dilution to the wells 4-11, for the blank and medium control add 100 µl of ES cell medium to the wells 2-3. Incubate the cells with drugs for three days.

(Day 12)

After incubating with cisplatin for three days 40 µl of Cell Titer Blue (Promega G8081) is added to each well. The plates are incubated for 4 hours at 37°C before recording the fluorescence (exitation: 560 nm, emission 590 nm) using a 96-well plate reader (e.g. the Tecan Infinite M200 plate reader).

Growth curves

The cells that are seeded for the cisplatin sensitivity assay are also used to generate growth curves. After switching and puromycin selection, cells are seeded in triplicate on a gelatinized 96-well plate at a density of 500 cells per well in 200 µl ES cell medium. Use the cell suspension of 60.000 cells / 6 ml made for the cisplatin sensitivity assay to seed 500 cells in 50 µl per 96-well which already contains 150 µl of medium. Prepare plates with cells for 5 time-points: T= 0, 1, 2, 3, 4 days after seeding the cells (T = 0 is 4 hours after seeding). At each time-point add 25 µl 50% TCA (trichloroacetic acid) and store the plate at 4°C.

When all time-points are collected the plates can be stained:

- Wash the plates 5 times with tapwater.
- Leave the plates to dry with open lids at room temperature.
- Add 50 µl SRB (0.4% sulforhodamine B in 1% acetic acid) and incubate 30 minutes at room temperature.
- Wash the plates 3 times with 1% acetic acid
- Leave the plates to dry with open lids at room temperature.
- When the plates are dry add 150 µl 10mM Tris to dissolve the SRB.
- Measure absorbance at 540 nm using a 96-well plate reader.

Genotyping

Brca1 SCo switching PCRs: to check for switching of the *Brca1*$^{SCo}$ allele in ES cells transfected with hBRCA1 variants, cell pellets before and after 4OHT treatment are

stored at -20°C. The pellets are lysed in 100 µl directlysis buffer (Viagen) including proteinase K (Sigma P6556) for 3 hours at 55°C. The proteinase K is inactivated by heating the samples for 15 minutes at 85°C.

Perform a Brca1 SCo/wt PCR to detect the non-switched *Brca1*$^{SCo}$ allele and to confirm the absence of a *Brca1*$^{wt}$ allele with the following primers and PCR mixture:

| | |
|---|---|
| PGKP-R | 5'-GCTGGTTCTCCTCTTCCTCATC-3' |
| m-Brca1-intron6-F | 5'-CACCTGCTCTGGCTGATG-3' |
| m-Brca1-intron6-R2 | 5'-AGGTCTGCCTGCCTCTACTTC-3' |

| | |
|---|---|
| 10x PCR buffer (MRC Holland) | 2.0 µl |
| 10 mM dNTP | 2.0 µl |
| Primermix (10 µM each) | 1.0 µl |
| Taq polymerase (Invitrogen) | 0.2 µl |
| DNA | 1.0 µl |
| $H_2O$ | 13.8 µl |

Perform a Brca1 ΔSCo PCR to detect the switched *Brca1*$^{SCo}$ allele with the following primers and PCR mixture:

| | |
|---|---|
| PURO-ar-F | 5'-GTGGGCTTGTACTCGGTCAT-3' |
| PGKP-R | 5'-GCTGGTTCTCCTCTTCCTCATC-3' |

| | |
|---|---|
| 10x PCR buffer (Invitrogen) | 2.0 µl |
| 10 mM dNTP | 2.0 µl |
| Primermix (10 µM each) | 1.0 µl |
| 50 mM $MgCl_2$ (Invitrogen) | 1.0 µl |
| Taq polymerase (Invitrogen) | 0.2 µl |
| DNA | 1.0 µl |
| $H_2O$ | 12.8 µl |

PCR program: 5' 94°C; 30x (30'' 94°C ; 30'' 62°C; 30'' 72°C); 5' 72°C. Product sizes: wt: 336 bp; SCo: 158 bp (Brca1 SCo PCR) and wt: -; SCo: -; ΔSCo: 125 bp (Brca1 ΔSCo PCR).

## References


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