Retrovirus Mediated Malignant Transformation of Mouse Embryonic Fibroblasts

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[Abstract] Cellular transformation is a widely used method to artificially induce cells to form tumours *in vivo*. Here, we describe the methodology for malignant transformation of mouse embryonic fibroblasts (MEFs) for transplantation into immunodeficient nude mice, as used in Leong *et al.* (2013). The two-step process involves: 1) down-regulation of *Trp53* expression using a short hairpin RNA (shRNA); and 2) overexpression of the oncogenic $HRas^{V12}$ protein. Reduction of *Trp53* expression leads to cell immortalisation, and the subsequent overexpression of oncogenic $HRas^{V12}$ results in malignant transformation of a cell.

Materials and Reagents

1. Source of tissue: body of embryonic day 13.5 mouse embryos, harvested fresh from pregnant females
2. Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Gibco®, catalog number: 41965-039)
3. Fetal Calf Serum (FCS) (Life Technologies, Gibco®, catalog number: 10437-028)
4. Trypsin (Life Technologies, Gibco®, catalog number: 25200056)
5. Dulbecco's Phosphate Buffered Saline (PBS), without Ca^{2+} and Mg^{2+} (Life Technologies, Gibco®, catalog number: 14190-144)
6. Retroviral supernatant containing LMP-p53.1224 shRNA construct (Dickins *et al*., 2005)
7. Retroviral supernatant containing pWZL-$HRas^{V12}$ cDNA construct (Serrano *et al*., 1997)
8. Hygromycin B (Life Technologies, catalog number: 10687-010)
9. Puromycin (Sigma-Aldrich, catalog number: P9620-10ML)
10. Hexadimethrine bromide/Polybrene (Sigma-Aldrich, catalog number: H9268)
11. Polybrene (1,000x stock) (see Recipes)

Equipment

1. Tissue culture flasks T75 (Greiner Bio-One, catalog number: 658175)
2. 10-cm tissue culture dishes (BD Biosciences, Falcon®, catalog number: 353003)

3. 21-gauge needles
4. 5 ml syringes
5. 37 °C 10% CO_2 cell culture incubator
6. Table-top centrifuge

Procedure

1. Retroviral supernatants are prepared as previously described, at a titer of 10^6 to 10^7 viral particle per ml of viral supernatant (Pear *et al*., 1993).
   Note: Do not freeze/thaw supernatant, and use within 6 months.
2. Primary MEFs are generated from embryonic day 13.5 (E13.5) embryos by passing the embryonic body (excluding head, liver and intestines) through a 21-gauge needle and syringe followed by repeated pipetting into a 10-cm tissue culture dish (1 embryo per dish) in 1 ml of DME medium containing 10% (v/v) FCS (DMEM/FCS). It is not necessary to obtain a single cell suspension at this stage, as trypsinisation at later stages will produce a single cell suspension and excessive manipulation at this stage promotes cell death. Add 9 ml of DMEM/FCS and mix to combine.
3. Primary MEFs are then incubated in 10% CO_2 incubator at 37 °C for 2-3 days undisturbed.
4. MEFs are washed once in PBS, trypsinised, trypsin inhibited with DMEM/FCS and pelleted at 485 *g* for 5 minutes.
5. MEFs are split ~1:2 into a T75 tissue culture flask and incubated in 10% CO_2 incubator at 37 °C overnight so that cells are ~60-70% confluent the following day.
6. On the next morning, aspirate the supernatant and wash once with PBS. Combine the retroviral supernatant containing LMP-p53.1224 shRNA, DMEM/FCS and polybrene using the following recipe:

Retroviral supernatant	1.5 ml (*i.e.*, ~1:7 dilution)
DMEM/FCS	8.5 ml
Polybrene (1,000x stock)	10 µl (4 µg/ml)
Total	10 ml

7. After ~7-8 h of infection, repeat step 6, and leave the fresh retroviral supernatant overnight.
8. On the next day, aspirate the supernatant, wash cells once with PBS, replace with fresh DMEM/FCS, and incubate at 37 °C overnight.
9. On the following day, replace medium with fresh DMEM/FCS containing 5 µg/ml puromycin (LMP-p53.1224 shRNA construct has a puromycin selectable marker), and leave for 2 days, if not confluent. Otherwise, split as necessary.

10. At the end of puromycin selection on day 3, cells are washed once with PBS, trypsinised and seeded so that cells are ~60-70% confluent in a T75 flask the following day. Culture cells in DMEM/FCS without puromycin and incubate overnight at 37 °C.
11. On the next day, repeat steps 6-8, but with retroviral supernatant containing pWZL-HRas^{V12} cDNA. The two tranductions should be performed sequentially, as suggested, so that p53 knockdown and immortalization precedes HRas^{V12} overexpression. This ensures the best efficiency of transformation since HRas^{V12} overexpression with inefficient p53 knockdown results in senescence.
12. On the following day, replace medium with fresh DMEM/FCS containing 300 µg/ml hygromycin (pWZL-HRas^{V12} cDNA construct has a hygromycin selectable marker) for 6 days. Replace with fresh hygromycin after 3 days, and split cells when necessary.
13. At the end of hygromycin selection on day 7, replace with fresh DMEM/FCS without hygromycin.
14. Passage cells as necessary for another 10-14 days to allow HRas^{V12} to drive cell proliferation. These transformed cells can now be used for *in vitro* or *in vivo* experiments. For example, cells can be injected subcutaneously into the flank of nude mice to assess tumour growth rate *in vivo*. The cells can be frozen and stored in liquid nitrogen, or can be continuously passaged, however extended passaging will result in additional genetic aberrations based on the knockdown of p53.

Recipes

1. 1,000x stock polybrene (4 mg/ml)
   Mix 0.2 g of hexadimethrine bromide with 50 ml Milli Q H_2O
   Filter sterilize (0.22 µm)
   Aliquot and store at -20 °C

Acknowledgments

This protocol was previously used and adapted from Leong *et al.* (2013).

References

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