Medicina Molecolare e Modelli Animali di Malattia

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LEZIONE 1
Generazione, screening e mantenimento di modelli murini geneticamente modificati “convenzionali”

Dept. of Molecular Medicine
Genetic manipulation in mammals

-1974 Jaewsh e Mintz: purified DNA of SV40 virus injected in mouse embryos isolated in the murine tissue after birth;

-1980 Anderson e Capecchi: high transformation efficiency of mammal cells in culture by microinjection of purified DNA into the nucleus;

-1982 Evans: generation of the first model of transgenic mouse for the rat Growth Hormone (‘giant mouse’);

-2007 Nobel Prize in Physiology or Medicine to M.R. Capecchi, M.J. Evans and O. Smithies “for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells”.
Animal models of human pathologies

✓ ANIMALS: Rodents (mouse, rat)

✓ ADVANTAGES:
- high homology to the human genome
  (99% of mouse genes have homologues in humans);
- simple handling (small dimensions);
- animal housing (Standard Pathogen Free conditions);
- reproductive and life cycles are short (sexual maturity at 40-60 days of age; lifespan: 1-3 years);
- high reproductive rate (gestation: 20 days; litter size: 5-10; weaning: 18-21 days);

✓ AIMS:
- studies on the etiopathology of human diseases;
- molecular pathogenetic analysis;
- screening/testing efficacy and/or side effects of new therapies and pharmaceutical compounds;
- genetic manipulation to understand the function and the mechanism of action of different genes (oncogenesis).
MURINE ANIMAL MODELS

SPONTANEOUS
- AUTOIMMUNE DISEASES:
  - LUPUS ERYTHEMATOSUS (SLE)
  - TYPE I AUTOIMMUNE DIABETES (NOD)

INDUCED
- GENETICALLY MODIFIED
  - TRANSGENIC ('Gain of function')
  - KNOCK-OUT ('Loss of function')
  - STREPTOZOTOCIN (Type I Diabetes)

TOXIC
Technical approaches to generate genetically modified mice

a) DNA microinjection into single cell embryos to produce transgenic mice containing one or more copies of randomly integrated expression vector DNA

b) Use of homologous recombination at defined genomic loci into embryonic stem cells (ES), to create gene knockouts (KO) or replacements
GENERATION OF GENETICALLY MODIFIED MICE
FLOW-CHART

STEP 1: Isolation and cloning of the gene of interest

STEP 2: In vitro manipulation of the gene to create a ‘transgene construct’

STEP 3: Integration of the genetically modified gene in the host germinal line
STEP 1: Isolation and cloning

Cut DNA with restriction enzymes

Insert fragments into vectors

Introduce vectors into bacteria
STEP 2: *In vitro* manipulation

Transgene construct

Coding Region/Functional Domain Sequence
+ insertion of an in frame TAG sequence to detect the exogenous protein
STEP 3: Integration of the ‘transgene’ by microinjection
MICROMANIPULATOR
MICROINJECTION FEATURES

ADVANTAGES:

- The only technique that allows the insertion of exogenous DNA at a single cell level;

- High efficiency (very small quantity of DNA needed: 100-200 copies)

- Different cell types can be microinjected with different types of DNAs;

DISADVANTAGES:

- Few fertilized eggs can be microinjected in a single experiment (few hundreds);

- Only 5% of the injected eggs will generate transgenic mice
MICROINJECTED TRANSGENE FEATURES

- huge gene dimensions (>50 Kb);

- the insertion usually occurs in multiple copies (‘head-to-tail’ concatamers), with possible ‘toxic’ effects;

- the exogenous DNA has the genetic behaviour of a ‘dominant’ gene (usually its effect is already visible in heterozygous mice);

- the insertion site of the exogenous DNA in the host genome is RANDOM and usually unique (possible insertion inside vital genes, oncosuppresors, etc);

- transgene expression variability in different transgenic lines, due to the ‘position effect’ (i.e. insertion close to enhancers or inside heterochromatic region);

IT IS MANDATORY TO ANALYZE THE PHENOTYPE OF DIFFERENT FOUNDERS
ANALYSIS OF GENETICALLY MODIFIED MICE
FLOW-CHART

STEP 1:
“GENOTYPING” of the newborn mice to detect the presence of the transgene at DNA level:
(a) DNA isolation from mouse tail (0.5 cm at 3-4 wks of age)

- Digestion buffer/ Proteinase K digestion o.n. at 55°C;
- Phenol/Chloroform extraction
- Isopropanol precipitation

OR use an automated DNA extractor
(based on magnetic beads)
ESTRAZIONE DNA dalle code

- Pulire P1000 e P20 con acqua, alcool e metterle sotto gli UV.
- Punte pulite: blu senza filtro, da 1000 cotonate, gialle. Sacchetto di carta argentata per le punte del fenolo.
- Prendere il fenolo (frigo stabulario 4°) e trasferire aliquota dalla fase in basso in una falcon sotto cappa chimica.
- Spinnare le code.
- Aggiungere isovolume di fenolo (660 µl).
- Agitare ⇒ 10 min in ruota.
- Ctg 12.500g 15min 4°.
- Nel frattempo preparare eppendorf e scrivere: dna, numero topo, data estrazione.
- Trasferire 300 µl dalla fase superiore (dove è il dna).
- Isovolume di isopropanolo (300 µl).
- Agitare bene finchè non si vede il dna.
- Ctg 12.000g 15min 4°.
- Levare il sopranatante e lasciare asciugare le eppendorf aperte sotto cappa chimica.
- Aggiungere 200-300 µl di H₂O a seconda della consistenza del pellet.
- Conservare in frigo a 4°.
Procedure

1. Cut 1.3 cm of each rodent tail tissue sample into small pieces. Place tissue samples into 1.5 ml microcentrifuge tubes, and add 100 μl Buffer ATL.
   All samples must have the same volume. If the volume of a sample needs to be increased, add the appropriate volume of Buffer ATL.
2. Add 30 μl proteinase K, close the microcentrifuge tubes, and incubate the samples at 56°C in a shaking incubator until the tissue is completely lysed.
   Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–2 h. If it is more convenient, samples can be lysed overnight; this will not affect the DNA quality.
   Optional: If Br obtained genomic DNA is required, omit di pF of Br over B (100 mg/ml), mix by swirling, and incubate for 2 min at room temperature (15–25°C).
3. Open the front door of the Bioprint 15 and slide out the tube strip tray.
4. Load up to fifteen 5-tube strips into the tube strip tray. One 5-tube strip is used per sample.
   If loading five 5-tube strips or fewer, we recommend loading them on a single column. If loading ten 5-tube strips or fewer, we recommend loading them on 2 columns.
   Load the 5-tube strips in the tube strip tray such that the tube of each 5-tube strip faces to the left.
   Make sure that the 5-tube strips are fully inserted into the tray and are not skewed.
5. Add reagents into each 5-tube strip according to Table 2.

   Table 2. Bioprint 15 Workstation Setup and Buffer Volumes

<table>
<thead>
<tr>
<th>Well</th>
<th>Reagent</th>
<th>Volume of reagents [μl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lysate*</td>
<td>650</td>
</tr>
<tr>
<td>2</td>
<td>Buffer AW1</td>
<td>700</td>
</tr>
<tr>
<td>3</td>
<td>Buffer AW2</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>Buffer AW5</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>Buffer AS</td>
<td>200</td>
</tr>
</tbody>
</table>

   Note: Well 1 is on the left of the 5-tube strip, well 5 is on the right.
6. Briefly centrifuge the microcentrifuge tubes to remove drops from the inside of the lid.
   Open the microcentrifuge lid and transfer the sample to well 1 of the 5-tube strip.
7. Vortex the master mix containing Buffer AL, Isopropanol, and Magnificat Suspension G (see “things to do before starting”) for 1 min. Add 450 μl of master mix to each sample in well 1 of each 5-tube strip.
   Notes: If using an Eppendorf Multi-PIPETTE, aliquot 450 μl master mix to each sample. The starting volume of master mix should be increased accordingly.
8. Switch on the Bioprint 15 at the power switch.
9. Load up to three 5-tube covers into the rod cover slots. Make sure all covers are a column of 5-tube strips.
   Notes: If necessary, remove the tube strip tray to allow easier loading of the 5-tube covers.
   IMPORTANT: Do not touch 5-tube covers further after they slide into place; otherwise, an instrument crash will occur.
10. Slide back the tube strip tray fully into the Bioprint 15.
11. Close the front door of the Bioprint 15.
   Closing the front and top doors protects the samples from contamination.
12. Select the protocol "BEL5 DNA Tissue" using the [ and ] keys on the Bioprint 15 workstation. Press "Start" to start the protocol run.
13. After the protocol run ends, slide out the tube strip tray, and transfer the eluted DNA from well 5 of each 5-tube strip to other tubes for long-term storage.
   Note: Well 5 is on the right of the 5-tube strip.
   Care should be taken to avoid any contamination. If新媒体 elements in eluates do not affect the downstream applications. If the risk of contaminating the sample, use a suitable reagent and the eluates transferred to clean tubes (see the appendix of the Bioprint DNA Manual).
14. Remove the 5-tube strips and 5-tube covers and discard them according to your local safety regulations.
15. Switch off the Bioprint 15 at the power switch.
16. Wipe the surface of the tube strip tray and adjacent surfaces with a soft cloth or tissue moistened with distilled water or a mild detergent solution. If infectious agents are split onto the tube strip tray, clean using 70% ethanol or other disinfectant.
   Notes: Do not use bleach or disinfectant. See "Safety Information" in the Bioprint DNA Manual.