TROUBLE SHOOTING GUIDE

The two most common problems encountered when generating knockout mice are ES cell differentiation and the inability to generate chimeras once an ES cell-targeting clone has been established.

Listed below are a number of common causes of ES cell differentiation and recommendations on how to help prevent differentiation occurring. Also listed are a number of reasons that often cause the slow growth of ES cell clones, and some causes for the lack of generating chimeras from the cell culture aspect.

Causes of differentiation		Recommendations
1.	Incorrect concentration of mLIF	ESGRO® (mLIF) should be used at the recommended concentration of 1000 Units / mL in ES cell media.
2.	Expiry date of LIF	Always check the date of each batch prior to use.
3.	Media	Media should be less than 4 weeks old as glutamine by-products are toxic to ES cells. ES cells should be fed with fresh media every 2-3 days or when media turns yellow.
4.	Serum	New batches of Fetal Bovine Serum should be tested for the affect of inducing differentiation. ES cell media usually requires a serum concentration between 10-20%.
5.	Lack of passaging	ES cells should be passaged every 2-3 days as frequent passaging removes differentiated cells. Only undifferentiated ES cells will survive frequent passaging. Refer to the attached figures 1, 2, 3, 4, 5, 6 and 7 for illustrations of ES cell confluency and differentiated ES cells.
6.	Disinfectants	Disinfectants such as Roccal or Lysol should be avoided in the Tissue Culture Room where ES cells are cultured. Some disinfectants have been suspected to cause differentiation by use in water baths and aerosols created by spray wiping. The use of 70% ethanol to clean tissue culture surfaces is recommended.

7.	Feeder cells	If differentiation cannot be controlled, it may be necessary to culture ES cells on a confluent layer of feeder cells. The recommended number of γ irradiated fibroblasts to use is $1x10^6$cells / 100mm dish; $5x10^5$ cells / 24, 12 or 6 well plate and $4.5x10^5$ cells / 96 well plate (spread evenly across the wells). Exogenous LIF should be at a concentration of 1000 units / mL to assist in maintaining the undifferentiated phenotype.
8.	Incubator settings	Ensure that the CO_2 incubator readings are correct at 37^{o}C and 5% CO_2.
9.	Growth inactivation of feeder cells	γ irradiation is recommended as residual mitomycin C may contribute to both the differentiation and even the death of ES cells.
10.	Rate of growth of ES cells	Slow growing ES cells will be most likely to undergo differentiation. Check above. Increase serum concentration if cells are not growing quickly enough.
11.	Concentration of ES cells	Low confluency of ES cells can result in differentiation. ES cells should be plated at a minimum density of $1x10^6$ cells / 100mm dish. Refer to the attached figures for illustrations of ES cell confluency. Refer to figs. 1, 2, 3, 4, and 5.
12.	Gelatin	It is preferable to use cell culture grade gelatin at all times (even if using feeder layers) as the gelatin minimises surface differences on the tissue culture plates.
13.	Low level of contamination	Regular use of Penicillin / Streptomycin in media can often mask a low level contamination with agents such as mycoplasma. It is recommended to have your cell lines tested for mycoplasma on a periodic basis.

Causes of Slow Growth of ES Cells		**Recommendations**
14.	Insufficient serum.	Ideally a concentration between 10-20% is routinely used and this will vary according to each ES cell strain. It is recommended to routinely testing new batches of serum to determine the optimum concentration required for fast growth without inducing too much differentiation.

15.	Low number of ES	ES cells grow best when slightly crowded. Plate a higher density from frozen stocks or passage cells to increase individual colony numbers. Refer to the attached figures 1, 2, 3, 4, 5, 6 and 7 for illustrations of ES cell confluency and differentiated ES cells.
Causes for the lack of chimeras		**Recommendations**
16.	Time taken to culture the ES cells	Once the targeted ES cell clone is identified, minimise the time that the ES cells remain in culture. It is preferable to passage ES cell lines 1-2 times prior to microinjection and / or aggregation.
17.	Karyotype the ES cells	Routine analysis of the ES cells karyotype is recommended, especially when the cell lines are reaching a high passage number.
18.	Undetected differentiation	Some degree of differentiation may go undetected by simple microscopic examination, and therefore regular passage of the ES cells is necessary.
19.	ES cell passage number	In general; the best chimeras are generated from low passage number ES cells. If cell lines have been cultured for a high number of passages, it is recommended to go back to a lower passage frozen stock.
20.	Number of ES cells microinjected	Most often ES cell lines are microinjected at approximately 8-12 cells / blastocyst.
21.	Time taken prior to microinjection	It is recommend that ES cells be microinjected as soon as possible after removing them from the tissue culture plates, and not left on ice or room temperature for extended periods.
22.	Compatibility of ES and mouse strain	Prior to any microinjection or aggregation techniques, please check the combination of embryo strains and ES cells as some strains are reported to be incompatible.

Figure 1: Ideal confluence of ES cells (10x)

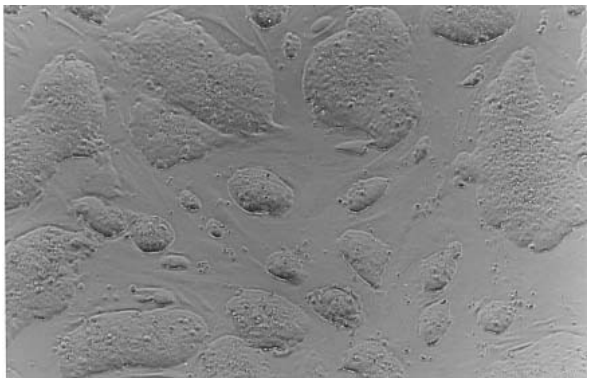


Figure 2: Over confluent ES cells (10x)

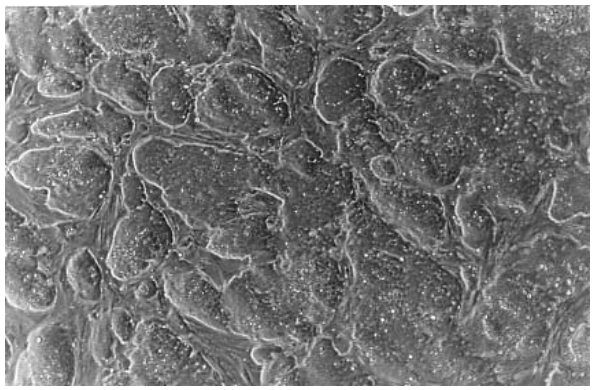


Figure 3: ES Cells at a density ready for passage (10x)

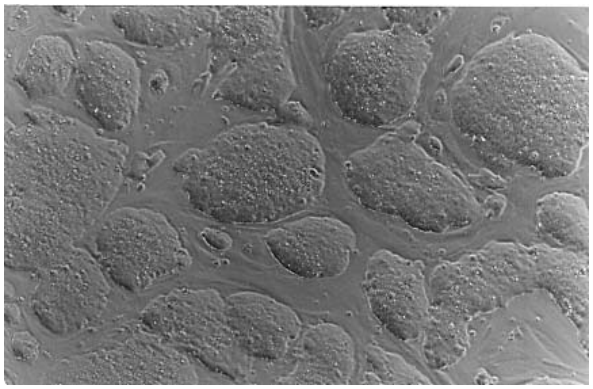
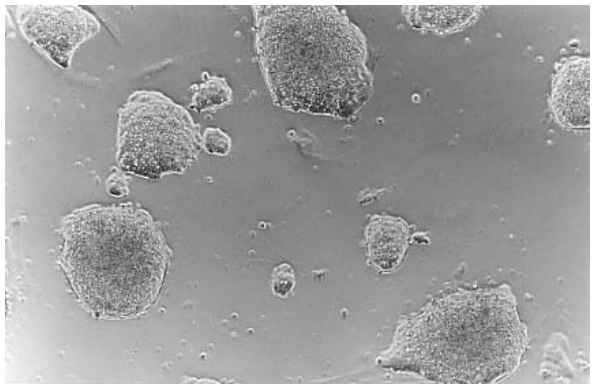
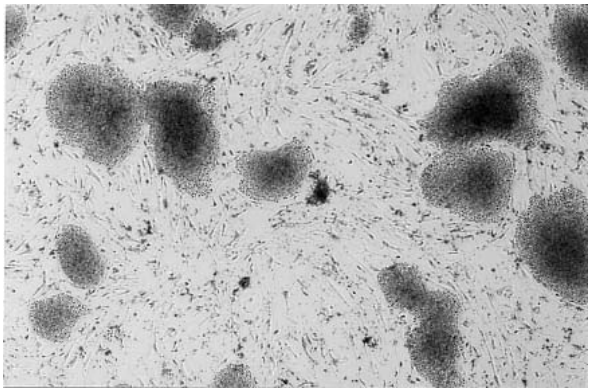


Figure 4: ES cells plated at low density (10x)



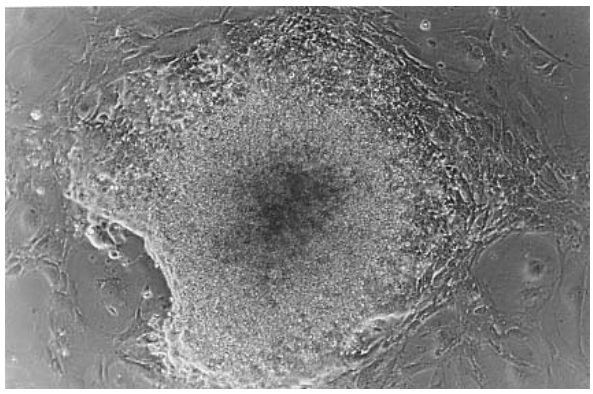
Cells require passage to increase individual colony numbers

Figure 5: ES cells plated at too low density with fibroblasts (10x).



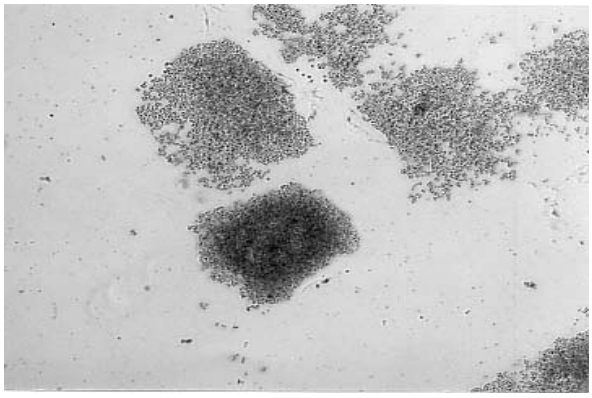
Cells require passage to increase individual colony numbers. Note the darkened areas in the centre of each colony where ES cells are dying.

Figure 6: Highly Differentiated ES cells (25x)



Note the loss of discreet ES cell colony border, the formation of “cobblestone” like cells and ES cells differentiating into fibroblasts that extend outwards from the colony. It is possible to save this colony by passaging several times, however it is not usually recommended.

Figure 7: Highly Differentiated ES cells – plated at too low density (10x)



Note – these cells are beyond rescue by passaging