THE USE OF 3i MEDIUM FOR THE DERIVATION OF BOVINE EMBRYONIC STEM CELLS

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INTRODUCTION
The derivation of true embryonic stem cells (ES cells) in large animals is still elusive. Several laboratories have attempted to obtain ES cells carrying all the properties of mouse ES cells, including chimera formation following blastocyst injection, with no or very limited success. A recently published method based on the use of specific inhibitors of signalling pathways known to drive ES differentiation in the mouse model, have provided a novel strategy to renew the efforts in large animal species (Ying et al., Nature, 453, 519-523, 2008). Such molecules include SU5402 or PD173074 (FGF tyrosine kinase receptor inhibitors), PD184352 (ERK 1-2 kinases inhibitor) and CH99021 (GSK3 kinase inhibitor). These compounds added to a serum free medium, N2B27, without additional growth factors, comprise the so-called 3i medium.

EXPERIMENTAL DESIGN
We applied the same approach to bovine ES derivation isolating inner cell mass (icm) cells from in vitro produced expanded blastocysts and plating them on inactivated mouse embryonic fibroblasts in N2B27 supplemented with 100nM PD173074, 100-800nM PD184352 and 200-2000nM CH99021.

RESULTS
At the maximum concentrations of inhibitors indicated above, that is used successfully in the mouse model for de novo ES derivation, we observed that icm cells remained viable for 3-4 days, maintaining Oct4 expression. However, in the following days the cells progressively underwent degeneration and disappeared over about a week, during which time OCT4 expression was maintained in the viable cells and no differentiated cells were present in the cultures (Fig.1). Reducing the concentrations of inhibitors we observed a progressive improvement of viability although accompanied by a parallel loss of OCT4 expression and by the initiation of neural differentiation. Following two passages the neural differentiation was widespread in the cultures with clear formation of neural rosettes accompanied by extensive apoptosis. We then tested another inhibitor of ERK kinases, PD032591 in association with CH99021 (so-called 2i medium) and Leukemia Inhibitory Factor. Also in this case the highest concentration of inhibitors recommended in the mouse model (0.8μM PD032591 and 3μM CH99021) induced cell death following a few days of culture. Reducing the concentration of PD032591 (Fig.2) allowed cell proliferation and the establishment of cell lines that maintain a ES-like growth pattern, were alkaline phosphatase and Sox2 positive - with a few Tuj1 positive cells – but Oct4 negative indicating a neural identity.
Fig. 1. Bovine embryo outgrowths 6 days post plating in medium N2B27 supplemented with different concentration of 3i inhibitors:
A) N2B27 only;
B) N2B27 with 100nM PD173074, 100nM PD184352 and 100nM CH99021;
C) N2B27 with 100nM PD173074, 100nM PD184352 and 500nM CH99021;
D) N2B27 with 50nM PD173074, 500nM PD184352 and 100nM CH99021;
E) N2B27 with 100nM PD173074, 500nM PD184352 and 100nM CH99021;
F) N2B27 with 100nM PD173074, 500nM PD184352 and 2000nM CH99021.

Fig. 2. A) bovine embryo outgrowth 6 days post plating on mitomycin inactivated mouse embryonic fibroblasts, in medium N2B27 supplemented with PD032591 (200nM) and CH99021 (μ2M); B-C) compact colonies at passage 1; D) Tuj1 staining at passage 2; E) morphology at passage 4; F) alkaline phosphatase staining at passage 4.
CONCLUSION

In conclusion, these results indicate that the combination of inhibitors (3i medium) tested at the concentrations used in the mouse model is efficiently blocking differentiation of bovine icm cells, but it does not allow proliferation and establishment of a cell line. By contrast PD032591, used at low concentration and in association with CH99021 (2i medium) and Leukemia Inhibitory Factor, allows derivation of alkaline phosphatase positive neural cell lines. Work is in progress to improve cell viability at higher inhibitor concentration in order to block neural differentiation.

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