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Ioannis J Limnios[1], Klaus I Matthaei[2], Margaret L Delbridge[1&3], Jennifer A Marshall Graves[1&3], Ernst J Wolvetang[4].

[1]Research School of Biology, ANU, Canberra.
[2]John Curtin School of Medical Research, ANU, Canberra.
[3]ARC Centre of Excellence for Kangaroo Genomics, Research School of Biology, ANU, Canberra.
[4]Australian Institute of Bioengineering and Nanotechnology, University of Queensland, Brisbane.

ABSTRACT

In vitro models of marsupial and monotreme embryogenesis would provide powerful platforms to study mammalian pluripotency and evolution. Despite several efforts, no marsupial embryonic stem (ES) cells have been established, and to our knowledge no attempts have been made to isolate monotreme ES cells. Advances in cell reprogramming offer functional alternatives to obtaining pluripotent stem cell lines: the induction of pluripotency in somatic cells[1 & 2]. We are investigating methods to generate and culture Induced Pluripotent Stem (iPS) cells from marsupials and monotremes.

This project faces two major challenges:
1) Generating iPS cells in species for which ES cells have not been previously isolated.
2) Identifying species-specific culture conditions and methods that maintain pluripotency *in vitro*.

We have begun studying the effects of over-expressing the human reprogramming factors (Oct4, Sox2, Nanog, LIN28, Klf4 and cMyc) in human, marsupial and monotreme fibroblasts, cultured under varied culture conditions.

Here we present preliminary data of reprogramming experiments which suggest partial reprogramming of marsupial and monotreme fibroblasts and highlights the need to empirically determine culture conditions that support the long-term maintenance of pluripotency *in vitro*.

METHODS

PRIMARY FIBROBLAST CELL CULTURE

Primary fibroblast lines used: Tammar Wallaby (M) p3, Tammar Wallaby (F) p3, Opossum (M) p3, Tasmanian Devil (M) p4 and Platypus (M) p2. Cells were cultured in AmnioMAX (Gibco) at 32.6^{C} and maintained between 50 – 90% confluence. Cells were seeded at 5x10^{4} cells/well (Falcon MULTIWELL) 12 hours before lentiviral transduction.

LENTIVIRUS PRODUCTION

293FT cells (Invitrogen) were grown in T175 flasks with DMEM high glucose medium (Gibco) supplemented with 10% FCS (JRH Sciences) and under Geneticin (Gibco) selection (500μg/ml) until 24hrs prior to transfection. Media was changed daily.

293FT cells (80 - 90% confluent) were transfected to produce lentivirus. Briefly, transfection complexes were made using a 3:1 ratio of Fugene6 to DNA (pLenti vectors carrying the open reading frames of human Oct4 (O), Sox2 (S), Nanog (N), Lin28A (L), Klf4 (K) or cMyc (M), and co-transfected with ViraPower) into separate 293FT flasks to generate individual lentiviral supernatants. Media was changed 16hrs post transfection, and subsequently harvested after 24hrs and 48hrs. Lentiviral containing media was syringe filtered (0.45μm) and concentrated using Fast-Trap (Millipore) lentivirus concentration kit before storage at -80^{C}.

FIBROBLAST TRANSDUCTION

For each fibroblast cell line, 3 wells seeded with 5x10^{4} cells were transduced with 1.5ml of AmnioMAX containing ~150μl of concentrated lentivirus, with equal ratios of each lentivirus (O,S,N,L,K & M) plus 6μg/ml polybrene. Cells were incubated at 32.6^{C} for 16hrs, then refed 2ml AmnioMAX.

iPS CELL CULTURE

48 hrs post transduction, cells were passaged with TrypLE Express (Gibco) onto full density MEFs (kindly provided by the Australian Stem Cell Centre's Core hESC laboratories (Stem Core), as shown in figure 1A. Cells were subsequently cultured in MEF conditioned KSR [KnockOut DMEM/F-12 media (Gibco), supplemented with 20% Knock-out Serum Replacement (Gibco)] media base, with 10ng/ml bFGF. Media was changed daily. Cells were cultured under 3 different conditions:

1) CM KSR + 10ng/ml bFGF *
2) CM KSR + 10ng/ml bFGF + 1000 U ml/ml LIF (Chemicon) *
3) CM KSR + 10ng/ml bFGF + 1000 U ml/ml LIF + 4μM/ml BIO (Stemgent) *
•*Media 1, 2 & 3 were supplemented with 1mM Valproic Acid from day 2 to day 14.*

(BIO – GSK3b inhibitor)

Figure 1B outlines the time line of various culture conditions used for each transduced fibroblast cell line.

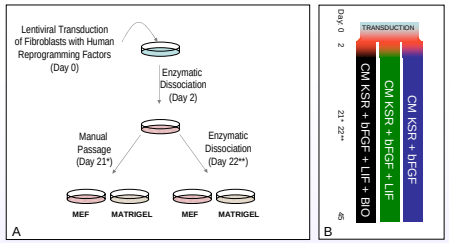

Figure 1: Culture regime of transduced marsupial and monotreme fibroblasts for iPS generation A) Differential culture of transduced fibroblast lines by either manual passage or enzymatic dissociation onto either full density MEFs or Matrigel (BD Scientific). B) Transduced fibroblasts were cultured under three different conditions. The combination of different passage methods, growth factors and surface substrate resulted in 12 different culture conditions for each cell line.

Colonies appearing by day 21 were manually passaged under dissection microscope (Leica M26), or enzymatically dissociated with TrypLE Express and replated on either fresh full density MEFs, or Matrigel.

Media was changed daily during all experiments.

PHOTOMICROSCOPY

Cells were observed and photographed under an Olympus IX81 (10x, 20x & 40x objective), with high resolution positioning controller (Corvus), incubator system (Solven Scientific) using Cell^R software package.

RESULTS AND DISCUSSION

MORPHOLOGICAL CHANGES IN TRANSDUCED MARSUPIAL & MONOTREME FIBROBLASTS

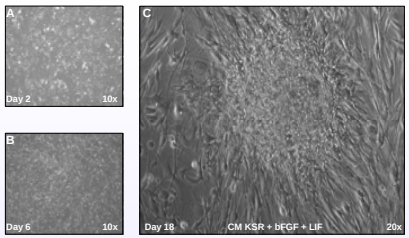

Figure 2: Phase contrast images of transduced Tammar Wallaby fibroblasts. A) Cells cultured on plastic prior to reseeding on MEFs B) Cells seeded on full density MEFs C) Formation of colonies with ESC-like morphology observed at day 18.

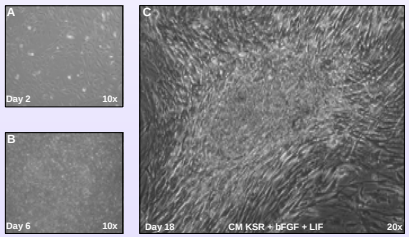

Figure 3: Phase contrast images of transduced Tasmanian Devil fibroblasts. A) Cells cultured on plastic prior to reseeding on MEFs B) Cells seeded on full density MEFs C) Formation of colonies with ESC-like morphology observed at day 18.

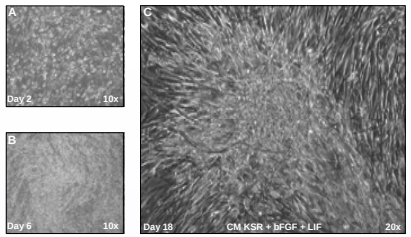

Figure 4: Phase contrast images of transduced Platypus fibroblasts. A) Cells cultured on plastic prior to reseeding on MEFs B) Cells seeded on full density MEFs C) Formation of colonies observed at day 18.

Transduced marsupial and monotreme fibroblasts underwent obvious morphological changes, forming small compact cells with ESC-like morphology and grew as distinct colonies. However, colonies stopped growing after one month, indicative of incomplete reprogramming. No colonies were formed from Opossum fibroblasts.

EFFECT OF CULTURE CONDITIONS ON PUTATIVE iPSC COLONY FORMATION

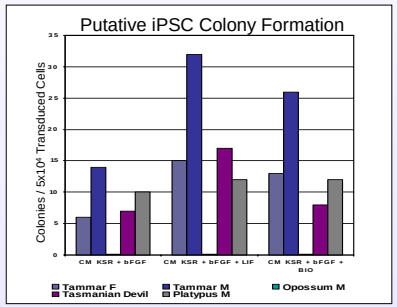

Figure 5: Number of colonies formed/5x10^{4} transduced fibroblasts from Tammar, Opossum, Tasmanian Devil and Platypus, under differential cytokine conditions.

For all species, colony formation was highest with the addition of LIF, consistent with mouse and chicken ES cells where it is required for proper maintenance of pluripotency. The addition of the GSK3b inhibitor BIO did not increase colony formation in any species, though this was not tested in the absence of LIF.

EFFECTS OF CULTURE CONDITIONS ON MORPHOLOGY

CULTURE MEDIA

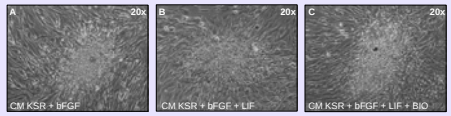

Figure 6: Morphology of transduced Tammar (M) fibroblasts grown in CM KSR + bFGF, CM KSR + bFGF + LIF, CM KSR + bFGF + LIF + BIO. Cells were grown on full density MEFs and photographed at day 18.

SURFACE SUBSTRATE & CULTURE METHOD

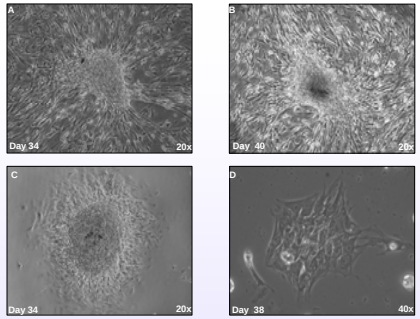

Figure 7: Morphology of transduced Tammar (M) fibroblasts cultured in CM KSR + bFGF + LIF. A) Manual passage of colonies onto MEFs B) Enzymatic passage onto MEFs C) Manual passage onto Matrigel D) Enzymatic passage onto MEFs

Culture media does not appear to affect colony morphology or size (fig 6). Manual passage of colonies onto MEFs retained original colony morphology (fig 7 A). Enzymatic passage onto MEFs demonstrated the ability of individual cells to reform colonies (fig 7 B). Manual passage onto Matrigel produced colonies with like hESC-like morphology (fig 7 C). Enzymatic culture of colonies onto Matrigel produced morphology of single cell adapted hESCs on Matrigel (not shown).

SUMMARY AND CONCLUSION

- Marsupial and monotreme fibroblasts transduced with human reprogramming factors formed colonies with hESC-like morphology.
- Colony formation was highest in media containing LIF, while BIO had no observable effect.
- Colony morphology was consistent between varied culture media, but not culture methods and surface substrate.
- Culture media could not support long term-culture of putative iPS cells.
- These results suggest partial reprogramming of fibroblasts and/or sub-optimal culture conditions to support marsupial & monotreme iPSCs.

ONGOING AND FUTURE WORK

- Work is currently underway to determine the best conditions to support the long-term culture of marsupial and monotreme iPSCs using combinations of growth factors and chemical inhibitors. We are also optimising transduction efficiency of fibroblasts by paired delivery of transcription factors.
- Additionally, we are exploring alternative cell reprogramming strategies, including repeated transient transfection, episome-based expression systems and protein extracts.
- Established iPS cell lines will be characterised by pluripotent gene expression profiling, embryonic surface markers and *in vitro* differentiation potential.

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