GENERATION AND CHARACTERISATION OF mIPS

by

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Abstract:

The use of embryonic stem cells to treat human disease has not reached its potential due to a number of problems including ethical issues and limited supply. The development of induced pluripotent stem (iPS) cells aims to address these issues by being ethically sound and unlimited in supply. Several methods for reprogramming have been developed. This project employed a recently described technique to generate iPS cells with and without the c-Myc gene (Sommer *et al.*, 2009). This method was chosen because the pluripotency cassette is flanked by loxP sites which allowed for Cre recombinase mediated excision from the genome to produce insert free iPS cells. iPS cells were generated with a 3 gene and 4 gene approach, the cells were tested for pluripotency by Immunofloursecense of pluripotency proteins including Nanog, oct4 and SSEA-1 in the iPS cells. The differentiation potential was studied as further confirmation of the generated iPS cells pluripotency. Expression of the proteins α-Smooth Muscle Actin (mesoderm), β-III-Tubulin (ectoderm) and Troma1 (endoderm) were analysed by Immunofloursecense to show that the iPS cells could differentiatiate into cells from each of the three germ layers. All tested iPS clones that were generated were observed to be pluripotent by these methods of measuring pluripotency.

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1. INTRODUCTION

1.1 Stem cells

Cells in the early embryo, up to the 8 cell stage, are totipotent, as they possess the ability to differentiate into any cell lineage including the extra embryonic tissue. Cells at this stage are the most plastic, as they differentiate they become less plastic through DNA methylation and other epigenetic changes. As an embryo develops an area called the inner cell mass is formed. This area contains cells which are pluripotent meaning that they are able to differentiate into all three of the germ layers ,mesoderm, endoderm and ectoderm, but not the extra embryonic tissues. These three germ layers will eventually form all of the cells required to populate the growing organism.

As a cell differentiates, its ability to change fates becomes limited. This limitation is a method of control which protects organisms by stopping cells growing out of control and leading to cancer formation. Differentiated cells also lose the ability to renew themselves, this is another control mechanism against cancer. There are some populations of adult cells which retain the ability to renew themselves and also remain in an undifferentiated state, these are called adult stem cells. To be classified as a stem cell a cell must be able to self-renew and also differentiate into a number of different cell lineages. Stem cells can be at different stages in their ability to differentiate into multiple lineages. The early embryonic stem cells are totipotent, the later embryonic cells derived from the blastocyst are

pluripotent and adult stem cells are generally multipotent. Multipotent cells are not able to differentiate into all lineages. The most differentiated cells are monopotent.

Cells which are pluripotent have a great therapeutic potential as they are able to differentiate into any cell type if they are stimulated with the correct endogenous and exogenous stimuli. There are ethical issues with the use of blastocyst derived embryonic stem (ES) cells because of the destruction of the embryo in the process of generation. This issue arises from the fact that the embryo could essentially grow to be a living organism and therefore the organism has to be sacrificed in order to be harvested for the cells. Another problem is that in order to transplant ES cells or tissue derived from them the tissue type of the donor and the patient must match. If the tissue types do not match then the donated cells will be recognised as foreign by the recipient's immune system and the cells will be rejected and destroyed. With the ethical issues and the tissue matching problems associated with the generation and use of ES cells, another source of pluripotent cells was required.

1.2 Induced pluripotent cells

In 2006 Takehashi & Yamanaka created the first induced pluripotent stem (iPS) cell. The group observed that introducing oct-4, sox2, klf4 and c-Myc genes into a population of murine fibroblasts (somatic cells) by transduction they were able to reverse the cells fate and to generate ES like cells. This discovery has sparked a lot of interest as if these iPS cells are similar enough to ES that tissue can be derived from them then they would address both major issues associated with ES cell work (Takahashi & Yamanaka, 2006). To determine if a

population of cells is pluripotent they can be injected into an immunodeficient mouse and a tumour, called a teratoma, will form which contains cells from all three of the germ layers or the ultimate proof of pluripotency, cells can be injected into a blastocyst and the resultant mice will be chimeras. The original iPS cells created by Takahashi *et al*.(2006) were able to form teratomas but were not able to generate chimeric mice. However repeating the iPS formation and then selecting cells, with high expression of the pluripotency marker nanog, to transplant led to formation of chimeric mice (Okita *et al.*, 2007).

However much like ES cells there are a few problems with iPS cells too. Firstly the use of a virus to transport the pluripotency genes into the cells can lead to incorrect integration into the genome and potential cancer formation if a proto-oncogene is disrupted. Evidence of this was seen when the first chimeric mice developed with iPS cells produced offspring which had a high rate of tumour formation (Okita *et al.*, 2007). This issue severely limits the possibility of using this technology to treat human disease. To address the tumour formation a number of groups have employed several different approaches to induce pluripotency.

One of the strategies was to transport the genes into the cell using a carrier which would not integrate with the hosts genomic DNA. Stradfeld *et al.* (2008) used an adenovirus, which would not integrate with the host genome. Okita *et al.* (2008) used a plasmid to express the genes, they were then able to check for integration with PCR. Two groups, Zhou *et al.* (2009) and Kim *et al.* (2009) synthesised the recombinant proteins from the pluripotency genes and introduced the proteins into the cells. Fusaki *et al.* (2009) developed a system

which employed an RNA virus to traffic the pluripotency genes in the form of RNA into the cells. Woltjen *et al.* (2009) showed that it is possible to induce pluripotency using a transposon system. Warren *et al.* (2010) used modified mRNA to express the pluripotency genes in target cells. These methods all deal with the problem of integration into the host cells genome by using methods, which are less likely to or cannot integrate into the genome.

Another problem with the iPS generation is that the transcription factor c-Myc, one of the four factors required for reprogramming, is a proto-oncogene (Duinsbergen *et al.*, 2009). The link to tumour formation and c-Myc means that iPS cells would not be suitable for use on humans. It is possible to reprogram cells without the c-Myc gene, however the efficiency of the induction is significantly lower (Nakagawa *et al.*, 2008).

There is also a concern that the iPS cells generated are not quite identical to that of ES cells (Marchetto *et al.*, 2009). The methylation of the genome has been shown to be not the same as ES cells, this could cause problems when the iPS cells are differentiated as methylation could potentially cause crucial genes to be silenced.

Some groups have begun to try to determine if other genes could be added into the pluripotency factor mix in order to increase efficiency or address the problems of using C-Myc. Maekawa *et al*. (2011) showed that a transcription factor called Glis1 could be used alongside Oct4, Sox2 and Klf4 to induce pluripotency. Moon *et al.* (2011) removed Sox2, Klf4 and C-Myc and replaced them with Bmi1 and were able to induce pluripotent cells. However Bmi1 is also a proto-oncogene, which will severely limit the possibility of using it in human iPS cell formation. There may be other combinations of genes, which are more suitable for inducing pluripotency.

1.3 AIMS

The aim of this project is to employ a recently described lentiviral (Fig 1.1, Sommer *et al.* (2009 & 2010)) method to induce pluripotency in primary mouse embryonic fibroblasts and to characterise and differentiate the resultant iPS cells. This vector was chosen because it incorporates a pluripotency cassette which contains all four of the key genes, oct-4, sox2, klf4 and c-Myc, which is flanked by loxP sites which will allow the pluripotency cassette to be excised in the presence of Cre recombinase. The vector can also be synthesised with the gene for mCherry flourochrome instead of c-myc, enabling the effect of c-myc in the reprogramming process to be studied.

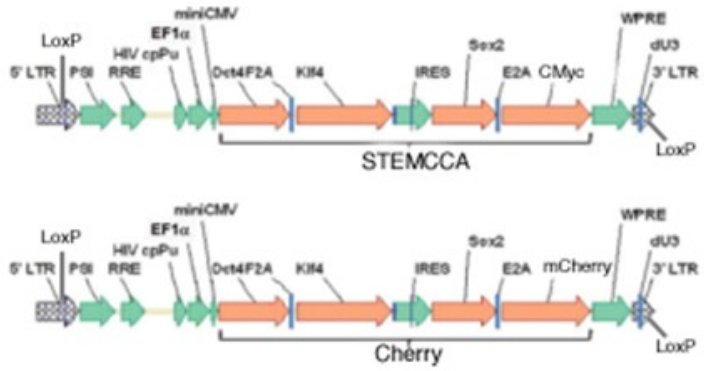


Fig 1.1 Map of STEMCCA and Cherry viral vectors used in this study
(Sommer *et al.* 2009, Sommer *et al.* 2010)

2. MATERIALS AND METHODS

2.1 Cell culture and growth conditions

Primary mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's modified eagle's medium (DMEM, Gibco) with 10-20% foetal bovine serum (FBS, Biosera), 2mM L-glutamine (Gibco), 1% Penicillin/Streptomycin (Gibco) and 0.1mM 2-mercaptoethanol (Sigma).

293 T-HEK cells were grown in DMEM with 10% FBS, 2mM L-glutamine and 1% Penicillin/Streptomycin.

Early iPS cells were grown in 141ml DMEM with 25ml ESC (embryonic stem cell) qualified FBS, 1.75ml L-glutamine, 1.75ml Penicillin/Streptomycin, $1.75x10^5$ units of LIF (Leukaemia inhibitory factor) and 1.19ul 2-mercaptoethanol.

Late iPS cells were grown in ES medium consisting of 410ml DMEM-KO 1% L-glutamine, 1% Penicillin/Streptomycin, 1% non-essential amino acids, 15% serum replacement, 10^3 units of LIF and 0.1mM 2-mercaptoethanol.

Cells to be frozen were centrifuged at 1100rpm, RT for 5 minutes and resuspended in 500µl of normal cell specific medium, then 500µl of 2x Freezing medium (40% cell specific medium, 40% FBS/serum replacement, 20% DMSO) was added drop by drop whilst mixing then stored at -80°C or -150°C until required.

2.2 Virus titration

Each well in a six well culture plate was seeded with around 10^6 293 T-HEK cells. The cells were allowed to grow for 24 hours before the titration. After the cells had grown for 24

hours the medium on each well was removed and replaced with 1ml 293 T-HEK medium plus 5µl/ml Polybrene. A serial dilution of the concentrated virus (cherry or green) was set up by adding 1µl of the concentrated virus to 9µl 293 T-HEK medium with Polybrene. 1µl of this solution was then added to another 9µl of medium with Polybrene. 1µl of each of these dilutions was added to two separate wells of the culture dish representing 0.1µl and 0.01µl of the concentrated virus respectively. 1µl of the concentrated virus was added to the next well. 10µl and 100µl of unconcentrated virus was added to two more wells and nothing was added to the final well. The plate was shaken to mix the virus with the cells. The no virus well's medium was aspirated and the cells were trypsinized, collected and counted using a haemocytometer. The culture plate was then incubated at 37°C, 5% CO_2 for 24 hours. After incubation the media was gently removed from the 5 wells and carefully replaced with 500µl of PBS. The PBS was removed gently and then replaced with 2ml of fresh 293 T-HEK medium without Polybrene. The plate was then incubated at 37°C, 5% CO_2 for a further 24-48 hours. After incubation the medium was carefully removed from the wells and 500µl of PBS was gently added to each well. The PBS was removed and 500µl of Trypsin-EDTA was added to each well and the plate was incubated for 2 minutes at room temperature. The trypsin was washed around the well, in order to extract as many cells as possible, and then added to a 1.5ml eppendorf tube. 800µl of 293 T-HEK medium was then added to the well, to remove any remaining cells, and then added to the same tube as the trypsin cell solution. This was repeated for the other wells. The tubes were centrifuged at 3000 rpm, room temperature for 5 minutes. The supernatant was discarded and the cells were resuspended in 300µl 293 T-HEK medium with EDTA then transferred to a FACS tube. The percentage of transfected cells was analysed using Dako CyAn (Beckman Coulter) flow cytometer.

The titre was calculated based on the equation:

Number of cells at time of infection x percentage fluorescent = number of virions per volume virus added

The titre was then used to calculate how much virus would be needed to infect the MEFs at a certain multiplicity of infection (MOI) using the following equation.

Number of viruses deposited = number of cells deposited x MOI

2.3 iPS cell generation

1ml gelatin (0.1%) solution was added to each well of a 6 well dish and incubated at 37°C for 30 minutes. The solution was removed and the plates were allowed at least an hour to dry before being used. Several plates were coated at one time.

10^5 fibroblasts were added to each required well of a gelatin coated 6 well dish. No virus control samples were kept in separate plates to each virus plate in order to reduce contamination risk. Each type of virus was also confined to individual plates.

The medium on the fibroblasts was removed and 1ml of MEF media with 5µg/ml Polybrene was added to each well containing fibroblasts. The desired MOI for each virus was chosen and the corresponding amount of each virus to achieve the MOI was added to each experimental well. Nothing was added to the non-virus control wells. The plates were incubated at 37°C for 24 hours. After 24 hours removing the media from the wells and adding 2ml iPS medium stopped the infection. The medium was changed at least every 2 days. When colonies were observed the medium was changed to ES medium and changed every day. Once colonies were large enough and rounded in shape the well was washed with PBS three times then covered with 1ml PBS. Using a microscope a single colony was

chosen then a 20µl pipette with a clean tip on it was used to cut away the MEFs surrounding the colony. Once the colony was liberated from the MEFs it was withdrawn into the pipette with 20µl of PBS. The colony was transferred to a well in a 96 well plate, and then 50µl of Trypsin-EDTA was added to the well. The 96 well plate was incubated at 37°C for 2 minutes before 100µl of ES medium with 1x Trypsin inhibitor was added to the well. The contents of the well were mixed by pipetting up and down twice before being transferred to a 24 well culture plate previously coated with gamma irradiated MEFs with wells containing 1ml of ES medium. This was repeated for as many colonies as possible and the medium was changed each day. Once the colonies had begun to grow the medum was aspirated, then the colonies were washed with PBS and then trypsinised for 2 minutes, then the trypsinistion was stopped by the addition of ES medium plus Trypsin inhibitor. The cell suspension was removed and centrifuged in a 1.5ml eppendorf tube at 3000rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1ml ES medium and then added to a well of a 6 well culture plate previously coated with gamma irradiated MEFs with wells containing 2ml of ES medium.

2.4 DNA extraction

The removal of the pluripotency cassette was confirmed by extracting the DNA from the transfected clones by adding 50µl of lysis buffer (25mM NaOH, 200µM EDTA) to the cells then incubating at 95°C for 20 minutes. After incubation 50µl of stop buffer (40mM Tris.HCl pH7.5) was added to each sample. 1-4µl of this was used in each PCR reaction.

2.5 PCR

The following procedure was used for pluripotency cassette removal confirmation PCR. 1-4µl of extracted DNA sample was added to 22.7µl of PCR Reddymix (Thermo scientific) plus 1µl forward and 1µl reverse primer. The primers used are shown in table 2.1. The PCR conditions used were as follows:

1. 95°C for 3 minutes
2. 94°C for 30 seconds
3. 60°C for 30 seconds
4. 72°C for 90 seconds
5. Goto step 2 x 44
6. 72°C for 5 minutes

The PCR product was analysed on a 2% agarose gel that was run at 120v for 1.5 hours. The gel was imaged using a SynGene Gene Genius Bio Imaging System.

Table 2.1 Primers used in this study

Primer name	Sequence	Concentration (µM)
miPSt-CreEx-F	GAAACTCTGGTGCATAAATCGATAG	10
hiPSC-CreEx-R	GGAGGCGGCCCAAAGGGAGATCCG	10

2.6 Competent bacteria transformation

2µl of purified plasmid DNA was added to 100µl of competent E.coli (Bioline) and the cells were incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 30 seconds, incubated for a further 20 minutes on ice then 1ml of sterile L-broth was added to the cells and they were incubated at 37°C for an hour. 100µl of this mixture was plated onto an agar plate containing a specific antibiotic (Kanamycin or Ampicillin) and incubated at 37°C overnight.

2.7 Plasmid DNA extraction

A single colony from an agar plate was picked with a clean pipette tip and used to inoculate 1ml L-broth plus the specific antibiotic used on the agar plate. The inoculated L-broth was incubated at 37°C with vigorous shaking (300rpm) for 8 hours. This pre-culture was used to inoculate a larger L-broth culture (200-500ml) plus the specific antibiotic used in the pre-culture. This larger culture was incubated at 37°C with vigorous shaking overnight.

The overnight culture was added to 500ml centrifuge tubes and spun at 6000rpm, 4°C for 30 minutes. The supernatant was discarded and the pellet was used in a maxi plasmid prep kit (Qiagen). The manufacturer's instructions were followed however the final pellet containing the purified plasmid DNA was resuspended in extra distilled H_2O (Sigma). DNA concentration was measured using a Nanodrop (Thermo scientific). Plasmid DNA was then stored at -20°C until required.

2.8 Nucleofection

Cells were transfected using an Amaxa Nucleofector for ES cells using the program A-024 (Lonza). The medium on the cells was refreshed on the day of the transfection and allowed

to incubate for at least one hour before the cells were collected from the plate. 3 million cells were resuspended in 100µl of Amaxa Nucleofector solution for ES/MEFs (Lonza). 10µg of pIRES2-EGFP plasmid DNA (fig 2.1) was added to a Nucleocuvette (Lonza) and then the cell suspension was added to the plasmid DNA. The Nucleocuvette was then sealed and placed into the Nucleofector device. The cells were removed from the Nucleocuvette using a small sterile Pasteur pipette (Lonza) and transferred into a 1.5ml eppendorf tube containing 500µl pre-warmed RPMI. The tube was incubated at 37°C for 1 hour then the contents were transferred to a 175cm^3 tissue culture flask containing 35ml pre-warmed MEF medium for MEFs or a 10cm tissue culture plate containing 10ml pre-warmed ES medium for ES cells. The transfected cells were then incubated at 37°C.

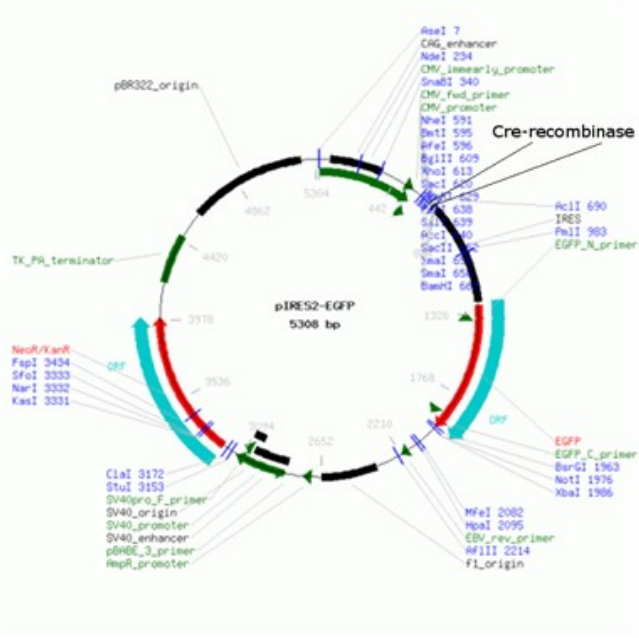


Fig 2.1 Map of pIRES2-EGFP plasmid
Modified pIRES2-EGFP plasmid containing the coding sequence for Cre recombinase. Diagram adapted from https://www.lablife.org

2.9 MEF extraction

Embryos at day 14 were separated from the mother. The heart, liver, gut and cerebellum were all removed from the embryo. Then the embryo was added to 3ml PBS. 300µl of 10x Trypsin was added to each embryo and then incubated at 37°C for at least 30 minutes with occasional shaking. After the incubation 1.5ml of the mixture was removed and added to a clean tube with 1.5ml DMEM. 1.5ml of 1x Trypsin was added to the tube with the embryo and incubated at 37°C for 15 minutes with occasional shaking. 1.5ml was removed from the embryo again and added to the second DMEM tube with another 1.5ml DMEM. This was repeated 5 more times. The DMEM tube was then centrifuged at 1100 rpm for 5 minutes, the resultant cell pellet was resuspended in 10ml MEF medium and added to a 10cm culture plate and incubated at 37°C.

2.10 Fluorescence activated cell sorting (FACS) and flow cytometry

FACS was used to isolate GFP positive cells after 48-72 hours after transfection. Medium was aspirated from cells to be sorted, and then the cells were washed with PBS. The cells were then trypsinised and centrifuged at 1100 rpm for 5 minutes. The cell pellet was resuspended in 300µl of cell specific medium plus 1mM EDTA. This cell suspension was added to a sterile FACS tube. The tube was then inserted into the XDP Moflow High Speed Cell Sorter (Beckman Coulter) and the GFP positive cells were isolated and collected into a sterile FACS tube containing 200µl cell specific medium.

Flow cytometry was used to assess the virus titration and to check expression of mCherry fluorochrome in iPS colonies. Medium was aspirated from cells to be analysed, the cells were washed with PBS. The cells were then trypsinised and centrifuged at 1100 rpm for 5

minutes. The cell pellet was resuspended in 300µl of cell specific medium plus 1mM EDTA. This cell suspension was added to a sterile FACS tube. The tube was then inserted into the Dako CyAn (Beckman Coulter) flow cytometer and the GFP expression was calculated for the virus titration, or mCherry fluorescence expression was calculated for the virus titration and iPS colony checking.

2.11 Immunofluorescence

Cells were grown on 1cm diameter round coverslips, which were transferred to a clean 4 well plate once the cells were confluent. The cells were washed with PBS then fixed with 4% formaldehyde methanol free in PBS for 20 minutes. Cells were washed twice in PBS then quenched with 50mM NH_4Cl in PBS for 10 minutes. The cells were then washed once in 200µl PBS and if the target protein was not located on the cell surface the cells were permeabilized with 0.25% TritonX-100 in PBS for 15 minutes. Cells were washed in PBS twice then non-specific antibody binding was blocked with 5%FBS/0.1% Tween-20 in PBS for 60 minutes. The slides were incubated overnight at 4°C. with primary antibody (table 2.2) diluted in 5%FBS/0.1% Tween-20. The following day cells were washed twice in 0.1% Tween-20 for 15 minutes. Secondary antibodies (table 2.3) were used at the dilution indicated in 5%FBS/0.1% Tween-20. The cells were incubated for 60 minutes. The cells were washed three times in PBS for 20 minutes. The PBS was then removed and the coverslips were placed cell side down onto 15µl of Vectashield/DAPI. Excess Vectashield/DAPI was removed and the coverslip was sealed with clear nail varnish. The slides were imaged using Zeiss LSM 510 Meta confocal microscope.

Table 2.2 Primary antibodies used in this study

Antibody	Ig	Stock concentration (µg/ml)	Dilution used	Supplier
c-Myc	Mouse IgG	200	1:100	Neo Markers
Klf4	Goat IgG	200	1:100	Santa Cruz
Sox2	Mouse IgG	500	1:100	R&D Systems
Oct4	Mouse IgG	200	1:200	Santa Cruz
Nanog	Rabbit IgG	200	1:100	Santa Cruz
SSEA1	IgM	200	1:100	Santa Cruz
Mouse isotype Rabbit isotype	Mouse IgG Rabbit IgG	400 400	1:200 1:200	Santa Cruz Santa Cruz
Goat isotype	Goat IgG	400	1:200	Santa Cruz
Troma-1	Rat IgG	400	1:100	Developmental Studies Hybridoma Bank
α-sma	Mouse IgG	2000	1:500	Sigma
β-III-tubulin	Mouse IgG	200	1:200	Sigma

Table 2.3 Secondary antibodies used in this study

Antibody	Fluorochrome	Dilution used	Supplier
Goat anti mouse IgG	488-Alexa	1:1000	Molecular Probes
Goat anti mouse IgG	594-Alexa	1:1000	Molecular Probes
Goat anti mouse IgG	633-Alexa	1:1000	Molecular Probes
Donkey anti goat IgG	488-Alexa	1:1000	Molecular Probes
Goat anti rabbit IgG	594-Alexa	1:1000	Molecular Probes
Goat anti mouse IgM	633-Alexa	1:1000	Molecular Probes

2.12 Alkaline Phosphatase staining

The medium was removed from cells to be stained and the cells were rinsed once with cold PBS. The PBS was removed and 10% Neutral formalin buffer (100ml 37% formalin, 900ml dH_2O, 4g sodium dihydrogen phosphate (monohydrate), 6.5g disodium hydrogen phosphate (anhydrous)) was added to each well to cover the cells and incubated for 15 minutes at room temperature. The cells were then washed once with cold dH_2O and then incubated in cold dH_2O for 15 minutes at room temperature. The dH_2O was removed and the cells were covered in fresh substrate (25ml dH_2O, 25ml 0.2M Tris-HCl pH8.3, 200µl N,N-Dimethylformamide, 0.005g Naphthol AS MX-PO_4, 0.03g Red Violet LB salt) and incubated for 30 minutes. The substrate was removed from the cells and washed with dH_2O then the cells were dried.

2.13 Differentiation of iPS cells

iPS cells to be differentiated were centrifuged at 1100rpm for 5 minutes, the cell pellet was washed in PBS. The PBS was removed and the cell was resuspended in 10ml ES differentiation medium (41ml DMEM-KO, 5ml L-Glutamine, 5ml Penicillin/Streptomycin, 5ml non-essential amino acids, 5ml FBS, 7.5ml serum replacement, 0.32µl 2-mercaptoethanol). The cell suspension was added to a 10cm non-coated culture dish. After 6 days the cell suspension was transferred to a 24 well plate with or without 0.1% gelatin coated coverslips in each well. After 48 hours the cells were stained as described in section 2.1. RNA was extracted from the cells in wells without coverslips and from any cells remaining in wells after the coverslips had been removed.

2.14 Karyotyping

The medium on the cells was refreshed on the day of the karyotyping and allowed to incubate for at least one hour before 100ng/ml of KaryoMAX colcemid solution (Invitrogen) was added. The cells were incubated for a further 2 hours with the KaryoMAX then the cells were collected and 1-2 $x10^4$ cells were washed twice in PBS and then the cell pellet was resuspended in 0.1M KCl, incubated for 10 minutes at room temperature. The cells were then added to a cytospin funnel, the funnel was then attached to a glass slide with a filter paper and cytospun at 1800rpm for 10 minutes. The slide was then submerged in KCM buffer (120mM KCl, 20mM NaCl, 10mM Tris/HCl pH8, 0.5mM EDTA, 0.1% Triton-x) and incubated for 10 minutes at room temperature. The cells were then fixed by submerging them in 4% formaldehyde in KCM buffer for 10 minutes. The slides were washed twice in PBS then 10µl DAPI was added to a coverslip and the cells were placed onto the coverslip.

The coverslip was then sealed with clear nail varnish and imaged using Zeiss LSM 510 Meta confocal microscope. The number of chromosomes of cells in metaphase were counted.

3. RESULTS

3.1 Virus titration

The two murine lentiviruses, STEMCCA and Cherry (fig 1.1), which were to be used to reprogram the MEFs, were titrated in order to calculate the amount of each virus that was required for the reprogramming. The STEMCCA virus contains the four factors, Oct4, Klf4, Sox2 and C-Myc. The Cherry virus contains the three factors, Oct4, Klf4 and Sox2 the C-Myc was replaced with mCherry fluorochrome. In order to titrate the STEMCCA virus an analogous GFP containing virus which was synthesised in parallel to the STEMCCA virus was used, as the STEMCCA virus contains no fluorescent marker. The titration was carried out as described in section 2.2. Fig 3.1 (A) illustrates the population of live cells which were gated and measured for their fluorescence intensities, the left panel represents the non-infected cells and the right panel represents the infected cells, shown to illustrate that the populations were identical in the non-infected and the infected cells. Fig 3.1(B) shows a dot plot representing the percentage of 293T-HEK cells which were expressing mCherry fluorochrome at 72 hours after being infected with 1µl of concentrated Cherry virus (right panel) compared to uninfected 293T-HEK cells (left panel). (C) shows a dot plot representing the percentage of 293T-HEK cells which were expressing GFP at 72 hours after being infected with 1µl of concentrated virus (right panel) compared to uninfected 293T-HEK cells (left panel). The values in table 3.1 show the number of fluorescent positive cells as a percentage of all the cells for the concentrated and unconcentrated virus samples. These percentages were used to calculate, using the equation in section 2.2, the amount of virus required for each MOI that was used in the following experiments.

Table 3.1 Titration of the two viral vectors

Virus added	**Percentage of fluorescent cells at 72h**
No virus	0
10µl unconcentrated GFP virus	20.41
100µl unconcentrated GFP virus	77.78
0.01µl concentrated GFP virus	1.63
0.1µl concentrated GFP virus	8.72
1µl concentrated GFP virus	71.00
10µl unconcentrated Cherry virus	0.09
100µl unconcentrated Cherry virus	0.52
0.01µl concentrated Cherry virus	0
0.1µl concentrated Cherry virus	0.04
1µl concentrated Cherry virus	0.54

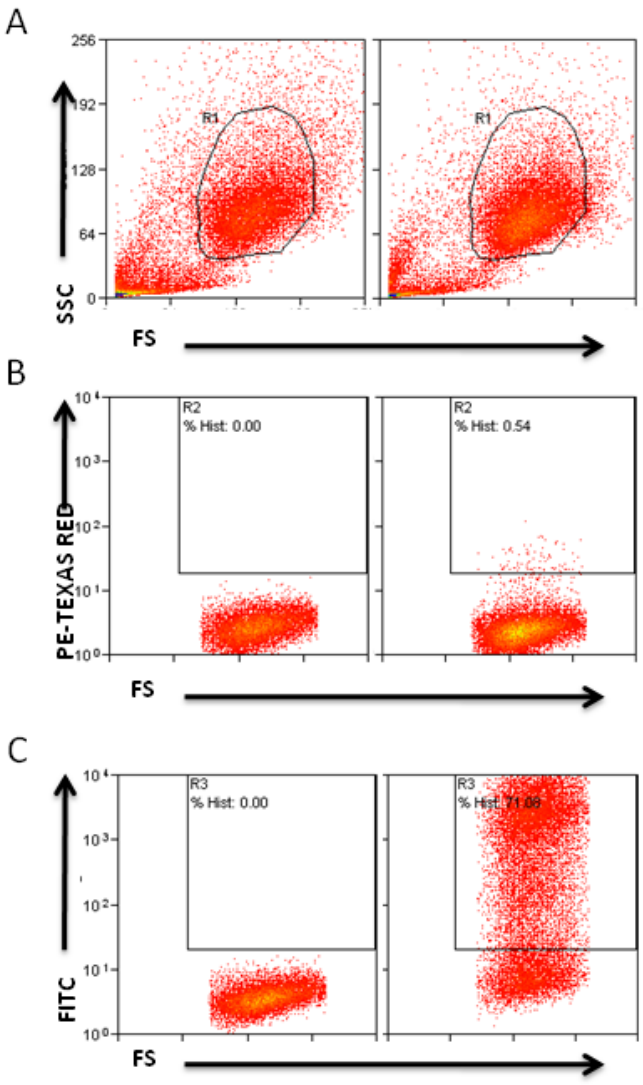


Fig 3.1 Titration of Cherry and STEMCCA lentivirus.
$1.5x10^6$ 293 T-HEK cells were infected with 1 µl concentrated Cherry virus or 1 µl concentrated GFP virus . (A) Dot plots showing the gated region (live cells). Left panel non-infected cells, Right panel viral infected cells . (B) dot plot showing percentage of infected cells with Cherry virus detected in the PE-texas red channel (right panel), non-inected cells (left panel). (C) dot plot showing percentage of infected cells with STEMCCA virus detected in the FITC channel (right panel), non-inected cells (left panel)

3.2 iPS cell generation

iPS cells were generated from MEFs as described in section 2.3. Fig 3.2 shows the growth of the colonies after infection with Cherry or STEMCCA virus at a MOI of 0.5. The STEMCCA infected cells were reprogrammed to form iPS cell colonies in half the time it took the Cherry infected cells. There were visibly more colonies formed in the STEMCCA infected cell compared to the Cherry infected cells. This can be observed in fig 3.3 which shows alkaline phosphatase staining of the colonies at MOI of 0.5, (A) shows the STEMCCA colonies and (B) shows the Cherry colonies. Visually the colonies stained the same no matter which virus was used to reprogram the MEFs. iPS cells generated with the Cherry virus were analysed by flow cytometry as described in section 2.10 for the expression of the mCherry fluorochrome, fig 3.4 (B) shows the expression of mCherry in one of the expanded Cherry clones (red line) compared to a STEMCCA clone (blue line). The Cherry clone's mean fluorescent intensity is half a magnitude higher than the control STEMCCA cells. One of the Cherry clones was also analysed on a confocal microscope. The clones were observed to be fluorescent red (fig3.4 (A) (right panel)) compared to the STEMCCA clones (left panel) which were not fluorescent.

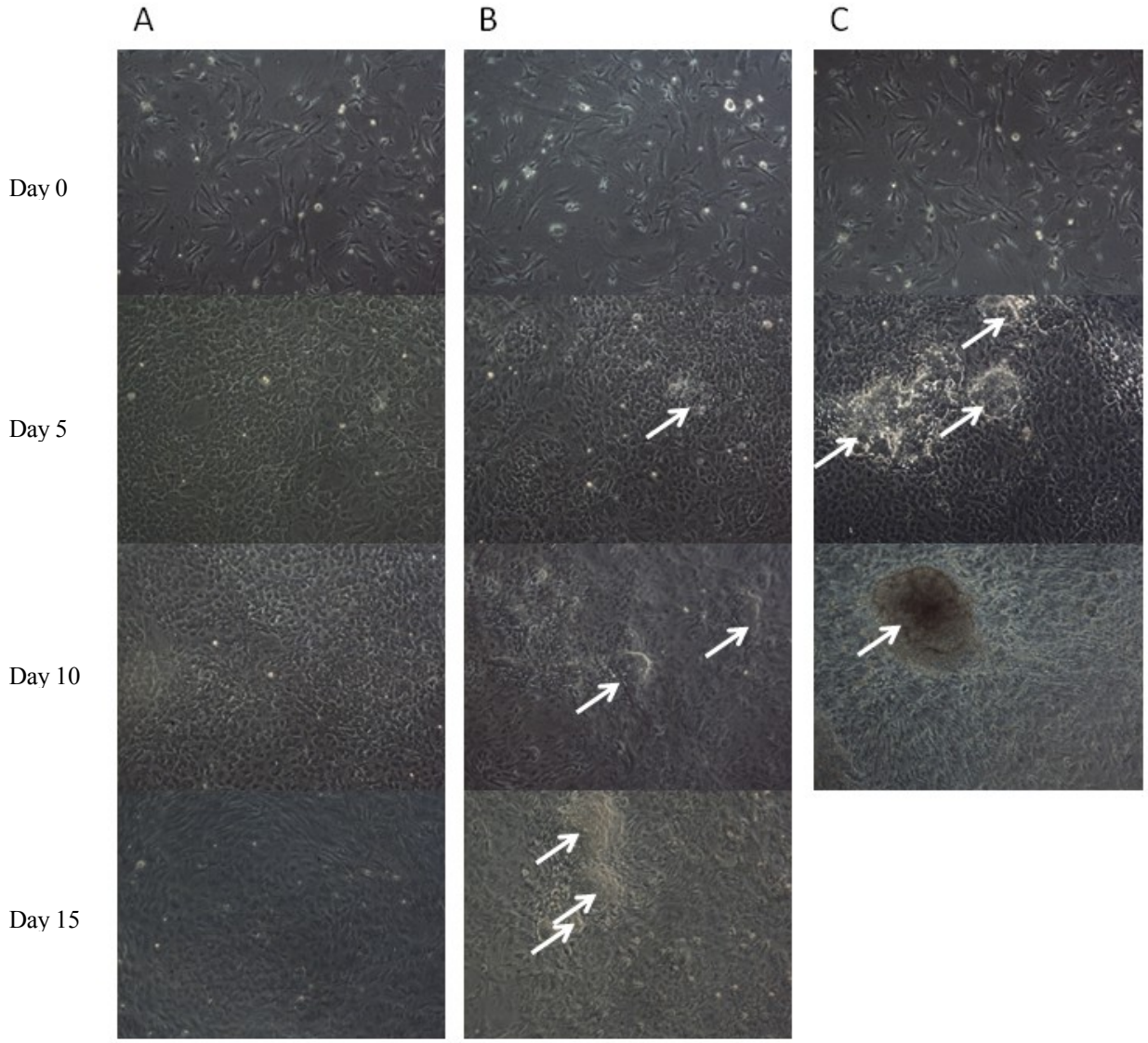
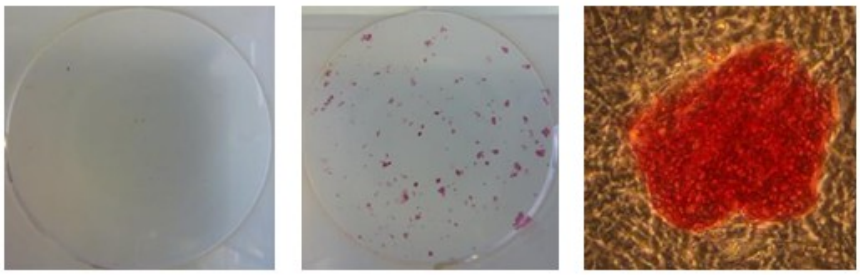


Fig 3.2 Development of iPS colonies after infection with Cherry or STEMCCA lentivirus
10^5 MEFs were infected with Cherry (B) or STEMCCA (C) lentivirus. (A) shows the non infected control MEFs at day 0, 5, 10 and 15 post infection. (B) shows the Cherry infected MEFs at day 0, 5, 10 and 15 post infection. (C) shows the STEMCCA infected MEFs at day 0, 5, 10 and 15 post infection. White arrows indicate the formation of iPS colonies. All images taken at 10x magnification

A



B

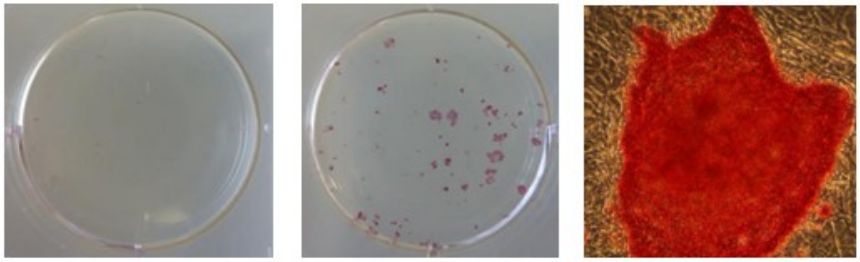
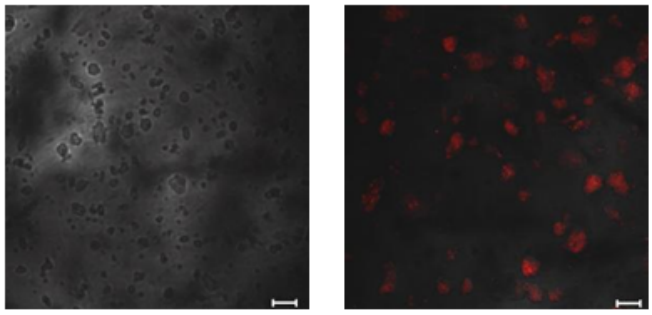


Fig 3.3 Alkaline Phosphatase (ALP) staining for Cherry and STEMCCA lentivirus infected MEFs

(A) shows the non infected MEFs (left panel) compared to the STEMCCA infected MEFs (middle panel) at day 11 post infection stained with ALP. The right panel shows a positive colony at 10x magnification. (B) shows the non infected MEFs (left panel) compared to the Cherry infected MEFs (middle panel) at day 18 post infection stained with ALP. The right panel shows a positive colony at 10x magnification

A



B

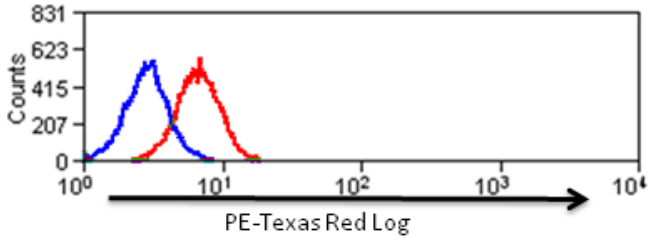


Fig 3.4 Expression of mCherry fluorochrome in live iPS cells

(A) shows STEMCCA iPS cells grown on a MEF feeder layer (left panel) and Cherry iPS cells grown on a MEF feeder layer (right panel), the red fluorescence represents the expression of the mCherry fluorochrome. Scale bar = 100µm. (B) shows the difference in fluorescence intensity between the STEMCCA iPS cells (blue line) and the Cherry iPS cells (red line)

3.3 Confirmation of pluripotency

Colonies were grown on coverslips for 3 days and then stained as described in section 2.11. Fig 3.5 to Fig 3.8 are cells from a Cherry clone which had been previously transfected with a plasmid containing the coding sequence for the protein Cre recombinase, which when expressed by the cells removed the pluripotency cassette due to the flanking LoxP sites which were engineered into the Cherry lentivirus. Fig 3.5 (A) all cells are expressing Oct4 at various levels, and there are also two cells which are expressing Nanog. Fig 3.5 (B) represents isotype controls for each of the specific antibodies used, the IgG Rabbit showed a lot of secondary antibody binding, however the IgG appears to be only located on the surface of the cells, in contrast to the positive Nanog cells in (A) where the Nanog expression is localised in the nucleus. Fig 3.6(A) shows all cells expressing the membrane localised protein SSEA-1. The isotype control (B) demonstrates that the fluorescence observed in (A) is due to specific binding of the antibody to ssea-1. Fig 3.7 (A) many of the cells were observed to be expressing the proteins Sox2 (red) and Klf4 (green). (B) shows the isotype controls to confirm the specific binding of the Sox2 and Klf4 antibodies. Fig 3.8 (A) represents the expression of c-Myc protein in the cells. (B) shows the isotype controls to confirm that the c-Myc antibody binding was specific.

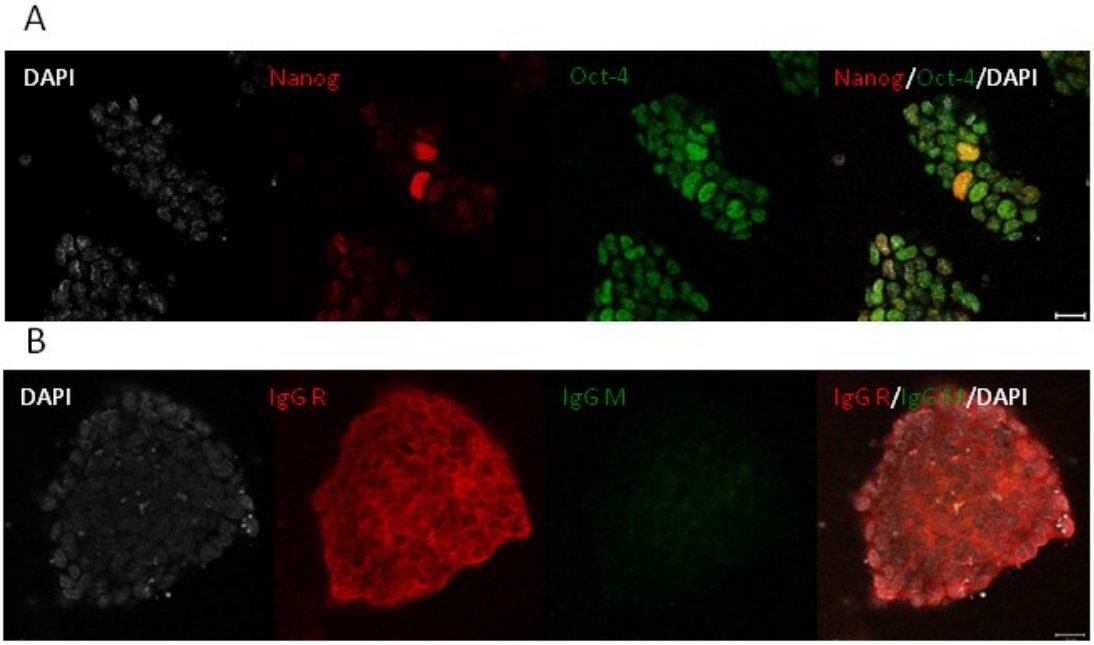


Fig 3.5 Nanog/Oct-4 Immunofluorescence of one the Cherry iPS clones isolated
Immunofluorescence of Cherry iPS cells which had been previously transfected with Cre recombinase to remove the pluripotency cassette. (A) Cherry iPS cells stained for expression of Nanog (red) and Oct-4 (green). DNA is stained with DAPI (white). (B) Isotype controls R=rabbit, M=mouse. Scale bar represents 20 µm.

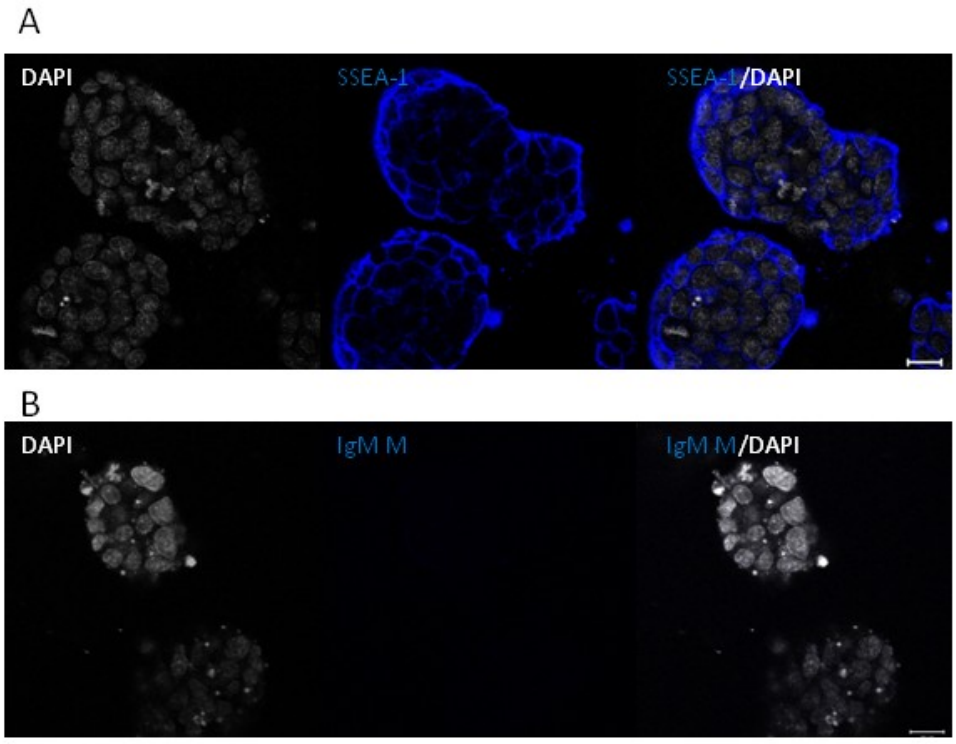


Fig 3.6 SSEA-1 Immunofluorescence of one the Cherry iPS clones isolated
Immunofluorescence of Cherry iPS cells which had been previously transfected with Cre recombinase to remove the pluripotency cassette. (A) Cherry iPS cells stained for expression of SSEA-1 (blue). DNA is stained with DAPI (white). (B) Isotype controls M=mouse. Scale bar represents 20 µm.

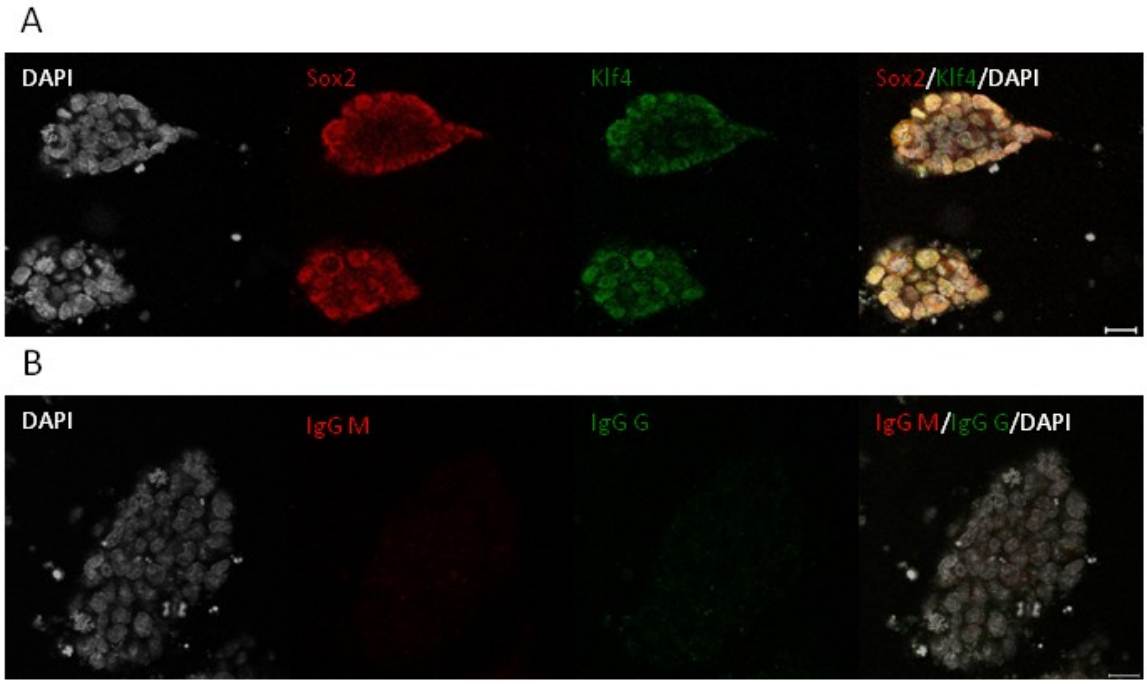


Fig 3.7 Sox2/Klf4 Immunofluorescence of one the Cherry iPS clones isolated
Immunofluorescence of Cherry iPS cells which had been previously transfected with Cre recombinase to remove the pluripotency cassette. (A) Cherry iPS cells stained for expression of Sox2 (red) and Klf4 (green). DNA is stained with DAPI (white). (B) Isotype controls M=mouse, G=goat. Scale bar represents 20 µm.

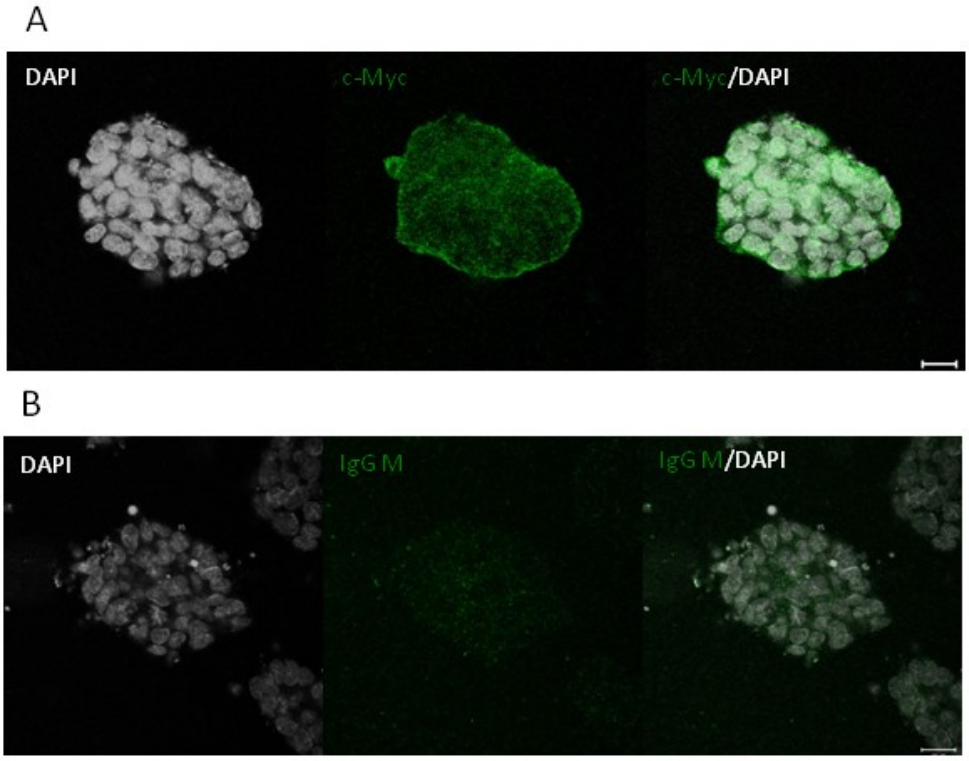


Fig 3.8 c-Myc Immunofluorescence of one the Cherry iPS clones isolated
Immunofluorescence of Cherry iPS cells which had been previously transfected with Cre recombinase to remove the pluripotency cassette. (A) Cherry iPS cells stained for expression of c-Myc (green). DNA is stained with DAPI (white). (B) Isotype controls M=mouse. Scale bar represents 20 µm.

3.4 Removal of the pluripotency cassette

The pluripotency cassette was removed following section 2.8 to transfect plasmid pIRES2-EGFP which had been sub cloned to contain the coding sequence for the protein Cre recombinase. The GFP marker allowed for cells, which had been transfected to be isolated as described in section 2.10. The removal of the pluripotency cassette was confirmed by extracting the DNA (section 2.4) from a sample of the sorted cells and then performing PCR on the DNA as described in section 2.5 with the primers in table 2.1. The results of the PCR are shown in fig 3.9. The primers were designed to amplify a section of the pluripotency cassette, therefore the clones which have a band on the agarose gel still contained the pluripotency cassette and were discarded.

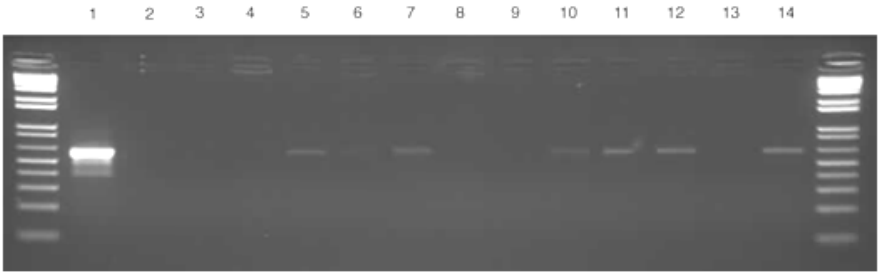


Fig 3.9 Confirmation of STEMCCA pluripotency cassette removal in iPS isolated clones
DNA was extracted from each picked colony. PCR using primers specific for a region in the pluripotency cassette were used on the plasmid DNA (lane 1), individual picked colonies (lane 2-14). Successful removal of the cassette was achieved in clones with no band.

3.5 Differentiation potential

Cells were differentiated as described in section 2.13. Fig 3.10 (A), 3.11 (A) and 3.12(A) show the staining of differentiated cells for markers of the three different germ layers, Troma-1for the endoderm (3.10), α-smooth muscle actin (α-SMA) for the mesoderm (3.11) and β-III-tubulin for the ectoderm (3.12). Fig 3.11 (B) and 3.12(B) show the isotype controls which had very little fluorescence signal, confirming that the antibodies are binding to the correct proteins and not binding non-specifically. These immunofluorescence experiments were repeated on two other clones, one Cherry and one STEMCCA, which still retained the pluripotency cassette in their genome (Fig S1-S4, Appendix).

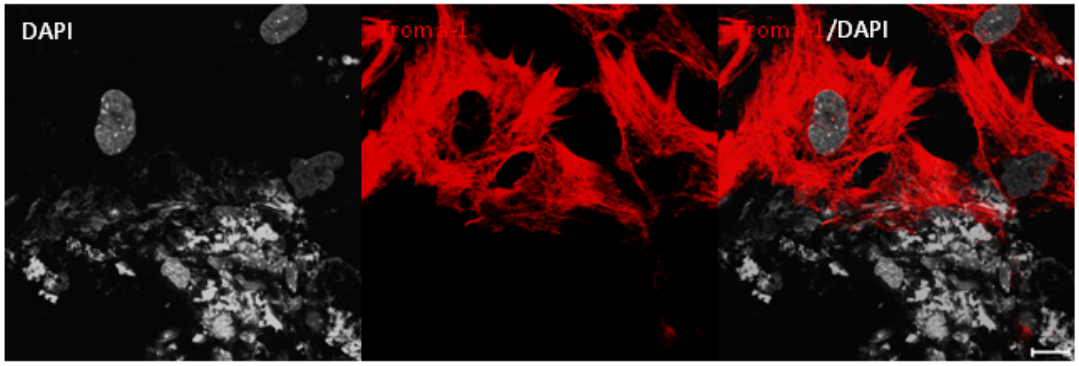


Fig 3.10 Troma-1 Immunofluorescence of one the Cherry iPS clones isolated and allowed to spontaneously differentiate
Immunofluorescence of Cherry iPS cells which had been differentiated by embryoid body formation in suspension culture (A) Cherry iPS cells stained for expression of Troma-1 (red). DNA is stained with DAPI (white). Scale bar represents 20 µm.

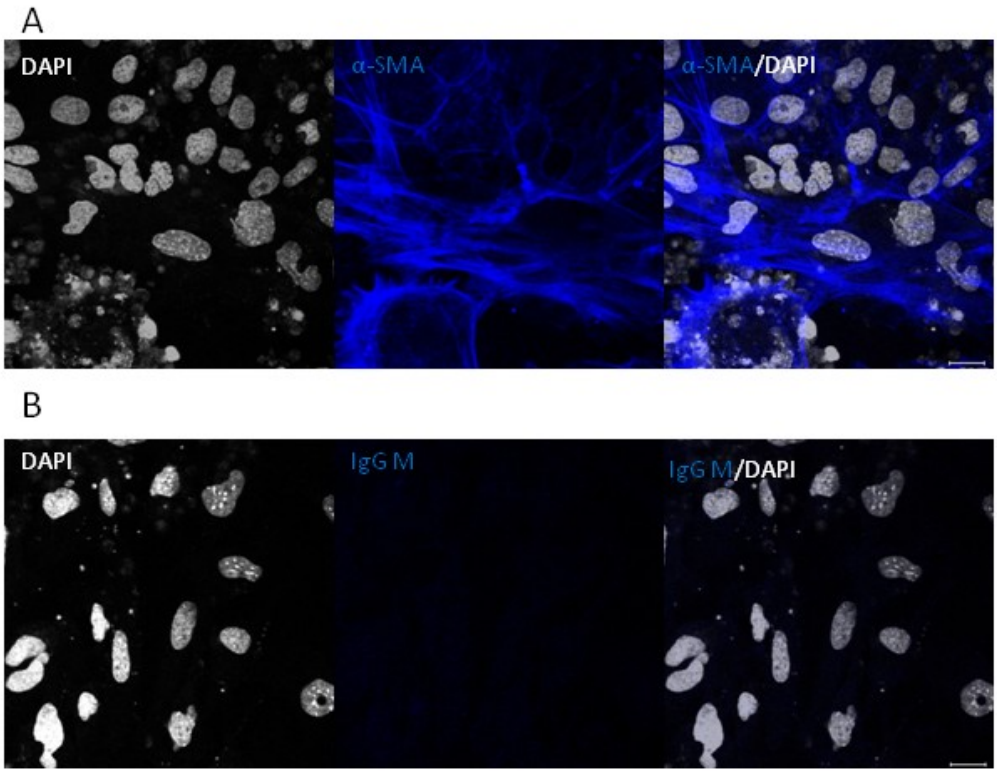


Fig 3.11 α-SMA Immunofluorescence of one the Cherry iPS clones isolated and allowed to spontaneously differentiate

Immunofluorescence of Cherry iPS cells which had been differentiated by embryoid body formation in suspension culture (A) Cherry iPS cells stained for expression of α-SMA (blue). DNA is stained with DAPI (white). (B) Isotype controls M=mouse. Scale bar represents 20 µm.

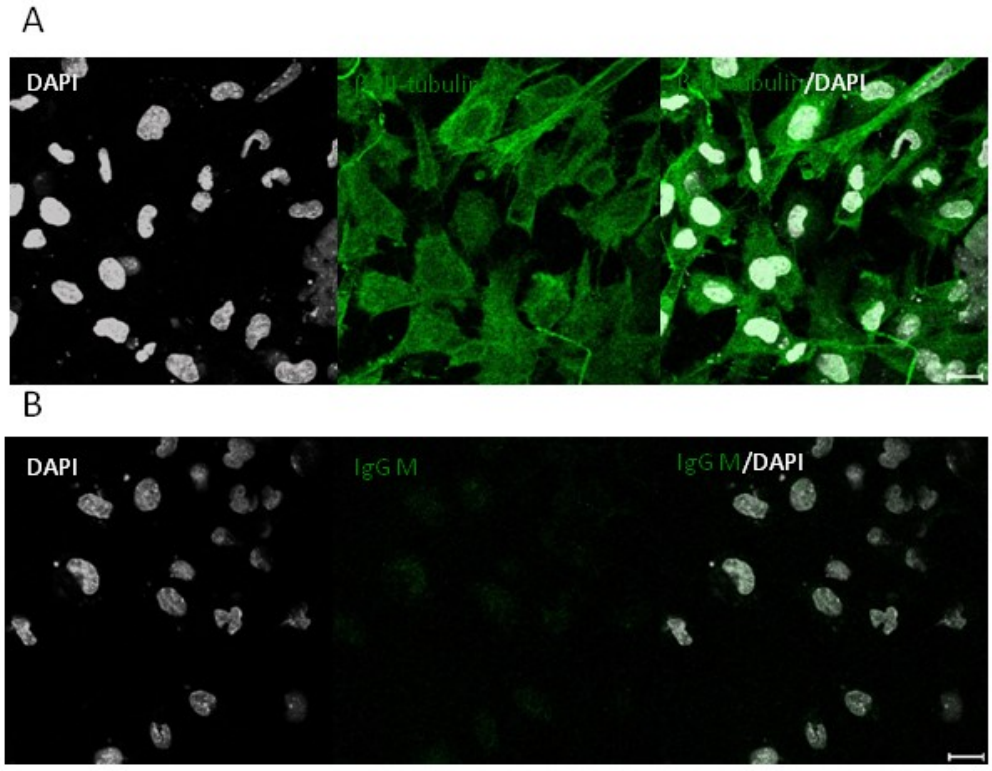


Fig 3.12 β-III-tubulin Immunofluorescence of one the Cherry iPS clones isolated and allowed to spontaneously differentiate

Immunofluorescence of Cherry iPS cells which had been differentiated by embryoid body formation in suspension culture (A) Cherry iPS cells stained for expression of β-III-tubulin (green). DNA is stained with DAPI (white). (B) Isotype controls M=mouse. Scale bar represents 20 µm.

3.6 Karyotyping

Karyotyping was completed on one of the STEMMCA iPS clones as described in section 2.14. The image in fig 3.13 (A) represents the chromosomes of one of 28 cells at metaphase which were counted. (B) shows the total numbers of chromosomes counted in each of the cells.

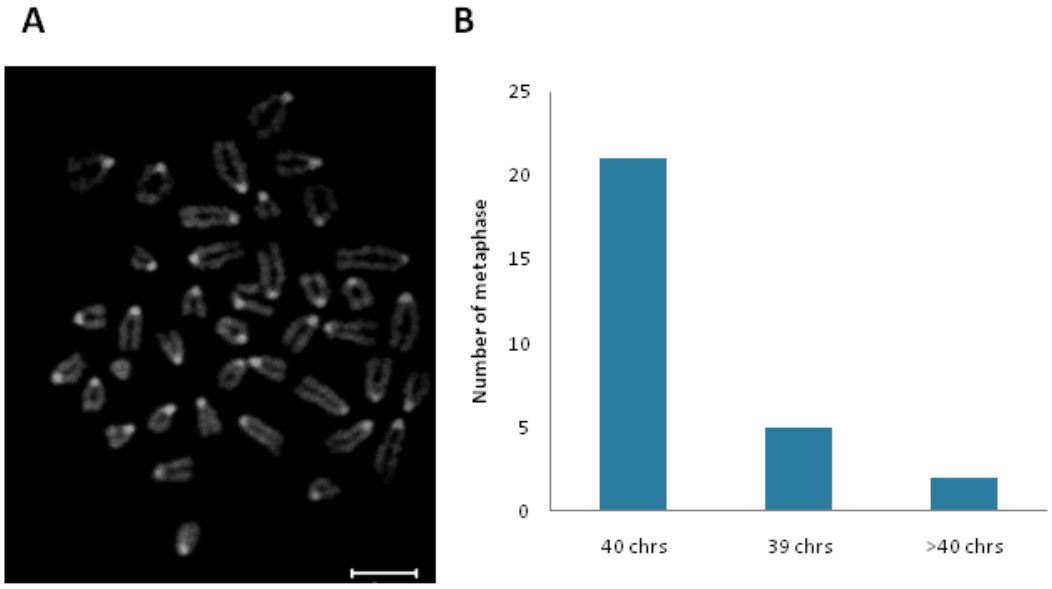


Fig 3.13 Karyotyping of iPS clone 16.3 after removal of STEMCCA pluripotency cassette
(A) Represents the chromosomes of an individual cell stained with DAPI after colcemid treatment and cytospin. Scale bar represents 5µm. (B) Bar graph showing the number of chromosomes in the 28 individual metaphases that were counted.

4. DISCUSSION

These data show that it is possible to generate iPS cells by reprogramming MEFs using the STEMCCA and Cherry lentiviral vectors (Sommer *et al.* 2009 & 2010).

The data is section 3.2 confirms that the removal of the c-Myc gene from the pluripotency cassette lowered the efficiency and speed of reprogramming as was previously observed by Nakagawa *et al.,* (2008).

It has been shown previously that the transcription factors Nanog, Oct4 and Sox2 are required by ES cells to function correctly (Boyer *et al.*, 2005), the fact that iPS cells generated in this study were expressing these factors (section 3.3) even after the pluripotency cassette was excised shows that the cells are pluripotent. The expression of Nanog is especially relevant as this factor was not included in the pluripotency cassette and therefore must have been activated by the reprogramming process. The Nanog expression has been shown to be crucial in forming chimeric mice (Okita *et al.*, 2007). The cell surface antigen SSEA-1 is also known to be important in ES cells (SOLTER & KNOWLES, 1978), and was observed to be expressed in the generated iPS cells even though the gene for SSEA-1 was not in the pluripotency cassette.

Differentiating the Cherry iPS after excision of the pluripotency cassette into the cell of each of the three germ layers demonstrates that at least some of these iPS cells are pluripotent (section 3.5). Both STEMCCA and Cherry iPS cells which still contained the pluripotency cassette were able to spontaneously differentiate into cells from each germ layer (Fig S1-S4, Appendix). There was is no clear difference in the expression of the proteins which were analysed demonstrating that the differentiation potential between the STEMCCA and Cherry iPS cells or the Cherry iPS cells without the pluripotency cassette were the same.

The karyotyping experiment (section 3.6) shows that the majority of the metaphases which were counted had the correct number of chromosomes for a healthy mouse cell. This is important as if the reprogramming process creates chromosomal abnormalities it will affect any cells which could be derived from these iPS cells.

4.1 Further work

The data in section 3 show the beginnings of characterisation of the clones which were generated, however most the data has been produced from a small number of the clones. With more time it would be possible to repeat each experiment several times for each of the clones. This would allow conclusions to be drawn between the two lentiviral vectors which were employed in this study, furthermore the effect of not excising the pluripotency cassette could also be gauged.

There are also more experiments which would further characterise the iPS clones. Firstly it would be useful to analyse the gene expression through reverse transcriptase PCR of the RNA extracted from iPS cells at different points in the reprogramming process which could be compared to ES cells and MEFs RNA (positive and negative controls of pluripotency respectively). If the reprogramming process were successful the gene expression pattern of the iPS cells should be the same as an ES cell. The genes for the proteins tested in section 3.4 could be used and any which are in the pluripotency cassette would likely be highly expressed in cells which still contained the pluripotency cassette. Other genes such as Nanog, Ecat1 and Cripto (Mitsui *et al.*, 2003), which have been shown to be important in ES cells, could be tested.

Furthermore the protein expression of some of these proteins could be analysed by western blotting as the RNA expression does not always correspond to the actual protein expression in the cells. The abundance of protein could be studied in each of the stages of reprogramming compared to ES cells in order to gain greater understanding of the reprogramming process.

The pluripotency of the clones could also be further analysed by the generation of teratomas by injecting cells from each of the individual iPS clones into nude mice. The resultant teratomas should contain cells from each of the germ layers further confirming the pluripotency of the generated clones.

The final method which would have been used if this work were to be continued would be the formation of chimeric mice by injection of an iPS clone cells into a mouse blastocyst. The ability to form chimeras would show that the iPS cells are germline-competent and would be the ultimate proof of pluripotency.

The time constraints of this project meant that it was not possible to carry out these methods, however it would be important to characterise all the isolated clones in these ways to be sure that they were truly pluripotent. Comparing the levels of pluripotency observed in each clone could lead to insights into the reprogramming process.

In addition to further characterising the clones which were generated previously it would be insightful to begin to look at the effect that other genes may have on the reprogramming process as has been previously shown (Maekawa *et al.*, 2011, Moon *et al.*, 2011). The gene b-Myb is important in DNA replication in mouse ES cells (Lorvellec *et al.*, 2010), and could therefore be an interesting gene to study in the context of cellular reprogramming.

5. Appendix

5.1 Supplementary figures

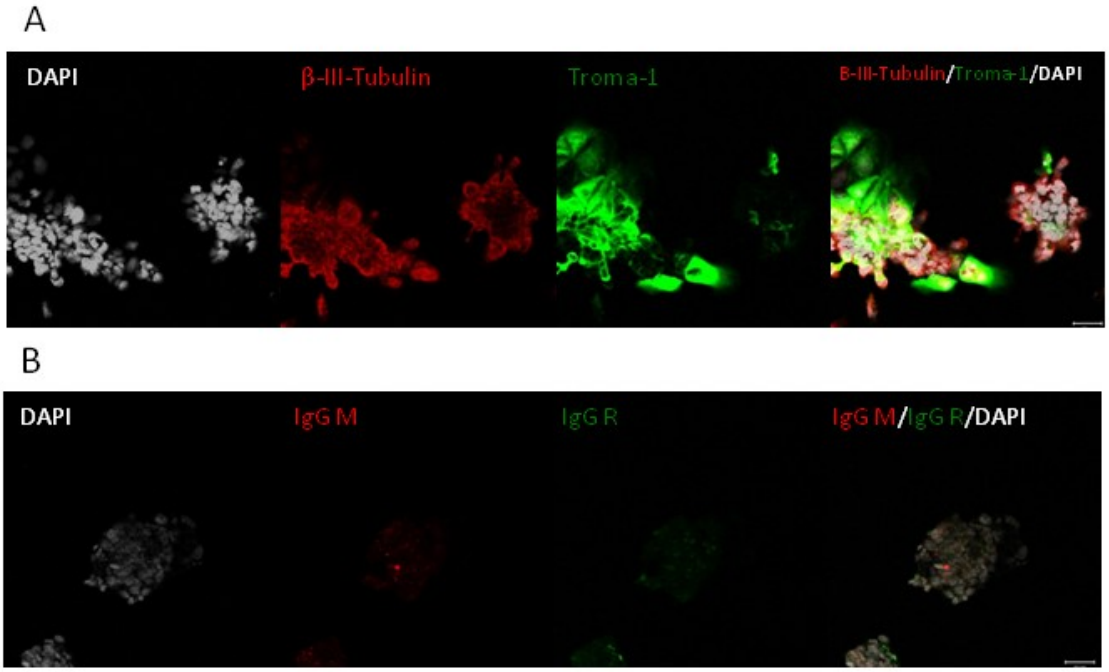


Fig S1 Troma-1 and β-III-Tubulin Immunofluorescence of one the Cherry iPS clones isolated and allowed to spontaneously differentiate
Immunofluorescence of Cherry iPS cells which had been differentiated by embryoid body formation in suspension culture (A) Cherry iPS cells stained for expression of β-III-tubulin (red) and Troma-1 (green). DNA is stained with DAPI (white). (B) Isotype controls R=rat, M=mouse. Scale bar represents 50 µm.

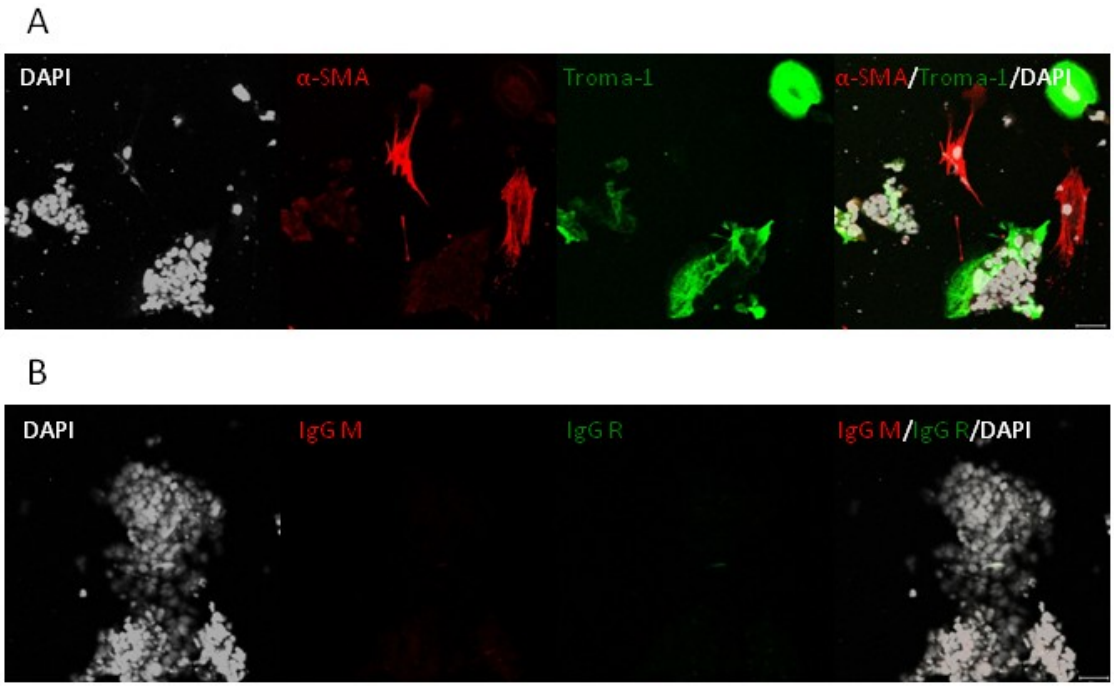


Fig S2 Troma-1 and α-SMA Immunofluorescence of one the Cherry iPS clones isolated and allowed to spontaneously differentiate
Immunofluorescence of Cherry iPS cells which had been differentiated by embryoid body formation in suspension culture (A) Cherry iPS cells stained for expression of α-SMA (red) and Troma-1 (green). DNA is stained with DAPI (white). (B) Isotype controls R=rat, M=mouse. Scale bar represents 50 µm.

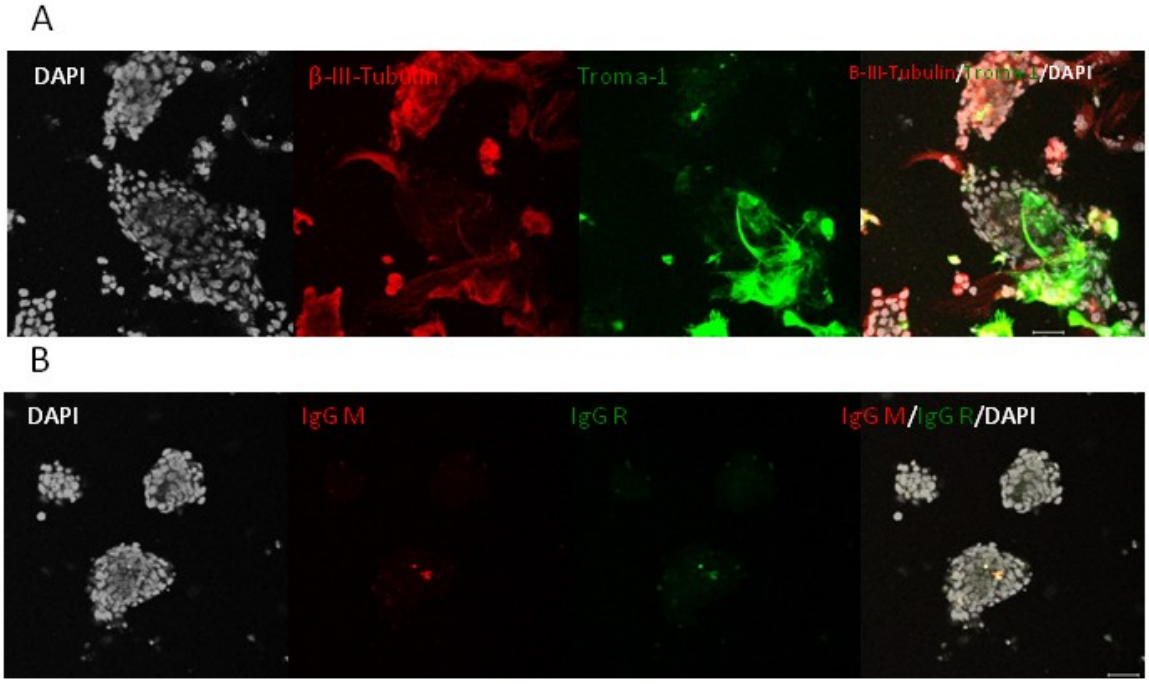


Fig S3 Troma-1 and β-III-Tubulin Immunofluorescence of one the STEMCCA iPS clones isolated and allowed to spontaneously differentiate

Immunofluorescence of STEMCCA iPS cells which had been differentiated by embryoid body formation in suspension culture (A) Cherry iPS cells stained for expression of β-III-tubulin (red) and Troma-1 (green). DNA is stained with DAPI (white). (B) Isotype controls R=rat, M=mouse. Scale bar represents 50 µm.

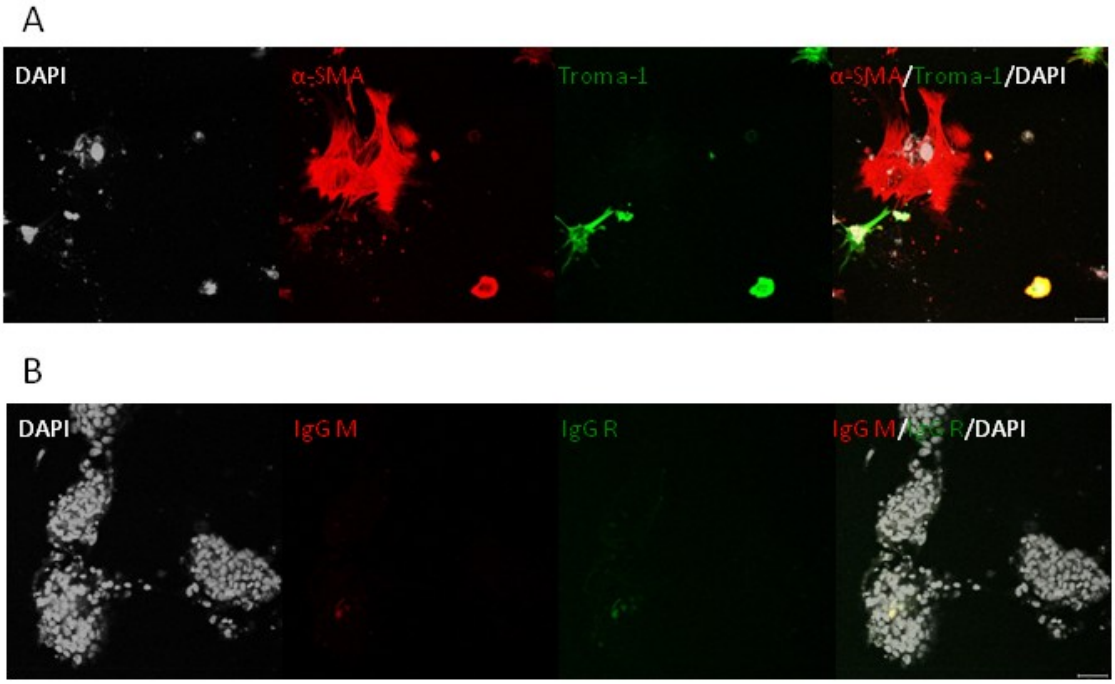


Fig S4 Troma-1 and α-SMA Immunofluorescence of one the STEMCCA iPS clones isolated and allowed to spontaneously differentiate

Immunofluorescence of Cherry iPS cells which had been differentiated by embryoid body formation in suspension culture (A) STEMCCA iPS cells stained for expression of α-SMA (red) and Troma-1 (green). DNA is stained with DAPI (white). (B) Isotype controls R=rat, M=mouse. Scale bar represents 50 µm.

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