Derivation of Induced Pluripotent Stem Cells from Rat Primary Cells

Utilising the Cre-Lox Lentiviral Delivery System

by

Michael James Smith

A dissertation submitted to the University of Birmingham for

the degree of Master of Research in Biomedical Research

School of Dentistry

College of Medical and Dental Sciences

University of Birmingham

August 2012

Contents

Derivation of Induced Pluripotent Stem Cells from Rat Primary Cells Utilising the Cre-Lox Lentiviral Delivery System

Abstract:

Somatic cells can be reprogrammed to an embryonic stem cell-like state, regaining the ability to differentiate into any cell in the body. Induced pluripotent stem cells (iPSCs) can be derived through the delivery and expression of exogenous transcription factors; Oct4, Sox2, Klf4 and c-Myc being the original and most commonly used. The project utilised the STEMCCA virus, a lentivirus delivery system comprising of all four reprogramming factors in a single cassette. Different media and culturing conditions were used in an attempt to derive rat iPSCs from adipose- and dental pulp derived stem cells (ADSCs and DPSCs, respectively). Staining for alkaline phosphatase, which is highly expressed in ESCs and iPSCs, was used to demonstrate whether colonies observed were potentially composed of iPSCs. Initial attempts using iPSC media, a feeder layer or ascorbic acid and lithium chloride supplementation, were unsuccessful in generating iPSC colonies. Medium supplemented with two inhibitors, CHIR-99021 and PD-0325901, was successful in generating iPSC colonies with an ESC-like morphology from rat embryonic fibroblasts. iPSC colonies failed to form using the same culture conditions with the ADSCs and DPSCs.

1. Introduction

Stem cells have the potential to regenerate and repair tissues whilst being able to self-replicate, which has led to scientists exploiting them to develop therapies, however not all stem cells are the same or have the same capacity to regenerate and repair tissues.

During development cells become increasingly committed to specific lineages; upon fertilisation until the 8-cell stage, cells of the developing embryo are totipotent due to their capacity to differentiate into both embryonic and extraembryonic tissues[1]. The embryo continues through several more cell divisions to the morula stage and then forms the blastocyst which consists of the trophoblast, inner cell mass (ICM) and blastocoele (Fig. 1). The blastocyst marks an early stage whereby cells commit to a specific lineage; cells of the ICM commit to give rise to the embryo and the trophoblastic cells become committed to form extraembryonic tissues, such as the placenta, which will support the development of the growing embryo. The cells of the ICM are pluripotent as they are able to differentiate into any cell from the three germ layers; the ectoderm which gives rise to the nervous system and epidermis; mesoderm which generates of the muscle, heart and blood; and the endoderm which forms of the lungs, gastrointestinal tract, endocrine organs and bladder.

Cells of the developing embryo become increasingly specialised and committed to specific lineages. Other than totipotent and pluripotent, cells can be termed multi-, omni- or uni- potent depending on their capacity to differentiate into different cell types. Adult stem cells, such as haematopoietic and neural stem cells, are traditionally defined as multipotent as they can form all the cells of that specific lineage but are unable to form cells from different germ layers. However some cells such as mesenchymal stem cells are challenging the traditional definitions of cell potency due to being able

to differentiate and form cells from different germ layers[2-4]. Omnipotent cells have a restricted

potential to differentiate into just a few cell types from a specific lineage whereas unipotent cells are

only able to give rise to one cell type. The reduction in cell potency is concurrent with the acquisition

of epigenetic changes, such as methylation of gene promoters, which are responsible for the stem

cell state or lineage specification[5].
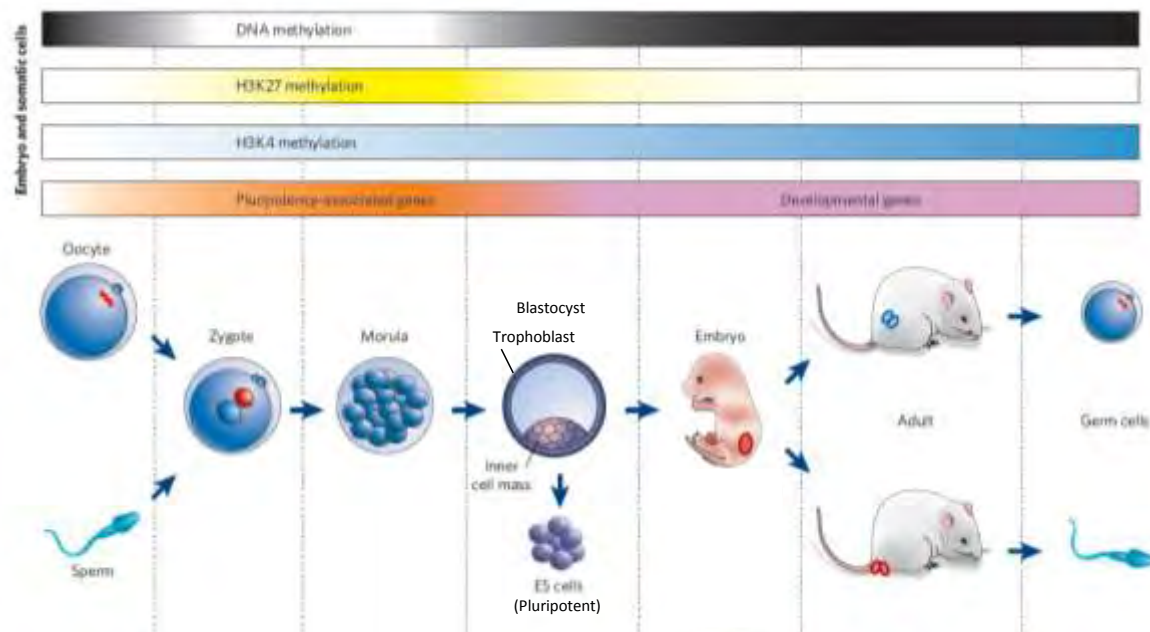
Figure 1: Overview of mammalian development. Diagram shows the crucial stages of development,

the stages pluripotency and developmental associated genes are expressed and the global

epigenetic patterning. ESCs can be successfully isolated from the inner cell mass of the blastocyst.

Global hypomethylation is present from the zygotic to morula stage, which corresponds to

expression of pluripotency associated genes. Epigenetic changes are accumulated, downregulating

pluripotency associated genes which results in developmental associated genes being expressed.

Image adapted from Reik *et al.,* (2007)[5].

The pluripotent cells from the ICM, otherwise known as embryonic stem cells (ESCs) have been

successfully isolated and cultured[6-8], which raises the possibility of them being used therapeutically

for regenerating and repairing damaged tissues due to their ability to self-replicate and potentially

form any cell in the body. However ethical and safety concerns regarding the use of human embryos,

and the potential for teratoma formation have limited their application. Ensuing research found that somatic cell nuclear transfer (SCNT), a technique whereby somatic cell nuclei are injected into enucleated oocytes, could generate pluripotent ESC-like cells from somatic cells[9, 10]. The importance of this discovery was that somatic cells not only retained the ability the form other cells but could also be reprogrammed back to a pluripotent state. However, the utility of SNCT for therapeutic purposes may be limited due to opposition to human cloning, the labour intensive nature of the derivation of ESC-like cells and, up to this point, the human ESC-like cells created being triploid[9].

The recent discovery that somatic cells can be reprogrammed into pluripotent cells with the addition of four transcription factors has ignited an interest in using these cells for therapeutic purposes (Fig. 2). Yamanka and Takahashi were able to create ESC-like cells from mouse embryonic fibroblasts (MEFs) using retroviruses to deliver a cocktail of candidate transcription factors[11], which had previously been shown to either be highly expressed in ESCs or important for their derivation or maintenance[12-17]. Systematic screening of the 24 candidate genes used identified that four factors; Oct4, Sox2, Krüppel like factor 4 (Klf4) and c-Myc, were required to reprogram a somatic cell to an ESC-like state. The cells that had been reprogrammed to an ESC-like state were termed induced pluripotent stem cells (iPSCs). iPSCs were characterised and shown to express embryonic genes important in maintaining pluripotency and at a similar level to ESCs. The iPSCs were demonstrated, by transplantation into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, to have the ability to form cells from all three germ layers. The initial paper by Takahashi and Yamanaka was unable to show iPSCs could contribute to chimera formation, however subsequent studies have confirmed pluripotency by demonstrating the generation of chimeras from iPSCs, moreover they have shown iPSCs could contribute to the germ line[18, 19].
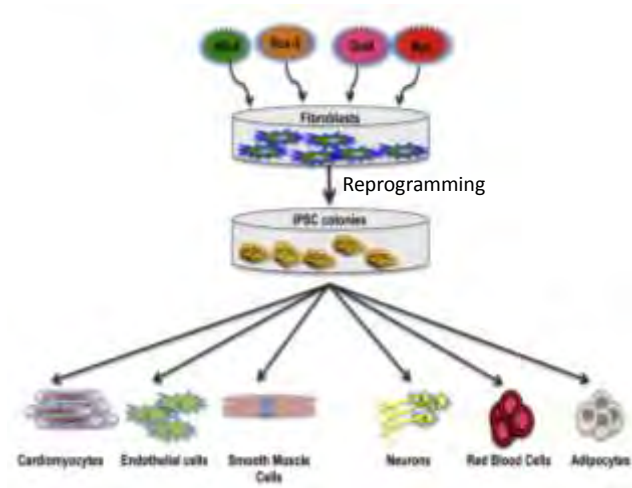
Figure 2: Reprogramming of somatic cells to iPSCs. Somatic cells (fibroblasts) can be reprogrammed using the four factors, Oct4, Sox2, Klf4 and c-Myc to generate iPSCs. iPSCs have the potential to differentiate into any somatic cell. Figure taken from Nsair *et al.,* (2011)[20].

Subsequent studies have derived iPSCs from different tissues, species, and using different methods. Initial iPSC derivation utilised genetically modified retroviruses[11, 21] and lentiviruses[22-24] to deliver the reprogramming factors, however concerns arose regarding the use of viruses if iPSCs were to be used therapeutically. Reprogramming via retroviruses and lentiviruses requires viral genes being integrated into the genome which could potentially cause aberrant mutations[25, 26]; the viral genes have also been shown to be persistently expressed albeit at low levels[27].

To overcome these obstacles scientists have modified different viruses to deliver the reprogramming factors. Adenoviruses and Sendai viruses have been used to deliver the reprogramming factors due to their advantage of not integrating into the genome. However they also have disadvantages such as a lower efficiency of reprogramming in the case of adenoviral vectors (0.001% opposed to up to 1%) and difficulty in removing all the Sendai viruses from the cells[27]. Others have developed excisable vectors, such as loxP-flanked lentiviral cassettes[28, 29] or transposons[30-32], which allow for the inducing genes to be removed after reprogramming, the disadvantage being the labour intensive screening required to verify successful removal of the vector. More recently, protocols have been developed to derive iPSCs using either proteins[33, 34], modified mRNA[35] or microRNAs[36, 37]. These

have the benefits of not having genomic integration and thus being therapeutically more relevant, however have a lower efficiency of generating iPSCs (efficiency of approximately 0.001-0.1%).

1.1 Reprogramming: The Factors and Mechanism

Originally, iPSCs were generated using the four factors Oct4, Sox2, Klf4 and c-Myc (OSKM)[11]. Developments in the reprogramming field have shown that not all of the OSKM factors are required for reprogramming, with all four able to be replaced to some extent. c-Myc was initially removed due to being an oncogene and found not to be critical for the generation of iPSCs, but rather increased the efficiency of derivation[38, 39]. Sox2 and Klf4 can be replaced with other factors or excluded altogether, however the efficiency of iPSC derivation is reduced[40-42]. The cell type used to derive iPSCs is important as factors can be removed if expressed endogenously, for example neural stem cells only require the transduction of Oct4 to be reprogrammed as they already express Sox2[41]. Oct4 has been described as the key factor that could not be replaced when reprogramming cells, however it was discovered that Nr5a2 can replace Oct4 [43]. Nanog, lin28, as well as other factors from the Sox, Klf and Myc family have also been used to reprogram somatic cells to iPSCs[38, 44, 45].

Reprogramming involves the downregulation and cessation of the somatic cell program and an induction and upregulation of the embryonic or pluripotency program. Somatic cell specific genes such as Thy-1 are downregulated and silenced and ESC genes such as stage specific embryonic antigen-1 (SSEA-1), alkaline phosphatase, Oct4 and nanog are upregulated[24, 46]. The OSKM reprogramming factors have been demonstrated to play different roles in reverting somatic cells back to an ESC-like state[47]. c-Myc was found to confer the most ESC-like expression changes required for reprogramming, even though it is not essential for reprogramming. c-Myc is able to repress the expression of somatic specific genes, which may be brought about by c-Myc binding to Mix-1 and repressing its activity. The c-Myc/Miz-1 complex has also been shown to recruit methyltransferases and deacetylases to gene promoters, which as explained later is important in

6

reprogramming, as well as repressing genes important in differentiation, most notable being the Hox genes[48]. OSK plays a greater role in activating ESC specific genes through binding to gene promoters. OSK have been shown in ESCs to co-occupy ESC specific genes, including their own promoters, and the same is believed to be true in iPSCs and reprogramming[49-52]. Oct4, a product of the POU5f1 (POU domain, class 5, transcription factor 1) gene, is essential to establish and maintain pluripotency [15]. The level of Oct4 needs to be tightly regulated to maintain pluripotency as low levels, excessive levels or no Oct4 can cause cells to lose their potency and differentiate[53]. Nanog is also important for maintaining the pluripotency of cells, however was found to be redundant in reprogramming cells to a pluripotent state[11].

Reprogramming also involves global epigenetic remodelling, which contributes to the downregulation of somatic genes and upregulation of ESC genes[5]. Histone methylation changes in ESC specific genes have been demonstrated during reprogramming, with histone H3 K27 trimethylation (lysine 27; a repressive mark) being replaced with the activating histone H3 K4 trimethylation mark(Fig. 1)[47]. iPSC generation can be promoted by using inhibitors of epigenetic enzymes such as valproic acid, a histone deacetylase inhibitor[54, 55]. The histone modifications are important in reprogramming as is the methylation status of promoters, with ESC gene promoters becoming demethylated allowing for transcription factors to bind and induce expression[21]. iPSCs and ESCs have similar global epigenetic patterning, however iPSCs retain epigenetic marks associated with the cells they were derived from[56]. Furthermore, this retention of somatic cell epigenetic marks has been shown to have an effect of iPSC differentiation, with a bias found towards differentiating along the lineage of derivation, however this can be mitigated by passaging iPSCs in vitro[57].

1.2 Derivation of iPSCs from different species

Since the original derivation of iPSC from MEFs, iPSCs have been generated from numerous different species including humans[21] ; non-human primates such as the rhesus macaque (*Macaca*

mulatta)[58]; domestic animals and livestock including pigs[59, 60] and sheep[61]; and endangered animals with two examples being the drill (*Mandrillus leucophaeus*) and the northern white rhinoceros (*Ceratotherium simum cottoni*)[62]. Derivation of iPSCs from different species has required the modification and development of culture and induction techniques to support iPSC formation. Human iPSCs (hiPSCs) were generated from adult fibroblasts using the same factors for mouse iPSC (miPSC) derivation, however required basic fibroblast growth factor (bFGF) rather than leukaemia inhibitory factor (LIF) to be supplemented to the culture media[21]. Derivation of hiPSCs also utilises media which has been developed to support the growth of ESCs, and therefore iPSCs, over the growth of somatic cells. iPSCs from other species requires the culturing conditions to be optimised and modified to support their growth. Due to the high conservation of the reprogramming factors the majority of iPSCs are reprogrammed using either the mouse or human genes.

1.3 Derivation of rat iPSCs

Derivation of rat iPSCs (riPSCs) has been a goal for research groups ever since miPSCs were generated. This is due to the rat being a valuable model organism for the study of human diseases like diabetes and hypertension as well as being important for the testing of regenerative therapies[63]. The derivation of riPSCs thus allows for easier production of genetically modified rats as well as being able to test the therapeutic suitability of iPSCs in rat models. riPSCs have been generated by only a few groups and have been generated from rat embryonic fibroblasts (REFs)[19, 64], adult rat fibroblasts[63], the rat cell line WB-F344[65] and rat neural precursor cells[66], however they have never been generated from dental pulp stem cells (DPSCs) or adipose derived stem cells (ADSCs).

1.4 Culture media development

The culture media iPSCs are cultured and reprogrammed in has a large impact on the success and efficiency of derivation. Small molecules have been used to improve the efficiency of reprogramming

which include inhibitors of differentiation pathways such as the glycogen synthase kinase 3β (GSK3β) inhibitor CHIR99021, the MAPK/ERK (MEK) inhibitor PD0325901 or TGFβ inhibitors; and small molecules that affect epigenetic marks such as valproic acid, 5' azacytidine, BIX-01294[67]. Others found the addition of ascorbic acid (vitamin C)[55] or lithium (in the form of lithium chloride)[68] facilitated reprogramming and therefore increased the efficiency of iPSC derivation. Ascorbic acid was demonstrated to alleviate cell senescence by downregulating p53[55] as well as promoting the transition of pre-iPSCs – cells which have ESC-like morphology but fail to completely activate pluripotency genes and cannot form chimeras[69] – into fully fledged iPSCs. Lithium prevents differentiation by inhibiting GSK3β, however culturing with CHIR99021 gives further improvements in efficiency suggesting lithium has other hitherto unknown roles in reprogramming[68]. Systematic screening of chemical compounds has been performed and used to develop media that can improve the efficiency and quality of iPSCs derived[70].

1.5 Reprogramming of adipose and dental pulp derived stem cells

Dental pulp and adipose tissue encompass a population of mesenchymal stem cells, which have increased the interest in the tissues. Adipose and dental pulp are easily available due to being in relatively high abundance owing to the loss of deciduous teeth, routine extraction of 3rd molars and the increasing application , especially in developed countries, of lipoaspiration (liposuction). Furthermore, adipose derived and dental pulp derived stem cells (ADSCs and DPSCs respectively) have been shown to be easier to reprogram with a greater efficiency of iPSC generation when using comparable transduction and culturing conditions[71, 72]. This is thought to be due to stem cells being more plastic than fully differentiated cells, therefore less reprogramming is required to revert a stem cell back to a pluripotent cell. Moreover, stem cells have also been shown to express genes required for maintaining or inducing pluripotency, for example, DPSCs have been revealed to express Oct4, Sox2 and c-Myc.

1.6 Aim of project

The aim of the project was to generate stable rat iPSCs from postnatal dental pulp and adipose cells using the Cre-lox lentiviral delivery system (Fig. 3) and to characterise them. riPSCs have not been generated from dental pulp or adipose tissue before and the STEMCCA ("stem cell cassette") has not previously been utilised to generate riPSCs.

2. Methods

2.1 Cell Culture

2.1.1 Adipose and Dental Pulp Stem Cells

ADSCs and DPSCs were harvested from the same six week old Wistar-Hann rats. ADSCs were isolated by digesting the inguinal fat pads in collagenase (8 mg collagenase (C1764, Sigma), 160 mg bovine serum albumin (A2153-100G, Sigma) and 8 ml phosphate buffered saline (PBS)) for 30 mins at 37 °C. α-MEM (LM-E1148/500, Biosera) with 20% foetal calf serum (FCS; A2153, Sigma) was added to the mixture to stop the reaction and then passed through a cell sieve. The mixture was then spun at 180 rcf for 5 mins; the pellet resuspended in 1 ml α-MEM with 20% FCS and then filtered through a 70 μm cell strainer. ADSC were cultured in T75 flasks in isolation media; α-MEM (Biosera), with 20% FCS and 1% penicillin/streptomycin (15070, Gibco). DPSCs were isolated from the dental pulp of the incisors of two rats (8 teeth in total). The dental pulp was removed using tweezers and then minced using a scalpel, wherein the pulp was digested in 4 ml 1x trypsin-EDTA (25200, Gibco) for 30 mins at 37 °C, after which the trypsin was quenched by adding 4 ml of isolation media. The digested mixture was then passed through a cell filter, pelleted by centrifugation at 180 rcf and the pellet resuspended in isolation media then cultured in a T25 flask. ADSC and DPSC were cultured in a humidified incubator at 37 °C in 5% CO_2 and used at passage 2 or 3.

2.1.2 Rat Embryonic Fibroblasts (REFs)

REFs were prepared by digesting E14.5 embryos in trypsin after removing the foetal heart, liver and cerebellum[11, 18]. Initially, the embryo was digested in 3 ml 1x trypsin-EDTA (15400-054, Gibco) for 30 mins at 37 °C with regular agitation. Subsequently, the embryos were digested in 6 successive rounds of 15 mins digestions at 37 °C with 1.5 ml of digested tissue removed and 1.5 ml 1x trypsin was replaced each iteration. Trypsin was inactivated using REF media (DMEM (41965, Gibco)

supplemented with 10% FCS (Sigma), 1% penicillin and streptomycin (Gibco), 2 mM L-glutamine (25030, Gibco) and 0.1 mM β-mecaptoethanol (M7522, Sigma)). The digested mixture was centrifuged at 218 rcf and the pellet and filamentous material resuspended in REF media. REFs were cultured in T75 flasks at 37 °C in a humidified incubator. REFs were used at passage 1.

2.2 Viral Titration

293T-HEK cells[73]– human embryonic kidney cell line that is highly transfectable - were seeded at a density of 1×10^6 per well in five wells in a six well plate and cultured in T-HEK medium (DMEM, 10 % Foetal bovine serum, 1 % penicillin/streptomycin and 2 mM L-glutamine). 293T-HEK cells were cultured in 5% CO_2 at 37 °C for 24 hours before being gently washed with PBS. Cells from one well were removed by digesting with 1x trypsin-EDTA and counted using a haemocytometer to determine the number of cells present for the titration. The remaining four wells were used for the viral titration and to each well 1 ml of media with polybrene (107689, Sigma; increases the infectability of cells). The ZsGreen virus, a virus genetically modified to produce green fluorescent protein (GFP), was used for the titration as the STEMCCA virus does not contain a fluorescent marker that could be used to determine the titres. The concentrated ZsGreen virus was diluted in media and polybrene to a tenth and a hundredth of the concentration. 1 µl of the concentrated and diluted virus was added individually to three wells, the fourth well was used as an uninfected control. The infection was stopped by replacing the media devoid of polybrene 20 hours after the initial infection. The T-HEK media was replaced every day. The cells were cultured for a total of 72 hours post-infection before being washed with PBS and removed using 1x tryspin-EDTA. Flow cytometric analysis was used to determine whether the cells had been infected, which was used to calculate the titres.

The titres were calculated using the formula:

Number of cells at time of infection	x	% of cells GFP positive	=	Number of virions per volume of virus added to the well

The average virions per volume was then calculated for the ZsGreen virus and multiplied by the number of virions required to infect 1 x 10^5 with a multiplicity of infection (MOI) of 1 (1×10^5 cells with a MOI of 1 equals 1×10^5 virions deposited). The MOI is the number of virions required to infect a set proportion of cells. The STEMCCA virus has a lower efficiency of infecting cells that the ZsGreen virus, therefore a conversion factor of x20 was used to calculate the volume of STEMCCA virus that is required to infect 1x105 cells with a MOI of 1 from the ZsGreen results.

2.3 Reprogramming

The project utilised a single lentivirus stem cell cassette delivery system, which had been developed to deliver all four reprogramming factors (OSKM) in a single integration. Efficient reprogramming using the STEMCCA ("stem cell cassette") virus is achieved due to the design of the STEMCCA cassette which produces OSKM factors with a stoichiometry (ratio to one another) that favours reprogramming. The reprogramming cassette becomes flanked by loxP sites during integration which allows for Cre recombinase mediated removal of the cassette following iPSC derivation.
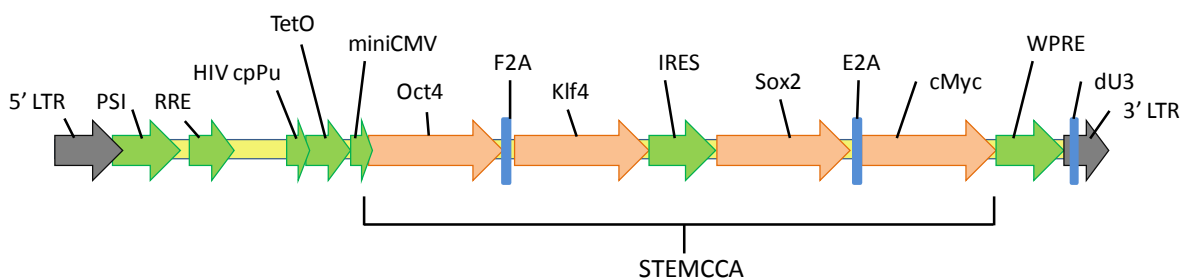
Figure 3: Schematic diagram showing the inducible STEMCCA developed by Sommer *et al.,* (2009)[29]. The STEMCCA virus used to transduce the cells in the project did not contain the inducible TetO element. The Oct4 and KLF4 and; Sox2 and c-Myc genes are separated by F2A and E2A sequences respectively which encode self-cleaving peptides. The 2A peptides allow for post-translational cleavage of the two proteins, which improves the levels of protein produced from the cassette. Abbreviations: LTR, long terminal repeat; PSI, packaging signal; RRE, rev responsive element; HIV, Human immunodeficiency virus; cpPU, central polypuryne tract; miniCMV, mini-cytomegalovirus; IRES, internal ribosome entry site; WPRE, woodchuck hepatitis virus post-translational regulatory element; dU3, deleted U3.

All cells prior to reprogramming were checked for mycoplasma using the MycoAlert kit (LT07, Lonza), as mycoplasma can influence stem cell behaviour and viability[74]. ADSC, DPSC and REFs were seeded on 0.1% gelatinised plates at a density of 1×10^5 cells per well in a six well plate the day before the intended infection and grown in their respective media (Fig. 4, A). On the day of infection the media was removed, the cells were washed with PBS and the medium replaced with the addition of polybrene; the STEMCCA virus was added to the cells at a MOI of 1. The following day the medium was removed to stop the infection and replaced with iPSC media; DMEM supplemented with 15% Hyclone ESC qualified FBS (03147, Thermo Scientific), 1 % penicillin/streptomycin (5000 units ml^{-1}), 2 mM L-glutamine, 0.1 mM β-mecaptoethanol and 0.01 % mouse LIF (1×10^6 units ml^{-1}; ESG1106, Millipore). The iPSC media was modified for some culture experiments with the addition of other compounds which include 50 μg ml^{-1} ascorbic acid (A4544, Sigma), 10 mM lithium chloride (L9650, Sigma) and the inhibitors CHIR-99021 (S1263-SEL, Stratech Scientific) at 3 μM and PD-0325901(S1036-SEL, Stratech Scientific) at 0.5 μM. The inhibitors when used together are known as 2i. Cells were cultured in iPSC medium until iPSC colonies were established.

Another method of iPSC derivation was performed which utilised a feeder layer of gamma irradiated MEFs and ESC media(Fig. 4, B). ADSC and DPSCs were infected with the STEMCCA virus and grown in iPSCs media as previously described. Five days post-transduction the infected ADSCs and DPSCs were washed with PBS and removed from the culture dish using 1x trypsin-EDTA. Cells were then seeded onto either the gamma-irradiated MEF feeder layer which had been seeded onto gelatinised 6 well plates the day before, or onto gelatinised 6 well plates devoid of irradiated MEFs. The medium at this point was changed to ESC media which consists of 410 ml knockout DMEM (10829, Gibco), 5 ml L-glutamine, 5 ml penicillin/streptomycin, 5 ml non-essential amino acids (M7145, Sigma), 75 ml knockout serum replacement (10828-028, Invitrogen), 50 μl LIF and 3.5 μl β-mecaptoethanol, as ESC media should support the growth of iPSCs.
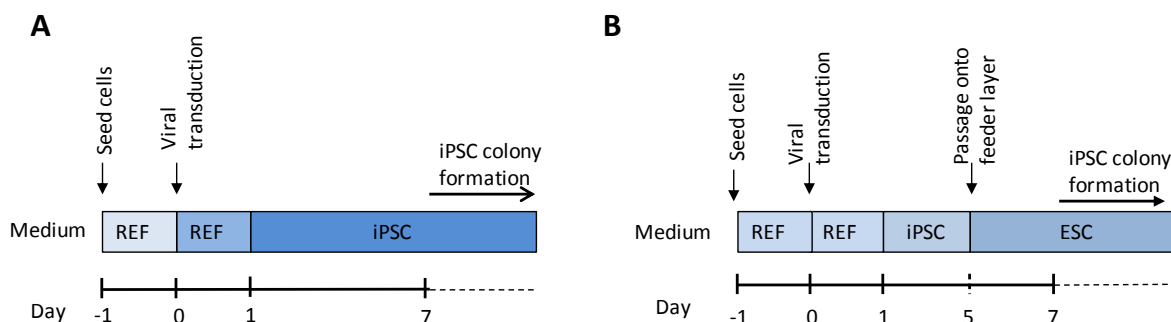
Figure 4: Schematic diagram showing the transduction time schedule and the media used. **A,** Example of method to derive iPSCs without re-seeding on feeder layer; **B,** method used to generate iPSCs using ESC media and an irradiated MEF feeder layer[21, 66]. Medium for initial culture was that suited to the cells being cultured, with REF media being shown as an example in the schematic diagram.

2.4 Alkaline Phosphatase staining

Media was removed from the presumptive iPSCs, rinsed with cold PBS and then fixed in cold 10% neutral formalin buffer (16g Na_2NPO_4, 4g $Na_2HPO_4.H_2O$, 1 L distilled water and 16 % formaldehyde methanol free) for 15 mins. The neutral formalin buffer was removed and the cells washed with cold distilled water and remained in cold distilled water for 15 mins. The distilled water was then removed and the substrate solution added and left at room temperature for 45 mins. The substrate solution consisted of 0.0025 g Naphtol AS MX-PO_4 (N5000, Sigma) dissolved in 100 µl N,N-dimethylformamide (D4551, Sigma) mixed with 12.5 ml 0.2M pH 8.3 Tris-HCl and 12.5 ml distilled water; fast red violet LB salt (F1625, Sigma) was then added to the mixture then filtered using Whatman's No. 1 filter paper. The substrate solution was then removed and rinsed with distilled water. Red staining indicates the presence of alkaline phosphatase; a marker for iPSCs.

2.5 DNA extraction

DNA was extracted from the excess cells from the feeder layer transfection protocol using the TRIzol extraction method. Briefly, cells were pelleted and then homogenised in 0.5 ml TRIzol reagent (15596, Gibco). 150 µl of 100% ethanol was then added and the tubes inverted to mix the reagents.

The samples were incubated at room temperature for 2-3 mins and then centrifuged at 2000 x g for 5 mins at 4 °C to pellet the DNA. The pellet was then washed with 0.5 ml sodium citrate/ethanol solution (0.1 M sodium citrate in 10% ethanol, pH 8.5) and incubated for 30 mins at room temperature with occasional inversion. The mixture was then centrifuged at 2000 x g for 5 mins at 4 °C and the supernatant then removed. This was repeated and then 1 ml of 75% ethanol added and the sample incubated for 20 mins at room temperature with the occasional inversion. The sample was then centrifuged at 2000 x g for 5 mins at 4 °C and the supernatant discarded, after which the DNA pellet was left to air dry for 5 to 10 mins. The DNA was resuspended in 10 μl of 8 mM NaOH and quantified using a Nanodrop (Thermo Scientific).

2.6 Polymerase Chain Reaction (PCR)

PCR was used to check that the STEMCCA cassette had been inserted into the genome. 12 ng of DNA was added to 22.7 μl of PCR Reddymix plus (AB-0608/LD, Thermo Scientific) and the volume made up to 25 μl using PCR water. The primers used were; miPSt-CreEx-F, GAAACTCTGGTGCATAAATCGATAG and miPSC-CreEx-R, GGAGGCGGCCCAAAGGGAGATCCG. The conditions of the PCR reaction were 95 °C for 3 mins, 94 °C for 30 secs, 60 °C for 30 secs, 72 °C for 1.5 mins, 40 cycles and a final heat of 72 °C for 5 mins. The PCR product was analysed by running on a 1.5 % agarose gel containing ethidium bromide for 2 hours at 100 V and then visualised using a Gel Doc XR+ (Bio Rad).

3. Results

3.1 Viral titration

A viral titration was initially performed to determine the amount of STEMCCA virus that would be used for the transduction. 293T-HEK cells were infected with the ZsGreen virus as a surrogate for the STEMCCA virus due to the ZsGreen virus expressing GFP and therefore allowing for cells infected with the virus to be determined using flow cytometry. 293T-HEK cells were infected with different concentrations of the ZsGreen virus (1 µl of concentrated virus (1 C) and a tenth (0.1 C) and hundredth (0.01 C) of this concentration), as well as control 293T-HEK cells that were not infected with the virus. Cells were analysed by forward scatter (FS; corresponds to cell volume) and side scatter (SS; indicates complexity or granulation of a cell) to select for alive cells (Fig. 5, A). These cells were gated and analysed for the expression of GFP (shown by FITC log on y-axis, Fig. 5, B). The no virus control was used to determine the gating that would be used to signify whether a cell had been infected with the ZsGreen virus (Fig. 5, Bi). The analysis showed the 90.07 % of cells were infected when 1 C of ZsGreen virus was used, 15.27 % with 0.1 C and 4.93 % with 0.01 C. These percentages of infection were then used to determine the number of virions per volume of virus added with the average being calculated to be 5.4×10^9 virions ml^{-1}. It was then calculated that to infect 1×10^5 cells with a MOI of 1, 0.0185 µl of concentrated ZsGreen virus was required. This was converted to the volume of STEMCCA virus required by multiplying by 20, giving 0.371 µl.
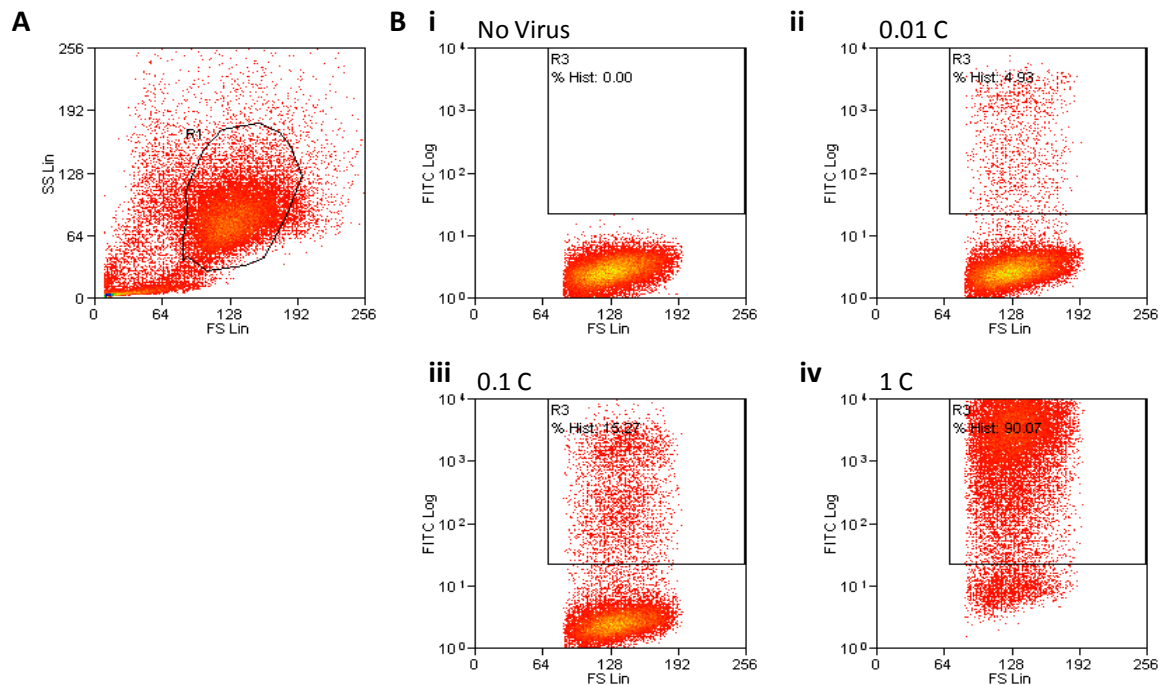
Figure 5: Flow cytometric analysis of 293T-HEK cells infected with ZsGreen virus to determine the quantity of STEMCCA virus required to transduce cells. A; Density plot showing an example of the gating (R1) used to select cells for further analysis. B; Plots showing the gating (R3) used to determine the percentage of cells infected with the ZsGreen virus. Plots shown for 293T cells infected with no virus (i), 0.01C (ii), 0.1C (iii) and 1C (iv) virus. The ZsGreen virus fluoresces green when stimulated with a 488 nm laser and the level of fluorescence is measured by FITC log (y-axis). Each dot on the density plot represents a cell; the colour denotes the number of cells in an area (red, low density; yellow, high density).

3.2 Initial transduction using iPSC media

ADSCs, DPSCs and MEFs were infected with the STEMCCA virus at a MOI of 1 and then cultured in iPSC media. MEFs which were used as a positive control formed iPSC colonies (Fig. 6), which can be seen by their morphology; "dome shaped and refractile colonies"[27], by day 6 (Fig. 6, E) and the colonies remained to day 10 (Fig. 6, F). The ADSCs and DPSCs were observed to form colonies on day 10 (Figs. 7, F and 8, C and F), however did not have the morphology of iPSC colonies and in the case of the DPSCs similar colonies were observed on the control culture which had not been infected with

the STEMCCA virus (Fig. 8, C). The colonies observed on day 10 remained until day 20 (Figs. 7 and 9,

H and K); in this time no colonies with an iPSC morphology were formed. Verification, by staining for

alkaline phosphatase confirmed that the colonies formed were not iPSCs (Figs. 7 and 8, H and K). The

DPSCs stained moderately for the expression of alkaline phosphatase, regardless of whether the cells

had been transduced or not (Fig. 8, H, I, K, and L). The ADSCs did not stain for alkaline phosphatase,

however the majority of cells detached from the culture dish prior to fixing and staining. Transduced

ADSCs and DPSCs were also cultured in DMEM based iPSC media with similar results observed (Figs.

S1 and S2)
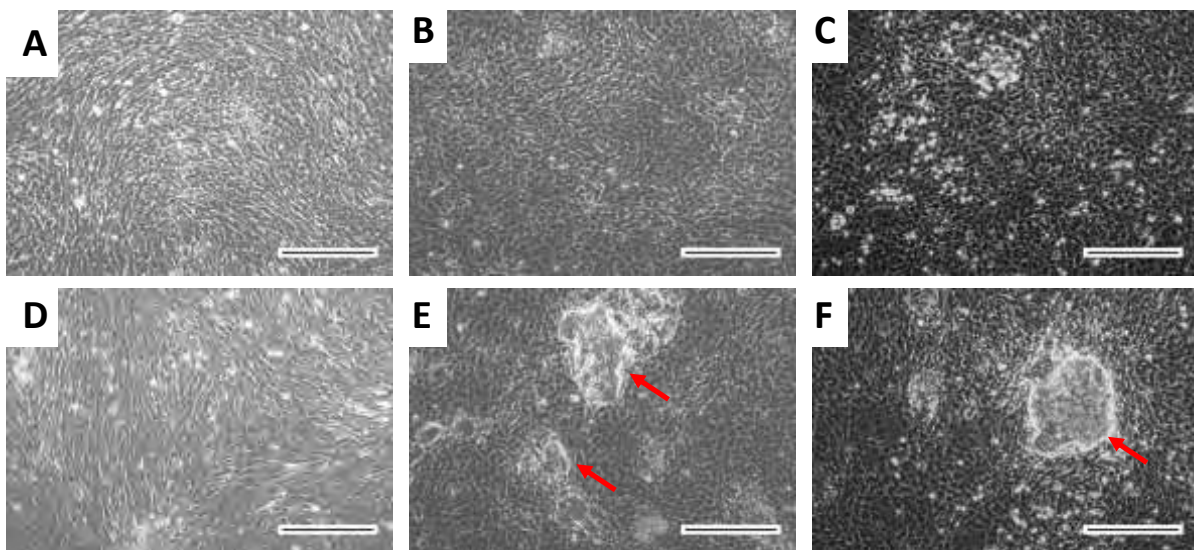
Figure 6: MEFs transduced with the STEMMCCA virus and cultured in iPSC medium. **A-C**, Control
MEFs which were not transfected with the STEMCCA virus; **D-F**, MEFs infected with the STEMCCA
virus. Images show transduced cells at days 4 (A and D), 6 (B and E), and 10 (C and F). Arrows indicate
iPSC colony formation and scale bars represent 100 μm.
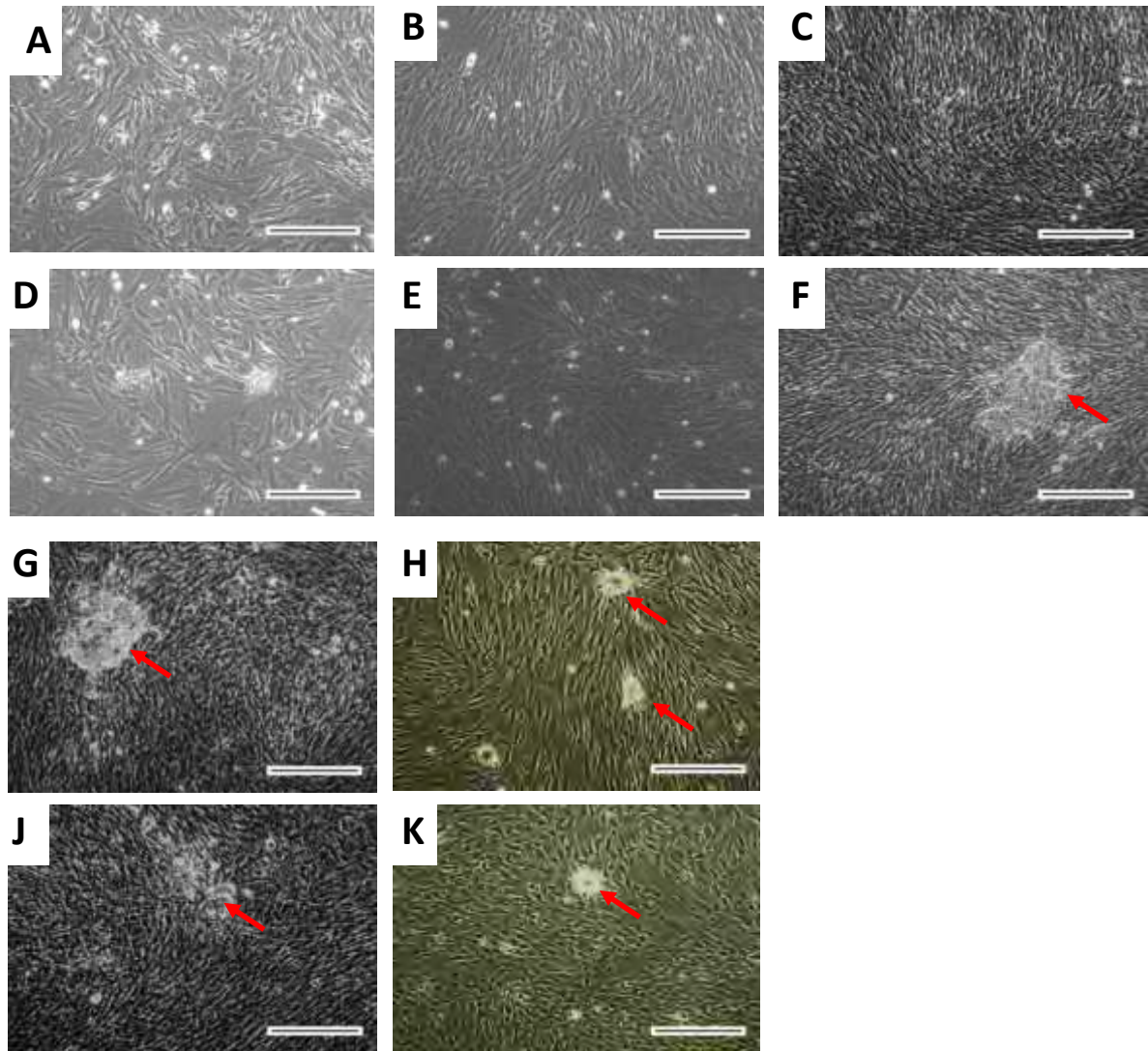
Figure 7: ADSCs transduced with the STEMMCCA virus and cultured in iPSC medium containing α-MEM. **A-C, G and H**, Control ADSCs that were not transfected with the STEMCCA virus; **D-F, J and K**, ADSCs when infected with the STEMCCA virus. Images show transduced cells at days 4 (A and D), 6 (B and E), 10 (C and F) and 18 (G and J). ADSCs were stained for alkaline phosphatase on day 20 (H and K). Scale bars represent 100 μm. Arrows indicate colonies which formed. Plates stained with alkaline phosphatase are not shown due to cells dissociating from culture dish.
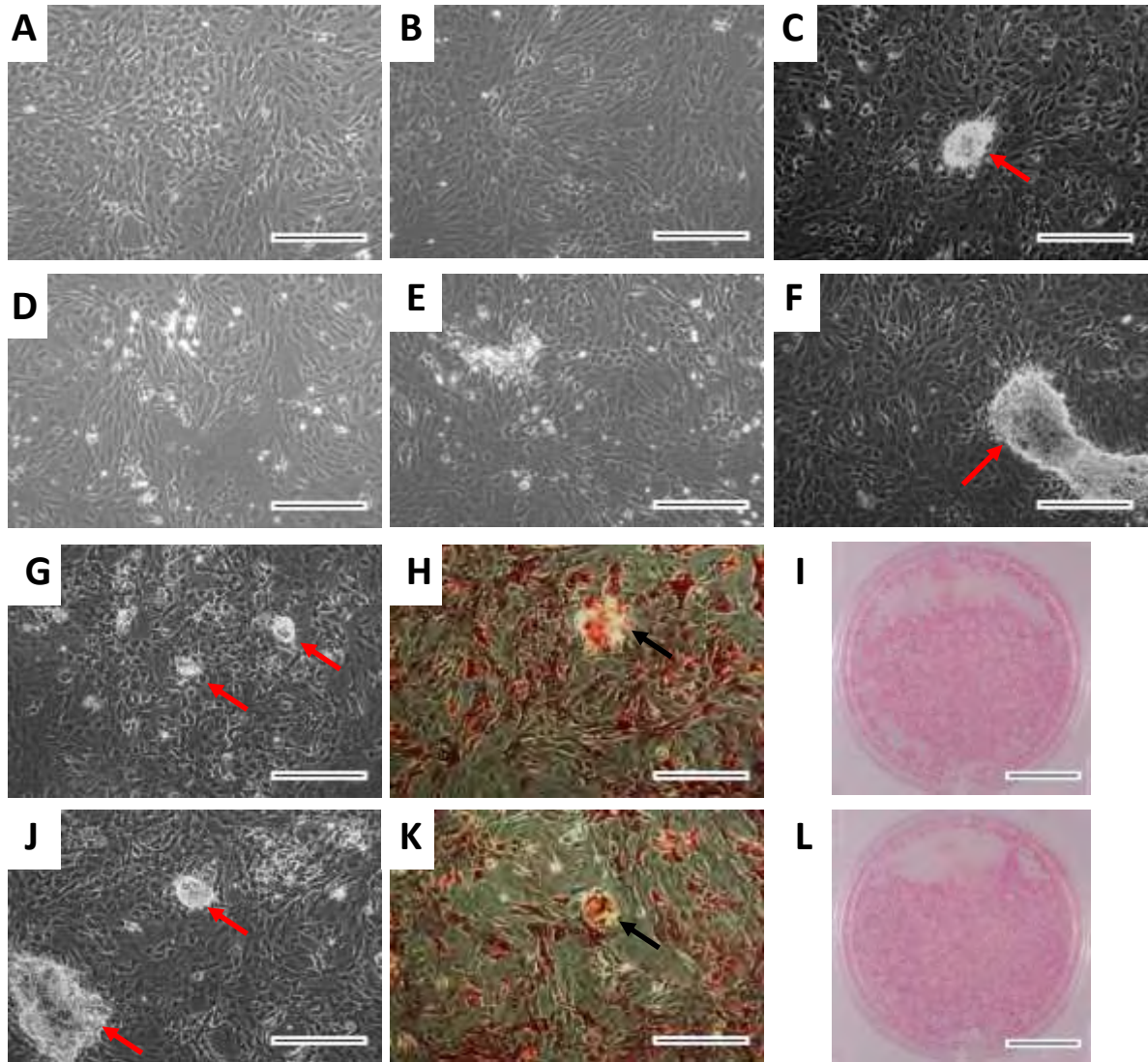
Figure 8: DPSCs transduced with the STEMMCCA virus and cultured in iPSC medium containing α-MEM. **A-C and G-I**, Control DPSCs; **D-F and J-L**, Transduced DPSCs. Images show transduced cells at days 4 (A and D), 6 (B and E), 10 (C and F) and 18 (G and J). DPSCs were stained with alkaline phosphatase on day 20 (H, I, K, and L). Scale bars represent 100 μm for A-H, J and K and 1 cm for I and L. Arrows indicate colony formation.

## 3.3 Feeder layer reprogramming method

Reprogramming using iPSC media failed to generate iPSC colonies from DPSCs and ADSCs therefore an alternative method used to derive iPSCs was performed(See Fig. 4). For the alternative reprogramming method, ADSCs and DPSCs were seeded onto an irradiated MEF feeder layer 5 days post-transduction and the media was changed to ESC media, which promotes the growth of ESCs

and iPSCs rather than somatic cells. ADSCs seeded onto the irradiated MEF feeder layer failed to form any colonies (Fig. 9), whereas the DPSCs initially formed colonies (Fig. 10, D and E) which did not establish into iPSC colonies (Fig. 10, F, I and J). Staining for alkaline phosphatase confirmed that no iPSC colonies had formed (Fig. 9 and 10 G-J), however some cells showed expression of alkaline phosphatase (Fig. 9 and 10, G and I). Cells were also cultured in ESC media but were not seeded onto an irradiated MEF feeder layer (Figs. S3 and S4). These cultures also failed to derive riPSCs from ADSCs and DPSCs.
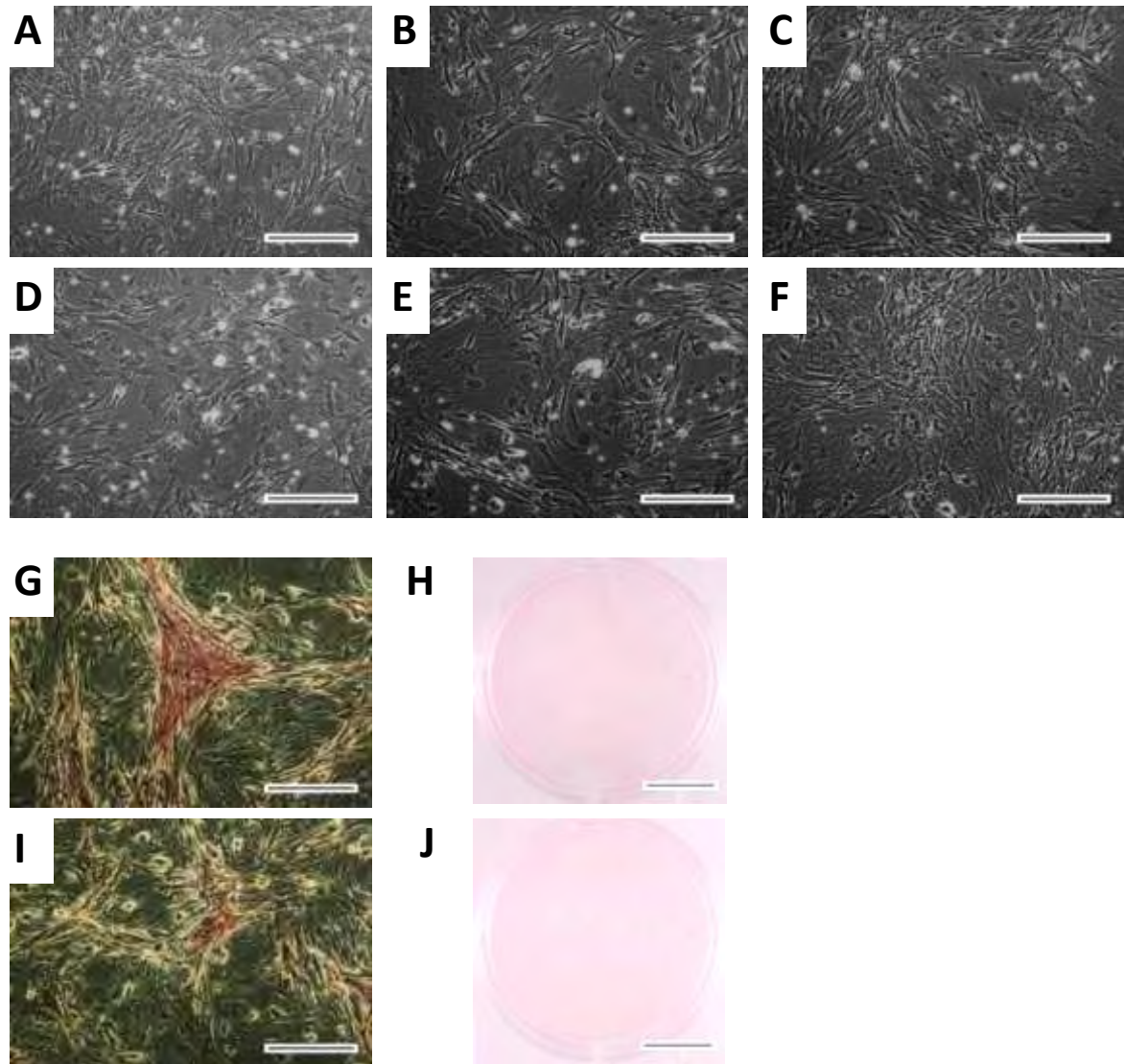
Figure 9: ADSCs infected with the STEMCCA virus and seeded on an irradiated MEF feeder layer. A-C, G and H, Control ADSCs; **D-F, I and J**, ADSCs infected with the STEMCCA virus. ADSCs were seeded on the irradiated MEF feeder layer on the 5th day post-infection. Images are from day 7 (A and D), day 9 (B and E), day 14 (C and F) and the cultures were stained on day 21 for alkaline phosphatase (G-J). Scale bars represent 100 µm (A-G and I) and 1 cm (H and J).
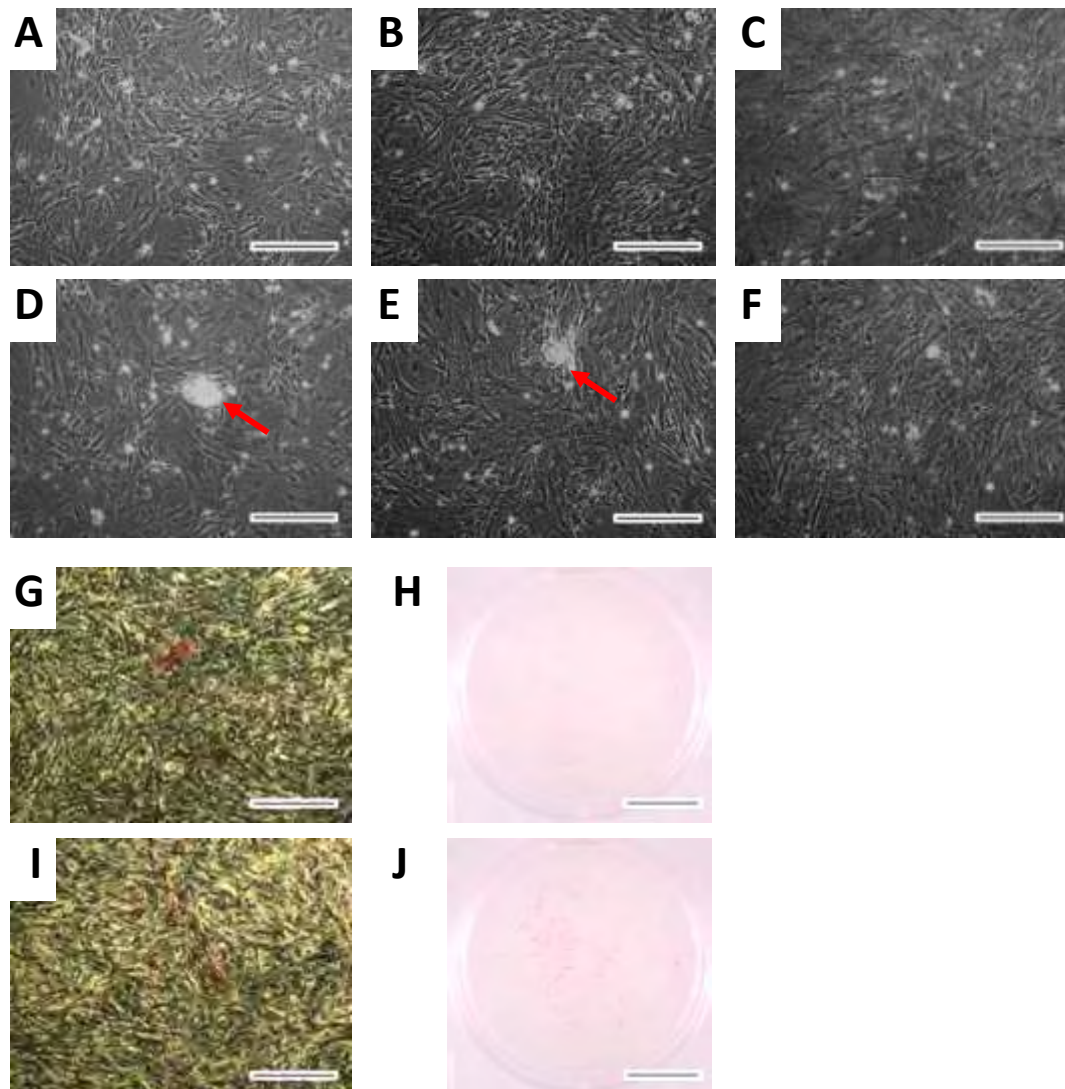
**Figure 10: DPSCs cultured on an irradiated MEF feeder layer post-infection with the STEMCCA virus.**

A-C, G and H, Control DPSCs not infected with the STEMCCA virus; **D-F, I and J**, Transduced DPSCs. Images taken on day 7 (A and D), day 9 (B and E), day 14 (C and F) and the cultures were stained on day 21 for alkaline phosphatase (G-J). Arrows indicate colonies and scale bars represent 100 μm (A-G and I) and 1 cm (H and J)

DNA from spare cells not required for the re-seeding onto the feeder layer was isolated and used to check that the STEMCCA had been inserted into the genome. PCR confirmed the presence of the STEMCCA cassette in the virally infected cells (Fig. 11). The band observed of the virally infected ADSCs was weak, which was probably due to the poor quality and quantity of DNA isolated.
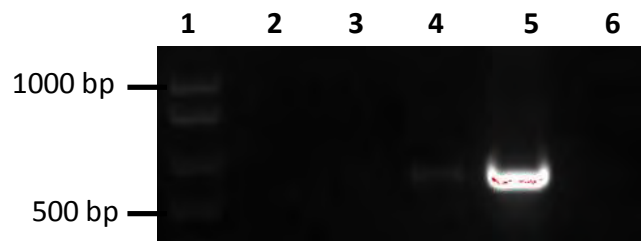
Figure 11: Confirmation by PCR of STEMCCA integration into genome of lentiviral infected cells. A product of approximately 600 bp is observed for the lentivirally infected cells. Lane 1, 1 kb plus ladder; lane 2, ADSCs; lane 3, DPSCs; lane 4, ADSCs infected with STEMCCA virus; lane 5, DPSCs infected with STEMCCA virus; lane 6, PCR control.

3.4 Transduction of REFs

REFs were isolated form E14.5 rat embryos and used in subsequent transduction experiments as they should be easier to reprogram and form iPSCs, which would then give an indication whether the culturing method would potentially work for ADSCs and DPSCs. Initially, REFs were transduced and cultured in the iPSCs medium (Fig. 4, A), which confirmed the finding with ADSCs and DPSCs that iPSCs could not be generated using that medium. REFs demonstrated a propensity to form colonies (Fig. 12, B, E, G, H, J and K); however the colonies did not have an iPSC morphology or stained for alkaline phosphatase (Fig. 12, H and K). Similar to the ADSCs, the majority of the REFs detached from the culture dish between day 18 and day 20.
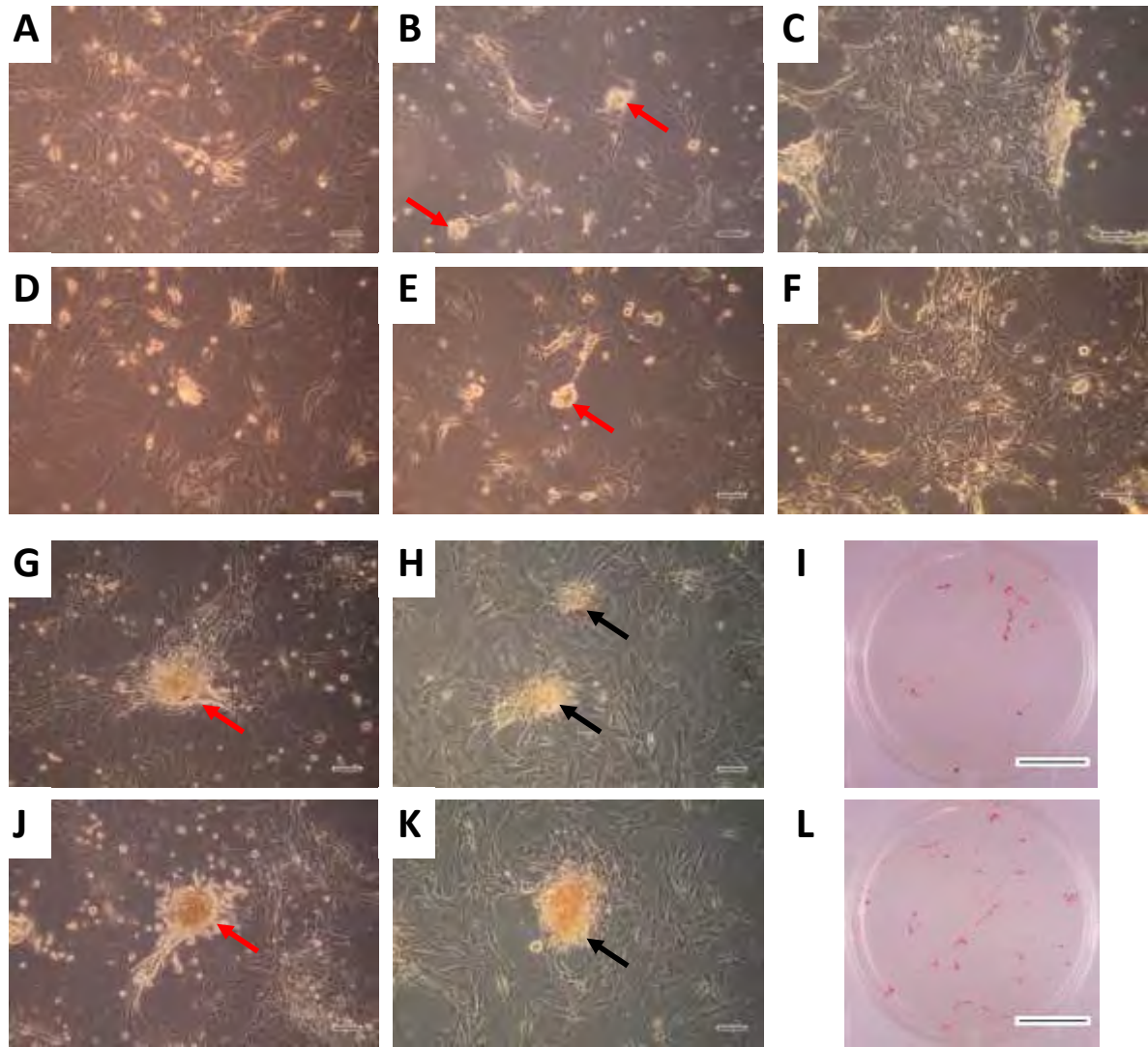
Figure 12: REFs derived from E14.5 embryos transduced with the STEMMCCA virus and cultured in iPSC medium. **A-C and G-I**; Uninfected control REFs; **D-F and J-L**, REFs infected with the STEMCCA virus. Images show transduced cells at days 4 (A and D), 6 (B and E), 10 (C and F) and 18 (G and J). REFs were stained with alkaline phosphatase on day 20 (H, I, K, and L). Scale bars represent 100 μm

3.5 Supplementation of iPSC media with Ascorbic Acid and Lithium chloride

Previous research has shown that supplementation of media with additives can improve the reprogramming efficiency[55, 68, 70], therefore REFs (Figs. 13 and 14), ADSCs (Fig. 15) and DPSCs (Fig. 16) were grown in iPSC media supplemented with just ascorbic acid (Fig. 13) or with ascorbic acid and lithium chloride(Fig. 14-16)[55, 68]. The iPSC media supplemented with ascorbic acid or the

combination of ascorbic acid and lithium chloride was unable to support or promote the derivation

of iPSCs (Figs. 13, 14 and 16, H, I, K and L; 15, I and J), however colonies formed again (Figs. 13, G, H,

J and K; 14, D-H, J and K; 15, D-F).
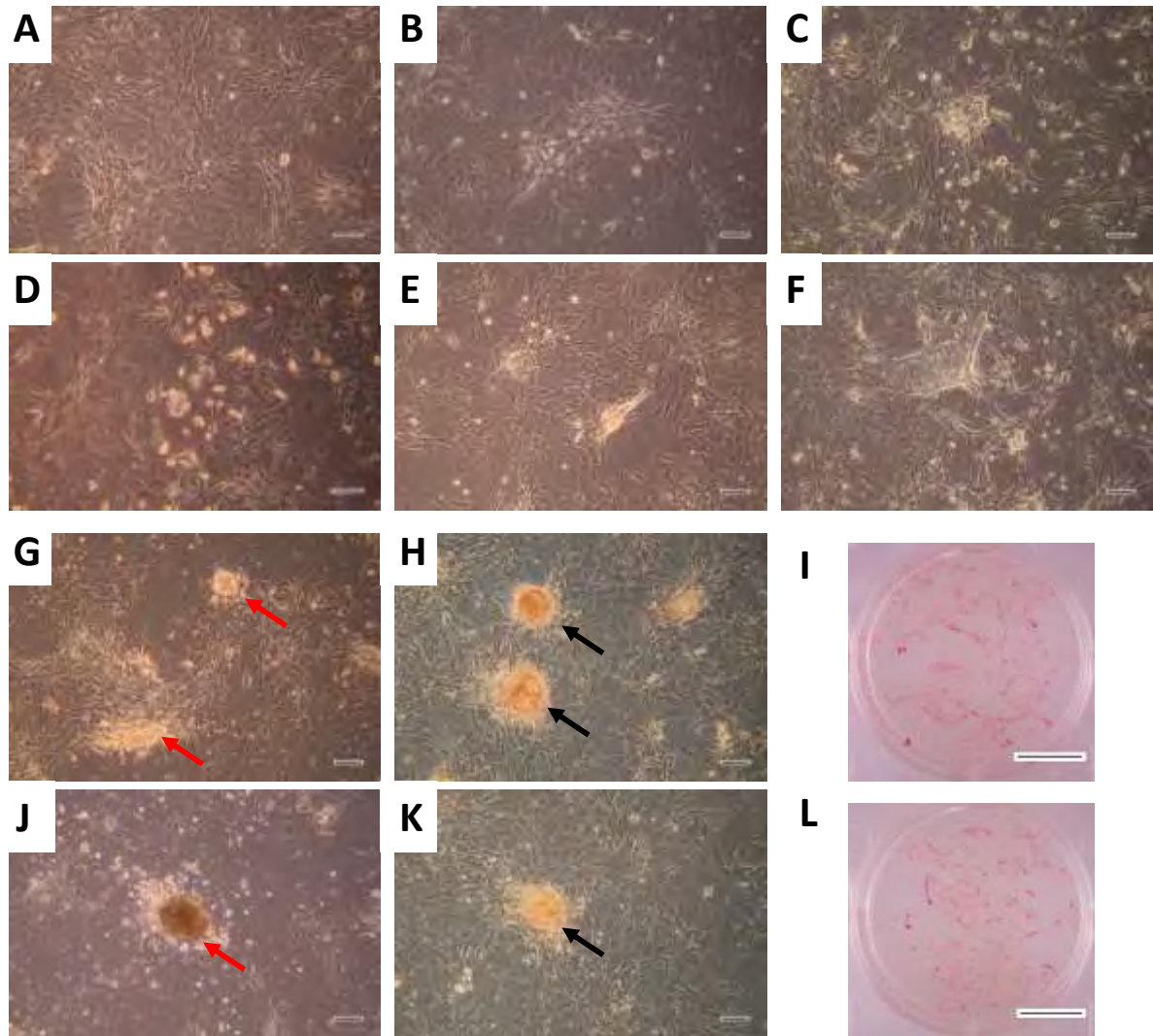
Figure 13: REFs cultured in iPSC medium supplemented with ascorbic acid after viral transduction. **A, B, C, G, H and I**; Control REFs that were not transfected with the STEMCCA virus. **D, E, F, J, K, and L**; REFs when infected with the STEMCCA virus. Images show transduced cells at days 4 (**A and D**), 6 (**B and E**), 10 (C and F) and 18 (G and J). REFs were stained with alkaline phosphatas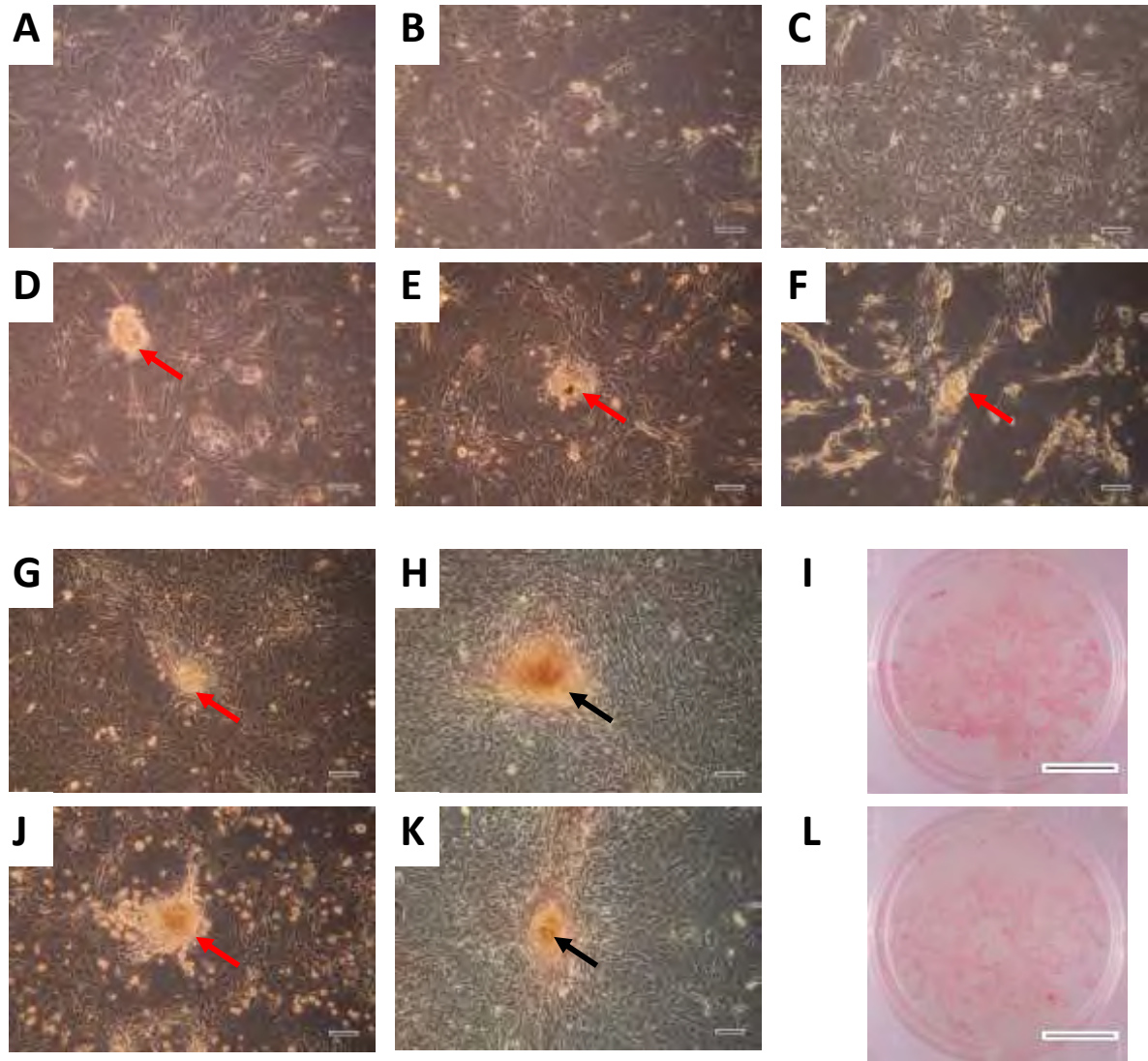e on day 20 (**H, I, K, and L**). Scale bars represent 100 µm for A-H, J and K and 1 cm for I and L. Arrows indicate colony formation.

Figure 14: REFs cultured in iPSC medium supplemented with ascorbic acid and lithium chloride following viral transduction. A-C and G-I; Control REFs**; D-F and J-L,** REFs lentivirally transduced with the STEMCCA and cultured in iPSC medium supplemented with the additives ascorbic acid and lithium chloride. Images show REFs at days 4 (A and D), 6 (B and E), 10 (C and F) and 18 (G and J). REFs were stained with alkaline phosphatase on day 20 (H, I, K, and L). Scale bars represent 100 μm (A-F, G, H J and K) and 1 cm (I and L). Arrows show colonies that have formed.
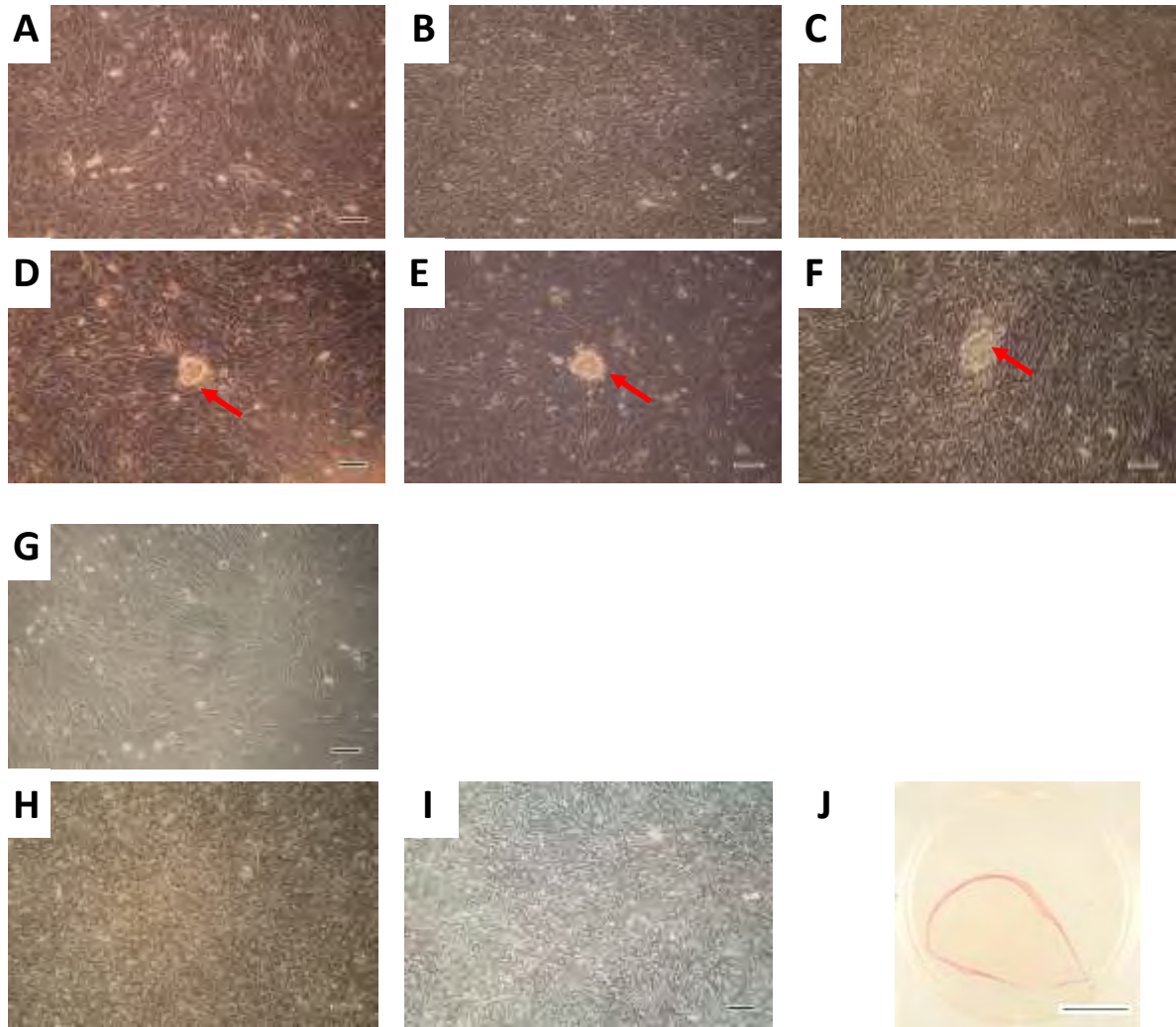
Figure 15: ADSCs cultured in iPSC medium supplemented with ascorbic acid and lithium chloride. Lentivirally infected (**D-F and H-I**) and control ADSCs (**A-C and G**). ADSCs shown from day 5 (A and D), 9 (B and E), 17 (C and F) and 21 (G-J). Cells stained for alkaline phosphatase (I and J). Images of the control ADSCs stained for alkaline phosphatase are not shown due to cells dissociating from culture dish. Arrows indicate colonies and scale bars represent 100 µm (A-I) and 1 cm (J).
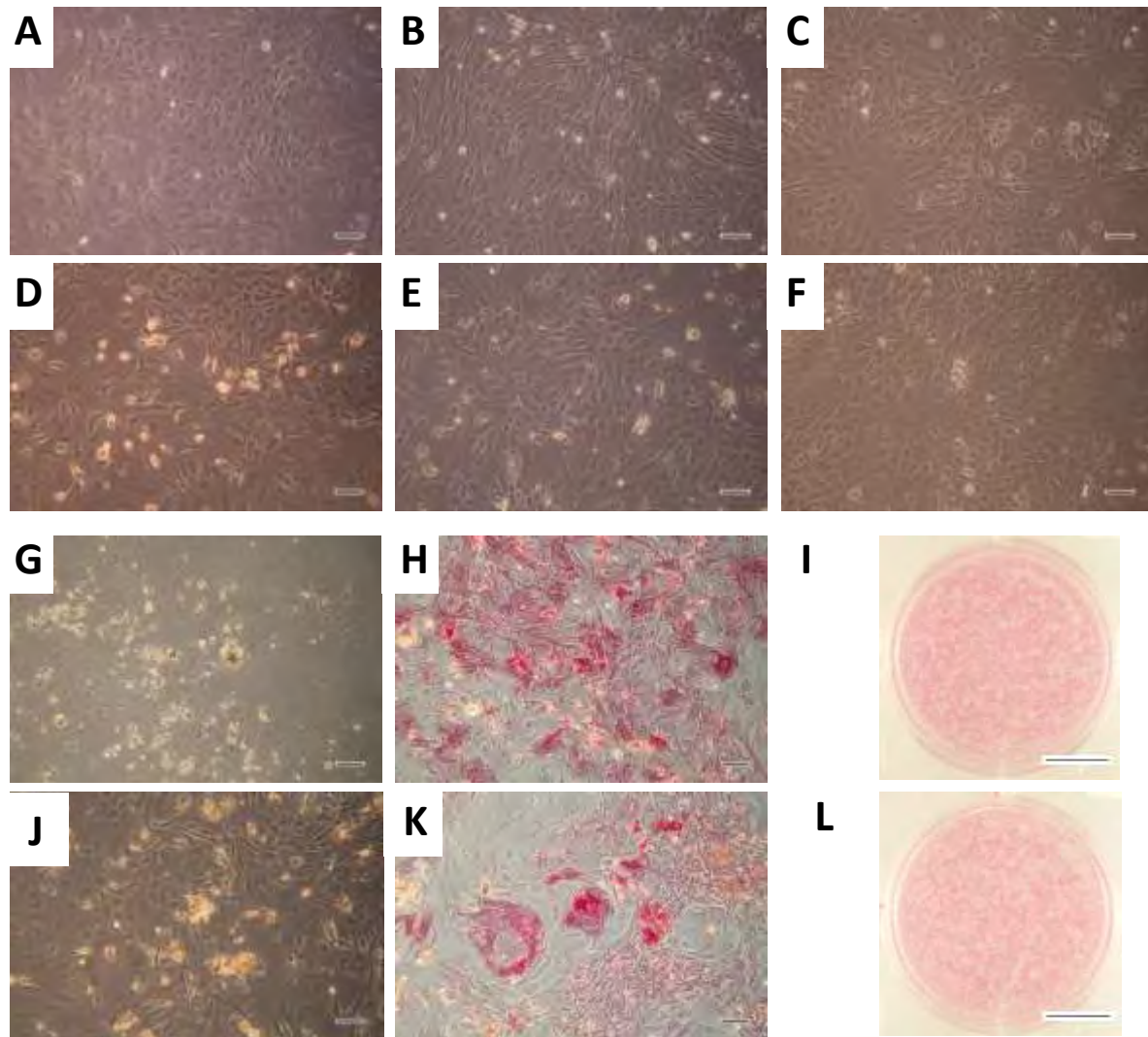
Figure 16: Culturing of DPSCs in iPSC medium supplemented with ascorbic acid and lithium chloride after lentiviral infection. DPSCs without lentiviral infection (**A-C and G-I**) and DPSCs infected with STEMCCA virus (**D-E and J-K**) from day 5 (A and D), 9 (B and E), 17 (C and F) and 21 (G-L). Cells stained for alkaline phosphatase (H, I, K and L). Scale bars: 100 μm (A-H, J and K) and 1 cm (I and L).

3.6 Using the inhibitors CHIR-99021 and PD-0325901

The inhibitors CHIR-99021 and PD-0325901 were added to the iPSC medium as they had previously been used to generate iPSCs [19]. REFs formed colonies after 6 days post-transduction (Fig. 17, B and F), with a typical iPSC morphology being observed after 8 days (Fig. 17, F). The colonies stained intensely for alkaline phosphatase and could be seen with the naked eye on the culture dish. ADSCs

and DPSCs were also cultured in iPSC medium with the addition of the two inhibitors without,
however without success of generating iPSC colonies (Figs 19 and 21).
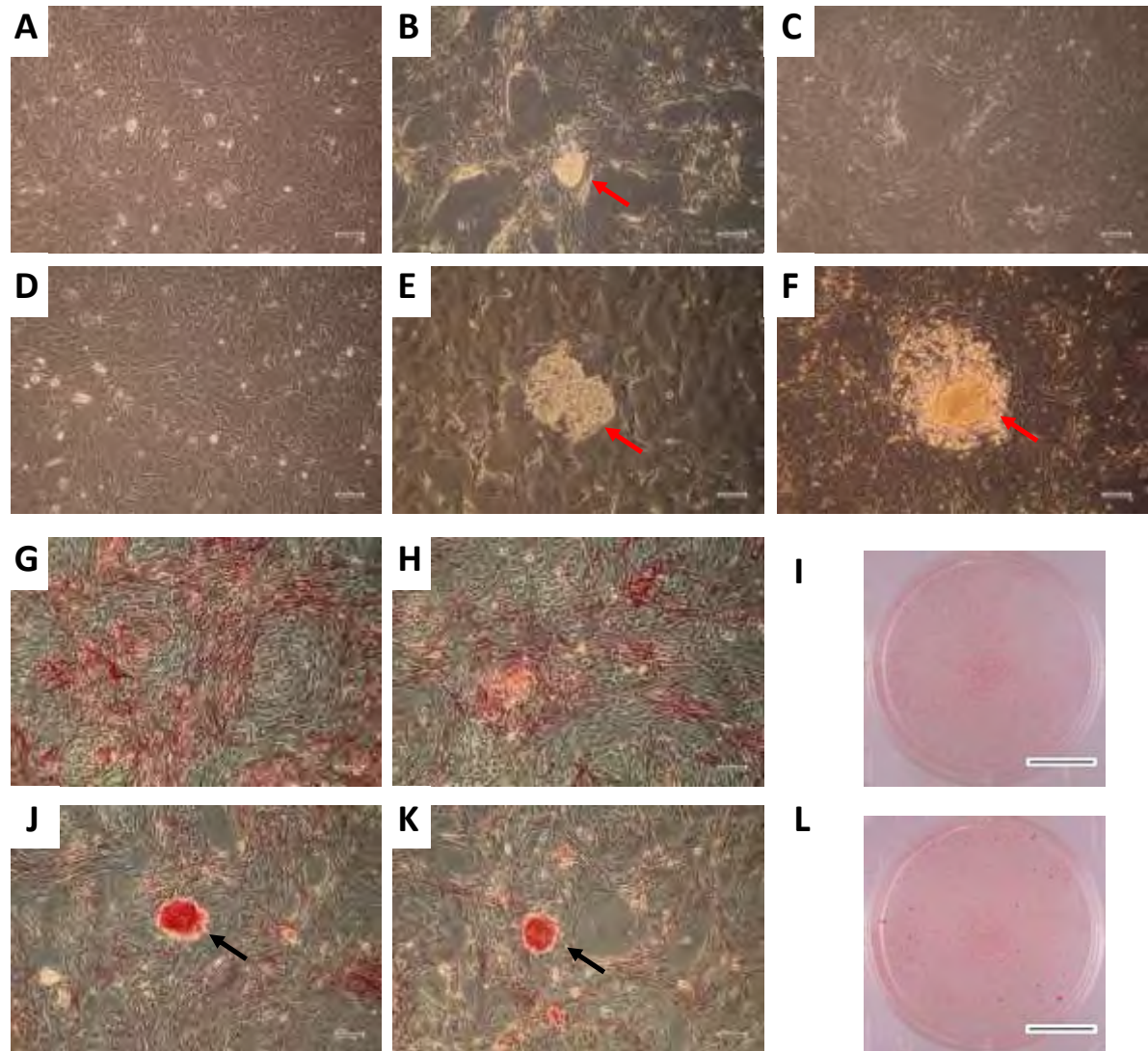
Figure 17: Derivation of presumptive riPSCs from REFs by viral transduction and culturing in 2i media. A-C and G-I, Control REFs that were not infected with the STEMCCA virus; **D-F and J-L**, REFs infected with the STEMCCA virus. Images show cells at days 0 (A and D), 6 (B and E), and 8 (C and F). REFs were stained for alkaline phosphatase on day 9 (G - L). Scale bars represent 100 µm for A-H, J and K and 1 cm for I and L. Arrows indicate colony formation.
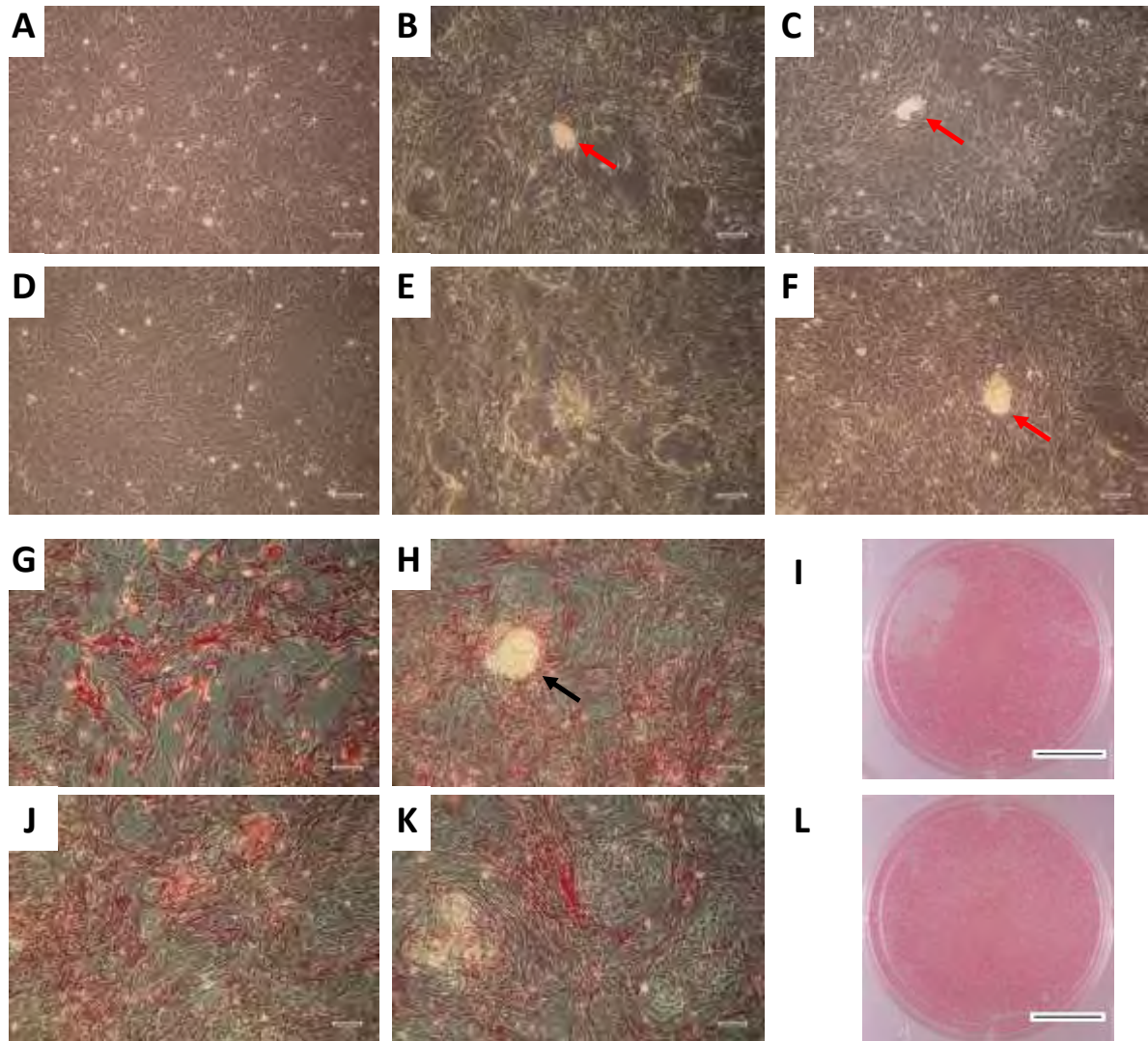
Figure 18: Transduction of REFs using the STEMCCA virus and culturing in 2i media supplemented with ascorbic acid and lithium chloride. A-C and G-I, Control REFs; **D-F and J-L**, STEMCCA virus infected REFs. Images show cells at days 0 (A and D), 6 (B and E), and 8 (C and F). REFs were stained with alkaline phosphatase on day 9 (G - L). Scale bars in A-H, J and K indicate 100 µm, and 1 cm in I and L. Arrows indicate colony formation.

Cultures of REFs, ADSCs and DPSCs were simultaneously transduced and grown in iPSC media containing the two inhibitors with the addition of ascorbic acid and lithium chloride to see if this would increase the efficiency of iPSC derivation (Figs. 18, 20 and 22). Colonies formed but did not show the iPSC morphology previously seen in the REF culture with just the two inhibitors added (Fig. 18, B, C F and H). Confirmation by staining for alkaline phosphatase showed that iPSC colonies did

not form (Figs. 18, 20 and 22, G-L). Ascorbic acid and lithium chloride combined with the iPSC media

and two inhibitors did not improve the outcome for the ADSCs and DPSCs with no iPSC colonies

forming (Figs. 20 and 22).
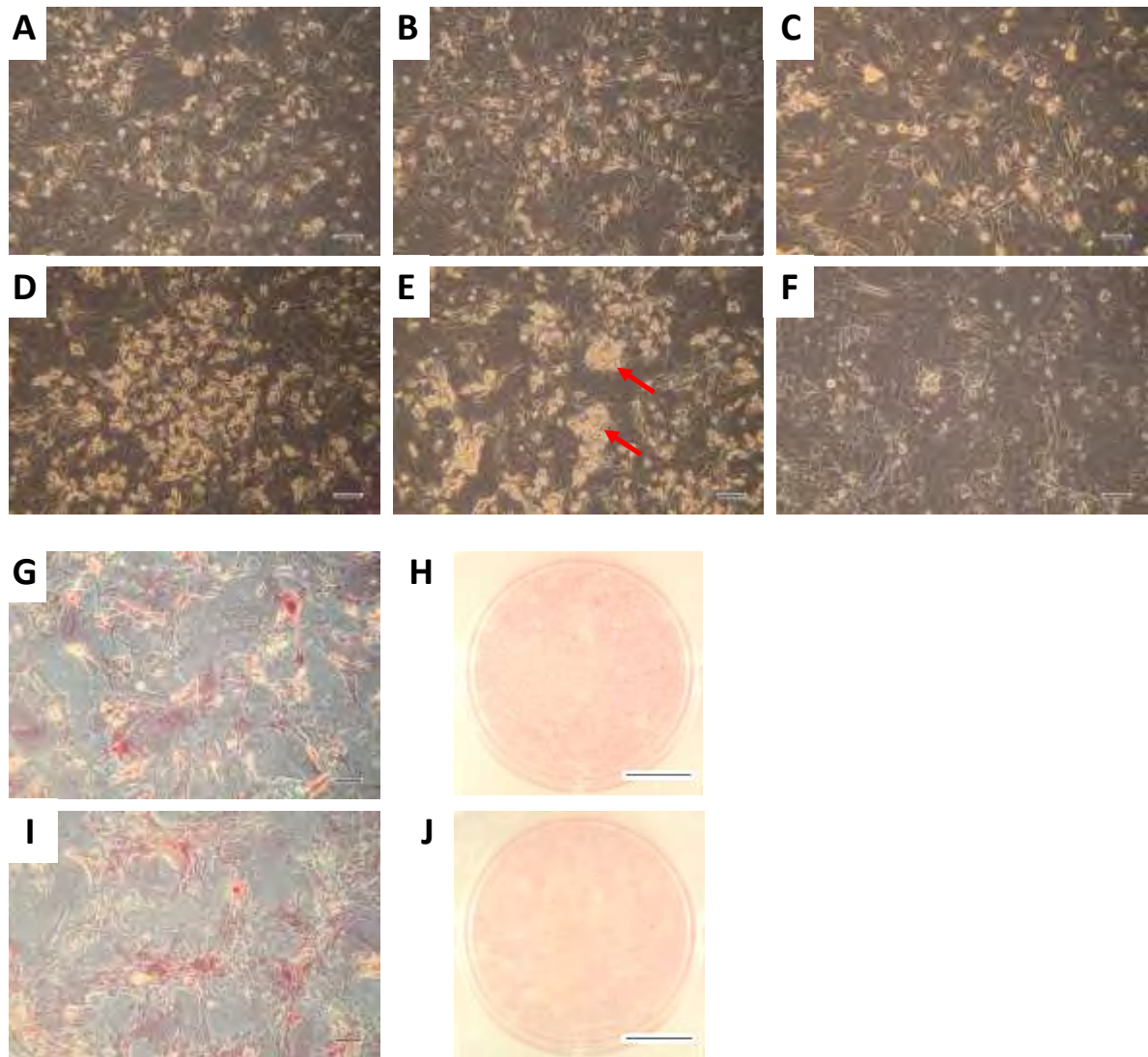
Figure 19: Culturing of ADSCs in iPSC medium containing the two inhibitors. ADSCs failed to form iPSC colonies when infected with the STEMCCA virus (**D-F, I and J**). Control cultures show ADSCs grown without viral transduction (**A-C, G and H**). Cells shown from days 0 (A and D), 6 (B and E), 14 (C and F) and stained for alkaline phosphatase on 21 (G-J). Scale bars represent 100 μm (A-G and I) and 1 cm (H and J). Arrow show potential colony formation.
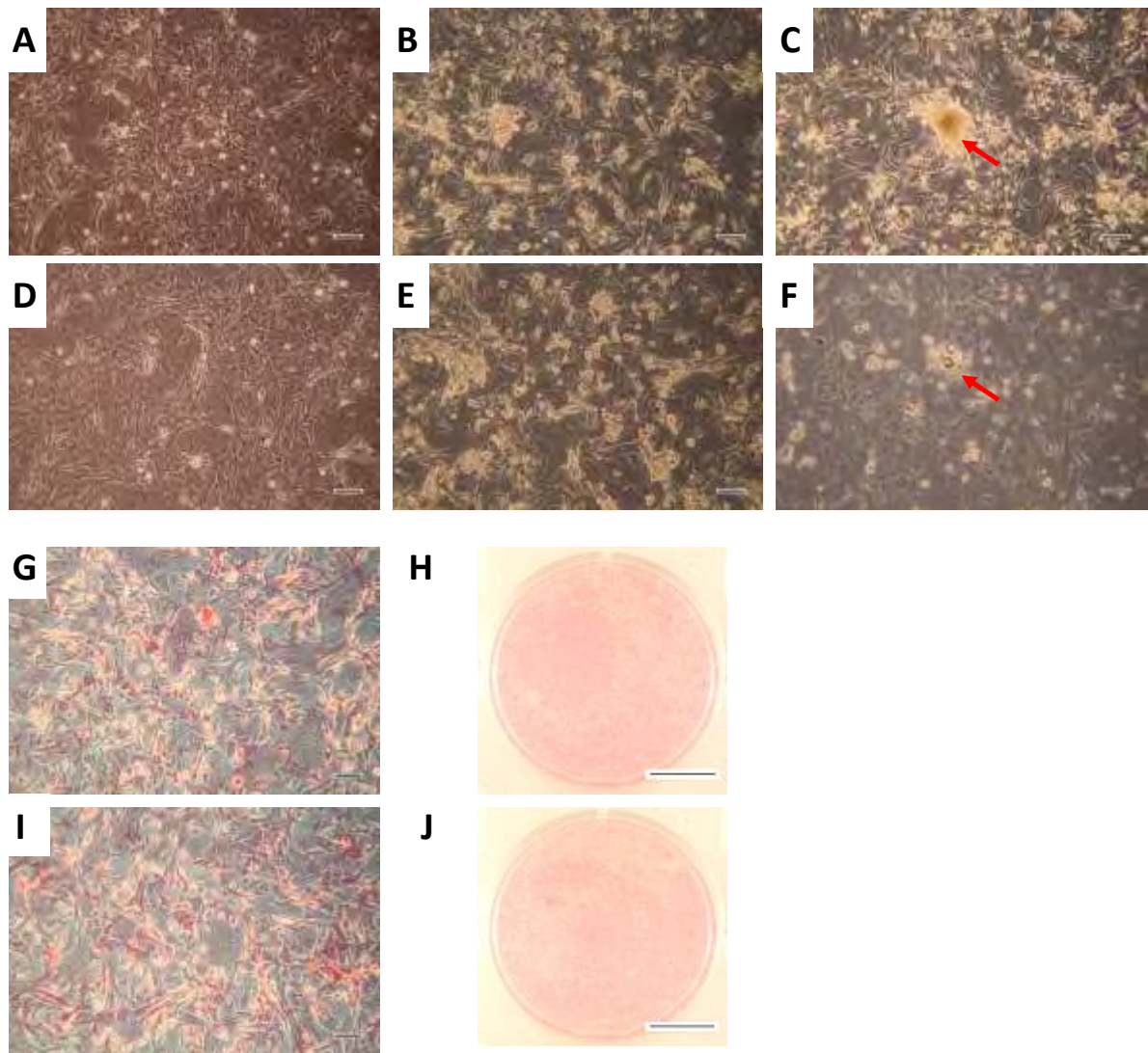
Figure 20: ADSCs cultured in iPSC medium containing 2i, ascorbic acid and lithium chloride. ADSCs infected with the STEMCCA virus (**D-F, I and J**) and control cultures of ADSCs (**A-C, G and H**). Cells shown from days 0 (A and D), 6 (B and E), 14 (C and F) and 21 (G-J). Staining for alkaline phosphatase (G-J). Scale bars represent 100 µm (A-G and I) and 1 cm (H and J). Arrow show colony formation.
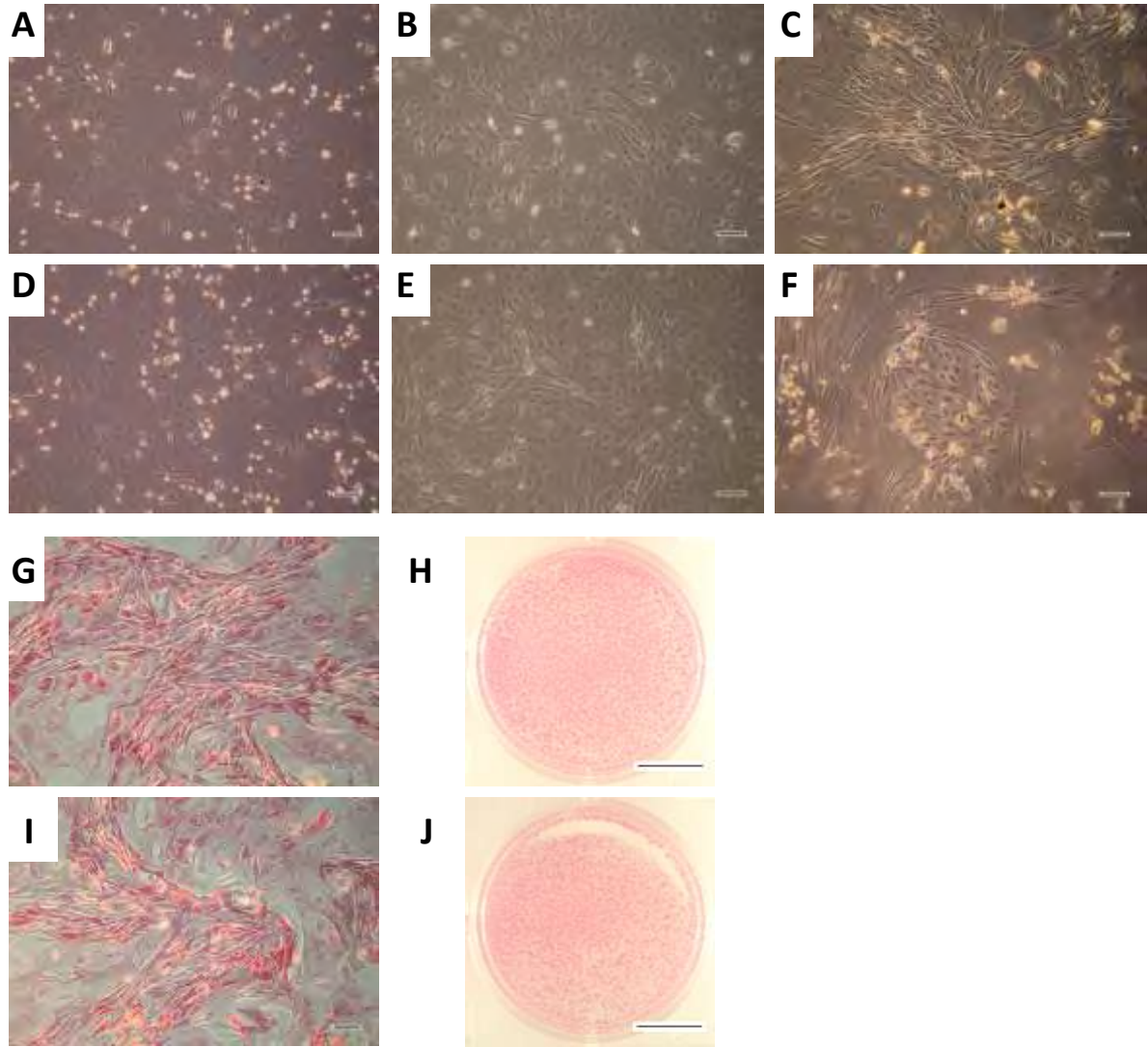
Figure 21: Culturing of DPSCs with CHIR-99021 and PD-0325901 in iPSC media. DPSCs lentivirally transduced (**D-F, I and J**) and control cells (**A-C, G and H**) from the day of infection (A and D), day 6 (B and E), day 14 (C and F) and day 21 (G-J). Alkaline phosphatase expression in DPSCs (G-J). 100 μm (A-G and I) and 1 cm are represented by the scale bars.
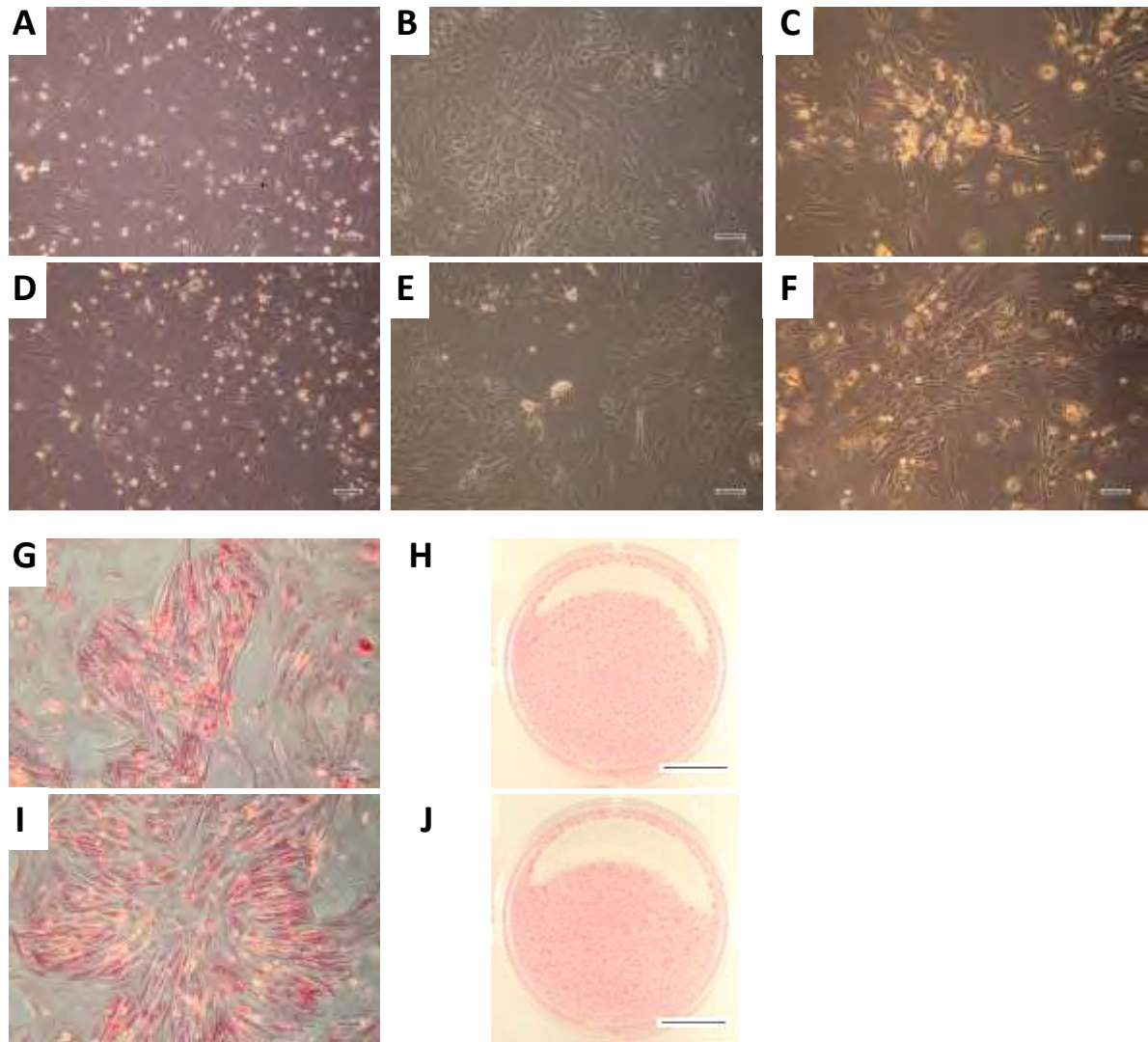
Figure 22: Attempted derivation of riPSCs from DPSCs using iPSC media supplemented with 2i, ascorbic acid and lithium chloride. DPSCs transduced with the STEMCCA (**D-F, I and J**) and control cells (**A-C, G and H**) from the day of infection (A and D), day 6 (B and E), day 14 (C and F) and day 21 (G-J). G-J, Cells stained for the expression of alkaline phosphatase. 100 μm (A-G and I) and 1 cm are represented by the scale bars.

4. Discussion:

Presumptive riPSCs were successfully generated from REFs using the STEMCCA lentivirus delivery system when cultured in iPSC media supplemented with the two inhibitors CHIR-99021 and PD-0325901 (Fig. 17), however further characterisation is necessary to demonstrate whether the colonies observed are *bona fide* iPSCs. riPSCs could not be generated from DPSCs or ADSCs using the same culturing conditions (Fig. 19 and 21).

The two inhibitors, CHIR-99021 and PD-0325901, have previously been demonstrated to be essential components of the culture media used to derive riPSCs[19, 63, 64, 66], in addition to being required to isolate and culture rESCs[75, 76]. CHIR-99021 inhibits glycogen synthase kinase 3β (GSK3β) which is involved in the wnt-signalling pathway. The role of wnt-signalling in ESCs and therefore iPSCs is a contentious issue with evidence supporting both a role in promoting differentiation as well as maintaining stem cell potency through enhanced expression of nanog[77, 78]. The inhibitor, CHIR-99021, however has been demonstrated to promote reprogramming in a dose dependent response[79]. PD-0325901 is an inhibitor of MAPK/ERK kinase (MEK)[80], which is involved in apoptosis, cell proliferation, differentiation, motility, metabolism and survival[81]. In ESCs inhibition of MEK prevents differentiation and promotes ESC growth and self-renewal[82]. Together the two inhibitors are now used extensively to culture and derive ESCs and iPSCs respectively[83].

The protocol using the two inhibitors in the iPSC medium was unable to generate riPSCs from the DPSCs or ADSCs, even though riPSCs were able to be generated from REFs using the same culture condition. Reprogramming adult cells has previously been demonstrated to take longer than reprogramming embryonic cells which could explain why iPSCs were not derived from ADSCs and DPSCs. The ADSCs and DPSCs failed to form iPSC colonies when cultured for up to 21 days after the viral transduction, which should, based on previous studies, be sufficient time to reprogram ADSCs and DPSCs [71, 84, 85]. The culture conditions may therefore require modifications to promote the derivation of iPSCs from ADSCs and DPSCs, which could include additional supplements or inhibitors

being added to the media. The DPSCs stained moderately for alkaline phosphatase (Figs. 8, 16, 21

and 22) regardless of whether reprogramming had taken place, demonstrating that alkaline

phosphatase expression is not exclusive to iPSCs and that further characterisation is required to

demonstrate cells have become iPSCs. The ADSCs in particular proved difficult to culture for

prolonged periods without passaging and frequently dissociated from the culture dish. It may be

preferable with ADSCs to culture using a feeder layer and ESC media as this represses the growth of

somatic cells while promoting the growth of iPSCs, which would circumvent the problems with the

ADSCs becoming overly confluent and dissociating from the dish. In ADSCs and DPSCs cultures,

colonies with a non-ESC like morphology were frequently observed which has previously been

described in the literature[71].

The other culture conditions used (iPSC media, ascorbic acid and lithium chloride supplementation

and seeding onto a feeder layer) were unable to generate iPSC colonies from either the REFs, ADSCs

or DPSCs. This was possibly due to the fundamental requirement of the two inhibitors to derive and

culture riPSCs. Interestingly, when ascorbic acid and lithium chloride (previously shown to improve

reprogramming and iPSC derivation) were also supplemented along with the two inhibitors, no iPSC

colonies formed from the REFs (Fig. 18). This suggested either ascorbic acid and/or lithium chloride

were incompatible with riPSC derivation and/or that they abrogated the effect of the two inhibitors.

Lithium chloride can also inhibit GSK3β, which could mean that lithium chloride and CHIR-99021 are

competitive inhibitors therefore reducing their efficacy to inhibit GSK3β. Ascorbic acid on the other

hand has been shown to enhance differentiation of stem cells, including ESCs, which could impede

reprogramming, however independent groups have had success with deriving iPSCs with media

supplemented with ascorbic acid. Why ascorbic acid has these contrary effects is hitherto unknown

due to the complex interactions ascorbic acid produces in cells. This shows that the culture media is

vital in successfully reprogramming cells and generating iPSCs. Further research is required to

optimise the culture media used to derive riPSCs, which may be different from that used for miPSCs

and hiPSCs derivation.

iPSC colonies took longer to generate from REFs, isolated from E14.5 embryos, when compared with iPSCs colonies derived from MEFs at the same embryonic stage. This could have been due to the culture and transduction conditions not being optimised to efficiently generate riPSCs, or could be due to fundamental differences between MEFs and REFs. The STEMCCA is composed of mouse OSKM genes, which could account for iPSCs taking a shorter time to derive from MEFs compared with REFs. Genes involved in pluripotency are highly conserved[86, 87], with the majority of iPSC derivation using either mouse or human OSKM genes, regardless of the species the iPSCs are being derived from[11, 19, 21, 62, 88]. Mouse LIF was added to the reprogramming media which again even though highly conserved (greater than 90 %; Blastp[89], accession numbers NP_032527 (mouse LIF) and NP_071532 (rat LIF)) could contribute to the extended time required to reprogram rat cells.

ADSCs and DPSCs may also be difficult to reprogram using the STEMCCA virus due to the stoichiometry of OSKM being inefficient or unfavourable to the reprogramming process. The STEMCCA virus is designed to produce the OSKM factors at levels to efficiently reprogram cells, however ADSCs and DPSCs have been demonstrated to express some of these factors endogenously which could affect the reprogramming efficiency. DPSCs have been shown to express Oct4, Sox2, c-Myc and Nanog [90-92] whereas ADSCs have been shown to expressOct4, Sox2, Klf4, c-Myc and other embryonic genes such as Rex1[93]. Other papers have derived iPSCs from ADSCs or DPSCs using vectors which deliver the reprogramming factors individually thus allowing for the levels of vectors added to be modified.

Further work is required to determine a media that supports riPSC derivation from ADSCs and DPSCs. Once riPSCs have been derived from ADSCs they will need to be characterised for future work which could include assessment of the suitability of iPSCs derived from ADSCs and DPSCs for use in regenerative therapies.

5. Conclusion

A method of generating riPSCs has been developed using the STEMCCA lentivirus delivery system. The media requires the addition of two inhibitors; CHIR-99021, which inhibits GSK-3β and PD-0325901, a MEK inhibitor. riPSCs were able to be derived from REFs, but hitherto unable to be derived from rat ADSCs or DPSCs. Subsequent work will require a method of deriving iPSCs form ADSCs and DPSCs and the riPSCs will need to be characterised for future use.

6. Acknowledgements

7. Supplementary Data
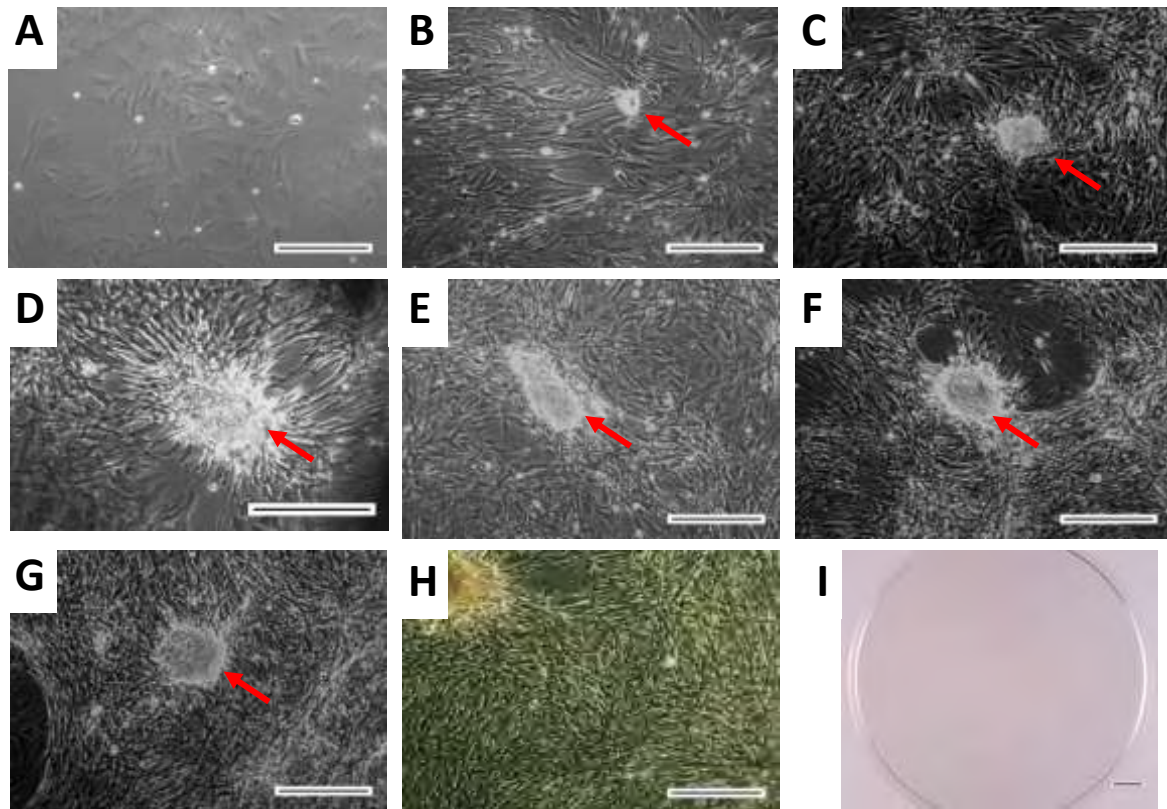
Figure S1: ADSCs infected with the STEMCCA virus and cultured in DMEM based iPSC media. Cells shown from days 0 (A), 5 (B), 7 (C), 9 (D), 11 (E), 23 (F), 27 (G) and stained for alkaline phosphatase on day 29 (H and I). Arrows show colony formation. Scale bars: 100 μm (A-H) and 1 cm (I).
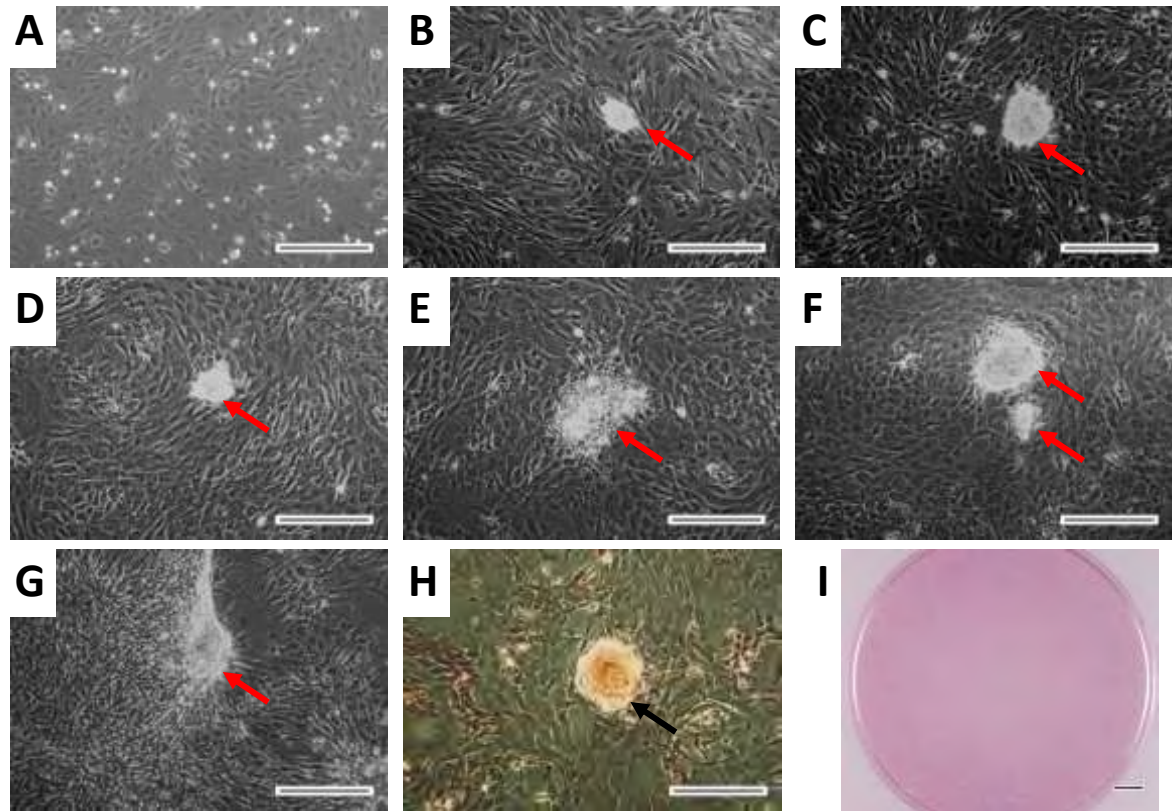
Figure S2: DPSCs infected and grown in DMEM based iPSC media. DPSCs shown from days 0 (A), 5 (B), 7 (C), 11 (D), 13 (E), 15 (F), 23 (G) and stained for alkaline phosphatase on day 29 (H and I). Arrows show colony formation. Scale bars: 100 μm (A-H) and 1 cm (I).
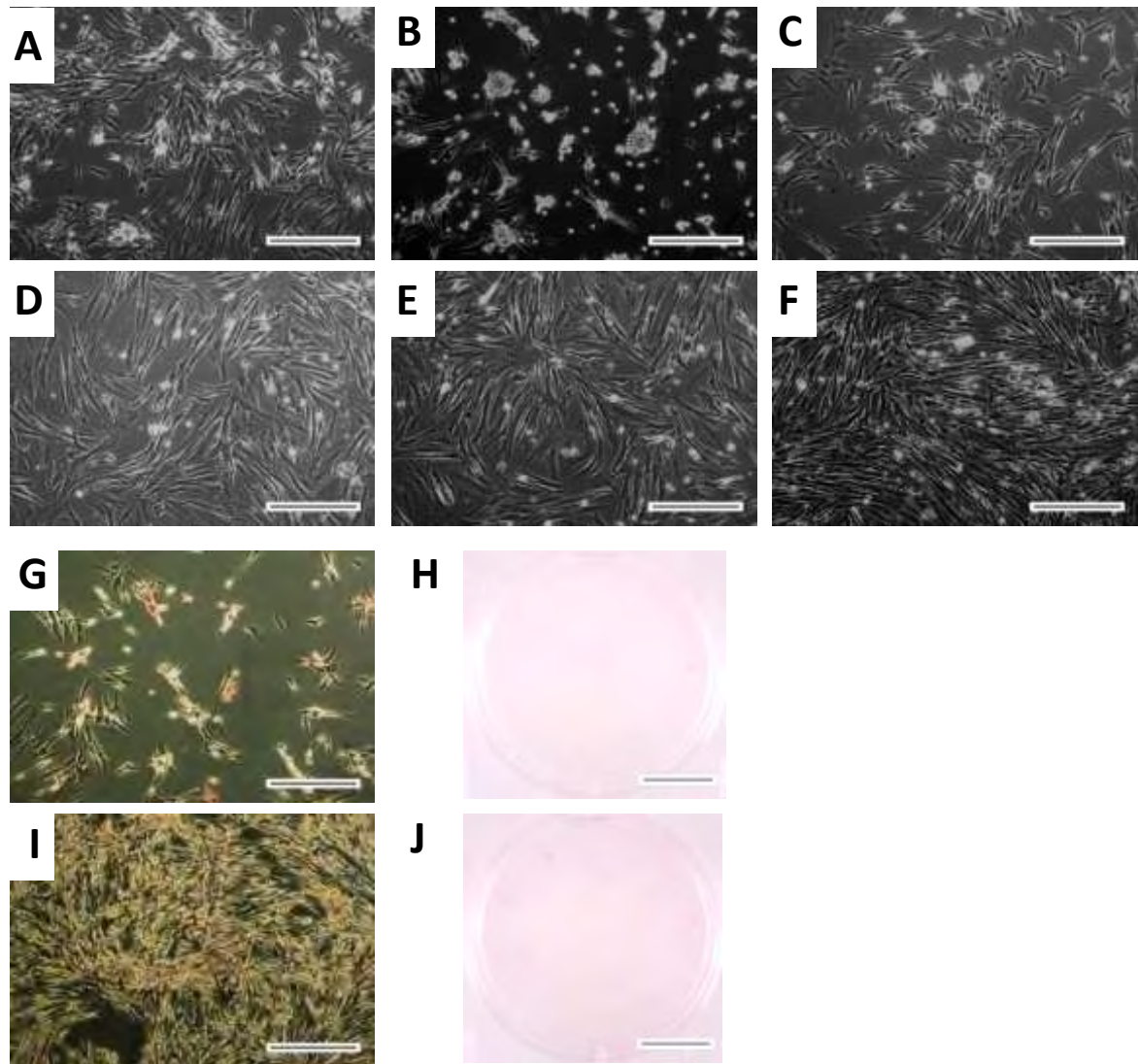
Figure S3: ADSCs cultured in ESC media without being seeded onto irradiated MEF feeder layer.
Images are from day 7 (A and D), day 9 (B and E), day 14 (C and F) and the cultures were stained on day 21 for alkaline phosphatase (G-J).
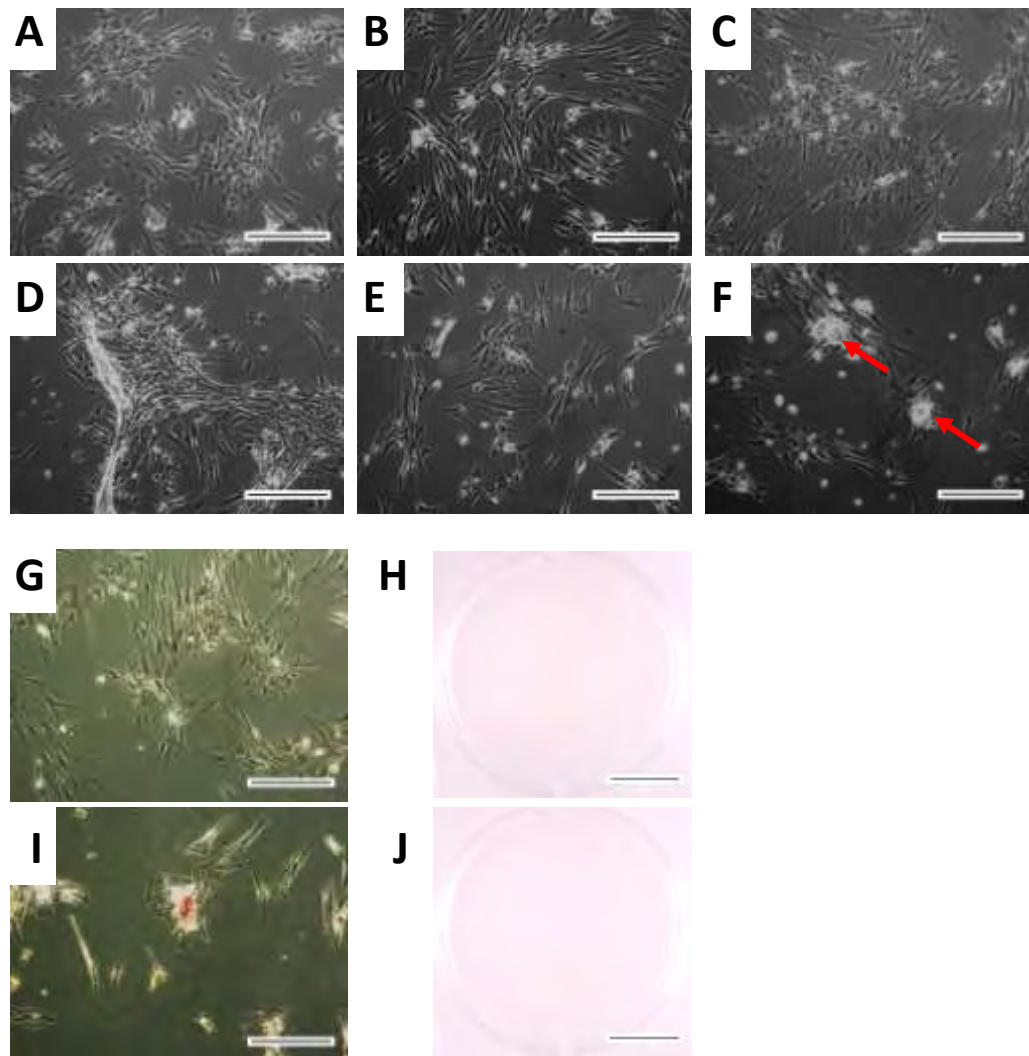
Figure S4: DPSCs cultured in ESC media without being seeded onto irradiated MEF feeder layer.
Images are from day 7 (A and D), day 9 (B and E), day 14 (C and F) and the cultures were stained on day 21 for alkaline phosphatase (G-J).

8. References

1. Johnson, M. H. and Mcconnell, J. M. L., *Lineage allocation and cell polarity during mouse embryogenesis.* Seminars in Cell & Developmental Biology, 2004. **15**(5): p. 583-597.
2. Pittenger, M. F., Mackay, A. M., Beck, S. C*., et al.*, *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-147.
3. Jiang, Y. H., Jahagirdar, B. N., Reinhardt, R. L*., et al.*, *Pluripotency of mesenchymal stem cells derived from adult marrow.* Nature, 2002. **418**(6893): p. 41-49.
4. Morrison, S. J., *Stem cell potential: Can anything make anything?* Current Biology, 2001. **11**(1): p. R7-R9.
5. Reik, W., *Stability and flexibility of epigenetic gene regulation in mammalian development.* Nature, 2007. **447**(7143): p. 425-432.
6. Evans, M. J. and Kaufman, M. H., *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-156.
7. Martin, G. R., *Isolation of a pluripotent cell-line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem-cells.* Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 1981. **78**(12): p. 7634-7638.
8. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S*., et al.*, *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-1147.
9. Noggle, S., Fung, H.-L., Gore, A*., et al.*, *Human oocytes reprogram somatic cells to a pluripotent state.* Nature, 2011. **478**(7367): p. 70-U81.
10. Wilmut, I., Schnieke, A. E., Mcwhir, J*., et al.*, *Viable offspring derived from fetal and adult mammalian cells.* Nature, 1997. **385**(6619): p. 810-813.
11. Takahashi, K. and Yamanaka, S., *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-676.
12. Avilion, A. A., Nicolis, S. K., Pevny, L. H*., et al.*, *Multipotent cell lineages in early mouse development depend on sox2 function.* Genes & Development, 2003. **17**(1): p. 126-140.
13. Chambers, I., Colby, D., Robertson, M*., et al.*, *Functional expression cloning of nanog, a pluripotency sustaining factor in embryonic stem cells.* Cell, 2003. **113**(5): p. 643-655.
14. Mitsui, K., Tokuzawa, Y., Itoh, H*., et al.*, *The homeoprotein nanog is required for maintenance of pluripotency in mouse epiblast and es cells.* Cell, 2003. **113**(5): p. 631-642.
15. Nichols, J., Zevnik, B., Anastassiadis, K*., et al.*, *Formation of pluripotent stem cells in the mammalian embryo depends on the pou transcription factor oct4.* Cell, 1998. **95**(3): p. 379-391.
16. Niwa, H., Burdon, T., Chambers, I*., et al.*, *Self-renewal of pluripotent embryonic stem cells is mediated via activation of stat3.* Genes & Development, 1998. **12**(13): p. 2048-2060.
17. Niwa, H., Miyazaki, J., and Smith, A. G., *Quantitative expression of oct-3/4 defines differentiation, dedifferentiation or self-renewal of es cells.* Nature Genetics, 2000. **24**(4): p. 372-376.
18. Okita, K., Nakagawa, M., Hong, H*., et al.*, *Generation of mouse induced pluripotent stem cells without viral vectors.* Science, 2008. **322**(5903): p. 949-953.
19. Hamanaka, S., Yamaguchi, T., Kobayashi, T*., et al.*, *Generation of germline-competent rat induced pluripotent stem cells.* Plos One, 2011. **6**(7).
20. Nsair, A. and Maclellan, W. R., *Induced pluripotent stem cells for regenerative cardiovascular therapies and biomedical discovery.* Advanced Drug Delivery Reviews, 2011. **63**(4–5): p. 324-330.

21.   Takahashi, K., Tanabe, K., Ohnuki, M.*, et al.*, *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-872.

22.   Maherali, N., Ahfeldt, T., Rigamonti, A.*, et al.*, *A high-efficiency system for the generation and study of human induced pluripotent stem cells.* Cell Stem Cell, 2008. **3**(3): p. 340-345.

23.   Stadtfeld, M., Brennand, K., and Hochedlinger, K., *Reprogramming of pancreatic beta cells into induced pluripotent stem cells.* Current Biology, 2008. **18**(12): p. 890-894.

24.   Stadtfeld, M., Maherali, N., Breault, D. T.*, et al.*, *Defining molecular cornerstones during fibroblast to ips cell reprogramming in mouse.* Cell Stem Cell, 2008. **2**(3): p. 230-240.

25.   Lister, R., Pelizzola, M., Kida, Y. S.*, et al.*, *Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells.* Nature, 2011. **471**(7336): p. 68-U84.

26.   Gore, A., Li, Z., Fung, H.-L.*, et al.*, *Somatic coding mutations in human induced pluripotent stem cells.* Nature, 2011. **471**(7336): p. 63-U76.

27.   Robinton, D. A. and Daley, G. Q., *The promise of induced pluripotent stem cells in research and therapy.* Nature, 2012. **481**(7381): p. 295-305.

28.   Sommer, C. A., Sommer, A. G., Longmire, T. A.*, et al.*, *Excision of reprogramming transgenes improves the differentiation potential of ips cells generated with a single excisable vector.* Stem Cells, 2010. **28**(1): p. 64-74.

29.   Sommer, C. A., Stadtfeld, M., Murphy, G. J.*, et al.*, *Induced pluripotent stem cell generation using a single lentiviral stem cell cassette.* Stem Cells, 2009. **27**(3): p. 543-549.

30.   Woltjen, K., Michael, I. P., Mohseni, P.*, et al.*, *Piggybac transposition reprograms fibroblasts to induced pluripotent stem cells.* Nature, 2009. **458**(7239): p. 766-U106.

31.   Kaji, K., Norrby, K., Paca, A.*, et al.*, *Virus-free induction of pluripotency and subsequent excision of reprogramming factors.* Nature, 2009. **458**(7239): p. 771-U112.

32.   Yusa, K., Rad, R., Takeda, J.*, et al.*, *Generation of transgene-free induced pluripotent mouse stem cells by the piggybac transposon.* Nature Methods, 2009. **6**(5): p. 363-U69.

33.   Kim, D., Kim, C.-H., Moon, J.-I.*, et al.*, *Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins.* Cell Stem Cell, 2009. **4**(6): p. 472-476.

34.   Zhou, H., Wu, S., Joo, J. Y.*, et al.*, *Generation of induced pluripotent stem cells using recombinant proteins.* Cell Stem Cell, 2009. **4**(5): p. 381-384.

35.   Warren, L., Manos, P. D., Ahfeldt, T.*, et al.*, *Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mrna.* Cell Stem Cell, 2010. **7**(5): p. 618-630.

36.   Miyoshi, N., Ishii, H., Nagano, H.*, et al.*, *Reprogramming of mouse and human cells to pluripotency using mature micrornas.* Cell Stem Cell, 2011. **8**(6): p. 633-638.

37.   Anokye-Danso, F., Trivedi, C. M., Juhr, D.*, et al.*, *Highly efficient mirna-mediated reprogramming of mouse and human somatic cells to pluripotency.* Cell Stem Cell, 2011. **8**(4): p. 376-388.

38.   Nakagawa, M., Koyanagi, M., Tanabe, K.*, et al.*, *Generation of induced pluripotent stem cells without myc from mouse and human fibroblasts.* Nature Biotechnology, 2008. **26**(1): p. 101-106.

39.   Wernig, M., Meissner, A., Cassady, J. P.*, et al.*, *C-myc is dispensable for direct reprogramming of mouse fibroblasts.* Cell Stem Cell, 2008. **2**(1): p. 10-12.

40.   Danwei, H., Osafune, K., Maehr, R.*, et al.*, *Induction of pluripotent stem cells from primary human fibroblasts with only oct4 and sox2.* Nature Biotechnology, 2008. **26**(11): p. 1269-1275.

41.   Kim, J. B., Sebastiano, V., Wu, G.*, et al.*, *Oct4-induced pluripotency in adult neural stem cells.* Cell, 2009. **136**(3): p. 411-419.

42.   Li, Y. Q., Zhang, Q. A., Yin, X. L.*, et al.*, *Generation of ipscs from mouse fibroblasts with a single gene, oct4, and small molecules.* Cell Research, 2011. **21**(1): p. 196-204.

43. Heng, J.-C. D., Feng, B., Han, J.*, et al.*, *The nuclear receptor nr5a2 can replace oct4 in the reprogramming of murine somatic cells to pluripotent cells.* Cell Stem Cell, 2010. **6**(2): p. 167-174.

44. Yu, J., Vodyanik, M. A., Smuga-Otto, K.*, et al.*, *Induced pluripotent stem cell lines derived from human somatic cells.* Science, 2007. **318**(5858): p. 1917-1920.

45. Nakagawa, M., Takizawa, N., Narita, M.*, et al.*, *Promotion of direct reprogramming by transformation-deficient myc.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(32): p. 14152-14157.

46. Brambrink, T., Foreman, R., Welstead, G. G.*, et al.*, *Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells.* Cell Stem Cell, 2008. **2**(2): p. 151-159.

47. Sridharan, R., Tchieu, J., Mason, M. J.*, et al.*, *Role of the murine reprogramming factors in the induction of pluripotency.* Cell, 2009. **136**(2): p. 364-377.

48. Varlakhanova, N., Cotterman, R., Bradnam, K.*, et al.*, *Myc and miz-1 have coordinate genomic functions including targeting hox genes in human embryonic stem cells.* Epigenetics & Chromatin, 2011. **4**.

49. Boyer, L. A., Lee, T. I., Cole, M. F.*, et al.*, *Core transcriptional regulatory circuitry in human embryonic stem cells.* Cell, 2005. **122**(6): p. 947-956.

50. Chen, X., Xu, H., Yuan, P.*, et al.*, *Integration of external signaling pathways with the core transcriptional network in embryonic stem cells.* Cell, 2008. **133**(6): p. 1106-1117.

51. Kim, J., Chu, J., Shen, X.*, et al.*, *An extended transcriptional network for pluripotency of embryonic stem cells.* Cell, 2008. **132**(6): p. 1049-1061.

52. Loh, Y. H., Wu, Q., Chew, J. L.*, et al.*, *The oct4 and nanog transcription network regulates pluripotency in mouse embryonic stem cells.* Nature Genetics, 2006. **38**(4): p. 431-440.

53. Rodriguez, R. T., Velkey, J. M., Lutzko, C.*, et al.*, *Manipulation of oct4 levels in human embryonic stem cells results in induction of differential cell types.* Experimental Biology and Medicine, 2007. **232**(10): p. 1368-1380.

54. Huangfu, D. W., Osafune, K., Maehr, R.*, et al.*, *Induction of pluripotent stem cells from primary human fibroblasts with only oct4 and sox2.* Nature Biotechnology, 2008. **26**(11): p. 1269-1275.

55. Esteban, M. A., Wang, T., Qin, B.*, et al.*, *Vitamin c enhances the generation of mouse and human induced pluripotent stem cells.* Cell Stem Cell, 2010. **6**(1): p. 71-9.

56. Kim, K., Doi, A., Wen, B.*, et al.*, *Epigenetic memory in induced pluripotent stem cells.* Nature, 2010. **467**(7313): p. 285-290.

57. Polo, J. M., Liu, S., Figueroa, M. E.*, et al.*, *Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells.* Nat Biotech, 2010. **28**(8): p. 848-855.

58. Liu, H., Zhu, F., Yong, J.*, et al.*, *Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts.* Cell Stem Cell, 2008. **3**(6): p. 587-590.

59. Esteban, M. A., Xu, J., Yang, J.*, et al.*, *Generation of induced pluripotent stem cell lines from tibetan miniature pig.* Journal of Biological Chemistry, 2009. **284**(26): p. 17634-17640.

60. Ezashi, T., Telugu, B. P. V. L., Alexenko, A. P.*, et al.*, *Derivation of induced pluripotent stem cells from pig somatic cells.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(27): p. 10993-10998.

61. Bao, L., He, L., Chen, J.*, et al.*, *Reprogramming of ovine adult fibroblasts to pluripotency via drug-inducible expression of defined factors.* Cell Research, 2011. **21**(4): p. 600-608.

62. Friedrich Ben-Nun, I., Montague, S. C., Houck, M. L.*, et al.*, *Induced pluripotent stem cells from highly endangered species.* Nat Meth, 2011. **8**(10): p. 829-831.

63. Liao, J., Cui, C., Chen, S. Y.*, et al.*, *Generation of induced pluripotent stem cell lines from adult rat cells.* Cell Stem Cell, 2009. **4**(1): p. 11-15.

64. Liskovykh, M., Chuykin, I., Ranjan, A*., et al.*, *Derivation, characterization, and stable transfection of induced pluripotent stem cells from fischer344 rats.* Plos One, 2011. **6**(11).

65. Li, W. L., Wei, W., Zhu, S. Y*., et al.*, *Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors.* Cell Stem Cell, 2009. **4**(1): p. 16-19.

66. Chang, M. Y., Kim, D., Kim, C. H*., et al.*, *Direct reprogramming of rat neural precursor cells and fibroblasts into pluripotent stem cells.* Plos One, 2010. **5**(3).

67. Li, W. L. and Ding, S., *Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming.* Trends in Pharmacological Sciences, 2010. **31**(1): p. 36-45.

68. Wang, Q., Xu, X. X., Li, J*., et al.*, *Lithium, an anti-psychotic drug, greatly enhances the generation of induced pluripotent stem cells.* Cell Research, 2011. **21**(10): p. 1424-1435.

69. Silva, J., Barrandon, O., Nichols, J*., et al.*, *Promotion of reprogramming to ground state pluripotency by signal inhibition.* PLoS Biol, 2008. **6**(10): p. e253.

70. Chen, J. K., Liu, J., Chen, Y*., et al.*, *Rational optimization of reprogramming culture conditions for the generation of induced pluripotent stem cells with ultra-high efficiency and fast kinetics.* Cell Research, 2011. **21**(6): p. 884-894.

71. Sun, N., Panetta, N. J., Gupta, D. M*., et al.*, *Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(37): p. 15720-15725.

72. Beltrao-Braga, P. C. B., Pignatari, G. C., Maiorka, P. C*., et al.*, *Feeder-free derivation of induced pluripotent stem cells from human immature dental pulp stem cells.* Cell Transplantation, 2011. **20**(11-12): p. 1707-1719.

73. Dubridge, R. B., Tang, P., Hsia, H. C*., et al.*, *Analysis of mutation in human-cells by using an epstein-barr-virus shuttle system.* Molecular and Cellular Biology, 1987. **7**(1): p. 379-387.

74. Markoullis, K., Bulian, D., Holzlwimmer, G*., et al.*, *Mycoplasma contamination of murine embryonic stem cells affects cell parameters, germline transmission and chimeric progeny.* Transgenic Research, 2009. **18**(1): p. 71-87.

75. Buehr, M., Meek, S., Blair, K*., et al.*, *Capture of authentic embryonic stem cells from rat blastocysts.* Cell, 2008. **135**(7): p. 1287-1298.

76. Li, P., Tong, C., Mehrian-Shai, R*., et al.*, *Germline competent embryonic stem cells derived from rat blastocysts.* Cell, 2008. **135**(7): p. 1299-1310.

77. Miki, T., Yasuda, S. Y., and Kahn, M., *Wnt/beta-catenin signaling in embryonic stem cell self-renewal and somatic cell reprogramming.* Stem Cell Reviews and Reports, 2011. **7**(4): p. 836-846.

78. Takao, Y., Yokota, T., and Koide, H., *Beta-catenin up-regulates nanog expression through interaction with oct-3/4 in embryonic stem cells.* Biochemical and Biophysical Research Communications, 2007. **353**(3): p. 699-705.

79. Li, W. L., Zhou, H. Y., Abujarour, R*., et al.*, *Generation of human-induced pluripotent stem cells in the absence of exogenous sox2.* Stem Cells, 2009. **27**(12): p. 2992-3000.

80. Lin, T., Ambasudhan, R., Yuan, X*., et al.*, *A chemical platform for improved induction of human ipscs.* Nat Meth, 2009. **6**(11): p. 805-808.

81. Kolch, W., *Coordinating erk/mapk signalling through scaffolds and inhibitors.* Nat Rev Mol Cell Biol, 2005. **6**(11): p. 827-837.

82. Burdon, T., Stracey, C., Chambers, I*., et al.*, *Suppression of shp-2 and erk signalling promotes self-renewal of mouse embryonic stem cells.* Developmental Biology, 1999. **210**(1): p. 30-43.

83. Ying, Q.-L., Wray, J., Nichols, J*., et al.*, *The ground state of embryonic stem cell self-renewal.* Nature, 2008. **453**(7194): p. 519-523.

84. Yan, X., Qin, H., Qu, C*., et al.*, *Ips cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin.* Stem Cells and Development, 2010. **19**(4): p. 469-480.

85.   Tat, P. A., Sumer, H., Jones, K. L*., et al.*, *The efficient generation of induced pluripotent stem (ips) cells from adult mouse adipose tissue-derived and neural stem cells.* Cell Transplantation, 2010. **19**(5): p. 525-536.

86.   Morrison, G. M. and Brickman, J. M., *Conserved roles for oct4 homologues in maintaining multipotency during early vertebrate development.* Development, 2006. **133**(10): p. 2011-2022.

87.   Fernandez-Tresguerres, B., Canon, S., Rayon, T*., et al.*, *Evolution of the mammalian embryonic pluripotency gene regulatory network.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(46): p. 19955-19960.

88.   Rajarajan, K., Engels, M. C., and Wu, S. M., *Reprogramming of mouse, rat, pig, and human fibroblasts into ips cells.* Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.], 2012. **Chapter 23**: p. Unit 23.15.

89.   Altschul, S. F., Gish, W., Miller, W*., et al.*, *Basic local alignment search tool.* Journal of Molecular Biology, 1990. **215**(3): p. 403-410.

90.   Liu, L., Wei, X., Ling, J*., et al.*, *Expression pattern of oct-4, sox2, and c-myc in the primary culture of human dental pulp derived cells.* Journal of Endodontics, 2011. **37**(4): p. 466-472.

91.   Govindasamy, V., Abdullah, A. N., Ronald, V. S*., et al.*, *Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth.* Journal of Endodontics, 2010. **36**(9): p. 1504-1515.

92.   Huang, A. H.-C., Chen, Y.-K., Chan, A. W.-S*., et al.*, *Isolation and characterization of human dental pulp stem/stromal cells from nonextracted crown-fractured teeth requiring root canal therapy.* Journal of Endodontics, 2009. **35**(5): p. 673-681.

93.   Sugii, S., Kida, Y., Kawamura, T*., et al.*, *Human and mouse adipose-derived cells support feeder-independent induction of pluripotent stem cells.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(8): p. 3558-3563.