[Frontiers in Bioscience E4, 1813-1822, January 1, 2012]

Conversion of mouse fibroblasts to sphere cells induced by AlbuMAXI-containing medium

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1. ABSTRACT

The reprogramming of fibroblasts to pluripotent stem cells and the direct conversion of fibroblasts to functional neurons has been successfully manipulated by ectopic expression of defined factors. We demonstrate that mouse fibroblasts can be converted into sphere cells by detaching the fibroblast cells by protease and then using the AlbuMAX I-containing culture medium without genetic alteration. AlbuMAX I is a lipid-rich albumin. Albumin-associated lipids arachidonic acid (AA) and pluronic F-68 were responsible for this effect. The converted colonies were positive for both alkaline phosphatase and surface specific embryonic antigen-1 (SSEA-1) staining. Global gene expression analysis indicated that the sphere cells were in an intermediate state compared with mES cells and MEF cells. The sphere cells were able to differentiate into tissues representing all three embryonic germ layers following retinoic acid treatment, and differentiated into smooth muscle cells following treatment with vascular endothelial growth factor (VEGF). The study presented a potential novel approach to transdifferentiate mouse fibroblast cells into other cell lineages mediated by AlbuMAX I-containing culture medium.

2. INTRODUCTION

Lineage commitment used to be considered an irreversible processe during development until it was demonstrated that adult somatic cells can be reprogrammed after fusion with a mature oocyte. Such reprogrammed cells have been used to produce cloned animals of different species (1, 2). Recent studies also shown that the reprogramming of mouse and human fibroblasts to a pluripotent state can be achieved *in vitro* by ectopic expression of defined factors, such as Oct4, Sox2, Nanog, c-Myc, or Klf4 (or Lin28). The DNA methylation, gene expression and chromatin state of such induced pluripotent stem (iPS) cells are very similar to embryonic stem (ES) cells (3, 4, 5, 6, 7). Furthermore, a more recent study has indicated that the expression of neural-lineage-specific transcription factors *Ascl1*, *Brn2* (also called *Pou3f2*) and *Myt1l* can directly convert fibroblasts into functional neurons *in vitro* (8). Mouse fibroblasts can be reprogrammed to an intermediate state of differentiation by chemical induction (9).

These studies demonstrate that fully differentiated cells can reverse their gene expression profile

to that of pluripotent stem cells or other cell lineages through genetic factors or chemical induction.

We report here that using the combination of detaching the fibroblast cells by protease and exposure them in an AlbuMAX I -containing culture medium can convert mouse fibroblast cells into sphere cells with differentiation potential without requiring genetic alteration. AlbuMAX I was isolated from bovine plasma through a chromatographic separation process. It uniquely retained naturally occurring lipids associated with the purified albumin, and is therefore an excellent choice to replace serum in media formulation. AlbuMAX I-containing Knock out Serum Replacer (KSR) medium has been used for the growth and maintenance of undifferentiated stem cells successfully under serum-free conditions (10, 11). ES cells grown in KSR-containing medium differentiate less than those grown in serum-containing medium, and the medium can improve the efficiency of establishing many ES cell lines from blastocysts, as well as increase the success rate of producing chimeric mice (12). Furthermore, KSR-containing medium has been shown to increase the efficiency of iPS cell production using the viral approach (13).

AlbuMAX I is a lipid-rich albumin. Several albumin-associated lipids have been identified in AlbuMAX I. Among them are arachidonic acid (AA) and pluronic F-68. AA is a polyunsaturated omega-6 fatty acid 20:4(ω-6), which is one of the essential fatty acids required by most mammals. This lipid has been marketed as an anabolic bodybuilding supplement. The metabolism of AA through lipoxygenase pathways leads to the generation of several different biologically active eicosanoids, which affect diverse biological processes including cell growth, cell survival, angiogenesis, and wound healing (14). Several studies also have shown that AA induces a calcium influx (15). Basic fibroblast growth factor (bFGF), widely used to prevent stem cell differentiation, can stimulate rapid release of AA (16). Another important albumin-associated lipid is the non-ionic surfactant pluronic F-68. Pluronic F-68 facilitates cell orientation and subsequent collagen synthesis and therefore promotes early wound healing. It has been used in early postsurgical wound healing to facilitate early attachment and enhance the growth rate of human gingival fibroblasts (17). Pluronic F-68 can also improve plant protoplast proliferation, cell proliferation, bud induction and shoot regeneration (18). Albumin-associated lipids have been shown to play a role in regulating human ES cell self-renewal, but little is known about the role of these lipids in cellular reprogramming (19).

3. MATERIALS AND METHODS

3.1. Constitution of the AlbuMAX I–containing Culture Medium

AlbuMAX I-containing stem cell medium consists of basal media and AlbuMAX I (12.5g/L, Invitrogen 11020-021). The basal media contains DMEM/F12 (Invitrogen 11330-032), 1x non-Essential Amino Acids (Invitrogen 11140-050), 1x L-Glutamine (Invitrogen 25030-018), 0.1mM β-mercaptoethanol, 4ng/ml bFGF (13256-029), thiamine (9mg/L, Sigma T1270), reduced L-glutathione (1.5mg/L, Sigma G6013), ascorbic acid -2-PO4 (50mg/L, Sigma A8960), transferrin (8mg/L, Sigma Tobb5), insulin (10mg/L, Sigma I6634), and Recombinant Leukemia inhibitory factor (ESGRO®, Chemicon ESG1107).

The Knockout Serum Replacer (KSR) contains DMEM/F12 (Invitrogen 11330-032), 1x non-Essential Amino Acids (Invitrogen 11140-050), 1x L-Glutamine (Invitrogen 25030-018), 0.1mM β-mercaptoethanol, and 4ng/ml bFGF (13256-029) and 20% Knockout Serum Replacer (10828-028) (20).

3.2. The Procedures of Conversion of Fibroblast Cells into Sphere Cells with AlbuMAX I-containing Medium

Mouse embryonic fibroblast (MEF) cells were ordered from Millipore (EmbryoMax® primary mouse embryo fibroblasts, Neo resistant, not mitomycin C treated, strain FVB/N, passage 3. Cat #PMEF-NL). The YFP Rosa26 MEFs were derived from a mouse carrying a YFP transgene derived from Andras Nagy's ES cell line YC5 mated to the Rosa26 mice originally made by Phil Soriano (21, 22). To convert MEF cells into sphere cells with the AlbuMAX I-containing stem cell medium, the early passage (< 5) of MEF cells were cultured in DMEM Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum (FBS) at 37°C, 10% CO2 in a 10 cm culture plate until confluent. The cells were trypsinized by adding 1.5 ml of trypsin-EDTA (Invitrogen 25200) and removing the trypsin completely within 1 minute. The trypsin-treated cells were incubated at room temperature for 2-3 minutes until they started to detach. The cells were then directly suspended in 3 ml AlbuMAX I-containing medium. The cell suspension was mixed and 0.5 ml of the cell suspension was added (about 10^5) to a 6-well plate, each well containing 3 ml AlbuMAX I-containing medium. The cells were cultured at 37°C, 10% CO_2. The AlbuMAX I -containing medium promoted the aggregation of the small round cells into granulated cells. Some of the granulated cells continued to grow into round, bright-edged sphere cells. The converted colonies could be cultured for many passages with or with out feeder.

3.3. Karyotyping

Standard G-banding chromosome analysis was carried out by the Cell Line Genetics Company (Madison, WI).

3.4. Alkaline-phosphatase (AP) and immunofluorescent staining

An AP detection kit (Chemicon SCR004) was used to examine the stem cell surface marker alkaline-phosphatase expression. The colonies were fixed with 4% paraformaldehyde after incubation for 1 minute. The fixed colonies were rinsed with 1x TBST (20 mM Tris-HCl, pH 7.4, 0.15 NaCl, 0.05% Tween-20) and stained with 0.5 mL Naphthol/Fast Red Violet Solution in the dark at room temperature for 15 minutes. The images were captured by inverted microscope (Olympus CK2) at 20X magnification.

Immunofluorescence staining for SSEA1 stem cell marker. The coverslips with the converted colonies were fixed with Formalde-Fresh (formadehyde 4%W/V, Methanol 1%W/V), permeabilized in PBS containing 1% NP40, and blocked with 10% horse serum for 1 hour. The coverslips with the cells were stained with SSEA 1 (Chemicon MAB4301) at 4^0C overnight. After washing with TBST, the cells were stained with fluorescent–labeled secondary antibody Alexa Fluor 488 (1:400, from Molecular Probes). The coverslips were mounted with Gelmount (Fisher Scientifics). Images were acquired with an inverted microscope (Nikon TS100) at 20X magnification using MetaMorph Imaging Software.

3.5. Microarray analysis

Microarray studies were processed by Asuragen, Inc. MEF, mouse ES cell J11, and two weeks old sphere cells total RNA were isolated from these cell lines, according to the company's standard operating procedures. The purity and quantity of total RNA samples were determined by absorbance readings at 260 and 280 nm using a NanoDrop ND-1000 UV spectrophotometer. The integrity of total RNA was qualified by Agilent Bioanalyzer 2100 capillary electrophoresis. Total RNA (300 ng per sample) was used for preparation of biotin-labeled targets (cRNA) using a MessageAmp™ II-based protocol (Ambion Inc., Austin, TX) and one round of amplification. The cRNA yields were quantified by UV spectrophotometry and the distribution of transcript sizes was assessed using the Agilent Bioanalyzer 2100 capillary electrophoresis system. Labeled cRNA was used to probe MouseWG-6 v1.1 Expression BeadChips, hybridization, washing, and scanning of the Illumina arrays were carried out according to the manufacturer's instructions. Raw data were extracted using Illumina BeadStudio software v 3.0. Following quality assessment, data from the replicate beads on each array were summarized into average intensity values and variances in an Excel report containing the project description (sample key), gene identifiers and corresponding probe IDs, table of detection *p*-values, and table of background-subtracted data. The background subtraction, expression summary, normalization, and log base 2 transformation of gene signals were carried out using Quantile Normalization (23). For statistical analysis, one-way ANOVA was used for multiple group comparison across all samples in the experiment, followed by multiple testing corrections to determine the false discovery rate. Genes with a FDR-adjusted *p*-value of < 0.05 were considered differentially expressed genes (DEG). The raw data for this study has been deposted in GEO (GSE 29277).

3.6. *In vitro* differentiation assay

To investigate whether the sphere cells have a differentiation potential, embryoid body (EB)-like colonies were formed from two weeks converted cells by culturing the cells in AlbuMAX I-containing medium without passing for two weeks. EB-like colonies continued to differentiate on gelatin coated plates and induced by 2μM trans-retinoic acid for an additional 10-15 days. Expression of endoderm-, mesoderm-, and ectoderm-specific markers was examined by using antibodies raised against alpha-fetoprotein (1:100, Sigma Inc. A8452), smooth muscle actin (1:100, Sigma Inc. A5228), and beta-tubulin III (1:100, Sigma, Inc. T5201), and Troponin C (1:100, Santa Cruze SC-48347), respectively, at 4°C overnight. After washing with TBST, the cells were stained with fluorescent–labeled secondary antibody Alexa Fluor 488 (1:400, Invitrogen A31620). The coverslips were mounted with vecta shield (Vector Laboratory, H 1000). Images were acquired with an inverted microscope (Nikon TS100) at 20X magnification using MetaMorph Imaging Software.

For smooth muscle differentiation, EB-like colonies were transferred to collagen IV-coated plate that contained vascular endothelial growth medium which contained 500 ml EGM-2 (Lonza CC-4173), 10 ml FBS (Lonza CC-4101A), 0.2 ml Hydrocortisone (Lonza CC-4112), 2 ml hFGF-B (Lonza CC-4113A), 0.5 ml VEGF (Lonza CC-4114A), 0.5 ml R3-IGF-1 (Lonza CC-4115A), 0.5ml hEGF (Lonza CC-4317A), 0.5 ml GA-1000 (Lonza CC-4381A), 0.5 ml Heparin (Lonza CC-4396A), and supplemented with 50ng/ml vascular endothelial growth factor (VEGF) (494-VE/CF R&D Systems) (24). Two weeks after culturing the converted cells in this medium at 37°C, 10% CO_2, the cell morphologies were examined through immunofluorescent staining with smooth muscle actin antibodies (Sigma Inc. A1978).

4. RESULTS

4.1. AlbuMAX I -containing culture medium promoted the conversion of the fibroblast cells into sphere cells

To investigate the possible role of lipid-rich albumin in promoting the conversion of fibroblast cells into other cell lineages, mouse embryonic fibroblast cells (MEFs) cells were cultured in a fibroblast growth medium until confluent. The cells were detached by trypsin and exposed to the AlbuMAX I-containing medium. Within a few hours, the medium promoted the aggregation of the small round cells into bright edged granulated cells as shown in Figure. 1A. Twenty-four hours later, some of the granulated cells grew into colonies. The conversion efficiency was between 50-80% (sphere cells/total cells). The colonies can be incubated and passed for many passages, with media changed every 3-5 days. The conversion process was repeated by using primary dermal fibroblasts derived from Rosa 26 YFP mouse adult skin indicated that sphere cells were not came from the rare stem cell like cells were present in the mouse embryonic fibroblast (Figure 1B). Neomycin PCR results indicated the converted cells were indeed derived from the Neo resistant MEF cells (Figure 1C). The karyotype of the converted cells indicated that there were no major translocations, amplifications or other chromosomal changes after the conversion (Figure 1D). These results indicate that an AlbuMAX I-containing medium has the ability to convert fibroblasts into sphere cells. In addition, detaching the MEFs with proteases such as trypsin or Accutase before adding the AlbuMAX I-containing medium played a very important role in the conversion process. If the fibroblast growth medium was simply replaced with AlbuMAX I-containing medium without trypsinization, the cells retained a fibroblast morphology

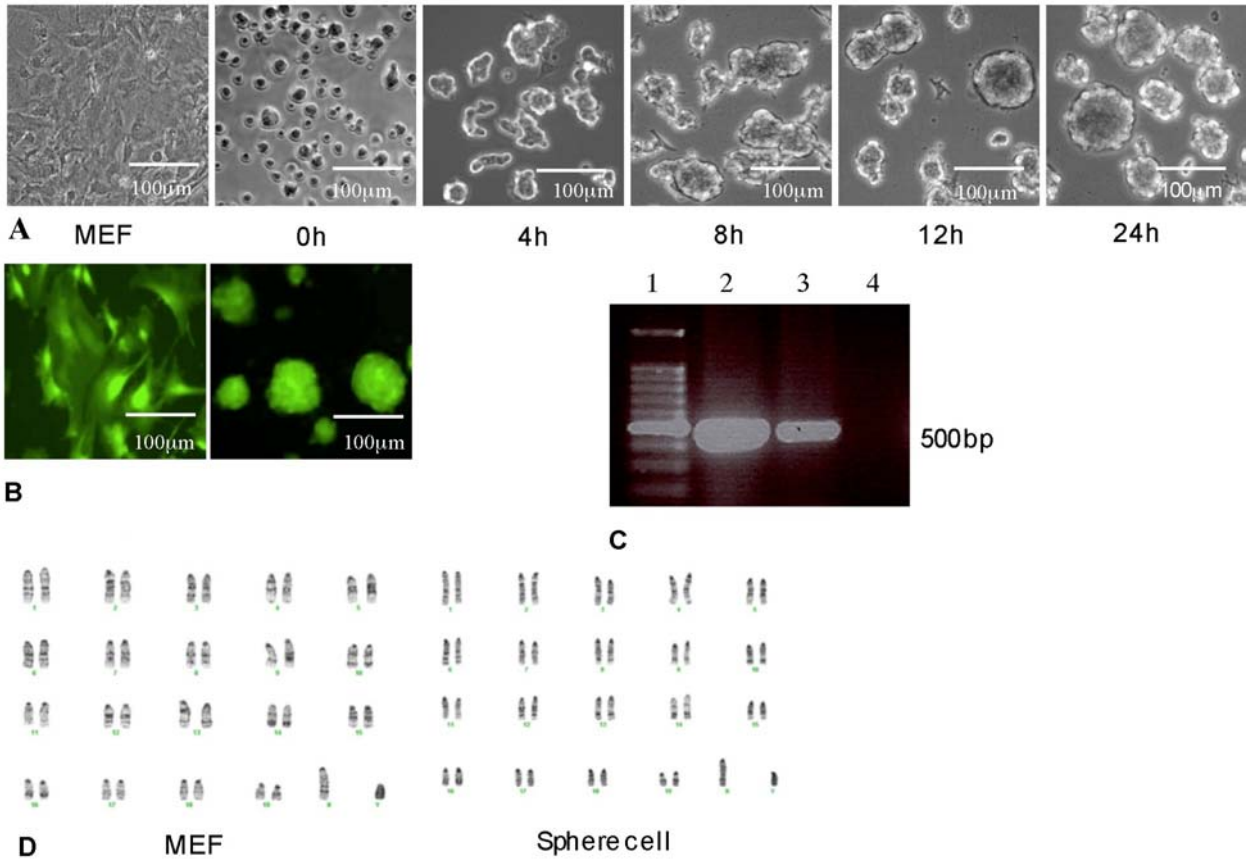

Figure 1. AlbuMAX I-containing medium induced the conversion of fibroblast cells into sphere cells. A. Phase contrast images document the sequential changes in morphology of MEF cells during the conversion process. From the left panel to the right: mouse embryonic fibroblast cell morphology (MEF) cultured in skin fibroblast cell medium (prior to trypsinization) or 0, 4, 8, 12, 24 hours after the trypsin-treatment and exposure to AlbuMAX I-containing medium. The images were acquired by inverted microscope (Nikon TS100) at 10X magnification using MetaMorph Imaging Software**.** B. Primary dermal fibroblasts derived from Rosa 26 GFP mouse adult skin was converted into sphere cells using AlbuMAX I-containing medium. C. Neomycin gene PCR analysis using the primer: Neof: ATTGAACAAGATGGATTGCAC and NeoR: TTCGTCCAGATCATCCTGATCGAC. From left panel to right, molecular weight marker, MEF cells genomic DNA, converted sphere cell line genomic DNA and mouse J11 embryonic stem cell genomic DNA. D. The G-banding chromosome analysis of MEF cells and the converted cells indicated that converted cells have a normal karyotype after conversion with AlbuMAX I-containing medium.

(Figure 2A). The conversion process was also very sensitive to cell density. Cell conversion occured at higher rates in cell densities of 10^5/ml to 10^6 /ml cells in each well of the six-well plate. However, a cell density exceeding 10^7/ml inhibited the conversion process (Figure 2B). Furthermore, the conversion process was also sensitive to serum and gelatin-coated plates. After trypsinization, if the cells were exposed to a small amount of serum-containing medium and then transferred to AlbuMAX I-containing medium, conversion was not observed (Figure 2C). In addition, the conversion appeared to favor the more acidic condition of 10% CO_2 as opposed to 5% CO_2 (result no show).

4.2. Albumin-associated lipids arachidonic acid (AA) and pluronic F-68 were the critical components in AlbuMAX I that are responsible for the conversion effect

An AlbuMAX I-containing medium consists of a combination of basal medium that contains DMEM/F12, non-Essential amino acids, L-Glutamine, beta-mercaptoethanol, thiamine, reduced glutathione, ascorbic acid-2-PO4, transferrin, insulin, trace elements, bFGF and AlbuMAX I (20). To further identify the key components promoting the conversion of MEF cells into sphere cells, we constituted the AlbuMAX I-containing medium and eliminated individual components. Results from these experiments indicated that AlbuMAX I was the critical component to convert the fibroblasts into sphere cells (Table 1).

AlbuMAX I is lipid-rich bovine serum albumin (BSA). To identify if the BSA or the lipids associated with BSA are responsible for the effect of conversion, the same amount of the low-lipid albumin, Cohn fraction V from Roche (9048-46-B) and human recombinant serum albumin (HAS, Valley Biomedical Corporte Inc., HS1021) were added to the basal medium. Low-lipid albumin from Roche or human recombinant serum albumin resulted in a

Table 1. AlbuMAXI is the key component in the medium promoting the conversion of skin fibroblast cells into sphere cells

Ingredient	Thiamine	Reduced Glutathione	Ascorbic acid-2	Transferrin	Insulin	AlbuMAX I	bFGF	Conversion
Concentration in 1X medium (mg/L)	9	1.5	50	8	10	12,500	4ng/ml	
Medium 1	-	+	+	+	+	+	+	Yes
Medium 2	+	-	+	+	-	+	+	Yes
Medium 3	+	+	-	+	+	+	+	Yes
Medium 4	+	+	+	-	+	+	+	Yes
Medium 5	+	+	+	+	-	+	+	Yes
Medium 6	+	+	+	+	+	-	+	**No**
Medium 7	+	+	+	+	+	+	-	Yes

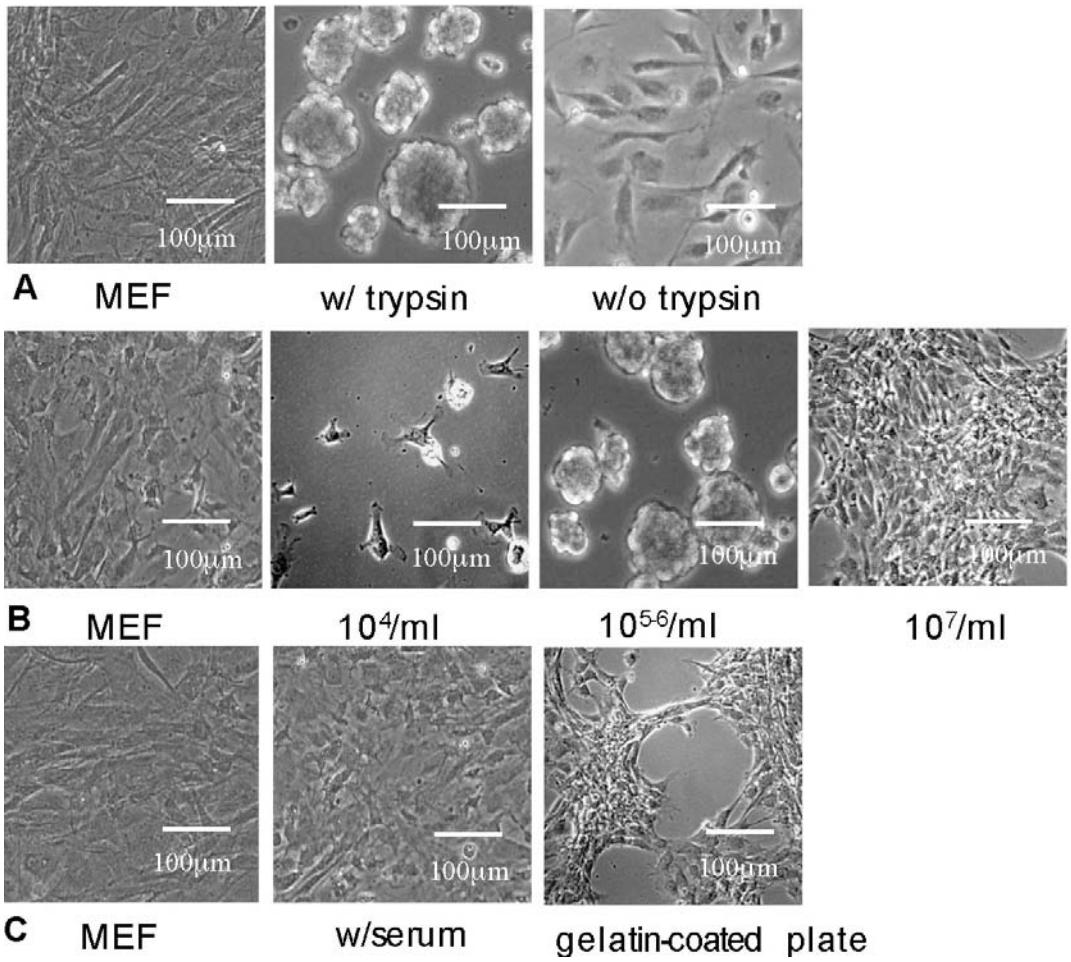

Figure 2. The culture strategy of the conversion process. A. Detachment of the MEF cells with protease is required for conversion with AlbuMAX I-containing medium. Left panel, mouse embryonic fibroblast cell morphology (MEF) cultured in the skin fibroblast cell medium; Middle panel, MEF cells converted into sphere cells 24 hours later after trypsinization and cultured in AlbuMAX I-containing medium. Right panel, the cells retained the skin fibroblast cell morphology if the skin fibroblast medium was simply replaced with AlbuMAX I-containing medium without trypsinization. B. The conversion process was sensitive to cell density. From left panel to right, the skin fibroblast cells were cultured in the skin fibroblast cell medium; and seeded at 10^4/ml; 10^5/ml, 10^6/ml in AlbuMAX I-containing medium**.** C. The conversion process was sensitive to serum and gelatin. The left panel, the skin fibroblast cells cultured in the skin fibroblast cell medium; middle panel, MEF cells were exposed to the medium that contained serum, and then transferred to the AlbuMAX I-containing medium; the right panel, MEF cells were seeded in a gelatin coated plate.

very limited occurrence of conversion (Figure 3A). The results indicated that albumin-associated lipids, not the albumin apoprotein, were responsible for the conversion effect. The next task was to identify the active lipids in AlbuMAX I responsible for the conversion. The low-lipid BSA was supplemented with several albumin-associated

Table 2. The pluripotency gene expression profiles in mouse sphere cells compared with MEF cells and mES cells

Accession	Symbol	MEF cells	mESC	Sphere cells	p-value (ANOVA)*
AB093574	Nanog	11.84894582	13.10859343	13.16134754	1.7246E-06
NM_028610	Dppa4	8.05944685	9.704797769	8.321144926	6.75217E-06
NM_181548	Eras	9.183113901	10.3528649	10.28675273	0.000265692
NM_009235	Sox 15	6.959398906	7.890107949	7.587105102	0.002638022
NM_015798	Fbxo15	11.18681274	11.84775424	11.9980483	0.008136962
NM_139218	Dppa3 / Stella	5.639786313	5.932065446	5.889343914	0.06397259
NM_031261	Fthl17	5.997181891	5.992566217	5.992566217	0.233925377

* The Analysis of variance

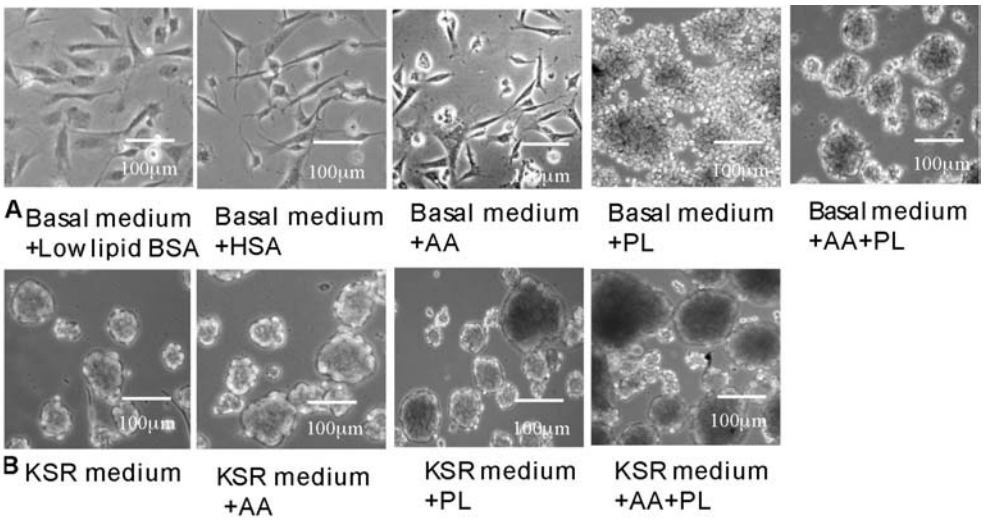

Figure 3. Albumin-associated lipids in AlbuMAX I was the critical components for conversion effect. A. From the left panel to the right**:** MEF cells were cultured in basal medium with low-lipid BSA; MEF cells were cultured in basal medium with human recombinant serum albumin; MEF cells were cultured in basal medium low-lipid BSA and supplemented with arachidonic acid (AA); MEF cells were cultured in basal medium low-lipid BSA and supplemented with pluronic F-68 (PL); MEF cells were cultured in basal medium low-lipid BSA and supplemented with arachidonic acid and pluronic F-68. B. Conversion can be recapitulated with KSR stem cell culture medium and supplemented the medium with Albumin-associated lipids enhanced the KSR conversion. From the left panel to the right**:** MEF cells were cultured in KSR medium; MEF cells were cultured in KSR medium supplemented with arachidonic acid (AA); MEF cells were cultured in KSR medium supplemented with pluronic F-68; MEF cells were cultured in KSR medium supplemented with arachidonic acid and pluronic F-68.

lipids with the concentration similar to naturally retained lipids associated with the purified albumin, such as arachidonic acid (AA) (2 mg/L), stearic acid (10mg/L), myristic acid (10mg/L), linoleic acid (10mg/L), oleic acid (10 mg/L), palmitic acid (10 mg/L), palmitoleic acid (10 mg/L), and a non-ionic surfactant pluronic F-68 (7.5g/L). The results indicated that pluronic acid F-68 has the ability to promote aggregation of the detached cells (Figure 3A). Addition of AA promoted the aggregated cells to form bright and shiny edge colonies similar to the AlbuMAX I-induced colonies. The conversion effect can be fully recapitulated with KSR stem cell culture medium form Invitrogen which contains AlbuMAX I. Supplemented KSR with AA (2 mg/L), and pluronic acid F-68 (7.5g/L) enhanced the conversion effect (Figure 3B).

4.3. Characterization of the Gene Expression Profile of Sphere Cells Induced by AlbuMAX I-Containing Medium

Up-regulation of fibroblast growth factor receptors 3 (FGFR3) plays an important role in the reprogramming of primordial germ cells into stem cells (25). We observed the up-regulation of the expression of fibroblast growth factor receptor 3 (FGFR3) in a time-dependent manner after transferring the fibroblast cells into the AlbuMAX I-containing medium (Figure 4A). Furthermore, the early pluripotent surface specific embryonic antigen-1 (SSEA-1) was up-regulated one day after the fibroblast cells exposure AlbuMAX I -containing medium (Figure 4B). The converted colonies were positive for both alkaline phosphatase and SSEA-1 staining (Figure 4C). To further characterize the gene expression profile of the sphere cells, the Illumina Microarray global gene expression analysis of the sphere cells compared with mES cells and MEF cells indicated that the sphere cells gene expression was relatively different from both mES cells and MEF. The results indicated that the AlbuMAX I-containing medium indeed promoted the changes of gene expression in fibroblast cells to an intermediate state. In addition, we examined the expression profiles of the 24 genes identified by Yamanaka as being related to induction of pluripotency (3). Seven of 24 genes were expressed with similar regulation between mESC and sphere cells; among them, the Nanog gene is up regulated in sphere cells compared with MEF cells, but the expression of Oct4 were not changed in sphere cells compared with MEF cells (Table 2).

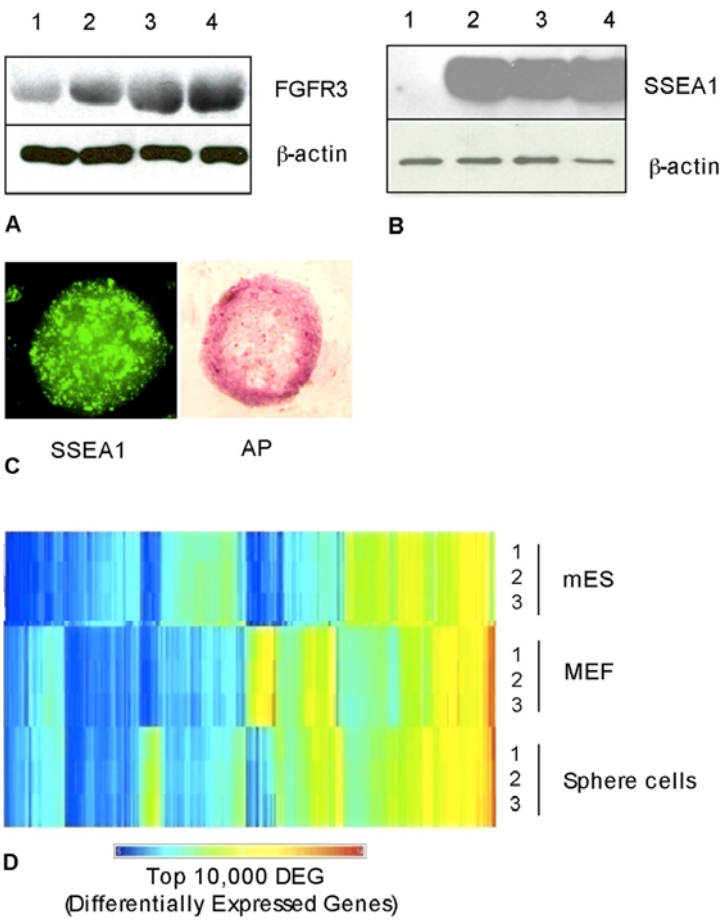

Figure 4. Characterization of the converted cells induced with AlbuMAX I-containing culture medium. A. FGF Receptor 3 was up-regulated induced by AlbuMAX I-containing medium. Western blotting of FGF Receptor 3 protein expression after transferring the fibroblast cells into AlbuMAX I-containing medium. Lane 1, 4 hours after culture in the medium; Lane 2, 8 hours after culture in the medium; Lane 3, 12 hours after culture in the medium; Lane 4, 24 hours after culture in the AlbuMAX I-containing medium. beta-actin was used as a loading control. B. Western blotting probed with stem cell marker SSEA1 (Chemicon MAB4301). Lane 1, cell lysate of fibroblast cell. Lane 2, cell lysate of 1 day old sphere cells. Lane 3, cell lysate of 2 day old sphere cells. Lane 4, cell lysate of a mouse embryonic stem cell. beta-actin was used as a loading control. C. Alkaline-phosphatase (AP) and Immunofluorescent staining for stem cell marker SSEA1. Left panel is AP staining, right panel is SSEA1 staining. D. The heat map of gene expression array comparing 2 weeks old sphere cells to MEF cells and mouse embryonic stem cells indicated that gene expression in sphere was different from MEF cells and mES cells.

4.4. The differentiation potential of the sphere cells

To investigate if the sphere cells induced by an AlbuMAX I-containing medium have developmental potential, we performed differentiation assays with sphere cells. Two week old sphere cells were cultured in AlbuMAX I-containing medium without FGF, and embryoid bodies formed. The embryoid bodies can be induced to differentiate into cell types normally derived from the three embryonic germ layers by 2μM trans-retinoic acid for an additional 10 days on gelatin coated plates. The expression of endoderm-, mesoderm-, and ectoderm-specific markers was confirmed by using antibodies raised against alpha-fetoprotein, smooth muscle actin, and beta-tubulin III, respectively, indicating that these sphere cells have the capacity to differentiate into different cell types (Figure 5A). In addition, the sphere cells can also be differentiated into smooth muscle cells after transferring to collagen VI-coated plate that contained EGM-2 vascular endothelial growth medium supplemented with 50ng/ml vascular endothelial growth factor (VEGF) (24). Two weeks after culturing the converted cells in this medium, the cell morphology and protein expression was examined by immunofluorescent staining and western blot with smooth muscle actin antibodies (Figure. 5B &5C).

5. DISCUSSION

We have demonstrated for the first time that a lipid-rich albumin-containing medium has the ability to convert mouse embryonic fibroblast cells into sphere cells, and these sphere cells have the potential to differentiate into other cell lineages. Sphere cells could be the intermediate early stage of iPS cells. As the sphere cells continue to be passed in the AlbuMAX I-containing

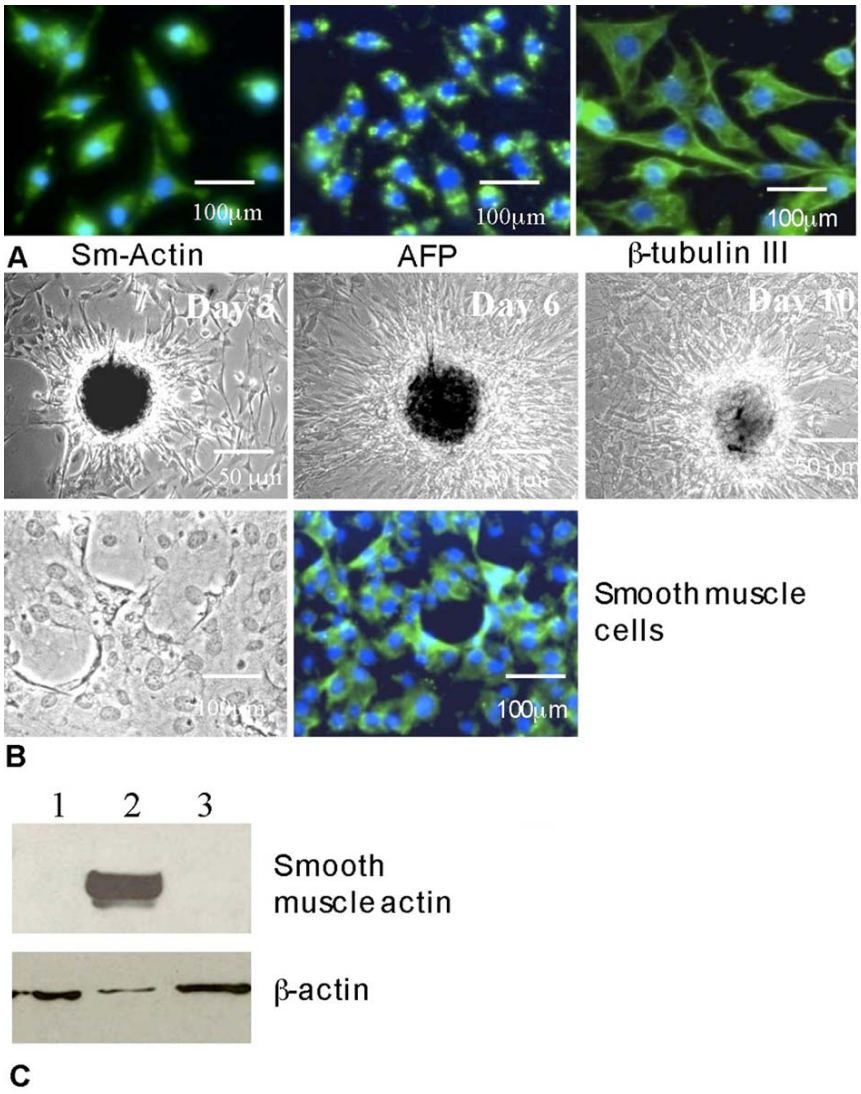

Figure 5. Differentiation potential of converted sphere cells. A. Embryoid bodies derived from sphere cells differentiated *in vitro* into three germ layers, induced by retinoic acid. Differentiated cells were stained with differentiation markers: endoderm alpha-fetoprotein (AFP), mesoderm smooth muscle (SM-Actin) (middle), and ectoderm beta-tubulin III. B. Sphere cells differentiated into smooth muscle cells induced by VEGF. Upper panel: The process of the differentiation. Lower panel: The cell morphology of smooth muscle was examined by immunofluorescent staining with smooth muscle actin antibodies. Images were acquired with an inverted microscope (Nikon TS100) at 20X magnification using MetaMorph Imaging Software. C. Smooth muscle actin expression was examined by Western blot analysis with smooth muscle actin antibodies. Lane 1, Cell lysate of MEF cells; Lane 2, Cell lysate of smooth muscle differentiated from sphere cells; Lane 3, cell lysate of mouse embryonic stem cell. beta-actin used as a loading control.

medium, a stable cell line can be established. The established stable cells expressed the same level of Oct4, Sox2 and Nang as mouse embryonic stem cells, and exhibited morphology similar to that of ES cells. The established stable cells were able to proliferate well, as shown in Figure 6. Over the course of this project, we have noticed that only a few conversions can result in establishing stable cell lines, with the majority of converted cells remaining in an intermediate stage (sphere cells), which may be due to the cells being partially reprogrammed. The partially reprogrammed intermediate cells did not express pluripotent proteins, like Oct4. This result indicated that less stringent Nanog and some other "stemness genes" might be sufficient to reprogram differentiated cells to an intermediate state, Oct 4 could be the ultimate factor that brings the cells to the complete state, a conclusion that is consistent with Dr Tian's funding (9). Interestingly, the partially reprogrammed cells can be differentiated into other cell lineages *in vitro*. The avenue of promoting the fully reprogramming of the sphere cells into iPS cells remains to be explored.

The conversion efficiency of MEFs to sphere cells is between 50-80% (sphere cells/total cells). Most of

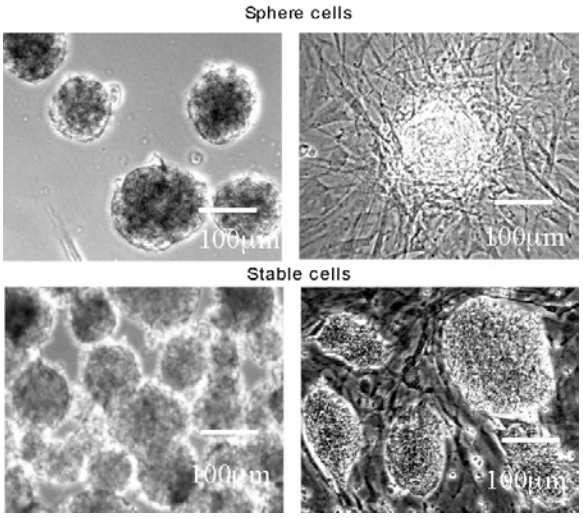

Figure 6. The morphology of the sphere cells and stable cell line cells. Phase contrast images of sphere cells and stable cell line cells were cultured in AlbuMAXI-containing medium with (right column) or without (left column) a feeder layer. Stable cells exhibited morphology similar to that of embryonic stem cells.

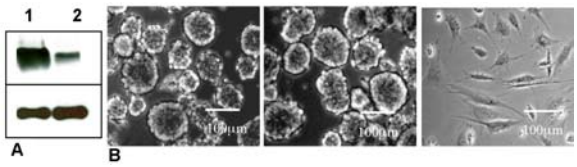

Figure 7. Reprogramming process and FGF Receptor 3 up-regulated induced by AlbuMAX I-containing medium were inhibited by Thapsigargin, a Ca^{2+}ATPase inhibitor. A. Thapsigargin (1ug/ml) inhibited the FGF receptor 3 expression. Lane 1, 24 hours after culture in the AlbuMAX I-containing medium; Lane 2, 24 hours after culture in the AlbuMAX I-containing medium with Thapsigargin (1ug/ml). beta-actin was used as a loading control.B. Thapsigargin (1μg/ml) inhibited the conversion of fibroblast cells into sphere cells. Left, AlbuMAX I-containing medium; Center, AlbuMAX I-containing medium with 1.5ul of DMSO; Right, AlbuMAX I-containing medium with Thapsigargin (1μg/ml), 1000X Thapsigargin stock was dissolved in DMSO. Images were acquired in the inverted microscope (Nikon TS100) at 10X magnification using the MetaMorph Imaging Software. Scale bar 100μm.

the MEFs that did not convert into sphere cells underwent apoptosis. We speculate that the conversion efficiency might be due to different stages of fibroblast cell cycle. We will answer the question through the synchronization of skin fibroblast cells at the different cell cycle stages in the future. In addition, the molecular mechanism of the reprogramming is unclear at this time. Since AlbuMAX I can stimulate rapid Ca^{2+} release from the sarcoplasmic (15), we speculate that the Ca^{2+} influx induced by AlbuMAX I might play an important role in the conversion process. Thapsigargin, a tight-binding inhibitor for sarco/endoplasmic reticulum Ca^{2+} ATPase was added to the AlbuMAX I-containing medium at a concentration of 1μg/ml (26). As shown in Figure 7, FGFR3 expression and the conversion of fibroblast cells into sphere cells were inhibited by Thapsigargin. These results suggested that the Ca^{2+} influx induced by AlbuMAX I might play a very important role in up regulation of the FGF signaling pathways during the conversion process.

Reprogramming with lipid rich albumin could be a revolutionary method for reprogramming or transdifferentiating one cell lineage to another cell lineage in an easy and low-cost manner without genetic alteration. The methodology described has the potential to greatly accelerate cell therapy research.

6. ACKNOWLEDGEMENTS

The project is funded by University of Dayton seed grant. The authors are indebted to Panagiotis A. Tsonis, Mary S. Connolly, John J. Rowe, and Jayne B. Robinson, Jay Johnson, Mathew E. Willenbrink, Michael V. McCabe and George Kouns for the valuable discussion and comments.

7. REFERENCES

1. Wilmut Ian, Schnieke Angelica E, McWhir Jim. Kind Alexander JH, Campbell Keith HS: Viable offspring derived from fetal and adult mammalian cells. *Nature* 385: 810-813 (1997).

2. Wakayama Teruhiko, Perry Ashlee CF, Zuccotti Maurizio DM, Johnson Ken R, and Yanagimachi Ryuzo: Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 394:369-374 (1998).

3. Takahashi Kazutoshi, Yamanaka Shinya Y: Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126 : 663-676 (2006).

4. Okita Keisuke, Ichiska Tomoko, and Yamanaka Shinya Y: Generation of germline-competent induced pluripotent stem cells. *Nature* 148: 313-317 (2007).

5. Wernig Marius D, Meissner Alex, Foreman Ruth K, Brambrink Tobias, Ku Manching Ching, Konrad Hochedlinger, Bernstein Bradley E, Jaenisch Rudolf: *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448: 318-324 (2007).

6. Takahashi Kazutoshi, Tanabe Koji, Ohnuki Mari, Narita Megumi, Ichisaka Tomoko, Yamanaka Shinya Y: Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131: 861-872 (2007).

7. Yu Junying, Vodyanik Maxim A, Smuga-Otto Kim, Antosiewicz-Bourget Jessica E, Frane Jennifer L, Tian Shulan, Nie Jeff and Thomson James AM: Induced

pluripotent stem cell lines derived from human somatic cells. *Science* 1917-1920 (2007).

8. Vierbuchen Thomas, Ostermeier Austin, Pang Zhiping P, Kokubu Yuko, Südhof Thomas C, Wernig Marius: Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463:1035-1041 (2010).

9. Joonghoo Park, Chul Kim, Yong Tang, Tomokazu Amano, Chih-Jen Lin, X. Cindy Tian: Reprogramming of mouse fibroblasts to an intermediate state of differentiation by chemical induction. *Cell Reprogram.* 13(2):121-131 (2011).

10. Skottman Heli, Stromberg Anne Marie, Matilainen Eija, Inzunza Jose, Hovatta Outi LL and Lahesmaa Riitta: Unique gene expression signature by human embryonic stem cells cultured under serum-free conditions correlates with their enhanced and prolonged growth in an undifferentiated stage. *Stem Cell* 24: 151-167 (2006).

11. Sperger Jamie M, Chen Xin Yuan, Draper Jonathan S, Antosiewicz Jessica E, Chon Christopher H, Jones Sunita B, Brooks James D, Thomson James Alexander: Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proc. Natl. Acad. Sci. U.S.A.*100:13350-13355 (2003).

12. Cheng Jun Sheng, Dutra Amalia S, Takesono Aya, Garrett-Beal Lisa J, Schwartzberg Pamela L: Improved generation of C57BL/6J Mouse Embryonic stem cells in a defined serum-free media. *Genetics* 39:100-104 (2004).

13. Okada Minoru, Oka Masahiro and Yoneda Yoshihiro. Effective culture conditions for the induction of pluripotent stem cells. *Biochem Biophys Acta.* 9:956-963 (2010).
14. Bazan Haydee EP. Cellular and molecular events in corneal wound healing: significance of lipid signaling. *Exp Eye Res*. 80:453-63 (2005).

15. Erriquez Jessica, Gilardino Alessandra, Ariano Paolo, Munaron Luca, Lovisolo Davide, Distasi Carla: Calcium signals activated by arachidonic acid in embryonic chick ciliary ganglion neurons. *Neurosignals* 14 (5):244-54 (2005).

16. Fafeur VeRonique, Jiang Zhi Ping, Böhlen Peter: Signal transduction by bFGF, but not TGF beta 1, involves arachidonic acid metabolism in endothelial cells. *J Cell Physiol*. 149 (2):277-83 (1991).

17. Hokett Steven D, Cuenin Michael F, O'Neal Robert B, Brennan William A, Strong Scott L, Runner Royce R, McPherson James C, and Van Dyke Thomas E: Pluronic Polyol effects on human gingival fibroblast attachment and growth. *Journal of Periodontology* 71: 803-809 (2000).

18. Kumar V, Laouar Leila, Davey MR, Mulligan Bernard J, Lowe KC: Effects of Pluronic F-68 on callus growth and protoplast plating efficiency of Solanum dulcamara. *Plant Cell Reports* 10:52-54 (1991)

19. Garcia-Gonzalo Francesc R, Belmonte, JCI: Albumin-associated lipids regulate human embryonic stem cells self-renewal. *PLos One* 1:1384-1397 (2008).

20. Price P, Goldsborough M, Tikins ML: Embryonic stem cell serum replacer. PCT/US98/00467:WO 98/30679.

21. Glenn Friedrich and Philippe Soriano: Promoter traps in embryonic stem cells: A genetic screen to identify and mutate developmental genes in mice. *Genes Dev* 5:1513-1523 (1991).

22. Anna-Katerina Hadjantonakis and Andras Nagy: FACS for the isolation of individual cells from transgenic mice harboring a fluorescent reporter protein. *Genesis* 27:95-98 (2000).

23. Bolstad Benjamin M, Irizarry Rafael A, Astrand Magnus, Speed, Terence P: A comparison of normalization methods for high density oligonucleotide array data based on bias and variance. *Bioinformatics* 19:185-193 (2003).

24. Schenke-Layland Katja, Rhodes Katrin E, Angelis Ekaterini, Butylkova Yekaterina, Heydarkhan-Hagvall Sepideh, Gekas Christos Ch, Zhang Rui Ming, and Maclellan William Robb: Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and hematopoietic lineages. *Stem Cell* 26:1537-1546 (2008).

25. Durcova-Hills Gabriela, Adams Ian R., Barton Sheila C, Surani M Azim, McLaren Anne: The role of exogenous fibroblast growth factor-2 on the reprogramming of primordial germ cells into pluripotent stem cells. *Stem Cell* 24: 1441-9 (2006) .

26. Mircetic Marko, Casado Natalia: Thapsigargin deplete calcium stores within the endoplasmic reticulum and decreases the peak EPSPs achieved in associated muscle cells after extended periods of high frequency stimulation. Pioneering Neuroscience 3: 27-30 (2003).

Abbreviations: AA: arachidonic acid, VEGF: vascular endothelial growth factor, FGF: Fibroblast growth factor, iPS cell: induced pluripotent stem cells, ES cell: embryonic stem cells, FGFR3: fibroblast growth factor receptos 3, KSR: Knockout Serum Replacer.

Key Words: Reprogramming, Transdifferentiation, Intermediate Cell, Sphere cell, AlbuMAX I-containing medium, KSR

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