CLONING AND STEM CELLS
Volume 11, Number 3, 2009

DOI: 10.1089/clo.2009.0015

Original Article

Induction of Stem Cell Gene Expression in Adult Human Fibroblasts without Transgenes

Raymond L. Page,[1,2,3,4] Sakthikumar Ambady,[1,2] William F. Holmes,[2] Lucy Vilner,[1] Denis Kole,[2] Olga Kashpur,[2] Victoria Huntress,[2] Ina Vojtic,[1] Holly Whitton,[2] and Tanja Dominko[1,2,4]

Abstract

Reprogramming of differentiated somatic cells into induced pluripotent stem (iPS) cells has potential for derivation of patient-specific cells for therapy as well as for development of models with which to study disease progression. Derivation of iPS cells from human somatic cells has been achieved by viral transduction of human fibroblasts with early developmental genes. Because forced expression of these genes by viral transduction results in transgene integration with unknown and unpredictable potential mutagenic effects, identification of cell culture conditions that can induce endogenous expression of these genes is desirable. Here we show that primary adult human fibroblasts have basal expression of mRNA for OCT4, SOX2, and NANOG. However, translation of these messages into detectable proteins and their subcellular localization depends on cell culture conditions. Manipulation of oxygen concentration and FGF2 supplementation can modulate expression of some pluripotency related genes at the transcriptional, translational, and cellular localization level. Changing cell culture condition parameters led to expression of REX1, potentiation of expression of LIN28, translation of OCT4, SOX2, and NANOG, and translocation of these transcription factors to the cell nucleus. We also show that culture conditions affect the *in vitro* lifespan of dermal fibroblasts, nearly doubling the number of population doublings before the cells reach replicative senescence. Our results suggest that it is possible to induce and manipulate endogenous expression of stem cell genes in somatic cells without genetic manipulation, but this short-term induction may not be sufficient for acquisition of true pluripotency. Further investigation of the factors involved in inducing this response could lead to discovery of defined culture conditions capable of altering cell fate *in vitro*. This would alleviate the need for forced expression by transgenesis, thus eliminating the risk of mutagenic effects due to genetic manipulation.

Introduction

Nuclear transplantation into oocytes (Campbell et al., 2007), hybrid formation with embryonic stem cells and embryonic carcinoma cells (Taranger et al., 2005), exposure to extracts from pluripotent cell types (Byrne et al., 2003; Miyamoto et al., 2007; Tada et al., 2001), and most recently viral transduction of stem cell genes coding for transcription factors (Maherali et al., 2007; Meissner et al., 2007; Takahashi et al., 2007b; Yu et al., 2007) have demonstrated that the developmental program of an adult somatic cell can be reversed. It has been shown that transfection of genes coding for transcription factors into fibroblasts may be sufficient to induce pluripotency in both mouse (Takahashi et al., 2007a) and human (Takahashi et al., 2007b; Yu et al., 2007) fibroblasts. This achievement has groundbreaking implications for cell therapy research because cells with nearly equivalent differentiation potential as embryonic stem cells (ESC) could be created from an autologous patient-specific source. Activation of OCT4, SOX2, NANOG, and LIN28 appears to be sufficient to set in motion a cascade of molecular events leading to acquisition of pluripotency with characteristics of ESCs (Yu et al., 2007). Transfection of OCT4 and SOX2 was absolutely required to achieve this transformation, whereas the addition of NANOG was beneficial, but not essential and alone not capable of achieving pluripotency (Yu et al., 2007). Interestingly, Takahashi et al. (2007b) showed that pluripotency could be induced in mouse fibroblasts by transduction with a different set of four factors, but included OCT4 and SOX2. Recently, pluripotency was achieved by addition of the histone deacetylase inhibitor valproic acid along with only OCT4 and SOX2 expressing trasngenes (Huangfu et al., 2008).

[1]CellThera, Inc., Worcester, Massachusetts.
[2]Department of Biology and Biotechnology, [3]Department of Biomedical Engineering, [4]Bioengineering Institute, Worcester Polytechnic Institute, Worcester, Massachusetts.

Maintenance of inherent (ESC) or induced [induced pluripotent stem (iPS)] pluripotency in human cells depends on continuous presence of FGF2 (Levenstein et al., 2006), as its withdrawal leads to spontaneous cell differentiation (Diecke et al., 2008). FGF2 induces differential expression of members of the transforming growth factor (TGF)-β family of proteins. Upregulation of TGF-β1, activin A (TGF-β receptor and ALK receptor ligand, respectively) and gremlin1 (BMP4 inhibitor), and downregulation of BMP4 leads to SMAD2/3-driven expression of OCT4, SOX2, and NANOG (Suzuki et al., 2006). These three transcription factors co-occupy promoters of several genes, including FGF2. Increased expression of FGF2 completes this autoregulatory loop that is perpetuated in the presence of exogenous FGF2. Interestingly, a very similar effect of FGF2 on expression of the TGF-β family members was observed in fibroblasts used to support growth and maintenance of hESCs (Greber et al., 2007a). In addition to supplementation with FGF2, reducing oxygen concentration during culture is becoming increasingly more appreciated in hESC laboratories (Ezashi et al., 2005). Adult and embryonic stem cells cultured in a reduced oxygen atmosphere have been shown to maintain their undifferentiated state more efficiently and increase the efficiency of their differentiation upon induction (Chakravarthy et al., 2001; Covello et al., 2006; Fink et al., 2004; Forsyth et al., 2006; Grayson et al., 2006, 2007; Pistollato et al., 2007). However, the effects of a combination of these easily controllable factors on the phenotype of cultured human adult fibroblasts have not been investigated. Therefore, we sought to evaluate the potential effects of these factors on the *in vitro* life span of primary human fibroblasts, and on the expression and localization of genes often associated with pluripotency.

Materials and Methods

Cell culture

Primary adult human dermal fibroblasts from connective tissue isolated from tissue biopsy from a below-knee amputation of a 24-year-old male (CRL-2352) were obtained from American Tissue Culture Collection (ATCC; Manassas, VA) at passage 2. Cells were cultured in medium consisting of DMEM:Ham's F12 (60:40, MediaTech) with 10% Fetalclone III (Hyclone, Logan, UT). The DMEM (without L-Gln or phenol red) was supplemented with 4 mM fresh L-Gln (MediaTech, Manassas, VA) prior to use. Cultures were carried out in a 37°C incubator in a humidified environment of 5% CO_2, 5% O_2, and 90% N_2. The number of population doublings was calculated as $\log_2$ (#final/#initial). Cells were seeded at 100,000 cells per T25 flask at each passage (Falcon, Oxnard, CA). When used, human recombinant FGF2 (Chemicon, Temecula, CA, or Protide, Lake Zurich, IL) and BMP-2 (R&D Systems, Minneapolis, MN) was supplemented into medium at 4 and 1 ng/mL, respectively. Human muscle fibroblasts were derived from surplus muscle tissue from the calf flexor muscle used for a surgical knee repair in a 59-year-old adult male (CT0706). Tissue was rinsed in Leibowitz L-15 medium (MediaTech) containing 10 μg/mL gentamicin (Invitrogen, Carlsbad, CA) and 2.5 μg/mL fungizone (Hyclone) and minced using a sterile scalpel and digested with 1800 units/mL collagenase Type IV for 1 h at 37°C. Cell lines were established by culture at 5% O_2 from the beginning and working stocks cryopreserved at passage 2. Teratocarcinoma cells (CRL-2073) were grown as recommended by the supplier (ATCC). Human embryonic stem cells (H9, WiCell, Madison, WI) were cultured on mitomycin C-treated mouse embryonic fibroblasts seeded onto 0.1% gelatin coated six-well plates using 80% Knockout™ DMEM (Invitrogen), 20% Knockout™ serum replacement supplemented with 2.0 mM L-Gln, 0.055 mM 2-mercaptoethanol, and 4.0 ng/mL FGF2, as recommended by the supplier.

Immunocytochemistry

Fibroblasts were seeded into 24-well plates (BD Falcon) onto 12-mm round glass coverslips (VWR, West Chester, PA) at 5000 cells per well in medium consisting of DMEM with 4 mM fresh L-Gln: Ham's F12 medium (60:40) supplemented with 1× TCH serum replacement (Protide Pharmaceuticals), 1X ITS-X (Invitrogen), 2% FetalClone III (Hyclone) and cultured at 37°C, 5% CO_2, 5% O_2 and 90% N_2. For FGF2 treatments, FGF2 (4 ng/mL) was added to the medium at the time of seeding. After 7 days, the cells were washed with DPBS w/o Ca/Mg (Mediatech) and fixed in methanol (−20°C) for 10 min, washed with DPBS, and stored in DPBS at 4°C until use. Cells were washed with phosphate-buffered saline (PBS)/Tween (PBS, MediaTech, with 0.05% Tween-20, Biorad, Hercules, CA) and blocked for 30 min at room temperature with PBS containing 0.05% Tween, 5% fetal bovine serum (FBS), and 1% bovine serum albumin (BSA). Primary antibodies (2.5 μg/mL) were added in blocking solution for 30 min at room temperature. Cells were washed four times in PBS/Tween and Alexafluor-568 labeled appropriate secondary antibody (4 μg/mL) in blocking solution added for 30 min. Cells were washed four times in PBS/Tween and stored in PBS at 4°C until image analysis. Antibodies used were: OCT4 (Abcam, Cambridge, MA, ab19857), SOX2 (Abcam, ab15830), NANOG (Abcam, ab21624), FGFR-1 (Abgent, San Diego, CA, AP7636a), FGFR-2 (Abgent, AP7636a), FGFR-3 (Abcam ab10651), FGFR-4 (Abcam ab 41948), FGF2 (Santa Cruz, Santa Cruz, CA, sc-1390 or sc-79). Coverslips were removed and mounted onto glass slides in 80% glycerol in PBS containing 0.1% Na-Azide and sealed with nail polish. Cells were visualized using an Olympus IX81 inverted microscope with epi-fluorescence using appropriate filters (Semrock, Inc., Rochester, NY) and phase contrast. Images were collected using a 12 bit Hamamatzu CCD camera and processed using Slidebook®.

Western blotting

Total protein was isolated from subconfluent fibroblasts with RIPA cell lysis buffer (Santa Cruz Biotechnology), supplemented with complete protease inhibitor cocktail (PIC, Santa Cruz Biotechnology) and 1 mM DTT. Lysates were incubated on ice for 30 min and vortexed every 10 min. Lysates were centrifuged at 13,000×g and supernatants stored at −80°C. Protein concentration was determined with Quant-iT protein assay kit (Invitrogen). Equal amounts of protein supernatant and denaturing 2× sample buffer (BioRad Laboratories) were mixed and heated to 95°C for 3 min. Proteins were separated on 4–20% gradient SDS-PAGE gels and transferred to nitrocellulose membranes (BioRad Laboratories) using Towbins transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.037% SDS). The membranes were blocked with Tween-Tris-buffered saline (TTBS: 25 mM Tris,

TABLE 1. DNA SEQUENCES USED FOR RT-PCR PRIMERS

Target gene	*Forward primer*	*Reverse primer*	*Amplicon size (bp)*	*Genomic size (bp)*	*Accession number*
OCT4	5′-GTTGATCCTCGGACCTGGCTA-3′	5′-GGTTGCCTCTCACTCGGTTCT-3′	646	5339	NM_002701
NANOG	5′-GTCTTCTGCTGAGATGCCTCACA-3′	5′-CTTCTGCGTCACACCATTGCTAT-3′	387	4811	NM_024865
KLF4	5′-CGATCAGATGCAGCCGCAAGTC-3′	5′-TGTGTAAGGCGAGGTGGTCCGA-3′	365	1545	NM_004235
TERT	5′-GCTTCCTCAGGAACACCAAGA-3′	5′-TGCAACTTGCTCCAGACACTC-3′	298	1657	NM_198253
REX1	5′-GGAGGAATACCTGGCATTGAC-3′	5′-CGGCTTCTCTCCAGTATGAAC-3′	270	270[a]	NM_174900
FGFR1	5′-TGAGTACGGCAGCATCAACCACAC-3′	5′-CCAGAACGGTCAACCATGCAGAGT-3′	377	4342	NM_023110
FGFR2	5′-CAATCACACGTACCACCTGGATG-3′	5′-GTCTGGCTTCTTGGTCGTGTTCT-3′	508	23,401	NM_000141
FGFR3	5′ -AGTGGCTCAAGCACGTGGAGGT-3′	5′-GAGCTCATGGACGCGTTGGACT-3′	442	106	NM_000142
FGFR4	5′-GCAATTCCATCGGCCTCTCCTA-3′	5′-TTGACTTGCCGGAAGAGCCTGA-3′	279	706	NM_002011
LIN28	5′-GGTTCGGCTTCCTGTCCATGA-3′	5′-GGTGGCAGCTTGCATTCCTTG-3′	313	14,769	NM_024674
FGF2	5′-CTGGCTATGAAGGAAGATGG-3′	5′-CAGCTCTTAGCAGACATTGG-3′	221	15,698	NM_002006
SOX2	5′-GCCGAGTGGAAACTTTTGTCG-3′	5′-GCAGCGTGTACTTATCCTTCTT-3′	154	154[a]	NM_003106
GAPDH	5′-ATCACCATCTTCCAGGAGCGA-3′	5′-TTCTCCATGGTGGTGAAGACG-3′	101	na	NM_002046

[a]Intronless gene.
na, reverse primer spans the intron–exon junction so it will not amplify the genomic DNA.

137 mM NaCl, 2.7 mM KCl, 0.2% Tween), 5% dry milk (Santa Cruz) and 5% FBS. The same buffer was used for primary and secondary antibody incubations. Antibodies used were: OCT4 (Abcam), SOX2 (Abcam), NANOG (Santa Cruz), and fibroblast specific protein FSP (Sigma, St. Louis, MO). HRP-conjugated secondary antibodies were used (Invitrogen). In between antibody incubations, membranes were washed three times with TTBS. Membranes were incubated in luminol (Santa Cruz Biotechnologies) and luminescence detected using a Kodak 4000MM Image Station. All images were acquired after 30-sec exposure and processed using Kodak imaging software.

Reverse Transcription PCR (RT-PCR)

Total RNA was isolated using Trizol (Invitrogen) following the manufacturer's protocol. Four micrograms of total RNA was used to perform first strand cDNA synthesis using Superscript (Invitrogen). For RT-PCR, 0.5 μL of the first-strand cDNA was used as a template. PCR was performed in Mg^{2+} free PCR buffer (TaKara, Shiga, Japan) supplemented with 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 25 pmol each of forward and reverse primers and 0.5 U of TaKara ExTaq polymerase per reaction. PCR cycling was done as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 15 sec; annealing at primer-specific annealing temperature for 1 min; and extension at 72°C for 1:30 min. Final extension was done at 72°C for 10 min and the samples held at 4°C until use. Amplification products were resolved on 2% agarose gels containing 0.5 μg/mL ethidium bromide in 1× TAE buffer and photographed using a Kodak 4000MM Image Station. Human OCT4 (POU5F1) gene transcribes two mRNA variants (NM_002701 and NM_203289), that translates to 360 amino acid and 265 amino acid proteins respectively. Of these, 225 amino acids at the C-terminal are common to both isoforms. The RT-PCR primers used in our study was specifically designed to amplify from the transcript for the 360 amino acid variant (NM_002701). This variant has been shown to be expressed by human ESCs, wheres the 265 amino acid variant was not, suggesting that the former is important for maintaining "stemness" in human ESCs (Cauffman et al., 2006). RT-PCR for the 265 aa variant in the dermal fibroblasts, including human embryonic carcinoma cells (ATCC, CRL2073) did not show any amplification. Primers used are listed in Table 1.

Quantitative reverse transcription-PCR (qRT-PCR)

RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol and quantified by spectrophotometry. Two micrograms of RNA was subjected to DNase I digestion, followed by a reverse transcription using a QuantiTech® Reverse transcription kit (Qiagen, Chatsworth, CA) with a mixture of oligo-dT and random hexamers primers. cDNA (50 ng/well) was used as a template in qPCR reactions with oligonucleotides specific for the genes of interest (Table 2). A nontemplate control and an RNA sample without reverse transcription for each sample were used to control for potential contaminating DNA. All qPCR reactions were performed in triplicate with the resultant values being combined into an average cycle threshold (C_T). The efficiency of qPCR was calculated from the slope of a relative standard

Table 2. DNA Sequences Used for qRT-PCR Primers

Target gene	Forward primer	Reverse primer	Amplicon size (bp)	Genomic size (bp)	Accession number
ACTIN	5′-AGAGCTACGAGCTGCCTGAC-3′	5′-GGATGCCACAGGACTCCA-3′	111	206	NM_001101
NANOG	5′-ATGCCTCACACGGAGACTGT-3′	5′-AAGTGGGTTGTTTGCCTTTG-3′	103	3287	NM_024865
LIN28	5′-GAAGCGCAGATCAAAAGGAG-3′	5′-GCTGATGCTCTGGCAGAAGT-3′	115	870	NM_024674
OCT4	5′-TCGAGAACCGAGTGAGAGG-3′	5′-GAACCACACTCGGACCACA-3′	125	385	NM_002701
REX1	5′-CATCGCTGAGCTGAAACAAA-3′	5′-CTGCTGGACTGTGAGCACTACT-3′	92	92[a]	NM_174900
SOX2	5′-TGATGGAGACGGAGCTGAA-3′	5′-GGGCTGTTTTTCTGGTTGC-3′	103	103[a]	NM_003601
TERT	5′-GCTGACGTGGAAGATGAGC-3′	5′-TTGGCCAGGATCTCCTCA-3′	108	797	NM_198253

[a]Intronless gene.

curve using GAPDH primers. Relative quantification was determined using a 7500 Real Time PCR system (Applied Biosystems, Bedford, MA) measuring SYBR green fluorescence (PerfeCTa™ SYBR Green FastMix, Low ROX, Quanta Biosciences, Gathersburg, MD). Expression profiles for the mRNA transcripts are shown as inverted cycle threshold (C_T) relative to those of ACTIN. This was done by subtracting the C_T value from each gene from the total number of cycles run (40), which enables the relative values to be plotted such that genes detected at larger cycles are accurately represented as being present in less abundance.

Severe combined immunodeficiency (SCID) mouse injections

Animal studies were done with IACUC approved protocols and in accordance with animal care and use procedures at Worcester Polytechnic Institute, Worcester, MA. One million of control and one million of FGF2-treated fibroblasts were mixed with 8–12 μm diameter carbon beads in sterile DPBS and injected into the hind leg muscle of SCID mice (Charles River Laboratories, Wilmington, MA). Animals were euthanized 6 weeks after injection, the muscle excised, and processed for histology. Tissues were fixed in 4% formaldehyde in DPBS and embedded in paraffin. Sections were stained with H&E and the injection site located by microscopic visualization of the carbon beads.

Results

Adult human fibroblasts grown in DMEM/F12, 10% serum substitute (Fetal Clone III, Hyclone), with FGF2 (4 ng/mL; Chemicon) at 37°C and 5% O_2, 5% CO_2, 90% N_2 cultured continuously with a rigorously controlled passage schedule underwent 70 population doublings (PDs) before reaching senescence compared to 33 PDs for cells cultured in atmospheric oxygen without FGF2 (Fig. 1). Cells cultured without FGF2 in 5% O_2 underwent 50 PDs. The addition of FGF2 and culture with atmospheric oxygen resulted in 50 PDs. The increase over the expected 33 PDs (according to the cell supplier) with FGF2 supplementation was accompanied by change in morphology to smaller cells with a more spindle

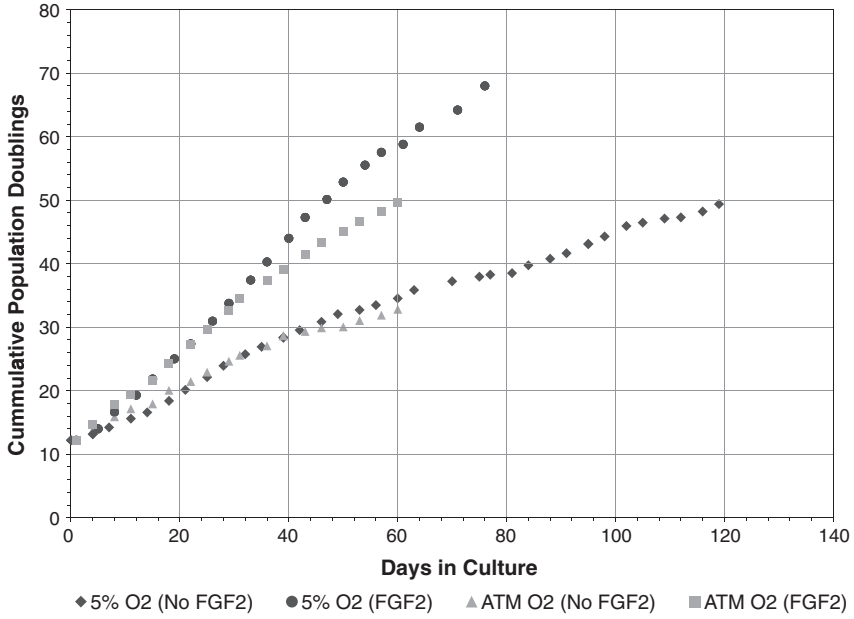

FIG. 1. Adult human dermal fibroblasts (CRL-2352) were grown with (FGF2) or without 4 ng/mL FGF2 (no FGF2), passaged at regular intervals and seeded at the same density. ATM, atmospheric oxygen.

shape. The FGF2/low oxygen-treated fibroblasts maintained normal karyotype at 65 PDs (44 XY) and entered replicative senescence as indicated by cessation of further cell division and β-galactosidase staining (not shown). These data indicated both an individual and synergistic role for both reduced oxygen and FGF2 supplementation for increasing cell life span, which prompted us to investigate expression of genes related to undifferentiated cells.

RT-PCR using primers designed to recognize embryonic forms of transcripts for the stem cell genes (Table 1) was performed on day 7 of the initial culture to obtain control baseline values for the cells' transcriptional activity. Contrary to the expected absence of stem cell gene transcripts, RT-PCR amplified detectable amounts of OCT4, NANOG, and KLF4 mRNA in fibroblasts grown under 5% O_2. Transcription of these genes did not change when cultures were supplemented with FGF2 and the amplified transcripts were of the same size as those present in human teratocarcinoma cell controls (Fig. 2). Other stem cell genes SOX2, REX1, and LIN28 showed transcriptional dependence on FGF2 (Fig. 2). Neither lowered oxygen nor FGF2 supplementation had an obvious effect on expression of OCT4, NANOG, KLF4, or hTERT, when compared to GAPDH controls (Fig. 2). To quantify and validate these observations, quantitative real-time PCR (qRT-PCR) was employed and the analysis confirmed our RT-PCR data (Fig. 3). The quantitation of mRNA expression from genes associated with pluripotency revealed that normal human fibroblasts have a basal expression of these genes (OCT4, SOX2, NANOG, and hTERT), albeit much lower than that expressed in hESCs (Fig. 3b).. However, REX1 and LIN28 transcript levels were induced by culture in reduced oxygen with additional upregulation of LIN28 in the presence of FGF2 (Fig. 3a). Reduced oxygen culture resulted in an increase in hTERT mRNA, which was not further affected by FGF2 (Fig. 3a). Similar trends were observed in a second somatic cell line (Fig. 3c). Primary muscle-derived human fibroblasts (CT0706) contained detectable amounts of NANOG, OCT4, and hTERT mRNA, which were not affected by reduced O_2 or FGF2. However, SOX2 mRNA was only detected by culture in low O_2 with FGF2. In contrast to the dermal fibroblasts, REX1 and LIN28 were not detected in the muscle derived fibroblasts (Fig. 3c).

To evaluate the potential significance of the presence of OCT4, SOX2, and NANOG mRNA in fibroblasts and to determine whether the messages were being translated, we performed a Western blot analysis. OCT4, NANOG, and SOX2 proteins were detected only in the presence of FGF2 (Fig. 4a). Their appearance was not a consequence of transcriptional upregulation as the levels of their mRNA remained unchanged in control untreated fibroblasts and did not increase after treatment (Fig. 3). In order to perform their function as transcription factors, OCT4, SOX2, and NANOG need to translocate to the nucleus. Immunocytochemistry revealed that when cultured on untreated glass coverslips in the presence of FGF2, OCT4, SOX2, and NANOG antigens were detected in the nucleus (Fig. 4b) and identical cultures on tissue culture plastic did not show this pattern of expression (Fig. 4b). Preliminary observations show that NANOG and SOX2 are colocalizing in the nuclei and the perinuclear region of expressing cells (Fig 4c). Not all of the cells within the FGF2-treated population responded by detectable nuclear expression of OCT4, SOX2, and NANOG. Expressing cell

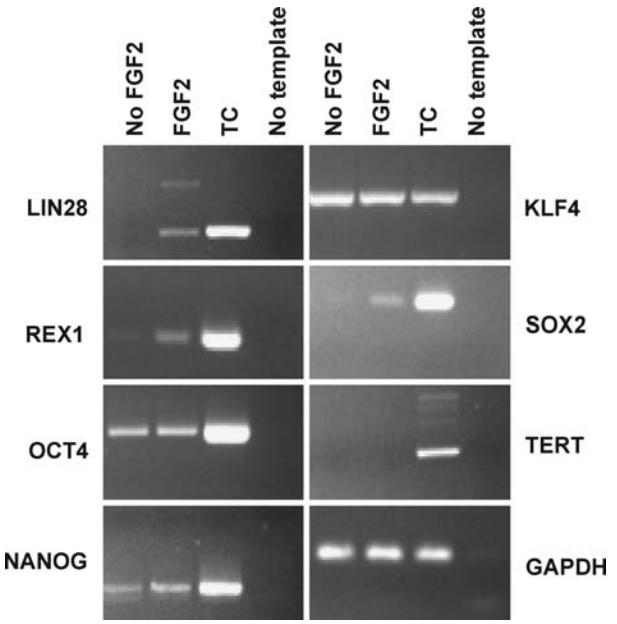

FIG. 2. Expression of stem cell genes in fibroblasts. RT-PCR of LIN28, REX1, OCT4, NANOG, KLF4, SOX2, TERT, and GAPDH in fibroblasts grown with (FGF2) or without FGF2 (no FGF2), teratocarcinoma cells (TC), and no template control. No reverse transcriptase controls were negative (not shown). Assays were performed after 7 days of culture. No FGF2 and FGF2 lanes contain fibroblasts grown under 5% O_2 conditions.

populations were clustered in discrete groups, indicating that distinct subpopulations of adult human dermal fibroblasts may be more susceptible to these changes. These observations were reproduced in an independent primary fibroblast cell population isolated from adult muscle tissue (CT0706).

The unexpected detection of apparent translation and nuclear translocation of OCT4, SOX2, and NANOG in fibroblasts due to FGF2 prompted us to evaluate the expression of FGF2 itself and its receptors in cells cultured under these conditions. Supplementation of FGF2 had no significant effect on transcription of FGF receptors or FGF2 itself regardless of the culture conditions (Fig. 5a). However, immunocytochemistry showed that in cells supplemented with FGF2, FGF2 itself, and both FGFR1 and FGFR2 translocated to the nucleus, whereas FGFR3 was detected in the nucleus in the absence of FGF2 (Fig. 5b). FGF2 addition had no effect on FGFR4 localization (Fig. 5b). Addition of BMP-2 (1 ng/mL) to the medium prevented FGF2-induced OCT4 nuclear detection (Fig. 6).

Discussion

Recent efforts in derivation of iPS cells have demonstrated that a relatively small number of genes when introduced into differentiated somatic cells have the ability to reprogram nuclear memory and cause acquisition of a pluripotent phenotype. These observations suggest that there may be only a few prime upstream regulators of the stability of differentiation phenotype, particularly in fibroblasts. We show that by manipulating culture conditions alone, we can achieve changes in fibroblasts that would be beneficial in development

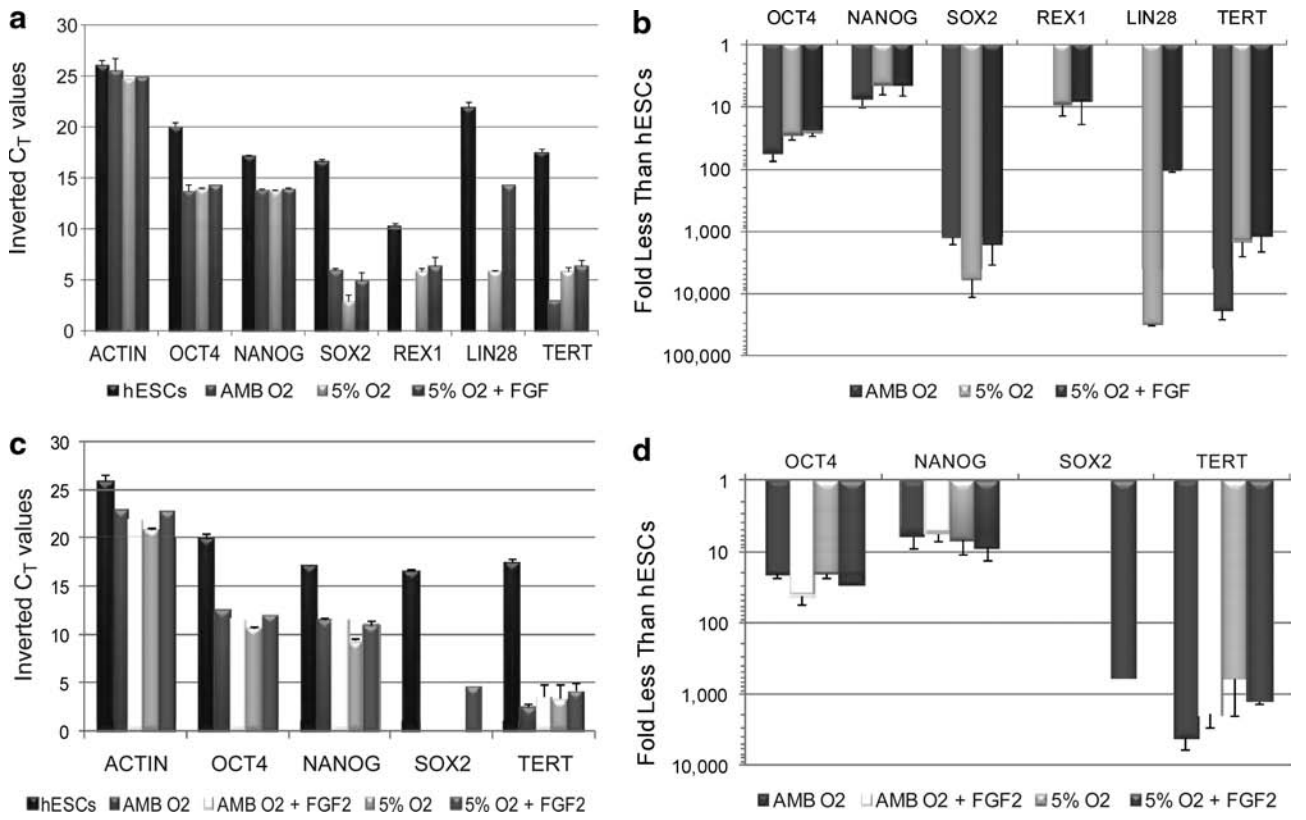

FIG. 3. qRT-PCR results comparing relative gene expression levels in two primary human fibroblast cell lines (**a,b**—CRL-2352, **c,d**—CT0706). Primary fibroblasts grown in ambient O_2 conditions were used as control cells. Experimental fibroblasts were grown at 5% O_2 with or without 4 ng/mL FGF2 (5% O_2 and 5% O_2 + FGF2, respectively), and compared to human embryonic stem cells H9 (hES). No template and no reverse transcriptase controls were performed under identical PCR conditions. Assays were performed after 7 days of culture. Error bars represent standard error of measurement (SEM).

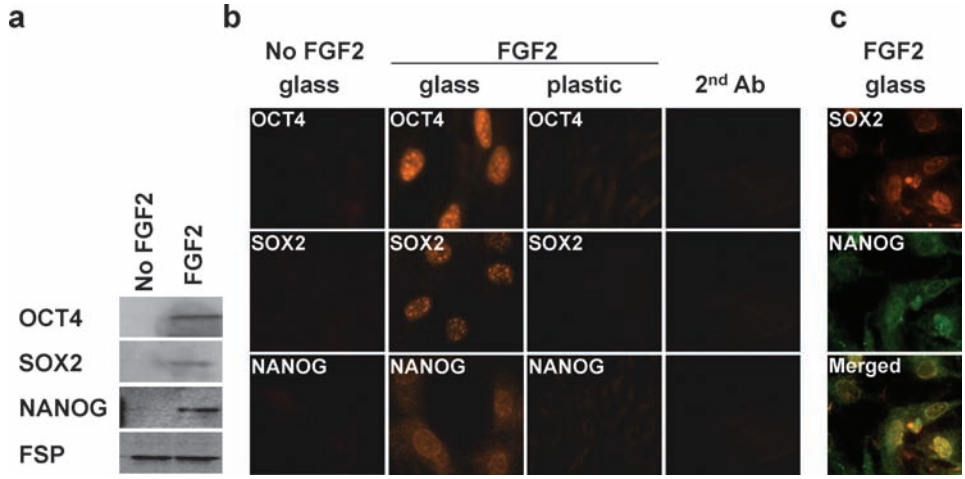

FIG. 4. Expression and localization of stem cell transcription factors in human fibroblasts. (**a**) Total protein was isolated from control or FGF2-treated fibroblasts. Western blots were probed with primary antibodies against OCT4 (Abcam), SOX2 (Abcam), and NANOG (Santa Cruz), and detected with HRP-conjugated secondary antibodies (Invitrogen). Fibroblast specific protein FSP (Sigma) was used as a loading control. All cultures were grown under 5% O_2. (**b**) For immunocytochemistry, control (no FGF2), or FGF2-treated fibroblasts were labeled with primary rabbit antibodies against OCT4 (Abcam), SOX2 (Abcam), and NANOG (Abcam). Alexafluor-568 conjugated goat antirabbit antibody (Invitrogen) was used for detection (red). Staining with secondary antibody alone was used as a negative control (2nd Ab). (**c**) FGF2-treated fibroblast grown on glass were double stained with anti-SOX2 (rabbit polyclonal, Abcam) and anti-NANOG antibody (goat polyclonal; Santa Cruz). SOX2 was detected with Alexafluor 568 conjugated goat antirabbit secondary antibody and NANOG was detected with Alexafluor488 conjugated donkey antigoat secondary antibody (both from Invitrogen). The images were overlaid to produce a composite (merged) image. Assays were performed after 7 days of culture. Images were captured using identical capture settings and processed using Slidebook®.

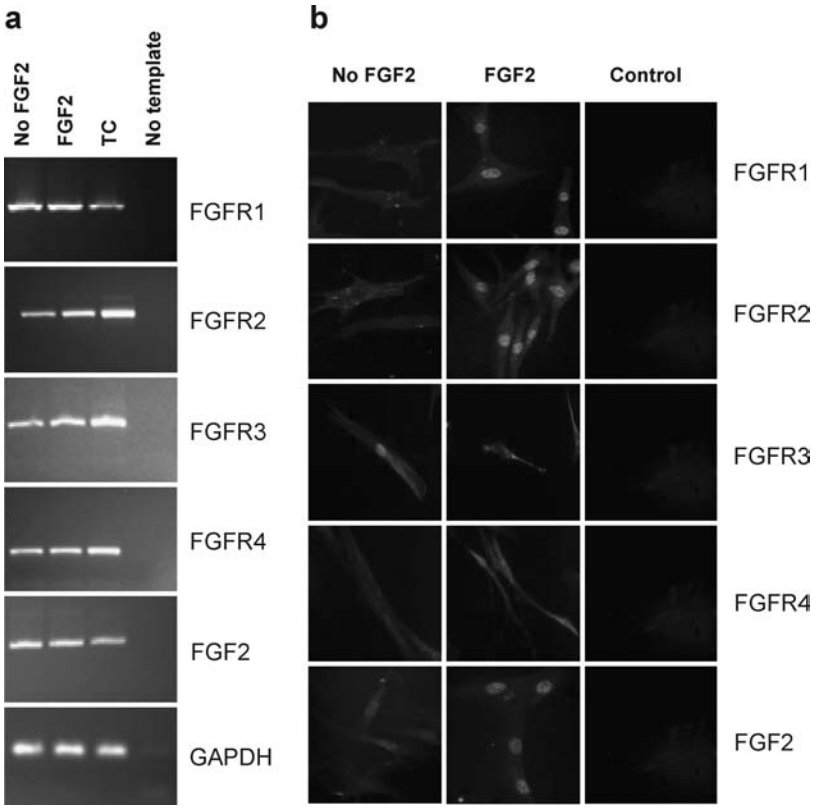

FIG. 5. Expression of FGF2 and its receptors in fibroblasts. (**a**) RT-PCR results in control fibroblasts, FGF2-treated fibroblasts, and teratocarcinoma cells (TC). No template control was performed under identical conditions. (**b**) Immunofluorescence staining of control (no FGF2) and FGF2-treated human adult dermal fibroblasts with antibodies against FGFR1 (Abgent), FGFR2 (Abgent), FGFR3 (Abcam), FGFR4 (Abcam), and FGF2 (Santa Cruz). Secondary antibodies were conjugated to Alexafluor 568. Secondary antibody incubations alone were used as negative controls (control). Assays were performed after 7 days of culture. Images were captured using identical capture settings and processed using Slidebook®.

of patient-specific cell therapy approaches. First, we demonstrate an increase in the number of population doublings of adult dermal fibroblasts *in vitro*; second, fibroblasts acquire expression and nuclear localization of several stem cell specific transcription factors, and third, the cells maintain low TERT levels and are not tumorigenic.

Experimental evidence gathered over the past decades for involvement of FGF2 in a number of cellular and developmental processes is extensive. Among others, FGF2 has been shown to regulate human ES cell self-renewal (Levenstein et al., 2006), is a potent mitogen and morphogen for a variety of cell types (reviewed in Ornitz and Itoh, 2001), has been

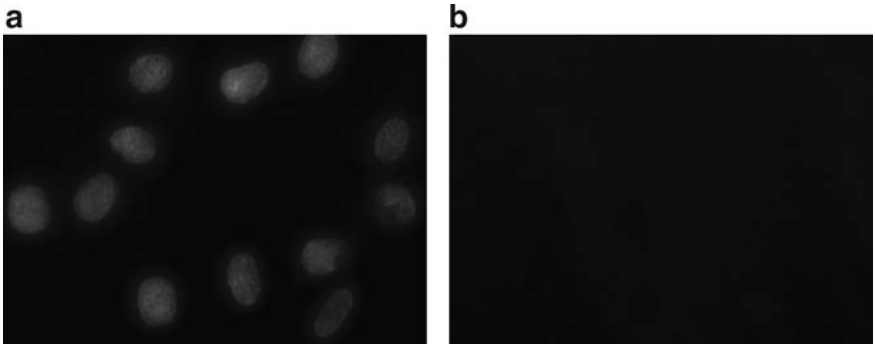

FIG. 6. BMP2 prevents FGF2-induced expression and nuclear localization of OCT4 in dermal fibroblasts. Immunofluorescence staining of adult human fibroblasts for OCT4 (Abcam) cultured with FGF2 (4 ng/mL) alone (**a**) and with FGF2 (4 ng/mL) and BMP-2 (1 ng/mL) (**b**). Secondary antibodies were conjugated to Alexafluor 568. Assays were performed after 7 days of culture. Images were captured using identical capture settings and processed using Slidebook®.

shown to initiate regeneration in the eye (Hayashi et al., 2004), and reprograms primordial germ cells to pluripotency (Durcova-Hills et al., 2006).

The increase in *in vitro* longevity of fibroblasts cultured in reduced oxygen were consistent with those reported previously (Saito et al., 1995). An increased life span has been associated with FGF2-facilitated selection and enrichment of multipotent cells (Bianchi et al., 2003; Quarto and Longaker 2006); however, FGF2 has not been shown to impact the *in vitro* life span of terminally differentiated cell types. The initial FGF2 effect is likely mediated via FGF receptors at the plasma membrane. Nuclear translocation of FGFR1 and FGFR2 upon FGF2 treatment indicates a possibility for involvement of both FGF2 and these receptors at the chromatin level. FGF receptors have been observed in 3T3 cells (Maher, 1996) and mammary epithelial cells (Bryant and Stow, 2005), and have been associated with FGF2 mitogenic activity, which may act through a number of FGFR isoforms. Therefore, the increased number of population doublings in our FGF2-treated fibroblasts could be a consequence of FGF2 mitogenic stimulation. The changes we observed in FGFR and FGF2 localization did not appear to coincide with an increase in their mRNA levels. In addition to their mitogenic effects, both FGFR1 and 2 have been associated with FGF2-mediated maintenance of pluripotency in hES cells (Babaie et al., 2007; Greber et al., 2007a). Although FGF2 has not been implicated previously in transcriptional activation of OCT4 or SOX2, it has been determined that the maintenance of expression of these genes and cell pluripotency is dependent on FGF2 (Levenstein et al., 2006). The proposed action of FGF2 involves induction of members of TGF-β pathway; TGF-β ligands maintain expression of OCT4, SOX2, and NANOG, which in turn, activate expression of endogenous FGF2 that completes this regulatory loop (Greber et al., 2007b). Our data is in agreement with previous hypothesis that this circuit can be initiated and perpetuated by exogenous FGF2, leading to autocrine signaling by endogenous FGF2 (Dvorak et al., 2005; Dvorak and Hampl, 2005; Greber et al., 2007a; Levenstein et al., 2006; Xu et al., 2005). BMP signaling has been shown to antagonize FGF2 signaling in maintaining the pluripotent state of human ES cells (Xu et al., 2008). Inhibition of OCT4 nuclear detection in our culture system in the presence of BMP-2 suggests that a similar pathway may be involved.

Recently, FGF2 has been shown to be involved in remodeling of the chromatin in rat cortical neuronal progenitor cells by methylation of histone H3K4 (K4me3) and repression of methylation of H3K9 (Song and Ghosh, 2004). Both of these posttranslational histone H3 modifications have been associated with transcriptionally active chromatin (Kimura et al., 2004).

It was surprising that detectable levels of stem cell transcription factors were present in control fibroblasts and that at least one of these genes (OCT4) represented the true embryonic form. Despite the presence of transcription factor mRNAs, however, no detectable level of these proteins could be found either by Western blotting or ICC. Although we have not yet determined the functional relationship between FGF2 and stem cell gene expression in dermal fibroblasts, it has become apparent that a combination of FGF2 supplementation, low oxygen culture conditions, and cell culture surface triggered translation of these proteins and their appropriate translocation to cells' nuclei. Our results suggest for the first time that alteration of cell fate may depend not only on induction of new transcription, but on posttranscriptional regulation as well.

The absence of tumor formation after injection into SCID mice indicates that despite stem cell gene expression, subsequent translation and appropriate nuclear localization after 7 days of culture, these cells have not yet acquired a pluripotent phenotype. Events downstream of OCT4, SOX2, and NANOG that may be critically important for acquisition and maintenance of pluripotency may require extended culture of cells under appropriated conditions (Takahashi et al., 2007b; Yu et al., 2007). Because only up to 30% of cells demonstrated stem-like nuclear localization of the transcription factors, absence of tumor formation may have been due to insufficient numbers of OCT4/NANOG/SOX2 positive cells injected.

The ability of lowered oxygen together with FGF2 to induce expression of stem cell genes in adult human fibroblasts without hTERT protein expression and with a significantly increased replication potential suggests that sufficient numbers of cells could be produced for therapeutic applications. Our data suggests that mechanisms regulating translation and posttranslational modifications may be critically important in induction of a stem cell phenotype. This suggests that there may be a subpopulation of fibroblasts capable of responding to FGF2 at the translational or signaling level. In addition, the possible synergistic effect of FGF2 and subatmospheric oxygen culture conditions warrants further exploration.

The mechanism of induction of key regulatory genes involved in pluripotency by nontransgenic methods to create truly pluripotent cells will require further investigation. The published studies on transgene induced pluripotency in fibroblasts show that forced expression of exogenous pluripotency genes is required for at least 30 days prior to detection of phenotypic changes in the cells followed by amplification of colonies of cells with truly pluripotent properties (Takahashi et al., 2007b; Yu et al., 2007). The long-term stability of this phenotype will likely involve introduction of extra cellular components and specialized media formulations similar to those employed for derivation and *in vitro* maintenance of hESCs and IPS cells, and possibly factors yet to be identified. However, this work suggests that it may be possible to dedifferentiate adult human somatic cells by modifying the *in vitro* culture conditions. The ability to dedifferentiate somatic cells to a less differentiated (not necessarily pluripotent) state by simply modifying the culture conditions may have value in the utilization of autologous or primary cells for cell therapy and diagnostic applications.

Acknowledgments

We thank Prof. Marsha Rolle and Prof. Charles Murry for reviewing the manuscript. We thank Sharon Shaw for assistance with histological analysis of injected muscle. We thank Amanda Blackwood and Cara Messier from Prof. G. Pins' lab at WPI for providing primary human dermal fibroblasts used as controls. We thank Professor Raymond Dunn and Dr. Ronald Ignotz for assistance with muscle tissue. This work was supported by the WPI start up funds to T.D., NIH R01GM085456, and by the Defense Advanced Research Projects Agency and US Army Research Office. The

content of the information does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred. Any intellectual property arising from this communication has been assigned to Worcester Polytechnic Institute.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Address correspondence to:
Raymond L. Page, Ph.D.
Biology and Biotechnology Department
Worcester Polytechnic Institute
100 Institute Road
Worcester, MA 01609

E-mail: rpage@wpi.edu