Defining the Diversity of Phenotypic Respecification Using Multiple Cell Lines and Reprogramming Regimens

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To better understand the basis of variation in cellular reprogramming, we performed experiments with two primary objectives: first, to determine the degree of difference, if any, in reprogramming efficiency among cell lines of a similar type after accounting for technical variables, and second, to compare the efficiency of conversion of multiple similar cell lines to two separate reprogramming regimens—induced neurons and induced skeletal muscle. Using two reprogramming regimens, it could be determined whether converted cells are likely derived from a distinct subpopulation that is generally susceptible to reprogramming or are derived from cells with an independent capacity for respecification to a given phenotype. Our results indicated that when technical components of the reprogramming regimen were accounted for, reprogramming efficiency was reproducible within a given primary fibroblast line but varied dramatically between lines. The disparity in reprogramming efficiency between lines was of sufficient magnitude to account for some discrepancies in published results. We also found that the efficiency of conversion to one phenotype was not predictive of reprogramming to the alternate phenotype, suggesting that the capacity for reprogramming does not arise from a specific subpopulation with a generally “weak grip” on cellular identity. Our findings suggest that parallel testing of multiple cell lines from several sources may be needed to accurately assess the efficiency of direct reprogramming procedures, and that testing a larger number of fibroblast lines—even lines with similar origins—is likely the most direct means of improving reprogramming efficiency.

Introduction

CELLULAR REPROGRAMMING, accomplished by either direct or indirect methods, is an inefficient process with many potential sources of variation. Several cellular characteristics, including cell type, species of origin, and age of the donor subject, are known to influence reprogramming efficiency. There is an emerging awareness, however, that even when these general cellular properties and technical aspects of the reprogramming regimen are held constant, variation may still be the rule rather than the exception. This is observed in reprogramming experiments using either indirect means such as somatic cell nuclear transfer [1–4], or direct methods such as plasmid transduction or infection with recombinant retroviruses expressing transcription factors [5–9]. One of the earliest studies of direct reprogramming, describing the conversion of mouse fibroblasts to skeletal muscle myotubes by transduction of the myogenic transcription factor *MyoD1*, found that reprogramming was not uniform across all cell lines. This was true even within a single cell type isolated from a single species [5]. A comparison of five mouse fibroblast cell

lines, C3H10T1/2, NIH3T3, Swiss 3T3, Swiss 3T3 clone 2, and L Cells, transfected with a *MyoD* expression plasmid and selected to produce colonies of stably transduced cells yielded colonies of both the input and the conversion phenotype. The conversion efficiency varied dramatically from a maximum of 53% myoblastic colonies in C3H10T1/2 cells to a minimum of 3% myoblastic colonies in L cells. Similarly, a report from Lattanzi et al. [10] comparing the myogenic conversion of fibroblasts from different tissue sources infected with a high-titer (MOI 2,000) *MyoD* adenovirus vector found that murine dermis-, muscle-, and bone marrow-derived fibroblasts converted at efficiencies of 59%, 43%, and 7%, respectively, and human fibroblasts derived from the same tissues at respective efficiencies of 54%, 36%, and 6%. Together, these reports indicate that reprogramming variation may be observed regardless of whether vector delivery is relatively inefficient (plasmid transduction) or highly efficient (adenoviral infection). More recently, variation in the input cell population was postulated to account for reprogramming disparity in the conversion of fibroblasts to functional cardiomyocytes reported by several groups [8,11–13].

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Although variation in reprogramming efficiency may be frequently observed and reported, whether the observed variation arises from technical differences or from undefined differences intrinsic to the target cell lines used remains unknown. If variation is still observed when technical elements are tightly controlled and factored into the calculation of reprogramming efficiency, it suggests that line-intrinsic characteristics play an important role in line-to-line variation.

Line-intrinsic variation in the number of cells amenable to respecification could arise through two hypothetical mechanisms. In the first, reprogrammed cells would have their origin in a subset of cells with general susceptibility to identity change. Similar to stem cells, these cells would be receptive to adoption of alternate fates, but might lack the active determinants that are associated with canonical stem cells. Alternatively, reprogrammed cells could arise through chance reprogramming of cells to a specific alternate fate, but with no overall enhanced capacity for respecification.

Within cell lines of the same general type, are line-dependent differences in reprogramming capacity observed? If so, do reprogrammed cells arise from a subpopulation with a generally weak “grip” on identity or from cells with an independent capacity for respecification to a given phenotype? If significant line-to-line differences are observed and cells with general lability of identity are the source, we hypothesized that we should observe a correlation in reprogramming efficiency using two separate conversion regimens applied to multiple target cell lines. Conversely, if cells with an independent capacity for respecification are the source of converted cells, we would observe no significant correlation between conversion regimens. To test this hypothesis, we used a rigorous measure of reprogramming efficiency that was robust across replicates within a specific reprogramming regimen and performed a parallel analysis of 19 primary fibroblast cell lines converted to two alternate and disparate identities—induced neural cells (iNCs) and induced skeletal muscle cells (iSMCs). The results presented next bring new insights into the nature of cells successfully converted to an alternate phenotype using methods of direct cellular reprogramming.

Materials and Methods

Fibroblast lines

Human primary fibroblasts were obtained from skin-punch biopsies or gingival explants under MSU-approved IRB protocols (ADF, E2F, EAF, and HSK) or were obtained from commercial sources, including the ATCC (FET and HDNF) and the Coriell Institute (RET, SAF, and AUT). Additional information on these lines is provided in Table 1. Mouse fibroblast lines were harvested from a single 5-month-old *nu/nu* mouse sacrificed by CO₂ overdose, the carcass disinfected with ethanol, and multiple tissues removed for dissection. 125 mm³ fragments of each target organ were removed, minced, and individual pieces placed in one well of a six-well plate for outgrowth. Primary outgrowths were plated in fibroblast medium [Dulbecco’s modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), and 1×antibiotic/antimycotic (Invitrogen)]; were left undisturbed except for medium changes for 2 weeks; and then passaged 1:1 to a new well. After an additional week of

growth, the lines were passaged 1:2. This process was repeated to passage 5 when the wells were compared to identify those with typical fibroblast morphology, an absence of obviously nonfibroblastic cells, and similar growth characteristics. Thirteen mouse fibroblast lines were selected for two additional rounds of passage and expansion and then frozen as multiple aliquots for use in experiments.

Fibroblast characterization

Fibroblast RNAs from the mouse lines at passage 6–7 and the human lines at “passages” 8–10 were purified using Trizol (Invitrogen) or the RNeasy kit (Qiagen), and 2 µg of purified RNA was converted to cDNA using Superscript II (Invitrogen), following the manufacturer’s guidelines. Quantitative polymerase chain reaction (qPCR) was performed on an ABI Prism 7000 analyzer using 1 µL of cDNA and normalizing against nuclear lamin A or ARHGAP mRNAs as internal controls. Other genes used as internal controls (*RPL27A*, *EED*, and *GR*) gave similar results. Primers for qPCR analysis are shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd).

Processing of human and mouse fibroblasts for immunocytochemical analysis to examine marker expression at the level of individual cells was performed as described next for iNC and iSMC analysis using primary antibodies as follows: anti-vimentin 1:1250 (Millipore AB5733), anti-fibronectin 1:750 (BD, 610077), anti-nestin 1:250 (Santa Cruz Biotechnologies; H-85), and anti-Sox2 1:250 (Santa Cruz Biotechnologies; Y-17). Multiple images of each immunostained line were captured, and approximately 1×10³ cells were examined at high magnification for the presence or absence of markers that is consistent with stem cell (Sox-2/nestin) or fibroblast identity (vimentin/fibronectin). NPCs used for comparison were fixed in parallel with fibroblasts and were generated as described next for iNC studies.

Relative infectivity for “Factor Expression Early” (FEE) was calculated by infecting 1×10⁵ actively growing cells with concentrated NITSC-NLS-YFP retrovirus at an MOI of approximately 0.5 and then counting the YFP-positive cells as a fraction of all cells at day 4 postinfection over three replicates. Since the precise cellular age of each line was to some degree unknown, to ensure that none of the lines analyzed was approaching cellular senescence which could influence reprogramming, each line was continuously passaged and counted for four rounds at the end of experiments to determine that all lines were still proliferative. One of the 13 mouse lines, LU3, failed by the third trial passage and was removed from the final analysis. Two human lines, AUT and HSK, produced iNCs and iSMCs and were partially characterized, but were not included in the final analysis because the “matching” frozen stocks were lost before the completion of some experiments due to freezer failure. Mouse lines TA6, KI2, and LU6 were not included in the experiments shown in Supplementary Fig. S3 for the same reason.

iNC induction—plasmids and virus production

cDNAs encoding human *ASCL1*, *POU3F2*, and *ZIC1* were obtained from Open Biosystems. *Myt1L* and *NeuroD1* ORFs were obtained from cDNA produced from human brain

TABLE 1. PROPERTIES OF HUMAN AND MOUSE FIBROBLAST LINES

Human fibroblast lines													
General	mRNA expression												
	Study ID	Line ID	Donor	Sex	Age	Tissue	COL1A2	FNECTIN	FIBR1	FIBU5	VIM	KER14	PECAM
Immunocytochemistry													
							FOXG1	SOX2	MYOD	MYF5	FNECTIN	VIM	SOX2
													NEST
FET	IMR90		Healthy	F	E16 wk	Lung	+	+	+	+	+	+	+
NWB	HDF		Healthy	M	Newborn	Skin	+	+	+	+	+	+	+
ADF	MSU-HUMGM		Healthy	M	44	Gingiva	+	+	+	+	+	+	+
RET	GM17880		Rett syndrome	F	5	Skin	+	+	+	+	+	+	+
E2F	MSU-HUMAG10		Healthy	M	73	Skin	+	+	+	+	+	+	+
EAF	MSU-HUMAG07		Healthy	M	71	Skin	+	+	+	+	+	+	+
SAF	GM01792		Schizophrenia	M	26	Skin	+	+	+	+	+	+	+
AUT	GM07992		idic(15) autism	F	3	Skin	ND	ND	ND	ND	ND	+	+
HSK	MSU-HUMSK		Healthy	M	41	Skin	ND	ND	ND	ND	ND	ND	ND

Mouse fibroblast lines													
HE4	N/A		nu/nu	M	5 mo	Heart	-	+	+	+	+	+	+
SM1	N/A		nu/nu	M	5 mo	Skel Musc	+	+	+	+	+	+	+
K12	N/A		nu/nu	M	5 mo	Kidney	+	+	+	+	+	+	+
K13	N/A		nu/nu	M	5 mo	Kidney	-	+	+	+	+	+	+
K15	N/A		nu/nu	M	5 mo	Kidney	+	+	+	+	+	+	+
K16	N/A		nu/nu	M	5 mo	Kidney	+	+	+	+	+	+	+
L16	N/A		nu/nu	M	5 mo	Liver	+	+	+	+	+	+	+
LU6	N/A		nu/nu	M	5 mo	Lung	+	+	+	+	+	+	+
TA4	N/A		nu/nu	M	5 mo	Tail Skin	+	+	+	+	+	+	+
TA6	N/A		nu/nu	M	5 mo	Tail Skin	+	+	+	+	+	+	+
TE4	N/A		nu/nu	M	5 mo	Testis	+	+	+	+	+	+	+
TE5	N/A		nu/nu	M	5 mo	Testis	+	+	+	+	+	+	+
LU3	N/A		nu/nu	M	5 mo	Lung	ND	ND	ND	ND	ND	ND	ND
MEF	N/A		FVB	F/M	E13 dy	Embryo	ND	ND	ND	ND	ND	ND	ND

General properties: Study ID, abbreviated line designation used this report; Line ID, line common name; Donor, donor health status or strain; Sex, sex of donor (male, female, or of mixed sex); Age, age of donor (years, unless otherwise noted); Tissue, fibroblast tissue of origin. mRNA Expression: results of quantitative PCR analysis of multiple markers. COL1A2, fibroblast marker collagen type I alpha 2; FNECTIN, fibroblast marker fibronectin; FIBR1, fibroblast marker fibrillin 1; FIBU5, fibroblast marker fibulin 5; VIM, fibroblast marker vimentin; KER14, keratinocyte marker keratin 14; PECAM, endothelial cell marker platelet endothelial cell adhesion molecule/CD31; FOXG1, neural progenitor marker forkhead box protein G1; SOX2, neural progenitor marker SRY (sex determining region Y)-box 2; MYOD, myogenic progenitor marker MyoD1; MYF5, myogenic progenitor marker myogenic factor 5. Immunocytochemistry: summary of immunocytochemical analysis of fibroblast-associated (fibronectin, vimentin) and stem cell-associated markers (Sox-2, nestin) in fibroblast lines. N/A, not applicable; ND, -not done; +, positive; ±, weakly positive; -, negative.

reference RNA (Applied Biosystems). NITSC was produced by introducing the BstEII-ClaI fragment containing Neo-IRES-TTA-TetO from NIT (Genbank Acc# AF311318) into BstEII-ClaI cut pMSCVneo (Clontech) and a polylinker for transgene expression, SfiI-MluI-PmeI-ClaI. Primers used for cloning of factors into NITSC are shown in Supplementary Table S1. The amplified YFP ORF with an MluI site inserted immediately before the stop codon was digested SfiI-PmeI and introduced into NITSC to produce the control vector NITSC-YFP. Remaining factor ORFs were PCR amplified with compatible MluI or AscI sites at the 5' end and ClaI at the 3' end for cloning into NITSC-YFP to produce the fusion protein constructs. NITSC recombinant MMLV particles were produced by three-way calcium phosphate transfection of HEK cells with gag-pol and VSV encoding plasmids to produce replication defective virus particles. Two days after transfection, viral supernatants were harvested, filtered, and introduced into fibroblast cultures using the carrier polybrene (8 μ g/mL) to improve infection efficiency essentially as described for lentiviral vectors in Suhr et al. [14]. Viral supernatants were frozen as aliquots and tested on MEFs to provide a rough titer and to establish the competence of viral preparations to produce iNCs before use with target cells. As indicated in the text, the YZIC/YASCL/YPOU3F (ZAP) combination appeared most potent on both mouse and human fibroblasts in preliminary experiments and was used for conversion unless otherwise noted. Equal volumes of each viral supernatant were used (i.e., for ZAP, typically 5 mL of each viral supernatant for a total of 15 mL infectious medium/10-cm plate).

iNC induction–infection and iNC conversion

For preliminary studies to determine the optimal timing and conditions for iNC conversion, approximately 1×10^6 growing mouse or human fibroblasts in fibroblast medium at equal confluency were infected with viral medium and kept overnight to allow infection. The next day, virus-infected cultures were passaged by trypsin treatment to six-well, 12-well, or 35 mm tissue culture plates and allowed to remain for 12–24 additional hours in fibroblast growth medium to attach and expand. After this time, the fibroblast medium was aspirated and replaced with iNC medium (DMEMF12 with N2 supplement and penicillin/streptomycin at 50 μ /mL) (Invitrogen). iNC medium was changed at 4–5 day intervals for the experimental duration, and the cells were kept in a 5% CO₂ environment at 37°C. Cell culture plates coated with polyornithine/laminin (PORN/lam) or with no coating were used for pilot experiments interchangeably with little noticeable impact on iNC formation. To determine the optimal time for counting of iNCs, factor-infected MEFs and adult mouse and human fibroblast cultures were fixed and immunostained with the neural TUJ1 antibody (see below) at 5–6 day intervals postinfection.

iNC conversion for determination of reprogramming efficiency across mouse and human cells lines was performed essentially as described earlier except that 1×10^5 target cells in one well of a six-well plate were infected with 3 mL of the iNC (ZAP) viral cocktail and 24 h later, passaged to three wells of a 12-well plate. The passaged cells were allowed to rest an additional day and were then shifted to iNC medium with medium changes every 3–4 days until fixation and

immunoprocessing on day 12 for mouse iNCs and day 24 for human iNCs. Experiments for quantification of reprogramming efficiency were performed in three separate replicates.

iNC induction–immunohistochemistry and imaging

Cells were fixed using 4% paraformaldehyde for 10 min followed by 3 $\times$ phosphate-buffered saline (PBS) washes for 10 min each. PBST (PBS with 0.3% Triton X-100) with 3% donkey serum (DS) was used for 30–60 min at room temperature to block, and was then replaced with PBST+1% DS with added primary antibody overnight at 4°C. Primary antibodies were used at the following dilutions: TUJ1-1:3000 (Santa Cruz; Cat# sc-58888), MAP2ab-1:300 (Sigma; Cat#M1406), Synapsin 1-1:400 (Millipore; Cat#AB1543P), pan-neurofilament-1:1000 (Covance; SMI311), Doublecortin-1:400 (Santa Cruz, Cat#sc-8066), GAD-1:250 (Santa Cruz; Cat#sc-7513), PSD95-1:250 (NeuromAb), and GABAR3-1:250 (NeuromAb). After overnight incubation with primary antibody, wells were washed with PBST+1%DS 3 $\times$ 10 min and incubated with PBST+1%DS with the appropriate secondary antibody (Jackson ImmunoResearch) for 30–60 min. Wells were then washed with PBS 3 $\times$ 10 min to remove excess secondary, stained briefly with PBS+1 μ g/mL bis-benzamide to label nuclear DNA, and rinsed again. All plates and wells were stored at 4°C in the dark until imaging. Imaging was performed on a Nikon Eclipse TE2000 inverted-stage fluorescence microscope.

Electrophysiology

For electrophysiological recordings, infected NPC neurons (see below) or iNCs were cultured on PORN/lam-coated 35 mm plates at a low density (2.5×10^5 cells/35 mm plate) as described earlier. All recordings were made using the whole-cell configuration of the patch-clamp technique [15]. Patch glass pipette electrodes were double pulled and heat polished. The electrode was brought into contact with visually identified iNC targets to produce a high-resistance seal between electrode tip and cell membrane, and the whole-cell configuration was achieved by applying suction to the back of the electrode. For voltage clamp experiments, electrode capacitance was compensated before achieving the whole-cell configuration, and membrane capacitance and series resistance were compensated after achieving this configuration. Membrane current and potential signals were amplified (List Electronic EPC-7), digitized (Digidata 14140A; Molecular Devices), and stored on a computer. Voltage steps and current injection pulses were generated, and potential and current signals were analyzed using a software written by Dr. John Dempster (Dept. Physiology, University of Strathclyde). In all voltage clamp recordings, the holding potential (V_h) was -80 mV. The presence and properties of voltage-gated current was examined during positive voltage steps (30 to 250 ms depending on the experiment) to test potentials (V_{test}) between -75 mV and $+50$ mV. To examine voltage-dependent, steady-state inactivation of voltage gated Na⁺ channels, a double voltage step was used: A step to a conditioning potential (V_{con}, -130 mV to $+40$ mV, 50 ms) was applied immediately before the step to the test potential (0 mV). To examine whether cells had the capacity to generate action potentials, membrane potential was measured under current clamp.

The extracellular solution contained NaCl 135 mM, KCl 5 mM, glucose 10 mM, MgCl₂·6H₂O 1 mM, CaCl₂·2H₂O 2 mM, and HEPES 20 mM (pH 7.3). For recordings of isolated voltage-gated Na⁺ current, the electrode solution contained CsCl 20 mM, cesium methanesulfonate 130 mM, MgCl₂·6H₂O 2 mM, glucose 10 mM, EGTA 10 mM, and HEPES 10 mM (pH 7.3). For recordings of mixed voltage-gated Na⁺ and K⁺ current, and for recordings of membrane potential and action potentials, the electrode solution contained KCl 20 mM, potassium methanesulfonate 130 mM, MgCl₂·6H₂O 2 mM, glucose 10 mM, EGTA 0.01 mM, and HEPES 10 mM (pH 7.3).

Human NPC culture and neuron derivation

For the control human neurons in Supplementary Fig. S5, H9 human ES cells were differentiated to NPCs as described [16]. H9-NPCs were propagated to passage 5 in iNC medium supplemented with 20 ng/mL FGF-2. For differentiation, NPCs were plated on PORN/lam plates, and FGF-2 was progressively withdrawn to a final concentration of 2 ng/mL by day 20–24, when the cells were processed for immunostaining to confirm neuronal identity and subjected to electrophysiological analysis. Undifferentiated NPCs at 30%–50% confluency were fixed and used as controls in the experiments shown in Supplementary Fig. S3.

iSMC conversion—plasmids and virus production

All factors for iSMC conversion were cloned from cDNAs produced from a piece of the donor mouse skeletal muscle not used for fibroblast derivation in culture. The primers for cloning of the full-length MyoD, MYF5, MYF6, and myogenin ORFs by the same YFP-fusion strategy as the iNC factors are shown in Supplementary Table S1. Equal volumes (0.75 mL) of viral supernatant for each of the four myogenic factors was used to infect target fibroblasts; iSMCs were induced using iSMC medium [(DMEM, 0.1% FBS, 1×antibiotic/antimycotic (Invitrogen)], and all iSMCs were generated on uncoated plates, but otherwise, virus production, infection, and conversion were performed as with iNCs.

iSMC induction—immunohistochemistry and imaging

All procedures were performed as with iNCs except for the use of skeletal muscle-specific antibodies sarcomeric anti- α actinin used at 1:1000 (Sigma; A7811) and anti-sarcomeric myosin used at 1:500 (DSHB at University of Iowa).

Quantification of phenotypic conversion

Quantification of iNC/iSMC conversion was done using Hoechst33342 staining for nuclei/DNA (Blue), YFP fluorescence of the tagged proteins to indicate relative factor expression (Green), and β -IIIITubulin/*TUJ1* (iNCs) or sarcomeric α -actinin (iSMCs) immunostaining to indicate phenotypic conversion (Red). Relative reprogramming efficiency was calculated either by dividing the red fluorescence value by the blue fluorescence or by dividing the red/blue value by the “green” fluorescence value to include a factor-expression component in the calculation. For iSMCs, total sarcomeric α -actinin fluorescence was taken as the red value; whereas iNC conversion was measured as the number of red

fluorescent cells that also had fibers of at least three soma lengths. For each well, five fields—one in the center of each well and one at each compass point approx 1 cm from the well edge—were imaged at 100X magnification for each separate channel and stored as a merged RGB image. For measurement of fluorescence, the RGB image was split into individual black and white channels and quantified using NIH ImageJ. For the graphs, the highest relative conversion value for iNCs or iSMCs for each group was set at 100, and the remaining values were calculated as a fraction of that maximum. Calculation of correlation, significance, and ANOVA were performed using SigmaPlot 12 software.

Results

We sought to control external variables in the reprogramming process to promote better measurement of relative reprogramming efficiency. In keeping with this concept, all procedures and analyses were performed in parallel on all cell lines within a group and in at least three separate experiments. Fibroblasts were selected as the input cell type, because they have been extensively used as the raw material for most reprogramming studies, are easily established and expanded for many passages (without the need of cellular transformation), are adherent, and can be efficiently cryogenically preserved. Fibroblast lines were obtained from two species—human and mouse. Human fibroblasts were selected for study, because they are of the most direct clinical relevance and mouse fibroblasts were selected because they have been shown to be capable of reprogramming to several output cell types and because we were able to establish multiple isogenic fibroblast lines from a single donor animal under highly controlled conditions. Using both groups of cells, we were able to compare fibroblast lines with similar morphological properties from multiple individuals with fibroblast lines of variable morphology and tissue of origin, but from a single subject. We chose to work primarily with nonembryonic cells to better relate our findings to applications in human or veterinary medicine, as older subjects are more likely to be the primary target of reprogramming-based therapy for cellular replacement or transplantation.

Establishment of input lines

Human fibroblast lines used for analysis are shown in Table 1 (top panel). Lines for analysis were selected based on several general criteria. The lines needed to display robust growth in culture, should be well established (>passage 8 (P8)), display a homogenous morphology (Supplementary Fig. S1A), and be at least four passages from mitotic senescence. Most of the lines were from donor subjects with no known disease, but two were included from individuals with diagnosed neurological disorders—Rett syndrome and schizophrenia. Seven human fibroblast lines were subject to complete characterization and analysis with two additional lines that were only subjected to partial analysis, because they did not satisfy all criteria for inclusion.

To examine the impact of genetic homogeneity on reprogramming efficiency, mouse fibroblast lines were generated from a single donor animal (Table 1, bottom panel). To ensure that the individual mouse lines did not functionally approximate secondary lines or subclones that could occur if all lines were derived from a single explant, we harvested

eight different mouse organs for establishment of fibroblast lines (Supplementary Fig. S1B) and then selected only those lines which displayed no evident nonfibroblastic cells and a consistently uniform fibroblastic morphology (Supplementary Fig. S1C). Lines also had to display a similar capacity for growth, passage, and survival of freeze/thaw that allowed them to be maintained side by side with other lines. In this way, approximately 50 lines were progressively winnowed to 12 lines that were the most typical based on their growth characteristics and morphology. Unlike the human lines, the mouse lines were generated and maintained identically from their first day in culture—passaged, fed, frozen, thawed, and, in every other regard, cultured side by side. Ultimately, these lines, derived from seven different tissue sources (brain tissue did not give rise to robust fibroblast cultures), were frozen in multiple aliquots at P8 for use in experiments.

The phenotype of human and mouse fibroblast lines was further established by quantitative real-time polymerase chain reaction (RT-PCR) analysis to confirm an abundance of fibroblast-associated mRNAs and the absence of significant signal for indicators of other differentiated or progenitor cell types. All fibroblast lines displayed an abundance of the fibroblast-associated markers collagen type 1 α 2, vimentin, fibronectin, fibrillin I, and fibulin V with two exceptions: mouse lines HE4 and KI3 repeatedly tested negative for mouse collagen type 1 α 2. HE4 also displayed weak vimentin immunopositivity (see below), but since both lines were positive for some fibroblast markers and were negative for indicators of stem cell types, both lines were kept in the study with this caveat in mind. mRNAs for other common contaminating cell types (i.e., keratinocytes (keratin 14) and endothelial cells (PECAM)) or for myogenic or neurogenic progenitor-type cells (i.e., MyoD, Myf5, and FoxG1, Sox2, respectively) were not detected (Supplementary Fig. S2, summarized in Table 1).

To further characterize our lines at the level of individual cells, each line was examined for the expression of fibroblast-associated markers or markers of stem cell types by fluorescent immunocytochemistry. Sox-2 and nestin were selected as stem cell markers, because these markers label multiple classes of stem cell, including pluripotent stem cells, neural stem cells, and muscle precursors such as mesen-

chymal stem cells and satellite cells, but are not expressed at significant levels in fibroblasts [17–23]. The results (also summarized in Table 1) revealed that essentially all cells appeared immunopositive for the fibroblast-associated markers vimentin and fibronectin and no cells appeared positive for the stem cell markers nestin and Sox-2 which were readily observed in control cultures of human neural stem cells (Supplementary Fig. S3A, B). A parallel analysis of selected mouse lines (Supplementary Fig. S3C, D) showed a result similar to the human lines, with the exception of HE4 that displayed weak vimentin staining. Additional RT-PCR and immunocytochemical analysis of lines such as ADF and AUT examining other markers of stem and progenitor cell types, including neural crest-derived cells and pluripotent cell types, did not reveal any indication of other progenitor cell types within our fibroblast lines.

Conversion to iNCs

iNCs were selected as a conversion cell type to test comparative direct reprogramming efficiency for several reasons. The first was because several independent laboratories had confirmed the production of iNCs using similar methodologies [7,24–27]. The second was that iNCs should display a distinct set of markers of neuronal phenotype and changes in cellular morphology that would allow them to be readily distinguished from input fibroblasts. Third, evidence to date suggested that since they were produced directly from input cells without generation of an intermediate cell type or the requirement for cell division and formation of a progenitor colony, each iNC represented an individual reprogramming event.

For iNC conversion, human and mouse fibroblast cultures were infected with combinations of an MMLV-based retroviral vector encoding the neurogenic factors ASCL1, POU3F2, ZIC1, MYT1L, or NeuroD1 [7,25] of human origin fused to YFP as shown schematically in Supplementary Fig. S4. As predicted, all of the YFP-fusion proteins localized primarily to the nucleus, some presenting a mottled appearance and others appearing more diffuse (Fig. 1A). ASCL1-YFP generally presented as weak diffuse nuclear fluorescence with one or two bright punctuate bodies. Pilot studies indicated that ASCL1 and POU3F2 combined with

FIG. 1. Induced neural cell (iNC) conversion of mouse and human fibroblast lines. **(A)** HEK cells transfected with YFP fusion protein for iNC conversion, as labeled. Phase-contrast images are in the upper panels, and corresponding fluorescent images are in the lower panels. **(B)** Mouse embryonic fibroblasts infected with the individual factors (as labeled) and stained for β -III-tubulin/TUJ1 in red (upper panels). Mouse (MEFs) or human fibroblasts (FET) infected with combinations of Zic1/Ascl1/Pou3f2 (ZAP) or Myt1L/Ascl1/Pou3f2 (MAP)+NeuroD1 (MAPN) (lower panels). Green fluorescence indicates expression of reprogramming factor(s). Blue color is bis-benzimide nuclear staining of DNA. Insets have the blue channel removed and the green channel intensified to show YFP-factor expression in the nucleus of all iNCs. **(C)** Mouse iNCs produced from MEFs by day 10–15 postinfection immunopositive for multiple neural markers (red), including MAP2, pan-neurofilament (NF), doublecortin (DCX), or synapsin I (SYN). iNCs produced from adult mouse fibroblast lines (as labeled) with typical iNC morphology immunostained for β -III-tubulin/TUJ1 (red). **(D)** Human iNCs with typical morphology at day 24–30 postinfection and immunostained for multiple neuronal markers as in **(C)** in addition to PSD95, GABA receptor β 3 (GABAR-B3), and GAD1 (as labeled). GABAR-B3 and GAD1 iNCs were labeled using immunoperoxidase secondary antibody coupled with DAB staining. Scale bars are 10 μ m unless otherwise labeled. **(E)** Relative conversion of mouse fibroblast lines to iNCs calculated as a function of cell number at the time of harvest. The highest efficiency of conversion within each group was set to a value of 100. Bars indicate standard error of the mean (SEM). **(F)** As in E, for the human fibroblast lines. **(G)** As in E, but showing relative conversion of mouse fibroblast lines factoring in factor expression early (FEE) or late (FEL). The Pearson product-moment correlation (r) and P -value (P) for the two methods is shown. ANOVA (INF) indicates the probability that the median value differences (MVDs) for iNC conversion of lines are by chance occurrence. **(H)** As in G, for the human fibroblast lines. Color images available online at www.liebertpub.com/scd

either ZIC1 or MYT1L/ND produced many TUJ1-positive iNCs in both human and mouse cultures using either fetal or adult fibroblasts, whereas cells infected with each neurogenic factor alone produced very few iNCs (Fig. 1B). Mock-infected cells or cells infected with a YFP vector only and

cultured under identical conditions produced no strongly immunoreactive TUJ1-positive cells with neuron-like morphology (Supplementary Fig. S5A). As previously described, the primary difference between the generation of mouse and human iNCs was the maturation rate [7,24–27]. While mouse

iNCs with relatively mature morphology were sometimes observed as early as day 4–5 and reached maximal differentiation by day 10–12 postinfection, human iNC maturation appeared more progressive, with cells at days 8–12 displaying a rounded cell soma and very short processes of 7–10 μm , longer processes at day 18–20, and long processes with more complex branching and spine-like projections by day 24–30 (Supplementary Fig. S5B, C). Mature iNCs that displayed elongated processes (>3 soma lengths) were positive for multiple markers of mature neurons in addition to TUJ1, including MAP2a/b, synapsin 1, doublecortin, neurofilament 300 kDa, and others as shown in Fig. 1C and D. iNCs also displayed electrophysiological properties such as TTX-sensitive Na^+ currents and action potentials that were essentially indistinguishable from human neurons generated under identical conditions from human ES cell-derived human neural progenitor cells (NPCs) (Supplementary Fig. S5D).

To determine whether, and to what degree, variation was observed in the reprogramming of primary fibroblast lines to neural cells, all lines were thawed, passaged, and plated at the same density for side-by-side infection and culture. On day 12 for mouse cells and day 24 for human cells, transduced and control cultures were harvested in parallel for immunochemical staining and quantification of converted cells. iNCs with morphological characteristics and processes essentially indistinguishable from neurons generated side by side from human ES-derived NPCs were observed in all mouse and human fibroblast lines tested (Supplementary Fig. S6A, B), although in some lines they were rare or sometimes displayed shorter, less mature processes (i.e., SAF, LU6, TE5). Conversion efficiencies calculated as a simple percentage of output cells versus unconverted cells were in line with published reports of iNC conversion using methods similar to those in this report [7,10,25]. Maximum conversion efficiency to iNCs was $0.72\% \pm 0.08\%$ for adult mouse fibroblasts (TA4), and $1.07\% \pm 0.18\%$ for human cells (RET)). These conversion efficiencies, calculated as a simple percentage of output cells versus unconverted cells, were less than published studies reporting 2%–18% conversion using the same or different combinations of factors [7,24–26]. A decrease in reprogramming efficiency may be the trade-off for using YFP fusion proteins to facilitate assessment of factor delivery. In any event, the number of cells returned in our experiments and the degree to which they displayed markers and characteristics of induced neurons was more than sufficient for purposes of comparison of iNC conversion among our fibroblast lines.

As shown in Fig. 1E and F, relative reprogramming efficiency could be calculated by dividing the red fluorescence value indicative of reprogrammed cells in each line by the Hoechst 33342 fluorescence value indicative of total cells present at the time of harvest. Relative reprogramming calculated in this way shows dramatic differences between cell lines of both mouse and human origin, and this is the method that is used for measuring reprogramming efficiency in most published reports. The limitation of this method of determining conversion efficiency is that an unknown degree of the disparity may be caused by differences in factor delivery and production. Viral transduction and factor expression was included in the calculation of reprogramming efficiency in two ways. The first, referred to as “FEE”, was

essentially a determination of relative infection efficiency. Each line used in the analysis was infected with a nuclear-localized YFP virus at an MOI of approximately 0.5 and then counted to determine the number of cells with yellow fluorescent nuclei as a fraction of all cells on day 4 postinfection. The relative capacity of each line to take up and express virus at levels sufficient to be scored as positive relatively early after infection could then be used as one means of including factor transduction efficiency into the calculation of reprogramming efficiency.

Factor expression late (FEL) was an alternate method of measuring factor expression that was determined by the level of YFP fluorescence produced by the transgenes at the time of harvest. The fluorescent signal at this stage was less uniform than in cells used in the calculation of FEE. By the time of harvest, the YFP signal in iNC cultures was often punctuate or faint and could not be quantified accurately as a percentage of positive cells; so, FEL was measured as the overall intensity of YFP signal at the time of harvest instead of as a percentage of positive cells. Both FEE and FEL have their own individual merits, but both were included in our analysis to address the possibility that factor expression measured at different stages of the reprogramming process might influence the final determination of reprogramming efficiency.

As shown in Fig. 1G and H, after factoring factor expression into the calculation of reprogramming efficiency, clear line-to-line variation in the reprogramming capacity of cells of both mouse and human origin was still observed, irrespective of how transgene expression was factored into the calculation of efficiency. There was a strong and significant correlation between the iNC reprogramming efficiency calculated using both standards in the mouse cell lines ($r=0.975$, $P<0.0001$), and a similar trend was observed in the human lines ($r=0.664$, $P=0.104$). More importantly, analysis of variance within human iNC cultures revealed that the differences in median reprogramming among lines using either FEE or FEL were significantly greater than would be expected by chance (FEE $P=0.005$, FEL $P=0.006$). Differences among iNC reprogramming efficiencies in the mouse lines were only significant to $P=0.150$ (FEE) and $P=0.108$ (FEL), but appeared to be trending in the same direction. It was noted that although including factor expression into the calculation of reprogramming efficiency did not dramatically change the values for the best and worst converters, lines in the middle of the pack were more dramatically affected. Likewise, lines with intermediate conversion efficiencies also displayed more variation depending on the use of either FEE or FEL.

Conversion to induced skeletal muscle

A second reprogramming regimen was used to determine whether or not line-to-line variation observed with iNC reprogramming would be observed using an alternate regimen and to determine whether the same lines most amenable to iNC respecification were also those most reprogrammable to a second fate. Reprogramming to iSMCs was performed using four myogenic factors that were known to promote skeletal muscle identity and expressed as YFP fusion proteins as with neurogenic factors (Fig. 2A). One factor was MyoD1, shown in multiple studies to induce the conversion of fibroblastic cells to myotube-like cells [5,10]. A second

FIG. 2. Induced skeletal muscle cells (iSMC) factors and induction of iSMC phenotype in mouse and human fibroblast lines. **(A)** HEK cells transfected with YFP fusion protein vectors for iSMC conversion, as labeled. Green fluorescence is the nuclear-localized myogenic factors and blue is bis-benzimide staining. **(B)** KI6 mouse fibroblasts infected with the four separate myogenic factor viruses (as labeled) and immunostained for sarcomeric α -actinin. **(C)** Example of the morphological change observed in human NWB or mouse KI6 fibroblasts after infection and expression of iSMC factors (as labeled). **(D)** Magnified image (400 $\times$) of phase-contrast (upper) and fluorescent (lower) image of bis-benzimide stained KI6 iSMC myotubes. Arrows indicate multiple nuclei within the fiber. **(E)** Control or iSMC-factor infected mouse and human cells stained for skeletal muscle antigens sarcomeric α -actinin (α -ACT) or sarcomeric myosin (SMYO). Mouse cells were processed at day 12, and human cells were processed at day 24 postinfection. Scale bars are 10 μ m. **(F–I)** Relative conversion of mouse and human fibroblast lines to iSMCs as labeled (after Fig. 1E–H). Color images available online at www.liebertpub.com/scd

factor was the myogenic transcription factor myogenin [28], which in our pilot studies also displayed some capacity to induce muscle marker expression and a shift to myotube-like morphology, though to a lesser extent than *MyoD* (Fig. 2B). We also included two additional factors—*MYF6* [29] and *MYF5* [30]—that displayed minimal capacity to induce conversion on their own, but are known to support skeletal muscle maturation. Recombinant virus stocks for each of these four myogenic factors were combined in equal quantity and used as a multi-factor mix for iSMC conversion.

A shift of virus-transduced cultures from fibroblast growth medium to iSMC medium (DMEM+0.1% FBS) 3–4 days postinfection was found to best support conversion and maintenance of the iSMC phenotype. While uninfected fibroblasts (or cells infected with YFP only) showed no indication of conversion of cells to a myotube-like morphology under iSMC growth conditions, both mouse and human cells transduced with the iSMC virus cocktail showed abundant evidence of cell fusion and myotube formation by 6–8 days postinfection, and appeared to complete morphological maturation by 10–12 days of culture for mouse cells and approximately 18–20 days for human cultures (Fig. 2C, D). Immunocytochemical analysis of control and myogenic-factor infected fibroblasts confirmed that no cells which were immunopositive for markers of skeletal muscle myotubes were observed in control cultures (Fig. 2E), whereas factor-transduced cultures in some test lines displayed numerous elongated, tube-like cells that were positive for the muscle markers sarcomeric myosin and α -actinin (Fig. 2E). These data indicated that the viral reagents and culture regimen we used for iSMC reprogramming produced cells with multiple strong indicators of the conversion cell phenotype that were essentially identical to induced skeletal muscle myotubes or myotubes produced from cultured myoblasts previously reported [5,10,31,32].

The experimental design for the analysis of iSMCs was essentially identical to the process used earlier for iNCs. Maximum conversion efficiency calculated as a percentage of iSMCs to unconverted cells was similar to other published conversion rates of mouse and human fibroblasts to iSMCs [5,7,10,25]. Maximum iSMC conversion for adult mouse fibroblasts was $20.3\% \pm 3\%$ (KI6) and $60.4\% \pm 6\%$ for human fibroblasts (FET).

iSMCs, identified as elongated cells with strong α -actinin immunopositive fluorescence, were observed in ten out of the twelve mouse fibroblast lines tested. Lines such as KI6 and TA6 produced an abundance of iSMCs; while lines such as HE4, SM1, and LU6 produced only rare cells and lines LI6 and TE4 produced no cells with the morphology and marker expression consistent with the output phenotype (Supplementary Fig. S7A). iSMCs were observed in all transduced human cultures (including the AUT and HSK lines not included in the full analysis), although immunopositive iSMCs were rare in the EAF and E2F lines derived from elderly donor subjects (Supplementary Fig. S7B).

As with iNCs, iSMC reprogramming efficiency calculated only as a function of converted cells relative to all cells at the time of harvest (Fig. 2F, G) or factoring-in transgene expression as either FEE or FEL (Fig. 2H, I) revealed substantial differences among the cell lines of both species. There was a strong and significant positive correlation between the reprogramming efficiencies calculated using both FEE and FEL

for mouse lines ($r=0.969$, $P < 0.001$) and human lines ($r=0.837$, $P=0.019$). More importantly, analysis of variance indicated differences in median iSMC reprogramming efficiencies significantly greater than would be expected by chance for both mouse ($P < 0.001$) and human fibroblast cell lines ($P < 0.05$) (Fig. 2H, I). These results confirm that although there are some differences in calculated reprogramming efficiencies depending on how or when factor transduction levels are measured, the overall results are similar and support the notion that there are substantial differences in the reprogramming capacity of individual lines, even of the same general type.

iNC versus iSMC reprogramming and factors controlling reprogramming efficiency

The reprogramming efficiencies for all cell lines and conversion regimens are combined in Fig. 3A–D. Analysis of the correlation between iNC and iSMC conversion normalizing with either FEE or FEL revealed no significant correlation, indicating that efficiency of conversion to one phenotype is not predictive of reprogramming to another identity.

Since successful infection with MMLV-based vectors requires active cell division and both reprogramming regimens produce a nondividing, growth-arrested cell phenotype, we sought to determine whether growth-related cellular characteristics—infectivity and mitotic index—correlated with conversion efficiency. As shown in Fig. 3E and F, comparing the reprogramming values without correction by FEE or FEL with relative growth and infectivity revealed no correlation with iNC conversion efficiencies; however, there was a significant positive correlation between infectivity/growth and conversion to iSMCs in both the human and mouse lines. Despite this correlation, there was still striking variability in the reprogramming efficiency of different lines, suggesting that multiple factors in addition to those regulating the cell cycle likely impact the reprogramming process.

Discussion

The initial impetus for this report arose from several observations in our laboratory. One observation was that cell lines of a similar origin and properties often appeared to differ significantly in their capacity to reprogram to an alternate phenotype, and, while this difference was generally attributed to technical variables, this attribution was rarely, if ever, systematically investigated. Another was that reprogramming regimens described in the literature were sometimes not as efficient as reported, even when components such as viral vectors, media formulations, and culture conditions were very similar or even identical. Though it is recognized that properties of the input cell such as species of origin, cellular age, and cell phenotype can have a dramatic impact on reprogramming efficiency, our own observations and published studies where differences in reprogramming efficiency were observed even where the potential for technical variation was minimal [5,10] led us question to what extent reprogramming efficiency could be expected to vary among cell lines of the same type when methodological and technical issues were deliberately controlled.

The comparison of two conversion regimens over a large number of cell lines also permitted us to determine whether

FIG. 3. Comparative conversion of mouse and human fibroblasts to iNCs and iSMCs. **(A)** Comparative conversion of human fibroblast lines to iNCs (black bars) and iSMCs (white bars) calculated as a function of FEE or **(B)** as a function of FEL. **(C)** As in **(A)** for mouse fibroblast lines. **(D)** As in **(B)** for mouse fibroblast lines. The correlation between iNC and iSMC conversion and significance of this value is shown for each group. **(E)** Line graph of mean relative iNC conversion (iNC), iSMC conversion (iSMC), mitotic rate (GROWTH), and infection efficiency (INFECTION) for each of the human fibroblast lines sorted by increasing infection efficiency. At the right is a plot of the trend lines. Correlations (r) with significance (P) of ≤ 0.05 are shown above. **(F)** As in **(E)**, for the mouse cell lines.

converting cells arose from a specific population of reprogramming-susceptible cells or whether they were likely derived from a “main” population of cells, each with their own independent capacity for adoption of a new identity. It was long thought likely that reprogrammed cells arose from cryptic populations of canonical stem cells of varying potency contaminating the main population, but direct reprogramming experiments from many sources to produce induced pluripotent stem cells [6,33–38], neurons [7,24–27,39,40], hepatocytes [9,41], cardiomyocytes [8,13,42], and other induced cell types have since largely disproved this proposition.

In our own study, considering the efficiency of reprogramming that we observed coupled with the probability of uptake of sufficient viral copies for conversion (particularly in the case of iNCs) and an absence of evidence of nonfibroblasts at the level of individual cells by immunocytochemical analysis, it is unlikely that contaminating non-fibroblasts contribute significantly to the converted cell pool. Therefore, if converted cells arise through a specific “susceptible” population, it is likely that this population is a subpopulation of the fibroblasts themselves. Such cells could not only be a purposeful biological variant, but they may also be defective cells that lack the determinants which allow

them to maintain a fixed identity when challenged with a stronger competing program. These lacking determinants may be genetic in nature, arising from point mutations that alter gene expression or function, epigenetic, arising from alterations in methylation or modification of histones that regulate gene silencing, or contextual, arising from some cellular characteristic that is determined by the cell’s interaction with other cells (perhaps akin to side-population cells) [43].

Our results are at odds with the idea that converted cells arise from a fixed subpopulation of cells with general susceptibility to identity respecification. When subjected to two separate reprogramming regimens, the efficiency of conversion to one phenotype did not predict reprogramming efficiency to a second phenotype. Although only two conversion regimens were tested, this result suggests that a mechanism such as a genome-wide failure of some aspect of the molecular machinery controlling cellular identity does not underlie phenotypic lability; instead, the process is likely to be stochastic, falling by chance on genes that permit the cell to respond only to specific transcription factors and to respecify only to some phenotypes. It implies that the fraction of reprogramming-receptive cells in any given population may arise from any number of discrete genetic or epigenetic mutations (possibly even single-base changes) in critical

determinants in or around the control regions of genes or classes of genes that are critical to the maintenance of a specific cell phenotype. These mutations would also only be present in a fraction of the population—daughter cells of the cell harboring the mutation—and would be heritable and account for the apparent “stability” of reprogramming to a given phenotype within most lines. Whether these alterations are epigenetic, genetic, or some combination of both remains to be elucidated. If only tiny changes in the genome or epigenome are responsible for susceptibility to conversion, it may be difficult to isolate or even identify individual cells that are “good reprogrammers” *a priori*. Very sensitive methods that combine sequencing with quantification, such as whole genome sequencing, genome-wide bisulfite sequencing, or RNAseq, may be able to identify even single-base changes that correlate with reprogramming efficiency and provide insights into gene targets and pathways which could be manipulated to improve phenotype-specific conversion.

Our results further indicated that when technical components were held constant, the direct reprogramming capacity of independent primary fibroblast cell lines was reproducible within a single line from experiment to experiment, but varied dramatically from line to line irrespective of whether the lines were derived from different donors or from a single subject. It was notable that the disparity in reprogramming efficiency between different lines—that in some instances differed by orders of magnitude—was more than sufficient to account for discrepancies in reproducing published reprogramming regimens if the target cells were not of very similar type. Indeed, our results suggest that the production of primary lines of “very similar” type for use in reprogramming experiments by independent laboratories may, in itself, be a very challenging proposition. Fibroblasts cultured from different body regions are known to have distinct differences in gene expression for transcription factors such as members of HOX gene clusters [44] despite sharing general characteristics and a common phenotype. It may be that propagation of tissue explants and repeated passage of resulting cells magnifies intrinsic differences such as those seen with HOX genes, even in lines derived from the same general tissue source. This would explain our results with cell lines such as KI2, KI3, KI5, and KI6 that displayed dramatic differences in reprogrammability despite having originated from proximal tissue fragments from a single kidney harvested from a single mouse.

Along these same lines, we observed clear reprogramming differences in fibroblasts derived from different tissue sources, but cannot conclude that specific tissues repeatedly give rise to cells with superior or inferior reprogramming capacity until a larger number of samples can be assayed. In our hands, skeletal muscle-derived fibroblasts were poor converters to iSMC identity, indicating that in at least some cases, the tissue of origin does not predict relative capacity to reprogram to that same tissue. A larger number of lines derived from more subjects will be needed to begin to see patterns in the reprogrammability of fibroblasts from different tissue sources.

Similarly, we observed that our two human cell lines isolated from patients with neurological disease (RET and SAF) displayed reprogramming efficiencies that were at opposite ends of the spectrum with regard to iNC conver-

sion, but were very similar with regard to iSMC conversion. While it is tempting to speculate that iNC conversion was in some way influenced by the determinants of disease, the variability in efficiency which we observed between other human and mouse lines suggests that until a much higher number of lines from multiple patients are compared directly, these differences as likely to arise by chance as from the determinants responsible for neural dysfunction. It will be interesting to determine in future experiments whether “qualitative” measures of converted cells display as much variability as the number of cells converted. For example, if converted neurons, regardless of their total numbers, display an essentially equivalent level of maturation and electrophysiological response, it may be that lines from a lower number of donor subjects would be sufficient to produce meaningful results.

Our results comparing the cell growth characteristics of individual lines and reprogramming efficiency suggest that there may be gross physical properties that either modulate or “predict” reprogramming efficiency. We expected and observed some correlation between infection efficiency/mitosis and reprogramming; however, a significant correlation was only observed with iSMCs and not with iNCs. With more cell lines and more detailed analysis of characteristics such as cell growth, the molecular determinants that account for this difference may be identifiable and may provide us with insights into the primary forces that govern phenotypic conversion.

Ultimately, our findings suggest that parallel testing of multiple cell lines from several sources may be needed to accurately determine the efficiency of direct reprogramming procedures, and, by extension, that the most direct means of improving reprogramming efficiency for a given regimen may be to simply test more fibroblast lines, even if these lines are from a single source.

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Author Disclosure Statement

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