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Generation of an Inducible Fibroblast Cell Line for Studying Direct Cardiac Reprogramming

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Abstract

Direct reprogramming of fibroblasts into induced cardiomyocytes (iCMs) through forced expression of cardiac lineage-specific transcription factors holds promise as an alternative strategy for cardiac regeneration. To facilitate research in iCM reprogramming, we generated a suite of new tools. We developed a transformed cell line derived from mouse embryonic fibroblasts (MEF). This fibroblast cell line (MEF-T) harbors an αMHC-eGFP reporter transgene for rapid detection of newly derived iCMs. The MEF-T cell line is highly proliferative and easily transfected and transduced, making it an ideal tool for transgene expression and genetic manipulation. Additionally, we generated a Tet-On inducible polycistronic iCM reprogramming construct for the temporal regulation of reprogramming factor expression. Furthermore, we introduced this construct into MEF-T and created an inducible reprogrammable fibroblast cell line. These tools will facilitate future research in cell fate reprogramming by enabling the temporal control of reprogramming factor expression as well as high throughput screening using libraries of small molecules, non-coding RNAs and siRNAs.

Keywords

Cell Reprogramming; Inducible Gene Expression; Fibroblast Cell Line; MGT

Results and Discussion

Direct cardiac reprogramming of fibroblasts into cardiomyocyte-like cells offers additional strategies for cardiac regeneration and disease modeling. The expression of three cardiac-lineage transcription factors – Mef2c, Gata4, and Tbx5 (MGT) – is sufficient to convert fibroblasts directly into induced cardiomyocytes (iCMs) *in vitro* (Ieda et al., 2010) and *in vivo* (Qian et al., 2012). A polycistronic construct with the three transcription factors separated by peptide cleavage sites yields stoichiometrically optimal ratios of the three reprogramming factors (Wang et al., 2015a). This polycistronic MGT construct produces improved reprogramming efficiency *in vitro* (Wang et al., 2015a) and improved reprogramming efficiency and cardiac function *in vivo* (Ma et al., 2015). To facilitate studies in direct cardiac reprogramming, we developed a suite of tools for iCM research including a

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transformed fibroblast cell line with a cardiac reporter, an inducible polycistronic reprogramming construct, and an inducible reprogrammable fibroblast cell line.

First we developed a cardiac reporter fibroblast cell line. We isolated mouse embryonic fibroblasts (MEF) at embryonic day 13.5 from α muscle heavy chain-green fluorescent protein (αMHC-GFP) cardiac reporter strain mice (Ieda et al., 2010; Qian et al., 2012) (Fig. 1a). This transgenic strain drives GFP reporter expression with the cardiac αMHC promoter. Cardiomyocytes from αMHC-GFP mice are GFP positive, while fibroblasts are GFP negative. Consequently, primary MEFs isolated from αMHC-GFP embryos are GFP negative; however, MEFs that have been reprogrammed into iCMs through the forced expression of cardiac lineage specific transcription factor cocktails are GFP positive. We transformed primary αMHC-GFP MEFs with retroviral delivery of SV40 large T antigen (Hahn et al., 2002) and selected transformed cells with Zeocin (Fig. 1a). The transformed mouse embryonic fibroblast cell line, MEF-T, is morphologically similar to parental primary MEF (Fig. 1b). However, MEF-Ts are more prolific than primary MEFs and have a shorter population doubling time (Fig. 1c). Accordingly, a higher proportion of MEF-Ts are active in the cell cycle compared to primary MEFs (Fig. 1d). Propidium iodide staining and DNA content analysis reveal that 31% of MEF-Ts are in S phase compared to only 9% of primary MEFs (Fig. 1d). Additionally, nucleotide analog EdU incorporation confirms that more MEF-Ts are actively synthesizing DNA than MEFs (Fig. 1e). A 2.5 hour EdU pulse labeled 49% of MEF-T cells compared to 19% of primary MEFs. The proportion of MEF-T cells positive for the proliferation marker Ki67 is also over 2-fold higher than primary MEFs (Fig. 1f). These data demonstrate that MEF-T is a transformed, highly proliferative fibroblast cell line.

We next determined the potential of the MEF-T cell line for transgene expression using three common laboratory techniques: a lipid-based transfection reagent (Lipofectamine3000), lentiviral transduction, and retroviral transduction. First, we used Lipofectamine3000 to transfect primary MEF and MEF-T. Following transfection with a GFP-expressing plasmid, 35% of MEF-T expressed the GFP transgene compared to 7% of primary MEF (Fig. 2a), indicating that MEF-T is significantly more susceptible to transfection using lipid-based transfection reagents than primary MEF. Second, we used a lentiviral vector to transduce primary MEF and MEF-T. A GFP-expressing lentivirus transduced 97% of MEF-T but only 57% of primary MEF (Fig. 2b), indicating that MEF-T is also significantly more susceptible to transduction by lentiviral vectors. Third, to test a large construct encoded in a retroviral construct, we took advantage of our polycistronic system. We designed a retroviral construct to serve as a control for our polycistronic reprogramming construct Mef2c-Gata4-Tbx5 (MGT) (Wang et al., 2015b) by replacing the transcription factor Mef2c with the fluorescent reporter GFP to create the polycistronic construct GFP-Gata4-Tbx5 (GGT). GGT transduced 94% of MEF-T but only 61% of primary MEF (Fig. 2c). MEF-T is significantly more susceptible to retroviral transduction, lentiviral transduction, and Lipofectamine transfection. Finally, we assessed the duration of transgene expression of constructs of different sizes in the MEF-T cell line. The small GFP lentivirus produced sustained transgene expression, while the large GGT retrovirus produced a transient transgene expression (Fig. 2d and e). The cardiac reporter fibroblast cell line MEF-T is the first reported of its kind and will be a valuable tool for *in vitro* studies involving activation of cardiac markers such as αMHC.

As an additional tool to facilitate studies in direct cardiac reprogramming, we incorporated the Tet-On inducible gene expression system (Das et al., 2004; Gossen et al., 1995; Zhu et al., 2002) into our MEF-T cell line to permit the temporal regulation of factor expression during the reprogramming process. With constitutive expression of the engineered reverse tetracycline-controlled transcriptional activator (rtTA), transcription of genes under the control of the tetracycline responsive element *tetO* in the promoter can be regulated by the addition or removal of the tetracycline derivative doxycycline. In the absence of doxycycline, rtTA does not bind to the tetracycline responsive element in the promoter and fails to initiate transcription of the target genes. However, in the presence of doxycycline, rtTA binds to the promoter and initiates transcription of the target genes. We generated a MEF-T cell line that constitutively expresses rtTA for the temporal regulation of transgene expression, called MEF-T-rtTA (inducible MEF line-iMEF). We tested the TetOn system in iMEF using lentiviral infection with a tetracycline responsive RFP reporter construct (Fig. 3a). iMEFs transduced with RFP do not express RFP in the absence of doxycycline; however, with the addition of doxycycline to the culture media, 87% of iMEFs robustly express the RFP reporter (Fig. 3b). The spectral peak of uninfected iMEF overlaps completely with RFP infected iMEFs in the absence of doxycycline, indicating that the TetOn system is not leaky (Fig. 3b and c). The iMEF cell line is a valuable tool for temporal regulation of gene expression during reprogramming. One application of the iMEF cell line is to control RNA interference during reprogramming. Tetracycline-responsive short hairpin RNA can be used in combination with standard retroviral MGT to test the role of a gene of interest by knocking it down at specific times during the reprogramming process. Additionally, the initiation and duration of reprogramming factor expression can be controlled in MEF-T-rtTA if tetracycline-responsive promoters are used to drive transcription factor transcription. This system can be applied to the standard MGT cocktail or direct cardiac reprogramming cocktails utilizing additional factors such as Hand2, myocardin, SRF, Nkx2.5, or miR-133 (Addis et al., 2013; Christoforou et al., 2013; Jayawardena et al., 2012; Muraoka et al., 2014; Protze et al., 2012; Song et al., 2012). Furthermore, the iMEF-cell line has broad applicability in direct lineage reprogramming over and above direct cardiac reprogramming, as it can be employed to regulate the temporal expression of reprogramming cocktails for other lineages such as hepatocyte or neuron.

To complement the iMEF cell line, we designed a tetracycline-responsive MGT reprogramming factor construct for use in the Tet-On inducible gene expression system. We put the polycistronic MGT construct (Ma et al., 2015; Wang et al., 2015b) under the control of a tetracycline responsive promoter for temporal control of MGT reprogramming factor expression, creating an inducible polycistronic MGT construct (iMGT) (Fig. 3d). In the absence of doxycycline, the reprogramming factors are not expressed; however, with the addition of doxycycline, stoichiometrically optimal ratios of the three transcription factors are expressed for optimal reprogramming efficiency (Fig. 3d). When iMGT is co-expressed with rtTA in HEK 293T cells by either transfection or lentiviral delivery and doxycycline is supplied, the reprogramming factor protein expression is similar to that from the retrovirally expressed polycistronic MGT construct (Fig. 3e). In the absence of doxycycline, the three reprogramming factors are not detected.

Finally, we incorporated the iMGT construct into the iMEF cell line to generate a new cell line (icMEF) that can be reprogrammed simply by the addition of doxycycline to the culture media. The αMHC-GFP cardiac reporter in the icMEF cell line indicates reprogramming with GFP reporter expression. Within three days of doxycycline addition, 23% of icMEF cells are GFP positive (Fig. 3f and g). With doxycycline addition, icMEF also upregulate expression of cardiac genes, including sarcomere components *Actc1* and *Tnnt2* and ion channel subunits *Slc8a1*, *Kcna5*, and *Scn5a* (Fig. 3h). Similarly, inducible MGT expression in primary MEFs turns on αMHC-GFP reporter expression and cardiac marker cTnT expression detected by immunocytochemistry (Fig. 3i). It is possible that the icMEFs that remain cardiac marker negative after doxycycline addition have undergone transdifferentiation to some extent but have not yet turned on these markers. Such phenomenon is inherent in cell fate reprogramming due to the existence of a series of molecular barriers and the asynchronous conversion among starting cells that are not completely identical at the molecular and epigenetic levels. Single cell genomics will be the ultimate approach to determine how cell heterogeneity and the asynchronous nature of this process impact cardiac fate acquisition in fibroblasts.

In iPSC reprogramming, increased cellular proliferation through the inhibition of cell cycle regulation improves reprogramming efficiency (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009; Zhao et al., 2008). In a similar manner, it is possible that SV40 T antigen-mediated transformation and increased proliferation of the icMEF cell line contributes to these relative higher rates of reporter expression and reprogramming efficiency when compared to the primary CF reprogramming. While we recently demonstrated that deletion of p53, p19, and p16 did not result in a significant increase in the percentage of generated iCMs (Zhou et al., 2016), the use of retroviruses to introduce the reprogramming factors inevitably links proliferation rate to reprogramming efficiency. Future experiments using quiescent fibroblasts will be performed to test the potential role of cellular proliferation in iCM reprogramming.

In summary, the icMEF cell line described here will facilitate high-throughput and combinatorial screening using libraries of small molecules, pharmacological reagents and non-coding RNAs to further accelerate research in the field. The rapid and simple single GFP reporter read-out is ideal for such purposes since it reduces potential noise and complications from additional markers. Primary screening using the icMEF cell line has the advantage of high thoughput and high yield, but can inevitably yield false positives. In addition, icMEFs cannot be reprogrammed into fully matured, beating cardiomyocytes. Candidate factors identified from large screens in icMEFs will need to be validated and further characterized through secondary screens in primary fibroblasts using additional read-outs, including supplemental markers and functional parameters. The icMEF cell line was designed as a tool for the rapid, high-throughput identification of novel factors involved iCM reprogramming for better understanding of CM biology and potential applications in regenerative medicine.

Methods and Materials

All cell lines and constructs described here will be made available to the research community.

Primary Cell Culture and Immortalization

Mouse embryonic fibroblasts (MEF) were isolated as previously described (Jozefczuk et al., 2012) from αMHC-GFP reporter mice (Ieda et al., 2010; Qian et al., 2012). Animal care was performed in accordance with the guidelines established by University of North Carolina, Chapel Hill. MEF were seeded at a density of 5×10^4 cells per well in a 6 well plate coated with 0.01% gelatin. The following day, cells were lentivirally infected with large T-antigen with Zeocin resistance (AddGene #1779). Two days later, cells were re-plated in media with 300 μg/mL Zeocin. Antibiotic selection was maintained until all uninfected cells in control wells had died.

Propidium Iodide Cell Cycle Analysis

MEF and MEF-T were seeded at a density of 4×10^4 cells per well in 24 well plates coated with 0.01% gelatin. After 48 hours of culture, cells were dissociated with 0.025% Trypsin-EDTA, washed once with 2% FBS in PBS and once with PBS, and fixed in 70% ethanol overnight at −20°C. Fixed cells were pelleted, washed twice in 1% BSA in PBS, resuspended in PBS with 10 μg/mL RNaseA and 50 μg/mL PI, and analyzed immediately by flow cytometry. Data was collected on an Accuri C6 cytometer (BetaDickson) and analyzed using FlowJo software (Tree Star).

Ki67 Nuclear Antibody Staining

MEF and MEF-T were seeded at a density of 4×10^4 cells per well in 24 well plates coated with 0.01% gelatin. After 48 hours of culture, cells were dissociated with 0.025% Trypsin-EDTA, washed once with 2% FBS in PBS and once with PBS, and fixed in 70% ethanol overnight at −20°C. Fixed cells were pelleted, washed in 1% BSA/PBS, resuspended in 50 μL rabbit anti-Ki67 antibody (1:500, Abcam) in 1% BSA/PBS for 30 minutes at room temperature, washed twice in 1% BSA/PBS, resuspended in 50 μL Alexa Fluor 488–conjugated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch, Inc.) in 1%BSA/PBS for 30 minutes at 4°C, washed twice in 1% BSA/PBS, resuspended in PBS, and analyzed by flow cytometry. Data was collected on an Accuri C6 cytometer (BetaDickson) and analyzed using FlowJo software (Tree Star).

EdU Incorporation and Staining

MEF and MEF-T were seeded at a density of 4×10^4 cells per well in 24 well plates coated with 0.01% gelatin. After 48 hours of culture, EdU was added to the cell culture media to a final concentration of 10 μM and incubated for 2.5 hours. For flow cytometry analysis, cells were harvested and EdU incorporation was visualized using a Click-iT Plus EdU Flow Cytometry Assay Kit (Life Technologies, C10632) according to the manufacturer's instructions. Cells were counted on an Accuri C6 cytometer (BetaDickson) and analyzed using FlowJo software (Tree Star). For immunocytochemistry, cultures were washed with 3% BSA in PBS three times and fixed with 4% paraformaldehyde (EMS) at room

temperature (RT) for 15 min. After permeabilization with 0.2% Triton in PBS for 20 min at RT, EdU incorporation was visualized with 500 μL of Click-iT Plus reaction cocktail (Life Technologies, C10632) according to the manufacturer's protocol incubated for 30 minutes. After washing with PBS three times, nuclei were stained with Hoechst 33342 (Life Technologies). Images were acquired using EVOS® FL Auto Cell Imaging System (Life Technologies).

Viral Transduction and Efficiency Determination

MEF and MEF-T were seeded at a density of 2×10^4 cells per well in 24 well plates coated with 0.01% gelatin and infected with virus the following day in iCM media (4:1 DMEM:M199 with 10% FBS) with 4 μg/mL polybrene. Three days post infection, cells were dissociated with 0.025% Trypsin-EDTA, washed and resuspended in PBS. Transduction efficiency was analyzed immediately by flow cytometry. Data was collected on an Accuri C6 cytometer (BetaDickson) and analyzed using FlowJo software (Tree Star).

Characterization of iMEF by Flow Cytometry

iMEF were seeded at a density of 2×10^4 cells per well in 24 well plates coated with 0.01% gelatin and infected with virus the following day in iCM media (4:1 DMEM:M199 with 10% FBS) with 4 μg/mL polybrene and 1 μg/mL doxycycline. Three days post infection, cells were dissociated with 0.025% Trypsin-EDTA, washed and resuspended in PBS. Transduction efficiency was analyzed immediately by flow cytometry. Data was collected on an Accuri C6 cytometer (BetaDickson) and analyzed using FlowJo software (Tree Star).

Inducible Polycistronic Reprogramming Construct (iMGT) Cloning

A modified pTRIPZ (Thermo Scientific, now Dharmacon) vector described previously (Zheng et al., 2014) was a kind gift from Qing Zhang. Our polycistronic MGT construct (Wang et al., 2015a) was cloned into the vector behind the tet operator sites and minimal CMV promoter using the AgeI and ClaI restriction enzyme sites. WPRE was amplified from the vector with primers to include flanking KpnI and MluI cleavage sites. Digestion of the vector with the enzymes MluI and KpnI removed a segment from base pairs 4064 to 8019 that included WPRE, rtTA3, PuroR, and the shRNAmir insertion site. This removal of the second KpnI site at bp 6543 leaves a single KpnI site at bp 8019. The WPRE PCR amplicon was digested with KpnI and MluI and ligated into the vector. The resulting construct contains polycistronic MGT in frame behind the tet operator sites and minimal CMV promoter and lacks rtTA3 and PuroR. For the co-expression of rtTA with the iMGT construct, a pTRIPZ vector was digested with the enzymes XbaI and MluI and re-ligated to remove the tet operator sequences and minimal CMV promoter. In this construct, rtTA3 is constitutively expressed under the human ubiquitin C promoter. The transcriptional activator rtTA3 is sensitive to doxycycline dosage and can be titrated to regulate transcriptional activity (Das et al., 2004).

Cardiac Reprogramming Using the icMEF Cell Line

icMEF were seeded at a density of 2×10^4 cells per well in 24 well plates coated with 0.01% gelatin. The following day, media was changed to iCM media (4:1 DMEM:M199 with 10%

FBS) with 1 μg/mL doxycycline. Three days post infection, cells were dissociated with 0.025% Trypsin-EDTA, washed and resuspended in PBS. Transduction efficiency was analyzed immediately by flow cytometry. Data was collected on an Accuri C6 cytometer (BetaDickson) and analyzed using FlowJo software (Tree Star).

Growth Curve

MEF-T were seeded at a density of 1×10^5 cells per well in 6 well plates coated with 0.01% gelatin. Wells were harvested in triplicate and cells counted by hemocytometer at 24 hour intervals for eight days.

Immunocytochemistry

Cells were washed with PBS three times and fixed with 4% paraformaldehyde (EMS) at room temperature (RT) for 15 min. After permeabilization with 0.2% Triton/PBS for 15 min and blocking in 5% BSA for 1 hour, cells were treated with primary antibody at 4°C overnight, secondary antibody for 1 hour at RT, and nuclei staining with Hoechst 33342 (Life Technologies). The following antibodies were used: Rabbit anti-Ki67 (1:500, Abcam), rabbit anti-GFP (1:500, Life Technologies), Alexa Fluor 488–conjugated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch, Inc.). RFP reporter fluorescence was imaged without antibody staining. Images were acquired using EVOS® FL Auto Cell Imaging System (Life Technologies).

Western Blotting

Cells were lysed in 2x SDS loading buffer (Bio-Rad). Proteins in cell lysate was separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the following antibodies: Mef2c (1:1000, Abcam), Gata4 (1:200, Santa Cruz Biotechnology), Tbx5 (1:200, Santa Cruz Biotechnology), or β-Actin (1:1000, Santa Cruz Biotechnology). The target proteins were detected by chemiluminescence (ECL, Thermo Fisher Scientific). The membranes were stripped with stripping buffer (Sigma) and re-probed with antibody against a second protein or β-Actin as a loading control.

Real Time Quantitative Polymerase Chain Reaction

Total RNA was harvested from icMEF cultures three days after doxycycline addition by standard phenol/chlorophorm isolation using TRIzol reagent (Thermo Fisher Scientific). cDNA was obtained by SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's protocol. RT-qPCR was performed on a ViiA 7 Real-Time PCR System (Applied Biosystems) with SYBR Green (Thermo Fisher Scientific) or TaqMan (Thermo Fisher Scientific) chemistry.

Statistical Analyses

The statistical significance of differences between groups was analyzed using a two way unpaired student's t-test. A p-value < 0.05 was regarded as significant. Error bars indicate standard deviation.

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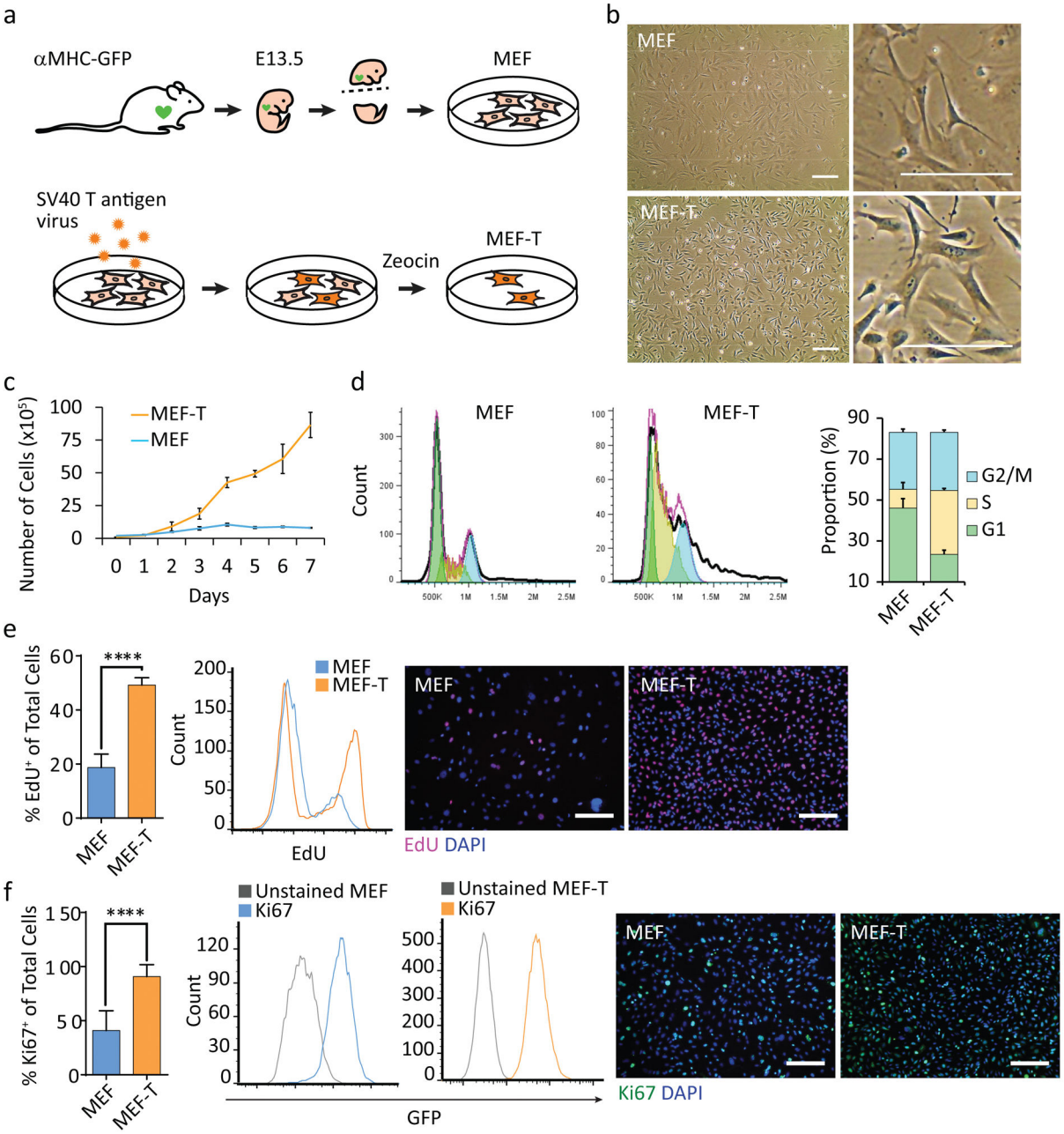

Figure 1.

Characterization of the MEF-T cell line. **(a)** Schematic of cell line development. Embryos are harvested from a pregnant female αMHC-eGFP mouse at embryonic day 13.5. Mouse embryonic fibroblasts (MEF) are isolated and transformed with lentiviral delivery of SV40 T antigen. Transformed cells are selected with Zeocin. **(b)** Morphology of primary MEF and MEF-T cell line. Scale bar is 200 μm. **(c)** Growth curve for MEF and MEF-T. **(d)** Propidium iodide staining and DNA content analysis. **(e)** Incorporation of nucleotide analog 5-ethynyl-2′-deoxyuridine (EdU). Scale bar is 200 μm. $P < 0.0001$. **(f)** Nuclear Ki67 proliferation marker staining. Scale bar is 200 μm. $P < 0.0001$.

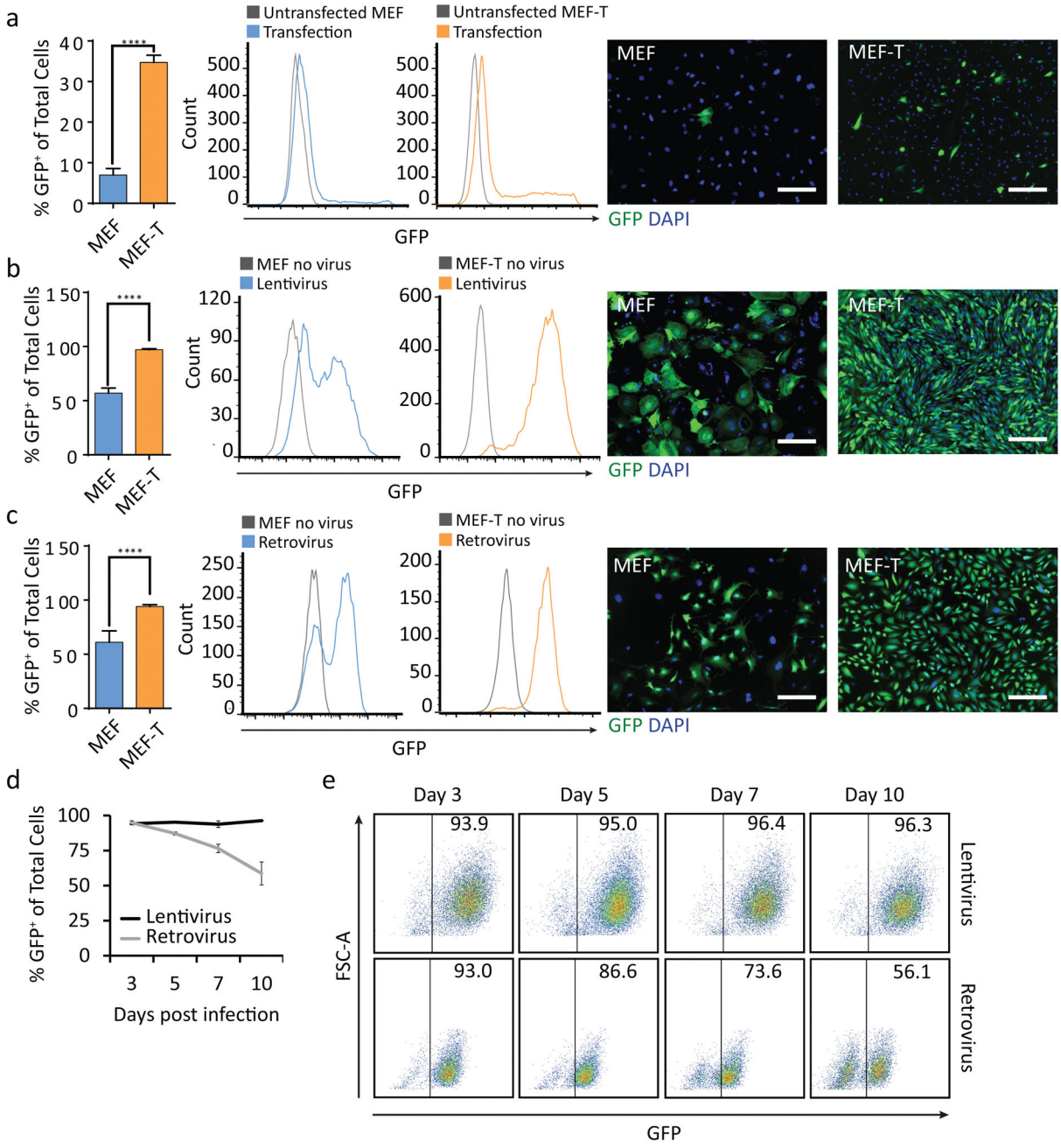

Figure 2.

Transgene expression in the MEF-T cell line. **(a)** Transfection with GFP transgene using Lipofectamine3000. Scale bar is 200 μm. $P < 0.0001$. **(b)** Lentiviral transduction with GFP transgene. Scale bar is 200 μm. $P < 0.0001$. **(c)** Retroviral transduction with GFP-Gata4-Tbx5 (GGT) transgene. Scale bar is 200 μm. $P < 0.0001$. **(d, e)** Sustained expression of GFP transgenes delivered by lentivirus and retrovirus.

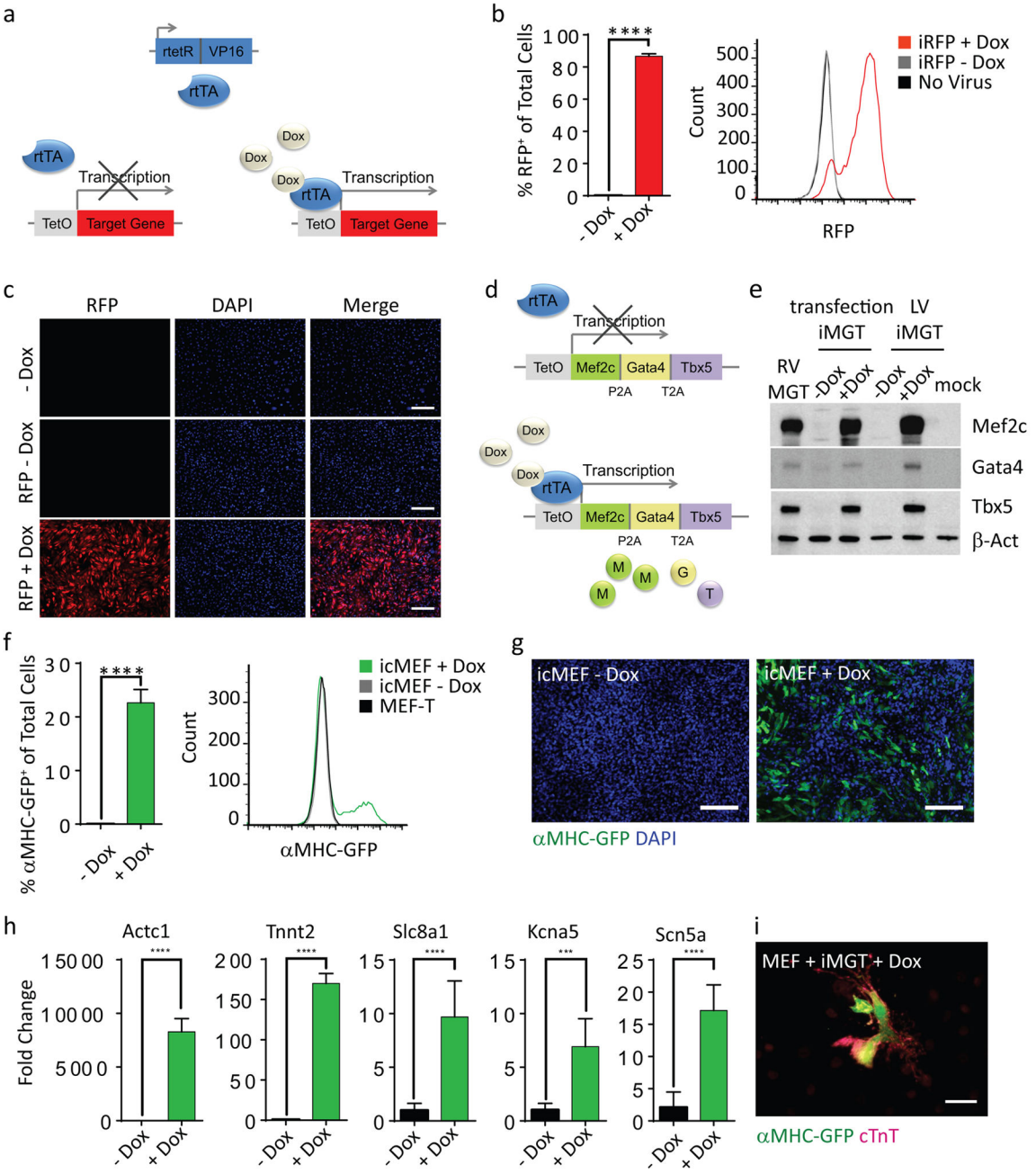

Figure 3.

Inducible reprogramming constructs and cell lines. **(a)** Schematic of the TetOn inducible gene expression system. **(b)** Validation of the iMEF cell line using a tetracycline responsive RFP reporter lentiviral infection. $P < 0.0001$. **(c)** Immunocytochemistry of controlled RFP reporter expression in iMEF. Scale bar is 200 μm. **(d)** Schematic of the inducible polycistronic MGT (iMGT) construct. **(e)** Western blot of reprogramming factor expression from the polycistronic and iMGT constructs. **(f)** icMEF cell line reprogramming efficiency at three days after doxycycline addition. $P < 0.0001$. **(g)** Immunocytochemistry of icMEF αMHC-eGFP reporter at three days after reprogramming with doxycycline. Scale bar is 200

μm. **(h)** Real time quantitative PCR of cardiac sarcomere protein and ion channel subunit upregulation in icMEF three days after doxycycline addition. **** $P < 0.0001$. *** $P = 0.0003$ **(i)** Immunocytochemistry of αMHC-eGFP reporter and cardiac Troponin T expression in primary MEF reprogrammed with iMGT and doxycycline. Scale bar is 20 μm.