Class IIa Histone Deacetylases and MEF2 proteins Regulate the Mesenchymal-to-Epithelial Transition of Somatic Cell Reprogramming*

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SUMMARY
Class IIa histone deacetylases (HDACs) and myocyte enhancer factor-2 (MEF2) proteins compose a signaling module that orchestrates lineage specification during embryogenesis. We show here that this module also regulates the generation of mouse induced pluripotent stem cells (iPSCs) by defined transcription factors. Class IIa HDACs and MEF2 proteins rise steadily during fibroblast reprogramming to iPSCs. MEF2 proteins tend to block the process by inducing the expression of Tgfβ cytokines, which impairs the necessary phase of mesenchymal-to-epithelial transition (MET).
Conversely, class IIa HDACs endeavor to suppress the activity of MEF2 proteins, thus enhancing the MET and colony formation efficiency. Our work highlights an unexpected role for a developmental axis in somatic cell reprogramming and provides new insight into how the MET is regulated in this context.

The reprogramming of somatic cells to iPSCs requires the removal of a series of endogenous (the somatic cell genetic program) and exogenous (the extracellular milieu) barriers that prevent the transition from intermediate states to pluripotency (1,2). Improved understanding of these barriers is important because it may facilitate higher fidelity of the conversion through experimental manipulation. In this regard, we and others have shown that a mesenchymal-to-epithelial transition or MET is an early requisite step of fibroblast reprogramming (3,4). This discovery helps explain why inhibiting Tgfβ signaling enhances reprogramming (5,6), as Tgfβ cytokines are highly secreted by fibroblasts and induce/sustain the epithelial-to-mesenchymal transition (or EMT) phenotype (7). Yet, the initiation of the MET by the reprogramming factors is still poorly understood except for the repression of Tgfβ-related genes by c-Myc (plus Sox2/Oct4) (3,8) and specific microRNAs (4,9-11), and the activation of an epithelial program by Klf4 (3).

In our search for signaling pathways that regulate reprogramming, we focused on class IIa HDACs because of their fundamental role in tissue specification during embryogenesis (12-15). This subfamily of HDACs consists of isoforms 4, 5, 7 and 9, and compared to other HDACs have little or no catalytic activity (16,17). Instead, they function by binding to and controlling the activity of transcription factors, including the MEF2 family of proteins (15,18,19). MEF2 was originally identified as a transcription factor capable of binding to the muscle creatine kinase promoter (20), but is now known to comprise 4 isoforms (A, B, C and D) in vertebrates, and considered a master regulator of metazoan development (15,21). Class IIa HDACs suppress the activity of MEF2 proteins by preventing the binding of co-activators (e.g., p300) and inducing the recruitment of co-repressor complexes through their catalytic domains (15,22). Class IIa HDACs also shuttle between nucleus and cytoplasm in response to extracellular signals, thus fine-tuning MEF2 activity (12-15). Despite this signaling pathway has been extensively studied in development, little is known about other contexts. Here we show that the interplay between class IIa HDACs and MEF2 proteins determines the efficiency of somatic cell reprogramming by controlling the expression of Tgfβ cytokines.

EXPERIMENTAL PROCEDURES

Cell culture and reprogramming experiments-OG2 embryonic fibroblasts were used in all reprogramming experiments unless otherwise mentioned. They were obtained by crossing OG2 male mice with 129/sv female mice (23). Embryonic fibroblasts, tail tip and mammary fibroblasts were isolated as described (23,24); these cells and HEK293T cells were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS; Hyclone), L-glutamine, non-essential amino acids, sodium pyruvate, penicillin/streptomycin, 20,000 cells were transduced twice in 12-well dishes using viral supernatants generated with PlatE cells (24, 25). The medium was changed to mouse ESC medium (DMEM supplemented with 15% FBS [Gibco], L-glutamine, non-essential amino acids, sodium pyruvate, penicillin/streptomycin, beta mercaptoethanol and 100 U/mL LIF [Millipore]) on day 2 post-infection, and renewed daily. Cells were not split on feeders except for colony expansion and characterization. Feeder layers consisted of mouse embryonic fibroblasts treated with mitomycin C. Doxycycline (Sigma) was added at 1 μg/mL for the times indicated. GFP+ colonies were visualized and counted using a
Zeiss SteREO Lumar V12 microscope. iPSCs generated in this study or produced in a previous report (23), and also mouse ESCs (generated by us from OG2 mice), were routinely cultured on feeders in KSR medium (contains the same recipe as mouse ESC medium but FBS is substituted by KSR [Knockout Serum Replacement; Gibco]). Karyotype analysis, DNA methylation analysis and chimeric mice production with newly generated iPSCs was done as described (3,23,26). Tgfβ receptor1 (TgfβR1) inhibitor and Tgfβ3 cytokine were purchased from Tocris and R&D Systems, respectively, and Vitamin C from Sigma.

Plasmids-pMXs vectors expressing the Yamanaka factors were purchased from Addgene; all other vectors were made by ourselves using either cDNA obtained from mouse fibroblasts or purchased from Fulengene. The doxycycline-inducible lentiviral system was also described before (26). All newly generated vectors have a Flag tag in the carboxy-terminal end of the protein for ease of detection. DNA mutagenesis/deletion was produced using suitable oligos and a PCR-based method. shRNA inserts were cloned into the pRetroSuper vector; the sequences were as follows (5'-3'): MEF2A, GCAGTTATCTCAGGGTTCAAA and GATTGAAATACTGGTGCAAA; MEF2C, GCCATCAGTGAATCAAAGGATA; MEF2D, CACATCAGCATCACTGATGAACGGAA; HDAC4, GCAGAGGATCCACCAGTTAAG and GGTACAATCTCTCTGCCAAAT; HDAC5, GACGCCTCCCTCCTACAAATT and CATCGCTGAGAACGGCTTTAC; HDAC7, AGACAAGAGCAAGCGAAGT and CCATGTTTCTGCCAAATGTTT; a sequence that targets the firefly luciferase gene transcript was used as control (3). Retroviral supernatants containing these constructs were produced as for the pMXs plasmids. The infection efficiency was near 100% (based on the use of a control GFP retroviral vector) but we added puromycin at day 3 post-transduction (it was maintained for 3 days) for selecting only those cells that contain the shRNA vectors. All new plasmids were verified by sequencing before use. The MEF2-responsive reporter was purchased from Panomics. Luciferase activity was measured using the Dual-Glo luciferase assay system (Promega); a Renilla luciferase plasmid was used for normalization.

PCR analysis, immunofluorescence, Western blotting and immunoprecipitation-qPCR analysis was performed using SYBR Green (Takara) and an ABI 7300 machine. Items were run in triplicate and values normalized on the basis of β-actin values. Primers used in this study were (5'-3') as follows: HDAC4, forward- AAACCTGCTGAGAACAGAG ATCTGA and reverse-CTGAGCTCTCAAGACA GACAAACA; HDAC5, forward-GGACGCCTCCTCCTACAAATTG and reverse-AGTTGGGT TCCAGAGGCGGCTTTAC; HDAC7, forward- GTGCGGAGGCTCTCAATGTCACGG and reverse-TCGAGCAATGGCATTACACCCTA; MEF2A, forward-CAGGTTGTTGCGATCTCTTG G and reverse-TCGTCTATCTTCTTGAGCATTCA AA; MEF2C, forward-ATCCCGATGCGACGATTCCAG and reverse-AACAGCACACAAATCT TTGCCCT; MEF2D, forward- CGAGATCAGCTGACGTCATCATT and reverse-AGCCGCTAGCGACAGATTCC; Tgfβ1, forward-CTCCGGTG CTTCTAGTGC and reverse-TCCCTAGATTGGCACAGATTG; Tgfβ2, forward-TGGCACTGATGCCGCG TCATCATCTT and reverse-AGCCGCTTAGAA CCCTTCTTCC; Tgfβ3, forward-CA CTGCTTAGTTCATGG and reverse-ATT TGACAGAGTCTG; Primers for iPSC characterization (qPCR), semi-quantitative RT-PCR and bisulfite sequencing were described before (23). Immunofluorescence microscopy was performed using a Leica TCS SP2 Spectral confocal microscope. Western blotting was performed using ECL and ECL plus (Amersham). Antibodies were purchased from the following suppliers: HDAC4, -5, -7, and MEF2A-D from Abcam, Flag and β-actin from Sigma, SSEA-1

SSEA-1
from R&D, E-cadherin and β-catenin from BD Biosciences; the Nanog antibody was made by us. DAPI was purchased from Sigma. Immunoprecipitation was done using monoclonal ANTI-FLAG M2 Affinity Gel and Flag peptide (Sigma).

Statistical analysis—The Student’s t-test was used throughout the manuscript.

RESULTS

Class IIa HDACs regulate somatic cell reprogramming. Given the fundamental role of class IIa HDACs in development (12-14), we speculated that they might regulate reprogramming as well. Supporting this idea, we observed that mouse ESCs/iPSCs display higher levels of HDAC4, -5 and -7 than fibroblasts by Western blotting (Fig. 1A); we did not study HDAC9 because qPCR analysis showed low expression (data not shown). This prompted us to test whether over-expressing HDAC4, -5 and -7 influences fibroblast reprogramming (see the validation of the expression vectors in Fig. 1B).

We used embryonic fibroblasts bearing a transgenic Oct4 promoter that produces GFP as indication of completed reprogramming (3,27). Besides, we employed both 3 (Oct4, Klf4, and Sox2, or OKS) and 4 (the same plus c-Myc or OKSM) Yamanaka factors (1), with the aim of identifying potential differences between the 2 methods. Reprogramming with OKS is less efficient than OKSM, which is caused at least in part by the existence of a stronger MET phase in the latter (3). Yet, OKS is preferred because of the higher risk of tumor formation using OKSM, which is related to the oncogenic effects of c-Myc (28). The 3 class IIa HDACs enhanced GFP+ colony formation with OKS (~10 fold) but not with OKSM (Fig. 1C). HDAC1 and -6, which belong to class I and IIb HDAC subfamilies, respectively (15), were used as controls and had no effect (Fig. 1C). Importantly, iPSC colonies generated with OKS and class IIa HDACs (only HDAC7 was tested) were bona fide according to standard characterization procedures (including qPCR analysis, bisulfite sequencing and normal karyotype), and produced chimeric mice with germline transmission (Fig. 2A-G). Similarly, we confirmed that HDAC7 improves reprogramming with OKS using 2 additional types of mouse fibroblasts (adult tail tip and mammary) (Fig. 1D). Hence, exogenous class IIa HDACs enhance fibroblast reprogramming using OKS but not with OKSM.

Next, we envisaged that the expression of HDAC4, -5 and -7 should augment during reprogramming to mimic the profile of ESCs/iPSCs (see Fig. 1A). As anticipated, all 3 isoforms increased steadily, but this was more noticeable in OKSM than OKS (Fig. 3A). Knocking down HDAC4, -5 and -7 using shRNA vectors (see the knockdown efficiency in Fig. 3B) reduced the reprogramming efficiency with OKS and OKSM (Fig. 3C). Therefore, endogenous class IIa HDACs are required for OKS and OKSM reprogramming. The circumstance that their individual knockdown decreases reprogramming efficiency reflects that their effect is synergistic. These experiments also suggest that the higher basal expression of class IIa HDACs in OKSM reprogramming makes the system less sensitive to over-expression (see Fig. 1B).

Class IIa HDACs regulate the MET of reprogramming. Although the above explanation to why exogenous class IIa HDACs fail to enhance OKSM reprogramming may be partly correct, we aimed to investigate additional possibilities. We considered a putative effect of exogenous class IIa HDACs on cell proliferation because this is an important aspect determining reprogramming efficiency (29) and is significantly lower in OKS than OKSM (30). However, class IIa HDACs only changed this parameter modestly with OKS (Fig. 4A). Interestingly, we also noticed that they significantly increase epithelial-like morphology.
Role of class IIa HDACs and MEF2 in reprogramming during the first days of OKS reprogramming (Fig. 4B, upper panel), suggestive of an accelerated MET. This phenomenon also existed in OKSM but was less obvious, in agreement with the MET happening quicker and being more substantial in this setting (3). To confirm our idea, we stained fibroblasts reprogrammed with OKS/OKSM and HDAC4 or -7 for 2 relevant cell-cell adhesion proteins (E-cadherin and β-catenin). Class IIa HDACs considerably increased the distribution of both molecules at intercellular junctions in cells reprogrammed with OKS compared with the control, and less notably with OKSM (Fig. 4B, middle and lower panel). This differential effect was verified by Western blotting for E-cadherin (Fig. 4C). Moreover, shRNA for HDAC4 and -7 decreased the accumulation of E-cadherin in OKS and OKSM reprogramming (Fig. 4D), demonstrating that endogenous class IIa HDACs are required for the MET phase of both methods. To further validate our hypothesis, we reprogrammed mammary epithelial cells with OKS and HDAC7, as in this context the MET phase is not necessary and the effect of HDAC7 should be redundant (3). As predicted, we observed no synergistic effect in GFP+ colony formation (Fig. 4E). We also verified that the HDAC7 transgene had effectively integrated in the resulting colonies (Fig. 4F).

We then wished to determine the time window of sensitivity to class IIa HDACs, as one would expect that it is restricted to the early phase. To test this, we prepared a vector producing HDAC7 in a doxycycline-inducible manner (iHDAC7-Dox) (Fig. 4G). Treatment with doxycycline between days 3 and 9 produced a sharp increase (~6 fold) of GFP+ colonies in cells transduced with OKS and iHDAC7-Dox, but there was a plateau if doxycycline was administered longer (Fig. 4H, left panel). We detected as well a reproducible increase (~2 fold) if doxycycline was added from days 3 to 7 to cells reprogrammed with OKSM and iHDAC7-Dox (Fig. 4H, right panel). Yet, this increase was reduced to baseline levels when doxycycline was maintained during the whole process (Fig. 4H, right panel). Hence, over-expression of exogenous class IIa HDACs only during the MET phase also potentiates OKSM reprogramming, albeit less remarkably than OKS. However, their sustained over-expression abrogates this response in OKSM by an unknown mechanism. It is possible that the different reprogramming kinetics between OKS and OKSM explain this phenomenon, as the molecular mechanisms induced in both settings are similar but not identical (31).

Class IIa HDACs regulate the expression of Tgfβ cytokines during reprogramming. We investigated how class IIa HDACs regulate the MET of reprogramming, with a focus on Tgfβ signaling. We chose this pathway because shutting down Tgfβ signals is a major mechanism used by c-Myc to enhance the MET in OKSM reprogramming (3). Notably, our qPCR analysis showed a decrease of Tgfβ cytokines (particularly Tgfβ2) in fibroblasts transduced with HDAC4 or -7 (Fig. 5A). We also detected a reduction of all 3 Tgfβ cytokines in fibroblasts reprogrammed with OKS and HDAC7 (Fig. 5B). Moreover, addition of recombinant Tgfβ3 (Tgfβ1, 2 and 3 have been reported to block reprogramming (3,6)) neutralized the improved reprogramming efficiency caused by HDAC7 in OKS (Fig. 5C). These results provide a mechanism to why class IIa HDACs potentiate fibroblast reprogramming, but do not demonstrate how this is mediated.

MEF2 proteins are downstream targets of class IIa HDACs in reprogramming. Next, we searched for putative effectors of class IIa HDACs that could explain the reduction of Tgfβ cytokines in reprogramming. We centered on MEF2 proteins because they are well-studied partners of class IIa HDACs (Fig. 6A) and have a prodifferentiation effect (15,21). We observed...
a progressive increase of MEF2 isoforms during reprogramming with OKS or OKSM (Fig. 6B). To define whether MEF2 proteins play a role in this process, we first created mutated forms of HDAC4, -5 and -7 that bear amino acid substitutions in the MEF2 binding site (32), and a version of HDAC7 with a deleted MEF2 binding site (24) (see scheme in Fig. 6A). These mutated forms lost the ability to bind to MEF2, as verified by co-expression with MEF2D and immunoprecipitation (Fig. 6C), and could not repress the activation of a MEF2-responsive luciferase reporter by MEF2D (Fig. 6D). As anticipated, the MEF2 binding-mutants of all 3 class Ila HDACs failed to enhance OKS reprogramming (Fig. 6E). These findings advocate but not demonstrate that class Ila HDACs regulate reprogramming by suppressing MEF2 proteins.

To determine a direct role for MEF2 proteins in reprogramming, we reduced MEF2 expression levels by using shRNA vectors (Fig. 6F). This enhanced the number of GFP+ colonies in OKS and OKSM (Fig. 6G), in agreement with the observation (see Fig. 4H) that restricted (inducible) over-expression of class Ila HDACs in the MET phase potentiates both types of reprogramming. Yet, knockdown of MEF2 proteins boosted OKS reprogramming less significantly than over-expressing class Ila HDACs (see Fig. 1C, right panel). This apparent discrepancy is perhaps due to the circumstance that, besides preventing the binding of co-activators to MEF2 proteins, class Ila HDACs recruit chromatin regulators to induce repressive modifications nearby MEF2 binding sites (22).

Elevated MEF2 proteins block reprogramming by inducing Tgfβ cytokines. We prepared expression vectors for each of the four isoforms of MEF2 (Fig. 7A), and overexpressed them individually in OKS/OKSM reprogramming. MEF2A, B, C, and more potently MEF2D, reduced GFP+ colony formation with either factor combination (Fig. 7B). MEF2 proteins could also block reprogramming in the presence of Vitamin C (Vc) (Fig. 7C), a natural compound that enhances colony formation efficiency significantly (23). Importantly, simultaneous over-expression of class Ila HDACs counteracted the reprogramming blockade triggered by exogenous MEF2 proteins (in the presence or absence of Vc) without altering their expression levels (Fig. 7D-F). Therefore, excessive MEF2 activity prevents fibroblast reprogramming to iPSCs.

Considering the role of class Ila HDACs in the MET of reprogramming, we envisaged that MEF2 should play an opposite role in this process. As expected, over-expression of MEF2A or MEF2D reduced the MET in OKS and OKSM reprogramming (Fig. 8A-B). Moreover, qPCR analysis showed an increase of Tgfβ cytokines (particularly Tgfβ2) in fibroblasts transduced with MEF2A or MEF2D (Fig. 8C) or reprogrammed with OKS and MEF2D (Fig. 8D). Importantly, the addition of a TgfβR1 inhibitor restored MET-like morphology changes and iPSC generation in fibroblasts reprogrammed with OKS/OKSM and MEF2D (Fig. 8E-F). We also observed that MEF2D over-expression prevents the increase in E-cadherin triggered by Klf4 alone (3), which could be relieved using TgfβR1 inhibitor (Fig. 8G). Therefore, MEF2 proteins block the MET phase of reprogramming by inducing Tgfβ cytokines, and this subsequently impairs colony formation efficiency.

DISCUSSION

From a conceptual perspective, reprogramming can be viewed as a reversal of tissue specification during embryogenesis. However, if this were true it would imply that developmental programs must become switched on during reprogramming. This in turn would tend to derail the epigenetic transformation and
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perhaps help explain the low efficiency of the process. In support of such idea, we have shown that MEF2 proteins, a family of transcription factors with key role in organogenesis (15,21) augment during reprogramming and impair iPSC generation (Fig. 9). This negative effect is counteracted in a rheostat-like manner (using the analogy with an electrical circuit) by a steady increase in class IIa HDACs, which are well-known regulators of MEF2 proteins activity (15,21) (Fig. 9).

MEF2 proteins block reprogramming by restraining the epithelial-like program driven by Klf4 (3,33), which is mediated at least in part by increasing the expression of Tgfβ cytokines (Fig. 9). This suggests that MEF2 proteins bind to DNA consensus sites regulating the transcription of Tgfβ cytokines, but an indirect mode of action is also plausible. A connection between MEF2 proteins and the MET/EMT had not yet been explored except for the induction of MEF2 by Twist during mesoderm specification in drosophila, and the observation that MEF2 is needed for proper craniofacial formation after neural crest delamination (21). It was previously reported that MEF2 proteins and Tgfβ-induced Smads physically interact between in myoblasts (34,35). Smad2 binds to and activates MEF2A (34), favoring myoblast terminal differentiation, while Smad3 binds to MEF2C and prevents it (35). We have shown here that MEF2 proteins induce Tgfβ cytokines in mouse fibroblasts, further adding complexity to the reported crosstalk. Given the numerous functions of MEF2 proteins in development (15,21), it seems probable that they impair reprogramming through additional prodifferentiation pathways. This phenomenon might help understand the paradoxical observation that on one side the Yamanaka factors drive cells to a pluripotent cell fate, and on the other they can be used to instruct specific lineages (e.g., neural and cardiomyocytes) (36,37). Favoring such idea, MEF2C is among the transcription factors used to induce the direct transdifferentiation of fibroblasts into cardiomyocytes (38). It is also interesting to think that a putative activation of lineage-specific signals by MEF2 proteins makes the resulting iPSCs more prone to retain epigenetic memory of those lineages (39).

Likewise, it is possible that during reprogramming class IIa HDACs target other transcription factors besides MEF2 proteins (18,19), further increasing the complexity of the presented network and its putative consequences.

Notably, our work also ascribes a positive role for a subfamily of HDACs in reprogramming. This contrasts with the general assumption that HDACs are negative in this process, which is based on the positive effect of valproic acid (VPA) in iPSC generation (40). However, the discrepancy can be explained by interference with a different type of HDACs (e.g., class I), as class IIa HDACs are not responsive to VPA (41). In this regard, we have observed that treatment with VPA synergizes with class IIa HDACs in OKS reprogramming (data not shown).

In summary, we have demonstrated that a signaling module that controls embryogenesis plays a fundamental role in somatic cell reprogramming by regulating the MET phase (Fig. 9). It is tempting to speculate that developmental transitions driven by class IIa HDACs/MEF2 proteins are orchestrated as well through changes in Tgfβ expression, and that this regulatory node participates in normal tissue homeostasis, fibrosis and tumorigenesis.
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FOOTNOTES
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5Abbreviations used are: EMT, epithelial-to-mesenchymal transition; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; HDAC, histone deacetylase; MEF2, myocyte enhancer factor-2; MET, mesenchymal-to-epithelial transition; Tgf\(\beta\), transforming growth factor-\(\beta\); Tgf\(\beta\)R1, Tgf\(\beta\) receptor 1; VPA, valproic acid.

FIGURE LEGENDS

FIGURE 1. Class IIa HDACs regulate somatic cell reprogramming. (A) Representative Western blot for endogenous HDAC4, -5 and -7 using lysates from the indicated cell types. \(\beta\)-actin was the loading control. (B) Representative Western blot with anti-Flag shows adequate over-expression of the indicated class IIa HDACs, HDAC1 and -6. (C) Number of GFP+ colonies produced in fibroblasts reprogrammed with OKS/OKSM and HDAC4, -5 and -7. Empty vector was used as control (Ctrl; also in D), also HDAC1 and -6. Colonies were counted at day 18 (also hereafter unless otherwise indicated). Mean values +/- standard deviation (SD) of a representative experiment with items in triplicate are shown (also hereafter for other colony formation experiments). **p value<0.01. (D) Number of GFP+ colonies in tail tip fibroblasts (TTFs) and mammary (MaFs) transduced with OKS and HDAC7.
FIGURE 2. Characterization of iPSCs generated by HDAC7 and OKS. (A) Phase contrast and immunofluorescence photographs of a representative iPSC clone produced with OKS and HDAC7; scale=50 μm. (B) Semi-quantitative PCR showing integration of the exogenous transgenes in the genome of selected iPSC clones. Untransduced fibroblasts and pMXs plasmids containing the corresponding cDNA were used as controls. (C) qPCR for endogenous (Endo) ESC-like markers in the indicated iPSC clones and mouse ESCs compared to fibroblasts. (D) qPCR for the exogenous (Exo) transgenes in the indicated iPSC clones; ESCs and reprogramming fibroblasts extracted at day 6 were the controls. (E) Normal karyotype of a selected iPSC clone generated with OKS and HDAC7. (F) DNA methylation status of the Oct4 proximal promoter in the indicated cell types. Empty circles represent demethylated CpGs. (G) Photograph of a representative chimeric mouse (blue arrow) generated with the same iPSC clone; transmission to the germline can be observed in the baby mouse with agouti skin color (red arrow).

FIGURE 3. Knockdown of endogenous class IIa HDACs impairs reprogramming. (A) Representative Western blot for HDAC4, -5 and -7 using lysates of a time course reprogramming experiment with OKS/OKSM in fibroblasts. Empty vector was used as control. D indicates day. (B) shRNA vectors (depicted as sh followed by the target gene) for HDAC4, -5 and -7 were infected in fibroblasts and the knockdown efficiency measured by qPCR. (C) Number of GFP+ colonies in fibroblasts reprogrammed with OKS/OKSM and the indicated shRNA vectors; shRNA against firefly luciferase was used as control (also hereafter in similar experiments). *p value<0.05.

FIGURE 4. Class IIa HDACs enhance reprogramming by accelerating the MET phase. (A) Measurement of proliferation at different times in fibroblasts reprogrammed as indicated. Empty vector was added to OKS as control for HDAC7. A representative experiment with triplicate items counted in duplicate (after detaching cells with trypsin) is shown. (B) Representative phase contrast and immunofluorescence photographs of fibroblasts reprogrammed with OKS/OKSM and HDAC4 or -7 or empty vector; scale=50 μm. E-cad indicates E-cadherin. (C-D) Representative Western blots for E-cadherin using lysates of fibroblasts reprogrammed with OKS/OKSM and the indicated expression vectors or shRNA constructs. Lysates were taken at day 6 in all cases. (E) Number of GFP+ colonies in mammary epithelial cells (MECs) reprogrammed with OKS and HDAC7 or empty vector. Colonies were counted at day 21. (F) Semi-quantitative PCR showing integration of the exogenous HDAC7 transgene in the genome of selected iPSC clones produced as in E. Untransduced MECs and pMXs plasmids containing the corresponding cDNA were used as controls. (G) Western blot of fibroblasts infected with iHDAC7-Dox and treated with doxycycline. (H) Number of GFP+ colonies in fibroblasts reprogrammed with OKS/OKSM and iHDAC7-Dox. Doxycycline (Dox) was administered for the indicated time points.

FIGURE 5. Class IIa HDACs modulate the expression of Tgfβ cytokines. (A) qPCR for Tgfβ cytokines using RNA lysates from fibroblasts transduced as indicated. Empty vector was used as control (also in C). (B) qPCR for Tgfβ cytokines using RNA lysates from fibroblasts reprogrammed with OKS and either empty vector or HDAC7. (C) Number of GFP+ colonies in fibroblasts reprogrammed with OKS/OKSM and HDAC7. Tgfβ3 was added as shown.

FIGURE 6. MEF2 proteins are downstream targets of class IIa HDACs during reprogramming. (A)
Scheme depicting the structure of HDAC4, -5 and -7, the location of the MEF2 binding site and the catalytic domain, and also the corresponding modifications performed to abolish their function. (B) Representative Western blot for MEF2 proteins using lysates of a time course reprogramming experiment with OKS/OKSM in fibroblasts. Empty vector was used as control (also in D, E and G). (C) Immunoprecipitation of MEF2D after co-expression with the indicated Flag-tagged class IIa HDAC variants in HEK293T cells. IP indicates immunoprecipitated samples. (D) Co-transfection of a MEF2 luciferase reporter gene and the indicated expression vectors in HEK293T cells. Activity was measured 48 hours post transfection; items were measured in triplicate and the mean values +/- SD of a representative experiment are shown. (E) Number of GFP+ colonies in fibroblasts reprogrammed with OKS and wild type or mutated forms of HDAC4, -5 and -7. (F) shRNA vectors for MEF2 proteins were infected in fibroblasts and the knockdown efficiency measured by qPCR. (G) Number of GFP+ colonies in fibroblasts reprogrammed with OKS/OKSM and the indicated shRNA vectors.

**FIGURE 7.** MEF2 proteins derail reprogramming by impairing the MET phase. (A) Representative Western blot with anti-Flag showing adequate over-expression of the indicated MEF2 proteins. (B) Number of GFP+ colonies in fibroblasts reprogrammed with OKS/OKSM and MEF2 proteins. Empty vector was used as control (also in C). (C) Number of GFP+ colonies in fibroblasts reprogrammed with OKS/OKSM and MEF2 isoforms. Cells were treated with vitamin C (Vc) to enhance the number of colonies and thus magnify differences (also in E). (D) Co-expression of HDAC7 rescues the reprogramming blockade in fibroblasts transduced with OKS/OKSM and MEF2C. A dose-response experiment is shown (also in E). The total amount of viruses (as per volume) was maintained constant by using empty vector (also in E). (E) Co-expression of HDAC7 rescues the reprogramming blockade in fibroblasts transduced with OKS/OKSM and MEF2D. (F) Western blot for HDAC7 and MEF2D using lysates from a dose-response experiment as indicated in E. Lysates were prepared at day 6 after infection.

**FIGURE 8.** MEF2 proteins impair the MET of reprogramming by inducing Tgfβ cytokines. (A) Representative phase contrast photographs at day 7 post-infection of fibroblasts transduced with OKS/OKSM and MEF2A or MEF2D. Empty vector was used as control (also in B-G). Scale=50 μm (also in E). (B) Representative Western blots for E-cadherin using lysates of fibroblasts reprogrammed as indicated. Lysates were taken at day 7 in all cases. (C) qPCR for Tgfβ cytokines using RNA lysates from fibroblasts transduced with MEF2A or MEF2D. (D) qPCR for Tgfβ cytokines of fibroblasts reprogrammed with OKS and MEF2A or MEF2D. (E) Representative phase contrast photographs of fibroblasts reprogrammed with OKS/OKSM and MEF2D treated or untreated with TgfβR1 inhibitor (TgfβR1i). (F) Number of GFP+ colonies in fibroblasts reprogrammed with OKS/OKSM and MEF2D. TgfβR1 inhibitor was added as indicated. (G) Western blot for E-cadherin of lysates from fibroblasts transduced with Klf4 and MEF2D treated or untreated with TgfβR1 inhibitor.

**FIGURE 9.** Scheme depicting how class IIa HDACs/MEF2 proteins regulate the expression of Tgfβ cytokines during reprogramming.
Figure 1

A

B

C

D

Role of class IIa HDACs and MEF2 in reprogramming
Figure 2

A. Phase
Oct4-GFP
Nanog
SSEA-1

B. Negative ctrl
Positive ctrl
3FH7-C3
3FH7-C6

C. Relative expression

D. Relative expression

E. 3FH7-C3

F. Fibroblasts
3FH7-C3
3FH7-C6
ESC5

G. 3FH7-C3

Role of class IIa HDACs and MEF2 in reprogramming
Figure 3

A

B

C

Role of class IIa HDACs and MEF2 in reprogramming

by guest on September 16, 2017 http://www.jbc.org/ Downloaded from
Figure 4

Role of class IIa HDACs and MEF2 in reprogramming

A) Cell number (x10^5) vs. time (days 0-9).

B) Phase and E-cad expression in OKS and OKSM cells.

C) β-catenin expression in OKS and OKSM cells.

D) E-cad and β-actin expression in OKS and OKSM cells.

E) GFP+ colonies in OKS cells with HDAC7 knockdown.

F) MEC-derived iPSCs with HDAC7 knockdown.

G) HDAC7 expression with Dox treatment.

H) GFP+ colonies in OKS and OKSM cells with HDAC7 knockdown over time.
Figure 5

A

Fibroblasts

Relative expression

Tgfβ1  Tgfβ2  Tgfβ3

HDAC4  HDAC7

HDAC7 -+ ++

7JIȕ QJP0 QJP0

--

B

OKS-D5

Relative expression

Tgfβ1  Tgfβ2  Tgfβ3

Ctrl  HDAC7

HDAC7 -+ ++

C

OKS

GFP+ colonies

HDAC7 -  +  +  +

Tgfβ3 -  -  10 ng/ml  20 ng/ml

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Figure 6

A

MEF2 binding site
Catalytic domain

HDAC4

HDAC5

HDAC7

Mutated

HDAC4-173L/A
HDAC5-179L/A
HDAC7-112L/A
HDAC7-ΔMEF

B

Ctrl
OKS
OKSM

MEF2A
MEF2B
MEF2C
MEF2D
β-actin

C

Input
Flag

MEF2D

Flag

IP: Flag

MEF2D

D

Relative MEF2 activity

MEF2D

Ctrl
+ HDAC4
+ HDAC4-173L/A
+ HDAC5
+ HDAC5-179L/A
+ HDAC7
+ HDAC7-112L/A

E

OKS

**

Ctrl
HDAC4
HDAC5
HDAC7
HDAC4-173L/A
HDAC5-179L/A
HDAC7-112L/A
HDAC7-ΔMEF

G

OKS

**

Ctrl
shMEF2A #1
shMEF2A #2
shMEF2B #1
shMEF2B #2

OKSM

**

Ctrl
shMEF2A #1
shMEF2A #2
shMEF2B #1
shMEF2B #2
Role of class IIa HDACs and MEF2 in reprogramming

Figure 7

A

B

C

D

E

F

Role of class IIa HDACs and MEF2 in reprogramming
Figure 8

A

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B

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E

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E-cad, β-actin
Figure 9

Role of class IIa HDACs and MEF2 in reprogramming
Class IIa Histone Deacetylases and MEF2 proteins Regulate the Mesenchymal-to-Epithelial Transition of Somatic Cell Reprogramming

Qiang Zhuang, Xiaobing Qing, Yue Ying, Haitao Wu, Christina Benda, Jiao Lin, Zhijian Huang, Longqi Liu, Yan Xu, Xichen Bao, Baoming Qin, Duanqing Pei and Miguel A. Esteban

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