Histone chaperone ASF1A is required for maintenance of pluripotency and cellular reprogramming

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by partial disruption of two processes may partly explain greater severity of lung disease in CF compared with primary ciliary dyskinesia, which obliterates MCT (*29*), because compromising one defense may accentuate the other defect. For example, mucus that fails to detach would impair MCT and provide a nidus for bacteria to grow under conditions that promote resistance to host defenses already weakened by CF (*28*, *30*). Conversely, reduced antibacterial activity could precipitate infection that triggers submucosal gland secretion, and defective mucus detachment would impair MCT. Inflammation resulting from both defects would evoke submucosal gland hypertrophy, further increasing the amount of static mucus. Because newborns are universally screened for CF in many countries, an opportunity for early intervention exists. Our data suggest that submucosal glands and the mucus tethered to them may be targets for early treatment and that MCT assays could report therapeutic efficacy.

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SUPPLEMENTARY MATERIALS

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CELL REPROGRAMMING

Histone chaperone ASF1A is required for maintenance of pluripotency and cellular reprogramming

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Unfertilized oocytes have the intrinsic capacity to remodel sperm and the nuclei of somatic cells. The discoveries that cells can change their phenotype from differentiated to embryonic state using oocytes or specific transcription factors have been recognized as two major breakthroughs in the biomedical field. Here, we show that ASF1A, a histone-remodeling chaperone specifically enriched in the metaphase II human oocyte, is necessary for reprogramming of human adult dermal fibroblasts (hADFs) into undifferentiated induced pluripotent stem cell. We also show that overexpression of just ASF1A and OCT4 in hADFs exposed to the oocyte-specific paracrine growth factor GDF9 can reprogram hADFs into pluripotent cells. Our Report underscores the importance of studying the unfertilized MII oocyte as a means to understand the molecular pathways governing somatic cell reprogramming.

Two major breakthroughs in the biomedical field occurred with the discovery that either oocytes or specific transcription factors can radically change a cell's phenotype from a differentiated to an embryonic state (*1*–*3*). A clear understanding of how this cellular reprogramming process takes place remains incomplete. Growing evidence suggests that the reprogramming capacity of the mammalian metaphase II oocyte yields superior results—when looking at epigenetic marks of the resulting cells—to those of the factor-based reprogramming approaches (*4*–*8*). One hypothesis that accounts for this enhanced reprogramming capacity posits that the current induced pluripotent stem cell (iPSC) generation strategies lack specific factors that the oocyte possesses. We hypothesized that by understanding the functions of genes present in the MII oocyte, we will be able to identify intra- and extracellular oocyte factors responsible for the oocyte's reprogramming capacity that may also have a role in dedifferentiation events such as generation of iPSCs.

Histone chaperones regulate all facets of histone metabolism. One such gene, antisilencing function 1 (ASF1), the most conserved histone 3 and histone 4 chaperone, has been implicated in replication, transcription, and DNA repair [reviewed in (*9*)]. ASF1 has been identified as a single protein in yeast (*10*), whereas in most vertebrates, it exists as two paralogs, termed ASF1A and ASF1B in mammals.

ASF1A is specifically enriched in the metaphase II human oocyte (*11*), and recent research using cross-species global transcriptional analysis has singled it out as a potential oocyte reprogramming factor (*12*). Most of the information about ASF1A comes from the work done in yeast and *Drosophila* (*9*, *13*). It has been characterized as a histone-remodeling chaperone that cooperates with histone regulator A (HIRA) and with chromatin assembly factor 1 (CAF-1), which plays a key role in remodeling chromatin in pluripotent embryonic cells (*14*). ASF1A is specifically required for H3K56 acetylation, a histone state shown to reflect more accurately the epigenetic differences between human embryonic stem cells (hESCs) and somatic cells—more so than other active histone marks, such as H3K4 trimethylation and

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H3K9 acetylation—suggesting the involvement of K56Ac in the human core transcriptional network of pluripotency (*15*–*18*). However, little is known about the role of ASF1A in human cells and, more specifically, about its role in the pluripotency state of a cell. Here, we report that ASF1A is necessary for the cellular reprogramming of human adult dermal fibroblasts (hADFs) into undifferentiated iPSCs.

Furthermore, we show that overexpression of ASF1A and OCT4 alone in somatic cells exposed to oocyte-specific growth factor GDF9 can reprogram hADFs into pluripotent cells. In addition, we identify transcriptional networks activated by ASF1A, OCT4, and GDF9 involved in the reprogramming process. Our study suggests that studying the unfertilized MII oocyte offers an important opportunity to elucidate the molecular pathways governing somatic cell reprogramming.

To determine whether ASF1A has a role in pluripotent stem cells and pluripotency acquisition, we investigated its expression in hESCs during differentiation. Gene expression and protein analyses show that during spontaneous differentiation, ASF1A expression decreases, as does the expression of the pluripotency-related genes OCT3/4, NANOG, SOX2, and DNMT3B (Fig. 1A and fig. S1A). We found the highest ASF1A expression levels in hESCs and the lowest in hADFs (fig. S1B).

To further investigate the role of ASF1A in somatic and embryonic stem cells, we tested whether forced expression of ASF1A in hESCs and hADFs would affect their differentiated states. We engineered H9 hESCs or hADF to overexpress either ASF1A or green fluorescent protein (GFP) by transducing these cells with a lentiviral vector (pWPI). H9 hESCs overexpressing ASF1A showed a tenfold increase in OCT4, NANOG, SOX2, and DNMT3B expression (fig. S2B) 6 days after transduction. We found that hADFs overexpressing ASF1A also showed a similar relative increase in pluripotency marker expression compared with GFP-transduced cells (fig. S2A). When we cultured hESCs overexpressing ASF1A as embryo bodies and then plated them into 10% fetal bovine serum media to promote spontaneous differentiation into endoderm, mesoderm, and ectoderm cell derivatives, ASF1A-overexpressing hESCs showed a clear resistance to differentiation by delaying the down-regulation of pluripotency-related genes and the onset of expression of differentiation markers (fig. S3, A and B). These results indicate that constitutive expression of ASF1A favors the maintenance of pluripotency, suggesting its role in pluripotency acquisition.

To determine whether ASF1A expression is required during cellular dedifferentiation into iPSCs, we blocked ASF1A expression using short hairpin RNA (shRNA) (fig. S4A) and subsequently transduced hADF with the four Yamanaka factors: OCT3/4, SOX2, KLF4, and c-MYC (OSKM). We used two different ASF1A shRNAs (ASF1A shRNA-147 and ASF1A shRNA-1234) or control shRNA. Down-regulation of ASF1A did not alter cell proliferation rates of hADF (fig. S4B). When shRNA-147 was used, we found a significant decrease in the number of TRA-1-60^{+} reprogrammed iPSCs colonies. However, when we used the most efficient of the two shRNAs (shRNA-1234), we completely precluded the appearance of TRA-1-60^{+} reprogrammed iPSC colonies (Fig. 1B). When the same ASF1A-shRNA vector was used to down-regulate ASF1A expression in hESC-H9, we observed a reduction in the expression of pluripotency markers (Fig. 1C), along with a change in colony morphology (fig. S5) as ASF1A decreased. These experiments show that ASF1A expression is required for pluripotency maintenance and for reprogramming hADFs into iPSCs.

To further analyze the role of ASF1A in the pluripotent state of a cell and its possible interaction with the master reprogramming genes, we overexpressed ASF1A along with the Yamanaka factors individually (OCT4, SOX2, and KLF4) and together (OSKM). One week after transduction, we observed no difference in the pluripotent gene expression pattern among the different combinations (fig. S6). Three to four weeks after transfection, however, the combination of ASF1A and OCT4 alone generated pre-iPSC–like colonies. Dermal fibroblasts transduced with OSKM plus ASF1A resulted in a slight increase in TRA-1-60^{+} iPSC-like colonies (fig. S7A) over fibroblasts transduced with OSKM alone.

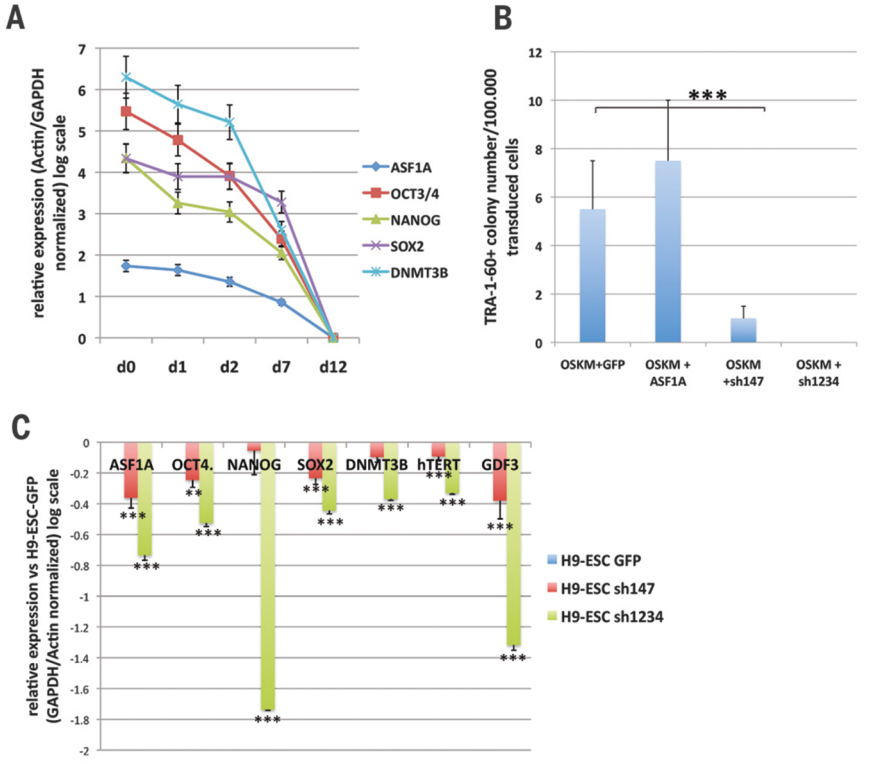

Fig. 1. ASF1A role during cell reprogramming. (**A**) H9 hESCs were cultured under conditions to promote spontaneous differentiation. ASF1A expression decreases as pluripotent cells differentiate. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) data for genes characteristic of undifferentiated stem cells was performed as indicated on mRNA collected at days 0, 1, 2, 7 and 12 during differentiation. Mean values ± SEM are plotted, indicating expression of the specific gene normalized to glyceraldehyde-phosphate dehydrogenase (GAPDH)/Actin relative to the expression on day 12, which was arbitrarily assigned a value of 0, in a logarithmic scale (1 unit means 10-fold change). (**B**) In the absence of ASF1A, somatic cells cannot reprogram into pluripotent cells when using the Yamanaka factors. Seventy-two hours after hADFs lentiviral transduction with GFP, ASF1A or two different shRNAs against ASF1A, hADFs were transduced with retroviral supernatants encoding OSKM factors for reprogramming. The graph shows the number of Tra-1-60^{+} colonies derived from 100,000 cells after OSKM overexpression in GFP (control), ASF1A, or the shRNAs 147 or 1234 expressing cells compared with control OSKM GFP-expressing fibroblasts. Mean values ± SEM. ***$P < 0.01$. (**C**) Down-regulation of ASF1A in H9-hESCs significantly decreases the expression of pluripotency-related genes. qRT-PCR data for ASF1A expression on mRNA were collected from H9-hESC cells expressing a lentivector encoding GFP or two different shRNAs against ASF1A (sh147 and sh1234). Mean values ± SEM are plotted indicating expression of the specific gene normalized to GAPDH/Actin relative to the expression of H9-hESC-GFP, which was arbitrarily assigned a value of 0, in a logarithmic scale. For all three panels, data correspond to the average of three independent experiments done in duplicate. ***$P < 0.001$, **$P < 0.05$, and *$P < 0.01$ compared with H9-hESC-GFP.

We hypothesized that other oocyte factors could be necessary to achieve complete iPSCs formation. We focused on paracrine factors secreted by the oocyte itself, which are known to have well-described signaling pathways in the mammalian MII oocyte. We tried seven different ligands in combination with the overexpression of ASF1A and OCT4 (table S1). Only GDF9, added during 48 hours after ASF1A/OCT4 transduction, could generate colonies with typical iPSC morphology ($5 \pm 2 \times 10^{-7}$% of transduced cells) (figs. S7, A and B, and S8). Overexpression of OCT4 alone or in the presence of GDF9 did not produce any reprogrammed colony. ASF1A-OCT4-GDF9 (AO9)–derived colonies were fully reprogrammed, showed normal karyotype (fig. S9) and expressed standard stem cell markers after culturing for six to ten passages (Fig. 2A and fig. S7B), and showed a gene expression profile similar to hESCs (Fig. 2B). We found no detectable expression of exogenous ASF1A/OCT4 from the retroviral vectors in the AO9-iPSC clones 65 days after transduction (fig. S10).

When induced to differentiate in vitro, fully reprogrammed AO9-iPSCs formed ecto-, endo-, and mesoderm cell lineages (figs. S11 and S12). Injection of AO9-iPSC lines into immunodeficient mice formed mature teratomas that had intestinal epithelium (endoderm), cartilage (mesoderm), and neural epithelium (ectoderm) (Fig. 2, C to E).

At the epigenetic level, overexpression of ASF1A on human dermal fibroblasts increased H3K56Ac significantly, and the acetylation was even higher when OCT4 was coexpressed in the same cells (Fig. 3A and fig. S13). We further confirmed the interaction between ASF1A and H3K56Ac in hADFs, hiPSCs, and hESCs (Fig. 3B), corroborating the findings described in yeast and drosophila (*19*, *20*). We analyzed hADF 72 hours after the overexpression of the ASF1A-OCT4 factors and observed that these two factors coimmunoprecipitate (Fig. 3C). Chromatin immunoprecipitation (ChIP) analysis confirmed that H3K56ac is found in regulatory regions of NANOG, OCT4, and SOX2 after overexpression of ASF1A (Fig. 3D and fig. S14). Our results strongly suggest that both ASF1 and OCT4 are capable of activating genes at the core of the pluripotency regulatory network, at least in part through the acetylation of H3K56.

In an effort to elucidate the signaling pathways involved in the AO9 reprogramming process, we analyzed global gene expression profiles of human dermal fibroblasts 48 hours after exposing cells to the individual factors both alone and all three combined—i.e., overexpression of just ASF1A, OCT4, or GDF9, or of AO9 (the three factors combined) (Fig. 4). Using Ingenuity Pathway Analysis (Redwood City, CA) (table S2), we found that AO9 overexpression regulates, among many other signaling pathways, p38 and interleukin-6 signaling. Our data show that GDF9 activates R-SMADs 2/3 phosphorylation on human dermal fibroblasts, but not extracellular signal–regulated kinase 1/2 (Fig. 4 and fig. S15). Detailed information of the specific comparisons can be found in the supplementary materials.

Our work has uncovered two specific factors present in the human oocyte, ASF1A and GDF9, which play crucial roles in somatic cell reprogramming. ASF1A expression is necessary for somatic cell reprogramming and maintenance of pluripotency. It functions by interacting with histone 3, promoting its acetylation at lysine 56. H3K56 acetylation mediated by ASF1A occurs mainly at the S phase in unstressed cells (*15*). The fact that H3K56ac is cell-cycle–dependent and marks less than 1% of total H3 (*17*, *20*) may explain why it was previously difficult to determine the role of ASF1A in mammalian cells. Here, our coimmunoprecipitation experiments show that this interaction is likely to be direct. H3K56ac presence correlates positively with binding of

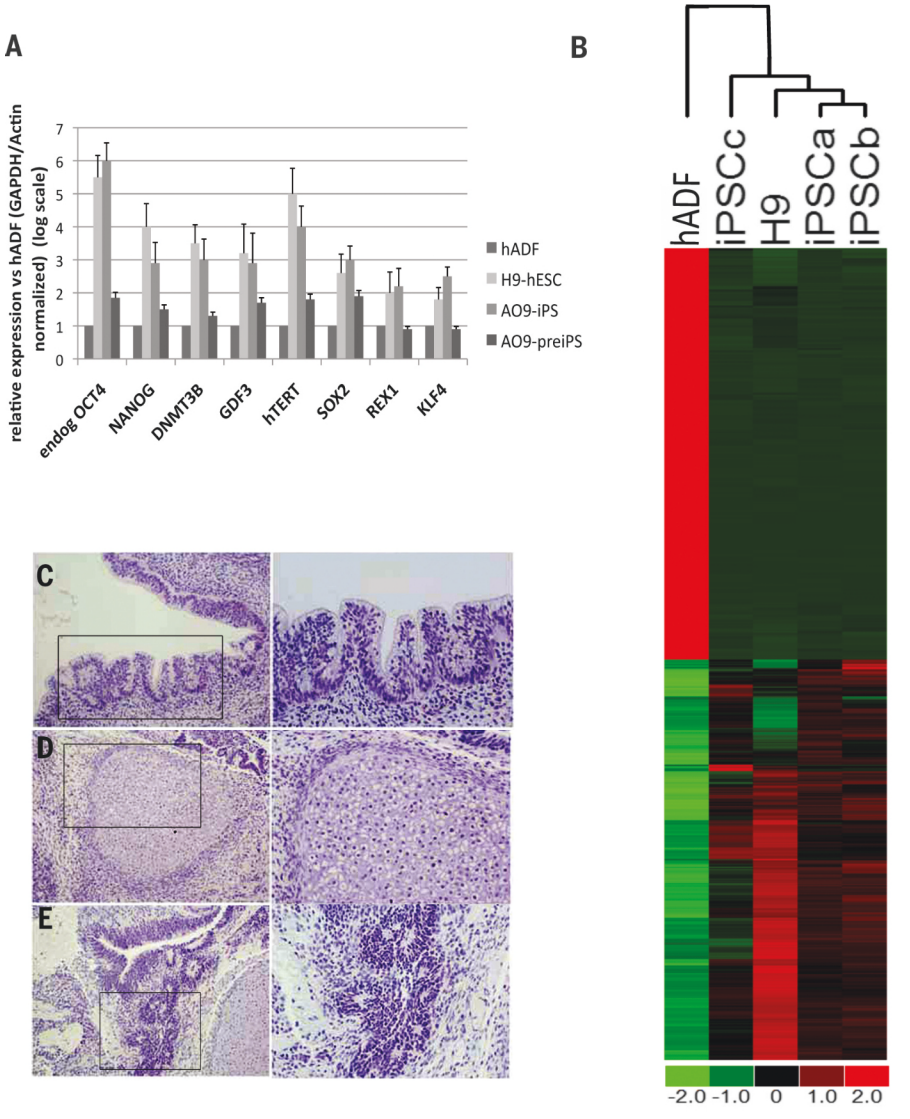

Fig. 2. ASF1A, OCT4, and GDF9 (AO9) combination is sufficient for reprogramming hADF to pluripotency. (**A**) qRT-PCR data for genes characteristic of pluripotent cells was performed as indicated on mRNA collected from hADF, H9 hESCs, and iPSCs obtained overexpressing ASF1A, OCT4 in the presence of GDF9 (AO9-iPSC). Values indicate expression of the specific gene normalized to GAPDH/Actin in a logarithmic scale relative to the hADF sample, which was arbitrarily assigned a value of 0. Data correspond to the average of three independent experiments done in duplicate. (**B**) Expression array data analysis of similarities between H9-ESCs and AO9-iPSCs (three independent lines: AO9-iPSCa, b, and c) compared with adult human dermal fibroblasts (hADF). Dendogram and heat map based on genes up- or down-regulated 10-fold or greater versus dermal fibroblasts to visualize similarly expressed group of genes. (**C** to **E**) AO9-iPSC differentiation capacity. Hematoxylin and eosin staining of representative matured AO9-iPS–derived teratomas exhibiting characteristic structure of (C) intestinal epithelium (endoderm), (D) cartilage (mesoderm), and (E) neural epithelium (ectoderm).

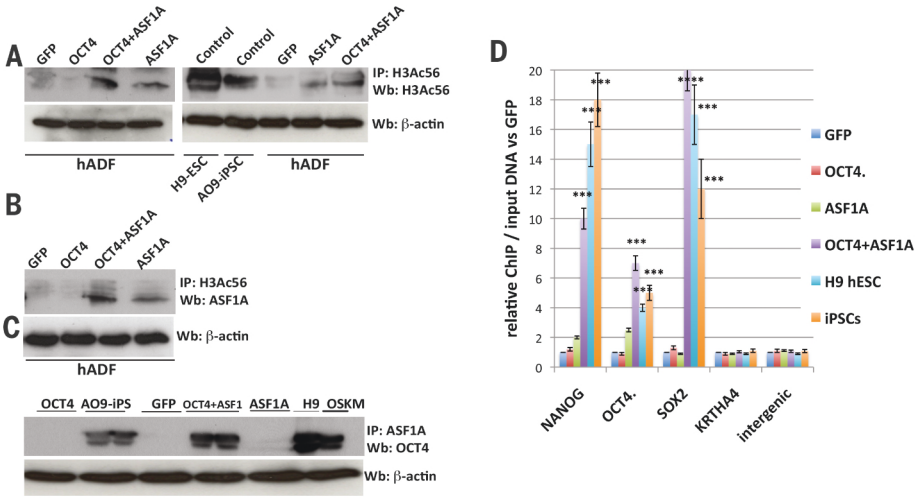

Fig. 3. ASF1A, OCT4, and H3K56 acetylation. (**A**) Retroviral-driven overexpression of ASF1A alone or ASF1A+OCT4 increases H3K56 acetylation in hADF shown by immunoprecipitation 72 hours after transduction using H3K56 antibodies (IP: H3Ac56) and gel blotted [Western blot (Wb)] with H3K56 antibody as well. H9-ESCs and AO9-iPSCs samples were used as positive control for immunoprecipitation. (**B**) IP and Wb using specific antibodies against H3K56ac (IP: H3Ac56) and ASF1A (Wb: ASF1A) demonstrate protein-protein interaction of ASF1A with acetylated H3K56 in transduced hADF. (**C**) Protein interaction is observed between ASF1A and OCT4 when ASF1A is immunoprecipitated in hADF overexpressing OCT4+ASF1A; in pluripotent cells H9-hESC; and in OSKM iPSCs and AO9-iPSC. Immunoprecipitated material was analyzed by Wb using the specified antibodies to detect OCT4 and ASF1A coimmunoprecipitation. Actin was used as a loading control. (**D**) ChIP assay in hADF overexpressing GFP, ASF1A, OCT4, both ASF1A and OCT4, and in H9 hESC and AO9-iPSCs using specific antibody against H3K56Ac. qRT-PCR was done using ChIP and input samples using the specific primers for NANOG, OCT4, and SOX2 promoters and two negative controls KRTHA4 (hypoacetylated gene) and an intergenic region primers. Mean values ± SEM are plotted indicating amplification of the specific gene region normalized to GFP sample, which was arbitrarily assigned a value of 1. Data correspond to the average of three independent experiments done in duplicate. Student's *t* test was applied for statistical significance. ***$P < 0.001$, **$P < 0.05$, and *$P < 0.01$ compared with ASF1A expressing hADF.

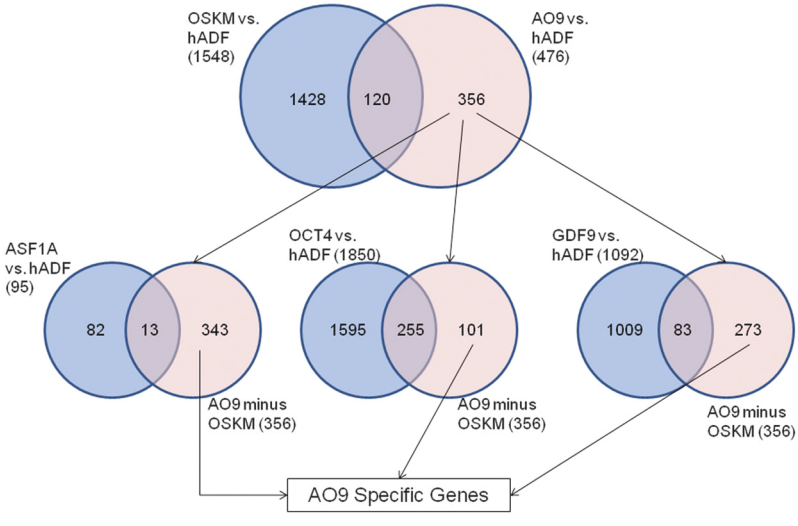

Fig. 4. Comparisons of differentially expressed genes 48 hours after overexpression of different factors in human dermal fibroblasts. Venn diagrams to select the genes that are differentially expressed in AO9 condition compared with OSKM (region II, upper diagram). Lower diagrams show three different comparison of previous region II with single-factor–specific up- or down-regulated genes.

NANOG, SOX2, and OCT4 transcription factors at their target gene promoters, and it binds specifically to these pluripotent factor promoters to increase their expression (*17*, *18*). ASF1A downregulation seems to mediate a reduction of histone 3 acetylation at lysine *56*, which in turn negatively impacts the expression of pluripotency-related markers and increases expression of differentiation-related ones (*18*).

In conclusion, we have identified two oocyte factors capable of—together with OCT4—reprogramming human dermal fibroblasts: ASF1A and GDF9. We found that GDF9 conditions somatic cells, activating SMAD 2/3 and likely making the cells more susceptible to reprogramming by OCT4 and ASF1A. We also found that ASF1A works by acetylating H3K*56*, affecting the expression of core pluripotency genes. It is quite possible that many other genes expressed in the MII oocyte are important for reaching and maintaining pluripotency. These genes may have a specific role during the first stages of the reprogramming process.

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SUPPLEMENTARY MATERIALS

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