Reprogramming to a muscle fate by fusion recapitulates differentiation

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Summary

Fusion of mammalian cells to form stable, non-dividing heterokaryons results in nuclear reprogramming without the exchange of genetic material. In this report, we show that reprogramming in somatic cell heterokaryons involves activation of the canonical skeletal muscle transcription factors as well as contraction-excitation genes. Thus, the effect of heterokaryon formation on gene expression is to induce a recapitulation of differentiation. Heterokaryons formed with a relatively refractory cell type, the hepatocyte cell line HepG2, revealed the importance of both MyoD expression and other unidentified cytoplasmic components, neither of which are sufficient for efficient muscle gene activation, but are synergistic. We provide evidence that de-repression by transient histone deacetylase inhibition can induce MyoD expression and increase the extent and efficiency of muscle gene transcription. Taken together, the results suggest that understanding the mechanistic basis, using a combination of approaches, and taking into account cell history, will facilitate an increase in the efficiency and fidelity of conversion from one differentiated phenotype to another desired cell type. Inherent advantages of the heterokaryon system merit further investigation in the pursuit of directed cloning.

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Introduction

We have extended our earlier studies of nuclear reprogramming in heterokaryons to enhance our understanding of the mechanistic basis as well as to determine whether gene activation occurs to sufficient extent to suggest potential practical utility. Recent interest in inducing changes in cell identity has increased as the therapeutic value of generating patient-specific cell types has become apparent (Higgs, 2008; Jaenisch and Young, 2008). Widely pursued approaches involve embryonic stem cell (ESC) formation by various methods, each with theoretical and practical advantages and disadvantages. Two general approaches have shown promise for converting somatic cells to ESCs; the first, nuclear transfer, involves the direct response of a nucleus to factors present in the oocyte, and the second involves transduction and expression of ESC-specific transcription factors to drive reprogramming (Aoi et al., 2008; Gurdon and Byrne, 2003; Hanna et al., 2007; Hochedlinger and Jaenisch, 2002; Takahashi et al., 2007; Wernig et al., 2007). A third approach, hybrid formation, involves elements of both paradigms in that the necessary factors are provided by fusion, but mixing of genetic material also occurs (Cowan et al., 2005). The interpretation or practical application of the latter approach is hindered by the mixing of same-species genetic material, precluding an analysis of gene expression from a particular nucleus (Pralong et al., 2006).

Experimental paradigms for activating expression of dormant, tissue-specific genes in somatic cells were developed using skeletal muscle as a model system. Certain cell types were directly converted to muscle by induced overexpression of the bHLH transcription factor MyoD, whereas others, such as hepatocytes, were not (Choi et al., 1990; Davis et al., 1990; Tapscott et al., 1988). Cell history, or the lineage of origin, influenced the cell response to MyoD, or its reprogrammability (Schafer et al., 1990). In parallel to early nuclear-transfer experiments, reprogramming was investigated in skeletal muscle by cell-cell fusion to alter the balance of factors to which a nucleus is exposed (Blau et al., 1985a; Blau et al., 1983; Blau et al., 1985b; Wright, 1984). The heterokaryon approach permitted such investigations in the absence of transgenes or hybrid-related genomic mixing, because the nuclei in heterokaryons remain distinct and intact. Nuclear fusion and mixing of genetic material does not occur because heterokaryon nuclei do not enter S phase or mitosis in the culture conditions used (Chiu and Blau, 1984; Zhang et al., 2007). In addition, controlling the nuclear ratio can provide an excess of cytoplasmic factors to drive cell-type-specific gene expression in a desired direction (Pavlath and Blau, 1986). A third advantage of the heterokaryon system for mechanistic studies is that muscle differentiation involves a well-characterized sequence of events in which expression of transcription factors and structural genes occurs predictably in turn. Thus, changes in gene expression can be examined against this well-known backdrop of events. The methods available previously permitted evaluation of only a very small number of genes, precluding investigation into the extent or underlying mechanisms.

Renewed interest in the alteration of cell identity has resulted in rapid advances in approaches to induced pluripotent stem (iPS) cell formation. Although current approaches are extremely promising, they also face significant challenges without clear solutions, including the incorporation of foreign genetic material, the

inefficiency of nuclear transfer, and ethical problems. We have revisited experimentation with somatic cell heterokaryons to assess whether heterokaryon formation is a feasible approach to understand the mechanisms underlying alteration of cell identity, and to determine how the unique characteristics of the heterokaryon system might be usefully exploited.

We show that heterokaryon formation between skeletal muscle cells and liver cells results in induction of the canonical skeletal muscle genes. We provide evidence that transient de-repression by histone deacetylase inhibition can both induce MyoD expression and increase the extent and efficiency of muscle gene activation, demonstrating the importance of muscle regulatory factor expression for downstream expression of the muscle differentiation program. The results provide evidence that gene activation in heterokaryons follows a sequence of essential steps of the differentiation program, with induction of determination and lineage-specific transcription factors constituting essential events for the efficient expression of stable differentiated phenotypes. As such, efforts to reprogram cells should target activation of endogenous determination factors for the desired cell type. These findings have implications for optimizing cloning or iPS cell strategies by showing that choice of the starting cell type should take into account the degree of existing repression of the desired expression profile.

Results

Previous studies in heterokaryons demonstrated the activation of skeletal muscle differentiation genes such as myosin heavy chain and neural cell adhesion molecule (NCAM) (Blau et al., 1985a; Blau et al., 1983). Cells from different embryonic lineages – for example, fibroblasts (mesoderm), keratinocytes (ectoderm) and hepatocytes (endoderm) – reprogram at different efficiencies, with fibroblasts and hepatocytes being the most and least efficient, respectively (Blau et al., 1985b). Although NCAM expression is a valuable downstream indicator of reprogramming on a cell-by-cell basis, we sought to investigate earlier events, to determine whether the process resembles the development or differentiation of muscle. We chose human hepatoma cells (HepG2), which are among the most refractory cell lines, to elucidate important obstacles to conversion to a muscle phenotype that are present in these cells. Human HepG2 cells were fused to differentiated mouse C2C12 muscle cells using polyethylene glycol (Fig. 1A).

MyoD expression occurs early during the course of muscle differentiation and, along with the other muscle regulatory factors (MRFs) Myf5, myogenin and Mrf-4, is responsible for activating downstream genes such as *NCAM* that are important for expressing the muscle phenotype (Kawahigashi et al., 1998; Yun and Wold, 1996). To provide insight into the effects of heterokaryon formation on gene expression, heterokaryon RNA was assayed by RT-PCR for the activation of the canonical myogenic bHLH genes that have a crucial role in muscle development and differentiation. Primers were designed to detect human but not mouse Myf-5, MyoD, MRF4 and myogenin mRNA. Each primer was first assayed on RNA from RH30, a human rhabdomyosarcoma line that expresses the MRFs (positive control), and mouse C2C12 cells that express all myogenic bHLH mRNA transcripts at high levels, to document human specificity (Fig. 1B). Heterokaryons were then formed by fusing differentiated murine myotubes with human HepG2 cells. RNA was prepared from cultures before PEG (pre-PEG) and from heterokaryons every 24 hours thereafter for 7 days, followed by RT-PCR to detect activation of the human muscle mRNA transcripts. Heterokaryon formation resulted in induction of all four bHLH

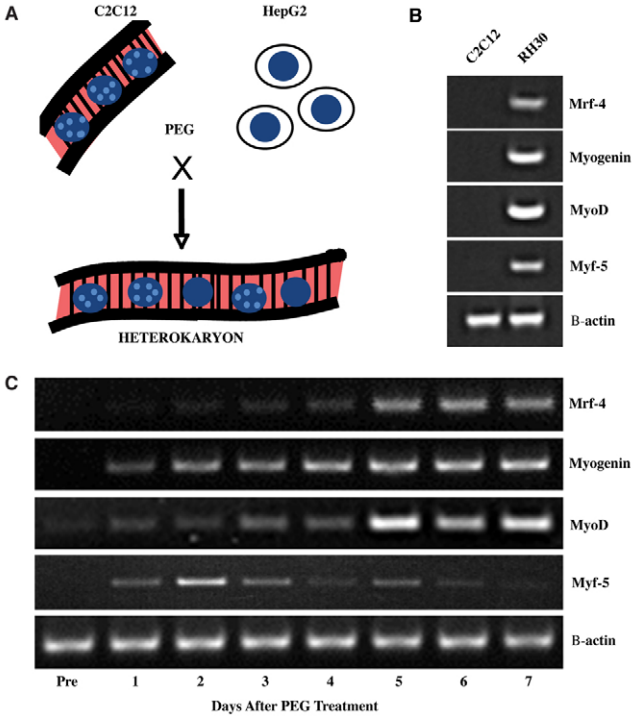

Fig. 1. Reprogramming of gene expression in heterokaryons formed between differentiating murine myoblasts and human hepatoma cells (HepG2). (A) HepG2 cells were fused with myotubes using polyethylene glycol, and resulting cultures were grown in differentiation medium supplemented with ara-c and ouabain. (B) Activation of muscle gene expression over time after fusion. RT-PCR using human-specific primers for genes encoding Myf-5, MyoD, myogenin and Mrf-4. Controls for primer specificity and absence of amplification in mouse muscle cells (C2C12, murine myoblasts; Rh30, human rhabdomyosarcoma cell line). (C) Time course of gene expression by hepatocyte nuclei before and at daily time points after fusion (β-actin, 30 cycles; Myf-5, 33 cycles; MyoD, 35 cycles; myogenin, 33 cycles; Mrf-4, 33 cycles).

mRNAs (Fig. 1C). Myf-5 reached maximal expression earliest, and then declined, followed by MyoD and myogenin. MRF4 reached maximal levels last. These results demonstrate that heterokaryon formation induces muscle transcription factors in non-muscle nuclei, and the pattern of gene activation resembles the expression of bHLH transcription factors during normal muscle differentiation, implying a recapitulation of the gene expression program exhibited during skeletal muscle differentiation.

The activation of MRFs detected in heterokaryons raised the possibility that their expression in non-muscle nuclei is important or necessary for the downstream changes in gene expression, such as NCAM activation. Ectopic MyoD expression can directly convert some non-muscle cells to muscle cells; however, HepG2 hepatocytes, which express the HNF family of liver transcription factors, are refractory to MyoD and are much less amenable to muscle gene activation in heterokaryons than are fibroblasts (Blakely, 1993; Blau et al., 1985b; Schafer et al., 1990). HepG2 cells induced to stably overexpress MyoD (HepG2-MyoD) did not convert to muscle cells when placed in low serum and did not express the muscle marker NCAM, when assayed by immunohistochemistry (Fig. 2A), nor was myogenin detectable by RT-PCR (data not shown). We hypothesized that in the case of HepG2 cells, MyoD expression level was important but insufficient by itself to drive a switch to the muscle gene expression profile.

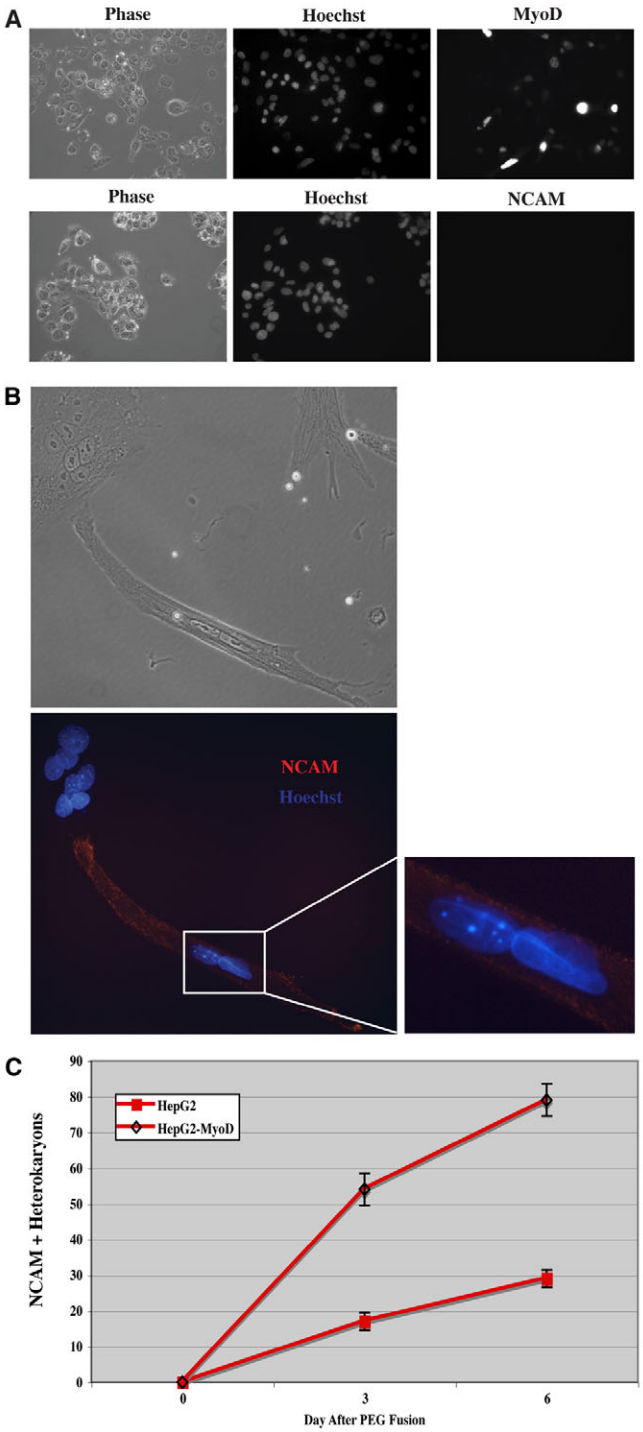

Fig. 2. Effects of overexpression of MyoD in HepG2 cells. (A) Morphology of HepG2-MyoD cells (Phase). HepG2-MyoD cells stained with anti-MyoD antibody and Alexa Fluor 488 anti-mouse secondary antibody show stable expression of MyoD (top row, MyoD). Cells stained with anti-NCAM antibody 5.1H11 and Alexa Fluor 488 secondary antibody as in heterokaryon experiments are shown in bottom row (NCAM). (B) Activation of NCAM protein expression in HepG2 or HepG2-MyoD nuclei after fusion with muscle cells. Skeletal muscle NCAM is expressed by hepatocyte nuclei only after fusion with muscle cells (NCAM, red). Individual cells are determined to be heterokaryons by the presence of nuclei derived from both human (uniform staining pattern) and mouse (punctate staining pattern) nuclei. (C) Frequency of muscle gene activation scored as the percentage of individual heterokaryons that express human NCAM (NCAM + heterokaryons). Error bars indicate the standard error of the proportion calculated from the binomial equation.

To test the hypothesis that MyoD expression is an important early event that requires cooperation with additional factors during the conversion to a muscle gene expression profile, heterokaryons were made with HepG2 or HepG2-MyoD cells. Using a combination of Hoechst 33258 staining to distinguish human (diffuse) from mouse (punctate) nuclei in a given cell and the human-specific NCAM antibody 5.1H11, the percentage of heterokaryons in which human muscle gene expression is reprogrammed (denoted NCAM + heterokaryons %) was scored as previously described (Blau et al., 1985b; Zhang et al., 2007) (Fig. 2B). Muscle gene activation was analyzed by NCAM expression 3 and 6 days after heterokaryon formation. After fusion with differentiated myotubes, the HepG2-MyoD cells expressed NCAM with a much higher efficiency than control HepG2 cells (17% and 29% vs 54% and 79%, at day 3 and 6, respectively) (Fig. 2C). The frequency of NCAM expression in HepG2-MyoD heterokaryons increased dramatically and approached that observed with MRC-5 fibroblasts (Blau et al., 1985b).

Although fusion with muscle cells was necessary for muscle gene activation, increased NCAM activation upon MyoD overexpression underscores the importance of the presence of MyoD in the non-muscle nuclei as a driving event. We therefore investigated parameters of activation of the endogenous MyoD gene (*MYOD1*). To test whether manipulation of the levels of histone acetylation could affect the conformation of the MyoD regulatory regions, cells were exposed to histone deacetylase inhibitors for 24 hours followed by chromatin immunoprecipitation using antibodies to acetylated His3 Lys9. PCR amplification of the MyoD gene promoter showed that it is maintained in a relatively repressed state in untreated cells. However, after exposure to 2.5 μM trichostatin-A (TSA), MyoD gene promoter amplification increased markedly following immunoprecipitation, indicating a shift from a closed to an open conformation (Fig. 3A).

Given the marked change in promoter configuration after TSA treatment, activation of *MYOD1* transcription was assayed by RT-PCR. HDAC inhibitor treatment alone without fusion to muscle cells resulted in a strong induction of *MYOD1* transcription. This effect was produced using different HDAC inhibitors, including sodium butyrate and MS-275. With TSA and MS-275 the induction of *MYOD1* transcription was dose-responsive at μM levels (Fig. 3B). However, NCAM expression was never detected (data not shown). In addition to *MYOD1*, transcription of the gene encoding Myf-5 (*MYF5*) and to a lesser extent Mrf-4 (*MRF4/MYF6*) was induced by TSA (Fig. 3C). However, myogenin gene (*MYOG*) expression remained silenced. To test whether HDAC inhibitor treatment caused lasting alterations in gene transcription, gene expression was analyzed at various time points after drug withdrawal. After removal of HDAC inhibitors, *MYOD1* transcription (as well as creatine kinase) was downregulated over time (Fig. 3D). Thus, brief HDAC inhibition is sufficient to activate transcription of the early myogenic bHLH genes in non-muscle cells, but insufficient to cause stable acquisition of the muscle gene expression profile.

Our finding that activation of the myogenic bHLH transcription factors occurs early after heterokaryon formation suggests their importance for downstream events, and prompted us to test whether inhibition of histone deacetylase activity could increase the efficiency of muscle differentiation gene expression in HepG2 cells by direct activation of the MRFs before fusion. HepG2 cells were treated with HDAC inhibitors while still in growth medium for 24 hours before drug withdrawal and fusion with muscle cells. Indeed,

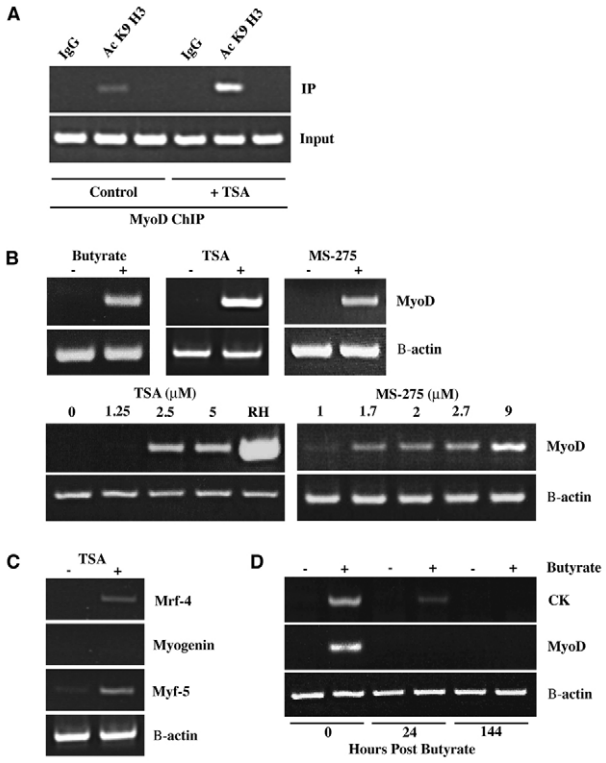

Fig. 3. De-repression of myogenic bHLH transcription factors by histone acetylation. (A) Chromatin immunoprecipitation was performed using HepG2 cells in the presence (24 hours) or absence of trichostatin-A (2.5 μM; TSA) with control antibody (IgG), acetylated histone antibody (Ac K9 H3) or dimethyl-histone H3 (Lys9) (unmarked lane), followed by semi-quantitative PCR amplification of the MyoD promoter from 10% of the immunoprecipitate (IP) or 2% of the input sample prior to immunoprecipitation (Input) (36 cycles). (B) Induction of *MYOD1* transcription in cells treated with various histone deacetylase inhibitors. Concentrations of HDAC inhibitors used in the top three panels were: 0.5 mM sodium butyrate (Butyrate); 2.5 μM trichostatin-A (TSA); 9 μM MS-275. For demonstration of dose response, TSA or MS-275 was used at the indicated concentration (bottom panels). In all cases, HDAC inhibitors were added to cells in growth medium for 24 hours before harvesting. β-actin was detected as a loading control. The rhabdomyosarcoma cell line Rh30 was used as a positive control for *MYOD1* transcription (RH). RT-PCR was performed on 500 ng RNA for 35 cycles. (C) Assay for induction of the four canonical myogenic bHLH transcription factors in HepG2 cells after exposure to TSA. Cells were exposed to 2.5 μM TSA for 24 hours followed by RT-PCR for the indicated transcripts (Myf-5, 33 cycles; MyoD, 35 cycles; Mrf-4, 34 cycles; myogenin, 34 cycles; β-actin, 30 cycles). (D) Return to baseline gene expression after withdrawal of histone deacetylase inhibitor. HepG2 cells were treated with sodium butyrate (5 mM) for 24 hours followed by a medium change. RT-PCR for MyoD and creatine kinase (CK) was performed on cells harvested after 24 hours of butyrate exposure or at the indicated time points after washout (39 cycles). β-actin was detected as a loading control (30 cycles).

in contrast to untreated controls, 24 hours of HDAC inhibition with butyrate, TSA or MS-275 resulted in an ~twofold increase in the percentage of NCAM-positive heterokaryons at day 3 and 6 after PEG fusion (Fig. 4A). Fusion of HepG2 cells with myotubes was essential for NCAM expression, even after HDAC inhibition, confirming that additional factors present in the muscle cytoplasm are required in hepatocytes after de-repression and activation of MyoD transcription. The HDAC-inhibited HepG2s did not activate NCAM as frequently as when MyoD was ectopically expressed.

Although butyrate, TSA and MS-275 are structurally different molecules that share the property of inhibiting specifically class I HDACs (Hess-Stumpp et al., 2007; Piekarz and Bates, 2004), it remained formally possible that the observed actions of these agents could arise from non-specific effects. Therefore, to demonstrate definitively that histone deacetylase inhibition was the relevant target of the pharmacological treatments, shRNA constructs were designed to knockdown *HDAC1* mRNA transcript levels. HepG2 cells were infected with retroviruses driving expression of two different short hairpin (sh)RNAs targeting *HDAC1*. Infected cells

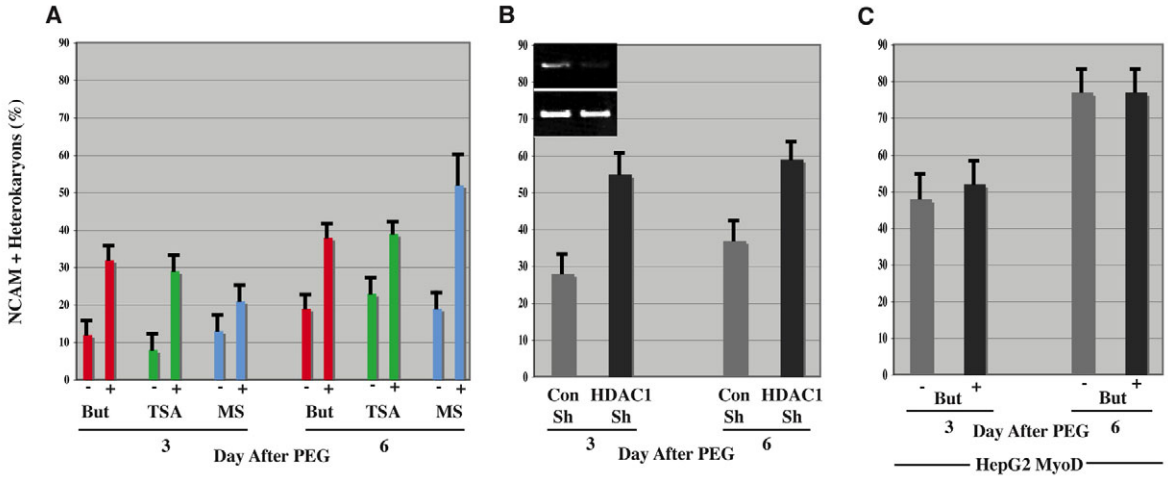

Fig. 4. Effects of HDAC inhibition on muscle gene activation in heterokaryons. Where indicated by (+), cells were treated with HDAC inhibitors for 24 hours, then washed, trypsinized and seeded for heterokaryon formation. Untreated controls are indicated by (–). (A) HepG2 cells were exposed to three different pharmacological HDAC inhibitors: 0.5 mM sodium butyrate (But), 2.5 μM Trichostatin-A (TSA) and 3.3 μM MS-275 (MS). (B) Specific inhibition of HDAC1 was tested by infecting HepG2 cells with a retrovirus expressing shRNAs targeting HDAC1 or control shRNA, followed by brief selection in puromycin and heterokaryon formation. Knockdown of *HDAC1* was confirmed by RT-PCR [23 cycles for both HDAC1 (inset, top row) and β-actin (inset, bottom row)]; the control sample is on the left and the HDAC shRNA sample on the right. (C) HepG2-MyoD cells were either untreated or treated with 0.5 mM sodium butyrate, followed by heterokaryon formation and assay for NCAM expression. NCAM expression was assayed at day 3 and day 6 after fusion. In all cases, reprogramming was scored as the percentage of heterokaryons with positive staining for human NCAM, using the antibody 5.1H11. Error bars indicate the standard error of the proportion calculated from the binomial equation. Experiments were performed at least in triplicate.

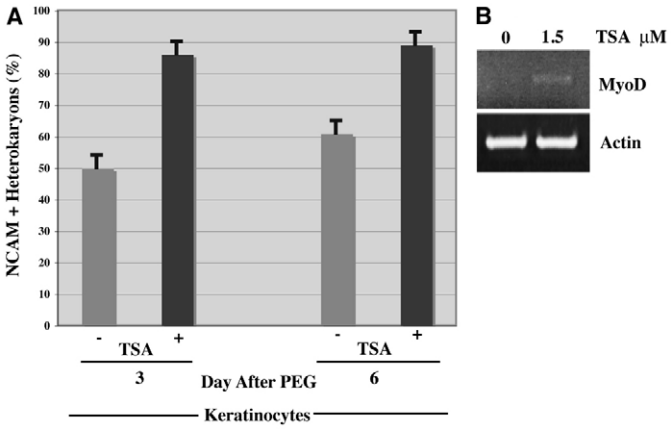

Fig. 5. Effect of HDAC inhibition on muscle gene expression in primary human keratinocytes. (A) Cells were exposed to TSA for 24 hours and then used to form heterokaryons as in the previous figure. Equal numbers of control and treated cells were seeded. Results are means ± standard error of the proportion. (B) Induction of *MYOD1* transcription after treatment with TSA.

were selected in puromycin, which was possible given the window of time during shRNA expression, but before a decrease in HDAC1 levels, which ultimately resulted in growth inhibition. RT-PCR using RNA from cells expressing the *HDAC1* constructs confirmed that compared with control constructs, *HDAC1* shRNAs significantly decreased *HDAC1* mRNA transcript levels. (Fig. 4B, inset). Knockdown of *HDAC1* significantly increased the efficiency of *NCAM* expression at day 3 and day 6 (Fig. 4B). Thus, HDAC1-related functions constitute a major mechanism of muscle gene repression in HepG2 cells.

It remained possible that inhibition of HDAC increases the efficiency of muscle gene activation by mechanisms that are distinct from and unrelated to initiating *MYOD1* activation, such as the direct de-repression of downstream genes. To test this possibility, HepG2-MyoD cells were exposed to HDAC inhibitors for 24 hours and then used to form heterokaryons. In this case, HDAC inhibition did not increase the frequency of NCAM expression (52% and 77%) compared with untreated HepG2-MyoD cells (48% and 77%) at day 3 and day 6 after fusion (Fig. 4C). Both treated and untreated groups demonstrated a high frequency of NCAM activation, but at an efficiency below that of fibroblasts (J.H.P. and H.B., unpublished data).

Finally, to test whether the effect of HDAC inhibition is specific to HepG2 cells, muscle gene activation in primary human keratinocytes was tested. Keratinocytes expressed NCAM at a frequency intermediate between HepG2 cells and fibroblasts, in agreement with previously reported experiments (Blau et al., 1985b; Zhang et al., 2007). In this cell type, exposure to TSA markedly increased the efficiency of NCAM expression (50% and 60% vs 85% and 89% at day 3 and day 6, respectively) to a level on par with fibroblasts (Fig. 5). In addition, exposure to micromolar concentrations of TSA resulted in induction of *MYOD1* transcription by keratinocyte nuclei, confirming that induction of *MYOD1* and increased activation of NCAM in response to HDAC inhibition are not specific to HepG2 cells (Fig. 5).

Although NCAM expression is a useful reprogramming marker, it was important to investigate whether other additional downstream muscle genes are activated in heterokaryons. To this end, we sought to develop an array-based system for differentially detecting human mRNA transcripts in mixed human and mouse samples. We reasoned that it would be possible to design an oligonucleotide array in which each oligonucleotide is designed to specifically detect a human muscle or liver transcript but not the corresponding mouse transcript. Oligonucleotides (70-mers) were designed for a complement of known human muscle-specific genes. A total of 75 known muscle genes were chosen, as well as 13 liver genes. Represented genes are characteristic of muscle differentiation, including contraction-excitation, metabolism, surface structure, sarcoplasmic reticulum and transcription factors (supplementary material Table S1). Bacterial probes (spike controls, Ambion) were also included in each array. Despite species-specific design, a significant number of spots exhibited hybridization to mouse C2C12 cDNA, necessitating an increase in wash stringency using tetra-ethyl-ammonium (TEA) salt (see Materials and Methods). As a result, a significant number of spots were species specific and hybridization occurred using human but not mouse muscle RNA. Spots that did not achieve species specificity were removed from the analyses. The total number of informative muscle elements was 65. Heterokaryons were formed as in the previous experiments and cDNA before and 6 days after fusion was assayed in HepG2, HepG2-MyoD and HepG2 cells pre-treated with TSA (Fig. 6A). We observed a slight relative decrease in total human RNA over days in culture and although this was insufficient to affect qualitative RT-PCR results, normalization was important for quantitative experiments and comparison between experimental groups. Therefore, the proportion of human cDNA in each sample was determined by quantitative PCR and mouse C2C12 cDNA was added to equalize the proportion of human cDNA in all samples. The three biological replicates for each sample were normalized by applying variance stabilization (Huber et al., 2002). To identify the relevant differentially expressed genes, the non-parametric Rank Product test was used (Hong et al., 2006). The heterokaryon samples at day 6 were compared with C2C12 + HepG2 pre-fusion (Pre-PEG) as a control. The threshold was taken as a fold change greater than or equal to 1.5 compared with the control ($P<0.05$). TSA treatment of HepG2 cells without fusion to muscle was sufficient to cause induction of a few muscle genes (3 of 65) to significant levels detectable on the array (C2 + HepG2 TSA compared with C2 + HepG2). These genes remained activated at day 6 after fusion, whereas additional genes were induced in heterokaryons with HepG2 exposed to TSA (C2 + HepG2 TSA) (16 of 65 muscle genes; 10 of 17 contraction-excitation genes detectable) (Fig. 6A, left and right upper panels). By contrast, MyoD overexpression resulted in detectable expression of only one gene before fusion (C2 + HepG2MyoD) (Fig. 6A, left, middle), but caused a concentrated, stronger expression of the contraction-excitation genes at day 6 (18 of 65 muscle genes; 12 of 17 contraction-excitation genes detectable) (Fig. 6A, right middle panel). Untreated HepG2 cells exhibited activation of the fewest genes in heterokaryons (C2 + HepG2) (10 of 65 muscle genes; 6 of 15 contraction-excitation genes detectable) (Fig. 6A, right lower panel). Because of the relatively low signal after stringent washing, only genes with very strong expression levels reach significance. Therefore this experiment probably represents an underestimate of the number of genes present on the array that were activated in heterokaryons. When compared with untreated HepG2 cells, both MyoD overexpression and TSA pre-treatment resulted in a relative increase in expression of contraction-excitation genes at 6 days after fusion. There was a decrease in expression of many liver genes, but the sensitivity of the method under stringent conditions did not reach statistical

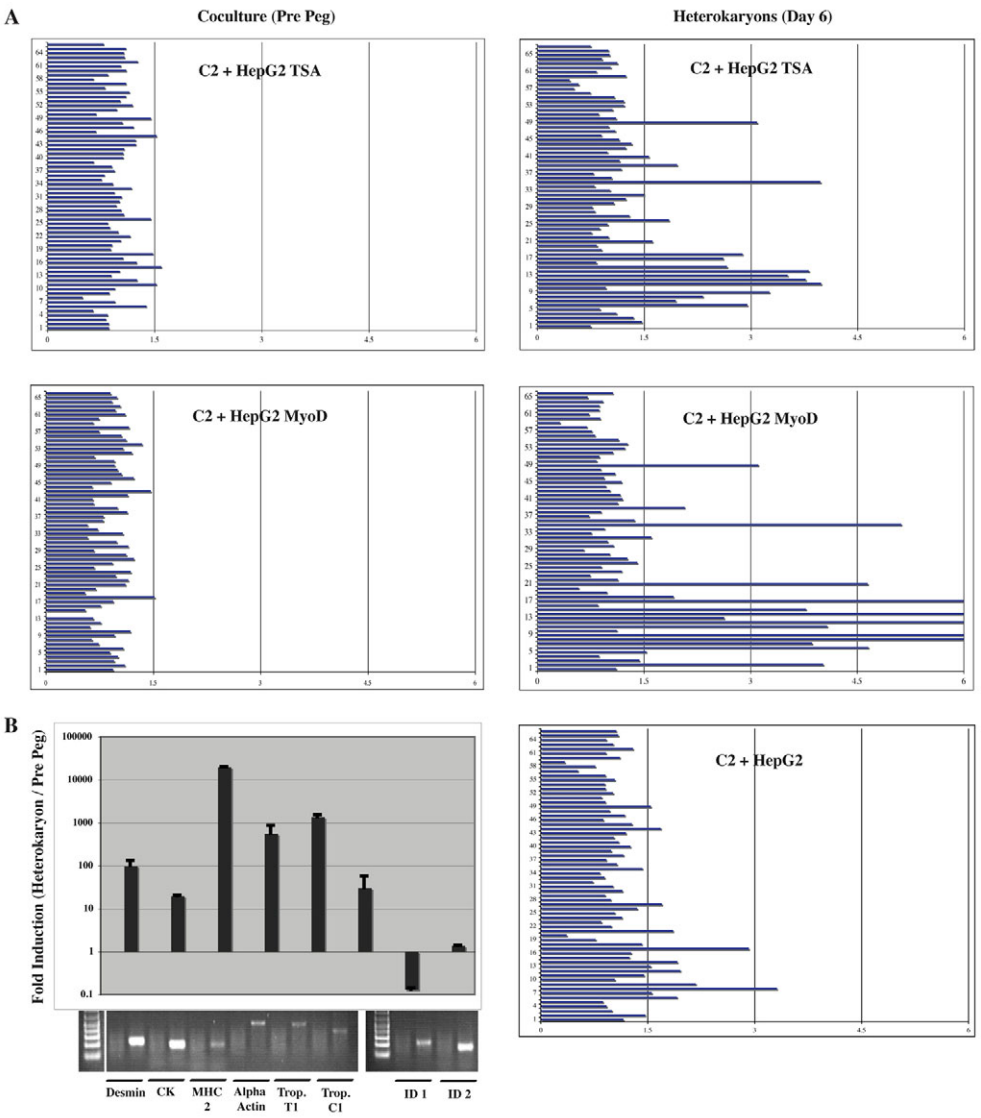

Fig. 6. Species-specific array hybridization for activation of muscle genes in non-muscle cell nuclei (A). Data displayed in bar graph format with fold change in expression level of the sample compared with control HepG2 cells before fusion along the *x*-axis. A value of 1.5 indicates significant fold change ($P<0.05$) calculated by the non-parametric Rank Product test. For each experiment, fold change in gene expression was evaluated after seeding but before fusion (PrePeg), and at 6 days after fusion (Day 6) compared with HepG2 PrePeg as a control. (B) Quantitative RT-PCR for human muscle genes. Human-specific primers for eight genes encoding desmin, creatine kinase (CK), myosin heavy chain 2 (MHC2), cardiac α-actin, troponin T1, troponin C1, ID1 and ID2 were designed and normalized to the three housekeeping genes encoding GAPDH, PMS and HRPT. Each sample was run in triplicate. The data are represented on a logarithmic scale as fold induction of expression of the indicated gene in heterokaryons compared with before fusion (top panel). The PCR products are shown demonstrating the species specificity; in each case, use of mouse C2C12 RNA resulted in failure to amplify a product (left lanes) and use of human muscle RNA resulted in amplification of a single band of the appropriate size (right lanes).

significance (data not shown). Levels of transcripts with a role in metabolism, the sarcoplasmic reticulum and transcription were generally below the level at which relative expression could be assayed with confidence (see supplementary material Table S1 for relative expression level and significance for each gene analyzed).

To confirm the validity of the array data, five muscle genes represented on the array and three additional genes [encoding inhibitor of differentiation (ID1 and ID2) and cardiac α-actin] were analyzed for activation by quantitative RT-PCR (Fig. 6B). Induction of each of the muscle differentiation genes in heterokaryons was confirmed. In addition, assay of the mRNA transcripts revealed that ID1 but not ID2 is significantly downregulated in heterokaryons.

Discussion

We investigated the extent and mechanism of muscle gene activation in somatic cell heterokaryons. The results show significant similarities between heterokaryon reprogramming and muscle differentiation. The pattern of activation of the muscle transcription factors is similar to that which occurs during the formation of mature muscle cells, and canonical muscle differentiation genes are activated. As in normal muscle differentiation, the importance of MyoD presence in the non-muscle nucleus is highlighted by the enhanced reprogramming observed when *MYOD1* is overexpressed or upon de-repression using HDAC inhibition.

HDAC inhibition was used for a brief period, prior to heterokaryon formation, in accordance with previous demonstrations that such treatment could enhance the action of muscle regulatory factors in myoblasts before fusion (Iezzi et al., 2002). We hypothesize that in this particular scenario, pre-fusion ‘relaxation’ of chromatin at muscle regulatory regions renders the non-muscle nuclei more susceptible to muscle cytoplasmic factors after fusion. HDAC inhibition did not result in stable reprogramming because of the transient effect of the agents. Moreover, whereas the experiments reported here arbitrarily assay muscle genes, it is likely that HDAC inhibition affects several genes in different lineages. Pre-treatment with HDAC inhibitors appears to increase the efficiency of reprogramming in cloning and in iPS cell generation (Ding et al., 2008; Huangfu et al., 2008; Iager et al., 2008; Shi et al., 2008). It has been elegantly shown that the presence of HDAC inhibitors during heterokaryon culture did not affect the activation of muscle genes when preventing repression of non-muscle genes (Terranova et al., 2006). When

considered from a mechanistic standpoint, the findings in that report point to separable aspects of reprogramming: the activation of subsets of genes along with the repression of others. Thus, continuous inhibition of HDAC activity did not promote conversion, but rather inhibited conversion by blocking the extinguishment of the non-muscle phenotype. In contrast to our study, low-dose HDAC inhibition was present throughout these experiments. In the present report, a brief pulse of HDAC inhibition before heterokaryon formation enhanced the downstream acquisition of the muscle gene expression profile days after washout of the drug. Together, the distinct but complementary observations among reports (Terranova et al., 2006) (and this study) reflect the specific context of HDAC inhibitor use and are in line with the known biochemical functions of the HDACs.

The observation that butyrate treatment before fusion did not further increase NCAM activation in cells that already express MyoD suggests that the important effect of HDAC inhibition in muscle reprogramming is activation of MyoD or other early bHLH factors. Activation of transcription of genes encoding Myf-5 and Mrf-4 occurred along with MyoD activation, and a significant contribution of these transcription factors to the process is possible. In particular, although the Mrf-4 gene is typically activated late in muscle differentiation, it acts as an early phase gene in certain contexts (Kassar-Duchossoy et al., 2004). The observation that *MYOG* remained silenced in the presence of HDAC inhibitors before fusion, suggests the dominance of other modes of repression, and is in accord with its known function of signaling a commitment to terminal muscle differentiation (Rudnicki and Jaenisch, 1995; Venuti et al., 1995). It remains possible that the increase in muscle gene activation does not require MyoD per se, because HDAC inhibition de-represses other MRFs as well as certain downstream genes. That MyoD alone is not sufficient is demonstrated by the inability of either MyoD or HDAC inhibition to activate many downstream muscle genes in these cells; other diffusible factors present in muscle cytoplasm are necessary. The facilitation of cell cycle exit afforded by heterokaryon formation under the culture conditions used might have an important role.

Differentiation involves unique chromatin fingerprint configurations for each cell type. Nuclear reprogramming by oocyte nuclear transfer or directly with ESC transcription factors, relies on the ability to erase one chromatin fingerprint and replace it with another, and has been shown to be an imperfect process (Mikkelsen et al., 2008). Studies of somatic cell heterokaryons and synkaryons have, until recently, only assayed gene expression without providing mechanistic insight into the levels of control and their reversal. It remained possible that gene expression changes that occur in heterokaryons are restricted to permissive or 'open' loci. However, chromatin remodeling in somatic cell heterokaryons was predicted by the observation of nuclear swelling in heterokaryons formed between mouse muscle and nucleated chicken erythrocytes (Ringertz and Savage, 1976) and more recently in vivo, in heterokaryons formed between bone-marrow-derived cells and Purkinje neurons, and by studies of chromatin structure in lymphocyte-muscle heterokaryons (Johansson et al., 2008; Terranova et al., 2006; Weimann et al., 2003). This study, and our previous report (Zhang et al., 2007) demonstrate that somatic cell reprogramming, like that in oocytes, is driven by remodeling of chromatin at key loci, to allow expression of, in this case, the muscle regulator, MyoD. MyoD, in concert with other diffusible muscle transcription factors then presumably drives the remodeling of chromatin at downstream muscle loci, enabling the ensuing alteration of global gene expression.

Despite the limited number of informative elements, the muscle array experiment confirms the relevance of and extends the NCAM data, supporting the concept that de-repression before fusion renders HepG2 nuclei more susceptible to activation of the muscle transcription profile. The detection of the majority of known contraction-excitation genes under very stringent conditions indicates that heterokaryon formation induces a robust, extensive activation of the muscle program. Future studies using heterokaryons will benefit greatly from methods that differentially detect human-specific gene expression on a global scale in mixed samples. Use of the species-specific oligonucleotide array method here was hampered by the difficulty of designing oligonucleotides that exhibit strong specificity for human over mouse mRNA transcripts. In the heterokaryon samples, the relative ratio of human to mouse total mRNA is low, making preferential detection of the human transcripts difficult. The advent of whole transcriptome sequencing or the development of alternative methods of species-specific transcript detection should facilitate studies of gene expression in heterokaryons.

The heterokaryon system offers insights fundamental to both the understanding of differentiation control in normal biology and for practical applications. From the standpoint of applications, this study supports the concept that somatic cells, like oocytes, contain machinery that drives remodeling of chromatin configuration as well as gene expression. It follows that fusion of somatic cells might ultimately represent a viable approach to directed cloning, for production of specified cell types, such as skeletal muscle. In addition, the mechanistic steps along the pathway to changes in cellular identity can be defined. In this case, reversal of histone deacetylation at the *MYOD1* locus is one such step. Ultimately, chromatin roadmaps might be developed that outline the context-specific hurdles for phenotypic conversion, and these could offer insight into which specific cell types are better or worse choices for production of a desired cell type. Such a mechanistic approach should also prove useful in iPS cell formation by defined factors or for oocyte cloning, in which efficient and complete reprogramming might depend, in part, on the degree of repression of a particular lineage in the starting cell type.

Materials and Methods

Cells and tissue culture

Mouse C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum and penicillin-streptomycin (DM). Myoblasts were seeded on collagen-coated plates in DMEM supplemented with 2% horse serum and antibiotics. After 24 hours, the medium was changed and ara-c (10^{-5} M) was added to eliminate dividing myoblasts. Medium supplemented with ara-c was replaced daily. After 48 hours, myotubes were used for the production of heterokaryons.

Human HepG2 cells were grown in DMEM supplemented with 10% FCS and antibiotics. HepG2-MyoD cells (Schafer et al., 1990) were maintained with the addition of G418. Human primary keratinocytes isolated from neonatal foreskin were grown in medium 154-CF (Cascade Biologics) supplemented with human keratinocyte growth supplement (Cascade Biologics) and 0.07 mM calcium. Keratinocytes were used at passage three or below.

Where indicated, HDAC inhibitors were added to growing HepG2 cells or keratinocytes for 24 hours before washing, harvesting for analysis or trypsinizing and seeding on myotubes for fusion. The HDAC inhibitors sodium butyrate (Sigma), Trichostatin-A (Sigma) and MS-275 (Calbiochem) were stored according to the manufacturer's protocol.

PEG fusion

Heterokaryons were produced by fusing myotubes and HepG2 cells or keratinocytes using polyethylene glycol. Non-muscle cells were resuspended in DM, counted, and within each experiment, equal numbers of non-muscle cells were plated in control

and experimental dishes. Non-muscle cells were plated onto 2-day myotube cultures. Four hours later, cultures were treated with PEG 1500 (50% w/v in HEPES) (Roche) for 60 seconds at 37°C, followed by three successive washes with DMEM. DM was added and replaced at 4-6 hours after PEG treatment along with addition of ara-c and ouabain (10^{-5} M).

Immunofluorescence

Heterokaryons were stained with the mouse monoclonal antibody, 5.1H11 (Developmental Studies Hybridoma Bank, University of Iowa). Cells were fixed in 1.5% formaldehyde in PBS for 10-15 minutes at room temperature, washed three times in PBS and exposed to the antibody undiluted in mouse ascites fluid for 60 minutes at room temperature. After washing three times in PBS, cells were incubated with the secondary antibody, Alexa Fluor 488 or Alexa Fluor 547 goat anti-mouse (Molecular Probes) at 1:500 in DM for 60 minutes at room temperature. Cells were washed with PBS three times and permeabilized with ice-cold methanol for 20 minutes at room temperature, washed three times in PBS and stained with Hoechst 33258 (0.12 μM) in PBS for 15 minutes, followed by four washes in PBS.

For MyoD staining, HepG2 cells were fixed as above, washed, and permeabilized in Triton X-100 for 10 minutes at room temperature. Blocking was with 20% fetal calf serum in PBS, followed by incubation with the antibody mouse anti-MyoD (PharMingen, Cat. no. 554130) diluted in blocking buffer for 45 minutes at room temperature. Cells were rinsed three times in PBS and then incubated with secondary antibody, Alexa Fluor 488 goat anti-mouse (Molecular Probes) at 1:500 dilution for 45 minutes, rinsed, stained with Hoechst 33258 and processed as above. Cells were visualized using a Zeiss Axioplan 2 fluorescence microscope equipped with a ×40 water immersion objective. Images were acquired using OpenLab software.

Analysis of gene expression

RNA was prepared from cells using TRIzol reagent (Invitrogen) and the RNeasy mini kit (Qiagen). 1 μg total RNA was reverse transcribed using oligo dT primers and the First Strand cDNA synthesis kit (Roche). 2 μl cDNA was amplified with gene-specific primers using Invitrogen PCR supermix. For Myf-5 gene analysis, RNA was first treated with DNase (Invitrogen). RT-PCR was performed on 200 ng RNA using SuperScript III (Invitrogen). Primer sequences: β-actin top, 5′-TTTGAGAC-CTTCAACACCCCAGCC-3′, bottom, 5′-AATGTCACGCACGATTTCCCGC-3′; Myf-5 top, 5′-ATGCACAGATAAAAACTCCTT-3′, bottom, 5′-AATCAATGGCC-CCCTATCAGAAA-3′; MyoD top, 5′-AGCACTACAGCGGCGACT-3′, bottom, 5′-GCGACTCAGAAGGCACGTC-3′; Myogenin top, 5′-GCGGGCGGCCACACTGA-3′, bottom, 5′-GGGGGCTCGCAAGGATG-3′; Mrf-4 top, 5′-TTCGATGC-CTTTCTTCCATC-3′, bottom, 5′-CATTAAATGCAACTTTCCCACT-3′; creatine kinase top, 5′-AAGAAGCTGCGGGACAAGGAGACT-3′, bottom, 5′-AG-GCCAGCAGCAGCGGGGACAC-3′; GAPDH top, 5′-GCCCCAGCAAGAGCA-CAAGAGG-3′, bottom, 5′-ACAAGGTGCGGCTCCCTA-3′.

Chromatin immunoprecipitation

1×10^6 HepG2 cells were treated with TSA followed by chromatin immunoprecipitation according to the manufacturer's protocol (Upstate) with modifications. Sonication was at 70% power for 10 pulses of 5 seconds each; pre-cleaning was increased to 1 hour; antibody incubation time was 2 hours; bead wash was performed twice and DNA was recovered using QIAquik columns (Qiagen). Samples were eluted in 40 μl, and 5 μl was used for PCR. The primer sequences used for amplification of the MyoD promoter were: forward, 5′-CCTGGGCTCCGGGGCGTTTAG-3′, reverse, 5′-GCGGAGCGGTGGCGACAGTAGC-3′. Antibodies used were normal Rabbit IgG (Upstate), anti-dimethyl-Histone H3 (Lys9) (Upstate), and anti-acetyl-Histone H3 (Lys9) (Upstate). PCR products were analyzed on a 2% agarose gel.

Knockdown of histone deacetylase 1

shRNA constructs targeting HDAC 1 were generated by cloning targeting and loop sequences into the pSuper (OligoEngine) vector. Five different constructs were tested by transient transfection (Fugene) in HEK293 cells and RT-PCR assay for *HDAC1* knockdown. Two constructs that gave >70% knockdown were selected. ΦA cells (Gary Nolan, Stanford University, Palo Alto, CA) were then used to produce amphotropic retrovirus expressing *HDAC1* shRNA or control shRNA (GFP). HepG2 cells were infected with retrovirus every 12 hours for 4 rounds, followed by selection in puromycin for 24 hours and then seeded onto myotube cultures for heterokaryon formation. The sequences used for the *HDAC1* shRNA were 21 top, 5′-GATCCCCGCAGCAGACGGACATCGCTTTCAAGAGAAGCGATGTCCGTCT-GCTGCTTTTTA-3′, bottom, 5′-AGCTTAAAAAGCAGCAGACGGACATCGCTT-CTCTTGAAAGCGATGTCCGTCTGCTGCGGG-3′; 24 top, 5′-GATCCCCGAC-GACCCTGACAAGCGCATTCAAGAGATGCGCTTGTCAGGGTCGTCTTTTTA-3′, bottom, 5′-AGCTTAAAAAGACGACCCTGACAAGCGCATCTCTTGAAT-GCGCTTGTCAGGGTCGTCGGG-3′. The sequences used for HDAC1 RT-PCR were top, 5′-GGGCTGGCAAAGGCAAGTATTATG-3′ and bottom, 5′-AACGGGAAGAATGGGTGGCAAG-3′.

Microarray analysis

Gene-specific 70-mer 3′-biased oligonucleotides were designed using OligoDesign software for human specificity. These 70-mers were subjected to a BLAST (Basic Local Alignment Search Tool) search against the mouse transcripts to enhance species specificity and to exclude cross hybridization with other sequences, and were synthesized by Operon. Lyophilized probes were dissolved in 3× SSC to 40 μM in preparation for spotting on Scott amino-silane glass slides by the Stanford microarray facility (www.microarray.org). The quality of the array was tested by first examining the printing efficiency using tracking dye and then by control hybridization.

Preparation of labeled cDNA, hybridization and scanning

Total RNA was isolated from samples grown on 60 mm plates using the RNeasy kit (Qiagen). Probes for hybridization were generated according to the Amersham Direct labeling kit. 20 μg total RNA was used to label the cDNA using Cy5-UTP for the samples and Cy3-UTP for human reference RNA. The human reference RNA was pooled from human cell lines. Cy3- and Cy5-labeled cDNAs were combined and co-purified using Qiaquik Mini-Elute columns (Qiagen). Prior to hybridization, the arrays were UV crosslinked using 6000 mJ, and incubated in prehybridization buffer (0.1 mg/ml BSA, 0.1% SDS, 5× SSC, 25% formamide) for 30 minutes at 42°C. Arrays were then washed for 5 minutes in 0.01× SSC buffer twice, followed by a 2-minute wash with distilled water and dried for 5 minutes at 600 r.p.m. in a centrifuge. Labeled cDNA was added to the hybridization buffer (6× SSC, 0.2% SDS, 0.4 μg/μl yeast tRNA, 0.4 μg/μl Poly-A, human Cot1 DNA and mouse Cot1 DNA), denatured for 5 minutes at 95°C, and applied to the arrays using a coverslip (LifterSlip, Erie Scientific). Arrays were placed in the hybridization chambers in a 42°C water bath for 20 hours. Arrays were then washed successively with: wash 1, 2× SSC, 0.1% SDS at 42°C; wash 2, 2.4 M tetraethylammonium Chloride 98% (Sigma) at 58°C twice for stringency to achieve species specificity; wash 3, cold 0.02× SSC for 3 minutes at 4°C, and were dried. Arrays were scanned using an Axon 400B scanner. Image processing and quantification of signal values of the spotted arrays were performed using Genepix 3.0 software (Axon Instruments). The proportion of human RNA in each sample was quantified using human GAPDH, and normalized amounts of RNA were used for labeling the samples. The data were normalized by applying variance stabilization (Huber et al., 2002) using the vsn package of bioconductor (http://www.bioconductor.org). To compare heterokaryons with the control HepG2 pre-fusion, we performed the non-parametric Rank Product test (http://cran.R-project.org).

Q-PCR

Quantitative RT-PCR was performed for five genes that were differentially regulated according to the microarray. Human-specific primers for eight genes encoding creatine kinase, cardiac α-actin, desmin, myosin heavy chain 2, TNNC1, troponin 1, ID1 and ID2 were designed. QRT-PCR was performed using the platinum SYBR Green qPCR Kit (Invitrogen). The mRNA transcript levels were normalized to three housekeeping genes GAPDH, PMS and HRPT. Each sample was run in triplicate. QPCR-primers: desmin F, 5′-GAGGTCCATACCAAGAAGACG-3′, R, 5′-GTGTCCTGGGATG-GAAGAA-3′; ID1 F, 5′-CGGATCTGAGGGAGAACAAG-3′, R, 5′-TGAGAAG-CACCAAACGTGA-3′; ID2 F, 5′-AAATGCCCTTTCTGCAGTTG-3′, R, 5′-GACT-GAATACTGGATCCTTCTGGTA-3′; TNNC1 F, 5′-ACCAAGGAGGAGCTGGAC-3′, R, 5′-GTCGCCGTCTTTCATCAGA-3′; TNNT1 F, 5′-AGCTCGTTGC-CTTGAAGGAG-3′, R, 5′-AATGTCCAGAGGCTTCTTACG-3′; MHC2 F, 5′-TGC-TAGTAATGTAGAAACGGTCTCC-3′, R, 5′-CTCTGCGCAGTCAGGTCA-3′; creatine kinase F, 5′-CCCACCAGAGTCCTGCTC-3′, R, 5′-GATATTTCATTG-GCCAGAATCC-3′; cardiac α-actin F, 5′-TGAACGTGAAATTGTCCGTGA-3′, R, 5′-GCCAGCAGATTCCATACCAATGA-3′; GAPDH F, 5′-TGTCCCCACTGC-CAACGTGTCA-3′, R, 5′-AGCGTCAAAGGTGGAGGAGTGGGT-3′.

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