Acquisition and extinction of gene expression programs are separable events in heterokaryon reprogramming

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Summary

Although differentiated cells normally retain cell-type-specific gene expression patterns throughout their lifetime, cell identity can sometimes be modified or reversed in vivo by transdifferentiation, or experimentally through cell fusion or by nuclear transfer. To examine the epigenetic changes that are required for the dominant conversion of lymphocytes to muscle, we generated heterokaryons between human B lymphocytes and mouse C2C12 myotubes. We show that within 2 days of heterokaryon formation lymphocyte nuclei adopt an architecture resembling that of muscle and then initiate the expression of muscle-specific genes in the same temporal order as developing muscle. The establishment of this muscle-specific program is coordinated with the shutdown of several lymphocyte-associated genes. Interestingly, erasing lymphocyte identity in reprogrammed cells requires histone deacetylase (HDAC) activity. Inhibition of HDAC activity during reprogramming selectively blocks the silencing of lymphocyte-specific genes but does not prevent the establishment of muscle-specific gene expression. Successful reprogramming is therefore shown to be a multi-step process in which the acquisition and extinction of lineage-specific gene programs are separable events.

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Key words: Reprogramming, Lymphocytes, Interspecies heterokaryons, Gene silencing

Introduction

The theory of nuclear equivalence – that specialised cells of metazoans possess a gene pool identical to that of the zygote nucleus – has been experimentally examined and debated for over a century (Spemann, 1938; Weismann, 1982). Demonstrations of somatic cell reprogramming, particularly through nuclear transfer and in heterokaryons, have shown that many types of differentiated cells retain flexible lineage potential (Baron and Maniatis, 1986; Blau et al., 1983; Gurdon, 1962; Hochedlinger and Jaenisch, 2002; Wilmut et al., 1997; reviewed by DiBerardino, 1988; reviewed by Surani, 2001). In addition, the constitutive expression of specific transcription factors by certain cell types can sometimes modify or override lineage outcome. For example, expression of individual muscle regulatory factors (MRF) of the MyoD family has been shown to convert a range of non-muscle cell types into muscle (Aurade et al., 1994; Choi et al., 1990; Davis et al., 1987; Weintraub et al., 1989). Similarly, high levels of the transcription factors C/EBPα and C/EBPβ can reprogramme committed mature B-lymphocytes to become macrophages (Xie et al., 2004).

In parallel with these demonstrations of dominant conversion, other studies have revealed that additional factors are necessary to maintain cell identity. These include trithorax and polycomb group proteins, required to retain early developmental decisions (reviewed by Orlando, 2003), and other chromatin modifiers such as MEP-1 and Mi-2 that are essential for maintaining soma-germline distinctions (Unhavaithaya et al., 2002). Collectively, these studies have suggested that differentiated cells require continuous and active regulation to maintain their identity (Blau and Baltimore, 1991). Whereas cell-type-specific gene expression programs are established through a network of transcriptional activators and repressors, epigenetic factors might also be required to maintain specification, perhaps by stabilising chromatin domains (Fisher, 2002; Orlando, 2003; Shin and Mello, 2003). Chromatin remodelling factors, including histone deacetylases (HDAC), are required for 'resetting' gene expression, for example by overriding germline-specific states in *C. elegans* (Shin and Mello, 2003), experimental reprogramming through nuclear transfer or using *Xenopus* egg extracts (Hansis et al., 2004; Kikyo et al., 2000; Rideout et al., 2001), and in the normal development of mammalian oocytes (Arney et al., 2002; Santos et al., 2002).

Later in ontogeny, cellular differentiation is characterised by the coordinated expression and extinction of genes at specific times. In developing lymphocytes, the regulated expression and silencing of the terminal deoxynucleotidyl transferase (*TdT*) locus, *Dntt*, provides a particularly well-studied example (Ernst et al., 1999). *Dntt* is expressed by immature lymphocytes but

is transcriptionally silenced in response to signalling through the T- or B-cell receptor. A temporal analysis of the chromatin modifications that accompany stable silencing showed that silencing is nucleated at the promoter by the ordered deacetylation of histone H3 Lys9, loss of methylation at H3 Lys4 and methylation at H3 Lys9. This was followed by repositioning of the *Dntt* locus to pericentric heterochromatin (Brown et al., 1999) and the bi-directional spreading of repressive histone modifications (Su et al., 2004). The importance of histone deacetylation for initiating the assembly of silent chromatin is underscored by a requirement for Ikaros, a DNA binding factor that is essential for lymphocyte development, that interacts with the HDAC-containing nucleosome remodelling NuRD complex (Koipally et al., 1999) and is required for the appropriate stage-specific silencing of many lymphocyte-associated genes (Sabbattini et al., 2001; Su et al., 2004).

In an experimental context, lymphocytes can be successfully reprogrammed to express early developmental markers such as *Oct4* when injected into *Xenopus* oocytes (Byrne et al., 2003) and occasionally upon transfer into mouse ooctyes (Hochedlinger and Jaenisch, 2002; Rideout et al., 2001). Dominant reprogramming of lymphocytes also occurs in cell hybrids generated with embryonic stem cells or germ cells (Tada et al., 1997; Tada et al., 2001). Despite this, we do not yet understand how genome function is epigenetically reset during reprogramming. In this study, we examined the dominant conversion of human lymphocytes to muscle in experimental heterokaryons and investigated the role of HDAC activity in this process. We show that heterokaryon formation results in a rapid increase in nuclear volume and the redistribution of constitutive heterochromatin within lymphocyte-derived nuclei. This is followed over 2-8 days by the de novo expression of human muscle genes in a temporal order that accurately recapitulates gene expression in normal developing muscle. Activation of muscle-specific genes in heterokaryons was associated with declining expression of several lymphocyte-specific genes including *CD45*, *PAX5*, *CD20* and *CD37*. Treatment with HDAC inhibitors selectively blocked lymphocyte gene extinction, a result that suggests that, although gene activation and silencing are coordinated events in reprogramming, they are mechanistically distinct.

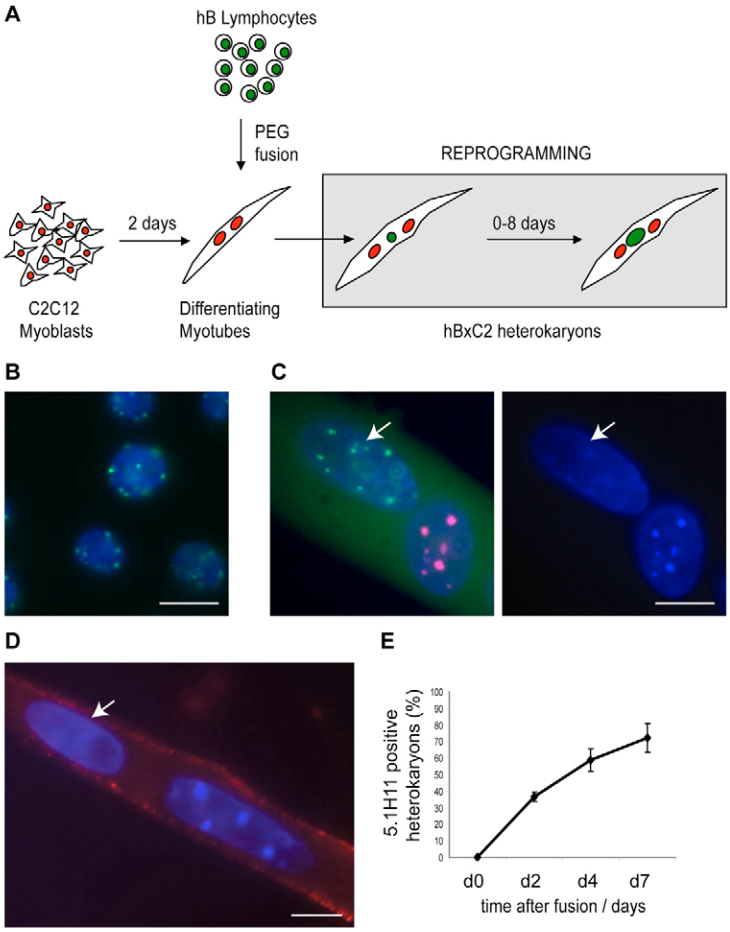

Fig. 1. Reprogramming human B-lymphocytes in mouse C2C12 heterokaryons. (A) Protocol used to generate interspecies (hB×C2) heterokaryons. Mouse C2C12 myoblasts were differentiated into myotubes and fused with human B lymphocytes (hB). Mouse and human nuclei were distinguished by FISH with probes specific for mouse γ-satellite DNA (red) or human α-satellite DNA (green), or by DAPI staining (blue). (B) Confocal image of hB nuclei before fusion. (C) Confocal images of a myotube containing a human (arrow) and a mouse nucleus. (D) Confocal section of a reprogrammed heterokaryon identified by hNCAM expression (5.1H11 antibody, red). One mouse (DAPI intense foci) and one human (arrows) nucleus are seen. Bars, 10 μm. (E) Expression-kinetics of human NCAM (detected by 5.1H11 antibody) by lymphocyte-derived nuclei (mean ± s.d., n=3, 50 nuclei per experiment).

Results

Increased nuclear volume and redistribution of constitutive heterochromatin are early events in the reprogramming of lymphocytes to muscle

The potential of human B lymphocytes to convert to muscle was examined by generating stable heterokaryons by using polyethylene glycol (PEG)-mediated fusion with mouse C2C12 myotubes (Blau et al., 1983; Chiu and Blau, 1984), as illustrated in Fig. 1A. Human and mouse nuclei within the resulting myotubes were distinguished by fluorescent in situ hybridisation (FISH) using differentially labelled probes that selectively recognise human γ-satellite or mouse α-satellite DNA. α-satellite DNA (green) was present in the nuclei of human B cells before (Fig. 1B) and after fusion with mouse myotubes (Fig. 1C) and allowed them to be readily discerned from mouse C2C12 nuclei (labelled red with γ-satellite probe). Human lymphocyte-derived nuclei were also identified by counter-staining with DAPI (Fig. 1C right panel, arrow), which highlights AT-rich regions that surround mouse centromeres and generates punctuate labelling that is selective for mouse nuclei. Reprogramming efficiency in interspecies heterokaryons was judged by expression of the human muscle marker NCAM, detected using a monoclonal antibody 5.1H11 (Fig. 1D, labelled in red) that recognises human but not mouse NCAM (Chiu and Blau, 1984). Using this antibody, 37% of heterokaryons expressed human NCAM 2 days after fusion, 59% after 4 days and 72% after 7 days (Fig. 1E), consistent with previously reported estimates of the reprogramming of

mesoderm-derived cells by C2C12 myotubes (Blau et al., 1985).

Dramatic and rapid changes in the size and architecture of human lymphocyte-derived nuclei were observed upon heterokaryon formation (Fig. 2). The volume of human B cell nuclei increased from approximately 600 μm^3 prior to fusion to 1500 μm^3 and 1800 μm^3 2 and 4 days after heterokaryon formation, respectively (Fig. 2A,B). The distribution of constitutive heterochromatin within these nuclei was also altered. Prior to fusion, human centromeres were clustered to form five to eight chromocentres per nucleus detected by Calcinosis, Raynaud's, Esophagus, Sclerodactyly and Telangiectasia antisera (CREST) labelling (Fig. 2C, green) (Alcobia et al., 2000; Brown et al., 2001; Weierich et al., 2003). Two days after heterokaryon formation, CREST signals were more compact, consistent with a reduced number of spatially discrete signals (supplementary material Fig. S1), and the number of chromocentres per nucleus was markedly reduced (Fig. 2C,D). This reorganisation mimicked the spatial distribution of constitutive heterochromatin seen in neighbouring C2C12 nuclei (supplementary material Fig. S1). Changes in nuclear size and architecture were evident before the expression of hNCAM, suggesting that nuclear reorganisation precedes human muscle gene expression (Fig. 2D). The rapid timing of the response implicates transacting factors derived from mouse myotubes in the nuclear remodelling of lymphocytes. Consistent with this possibility, myogenin protein was detected in many human nuclei as early as 24 hours after heterokaryon formation (Fig. 2E), several days before endogenous human myogenin transcripts were expressed (Fig. 3A).

Reprogramming initiates a temporally ordered activation of human myogenic regulatory factors by lymphocyte-derived nuclei in C2C12 heterokaryons

To determine the kinetics of gene activation and silencing induced by heterokaryon formation and reprogramming of human lymphocytes, we analysed the expression of a panel of human muscle- and B cell-associated genes using reverse transcriptase (RT)-PCR and primer combinations that were specific for human transcripts. This showed that muscle gene expression was efficiently initiated in human B cell nuclei 2 days after heterokaryon formation (Fig. 3A). Interestingly, human myogenic regulatory factor (MRF) genes were expressed in a sequence that accurately reflects their normal temporal order (Pownall et al., 2002), so that h*MYF5* was transiently expressed at days 2-3, followed by h*MYOG* (day 3 onwards) and h*MRF4* later (day 4 onwards). The establishment of this new gene program by human B-cell-derived nuclei was accompanied by declining expression of the B-cell-specific regulator h*PAX5* and the leukocyte-associated gene h*CD45*. Expression of human *GAPDH*, a ubiquitously expressed gene, remained constant throughout these analyses.

HDAC activity is required for the silencing of lineage inappropriate genes during reprogramming

To assess the importance of HDAC activity in the dominant reprogramming of lymphocytes to muscle, we treated

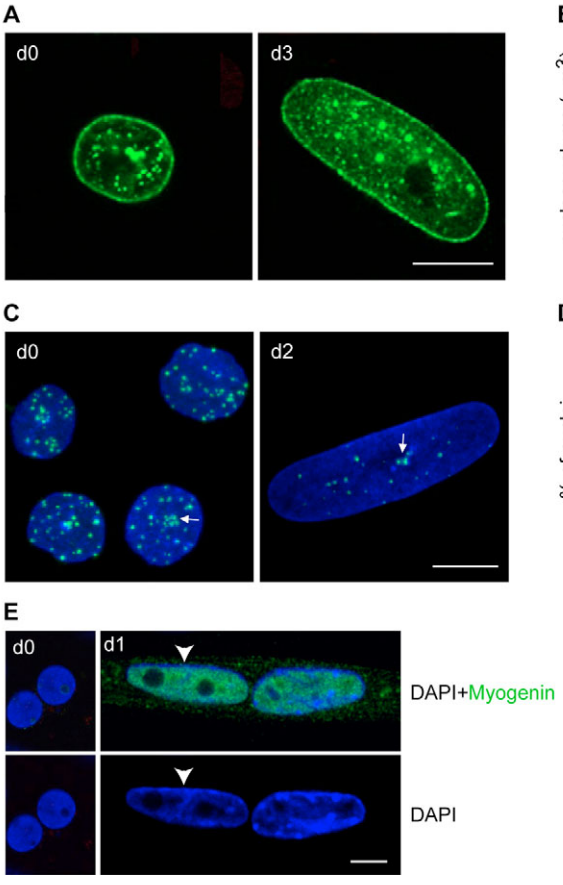

Fig. 2. Nuclear reorganisation is an early event in lymphocyte reprogramming. (A) Single optical section showing lamin A/C immunofluorescence-labelling of human B lymphocyte nuclei (hB) before day 0 (d0) and three days (d3) after fusion with C2C12 myotubes. (B) Volume of hB nuclei before (d0), and 2 and 4 days after heterokaryon formation (d2 and d4, respectively) compared with C2C12 myotubes 2 and 4 days after serum withdrawal and differentiation (d2 and d4, respectively). Nuclear volume was estimated as described in Materials and Methods (mean ± s.d., n=3 with 50 nuclei per experiment). (C) CREST antisera (green) reveal the centromere distribution in hB nuclei (d0) and two days after heterokaryon formation (d2), where individual chromocentres are indicated (arrowed). Confocal images are maximal projection of multiple optical z-sections. (D) Distribution of constitutive heterochromatin in hB nuclei before (d0, open bars) and 2 days after fusion in hNCAM expressing (d2, black bars) and hNCAM-negative (not yet reprogrammed) heterokaryons (grey bars) was compared by assessing the number of discrete CREST signals and number of chromocentres per nucleus (n=100). (E) Myogenin protein (green) in a human nucleus (arrowheads) 1 day after fusion. Images show single optical sections. Bars, 10 μm.

heterokaryons with the HDAC inhibitor TSA. Application of low doses of TSA to differentiating C2C12 or to primary cultures of mouse muscle has been shown to increased histone H3 Lys9 and histone H4 Lys20 trimethylation at pericentric

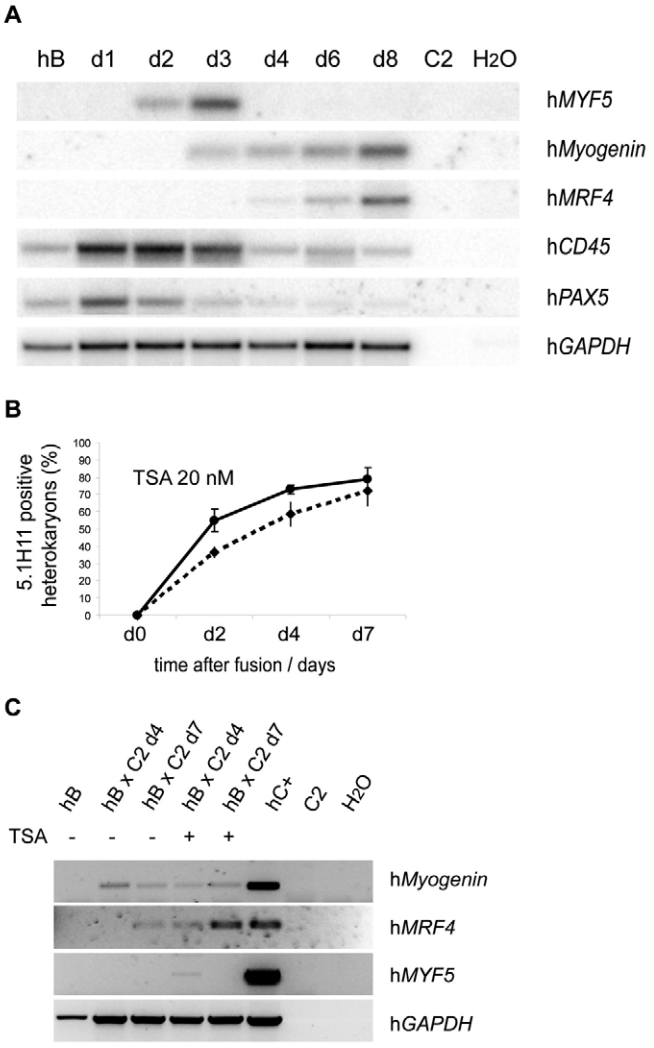

Fig. 3. Ordered expression of human myogenic genes by lymphocyte-derived nuclei. (A) Expression of human muscle-specific (h*MYF5*, h*MYOG*, h*MRF4*) and human lymphocyte-specific (h*CD45*, h*PAX5*) genes detected by RT-PCR. Prior to fusion, human B cells expressed h*GAPDH*, h*CD45* and h*PAX5*, but not muscle-specific genes. Following heterokaryon formation, human *MYF5*, *MYOG* and *MRF4* expression was initiated and the expression of human lymphocyte-specific genes *CD45* and *PAX5* declined. Mouse C2C12 samples (C2) were used as negative controls to confirm the specificity of primers to human transcripts and h*GAPDH* was used to standardise input. (B) Expression kinetics of human NCAM in hBxC2 heterokaryons in the presence (solid line) or absence (broken line) of TSA (20 nM). For each time point, 100 heterokaryons were analysed in two separate experiments and values shown are the mean ± s.d. (C) h*MYOG*, h*MRF4*, h*MYF5* and h*GAPDH* gene expression in human B lymphocytes (hB) and in day 4 (d4) and day 7 (d7) hBxC2 heterokaryons cultured in the presence (+) or absence (–) of 20 nM TSA. As positive controls for PCR analysis (hC+) RNA was isolated from differentiating human muscle. Mouse C2C12 cells (C2) were used as negative control. h*GAPDH* was used to standardise input.

DNA and prevent centromeric clustering (Terranova et al., 2005), suggesting a role for HDACs in the formation of repressive heterochromatin environments during terminal differentiation. To assess their importance during lineage conversion, heterokaryons established between human B lymphocytes and C2C12 cells were treated with low doses of trichostatin A (TSA, 20 nM). This did not affect the success of heterokaryon formation or the proportion of 5.1H11-positive reprogrammed nuclei 7 days after fusion (79±6.7%, compare with 72±8.7% in absence of TSA), although a slightly enhanced induction of human NCAM expression was seen in the presence of TSA (Fig. 3B). A comparison of *MYF5*, *MYOG* and *MRF4* expression in heterokaryons that were treated with TSA or not (Fig. 3C) confirmed that acquisition of muscle gene expression by human lymphocyte-derived nuclei was not significantly compromised. *MYOG* expression was detected at days 4 and 7, *MRF4* was most abundant at later stages, whereas *MYF5* was only detected early after heterokaryon formation (days 2-3). TSA treatment resulted in a slight advance in the timing of h*MRF4* expression, a result that is consistent with a global increase in histone acetylation (Fig. 4D) and chromatin accessibility induced by TSA and a previous report that showed precocious expression of m*MRF4* in primary mouse-muscle treated with HDAC inhibitors (Terranova et al., 2005). TSA did, however, have a dramatic effect on the temporal extinction of human lymphocyte gene expression in interspecies heterokaryons (Fig. 4A). Whereas expression of h*CD45*, h*PAX5*, h*CD20* and h*CD37* was extinguished (or significantly diminished in the case of h*CD45*) in heterokaryons by day 7, sustained expression of these genes was evident in TSA-treated heterokaryons (highlighted in green, compare left and right panels, Fig. 4A). Human *GAPDH,* a gene that is expressed in all human tissues, was detected throughout in both TSA-treated and untreated cultures, and is shown for comparison.

To evaluate further the selectivity of the TSA response, we analysed the expression of other human genes including hPAX6, h*REX1*, h*TERT*, h*NANOG* and h*OCT4*. Most genes were not significantly upregulated in heterokaryons upon TSA treatment (Fig. 4B). Similarly, TSA-treated mouse myoblasts and mouse myotubes did not inappropriately express most lymphocyte-associated and neural-associated genes (Fig. 4C), although a minority (two of 11) showed slight upregulation. This result is consistent with estimates that approximately 10% of genes are sensitive to HDAC inhibition in myoblasts (Iezzi et al., 2004). The selectivity of this response is also supported by the finding that h*PAX5* extinction in heterokaryons was not associated with a decline in the expression of *PAX5* upstream positive regulators (h*E2A* and h*EBF*) and that h*PAX5* (but not h*E2A* or h*EBF*), was sensitive to TSA treatment (supplementary material Fig. S2).

Inhibition of HDAC activity results in the coexpression of h*CD20* and h*NCAM* in individual reprogrammed nuclei

The observation that TSA prevents the silencing of lymphocyte-associated genes but allows the acquisition of muscle gene expression predicts that TSA treated heterokaryons would contain nuclei that simultaneously express lymphocyte and muscle-associated genes. To verify this at the level of individual nuclei, RNA-FISH analysis was performed to detect human muscle- (*hNCAM*-biotin) and lymphocyte- (*hCD20-DIG*) specific transcripts following TSA

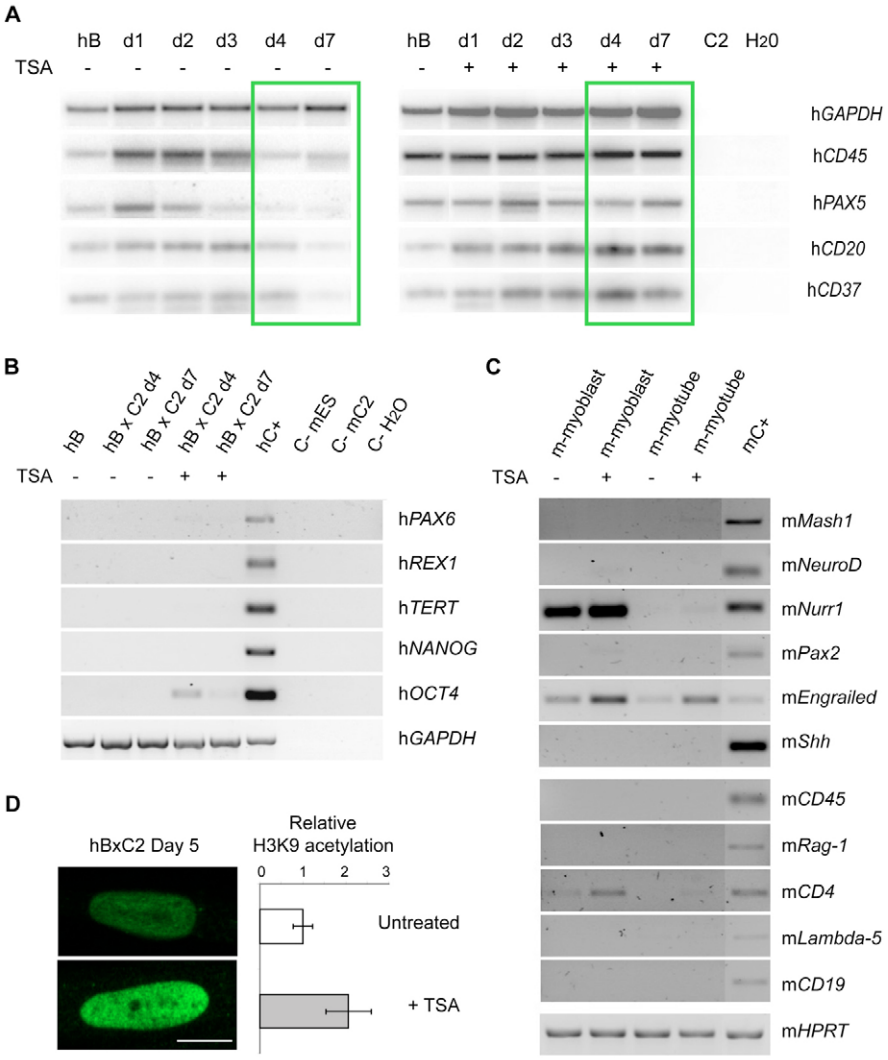

Fig. 4. HDAC inhibition prevents the shutdown of lymphocyte-specific genes during reprogramming. (A) Impact of 20 nM TSA treatment on the expression of h*CD45*, h*PAX5*, h*CD20* and h*CD37* in interspecies hB×C2 heterokaryons. Green boxes highlight that lymphocyte-associated genes are not extinguished following TSA treatment. (B) Expression of h*PAX6*, h*REX1*, h*TERT*, h*NANOG* and h*OCT4* in human B lymphocytes (hB) and in day 4 (d4) and day 7 (d7) hB×C2 heterokaryons cultured in the presence (+) or absence (–) of 20 nM TSA. As positive controls for PCR analysis (hC+), RNA was isolated from human embryonic stem cells (for h*OCT4*, h*NANOG*, h*REX1*, h*TERT*) and human neuronal progenitor cells (for h*PAX6*), and h*GAPDH* was used to standardise input. Mouse ES cells (C- mES) and mouse C2C12 cells (C- mC2), used as negative controls to confirm the specificity of primers to human transcripts, are shown on the right. (C) RT-PCR analysis of mouse transcripts for m*Mash1*, m*NeuroD*, m*Nurr1*, m*Pax2*, m*Engrailed1*, m*Shh* (neural-associated) and m*CD45*, m*Rag1*, m*CD4*, m*Lambda-5*, m*CD19* (lymphocyte-associated) in mouse myoblasts and myotubes cultured in absence or in presence of 20 nM TSA. Positive controls in this analysis include RNA isolated from mouse brain and foetal liver (mC+); m*HPRT* was used to standardise input. The sequence of human- or mouse-specific pairs of primers used for PCR amplification is available upon request. (D) Increased levels of histone H3 Lys9 acetylation (H3K9ac) in human nuclei in response to treatment of heterokaryons with 20 nM TSA (+). Confocal microscope settings and laser power were kept constant so that the relative abundance of H3K9 acetylation could be directly compared (and quantified, right hand panel) to untreated control (mean ± s.d., 50 nuclei per experiment). Bar, 10 μm.

treatment. The specificity of probes for *hCD20* and *hNCAM* transcripts was verified by the detection of two RNAse-sensitive *hCD20* signals (green) in human B lymphocyte nuclei and two *hNCAM* signals (red) exclusively in primary human myoblasts (Fig. 5A and B, respectively). Using these probes, RNA-FISH analysis was used to score nuclei expressing the muscle gene *hNCAM* alone, the lymphoid gene *hCD20* alone, or coexpressing both *hNCAM* and *hCD20* within heterokaryons at day 5 (illustrated in Fig. 5C, values shown in Fig. 5D and supplementary material Table 1). In untreated heterokaryons, most nuclei (75%) in which human RNA signals were found expressed *hNCAM* and only a minority (32%) expressed *hCD20*. Nuclei in which both transcripts were detected simultaneously were rare (7%). Addition of TSA or the structurally unrelated HDAC inhibitor valproic acid (VPA) did not affect muscle-specific *hNCAM* signals (red histograms) but had a dramatic effect on the proportion of lymphocyte-derived nuclei that retained *hCD20* expression (green histograms, Fig. 5D). Consequently, and in contrast to untreated cultures, many lymphocyte-derived nuclei within TSA- or VPA-treated heterokaryons coexpressed both *hCD20* and *hNCAM* (28 and 29%, respectively, Fig. 5D). These results confirm the importance of HDAC activity for silencing lymphocyte-specific genes during reprogramming.

Discussion

Previous studies have shown that lymphocytes can be reprogrammed by injection into *Xenopus* (Byrne et al., 2003) or mouse oocytes (Hochedlinger and Jaenisch, 2002) or by generating cell hybrids with embryonic stem cells or germ cells (Tada et al., 1997; Tada et al., 2001). In this study, we show that the dominant conversion of human lymphocytes to muscle is a multi-step process that includes changes in nuclear size, architecture and gene activity. In muscle heterokaryons, where chromosome replication is precluded, these steps can be distinguished on the basis of their timing and a requirement for HDAC activity. Within the first 48 hours of heterokaryon formation, lymphocyte nuclei increase in size and heterochromatin domains are redistributed to mimic the spatial arrangement of neighbouring mouse myocytes.

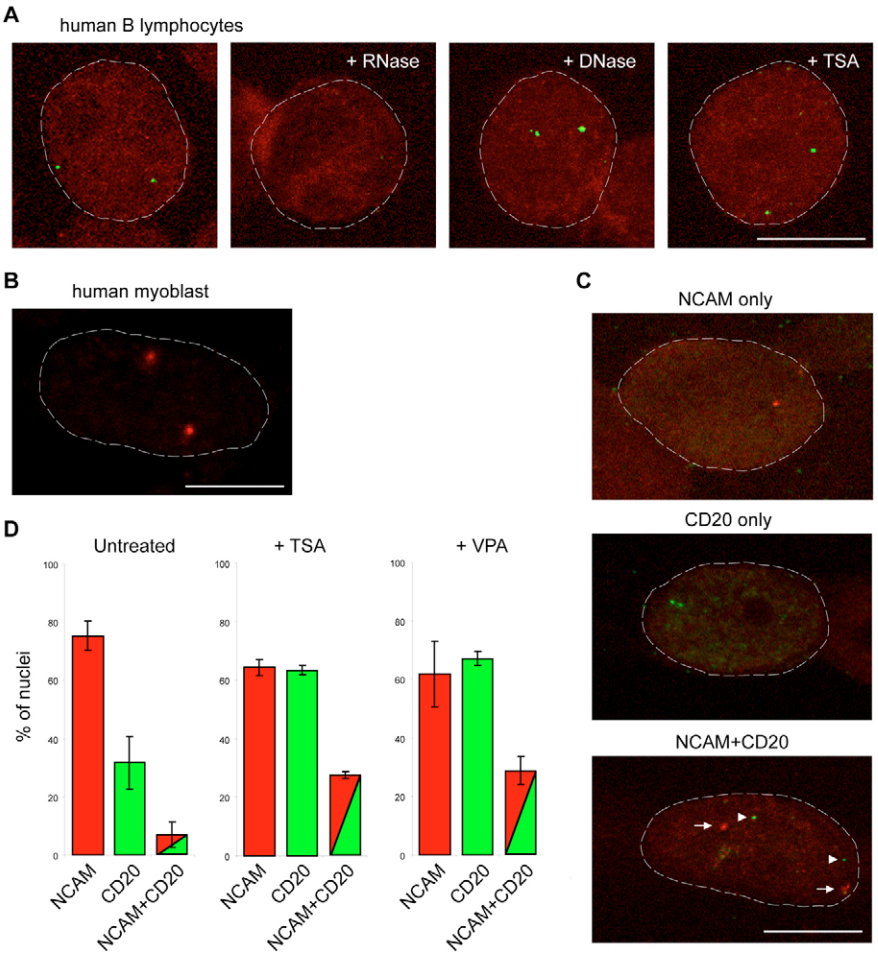

Fig. 5. Coexpression of lymphocyte- and muscle-specific genes in individual nuclei treated with HDAC inhibitors TSA and VPA. (A) Human B lymphocytes were analysed for h*CD20* (green) and h*NCAM* (red) transcripts using RNA FISH. Two foci of h*CD20* signal are evident in hB cell nuclei that were removed by pre-treatment of samples with RNAse A (+RNase), but not DNAse (+DNAse). Application of 20 nM TSA did not impair h*CD20* signal detection (+TSA). (B) Human *NCAM* transcripts (red) were detected in human myoblast nuclei but not in lymphocytes. (C) RNA FISH showing examples of nuclei that express h*CD20* transcripts alone (green) and h*NCAM* transcripts alone (red) or both in lymphocyte-derived nuclei within day 5 hB×C2 heterokaryons. Confocal images are maximal projection of multiple optical *z*-sections (Bars, 10 μm). (D) the proportion of nuclei expressing h*NCAM* (red), h*CD20* (green), or both h*NCAM* and h*CD20* (red/green) signals in heterokaryons cultured in the absence (untreated) or presence of 20 nM TSA or 1 mM VPA is expressed as a proportion of human nuclei in which RNA transcripts were detected (see supplementary material Table S1).

Importantly, this reorganisation precedes the activation of endogenous human muscle-specific genes. It is possible that these changes in nuclear architecture are the result of physical constraints or dominant factors within myotubes that impose a muscle-specific organisation. However, because cycling and non-cycling lymphocytes display different constitutive heterochromatin organisation (Brown et al., 1999; Solovei et al., 2004) and cell-cycle withdrawal is a characteristic feature of myogenic differentiation (Ait-Si-Ali et al., 2004; Shen et al., 2003; Walsh and Perlman, 1997), it is also possible that nuclear reorganisation is a consequence of cell-cycle arrest. Previous studies have shown that fusion of nucleated chicken erythrocytes with rat myoblasts results in pronounced nuclear enlargement and chromatin redistribution prior to gene reprogramming (Dupuy-Coin et al., 1976) and similar effects have been reported for mouse lymphocytes injected into *Xenopus* oocytes (Byrne et al., 2003) and bone marrow cells that form stable heterokaryons with Purkinje neurons (Weimann et al., 2003). More recently, chromatin redistribution in developing muscle was shown to be dependent on the methyl-CpG-binding proteins MeCP2 and MBD2 (Brero et al., 2005). Taken together, these results suggest that myogenic regulators and chromatin remodelling machinery both have important roles in conveying dominant cell-type-specific nuclear organisation during reprogramming.

Our results show that de novo expression of an endogenous muscle gene program by lymphocyte-derived nuclei begins 2 days after heterokaryon formation and coincides with the reduced expression of several lymphoid-associated genes (h*CD45*, h*PAX5*, h*CD20*, h*CD37*). Extinction of lymphocyte-specific gene expression begins after 2-3 days and continues over a 7-day period with similar kinetics to the reported shut-down of human albumin expression during hepatocyte reprogramming to muscle (Miller et al., 1988). Remarkably, we show that inhibition of HDAC activity by low doses of TSA or VPA prevents the silencing of lineage-inappropriate genes without affecting ongoing conversion to muscle. This shows that the establishment of muscle gene expression and the extinction of lymphocyte identity are distinct components of lineage conversion. Remarkably, by inhibiting HDAC activity in experimental heterokaryons nuclei are seen to coexpress two different lineage-associated gene programs. This underscores the importance of gene silencing for successful reprogramming and suggests that in future it might be possible to preserve certain characteristics of donor cells through lineage conversion, by restricting the availability of HDACs.

Materials and Methods

Cell culture

C2C12 cells (Blau et al., 1983; Yaffe and Saxel, 1977) were maintained as undifferentiated myoblasts in growth medium (GM); Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% foetal calf serum (FCS), 4 mM glutamine and 10 μg ml^{-1} penicillin and streptomycin. Myotube formation was induced by culturing in low-serum medium (DM: DMEM containing 2% horse

serum (HS), 4 mM L-glutamine and antibiotics). C2C12 cells were cultured for 1 day in DM, re-plated on gelatin-coated dishes (or Thermanox® coverslips) at 3×10^4 cells per cm^2 and proliferating cells were later eliminated by the addition of 10^{-5} M cytosine β-D arabino furanoside (Ara-C, Sigma). EBV-transformed human B lymphocytes (hB) clones were maintained in RPMI medium (RPMI) supplemented with 10% FCS, 2 mM L-glutamine and antibiotics. Human foetal muscle 16806 (HFM 16806) cells were maintained as undifferentiated myoblasts in Ham F10 media supplemented with 20% FCS, 4 mM L-glutamine and antibiotics. Differentiation was induced by culturing in DMEM with 4% FCS, 4 mM L-glutamine and antibiotics.

Experimental heterokaryons

Heterokaryons were generated by fusing C2C12 myotubes and hB cells according to the protocol of Chiu and Blau (Blau et al., 1983; Chiu and Blau, 1984) using 50% polyethylene glycol pH 7.4 (PEG 1500, Roche). Briefly, C2C12 myoblasts were induced to differentiate in DM for 2 days, non-adherent hB lymphocytes were added (7×10^6 per 35-mm dish), centrifuged (400 ***g*** for 15 minutes) to promote cell contact and fused with PEG 1500 for 60 seconds at 37°C. After rinsing, cells were maintained in DM supplemented with 10^{-5} M Ara-C and 10^{-5} M ouabain (to eliminate proliferating cells and non-fused human B cells) for 24 hours before culturing for a further 1-7 days in DM containing Ara-C. Where stated, cells were treated immediately after fusion with HDAC inhibitors; 20 nM trichostatin A (TSA) or 1 mM valproic acid (VPA). Mouse and human nuclei were distinguished in the resulting heterokaryons by counterstaining with 4,6-diamidino-2-phenylindole (DAPI).

Antibodies, immunostaining and microscopy

Indirect immunofluorescence labelling was performed using the following primary reagents: Mouse 5.1H11 (Developmental Studies Hybridoma Bank, DSHB), Human CREST autoimmune serum (kind gift of Geoffrey Brown, University of Birmingham Medical School, UK); anti-human Lamin A/C (Novocastra Laboratories Ltd); anti-myogenin (DAKO), rabbit anti-H3K9ac (Upstate). Secondary antibodies were purchased from Molecular Probes and used at appropriate dilutions. Staining with the mouse monoclonal antibody 5.1H11 was performed on unfixed samples labelled for 1 hour at 37°C. 5.1H11-labelling was revealed using biotin anti-mouse IgG antibody (1 hour at 37°C) followed by Texas-Red-coupled avidin (15 minutes at 37°C). All washes were performed at room temperature in DM. 5.1H11-labelled samples were fixed in 2% paraformaldehyde in PBS for 20 minutes. For nuclear staining, fixed samples were permeabilised in 0.4% Triton X-100, incubated for 30 minutes in a blocking solution (2.5% BSA, 0.05% Tween 20 in PBS), incubated in primary antibody (1.5 hours at room temperature), washed and incubated in fluorochrome-labelled secondary antibody diluted in blocking solution (45 minutes at room temperature). Where appropriate, nuclei were counterstained with DAPI (1 μg/ml); slides were mounted in vectashield before analysis with a TCS Leica laser-scanning confocal microscope. To estimate nuclear volumes, *z*-stacks spanning individual nuclei were collected on the laser scanning confocal microscope. The volume of each section was obtained by multiplying the *xy*-surface of each confocal plane by the thickness of sections along the *z*-axis. To investigate chromocentre organisation, image processing softwares (Leica Confocal Software and Metamorph 4.0) were used to perform 3D reconstructions and maximal projections of *z*-series collected through individual nuclei after CREST immunostaining. The number of chromocentres, the number of centromeric signals per chromocentre, and the total number of individual signals per nucleus were analysed in reconstructed projections.

Probe preparation and fluorescence in situ hybridisation (FISH)

Mouse major-satellite (γ-satellite)-DNA probe was directly labelled with Fluoro-Red and used in combination with DIG-coupled human α-satellite-DNA probe (Q-Biogen). α-satellite DNA was detected with anti-digoxygenin fluorescein isothiocyanate (AD-FITC) raised in sheep (Boehringer), followed by anti-sheep FITC (Vector Laboratories). DNA-FISH labelling of whole cells was performed as described previously by Brown et al. (Brown et al., 1999; Brown et al., 1997) where samples were treated to preserve the 3D architecture of nuclei. RNA-FISH was performed as described previously by Lawrence et al. (Lawrence et al., 1989) with probes synthesised by PCR. h*CD20* (a 6.1 kb genomic DNA fragment spanning the last six exons of the *CD20* gene) was labelled with digoxygenin-11-dUTP (Roche), and detected as described above. h*NCAM* (a 5.6kb genomic DNA fragment spanning exon 2 to exon 7 of the *NCAM* gene), was labelled with biotin-16-dUTP (Roche) and detected with Avidin-Texas-Red (Vector Laboratories), followed by a biotinylated goat anti-avidin antibody (Vector Laboratories) and Avidin-Texas-Red antibody.

Reverse transcriptase (RT)-PCR analysis

Total RNA was extracted with RNAzol B, DNA was eliminated using the DNA-free kit (Ambion) and cDNA synthesis was performed using either oligo $(dT)_{12\text{-}18}$ or random primers (Invitrogen). PCR amplification was performed with HotStarTaq™ DNA polymerase (Qiagen) using the following program: 94°C for 15 minutes once, then *n* cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 60 seconds. The sequence of the human-specific primers and the number of cycles (*n*) are indicated in supplementary material Table S2. For each reaction PCR amplification, conditions were within the dynamic range (i.e. non-saturating). PCR products were separated on agarose gels and blotted on Hybond-N nylon membrane (Amersham Pharmacia Biotech) before hybridisation with ^{32}P-labeled probes (Prime-Lt II kit, Stratagene). Real-time PCR analysis of h*PAX5*, h*EBF* and h*E2A* was carried out on a Chromo4™ DNA engine using Opticon Monitor software (MJ Research Inc.), running the following program: 95°C for 15 minutes once, then 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. PCR reactions included 2× Sybr-Green PCR Mastermix (Qiagen), 300 nM primers and 2 μl of template in a 35 μl reaction volume. Each measurement was performed in triplicate and data were normalised according to human *GAPDH* expression.

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