Lysine-specific demethylase 2A (KDM2A) normalizes human embryonic stem cell derived keratinocytes

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Studies on human lysine-specific demethylase 2A (KDM2A) by others have recently begun. To date, the demethylase activity has been known to reduce expression of genes and eventually inhibit proliferation of cells. However, while attempting to improve proliferation of hES-cell–derived Nod keratinocytes, which grow poorly and have a short life span, we found that high expression of the KDM2A gene improves the poor proliferation of the cells. Of the four isomer cDNAs that we prepared from alternatively spliced KDM2A transcripts, only one stimulates the proliferation. This (KDM2A-N782) encodes the 782AA protein containing the JmjC, CXXC, and Ring domains, but not the F-box and AMN1 domains, unlike KDM2A, which has been studied by other groups. Our results not only show that differently spliced transcripts from a gene result in totally opposite outcomes, but also present critical evidence of the complicated activities of KDM2A, which contains all of the five domains.

development | differentiation | epigenetics | transcript isomers | expression vector

It is generally assumed that the differentiated cell types generated from ES cells in vitro are identical to those produced by normal embryogenesis. This is far from true for keratinocytes. There are good reasons for this: the rapidity of differentiation in vitro and the lack of signals operating in embryogenesis, such as polarity and gradients.

No other single cell type isolated from differentiation of ES cells in culture has been shown to be identical to the corresponding cell type produced by embryogenesis.

We first obtained keratinocytes from human embryonic stem cells (hES) by allowing the cells to differentiate after injecting them into SCID (immune deficient) mice, in which the cells formed nodules (1). Hence we refer to the cells as Nod cells. The Nod cells gave rise to keratinocytes, which expressed the same marker proteins as postnatal keratinocytes; but they proliferated more slowly and their life span was much shorter. Also their colonies were not compact as are those of postnatal keratinocytes but instead underwent fragmentation (2). When the Nod keratinocytes were transduced with the E6E7 genes of HPV16, their proliferation was greatly increased and they became immortalized, as expected from the oncogenic effect of the E6E7 gene (2).

To improve the proliferation of Nod keratinocytes by less drastic means, we approached the problem in a different way. The approach required two steps: (*i*) preparation of a retrovirus-based cDNA library of postnatal keratinocytes and (*ii*) selection of Nod keratinocytes whose proliferation was improved by one of the constructs. After obtaining cells with improved proliferation, we identified the gene and the transcript responsible for the improvement.

We carried out the two steps, and eventually found that the improvement was caused by insertion of a genetically modified retrovirus at a position upstream of the lysine-specific demethylase 2A (KDM2A) gene, which also has 10 other synonyms including *FBXL11* and *JHDM1A* (refs. 3, 4, see also NM_012308 at the National Center for Biotechnology Information, NCBI). Although this product has been shown to retard proliferation of human colon cancer cell HT29 by inhibiting a gene activator NFκB (5, 6), the insertion into the genome of our Nod keratinocytes caused higher expression of the KDM2A gene and improved growth. The KDM2A transcript must be important for development and maintenance of keratinocyte multiplication. Only one of four cDNAs that we constructed from four differently spliced transcripts of the gene stimulated proliferation of Nod keratinocytes.

Results and Discussion

Improvement of Proliferation in Nod Keratinocytes by Infection of Retroviral Constructs. To facilitate genetic manipulation, we constructed a retrovirus-based expression vector pMarXG (Fig. 1), in which the Gateway recombination cassette for cloning (Invitrogen) was ligated to pMaRX IV puro in place of the conventional cloning site (7, 8). Then, cDNAs of postnatal keratinocytes were integrated at the *attP1* and *attP2* sites of this plasmid by the catalysis of recombinase-mixture Clonase II. This reaction released the *ccdB* cm^R region from pMarXG. Because the *ccdB* encodes a DNA gyrase inhibitor for *Escherichia coli* (9), pMarXG suppressed growth of the host *E. coli* but the cDNA-recombinant plasmids allowed the host to grow. On the basis of these characteristics, the recombinant plasmids were enriched in a population of the transformed *E. coli*. From such bacterial cultures, cDNA library plasmids were extracted. The library was transfected to a packaging cell line 293GPG (10), and an infectious retroviral cDNA library was prepared from the culture supernatants.

Four Nod-keratinocyte cultures, each of which contained 1–2 × 10^5 cells, were infected with the retroviral cDNA library. The cultures were incubated for 3 d for expression of the $Puro^R$ protein and then selected for 4 d for resistance to 1 μg/mL puromycin. After the selection, cultures were incubated without puromycin and periodically trypsinized and transferred to a new dish for further proliferation. At each trypsinization, the number of cells was counted. We found that one of four cultures grew significantly faster than the others (Fig. 2). The improved proliferation was observed in the first cultivation, and the property became increasingly obvious in the following cultivations. The doubling time of the culture fell below 40 h, which was about three-fifths that of the other three cultures. The culture also had an extended life span, i.e., 32 doublings, twice that of the other cDNA-infected cultures. A control culture, infected with pMarXG alone, did not grow at all.

Identification of the Gene That Improves Proliferation of Nod Keratinocytes. This improvement of proliferation was most likely caused by a cDNA of postnatal keratinocytes. To confirm this presumption, the rapidly proliferating culture was cultivated up to almost the growth limit (for 74 d), and the genomic DNA was extracted. With the DNA as a template, PCR amplified a 1.7-kb fragment from the cloning site (Fig. 3). The same fragment was also obtained with the genomic DNA of a culture expanded from a single colony that exhibited faster proliferation

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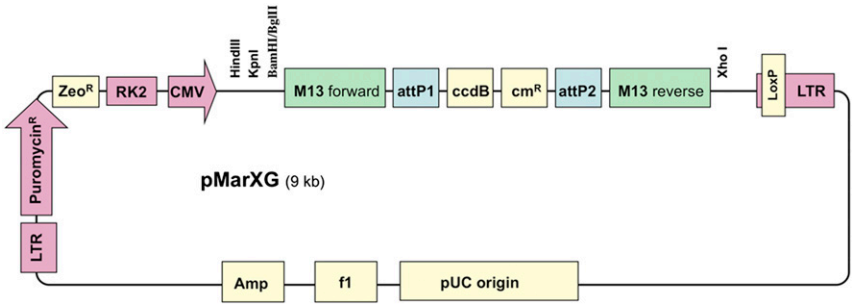

Fig. 1. Retrovirus-based expression vector, pMarXG. pMarXG has a Gateway recombination cassette spanning from M13 forward to M13 reverse on the founder plasmid, pMaRX IV puro, provided by J. Gil (Cell Proliferation Group, MRC Clinical Sciences Centre, Imperial College London, Hammersmith Campus, London, UK) and D. Beach (Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry, London, UK). Genes and elements functioning in *E. coli* are marked with yellow boxes, and those functioning in mammalian cells are marked with red boxes. However, *zeo*^{R} can be expressed in both bacterial and mammalian cells and the *loxP* can function in the presence of Rec recombinase. Recombination of the *attP1* and *attP2* sites occurs in the presence of recombinase-mixture Clonase II.

as early as 16 d after inoculation. These results implied that, after the prolonged cultivation, the mass culture appeared to contain only a single fast-proliferating cell line that was created by a single genetic event: the culture appeared "genetically pure." This DNA was not amplified with chromosomal DNAs of three other cultures. Therefore, the 1.7-kb fragment was likely to be derived from the postnatal keratinocytes' cDNA that improved proliferation of Nod keratinocytes.

Contrary to our expectation, sequencing of the DNA fragment revealed that the DNA was the vector's cloning region with an 854-bp deletion extending through the *ccdB*. The deletion eliminated 77 of the 102 *ccdB* codons. This retrovirus must have occurred during preparation of the cDNA library and been inserted into a specific site of the genome in the following experiments. The result suggested that the improved proliferation was caused by the insertion of the Δ*ccdB* retrovirus.

We identified the insertion site by inverse genomic PCR followed by sequencing (Fig. 3*B*). To this end, a fragment of the genomic DNA fused to the 3′ LTR was excised with DraI and EcoRI and purified (11). It was circularized with ligase after both restriction ends were blunted and then used as a template for PCR. This yielded a unique 0.6-kb DNA fragment (Fig. 3*B*). No PCR product was obtained when either the Δ*ccdB*-retrovirus–harboring genomic DNA was digested with DraI alone (Fig. 3*B*, lane 2) or insertion-free postnatal-keratinocyte DNA was used (Fig. 3*B*, lanes 3 and 4). Sequencing of the 0.6-kb fragment and

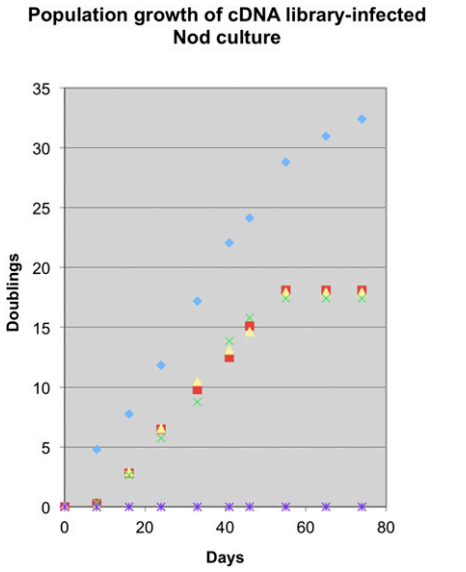

Fig. 2. Cell growth of Nod keratinocytes infected with a retroviral cDNA library. Four cultures (blue, red, yellow, and green) were infected with retroviral cDNA library, and one (purple) with empty retrovirus. The number of cell doublings after selection with puromycin was plotted as a function of time. One of the cultures (blue) grew more rapidly than the others. We later found that a retrovirus was inserted upstream of the KDM2A gene in this culture.

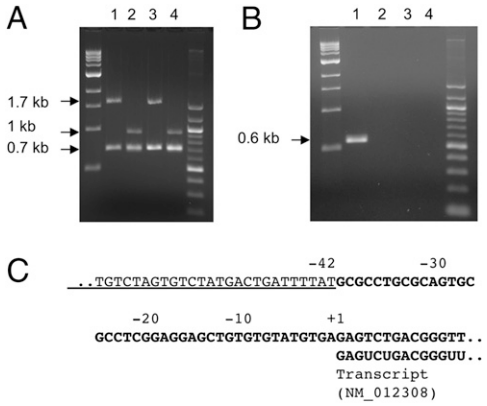

Fig. 3. Identification of the gene responsible for the fast proliferation of Nod keratinocytes. (*A*) The 1.7-kb fragment associated with rapidly growing Nod keratinocytes. The 1.7-kb fragment was amplified by PCR. Template in lanes 1 and 2 was chromosomal DNA derived from the mass culture of the rapidly growing Nod keratinocytes (grown up to 74 d, Fig 2) and in lanes 3 and 4, chromosomal DNA derived from a colony consisting of the most rapidly proliferating cells at day 16. Identity of the two 1.7-kb fragments was confirmed by digestion of the fragments with EcoRI-HF: both fragments were cleaved into 1-kb and 0.7-kb fragments (lanes 2 and 4). The 0.7-kb fragments were not visible because they were confounded with a 0.7-kb noise band. (*B*) Fragment of the gene responsible for the fast proliferation. The 0.6-kb fragment was identified by inverse PCR. Template for lanes 1 and 2 was prepared from Nod-keratinocyte genomic DNA with the ΔccdB retroviral insertion and that of lanes 3 and 4 was prepared from the postnatal-keratinocyte genomic DNA free of the virus. Templates for lanes 1 and 3 were prepared by digestion with both DraI and EcoRI-HF and those of lanes 2 and 4 by digestion with DraI only. The 0.6-kb fragment obtained by PCR (lane 1) was sequenced. (*C*) Insertion of the Δ*ccdB* retrovirus in the KDM2A gene. The KDM2A (human lysine-specific demethylase 2A) gene of the more rapidly growing Nod keratinocytes contained an insertion of the Δ*ccdB* retrovirus at position −42. The 3′ end of the retrovirus is underlined, and the KDM2A gene on the genome is in bold type. The KDM2A transcript NM_012308 begins at G (+1). The entire transcript is 7,393 bp long and the coding region extends from position 865 to position 4,353.

BLAST search for the sequence against the human genome plus transcript database of NCBI demonstrated that the Δ*ccdB* retrovirus was inserted 42 bp upstream of the transcription start site of the lysine-specific demethylase 2A gene (*KDM2A*$^{-42}$::Δ*ccdB* retrovirus, Fig. 3*C*).

Insertion of Δ*ccdB* Retrovirus Enhanced Expression of the KDM2A Gene. To demonstrate the consequence of the insertion on KDM2A transcription, we estimated the level of the KDM2A transcripts by RT-PCR. For this object, first-strand cDNAs were synthesized with an oligo dT containing randomized nucleotides at its 5′ end. Then the KDM2A transcripts and the control transcripts (KRT14) were amplified to levels visible by ethidium bromide staining.

In the initial RT-PCR, amplification of the KMD2A and KRT14 cDNAs were carried out independently in two separate reactions. It can be seen on Fig. 4*A* that faster-proliferating Nod keratinocytes bearing the *KDM2A*$^{-42}$::Δ*ccdB* retrovirus contained more KDM2A transcripts than one of the three Nod-keratinocyte cultures that were infected with an aliquot of the same retroviral stock but did not have the same insertion (red in Fig. 2). To analyze KDM2A transcription more precisely, RT-PCR was performed under stricter conditions. Namely, PCR was carried out in a reaction mixture containing two pairs of primers: one for KDM2A and one for KRT14, using the serially diluted first-strand cDNAs as template (Fig. 4*B*). This result made it clear that Nod keratinocytes with *KDM2A*$^{-42}$::Δ*ccdB* retrovirus had about twice the level of KDM2A transcripts of the nonstimulated Nod keratinocytes (Fig. 4 *B* and *C*). Although KDM2A transcripts were more highly amplified at lower concentrations of template than KRT14, it seems that the phenomenon merely reflected a higher affinity of the Taq polymerase for the KDM2A template than the KRT14 template (Fig. 4*C*).

We then wondered whether the transcript level of the poorly growing Nod keratinocytes was suboptimal and measured its level in postnatal keratinocytes. It turned out that the transcript level of the poorly growing Nod keratinocytes was similar to that of fast-growing postnatal keratinocytes.

We concluded that Nod keratinocytes do not grow well even though the cells maintained a KDM2A transcript level comparable to that of postnatal keratinocytes, but that the poor proliferation of Nod can be overcome by a higher level of KDM2A transcripts. Later, we found that the KDM2A transcripts are in fact the sum of multiple differently spliced transcripts including four variants that we cloned, and we showed that only one of the four variants is effective in improving the poor growth of Nod keratinocytes.

Identification of Four Differently Spliced KDM2A Transcripts. To confirm the role of the KDM2A in the improvement of cell proliferation, it was desirable to express the cDNA in the poorly growing cells. For this object, we decided to clone the KDM2A

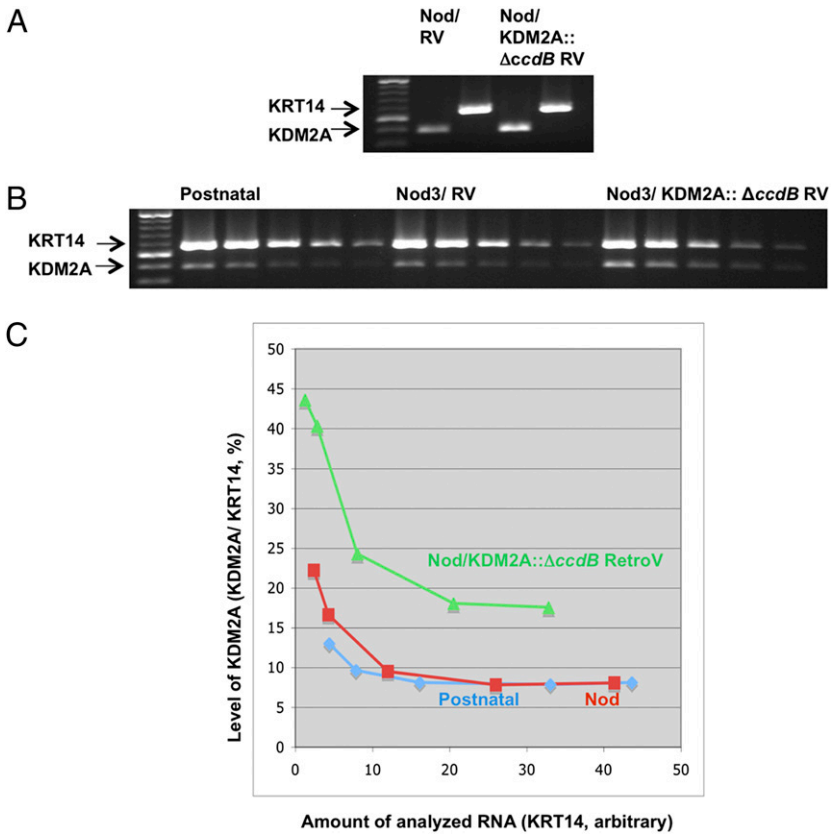

Fig. 4. Expression of the KDM2A transcript in Nod keratinocytes. DNA fragments amplified by RT-PCR were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The amount of DNA in each band was estimated with AlphaImager EP. The KDM2A fragment migrated with the 400-bp marker and the KRT14 fragment with the 600-bp marker. (*A*) PCR was performed in separate reactions for KD2A and KRT14. Expression of KDM2A gene was higher in Nod/*KDM2A*::ΔccdB RV (RV, retrovirus) keratinocytes than in nonstimulated Nod/RV keratinocytes. (*B*) RT-PCR was carried out with two pairs of primers: one for KDM2A and one for KRT14. (*C*) Relative levels of KDM2A in *B* are given as ratios of KRT14. Nod keratinocytes, which grow rapidly due to the retrovirus insertion (green) also show higher expression of KDM2A than their insertion-free counterpart (red). Because insertion-free Nod keratinocytes have the same level of the transcript as postnatal keratinocytes, the cause for the slow growth of the Nod keratinocytes is not a lower level of the KDM2A transcript. However, overexpression of the KDM2A can overcome this cause, whatever it may be.

cDNA. RNA was prepared from the Nod keratinocytes harboring *KDM2A*$^{-42}$::Δ*ccdB* retrovirus, and a cDNA consisting of the KDM2A-coding region was synthesized by RT-PCR from the RNA, using as reference the sequence of the KDM2A transcript deposited at NCBI under NM_012308. Then the cDNA was cloned into pMarXG by recombination. The experiment was straightforward but we soon realized that the transcript consists of several variants: the prepared cDNA migrated on agarose gel as a wider band than expected from a homogeneous DNA. Accordingly, 20 plasmids were extracted from single *E. coli* colonies transformed with the cDNA-containing pMarXG. From the sequence of NM_012308, the KDM2A cDNA was predicted to have the BamHI and XhoI restriction sites as shown in Fig. 5*A*. Then, all 20 plasmids were digested with the two enzymes and subjected to gel electrophoresis to sort them. This survey identified four variant KDM2A cDNAs (Fig. 5*B*), and a representative of each variant was sequenced. As shown in Fig. 5*A*, pKDM2A contained the 3,544-bp sequence that has the entire coding region of NM_012308. Three other plasmids contained splicing variants of the coding region: pKDM2A-ΔN contained a 122-bp additional exon in the α nucleotide fragment (Fig. 5 *A* and *B*), pKDM2A-N782 lost exons spanning 452 bp in the δ fragment, and pKDM2A-C620 contained two additional exons (98 bp and 96 bp) in the α fragment, although losing 25-bp and 133-bp exons in the δ fragment. Nucleotide sequencing of the coding regions also provided information on the ORFs and the Kozak sequences (12). It appeared that the KDM2A cDNA encodes the 1162AA full-length protein (Fig. 5*C*), the KDM2A-ΔN cDNA encodes the 1145AA protein lacking a small portion at the N terminus due to a frameshift, the KDM2A-N782 cDNA encodes the 782AA protein without the C-terminal domains, and the KDM2A-C620 cDNA encodes only the C-terminal 620AA protein (Fig. 5*C*). Notably, these four clones encoded proteins with unique combinations of domains. It has been proposed that JmjC, C2 (CXXC-ZF), Ring (PHD), F-box, and AMN1 domains have activities for histone H3 lysine36 dimethyl (H3K36me2) demethylase, for binding to CpG islands, for association with proteins, for association with S-phase kinase-associated protein 1A (SKIP1), and for inhibition of cell division, respectively (4), (13–15). Thus, these transcripts appeared to have distinct effects on proliferation of cells.

Of the twenty plasmids, two had no relation to the KDM2A transcript. One contained the mouse-feeder's Kdm2a gene and another had lost the cloning site. And of the 18 plasmids, 10, 4, 3, and 1 contained KDM2A, KDM2A-ΔN, KDM2A-N782, and KDM2A-C620, respectively. Therefore, the percentage of the number of each cDNA was 56% (10/18) for KDM2A, 22% (4/18) for KDM2A-ΔN, 17% (3/18) for KDM2A-N782, and 5% (1/18) for KDM2A-C620. This ratio indicated the approximate molar ratio of KDM2A transcripts in the Nod keratinocytes bearing the *KDM2A*$^{-42}$::Δ*ccdB* retrovirus.

Many short KDM2A transcripts are listed in the Ensembl database. We did not find such transcripts simply because those do not possess the 5′ region needed for our PCR.

KDM2A-N782 Is the only Splicing Isoform That Promotes Proliferation of Nod Keratinocytes. We transduced Nod keratinocytes with retrovirus bearing each clone we isolated to investigate which clones stimulate the proliferation. After the transduction, the cultures were selected for 2–3 d for resistance to 0.5 μg/mL puromycin and then their proliferation was examined. Of the four clones examined, the KDM2A-N782 clone, which encodes the KDM2A variant protein possessing the JmjC, C2 (CXXC), and Ring domains but lacking both F-box and AMN1 domains of

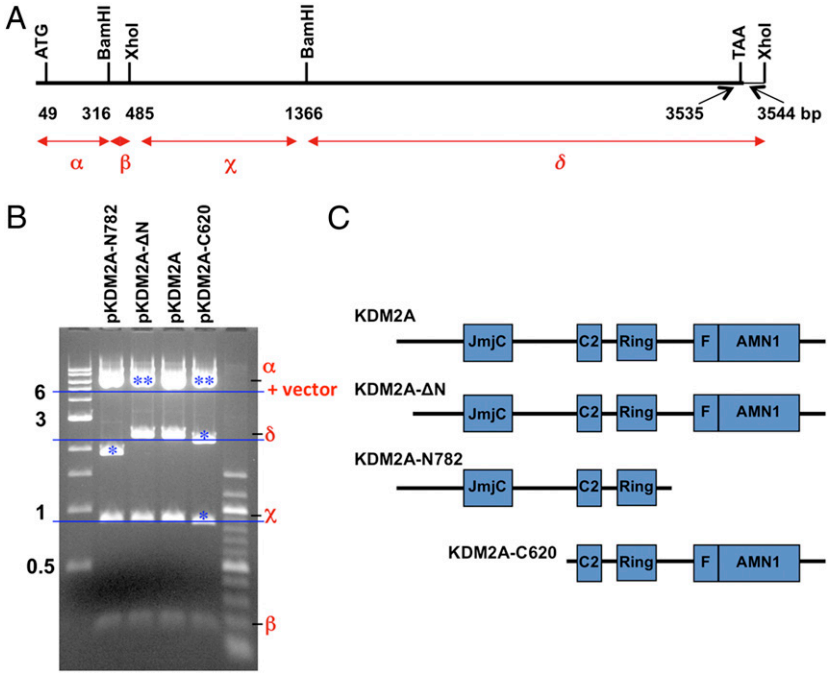

Fig. 5. Four different clones of the KDM2A transcript variants. (*A*) Restriction map of the KDM2A cDNA cloned into pMarXG. The 3544-bp KDM2A cDNA has unique BamHI and XhoI restriction sites. Although the right XhoI site is located within the vector, the site is useful for this analysis. The fragments resulting from the double digestion were named α, χ, β, and δ. (*B*) Fragments produced by BamH and XhoI of four splicing isoforms identified in this study. To present all of the restriction fragments, which range from 169 to 6,700 bp, electrophoresis had to be terminated before the smallest bands became invisible. Even under this condition, the differences in migration of the variant fragments from those of the KDM2A cDNA were manifested mostly by the aid of the drawn blue lines. The fragments derived from the four splicing isoforms are illustrated by blue asterisks. One (*) and two asterisks (**) indicate fragments migrating faster and more slowly than the equivalent fragments of pKDM2A, respectively. (*C*) KDM2A mRNAs and the encoded proteins were identified by DNA sequencing of the four clones. Only clone KDM2A-N782 promotes proliferation of Nod keratinocytes.

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1         10         20         30         40         50         60
MEPEEERIRYSQRLRGTMRRRYEDDGISDDEIEGKRTFDLEEKLHTNKYNANFVTFMEGK
DFNVEYIQRGGLRDPLIFKNSDGLGIKMPDPDFTVNDVKMCVGSRRMVDVMDVNTQKGIE
MTMAQWTRYYETPEEEREKLYNVISLEFSHTRLENMVQRPSTVDFIDWVDNMWPRHLKES
QTESTNAILEMQYPKVQKYCLMSVRGCYTDFHVDFGGTSVWYHIHQGGKVFWLIPPTAHN
LELYENWLLSGKQGDIFLGDRVSDCQRIELKQGYTFVIPSGWIHAVYTPTDTLVFGGNFL
HSFNIPMQLKIYNIEDRTRVPNKFRYPFYYEMCWYVLERYVYCITNRSHLTKEFQKESLS
MDLELNGLESGNGDEEAVDREPRRLSSRRSVLTSPVANGVNLDYDGLGKTCRSLPSLKKT
LAGDSSSDCSRGSHNGQVWDPQCAPRKDRQVHLTHFELEGLRCLVDKLESLPLHKKCVPT
GIEDEDALIADVKILLEELANSDPKLALTGVPIVQWPKRDKLKFPTRPKVRVPTIPITKP
HTMKPAPRLTPVRPAAASPIVSGARRRRVRCRKCKACVQGECGVCHYCRDMKKFGGPGRM
KQSCVLRQCLAPRLPHSVTCSLCGEVDQNEETQDFEKKLMECCICNEIVHPGCLQMDGEG
LLNEELPNCWECPKCYQEDSSEKAQKRKMEESDEEAVQAKVLRPLRSCDEPLTPPPHSPT
SMLQLIHDPVSPRGMVTRSSPGAGPSDHHSASRDERFKRRQLLRLQATERTMVLRQETLD
KN
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Fig. 6. The predicted amino acid sequence of clone KDM2A-N782. Underlines indicate JmjC, CXXC-ZF, and PHD (Ring) domains from top to bottom.

the C terminus (Fig. 6), stimulated the proliferation from the beginning and maintained the stimulation thereafter (Fig. 7). A similar stimulation with this clone was also observed without selection with puromycin. Unexpectedly, the KDM2A clone encoding all of the five domains did not significantly improve the proliferation. Neither did the KDM2A-ΔN clone, which only lacks a small N-terminal sequence but encodes all of the functionally important domains. It was therefore clear that the stimulatory activity present in the KDM2A-N782 was counteracted by the C terminus containing the F-box and AMN1 domains. Because the demethylase activity of the JmjC domain removes histone 3 from the chromosomes (4), the domain is likely to be the most critical factor to stimulate the keratinocyte growth. We concluded that the demethylase activity of the JmjC domain is essential to stimulate the poor growth of Nod keratinocytes but the activity of the full-length protein KDM2A is counteracted by the C-terminal domains, F-box and AMN1. The absence of effect of KDM2A-C620 on the keratinocyte grow was compatible with this conclusion.

A simple explanation for our findings is that the C-terminal domains directly antagonize the JmjC demethylase activity. However, the KDM2A protein may not be so simple. Evidence for the complexity of the protein has been emerging. KDM2A binds to many CpG islands of numerous gene promoters through the C2 (CXXC) zinc finger domain, and removes the associating H3K36me2 from the promoters converting it to H3K36me1 by the JmjC's demethylase activity (13). However, Bartke et al. have demonstrated that the KDM2A binds to H3K9me3-associated promoters by participation of an additional factor, HP1 (16). This alternative binding to H3K9me3 appears to need the C-terminal domains as the critical factor, because SF-KDM2A, a protein similar to KDM2A-C620, binds to the target even in the absence of HP1. An example of such an alternative recognition occurs in the promoter of the ribosomal RNA gene (16), whose transcription has been shown to be inhibited by KDM2A in MCF7 cells (17). And this inhibition has been shown to result in retardation of the cell proliferation (17). It is therefore evident that KDM2A has dual demethylase activities: the primary demethylase activity to stimulate cell growth through the demethylation of H3K36me2 and the secondary demethylase activity to inhibit cell growth through the demethylation of H3K9me3. However, further complexity comes from another specificity of the JmjC domain, which recognizes K218- and K221-methylated RelA (p65), a member of the NF-κB family. The demethylation of the two-lysine residues results in inhibition of HT29 cell growth (6). This specificity might require the C-terminal domains of KDM2A and another protein yet to be discovered. We discovered the simple aspect of the KDM2A demethylase activity, i.e., stimulation of cell growth by isolating and expressing the alternatively spliced transcript KDM2A-N782 as well as its isomers. The KDM2A-N782 transcript may also have a similar effect on other cells.

We propose a simple model of how KDM2A-N782 improves the proliferation of Nod keratinocytes (Fig. 8). Nod keratinocytes produce smaller amounts than postnatal keratinocytes of a protein that activates genes participating in cell growth. When more KDM2A-N782 protein is produced by either promoter insertion or transduction, the protein removes H3K36me2 from the promoter of growth-promoting genes and thus increases binding of the activator protein to these genes. This higher binding increases transcription of the growth-promoting genes and eventually causes increased cell multiplication.

Finally, although the retrovirus is a powerful tool for new discoveries, it cannot be used to develop cell types that are intended to be used in humans. Cells prepared by infection with retroviruses, such as induced pluripotent stem cells and cell types differentiated from them, may contain potentially harmful cells that resulted from virus insertion. Although these cells may be a tiny fraction of a large mass of cells, once patients receive

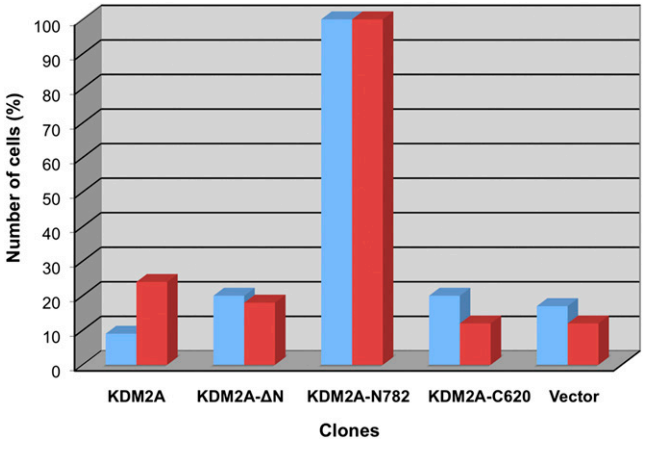

Fig. 7. Proliferation of Nod keratinocytes transduced with different clones. Nod keratinocyte cultures were transduced with viral clones encoding KDM2A, KDM2A-ΔN, KDM2A-N782, and KDM2A-C620, respectively, as well as with the empty retrovirus and incubated for 30 d. Red and blue show results of two independent experiments. Only clone KDM2A-N782 increases proliferation of Nod keratinocytes.

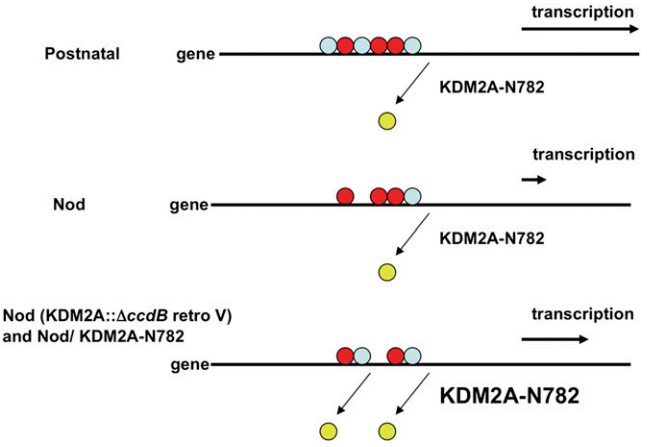

Fig. 8. A simple model of how KDM2A increases proliferation of Nod keratinocytes. The overexpressed KDM2A-N782 demethylates H3K36me2 (red) to H3K36me1 (yellow) and removes it from a promoter necessary for rapid growth of Nod keratinocytes and thus facilitates access of an activator protein (blue) to this promoter. Higher amounts of bound activator explain why KDM2A-N782–overexpressing Nod keratinocytes grow faster than cells that do not overexpress the KDM2A splicing isoform.

retrovirus-manipulated cells for a treatment, the cells could come to dominate other cells of the patients' over the years and accumulate sufficiently to cause unintentional health problems, such as cancer.

Addendum

For those scientists who are enthusiastic about the possibilities of human therapy with embryonic stem cells, we suggest that caution if not skepticism would be the most realistic view of the future.

Materials and Methods

Cultivation of Keratinocytes. Keratinocytes were cocultivated with lethally irradiated 3T3 in FAD medium (18, 19). For experiments of proliferation, 1–5 $\times$ 10^4 cells were initially inoculated in a 6-cm dish. These cultures were trypsinized when the cells grew sufficiently and they were transferred to new dishes with the same dilution (typically 20-fold).

Preparation of pMarXG. Gateway recombination cassette (2.5 kb) for the *ccdB cm^R* genes and *attP1* and *attP2* recombination sites flanked by M13 forward and M13 reverse was amplified by PCR from a template pDONR221 (Invitrogen), and the amplified DNA (Fig. 1) was ligated to BamHI and XhoI sites in the multicloning site of pMaRX IV puro (7, 8). For primer oligonucleotides and conditions of PCR, see Tables S1 and S2. The ligated plasmids were introduced into one shot ccdB survival 2 T1R competent cells (Invitrogen) and selected for resistance to chloramphenicol. A plasmid isolated from a pure culture resistant to chloramphenicol was named pMarXG. This plasmid, when introduced to *E. coli* NEB 10-beta (New England Biolabs), did not allow the bacteria to grow on LB agar as expected from the inhibition by the *ccdB* gene product.

Preparation of a Retrovirus-Based cDNA Library. Total RNA was extracted from postnatal keratinocytes (YF29) with RNeasy Mini kit (Qiagen), and the mRNA was extracted with Oligotex mRNA Mini kit (Qiagen) and used as the source for construction of a cDNA library. All of the procedure was carried out according to the manufacturer's protocol (CloneMiner II cDNA Library Construction; Invitrogen). The constructed library was introduced to NEB 10-beta competent cells and selected on LB agar containing 0.25% NaCl and 25 μg/mL zeocin at 30 °C. Seventy-six thousand independent colonies were collected, and then a pool of cDNA-library plasmids was extracted from the bacterial culture.

Preparation of Retroviral cDNA Library and Retroviral Clones. Ten micrograms of plasmids was transfected to packaging cell line 293GPG (10) cultivated to 70–80% confluent in a 10-cm dish, after mixed with FuGENE 6 or X-tremeGENE 9 DNA transfection reagent (Roche Biochemicals). The cultural supernatants were collected every 2 d up to 9 d after the transfection. All of the supernatants were combined, filtrated with 0.45-mm Millex-HV (Millipore), and ultracentrifuged for 2 h at 20,000 rpm in a SW28 rotor of Beckman. The collected viruses were suspended in 1/100th the original volume.

Infection of Retroviral Preparations. A culture of Nod keratinocytes was added with retrovirus (1/100th the culture volume) and incubated for 2 h in the presence of 8 μg/mL polybrene. Then the cultures were incubated overnight in the presence of 2 μg/mL polybrene.

PCR and RT-PCR. Genomic DNA was extracted with Gentra puregene (Qiagen), and total RNA was extracted with RNeasy Mini kit (Qiagen). PCR was routinely carried out with Phusion Hot Start (Finnzymes), but estimation of the level of the KDM2A transcript was conducted with the Taq polymerase included in the ProtoScript AMV Long Amp Taq RT-PCR kit (NEB). For cloning of the KDM2A transcripts, the first strand of cDNA was synthesized with ProtoScript Avian Myeloblastosis virus reverse transcriptase of the NEB kit and then amplified with Phusion Hot Start II. For primer oligonucleotides and conditions of PCR, see Tables S1 and S2.

Excision and Purification of Genomic DNA Linked to Δ*cddB* Retrovirus. Genomic DNA (2 μg) was digested overnight with DraI and EcoRI-HF. The digest was mixed with 80 ng of 5′-end biotin-labeled oligo nucleotide complimentary to the retroviral 3′ LTR sequence (Olit143) and annealed in the presence of blockers (80 ng of Olit141and 80 ng of Olit142). Then the annealed DNA was captured with streptavidin magnetic beads (NEB), washed, and finally collected in 10 μL of 0.5× TE by heating for 3 min at 80 °C. For primer oligonucleotides see Table S1.

DNA Sequences. DNA was sequenced by a group at the biopolymer facility at Harvard Medical School. Sequences of the KDM2A transcripts cloned in this experiment were deposited at GenBank. Accession nos. are JQ710741 for KDM2A, JQ710742 for KDM2A-ΔN, JQ710743 for KDM2A-N782, and JQ710744 for KDM2A-C620.

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