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siRNA-Mediated Allele-Specific Inhibition of Mutant Type VII Collagen in Dominant Dystrophic Epidermolysis Bullosa

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TO THE EDITOR

Dominant dystrophic epidermolysis bullosa (DDEB) is a blistering disease of the skin and mucosae, in which mutant type VII collagen monomers exert dominant-negative interference on normal α1(VII) chains upon homotrimer assembly (Burgeson, 1993). Specific inhibition of the mutant mRNA has been achieved recently in other dominant skin disorders, including epidermolysis bullosa simplex and pachyonychia congenita, using small interfering RNAs (siRNAs) targeting mutations in the keratin 5 and the keratin 6A genes, respectively (Hickerson *et al.*, 2008; Atkinson *et al.*, 2011). We have investigated allele-specific RNA interference as a new therapeutic approach for DDEB, by targeting in-frame skipping of exon 87 (Δ87) of *COL7A1* (OMIM *120120) caused by several intronic or exonic mutations, some of which are recurrent mutations, underlying DDEB pruriginosa (Supplementary Table S1 online; Sakuntabhai *et al.*, 1998; Mellerio *et al.*, 1999; Covaciu *et al.*, 2011).

We developed a fluorescence-based screen for siRNAs selectively blocking the mutant mRNA, using cultured cells transfected with a plasmid vector encoding either the mutant ($COL7A1_{\Delta 87}$) or wild-type ($COL7A1_{WT}$) mRNA upstream of an internal ribosome entry site (IRES)-firefly luciferase reporter (Figure 1a). The abnormal exon 86–exon 88 splice junction was scanned with 21 siRNAs; some were 5′-end modified to increase efficiency (Grimm, 2009; Figure 1b). Positive (siwt, targeting *COL7A1* exon 22) and negative control (NC) siRNAs were used in parallel at 1–10 nM with no observed dose effect (data not shown). Eleven siRNAs, siCOL7Δ87mut3, −9 to −11, −14 to −19, and −21, displayed over 40% inhibition of $COL7A1_{\Delta 87}$ (Figure 1c).

Best differential inhibition was observed with siCOL7Δ87mut3, 45% (1 nM), and siCOL7Δ87mut18, 55% (2 nM), versus 14% inhibition of $COL7A1_{WT}$. High specificity for the mutant mRNA was confirmed by transfecting these siRNAs into fibroblasts and keratinocytes from patients carrying a Δ87 mutation, NM_000094.3:c.6900 + 4A > G (Drera *et al.*, 2006); mRNA extinction was measured by Δ87-specific PCR amplification (Figure 2). The positive control siRNA achieved 55% inhibition of $COL7A1_{WT}$ or $COL7A1_{\Delta 87}$ mRNA (Figure 2a and b), whereas the NC siRNA had no significant effect. In patient fibroblasts, siCOL7Δ87mut3 inhibited $COL7A1_{\Delta 87}$ by 38% (1 nM), 36% (2 nM), 31% (5 nM), and 41% (10 nM), versus 2–8% $COL7A1_{WT}$ inhibition in healthy control fibroblasts (Figure 2b). In patient keratinocytes, $COL7A1_{\Delta 87}$ extinction by siCOL7Δ87mut3 was 38, 58, 45, and 54%, respectively. Conversely, no depression but enhancement of $COL7A1_{WT}$ levels was observed in healthy control keratinocytes, consistent with luciferase assay data (Figure 1). siCOL7Δ87mut18 inhibited $COL7A1_{\Delta 87}$ by 47% (1 nM), 58% (2 nM), 54% (5 nM), and 65% (10 nM) in patient fibroblasts, and by 53, 41, 35, and 52%, respectively, in patient keratinocytes. Experiments on healthy control cells showed no significant reduction of $COL7A1_{WT}$ levels by siCOL7Δ87-mut18 (Figure 2c).

siCOL7Δ87mut18, which shows the strongest specific $COL7A1_{\Delta 87}$ inhibition, carries a 5′-terminal amine modification of the sense strand to promote antisense strand incorporation into the RNA-induced silencing complex (RISC). siRNA duplex thermodynamics determine which strand enters RISC as the guide strand, and only the antisense strand can direct cleavage of the sense mRNA targets (Khvorova *et al.*, 2003). Avoidance of off-target effects (Grimm, 2009) is another potential benefit of sense-strand suppressive modification, which was not specifically addressed here.

Earlier studies of allele-specific siRNAs targeting keratin or collagen genes achieved 70–95% inhibition of the mutant allele (Hickerson *et al.*, 2008; Lindahl *et al.*, 2008; Atkinson *et al.*, 2011). The structure of the target mRNA could explain the difficulty in achieving similarly high inhibitory activity and specificity here. First, the G/C content of the Δ87 region attains 68–73%,

Abbreviations: COL7A1Δ87, COL7A1 mRNA with deleted exon 87; COL7A1WT, wild-type COL7A1 mRNA; DDEB, dominant dystrophic epidermolysis bullosa; Δ87, in-frame skipping of COL7A1 exon 87; IRES, internal ribosome entry site; NC, negative control siRNA; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; siwt, positive control siRNA; WT, wild type

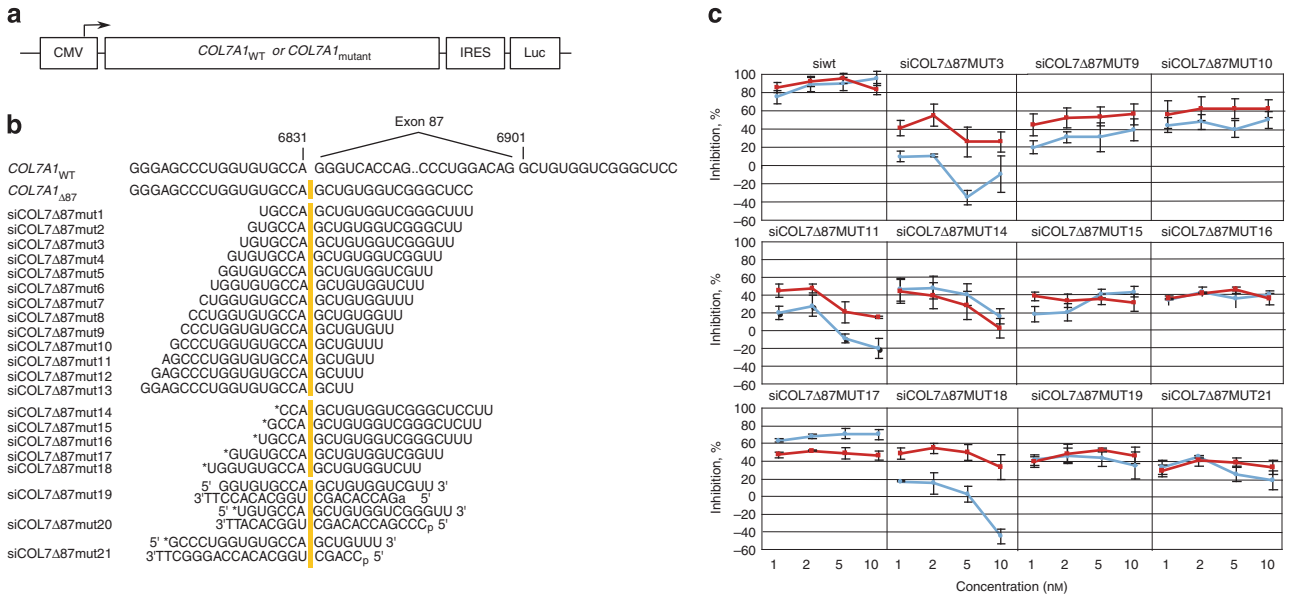

Figure 1. Assay for COL7A1 mRNA inhibition by siRNAs. (**a**) Bicistronic reporter cassettes. The IRES enables expression of the firefly luciferase gene downstream from the wild-type (WT) or mutant *COL7A1* complementary DNA and the cytomegalovirus (CMV) promoter/enhancer. (**b**) siRNAs were designed to scan 15 successive 19-base windows encompassing the exon 86–exon 88 splice junction ($COL7A1_{\Delta 87}$). Several siRNAs carried a 5′-terminal amine (asterisk) or phosphate (p) modification. The sequence of the target mRNA region in the WT and the exon 87 deletion allele is shown at the top. (**c**) Screening of the siRNA panel in the luciferase reporter system. Inhibition of the $COL7A1_{WT}$ (blue) and $COL7A1_{\Delta 87}$ (red) transcripts was measured in parallel. Results are shown for siRNAs with >40% inhibition of $COL7A1_{\Delta 87}$. Error bars denote the standard deviation. IRES, internal ribosome entry site; siRNA, small interfering RNA; siwt, positive control siRNA.

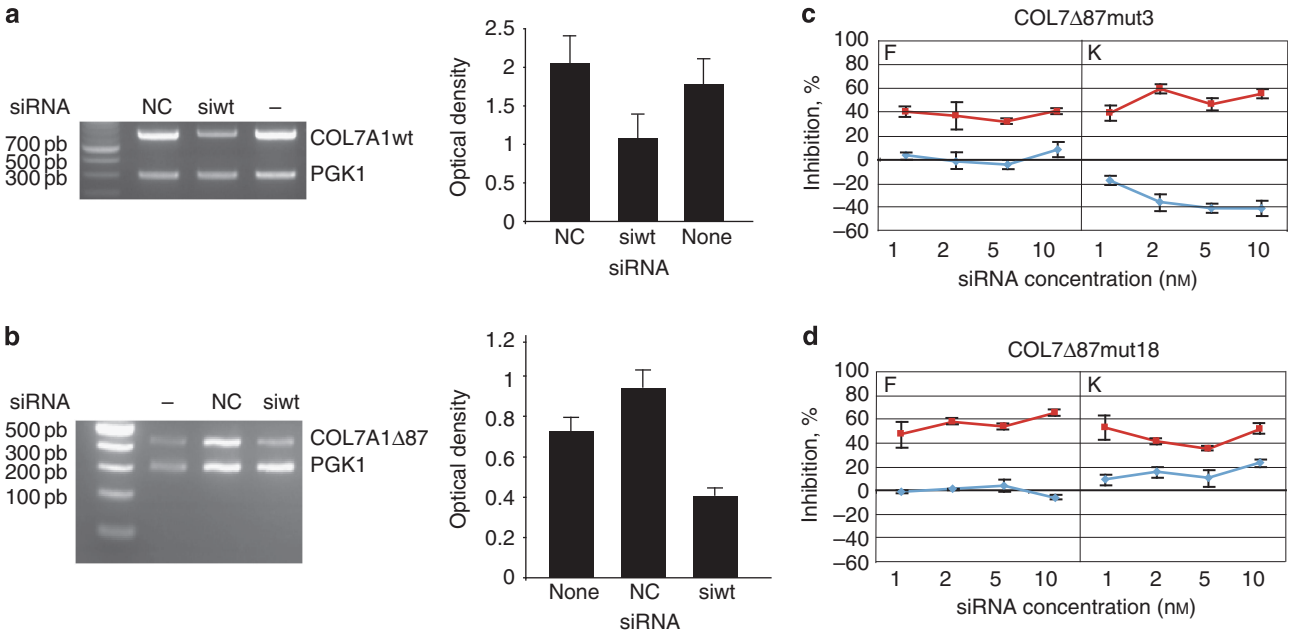

Figure 2. Allele-specific inhibition of endogenous *COL7A1Δ87* mRNA. Patient or healthy cells were transfected with siRNA. mRNA levels were determined by RT–PCR and normalized to the internal control amplification (*PGK1* mRNA). (**a**, **b**) Healthy fibroblasts (**a**) and Δ87 DDEB fibroblasts (**b**) were transfected with either siwt or NC siRNA. The siwt inhibited $COL7A1_{wt}$ by 50% and $COL7A1_{\Delta 87}$ by 55% as assessed by allele-specific RT–PCR. (**c**, **d**) Fibroblasts (F) and keratinocytes (K) from a DDEB patient (red) and from a control (blue) were transfected with siCOL7Δ87mut3 (**b**) or siCOL7Δ87mut18 (**d**). In fibroblasts, siCOL7Δ87mut3 and siCOL7Δ87mut18 achieved inhibition rates of 40 and 65%, respectively, against $COL7A1_{\Delta 87}$, in contrast with a maximum inhibitory effect of 12% toward $COL7A1_{wt}$. In keratinocytes, the respective inhibition rates reached 53 and 65%. The maximum inhibition of $COL7A1_{WT}$ observed with siCOL7Δ87mut18 was 23%. Error bars denote the standard deviation. DDEB, dominant dystrophic epidermolysis bullosa; NC, negative control; RT–PCR, reverse transcription–PCR; siRNA, small interfering RNA; siwt, positive control siRNA; WT, wild type.

whereas 30–50% G/C in siRNA sequences is considered optimal for allelic discrimination (Grimm, 2009). Second, the target lies within the mRNA region encoding the collagenous domain of type VII collagen. This region is rich in short direct repeats (Supplementary Figure S1 online), which may act as spurious targets of a given siRNA. Together with the high overall G/C content, this also predicts a tendency for mRNA to form secondary structures potentially masking siRNA targets (Vert *et al.*, 2006). Finally, analysis of siRNA sequences with the Sfold software (http://sfold.wadsworth.org; Ding and Lawrence, 2003) predicted the thermodynamics of their secondary structures to be uniformly unfavorable. Most recurrent mutations in DDEB affect the collagenous domain, including Δ87 mutations and the well-known single-nucleotide mutation p.Gly2043Arg (Supplementary Table S1 online). This mutation was addressed using 24 siRNAs, of which 9 displayed over 40% inhibition, and none clearly discriminated between the wild-type (WT) and mutant alleles (Supplementary Figure S2a online). The highest specificity to the p.Gly2043Arg allele was observed (at 5 nM) for a siRNA with the mismatch at nucleotide 19; however, in healthy control cells, this showed severe inhibition of endogenous $COL7A1_{WT}$ as well (Supplementary Figure S2b online).

In pachyonychia congenita and epidermolysis bullosa simplex, two dominant genodermatoses, reducing the mutated protein by 50% allowed phenotypic reversion (Cao *et al.*, 2001; Hickerson *et al.*, 2008). In DDEB, Fritsch *et al.* (2009) have shown that increasing the ratio of WT to mutant protein improves thermal stability in the collective of type VII collagen trimers. Similarly, an incomplete effect of the splice-site mutation may account for observed Δ87 DDEB cases with milder or pretibial presentations contrasting with the more frequent, severe pruriginosa phenotype (Supplementary Table S1 online). The rationale of allele-specific siRNA usage is thus to deplete mutant mRNA levels to reduce the stoichiometry of mutant protein monomers during trimer assembly, and increase functional type VII collagen levels accordingly. The 58% reduction in mutant mRNA achieved against $COL7A1_{\Delta 87}$ predicts 35% of new α1(VII) triplets to exclude mutant chains, versus only 13% initially (Supplementary Materials and Methods). At this quantitative threshold, treatment of patients carrying Δ87 mutations would be expected to result in a milder blistering phenotype. Further investigations of the functional effects of siRNAs at the protein and tissue levels will be required, using animal models engrafted with human skin equivalents engineered with DDEB patient cells (Garcia *et al.*, 2011). Indeed, inhibition of the mutant *COL7A1* allele was analyzed only at the mRNA level in our study, and the expected reduction in the levels of the protein and its effects on *in vivo* function need to be formally confirmed.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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