siRNA based strategy to inhibit mutant keratin 5 or keratin 14 expression in Epidermolysis Bullosa Simplex

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I. Background

Epidermolysis Bullosa Simplex (EBS) is a rare genodermatose characterized by intra-epidermal detachment following mild trauma, palmoplantar keratoderma and nail dystrophy. It is most often caused by dominant missense variants in the *KRT5* or *KRT14* genes encoding keratin 5 (K5) or keratin 14 (K14) respectively (1). Keratin 5 and 14 form obligate heterodimers that assemble to produce the intermediate filament network of basal keratinocytes. The most severe forms are caused by pathogenic variants in the helix initiation and helix termination peptides (HIP and HTP) at each ends of the central rod domain affecting proper assembly of heterodimers (Figure 1). The incorrect assembly results in keratin aggregate formation evident by transmission electron microscopy or confocal microscopy following keratin 5/14 immunostaining (2). Small interfering RNA (siRNA) technology has the potential to induce post translational silencing of transcripts carrying specific dominant variants (3). It is thus a promising strategy for a therapeutic approach in EBS caused by dominant mutations in *KRT5* or *KRT14*.

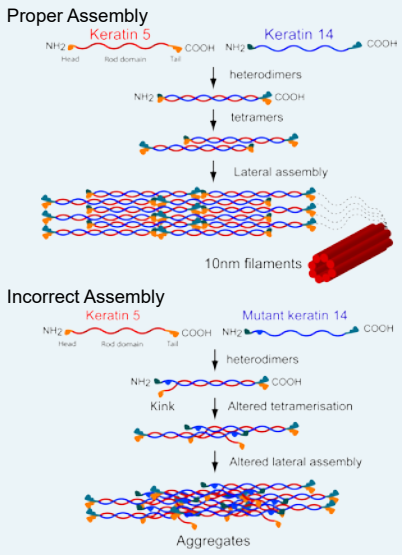


Figure 1. Proper and incorrect assembly of keratin 5 and keratin 14 into filaments.

II. Aim of the study

We designed allele-specific siRNAs targeting frequent pathogenic *KRT5* and *KRT14* variants (p.Glu477Lys and p.Arg125Cys respectively) underlying severe EBS. Candidate siRNAs were tested *in vitro* on primary keratinocytes of patients carrying these variants and their efficacy was assessed by counting keratin aggregates using confocal microscopy and 3D live imaging (holotomography).

III. Methods

Primary keratinocytes were transfected with siRNAs and subjected to a hypoosmotic shock using 150 mM urea to induce keratin filament network reformation. Cells were then processed for K14 and K5 immunostaining to visualize keratin aggregates by confocal microscopy or were observed using 3D live cell imaging. Aggregates from IF staining were counted automatically using a machine learning CellPose cyto2 model, Ilastik and FIJI (4,5).

IV. Imaging of EBS keratinocytes

Candidate siRNAs were transfected in primary healthy and EBS patients keratinocytes using lipofectamine RNAimax and incubated for 48h. Cells were then subjected to a hypoosmotic shock followed by K5 and K14 immunostaining and laser scanning confocal microscopy or were analysed by live holotomography using Nanolive microscope (Figure 3). Each experiment was repeated 3 times to ensure statistical power.

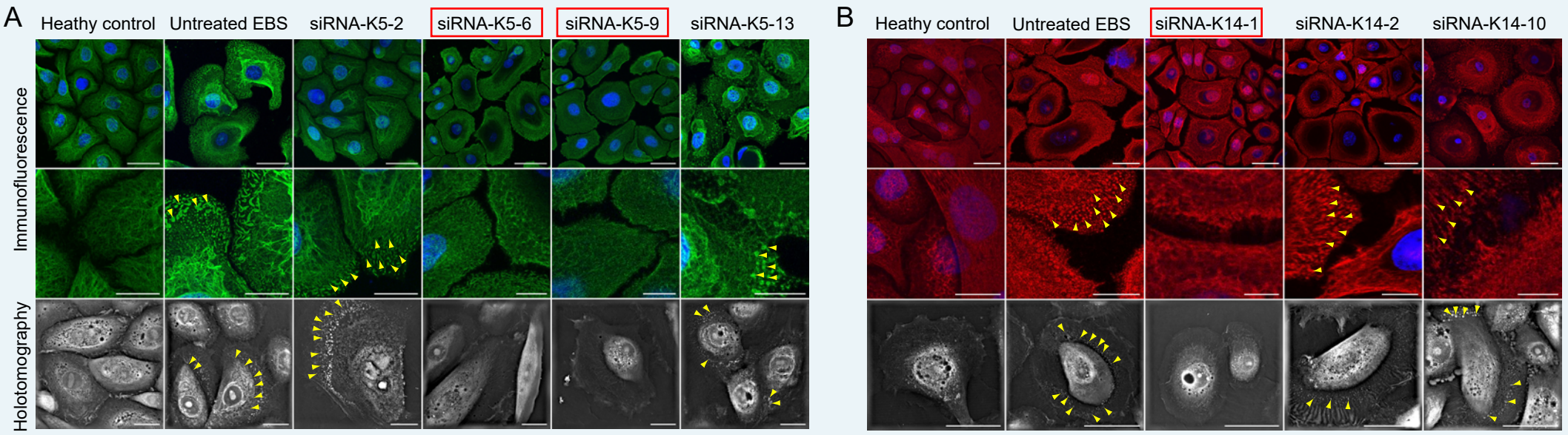


Figure 3. Immunofluorescence and holotomography images of EBS-KRT5-E477K (A) and EBS-KRT14-R125C (B) primary keratinocytes treated with 10nM of candidate siRNAs targeting the p.Glu477Lys variant in KRT5 or the p.Arg125Cys variant in KRT14, respectively. Keratin aggregates are indicated by arrowheads. Boxed siRNA show a strong reduction in detectable keratin aggregates. Scale bars = 20 µm.

V. Keratin aggregate counting

Keratin aggregates were counted using the developed FIJI macro for each conditions. Data were processed and statistical tests were performed with GraphPad Prism (Figure 4).

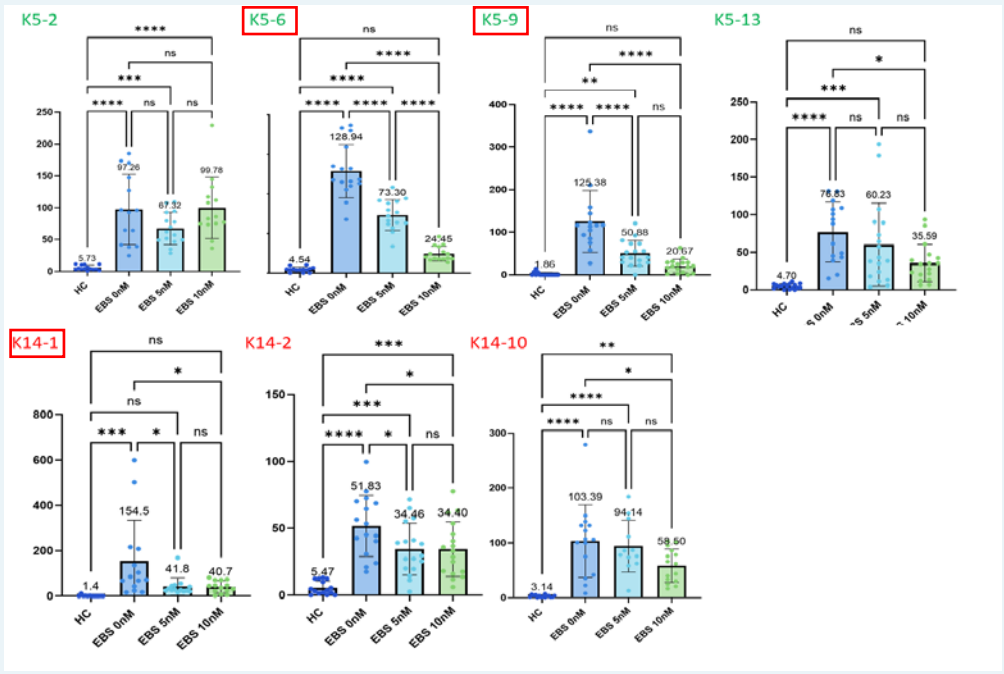


*Figure 4. Keratin aggregate counts per cell in EBS primary keratinocytes treated with the candidate siRNAs. Each dot respresents the average quantity of aggregates per cell. About 100 individual cells were counted for each condition. Boxed siRNA showed the best efficacy. Statiscal analysis was performed using graphpad Prism software (Mann-Whitney test, *:p<0.05; **:p<0.01; ***:p<0.001; ****:p<0.0001.)*

VI. Conclusion and perspectives

Candidate siRNAs were tested in patients' primary keratinocytes carrying the p.Glu477Lys or p.Arg125Cys pathogenic variants. A bioinformatic pipeline was developped to automatically recognize and count keratin aggregates using AI-based softwares. The results indicate that siRNA-K5-6 and siRNA-K5-9 demonstrated the best reduction in aggregate number in *KRT5* mutated EBS keratinocytes (respectively 81% and 83 % reduction compared to untreated cells, p<0.001), and that EBS-K14-1 had the best reduction in number of aggregates in *KRT14* mutated EBS keratinocytes (73% reduction compared to untreated cells, p<0.05). 3D live holotomography allowed to visualize keratin aggregate formation and dynamic in live cells and results were consistent with immunostaining results. Interestingly, the two different mutations led to the formation of aggregates of different size and shape. Selected siRNAs will be further tested *in vivo* in a xenografted nude mice model using systemic or local delivery of naked, conjugated or LNP-formulated siRNAs.

References

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