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## Persistent failures in gene repair

To the editor:

Several recent reports describe the use of chimeric RNA/DNA oligonucleotides (RDOs) to alter DNA sequences. This targeted gene correction strategy, also called chimeraplasty, initially was shown to change episomal sequences<sup>1</sup>, but various examples of altering genomic sequences in both mammalian<sup>2-5</sup> and plant cell systems<sup>6,7</sup> have since been described. DNA sequence alterations have also been achieved in nuclear or cell-free extracts<sup>8,9</sup>. This novel RDO technology holds promise as a means to correct point mutations in disease genes and would have several advantages over conventional gene therapy strategies relying on gene addition. Although the number of papers reporting successful usage of the RDO technology is slowly growing, the number of independent groups from which these studies derive does not.

The basic design of a chimeric oligonucleotide is the same in all studies: double-hairpin folded 68-mers with a chimeric DNA and 2'-O-methyl RNA backbone. The ability to form intramolecular hybrids should protect the RDOs against cellular exonucleases; the RNA residues are methylated, which also prevents degradation. Once transported into the nucleus, the RDO is thought to bind to the DNA target on the basis of a homology region 25 base pairs in length. It is postulated that the presence of the RNA residues makes base pairing more effective. Recombinase activity may then form intermediate structures, and non-matching base pairs are assumed to attract the mismatch-repair protein machinery. The exact mechanism of RDO-mediated sequence exchange, however, is still unknown and needs to be clarified.

Two recent reports describe modifications of the original RDO design and its effects as measured by *in vitro* reactions in nuclear extracts<sup>10,11</sup>. These studies indicate that a mismatching base in the all-DNA strand alone is capable of inducing sequence exchange, whereas a sole mismatch in the RNA residue-containing strand is not. It was also observed that 68-mers only consisting

of DNA residues could alter sequences *in vitro*, whereas the same constructs failed *in vivo*.

To investigate the potential of chimeric oligonucleotides in the therapy of heritable skin diseases, we have studied RDO technology in immortalized keratinocytes derived from two patients with epidermolysis bullosa who had homozygous mutations in the keratin 14 (*KRT14*) and the type XVII collagen gene (*COL17A1*), respectively (see Fig. 1). Both mutations result in absence of the corresponding proteins. Therefore, our immunofluorescence microscopy-based assay system, which uses specific monoclonal antibodies for detecting corrected cells, is of very high sensitivity. For both lines, we established efficient transfection protocols by testing several transfection agents and monitoring the nuclear uptake of fluorescently labeled oligonucleotides by laser-scanning fluorescence microscopy. Over an extended period of time, we carried out several RDO transfection-correction experiments with both the keratinocyte cell lines. These also included experiments with UVB-irradiated cells in an attempt to activate the DNA repair machinery.

To date, no mutation corrections have been observed. Attempts to alter the same epidermolysis bullosa genes in lymphocytes also failed. In addition, efforts to reproduce RDO experiments described in the literature, such as  $\beta$ -globin in lymphocytes and coagulation Factor IX in liver cells, have also been unsuccessful. In these latter cases, however, the less sensitive PCR/restriction-fragment-length polymorphism analysis system was used to

detect sequence alterations.

Nevertheless, during a working visit to Kyonggeun Yoon's laboratory at Thomas Jefferson University (Philadelphia, PA), one of us (G. van der Steege) has obtained limited success with a melanocyte cell line derived from an albino mouse and a RDO designed to correct a mutation in the tyrosinase gene. Yoon and colleagues, who are gratefully acknowledged, have successfully applied the RDO technology in several cell systems, including this albino melanocyte cell line<sup>5,12</sup>. The above-mentioned correction of the tyrosinase mutation occurred only once in a particular series of five experiments, as demonstrated by pigmentation of a couple of cells in the culture dish. This success was achieved with an RDO synthesized by Eurogentec (Seraing, Belgium), our regular supplier of RDOs. This particular experiment thus validated the quality of the RDOs derived from Eurogentec. An unexpectedly high variability of correction frequencies with the melanocyte line has been described but, despite using the very same cell line and RDO, we were in all our attempts thus far unable to reproduce any positive result in our laboratory in Groningen.

The reasons for the persistent failure of the RDO technology are unknown. Insufficient quality of the synthesized RDO is unlikely to be the major problem, in view of the tyrosinase correction results. A good RDO quality (e.g., correct synthesis length and purity) is an obvious prerequisite, but poor RDO quality cannot entirely explain the lack of success experienced by others and us. It may be that the choice of keratinocytes as the study system is not opti-



**Figure 1.** Sequences of the genomic targets and the RDOs used in keratinocyte correction experiments. The cell line with the COL17A1 mutation (A) is homozygously deleted for a GC base pair at position 2342 (GenBank accession no. M91669), leading to absence of type XVII collagen. The 68-mer C17-RDO sequence is designed to align with the genomic sequence around the mutated position and to re-introduce the deleted base pair. The keratin 14 cell line (B) carries a homozygous mutation in the 3' splice site of intron 1 of *KRT14*, leading to aberrant splicing and truncated, if any, protein. The K14-RDO should correct the mutated base pair.



mal. Variation among cell types and a lower responsiveness of keratinocytes with respect to RDO-mediated sequence changes have been described<sup>13</sup>. However, this does not explain the failure to be complete, as an "all-or-nothing" principle in this is unlikely. Our ongoing experiments include *in vitro* reactions using nuclear extracts and the development of a mutated reporter gene system, enabling sensitive monitoring of correction frequencies in different cell lines and systems. However, preliminary results with this latter, sensitive system, used to study episomal correction in CHO cells, also indicate complete failure of the RDO technology.

We believe that the persistent failure to implement the RDO technology is noteworthy. The complete lack of success hampers further studies and frustrates the usage of this theoretically tempting method. We would like to stress that, despite our disappointing experiences, we do not denounce the RDO technology as being invalid or objectionable. However, it may be of general concern that a broad application of this technique is still to be awaited, despite the number and the extent of positive reports, especially of some *in vivo* studies<sup>14-16</sup>. An international collaboration with free exchange of results, cell lines, and RDOs may not only speed up the elucidation of the still unknown mechanism behind RDO-mediated sequence change, but also prove (or disapprove) its applicability. Such a call for a "chimeraplasty consortium" of course includes an appeal to "the happy few" who have positive experiences with this technology to participate.

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### Immunex's Enbrel enrollment program

To the editor:

I am deeply concerned that the article entitled, "Immunex takes premature step to guarantee Enbrel market share," which appeared in the February issue of *Nature Biotechnology*, contained serious factual errors. These errors and the inaccurate conclusions of the article's author, Debra Robertson, are misleading and damaging.

Robertson states that the enrollment program was developed to boost short-term sales of Enbrel. The opposite is true. At this time, only current Enbrel users have access to the drug in order to manage demand during this time of temporarily limited supply. We feel the three following points clarify Immunex's enrollment program and address the major inaccuracies in the article:

(1) Demand for Enbrel has already reached current supply, necessitating the enrollment program to help ensure uninterrupted therapy for current Enbrel users. The program was not designed to increase year-end sales (as erroneously stated in the story).

(2) Enbrel can be used alone, or in combination, with other disease-modifying antirheumatic drugs (DMARDs). The article incorrectly states that the company is limiting patients to the exclusive use of Enbrel.

(3) The Rhode Island facility, when complete, will double current capacity, not produce \$1.5 billion of Enbrel alone, as indicated in the story.

Immunex has communicated with thousands of doctors, pharmacists, patients, and patient organizations to help patients through

this challenging situation. It is irresponsible that neither the writer nor the journal checked with the company or any of these sources when crafting the story and got it so wrong.

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Debra Robertson replies:

Several sources were used in preparing this article, including interviews with Immunex spokeswoman Robin Shapiro and Caroline Copithorn, a biotechnology analyst at Morgan Stanley Dean Witter (New York), who specializes in Immunex. I spoke at length to Shapiro about the enrollment program and its manufacturing plans. When questioned about the timing of the program, sales numbers, and production capacity, Shapiro was vague and indicated that supply and demand were fluid and that "anything could happen." Unsatisfied, I talked to Copithorn about the numbers, which still failed to add up. According to Copithorn's calculations, the enrollment program would lead to an increase in fourth-quarter sales, and the projected sales would likely outstrip Immunex's ability to manufacture the drug using its present facilities.

Although Immunex argues that the goal was not to limit access of patients to the drug, there was a general feeling among physicians that they had to sign their patients up before the end of the year (and thus purchase the drug) to ensure future access to the treatment. Indeed, in the 27 November 2000 release of "The Pink Sheet," Immunex themselves state, "Patients considering initiating therapy with Enbrel are advised by

Enrollment program materials to discuss with their physicians whether they should be registered now." Prospective patients and their doctors "should be aware that supplies of Enbrel will likely be limited in the future."

With regard to the repercussions of the enrollment program on the use of other DMARDs, Immunex stipulated that enrolled patients had to use the drug continuously to remain in the program. If a patient left the program to try another treatment and it did not work, the patient was then required to go to the bottom of the list. Patients are not likely to try other treatments under such circumstances.

It was interesting to learn about current capacity at the Rhode Island facility. Immunex should brief their spokesperson so that figures such as these are provided when requested by journalists.

