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van den Akker, Peter

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Chapter 10

A ‘late-but-fitter revertant cell’ explains the high frequency of revertant mosaicism in epidermolysis bullosa

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Revertant mosaicism (RM), or ‘natural gene therapy’, is the phenomenon in which germline mutations are corrected by somatic events. In recent years, RM has been identified in all major types of epidermolysis bullosa (EB). Moreover, RM was proven in 60% of Dutch patients with generalized non-Herlitz junctional EB (JEB-nH-gen) and, based on clinical inspection, seems to be present in all JEB-nH-gen patients. We therefore hypothesize that RM should be expected in all patients with recessive EB (REB). The mathematical developmental model we used to test this hypothesis shows that the probability $P$ that single nucleotide reversions occur in REB patients’ skin approaches 1. Reverse mutations are expected to occur frequently (estimated $216\times$). However, they should occur early in embryogenesis to produce recognizable revertant patches, which is expected in only one per ~10,000 patients. To explain this underestimate compared to our clinical observations, we postulate the ‘late-but-fitter revertant cell’ hypothesis: reverse mutations arise at later stages of development, but provide revertant cells with a selective growth advantage that drives the development of recognizable patches. Revertant cells will theoretically also be present in other genetic diseases, which might offer novel therapeutic options if these cells can be identified and isolated.
Revertant mosaicism was first reported in Lesch-Nyhan syndrome and subsequently in several other genetic syndromes. In 1997, RM was first reported in a skin disorder, EB, a group of heritable blistering disorders caused by mutations in the components of the epidermal-dermal adhesion complex. Although long considered extraordinary, it has become evident that RM is common in REB. In this study, we mathematically investigated the hypothesis that RM can be expected in all REB patients.

We used a mathematical developmental model in which the number of basal keratinocytes (BKs), $n$, starting with a single embryonic ectodermal progenitor cell, increases exponentially, $n = 2^y$, each generation, $y$. We focused on single nucleotide corrections, as more than half of the reversions observed in REB involve such mutations. In our model, the probability that reverse mutations occur depends on four factors: (1) the total number of BKs in an adult human body ($36 \times 10^9$, $\sim 2^{25}$), (2) the number of mitoses required to obtain this number ($36 \times 10^9 - 1$), (3) the probability of a nucleotide alteration per nucleotide per mitosis ($\sim 1 \times 10^{-9}$), and (4) the number of nucleotides that correct the germline mutation when altered (assumed 6). See Supplementary Methods and Table 1 for the mathematical calculations and quantitative estimates used. Our calculations show that, during the total number of mitoses, the probability $P$ that at least one reverse mutation occurs approaches 1 and reverse mutations are expected to occur 216 times in an adult human body. This indicates that reverse mutations should not be considered extraordinary, but rather an event that can be expected to occur with mathematical certainty in REB patients carrying mutations that are correctable by single nucleotide mutations.

The question is whether the revertant cells will induce revertant skin patches that are clinically recognizable. To induce recognizable patches, i.e. patches covering at least 1 cm$^2$ (corresponding to $2 \times 10^6$ revertant BKs) (Figure 1), reverse mutations in our model should arise in

![Figure 1. The smallest revertant patch observed in any of our patients. Photograph of the revertant patch on the dorsal middle finger of patient EB093-01 with JEB-nH-gen due to compound heterozygous COL17A1 mutations. The size of the patch was approximately 2 cm$^2$ and it allowed him to wear a wedding ring. Revertant patches should therefore be 1 cm$^2$ minimum in order to be clinically recognizable.](image)
the 14th cell generation the latest \(2^{14} = 16,384\) BKs (Supplementary Methods, Table 1). This is supposed to be to be before the 4th week of embryogenesis.\(^{24}\) The probability \(P\) that at least one reverse mutation will occur in the first 14 generations is \(~0.0001\). A clinically recognizable revertant skin patch is therefore expected in only one per \(~10,000\) patients (\(~1/0.0001\)).

### Table 1. Quantitative estimates and calculated values in the mathematical developmental model \(n = 2^n\).

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</tr>
<tr>
<td>0.070 mm</td>
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<td>18</td>
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<tr>
<td>1.348 mm</td>
<td>DEJ length corresponding to 1 mm of skin length</td>
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<td>20</td>
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<tr>
<td>6 mutations</td>
<td>Expected number of novel point mutations in diploid genome per mitosis</td>
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<td>216x10(^7) mutations</td>
<td>Expected number of point mutations in basal keratinocytes of adult human body</td>
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<td>–1</td>
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<td>Calculated in this study</td>
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<td>25</td>
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</table>

DEJ, dermo-epidermal junction; REB, recessive epidermolysis bullosa
This conclusion strongly contradicts our clinical observations that revertant patches are present in all Dutch JEB-nH-gen patients. This underestimate can only be partly explained by the developmental model itself. First, the model ignores cell loss due to biologically important processes like apoptosis and differentiation. As the probability of reverse mutations depends on the number of mitoses needed to obtain final cell numbers, integrating cell loss increases the probability of reverse mutations by means of increasing the total number of mitoses. To estimate the possible effect of cell loss, we used a conservative model in which 90% of new cells in each generation are lost, $n = 1.1^y$, as the exact cell loss rate is unknown. In this model 359×10^9 mitoses (255 generations) are required to obtain the 36×10^9 BKs of an adult human body and 19.6×10^6 mitoses (152 generations) for the 2×10^6 cells of a recognizable revertant patch, assuming identical growth rates for mutant and revertant cells. The reverse mutation should then occur no later than in the 103rd generation (18×10^3 BKs, 180×10^3 mitoses). Loss of 90% of cells in each generation thus increases the required number of mitoses ~10× compared to the $n = 2^y$ model. The probability of recognizable patches ($P = 0.0011$) consequently also increases a factor 10. Hence, even with 90% cell loss in each generation, recognizable revertant patches could be expected in only one per ~909 patients (~1/0.0011).

Second, we assumed that all BKs are equal and need to be revertant. In reality, a hierarchy among BKs is accepted: long-term proliferating epidermal stem cells produce clonogenic transient amplifying cells that provide the other (transient amplifying) BKs to the epidermal proliferating units, which collectively form skin patches. Only a limited number of epidermal stem cells thus need to be revertant in order to acquire recognizable revertant patches. As the exact number of epidermal stem cells per mm^2 is unknown, the critical number of revertant stem cells needed for recognizable patches is unknown, but it will be smaller than the number used. To estimate the possible effect of BK hierarchy, we assumed an epidermal stem cell:‘other’ BK ratio of 1:1,000 (based on review by Strachan and Ghadially, ref. 25). This reduces the number of mitoses in which reverse mutations can occur 1,000-fold. Consequently, the probability $P$ of at least one reverse mutation decreases substantially to 0.19 (vs. ~1). As the ratio of cells in total skin vs. the recognizable patch remains unchanged, both are reduced 1,000-fold by considering epidermal stem cells, the probability of a recognizable patch is unchanged, i.e. one per ~10,000 patients.

Third, we used the average human somatic cell mutation rate (1×10^-9/nt/mitosis), as data on the actual mutation rate for BKs are not available, which could be substantially higher. However, only an unrealistically 100,000-fold increase in mutation rate to at least 1×10^-4/nt/mitosis could explain the occurrence of reverse mutations in the 14th cell generation in every patient.

Another, more likely, explanation is that revertant cells possess a selective growth advantage over their non-revertant neighbors. If revertant cells are able to survive longer or to divide more times, reverse mutations would be allowed to occur at later stages of development when their occurrence is more likely (Figure 2). In blood diseases involving hematopoietic bone marrow stem cells, such a growth advantage is believed to explain the ability to detect reverse mutations.
and clinical improvement.\textsuperscript{27-32} As skin is also a regenerating organ, similar mechanisms may be active. Additionally, growth potential differences between wild-type and mutant cell lineages were shown for other diseases like neurofibromatosis type I and Wiskott-Aldrich syndrome.\textsuperscript{33,34}

Several arguments support a selective growth advantage of revertant REB cells. First, revertant patches are not confined to the lines of epidermal development, Blaschko’s lines.\textsuperscript{35,36} Rather, they appear to develop in one Blaschko-line segment and then expand centrifugally into adjacent segments (Figure 3). This observation indicates that reverse mutations occur when epidermal formation from Blaschko-lines is completed, i.e. after the end of the 4\textsuperscript{th} week.\textsuperscript{24} This is in line with our conclusion that reverse mutations likely occur in later stages when the body of cells has already reached a critical amount.

Second, a segmental phenotype due to somatic second-hit mutations has not been reported in carriers of REB mutations. As there are more target nucleotides for gene silencing than for correction, the probability that the wild-type allele is silenced in carriers far exceeds the probability of reverse mutations in REB patients.\textsuperscript{15} We speculate that the lack of a segmental phenotype in carriers is due to a significant growth disadvantage of cells harboring second-hit mutations. Third, the expanding revertant patches in ichthyosis with confetti seem to perfectly demonstrate a growth advantage of revertant skin cells.\textsuperscript{37,38}

The 29\textsuperscript{th} cell generation is the first where more than one reverse mutation is expected to occur. Revertant cells need another 21 generations to acquire the $2\times10^6$ cells of a minimal recognizable patch. They thus need to go through at least 15 (21 vs. 6) more generations than

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**Figure 2.** Selective growth advantage may explain the occurrence of clinically recognizable revertant patches. A. Schematic representation of the ‘bell-shaped’ increase in cell number according to the $n = 2^y$ model starting with a single progenitor. In the later generations, the probability of a reverse mutation approaches 1 asymptotically. However, a reverse mutation that occurs in later generations cannot result in a clinically recognizable patch since the revertant cell cannot go through the required number of mitoses. B. We therefore propose that revertant cells have a selective growth advantage, e.g. they possess the ability to go through more generations than their mutant neighbors. This would allow reverse mutations to occur at later stages and still result in visible patches. Green area: revertant area. Horizontal bar: size of minimal clinically recognizable patch ($\geq 1$ cm$^2$). Gen, generation of cells. $N_{\text{BK0}}$, number of basal keratinocytes. $N_{\text{som mut}}$, expected total number of somatic mutations. Mut/Nt, average number of mutations per nucleotide. $P_{\text{no rev}}$, probability of no reverse mutation. $P_{\geq 1 \text{ rev}}$, probability of at least one reverse mutation.
their mutant neighbors. Future studies need to clarify whether the revertant growth advantage is indeed of this order of magnitude. Two different mechanisms may act synergistically in providing the enormous revertant growth advantage: first, revertant cells may survive longer and, second, may possess the ability to go through more cell divisions.

Despite their likely growth advantage, the majority of patients do not report expansion of revertant patches in adulthood. This suggests a limited time frame of growth advantage with an unknown end point. In a recently reported child with Kindler syndrome the revertant patch expanded relatively to body size until the age of 8 years.\textsuperscript{6} This indicates that revertant patches may have the ability to expand at least until this age. Why they lose their ability to expand further after a certain time point is unknown. Further studies comparing the genetic/epigenetic constitution of revertant and non-revertant cell lineages could help solve this issue.

In conclusion, our developmental model suggests that REB patients with mutations that are correctable by single point reversions carry multiple reverse mutations in their skin. Reverse mutations should, however, arise early during embryogenesis to produce clinically recognizable revertant patches, which is expected in only one per ~10,000 patients. We therefore postulate the ‘late-but-fitter revertant cell’ hypothesis: reverse mutations arise at later stages of development, but provide the revertant cells with a selective growth advantage that drives the development of revertant patches which are common among REB patients. Moreover, our results indicate that revertant cells will also be present in the affected tissues in many other genetic diseases, provided they contain comparable (stem) cell numbers to skin. If ways can

\textbf{Figure 3.} A selective growth advantage of revertant cells is supported by the patterns of the revertant skin patches. \textbf{A.} Dorsal forearm of an 8-year old boy (EB134-01) with generalized JEB-nH due to the compound heterozygous \textit{COL17A1} mutations c.1260delC/c.3495_3496delCT showing a revertant patch (indicated by blue line). \textbf{B.} The lines of Blaschko on the dorsal forearm as deduced by Blaschko and Happle.\textsuperscript{35,36} \textbf{C.} The lines of Blaschko projected on the revertant patch on the dorsal forearm of patient EB134-01. The revertant patch has clearly exceeded the boundaries of the Blaschko lines and has grown into adjacent Blaschko line segments.
be found to identify and isolate these cells, this could offer novel opportunities for autologic revertant cell therapy, a promising approach already under investigation for REB.39

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SUPPLEMENTARY METHODS

The occurrence of reverse mutations in basal keratinocytes

We have used an exponential model in which the total number of basal keratinocytes (BKs), \( n \), starting with a single embryonic ectodermal progenitor cell, doubles with every generation of cells, \( y \), according to \( n = 2^y \). The total number of BKs, \( n_{\text{total}} \), in an average adult human body is a function of the total body dermo-epidermal junction (DEJ) surface \( S_{\text{DEJ}} \) and the number of BKs per unit DEJ. The \( S_{\text{DEJ}} \) is not identical to the human skin surface \( S_{\text{skin}} \), as it is greatly increased by the presence of rete ridges and dermal papillae. Assuming an average of 6 rete ridges-dermal papillae complexes per mm skin length \((L_{\text{skin}})\), with an average height of 0.070 mm, the DEJ can be described as a sinus function with amplitude 0.035 mm and period 12\(\pi\) (Table 1):

\[
f(x) = 0.035 \cdot \sin(12 \cdot \pi \cdot x)
\]

The exact length of the line described by this sinus, i.e. the actual DEJ length \( L_{\text{DEJ}} \), is calculated by solving the integral of the function (Mathematica v8, Wolfram Research Inc., Champaign, IL)

\[
L_{\text{DEJ}} = F(x) = \int_{a}^{b} \sqrt{1 + (0.42 \cdot \pi \cdot \cos(12 \cdot \pi \cdot x))^2} \, dx
\]

Using \( a = 0 \) and \( b = 1 \) mm \( L_{\text{skin}} \) reveals that 1 mm of \( L_{\text{skin}} \) corresponds to 1.348 mm \( L_{\text{DEJ}} \). Consequently, since the ‘finger like appearance’ of dermal papillae resembles two interfering sinus waves, 1 mm\(^2\) \( S_{\text{skin}} \) corresponds to \((1.348)^2 \approx 1.817\) mm\(^2\) \( S_{\text{DEJ}} \). Rete ridges thus theoretically expand the dermal-epidermal surface contact by 81.7%.

The number of BKs was measured to be between 15,000 and 20,000 to 30,000 per mm\(^2\) skin.\(^{20,21}\) Assuming that BKs have a circular base of diameter \(~10\) \(\mu\)m (ref. 19, own observations, data not shown) and surface \(~78.5\) \(\mu\)m\(^2\), approximately 12,739 BKs are expected per mm\(^2\) \( S_{\text{DEJ}} \) (1 mm\(^2\) \( S_{\text{DEJ}} \) / 78.5\(\times\)10\(^{-6}\) mm\(^2\) per BK) and 23,146 BKs per mm\(^2\) \( S_{\text{skin}} \). Taken together, we decided to use 20,000 BKs per mm \( S_{\text{skin}} \) in further calculations.

Assuming a total human adult body surface of 1.8 m\(^2\) (corresponding to 3.3\(\times\)10\(^6\) mm\(^2\) \( S_{\text{DEJ}} \)) the total number of BKs in an adult human body \( n_{\text{total}} = 1.8\times10^6\) mm\(^2\) \( \times \) 20,000 BKs/mm\(^2\) = 36\(\times\)10\(^9\). This number is reached in the 35\(^{\text{th}}\) generation, after a total of 36\(\times\)10\(^9\) – 1 mitoses. Given a per nucleotide point mutation rate of approximately 1\(\times\)10\(^{-9}\) per mitosis\(^{14-16}\) and 6\(\times\)10\(^9\) nucleotides per diploid genome,\(^{22}\) ~6 novel point mutations may be expected in the daughter cell’s genome in each cell division. In the 35\(^{\text{th}}\) generation of cells, the total expected number of point mutations that have occurred in the body’s BKs is approximately 6 \(\times\) total number of mitoses = 216\(\times\)10\(^9\), indicating that each single nucleotide will be mutated on average 36 times in the adult human skin.
Alteration of one of the nucleotides of a nonsense codon will usually change the nonsense into a sense codon. If nonsense mutations are present on both alleles, there are therefore 6 target nucleotides for a reversion, thereby ignoring the small probability that the nonsense codon will be changed into one of the other two possible nonsense codons. For frame-shift and splice-site mutations, the number of potential target nucleotides for reversion events are usually unknown but theoretically numerous. For missense mutations this number is probably less than 6. We therefore decided to use the number of 6 target nucleotides for nonsense codons in our calculations. A larger or smaller number of target nucleotides would not affect the order of magnitude of the probability of the occurrence of reverse mutations. The probability of a reverse point mutation per mitosis in case of 6 target nucleotides is thus \( p_{rev} = 6 \times 1 \times 10^{-9} = 6 \times 10^{-9} \). The probability that no reverse mutation will occur during the total number of mitoses until the 35th generation \( (P_{not}) \) is

\[
P_{not} = \left(1 - p_{rev}\right)^{\text{total number of mitoses}} = \left(1 - 6 \cdot 10^{-9}\right)^{(36 \times 10^9 - 1)} = 1.6 \cdot 10^{-94}
\]

Therefore, the probability \( P \) that at least one reverse mutation occurs during these mitoses is

\[
P = 1 - P_{not} \approx 1
\]

The expected number of reverse mutations in \( 36 \times 10^9 - 1 \) mitoses is \( (36 \times 10^9 - 1) \times 6 \times 10^{-9} = 216 \).

**Clinically recognizable revertant patches**

One cm² of revertant \( S_{skin} \) corresponds to 100 mm² × 20,000 BKs/mm² = 2×10⁶ revertant BKs. According to the \( 2' \) model, it takes 21 generations to obtain this number from one single revertant ancestor. This implies that a reverse mutation should have arisen at the latest in the \( y = 35-21 = 14^{th} \) generation, corresponding to a stage of \( 2^{14} = 16,384 \) BKs. Considering that in early embryonic stages rete ridges and dermal papillae are not yet developed,24 the reverse mutation should have arisen at the embryonic stage where the embryo’s body surface was less than 2 mm². For comparison, a 4-week embryo measures approximately 5 mm in length and, if compared to a cylinder with height = 5 mm and radius = 0.5 mm, its body surface is in the order of 17 mm², indicating that a reverse mutation should arise before the 4th week of embryonic development in order to give rise to a clinically recognizable revertant skin patch.

The probability \( P_{not} \) that a reverse mutation does not arise in the first 14 generations is

\[
P_{not} = \left(1 - 6 \cdot 10^{-9}\right)^{(16,384+1)} = 0.9999
\]

and the probability \( P \) that at least one reverse mutation arises in the first 14 generations is
The probability that a reverse mutation does not arise in the first 14 generations is
\[ P = 1 - 0.9999 = 0.0001 \]

Therefore, a clinically recognizable revertant skin patch is expected once in only \( \sim 1/0.0001 = 10,000 \) patients.
REFERENCES

Mathematics of revertant mosaicism


