Epigenetic editing

Goubert, D.; Beckman, W. F.; Verschure, P. J.; Rots, M. G.

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Epigenetic Editing: towards realization of the curable genome concept

D Goubert[1], WF Beckman[2], PJ Verschure[2] and MG Rots[1]

[1]Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713 GZ Groningen The Netherlands (m.g.rots@umcg.nl)

[2]Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, the Netherlands (p.j.verschure@uva.nl)

Recent developments in biotechnology have enabled us to modulate DNA sequences in a very precise way. Moreover, these technologies enable us to alter the epigenetic composition of the genome thereby changing gene expression patterns, leaving the primary DNA sequence intact. This new approach, so-called Epigenetic Editing, holds great promise to permanently reprogram cell identity. Epigenetic editing refers to the modification of epigenetic marks of defined genes for instance to re-express epigenetically silenced genes. Reprogramming the epigenetic composition and hence gene expression patterns of differentiated cells is no longer science fiction. Of course it is important to place such approaches into societal context and consider to what extent society accepts interference at the epigenetic level. The question remains: do we envision this development as an ethical obligation towards future generations, enabling us to improve mankind and human health?; and more importantly: do we need to consider restricting the use of any novel tool that allows these implications? Unfortunately, this technology could be used by so called 'biohackers' possibly provoking unwanted biological alterations. Clearly this biotechnological evolution is causing science friction, urging society, scientists, ethicists and lawyers to enter debates and to change policies. At the same time, we need to increase our awareness and not let policies impede with technological breakthroughs having such a promising impact on therapeutic tools and disease cures.

Epigenetic Editing

In 2012, Jennifer Doudna and Emmanuelle Charpentier, professors from the States and Sweden/Switzerland described how the bacterial immune system, called CRISPR-Cas, can be utilised to inactivate genes (1). This prokaryotic immune system is directed against foreign, invasive genetic elements (e.g. from viruses or phages). When bacterial cells are faced with an invasive pathogen, copies of the exogenous DNA are made and a small sequence of the foreign DNA, known as a spacer, is integrated into the CRISPR locus of the bacterial genome. Spacers are transcribed into a set of small RNA guides. Subsequently, should the bacterial cell encounter the same pathogen, any foreign DNA

will be detected by small RNAs that also guide for its destruction. The small RNA binds to the invading DNA and directs its cleavage by Cas9 nucleases. The Cas9 enzyme introduces DNA double strand breaks at its binding site which are then repaired by the cell in an inaccurate way often resulting in gene inactivation. Importantly, when in addition, DNA fragments that are homologous to the recognized sequence are introduced into the cell, the cell can repair the damage by homologous recombination. Researchers have shown that this CRISPR-Cas prokaryotic immunity can also be used in eukaryotic cells to deliver the Cas9 nuclease together with a customizable single guide RNA (sgRNA), to target defined genes. Using this CRISPR-Cas immune/response system researchers can correct genetic mutations or introduce completely new pieces of DNA by designing the proper sgRNA and homologous DNA sequences. The CRISPR-Cas- approach has revolutionized biomedical sciences and a patent war is currently ongoing between the inventors of the approach.

Epigenetics concerns the study of heritable changes in gene expression, that are affected by other mechanisms than changes in the primary DNA sequence. Epigenetic editing is one of many possibilities realized by precise genome engineering, providing a way to re-write epigenetic marks and thereby alter cellular gene expression patterning. Epigenetic gene regulation is crucial for cell type specific gene expression patterns in higher eukaryotes, conferring stability of the cellular phenotype, while allowing changes in expression in response to environmental or developmental cues. Derangements in epigenetic gene regulation have severe effects on cell behaviour and contribute to the maintenance of a diseased state. Nowadays, it is well accepted that many diseases are associated with an altered epigenetic landscape. Intriguingly, epigenetic marks are considered to be stably maintained (as they underlie cell identity), whereas epigenetic enzymes driving the epigenetic composition are in principle largely influenced by environmental conditions and thus able to evoke a change in the epigenetic state. This explains the recent observations that nutrition and life style choices are associated with alterations in the composition of the epigenomic landscape. Moreover, since the epigenetic composition is reversible it opens opportunities for therapeutic intervention at the epigenetic level. Many efforts are ongoing to design inhibitors of epigenetic enzymes (2). FDA-approved and novel epigenetic drugs (acting in a genome-wide, unspecific manner) have shown preclinical effectiveness at restoring therapy sensitivity for the treatment of haematological malignancies. However, these epigenetic drugs bring along genome-wide effects and influence unwanted targets, preventing their wide spread application. Gene specific epigenetic alterations can be induced by targeting epigenetic enzymes, i.e. so-called epigenetic writers or erasers to a given genomic location using DNA binding platforms, such as CRISPR-Cas. This novel technology is referred to as Epigenetic Editing (3).

The Epigenetic Editing laboratory headed by professor Rots at the University Medical Centre Groningen, studies epigenetic deregulation of various diseases with a focus on identifying which

epigenetic alterations are associated with phenotypic disease deregulations and can serve as targets for therapeutic intervention (4-7). To validate the candidates, molecular tools are designed that consist of at least two components, i.e. a DNA binding platform component and an epigenetic effector domain component (3, 8, 9) (Figure 1). The DNA-binding platform is designed such that it binds to a number of base pairs of the desired gene. This DNA-binding platform thus serves as a GPS to find its desired destination. The effector domain is designed such that it consists of an epigenetic writer or an eraser that is able to alter defined epigenetic marks. The writer or eraser thus rewrites the epigenetic marks at the given location to turn off or on the gene that is instructed by the epigenetic marks. Epigenetic editing enables the reprogramming of epigenetic composition and alters genome functioning without affecting the DNA-sequence itself (10). Therefore, it possess an advantage over genetic editing and this technique is considered less radical as the effects are less likely to be inherited by the next generation.

Epigenetic reprogramming

To illustrate the potential impact of gene-specific epigenetic interference technologies: in cancer cells tumor suppressor genes are often genetically mutated, which makes them unable to perform their function to suppress tumor growth. Even more frequently though these tumor suppressor genes are epigenetically altered such that these tumor suppressor genes are not transcribed. This implicates that the tumor is no longer suppressed and grows without control. Conversely, in cancer cells so-called oncogenes that ensure continuous tumor cell division are often deregulated and permanently switched on. If we could correct the epigenetic mechanisms underlying such cancer gene expression pattern alterations we might contribute to reprogramming cancer into a chronic yet treatable disease instead of leading to a terminal disease state (11).

Alternatively, we might design tools to actively interfere with the development of resistance: Most women with early ER positive breast cancer are treated with oral adjuvant endocrine therapy. In pre- and postmenopausal women different clinical strategies are employed to prevent endocrine signalling and cell proliferation. Selective ER modulators and downregulators (SERMs and SERDS) are the treatments of choice for premenopausal women and aromatase inhibitors (AIs) for postmenopausal women (12). Often, these medications are effective in preventing disease relapse and death from the primary tumor, however in 30-40% of the initially responsive patients, relapses and a progression to metastatic disease can occur, resulting in a poor prognosis. Acquired resistance reflects tumour cell adaptation involving molecular changes that allow continued cell proliferation providing cells with a selective advantage. Acquired resistance to endocrine therapies is a long-term process in which genetic alterations act synergistically with epigenetic changes. The acquired resistance is the result of a complex interplay of factors being involved in various signalling pathways (13).

Epigenetic reprogramming, more specifically the epigenetic composition of regulatory elements (e.g. enhancers), is an integral component of cellular differentiation that facilitates lineage-specific transcriptional programs (14). Recently it has been demonstrated that genome-wide epigenetic reprogramming (DNA methylation, posttranslational histone modifications and chromatin compaction) induces changes in gene regulatory networks and underlies long-term endocrine treatment resistance development (15-17). One example of this was published in 2015 by Magnani *et al*., where it was shown that endocrine therapy resistant cells were capable of activating endogenous cholesterol pathways through alterations in epigenetic histone modifications involving large topological domains and the activation of superenhancers, both *in vivo* and *in vitro* (18). The overall effect of increased cholesterol concentration was to activate the estrogen receptor, circumventing the cells reliance on estrogen. Other, epigenetic mechanisms of achieving this include DNA hypermethylation of *ESR1* (19) and overexpression of HDAC1 (20), both of which silence the expression of the estrogen receptor, and allow other growth pathways to become dominant.

Resistance to endocrine therapy is an urgent medical problem. To proceed in this field, we need to identify and target cancer-specific epigenetic changes in individual patients during the course of resistance development and for this we need diagnostic tools (e.g. tissue biopsies or serum) to monitor, evaluate and predict the epigenetic component of treatment outcome.

From 2015 on, Dr. Verschure at the University of Amsterdam (UvA) started as coordinator of an international research consortium (an Innovative Training Network (ITN) funded by EU H2020 MSCA-ITN-2014) to focus on uncovering the role of epigenetic regulation in resistance development for endocrine therapy in estrogen receptor (ER) positive breast cancer. The EU research consortium entitled 'Epigenetic regulation of endocrine therapy resistance in breast cancer: A systems medicine approach to Predict treatment outcome' (Acronym: EpiPredict) consists of 15 parties, academic institutes and private companies, from 8 different countries, training a multidisciplinary group of 12 PhD students. The PhD students perform their research at 8 different laboratories, i.e. UvA, Imperial College London, the Deutsches Krebs-forschungs Zentrum, the University of Milano-Bicocca, the UMCG, the Hungarian Academy of Sciences, Epiontis GmbH, the Eidgenössische Technische Hochschule Basel.

Within EpiPredict, a systems medicine approach is employed to obtain mechanistic, detailed insights in to how changes of a patient's epigenome can affect gene expression, pathway activation and metabolic rewiring through a defined set of resistance involved pathways. We combine multidisciplinary research strategies and next generation technologies (epigenetic, gene expression, protein pathway activation, metabolic pathway profiling, gene-specific epigenetic interference

technologies and computational approaches). The complex dynamic interactions that determine treatment resistance are virtually impossible to understand from only genome-wide experiments and bioinformatics analysis. Therefore, we establish mechanistic models (21, 22) from research/clinical data enabling re-iterative *in-silico* experimentation. These models predict (dynamic) phenotypic responses upon changes in biological parameters (e.g., availability of ligands) thereby generating new hypotheses and wet-lab experiments to be tested with epigenetic interference technologies to further refine the model. The scientific mission of EpiPredict is to utilize mechanistic understanding of the involved epigenetic regulation and cell type switching underlying endocrine therapy resistance development to explore (i) robust diagnostic/prognostic tools to stratify patients for their likelihood of developing endocrine resistance and (ii) prediction measures for effectiveness of additional drugs counteracting resistance an important step towards tailored treatment-monitoring schedules. We will determine cellular heterogeneity and sub-cell type epigenetic state switching (23) and use CRISPR/Cas-based Epigenetic editing (24) to locally overwrite epigenetic signatures and verify the impact of defined alterations in epigenetic regulation on the ability of cells to end-up in an endocrine resistant state due to a concrete phenotypic switch. Epigenetic diagnostic tools to predict and monitor treatment outcome will open-up an unexplored field of research with great potential for personalized medicine (25).

Dogmas

The Epigenetic Editing group of professor Rots was one of the first laboratories in the world promoting the concept of epigenetic editing (3). At that time, 10 years ago, the general concept of epigenetic editing was treated with quite some controversy making it inherently difficult to overcome existing dogmas. In general, four major objections were raised against the concept: First of all, it was considered to be impossible to re-express epigenetically silenced genes, since these genes were supposed to be inaccessible. Silenced genes were believed to be located in compacted, epigenetically silenced genomic regions, and the transcription machinery was considered unable to access these tightly packed chromatin regions. Secondly, it was unclear whether gene expression is instructed by epigenetic marks or simply an indirect effect of an established gene expression pattern. In principle epigenetic marks were seen as a means of cells to remember their transcriptional program after cell division. Thirdly, at that time it was still inconceivable to consider a way to enable true gene-specific intervention. Luckily, the CRISPR-Cas system provided clear examples of gene-specific modulation of gene expression patterns. In the end, even when the first three considerations would be proven surmountable, it was thought to be impossible to actually tip the gene expression balance from silenced to re-expressed state in a long lasting manner.

One by one, existing dogmas were refuted worldwide: Also the Rots laboratory showed that silent genes are accessible and can be re-expressed (26-31). Moreover, it was shown that genes can be turned off by writing repressive epigenetic marks on, for example, the actively transcribed oncogene *her2/neu* (32-34). This study also refutes the dogma that epigenetic marks are not instructive in determining gene expression levels. Moreover, it was demonstrated that the local removal of DNA methylated CpG sites is sufficient to re-express an epigenetically inactivated gene (28, 35). At this moment the concept of epigenetic editing is reaching acceptance using the easy and cheap CRISPR-dCas approach (36).

There are now several publications demonstrating that placing methyl groups on a gene is able to downregulate gene expression of that gene (37). The pending question is: can we accomplish a permanent reprogramming using a 'one and done' approach. The ambition would be to treat cells one-time, possibly with a combination of targeted epigenetic writers and erasers, allowing rewritten epigenetic marks to be memorized by the cell in a permanent manner. So far, the outcome of such studies focusing on sustained epigenetic editing are controversial (9, 38). The Rots laboratory recently provided the first indications showing sustained re-expression of epigenetically silenced genes by targeting defined epigenetic modifications to the gene of interest, i.e. histone H3 lysine 4 methylation (H3K7me) in combination with or without H3K4me (24).

Currently, many diseases have no clear clinical treatment or cure, however for several diseases we do have in depth knowledge relating to changes in gene expression profiles of disease-associated target genes and downstream genes, for example. In principle epigenetic editing would enable us to reprogram the epigenetic profile of such involved genes, potentially reversing the diseased phenotype. Of course, it is crucial to understand the network wiring of involved genes which is often not straightforward to determine due to the complex behaviour of gene regulatory networks.

Pros and cons

Scientific progression will always be associated with ethical dilemmas: The Good (clinical cures), the Bad (bioterrorism) and the Ugly (“designer babies”). Communication within society is of utmost importance. The public needs to be well informed about new technologies to avoid a situation where new technologies are restrictd or abandoned due to fear, as was the case when gene therapy was first introduced for disease treatment. The first clinical trials using gene therapy focused on correcting for a genetic mutation that caused the phenotype of severe combined immunodeficiency (SCID), so-called “bubble boys”. SCID is an extremely rare genetic disorder characterized by disturbed development of functional T and B cells, resulting in an ineffective immune system. Disease victims (males) are extremely vulnerable to infections and permanently live in quarantine (a plastic house or bubble). By

the end of the last century, twenty SCID boys were treated with viral transduced gene therapy transferring a copy of the healthy gene to the immune cells. Nineteen of the treated SCID boys were eventually cured(39). Although this by itself was a great success (the good), the trial is certainly not recognized as such. As a matter of fact, of the twenty SCID boys that were treated, the gene therapeutic viruses caused leukemia in five patients (the bad). For society, this proved that gene therapy is very dangerous. Four out of five SCID boys with leukaemia were eventually cured of the condition, but unfortunately one deceased. Despite the tragic end of this one patient, the rest of the participants of the first gene therapy trial enjoy a normal life, which without the gene therapy had not been possible. Unfortunately, it seemed that funding agencies and companies back in the early 2000s wanted to stay far away from viral gene therapy, also because of biological warfare and ethical objections ('Playing God'), which articulates 'the ugly' side of gene therapy.

Also the CRISPR-Cas approach, as well as other novel biotechnological applications to interfere with the (epi)genetic composition, promise a lot of “good” aspects. The “ugly” (designer babies) and the “bad” (bioterrorism) -sides of such approaches deserve solid policies. It is important to also fully realize the 'good' side, whatever the impact may be when such technology falls into the wrong hands. Obviously, we need to control adverse applications, but without destroying investments to strengthen the good side. Reprogramming of genes to restore protein expression networks to a healthy situation would be a breakthrough for many diseases for which no therapy is available at the moment. In other words, “the curable genome shines on the horizon”.

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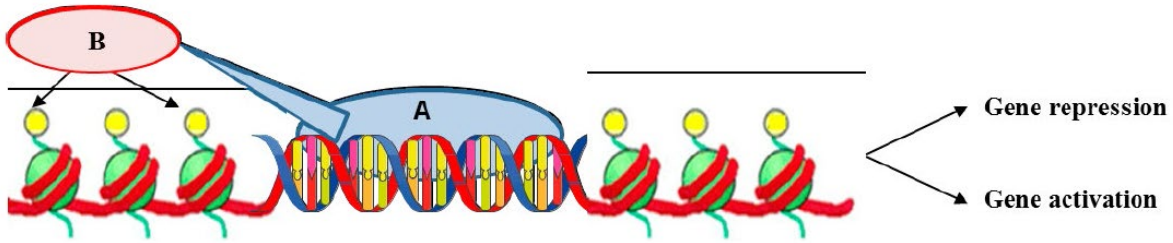

Figure 1. The concept of epigenetic editing