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Background information on the award of the
Paul Ehrlich and Ludwig Darmstaedter Prize 2022
to Prof. Dr Katalin Karikó, PD Dr Özlem Türeci and Prof. Dr Uğur Şahin

The talented courier from the centre of the cell

For decades, messenger RNA (mRNA) lived in the shadow of its big mother DNA and some of its more popular siblings in the RNA family. It was considered far too unstable, to ever become a suitable candidate for drug development and medical applications. The fact that this inconspicious Cinderella was able to outwit all other molecular competitors during the Corona pandemic is largely due to the achievements of the three prize winners. Thanks to their uncompromising basic research efforts, their persistent development work and their determined and fast reaction to the emergence of SARS-CoV-2, they developed a vaccine to effectively combat the raging pandemic. In this way, they proved the enormous potential of preventive and therapeutic use of mRNA both to the *scientific community* and to the general public.

mRNA was discovered late - only in 1960, 16 years after DNA had been identified as the carrier of genetic information, and seven years after the DNA´s double helical structure had been deciphered. Until the discovery of mRNA, the relationship between the two nucleic acids DNA and RNA, and the proteins in living cells remained a mystery. It was known for some time that there is a particularly large amount of RNA at the sites where protein synthesis takes place. In addition, ribosomes had been discovered in the mid-fifties as possible protein factories (they were not given this name until 1958). However, the evidence for the widespread assumption "that the nucleic acids are in some way responsible for the control of protein synthesis, either directly or indirectly" was "rather meagre", as Francis Crick remarked in his famous lecture on protein synthesis in 1957. In this lecture, he nevertheless formulated two hypotheses that are still valid today: First, the blueprint for a specific protein depends solely on the sequence of nucleotides of a specific segement of nucleic acid. Secondly, once it has been transmitted, this genetic information cannot flow back out of the protein towards the nucleic acid. However, he was mistaken in assuming that each ribosome was assigned to one specific RNA template only.[1]

A sudden inspiration

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The eureka moment came for him and Sydney Brenner on 15 April 1960, when the French researcher Francois Jacob visited them at King's College, Cambridge[2]. Jacob reported on an experiment whose results contradicted Crick's template theory. His group had worked with two types of bacteria. In a culture medium containing lactose, one bacterial strain immediately began to synthesise an enzyme capable of digesting this sugar; the other strain lacked this enzyme. However, if the gene for the missing enzyme was introduced to the latter by bacterial conjugation, within minutes, it began to produce it at full speed. The gene - i.e. its DNA - apparently had triggered the production of a signal molecule, because the bacteria would not have been able to produce new ribosomes so quickly. The French called this molecule X.

A universal signalling molecule

Then Brenner and Crick saw the light. They leaped to their feet, gesticulating wildly and talking at once. During Jacob's lecture, they had suddenly recalled an experiment by two American colleagues. Two years earlier, these colleagues had infected bacteria with bacteriophages. With the help of radioactive markers, they had subsequently observed that the infected bacteria briefly produced an RNA that had the same nucleotide sequence as the DNA of the infecting phages. However, they failed to find a satisfactory explanation for their finding of a "DNA-like RNA". It dawned on Brenner and Crick that this RNA had to be identical to the signal molecule X. If this were so, they concluded, a ribosome would not contain the recipe for the production of a specific protein in the form of a template, as had previously been assumed, but would rather be a universal reader for all possible protein recipes.

No call from Stockholm

To prove this assumption, they had to experimentally disprove that molecule X led to the formation of new ribosomes. During a summer visit, they succeeded in doing so with the help of Mathew Meselson´s ultracentrifuges had at Caltech in Pasadena. Instead of new ribosomes, it was a small RNA molecule that temporarily associated with the old ribosomes of the bacterium. Meanwhile, at the Cold Spring Harbor Laboratories, James Watson was using other methods that gave essentially similar results. When he heard of his colleagues' breakthrough, he urged them to postpone their publication. It was therefore not until 13 May 1961 that two articles appeared in *Nature* reporting the discovery of this new type of RNA accounting for only 3% of all RNA present in a cell; ribosomal RNA accounts for another 80%. A month later, it received its name in a review paper by Francois Jacob and Jacques Monod, who called it " messenger RNA".[3] Despite the importance of this discovery, which also played a key role in the subsequent efforts to crack the genetic code, it was never rewarded with a Nobel Prize, probably because there were clearly more than three researchers involved in it. However, in the following years, most of the scientists involved in this game-changing discovery received this honour in Stockholm for other achievements.

A change of perspective

With the discovery of mRNA, the question of how living organisms translate genetic information into biological function was finally settled. It is remarkable that Jacob and Monod called the newly discovered molecule a "messenger". For this expresses a change of perspective. In molecular biology, too, the bricks and motar of molecular biology began to assume a backstage role, whereas transmitters of information began to

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occupy centre stage. Karikó, Türeci and Şahin took this change of perspective to heart and managed to implement it with great success.. For their goal - already partially realized - is to transfer a defined genetic message into the cells of a patient by means of mRNA, producing desired proteins from within. Informational help for self-help, so to speak.

Beyond the bacteria

Yet, this goal was out of reach for a long time. It required knowledge about the function of mRNA in eukaryotes whose cells have a cell nucleus, in contrast to bacteria, which lack such a structure. In the 1970s, three major differences to the nucleus-free bacteria were discovered for the transcription in eukaryotic organisms. Although the nucleotide thymidine of the DNA is replaced by the nucleotide uridine in the mRNA of all organisms, the eukaryotic mRNA is additionally provided with a molecular cap of a modified guanine residue at its front end, a process referred to as "capping". Secondly, after the end of transcription, a tail consisting of a string of adenine nucleotides is attached to the transcript ("polyadenylation"). Thirdly, transcription yields only an immature form of the final mRNA. This is because the coding parts of eukaryotic genes are disrupted by non-coding streches of sequence. These introns must be removed and the coding exons joined together by the spliceosome, a sophisticated molecular machinery of the cell nucleus. Only when it has gone through the processes of capping, poyladenylation and splicing is the newly transcribed mRNA exported from the cell´s nucleus into the cytosol. There it associates with ribosomes and instructs them to synthesise a protein; each sequence of three nucleotides ("codon") provides the information for the addition of a particular amino acid to the growing protein chain. In addition to the recipe for building a protein, the RNA contains sequence elements that are not translated into the protein but carry important instructions for regulating the amount and duration of production of the mRNA-encoded protein in particular cell types. Sequences that support protein production in muscle cells may not necessarily have the same effect in other cells of the body.

Three key technologies

Realizing the production of large amounts of a specific mRNA became possible only after three essential technologies for working with nucleic acids were invented and brought to the market in the 1980s. The invention of the polymerase chain reaction (PCR) made it possible to amplify even tiny amounts of DNA in a short time, which could be used as a template for the second essential step, in vitro transcription (IVT). This latter process made use of RNA polymerases, such as that of the bacteriophage SP6, to produce large quantities of mature mRNA in test tube. Lastly, the efficient packaging of nucleic acids in fat droplets (liposomes), whose outer structure corresponds to that of cell membranes for efficient fusion, enabled the transport of in vitro-transcribed mRNA (IVT mRNA) into the interior of eukaryotic cells where they are protected from RNA-degrading enzymes (RNAses).

The dominance of DNA

Hence, the stage was set for preclinical testing of mRNA for therapeutic use as early as the late 1980s. Yet, even the research group that in 1990 for the first time had shown that the intramuscular injection of IVT mRNA in mice led to the expression of the desired protein stopped this line of research. For, at a time when the worldwide human

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genome project began to decipher the entire human genetic information, the therapeutic use of DNA seemed a far better prospect. Thanks to genetic engineering, many therapeutic proteins such as insulin could already be produced in recombinant fashion in the laboratory, and the development of the first monoclonal antibodies into drugs was imminent. Particular hopes were associated with DNA-based gene therapies, based on the introduction of "healthy" genes into cell nuclei by means of viral ferries. This strategy was considered the optimal solution to permanently correct pathological mutations, such as that causing cystic fibrosis. This disease is caused by a misfolded ion channel that is formed by a protein consisting of 1480 amino acid building blocks. The *CFTR* gene, in which a tiny deleterious mutation occurs, was discovered in 1989, and, along with some monogenetic immunodeficiency diseases, became the target of the first gene therapy trials. To date, gene therapies based on DNA transfer have yet to fully meet the high expectations, mainly because of side effects associated with certain types of viral gene ferries.

Undeterred despite lack of funding

Katalin Karikó, who had been working at the University of Pennsylvania since 1989, was an outsider in the era of DNA. Her application to fund a research project aimed at introducing the blueprint of a healthy ion channel into affected cells via mRNA transfer was rejected. At that time, nobody wanted to fund RNA-centered projects. The main argument was that mRNAs are very unstable and hence were considered unfit for the purpose of obtaining sufficient amounts of protein and extended time periods of protein synthesis. Their intracellular half-life ranges from a few minutes to several hours. Extracellularly, they are threatened with immediate destruction by ubiquitously present RNA-degrading enzymes (RNAses). Those who worked with DNA in the laboratory also considered them to be annoying contaminants in their own experiments. Because she could not raise enough funding for her work, Karikó was demoted from Research Assistant Professor to Senior Research Investigator in 1995. Undeterred, she nevertheless remained true to her goal of developing protein replacement therapies based on the injection of IVT mRNA. From the very beginning of her scientific career, she was convinced of the potential of mRNA. Already as a post-graduate student, after her biology studies at the Hungarian University of Szeged, she had conducted research in the RNA laboratory there. One of her tasks was to isolate RNA, which at that time could not yet be produced artificially in the test tube. Thus, she became a specialist in RNA biochemistry, an expertise with which she emigrated to the USA in 1985.

The advantages of mRNA

Karikó was always convinced of the advantage of mRNA protein replacement therapies. Because unlike DNA therapeutics, mRNA does not have to enter the cell nucleus to exert its effect. Unlike DNA therapeutics, mRNA does not have to enter the cell´s nucleus to exert its effect. Unlike DNA therapeutics, which carry a risk of mutagenicity, RNA does not integrate into the genome of its target cell; moreover, because it is rapidly degraded, its temporal effects can be well controlled. In the 1990s, she almost single-handedly proved that even highly complex, extensively post-translationally modified proteins are immediately produced functionally in large quantities in cell cultures after introducing the relevant IVT mRNA. As an example, she used the urokinase receptor, a membrane protein that is a switch point at the beginning of a proteolytic cascade that can be involved in various vascular diseases as well as in the

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progression of tumours. Its overexpression by IVT mRNA facilitated a better understanding of the physiological functions of this cascade and the exploration of possible pharmacological interventions[4].

A barely noticed breakthrough

Preclinically, Karikó focused on mRNA therapies for the treatment of strokes and cerebral diseases. For example, she attempted to counteract the rampant and ultimately lethal cerebral vasoconstriction after a stroke by local injection of the mRNA encoding the enzyme NO synthase, which catalyses the formation of the vasodilatory nitric oxide. However, in those experiments a biological effect couldn't be measured, likely due to the fact that the amount of protein produced from the mRNA was not sufficient. Karikó, working in the laboratory largely on her own for lack of funding, or together with her colleague, Drew Weissman, began to study this phenomenon in detail. She found that externally applied mRNA activates three Toll-like receptors (TLR) that are active in the human innate immune system, and whose signals are relayed to the adaptive immune system via dendritic cells. She started from the hypothesis that this immune response can be prevented by the incorporation into the RNA of modified nucleotide building blocks. She based this on a natural model: DNA that is modified at certain bases by methyl groups is not immunogenic. In years of experiments, she tested one modified building block after the other. Eventually, she found that the immunogenicity of IVT mRNA could be mitigated particularly effectively by modifying the nucleotide uridine, either by replacing it with the naturally occurring isomer pseudouridine or by adding a sulphur moieties or methyl groups to the pyrimidine ring. This discovery was the decisive breakthrough on the way to realising mRNA therapies. However, when these results [5] were first published in 2005, they didn´t make headlines. At that time, most RNA researchers were more interested in exploring the therapeutic potential of RNA interference using small interfering RNA molecules (siRNAs), which had been discovered a few years earlier. The interest towards the pseudouridine-containing mRNA didn't increase even after demonstrating in mice that such mRNA can be translated into protein at very high level without immune activation.[6]

From tumour antigens to cancer vaccines

Uğur Şahin and Özlem Türeci's path to mRNA therapies began in cancer research. The main focus of the two scientists was the development of therapeutic cancer vaccines to activate the patient`s immune system for a precise yet potent attack against their tumour. They started in the 1990s by developing various methods to systematically identify tumor antigens, i.e. those molecular recognition features that distinguish a cancer cell from a normal cell. To develop an ideal cancer vaccine, Uğur Şahin and Özlem Türeci faced three major challenges that were considered insurmountable at the time. First, even quite small tumors consist of billions of cancer cells. To attack such a large number of cancer cells, the vaccine had to be extraordinarily effective and able to generate billions of immune cells. Second, the tumour characteristics that are recognized by the immune system are different for each and every individual patient. Şahin and Türeci recognized that an individualized vaccine technology was needed to tailor vaccines to each patient's antigen profile. Further, such an individualized vaccines had to be produced very quickly so that they could be administered in a timely manner to patients awaiting treatment before the cancer spread further. From their preclinical studies, the two physicians concluded that developing a vaccine with all these properties

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was possible, in principle, with IVT RNA. Over the next two decades, they achieved a series of scientific and technological breakthroughs to systematically unlock the full potential of this type of molecule for cancer vaccination.

Precision work and entrepreneurial courage

At the end of the 1990s, Şahin and Türeci set about optimising IVT mRNA so that it could, in principle, act as a highly potent vaccine capable of shrinking existing cancer masses, To this aim, they optimized the RNA molecules structurally and, further, developed methods to deliver the mRNA vaccine to the right cells in the human body. In years of research, they solved the basic mRNA-associated problem, namely the low and short-lived protein production, in a different way than Katalin Karikó. Şahin and Türeci optimized each of the structural components of mRNA (cap, the poly-A tail and its untranslated regions) to achieve increased intracellular stability of synthetic mRNA and translation especially in immune cells. The combination of these improved elements dramatically increased antigen yield. With the results of this work (published in 2006[7]), they emerged as the winners of the first Go.Bio competition of the Federal Ministry of Education and Research in the same year, which motivated them to found their company BioNTech in 2008, the financial foundation of which was the investment of the Strüngmann brothers' family office.

In the years to follow, they further improved vaccine efficacy by developing strategies to deliver mRNA into dendritic cells (DCs), specifically those DCs located in lymphoid tissues. This particular DC species are the "high-performance trainers" of the immune system. Uğur Şahin and Özlem Türeci discovered the mechanism of selective mRNA uptake into these DCs and, with their team, developed a lipid nanoparticle formulation that exploited this mechanism to target RNA into these cells[8 9]. For these studies, they used immunogenic RNA, which, in addition to instructing the synthesis of vaccine antigens, also contributes the pro-inflammatory adjuvant effect desired for any vaccine; as a result, it further enhances the immune response triggered by the mRNA. This simultaneously allowed large numbers of dendritic cells to be targeted to generate a correspondingly large number of immune cells that precisely recognized only the cancer cells. These breakthrough improvements formed the basis for the successful use of mRNA for various human applications. The principle of targeting DCs in lymphoid tissues was later used by the two physicians in the development of the COVID-19 vaccine.

Step by step to clinical application

BioNTech has since made great progress in the clinical application of cancer vaccines, also thanks to the development of suitable lipid nanoparticle formulations for the delivery of mRNA. Several of BioNTech's cancer vaccines have successfully passed the first phase of clinical trials, including in black skin cancer (melanoma)[10].

To successfully realize their original vision of an individualized vaccine technology, Uğur Şahin and Özlem Türeci developed a breakthrough approach that can be universally applied to different types of cancer. This encompasses all steps from genomic analysis of the patient's tumor, computer-assisted vaccine design tailored to the patient's individual antigen profile, as well as optimized processes for rapid and reliable on-demand production and quality control of the mRNA vaccine [11 12 13].

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As a result of years of research, individualized mRNA vaccines can be made available on average in a few weeks for patients treated in the clinical trials. The knowledge and know-how gained in this process should later contribute decisively to the rapid availability of the COVID-19 mRNA vaccine. Moreover, BioNTech's COVID-19 vaccine greatly benefited from the incorporation of the optimized designs for various structural components of mRNAs originally developed by Uğur Şahin and Özlem Türeci for their cancer vaccines.

Katalin Karikó joined the company in 2013. The three award winners published a comprehensive overview of the possibilities of the mRNA platform, for which they played a major role in creating, in *Nature Reviews Drug Discovery in* 2014.[14]

For the development of protein replacement therapies, the non-immunogenic mRNA molecules discovered by Karikó are indispensable, because relatively large quantities of proteins have to be produced for therapeutic replacement purposes. While conventional protein replacement therapies only replace extracellular proteins, mRNA-based methods can also replace faulty or missing intracellular proteins, as would be necessary in cystic fibrosis, for example. As suggested by recently published preclinical research results of the three prize winners mRNA could also one day be used to treat an autoimmune disease, such as multiple sclerosis[15].

The basis of the first two mRNA vaccines

The development of a vaccine does not depend on the use of non-immunogenic mRNA. This is because a certain immune stimulation, a tickling of the immune system, so to speak, is necessary for every vaccination. This also applies to vaccines against SARS-CoV-2. However, the lipid nanoparticles can take over the immune stimulation in the vaccines that are currently in use. With unmodified mRNA, the development of a highly effective COVID-19 vaccine takes considerably longer than with non-immunogeneic RNAs. It is no surprise then that the mRNA vaccines that have been so successful in the fight against the corona pandemic incorporate Katalin Karikó's discovery alongside of other optimized mRNA components.

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[1] Cf. Crick, F.H. (1958) On protein synthesis. *Symp Soc Exp Biol*.12: 138-163, p. 153. "The Central Dogma... states that once 'information' has passed into protein it cannot get out again. In more detail, the transfer of information from nucleic acid to nucleic acid, or from nucleic acid to protein may be possible, but transfer from protein to protein, or from protein to nucleic acid is impossible. ”

[2] See Cobb, M. (2015) Who discovered messenger RNA? *Current Biology* 25: R526-R532, and Michel Morange (2020). The black box of biology. A history of the molecular revolution. Harvard University Press: 138-147

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