Covid Vaccine and MRNA Singularity
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Moderna Will Develop mRNA Vaccines for 15 of the World's Worst Diseases

By **Vanessa Bates Ramirez**

To much of the world, it seemed like the Covid-19 vaccines were whipped up in less than a year—an amazing feat of science and biotechnology if ever there was one. While the vaccines did get fast-tracked through clinical trial and regulatory approval phases, the truth is that the technology behind the Pfizer and Moderna vaccines—mRNA—had been in the works for decades. Now Moderna is leveraging that same technology to fight several other viruses.

This week Moderna announced plans to develop vaccines against 15 different pathogens that either have the potential to cause a pandemic or are an ongoing thorn in humanity's side. Among them are chikungunya, dengue, ebola, malaria, and Covid's predecessor, Middle East Respiratory Syndrome (MERS).

Traditional vaccines use a weakened piece of a virus to expose our bodies to it so they can get some practice launching a counter-attack before being fully exposed. mRNA vaccines work by training our cells to create proteins to fight viruses. DNA makes mRNA, which acts as a "messenger" by instructing our cells to make proteins (and those proteins in turn control pretty much everything that happens in our cells).

The "workshop" where the proteins get made is the cell's ribosome. Scientists at the University of Pennsylvania were able to create mRNA that could get past cells' defenses without triggering an immune response, but still be recognized by the ribosome. In the case of the Covid vaccines, the modified RNA was programmed to get the ribosome to make the virus's telltale spike protein.

The immune system tags this protein as an invader and launches a response, producing antibodies to fight what seems like an infection. When a vaccinated person comes into contact with the real virus, his or her cells know how to fight it before it takes over the body.

The mRNA, then, is essentially a delivery vehicle, a Trojan horse that can be made to smuggle in instructions to make any protein of scientists' choosing. They identify a vaccine target by sequencing a virus's genome—the low cost and fast turnaround of genome sequencing is another key part to this technology—then encode mRNA for the relevant protein.

As Drew Weissman, one of the physician-scientists who helped develop mRNA vaccine technology put it, "mRNA vaccines are essentially plug and play. We believe you can change the part of the mRNA that encodes a protein, plugging in new code specific to the virus we hope to protect against, and cause one's body to produce proteins that match that virus' proteins. We do not have to develop and manufacture an entirely new formula."

On top of that, mRNA is easy to scale in production at a relatively low cost, making it easier and more cost-effective to screen multiple vaccine candidates quickly.

Creating 15 new vaccines, then, may not be as much work as it sounds like (though getting them through clinical trials and ultimately bringing them to market probably still will be). The company plans to prioritize work on viruses classified as "persistent global health threats,"

including HIV, tuberculosis, and malaria, and aims to have vaccines for all 15 pathogens in clinical trials by 2025 (its HIV vaccine already started human trials last year).
Two other significant pieces of news were included in Moderna's release this week. First, the company said it would permanently wave its Covid-19 vaccine patents in low- and middle-income countries, sticking to a pledge made early in the pandemic. This means labs in these countries can use the company's technology to produce local versions of a Covid vaccine. 92 countries are included, all of them part of the Gavi COVAX Advance Market Commitment.
Moderna also said it plans to build a $500 million facility for manufacturing mRNA vaccines in Kenya, and will supply up to 500 million doses a year of mRNA vaccines to the African continent.

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(Note abortive cells are from aborted baby fetus.)

Are we facing the next, very rapid stage of evolution, via AI?

By Eric Holloway

These are certainly heady times in the biotech world. With the new mRNA vaccine being created in just a few days in January 2020, someone can mass produce DNA in their garage for the price of a hamburger, and Alpha Fold 2 can predict proteins from DNA with accuracy, rivalling wet lab results, it seems we are on the cusp of something extraordinary. Most viral infections will cease if all we need to do to roll out a new vaccine is sequence the virus genome and mass produce the portion that binds to human cells.
On the darker side, it will also likely mean a greater threat of biowarfare. Creating a new virus may just be a matter of downloading a sequence from one of the many online databases, and having it delivered to your doorstep. Pair this with enormous advances in computational power, perhaps even quantum computing, and the possibilities seem limitless.
Will this be the beginning of Superman? And will it be the Superman of the comic books or Nietszche's unhinged anti-hero?
These are crazy questions for a crazy era in human history. One might, surveying the scene, believe that we are on the edge of the next step of evolution. Professor Mark Alan Walker *(pictured)* of New Mexico State University certainly thinks we are. He believes that the technological changes we are facing are so great that "The potential radical change of creating noetic beings overshadows all of the revolutions in human life combined."
At the same time, there is an interesting paradox underlying his revolution. As Walker also states: "Person-engineering technologies will make it possible to accomplish in a matter of years what evolution would take thousands of millennia to achieve."
This sort of observation led Ray Kurzweil to propose his notion of a the Singularity. Kurzweil noticed that technologies form a self-feedback loop, resulting in ever more powerful technologies operating at ever greater speeds. And at the core of this technological feedback loop lies the human person, a combination of extraordinary and exquisite bio-computational technology. The logical conclusion is: the human is the last puzzle to unlock. At that point, in his view, we merge with machines.

On this view, the fantastic catalytic properties that allow us to catapult technological progress into the future is the final frontier of technology. We must live longer, be healthier, think smarter, and focus all our best resources to capture our élan vital in a little grey philosopher's stone with the Windows chime and the (soon, we are told) self-aware Clippy.

On the two sides of this debate lie the conservatives and the progressives. The conservatives worry about the Pandora's Box *(pictured)* of potential evils we are about to open, looking back at the past couple of centuries of untold human destruction. Progressives, meanwhile, marvel at the technotopia that promises to make on earth for us a heaven. In their view, the collateral damage pales in comparison to the infinite vista unfolding right here and right now. We must gird up our loins and immanentize the eschaton! Apotheosis (becoming gods) [11] is within our grasp!

But there is a third possibility that neither side is considering. And that is: a Singularity is merely logically impossible. Despite the giddy technological progress we have seen in the past couple centuries, even though we have entered an era where many of the poorest among us live like the kings of old, our basic human nature lies unchanged. In fact, some smart people say we have regressed.

Thus, no matter what spectacles the future may bring, as far as the fundamentals are concerned when it comes to ourselves and the world around us, we will be at our limit merely making sure we don't screw up our what our ancestors have gifted to us through untold labor and sorrow.

Why do I say such a naive thing? Who could possibly think in this day and age nothing fundamentally different is about to happen?

Let's take a step back. Most of our problems come down to mathematics and the human will, two things which no amount of genetic and silicon gadgetry can change.

Mathematics is the cause of the second law of thermodynamics, the observation that everything in the universe runs down. This amounts merely to saying that what is most likely to occur is most likely to occur.

Disorder and decay are the typical states of the universe. The complex organization of energy we humans see around us in our verdant fertile nest is enormously atypical. This fundamental law drives right through the heart of any technology, genetic or otherwise, that we might invent.

The human will also faces a similar dilemma. No matter how much money, education, affection and attention you give a person, their will is still free to do good and to do evil. No matter how much torture, despair, horror, and sorrow you inflict on a person, their will is still free to do good and to do evil. This is the most incredible and scary fact of our existence. There is nothing anyone can do to conquer a person's will. The crowning achievements and utterly detestable atrocities of our history have made this clear.

At every period in history, regardless of circumstances, individuals have of their own volition performed acts that show even if there are not angels and devils in an unseen spiritual world, they certainly walk among us in human form. Nothing that will ever occur in the future will change this fundamental fact of human existence.

This brings us back to the paradox that Professor Walker mentions: "Person-engineering technologies will make it possible to accomplish in a matter of years what evolution would take thousands of millennia to achieve."

Think about this statement for a second. We are about to do something in blink of an eye at which evolution, we are told, has labored away slavishly and wastefully for untold eons. And yet we ourselves are the product of this unmitigatedly stupid (for lack of a better word) physical process. There are two possible explanations for this extreme discontinuity. Either a blind marble

rolling around an infinitely complex maze in the dark accidentally hit the single light switch or our origin and destination lie beyond this physical plane. Neither explanation makes the rise of Walker's godlike beings plausible.

Best case scenario: we would end up with a civilization of 100-year-olds watching cat videos in virtual reality.

Most likely scenario: we will accelerate the genetic entropy of our genome even more, crippling future generations in our blind rebellion against reality.

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Developing mRNA-vaccine technologies

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Abstract

mRNA vaccines combine desirable immunological properties with an outstanding safety profile and the unmet flexibility of genetic vaccines. Based on in situ protein expression, mRNA vaccines are capable of inducing a balanced immune response comprising both cellular and humoral immunity while not subject to MHC haplotype restriction. In addition, mRNA is an intrinsically safe vector as it is a minimal and only transient carrier of information that does not interact with the genome. Because any protein can be expressed from mRNA without the need to adjust the production process, mRNA vaccines also offer maximum flexibility with respect to development. Taken together, mRNA presents a promising vector that may well become the basis of a game-changing vaccine technology platform. Here, we outline the current knowledge regarding different aspects that should be considered when developing an mRNA-based vaccine technology.

Keywords: mRNA, adjuvant, vaccine, mRNA production, mRNA design, mRNA uptake, formulation, protein expression

Introduction

RNA is considered as notoriously unstable making its therapeutic use a provocative idea. Despite the sensitivity of the molecule to the virtually omnipresent ribonucleases (RNases),[1] mRNA as a therapeutic was first promoted in 1989 after the development of a broadly applicable in vitro transfection technique.[2] Only a couple of years later, mRNA was advocated as a vaccine platform, perhaps being ideal in the sense that it brings together the immunological features of live attenuated vaccines such as endogenous antigen expression and T cell induction with those of killed or subunit vaccines like defined composition and safety.[3,4]

Particularly compared with DNA as a therapeutic or more specifically as a vaccine, mRNA offers strong safety advantages.[5] As the minimal genetic construct, it harbors only the elements directly required for expression of the encoded protein. Moreover, while recombination between single-stranded RNA molecules may occur in rare cases,[6,7] mRNA does not interact with the genome. Thus, potentially detrimental genomic integration is excluded. Finally, this lack of genomic integration in combination with mRNA being non-replicative as well as metabolically decaying within a few days[8] makes mRNA a merely transient carrier of information.

mRNA as the technological basis of therapeutics and vaccines is characterized by a great flexibility with respect to production and application. Any protein can be encoded and expressed by mRNA, in principle enabling the development of prophylactic and therapeutic vaccines fighting diseases as diverse as infections and cancer as well as protein replacement therapies. Since changes of the encoded protein just alter the sequence of the RNA molecule, leaving its physico-chemical characteristics largely unaffected, diverse products can be manufactured using the same established production process without any adjustment, saving time and reducing cost compared with other vaccine platforms. In terms of efficacy, mRNA-based therapeutics profit from the fact that they do not need to cross the nuclear envelope as opposed to DNA. In contrast to peptides, mRNA vaccines lack MHC haplotype restriction. In addition, mRNA binds to pattern recognition receptors and mRNA vaccines may be designed to be self-adjuvanting,[9] a property which peptide- and protein-based vaccines lack.

All in all, mRNA presents a promising, even if challenging, class of therapeutic molecules that has the potential to become the basis of a "disruptive technology."[10] In the following we are casting light on what has to be considered when developing an mRNA-vaccine technology touching important topics such as mRNA manufacturing and quality, mRNA format and formulation as well as antigen/protein expression and immunological properties of mRNA-vaccines.

mRNA synthesis

Functional synthetic mRNA may be obtained by in vitro transcription of a cDNA template, typically plasmid DNA (pDNA), using a bacteriophage RNA polymerase.[11] Hence, the preparation of pDNA is the first step in the production of mRNA. Manufacture of mRNA might thus appear to require more effort than manufacture of pDNA. However, unpolished pDNA contains traces of bacterial genomic DNA and three forms of pDNA (supercoiled, relaxed circle or linear) in variable proportions. Hence, the reproducible preparation of pure and invariant pDNA, as required for a vaccine, is demanding. Remains of bacterial DNA and the heterogeneity of pDNA are not a concern, on the other hand, if linearized pDNA is transcribed using bacteriophage RNA polymerase,[5] because all DNA is removed during further processing steps (see below).

Synthetic mRNA contains a protein-encoding open reading frame (ORF) flanked at the minimum by two elements essential for the function of mature eukaryotic mRNA: a "cap," i.e., a 7-methyl-guanosine residue joined to the 5′-end via a 5′-5′ triphosphate,[12] and a poly(A) tail at the 3′-end.[13] Accordingly, a pDNA template for in vitro transcription contains at least a bacteriophage promoter, an ORF, optionally a poly(d(A/T)) sequence transcribed into poly(A) and a unique restriction site for linearization of the plasmid to ensure defined termination of transcription (the cap is not encoded by the template).

The linearized pDNA template is transcribed into mRNA in a mixture containing recombinant RNA polymerase (T7, T3 or SP6) and nucleoside triphosphates. It is possible to obtain capped mRNA by transcription. To this end, a cap analog like the dinucleotide m^7G(5′)-ppp-(5′)G (called "regular cap analog" in the following) may be included in the reaction.[14] If the cap analog is in excess of GTP, transcription initiates with the cap analog rather than GTP, yielding capped mRNA.[15] Alternatively, the cap may be added enzymatically post transcription. A poly(A) tail may also be added post transcription if it is not provided by the pDNA template. Following transcription, the pDNA template as well as contaminating bacterial DNA is digested by DNase.

mRNA purification

At this point, the sample contains the desired mRNA transcript within a complex mixture including various nucleotides, oligodeoxynucleotides, short abortive transcripts from abortive cycling during initiation,[16] as well as protein. These contaminants may be removed from the sample by a combination of precipitation and extraction steps.

However, the sample includes additional contaminating RNA species that cannot be separated from the correct transcript by simple means: Shorter than designated transcripts arise from premature termination during elongation. Longer than designated transcripts arise from template DNA linearized with an enzyme that leaves a 3′-overhang[17] or from traces of nonlinearized template DNA. Undesirable transcripts are also produced due to the RNA-dependent RNA polymerase activity of bacteriophage polymerases.[18] Accordingly, to be used as a drug substance, mRNA will have to be purified further to remove such contaminating transcripts.

A single chromatographic step that separates mRNA according to size removed both shorter and longer transcripts, yielding a pure single mRNA product.[19] Implementation of such a chromatographic purification within a GMP production process for mRNA increased the activity of mRNA molecules several-fold in terms of protein expression in vivo.[8]

Increased protein expression as a result of stringent purification of mRNA was also observed when transcripts coding for luciferase or erythropoietin were purified by HPLC.[20] The increase in protein expression was much higher than would be expected simply based on the removal of incorrect transcript. The authors demonstrated that increased protein expression after HPLC purification was also due to the removal of contaminating, e.g., double-stranded, RNA that activates innate immune sensors, thereby reducing protein expression.

Synthetic mRNA for therapy is in general designed following the blueprint of eukaryotic mRNA. Cap and poly(A) tail are essential elements because they are required for efficient translation.[2,15,21] Positioned at the very 5′- and 3′-end of mRNA, Cap and poly(A) tail are also required to stabilize mRNA in the cytosol, where decay is catalyzed predominantly by exonucleases.[22,23]

However, to further increase both translation and stability, mRNA requires 5′ and 3′ untranslated regions (UTRs) to flank the ORF.[24-27] UTRs have to be carefully chosen because they may also impair translation or mRNA stability.[28] In particular, specific cis-acting destabilizing sequences like AU-rich elements[29] and miRNA binding sites[30,31] mostly reside in UTRs, although they may also be found in ORFs.[32] Care must be taken to avoid such destabilizing signals.

Following these considerations, efforts have been made to identify beneficial mRNA elements in order to improve translation and stability of synthetic mRNA molecules inside the cell. Improved mRNA formats thus identified will likely also yield better mRNA vaccines, as it is widely assumed that the efficacy of an mRNA vaccine will rise as protein expression is increased and prolonged.

Cap

mRNA may be capped during transcription by including a cap analog in the reaction. However, it has been found that the regular cap analog is often incorporated in the reverse orientation so that the m^7G nucleotide does not constitute the cap but is instead the first transcribed nucleotide. As a result, about one third of mRNA molecules are not methylated at their cap.[33] Such mRNA lacking methylation of the cap base is not translated.[34]

In order to avoid unmethylated cap by reverse orientation, mRNA may be transcribed without cap analog and subsequently capped using the vaccinia virus capping complex.[35] This complex with triphosphatase, guanylyltransferase and (guanine-7-)methyltransferase activity adds a

natural cap to the 5′-triphosphate of an RNA molecule. However, an additional enzymatic step may complicate production, particularly at an industrial scale.

Alternatively, a cap exclusively in the correct orientation is obtained with the use of “anti-reverse” cap analogs (ARCAs). In the most common ARCA, 3′-O-methylation of the base-methylated guanosine only allows addition of a nucleotide at the non-methylated guanosine. ARCA-capped mRNA was translated at more than doubled efficiency in rabbit reticulocyte lysate compared with mRNA capped by regular cap analog.[34] In addition, it has been shown that mRNA transcribed in vitro with ARCA also has a longer half-life in cultured cells.[36] In an independent study, ARCA-capped mRNA has been reported to both increase and prolong protein expression in cultured cells.[37]

Protein expression from in vitro transcribed, enzymatically capped mRNA can be further increased by enzymatic 2’-O-methylation of the first transcribed nucleotide, resulting in protein expression comparable to that from mRNA capped with ARCA co-transcriptionally.[38]

ARCAs have been further modified within the triphosphate linkage in order to inhibit decapping of the corresponding mRNA and increase binding of eukaryotic initiation factor 4E involved in the recruitment of ribosomes. Modifications either substituted for a bridging oxygen (e.g., (methylenebis)phosphonate and imidodiphosphate) or a non-bridging oxygen (e.g., phosphorothioate, phosphoroselenoate and boranophosphate).[39] Phosphorothioate-modified ARCAs yielded mRNA with both further increased translation efficiency and elongated half-life in cultured cells compared with ARCA.[40] However, phosphorothioate-modified ARCAs are obtained as a mixture of two diastereomers that must be separated after synthesis because of their different biological activity.

Poly(A) tail

When the poly(A) tail was unveiled to enhance translation initiation, it was noted that the efficiency of polysome formation increased with increasing length of the poly(A) tail up to 68 residues.[15] Translation of in vitro transcribed mRNA transfected into cultured cells still increased slightly by lengthening the poly(A) tail from 54 to 98 residues.[41] This study was further extended by investigating the effect of even longer poly(A) tails on protein expression.[38] The peak protein level, reached one day after electroporation of mRNA into cells, was doubled when the poly(A) tail was extended from 64 to 150 residues. Further extension of the poly(A) tail by enzymatic polyadenylation led to an additional moderate increase in peak expression. By contrast, upon transfection of UMR-106 cells, protein levels 16 h post transfection increased with increasing length of the poly(A) tail only up to 60 residues, but declined with further increasing poly(A) tail length.[42] In practical terms, it is noteworthy that maintenance of long poly(d(A/T)) sequences is demanding and strongly dependent on the bacterial strain.[42]

UTRs

Already early on, in vitro transcribed mRNA contained 5‘- and 3‘-UTRs, specifically those of the β globin gene of Xenopus.[11] Both the Xenopus β globin 5′- and 3′-UTRs were demonstrated to impart much greater translational efficiency on heterologous mRNA in the mouse NIH 3T3 fibroblast cell line.[2] A combination of the β globin 5′-UTR, improving translation and the α globin 3′-UTR, known to stabilize mRNA,[27] has been used in the construction of a library from amplified tumor-derived cRNA for use as vaccines against metastatic melanomas.[43] Globin UTRs are still in widespread use in in vitro transcribed mRNA including RNA for immune therapy.[38,44,45]

UTRs from non-globin genes have also been included in in vitro transcribed mRNAs used for investigations of the therapeutic value of mRNA. The 5′-UTR of tobacco etch virus enhances

translation of in vitro transcribed mRNA in mammalian cells[46] and has been included in mRNA expressing erythropoietin in different cell types[20] and mice.[47] Furthermore, a structure of the 5′-UTR of human heat shock protein 70 enhanced translation of mRNA in mammalian cells and was predicted to be valuable in the context of genetic vaccination.[48]

Inclusion of an internal ribosomal entry site (IRES) in in vitro transcribed mRNA can be an alternative and/or complementary means to achieve expression of therapeutic proteins. For instance, the EMCV IRES was included in mRNAs coding for four transcription factors used to reprogram fibroblasts to pluripotent stem cells.[49] The EMCV IRES has even been used successfully to direct protein expression from mRNA lacking a cap.[50] Vaccination with dendritic cells transfected with such IRES-containing, cap-less mRNA protected mice from metastasis upon intravenous injection of melanoma cells.

Completely novel UTRs may be provided by screening whole transcriptomes for sequence elements that either increase translation or mRNA stability.[51]

ORF

Codon usage is also considered as a factor affecting the efficiency of translation in many species. However, in humans codon usage bias does not correlate with tRNA levels and gene expression.[52,53] In conclusion, codon optimization cannot be expected to (generally) improve mRNA translation in humans, particularly if the ORF is already of human (or even mammalian) origin.

Obviously, the start codon should be part of a Kozak sequence[54] and the sequence surrounding the stop codon may be optimized.[55] In addition, no upstream start codons, preceding the correct start codon, should be present in the mRNA.

Combinatorial design

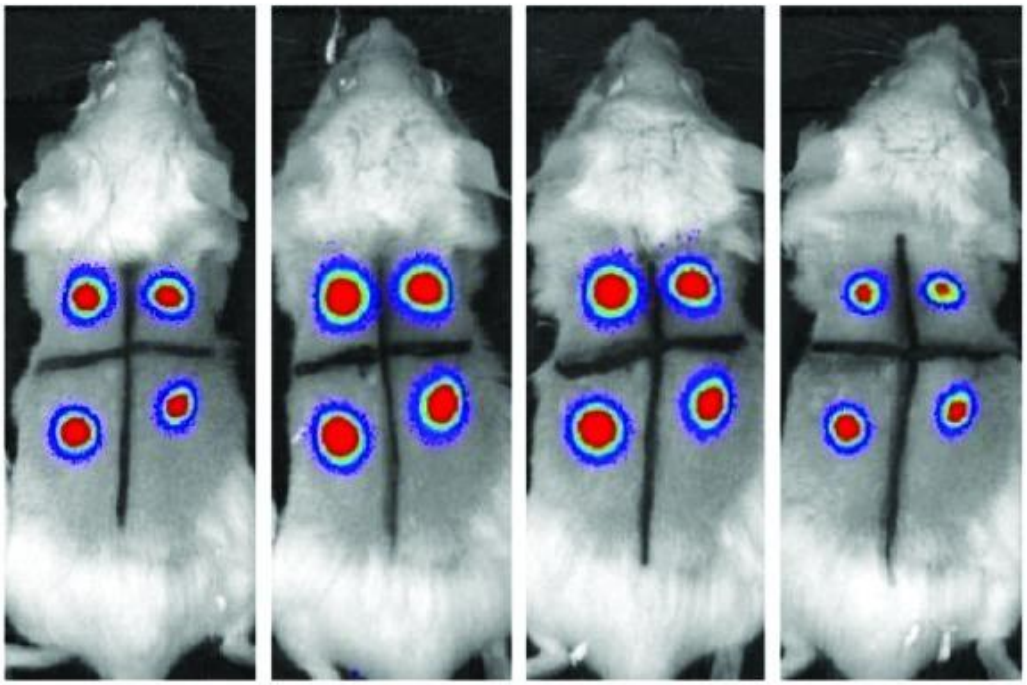
In order to obtain effective vaccine platforms, different beneficial mRNA elements have been joined.

Capping with ARCA has been combined with a long transcribed poly(A) tail of 100 residues. Such luciferase-encoding mRNA was tested in immortalized cell lines (JawsII, HepG2, HeLa) as well as immature and mature human dendritic cells.[56] Compared with mRNA capped with regular cap analog and ending with a shorter A64 poly(A) tail, a very substantial improvement in protein expression was seen in all tested cell types. The magnitude of the rise in protein level afforded by either element alone or their combination was strongly dependent on the cell type.

Sahin and coworkers combined two consecutive β globin 3‘-UTRs, a rather long transcribed poly(A) tail of 120 residues[57] and a phosphorothioate modified anti-reverse cap.[58] This resulted in increased and prolonged protein expression in transfected dendritic cells. Upon injection of mRNA into the lymph node, protein expression peaked at 8 h and was demonstrated up to 72 h after mRNA injection.

Using our proprietary mRNA technology modifying coding and noncoding parts of the molecule, we were able to improve both the level and duration of expression, increasing total protein expression by several orders of magnitude.[59] Upon intradermal mRNA injection, strongly prolonged translation gives maximum protein levels 24 to 48 h after mRNA injection and lasts for many more days (Fig. 1).

A


max
min
8 hours
24 hours
48 hours
72 hours

B

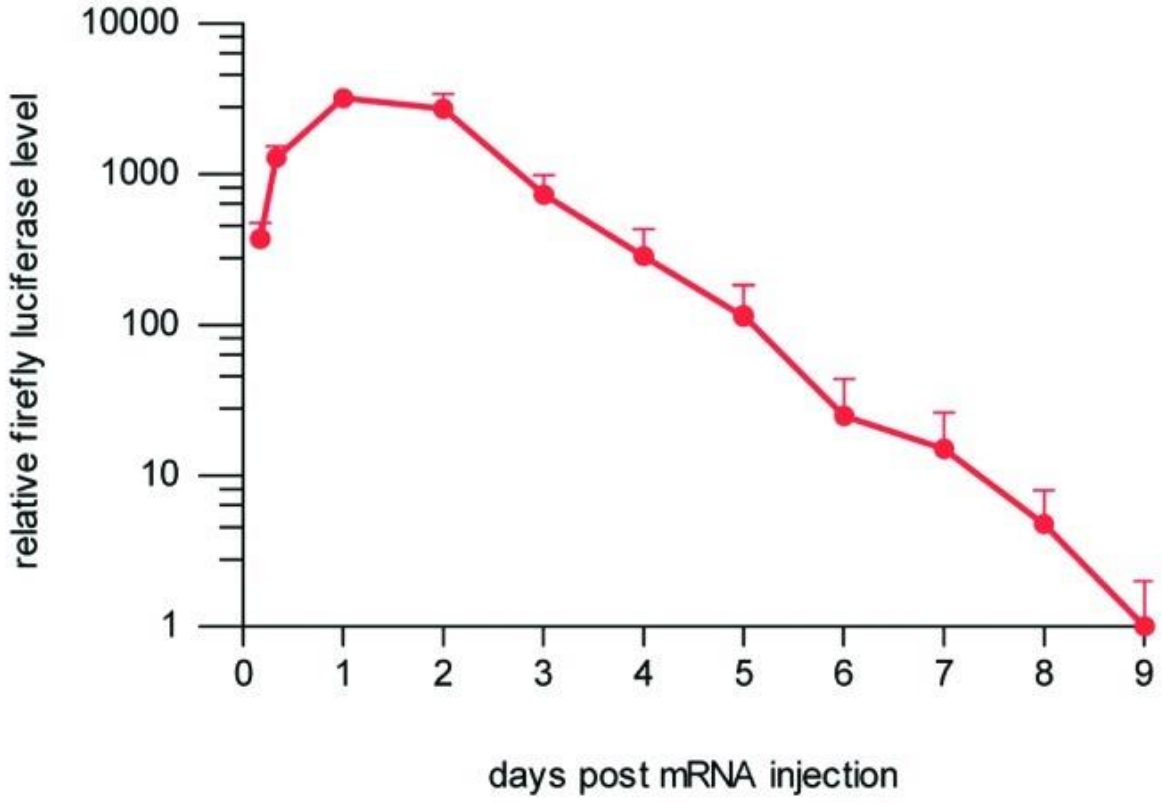

firefly luciferase expression from mRNA in vivo
relative firefly luciferase level
10000
1000
100
10
1
0
1
2
3
4
5
6
7
8
9
days post mRNA injection

Figure 1. Protein expression in vivo is strongly prolonged using CureVac's proprietary mRNA technology and lasts for many days. Firefly luciferase-encoding mRNA, optimized for translation and stability, was injected intradermally in a BALB/c mouse (4 injection sites). At various time points after mRNA injection, luciferase expression was visualized in the living animal by optical imaging. (**A**) Visualization of luciferase expression at selected time points, showing maximal protein levels 24 to 48 h after mRNA injection. (**B**) Time course of luciferase expression until 9 d after mRNA injection. Background signal was set to 1.

To be translated and elicit an antigen-specific immune response, an mRNA-vaccine has to reach the cytosol of target cells. However, as opposed to DNA vaccines, RNA vaccines only have to cross the plasma membrane, but not the nuclear envelope which may improve the probability of successful in vivo transfection.[60] As early as 1990, the uptake of mRNA by mouse muscle cells upon simple injection, i.e., without any additional help from special delivery systems, was demonstrated.[61] Later on, numerous studies confirmed that locally administered naked mRNA is taken up by cells in target tissues.[8,62-65] The mechanism by which naked mRNA enters cells remained unclear initially. However, elucidating and understanding the uptake route is important to facilitate the development of more efficient mRNA-vaccines.

A plethora of studies investigated the cellular entry of nucleic acids. Most of them looked into the uptake routes of pDNA, DNA oligonucleotides, siRNA or long dsRNA and a complex picture emerged. The molecules entered cells by diffusion controlled mechanisms or diverse endocytic pathways, often strongly dependent on the respective cell type or species and frequently showed a vesicular localization, i.e., an entrapment in endocytic or lysosomal compartments.[66-73] However, mRNA differs from these types of molecules due to its unique combination of physico-chemical and structural parameters. In contrast to DNA, mRNA contains uridine instead of deoxythymidine, preferentially adopts a C3′-endo conformation and is hydroxylated at the 2′-position of the ribose. The single-stranded nature lets mRNA fold into complex secondary and tertiary structures, completely unknown from double-stranded DNA and RNA molecules, respectively. Finally, its length of a few hundred to several thousand nucleotides distinguishes mRNA from other single-stranded RNAs like antisense RNA or aptamers.

First insight into the uptake mechanism of naked mRNA was gained by a mouse study investigating intradermal administration by injection.[8] Local entry into cells of the dermis which were not exclusively professional antigen presenting cells (pAPCs) turned out to be saturable, improvable by calcium and associated with the movement of vesicles. More elaborate work in vitro revealed that uptake of naked mRNA is a widespread phenomenon among primary cells and cell lines of diverse types.[74] These efforts confirmed saturability of uptake and demonstrated that it is also temperature and dose dependent. Most of the mRNA appeared to enter cells via caveolae/lipid rafts,[74] most likely mediated by (a) scavenger-receptor(s) which are known to concentrate in caveolae and to preferentially recognize and facilitate internalization of negatively charged macromolecules.[75-78]

To a minor degree, macropinocytosis also appeared to be involved in mRNA uptake of different primary cells and cell lines.[74] By contrast, macropinocytosis apparently predominates mRNA uptake by dendritic cells upon intranodal injection.[79] The picture becomes even more complicated when looking at formulated mRNA vaccines. For instance, a recently developed two-component vaccine consisting of naked and protamine-complexed mRNA reveals different

routes and kinetics of uptake for the two components, albeit both are taken up via an endosomal pathway.[9,80]

mRNA uptake and expression in vivo is quite efficient (much more efficient than spontaneous uptake by cells in vitro) and comparable even with cells transfected in vitro under optimal conditions.[8,61] In part, hydrodynamic pressure may contribute to target cell transfection in case of local injections[81] as it does upon intravenous administration.[82] However, the correlation between pressure and transfection efficiency/protein expression may not be linear but show an optimum.[83] Anyway, a large amount of the mRNA appears to stay trapped in endosomal vesicles. Hence, mRNA vaccines may profit strongly from approaches increasing the fraction of mRNA that reaches the cytosol.

mRNA is threatened by rapid degradation by ubiquitous extracellular ribonucleases before being taken up by cells.[84] Thus, the efficacy of mRNA vaccines may benefit significantly from complexing agents which protect RNA from degradation. Complexation may also enhance uptake by cells and/or improve delivery to the translation machinery in the cytoplasm. To this end, mRNA is often complexed with either lipids or polymers.

Importantly, not all complexing agents that promote transfection of DNA are suitable for complexation of mRNA. Different large polycations, all proven DNA transfection reagents, were shown to strongly inhibit translation of mRNA in cell-free translation systems as well as inside cells. Only much smaller polycations allowed for efficient translation. Likely, mRNA is not released in the cytosol if bound to large polycations.[85] Interestingly, DNA may be released in the cytosol from large polycations by endogenous RNA.[86]

In line with the general conception that mRNA should be protected and uptake enhanced, the first report demonstrating the induction of an immune response upon direct injection of mRNA in vivo used mRNA encapsulated in liposomes.[87] Common is the use of cationic lipids, for instance used for the intradermal and intravenous injection of antigen-encoding mRNA.[88] However, complexation of mRNA with protamine, a small arginine-rich nuclear protein which stabilizes DNA during spermatogenesis, was shown to also efficiently stabilize mRNA against degradation by serum components.[63]

In addition, complexing agents rationally designed to further improve delivery of nucleic acids to the cytosol have been used for formulation of mRNA vaccines. Hemagglutinating virus of Japan (HVJ)–liposomes have been reported to deliver their cargo directly into the cytoplasm of host cells in vivo by means of a virus–cell fusion mechanism. Such liposomes were used to inject mRNA (replicating in this case) encoding melanoma antigen gp100 into the spleen of mice.[89] Alternatively, vectors improving cytosolic nucleic acid delivery by means of permeation of endosomal membranes due to their high histidine content have been used to formulate antigen-encoding mRNA.[90]

Complexing agents may have to be tailored to the specific route of delivery. Due to the abundance of professional antigen presenting cells in the skin,[91] this organ may be particularly suitable for vaccination. However, delivery of a DNA vaccine into mouse skin by tattooing failed when formulated into cationic nanoparticles but was successful upon PEGylation of the nanoparticles to shield their surface charge.[92] Likely, adsorption of the cationic nanoparticles to the negatively charged extracellular matrix in the skin prevented their uptake by cells. In

addition, the use of complexing agents in vivo is often hampered by toxicity, particularly for high molecular weight compounds.[93] Still, progress in the drug delivery field is steady,[94,95] including innovative approaches to targeting of drugs to particular cell types.[96] Looking ahead, improved delivery is certain to contribute to increased efficacy of mRNA vaccines.

As an intermediate carrier of genetic information, endogenous mRNA is used as template for protein expression. Hence, like DNA, mRNA is at least in principle an attractive means to force cells to produce proteins of interest by introducing exogenous nucleic acid molecules. For mRNA, this concept was first applied in the early 1970s. Microinjection of preparations of rabbit hemoglobin mRNA and encephalomyocarditis virus RNA, respectively, into oocytes from *Xenopus laevis* provided clear evidence that these molecules gave rise to the expression of RNA-encoded proteins.[97,98] More than ten years later, in vitro transcribed RNA from brome mosaic virus (BMV) and poliovirus cDNA were shown to be infectious, an unequivocal indication of protein expression from those RNAs.[99,100] However, at that time viable techniques allowing use of mRNA as a general tool for protein expression were still missing. This changed with the adaptation of efficient transfection methods such as electroporation and cationic lipofection for the delivery of RNA.[2,101] Further developments and insights into mRNA biology enabled significant overexpression of proteins after delivery.[102] Finally, the in vitro use of mRNA culminated in the establishment of cell reprogramming protocols that may be of some medical relevance in the future.[49,103]

Whereas all these examples cover mRNA-mediated protein expression exclusively taking place in vitro, meanwhile, cell based approaches of mRNA-mediated protein expression have expanded into in vivo settings. On the one hand, mRNA injection into fertilized oocytes or early embryos became a well-established tool in developmental biology.[104] On the other hand, loading of dendritic cells with antigen-encoding mRNA originally described by Boczkowski et al.[105] became a widely used approach in immunology and was investigated in several clinical trials in humans (see section mRNA-based vaccines).

Since these semi-in vivo applications introducing the mRNA ex vivo are laborious and technically very demanding, scientists were interested in direct in vivo application early on. First efforts demonstrated that local injection of naked mRNA can lead to expression of different proteins in mouse muscle tissue.[61,62] In an attempt to improve mRNA delivery, a particle-mediated administration via gene gun was developed and demonstrated to give rise to protein expression in liver and epidermis.[106] Later, successful protein expression upon intradermal injection in mice was proven.[63] Using this administration route, it was shown that (perhaps various) MHC class II-negative non-pAPCs take up and express mRNA.[8] Together, these findings suggest that mRNA can be taken up and expressed by different cell types in vivo, which is consistent with in vitro data.[74]

These results conclusively show that mRNA-mediated protein expression in vivo is generally possible. In addition, they demonstrate that expression is sufficient to raise detectable immune responses. However, raising an effective immune response and, even more, achieving a therapeutic effect by mRNA-mediated protein supply may be more demanding in terms of the required level of protein expression. Using our proprietary mRNA-technology, we could demonstrate that a single intramuscular injection of erythropoietin (Epo)-encoding mRNA led to a biologically relevant increase of reticulocytes in mice (Fig. 2). Therapeutic effects using Epo-

mRNA were confirmed by two independent studies.[47,65] The potency of mRNA-mediated protein expression was further underlined by an analysis of protein complementation in a surfactant protein B-deficient mouse model.[65] However, in contrast to our work, these studies deployed mRNA harboring modified nucleotides to increase protein expression. While such modifications can enhance translation of the mRNA[107,108] and may be beneficial for protein replacement therapies, they interfere with the design of mRNA-vaccines with self-adjuvanticity, an important feature required for a potent vaccine (see next section).

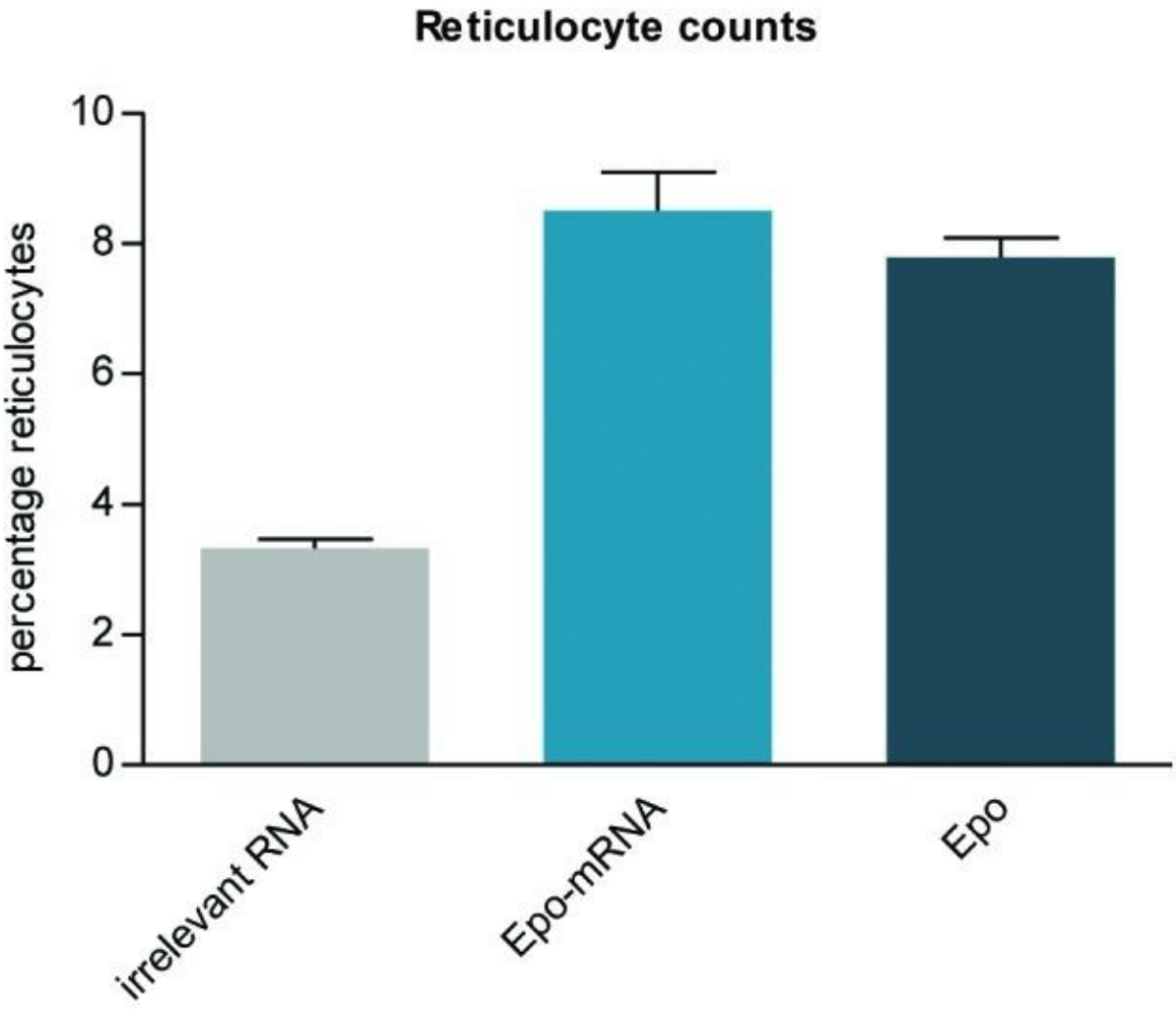


Figure 2. A biologically relevant increase of reticulocytes is induced in mice using CureVac's proprietary mRNA technology. A single intramuscular injection in BALB/c mice of erythropoietin (Epo)-encoding mRNA, optimized for translation and stability, causes the expression of functional Epo. Reticulocyte levels are raised comparably by mRNA and recombinant protein injected intramuscularly.

To be efficient, vaccines should contain a strong adjuvant supplying a danger signal for the initiation and support of the adaptive immune response in addition to an appropriate

antigen.[109] The immunostimulatory properties of RNA were first discovered by the observation of interferon induction upon exposure of cells to exogenous RNA extracted from viruses.[110] Further support came from synthetic double-stranded RNA inducing interferon upon intravenous injection into rabbits.[111] However, severe side effects of these early RNA adjuvants soon limited their further use.[112] The idea of synthetic RNA, mainly produced by in vitro transcription, as immunostimulant was then re-stimulated particularly during the last decade bringing forth a plethora of studies.

In 2004, in vitro transcribed mRNA was shown to serve as an adjuvant, if it was stabilized by either complexation or chemical modification.[113] One year later, a strong danger signal was ascribed to protamine-condensed mRNA leading to TNFα and IFNα secretion by various cells.[114] A thorough analysis of complexes of single-stranded RNA and protamine indicated that cell activation in terms of cell selectivity and induced cytokine pattern may depend on particle size.[115] Recently, research on protamine-complexed RNA culminated in a simplified vaccine approach, combining naked and protamine-formulated mRNA.[9] The resulting mRNA vaccine consists of two components complementing each other; while antigen supply is mainly driven by the naked mRNA, the protamine complexes contribute a strong immunostimulatory signal. Of note, protamine-formulated RNA can also confer adjuvanticity to, e.g., protein vaccines.[116]

Among potent adjuvant targets, RNA-sensing receptors are a particularly diverse class of molecules evolved to detect and counteract viral infections by orchestrating the innate and adaptive arms of the immune system.[117,118] Single- and double-stranded RNAs are recognized by toll-like receptors (TLR) 7/8 and 3, respectively, in the endosome.[119-121] However, TLR3 is not only activated by double-stranded RNA, an intermediary for many viruses, but also by mRNA either released from cells or produced by in vitro transcription.[122] Protamine-complexation of RNA appears to preserve its interaction with TLRs as indicated by the stimulation of several blood cell types.[114] For mRNA vaccines it was demonstrated that activation of TLR7 and potentially TLR3 is critical for priming immune responses.[9,45] Notably, optimal sequence motifs for receptor binding could be identified for single-stranded RNA.[123-126]

Other pattern recognition receptors may be important for the functionality of an RNA-based adjuvant (and vaccine) as well. The cytosolic helicase RIG-I recognizes uncapped RNA molecules harboring a 5′-triphosphate moiety.[127-129] Together with the homologous proteins MDA5 and LGP2, RIG-I forms a receptor family whose members can all bind double-stranded RNA,[130-132] but do have additional recognition patterns. For example, MDA5 is involved in the discrimination of RNAs based on the ribose 2′-O-methylation status of the cap structure.[133] For the sake of completeness, the cytoplasmic RNA sensors PKR and 2′-5′-oligoadenylate synthetase, inhibiting translation by phosphorylation of eIF-2α and activating RNase L, respectively, should be mentioned here as well.[134,135] However, the contribution of these non-TLR RNA-sensors to the immunostimulation by RNA-based adjuvants (and vaccines) is still a matter of debate. Notably, as far as investigated, the interaction between endosomal as well as cytoplasmic receptors and RNA is impaired if the RNA harbors distinct nucleotide modifications.[108,136,137] As a consequence, such modified RNA impairs the design of self-adjuvanting mRNA-vaccines.

Go to:

mRNA-Based Vaccines

After in vivo administration of mRNA was proven to be feasible,[61] the concept of using mRNA as a basis for vaccines was pursued almost immediately. First success was reported in 1993 when subcutaneous injection of liposome-encapsulated mRNA encoding the nucleoprotein (NP) of

influenza virus was demonstrated to elicit NP-specific cytotoxic T cells (CTLs).[87] By contrast, naked mRNA failed to raise specific CTLs in this setting. Shortly afterwards, the use of naked mRNA triggered the induction of antigen-specific antibodies in response to a heterologous prime-boost schedule (repeated intramuscular RNA vaccination, challenge with tumor cells).[62] However, none of the animals was protected against tumor challenge. An antigen-specific antibody response induced solely with mRNA was demonstrated first using particle-mediated mRNA delivery into mouse epidermis.[106]

In 2000, the field of mRNA vaccines was advanced by introducing a new protocol for vaccination allowing the administration of naked mRNA via intradermal injection.[63] This basic vaccination design did not require any transfection reagents, special equipment or heterologous boost, yet could elicit a complete adaptive immune response consisting of antigen-specific antibodies and T cells with lytic activity against the model antigen β-galactosidase. Directly thereafter, intradermal injection of total RNA isolated from the S1509 tumor cell line was shown to induce immunity to a subsequent challenge with the tumor.[138] Tumor growth inhibition was also achieved by intradermal as well as intravenous injection of in vitro transcribed and lipid-complexed mRNA encoding the model antigen ovalbumin (OVA).[88] However, analogous vaccination with mRNA coding for a model tumor/self-antigen was not sufficient to break tolerance to this self-antigen in TRAMP mice. A similar approach using histidylated lipopolyplexes for systemic injection revealed that MART1 mRNA could not only prevent B16 melanoma from progression but also from metastasis.[90]

In a comparison of different administration routes for the delivery of naked mRNA vaccines good immunogenicity against ovalbumin and influenza A virus hemagglutinin could be demonstrated after repeated and frequent injections into the lymph node.[45] In order to optimize the vaccine's potency, the authors engineered the antigen by adding an MHC class I molecule trafficking signal for increased antigen presentation.[44] Unlike with intranodal injection, the authors could not elicit such immune responses upon perinodal, subcutaneous or intradermal injections.[45] Recently, an alternative, simplified approach was described leading to successful immunization by intradermal injection.[9] Combining naked mRNA with protamine-formulated mRNA results in a two-component vaccine capable of inducing strong immune responses and tumor protection in prophylactic as well as therapeutic settings in mice. In this vaccine, the two components fulfill complementary functions: while the naked mRNA confers optimal antigen expression, the protamine-complexed mRNA contributes strong immunostimulatory effects. Notably, for tumor treatment this new type of mRNA vaccine can be combined with other, standard, therapies such as chemotherapy, thereby achieving improved effects as compared with each treatment alone.[59]

As an alternative to direct injection of mRNA, an immune response may also be induced by vaccination with pAPCs transfected with mRNA ex vivo. mRNA-transfected murine dendritic cells (DCs) were shown to elicit anti-tumor immunity in EG.7-OVA and B16 melanoma models.[105] Tumor growth was also significantly reduced upon injection of epidermal cells enriched for Langerhans cells, which belong to the group of pAPCs, that had been transfected with total RNA derived from tumor cells.[138] Using human DCs, transfection with mRNA encoding CEA or the E6 antigen of human papillomavirus type 16 induced a primary CTL response in vitro.[139] Today, ex vivo mRNA transfection of pAPCs is the most frequently used approach for mRNA vaccination in the clinic. For instance, a clinical trial utilizing telomerase mRNA-transfected DCs demonstrated the capability of such applications to stimulate antigen-

specific cellular immune responses.[140] However, the underlying procedure is very time consuming, laborious and needs patient-specific (autologous) cell preparations.
Very few clinical studies of the direct administration of mRNA-based vaccines have been published. The first trial deployed autologous mRNA libraries derived from melanoma lesions, whereas in a later study a cocktail of protamine-complexed mRNAs encoding six different antigens was given intradermally using an intensified treatment regimen.[43,141,142] A further clinical trial with patients with renal cell carcinoma stage IV included the administration of GM-CSF as adjuvant 24 h after vaccination with six antigens,[143] an approach that will be discussed in more detail in the next section.

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Adjuvanted mRNA-Based Vaccines

As discussed, mRNA-vaccines can be designed to possess self-adjuvanticity contributing to their excellent performance. Although pDNA vaccines also show native immunogenicity, great efforts were made to improve the immune response by co-delivery or, more elegantly, co-expression of co-stimulatory molecules and cytokines. Indeed, inclusion of adjuvant molecules encoded as DNA could enhance DNA vaccines. Co-injection of DNA encoding tumor or viral antigens and the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF) improved T and B cell responses.[144-147] As a further example, DNA expressing a recombinant soluble multi-trimeric TNF superfamily ligand enhanced the immune response to an HIV-1 Gag DNA vaccine.[148] Moreover, DNA encoding the cytokine IL-2 was demonstrated to affect the polarization of the immune response, an important vaccine parameter the optimum of which differs among indications. While a DNA vaccine for *Helicobacter pylori* elicited a strong Th2 response, it was shifted toward a Th1-biased response by IL-2.[149] In addition to directly administering a DNA encoding the adjuvant molecule, more indirect approaches are also feasible. For instance, the co-delivery of a CD40-expressing plasmid induced anti-CD40 antibodies, part of which were capable of activating CD40 which in turn led to an improved immune response to an HBV DNA vaccine.[150]
In an analogous fashion, RNA vaccines supplemented with additional adjuvant molecules have been the object of various investigations. In mice, recombinant GM-CSF enhanced the immune response to the model antigen β-galactosidase and affected polarization of immunity by shifting a Th2 to a Th1 response.[64] In addition, GM-CSF as a supplement of an mRNA vaccine was already tested in a clinical trial.[143] An improved anti-tumor effect of a naked mRNA vaccine in mice was demonstrated for human FLT3 ligand protein fused to human IgG4-Fc fragment.[151] However, in light of getting good protein expression upon mRNA administration in vitro and in vivo (see section mRNA-mediated protein expression), providing auxiliary adjuvant molecules via mRNA rather than as protein appears to be a feasible and much more elegant approach. First support for this idea came from a study investigating the effect of GM-CSF, IL-2 and CD80, all encoded by mRNA, on the potency of model mRNA vaccines.[88] Among these adjuvants, GM-CSF mRNA improved the induction of CTL activity in a dose-dependent manner. Moreover, a more durable CTL response indicated an enhanced generation of memory cells. We have recently investigated the anti-tumor effect of an accessory adjuvant molecule encoded by mRNA. To this end, we included CD40 ligand as a co-stimulatory molecule activating pAPCs[152] which may establish an autoregulatory circuit improving the immune response by enhancing antigen presentation. CD40 ligand supplied as mRNA significantly improved tumor growth reduction as compared with the non-adjuvanted two-component mRNA vaccine (Fig. 3).
In summary, these data suggest that the combination of mRNA vaccines and additional mRNAs

encoding auxiliary adjuvant molecules is a very promising approach which, however, remains challenging in terms of arriving at suitable, i.e., effective and practical, treatment regimens.

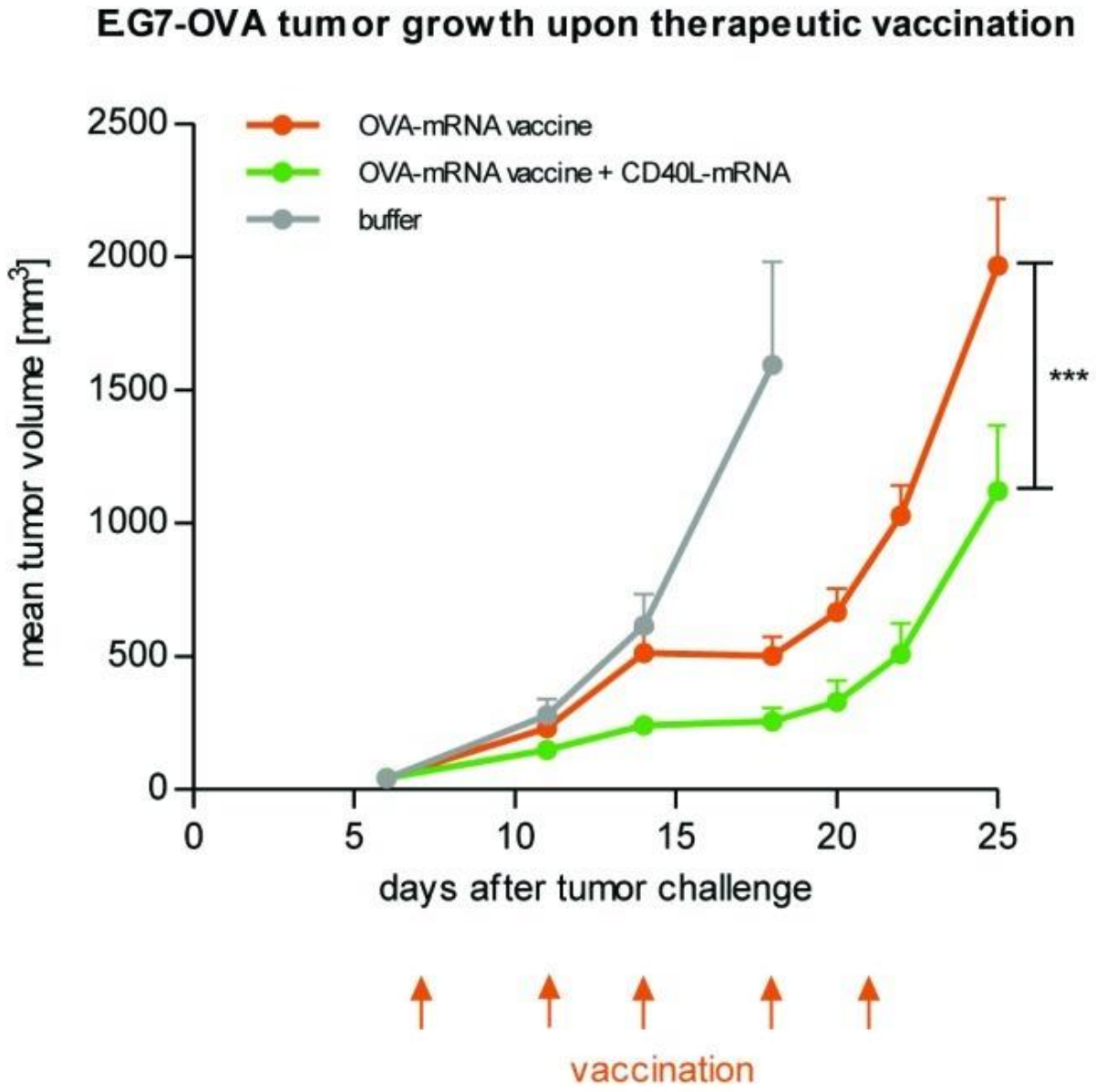

Figure 3. CD40 ligand as an accessory adjuvant molecule encoded by mRNA increases the anti-tumor effect of a two-component mRNA vaccine. Mice (n = 8 per group) were challenged subcutaneously with syngenic E.G7-OVA tumor cells on day 0. Commencing on day 7, mice were vaccinated intradermally with either OVA-mRNA vaccine alone or in combination with mRNA coding for CD40 ligand according to the indicated schedule. Mice treated with buffer served as the control. The combination of CD40 ligand-encoding mRNA together with OVA mRNA vaccination increases the efficacy of the therapeutic anti-tumor mRNA vaccination.

Concluding Remarks

About two decades after the first successful administration of mRNA in vivo, mRNA-based vaccines promise to become a game-changing vaccine technology platform for therapeutic as well as prophylactic applications. Today, the scientific community is eagerly waiting for first clinical efficacy data. But there is still a wide field for further development/improvements of mRNA-based vaccines. As discussed, the format and uptake of the mRNA are critical parameters for efficient antigen expression which can be influenced by novel RNA designs as well as mRNA formulation and administration. However, any changes to these parameters may have major implications on mRNA production and/or its interactions with RNA-sensors and should be carefully considered early on. For instance, in addition to the previously mentioned nucleotide modifications, novel delivery modes may severely affect vaccine adjuvanticity. While direct delivery into the cytosol would certainly enhance antigen expression, the lack of interaction with endosomal RNA receptors may severely weaken immunostimulation by the vaccine and this issue would likely have to be addressed. The inclusion of accessory mRNA molecules into an mRNA vaccine may be an interesting option for achieving optimal effects in case of particularly challenging treatments. Moreover, the combination with other anti-tumor therapies will most likely yield the greatest potency. However, this would increase the complexity of the vaccine and/or the treatment regimen making the development more challenging. Taken together, mRNA offers a promising vaccine vector in the light of being flexible, effective and safe. Hence, it could become a "disruptive technology" not just for cancer immunotherapy, but also for vaccination, either prophylactic or therapeutic, against infectious diseases.

Retrieved July 31, 2022 from Developing mRNA-vaccine technologies - PMC (nih.gov)

Question:
Do COVID-19 vaccines contain fetal cells? Were abortions performed to make the vaccines?
Answer:
Vaccines do not contain fetal cells, and no abortions are performed to make vaccines. This includes COVID-19 vaccines.

Some vaccines are made by growing the vaccine viruses in human fetal cell lines. However, the vaccines themselves do not contain fetal cells or tissue. The purification process removes nearly all the cell components so that only trace amounts of DNA and protein may be present in the vaccine.

Fetal cell lines are used to test and develop many common over-the-counter and prescribed medications, including antacids and cold medications.

Are COVID-19 vaccines made using fetal cell lines?

- Fetal cell lines were used to make the Janssen (Johnson & Johnson) vaccine and the AstraZeneca (Vaxzevria) vaccine. However, the vaccines themselves do not contain fetal cells or tissue. The purification process removes nearly all the cell components so that only trace amounts of DNA and protein may be present in the vaccine.
- Fetal cell lines were not used to make the Moderna (Spikevax) and Pfizer-BioNTech (Comirnaty) COVID-19 mRNA vaccines. However, the cell lines were used in the

very early stages of research and development of these vaccines to test 'proof of concept' (to test that the vaccines could work).

What fetal cell lines are used?

- The AstraZeneca (Vaxzevria) vaccine uses the HEK 293 fetal cell line, and the Janssen (Johnson & Johnson) vaccine uses the PER.C6 fetal cell line. However, the vaccines themselves do not contain fetal cells or tissue. The purification process removes nearly all the cell components so that only trace amounts of DNA and protein may be present in the vaccine.
- The Moderna (Spikevax) and Pfizer-BioNTech (Comirnaty) COVID-19 vaccines used the fetal cell line HEK 293 in the very early stages of research and development. It was not used to make these vaccines.
- The HEK 293 and PER.C6 fetal cell lines descend from cells taken from fetuses aborted in the 1970s and 1980s. The fetuses were not aborted to make vaccines.

Why are fetal cell lines used?

Fetal cell lines are used with some vaccines because viruses need to be grown in cells and human cells are often better than animal cells at supporting the growth of human viruses.
Vaccine manufacturers may use these fetal cell lines during the following two phases:

- Research and development
- Production and manufacturing

Fetal cell lines are used in scientific and medical research and in the research and development of most medical products available today.

What are fetal cell lines?

Fetal cell lines are cells that are grown in a laboratory. They descend from cells taken from fetuses aborted in the 1970s and 1980s that have since multiplied into many new cells over the past four or five decades, creating the fetal cell lines. The fetuses were not aborted to make vaccines. Current fetal cell lines are thousands of generations removed from the original fetal tissue. They do not contain any tissue from a fetus.

What does the Catholic church say about the use of fetal cell lines in vaccines?
The Vatican's Pontifical Academy for Life declared in 2005 and reaffirmed in 2017 that in the absence of alternatives, Catholics could, in good conscience, receive vaccines made using historical human fetal cell lines. In December 2020, the Congregation for the Doctrine of the Faith provided a note on the morality of using some anti-Covid-19 vaccines.

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