A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs

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Karl-Josef Kallen and Andreas Theß

Abstract: Recent advances strongly suggest that mRNA rather than DNA will be the nucleotide basis for a new class of vaccines and drugs. Therapeutic cancer vaccines against a variety of targets have been developed on this basis and initial clinical experience suggests that preclinical activity can be successfully translated to human application. Likewise, prophylactic vaccines against viral pathogens and allergens have demonstrated their activity in animal models. These successes could be extended preclinically to mRNA protein and gene replacement therapy as well as the induction of pluripotent stem cells by mRNA encoded transcription factors. The production of mRNA-based vaccines and drugs is highly flexible, scalable and cost competitive, and eliminates the requirement of a cold chain. mRNA-based drugs and vaccines offer all the advantages of a nucleotide-based approach at reduced costs and represent a truly disruptive technology that may start a revolution in medicine.

Keywords: mRNA-based vaccines, replicons, RNActive vaccines, therapeutic mRNA

Introduction

The technological advances of molecular biology in recent decades have dramatically changed our understanding of human diseases as well as their diagnosis and treatment. The identification of crucial factors in the pathophysiology of human diseases, made possible by genetic screening, transgenic or knockout animal models to name only a few, also allowed us to develop highly selective, small molecule inhibitors of specific targets that sometimes completely changed the natural course of inexorable diseases, for example, the inhibition of the oncogenic fusion protein bcr-abl in chronic myelogenous leukemia by imatinib, dasatinib, and nilotinib [Panjarian *et al.* 2013; Quintas-Cardama and Jabbour, 2013]. Our increasing ability to manipulate the genetic code of proteins in question, that is, their DNA, in many instances enabled us to bypass the development of small molecule inhibitors and instead to exploit proteins such as monoclonal antibodies, recombinant naturally occurring or highly engineered proteins for diagnostic and therapeutic purposes to an extent that was almost unimaginable just two decades ago (examples given in the following references) [Bargou *et al.* 2008; Bendtzen, 2012; Day *et al.* 2013; Lindsay *et al.* 2013; Ljung, 2013; Moots and Naisbett-Groet, 2012; Robak, 2012; Sellam *et al.* 2013; Specenier and Vermorken, 2013]. However, the use of proteins for medical purposes often requires good manufacturing practice (GMP) production processes that are technically and financially very demanding and not very flexible in that each protein requires its own production facility.

It was recognized early on that proteins could principally be expressed either by direct injection of DNA or messenger RNA (mRNA) into target organs [Wolff *et al.* 1990]. The initial efforts to exploit this effect focused on the development of nucleotide-based vaccines, since the capacity to express proteins on this basis was considered limited. While the first reports on nucleotide-based vaccines showed that vaccines produced on a DNA or mRNA basis had similar activity [Conry

Correspondence to:
Karl-Josef Kallen MD, PhD
CureVac GmbH, Paul Ehrlich Str. 15, 72076 Tübingen, Germany
kl@curevac.com

Andreas Theß, PhD
CureVac GmbH, Tübingen, Germany

et al. 1995; Martinon *et al.* 1993; Tang *et al.* 1992], most researchers focused on the development of DNA vaccines in the coming decades. The main reasons for this attitude were a perceived instability of mRNA, difficulties to ensure long-term storage, and importantly, the cost of manufacturing GMP grade material.

Roughly 10 years ago though, Pascolo pointed out that the latter view was erroneous and that the costs of manufacturing mRNA on a large scale would be lower than those to produce DNA [Pascolo, 2004]. Nucleotide vaccines based on mRNA offer the flexibility to encode virtually any protein as antigen in a very short time span, but could be produced with the same production process in the same production facilities. Thus novel vaccines could be made in a very short time with limited financial investments, which is of great importance for pandemic scenarios in infectious diseases and for the possibility of making cancer vaccines against patient-specific cancer-associated or mutated antigens [Kreiter *et al.* 2012; Petsch *et al.* 2012]. Moreover, mRNA carries no risk of genomic integration, which might not just be a theoretical risk for DNA. This gives mRNA an inherent safety advantage over DNA-based therapeutics.

This article argues that, contrary to former expectations, it is not DNA that emerges as the nucleotide basis for vaccines, but mRNA might be the ideal basis for the development of new vaccines against infectious pathogens [Petsch *et al.* 2012] and for cancer vaccines [Kübler *et al.* 2011; Sebastian *et al.* 2011, 2012]. With the possibility to do more at reduced costs, mRNA vaccination technology would constitute a truly disruptive technology [Christensen, 1997]. Recent technological advances in understanding the properties of mRNA have widened the conceivable applications of mRNA beyond immunization purposes to the production of proteins *in situ* [Kariko *et al.* 2012; Kormann *et al.* 2011] and to autologous cell therapies based on mRNA-induced pluripotent stem cells [Mandal and Rossi, 2013]. Hence, it may well be that the first drug approved designed on a nucleotide basis will rely on mRNA rather than the seemingly less complicated DNA [Geall *et al.* 2013; Gilboa, 2012].

DNA vaccines

Vaccines based on nucleotides appeared as the ideal basis to move beyond empirical to rational design of vaccine research and development, and to stimulate the immune system in a directed manner that also generates cellular immunity [Ulmer *et al.* 2012]. In addition, manufacturing was considered to be relatively simple, inexpensive and scalable with the production process readily adaptable from one vaccine to another or rather from one nucleotide code to another. Though early efforts to develop vaccines based on nucleotides used both DNA [Tang *et al.* 1992; Ulmer *et al.* 1993] and RNA [Martinon *et al.* 1993] for vaccination, the next two decades were largely dominated by research on DNA vaccines, due to the perceived greater ease of use. DNA vaccines were able to induce potent T- and B-cell immune responses in animals against a variety of antigens. This culminated in the development and commercialization of plasmid DNA-based vaccines for animal usage: a melanoma cancer vaccine was approved for dogs [Grosenbaugh *et al.* 2011], an infectious hematopoietic necrosis virus vaccine for fish and a vaccine for West Nile virus for horses [Davis *et al.* 2001; Garver *et al.* 2005; Kurath *et al.* 2006].

However, the development of DNA vaccines for humans has not been similarly successful thus far. While a number of clinical trials demonstrated the principle ability of DNA vaccines to induce cellular T- and B-cell responses in humans, the strength of these immune responses was rather lower than that achieved by more conventional approaches. The reasons for this are not quite clear, but might be related to the necessity of DNA vaccines to cross at least two cell membranes, the nuclear membrane in addition to the plasma membrane to achieve antigen expression. Extensive efforts have therefore been directed at various techniques to facilitate the physical delivery of DNA that include sophisticated electroporation (EP) techniques, needle-free approaches, such as particle bombardment and high-pressure delivery, and dermal patches, but also activation of the immune system by encoded immunostimulatory molecules (genetic adjuvants) [Sardesai and Weiner, 2011; Ulmer *et al.* 2012]. Recent studies with new EP devices reignited hope for DNA vaccines. A therapeutic human papillomavirus (HPV) 16/18 candidate vaccine, VGX-3100, induced robust immune responses to antigens from high-risk HPV serotypes after *in vivo* EP [Bagarazzi *et al.* 2012]. It was argued that these contribute to elimination of HPV-infected cells and subsequent regression of the dysplastic process. Malaria DNA vaccine

candidates were recently generated from synthetic sequences of four antigens from *Plasmodium falciparium* (circumsporozoite protein, liver stage antigen 1, thrombospondin-related anonymous protein, and cell-traversal protein for ookinetes and sporozoites), administered *in vivo* and studied preclinically for their immunogenicity in mice and nonhuman primates (NHPs). Humoral and cellular responses comprising interleukin (IL)-2, interferon γ (IFNγ) and tumor necrosis factor α (TNFα) producing CD4+ and CD8+ T cells were detected against all antigens encoded in mice [Ferraro *et al.* 2013]. The cellular responses were also found in NHPs, and importantly, antigen-specific CD8+ Granzyme B+ T cells could be detected. An alternative approach by Lu and colleagues showed that priming with DNA vaccines followed by protein boost vaccination regimes results in remarkable immune responses [Vaine *et al.* 2008; Wang *et al.* 2012], but this approach gives up some of the advantages of a DNA vaccination only. Against this background of advances in the DNA vaccine field, the recent failure of Allovectin-7 [Bedikian *et al.* 2010], a bicistronic plasmid encoding human leukocyte antigen (HLA)-B7 and β2 microglobulin formulated with a cationic lipid system, to meet its primary endpoint to achieve durable responses in advanced melanoma in a phase III trial came as a disappointment, but detailed results of the study have not yet been published (http://www.vical.com/). Yet, a phase II trial with TransVax, a therapeutic DNA vaccine targeting cytomegalovirus glycoprotein B and phosphoprotein 65 formulated with poloxamer CRL1005 and benzalkonium chloride, yielded encouraging results [Kharfan-Dabaja *et al.* 2012]. Cytomegalovirus-seropositive patients after allogeneic hemopoietic stem-cell transplantation showed a reduction in the rate of clinically significant viremia requiring cytomegalovirus-specific antiviral therapy which, however, did not reach statistical significance. A longer follow up revealed a significant reduction of occurrence and recurrence of cytomegalovirus viremia and improved the time to event for viremia episodes compared with placebo. The DNA vaccine was well tolerated and is presently being studied in a phase III trial (http://www.vical.com/). Hence, there clearly is a silver lining on the horizon for DNA vaccines, but despite these recent progresses it remains to be seen whether DNA vaccines can now achieve the breakthrough not seen in the past two decades of research on DNA vaccines.

In addition to the challenges mentioned above, DNA vaccines carry the principle risk of genomic integration as exemplified by the generation of stably transfected cell lines which results from random genomic integration of the transfected DNA which is afterwards selected for. This risk of genomic integration is generally considered to be very small. However, hundreds of millions of doses of a successful prophylactic vaccine against infectious pathogens might be administered to healthy recipients over years. Under these conditions, even a very rare event might become a potentially serious safety problem, particularly in view of the vaccination fatigue in western countries [Finnegan, 2012]. Another issue is the duration of antigen expression which can last for several months. Prolonged antigen expression *per se* may not necessarily correlate with good immune responses and may even be detrimental to the intended immune effect and lead to exhaustion of T cells [Han *et al.* 2010; Shin and Wherry, 2007; Wherry *et al.* 2003]. A different explanation for the 'underperformance' of DNA vaccines in humans may be a weaker than presumed built in adjuvanticity of these vaccines. The importance of cytoplasmic DNA sensors for the induction of DNA-dependent immune responses has been increasingly recognized recently [Aoshi *et al.* 2011; Desmet and Ishii, 2012; Marichal *et al.* 2011]. It is conceivable that without the assistance of sophisticated delivery methods, not enough DNA might end up in the cytoplasm or that species differences exist in the sensitivity of these sensors to stimulation by DNA. Both effects might impair the efficacy of DNA vaccines in humans.

The promise of mRNA-based vaccines

The potential of mRNA to be used for protein expression was first demonstrated by Wolff and colleagues with the successful expression of a variety of proteins after direct injection of their mRNA into the muscle of mice [Wolff *et al.* 1990]. This was followed by the first report of a successful mRNA vaccine that demonstrated the induction of anti-influenza cytotoxic T lymphocytes *in vivo* by immunizing mice with liposomes containing mRNA encoding the influenza virus nucleoprotein [Martinon *et al.* 1993]. However, liposomal protection of mRNA is not essential as was demonstrated by the repeated injection of an unprotected mRNA coding for carcinoembryonic antigen (CEA) directly into the muscles of mice [Conry *et al.* 1995]. Mice immunized with

unprotected CEA encoding mRNA developed an antibody response to CEA upon challenge with a CEA-expressing tumor cell line, while control mice not receiving mRNA injections did not develop antibodies to CEA. Yet, the immune response required not just administration of the mRNA, but also expression of the encoded antigen by the tumor cell line used for challenge. Five years later, a major leap forward was made by the demonstration that substantial humoral and cellular immune responses could be directly induced by the injection of antigens encoded as naked (unprotected) or protamine-protected mRNA into the ear pinna of mice [Hoerr *et al.* 2000]. The induced immune response was antigen specific and importantly also functional as indicated by the capacity of induced T cells to lyse cells expressing the antigen. These initial experiments established that mRNA could principally serve as the basis for gene therapy or gene replacement and for the induction of protective cellular or humoral immunity. It was suggested that this approach might be particularly useful for induction of an immune response against a proto-oncogene product or growth factor likely to elicit malignant transformation upon prolonged expression [Conry *et al.* 1995].

The challenges posed by production

Common laboratory experiences have taught most researchers that RNA is a highly unstable molecule and difficult to work with. This view is heavily influenced by the ubiquitous presence of RNases that can indeed rapidly degrade RNA and thus destroy experiments when appropriate precautions are neglected. However, when looking at its chemical characteristics RNA is in fact a very stable molecule under physiological conditions. At CureVac, the team of F. von der Mülbe demonstrated that it can easily be produced in a process involving the transcription of target RNA by RNA polymerases from a linearized plasmid DNA template, followed by enzymatic destruction of the DNA template by DNases and purification of the resulting mRNA by precipitation and chromatographic methods according to size (Figure 1; detailed description given by Ketterer and colleagues and Pascolo) [Ketterer *et al.* 2008; Pascolo, 2004, 2006]. This process results in highly pure RNA products and works very well for standard mRNA sizes of a few kilobases, but has also been used successfully to produce mRNAs of sizes up to 15 kb.

Thermal stability of vaccines can pose a major logistical problem for vaccines, particularly in countries where the infrastructure makes it difficult to maintain the cold chain. The problem could be solved, however, by demonstrating that the mRNA-based vaccines can be lyophilized and that the lyophilized vaccines retain their full biological activity [Petsch *et al.* 2012]. This could also be demonstrated for lyophilized mRNA-based vaccines exposed to thermal stress under *International Conference on Harmonisation* conditions at temperatures of 25 and 40°C for periods of several years or months, respectively. Even under extreme stress conditions at a temperature of 60°C, stability could be shown for several months. Ongoing experiments suggest that these periods can still be substantially extended (F. von der Mülbe, personal communication).

The production process avoids the use of problematic starting materials such as animal-derived key components, and results in high batch-to-batch reproducibility. Furthermore, the same production process can be used for many different vaccines. This platform characteristic of the production process avoids many costly steps caused by the requirement to fulfill regulatory demands for product-specific validations. The process can be easily adapted to GMP conditions which allowed us to build the first GMP production facility for the production of clinical material. The total production process is highly flexible and scalable, meaning that the process can be easily changed to the production of a new vaccine within a few days and that the process can be scaled to the production of millions of vaccine doses either in one big facility or in several smaller ones. A sensitivity analysis performed with the help of several production experts revealed that vaccines produced on an industrial scale in such a process could be produced at costs that would be competitive in commodity markets, such as the one for influenza vaccines [Petsch *et al.* 2012]. In fact, contrary to common beliefs, mRNA-based immunotherapy appears to be no more costly than other strategies, such as protein, peptide, DNA, cell or recombinant pathogen based strategies, and might actually be less expensive [Pascolo, 2004].

Ex vivo transfection of dendritic cells with mRNA

Practically any cell type can be transfected with mRNA, including dendritic cells (DCs) [Breckpot

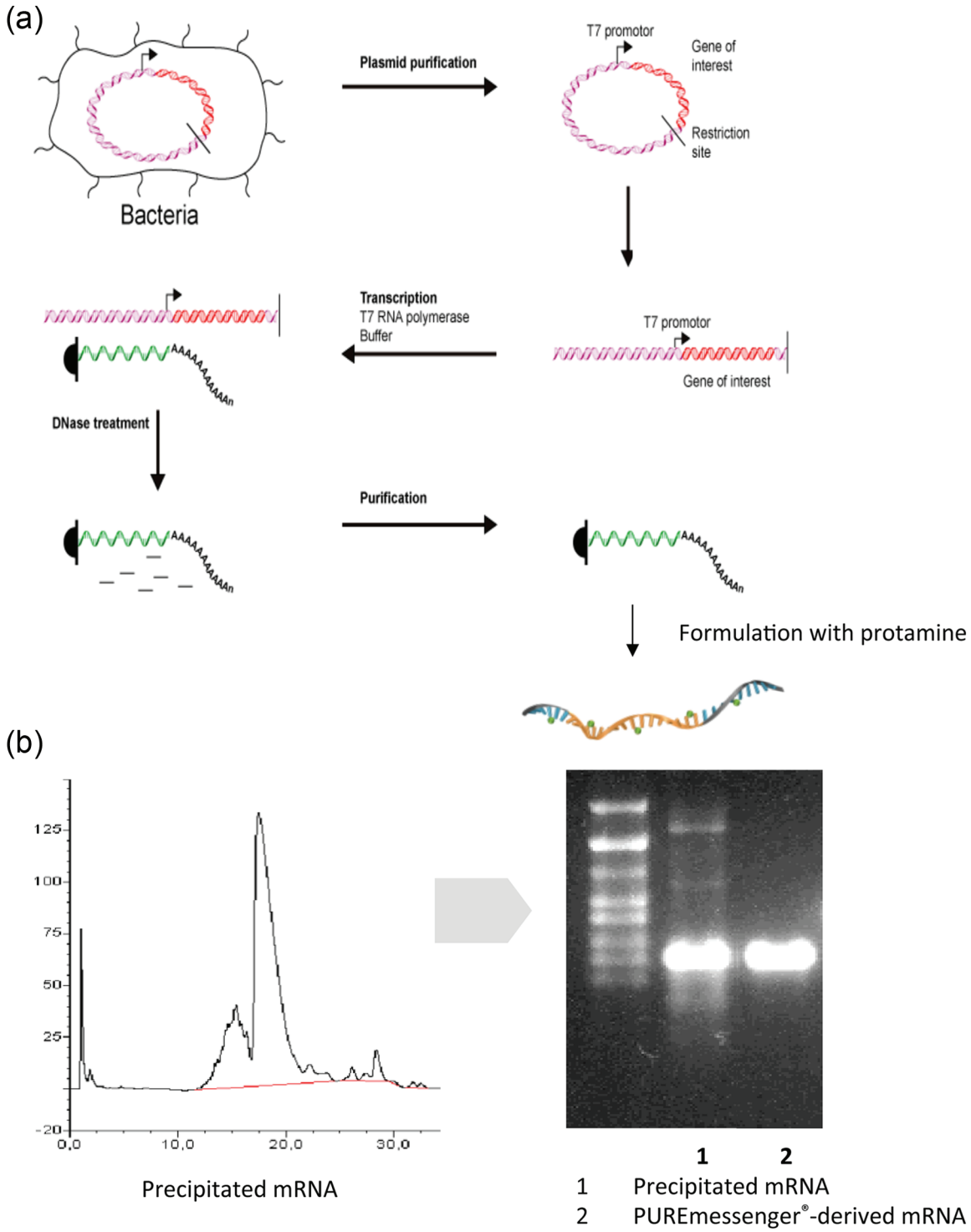

Figure 1. Production and thermal stability of messenger RNA (mRNA). (a) Basic structure of CureVac's good manufacturing practice (GMP) production process of mRNA. All individual steps of the process are performed under GMP conditions. The production of a large number of vaccines in parallel is possible under these conditions. The process can be completed in a few weeks, including more than 39 quality controls demanded for GMP production. Importantly, the process is highly scalable and would allow production of a given vaccine either in one large facility or in several small ones. Costs would be a fraction of those required by a vaccine production site today and can be easily adapted to the production of a new vaccine within days (b) CureVac has developed a scalable, proprietary mRNA purification process (PUREmessenger). Impurities are removed by a chromatography procedure that results in notably purer mRNA than obtainable by standard methods. The highly purified mRNA has also enhanced expression capacity.

et al. 2004]. DCs and other professional and semi-professional antigen-presenting cells (APCs) are critically important for initiating effective immune responses priming CD4 and CD8 T cells against mRNA-encoded antigens. Due to the flexibility of mRNA to encode any antigen and the perceived problems to produce mRNA at a large scale under GMP conditions, efforts to exploit this flexibility for the industrial development of vaccination strategies focused on the *ex vivo* transfection of APCs early on [Boczkowski *et al.* 1996; Gilboa and Vieweg, 2004; Sullenger and Gilboa, 2002]. These included the transfection of mRNA-encoded chimeric antigens targeting the antigens to endosomes to enhance major histocompatibility complex (MHC) class II presentation, the combination of various mRNA-encoded antigens and the use of APCs other than DCs for stimulation of the immune system [Bonehill *et al.* 2003; Breckpot *et al.* 2003; Gilboa, 2007; O'Neill *et al.* 2004; Okada *et al.* 2005; Van den Bosch *et al.* 2006]. However, a failed trial comparing *ex vivo* generated, peptide-loaded DC immunotherapy to standard chemotherapy in patients with advanced melanoma was a major setback for DC-based vaccination in general [Schadendorf *et al.* 2006]. Yet it was argued that DC activation would remain a cornerstone of a successful immunotherapy and novel approaches were fostered (detailed analysis given by Gilboa and Van Lint and colleagues) [Gilboa, 2007; Van Lint *et al.* 2013].

One of these was developed by Thielemann and colleagues. They developed a cocktail of three different immunostimulatory molecules to systematically alter the activation status of DCs [Bonehill *et al.* 2008]. The immunostimulatory cocktail consisted of a constitutively active toll-like receptor 4 (TLR4) variant (caTLR4), CD40L for activation of T cells by binding to CD40 and CD70 that provides a survival and proliferation signal to T cells by binding to CD27 and was coadministered with the antigen of interest linked to an MHC class II sorting signal. DCs matured with this so-called 'TriMix' mRNA were able to induce specific T cells against the encoded antigen more than 200-fold more effectively than DCs generated with the classical stimulatory cytokine cocktail consisting of IL-1β, TNFα, IL-6 and prostaglandin E_2 [Bonehill *et al.* 2008]. Recent publications suggested that a certain threshold antigen dose is required for the induction of a productive immune response involving antigen-specific T cells [Henrickson *et al.* 2008], but also that uptake of mRNA and subsequent antigen expression occurs only in immature and not matured DCs [Diken *et al.* 2011]. A successful DC maturation with the TriMix cocktail would therefore not necessarily have been expected to result in good immunogenicity due to a potentially impaired mRNA uptake by matured DCs. However, this would not pose a logical problem, if the way in which DCs are stimulated and matured has a decisive impact on whether nucleotide-encoded antigens are expressed or not and consequently whether or not immune responses can be generated successfully. Hence, not all activators of the immune system lead to maturation of DCs in a manner detrimental to use in combination with mRNA-expressed antigens [Van Lint *et al.* 2012].

The first trial in patients with advanced melanoma was performed with a TriMix-DC therapy encoding the melanoma-associated antigens (MAAs) MAGE-A3, MAGE-C2, tyrosinase or gp100, all linked to a HLA class II sorting signal [Van Nuffel *et al.* 2012b; Wilgenhof *et al.* 2011b]. MAGE-A3 and -C2 were linked at the N terminus to the signal peptide of lamp1 to ensure transport to the endoplasmic reticulum. Functional CD8+ and CD4+ T cells were elicited, recognizing epitopes derived from encoded antigens that were presented by several HLA types. Importantly, neoepitopes created by the fusion process were also recognized by CD8+ and CD4+ T cells. This proved that DCs generated *ex vivo* with TriMix mRNA can induce effector CD8+ and CD4+ T cells from the naive T-cell repertoire of patients with melanoma [Van Nuffel *et al.* 2012b].

The sequence of four intradermal vaccinations with this TriMix-DC-MEL therapy followed by IFNα2b treatment was studied in another trial [Wilgenhof *et al.* 2011b]. Adverse events were mild, comprising grade II injection site reactions in all 29 patients and grade II lethargy and fever in two patients. An antigen-specific response could be determined in 51.7% (12/21) patients after the fourth vaccination. One partial remission and five stable diseases (lasting more than 6 months with regression of metastases) were observed in 17 patients with evaluable disease at baseline [Wilgenhof *et al.* 2011b]. The clinical course of some patients treated with the autologous RNA DC therapy followed the recently defined immune response related criteria, suggesting that the immunotherapy established a new equilibrium between tumor (growth rate) and cancer immunosurveillance [Dunn *et al.*

2004; Gulley and Drake, 2011; Madan *et al.* 2012; Wilgenhof *et al.* 2011a]. Present efforts strive to augment the antitumor effect by combining the RNA DC therapy with the recently approved checkpoint inhibitor ipilimumab and yielded encouraging results [ClinicalTrials.gov identifier: NCT01302496] [Neyns *et al.* 2012].

The combined intravenous and intradermal administration of TriMix-DC-MEL therapy was also investigated. A particularly favorable outcome was observed in a patient with stage IVc melanoma and lung, lymph node, bone, and liver metastases whose condition was resistant to darcarbazine. A broad CD8+ and CD4+ T-cell response to the MAA could be measured and the patient developed a sustained objective clinical response [Van Nuffel *et al.* 2012a]. The complete phase Ib trial in 15 patients with pretreated advanced melanoma resulted in two complete and two partial remissions. The objective responders remained progression free for 24+, 28+, 33+, and 34+ months respectively [Wilgenhof *et al.* 2013]. An immunoanalysis of the clinical trials performed with TriMix-MEL suggested that functional specific CD8+ T cells distribute both to the skin and peripheral blood of patients. However, in some patients the majority of epitopes were only recognized by CD8+ T cells derived from either skin biopsies or peripheral blood, indicating that some compartmentalization might also be significant in the immune response induced by TriMix-DC-MEL therapy [Benteyn *et al.* 2013; Van Nuffel *et al.* 2012b].

An important step forward was achieved in a recent analysis that compared direct intranodal injection of TriMix mRNA targeting trp2, WT1 or ovalbumin to the injection of DCs stimulated *ex vivo* with TriMix mRNA [Van Lint *et al.* 2012]. Injection into the lymph nodes created a proinflammatory environment with the expression of MHC class II molecules, inflammatory cytokines and chemokines such as IL-6, IL-15, IFNγ, monocyte chemotactic protein 1 and Interferon-inducible protein 10, as well as granzyme B leading to the induction of antigen-specific CD4+ and CD8+ T cells. Contrary to maturation of DCs with the classical cytokine cocktail, maturation induced by TriMix did not hamper uptake of mRNA by CD11c+ DCs. Direct intranodal vaccination with TriMix mRNA was as effective as TriMix-DC therapy in the induction of cytolytic T lymphocytes and therapeutic responses in a variety of different mouse models [Van Lint *et al.* 2012]. Since clinical benefits have already been reported after TriMix-DC therapy (see above), one may expect that intranodal administration of a TriMix-based mRNA cocktail would match the efficacy of therapies based on autologous DCs manipulated *ex vivo* with mRNA. This would make this approach particularly interesting and a newly founded biotech, eTheRNA (Prof K. Thielemans, Free University of Brussels, Brussels, Belgium), strives to build on these results.

DC-based vaccination approaches have also been suggested as a way to achieve functional cure of human immunodeficiency virus (HIV) when traditional treatment regimens have first largely reduced viral load [Vanham and Van Gulck, 2012]. The principle feasibility of this idea has already been demonstrated in humans [Van Gulck *et al.* 2012]. Experiments with mRNA-based vaccines against HIV target antigens encapsulated by lipoplexes prepared from cationic lipids demonstrated that such lipoplex/mRNA vaccines could be used for subcutaneous vaccination [Pollard *et al.* 2013]. This procedure is reminiscent of the approach already used in the first report on successful vaccination with an mRNA-based vaccine [Martinon *et al.* 1993]. The TriMix approach described in more detail above demonstrates that recent technological advances have allowed us to transfer knowledge gained by encouraging results with DC vaccinations in the past to more direct administration of novel mRNA vaccines, which could make the benefits of DC vaccination available to a much wider patient population.

Intranodal applications of mRNA-based vaccines

In the logic of this analysis, Sahin and colleagues opted to engineer mRNA molecules that had characteristics allowing direct administration *in vivo* to circumvent the demands of DC vaccination. The translational efficacy of RNA-encoded antigens was optimized by modifications of the length and structure of the poly(A) tail as well as the 3` untranslated region (UTR) between open reading frame (ORF) and poly(A)-tail [Holtkamp *et al.* 2006]. A length of 120 nucleotides of the poly(A) tail that had to end unmasked, that is, not followed by irrelevant nucleotides stemming from the cloning procedure, was reported to be optimal. Furthermore, a 3' UTR consisting of two sequential β-globin regions cloned head to tail between the coding region and the poly(A) tail each independently enhanced RNA stability and translation [Holtkamp *et al.* 2006]. A further improvement of antigen presentation was achieved

by adding the MHC class I signal peptide to the N terminus and the MHC class I trafficking signal (MITD) to the C terminus of the antigen [Kreiter *et al.* 2008]. DCs transfected with RNA encoding such antigen–MITD fusion proteins showed a distinctly higher stimulatory capacity than wild-type controls. Interestingly, not only was the presentation of MHC class I in human and murine DCs improved, but also that of class II epitopes. This was interpreted as a result of the mimicking of the dynamic trafficking of MHC molecules in immature and mature DCs. The role of the secretion signal attached to the fusion proteins was emphasized, which might improve antigen processing by a better concert of protein degradation by endoplasmic reticulum adjacent proteasomes and access to the transporter associated with antigen processing (TAP) molecules. Importantly, the improved antigen presentation led to a polyepitopic expansion of CD4+ and CD8+ T cells, which resulted in distinct CD8+ T-cell specificities and a broad and variable antigen-specific CD4+ repertoire.

As the level and duration of antigen expression are critically important to generate sustained antigen-specific immune responses, modifications of the mRNA cap structure were tried to achieve further improvement [Kuhn *et al.* 2010]. The $m_2$7,2′-OGppspG (β-S-ARCA) phosphorothioate caps enhanced RNA stability and translation efficacy particularly well in immature, but not mature DCs. This is consistent with the already mentioned observation by the same group that the maturation status of DCs has an essential impact on the uptake of mRNA [Diken *et al.* 2011]. Kreiter and colleagues reported that such modified mRNA led to the priming of antigen-specific CD4+ and CD8+ T cells from naive T cells after repeated intranodal administration, but not after subcutaneous, intradermal or near nodal administration. The mRNA was selectively taken up by resident lymphatic DCs, propagated in an immunostimulatory environment and generated memory T cells in addition to cytolytic effector T cells. The immunological response translated into a good antitumor response in different therapeutic mouse models and increased survival substantially [Kreiter *et al.* 2010]. The activity of this regimen could be further enhanced by pretreatment of mice by a fusion protein of the extracellular domain of human fms-like tyrosine kinase 3 (FLT3) ligand with the heavy chain constant regions 2 and 3 (CH2–CH3 domain) of human immunoglobulin (Ig)-G4 leading to much increased therapeutic effects of the mRNA. Plasmacytoid DCs that were kept in an immature state by the administration of the FLT3 ligand/IgG4 fusion protein thus allowing increased uptake of the mRNA were essential for the effect [Kreiter *et al.* 2011]. The approach is currently being tested in a phase I dose escalation trial in advanced melanoma, analyzing the safety and tolerability of intranodal administration of a mRNA-based vaccine targeting tumor-associated antigens [ClinicalTrials.gov identifier: NCT01684241]

The potential of such optimized mRNA-based vaccination procedures to develop much more patient-centered cancer immunotherapies became clear in a recent analysis of the genome of murine B16/F10 melanoma cells. It was found that murine B16/F10 melanoma cells harbor around 962 nonsynonymous mutations, 563 of which are located in expressed genes [Castle *et al.* 2012]. A detailed analysis of a subgroup of 50 mutations revealed that one-third of these mutations might be immunogenic and that 60% of immune responses are preferentially directed against the mutated sequence rather than the wild-type parent gene. It was suggested that an mRNA-based vaccination platform might be well suited to induce a broad immune response against the mutanome of a patient, opening the way to highly individualized cancer treatment [Diken *et al.* 2013; Kreiter *et al.* 2012]. Technology breakthroughs such as deep sequencing have allowed us to clarify the genome and mutations therein of an increasing number of human tumors [Biankin *et al.* 2012; Killela *et al.* 2013; Vogelstein *et al.* 2013]. Despite these advances, as of today it is not yet possible to reliably identify the crucial mutations in an individual patient's tumor genome (Ultan McDermott, Wellcome Trust Sanger Institute, Cambridge, UK, personal communication). However, in the future hopefully this will be possible, by which time mRNA vaccination technology and also the regulatory requirements might have advanced enough to rapidly generate patient individual cancer vaccines.

Achieving enhanced protein expression with RNA-based replicons

To enhance *in vivo* expression of foreign proteins encoded by nucleotides, the self-amplifying characteristics of certain RNA viruses, most often members of the α virus family were exploited early on [Johanning *et al.* 1995; Xiong *et al.* 1989; Zhou *et al.* 1994]. The structural genes of such RNA

viruses are replaced by the genes of interest while the nonstructural proteins are left intact to ensure replication and protein translation. Liljeström and colleagues described a plasmid-encoded replicon system called DREP (DNA replicon) which was based on self-replicating Semliki Forest virus vectors (replicons) to immunize successfully against lethal challenges with influenza [Berglund *et al.* 1998, 1999]. Other viruses have been utilized as the basis for such replicons too [Anraku *et al.* 2002; Dubensky *et al.* 1996; Hariharan *et al.* 1998; Kirman *et al.* 2003]. Once the replicon genes including the gene of interest are read from the plasmid, self-amplification of the replicon leads to powerful protein expression allowing a dose-sparing effect over DNA vaccines. The increased immunogenicity of replicon DNA vaccines in addition to enhanced antigen expression levels may reflect the provision of immunostimulatory ligands by the replicating RNA in the transfected cell that activate pattern recognition receptors such as TLR3, RNA-activated protein kinase (PKR), and melanoma differentiation-associated protein 5 (MDA5) or retinoic acid inducible gene I [(RIG-I) [Diebold *et al.* 2009; Johansson *et al.* 2012; Leitner *et al.* 2000, 2003; Pichlmair *et al.* 2006; Rehwinkel *et al.* 2010; Schulz *et al.* 2005]. In an extension of this work, RNA-based replicons were developed (RREPs) as a safer solution, excluding persistence of the plasmid and a possible genomic integration [Fleeton *et al.* 2000]. Intramuscular delivery of *in vitro* transcribed naked RREPs could induce protective immune responses *in vivo* [Fleeton *et al.* 2001]. DREPs can also induce potent immune responses after intradermal delivery [Berglund *et al.* 1998]. This could also be demonstrated for RREPs administered intradermally, but not for naked mRNA administered thus. The immune response elicited by RREPs was comparable to that induced by DREPs, however EP could enhance the effects of intradermally administered RREPs twofold and that of DREPs 12-fold. This might reflect the enhanced entry into the nucleus of DREPs after EP. Since EP for intradermal administration is less cumbersome than after intramuscular administration, this might offer a biosafe alternative for nucleotide-based vaccination in the future.

The efficacy of the administration of naked, not encapsulated RNA replicons can be enhanced by the use of viral replicon delivery systems. While these facilitate delivery to target cells and enhance immunogenicity, they encounter the principle problem of vector immunity [Fleeton *et al.* 2001]. Apparently, though, this might be overcome by increased dosing of the virus. High titers of neutralizing antibodies and elevated T-regulatory-cell levels after repeated administration of an α virus vector expressing the tumor antigen CEA and encapsulated in virus-like replicon particles (VRPs) to patients with metastatic cancer could be overcome to induce clinically relevant CEA-specific T-cell and antibody responses by increased dosing [Morse *et al.* 2010]. However, in a recently reported phase I dose escalation trial of VRPs expressing prostate-specific membrane antigen (PSMA) in patients with prostate cancer, weak PSMA-specific enzyme-linked immunosorbent assay signals were detected only in the lower of two dose cohorts. Cellular immune responses could not be found in either of the two dose cohorts. Neutralizing antibodies were found in both cohorts which might indicate that dosing was either suboptimal or that vector immunity annihilated an antigen-specific immune response [Slovin *et al.* 2013].

Alternatives to VRPs have recently been found in clinically suitable delivery systems for siRNA. A self-amplifying α virus derived mRNA vector system termed SAM (self-amplifying messenger RNA) was developed to encode the F protein of respiratory syncytial virus and encapsulated with a synthetic lipid nanoparticle [Geall *et al.* 2012]. The authors demonstrated that at very low mRNA doses, very high titers of IgG1 antibodies against the F protein as well as IFNγ-producing CD4+ and CD8+ T cells could be induced in mice after intramuscular administration. The results could be replicated in immunogenicity studies in cotton rats in which the nonviral delivery methods provided protection levels comparable to those achieved with vectors that embedded the self-amplifying RNA in VRPs [Geall *et al.* 2012]. The authors also showed that lipid nanoparticle encapsulated SAM vaccines elicited functional immune responses against antigens from HIV [Geall *et al.* 2012]. The SAM system allows rapid production of a functional vaccine against novel viral threats within a very short time interval after the gene sequence of the relevant target is published, as demonstrated by the completion of an immunogenic vaccine against influenza strain H7N9 in a matter of days to very few weeks [Hekele *et al.* 2013]. Apparently, though, the best immunogenicity is achieved when an interval of at least 8 weeks is used between different vaccinations with the SAM technology. Nevertheless, this approach might represent a viable way to use RNA-based

replicons for widespread vaccination against a multitude of infectious diseases [Geall *et al.* 2013].

An interesting, often overlooked field for the application of mRNA-based vaccines is allergy. The incidence of allergy has risen dramatically in recent years and now affects up to 25% of the population in western countries, probably a consequence of a more urban rather than the traditional rural lifestyle that leads to a reduced microbial exposure early in life and reduced expression of genes typical of activation of T helper (Th)-1 lymphocytes [Weiss *et al.* 2012]. The allergic immune response is consequently characterized by a preponderance of allergen specific Th2 cells which secrete the 'Th2'-type cytokines IL-4, IL-5, and IL-13. These govern alterations in the B-cell switch that lead to production of allergen-specific IgE antibodies that bind to FcεR receptors on mast cells and basophilic granulocytes on contact with the allergen [Gould and Sutton, 2008]. IL-5 is key to the induction and propagation of eosinophilic granulocytes that migrate from the mucosal surface where initial contact with allergens occurs in tissues deeper in the airways in a process called the 'allergic march', leading to severe allergic pathophysiologies such as asthma [Takatsu and Nakajima, 2008]. In a series of papers, Thalhamer and colleagues established that allergen-specific Th1 polarization induced by DNA vaccines encoding a variety of different allergens could prevent allergy [Bauer *et al.* 2006; Scheiblhofer *et al.* 2006a, 2006b; Weiss *et al.* 2006]. While promising, antiallergy DNA-based vaccines would encounter the same safety considerations as prophylactic vaccines against infectious diseases. However, the same group was able to demonstrate that RNA-replicons were as effective as DNA vaccines in preventing allergies in a series of experiments testing up to 29 different allergens [Roesler *et al.* 2009]. While RNA replicons appeared initially superior to naked RNA compared on a weight basis, a fivefold dose increase of the naked mRNA annihilated the difference. Thus, mRNA-based vaccines appear as a promising path to prevent allergies and ultimately might even find their way into therapy of allergies [Weiss *et al.* 2012]. An mRNA-based vaccine would be attractive to encode engineered hypoallergic allergens [Thalhamer *et al.* 2010; Wallner *et al.* 2011] and might also profit from recently developed laser microporation devices for painless delivery of prophylactic and therapeutic vaccines to the skin [Scheiblhofer *et al.* 2013].

The use of nonencapsulated mRNA-based vaccines: the RNActive approach

The approaches described above attempted to increase the effectiveness of mRNA-based vaccines either by improving DC maturation (coadministration of costimulatory molecules, class I or II targeting) or increasing the expression capacity (replicon-based approach). A fundamentally different path was followed by the so-called RNActive technology: the expression capacity of mRNA-encoding proteins was greatly enhanced by sequence modifications of the mRNA and immunogenicity of mRNAs created by complexation with protamine.

The minimal mRNA structure is characterized by a protein-encoding ORF flanked by two essential elements at the 5' and 3' end: the 'cap', a 7-methylguanosine residue bound to the 5' end of the RNA via a 5'–5' triphosphate bond, and the poly(A) tail at the 3' end [Banerjee, 1980; Wickens, 1990]. Additionally, UTRs at the 5' (between cap and ORF) and the 3' end [between ORF and poly(A)-tail] of the ORF affect protein expression [Schlake *et al.* 2012]. This minimal structure is sequence engineered by use only of the naturally occurring nucleotides A, G, C and U (T) without affecting the primary amino acid sequence encoded by the ORF. The optimization procedure includes experimental procedures for the identification of novel 5' and 3' UTRs with favorable impact on mRNA translation and stability, an algorithm optimizing the sequence of the ORF as well as the use of a poly(A)-tail with defined length. Together with ultra-high purification of the mRNA, the expression of luciferase, commonly used as a reporter gene due to its short half life (~2 h), could be increased by four to five orders of magnitude in various test systems (Figure 2). The expression kinetics of encoded proteins were also changed dramatically: peak expression now occurs after 24–48 h with the expression after 72 h matching that of the early time points. Thus the protein expression kinetics of CureVac's enhanced RNA molecules have started to mimic that of proteins after an influenza virus infection [Julkunen *et al.* 2001].

'Self adjuvanticity' was bestowed on this sequence-engineered, expression-enhanced mRNA by complexation with protamine. The mRNA/protamine complexes form larger particles than uncomplexed mRNA (~250–350 nm *versus* ~50 nm) and activate the immune system in a process involving the endosome resident TLR7 [Fotin-Mleczek *et al.*

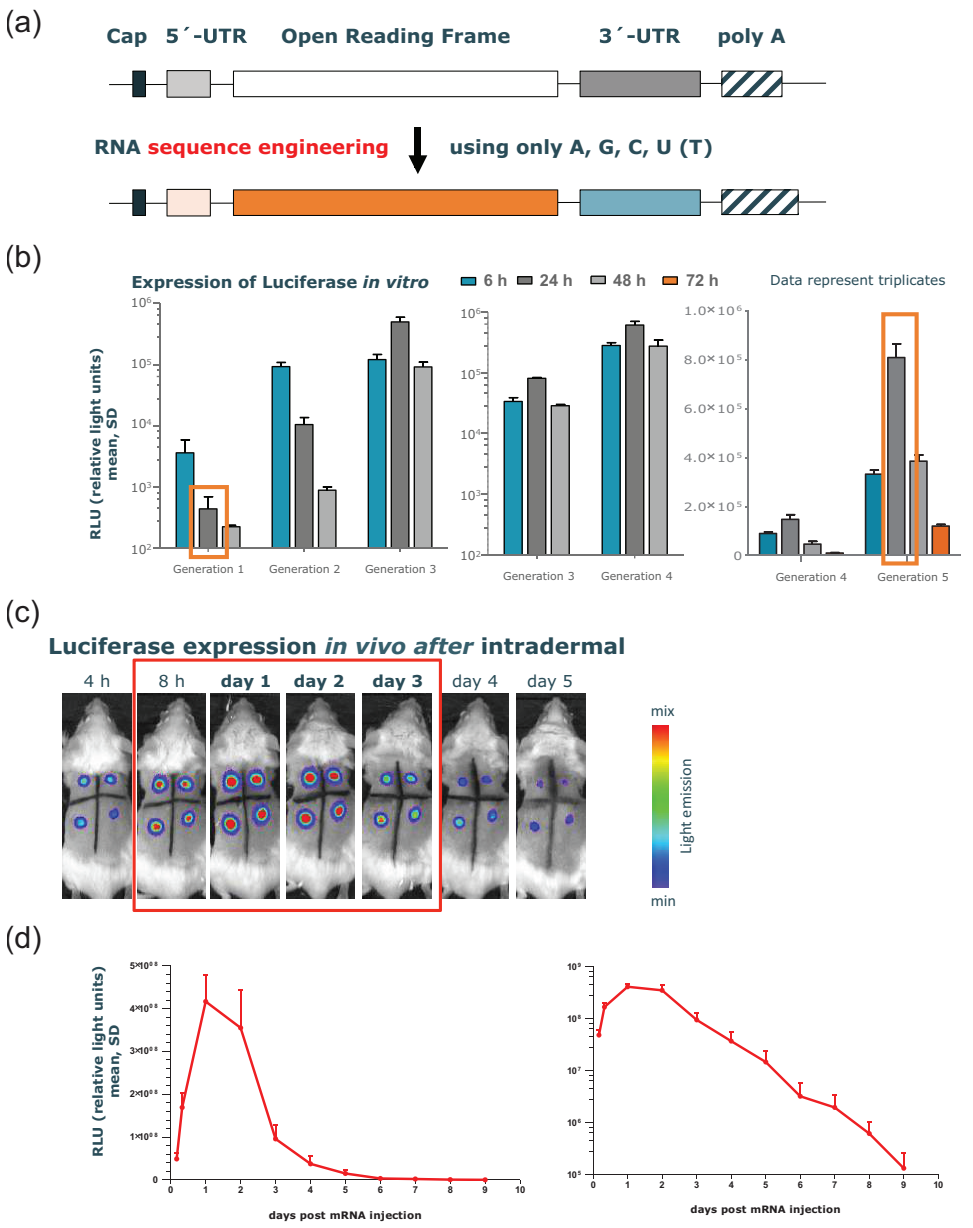

Figure 2. Effects on protein expression by sequence engineering of the principle messenger RNA (mRNA) structure. (a) The classical structure of an mRNA molecule consists of a cap region, followed by an (optional) 5' untranslated region (UTR), the open reading frame (ORF), an (optional) 3' UTR, and the poly(A)-tail. Sequence engineering of each subunit of an mRNA molecule with only the naturally occurring nucleotides A, G, C, U that do not affect the primary amino acid sequence encoded by the ORF are the basis of optimized mRNA molecules used in RNActive vaccines. (b) Effect of different generations of sequence-engineered mRNAs (e.g. generated by optimization of the nucleotide content of the ORF or incorporation of different 3' or 5' UTRs or combinations thereof) encoding PpLuc produced over the last few years on *in vitro* expression of luciferase. The mRNA generations encoding Firefly luciferase were electroporated into HeLa cells (generation 1–4) or transfected into human dermal fibroblasts by lipofection (generation 4 and 5) and compared for their *in vitro* expression of luciferase. The luciferase level was determined at 6, 24 and 48 h, or 72 h post transfection. The dynamic range of the assay does not allow us to compare all mRNA molecules in one experiment. (c) Firefly luciferase encoding mRNA, optimized for translation and stability, was injected intradermally into a BALB/c mouse (four injection sites). The luciferase expression was visualized in the living animal by optical imaging at various time points after mRNA injection and showed maximal protein levels 24–48 h after mRNA injection. (d) Quantitative expression of luciferase over time until 9 days after mRNA injection. Results are shown on a linear scale (left-hand panel) or on a semi-logarithmic scale (right-hand panel). The figure is adapted from Schlake and colleagues [Schlake *et al.* 2012], details therein.

2011; Kallen *et al.* 2013; Scheel *et al.* 2005]. *In vitro* experiments revealed that the uncomplexed mRNA is taken up by an adenosine triphosphate dependent process and could subsequently be detected in cytoplasm and lysosomes [Lorenz *et al.* 2011]. The final formulation of an RNActive vaccine is obtained by mixing the immunostimulating mRNA/protamine complexes with the antigen-expressing 'naked' sequence-engineered mRNA. An optimal ratio between the two components could be established that ensures both good antigen expression and good immunostimulation after intradermal administration [Fotin-Mleczek *et al.* 2011].

The two-component, self-adjuvanted RNActive vaccines generated by this technology elicit strong and balanced immune responses: Th1 and Th2, humoral and cellular as well as effector and memory responses are induced. Highly effective vaccines could be engineered on this basis that induce powerful CD4+ and CD8+ T-cell responses and work well in different tumor models after repeated, frequent administration [Fotin-Mleczek *et al.* 2011, 2012] as well as vaccines against infectious diseases such as influenza that induce protective, long-lived humoral immune responses and only require administration as prime, boost [Petsch *et al.* 2012]. Importantly, in species varying from mice over ferrets to large pigs, RNActive vaccines were effective after the easy intradermal administration and did not require the more complicated intranodal administration [Kreiter *et al.* 2010, 2011; Van Lint *et al.* 2012]. In fact, intradermal administration of RNActive vaccines was as immunogenic as intranodal administration of a conventional mRNA vaccine [Kallen *et al.* 2013].

This difference between RNActive vaccines and other mRNA-based vaccines may result from the enhanced protein expression capacity of RNActive vaccines and a favorable way of activating the immune system. Activation of TLR7/8 proved to be a critical component of a new, largely improved cocktail for DC maturation that led to strong Th1 responses [Spranger *et al.* 2010, 2012]. Moreover, newly developed small molecule TLR7/8 agonists were shown to colocalize to an MCH class II containing compartment of human plasmacytoid DCs [Iavarone *et al.* 2011; Russo *et al.* 2011]. The production of the type I interferons IFNα and IFNβ by these cells appears to be instrumental for generation of strong immune responses, particularly Th1 and memory responses, that are required to reject tumors [Desmet and Ishii, 2012; Diamond *et al.* 2011]. Activation of TLR7/8 might therefore be a particularly useful way to activate the immune system not just to combat cancer, but also for successful vaccination strategies against chronic infections [Bernstein *et al.* 2012; Mbow *et al.* 2010; Walsh *et al.* 2012]. Furthermore, the expression of different TLRs is cell type specific, meaning that different APCs react specifically to activators such as pathogen-associated molecular patterns or endogenous damage associated molecular patterns (extensively reviewed by Desmet and Ishii) [Desmet and Ishii, 2012], For example, TLR7 expression occurs mostly in plasmacytoid DCs and B cells in humans, while TLR8 is expressed in monocytes, macrophages, and conventional DCs, but not plasmacytoid DCs. Since DC subsets appear to have different preferred localizations that can also be affected by the inflammatory status of a patient [Hartmann *et al.* 2003, 2006; Naik *et al.* 2006; Wollenberg *et al.* 2002], the route of administration might also affect the vaccination efficiency. Translation of experimental results from animal models to humans thus also requires consideration of route of administration used and an analysis of the specific challenges or advantages posed by it.

Clinical experiences with mRNA-based vaccines

Clinical trials that used direct injection of mRNA instead of *in vitro* treatment of DCs and the subsequent administration of these modified cells to patients were first initiated around 10 years ago. The first trial included 15 patients with melanoma stage III or IV [Weide *et al.* 2008]. A cDNA library was prepared from the metastatic lesions of patients and transcribed into an mRNA library. The autologous mRNA library was then administered to patients with granulocyte macrophage colony-stimulating factor (GM-CSF) as adjuvant. The repeated injection of the mRNA library vaccine was feasible and safe. The immunoanalysis, hampered by the technological difficulties to measure immune responses against not strictly defined targets, suggested that humoral responses could be induced in four patients and that there might have been transient increases in the frequencies of CD4+ and CD8+ T cells. An objective response could not be observed, but two patients had a mixed response and a favorable clinical course was observed in five patients.

In the next trial, protamine-stabilized mRNAs coding for Melan-A, tyrosinase, gp100, Mage-A1, Mage-A3 and survivin were injected intradermally into 21 patients with metastatic melanoma with GM-CSF as adjuvant [Weide *et al.* 2009]. In 10 patients keyhole limpet hemocyanin (KLH) was added to the vaccine. No adverse event greater than grade II was observed. Two of four immunologically evaluable patients showed an antigen-specific T-cell response. Interestingly, a decrease of Foxp3+/CD4+ T-regulatory cells was observed in patients also receiving KLH, whereas myeloid suppressor cells (CD11b+HLA-DRlo monocytes) were reduced in the patients not receiving KLH. One of seven patients with measurable disease showed a partial response of lung metastases after 12 vaccinations. A histopathologically proven bone metastasis detected in this patient 16 months after the start of vaccination was surgically removed and this patient remained relapse free.

Thirty patients with stage IV renal cell cancer were treated with naked mRNA coding for the tumor-associated antigens mucin 1 (MUC1), CEA, human epidermal growth factor receptor 2 (Her-2/neu), telomerase, survivin, and melanoma-associated antigen 1 (MAGE-A1) with GM-CSF as adjuvant [Rittig *et al.* 2011]. The first 14 patients received a less intensive induction schedule than the consecutive 16 patients. Both cohorts received monthly vaccinations afterwards and in both groups the vaccinations were well tolerated. Immunologically evaluable material was available for 17 patients, of which 12 showed an immune response. Six patients in the first and nine in the second cohort had stable disease lasting longer than 3 months; one patient in the first cohort had a confirmed partial response with shrinkage of cervical and mediastinal lymph nodes. One patient in the second cohort who required abdominal paracentesis every other day had a decline of the paracentesis frequency in line with a decline of the tumor marker CA-125 and regression of abdominal tumor sites. Ultimately he remained free of paracentesis for over 3 months.

The first clinical trials with self-adjuvanted RNActive® vaccines (CureVac GmbH, Tübingen, Germany) were performed in patients with advanced castrate-resistant prostate carcinoma and stage IIIB/IV non-small cell lung cancer (NSCLC) that was at least stable after first-line platinum-based chemotherapy or chemoradiation. Both studies suggested that the application of RNActive vaccines to humans was safe. The four tumor associated antigens prostate specific antigen, prostate stem-cell antigen, prostate-specific membrane antigen, and six transmembrane epithelial antigen of the prostate 1 were selected for the first-in-man phase I/IIa study in patients with prostate cancer and designated CV9103 [Kübler *et al.* 2011]. The NSCLC antigen cocktail CV9201 consisted of five tumor-associated antigens: MAGE-C1, MAGE-C2, NY-ESO-1, survivin, and 5T4 [Sebastian *et al.* 2011, 2012].

The phase I/IIa prostate-carcinoma study with CV9103 showed an unexpectedly high level of antigen-specific T cells after five intradermal vaccinations administered over a 6-month period in around 80% of immunologically evaluable patients with prostate carcinoma independent of their HLA background [Kübler *et al.* 2011]. Others have also advocated mRNA-based vaccination as a method to overcome HLA restriction of patients with tumor [Van Nuffel *et al.* 2012c]. Antigen-specific T-cell immune responses were detected against all antigens independent of their cellular localization. Less clear results were observed for antigen-unspecific B cells that appeared to be increased and natural killer cells that seemed to show a slightly increased activation. The majority of patients showing an antigen-specific immune response reacted against more than one antigen, a phenomenon recently associated with increased survival in a study of a peptide vaccine in patients with renal cell carcinoma [Walter *et al.* 2012]. While this study was not designed to assess clinical efficacy, individual patients showed interesting clinical courses that might indicate clinical benefit. The overall survival of vaccinees and its correlation with immune responses is presently being analyzed (manuscript in preparation).

Based on these initial results, a controlled phase IIb study has opened enrolment of patients with castrate-resistant prostate carcinoma with asymptomatic or minimally symptomatic metastasis to ascertain the clinical efficacy of CV9104, a further developed version of CV9103, in a systematic manner. The study will employ more frequent vaccination in the induction phase followed by maintenance vaccinations at prolonged intervals. The primary endpoint of this study of around 180 patients in nine European countries is overall survival. Additionally a number of secondary endpoints will investigate the mechanism of action,

associated with improved survival and the impact on subsequent therapies [ClinicalTrials.gov identifier: NCT01817738].

Similarly to the prostate carcinoma study, the number of vaccinations in the phase I/IIa trial of patients with NSCLC was limited to five intradermal immunizations, but the more life-threatening disease of these patients required a more intensive vaccination schedule. Similar to CV9103, the NSCLC cocktail CV9201 showed a favorable safety profile. An antigen-specific humoral and cellular immune response was determined in roughly two-thirds of the treated patients. A significant increase of pregerminal center B cells by a factor of at least two was observed in more than half of the patients and associated with an increase in total CD4+ effector T cells during treatment. Together, more than 80% of the treated patients with NSCLC had a detectable antigen-specific immune response or an increase in germinal center B cells despite their heavy pretreatment with platinum-based chemotherapy (Sebastian, manuscript in preparation). Based on these results, a phase Ib study has been launched in patients with metastatic NSCLC to ascertain an assumed synergistic effect between vaccination and radiation [ClinicalTrials.gov identifier: NCT01915524].

Novel options: gene therapy

Wolff and colleagues demonstrated that mRNA can be used to express foreign proteins *in vivo* [Wolff *et al.* 1990]. The potential of self-amplifying RNA replicons to improve this was recognized early on [Johanning *et al.* 1995; Zhou *et al.* 1994]. However, while self-amplifying RNA replicons drive up protein expression, the much enhanced protein expression might also lead to immunogenic suicidal cell death, a problem that could be aggravated by activation of proteins sensing double-stranded RNA such as double-stranded PKR (dsPKR) [Berglund *et al.* 1998; Diebold *et al.* 2009]. This would probably increase the potency of a vaccine, but would clearly be unwanted for the expression of proteins for nonimmunogenic purposes. Mammalian mRNA, however, not only contains the nucleotides A, G, C, U but also modified nucleosides such as 5-methylcytidine and *N*6 methyladenosine [Kariko and Weissman, 2007]. These as well as another prominent modified nucleoside, pseudouridine (ψ), reduce activation of the immune system through RNA sensors such as TLR3, TLR7, TLR8, and dsPKR [Kariko and Weissman, 2007]. Bacteria lack such modified nucleosides, but they are abundant in viruses where they are thought to contribute to immunoavoidance of the virus [Kariko *et al.* 2005, 2008; Koski *et al.* 2004]. Nucleoside modifications can limit activation of 2'-5'-oligoadenylate synthetase and slow cleavage by RNase L. Activation of PKR could be diminished by incorporation of pseudouridine (ψ) into mRNA which resulted in enhanced protein expression, possibly due to the reduced inhibitory impact of immune stimulation on protein translation [Anderson *et al.* 2010].

Pseudouridine (ψ) was therefore used to prepare mRNA coding for erythropoietin. Injection of the erythropoietin encoding pseudouridine (ψ) mRNA intraperitoneally could substantially increase reticulocyte counts and hematocrit mice [Kariko *et al.* 2012]. Neither increased cytokine levels nor newly generated antierythropoietin antibodies levels could be measured in mice after repeated injection of the erythropoietin pseouridine (ψ) mRNA. In a pilot study in macaques, elevated erythropoietin levels were found after a single intraperitoneal administration. An internal analysis at CureVac, however, revealed that the enhanced, sequence-engineered mRNA used in the RNActive vaccines does not cause relevant immune activation when the mRNA complex with protamine is absent (unpublished observation). A mRNA thus prepared coding for erythropoietin led to a clear, biologically relevant increase in reticulocytes in mice upon a single intramuscular injection. Corresponding pseudouridine (ψ)-containing mRNA was not any more effective at increasing reticulocytes (unpublished observation). Other researchers replaced a relatively small proportion of only 25% of uridine and cytidine with 2-thiouridine and 5-methyl cytidine which caused a synergistic decrease of mRNA binding to pattern recognition receptors and reduced the release of immunogenic cytokines [Kormann *et al.* 2011]. A single intramuscular injection of such a modified erythropoietin mRNA raised the hematocrit from 51.5% to 64.2 % after 28 days. Importantly, the authors could also demonstrate that they could rescue a lethal congenital phenotype of a mouse strain caused by lack of surfactant protein B (SP-B) by aerosolic administration of modified SP-B mRNA. A very recent paper showed that direct intramyocardial injection of a synthetic modified RNA (modRNA) encoding human vascular endothelial growth factor A (VEGF-A) with lipofectamine as vehicle increased

functionality and long-term survival in the expansion and directed differentiation of endogenous heart progenitors in a mouse myocardial infarction model [Zangi *et al.* 2013]. VEGF-A prepared as a nonmodified mRNA was ineffective and VEGF-A prepared as a DNA vector had even detrimental effects in some readouts, possibly due to the persisting expression of VEGF-A. In contrast, VEGF-A modRNA showed a pulse-like cardiac expression profile *in vivo* with a rapid onset of protein expression after a few hours and a peak after 18 h, which might be ideal to mimic the often transient nature of paracrine signals. Conspicuously, the *in vivo* expression profile of a luciferase modRNA closely resembled that of luciferase encoded by CureVac's enhanced engineered mRNA (see above) after direct injection without vehicle into the mouse dermis [Figure 2(c)], while the *in vitro* expression of VEGF-A modRNA appeared to be shorter than that of luciferase expressed *in vitro* with the enhanced engineered mRNA [Figure 2(b)] [Zangi *et al.* 2013]. This may indicate that a sequence-engineered, expression-enhanced mRNA prepared from nonmodified nucleotide might match or even surpass synthetic modified mRNA as a delivery system for proteins.

The use of modified mRNA, as described, pursued commercially by companies such as Ethris or ModeRNA, also allowed another breakthrough discovery. Warren and colleagues were able to develop a protocol to induce pluripotent stem cells by the repeated transfection of cells with a cocktail of modified mRNAs based on the four canonical stem-cell defining transcription factors (Yamanaka factors) [Takahashi *et al.* 2007; Takahashi and Yamanaka, 2006]: KLF4, c-MYC, OCT4, and SOX2, as well as a modRNA coding for LIN28 (KMOSL) [Warren *et al.* 2010]. The latter had previously been shown to facilitate reprogramming [Hanna *et al.* 2009; Yu *et al.* 2007]. RNA-induced pluripotent stem cells (RiPSCs) could be generated from multiple independent derivations and all showed robust expression of the pluripotency-associated transcripts OCT4, SOX2, NANOG, and hTERT. Since modRNAs generated iPSCs at very high efficiency, these results could be the starting point for patient-specific therapies. In contrast to these works, it was also shown that five consecutive transfections of *in vitro* produced, nonmodified mRNA encoding the transcription factors Oct4, Lin28, Sox2, and Nanog into human foreskin fibroblasts resulted in continuous protein expression that led to the formation of induced pluripotent cell colonies that expressed alkaline phosphatase and several embryonic stem cell markers [Yakubov *et al.* 2010]. A more recent publication even suggested that activation of inflammatory pathways, in particular TLR3 (a sensor of double-stranded RNA), is required for efficient nuclear reprogramming and the induction of pluripotency [Lee *et al.* 2012]. Hence, nonmodified mRNA might be an alternative to modified mRNA for the induction of RiPSCs.

Therapies based on RiPSCs will most likely require adaptation to diverse cell types [Warren *et al.* 2010]. The flexibility of the mRNA format and the transient nature of protein expression mediated by mRNA allows one to experimentally activate developmentally important transcriptional programs by expression of relevant transcription factors in a temporally controlled and stage-specific sequence to direct RiPSC cells to diverse fates [Mandal and Rossi, 2013].

The interest in mRNA-based technologies to express proteins *in vivo* has been further highlighted by a recent call issued by the Defense Advanced Research Projects Agency (USA) searching for technologies able to express antibodies at biologically active levels *in vivo* that have no risk of genomic integration.

Conclusion

An mRNA-based approach to express biologically active protein levels has principle advantages over DNA-based approaches. Even a minimal risk of genomic integration can be excluded, mRNA has to cross only one cellular membrane to be active in the cytoplasm, there is no need for a promoter and mRNA is also active in nondividing cells. mRNA constructs can be engineered to reduce and even eliminate vector-induced immunogenicity, thus allowing repeated administrations. Impressive preclinical results have been achieved with mRNA-based vaccines in fields as diverse as oncology, infectious diseases, and allergy. The first clinical studies in oncology suggest that it is possible to translate these preclinical results to humans. mRNA-based vaccines can be produced at low costs in a highly flexible and scalable production process and would constitute a revolutionary, disruptive technology in vaccinology. Equally impressive results have been achieved in stem cell research and diseases caused by gene defects. It appears that mRNA technology has now reached a level where protein or gene

replacement therapies based on mRNA become conceivable. Hence, mRNA rather than DNA which was favored for decades will be the basis of a new class of drugs based on nucleotides. This might well be the beginning of a revolution in medicine based on mRNA.

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