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► **To cite this version:**

Florence Faurez, Daniel Dory, Vincent Le Moigne, Rodolphe Gravier, André Jestin. Biosafety of DNA vaccines: New generation of DNA vectors and current knowledge on the fate of plasmids after injection.. *Vaccine*, 2010, 28 (23), pp.3888-95. 10.1016/j.vaccine.2010.03.040 . hal-00493412

HAL Id: hal-00493412

<https://anses.hal.science/hal-00493412>

Submitted on 18 Jun 2010

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**Biosafety of DNA vaccines: new generation of DNA vectors
and current knowledge on the fate of plasmids after
injection**

Mansucript ID JVAC-D-09-01542, revision 2

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40 **Abstract**

41

42 DNA vaccination has been widely studied to develop new, alternative, efficient and safe
43 vaccines for humans and animals. Many efforts have been made to increase the immunising
44 potential of these vaccines and three veterinary vaccines are now available on the market.
45 Much work is also being dedicated to develop effective DNA vaccines for humans. However,
46 this new vaccination technique raises issues concerning biosafety due to the nature of the
47 vector, i.e. a DNA molecule that contains sequences of prokaryotic origin (e.g. genes for
48 antibiotic resistance). This review describes the development of the new generation of DNA
49 vectors that are partially or completely devoid of elements of prokaryotic origin and outlines
50 the results of studies on the fate of plasmids after their injection *in vivo*.

51

52 **I. From discovery to industrial application**

53 In 1990, Wolff *et al.* showed that the injection of a DNA plasmid in mouse muscle resulted in
54 a significant expression of the protein encoded by the plasmid [1]. Starting with this
55 discovery, various antigens encoded by plasmids have been successfully used to induce the
56 production of antibodies [2, 3] and cytotoxic T lymphocytes [4], thereby demonstrating the
57 potential of this strategy for DNA vaccination and gene therapy. Progress in this field has
58 resulted in the development and the marketing of three veterinary DNA vaccines since 2005.
59 Two of them are authorised for use in the United States: one targets the West Nile virus
60 infection in horses and the other targets canine malignant melanoma. The third vaccine,
61 authorised for use in Canada on salmon, is directed against the infectious hematopoietic
62 necrosis virus.

63

64

65 **II. First- and new generation of DNA vectors for DNA vaccination**

66 **II.1 First-generation of plasmids bearing prokaryotic elements**

67

68 DNA vaccines are basically composed of plasmids encoding the vaccine antigens and saline
69 solutions. The plasmids contain an antibiotic resistance gene controlled by a prokaryotic
70 promoter and a prokaryotic origin of replication that allow for selection and replication of
71 plasmids in transformed bacteria. The plasmid transcription unit is usually composed of a
72 strong and ubiquitous viral promoter that confers optimal expression of the gene of interest in
73 eukaryotic cells, as well as a termination-polyadenylation sequence usually derived from the
74 simian virus 40 (SV40) genome or from the bovine growth hormone gene [5, 6]. The
75 prokaryotic sequences of conventional DNA vaccines have occasionally been shown to
76 induce negative effects *in vitro* and *in vivo*. For example, the expression of the neomycin
77 resistance gene has been reported to hinder the expression of the gene of interest in
78 mammalian cells [7]. Likewise, ampicillin gene expression has been shown to reduce
79 transgene expression *in vivo* compared to kanamycin gene expression [8]. Moreover,
80 unmethylated CpG sequences that are present in certain plasmids are recognised by the Toll-
81 like receptor 9 (TLR9), which leads to an adjuvant effect through the activation of the innate
82 immune system [9]. However, studies have shown that TLR9 or CpG are not necessarily
83 essential for the induction of innate immunity after the injection of a DNA vaccine [10, 11].
84 In fact, CpG can play a role independently of TLR9 in primary B cells [12] and TANK-
85 binding kinase-1 signalling pathways can be activated by dsDNA *in vitro* [13, 14] and *in vivo*

86 [15]. dsDNA can thus mediate adjuvanticity and, because it induces T-cell responses, dsDNA
87 is essential for the immunogenicity of DNA vaccines [16].
88 Stimulation of the immune system may be beneficial and expected when plasmid DNA is
89 intended for vaccination purposes, but not for gene therapy.

90
91

92 **II.2 New generation of DNA vectors partially or totally devoid of prokaryotic** 93 **elements**

94
95 To minimise all the adverse effects described, plasmids partially or totally devoid of
96 prokaryotic elements have been developed. Selection of bacteria transformed by the plasmids
97 during the production process was initially based on antibiotic resistance and has now been
98 replaced by antibiotic-free selection systems. In these selection systems, the prokaryotic
99 origin of replication has been maintained. For example, selection mechanism can be based on
100 an RNA/RNA antisense interaction involving the naturally occurring RNA I derived from the
101 origin of replication of the plasmid [17] or on operator-repressor titration [18, 19]. The latter
102 system has been implemented in a DNA vaccine [20], tested in many clinical trials and has
103 been shown to be safe in a good laboratory practice toxicology study [21].

104 In addition, other vectors that are totally devoid of prokaryotic elements, such as
105 plasmids or linear dumbbell-shaped expression cassettes, have also been developed. In 1997,
106 Darquet *et al.* [22] developed a plasmid that does not contain any prokaryotic elements by
107 producing supercoiled recombinant circular DNA molecules called minicircles. These
108 molecules do not contain either the prokaryote origin of replication or antibiotic resistance
109 genes, both having been eliminated during site-specific recombination. Following
110 recombination, minicircles are purified from miniplasmids, which are circular DNA
111 molecules containing prokaryotic elements, and from parental plasmids that may still be
112 present. Various types of site-specific recombination techniques have been used to improve
113 the yields of minicircles. The first type of site-specific recombination technique uses
114 bacteriophage λ integrase to mediate recombination between the *attP* and *attB* sites. The yield
115 of recombined plasmids is low, corresponding to roughly 60% of starting material [22, 23].
116 The second recombination technique uses Cre recombinase [24]. The yield in minicircles for a
117 7 kbp parental plasmid is roughly 200 $\mu\text{g/l}$ culture, while 20 kbp parental plasmid yields only
118 40 $\mu\text{g/l}$ culture. Purification of minicircles is done using caesium chloride density gradients.
119 The density gradient separates minicircles from both miniplasmids and non-recombined

120 plasmids. However, undesirable dimeric, concatemeric and relaxed minicircles are frequently
121 copurified along with the targeted supercoiled minicircles. The third technique utilises phage
122 ϕ 31 integrase to catalyse recombination and produce minicircles; the resulting product is then
123 purified on commercially available affinity columns. This technique does not require either
124 digestion with enzymes or purification on a caesium chloride gradient. The I-SceI site and the
125 expression of I-SceI endonuclease gene by the plasmid linearises non-recombined plasmids
126 and miniplasmids, which are then eliminated by exonucleases. The yield of minicircles is nine
127 times higher than with the original protocol, or 1.8 mg/l culture with a purity of 97%.
128 However, concatemers and miniplasmids are still present. A fourth technique was developed
129 in 2008 by Mayrhofer *et al.* [25]. This technique is called recombination-based plasmid
130 separation technology (RBPS technology). It consists of using ParA resolvase to mediate
131 recombination and affinity chromatography based on protein-DNA interactions for
132 purification. Using this technique, recombination efficiencies of 99.57% can be attained.
133 Unlike other techniques, neither multimers nor concatemers are copurified and the final
134 purified product contains 98.8% minicircles, 1% miniplasmids and 0.2% parental plasmids.
135 Although minicircle DNA molecules have low immunogenicity since most of the
136 unmethylated CpG motifs have been eliminated, they may nonetheless be interesting for use
137 in vaccines. The first advantage is their small size that may improve the dissemination of
138 DNA and their entry into the nucleus. Moreover, Molnar *et al.* have shown that plasmid size
139 has an inverse relationship with the level of transgene expression [26]. In the case of
140 minicircles, it has been demonstrated that transfection and expression levels are higher
141 compared to conventional plasmids [22, 23, 27, 28]. The second advantage is the absence of
142 antibiotic resistance genes. The third advantage is the reported reduction in the risk of plasmid
143 transfer to local bacteria owing to the absence of the prokaryotic origin of replication.
144 The other type of vector completely free of prokaryotic elements, linear dumbbell-shaped
145 expression cassettes, is constructed using one of two methods described thus far: linear
146 dsDNA molecules generated *in vitro* either by polymerase chain reaction (PCR) amplification
147 or by endonuclease processing from plasmids. In the case of PCR amplification from
148 plasmids, the PCR product is end-protected by ligation with hairpin oligodeoxynucleotides
149 (ODN). Hirata *et al.* have demonstrated that, after electroporation, PCR fragments show better
150 expression compared to the plasmids carrying the same gene [29]. Moreover, PCR fragments
151 can induce protection against influenza A challenge in mice [30]. The endonuclease
152 processing technique has been used by the Mologen AG pharmaceutical company, which has
153 developed a minimalistic immunogenic defined gene expression vector (MIDGE). Plasmids,

154 which contain the expression cassette of interest, are digested by restriction endonuclease like
155 *Eco3I* I [31], the ends ligated with hairpin ODN and DNA is purified by anionic exchange
156 column chromatography. Hairpin ODNs can be used to bind molecules such as peptides,
157 proteins or sugars to DNA. For example, nuclear localisation signal (NLS) peptides can be
158 added to direct the DNA to the nucleus [32]. MIDGE vectors and MIDGE-NLS vectors
159 successfully support expression *in vitro* and *in vivo* [31, 33, 34], to support immune responses
160 *in vivo* [31-33], protecting cats or mice against challenges with feline immunodeficiency virus
161 [35] or *Leishmania major* [34], respectively, and to enhance protective anti-tumor immunity
162 [36].

163

164 **III. The different routes of administration**

165 Before describing the fate of plasmids after injection, it appears appropriate to recall briefly
166 the different routes of administration thereof. There are two main routes of administration for
167 DNA vaccines: intramuscular injection and intradermal injection. Subcutaneous injection,
168 application to mucosal surfaces, intravenous and intranodal injections are other possible
169 routes of administration, but they have been used much less frequently. Plasmid DNA can be
170 directly injected in an aqueous solution — in which case it is called naked DNA — ,
171 complexed with liposomes [37] or packaged in viral [38, 39] or bacterial [40] vectors. In this
172 review, we will focus on the routes of administration for delivering naked DNA.

173 The most widely employed delivery method is intramuscular injection. Naked DNA in saline
174 is injected directly into skeletal muscle tissue using a hypodermic needle. It has been
175 demonstrated that the induced immune response is primarily a T_{H1} cell-mediated immune
176 response [41]. From 95 to 99% of intramuscularly injected plasmids, which are found in the
177 interfibrillar space, are degraded in the muscle tissue within 90 min post-administration [42].
178 To counter this low transfection rate, injection of naked DNA can be assisted by
179 electroporation [43]. Electroporation consists in creating an electric field by applying an
180 electric pulse between electrodes placed in the muscle. This technique increases the intensity
181 of the humoral immune response, although the cell-mediated immune response remains
182 predominant [41, 44, 45]. Another technique that increases the intensity of the humoral
183 immune response by intramuscular administration is a high-pressure liquid injection using a
184 BiojectorTM [46].

185 Naked plasmid DNA administered by intradermal injection is often injected with a
186 hypodermic needle. However, other delivery methods are also used, such as the gene gun that
187 propels DNA adsorbed on gold beads [47], BiojectorTM [48, 49], topical application of naked

188 DNA [50], or tattooing [51]. These different methods primarily induce humoral immunity
189 characterised by a T_H2 response and a pronounced production of IgG1 antibodies [52-57].
190 However, intradermal plasmid DNA injection methods cannot be qualified as inducing a
191 predominantly humoral immune response. Electroporation following intradermal injection of
192 naked DNA and intradermal injection of MIDGE-NLS may induce a predominant cell-
193 mediated immune response [33, 34, 58].

194 Other routes of administration involve subcutaneous injection and mucosal surface delivery.
195 In the case of subcutaneous methods, naked DNA can be injected by a needle [59] or by high-
196 pressure gas [60]. In the case of mucosal delivery, naked DNA can be deposited in the nasal
197 [61] or buccal [61, 62] cavity or delivered by topical application on, for example, the eye [63]
198 or vaginal mucosa [64]. Unlike intramuscular and cutaneous (intradermal and subcutaneous)
199 administration, mucosal surface delivery induces systemic and mucosal immunity [61, 63].

200

201 **IV. Fate of plasmid DNA after injection**

202 **IV.1. Plasmid DNA uptake mechanisms following intramuscular injection**

203 Induction of an immune response after immunisation by naked plasmid DNA is possible if the
204 gene of interest coded by the plasmid DNA is expressed in the nucleus of the host cell. Before
205 the gene of interest can be expressed, injected plasmid DNA must overcome three obstacles.

206 The first obstacle involves crossing the plasma membrane. DNA is a polyanion and thus
207 cannot interact with cell plasma membranes which are negatively charged. However, Wolff *et*
208 *al.* indirectly showed that naked DNA can enter cells after injection in mouse muscle tissue
209 [65]. Several studies have been conducted to explain this result. Studies on the role of
210 disrupted plasma membranes or on transient membrane pores induced during injection have
211 been inconclusive compared to experiments that have demonstrated active cellular
212 mechanisms of DNA uptake by using low temperature conditions to inhibit energy production
213 [66, 67]. There are two main active uptake mechanisms: fluid-phase endocytosis, i.e. not
214 mediated by a receptor, and adsorptive endocytosis. DNA uptake is inhibited in excesses of
215 non-coding DNA or dextran sulphate, illustrating the importance of the negative charge of
216 DNA in the uptake mechanism [66, 68]. These results reject the hypothesis of uptake by fluid-
217 phase endocytosis. A large number of membrane receptors may be involved in
218 oligonucleotide capture by cells. However, none of them have been shown to be necessary
219 and sufficient for DNA uptake. Moreover, given the concentration of membrane receptors on
220 the cell surface, it is highly likely that plasmid DNA interacts with several receptors

221 simultaneously. Not all cell types take up plasmid DNA in the same way. This suggests that
222 uptake mechanisms depend on certain sequences that may be specific to cell type [69, 70].
223 The second obstacle involves crossing the cytoplasm. The cytoplasm is made up of a network
224 of microfilaments, microtubules and a variety of subcellular organelles that float in the
225 cytosol. The cytoskeleton generates mechanical resistance in the cell and transports organelles
226 and large complexes. The structure of the cytoskeleton, the presence of organelles and the
227 high concentration of proteins limit the diffusion of large macromolecules, such as plasmid
228 DNA [71]. Thus, it is thought that the cytoplasm hinders plasmid transport to the nucleus.
229 However, plasmid DNA has a mechanism to circumvent this impediment. Studies using
230 microinjection or electroporation delivery methods have shown that plasmid DNA associates
231 with proteins that resemble dyneins whereby the microtubule network helps transport plasmid
232 DNA to the nucleus [72, 73]. Plasmid DNA can also cross the cytoplasm another way. Studies
233 have demonstrated that labelled plasmid DNA co-localises with endosomal markers [74, 75],
234 confirming plasmid DNA uptake by endocytosis. When endosomes engulf a plasmid, plasmid
235 DNA is protected from degradation for most of its journey to the nucleus. While little is
236 known on how naked DNA is released from the endolysosome, studies with complexed DNA
237 have shown that a few plasmid DNA molecules are released into the cytoplasm, while the rest
238 are degraded [76]. To curtail its metabolic instability in the cytoplasm, the DNA molecule
239 must be escorted up to the nuclear membrane. A study on the behaviour of different plasmids
240 in different cells showed a half-life of about 90 min after micro-injection in the cytoplasm and
241 only 0.1% of plasmid DNA that was micro-injected actually entered the nucleus. The
242 characterisation of the mechanisms involved in this rapid elimination showed that DNA is
243 digested by DNase enzymes in the cytoplasm [77].

244 The third obstacle involves crossing the nuclear membrane. There are three possible ways for
245 plasmid DNA to enter the nucleus. DNA can be imported into the nucleus through nuclear
246 pores by simple diffusion, be incorporated during disassembly of the nuclear membrane
247 during mitosis or enter via nuclear pores by facilitated diffusion. The nuclear membrane
248 possesses nuclear pore complexes (NPC) that play an important role in protein transport to
249 and from the cytoplasm and the nucleoplasm. DNA fragments smaller than 250 bp are capable
250 of diffusing passively into the nucleus via the NPCs while fragments longer than 250 bp
251 cannot [78]. Several research groups have demonstrated the importance of nuclear membrane
252 disassembly in the transfection efficiency for DNA larger than 250 bp. However, cellular
253 division has a relatively weak effect on expression. There are three possible explanations for
254 this weak effect. First, the plasmid may be degraded by the time mitosis occurs. Second,

255 plasmids may be excluded from re-forming nuclei. Third, plasmids may be differentially
256 partitioned to each daughter cell [79]. Plasmid DNA may finally enter the nucleus in two
257 ways: either by taking advantage of nuclear membrane disassembly in dividing cells or by
258 crossing an intact membrane by facilitated diffusion via NPCs [79]. In the latter case, it has
259 been suggested that DNA molecules are associated with polypeptides, such as transcription
260 factors, that contain a nuclear localisation signal (NLS) [80]. Transcription factors not only
261 have an NLS sequence that interacts with nuclear import receptors, but also possess amino
262 acid sequences that recognise the promoter or enhancer sequences of the plasmid DNA. Dean
263 *et al.* showed that a plasmid containing early enhancer-promoter SV40 sequences interact
264 with transcription factors via a 72 bp early enhancer SV40 sequence, termed DNA nuclear
265 targeting sequence (DTS), and that these transcription factors transfer the plasmid to the
266 nucleus [81, 82]. Inclusion of DTS elements into minicircle DNA causes a 30-fold increase in
267 efficiency in cells and a 6-fold increase in mouse lungs [27]. DTS elements can be used to
268 enhance levels of gene transfer in a cell-specific manner in non-dividing cells, however, the
269 presence of DTS elements does not influence levels of expression in some tissues [83].

270

271

272 **IV.2. Cellular distribution of plasmid DNA after intramuscular injection**

273 Striated muscle tissue is made up of muscle cells, called myocytes, satellite cells that induce
274 the regeneration of muscle cells and connective tissue. Myocytes are large multinucleated
275 cells (syncytia). They contain around a hundred nuclei that are located under the plasma
276 membrane and their cytoplasm contains contractile proteins (myofilaments). Myocytes and
277 satellite cells are enveloped in a basal lamina and a layer of connective tissue, the
278 endomysium [84]. It has been demonstrated that 5 minutes after intramuscular injection with
279 rhodamine-labelled DNA, the DNA was localised in and between myocytes [85]. Myocytes
280 containing the plasmid DNA express the gene of interest [85].

281 The connective tissue spans three levels of organisation and includes the epimysium that lines
282 the whole muscle, the perimysium that forms a sheath around bundles of myocytes and the
283 endomysium that surrounds each myocyte [86]. Several research groups have shown that
284 macrophages and dendritic cells are always present at all three organisational levels of
285 connective tissue [87-89]. Plasmid DNA that has been administered intramuscularly and
286 traced with DNA labels or with green fluorescent protein (GFP) expression coded by the
287 plasmid DNA is not only found in myocytes, but also in antigen-presenting cells, such as
288 macrophages and B cells [75, 85, 90, 91]. However, no plasmid DNA-coded proteins have

289 been reported in dendritic cells, although transplantation experiments in mice suggest that the
290 transfer of antigens from muscle cells to the major histocompatibility complex (MHC) class I
291 molecules of antigen-presenting cells — and therefore also dendritic cells — can occur [92].
292 Myeloid CD11c⁺ (dendritic) cells that have been transfected during intramuscular
293 immunisation appear to make up 2% of all myeloid cells present in mouse muscle [75].

294

295

296 **IV.3. Tissue distribution of plasmid DNA after intramuscular injection**

297 Differences in distribution and persistence of a plasmid can be attributed to several factors
298 such as the route of administration, the number of injections, the quantity of plasmid DNA
299 and the animal species under study [93]. Given the variability of these factors, it is difficult to
300 compare the results from different studies in detail. However, it is possible to draw some
301 general conclusions. Whatever the route of administration, the site of injection is the area
302 where the plasmid remains the most concentrated and persists the longest. However, all
303 organs under observation have shown evidence of plasmid DNA as of the first minutes
304 following injection, but with lower quantities of plasmids and for shorter periods [46, 93-
305 104].

306 In particular, it has been shown that more than 90% of injected plasmids were found in mouse
307 thigh muscles 5 minutes after intramuscular injection of less than 20 µg of plasmid DNA
308 coding for the hAAT protein and that, throughout the duration of the experiment, less than
309 10% of plasmid DNA was present in the observed organs at any given point in time [68].
310 Persistence of the plasmid at the point of injection varies with the animal model and the
311 identity and amount of plasmid DNA injected. However, in most studies, the determination of
312 plasmid DNA persistence times has been limited by the duration of the study. It has been
313 shown that plasmid DNA persists more than 2 years in mice [105], at least 28 days in rats
314 [94], 54 days in sheep [100], 70 days in goldfish [106], 90 days in rainbow trout (Kurath,
315 2006 and [103]), 10 weeks in turkeys [93] and in one out of three pigs after 4 weeks [101].
316 Plasmid concentrations are maximal after several minutes, for example 12 min in pigs [101],
317 then decrease to trace amounts after several hours. In fact, more than 98% of plasmids are
318 eliminated after 9 h at the injection site in mice [68]. After several minutes, plasmid DNA is
319 also present at low quantities in other organs for short periods of time. The organs tested after
320 intramuscular injection include all vascularised organs [97], but most studies test for plasmid
321 presence in blood, liver, spleen, kidneys, lungs, draining lymph nodes, the opposite muscle
322 and gonads. Blood-brain barriers and blood-gonad barriers do not seem to act on plasmid

323 DNA since gonads and the brain show evidence of plasmid DNA in several studies after
324 intramuscular injection [96, 97, 107-109]. The mechanism involved in the dissemination of
325 plasmid DNA throughout the body has not been completely determined. However, the
326 presence of plasmids in immune cells [85, 91, 95], in lymph [85, 96, 100, 110] and blood [95]
327 may contribute to their dissemination.

328 Plasmid DNA is generally detected by PCR, Southern blot or by quantitative PCR. These
329 methods do not differentiate functional plasmid DNA, which expresses the protein of interest,
330 from degraded plasmids. One study showed that it was possible to rescue plasmid DNA up to
331 6 months post-injection via intramuscular administration of 100 µg of plasmids in mice [111].
332 In rainbow trout, DNA is detected for at least 90 days in muscle tissue, whereas the
333 expression of the protein of interest is greatest at 14 days in muscle, kidneys and thymus
334 glands and can no longer be detected after 28 days [103]. However, studies in mice and
335 turkeys have shown that the plasmid expresses the protein of interest in muscle tissue as long
336 as it persists [93, 105]. Antigen expression does not only depend on the state of the plasmid,
337 but also on the animal model employed, the identity of the antigen, the viral promoter, and the
338 delivery method [93, 112-114].

339
340

341 **IV.4. Clearance of plasmid DNA after injection**

342 After intramuscular injection, plasmid DNA is either transfected into cells or it remains free in
343 the body. According to the route it takes in the host, plasmid DNA will be eliminated
344 differently. It is difficult to differentiate between the fate of transfected plasmid DNA and the
345 fate of free plasmid DNA. Antigen clearance can be studied to determine the fate of
346 transfected plasmids and the fate of free plasmid DNA can be determined by studying the
347 general circulation.

348 Little is known about the host factors that limit long-term plasmid DNA expression.
349 Elimination of myocytes expressing the antigen appears to depend on the immunogenicity of
350 the antigen and the presence of a functional immune system [115, 116]. One study showed
351 that the innate immune system, mediated by macrophages and natural killer (NK) cells may
352 be involved in the elimination of antigen expression [117]. Payette *et al.* showed that the
353 decrease in HB antigen expression after intramuscular injection is mediated by mechanisms
354 that induce the major histocompatibility complex II (MHC II) and antibodies [118]. However,
355 because it induces a high humoral immune response, the HB antigen model is not appropriate
356 for studying the role of cell-mediated immune responses during antigen expression. The role

357 of cell-mediated immune responses in antigen clearance has been clarified by Letvin *et al.*
358 [115, 119]. After establishing a correlation between the decrease in antigen expression and
359 cell-mediated immune response [115], they determined that CD4⁺ cells, but not NK cells,
360 macrophages or CD8⁺ cells, are largely responsible for this decrease via the Fas/FasL
361 mechanism and via their MHC II molecules. Plasmid DNA may then be degraded by
362 apoptosis or, in the case of necrotic cells, by DNases present in the serum such as, for
363 example, murine DNase1I3 that is secreted by macrophages [120, 121].

364 Due to variability in quantities of plasmid isomers, variability in plasmid size and variability
365 in the animal models used, the different studies cannot be directly compared in detail.
366 However, the clearance of free plasmid DNA may be induced by different factors. The
367 presence of endonucleases in mouse muscle mediated the degradation of 95 to 99% of naked
368 plasmid DNA within 90 min of intramuscular injection, which most likely occurred in the
369 extracellular space [42]. After intramuscular injection in pigs, plasmid DNA bioavailability —
370 i.e. the quantity of plasmid DNA that reaches the general blood circulation — is low, around
371 10% [101]. The highest concentrations of plasmid DNA are detected in the blood around 15
372 min post-injection and they rapidly decrease thereafter [101, 122]. Blood circulation appears
373 to play a role in the dissemination of plasmid DNA to tissues other than muscle. Moreover,
374 one study showed that higher quantities of plasmid DNA are found in blood than in organs
375 [96]. Some plasmids in the bloodstream may be degraded by nucleases present in blood. The
376 degradation rate of supercoiled plasmids found in mouse blood, after intramuscular injection,
377 is 20.9% after 10 min, 34% after 1 h, 86.8% after 1 day and 97.8% after 1 week [96].
378 Moreover, studies showed that free plasmid DNA present in the blood may be internalised
379 and degraded by liver non-parenchymal cells after intravenous injection [123, 124]. Some
380 plasmid DNA, intramuscularly injected in mice, is eliminated in urine and faeces. Urine
381 collected 10 minutes post-injection already showed a strong signal that continued to increase
382 up to the end of the 1 week study. At 10 minutes post-injection, faeces only showed a weak
383 signal, while after 1 h, the signal became much stronger [96]. However, no studies to detect
384 the presence of encoded proteins in urine and in faeces have been conducted to date.

385

386 **IV.5. Potential risk of plasmid DNA genomic integration**

387 The main safety concern for DNA vaccination is that partial or complete plasmid genome
388 integration could result in insertional mutagenesis and possibly in the activation of oncogenes
389 or inactivation of tumor suppressor genes [125]. Few studies have been undertaken on this
390 subject until recently and most have concluded that the rate of plasmid genomic integration is

391 negligible [46, 108, 126-130], because plasmid integration could not be unambiguously
392 detected. Furthermore, the strategies used in these studies could not clearly distinguish
393 between integrated plasmids and non-integrated residual genome-associated plasmids. Thus,
394 the plasmid sequences detected in these studies were assumed to be host genome-integrated
395 copies; based on this assumption, integration appears to occur at rates that are at least three
396 orders of magnitude below the spontaneous mutation rate in mammalian genomes [131]. To
397 confirm plasmid integration, Wang *et al.* [132] designed an elegant and efficient method to
398 detect and identify insertion sites after plasmid delivery. To detect rare integration events,
399 their approach is based on PCR amplification of a sequence found between a repeated genome
400 sequence and a known plasmid sequence. Furthermore, to increase the probability of potential
401 integration, they used the more potent electroporation delivery technology. They thereby
402 effectively detected four independent plasmid integration events in the mouse genome and
403 this frequency of events is lower than the rate of spontaneous mutation in mammalian
404 genomes [131].

405

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408

409 **VI. Conclusion:**

410 DNA vaccination is an elegant and simple concept. Many efforts continue to be made to
411 better understand the mechanisms behind DNA vaccination and to find new strategies to
412 improve vaccination efficiency. Progress in this field has resulted in three veterinary vaccines
413 that have been authorised for use. However, biosafety of DNA vaccination remains a major
414 issue. Concerns specific to the DNA vaccination concept stem from the use of injected DNA
415 molecules, which can potentially integrate the host genome. Host gene expression can be
416 affected if integration occurs in host genes or if genes of prokaryotic origin, such as those that
417 confer resistance to antibiotics, integrate into the host genome. One way to limit these risks is
418 to develop and use plasmids or other DNA molecules that lack the genes for antibiotic
419 resistance and the prokaryotic elements needed for their replication in bacteria. As described
420 above, these kinds of vectors already exist and their vaccine efficacies are currently being
421 tested in several large animal models.

422 There is a pressing need to understand how integration events occur. For instance, integration
423 events may depend on the presence of specific nucleic acid sequences or on the concentration
424 of plasmids in a specific tissue type or near the host genome. To date, only one study showing

425 random integration events has been published [132]. Understanding the mechanisms involved
426 in integration would be useful for developing strategies to limit these integration events.
427 Another goal in the future is to develop a technique that can detect, or at least increase the
428 probability of detecting, integration events, which are described as rare. The above-described
429 method (see IV.5) for detecting actual integration events has limitations. In fact, under these
430 technical conditions, the absence of amplification is not sufficient proof that integration has
431 not occurred. The drawback of this PCR technique that detects integration events is that the
432 distance between the plasmid sequences targeted by the primers is high. Therefore, if the
433 integrated plasmid fragment does not contain one of the primer sequences, it will not be
434 detected. One way to enhance the probability of detecting integration events is to reduce the
435 distance between the plasmid-targeted primers by using new techniques such as high-
436 throughput sequencing with primers targeting sequences distributed throughout the plasmid.
437 The Food and Drug Administration recommends evaluating whether the DNA plasmid has
438 integrated the genome of the vaccinated animals when the plasmids persist at levels exceeding
439 30,000 copies per μg of host DNA [133]. The European Medicines Agency (EMA)
440 recommends using the most sensitive available methods for this purpose [134]. The
441 development of new and more efficient methods to assess plasmid integration into host
442 genomes strives to satisfy these recommendations.

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448 **References**

449

- 450 [1] Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer
451 into mouse muscle in vivo. *Science* 1990;247:1465-8.
- 452 [2] Tang D, DeVit M, Johnson SA. Genetic immunization is a simple method for eliciting an
453 immune response. *Nature* 1992;356:152-4.
- 454 [3] Cox GJM, Zamb TJ, Babiuk LA. Bovine herpesvirus 1: immune response in mice and cattle
455 injected with plasmid DNA. *J Virol* 1993;67:5664-7.
- 456 [4] Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dworki VJ, et al. Heterologous
457 protection against influenza by injection of DNA encoding a viral protein. *Science* 1993;259:1745-9.
- 458 [5] Gurnathan S, Klinman DM, Seder RA. DNA vaccines: Immunology, application, and
459 optimization. *Annual Review of Immunology* 2000;18:927-74.
- 460 [6] Feltquate DM. DNA vaccines: Vector design, delivery, and antigen presentation. *J Cell*
461 *Biochem* 1998;S30/31:304-11.
- 462 [7] Valera A, Perales JC, Hatzoglou M, Bosch F. Expression of the neomycin-resistance (neo)
463 gene induces alterations in gene expression and metabolism. *Hum Gene Ther* 1994;5:449-56.
- 464 [8] Hartikka J, Sawdey M, Cornefert-Jensen F, Margalith M, Barnhart K, Nolasco M, et al. An
465 improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum Gene Ther*
466 1996;7:1205-17.
- 467 [9] Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen M-D, et al. Immunostimulatory DNA
468 sequences necessary for effective intradermal gene immunization. *Science* 1996;273:352-4.
- 469 [10] Babiuk S, Mookherjee N, Pontarollo R, Griebel P, van Drunen Littel-van den Hurk S, Hecker
470 R, et al. TLR9^{-/-} and TLR9^{+/+} mice display similar immune responses to a DNA vaccine.
471 *Immunology* 2004;113:114-20.
- 472 [11] Spies B, Hochrein H, Vabulas M, Huster K, Busch DH, Schmitz F, et al. Vaccination with
473 plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient
474 mice. *J Immunol* 2003;171:5908-12.
- 475 [12] Zhu P, Liu X, Trembl LS, Cancro MP, Freedman BD. Mechanism and regulatory function of
476 CpG signaling via scavenger receptor B1 in primary B cells. *J Biol Chem* 2009;284:22878-87.
- 477 [13] Stetson DB, Medzhitov R. Recognition of cytosolic DNA activates an IRF3-dependent innate
478 immune response. *Immunity* 2006;24:93-103.
- 479 [14] Peters KL, Smith HL, Stark GR, Sen GC. IRF-3-dependent, NFkappa B- and JNK-
480 independent activation of the 561 and IFN-beta genes in response to double-stranded RNA. *Proc Natl*
481 *Acad Sci U S A* 2002;99:6322-7.
- 482 [15] Shirota H, Petrenko L, Hattori T, Klinman DM. Contribution of IRF-3 mediated IFNbeta
483 production to DNA vaccine dependent cellular immune responses. *Vaccine* 2009;27:2144-9.
- 484 [16] Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, et al. TANK-binding kinase-1
485 delineates innate and adaptive immune responses to DNA vaccines. *Nature* 2008;451:725-9.
- 486 [17] Mairhofer J, Pfaffenzeller I, Merz D, Grabherr R. A novel antibiotic free plasmid selection
487 system: advances in safe and efficient DNA therapy. *Biotechnol J* 2008;3:83-9.
- 488 [18] Garmory HS, Leckenby MW, Griffin KF, Elvin SJ, Taylor RR, Hartley MG, et al. Antibiotic-
489 free plasmid stabilization by operator-repressor titration for vaccine delivery by using live *Salmonella*
490 *enterica* Serovar typhimurium. *Infect Immun* 2005;73:2005-11.
- 491 [19] Williams SG, Cranenburgh RM, Weiss AM, Wrighton CJ, Sherratt DJ, Hanak JA. Repressor
492 titration: a novel system for selection and stable maintenance of recombinant plasmids. *Nucleic Acids*
493 *Res* 1998;26:2120-4.

- 494 [20] Hanke T, McMichael AJ. Design and construction of an experimental HIV-1 vaccine for a
495 year-2000 clinical trial in Kenya. *Nat Med* 2000;6:951-5.
- 496 [21] Hanke T, McMichael AJ, Samuel RV, Powell LA, McLoughlin L, Crome SJ, et al. Lack of
497 toxicity and persistence in the mouse associated with administration of candidate DNA- and modified
498 vaccinia virus Ankara (MVA)-based HIV vaccines for Kenya. *Vaccine* 2002;21:108-14.
- 499 [22] Darquet AM, Cameron B, Wils P, Scherman D, Crouzet J. A new DNA vehicle for nonviral
500 gene delivery: supercoiled minicircle. *Gene Therapy* 1997;4:1341-9.
- 501 [23] Darquet AM, Rangara R, Kreiss P, Schwartz B, Naimi S, Delaere P, et al. Minicircle: an
502 improved DNA molecule for in vitro and in vivo gene transfer. *Gene Therapy* 1999;6:209-18.
- 503 [24] Bigger BW, Tolmachov O, Collombet JM, Fragkos M, Palaszewski I, Coutelle C. An araC-
504 controlled bacterial cre expression system to produce DNA minicircle vectors for nuclear and
505 mitochondrial gene therapy. *Journal of Biological Chemistry* 2001;276:23018-27.
- 506 [25] Mayrhofer P, Blaesen M, Schlee M, Jechlinger W. Minicircle-DNA production by site
507 specific recombination and protein-DNA interaction chromatography. *J Gene Med* 2008;10:1253-69.
- 508 [26] Molnar MJ, Gilbert R, Lu Y, Liu AB, Guo A, Larochelle N, et al. Factors influencing the
509 efficacy, longevity, and safety of electroporation-assisted plasmid-based gene transfer into mouse
510 muscles. *Mol Ther* 2004;10:447-55.
- 511 [27] Vaysse L, Gregory LG, Harbottle RP, Perouzel E, Tolmachov O, Coutelle C. Nuclear-targeted
512 minicircle to enhance gene transfer with non-viral vectors in vitro and in vivo. *J Gene Med*
513 2006;8:754-63.
- 514 [28] Zhang X, Epperly MW, Kay MA, Chen ZY, Dixon T, Franicola D, et al. Radioprotection in
515 vitro and in vivo by minicircle plasmid carrying the human manganese superoxide dismutase
516 transgene. *Hum Gene Ther* 2008;19:820-6.
- 517 [29] Hirata K, Nishikawa M, Kobayashi N, Takahashi Y, Takakura Y. Design of PCR-amplified
518 DNA fragments for in vivo gene delivery: size-dependency on stability and transgene expression. *J*
519 *Pharm Sci* 2007;96:2251-61.
- 520 [30] Vilalta A, Jimenez G, Rusalov D, Planchon R, Lalor P, Carner K, et al. Vaccination with
521 polymerase chain reaction-generated linear expression cassettes protects mice against lethal influenza
522 A challenge. *Hum Gene Ther* 2007;18:763-71.
- 523 [31] Schirmbeck R, Konig-Merediz SA, Riedl P, Kwissa M, Sack F, Schroff M, et al. Priming of
524 immune responses to hepatitis B surface antigen with minimal DNA expression constructs modified
525 with a nuclear localization signal peptide. *J Mol Med* 2001;79:343-50.
- 526 [32] Zheng C, Juhls C, Oswald D, Sack F, Westfehling I, Wittig B, et al. Effect of different nuclear
527 localization sequences on the immune responses induced by a MIDGE vector encoding bovine
528 herpesvirus-1 glycoprotein D. *Vaccine* 2006;24:4625-9.
- 529 [33] Moreno S, Lopez-Fuertes L, Vila-Coro AJ, Sack F, Smith CA, Konig SA, et al. DNA
530 immunisation with minimalistic expression constructs. *Vaccine* 2004;22:1709-16.
- 531 [34] Lopez-Fuertes L, Perez-Jimenez E, Vila-Coro AJ, Sack F, Moreno S, Konig SA, et al. DNA
532 vaccination with linear minimalistic (MIDGE) vectors confers protection against *Leishmania major*
533 infection in mice. *Vaccine* 2002;21:247-57.
- 534 [35] Leutenegger CM, Boretti FS, Mislin CN, Flynn JN, Schroff M, Habel A, et al. Immunization
535 of cats against feline immunodeficiency virus (FIV) infection by using minimalistic immunogenic
536 defined gene expression vector vaccines expressing FIV gp140 alone or with feline interleukin-12 (IL-
537 12), IL-16, or a CpG motif. *J Virol* 2000;74:10447-57.
- 538 [36] Choi Y, Jeon YH, Kang JH, Chung JK, Schmidt M, Kim AC. MIDGE/hNIS vaccination
539 generates antigen-associated CD8+IFN-gamma+ T cells and enhances protective antitumor immunity.
540 *Int J Cancer* 2007;120:1942-50.
- 541 [37] Bacastrada ME, Foldvari M, Babiuk SL, Babiuk LA. Vaccine delivery: lipid-based delivery
542 systems. *Journal of Biotechnology* 2000;83:91-104.

- 543 [38] Lundstrom K. Alphavirus vectors: Applications for DNA vaccine production and gene
544 expression. *Intervirology* 2000;43:247-57.
- 545 [39] Lundstrom K, Schweitzer C, Rotmann D, Hermann D, Schneider EM, Ehrenguber MU.
546 Semliki Forest virus vectors: efficient vehicles for in vitro and in vivo gene delivery. *FEBS Letters*
547 2001;504:99-103.
- 548 [40] Mielcarek N, Alonso S, Loch C. Nasal vaccination using live bacterial vectors. *Advanced*
549 *Drug Delivery Reviews* 2001;51:55-69.
- 550 [41] Sin JI, Bagarazzi M, Pachuk C, Weiner DB. DNA priming-protein boosting enhances both
551 antigen-specific antibody and Th1-type cellular immune responses in a murine herpes simplex virus-2
552 gD vaccine model. *DNA and Cell Biology* 1999;18:771-9.
- 553 [42] Barry ME, Pinto-Gonzalez D, Orson FM, McKenzie GJ, Petry GR, Barry MA. Role of
554 endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after
555 naked DNA injection. *Hum Gene Ther* 1999;10:2461-80.
- 556 [43] Widera G, Austin M, Rabussay D, Goldbeck C, Barnett SW, Chen MC, et al. Increased DNA
557 vaccine delivery and immunogenicity by electroporation in vivo. *Journal of Immunology*
558 2000;164:4635-40.
- 559 [44] Scheerlinck JP, Karlis J, Tjelle TE, Presidente PJ, Mathiesen I, Newton SE. In vivo
560 electroporation improves immune responses to DNA vaccination in sheep. *Vaccine* 2004;22:1820-5.
- 561 [45] Wu CJ, Lee SC, Huang HW, Tao MH. In vivo electroporation of skeletal muscles increases
562 the efficacy of Japanese encephalitis virus DNA vaccine. *Vaccine* 2004;22:1457-64.
- 563 [46] Sheets RL, Stein J, Manetz TS, Duffy C, Nason M, Andrews C, et al. Biodistribution of DNA
564 plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile virus is
565 similar, without integration, despite differing plasmid backbones or gene inserts. *Toxicol Sci*
566 2006;91:610-9.
- 567 [47] Barry MA, Johnston SA. Biological features of genetic immunization. *Vaccine* 1997;15:788-
568 91.
- 569 [48] Ren S, Li M, Smith JM, DeTolla LJ, Furth PA. Low-volume jet injection for intradermal
570 immunization in rabbits. *BMC Biotechnol* 2002;2:10.
- 571 [49] Choi AH, Smiley K, Basu M, McNeal MM, Shao M, Bean JA, et al. Protection of mice
572 against rotavirus challenge following intradermal DNA immunization by Biojector needle-free
573 injection. *Vaccine* 2007;25:3215-8.
- 574 [50] Kang MJ, Kim CK, Kim MY, Hwang TS, Kang SY, Kim WK, et al. Skin permeation,
575 biodistribution, and expression of topically applied plasmid DNA. *J Gene Med* 2004;6:1238-46.
- 576 [51] van den Berg JH, Nuijen B, Beijnen JH, Vincent A, van Tinteren H, Kluge J, et al.
577 Optimization of intradermal vaccination by DNA tattooing in human skin. *Hum Gene Ther* 2008.
- 578 [52] Fan H, Lin Q, Morrissey GR, Khavari PA. Immunization via hair follicles by topical
579 application of naked DNA to normal skin. *Nature Biotechnology* 1999;17:870-2.
- 580 [53] Boyle JS, Silva A, Brady JL, Lew AM. DNA immunization: induction of higher avidity
581 antibody and effect of route on T cell cytotoxicity. *Proc Natl Acad Sci U S A* 1997;94:14626-31.
- 582 [54] Hahn UK, Alex M, Czerny CP, Bohm R, Beyer W. Protection of mice against challenge with
583 *Bacillus anthracis* STI spores after DNA vaccination. *Int J Med Microbiol* 2004;294:35-44.
- 584 [55] Zhu W, Thomas CE, Sparling PF. DNA immunization of mice with a plasmid encoding
585 *Neisseria gonorrhoea* PorB protein by intramuscular injection and epidermal particle bombardment.
586 *Vaccine* 2004;22:660-9.
- 587 [56] Pokorna D, Rubio I, Muller M. DNA-vaccination via tattooing induces stronger humoral and
588 cellular immune responses than intramuscular delivery supported by molecular adjuvants. *Genet*
589 *Vaccines Ther* 2008;6:4.

590 [57] Pertmer TM, Roberts TR, Haynes JR. Influenza virus nucleoprotein-specific immunoglobulin
591 G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector
592 DNA delivery. *J Virol* 1996;70:6119-25.

593 [58] Vandermeulen G, Staes E, Vanderhaeghen ML, Bureau MF, Scherman D, Preat V.
594 Optimisation of intradermal DNA electrotransfer for immunisation. *J Control Release* 2007;124:81-7.

595 [59] Bohm W, Mertens T, Schirmbeck R, Reimann J. Routes of plasmid DNA vaccination that
596 prime murine humoral and cellular immune responses. *Vaccine* 1998;16:949-54.

597 [60] Rao S, Kong WP, Wei CJ, Yang ZY, Nason M, Styles D, et al. Multivalent HA DNA
598 vaccination protects against highly pathogenic H5N1 avian influenza infection in chickens and mice.
599 *PLoS ONE* 2008;3:e2432.

600 [61] Etchart N, Buckland R, Liu MA, Wild TF, Kaiserlian D. Class I-restricted CTL induction by
601 mucosal immunization with naked DNA encoding measles virus haemagglutinin. *J Gen Virol*
602 1997;78:1577-80.

603 [62] Jia R, Guo JH, Fan MW, Bian Z, Chen Z, Peng B, et al. Mucosal immunization against dental
604 caries with plasmid DNA encoding pac gene of *Streptococcus mutans* in rats. *Vaccine* 2004;22:2511-
605 6.

606 [63] Daheshia M, Kuklin N, Manickan E, Chun S, Rouse BT. Immune induction and modulation
607 by topical ocular administration of plasmid DNA encoding antigens and cytokines. *Vaccine*
608 1998;16:1103-10.

609 [64] Kanazawa T, Takashima Y, Hirayama S, Okada H. Effects of menstrual cycle on gene
610 transfection through mouse vagina for DNA vaccine. *Int J Pharm* 2008;360:164-70.

611 [65] Wolff JA, Ludtke JJ, Acsadi G, Williams P, Jani A. Long-term persistence of plasmid DNA
612 and foreign gene expression in mouse muscle. *Hum Mol Genet* 1992;1:363-9.

613 [66] Budker V, Budker T, Zhang G, Subbotin V, Loomis A, Wolff JA. Hypothesis: naked plasmid
614 DNA is taken up by cells in vivo by a receptor-mediated process. *J Gene Med* 2000;2:76-88.

615 [67] Nakamura M, Davila-Zavala P, Tokuda H, Takakura Y, Hashida M. Uptake and gene
616 expression of naked plasmid DNA in cultured brain microvessel endothelial cells. *Biochem Biophys*
617 *Res Commun* 1998;245:235-9.

618 [68] Levy MY, Barron LG, Meyer KB, Szoba Jr FC. Characterization of plasmid DNA transfer
619 into mouse skeletal muscle: evaluation of uptake mechanism, expression and secretion of gene
620 products into blood. *Gene Therapy* 1996;3:201-11.

621 [69] Lehmann MJ, Sczakiel G. Spontaneous uptake of biologically active recombinant DNA by
622 mammalian cells via a selected DNA segment. *Gene Ther* 2005;12:446-51.

623 [70] Wheeler M, Cortez-Gonzalez X, Frazzi R, Zanetti M. Ex vivo programming of antigen-
624 presenting B lymphocytes: considerations on DNA uptake and cell activation. *Int Rev Immunol*
625 2006;25:83-97.

626 [71] Luby-Phelps K. Cytoarchitecture and physical properties of cytoplasm: volume, viscosity,
627 diffusion, intracellular surface area. *Int Rev Cytol* 2000;192:189-221.

628 [72] Vaughan EE, Dean DA. Intracellular trafficking of plasmids during transfection is mediated
629 by microtubules. *Mol Ther* 2006;13:422-8.

630 [73] Vaughan EE, Geiger RC, Miller AM, Loh-Marley PL, Suzuki T, Miyata N, et al. Microtubule
631 acetylation through HDAC6 inhibition results in increased transfection efficiency. *Mol Ther*
632 2008;16:1841-7.

633 [74] Trombone AP, Silva CL, Lima KM, Oliver C, Jamur MC, Prescott AR, et al. Endocytosis of
634 DNA-Hsp65 alters the pH of the late endosome/lysosome and interferes with antigen presentation.
635 *PLoS ONE* 2007;2:e923.

636 [75] Coelho-Castelo AA, Santos Junior RR, Bonato VL, Jamur MC, Oliver C, Silva CL. B-
637 lymphocytes in bone marrow or lymph nodes can take up plasmid DNA after intramuscular delivery.
638 *Hum Gene Ther* 2003;14:1279-85.

- 639 [76] Lechardeur D, Verkman AS, Lukacs GL. Intracellular routing of plasmid DNA during non-
640 viral gene transfer. *Adv Drug Deliv Rev* 2005;57:755-67.
- 641 [77] Lechardeur D, Sohn KJ, Haardt M, Joshi PB, Monck M, Graham RW, et al. Metabolic
642 instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Ther* 1999;6:482-
643 97.
- 644 [78] Lechardeur D, Lukacs GL. Nucleocytoplasmic transport of plasmid DNA: a perilous journey
645 from the cytoplasm to the nucleus. *Hum Gene Ther* 2006;17:882-9.
- 646 [79] Ludtke JJ, Sebestyen MG, Wolff JA. The effect of cell division on the cellular dynamics of
647 microinjected DNA and dextran. *Mol Ther* 2002;5:579-88.
- 648 [80] Hebert E. Improvement of exogenous DNA nuclear importation by nuclear localization signal-
649 bearing vectors: a promising way for non-viral gene therapy? *Biol Cell* 2003;95:59-68.
- 650 [81] Dean DA. Import of plasmid DNA into the nucleus is sequence specific. *Exp Cell Res*
651 1997;230:293-302.
- 652 [82] Dean DA, Dean BS, Muller S, Smith LC. Sequence requirements for plasmid nuclear import.
653 *Exp Cell Res* 1999;253:713-22.
- 654 [83] Miller AM, Dean DA. Tissue-specific and transcription factor-mediated nuclear entry of
655 DNA. *Adv Drug Deliv Rev* 2009;61:603-13.
- 656 [84] Hohlfeld R, Engel AG. The immunobiology of muscle. *Immunol Today* 1994;15:269-74.
- 657 [85] Dupuis M, Denismize K, Woo C, Goldbeck C, Selby MJ, Chen MC, et al. Distribution of
658 DNA vaccines determines their immunogenicity after intramuscular injection in mice. *Journal of*
659 *Immunology* 2000;165:2850-8.
- 660 [86] Fang SH, Nishimura T, Takahashi K. Relationship between development of intramuscular
661 connective tissue and toughness of pork during growth of pigs. *J Anim Sci* 1999;77:120-30.
- 662 [87] Pimorady-Esfahani A, Grounds MD, McMennamin PG. Macrophages and dendritic cells in
663 normal and regenerating murine skeletal muscle. *Muscle Nerve* 1997;20:158-66.
- 664 [88] McLennan IS. Degenerating and regenerating skeletal muscles contain several subpopulations
665 of macrophages with distinct spatial and temporal distributions. *J Anat* 1996;188 (Pt 1):17-28.
- 666 [89] Honda H, Kimura H, Rostami A. Demonstration and phenotypic characterization of resident
667 macrophages in rat skeletal muscle. *Immunology* 1990;70:272-7.
- 668 [90] Gronevik E, Tollefsen S, Sikkeland LI, Haug T, Tjelle TE, Mathiesen I. DNA transfection of
669 mononuclear cells in muscle tissue. *J Gene Med* 2003;5:909-17.
- 670 [91] Chattergoon MA, Robinson TM, Boyer JD, Weiner DB. Specific immune induction following
671 DNA-based immunization through in vivo transfection and activation of macrophages/antigen-
672 presenting cells. *J Immunol* 1998;160:5707-18.
- 673 [92] Ulmer JB, Deck RR, Dewitt CM, Donnhy JI, Liu MA. Generation of MHC class I-restricted
674 cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-
675 muscle cells. *Immunology* 1996;89:59-67.
- 676 [93] Loots K, Vleugels B, Ons E, Vanrompay D, Goddeeris BM. Evaluation of the persistence and
677 gene expression of an anti-*Chlamydothila psittaci* DNA vaccine in turkey muscle. *BMC Vet Res*
678 2006;2:18.
- 679 [94] Tuomela M, Malm M, Wallen M, Stanescu I, Krohn K, Peterson P. Biodistribution and
680 general safety of a naked DNA plasmid, GTU-MultiHIV, in a rat, using a quantitative PCR method.
681 *Vaccine* 2005;23:890-6.
- 682 [95] Winegar RA, Monforte JA, Suing KD, KG OL, Rudd CJ, Macgregor JT. Determination of
683 tissue distribution of an intramuscular plasmid vaccine using PCR and in situ DNA hybridization.
684 *Hum Gene Ther* 1996;7:2185-94.
- 685 [96] Zhang HY, Sun SH, Guo YJ, Chen ZH, Huang L, Gao YJ, et al. Tissue distribution of a
686 plasmid DNA containing epitopes of foot-and-mouth disease virus in mice. *Vaccine* 2005;23:5632-40.

- 687 [97] Parker SE, Monteith D, Horton H, Hof R, Hernandez P, Vilalta A, et al. Safety of a GM-CSF
688 adjuvant-plasmid DNA malaria vaccine. *Gene Therapy* 2001;8:1011-23.
- 689 [98] Parker SE, Borellini F, Wenk ML, Hobart P, Hoffman SL, Hedstrom R, et al. Plasmid DNA
690 malaria vaccine: tissue distribution and safety studies in mice and rabbits. *Hum Gene Ther*
691 1999;10:741-58.
- 692 [99] Pal R, Yu Q, Wang S, Kalyanaraman VS, Nair BC, Hudacik L, et al. Definitive toxicology
693 and biodistribution study of a polyvalent DNA prime/protein boost human immunodeficiency virus
694 type 1 (HIV-1) vaccine in rabbits. *Vaccine* 2006;24:1225-34.
- 695 [100] Mena A, Andrew ME, Coupar BEH. Rapid dissemination of intramuscularly inoculated DNA
696 vaccines. *Immunology and Cell Biology* 2001;79:87-9.
- 697 [101] Gravier R, Dory D, Laurentie M, Bougeard S, Cariolet R, Jestin A. In vivo tissue distribution
698 and kinetics of a pseudorabies virus plasmid DNA vaccine after intramuscular injection in swine.
699 *Vaccine* 2007;25:6930-8.
- 700 [102] Son MK, Choi JH, Lee DS, Kim CY, Choi SM, Kang KK, et al. Pharmacokinetics and
701 biodistribution of a pGT2-VEGF plasmid DNA after administration in rats. *J Cardiovasc Pharmacol*
702 2005;46:577-84.
- 703 [103] Garver KA, Conway CM, Elliott DG, Kurath G. Analysis of DNA-vaccinated fish reveals
704 viral antigen in muscle, kidney and thymus, and transient histopathologic changes. *Mar Biotechnol*
705 (NY) 2005;7:540-53.
- 706 [104] Kim N, Kwon SS, Lee J, Kim S, Yoo TJ. Protective effect of the DNA vaccine encoding the
707 major house dust mite allergens on allergic inflammation in the murine model of house dust mite
708 allergy. *Clin Mol Allergy* 2006;4:4.
- 709 [105] Armengol G, Ruiz LM, Orduz S. The injection of plasmid DNA in mouse muscle results in
710 lifelong persistence of DNA, gene expression, and humoral response. *Mol Biotechnol* 2004;27:109-18.
- 711 [106] Kanellos T, Sylvester ID, Ambali AG, Howard CR, Russell PH. The safety and longevity of
712 DNA vaccines for fish. *Immunology* 1999;96:307-13.
- 713 [107] Kim BM, Lee DS, Choi JH, Kim CY, Son M, Suh YS, et al. In vivo kinetics and
714 biodistribution of a HIV-1 DNA vaccine after administration in mice. *Arch Pharm Res* 2003;26:493-8.
- 715 [108] Manam S, Ledwith B, Barnum AB, Troilo PJ, Pauley CJ, Harper LB, et al. Plasmid DNA
716 vaccines: Tissue distribution and effects of DNA sequence, adjuvants and delivery method on
717 integration into host DNA. *Intervirology* 2000;43:273-81.
- 718 [109] Oh YK, Kim JP, Hwang TS, Ko JJ, Kim JM, Yang JS, et al. Nasal absorption and
719 biodistribution of plasmid DNA: an alternative route of DNA vaccine delivery. *Vaccine*
720 2001;19:4519-25.
- 721 [110] Liu C, Fan M, Xu Q, Li Y. Biodistribution and expression of targeted fusion anti-caries DNA
722 vaccine pGJA-P/VAX in mice. *J Gene Med* 2008;10:298-305.
- 723 [111] Coelho-Castelo AA, Trombone AP, Rosada RS, Santos RR, Jr., Bonato VL, Sartori A, et al.
724 Tissue distribution of a plasmid DNA encoding Hsp65 gene is dependent on the dose administered
725 through intramuscular delivery. *Genet Vaccines Ther* 2006;4:1.
- 726 [112] Watkins C, Hopkins J, Harkiss G. Reporter gene expression in dendritic cells after gene gun
727 administration of plasmid DNA. *Vaccine* 2005;23:4247-56.
- 728 [113] Jeon YH, Choi Y, Kang JH, Chung JK, Lee YJ, Kim CW, et al. In vivo monitoring of DNA
729 vaccine gene expression using firefly luciferase as a naked DNA. *Vaccine* 2006;24:3057-62.
- 730 [114] Garmory HS, Brown KA, Titball RW. DNA vaccines: improving expression of antigens.
731 *Genet Vaccines Ther* 2003;1:2.
- 732 [115] Greenland JR, Geiben R, Ghosh S, Pastor WA, Letvin NL. Plasmid DNA vaccine-elicited
733 cellular immune responses limit in vivo vaccine antigen expression through Fas-mediated apoptosis. *J*
734 *Immunol* 2007;178:5652-8.

735 [116] Davis HL, Millan CL, Watkins SC. Immune-mediated destruction of transfected muscle fibers
736 after direct gene transfer with antigen-expressing plasmid DNA. *Gene Ther* 1997;4:181-8.

737 [117] Yokoyama M, Hassett DE, Zhang J, Whitton JL. DNA immunization can stimulate florid local
738 inflammation, and the antiviral immunity induced varies depending on injection site. *Vaccine*
739 1997;15:553-60.

740 [118] Payette PJ, Weeratna RD, McCluskie MJ, Davis HL. Immune-mediated destruction of
741 transfected myocytes following DNA vaccination occurs via multiple mechanisms. *Gene Therapy*
742 2001;8:1395-400.

743 [119] Geiben-Lynn R, Greenland JR, Frimpong-Boateng K, van Rooijen N, Hovav AH, Letvin NL.
744 CD4+ T lymphocytes mediate in vivo clearance of plasmid DNA vaccine antigen expression and
745 potentiate CD8+ T-cell immune responses. *Blood* 2008;112:4585-90.

746 [120] Napirei M, Wulf S, Eulitz D, Mannherz HG, Kloeckl T. Comparative characterization of rat
747 deoxyribonuclease 1 (Dnase1) and murine deoxyribonuclease 1-like 3 (Dnase1l3). *Biochem J*
748 2005;389:355-64.

749 [121] Baron WF, Pan CQ, Spencer SA, Ryan AM, Lazarus RA, Baker KP. Cloning and
750 characterization of an actin-resistant DNase I-like endonuclease secreted by macrophages. *Gene*
751 1998;215:291-301.

752 [122] Kang ES, Kim CY, Kim SB, Im SJ, Yang SH, Sung YC, et al. In vivo kinetics and
753 biodistribution of HB-110, a novel HBV DNA vaccine, after administration in mice. *Arch Pharm Res*
754 2007;30:355-60.

755 [123] Hisazumi J, Kobayashi N, Nishikawa M, Takakura Y. Significant role of liver sinusoidal
756 endothelial cells in hepatic uptake and degradation of naked plasmid DNA after intravenous injection.
757 *Pharm Res* 2004;21:1223-8.

758 [124] Liu F, Shollenberger LM, Conwell CC, Yuan X, Huang L. Mechanism of naked DNA
759 clearance after intravenous injection. *J Gene Med* 2007;9:613-9.

760 [125] Nichols WW, Ledwith BJ, Manam SV, Troilo PJ. Potential DNA vaccine integration into host
761 cell genome. *Annals of the New York Academy of Sciences* 1995;772:30-9.

762 [126] Kang KK, Choi SM, Choi JH, Lee DS, Kim CY, Ahn BO, et al. Safety evaluation of GX-12, a
763 new HIV therapeutic vaccine: investigation of integration into the host genome and expression in the
764 reproductive organs. *Intervirology* 2003;46:270-6.

765 [127] Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, 2nd, et al. Plasmid
766 DNA vaccines: assay for integration into host genomic DNA. *Dev Biol (Basel)* 2000;104:33-43.

767 [128] Liang A, Cao S, Han L, Yao Y, Moaen-Ud-Din M, Yang L. Construction and evaluation of
768 the eukaryotic expression plasmid encoding two copies of somatostatin genes fused with hepatitis B
769 surface antigen gene S. *Vaccine* 2008;26:2935-41.

770 [129] Martin T, Parker SE, Hedstrom R, Le T, Hoffman SL, Norman J, et al. Plasmid DNA malaria
771 vaccine: the potential for genomic integration after intramuscular injection. *Hum Gene Ther*
772 1999;10:759-68.

773 [130] Vilalta A, Mahajan RK, Hartikka J, Rusalov D, Martin T, Bozoukova V, et al. I. Poloxamer-
774 formulated plasmid DNA-based human cytomegalovirus vaccine: evaluation of plasmid DNA
775 biodistribution/persistence and integration. *Hum Gene Ther* 2005;16:1143-50.

776 [131] Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, et al. Plasmid DNA
777 vaccines: Investigation of integration into host cellular DNA following intramuscular injection in
778 mice. *Intervirology* 2000;43:258-72.

779 [132] Wang Z, Troilo PJ, Wang X, Griffiths TG, Pacchione SJ, Barnum AB, et al. Detection of
780 integration of plasmid DNA into host genomic DNA following intramuscular injection and
781 electroporation. *Gene Ther* 2004;11:711-21.

782 [133] The US Food and Drug Administration. Guidance for Industry. Considerations for Plasmid
783 DNA Vaccines for Infectious Disease Indications. 2007.

784 [134] The European Medicines Agency (EMA). Note for guidance: DNA vaccines non-
785 amplifiable in eukaryotic cells for veterinary use. CVMP/IWP/07/98-FINAL. 2000.
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787
788