THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Genome editing for disease resistance in livestock

Citation for published version:

Proudfoot, C & Tait-Burkard, C 2017, 'Genome editing for disease resistance in livestock', *Emerging Topics in Life Sciences*, vol. 1, no. 2, pp. 209. <https://doi.org/10.1042/ETLS20170032>

Digital Object Identifier (DOI):

[10.1042/ETLS20170032](https://doi.org/10.1042/ETLS20170032)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Emerging Topics in Life Sciences

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Genome Editing for Disease Resistance in Livestock

Chris Proudfoot¹ and Christine Burkard^{1,*}

1 The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, EH25 9RG Midlothian, United Kingdom.

* Corresponding author

E-mail: Christine.burkard@roslin.ed.ac.uk

1 **Abstract (250 words)**

2 One of the major burdens on the livestock industry is loss of animals and decrease of production
3 efficiency due to disease. Advances in sequencing technology and genome editing techniques provide
4 the unique opportunity to generate animals with improved traits. In this review we discuss the
5 techniques currently applied to genetic manipulation of livestock species and the efforts in making
6 animals disease resistant or resilient.

7	<u>Abbreviations list</u>	
8	ASFV	African swine fever virus
9	BSE	Bovine spongiform encephalopathy
10	bTB	Bovine Tuberculosis
11	Cas	CRISPR-associated gene
12	CPI	Cytoplasmic microinjection
13	CRISPR	Clustered regularly interspaced short palindromic repeats
14	DBD	DNA binding domain
15	DSB	Double strand break
16	GeCKO	Genome-wide CRISPR knock-out
17	GWAS	Genome-wide association study
18	HDR	Homology directed repair
19	HE	Homing endonuclease
20	iPSC	induced pluripotent stem cell
21	IVF	<i>In vitro</i> fertilisation
22	KO	Knockout
23	NHEJ	Non-homologous end joining
24	PGC	Primordial germ cell
25	PNI	Pronuclear microinjection
26	PrP	Prion protein
27	PRRS	Porcine reproductive and respiratory syndrome
28	PRRSV	Porcine reproductive and respiratory syndrome virus
29	SCNT	Somatic cell nuclear transfer
30	siRNA	small interfering RNA
31	SMGT	Sperm-mediated gene transfer
32	SSC	Spermatogonial stem cells
33	ssRNA	single-stranded RNA
34	TALE	Transcription activator-like effector

- 35 **TALEN** Transcription activator-like effector nuclease
- 36 **ZF** Zinc finger
- 37 **ZFN** Zinc finger nuclease

38 **Genome editing for disease resistance in livestock**

39

40 Chris Proudfoot and Christine Burkard

41

42 **Introduction**

43 With the world population predicted to reach almost 10 billion by 2050 there are a number of challenges
44 in sustainable management of finite resources. The rising demand for food requires improved
45 productivity of agricultural systems. One of the major burdens on the livestock industry is loss of
46 animals and decrease of production efficiency due to disease. Furthermore, it is important to improve
47 the health and welfare of animals by reducing and preferably preventing the effects of disease. Advances
48 in sequencing technology and genome editing techniques provide the unique opportunity to generate
49 animals with improved traits. In this review we will discuss the techniques currently applied to genetic
50 manipulation of livestock species and the efforts in making animals disease resistant or resilient.

51 **The tools**

52 In 1982 Plamiter and Brinster (1) set the stage for sequential advances in our ability to modify and
53 improve mammalian genomes for desirable traits. Whereas previous work by others showed that
54 foreign DNA fragments could be integrated into the genome of embryos by pronuclear microinjection
55 (PNI), their work demonstrated a functional application; introduction of a growth hormone gene into
56 mouse embryos resulted in rapid growth of the animals. Beyond utilizing cell-based approaches, early
57 genome modification was restricted to the injection of plasmids or gene fragments into the pronucleus
58 of embryos. More efficient integration of foreign DNA fragments into the target genome was
59 subsequently achieved using transposons or retroviral vectors (2, 3). Early specific edits in cells could
60 be achieved with homing endonucleases (HE), natural meganucleases, which introduce double strand
61 breaks (DSBs) at target recognition sites of 14-40bp (4). However, engineering of HEs has been
62 challenging and they are prone to off-target cutting, wherefore there are currently still very few *in vivo*
63 applications (5). The development of the zinc finger nuclease (ZFN) (6), the first genome editing tool,
64 increased the repertoire of programmed modifications, allowing precise cutting and repair to any target
65 genome. The intervening two decades have borne witness to the continued development of the editing
66 toolbox, with improvements in adaptability and efficiency, coupled with reduced costs and facile in-
67 house assembly platforms, resulting in an almost exponential uptake of the technology in the last five
68 years (Figure 1).

69 Zinc Fingers (ZFs) are amongst the most well-characterised protein DNA binding domains (DBDs) found
70 in nature. Each ZF binds a triplet of nucleotides, with synthetic arrays of ZFs constructed to improve
71 specificity to a desired target sequence (typically 9-18 bases). ZFNs are chimeric enzymes created by

72 fusing a modular ZF array to the nuclease domain of the restriction enzyme FokI. This nuclease domain
73 has no innate sequence specificity, with target site delineated by the ZF array. The FokI nuclease domain
74 requires dimerization to function, so paired ZFNs are typically employed to generate a targeted DNA
75 DSB, further increasing site specificity. Unfortunately, the design of ZFNs remains a complicated and
76 technically challenging process. Only a small number of companies have the expertise required to
77 produce reliable ZFNs, and, as a result, these reagents have remained relatively expensive, curbing their
78 wider use.

79 Transcription activator-like effectors (TALEs) are a second group of naturally occurring proteins
80 containing DBDs. Produced by proteobacteria of the genus *Xanthomonas*, TALEs bind host DNA and
81 thereby alter the transcriptional profile of infected cells. The DNA binding portion of each TALE is
82 composed of a repeated modular array, with each module having sequence preference for a single DNA
83 base (7). This simple 1-to-1, module-to-base, relationship makes design of functional synthetic DBDs
84 straight forward, and kits can be purchased to allow their assembly in a standard molecular biology lab
85 in less than two weeks. Typically designed to recognise 12-20 bases, arrays are fused with FokI to give
86 a TALE nuclease (TALEN), and, as with ZFNs, these are employed in pairs to allow FokI dimerization and
87 increase specificity. Two sequential modifications to the FokI domain further reduced the potential for
88 off-target cutting by both TALENs and ZFNs; conversion of the homodimer into an obligate heterodimer,
89 and conversion from a nuclease to a nickase by mutation of a catalytic domain (8). However, while
90 TALENs were certainly more widely utilised than ZFNs, they have since been superseded by the most
91 recent tool.

92 Developed from an innate bacterial antiviral mechanism, the latest addition to the genome editor
93 toolbox is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated
94 (Cas) system. With target specificity directed by a short single-stranded RNA (ssRNA) molecule(s), this
95 represents a departure from the preceding genome editors that utilise a protein-based DBD. As with the
96 FokI nuclease domain, Cas nucleases lack innate sequence specificity but are instead guided to their
97 target site by Watson-Crick base pairing between their complexed ssRNA and the cognate DNA
98 sequence. This constitutes a major advantage for this tool; the Cas nuclease is not covalently fused to a
99 DBD so the same protein can be utilised to target multiple different target sites simply by combining it
100 with different combinations of ssRNA. Furthermore, while all of the reagents required can be produced
101 in almost any molecular biology lab, both the Cas nuclease and the ssRNA molecules can also be
102 purchased from multiple vendors. The ease with which reagents can be designed, coupled with
103 economical availability, has resulted in huge uptake of this tool and an explosion of publications in this
104 field. (9, 10)

105

106 **The possibilities**

107 Genome editors can break DNA at specific target sites; it is through the subsequent repair of these
108 breaks that scientists can introduce desired changes at the target locus. Most cell types preferentially
109 utilise the non-homologous end-joining (NHEJ) pathway, an error-prone process that typically results
110 in small insertions or deletions at the site of repair. By creating a DNA break in the coding sequence of a
111 gene, this form of repair often generates a frameshift mutation and thereby truncation of the encoded
112 protein or functional gene knockout (KO). Alternatively, by flooding the target cell with a DNA repair
113 template, it is possible to trigger the homology-directed repair (HDR) pathway. This allows the
114 introduction of precise sequence changes proximal to the cut site, ranging from single base changes to
115 the insertion of transgenes. Researchers commonly use synthetic single stranded oligodeoxynucleotides
116 to introduce small changes, or plasmid/dsDNA templates for larger insertions. Finally, by creating two
117 simultaneous breaks on a chromosome the intervening DNA sequence can be deleted; this approach can
118 be used to alter transcriptional profiles by removing regulatory elements, or to delete exons thereby
119 removing protein domains while leaving the remaining reading frame intact. (11)

120 As a result of the evolution of this technology and a greater understanding of how to harness its potential
121 we are now able to introduce extremely precise changes to the genome with greater accuracy and
122 efficiency than has ever been possible. We are now at a stage where we are limited more by our
123 imagination than the technology available.

124 **The industry**

125 The long history of livestock domestication has relied on the sequential selection of animals based on
126 desirable traits, with generational improvements in their ability to thrive in the varied habitats occupied
127 by the communities farming them. Traditionally, selective breeding focused on handling and
128 productivity traits, such as docility, feed conversion, and fertility, with modern breeding goals
129 incorporating animal health and welfare. Selecting for disease resistance and resilience is not only
130 important from an animal health and welfare perspective but has significant economic impacts. For
131 instance, it is estimated that endemic diseases incur added costs of €30-40 per slaughter pig in the
132 European Union, adding to up to €10.5bn per year ((12), EU28 2016 (13)).

133 Livestock breeding companies incorporate genetic improvement into their programmes by assigning
134 values to various traits of individual animals and incorporating only those with the highest overall merit
135 into their nucleus herd. Advances in affordable genotyping tools allow direct linkage between
136 physiological characteristics and genome-wide association studies (GWAS) (14), resulting in
137 increasingly efficient and productive breeding populations. Compilation of phenotypic information is
138 relatively straightforward when traits can be measured accurately under normal husbandry conditions.
139 By contrast, disease susceptibility, or lack thereof, is a trait that is difficult to quantitatively assess as
140 exposure to pathogens within a population of animals is rarely uniform and the deliberate exposure of

141 large numbers of animals under experimental conditions is both ethically questionable and very
142 expensive (15). Even if such variance could be readily identified, the merit gains achieved by recent
143 breeding programmes may prove a barrier to propagation within a nucleus herd; the allelic variant
144 might be present in low abundance, recessive, or associated with animals that would otherwise be
145 considered of low merit. In this scenario genome editing offers an opportunity to contribute to the
146 natural breeding process, introducing newly identified genetic features into the progeny of elite nucleus
147 animals without negatively impacting other highly desirable traits (16). Such an approach could also
148 contribute to genetic improvement if relevant polymorphisms were identified in related breeds or even
149 other species (Figure 2). As such, genome editors have great potential in allowing the introduction of
150 novel traits that improve animal welfare, increase production, reduce food waste in the production
151 chain, improve food security, and contribute to the economic security of small holder farmers.

152 **The animals**

153 Editing in **cattle** poses a significant challenge due to cost, small number of offspring, and long generation
154 time (9 months gestation, 12-18 months to reach sexual maturity). As a consequence, there is significant
155 pressure for editing techniques to be highly efficient to ensure intended offspring. While somatic cell
156 nuclear transfer (SCNT) of confirmed edited cells is often the preferred option (17, 18) cytoplasmic
157 microinjection (CPI) and PNI into *in vitro* fertilised oocytes have also been employed (19). While
158 generation of chimeras by microinjection of edited induced pluripotent stem cells (iPSCs) into
159 blastocysts has been demonstrated in multiple papers, so far no germline transmission was reported
160 (20, 21). A potential future editing technique in cattle may also be the editing of spermatogonial stem
161 cells (SSCs). Long-term cultivation methods have been recently published and advances in
162 transplantation of these cells and sterile recipients could provide a promising avenue for generating
163 genome edited cattle (22, 23).

164 Editing **goats and sheep** is less restricted than cattle; they are smaller, cheaper, and produce more
165 offspring and, with gestation times of 5 months and sexual maturity at 6-8 months, the generation times
166 are significantly lower. Oocytes can be collected from abattoir samples or by laparoscopic ovum pick-
167 up, with *in vitro* fertilization (IVF) and microinjection of zygotes (24). In all ruminants blastocysts can
168 be re-implanted into a recipient, allowing testing embryo viability and genotype prior to the
169 implantation. Alternatively, small ruminants have also been generated by SCNT (25-27) and goats by
170 sperm-mediated gene transfer (SMGT) (28). The generation of chimeric embryos from (non-edited)
171 iPSCs has been demonstrated in sheep (29), whilst in goats the generation of iPSCs has been reported,
172 no editing or chimeric integration has been demonstrated yet (30, 31).

173 **Pigs** have several advantages over ruminants for genome modification; they have large litter sizes, a
174 short gestation of less than 4 months, and can reach sexual maturity at 5-6 months. While many groups
175 have successfully used SCNT to generate modified pigs, microinjection and transfer of zygotes is an

176 efficient alternative. As pigs are a multiparous species that self-limit the number of embryos they carry
177 to term, an excess of manipulated zygotes can be transferred to each recipient to improve pregnancy
178 rates. Zygotes are generally harvested from donor animals as polyspermy associated with IVF remains
179 a significant issue (32). Genome modified animals have been produced using a variety of techniques,
180 including SCNT and PNI and CPI of zygotes (comprehensive lists of modified animals can be found (33,
181 34)). Germline transmission has been demonstrated in chimeric animals generated from iPSCs (35,
182 36). A variety of cultivation methods for SSCs have been described in pigs, whereas long-term cultivation
183 still remains a challenge (reviewed in (37)). Furthermore, genome edited, sterile recipients for the
184 transfer of (edited) SSCs have been described in pigs (38).

185 Editing in **chicken** has proven challenging compared to mammals due to significant differences in
186 reproductive physiology. The germinal disk of a laid egg consists of approximately 50,000 cells leaving
187 no clear route for efficient modification. Editing in chicken currently relies on primordial germ cells
188 (PGCs) that are edited *in vitro* and transferred into recipient embryos. PGCs can be isolated, cultivated
189 and genetically modified whilst maintaining their PGC status (39, 40). Transfer of PGCs to the blood
190 stream of recipient embryos results colonisation of the developing gonad and subsequent germline
191 transmission (41). With PGCs it is now possible to manipulate the genome of the chicken in culture and
192 to use those cells to establish an edited chicken line. Furthermore, chicken PGCs may be modified by
193 microinjection of transfection reagents and transposons into the blood stream of embryos to generate
194 germline-modified animals (42).

195 Whilst there has been a long history in management and breeding of the aforementioned animals, there
196 are in fact numerous other livestock species that confer significant economic impact to the agricultural
197 sector.

198 The global consumption of **farmed fish** has recently overtaken beef and is the fastest growing animal
199 protein production sector. Whilst selective breeding in fish is utilised for many species, targeted genome
200 modification is in its infancy (43). Fish lay large numbers of eggs that are externally fertilised and
201 thereby readily accessible for genetic manipulation. Early examples of transgenic fish are growth
202 hormone transgenic salmon and Nile tilapia (44, 45). Genome edited salmon have been produced using
203 CRISPR/Cas9 to induce albinism (46) or infertility (47). Both of these traits are of potential utility for
204 Salmon breeders as albinism can serve as a visual editing indicator and sterility would prevent
205 interbreeding of edited with wild salmon. Catfish were edited for enhanced growth by using
206 CRISPR/Cas9 to knock out the myostatin gene (48). No genome editing for disease resistance has yet
207 been reported in fish, however multiple GWAS studies are currently being conducted to identify such
208 targets. A potential target region has been identified in salmon conferring resistance against pancreatic
209 necrosis (49).

210 FAO lists **honeybees** as livestock as they are integral to many agricultural practices. Global impact of
211 honeybees as pollinators in crop production is significant (50, 51). Selective breeding in bees is
212 employed to select for hygienic, low disease burden colonies (52, 53). Edited or transgenic bees can be
213 generated by microinjection of embryos (54, 55). As has been the case in many agricultural species early
214 work involved the introduction of fluorescence markers into the genome (54). Application of genome
215 editors thus far has been to identify gene function rather than to address disease resistance (55). With
216 improvements in the understanding of the bee genome and more detailed association studies it is
217 anticipated that genome editing for disease resistance in bees is in the future (56).

218 **The diseases**

219 Mastitis has a huge impact in the dairy industries. In the US it is the most common disease in dairy cattle
220 resulting in estimated annual losses of \$2bn. Globally, small ruminants also play an important role in
221 the dairy industries with mastitis conferring a significant economic burden. *Staphylococcus aureus* is the
222 most common pathogen to cause mastitis and there is a very low natural heritability of resilience to
223 infection. In ruminants a number of similar transgenic strategies have been employed by generating
224 animals producing enzymes inhibiting the growth of bacteria in the mammary gland. In cattle the
225 antibiotic lysostaphin was introduced by SCNT resulting in secreted protein in their milk, capable of
226 killing *S.aureus* (57). The milk from goats expressing human lysozyme was shown to inhibit the growth
227 of mastitis-causing bacteria and *Pseudomonas fragi*, responsible for the cold-spoilage of milk (58, 59).
228 Importantly, the growth of *L. lactis*, required for the making of processed dairy products, such as cheese,
229 was not inhibited.

230 Misfolding of the prion protein (PrP) is associated with neurodegenerative diseases in many mammals.
231 The accumulation of misfolded PrP plaques results in bovine spongiform encephalopathy (BSE) in cattle
232 and scrapie in sheep and goats. A number of different groups have knocked out the PrP gene as a strategy
233 to circumvent such diseases. While a transgenic approach has been used to achieve this goal, application
234 of genome editors could be used streamline this process. In addition to value to the agricultural sector,
235 interest in PrP KO livestock extends to biopharmaceuticals, as this is considered an appropriate safety
236 measure of products destined for human applications (60-63).

237 Early efforts are ongoing to make ruminants resilient to *Mannheimia (Pasteurella) haemolytica* infection,
238 which causes epizootic pneumonia (shipping fever) and may also contribute to enzootic pneumonia in
239 calves and lambs as well as peritonitis in sheep. The pathogen can produce a cytotoxic leukotoxin, which
240 is largely responsible for the pathogenicity of the bacteria. In ruminants the leukotoxin binds to the
241 uncleaved signal peptide of the CD18 protein present on the cell surface leukocytes (64). In other
242 species, including mouse and human, the mature CD18 lacks the signal peptide as a result of proteolytic
243 processing. Based on the human sequence, ZFNs were used to introduce a single amino acid change.

244 Leukocytes from the resultant fetuses were resistant to cytotoxicity associated with *M.haemolytica*
 245 leukotoxin (65).

246 Bovine tuberculosis (bTB) has a direct effect on productivity in cattle and buffalo, impacts international
 247 trade, and poses a significant human health risk. Polymorphisms in the NRAMP1 gene, also known as
 248 SLC11A1, in cattle have been associated with varying levels of resilience to bTB infection (66).
 249 CRISPR/Cas9 was used for targeted insertion of an NRAMP1 variant associated with resilience to bTB
 250 infection into the cattle genome. *Ex vivo* challenge of peripheral blood monocytes showed reduced
 251 pathogen growth in exogenous NRAMP1 expressing cells. An *in vivo* study in the transgenic animals
 252 reported diminished interferon response to TB infection but did not assess pathogen burden (67).

253 African Swine Fever Virus (ASFV) is a disease endemic to huge swathes of sub-Saharan Africa. Native
 254 suid hosts, including the warthog, are resilient to the infection, while domestic pigs develop a lethal
 255 haemorrhagic fever. Species-specific variation of the *RELA*, a component of the transcription factor NF-
 256 kb, between native and domestic suids were postulated to underlie this host genetic variance (68). Using
 257 a ZFN pair with a plasmid template for HDR, researchers converted the encoded domestic pig protein
 258 sequence to the warthog equivalent (69). Data to show resilience of the animals to ASFV infection has
 259 yet to be reported. It is important to differentiate between disease resistance, the ability of an animal to
 260 suppress the establishment and/or development of an infection, and disease resilience where an
 261 infected host manages to maintain an acceptable level of productivity despite challenge pressure (70).
 262 Should these pigs prove to be resilient to ASFV infection it is likely that their use may not be permitted
 263 in many jurisdictions, since they could act as reservoirs of infection. However, in environments where
 264 the disease is endemic use of such animals could be beneficial.

265 The most economically important pig disease worldwide is porcine reproductive and respiratory
 266 syndrome (PRRS). *In vitro* experiments showed that entry of the causative agent of the disease, PRRS
 267 virus (PRRSV), into host cells relies on two proteins, CD163 and CD169 (71). It was further
 268 demonstrated that subdomain 5 of CD163 was essential for PRRSV entry (72). Surprisingly, SCNT with
 269 fibroblasts lacking CD169 resulted in pigs that were not resistant to PRRSV infection (73). Functional
 270 CD163 KO animals were generated using a CRISPR/Cas9 to induce a NHEJ-mediated premature stop
 271 codon (74). The CD163 KO animals were shown to be resistant to PRRSV infection both *in vitro* and *in*
 272 *vivo* (75). CD163 has a wide variety of important biological functions in inflammation and immune
 273 response. To retain these functions precise deletion of only CD163 subdomain 5 has been carried out.
 274 Subdomain 5 is encoded by exon 7, which was excised from the genome using CPI of two guide RNAs
 275 targeting the flanking intronic sequence. Cells from the resulting animals are resistant to PRRSV
 276 infection and maintain their biological function (76).

277 Reactivation of endogenous retroviruses is a potential barrier to the use of livestock as tissue and organ
278 donors. Genome editors have been used to permanently inactivate porcine endogenous retrovirus in
279 pigs presenting a potential solution to this human health threat (77).

280 Avian influenza poses a significant threat to the global poultry industry and to human health, as zoonotic
281 transmission is frequently observed. The control of outbreaks requires the culling of infected and
282 neighbouring flocks and the implementation of strict biosecurity measure to prevent the further spread
283 of the virus. Transgenic chickens were generated by microinjection of eggs with retrovirus to
284 incorporate a small decoy RNA fragment under a U6 promoter into the chicken genome (78). The decoy
285 RNA fragment expressed in chickens interferes with the formation of infectious influenza particles,
286 thereby preventing spread to co-housed birds. This approach has yet to be evaluated in other species
287 susceptible to influenza.

288 **Discussion and Outlook**

289 Application of genome editors allows easy-to use, targeted strategies for genome modification in
290 livestock. To improve disease resistance traits, editing targets are identified by investigation of in vitro
291 host-pathogen interactions, species variation, or GWAS studies (Figure 3). The field of genome editors
292 is fast evolving and sequential improvements coupled with a better understanding of DSB repair
293 mechanisms will inevitably result in an expanded range of editing opportunities. Advances in delivery
294 techniques, such as editing gametes or spermatogonial, embryonic, or induced pluripotent stem cells,
295 will streamline the production of edited animals and make it applicable to a wider range of species.
296 Generating disease resistant animals will not only help to feed the world but also improve animal
297 welfare and aid in the reduction of antimicrobial use.

298 Most of the examples discussed in this review are still at early stages and integration of genome edited
299 animals into highly productive elite breeding lines will take time. Furthermore, approval of edited
300 animals for human consumption relies on national and multi-national legislation, which is currently at
301 early stages. And in the end, also the consumer will decide on the success of genome edited animals in
302 livestock production.

303

304

305 **References**

- 306 1. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, et al.
307 Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth
308 hormone fusion genes. *Nature*. 1982;300(5893):611-5.
- 309 2. Huszar D, Balling R, Kothary R, Magli MC, Hozumi N, Rossant J, et al. Insertion of a bacterial gene
310 into the mouse germ line using an infectious retrovirus vector. *Proc Natl Acad Sci U S A*.
311 1985;82(24):8587-91.
- 312 3. Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. Molecular reconstruction of Sleeping Beauty, a Tc1-
313 like transposon from fish, and its transposition in human cells. *Cell*. 1997;91(4):501-10.
- 314 4. Chevalier BS, Stoddard BL. Homing endonucleases: structural and functional insight into the
315 catalysts of intron/intein mobility. *Nucleic Acids Res*. 2001;29(18):3757-74.
- 316 5. Guha TK, Wai A, Hausner G. Programmable Genome Editing Tools and their Regulation for
317 Efficient Genome Engineering. *Comput Struct Biotechnol J*. 2017;15:146-60.
- 318 6. Belfort M, Bonocora RP. Homing endonucleases: from genetic anomalies to programmable
319 genomic clippers. *Methods Mol Biol*. 2014;1123:1-26.
- 320 7. Moore R, Chandrasekhar A, Bleris L. Transcription activator-like effectors: a toolkit for synthetic
321 biology. *ACS synthetic biology*. 2014;3(10):708-16.
- 322 8. Joung JK, Sander JD. TALENs: a widely applicable technology for targeted genome editing. *Nature*
323 reviews Molecular cell biology. 2013;14(1):49-55.
- 324 9. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-
325 Cas9 system. *Nature protocols*. 2013;8(11):2281-308.
- 326 10. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with
327 CRISPR-Cas9. *Science*. 2014;346(6213):1258096.
- 328 11. Shao Y, Guan Y, Wang L, Qiu Z, Liu M, Chen Y, et al. CRISPR/Cas-mediated genome editing in the
329 rat via direct injection of one-cell embryos. *Nature protocols*. 2014;9(10):2493-512.
- 330 12. Niemi J, Jones P, Tranter R, Heinola K. The economic significance of production diseases and their
331 control in pig farms. PROHEALTH conference on sustainable control of pig and poultry
332 production diseases. 2016;<http://www.fp7-prohealth.eu>.
- 333 13. Statistical Office of the European Communities (EUROSTAT). Agricultural production statistics.
334 Accessed 01/08/2017;<http://ec.europa.eu/eurostat/web/agriculture/data/main-tables#>.
- 335 14. Goddard ME, Hayes BJ. Mapping genes for complex traits in domestic animals and their use in
336 breeding programmes. *Nature reviews Genetics*. 2009;10(6):381-91.
- 337 15. Hermes S, Dominik S. Breeding disease resistant pigs. *Breeding Focus 2014 - Improving*
338 *Resilience*. 2014;Pork CRC., Animal Genetics and Breeding Unit, UNE.
- 339 16. Jenko J, Gorjanc G, Cleveland MA, Varshney RK, Whitelaw CB, Woolliams JA, et al. Potential of
340 promotion of alleles by genome editing to improve quantitative traits in livestock breeding
341 programs. *Genet Sel Evol*. 2015;47:55.
- 342 17. Luo J, Song Z, Yu S, Cui D, Wang B, Ding F, et al. Efficient generation of myostatin (MSTN) biallelic
343 mutations in cattle using zinc finger nucleases. *PLoS One*. 2014;9(4):e95225.
- 344 18. Yu S, Luo J, Song Z, Ding F, Dai Y, Li N. Highly efficient modification of beta-lactoglobulin (BLG)
345 gene via zinc-finger nucleases in cattle. *Cell Res*. 2011;21(11):1638-40.
- 346 19. Proudfoot C, Carlson DF, Huddart R, Long CR, Pryor JH, King TJ, et al. Genome edited sheep and
347 cattle. *Transgenic Res*. 2015;24(1):147-53.
- 348 20. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, et al. Transgenic bovine chimeric
349 offspring produced from somatic cell-derived stem-like cells. *Nat Biotechnol*. 1998;16(7):642-6.
- 350 21. Iwasaki S, Campbell KH, Galli C, Akiyama K. Production of live calves derived from embryonic
351 stem-like cells aggregated with tetraploid embryos. *Biol Reprod*. 2000;62(2):470-5.
- 352 22. Oatley JM. Spermatogonial stem cell biology in the bull: development of isolation, culture, and
353 transplantation methodologies and their potential impacts on cattle production. *Society of*
354 *Reproduction and Fertility supplement*. 2010;67:133-43.
- 355 23. Oatley MJ, Kaucher AV, Yang QE, Waqas MS, Oatley JM. Conditions for Long-Term Culture of
356 Cattle Undifferentiated Spermatogonia. *Biology of reproduction*. 2016;95(1):14.

24. Baldassarre H, Wang B, Kafidi N, Gauthier M, Neveu N, Lapointe J, et al. Production of transgenic goats by pronuclear microinjection of in vitro produced zygotes derived from oocytes recovered by laparoscopy. *Theriogenology*. 2003;59(3-4):831-9.
25. Keefer CL, Baldassarre H, Keyston R, Wang B, Bhatia B, Bilodeau AS, et al. Generation of dwarf goat (*Capra hircus*) clones following nuclear transfer with transfected and nontransfected fetal fibroblasts and in vitro-matured oocytes. *Biology of reproduction*. 2001;64(3):849-56.
26. Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, et al. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science*. 1997;278(5346):2130-3.
27. Baldassarre H, Wang B, Keefer CL, Lazaris A, Karatzas CN. State of the art in the production of transgenic goats. *Reprod Fertil Dev*. 2004;16(4):465-70.
28. Zhao Y, Yu M, Wang L, Li Y, Fan J, Yang Q, et al. Spontaneous uptake of exogenous DNA by goat spermatozoa and selection of donor bucks for sperm-mediated gene transfer. *Molecular biology reports*. 2012;39(3):2659-64.
29. Sartori C, DiDomenico AI, Thomson AJ, Milne E, Lillico SG, Burdon TG, et al. Ovine-induced pluripotent stem cells can contribute to chimeric lambs. *Cellular reprogramming*. 2012;14(1):8-19.
30. Ren J, Pak Y, He L, Qian L, Gu Y, Li H, et al. Generation of hircine-induced pluripotent stem cells by somatic cell reprogramming. *Cell Res*. 2011;21(5):849-53.
31. Sandmaier SE, Nandal A, Powell A, Garrett W, Blomberg L, Donovan DM, et al. Generation of induced pluripotent stem cells from domestic goats. *Mol Reprod Dev*. 2015;82(9):709-21.
32. Romar R, Funahashi H, Coy P. In vitro fertilization in pigs: New molecules and protocols to consider in the forthcoming years. *Theriogenology*. 2016;85(1):125-34.
33. Tan W, Proudfoot C, Lillico SG, Whitelaw CB. Gene targeting, genome editing: from Dolly to editors. *Transgenic Res*. 2016;25(3):273-87.
34. Tan WS, Carlson DF, Walton MW, Fahrenkrug SC, Hackett PB. Precision editing of large animal genomes. *Advances in genetics*. 2012;80:37-97.
35. West FD, Terlouw SL, Kwon DJ, Mumaw JL, Dhara SK, Hasneen K, et al. Porcine induced pluripotent stem cells produce chimeric offspring. *Stem cells and development*. 2010;19(8):1211-20.
36. West FD, Uhl EW, Liu Y, Stowe H, Lu Y, Yu P, et al. Brief report: chimeric pigs produced from induced pluripotent stem cells demonstrate germline transmission and no evidence of tumor formation in young pigs. *Stem cells*. 2011;29(10):1640-3.
37. Zheng Y, Zhang Y, Qu R, He Y, Tian X, Zeng W. Spermatogonial stem cells from domestic animals: progress and prospects. *Reproduction*. 2014;147(3):R65-74.
38. Park KE, Kaucher AV, Powell A, Waqas MS, Sandmaier SE, Oatley MJ, et al. Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. *Sci Rep*. 2017;7:40176.
39. van de Lavoie MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, et al. Germline transmission of genetically modified primordial germ cells. *Nature*. 2006;441(7094):766-9.
40. Dimitrov L, Pedersen D, Ching KH, Yi H, Collarini EJ, Izquierdo S, et al. Germline Gene Editing in Chickens by Efficient CRISPR-Mediated Homologous Recombination in Primordial Germ Cells. *PLoS One*. 2016;11(4):e0154303.
41. Macdonald J, Glover JD, Taylor L, Sang HM, McGrew MJ. Characterisation and germline transmission of cultured avian primordial germ cells. *PLoS One*. 2010;5(11):e15518.
42. Tyack SG, Jenkins KA, O'Neil TE, Wise TG, Morris KR, Bruce MP, et al. A new method for producing transgenic birds via direct in vivo transfection of primordial germ cells. *Transgenic Res*. 2013;22(6):1257-64.
43. Gjedrem T, Robinson N. Advances by Selective Breeding for Aquatic Species: A Review. *Agricultural Sciences*. 2014;Vol.05No.12:7.
44. Du SJ, Gong ZY, Fletcher GL, Shears MA, King MJ, Idler DR, et al. Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. *Biotechnology (N Y)*. 1992;10(2):176-81.

- 410 45. Rahman A, Maclean N. Production of lines of growth enhanced transgenic tilapia (*Oreochromis*
411 *niloticus*) expressing a novel piscine growth hormone gene. *New Developments in Marine*
412 *Biotechnology*. 1998;19-28.
- 413 46. Edvardsen RB, Leininger S, Kleppe L, Skaftnesmo KO, Wargelius A. Targeted mutagenesis in
414 Atlantic salmon (*Salmo salar* L.) using the CRISPR/Cas9 system induces complete knockout
415 individuals in the F0 generation. *PLoS One*. 2014;9(9):e108622.
- 416 47. Wargelius A, Leininger S, Skaftnesmo KO, Kleppe L, Andersson E, Taranger GL, et al. Dnd
417 knockout ablates germ cells and demonstrates germ cell independent sex differentiation in
418 Atlantic salmon. *Sci Rep*. 2016;6:21284.
- 419 48. Khalil K, Elayat M, Khalifa E, Daghash S, Elasad A, Miller M, et al. Generation of Myostatin Gene-
420 Edited Channel Catfish (*Ictalurus punctatus*) via Zygote Injection of CRISPR/Cas9 System. *Sci*
421 *Rep*. 2017;7(1):7301.
- 422 49. Houston RD, Gheyas A, Hamilton A, Guy DR, Tinch AE, Taggart JB, et al. Detection and
423 confirmation of a major QTL affecting resistance to infectious pancreatic necrosis (IPN) in
424 Atlantic salmon (*Salmo salar*). *Dev Biol (Basel)*. 2008;132:199-204.
- 425 50. Great Britain. National Audit Office. Department for Environment, Food, and Rural Affairs : The
426 health of livestock and honeybees in England. London: The Stationery Office; 2009. 54 p. p.
- 427 51. Calderone NW. Insect pollinated crops, insect pollinators and US agriculture: trend analysis of
428 aggregate data for the period 1992-2009. *PLoS One*. 2012;7(5):e37235.
- 429 52. Fries I, Lindström A. Breeding disease resistant honeybees. Swedish University of Agricultural
430 Sciences. 2010.
- 431 53. Spivak M, Reuter GS. Varroa destructor infestation in untreated honey bee (Hymenoptera:
432 Apidae) colonies selected for hygienic behavior. *J Econ Entomol*. 2001;94(2):326-31.
- 433 54. Schulte C, Theilenberg E, Muller-Borg M, Gempe T, Beye M. Highly efficient integration and
434 expression of piggyBac-derived cassettes in the honeybee (*Apis mellifera*). *Proc Natl Acad Sci U*
435 *S A*. 2014;111(24):9003-8.
- 436 55. Kohno H, Suenami S, Takeuchi H, Sasaki T, Kubo T. Production of Knockout Mutants by
437 CRISPR/Cas9 in the European Honeybee, *Apis mellifera* L. *Zoolog Sci*. 2016;33(5):505-12.
- 438 56. McAfee A, Harpur BA, Michaud S, Beavis RC, Kent CF, Zayed A, et al. Toward an Upgraded Honey
439 Bee (*Apis mellifera* L.) Genome Annotation Using Proteogenomics. *J Proteome Res*.
440 2016;15(2):411-21.
- 441 57. Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, et al. Genetically enhanced
442 cows resist intramammary *Staphylococcus aureus* infection. *Nat Biotechnol*. 2005;23(4):445-
443 51.
- 444 58. Maga EA, Sargent RG, Zeng H, Pati S, Zarling DA, Oppenheim SM, et al. Increased efficiency of
445 transgenic livestock production. *Transgenic Res*. 2003;12(4):485-96.
- 446 59. Maga EA, Cullor JS, Smith W, Anderson GB, Murray JD. Human lysozyme expressed in the
447 mammary gland of transgenic dairy goats can inhibit the growth of bacteria that cause mastitis
448 and the cold-spoilage of milk. *Foodborne pathogens and disease*. 2006;3(4):384-92.
- 449 60. Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T, Vargas F, et al. Production of cattle
450 lacking prion protein. *Nat Biotechnol*. 2007;25(1):132-8.
- 451 61. Denning C, Burl S, Ainslie A, Bracken J, Dinnyes A, Fletcher J, et al. Deletion of the
452 alpha(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. *Nat*
453 *Biotechnol*. 2001;19(6):559-62.
- 454 62. Yu G, Chen J, Yu H, Liu S, Chen J, Xu X, et al. Functional disruption of the prion protein gene in
455 cloned goats. *The Journal of general virology*. 2006;87(Pt 4):1019-27.
- 456 63. Yu G, Chen J, Xu Y, Zhu C, Yu H, Liu S, et al. Generation of goats lacking prion protein. *Molecular*
457 *reproduction and development*. 2009;76(1):3.
- 458 64. Shanthalingam S, Srikumaran S. Intact signal peptide of CD18, the beta-subunit of beta2-
459 integrins, renders ruminants susceptible to *Mannheimia haemolytica* leukotoxin. *Proc Natl Acad*
460 *Sci U S A*. 2009;106(36):15448-53.
- 461 65. Shanthalingam S, Tibary A, Beever JE, Kasinathan P, Brown WC, Srikumaran S. Precise gene
462 editing paves the way for derivation of *Mannheimia haemolytica* leukotoxin-resistant cattle.
463 *Proc Natl Acad Sci U S A*. 2016;113(46):13186-90.

464 66. Cheng X, Wang H. Multiple targeting motifs direct NRAMP1 into lysosomes. *Biochem Biophys*
465 *Res Commun.* 2012;419(3):578-83.

466 67. Gao Y, Wu H, Wang Y, Liu X, Chen L, Li Q, et al. Single Cas9 nickase induced generation of NRAMP1
467 knockin cattle with reduced off-target effects. *Genome biology.* 2017;18(1):13.

468 68. Palgrave CJ, Gilmour L, Lowden CS, Lillico SG, Mellencamp MA, Whitelaw CB. Species-specific
469 variation in RELA underlies differences in NF-kappaB activity: a potential role in African swine
470 fever pathogenesis. *Journal of virology.* 2011;85(12):6008-14.

471 69. Lillico SG, Proudfoot C, King TJ, Tan W, Zhang L, Mardjuki R, et al. Mammalian interspecies
472 substitution of immune modulatory alleles by genome editing. *Sci Rep.* 2016;6:21645.

473 70. Albers GA, Gray GD, Piper LR, Barker JS, Le Jambre LF, Barger IA. The genetics of resistance and
474 resilience to *Haemonchus contortus* infection in young merino sheep. *Int J Parasitol.*
475 1987;17(7):1355-63.

476 71. Van Gorp H, Van Breedam W, Delputte PL, Nauwynck HJ. Sialoadhesin and CD163 join forces
477 during entry of the porcine reproductive and respiratory syndrome virus. *The Journal of general*
478 *virology.* 2008;89(Pt 12):2943-53.

479 72. Van Gorp H, Van Breedam W, Van Doorselaere J, Delputte PL, Nauwynck HJ. Identification of the
480 CD163 protein domains involved in infection of the porcine reproductive and respiratory
481 syndrome virus. *Journal of virology.* 2010;84(6):3101-5.

482 73. Prather RS, Rowland RR, Ewen C, Tribble B, Kerrigan M, Bawa B, et al. An intact sialoadhesin
483 (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine
484 reproductive and respiratory syndrome virus. *Journal of virology.* 2013;87(17):9538-46.

485 74. Whitworth KM, Lee K, Benne JA, Beaton BP, Spate LD, Murphy SL, et al. Use of the CRISPR/Cas9
486 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos.
487 *Biology of reproduction.* 2014;91(3):78.

488 75. Whitworth KM, Rowland RR, Ewen CL, Tribble BR, Kerrigan MA, Cino-Ozuna AG, et al. Gene-edited
489 pigs are protected from porcine reproductive and respiratory syndrome virus. *Nat Biotechnol.*
490 2016;34(1):20-2.

491 76. Burkard C, Lillico SG, Reid E, Jackson B, Mileham AJ, Ait-Ali T, et al. Precision engineering for
492 PRRSV resistance in pigs: Macrophages from genome edited pigs lacking CD163 SRCR5 domain
493 are fully resistant to both PRRSV genotypes while maintaining biological function. *PLoS*
494 *pathogens.* 2017;13(2):e1006206.

495 77. Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, et al. Inactivation of porcine endogenous
496 retrovirus in pigs using CRISPR-Cas9. *Science.* 2017.

497 78. Lyall J, Irvine RM, Sherman A, McKinley TJ, Nunez A, Purdie A, et al. Suppression of avian
498 influenza transmission in genetically modified chickens. *Science.* 2011;331(6014):223-6.

499

501

502 **Figure 1: Genome editing tools.** By transfection of plasmids, transposons or DNA fragments and
503 random or homology-directed integration foreign DNA fragments or modifications may be introduced
504 into a target genome. Retroviruses or single-round infectious retroviruses may be used to integrate
505 foreign genes at random sites into the target genome. Homing endonucleases, zinc finger nucleases
506 (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced
507 palindromic repeats (CRISPR)/Cas9 are genome editors relying on enzymatic activity to introduce a
508 targeted double strand break in the genome. Repair of these double strand breaks can lead to non-
509 homologous end joining events, resulting in insertion or deletions. A combination of genome editors
510 with DNA fragments, plasmids or transposons can be used to enhance the efficiency of homology-
511 directed repair to integrate foreign genes or modify single base pairs at a specific locus in the genome.

Figure 2: Techniques to generate genome-manipulated animals. Using genome editing tools a variety of techniques are available for the generation of genome edited animals. Sperm-mediated gene transfer (SMGT) is used for the delivery of genome editing reagents to the zygote. Furthermore, spermatogonial stem cell (SSC) or primordial germ cell (PGC) manipulation offer new avenues to generate edited animals. Pronuclear and cytoplasmic injection of genome editors into zygotes and subsequent transfer to surrogates or maturation in incubators prior to transfer may be used to generate genome-manipulated animals. Somatic cell nuclear transfer allows the selection of specific edits in somatic cells prior to nuclear transfer to surrogates or maturation in incubators. The injection of edited embryonic or induced pluripotent stem cells into blastocysts can also be used to generate chimeric edited animals.

524

525 **Figure 3: Research paths to identify editing targets for disease resistance.** Identification of
 526 sequence variation associated with susceptibility to disease state allows for the identification of
 527 potential editing targets. Genome-wide association studies (GWAS) allow high throughput comparisons
 528 between large numbers of animals of the same species. Hypothesis-driven analysis of specific gene
 529 sequences can be used to identify species variance associated with disease resistance. In a laboratory
 530 setting *in vitro* host-pathogen interaction studies can be used to identify pathways amenable to
 531 interventions. Genome-wide screening methods, such as small-interfering RNA (siRNA) or genome-
 532 scale CRISPR knock-out (GeCKO), allow for the identification of a large number of proteins required for
 533 pathogen replication. Hypothesis-driven strategies rely on a prior knowledge of specific host-pathogen
 534 interactions.