Regulation and safety considerations of somatic cell nuclear transfer-cloned farm animals and their offspring used for food production

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Revised

REGULATION AND SAFETY CONSIDERATIONS OF SOMATIC CELL NUCLEAR TRANSFER-CLONED FARM ANIMALS AND THEIR OFFSPRING USED FOR FOOD PRODUCTION

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Abstract

This document discusses recent developments in cloning of husbandry animals through somatic cell nuclear transfer, particularly with a view on improvements in their efficacy. Commercial developments in North and South America, Australia-New Zealand, and China are noted. The regulations and safety aspects surrounding the use of clones and their offspring for the purpose of food production are discussed. It is generally considered that foods from offspring of clones are no different than similar foods from conventional animals, yet besides safety, also ethical and animal welfare considerations come into play at the policy level. The related topic of detection and traceability of clones is discussed, which covers both molecular and documentary methods.

Key Words: animal cloning, animal husbandry, food safety, regulation, risk assessment, somatic cell nuclear transfer, traceability

1. Introduction

Animal husbandry has seen significant developments in the last several decades. Especially during the second half of the 20th century, producers increasingly used selective breeding techniques for the creation of livestock (cross)breeds to increase production yields. Selective breeding aims to generate breeds with beneficial traits, for instance animals with increased muscle content, through inbreeding (breeding of genetically closely related individuals), linebreeding (breeding of individuals of the same breed) or crossbreeding (breeding of individuals of differing breeds). Advances in animal breeding technologies, such as artificial insemination, and genetics have increased both breeding efficiency as well as our understanding of the inheritance of genetic traits [1,2]. A downside of selective breeding is a decrease in genetic diversity in the resulting livestock due to inbreeding, which may have a negative effect on other traits, such as disease resistance, and may cause genetic disorders [3]. Traditional selective breeding strategies did, however, result in "elite" livestock breeds, such as Holstein Friesian-type cattle that produce large quantities of milk, the popular Aberdeen Angus beef cattle and the Yorkshire pigs, one of the most numerous pig breeds worldwide [4].

Since the 1940s, techniques such as artificial insemination and later embryo transfer have frequently been used to ensure that offspring is regularly produced as well as to improve herd genetics. As a relatively new technique serving the same purpose, cloning through transfer of a cell nucleus into an enucleated oocyte has been used experimentally in various animal species since the 1980s and has recently entered commercial practice in some countries. It is important to note that, in the following, "cloning" refers solely to somatic cell nucleus transfer (SCNT) for the purpose of animal cloning, hence this does not encompass other cloning methods such as the splitting of embryos or molecular cloning of DNA. Cloning by SCNT is also an essential step of procedures to permanently genetically modify animals, either with recombinant DNA containing foreign genes ("transgenes") or with mutations introduced with precise genome editing techniques.

Given the rapid pace in recent developments, this review will therefore explore the developments in the cloning of agricultural livestock, the application of genetic engineering and gene editing in livestock as well as the current regulation and potential safety issues of cloned livestock and the potential release on the market of cloned livestock products.

2. Cloning of livestock animals through SCNT

As early as in 1984, Steen Malte Willadsen at the Institute of Animal Physiology in Cambridge cloned the first mammal, a sheep, using nucleus transfer with embryonic cells [5]. In 1996, researchers of the group of Ian Wilmut used somatic cells for the nucleus transfer, instead of embryonic cells, to clone the famous sheep Dolly [6].

2.1. Challenges for successful cloning through SCNT

It became apparent that cloning of animals via somatic cell nuclear transfer (SCNT) is an inefficient process. Of 277 sheep cloning attempts, Dolly was the only lamb that survived to adulthood, which corresponds to a success rate of 0.4% [7]. Upon transfer of a somatic cell nucleus into an enucleated oocyte, the somatic cell

genome will get reprogrammed by dedicated machinery present in the oocyte cytoplasm. This may result in reprogramming to the so-called totipotent state that is similar to a fertilized oocyte, however this process is prone to errors and often fails [8]. Also technically, the process of successfully performing nucleus transfer and embryo reconstruction is demanding and difficult. Developments such as zona-free cloning methods, including handmade cloning, have increased the throughput of the cloning procedure [9–12]. Zona-free cloning techniques are based on the same principles as SCNT, however the nucleus transfer is performed using zona-free oocytes. The zona pellucida is a protective glycoprotein layer surrounding the oocyte, which is generally left intact during regular SCNT procedures. These cloning techniques eliminate the need to use micromanipulators during the nucleus transfer procedure and studies using handmade cloning reported that this method improves *in vitro* embryo development [9,13]. Despite advances made in cloning technology, SCNT and handmade cloning increase the risk of fetal and placental abnormalities as well as welfare concerns for the surrogate dam because of frequent miscarriage, difficult births and neonatal death. Abnormalities observed after SCNT include prolonged gestation, higher post-natal mortality rates and growth defects [14,15]. These anomalies, however, are not specific only to animal cloning, they are also observed after artificial reproduction methods using *in vitro* produced (IVP) embryos, albeit at lower frequencies [16]. It is important to note that the application of IVP and SCNT instead of natural mating increased the occurrence of congenital anomalies significantly [17,18]. For instance, the abnormal phenotype hydrops allantois (hydroallantois), a condition that leads to the excessive accumulation of fluid in the allantoic space, is seen sporadically in cattle with approximately 1 in 7,500 (0.01%) pregnancies affected [19]. In IVP pregnancies the occurrence of hydroallantois is significantly higher with approximately 1 case in 200 (0.5%) pregnancies affected [19]. In comparison, the prevalence of hydroallantois after somatic cloning is increases to approximately 12-15% [20,21]. Hydrops allantois is a severe condition and often results in death of the calf or the recipient dam. This poor prognosis for both the calf and the dam necessitates induced parturition or abortion, both of which are a severe burden on animal welfare [22]. Health and welfare issues such as these may raise ethical concerns underlying the decisions of governments to put in place strict regulations on the use of animal cloning technologies.

It is believed that problems with cellular reprogramming and epigenetic mechanisms contribute to congenital anomalies after SCNT. Cellular programming occurs when the donor nucleus is transferred to the recipient oocyte, however this reprogramming is often incomplete or inefficient, resulting in incorrect epigenetic modalities such as in histone deacetylation and DNA methylation [23,24]. These factors might lead to abnormalities in embryo development and cause adverse effects for the surrogate dam, though the exact cause of these anomalies has yet to be elucidated. Animal cloning has the potential to benefit animal husbandry by offering a way to multiply top-producing animals. Farmers have the possibility to preserve genetic material of elite animal breeds and this material may then be used to create clones. Care should be taken to avoid animal health and welfare-related problems of cloning which may occur in the founder generation but are generally absent in clone progeny [25,26]. In addition, healthy clones of top-producing animals may be selected for use as founders in conventional breeding programs to expand the population of top-producers.

2.2. SCNT, transgenesis, and genome editing

Somatic cell nuclear transfer has created the potential to more easily generate transgenic animals. Genetic modification of animals has, before the development of SCNT, been achieved via the microinjection of genetic material, i.e. a transgene, into the pronuclei of zygotic cells. The microinjection technique is, however, a rather inefficient procedure and the integration of DNA via microinjection results in mosaicism with the resulting organisms containing cells with different genotypes – with or without the transgene [7]. SCNT has the benefit that donor nuclei can be used with specific genetic modifications for the creation of cloned animals. These donor nuclei can either be derived from transgenic animals with the relevant genetic change or from cultured donor cell lines harboring the genetic modification [7]. Donor cells can be analyzed for their genetic make-up, to ensure that the appropriate genetic modification is present before using the cells in the cloning procedure. This screening of donor cells is an additional safeguard to ascertain the surrogate mothers are implanted only with the correct genetically modified embryos.

Different techniques can be used to confer genetic changes in agricultural livestock. The aforementioned microinjection technique is a classical method that has been used extensively to create transgenic organisms. Recently, more targeted approaches have been developed and employed to specifically modify an organisms' genetic content. Targeted genetic modification, contrary to recombinant-DNA techniques using DNA elements such as plasmids and transposons, starts with the creation of single-strand nicks or double-strand breaks in the hosts' DNA. Following the generation of double-strand breaks, DNA repair mechanisms will be activated to mend the gap, either by non-homologous end joining or homologous recombination. Molecular scissors have been developed to cut DNA in a precise manner and with high efficiency. The molecular scissors used for targeted genetic engineering of organisms comprise site-directed nucleases (SDN), including zinc finger nucleases (ZFN), transcription activator-like endonucleases (TALEN) and clustered regularly interspaced short palindromic repeats with associated protein 9 (CRISPR-Cas9). These SDNs can be designed to specifically cleave the DNA at desired locations and, following the creation of a double-strand break, endogenous DNA repair mechanisms can be exploited to introduce genetic modifications. DNA repair of double-strand breaks may have different outcomes ranging from small insertions or deletions of a single basepair and potentially deletions or insertions of large stretches of DNA depending, amongst others, on the amount of breaks made and the presence of DNA inserts containing sequences homologous to the regions flanking the double-strand break [27]. These novel technologies allow for the creation of precise genetic changes in livestock animals and may be used to re-create naturally occurring genetic variants. Furthermore, precise gene edits are indistinguishable from natural mutations and these technologies do not rely on random integration of recombinant DNA.

Precise editing of the genome of farm animals has the potential to enhance beneficial traits, such as improved disease resistance, increased muscle tissue and faster growth, but also to suppress certain traits, such as in cattle without horns [28]. Combining the power of these novel gene editing technologies with optimized cloning techniques allows for faster development of genetically enhanced livestock breeds, compared to

traditional crossbreeding. However, not much information is available on the potential negative side effects of off-target gene editing, related to the application of these technologies, on animal health and welfare.

3. Developments in livestock cloning

Somatic cell nuclear transfer has significantly been improved since its first successful application in the 1980s. Strategies have been developed to improve the efficiency of the SCNT procedure, for instance embryo aggregation to stimulate cell-cell interactions [29,30] and the utilization of histone deacetylase inhibitors to improve reprogramming [31]. Other potential strategies include, amongst others, antioxidant treatment to protect embryos from reactive oxygen species stress [32] as well as selection of embryos with normal chromosomal segregation [33]. However the beneficial effects conveyed by the latter two methods on cloning efficiency have primarily been observed *in vitro*. Precautionary measures are taken to prevent the transmission of diseases during the SCNT procedure, for instance contamination with viruses (e.g. bovine viral diarrhea virus, porcine reproductive and respiratory syndrome virus [34–36]. Abnormalities, in particular large offspring syndrome, often observed after artificial reproduction techniques that employ embryos from in vitro fertilized oocytes have largely been abolished due to improved methods such as serum-free embryo culturing conditions [37]. However, fetal and placental anomalies are often observed despite improvements in SCNT, and these problems might be caused, in part, by errors in epigenetic modifications that persist throughout the SCNT procedure [16]. The deficiencies in placental development might contribute to the high amount of failed SCNT pregnancies and high postpartum mortality rate [38–41]. Success rates of SCNT remain low, recent studies in goats have shown just 2.9% SCNT efficiency (calculated as live offspring/reconstructed embryo transferred) [42] and studies by Ao et al. revealed that 65% of live-born SCNT-derived piglets died within 4 days [38]. The efficiency of SCNT depends on the livestock species, for instance in cattle SCNT seems to be more efficient than in other mammals, as well as on attempts to perform genetic engineering of donor cells used for the reconstructed embryos, which have a negative effect on cloning efficiency [43]. Contrary to genetic engineering in somatic donor cells used for nucleus transfer, genome editing of embryos using site-directed nucleases such as CRISPR-Cas9 is more precise and efficient and avoids the cellular reprogramming problems associated with SCNT [43,44].

Despite the progress made in the SCNT technique only a small percentage of the reconstructed embryos develop into live offspring (1-5%) [45,46]. Kent-First et al, report that besides inefficient nuclear reprogramming, other factors such as inefficient synchronization of donor cells to the correct cell cycle, high attrition rate of late term pregnancies and a compromise to the health of neonatal calves contribute to the low cloning efficiency [46]. A survey on SCNT cloned cattle investigated the death loss of over 500 cloned cattle and their progeny [47]. Their results show that death loss to fetal death in SCNT calves was 16.4% compared to 8.9 and 4.6% for clone progeny and conventionally bred cattle, respectively. Furthermore, neonatal death within 24h after birth for SCNT calves was 14.4% compared to 0.8 and 1.9% for clone progeny and conventionally bred cattle, respectively. However, death loss due to diseases, such as respiratory problems, up to 200 days after birth, were not significantly different from conventionally bred cattle. The findings of this study show that SCNT cattle have a higher chance of fetal death and the compromised health of the calves

might result in neonatal death, however SCNT clone progeny have a death loss comparable to conventionally bred cattle. These findings are in accordance with similar studies on the health of clones and their progeny [48,49].

A report on the developments in animal cloning by the European Food Safety Authority (EFSA) found that, while there have been improvements in cloning techniques, as stated before the health and welfare of surrogate dams and clones are often adversely affected by the cloning process [36]. Improvements in cloning procedures have contributed to an increased cloning efficiency in mice, however SCNT efficiency in livestock cloning has remained low [45]. Despite the fact that advances in cloning efficiency have somewhat reduced the occurrence of various abnormalities, as described above, not all the factors contributing to these anomalies have been elucidated, meaning that health and welfare issues still persist with the cloning procedure.

3.1. Commercial livestock cloning

As aforementioned, the cloning of top-producing livestock has potential to ensure that farmers have a way to improve herd genetics and generate more elite farm animals. For farmers it may also be economically beneficial to pursue animal cloning, since a clone will double a farmer's production and the fees for cryopreservation and cloning of elite breeds are economically attractive and might lower in cost when the cloning technology gets improved upon and becomes more readily available. Nowadays, several companies have started to specialize in the cloning of farm animals and the cryopreservation of genetic material. Examples include the Australian companies Reinclonation and Clone International, the US based Viagen / Trans Ova Genetics and Pregenex, as well as Biosidus, Nuevo Milenium (particularly its Germinal Biotech laboratory), Goyaike in Argentina and Geneal, In Vitro Brasil, Clonagem Animal, and Vitrogen YVF Biotech in Brazil. Furthermore, Chinese companies and institutes such as Boyalife Genomics and BGI Ark Biotechnology [50] have set up cloning factories to provide cloning services to improve agricultural yields as well as to perform research on transgenic farm animals.

The cloning procedures employed by these companies are as follows: i) somatic cells are harvested from the donor animal, generally epidermal cells from an ear notch biopsy, ii) epidermal cells are cultured, cultured cells may be stored in liquid nitrogen for cryopreservation, iii) nuclei from the somatic cells will be isolated and fused with enucleated oocytes, using traditional SCNT or HMC technology, iv) reconstructed embryos are cultured till the blastocyst stage and transferred to a recipient surrogate. Subsequently, the clone will be carried to term and delivered. The resulting cloned animals can then be used for further breeding to enhance herd genetics. Cloning factories in China primarily focus on the mass production of cloned non-modified animals for agriculture as well as on transgenic animals with enhanced traits (Sullivan & Liu, 2015, p44-45 [51]). As of this writing, globally no transgenic mammalian animals have been approved for commercialization for food production. Recently, Recombinetics Inc. succeeded in gene editing an Angus cattle cell line with the *SLICK* genotype [52] and subsequently used this cell line for cloning of a thermotolerant Angus calf. Gene edited cattle with a *SLICK* phenotype [53] have less dense "slick" hair as well as an increased rate of thermal sweating and therefore a higher thermotolerance in tropical conditions [54]. Whilst there is an increasing demand for the popular tender Angus beef in Brazil, Angus cattle normally do not thrive in hot climates. Reports are that

the gene edited Angus calf is healthy and performing well in the tropical environment. Since genome edited cows are regarded as conventional animals under Normative Resolution #16 in Brazil, as previously was the case for hornless cattle [55], *SLICK* Angus is a likely candidate for commercialization in the near future for food production depending on a case-by-case assessment by the National Biosafety Technical Commission (CTNBio).

4. Safety evaluation of cloned livestock food products

Both the US Food and Drug Administration (FDA) and EFSA have performed comprehensive safety assessments on food products from cloned animals. When the application of animal cloning for the breeding of livestock became more common in the early 2000s, the FDA requested producers to keep food products from clones off the market and conducted an evaluation on the safety of food products from cloned animals and the risk to animal health and welfare. A final risk assessment was issued by the FDA in 2008 [56], investigating in detail cloning procedures, epigenetic reprogramming in clones and their progeny, animal health risks as well as food consumption risks. Similarly, EFSA performed risk assessment on animal cloning, following a request by the European Commission. In 2008, EFSA issued their opinion on food safety, animal health & welfare and the environment providing scientific advice for the EC on this matter [57]. In 2009 and 2010 EFSA made further statements on animal cloning that was in accordance with their 2008 opinion [58,59] and in 2012 EFSA issued an update on the risk assessments and the developments in animal cloning [36]. Both evaluations, by FDA and EFSA, came to the conclusion that, in terms of food safety, there is no difference in food products derived from animal clones and their progeny compared to conventionally bred animals. Research results have shown that food products from cloned cattle and pigs and their progeny are not different in composition compared to reference meat and milk, although only limited data is available for other farmed species such as goats, sheep and horses [36]. Furthermore, animal clones are most likely going to be used for breeding purposes and food products from clones will therefore probably only sporadically enter the market.

Recently, a literature survey was performed that covered risk assessment studies from the last decade on the safety of cloned animal products [60]. Numerous reports were analyzed, including from the USA and EU, which studied the composition of meat and milk, quality parameters, genotoxicity and allergic reactions to meat and milk from cloned animals and progeny, as well as reproduction and adverse breeding effects. No evidence was found that meat or milk derived from cloned animals and their progeny is different from meat and milk of their nonclone counterparts. Furthermore, the numerous studies analyzed in this survey showed no evidence that meat and milk from cloned animals pose a food safety risk, which is in accordance with the evaluations from FDA and EFSA.

It is important to note that for production of biopharmaceuticals such as monoclonal antibodies, vaccines and hormones, the use of transgenic, cloned animals nowadays is common practice [61,62]. The production of antithrombin alfa by transgenic goats [63], for instance, has been approved for usage as a drug by the US FDA (FDA biologics license 125284). Transgenic animals that are used for the production of biopharmaceuticals in the US fall under the regulation of new animal drugs, act FFDCA (21 U.S.C. 321 et seq.), and these animals are strictly segregated from animals for food production. Food products from these genetically engineered animals

are prohibited from entering the food or feed supply without prior FDA authorization. Similarly, there has been no approval yet in the EU for introduction of food products from transgenic animals into the food supply, whereas several biological medicines that have been produced by transgenic animals in the US have been approved for therapeutic uses in the EU. Examples of these biopharmaceuticals are sebelipase alfa, which is expressed in transgenic chickens and purified from egg white [64], and antithrombin alfa, which is expressed in transgenic goats and purified from goat milk [65]. The regulation of cloned animals as well as food and feed products from animal clones will be further explained below.

5. Regulation of livestock cloning

Animal cloning is a form of non-traditional breeding that may have a serious effect on animal welfare, because of health anomalies in the animal clone and adverse health risks for the surrogate dam. Because of this, animal cloning may be subject to stringent regulations. The regulation of animal cloning for agricultural purposes in the USA, the EU, and important meat exporting countries, such as Argentina and Brazil will be outlined here.

Regulation in the European Union

In the EU the placing on the market of food products derived from cloned animals is regulated under Regulation (EU) 2015/2283 for novel foods and novel food ingredients. Prior to the introduction of these amendments that also explicitly addressed products from cloned animals as “novel foods”, the European Commission had received advice from the European Group on Ethics in Science and New technologies (EGE) on the safety and ethical aspects of the cloning of animals through SCNT for food production. The EGE considered four general ethical concerns, also drawing on the outcomes of the previously cited findings of experts on health and welfare implications of cloning through SCNT for EFSA [57], namely [66]:

- Concerns for the cloned animals themselves, such as welfare, health, “integrity”, and their use for man’s benefit. More specifically, the EGE’s opinion referred to five rights defined by the World Organization for Animal Health, namely freedom from hunger, thirst, and malnutrition; from fear and distress; from discomfort (thermal, physical); and from suffering (pain, injury, disease), as well as the ability to express normal animal behavior. It was recognized that animals have to be regarded as “sentinel beings”. Any constraints on these criteria would need to be justified with important benefits. The group also recognized that cloning could help to maintain rare breeds of farmed animal species, hence preserving farm animal biodiversity. The latter could help reduced or prevent the occurrence of inbreeding and widespread sensitivity towards animal diseases, for example.
- Concerns for humans, such as the safety of cloned animals for humans and also the possible extension of animal technology to misuse in human
- Environmental concerns, such as loss of biodiversity, biosafety issues, sustainability, and contamination
- Societal issues, such as desirability and acceptance, consumer information, intellectual property , industrialization of agriculture.

Considering these factors, the EGE expressed its doubts on whether the cloning of animals for food production would be justified due to the reported level of suffering and health issues of surrogate dams and clones. It recommended that a number of conditions be met before food products from cloned animals or their offspring could be marketed within the EU. These included guaranteed food safety of these products, fulfilment of animal rights, traceability, and also enforcement and verification of these requirements for imported products. It also advised to perform further research into the impacts of cloning on health and welfare of clones and their offspring as well as surveys of public perception, clarification of the status of cloning within EU legislation on patenting, the maintenance of animal breed diversity, ascertainment that consumer rights will be respected in case of international trade in products from cloned animals, and particularly the fostering of public participation in the discussions surrounding the cloning of farm animals [66].

"Novel Foods Regulation" [Regulation (EU) 2015/2283] specifies that food from animal clones has been obtained by non-traditional breeding practices and therefore falls under the scope of this Regulation until specific legislation regarding food from animal clones comes into force. The European Commission has presented proposals for regulations to its member states regarding animal cloning, including cloning techniques (somatic as well as embryonic cell nucleus transfer-mediated cloning methods) and the placing on the market of food from animal clones. Proposal "COM(2013) 892 final" covers the use of the cloning technique and proposes to provisionally prohibit the use of animal cloning for farm purposes because of the impact on health and welfare of farm animals. It should be noted that animal cloning for research purposes, medical purposes and the preservation of rare breeds and endangered species are not included in this directive. Furthermore, proposal "COM(2013) 893 final" covers the placing on the market of animal food products from clones and calls for a ban in the EU on placing on the market products derived from animal clones, based on ethics and animal welfare considerations. After debates in the European Parliament about the proposals on animal cloning, amendments were suggested to the proposed regulation. The EC responded to the suggested amendments and further clarified the scope of the ban on animal cloning, which does not include food from animal clone progeny and germinal products, such as semen, ova and embryos derived from animal clones [67]. However, germinal products, descendants, and food products from animal clone progeny may still require approval if the amendments proposed by the European Parliament for specific legislation of clones from cattle, goat, sheep, pigs and horses are to become adopted [68].

Regulation in non-EU countries

A concise overview of progress and regulations in various countries across the globe has been provided previously by [69]. Interestingly, a consortium of countries brought out a statement on the cloning of animals for the purpose of agricultural production, at a meeting in Buenos Aires in 2011 [70]. The signatories included the USA, Canada, Argentina, Brazil, Paraguay, and New Zealand. As major beef-producing and –trading countries [*e.g.* [71]], they had presented this statement to the World Trade Organization, indicating their willingness to litigate against any trade barrier imposed against these products. The intergovernmental statement features five points, stressing that: i) the regulation of clones should be science-based, ii) there has been no evidence showing that foods from progeny of clones would be less safe than from other animals, iii)

progeny of clones obtained through mating are not clones themselves, iv) bans and labelling requirements imposed on foods from such progeny could impact negatively on international trade, and v) any enforcement applied to such progeny would be disproportionate and illegitimate [70].

Regulation in the United States of America

After extensive risk assessments the FDA concluded in 2007 that meat and milk from cloned cows, pigs, goats and their progeny are as safe as food from conventionally bred farm animals [72]. As mentioned before, in 2008 the FDA issued a risk assessment on animal cloning [56] and guidance was released for industry to manage and reduce the risks associated with animal cloning. Because animal clones are primarily being used as breeding stock and meat & milk being safe for consumption, there is no special regulation for meat and milk from animal clones. Contrary to the EU, animal cloning is not prohibited in the US and the FDA has issued a risk management plan as well as guidance for the industry [72–74]. To address ethical considerations, such as the burden of the animal cloning procedure on animal health and welfare, the FDA worked with the International Embryo Transfer Society and issued a manual with animal care standards [75]. This manual contains guidance for cloning practitioners to reduce the frequency and impact of anomalies, such as the aforementioned hydroallantois, in the surrogate dam, fetus as well as newborn and juvenile clones.

Regulation in Argentina

Argentina, one of the signatories of the statement on the cloning of animals for the purpose of agricultural production of 2011, the National Food Safety and Quality Service (SENASA) published a document in 2012 on how to assess products derived from clones and their progeny for food use. It acknowledged that most of the clones of food-producing animals are generated for reproductive purposes, namely for further breeding and therefore are not intended to be sold as food. With the increasing number of clones of production animals being developed worldwide, the authors foresaw their potential entry into the food production chain. Following a concise review of foreign governments' regulatory approaches and scientific evidence on the safety of foods from clones and their progeny, it concluded that these are as safe and nutritious as from animals obtained through sexual reproduction techniques, and that therefore there was no reasonable motivation to regulate their commercialization [76,77].

In January 2018, the Argentinean government announced the adoption of a new decree implementing a law from 2007 aiming to promote the research, technology transfer and application of biotechnologies, stimulation of business activities through tax benefits, as well as the study of the impacts of modern biotechnology at the national level. Whilst the decree does not refer to specific biotechnological techniques, a news release from the Argentinean government mentions animal cloning as one of the fields in which Argentinean biotechnological enterprises have a leading role [78–80]. The Decree aligns with a public-private sectoral agreement on the development of biotechnology signed in 2017 [81].

Regulation in Brazil

In Brazil in 2001, "Vitória da EMBRAPA", a cow of the Simmental (Swiss "Fleckvieh") breed, became the first successfully cloned bovine of Latin America [82]. Eight years later, in 2009, the three-month old Zebu breed

calf Divisa Mata Velha TN 1 became the first to be entered as a clone into the Genealogical Registry of Zebu Breeds in 2009 [83]. Cloned animals will also be assessed for their suitability for reproduction and, for cows (females), as oocyte donors [83].

A bill introduced in Brazil in 2007 seeks to establish a law that requires the mandatory registration of activities involving the cloning of domestic animals used for zootechnical purposes. The activities covered by the proposal included all stages from research via animal production, import, and environmental release towards commercialization. Amongst its provisions, it was stated that those who introduce cloned animals into the human food production chain without permission were to be penalized. It was also foreseen that different authorities would have to handle different types of application of cloned animals. For example, the Ministry of Agriculture, Livestock, and Supply was to handle the registry of cloned production animals. Moreover, if animals were to be used for the production of pharmaceuticals, then this would come also under the authority of the National Agency of Health Surveillance (ANVISA) whilst clones that have also been genetically modified would need to be assessed as well by CTNBio [55,84]. After the proposal became a draft law in 2013, it has since been approved by the Committee on Technology, Communication and Informatics of the Chamber of Deputies, while still being discussed by the Committee for the Environment and Sustainable Development [85].

Regulation in other countries

In Australia and New Zealand, no specific requirement for pre-market approval exists for foods derived from cloned production animals and from their progeny. Food Standards Australia New Zealand, which oversees the food safety in both countries, reported in 2016 the existence of approximately 30-40 cloned cattle in Australia, used for breeding purposes but not for food. It also acknowledged that food products from their progeny probably occurred in the food supply [86].

In Canada, foods derived from cloned production animals obtained through somatic cell nuclear transfer, and from the progeny of these animals, are considered a “novel food”, under an interim policy defined in 2003 [87].

Whereas cloning of humans is expressly prohibited in PR China, various news reports and interviews (*e.g.* [88]) indicate that there is still a lack of regulation specifically for the cloning of animals. Moreover, PR China is global player in the field of animal biotechnology while the development of GM variants of various livestock species is proactively promoted through research funding by the Chinese government. No approvals for the commercial use of cloned and/or GM animals have been granted, though [89].

5.1. Cloned animal products on the market

Since animal cloning for food production breeding purposes is allowed in certain countries, such as in Argentina, Brazil and the US, products from cloned livestock might enter the European food supply, where animal cloning is prohibited. The EU for instance, where animal cloning for direct or indirect food production is currently banned, is a net-importer of beef, veal, sheep and goat meat [90], according to data from Eurostat. If indeed animal clone food products are imported, then these would be difficult to identify with current detection methods. When animal cloning becomes globally more prevalent the probability of animal clone-derived products entering the food chain in Europe will increase as well. Not only animal derived food products

386 are traded but also live animals and germinal products, such as semen, oocytes and embryos. Reports by the
387 European Commission have indicated that imports of reproductive material into the EU is limited but does
388 occur. For instance for cattle, reproductive material is imported but only limited amounts of this material is
389 intended for the reproduction of beef breeds [90]. Furthermore, porcine reproductive material, mainly semen,
390 is rarely imported because preferably fresh semen is used for artificial insemination [91]. As regards live animal
391 trading in general, an EU system is in place for the individual identification of live animals through the use of
392 double ear tags and/or electronic identification and certification on methods of production. The latter system
393 may allow the tracing of cloned animals, once identified. However, not all third countries use individual
394 identification and the background and parentage of imported livestock will generally not be known. EU
395 member states do not monitor if imported livestock and reproductive material have a cloned animal origin,
396 therefore it is not possible to assess whether products derived from cloned animals have entered the market.

397 ## 5.2. Detection and traceability of animal clones and clone derived products

398 With the possibility of cloned animal products appearing on the European market, where these products are
399 banned, it is necessary to detect and trace potential products with an animal clone origin. The composition and
400 quality of animal products, such as meat and milk, are indistinguishable from products derived from non-
401 cloned livestock. Therefore, scientists have investigated whether cloned animals can be detected in other ways.
402 It has been reported that incomplete reprogramming of the genetic content, including epigenetic modifications
403 and telomere length, of cloned animals contributes to abnormalities and influences aging [92]. These
404 incomplete reprogramming features, in particular telomere length, have been investigated as potential targets
405 for detection methods. Numerous studies, however, have shown that the telomere length of animal clones is
406 not always dissimilar to telomere lengths of control animals [93], which might depend on the donor cell type
407 used for the SCNT, age of the donor animal and cell culturing conditions. Furthermore, every study on telomere
408 length of animal clones indicates that animal clone offspring have telomere lengths that are similar to those
409 from their nonclone counterparts. The differing findings on telomere length of 1st generation animal clones
410 show that a detection method based on telomere length alone is unspecific, but might be used as an indication
411 for further characterization. Furthermore, epigenetic changes, such as DNA methylation and histone
412 modifications (e.g. acetylation and methylation), in embryos created through SCNT have been documented to
413 differ significantly from the control embryos [23,94]. Recent studies on bovine clones have been performed
414 that show aberrant methylation profiles of satellite loci of young fetuses, but not in individuals with a longer
415 lifespan [94]. Moreover, recent studies on SCNT cloned bulls reported normal DNA methylation [95]. Besides
416 nuclear DNA features, researchers have also investigated mitochondria of cloned animals. Contrary to nuclear
417 DNA, which is transmitted in a Mendelian fashion, mitochondrial DNA (mtDNA) is inherited solely from the
418 dam. During the SCNT procedure somatic cells are merged with oocytes via electrofusion, which might cause
419 the transfer of mtDNA to the oocyte. A study by Burgstaller et al reported the presence of mitochondrial
420 heteroplasmy in sheep clones, however not all clones exhibited heteroplasmy [96]. Of the sheep clones that
421 were positive of mitochondrial heteroplasmy, all but one of the sheep contained less than 1% mtDNA
422 originating from the nuclear donor cell. It remains to be elucidated whether molecular detection techniques
423 that measure telomere length, epigenetics and mitochondrial DNA heteroplasmy are robust enough for the

detection both in direct samples, such as meat, embryos and tissue from potential clones, as well as processed samples or samples where products from different individual animals have been pooled together (for instance milk from cloned and non-cloned cattle combined). Furthermore, a combination of molecular techniques for routine screening purposes is not cost-effective and will therefore probably not be an interesting and practical solution for regulators.

As regards traceability, documentation accompanying live animals and animal products, such as individual identification methods (animal passports, pedigree, certificates of origin, etc.) may aid regulators to define whether animal clone products enter the market, provided that the registration of cloned animals will be harmonized. Moreover, documentation and protocols stating the origins of ingredients used in processed food products will confer transparency about the contents of these products and whether ingredients from cloned livestock, once identified, have been added. Individual animal identification systems are already in place in EU member states for certain farm animals, such as cattle and horses, as well as in several third countries, however there is no compulsory system for ancestry recording in the EU. Current traceability systems in the EU do not allow products from a potential animal clone, such as meat and milk, to be traced back to an individual animal, but rather to a group of animals [90]. Some animals (e.g. pigs) and animal products are identified on a batch basis and this batch may contain animal products from several different farms, this allows the identification of these holdings that supplied the products but not specifically the identification of individual animals from which the product originated. The Brazilian national Zebu breed registry system described above would provide an example of how the cloned nature of an animal is highlighted and preserved with a uniform affix “TN” to the animal’s name, not only in the data records but also in the ear tattoo and brand applied to the animal’s skin [83].

Current identification systems for livestock do not always allow for the precise identification of the origin of animal products, depending on the identification method of animal species in their country of origin. However, in case of animal products that are suspected to be of an animal clone origin the utilization of improved detection methods, for instance on telomere length, mitochondrial heteroplasmy, epigenetics analysis, or a combination of these techniques may help regulators to determine if the product indeed has an animal clone origin. An initiative for the identification of cloned livestock was started in 2007, when the US-based Viagen and Trans Ova companies set up a Supply Chain Management Program. The program was run from 2008 until 2012 without further participation from competitor companies and without being accessed by industry throughout its lifetime [97].

6. Final Remarks

As discussed before, animal cloning has the potential to aid in the improvement of herd genetics and genetic material of top producing livestock may be cryopreserved for future cloning attempts. Advances in the SCNT methodology have increased its efficiency as well as reduced the prevalence of abnormalities associated with the SCNT technique. However, the adverse effects to health and welfare of clones and surrogate dams is still an issue and therefore, cloning is currently prohibited in the EU.

Earlier as well as more recent safety assessments have reported that food products derived from animal clones and their progeny are no different in composition and quality compared to products from their traditionally bred counterparts. It should be noted that cloned livestock are generally used for breeding purposes instead of meat production, it is therefore likely that food products from the original clones will only rarely enter the food chain. Food products from clone progeny, however, are more likely to find its way to consumers. Current regulation in the EU has placed a ban on food products from animal clones, given, amongst others ethical considerations regarding animal welfare. This ban does not cover products from their progeny, which are considered to be indistinguishable from traditionally bred livestock.

Current detection methods do not allow enforcement laboratories to reliably detect whether a food product originates from a cloned animal in a cost-efficient way. Enforcement therefore basically will have to rely on documentation control and general regulatory requirements for traceability in food and feed production chains. Individual animal identification is already common for cattle and horses, but for other animals, e.g. pigs and poultry, often only batch identification is being implemented. Batch identification allows identification of the holdings from which animal products originated. These systems may be of use once it is internationally agreed to exchange information on the clone nature of exported animals.

Future advances in animal cloning might reduce the risks of this technology to animal health and welfare, in which case governments might re-evaluate regulation.

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