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A journey through horse cloning

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Abstract. Interest in equine somatic cell nuclear transfer technology has increased significantly since the first equid clones were produced in 2003. This is demonstrated by the multiple commercial equine cloning companies having produced numerous cloned equids to date; worldwide, more than 370 cloned horses have been produced in at least six different countries. Equine cloning can be performed using several different approaches, each with different rates of success. In this review we cover the history and applications of equine cloning and summarise the major scientific advances in the development of this technology in horses. We explain the advantages and disadvantages of different procedures to produce cloned equine embryos and describe the current status of equine clone commercialisation, along with observations of differences in regional breed association registration regulations.

Additional keywords: embryo, equine, somatic cell nuclear transfer.

Introduction

Two decades have passed since 'Dolly', the first cloned mammal derived from an adult somatic cell, was born (Wilmut *et al.* 1997). However, the scientific community and general public are still in awe of cloning technology, which, in the years following Dolly, has been applied to most domestic mammals. Despite new achievements in the reprogramming and cloning area in recent years, we are still far from having a complete understanding of all the mechanisms behind the transformation of a single differentiated somatic cell into an entire organism. Particularly in the horse, the potential to preserve the tissue of an animal with valuable genetics has been shown to be of reproductive advantage when used to replicate exceptional geldings or individuals that die unexpectedly. This further allows the possibility for equine clones to be used in breeding sport horses, whereas they otherwise would not have a chance to contribute their genetics to future generations. In addition, cloning can partially help overcome the lack of an efficient superovulation protocol in mares by providing an increased number of embryos or foals from a desirable mare, born through her clones. This review summarises the main scientific achievements in the development of horse cloning technology and describes the present status of international commercialisation of horse cloning.

History of horse cloning

Relatively late compared with other domestic species, and 36 years after the first report of *in vitro* equine oocyte maturation (Fulka and Okolski 1981), the first equid clones produced by somatic cell nuclear transfer (SCNT) were born in 2003 (Galli *et al.* 2003; Woods *et al.* 2003). However, these were not the first reports of the artificial production of genetically identical foals. In the 1980s, genetically identical foals were produced by embryo splitting (Allen and Pashen 1984; McKinnon *et al.* 1989; Skidmore *et al.* 1989). The first equid cloned using SCNT was a mule (Fig. 1*a*) derived from a cell from a 45-day-old fetus created using *in vivo*-matured oocytes and immediate oviducal embryo transfer (Woods *et al.* 2003). In 2003, the group lead by Cesare Galli of the Laboratory of Reproductive Technologies in Cremona, Italy, reported the cloning of an adult horse (Fig. 1*b*) using a complete *in vitro* procedure from oocyte to blastocyst stage (Galli *et al.* 2003). The cloned offspring, named 'Prometea', was genetically identical to the mare that carried it, thereby challenging the idea that maternal immunological recognition of antigens derived from the fetus is necessary for a successful pregnancy (Szekeres-Bartho 2002). Achievements in equine cloning started to accelerate in 2005 after the group led by Katrin Hinrichs reported the first horse clone (Fig. 1*c*) produced in the

Fig. 1. First equine clones produced in different countries worldwide. (*a*) 'Idaho Gem', the cloned mule produced in 2003 in the US (Woods *et al.* 2003). (*b*) 'Prometea' the cloned horse produced in 2003 in Italy (Galli *et al.* 2003). (*c*) Cloned horse produced in 2005 in the US (Hinrichs 2005; image from http://vetmed.tamu.edu/news/press-releases/first-cloned-horse-in-north-america-born-at-texas-am, accessed 5 October 2017) Larry Wadsworth, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University. (*d*) 'Ñandubay Bicentenario', the cloned horse produced in Argentina in 2010 (Gambini *et al.* 2012). (*e*) A cloned horse produced in Korea in 2010 (Lee *et al.* 2015). (*f*) 'Turbante', the cloned foal produced in Brazil by *In vitro* Brasil Clonagem in 2012. (*g*) The cloned foal produced in Colombia by GenesCol in 2016 (image provided by M. Martinez Diaz, GenesCol).

US (Hinrichs 2005). The Galli group also reported the production of foals in 2005 (Lagutina *et al.* 2005), and this group has produced a total of 20 cloned foals up to June 2017 (C. Galli, pers. comm.). More cloned horses were produced by the Hinrichs group (Hinrichs *et al.* 2006, 2007; Choi *et al.* 2009, 2013, 2014, 2015) and, up to June 2017, they had produced 21 live foals, of which 18 survived and were healthy (K. Hinrichs, pers. comm.). At the beginning of 2006, commercial horse clone production was initiated within the US (Hinrichs 2006) and since then 'some hundreds' of clones have been commercially produced (ViaGen; www.viagen.com, accessed 4 May 2017).

In 2008, first cloned horse was produced in Argentina but, unfortunately, it only survived for a couple of hours after birth (Miragaya *et al.* 2011). In the same year the first cloned horse, Prometea, gave birth to her first foal, named 'Pegasus', conceived by AI. Two years later, in August of 2010, Daniel Salamone's group in Argentina produced the first healthy cloned foal in South America (Gambini *et al.* 2012), an Argentinean Criollo horse named 'Ñandubay Bicentenario' in honour of Argentina's bicentenary (Figs 1*d*, 2). Cloned foal production continued in Argentina between 2012 and 2016 (Gambini *et al.* 2012, 2014; Olivera *et al.* 2016) and approximately 120 cloned horses have been born in Argentina, being produced by two private companies since 2013 (R. Olivera (Kheiron) and A. Mutto (Crestview Genetics), pers. comm., 2017). In 2010, a cloned foal produced using ovum pick-up (OPU)-derived oocytes was born in Korea (Fig. 1*e*; Lee *et al.* 2015), and the birth of further OPU-derived cloned foals has been reported since 2013 (Choi *et al.* 2013, 2014). In 2012, Brazil reported their first cloned horse, a Mangalarga Marchador horse (Fig. 1*f*), produced by *In Vitro* Brasil Clonagem Animal, which has produced a total of 15 cloned foals since. Between two and five cloned foals are born per year in Europe (Reis 2015). In 2016, Colombia, another South American country, produced its first cloned horse, a copy of a Paso Colombiano horse, born on September 2016 (Mario Martinez Diaz (GenesCol), pers. comm., 2017; Fig. 1*g*). In Australia, cloned equine embryo production has recently been reported (Gambini *et al.* 2016*b*) and, at the time of writing (April 2017), equine cloned embryo transfer is being performed. At 14 years of age, 'Prometea', the oldest horse clone, is still alive and well (C. Galli, pers. comm., 2017) and horse cloning has been successfully performed on almost every continent (Fig. 3).

Major scientific advances

Cloning technique

SCNT can be achieved by different procedures. 'Traditional cloning', as used to produce Dolly the sheep, involves enucleation of a matured oocyte and placement of the donor cell into

Fig. 2. 'Ñandubay Bicentenario', the first viable cloned horse born in Argentina and produced by Daniel Salamone's group at the University of Buenos Aires in 2010. (*a*) Photograph of 'Ñandubay Bicentenario' taken on September 2010 together with the surrogate mother. (*b*) Photograph taken on June 2017. The foal was derived from a blastocyst generated by the aggregation of two early embryos (Gambini *et al.* 2012). Currently, the foal is in perfect health.

the perivitelline space, followed by electrofusion, activation and *in vitro* culture. Subsequently, it was shown that electrofusion can be bypassed if the donor cell is injected directly into the enucleated ooplasm (Wakayama *et al.* 1998).

SCNT can also be performed by removing the zona pellucida (ZP). This facilitates some steps of traditional cloning, such as enucleation (even without the need for a micromanipulator) and cell fusion, but it also requires individual manipulation of reconstructed embryos and a special culture system to keep the blastomeres in proximity during embryo development (Vajta *et al.* 2008). Another approach for the commercial production of cloned horses uses donor fibroblasts synchronised in G_2 of the cell cycle paired with OPU-derived activated oocytes in telophase II of meiosis (Maserati and Mutto 2016).

Similar blastocyst rates can be obtained using either traditional or ZP-free cloning in horses. However, pregnancy rates following the transfer of cloned horse blastocysts produced by traditional SCNT (Choi *et al.* 2014, 2015) seem to be more consistent than those reported from for ZP-free cloning (range 5–50%; Lagutina *et al.* 2005; Gambini *et al.* 2012, 2014; Olivera *et al.* 2016). The presence of the ZP could be an advantage for traditionally cloned embryos by allowing a better embryo capsule to be formed *in vitro* and/or to keep embryonic cells isolated until a proper embryo capsule develops *in vivo*. This hypothesis is supported by the absence of a confluent equine embryo capsule in hatched areas of equine intracytoplasmic sperm injection (ICSI) blastocysts (Smits *et al.* 2012). Conversely, an important advantage of using ZP-free cloning is that embryos can be easily aggregated, and this has been shown to improve *in vitro* and *in vivo* embryo development in the horse (Gambini *et al.* 2012, 2014) and other mammals (Boiani *et al.* 2003; De Souza Ribeiro *et al.* 2009; Buemo *et al.* 2016). The techniques used to clone equine embryos are shown in Fig. 4.

Equine donor cell

The somatic donor cell will contribute the genome of the new embryo, although its importance in cloning is often underestimated. Subcutaneous connective tissue-derived fibroblasts are common sources of donor cells used in horse cloning due to the simplicity of their recovery and the conditions required for cell culture and cryopreservation. In addition to the source of the donor cell, cell cycle synchronisation, culture conditions, reprogramming status and the number of cell divisions, among others, are variables that should be considered when trying to improve animal cloning success. It has been shown in horses, as well as in other species, that the development rate of SCNT embryos is greatly affected by the cell line (Lagutina *et al.* 2005) and the tissue cell line (Choi *et al.* 2009). A few reports have investigated the effects of different methods used for cell cycle synchronisation (Hinrichs *et al.* 2006) or have explored the use of different types and sources of donor cells (Lagutina *et al.* 2005; Olivera *et al.* 2016). Roscovitine treatment, growth to confluence and/or serum starvation were all found to produce healthy cloned foals when the G_0/G_1 cell cycle stage is needed. Using a different approach, roscovitine has also been shown to synchronise donor cells in G_2 with telophase II activated oocytes for cloned embryo production (Bordignon and Smith 2006; Maserati and Mutto 2016). In general, there is a tendency to use cells that have undergone a low number of passages in the horse because, with an increasing number of passages, the rates of nuclear remodelling decrease (Li *et al.* 2003). Generation of induced pluripotent stem (iPS) cells (Takahashi and Yamanaka 2006) opened a whole new world in the reprogramming area, and this type of nuclear reprogramming could be usefully applied to SCNT. However, iPS cells as nuclear donor cells did not produce embryos to the blastocyst stage, presumably because of the persistent expression of pluripotency-inducing genes (Olivera *et al.* 2016). In addition, the use of fetal rather than adult fibroblasts is likely to be more efficient for cloning in the horse (Li *et al.* 2002; Lagutina *et al.* 2005; Olivera *et al.* 2016) because this has been demonstrated for other species (Lagutina *et al.* 2005). Cumulus cells and umbilical cord-derived mesenchymal stromal cells have also been used successfully to produce cloned horse embryos, although no live

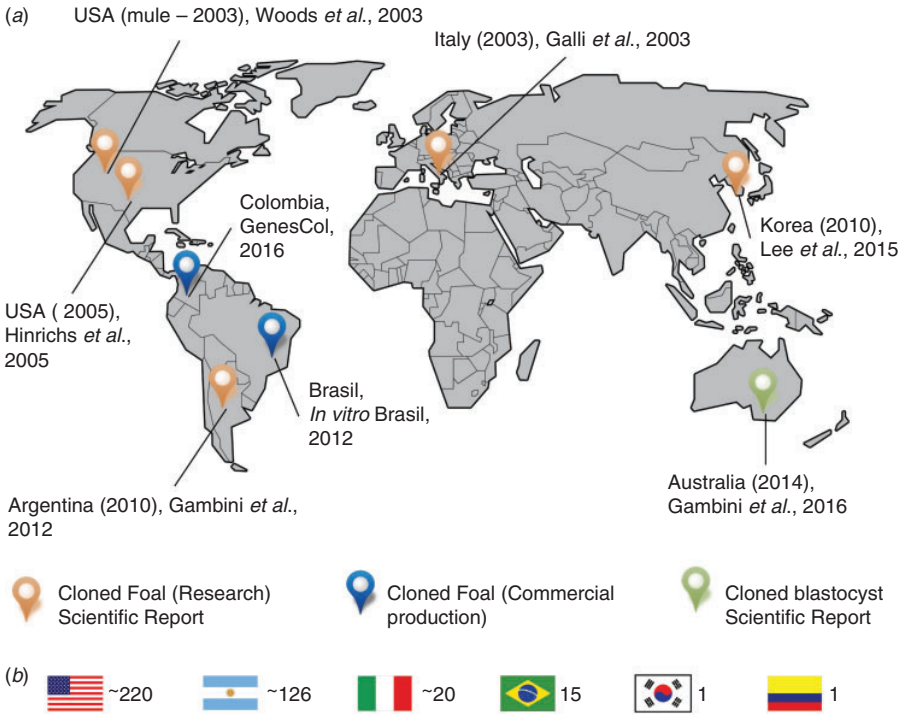

Fig. 3. (*a*) Worldwide distribution of reports of the first equine clones in different countries. (World map image from https://commons.wikimedia.org/wiki/File:BlankMap-World-1985.png, accessed 8 October 2017; licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license, modified.) (*b*) Estimated number of cloned horses produced in different countries by 2016.

foals were obtained (Vanderwall *et al.* 2004; Lagutina *et al.* 2005; Olivera *et al.* 2016).

Artificial activation and embryo culture

Calcium oscillations can be triggered in horses by using most of the methods that have been tested previously in other species, including ionomycin, electroporation, ethanol, thimerosal and injection of sperm extract (Li *et al.* 2000; Choi *et al.* 2001, 2002; Bedford *et al.* 2003; Hinrichs *et al.* 2007). Of these, only incubation with ionomycin and injections of stallion sperm extract have been used successfully in the production of horse clones. Injection of murine phospholipase C-zeta cRNA did not improve blastocyst production compared with injection of sperm extract (Choi *et al.* 2009). Artificial induction of calcium oscillations must be followed by kinase inhibition and/or inhibition of protein synthesis to optimise rates of oocyte activation in addition to inhibiting formation of the second polar body. In horses, 6-(Dimethylamino)purine (6-DMAP) is commonly used alone or in combination with cycloheximide, with this combination being shown to be more efficient in inducing activation than either chemical alone (Galli *et al.* 2007). As in other species, oocyte activation of SCNT embryos in the horse affects not only preimplantation embryo development, but also pregnancy outcomes (Hinrichs *et al.* 2007). Therefore, exploring new and better activating methods in horses is crucial to improving the efficiency of horse cloning and to avoid the toxic effects reported for the chemicals used in oocyte activation (Oliveira *et al.* 2014). Direct injections of roscovitine have been used to induce equine oocyte activation, although such treatment did not increase the rate of blastocyst development and resulted in a conceptus that developed abnormally in early gestation before being aborted at 114 days gestation (Hinrichs *et al.* 2006*b*). Furthermore, the roscovitine-induced parthenogenetic activation rate is lower than that obtained using 6-DMAP (Fernandes *et al.* 2014). Early activation (up to 1 h after fusion) of reconstructed cloned embryos did not improve embryonic development compared with that achieved following a time delay of 2 h (Choi *et al.* 2004; Olivera *et al.* 2016). Recently, a blastocyst development of 30% was obtained by manipulating the timing of maturation and prolonging the delay between reconstruction and activation by 5–8 h (Choi *et al.* 2015).

For *in vitro* culture, SCNT embryos are cultured in media previously tested using ICSI embryos. Synthetic oviducal fluid (SOF) (Lagutina *et al.* 2005), Dulbecco's modified Eagle's medium : nutrient mixture F-12 (DMEM/F12) (Choi *et al.* 2009) and, more recently, Global medium (Choi *et al.* 2015) have all been shown to support SCNT embryo development and foal production.

Health of SCNT pregnancies and foals

Pregnancy and foaling rates following non-surgical transfer of SCNT blastocyst embryos into the uteri of synchronised mares have been variable depending upon the cloning technique,

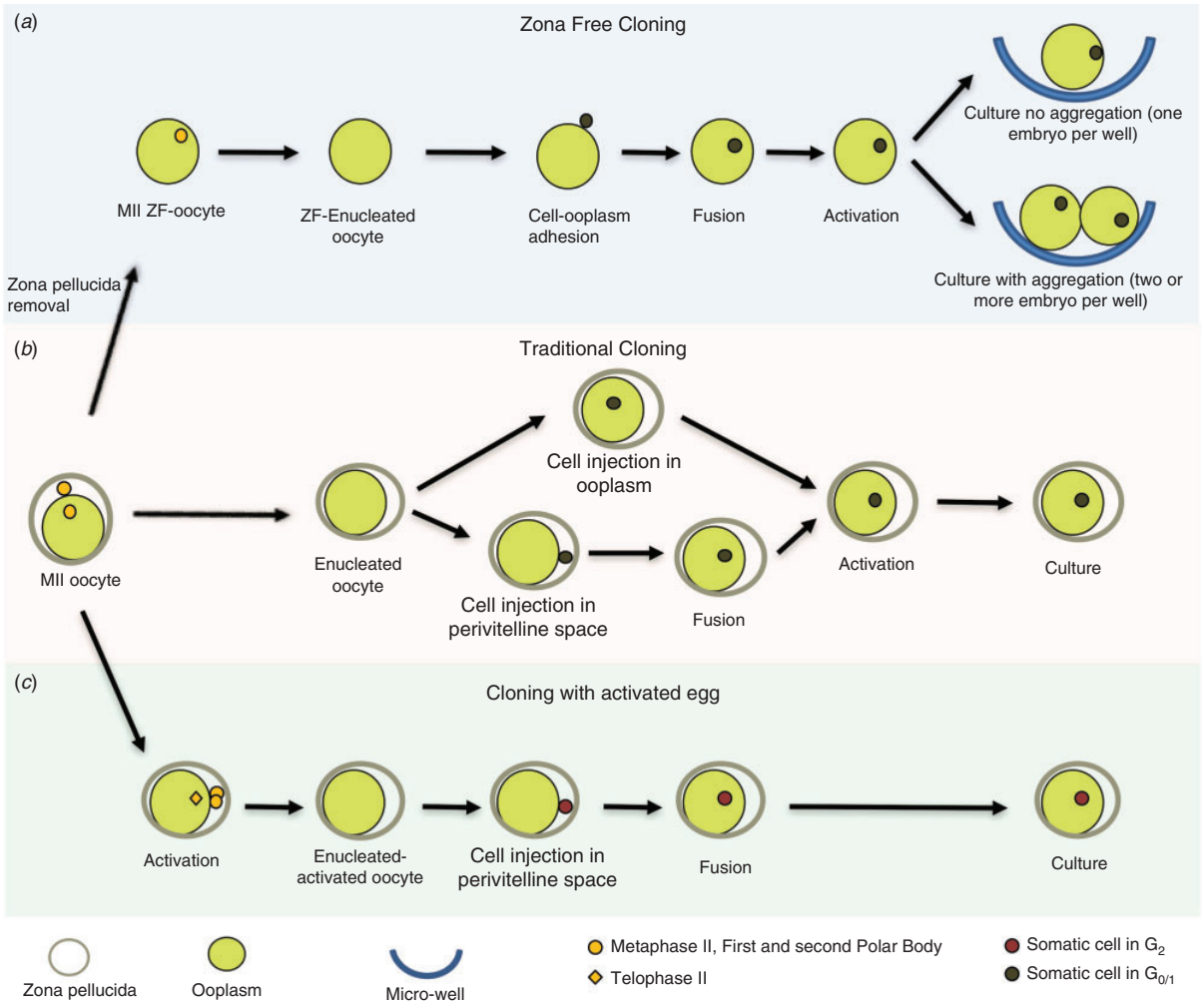

Fig. 4. Horse somatic cell nuclear transfer (SCNT) procedures. (*a*) Zona pellucida (ZP)-free cloning. MII-arrested oocytes are subjected to removal of the ZP, usually by protease treatment. ZP-free (ZF) matured oocytes are enucleated by micromanipulation using a blunt pipette and a closed holding pipette, followed by adhesion and electrofusion with a somatic cell synchronised in G_1/G_0 of the cell cycle. Artificial activation occurs after 2–8 h of successful fusion. ZF reconstructed embryos are cultured in microwells by placing one (no aggregation) or more than one (aggregated embryos) ZF-reconstructed embryos in each microwell. (*b*) Traditional cloning. MII oocytes are subjected to enucleation and a G_1/G_0 somatic cell is injected into the ooplasm (with or without the assistance of the Piezo drill), or into the perivitelline space and then electrofused. Artificial activation follows after a delay of 2–8 h of successful fusion or injection. (*c*) Cloning with an activated egg. MII oocytes are subjected to artificial activation with ionomycin. Telophase II oocytes are enucleated and a G_2 somatic cell is injected into the perivitelline space (as an alternative, the cell could also be injected directly into the ooplasm) and then fused into the activated oocyte. Embryos are usually cultured for 7–10 days and blastocysts are prepared for embryo transfer or cryopreservation.

the donor cell line, the number of embryos transferred and many other factors. The maternal recognition of pregnancy signal in the horse has not yet been elucidated (for a review, see Klein 2016), and the need for an intact embryo capsule may be one of the reasons why SCNT embryos fail to survive *in vivo* (Tremoleda *et al.* 2003; Gambini *et al.* 2012). The pregnancy loss rate in mares carrying clones is also variable, but is far higher than in mares carrying embryos generated by *in vivo* techniques or produced by ICSI. Looking at recent SCNT reports using adult fibroblasts, pregnancy rates can vary from as low as 9% (Olivera *et al.* 2016) up to 100% (Choi *et al.* 2015). In the study of Choi *et al.* (2015), of a total of 15 pregnancies only four foals were born alive (26.66%), whereas Olivera *et al.* (2016) reported that 22 viable cloned foals resulted from 78 pregnancies (28.20%). In yet another report, one viable foal was obtained (25%) from four cloned pregnancies (Gambini *et al.* 2014). In view of these results, it seems that an average of three to four pregnancies need to be obtained to produce one cloned foal. Of the potential causes for pregnancy failure, inadequate placental development has been reported in cloned equine

pregnancies (Gambini *et al.* 2012; Pozor *et al.* 2016). Therefore, ultrasound evaluation of cloned pregnancies is recommended to detect signs of placental abnormality, such as an increased thickness of the combined uterus and placenta or the existence of a placental separation. In cases where signs of placental abnormalities or placentitis are detected, various therapies may be implemented and exogenous progesterone administration has been used prophylactically by different groups (Gambini *et al.* 2012; Olivera *et al.* 2016).

In the first report of equine cloning, the three cloned mules were born normal and healthy (Woods *et al.* 2003). In that study, the oocytes had been matured *in vivo* and reconstructed embryos using fetal fibroblast were surgically transferred early in embryo development, which could be the underlying reason for the good health of the pregnancies and resulting cloned animals (Vanderwall *et al.* 2004*b*). Subsequently, it was shown that approximately 50% of the live-born cloned foals derived from adult fibroblast exhibit some degree of pathology, including neonatal maladjustment syndrome, an enlarged umbilical cord and leg tendon contractures (Johnson *et al.* 2010). A more recent study obtained 29 cloned offspring, of which only five died after birth (Olivera *et al.* 2016). However, all these animals were born at an equine hospital where neonatal care was provided. Therefore, the general consensus is that supportive therapy, such as supplemental oxygen, guaranteed colostrum intake and prophylactic antibiotic administration, is desirable for all newborn cloned foals (Johnson and Hinrichs 2015). Dystocia caused by 'large offspring syndrome' has been reported in cloned cattle (Young *et al.* 1998), but has not been noted in horses, where equine cloned pregnancies usually finish with normal parturition.

Cryopreservation

SCNT embryos frozen conventionally in 10% glycerol show no difference in pregnancy rate compared with unfrozen embryo transfer (Galli *et al.* 2007). A recent study reported five pregnancies and the birth of two foals after the transfer of seven SCNT vitrified–warmed blastocysts, and four pregnancies resulting in one foal from the further transfer of five SCNT vitrified–warmed blastocysts (Choi *et al.* 2015). The small size of the *in vitro*-produced equine blastocysts could be one reason for the relative success of the cryopreservation method, which enables the production of embryos in places and times of the year when recipient mares are not available and facilitates the transport of embryos to different locations. To the best of our knowledge, no reports of cloned equine embryo development to the blastocyst stage have been published using vitrified oocytes, but because it was reported recently that methods of immature oocyte vitrification allowed the birth of a healthy foal after ICSI (Ortiz-Escribano *et al.* 2017), oocyte vitrification will probably be also available soon for cloned embryo production.

Scientific curiosities of horse cloning

Heterospecific horse cloning (2006–16)

Until now, cloned equine blastocyst like structures have been obtained by using bovine (Tecirlioglu *et al.* 2006) and domestic cat (Gambini *et al.* 2016) enucleated oocytes as hosts for an equine fibroblast. Because heterospecific embryo transfer among equids species was shown to be successful (Summers *et al.* 1987; Allen *et al.* 2011), SCNT using a mare's oocyte as the host for a genome derived from another equid could potentially produce a viable embryo. Therefore, cloning could also be used for saving endangered or extinct equine species (Smits *et al.* 2012*b*).

Horse clone foal produced using MI oocytes (2009)

It is possible to produce healthy cloned foals by using MI oocytes as a host for the donor cell (Choi *et al.* 2009). Blastocyst development rates did not differ between oocytes in MI or MII at the time of nuclear transfer.

Aggregated cloned embryos (2012)

ZP-free cloning allows the aggregation of embryos when performed before the morula stage. This technique has been used to produce healthy cloned foals in South America (Gambini *et al.* 2012), and is now being used for the large-scale production of clones (Olivera *et al.* 2016). The aggregation of up to four embryos is beneficial to cloning efficiency (Gambini *et al.* 2014) and, interestingly, aggregation of cloned embryos may lead to a generation of mitochondrial chimeric embryos, which could be beneficial because cells with any grade of mitochondrial dysfunction could be potentially replaced by cells with normal mitochondria. Moreover, different cell reprogramming and compensation for epigenetic defects may be another reason for the improved developmental competence of aggregated embryos (Eckardt and McLaughlin 2004).

Day 17 SCNT embryos (2012)

A surprising aspect of equine SCNT blastocysts is their ability to expand and grow *in vitro*. Embryos larger than 3500 µm were obtained on Days 16–17 of *in vitro* culture (Gambini *et al.* 2012). Embryo quality in horses could be potentially assessed by measuring *in vitro* embryo growth rates.

Genetically identical SCNT horse (2014)

Using SCNT, it is possible to produce a horse with the same genomic DNA of a previously existing horse, although the cells of the resulting clone will possess mitochondrial (mt) DNA from the abattoir-derived oocyte used as a host during the cloning procedure. However, it is possible to produce a 100% genetically identical cloned foal by using host-derived oocytes from the maternal line of the donor cell (Choi *et al.* 2014). A female clone will then pass the mtDNA to her offspring, although this would not be the case for a male clone. Theoretically, if oocytes with any degree of mitochondrial dysfunction are used as the host for SCNT, it is possible to adversely affect early embryo development; conversely, mitochondria of known high performance potential could possibly be used to improve performance or correct existing mitochondrial disease in equine with high nuclear genetic value.

Limitations for faster scientific progress

Although horse cloning has important scientific and commercial applications, only a few laboratories worldwide have reported

Table 1. Some of the main breeds of horses cloned reported until 2016

Breed	Reference
Criollo Argentino	Gambini *et al.* (2012)
Polo pony	Gambini *et al.* (2014), Olivera *et al.* (2016), Kheiron[A], Crestview Genetics[A]
Haflinger	Galli *et al.* (2003), Lagutina *et al.* (2005)
Warmblood	Lee *et al*. (2015)
Arabian horse	Lagutina *et al.* (2005), Crestview Genetics[A]
Thoroughbred	Kheiron[A]
Jumping horse	Gambini *et al.* (2014), ViaGen[B], Kheiron[A]
Paso colombiano	GenesCol[A]
Paso fino	ViaGen[B]
Cutting horse	ViaGen[B]
Mangalarga marchador	*In vitro* Brasil Clonagem[A]
Campolina	*In vitro* Brasil Clonagem[A]
Bucking horse	ViaGen[B]
Barrel racing horse	ViaGen[B]
Quarter horse	ViaGen[B]

[A]Personal communication, June 2017.
[B]See 'Our Equine Client Stories' at http://www.viagen.com, accessed 8 October 2017.

the birth of viable equine clones. A major limitation to the progress of equine cloning has been the availability of adequate numbers of quality oocytes. This is due to regional restrictions regarding the slaughter of horses and limitations on the collection of oocytes in the mare compared with other domestic species, together with the rapid decline in oocyte viability (Gambini *et al.* 2014*b*). Mares' ovaries vary in size depending upon follicular activity and they are enclosed within a very tough and fibrous tunica albuginea (Ginther 1992). Moreover, it is necessary to scrape each follicle due to the strong attachment of the oocyte to the follicular wall (Hawley *et al.* 1995). Together, these aspects, along with the large volume of follicular fluid, result in a low recovery of oocytes compared with other animals, and the same limitations apply to *in vivo* follicle aspiration.

Commercial interest in horse cloning is also an important factor and the lack of scientific publications from most private companies, together with their operating activities, which limit access of research institutions to the necessary biological material, seriously compromises the development of research groups in the field. The lack of an efficient method for traditional IVF in the horse may be another reason why there is a lack of adequate *in vitro* culture systems for horse embryos. Currently, ICSI is the most efficient method for *in vitro* embryo production, which limits the production of equine embryos to laboratories that have the equipment and technical expertise to perform ICSI.

Current status of horse cloning commercialisation

Horse cloning is commercially available today in North America (US), South America (Argentina, Brazil and Colombia), Europe (Italy) and Australia. An estimated minimum number of 375 cloned horses are now present worldwide. Production of cloned horses by country is as follows: US (~220), Argentina (~126), Brazil (~15), Italy (~20), Colombia (1) and South Korea (1). The actual total number of horse clones could be higher considering the births of some cloned equines that have not been announced by private companies.

According to the information revealed by the horse cloning companies that agreed to release production data for this review (four companies in total from Argentina, Brazil and Colombia), all of them culture embryos until the blastocyst stage, with a blastocyst rate ranging from 18% to 40%, pregnancy rates ranging from 20% to 40% and foaling rates ranging from 50% to 95%. The main breeds that have been cloned so far are summarised in Table 1.

Acceptance of horse cloning by breeding societies

Cloning and any other form of assisted reproductive technology (ART) is not permitted by international studbooks that register racing thoroughbreds. The American Quarter Horse Association, the largest horse breed in the US (Squires 2015), is another example of a studbook that does not allow the registration of clones. In contrast, the manifest of the Argentine Polo Pony Breeder's Association (AACCP) is 'to promote the research and the application of artificial insemination, embryo transfer and any other technique to improve animal breeding' (2nd article, AACCP Statute; www.criapoloargentino.com.ar, accessed 4 May 2017).

In general, warmblood studbooks and the World Breeding Federation for Sport Horses are willing to register cloned horses. The Federation Equestrian Internationale recently recognised that cloning does not affect fair play and authorised the participation of clones and their offspring in official competitions, noting that these events are a competition with two athletes, the horse and the rider working together (Reis 2015). Cloning is also applied to horses in which the value of the progeny does not depend on registration with a breed association (Campbell 2016).

In December 2013, the European Commission tabled proposals to ban the use of the cloning technique for farm animals in the European Union and the import of such animal clones (IP/13/1269 18/12/2013; http://europa.eu/rapid/press-release_IP-13-1269_en.htm, accessed 8 October 2017). However, in

October 2015, the European Parliament amended the European Commission's proposal to remove the exemption from the ban for sporting and cultural events (Campbell 2016).

Another interesting regulation is that the Asociación Rural Argentina (SRA) does not allow the registration of 100% identical clones and requires an mtDNA study to distinguish between the cloned animal and the donor animal. If that differentiation cannot be performed, the clone cannot be registered with the SRA. This regulation differs from that in Brazil, where oocytes must be sourced from animals of the same breed. The testing of mtDNA is not yet required in Brazil. Because regulations specifying the mitochondrial strain used in equine cloning do not exist outside South America, cloned embryos produced internationally for embryo transfer and birth within Argentina and/or Brazil may not meet the requirements for breed association registration in these countries.

Horse clones in the food chain

Because cloning in horses has been applied mostly to high-value horses and is relatively new (the oldest horse cloned is 14 years old), the possibility of introducing cloned animals into the food chain remains unlikely. Cloning technology used in equines is now being applied in many different countries and as the numbers of cloned horses increase, regulation of cloned equine meat could be proposed in the future under novel food guidelines (http://europa.eu/rapid/press-release_IP-13-1269_en.htm, accessed 8 October 2017).

Perspectives and conclusion

The combination of commercial interests with the scarcity and high costs of obtaining equine oocytes are reflected in the low number of scientific groups researching equine cloning. Despite these limitations, the overall efficiency of horse cloning has improved significantly in the past few years, resulting in an increased number of cloned equines produced today. The apparently normal health of ICSI pregnancies and newborn foals suggest that most of the problems observed in cloned horse embryos are due to inappropriate genomic reprogramming, epigenetic state of the donor nuclei or suboptimal artificial activation protocols. Therefore, more research in these areas is required to improve the efficiency of horse cloning.

Commercially, there is a need to standardise the regulations controlling the registration of cloned horses between countries as well as breed associations. As new tools are created for the genetic advancement of equine breeds, more emphasis should be placed on breed registration regulation that places the overall health and safety of the animal first, focusing on sound biological principals for the promotion and growth of international markets.

Conflicts of interest

The authors declare no conflicts of interest.

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