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Emerging applications of sperm, embryo and somatic cell cryopreservation in maintenance, relocation and rederivation of swine genetics

H. Men^{1,3}, EM. Walters^{2,3}, H. Nagashima⁴, and RS. Prather^{2,3,*}

¹Department of Veterinary Pathobiology, University of Missouri, Columbia, MO 65211, USA

²Division of Animal Sciences, University of Missouri, Columbia, MO 65211, USA

³National Swine Resource and Research Center, University of Missouri, Columbia, MO 65211, USA

⁴Meiji University International Institute for Bio-Resource Research, Tama, Kawasaki 214-8571, Japan

Abstract

Advances in porcine assisted reproductive technology (ART) make it possible to use cryopreserved sperm, embryos and somatic cells in the maintenance, relocation and regeneration of swine genetics. In this review, development of key application-limiting technology is discussed in each cell type, focusing on the efficiencies, ease of storage and transportation, and minimization of pathogen transmission. Methods to regenerate swine genetics and/or models using frozen sperm, embryos and somatic cells in combination with other porcine ARTs, such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and somatic cell nuclear transplantation (SCNT), are also discussed. The applications of these ARTs utilizing cryopreserved cells will greatly increase the efficiency as well as biosecurity for maintenance, relocation and rederivation of swine genetics/models.

Keywords

Cryopreservation; ART; Swine genetics/models; Maintenance; Rederivation

1. Introduction

As an important food animal, pigs have been subjected to continued efforts to advance genetics to increase their productivity, performance and adaptivity to local environments [1]. Like other animals, maintenance of genetic diversity in pigs is important for their adaptation to the environments as well as their population growth [2–4]. Secondly, similarities in structure and function between pigs and human make this species the ideal model for the study of human diseases and likely candidates for xenotransplantation [5,6]. As the

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*Corresponding author: Randall S. Prather, PhD, Curators' Professor and Distinguished Professor of Reproductive Biotechnology, Division of Animal Sciences, E125 Animal Science Research Center, 920 East Campus Drive, University of Missouri-Columbia, Columbia, Missouri 65211 USA, Phone: 573-882-6414; FAX: 573-884-7827; pratherr@missouri.edu.

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popularity of swine as models of human disease and health increases, a rapid growth of swine models is expected. Therefore, it is important to maintain and safeguard swine genetics/models in an efficient and cost-effective manner. As germplasm cryopreservation has become a major tool for the maintenance of rodent models [7], current advances in swine germplasm cryopreservation-related ART technology make it possible to use similar approaches for maintenance and relocation of swine genetics/models.

Development of animal ARTs has revolutionized the genetic improvement, exchange and conservation of animal resources of agricultural and biomedical importance. For example, ARTs have been used in the cattle industry for genetic improvement and global exchange of cattle genetics for several decades. Frozen semen and embryos are currently the major methods to move cattle genetics worldwide. Germplasm cryopreservation has become a major tool for the maintenance of rodent models to reduce the cost and space requirement associated with maintenance of live animals [7]. However, similar technology has not gained wide applications for maintenance and reconstruction of swine genetics/models due to the suboptimal post-cryosurvival of pig sperm and embryos. Currently, artificial insemination (AI) in swine industry depends mainly on liquid stored semen. Although frozen sperm is also being used in some circumstances, live animals are still a major method for globally exchange of porcine genetics. The relatively high cost of transporting living animals, as well as the associated cost of quarantine for disease prevention measurements, has limited global exchange of porcine genetics. Costs and the risks can be minimized through transportation of cryopreserved porcine semen, embryos and somatic cells.

Minimizing pathogen transmission during germplasm cryostorage is essential for animal genetic maintenance, exchange and rederivation. Although risks are low if recommended sanitary methods and procedures [8,9] are followed, there is documented evidence of contamination by direct contact with liquid nitrogen (LN₂) and cross-contamination in shared LN₂ tanks [9–11]. Therefore, porcine sperm, embryos and somatic cells cryopreserved in closed systems are essential for the maintenance, relocation and rederivation of swine genetics/models.

The purpose of this review is to promote the use of germplasm cryopreservation-related porcine ART for the maintenance, exchange and regeneration of swine genetics/models. In this regard, the discussion will focus on the ability to preserve and regeneration of swine genetics. Efficiencies, ease of storage and transportation, and minimization of pathogen transmission of porcine sperm, embryos and somatic cells, as well as their combination with other porcine ARTs, such as IVF, ICSI, and SCNT for the regeneration of swine genetics and/or models, will be discussed. For basic aspects of sperm, embryo cryopreservation and porcine SCNT, readers are referred to several recent excellent reviews [2,12–15].

2. Role of frozen sperm

Sperm are haploid cells that only contain half of the genetic material of an individual. This genetic characteristic determines the unique roles of frozen sperm in animal genetics maintenance, relocation and rederivation. Although frozen sperm may not be a good candidate for maintenance of animals if the whole genome or multiple traits need to be preserved unless oocyte cryopreservation technique is available for the particular breeds [16], they are ideal for preservation and regeneration of animal genetics if only a single trait is desirable, or there is a single gene mutation in animal models. In fact, sperm cryopreservation has been used for maintenance and reconstruction of animal models with single locus mutation in laboratory animals, such as mice and rats, for many years [7].

Frozen boar sperm have significantly reduced fertility due to their high sensitivity to freezing. Traditional cervical AIs using frozen/thawed sperm usually require a large number

(5 to 6×10^9 sperm/AI-dose vs 2 to 3×10^9 sperm/AI-dose with liquid stored sperm) of sperm to be inseminated two or three times during an estrus cycle [17,18]. Even using this insemination strategy, efficiencies in terms of pregnancy and piglet rates resulting from frozen/thawed sperm are not comparable to those from liquid stored sperm (Table 1); which usually results in > 80% farrowing rates and > 10 piglets per litter, in commercial applications [18,19].

Improving fertility of frozen/thawed boar sperm has been an objective of both the scientific community and the swine industry. Major improvements have been made in the following areas. Newly designed cryogenic containers have improved fertility of frozen/thawed boar sperm. Traditionally, boar sperm were frozen in 5 mL straws. The large volume of extended semen precludes uniform rates of cooling and thawing in the center and periphery of the straw. To overcome these uneven cooling and thawing rates within 5 mL straws, a FlatPack packaging system has been developed for boar sperm freezing [17,20]. The FlatPack is a 5 mL, flat plastic package that allows more uniform cooling of the enclosed sperm sample, due to an increased surface area to volume ratio. Consequently, sperm frozen using FlatPacks had significant improvements in motility and fertility compared to those cryopreserved in conventional 5 mL straws. Recently, sperm are frozen at high concentration (1 to 2×10^9 sperm/mL) in small volume (0.7 mL) in newly designed MiniFlatPacks [21]. Sperm motility resulting from the new packing system is comparable to sperm frozen in 0.5 mL straws. This high sperm concentration in a small volume has allowed the development of new insemination techniques, such as intra-uterine insemination or deep uterine insemination (DUI) [18,21–25], that in turn increase the efficient use of frozen/thawed boar sperm. As frozen/thawed boar sperm have a shorter lifespan (< 8 h) than liquid stored sperm, the time of insemination is critical to maximize pregnancy and piglet rates. The efficiency of AI with frozen/thawed sperm increased when insemination at a fixed time after sows were hormonally synchronized [22,26,27]. Finally, the discovery that boar sperm are highly sensitive to oxidative stress during cryopreservation due to the high content of unsaturated fatty acids in the plasma membrane [28] has led to development of a freezing protocol that incorporates antioxidants, thereby improving the cryosurvival of frozen/thawed boar sperm [14,29].

Control of pathogen transmission as a result of semen distribution is also crucial when frozen sperm are used, either for production purposes or as a tool for model maintenance. Good practices for maintenance of a boar stud with high level of biosecurity, as well as sanitation protocols including the use of antimicrobial agents during semen processing, are suggested [30]. In addition, packaging systems are also crucial for minimizing the chance of pathogen transmission. Historically, there are two packaging systems for frozen boar sperm: an open system in pellet form [31] and closed packaging systems, which vary in volumes and shapes [20,32,33]. The closed system offers a clear advantage over the open system for minimizing pathogen transmission and is the dominant packaging form for frozen sperm. The ability to keep frozen sperm in liquid nitrogen for an extended interval also provides opportunity to conduct comprehensive pathogen screening tests before use, thereby further increasing the biosecurity of swine genetics exchange through frozen sperm [16].

Frozen boar sperm have been used for distribution of superior swine genetics nationally and internationally for many years to improve the global swine population [12,34]. Recent development of porcine ARTs provides even more flexibilities for the purpose of this discussion. For preservation, sperm may be frozen at packaging systems of various sizes: 0.25 mL, 0.5 mL, 5 mL maxi straws or FlatPacks. To regenerate animals using frozen sperm, traditional AI can be used for sperm frozen in larger sizes of packaging systems, such as 5 mL maxi straws or FlatPacks. Sperm frozen in smaller packaging systems can be used for IVF and subsequent embryo transfer. In this case, oocytes can be obtained from

superstimulated gilts or sows from the existing herd using oocyte pickup (OPU) if specific breeds are desired [35]. Abattoir ovaries can be another source of oocytes if the desired traits are carried by the sperm. For boars with low post-thaw motility or a very limited supply of sperm, intracytoplasmic sperm injection (ICSI) may be used [36,37]. Although ejaculated sperm is normally used for germplasm banking, epididymal sperm may also be cryopreserved in some cases, such as unexpected death or boars with conformational issues preventing routine collection. In this case, IVF or ICSI other than traditional AI may be required for animal regeneration, as the supply of epididymal sperm is usually limited. An efficient IVF protocol using epididymal sperm has been established and transgenic piglets were generated using epididymal sperm from transgenic boars with this protocol [38]. Similarly, frozen sperm can also be used for maintenance of swine models of human diseases and health, especially for those involving genetic modification of a single locus on a chromosome. In that regard, sperm from the swine models at the National Swine Resource and Research Center are cryo-banked for maintenance purpose.

3. Role of cryopreserved embryos

Embryos contain diploid cells and have the complete set of genetic information of an individual. They are able to develop into individual animals after being transferred into the uteri of synchronized surrogates. Therefore, cryopreserved embryos are ideal for the maintenance, relocation and rederivation of swine genetics. Similar to frozen sperm, the efficiencies, ease of storage and handling, and pathogen transmission are of concern for cryopreservation of porcine embryos.

Similar to boar sperm, porcine pre-implantation stage embryos are very sensitive to low temperature and are very difficult to cryopreserve using conventional slow cooling or vitrification without special handling of the embryos, with the exception of peri-hatching or hatched blastocysts [3,39–41]. The sensitivity to low temperature is due mainly to their high level of intracellular lipids [40,42,43]. Progress towards the efficient cryopreservation of porcine embryos was not made until the mid-1990s when a centrifugation-based, mechanical delipidation method, a technique to reduce the cryosensitivity of porcine embryos, was developed [42,43]. With this method, intracellular lipids are initially polarized to one side of the embryos through centrifugation and then the polarized lipid droplets are removed from the embryos through micromanipulation. This delipidation method significantly increases cryotolerance of porcine embryos without compromising developmental potential [42,43]. Porcine embryos at 2 to 8 cell and later stages can be successfully frozen with conventional slow cooling methods after mechanical delipidation, and live births have resulted from transfer of the cryopreserved embryos [42,44]. This breakthrough has formed the basis of increasing the cryotolerance of porcine embryos; several recent reports in porcine embryo cryopreservation, especially early stage embryos, have been based on this principle [44–52].

Progress has also been made employing another recently developed vitrification technology: vitrification at ultrarapid cooling rates [53,54]. This has been achieved by decreasing the volume of vitrification solution to approximately 0.1 to 2 μ L using special packaging systems, such as an electron microscope grid, open pulled straw (OPS), cryotop and cryoloop, and then cooling the sample by directly plunging the sample into LN₂ [53–55]. The ultrarapid cooling rate ensures oocytes or embryos to quickly bypass the temperature-sensitive zone to minimize the cryodamage during vitrification. Using this approach, porcine embryos at the morula and early blastocyst stages were successfully cryopreserved using OPS vitrification without prior delipidation and thus intact zona pellucida were retained. Live piglets were also obtained from both vitrified morula and blastocysts following embryo transfer [56–59]. Cryopreservation of earlier stage porcine embryos using this approach is still challenging [60]. A combination of delipidation and vitrification at ultrarapid cooling

rates provides even better protection for porcine embryos [45,46]. Porcine embryos generated in vitro have also been able to survive cryopreservation with this approach [48–51].

For both agricultural and biomedical research purposes, embryo cryopreservation requires embryos being cryopreserved with intact zona pellucida and in a closed system to maintain a high level of biosecurity. However, both mechanical delipidation and vitrification at ultrarapid cooling rates increase the risk of pathogen transmission. Mechanical delipidation uses a beveled glass needle to remove the polarized lipid droplets through a puncture on the zona pellucida; therefore, it substantially increases the likelihood of pathogen contamination of the embryos [8]. Vitrification at ultrarapid cooling rates requires direct contact of the sample with LN₂ and also increases the chances of pathogen transmission during embryo cryopreservation and storage [10,61,62]. To overcome the disadvantages associated with these methods, several recent modifications to the centrifugation-based delipidation method have result in the cryopreservation of porcine embryos with an intact zona pellucida. In these methods, intracellular lipids were either polarized through centrifugation without removal [45,46] or completely disassociated from blastomeres after centrifugation as a result of increased perivitelline space achieved by partial digestion of zona pellucida before centrifugation [50] or exposure to hypertonic solution to reduce the size of blastomeres (thus the increased perivitelline space) during centrifugation [49]. By taking advantage of the relatively large perivitelline spaces in 4 to 8 cell stage embryos, complete separation of lipid droplets is also achieved through prolonged centrifugation at high speed [52]. Although most of the reports employed vitrification through direct contact of samples with LN₂ to achieve ultrarapid cooling rate, delipidated porcine embryos at the early blastocyst to blastocyst stage (Day 5) can survive conventional vitrification using 0.25 mL mini-straws at rates comparable to vitrification at ultrarapid cooling rates. Blastocysts vitrified by the two packaging systems (OPS and 0.25 mL straws) also have the ability to develop to term after embryo transfer into synchronized surrogates [52]. Chemical delipidation using lipolytic agents has also been attempted to avoid mechanical damage to the zona pellucida [63]. However, chemical delipidation did not gain practical application, because porcine embryos can only be partially reduced through lipolysis, due to the reversible nature of the biochemical reaction, whereas complete removal of intracellular lipids are essential to maximize the cryosurvival of porcine embryos [43]. Major achievements and efficiencies for the cryopreservation of porcine embryos using various approaches are listed (Table 2).

Cryopreservation of porcine embryos in 0.25 mL mini-straws provides several benefits over cryopreservation using vitrification at ultrarapid cooling rates. The 0.25 mL straws can be sealed at both ends, thereby substantially reducing the chances of pathogen transmission during storage or transportation [10,61,62]. There are also existing systems for using straws as packaging containers. For example, straws can be easily labeled using commercially available straw printers before embryo packaging and cryopreservation for easy identification. The storage and transportation containers are also available. Therefore, the ability to efficiently cryopreserve zona-intact porcine embryos in a conventional 0.25 mL mini-straw system provides the opportunity to use cryopreserved embryos for swine genetic maintenance, relocation and regeneration.

For animal preservation via embryo cryopreservation, embryos at the blastocyst stage are recommended, due to the increased cryotolerance compared to earlier stage embryos. Embryos can be vitrified in 0.25 mL straws. To ensure regeneration of breeding pairs, both male and female embryos need to be cryopreserved; this can be achieved through cryobanking a relatively larger number of embryos. Animal regeneration using cryopreserved embryos can be achieved through surgical embryo transfer.

4. Role of cryopreserved somatic cells

The new opportunity for maintenance, relocation and rederivation of swine genetics via somatic cell cryopreservation is mainly depended on development of SCNT in pigs [5,64–66]. Similar to embryos, somatic cells also contain the complete genetic complements of both parents and therefore are able to regenerate animals with a nearly intact parent genome if oocytes from same or closely related strain are used as recipient oocytes in SCNT, thus minimizing the effect of mitochondrial heteroplasmy [67].

Although current porcine SCNT efficiencies (piglets per embryos transferred) are low and also depend on the stages at which cloned embryos are transferred (Table 3), this technology is able to generate piglets from somatic cells of various sources with varying degrees of differentiation [15]. Among the cell types used, fetal fibroblasts are the most widely used cell type [5,64,66]. Piglets have also been generated using SCNT procedures using fetal genital ridge cells, fetal somatic stem cells, granulosa cells, preadipocytes, heart cells; epithelial cells, salivary gland derived progenitor cells, skin derived stem cells, as well as adult fibroblasts [65,68–74]. Due to their relative ease in collection and culture, as well as their ability to be genetically modified in vitro, fetal fibroblasts have also been the preferred cell type for production of pig models with targeted mutations through SCNT [5,66]. Several somatic cell types, such as fetal fibroblasts, skin epithelial cells, as well as adult fibroblast cells etc., from pigs are relatively easy to cryopreserve compared to sperm and embryos. Therefore, the cryopreserved form of these cells can be used for swine genetic maintenance, exchange and regeneration.

Similar to sperm and embryo cryopreservation, minimizing potential pathogen transmission is also critical for the cryopreservation of somatic cells. Due to the relative ease in cryopreservation, cryovials of various sizes are traditionally used as packaging systems for cryopreservation of somatic cells. However, there is a risk of explosion during thawing as a result of LN₂ leaking into cryovials. Therefore, straws, such as 0.5 mL straws, can be used as a replacement of cryovials for cryopreservation of somatic cells. As discussed above, straws can be tightly sealed at both ends and thus provide a closed barrier for potential pathogen contamination from surrounding sources such as liquid nitrogen. The use of 0.5 mL straws provides additional benefits for easy labeling and categorization for storage and transportation.

Overall, cloned pigs have similar growth characteristics, physiology, reproductive performance and general health compared to the non-cloned pigs [75,76]. Although some congenital abnormalities and defects, as in other cloned animals, have been observed in pigs, the incidence is relatively low and some of these abnormalities can be corrected in the next generation as they are likely the result of aberrant DNA methylation [15]. Currently, SCNT efficiencies are in general very low across several mammalian species, including pigs [77]. The low efficiencies may prevent it from commercial application; however, it is feasible to use it for maintenance, relocation and regeneration of important swine genetics and/or models, especially if recipient oocytes from same or genetically closely related breeds are available.

Currently, SCNT is the major method for the production of genetically modified swine models for biomedical research. It can also be used for the regeneration of rare or important swine genetics. For the purpose of swine model maintenance, selection of cell types to be cryopreserved should depend on the models. For those with several breeding pairs, fetal fibroblasts may be an option for cryopreservation. However, if there are a very limited number of animals of a particular model, other type of cells, such as ear fibroblasts, should be used since collection of fetal fibroblasts requires death of fetuses and sometimes the

founder animals. Moreover, similar to embryo cryopreservation, somatic cells from both sexes need to be cryobanked in order to produce breeding pairs.

5. Additional considerations

To ensure animal reconstruction, there are several additional considerations for cryobanking sperm, embryos and somatic cells. Cryobanking of multiple cell types, such as sperm, embryos as well as somatic cells, are recommended for each strain or model, so that animals can be reconstructed with at least one type of cells. Maintenance of these cells at two different locations is highly recommended to prevent loss due to natural catastrophic events. For cost-effective regeneration of animals, frozen sperm is the best choice for animals with a single desired trait or single mutation. For animals with multiple traits or mutations, cryopreserved embryos may be the best choice.

With development of new technology, there will be more opportunities for animal genetic maintenance and regeneration. For example, induced pluripotent (iPS) stem cell technology may eventually become applicable for this purpose [78–81]. While it still remains to be determined if the pluripotent feature of iPS cells can produce animals with fewer or no congenital defects compared to somatic cells. In addition, animals may also be able to regenerate through chimeric animal production using reprogrammed pluripotent stem cells.

6. Conclusion

In this review, we provide a discussion of potential applications of cryopreserved sperm, embryos and somatic cells for maintenance, relocation and regeneration of swine genetics. Although some of the techniques discussed here require laboratory settings and trained personnel, the substantial advantages associated with cryopreservation-related technologies, such as cost-effectiveness, a high level of biosecurity, and ease of storage and transportation, provide many benefits over traditional methods, and therefore, are highly recommended for future applications in these areas.

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Table 1
Efficiencies for the production of offspring derived from frozen porcine sperm in various cryogenic containers.

Cryogenic container	AI ^a	AI dose (× 10 ⁶)	AI (no.)	Pregnancy rate (%)	Farrowing rate (%)	Litter size	Reference
Pellet	CAI ^b	600	1	--	47.0	7.40	[82]
0.5 mL straws	DUI ^c	100	1	--	77.55	9.31 ± 0.41	[22]
5 mL straws	CAI	300	2	80	--	8.00	[83]
FlatPacks	CAI	500	1	--	72	10.7 ± 3.6	[17]
MiniFlatPacks	DUI	200	1	40	ND ^d	ND	[23]

^a AI or IVF

^b Cervical AI

^c Deep uterine insemination.

^d Not determined.

Major technical development and piglet efficiencies of porcine embryo cryopreservation in related to packaging systems and cryopreserving protocols.

Table 2

Embryo	Treatment	Packaging system	Cryopreservation protocol	Piglets (%)	Reference
IVD ^a morulae and blastocysts	None	Ministraws	Slow cooling (only to -35 °C)	+	[84]
IVD blastocysts	None	Ministraws	Vitrification	-	[85]
IVD 4-8 cell embryos	Mechanical delipidation	Ministraws	Slow cooling	3 (7.69)	[42]
IVD expanded blastocysts	Cytoskeleton stabilization	Ministraws	Vitrification	29 (12.95)	[86]
IVD early blastocysts	Mechanical delipidation	OPS ^b	Vitrification	5 (2.78)	[45]
IVD blastocysts	None	OPS	Vitrification	38 (9.50)	[56]
IVD morulae	None	OPS	Vitrification	26 (13.00)	[57]
Cloned blastocysts	Mechanical delipidation	OPS	Vitrification	10 (6.13)	[48]
IVP ^c blastocysts	Chemical delipidation	OPS	Vitrification	-	[63]
IVD 2-4 cell embryos	SOPS ^d and none	vit-Master	Vitrification	-	[60]
IVP 4-8 cell embryos	Mechanical delipidation	Cryotop	Vitrification	9 (2.54)	[51]
IVP zygotes	Mechanical delipidation	SSD	Vitrification	17 (2.27)	[87]
IVP blastocysts	Mechanical delipidation	OPS	Vitrification	6 (2.98)	[52]
		Ministraws	Vitrification	7 (3.30)	[52]

^aIn vivo derived.

^bOpen pulled straw.

^cIn vitro produced.

^dSuperfine OPS.

Table 3
Efficiencies of porcine somatic cell nuclear transfer (SCNT) using various cell types in production of live piglets.

Cell type	Genetic modifications	Nuclear transfer	Embryo stage at transfer	No. embryos transferred	Piglets (% ^d)	Reference
Fetal fibroblasts	-	TC ^b	2-8 cells	110	1 (0.9)	[64]
	-	TC	1-cell	143	2 (1.4)	[68]
	GFP	TC	1-2 cells	308	5 (1.6)	[66]
	α-1,3-gal KO	TC	1-2 cells	338	7 (2.1)	[5]
	-	HMC ^c	Blastocyst	58	10 (17.2)	[88]
	-	TC	Blastocyst	20	3 (15.0)	[88]
	Fat-1	TC	1-2 cells	1633	12 (0.7)	[89]
Fetal somatic Stem cells	-	TC	1-cell	(70-100) × ^d	7 (1.43-1)	[73]
Genital ridge cells	-	TC	4-6 cells	164	2 (1.2)	[68]
Granulosa cells	-	TC	1-cell	401	5 (1.2)	[65]
Heart cells	-	TC	2-4 cells	1439	8 (0.56)	[69]
Preadipocytes	-	TC	1-4 cells	732	5 (0.68)	[71]
Salivary gland-derived progenitor cells	-	TC	1-4 cells	615	12 (1.95)	[72]
Skin fibroblasts	Transgenic sow (aLApLF+aLA-hFIX)	TC	1 cell	685	4 (0.58)	[70]
Skin stem cells	-	TC	1-2 cells	443	3 (0.68)	[74]

^aNo. of piglets/no. of embryos transferred

^bTraditional cloning

^cHandmade cloning

^dRange of embryo numbers transferred per recipient, multiplied by the number of recipients (as indicated in the original report)