

## Cloning Efficiency and a Comparison between Donor Cell Types

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### Abstract

Dolly the sheep cloning “the first cloning process” was derived by somatic cell nuclear transfer and it had reported that the clone got premature senescence as a result of continues telomere shortening and ongoing decrease in telomerase activity by time; thus majority of recent researches is directed to use other types of donor cells to be transferred into the recipient enucleated oocyte to maintain full-term and healthy postnatal development for the clone. Embryonic cells such as embryonic stem cell and primordial germ cell were on top as a product of high activity of telomerase, but it cannot be used to clone an adult and primordial germ cell could only be used in nuclear transfer in early stages of embryonic development. Adult stem cell and adult germ cell also had shown increase in telomerase activity but using germ cell as a donor cell at specific day postcoitum had shown incompetence to satisfy full-term development of embryo making it inadequate to be donor cell in cloning process. Another effective cell that could reverse cellular aging in cloning is fibroblast cell. Sertoli cell in male and cumulus cell in female had shown the high efficiency to be donor cells in nuclear transfer. As we suppose that cloning will soon be applicable in many fields such as pharmacy, agriculture, biotechnology and medicine, here, we review current progress in cloning including several types of donor cells had been used in cloning.

**Keywords:** Nuclear transfer (NT); Telomerase; Donor cell; Enucleated oocyte

### Introduction

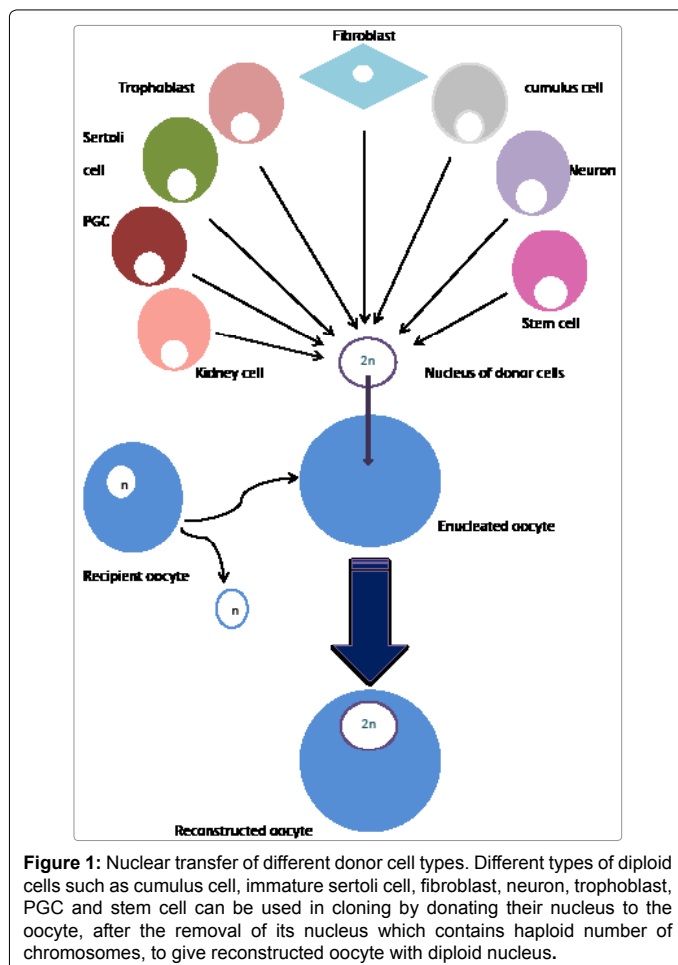
When scientists first discovered anesthesia, atomic energy, and recombinant DNA, we did not know if these breakthroughs might lead

to deleterious applications. The choices we make for the application of knowledge reside in ethical decisions by humans. Animal cloning, like other researches, was initiated to seek fundamental knowledge for the benefit of humankind.

Cloning animals through somatic cell nuclear transfer (SCNT) techniques, in its simplest forms, involves the isolation of a diploid nucleus of a donor somatic cell taken from the animal and then it is injected or fused to a recipient enucleated oocyte (Figure 1). Nucleus is then reprogrammed by the recipient oocyte and acquires totipotency. The reconstructed oocyte is then stimulated by an electric shock to begin the mitotic division and the blastocyst, which is an early stage of embryonic development, is formed. Blastocyst then is implanted into a foster mother to develop to term. Finally, the animal is borne with identical genome to the original organism so it is called a clone.

In fact, SCNT is simply inefficient. The success rate ranges from 0.1 percent to 3 percent [1], which means that for every 1000 tries, only one to 30 clones are made. Some reasons of this high rate of failure are the enucleated egg and the transferred nucleus may not be compatible or the egg with a newly transferred nucleus may not begin to divide or develop properly [2].

As cells divide, their chromosomes get shorter. This is because the DNA sequences at both ends of a chromosome, called telomeres, shrink in length every time the DNA is copied, the cause is supposed to be incomplete replication of telomere and other factors may contribute in telomere shortening such as exonuclease, DNA metabolism and associated processes [3,4]. So, what happens to the clone if its transferred nucleus is already pretty old? Will the shortened telomeres affect its development or lifespan? Dolly “the first cloned animal” was borne in



**Figure 1:** Nuclear transfer of different donor cell types. Different types of diploid cells such as cumulus cell, immature sertoli cell, fibroblast, neuron, trophoblast, PGC and stem cell can be used in cloning by donating their nucleus to the oocyte, after the removal of its nucleus which contains haploid number of chromosomes, to give reconstructed oocyte with diploid nucleus.

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1997 by SCNT cloning. Genetic material of somatic cell was transferred into an enucleated oocyte, as it was transferred from an adult somatic cell; it had shown decrease in telomere length and telomerase activity. Dolly the sheep's chromosomes had shorter telomere lengths than normal [5]. This means that Dolly's cells were aging faster than the cells from a normal sheep. Telomere shortening does not take place during gametogenesis leading to the production of embryos "from natural mating" with normal telomere lengths [6]. Although the efficacy of cloning is low, it appears that at least some somatic cell genomes can acquire totipotency following transfer into enucleated oocytes.

Current progress is attributed to developments of donor cells such as using embryonic cells instead of adult cells for nuclear transfer (NT) in order to maintain high activity of telomerase and long telomeres in the clonal cells. Stem cells are also used as they show increase in telomerase activity. Fibroblast and cumulus cells could reverse cellular aging [7]. All of them share the same goal to restore the clone's cells to youthful

state. Whatever the source of donor nucleus is, reprogramming by NT is required to facilitate embryonic development properly [8].

Somatic cell cloning is now being applied extensively in the production of clones of individuals, generation of animals with genetic modifications for agricultural and pharmaceutical purposes, it is also used in a wide range in biomedical research and regenerative medicine "therapeutic cloning" to grow human cells and tissues from the cloned ESC [9,10]. One of the most important applications of nuclear cloning is conservation of animals particularly animals that are on the edge of extinction or even extinct. An example on that is the rescue of burcardo (*Capra pyrenaica pyrenaica*) which is an extinct wild goat, but the clone died few minutes after birth [11]. Also cloning endangered species such as the gaur (*Bos gaurus*) which is a large wild ox [12] and mouflon (*Ovis orientalis musimon*), which is a subspecies group of the wild sheep, that was cloned using a post-mortem somatic cell [13]. Cloning applications are discussed in more details in [14].

Authors	Donor cell	Percentage of cloned animals per ET			Reference
		1.5-2% at 8.5 dpc	4.5% at 9.5 dpc	1.3% at 10.5 dpc	
Yamazaki et al.	PGC				8
Cibelli et al.	Adult fibroblast	10.7%			42
Ogura et al.		2.6%			32
Inoue et al.		3%			20
Inoue et al.		1.9% male	0.7% female		21
Ogura et al.	Fetal fibroblast	1.3%			32
Ono et al.		0.7%			44
Ogura et al.		0%			31
Wakayama et al.	Cumulus cell	2-0.8%			49
Lanza et al.		1%			33
Zou et al.		1.3%			52
Kato et al.		83%			46
Inoue et al.		1.5%			21
Makino et al.		7.1%			76
Mizutani et al.		5.7%			36
Ogura et al.		5.7%			31
Ogura et al.		6.5%			32
Inoue et al.		4.7%			21
Mizutani et al.	Sertoli cell	6%			36
Wakayama et al.	ESC	4.9%			16
Saito et al.		43%	34% genetically modified		68
Eggan et al.		SCNT	F1		17
	inbred				
Eggan et al.	Tetraploid blastocyst complementation	17%		8%	
		6%			
Kato et al.	MSC	7.6%			47
Inoue et al.	HSC	0.7% male	0% female		21
Mizutani et al.	NSC	0.5%			19
Inoue et al.		1.6%			20
Makino et al.	Cerebral cortex neuron	0%			76
Mizutani et al.	Neural cell	male		female	36
		Pyramidal cell	10.2%	4.6%	
		Purkinje cell	0%	1%	
		Dentate gyrus cell	3.8%	2.8%	
		Cerebral cortex cell	2.4%	2.9%	

Table 1: Comparison between donor cell types.

Herein, we attempt to summarize our current understandings of cloning techniques and discuss several types of donor cells for NT showing their effect on embryonic development and its healthy life (Table 1).

### Cloning; current progress

Nuclear cloning technology has wide applicability to be developed, however inefficiency of the first cloning process, thought to be caused by the donor nucleus remains to be a major issue. Accordingly, many attempts were made to find an appropriate type of donor cells to achieve better results in NT.

Within the variety of cells that can be nucleus donors, there were some ongoing trials on primordial germ cell depending on the fact that undifferentiated cells are more efficient donors than differentiated somatic cells in NT [15], but this is provided at a certain day postcoitum (dpc) at which primordial germ cells show pluripotency which makes these cells effective nucleus donors, after that time their efficiency in cloning decrease because of their differentiated state [8], so adult germ cells might not be an efficient donor cell despite their high telomerase activity.

Other types of cells with high telomerase activity are stem cells which are undifferentiated cells with multiple differentiation capacities. Embryonic stem cell (ESC) which is a pluripotent cell had shown efficiency to be nucleus donor in cloning [16-18]. Multipotent stem cells can be neural, mesenchymal or hematopoietic stem cells (HSC). Neural stem cell (NSC) [19,20] and HSC [21] were thought to be suitable sources in cloning for their undifferentiated nature, but experimental results had proved the inefficiency of these cells to be nucleus donors however, the developmental rate was higher in embryos derived from mesenchymal stem cell (MSC) [22-25]. Skin-derived stem cell had also shown high efficiency when used as a source of nucleus in NT [26]. Certain types of stem cells had proved to have the longest telomeres over body cells of the mouse [27].

Other successful studies were applied on cumulus oophorus cells in female and sertoli cells in male. Cumulus cell, also called discus proligerus, are cluster of cells surrounding the oocyte as a protective function and also it induces oocyte maturation [28]. These cells are the standard donor cells in female mouse cloning [21] furthermore, they could reverse cellular aging of the clone [7,29]. Sertoli cells, also called mother or nurse cell, are cells of the testicles which is a part of seminiferous tubule, its main function is to nourish sperm development through spermatogenesis stages [30]. Immature sertoli cells had shown higher efficiency in cloning than other donor cell types such as fetal fibroblast [31,32], adult fibroblast [21,32] and HSC [21].

Fibroblast had been also used in a study to be the nucleus donor. Fibroblast is the most common cell in animal connective tissue; it has the ability to synthesize the extra cellular matrix and collagen. Senescent fibroblast was used in a study of cloning related to telomere length and telomerase activity to see the effect of senescence of donor cells in cloning efficiency, although the nucleus to be transferred is senescent; effectively normal healthy animals were cloned with elongation in their telomere length and extension of life-span [33]. Developmental rate of offspring cloned from adult fibroblast per embryos transferred was less than that of immature sertoli cells and cumulus cells [21]. Fetal fibroblast efficacy is also less than mesenchymal stem cells [22,25] but it had proved to be more efficient than other cells such as mammary epithelial cells [34].

Neural cells are the main component of the central and peripheral

nervous system. They process and transmit information in the form of signals. Only certain types of neural cells could support embryonic development and had good cloning efficiency such as mouse olfactory sensory neurons [35] and pyramidal cells [36], however cerebral cortex cells and dentate gyrus had lower cloning efficiency [36].

Trophoblast cells are the outer layer cells of a blastocyst which are first to differentiate from the fertilized egg. Trophoblast had been used in cloning cow [37], mouse [38] and buffalo [39]. The authors had accounted that trophoblast cells did not offer any advantage in cloning over fetal or adult fibroblast.

Porcine primary kidney cell had been successfully used to produce transgenic pigs [40]. It had shown higher transfection efficiency, proliferation capacity and blastocyst formation than fibroblasts.

### Germ cell as a source for cloning

Germ cells are diploid cells that give rise to gametes with haploid genome through several series of meiosis and mitosis division. In animals, it gives sperm in male through spermatogenesis and gives oocyte in female through oogenesis. In mouse embryo, cells of pluripotent epiblast are induced to be primordial germ cells (PGCs) that migrate by 8.5–9.5 dpc from the epiblast, proliferating through migration, reaching the developing genital ridge by 10.5 – 11.5 dpc in a mouse [8]. Germline genome initiates epigenetic reprogramming at the 10.5 dpc by methylation of the H19 paternal allele-specific and is completed by 13.5 dpc [8,41].

Germ cell when used as a donor cell for NT, experiments were carried out on the fetal mouse PGC at several stages of embryonic development beginning with migration from pluripotent epiblast till reaching the genital ridge, within this short period PGC had shown development in its genome potency and epigenetic modifications that are important to assess whether if these cells are adequate to be nucleus donors or not [8]. PGC is a good choice for cloning for, being embryonic cells, they are characterized by long telomeres and high telomerase activity, this should give the clone normal life time identical to the age-matched non-clone and another considerable reason is that PGC at 8.5-9.5 dpc can express genes characteristic of the pluripotent embryonic cells [8] making these cells successful donors [8,15].

Potential development of PGC genome is necessary to indicate the efficiency of the cell in NT as it is thought that the more potency, the less reprogramming and the more efficacy [15]. When a study compared between PGCs in different developmental stages of mouse embryo, they found that the most efficient cell stage is at 9.5 dpc that had given normal embryo with normal forelimb buds [8,41]. Gross morphology of embryos was examined on the day 10.5 of pregnancy and it had confirmed that PGC when used by 10.5 dpc, had shown normal development [41], but in another study on the same animal, the clone only lived two days after birth [8]. Other stages of PGCs when used at (11.5-15.5) dpc, had shown variation in retardation of embryonic development from slightly to sever retardation, the most retarded was the animal embryo cloned from PGC at 15.5 dpc [41].

As shown, using PGC in cloning is more efficient at a particular stage of primordial cells (9.5 dpc) for its pluripotent genome and then the more chance to have normal healthy embryo so from self-evident, adult germ cells –like PGC at late stages–is less efficient to be nucleus donors, in other words, these studies are restricted for cloning embryos only and it cannot be used to clone an adult. Several applications of cloning such as agriculture, conservation of certain animals or regenerative medicine, cloning an adult is required, so further type of adult somatic cells is needed that can acquire totipotency and achieve

high cloning efficacy to produce normal healthy clones.

### Fibroblast as a source for cloning

Fibroblasts, the active form of fibrocyte, are large, flat, elongated spindle-like cells which possess many processes extending out from its body. They are mainly forming the most common cells of connective tissue. Fibroblasts produce collagen, and all component of extracellular matrix, the ground substance which is an amorphous gel-like matrix fills the spaces between cells in tissue and variety of fibers. Fibroblasts appear to play important role in wound healing. In embryo, fibroblasts, like any type of connective tissue cells, are derived from primitive mesenchyme.

Fibroblasts were used to be the source of nucleus in studies related to mammalian aging which could reverse cellular ageing by NT. Donor cells used in these studies were senescent cells where fetal fibroblasts were isolated from female bovine embryo and cultured until senescence [33,42] or directly from adult cells which were cultured briefly [7]. Senescent fibroblasts had shown a surprising behavior. Although they were old, results were unexpected; the cloned calves were borne with more extended telomere and other signs proving that they were younger than age-matched controls [33]. They had shown longer telomeres than the donor cows and similar to age-matched *in vitro* fertilized embryos [7]. Three healthy cloned calves were generated out of 28 embryos transferred, so efficiency of senescent fibroblast to be nucleus donor would be 10.7% [42]. The authors had accounted that NT could reverse cellular aging and extend life-span of senescent cell and that telomere restoration usually occurs during embryogenesis by the increase in telomerase activity [7,33,42].

Adult fibroblast was also used by a study for cloning that compared between efficiency of certain donor cells in cloning. Cells were collected from mouse tail-tip tissue at 2-4 months age, the percentage of developed offspring per embryo transferred (ET) in male had been 1.9% and 0.7% in female, this percentage is quite efficient compared to other cell types used (1.5% for cumulus cell and 4.7% for sertoli cell) [21].

Fetal fibroblast (FF) efficiency was also examined by other studies carried on porcine female fetus on day ~30 in comparison to another cell type (MSC). The rate of blastocyst formation was a relatively low comparing to MSC and the rate of apoptosis was relatively high [22,25]. The obtained results show that MSCs are more efficient than FFs in cloning, but comparing to other cell types such as mammary epithelial cells, it had shown relatively higher efficiency in a study for cloning lambs which used FF derived from a day-26 ewe fetus [34]. Using FF as a donor cell is restricted for cloning embryos only but not for cloning adults and its economic benefit is not predictable.

Although it is known that cloning from donor cell in G0 or G1 phase of the cell cycle is necessary for embryonic development [34,43] for the proper reprogramming by NT and it is difficult to use a donor cell arrested at meta phase owing to its relatively large size and difficulty to be reprogrammed by NT for its highly condensed chromatin, a study could successfully clone mouse from fetal fibroblast arrested at metaphase of the cell cycle by serial nuclear transfer technique, in which nucleus is fused to an enucleated oocyte, after activation, it is transferred to an enucleated fertilized one-cell stage embryo to facilitate reprogramming of the implanted nucleus [44]. This method might be applied on HSC as it is thought that its genome is resistant to reprogramming [21]. However, cloning efficiency is relatively low as only two pups per 272 ET (0.7%) could reach adulthood and other two pups died for umbilical hernia and respiratory defects. No healthy offspring produced from single nuclear transfer technique and all of

the clones from both techniques had shown placental defects [44]. This confirms the ability to use a donor cell arrested at metaphase of the cell cycle, but efficiency is quite low. A study could improve injection technique to allow injection of metaphase nuclei by "the hole removal technique" in which the membrane of the pipette located at the entry site is removed to make it easier to inject large size nuclei [45]. This could increase cloning efficacy and increase survival rate of reconstructed oocytes.

### Cumulus cell as a source for cloning

Cumulus cells are cluster of cells surrounding oocyte in both ovarian follicle and ovulation. The innermost layer of these cells must be penetrated by spermatozoa for fertilization. The main function of cumulus cells is to protect oocyte and induce its maturation [28]. When oocyte is isolated, a group of cumulus cells is found surrounding it. This is usually called oocyte-cumulus complex.

Cumulus cells are the most common cells used in female animal cloning. These cells are appropriate donors due to their high efficiency shown in cloning and they can be easily obtained without causing injury for animals to be cloned [46] and easily injected to recipient oocyte owing to their small size [31]. Generally, it is accepted that relatively high efficiencies of cloning can be achieved by using donor cell from female reproductive system [47] such as oviduct, granulosa or cumulus cells. More than 90% of cumulus cells surrounding ovulated oocytes are in the G0/ G1 phase of the cell cycle [48], so it is unnecessary to synchronize their cell cycle phase before NT. Talking about cumulus cells as source of nucleus in NT is an indication of cloning female animals only.

Many studies carried out on cumulus cells were applied on different animal types. Some of them were applied on mice; the proportion of cloned mice developed to term per blastocyst transferred was (2-2.8%) with normal phenotype [49], but a lower percentage had been reported by another study that had reported that only 5 normal mice developed to terms with a percentage 1%, one of them died at the first day, two lived for only two weeks, the fourth lived for a month and the fifth cloned mice lived for fourteen months [33]. Obesity is a common feature that characterizes mice cloned by cumulus cells [33,50,51]. That phenotype appears in adulthood and not transmitted to their offspring [33,50]. It is suggested that the cause of obesity is the expression of agouti gene (encodes the agouti protein) that mouse carry, however, mouse that did not carry agouti gene were also heavier than control [51], further studies are required to explain the obtained results.

Cumulus cells were used as a source of nucleus in cloning goats by a study which had reported that the proportion of offspring developed to term per ET had been 1.3%. They had obtained 3 goats from cloning, but two of them died shortly after birth for breathing difficulties although they had normal birth weight with no obesity shown, they were derived from *in vitro* cultured cells without starvation. The third goat could survive for longer period, it had shown obesity as the weight of birth was 37% overweight, the goat was derived from starved cumulus cell [52]. Both donor cells produced from serum starvation and without starvation could support embryonic development. The authors assumed that any cells rather than the G0 stage can be reprogrammed with exception of cells in S phase for the replication of certain regions of genes at this phase of cell cycle which may lead to abnormalities.

Another study used cumulus cells in comparison with oviduct cells for cow cloning Successfully, number of cloned cows was large number per ET with a percentage 80% for both cumulus and oviduct cells. For cumulus cells, five out of six ET were borne so efficiency



would be 83% as separate, but three of them died at or immediately after birth. Postmortem analysis had reported that death was caused by environmental factors and it did not reveal any abnormality. This high percentage of efficiency might be related to culture system used, quality of transferred blastocyst and another important factor is that nucleo-cytoplasmic interaction between bovine cumulus nucleus and oocyte cytoplasm might be compatible and suitable for latter embryonic development [46].

Like fibroblast, telomere lengths in the cells of animals cloned from cumulus cells were not different from that of age-matched controls and there were no premature aging of the cloned cows [7] and mice [29]. The study for cloning mice had investigated telomere lengths in the cloned mice to six successive generations using two independent mouse cell lines. The total percentage of cloned pups per ET had been 0.6% and 1.1% and all of them had shown increase in telomere lengths [29]. These results, besides senescent fibroblast results, might be explained by the ability of NT to reverse cellular aging as general or due to the high telomerase activity in cumulus cells used in this study as special.

A study for evaluation of telomere length in cumulus cells had reported that telomere length in these cells is associated with oocyte maturation. Shorter telomeres were found in cumulus cells surrounding immature oocyte and poor-quality embryo, but longer telomeres were found in cumulus cells surrounding mature oocyte and high-quality embryo [53]. If the ability of cumulus cells to produce embryos with long telomere is attributed by its own telomerase activity, this might give the proper selection of cumulus cells before being used in order to maintain longer life span for the clone.

The difference between these studies and other studies of cloning might be explained by difference in donor cell types or types of animals that are the providers of donor cells [7,53]; however, it is still unclear which of them is the general rule and which is the exception. The ability of fibroblasts and cumulus cells to produce embryos with long telomere and high telomerase activity might have various applications in regenerative medicine to grow human young cells, tissues and organs from the cloned ESC. This also could be an effective factor in agriculture field to produce healthy cloned animals with more extended life-span. Further investigations on other types of cells are required to assess whether if cellular aging can be reversed by the NT of any donor cell generally or by cumulus cell and fibroblast only.

### Sertoli cell as a source for cloning

Sertoli supporting cells are located within the seminiferous tubule in the testis in male and sit on the basal membrane. Their main task is the nourishment of spermatozoa, so they are called mother or nurse cells [30]. In sertoli cell-only syndrome, a type of azoospermia, in which only sertoli cells line the seminiferous tubules in the testes leading to sterility. Immature sertoli cells are the counterpart of cumulus cells in male animal cloning. It is the most favored donor cells in male-mice cloning; this is due to, beside their high efficiency, they are easily reprogrammed by NT and more successful gene activated, this might be due to their small size [54].

A study used fresh, cultured, cryopreserved and transfected sertoli cell as a source for mouse cloning, the proportion of the clones per blastocyst transferred when fresh sertoli cells were used at day 3-5 of the donor animal age had been 4.5%, but when used at day 8-10, it had been 1.2% [31]. This shows that the ability of sertoli cells as donor cells decreases by increasing the age of the animal to be cloned. This obtained result had been confirmed by another study that used sertoli cells isolated from mature testes of male which could not develop any

offspring [49]. Immature sertoli cells efficiency after a weak culture was not different than fresh cells as it had been 3.1% and 6.7% after cryopreservation, but transfected cells with gene encoding a green fluorescent protein had failed to support embryonic development, this is supposed to be due to the exogenous gene or prolonged in vitro culture which might have caused abnormalities. Most of developed offspring were healthy and normal [31].

Immature sertoli cells had shown high efficiency among different donor cell types such as fetal fibroblast with a percentage 5.7% versus 0% [31] and 6.5% versus 1.3% [32], adult fibroblast with a percentage 7.4% versus 1.9% [21] and 6.5% versus 2.6% [32] and HSC with a proportion 7.4% versus 0.7% [21]. Beside this high efficiency, most of the cloned mice had shown several diseases such as pneumonia, hepatic failure, reduction in antibody productivity [32,52], body weight increase of some of them which is suggested to be resulted from agouti gene, early death of some of them [32], leukemia, lung cancer and high rate of death as out of 12 cloned mice, they started to die 311 day after birth and 10 died before 800 day after birth [55].

Less than 70% of immature sertoli cells are in G0/ G1 phase of the cell cycle [56], while mature sertoli cells usually do not divide and remain in G0 phase of the cell cycle [49]. Thus, it is predictable that mature sertoli cells would also be efficient in cloning, but the fact is that the more age the less efficiency of sertoli cells to be nucleus donor [31,49]. This is due to the large size of mature sertoli cells which makes it hard to be injected without causing damage to its nucleus and enucleated recipient oocyte [31]. Also mature sertoli cells had shown decrease in telomerase activity which had been reported in a study that measured telomerase activity in sertoli cell-only testes [57] and it is known that telomerase activity is higher in younger ages, so immature sertoli cells are more efficient to be the source of nucleus to confirm supporting longer healthy life for the clones. To have the ability of cloning only young ages is a disadvantage of sertoli cells because this way, adult animal cannot be cloned by these cells which might be required in many fields such as agriculture and biotechnology.

### Stem cell as a source for cloning

Stem cells are undifferentiated cells characterized by their high telomerase activity and long telomeres [27], classified according to their origin into: Embryonic stem cells (ESC) and adult stem cells. Generally stem cells are used in wide range in cloning as a source of nucleus according to the fact that the less differentiation, the easier of reprogramming and the more efficiency [15]. ESCs were used by many investigations as a nucleus donor cells with a high rate of success [16-18], however, many chromosomal abnormalities [58] and gene expression defects [15,59,60] were observed in the clones. MSCs had shown higher efficiency in cloning than many other donor cell types [22-25]. Fetal porcine skin-derived stem cell [26] and deer antler stem cell [61] were also used as nucleus donor cells in cloning. HSC [21] and NSC [19,20] had the lowest efficiency among the different stem cell types.

### Embryonic stem cell as a source for cloning

Embryonic stem cells are pluripotent cells [62] which can be isolated from the inner cell mass of a blastocyst at early stages of embryonic development. ESCs are regarded as one of the most efficient donor cells in cloning for its pluripotent genome [15]. Further technique had been developed for cloning from ESC [15,17,63] and induced pluripotent stem cell (ips) [64,65] rather than SCNT technique. It is the tetraploid blastocyst complementation, in which ESCs or ips cells are injected to form the inner cell mass of tetraploid embryo which cannot contribute

embryonic development so, genetic material would be from the injected stem cell only. This method has been applied on mouse ESCs [15,17], cow ESCs [63], mouse mature T-cells and B-cells by two-step cloning procedure in which clonal ESCs were established from the blastocyst, which had been cloned by SCNT, and then injected to the tetraploid embryo [66] and mice ips cells in which embryonic fibroblast were reprogrammed into ips cells by the reprogramming factors “Oct4, Sox2, c-Myc and Klf4” and then injected to the tetraploid blastocyst embryo [64,65].

The advantage of using this technique instead of SCNT is to achieve more adequate reprogramming of the donor nucleus and also it gives the ability to have the total genome including mitochondrial DNA which is an absent advantage in SCNT [67]; however, this advantage cannot be achieved by the two-step cloning procedure due to the presence of the mitochondrial DNA of the enucleated oocyte in the first step performed by SCNT technique.

A study could successfully clone mice using ESC at late passage transferred to enucleated metaphase II oocyte. The total rate of survived clones for both cell lines (E14 and R1) per ET was 4.9% (1.6% for E14 and 8.3% for R1) [16]. The authors had accounted that these ESCs used at late passage could support embryonic development to produce fertile cloned mice even after prolonged culture. This is a useful feature of ESCs at late passage that can be used in gene modification through cloning to produce transgenic animals. It had failed in immature sertoli cells [49].

All cloning reports had demonstrated that early cell passage is required to be a source of nucleus [68] so, there is a need for cells that can support development to birth and beyond after gene modification and can be used in early passage. A study could produce cloned calves with efficiency 34% for transgenic calves and 43% for non-transgenic using ES-like cells [68]. Rate of fusion of oocyte and cleavage of embryo were not affected by number of passages, however, rate of blastocyst formation was not high (4%); this might be due to the inadequate cell cycle of the donor cells.

ESC in G1 phase of the cell cycle is suggested to support postimplantation development; however, approximately 60% of ESCs are in the S phase of the cell cycle [69]. S phase is a stage of cell cycle in which the cell is inadequate to be in donor cell in NT because parts of genes replicates at this phase leading to less efficiency of blastocyst formation [52,69] due to defective reprogramming [52]. This might explain the abnormalities in clonal animals from ESCs [15,17,58,59]. Even if the clone could appear normal, gene abnormalities might be present leading to undetectable physiological abnormalities which were demonstrated by a report that examined gene expression in cloned mice from ESC at early passage by SCNT and tetraploid blastocyst complementation techniques to see if defects are from the technique or ESC line [15]. The authors assumed that ESCs have an unstable genome and that the resulted abnormalities do not affect fetal growth.

Another study compared between both technique for two different cell lines (F1 and inbred ES cell lines), applied on mice, to assess whether heterozygosity of donor ESC genome would affect cloning efficiency or not [17]. The percentage per ET of the cloned mice developed to terms from F1 ES cell line by SCNT had been 17% and most of them reached adulthood, but there were increase in placental birth weight, 18% were produced by tetraploid blastocyst complementation with normal weight and most of them developed into fertile adults. This is quite high percentage comparing to SCNT of inbred ES cell lines in which 8% of ET lived to terms but none of them reached adulthood for

respiratory distress and also there were increase in placental weight, 6% were produced by tetraploid blastocyst complementation with normal weight, but most of them died also for respiratory failure. The authors assumed that death of mice cloned from inbred ES cell lines is due to the decrease of developmental potency than F1 ES cell lines. This shows that heterozygosity of ESC genome is an important parameter for postnatal survival of the clones. This result had been also reported by another study for SCNT of mice inbred ES cell lines, however, 27.8% of the clones could survive to adulthood [18].

In order to increase ESC cloning efficacy, one might speculate that using growth inhibitors, to arrest cells at G0/ G1 rather than S phase of most of ESCs, such as serum starvation [59] or seeding cells at high confluency [18,59]. In serum starvation, 59% of starved cells arrested at G0/ G1 were obtained rather than 25% of the non-starved [59]. Beside the high efficiency of using high confluence ESCs (45% blastocyst formation) [18], overexpression of insulin-like growth factor II (Igf2) and H19 genes were observed in animals cloned from high confluence and serum starved cells which might lead to overgrowth defects [59].

Cloning adult animals for many applications such as agriculture to preserve needed characters or conservation of certain animals cannot be achieved by ESCs, except for the two-step technique using adult cell at the first step and cloned ESCs in the second [66]. However, using ESCs at late passage [16] or ES-like cells at any passage [68] can produce transgenic animals with the required characters. Producing transgenic animals is important for many fields such as agriculture and biotechnology.

### Mesenchymal stem cell as a source for cloning

Mesenchymal stem cells are adult multipotent cells that can be derived from different sources. MSC had shown greater potential to be a nucleus donor in cloning than both fetal fibroblast [22,25] and adult fibroblast [24,70]. Majority of MSCs are arrested at G0/ G1 phase of the cell cycle [22] which is a required feature in the donor cells.

MSCs had been used as a donor cell to clone pigs [22,24,25,70] and cows [23,70] with high rate of success achieved by number of factors: MSC had shown facility to be reprogrammed by NT [24] due to its undifferentiated genome, it had shown ability to acquire totipotency after NT [23,24], higher rate of blastocyst formation than fetal fibroblast [22,25] and adult fibroblast [24,70] with no difference from in vitro fertilized (IVF)embryos [22,25], lower levels of apoptosis than fetal fibroblast [22] and G0/ G1 arrested phase of the cell cycle of majority of them [22]. One healthy cloned pup out of 13 ET was obtained from MSC nuclear transfer, so the percentage would be 7.6% success [23].

One of the most important features of MSC is the stability of its genome thus, the ability to be genetically engineered. It could have been successfully modified by viral and non-viral vectors [24]. This would be a key for producing transgenic animals used in agriculture, biotechnology and other fields.

### Hematopoietic stem cell as a source for cloning

Hematopoietic stem cells are adult multipotent stem cells located in the bone marrow. They give rise to all other blood cell types. However it is known that the undifferentiated cells has the most affinity to be reprogrammed by NT and give the most cloning efficiency [15], HSC had broken that thought.

A study for cloning had compared between HSC and other donor cells (cumulus, immature sertoli and adult fibroblast) [21]. Unexpectedly, HSC had the lowest rate of blastocyst formation and

lowest efficiency among other donor cells. However, two male cloned mice were obtained with normal appearance (0.7% per ET), but no offspring were obtained from female HSC. The authors demonstrated that HSC could initiate zygotic gene activation (ZGA), but failed to activate 5 out of 6 necessary embryonic genes which might reflect the resistant of its genome to reprogramming.

### Skin stem cell as a source for cloning

Skin stem cells are multipotent cells [71] found in skin, they have the ability of differentiation and production of different skin cell lineages. They are active during skin renewal and repair after injury. Fetal porcine skin-derived cells had shown high efficiency in cloning with high quality of embryos produced [26,72].

Porcine fetal skin-derived stem cells are arrested at the G0/ G1 of the cell cycle for majority of them [26], which is a good feature for donor cell. In a comparison to fetal fibroblast, it had been reported that blastocyst formation rate was higher in the fetal skin stem cell than fibroblast [26,72].

Porcine skin-originated sphere (PSOS) stem cell derived from fetal skin had shown great genome stability making these cells capable of genetic engineering to produce genetically modified animals [26] which can be used in agriculture, biotechnology or other biological fields. The ability of fetal PSOS to produce offspring with low rate of abnormalities may be a good choice to help in developing porcine cloning technique [26]. However, further studies are needed to investigate the ability of them to support embryonic development in other species of animals.

### Antler stem cell as a source for cloning

Red deer had been cloned from the multipotent antler stem cells, which is present in the horn of male deer, and their differentiated progeny (putative bone and adipocyte). They were successfully reprogrammed and could produce eight healthy cloned red deer calves that could survive to weaning and beyond. They were genetically identical to the donor cells. Two of them were cloned from the undifferentiated antler stem cell [61].

The rate of blastocyst formation and developmental rate to weaning of the undifferentiated antler stem cell was similar to differentiated donor cells. Contrary to the previous stem cell cloning studies, this study reported that there is no correlation between differentiation degree of the donor cell, reprogramming and consequently cloning efficiency.

These obtained results might explain the inefficiency of the multipotent HSC to be reprogrammed and to produce offspring despite its undifferentiated genome [21]; however, they are contrary to the obtained results of cloning from MSCs [22,24,25,70] and skin-derived stem cells [26,72] with higher efficiency than differentiated somatic cells.

### Neural stem cell as a source for cloning

NSC are self-renewing multipotent stem cells that give rise to neurons, astrocytes (star-shaped glial cell in the brain and spinal cord) and oligodendrocytes (a type of neuroglia that supports neurons in the central nervous system) [73].

Like HSC, NSC did not show high efficiency in cloning [19,20]. A study for cloning used NSC isolated from porcine brain in comparison to differentiated NSC, ESC, immature sertoli and cumulus cell [19] and MSC and adult fibroblast in another study for cloning mice [20]. The rate of development to the two-cell stage of the undifferentiated NSC

was relatively high (73% [19], 76% [20]), however, the rate of blastocyst formation was very low (7%) [19]. The proportion of viable offspring produced by cloning per ET had been 0.5%. This is a low percentage comparing to ESC (3.5%), cumulus cell (2.7%), and immature sertoli cell (2.2%), but differentiated NSC had failed to produce any viable offspring [19]. NSC had shown higher efficiency than MSC (1.6% versus 0%), but lower than adult fibroblast (3%) [20].

The authors had demonstrated that cloning efficiency depends on epigenetic and genetic status of the original genome of the donor cell as MSC could not produce any offspring and the rate of embryonic death was high because it had shown chromosomal abnormalities in its original genome [20]. This might explain the low efficiency of some types of stem cells such NSCs and HSCs despite their undifferentiated genome. The ability of NSCs to produce full-term development of the cloned offspring and their high proliferation rate make these cells a good choice to be used in gene modification and transgenesis [19], but efficiency is very low.

### Neural cell as a source for cloning

Neurons or nerve cells are post-mitotic cells that have no ability to proliferate after embryonic development [74]. Neurons are the main component of the brain and spinal cord in the central nervous system and ganglia in the peripheral nervous system.

Studies for cloning had reported that cloning by using fetal (at 17.5 dpc) [75], postnatal [76] and adult [77,78] cerebral cortex is quite inefficient; however, fetal cells at 15.5 dpc had shown higher efficiency. These results suggest that neural cells lose their developmental totipotency by neurogenesis advancement [75,76]. This can be confirmed by the study in which the NSC had more efficiency than differentiated NSC that could not produce any viable offspring [19].

In contrast, a study could produce fertile mouse using post-mitotic olfactory sensory neurons (OSN). The authors demonstrated that OSN could acquire totipotency by NT and that post-mitotic cells have the ability to enter the cell again by the action of the oocyte environment. The obtained result had been confirmed by applying tetraploid blastocyst complementation technique to the cloned ESC which could produce viable offspring. This is an indication that OSN had acquired totipotency [35].

Another study for cloning mice could produce viable offspring by using adult (6-8 week) male and female neural cells in comparison with other somatic cells (sertoli and cumulus cells). Pyramidal cells (derived from the hippocampus) had shown the highest efficiency among the donor cell types used (10.2% in male and 4.6% in female). Purkinje cells (in the cerebellum) had failed to produce any offspring due to its large size which had led to developmental arrest (0% in male and 1% in female). Dentate gyrus (from the hippocampus) had lower efficiency than pyramidal cells (3.8% in male and 2.8% in female). Cerebral cortex also had low efficiency (2.4% in male and 2.8% in female), while sertoli cell efficiency had been 6% and cumulus cell had been 5.7%. The authors suggested that cells with reduced amount of repressive histone marks might increase cloning efficiency [36]. This might explain the low efficiency of fetal, postnatal and adult cerebral cortex neurons and dentate gyrus to be nucleus donors in cloning.

### Trophoblast cell as a source for cloning

Trophoblasts are the cells that form the outer layer of blastocyst which provide nutrients to embryo. They are the first cells to differentiate from the fertilized egg. These cells are the precursor of the placenta so



any defect in them might lead to placental dysfunction.

Trophoblast cells had been used in cloning cow [37], mouse [38] and buffalo [39]. The study for cloning cow by Trophoblast cell that expresses interferon tau (IFN- $\tau$ ) in comparison with adult fibroblast had demonstrated that both cells were reprogrammed efficiently [37]; however, trophoblast stem cell had shown low programmability in mouse cloning, low rate of blastocyst formation (0-15.9%) [38] and lower developmental competence and quality than adult and fetal fibroblast in buffalo cloning [39]. Expression of (IFN- $\tau$ ) in the trophoblast-derived blastocyst had been reported to be higher than the blastocysts derived from adult fibroblast and IVF embryo [37], but it was the same of adult and fetal fibroblast in buffalo cloning [39].

In the study for cloning buffalo, the authors had accounted that there were many differences in gene expression between the trophoblast cells derived from cloned and IVF embryo. This difference in gene expression might be the cause of placental defects of many cloned embryos. Also the nature of the donor cell seems to affect gene expression of the cloned blastocyst as the expression of many important genes in the blastocyst derived from trophoblast cells were different from adult and fetal fibroblast [39]. The authors assumed that using trophoblast cell as a donor cell did not offer any advantage over adult or fetal fibroblast in cloning.

### Kidney cell as a source for cloning

Porcine Primary kidney cell (PKC) had been used as a nucleus donor cell in NT to study the efficiency of these cells to produce genetically modified pigs comparing to porcine fetal fibroblast (PFF) and porcine ear fibroblast (PEF) [40].

PKC is a suitable source for cloning and gene modification for its higher proliferation capacity and subsequently higher blastocyst formation, higher transfection efficiency and more ability to afford prolonged culture than PFF and PEF [40].

There are different methods for transfection of primary mammalian cells can be used including the chemical methods which are calcium phosphate precipitation [79], nanofection [80] and lipofection [81], physical methods that include electroporation [82], microinjection [83] and nucleofection [84] and viral transduction [85]. By applying lipofection, nanofection, electroporation and nucleofection to the PKC, best results were obtained by the nucleofection technique as it had shown the highest transfection efficiency (70%-89%) with high fluorescence intensity of the transfected cells, good cell proliferation and low cytotoxicity [40].

Pig is always a good choice for biomedical research in general for its similarities with human in physiology, size, metabolism and pathology [86], but further studies on other species of animals are required to investigate the ability of kidney cells to produce viable transfected and non-transfected offspring by SCNT cloning.

### Conclusion and perspective

An efficient cloning would provide opportunities to develop agriculture, human medicine) and animal conservation. The success of cloning process is mainly related to proper selection of a donor cell and oocyte, cell culture and the technique used also play an important role in cloning efficacy.

In fact, any animal cloned by SCNT is not truly identical to the donor animal because some of the clone's genetic material comes from the mitochondrial DNA in the cytoplasm of the enucleated oocyte. This

can be avoided by the tetraploid blastocyst complementation technique using ESC [15,17,63] or ips cell [64,65]. Further techniques had been developed to improve reprogramming of the donor nucleus including, serial NT [44], chromatin transfer [87], sperm mediated-activation [88], aggregating somatic cell NT embryos [89], or altering the donor cells' epigenetic marks by treating the donor cell with pharmacological agents [90].

There is a number of considerations that should be taken into account about the donor cell to achieve successful cloning. Donor cell is better to be arrested at G0/ G1 [34,43] or even metaphase [44,45] but never to be arrested at S phase such as ESC [52,69] to achieve successful reprogramming of the implanted nucleus. In order to avoid damage of the donor nucleus and enucleated oocyte during NT, donor cell would better to be small in size, however, cells with large size could be used by the hole removal technique which had been applied on metaphase-arrested nuclei [45], which are characterized by their relative large size, without causing any damage to the donor nucleus and the recipient oocyte. Because it is still unclear whether telomerase activity and telomere length of the donor cell affects the health of the clone and its subsequent life time or not, it might be better to select a donor cell with long telomere and high telomerase activity such as embryonic cells, stem cells [27], cumulus cells surrounding mature oocyte or high-quality embryo [53] or using fibroblast for its ability to reverse cellular aging [7,33,42]. Another important parameter that may affect cloning efficiency is cell differentiation as the undifferentiated cells such as stem cells [15] and pluripotent PGCs at 8.5-9.5 dpc [8] shows more facility to be reprogrammed by NT. It is necessary for the donor cell to achieve the aim of cloning; for example, cloning for producing transgenic animals can be achieved by donor cell that can be genetically engineered and affords prolonged culture to allow gene modification such as ESC at late passage [16], ES-like cell [68], MSC [24], NSC [19], PSOS [26], and PKC [40]. Cloning adult animals for several applications is achieved by using their adult somatic cells to be nucleus donors such as adult stem cells, adult fibroblasts, neural cells, kidney cells or cumulus cells (in female animals) and ESC can also be used by two-step procedure in which the cloned ESCs produced from the first NT cloning step is injected to a tetraploid embryo in the second step [66].

Accordingly, there might be a possibility to clone animals by using the adult germ line stem cell as a source for nucleus for being undifferentiated cell, also it shows the longest telomere over the mouse body cells [27] and being derived from female genital organ, it might have the ability to support embryonic development of the clone [47], but further investigation is needed to confirm this hypothesis.

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