Review Article

Intra and Interspecies Somatic Cell Cloning in Rabbits, Cats and Goats

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Abstract

Nuclear transfer from adult somatic cells is an advanced reproductive biotechnology. This technique can be applied in animal production for producing a high genetically identical copy for endangered species conservation and for therapeutic cloning for human medicine. Our three-year preliminary study indicates the possibility of somatic cell cloning by using the rabbit as model of technical development. Later, interspecies cloning in wild cats and domestic cats was performed successfully in terms of cloned embryo production. Meanwhile, goat cloning served as the model for farm animals.

Keywords : Somatic cell nuclear transfer, development, interspecies, intraspecies.

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บทคัดย่อ

การโคลนนิ่งในสปีชีส์และต่างสปีชีส์ในกระต่าย แมวและแพะ

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การย้ายฝากนิวเคลียสด้วยเซลล์ของร่างกายเป็นเทคนิคใหม่ทางเทคโนโลยีชีวภาพทางระบบสืบพันธุ์ เทคนิคนี้มีประโยชน์ต่อการผลิตสัตว์ที่พันธุกรรมเหมือนกันหลาย ๆ ตัว ช่วยในการรักษาพันธุกรรมของสัตว์ป่าที่ใกล้สูญพันธุ์ และมีประโยชน์ในมนุษย์ในแง่การนำไปรักษา บทความนี้เป็นส่วนหนึ่งของงานวิจัยตลอดระยะเวลา 3 ปี ที่ศึกษาความเป็นไปได้ในการพัฒนาเทคนิคการโคลนนิ่งโดยใช้กระต่ายเป็นต้นแบบ และได้นำไปศึกษาการศึกษาการโคลนนิ่งข้ามสายพันธุ์ ระหว่างแมวป่าและแมวบ้าน ในขณะเดียวกันได้ทำการศึกษาในแพะที่ใช้เป็นต้นแบบในปศุสัตว์

คำสำคัญ: การย้ายฝากนิวเคลียส การพัฒนา Interspecies Intraspecies

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Introduction

There are several applications for nuclear transfer (NT) in the field of animal production and human medicine. For animals, NT helps to increase the production of milk and meat as well as helping better growth performance and disease resistance. NT will also assist in the production of genetically identical twins and more rapid genetic improvement. NT can be used for the preservation and propagation of companion animals such as dogs, cats and horses and as a tool for the conservation of genetic materials from endangered or unique animals (Long et al., 2003). In human medicine, NT technology provides an alternative method for cell-based transgenesis using domestic species by offering genetic modification. The genetically modified livestock can produce human therapeutic proteins in their milk and organs for xenotransplantation.

This paper describes our three-year study on the development of the technique of somatic cell cloning in 3 different species, rabbits, cats and goats by inter and intraspecies cloning with the collaboration of medical, agricultural and zoological conservation organizations in the country.

Rabbits: The development phase

In the development phase of NT, we used rabbits as a model for both recipient oocytes and donor cells. Matured oocytes at metaphase II were collected from superovulated females by oviductal flushing. The rabbit is an interesting animal model as it provides a high number of recipient oocytes per donor. The *in vitro* development of cloned embryos using adult or fetal fibroblast, cumulus cells as donor nuclei was studied.

1. The NT technique

The NT technique in rabbits was developed with the collaboration of Patrick Chesne and Dr. Xavier Vignon from INRA, Jouy-en Josas, France in year 2000. All processes are demonstrated in Fig. 1.

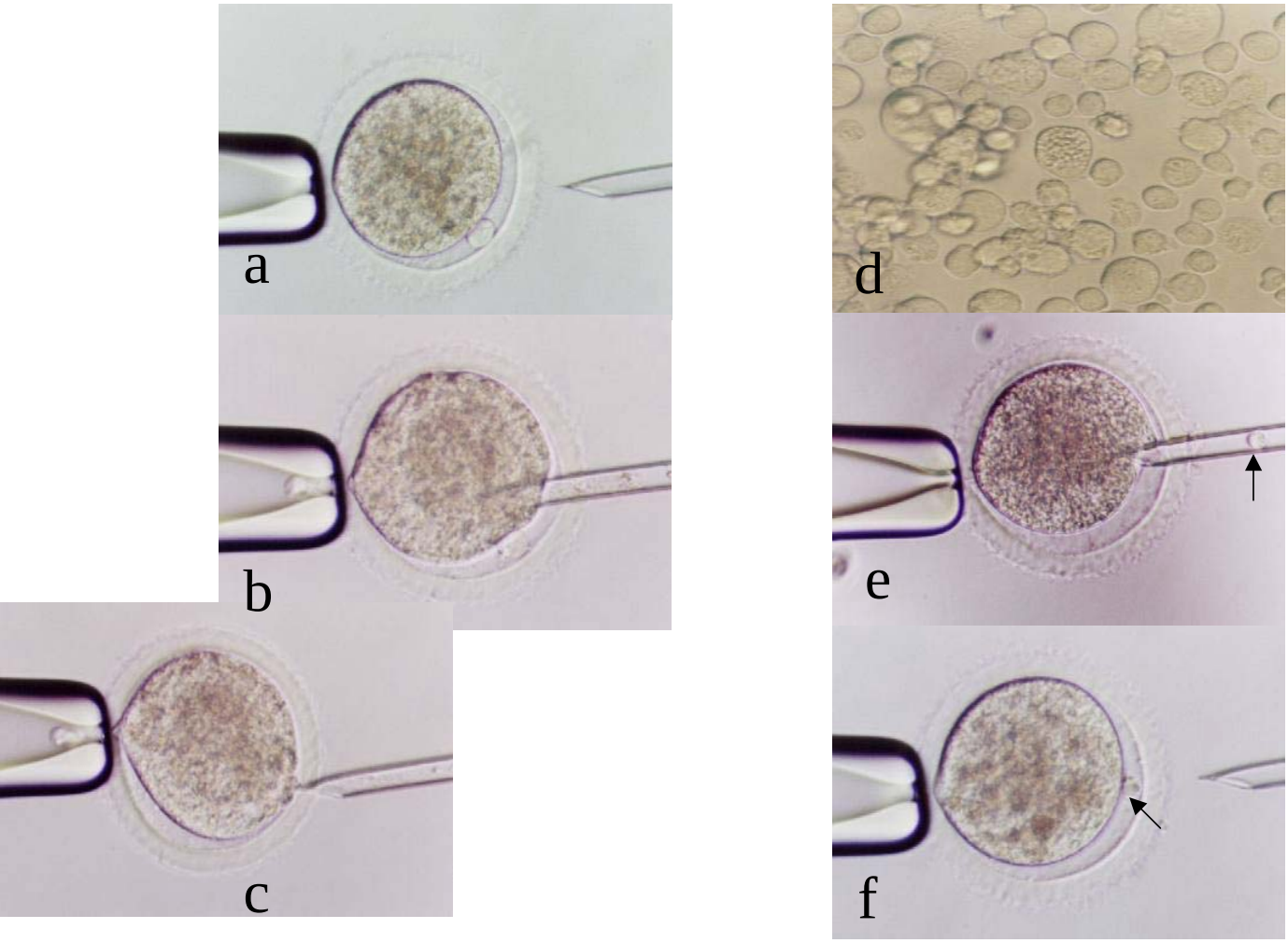

Figure 1 The process of somatic cell cloning in rabbits (x300) (Techakumphu et al., 2004).

a: A matured oocyte with a 1st polar body on the surface of the cytoplasm (arrow). The oocyte was maintained by a holding pipette, with an internal diameter (ID) of 20 m, and the polar body was placed between 4 and 5 o'clock, facing the beveled pipette (BP) of 20 m ID.

b: The BP was penetrated, through the zona pellucida into the cytoplasm of the oocyte, above the polar body. The surrounding cytoplasm, around 15-20%, was aspirated by delicate, slow aspiration.

c: The BP was later pulled back after enucleation had occurred.

d: A group of fibroblast cells as donor cells.

e: The single fibroblast cell (arrow) was aspirated into the BP and its tip penetrated through the same enucleation opening.

f: The donor cell (arrow) was expelled into the oocyte under the zona pellucida and attached to the cytoplasm.

2. In vitro development of rabbit cloned embryos

2.1 using adult fibroblast cells as donor nuclei

The objective of the study was to elucidate the technique of somatic cell nuclear transfer by using double electrical activation and post-activation with two drugs; cyclohexamide (CHX; a protein synthesis inbibitor) and 6-dimethylaminopurine (6-DMAP, a kinase inhibitor) after oocyte reconstruction. The metaphase II and 1st polar body of the oocyte were aspirated using an enucleating micropipette under an inverted microscope using the technique described in Fig. 1. Cultivated fibroblast cells from passage 9 to 15 were transferred to the enucleated oocytes. Reconstructed oocytes were later activated by two sets of electrical stimulation, 1 h apart, (3 DC pulses of 1.2 $Kv.cm^{-1}$ for 20 sec each in mannitol 0.3 M). After the second set of electrostimulation they were incubated in cyclohexamide 5 $g.ml^{-1}$ and 6-DMAP 2 mM in M199 for 1 h then washed and cultured in M199, supplemented with 10% FCS. The cleavage, morula and blastocyst rates were recorded every 24 h for 7 days. The study showed that the cloned morula and blastocysts can be obtained using this protocol.

2.2 using cumulus cells, serum-starved and non-starved fibroblast cells, as donor nuclei

It was found that the type and stage of the cell cycle of donor cell influenced the efficiency of nuclear transfer (Stice et al., 1993) and that serum starvation can induce the presumed G0 stage (Wilmut et al., 1997). The result from G0 stage donor nuclei provided a higher result in somatic cell cloning than other stages. Following on knowledge, this experimental work was designed to study the *in vitro* development of cloned rabbit embryos, by using cumulus cells and cultured muscular fibroblast cells, in serum-starved and non-starved conditions.

In our study, it was demonstrated that cumulus cells and cultivated fibroblast were totipotent and could be used as donor cells (Table 2). This finding was in agreement with reports in the literature (Chesne et al., 2001; Dinnyes et al., 2001; Kuhholzer et al., 2001). It was notified that the size of the cumulus cells was small, around 50% of the size of fibroblast cells and this may cause a poorer fusion rate, compared with that from fibroblast cells.

Table 2 The *in vitro* development of cloned rabbit embryos using cumulus cells, serum-starved and non-starved fibroblast cells, as the donor nucleus (Techakumphu et al., 2004).

Trial	Type of cell	Replica-tes	Recons-tructed oocytes	Fused-cultivated embryos (%)	Cleaved embryos (%)	4 cell (%)	8-16 cell (%)	M (%)	B (%)
1	CUMU LUS	12	140	103 (73.5)	74 (71.8)	12 (11.7)	8 (7.8)	36 (35.0)	16 (15.5)
2	SFB	10	92	84 (91.3)	74 (88.1)	14 (16.6)	8 (9.5)	31 (36.9)	21 (25.0)
	NSFB	9	79	70 (88.6)	58 (82.9)	8 (11.4)	7 (10.0)	22 (31.4)	20 (28.6)

SFB: serum-starved fibroblast, NSFB: non-starved fibroblast

CM: compact morula, B: blastocyst, HB: hatched blastocyst

Values with different letters (a, b) in the same column differ significantly (chi-square analysis, $p < 0.05$).

Table 3 *In vitro* development of cloned rabbit embryos using adult fibroblasts and starved and non starved foetal fibroblasts (Techakumphu et al., 2004).

Group	Oocytes	Recon structured oocytes	Cultured oocytes	Cleavage (%)	Development(%)			
					4 cell (%)	8-16 cell (%)	M	B to HB
AF	25 (10)*	251	136	104 (76.5)[a]	43 (31.6)	26 (19.1)	19 (14.0)	16 (11.8)
SFF	182 (8)*	160	64	37 (57.8)[b]	18 (28.1)	5 (7.8)	0	3 (4.7)
NSFF	40 (5)*	40	23	11 (47.8)[b]	5 (21.7)	4 (17.4)	0	1 (4.3)

( )* replications, % of cleavage calculated by no. of cultured oocytes.

AF: adult fibroblasts, SFF: starved fibroblasts, NSFF: non starved fibroblasts.

The fibroblast cells were easily obtained by the primary culture of biopsied muscle. From our experience, muscle biopsy provided a better fibroblast cell outgrowth from the explant, approximately 2 weeks after seeding and higher multiplication, than skin biopsy. It has been suggested that serum starvation can assist the cell into entering G0 stage of the cycle and these cells provide a higher success rate for somatic cell cloning when correct and complete reprogramming is done, after transfer to the oocyte cytoplast (Wilmut et al., 1997). From our results, the *in vitro* development of cloned embryos displays no significance difference between non-starved versus serum-starved fibroblast cells. Dinnyes et al. (2001) found that the cleavage rate of embryos produced from starved cells, was significantly higher than that from non-starved cells, however, the blastocyst rate showed no difference between the two treatments. Kuhholzer et al. (2001), found that the use of serum-starved cells (G0) exhibited no positive effect on the development of cloned embryos *in vitro*, when compared to cycling cells. It can be concluded that cumulus cells and muscular fibroblast cells can be used as donor nuclear cells for rabbit cloning and there is no effect of serum starvation on the donor nucleus on the *in vitro* development of cloned embryos.

Table 1 *In vitro* development of cloned rabbit embryos (Techakumphu et al., 2004).

No. passages	Reconstructed embryos	Cleaved embryos(%)	M/B(%)
9	12	10(83.3%)	0
10	29	18(62.1%)	15(51.7%)
11	27	22(80.9%)	6(22.2%)
12	46	27(58.7%)	7(15.2%)
13-15	45	32(71.1%)	12(28.9%)
Total	**159**	**109(68.6%)**	**46(28.9%)**

% morula and blastocysts calculated from the number of reconstructed embryos.

2.3 using adult vs foetal rabbit fibroblast cells

Nowadays differentiated cells are used, such as fresh cumulus cells (Chesne et al., 2002), cultivated cumulus cells (Yin et al., 2000) and adult fibroblasts (Dinnyes et al., 2001). The fibroblast cells can be obtained not only from adults but also from foetuses and can be used as donor cells in a nuclear transfer program. In this part, we report on the *in vitro* development of cloned rabbit embryos, using adult and foetal fibroblast cells, as donors.

Our findings show that the percentage of successful *in vitro* development was lower for the foetal fibroblast group than for the adult fibroblasts (Table 3). Only 2.3% of cultured oocytes in the former group reached the blastocyst stage compared with 11.8% in the latter group, which is comparable to that of Dinnyes et al. (2001) (16-18%). This result seems to be lower than reported in our previous result when using cumulus cells as donor cells (Table 2). The cell cycle phase of the donor cells, at the time of nuclear transfer, is one of the factors affecting the efficiency of nuclear transfer (Fulka et al., 1998; Thibault, 2003). The G0-stage foetal fibroblast may not be suitable for cloning and further experiments comparing G0 and interphase stage foetal fibroblasts need to be conducted to investigate the effect of the cell cycle phase on development.

As shown in the above results, it was demonstrated that rabbits can be used as a model to develop the technique of somatic cell nuclear transfer in animals.

Cats: Interspecies studies

Interspecies SCNT (iSCNT) has become established due to a lack of oocytes from the wild animal to produce cloned offspring. Accordingly, host oocytes from both close related and unrelated animals are preferred for iSCNT studies. Domestic cat oocytes collected after routine ovariohysterectomy are normally used for endangered felid cloning. The use of rabbit oocytes was been shown in many reports to enable the production of giant panda (Li et al., 2002), bovine (Techakumphu et al., 2005), elephant (Numchaisrika et al., 2005), human (Chen et al., 2003), and cat (Wen et al., 2003) cloned embryos. In addition, many attempts have been made to observe the capacity of bovine oocytes to reprogram the donor cells of several species including rats, sheep, pigs (Dominko et al., 1999), buffaloes (Kitiyanant et al., 2001), gaurs (Lanza et al., 2000; Hammer et al., 2001), monkeys (Dominko et al., 1999; Simerly et al., 2004), whales (Ikumi et al., 2003), and humans (Chang et al., 2003). In Thailand, there are some wild cats considered to be nearly extinct such as the marbled cat (*Felis marmorata* or *Pardofelis marmorata*) and the flat-headed cat (*Prionailurus planiceps*). Our study was conducted to study the possibility for producing cloned embryos from interspecies cloning between two wild cats, marbled and flat-headed cat-domestic cat and wild cat-rabbit. The experiments were conducted using domestic cat oocytes and rabbit oocytes as recipients to receive the marbled cat and flat-headed cat.

The interspecies cloning showed that the development of cloned MC-DC and DC-DC embryos to the 4-8 cell, morula and blastocyst stages (Fig. 2d) was not significantly different (the 4-8 cell; 56% vs. 50%, the morula; 8% vs. 8.3% and the blastocyst stages; 0% vs. 4.2%, respectively). The development of IVF embryos reaching the morula and blastocyst stages was greater than those of cloned MC-DC and DC-DC embryos (Table 4).

Table 4 The development of MC- and DC-DC cloned embryos (Thongpakdee et al., 2005).

Donor cell-oocyte	n	fused	4-8	M	B
		n(%)			
MC-DC	63	25(40)	14(56)	2(8)[a]	0[a]
DC-DC	60	24(40)	12(50)	2(8.3)[a]	1(4.2)[a]
IVF (control)	53	-	28(52.8)	12(22.6)[b]	5(9.4)[b]

Values within a column with different letters differ (p <0.05).

MC: Marbled cat, DC: Domestic cat.

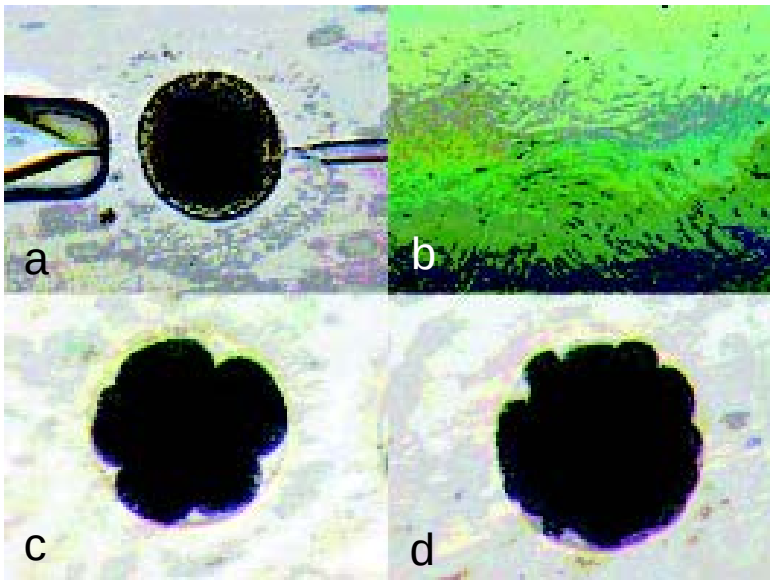

Figure 2 Enucleation (cat oocyte) (a) (x200), marbled cat fibroblast cells(b) (x100), MC-DC cloned embryos at the 8 cell stage(c) and a compact morula(d) (x300) (Thongpakdee et al., 2005).

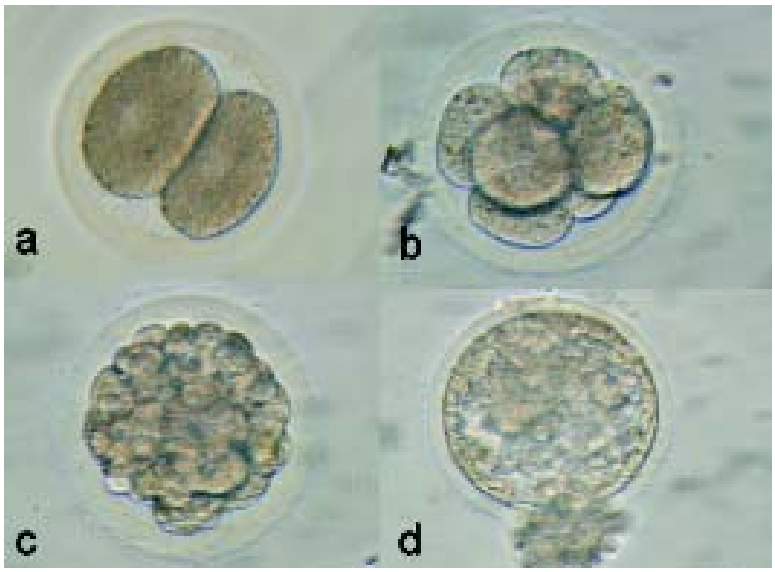

Figure 3 MC-RB cloned embryos at different stages: 2-cell stage (a), 8-cell stage (b), compact morula (c), hatching blastocyst (d) (x300) (Thongpakdee et al., 2005).

The cloned MC-DC (Fig. 2a-d) and MC-RB (Figs. 3a-d) embryos can develop to the blastocyst stage at no different rate.

The rabbit oocyte can be served for interspecies NT as showed in Table 5, the cleavage rates of domestic cat-rabbit and marbled cat-rabbit were comparable. The blastocyst rates were 4 to 6% respectively. These percentages were lower than those of the control (rabbit-rabbit).

Recently donor cells from the flat-headed cat were also introduced into domestic cat or rabbit recipient oocytes. The results demonstrated that flat-headed cat embryos are successfully produced by inter-generic SCNT (Thongpakdee et al., 2005).

Table 5 *In vitro* development of cloned embryos using domestic cat and marbled cat fibroblasts as the donor cells compared to foetal rabbit fibroblasts introduced in rabbit oocytes.

Donor cell-oocyte	n	2-8 cell	>8-16 cell	M	B
		n(%)			
DC	46	22(48)	8(17)	6(13)	2(4)
MC	47	25(53)	7(15)	5(11)	3(6)
RB	20	14(70)	7(35)	5(25)	3(15)

DC, domestic cat; MC, marbled cat; RB, rabbit donor cells.

Table 6 Effects of oocyte sources and activation protocols on *in vitro* development of NT embryos (Apimeteetumrong et al., 2004).

Oocyte sources	Activation protocols	Cultured *n*	Fused *n*(%)	Cleaved *n*(as % fused)	M/B *n*(as % fused)
In vivo matured	CHX	73	57 (78.1)	50(87.7)	0 (0)[a]
	ETOH	67	46(68.7)	40(87.0)	7(15.2)[b]
In vitro matured	CHX	98	77(78.6)	62(80.5)	0(0)[a]
	ETOH	67	56(83.6)	47(83.9)	4(7.1)[c]

a,b,c Values with different superscripts within the same column differ significantly ($p < 0.05$).

Goats: for producing transgenic goat in the future

Nuclear transfer (NT) or cloning technique has been used for producing transgenic goat, cloned farm animal offspring. These animals are capable of producing valuable proteins such as fibrinogen, human clotting factor IX and antithrombin III, which could have a marked impact on the pharmaceutical industry The study of activation protocols was performed using cycloheximide (CHX) or ethanol (ETOH) as activation agents in several species (Loi et al., 1998; Vignon et al. 1998; Baguisi et al., 1999;). A comparison of the development of NT goat embryos with either CHX or ETOH has not been reported. The aim of this study was to compare the development to morula and blastocyst stages after either CHX or ETOH activation in somatic NT goat embryos derived from *in vivo* and *in vitro* matured oocytes. The results showed that activation with ethanol affected the developmental competence of cloned goat embryos and the oocyte sources had no effect on fusion and cleavage rates (Table 6). Four from six recipients

were pregnant at 2.5 mts from nuclear transfer but only one went to term with a mummified fetus.

Conclusion

Up to now, the technique of somatic nuclear transfer has been developed in our group by using rabbits as a model. Our future objectives are concern its application in humans for therapeutic cloning and for endangered conservation in collaboration with The Zoological Park of Thailand. Meanwhile, the goat serves as a model for NT development in farm animals.

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