EUROPEAN ASSOCIATION FOR ANIMAL PRODUCTION
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Scientific developments in animal clonig

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Presentation outline

- Hystorical perspective
- Science & Technology
- Results
- GMO clones
- Pregnancy and birth
- Offspring of clones
- Safety of clones' derived products

Reproductive biotechnologies in farm animals

- It started with artificial insemination (‘50)
- It continued with embryo transfer (‘70)
- Then in vitro embryo production (‘80)
- Nuclear transfer (‘90)
- Genetic modification (‘00)

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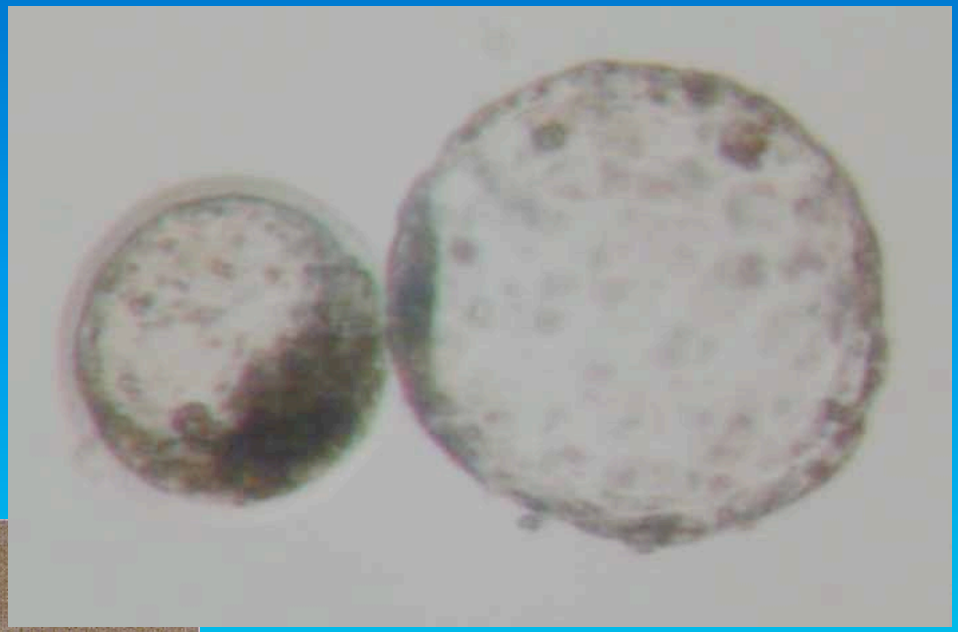
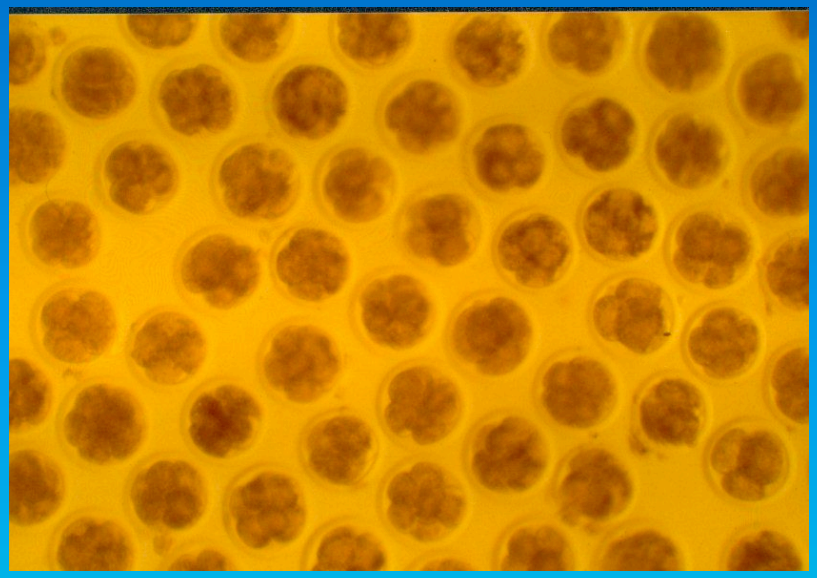




letters to nature

Nature **320**, 63 - 65 (06 March 1986); doi:10.1038/320063a0

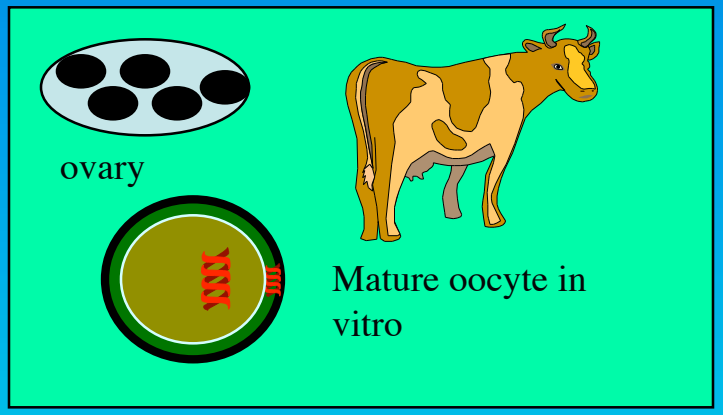
Nuclear transplantation in sheep embryos

S. M. WILLADSEN

AFRC Institute of Animal Physiology, Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ, UK

Nuclear transplantation and cell fusion techniques have proved valuable for embryological studies in several non-mammalian animal species[1]. More recently these procedures have been used successfully in small laboratory mammals, notably the mouse, to investigate the ability of nuclei and cytoplasm from various sources to produce viable embryos when combined[2–6]. The use of a similar approach to study the developmental biology of large domestic animals presents a number of technical and practical difficulties, and so far there has been no report of attempts to perform nuclear transplantation in sheep embryos. Here I describe such a procedure and its use to investigate the development of embryos in which whole blastomeres from 8- and 16-cell embryos were combined with enucleated or nucleated halves of unfertilized eggs. The procedure involves bisection of single-cell eggs in a medium containing cytochalasin; fusion of egg halves with single blastomeres, induced using Sendai virus or an electrofusion apparatus; and embedding in agar, followed by culture of the reconstituted embryos in the ligated oviducts of ewes in dioestrus. I show that fully viable embryos may be obtained by this procedure.

Cloning techniques by embryonic cloning



Nuclear transfer

enucleation

activation

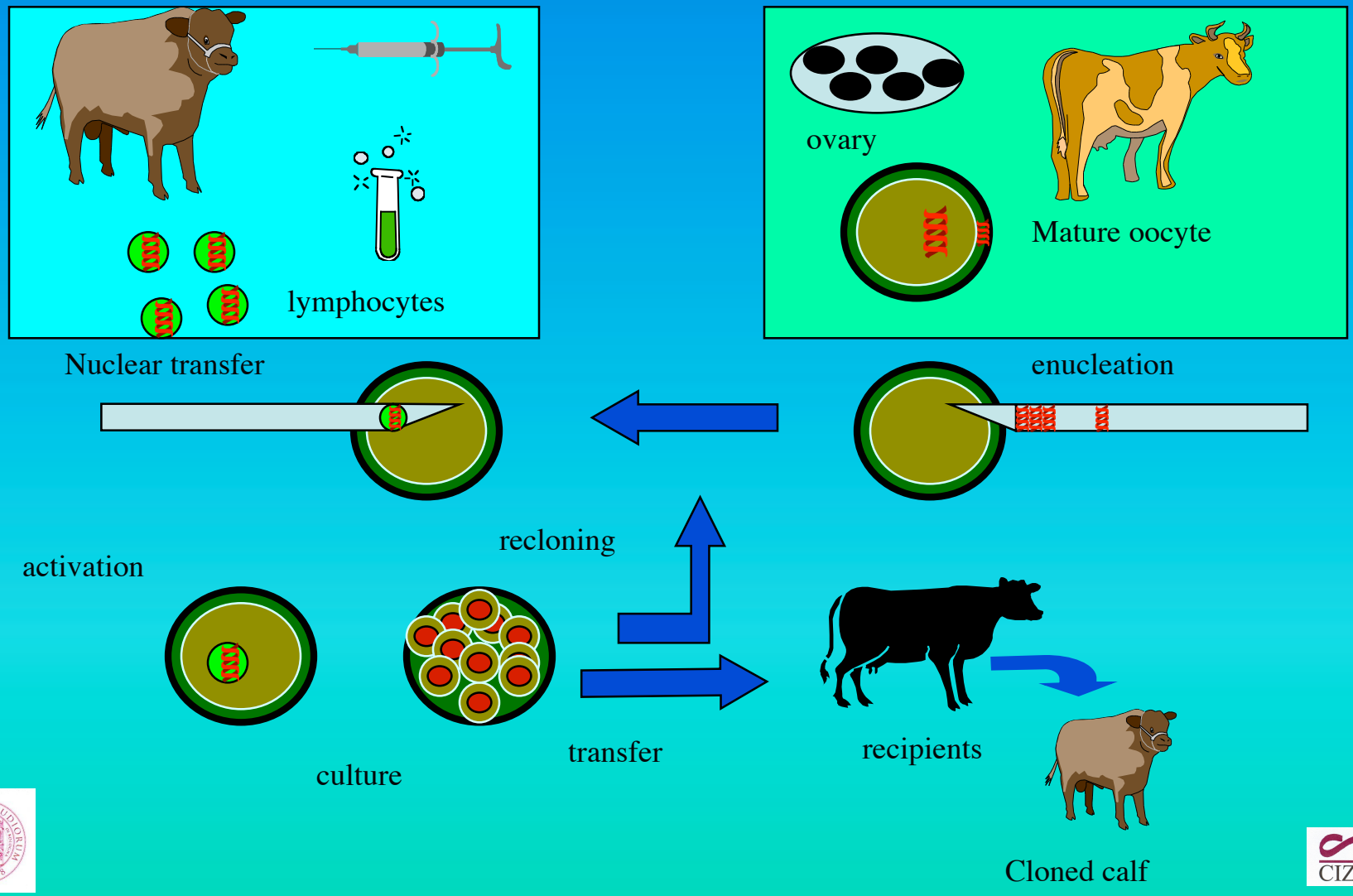
colture

transfer

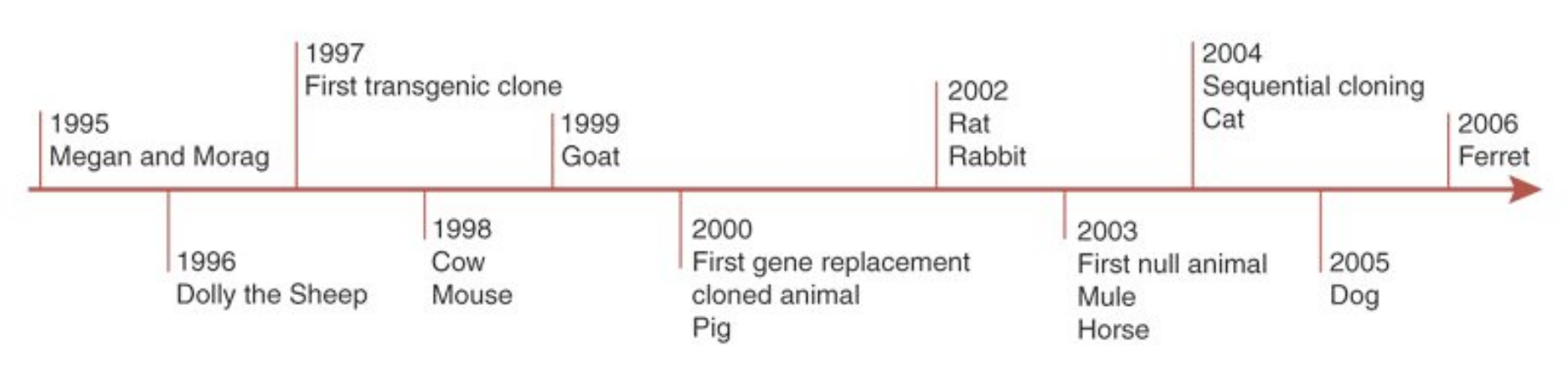
recipients

Cloned calf

Cloning technique by somatic cell nuclear transfer

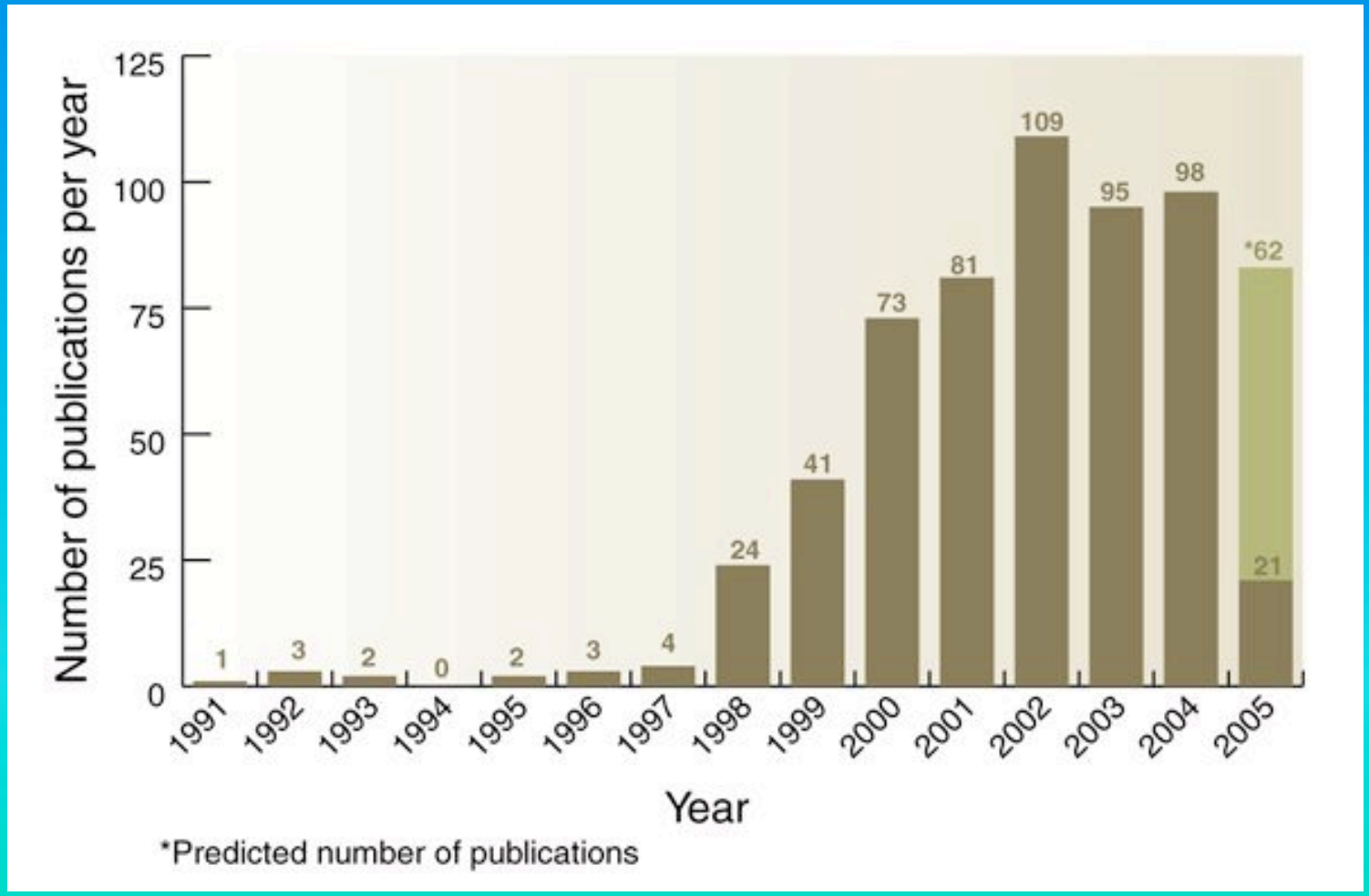


Timeline of somatic cell nuclear transfer (SCNT)



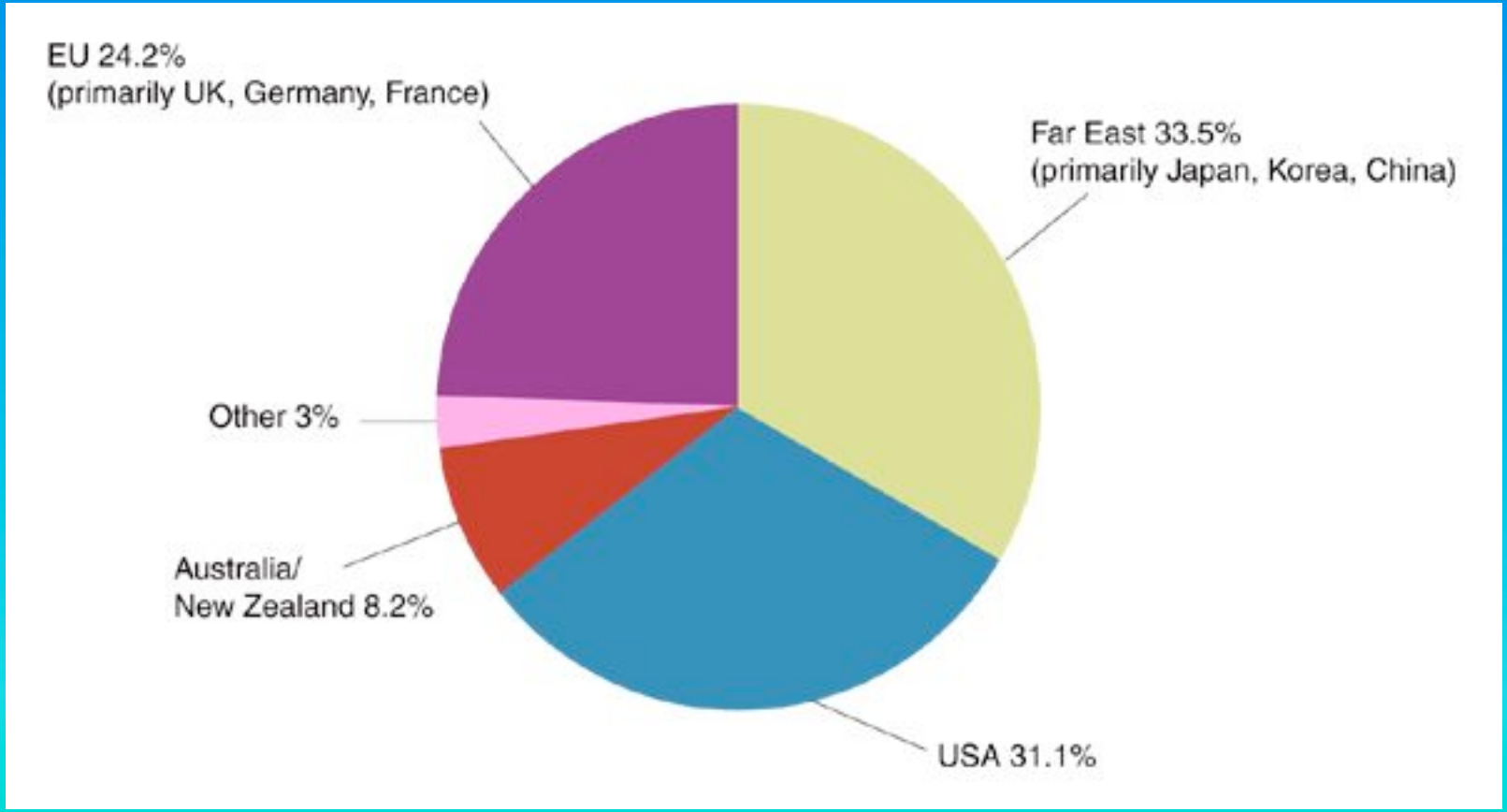
Suk et al, 2007 Nat. Biotech. 25: 47-53

Publication in NT in mammals excluding the mouse



Suk et al, 2007 Nat. Biotech. 25: 47-53

Geographical location of NT activities



Suk et al, 2007 Nat. Biotech. 25: 47-53

Cell line effect on live birth success

	N of embryos	n of recipients	n of pregnancies	n to term	n alive after birth
bull A	50	50	28	1	1
bull B	38	38	21	5	3
Bull C	47	25	12	0	0
Bull D	28	14	5	1 over 7 months	
Cow A	24	24	14	2	2
Cow B	24	24	12	1	1
Mare A	9	5	2	1	1
Stallion A	8	4	2	0	0
Stallion A fetal	35	20	1	0	0
Stallion B	71	23	6	2	2

Cell line effect on cloning success

Distribution of cell line efficiency in bovine cloning, expressed as number (%) of calves alive >150 days after birth over number of recipients pregnant at 30 days (in three countries)

		USA	ARG	BRA	Total
Cell lines (*n*)		80	11	6	97
Efficiency of a cell line (%)	0	19 (24)	3 (27)	1 (17)	23 (24)
	1–10	10 (12)	2 (18)	0 (0)	12 (12)
	11–20	23 (29)	3 (27)	0 (0)	26 (27)
	>20	28 (35)	3 (27)	5 (83)	36 (37)

Panarace et al, Theriogenology 67 (2007) 142-151

Development of bovine nuclear transfer (NT) embryos derived from adult fibroblasts, mesenchimal stem cells (MSC) and osteocytes, differentiated from MSC

Cell type	N of NT embryos	Cleavage N(%)	MC D6 N(%)	BL D7 N(%)	BL D8 N(%)
Adult fibroblasts	63	62(98.4)	36 (57.1)	33 (52.4)	42 (66.7)
MSC	102	102 (100)	58 (56.9)	65 (63.7)	67 (65.7)
Osteocytes	102	102 (100)	52 (51.0)	55 (53.9)	54 (52.9)

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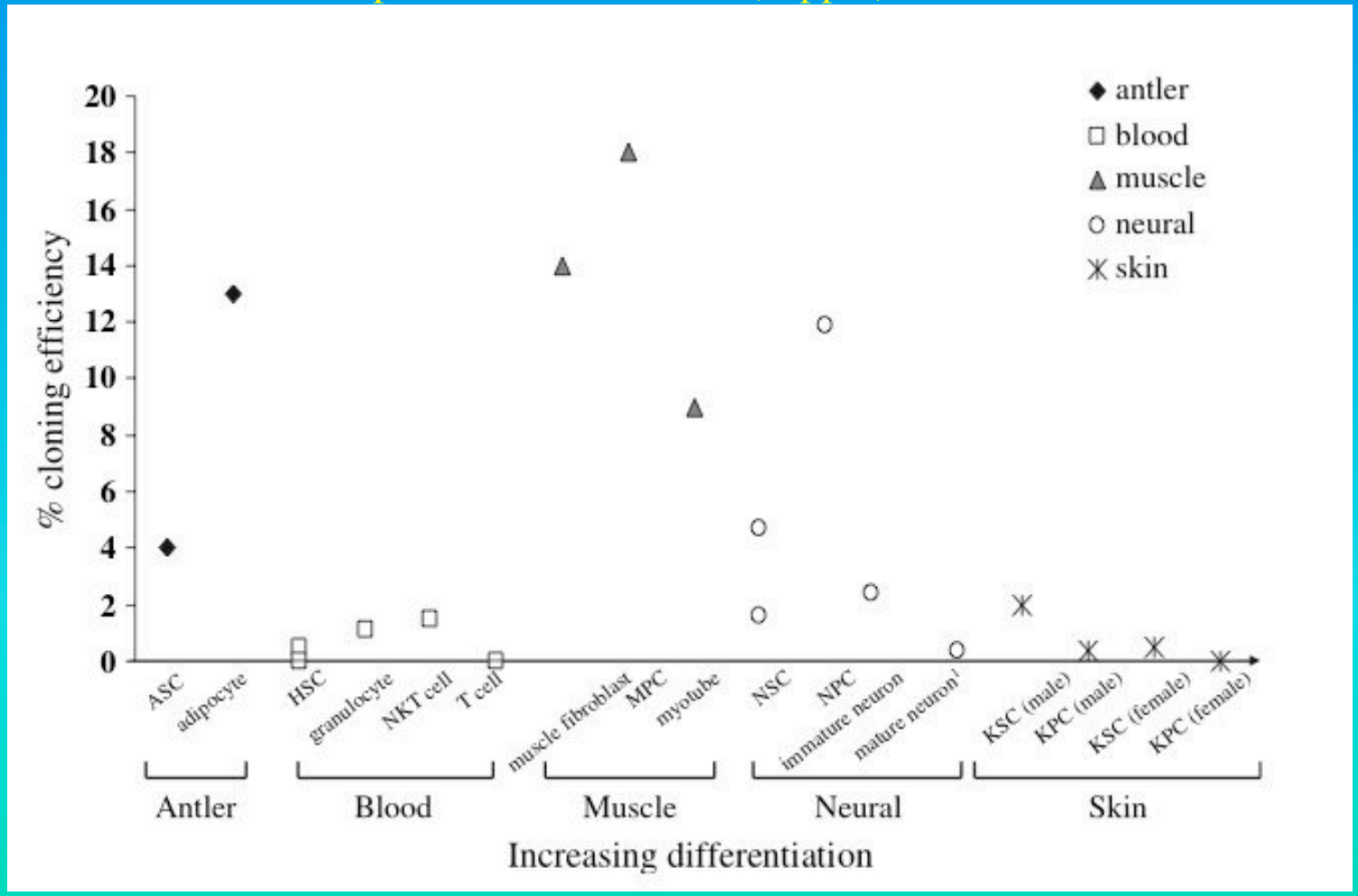
Somatic cloning efficiency and donor cell type

Oback, Reprod. Dom. Anim 43 (supp 2) 407-416, 2008



Nuclear transfer procedure

- Donor nucleus pre-conditioning
  - Drugs (TSA, Azacytidine, etc)
  - Cell extracts (tumor cell lines, xenopus eggs, etc)
- Recipient cytoplasts
  - Methapase II
  - Zona free
  - Zygotes
- Activation
  - Chemical
  - Sperm or sperm exctracts

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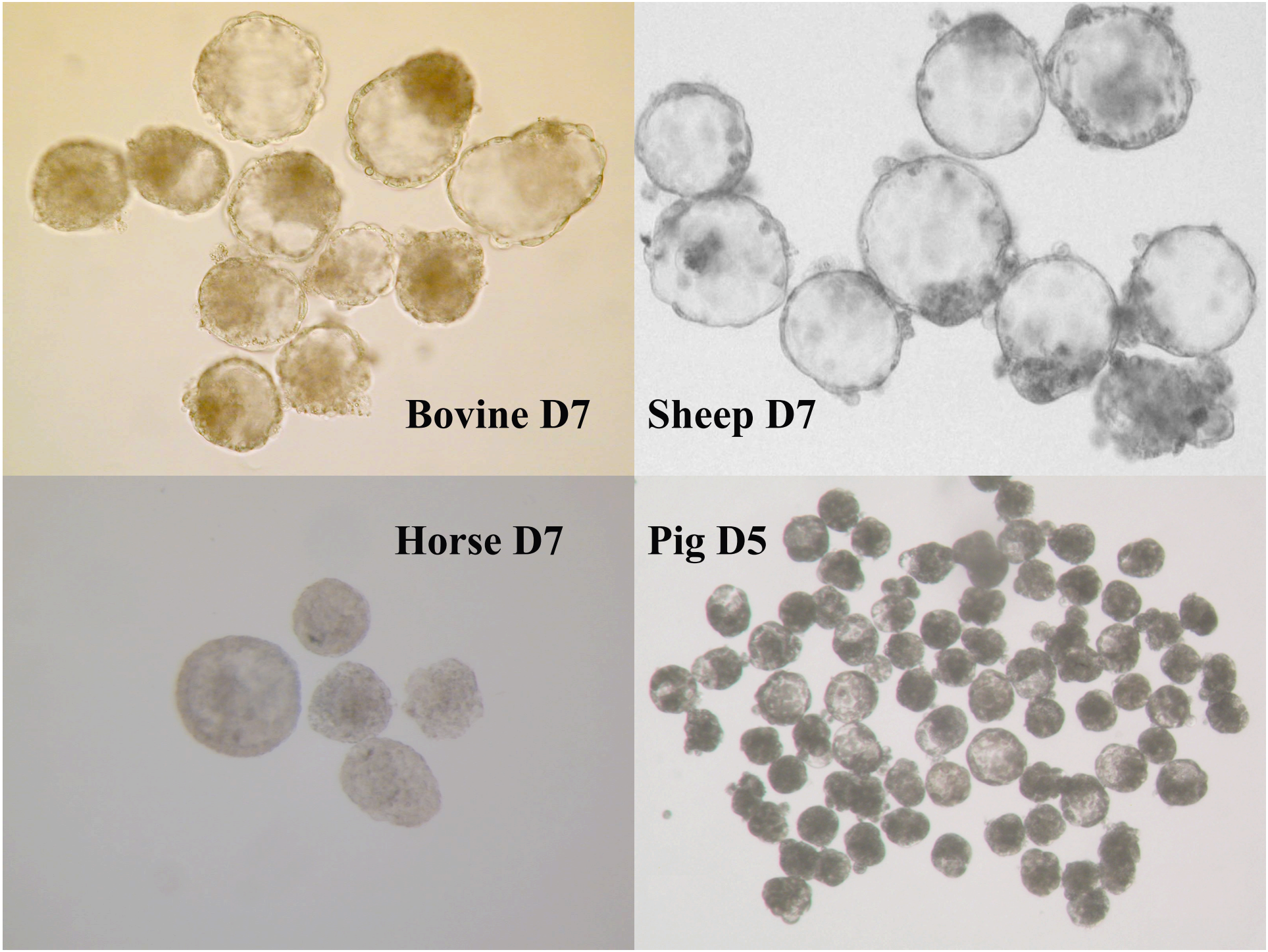
Bovine pre-implantation embryo development after NT

Galli et al, C&SC 4, 189-196, 2002

cell type	method (activation)	N. of constructs	N. fused or success. injected (%)	N. cleaved (%)	N. blastocysts D+7 (%)
lymphocytes	injection (DMAP)	353	338 (95.6)a	289(85.5)c	54 (16.0)f
"	injection (CHX)	234	224 (95.7)a	134 (59.8)d	24 (10.7)g
granulosa cells	fusion (DMAP)	253	177 (70.0)b	164 (92.7)c	70 (39.5)h
"	injection (DMAP)	273	250 (91.6)a	189 (75.6)d	46 (18.4)f
adult fibroblasts	fusion (DMAP)	227	139 (61.2)b	123 (88.5)c	89 (64.0)i
"	fusion (CHX)	192	117 (61.0)b	79 (67.5)cd	44 (37.6)h
"	injection (CHX)	722	696 (96.4)a	459 (65.9)c	78 (11.2)g


Bovine D7
Sheep D7
Horse D7
Pig D5

Use of zygotes as recipient cytoplasts

Group	*N*	*n*	Development (%) to day 35[a]	Development (%) to term[b]	Development (%) to weaning[b]	Age at birth (days ± S.E.M.)	Birth weight (kg ± S.E.M.)	Breed
AI	1	12	8 (67)*	8 (67)*	8 (67)*	$272 \pm 2^{b*}$	$39 \pm 3^{b*}$	Hereford × Friesian
NT	4	41	17 (41)†	4 (10)†	3 (7)†	$281 \pm 2^{\dagger}$	$47 \pm 2^{\dagger}$	Friesian
IVF-NT	4	49	30 (61)*	9 (18)†	6 (12)†	$277 \pm 1^{\dagger}$	49 ± 2^{y}	Friesian

N, Total number of independent NT or AI experiments; *n*, total number of embryo transferred (nET) or artificially inseminated (nAI). *†Rows with different superscripts differ significantly ($P<0.05$).
[a]Proportion of total number of nET or nAI that developed into fetuses and live calves at day 35 of gestation, term or weaning. [b]Only the four male calves were included in the weight and age analysis.

Schurmann et al, 2006

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Horse cloning
sperm exctract activation
(Hinrichs et al, Reproduction, 134, 319, 2007)

Activation treatment[a]	No. cultured	Cleavage (%)	Blastocysts (%)	Embryos transferred	Pregnancies	Live foals
Ionomycin	86	67 (78)	5 (5.8)	3	1 (33%)[b]	0
2 x I	87	68 (78)	4 (4.6)	3	2 (66%)[c]	1
I + SE	83	77 (93)	4 (4.8)	2	1 (50%)	1
SE + I	82	71 (87)	6 (7.3)	5	3 (60%)[d]	1[e]

Activation treatment[a]	No. cultured	Cleavage (%)	Blastocysts (%)	Embryos transferred	Pregnancies	Live foals
Sperm extract	59	40 (68)	2 (3.4)*,†	2	2 (100%)[b]	0
Ionomycin	65	52 (80)	5 (7.7)*,†	5	2 (40%)	2
I/6D/I	71	53 (75)	2 (2.8)*	1	1 (100%)[c]	0
SE + I	56	52 (93)	7 (12.5)†	5	4 (80%)	4[d]

Three donors used

NT embryo development

Galli et al. 2002Cloning & Stem Cells 4, 189-196

origin of blastocysts	method (activation)	N. of transfer	N. of pregnancies				
			D+35 (%)	D+60	D+120	D+180	term
lymphocytes	injection (DMAP)	71	41 (58)	24	6	5	1
"	injection (CHX)	14	5 (36)	2	0	0	0
granulosa cells	fusion (DMAP)	9	4 (44)	0	0	0	0
adult fibroblasts	fusion (DMAP)	20	10 (50)	6	2	1	0
"	fusion (CHX)	14	3 (21)	1	1	1	1
"	injection (CHX)	13	7 (54)	5	5	5	4
total	total	141	70 (50)	38	14	12	6

Cattle SCNT success

Efficiency of cloning cattle, including calf survival, in three countries

		USA	ARG	BRA	Total
No. embryos transferred		2662	623	89	3374
No. recipients		1760	617	89	2466
No. (%) embryos at 30 days		1120 (42)	224 (36)	38 (43)	1382 (41)
No. (%) twins		133 (13)	7 (3)	0 (–)	140 (11)
No. (%) singles		854 (87)	210 (97)	38 (100)	1102 (89)
No. (%) pregnant recipients	30 days	987 (37)	217 (35)	38 (43)	1242 (37)
	60 days	625 (23)^{a}	93 (15)^{b}	34 (38)^{c}	752 (22)
	90 days	475 (18)^{a}	73 (12)^{b}	32 (36)^{c}	580 (17)
	120 days	440 (17)^{a}	72 (12)^{b}	27 (30)^{c}	539 (16)
	150 days	411 (15)^{a}	66 (11)^{b}	26 (29)^{c}	503 (15)
	180 days	386 (15)^{a}	63 (10)^{b}	25 (28)^{c}	474 (14)
	210 days	360 (14)^{a}	58 (9)^{b}	25 (28)^{c}	443 (13)
	240 days	323 (12)^{a}	51 (8)^{b}	24 (27)^{c}	398 (12)
	270 days	304 (10)^{a}	47 (8)^{b}	20 (22)^{c}	371 (11)
	Term	294 (11)^{a}	44 (7)^{b}	20 (22)^{c}	358 (11)
No. (%) calves born		326 (12)^{a}	42 (7)^{b}	20 (22)^{c}	388 (11)
No. (%) live calves		261 (10)^{a}	38 (6)^{b}	18 (20)^{c}	317 (9)
No. (%) calves alive 24 h after birth		229 (9)^{a}	32 (5)^{b}	17 (19)^{c}	278 (8)
No. (%) calves alive >150 days		182 (7)^{a}	30 (5)^{a}	13 (15)^{c}	225 (7)

Within a row, values with different superscripts letters (a–c) differ ($P < 0.05$). Values for twins and singles were calculated over the number of pregnant recipients ($N = 987$ and 217 in USA and ARG, respectively).

Panarace et al, Theriogenology 67 (2007) 142-151

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Cattle SCNT success

TABLE 2. OVERVIEW OF SOMATIC CLONING SUCCESS RATES IN CATTLE

Donor cells	*Culture condition*	*Pregnancy established*[a]	*Development to term*[b]	*Development to weaning*[b]	*Reference*
Fetal genital ridge cells, fetal body/adult skin fibroblasts (34 lines)[c] (TG/Non-TG)[d]	Confluent	535/2170 (25%)	117/4340 (3%)[e]	82/4340 (2%)[e]	(Pace et al., 2002)
Fetal lung fibroblasts (5 lines) (TG/Non-TG)	Serum-starved or proliferating	128/318 (40%)	55/318 (17%)	33/318 (10%)	(Wells et al., 2003)
Adult skin fibroblasts and follicular cells (6 lines) (Non-TG)	Serum-starved	72/165 (44%)	32/165 (19%)	26/165 (16%)	Wells DN, unpublished data
Fetal fibroblasts (at least 3 lines) (TG)	Proliferating	110/247 (45%)	30/496 (9%)	24/496 (5%)	(Lanza et al., 2001)
Fetal lung/adult skin fibroblasts (7 lines) (TG/Non-TG)	Serum-starved	92/194 (47%)	27/194 (14%)	17/194 (9%)	(Oback et al., 2003) & this publication
Fetal/newborn/adult skin fibroblasts; adult cumulus, oviduct, uterine cells (20 lines) (Non-TG)	Serum-starved or confluent	50/134 (37%)	24/172 (14%)	13/172 (8%)	(Kato et al., 2000)
Fetal fibroblasts (TG/Non-TG) (4 lines)	Serum-starved	49/103 (48%)	25/208 (12%)	11/208 (5%)	(Zakhartchenko et al., 2001)
Fetal/adult skin fibroblasts (at least 5 lines) (Non-TG)	Serum-starved or proliferating	56/173 (32%)	15/173 (9%)	10/173 (6%)	(Heyman et al., 2002a)
Adult skin fibroblasts, cumulus cells, oviduct, uterine cells (4 cell lines) (Non-TG)	Serum-starved	23/44 (52%)	12/59 (20%)	5/59 (8%)	(Cho et al., 2002)
Fetal/adult skin fibroblasts (number of lines not reported) (Non-TG)	Confluent or proliferating	44/100 (44%)	5/200 (3%)	5/200 (3%)	(Kasinathan et al., 2001b)
Adult skin fibroblasts, Leukocytes, follicular cells (3 cell lines) (Non-TG)	Serum-starved or confluent	70/141 (50%)	6/141 (4%)	4/141 (3%)	(Galli et al., 2002)
Adult skin fibroblasts (1 line) (Non-TG)	Serum-starved or proliferating	15/36 (42%)	6/36 (17%)	4/36 (11%)	(Kubota et al., 2000)
Adult cumulus, oviduct cells (2 cell lines) (Non-TG)	Serum-starved	5/5 (100%)	8/10 (80%)	4/10 (40%)	(Kato et al., 1998)

[a]Proportion of total number of recipients that were classified pregnant between D17-D40 after ET; [b]proportion of total number of embryos transferred that developed to calves at term or weaning; [c]number of independently derived primary cell lines or clonal strains; [d]TG = transgenic. [e]Exact number of embryos is uncertain since on a few occasions 1 or 3 instead of 2 embryos were transferred.

Oback et al, C&SC, 2003

Horse embryo development after transfer (zona free)

Lagutina et al. 2005 Reproduction 130: 559

Donor cells	Horse	N embryos	N recipients	Pregnancy	35 days	3 months	6 months	Offspring
				n (%)	n (%)	n (%)	n (%)	n (%)
cumulus 22 h IVM	*	3	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
fetal fibroblasts §	C	33	18	1 (5.6)	1 (5.6)	1 (5.6)	1 (5.6)	0 (0)
adult fibroblasts	A	4	2	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)
adult fibroblasts	B	71	23	6 (26.1)	4 (17.4)	3 (13)	2 (8.7)	2 (8.7)
adult fibroblasts	C	26	12	2 (16.7)	2 (16.7)	1 (8.3)	1 (8.3)	0 (0)
adult fibroblasts	Total	101	37	9 (24.3)	6 (16.2)	4 (10.8)	3 (8.1)	2 (5.4)

Pig nuclear transfer

type of cells	no. recipients pregnant/ implanted	no. embryos implanted (per recipient)	total no. of fetuses* or offspring	average litter size
fibroblasts wild type	2/4	244 (61)	14	7
fibroblasts transgenic	4/4	203 (51)	24*	6

* some pregnancies were not allowed to go to term

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Why SCNT work the way it works

- Failure to reprogramme the genome
- Species differences
- Abnormalities and welfare problems

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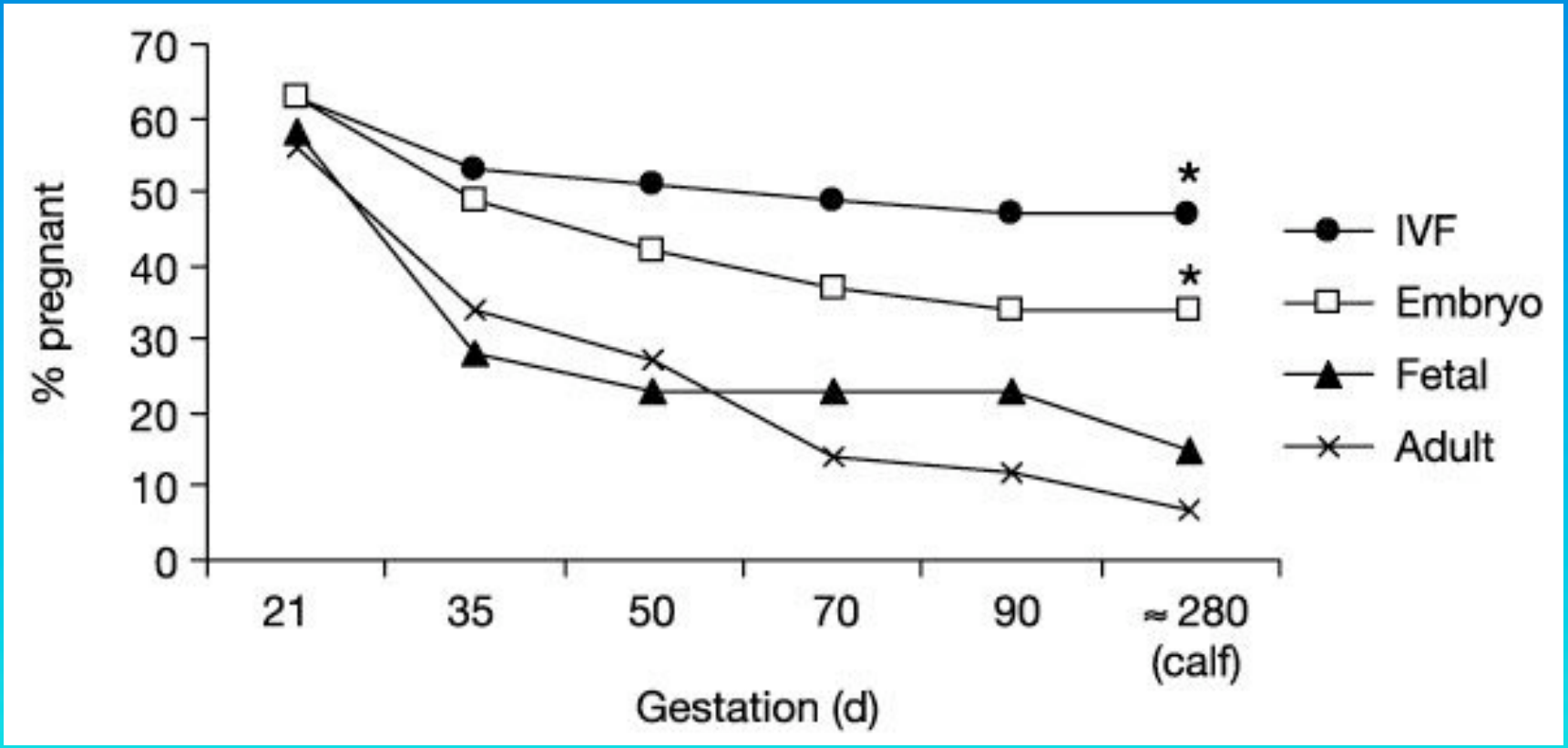
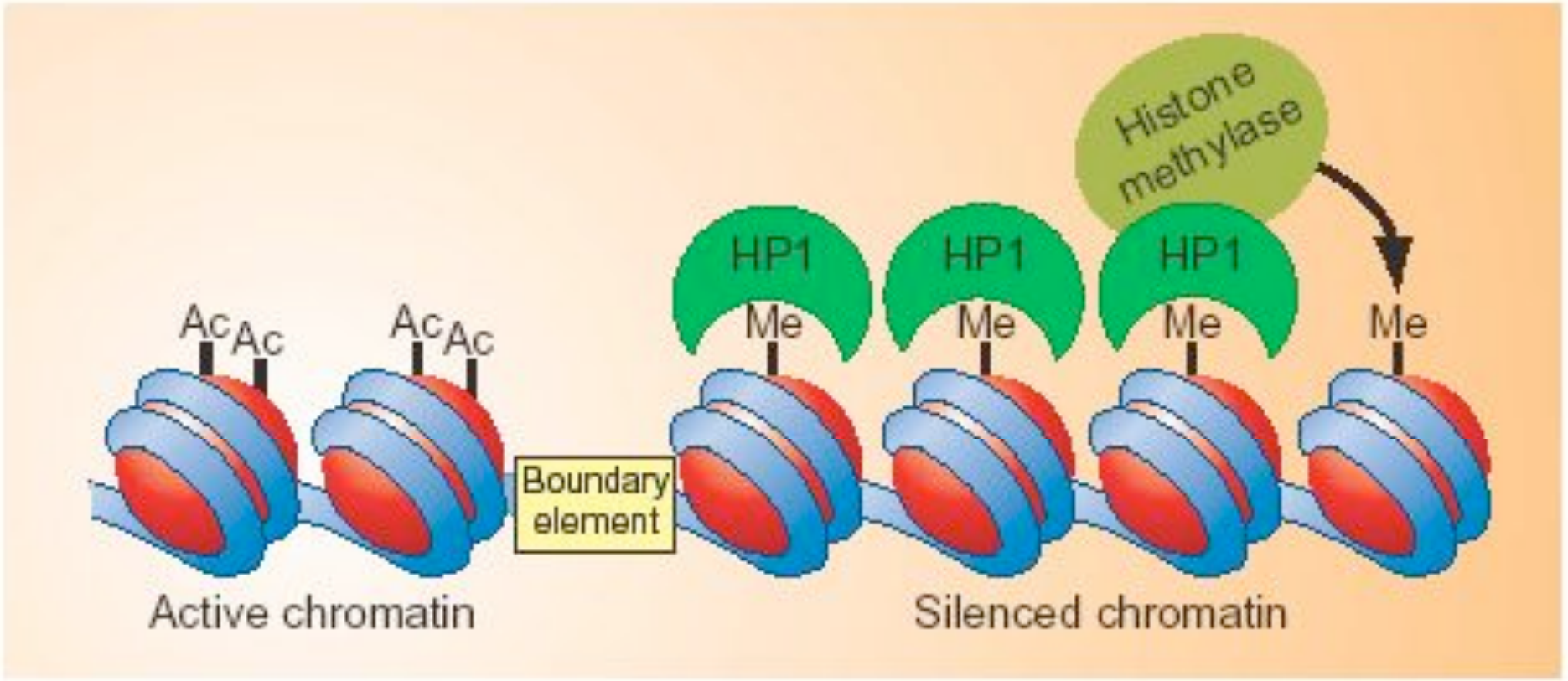
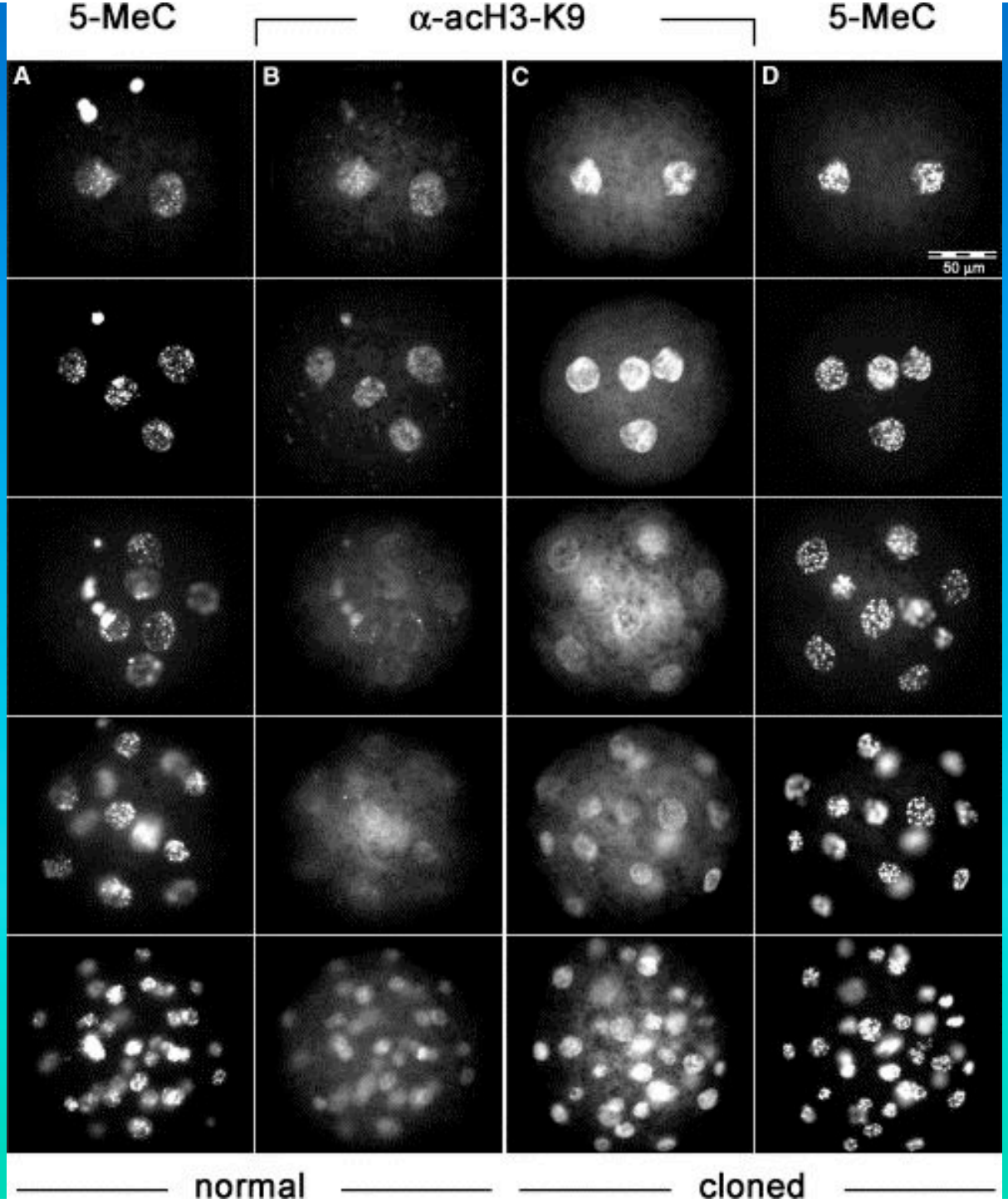
Comparison of pegnancy losses



Heyman, Y. *et al.* Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biol. Reprod.* **66**, 6-13 (2002)

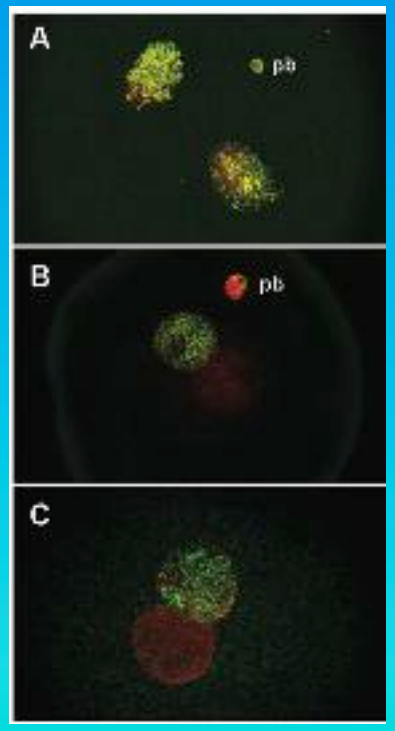


Chromatin chemistry. Chemical modifications—acetylation (Ac) or methylation (Me)—of histone proteins determine whether genes on the surrounding DNA are active. HP1 is a transcription-inhibiting protein.



Santos F, Zakhartchenko V, Stojkovic M, Peters A, Jenuwein T, Wolf E, Reik W, Dean W.
Epigenetic marking correlates with Developmental potential in cloned bovine preimplantation embryos.
Curr Biol 2003; 13: 1116-1121

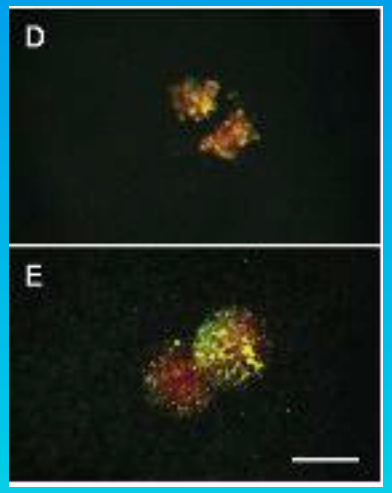
Methylation pattern at the pronuclear stage



sheep

mouse

human



rabbit

bovine

Beaujean N, Hartshorne G, Cavilla J, Taylor J, Gardner J, Wilmut I, Meehan R, Young L.
Non-conservation of mammalian preimplantation methylation dynamics. Curr Biol 2004; 14: R266-267

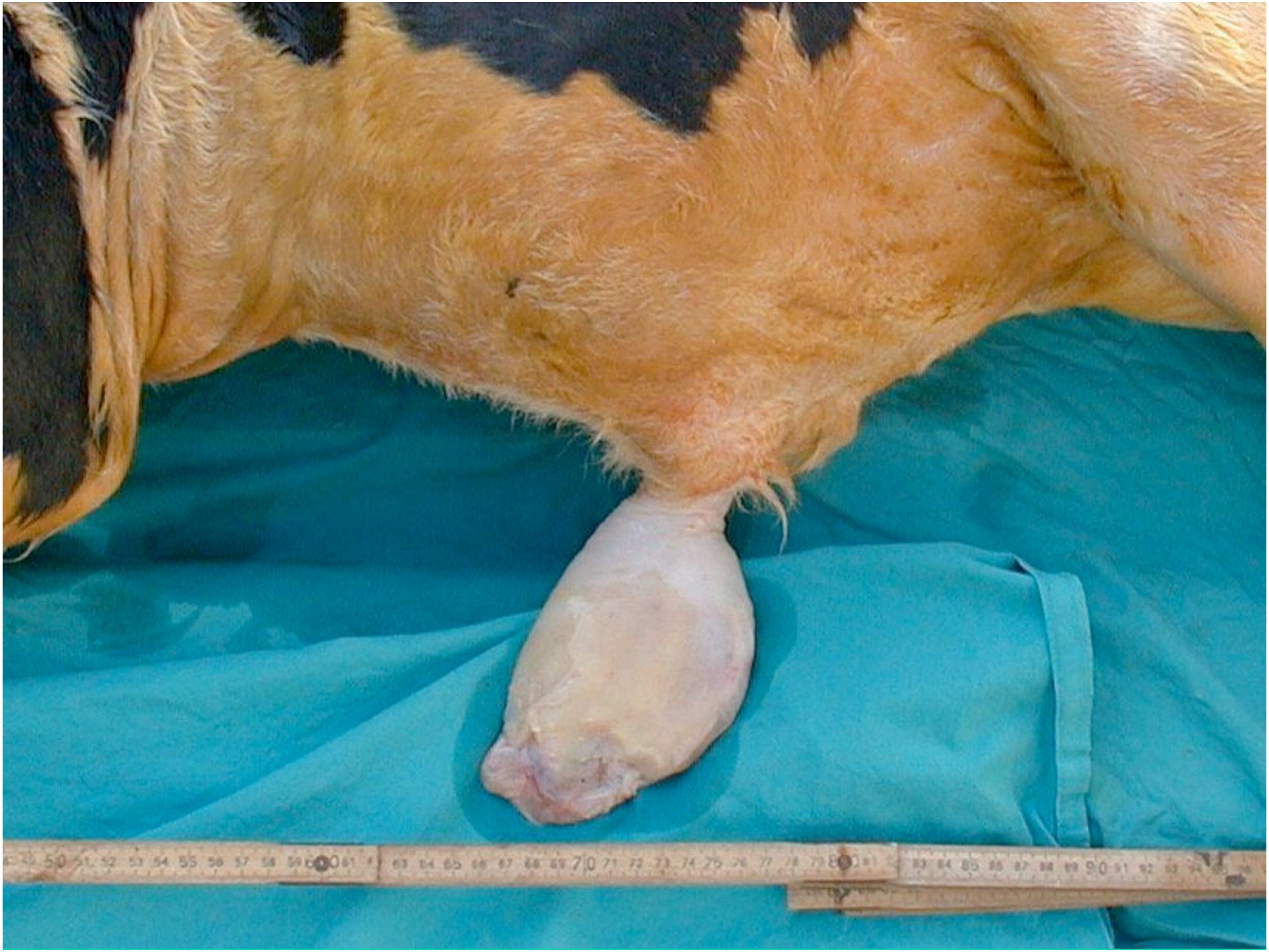
Most common abnormalities in cloned calves

Most common abnormalities in cloned calves during the perinatal period (in three countries)

Syndrome or illness	USA	ARG ($N = 36$)	BRA ($N = 20$)	Total
No. (%) with respiratory problems	22/98 (22)	6 (17)	1 (5)	29/154 (19)
No. (%) with hyper/ hypothermia	17/88 (19)	7 (19)	0 (0)	24/144 (17)
No. (%) calves treated with antibiotics	72/96 (75)	18 (50)	7 (20)	64/172 (37)
No. (%) with enlarged umbilical cord	50/116 (43)	8 (22)	6 (30)	64/172 (37)
No. (%) depressed/ prolonged recumbency	21/87 (24)	7 (19)	1 (5)	29/143 (20)
No. (%) with contracted flexor tendons	22/109 (20)	9 (25)	0 (0)	31/145 (21)
No. (%) with persistent urachus	4/95 (4)[a]	8 (22)[b]	3 (15)[ab]	15/151 (10)

Within a row, values with different superscripts letters (a and b) differ ($P < 0.05$). Data from USA is shown according to number of cases studied for each parameter.

Panarace et al, Theriogenology 67 (2007) 142-151



Animal welfare guidelines

Health Assessment and Care for Animals Involved in the Cloning Process

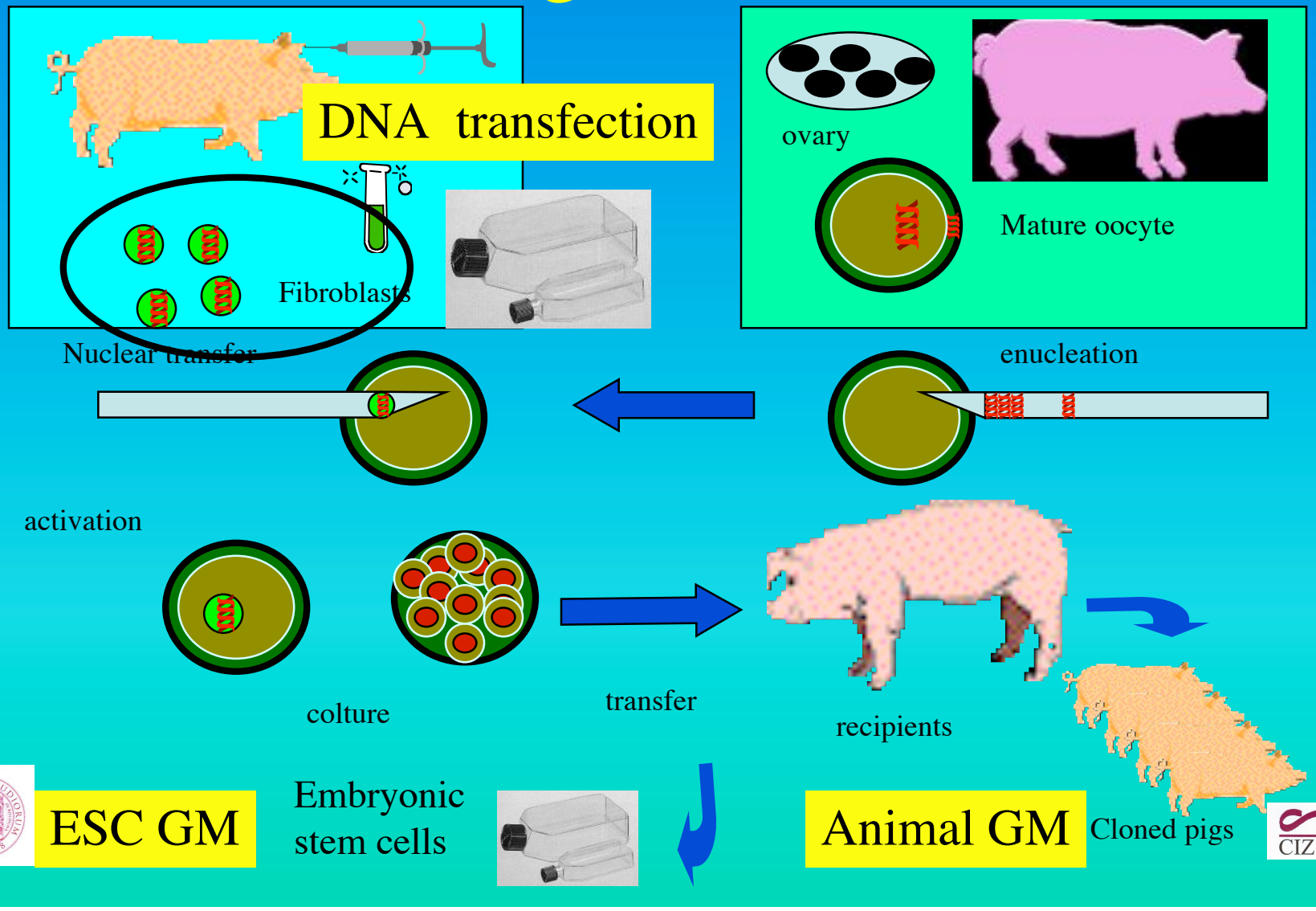
A consensus recommendation from the International Embryo Transfer Society

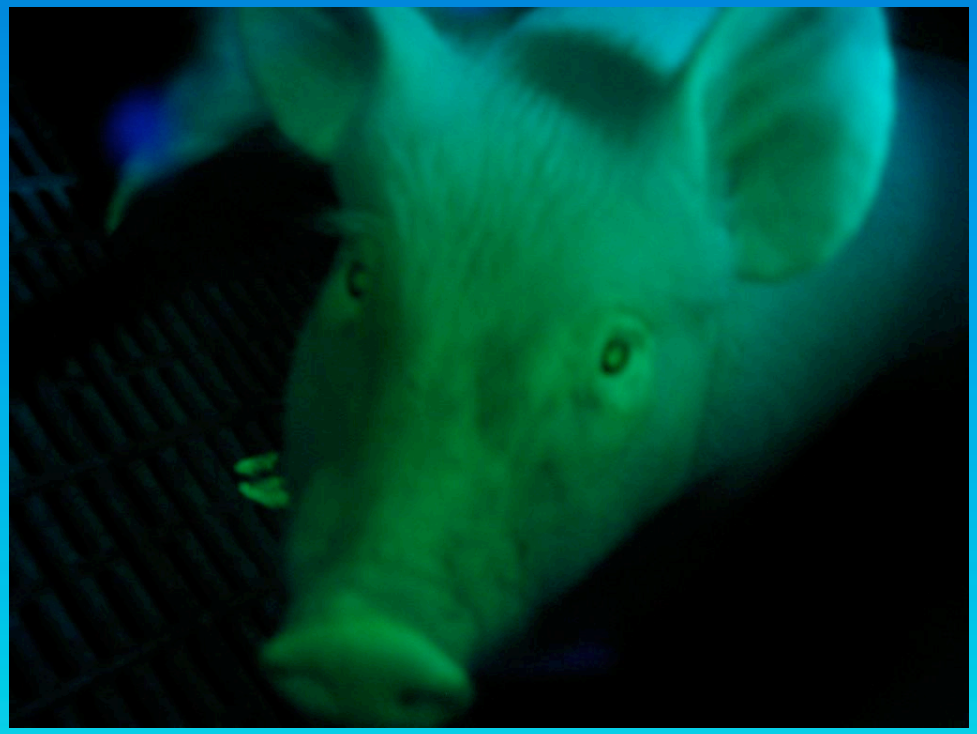
Draft # 3, May 2007

Genetically modified animals (GMO)

- Completely different category of animals
- Produced through genetic engeneering and nuclear transfer
- For biomedical applications (current)
- For food production (proof of principle)

Cloning technique for transgenesis





PNAS

Transgenic engineering of male-specific muscular hypertrophy

Dimitri Pirottin*, Luc Grobet†, Antoine Adamantidis‡, Frédéric Farnir*, Christian Hanset§, Henrik Daa Schroder¶, and Michel Georges*‖

*Unit of Factorial and Molecular Genetics, Department of Animal Production, and †Unit of Embryology, Department of Pathology and Morphology, Faculty of Veterinary Medicine, University of Liège (B43), 20 Boulevard de Colonster, B-4000 Liège, Belgium; ‡Research Center for Cellular and Molecular Neurobiology, University of Liège, 17 Place Delcour, B-4020 Liège, Belgium; §Center for Human Genetics, Cytogenetics, University of Liège, Centre Hospitalier Universitaire (CHU), Tour de Pathologie, B23, Sart Tilman, B-4000 Liège, Belgium; and ¶Institute of Pathology, Odense University Hospital, DK-5000 Odense C, Denmark

Communicated by James E. Womack, Texas A&M Un

Using a two-step procedure involving insert
and recombinase-mediated cassette exchang

RESEARCH ARTICLE

Cloned transgenic cattle produce milk with higher levels of β-casein and κ-casein

Brigid Brophy[1], Grant Smolenski[1], Thomas Wheeler[1], David Wells[1], Phil L'Huillier[1,2], and Götz Laible[1]*

Published online 27 January 2003; doi:10.1038/nbt783

To enhance milk composition and milk processing efficiency by increasing the casein concentration in milk, we have introduced additional copies of the genes encoding bovine β- and κ-casein (*CSN2* and *CSN3*, respectively) into female bovine fibroblasts. Nuclear transfer with four independent donor cell lines resulted in the production of 11 transgenic calves. The analysis of hormonally induced milk showed substantial expres-

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ARTICLES

nature biotechnology

Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection

Robert J Wall[1], Anne M Powell[1], Max J Paape[2], David E Kerr[3], Douglas D Bannerman[2], Vernon G Pursel[1], Kevin D Wells[4], Neil Talbot[1] & Harold W Hawk[1]

Mastitis, the most consequential disease in dairy cattle, costs the US dairy industry billions of dollars annually. To test the feasibility of protecting animals through genetic engineering, transgenic cows secreting lysostaphin at concentrations ranging

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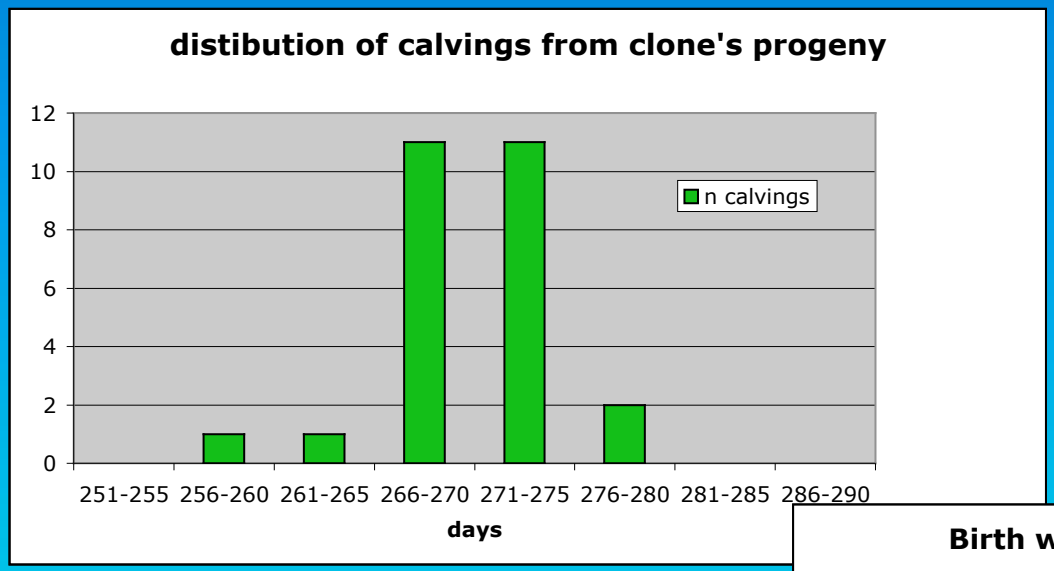
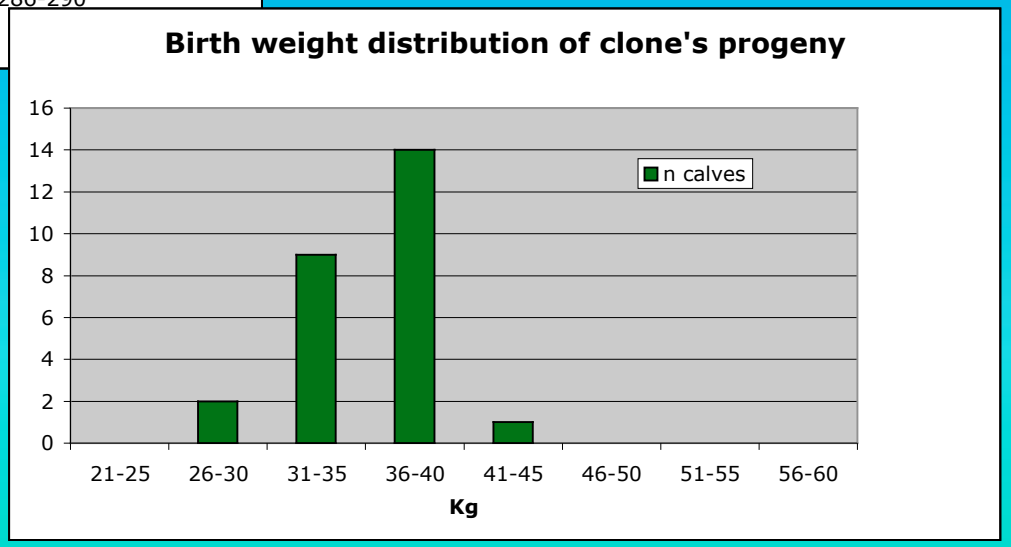
Artificial insemination results with frozen semen from Mtoto clone 2

Farms	n. of AI	n. pregnant	n. pregnancies lost (at 90 days)	% pregnant	% losses
Farm 1 (first round)	20	14	2	70	14
Farm 1 (second round)	10	8	0	80	0
Farm 2	20	10	0	50	0
farm 3	3	1	0	33	0
farm 4	10	8	0	80	0
total	63	41	2	65	5

Heyman et al, C&SC 2004, 6: 111-120

Progeny of clones





Heyman et al, C&SC 2004, 6: 111-120

Offspring from clones

Pegaso born on march 16th 2008 through AI

Piearazade du Vialaret
Offspring of Pieraz through A.I.

Final considerations

- SCNT is young technique with great potentials and it is here to stay
- It raises many more questions than that it can answer at present
- It will be a research intensive area of investigation
- There are now sufficient information to prevent animal welfare problems (observed mainly in ruminants)
- With current efficiency it is justified only to generate animals with high added value (breeding stock or transgenic animals)
- Therefore in the near future we will be dealing with the offspring rather then the clones themselves

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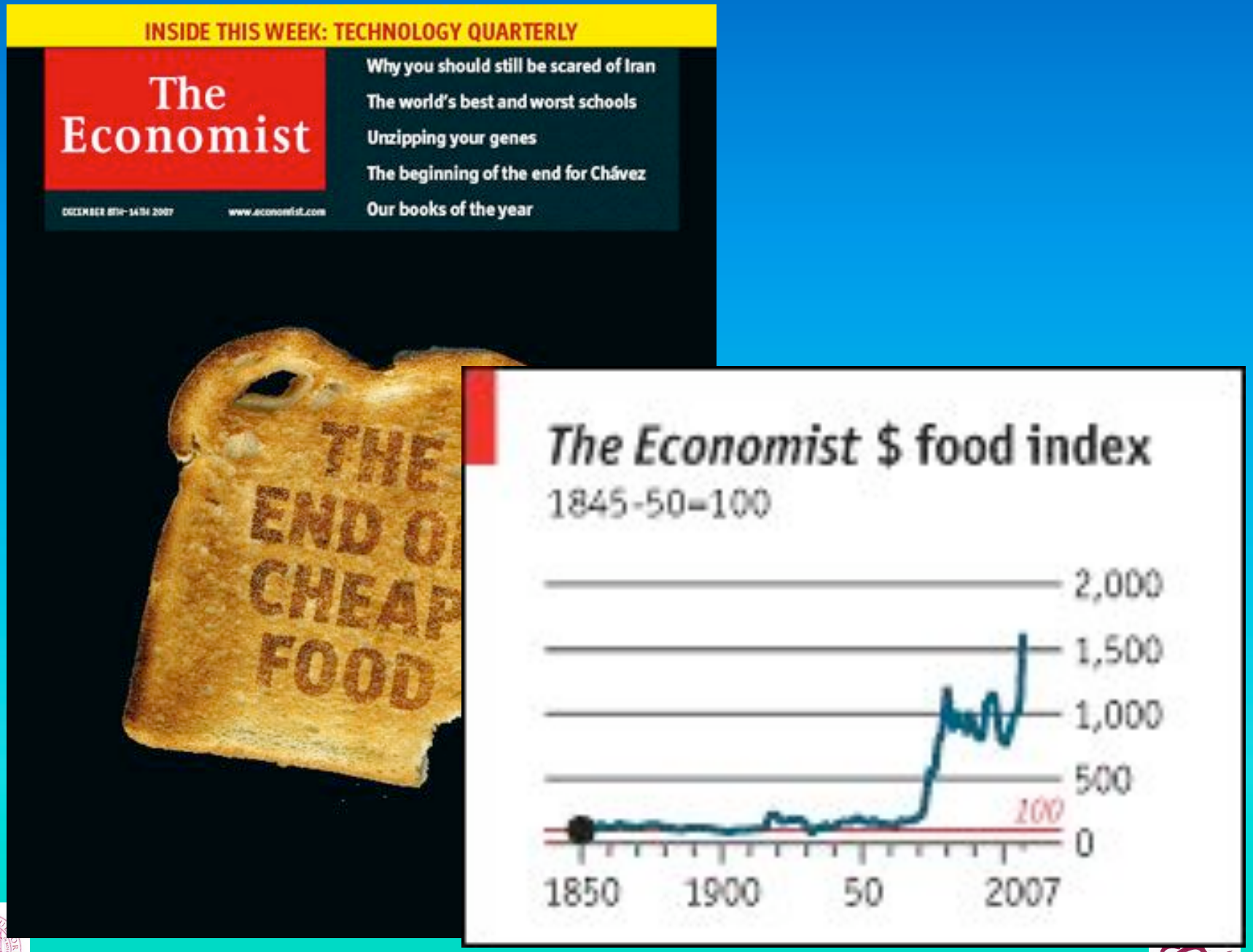

Do not throw away the baby with the dirty water!