Applying basic research in Animal Reproduction

Mouflon
Ovis ammon musimon

Farm animal domestication started 11000 years ago

Reproduction Biotechnoplogy accelerate genetic progress

Oestrus Synchromnization and artificial insemination AI

Superovulation and Embryo Transfer

Oocyte/embryo freezing

In vitro embryo production

Embryo multiplication: Cloning

Blsstomere sepsrationSeparazione di blastomeri
Embryo "splitting"
Nuclear Transfer with embryonic cells
Nuclear Transfer with somatic cells

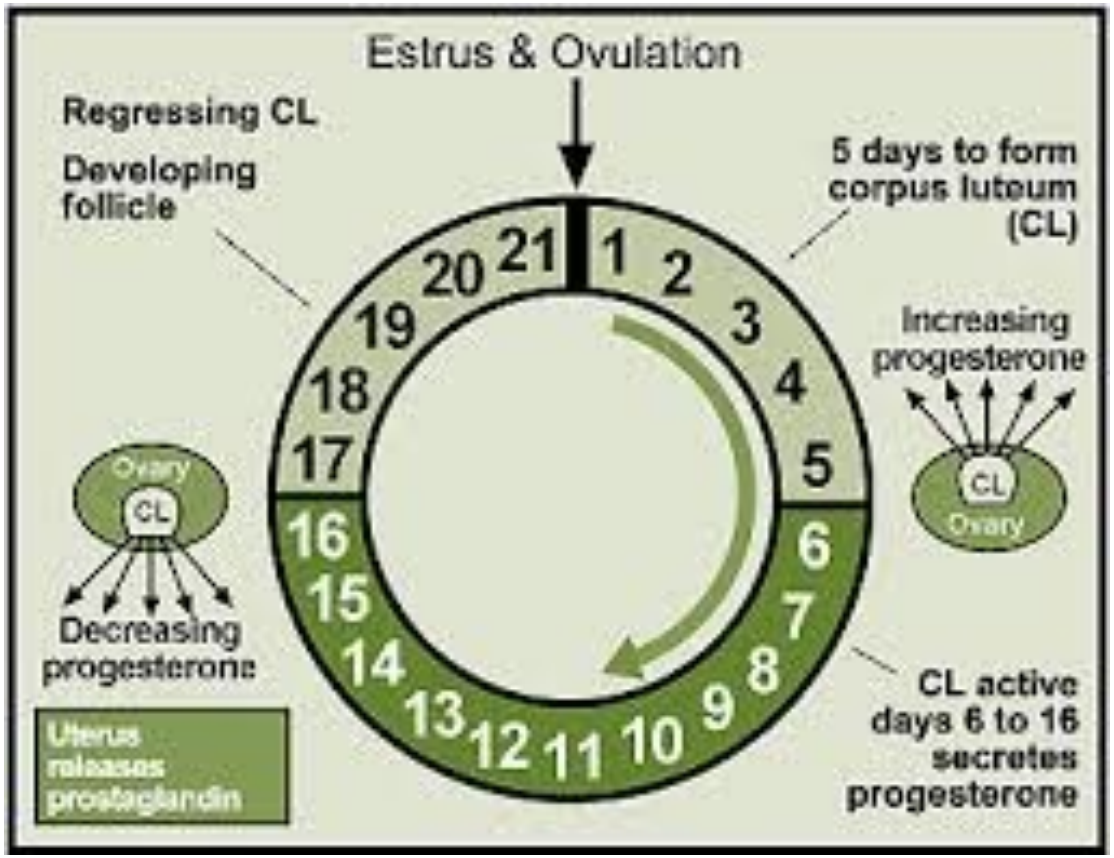
Embryo Sexing

Sex pre determination

Transgenic Animals

Embryonic/adult Stem Cells

Sintesi di un ciclo ovarico per studenti di Biotecnologie della Riproduzione



Biotecnologie embrionali

Sincronizzazione della attività ovarica e fecondazione artificiale

Superovulazione ed embryo transfer

Congelamento di gameti ed embrioni

Produzione embrionale in vitro

clonazione

Separazione di blastomeri
"splitting" (divisione embrionale) di embrioni
Trapianto nucleare con cellule embrionali
Trapianto nucleare con cellule somatiche

Sessaggio embrionale

Predeterminazione del sesso (sessaggio degli spermatozoi)

Animali transgenici

Cellule staminali embrionali

Controllo ciclo estrale:

Trattamento per 13/14 giorni con analoghi di Sintesi del progesterone

Sincronizzazione della attività ovarica e fecondazione artificiale
Ovino modello primario

Controllo del ciclo ovarico –
Giovanni Manunta – Università di Sassari

Ovino modello sperimentale fondamentale

Robinson JJ. Estrus synchronization in sheep by progesterone_impregnated intravaginal sponges. Nature 1962, 324

Tecnologia del materiale seminale – fecondazione artificiale
Pietro Cappai , Istituto Zootecnico e Caseario, Sassari

Riproduzione e biotecnologie negli ovini. ***Loi P.***, *Cappai P.* L' allevatore di Ovini e Caprini, XVI, 2, 11-14 (1999)

Effetto ariete sull'attività ovarica nella pecora di razza Sarda dopo il parto. *Naitana S* e ***Loi P.***Atti VIII SIPAOC, 1, 9-16 (1988)

Influenza del periodo del parto sulla durata dell'anaestro. *Naitana S., Cappai P., Branca A.,* ***Loi P.****, Filia F., Ledda S.* Meeting sullo studio della efficienza riproduttiva degli animali di interesse zootecnico 95, Bergamo 101-105 (1990

1) Prelievo e conservazione del materiale seminale

Andamento circannuale delle caratteristiche quanti-qualitative dell'eiaculato di muflone. ***Loi P.***, *Filia F., Ledda S., Cappai P., Marongiu A.* Atti IX Sipaoc 4, 10, grado (1990).

Osservazioni preliminari sulla possibilità di impiego del seme di muflone. *Naitana S.,* ***Loi P.****, Ledda S., Filia F., Cappai P., Branca A.* XI Convegno allevamento selvaggina, 217-219 (1989).

Inseminazione intrauterina con seme fresco e congelato di muflone. ***Loi P.****, Ledda S., Filia F., Naitana S., Cappai P.* Atti S.I.S.Vet XLIII, 323-326 (1989).

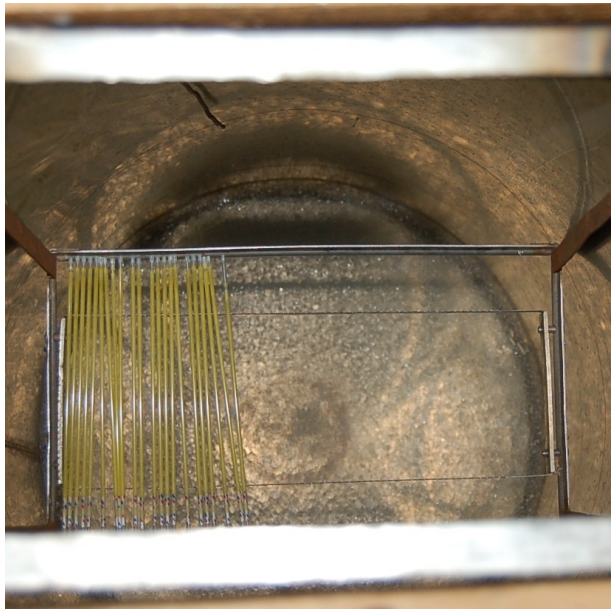
Costituita una banca di seme congelato (~3000 dosi) in azoto liquido

Crioconservazione del seme ovino – metodo Colas- Cappai

Medium per il congelamento del seme ovino:

medium base		**medium a 30° C**		**medium a 4° C**	
TRIS Acido Citrico Fruttosio Penicillina Streptomicina H_2O Milli Q	 2,42 g 1,36g 1,00 g 100.000 U.I. 100 mg 67,20 ml	Medium base H_2O Milli Q Tuorlo d'uovo	33,60 ml 6,40 ml 10,00 ml	Medium base Glicerolo Tuorlo d'uovo	33,60 ml 6,40 ml 10,00 ml

Crioconservazione del seme ovino – metodo Cogniè - Cappai



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Trapianto

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Predeterminazione del sesso (sessaggio degli spermatozoi)

Animali transgenici

Cellule staminali embrionali

Superovulazione – embryo transfer di femmine d'èlite

Aumento del tasso di ovulazione con ormone follicolo stimolante

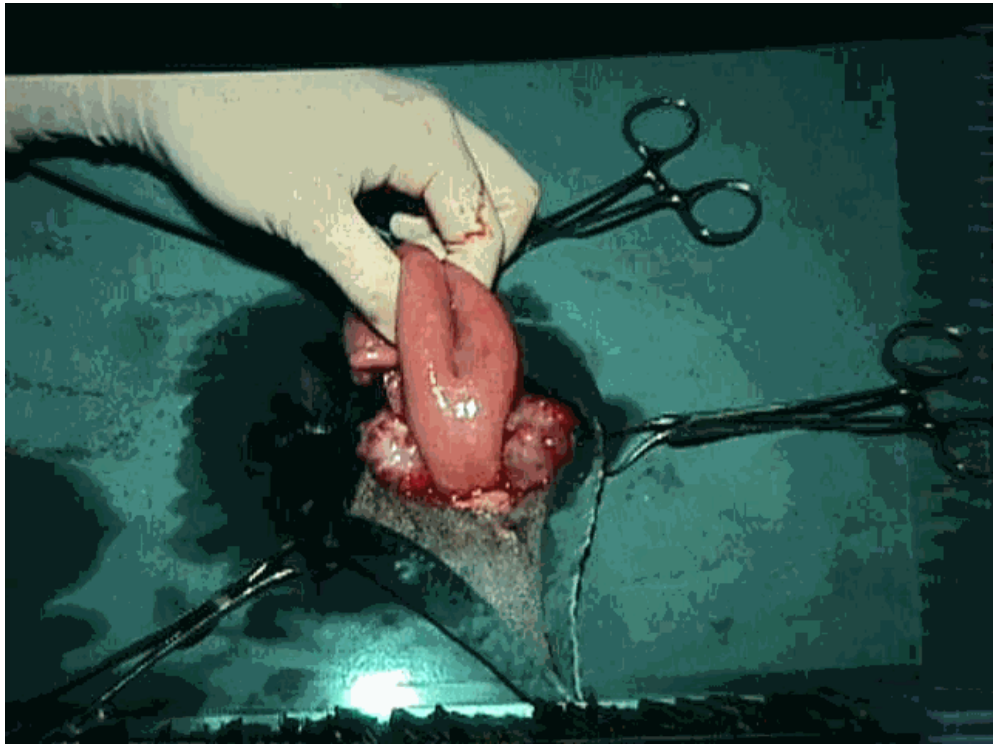
Formulazione ormonali testate su pecora Sarda

PMSG (Pregnant Mare Serum Gonadotropin)
Follicle Stimulating Hormone (FSH): Schering,
Follicle Stimulating Hormone (FSH): Sigma
Follicle Stimulating Hormone (FSH): Y. Conbarnous
Follicle Stimulating Hormone (FSH): Fatropin
Follicle Stimulating Hormone (FSH):Pluset- Serono
Follicle Stimulating Hormone (FSH):Ovagen, ICP, New Zealand

Impiego dell'HMG nella superovulazione della pecora di razza Sarda.
Naitana S., Ledda S., ***Loi P.****, Filia F., Branca A., Cappai P* Atti IX Sipaoc 7, 3, Grado (1990)

Risposta superovulatoria nelle pecore di razza sarda trattate con una singola somministrazione di pFSH in PVP.
Dattena M., ***Loi P.****, Branca A., Cappai P., Naitana S., Ledda S.*Atti XI S.I.P.A.O.C. 323-325 (1994)

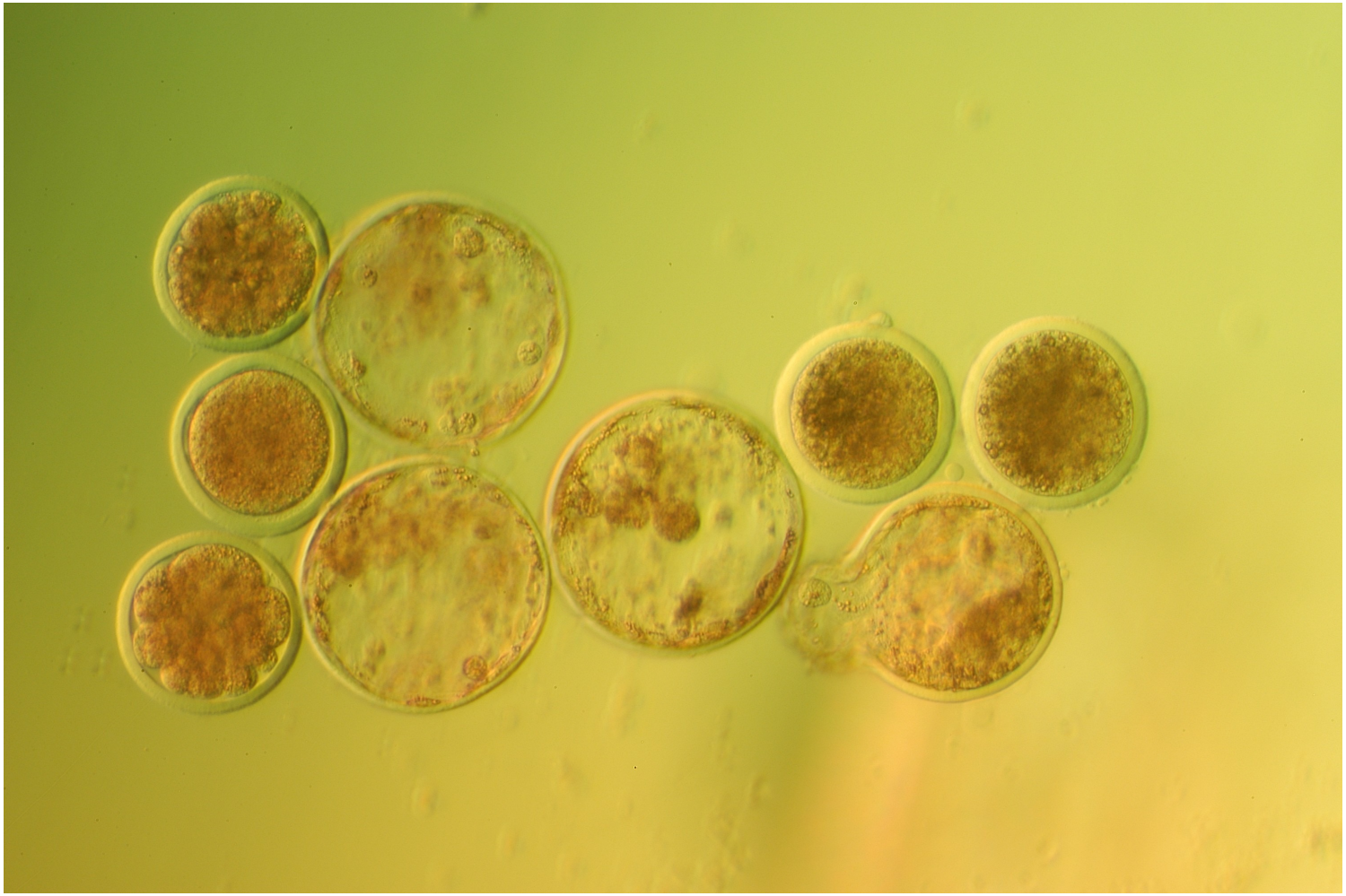
Recupero embrionale chirurgico
Trasferimento: chirurgico o laparoscopico



Catetere di Foley usato per il flushing uterino (20 ml c/a di medium 199 o PBS)

Superovulazione ed Embryo transfer……

Embrioni di pecora 7 giorni dopo la fecondazione (G. Ptak)



1) Superovulazione ed embryo transfer

Attività ovarica in
Filia F., Naitana S

Risposta del muflo
dell'anno. *Ledda S*
XII Convegno Alle

Embryo-transfer c
Naitana S., Ledda

Embryo recovery
and viability after
Dattena M., Gallu
Animal Reprod. Sc

LOI P., PTAK G., DATTENA M., LEDDA S., NAITANA S., CAPPAI P (1998)
. 26. Embryo transfer and related technologies in sheep reproduction. *REPRODUCTION NUTRITION DEVELOPMENT.*
vol. 38 pp. 615-628 ISSN: 0926-5287

Superovulazione ed embryo transfer nella pecora di Razza Sarda

n. Ovulazioni (Media)	Embrioni recuperati (Media)	Embrioni trasferibili (Media)	Agnelli nati (Media)
11,6	9,1	7.0	5,2

Dati medi prodotti da 2360 interventi di superovulazione ed embryo transfer su pecore di razza Sarda (1984-1998)

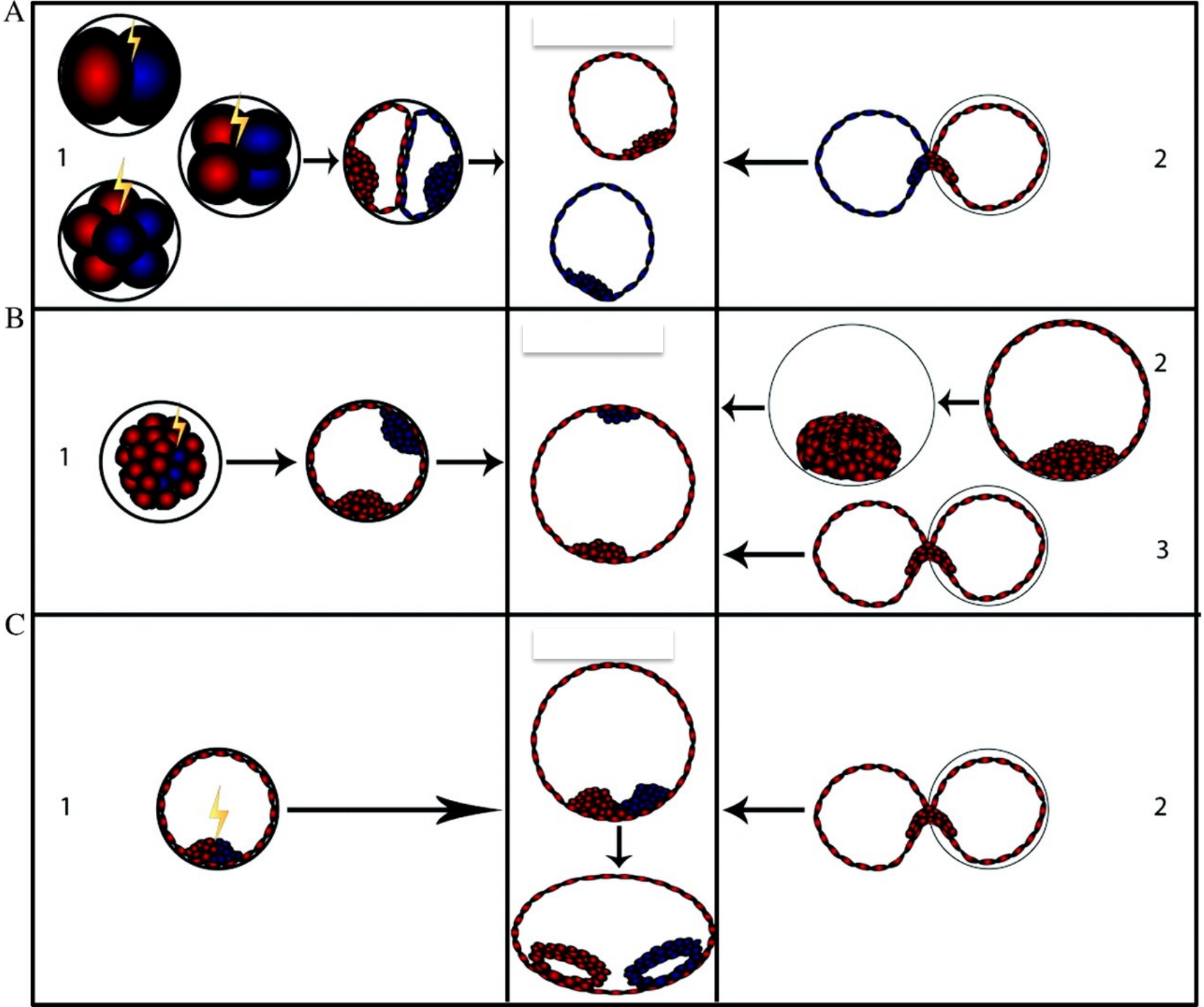
Embryo transfer and related technologies in sheep reproduction.
Loi P., Ptak G., Dattena M., Ledda S., Naitana S., Cappai P. Reprod. Nutr. Dev. 38, (1998)

Moltiplicazione di embrioni

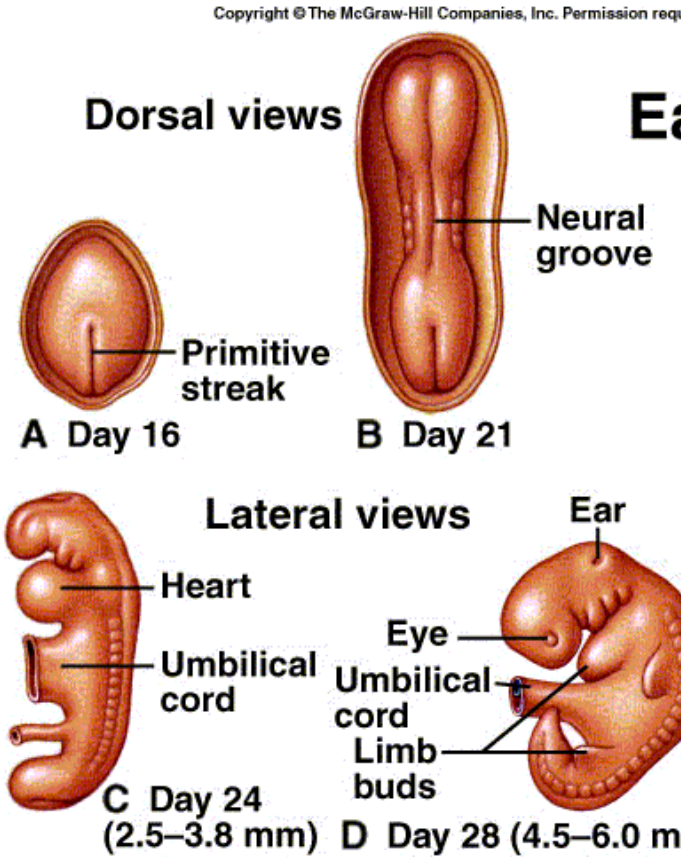
“splitting” (divisione embrionale) di embrioni
Separazione di blastomeri

Embryo splitting: produzione “artificiale” di gemelli monozigoti

Cosa sono i gemelli monoovulari?


A
1
2
B
1
2
3
C
1
2

..altra modalità di generazione di gemelli monovulari

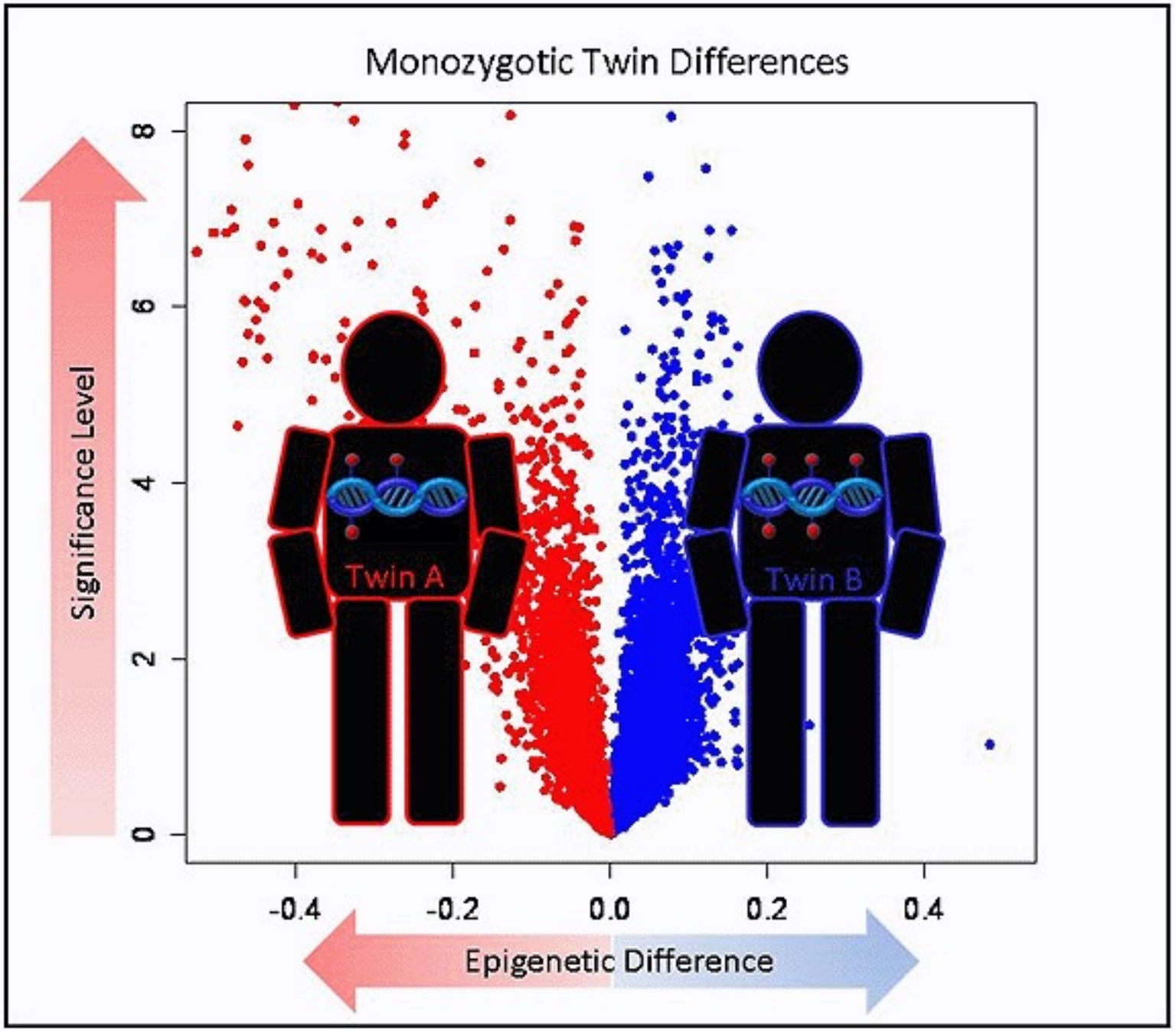


Duplicazione della linea primitiva

Duplicazione incompleta o parziale= gemelli siamesi

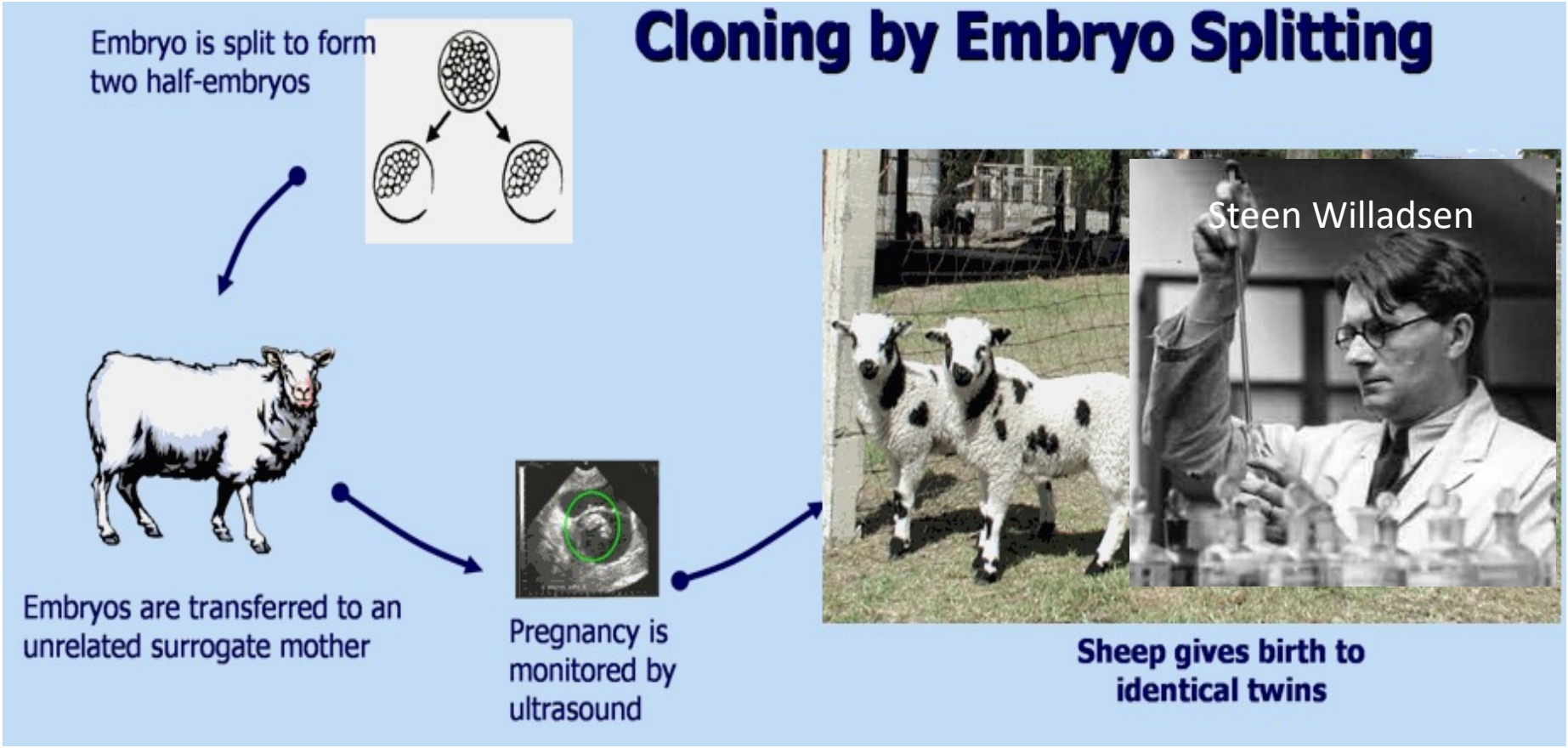
Gemelli monoovulari totalmente identici?

Fenotipicamente sì, epigeneticamente no


Monozygotic Twin Differences
Significance Level
0
2
4
6
8
Twin A
Twin B
-0.4
-0.2
0.0
0.2
0.4
Epigenetic Difference

Tipi di micromanipolazione e stadio embrionale per lo splitting

schema procedurale



Willadsen S. A method for culture of micromanipulated **sheep embryos** and its use to produce monozygotic twins Nature. 1979 Jan 25;277(5694):298-300

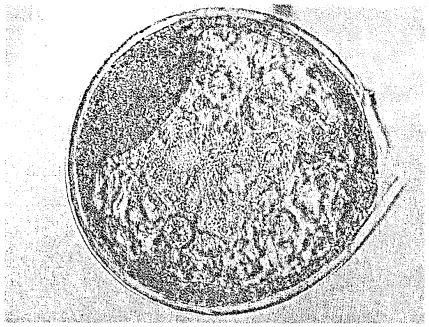


FIG 1: Fully expanded sheep blastocyst collected day 7½ after onset of oestrus. The diameter of the embryo is approximately 0·16 mm. Note the inner cell mass. Note also the rupture in the zona pellucida

Embryo micromanipulation

Only embryos which were considered to have developed normally up to the time of collection were used for micromanipulation. The micromanipulation system consisted of a Leitz micromanipulator (left and right unit) and a Wild M5 stereomicroscope. The left micromanipulator was fitted with a capillary holding pipette attached to a 20 ml syringe, while the right manipulator unit was fitted with a fine glass needle. During micromanipulation the embryo was held in the lid of a 9 cm petri dish containing phosphate buffered saline. A description of the manufacture of the microinstruments and of their arrangement for embryo manipulation has been given previously (Willadsen 1982).

Morulae.— The egg was held on the tip of the holding pipette by suction, while the point of the needle pierced the zona pellucida, traversed the perivitelline space, gently pushing the embryo itself aside, to penetrate the zona again at the opposite pole. Suction was discontinued, and the egg, still speared on the needle, was gently rolled across the top of the holding pipette until the embryo itself was placed between the needle and the glass wall with approximately half of its cells on either side of the needle. The needle was lowered so that a deep furrow was formed in the cell mass, dividing it into two halves connected by a thin cellular bridge. The egg was then rubbed against the wall of the holding pipette until the needle had cut through both the cell bridge and the zona pellucida, leaving two separate cell groups enclosed in a zona pellucida with a 180° tear from one pole to the other.

Blastocysts (Fig 1). — The embryo was held either by its zona pellucida (early and expanding blastocysts) or by the trophectoderm (hatched blastocysts) and pierced from pole to pole with the needle through the blastocoele, avoiding the inner cell mass (Fig 2a). The embryo was then rolled on the top of the holding pipette so that the inner cell mass was positioned between the needle and the glass wall with approximately half of its cells on either side of the needle (Fig 2b). The needle was lowered and the embryo was rubbed against the wall of the pipette until the inner cell mass and the overlying trophectoderm had been severed (Fig 2c). Similarly, the trophectodermal hemisphere opposite the inner cell mass was bisected (Fig 2d). In most instances unhatched blastocysts were released from the zona pellucida during bisection. If the zona was still present, a 180° tear was made in it between opposite poles. By this procedure, each blastocyst was divided into two embryos each consisting of approximately half of the inner cell mass and half of the trophectoderm. The actual bisection of the embryo took only a few minutes, but the operations were deliberately carried out at a relatively slow pace, the ewes being under general anaesthesia for an average of approximately one hour.

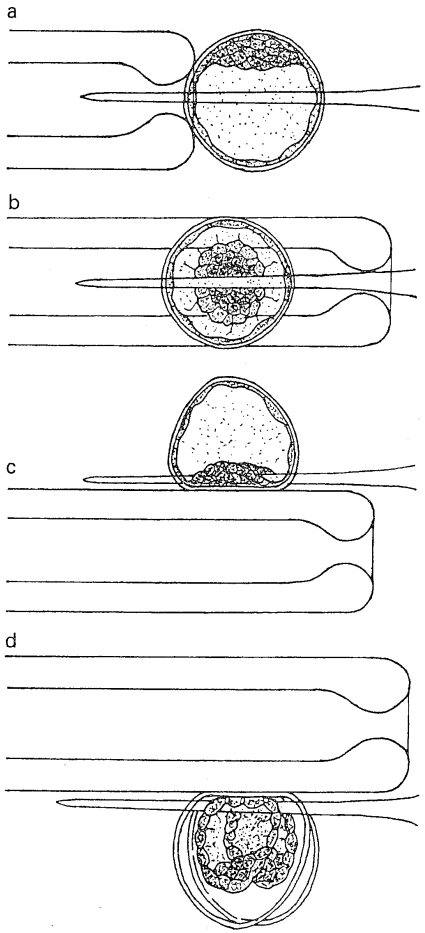


FIG 2: Bisection of a blastocyst. For description see text

Results

The results are presented in Table 1. Thirty-one (86 per cent) of the 36 eggs ovulated were recovered and among these were all the eggs ovulated in 14 (78 per cent) of the 18 ewes. Twenty-seven (87 per cent) of the recovered eggs had developed normally and at least one normally developing embryo was recovered from each of 16 (89 per cent) of the ewes. The two ewes from which no normally developing embryos were collected were used as recipients for surplus embryos from ewes which had come into oestrus on the same day. Thus, each of the 18 ewes in the study received one bisected embryo (ie, two half embryos).

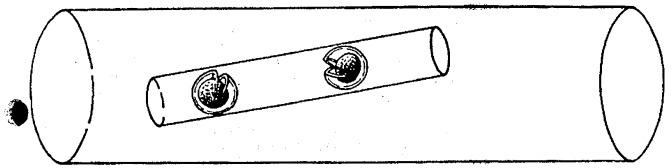


Fig. 1 Diagram of a pair of micromanipulated single blastomere eggs and agar embedding.

blastomeres to pass through. (3) The blastomeres were separated by gentle suction with a Pasteur pipette of smaller tip bore, allowing the passage of only one, slightly compressed, blastomere at a time. (4) Single blastomeres were injected with a capillary pipette into zonae evacuated as described above and held by suction. (5) A 1.2% solution of agar (Difco) in 0.9% NaCl in distilled water was prepared. While the solution was left to cool, the eggs to be embedded were transferred from PBS to sheep serum. (6) When the agar had cooled to 38–36 °C a few ml were poured into a Petri dish, and a monozygotic pair of single blastomere eggs was immediately transferred to the agar solution with a fine-bore Pasteur pipette to be picked up a few seconds later in a small amount of agar solution. The agar containing the eggs was held in the tip of the pipette for about half a minute and then expelled as a solid cylinder into PBS. The ends were cut off the cylinder so that the eggs were contained in a segment measuring 0.15×0.5–1.0 mm. (7) This tiny cylinder was in turn embedded by a similar procedure into a larger one, measuring 0.7×2.0–2.5 mm (Fig. 1). Procedures (1) and (4) were carried out with the aid of a Leitz micromanipulator and De Fonbrune suction/injection devices.

One to four agar cylinders, each containing a pair of monozygotic eggs, were transferred to each oviduct of ewes on days 1 or 2 of their oestrous cycle. To facilitate collection of eggs, the oviducts of recipient ewes were ligated at the utero-tubal junction. After $3\frac{1}{2}$ to $4\frac{1}{2}$ d the eggs were flushed from the oviducts and examined. Those embryos which had reached the late morula–early blastocyst stage were considered to have developed at a normal rate. Among the rest a distinction could be made between those which had undergone some development but were retarded and those which were completely degenerate (Fig. 2).

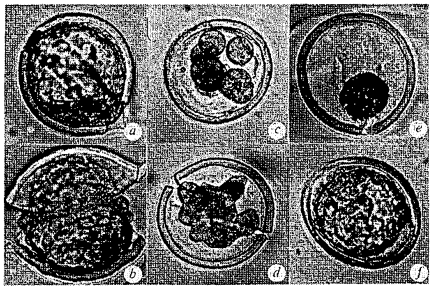


Fig. 2 Eggs from single blastomeres of two-celled sheep embryos after culture in sheep oviducts but still embedded in agar (~×400). *a*, *b*, Monozygotic pair after $4\frac{1}{2}$ days' culture. Both eggs have developed at a normal rate and are early blastocysts. *c*, *d*, Monozygotic pair after $3\frac{1}{2}$ days' culture. *c* Is retarded in its development, whereas *d* has developed at a normal rate and undergone compaction. After fixation and staining, *c* was found to contain 8 cells, *d* 16 cells, all with morphologically normal nuclei. Normally, compaction is only evident in sheep embryos containing 30 or more cells. *e*, *f*, Monozygotic pair after $4\frac{1}{2}$ days' culture. *e* Is completely degenerate, whereas *f* is an early blastocyst. The large defects in the zonae pellucidae are visible in *a*, *b* and *d*. The main reason for inserting the single blastomeres into evacuated zonae was that the presence of the zona made it easier to remove the agar after culture, without damaging the embryo.

The results of this part of the experiment are summarised in Table 1. It is evident that very considerable losses of embryos resulted from inefficient micro- and macrosurgery on two-celled embryos and recipient ewes, respectively, but few embryos were lost in the course of the embedding procedure. Furthermore, all embryos recovered from the recipients had intact agar coatings, which made it a simple matter to recognise not only the original pairs, but also individual embryos within these pairs. The overwhelming majority of embryos recovered had also developed at an apparently normal rate. As observed in investigations of single blastomere mouse eggs[1,16], the time at which compaction and blastulation occurred reflected the age of the embryo rather than the number of cells it contained.

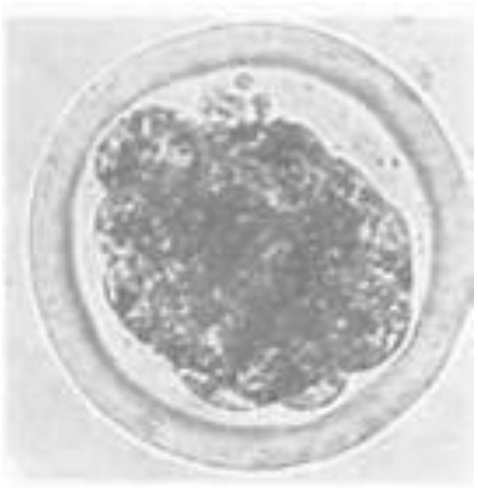
Table 1 Results of manipulation experiment

Pairs of monozygotic blastomeres	
Start of experiment (~no. of two-celled embryos)	61
After removal of zona pellucida, blastomere separation and injection into evacuated zonae	33
After embedding in agar	31
Transferred to ewes	31
Recovered from ewes	20
Stage of development of recovered single blastomere eggs	
Late morula or early blastocyst	35
Retarded	1
Degenerate	4
Monozygotic pairs in which both embryos had developed at a normal rate	16

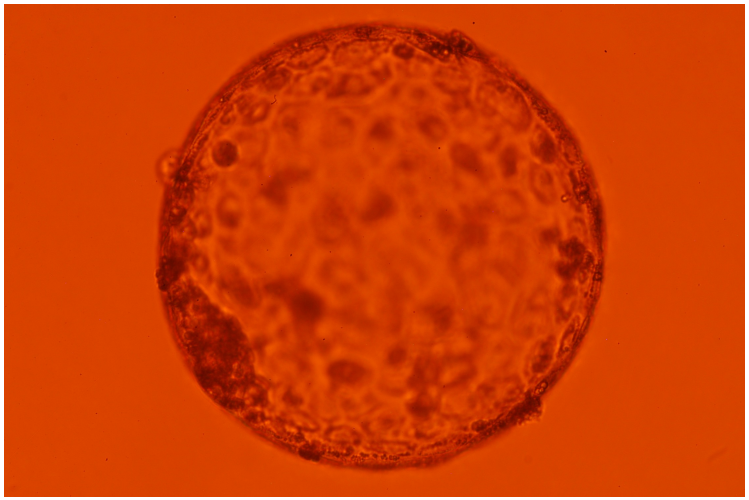
Thirty-two late morulae and early blastocysts, constituting 16 sets of monozygotic embryos, were manipulated out of the agar with a pair of 24-g hypodermic needles, and transferred to ewes on days 5, 6 or 7 of their oestrous cycle. Each ewe received one pair of monozygotic embryos. Of 16 recipients, 11 became pregnant. One aborted a single fetus on day 100. The remaining 10 went to term, producing, in all, five sets of twins (Fig. 3) and five single lambs. This result represents an embryonic survival rate 10–15% below that expected following transfer of ordinary embryos on days 5–7 in sheep[12,13]. It is likely that damage resulting from the manipulations necessary to remove the agar before transplantation was responsible for the lower viability of embryos in the present experiments, and when the twinning rate is taken into consideration, it seems reasonable to conclude that each blastomere of a normal two-celled sheep embryo has the potential to develop into an entire lamb. In view of the work of Nicholas and Hall[14], Seidel[15] and Tarkowski[16] on single blastomeres of two-celled embryos of rat, rabbit and mouse, this result was not surprising, although the experiments of Mullen, Whitten and Carter[17], in which only one pair of monozygotic twins was obtained from the separated blastomeres of an unspecified number of two-cell embryos, suggested low viability of such 'half embryos'.

Tipi di micromanipolazione e stadio embrionale per lo splitting: Quale stadio embrionale?

Morula compatta



Blastocisti



Risultati simili, entrambe pratiche: recupero dall'utero

Esempi di micromanipolazione per realizzare lo splitting
In morule e blastocisti

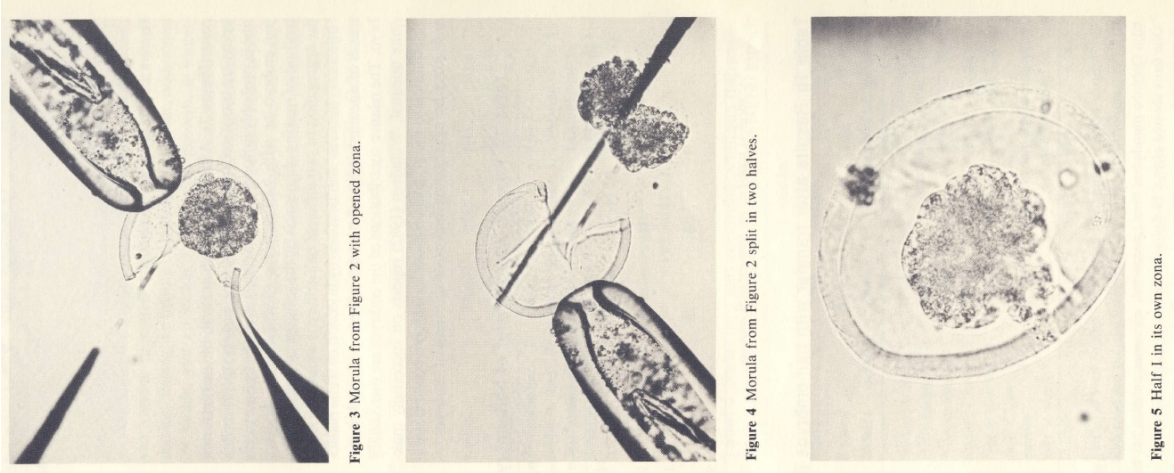
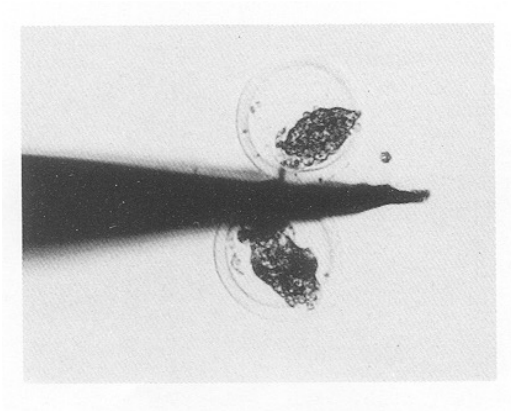
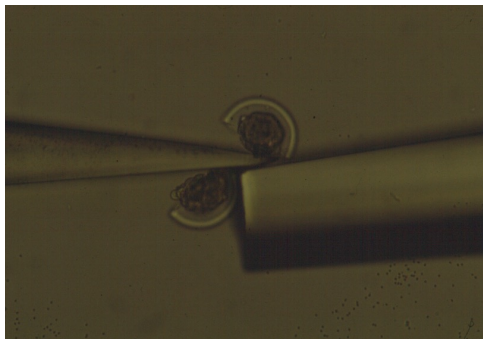


Figure 3 Morula from Figure 2 with opened zona.

Figure 4 Morula from Figure 2 split in two halves.

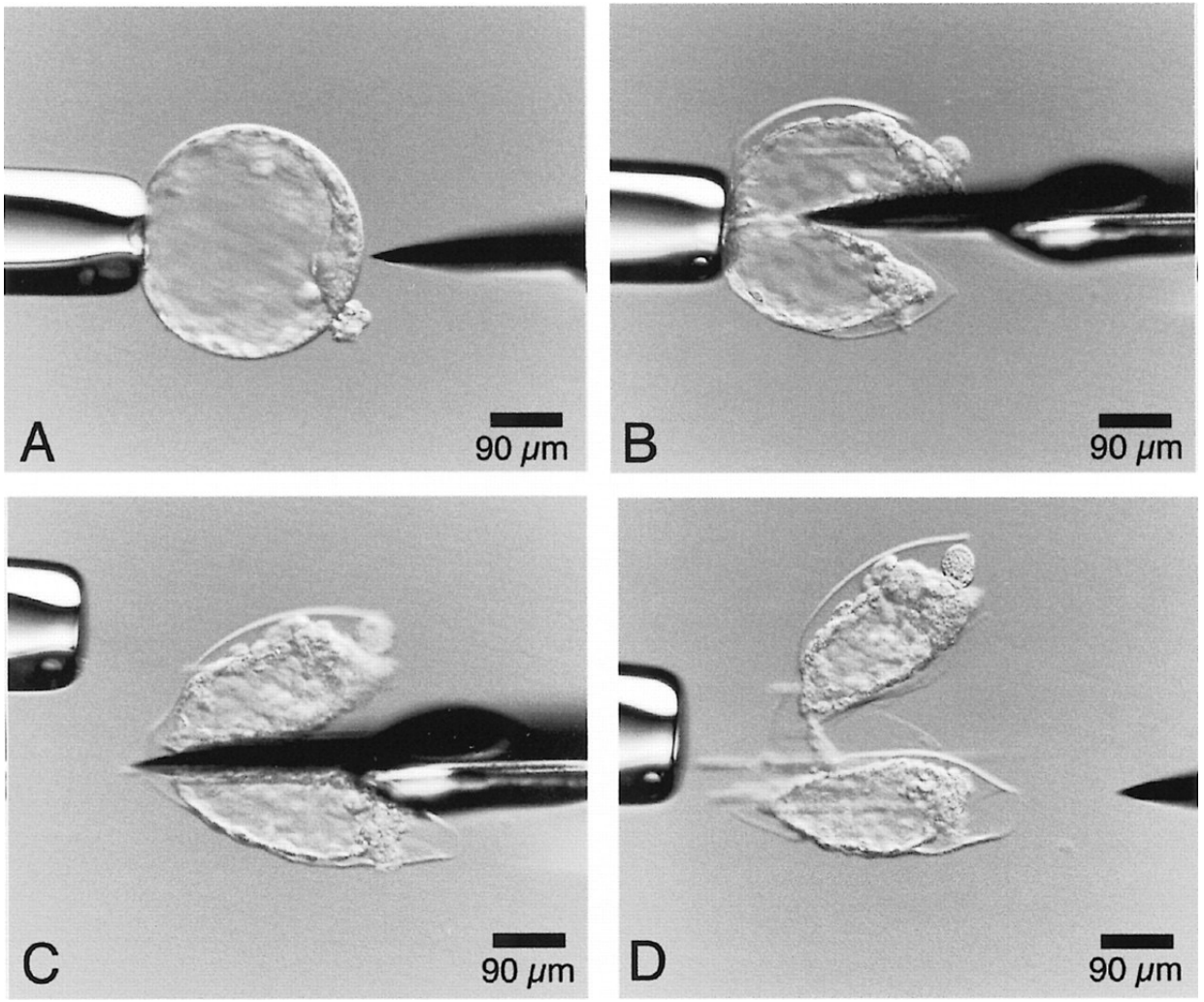
Figure 5 Half 1 in its own zona.





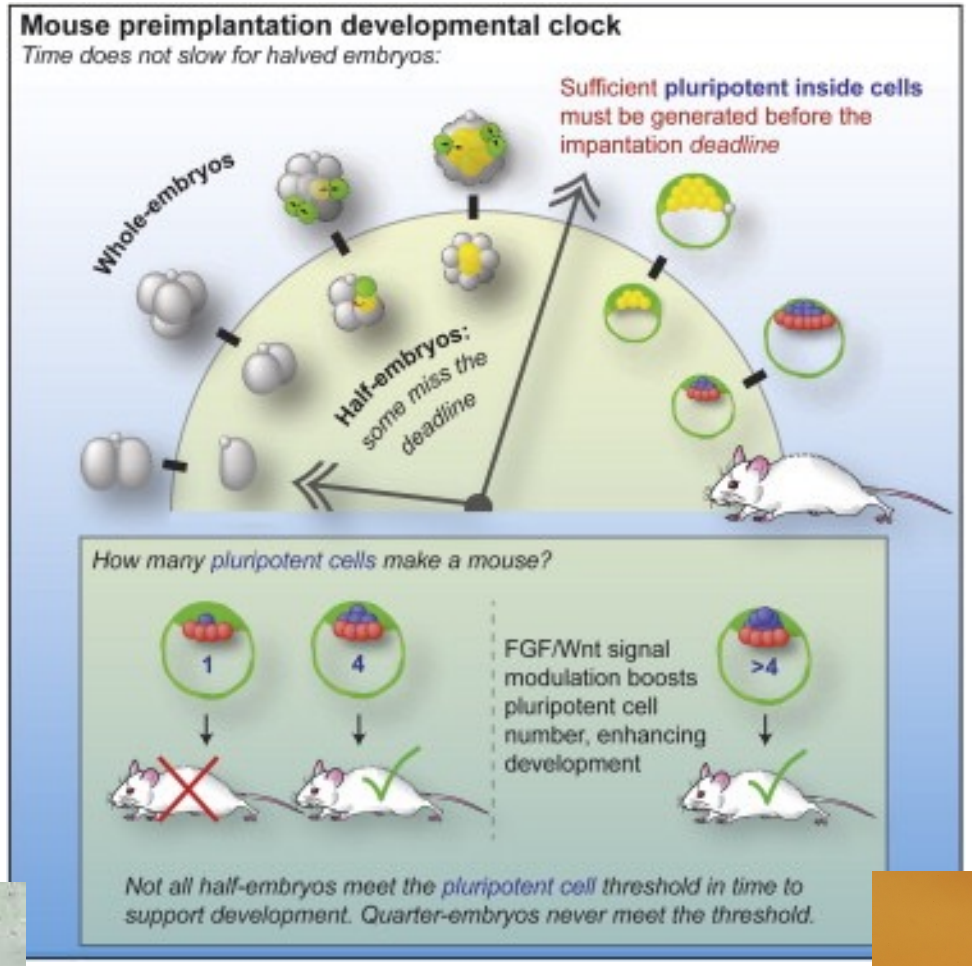
Emi-embrioni riposizionati in zone pellucide vuote

Embryo twinning by blastocyst bisection.

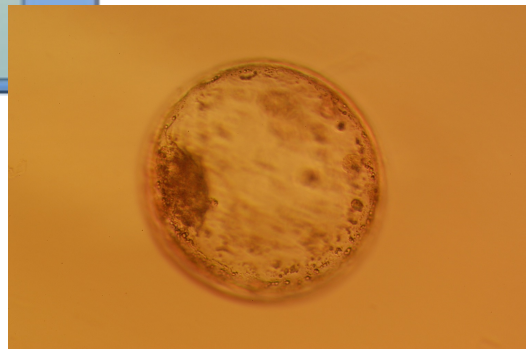


Mitalipov S M et al. Biol Reprod 2002;66:1449-1455

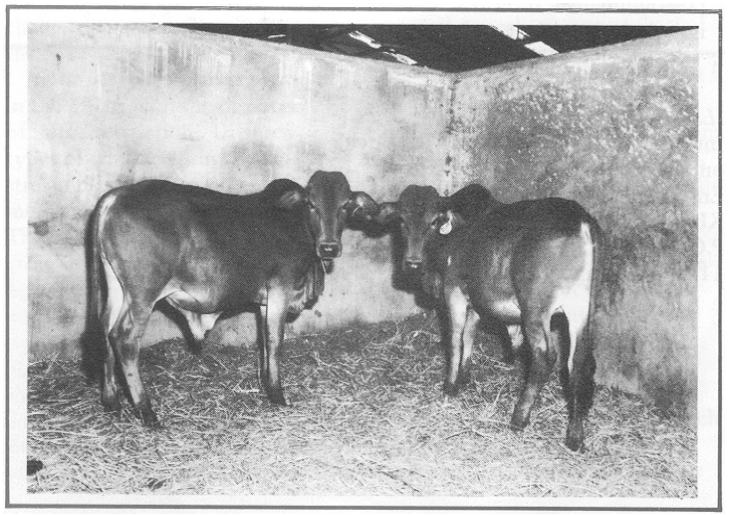
Necessario un numero minimo di cellule ICM per generare un feto!



Embrione divisibile solo in due!



Identical twins prodotti in quasi tutte le specie da reddito e nel Resus Macaca



Identical twins – gemelli monozigotici
rilevanza

Moltiplicazione genotipi altamente produttivi
o Particolari (specie da reddito!)

Produzione di modelli animali sperimentali
- 1 coppia di twins = 40 animali random

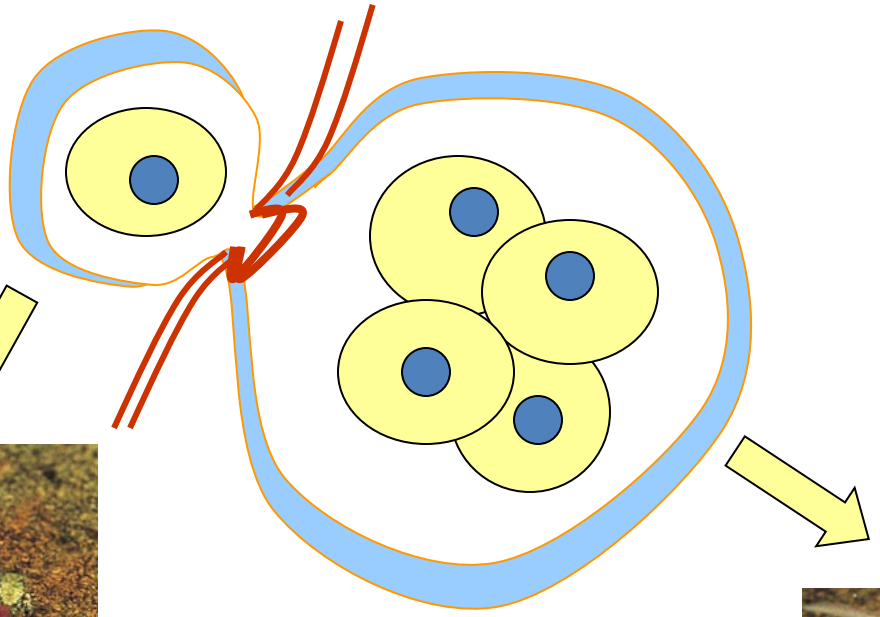
Importanti modelli per studi di epigenetica

Efficienza limitata nell'embryo splitting, solo 2, si può fare di più?

Blastomere separation

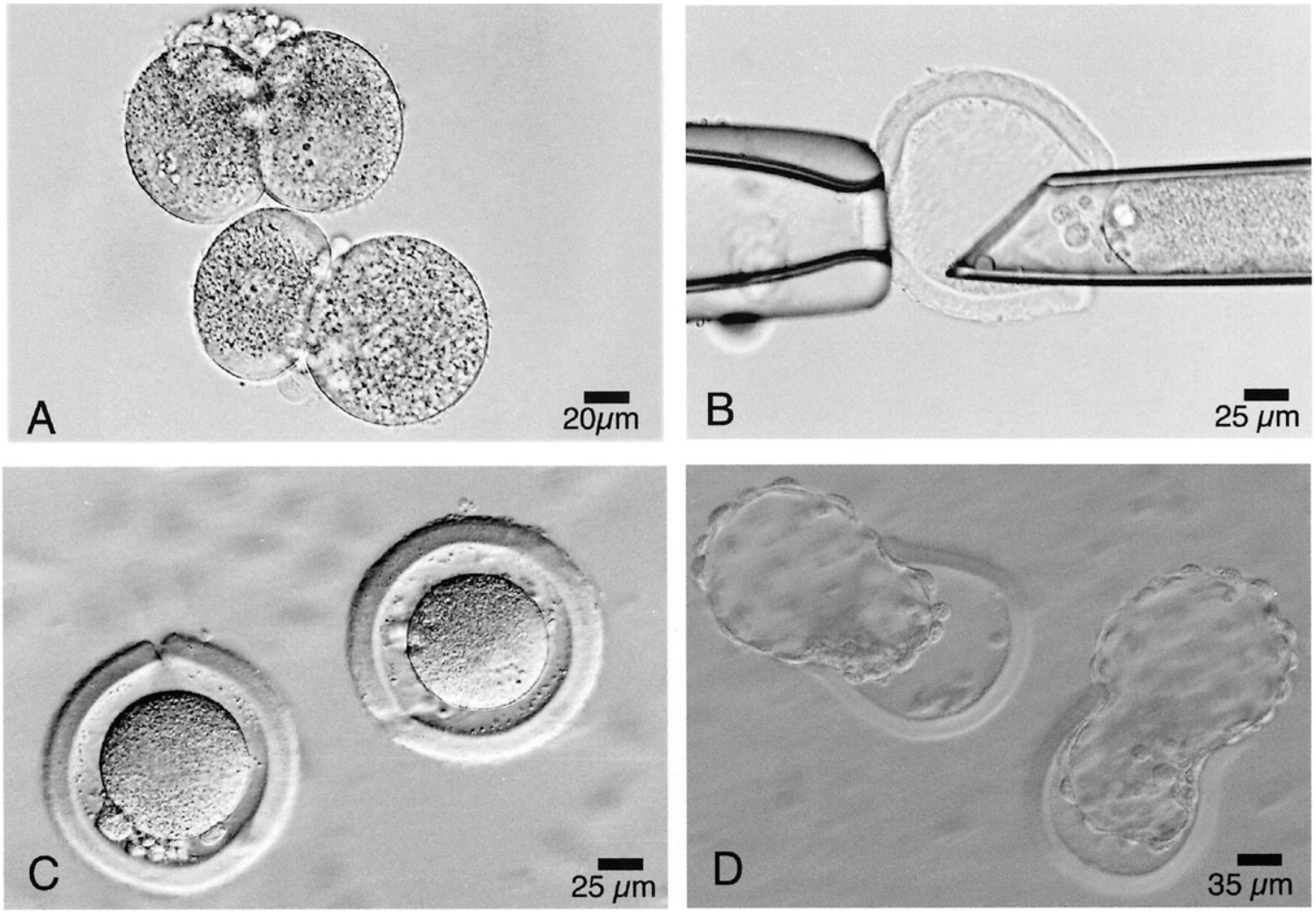
Blastomeri di un embrione sono totipotenti
Totipotenza: capacità di generare tutti i tipi cellulari,
Trofoblasto compreso

Hans Spemann's experiment (1928)



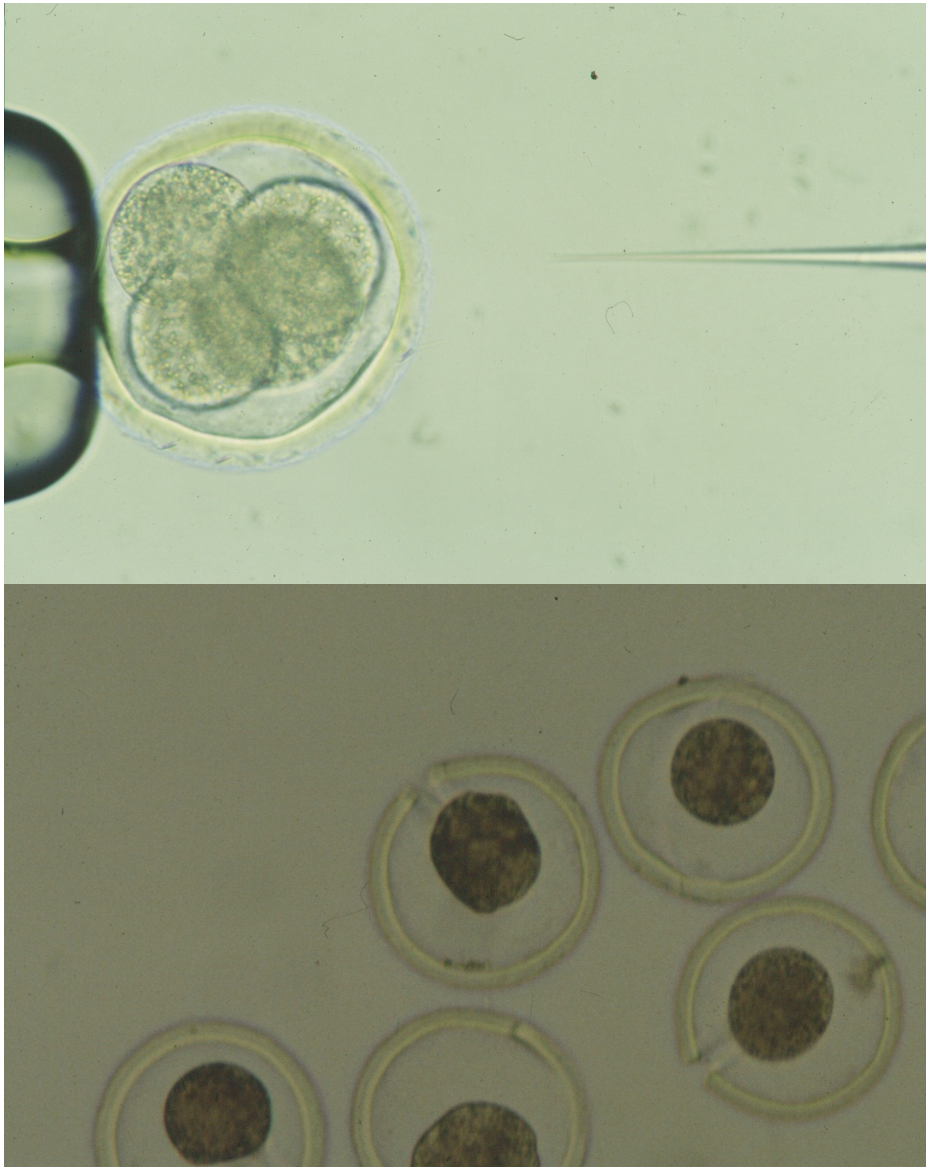
Blastomeri prima della Attivazione genoma embrionario Sono totipotenti

Embryo twinning by blastomere separation
Resus embryos.



Mitalipov S M et al. Biol Reprod 2002;66:1449-1455

Separazione di blastomeri in embrioni di pecora. Loi 1991



Separazione di blastomeri:
workflow

Siliconizzare le pipette di micromanipolazione

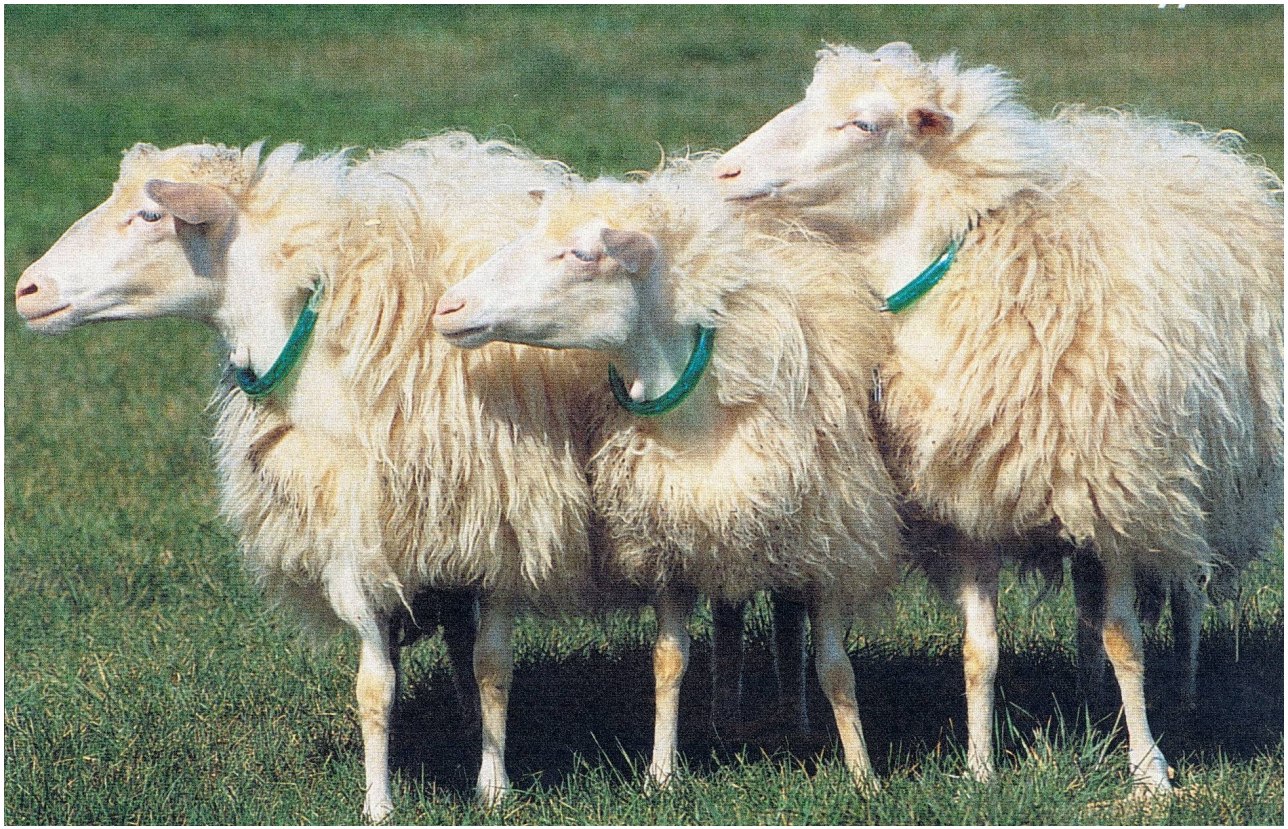
Rimozione Zona Pellucida – acid tyrode, proteasi
Differenze tra specie-
Ago di vetro - quest'ultimo mio preferito

Effettuare la separazione di blastomeri in medium
Senza FCS e Ca^{++} free!

Effettuare le micromanipolazioni in presenza di inibitori
Dell'actina (Citocalasina B)

Clone di tre agnelle identiche (Loi 1991)

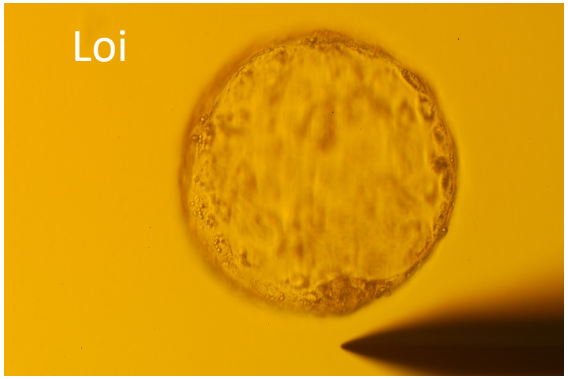
Vantaggi……

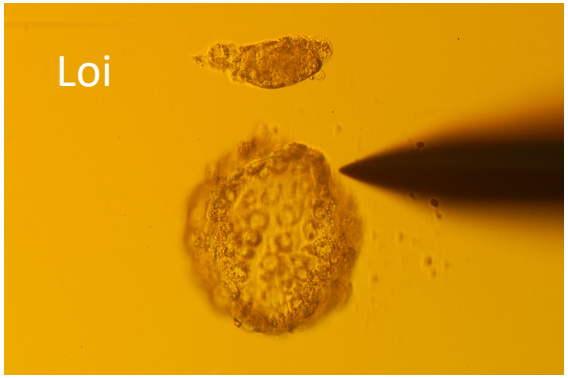


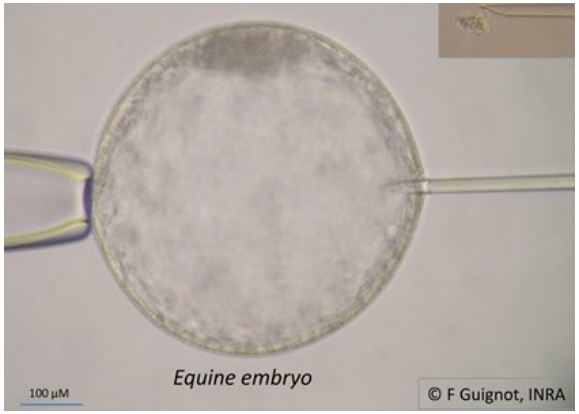
Ottimizzazione delle tecniche di micromanipolazione: Sessaggio/predeterminazione del sesso dell'embrione

Sessaggio dell’ embrione
Con Polimerase Chain Reaction…(?)
Come si fa?

Primo, biopsia embrionale

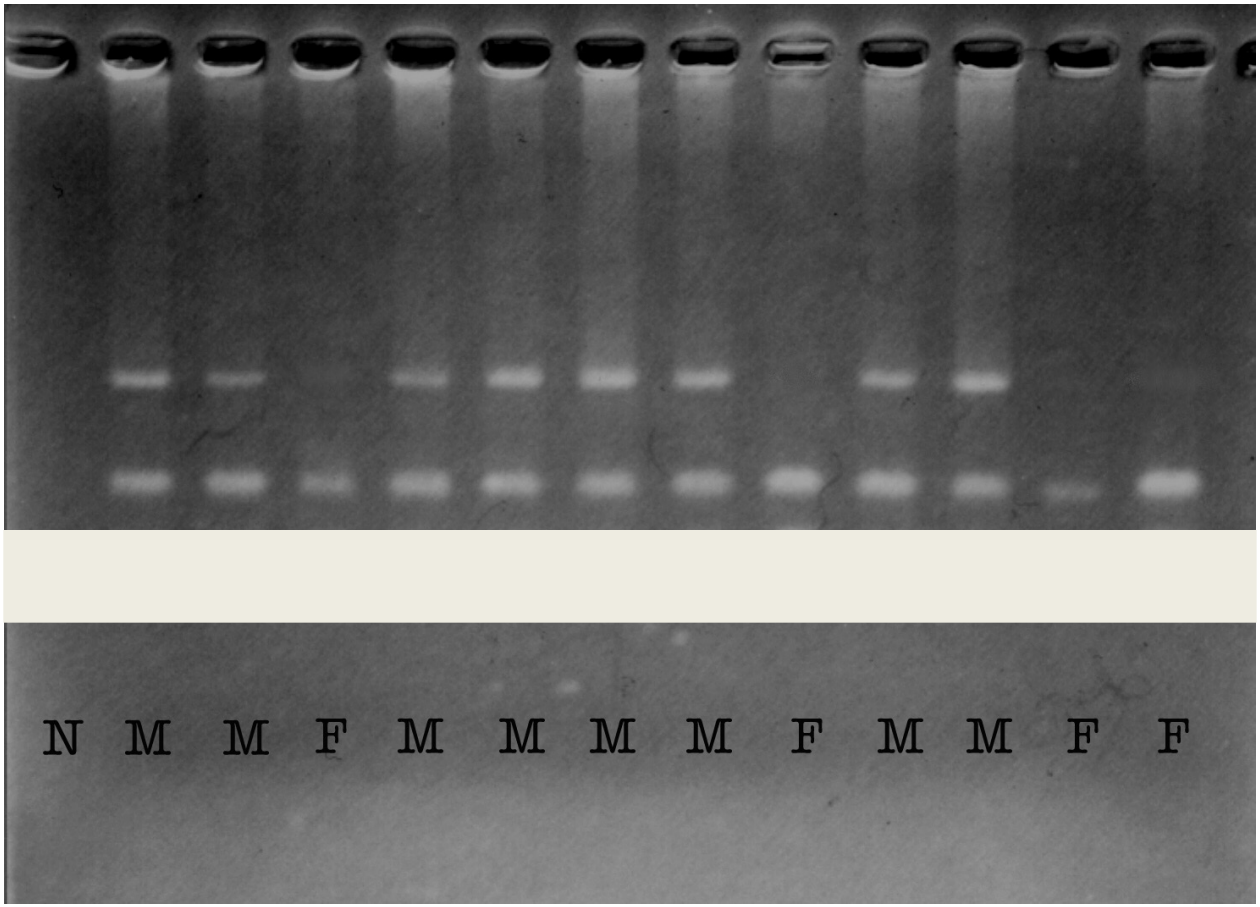






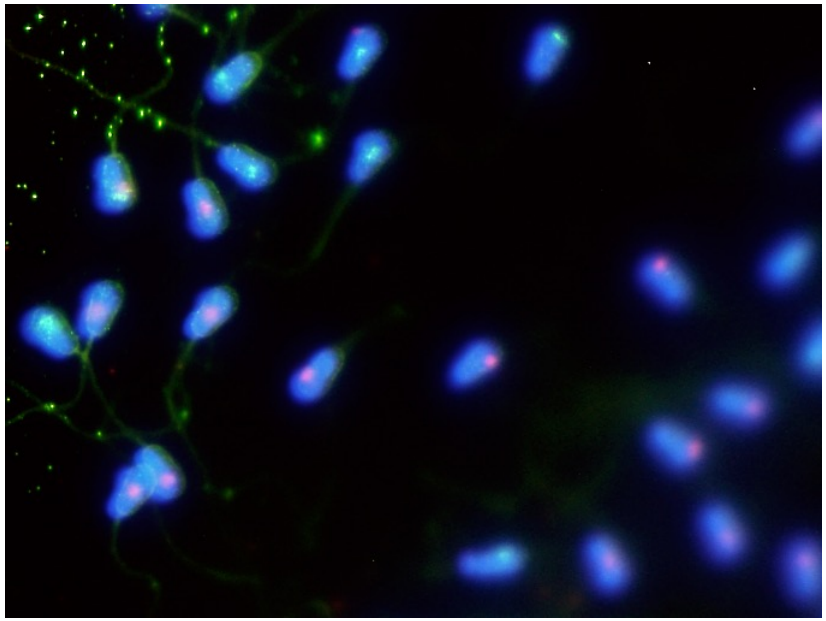
Analisi del frammento bioptico con PCR

Risoluzione del DNA amplificato in elettroforesi

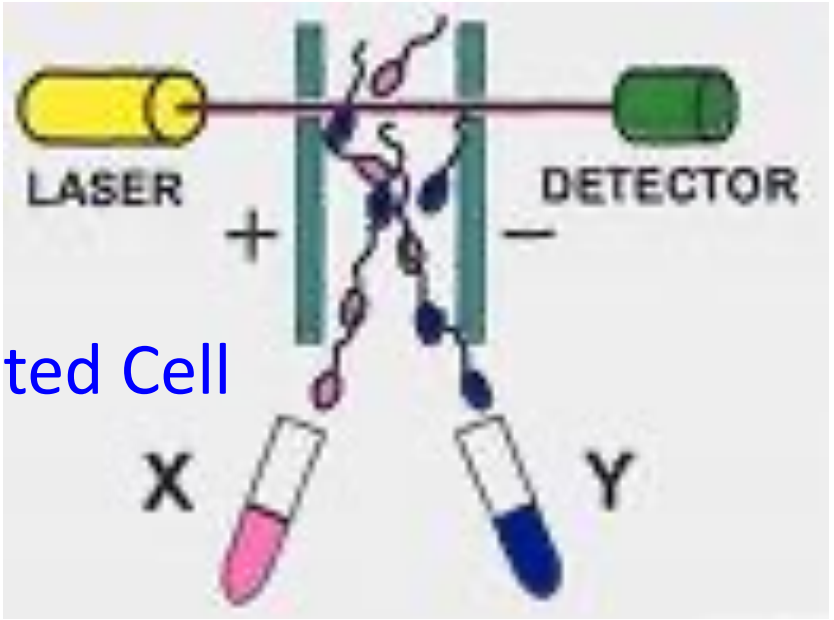


In ogni test-tube:
Primer housekeeping
Actina
Primer per la sequenza
SRY del cromosoma Y

Predeterminazione del sesso

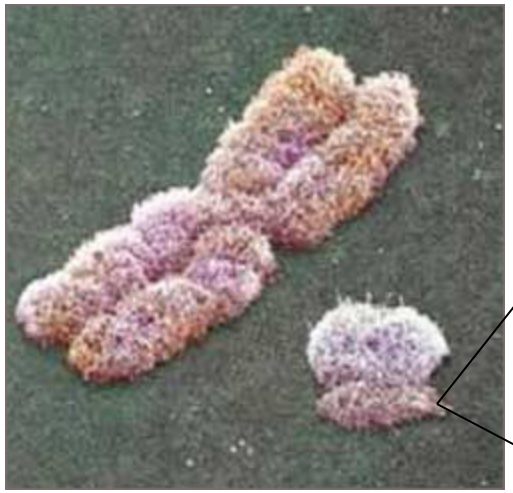


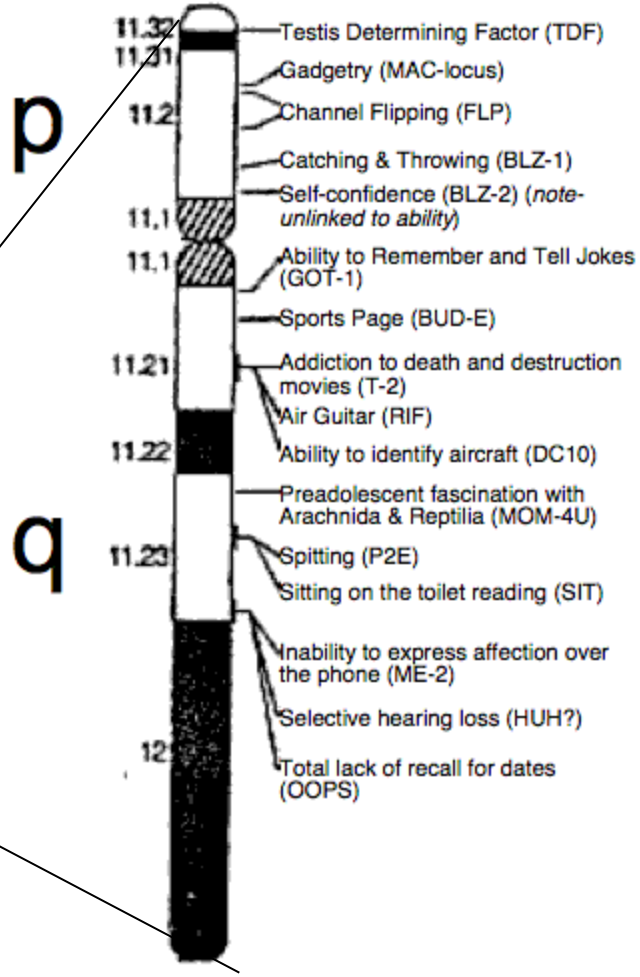
Spermatozoi
Con Y hanno il 10%
In meno di DNA
Di quelli X
Differenze tra specie



FACS = Fluorescence Activated Cell Sorter

Detentore del brevetto: X & Y limited, USA




p
q
11.32
11.31
11.2
11.1
11.1
11.21
11.22
11.23
12
Testis Determining Factor (TDF)
Gadgetry (MAC-locus)
Channel Flipping (FLP)
Catching & Throwing (BLZ-1)
Self-confidence (BLZ-2) (note-unlinked to ability)
Ability to Remember and Tell Jokes (GOT-1)
Sports Page (BUD-E)
Addiction to death and destruction movies (T-2)
Air Guitar (RIF)
Ability to identify aircraft (DC10)
Preadolescent fascination with Arachnida & Reptilia (MOM-4U)
Spitting (P2E)
Sitting on the toilet reading (SIT)
Inability to express affection over the phone (ME-2)
Selective hearing loss (HUH?)
Total lack of recall for dates (OOPS)

Aggregazione di blastomeri/ICM prima modalità sperimentale per produrre chimere

Fehilly CB, Willadsen SM, Tucker EM.Experimental chimaerism in **sheep**.
J Reprod Fertil. 1984 Jan;70(1):347-51.

Created by splitting embryos, "Geeps" prominently display characteristics of both parents.

Fehilly CB, Willadsen SM, Tucker Interspecific chimaerism between sheep and goat. Nature. 1984 Feb 16-22;307(5952):634-6.

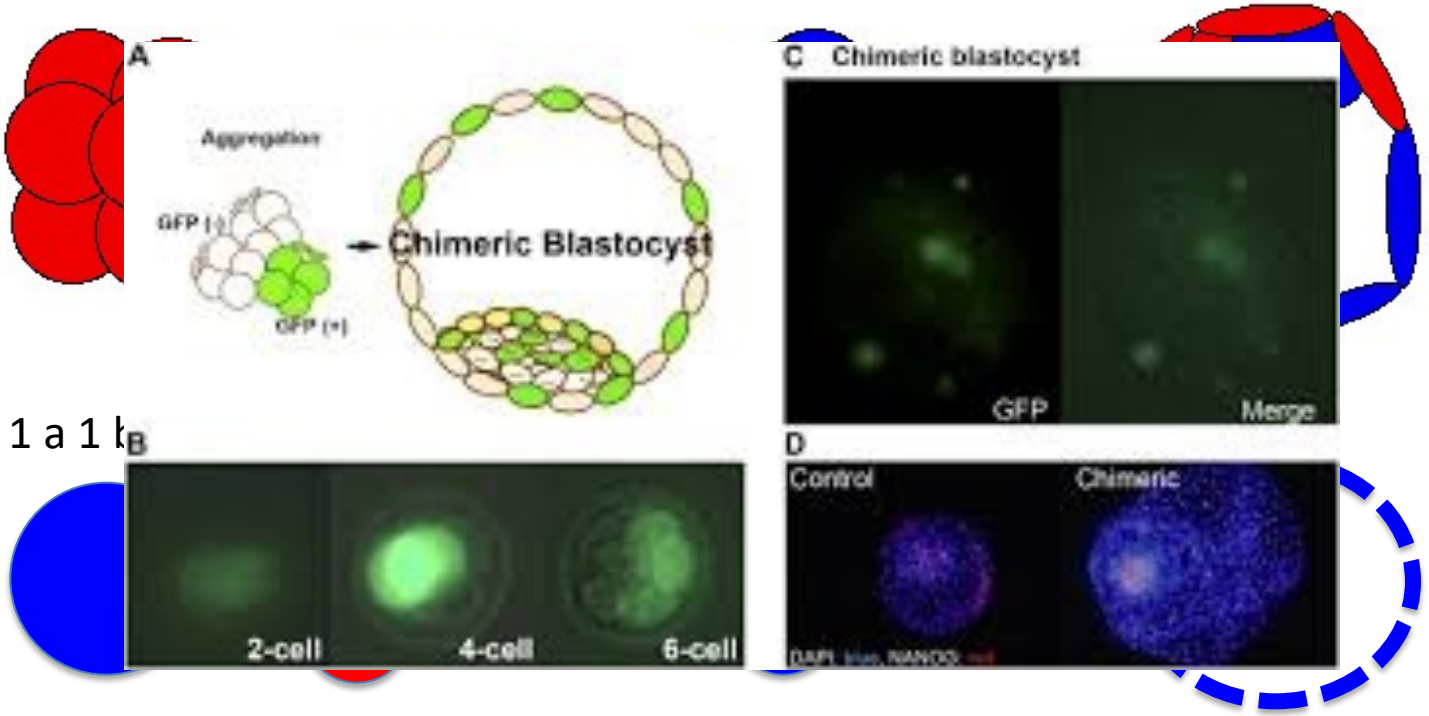
Chimere prodotte con:

Aggregazione di blastomeri di embrioni pre-impianto

Iniezione di ICM in blastocisti

Iniezione di cellule staminali embrionali in blastocisti/morule

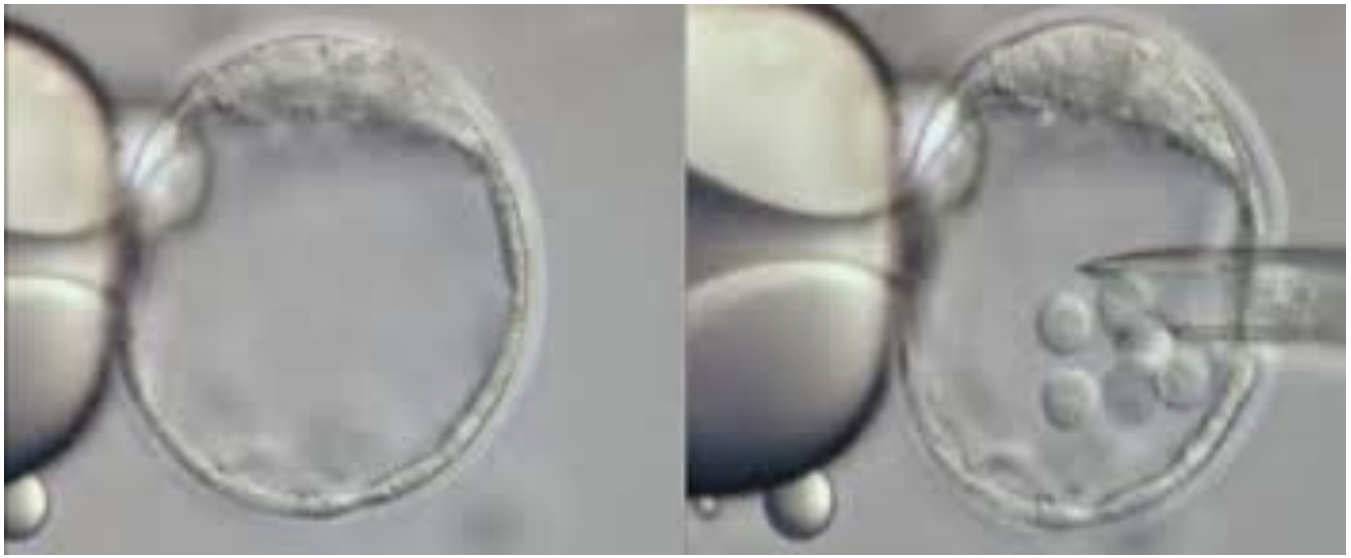
Aggregazione di blastomeri di embrioni pre-impianto



1 a 1

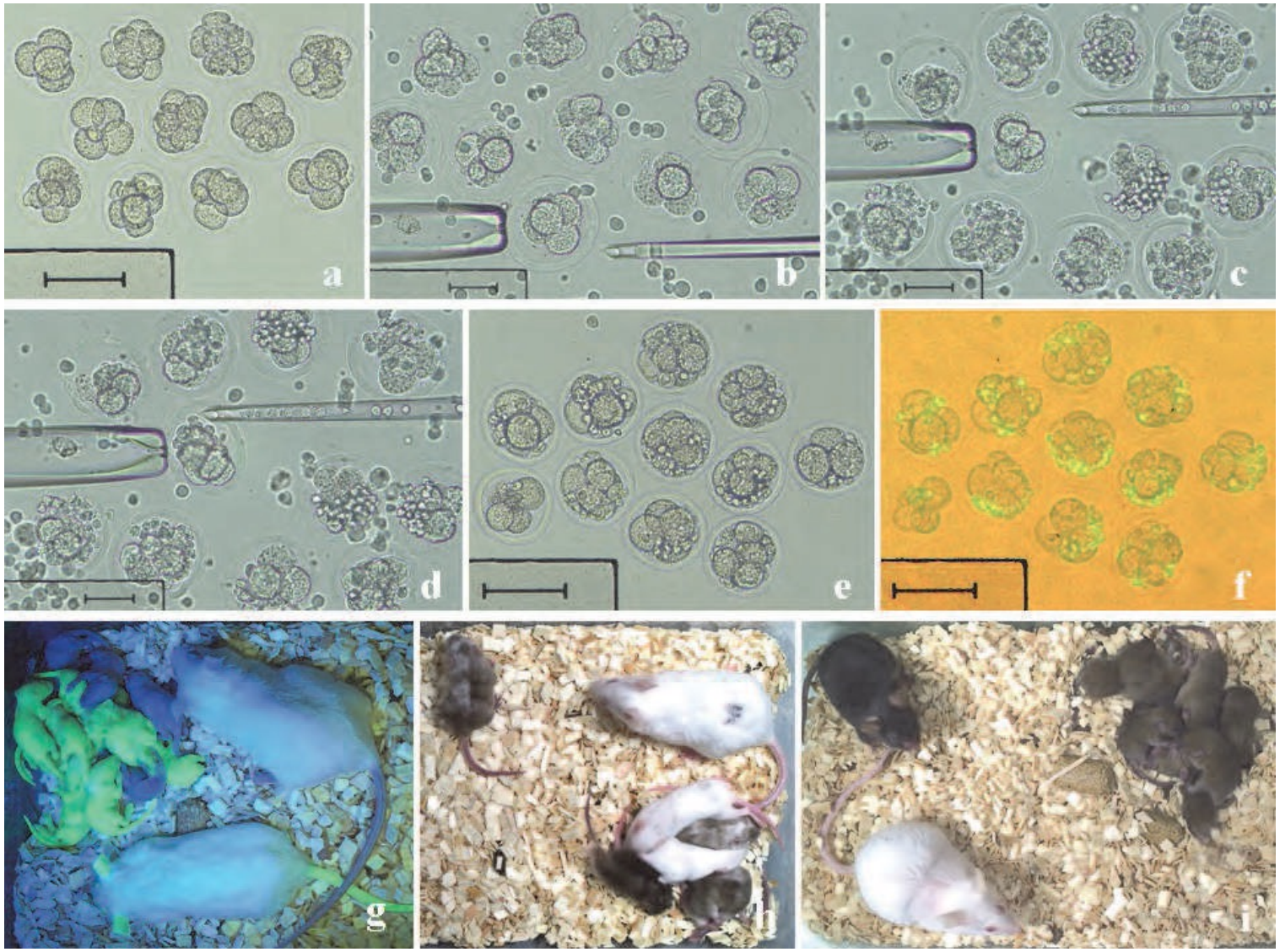
Stadi più avanzati di sviluppo tendono a contribuire alla ICM

Produzione di chimere con iniezione di ICM

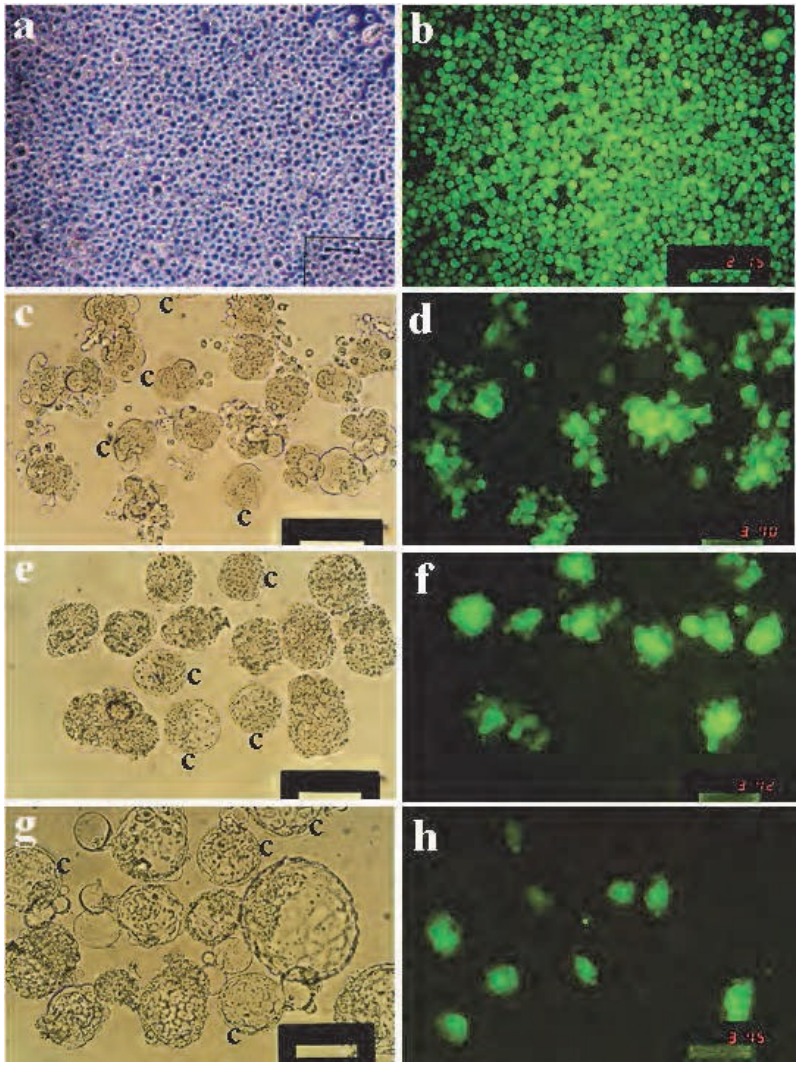


http://wn.com/Blastocyst_Microinjection

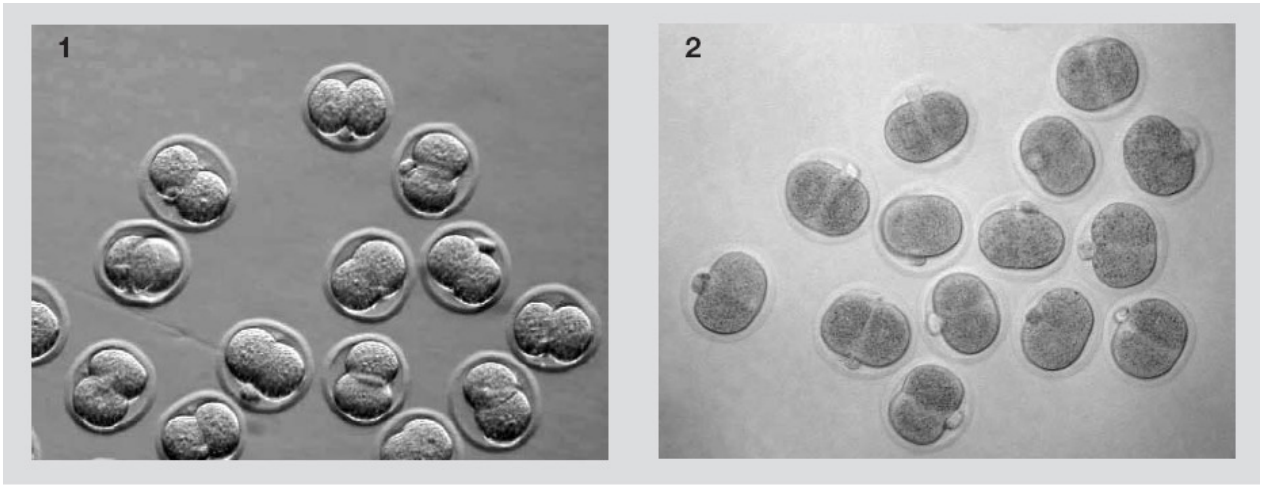
Produzione di chimere: iniezione di Stem Cells in morule

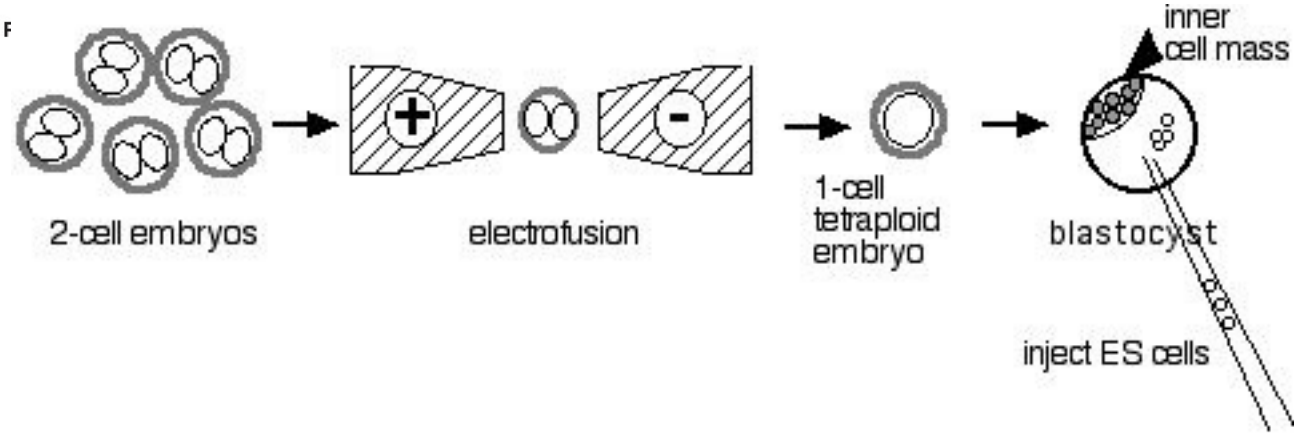


Produzione di chimere: aggregazione tra stem cells e morula
-fitoemoagglutinina-

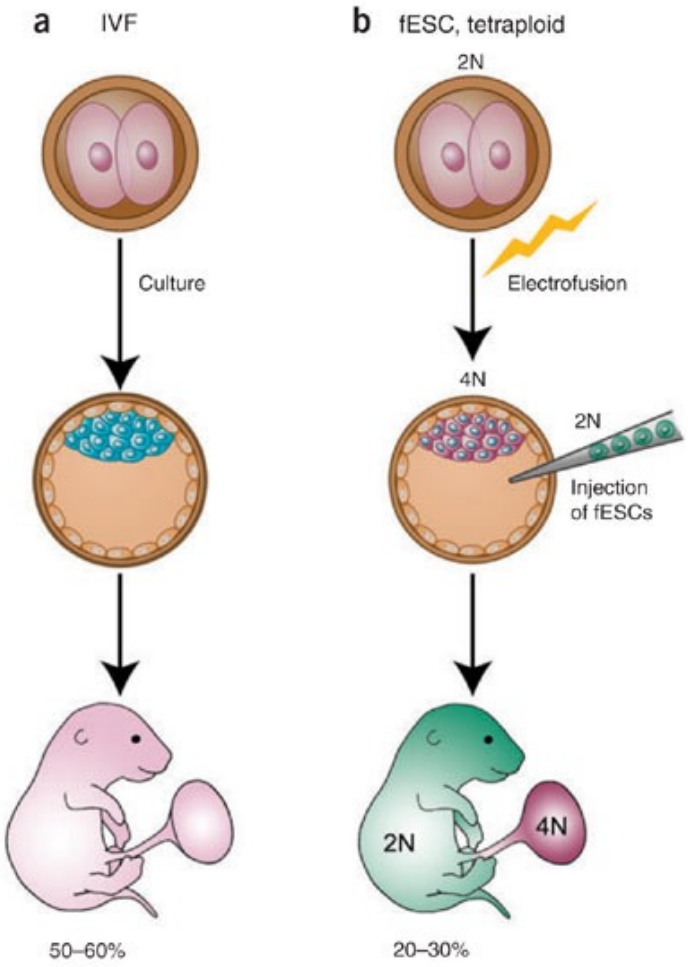


Produzione di feti interamente da stem cells: Tetraploid Complementation

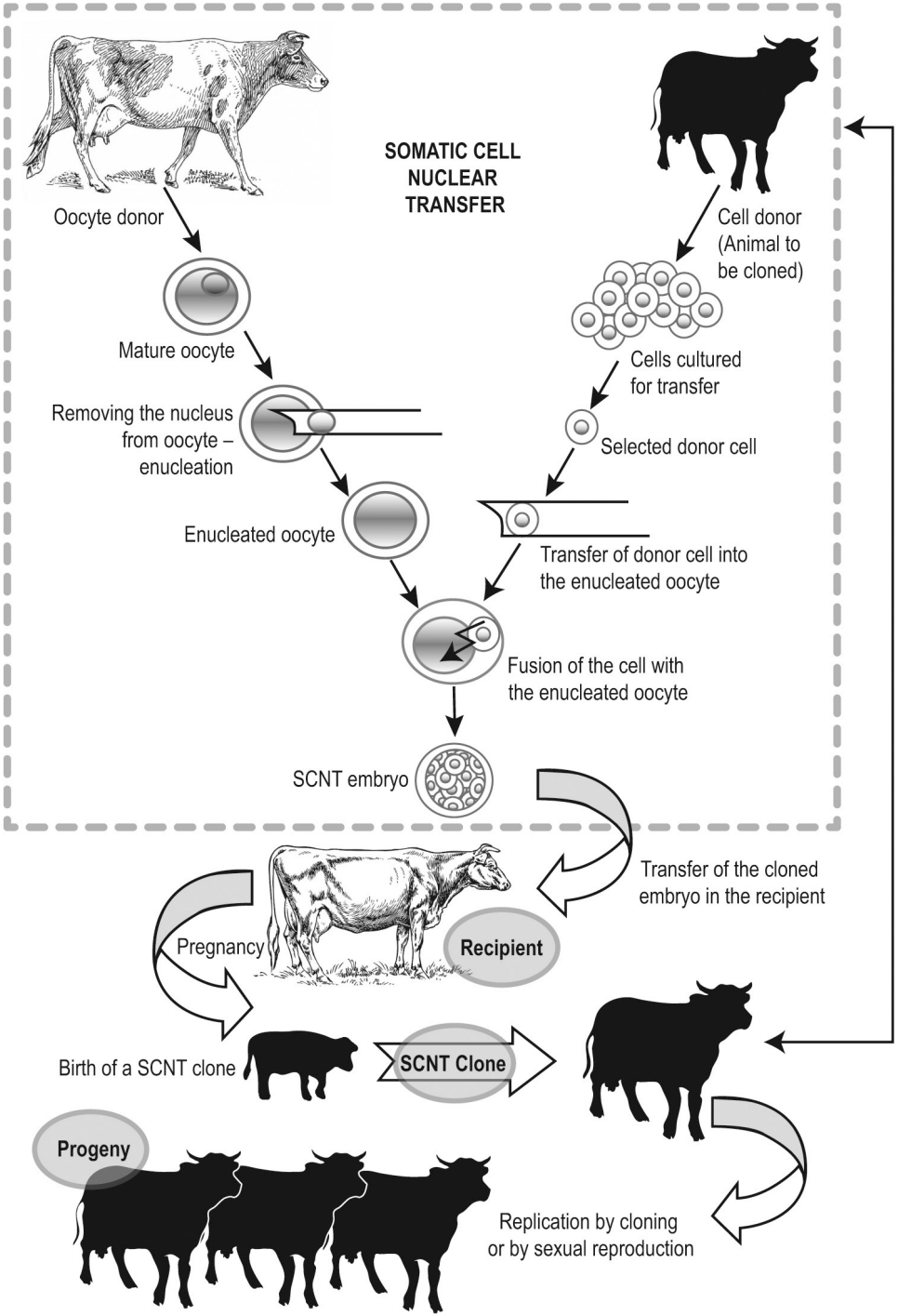




Cellule tetraploidi sono confinate nel trofoblasto (ricorda!)
Le cellule staminali formano l'ICM e quindi il feto



Produzione di chimere fondamentale per la produzione di animali transgenici


SOMATIC CELL NUCLEAR TRANSFER
Oocyte donor
Cell donor (Animal to be cloned)
Mature oocyte
Cells cultured for transfer
Removing the nucleus from oocyte – enucleation
Selected donor cell
Enucleated oocyte
Transfer of donor cell into the enucleated oocyte
Fusion of the cell with the enucleated oocyte
SCNT embryo
Transfer of the cloned embryo in the recipient
Pregnancy
Recipient
Birth of a SCNT clone
SCNT Clone
Progeny
Replication by cloning or by sexual reproduction

Nuclear transplantation in **sheep embryos**.
Willadsen SM. Nature. 1986 Mar 6-12;320(6057):63-5.