Jiří K a ň k a

Klonování embryí savců -

reprogramace přeneseného jádra

Ústav živočišné fyziologie a genetiky
Akademie věd České republiky
Rumburská 89
277 21 Liběchov

tel.: 315 639551
fax.: 315 697186
e-mail :kanka@iapg.cas.cz

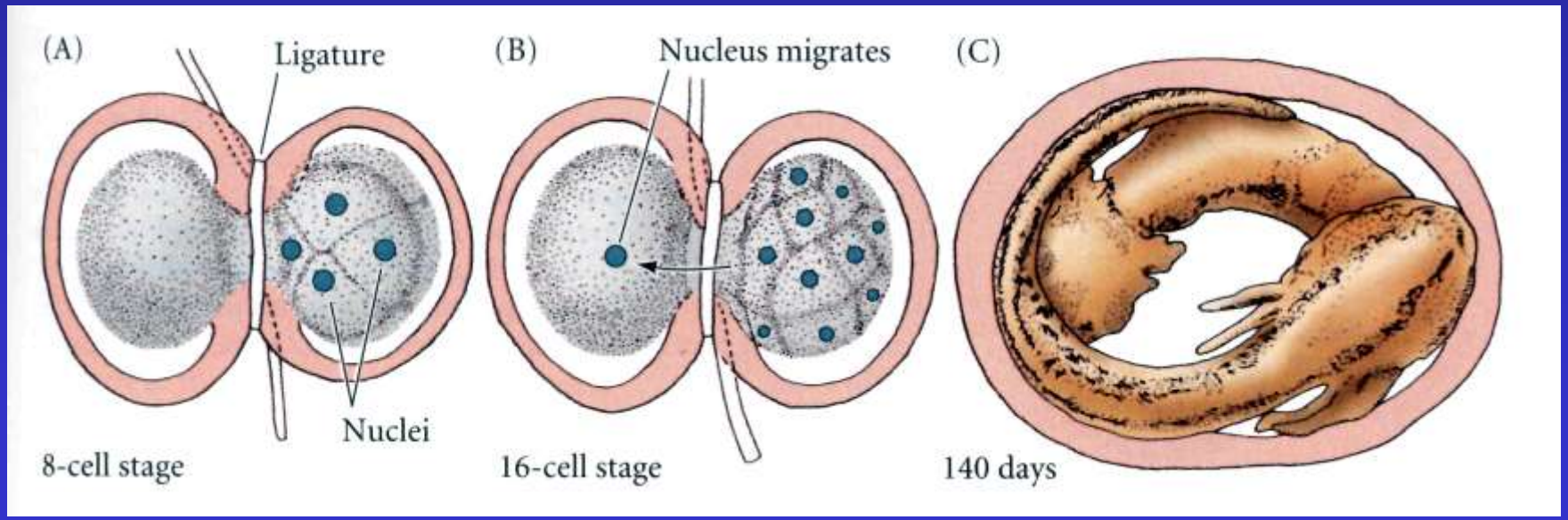
Dolly 1997

Wilmut, I.//Schnieke, A.E.//McWhir, J.//Kind, A.J.//Campbell, K.H.S., Nature, 1997

Historie klonování obratlovců

1885 - August Weissmann předpokládá, že příčinou diferenciace je diferenční dělení jader, která již od prvních dělení ztrácejí část genetické výbavy (Ascaris megalocephala).

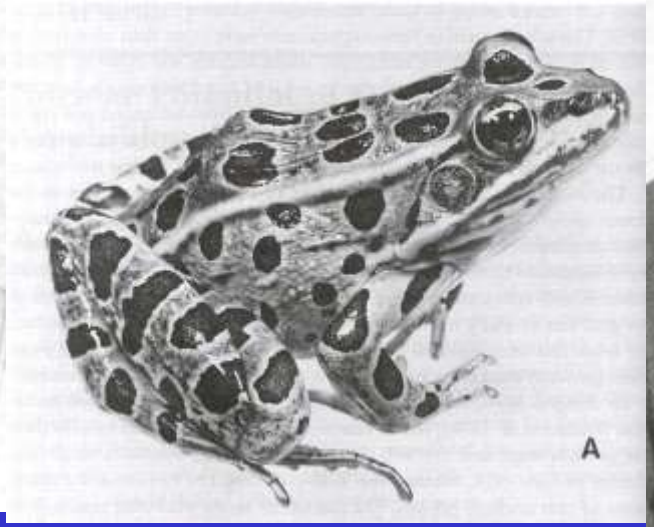
1914 - Hans Spemann rozdělil vajíčko čolka na dvě části



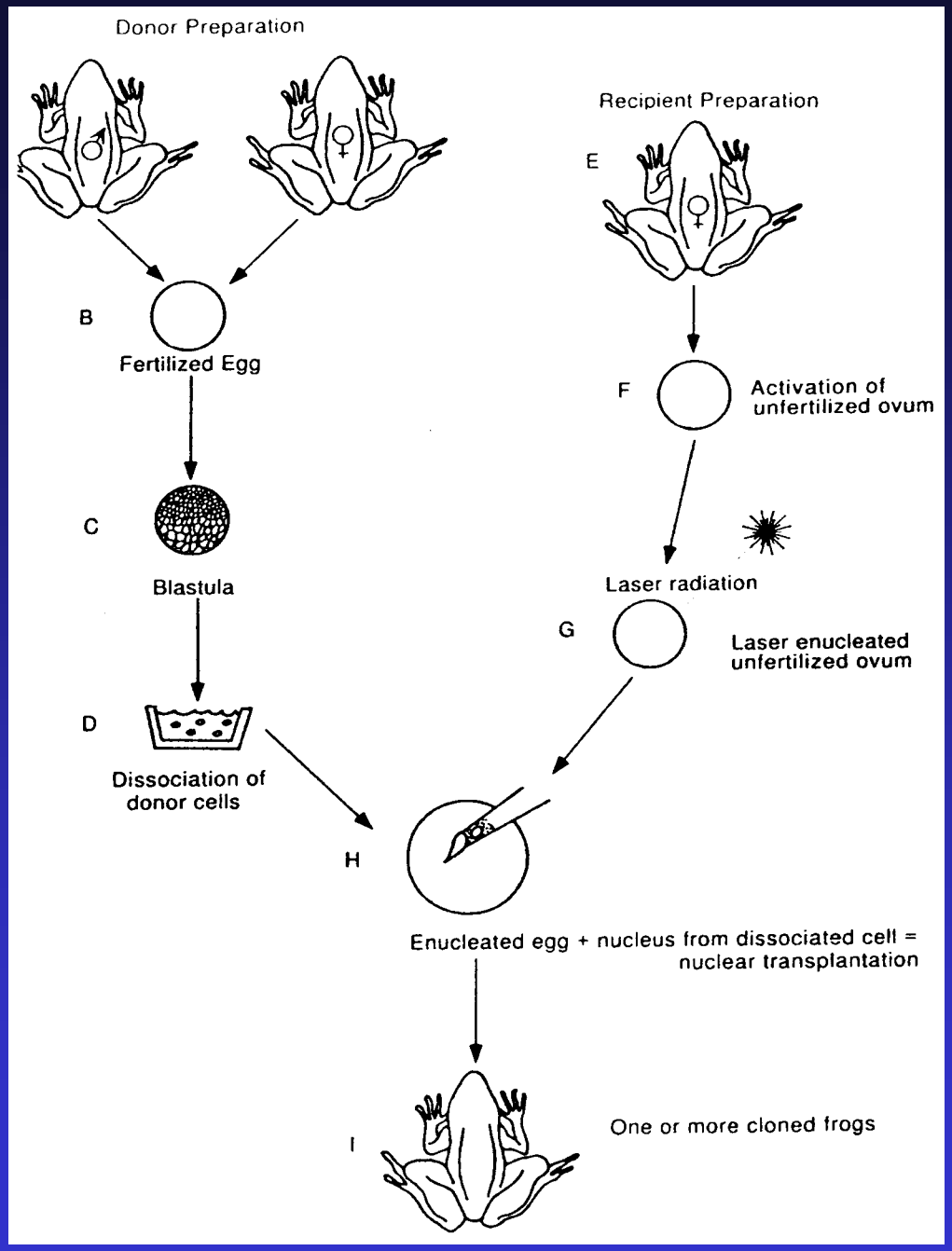
1952 - R. Briggs a T. J. King klonovali žábu Rana pipiens

Robert Briggs

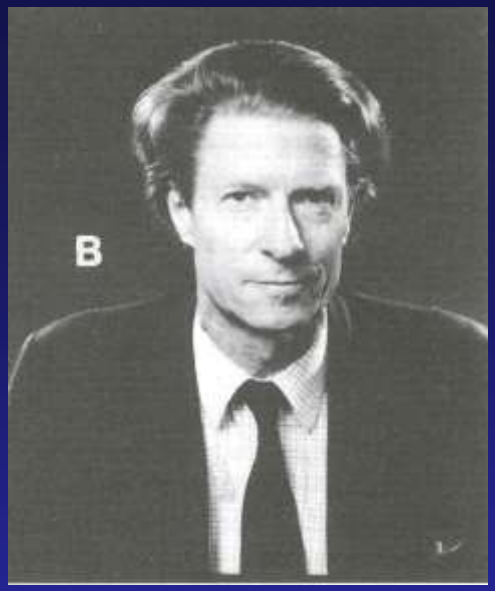
Rana pipiens



Thomas J. King

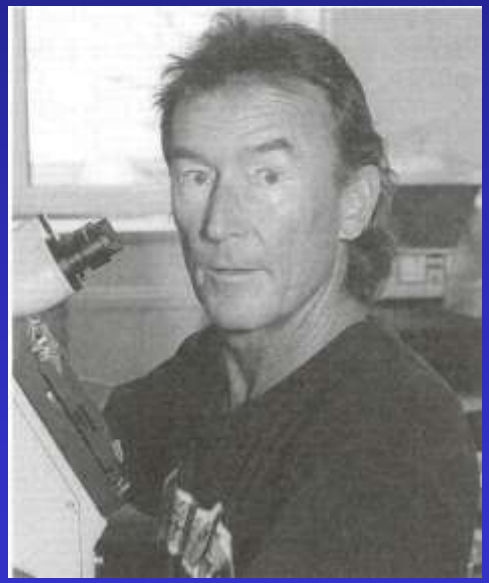

Donor Preparation
Recipient Preparation
E
B
Fertilized Egg
F
Activation of unfertilized ovum
C
Blastula
Laser radiation
G
Laser enucleated unfertilized ovum
D
Dissociation of donor cells
H
Enucleated egg + nucleus from dissociated cell = nuclear transplantation
I
One or more cloned frogs

1966 – J. Gurdon publikuje klonování žáby Xenopus laevis z buněk střevního epitelu



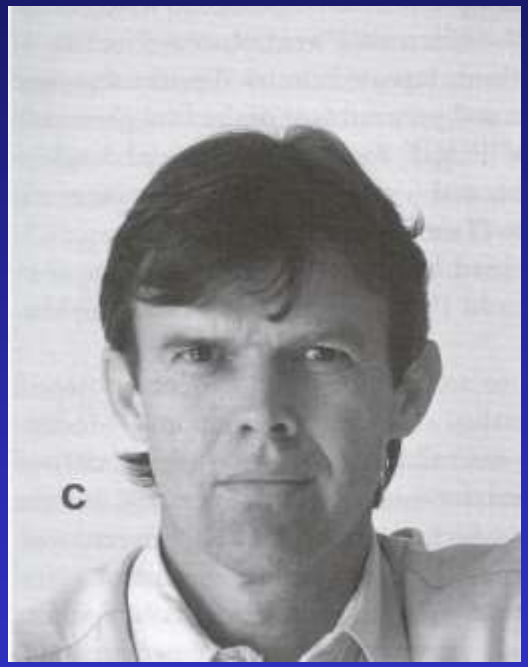
1981 - K. Illmensee tvrdí, že klonoval tři myši z buněk embryoblastu

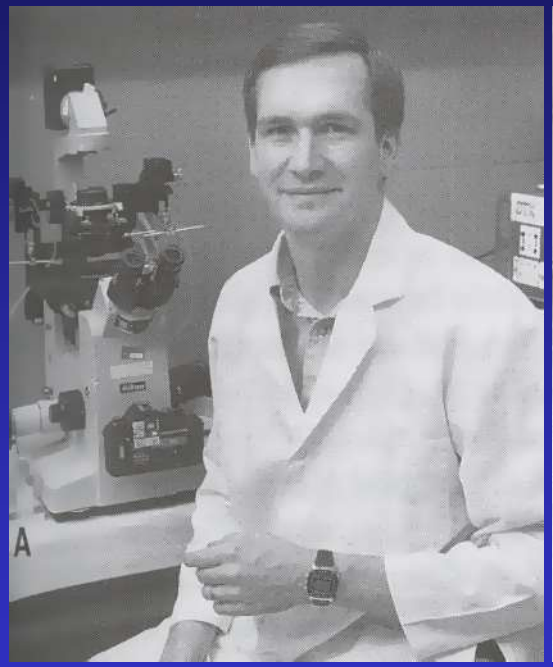
1983 - J. McGrath a D. Solter vyvinuli novou mikromanipulační metodu



1986 - S. Willadsen klonuje ovci z embryonálních buněk

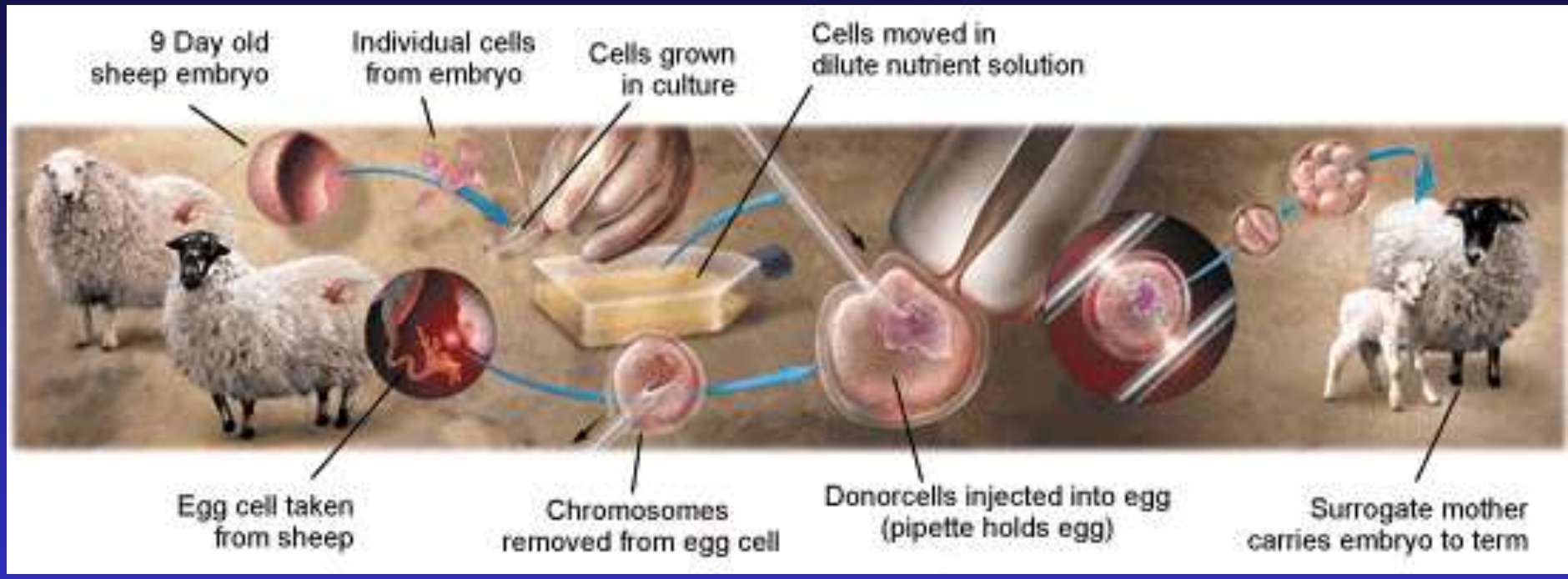
1986 - N. First, **R.S. Prather** a **W. Eystone** klonují skot z embryonálních buněk.





Steen Willadsen **Randy Prather** **Jim Robl**

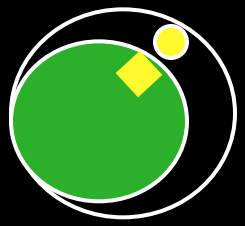
1995 - I. Wilmut a K. Campbell klonují ovce z diferencovaných fibroblastů kultivovaných in vitro (Campbell a kol., 1996a).



1996 - narodila se ovce Dolly, první savec klonovaný ze somatických buněk dospělého jedince

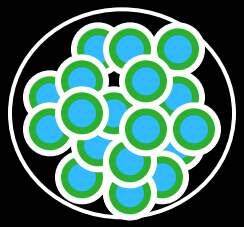
Wilmut, I.//Schnieke, A.E.//McWhir, J.//Kind, A.J.//Campbell, K.H.S., Nature, 1997

NUCLEAR TRANSFER IN RABBIT



unfertilized egg

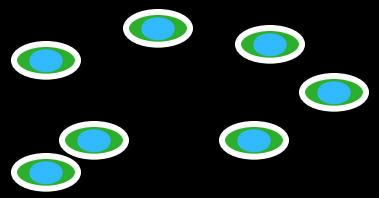
DNA stain, cytochalasin B



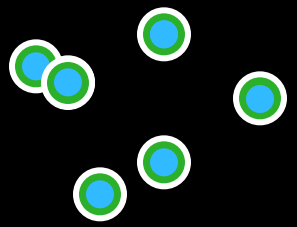
32 cell stage

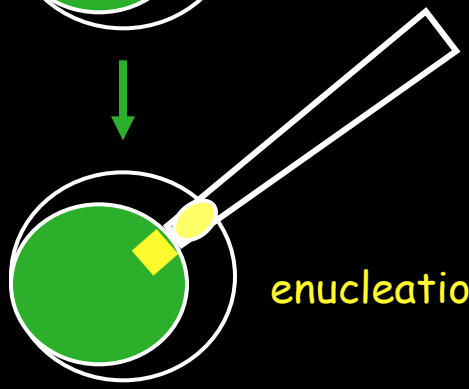
fibroblasts

trypsin

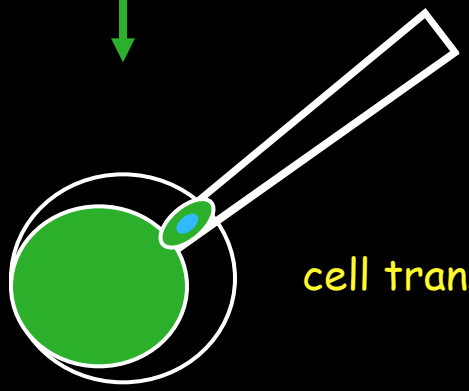


Liquid nitrogen
thawing
pronase treatment
Ca++ free medium





enucleation



cell transfer

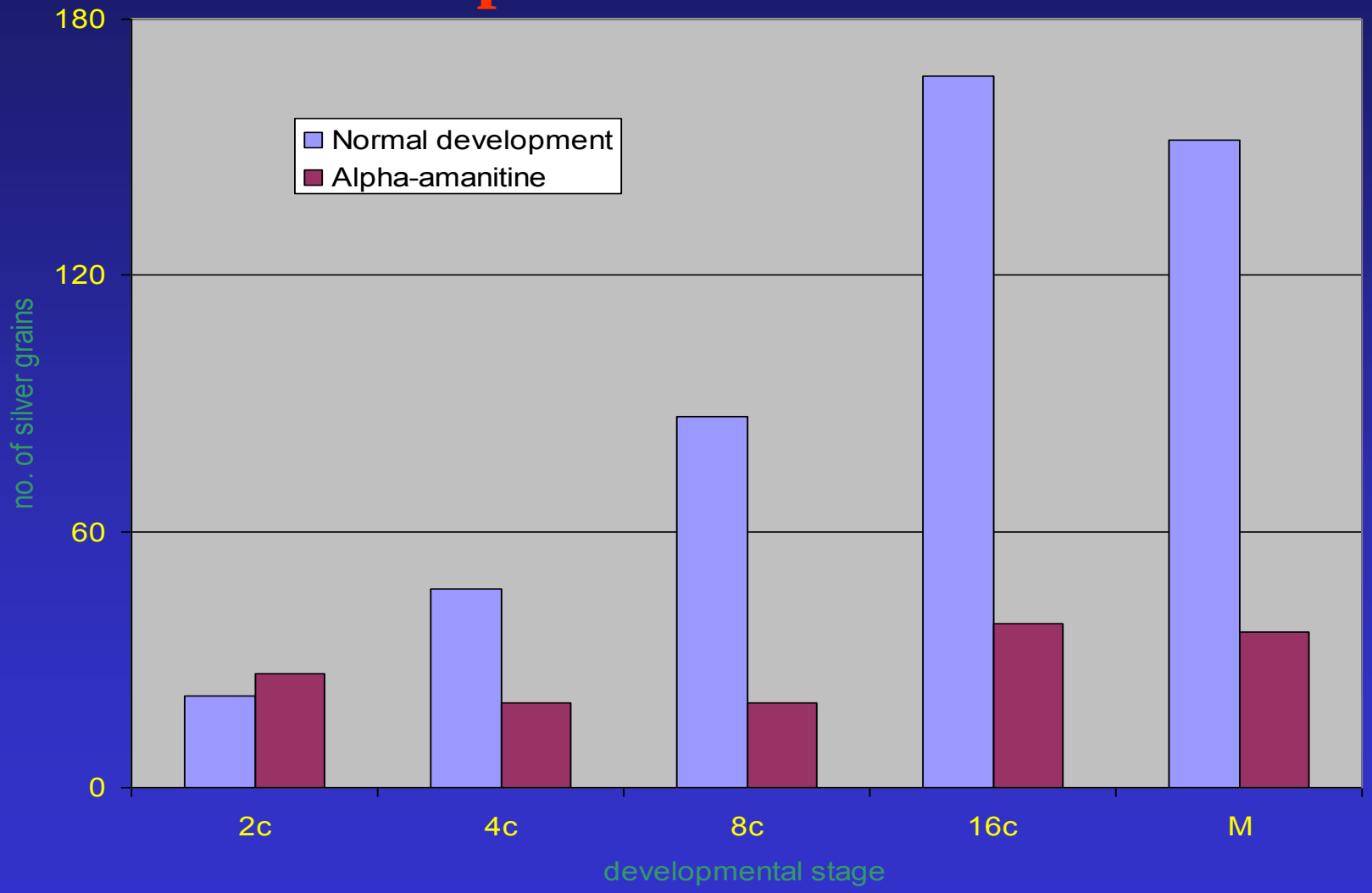
Electrofusion, in vitro cultivation

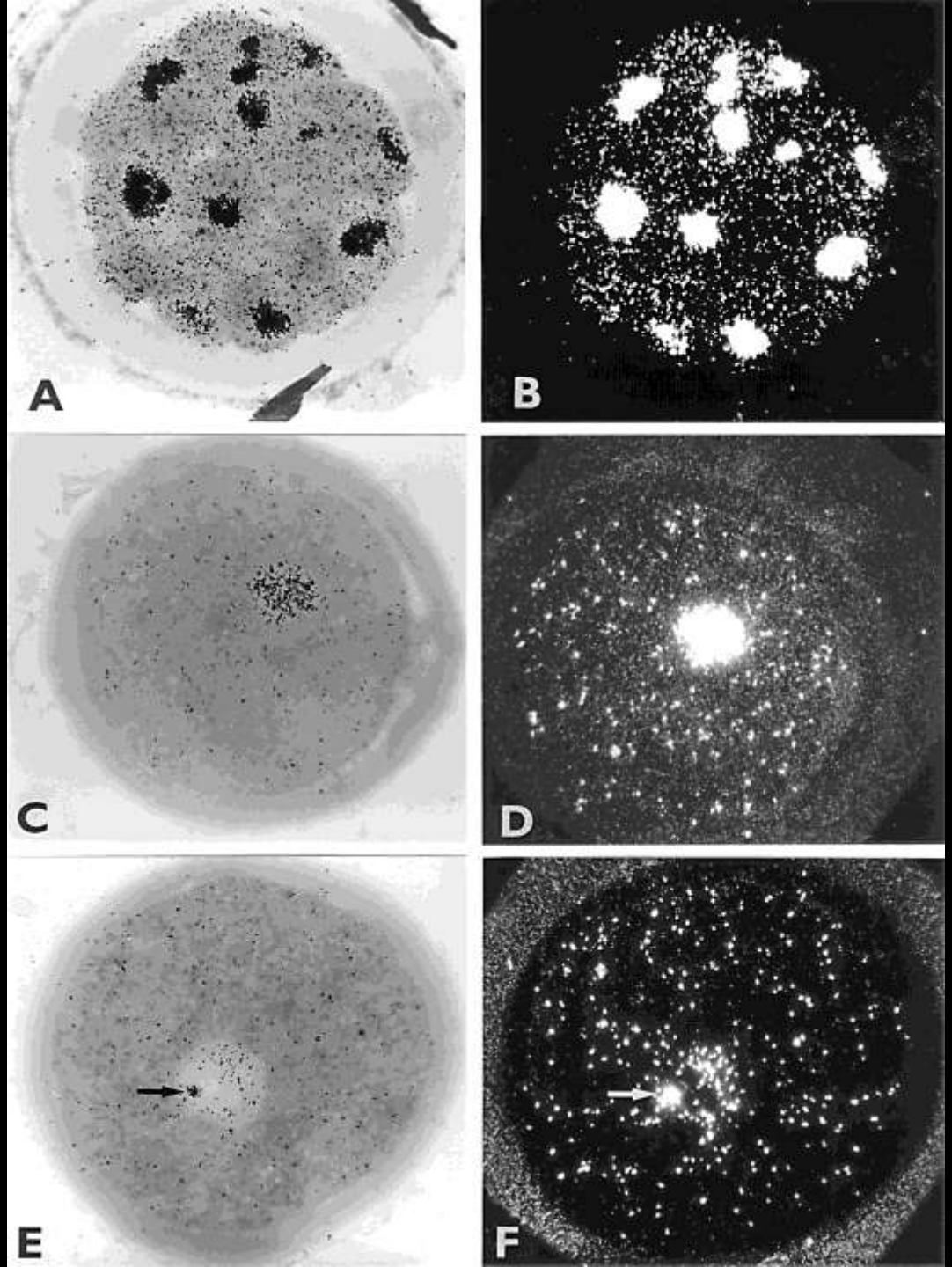
METODY

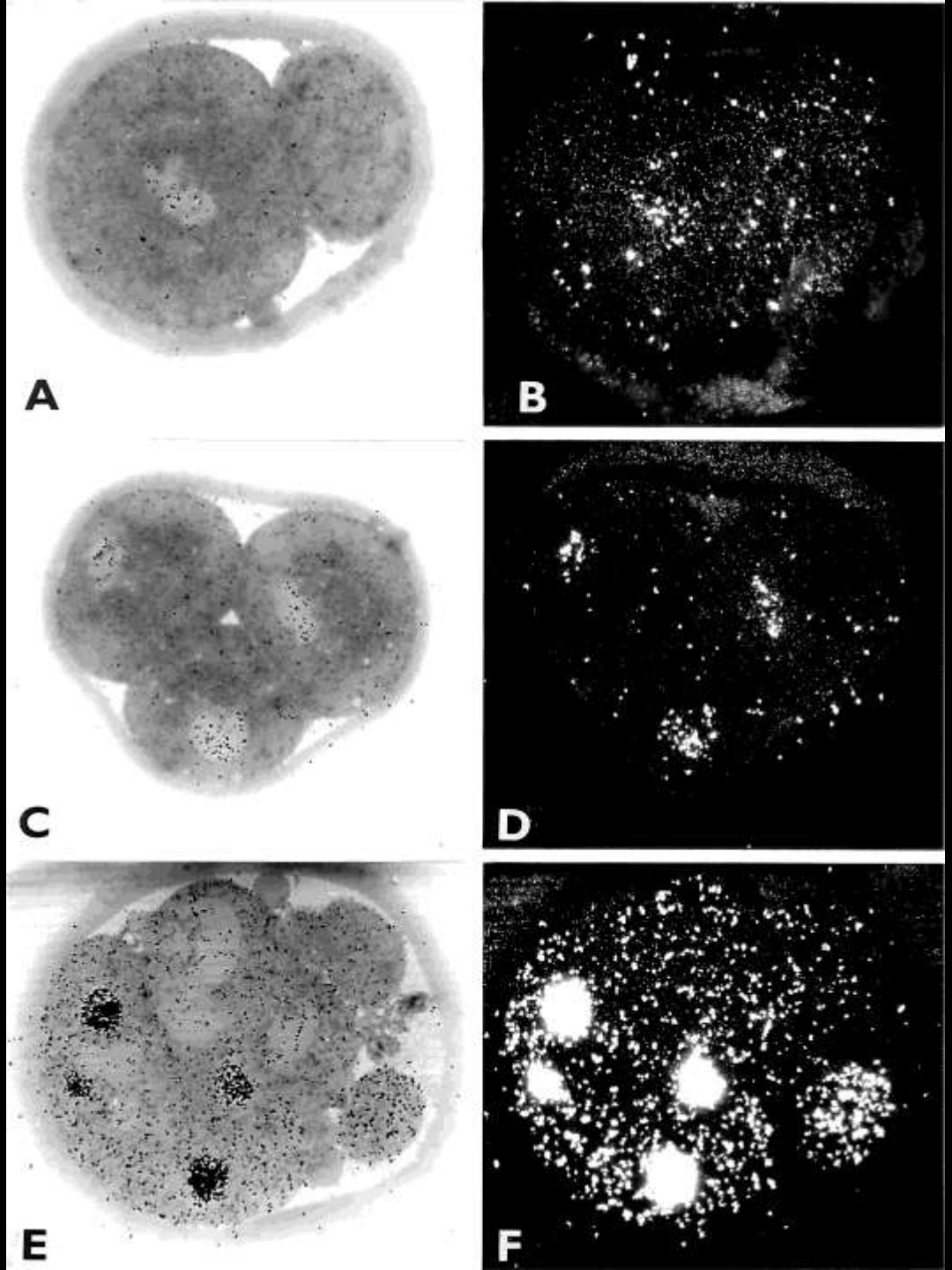
V následujících experimentech byla RNA syntéza u normálních a klonovaných embryí sledována pomocí:

- **Autoradiografie (ARG)**
- **Elektronová mikroskopie**
- **mRNA differential display a následná RT-PCR**

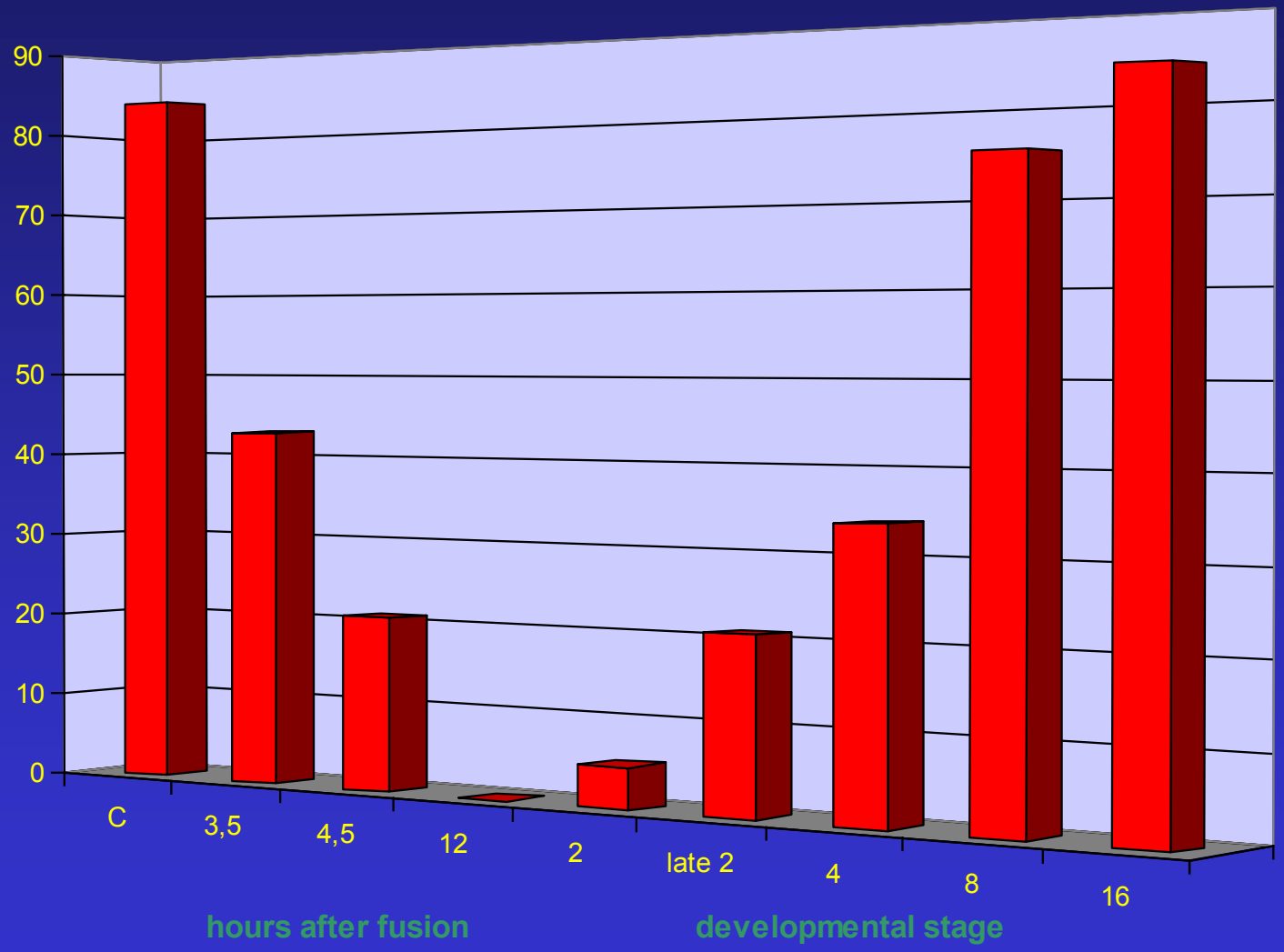
Incorporation of ^{3}H-uridine



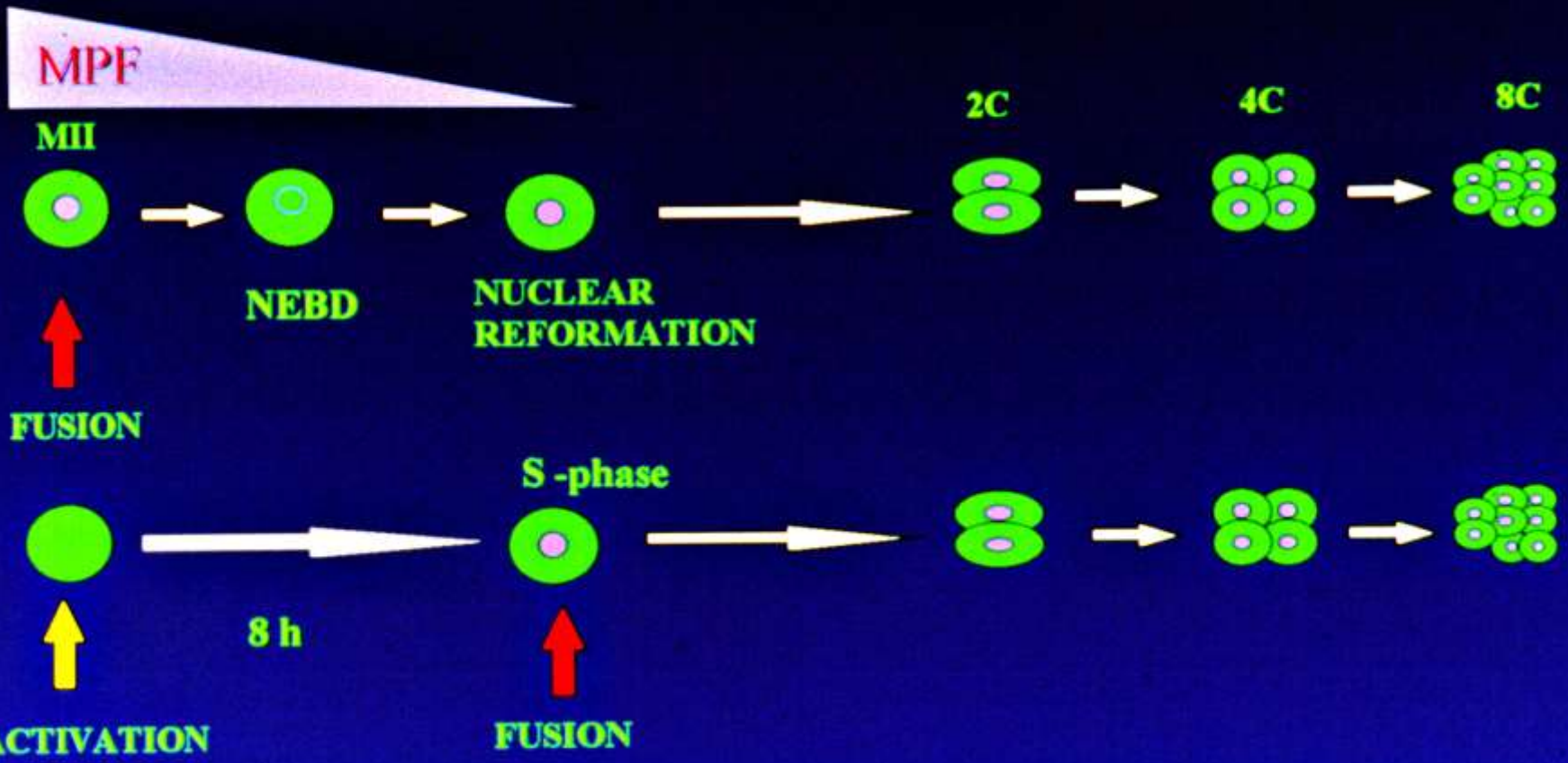

A
B
C
D
E
F


A
B
C
D
E
F

RNA synthesis in the nuclei of reconstructed embryos

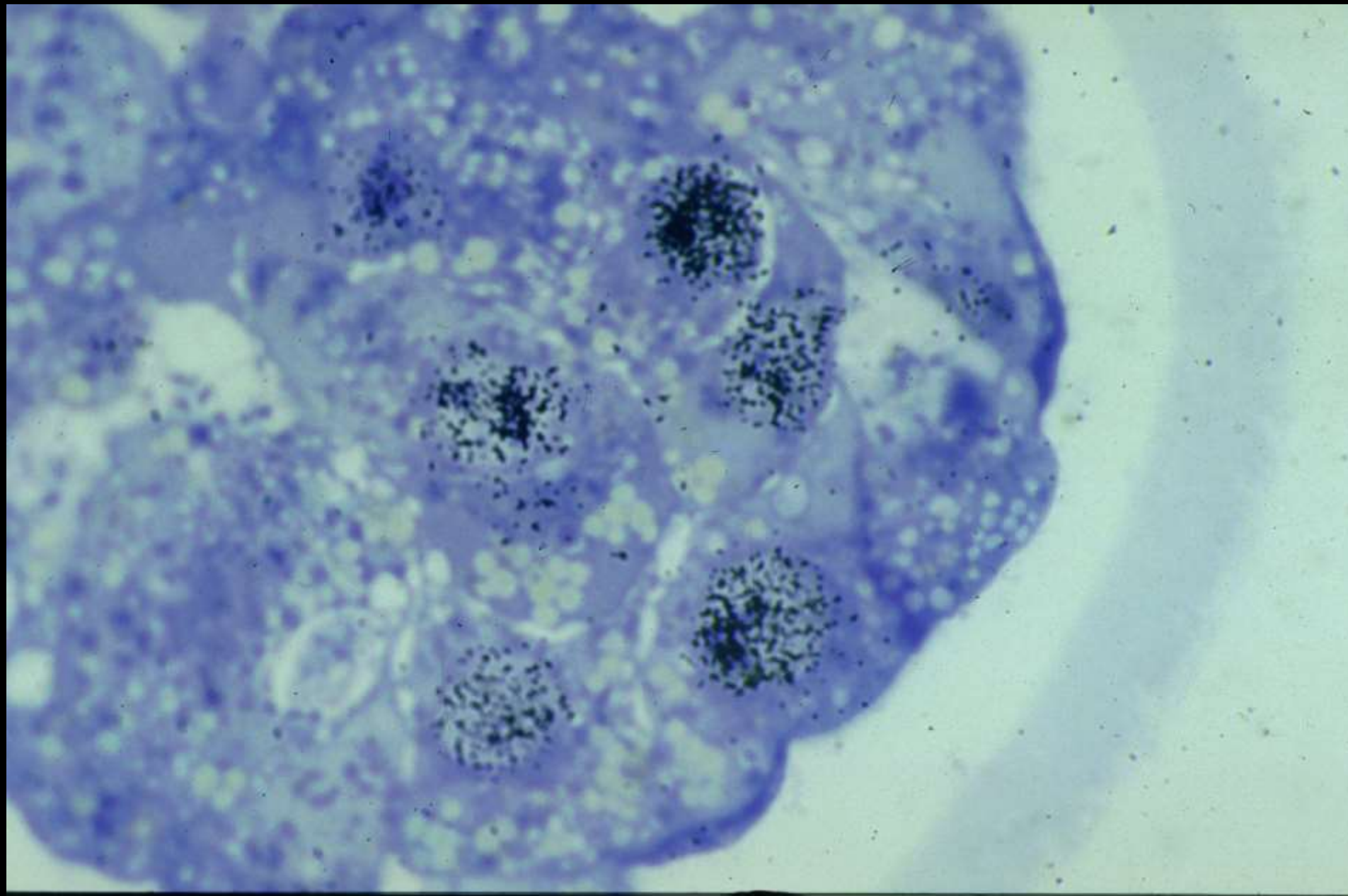


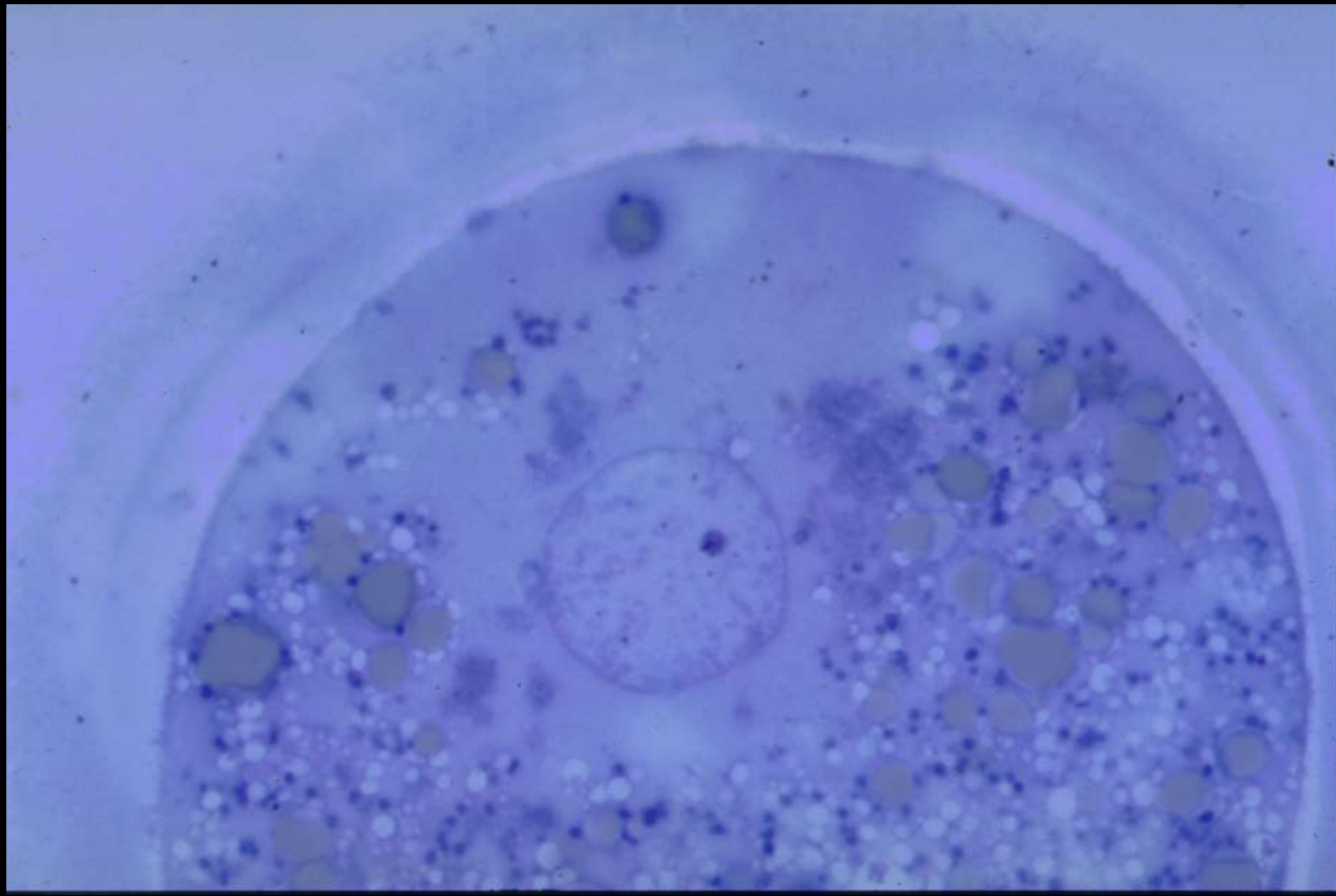
METHODS

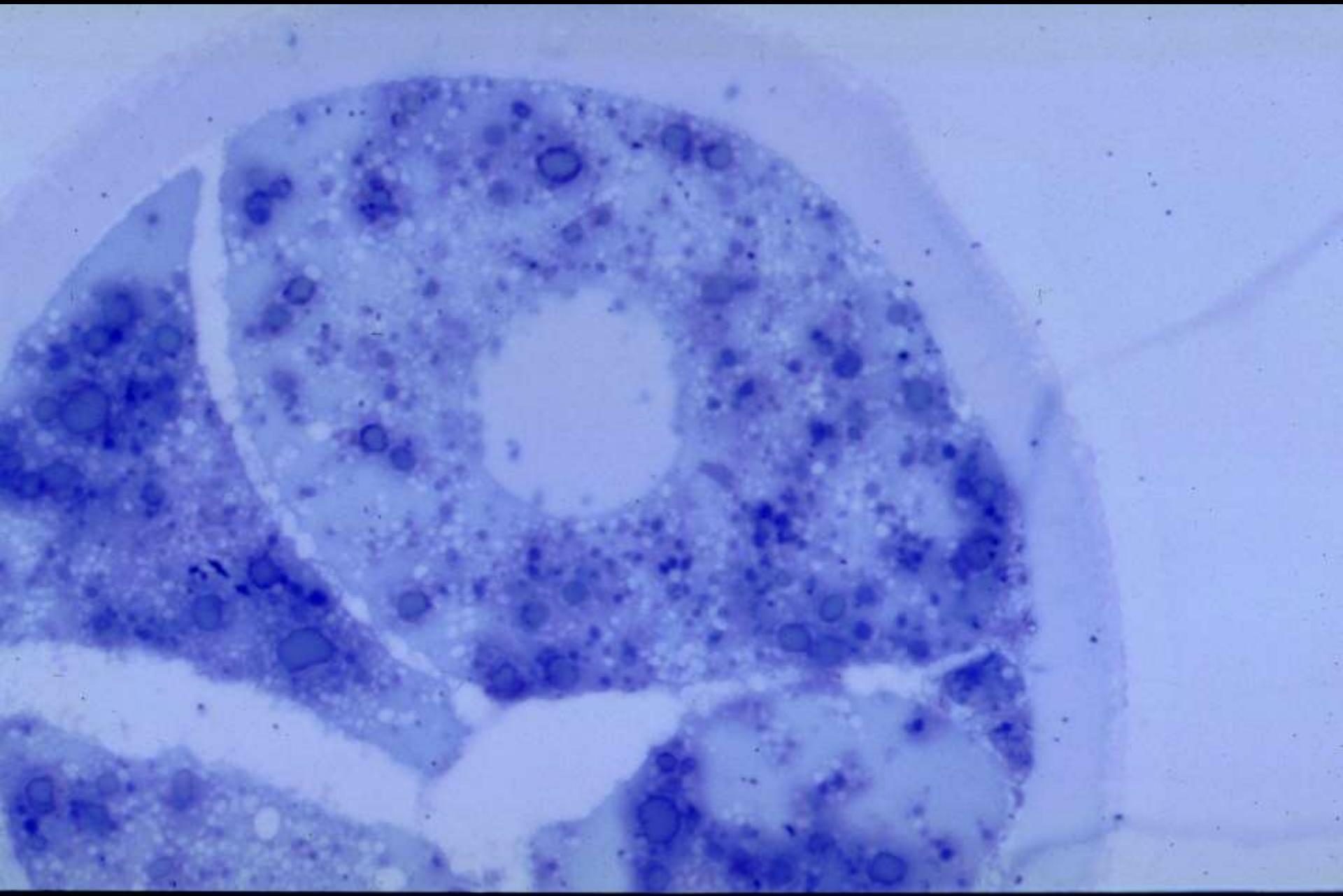


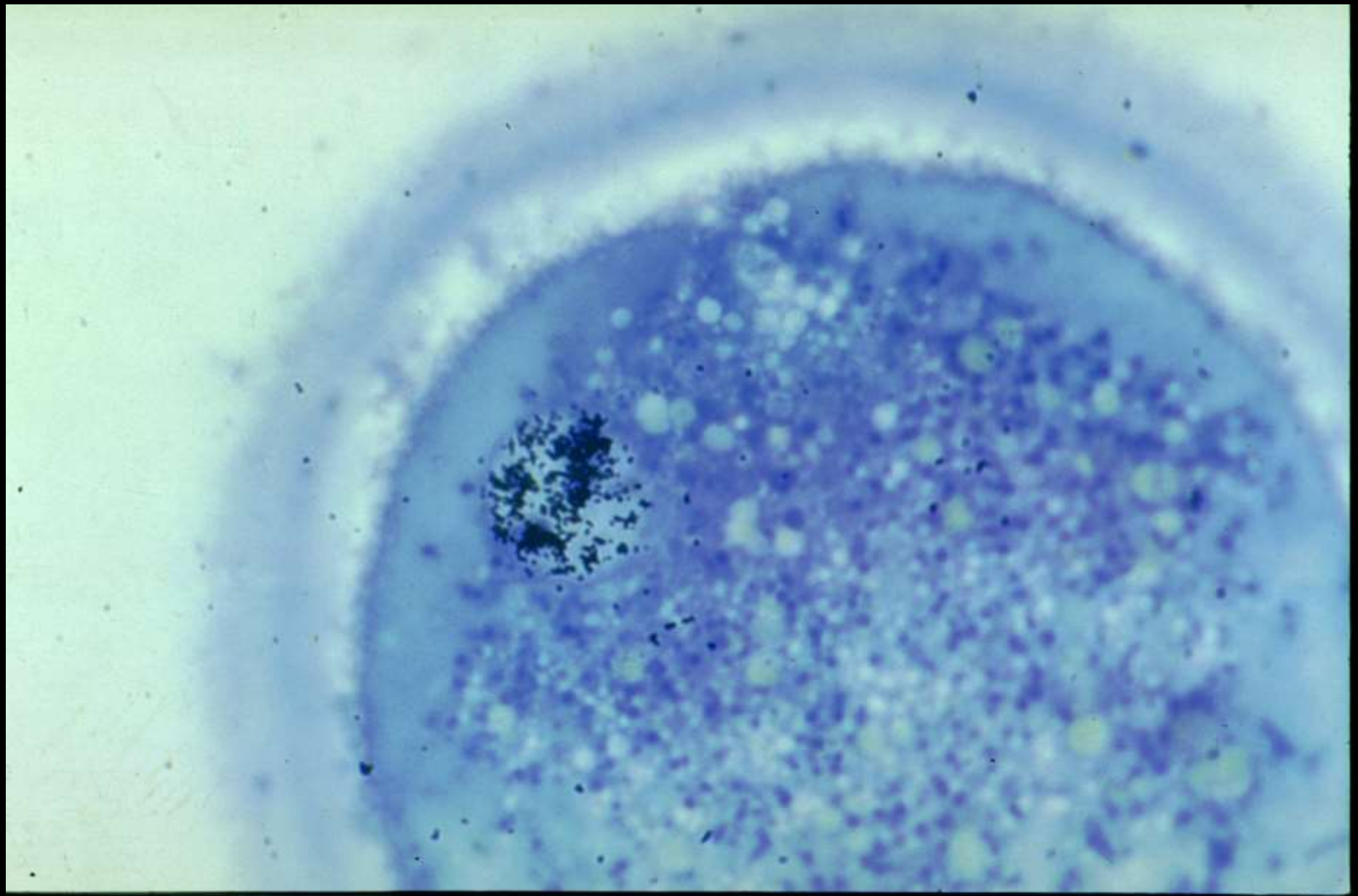
NTE were produced using either a MII phase (nonactivated) cytoplasts or S phase (activated) cytoplasts activated with calcium ionophore A23187 and cycloheximide treatment 8 h prior to fusion with a blastomere from an *in vitro* produced morula stage embryo. Control *in vitro* produced embryos were ^{3}H-uridine labelled at the 2-, 4-, early 8- and late 8-cell stages.

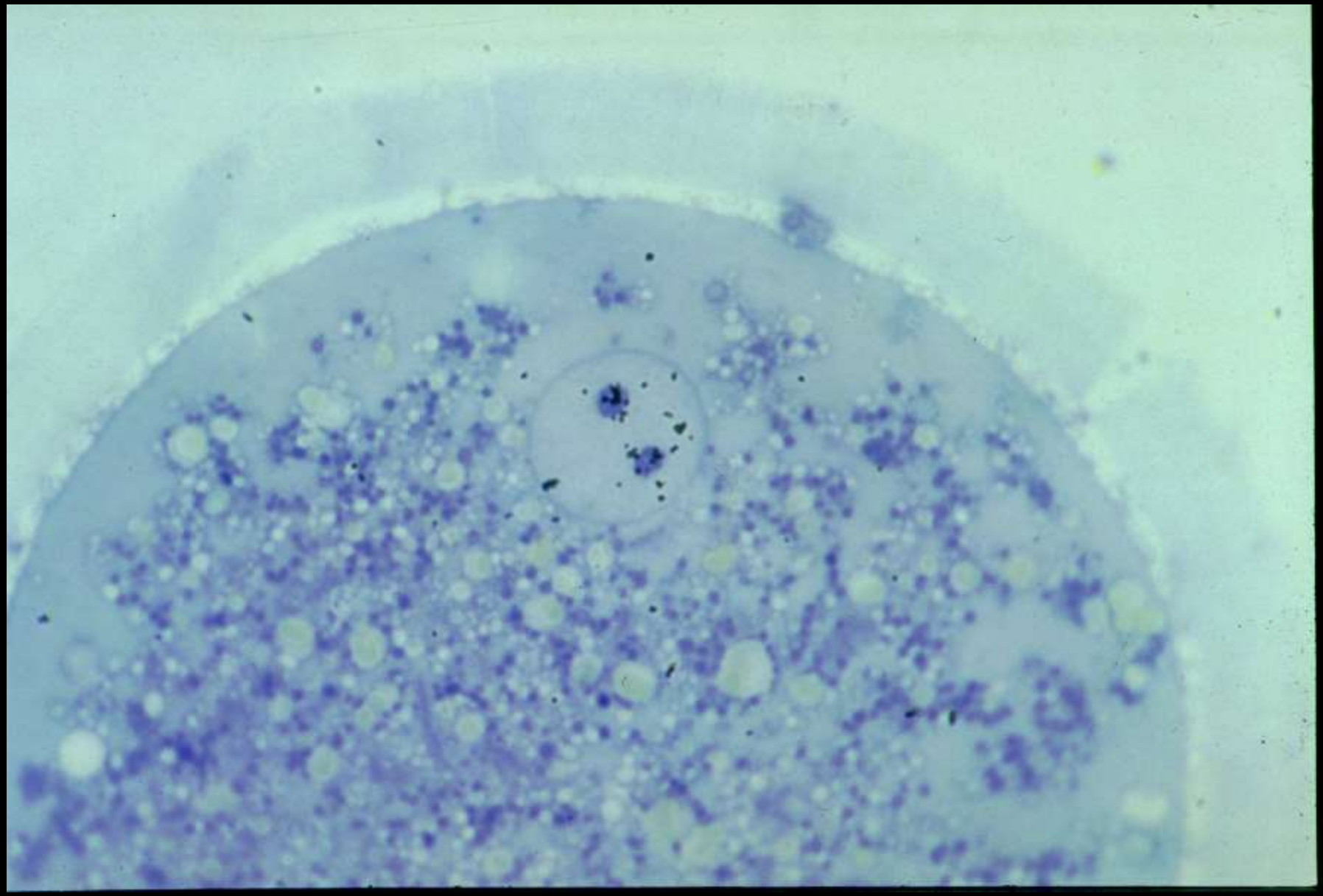
MPF – maturation promoting factor, p34^{cdc2} catalytic subunit +cyclin B regulatory component

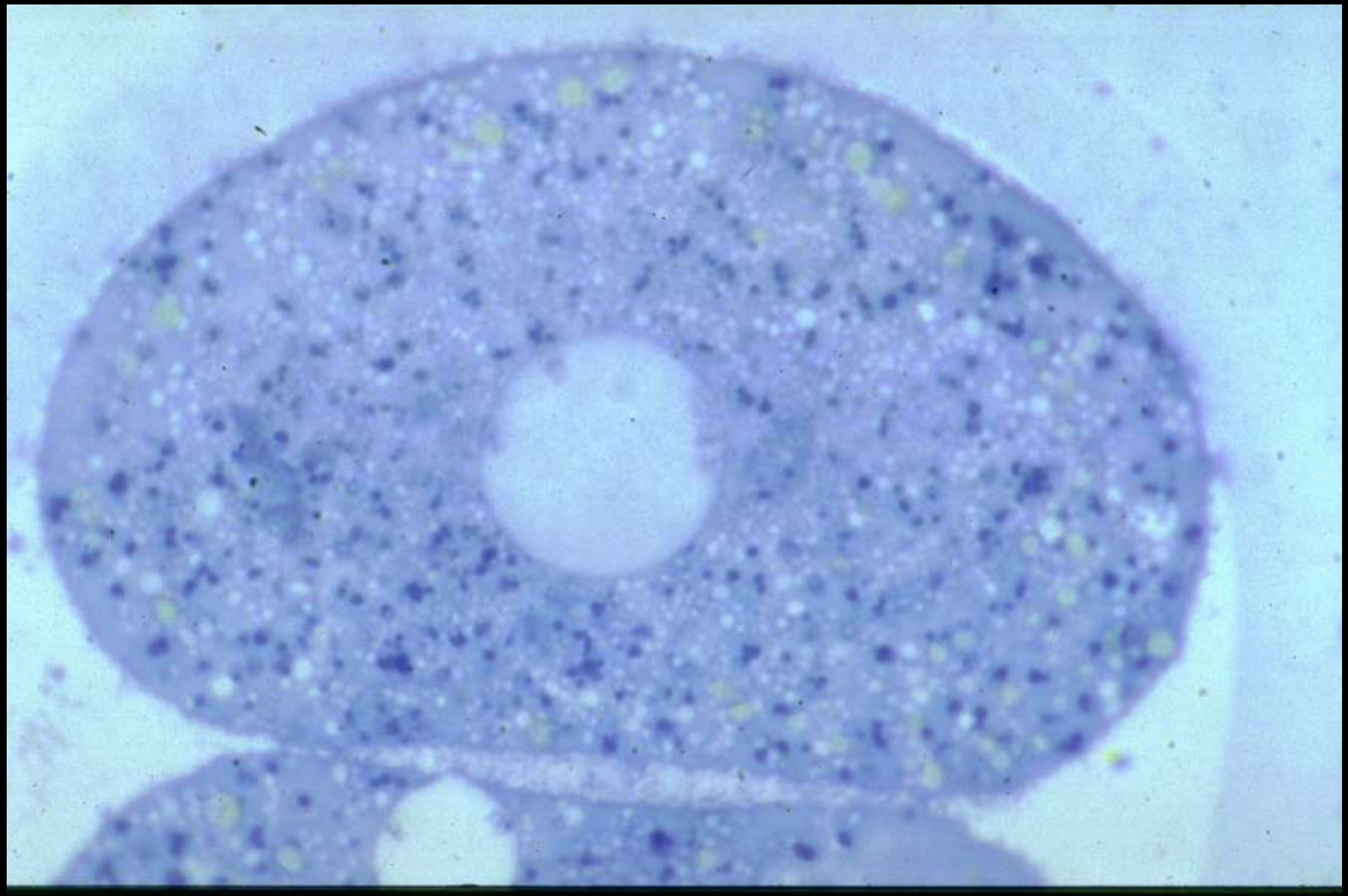


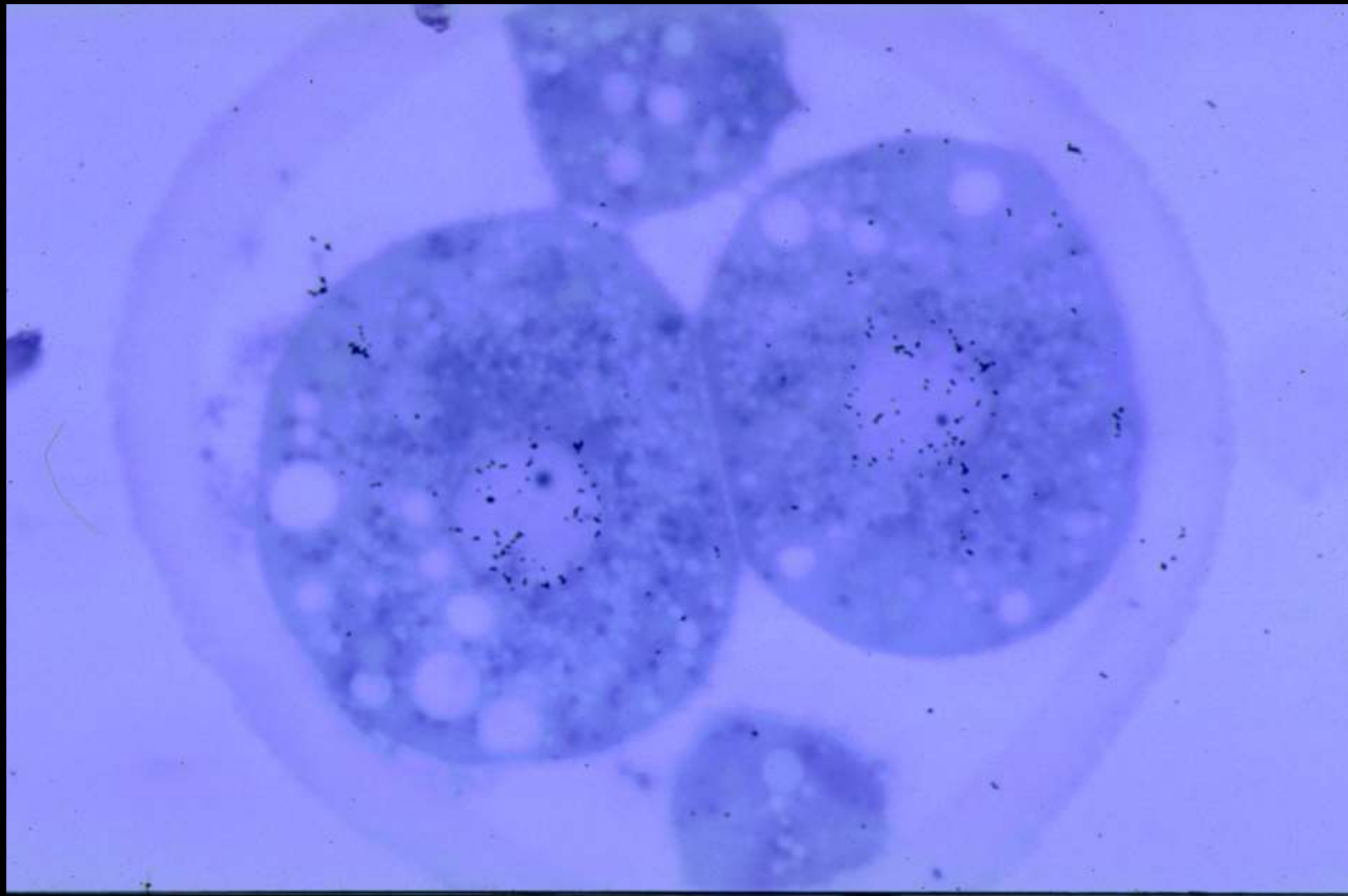


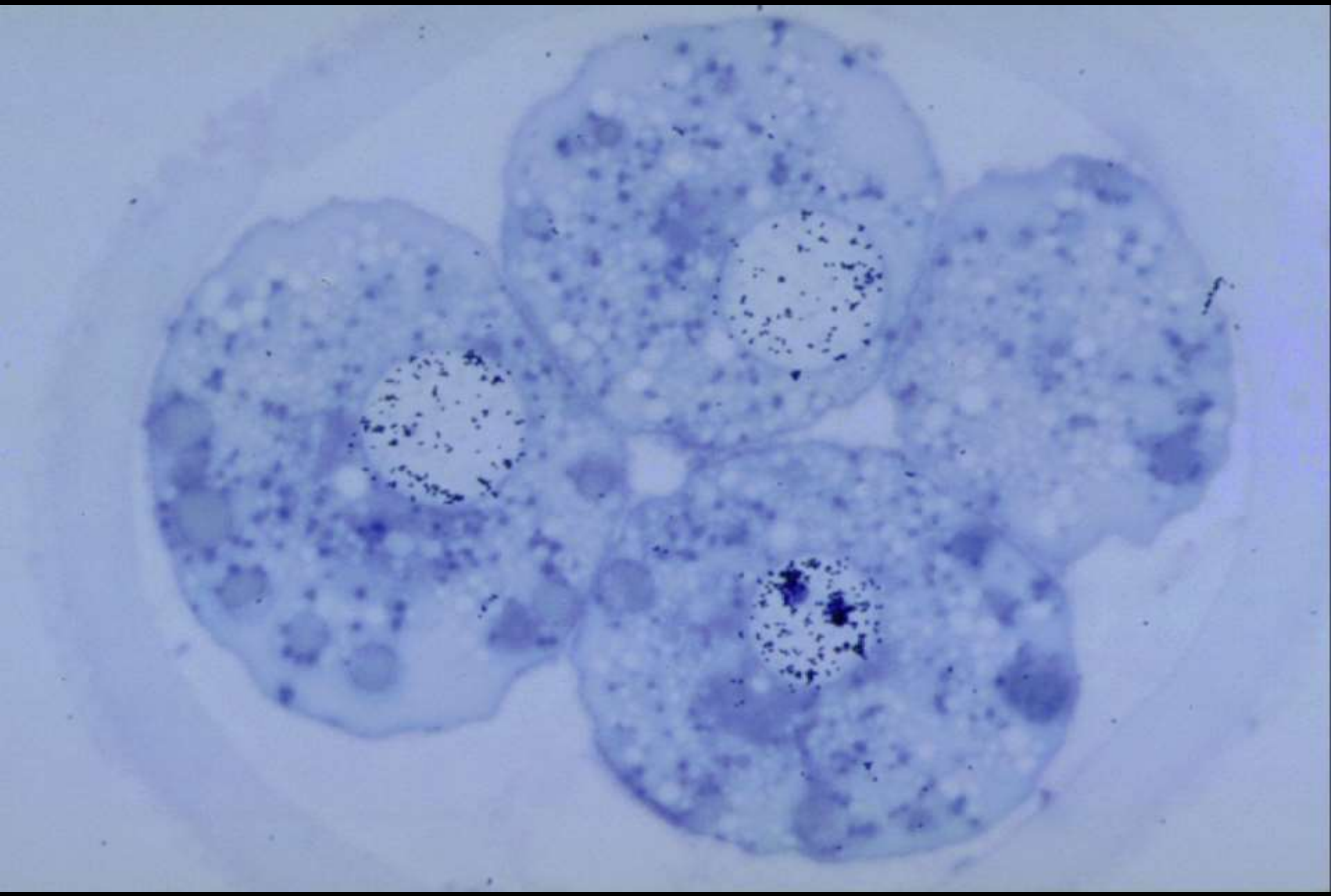












Výsledky

V kontrolních embryích skotu RNA syntéza začíná na stadiu pozdních 8 buněk, v časnějších stadiích není RNA synteza detekovatelná

Embrya rekonstruovaná z neaktivovaných cytoplastů (MII) :

- rychlý pokles syntezy RNA 1 a 3 hod. po fúzi, absence syntézy 20 hod. po fúzi
- RNA syntéza není přítomna na 2, 4 ani 8 buněčném stadiu

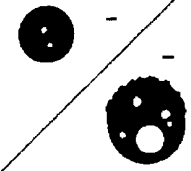
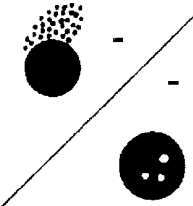
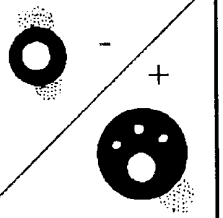
Výsledky

Embrya rekonstruovaná z aktivovaných cytoplastů (S fáze) :

- pozvolný pokles RNA syntézy po fúzi, absence syntézy RNA 20 hod. po fúzi a v 2 buněčném stadiu

- počátek syntézy RNA ve 4 buněčném stadiu, tedy o jeden buněčný cyklus dříve než u kontrolních embryí

TABLE 1. Nucleolar Ultrastructure and Nuclear RNA Synthesis

	CONTROL IN VITRO	NTE NONACTIVATED CTP	NTE ACTIVATED CTP
1 CELL	 -	 -/+	 ++
2 CELL	 -	 - -	 - -
4 CELL	 -	DEGENERATION	 +
8 CELL	 - +		 ++

+ POUL HYTTEL 98 +

Calf n°8803 «MARGUERITE»
Born February. 20, 1998
Dead April 4, 1998

Calf n°7810 «NARCISSE»
Born March 6, 1998

Celkové shrnutí

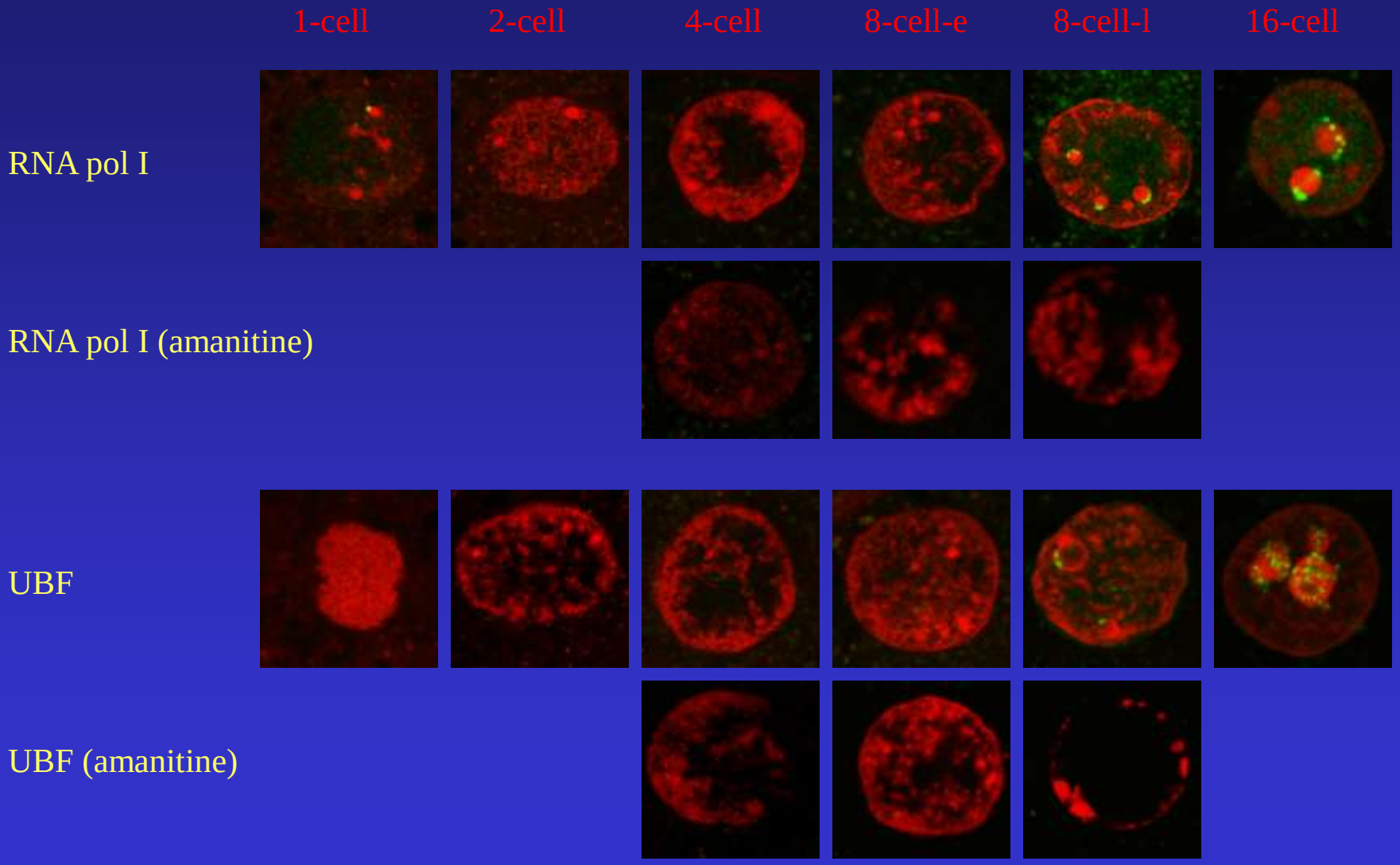
Určitý stupeň reprogramace jádra po přenosu je nezbytný k úspěšnému vývoji

Naprosto přesná časová posloupnost a kvantita exprese jednotlivých genů po přenosu jader není nezbytně nutná

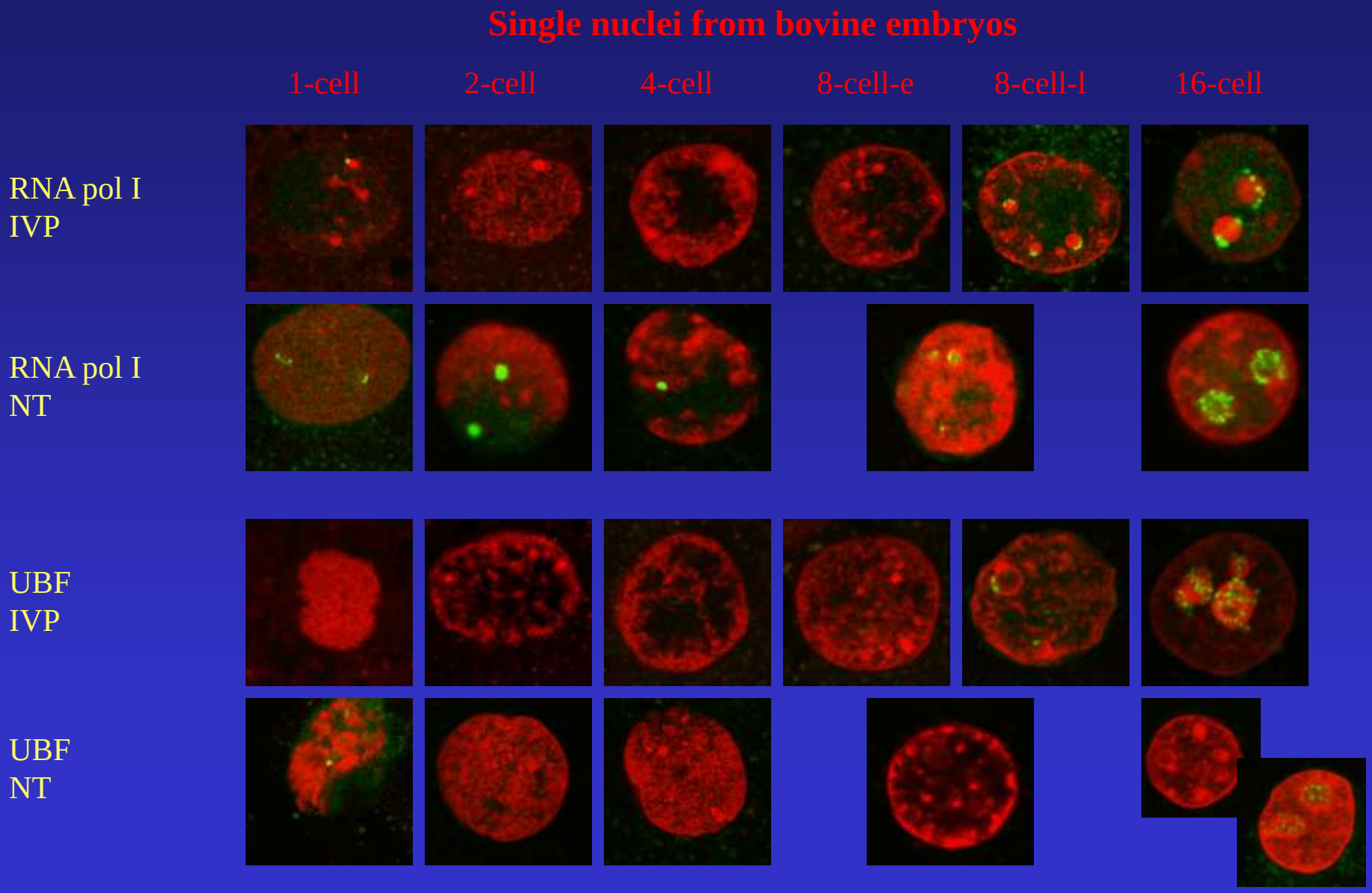
Sledování exprese jednotlivých genů v budoucnu poskytne užitečné informace o vývoji normálního i klonovaného embrya savců

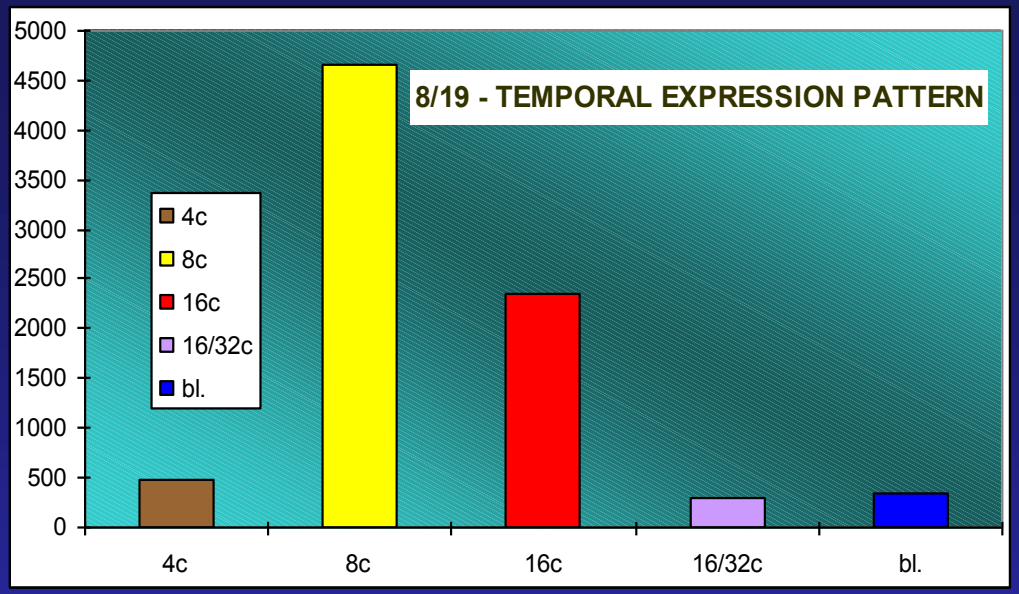
Nucleolar proteins in bovine embryos

Single nuclei from bovine embryos



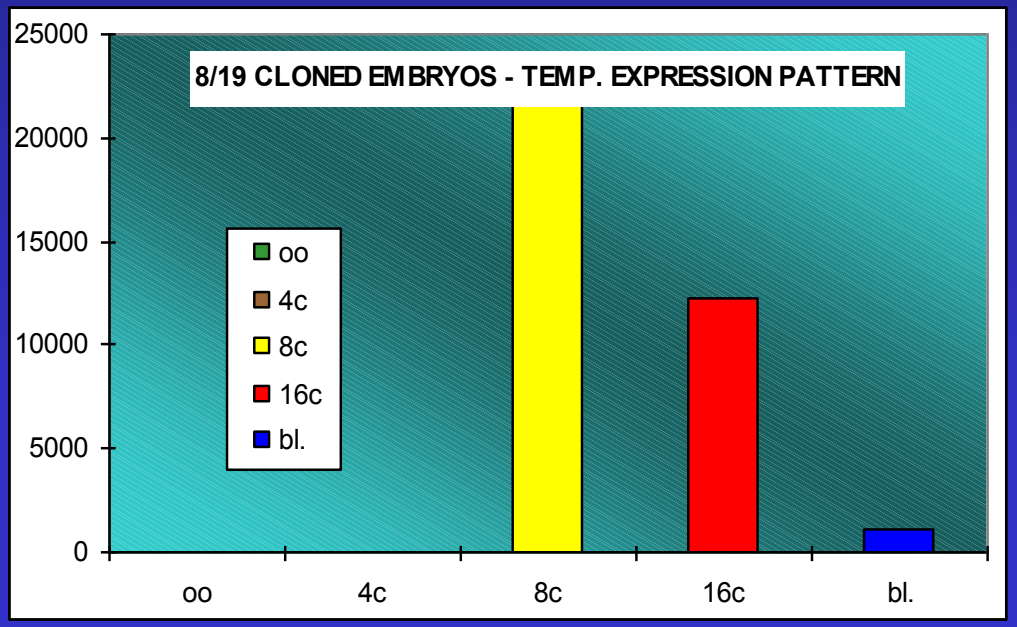
Nucleolar proteins in bovine embryos

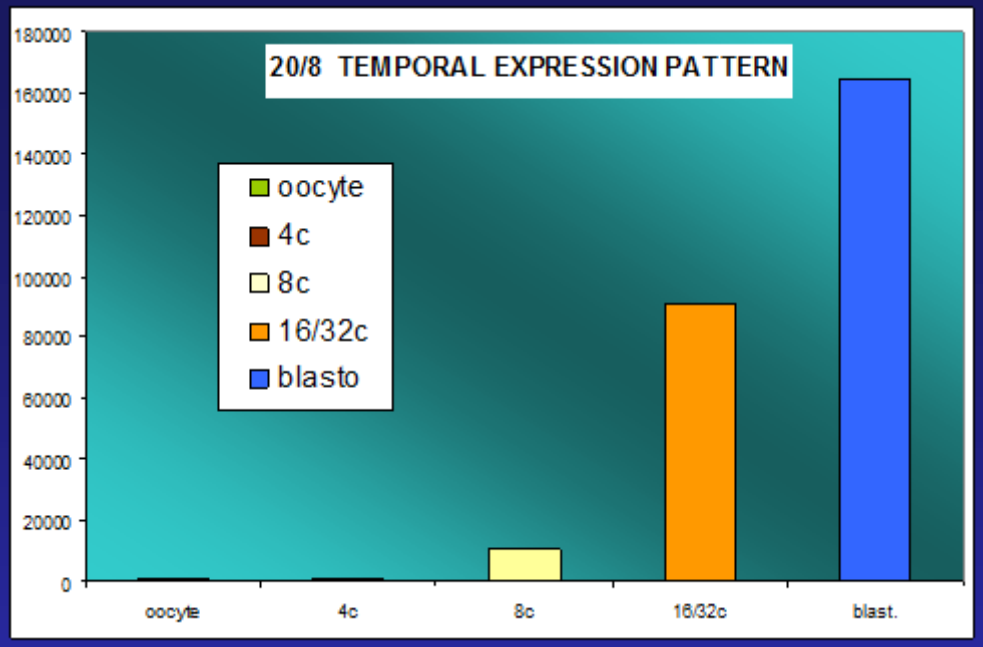


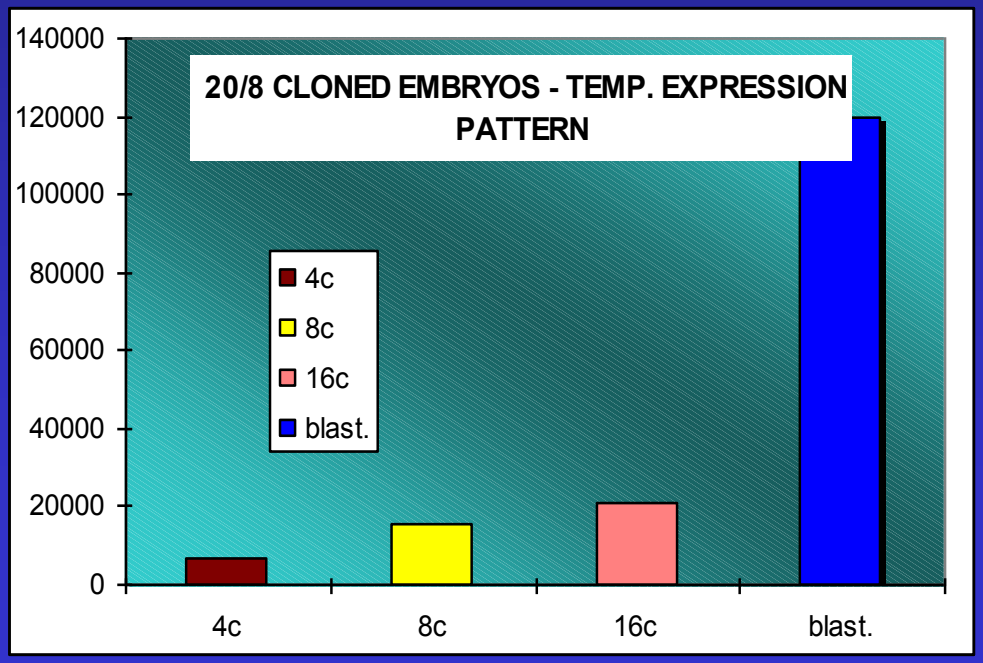


No. 8/19

mRNA for proline rich proteine

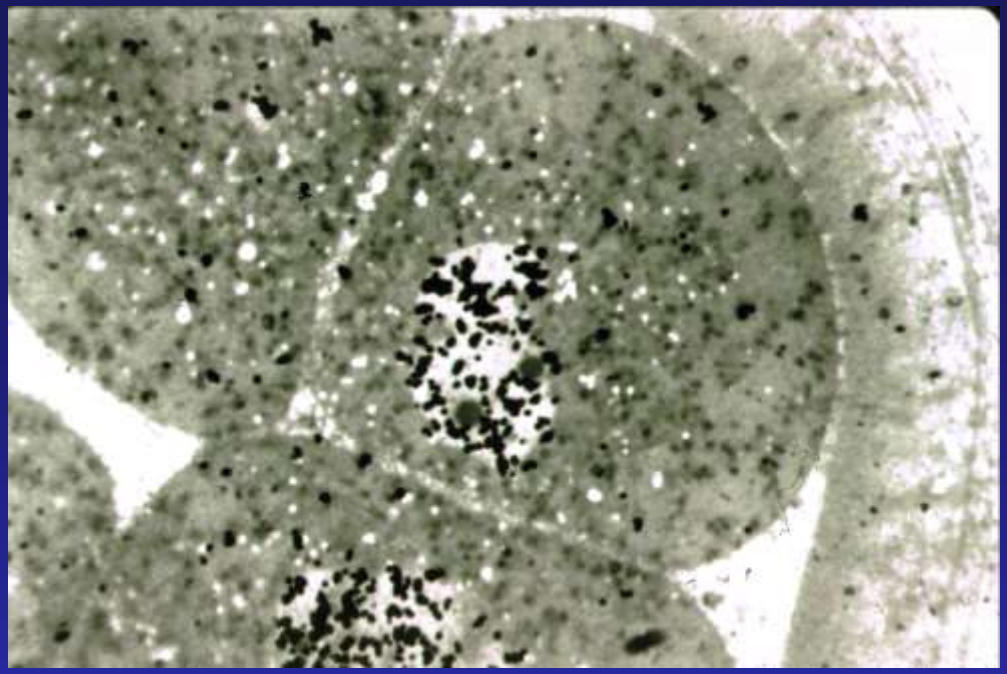




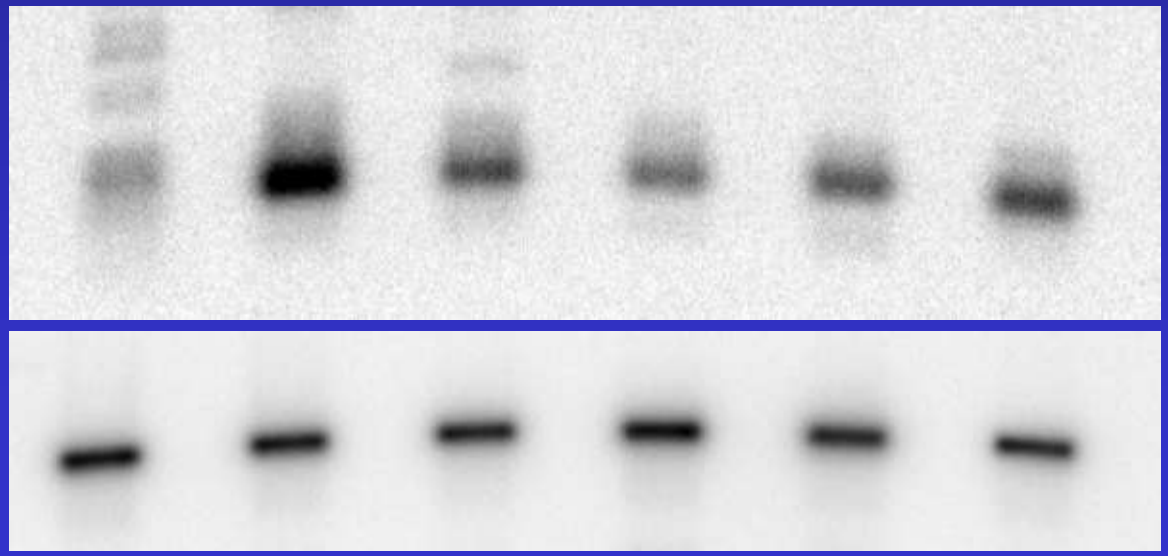


No. 20/8

mRNA for S3A ribosomal protein



Srovnání ARG a semikvantitativní RT-PCR



1998 - T. Wakayama (skupina R.Yamagimashiho) vytvořil více než 50 klonovaných myší z kumulárních buněk

1999 - T. Wakayama a R. Yamagimashi klonují samce myši ze somatických buněk

- D. Wells klonuje s vysokou úspěšností telata z buněk granulosy

1998 - **J.A. Thomson** (Univ. of Wisconsin) - embryonální kmenové buňky člověka

J.A. Gearhart (Johns Hopkins Univ., Baltimore) – primordiální embryonální kmenové buňky člověka

1999 - **Genzyme Transgenics** oznamuje narození kozy, klonované ze somatických buněk, transgenních pro recombinant human antithrombin III.

Březen 2000 - PPL Therapeutics oznamuje narození 5 selat, klonovaných ze somatických buněk.

Millie, Christa, Alexis, Carrel and Dotcom, the first cloned pigs

August 22, 2002

PPL Therapeutics

Double knock-out pigs

Alpha 1,3 galactose

Noah

Advanced Cell Technology (ACT) in Massachusetts, January 2001

Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells

Loi P, Ptak G, Barboni B, Fulka J, Cappai P, Clinton M

NATURE BIOTECHNOLOGY 19: (10) 962-964

OCT 2001

Science. 2003 Aug 22;301(5636):1063.

A mule cloned from fetal cells by nuclear transfer.

Woods GL, White KL, Vanderwall DK, Li GP, Aston KI, Bunch TD, Meerdo LN, Pate BJ.

Northwest Equine Reproduction Laboratory, Department of Animal and Veterinary Science, University of Idaho, Moscow, ID 83844, USA.
gwoods@uidaho.edu

IDAHO GEM

A cloned horse born to its dam twin
(vol 424, pg 635, 2003)
Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R, Lazzari G
NATURE

425 (6959): 680-680 OCT 16 2003

PROMETHEA

Science. 2003 Sep 25

Generation of Fertile Cloned Rats by Regulating Oocyte Activation.

Zhou Q, Renard JP, Le Friec G, Brochard V, Beaujean N, Cherifi Y, Fraichard A, Cozzi J.

Developmental Biology and Reproduction Unit, INRA, Jouy en Josas, France; Institute of Zoology, CAS, Beijing, RP of China.

RALPH

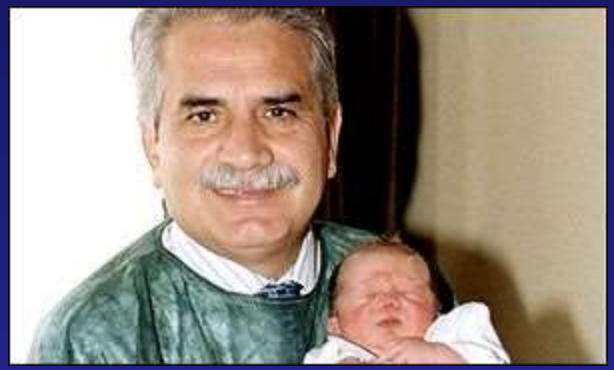
Efficiency of nuclear transfer

Species	Donor cell type	Morulae or blastocysts per fussed or injected oocytes	Offspring per transferred embryos	Reference
Cattle	Cumulus cells	18/47 (38%)	5/6 (83%)	Kato et al., 1998
	Oviductal cells	20/94 (21%)	3/4 (75%)	Kato et al., 1998
	Granulosa cells	383/552 (69%)	10/100 (10%)	Wells et al., 1999
	Mammary epithelial cells	36/140 (26%) 48/149 (32%)	1/4 (25%) 2/45 (4%)	Zakhartchenko et al., 2000 Kishi et al., 2000
	Fetal fibroblasts	24/92 (26%)	7/8 (87%)	Urukawa et al., 2001
	Calf ear skin cells	n.d. (n.d.)	1/6 (17%)	Renard et al., 1999
	Adult ear fibroblasts	49/82 (60%) 80/197 (41%)	1/16 (6%) 2/43 (4,7%)	Zakhartchenko et al., 1999 Kishi et al., 2000
	Adult fibroblasts (17 yr)	131/440 (30%)	6/54 (11%)	Kubota et al., 2000
	Transfected fetal fibroblasts	33/276 (12%) 87/1896 (5%)	4/28 (14%) 6/79 (8%)	Cibelli et al., 1998 Lanza et al., 2000
	Fetal, newborn, adult cells	980/2529 (39%)	24/172 (14%)	Kato et al., 2000
Overall efficiency		21-69%	4-83%	

Efficiency of nuclear transfer

Mouse	Cumulus cells	1385/2468 (56%)	16/1385 (1%)	Wakayama et al., 1998
	Fetal ovary cells	108/191 (56%)	4/108 (3,7%)	Wakayama and Yanagimachi, 2001
	Fetal testis cells	114/212 (54%)	2/112 (1,8%)	Wakayama and Yanagimachi, 2001
	Immature Sertoli cells	210/646 (33%)	7/215 (3,3%)	Ogura et al., 2000
	Fetal fibroblasts	278/932 (30%)	5/272 (1,8%)	Ono et al., 2001
	Tail tip fibroblasts	341/633 (54%)	3 (0,5%)	Wakayama and Yanagimachi 1999
	Transfeeted tail tip fibroblasts	281/832 (34%)	8/301 (2,7%)	Ogura et al., 2000
Overall efficiency		30-59%	0,5-3,7%	
Rabbit	Cumulus cells	371/612 (61%)	6/371 (1,6%)	Chesne et al., 2002
	Cumulus cells	n.d. (n.d.)	1/87 (1,1%)	Shin et al., 2002

Dr. Severino Antinori



Dr. Panos Zavos

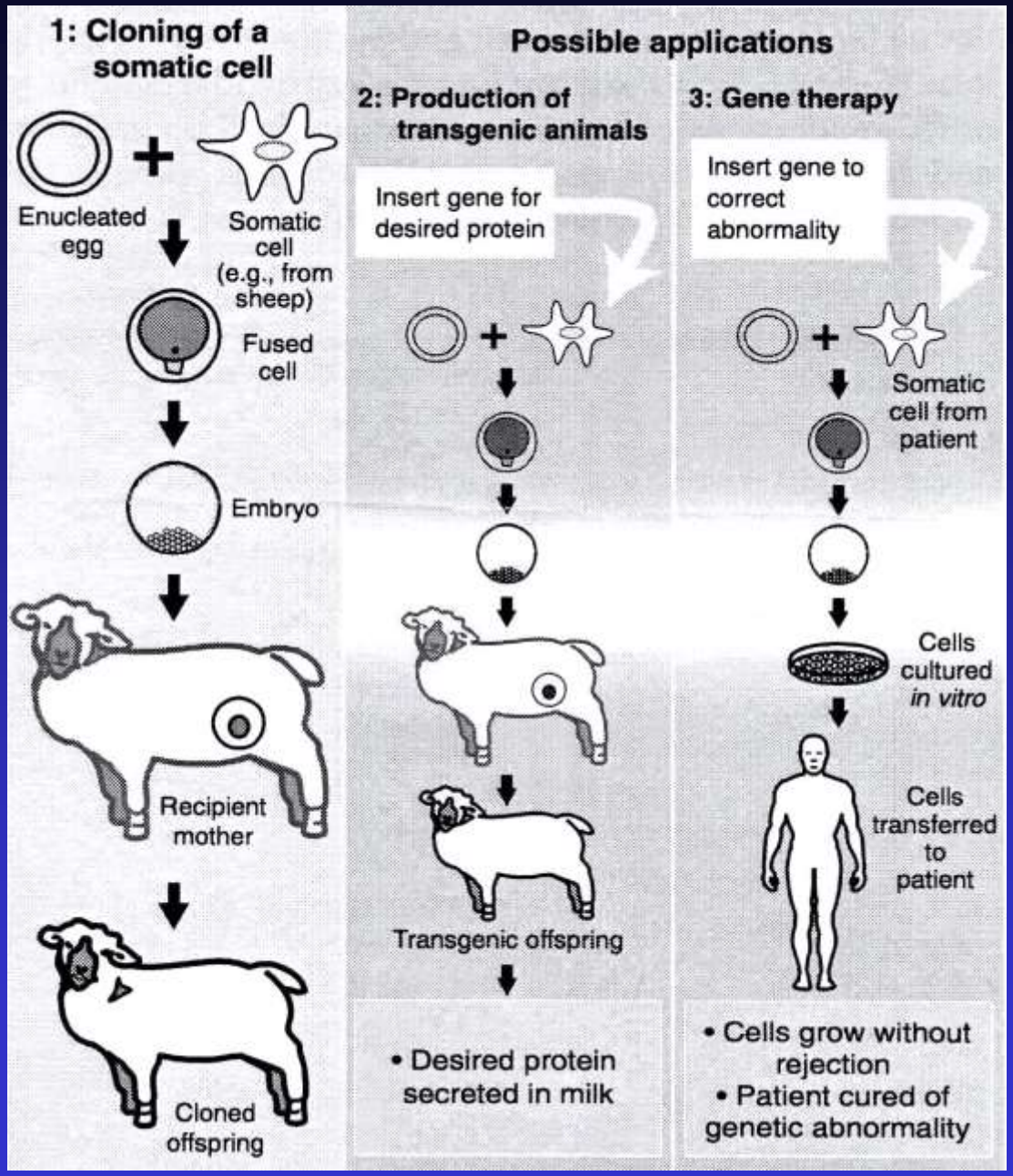
November 26, 2001

Advanced Cell Technology Inc. (ACT), Worcester, Massachusetts

ACT President Michael West

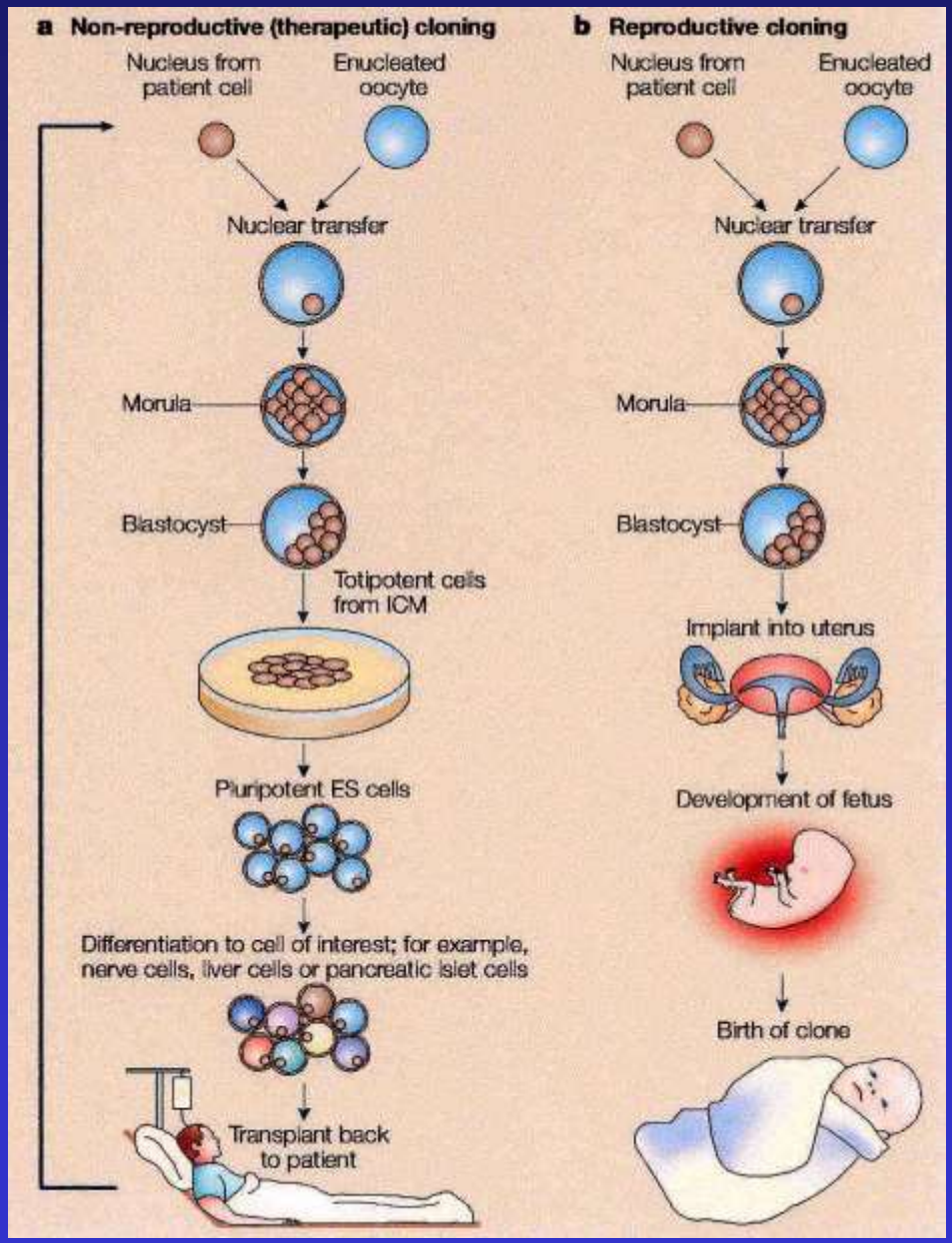
Jose B. Cibelli, Ph.D., D.VM., Vice-President of Research at ACT

CLONING - POTENTIAL BENEFITS

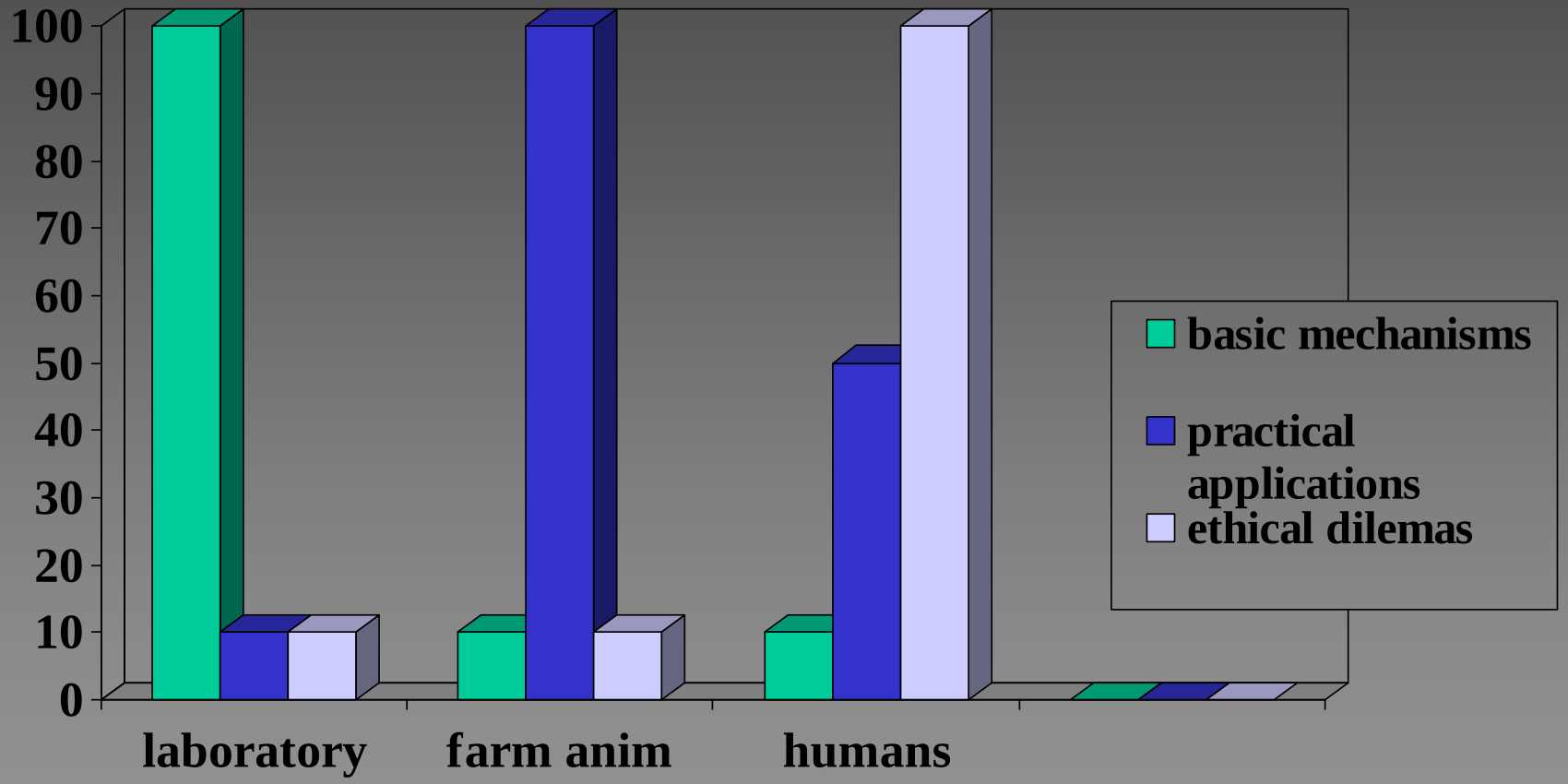


Trounson, A. ; MJA 167:568-569 ; 1997

Reproductive versus non-reproductive cloning



CLONING AND EMBRYONIC STEM CELLS



Solter, D. ; Croatian Medical Journal 40(3):309-318 ; 1999

J. Fulka Jr.
Inst. of Animal Production, Prague, Czech Rep.

Heyman Y., Chesne P., Renard J.-P., Fléchon J.-F., Duranthon V.
Biologie Cellulaire et Moléculaire, INRA, 78352 Jouy-en-Josas Cedex, France

H. Callesen, P. Holm, S. D. Smith, E. Soloy
Embryo Technology Center, Danish Institute of Animal Science, DK-8830 Tjele, Denmark

P. Hyttel
Royal Veterinary and Agricultural University, Section for Reproduction, DK-1870 Frederiksberg C, Copenhagen, Denmark

Ouhibi N., Moor R. M.
Laboratory of Protein Function, The Babraham Inst., Cambridge CB2 4AT, UK