PRIDOBIVANJE EMC BREZ UNIČENJA ZARODKA

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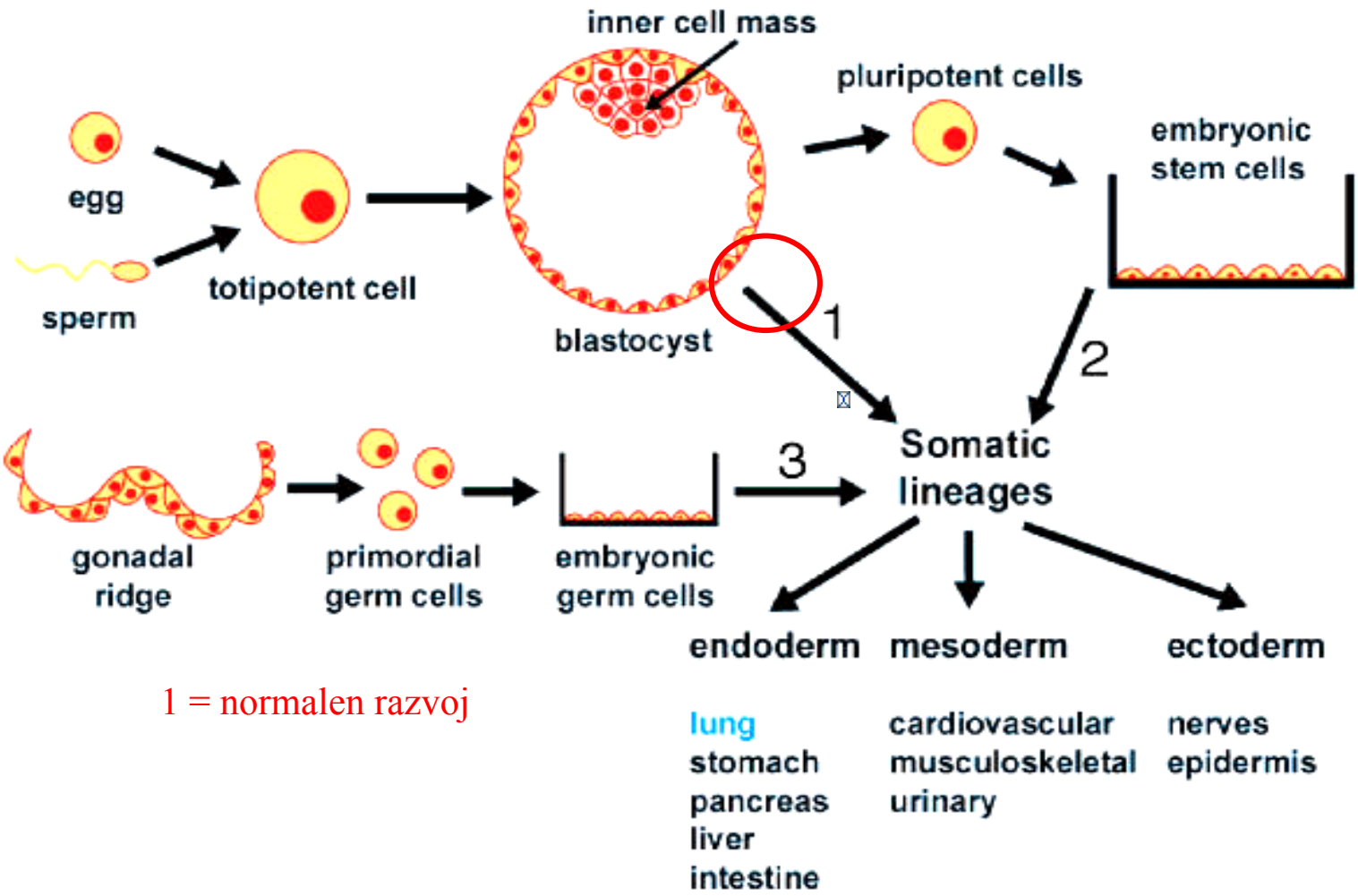
Zavod RS za transfuzijsko medicino

Ljubljana, januar 2009

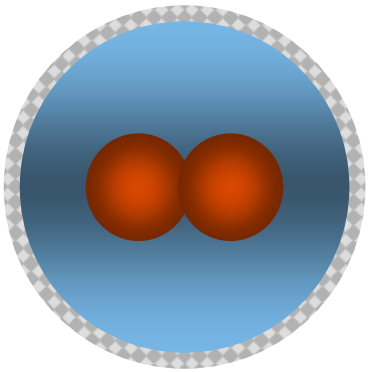
VSEBINA

- EMBRIONALNA MATIČNA CELICA - ODKRITJE IN LASTNOSTI EMC
- ALTERNATIVE PRIDOBIVANJA EMC
- NEVARNOSTI IN STRANSKI UČINKI

EMBRIONALNE MATIČNE CELICE (EMC)

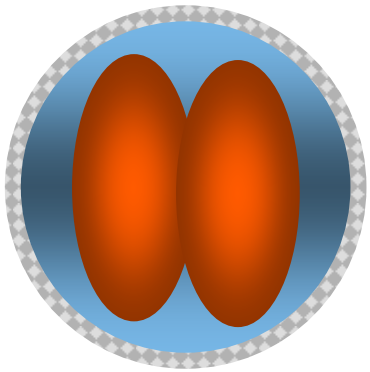


1 = normalen razvoj



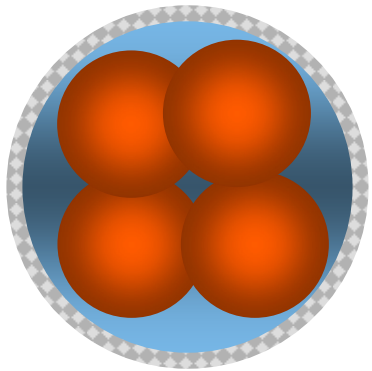
dan 1

1 celica



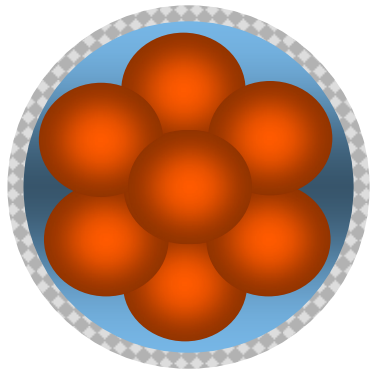
dan 2

2 celici



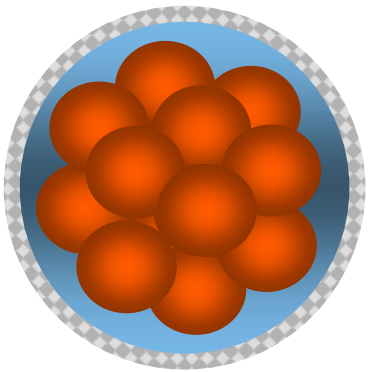
dan 2-3

4 celice



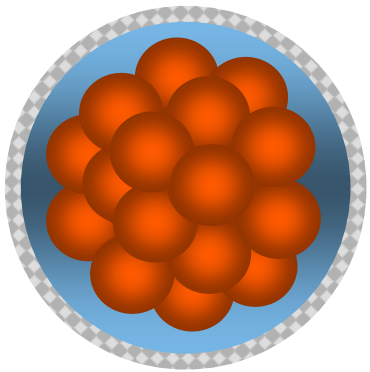
dan 3-4

8 celic



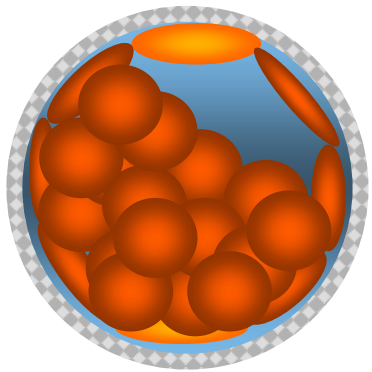
dan 3-4

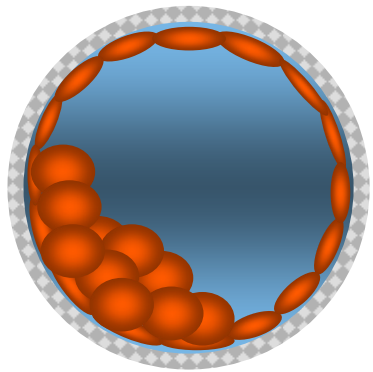
16 celic



dan 4-5

morula



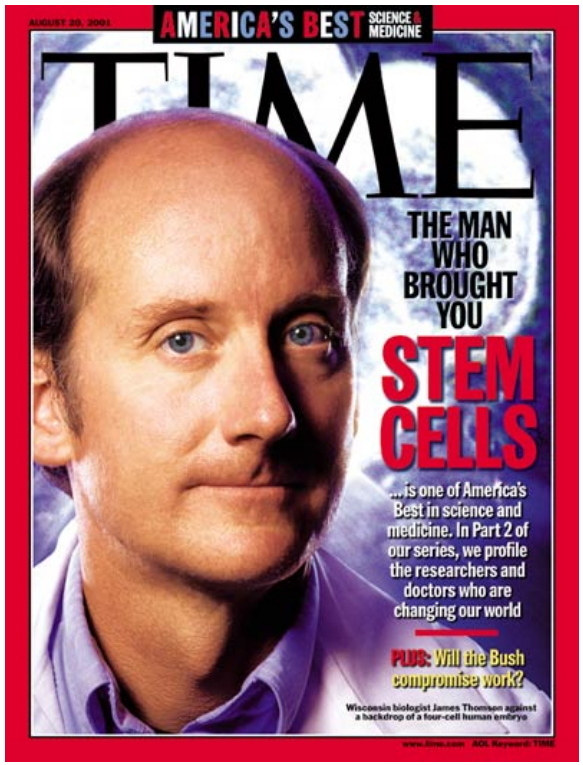


dan 4-6

zgodnja in zrela blastocista

EMC – ODKRIVANJE

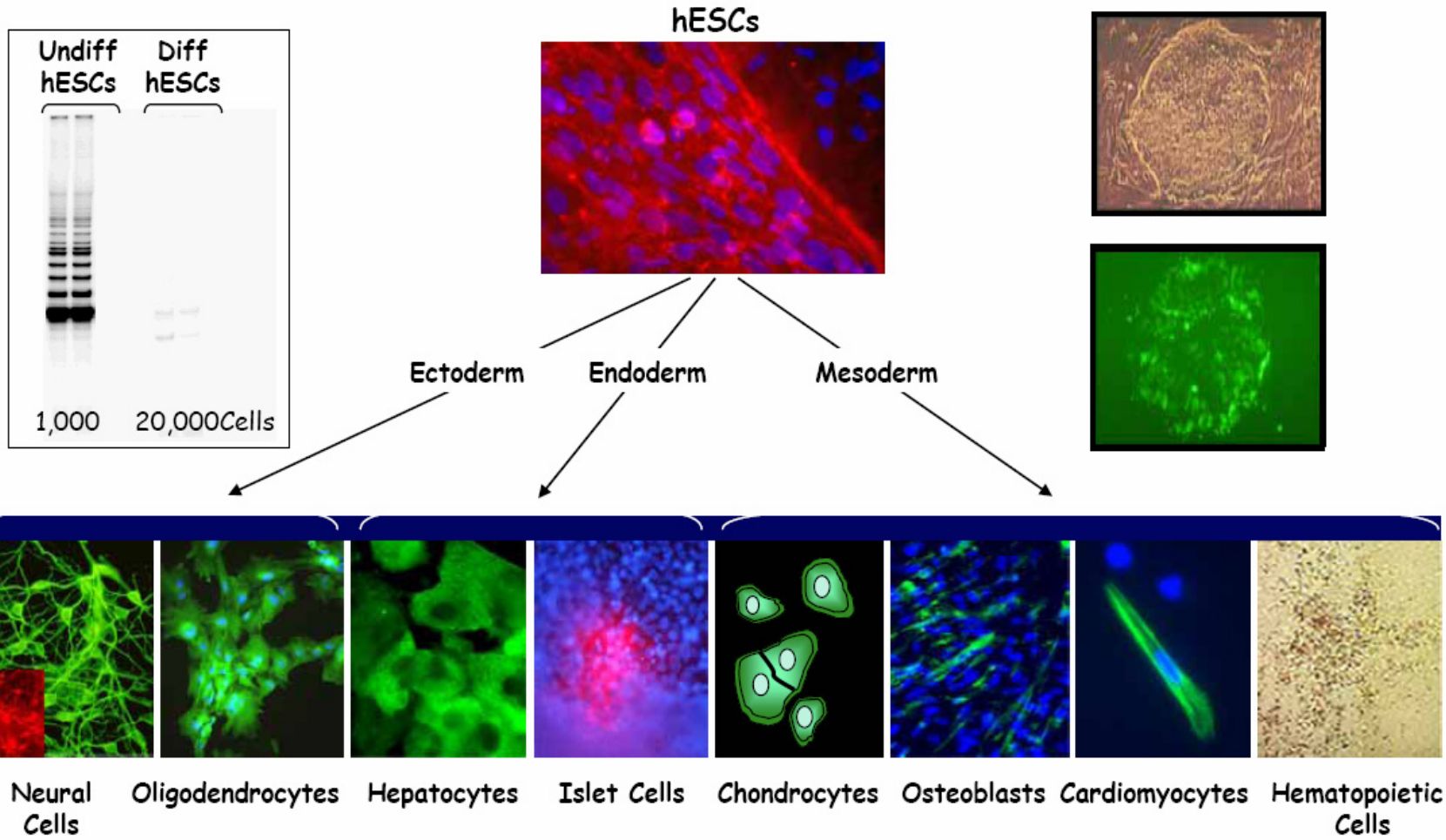
- miši - 1981 (Kaufman, Evans)
- primati - 1995
- humane - 1998 (James Thomson)



EMC –LASTNOSTI

- sposobnost neomejenega samo-obnavljanja
- pluripotentne - tvorijo celice vseh tkiv, tudi spolne
- možen razvoj v različne celične tipe vseh kličnih listov
- v kulturi tvorijo embrioidna telesca
- antigeni: SSEA-3, SSEA-4, Tra-1-60, Tra-1-80, Oct-4, alk. fosfataza
- genski profil: ACTB, NANOG, DNMT3B, REX1, SOX2, OCT3/4 (POU5F1), FGF-4

EMC - diferenciacija v različne celične vrste



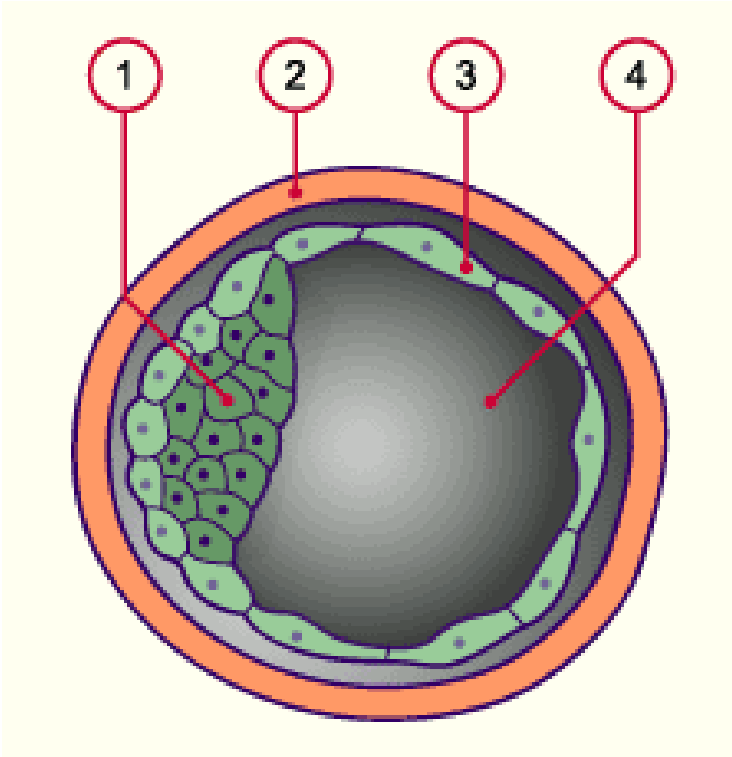
PRIDOBIVANJE EMC – KLASIČNO

mehanična odstranitev celic 1

(kemična odstranitev trofoblasta 3)

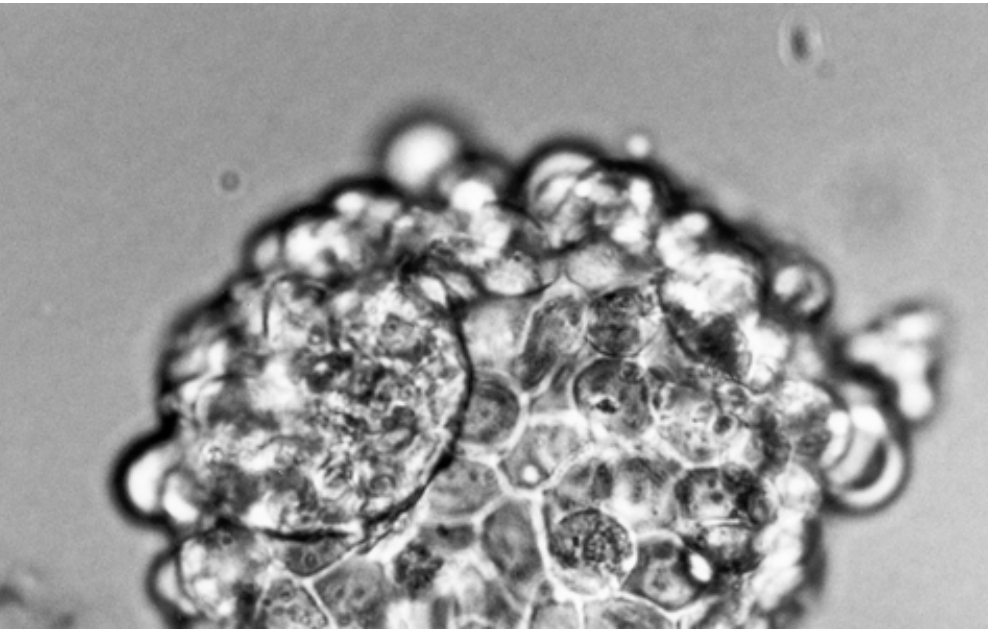
→ imunska izolacija EMC

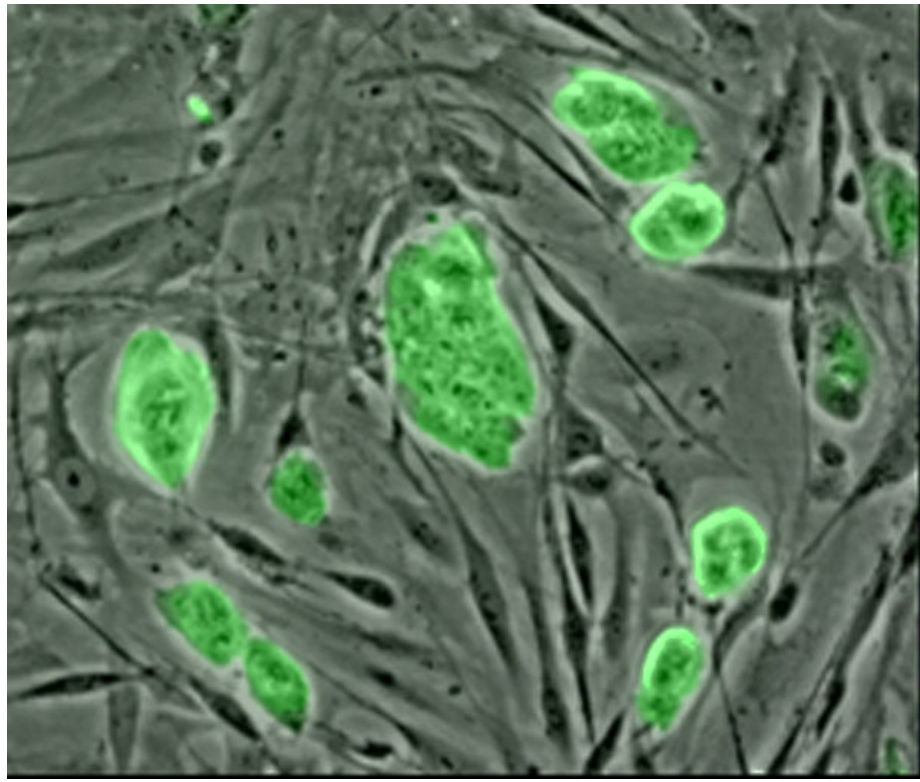
(oboje uniči embrij)



1.Embrioblast
2.Zona pellucida
3.Trofoblast
4.Blastocistna votlina

Gojenje EMC v kulturi





EMC - MOŽNI NAČINI UPORABE

- Regenerativna medicina
- Raziskave raka
- Raziskave neplodnosti in reprodukcije
- Individualizirane celične terapije
- Genska terapija
- Farmakologija – testiranje zdravil

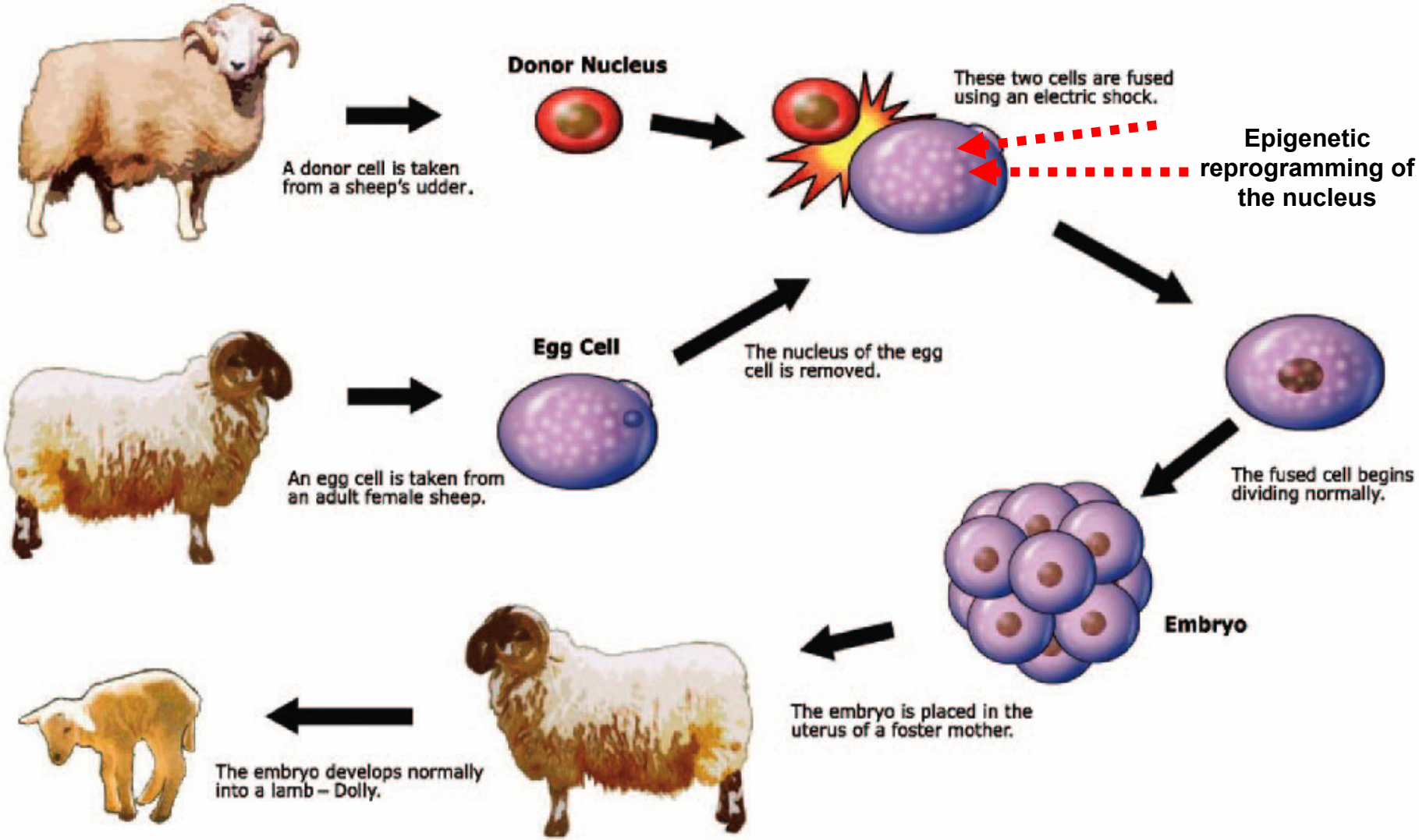
PRIDOBIVANJE – ALTERNATIVNI NAČINI

1. Tehnika prenosa jedra (nuclear cell transfer)
   - reproduktivno kloniranje
   - terapevtsko kloniranje
2. Ostanki pri postopkih IVF
3. Odvzem ene celice iz morule – blastomere - embrij se ne poškoduje
4. Uporaba partenogenetskih blastocist
5. Dediferenciacija odrasle celice (epigenetsko reprogramiranje)
   - gojenje in fuzija z MC
   - gojenje z ekstraktom oocitov
   - uporaba kemikalij, agensov, rastnih dejavnikov
   - dolgotrajno gojenje
   - usmerjena aktivacija dediferenciacijskih genov – iPSC (Induced Pluripotent SC)
6. EMC podobne celice

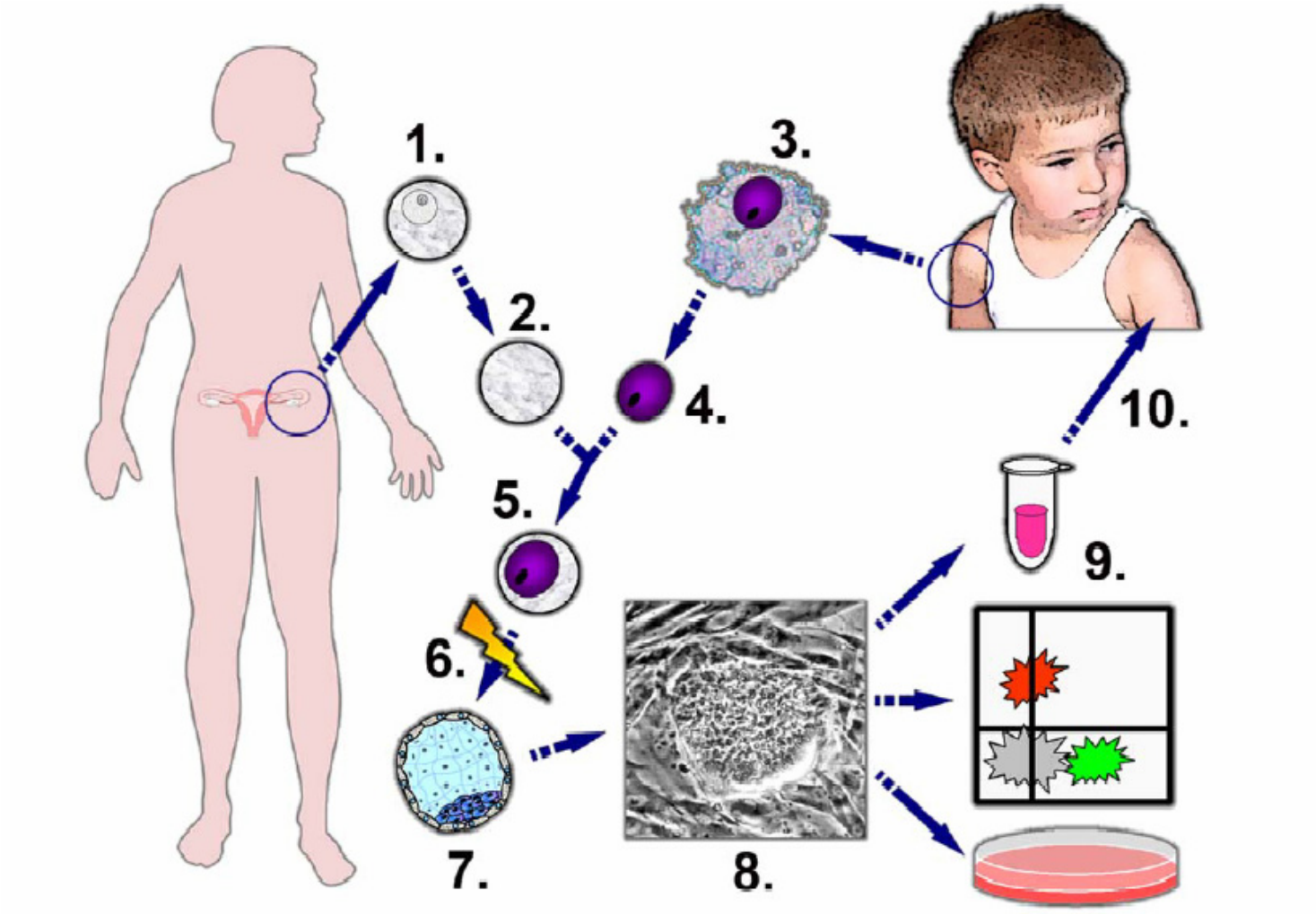
   EMC podobne celice iz fetusa ali UCB – npr. MLPC
7. EMC odraslega - ESC-A

1. SCNT – Prenos jedra somatske celice

a) reproduktivno kloniranje



SCNT - b) terapevtsko kloniranje



Težave pri kloniranju

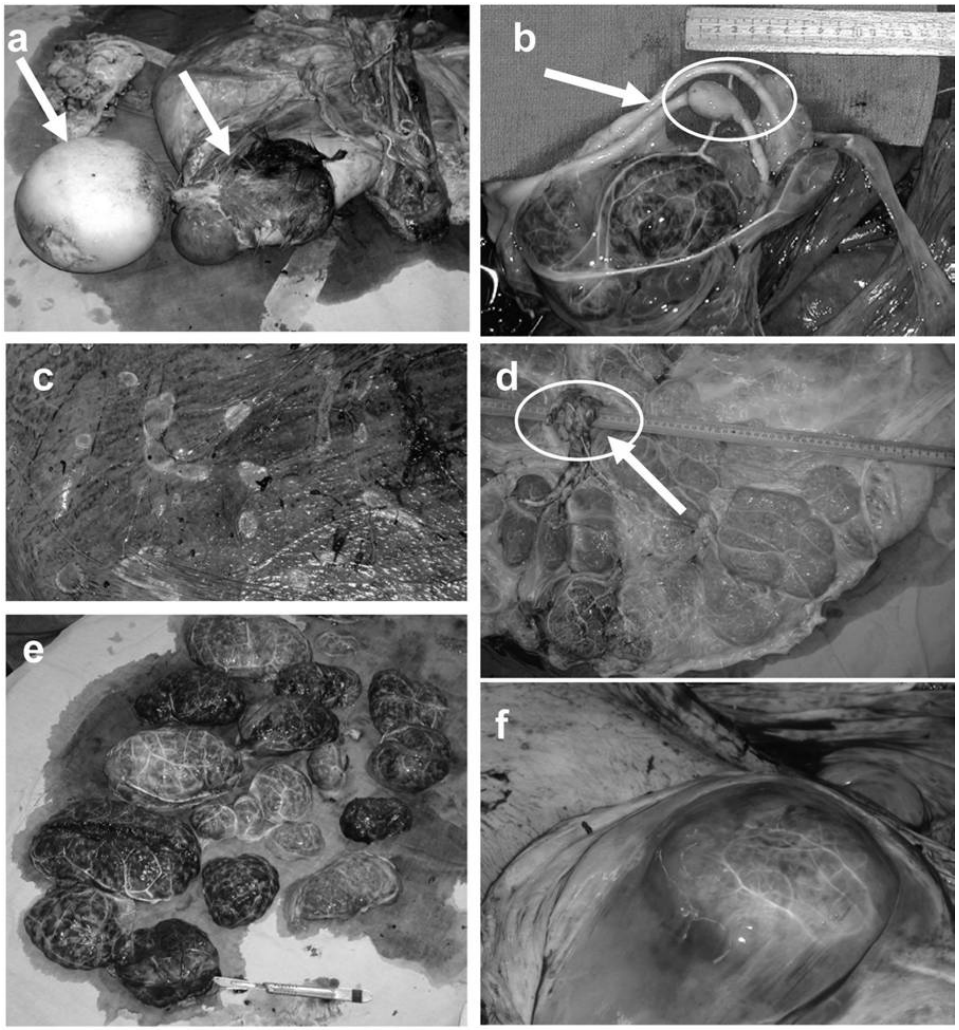
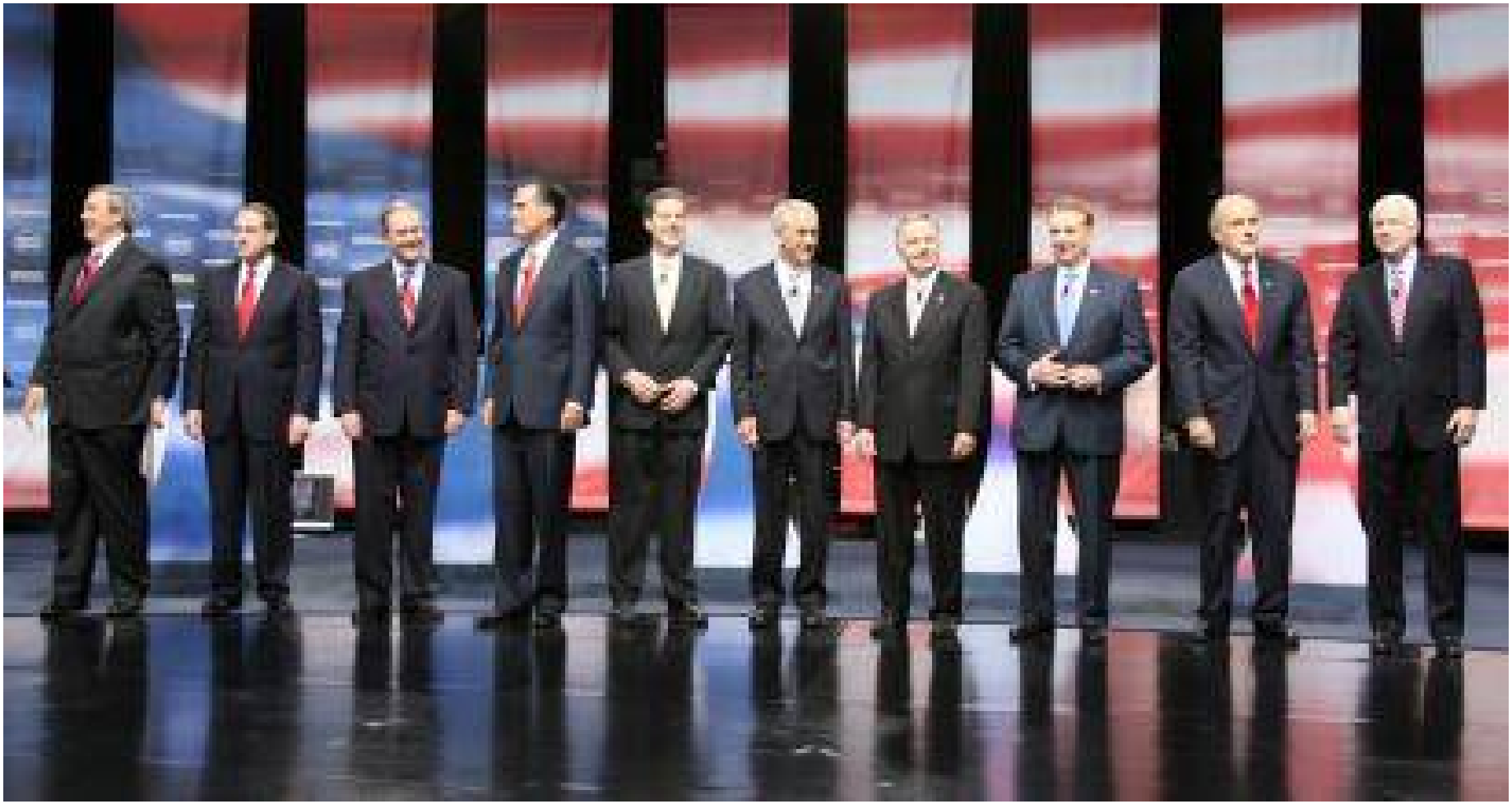


FIG. 8. Representative photographs of anomalies in term cloned placentas. (**a**) Acardiac amorphous globosus (arrows); (**b**) aneurysm in placental vasculature (see arrow); (**c**) epithelial plaques in region devoid of placentomes; (**d**) enlarged umbilical stalk (arrow); (**e**) placentomes collected from a single cloned placenta; (**f**) enlarged placentome surrounded by gelatinous edema.

Perinatal Physiology in Cloned And Normal Calves

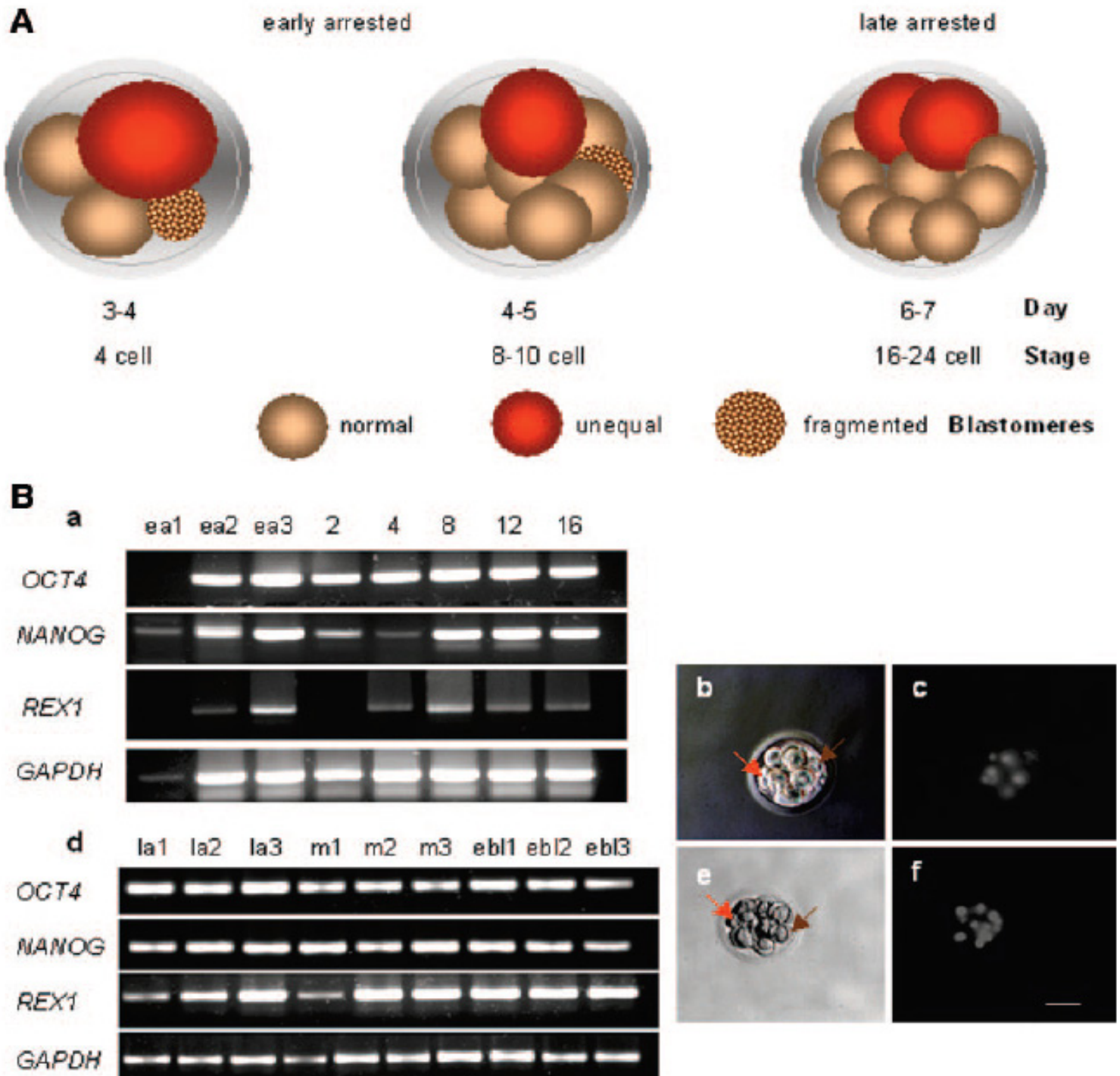
Cynthia A. Batchelder et al. Cloning and Stem Cells Mar 2007

NEVARNI KLONI: “US shock at human clones”

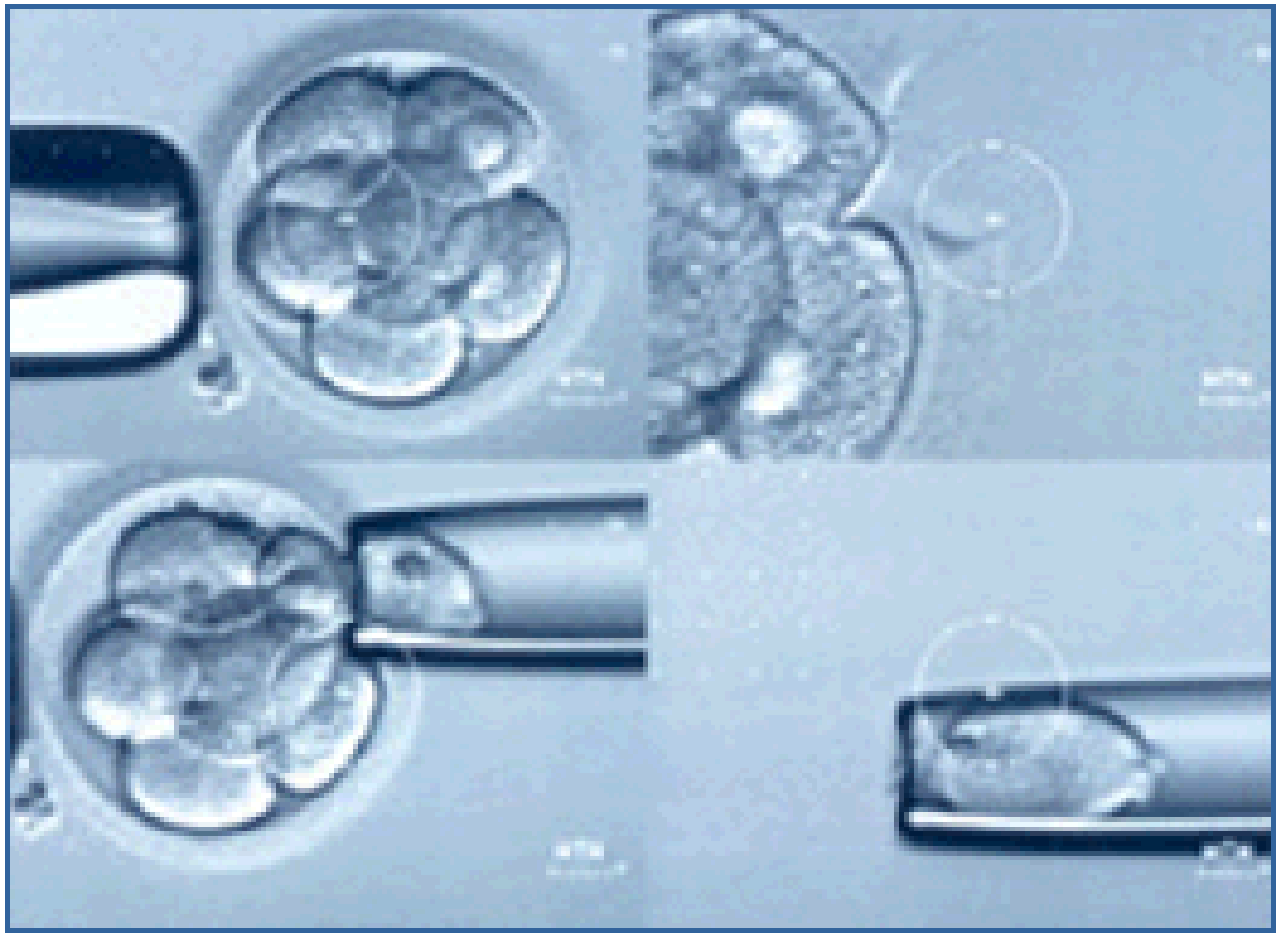


2. OSTANKI PRI POSTOPKIH IVF

- možnost uporabe pretečenih blastocist >5-10 let
- uporaba delno okvarjenih blastocist iz IVF postopka (arrested embrios, neenake blastomere)


A
early arrested
late arrested
3-4
4 cell
4-5
8-10 cell
6-7
16-24 cell
Day
Stage
normal
unequal
fragmented
Blastomeres
B
a
ea1 ea2 ea3 2 4 8 12 16
OCT4
NANOG
REX1
GAPDH
d
la1 la2 la3 m1 m2 m3 ebl1 ebl2 ebl3
OCT4
NANOG
REX1
GAPDH
b
c
e
f

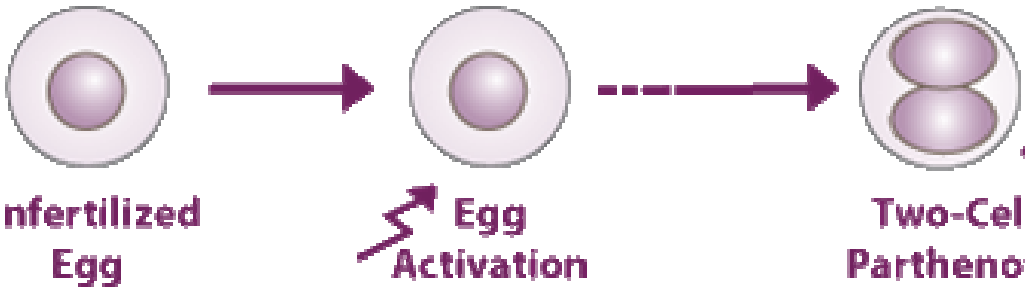
3. ODVZEM BLASTOMERE IZ MORULE



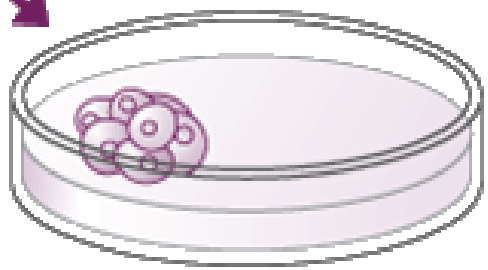
Klimanskaya I et al. Human embryonic stem cell lines derived from single blastomeres. Nature. 2006 Nov 23;444(7118):481-5.

4. Uporaba partenogenetskih blastocist

Parthenote Stem Cells



After approximately 6 days the parthenote reaches the 150 cell Blastocyst stage and the stem cells can be moved to a separate dish to grow in a colony.



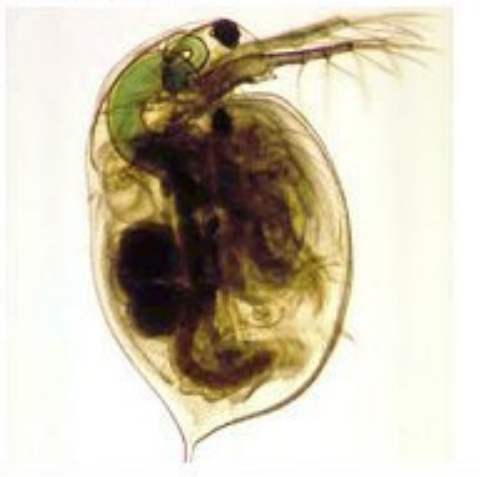
Egg Activation happens spontaneously in nature, but the process is not well understood. Research has found that unfertilized eggs can be activated either by (1) an electrical jolt or by (2) chemical stimulation If successfully activated, the egg undergoes early cell divisions similar to fertilized eggs, but cannot give rise to an offspring.

Partenogeneza (deviško rojstvo) - primeri

Komodo dragon

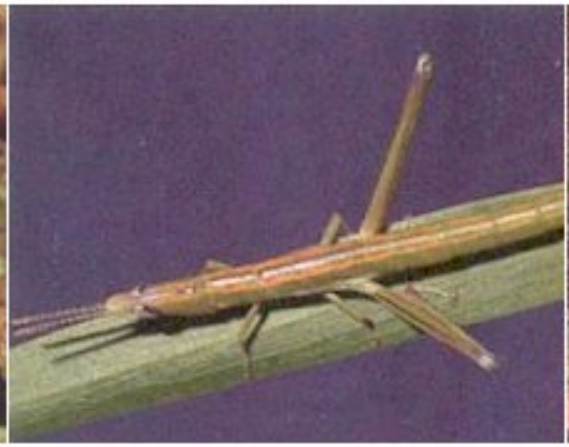
Bonnethead shark

Water flea



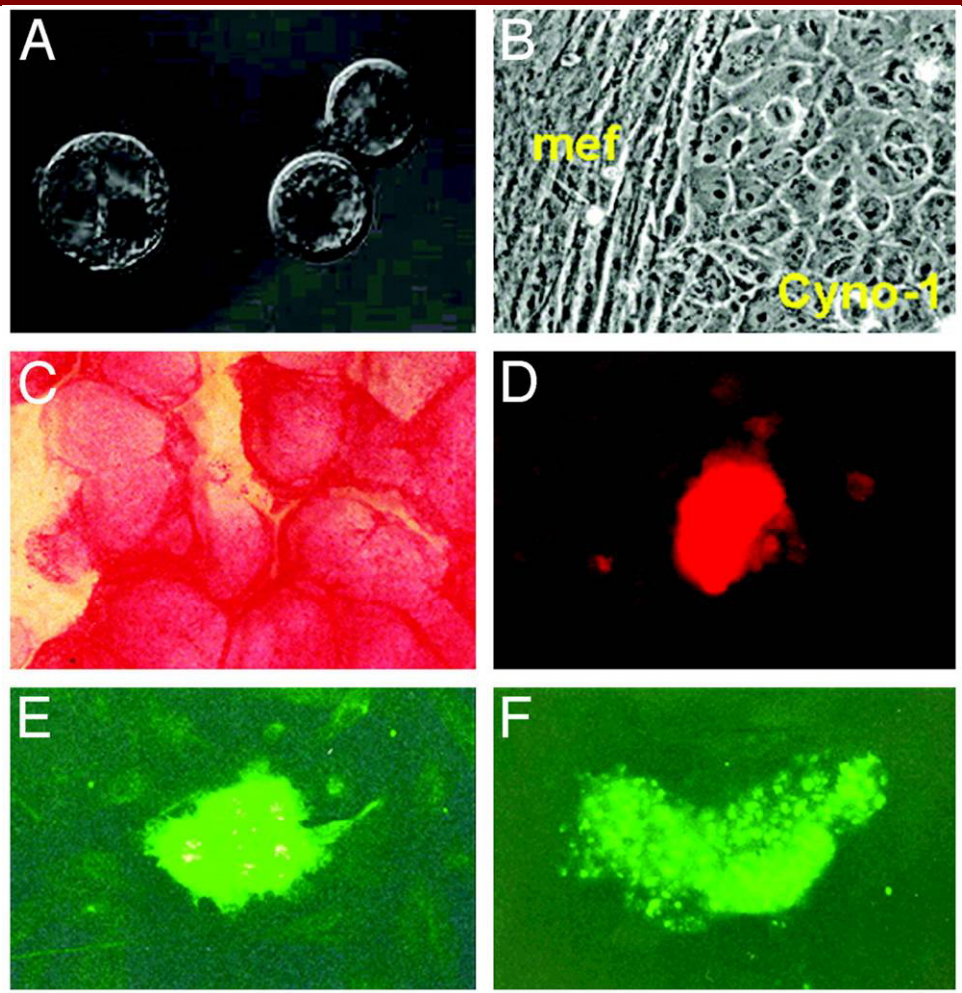
Bynoe's gecko

Warramaba virgo grasshopper



Mulga trees

Characterization of parthenogenetic embryos and derived cell lines



Vrana K. E. et.al. PNAS 2003;100:11911-11916

PNAS

5. EMC-am podobne celice

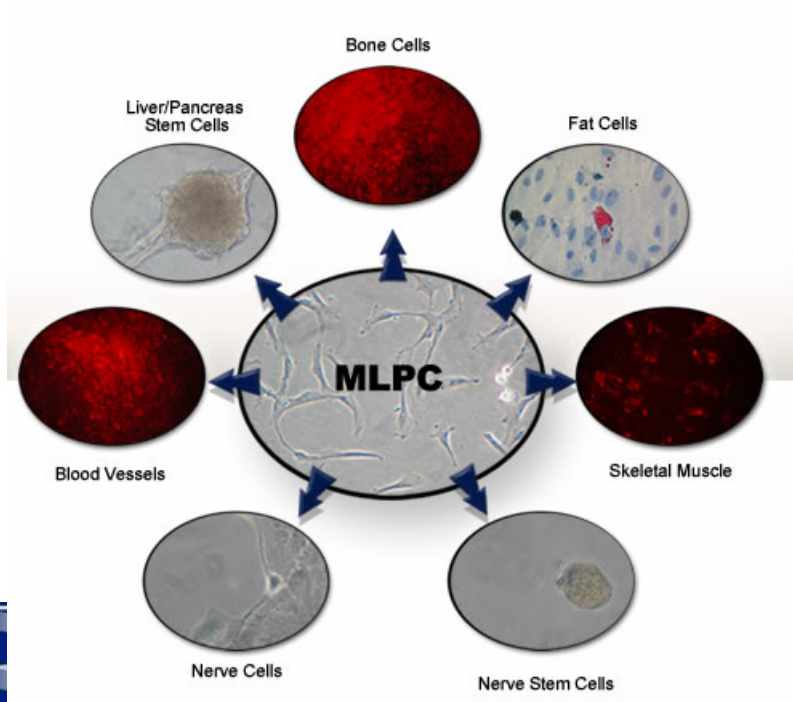
Multi-lineage Progenitor Cell – MLPC (by BioE)

- klonska ekspanzija iz popkovnične krvi
- etično nesporna celična linija
- normalen nabor kromosomov
- vse značilnosti EMC
  - diferenciacija v vse vrste celic
  - antigeni - (glej Tabela)
  - geni: TERT, OCT-4, SOX-2, GATA-4, PTEN, PUM-2, TBX-3

McGuckin et al. Production of stem cells with embryonic characteristics from human umbilical cord blood. Cell Prolif. 2005 Aug;38 (4):245-55.

MLPC

- primerne za bazične raziskave
- primerne za celično Th



Cell Marker	MLPC Leukocyte Phase	MLPC Fibroblast Phase	Cord Blood HSC	Bone Marrow MSC
CD2	Negative	Negative	Negative	Negative
CD3	Negative	Negative	Negative	Negative
CD4	Negative	Negative	Negative	Negative
CD5	Negative	Negative	Negative	Negative
CD7	Negative	Negative	Negative	Negative
CD8	Negative	Negative	Negative	Negative
CD9	Positive	Positive	Negative	Negative
CD10	Negative	Negative	Negative	Negative
CD13	Positive	Positive	Negative	Positive
CD14	Negative	Negative	Negative	Negative
CD15	Negative	Negative	Negative	Negative
CD16	Negative	Negative	Negative	Negative
CD19	Negative	Negative	Negative	Negative
CD20	Negative	Negative	Negative	Negative
CD22	Negative	Negative	Negative	Negative
CD29	Positive	Positive	Positive	Positive
CD33	Negative	Negative	Variable	Negative
CD34	Positive	Negative	Positive	Negative
CD36	Negative	Negative	Negative	Negative
CD38	Negative	Negative	Variable	Negative
CD41	Negative	Negative	Negative	Negative
CD44	Positive	Positive	Positive	Positive
CD45	Positive	Negative	Positive	Negative
CD61	Negative	Negative	Variable	Negative
CD73	Positive	Positive	Negative	Positive
Anti-HLA-DR	Negative	Negative	Variable	Negative
CD90	Positive	Positive	Positive	Positive
CD105	Positive	Positive	Negative	Positive
STRO-1	Positive	Negative	Negative	Negative
SSEA-3	Positive	Negative	Negative	Negative
SSEA-4	Positive	Negative	Negative	Negative
SCF	Positive	Negative	Negative	Negative
Glycophorin A	Negative	Negative	Negative	Negative
CD133	Positive	Negative	Positive	Negative

6. DEDIFERENCIACIJA SOMATSKE CELICE

Epigenetsko reprogramiranje - proces diferenciacije zavrtimo nazaj

- gojenje in fuzija z drugimi MC
- gojenje z ekstraktom oocitov
- uporaba kemikalij, agensov, rastnih dejavnikov
- dolgotrajno gojenje
- usmerjena reprogramiranje - aktivacija dediferenciacijskih genov
- vsEMC – golden nuggets

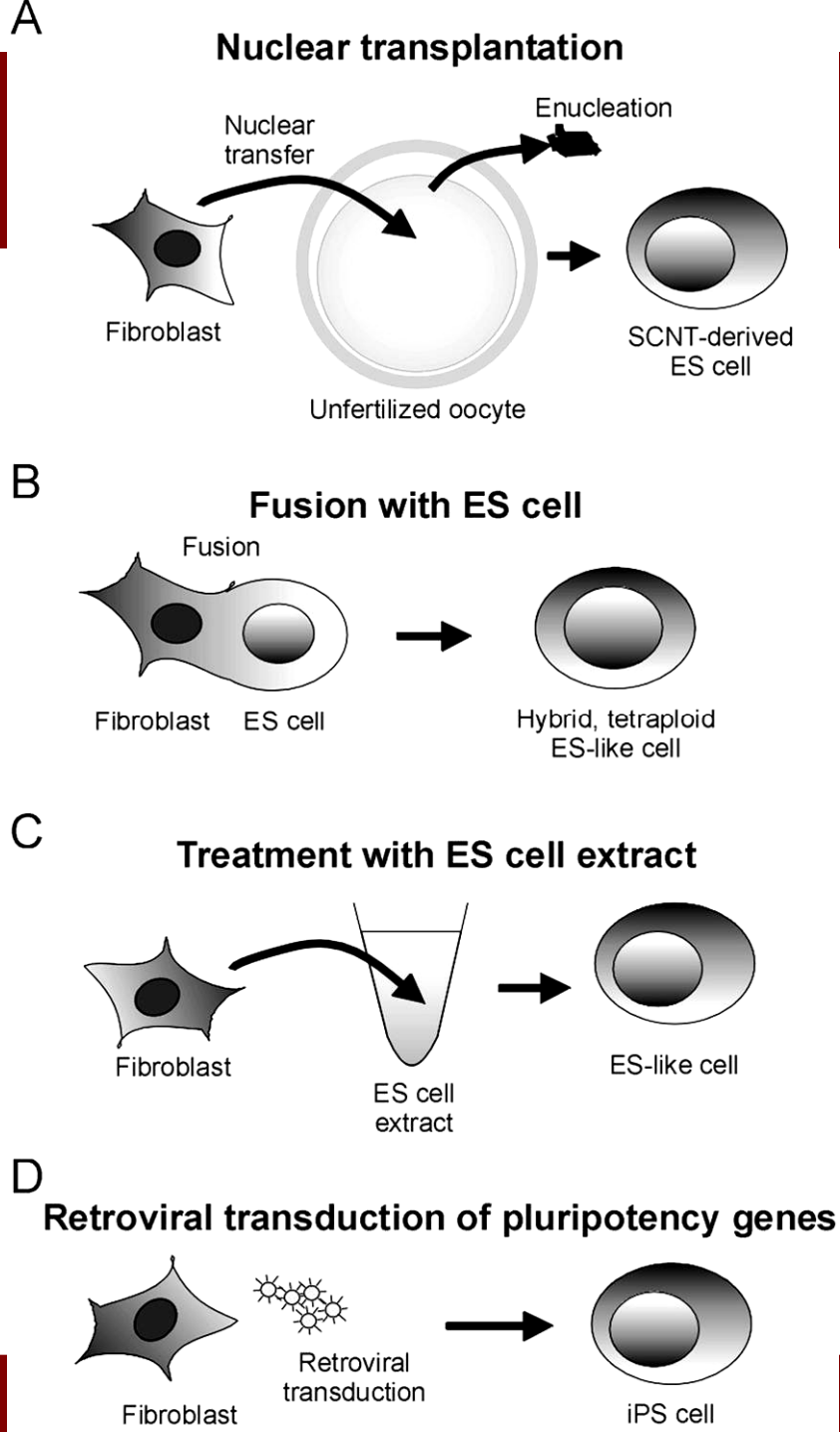


FIG. 1. Current strategies for reprogramming somatic cells to pluripotency.

(A) Transplantation of a somatic cell nucleus into an unfertilized oocyte (cloning). Growth of cloned embryos to the blastocyst stage and derivation of somatic cell nucleus transfer embryonic stem cells (SCNT ESCs) may be one option to create genetically matched replacement cells.

(**B**) Fusion of somatic cells with ESCs results in tetraploid hybrids with ESC properties.

(**C**) Transient incubation of somatic cells with extracts of ESCs elicits some nuclear reprogramming events and enhances pluripotency *in vitro*.

(**D**) Retroviral transduction of ESC transcription factors (Oct4, Sox2, Klf4, and c-Myc) is sufficient to evoke stemness in fibroblasts and generate induced pluripotent stem cells (iPS cells).

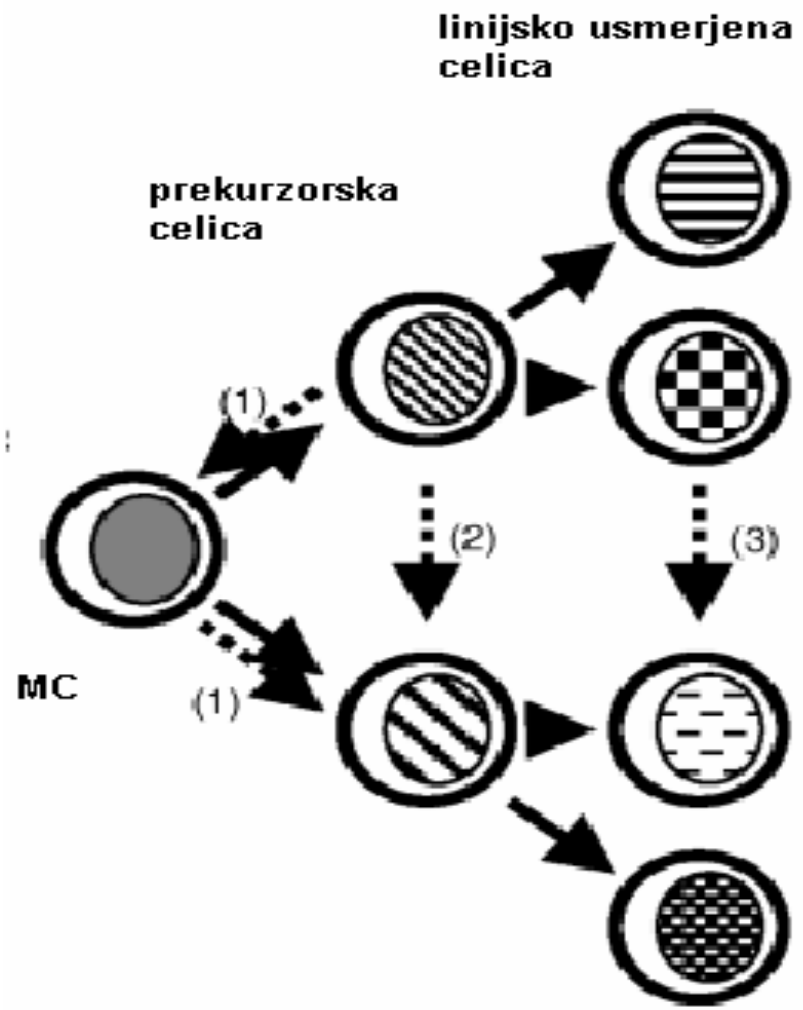
dolgotrajno gojenje

- pluripotentne MC iz popkovnične krvi

ali

- tkivne MC

dediferenciacija (1)

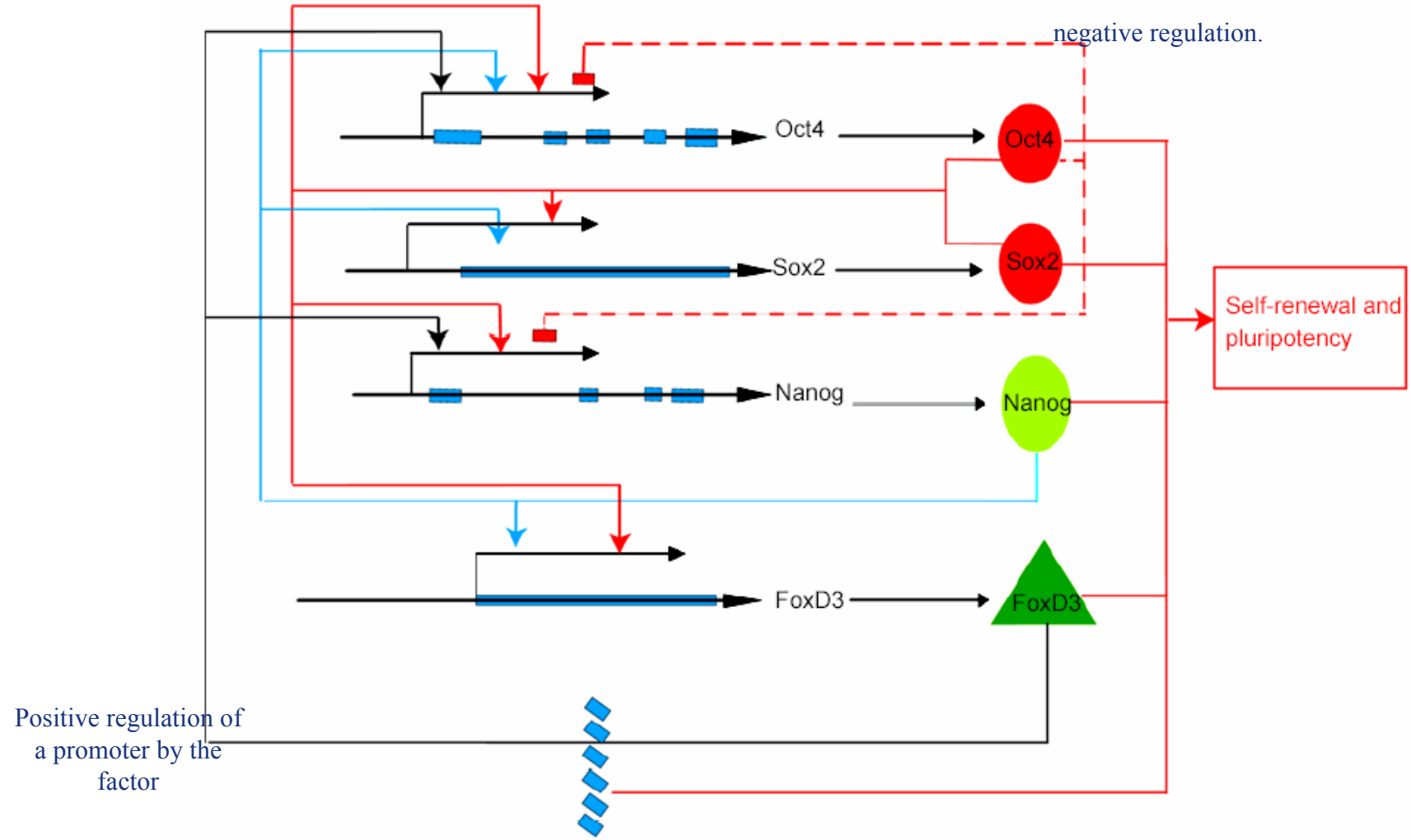


epigenetsko reprogramiranje 2 - dediferenciacija

- Transkripcijski faktorji pluripotentnosti v zgodnjem embriju (1-13 dni): OCT3/4, NANOG, REX1, GAPDHACTB, DNMT3B, SOX2
- Ti geni sodelujejo med seboj v mreži
- Regulatorna mreža transkripcije sproži ali prekine pluripotentnost/ samoobnavljanje
- Možna pozitivna in negativna regulacija teh genov

Hochedlinger K, Jaenisch R.. Nuclear reprogramming and pluripotency. Nature. 2006 Jun 29;441(7097):1061-7.

Regulatory network of key transcription factors in maintaining ES cell pluripotency and self-renewal.

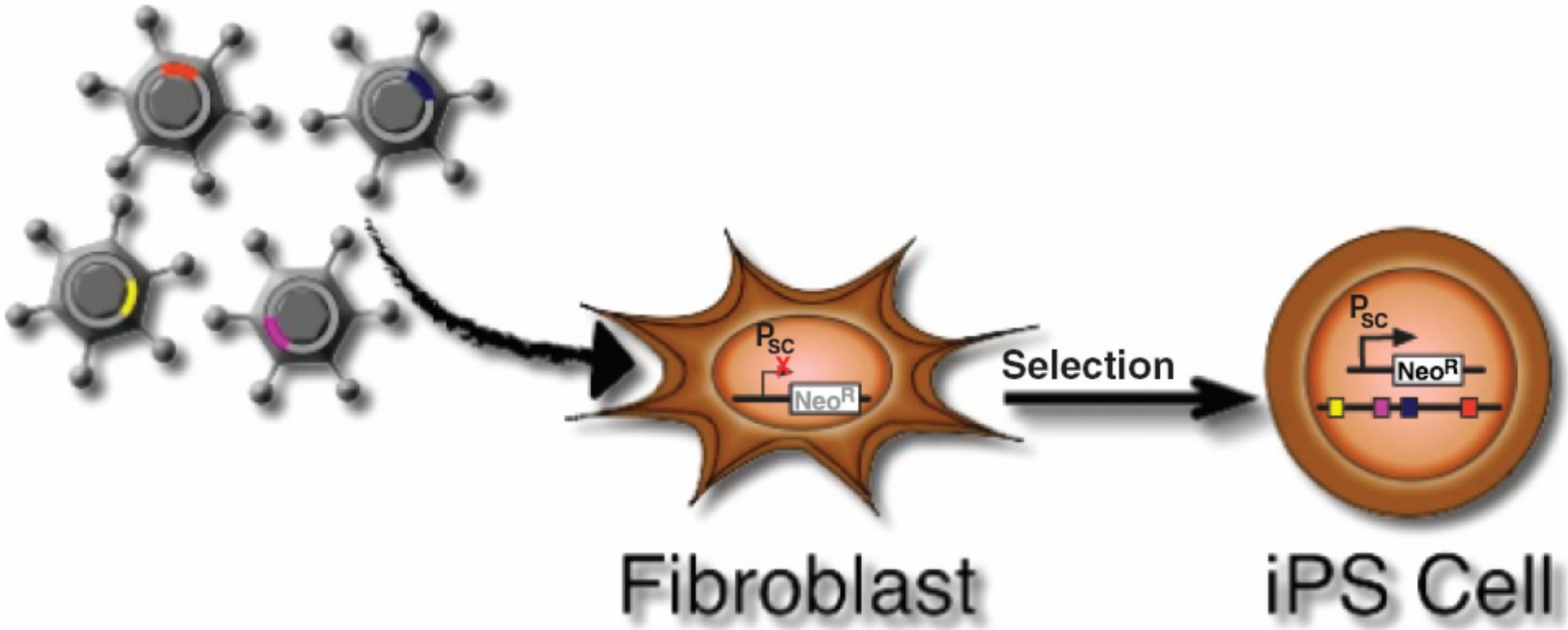


Cell

Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors

Kazutoshi Takahashi,[1] Koji Tanabe,[1] Mari Ohnuki,[1] Megumi Narita,[1,2] Tomoko Ichisaka,[1,2] Kiichiro Tomoda,[3] and Shinya Yamanaka[1,2,3,4,*]

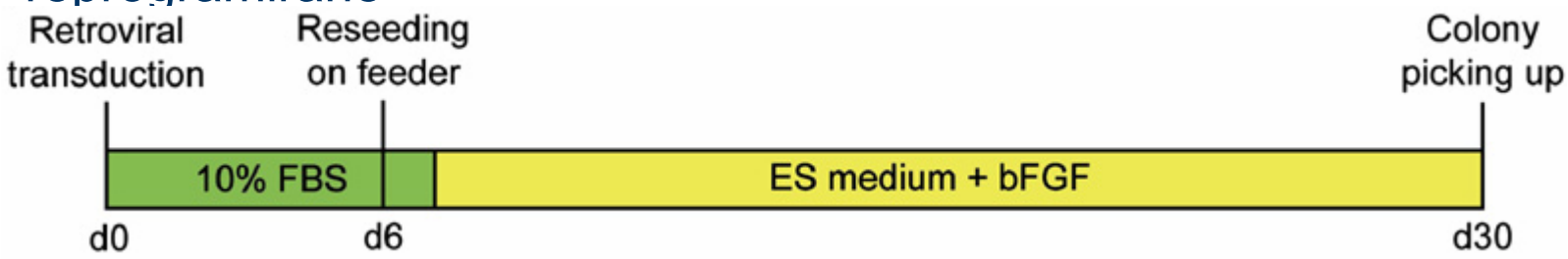
Cell *131*, 1–12, November 30, 2007 ©2007 Elsevier Inc. 1



Rodolfa et al. Defined reprogramming: a vehicle for changing the differentiated state. Differentiation (2007) 75:577–579

Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors

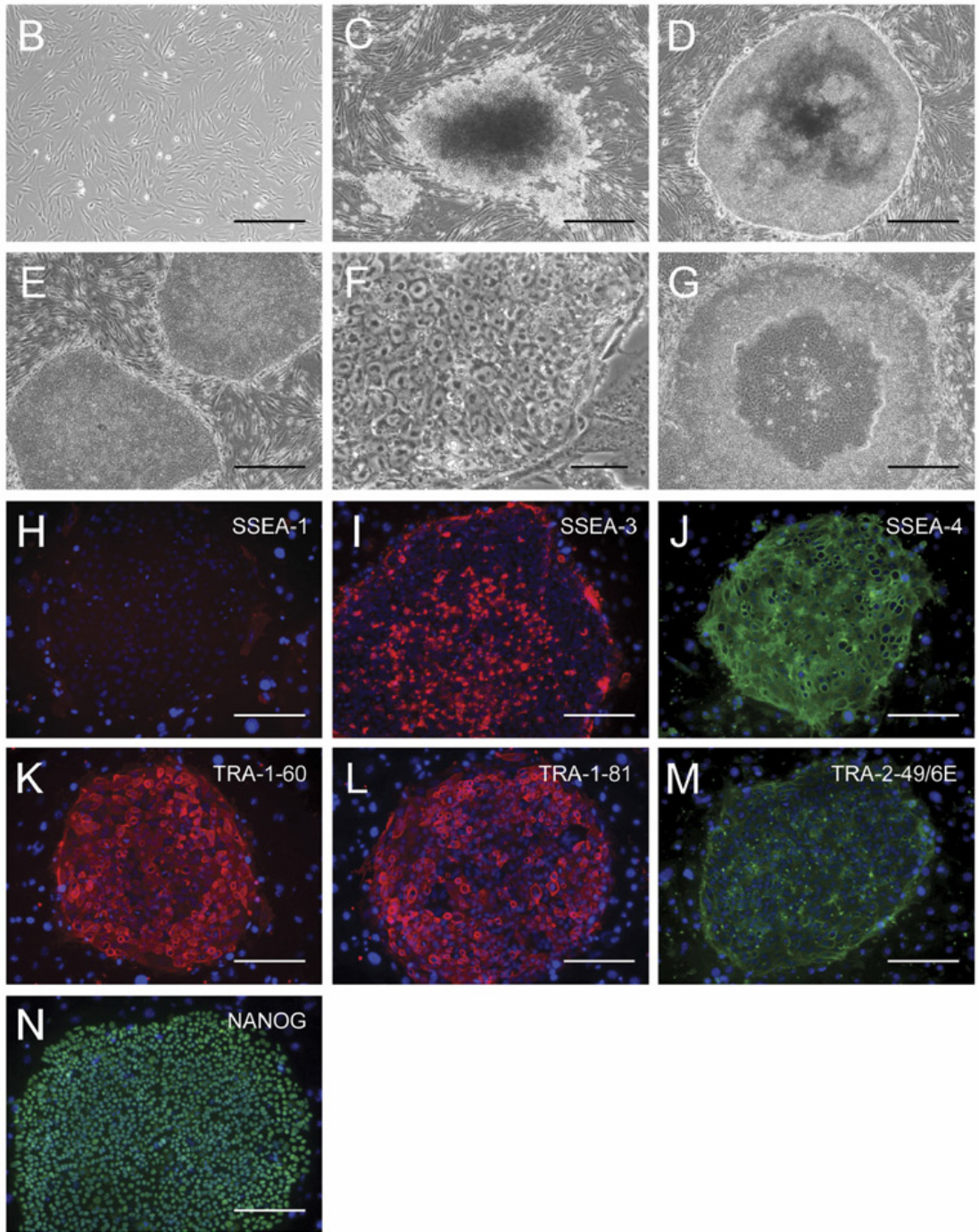
- Dediferenciacija odraslega človeškega bibroblasta (HDF)
- Uporaba samo 4 faktorjev: Oct3/4, Sox2, Klf4, c-Myc
  - Oct3/4 in Sox2 → ključna transkripcijska faktorja
  - C-Myc in Klf4 vplivata na strukturo kromatina, da se Oct3/4 in Sox2 lahko vežeta
- Vnos z retrovirusom (pLenti6/UbC-Slc7a1, lentivirus – družina HIV, SIV, FIV)
- Dobimo iPSC celice (induced pluripotent stem cells)
- Vsi štirje retrovirusi so v človeških iPS utišani – pomeni, da lastnosti celic niso odvisne od kontinuiranega izražanja transgenov, ampak so reprogramirane



Takahashi et al., Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors, Cell (2007)

iPS celice

Zaradi retroviralnih vključkov so iPS celice bolj tumorigene – potrebno iskati nevirusne metode vnosa genov.



Takahashi et al., Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors, Cell (2007), doi:10.1016/j.cell.2007.11.019

iPS celice so podobne hMEC

- Enaka morfologija
- Sposobnost proliferacije
- Izražanje neakih površinski antigenov
- Ekspresijski profil
- Epigenetski profil genov za pluripotentnost
- Visoka aktivnost telomeraze
- *In vivo* in *in vitro* sposobnost diferenciacije v celice iz vseh treh zarodnih plasti
- Vzorec metilacije – bivalentne modifikacije histonov
- Če jih vbrizgamo v blastocisto, prispevajo k himerizmu zarodka

Učinkovitost metode iPSC je slaba

- ~ 10 iPS kolonij iz 5×10^4 transduciranih fibroblastov
- To ni problem z vidika uporabnosti metode
- Z znanstvenega vidika se pojavlja nekaj pomislekov:
  - Mogoče iPS celice izhajajo iz nediferenciranih matičnih ali progenitorskih celic, ki so bile kot kontaminacija prisotne v kulturi fibroblastov.
  - Mogoče je potrebna integracija virusa na specifično mesto v genomu.
  - Za nastanek iPS so lahko potrebne majhne genetske ali epigenetske spremebe, ki jih ni mogoče detektirati s kariotipom.

7. Embrionalnim podobne celice odraslega (ESC-A)

(i) Endothelial Progenitor Cells **(EPCs)** (Asahara et al. 1997; Shi et al. 1998),

(ii) Mesenchymal Stem Cells **(MSCs)** (Peister et al. 2004; Prockop et al. 1997),

(iii) Multipotent Adult Progenitor Cells **(MAPCs)** (Jiang et al. 2002)

(iv) Marrow-isolated Adult Multilineage Inducible **(MIAMI)** cells (D'Ippolito et al. 2004).

(v) McGuckin et al. 2005 - Cell Prolif. cord blood-derived embryonic-like stem cells **(CBE)**

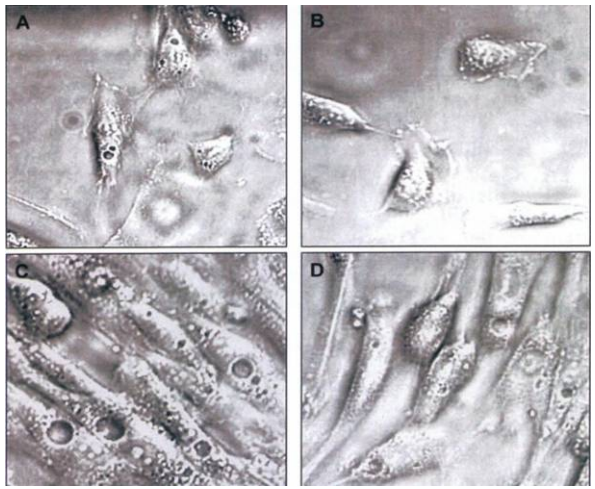
(vi) Precursors of germ cells (oocytes and spermatogonial cells) in BM (Johnson et al. 2005; Nayernia et al. 2006).

(vii) Very Small Embryonic-like Stem Cells **(VSELs)** (Kucia et al. 2006)

(viii) Embryonic Stem Cells of an Adult **(ESC-A)** Virant Klun et al. 2008

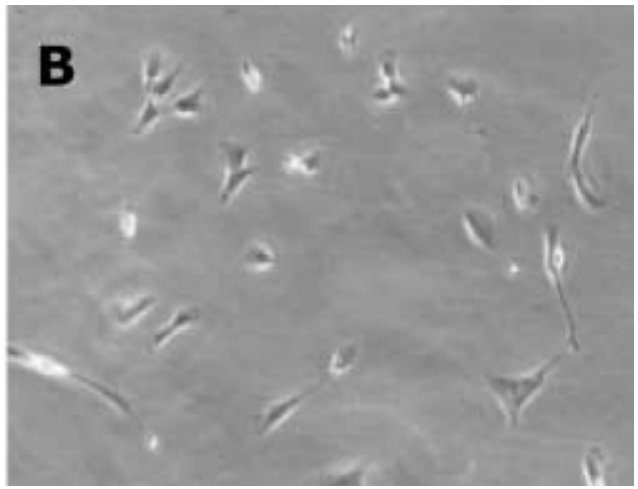
PRI ODRASLEM ČLOVEKU SO ODKRILI RAZLIČNE TIPE PLURIPOTENTNIH CELIC

MAPC



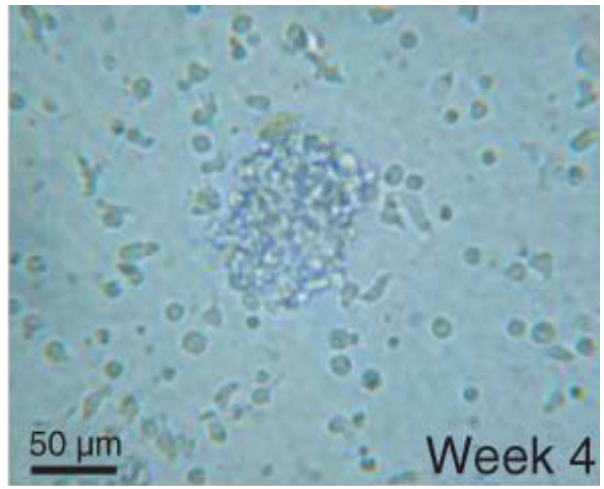
(Reyes in sod., 2001)

MIAMI (7-10 µm)



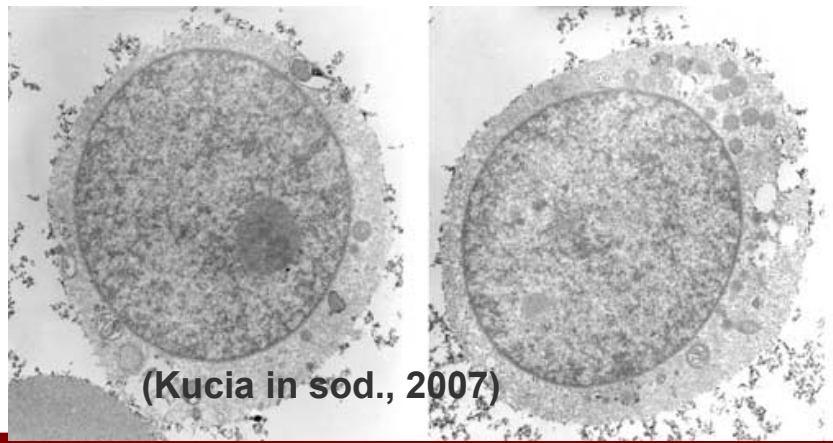
(D'Ippolito in sod., 2004)

CBE



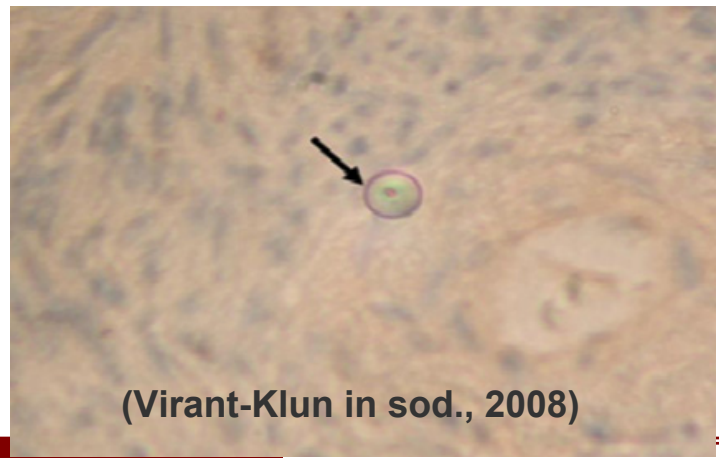
(McGuckin in sod., 2005)

VSEL (3 - 5 µm)



(Kucia in sod., 2007)

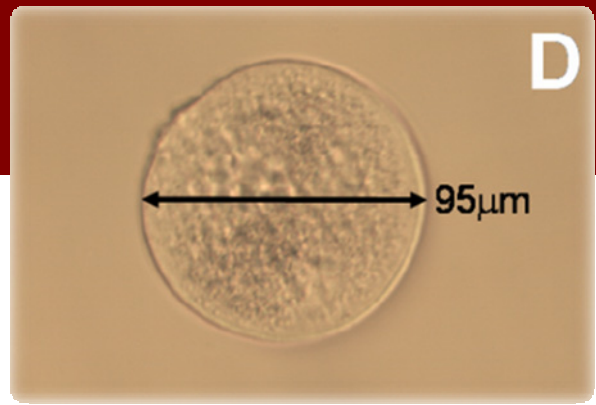
ESC-A (2 - 4 µm)

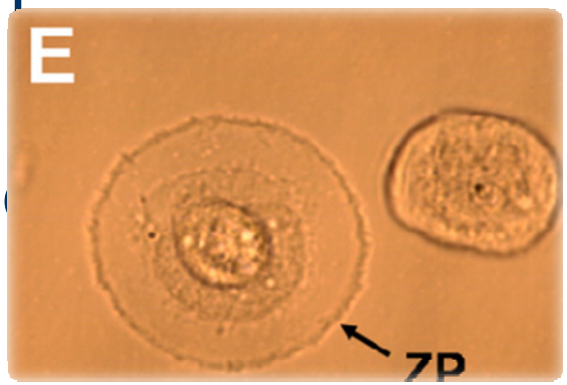


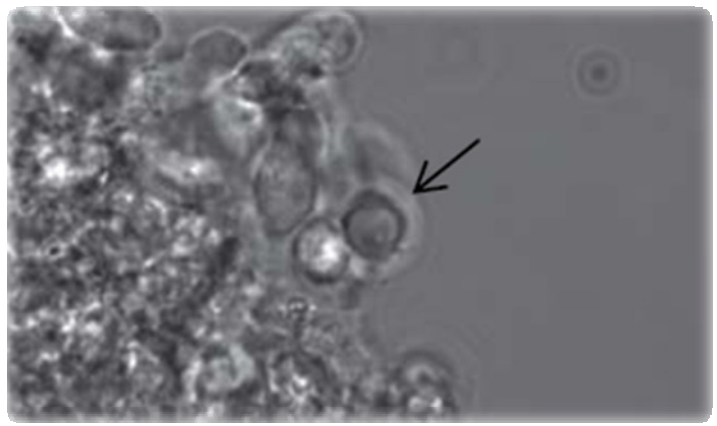
(Virant-Klun in sod., 2008)

CELICE ESC-A SO TUDI V HUMANEM OVARIJU

- Majhne 2-4 µm, okrogle
- Sposobne diferenciacije v oocitom podobne celice
- Rumenkaste barve (“golden nugg
- Embrionalni markerji:
  - SSEA-4
  - Oct-4
  - Nanog
  - Sox2
  - c-kit

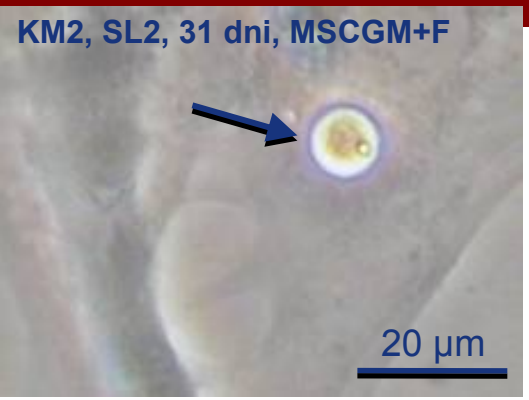


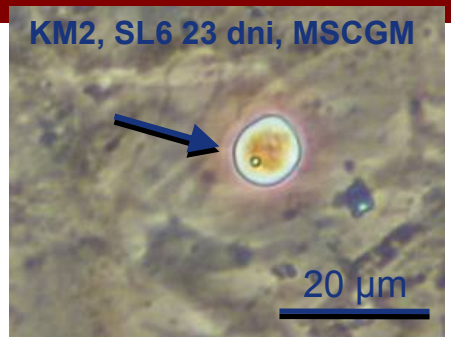


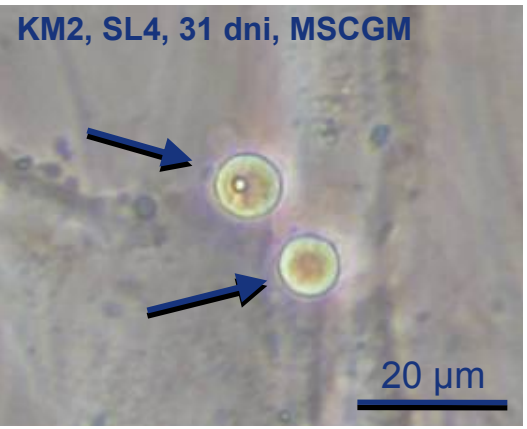


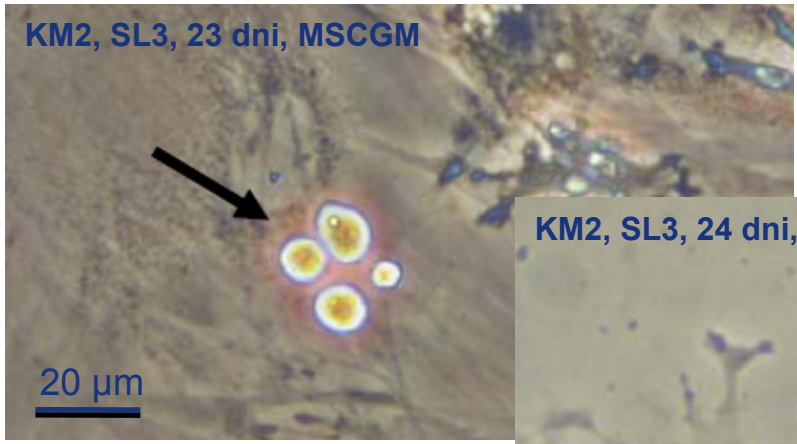
(Virant-Klun in sod., 2008)

ZGODNJE MATIČNE CELICE SMO ODKRILI V VSEH SLOJIH KM



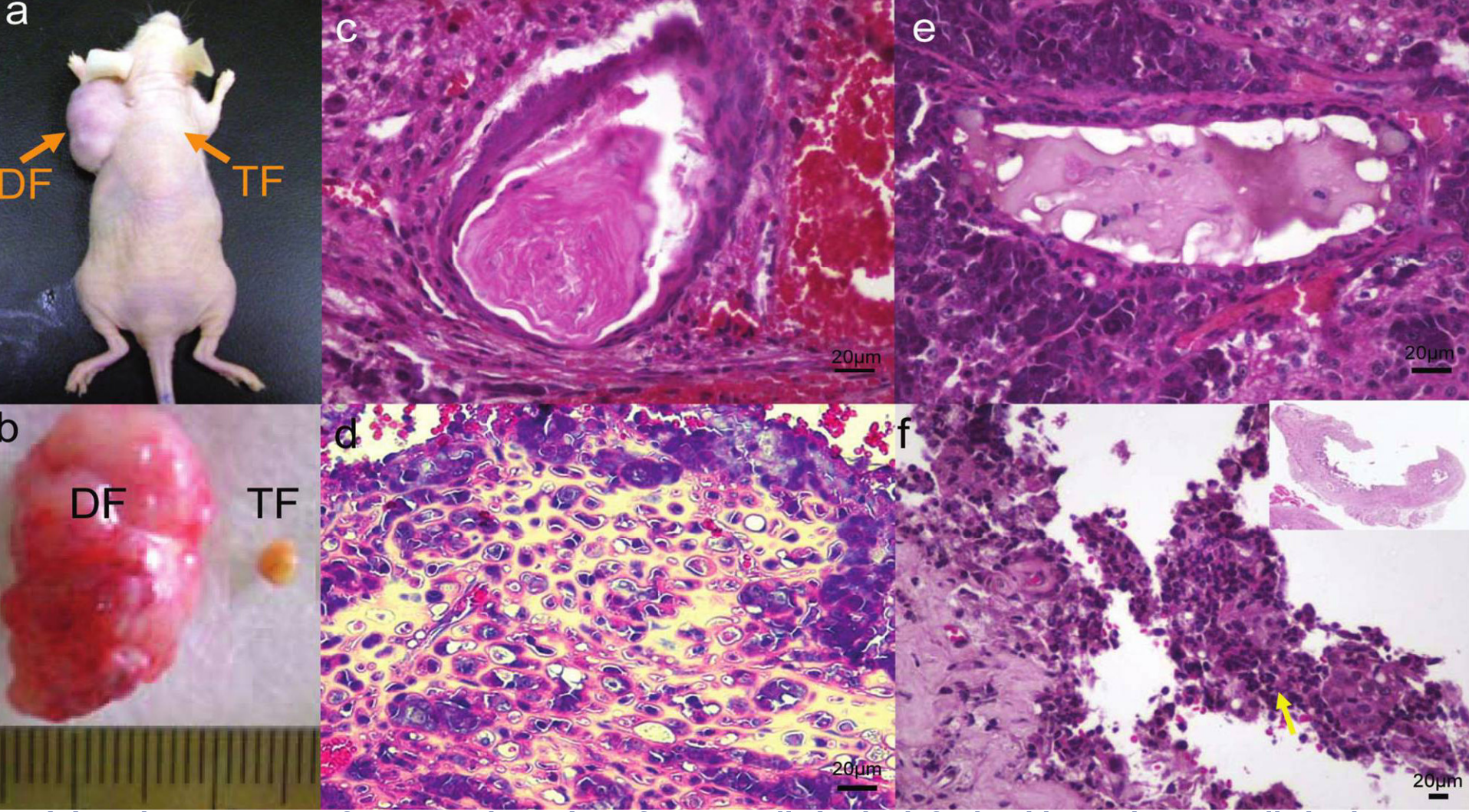






NEVARNOSTI UPORABE EMC

- Neprimerna fenotipska diferenciacija
- Vplivi medija za gojenje
- Nediferencirane EMC povzročajo teratome
- Lobstein (1829), Virchow (1853), Cohnheim (1889):
  - TU kosti izvirajo iz hrustančnih celic rastne ploščice
  - embrionalni ostanki generirajo vse oblike raka



(a) Nude mouse 5w after transplantation of ES-DF cells in the left shoulder and ES-TF cells in the right shoulder. The animal was treated with ganciclovir (GCV) from w2-w5.
(b) Teratomas derived from ES-DF and ES-TF cells showing the marked discrepancy in size and vascularity after GCV treatment. Hematoxylin and eosin (H&E) staining of the teratoma from ES-DF cells showing: (c) squamous cell differentiation with keratin pearl (400), (d) osteoid (nonmineralized bone) formation (400), and (e) respiratory epithelium with ciliated columnar and mucin-producing goblet cells (400). (f) In contrast, H&E staining of the teratoma from ES-TF cells after GCV (inset) treatment showed massive necrosis in the center (arrow) (200)

ESC lines: some have signs of neoplastic progression

- Expressing pluripotency markers at high levels
- High proliferative capacity
- Growth factor independence
- Increase of frequency of tumor initiating cells
- Niche independence
- Chrosmosomal abnormalities as revealed by genomic hybridisation techniques (not by standard cytogenetics)

hESC – razvoj v krvne celice

- Eritroidne celice (Epo + Kit ligand)
- Megakariociti (OP9 stroma + Tpo)
- Granulociti (OP9 stroma + citokini)
- Mastociti (IL3 + Kit ligand)
- Eozinofilci (OP9 stroma + IL5 ali IL3
- B Ly (OP9 stroma + IL7)
- T Ly (OP9 stroma + kultura priželjca)
- Makrofagi: (EB ali OP9 kultura)
- Dendritske celice: (IL3 + GM-CSF + TNF alfa + IL4 . ..)
- NK celice
- Osteoklasti: M-CSF + RANKL

V PRIHODNOSTI: