Cloning in eukaryotes: cell culture and stem cells

1) Animal cell culture

2) Stem cells: easy genetic manipulation, and capable of differentiation into other cell types

3) ES cells derived from Somatic Cell Nuclear Transfer (SCNT)

4) Induced pluripotent stem (iPS) cells, reprogrammed by directed gene expression

Guide to readings:

1)ATCC Animal Cell Culture Guide. Basic guide for animal cell culture from the American Type Culture Collection

2)Stem Cell Basics 2020. Guide to stem cell biology from the National Institutes of Health

3) Review: Pluripotent stem cells 2010. Methods for getting pluripotent stem cells, and the clinical implications.

4)ES cells from SCNT 2013. Perspective on pioneering human cloned stem cell work, done at OHSU.

5) *Stem cell comparison 2014.* Comparison between methods of stem cell reprogramming.

6) iPS and organoids in disease modeling 2019.

Gene transfer to animal cells

- Studying complex eukaryotes
  - Work with isolated cells to simplify cell biology studies
  - Human cell lines allow convenient studies of basic human cell & molecular biology

- Cells in culture can be used to establish conditions for gene therapy – treatment of genetic disorders by restoration of gene function

- Large scale animal cell culture can be useful in production of recombinant animal proteins: accurate post-translational modifications

Growth of mammalian cells: tissue culture

Culture media contains

- Glucose, amino acids, vitamins
- Salts/buffers
- antibiotics (anti-bacterial, anti-fungal)
- whole serum (undefined, provides necessary growth factors and other macromolecules)
- Or, specific growth factors, plus transferrin, and insulin (if no serum is added)

Grow at 37°C, in 5% CO_2

- Some cell types require a “feeder layer” of other, non-dividing cells (DNA-damaged to prevent cell division). Feeder cells provide a surface for growth, as well as some nutrients

Mammalian cell culture: 3 Types

1. Primary cell culture/finite cell line/cell strain, not derived from tumors
    a. Released from tissues by enzymatic digestion (which breaks up the extracellular connections)
    b. Limited number of cell divisions: 40 to 60 divisions, the "Hayflick Limit", followed by senescence

2. Continuous cell line, 'transformed', may be from a tumor
    a. Earliest example: HeLa (also, lots of others exist)
    b. Unlimited number of cell divisions: "immortalized"

3. Embryonic/pluripotent stem cells
    a. Derived from early embryo (blastocyst) or de-programmed adult cell
    b. Undefined cell type, but changes can be induced
    c. Potential for unlimited number of cell divisions

Biology of cells in culture

1. Primary cultures, non-tumor derived
    a. Essentially normal biology
    b. New cultures must constantly be made

2. Tumor cell lines
    a. Unlimited proliferation (not normal biology, in that respect)
    b. Mutations accumulate over time – how does the biology compare to non-tumor cells?

3. Embryonic stem cells
    a. Normal biology -- differentiation into various cell/tissue types can be induced
    b. Cells may be engineered, eg. to correct genetic defect

HeLa cells: the first human tumor cell line

- Henrietta Lacks was an African American woman, and a cancer patient at the Johns Hopkins Hospital in Baltimore. She died from the disease in 1951, at the age of 31
- A tissue sample from her cervical tumor was given to cell biologist George Gey, *without patient or family consent*
- Gey was able to produce the first immortalized tumor cell line from this sample, and shared the HeLa cell culture with labs worldwide
- HeLa cells became one of the central model systems for human cell and molecular biology

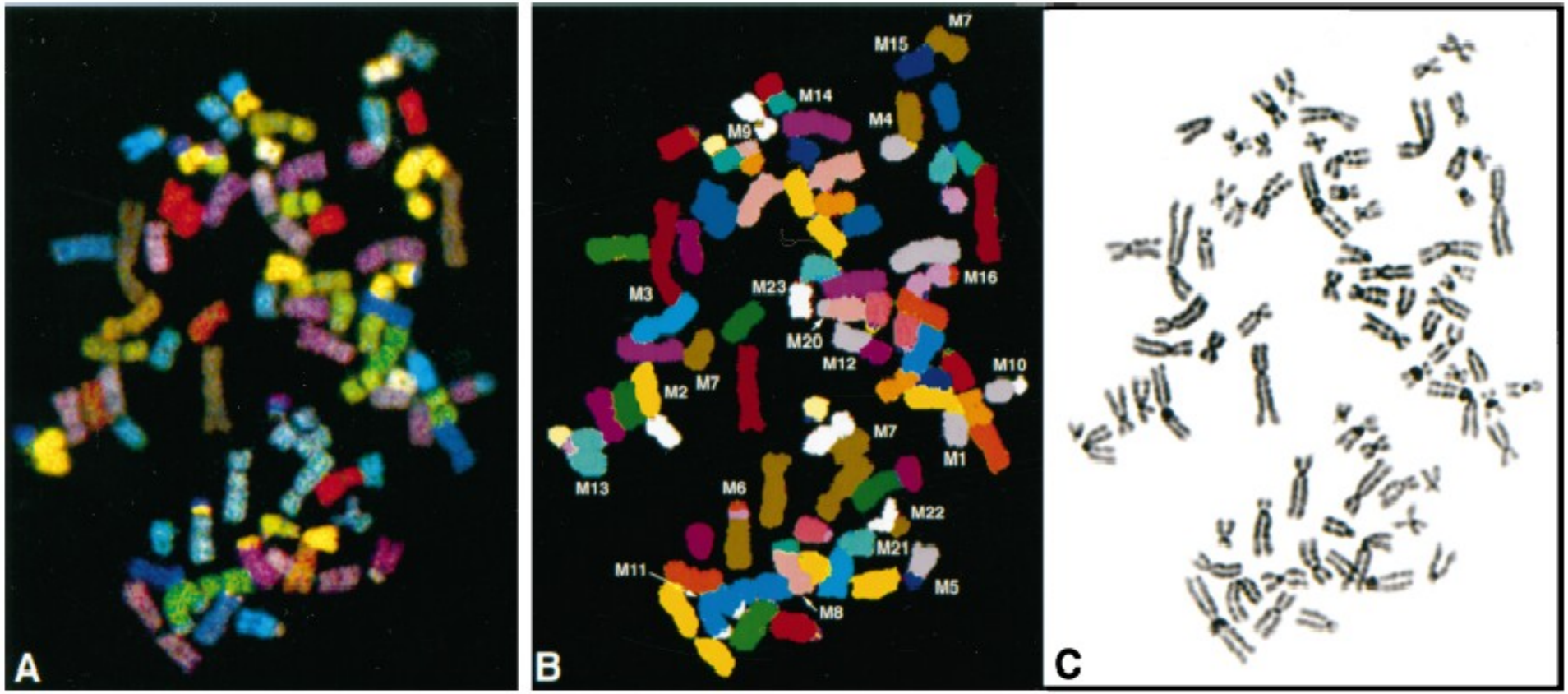
- Henrietta Lacks' surviving family members were unaware of the fate of her cells until more than 20 years later
- Her family has only recently received acknowledgment of ethical lapses surrounding the use of her cells for so long, and in many cases for profit

Proposed research & consent policy changes inspired by the case of Henrietta Lacks and her cells

- Acknowledge whom HeLa cells came from, that she was an African American woman, with a family and her own story
- Require consent for use of cells in research, even if those cells are 'de-identified'
- Acknowledge and undo disparities that exist in basic research, particularly as affected by past and current systemic racism
- Offer financial compensation to help make amends for past injustices

- See editorial "Henrietta Lacks: science must right a historical wrong"

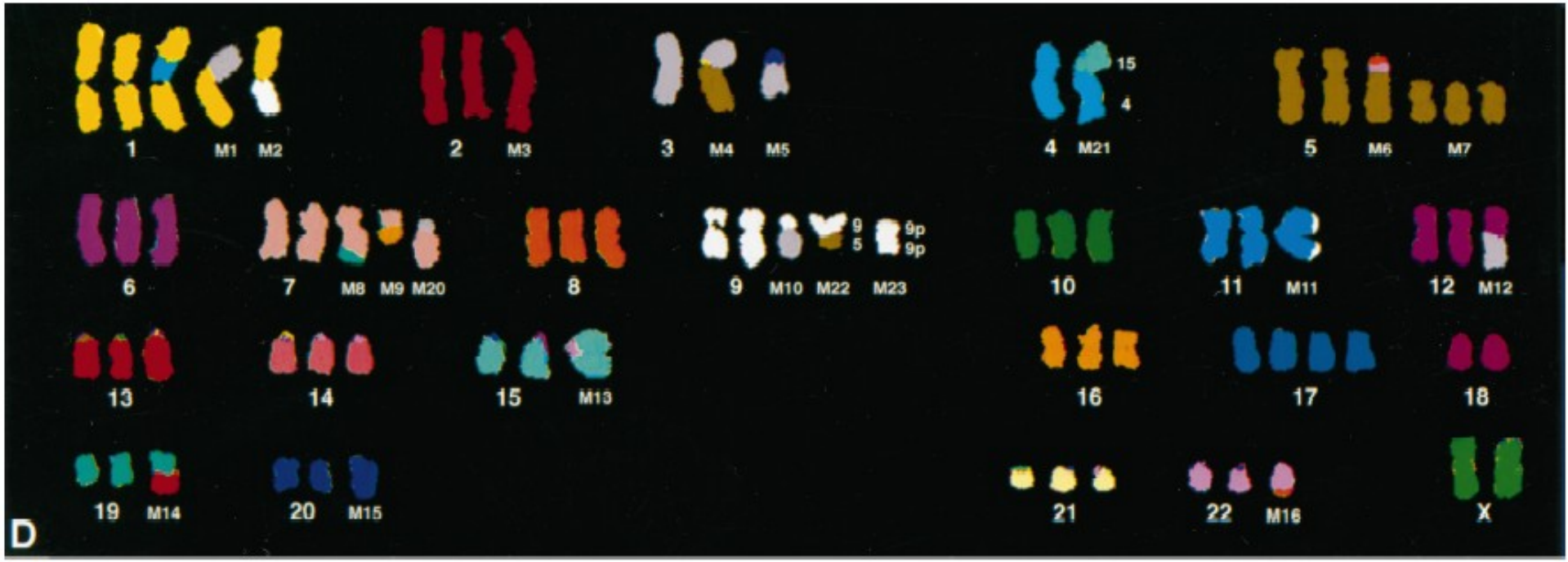
Tumor cell line biology: HeLa chromosomes are very different from those in normal human cells



"spectral karyotype" (SKY)
Helps to identify chromosomes

DAPI banding
(general fluorescent stain)

HeLa: chromosome number and forms are altered



Many chromosomes duplicated or rearranged, & numerous "derivative" chromosomes present

[CANCER RESEARCH 59, 141–150, January 1, 1999]

Comprehensive and Definitive Molecular Cytogenetic Characterization of HeLa Cells by Spectral Karyotyping

Merryn Macville,[1] Evelin Schröck, Hesed Padilla-Nash, Catherine Keck, B. Michael Ghadimi,[2] Drazen Zimonjic, Nicholas Popescu, and Thomas Ried[3]

Genome was sequenced in 2013:

- confirms gene duplications, rearrangement, & mutations
- Altered gene regulation leads to questions re. general relevance of biology to normal human cells

Stem cells: what they are

- Unspecialized, undifferentiated cells
- " Renewable" through cell divisions, capable of dividing many times
- Can be induced to differentiate into specialized cell types, e.g. cardiac, neural, skin, etc.
- http://stemcells.nih.gov/

Three types:

  - Embryonic stem (ES) cells: from embryos, pluripotent (giving rise to any cell type), also totipotent? (able to develop into a new individual organism?)
  - Adult stem (AS) cells: from adult tissues, multipotent (giving rise to specific cell types)
  - Induced pluripotent stem (iPS) cells: somatic cells reverted to an undifferentiated, pluripotent state

Kinds of stem cells

Totipotent: Able to give rise to all the cell types of the body, plus all of the cell types that make up the extra-embryonic tissues such as the placenta. Only zygote → ~4 cell stage cells are totipotent

Pluripotent: Able to give rise to all of the various cell types of the body. Pluripotent cells cannot
make extra-embryonic tissues such as the amnion, chorion, and other components of the placenta. <blastocyst inner cell mass>

Multipotent: Able to develop into more than one cell type of the body. <eg: hematopoeitic stem cells>

Why stem cells are important

Medicine

- ES and iPS cells are pluripotent, and could be used to produce new tissues for “ regenerative” medicine
- Cloned ES and iPS cells could be used to generate engineered cells and tissues that could be modified by gene therapy and not rejected by the recipient
- ES- and iPS-derived cells can be induced to grow ‘organoids’

Basic science

- How do stem cells remain unspecialized in culture?
- What are the signals that cause specialization in stem cells, and how do these signals function?
- Stem cell development could provide models for human tissue development (and developmental disease)

Where are stem cells found?

- ES cells: from inner cell mass of early (3 to 5 day old) embryo
  - human ES cells first cultured in 1998, using donated embryos (with consent) created for fertility purposes

- Reprogrammed somatic cells
  - ES cells from cloned somatic cells (SCNT)
  - Cell fusion: somatic cell with ES cell
  - induced pluripotent stem (iPS) cells

- AS cells: from adult tissues

How do you know if you have ES/iPS cells?

1) Growth capacity: ES cells are capable of lots of cell divisions in culture without differentiation
2) Cell-type " markers" tell you what kind of a cell you have: Oct-4 protein expression is high in ES cells but not in differentiated cells
3) Chromosomes should be normal: Check the karyotype (many immortalized cell lines are cancer-derived, and often have abnormal karyotypes)
4) The cells must be differentiatable
   A) Allow natural differentiation
   B) Induce differentiation
   C) Check for teratoma formation in SCID mice
      (Teratoma: tumor containing all three germ layers)
      (SCID: Severe combined immunodeficiency)

Federal regulations involving human ES cells

1998: The first human ES cell lines were derived.
www.sciencemag.org/content/282/5391/1145

2001: US President (George W. Bush) restricts federally funded research on ES cell lines. Only cell lines made prior to 2001 could be used in federally funded research. Non-federally funded research is exempt from this regulation.

2004: California sets up California Institute for Regenerative Medicine http://www.cirm.ca.gov/about-cirm/our-history

2009: US President (Barack Obama) revokes the 2001 Bush policy, allowing federal funding for creation and study of ethically derived, new human ES cell lines.
https://stemcells.nih.gov/policy/2009-guidelines.htm
http://www.nature.com/news/2009/090309/full/458130a.html

Alternatives to embryos for "ES-like" cells?

Some adult stem cells are multipotent (& possibly pluripotent?)

1) hematopoeitic: → develop into all types of blood cells
2) bone marrow stromal cells: → bone, cartilage, connective tissue, fat cells
3) neural: → brain and nerve cells
4) epithelial: → cell types lining digestive tract
5) skin: → epidermis, follicles
6) germ-line: → sperm, eggs

Some of these stem cell types can do more: brain stem cells have been seen to differentiate into some types of blood or skeletal muscle cells

AS versus ES cell: important differences

• AS cells are hard to find, generally difficult to isolate, cannot yet be cultured efficiently

• AS cells are generally limited to producing cells from the tissue type that they are found in (multipotent)

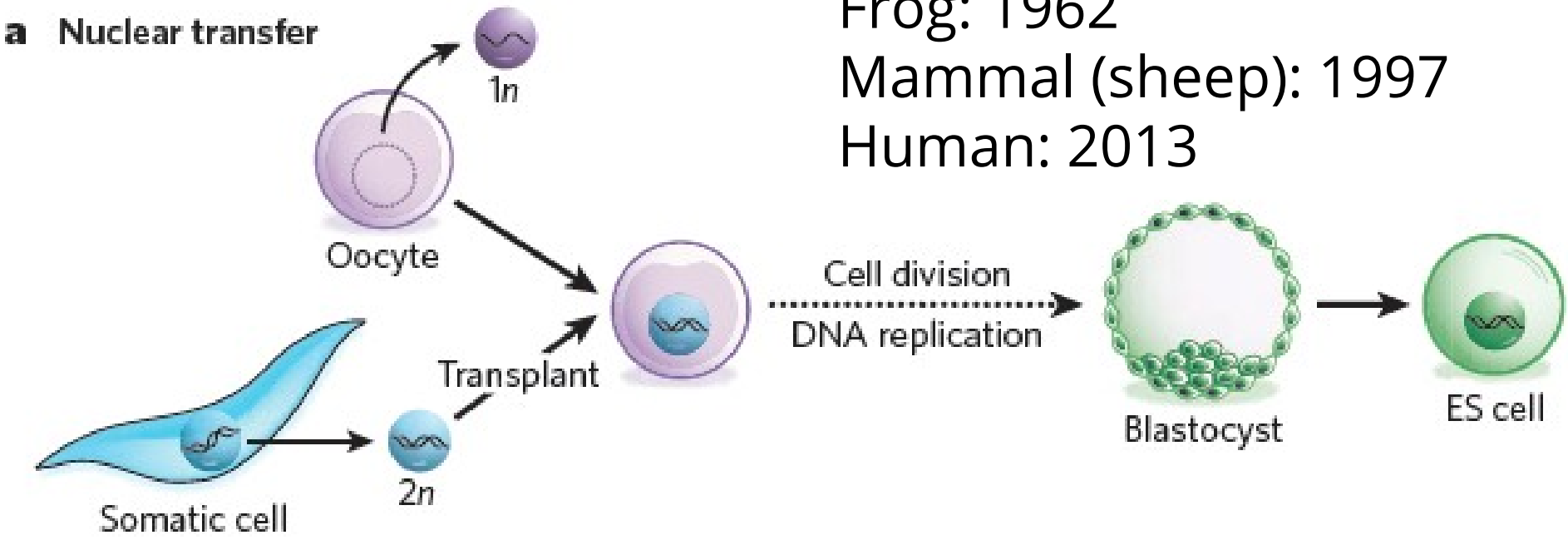
• ES cells divide a lot in culture (easy to manipulate and propagate)

• ES cells are pluripotent

Somatic cell nuclear transfer (SCNT): a route to patient-derived ES cells

- Human egg (donated, ethical issue) has its haploid genome removed, and provides cytoplasm with reprogramming factors
- Adult, differentiated cell provides diploid nucleus
- Diploid egg begins to divide, forming an embryo
- The embryo develops to blastocyst stage. ES cells are taken from the inner cell mass, destroying the clone embryo (ethical issue)
- Could embryo develop to become viable human? (ethical issue)

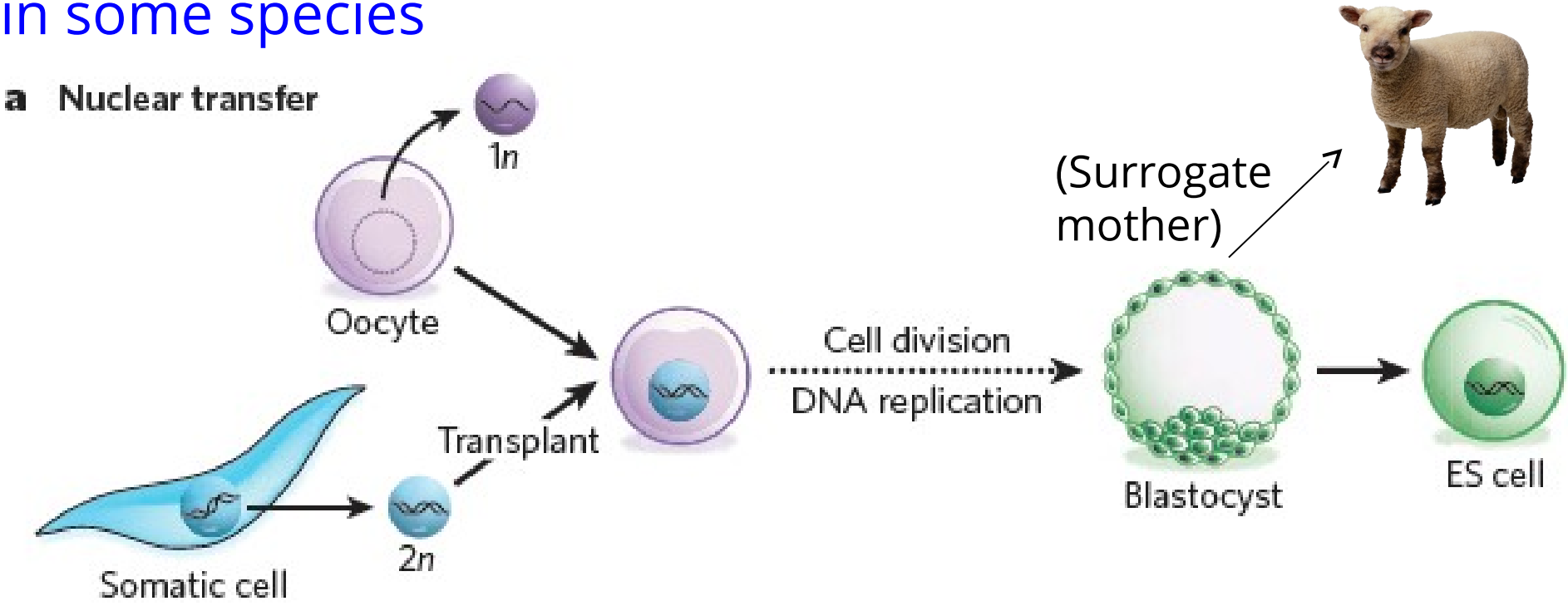
Reprogramming for pluripotency: Somatic cell nuclear transfer (SCNT)



Frog: 1962
Mammal (sheep): 1997
Human: 2013

Recipient cell:
- can be oocyte (n), or fertilized egg (2n)
- Recipient nucleus contains reprogramming factors
- condensed chromosomes and spindle are removed from recipient cells at a stage of the cell cycle when nuclear membrane is gone (leaves the normally nuclear reprogramming factors for the somatic cell nucleus)

SCNT blastocyst can develop to become cloned organism in some species



Cloned animals may show abnormalities:

- altered gene expression in embryo
- elongated telomeres
- impaired immune system
- tendency to obesity
- increased cancer rates

Presumably because of failure to erase “epigenetic memory”

2013

Human Embryonic Stem Cells Derived by Somatic Cell Nuclear Transfer

Masahito Tachibana,[1] Paula Amato,[2] Michelle Sparman,[1] Nuria Marti Gutierrez,[1] Rebecca Tippner-Hedges,[1] Hong Ma,[1] Eunju Kang,[1] Alimujiang Fulati,[1] Hyo-Sang Lee,[1,6] Hathaitip Sritanaudomchai,[3] Keith Masterson,[2] Janine Larson,[2] Deborah Eaton,[2] Karen Sadler-Fredd,[2] David Battaglia,[2] David Lee,[2] Diana Wu,[2] Jeffrey Jensen,[1,4] Phillip Patton,[2] Sumita Gokhale,[5] Richard L. Stouffer,[1,2] Don Wolf,[1] and Shoukhrat Mitalipov[1,2,*]

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http://dx.doi.org/10.1016/j.cell.2013.05.006

Somatic cell nuclear transfer (SCNT) 2013

- Somatic cells (fetal fibroblasts) provided the diploid nuclei
- Donated oocytes had spindle/condensed chromosomes removed in the presence of caffeine (helps maintain meiotic arrest)
- Fibroblasts were fused with oocyte
- Cells were activated with electroporation, and then grown to early embryo stage on feeder layer cells
- Inner cell mass removed and cells cultured
- Colonies resembling ES cells were collected for analysis
- Efficiency of ES cell production high (about 1 in 10 SCNT

Conclusions:

Human ES cells can be derived by SCNT (cloning)

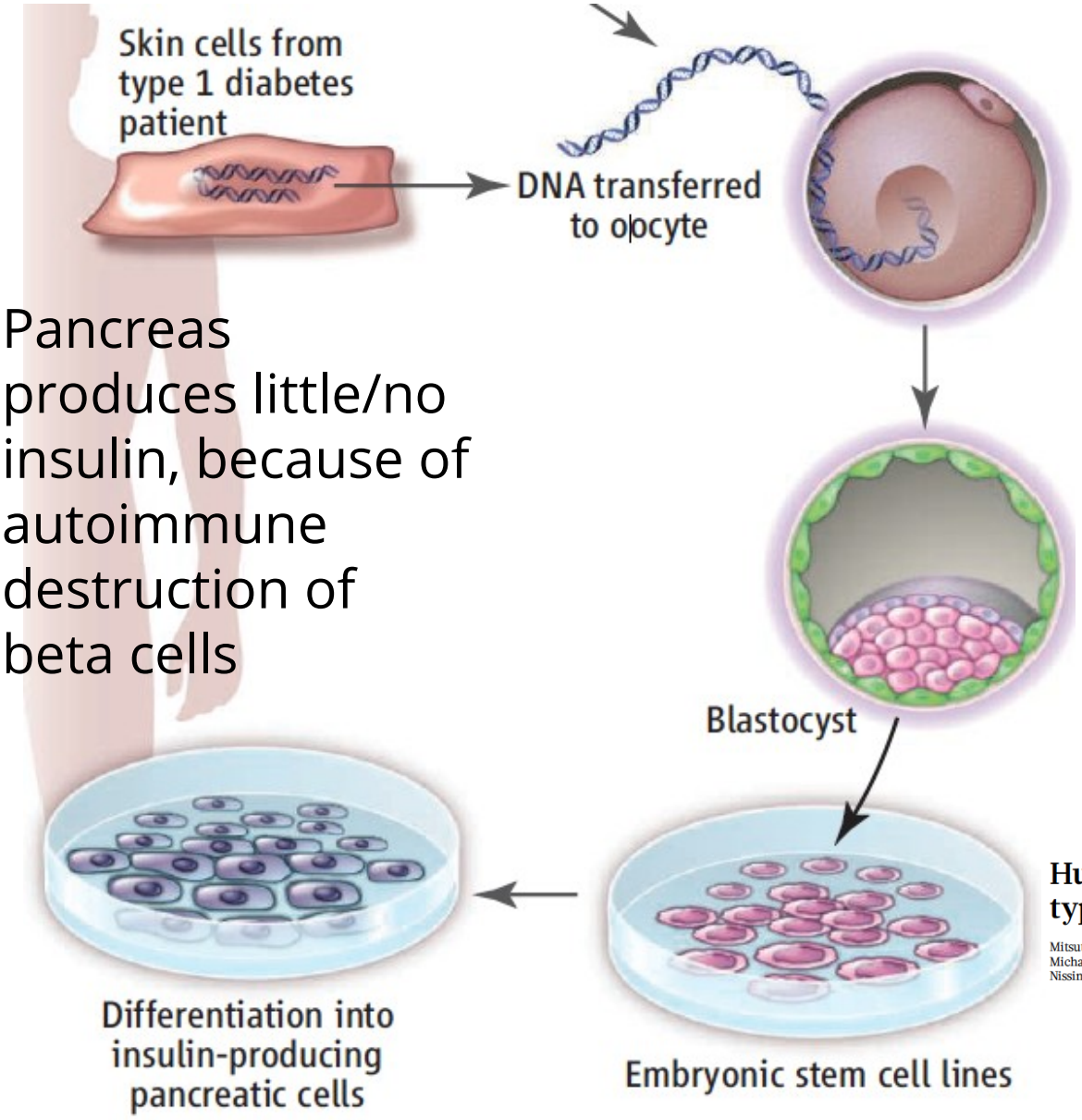
- cells display ES cell markers
- cells can form teratomas
- normal karyotype
- genotypes as expected from the fusion

The process is fairly efficient

* *How do these cells compare with iPS cells?*

Applications for human SCNT-derived ES cells

Example: patient-derived therapy for type I diabetes



Pancreas produces little/no insulin, because of autoimmune destruction of beta cells

Adult cells provide the nucleus, allows to make more beta cells

In mice: new beta cells make insulin, respond to glucose levels

How well might it work in humans?

Human oocytes reprogram adult somatic nuclei of a type 1 diabetic to diploid pluripotent stem cells

Mitsutoshi Yamada[1]*, Bjarki Johannesson[1]*, Ido Sagi[2], Lisa Cole Burnett[3], Daniel H. Kort[4,5], Robert W. Prosser[4,5], Daniel Paull[1], Michael W. Nestor[1], Matthew Freeby[3], Ellen Greenberg[3], Robin S. Goland[3], Rudolph L. Leibel[3], Susan L. Solomon[1], Nissim Benvenisty[2], Mark V. Sauer[4,5] & Dieter Egli[1]

doi:10.1038/nature13287

Ethical issue:
what about the oocytes/eggs required for somatic cell nuclear transfer?

- Availability of human eggs is limited
- Egg donation is a medical procedure
- Should egg donors be paid? How much?
- Can human eggs be produced by animal chimeras?

Other things to consider:

-- SCNT ES cell derivation requires destruction of an embryo (a clone embryo, but still, a theoretically viable human embryo)

-- Are cloned ES cells totipotent (giving rise to a whole person)?

-- What if someone attempts to clone humans? Lots of ethical problems arise

- What's the success rate of the process?
- Who raises the cloned child?
- Would a cloned person be able live a normal life, or would there be health complications derived from the reprogramming/embryogenesis process?
- What's the relationship between a clone and its original?
- …

Another method for reprogramming somatic cells to ES cell-like state

" Induced pluripotent stem cells" (iPSCs)

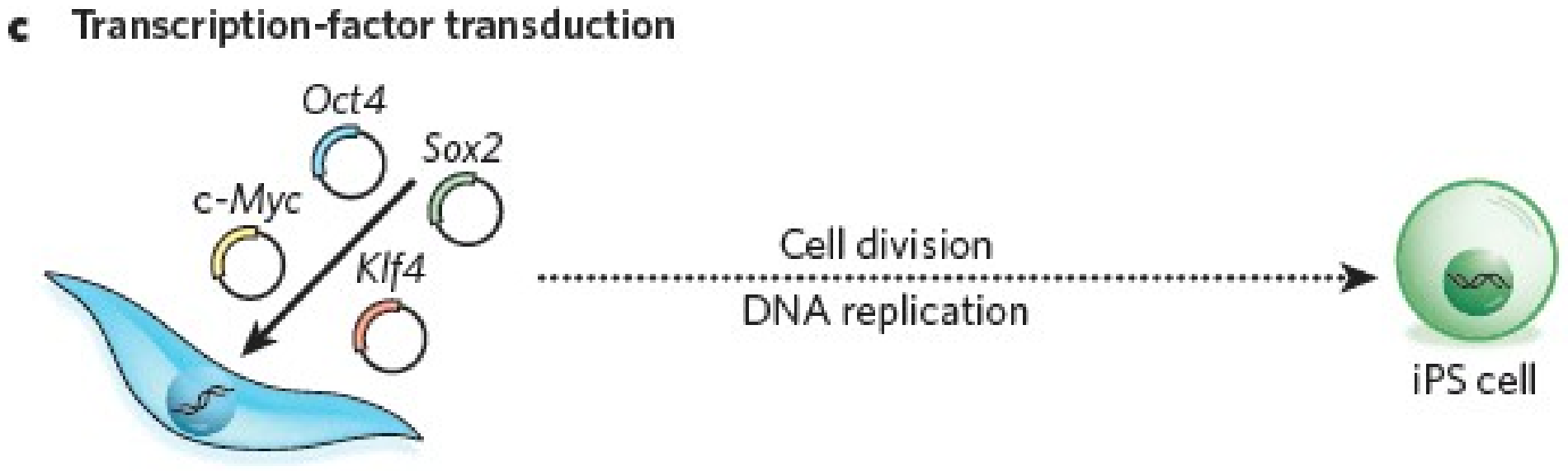
In SCNT, mammalian differentiated cells can be reprogrammed to an undifferentiated state by factors present in oocyte, but what are those factors?

Mouse cells can be converted to iPSCs by the action of four transcription factors:

Oct4
Sox2
c-myc
Klf4

(Takahashi and Yamanaka, Cell. 2006 Aug 25;126(4):663-76.)

Transcription factor induction of iPS cells from adult somatic cells



- Four transcription factors: Oct4, Sox2, c-Myc,& Klf4 are sufficient to reprogram to an ES-cell like state
- Other factors can substitute
- Efficiency of reprogramming is low
- There may be a stochastic (random) element to the reprogramming process (in addition to the factors)

Some issues with iPSCs

- Reprogramming can be inefficient
- Reprogrammed cells can accumulate mutations during the reprogramming process, show signs of premature aging
- Reduced differentiation into some cell types, eg. neural or heart tissue (probably from‘ epigenetic memory’)
- iPS cells can cause immune response when returned to same mouse from which cells originally came from

How do ESCs, NT ESCs, and iPSCs compare?

- Studies have directly compared:
  - Gene expression patterns
  - Epigenetic modifications (methylation)
  - Mutations

- Does it matter if a nucleus is reprogrammed by an egg cell, or whether it is reprogrammed by individual genes?

- Which method is more promising for production of patient-derived stem cells?

How do ESCs, NT ESCs, and iPSCs compare?

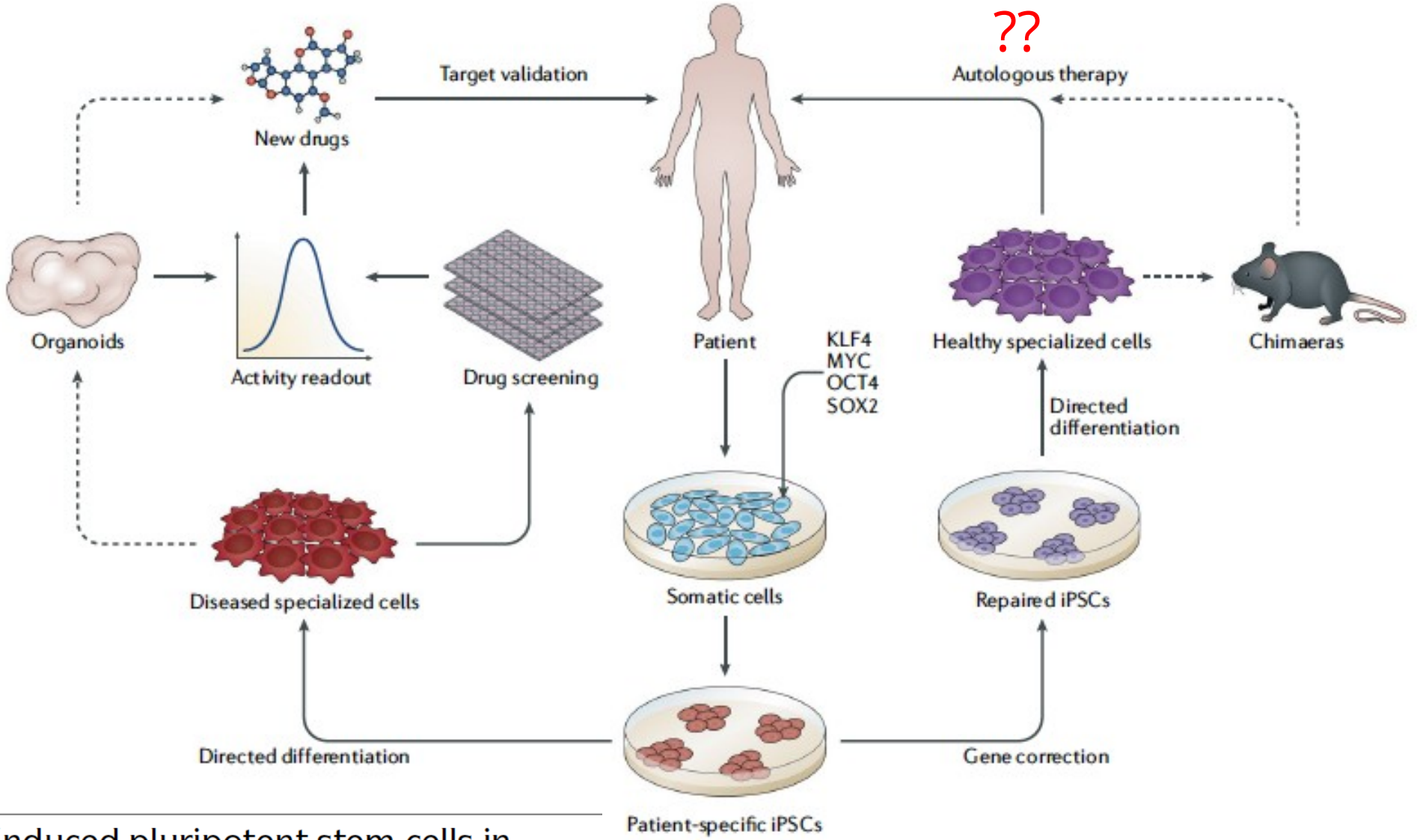
- Johannesson et al. (Egli, also Mitalipov) 2014
  - Cell type used for conversion: adult and neonatal human fibroblasts
  - iPSC conversion method: transient RNA transfection

- The number of methylation, mutation, and gene expression changes is very similar between these iPSCs and NT-ESCs, with both having more than ESCs

- The methylation and gene expression changes vary from one reprogramming to another: therefore the process is naturally error prone

- Neither reprogramming method works better than the other

Development and application of stem cells

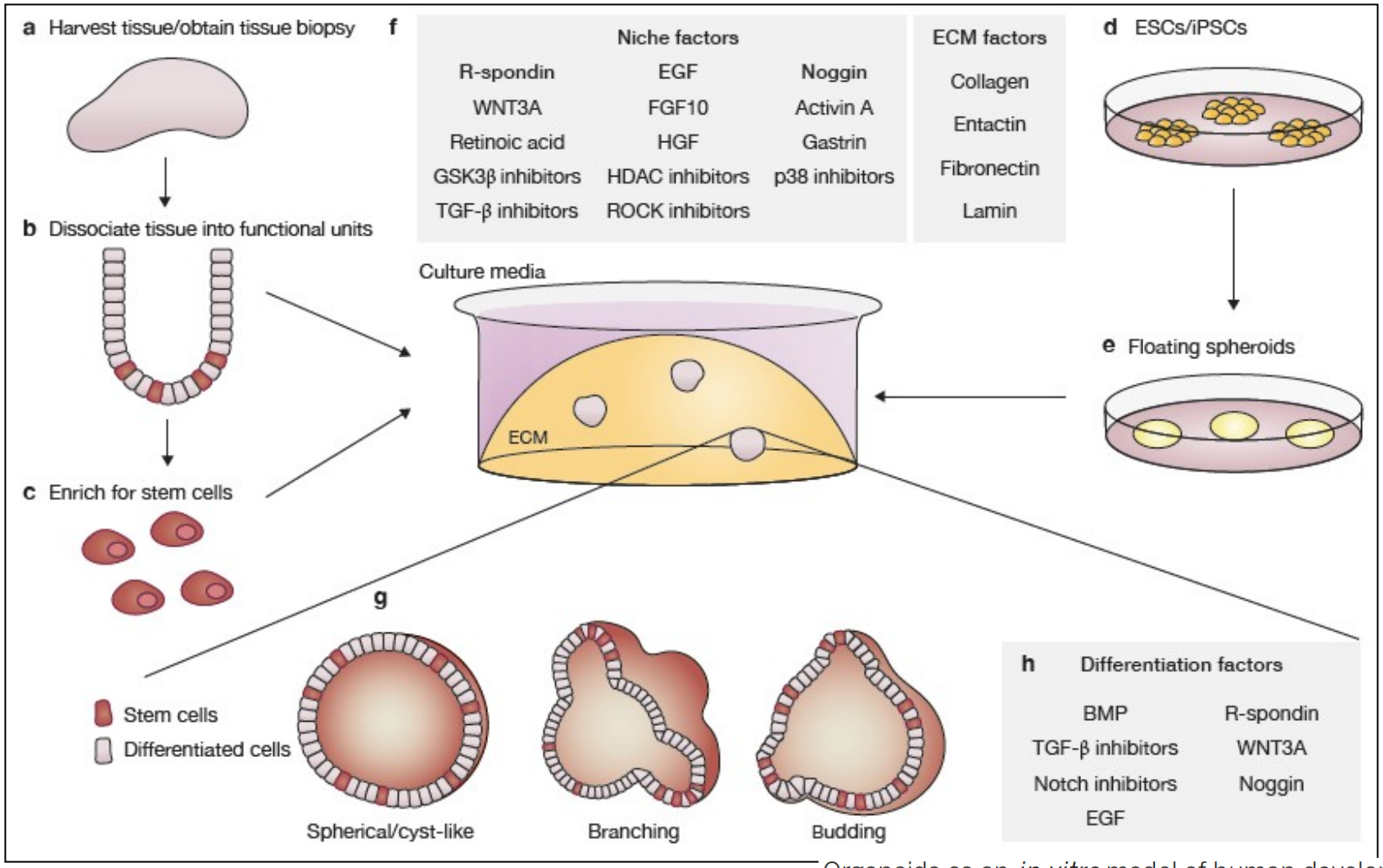


Induced pluripotent stem cells in disease modelling and drug discovery

R. Grant Rowe and George Q. Daley *

Development and application of stem cells

Organoids: *in vitro*-grown tissue clusters derived from primary tissues, or stem cells (ES or iPS)



Organoids as an *in vitro* model of human development and disease

Aliya Fatehullah[1], Si Hui Tan[1] and Nick Barker[1,2,3,*]

Stem cells: a cure all?

You have to be cautious…

https://sciencebasedmedicine.org/?s=stem+cell+therapy&category_name=&submit=Search

Stories linked above provide a discussion of stem cell therapies, hype, and unethical or outright fraudulent claims

More good stuff at https://sciencebasedmedicine.org

Summary

1) Animal cell culture

1) Stem cells: culturable, easy genetic manipulation, and capable of differentiation into other cell types

2) ES cells and their derivation by SCNT

3) Induced pluripotent stem (iPS) cells and their derivation