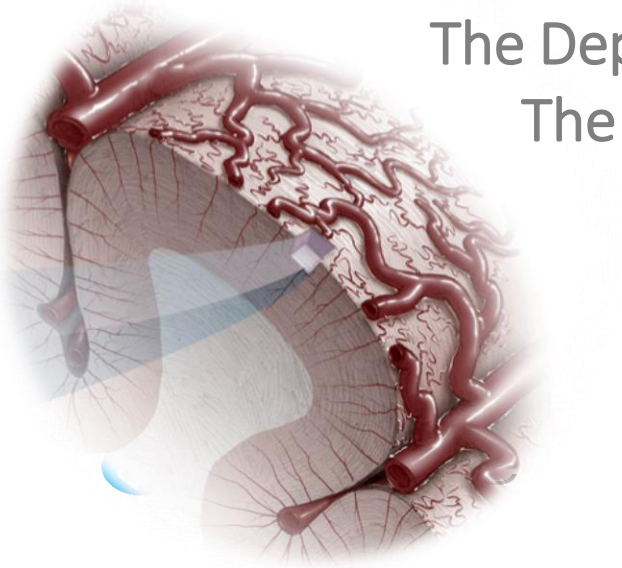
In vitro models for neuroimmunology

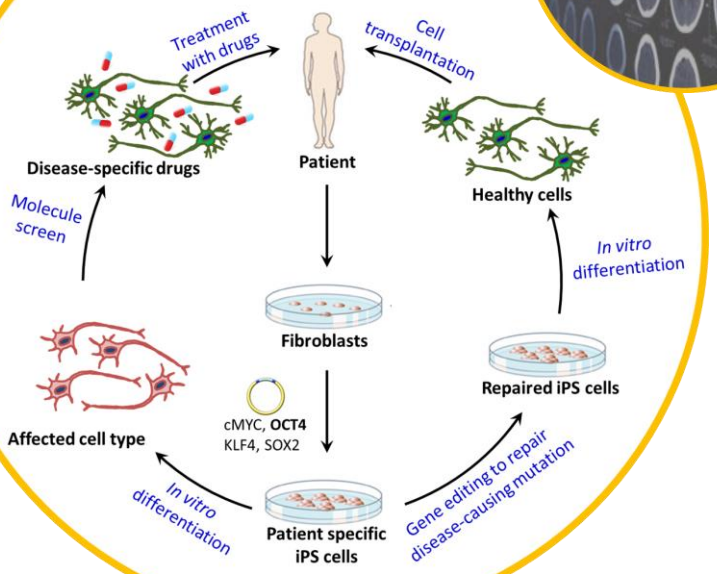
Gad D Vatine
The Department of Physiology and Cell Biology, Faculty of Health Sciences
The Regenerative Medicine and Stem Cell (RMSC) Research Center

www.vatinelab.com

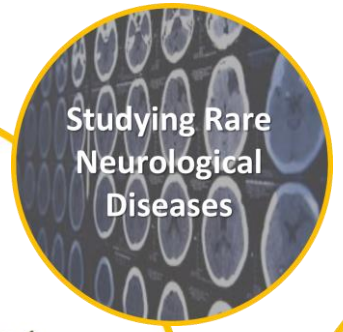


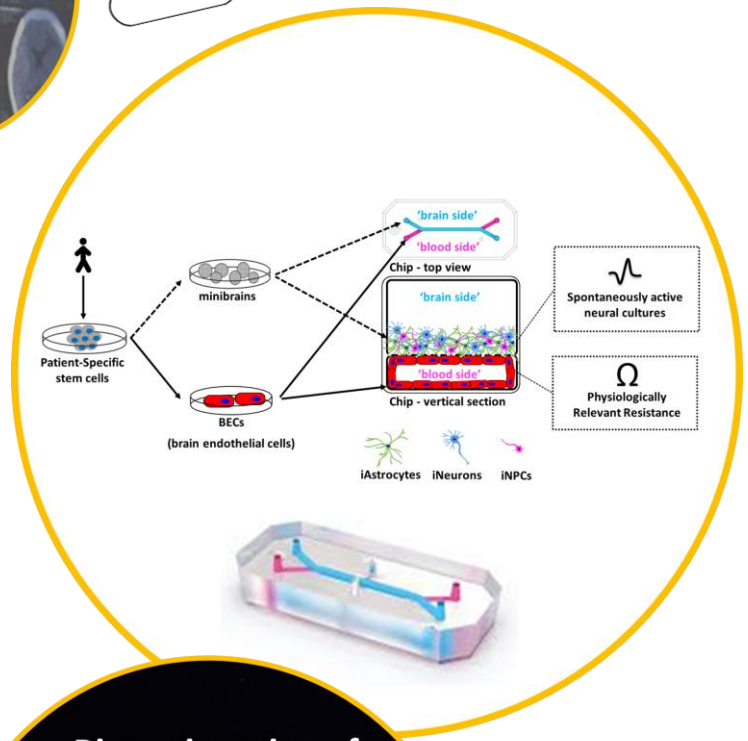
VatineLAB

Patient-Specific Stem Cells

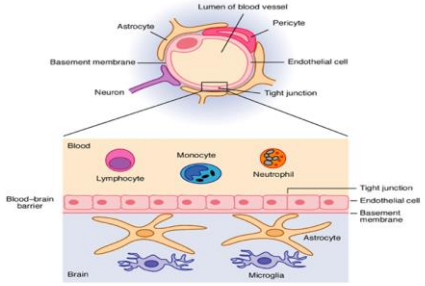


Studying Rare Neurological Diseases

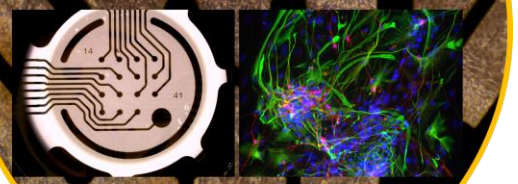




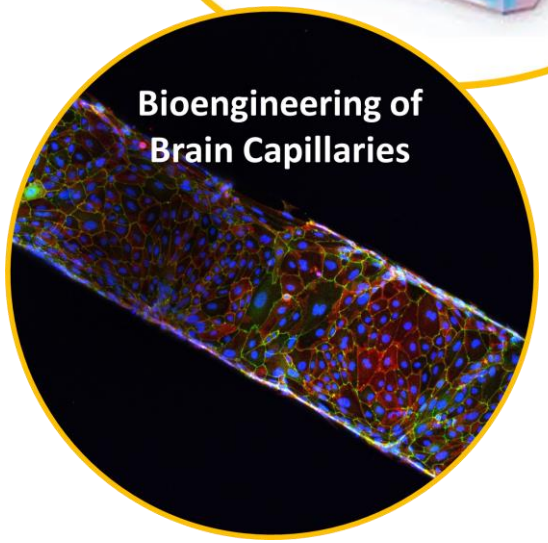
The Blood Brain Barrier (BBB)



Testing Neuronal Functions in Human Neurons



Bioengineering of Brain Capillaries



The Role of the BBB in Health and Disease

Pre-Clinical BBB-Targeted Gene Therapy

Yad La-Neshima

Awaking for hope

CONGENITAL CENTRAL HYPOVENTILATION SYNDROME NETWORK

Maia Drier

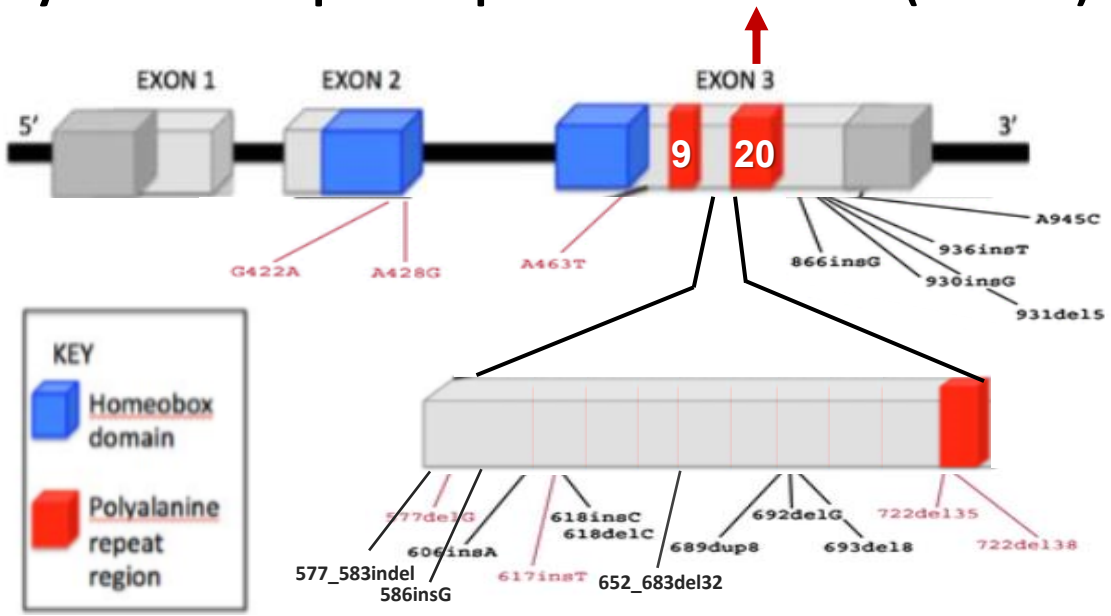
Dr Avital Adato

Heterozygous PHOX2B dominant pathogenic mutations underlie CCHS

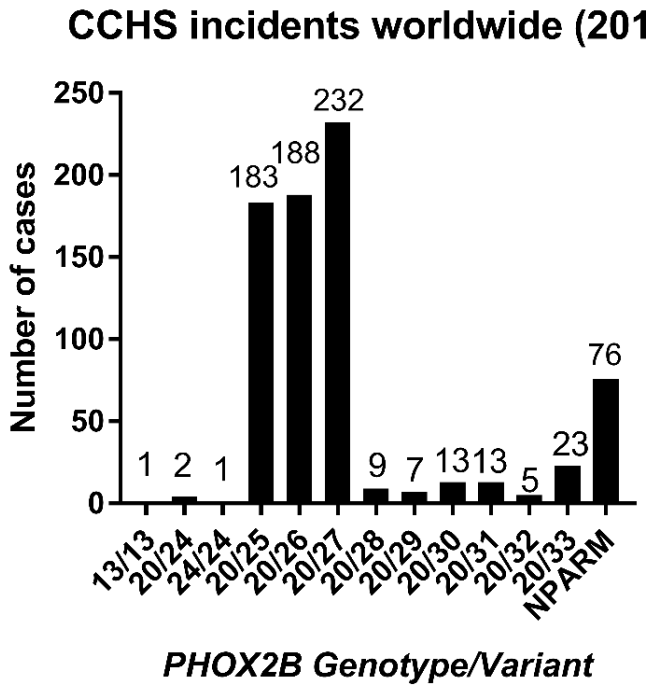
- *PHOX2B* is a 3 exons **transcription factor**
- *PHOX2B* CCHS-underlying mutations are autosomal dominant

- ~90% of CCHS patients have PARMs in the second polyalanine stretch (+ 4-13 ala)
- Disease severity correlates with PARMs length

Poly-Alanine Repeat expansion Mutations (PARMs)

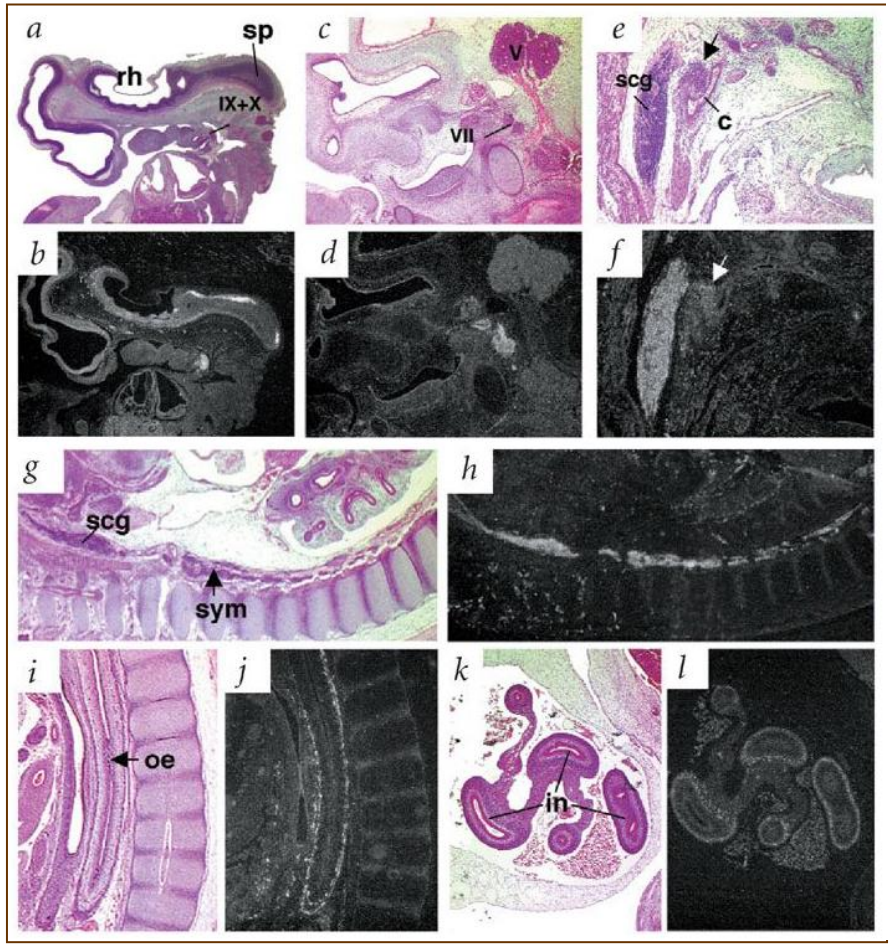


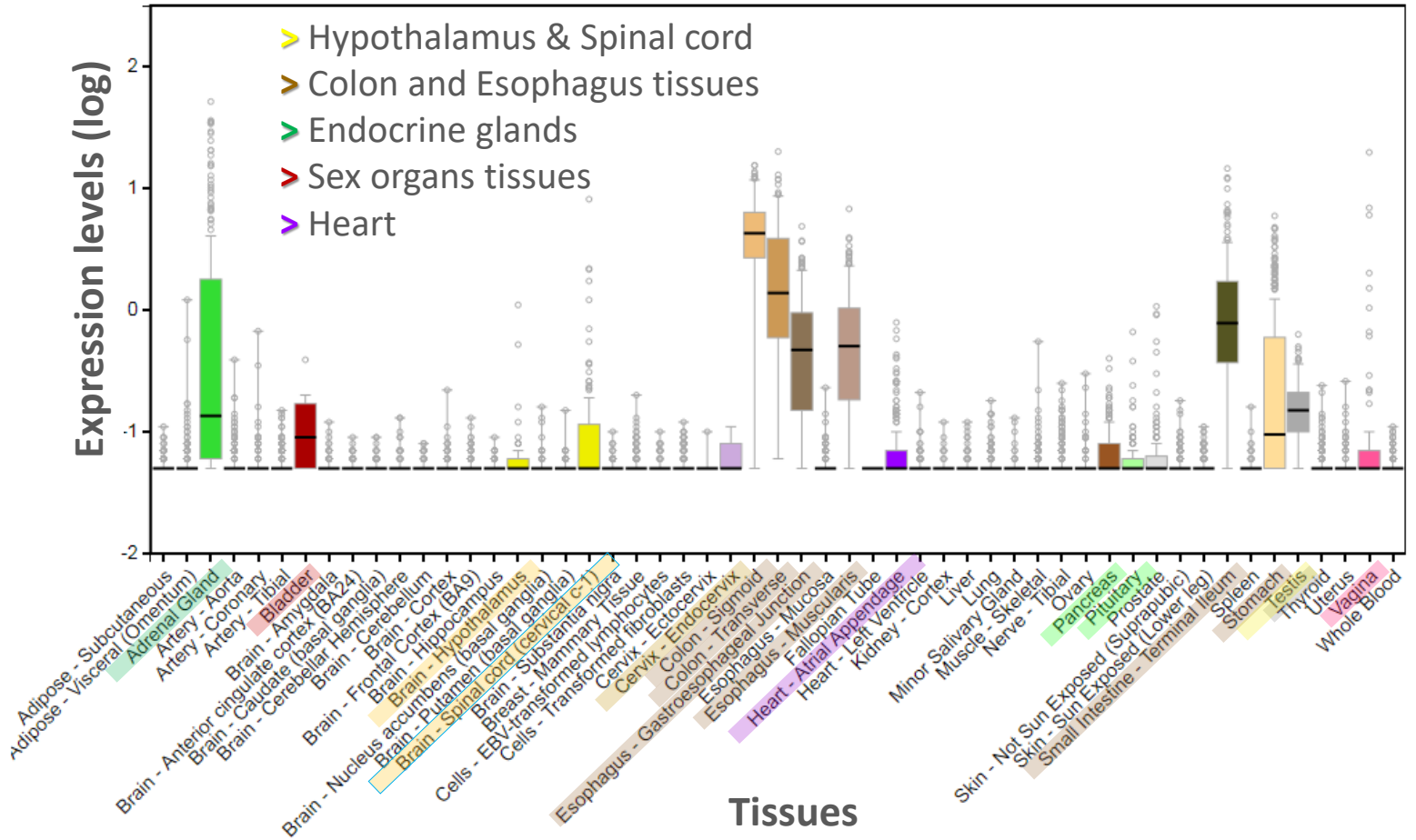
Non Poly-Alanine Repeat expansion Mutations (NPARMs)



Weese-Mayer, Berry-Kravis, Ceccherini, Keens, Loghmanee, Trang, ATS Statement 2010

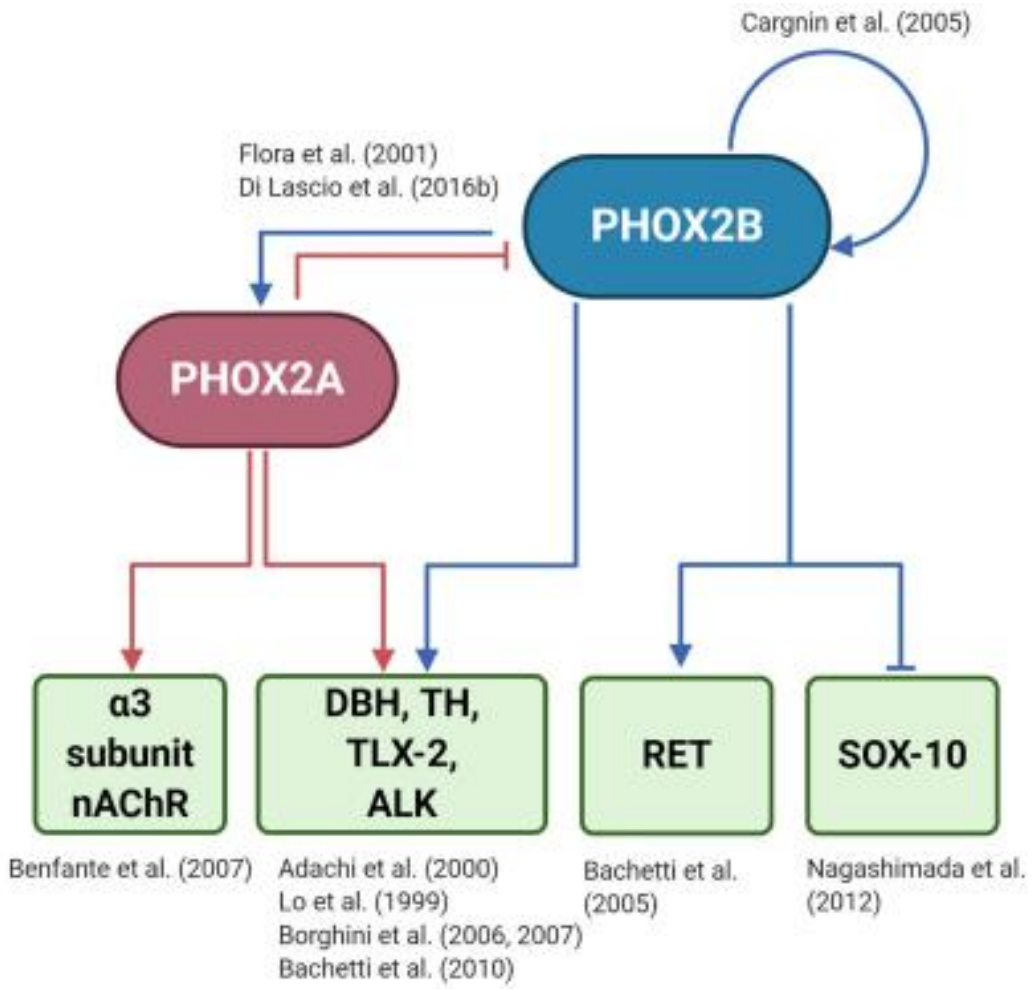
Where is PHOX2B expressed?

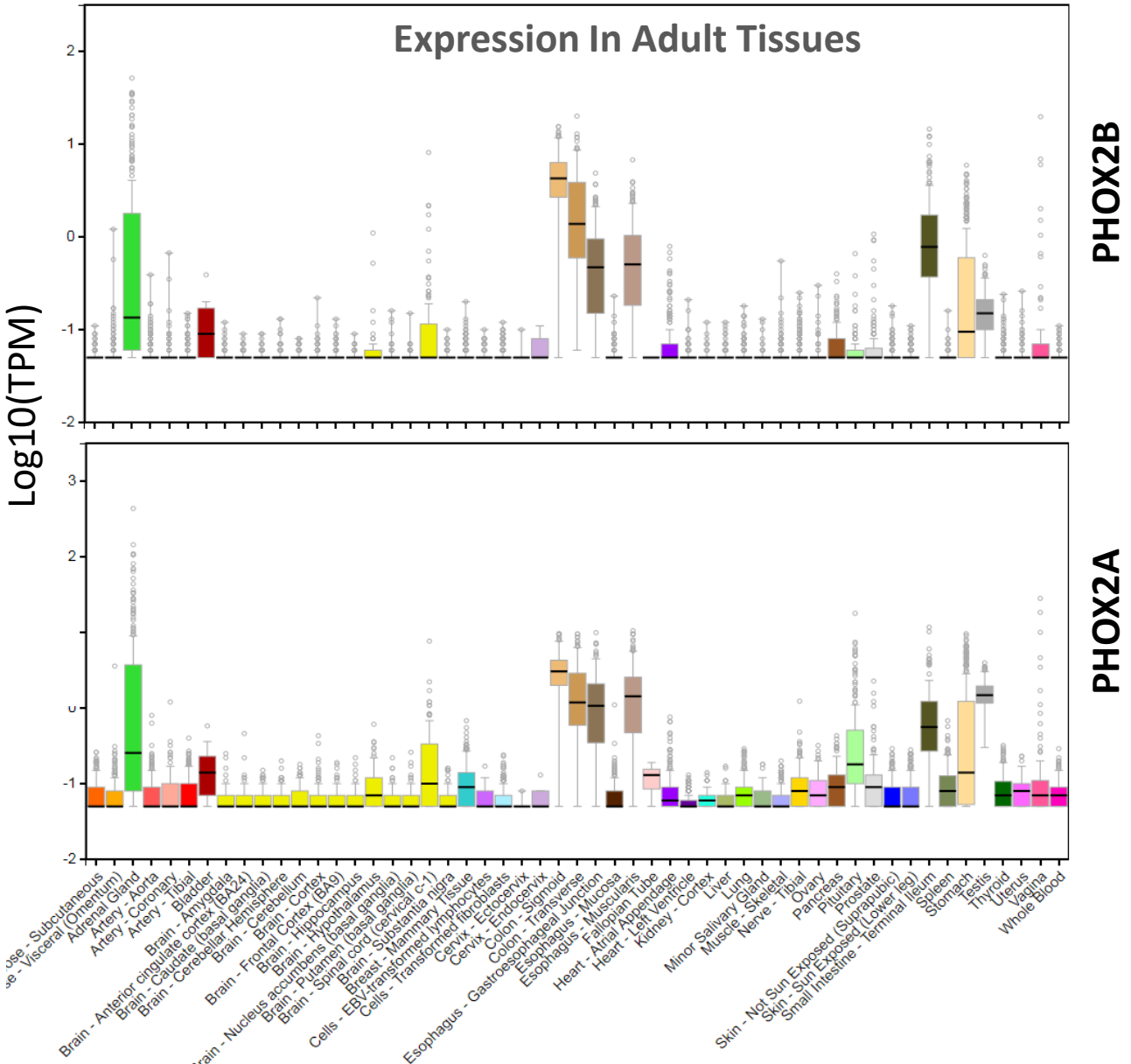




- PHOX2B expression in postmortem samples (Amiel et al., 2003)
- PHOX2B is expressed in the human nervous system and additional tissues

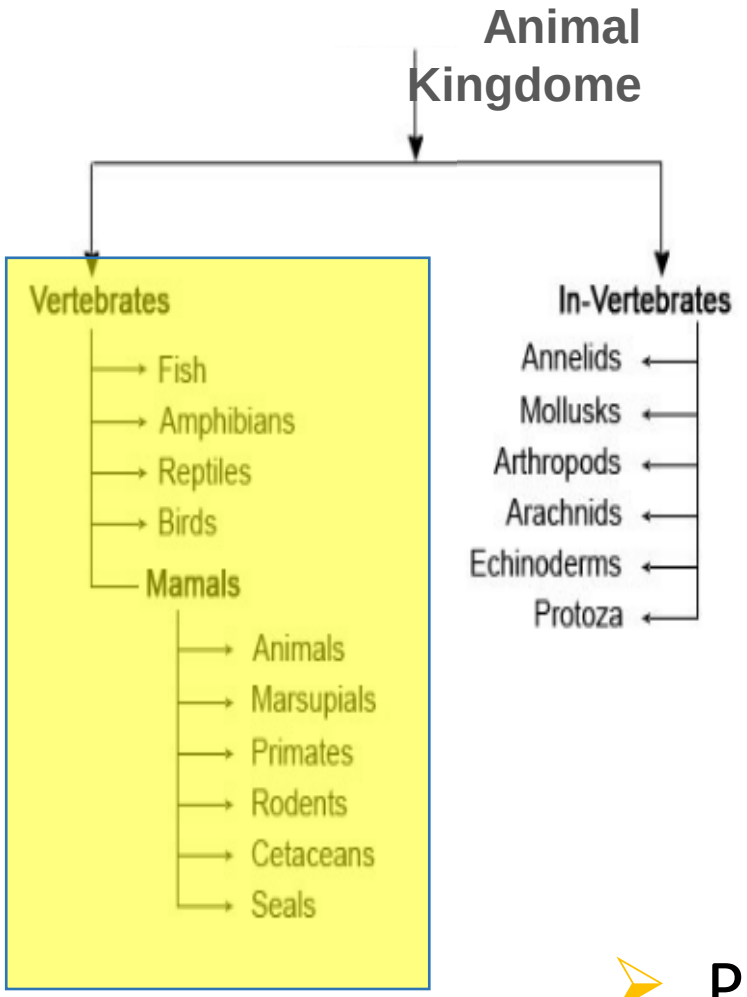
What genes are regulated by PHOX2B?





- Some of these genes are expressed in the autonomic nervous system (ANS)

How can we study PHOX2B?



Cat

rm homeobox protein 2B [Felis catus]

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Range 1: 1 to 315 GenPept Graphics

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Mouse

paired mesoderm homeobox protein 2B [Mus musculus]

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See 11 more title(s)

Range 1: 1 to 314 GenPept Graphics

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Sbjct  301  PNGAKAALVKSSMF  314
```

- PHOX2B is conserved across vertebrates

What can we learn from mouse models?

Mice mutants	Findings	Faith
Phox2b knock-out -/- Pattyn *et al.*, 1999	Phox2b is required for embryonic development of most neuronal types in the **peripheral nervous systems**	Die in the uterus around embryonic day 14
Phox2b −/+ Ramanantsoa *et al. 2006*	**Transient** chemosensitivity disorders: ➢ Sleep apneas (~6-fold increase than WT) ➢ Reduced sensitivity to hypercapnia	At **P10** differences from WT are no longer evident. The mutants survive and are fertile
Phox2b 27Ala/+ Ramanantsoa *et al. 2006*	➢ Reduced response to hypercapnia ➢ Highly unstable breathing interrupted by apneas ➢ No RTN neurons were present ➢ Loss of parafacial interneurons (pFRG)	Die within the first few postnatal hours

- PHOX2B is essential for embryonic development
- A single copy of the gene is enough for normal postnatal development and survival
- The mutated allele seems to disturb the function of the normal copy
  → **dominant toxic gain of function**

UCONN | UNIVERSITY OF CONNECTICUT

JUNE 27, 2024

UConn Today

MENU

June 25, 2024 | *Kim Krieger - UConn Communications*

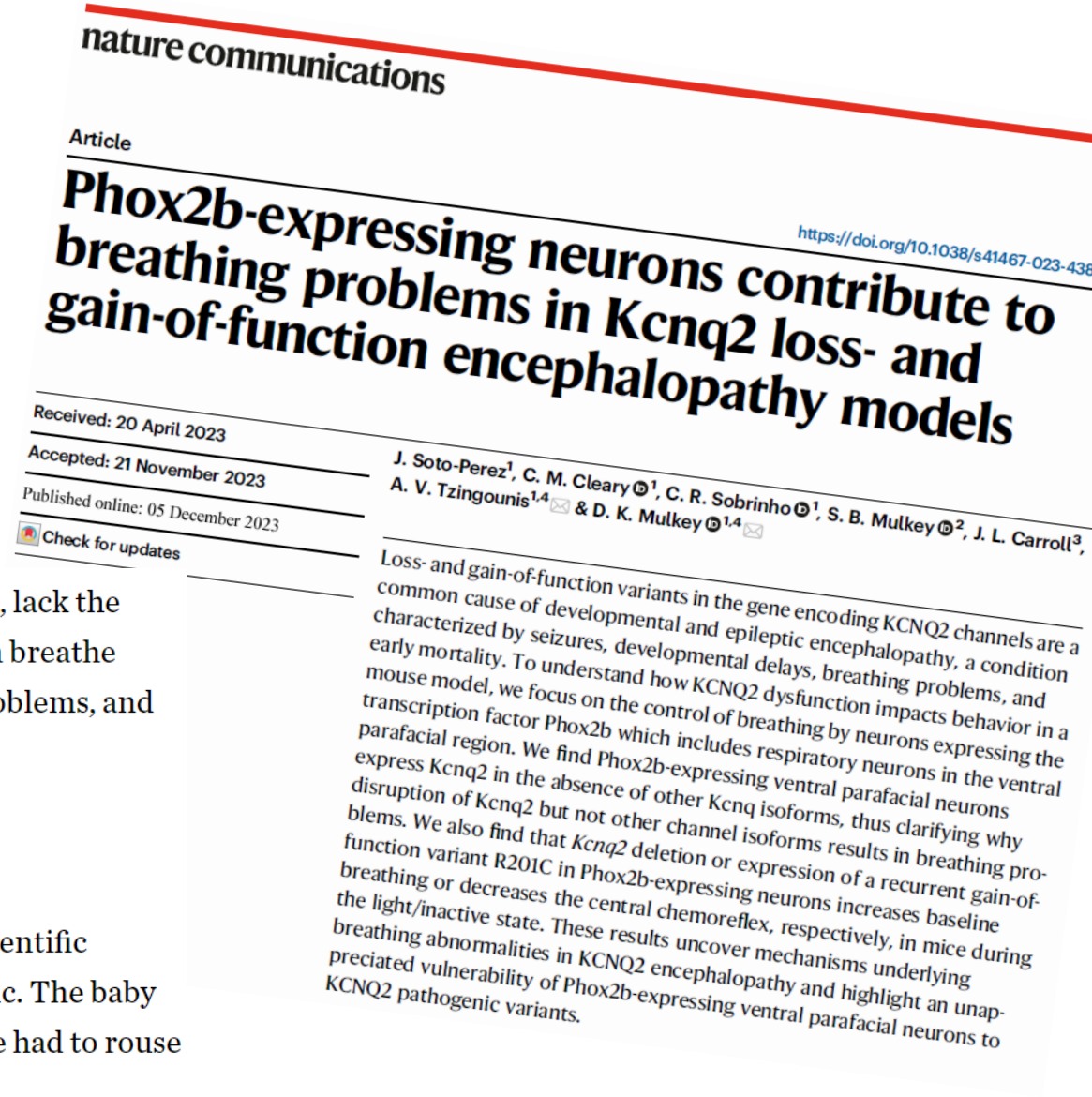
Neurobiologists Reveal a Secret of Ondine's Curse

People with Ondine's curse, also known as congenital central hypoventilation syndrome, lack the ability to regulate breathing unconsciously.

People with Ondine's curse, also known as congenital central hypoventilation syndrome, lack the ability to regulate breathing unconsciously. Such people must have a machine help them breathe when they sleep, or risk death. Poor sleep and less oxygen lead to health and learning problems, and people with the condition often die young.

The condition is also terribly stressful for the family of a person with Ondine's curse.

Dan Mulkey, a neurobiologist at UConn, describes how a colleague hitched a ride to a scientific conference with the family of a child who had the disorder. It was at night. They hit traffic. The baby would periodically fall asleep. The scientist sat in the backseat and every few minutes, he had to rouse the baby.



nature communications

Article

https://doi.org/10.1038/s41467-023-438

Phox2b-expressing neurons contribute to breathing problems in Kcnq2 loss- and gain-of-function encephalopathy models

Received: 20 April 2023

Accepted: 21 November 2023

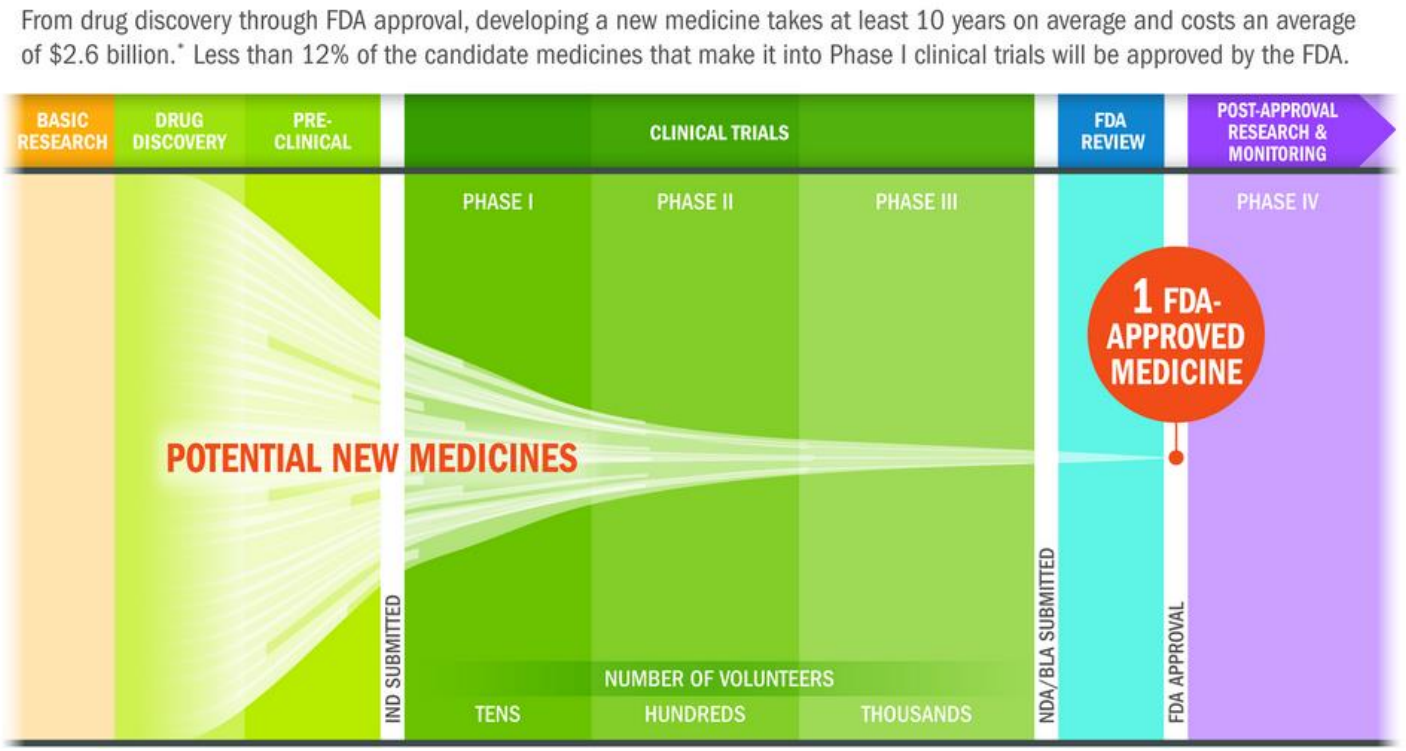
Published online: 05 December 2023

Check for updates

J. Soto-Perez[1], C. M. Cleary[1], C. R. Sobrinho[1], S. B. Mulkey[2], J. L. Carroll[3], A. V. Tzingounis[1,4] & D. K. Mulkey[1,4]

Loss- and gain-of-function variants in the gene encoding KCNQ2 channels are a common cause of developmental and epileptic encephalopathy, a condition characterized by seizures, developmental delays, breathing problems, and early mortality. To understand how KCNQ2 dysfunction impacts behavior in a mouse model, we focus on the control of breathing by neurons expressing the transcription factor Phox2b which includes respiratory neurons in the ventral parafacial region. We find Phox2b-expressing ventral parafacial neurons express Kcnq2 in the absence of other Kcnq isoforms, thus clarifying why disruption of Kcnq2 but not other channel isoforms results in breathing problems. We also find that *Kcnq2* deletion or expression of a recurrent gain-of-function variant R201C in Phox2b-expressing neurons increases baseline breathing or decreases the central chemoreflex, respectively, in mice during the light/inactive state. These results uncover mechanisms underlying breathing abnormalities in KCNQ2 encephalopathy and highlight an unappreciated vulnerability of Phox2b-expressing ventral parafacial neurons to KCNQ2 pathogenic variants.

Why do pre-clinical models fail to predict clinical relevance?



In Vivo

Animal models

* Not a human

+

In Vitro

Cell cultures

* Do not represent the physiological environment

=

Clinical trials

SCIENCE

The FDA no longer requires all drugs to be tested on animals before human trials

A new U.S. law has eliminated the requirement that drugs in development must undergo testing in animals before being given to participants in human trials.

Animal rights advocates have long pushed for such a move, and some in the pharmaceutical industry have argued that animal testing can be ineffective and expensive.

PETA cheered the new law as a "radical shift" in how new drugs and treatments will be created.

Signed by President Biden in December as part of a larger spending package, the law doesn't ban the testing of new drugs on animals outright.

There are a slew of other methods that drugmakers employ to assess new medications and treatments, such as computer modeling and "organs on a chip," thumb-sized microchips that can mimic how organs' function are affected by pharmaceuticals.

But Aliasger Salem, a professor at the University of Iowa's College of Pharmacy, told NPR that companies opting to use these alternative testing methods as a replacement for animal testing must be aware of the methods' limits to ensure their drugs are safe.

How can we perform research on humans?

Post mortem tissue

- Limited in scale and availability
- Limited possibility to perform experiments

Human fibroblasts and cell lines

- Scalable cell source
- Non-physiologically relevant

Primary human cells

- Limited in scale and availability
- Naturally abnormal

Human stem cells

- Scalable cell source
- Can potentially differentiate into any cell type

What is a Stem Cell? Ask Google

U.S. Department of Health and Human Services

FDA U.S. FOOD & DRUG ADMINISTRATION

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FDA Warns About Stem Cell Therapies

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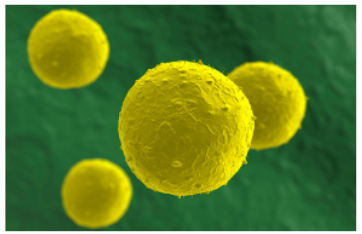


Researchers hope stem cells will one day be effective in the treatment of many medical conditions and diseases. But unproven stem cell treatments can be unsafe—so get all of the facts if you're considering any treatment.

Stem cells have been called everything from cure-alls to miracle treatments. But don't believe the hype. Some unscrupulous providers offer stem cell products that are both unapproved and unproven. So beware of potentially dangerous procedures—and confirm what's really being offered before you consider *any* treatment.

The facts: Stem cell therapies may offer the potential to treat diseases or conditions for which few treatments exist. Sometimes called the body's "master cells," stem cells are the cells that develop into blood, brain, bones, and all of the body's organs. They have the potential to repair, restore, replace, and regenerate cells, and could *possibly* be used to treat many medical conditions and diseases.

But the U.S. Food and Drug Administration is concerned that some patients seeking cures and remedies are vulnerable to stem cell treatments that are illegal and potentially harmful. And the FDA is increasing its oversight and enforcement to protect people from dishonest and unscrupulous stem cell clinics, while continuing to encourage innovation so that the medical industry can properly harness the potential of stem cell products.

To do your part to stay safe, make sure that any stem cell treatment you are considering is either:

- FDA-approved, or;
- Being studied under an Investigational New Drug Application (IND), which is a clinical investigation plan submitted and allowed to proceed by the FDA.

What is a Stem Cell? Ask ChatGPT

ChatGPT

What is a stem cell?

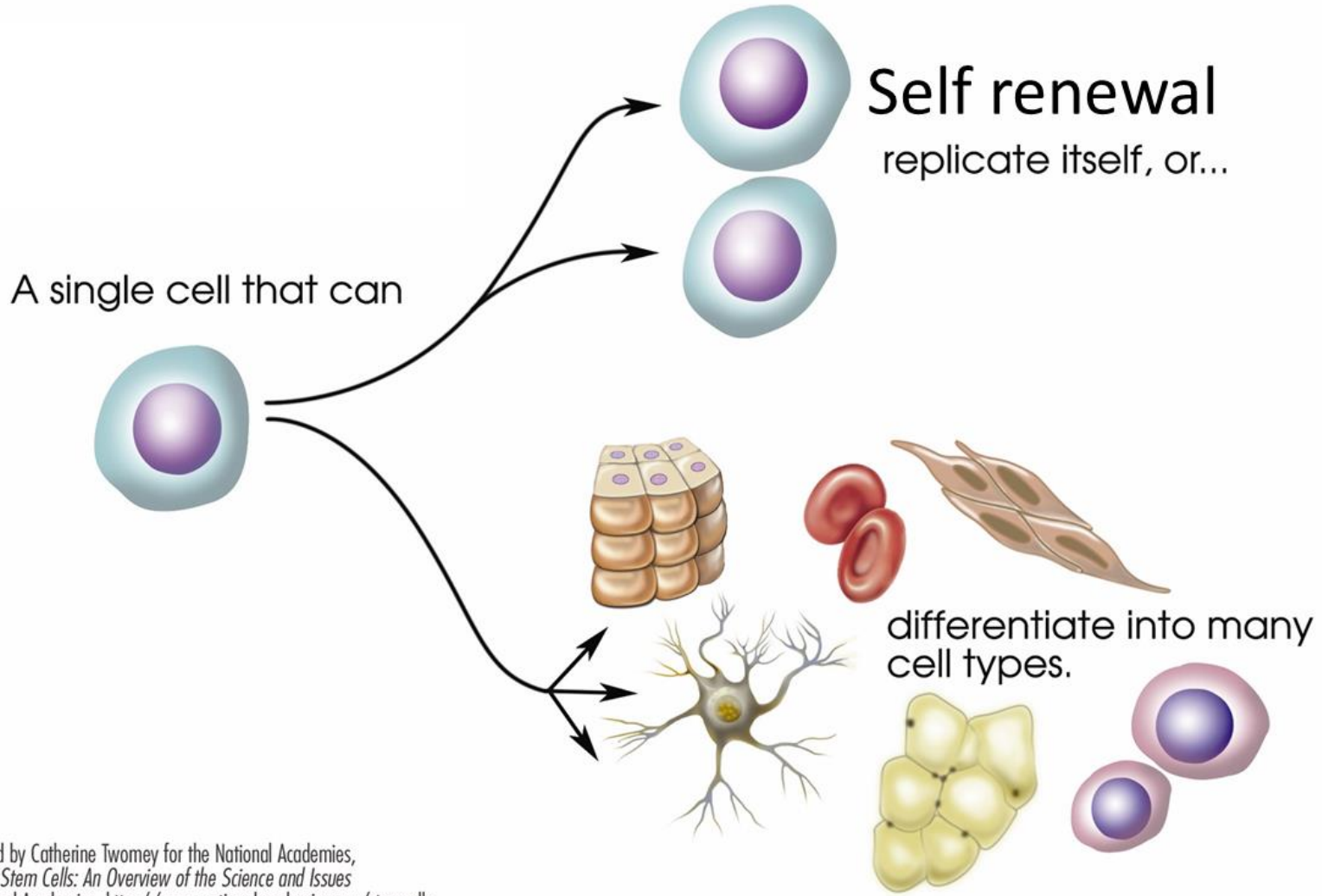
A stem cell is a type of cell with the unique ability to develop into many different cell types in the body during early life and growth. Additionally, in many tissues, they serve as a sort of internal repair system, dividing essentially without limit to replenish other cells as long as the person or animal is still alive. Here are some key characteristics and types of stem cells:

Key Characteristics of Stem Cells:

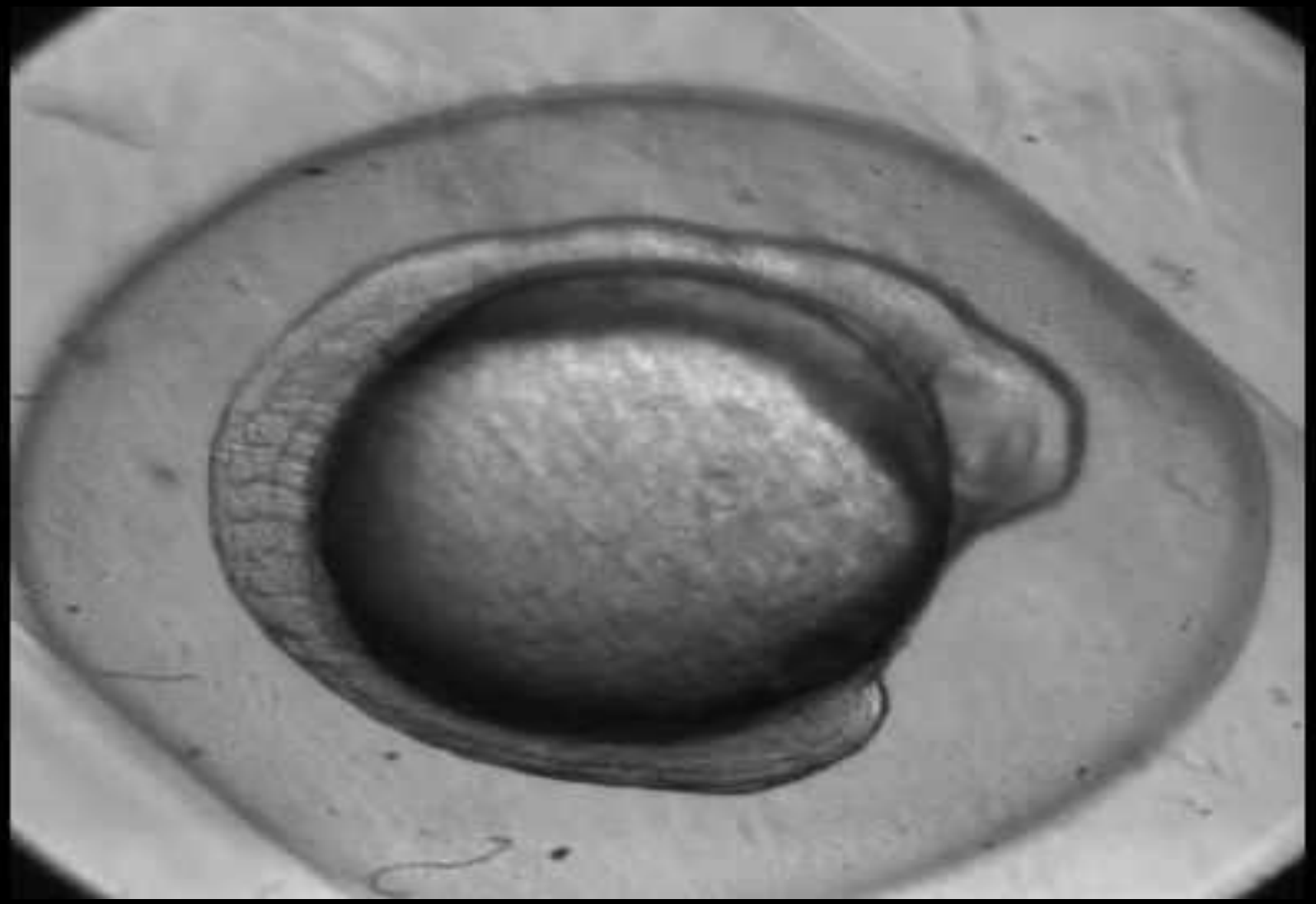
1. **Self-Renewal**: Stem cells can divide and replicate for long periods.
2. **Potency**: They have the potential to differentiate into various specialized cell types. The degree of this potential is categorized as:
    - **Totipotent**: Can form all cell types in a body, plus the extra-embryonic, or placental, cells.
    - **Pluripotent**: Can give rise to all cell types that make up the body.
    - **Multipotent**: Can develop into more than one cell type, but are more limited than pluripotent cells.

Message ChatGPT

What is a Stem Cell?

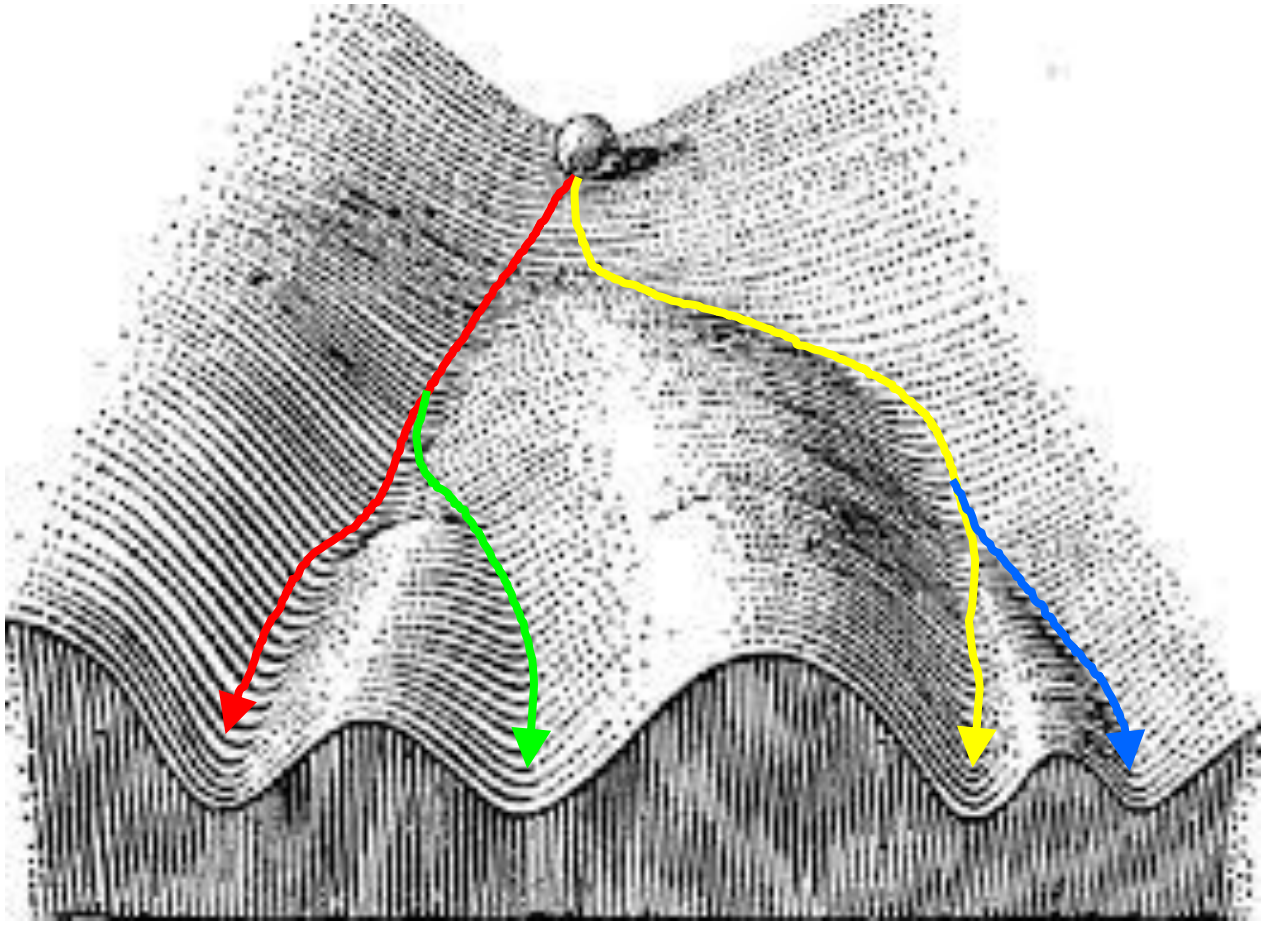


Embryonic development starts from a single cell that proliferates and differentiates into all cell types of our body



Cellular decision-making during development

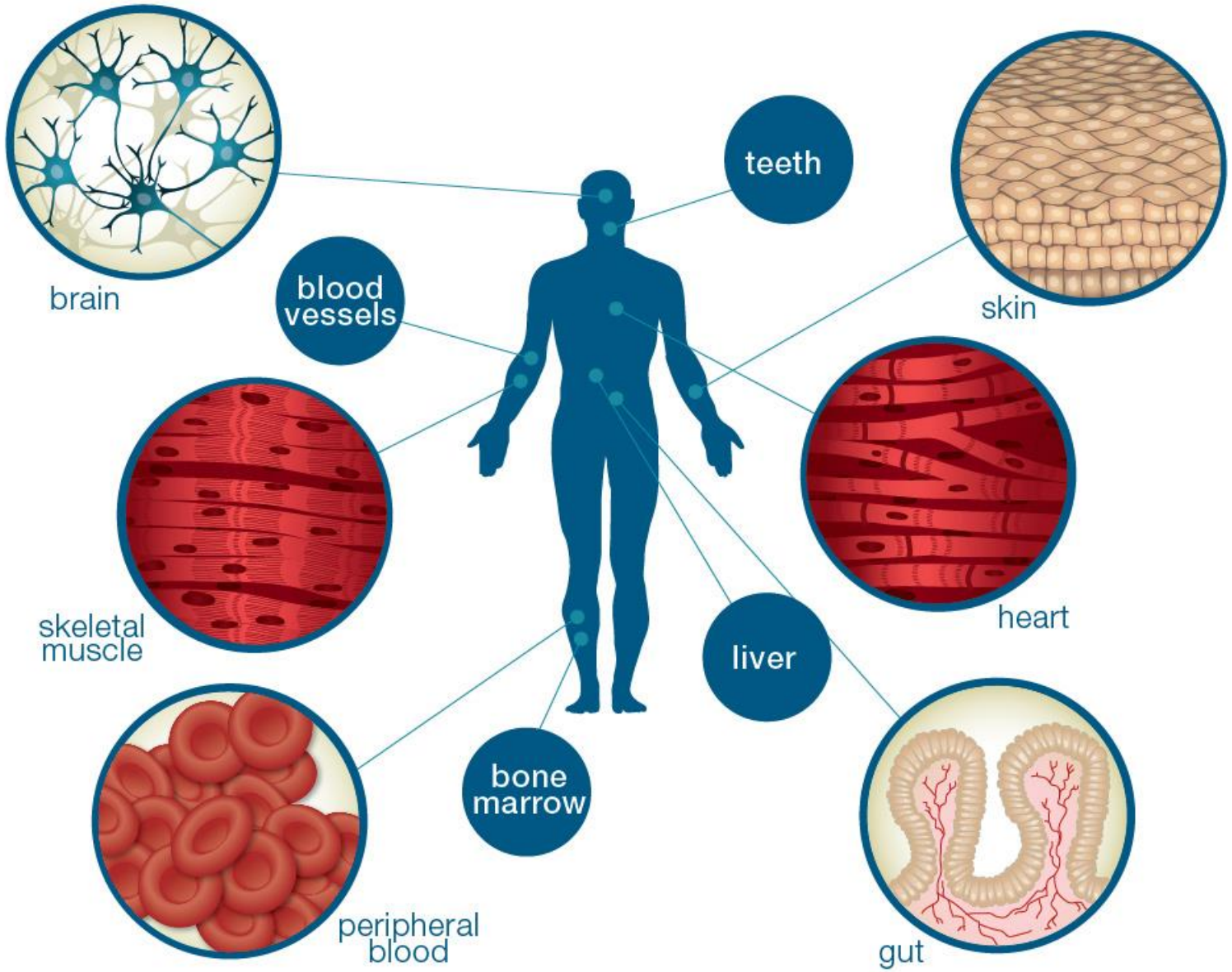
Conrad Waddington
(1905-1975)



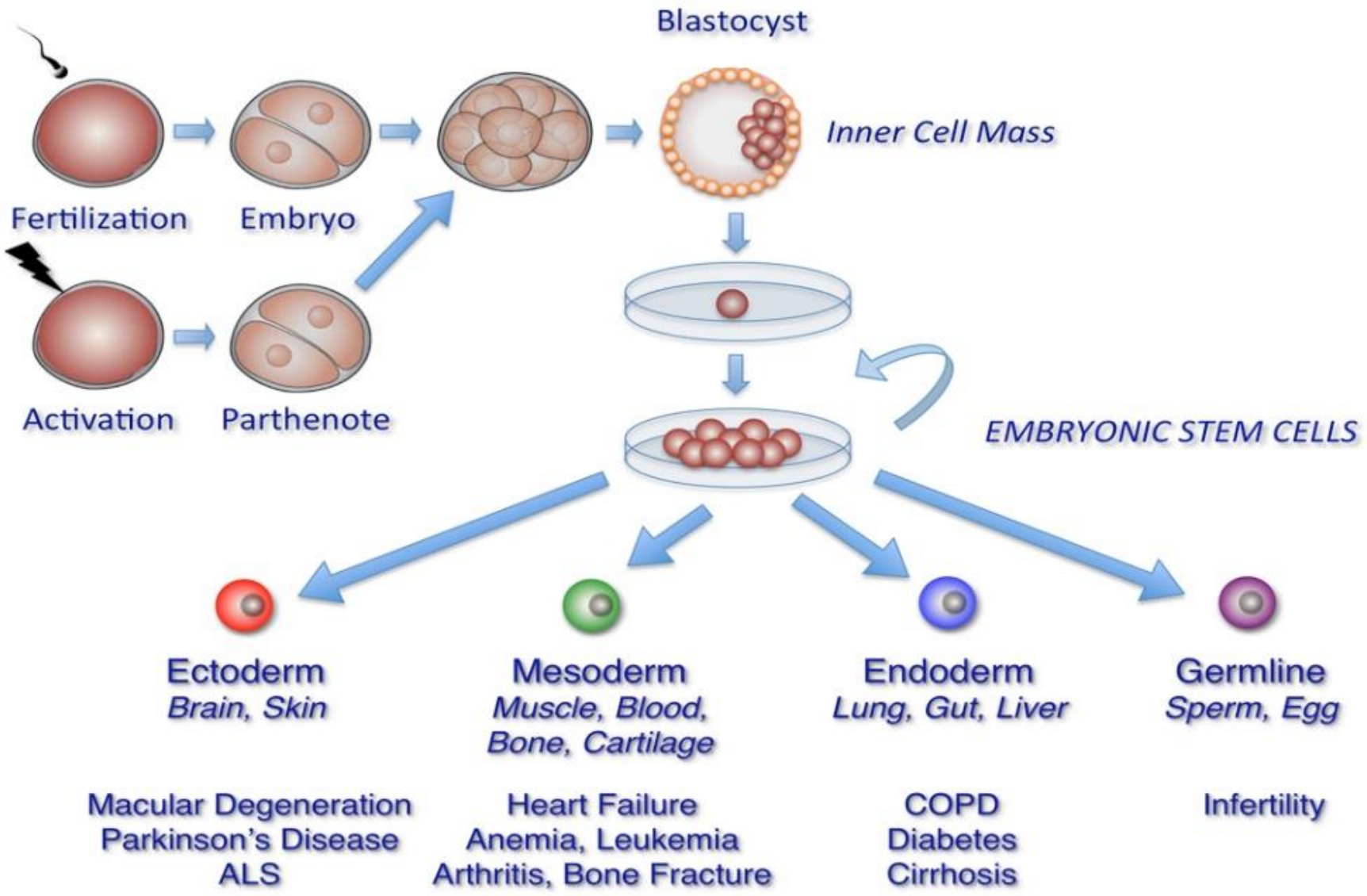
Adult stem cells

- Many **cells in our body are constantly replaced**. Adult stem cells provide an indispensable renewable source of cells

- Adult stem cells are unable to differentiate to all cell types (they are multipotent but not pluripotent)



Embryonic Stem Cells



The Nobel Prize in Physiology or Medicine 2007

Mario R. Capecchi
Sir Martin J. Evans
Oliver Smithies

Advanced information

Nobelförsamlingen
The Nobel Assembly at Karolinska Institutet

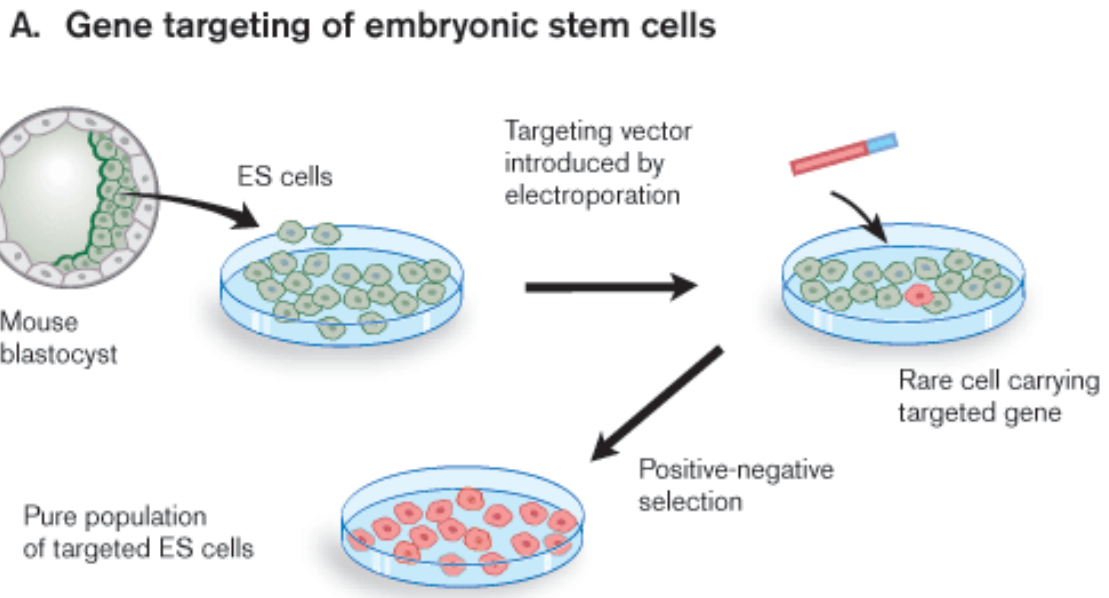
Karolinska Institutet

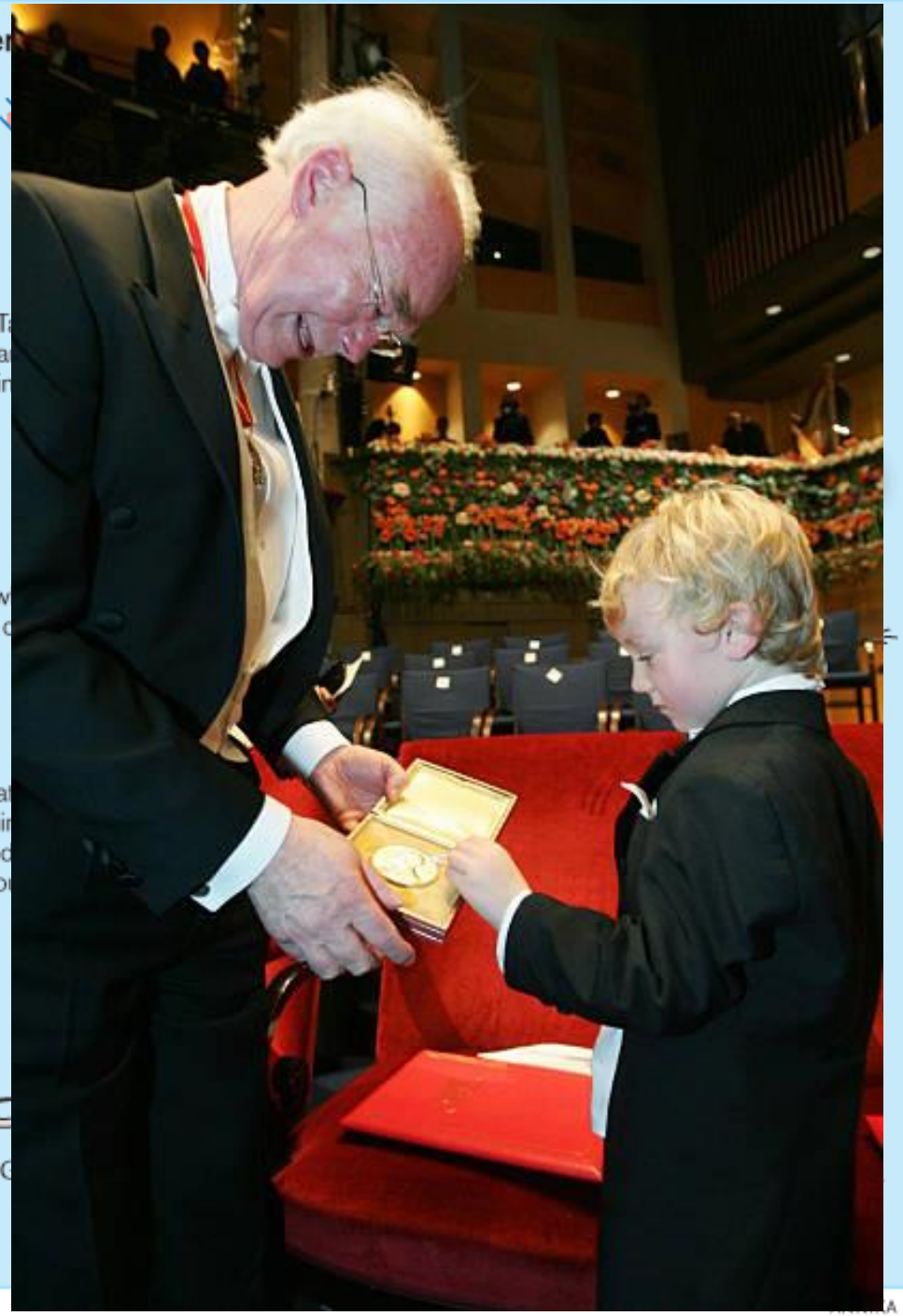
Advanced information [pdf]

Gene Modification in Mice

Introduction

The 2007 Nobel Prize in physiology or medicine is awarded to Drs Mario R. Capecchi, Martin J. Evans and Oliver Smithies for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells. Their work has made it possible to modify specific genes in the germline of mammals and to raise offspring that carry and express the modified gene. The toolbox of experimental genetic methods developed by Capecchi, Evans and Smithies, commonly called the knockout technology, has permitted scientists to determine the role of specific genes in development, physiology, and pathology. It has revolutionized life science and plays a key role in the development of medical therapy.



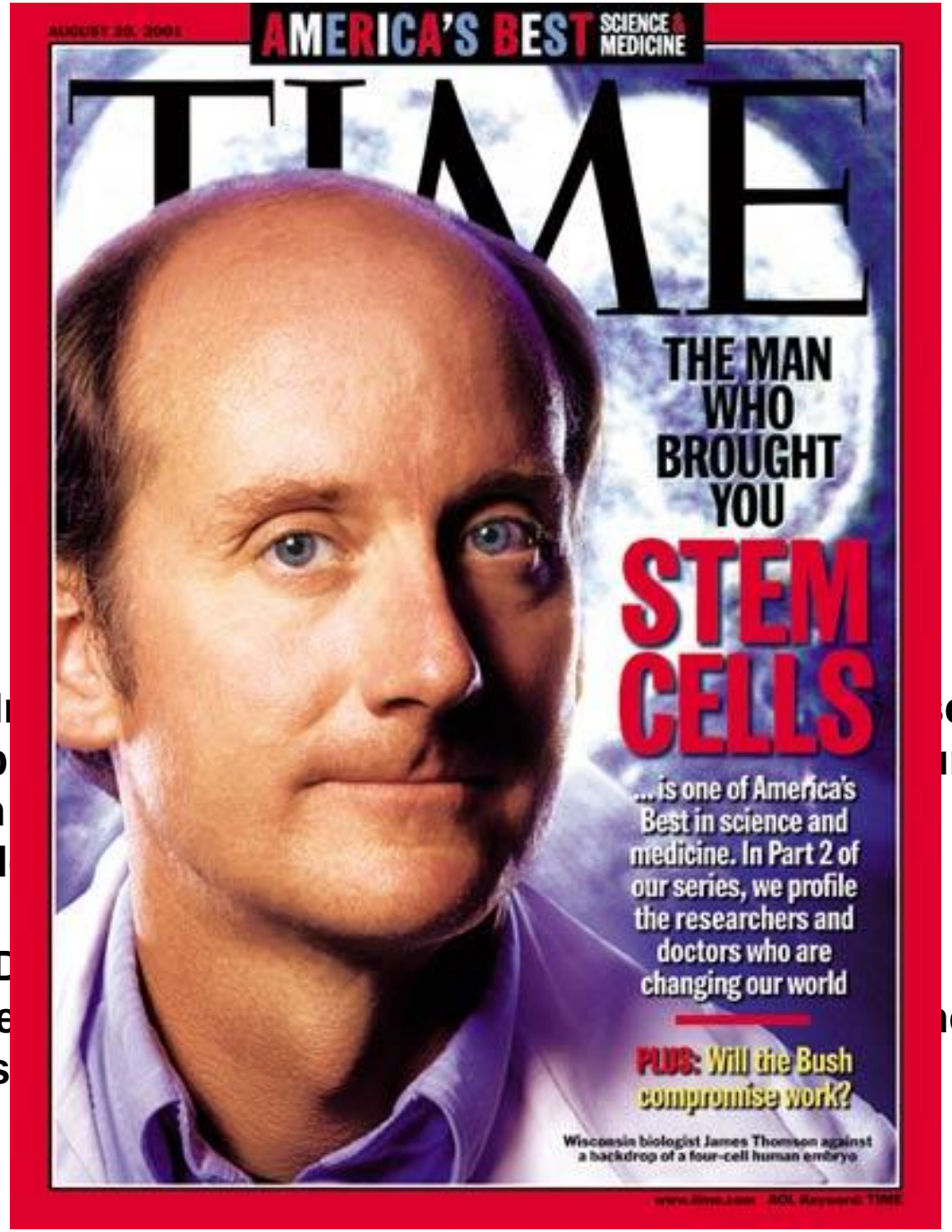


Embryonic Stem Cell Lines Derived from Human Blastocysts

James A. Thomson,* Joseph Itskovitz-Eldor, Sander S. Shapiro, Michelle A. Waknitz, Jennifer J. Swiergiel, Vivienne S. Marshall, Jeffrey M. Jones

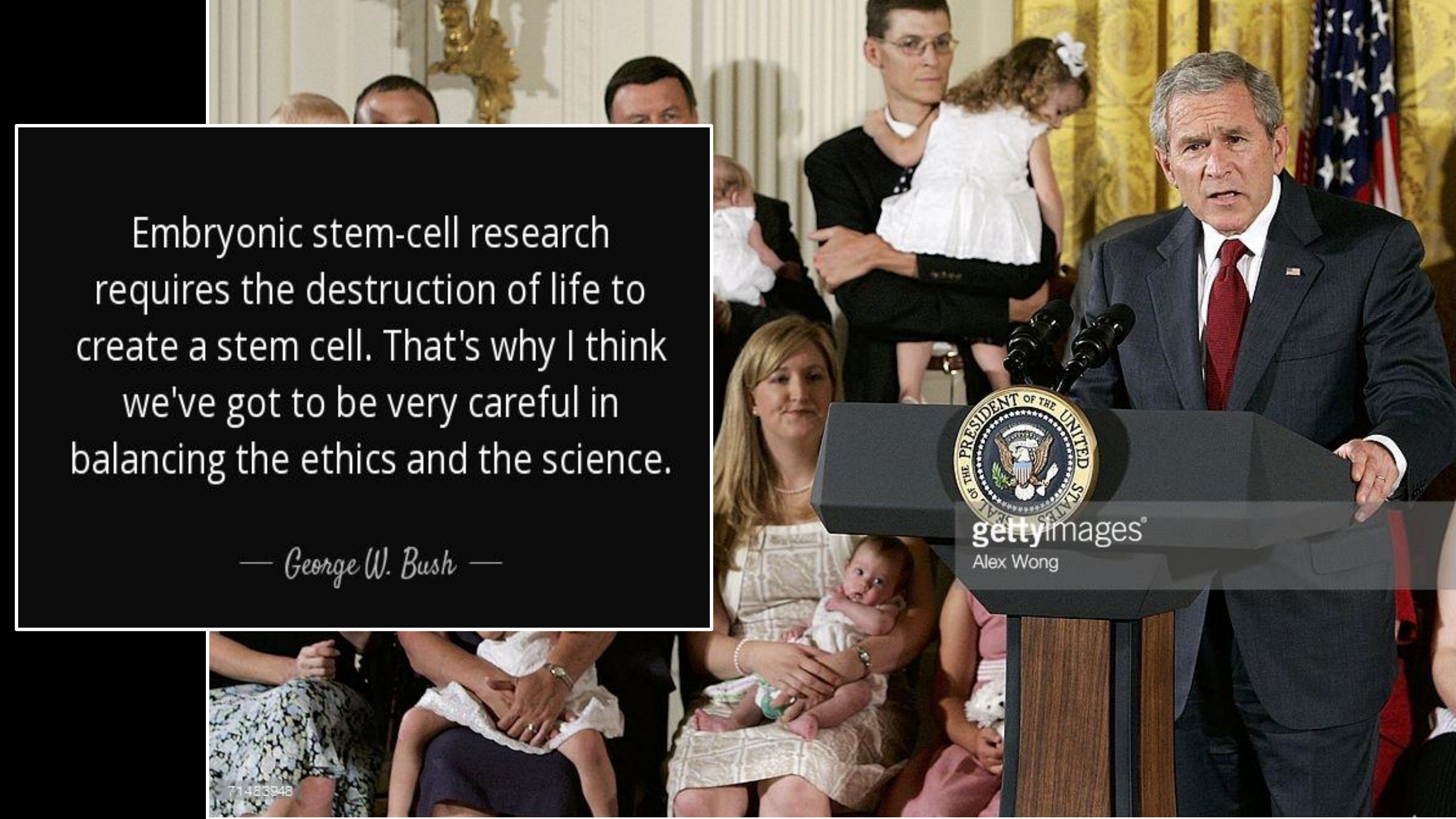
Human blastocyst-derived, pluripotent cell lines are described that have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages. After undifferentiated proliferation in vitro for 4 to 5 months, these cells still maintained the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). These cell lines should be useful in human developmental biology, drug discovery, and transplantation medicine.

- The generation of hESCs involves destruction of the embryo and is therefore **controversial**

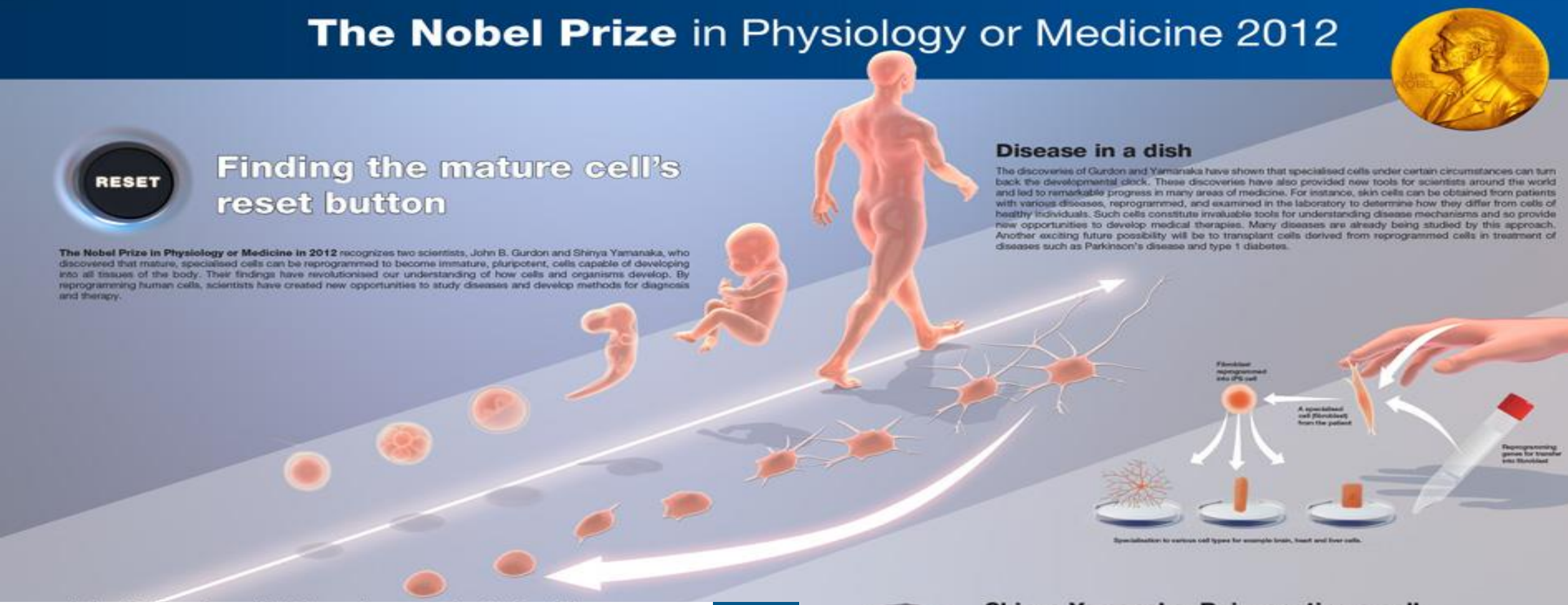


(A) I… emb… 24 h… Scal…

(B) D… abse… pres…


Embryonic stem-cell research requires the destruction of life to create a stem cell. That's why I think we've got to be very careful in balancing the ethics and the science.
— George W. Bush —
gettyimages
Alex Wong

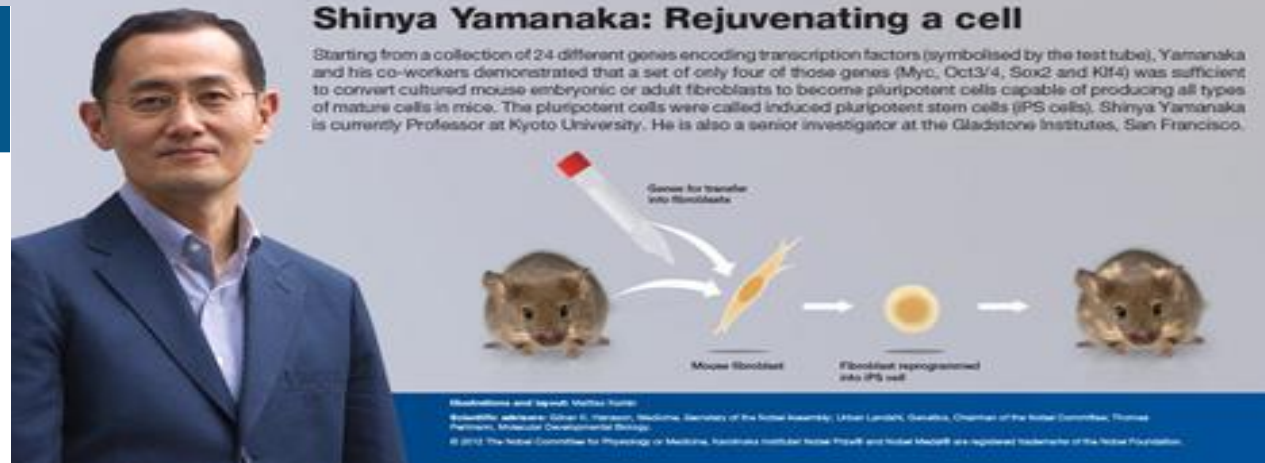
The Nobel Prize in Physiology or Medicine 2012



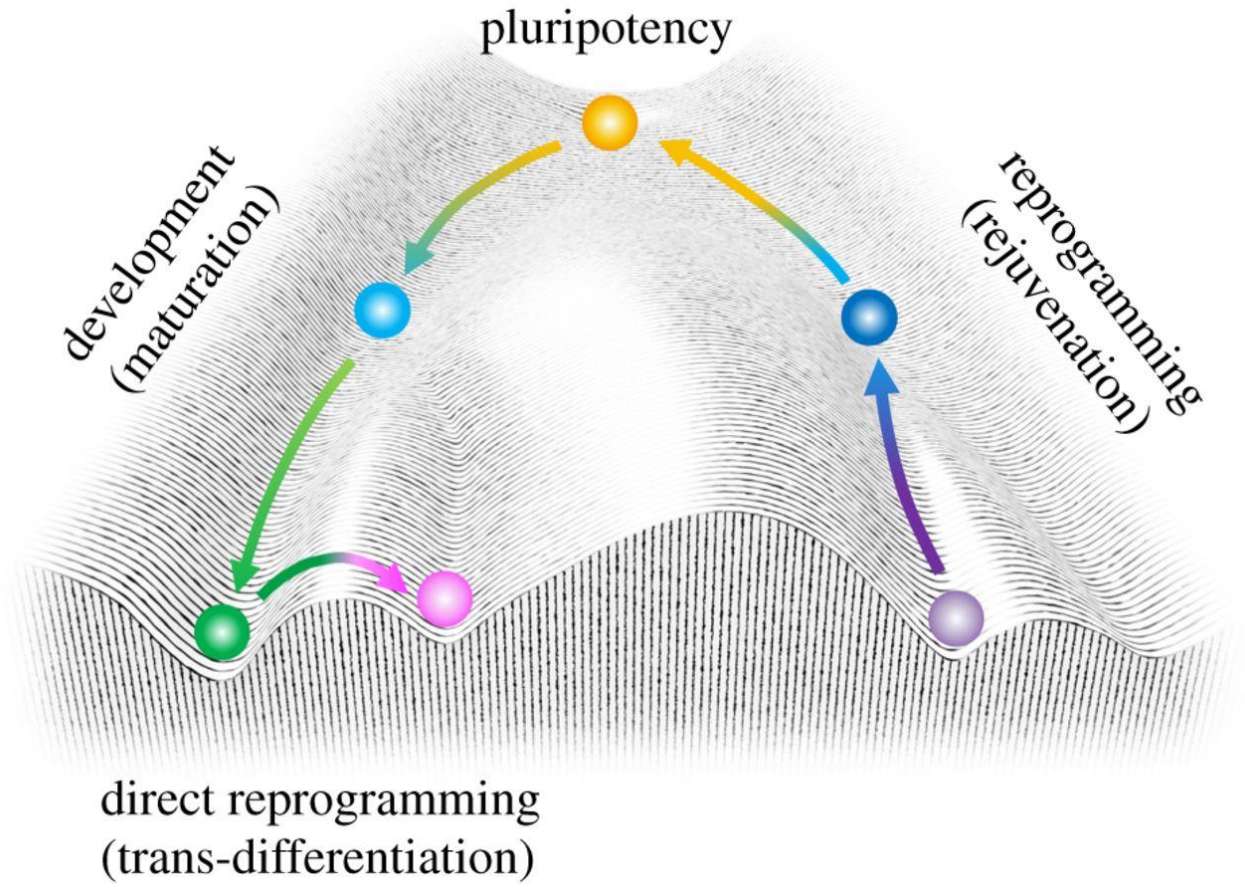
Cell

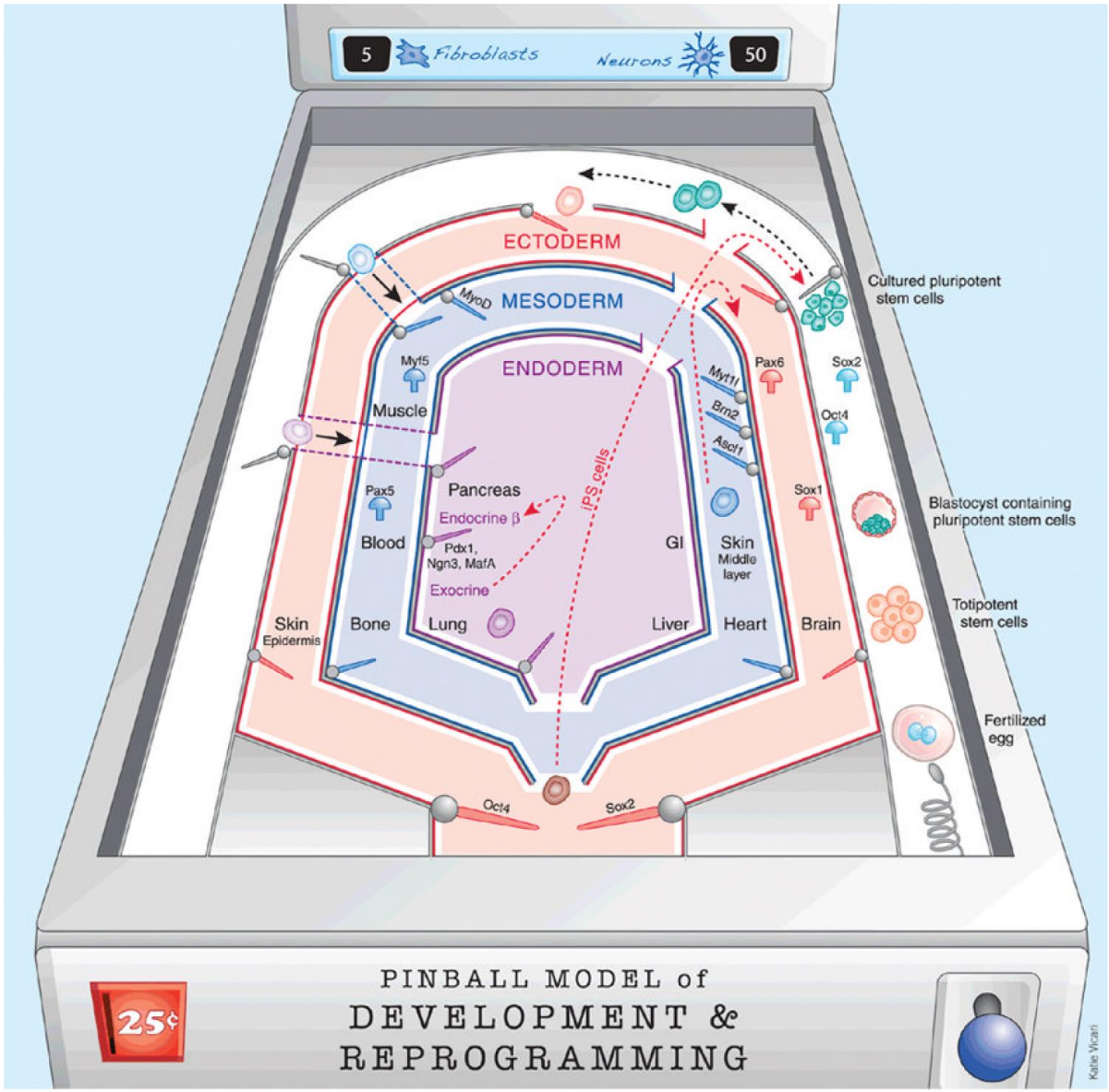
Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors

Kazutoshi Takahashi[1] and Shinya Yamanaka[1,2,*]
[1]Department of Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan
[2]CREST, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan
*Contact: yamanaka@frontier.kyoto-u.ac.jp
DOI 10.1016/j.cell.2006.07.024



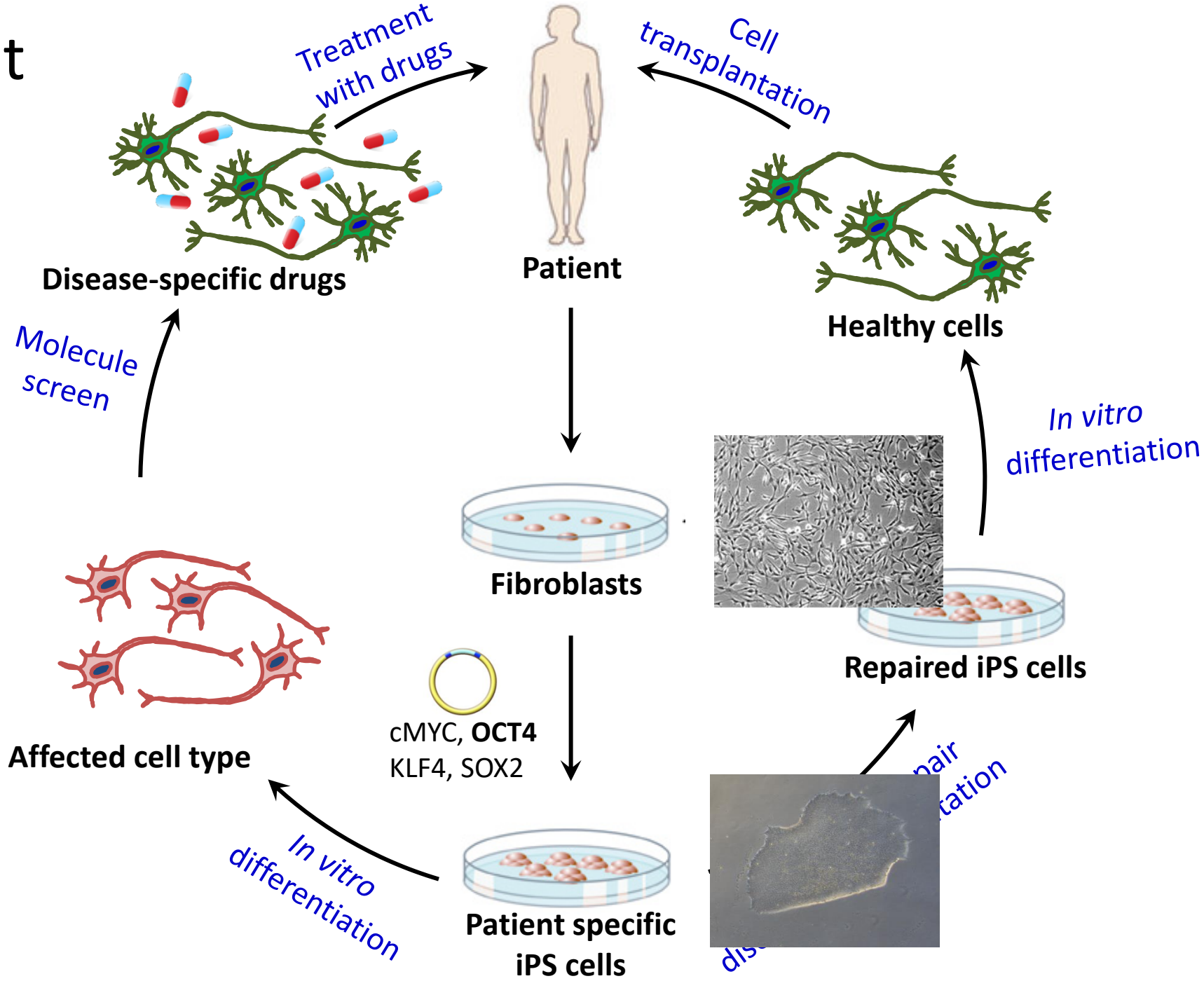
Reprogramming – a game changer in human research





Induced pluripotent stem cells (iPSCs)

- Personalized embryonic-**like** stem cells without ethical controversy
- A **disease-in-a-dish** model to study human disorders in a **personalized** manner



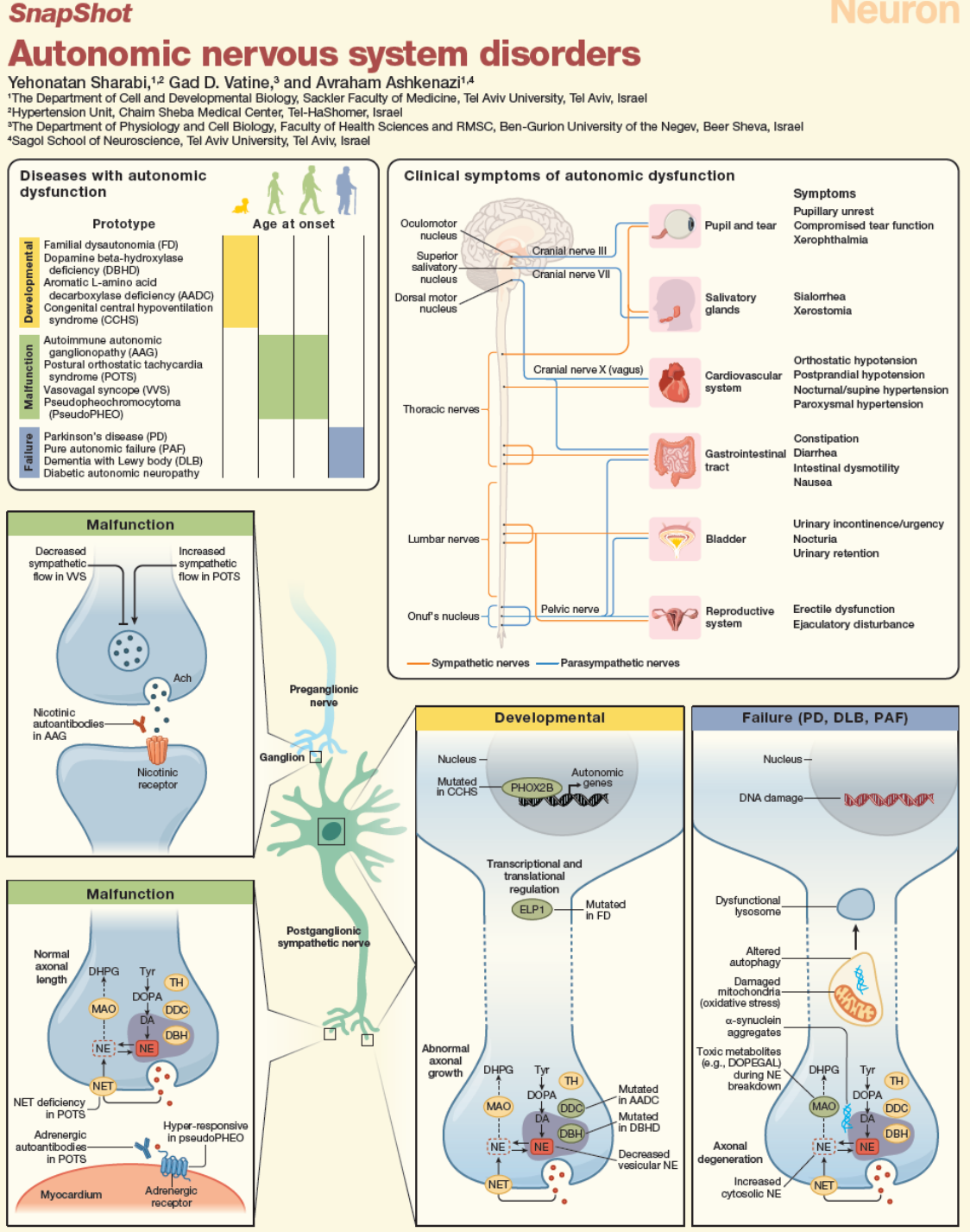
Sympathetic neurons malfunction in autonomic nervous system (ANS) disorders, including CCHS

Dr Avi Ashkenazi

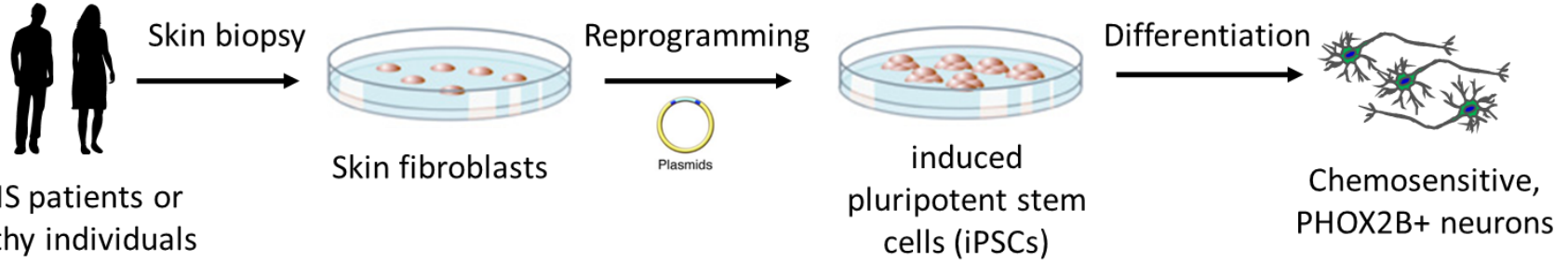
Fatima Amer-Sarsour

Dr Yevgeny Berdichevsky

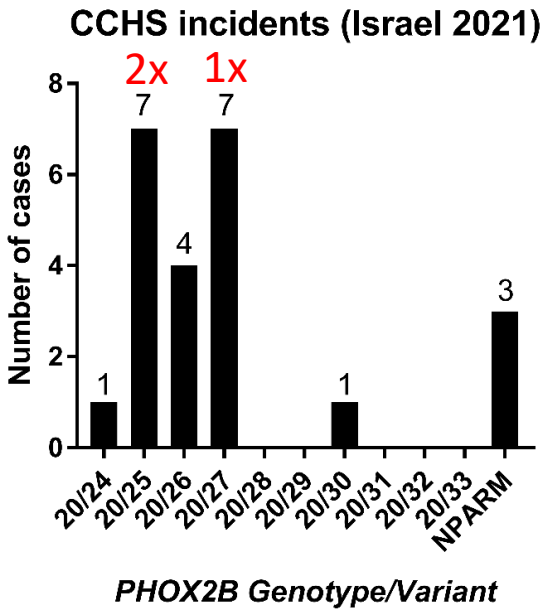
TEL AVIV UNIVERSITY

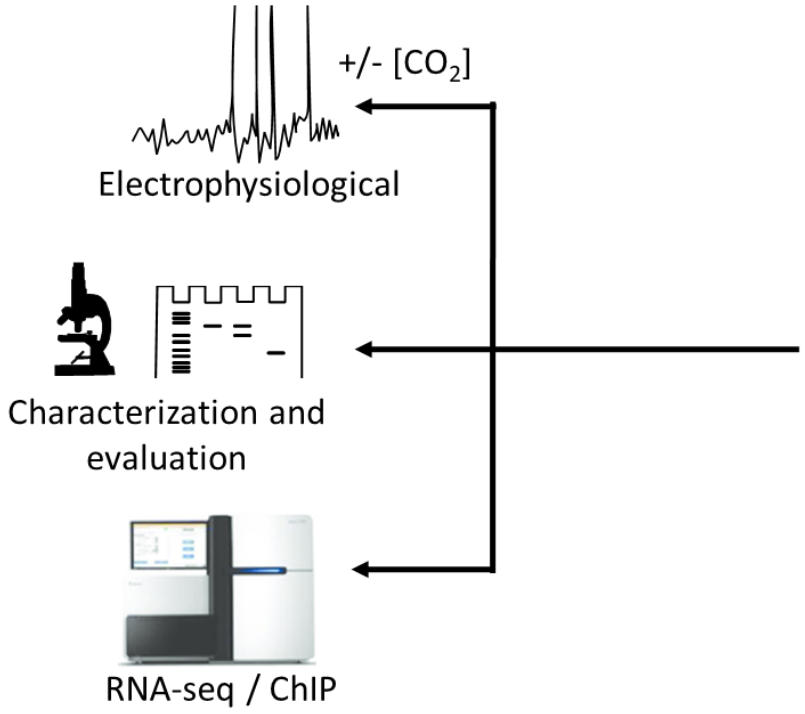


Generating a human-relevant disease-in-a-dish model for CCHS

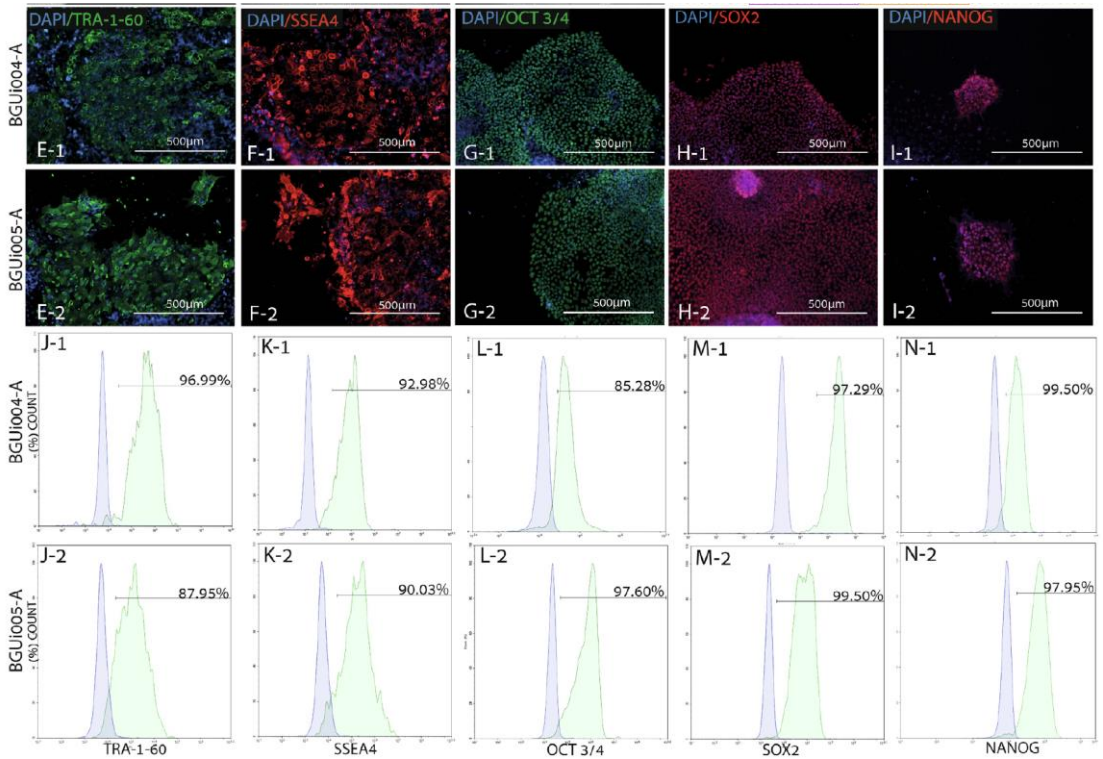


Daniel Falik

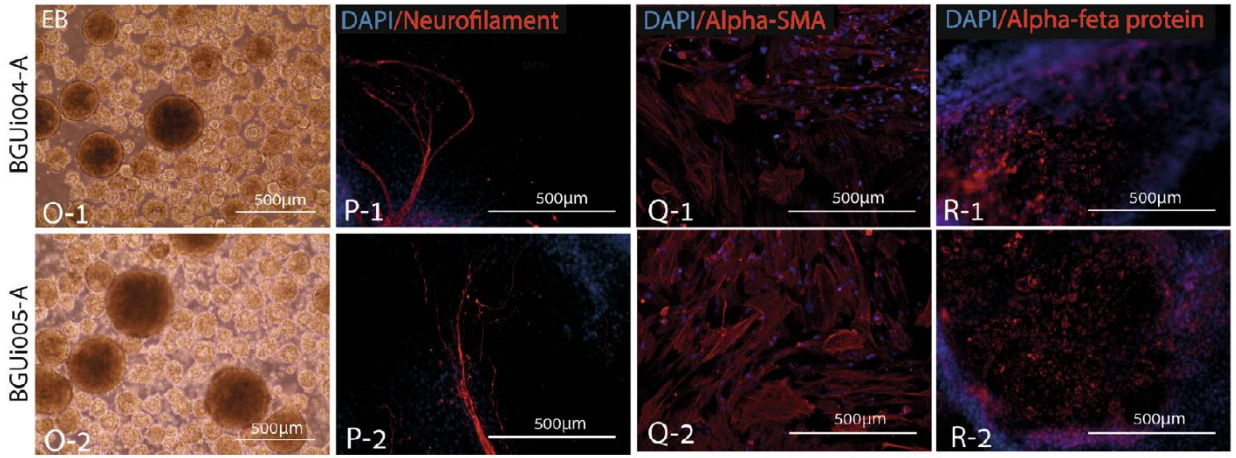




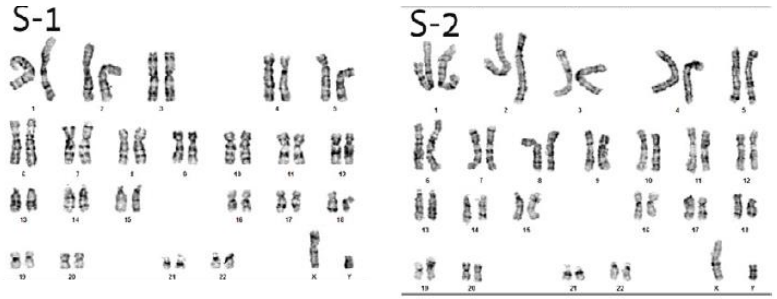
iPSCs from two 25+ CCHS patients were generated and characterized



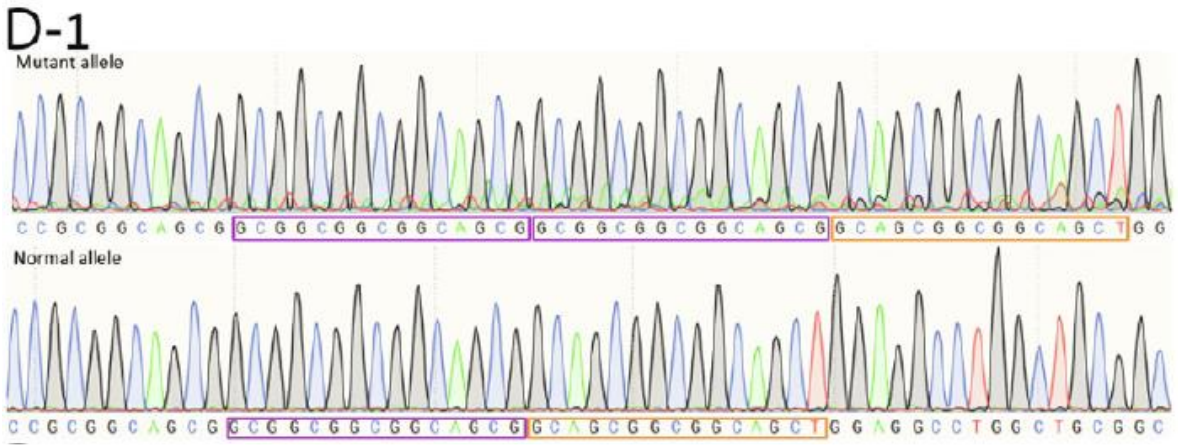
iPSCs express pluripotency markers



iPSCs can differentiated into the three germ layers



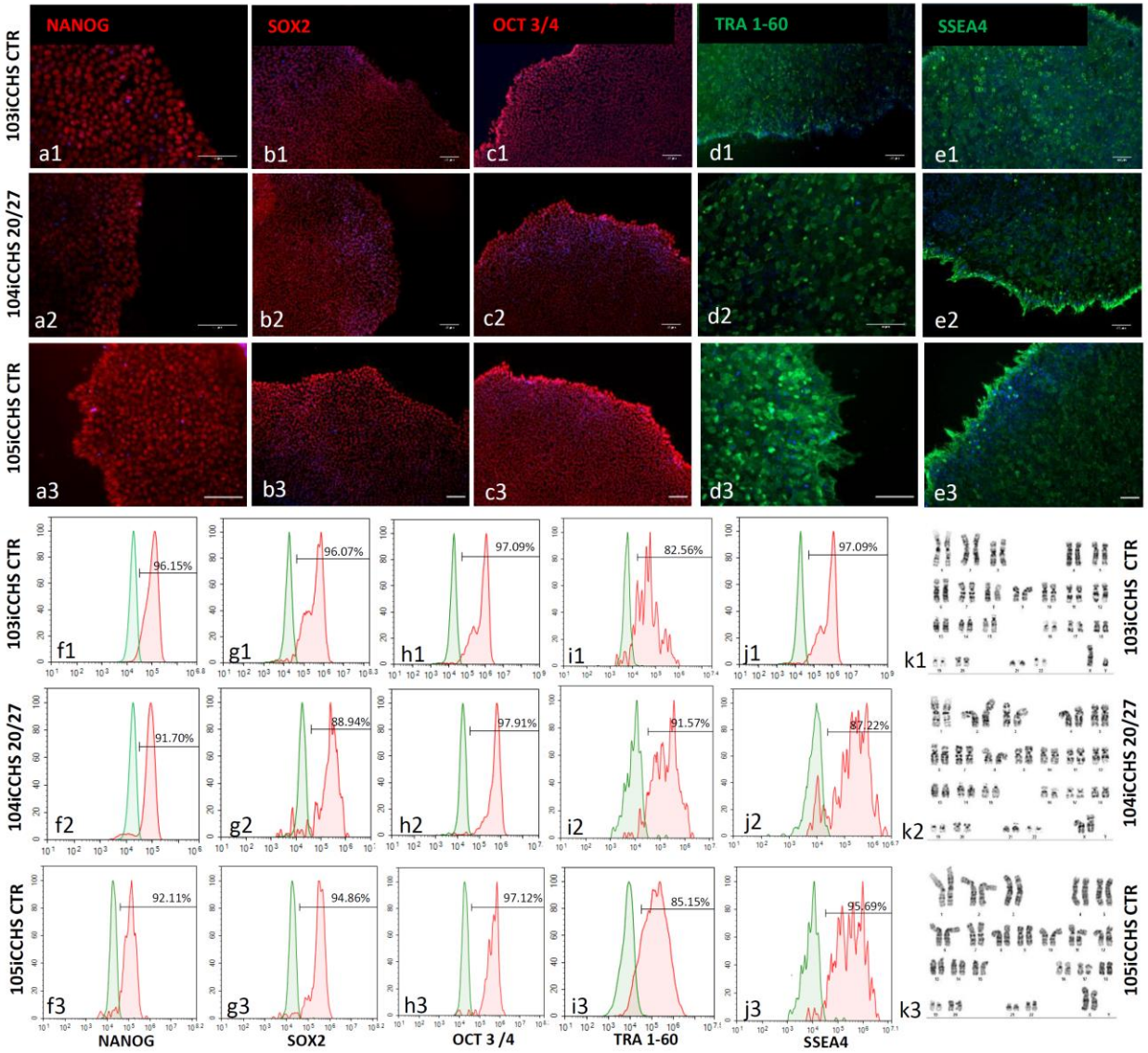
iPSCs display a normal karyotype

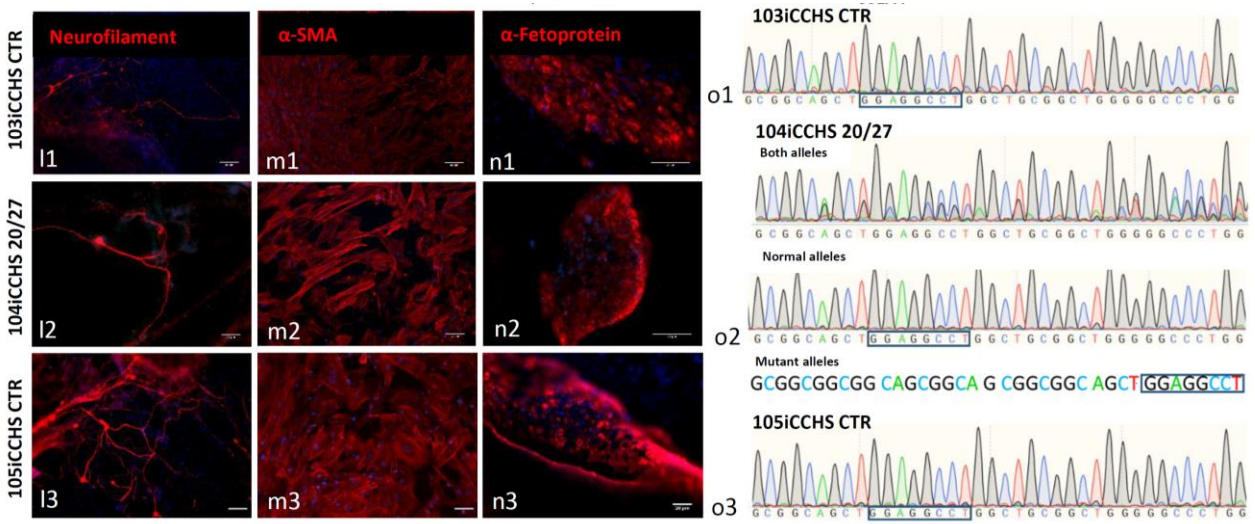


Genotyping of +5 polyalanine expansion

Falik et al., *Stem Cell Res* 2020

iPSCs from a 27+ CCHS patient and healthy controls were generated

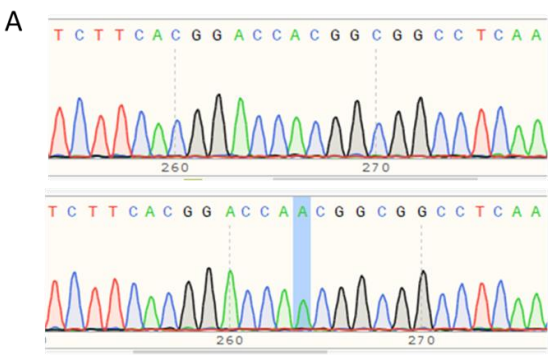


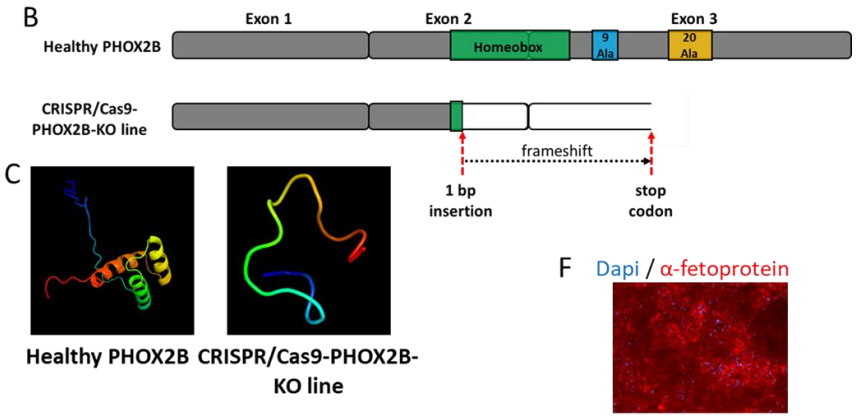


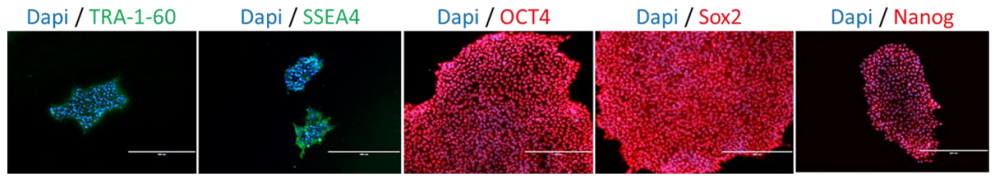
Amer-Sarsour, Falik et al., 2024

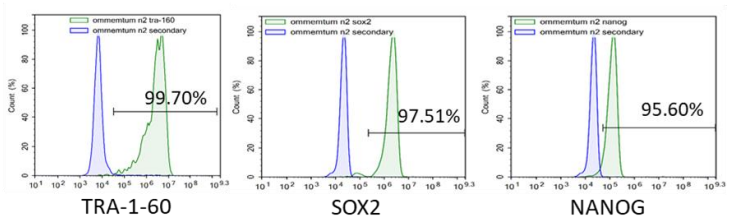
Meshi Zorski

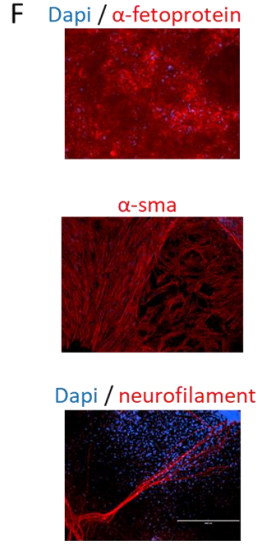
A CRISPR/Cas9-mediated isogenic iPSC line

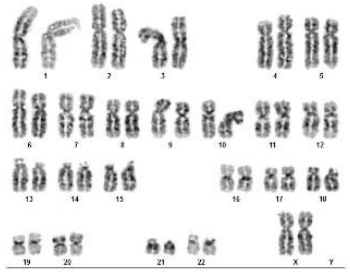










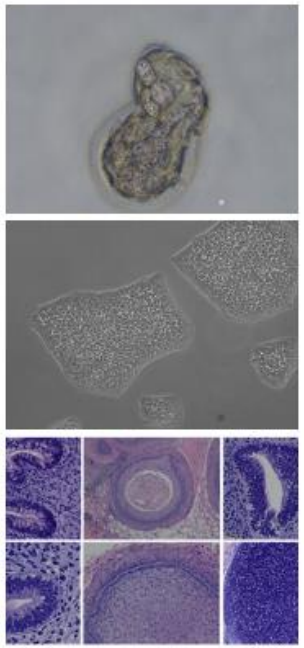


A comprehensive set of iPSCs to study CCHS

המרכז הרפואי
שערי צדק
SHAARE ZEDEK
MEDICAL CENTER
120 שנות מצוינות

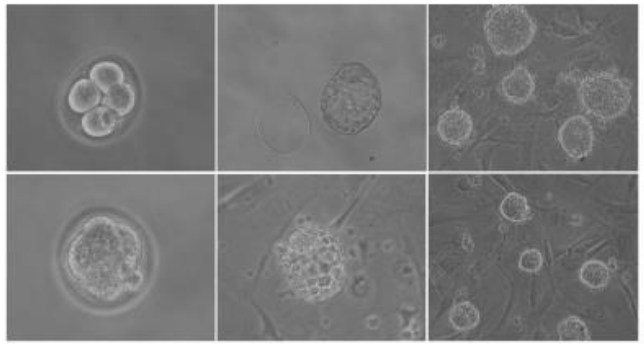
Stem Cell Research Laboratory

About | Staff | Research Topics | List of Publications | Funding | Available hESC Lines



General Introduction

The Stem Cell Research Laboratory in Shaare Zedek, headed by Dr. R. Eiges, was found in 2008 as part of the Medical Genetics Institute. The laboratory is mainly engaged with basic research studies related to genetic disorders, using mutant human embryonic stem cells as a model system. Specifically, we are focusing on the research of genetic conditions that are associated with unstable repeat expansions (microsatellites) in the DNA like fragile X syndrome, myotonic dystrophy type 1, a heritable form of ALS, as well as Dyskeratosis Congenital. In addition, it is charged with establishing and providing diseased human embryonic stem cell (HESC) lines as a universally available resource.



Principal Investigator
Prof. Rachel Eiges, PhD

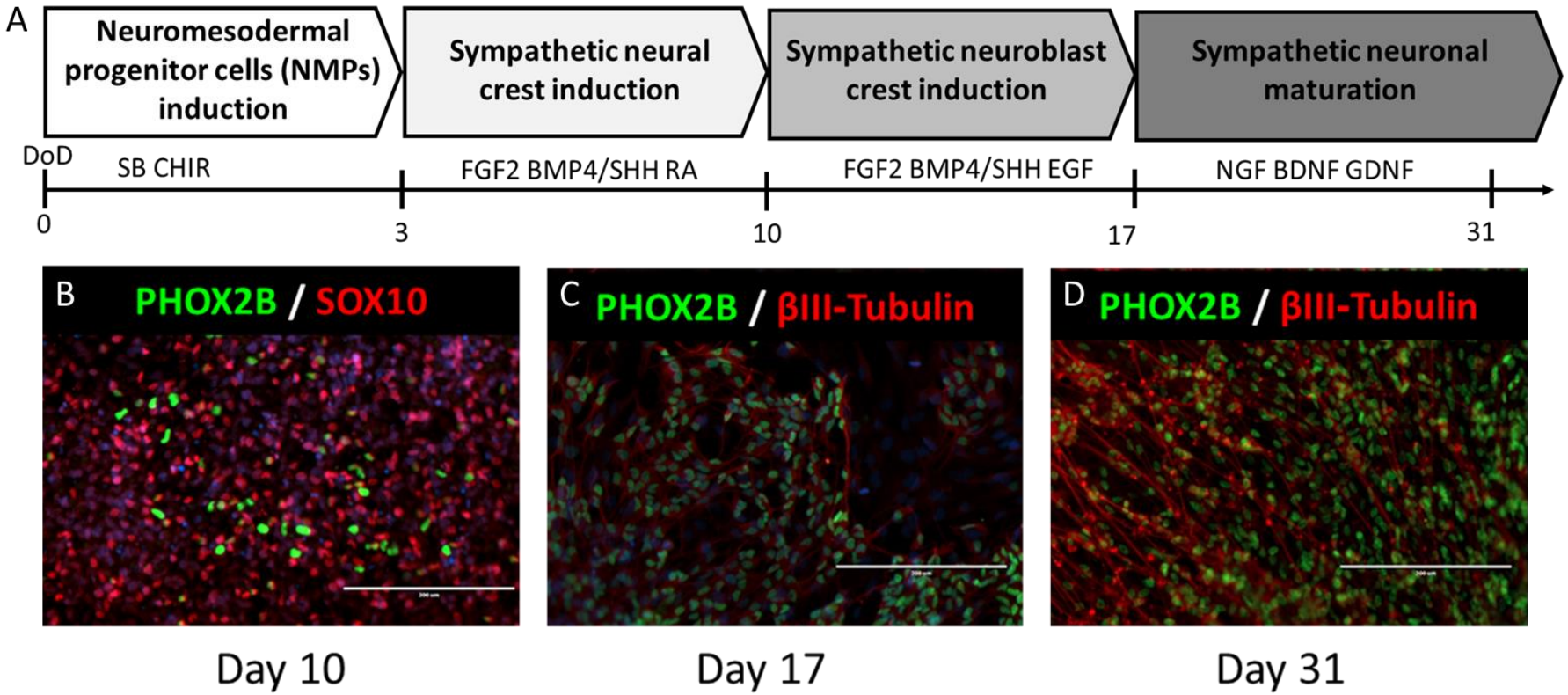
Tel: 972-2-6666721
Fax: 972-2-6666935
Email: rachela@szmc.org.il

Location
Main Building, 5th Floor

Prof Rachel Eiges

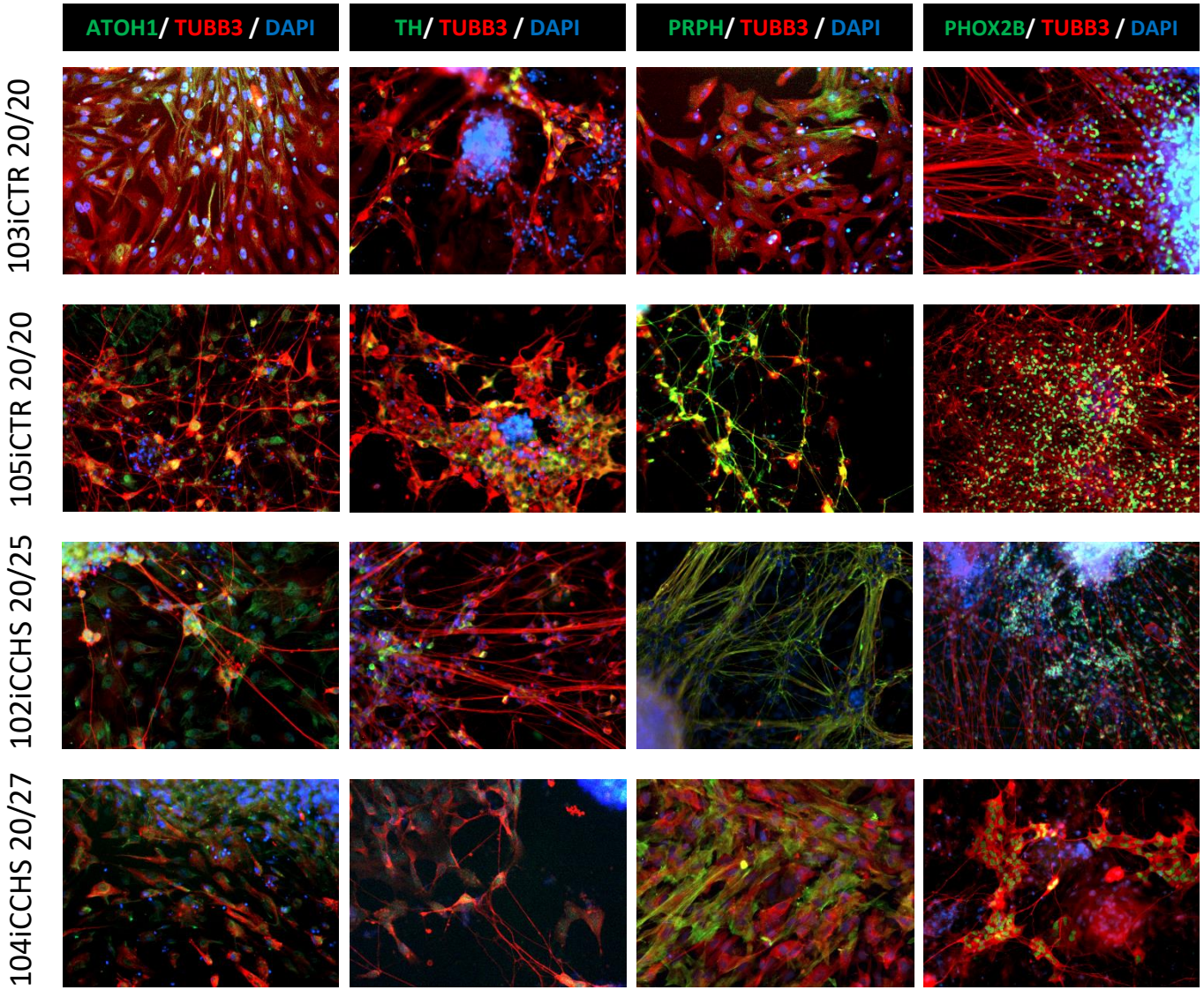
Establishment of a protocol to differentiate iPSCs into disease-relevant neurons

Dr Reut Eshel



iPSCs are differentiated into PHOX2B-positive neural crest progenitors (Day 10) and then to sympathetic neurons (Day 31)

CCHS and CTR lines differentiated into sympathetic neurons



<u>Differentiated neurons express</u>:

The pan-neuronal marker βIII-tubulin (TUBB3)

The chemosensitive marker ATOH1

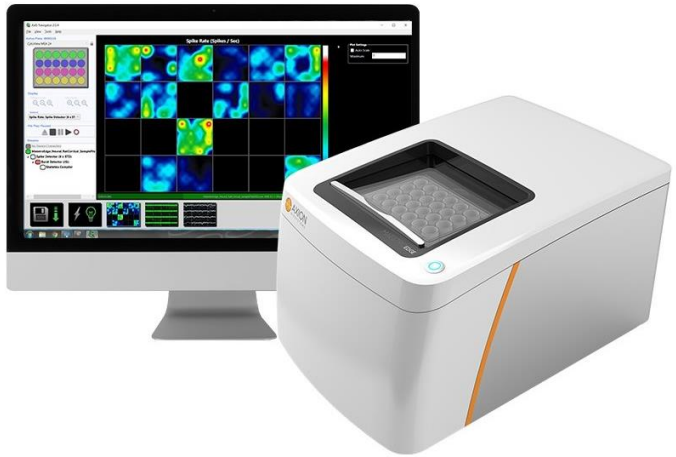
The sympathetic marker TH

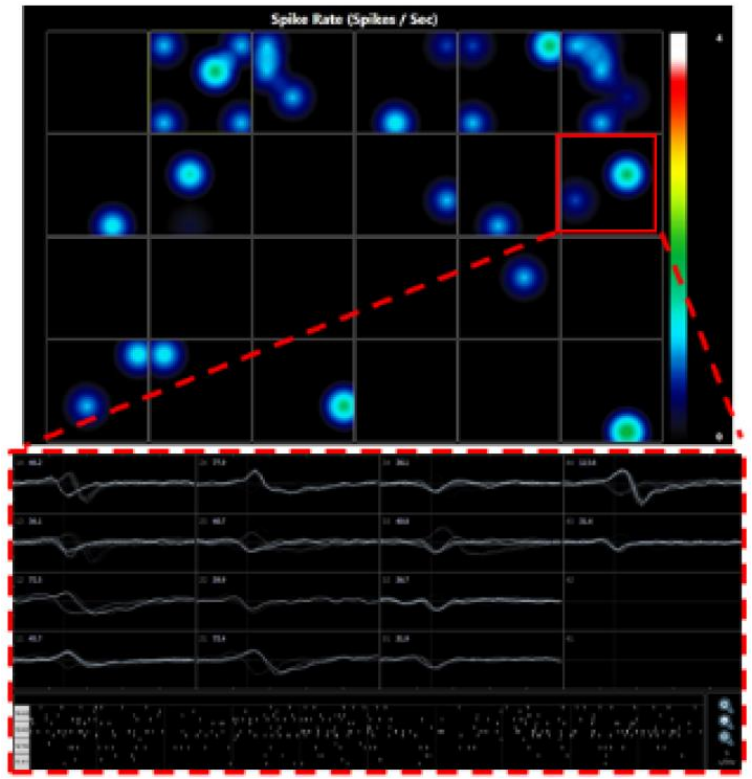
The peripheral neuronal marker PRPH

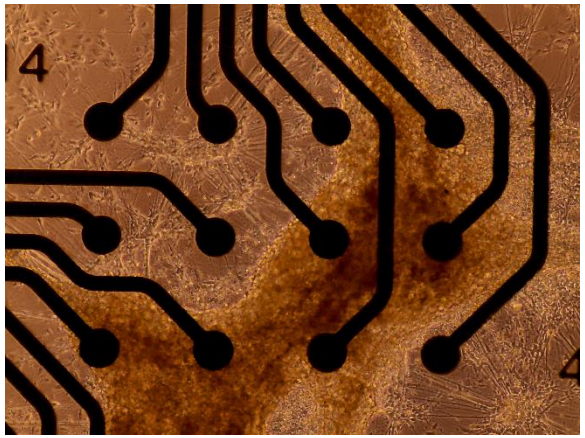
PHOX2B

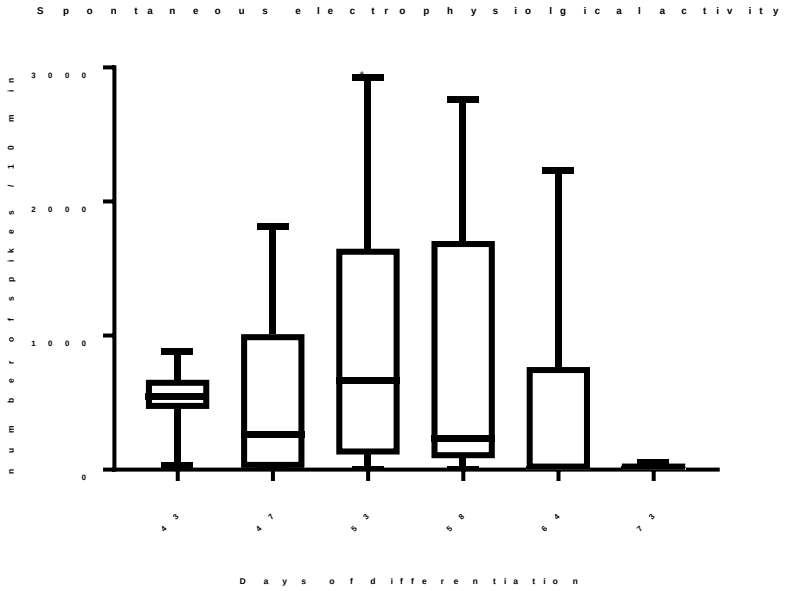
Amer-Sarsour, Falik et al., *2024*

Continuous extracellular MEA recordings of differentiating neurons









Differentiated iPSC-derived sympathetic mature to spontaneously active neurons

Stem Cell Reports
Article
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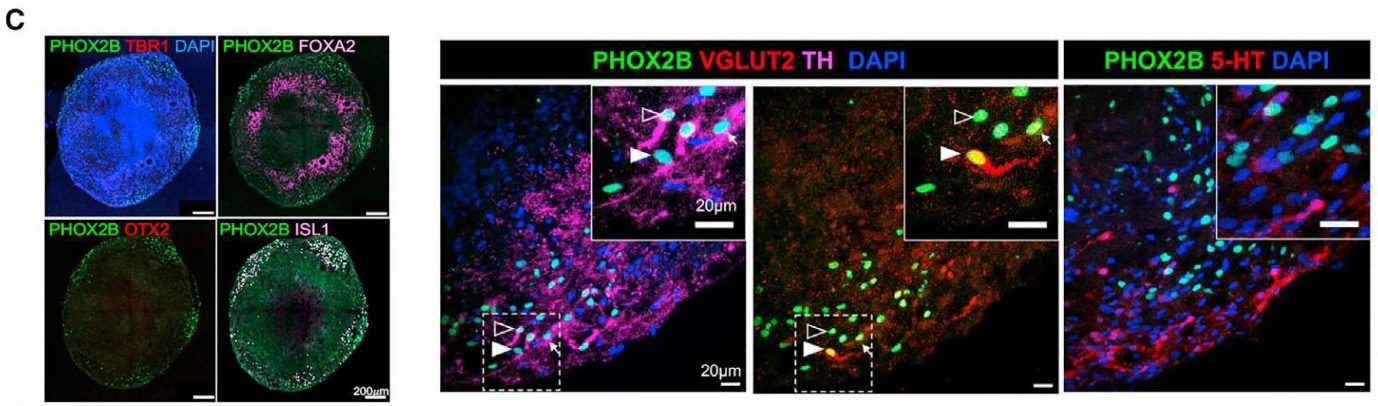
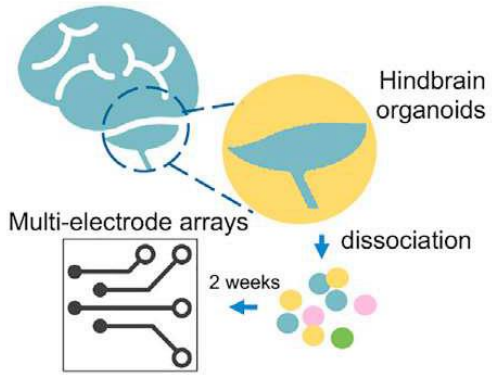
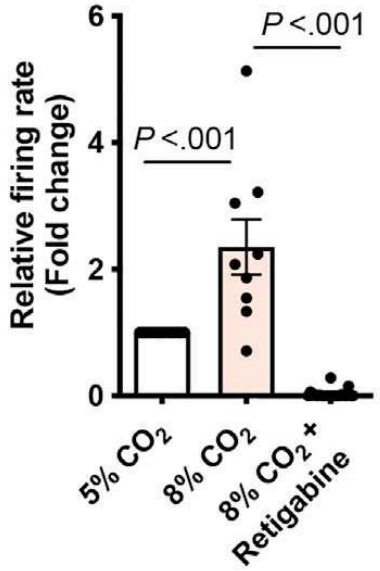
Organoid models of breathing disorders reveal patterning defect of hindbrain neurons caused by PHOX2B-PARMs

Kathy Nga-Chu Lui,[1] Zhixin Li,[1] Frank Pui-Ling Lai,[1] Sin-Ting Lau,[1] and Elly Sau-Wai Ngan[1,*]
[1]Department of Surgery, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR
*Correspondence: engan@hku.hk
https://doi.org/10.1016/j.stemcr.2023.05.020





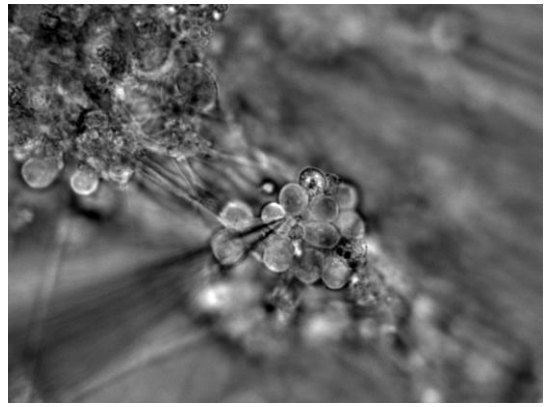


Preliminary electrophysiological characterization of CCHS-iPSC-neurons

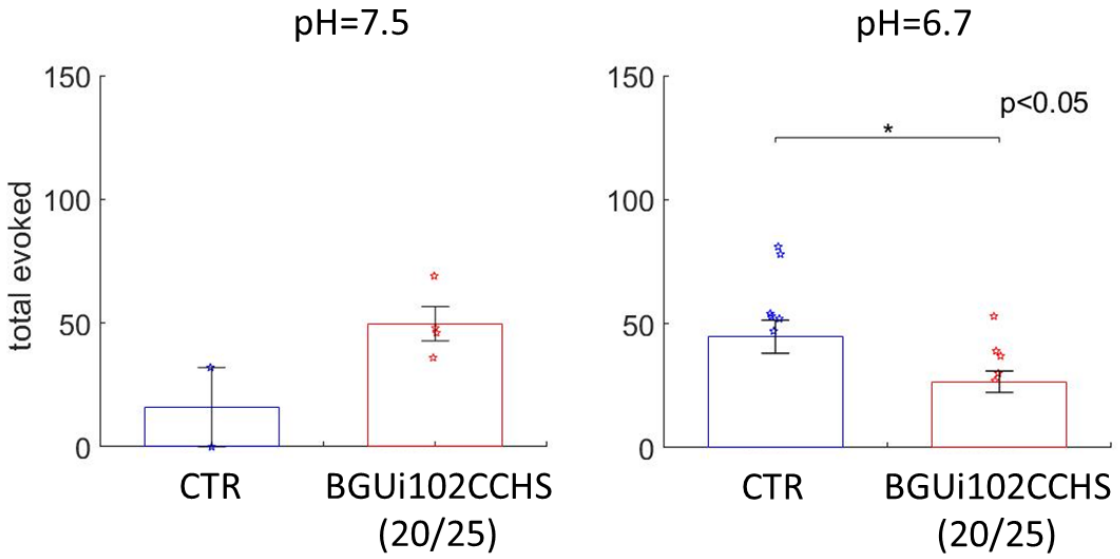
Dr Shani Stern

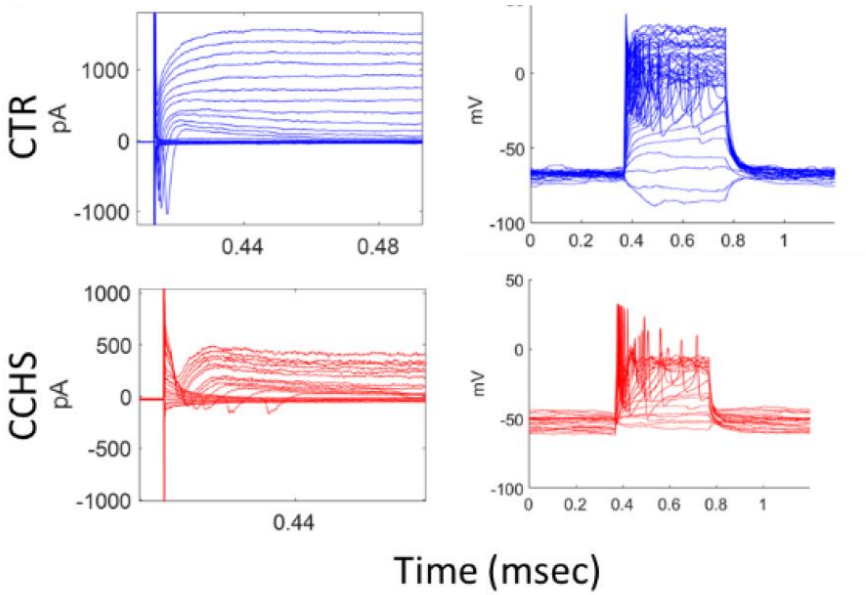
Idan Rosh

אוניברסיטת חיפה
University of Haifa
جامعة حيفا



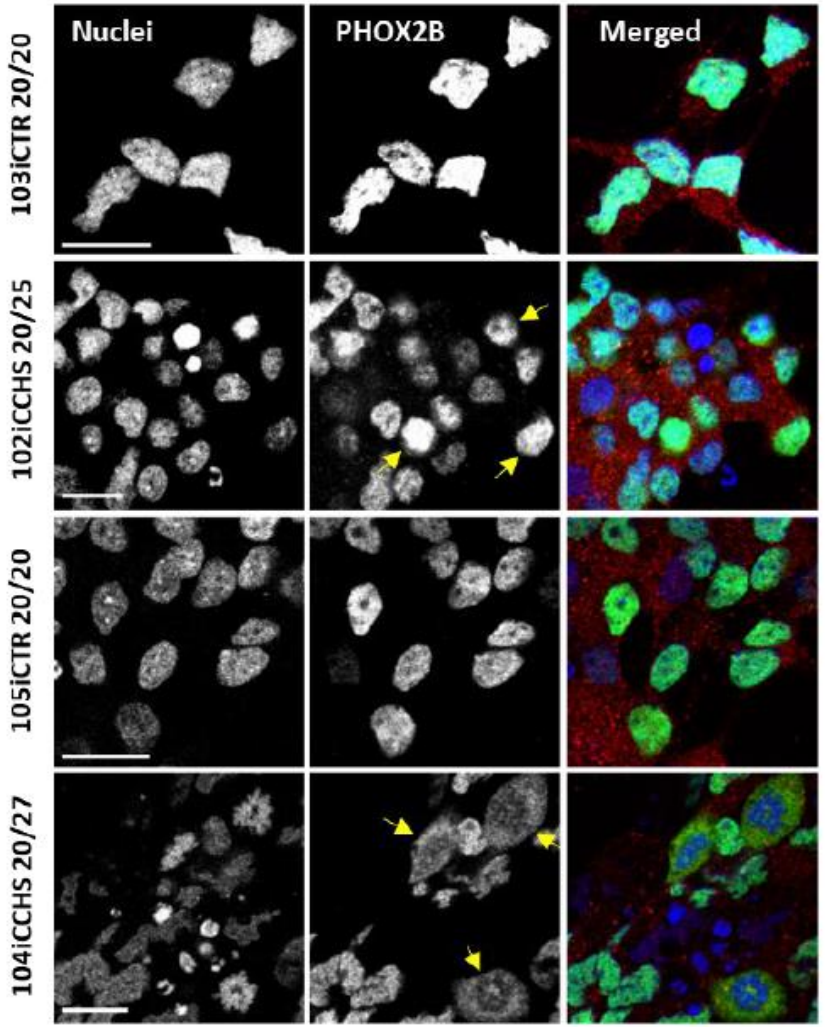
Voltage clamp in iPSC-derived neurons

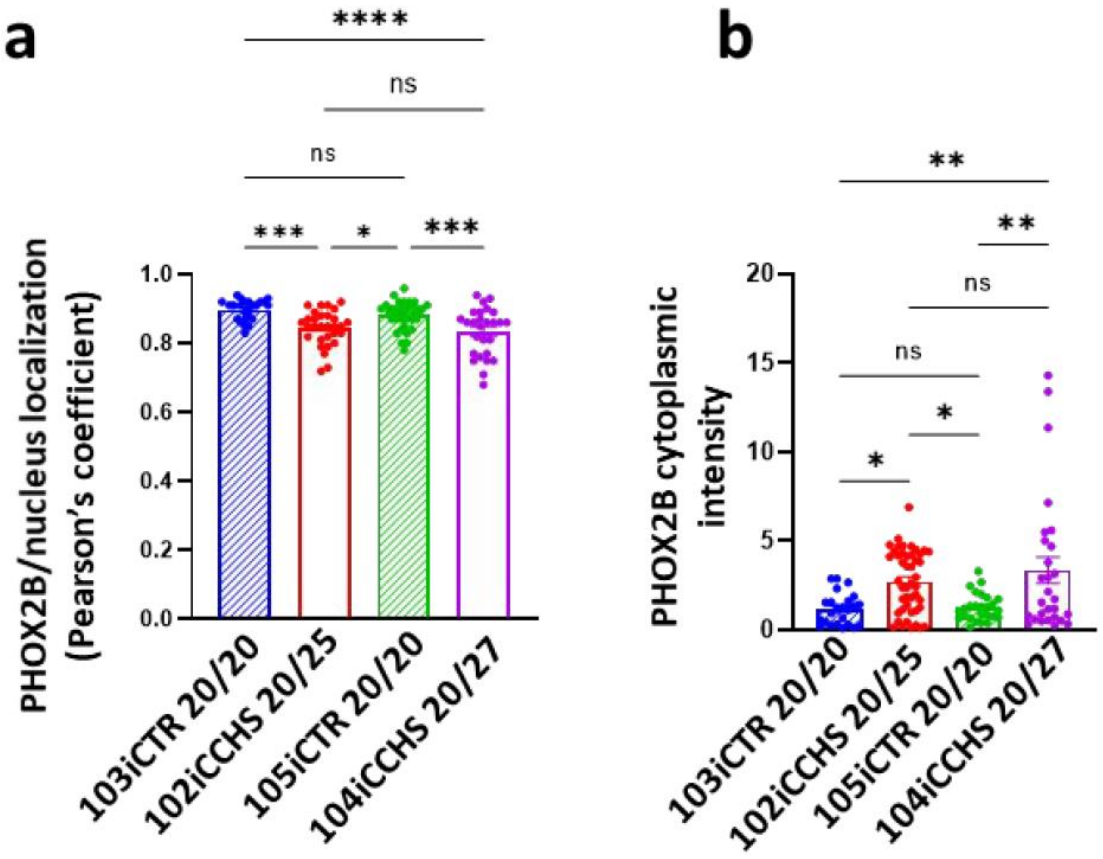




- CCHS neurons are hypo-excitable under low pH

Cellular PHOX2B mislocalization in CCHS neurons





Amer-Sarsour, Falik et al., 2024

PARMs impair PHOX2B nuclear translocation, leading to undesired cytoplasmic interactions with ubiquitin enzyme (UBA6)

The Ubiquitin system is involved in regulating protein degradation

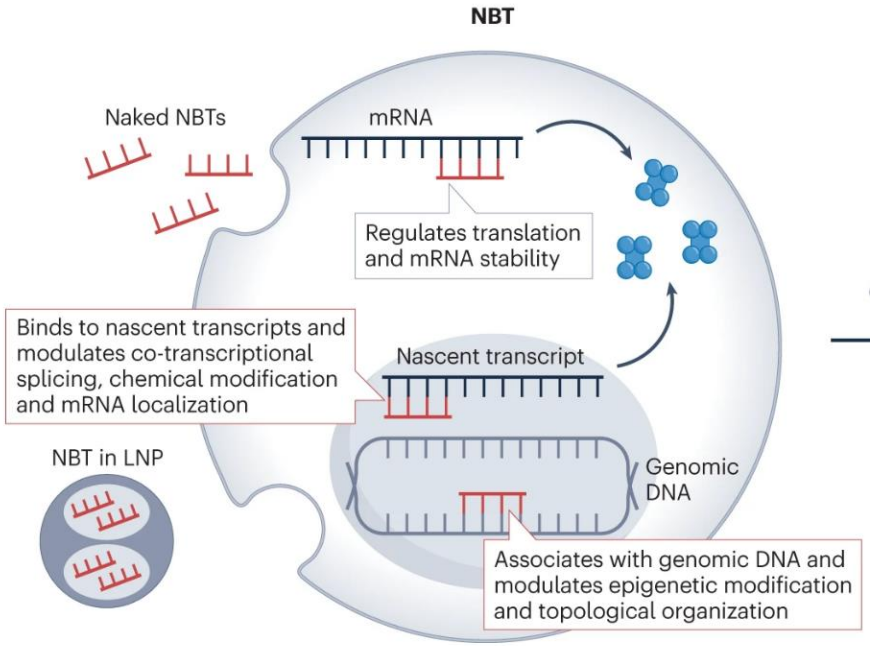
- Ubiquitin failure may lead to “traffic jams” in neurons, leading to cell death
- Exploiting the Ubiquitin system may rescue cellular toxicity

Harnessing antisense oligonucleotides (ASOs) for modulating UBA6 expression

Ori Kahiri

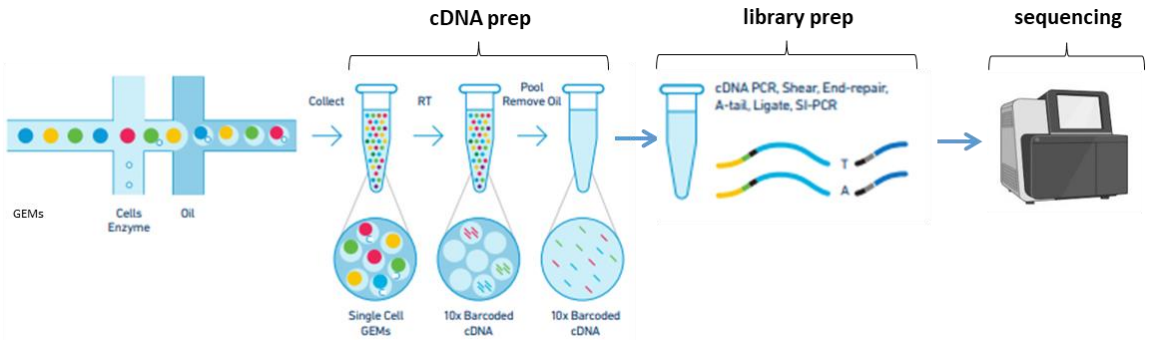
Dr Avital Adato

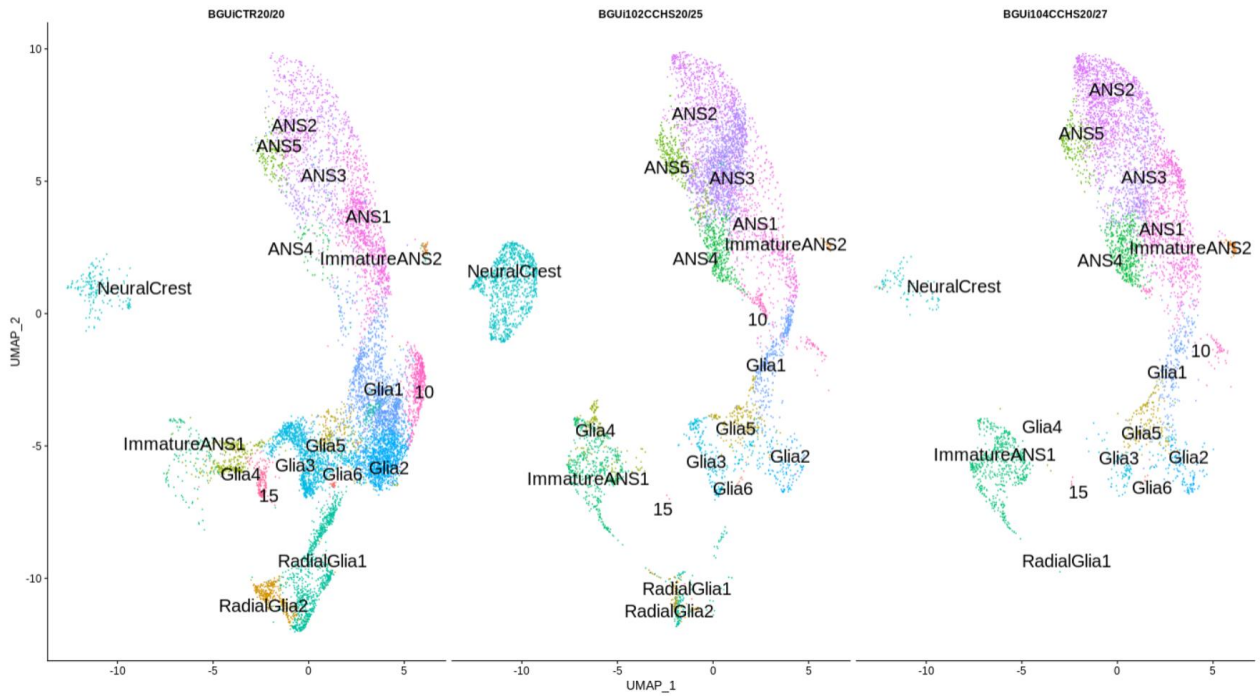
- ASOs are short synthetic single-stranded nucleotides sequence
- Modulate gene expression
- Increasingly used for ‘next-generation therapeutics’

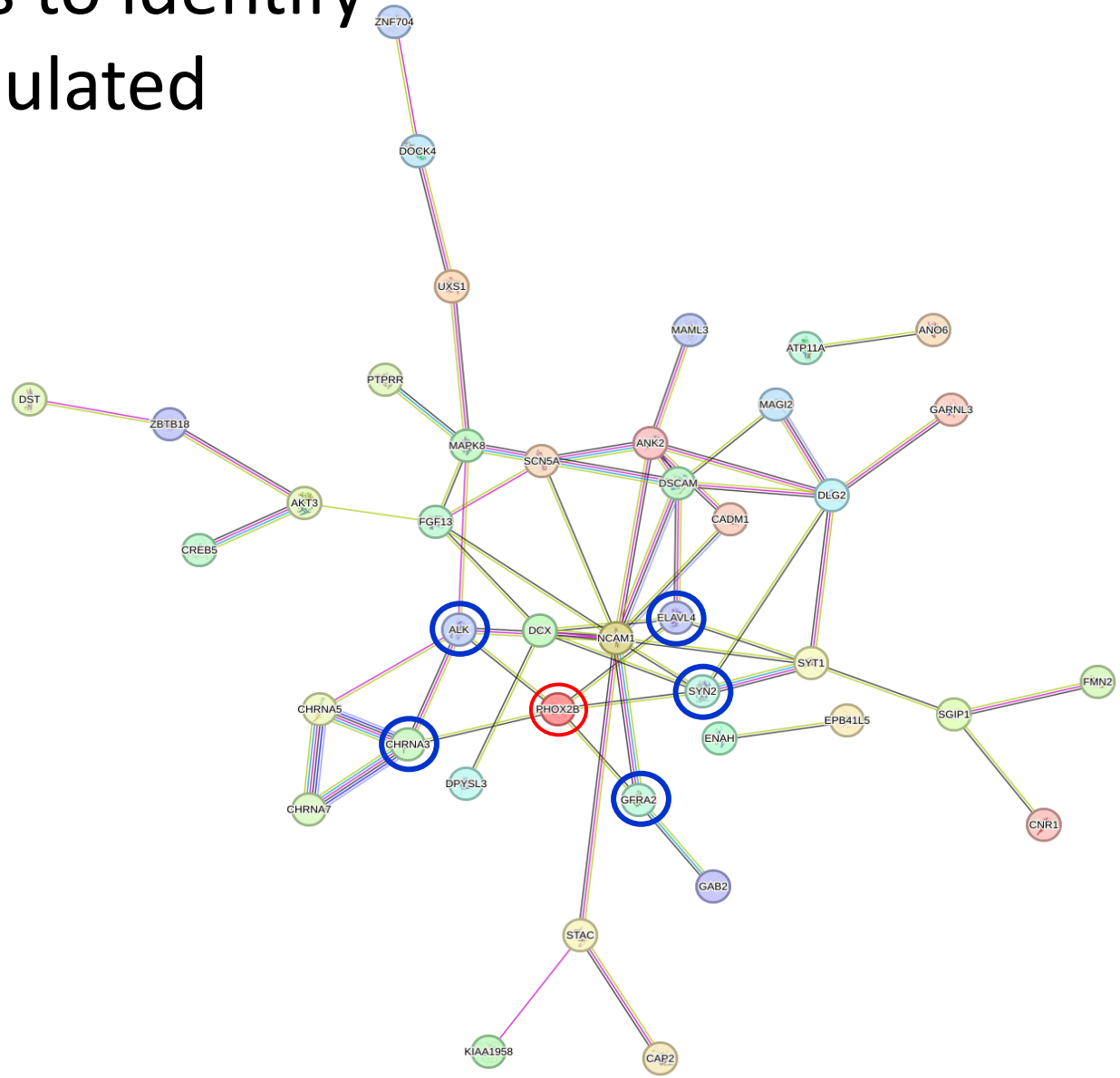


The 1st Israeli CCHS meeting

Single-cell RNA-sequencing analysis to identify affected pathways that can be modulated





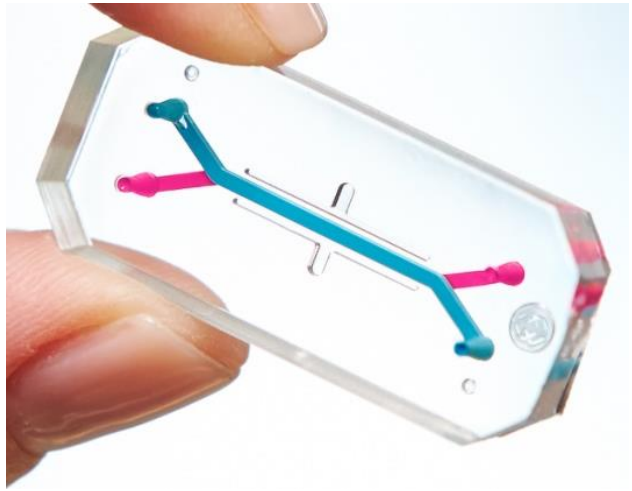


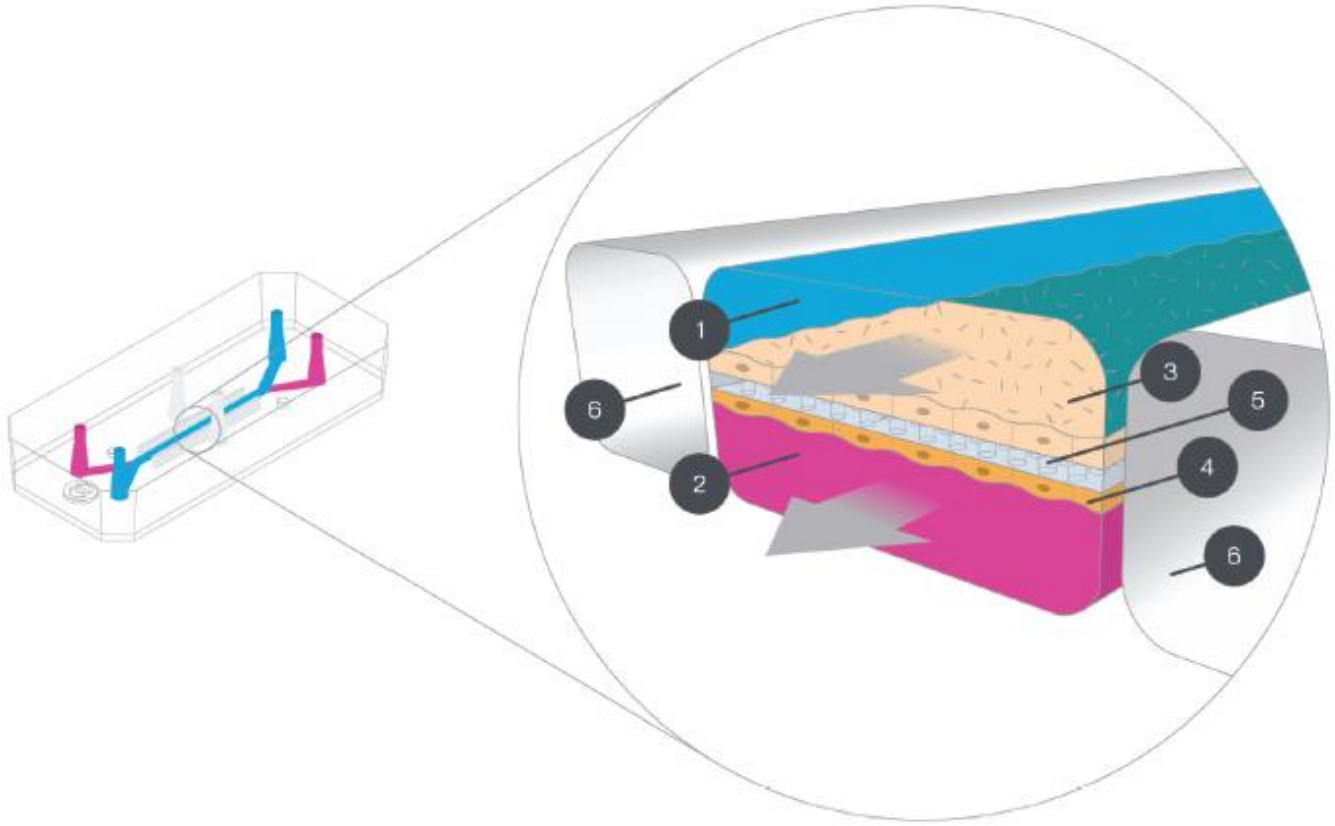
- Impaired differentiation of CCHS neurons
- Identifying affected molecular pathways

Summary

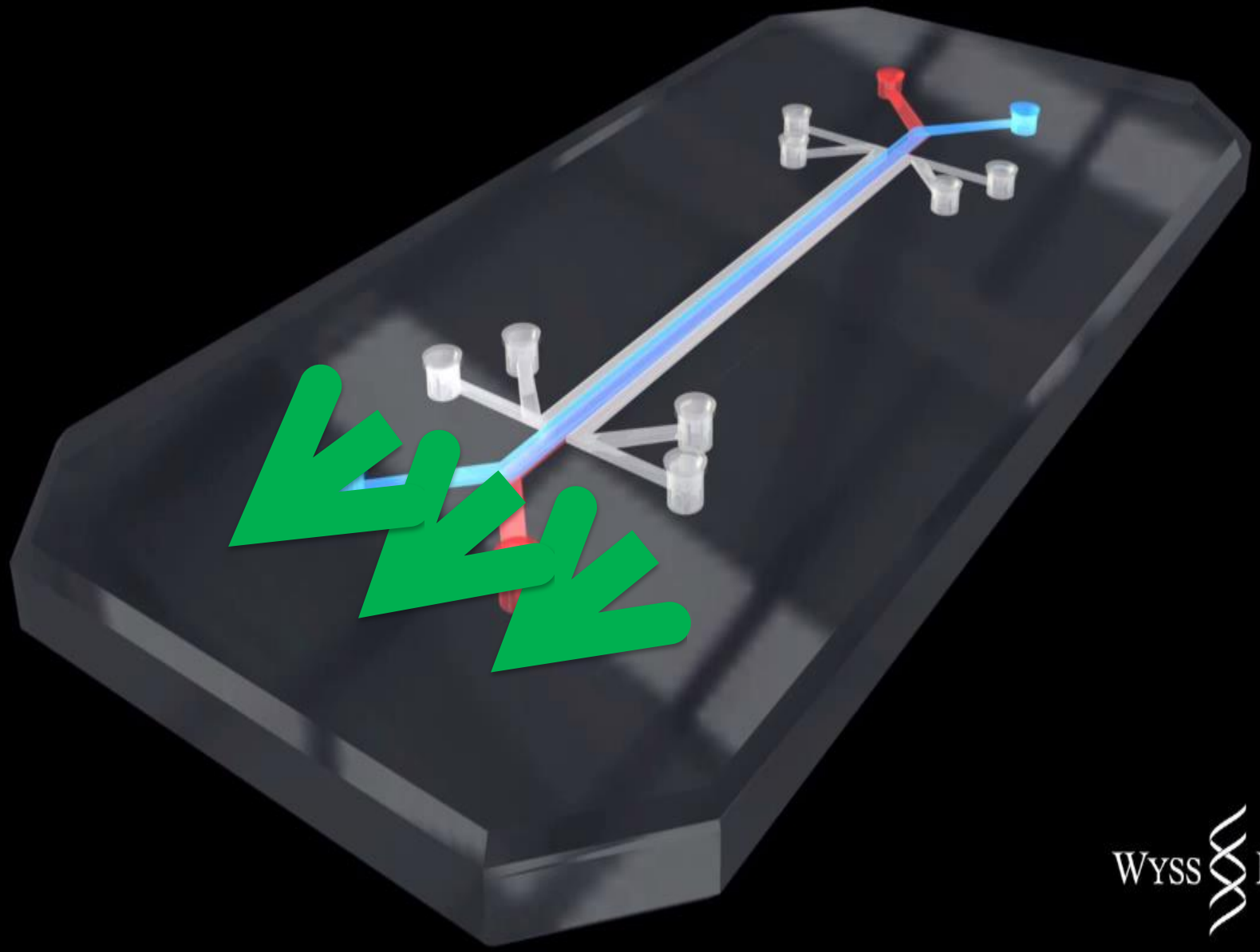
- iPSCs were generated and characterized from CCHS patients and their healthy family relatives
- iPSCs were differentiated into spontaneously active sympathetic neuron-like cells
- CCHS autonomic neurons are hypo-excitable under low pH
- PHOX2B nuclei translocation is impaired by PARMs
- Molecular analyses identified impaired PHOX2B-dependent transcriptional pathways and alterations in CCHS autonomic neurons

A microfluidic Organ-on-Chip system can mimic organ-level functionality





- Recreates intracellular interactions
- Mimics the dynamic environment
- Permits laminar perfusion – <u>not currently possible in organoids</u>



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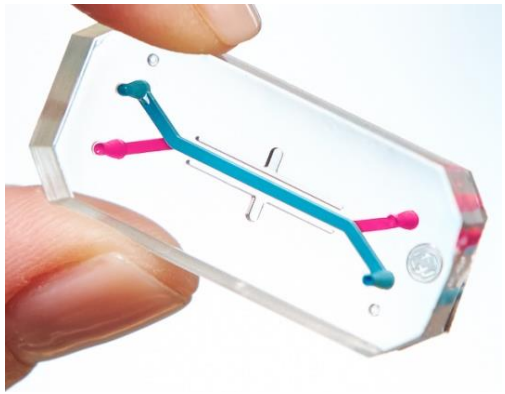
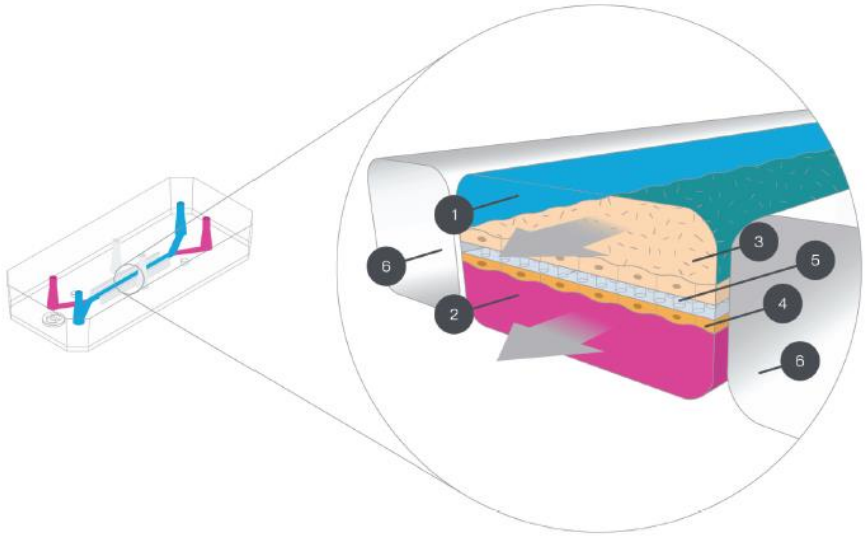
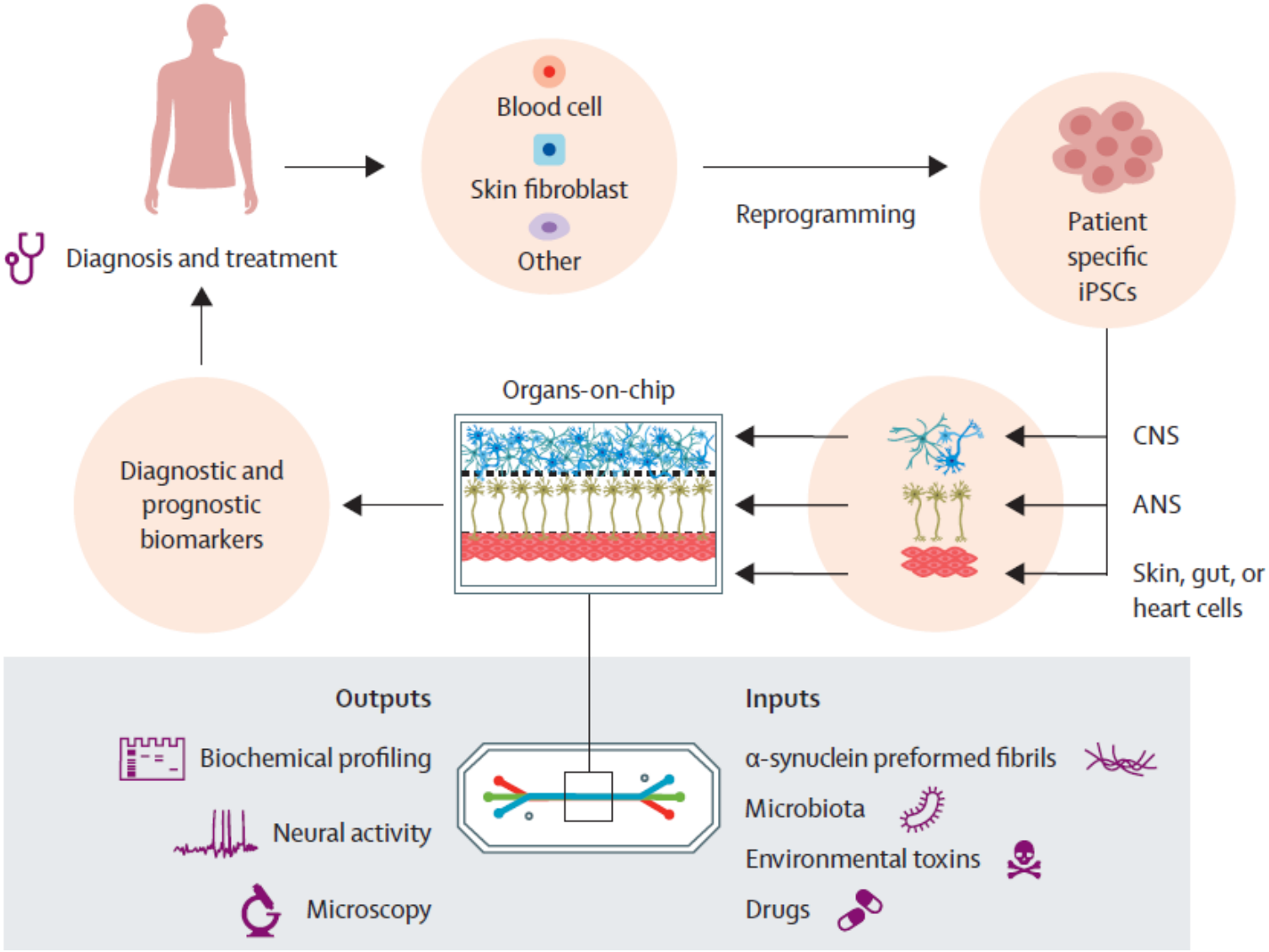
A microfluidic Organ-on-Chip system can mimic system-level functionality in the context of CCHS







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