

Twenty years on from the isolation of human embryo stem cells:

Fact, Fiction and Challenges Remaining To Realize Their Therapeutic Potential

British Blood Transfusion Society Annual Conference 2017
Scottish Event Campus, Glasgow, UK

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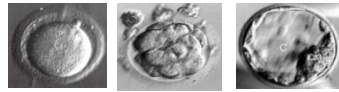
Co-founder, Chief Scientist
Roslin Cells Ltd



Disclosure

- Full-time employee of the University of Edinburgh
- Paid-consultant for
 - Roslin Cells Ltd
 - Roslin Cell Therapies Ltd
- Shareholder & non-executive director CENSO Biotechnologies

Stem Cells



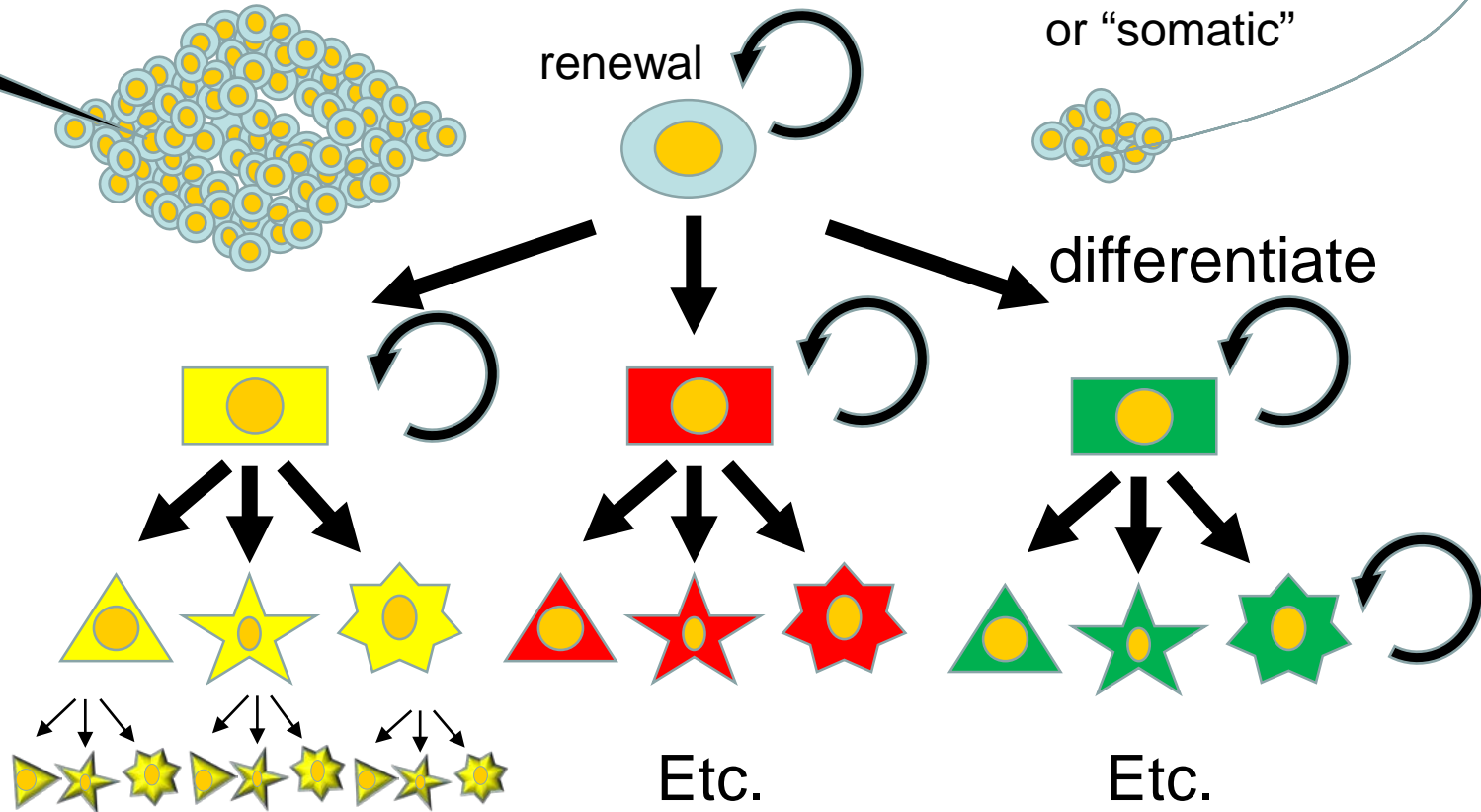
“Embryonic”



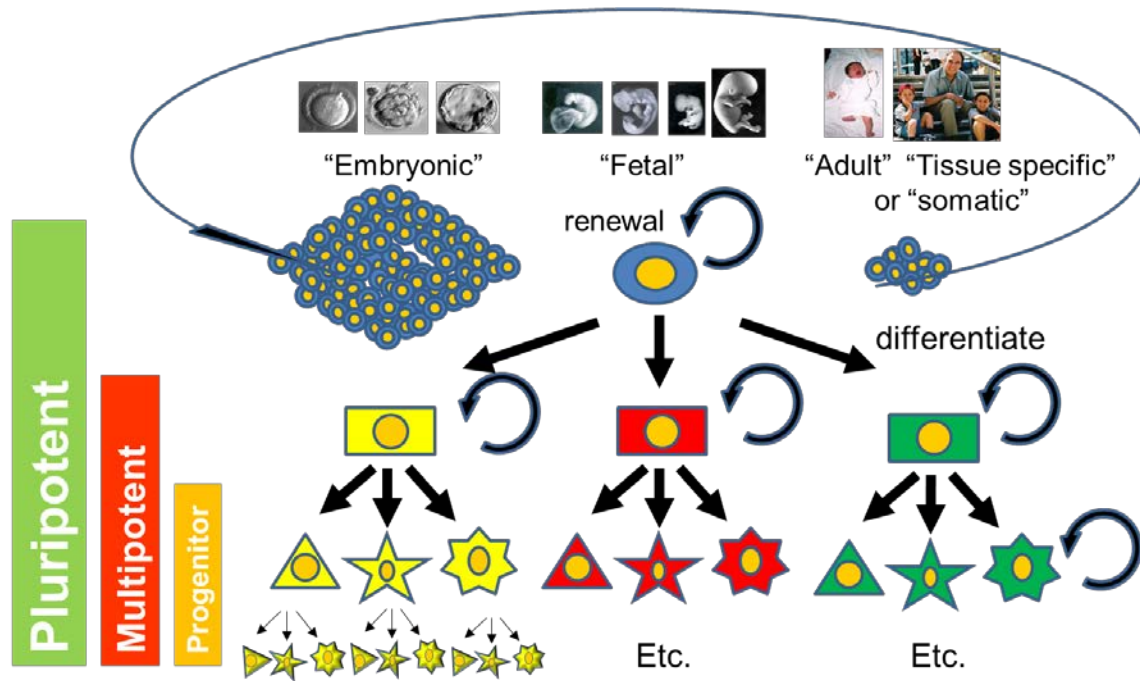
“Fetal”



“Adult” “Tissue specific”
or “somatic”



Why Pluripotent Stem Cells?



✓ Growth

✓ Differentiation

Scalable....

Renewable.....

Genotype specific.....

Otherwise inaccessible or limiting cell... supply.

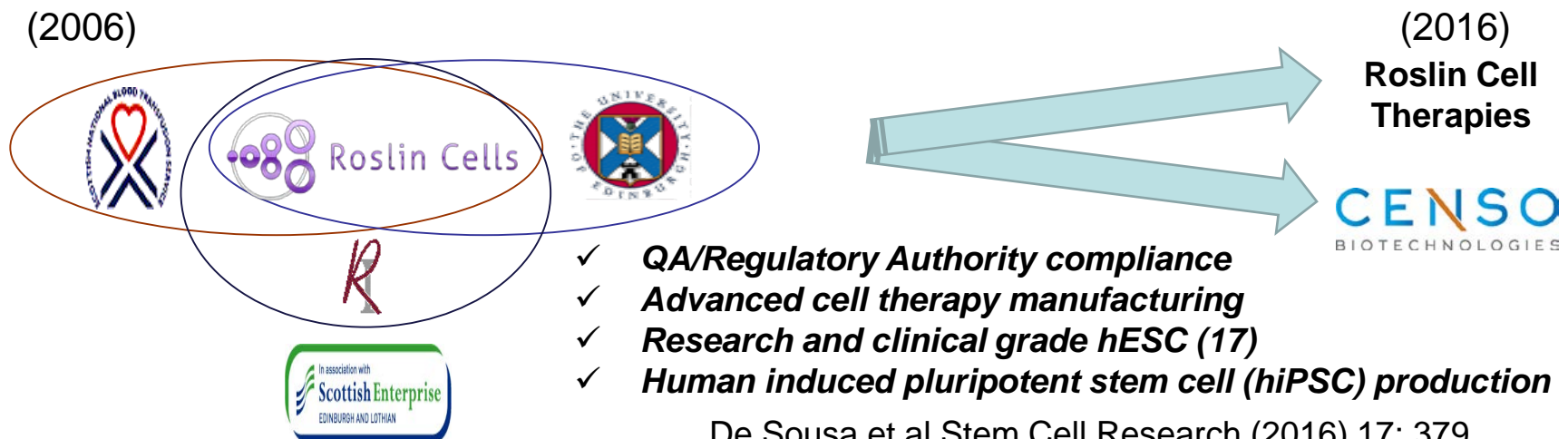
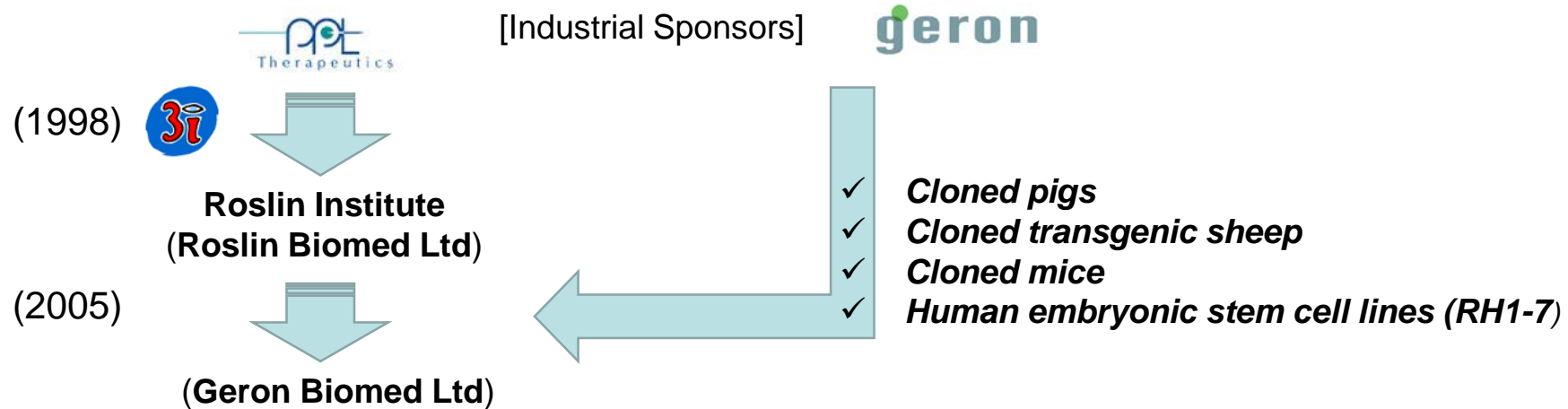
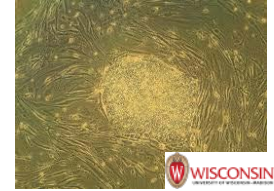


From discovery to clinical translation in Scotland, 20 years on



Viable offspring derived from fetal and adult mammalian cells. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Nature. 1997 Feb 27;385(6619):810-3.

Embryonic stem cell lines derived from human blastocysts. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Science. 1998 Nov 6;282(5391):1145-7.



hESC therapy development support

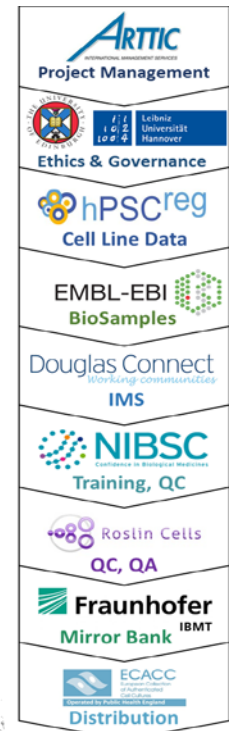
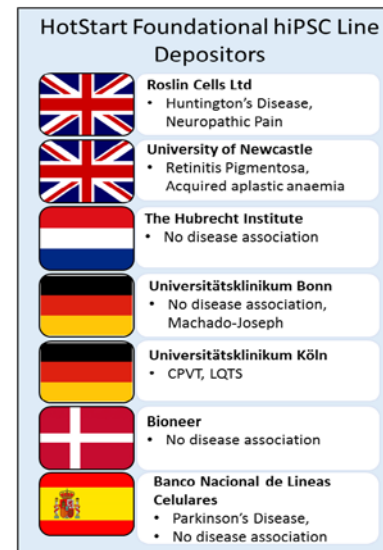
- Keratinocytes (Ulceration)
- Retinal pigmented epithelium (Macular degeneration)
- Neuronal committed progenitors (Huntingtons Disease)
- Neuronal committed progenitors (Parkinsons Disease)
- Haematopoietic progenitors
- Endothelial cells



NeuralstemcellRepair











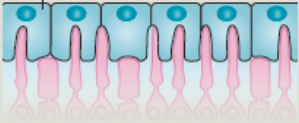






Standardised hiPSC research discovery/drug development



Distribution for academic and commercial research worldwide

Global advances in clinical evaluation of hESC based cell therapy

Disease	Age-related macular degeneration	Parkinson disease	Spinal cord injury	Diabetes	Myocardial infarction
iPSCs and/or ES cells					
Robust differentiation					
Cell type	Retinal pigment epithelium 	A9 dopaminergic neuron 	Oligodendrocyte progenitor 	Pancreatic islet β -cell progenitor 	Cardiomyocytes 
Current stage	Clinical Phase I and Phase II	Clinical Phase I	Clinical Phase I	Clinical Phase I-II	Clinical Phase I
Sponsors	8	2	1	1	1
Countries (Clinical)	US, UK, China, Korea, Brazil, Japan (<i>hiPSC</i>)	China, Australia	US	US	France
Dose/patient	$\sim 10^6$	$\sim 10^7$	$\sim 10^{7-8}$	$\sim 10^{7-8}$	$\sim 10^9$

Sources:

www.clinicaltrials.gov ; Trounson and DeWitt 2016 Nature Reviews, Molecular 17: 195-200.

And the journey taken.....

Process

Pre- Clinical

Manufacture

Clinical

Marketing

Pre-Clinical Challenges

- **Relevance of animal model** (small /large/multiple) –
 - clinical indication / route of administration / delivery device required / cell dose range / immunosuppression for engraftment
- **Bio-distribution studies** -
 - To mimic clinical administration route (labelled cells)
 - Mode of delivery (labelled cells).
- **Testing Clinical Target Material Versus Analogous Model-**
 - Matched Material (human cell in pig or analogous pig in pig)
- **Tumorigenicity** –
 - cell transformation, integration to genome
 - how to test and duration of study
- **Potency** –
 - Complex. Assay required to monitor product activity in vitro in human cells and/or in animal models.
 - Validation required for manufacture and release.
- **Defining Dose** –
 - Evidence generated from allometric scaling based on animal studies
 - Similar products may indicate feasibility of dose preparation /delivery, or early clinical data from similar types of products.
- **Sterility / Safety** –
 - microbiological testing bacteria and fungi and mycoplasma virus endotoxin free
 - Comprehensive screening of donor material
 - Comprehensive screening of donor material (virus)

Courtesy Dr Lindsay Fraser, In; Fraser, Bruce, Campbell, De Sousa. Quality assessment and production of human cells for clinical use. Methods in Molecular Biology – Huntingtons Disease (S Rowlands, S Dunnett, A Rosser Eds) Submitted.

Process

Pre- Clinical

Manufacture

Clinical

Marketing

Manufacturing challenges

- **GMP Qualification of raw materials –**
 - (COA/CoC/CoO/ TSE declaration / RA
 - Continuity of material supply (scale up)
 - Internal audit & supplier approval
- **Qualifying Clean Room Environment-**
 - (Grade D to A)
 - Impact on other CR procedures
- **Process**
 - Process validation/PST
 - Reducing variability
 - Development of GMP documentation
 - Procurement of donor samples (screening) and traceability
 - Quality related incident reporting / Change / Risk Management system
 - Scale up
 - Cryopreservation (Viability; Pre/post cryo consistency)
 - Transport
 - Shelf life/storage
 - Method of delivery and device required
- **Validating assays -**
 - Complex assays (characterisation / potency / Correlating expression to function)
 - Lack of standard controls
 - Defining release criteria / specifications

Courtesy Dr Lindsay Fraser, In; Fraser, Bruce, Campbell, De Sousa. Quality assessment and production of human cells for clinical use. Methods in Molecular Biology – Huntingtons Disease (S Rowlands, S Dunnett, A Rosser Eds) Submitted.



Pre- Clinical

Manufacture

Clinical

Marketing

The following parameters must be investigated;

- **Pharmacodynamics –**

- Appropriate functional test to demonstrate intended effect of the cell therapy.

- **Pharmacokinetics –**

- absorption, distribution, metabolism, and excretion studies.
- viability monitoring, proliferation/differentiation/distribution in the recipient, migration and functionality throughout the life span of the therapy.

- **Dose finding studies –**

- Based on pre-clinical study during product development
- Dose may be linked to the patient characteristics (variability)
- Identify minimal effective / optimal effective dose / maximal dose

- **Clinical efficacy –**

- Demonstration of efficacy in the target patient with clinically relevant specifications
- Determination of appropriate dose schedule with optimal therapeutic effect.
- Balance benefit versus cost to the recipient

- **Clinical safety –**

- Detection of adverse events
- Adequate patient follow-up
- Consider effect of repeat infusions

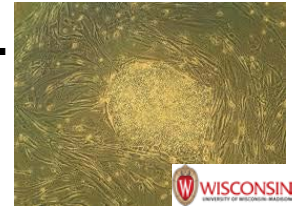
- **Pharmacovigilance and Risk management Plan –**

- Develop a Risk Management Plan (RMP)

Returning to the science of the beginning, where are we today?

Embryonic stem cell lines derived from human blastocysts.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM.
Science. 1998 Nov 6;282(5391):1145-7.



- (Resemblance to Mouse Embryonic Stem Cells)
- Derived from pre-/peri-implantation embryo
- Prolonged undifferentiated proliferation
- Immortal (high Telomerase)
- Karyotypic stability
- Stable developmental potential (3 germ layers, + extraembryonic)
- Non-clonal (potential variation in lineage potency)

Pluripotency States – naïve to primed

Pluripotent cells in developing embryos

Pluripotency is a transient state *in vivo*. It is acquired within the ICM of developing pre-implantation blastocysts, when cells of the ICM segregate into PE and pluripotent pre-implantation naive epiblasts, and is gradually lost during early post-implantation development, before cells differentiate into somatic lineages. This transition from a pre-implantation pluripotent state to a post-implantation pluripotent state, which are referred to as naive and primed states, respectively, is associated with changes in molecular and functional characteristics.

Differences between human and mouse pre- and post-implantation embryos may be reflected by the different characteristics of naive and primed pluripotent cells *in vitro* and by the different requirements for their maintenance.

Gene expression in pre-implantation epiblasts

Gene	Human	Mouse
KLF2	No	Yes
KLF17	Yes	No
ERAS	No	Yes
XIST	Low	No
DNMT3L	High	Low

Lacks extra-embryonic ectoderm and ectoplacental cone

Flat disc shaped (like most other mammals)

Epiblast

Extra-embryonic endoderm

TE

Post-implantation embryo (E9–E12)

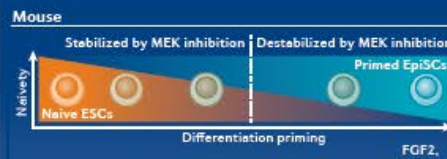
Naive and primed properties of pluripotent cells *in vitro*

Naive and primed states can be classified on the basis of multiple characteristics that each state can retain *in vitro*. Different combinations of exogenous factors confer distinct characteristics to pluripotent stem cells *in vitro*. As a result, cells acquire a distinct set of naive and primed properties. In mice, ESCs cultured in a medium supplemented with 2i (two inhibitors of MEK and GSK3) and LIF, and EpiSCs cultured in a medium containing FGF2 and activin A, constitute the two extremes of the naive and primed pluripotency spectrum; cells maintained in other media are in 'intermediate states' that display a mixture of naive and primed features. Human 'conventional' ESCs, which are considered to be 'primed', are distinct from mouse primed EpiSCs and have various naive features. Optimizing conditions to derive and maintain human naive cells with properties identical to mouse naive pluripotent cells is an ongoing challenge. Moreover, primed cells can be stabilized in a distinct pluripotent state in the presence of FGF2 and WNT inhibitors.

Pluripotency-associated property

Naive

Primed



Naive	Primed
2i, LIF	FGF2, activin A or FGF2, TGFβ
FBS, LIF	
SRG1, MEK1, GSK3βi, LIF	
BMP4, LIF	
GSK3βi, AXINs	

Mouse	Rat	Human	Rhesus
Naive ESCs	2i, LIF, MEK1, PKCi	2i, LIF, p38i, JNKi, PKCi, ROCKi, FGF2, activin A or FGF2, TGFβ	2i, LIF, p38i, JNKi, PKCi, ROCKi, FGF2, activin A or FGF2, TGFβ
Primed EpiSCs	FGF2, activin A or FGF2, TGFβ	2i, LIF, p38i, JNKi, PKCi, ROCKi, FGF2, activin A or FGF2, TGFβ	2i, LIF, p38i, JNKi, PKCi, ROCKi, FGF2, activin A or FGF2, TGFβ

Human

Mouse

Zygote

Morula

Early blastocyst

ICM

TE

Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

Post-implantation embryo (E9–E12)

Epiblast

PE

TE

Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

Post-implantation embryo (E9–E12)

Epiblast

PE

TE

Naive ESCs

Primed conventional ESCs

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Epiblast

PE

TE

Naive ESCs

Primed conventional ESCs

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Post-implantation embryo (E9–E12)

Epiblast

PE

TE

Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

Post-implantation embryo (E9–E12)

Epiblast

PE

TE

Naive ESCs

Primed conventional ESCs

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PE

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Epiblast

PE

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Naive ESCs

Primed conventional ESCs

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Epiblast

PE

TE

Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

Post-implantation embryo (E9–E12)

Epiblast

PE

TE

Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

Post-implantation embryo (E9–E12)

Epiblast

PE

TE

Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

Post-implantation embryo (E9–E12)

Epiblast

PE

TE

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Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

Post-implantation embryo (E9–E12)

Epiblast

PE

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Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

Post-implantation embryo (E9–E12)

Epiblast

PE

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Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

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Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

Post-implantation embryo (E9–E12)

Epiblast

PE

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Naive ESCs

Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

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Naive ESCs

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Primed conventional ESCs

Pre-implantation blastocyst (E6–E7)

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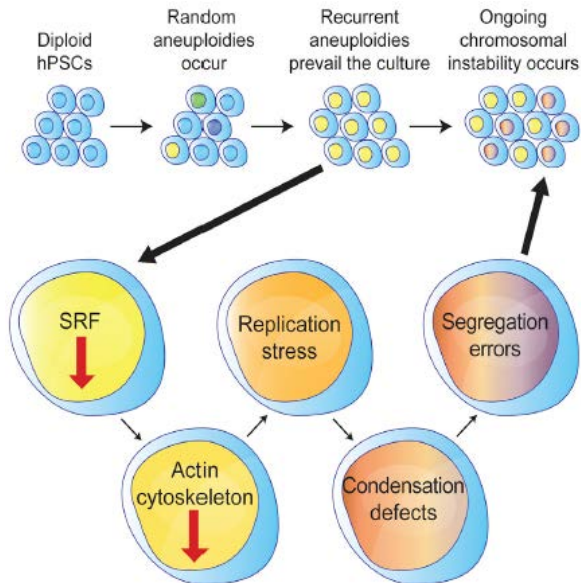
Epiblast

Genetic (*In*)stability

Cell Stem Cell

Genomic Instability in Human Pluripotent Stem Cells Arises from Replicative Stress and Chromosome Condensation Defects

N Lamm, U Ben-David, T Golan-Lev, Z Storchova, N Benvenisty, B Karem



- Aneuploid hPSCs exhibit replication stress resulting in condensation defects
- Partially condensed chromosomes lead to segregation errors in aneuploid hPSCs
- Levels of actin genes and their common regulator SRF in aneuploid hPSCs are decreased
- Cytoskeleton impairment perturbs replication and drives ongoing instability

Cell Stem Cell (2016) 18, 253-61

LETTER

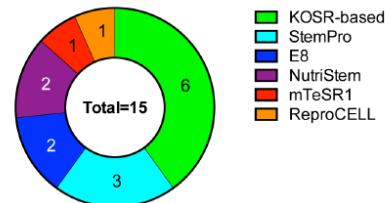
doi:10.1038/nature22312

Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations

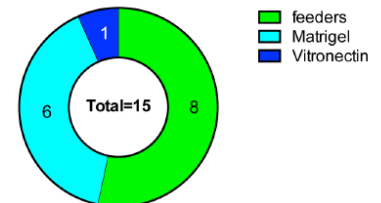
Florian T. Merkle^{1,2,3,4,*†}, Sulagna Ghosh^{1,2,3,4*}, Nolan Kamitaki^{3,5,6}, Jana Mitchell^{1,2,3,4}, Yishai Avior⁷, Curtis Mello^{3,5,6}, Seva Kashin^{3,5,6}, Shila Mekhoubad^{1,2,4†}, Dusko Ilic⁸, Maura Charlton^{1,2,3,4}, Genevieve Saphier^{1,3,4}, Robert E. Handsaker^{3,5,6}, Giulio Genovese^{3,5,6}, Shiran Bar⁷, Nissim Benvenisty⁷, Steven A. McCarroll^{3,5,6} & Kevin Eggan^{1,2,3,4}

- Whole exome sequence 140 hESC lines (26 GMP):
 - Six TP53 mutations in 5 unrelated lines
- Data mining published RNAseq of 117 hESC lines
 - Nine TP53 mutations

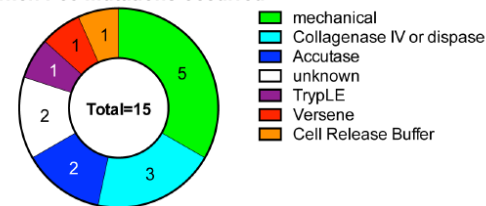
a Tissue culture media in which P53 mutations occurred



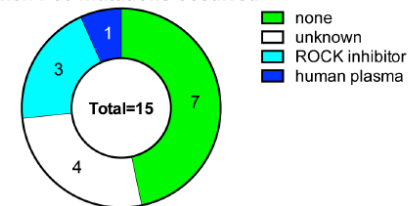
b Tissue culture substrates in which P53 mutations occurred



c Cell passaging method used when P53 mutations occurred



d Cell passage supplement used when P53 mutations occurred



Nature (2017) 545, 229-61

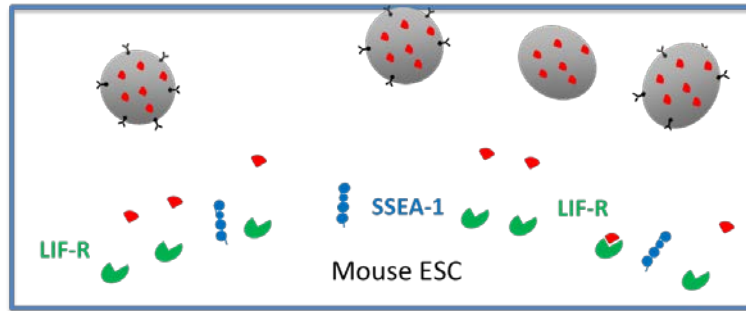
Pluripotent Stem Cell Production Hurdles

- Purified biologicals (variable/adventitious pathogens)
- Dynamic instability (cellular heterogeneity)
- Artefactual entity/culture (autocrine/paracrine signalling?).
- Costly (daily replenishment of bio-active factors)
- Non-scalable tools
- Immaturity (fetal equivalence) of differentiated derivatives
- Lack of predictive biomarker of potency

Production Solutions (De Sousa lab 2006-2016)



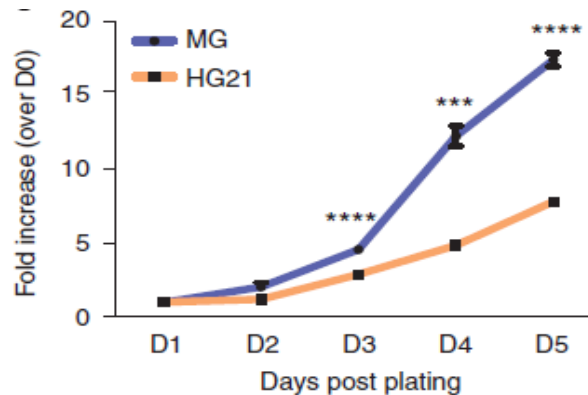
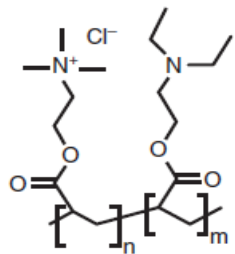
Nanoparticle mediated paracrine stimulation



SELF RENEWAL

Biomaterials 2012; 33 (28): 6634.

Thermomodulatable polymer substrates



Nature Comm 2013; 4: 1335

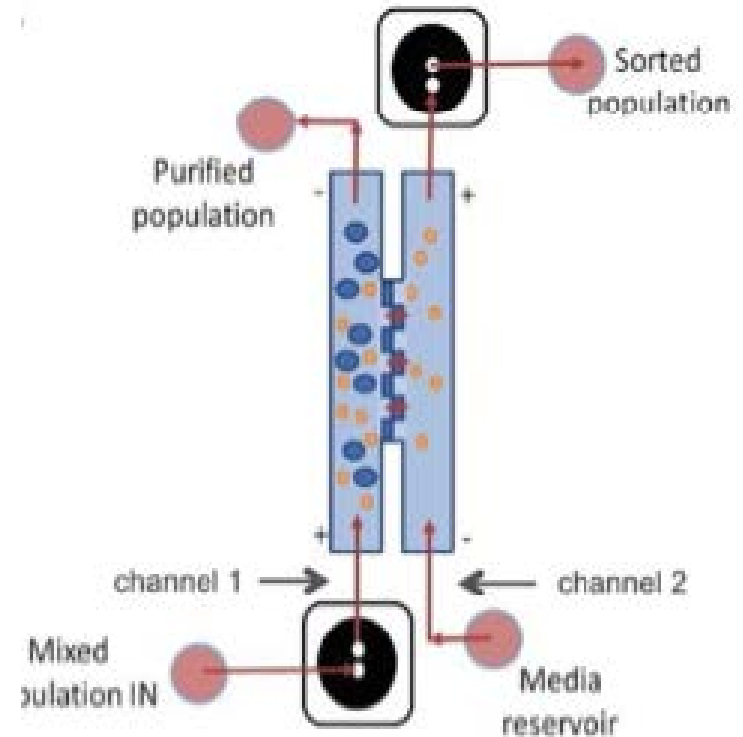
Advanced Healthcare Materials 2013;

Biomaterials 2014; 35: 599

Biomaterials Science 2014; 2: 1683

Biomaterials Science 2015; 3: 1371

Scalable label free cell separation



J of Biomed Eng 2011; 133: 101009

Biomechanics 2016; 14:014107

Immunogenicity

- Undifferentiated hESC:
 - Low MHC/failure to stimulate T-cell proliferation
 - Suppression of T-cell cytokines (arginase I dependent)
 - Inhibit Natural Killer cell mediated cytotoxicity
 - Low Toll-Like-Receptor expression and responses
 - IFN- γ induced upregulation of MHC I
 - Differentiation induced upregulation of MHC I & II
- Cell type specific /context dependent variation

Sources:

Trounson and DeWitt (2016) Nature Reviews, Molecular 17: 195-200.

English and Wood (2011) Current Opinion in Organ Transplantation. 16: 90-95.

Adventitious Pathogens

Considerations:

- One “immortal” source to many recipients
- Reliance on donor sources, biological reagents, extensive bioprocessing in absence of:
 - Validated screens for known/unknown pathogens
 - Limitations in understanding of risk
 - Eg. Prion Diseases

Prion diseases?

- Degenerative CNS disorders caused by transmissible pathogenic isoforms of prion protein (PRP).
 - Animal (Scrapie/BSE/CWD) & Human (CJD/Kuru) forms.
- Creutzfeldt-Jakob Disease (CJD) most common in humans (0.5-1.5 cases/million per year).
- CJD forms: (Different etiology /epidemiology / manifestation)
 - Sporadic (85%) – random misfolding? Spontaneous mutation?
 - Familial (10-15%) – mutations.
 - Iatrogenic (1%) – inadvertent human to human.
 - Variant (UK & France) – bovine to human.
- Global issue – NOT UK specific (!)

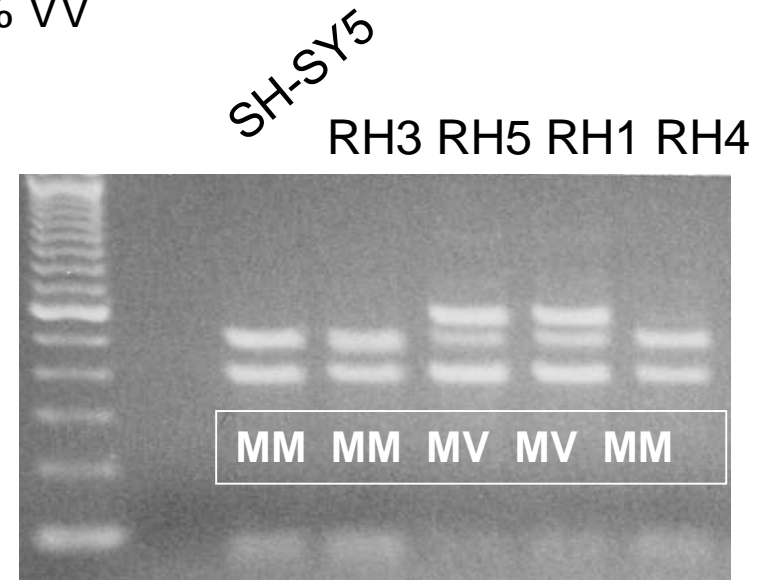
HESC susceptibility/manifestation of prion diseases

Factors:

- ✓ Genetic susceptibility polymorphisms
(eg. 177/178 vCJD cases are codon 129 MM)
- ✓ Expression of constitutive normal PRPc isoform.
- ✓ Tissue/cell-specific susceptibility to infection.
(uptake, amplification, transmission)
- ✓ Exposure to pathogenic isoforms (variant/Iatrogenic).
ie. bovine/human sourced reagents in culture.
- ✓ (Pathogenic mutations)

Susceptibility polymorphisms

- PRNP codon 129 polymorphisms x type underlie phenotype.
 - ie. sporadic CJD incubation period and symptoms.
 - Caucasians ~51% MV, ~37% MM, ~11% VV
 - vCJD cases 129 MM.

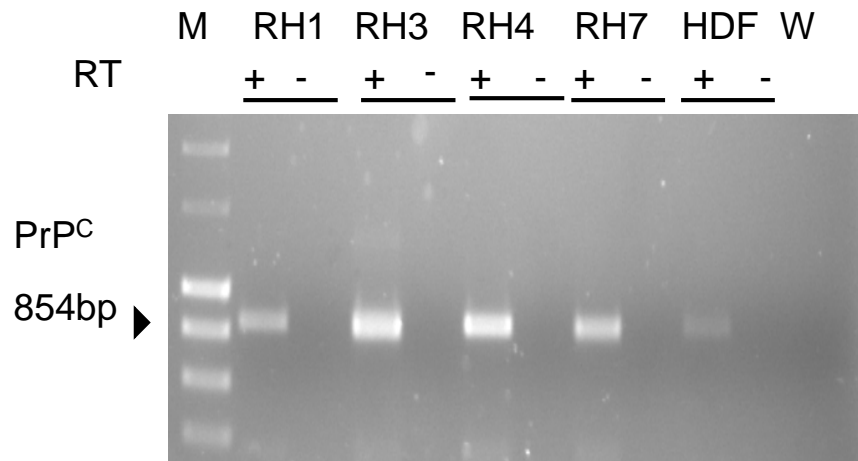


Recommendation:

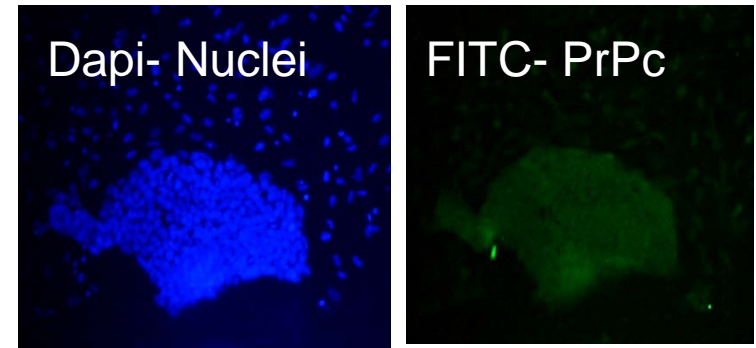
Selection of 129 MV hESC for therapy & PrP locus specific sequencing to confirm absence of pathogenic mutations..

hESC PrP Expression

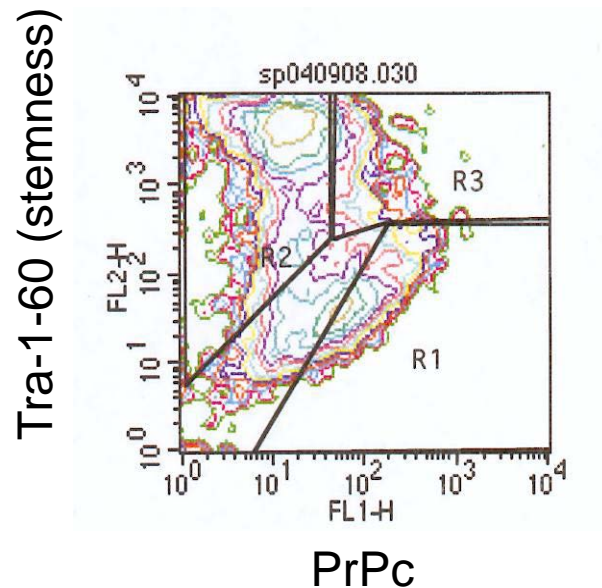
A



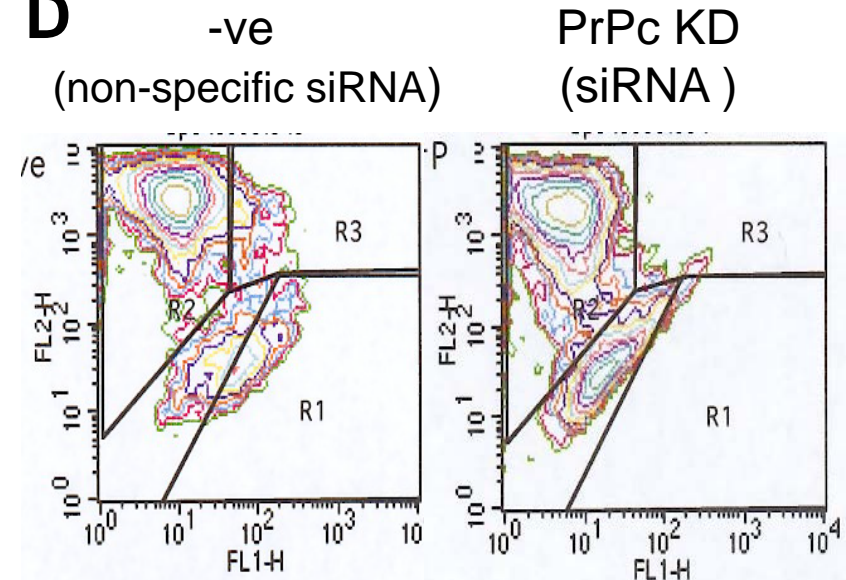
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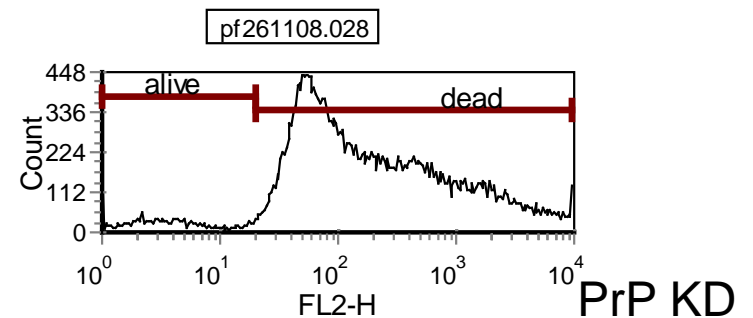
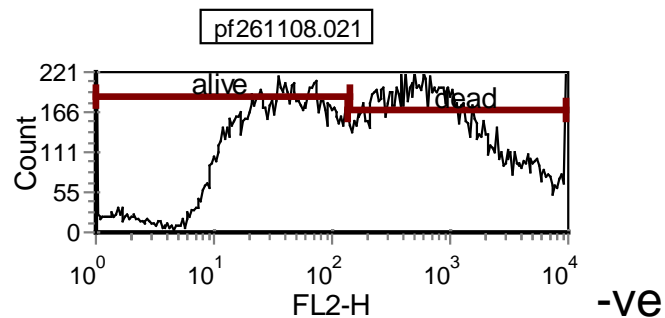
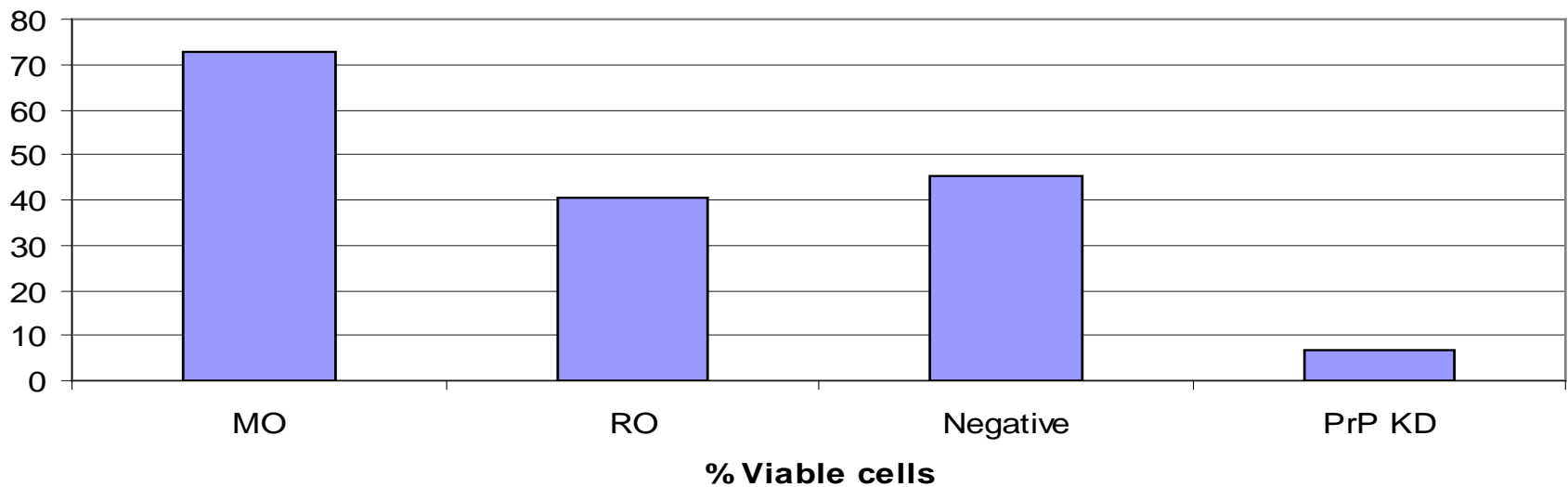


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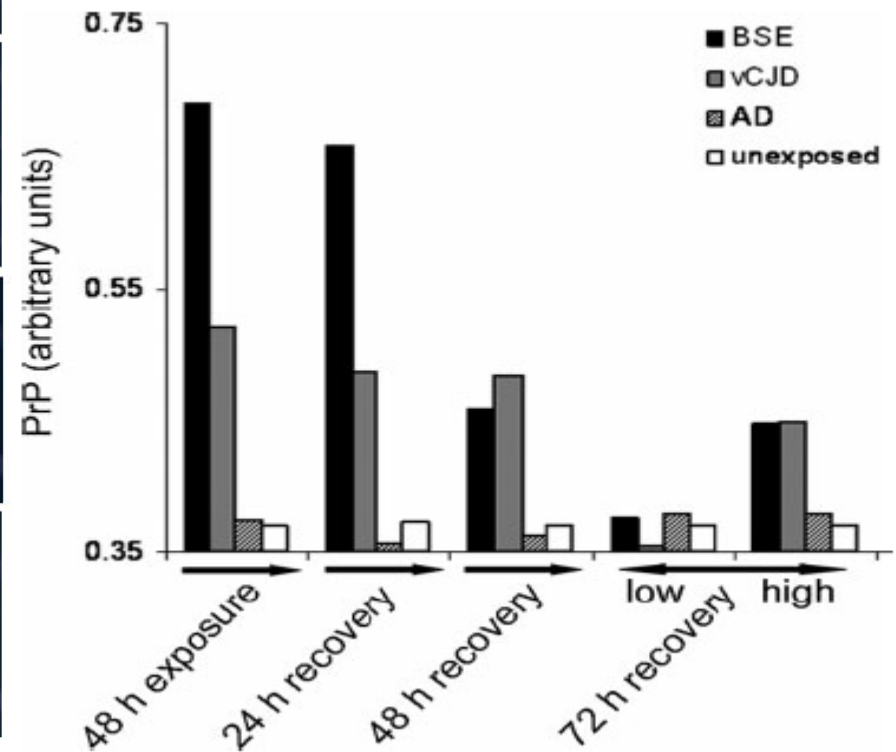
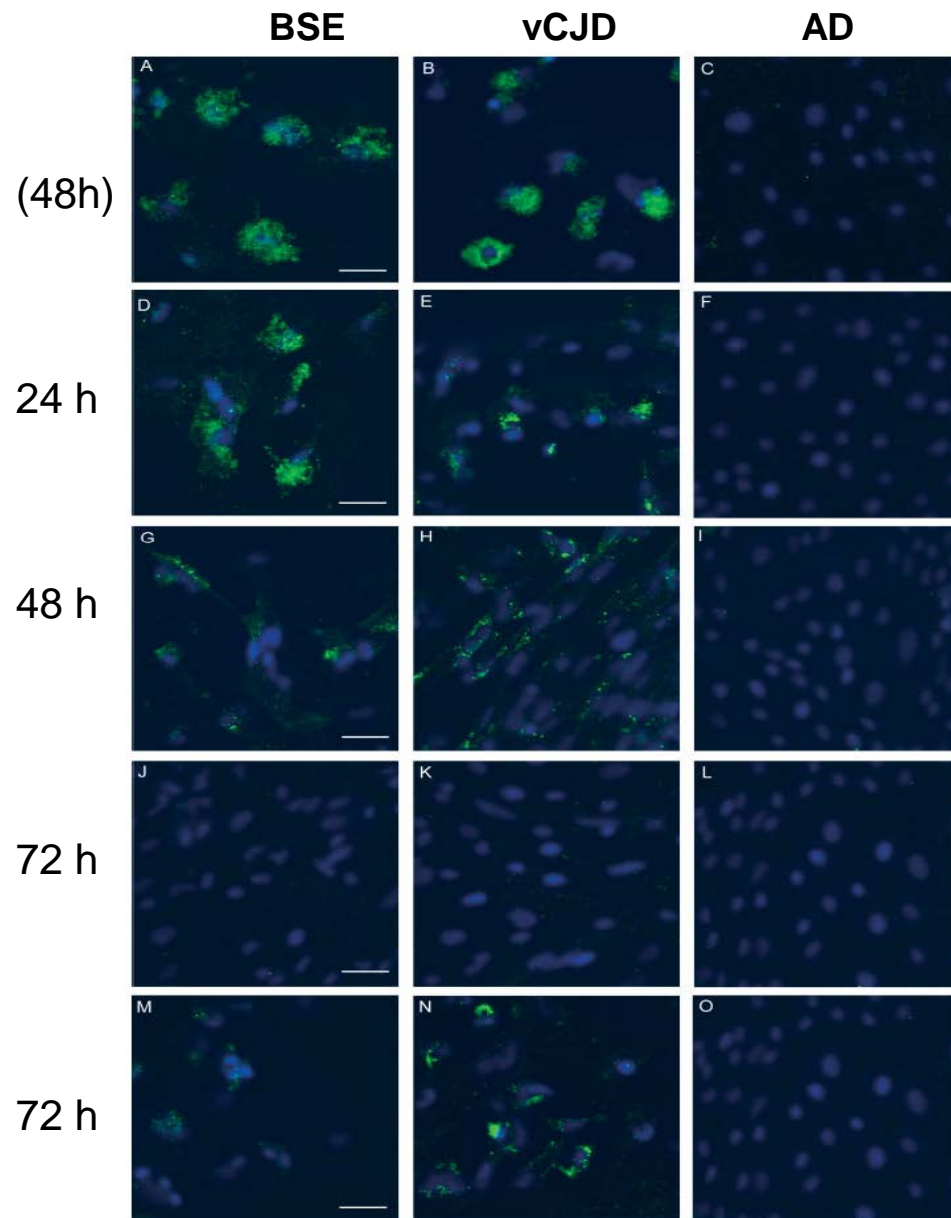


PrPc: A Survival Factor?

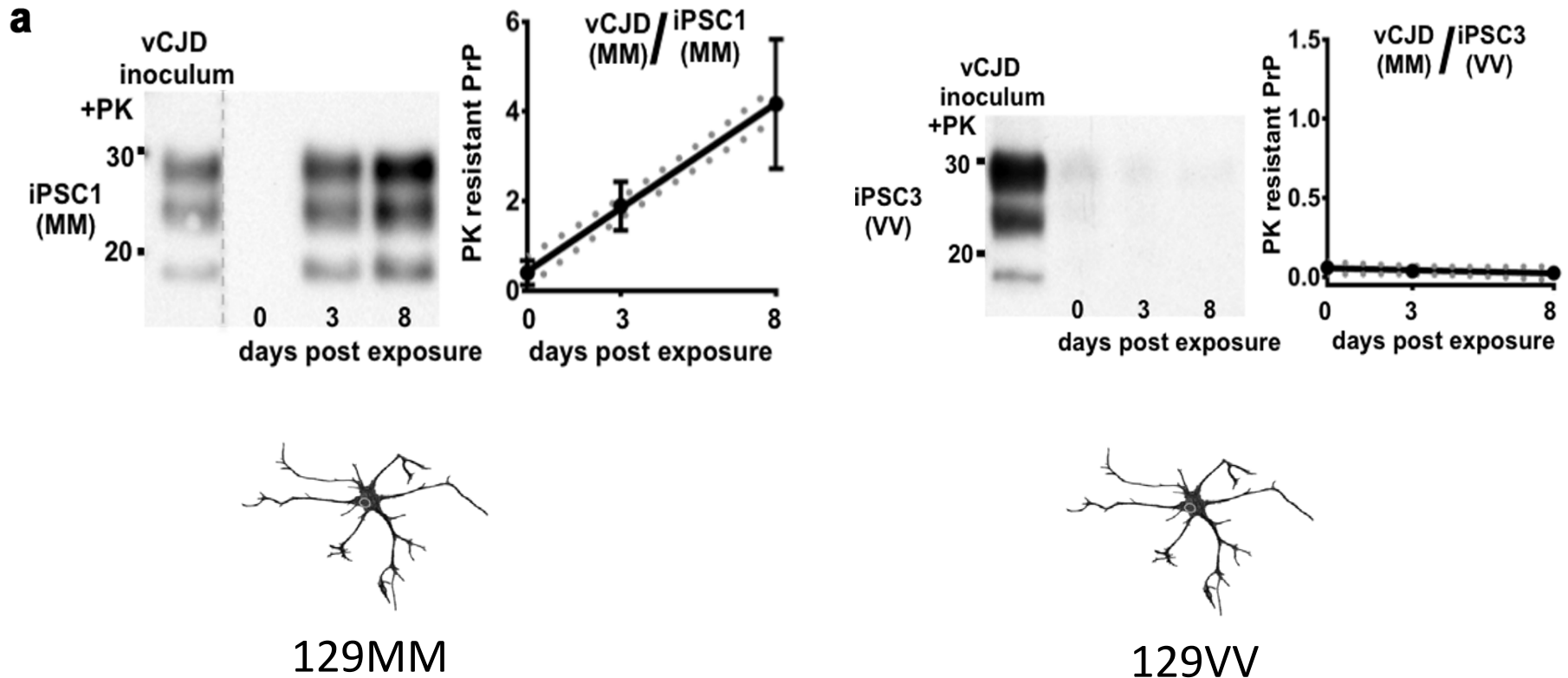
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hESC Prion Clearance



Human iPSC derived astrocytes expressing codon 129 MM genotype are susceptible to vCJD replication but MV and VV genotypes are comparatively resistant



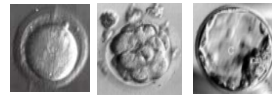
Courtesy of Dr James Alibhai

Krejciova Z, Alibhai J..., Head MW, Chandran S, JEM *in press*

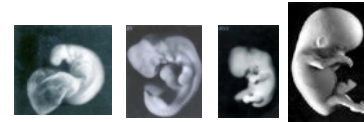
Epigenetic determinism

Stem cells & lineage specification in ~~principle~~ practice

In vivo



“Embryonic”

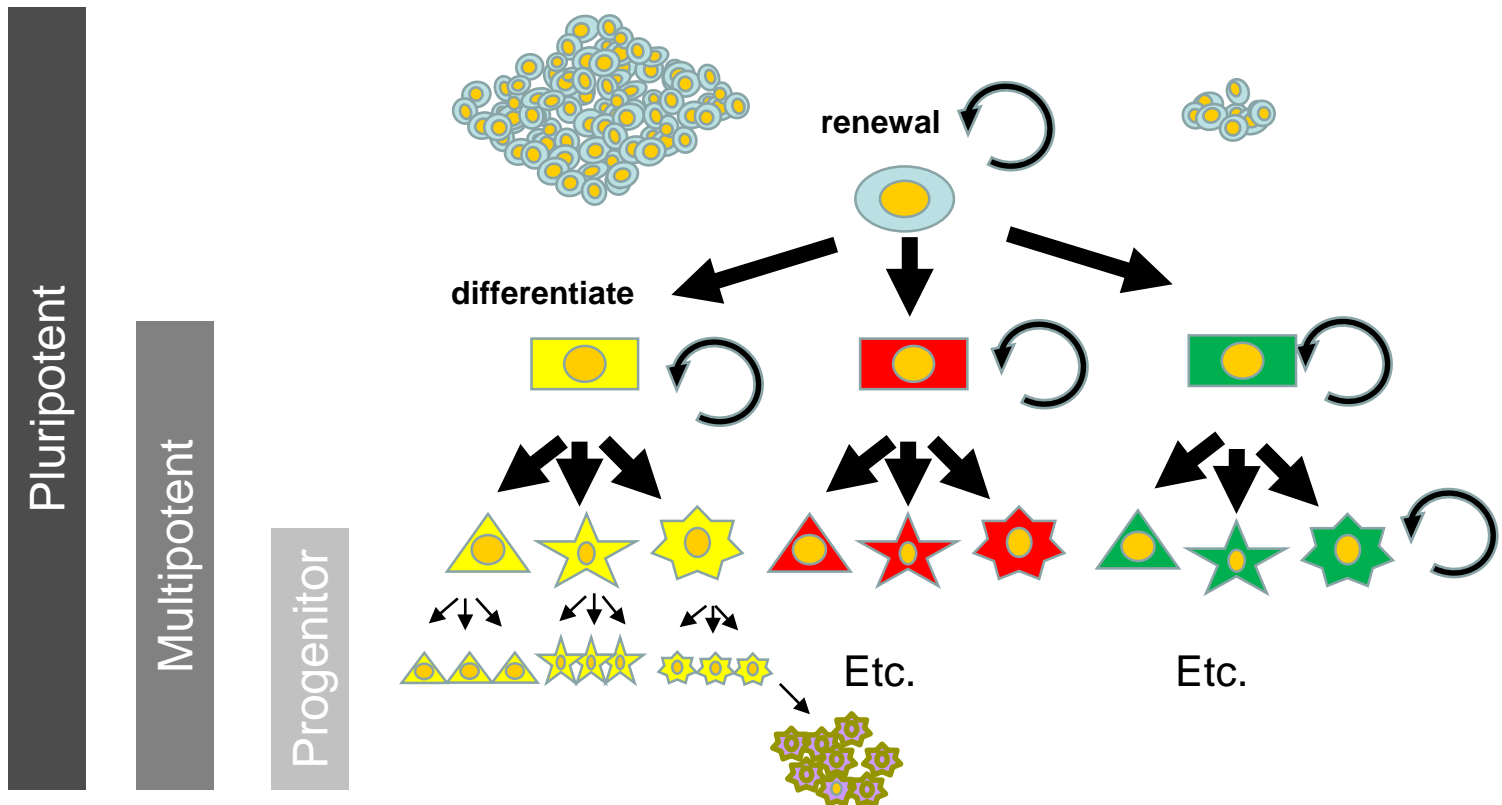


“Fetal”



“Adult” / “Tissue specific”

In vitro

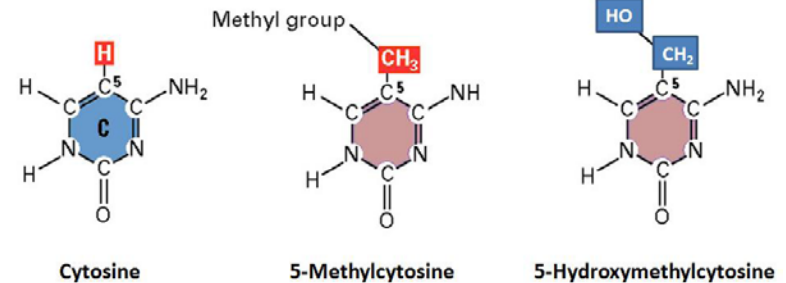


Epigenetic mechanisms

➤ Sequence (ACGT) not altered.

➤ Includes:

- DNA (Hydroxy)-methylation.
- Histone methylation/acetylation (A.A. residue specific)
- Histone variants
- Restrictive (Polycomb) / Permissive (Trithorax) complexes
- Global Chromatin Modifiers (Swi/Snf, Polycomb)
- Micro-RNAs



Epigenetic features of a PSC

- Dynamic/Permissive chromatin
- DNase I hypersensitivity
- Bivalent domains
- Hydroxymethylation (~1%)
- Variable methylation

Epigenomic biomarkers of pluripotency?

- Which genes conservatively modified?
- Functional relevance?
- Environmental responsiveness?

Global CGI-methylation patterns?

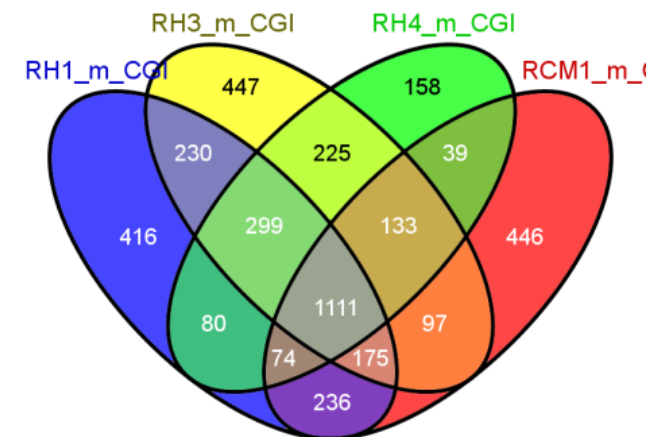
- DNA rich in CpG dinucleotides (1/10 vs 1/100 bp).
- Associated with 60-70% human gene promoters.
- Normally unmethylated (vs methylated if non-CGI).
- Contexts:
 - CGI-m p53 promoter → silencing → neoplasia.
 - Parental imprints (ie. H19, SNRPN, MEG3 and IGF2).
 - X-chromosome inactivation.

Is there an hESC specific consensus CGI-m?

	hESC	Adult
Total Common CGI-m (%)	1111 (6%)	821
Common Gene-Assoc. CGI-m	888	606
Genomic Distribution	Uniform	More on XY, less on Ch. 1-5.
GA CGI-m only in hESC	150	-
GA CGI-unmethyl in hESC	90	-

hESC ga-CGI	mRNA	No. of Genes**
Methylated (150)	+	96
	-	34
	Diff. (≥ 1)	20
Unmethylated (90)	+	54
	-	25
	Diff. (≥ 1)	11

hESC Epigenetic Signature?



**

Methylated – enriched for Transcriptional Repressors
Unmethylated – enriched for Transcriptional Activators.

Candidate epigenomic biomarkers?

HESC expressed genes with conserved & cell specific CGI-m:
Iron (Fe^{2+}) and 2-oxoglutarate dioxygenases (O_2 dependent)

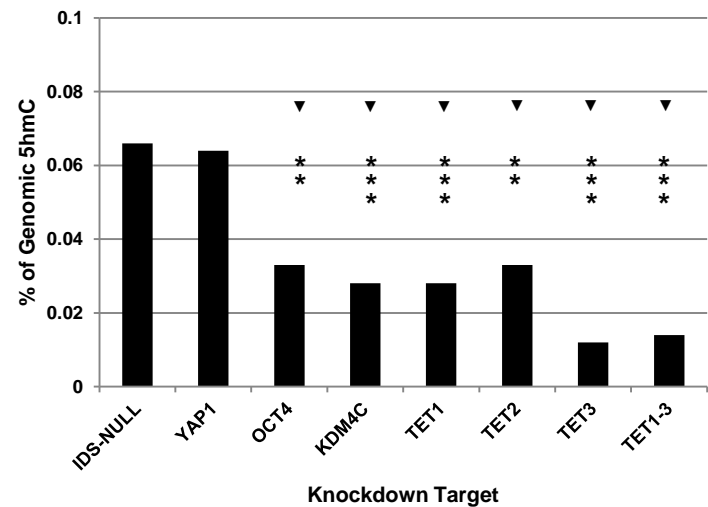
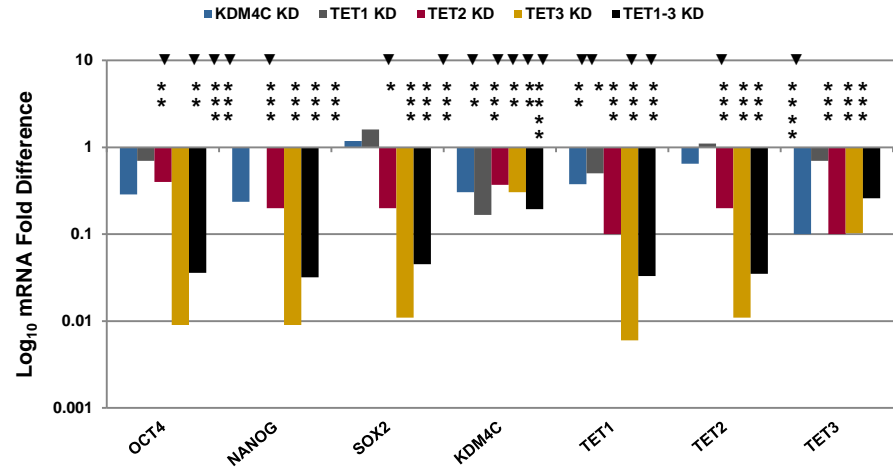
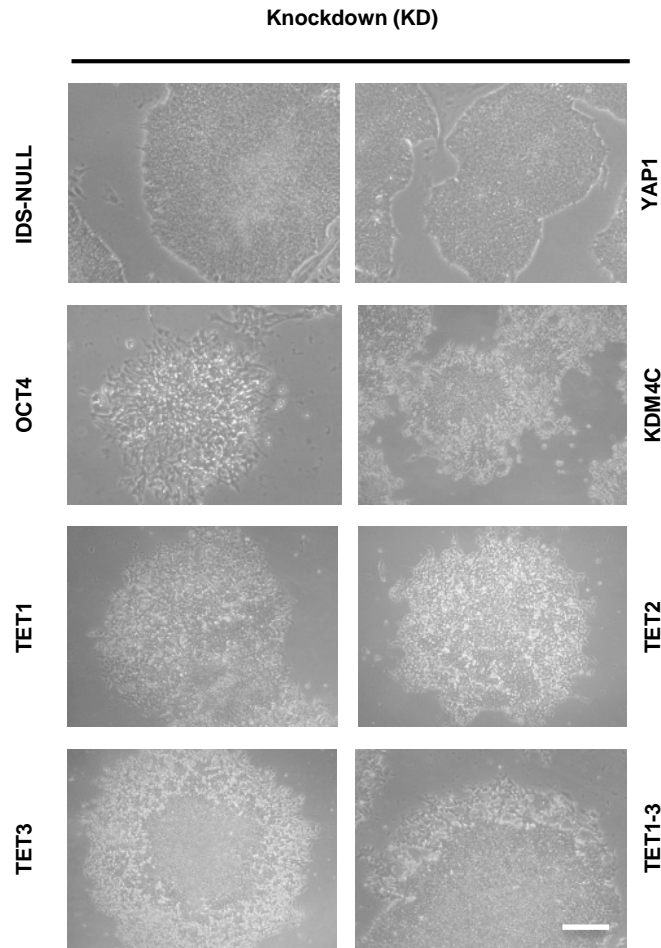
“Oxygen sensing dioxygenases”

- TET1** Ten Eleven Translocation 1 (1 of 3 members)
- 5mC \rightarrow 5HMC conversion catalysed by TET oncogenes.
 - (Mouse ESC) depletion \rightarrow Nanog CGI-m \rightarrow Silencing^{1,2}.
 \rightarrow mesendoderm/trophoblast specification.
 - Regulated by core pluripotency TF

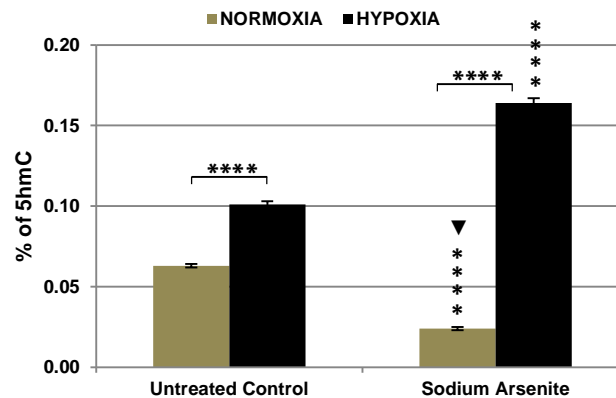
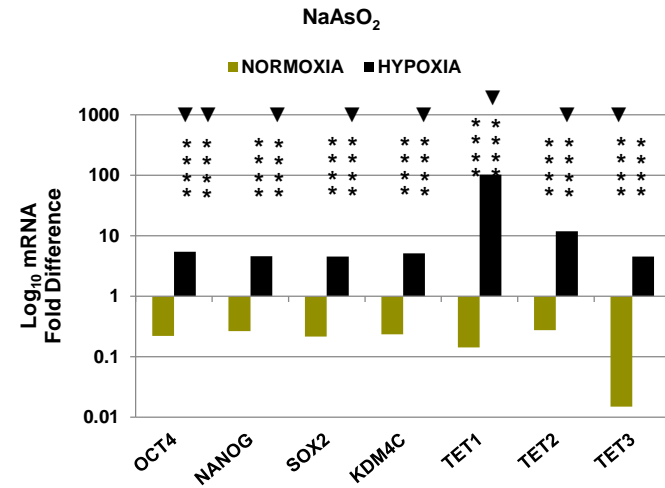
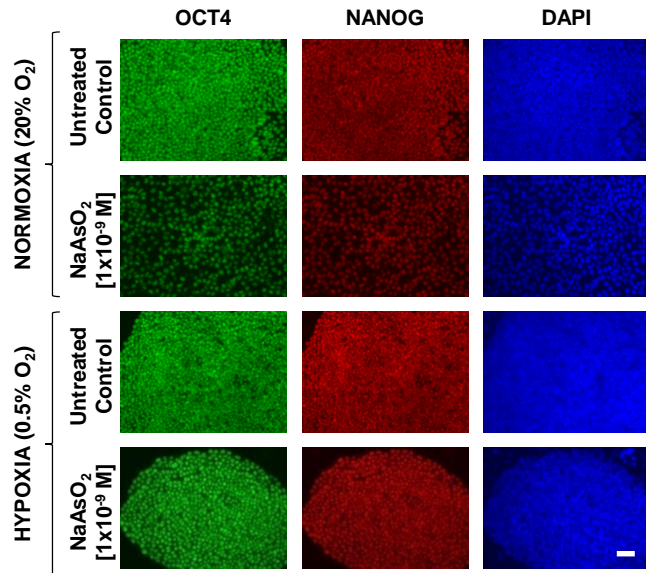
- KDM4C** Jumonji domain 2 contain Histone Lysine Demethylase
- chromatin remodelling enzyme
 - (Mouse ESC) depletion \rightarrow differentiation.
 - Regulated by core pluripotency TF

1. Ito et al., 2010, Nature doi:10.1038/nature09303
2. Koh et al., 2011, Cell Stem Cell 8(2):200-13
3. Loh et al., 2007, Genes Devel 21: 2545-2557

Interference with TETs & KDM4C induces hESC differentiation & 5hmC loss



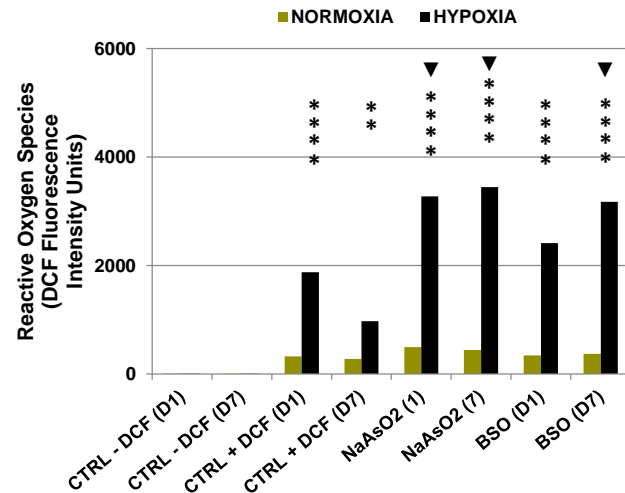
O₂ dependent trace level organometallic (NaAsO₂) induction of hESC differentiation, loss of dioxygenases and 5hmC



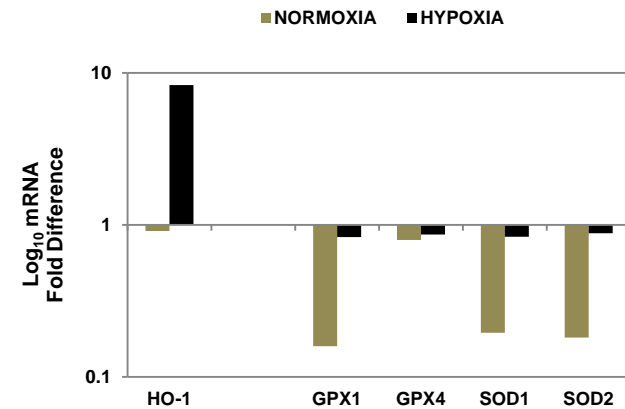
Protective Effect of Hypoxia

Differential O₂ dependent trace level organometallic (NaAsO₂) induced oxidative stress

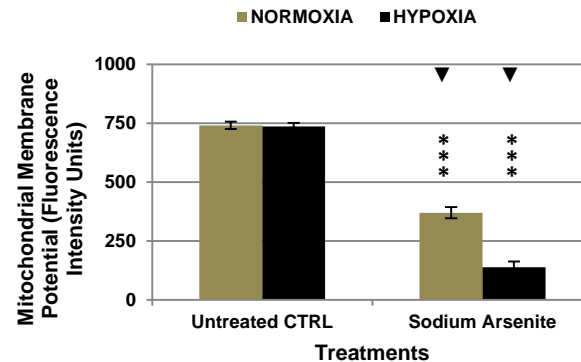
Reactive Oxygen Species



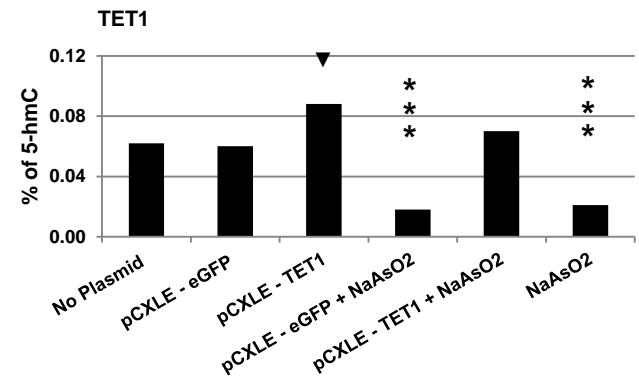
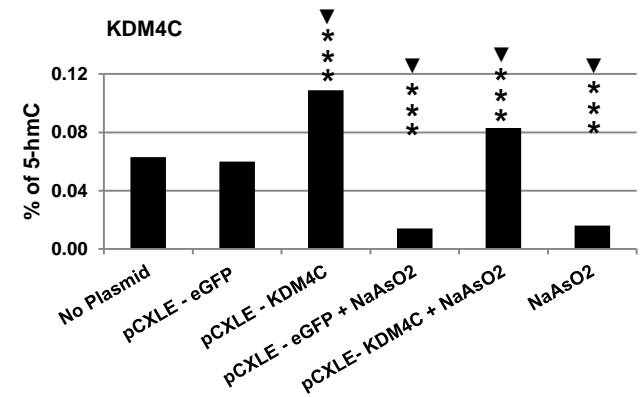
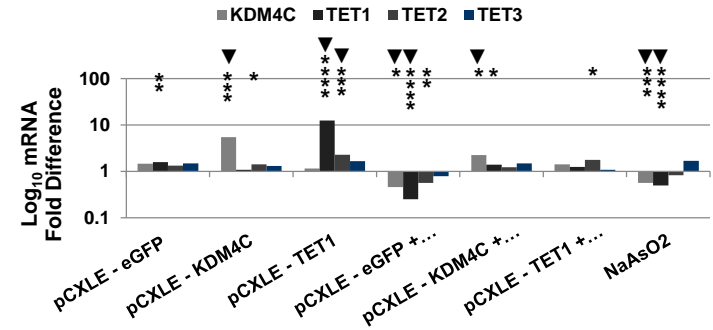
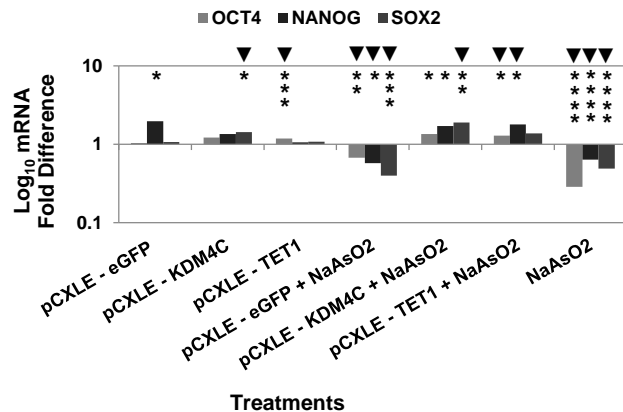
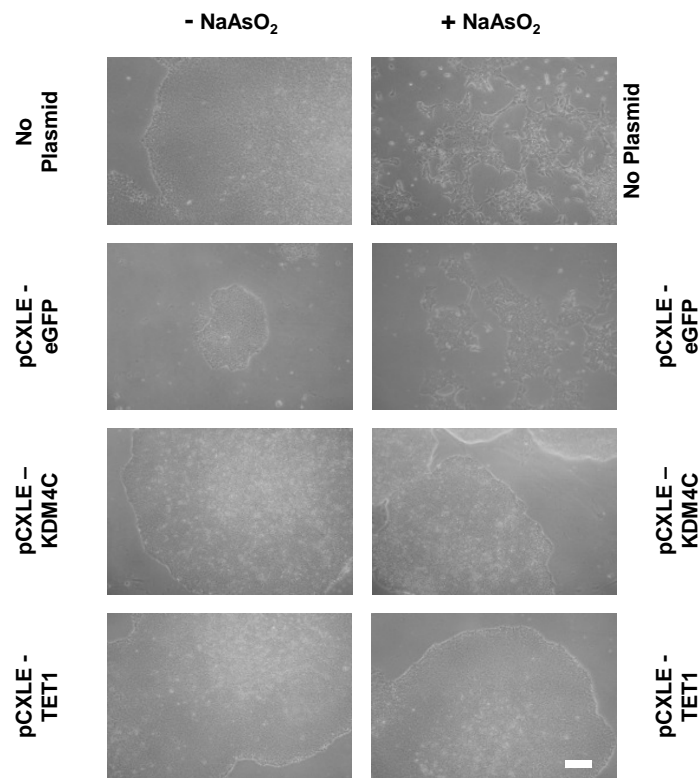
Antioxidant Gene Expression



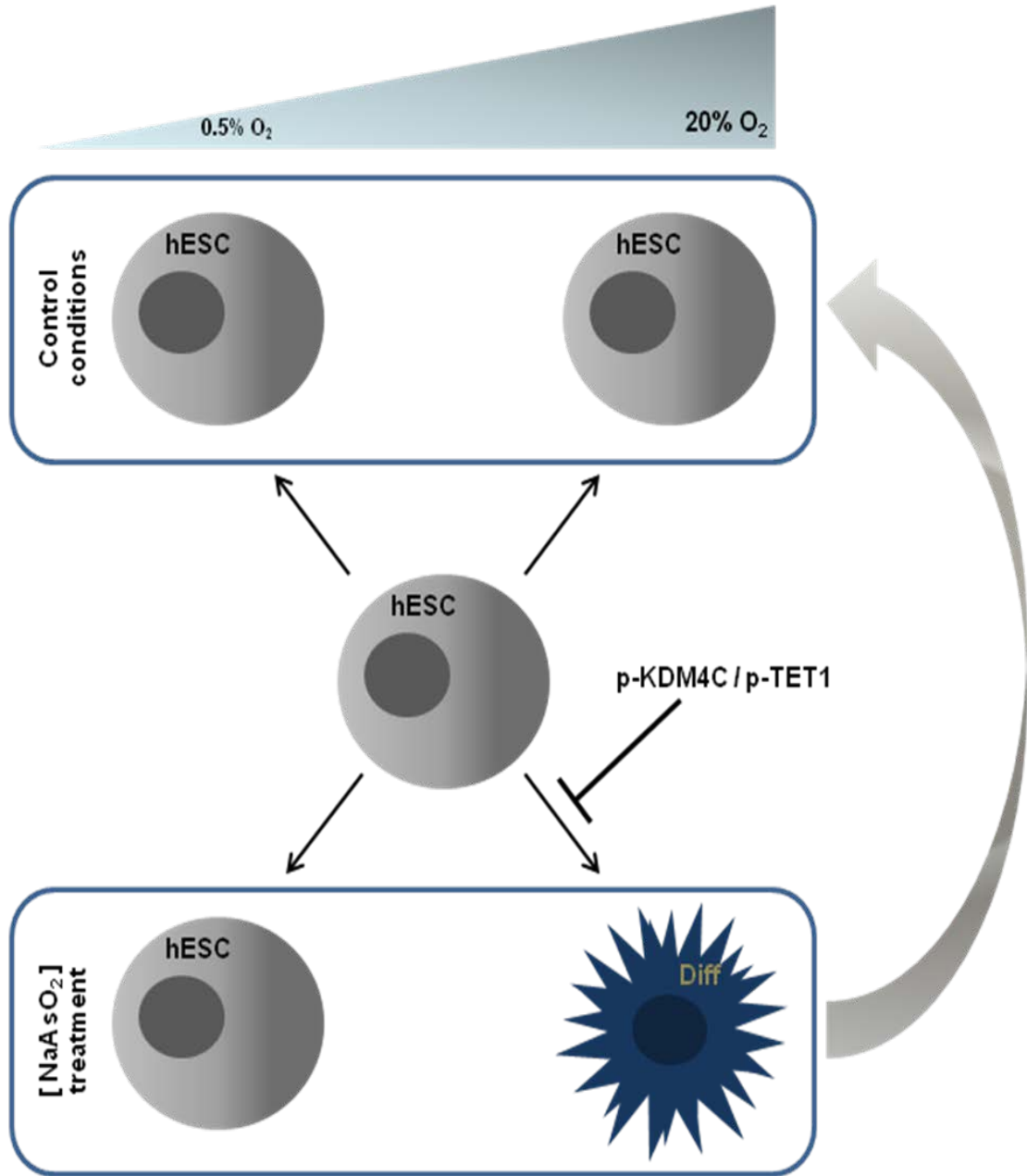
Mitochondrial function (uncoupling ATP/OxPhos)



Transient expression of O₂ sensing dioxygenases protects against trace level organometallic (NaAsO₂) induced differentiation

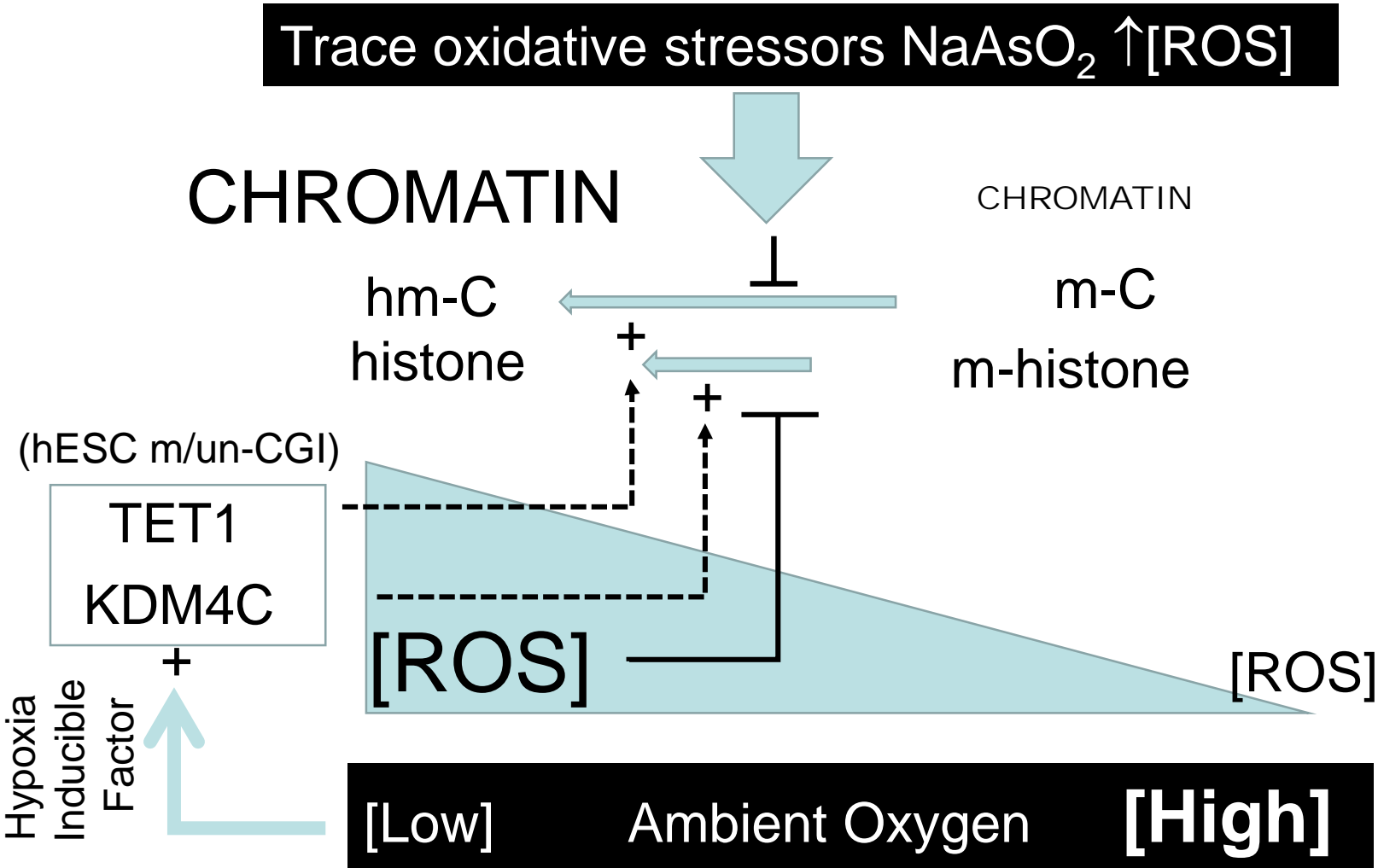


Hypoxia induced O₂ sensing dioxygenases sufficient to block trace chemical oxidative stress induced differentiation



Model – Environmental/Epigenetic determination of **stemness**

SELF-RENEWAL  *DIFFERENTIATION*



20 years on – hESC therapy facts, fiction and challenges

- ❖ Long road of public/privately sponsored innovation
- ❖ Further to travel before clinical practice
- ❖ Vital to augment understanding of risk & cell-type validated screening for adventitious pathogens such as Prions

(Critical in light of initiated Ocular/Brain/CNS transplants recalling past history of transmission via other advanced therapies – Human Growth Hormone)

- ❖ Ambient oxygen as an epigenetic mediator of stemness – implications for hESC production.

Acknowledgements



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Thank you for your attention.