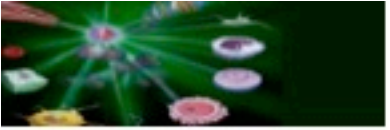
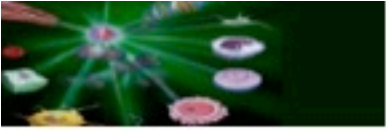
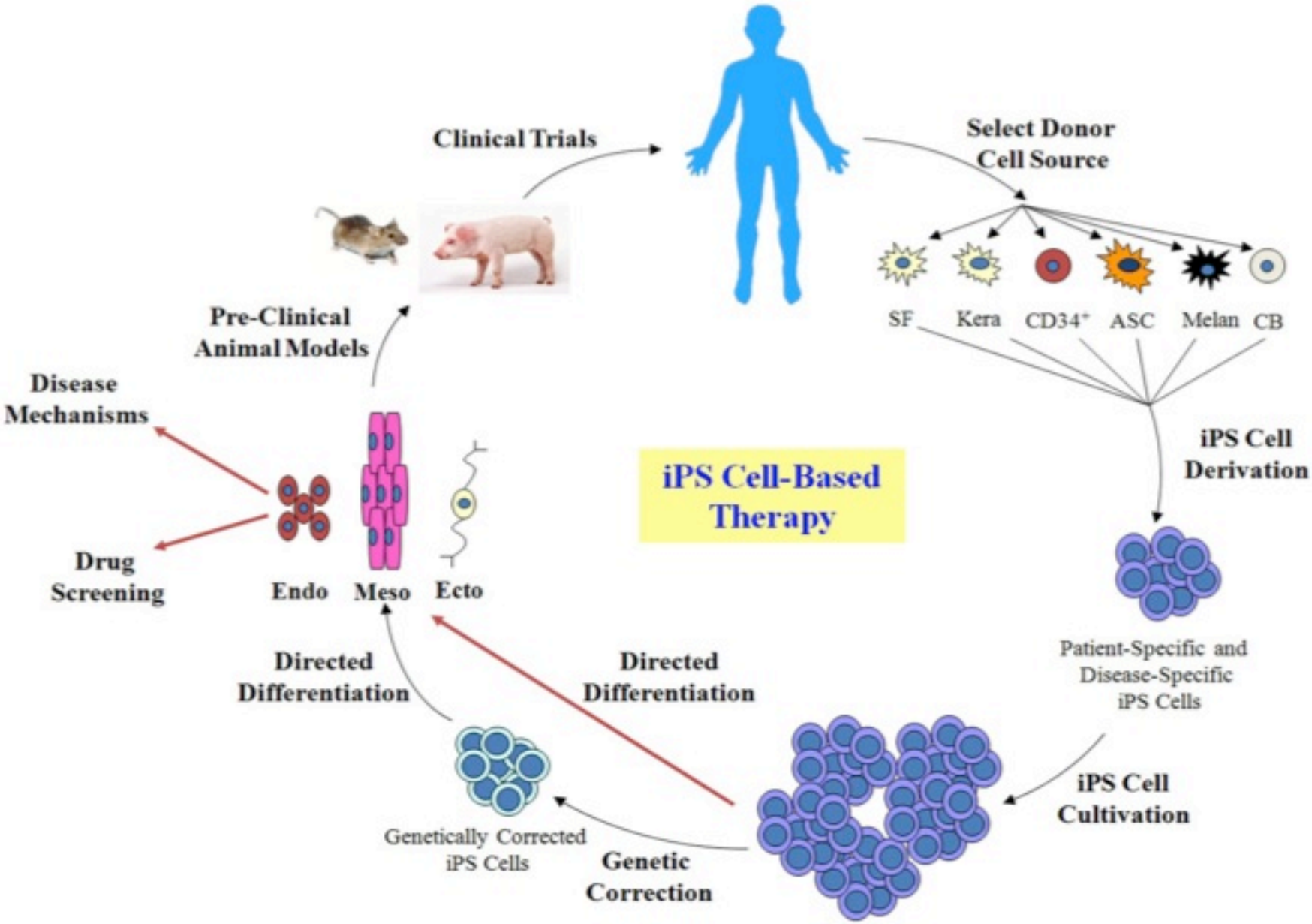


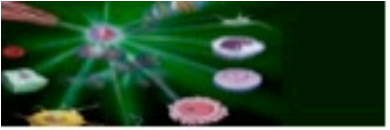
iPSC

Mahendra Rao Director
NIH CRM



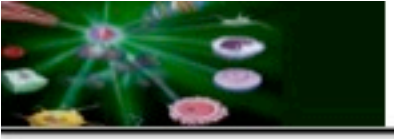
iPSC-Based Regenerative Medicine





iPSC- Possible differences with ESC

- Tissue source bias
- Incomplete reprogramming with consequent stability and heritability of the reprogramming cues
- Culture and propagation based differences
- Vector integration silencing and reactivation introduced differences
- miRNA, methylation and other epigenetic differences
- Telomere length differences
- Mitochondrial heritability differences
- Imprinting and X chromosome inactivation or activation process based differences
- Clonal selection bias for disease models
  - (PolyQ, mitochondrial, Chromosome abnormalities, X chromosome related diseases, etc)
- Immune response differences



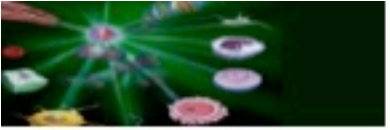
PSC based screening

ENGINEERING

- •Tissue Sourcing
- ↓
- •PSC derivation
- ↓
- •PSC derived product manufacture
- ↓
- •Developing an assay
- ↓
- •Determining scale of screen and type of screen

- Not a viable choice for standard screening
- Not a viable choice if enough adult cells available
- Useful when adult cell type or disease model in appropriate context unavailable
- Useful for measuring allelic variability in Toxicology
- Gene mapping and functional validation
- Predictive of functional utility of a therapy
- Understanding mechanism of action at high resolution
- Other specific uses

Develop panels of lines with other stake holders in a public private partnership

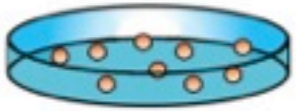


Detailed Process

Adult cells

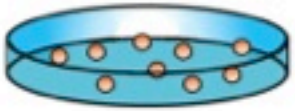
Around 1-5M

Reprogramming



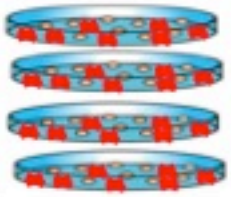
1-6 rounds of infection

Around 21-28 days



Around 50-150 colonies

Select clones



10-30 clones picked

culture

Around 6 weeks

Identify iPSC clones from partially programmed

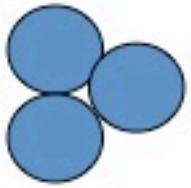
Multiple strategies with advantages and disadvantages

Candidate ESC like line

Pick 3 to be safe per parent line

ESC like line

Around 6 weeks



Grow propagate and bank

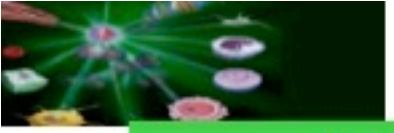
Test for pluripotency

Around 8 weeks-12 weeks

Test for differentiation

Differentiated cell type

Around 5 weeks for EB stage



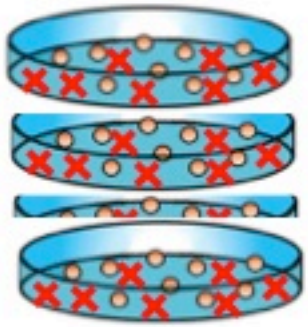
Controllable expression of reprogramming factors

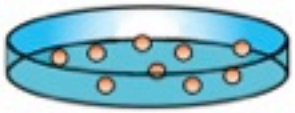
Adeno
Bacculo
Sendai
Protein transduction
Plasmids
etc

Multiple clones selected and evaluated

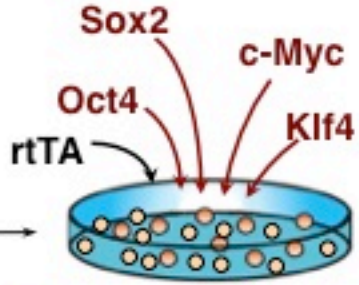
Functional evaluation

- doxycycline





Fibroblasts



lentiviral
Infection
+ doxycycline
Piggybac
HR +CRE-LOX
etc

Transgenes on

100s of colonies (mix of iPS and transformed cells)

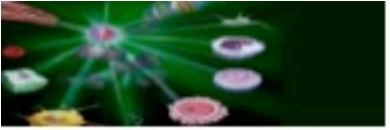
Transgenes off
Selection etc

iPS cells survive. Non-iPS cells die or differentiate

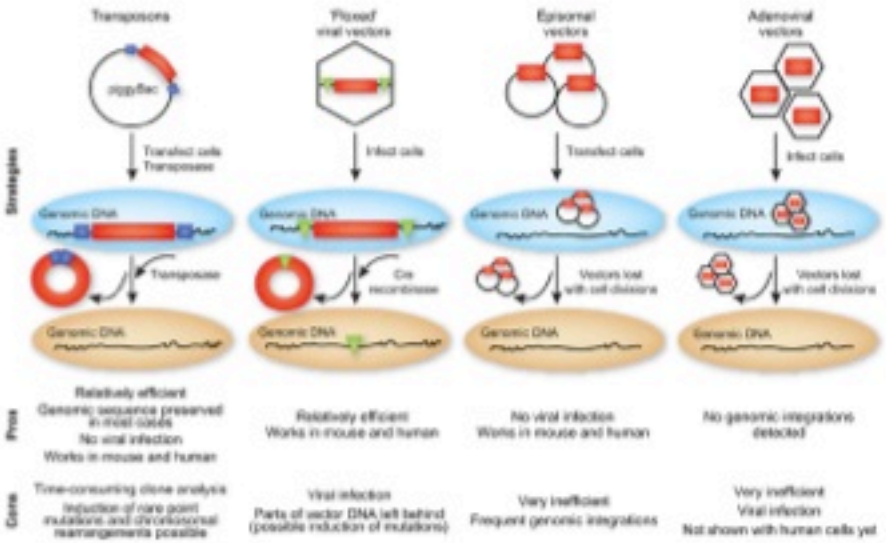
No tumors

…as seen in 20% of retro-iPS chimeras

9 months



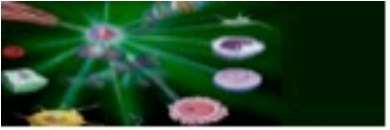
Other Integration free methods



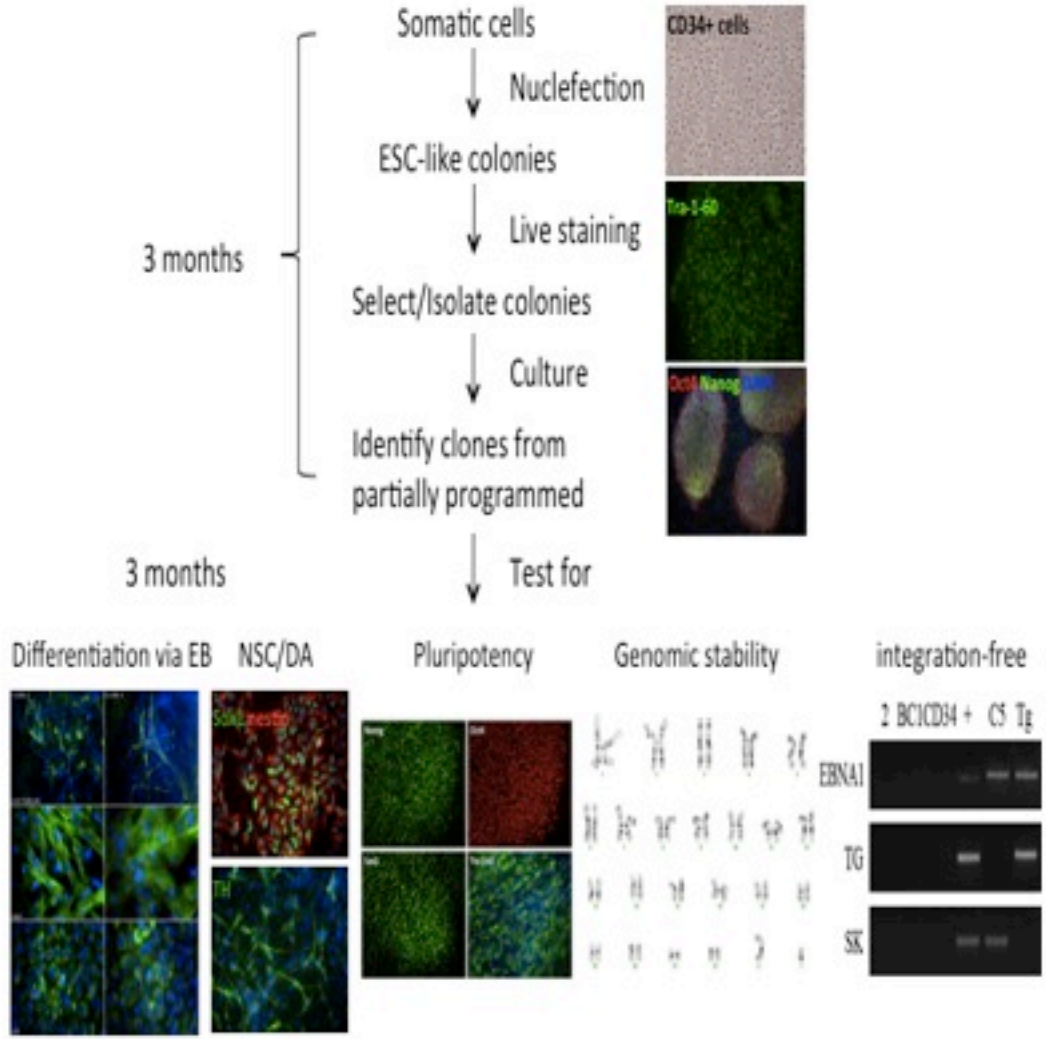
Methods include

- Protein transduction
- Media from pluripotent cells
- RNA
- miRNA
- Small molecules
- adenovirus

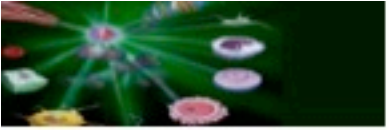
- Generally more expensive technology
- More steps and currently less efficient
- Large amounts of material and cells required
- Does not work with all cells
- Patent issues related to genes used, process used and vector used.
- However truly integration free and may be the option in the future especially for direct differentiation or transdifferentaition



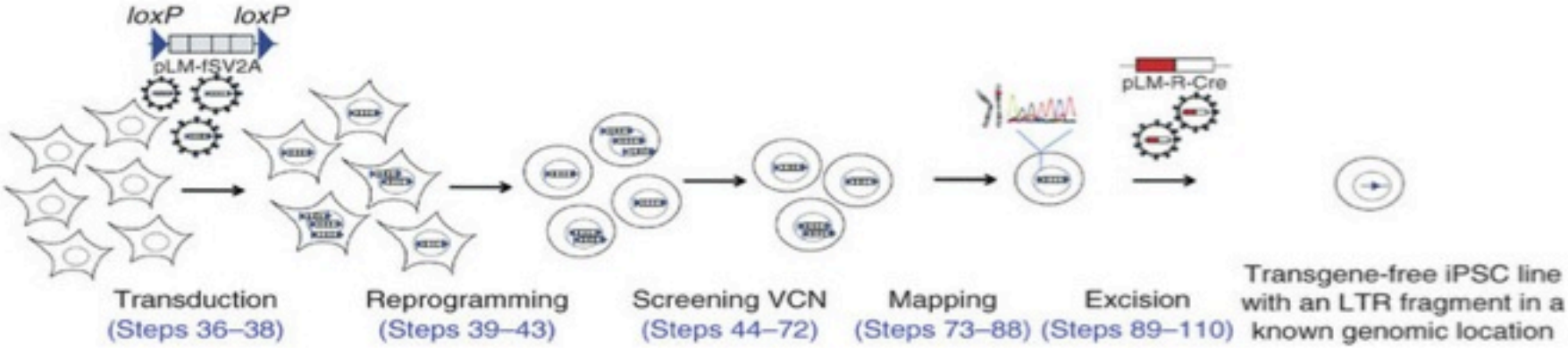
Derivation Using Plasmids and minicircles

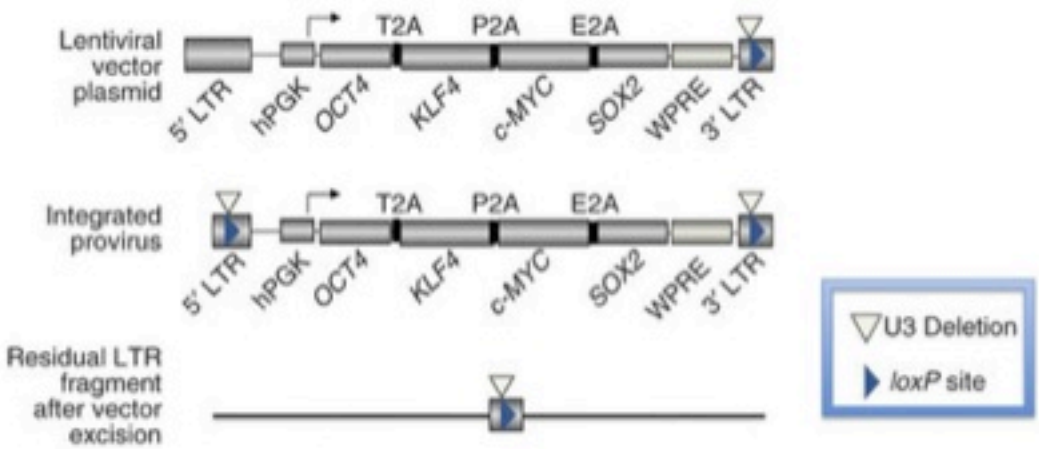


- Plasmid and mini circles work well and this is a cheap and reliable technology
- Electroporation adds steps and it is difficult to automate process
- Small amount of starting material required
- Works with fewer cell types than lentivirus
- Need to verify that no integrants present.
- Truly integration free
- Patent issues related to genes used but no process or vector issues to our knowledge

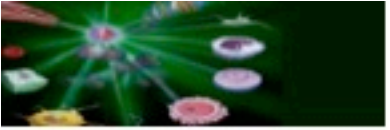


Derivation Using an excisable vector



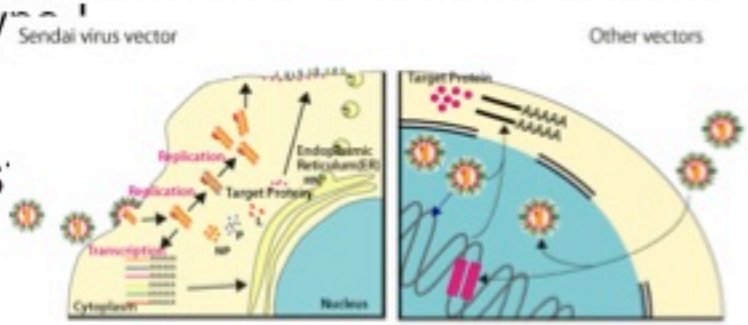


- Single vector method has proven robust and possible to automate
- Small amount of starting material
- Works with most cell types
- Cheap and reliable technology
- Excision necessary and this adds steps
- Small residue remains
- Patent issues related to genes used, vector used and process used.

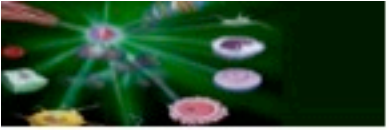


Background on Sendai Virus

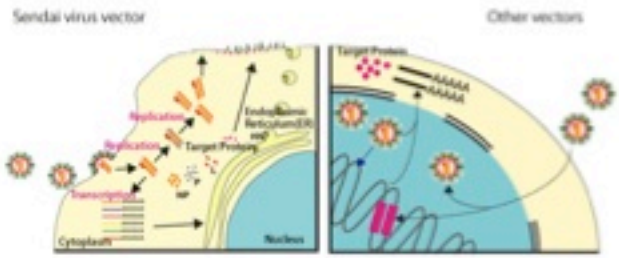
- Sendai Virus:
  - A respiratory virus of mouse and rat, belonging to the *Paramyxoviridae* family, classified as mouse parainfluenza virus ty
  - Genome of virus is RNA (minus sense).
    - No possibility of altering hos
  - Replicates exclusively in the cytoplasm.
  - Non-pathogenic to humans.



- Sendai Virus Vectors:
  - Fusion (F) gene has been deleted; virus retains infectivity but cannot produce infectious particles.
  - Capable of transducing a wide range of animal cells.
  - High level of expression of transgenes.
  - Short contact time of virus with target cells (~24 hours).
  - High transduction efficiency with low multiplicity of infection (MOI).
  - Zero footprint: the vectors and transgenes can be eliminated from host cells.
  - Product of DNAVEC Corp.



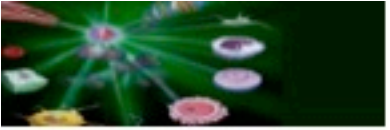
Derivation Using Sendai virus



1. RNA virus; number of transformed colonies significantly reduced,
   resulting in low background of non-iPS colonies
2. High rate of iPSC conversion (only 1/41 colony failed to expand)
3. Much higher efficiency than Lenti/episomal/protein methods
4. Non integrating; RNA virus; zero footprint
5. Virus can infect a wide varieties of cell types
6. Transduction only once, does not require multiple rounds of transduction

- more expensive technology
- Patent issues related to genes used, process used and vector used.

	Sendai	Lenti/ Retro	Adeno	Episomal/ Minicircle	Protein	Modified mRNAs
Efficiency	0.1-1%	0.001-0.01%	0.0001%	0.0001%	0.00001%	>1%
Integration	No	Yes	No (DNA)	No (DNA)	No	No
Multiple Transfections/ Transductions	No	No	No	No	Yes	Yes
Non-iPS background	low	high	?	?	?	?



Comparison of published methods for the reprogramming human somatic cells to induced pluripotent stem cells.

Method	Integrating1	Time (days)	Efficiency2
Retroviral	Yes	25-35	0.02-0.08
Lentiviral	Yes	20-30	0.02-1 %
Lentiviral (miRNA)	No	18-26	10.4-11.6%
Adenoviral	No	25-30	0.0002 %
Sendai virus	No	25	0.5-1.4%
mRNA	No	20	0.6-4.4 %
Protein	No	56	0.001 %
Episomal	No	30	0.0006-0.003 %
Piggybac	Yes	14-28	1%
Minicircles	No	14-16	0.005 %

- It is difficult to compare absolute reprogramming efficiencies as not all published studies use same criteria to calculate this parameter. Efficiency is also affected by number of factors infected/transfected, starting cell type, and use of small molecules.
- For all integrating methodologies there are strategies to remove the integrants.

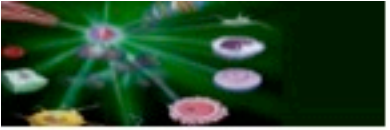
Generating iPSC from a small volume of peripheral blood.

Background: Steady-state peripheral blood contains about 1500 CD34+ hematopoietic stem (HSC) cells per ml (Sekhsaria S et al. Blood. 1996 98:1104) that are an ideal target for generation of iPSC.

Process:

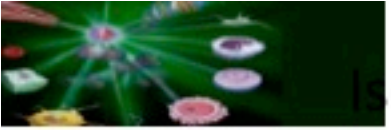
- **Gradient separate mononuclear cells (MNC) from 15ml peripheral blood.**
- **Cryopreserve MNC for later processing, or use freshly isolated MNC for anti-CD34 magnetic immunobead purification of CD34+ HSC.**
- **Place in HSC culture medium with HSC growth factors for 48hr, and collect non-adherent cells (further enriches for CD34+ HSC).**
- **Transduce these cells overnight with STEMCCA-loxP, a CRE excisable single lentivector expressing a c-Myc/Sox2/KLF4/OCT4 cassette (Sommer C et al. Stem Cells 2010 28:64) continuing to maintain in HSC culture conditions for 48 hrs from start of transduction.**
- **Transfer transduced CD34+ HSC to standard iPSC media on feeder cells.**
- **Yield of iPSC: 3-10 iPSC clones per 15ml whole blood with vector titer adjusted to yield primarily single copy inserts in resultant clones.**
- **Choose clones with single copy insert and normal karyotype for further processing: transduce with CRE expressing plasmid to excise vector.**

Comment: Works equally well with fresh blood or cryopreserved MNC; stored MNC from patients can serve as an archive source for iPSC generation.



iPSC from cord blood

- NYCBB
  - Confirm consent
  - Data Anonymization Issues
  - HLA typing data
- Fresh Harvest
- At time of use of unit
- From designed bag or tubing
- CD34+ cells or mononuclear fraction
  - Shipping
  - Tracking
  - Identity
- Receive at NYSCF
- iPSC generation
  - 50,000 cells
  - Sendai
  - Electroporation
  - Vectors
  - IP issues
  - 96 well format
  - Media and timeline
- iPSC testing
  - Pluripotency
  - Differentiation
  - Karyotype
- Ship and store →



Is a hematologic source a better choice?

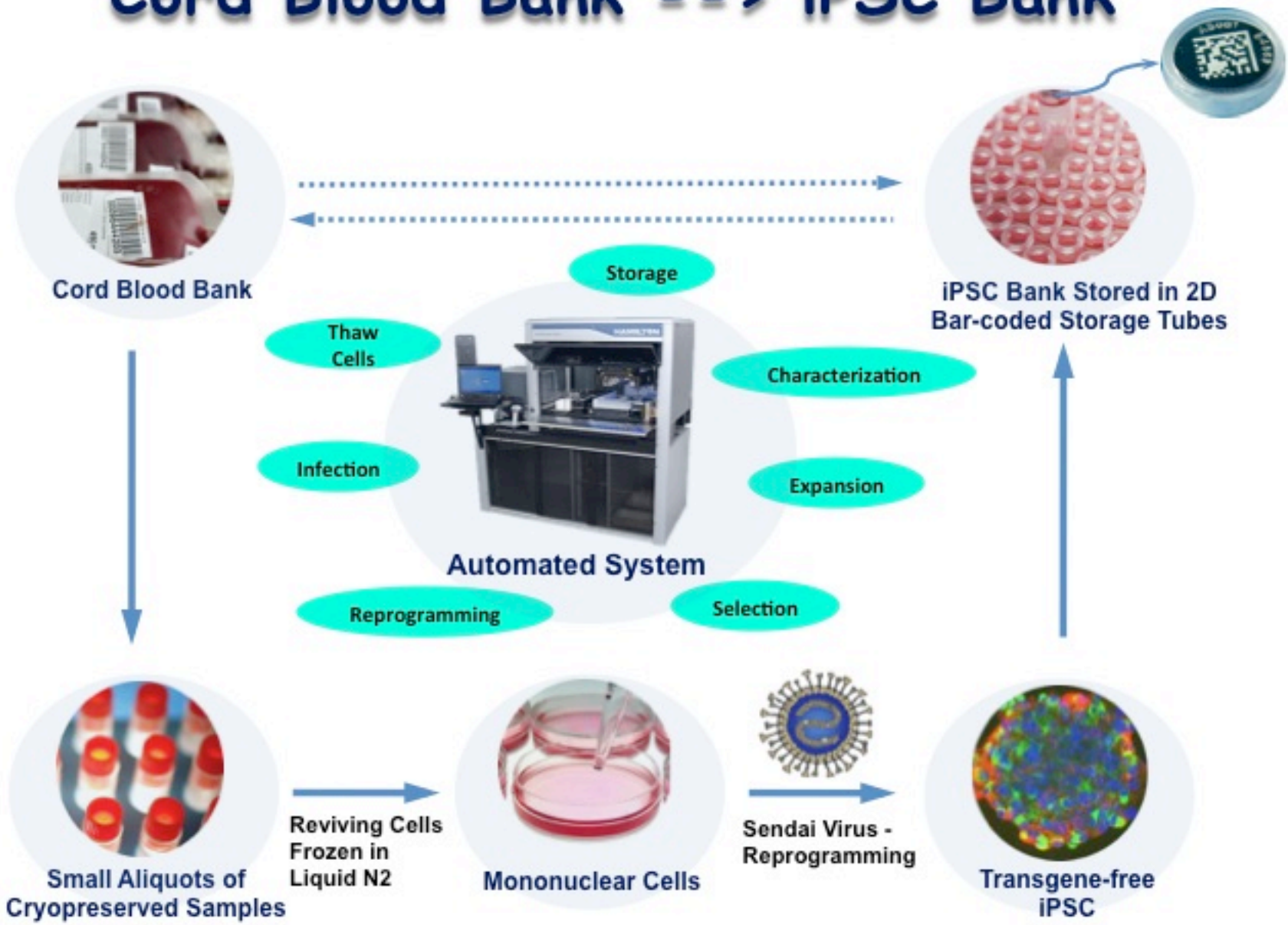
Pros

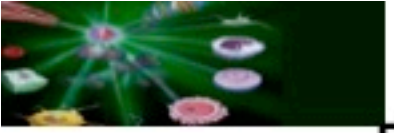
1: No of cells is very large so save time in the initial culture period
2: Stem cell populations may be easier to make iPSc
3: Cord blood cells less exposed to environmental damage
4: Reported efficiencies higher
5: Electroporation works well
6: Large scale flow based technologies for electroporation available (Maxcyte)
7: Cord and blood banks have optimized collection and storage procedures
8: Collection itself is relatively painless
9: Large no's of ethnically diverse HLA typed samples available today

Cons

1: Private cord blood banks do not type and store in single use bags
2: Lentivirus and other transduction technologies not effective in stem cell populations
3: May be biased to mesodermal lineages
4: Some zerofootprint technologies may not work (? Sendai)
5: Propagation and amplification of HSC is difficult

Cord Blood Bank --> iPSC Bank

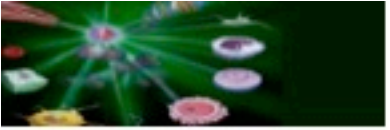




Possible reference or control lines

Lines	Repository	Distribution (Academic)	Distribution (Commercial)
CDI- 1 (from CDI)	?	yes	Yes via CDI
ND1 &ND2 (from Dr Thomson)	Coriell	Yes (MTA)	Yes (BLM)
NL 1-5 (from NIH CRM)	Coriell	Yes (MTA)	Yes (BLM)
NHLBI 1-6 (from Dr. Boehm)	ATCC	Yes (MTA)	Yes (BLM)
BC1 and BC1 GFP subclone	ATCC	Yes (JHU MTA)	Yes (JHU BLM)

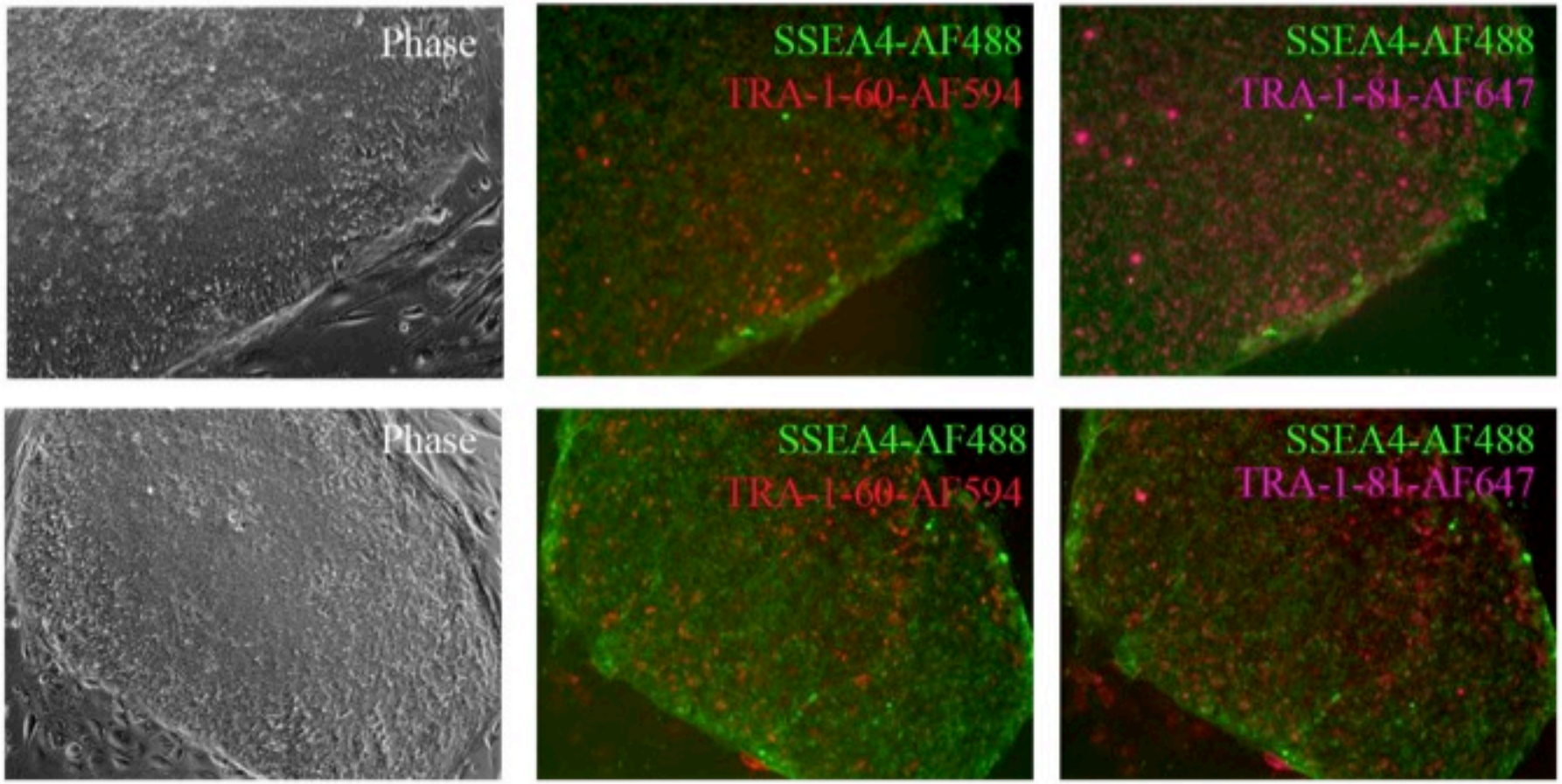
- Lines are integration free except for the NHLBI lines which were derived using Lentiviral vectors
- All except the NHLBI lines were derived from CD34+ cells. The NHLBI lines were derived from fibroblasts
- Plasmid vectors and electroporation was used to derive all the lines
- All lines have been successfully adapted to feeder free conditions and all lines are karyotypically normal and can differentiate into all three germ layers
- Gene expression data is available for all lines and currently BC1 is the best characterized



Culture System Choices for hESCs and iPSCs

	Media	Substrate	Passaging
Serum Free	• KO DMEM • KO DMEM/F-12 • KSR	• mEFs • Geltrex™ hESC (with conditioned media)	•TrypLE™, Accutase®, Collagenase •StemPro® EZ Passage™
Defined, Serum Free, Feeder free	• StemPro® hESC SFM	• Geltrex™ hESC (with conditioned media) • CELLstart™ • Suspension • mEFs*	• TrypLE, Accutase, Collagenase • StemPro® EZ Passage™
Xeno Free	• KO DMEM • KO DMEM/F-12 • KSR XF	• hFFs	• TrypLE™ • Collagenase • StemPro® EZ Passage™
Xeno Free, Feeder Free	•KO DMEM •KO DMEM/F-12 •KSR XF + Growth factor Cocktail	•CELLstart™ •Suspension	•TrypLE™ •Collagenase •StemPro® EZ Passage™

Live Multiplexing with Alexafluor Conjugated Antibodies



Antibodies conjugated to spectrally distinct Alexafluor dyes can be multiplexed

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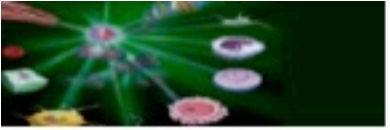
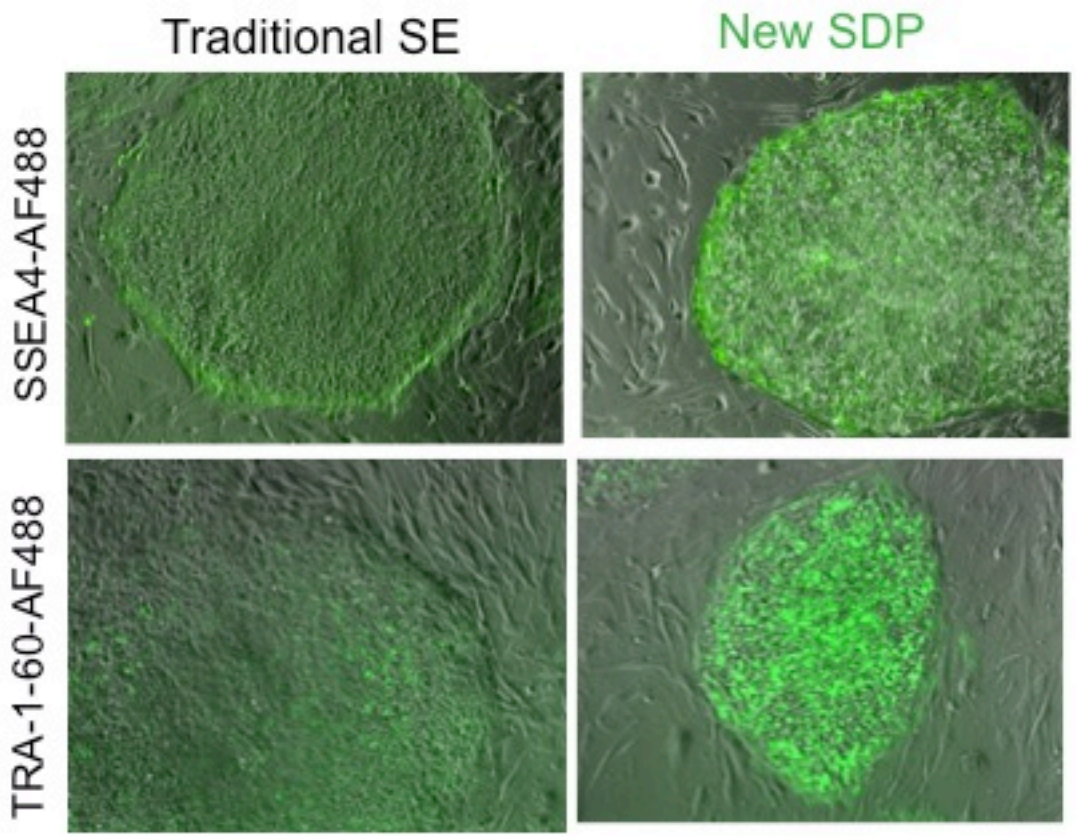
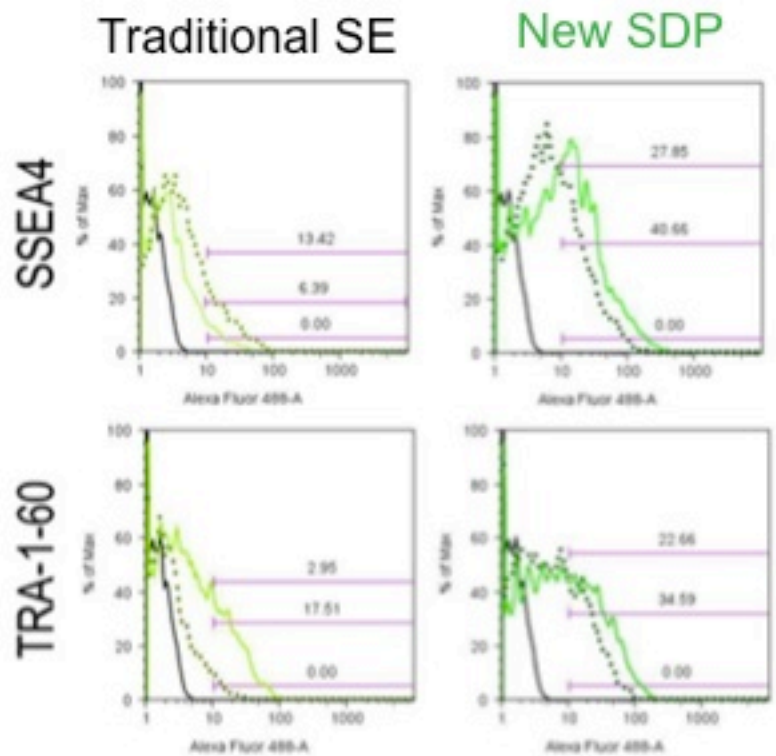


Better labeling Chemistry





New and improvised labeling methods allow generation of dye-conjugated antibodies that are more robust compared to traditional labeling methods.

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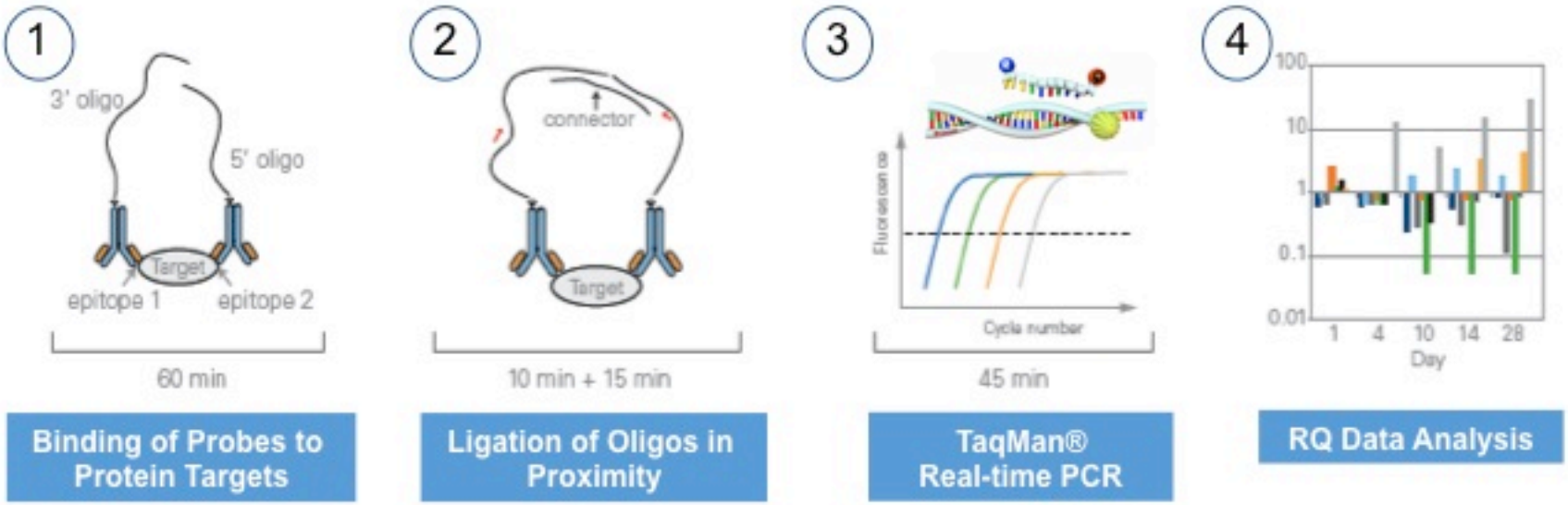
TaqMan® Protein Assays Utilize Proximity Ligation Assay (PLA™) Technology

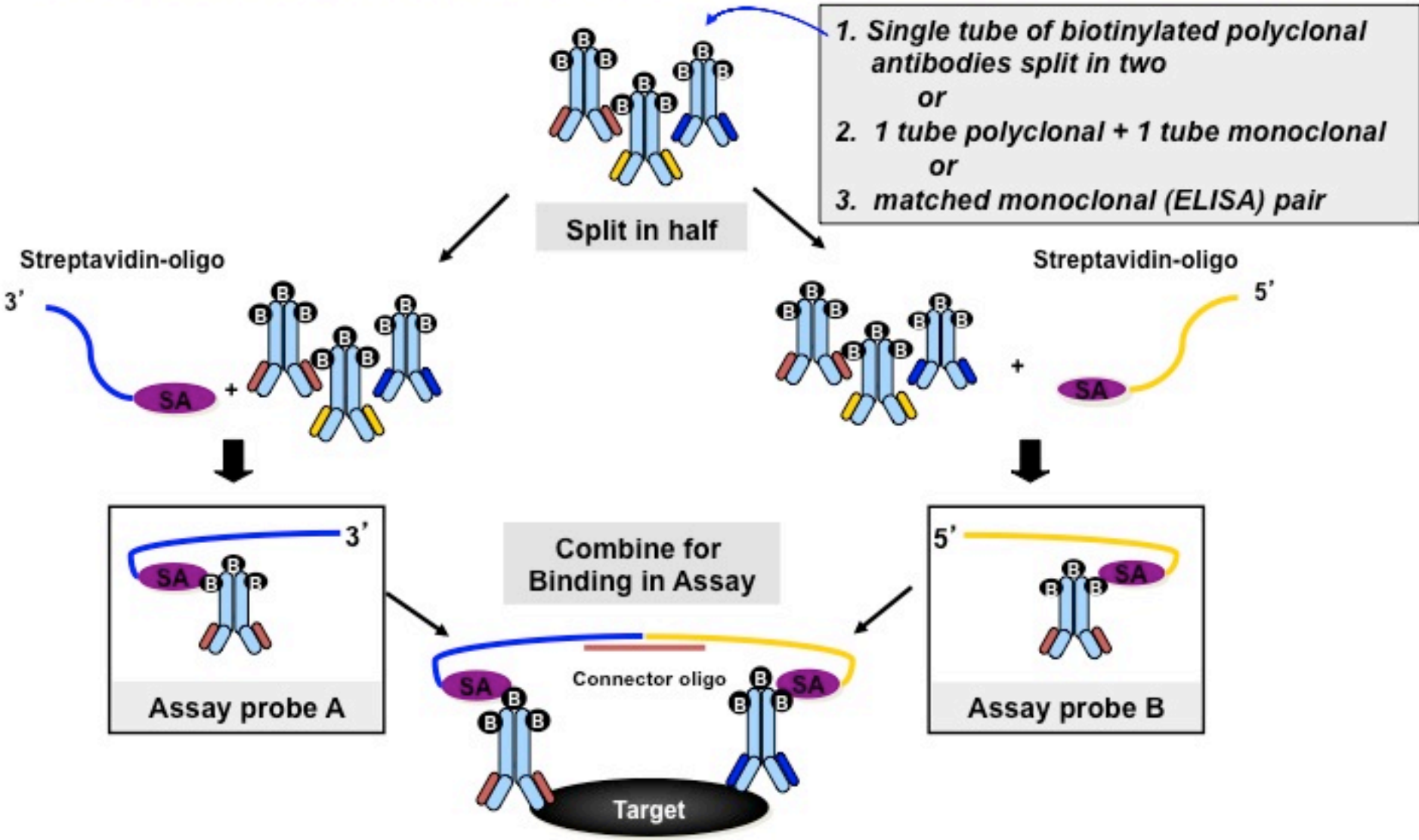


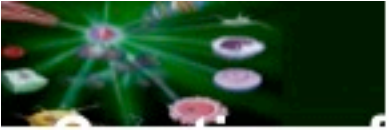
- Homogeneous assay based on two antibodies, each conjugated to a different oligonucleotide (one 3' oligo, one 5' oligo)
- When the two conjugated antibodies bind and are in close proximity, the oligonucleotides can be ligated, serving as the template for real-time PCR amplification and quantification

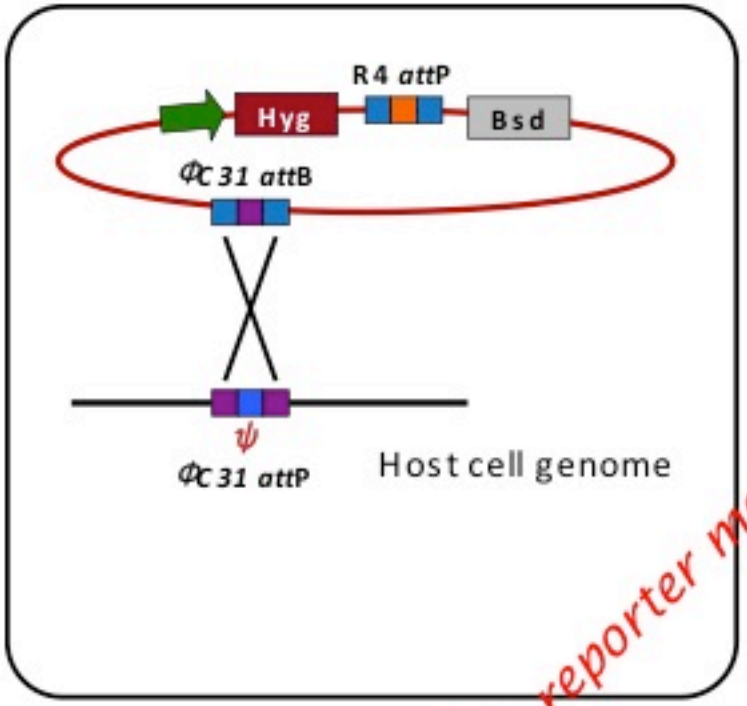
Invented by Professor Ulf Landegren, Simon Fredriksson and colleagues at Uppsala University/Olink Biosciences

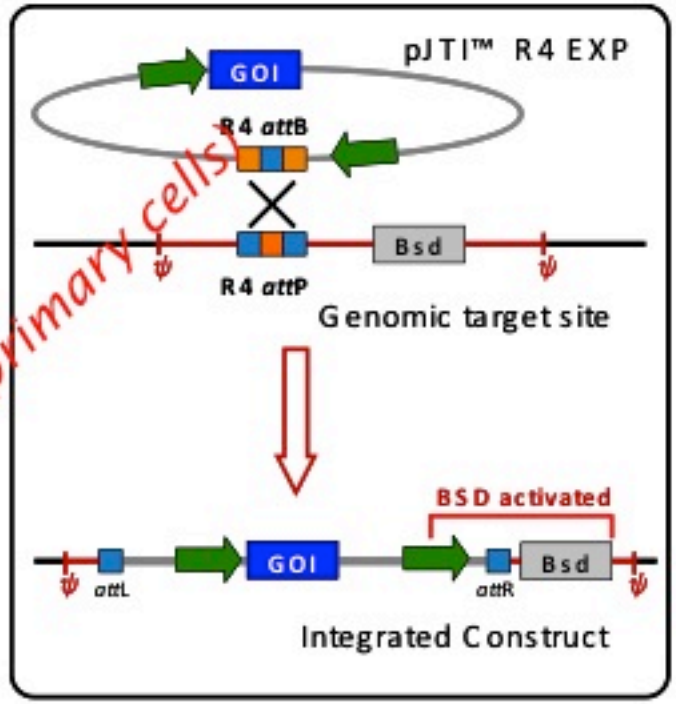
Fredriksson, S., et al. *Nature Biotechnology* **20**, 473 - 477 (2002)

Assay probes are made from biotinylated-antibodies & streptavidin-linked oligonucleotides







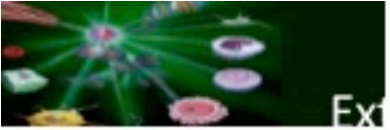


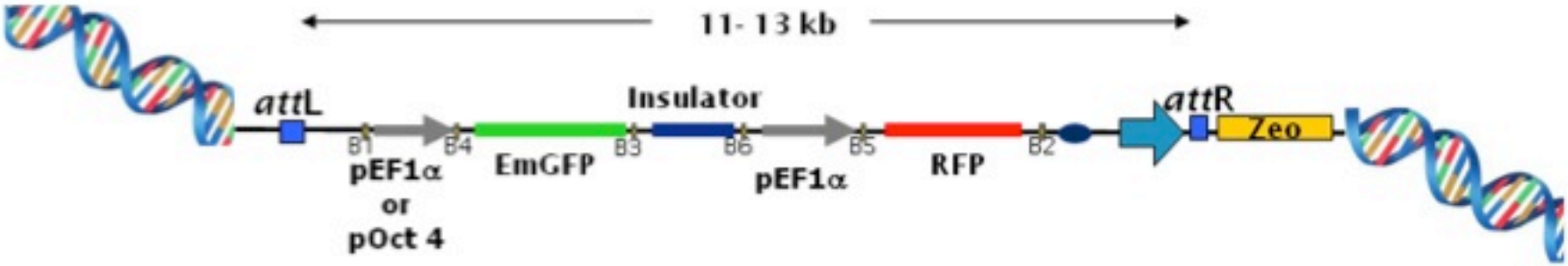
Proprietary reporter models in primary cells!

- First step involves creation of a target line mediated by PhiC31 integrase where the R4 target site and a promoter-less drug resistance gene is targeted to specific sites on the mammalian genome

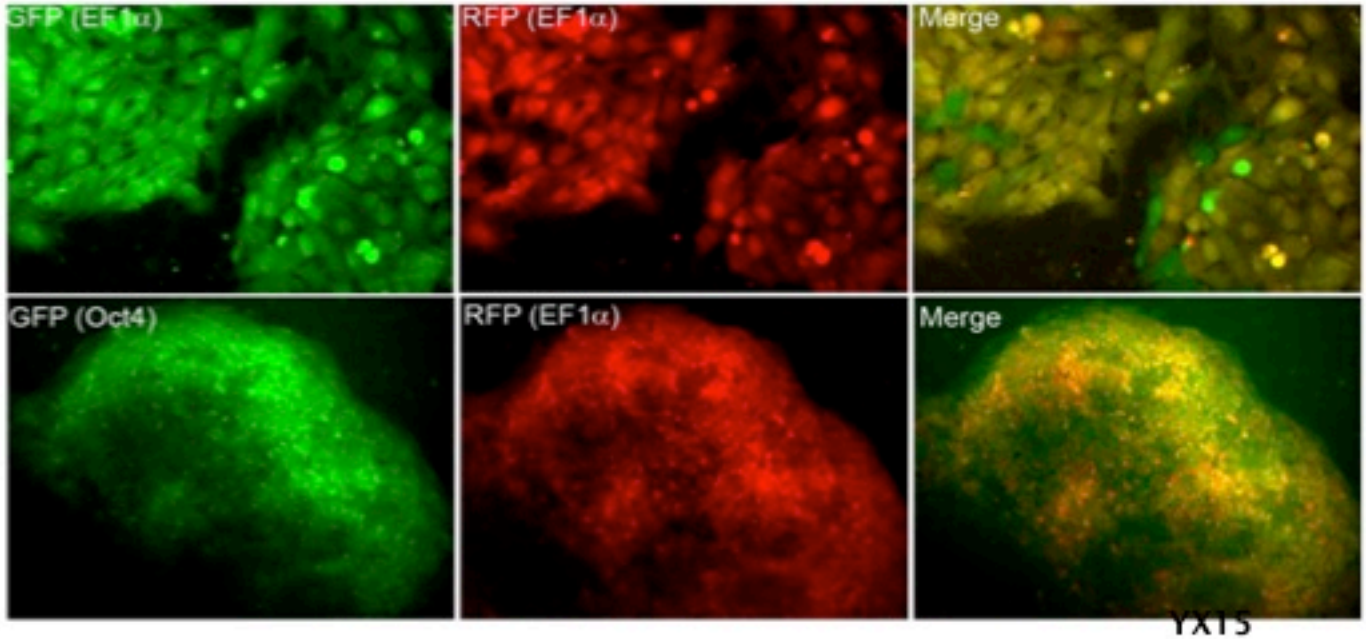
Liu et al. Stem Cells and Development (2009)

- Second step is R4 integrase mediated retargeting of a vector carrying a promoter along with the promoter-gene of interest. Precise recombination mediated by R4 results in reconstitution of the drug resistance gene which can be used to select for true retargeted clones



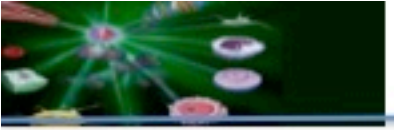


GFP Promoter



'Unlike lentivrus R4/13' platform accepts large, multigene expression constructs

Bhaskar Thyagarajan/ Haipeng Xue



Cell types currently available for screening

- Fetal
- Neonatal
- Adult
- Cadaveric

→ **Adult** → Differentiate

PACT
- Commercial
- Repositories

→ **PSC** → Differentiate → Intermediate Stage → Differentiated Cell

Engineer

- MSC
- HSC
- Hepatocytes (not great)
- Cardiomyocytes
- Keratinocyte
- Neural CNS& PNS
- RPE
- Islets

- MSC derived
- Bone
- Cartiliage
- Tendodn
- Adipocyte
- Smooth Muscle

- HSC derived
- T cells
- B cells
- Dendritic cells
- Macrophages
- RBC's

- NSC derived
- Dopaminergic
- Motoneurons
- Neurons
- Astrocytes
- Oligodendrocytes (no myelination model)
- Schwann Cells
- Sympathetic N
- Sensory N

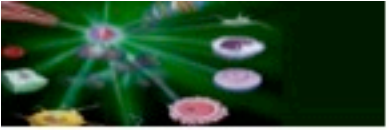
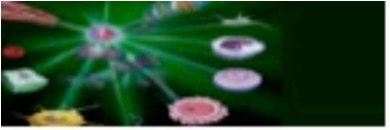
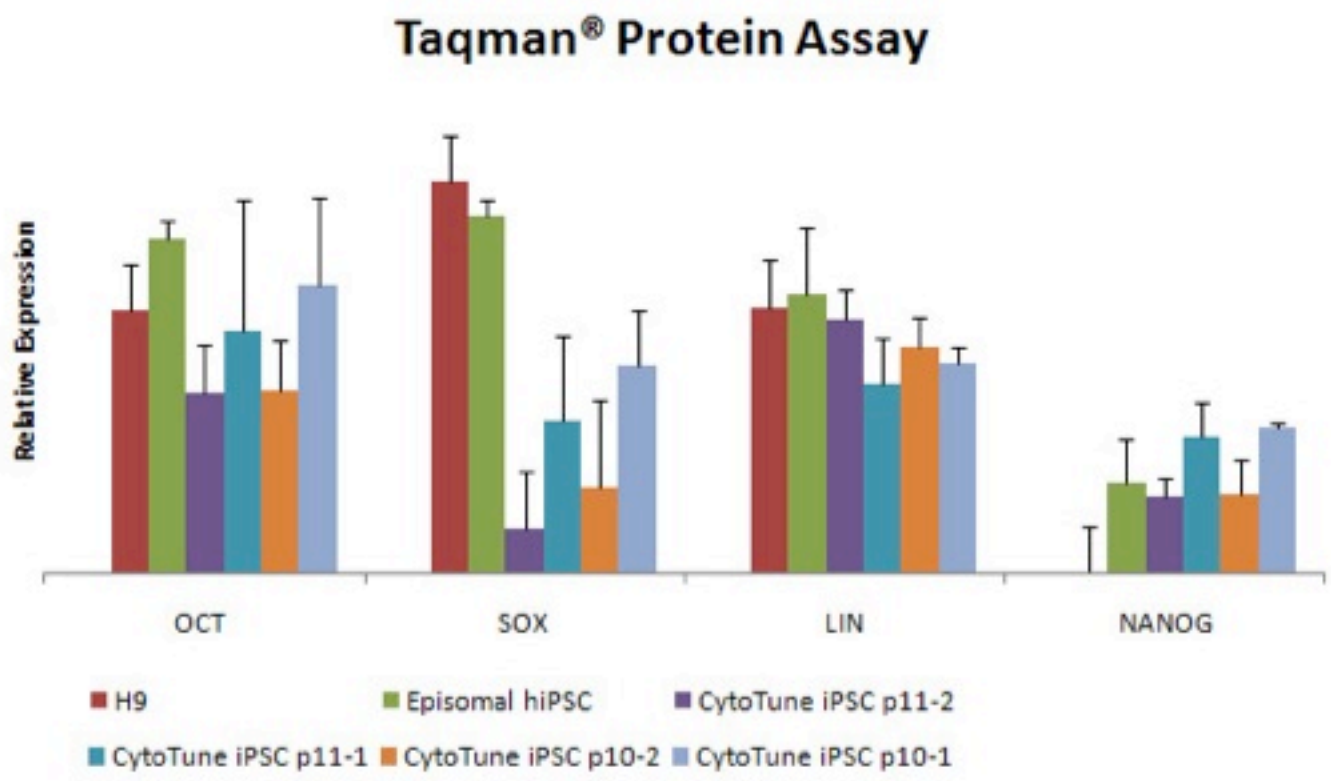
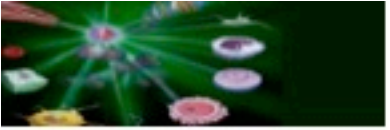


Table 1. Current Roadblocks to Stem Cell Therapy	
1.	Periods of limited government involvement due to legal, political and ethical issue
2.	Lack of mature regulatory policy
3.	Absence of uniform regulations and activity across countries
4.	Absence of uniform global patent interpretations on pluripotent stem cells
5.	Absence of standards and controls
6.	Lack of successful business models thus far for autologous therapy
7.	Limited availability of investment in new business models
8.	Issues of consent and sourcing related to cell-based manufacture
9.	Limited expertise in scaled-up cell manufacturing
10.	Issues of risk management, reimbursement, and long-term follow-up in cell-based therapy trials



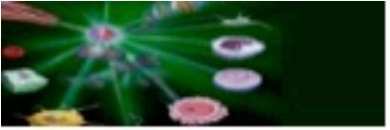
Characterizations of iPSC lines by TaqMan Protein Assays





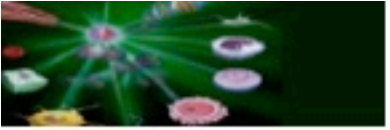
What is important for efforts in the mouse field

- Making iPSC from transgenic and other difficult strains
- Identifying the barriers to germ line transmission
- Looking at reactivation and epigenome issues in vivo
- Examining the potential immune issues with matched transplants and iPSC
- Providing a panel of reference lines for Tox efforts



Potential Breakthroughs in the iPSC field

- Zinc finger nuclease, Talens and R4 integrase methodologies for gene correction
- Is there a primed state?
- Development of novel differentiation methodologies
- Sorting and depletion protocols
- Direct differentiation
- Direct differentiation in vivo



Thank You

Questions- email

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