Neuronal Reprogramming via Nanochannel Electroporation
Honors Research Thesis
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Literature Review
Embryonic Development

- Blastocoele
- Blastula
- Gastrula
  - Mesoderm
  - Ectoderm
  - Endoderm

Blastopore
Embryonic Development
Embryonic Development

- All cells contain complete genetic information
- To result in different cell types, cells repress unnecessary genes and activate genes they do need
  - Transcriptional enhancers and repressors
  - Genomic regulation through epigenetics
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Effect of Epigenetics

- Altering gene expression without changing DNA sequence
DNA methylation

- A methyl group is added to a cytosine by DNA methyltransferase
- Cytosine methylation mainly occurs as a DNA transcription inhibitor
Histone Modification

- DNA wraps around the protein histones to form nucleosomes, which wind together to form condensed chromatin.
- Modification is most common via methylation or acetylation.
- Acetylation consists of adding an acetyl group to a lysine, reducing the attraction between the histone and the DNA sequence.
- Deacetylation results in more intermolecular forces between the DNA and the histone, resulting in a tighter structure.
Chromatin Structure

- Heterochromatin is chromatin tightly bound in complex forms; thus genes are inactive.
- Euchromatin is less densely packed, containing the majority of coding genes.
- This highly complex structure allows for efficient DNA packaging, prevents DNA damage, aids in DNA replication, and regulates gene expression.
Cloning and Cell Reprogramming History

- In 1952, a nucleus from a tadpole embryo transferred into an enucleated frog egg developed into a tadpole (Briggs & King, 1952)
- Then, for the first time somatic cell nuclear transfer (SCNT), injection of a nucleus from a fully differentiated adult cell into an enucleated egg, resulted in an embryo (Gurdon et al., 1958)
- In 2006, mouse fibroblasts were dedifferentiated into induced pluripotent stem (iPS) cells with expression of only four exogenous transcription factors (Takahashi & Yamanaka, 2006)
- Then three transcription factors, Brn2, Ascl1, and Myt11 (BAM), converted mouse fibroblasts into induced neurons (Vierbuchen et al., 2010)
Embryonic Development and Cell Reprogramming Overview
Natural Process of Cell Reprogramming

1N
CH₃CH₃CH₃
CH₃CH₃CH₃CH₃

CH₃
2N
CH₃
CH₃CH₃CH₃
CH₃CH₃CH₃CH₃

HIGHLY METHYLATED GAMETES

POORLY METHYLATED PLURIPOTENT STEM CELL

HIGHLY METHYLATED SOMATIC CELL

EXPERIMENTAL REPROGRAMMING

PHYSIOLOGICAL PROGRAMMING
Cell Reprogramming Components

- Machinery to reprogram, like enzymes that modify DNA and histones
- Pioneer factors that can identify their DNA sequence independent of the epigenetic state
- The presence of patterning genes to instruct the differentiation of cells
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Hindbrain Development

- Segmented into seven rostro-caudal (RC) rhombomeres with specific functional roles
- Each rhombomere has corresponding progenitor cells with patterning genes
- There is also dorso-ventral (DV) patterning
- RC-DV patterning factors form axes
Gradient Morphogen Hypothesis

- Morphogens act as positional cues for embryonic development
- They form a signaling gradient to regulate differential gene expression in a concentration dependent manner (Ashe & Briscoe, 2006)
Effect of Gradient Morphogen Hypothesis

- An example of the effect of morphogens on transcription factors produced
- *Krox20* is present *in situ* only very specifically in r3 and r5 as a result of morphogens
Transfection

- Artificial introduction and uptake of exogenous nucleic acids into cells

Diagram:
- Lipid Mediated
  - liposome-DNA complexes
  - heparan sulfate
- Viral Transduction
- Electroporation
- Polymers (e.g., Calcium Phosphate)
- Microinjection via microneedles
- Nuclear pore complex
- Cytosol
- Nucleus
Chemical Methods

- Diethylaminoethyl (DEAE)-dextran
  - Binds to nucleic acids, resulting in a complex be taken up by endocytosis
  - Advantages: relatively simple and low cost
  - Disadvantages: only results in transient transfection, high cytotoxicity, and low transfection efficiency

- Calcium phosphate co-precipitation
  - Nucleic acid and calcium chloride precipitate in a saline-phosphate solution which is taken up by cultured cells
  - Advantage: both transient and stable transfection, wide range of cell types
  - Disadvantages: high sensitivity to conditions, no \textit{in vivo} gene transfer, low efficiency
Lipids

- Artificial liposomes
  - Historically made to envelop nucleic acids then fuse with the plasma membrane

- Artificial cationic lipids
  - The lipid interacts with the nucleic acid to form a complex taken up by endocytosis
  - Advantages: broad size of nucleic acids, transient and stable transfections, and higher relative efficiency
  - Disadvantages: dependency on the cell type and culture conditions
Viral Methods

- Adenoviral and retroviral vectors infect cells with nucleic acids
- Advantages: *in vivo*, stable transfection, less cytotoxicity, specific cell targeting, and high efficiency rates
- Disadvantages: high costs, complex protocols, limit of size that can be incorporated, variation in virus tropism, and safety concerns
Physical Methods

- **Direct microinjection**
  - Extremely time-consuming and laborious

- **Bulk electroporation (BEP)**
  - A pulse of high voltage applied to cells in a solution forms transient nanopores
  - Advantages: once optimal conditions established it is simple, cost-effective, and can transfect a large number of cells at one time
  - Disadvantages: high cytotoxicity and the damage to the cell membrane
Current Transfection Drawbacks

- Although there are many methods of transfection, there are also many drawbacks
- High cytotoxicity and low transfection efficiency
- All of the methods discussed have another flaw: random nature of transfection
- Multicistronic vectors with co-expression of multiple genes partially resolve the problem, but a limit on the size of plasmids also restricts this method
Experimental Questions
Experimental Questions

- Can adding patterning genes to BAM-mediated neuronal reprogramming increase efficiency rates?
- What is the role of the cell cycle in neuronal reprogramming?
- What stages do cells go through during neuronal reprogramming?
- What are the reasons for such low efficiency rates in neuronal reprogramming?
Methodology
Nanochannel Electroporation (NEP)

- An optimal gene delivery technology capable of introducing complex combinations of DNA into a large number of individual cells through electroporation using a 3D array of nanochannels in a modified Transwell insert.

- Results in high transfection efficiencies and low cell-to-cell variability (Boukany et al., 2011).
NEP for Neuronal Reprogramming

- Prepared exogenous plasmids utilizing restriction enzymes and DNA ligase
- E12 MEFs (mouse embryonic fibroblasts) underwent NEP with a 1:2:1 ratio of *Brn2*, *Ascl1*, and *Myt1l* (BAM)
- Cells stained via fluorescent immunohistochemistry techniques

- Images viewed with fluorescence microscopy
- Neuronal reprogramming confirmed by staining for TUJ1, a neuronal marker
Results
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Addition of \textit{PHOX2B}, \textit{PHOX2A}, and \textit{RUNX3} (PRP) to BAM-Mediated Reprogramming

- Transcription factors \textit{PHOX2B}, \textit{RUNX3}, and \textit{PHOX2A} (PRP) are involved in the patterning of autonomic hindbrain neurons (Levanon et al., 2001, Brunet & Pattyn, 2002)

- We added PRP plasmids with BAM to the NEP cocktail and measure the relative efficiency of neuronal reprogramming
BAM versus BAM-PRP NEP results in increased neuronal reprogramming efficiency and complexity.
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Cyclin A2 Deletion

- Cyclins regulate the cell cycle by activating cyclin-dependent kinases.
- Cyclin A2 (CCNA2) promotes DNA replication, particularly for the onset of the S-phase (Girard et al., 1991).
- To delete CCNA2, we acquired CCNA2^{fl/fl} MEFs which results in removal of the gene after cre-mediated recombination.
Early CCNA2 Deletion Decreases Induced Neurons

- Onset of S-phase or other CCNA2 functions may be required for neuronal reprogramming.
Experimental Questions

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Astrocytes Present After NEP

- MEFs may be undergoing dedifferentiation and then pass through a neural stem cell phase
- Test for astrocytes, another product of neural progenitors, after NEP by staining for GFAP
Nestin Present in GFP-positive cells after NEP

- To test the possibility that the MEFs undergo a neural progenitor cell stage, we tested for nestin, an intermediate filament expressed in neural stem cells.
GFP-Positive Cells had Previous Nestin-Positive Stage

- To confirm that nestin-positive cells underwent reprogramming, MEFs were made with two loci, *nestin-cre-ER* and *Rosa<sub>mtDTomato/mGFP</sub>*

- Alone these MEFs express TdTomato, but with functional *cre*, deletion of TdTomato and expression of GFP occurs

- *Cre* recombinase will only function in nestin-positive cells that have been treated with 1 μM 4-OH (tamoxifen)
GFP-Positive Cells had Previous Nestin-Positive Stage
A Subset of Induced Neurons Undergo a Nestin-Positive Stage

![Graph and images showing GFP positive cells under different conditions.](image)
Discussion
Experimental Questions

- Can adding patterning genes to BAM-mediated neuronal reprogramming increase efficiency rates?
- What is the role of the cell cycle in neuronal reprogramming?
- What stages do cells go through during neuronal reprogramming?
- What are the reasons for such low efficiency rates in neuronal reprogramming?
The increase in induced neurons using PRP was relatively small, so they may be other factors that regulate induced neuron efficiency downstream of exogenous plasmid delivery.

Since early deletion of *CCNA2* resulted in a decrease of induced neurons, perhaps only MEFs are in S phase at the time of NEP can undergo directed differentiation.

Because some MEFs undergo a nestin-positive neuronal progenitor-like stage, cells first may undergo dedifferentiation and then re-differentiation, and there are with different pathways they may follow, such as to neurons or astrocytes.
Future Direction

- Measures must be taken during neuronal reprogramming to reduce the random nature of directed differentiation
- NEP can use more than DNA for cell reprogramming; any charged molecule utilized in reprogramming such as RNA (Warren et al., 2010), miRNA, and drugs (Guo et al., 2013)
- Improving the NEP platform, to control even more precisely the DNA intake of cells.
- Cell reprogramming has many implications for use in regenerative medicine and clinical applications using these techniques
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Questions?