Center for Orthopedic & Regenerative Medicine Science

Neucleated Plasma Particles (NucP-2): A new era in Regenerative Medicine

Michael N. Brown, MD, DC, DABPMR-PAIN

Disclosures
Corporate Associations

• Executive with CellPar Tech, LLC
  – Stem cell & regenerative medicine technology and research.
• FASER:
  – Force Assisted Soliton Energy Resonance
  – R&D team of physicist and engineers working on PEMF use in medicine
  – Green tech

Notification
Title change...

• If you have the right seeds you do not need as much fertilizer.
  – Washington is greener than Texas
A product of repeated clinical failures

Michael N. Brown, MD

Where have we been… where are we going?

- As development proceeds from a "totipotent" zygote cells proliferate and segregate by lineage-progression, resulting in formation of differentiated phenotypes.
- Processes begin with a totipotent zygote and continues throughout life.
- As development proceeds from totipotent zygote cells proliferate and segregated by lineage-commitment into two pluripotent layers:
  - TROPHBLAST \(\rightarrow\) Extra-embryonic membranes of placenta and the inner cell mass which becomes embryo.
  - INNER CELL MASS \(\rightarrow\)
    - Hypoblast
    - Epiblast

Hypoblast & Epiblast

- Hypoblast and Epiblast:
  - Segregates into pluripotent primary germ layers:
    - Ectodermal
    - Mesoderm
    - Endoderm
- Further segregation through lineage-commitment:
  - Multipotent
  - Tripotent
  - Bipotent
- Eventually \(\rightarrow\) Unipotent lineages further defines differentiation pathways to become quiescent reserve stem cells.
- Fxn: maintenance and repair of tissues of postnatal organism.
Precursor Cells in Adults

Totipotent SCs: Embryo, placentia, Gamates (sperm & ovum)

Pluripotent SCs: form ectodermal lineage cells, mesoderm lineage cells, and endoderm lineage cells. → All cells of the embryo but not the placenta and gamates.

Ultra-small nucleated plasma particle (u-NP2)
Small-nucleated plasma particle (s-NP2)
Intermediate-nucleated plasma particle (i-NP2)
Large-nucleated plasma particle (l-NP2)

Germ Layer Lineage SCs

GL-EctoSCs, GL-MesoSCs*, GL-EndoSCs

Progenitor Cells

Multipotent PCs: → hematopoietic
Tripotent PCs, → chondral-osteo-adipogenic
Bipotent PCs: → adipo-fibrogenic
Unipotent PCs: → myoblast, fibroblast, adipoblast, etc.

Embryonic Stem Cell Matching

• Embryonics: HLA mismatch
  → Donor cells are rejected with mismatch
• Some have got around this with therapeutic cloning.
  → Cell from recipient nucleus into donor cell and thus recognized as self.
  → Very expensive
  → Cells have limited life span and do not have same plasticity.
  → Can not transplant male to female cells

Holy grail

Gold Silver Bronze
Unipotent Reserve Stem Cells:

- Examples:
  - Unipotent myosatellite myoblasts of muscle (Mauro, 1961; Campion, 1984; Grounds et al., 1992).
  - Unipotent adipoblast cells of adipose tissue (Allhaud et al., 1992).
  - Unipotent chondrogenic cells and osteogenic cells of perichondrium and periosteum (Cruess, 1982; Young et al., 1995).
  - Bipotent reserve mesenchymal stem cells: adipofibroblasts (Vierck et al. 1996; Beresford, 1989; Caplan et al. 1997.) and others.

Multipotent cells

- Multipotent stem cells of marrow:
  - Owen, 1988; Beresford, 1989; Rickard et al., 1994; Caplan et al., 1997; Prockop, 1997).
- Tripotent preserved mesenchymal stem cells:
  - Cardiogenic/osteogenic/adipogenic stem cells of marrow. (Pittenger et al., 1999).
- Multipotent hematopoietic stem cells of marrow:
  (Palis and Segel, 1989; Maguire, 1998; Ratajczak et al., 1998)

Adult pleuripotent cells?

- Pluripotent mesenchymal stem cells initially found in connective tissues of prenatal avians, mice and rats (Grigoriadis et al., 1988, Young et al., 1993, 1998; Rogers et al., 1995).
- Pluripotent stem cells are not committed to any particular tissue lineage.
  - Can generate into multiple lineage-committed progenitor cells from a single clone, thus forming any tissue within a particular germ layer lineage.
  - Isolated from postnatal animals-capable of extended self renewal as long as they remain uncommitted to a particular lineage.
- Example: PP mesenchymal stem cell clone → Myotubes, fat, cartilage nodules and bone after 690 cell doublings.
Adult pleuripotent cells?

• Unlike progenitor stem cells, pluripotent stem cells are not affected by progression factors such as insulin, IGF-1, IGF-II.
• Differentiation factors such as dexamethasone, BMP, muscle morphogenic protein (MMP) does induce lineage commitment in these cells.
• Once PPSCs commit to a particular lineage they assume the characteristics of lineage-committed progenitor cells.
  – Their ability to replicate reduces from $600 \times 60 \times$ before becoming senescent.

• Really? Can we do this?
• Studies and postnatal rats, rabbits and humans suggest that resident stem cells consistent both lineage-committed cells and lineage non-committed pluripotent stem cells.
  – (Pate et al. 1993; Lucas et al., 1995, 1996; Young et al., 1999).
• In addition there are established populations of lineage-committed progenitor stem cells.
  – (Mauro, 1961; Cruess, 1982; Campion, 1984; Owen, 1988; Beresford, 1989; Alhadj et al. 1992; Grounds et al., 1992; Rickard et al., 1994; Young et al., 1995; Vorck et al., 1996; Caplan et al., 1997; Prolopo, 1997; Frals and Segel, 1998; Maguire, 1998; Paraczyk et al., 1998; Pittsenger et al., 1999).

Adult pleuripotent cells?

• 2001: Clonogenic Analysis of Reserve Stem Cells in Postnatal Mammals: Isolation from muscle dissection → Once isolated and incubated with insulin, less than 1% of the cells displayed phenotypic markers for differentiated cells of various mesodermal clininc ages.
• Thus, the majority of progenitor stem cells were removed from the population by propagating the cells for more than 50 cell doublings some prior to cloning.
  – 2001: the larger cells with low ratios of nucleus to cytoplasm were observed to undergo apoptosis between 40-50 cell doublings.
  – The remainder of cells that continued to divide were smaller with low CP:Nuc ratio.
Totipotent & pluripotent cultures vs. progenitor cells

- Regenerative cells expand to confluence.
- Toti and Pluri form layers and not inhibited by contact inhibition.

Precursor cells formed during embryogenesis

- Remarkably, while the vast majority of developing blastomeres transition through the sequence of developmental and differentiation, a few cells become reserve precursor cells that provide for continual maintenance (progenitor cells) and repair (stem cells) of the organism.
- The totipotent and pluripotent stem cells can be seen as early as the morula stage in human embryonic development.

Precursor Cells ESCs

- **Totipotent stem cells**: equivalent in differentiation potential to the blastomeres of the morula and blastocyst.
- **Pluripotent stem cells**: are equivalent in differentiation potential to the cells of the epiblast
- **Germ layer lineage stem cells**:
  - **ENDODERM** Stem cells gastrointestinal epithelium, hepatocytes, and pancreatic cells (α-cells, β-cells, δ-cells, and ductal cells)
  - **ECTODERM** Stem Cells neurons, neuroglia, epidermis, hair, teeth, and nails
  - **MESODERM** Stem Cells muscle, fat, cartilage bone, connective tissues, endothelial cells and blood cells
Embryonic stem cell & Teratomas

- When ESCs were placed into an animal they can form a mass of tissue that contained a variety of cell types in a non-spatially arranged jumbled fashion.
- This configuration is reminiscent of the sacrococcygeal teratoma that can form during embryogenesis if residual primitive streak cells remain after gastrulation at the caudal end of the developing embryo, located caudal to the notochord (the primary inducer of the embryo).

Teratoma in APPSC * ATPSC ?

- Adult PPSC and TPSC cells once placed in environment commit to a particular tissue/cell lineage lose the telomerase enzyme.
- Thus assume all attributes of tissue-committed progenitor cells, including a defined biological lifespan of 8–10 population doublings for rodents and 50–70 population doublings for humans.
- Thus… No Teratomas!


Embryonic SCs discovery

- The reports of mouse embryonic stem cells spurred the development of embryonic stem cells in other species.
- ESCs utilizing similar technologies have been verified by other labs in mice and generated from blastocysts of rat, golden hamster, rabbit, mink, pig, sheep, cow, horse, marmoset, non-human primate (Rhesus monkey), human, chicken, medaka, zebra fish and gilthead sea bream.

Alexander A. Maximow. He was a Russian-American physician published from 1896 until 1902, first person to use the term "stem cells"
Adult Precursor Cells

- Due to their appearance early in development and segregation with the developing blastomeres, precursor cells with similar differentiation potentials as those described for embryonic cells have been postulated to occur in most organs and tissues of the body throughout the lifespan of the individual.

- **Adult precursor cells:**
  - ID’d by isolation from solid organs, fluids, etc. via cryosection and immunocytochemical staining for cell precursor cell-specific antigen markers.
  - ID’d in mouse, rat, rabbit, cat, dog, goat, sheep, pig, cow, horse, and human.

References

Adult precursor cells

- These precursor cells were composed of:
  - Progenitor cells
  - Stem cells.

- The stem cells included:
  - Totipotent stem cells
  - Pluripotent stem cells
  - Germ layer lineage
    - ectodermal stem cells,
    - mesodermal stem cells,
    - endodermal stem cells

References

How did we find the cell???

- Histology
- Energy-dispersive spectral analysis coupled with scanning electron microscopy
- Carbohydrate biochemistry
- Qualitative and quantitative enzyme-linked immunoculture assays
- Differential cryopreservation
- Repetitive limiting serial dilution clonogenic analysis
- Cluster of differentiation (CD) marker analysis
- Fluorescent activated cell sorting
- Magnetic bead cell sorting
- Molecular analyses for telomerase activity and gene expression
- Bioactive factor pluripotency expression analyses
- Differential centrifugation
- Differential isolation and plating
- Immunocytochemistry
- Carbohydrate histochemistry

Types of TPSCs & PPSCs

- 2 Totipotent stem cell types
- 6 pluripotent stem cell types

Clinical application of TPSCs, PPSCs & MSCs

- 2 Totipotent stem cell types
- 6 pluripotent stem cell types
- Mesenchymal
### Pluri and Toti Isolation

- **Requires:**
  - Agent to expand cell numbers in the patient (in vivo) 3 x by 30 days.
  - Mobilization agent:
    - Reversed vascular diapedesis → 2-3 x yield
- **Combination results in isolation of cells similar to seen in culture and expansion**

### TPSC & PPSC Behavior

- **Activation required to use clinically**
  - Isolate the cells
  - Inject the cells without activation and they simply reseed the tissues and remain quiescent !!!
  - Activation process will meet minimal manipulation criteria

### Cytochemistry ID

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**TPSCs:**
- Transitional Blastomere-Like Stem Cell
- Epiblast Like Stem Cells;
- Tr-GLSCs: Transitional Germ Layer

**PPSCs:**
- Lineage Stem Cells;
  - MesoSCs: Mesodermal Germ Layer
  - Lineage Stem Cells; Mes PCs
  - Mesenchymal Progenitor Cells
Progenitor Cells

- By definition = lineage/tissue/cell-committed progenitor cells.
  - No inherent plasticity, but rather have both a defined differentiation potential
  - Limited replicative lifespan.
    • Ie: unipotent myosatellite myoblast progenitor cells will only form skeletal muscle.
    • Bipotent adipose-fibroblast progenitor cells will only form adipocytes (fat cells) and fibroblasts.
    • Tripotent chondroblast-osteoblast-adipoblast progenitor cells will only form cartilage, bone, and fat cells.
  - While multipotent hematopoietic progenitor cells will form the myriad of cell types within the hematopoietic lineage but not any cell type outside that lineage.
- Progenitor limited population doublings before pre-programmed senescence and cell death occurs.
- 8-10 population doublings for rodents and 50-70 population doublings for humans.

References


Endogenous Stem Cells

- In contrast to adult-derived lineage/tissues/cell-committed progenitor cells, adult-derived lineage-uncommitted stem cells demonstrate:
  - inherent plasticity, being capable of replicating themselves and forming their respective downstream cell types.
- Embryonic stem cells ➔ preprogrammed to spontaneously differentiate into tissues of the embryo/fetus or lineage-committed progenitor cells.
Endogenous Stem Cells

- **Adult Endogenous Stem cells** → uncommitted adult stem cells are not preprogrammed to form anything.
  - respond to environmental cues (i.e., inductive agents) to differentiate into specific cell types based on the particular activity of an inductive agent.
  - Ie.
    - nerve growth factor induces totipotent stem cells, pluripotent stem cells and endodermal stem cells to form neurons;
    - bone morphogenetic protein 2 induces totipotent stem cells, pluripotent stem cells and mesodermal stem cells to form bone;
    - while hepatocyte growth factor induces totipotent stem cells, pluripotent stem cells and endodermal stem cells to form hepatocytes.

References


Endogenous Stem Cells

- Unidirectional differentiation pathway for endogenous precursor cells, i.e., stem cells and progenitor cells. BLSCs, blastomere-like stem cells; ELSCs, epiblast-like stem cells; EctoSCs, ectodermal stem cells; MesoSCs, mesodermal stem cells; Endo SCs, endodermal stem cells; M, multipotent; T, Tripotent; B, Bipotent; U, Unipotent.
Histochem of stem cells

**Totipotent cells**

**Pluripotent cells**

Collectively all now called Nucleated Plasma Particles → **NucP2**

Endogenous Stem Cells

- **Totipotent stem cells:**
  - Will replicate themselves as well as forming similar downstream cell types, similar to embryonic blastomeres of the morula stage of development.
  - Will form all somatic tissues within the embryo proper including spermatogonia.
  - Equivalent in differentiation potential to the blastomeres of the morula and blastocyst.

- **Pluripotent stem cells:**
  - Will replicate themselves and form similar downstream cell types as embryonic epiblast cells (all somatic cells of the embryo but not the gametes).
  - Equivalent in differentiation potential to the cells of the epiblast.

**Germ layer lineage stem cells** (ectodermal, mesodermal and endodermal stem cells) will replicate themselves as well as form downstream cell types belonging to their respective germ layer lineages.

They are thus equivalent in differentiation potential to the cells comprising the ectodermal, mesodermal, and endodermal germ layer lineages, respectively.

Precursor cells that are “true” stem cells and not misnamed progenitor cells (lineage-uncommitted cells rather than lineage-committed cells) contain the telomerase enzyme as long as the cells remain lineage-uncommitted.
MSCs...

- “Caplan cells”:
  - CD 105, CD 123, CD 166
  - Multi potent progenitor cells
  - Bone, cartilage, fat
- Blood derived mesenchymal cells:
  - More homogeneous mesenchymal cell population.
  - CD 90, CD 13
  - Bone marrow, fat, cartilage, blood, endothelial cells, connective tissue, tendon, ligament, kidney, spleen, or trabeculae.

The Telomere & Teleomerase Enzyme

Critical for lineage uncommitted stem cell replication

Teleomerase

- Telomerase reverse transcriptase (TERT), telomerase RNA (TR or TERC)
- TERT has a ‘mitten’ structure that allows it to wrap around the chromosome to add single-stranded telomere repeats
- In normal circumstances, without the presence of telomerase, if a cell divides recursively, at some point all the progeny will reach their Hayflick limit
- With the presence of telomerase, each dividing cell can replace the lost bit of DNA, and any single cell can then divide unbounded
Endogenous Stem Cells

The telomerase enzyme adds telomeres to the ends of the chromosomes, protecting the chromosomes from degradation due to increased mitotic divisions.

Protection from chromosomal degradation gives these postnatal stem cells the capability for extensive proliferation.

The preprogrammed biological clock for endogenous stem cells does not begin at birth of the individual, but rather when the stem cells commit to a particular progenitor tissue/cell lineage.

The rub...


• au contraire !:
  – sufficient number of separate laboratories have proven that endogenous pluripotent stem cells do exist and can be readily isolated, characterized and utilized.

Amniotic Fluid-Derived Pluripotent Stem Cells

• The amniotic fluid contains a mixture of predominantly epithelial cells. It is thought that they are sloughed off from the epidermis, gastrointestinal, and urinary epithelia. They range from six to fifty microns in size and express cell surface markers from all three primary germ layer lineages.

• Small percentage of cells that display the c-kit cell surface marker by FACS analysis. This c-kit-positive population of cells were expanded to sub-confluence every 48-72 hours.

• These c-kit-positive cells derived from the amniotic fluid demonstrate greater than 300 population doublings, which is far greater than Hayflick’s limit.


Multipotent Adult Progenitor Cells (MAPCs)

• Multipotent Adult Progenitor Cells (MAPCs) were isolated from the bone marrow, muscle and brain of mice by Reyes M and colleagues.

• Originally, these stem cells were designated as mesenchymal because they appeared to resemble those described by Caplan.

• MAPCs were later demonstrated to form neurons (ectodermal germ layer lineage), and hepatocytes (endodermal germ layer lineage). Thus, MAPCs can differentiate into cell types from all three primary germ layer lineages.

• Thus MAPCs appear to be pluripotent stem cells rather than mesenchymal stem cells as they were first designated.

• Along the stem cell developmental continuum from totipotent stem cells (which grow in suspension culture to adherent culture) to unipotent progenitor cells (which grow as adherent cultures), MAPCs grow as adherent cultures.

Very Small Embryonic-Like Stem Cells

• Very small three to five micron cells were originally isolated from adult murine bone marrow (Sca1+Lin-CD45-), and human cord blood (CD133+Lin-CD45-).

• These cells were detectable with early developmental markers, such as cell surface SSEA, and nuclear Oct4 and Nanog transcription factors.

• These particular cells have a high nuclear to cytoplasmic ratio and demonstrate a preponderance of euchromatin rather than heterochromatin.

• Cells with these same characteristics have been found in:
  • skeletal muscle,
  • gonads,
  • heart,
  • brain.
**Very Small Embryonic-Like Stem Cells**

- In the appropriate in vivo model systems, these cells have been shown to differentiate into long-term repopulating:
  - mesenchymal stem cells,
  - skeletal muscle,
  - endothelial cells,
  - cardiac myocytes,
  - hematopoietic stem cells,
  - lung epithelial cells,
  - tumor stromal cells
  - oocytes.

Because of their small size, embryonic markers, and ability to form multiple primitive cell layers of the conceptus, these cells have been designated as very small embryonic-like stem cells (VSELs).

Very low numbers of VSELs circulate throughout the vasculature under steady-state conditions.

Additional VSELs can be mobilized in patients during myocardial infarction, stroke, acute burns, active inflammatory bowel disease, and cancer and with the use of granulocyte-colony stimulating factor.

**Adult Pluripotent Stem Cells**

- Originally isolated from mouse, rat and human skeletal muscle of newborn and adult.
- These cells were detectable with early developmental markers, such as cell surface SSEA (stage specific embryonic antigen) and CEA-CAM-1 (carcinoembryonic antigen-cell adhesion molecule-1), nuclear transcription factors Nanog, Nanos, Bcl-2, CXCR4, Oct4, and telomerase.
- These particular cells have a high nuclear to cytoplasmic ratio.
- These cells display a normal karyotype after population doublings well in excess Hayflick’s limit.
Adult Pluripotent Stem Cells

- Six to eight micron cells (SSEA+/CD10+) and 0.2 to 2.0 micron cells (CEA-CAM-1+/CD66e+) were originally isolated from mouse, rat and human skeletal muscle of newborn, adolescent, sexually mature and geriatric-aged individuals.
- These cells were detectable with early developmental markers, such as cell surface SSEA (stage specific embryonic antigen) and CEA-CAM-1 (carcinoembryonic antigen-cell adhesion molecule-1) nuclear transcription factors Nanog, Nanos, Bcl-2, CXCR4, Oct4, and telomerase.
- These particular cells have a high nuclear to cytoplasmic ratio. These cells display a normal karyotype after population doublings well in excess Hayflick's limit.
- Cells with these same characteristics have been found in 37 different tissues and organs thus far, including blood, bone marrow, brain, adipose tissue, and skeletal muscle.
- In the appropriate in vivo and in vitro model systems, these cells have been shown to differentiate and demonstrate phenotypic expression markers for 63 (6-8 µm cells) and 66 (0.2-2.0 µm cells) distinct cell types, including neurons, glial cells, keratinocytes, muscle, fat, cartilage, bone, connective tissues, hematopoietic cells, GI epithelium, liver cells, pancreatic cells, and spermatogonia.

Adult Pluripotent Stem Cells

- Because of their small size, presence of embryonic markers and their ability to form cells from the three primary germ layer lineages as well as spermatogonia, these cells were named according to parallel structures found within the developing zygote, i.e., epiblast-like stem cells (PPSC, 6-8 µm cells) and blastomere-like stem cells (TPSC, 0.2-2.0 µm cells).
- Moderate numbers of these cells circulate throughout the peripheral vasculature under steady state conditions.
- Additional PPSC and TPSC can be mobilized and harvested following exercise, severe trauma and after ingestion of a nutraceutical.
**Additional Pluripotent Stem Cells**

- Following an isolation and propagation protocol similar to that used for the MAPCs, a cell termed "unrestricted somatic stem cell (USSC)" was isolated from bone marrow in 1-3 weeks, but with only three cell passages.
- The cells showed the capability to form ectodermal, mesodermal and endodermal phenotypes.
- Marrow-isolated adult multilineage inducible (MIAMI) cells were also derived in a manner similar to MAPCs.
- Their derivation is based on seeding densities at either clonal or sparse dilutions.
- MIAMI cells demonstrated differentiation into ectodermal, mesodermal and endodermal phenotypes.

**Additional Pluripotent Stem Cells**

- Human bone marrow-derived multipotent stem cells (hBMSCs) have been reported.
- As the name implies, these cells were isolated from human bone marrow and selected from adherent cultures.
- Differentiation studies showed that these cells form phenotypes belonging to all three germ layer lineages. Fetal somatic stem cells (FSSCs) have been reported.
- The cells, derived from fetal soma, demonstrate a wide range of differentiation potentials.

**Reprogrammed Pluripotent Stem Cells**

- The idea of cloning animals utilizing nuclear transfer was first proposed by Spemann.
- The first demonstration that Spemann’s proposal was even possible for cloning adult animals was shown by Gurdon.
- Indeed, the concept of reprogramming a patient’s somatic cells into totipotent/pluripotent stem cells was conceived based on four independent breakthroughs in the field of developmental embryology in the late 1900’s, i.e., somatic cell nuclear transfer in amphibians; success of cloning of sheep (Dolly) by somatic cell nuclear transfer; assisted reproductive technologies for live human births (Mary Louise Brown); and derivation of human embryonic stem cells.
Somatic Cell Nuclear Transfer (SCNT)

- The process of somatic cell nuclear transfer, i.e., reprogramming of somatic cell nuclei into a totipotent embryonic stem cell capable of forming an organism, was first proposed by Hans Spemann when he referred to a "fantastical experiment" in his book, Embryonic Development and Induction.
- However, Spemann's process was first demonstrated by Gurdon.
- He utilized the nuclei from the intestinal epithelial cells of *Xenopus laevis* tadpoles and transferred the nuclei into the cytoplasm of enucleated eggs to demonstrate the development of an adult frog.
- His work demonstrated that the ova contained maternal factors within its cytoplasm with the inherent ability to reprogram gene expression of a differentiated somatic cell nucleus and that that nucleus had the capability of forming a new organism of the same genetic makeup as the host organism.
- His experiment represented the first reported example of a somatic cell being reprogrammed back to a totipotent state by an enucleated egg and developing into a live, viable offspring.

Somatic Cell Nuclear Transfer (SCNT)

- Therapeutic cloning, or SCNT, begins with the same process used to create Dolly.
- A diploid donor somatic cell from a body tissue, such as a fibroblast from skin, is stripped of its plasma membrane and cytoplasm and fused with an enucleated unfertilized ovum. The cytoplasm within the ovum reprograms the DNA within the diploid donor nucleus to an embryonic state.
- The fused cell (modified blastomere) is allowed to rest for a defined length of time and then induced to proliferate until it reaches the early blastocyst stage, composed of the inner cell mass and trophoblast. The inner cell mass cells are harvested and cultured to create a stable cell line that is genetically matched to the donor cells. The cells are pluripotent in that they:
  i. Have a capacity for unlimited cellular proliferation,
  ii. Will form cells from all three primary germ layer lineages and 
  iii. Will form teratomas. These are the hallmarks of embryonic pluripotent stem cells.

Is it the wave of the future?
Induced Pluripotent Stem Cells (iPSCs)

- The most widely accepted method to generate induced pluripotent stem cells (iPSCs) is the retroviral vector introduction of four genes (Oct4, Sox2, Klf4 and c-Myc), a.k.a., the Yamanaka factors, into more differentiated cell types, such as fibroblasts.
- Following the initial landmark work for which Yamanaka received the 2013 Nobel prize, iPSCs have since been generated from differentiated fetal and adult fibroblasts; hepatocytes; stomach cells; keratinocytes; cord blood; peripheral blood; fully differentiated B- and T-lymphocytes; dental pulp cells; and kidney cells.

Precursor Cell Definitions

Stem Cells
1) Stem cells ARE “Plastic” – can form any downstream cell type (unidirectional only).
2) Stem cells are NOT committed to any specific cell / tissue type.
3) Stem cells have unlimited proliferation potential
4) Stem cells maintain a biological “clock” of zero until differentiation begins.

Progenitor Cells
1) Progenitor cells are NOT “plastic” – have a defined differentiation potential.
2) Progenitor cells ARE committed to a specific cell / tissue type.
3) Progenitor cells have a limited life span, i.e., humans: 50-70 population doublings, before senescence and death.
4) Progenitor cells’ Biological “clock” starts at birth.

Totipotent Stem Cell
- Form embryo, placenta, & germ Cells (sperm & ovum)

Pluripotent Stem Cell
- Form all cells of the embryo, but not will not form placenta or germ cells

Germ Layer Lineage SCs
- Form ectodermal lineage cells; mesodermal lineage cells; & endodermal lineage cells

Bipotent (ex: myoblast, fibroblast, adipoblast, etc.)
**Precursor Cells in Adults**

**Totipotent SCs**  
Blastomere-Like Stem Cell (BLSC) *

**Pluripotent SCs**  
Epiblast-Like Stem Cell (ELSC) *
- Ultra-small nucleated plasma particle (u-NP2)
- Small-nucleated plasma particle (s-NP2)
- Intermediate-nucleated plasma particle (i-NP2)
- Large-nucleated plasma particle (l-NP2)

**Germ Layer Lineage SCs**  
GL-EctoSCs, GL-MesoSCs*, GL-EndoSCs

**Propagator Cells**  
Multipotent PCs, Tripotent PCs, Bipotent PCs, & Unipotent PCs

*Isolated and repetitively cloned from single cells

**Totipotent Adult Stem Cells**

![Diagram of Totipotent Adult Stem Cells]

- Embryonic cells
- Neural stem cell systems (NSCS)
- Extraembryonic cells
- Other cells

**u-NP2**

**i-NP2**

**s-NP2**

**l-NP2**
Differentiated Phenotypic Expression Markers

(expression of extracellular & intracellular markers via monoclonal antibodies with immunocytochemistry and selective agents (i.e., enzymes, hydrocarbons, etc.) combined with histochemical staining)

Nucleated Plasma Particle Characteristics

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What we are doing...

Center for Orthopedic & Regenerative Medicine Science
What we are doing....

• With current regulatory burden in the United States preventing physicians from culture and expansion of mesenchymal stem cells.
• Physicians have had to focus on:
  – Improved tissue extraction techniques
  – Laboratory cell processing to improve cell and viability counts to improve clinical outcomes.
    • Example:
      – Adipose tissue – Liberase (collagenase)
        • Designed initially for pancreatic tissue digestion
        • Not well designed for adipose tissue digestion

Adipose tissue prep

• Adipose tissue SVF prep:
  – Issues with the presence of trypsin
  – There are methods to bind trypsin and improve preparation.
  – There are better enzymes to utilize with adipose tissue.
  – We will be submitting for publication involving these techniques in the fall of 2015.

What are we doing...

• Stopped using SVF
• Compressed fat for graphs only
• Stopped using BMAC
• MSCs blood – homogenous population
• TPSCs – Neurological, neuropathic pain states.
• PPSCs – Orthopedic
• TPSCs & PPSCs mixed preps
What we are doing

• Toti's
  - Neuro, neurodeg, PD, SCI
  - CIDP, Neuropathic pain
  - Demyelination, etc.
  - Lumbar disc

• Ortho:
  - Pluri ↔ Toti
  - Pluri + Mesos

• IV:
  - Depends on protocol and indication
    - Pulm, cardio, neuro, ortho

• Intranasal:
  - Toti only

• Nebulized:
  - Pluri and toti

The lumbar disc - DDD

• Totipotent genome tagged with insect betagalactosidase.
  - Now progeny will express beta-gal and thus you can monitor where the go and the progeny they produce.
  - Retrodiscal
  - Intradiscal
  - Scaffolding HA
  - Other...

Induction of Specific Cell Types

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Inductive agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurospheres</td>
<td>Neurobasal-A, B-27, b-FGF, EGF</td>
</tr>
<tr>
<td>Neurons</td>
<td>Laminin, Nb-27, Nb-N2, b-FGF, EGF, NT3, BDNF</td>
</tr>
<tr>
<td>Neuroectoderm</td>
<td>Butylated hydroxyanisole, DMSO, KCl, Hydrocortisone, Forskolin, N2, Insulin, Valproic acid, Nb-A, TGF-β, b-FGF</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>TGF-β, b-FGF</td>
</tr>
<tr>
<td>Cartilage/Bone</td>
<td>BMP-2</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>VEGF, α-FGF, BMP-4</td>
</tr>
<tr>
<td>Red Blood Cells</td>
<td>SCF, IL-3, IL-6, EPO</td>
</tr>
<tr>
<td>Pancreatic β-Cells</td>
<td>KGH, HGF, Insulin, Nicotinomide</td>
</tr>
<tr>
<td>All cell types</td>
<td>Dexamethasone</td>
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</table>
Study Rationale

• NucP2 stem cell isolation and deployment technology
• NucP2 may offer significant advantage over techniques currently being utilized in regenerative medicine practice.
• NucP2 may be able to overcome some of the significant limitations in using these types of cellular interventions.
• Another limitation of utilizing autologous bone marrow and adipose tissue derived mesenchymal stem cell preparations is that there are few multipotent stem cells available within the cellular preparation.

Study Rationale

• The majority of the cells that we are currently using in common orthopedic procedures today are predominantly composed of progenitor cells, which are lineage committed and having limited capacity for replication and plasticity.
• What is desired is a regenerative medicine technique that can utilize autologous cells in order to remain compliant with FDA regulation and also fall within the guidelines of minimal manipulation also a requirement of FDA regulation.

Deployment technique experts

Physicians with a foundation in basic regenerative medicine techniques such as prolotherapy have an advantage.
Deployment techniques are highly specialized skills.
Physicians highly skilled interventional pain technology may have better outcomes.
Case examples #1

• JM: 72 yo CF riding bike to work in Seattle, WA. Struck by car:
  - Significant right hip pain unresponsive to conservative care.
  - MRI – early degenerative changes and acetabular labrum tear.
  - S/P arthroscopic debride ment of labrum tear without improvement and worsening hip pain.
  - C/O of chronic low back pain at LS spine and right SI joint region.
  - Numerous medical subspecialty consultations.
  - University of Washington pain medicine Department consultations, pharmaceutical pain management,
    - Nonresponsive to epidural injections
  - Ongoing physical therapy
  - Patient ambulating with a cane/ walking stick
  - Patient is a store owner having to reduce hours and hire more staff to handle responsibilities.
  - 4 years in chronic pain without improvement
  - Anxious lawyers wanting to settle or PI case

Case examples #1

• JM: 72 yo CF riding bike to work in Seattle, WA. Struck by car:
  - X-rays of lumbar spine – age-related degenerative changes
  - MRI lumbar spine – age-related degenerative changes
  - Patient now seen for consultation after 4 years post MVA in chronic pain.
  - C/O
    - Severe low back pain across the lumbosacral spine
    - Severe SI joint pain
    - Severe hip pain
    - Pain radiation into the anterolateral thigh, buttock and posterior thigh
    - Significant sensation of muscle tension in the TFL
  - Complete relief of hip pain and TFL and anterior thigh pain with intra-articular local anesthetic injection under ultrasound guidance

Case examples #1

• JM: 72 yo CF riding bike to work in Seattle, WA. Struck by car:
  - Symptomatic relief of back pain from lumbar facet injections/medial branch block
  - Symptomatic relief from SI joint pain with SIJ block
  - Prolotherapy 3 modest improvements of back pain and recurrent SIJ pain.
  - PRP injection directed to acetabular labrum, intra-articular hip, lumbar facet, and SIJ with modest improvement in recurrent symptoms.
  - Repeated PRP injection’s resulted in same modest improvements and recurrent symptoms
Case examples #1

- JM: 72 yo CF riding bike to work in Seattle, WA. Struck by car:
  - Adipose tissue SVF directed to intra-articular hip and acetabular labrum resolved all hip pain complaints.
  - Patient no longer having to ambulate with cane
  - Despite hip improvements persistent low back pain continued to create functional and work disability issues.
- NucP: 2 stem cell:
  - Lumbar facet injections and right sacroiliac joint injection
  - IV infusion
  - 3 days later patient attended market walking the entire market for business purchases in New York without pain.
  - Patient now reporting complete resolution of chronic low back pain and SIJ pain

Case example #2

- 68-year-old CF with tricompartmental osteoarthritis.
  - Progressive worsening knee pain and disability.
  - Now reporting poor walking tolerance, reduced recreational activity tolerance.
  - Modest response to platelet releasate injection
  - Status post adipose tissue SVF injection with no significant improvement after 6 months.
  - Patient now facing consideration of TKA

Case example #2

- 68-year-old CF with tricompartmental osteoarthritis.
  - Patient consulted to explore nonoperative options.
  - NucP: 2 stem cell:
    - Marked improvement
    - Improved functional activity tolerance
    - Return to some recreational activities
  - More questions and answers:
    - Will this have to be repeated?
    - How often
    - Post MRI one year pending WORMS, BLOCKS, CaLs... T3 magnet vs. T1.5 magnet, etc.
Case example #3

- 69-year-old CF with long-standing history of CIDP with severe neuropathic pain especially right lower extremity.
  - Patient met diagnostic criteria
  - Patient had an progressive worsening of pain and neurologic function.
  - Multiple medical consultations.
  - Corticosteroids, Imuran, IVIG, gabapentin, nortriptyline etc.
  - Patient not wanting to have chemotherapy i.e. CellCept, Cytoxan, cyclosporine,

- Patient seen 4 years prior to participate in pilot study:
  - Isolation of TPSCs deployed via intranasal route overlying cribriform plate
  - Isolation of PPSCs deployed via IV infusion
  - Single administration of intranasal and IV deployment resulted in remission of neuropathic pain, improved gait and functional activity performance 14 months.
  - Patient then started to regress and requesting tx.

Case of Parkinson’s Disease

- 66 YO CM with PD
  - Movement disorder
  - Speech dysarthria
  - Social isolation
  - On Sinemet (Carbidopa-Levodopa) and Stalevo which is becoming less effective

- Pt had totipotent adult autologous stem cell intranasal infusion and IV pluripotent stem cell infusion

- Outcome: 1.5 month – patient on ½ dose of medication
  - Better movement control
  - Reduced dysarthria
  - No more social isolation and more confident
Local anesthetics

- Marcaine, Liocaine, Prilocaine, Carbocaine, Procaine – ALL kills stem cells.
- Naropin (Ropivicaine):
  - Only anesthetic that is non-toxic to stem cells.
  - 100% stem cell viability
- Suspension of cells in saline in pain sensitive patients also helps.

Use of platelet releaseates...

<table>
<thead>
<tr>
<th>System</th>
<th>Platelets (x10^3/μL)</th>
<th>WBCs (x10^3/μL)</th>
<th>PDGF-AB (ng/mL)</th>
<th>PDGF-BB (ng/mL)</th>
<th>TGF-B1 (ng/mL)</th>
<th>VEGF (ng/mL)</th>
<th>P values*</th>
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<tbody>
<tr>
<td>Cascade*</td>
<td>343.5</td>
<td>1.1</td>
<td>9.7</td>
<td>14.6</td>
<td>0.1</td>
<td>0.3</td>
<td>&lt;0.0001</td>
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<tr>
<td>MTF</td>
<td>24.7</td>
<td>0.2</td>
<td>3.6</td>
<td>2.5</td>
<td>0.08</td>
<td></td>
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<tr>
<td>GPS III*</td>
<td>556.2</td>
<td>34.4</td>
<td>18.7</td>
<td>23.1</td>
<td>0.1</td>
<td>2.4</td>
<td>0.006</td>
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<tr>
<td>Biomet</td>
<td>292.6</td>
<td>13.6</td>
<td>12.8</td>
<td>10.1</td>
<td>0.08</td>
<td>1.1</td>
<td></td>
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<tr>
<td>Magellan*</td>
<td>780.2</td>
<td>11.0</td>
<td>34.4</td>
<td>33.0</td>
<td>0.1</td>
<td>1.2</td>
<td>0.01</td>
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<tr>
<td>Artericy</td>
<td>246.5</td>
<td>8.2</td>
<td>10.7</td>
<td>8.2</td>
<td>0.1</td>
<td>0.8</td>
<td></td>
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<tr>
<td></td>
<td>0.0001</td>
<td>0.006</td>
<td>0.009</td>
<td>0.371</td>
<td>0.005</td>
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</tbody>
</table>
Study objective: to investigate effects of leukocytes on PRP

Create leukocyte rich PRP (LR-PRP)

Create Leukocyte poor PRP (LP-PRP)

Inject into healthy patellar tendon of New Zealand White rabbits

Harvest patellar tendons and analyze histology

Results - Inflammatory Mediators

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-4</th>
<th>IL-10</th>
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<tr>
<td>TBC</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>LR-PRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LP-PRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

- LR-PRP causes significant increases in pro-inflammatory mediators
- LP-PRP causes significant increase in anti-inflammatory mediators

Use of platelet releaseates…

- Activated platelet
  - Alpha granules
  - PD growth factors

This is PRP

Super mix

1st Generation PRP

2nd Generation PRP

ADSC Induction Media
- IGF-1
- TGF-β1
- GH
- FGF-2

Platelet Rich Plasma
- IGF-1
- TGF-β1
- GH
- FGF-2
- PDGF
- VEGF
- IGF-BP2
- IGF-BP3
2nd Generation PRP

Neutralize specific PRP growth factors with antibodies
- VEGF
- IGFBP-2
- IGFBP-3
- EGF
- PDGF

Confirm absence of neutralized factors and presence of key growth factors by ELISA

2nd Generation PRP

Sterile inflammation, caused by DAMPs and mediated by IL-1β or TNF in the absence of infection, involves canonical NFκB activation and proinflammatory signalling. In OA, molecules from cartilage breakdown and intracellular components o...
mPRP

Task: Myostatin and TGF-β1 removal using antibodies bound to protein A/G agarose in sterile conditions

Protein A beads

TGFβ1 antibodies

myostatin antibodies

Protein G beads

Incubate 1 hour, RT
Add Ab-s
PBS buffer

Disc disease and pathological changes in the neuroforamen

- Disc leak
  - PLA2
  - Immune mediated responses
  - Inflammation
- Ischemia
- Demeylination
- Inflammatory membrane

PRP and PR
Orthopedic Spine Applications

- SI joint instability syndromes
- Accessory ligaments of SI joint
- Lumbar facet joints
- LD fascia
- IL ligament
- Platelet releaseate epidural
- Intradiscal PRP with or without fibrin
- Never had a Toti-Potent or Pleuri-Potent stem cell population to use until now…
Adipose derived MSCs

- Promotion of angiogenesis: vascular endothelial growth factor (VEGF), insulin like growth factor 1 (IGF-1), monocyte chemoattractant protein 1 (MCP1), basic fibroblast growth factor (bFGF) and interleukin 6 (IL6).
- 2) Stem cell growth and differentiation: stem cell factor (SCF), leukemia inhibitory factor (LIF), macrophage colony stimulating factor (MCSF), stromal derived factor 1 (SDF1), angiopoietin1 and activin A.
- 3) Inhibition of fibrosis: hepatocyte growth factor (HGF), bFGF, adrenomedullin (ADM).
- 4) Inhibition of apoptosis: VEGF, HGF, IGF1, transforming growth factor (TGFp), bFGF, granulocyte macrophage colony stimulating factor (GMCSF), activin A and thrombospondin 1. Immune mediated effects include the following (5 to 8).
- 5) Suppression of T and B cells: human leukocyte antigen G5 (HLA-G5), HGF, inducible nitric oxide synthase (iNOS), indoleamine2,3 dioxygenase (IDO), prostaglandin E2 (PGE2), bFGF and TGFp.
- 6) Induction of regulatory T cells (Treg) differentiation and expansion by TGFp expression.
- 7) Inhibition of natural killer (NK) cells by secretion of IDO, PGE2 and TGFp.
- 8) Inhibition of dendritic cell (DC) maturation by secretion of PGE2.
Adipose has more MSC CFUs

• The number of CFU-F calculated at the basis of 106 initially plated cells was highest for AT (557 ± 673), followed by BM (83 ± 61);


Cell markers show similar phenotype of AT and BM MSCs

Table 2. Comparison of the expression of surface proteins of mesenchymal stem cells derived from BM, Umbilical Cord Blood, and AT as analyzed by flow cytometry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>BM (%) (n 9)</th>
<th>UCB (%) (n 10)</th>
<th>AT (%) (n 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>97.5 ± 5.1</td>
<td>99.7 ± 0.5</td>
<td>99.8 ± 0.2</td>
</tr>
<tr>
<td>CD73</td>
<td>90.0 ± 0.0</td>
<td>99.8 ± 1.3</td>
<td>99.9 ± 0.5</td>
</tr>
<tr>
<td>CD90</td>
<td>91.1 ± 2.8</td>
<td>97.5 ± 7.3</td>
<td>99.3 ± 1.0</td>
</tr>
<tr>
<td>CD14</td>
<td>1.2 ± 1.7</td>
<td>1.0 ± 1.0</td>
<td>2.4 ± 3.0</td>
</tr>
<tr>
<td>CD16</td>
<td>1.6 ± 1.4</td>
<td>1.2 ± 1.5</td>
<td>6.0 ± 4.1</td>
</tr>
<tr>
<td>CD45</td>
<td>52.3 ± 3.7</td>
<td>3.6 ± 3.6</td>
<td>3.9 ± 5.1</td>
</tr>
<tr>
<td>CD105a</td>
<td>88.1 ± 7.4</td>
<td>72.4 ± 20.0</td>
<td>90.4 ± 5.9</td>
</tr>
<tr>
<td>CD106b</td>
<td>1.3 ± 1.1</td>
<td>2.9 ± 7.5</td>
<td>2.9 ± 15.5</td>
</tr>
<tr>
<td>CD29</td>
<td>99.0 ± 1.5</td>
<td>99.8 ± 0.4</td>
<td>99.9 ± 1.3</td>
</tr>
<tr>
<td>HLA I</td>
<td>95.2 ± 6.0</td>
<td>94.3 ± 6.8</td>
<td>98.8 ± 2.8</td>
</tr>
<tr>
<td>CD105b</td>
<td>66.3 ± 20.7</td>
<td>70.0 ± 23.6</td>
<td>30.3 ± 18.6</td>
</tr>
<tr>
<td>CD144</td>
<td>4.5 ± 3.4</td>
<td>0.8 ± 2.1</td>
<td>4.4 ± 5.2</td>
</tr>
</tbody>
</table>

The table shows mean values of the percentage of positive cells ± standard deviation to the total number of cells analyzed.

Proinflammatory "licensing" of MSCs

• MSCs have been dubbed as "smart" immune modulators since their suppressive effects require a previous licensing step that occurs in the presence of an inflammatory environment and is mediated by the secretion of specific cytokines (Jones et al., 2007, Jorgensen, 2010). Thus, IFN-γ, alone or together with tumor necrosis factor (TNF)-α, IL-1α or IL-1β, are required to trigger the expression by MSCs of high levels of soluble factors involved in immunosuppression such as IDO, HGF, TGF-β, and NO (Aggarwal and Pittenger, 2005; Ryan et al., 2007; Krampera et al., 2006, Ren et al., 2008).
• The need for this activation step has been confirmed in a model of GVHD, since recipients of IFN-γ- T cells did not respond to MSC treatment, evolving into fatal GVHD (Polchart et al., 2008).
• Others have attributed the immunomodulatory function of MSCs mainly to IL-6-dependent secretion of prostaglandin E2 (PGE2) (Boufi et al., 2010).
Stromal Vascular Fraction

BMAC cellular therapy

- BMAC
  - Marrow aspiration
  - MSCs
  - Obtained by centrifuge technique similar to PRP

Bone Marrow Aspirate Concentrate Cellular Therapy

Use of Adult Nucleated Plasma Particles For Treatment

Neurological Cardiovascular Pain Respiratory Musculoskeletal Diabetes

Where are we going...?
Plans for 2015-2016

Cell Par Technologies
Knee: Meniscus & DJD

• We are now questioning meniscus debreeding...

• Biomechanical effects:
  – Increase contact surface
  – Increased translational instability
  – Rapid progress of OA

Posterior horn set up...

• Biomechanics are at the root
• Ligaments:
  – Coronary ligaments, capsule

Coronary ligaments and capsular ligaments of posterior horn

• Researchers have proposed that the small coronary ligaments previously described attached to the meniscus (ligaments of Humphrey and Wrisburg) may play an important role in osteoarthritis progression.20

Osteoarthritis pathophysiology

- Small molecules of necrotic cell material and fragments of degenerating cartilage can activate the immune response resulting in inflammation and thickening of this membrane and result in pain and swelling.\(^1\)
- It is this inflammation that is linked to both initiation and progression of osteoarthritis.\(^2\)
- Inflammatory & cytokine soup... bad for the joint!
- How can we modulate the DISEASE PROCESS?


OCDs - microfracture

- Microfracture: some short-term benefit with no significant long-term results.\(^3\)


MSCs for OA knee

- Mesenchymal stem cells:
  - potential to become many other types of cells when exposed to specific growth factors or environments.
  - Cells ability to differentiate into cartilage lineage has great potential for cell based articular cartilage repair.\(^1-3\)

MSCs developmental plasticity

• Mesenchymal stem cells:
  – Has properties of “developmental plasticity” which means they can change to other tissues when placed in the right environment. 1,2.


Improved red & white zone cartilage volume

• Studies emerging demonstrating increased extracellular matrix, the substance that cartilage cells make, that appear to be restoring a meniscus-like tissue even in the avascular zone of the cartilage. 38-41


Adipose tissue derived MSCs
SVF in OA of the knee

• Arthroscopic second look after SVF:
  – SVF vs NaCl control:
    • Almost all patients showed significant improvement in all clinical outcomes of follow-up.
    • All of the patients in this study improved at 2 years compared to the 12 month follow-up. 87.5% of elderly patients greater than 65 years of age (14 out of 16) improved or maintained cartilage status at least 2 years postoperatively.
    • More importantly none of these patients underwent total knee arthroplasty (joint replacement) during the 2 year follow-up.1


Chondromalacia / Patellofemoral disease

• Can we really make a difference:
  – Life style modifications
  – Patellofemoral knee braces
  – Patellofemoral straps
  – VMO and quad strengthening
  – Improved biomechanics
  – Orthotics
  – Physical therapy
  – Soft tissue mob

SVF in patellofemoral arthritis

• Pak et al.
  – In a recent study one month after injection of autologous adipose derived stem cells patient’s pain improved 50-70% and after 3 months patient’s improved 80-90% and continued to improve over 1 year.44 More importantly the MRI findings demonstrated recovery of the articular cartilage.1
  – MRI changes were documented...!
  – After SVF injection: Pain improved 50-70% and after 3 months patient’s improved 80-90% and continued to improve over 1 year

ADSCs have been shown to be OA protective...

- Protects cartilage cells in osteoarthritis against cell death and progression of degeneration.
- OA involves increase in cytokines, metalloproteinases, reactive oxygen species which are present in osteoarthritis joints.
- MSCs downregulate pro-inflammatory cytokines.
- BMAC MSCs have similar anti-inflammatory effects in osteoarthritis.


The hip

- Hip is treated with similar principles as knee.
- Acetabular labrum
- FAI
- Peritrochanteric pain:
  - G. medius and hip RTC

Peritrochanteric hip pain
Rotator cuff tears

- Rotator cuff tears:
  - Impingement
  - Deg. Tendinopathy
  - Tendinosis
  - Insertional tears
    - Subarticular
    - Bursal surface
    - Interstitial tears

Image guided deployment

- Requires detailed understanding of anatomy.
- Understanding of pathology
- Understanding of image guided procedures
- Cell deployment techniques are completely different than interventional pain procedures and require special training.

The search for neuronal stem cells has ended!

Neurodegenerative disease stem cell therapy.
- Finally a pathway into the brain
Let the Body Induce the Preferred Cell Type
By Injecting Naïve Nucleated Plasma Particles
into a Particular Site…

Michael N. Brown, MD

**Parkinson’s Disease Neuronal Repair**

**Domaminergic neurons**

- Dopaminergic neurons, which have their cell bodies located in the *substantia nigra pars compacta* (SNpc).

- The progressive loss of these cells results in the gradual decrease over time of striatal dopamine levels, which in turn produces a decrease in striatal output to the thalamus.

- Selective dopaminergic neurotoxin 6-hydroxydopamine into the corpus striatum, middle forebrain bundle, or substantia nigra pars compacta.

- 6-hydroxydopamine (6-OHDA) is a selective dopamine neurotoxin which is taken up by transporter proteins at the nerve terminals within the corpus striatum and transported in a retrograde manner to the cell bodies in the substantia nigra.
**Dopaminergic neurotoxin 6-hydroxydopamine**

- Bilateral 6-OHDA lesions of the adult rat brain result in a partial progressive loss of dopaminergic terminals within the corpus striatum ipsilateral to the injection site and the subsequent death of the dopaminergic neurons projecting from the substantia nigra to the corpus striatum.25

Because of the similarities between the neurochemical and neuropathological changes elicited by the local injection of 6-OHDA to those found in Parkinson's disease, this particular experimental animal model has often been used to anticipate the relevance of a given treatment in the clinical management of the symptoms of Parkinson disease in humans.


**Stem cells for PD?**

- Lindvall proposed that four different cellular sources could be used to form dopaminergic neurons for neural transplantation for Parkinson disease:
  - (a) embryonic stem cells from a fertilized egg;
  - (b) neural stem cells from an embryonic brain;
  - (c) neural stem cells from an adult brain; or
  - (d) stem cells from other tissues.
- The crucial issue is whether the transplanted cells would form functional dopaminergic neurons, regardless of the source of the stem cells.

Stem Cells for PD

• Young et al. reported the isolation and single cell cloning of adult-derived pluripotent stem cells from the connective tissue stroma of multiple organs in animals and humans.
• They demonstrated that a clonal population of adult-derived pluripotent cells was capable of objectively forming 63 of the 220+ possible cells of the body, including multiple types of neurons, oligodendrocytes, astrocytes and capillaries.


Induce loss of dopaminergic neurons with stereotactic injection of neurotoxin 6-hydroxydopamine

stereotactically infused unilaterally into 6-OHDA hemi-lesioned out-bred Sprague-Dawley adult rat brains on the ipsilateral side.

The contralateral side received 0.02% ascorbate-saline buffer only, as the operational control.

• The sham control hemi-brain (receiving an infusion of saline-ascorbate buffer) at two weeks post infusion = tyrosine hydroxylase-positive cells were present throughout the striatum
• The 6-OHDA control hemi-brain (receiving an infusion 6-OHDA) two weeks post infusion = loss of tyrosine-hydroxylase positive cells in a central area within the striatum.

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Bright field microscopy of adult rat brain lesioned stereotactically with 6-
hydroxydopamine and then injected two weeks later with saline-ascorbate control buffer solution. Note needle track (denoted by lines and arrows) devoid of immunoreactivity for tyrosine hydroxylase activity.

Adult rat brain lesioned stereotactically with 6-hydroxydopamine and then injected two weeks later with Lac-Z transfected clone of adult-derived pluripotent stem cells. Note needle tract (outlined with black bars) containing cells that express immunoreactivity for tyrosine hydroxylase, as well as the presence of cells immunoreactive for tyrosine hydroxylase in adjacent tissue.

- Injected Vehicle only
- Tyrosine Hydroxylase (negative)

- Green Cells belong to Glial Scar
- Needle Track

- Injected Vehicle & Naive Pluripotent Stem Cells
- Tyrosine Hydroxylase (positive) Cells

- Tyrosine Hydroxylase-positive (brown) Cells
- Needle Track
Summary Slide
- Injected primitive PSCs formed dopaminergic neurons within substantia nigra

Unexpected results
- Primitive PSCs also migrated along needle track and regenerated normal tissues and cells in cerebral cortex damaged during stereotactic injections:
  Glial cells, Pyramidal neurons, & Capillaries (containing RBCs)

Stem cells for PD
- Adult rat hemi-brains from Parkinson study were examined for the presence of cells immunoreactive for beta-galactosidase. The Lac-Z transfected Scl-40beta pluripotent stem cell clone derived from adult rats was identified in stem cell phase and differentiation phase using an antibody to beta-galactosidase. The tissue harvested was from needle tracks created by previous stereotactic injections. The tissue was harvested and stained with antibody to beta-galactosidase (brown color, donor cells) and counterstained with methyl green (green color, host cells). Immunoreactivity to beta-galactosidase was expressed within the cytoplasm of differentiated cells, i.e., glia, pyramidal neurons, interneurons, and endothelial cells lining newly formed RBC-filled capillaries.

[Image of stem cell slide]
Post stem cell for PD

• Results from the animal study demonstrated:
  – replacement of dopaminergic neurons in the area of the 6-OHDA lesion
  – replacement of damaged neuronal cells, damaged neuronal supportive cells and damaged vascular structures caused by the needle injections.

The pathway to the brain

Parkinsons disease pilot

• Induction – extraction, isolation of TPSCs and PPSC, intra-nasal infusion of autologous totipotent stem cells and intravenous infusion of autologous pluripotent stem cells.
• Serial washing of cells with to remove plasma proteins.
• Tx: intra-nasal infusion of TPSCs + IV Inf.
• Intranasal infusion in modified Trendelenberg position.
• 5 min to insure the deposition of the stem cells on the olfactory mucosa with migration between the mucosal cells, along the olfactory processes, through the cribriform plate, to the olfactory bulb, and posteriorly along the olfactory nerves to gain entrance underneath the blood-brain barrier and to the sub-arachnoid cisterns of the brain.

Outcome measures

• Motor via UPDRS-III;38
• Cognition via Trail Making Part A and B;39
• Affect via Beck Depression Scale-II (BDI-II);40
• Schwab and England disability scale42 and Hoehn-Yahr Scale;43
• Sleep: Epworth Sleepiness Scale (ESS);
• Overall clinical improvement with the CIBIC-Plus (Clinician’s Interview-Based Impression of Change)
• Caregiver input; and Caregiver burden: Zarit Burden Scale
• Eval & FU: baseline,  2 weeks 3 months, 4 months, and three months post baseline (prior to the intranasal

Neurological conditions treated with nucleated plasma particles (NP2 / ASCs) and/or mobilizing agent

1) Parkinson disease (PD)
   1) mild cases, return of function (MA)
   2) More severe cases – see below (MA + NP2)
2) Chronic idiopathic demyelinating polyneuropathy (CIDP) – return of function and loss of symptoms (MA + NP2)
3) Multiple sclerosis (MS) – return of function (MA + NP2)
4) Amyotrophic lateral sclerosis (ALS) – stabilization of disease process (MA + NP2)
5) Neurogenic stroke – return of function (MA + NP2)
6) Alzheimer disease (AD) – return of function (MA + NP2)
7) Neuropathies – loss of symptoms (MA + NP2)
8) Epilepsy – those individuals with clinically defined epilepsy showed a decrease in epileptic seizures with a concurrent decrease in epileptic medications. (MA)

PD Stem Cell Pilot Study

• 10 participants, with 8 completing the study.
• We chose subjects who had a Modified Hoehn and Yahr Staging 1.5 to 4 (Severe Disability)
• allowing for a “middle” range of the disease process. Participants were tested at baseline (pre-treatment), at treatment, and at 1 month, 7 months and 14 months post-treatment.
• At one month post-treatment, all participants demonstrated an increase in their respective Hoehn-Yahr scores. Four showed an increase to 6.0-8.0, while four showed an increase to 9.0 on a 10.0 scale.
Neuropathic pain states

- Intranasal infusion
  - CIDP
  - Neuropathic pain states
  - Neurofascial hydrodistension

Myocardial Infarction Repair

Let the Body Induce the Preferred Cell Type
By Injecting Pluripotent Stem Cells:
  - Directly into site of lesion
  - Systemically via Tail Vein
Direct Injection of naïve PSCs (green)

Vasculature
Myocardium
Connective Tissues

Systemic Tail Vein Injection of naïve PSCs (green)

Connective Tissues
Mycocardium

Day 3
Day 14
Day 28
PSCs in Coronary Artery (red)

Creation of Pancreatic Islet Organoids for Type-I & Type-II Diabetes

Steps to creating a new pancreas
Build a Pancreatic Islet Organoid

1) Decellularized Matrix
2) TSCs & PSCs
3) Donor Islets
Pre-Glucose Incubation

Post-Glucose Incubation

Islets increase integrity

Islets slightly increase in integrity

Islets lose integrity
Parameters for Glucose Challenge

Native Pancreatic Islets (alone)

Pancreatic Organoids with Matrix-A

Pancreatic Organoids with Matrix-B

Measured insulin secretion sequentially in response to a glucose challenge in vitro

5 mM glucose for 24 hr
5 mM glucose for 1 hr
25 mM glucose for 1 hr

(*Lumelsky et al., Science 292:1389-1393, 2001)

Measured secretion with Rat-specific Insulin-RIA

Insulin Secretion in Response to a Glucose Challenge (n=6)
Respiratory Diseases
1) Individuals with COPD and having a FEV1 of greater than 50% showed benefit with ingestion of mobilizing agent only, in some instances increasing their FEV1 to greater than 85%.

2) However, for those individuals with an FEV1 of less than 50%, no increase in FEV1 was noted after long term ingestion of mobilizing agent only.

3) Individuals with FEV1s less than 50% noted improvement, i.e., increase in FEV1, after treatment with MA + autologous NP2.

Musculoskeletal
1) Acceleration (2x) of the normal healing process occurred in an individual with a torsion fracture of the fibula with ingestion of mobilizing agent only and simple casting of the lower extremity.

2) Individuals with rotator cuff injuries necessitating surgery demonstrated healing of their rotator cuff injuries after long term ingestion of mobilizing agent.

3) Other individuals reported less painful osteoarthritic joints after long term ingestion of mobilizing agent.

Other Conditions
1) Type-I diabetic with brown recluse spider bite, completely healed in 2 weeks with MA + single autologous NP2 transplant

2) Individual with SLE, Celiac disease and multiple allergies, MA + multiple autologous & allogeneic NP2 transplants, reverted from SLE-Type-IV to SLE-Type-II, and lost allergies to gluten, eggs, fowl, tree nuts.
Regenerative Medicine Characteristics

<table>
<thead>
<tr>
<th>ESCs or iPSCs</th>
<th>Adult NP2s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>Unlimited</td>
</tr>
<tr>
<td>Form cell types</td>
<td>Unlimited</td>
</tr>
<tr>
<td>Naïve in vitro</td>
<td>Spontan. Differen.</td>
</tr>
<tr>
<td>Naïve in vivo</td>
<td>Teratomas (Cancerous cells)</td>
</tr>
<tr>
<td>Implant in vivo</td>
<td>Differentiated Cells</td>
</tr>
<tr>
<td>HLA mismatch</td>
<td>Allogeneic</td>
</tr>
<tr>
<td>HLA match</td>
<td>Therapeutic cloning</td>
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</tbody>
</table>

In summary…

• A new era of orthopedic & regenerative medicine:
  – A new technology: From autologous cell mix techniques to true TP / PP stem cell therapies.
  – A new subspecialty is emerging created by clinical hybrids
    • Offers patients with spine and orthopedic difficulties new hope
    • A new change for the baby boomer population who want to stay active
    • Vascular disease
    • Neurological disease
    • Cardiac disease
    • Respiratory disease

Center for Orthopedic & Regenerative Medicine Science

THANK YOU

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