Aalto University
School of Chemical Technology

CHEM-E3225, Cell- and Tissue Engineering, 5 cr

Topic 4

Culturing (stem) cells for tissue engineering; see Birla CHP 2 section2.12. – this lecture also contains additional information not in Birla)

1) (Stem) cell culture conditions

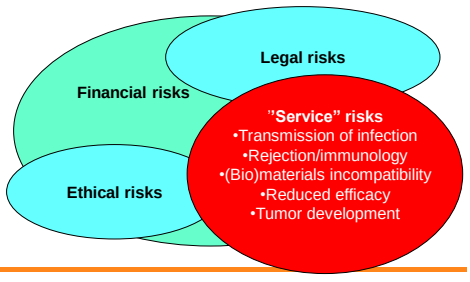
2) Infection control and good manufacturing practise, SOPs = Standard Operating Procedures

3) Characterization of stem cells (Are they safe to use ?)

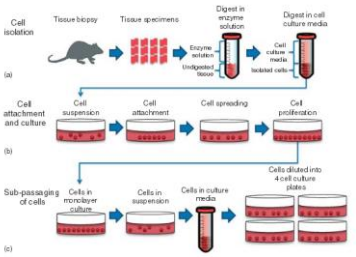
1. (Stem) Cell culture conditions

- the culture environment; culture media
- feeder cells in cell culture

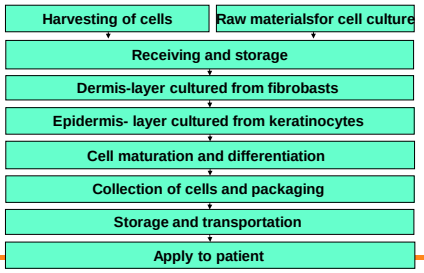
Risks involved with TE-products



Cell culture procedure



Production process (Apligraf)



What is cell culture?

- Cell culture is a process by which cells are grown under controlled conditions usually outside of their natural environment.
  - eukaryotes
  - plant tissue culture
  - fungal culture
  - bacterial culture
  - viral culture

Considerations for cell sourcing

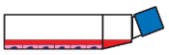
- Autologous vs. allogeneic
- Animal-derived vs. human-derived cells
- Cell lines
- Stem cells
- Stem cell engineering

Cell culture basics

- Confluence: cells proliferated under the appropriate conditions until they occupy all of the available substrate.
- Passage: cells have to be subcultured by transferring them to a new vessel with fresh growth medium to provide more room for continued growth.

1. Static culture (conventional system)

- The 1st *in vitro* cell culture was established in 1907.



Simple setup:

1. The upper side of cells gets nutrients but not the bottom side.
2. Mass transfer occurs by molecular diffusion.
3. Cell-substrate interaction is non-specific, mediated by the adsorbed proteins.

More advanced static culture systems:

1. Transwell insert allows the cells to have access to the nutrients from below.
2. The substrate is coated with ECM or biomaterials before seeding cells to induce specific interaction.

2. 3D culture

- The significant difference between 2D and *in vivo* was first realized by cancer biologists, followed by tissue engineers and stem cell biologists.
- 3D culture system is ”something between a Petri dish and a mouse”, meaning it is more predictive of *in vivo* system than *2D*
- In 3D culture, cells are cultured in a biomaterial scaffold. For example: cells cultured within Matrigel, within a polymeric hydrogel, or within a porous scaffold

What do cells need?

- Culture conditions vary widely for each cell type, but the artificial environment in which the cells are cultured invariably consists of a suitable vessel containing the following:
  - a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals)
  - growth factors } Usually from serum
  - hormones }
  - gases (O_2, CO_2)
  - a regulated physico-chemical environment (pH, osmotic pressure, temperature)
  - plating density
  - typically 37 °C, 5% CO_2 for mammalian cells
- Variation of conditions for a particular cell type can result in different phenotypes.

Cell culture media

- Basal culture media:
  - amino acids, vitamins, inorganic salts and carbon source (such as glucose) for cells. Often supplemented with serum.
- Serum (contains) growth and adhesion factors, hormones, lipids and minerals for cells
  - Also regulates cell membrane permeability
  - Disadvantages: high cost, difficult to standardize, specificity, variability, unwanted cellular effects, ANIMAL PRODUCT
  - There are, however, golden standard” – sera, which are safe but very expensive

  Alternatives to serum
  - Reduced-serum media
  - Inactivation of serum
  - Serum-free media

Adherent cells

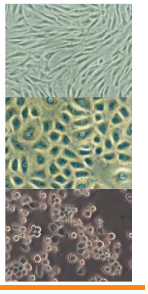
- Most cells are adherent and must be cultured while attached to a solid or semi-solid substrate (monolayer culture).
  - Adherent cells require a surface, such as tissue culture plastic or microcarrier.
  - The surface can be coated with extracellular matrix (such as collagen and laminin) components to increase adhesion properties and provide other signals needed for growth and differentiation.
- Organotypic culture is a type of adherent culture. This technique involves growing cells in a three-dimensional (3-D) environment as opposed to 2D culture dishes. 3D culture system is biochemically and physiologically more similar to *in vivo* tissue, but is technically challenging to maintain because of many factors.

Cell types

- Primary cells: Cells are isolated from the tissue and cultured under specific considitions to maintain their morphology and functionality.
- Cell line: Primary culture is known as cell line after the first subculture. Cell lines derived from primary cultures have a limited life span and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.
- Finite cell line: Normal cells usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as senescence.
- Continuous cell line: A finite cell line that undergoes transformation and acquires the ability to divide indefinitely. Transformation, which can occur spontaneously or can be chemically or virally induced.

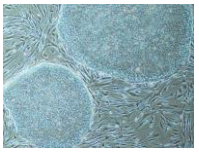
Cell morphology

- Fibroblastic (or fibroblast-like) cells are bipolar or multipolar, have elongated shapes, and grow attached to a substrate.
- Epithelial-like cells are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches.
- Lymphoblast-like cells are spherical in shape and usually grown in suspension without attaching to a surface.



Mimic cells in stem cell niche by using **Feeder cells**

- Pluripotent stem cells usually grow best when attached to other cells or to an extracellular matrix (ECM) and grow traditionally in cultures with feeder layers
- Feeder cells maintain embryonic stem (ES)/induced pluripotent stem (iPSc) cells in an undifferentiated state, without losing their pluripotency
  - Typically mouse embryonic fibroblasts can be used as feeder cells
  - The specific mechanisms remain unclear, but it is thought to be a result of secretion of soluble factors and direct interactions (feeder cells – stem cells)



Two colonies (islands) of human embryonic stem cells surrounded by a sea of supporting mouse feeder cells. ©Wong Xianrong

Feeder cells in cell culture

- Often necessary, but overall not desirable
  - Labour-intensive procedure
  - Necessity for xeno-free culture for the cultivation of hES cells
  - High risk of contamination during passages
- Many researchers are developing new culture systems to avoid using feeder cells:
  - external factors have been identified as important for the maintenance of the self-renewal ability
  - chemically defined media
  - culture surfaces, including intelligent materials, immobilization of proteins, and synthetic polymer coating
  - However, these methods are still troublesome or have limitations (cell type specification, high cost)

Cells in suspension

- Cells can also be cultured in suspension culture where cells are grown floating in the culture medium.
  - Some cells naturally live in suspension, without being attached to a surface, such as cells that exist in the bloodstream.
  - There are also cell lines that have been modified to be able to survive in suspension cultures so they can be grown to a higher density than adherent conditions would allow.

Supplements to cell culture media

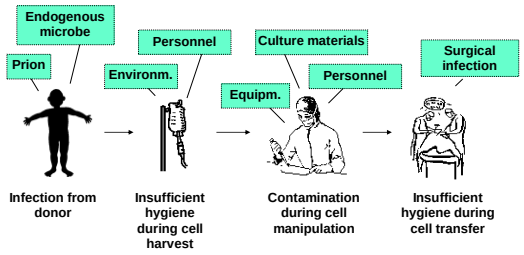
- Amino acids (i.e. MEM non-essential amino acids, GlutaMAX)
- Growth factors (i.e. human basic fibroblast growth factor)
- Hormones (depending on the cell line, i.e. insulin for hepatocytes)
- β-mercaptoethanol to reduce the production of reactive oxygen species (ROS)

2) Infection control and good manufacturing practise, SOPs = Standard Operating Procedures

What is microbial / biological* contamination ?

- Microbes are everywhere
- Most common contamination in cell laboratories comes from humans (hands, noses, hair, clothing etc.)
- Also from the environment and surfaces
- Most difficult organism for cell and tissue work is *Mycoplasma*
- Mycoplasma is hard to detect untill it already is causing damage to the cells; it has no cell wall, so antibiotics don't really work, it persists and is hard to get rid of in the lab
- *Biological contamination means that the contaminant can also be a) another cell line (cross contamination), b) biological substances of eg. c) pollen and other airborne proteins etc.

Microbial risk of contmination is not only in the lab, but across the transplantation process

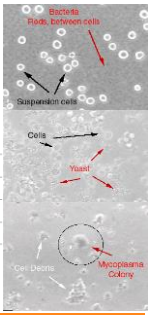


Microbial contamination

- Easy to detect:
  - Bacteria, molds, yeast
- Difficult to detect:
  - viruses, insects, mycoplasma, other cell lines

Number of Cultures Tested	Number Positive
Food and Drug Administration (FDA) (1970's to 1990's) (11) 20,000 cultures tested	over 3000 (15%)
Bionique Testing Laboratories (several years prior to 1993) (41) 11,000 cultures tested	1218 (11.1%)
Microbiological Associates (1985 to 1993) (13) 2,863 cultures tested	370 (12.9%)
ATCC (1989 to 1994) (42) 5,362	752 (14%)

Table: Corning Technical Bulletin 2008



Pictures: University of North Carolina at Chapel Hill School of Medicine 2015

Cell culture contamination

- Consequences of contamination:
  - Loss of time, money and effort
  - Adverse effects on the cultures -> cleaning of the whole cell lab
  - Inaccurate experimental results
  - Loss of valuable products
- Major contaminants:
  - Chemical contamination (from media, sera, water, incubators, storage vessels etc.)
  - Biological contamination (nonsterile work practice, supplies or media, airborne particles, accident etc.)

- **The BIG question is** – How do you prevent microbial contamination - without killing your cells - or destroying the nutrients in your cell culture media ?
- There are many methods by which microbial contamination can be prevented / microbes can be killed
  - Using heat (steam and dry heat)
  - Using chemicals (e.g. antibiotics)
  - Using UV light and irradiation
  - Filtering etc.

But these methods (all except filtering of media and solutions) and possibly irradiation, would kill the cells !!

Microbial hazard control

I. Currently no methods available for 100% removal of possible contaminants

- conventional sterilization can not be used (compromises cell viablity → product efficacy)

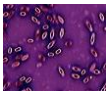
II. Possible infections can only be controlled by:

- adequate staff training
- monitoring of process environments
- donor, starting and raw material testing

III. Successfull infection control requires:

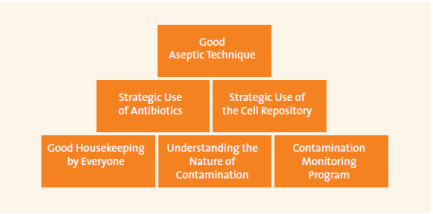
- application of existing GMP and other guidelines related to biological production
- utilization of clean rooms when possible/necessary

Infection risks



- Criteria for cell donation must be strict
  - testing and interviewing
  - bacterial and fungal contamination can be detected
  - viruses and prions – detection is a challenge
- Clean rooms and aseptic technique necessary
- Production facility contamination can have long-term consequences
- GMP generally OK with producers, but may be difficult to comply

Reducing contamination problems



Corning Technical Bulletin 2008

Good Manufacturing Practice

- **Good manufacturing practices** (**GMP**)
- For the safety of the consumer/patient/end user; for prevention of risks of microbial, biological and chemical contamination
  - practices required in order to conform to the guidelines recommended by agencies that control authorization and licensing for manufacture and sale of food, drug products, and active pharmaceutical products.
  - guidelines provide minimum requirements that a pharmaceutical or a food product manufacturer must meet to assure that the products are of high quality and do not pose any risk to the consumer or public.
- There are also good agricultural practices, good laboratory practices, good clinical practices etc
- There are similarly also guidelines and standards for many professions, which are embedded into standards (such as ISO) and other generally accepted harmonized agreements

GMP guidelines follow a few common principles

- Manufacturing facilities
  - Must maintain a clean and hygienic manufacturing area.
  - Must have controlled environmental conditions in order to prevent cross contamination of food or drug product from adulterants that may render the product unsafe for human consumption.
- Manufacturing processes are clearly defined and controlled. All critical processes are validated to ensure consistency and compliance with specifications.
- Manufacturing processes - any changes to the process are evaluated. Changes that have an impact on the quality of the drug (or product) are validated as necessary.
- Instructions and procedures are written in clear and unambiguous language = good documentation practices.
  - Operators are trained to carry out and document procedures.

GMP – continued

- Cross contamination with possible allergens is prevented.
- Keep records, manually or by instruments, during manufacture
  - Deviations are investigated and documented.
- Records of manufacture (including distribution) for traceability for every batch; recall procedures in place
  - Complaints about marketed products examined, defects investigated, measures taken to recall defective/hazardous products

3. Stem cell characterization – safety of the cells for use in tissue engineering

tests for stem cell potency, characterization chart

stem cell markers

Today we will look at:

- Cell Culture Conditions
- Stem cell characterization

WHY do we need to understand these ?

- Because the ultimate goal is to use our understanding of the biology of the cells and the ECM to produce some type of implant or cell product; so we need to understand how to control cell culture
- Because stem cells that are used must be safe and healthy
- Because producing engineered tissues require, that 1) we know how to cultivate cells 2) we know how they grow on biomaterials and 3) we know how they grow to form tissues eg in bioreactors
- (we will look at biomaterials and bioreactors later)

Stem cell line characterization

1. Pluripotency tests
2. Differentiation tests both *in vitro* and *in vivo*
3. Karyotype analysis
   Assure that new lines generated have maintained genetic stability, prolonged culture of pluripotent cell lines can result in genetic abnormalities, commonly causing aneuploidy
4. Cell identity determination
   – DNA fingerprinting and HLA analysis
5. Gene expression profiling via a stem cell array, detects expression of a common set of genes expressed in undifferentiated cells downregulated upon differentiation
6. Microbiological tests to ensure that the cultures are free of any contaminants

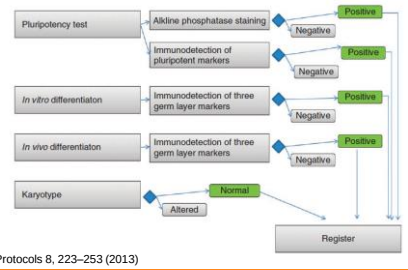
In vitro studies in culture systems in tissue engineering

- Include culture systems, scaffold systems and bioreactors.
- Studies are performed to characterize tissue engineered materials to understand its performance *in vivo* and to make sure that the materials are safe to use.
- The levels of cell culture in tissue engineering
  – 1. Static level (traditional cell culture)
  – 2. 3D culture (scaffolds)
  – 3. Dynamic cell culture
  – 4. Bioreactors – will be covered in more detail also in topic 5

Characterization of stem cells

- ES and iPS cells can be functionally characterized by the ability to differentiate into cells of the three germ layers.
- In addition, a number of molecular markers have been identified to verify the pluripotent status of stem cells.
- For example, human pluripotent stem cells express the cell surface proteins SSEA-4 and Alkaline Phosphatase and the transcription factors Oct-3/4 and Nanog.

Characterization flow chart



Nature Protocols 8, 223–253 (2013)

Stem cell markers

- The ability to verify stem cell pluripotency using established markers at the start of an experiment ensures that further stem cell proliferation and differentiation studies are conducted on high quality.
- Markers vary between cell types
  - For example: According to ISCT guideline, MSCs should be positive for CD105, CD73 and CD90, and negative CD45, CD34, CD14 (or CD11b), CD79α (or CD19) and HLA-DR surface molecules
- Methods used to test these markers: Immunostaining, flow cytometry and commercial kits

Summary

1. The Cell culture conditions, the characterization of cells and the safety of cells are all also connected to their use in Tissue Engineering in combinaiotn with biomaterials and scaffolds
2. We will cover biomaterials and scafflods in TOPIC 5 and also cover issues related cell-biomaterial interactions.
3. The culture conditions are also important to remember when looking at TOPIC 6 on Bioreactors

Summary

- *In vitro* studies in tissue engineering refer to culture systems, scaffold systems and bioreactors.
- Studies are performed to characterize tissue engineered materials to understand its performance *in vivo* and to make sure that the materials are safe to use.
- The levels of cell culture in tissue engineering
  - 1. Static level (traditional cell culture)
  - 2. 3D culture (scaffolds)
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  - 4. Bioreactors