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ANIMAL BIOTECHNOLOGY

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Animal biotechnology is a broad field encompassing the polarities of fundamental and applied research, including molecular modeling, gene manipulation, development of diagnostics and vaccines and manipulation of tissue. It accounts for the use of biotechnology tools, including molecular markers, stem cells, and tissue engineering. Molecular markers are increasingly being used to identify and select the particular genes that lead to desirable traits and it is now possible to select superior germ plasma and disseminate it using artificial insemination, embryo transfer and other assisted reproductive technologies. These technologies have been used in the genetic improvement of livestock. Transgenesis offers considerable opportunity for advances in medicine and agriculture. In livestock, the ability to insert new genes for such economically important characteristics as fecundity, resistance to or tolerance of other environmental stresses would represent a major breakthrough in the breeding of commercially superior stock. Another opportunity that transgenic technology could provide is in the production of medically important proteins such as insulin and clotting factors in the milk of domestic livestock. A comprehensive evaluation of strategies for developing, testing, breeding and disseminating transgenic livestock in the context of quantitative improvement of economic traits is being done. Genetic improvement of livestock depends on access to genetic variation and effective methods for exploiting this variation. Genetic diversity constitutes a buffer against changes in the environment and is a key in selection and breeding for adaptability and production in a range of environments. Animal cell culture technology in today's scenario has become indispensable in the field of life sciences, which provides a basis to study regulation, proliferation, differentiation, and to perform genetic manipulation. It requires specific technical skills to carry out successfully. Application of tissue culture includes the study and understanding of intracellular activity, intracellular flux, pharmacology, cell-cell interaction, cell products, toxicology, tissue engineering, genomics, and immunology. Knowledge acquired from these studies can be used in

the biomedical applications.

Culture Media: The culture medium is the most important component of the culture environment, because it provides the necessary nutrients, growth factors, and hormones for cell growth, as well as regulating the pH and the osmotic pressure of the culture. Although initial cell culture experiments were performed using natural media obtained from tissue extracts and body fluids, the need for standardization, media quality, and increased demand led to the development of defined media. The three basic classes of media are basal media, reduced-serum media, and serum-free media, which differ in their requirement for supplementation with serum.

Media Components Balanced Salt Solutions: A balanced salt solution (BSS) is composed of inorganic salts and may include sodium carbonate and, in some cases, glucose. Commercial complete media will list which BSS formulation was used.

Serum: Serum is vitally important as a source of growth and adhesion factors, hormones, lipids and minerals for the culture of cells in basal media. In addition, serum also regulates cell membrane permeability and serves as a carrier for lipids, enzymes, micronutrients, and trace elements into the cell. However, using serum in media has a number of disadvantages including high cost, problems with standardization, specificity, variability, and unwanted effects such as stimulation or inhibition of growth and/or cellular function on certain cell cultures. If the serum is not obtained from reputable source, contamination can also pose a serious threat to successful cell culture experiments. Always check new batches of serum before use. The quality and the composition can vary greatly from batch to batch. Serum is inactivated by incubating it for 30 min at +56^{o}C. Originally, heating was used to inactivate complements for immunoassays, but it may also have other, undocumented effects.

Other Supplements: In addition to serum, tissue extracts and digests have traditionally been used to supplement tissue culture media. The most common ones are amino acid hydrolysates (from beef heart) and embryo extract (chick embryo).

Basal Media: The majority of cell lines grow well in basal media, which contain amino acids, vitamins, inorganic salts, and a carbon source such as glucose, but these basal media formulations must be further supplemented with serum.

Reduced-Serum Media: Another strategy to reduce the undesired effects of serum in cell culture experiments is to use reduced-serum media. Reduced-serum media are basal media

formulations enriched with nutrients and animal-derived factors, which reduce the amount of serum that is needed.

Serum-Free Media: Serum-free media (SFM) circumvents issues with using animal sera by replacing the serum with appropriate nutritional and hormonal formulations. Serum-free media formulations exist for many primary cultures and cell lines, including recombinant protein producing lines of Chinese Hamster Ovary (CHO), various hybridoma cell lines, the insect lines Sf9 and Sf21 (*Spodoptera frugiperda*), and for cell lines that act as hosts for viral production (e.g., 293, VERO, MDCK, MDBK), and others. One of the major advantages of using serum-free media is the ability to make the medium selective for specific cell types by choosing the appropriate combination of growth factors. Using serum in a medium has a number of disadvantages: the physiological variability, the shelf life and consistency, the quality control, the specificity, the availability, the downstream processing, the possibility of contamination, the growth inhibitors, the standardization and the costs. Using serum-free media and defined media supplements (Nutridoma-CS, Nutridoma-SP, Transferrin) offers three main advantages: The ability to make a medium selective for a particular cell type. The possibility of switching from growth-enhancing medium for propagation to a differentiation-inducing medium. The possibility of bioassays (e.g., protein production) free from interference with serum proteins (easier downstream processing).

Media Recommendations: Many continuous mammalian cell lines can be maintained on a relatively simple medium such as MEM supplemented with serum, and a culture grown in MEM can probably be just as easily grown in DMEM or Medium 199. However, when a specialized function is expressed, a more complex medium may be required. Information for selecting the appropriate medium for a given cell type is usually available in published literature, and may also be obtained from the source of the cells or cell banks. If there is no information available on the appropriate medium for your cell type, choose the growth medium and serum empirically or test several different media for best results. In general, a good place to start is MEM for adherent cells and RPMI-1640 for suspension cells. The conditions listed below (Table 1) can be used as a guide line when setting up a new mammalian cell culture. Insect cells are cultured in growth media that are usually more acidic that those used for mammalian cells such as TNM-FH and Grace's medium.

Table 1: Mammalian Cell Culture and medium

Cell Line	Cell Type	Species	Tissue	Medium*
293	Fibroblast	Human	Embryonic kidney	MEM and 10% FBS
3T6	Fibroblast	Mouse	Embryo	DMEM, 10% FBS
A549	Epithelial	Human	Lung carcinoma	F-12K, 10% FBS
A9	Fibroblast	Mouse	Connective tissue	DMEM, 10% FBS
AtT-20	Epithelial	Mouse	Pituitary tumor	F-10, 15% horse serum, and 2 .5% FBS
BALB/3T3	Fibroblast	Mouse	Embryo	DMEM, 10% FBS
BHK-21	Fibroblast	Hamster	Kidney	GMEM, 10% FBS, or MEM, 10% FBS and NEAA
BHL-100	Epithelial	Human	Breast	McCoy'5A, 10% FBS
BT	Fibroblast	Bovine	Turbinate cells	MEM, 10% FBS, and NEAA
Caco-2	Epithelial	Human	Colon adeno carcinoma	MEM, 20% FBS, and NEAA
Chang	Epithelial	Human	Liver	BME, 10% calf serum
CHO-K1	Epithelial	Hamster	Ovary	F-12, 10% FBS
Clone 9	Epithelial	Rat	Liver	F-12K, 10% FBS
Clone M-3	Epithelial	Mouse	Melanoma	F-10, 15% horse serum, and 2 .5% FBS
COS-1, COS-	Fibroblast	Monkey	Kidney	DMEM, 10% FBS
CRFK	Epithelial	Cat	Kidney	MEM, 10% FBS, and NEAA
CV-1	Fibroblast	Monkey	Kidney	MEM, 10% FBS
D-17	Epithelial	Dog	Osteosarcoma	MEM, 10% FBS, and NEAA
Daudi	Lymphoblast	Human	Blood from a lymphoma	RPMI-1640, 10% FBS
GH1, GH3	Epithelial	Rat	Pituitary tumor	F-10, 15% horse serum, and 2 .5% FBS
H9	Lymphoblast	Human	T-cell lymphoma	RPMI-1640, 20% FBS
HaK	Epithelial	Hamster	Kidney	BME, 10% calf serum
HCT-15	Epithelial	Human	Colorectal adenocarcinoma	RPMI-1640, 10% FBS
HeLa	Epithelial	Human	Cervix carcinoma	MEM, 10% FBS, and NEAA (in suspension, S-MEM)
HEp-2	Epithelial	Human	Larynx carcinoma	MEM, 10% FBS
HL-60	Lymphoblast	Human	Promyeolocytic leukemia	RPMI-1640, 20% FBS
HT-1080	Epithelial	Human	Fibrosarcoma	MEM, 10% HI FBS, and NEAA
HT-29	Epithelial	Human	Colon adenocarcinoma	McCoy's 5A, 10% FBS
HUVEC	Endothelial	Human	Umbilical cord	F-12K, 10% FBS, and 100 µg/mL heparin
I-10	Epithelial	Mouse	Testicular tumor	F-10, 15% horse serum, and 2 .5% FBS
IM-9	Lymphoblast	Human	Marrow (myeloma patient)	RPMI-1640, 10% FBS

JEG-2	Epithelial	Human	Choriocarcinoma	MEM, 10% FBS
Jensen	Fibroblast	Rat	Sarcoma	McCoy's 5A, 5% FBS
Jurkat	Lymphoblast	Human	Lymphoma	RPMI-1640, 10% FBS
K-562	Lymphoblast	Human	Myelogenous leukemia	RPMI-1640, 10% FBS
KB	Epithelial	Human	Oral carcinoma	MEM, 10% FBS, and NEAA
KG-1	Myeloblast	Human	Marrow (erythroleukemia)	IMDM, 20% FBS
L2	Epithelial	Rat	Lung	F-12K, 10%FBS
LLC-WRC	Epithelial	Rat	Carcinoma	Medium 199, 5% horse serum
McCoy	Fibroblast	Mouse	Unknown	MEM, 10% FBS
MCF7	Epithelial	Human	Breast adenocarcinoma	MEM, 10% FBS, NEAA, and 10 μg/mL
WI-38	Epithelial	Human	Embryonic lung	BME, 10% FBS
WISH	Epithelial	Human	Amnion	BME, 10% FBS
XC	Epithelial	Rat	Sarcoma	MEM, 10% FBS, and NEAA
Y-1	Epithelial	Mouse	Tumor from adrenal	F-10, 15% horse serum, and 2 .5% FBS

BME: Basal Medium Eagle; **DMEM**: Dulbecco's Modified Eagle Medium; **FBS**: Fetal Bovine Serum; **GMEM**: Glasgow Minimum Essential Medium; **IMDM**: Iscove's Modified Dulbecco's Medium; **MEM**: Minimum Essential Medium; **NEAA**: Non-Essential Amino Acids Solution; **TNM-FH:** *Trichoplusia ni* Medium-Formulation Hink (i.e. Grace's Insect Medium, Supplemented)

Cell Culture

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. It is also used in drug screening and development, and large scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins). The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. When the cells are removed from the organ fragments prior to, or during cultivation, thus disrupting their normal relationships with neighboring cells, it is called cell culture.

Tissue culture is the general term for the removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they

may be derived from a cell line or cell strain that has already been already established. The culture of whole organs or intact organ fragments with the intent of studying their continued function or development is called organ culture.

Primary Culture: Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). There are two basic methods for doing this.

i. **Explant Cultures**, small pieces of tissue are attached to a glass or treated plastic culture vessel and bathed in culture medium. After a few days, individual cells will move from the tissue explant out on the culture vessel surface or substrate where they will begin to divide and grow.
ii. **Enzymatic Dissociation** more widely used method speeds up this process by adding digesting enzymes, such as trypsin or collagenase, to the tissue fragments to dissolve the cement holding the cells together. This creates a suspension of single cells that are then placed into culture vessels containing culture medium and allowed to grow and divide.

Subculturing: When the cells in the primary culture vessel have grown and filled up all of the available culture substrate, they must be subcultured (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth.

Buying and Borrowing

An alternative to establishing cultures by primary culture is to buy established cell cultures from organization such as the American Type Culture Collection (ATCC) or the Coriell Institute for Medical Research.

Cell Line: After the first subculture, the primary culture becomes known as a cell line or subclone. Cell lines derived from primary cultures have a limited life span (i.e., they are finite), and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.

Cell Strain: If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a cell strain. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line.

Finite vs. Continuous Cell Lines: 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; these cell lines are known as **Finite**. However, some cell lines become immortal through a process called transformation, which can occur spontaneously or can be chemically or virally induced. When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a **Continuous** cell line.

There are two basic systems for growing cells in culture, as monolayers on an artificial substrate (i.e., **adherent culture**) or free-floating in the culture medium (**suspension culture**). The majority of the cells derived from vertebrates, with the exception of hematopoietic cell lines and a few others are anchorage-dependent and have to be cultured on a suitable substrate that is specifically treated to allow cell adhesion and spreading (i.e., tissue-culture treated). However, many cell lines can also be adapted for suspension culture. Similarly, most of the commercially available insect cell lines grow well in monolayer or suspension culture. Cells that are cultured in suspension can be maintained in culture flasks that are not tissue-culture treated, but as the culture volume to surface area is increased beyond which adequate gas exchange is hindered (usually 0.2–0.5 mL/cm2), the medium requires agitation. This agitation is usually achieved with a magnetic stirrer or rotating spinner flasks.

Adherent Culture	Suspension Culture
Appropriate for most cell types, including primary cultures.	Appropriate for cells adapted to suspension culture and a few other cell lines that are nonadhesive (e.g., hematopoietic)
Requires periodic passaging, but allows easy visual inspection under inverted microscope.	Easier to passage, but requires daily cell counts and viability determination to follow growth patterns; culture can be diluted to stimulate growth.
Cells are dissociated enzymatically (e.g., TrypLE™ Express, trypsin) or mechanically.	Does not require enzymatic or mechanical dissociation.
Growth is limited by surface area, which may limit product yields.	Growth is limited by concentration of cells in the medium, which allows easy scale-up.
Requires tissue-culture treated vessel.	Can be maintained in culture vessels that are not tissue-culture treated, but requires agitation (i.e., shaking or stirring) for adequate gas exchange.
Used for cytology, harvesting products continuously, and many research applications.	Used for bulk protein production, batch harvesting, and many research applications

Maintenance: Once a culture is initiated, whether it is a primary culture or a subculture, it will need periodic medium changes. For example, HeLa cells are usually subcultured once per week. Other cell lines may be subcultured only every two, three or even four weeks.

Modification of Cell Morphology: Prior to use, cells should always be checked for any signs of deterioration, such as granularity around the nucleus, cytoplasmic vacuolation, or rounding of the

cells with detachment from substrate. Such signs may imply that the culture requires a medium change or may indicate a more serious problem (inadequate or toxic serum/medium, microbial contamination or senescence of the cell line).

Replacement of the Medium: Four factors indicate the need for the replacement of culture medium,

1 **Drop in pH**: Most cells stop growing as the pH falls from pH7.0 to pH 6.5 and start to lose viability between pH 6.5 and pH 6.0. *(As the pH drops, the indicator in the medium changes from red through orange to yellow*.)

2 **Cell Concentrations**: High cell concentrations exhaust the medium faster than low concentrations.

3 **Cell Type**: Normal cells usually stop dividing at high density due to cell crowding, growth factor depletion, etc. The cells arrest in the G1 phase of the cell cycle and deteriorate very little, even if left for two to three weeks (or longer).

4 **Deterioration of Morphology**: This factor should be checked frequently. You should always be aware of the morphology since this may reveal the presence of contamination.

Criteria for Subculture

Density of the Culture: Cells should be subcultured prior to confluence. The ideal method for determining the correct seeding density is to perform a growth curve at different seeding concentrations. This allows you to determine the minimum concentration that will give a short lag period and early entry into rapid logarithmic growth.

Exhaustion of Medium: Medium requires periodic replacement. If the pH falls too rapidly, subculture may be required. Time since Last Subculture or Routine subculture is best performed according to a strict schedule, so that reproducible behavior is achieved. It is essential to become familiar with the growth cell cycle for each cell line. Cells at different phases behave differently with respect to proliferation, enzyme activity, glycolysis and respiration, synthesis of specialized products, etc.

Requirements for Other Procedures: When cells require operations other than routine propagation (e.g., increasing stock, changing vessel or medium), this procedure should ideally be done at the regular subculture time. Cells should not be subcultured while still in the lag phase; cells should always be taken between the middle of the log phase and the plateau phase as

determined during a previous subculture Fig. 1 (unless experimental requirements dictate different timing)

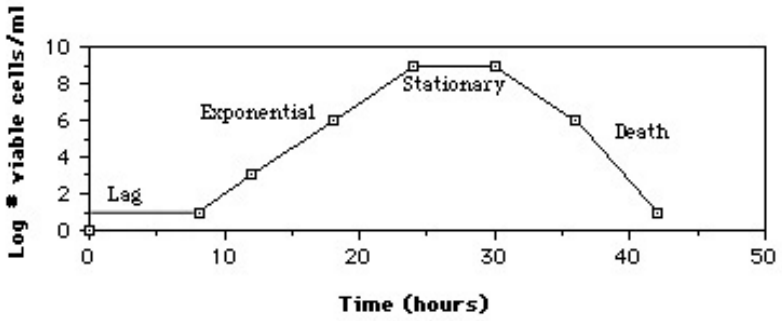

Figure 1: Growth curve of cell culture

Culture Conditions

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 a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O2, CO2), and regulates the physicochemical environment (pH, osmotic pressure, temperature).

Mammalian Cell: Morphology Most mammalian cells in culture can be divided in to three basic categories based on their morphology (Fig. 2)

(1) Fibroblastic (or fibroblast-like) cells are bipolar or multipolar and have elongated shapes. They grow attached to a substrate.

(2) Epithelial-like cells are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches.

(3) Lymphoblast-like cells are spherical in shape and they are usually grown in suspension without attaching to a surface.

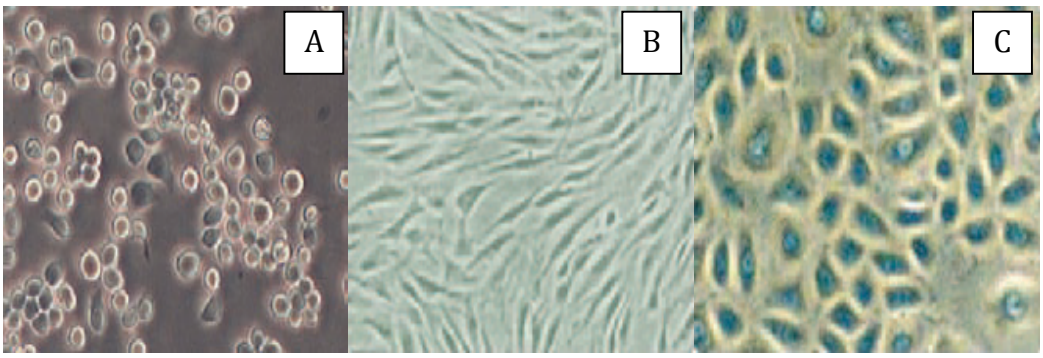

Figure 2: Mammalian cell line (A) Fibroblast (B) Epithelial and (C) Lymphoblast

In addition to the basic categories listed above, certain cells display morphological characteristics specific to their specialized role in host.

Aseptic Techniques

To minimize the risk of contamination, follow these 5 rules:

1. Always check the cells carefully before handling (by eye and on a microscope). Become familiar with the indicators of abnormal cell growth.
2. Whenever possible, maintain cultures without antibiotics for at least part of the time, to reveal cryptic contamination.
3. Check sterility of all reagents before use.
4. Use dedicated media and reagents; do not share with other cell lines.
5. Maintain a high standard of sterility at all steps.

Mycoplasma contamination, which may slow cell growth, cannot be checked under a regular microscope. To confirm or rule out such contamination, use a mycoplasma test (e.g. Roche Applied Science Mycoplasma PCR ELISA Kit).

Environment: There should be a laminar flow hood in the room dedicated to cell culture, and this hood should be used for all culture manipulations and storage of all equipment. The hood must be placed away from traffic or equipment that might generate air currents (e.g., centrifuges, refrigerators and freezers). Always carefully clean the hood before and after your procedure. Remove all unneeded items. It is crucial to always keep the work surface clean and tidy. To achieve this, follow these rules:

- Use 80% ethanol to clean the surface before starting.
- Place and keep on this surface only the items required for your procedure. This will reduce the possibility of contact between sterile and non-sterile items and facilitate culture manipulations.

- Clear space in the center of the bench, not just the front edge.
- Avoid spills, if they happen immediately clean the area.
- Remove everything when you are done, and again clean the work surface.

Reagents and media obtained from commercial suppliers will already have undergone strict quality testing. Most of the bottles are wrapped in polyethylene. The wrapping should be removed outside the hood. Unwrapped bottles should be cleaned with 80% ethanol whenever they are removed from the refrigerator or from a water bath. Regularly clean the refrigerator, the incubator and the water bath to avoid growth of mold or fungi. Imported cell lines should always be quarantined before being incorporated into your main stock. Do not perpetually use antibiotics; they will suppress some contaminants, but will not eliminate them.

Handling: Special care should be taken with caps. Use deep screw caps in preference to stoppers. When working on an open bench, flame glass pipettes and necks of the bottles before and after each use. Always use the pipettes which are best adapted your procedure; regularly clean them and check their calibration. Use a multi-channel pipette instead of a single pipette if you are working with multiwell plates. This will reduce both the time required to perform the procedure and the probability of contamination. Prepare as many reagents and equipment as possible in advance, to reduce the time the cultures are kept out of the incubator.

Cell Lines (contamination, cryopreservation); Cryopreservation Cell lines in continuous culture are prone to genetic drift, finite cell lines are fated for senescence, all cell cultures are susceptible to microbial contamination, and even the best-run laboratories can experience equipment failure. Because an established cell line is a valuable resource and its replacement is expensive and time consuming, it is vitally important that they are frozen down and preserved for long-term storage. As soon as a small surplus of cells becomes available from subculturing, they should be frozen as a seed stock, protected, and not be made available for general laboratory use. Working stocks can be prepared and replenished from frozen seed stocks. If the seed stocks become depleted, cryopreserved working stocks can then serve as a source for preparing a fresh seed stock with a minimum increase in generation number from the initial freezing. The best method for cryopreserving cultured cells is storing them in liquid nitrogen in complete medium in the presence of a cryoprotective agent such as dimethylsulfoxide (DMSO). Cryoprotective

agents reduce the freezing point of the medium and also allow a slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death.

Note: DMSO is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with local regulations.

Guidelines for Cryopreservation Following the guidelines below is essential for cryopreserving your cell lines for future use. As with other cell culture procedures, we recommend that you closely follow the instructions provided with your cell line for best results.

- Freeze your cultured cells at a high concentration and at as low a passage number as possible. Make sure that the cells are at least 90% viable before freezing. Note that the optimal freezing conditions depend on the cell line in use.
- Freeze the cells slowly by reducing the temperature at approximately 1^{o}C per minute using a controlled rate cryo-freezer or a cryo-freezing container such as "Mr. Frosty," available from NALGENE labware (Nalgene Nunc)
- Always use the recommended freezing medium. The freezing medium should contain a cryoprotective agent such as DMSO or glycerol.
- Store the frozen cells below –70^{o}C; frozen cells begin to deteriorate above –50^{o}C.
- Always use sterile cryovials for storing frozen cells. Cryovials containing the frozen cells may be stored immersed in liquid nitrogen or in the gas phase above the liquid nitrogen.
- Always wear personal protective equipment.
- All solutions and equipment that come in contact with the cells must be sterile. Always use proper sterile technique and work in a laminar flow hood.

Freezing Medium: Always use the recommended freezing medium for cryopreserving your cells. The freezing medium should contain a cryoprotective agent such as DMSO or glycerol.

Cryopreservation Medium

Recovery™: Cell Culture Freezing Medium is a ready-to-use complete cryopreservation medium for mammalian cell cultures, containing an optimized ratio of fetal bovine serum to bovine serum for improved cell viability and cell recovery after thawing.

Synth-a-Freeze: Cryopreservation Medium is a chemically defined, protein free, sterile

cryopreservation medium containing 10% DMSO that is suitable for the cryopreservation of many stem and primary cell types with the exception of melanocytes.

Protocol for Cryopreserving Cultured Cells

The following protocol describes a general procedure for cryopreserving cultured cells. For detailed protocols, always refer to the cell-specific product insert.

1. Prepare freezing medium and store at 2°C to 8°C until use. Note that the appropriate freezing medium depends on the cell line.
2. For adherent cells, gently detach cells from the tissue culture vessel following the procedure used during the subculture. Resuspend the cells in complete medium required for that cell type.
3. Determine the total number of cells and percent viability using a hemacytometer, cell counter and Trypan Blue exclusion, or the Countess, Automated Cell Counter. According to the desired viable cell density, calculate the required volume of freezing medium.
4. Centrifuge the cell suspension at approximately 100–200 g for 5 to 10 minutes; aseptically decant supernatant without disturbing the cell pellet.

Note: Centrifugation speed and duration varies depending on the cell type.

5. Resuspend the cell pellet in cold freezing medium at the recommended viable cell density for the specific cell type.
6. Dispense aliquots of the cell suspension into cryogenic storage vials. As you aliquot them, frequently and gently mix the cells to maintain a homogeneous cell suspension.
7. Freeze the cells in a controlled rate freezing apparatus, decreasing the temperature approximately 1°C per minute. Alternatively, place the cryovials containing the cells in an isopropanol chamber and store them at –80°C overnight.
8. Transfer frozen cells to liquid nitrogen, and store them in the gas phase above the liquid nitrogen.

Thawing Frozen Cells

Protocol for Thawing Frozen Cells

The following protocol describes a general procedure for thawing cryopreserved cells. For detailed protocols, always refer to the cell-specific product insert.

1. Remove the cryovial containing the frozen cells from liquid nitrogen storage and immediately place it into a 37°C water bath.
2. Quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37°C water bath until

there is just a small bit of ice left in the vial.

3. Transfer the vial it into a laminar flow hood. Before opening, wipe the outside of the vial with 70% ethanol.

4. Transfer the thawed cells drop wise into the centrifuge tube containing the desired amount of pre-warmed complete growth medium appropriate for your cell line.

5. Centrifuge the cell suspension at approximately 200~ g for 5–10 minutes. The actual centrifugation speed and duration varies depending on the cell type.

6. After the centrifugation, check the clarity of supernatant and visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet.

7. Gently re-suspend the cells in complete growth medium, and transfer them into the appropriate culture vessel and into the recommended culture environment.

Note: The appropriate flask size depends on the number of cells frozen in the cryovial, and the culture environment varies based on the cell and media type.

Biological Contamination

Contamination of cell cultures is the common problem encountered in cell culture laboratories, sometimes with very serious consequences. Cell culture contaminants can be divided into two main categories, **chemical contaminants** such as impurities in media, sera, and water, endotoxins, plasticizers, and detergents, and **biological contaminants** such as bacteria, molds, yeasts, viruses, mycoplasma, as well as cross contamination by other cell lines. While it is impossible to eliminate contamination entirely, it is possible to reduce its frequency and seriousness by gaining a thorough understanding of their sources and by following good aseptic technique. This section provides an overview of major types of biological contamination. Bacterial contamination is easily detected by visual inspection of the culture within a few days of it becoming infected; infected cultures usually appear cloudy (i.e., turbid), sometimes with a thin film on the surface. Sudden drops in the pH of the culture medium are also frequently encountered. Under a low power microscope, the bacteria appear as tiny, moving granules between the cells, and observation under a high-power microscope can resolve the shapes of individual bacteria. The simulated images below show an adherent 293 cell culture contaminated with *E. coli* (Fig. 3).

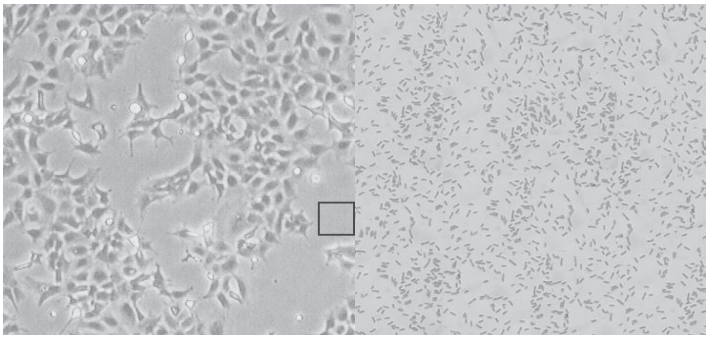

Figure 3: Simulated phase contrast images of adherent 293 cells contaminated with *E. coli*. The spaces between the adherent cells show tiny, shimmering granules under low power microscopy, but the individual bacteria are not easily distinguishable (panel A). Further magnification of the area enclosed by the black square resolves the individual *E. coli* cells, which are typically rod-shaped and are about 2 µm long and 0.5 µm in diameter. Each side of the black square in panel A is 100 µm.

Yeasts are unicellular eukaryotic microorganisms in the kingdom of Fungi, ranging in size from a few micrometers (typically) up to 40 micrometers (rarely). Like bacterial contamination, cultures contaminated with yeasts become turbid, especially if the contamination is in an advanced stage. There is very little change in the pH of the culture contaminated by yeasts until the contamination becomes heavy, at which stage the pH usually increases. Under microscopy, yeast appears as individual ovoid or spherical particles, which may bud off smaller particles. The simulated image below shows adherent 293 cell culture 24 hours after plating that is infected with yeast (Fig. 4).

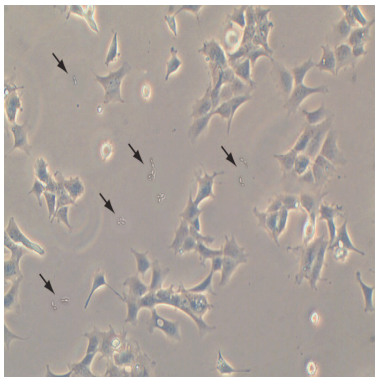

Figure 4: Simulated phase contrast images of 293 cells in adherent culture that is contaminated with yeast. The contaminating yeast cells appear as ovoid particles, budding off smaller particles as they replicate.

Similar to yeast contamination, the pH of the culture remains stable in the initial stages of contamination, then rapidly increases as the culture become more heavily infected and becomes turbid. Under microscopy, the mycelia usually appear as thin, wisp-like filaments, and

sometimes as denser clumps of spores. Spores of many mold species can survive extremely harsh and inhospitable environments in their dormant stage, only to become activated when they encounter suitable growth conditions. Viruses are microscopic infectious agents that take over the host cells machinery to reproduce. Their extremely small size makes them very difficult to detect in culture, and to remove them from reagents used in cell culture laboratories. Because most viruses have very stringent requirements for their host, they usually do not adversely affect cell cultures from species other than their host. However, using virally infected cell cultures can present a serious health hazard to the laboratory personnel, especially if human or primate cells are cultured in the laboratory. Viral infection of cell cultures can be detected by electron microscopy, immunostaining with a panel of antibodies, ELISA assays, or PCR with appropriate viral primers.

Protocol for Microbial Decontamination

1. Collect the contaminated medium carefully. If possible, the organism should be tested for sensitivity to a range of individual antibiotics. If not, autoclave the medium or add hypochlorite.
2. Wash the cells in DBSS (Hanks BSS without bicarbonate, with Penicillin, Streptomycin, Amphotericin B and Kanamycin or Gentamycin). For monolayers, rinse the culture 3 times with DBSS, trypsinize, and then wash the cells twice more in DBSS by centrifugation and re-suspension. For suspension cultures, wash the culture five times (in DBSS) by centrifugation and re-suspension.
3. Reseed a fresh flask at the lowest reasonable seeding density, depending on cell type.
4. Add high-antibiotic medium and change the culture every 2 days.
5. Subculture in a high-antibiotic medium. Repeat Steps 1 to 4 for three subcultures.
6. Remove the antibiotics, and culture the cells without them for a further three subcultures.
7. Recheck the cultures (phase-contrast microscopy, Hoechst staining).
8. Culture the cells for a further two months without antibiotics, and check to make sure that all contamination has been eliminated.

Mycoplasma are simple bacteria that lack a cell wall, and they are considered the smallest self-replicating organism. Because of their extremely small size (typically less than one micrometer), mycoplasma are very difficult to detect until they achieve extremely high densities and cause the

cell culture to deteriorate; until then, there are often no visible signs of infection. Chronic mycoplasma infections might manifest themselves with decreased rate of cell proliferation, reduced saturation density, and agglutination in suspension cultures; (Fig. 5) however, the only assured way of detecting mycoplasma contamination is by testing the cultures periodically using fluorescent staining (e.g., Hoechst 33258), ELISA, PCR, immunostaining, autoradiography, or microbiological assays.

Figure 5: Photomicrographs of mycoplasma-free cultured cells (A) infected with mycoplasma (B and C).

Protocol for Treating Mycoplasma-contaminated Cell Cultures with BM Cyclin

Remove culture medium from culture vessels by aspiration. Add new culture medium containing BM Cyclin 1 (4 µl of stock solution/ml, final concentration 10 µg/ml). Cultivate the cells for 3 days as usual. Remove culture medium, add new culture medium containing BM Cyclin 2 (4 µl of stock solution/ml, final concentration 5 µg/ml). Cultivate the cells for 4 days, repeat the above cycle twice. Cross-Contamination While not as common as microbial contamination, extensive cross-contamination of many cell lines with HeLa and other fast growing cell lines is a clearly-established problem with serious consequences. Obtaining cell lines from reputable cell banks, periodically checking the characteristics of the cell lines, and practicing good aseptic technique are practices that will help you avoid cross-contamination. DNA fingerprinting, karyotype analysis, and isotype analysis can confirm the presence or absence of cross contamination in your cell cultures. Using Antibiotics Antibiotics should not be used routinely in cell culture, because their continuous use encourages the development of antibiotic resistant strains and allows low-level contamination to persist, which can develop into full-scale contamination once the antibiotic is removed from media, and may hide mycoplasma infections and other cryptic contaminants. Further, some antibiotics might cross react with the cells and interfere with the cellular processes under investigation. Antibiotics should only be used as a last resort and only for short term applications, and they should be removed from the culture as soon as possible. If

they are used in the long term, antibiotic-free cultures should be maintained in parallel as a control for cryptic infections.

Cell Viability; (quantitation, cytotoxicity): The measurement of cell viability plays a fundamental role in all forms of cell culture. Sometimes it is the main purpose of the experiment, such as in toxicity assays. Alternatively, cell viability can be used to correlate cell behaviour to cell number, providing a more accurate picture, for example anabolic activity. There are wide arrays of cell viability methods which range from the most routine trypan blue dye exclusion assay to highly complex analysis of individual cells, such as using RAMAN microscopy. The cost, speed, and complexity of equipment required will all play a role in determining the assay used. This chapter provides an overview of many of the assays available today. Cell viability is a determination of living or dead cells, based on a total cell sample. Viability measurements may be used to evaluate the death or life of cancerous cells and the rejection of implanted organs. In other applications, these tests might calculate the effectiveness of a pesticide or insecticide, or evaluate environmental damage due to toxins.

Factors to Consider When Choosing a Cell Viability Assay: Among the many factors to consider when choosing a cell-based assay, the primary concern for many researchers is the ease of use. Homogeneous assays do not require removal of culture medium, cell washes or centrifugation steps. When choosing an assay, the time required for reagent preparation and the total length of time necessary to develop a signal from the assay chemistry should be considered. The stability of the absorbance, fluorescence or luminescence signal is another important factor that provides convenience and flexibility in recording data and minimizes differences when processing large batches of plates. Another factor to consider when selecting an assay is sensitivity of detection. Detection sensitivity will vary with cell type if you choose to measure a metabolic marker, such as ATP level or MTS tetrazolium reduction. The signal-to-background ratios of some assays may be improved by increasing incubation time. The sensitivity not only depends upon the parameter being measured but also on other parameters of the model system such as the plate format and number of cells used per well. Cytotoxicity assays that are designed to detect a change in viability in a population of 10,000 cells may not require the most sensitive assay technology. For example, a tetrazolium assay should easily detect the difference between 10,000 and 8,000 viable cells. On the other hand, assay model systems that use low cell numbers

in a high-density multiwell plate format may require maximum sensitivity of detection such as that achieved with the luminescent ATP assay technology. For researchers using automated screening systems, the reagent stability and compatibility with robotic components is often a concern. The assay reagents must be stable at ambient temperature for an adequate period of time to complete dispensing into several plates. In addition, the signal generated by the assay should also be stable for extended periods of time to allow flexibility for recording data. For example, the luminescent signal from the ATP assay has a half-life of about 5 hours, providing adequate flexibility. With other formats such as the MTS tetrazolium assay or the LDH release assay, the signal can be stabilized by the addition of a detergent-containing stop solution. In some cases the choice of assay may be dictated by the availability of instrumentation to detect absorbance, fluorescence or luminescence. The Promega portfolio of products contains an optional detection format for each of the three major classes of cell-based assays (viability, cytotoxicity or apoptosis). In addition, results from some assays such as the ATP assay can be recorded with more than one type of instrument (luminometer, fluorometer or CCD camera).

Cost is an important consideration for every researcher; however, many factors that influence the total cost of running an assay are often overlooked. All of the assays described above are homogeneous and as such are more efficient than multistep assays. For example, even though the reagent cost of an ATP assay may be higher than other assays, the speed (time savings), sensitivity (cell sample savings) and accuracy may outweigh the initial cost. Assays with good detection sensitivity that are easier to scale down to 384 or 1536well formats may result in savings of cell culture reagents and enable testing of very small quantities of expensive or rare test compounds. The ability to gather more than one set of data from the same sample (i.e., multiplexing) also may contribute to saving time and effort. Multiplexing more than one assay in the same culture well can provide internal controls and eliminate the need to repeat work. For instance, the LDH-release assay is an example of an assay that can be multiplexed. The LDH-release assay offers the opportunity to gather cytotoxicity data from small aliquots of culture supernatant that can be removed to a separate assay plate, thus leaving the original assay plate available for any other assay such as gene reporter analysis, image analysis, etc. Several of our homogeneous apoptosis and viability assays can be multiplexed without transferring media, allowing researchers to assay multiple parameters in the same sample well.

Reproducibility of data is an important consideration when choosing a commercial assay. However, for most cell-based assays, the variation among replicate samples is more likely to be caused by the cells rather than the assay chemistry. Variations during plating of cells can be magnified by using cells lines that tend to form clumps rather than a suspension of individual cells. Extended incubation periods and edge effects in plates may also lead to decreased reproducibility among replicates and less desirable Z'-factor values.

Cell Viability Assays that Measure ATP
CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture. Detection is based on using the luciferase reaction to measure the amount of ATP from viable cells. The amount of ATP in cells correlates with cell viability. Within minutes after a loss of membrane integrity, cells lose the ability to synthesize ATP, and endogenous ATPases destroy any remaining ATP; thus the levels of ATP fall precipitously. The CellTiter-Glo® Reagent does three things upon addition to cells. It lyses cell membranes to release ATP; it inhibits endogenous ATPases, and it provides luciferin, luciferase and other reagents necessary to measure ATP using a bioluminescent reaction. The unique properties of a proprietary stable luciferase mutant enabled a robust, single-addition reagent. The "glow-type" signal can be recorded with a luminometer, CCD camera or modified fluorometer and generally has a half-life of five hours, providing a consistent signal across large batches of plates. The CellTiter-Glo® Assay is extremely sensitive and can detect as few as 10 cells. The luminescent signal can be detected as soon as 10 minutes after adding reagent or several hours' later, providing flexibility for batch processing of plates.

Cell Viability Assays that Measure Metabolic Capacity

CellTiter-Blue® Cell Viability Assay (resazurin)

The CellTiter-Blue® Cell Viability Assay uses an optimized reagent containing resazurin. The homogeneous procedure involves adding the reagent directly to cells in culture at a recommended ratio of 20µl of reagent to 100µl of culture medium. The assay plates are incubated at 37°C for 1–4 hours to allow viable cells to convert resazurin to the fluorescent resorufin product. The conversion of resazurin to fluorescent resorufin is proportional to the number of metabolically active, viable cells present in a population. The signal is recorded using

a standard multiwell fluorometer. Because different cell types have different abilities to reduce resazurin, optimizing the length of incubation with the CellTiter-Blue® Reagent can improve assay sensitivity for a given model system. The detection sensitivity is intermediate between the ATP assay and the MTS reduction assay.

Cytotoxicity Assays: Determining the Number of Live and Dead Cells in a Cell Population, MultiTox-Fluor Multiplex Cytotoxicity Assay: Cell-based assays are important tools for contemporary biology and drug discovery because of their predictive potential for in vivo applications. However, the same cellular complexity that allows the study of regulatory elements, signaling cascades or test compound bio-kinetic profiles also can complicate data interpretation by inherent biological variation. Therefore, researchers often need to normalize assay responses to cell viability after experimental manipulation. Although assays for determining cell viability and cytotoxicity that are based on ATP, reduction potential and LDH release are useful and cost-effective methods, they have limits in the types of multiplexed assays that can be performed along with them. The MultiTox-Fluor Multiplex Cytotoxicity Assay (Cat. No. G9200, G9201, G9202) is a homogeneous, single-reagent-addition format that allows the measurement of the relative number of live and dead cells in a cell population. This assay gives ratiometric, inversely proportional values of viability and cytotoxicity that are useful for normalizing data to cell number. Also, this reagent is compatible with additional fluorescent and luminescent chemistries.

Assays to Detect Apoptosis: A variety of methods are available for detecting apoptosis to determine the mechanism of cell death. The Caspase-Glo® Assays are highly sensitive, luminescent assays with a simple "add, mix, measure" protocol that can be used to detect caspase-8, caspase-9 and caspase-3/7 activities. If you prefer a fluorescent assay, the Apo-ONE® Homogeneous Caspase-3/7 Assay is useful and, like the Caspase-Glo® Assays, can be multiplexed with other assays. A later marker of apoptosis is TUNEL analysis to identify the presence of oligonucleosomal DNA fragments in cells. The DeadEnd™ Fluorometric and the DeadEnd™ Colorimetric TUNEL Assays allow users to end-label the DNA fragments to detect apoptosis

Cell Counter: A cell counter is essential for quantitative growth kinetics, and a great advantage

when more than two or three cell lines are cultured in the laboratory. The Countess; Automated Cell Counter is a bench-top instrument designed to measure cell count and viability (live, dead, and total cells) accurately and precisely in less than a minute per sample, using the standard Trypan Blue uptake technique. Using the same amount of sample that you currently use with the hemacytometer, the countess. Automated Cell Counter takes less than a minute per sample for a typical cell count and is compatible with a wide variety of eukaryotic cells.

Multiplexing Cell Viability Assays: The latest generation of cell-based assays includes luminescent and fluorescent chemistries to measure markers of cell viability, cytotoxicity and apoptosis, as well as to perform reporter analysis. Using these tools researchers can investigate how cells respond to growth factors, cytokines, hormones, mitogens, radiation, effectors, compound libraries and other signaling molecules. However, researchers often need more than one type of data from a sample, so the ability to multiplex, or analyze more than one parameter from a single sample, is desirable.

Counting Cells in a Hemacytometer: Hemacytometers may be obtained from most major laboratory suppliers (e.g., Baxter Scientific). The procedure below provides some general directions on how to use the hemacytometer.

1. Clean the chamber and cover slip with alcohol. Dry and fix the coverslip in position.
2. Harvest the cells. Add 10 μL of the cells to the hemacytometer. Do not overfill.
3. Place the chamber in the inverted microscope under a 10X objective. Use phase contrast to distinguish the cells.
4. Count the cells in the large, central gridded square (1 mm^2). The gridded square is circled in the graphic below. Multiply by 10^4 to estimate the number of cells per mL. Prepare duplicate samples and average the count.

Trypan Blue: Exclusion The following procedure will enable you to accurately determine the cell viability. Cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemacytometer. If cells take up trypan blue, they are considered non-viable.

1. Determine the cell density of your cell line suspension using a hemacytometer.
2. Prepare a 0.4% solution of trypan blue in buffered isotonic salt solution, pH 7.2 to 7.3 (i.e.,

phosphate-buffered saline).

3. Add 0.1 mL of trypan blue stock solution to 1 mL of cells.

4. Load a hemacytometer and examine immediately under a microscope at low magnification.

5. Count the number of blue staining cells and the number of total cells. Cell viability should be at least 95% for healthy log-phase cultures. Remember to correct for the dilution factor.

To calculate the number of viable cells per mL of culture, use the formula below.

Live cell count/ Total cell count =Viability

Determine total viable cell yield using the formula below.

Viable cell count/ Quadrants counted x Dilution factor x Hemocytometer factor x Current volume (mL) = Viable cell yield.

Concentrating Cells: To concentrate cells from a suspension culture (or resuspended cells from monolayer culture):

1. Transfer the cell suspension to a sterile centrifuge tube of appropriate size and centrifuge for 10 minutes at 800 ~ g.

Note: Certain cell lines are very sensitive to centrifugal force.

2. Carefully remove the supernatant without disturbing the cell pellet.

3. Add the desired volume of fresh medium gently to the side of the tube and slowly pipette up and down 2 to 3 times to resuspend the cell pellet.

4. Transfer the cells to the desired, sterile container.

Cell Proliferation: An alternative way to determine the health of a culture is to perform a cell proliferation assay, i.e. to determine the number of dividing cells. One way of measuring this parameter is by performing clonogenic assays. In these assays, a defined number of cells are plated onto an appropriate matrix and the numbers of colonies that form are counted after a period of growth. Drawbacks to this type of assay are that it is tedious and it is not practical for large numbers of samples. Another way to analyze cell proliferation is to measure DNA Synthesis. In these assays, labeled DNA precursors (4H-thymidine or bromodeoxy-uridine, BrdU (e.g., Roche Applied Science Cell Proliferation ELISA, BrdU (chemiluminescent) Kit) are added to cells and their incorporation into DNA is quantified after incubation. The amount of labeled

precursor incorporated into DNA is quantified either by measuring the total amount of labeled DNA in a population, or by detecting the labeled nucleimicroscopically. Cell proliferation can also be measured using more indirect parameters. In these techniques, molecules that regulate the Cell Cycle (also called proliferation markers) are measured either by their activity (e.g., CDK kinase assays) or by quantifying their amounts (e.g., Western blots, ELISA, or immunohistochemistry).

Cell Cycle: The cell cycle is made up of four phases (Fig. 6). In the M phase (M=mitosis), the chromatin condenses into chromosomes, and the two individual chromatids, which make up the chromosome, segregate to each daughter cell. In the G1 (Gap 1) phase, the cell either progresses toward DNA synthesis or another division cycle or exits the cell cycle reversibly (G0) or irreversibly to commit to differentiation. During G1, the cell is particularly susceptible to control of cell cycle progression; this may occur at a number of restriction points, which determine whether the cell will re-enter the cycle, withdraw from it, or withdraw and differentiate. G1 is followed by the S phase (DNA synthesis), in which the DNA replicates. S in turn is followed by the G2 (Gap 2) phase in which the cell prepares for reentry into mitosis. Checkpoints, at the beginning of DNA synthesis and in G2, determine the integrity of the DNA and will halt the cell cycle to allow either DNA repair or entry into apoptosis if repair is impossible. The Phospho Histone H3 Imaging Kit (Roche) is a convenient method for fast cell cycle analysis by quantification of mitotic cells. Apoptosis, or programmed cell death, is a regulated physiological process whereby a cell can be removed from a population. Characterized by DNA fragmentation, nuclear blebbing, and cell shrinkage, apoptosis can be detected via a number of marker enzymes and kits (see Roche Applied Science products). Roche DNA Fragmentation Imaging Kit is a TUNEL assay-based method for accurate and fast quantitative fluorescence detection of apoptosis in medium to high throughput cellular workflows.

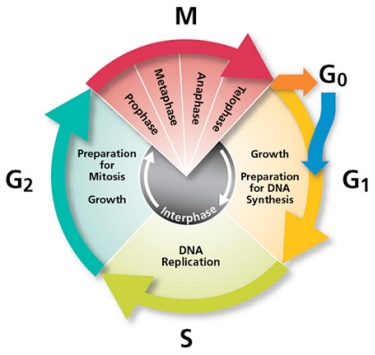

Figure 6: Cell cycle phases

Cytotoxicity: Cell viability and toxic effects can be assayed using Roche s easy-to-apply one-step Cell Viability Imaging Kit. The indicators of cytotoxicity can vary, depending on the study performed (e.g., Roche Applied Science Cytotoxicity Detection Kit Plus, (LDH)). The cytotoxicity effect can lead to the death of the cells or just to an alteration of their metabolism. This toxic effect can be initiated by addition of compounds or by addition of effector cells. Demonstrating the lack of toxicity of a given compound may require subtle analysis of its interaction with specific targets, e.g. a study of its ability to alter cell signaling or to initiate cell interactions that would give rise to an inflammatory or allergic response.

To test the potential cytotoxicity of compounds/cells, consider the following parameters:

Concentration of Compound: A wide range of concentrations should be tested to determine the survival curve.

Medium/Serum: In some cases, the serum may have a masking effect and lead to an underestimation of the cytotoxicity effect.

Duration of the Exposure: The action of one compound can happen within a few seconds or over several hours. Cell Density For most of the assays, confluent cells is not used. However, if you want to study the endothelial barrier function, you will need confluent cells in order to see an effect.

Colony Size: Some agents are cytostatic, i.e. they inhibit cell proliferation but are not cytotoxic. During continuous exposure they may reduce the size of colonies without reducing the number of colonies. In this case, the size of the colonies should be determined by densitometry, automatic colony counting or counting the number of cells per colony with the naked eye.

Solvents: Some agents to be tested have low solubilities in aqueous media, and it may be necessary to use an organic solvent to dissolve them. Ethanol, propylene glycol and dimethyl sulfoxide have been used for this purpose, but may themselves be toxic to cells. The final concentration of solvent should be maintained as low as possible (<0.5 %) and a solvent control must always be included in the study. Be aware that some organic solvents are not compatible with plastics.

The Dose-response relationship describes the biological effect induced by different

concentrations of a substance (Fig. 7). This curve should be determined whenever a new study is initiated, in order to fix the optimal conditions for the assay.

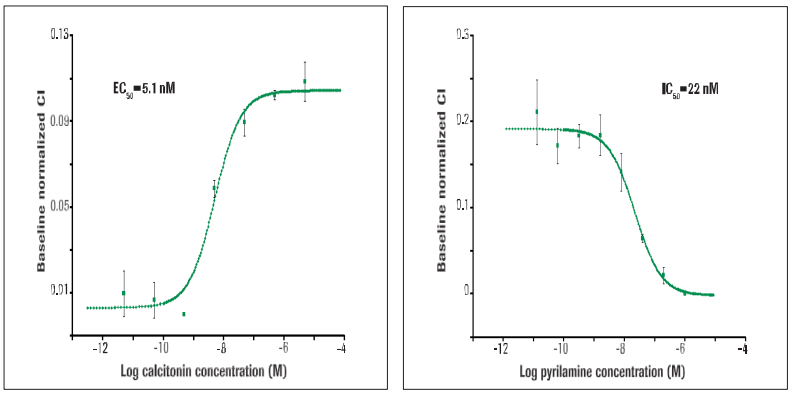

Figure 7: Dose-response curves.

The half-maximal effective concentration, or EC50, refers to the concentration of a compound which induces a response halfway between the baseline and the maximum. The EC50 represents the concentration of a compound where 50% of its maximal effect is observed. The half-maximal inhibitory concentration, or IC50, is the concentration of a compound required to inhibit a process by half. IC50 represents the concentration of a compound that is required for 50% inhibition *in vitro*. The median lethal dose, LD50 (abbreviation for "Lethal Dose, 50%"), or LCt50 (Lethal Concentration & Time) of a toxic compound is the dose required to kill half the tested population.

Applications of cell culture

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. It is also used in drug screening and development, and large scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins). The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

Model systems: Cell cultures provide a good model system for studying 1) basic cell biology and biochemistry, 2) the interactions between disease-causing agents and cells, 3) the effects of drugs on cells, 4) the process and triggers for aging, and 5) nutritional studies.

Toxicity testing: Cultured cells are widely used alone or in conjunction with animal tests to

study the effects of new drugs, cosmetics and chemicals on survival and growth in a wide variety of cell types. Especially important are liver-and kidney-derived cell cultures.

Cancer Research: Since both normal cells and cancer cells can be grown in culture, the basic differences between them can be closely studied. In addition, it is possible, by the use of chemicals, viruses and radiation, to convert normal cultured cells to cancer causing cells. Thus, the mechanisms that cause the change can be studied. Cultured cancer cells also serve as a test system to determine suitable drugs and methods for selectively destroying types of cancer.

Virology: One of the earliest and major uses of cell culture is the replication of viruses in cell cultures (in place of animals) for use in vaccine production. Cell cultures are also widely used in the clinical detection and isolation of viruses, as well as basic research into how they grow and infect organisms.

Cell-Based Manufacturing: While cultured cells can be used to produce many important produces, three areas have generating the most interest. **First** is the large-scale production of viruses for use in vaccine production. These include vaccines for polio, rabies, chicken pox, hepatitis B and measles. **Second**, is the large scale production of cells that have been genetically engineered to produce proteins that have medicinal or commercial value. These include monoclonal antibodies, insulin, hormones, etc. **Third**, is the use of cells as replacement tissues and organs. Artificial skin for use in treating burns and ulcers is the first commercially available product. However, testing is underway on artificial organs such as pancreas, liver and kidney. A potential supply of replacement cells and tissues may come out of work currently being done with both embryonic and adult stem cells. These are cells that have the potential to differentiate into a variety of different cells types. It is hoped that learning how to control the development of these cells may offer new treatment approaches for a wide variety of medical conditions.

Genetic counseling: Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from pregnant women, has given doctors an important tool for the early diagnosis of fetal disorders. These cells can then be examined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting and other molecular techniques.

Genetic Engineering: The ability to transfect or reprogram cultured cells with new genetic material (DNA and genes) has provided a major tool to molecular biologists wishing to study the cellular effects of the expression of theses genes (new proteins). These techniques can also be

used to produce these new proteins in large quantity in cultured cells for further study. Insect cells are widely used as miniature cells factories to express substantial quantities of proteins that they manufacture after being infected with genetically engineered baculoviruses.

Gene Therapy: The ability to genetically engineer cells has also led to their use for gene therapy. Cells can be removed from a patient lacking a functional gene and the missing or damaged gene can then be replaced. The cells can be grown for a while in culture and then replaced into the patient. An alternative approach is to place the missing gene into a viral vector and then "infect" the patient with the virus in the hope that the missing gene will then be expressed in the patient's cells.

Drug Screening and Development: Cell-based assays have become increasingly important for the pharmaceutical industry, not just for cytotoxicity testing but also for high throughput screening of compounds that may have potential use as drugs. Originally, these cell culture tests were done in 96 well plates, but increasing use is now being made of 384 and 1536 well plates.

What are stem cells, and why are they important?

Stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth. In addition, in many tissues they serve as a sort of internal repair system, dividing essentially without limit to replenish other cells as long as the person or animal is still alive. When a stem cell divides, each new cell has the potential either to remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell. Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs, such as the gut and bone marrow, stem cells regularly divide to repair and replace worn out or damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions. Until recently, scientists primarily worked with two kinds of stem cells from animals and humans: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells. The functions and characteristics of these cells will be explained in this document. Scientists discovered ways to derive embryonic stem cells from early mouse embryos nearly 30 years ago, in 1981. The detailed study of the biology of mouse stem cells led to the discovery, in 1998, of a method to derive stem cells from human

embryos and grow the cells in the laboratory. These cells are called human embryonic stem cells. The embryos used in these studies were created for reproductive purposes through *in vitro* fertilization procedures. When they were no longer needed for that purpose, they were donated for research with the informed consent of the donor. In 2006, researchers made another breakthrough by identifying conditions that would allow some specialized adult cells to be "reprogrammed" genetically to assume a stem cell-like state. This new type of stem cell, called induced pluripotent stem cells (IPSCs), will be discussed in a later section of this document.

Stem cells are important for living organisms for many reasons. In the 3- to 5-day-old embryo, called a blastocyst, the inner cells give rise to the entire body of the organism, including all of the many specialized cell types and organs such as the heart, lungs, skin, sperm, eggs and other tissues. In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease.

Given their unique regenerative abilities, stem cells offer new potentials for treating diseases such as diabetes and heart disease. However, much work remains to be done in the laboratory and the clinic to understand how to use these cells for cell-based therapies to treat disease, which is also referred to as regenerative or reparative medicine.

Laboratory studies of stem cells enable scientists to learn about the cells' essential properties and what makes them different from specialized cell types. Scientists are already using stem cells in the laboratory to screen new drugs and to develop model systems to study normal growth and identify the causes of birth defects.

Research on stem cells continues to advance knowledge about how an organism develops from a single cell and how healthy cells replace damaged cells in adult organisms. Stem cell research is one of the most fascinating areas of contemporary biology, but, as with many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries.

What are the unique properties of all stem cells?

Stem cells differ from other types of cells in the body. All stem cells regardless of their source have three general properties: 1) they are capable of dividing and renewing themselves for long periods; 2) they are unspecialized; and 3) they can give rise to specialized cell types.

Stem cells are capable of dividing and renewing themselves for long periods. Unlike muscle cells, blood cells, or nerve cells which do not normally replicate themselves, stem cells may replicate many times, or proliferate. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal. Scientists are trying to understand two fundamental properties of stem cells that relate to their long-term self-renewal: Discovering the answers to these questions may make it possible to understand how cell proliferation is regulated during normal embryonic development or during the abnormal cell division that leads to cancer. Such information would also enable scientists to grow embryonic and non-embryonic stem cells more efficiently in the laboratory. The specific factors and conditions that allow stem cells to remain unspecialized are of great interest to scientists. It has taken many years of trial and error to learn to derive and maintain stem cells in the laboratory without them spontaneously differentiating into specific cell types. For example, it took two decades to learn how to grow human embryonic stem cells in the laboratory following the development of conditions for growing mouse stem cells. Likewise, scientists must first understand the signals that enable a non-embryonic (adult) stem cell population to proliferate and remain unspecialized before they will be able to grow large numbers of unspecialized adult stem cells in the laboratory.

Stem cells are unspecialized: One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. For example, a stem cell cannot work with its neighbors to pump blood through the body (like a heart muscle cell), and it cannot carry oxygen molecules through the bloodstream (like a red blood cell). However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

Stem cells can give rise to specialized cells: When unspecialized stem cells give rise to specialized cells, the process is called differentiation. While differentiating, the cell usually goes through several stages, becoming more specialized at each step. Scientists are just beginning to understand the signals inside and outside cells that trigger each step of the differentiation process. The internal signals are controlled by a cell's genes, which are interspersed across long strands of DNA and carry coded instructions for all cellular structures and functions. The external signals for cell differentiation include chemicals secreted by other cells, physical contact

with neighboring cells, and certain molecules in the microenvironment. The interaction of signals during differentiation causes the cell's DNA to acquire epigenetic marks that restrict DNA expression in the cell and can be passed on through cell division. Many questions about stem cell differentiation remain. For example, are the internal and external signals for cell differentiation similar for all kinds of stem cells? Can specific sets of signals be identified that promote differentiation into specific cell types? Addressing these questions may lead scientists to find new ways to control stem cell differentiation in the laboratory, thereby growing cells or tissues that can be used for specific purposes such as cell-based therapies or drug screening.

Adult stem cells typically generate the cell types of the tissue in which they reside. For example, a blood-forming adult stem cell in the bone marrow normally gives rise to the many types of blood cells. It is generally accepted that a blood-forming cell in the bone marrow which is called a hematopoietic stem cell cannot give rise to the cells of a very different tissue, such as nerve cells in the brain. Experiments over the last several years have purported to show that stem cells from one tissue may give rise to cell types of a completely different tissue. This remains an area of great debate within the research community. This controversy demonstrates the challenges of studying adult stem cells and suggests that additional research using adult stem cells is necessary to understand their full potential as future therapies.

Embryonic stem cells

Embryonic stem cells, as their name suggests, are derived from embryos. Most embryonic stem cells are derived from embryos that develop from eggs that have been fertilized *in vitro* in an *in vitro* fertilization clinic and then donated for research purposes with informed consent of the donors. They are *not* derived from eggs fertilized in a woman's body.

Embryonic stem cells grown in the laboratory

Growing cells in the laboratory is known as cell culture. Human embryonic stem cells (hESCs) are generated by transferring cells from a preimplantation stage embryo into a plastic laboratory culture dish that contains a nutrient broth known as culture medium. The cells divide and spread over the surface of the dish. The inner surface of the culture dish is typically coated with mouse embryonic skin cells that have been treated so they will not divide. This coating layer of cells is called a feeder layer. The mouse cells in the bottom of the culture dish provide the cells a sticky surface to which they can attach. Also, the feeder cells release nutrients into the culture medium.

Researchers have devised ways to grow embryonic stem cells without mouse feeder cells. This is a significant scientific advance because of the risk that viruses or other macromolecules in the mouse cells may be transmitted to the human cells.

The process of generating an embryonic stem cell line is somewhat inefficient, so lines are not produced each time cells from the preimplantation stage embryo are placed into a culture dish. However, if the plated cells survive, divide, and multiply enough to crowd the dish, they are removed gently and plated into several fresh culture dishes. The process of re-plating or subculturing the cells is repeated many times and for many months. Each cycle of subculturing the cells is referred to as a passage. Once the cell line is established, the original cells yield millions of embryonic stem cells. Embryonic stem cells that have proliferated in cell culture for six or more months without differentiating, are pluripotent, and appear genetically normal are referred to as an embryonic stem cell line. At any stage in the process, batches of cells can be frozen and shipped to other laboratories for further culture and experimentation.

Embryonic stem cells stimulated to differentiate

As long as the embryonic stem cells in culture are grown under appropriate conditions, they can remain undifferentiated (unspecialized). But if cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously. They can form muscle cells, nerve cells, and many other cell types. Although spontaneous differentiation is a good indicator that a culture of embryonic stem cells is healthy, the process is uncontrolled and therefore an inefficient strategy to produce cultures of specific cell types. So, to generate cultures of specific types of differentiated cells heart muscle cells, blood cells, or nerve cells, for example: scientists try to control the differentiation of embryonic stem cells. They change the chemical composition of the culture medium, alter the surface of the culture dish, or modify the cells by inserting specific genes. Through years of experimentation, scientists have established some basic protocols or “recipes” for the directed differentiation of embryonic stem cells into some specific cell types (Fig. 8).

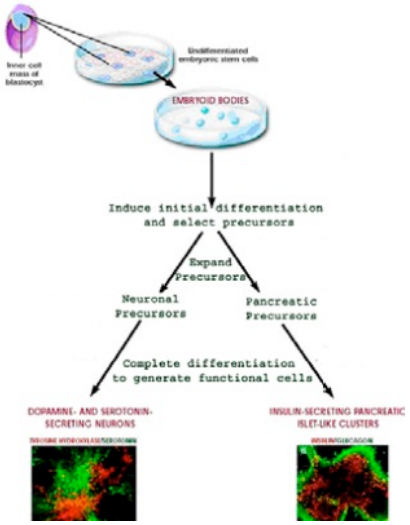

Figure 8: Directed differentiation of mouse embryonic stem cells

If scientists can reliably direct the differentiation of embryonic stem cells into specific cell types, they may be able to use the resulting, differentiated cells to treat certain diseases in the future. Diseases that might be treated by transplanting cells generated from human embryonic stem cells include diabetes, traumatic spinal cord injury, Duchenne's muscular dystrophy, heart disease, and vision and hearing loss.

Adult stem cells: An adult stem cell is thought to be an undifferentiated cell, found among differentiated cells in a tissue or organ that can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Scientists also use the term somatic stem cell instead of adult stem cell, where somatic refers to cells of the body (not the germ cells, sperm or eggs). Unlike embryonic stem cells, which are defined by their origin (cells from the preimplantation-stage embryo), the origin of adult stem cells in some mature tissues is still under investigation. Research on adult stem cells has generated a great deal of excitement. Scientists have found adult stem cells in many more tissues than they once thought possible. This finding has led researchers and clinicians to ask whether adult stem cells could be used for transplants. In fact, adult hematopoietic, or blood-forming, stem cells from bone marrow have been used in transplants for 40 years. Scientists now have evidence that stem cells exist in the brain and the heart. If the differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of transplantation-based therapies.

The history of research on adult stem cells began about 50 years ago. In the 1950s, researchers discovered that the bone marrow contains at least two kinds of stem cells. One population, called hematopoietic stem cells, forms all the types of blood cells in the body. A second population, called bone marrow stromal stem cells (also called mesenchymal stem cells, or skeletal stem cells by some) were discovered a few years later. These non-hematopoietic stem cells make up a small proportion of the stromal cell population in the bone marrow and can generate bone, cartilage, and fat cells that support the formation of blood and fibrous connective tissue. In the

1960s, scientists who were studying rats discovered two regions of the brain that contained dividing cells that ultimately become nerve cells. Despite these reports, most scientists believed that the adult brain could not generate new nerve cells. It was not until the 1990s that scientists agreed that the adult brain does contain stem cells that are able to generate the brain's three major cell types - astrocytes and oligodendrocytes, which are non-neuronal cells, and neurons, or nerve cells.

Adult stem cell differentiation: As indicated above, scientists have reported that adult stem cells occur in many tissues and that they enter normal differentiation pathways to form the specialized cell types of the tissue in which they reside. Normal differentiation pathways of adult stem cells. In a living animal, adult stem cells are available to divide for a long period, when needed, and can give rise to mature cell types that have characteristic shapes and specialized structures and functions of a particular tissue. The following are examples of differentiation pathways of adult stem cells (Fig. 9) that have been demonstrated *in vitro* or *in vivo*.

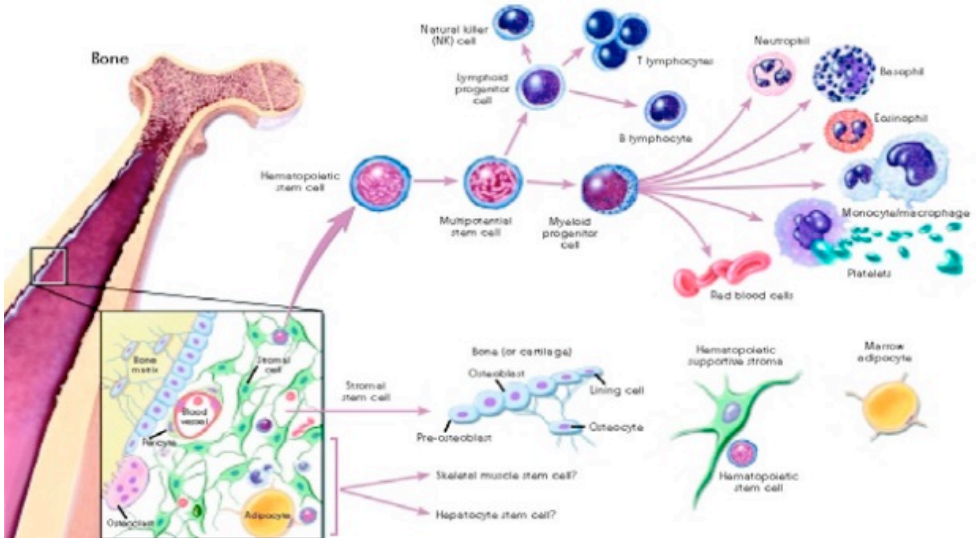

Figure 9: Hematopoietic and stromal stem cell differentiation

- **Hematopoietic stem** cells give rise to all the types of blood cells: red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, and macrophages.
- **Mesenchymal stem cells** have been reported to be present in many tissues. Those from bone marrow (bone marrow stromal stem cells, skeletal stem cells) give rise to a variety

of cell types: bone cells (osteoblasts and osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and stromal cells that support blood formation. However, it is not yet clear how similar or dissimilar mesenchymal cells derived from non-bone marrow sources are to those from bone marrow stroma.

- **Neural stem cells** in the brain give rise to its three major cell types: nerve cells (neurons) and two categories of non-neuronal cells - **astrocytes** and **oligodendrocytes**.
- **Epithelial stem cells** in the lining of the digestive tract occur in deep crypts and give rise to several cell types: absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells.
- **Skin stem cells** occur in the basal layer of the epidermis and at the base of hair follicles. The epidermal stem cells give rise to keratinocytes, which migrate to the surface of the skin and form a protective layer. The follicular stem cells can give rise to both the hair follicle and to the epidermis.

Transdifferentiation: A number of experiments have reported that certain adult stem cell types can differentiate into cell types seen in organs or tissues other than those expected from the cells' predicted lineage (i.e., brain stem cells that differentiate into blood cells or blood-forming cells that differentiate into cardiac muscle cells, and so forth). Although isolated instances of transdifferentiation have been observed in some vertebrate species, whether this phenomenon actually occurs in humans is under debate by the scientific community. Instead of transdifferentiation, the observed instances may involve fusion of a donor cell with a recipient cell. Another possibility is that transplanted stem cells are secreting factors that encourage the recipient's own stem cells to begin the repair process. Even when transdifferentiation has been detected, only a very small percentage of cells undergo the process.

In a variation of transdifferentiation experiments, scientists have recently demonstrated that certain adult cell types can be "reprogrammed" into other cell types *in vivo* using a well-controlled process of genetic modification. This strategy may offer a way to reprogram available cells into other cell types that have been lost or damaged due to disease. For example, one recent experiment shows how pancreatic beta cells, the insulin-producing cells that are lost or damaged in diabetes, could possibly be created by reprogramming other pancreatic cells. By "re-starting" expression of three critical beta cell genes in differentiated adult pancreatic exocrine cells, researchers were able to create beta cell-like cells that can secrete insulin. The reprogrammed

cells were similar to beta cells in appearance, size, and shape; expressed genes characteristic of beta cells; and were able to partially restore blood sugar regulation in mice whose own beta cells had been chemically destroyed. While not transdifferentiation by definition, this method for reprogramming adult cells may be used as a model for directly reprogramming other adult cell types.

In addition to reprogramming cells to become a specific cell type, it is now possible to reprogram adult somatic cells to become like embryonic stem cells (**induced pluripotent stem cells, iPSCs**) through the introduction of embryonic genes. Thus, a source of cells can be generated that are specific to the donor, thereby avoiding issues of histocompatibility, if such cells were to be used for tissue regeneration. However, like embryonic stem cells, determination of the methods by which iPSCs can be completely and reproducibly committed to appropriate cell lineages is still under investigation.

Similarities and Differences between Embryonic and Adult stem cells

Human embryonic and adult stem cells each have advantages and disadvantages regarding potential use for cell-based regenerative therapies. One major difference between adult and embryonic stem cells is their different abilities in the number and type of differentiated cell types they can become. Embryonic stem cells can become all cell types of the body because they are pluripotent. Adult stem cells are thought to be limited to differentiating into different cell types of their tissue of origin. Embryonic stem cells can be grown relatively easily in culture. Adult stem cells are rare in mature tissues, so isolating these cells from an adult tissue is challenging, and methods to expand their numbers in cell culture have not yet been worked out. This is an important distinction, as large numbers of cells are needed for stem cell replacement therapies. Scientists believe that tissues derived from embryonic and adult stem cells may differ in the likelihood of being rejected after transplantation. We don't yet know for certain whether tissues derived from embryonic stem cells would cause transplant rejection, since relatively few clinical trials have tested the safety of transplanted cells derived from hESCS. Adult stem cells, and tissues derived from them, are currently believed less likely to initiate rejection after transplantation. This is because a patient's own cells could be expanded in culture, coaxed into assuming a specific cell type (differentiation), and then reintroduced into the patient. The use of adult stem cells and tissues derived from the patient's own adult stem cells would mean that the cells are less likely to be rejected by the immune system. This represents a significant advantage,

as immune rejection can be circumvented only by continuous administration of immunosuppressive drugs, and the drugs themselves may cause deleterious side effects.

Induced pluripotent stem cells (iPSCs) are adult cells that have been genetically reprogrammed to an embryonic stem cell–like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells. Although these cells meet the defining criteria for pluripotent stem cells, it is not known if iPSCs and embryonic stem cells differ in clinically significant ways. Mouse iPSCs were first reported in 2006, and human iPSCs were first reported in late 2007. Mouse iPSCs demonstrate important characteristics of pluripotent stem cells, including expressing stem cell markers, forming tumors containing cells from all three germ layers, and being able to contribute too many different tissues when injected into mouse embryos at a very early stage in development. Human iPSCs also express stem cell markers and are capable of generating cells characteristic of all three germ layers.

Although additional research is needed, iPSCs are already useful tools for drug development and modeling of diseases, and scientists hope to use them in transplantation medicine. Viruses are currently used to introduce the reprogramming factors into adult cells, and this process must be carefully controlled and tested before the technique can lead to useful treatments for humans. In animal studies, the virus used to introduce the stem cell factors sometimes causes cancers. Researchers are currently investigating non-viral delivery strategies. In any case, this breakthrough discovery has created a powerful new way to "de-differentiate" cells whose developmental fates had been previously assumed to be determined. In addition, tissues derived from iPSCs will be a nearly identical match to the cell donor and thus probably avoid rejection by the immune system. The iPSC strategy creates pluripotent stem cells that, together with studies of other types of pluripotent stem cells, will help researchers learn how to reprogram cells to repair damaged tissues in the human body.

Potential uses of human stem cells and the obstacles

There are many ways in which human stem cells can be used in research and the clinic. Studies of human embryonic stem cells will yield information about the complex events that occur during human development. A primary goal of this work is to identify how undifferentiated stem cells become the differentiated cells that form the tissues and organs. Scientists know that turning genes on and off is central to this process. Some of the most serious medical conditions,

such as cancer and birth defects, are due to abnormal cell division and differentiation. A more complete understanding of the genetic and molecular controls of these processes may yield information about how such diseases arise and suggest new strategies for therapy. Predictably controlling cell proliferation and differentiation requires additional basic research on the molecular and genetic signals that regulate cell division and specialization. While recent developments with iPS cells suggest some of the specific factors that may be involved, techniques must be devised to introduce these factors safely into the cells and control the processes that are induced by these factors.

Human stem cells are currently being used to test new drugs. New medications are tested for safety on differentiated cells generated from human pluripotent cell lines. Other kinds of cell lines have a long history of being used in this way. Cancer cell lines, for example, are used to screen potential anti-tumor drugs. The availability of pluripotent stem cells would allow drug testing in a wider range of cell types. However, to screen drugs effectively, the conditions must be identical when comparing different drugs. Therefore, scientists will have to be able to precisely control the differentiation of stem cells into the specific cell type on which drugs will be tested. Current knowledge of the signals controlling differentiation falls short of being able to mimic these conditions precisely to generate pure populations of differentiated cells for each drug being tested. Perhaps the most important potential application of human stem cells is the generation of cells and tissues that could be used for cell-based therapies. Today, donated organs and tissues are often used to replace ailing or destroyed tissue, but the need for transplantable tissues and organs far outweighs the available supply. Stem cells, directed to differentiate into specific cell types, offer the possibility of a renewable source of replacement cells and tissues to treat diseases including Alzheimer’s disease, spinal cord injury, stroke, burns, heart disease, diabetes, osteoarthritis, and rheumatoid arthritis. For example, it may become possible to generate healthy heart muscle cells in the laboratory and then transplant those cells into patients with chronic heart disease. Preliminary research in mice and other animals indicates that bone marrow stromal cells, transplanted into a damaged heart, can have beneficial effects. Whether these cells can generate heart muscle cells or stimulate the growth of new blood vessels that repopulate the heart tissue, or help via some other mechanism is actively under investigation. For example, injected cells may repair by secreting growth factors, rather than actually incorporating into the heart. Promising results from animal studies have served as the basis for a small number

of exploratory studies in humans. Other recent studies in cell culture systems indicate that it may be possible to direct the differentiation of embryonic stem cells or adult bone marrow cells into heart muscle cells (Fig. 10).

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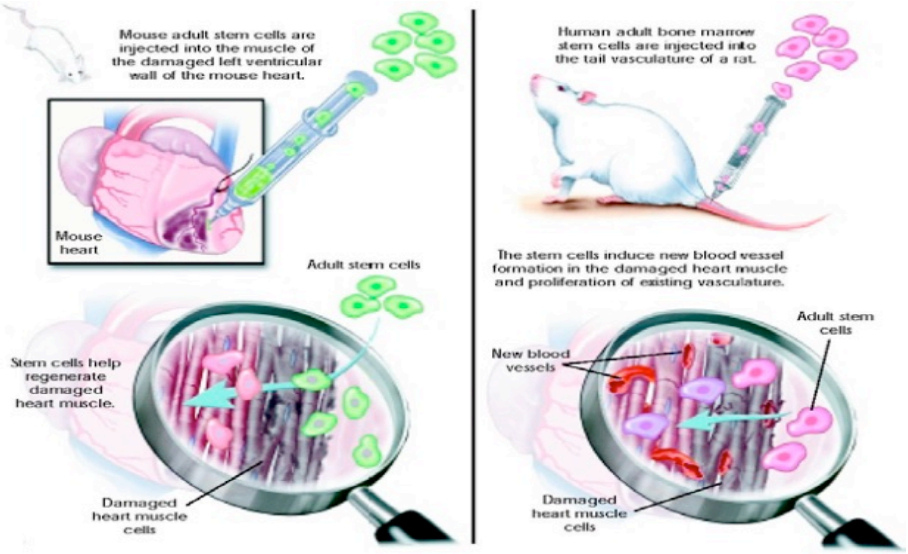

Figure 10: Strategies to repair heart muscle with adult stem cells

To realize the promise of novel cell-based therapies for such pervasive and debilitating diseases, scientists must be able to manipulate stem cells so that they possess the necessary characteristics for successful differentiation, transplantation, and engraftment. The following is a list of steps in successful cell-based treatments that scientists will have to learn to control to bring such treatments to the clinic. To be useful for transplant purposes, stem cells must be reproducibly made to:

- Proliferate extensively and generate sufficient quantities of cells for making tissue.
- Differentiate into the desired cell type(s).
- Survive in the recipient after transplant.
- Integrate into the surrounding tissue after transplant.
- Function appropriately for the duration of the recipient's life.
- Avoid harming the recipient in any way.
- Also, to avoid the problem of immune rejection, scientists are experimenting with different research strategies to generate tissues that will not be rejected.

To summarize, stem cells offer exciting promise for future therapies, but significant technical hurdles remain that will only be overcome through years of intensive research.

Genetic manipulation of animals (animal models in research)

A transgenic animal is an animal whose hereditary DNA has been augmented by addition of DNA from a source other than parental germplasm through recombinant DNA techniques. Transfer of genes or gene constructs allows for the manipulation of individual genes rather than entire genomes. There have been dramatic advances in gene transfer technology in the last two decades since the first successful transfer was carried out in mice in 1980 (Palmiter *et al*., 1982; Jaenisch, 1988). The technique has now become routine in the mouse and resulting transgenic mice are able to transmit their transgenes to their offspring thereby allowing a large number of transgenic animals to be produced. Successful production of transgenic livestock has been reported for fish, pigs, sheep, rabbits and cattle. The majority of gene transfer studies in livestock have, however, been carried out in the pig. Although transgenic cattle and sheep have been successfully produced, the procedure is still inefficient in these species (Nieman *et al*., 1994). Why are these animals being produced? How can man benefit from such modifications?

To know some of the common reasons:

For Medical Purpose:

Normal physiology and development: Transgenic animals can be specifically designed to allow the study of how genes are regulated, and how they affect the normal functions of the body and its development, e.g., study of complex factors involved in growth such as insulin-like growth factor. By introducing genes from other species that alter the formation of this factor and studying the biological effects that result, information is obtained about the biological role of the factor in the body.

Study of disease: Many transgenic animals are designed to increase our understanding of how genes contribute to the development of disease. These are specially made to serve as models for human diseases so that investigation of new treatments for diseases is made possible. Today transgenic models exist for many human diseases such as cancer, cystic fibrosis, rheumatoid arthritis and Alzheimer's.

Biological products: Medicines required to treat certain human diseases can contain biological products, but such products are often expensive to make. Transgenic animals that produce useful biological products can be created by the introduction of the portion of DNA (or genes) which codes for a particular product such as human protein (**α**-1-antitrypsin) used to treat emphysema.

Similar attempts are being made for treatment of phenylketonuria (PKU) and cystic fibrosis. In 1997, the first transgenic cow, Rosie, produced human protein-enriched milk (2.4 grams per litre). The milk contained the human alpha-lactalbumin and was nutritionally a more balanced product for human babies than natural cow-milk.

Vaccine safety: Transgenic mice are being developed for use in testing the safety of vaccines before they are used on humans. Transgenic mice are being used to test the safety of the polio vaccine. If successful and found to be reliable, they could replace the use of monkeys to test the safety of batches of the vaccine.

Chemical safety testing: This is known as toxicity/safety testing. The procedure is the same as that used for testing toxicity of drugs. Transgenic animals are made that carry genes which make them more sensitive to toxic substances than non-transgenic animals. They are then exposed to the toxic substances and the effects studied. Toxicity testing in such animals will allow us to obtain results in less time.

Animal Breeding and Transgenic Animals

Transgenesis offers considerable opportunity for advances agriculture. In livestock, the ability to insert new genes for such economically important characteristics as fecundity, resistance to or tolerance of other environmental stresses would represent a major breakthrough in the breeding of commercially superior stock. Another opportunity that transgenic technology could provide is in the production of clotting factors in the milk of domestic livestock. The genes coding for these proteins have been identified and the human factor IX construct has been successfully introduced into sheep and expression achieved in sheep milk (Clark *et al*., 1990). Moreover, the founder animal has been shown to be able to transmit the trait to its offspring (Nieman *et al*., 1994). To date, the majority of genes transferred into sheep have been growth hormone encoding gene constructs. Unfortunately, in most cases the elevated growth hormone levels have resulted into a clinical diabetes situation leading to an early death of the transgenic sheep (Rexroad *et al*., 1990). The first reports of the production of transgenic animals created a lot of excitement among biological scientists. In the field of animal breeding, there were diverse opinions on how the technology might affect livestock genetic improvement programmes. Some (Ward *et al*., 1982) believed that it would result in total re-organization of conventional animal breeding theory while others (Schuman and Shoffner, 1982) considered the technology as an extension of current

animal breeding procedures which, by broadening the gene pool, would make new and novel genotypes available for selection. Application of the technology in animal improvement is still far from being achieved. However, consideration needs to be given to its potential role in this field. Smith *et al* (1987) presented a comprehensive evaluation of strategies for developing, testing, breeding and disseminating transgenic livestock in the context of quantitative improvement of economic traits. An important contribution of transgenic technology is in the area of basic research to study the role of genes in the control of physiological processes. The understanding of the molecular control of life processes has important implications for both medicine and agriculture. For example, the generation (through mutation of an endogenous gene) of an organism which lacks a specific gene is a powerful tool to investigate the function of the gene product. This type of genetic analysis has been facilitated by the availability of *in vitro* cultures of embryonic stem cells from mice (Bradley, 1994).

Recent advances in *in vitro* technology (*in vitro* fertilization and maturation) will increase the number of zygotes available for gene transfer purposes. This, plus the utilization of embryonic stem cell (Stice *et al*., 1994) and primodial germ cell technologies should enhance the efficiency of gene transfer in cattle and sheep considerably.

Methods of genetic manipulation in animals: A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. Two methods of producing transgenic animals are widely used: (1) transforming embryonic stem cells (ES cells) growing in tissue culture with the desired DNA and (2) injecting the desired gene into the pronucleus of a fertilized egg. (3) Desirab Retrovirus-mediated transgenesis. (4) Pronuclear microinjection (Fig. 11). (5) Sperm-mediated transfer.

Genes from one species are transferred to other animals or species to improve the productivity of livestock. Faster growth rates, leaner growth patterns, more resistance to disease, increased milk production, more efficient metabolism, and transferring antimicrobial genes to farm animals are some of the goals of transgenic animal researchers. These include laboratory culture of large numbers of viable embryos for nonsurgical transfer to surrogate mothers, development of methods for sexing sperm and embryos, cloning embryos by nuclear transplantation and gene transfer to create livestock with superior performance traits. In all cases material progress will

depend upon a deeper understanding of the underlying physiological and developmental control mechanisms and public confidence that due regard is being paid to animal welfare, and to social and environmental implications. Genetic improvement of livestock depends on access to genetic variation and effective methods for exploiting this variation. Genetic diversity constitutes a buffer against changes in the environment and is a key in selection and breeding for adaptability and production on a range of environments.

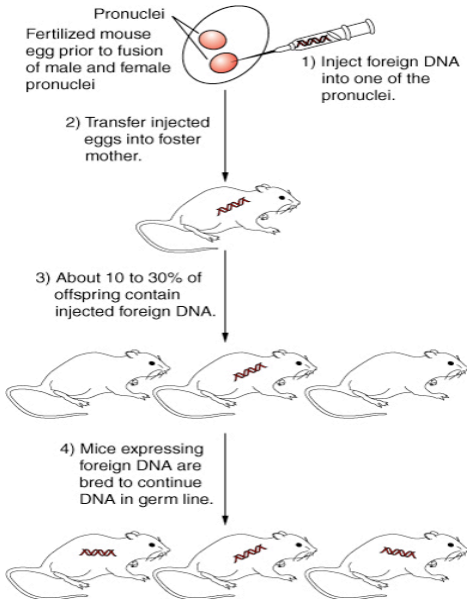

Figure 11: Pronuclear microinjection

In developed countries, breeding programmes are based upon performance recording and this has led to substantial improvements in animal production. Developing countries have distinct disadvantages for setting up successful breeding programmes: infrastructure needed for performance testing is normally lacking because herd sizes are normally small and variability between farms, farming systems and seasons are large; reproductive efficiency is low, due mainly to poor nutrition, especially in cattle; and communal grazing precludes implementation of systematic breeding and animal health programmes.

A brief history of cloning

1938 – First idea of cloning: Hans Spemann proposes a "fantastic experiment" to replace the nucleus of an egg cell with the nucleus of another cell and to grow an embryo from such an egg;

1952 – An attempt to clone a *Rana pipiens* frog: Robert Briggs and Thomas King; the scientists collect the nucleus from a frog egg cell with a pipette and replace it with the nucleus taken from a cell of a frog embryo; the experiment is not successful;

1970 – *Xenopus laevis* frog: John B. Gurdon is successful: he clones a frog, but its development only reaches the stage of tadpole. Despite attempts, he never manages to obtain an adult specimen. For many years, his achievement is questioned, especially in light of unsuccessful attempts to clone mammals;

1981 – Karl Illmenese and Peter Hope clone a mouse: They take the nucleus not from an adult specimen, but from a mouse embryo;

1994 – Neal First tries to clone a sheep: He takes the nucleus from an embryonic cell. He obtains a sheep embryo that develops 120 cells;

1995 – Two sheep are cloned (Moran and Megan): These had been the first animals cloned from differentiated cells obtained by means of a pioneering method of nuclei transfer. However, the cells from which the nucleus was taken did not come directly from another living animal, but from a cell culture. The ones who achieved that were Ian Wilmut and Keith Campbell;

1996 – The first mammal cloned from a cell taken from an adult animal – Dolly the sheep. Creators: Ian Wilmut and Keith Campbell;

1998 – The first cloned mouse (it was called Cumulina);

2000 – The first cloned rhesus monkey;

2000 – The first cloned pig (or even five pigs);

2001 – A buffalo and a cow cloned;

2001 – A cat cloned (it was called Copy Cat);

2002 – Konrad Hochedlinger and Rudolf Jaenisch clone **mice from T lymphocytes**;

2003 – A rabbit is cloned in France and Southern Korea;

2003 – A mule is cloned. It was achieved by the companies Idaho Gem and Utah Pioneer;

2003 – A deer (Dewey), a horse (Prometea) and a rat (Ralph) cloned;

2004 – Fruit flies cloned;

2005 – An Afghan hound (Snuppy) cloned;

2007 – A wolf cloned; South Korean scientists obtained two female wolves (Snuwolf and Snuwolffy);

2008 – A Labrador dog cloned;

2009 – The first animal from an extinct species cloned: Pyrenean ibex. The animal lived seven minutes. It died of lung malformations;

2009 – A camel female cloned (Injaz); Injaz was created from ovarian cells of a female killed for meat in 2005.

2009 - Samrupa, the world's first Murrah buffalo calf cloned using a simple "Hand guided cloning technique" was born in February, 2009 at National Dairy Research Institute (NDRI), Karnal, India, but died due to a lung infection five days after she was born.

2009– Garima-I, a buffalo calf cloned using an "Advanced Hand guided Cloning Technique" was born in June, 2009 at the NDRI. Two years later in 2011, she died of a heart failure.

2009 - Garima-II, another cloned calf was born in August, 2010. **A cloned male buffalo** calf Shresth was born in August, 2010 at the NRDI.

2010 - Got, the first Spanish Fighting Bull was cloned by Spanish scientists.

2013 - This buffalo was inseminated with frozen-thawed semen of a progeny tested bull and gave birth to a female calf, Mahima in January, 2013.

Cloning – the procedure for obtaining organisms with the same genetic information. You need to collect an egg cell from a donor (a female sheep, mouse or cat). Then you need to carefully remove the nucleus from the cell and collect another cell from the skin, udder or other tissue from another male or female donor of the same species (i.e. from another sheep, mouse or cat). From this cell, you also need to remove the cell nucleus and place it in the empty egg. The egg obtained in such a way needs to be treated with a gentle electric shock. The egg should begin to divide and grow into a multicellular embryo. At this stage, the embryo needs to be implanted into the uterus of a surrogate mother. If the pregnancy develops and the animal is born (Fig. 12).

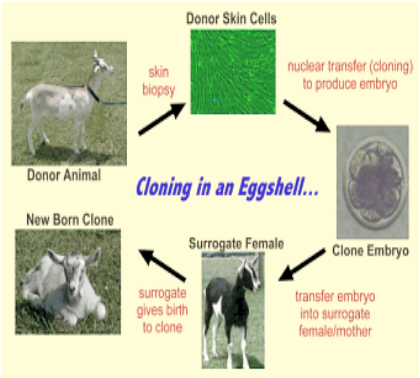

Figure 12: Steps in cloning simplified

Creating a clone of your favorite animal seems like a great way to insure your pet will be with

you forever. Although this might be a goal of cloning, it is not the primary focus of biotech specialists. Commercialization of cloning allows desirable traits to be reliably propagated. Animal breeders are able to clone animals with superior traits such as cows with high milk production or champion racehorses. Embryo twinning (splitting embryos in half) was the first method of cloning used to produce identical twin cattle. Since the twins are the result of mixing the genetic material from two parents, the exact genetic make-up of the animal is not known until it has matured. Dolly (the very famous sheep that was the first mammal ever cloned in the lab), however, was created from a single cell, not an embryo. DNA from a donor cell is inserted into an egg that has had its own DNA removed. It is a very delicate and difficult process. So far, animals successfully cloned include sheep, goats, pigs, cattle, cats, deer and dogs.

One can imagine future uses of cloning that could include using preserved DNA to help maintain endangered species or even recover extinct species!

Limits to Cloning: The donor cell must come from a living organism: an organism is also shaped by its environment, success rate for cloning is very low and clones may be old before their time.

The future of cloning: preservation of endangered animals, studying the effect of drugs etc on duplicates, improve agricultural production (Fig. 13)

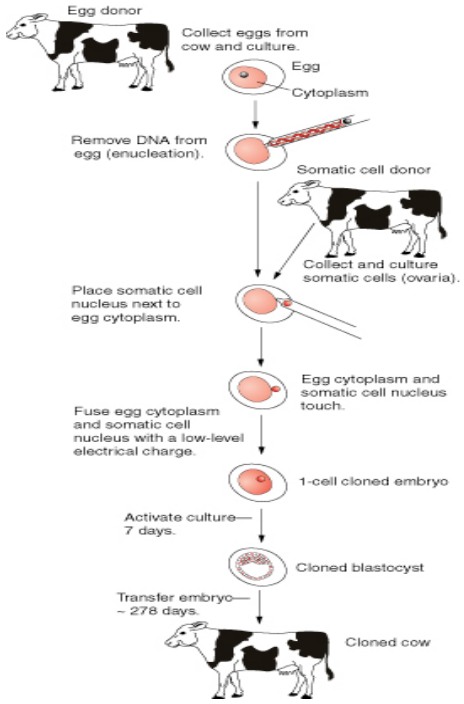

Figure 13: Cloning for improved agricultural production

Applications of transgenic animals.

Use of Animals in Research

- Animals play a vital role in primary research. The use of animal models permits more rapid assessment of the effects of new medical treatments and other products. Computer models and *in vitro* studies of cell cultures are often used as supplements to animal research, but they can't entirely duplicate the results in living organisms. Recent developments in animal biotechnology have changed medicine, agriculture, and the efforts to preserve endangered animals.
- For a new product to be approved for human use, the manufacturer must first demonstrate that it is safe for use. Trials are required on cell cultures, in live animals, and on human subjects. Testing on live animal models requires that two or more species be used because different effects are observed in different animals. If problems are detected in the animal tests, human subjects are never recruited for trials. The animals used most often are pure-bred mice and rats, but other species are also used. Another extremely valuable research animal is the zebrafish, a hardy aquarium fish. Dogs are used for the study of cancer, heart disease and lung disorders. HIV and AIDS research is conducted on monkeys and chimpanzees.
- Animal research is very heavily regulated. The Animal Welfare Act sets standards concerning the housing, feeding, cleanliness and medical care of research animals. Veterinarians also conduct research which has led to new cancer treatments for pets and studies in their adaptations for humans.
- Animal Models
- Mice
- Rats
- Zebrafish (3 month generation time, 200 progeny, complete embryogenesis in 120 hrs)
- Dogs (lungs and cardiovascular system)
- Cats
- Pigs (PPL Therapeutics- delete a gene which causes hyperacute rejection of pig-to-human organ transplantation)
- Primates (HIV and AIDs research, geriatric research)
- Bioengineering Mosquitoes to Prevent Malaria

- Cloned in a gene that prevents the parasite from traversing the midgut; blocking the continuation of its life cycle
- Developed an antibody that prevents the parasite from entering the mosquito's salivary gland

Pharming: not just a misspelled word! The term "pharming" comes from a combination of the words "farming" and "pharmaceuticals" - a blending of the basic methods of agriculture with advanced biotechnology.

Gene pharming is a technology that scientists use to alter an animal's own DNA, or to splice in new DNA, called a transgene, from another species. In pharming, these genetically modified (transgenic) animals are mostly used to make human proteins that have medicinal value. The protein encoded by the transgene is secreted into the animal's milk, eggs or blood, and then collected and purified (Fig. 14). One interesting GMO organism that has been in the news lately is the "glowing fish." GloFish ™ fluorescent zebra fish were specially bred to help detect environmental pollutants.

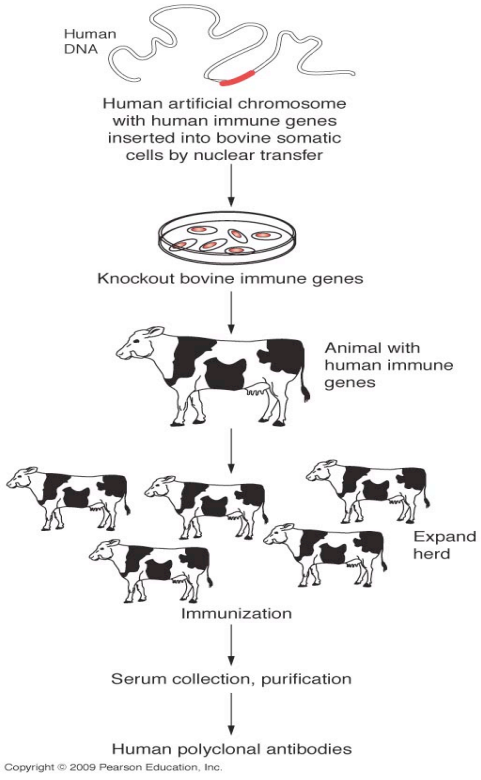

Figure 14: Pharming- Animal used to produce human polyclonal antibodies

Improving Agricultural Products with Transgenics

- Faster growth rates or leaner growth patterns (improve the product), more product
- Increase nutritional content-lactoferrin
- Turning the animals into efficient grazers
- Transfer antimicrobial genes to farm animals

Transgenic Animals as Bioreactors

-Biosteel otherwise known as spider silk, cloned into goat milk ("silkmilk" goats) (Fig. 15).
-Goats reproduce faster than cows and are cheaper than cows.
-Hens also make good bioreactors in that they are cheap and a lot of eggs are produced at one time.

Figure 15: Goats act as bioreactors

Genetically Engineered Insulin: Management of adult-onset diabetes is possible by taking insulin at regular time intervals. What would a diabetic patient do if enough human-insulin was not available? If you discuss this, you would soon realise that one would have to isolate and use insulin from other animals. Would the insulin isolated from other animals be just as effective as that secreted by the human body itself and would it not elicit an immune response in the human body? Now, imagine if bacterium were available that could make human insulin. Suddenly the whole process becomes so simple. You can easily grow a large quantity of the bacteria and make as much insulin as you need. Think about whether insulin can be orally administered to diabetic people or not. Why? Insulin used for diabetes was earlier extracted from pancreas of slaughtered cattle and pigs. Insulin from an animal source though caused some patients to develop allergy or other types of reactions to the foreign protein. Insulin consists of two short polypeptide chains: chain A and chain B, which are linked together by disulphide bridges (Fig. 16). In mammals, including humans, insulin is synthesised as a pro-hormone (like a pro-enzyme, the pro-hormone

also needs to be processed before it becomes a fully mature and functional hormone) which contains an extra stretch called the C peptide. This C peptide is not present in the mature insulin and is removed during maturation into insulin.The main challenge for production of insulin using rDNA techniques was getting insulin assembled into a mature form. In 1983, Eli Lilly an American company prepared two DNA sequences corresponding to A and B, chains of human insulin and introduced them in plasmids of E. coli to produce insulin chains. Chains A and B were produced separately, extracted and combined by creating disulfide bonds to form human insulin.

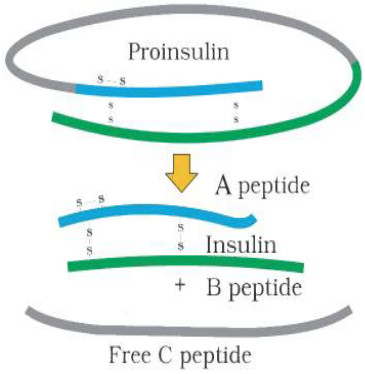

Figure 16: Genetically engineered insulin

Transgenics and cloning (Translational Significance): The reality its very expensive technology. Technologies still need to be refined large numbers of repetitions required to produce viable offspring in animals. Applications currently very limited predominantly used for biomedical purposes.

Gene Therapy:

If a person is born with a hereditary disease, can a corrective therapy be taken for such a disease? Gene therapy is an attempt to do this. Gene therapy is a collection of methods that allows correction of a gene defect that has been diagnosed in a child/embryo. Here genes are inserted into a person's cells and tissues to treat a disease. Correction of a genetic defect involves delivery of a normal gene into the individual or embryo to take over the function of and compensate for the non-functional gene. In simple, Genes are inserted into cells and tissues of an individual to correct certain hereditary diseases.It involves the delivery of a normal gene into the individual or embryo to replace the defective mutant allele of the gene.Viruses which attack the host and introduce their genetic material into host are all used as vectors.The first clinical gene

therapy was given in 1990 to a four year old girl with adenosine deaminase (ADA) deficiency. ADA deficiency can be cured by bone marrow transplantation in some children but it is not completely curative. For gene therapy, lymphocytes were grown in a cultural and functional ADA. cDNA is then introduced into these lymphocytes. These lymphocytes are then transferred into the body of the patient; the patient requires periodically infusion of such genetically engineered lymphocytes. If a functional gene is introduced into the bone marrow cells at early embryonic stage, it would be permanent cure.

Monoclonal Antibodies: Production of Monoclonal antibodies (Mabs) (Fig. 17). Used to treat cancer, heart disease, and transplant rejection. HUMANIZED monoclonal antibodies were developed to prevent the human anti-mouse antibody (HAMA) response

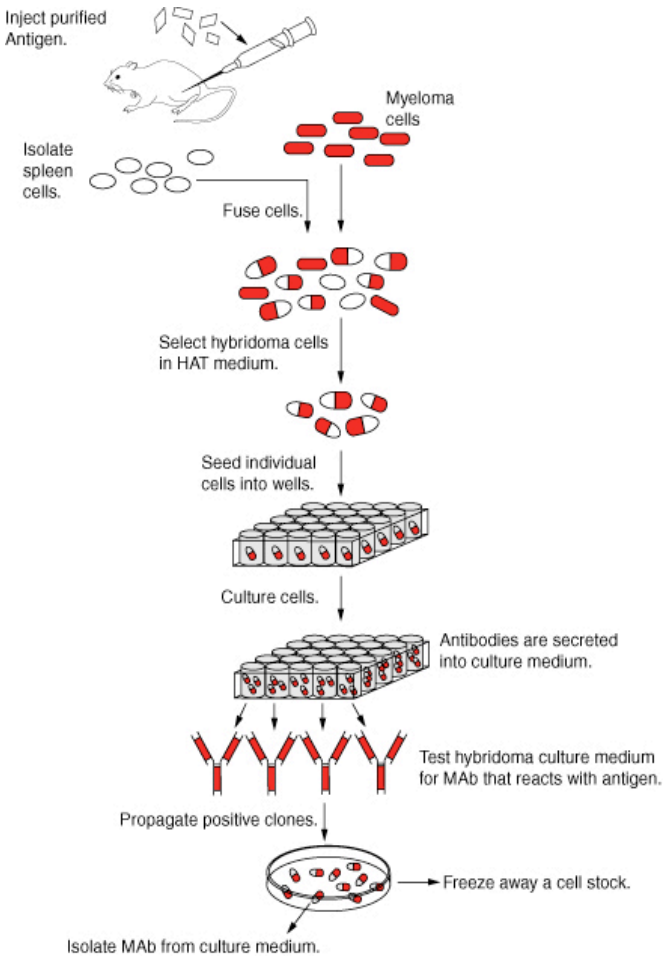

Figure 17: Production of monoclonal antiboides

Economic impact in developing countries: The developing world is grossly unprepared for the new technological and economic opportunities, challenges and risks that lie on the horizon. Although it is hoped that biotechnology will improve the life of every person in the world and allow more sustainable living, crucial decisions may be dictated by commercial considerations and the socioeconomic goals that society considers to be the most important. The use of biotechnology will lead to a distinct shift in the economic returns from livestock. Though the role of livestock in ensuring nutritional security is recognized in mixed crop-livestock systems, the importance of livestock goes beyond direct food production. Livestock supply draught power and organic manure to the crop sector, and hides, skins, bones, blood and fiber are used in many industries. Thus, livestock are an important source of income and employment, helping to alleviate poverty and smooth the income distribution among small landholders and the landless, who constitute the bulk of the rural population and the majority of livestock owners. In addition, livestock can easily be converted into cash and thus act as a cushion against crop failure, particularly in less favored environments. By enabling crop residues and by-products to be used as fodder, livestock production contributes positively to the environment. In developed countries livestock accounts for more than half of agricultural production, while in developing countries the share is about one-third. This latter share, however, is rising quickly because of rapid increases in livestock production resulting from population growth, urbanization, changes in lifestyles and dietary habits and increasing disposable incomes.

In most developing countries, biotechnological applications relating to livestock need to be suitable for animal owners who are resource-poor small-scale operators who own little or no land and few animals. Using technology to support livestock production is an integral part of viable agriculture in multi-enterprise systems. Livestock are part of a fragile ecosystem and a rich source of animal biodiversity, as local species and breeds possess genes and traits of excellence. Molecular markers are increasingly being used to identify and select the particular genes that lead to these desirable traits and it is now possible to select superior germ plasm and disseminate it using artificial insemination, embryo transfer and other assisted reproductive technologies. These technologies have been used in the genetic improvement of livestock, particularly in cattle and buffaloes, and the economic returns are significant. However, morbidity and mortality among animals produced using assisted reproductive technologies lead to high economic losses,

so the principal application of animal biotechnology at present is in the production of cheap and dependable diagnostic kits and vaccines. Several obstacles limit the application of biotechnology at present: there is a lack of infrastructure and insufficient manpower, so funding is needed if resource-poor farmers are to benefit from biotechnology.

Ethical Issues; (biosafety and regulatory issues):

Biosafety and Ethics

All procedures involved in the collection of human material for culture must be passed by the relevant hospital ethics committee. A form will be required for the patient to sign authorizing research use of the tissue, and preferably disclaiming any ownership of any materials derived from the tissue [Freshney, 2002, 2005]. The form should have a brief layman's description of the objectives of the work and the name of the lead scientist on the project. The donor should be provided with a copy. All human material should be regarded as potentially infected and treated with caution. Samples should be transported securely in double-wrapped waterproof containers; they and derived cultures should be handled in a Class II biosafety cabinet and all discarded material autoclaved, incinerated, or chemically disinfected. Each laboratory will have its own biosafety regulations that should be adhered to, and anyone in any doubt about handling procedures should contact the local safety committee (and if there is not one, create it!). Rules and regulations vary among institutions and countries, so it is difficult to generalize, but a good review can be obtained in Caputo [1996]. Genetic modification of organism can have unpredictable/ undesirable effects when such organisms are introduced into the ecosystem. The modification and use of such organism for public service has also resulted in problems with the granting of patents. Hence, the Indian Government has set up organizations which are authorized to make decisions regarding the validity of genetic modification and the safety of introducing genetically modified organisms fro public services. One such organization is the Genetic Engineering approval committee (GEAC).

Biosafety Levels

The regulations and recommendations for biosafety in the United States are contained in the document Biosafety in Microbiological and Biomedical Laboratories, prepared by the Centers for Disease Control (CDC) and the National Institutes of Health (NIH), and published by the

U.S. Department of Health and Human Services. The document defines four ascending levels of containment, referred to as biosafety levels 1 through 4, and describes the microbiological practices, safety equipment, and facility safeguards for the corresponding level of risk associated with handling a particular agent.

Biosafety Level 1 (BSL-1)

BSL-1 is the basic level of protection common to most research and clinical laboratories, and is appropriate for agents that are not known to cause disease in normal, healthy humans.

Biosafety Level 2 (BSL-2)

BSL-2 is appropriate for moderate-risk agents known to cause human disease of varying severity by ingestion or through percutaneous or mucous membrane exposure. Most cell culture labs should be at least BSL-2, but the exact requirements depend upon the cell line used and the type of work conducted.

Biosafety Level 3 (BSL-3)

BSL-3 is appropriate for indigenous or exotic agents with a known potential for aerosol transmission, and for agents that may cause serious and potentially lethal infections.

Biosafety Level 4 (BSL-4)

BSL-4 is appropriate for exotic agents that pose a high individual risk of life-threatening disease by infectious aerosols and for which no treatment is available. These agents are restricted to high containment laboratories. For more information about the biosafety level guidelines, refer to Biosafety in Microbiological and Biomedical Laboratories, 5th Edition, which is available for downloading at www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm.

Biopiracy: The industrialized/ developed nations are rich financially, bur poor in biodiversity and traditional knowledge, while the developing and underdeveloped countries are rich in bioresources and traditional knowledge. Some such developed countries use the bio resources and traditional knowledge of other countries without proper authorization and/ or compensation to the countries concerned (Biopiracy). Eg: Basmati rice grown in India is distinct for its unique flavor and aroma, but an American company got patent rights on Basmati through the US patent and trademark office; the new variety of Basmati has been developed by this company by crossing an Indian variety with semi-dwarf varieties. Now some nations are developing laws to prevent such unauthorized exploitation of their bioresources and traditional knowledge

Ethical issues associated with transgenics and cloning.

Technology isn't perfected yet with very low success rate, the animal developed has high mortality rates.

Safety/risk of consumption

According to the U.S. Food and Drug Administration cloned animals probably safe to raise and eat. Transgenic ones may not be safe to consume.

Animal welfare

Birth weights, longer gestation, difficult births in clones. Poor survival rate of fetuses using some techniques. Anatomical, physiological, behavioral abnormalities

Suffering of transgenic animals

Case of Beltsville pigs (human GH introduced): High mortality, arthritis, gastric ulcers, degenerative joint disease, infection, lethargy. Cloned animals found to have shortened life spans with health problems.

Implications for application of technologies to humans

Moral concerns: "are we playing God?" Impact on ecosystems and genetic diversity –What if GE organisms escape reproduce? What might be the impact of limited gene pools on livestock faced with new (deadly) pathogens? Potential for GE animals to move into areas previously unused for agriculture disrupt fragile ecosystems habitat preservation issues for wild animals.Lack of controls to prevent GE animals from entering the food chain (e.g., cows that produce drugs in their milk)

Animal biotechnology and law

•"Any food system practice that does not allow individuals who do not want to consume meat or milk from clones to act upon their values at a reasonable cost is ethically unacceptable and ought to be illegal." (Thompson, 1997)

Conclusion:

Biotechnology has given to humans several useful products by using microbes, plant, animals and their metabolic machinery. Animal/Plant tissues can be dissociated into their component cells, from which individual cell types can be purified and used for biochemical analysis or for the establishment of cell cultures. Many animal and plant cells survive and proliferate in a

culture dish if they are provided with a suitable medium containing nutrients and specific protein growth factors. Although many animal cells stop dividing after a finite number of cell divisions, cells that have been immortalized through spontaneous mutations or genetic manipulation can be maintained indefinitely in cell lines. Clones can be derived from a single ancestor cell, or by fusing two cells to produce heterocaryons with two nuclei, enabling interactions between the components of the original two cells to be examined. Heterocaryons eventually form hybrid cells with a single fused nucleus. One type of hybrid cell, called a hybridoma, is widely employed to produce unlimited quantities of uniform monoclonal antibodies, which are widely used to detect and purify cellular proteins.

DNA technology has made it possible to engineer microbes, plants and animals such that they have novel capabilities. Genetically Modified Organisms have been created by using methods other than natural methods to transfer one or more genes from one organism to another, generally using techniques such as recombinant DNA

technology. Since the recombinant therapeutics are identical to human proteins, they do not induce unwanted immunological responses and are free from risk of infection as was observed in case of similar products isolated from non-human sources. Transgenic animals are also used to understand how genes contribute to the development of a disease by serving as models for human diseases, such as cancer, cystic fibrosis, rheumatoid arthritis and Alzheimer's. Gene therapy is the insertion of genes into an individual's cells and tissues to treat diseases especially hereditary diseases. Viruses that attack their hosts and introduce their genetic material into the host cell as part of their replication cycle are used as vectors to transfer healthy genes or more recently portions of genes.

Although animal production is being changed significantly by advances made in thousands of biotechnology laboratories around the world, benefits are reaching the developing world in only a few areas of conservation, animal improvement, healthcare and the augmentation of feed resources.

Adopting biotechnology has resulted in distinct benefits in terms of animal improvement and economic returns.

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