Chapter 1

General Introduction
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Development and differentiation

The development of multicellular organism begins with single cell 'the fertilized egg' or zygote, which divides mitotically to produce all the cells of the body. During the earliest phase of development of mouse embryo, cells maintain identical and completely undifferentiated characteristics. Cleavage occurs immediately after fertilization. Cleavage is a series of rapid mitotic divisions wherein zygotic cytoplasm is divided into numerous smaller cells. These cells are called blastomeres, which are grouped to a sphere shaped structure known as BLASTULA. After the rate of mitotic division has slowed down, the blastomeres undergo dramatic movements wherein they change their position relative to one another. This series of extensive rearrangement of cell is called GASTRULATION. As a result of gastrulation a typical embryo contains three cell regions, called germ layers. The outer layer - The ectoderm- produces the cells of epidermis and nervous system, the inner layer - the endoderm- produces the lining of digestive tube and its associated organs (pancreas, liver, etc.), and the middle layer - the mesoderm- gives rise to several organs (Heart, kidney, gonads), connective tissues (Bone, muscle, tendons), and the blood cells. Once the three germ layers are established, the cells interact and rearrange themselves to produce the organs. Many organs contain cells from more than one germ layer.

Thus, a single cell, the fertililized egg, gives rise to all cell types. The generation of cellular diversity is called differentiation. Differentiation is the result of complex interplay of proliferation, cell-cell communication and inductive events. Differentiation involves changes in numerous aspects of cell physiology; size, shape, polarity, metabolic activity and responsiveness to signals. The molecular and biochemical changes that cells undergo
during the commitment to new phenotypes, has long fascinated developmental biologists. It is widely held that stable changes in the cell are accompanied by the changes in gene expression at the cellular states during development.

Requirement of a model system

The complexities involved in the study of development and differentiation of higher eukaryotes, especially mammals, have led to the use of tissue culture system (Martin, 1974). Studying differentiation in embryos in vivo provides a complex picture in which, deciphering the mechanism involved in defining a specific lineage is a difficult task. The tissue culture system provides a tool to analyze a particular differentiation process more specifically and analytically. A model system, in which developmental events can be studied, is provided by embryonal carcinoma cells (EC). Culture of EC cells, the stem cells of teratocarcinomas has allowed the analysis of early events resulting in commitment to differentiation. Often these events are similar to those, which occur in a small number of cells in the early mammalian embryos. The EC cell culture system is unique in a variety of lineages.

Embryonic stem (ES) cells, which are derived from embryos have normal karyotype, differentiate independent of chemicals, and can be used for generating knockout and transgenic mice. However growth of undifferentiated ES cells depends on the presence of a feeder layer and Leukemia Inhibitory Factor (LIF), making it less attractive for bulk biochemical approaches or well-defined culture conditions compared to EC cells.

Origin of Embryonal Carcinoma cells

Teratomas are tumors composed of various tissues foreign to their site of origin. Benign teratomas simply known as teratomas are composed of various types of differentiated somatic tissues, which have rather limited capacity for growth. In contrast malignant teratomas or teratocarcinomas, contain undifferentiated malignant stem cells which have
an unlimited proliferative ability and a tendency to metastasize (Stevens, L.C, 1967, Damjanov and Solter 1974, Martin 1980). Benign teratomas can be easily generated in many animal species by simply transplanting embryos or part of embryos in extra uterine site (Skreb, et.al., 1971), while teratocarcinomas can be produced efficiently only in mice. Permanent EC cell lines can be established from these teratocarcinomas (Stevens. 1970, Solter, et. al., 1970, Damjanov 1978).

Isolation of cell lines from teratocarcinoma tumours
Teratocarcinoma cells can be obtained in culture either from solid tumors or from embryoid bodies found in the ascites formed after injection (intra-peritonial) of tumor fragments. Two types of embryoid bodies can be distinguished: (a) Cystic form composed of a single layer of a parietal yolk sac like cells arranged around a fluid filled cavity and (b) a solid form composed of a layer of endodermal cells surrounding a core of EC cells. When cultured, both fragments of solid tumours containing EC cells and embryoid bodies grow and differentiate into a variety of cell types. From such cultures, cell lines can be isolated and established in vitro. A series of differentiated cell lines, such as myocard (PCD1), Myoblast (PCD2) and neuroepithelial cell lines, have been isolated and established in culture. Similar to other cell types established in culture from mice, most of these differentiated lines are aneuploid. Although obtained from teratocarcinomas, they are generally not tumorigenic and behave like similar cells isolated directly from embryonic or adult mice. For instance, the teratoma derived myoblast line fuses and produces myotubes in culture under conditions similar to those used with either primary cultures of mouse myoblasts or a cell line derived from rat embryo. A number of EC cell lines have also been established in vitro, all of which give rise to tumors upon injection into syngenic mice. Two classes have been distinguished according to the cell types present in the tumors. Nullipotential cell lines are supposed to give rise to tumors.
containing almost exclusively EC cells. This is the case for F9, which has been extensively
used for immunological studies. Both in vivo and in culture it produces undifferentiated
EC cells. These are accompanied sometimes by a few primitive endoderm like cells.
Similar lines have been isolated either from testicular or experimental teratocarcinomas or
from ovarian teratocarcinomas. In contrast multipotential EC cells like PCC3, PCC4,
P19, give rise to tumors containing a large variety of differentiated cell types. This has
been repeatedly and unambiguously shown both by injection in vivo of a single cell and by
in vitro differentiation of single cell (Ilmensee and Mintz 1976, Kleinsmith and Pierce
1964). A single multipotential EC cell can produce derivatives of all the three germ layers.

**PCC4 cells**

PCC4 is a multipotential EC cell line, which was isolated from the transplantable
teratocarcinoma OTT 6050, generated by Dr Stevens from a graft of a 6 day 129 embryo
into the testis of a 129 mouse (Strain 129 is known for the high incidence of spontaneous
testicular teratomas).

**Properties of EC (PCC4) cells**

PCC4 cells exhibit three important properties (1) they are malignant (2) they can
differentiate into derivatives of all 3 germ layers, can even be redirected and participate in
the production of normal mice when injected into a blastocyst (3) their derivatives are
generally nonmalignant.

**EC cells and differentiation**

Different EC cell lines differ in their predisposition to spontaneous differentiation. Using
different combinations of EC cell lines and chemical inducers, one can produce different
cell types. Thus exploiting different EC cell lines and chemical inducers, we can address
different types of biological events.
In vivo differentiation of EC cells consists of at least two separable events. The first is the induction of differentiation per se, that is irreversible withdrawal from continuous proliferation and loss of tumorigenicity. The second event or series of events are those, in which EC cells differentiate while forming a tumour. Differentiation in vitro however has been proven much more amenable to experimental analysis because of the inherent benefits and control afforded by tissue culture. Differentiation can be induced in vitro by various physical manipulations of culture conditions as well as by the addition of chemicals to the media. Rate of cell differentiation appears to be increased when EC cells are maintained at very high density (Nicolas et al., 1975). Culture feeder dependent-EC cells differentiate when feeder cells are removed. Some feeder dependent cells also differentiate when plated at low density in sufficiently rich medium, which allows colony formation. The spontaneous differentiation can also occur, but it is asynchronous and the resulting cells are highly heterogeneous. The problem of asynchrony and to some extent the heterogeneity can be solved with the use of certain chemicals to induce differentiation. The drugs are used at concentrations, which are not toxic so that only the effect of induced differentiation is observed and not that of the preexisting differentiated cells.

Several inducers of differentiation have been used for inducing differentiation in EC cells and to study the molecular mechanism behind it. Retinoids are most common inducers - the most potent is all trans retinoic acid - of differentiation (Strickland et al. 1978). Bipolar compounds such as Dimethyl Sulfoxide (DMSO) and hexamethylene-bisacetamide (HMBA) also induce differentiation of EC cells.

**Heat induced differentiation**

The concept of stress-induced differentiation was introduced first by Richards et al (1988). They showed that a heat shock at 42.5°-43.5° C for 1 hour applied to HL60 promyelotic leukemia cells induced differentiation in 34% cells, which showed the characteristics of
mature metamyelocytes. The treatment of HL60 cells with a variety of agents, which are known to induce stress, thermostolerance or heat shock like response, also induce granulocyte like differentiation. Treatment with ethanol, sodium arsenite, cadmium sulfate, lidocaine, and procaine induced differentiation of 73, 54, 14, 54 and 55% of HL60 cells respectively. All the treatments caused variation in the levels of heat shock proteins, showing that changes in heat shock protein synthesis may be an important element during the induction of differentiation. Later in 1994 it has been demonstrated that embryonal carcinoma cells PCC4 also differentiate upon heat shock accompanied with the changes in heat shock proteins profile (Bisht et al. 1994). Tatsuya et al (1996) have also shown that human embryonal carcinoma cells differentiate into trophectoderm lineage upon heat shock, similar to retinoic acid treatment. HSP 90, HCG, and cytokeratin expression profile is similar in the case of both RA-induced as well as heat-induced differentiation. Heat shock proteins being the interest of our lab, understanding the molecular mechanism of stress-induced differentiation in embryonal carcinoma cell lines was my objective. We have already shown that albumin, an adult liver specific protein, which is absent in embryonal liver of rats, is induced upon heat shock in embryonal liver (Usha et al., 1987).

Does stress make the cells mature (more differentiated)? Can stress trigger differentiation in embryonal cells? These are some of the relevant questions asked here.

Thus, the current study was initiated with following major aims:

2. Identification of the differentiated cell-type using known markers.
4. Molecular and functional characterization of selected gene/s identified by DDRT-PCR.