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The study model that we used for cell differentiation is the Zebrafish embryo, as it features regulators that are maintained during phylogeny.

This simplifies research significantly and does not pose insurmountable ethical problems.

We trust that the publication of the following results may prompt other work groups to continue these studies. The fight against cancer is difficult and long: it involves 130 different diseases, each of which requires a specific regulation therapy. We have blazed a new trail that we trust will be further explored by others. If this happens, credit should be given to the role played by the Editorial Committee of the Journal of Tumor Marker Oncology that actively brings this research work to the attention of the international community. My thanks therefore go to Professor Klavins, Professor Birkmayer and the other members of the Journal's Editorial Committee for their keen interest in our research findings.

Pier Mario Biava
Foundation for Research into the Biological Therapy of Cancer
Sesto S. G. Hospital, Viale Matteotti 83 - (Milano)-Italy

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Cancer and Cell Differentiation: A Model to Explain Malignancy

Pier Mario Biava*, Daniele Bonsignorio*

*Foundation for Research into the Biological Therapy of Cancer. Sesto S.G. Hospital, Viale Matteotti 83, Milano – Italy

Introduction

The evidence obtained from studying the interactions between tumor cells and embryonic tissues suggests that tumor development in embryos is reduced or suppressed when differentiation processes are in progress (1,2).

The administration of known carcinogens during cell differentiation in embryos causes malformations in offspring, but not tumor induction. Once organogenesis is complete, the frequency of tumor induction rises concurrently with the decrease in the rate of malformations (3,4,5).

These findings could indicate that cancer can be viewed as a developmental deviation that may be controlled by cell differentiation regulators.

Based on the foregoing, experiments on animals were carried out. Past experiments have demonstrated that factors present during cell differentiation can stop or delay tumor growth in animals. These factors are present in the pregnant uteri of mammals (6) and in the embryos of ovipara (7). More recent experiments in vitro have shown that pregnant pig and mouse uterus extracts slow down the proliferation rate of several established human tumor cell lines (8). It was clarified that the abnormal growth of cell clones during embryo organogenesis in mammals is prevented by low molecular weight substances present in the pregnant uterus microenvironment. A 5kDa fraction isolated in our laboratory from the pregnant uterus of mammals, named "Life- Protecting Factor", inhibited the cell proliferation curves of all treated human tumor cell lines as well as the crude pregnant uterus extracts. Therefore, the interactions between mother and embryo seem to be important for the normal development of the embryo and for preventing pathological cell growth. The embryo itself seems to prevent the abnormal multiplication of tumor cells. In fact it was demonstrated that different tumor cell lines responded with a significant slowdown in proliferation when treated with extracts taken during the stages of cell differentiation, while no slowing effect was observed when they were treated with the extracts taken from a merely multiplicative stage (9). Thus, cell differentiation is a key process in understanding the behavior of both normal and tumor cells. The fact that embryonic development and tumorigenesis are closely correlated is now accepted: they both share several pathways and molecules that can regulate some important genes of the cell cycle. The main effect of the in vitro treatment of tumor cell lines with the extracts of oviparous embryos is the activation of p53

expression, as observed by immunohistochemical and flow cytometry techniques after treating different tumor cell lines with fish embryo extracts (10). In addition, in another article of this issue, we have reported the induction of a post-translational regulation of pRb by zebrafish embryonic extracts, which is probably responsible for the observed slowdown of kidney adenocarcinoma proliferation curves in vitro. Embryonic differentiation and tumorigenesis, although they share several metabolic pathways seem to be opposite processes: the same molecules, which cause cell differentiation in embryos, seem to be able to inhibit cancer growth. In order to explain the mechanisms involved in these two different processes, it is necessary to illustrate an outline and a model of embryonic differentiation and cancerogenesis.

An outline and a model of embryonic differentiation

The differentiation processes begin shortly after fertilization, generally in the middle-blastula-gastrula period. There are three postulates of cell differentiation:

1. every cell nucleus contains the complete genome established in the fertilized egg. In molecular terms the DNAs of all differentiated cells are identical;
2. the unused genes in the differentiated cells are not destroyed or mutated and they retain the potential for being expressed;
3. only a small percentage of the genome is expressed in each differentiated cell and a portion of the synthesized RNA is specific for that cell type.

Briefly, the differentiation, which leads pluripotent embryonic stem cells to specialization, consists in a differential regulation of genes that restricts the expressed genome. The gene configurations of the cells after each stage of differentiation differ from the progenitors for some thousands of expressed genes.

Regulators are generally factors that cooperate in a network and this network promotes and controls the differentiation of each cell type. All cells communicate with each other through this network.

Cell differentiation is a very complex process that takes place at different levels:

A) a differential gene transcription which regulates how the nuclear genes are transcripted into RNA;
B) a selective nuclear RNA processing which regulates how the transcript RNA's get into the cytoplasm to become messenger RNA's;
C) a selective messenger RNA translation that regulates how messenger RNA's in the cytoplasm is translated into proteins;
D) a differential modification of proteins, which regulates how proteins are allowed to function in the cells.

Transcription factors are very important in controlling the differential expression of genes, but in eukariotes selective nuclear RNA's processes are more important. These selective processes clarify how the same gene can produce two different proteins in different cells or in the same cell at different times.

Moreover selective degradations or, otherwise, selective stabilizations of the messenger RNAs are responsible for further protein specifications.

Today we have a dynamic vision of the regulation of gene expression.

A gene is not thought to be an independent and autonomous control center of protein synthesis.

A gene is also controlled directly or indirectly by the synthesized proteins.

The interactions between nucleus and cytoplasm and between cytoplasm and microenvironment are so extensive that they constitute a marvelous example of complexity.

The developing embryo is an excellent example of "complex adaptive systems".

In fact the embryo is, 1) a network of many cells acting in parallel, 2) has many levels of a constantly revising and rearranging organization, 3) has an implicit prediction encoded in its genes and 4) is always in transition and is characterized by ongoing innovations.

Cell differentiation can be better understood in a model described here, which is consistent with the real situation. In this model the number of final gene configurations of cells in the human body (number of types of completely differentiated cells) can be predicted, if we retain that each kind of progenitor cell produces 3 different daughter cells (3 different "gene configurations") and that there are 5 stages of differentiation (fig. 1).

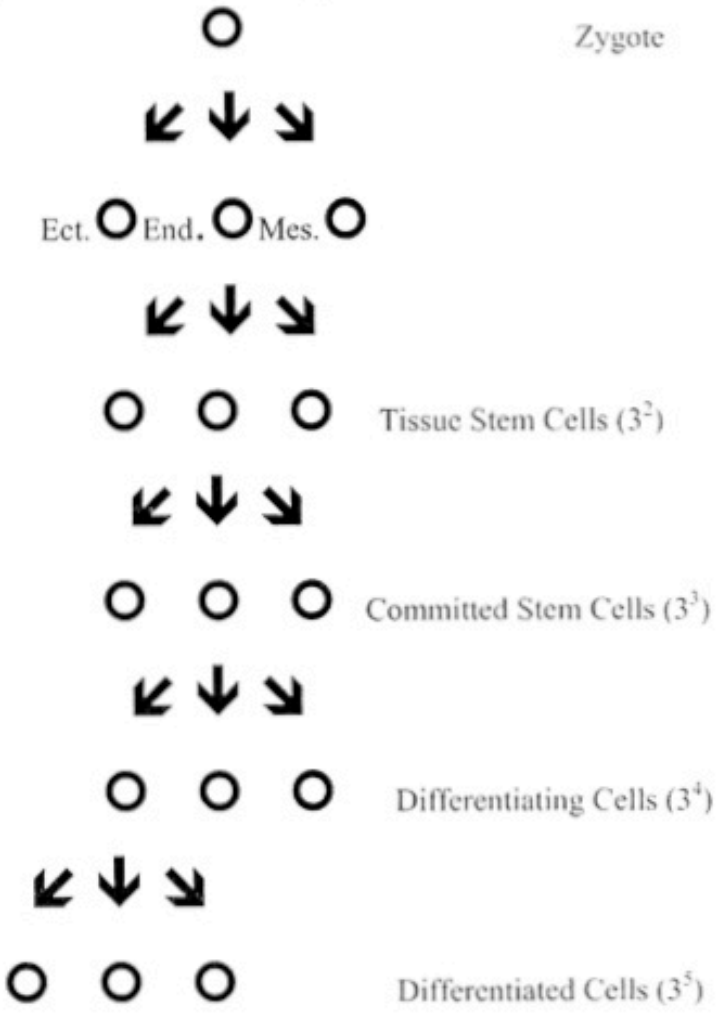

Fig. 1: Model of Cell Differentiation

This corresponds to the real situation: in fact the embryo, after segmentation (morula), is differentiating in three layers: ectoderm, endoderm and mesoderm. Gametes are differentiating in a different pathway compared with somatic cells. After gastrulation, there are another four stages of cell differentiation. For example, on the basis of precise data about some cell lines, such as hematopoietic cells, the stages of differentiation are: a) stem cell stage, b) committed stem cell stage, c) differentiating cell stage, d) differentiated cell stage. If we include the ectodermal, endodermal and mesodermal cell lines, there are five stages of differentiation. Therefore the mathematical formula to calculate the number of differentiated cells is:

$$N= 3^5.$$

The result is 243, which is the number of the different somatic differentiated cells. To calculate the final number of the differentiated cells we must add the number of gametes. The sex cells are 5 in males (spermatogonium, spermatocyte of the first order, spermatocyte of the second order, spermatid, spermatozoon) and 4 in females (ovogonium, ovocyte of the first order, ovocyte of the second order, egg cell).The final result is 252, which is the number of the different kinds of cells effectively counted in humans. Life is organized through very simple algorithm!

Cancer as undifferentiated mutated cells. A model to explain malignancy

The tumoral transformation of normal cells is a process with a minimal number of stochastic mutational events, between 4 and 7 (11). If mutations are introduced into normal cells in a non-stochastic manner, i.e., triggering at precise genes, the number is reduced (12). The preferred targets of these mutations are genes encoding for key-role effectors of cell cycle regulation and cell signaling, and for growth factors and their receptors; mutations are either gain-of-function, in the case of proto-oncogenes, or loss-of-function, in the case of tumor suppressor genes.

Defining the tumoral transformation of a cell simply as the outcome of a sum of gene mutations may be restrictive. For normal cells to become cancerous, transformation also depends on a complex network of surrounding microenvironmental signals from cell-to-cell "cross-talking" or from soluble extracellular factors. For example, it has been demonstrated that fibroblasts adjacent to prostate epithelium carcinoma cells are capable of directing tumor progression (13), that stromal neighbor cells are capable of promoting malignant transformation of immortalized keratinocytes by releasing proliferative stimuli (14), and that inflammatory cells can sustain, instead of fight, tumor growth (15). Even proinflammatory cytokines were shown to promote cancer cell proliferation by inhibiting tumor suppression pathways (16). Thus, the whole context is decisive in determining cell fate in line with a "heterotypic" view of cell biology, as it was called in a recent review (17). According to this view, defining tumorigenesis as a microevolutive process is now safe. A cancer cell acquires, as a consequence of this process, some capabilities: 1) self-sufficiency in growth signals, 2) insensitivity to antigrowth signals, 3) the ability to evade apoptosis, 4) limitless replicative potential, 5) the ability to sustain angiogenesis, 6) the ability to invade tissues and give metastasis. The acquisition of these capabilities during the course of tumor progression is usually the consequence of a great variability of the mechanisms used by cells to become malignant. Nonetheless the hypothesis presented here is that regardless of how the steps in these genetic pathways are arranged, the development of all types of human tumor cells is governed by a final common process. Some authors define "early crisis" and "genetic catastrophe" of cells some of the steps that enable the evolving population of premalignant cells to reach malignancy (18). As a result of these crises, during which a telomere dysfunction and DNA damage take place: 1) cells die or 2) cells survive after each crisis. The final results are adaptive responses and telomere maintenance in the case of cell survival. Surviving malignant cells have A) not only increased the level of telomerase, but B) have also activated proto-oncogenes or oncogenes, C) produce growth factors, D) are insensitive to anti-growth signals, E) have several surface antigens, also known as oncofetal antigens, maintained during phylogeny (19), most of which have been identified in the last 30 years (20-30). In other terms the cells that survive a period of genetic

instability become malignant through the achievement of a new stable gene configuration very similar to those present in the embryo during periods of multiplication. Cancer cells and embryonic cells share some molecular pathways and their key-role effectors: e.g. the APC/ β-catenin/ TCF/ Wnt pathway (31,32) and the Hedgehog/Smoothened/Patched pathway (33). In embryonic development these pathways lead cells to successful differentiation, in tumorigenesis their mutated counterparts lead cells to constant multiplication. This happens because a cancer cell is an undifferentiated cell in which the mutations present in its genome do not allow the cell to complete the whole program of differentiation and development. It is stopped in a step of the multiplication process, comprised between two stages of differentiation. Figure 2 shows the development of an embryonic cell line.

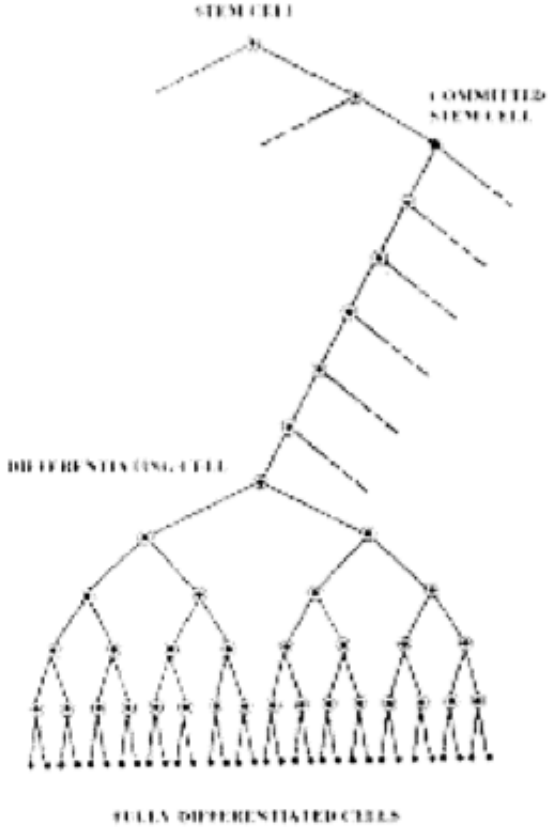

Figure 2: Schematic Model of Differentiation of a Non-Specified Cell Type starting from its progenitor cell

It is possible to see that there are some steps of multiplication between two stages of differentiation. A cancer cell can be defined as an "undifferentiated mutated cell", in which the differentiation and multiplication programs are uncoupled. It is like a computer in loop, repeating always the same instructions. Cancer is probably an example of deterministic chaos. It is a branching process, that leads the cell, since it does not die, to rampant genetic instability: the final attractor is a new stable "gene configuration" similar to that present in the embryo during the steps of multiplication, between two stages of differentiation (fig. 3). In line with this, considering the model of cell differentiation previously mentioned, the number of different types of cancer deriving from somatic cells can be predicted with the formula:

$$N = 3 + 3^2 + 3^3 + 3^4 = 120$$

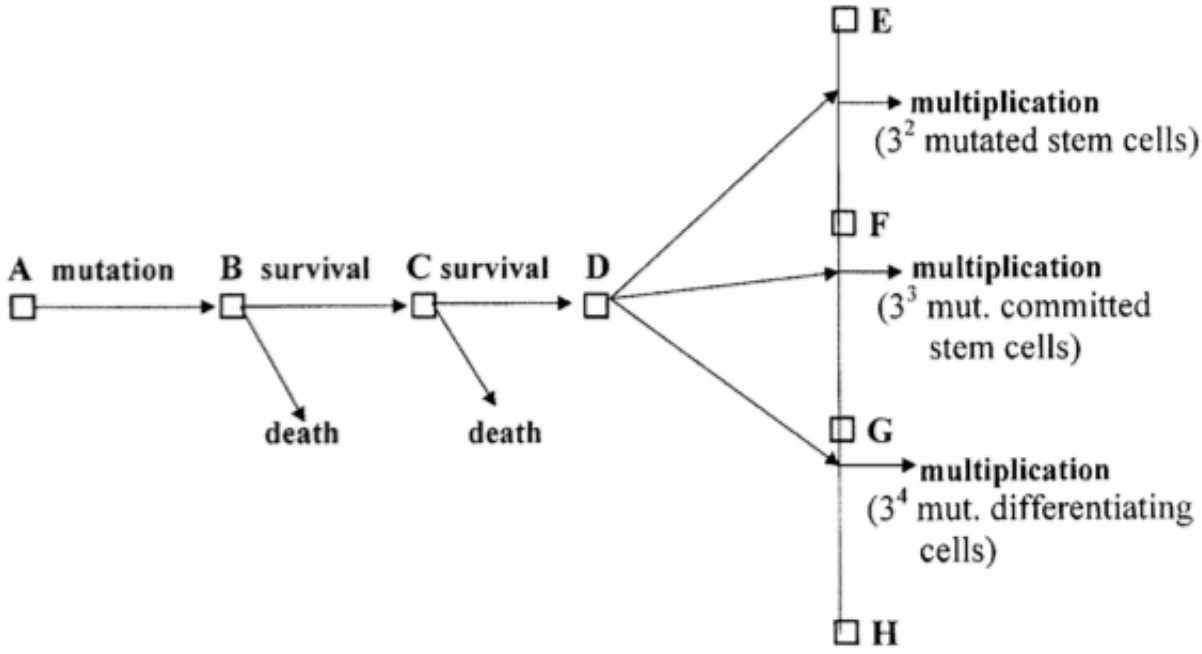

Figure 3: Cancer as a process of deterministic chaos. A: normal cell; B: cell with damaged DNA; C: cell with rampant genetic instability, D: cancer cell: stable gene configuration with uncoupled steps of cell multiplication and differentiation. E: stem cell; F: committed stem cell; G: differentiating cell, H: differentiated cell.

In order to calculate the final number of different kinds of tumors, it is necessary to add the number of tumors coming from sex cells and from different embryonic tissues (teratocarcinoma, embryonic carcinoma, corioncarcinoma). Therefore, the final amount of all different kinds of tumor is about 130. With regard to malignancy, the most aggressive tumors are represented by cells with "gene configurations" present at the early stages of differentiation, that carry out the multiplication program at an impressive speed. The current classification of tumors is redundant because it does not consider that the most malignant types of tumors come from cells that have the same "gene configurations". Finally, some types of tumors come from different cell clones with "gene configurations" arising from different stages of differentiation.

The regulation of cancer growth: a model of complexity

The cancer model above is not merely theoretical, but is based on the results of laboratory experiments. These experiments have shown that molecular factors present during precise stages of cell differentiation are able to inhibit tumor growth. This was demonstrated both *in vivo* on Lewis Lung carcinoma and *in vitro* on several human tumor cell lines. On the contrary, substances present during merely proliferative stages are ineffective in delaying the growth curves of several types of tumors. Thus, cell differentiation is a key-process in explaining the behavior of both undifferentiated normal and tumor cells. Cell differentiation mechanisms are based on a multigenic regulation, so that a more differentiated cell differs from a less differentiated one because of the expression of a great number of genes. Furthermore, according to the model, tumor cells have lost an important portion

of the program of cell differentiation in a progressive manner.

Therefore, if the ultimate goal is not to destroy the tumor cell, but its regulation, this can clearly be achieved only by providing the cell with all the factors that can bring it to differentiation. These factors can be found, but only when life is forming. In fact, during organogenesis the whole repertoire of regulatory molecules is present, which includes 1) DNA trascriptional factors; 2) nuclear RNA selection factors; 3) mRNA translational factors; 4) post-translational protein regulatory factors. We have seen that these factors can be used for the genic regulation of tumor cells. A p53-mediated transcriptional regulation and a pRb post-translational regulation were demonstrated, depending on the type of tumor. Thus, it was demonstrated that it is possible to regulate tumor cells, by-passing the mutations that give rise to malignancy. This happens only when the network of differentiation is complete enough. As a result, the focus should be on the microenvironment and networks of the biological structures, rather than on the single subjects of punctual mechanisms. This does not mean that research into molecular mechanisms should be disregarded, but that the single partial mechanisms should be put in a more integrated vision of the biological processes. Indeed, the difficulty in bridging the gap to a new scientific paradigm, that is, shifting our views from reductionism to complexity, has been the main barrier to acquiring a deeper and more complete knowledge of cancer. Studies and researches on stem cell differentiation are proceeding worldwide and the scientific community is ready to accept a new paradigm. These studies will be able to show that differentiation mechanisms are based on specific differentiative networks. The embryonic microenvironment during precise stages of development is fundamental not only for the differentiation of normal stem cells, but also for the differentiation of tumor cells. Embryo, during organogenesis, is never affected by carcinogenetic processes because, while the life program is under transcription, systems of correction in case of mutations are also active. In fact, it has been demonstrated that during cell differentiation, the administration of known carcinogens fails to induce the growth of tumors, perhaps because the genome control system is always working.

According to recent studies, the p53 function in embryo is to prevent malformations, and some authors have called p53 the "guardian of babies", as a gene that suppresses the onset of malformations (34, 35). However, when there is too much stress and mutations are too numerous, the p53 is no longer able to repair the DNA and causes apoptosis in all cells (abortion). These processes also occur in tumor cells when the p53 is activated. This happens when tumor cells are put in contact with embryonic differentiating factors.

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