The Gordian Knot of Quiescence, Commitment, and Clonal Expansion *

Erik A. Cerven*

*www.scienceandresearchdevelopmentinstitute.com

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Abstract

Perspectives and approaches of importance for understanding the gene kinetics involved in physiological and therapeutic regulation of cell proliferation are outlined. For this purpose a convenient notation is introduced that formalizes verbal information about gene expression similarly to equations. The notation is used explicitly for the genome regulators p300/CBP, c-Myc and Cyclin D_1 based on a literature survey. These proteins are capable of effectuating a metabolic switch between cell differentiation and proliferation. By taking into account the likely sequence of evolution of cellular faculties a better discrimination of central and peripheral gene regulatory elements can be achieved. Hence, proliferation is seen as an ancient primordial faculty that later has come, first under inhibitory, then under stimulatory control. This approach is also used for evaluating the various therapeutic approaches to proliferative disorders.

Keywords: Gene Expression, Gene Regulation, Cell Proliferation, Metabolism

1 Staging the Quiescence of a Stationary Cell and Commitment to Division

When Terrestrial Life invented itself billions of years ago the nutritional environment must have been plentiful enough to allow its ultimate expansion into all ecological niches, as is now observed. Therefore, one expects metabolism to be built on a core of pathways promoting unrestricted cell proliferation. When nutrients became exhausted new protective mechanisms evolved such as sporulation of single cells, motility, nutrient storage, and metabolic restraint within the framework of multi-cellular aggregates.

The main classes of low molecular weight constituents, namely lipids, carbohydrates, amino acids, and nucleic acids are shared by all forms of Terrestrial life, unicellular, multi-cellular, plants and animals. This also applies to several of the catalytic functions of enzymes active in primary metabolism. Cellular functions that are linked to proliferation and hence must have existed already at the unicellular stage of evolution include anabolism with membrane synthesis and template metabolism such as that

Email: cerven@scienceandresearchdevelopmentinstitute.com

Table I

UNICELLULAR STAGE
Electron transport - chemical energy (oxidative phosphorylation - glycolysis)
Membrane gradients
Nutrient transport
Primary metabolism
Metabolic oscillations e.g. circadian osc.
Cell multiplication
Anabolism
Membrane synthesis
Template metabolism (DNA, RNA)
Starvation response
Maintenance
Motility
Nutrient depositing
Stress responses
Heath
Radiation
Oxidative damage
MULTICELLULAR STAGE
Maintenance, controlled starvation making cells subordinate to organism
Cell differentiation, species evolution
Contact signaling
Chemical signaling
Circulatory system
Immune system effective against
viruses
bacteria
aberrant self
PROCHARYOTIC TO EUCHARYOTIC TRANSITION
Nuclei
Mitochondria
Chloroplasts
Endoplasmic reticulum
Golgi apparatus

Table I. Faculties of cellular life as probably having evolved at the uni- and multicellular stages of the evolution of life.

of DNA and RNA. The core of primordial metabolic responses common to all life is also expected to include various responses to environmental stress such as heath, radiation, and oxidative damage. All cells are capable of selecting glycolysis or electron transport as a means of generating convertible chemical energy, the latter usually coupled to oxygen consumption. Furthermore, all cells are capable of metabolic oscillations. These may have evolved as a means of probing the environment and include circadian rhythms. Some plants' diurnal shift of metabolic focus between nocturnal primary carbon dioxide assimilation and photo-assimilation (cf. [1]) has a counterpart in circadian oscillations of glycogen synthase kinase in the animal kingdom [2], hinting at the possibility of a common very

ancient ancestry of such oscillations.

Multicellular organisms likely evolved in response to nutritional deprivation since well structured adjacent and quiescent cells not only preserve structure but also channel the metabolic flux into specific functions while saving energy and nutrients. This principle is demonstrated by amoebae, which are capable of transforming between unicellular and multicellular forms of existence [3]. Several new metabolic responses evolved when Life entered the multicellular stage. First of all, cellular proliferation ceased to be a random spatial expansion and turned into an ordered process, giving rise to differentiation and embryogenesis, the basis of development and evolution. This required contact signaling, chemical signaling, and ultimately, a circulatory system. The circulatory system set the stage for an immune system in higher organisms. The immune system is capable of recognizing non-self matter such as viruses, bacteria and also detects non-functional self cells.

It is not known if the transition from procharyotic to eucharyotic cell occurred before or after the ascent of multicellularity. The organelles (nuclei, mitochondria, chloroplasts, endoplasmic reticulum, Golgi apparatus) then appeared, each acquiring specific functions and all perhaps relying on a modification of lipid metabolism effectuating the formation of the extra membrane needed.

The categories of functionality mentioned above are listed in Table I. This list is more than an epistemological classification, it represents indispensable faculties of life all having the mandate to enforce any molecular-genomic representation to serve their logic purpose. One may define an 'evolon' as a logic (as opposed to material) integral element of evolution coded for by a gene or a group of genes. In the course of evolving more complexity, existing molecular vehicles of these faculties were either maintained as such, substituted for others, or maintained with additional levels of control overlaid on them. In parallel with this evolution of functionality, mutations occurred that mostly left the original function undamaged allowing life to build on its previous successes rather than revolutionizing itself over and over again.

2 Introduction to Notations Used Herein for Gene Kinetical Events

In order to characterize enzyme-catalyzed substrate-product metabolism, certain notations are in use that simplify the perception of what is going on in the cell. Namely, enzymatic conversions are represented by arrows, like in

$$A \rightarrow B + C \rightleftharpoons D + E, \tag{1}$$

denoting that the substrate A is converted irreversibly into B and C and the latter are in equilibrium with products D and E. In gene kinetics, however, there is obviously no widely used notation representing the dynamics of expression of genes. Various processes are still described using words even though much could be gained by using a common formal language, searchable as binary code. In the present paper, the following notations will be used: A material factor, F, causes an increase in chemical activity of another factor G,

$$F > G \ / \tag{2}$$

and/or it causes the gene G to be increasingly transcribed,

Table II

Notations	Explanations
>	causes
<	dissipates into
/	increased chemical activity
/ /	increased transcription of prior factor or gene
\	decreased chemical activity
\ \	decreased transcription of prior factor or gene
> / /	(a transcription factor or co-activator) causes generally increased transcription of its controlled genes
> \ \	(a transcription factor or cosuppressor) causes generally decreased transcription of its controlled genes
-	complex or covalent ligand, e.g. X-Y
+ +	factors or genes work jointly to bring an effect, e.g. X + + Y
=	associated with, seen with
, \ X	the prior process is inhibited by X
, / X	the prior process is enhanced by X
, , \ X	the prior factor is inhibited by X
, , / X	the activity (expression) of prior factor is increased by X
->	converts into (eqv. of →)
<->	is in equilibrium with
*	used after > and < to indicate results of original bench-top research, see Table legend and two different processes
or	non-exclusive or
Ac	Acetyl group
P	Phosphate group(s)
Me	Methyl group
SUMO	Sumoylyl group
Ubq	ubiquitin ligand(s)
Hi	histone
EMT	Epithelio-mesenchymal transition
Diff	Differentiation
Cecy	Cell cycle in its phases, manifest cell proliferation
NI	Nuclear import, alt. localization in nucleus
NE	Nuclear export, alt. localization in cytoplasm
GAP	Gene-activating principle, material factor
GIP	Gene-repressing (inhibitory) principle, material factor
( )	with no added symbols of gene kinetics: mediator of prior or subsequent process, alt. verbal explanation
( )	with added symbols of gene kinetics: process taken as a whole and reintegrated within a higher hierarchy of processes

Table II. Summary of notations used for describing gene-kinetical events. The notations are used with the following additional rules:
‘,’, <, <, /, \, - and +, are separated from nearest neighbor by single space
factors of cause and effect closest to center at > or <
ligands including NE and NI furthest from center at > or <
identical factors closest to center at <–>
Original bench-top results are distinguished from quotations and theory using a star, *, directly after the < or the >, placed once in the summary as a kind of keyword, for example F > * G / .

$$F > G \; / \; / \tag{3}$$

(if G / / one may usually take for granted that G /), and this causes a response,

$$F > G \; / > H \; / \tag{4}$$

G may be a gene-activating principle (GAP), such as an inducer or a transcription-activating factor (alternatively a gene-inhibitory principle, 'GIP' causing less expression of H, H \ ). All letters, freely selected, may denote material factors, genes and/or pathways or faculties like in Table I. Just like the arrows denoting the metamorphosis of metabolites neglect for example intermediates of enzymatic conversions various intermediaries may be ignored here. Specific characterization may be put within parentheses before or after the core element of the notation in order to maintain its searchability as binary string.

G may dissipate through, for example, phosphorylation

$$G < G - P \; (ubiquitination) \tag{5}$$

or through complex formation (titration)

$$G < G - T. \tag{6}$$

Hyphens, separated by spaces, are reserved for various intermolecular bonds, covalent or non-covalent, and complexes including ligands, like phosphate (- P) and acetyl groups (- Ac) etc. such that the factor c-myc, for example is denoted cmyc. The slash symbol, '/' , denotes increase in chemical activity measured using some arbitrary method, double slash, '/ /' , enhanced transcription usually with increase in chemical activity, backslash '\' stands for decrease in chemical activity, and double backslash '\ \' stands for less transcription usually implying decreased chemical activity. The symbol '>' reads 'causes' while '<' reads 'dissipates through' (in the sense that the prior material factor shows less chemical activity). The symbol '=' reads 'associated with' in the sense of 'seen together with'. The notations used here are summarized in Table II.

Negative feedback at the gene level through the action of factor 'X' induced by and comprised in 'G', for example, is described respectively by

$$F > G \; / > X \; / > G \setminus \setminus \;\; and \;\; F > G \; / \; / = X \; / > G \setminus \setminus \, , \tag{7}$$

where in the latter case the up-regulation of G by F is supported by increased transcription.

Furthermore, slashes and backslashes before the names of additional factors or genes are taken to specify which agent enhances or inhibits (reverses) the process in question, for example

$$(EGF) \;\; CDK4 - cyclinD1 > E2F \; / \; , \; \setminus \; p16INK4a = E2F - Rb \;\; (< E2F + Rb - P) \tag{8}$$

wherein the core string element of notation to the extent possible is maintained and the added information made searchable *via* its core context. In the above expression the means by which the gene kinetics is effectuated (the enzymatic catalysis, phosphorylation, associated with the signaling cascade) is put in parenthesis. One should then be able find the process above in a literature search for i.a. 'E2F / , \' or 'CyclinD1 >' depending on interest.

Many transcription factors behave as activators or inhibitors depending on context (for instance YY1, E2Fs, Rb, cMyc, p300/CBP). This may in principle be caused by epigenetic influences, distinct complex-bound co-regulators, or one factor first building up the regulatory complex then titrating it apart,

$$F > G \;/\; > A - B - C - G < A - B - C + C - G. \tag{9}$$

3 What causes a gene to be more expressed?

When comparing the gene expression in a hostile clonal expansion with its 'normal' counterpart assumed to be some typical cell of the tissue affected one often makes the observation that a gene is more (or less) expressed in comparison to the reference. What may be the cause of, say, an increased gene expression, G /, connected with some faculty, H, G > H, characterized by factors X, Y, and Z,

$$F > G \;/\; > H = X = Y = Z \;? \tag{10}$$

A regulatory factor F may increase and cause G / , of course,

$$F > G \;/\; / = G \;/, \tag{11}$$

for example due to the release of F from binding,

$$B - F < B + F > G \;/\; / \tag{12}$$

or the dissipation, D, of products of the gene G may decrease

$$(G < G - D_G) \setminus > G \;/. \tag{13}$$

In the case of negative feedback on G by its own products, alternatively a downstream factor (or response), say 'X'

$$G \;/\; > H \;/ = X > G \setminus \setminus, \quad \textit{alternatively} \quad G \;/\; > H \;/\; > X \;/\; > G \setminus \tag{14}$$

the latter's dissipation may be increased,

$$(X < X - D_X) \;/\; > G \;/. \tag{15}$$

Another possibility of eliciting the response H via G / as an effect of F binding to the genome is by competition between two or more genes sharing the factor F. Namely, epigenetic conditions (such as the acetylation status) may cause a global release of F and yet favor the response H,

$$DNA - F < DNA + F > G \;/\; / = G \;/ \tag{16}$$

There is also the possibility of plain titration by a soluble factor A in equilibrium with G,

$$A - G \longrightarrow A + G > G \;/\; /, \tag{17}$$

the equilibrium may be affected by physicochemical properties of the microenvironment, and A may or may not be part of a gene regulatory complex.

Furthermore, compartmentalization of the factor F is possible. It may, in principle, be observed predominantly in the cytoplasm, the nucleus, the nucleolus, in euchromatin or in heterochromatin and (but usually not) in the mitochondria. Its regulatory effect, however, may not necessarily be confined

to where it is predominantly observed, which invalidates any correlation between its measured amount and its effect. In such cases active shuttling mechanisms and their vehicles (for example, NLS, the nuclear localization signal) rightfully capture the focus of the attention. For example, a gene may turn repressed by the nuclear export (NE) of an activating factor, C_A,

$$C_A < C_A - NE > G \setminus \setminus = G \setminus \tag{18}$$

or by the nuclear import (NI) of an inhibiting factor

$$NI - C_I > G \setminus \setminus = G \setminus \tag{19}$$

Another gene may be activated by the import of an activator,

$$NI - C_A > G / / = G / \tag{20}$$

or by the nuclear export of a repressor into the cytoplasm,

$$C_I < C_I - NE > G / / = G /. \tag{21}$$

The processes (10) to (21) represent various means by which the expression of G may increase.

In the printed literature there is a tendency to regard any gene that is increasingly expressed during cell proliferation and so also in tumor cells as a tumor promoter but the exercise above shows that this may not always be correct. Namely, the reason for an increased transcription may be that dissipation is slowed down (13), that there is inefficient negative feedback (14) - (15), a permissive epigenetic environment (16), or that there is compartmentalization (18) - (21). Any of these causes may represent a more central regulatory element than the actual gene itself. In fact, even if the gene is proven to cause tumors when tested as an exogene-clone implant in laboratory animals the titration process (9) above shows that the notion of tumor promoter may still be debatable in such cases and in practice, results obtained based on gene regulatory multi-component complexes are sometimes at variance with those of the individual components [4]. Similar arguments and precautions apply to 'tumor suppressors'.

The strategy of exploration after observing an enhanced gene expression in a clonal expansion is often to manifest that it is a tumor promoter by analyzing some of the possibilities represented in processes (10) to (21) above, then to search for inhibitors of its activity, try the pharmacological agent in laboratory animals and then in clinical trials hoping that it will be capable of grabbing all the metabolic plasticity of the clone and escaping that of the host. This does not happen of course, so the question arises how to avoid making the same mistake over and over again in different molecular landscapes. Some answers to that question will be provided in Ch. 6.

4 The Evolution of Gene Regulation

A pathway or functionality, H, has to exist before it can be controlled. Therefore, it is natural to think that negative (inhibitory) control, C_I, first evolved,

$$C_I > H \setminus, \tag{22}$$

and later was supplemented with up-regulatory (activating) elements, C_A, sensed by some effector molecule or condition, E,

$$E = C_A > C_I \setminus > H \; / \tag{23}$$

dissipating *via*,

$$C_A < C_A - D_A. \tag{24}$$

In classical notation, an 'inducer' is an 'effector' in the sense used here but the latter term is intended to be wider. An inducer is understood to be a low molecular weight compound, usually the initial substrate of a metabolic chain, that causes a gene to be expressed. An 'effector' may be defined more widely as a material substance associated with a metabolic/biophysical state of the cell that causes a gene to be expressed (by binding to it if known, indirectly if binding factor is unknown), thereby promoting a metabolic/biophysical effect due to the gene's expression. This sense has the advantage of bringing to life the gene's physiological role irrespective of what kind of molecular vehicle evolution has chosen for doing so. Various co-activators and phosphorylations may also play the role of an effector. Furthermore, one may also in general assume the existence of a reporter substance, R, telling C_A that H is no longer needed (alternatively that it is over-expressed).

$$H > R \; / > C_A \setminus > C_I \; / > H \setminus. \tag{25}$$

This is of course distinct from the 'reporter gene' used in genetic engineering and also from the notion 'checkpoint', which implies a process going forward to the next set of genes, usually within the cell cycle. The ICER protein in granulosa cells [5] may be taken as an example of a reporter substance since in this tissue FSH > PKA > CyclinD2 / / , \ ICER and LH > cAMP / > ICER / / > Cyclin D2 \ \ . In immortalized breast epithelial cells, p300 and CBP down regulating c-Myc [6] may provide another example since they tell c-Myc that differentiation is being accomplished (see below). A classical repressor, i.e. a low molecular weight metabolite that binds to a co-repressor thereby causing a gene to be less expressed is also an example of a 'reporter'. Focussing on 'effectors' and 'reporters' has the potential of early lifting to the foreground the physiologically important aspects of the regulation of a gene such that applications can be found before a full mechanistic characterization is undertaken.

Since a pathway must exist before it can be controlled one may formulate a rule of the reversal of control states of a gene, namely that it is caused to be

$$active \rightarrow inactive \rightarrow active \; (\rightarrow inactive \rightarrow active..) \qquad (rule) \tag{26}$$

while evolution at the molecular level adds more complexity to its control as time goes by. This rule may be expected to hold unless an exception can be proved. It has a spatial counterpart for ligands in that one ligand must be capable of binding to DNA before its effect can be modulated by another ligand. Hence,

$$closest\ is\ first \qquad (rule) \tag{27}$$

unless proven otherwise. Exceptions may exist where mutations of the first ligand may enable binding of already existent regulatory factors. A regulatory complex often has several points of contact with DNA. These rules lead to that single activating factors require past or present repressing factors elsewhere in the promoter of a gene. Deacetylated histone or methylated DNA may be regarded as such repressed states.

Tracing molecular evolution backwards in time may be as difficult as looking backwards in cosmological space, especially in its early stages but it is important for judgements about plausible applications of the genes in life sciences. Some primordial epigenetics may vaguely serve as an example of the processes (22) - (24) above: Assume that the expression of some gene, G, is associated with bare

DNA and that it is hindered by histone binding, DNA - Hi > G \ (Process(22)). This is reversed by histone acetylation catalyzed by a transferase HAT > DNA + Hi-Ac / > G / (Process (23)) and the acetylation in its turn is dissipated through the action of a histone deacetylase, Hi - Ac < Hi + Ac (HDAC).

When examining damaged faculties that characterize a hostile clonal expansion within an organism it is important to keep in mind how these faculties likely evolved sequentially. Otherwise one is set for surprises like when decades of research into the control of cellular proliferation by the important E2F transcription factors culminated in the conclusion that these factors are not mandatory for proliferation anyway [7]. Cells can even survive and multiply with biallelic deletions of the central $G_0 - G_1$ regulator Cyclin D_1 [8] and the histone acetylases CBP/p300 [9]. Cell proliferation is a primordial functionality that is capable of ignoring control mechanisms imposed on it during later stages of evolution. Accordingly tumor-linked mutations are seldom seen in the molecular vehicles of the phases of the cell cycle, the cdk-cyclins that are shared with procharyotes. The mutations rather occur in the genes, characteristic of eucharyotes, that control them (quoted in [10]). What is actually remarkable about clonal expansion within the nutritious internal environment of an organism is not that it occasionally takes place but that it mostly doesn't. Once a cell lineage has broken the code of conduct that maintains the organism's structure it is likely to find more and more loopholes in its own and its host's regulatory network to promote its own narrow Darwinian intent. This takes place via 1) changes of gene expression and structure within the cell lineage itself, 2) changes in its microenvironment, and 3) overload or deterioration of the host's immune defense system.

5 Proliferating *versus* Stationary Cell

Clearly[1], there is a conflict of interest between the organism relying on gene regulation promoting differentiated functions in its component cells and the suppressed Darwinian intent of the systemic cells themselves of multiplying as long as nutrients are available. In tumor cells these control mechanisms are set aside [11] allowing glycolysis as well as oxidative phosphorylation to proceed at maximal rates thus both supplying intermediates in support of anabolism and fuelling electrochemical membrane gradients [11, 12, 13]. The latter are used for enhanced biological transport to the effect that the electrochemical gradients must be regenerated at a faster rate and in fact, sustained depolarization may itself serve as a standalone trigger for commitment [14]. One may define such a state in which there is gradient reflux at the plasma membrane as an 'open' state, O (in contrast to 'closed', C) and associate it with cell proliferation and the cell's detachment from physical contact with its environment (in contrast to quiescence and multicellularity),

$$proliferating\ cell = open = O \longleftrightarrow C = closed = quiescent\ cell \tag{28}$$

[1] Abbreviations
FSH, follicle-stimulating hormone
LH, luteinizing hormone
PKA, phosphokinase A
HIF, hypoxia-inducible factor
ICER, cAMP early inducible repressor
ES embryonic stem cells
HSC, hematopoietic stem cells
VEGF, vasculoendothelial growth factor
CRE, cyclic AMP responsive element
CBP, CREB-binding protein
HAT, histone acetyltransferase
LDH, lactate dehydrogenase

The open state confers enhanced metabolism and motility to the cell. Acetyl groups associated with oxidative phosphorylation provide a logical mechanistic marker for the open state in that they a) are associated with nutrient feeding and anabolism, b) are capable of making DNA accessible for transcription and replication through acetylation of histones and c) provide building blocks for lipid and *de novo* synthesis of membrane. These functions all serve cell multiplication. The open state provides an example of the rule that a faculty (set of genes) must exist before it can be controlled. In this case it is the faculty 'proliferation' that must exist before it can be controlled. In the absence of proliferation-sustaining pathways the cell can not exist since its ancestry would be gone. Even if molecular evolution has substituted plain acetyl groups for more complex regulatory factors the same arguments hold. The availability of acetyl groups is expected to be higher under hypoxia (since they are less efficiently consumed by the mitochondria) to the effect that hypoxia is expected to favor the open state. In accordance, histone acetyltransferase activity in liver reflects systemic ethanol levels and is characterized by p300 + HIF1α > / / [15] and, expectedly, ethanol <-> Acetyl - CoA. The notion of a positive link between general metabolism and proliferation in the context of acetylation is supported by data on the p300/CBP -coactivated CREB protein (reviewed in ref. [16]) which regulates gluconeogenesis in liver among several key genes in primary metabolism that contain CRE-sequences (i.a. phosphoenolpyruvate carboxykinase, pyruvate carboxylase, ornithine decarboxylase, lactate dehydrogenase [2]) since CREB overexpression is positively linked to myeloproliferative disorders both phenomenologically [16] and by transgenic engineering [17] causing enhanced proliferation distinct from outright (unregulated) leukemia [17].

A proliferating cell is always solitary. If derived from a systemic multicellular cluster held together by intercellular junctions it must detach before or at least while dividing. The nutrient status *per se* is capable of effectuating such behavior. Namely, pancreatic Langerhan's islets under sustained high glucose levels or palmitate down-regulate the junction protein Connexin36 according to > PKA > ICER1γ / / > Connexin36 \ \ [18]. This serves as an example of the rule (cf. Table I) that the multi-cellular stage is associated with environmental or self-regulated nutrient deprivation[3] The release from such contact inhibition in the presence of excess nutrients is associated with cell proliferation. The Wnt signaling pathway constitutes another molecular vehicle of this rule. Here, the complex cytoskeleton - βcatenin - cadherin provides anchorage (equivalent of multicellularity) which is dissolved on Wnt signalling leading to Wnt > bcat - NI > cmyc / / , \ bcat - Ac (CBP) [19] (see also [20]) characterized by nutrient uptake, one of the hallmarks of the open state. Also the ancient [21] c-Myc protein is involved in this central element of the control of proliferation [22]. One may thus easily find examples of the rule that starvation stabilizes an organism whereas excess nutrients destabilize it by favoring the dissolution of intercellular structure. The increased life span of starved mono- and/or multicellular organisms in comparison with *ad libitum* -fed ones has been linked among other things to mitochondria [23], chromatin [24] and the expression of CBP [25], the latter a HAT and/or HAT-anchor with specific roles in particular genes as well as capable of altering chromatin structure, of course intimately connected with the intracellular acetyl group pool.

The histone acetyltransferase CBP and its close analogue p300 (CBP/p300) are conserved in multicellular eucharyotes [37, 38, 39] and are induced by hypoxia in mammals where, i.a., HIF1α - p300/CBP > VEGF / / [40]. There are several known examples where they are down-regulated by titration [41] or titrated by competing factors [42, 43, 44] suggesting limiting amounts in the nucleus but they are

[2]LDH is a key enzyme enabling a high rate of metabolism under hypoxic conditions, especially in tumors

[3]In the pancreas of mammals however, it is likely that this molecular switch has been tamed to allow a respite for the Langerhan cells to renew their reservoir of insulin (in that exocytosis also is down-regulated). It is interesting from an evolutionary point of view that failure of the systemic cells to take up glucose (as is seen in pathological hyperglycemia) deprives them of nutrients, which adds further pressure on their already subdued molecular vehicles of proliferation. This added protection against proliferative disorders may partly explain why such a disease burden not has been eradicated in the course of evolution.

also present in the cytoplasm where they may catalyze distinct reactions, such as ubiquitination (ref. [45]: NE - p300/CBP > p53 - UBQ and NI - p300/CBP > p53 - Ac). A characteristic feature of p300/CBP is their association with gene expressions seen in terminal differentiation (Table III). Since they *1)* promote differentiation, *2)* their down-regulation trigger c-Myc expression [6, 46] leading to proliferation because of cMyc > CyclinD1 / / > Cecy [47] and *3)* biallelic deletions of either one make animals tumor-prone [36] they are often regarded as tumor suppressors, as has been shown for p300 [48] (see also [49]), but in practice they can be seen at high levels in tumor cells [50] and have been linked progressively to the characteristic nuclear morphology [51] of such cells. These factors can also

Table III

Ref.	Process
[26]	cAMP + p300 > renin / / = Diff
[27]	p300 - MyoD > Diff / = p21 / > Cycl - cdk \
[28]	curcumin > p300 \ = HAT \ > heart hypertrophy \
[29]	TGFβ > Smad3 - SOX9 - p300/CBP > chondrogenesis / /
[30]	TGFβ + Smad4 + p300 > COL1A2 / / = collagenesis , \ E1A - p300 (adenovirus)
[31]	p300 - JunB > DMP1 / / = mineralization
[32]	p300 \ \ > EMT
[33]	cMaf - p300/CBP > crystallin / / (-Ac) = lens differentiation
[34]	EGF > ERK1/2 p - p300 - sp1 > Keratin16 / /
[35]	(embryoid body) EB + + p300 > (ES - >Diff) / (p300 > Nanog / /)
[36]	CBP > HSC - > Cecy but conversely p300 > HSC - > Diff

Table III. List of examples of the co-regulator p300 promoting cellular differentiation. Unless generally known or explained in Table II or under heading 'Notations' the abbreviations refer to genes

be seen hijacked for proliferative purposes by neoplastic cells [8], [4] [10], [5]. Their absence is oncogenic but their presence need not be oncosuppressive. Therefore they serve as an example of a rule for oncogenic/oncotrophic and oncoprotective/oncosuppressive gene regulators stating that:

$$causative\ agent \neq sustaining\ agent \qquad (rule) \tag{29}$$

This may seem obvious to the molecular biologist but has neither penetrated into medical practice nor into health care regulation. In medicine, the causative agent is usually the target of therapy as exemplified by infections, inflammations, hormone disturbances, surgery, etc. For proliferative disorders the standard treatment is accordingly still agents inhibiting DNA metabolism, which is seen as the focal point of cell division. This old doctrine also haunts strategical thinking about new molecular approaches in oncotherapautics in that 'highly expressed in proliferation' means 'bad' and consequently a potential pharmaceutical target. In Ch. 6 a more nuanced approach will be outlined.

The c-Myc gene controls not only anabolism but also cell differentiation [22], which was among the first genome-controlled faculties associated with the multi-cellular stage (Table I). Therefore, many footprints of past molecular evolution are expected to be seen in the gene. For example, one may use the acetylation example above for testing the rules (26) and (27). One sees among the gene's control elements an inhibitory factor, YY1, next to DNA. YY1 in its turn binds to p300 which is a HAT (histone acetyltransferase), a *bona fide* activator of genes via acetylation although in this ancient gene it has

[4] cJun - p300 > cyclinD / /

[5] cdk3 > ATF - P > CBP - ATF - P > / / (cJun, cFos CyclinD among targets)

turned into a co-repressor. P300 bridges to HDAC3, which again down-regulates the gene by deacetylation. These data (6) are indicative of the rules 'closest is first' to make active-inactive-active-inactive.

Since *1)* p300 > cMyc / / > CyclinD1 / / [46, 6], *2)* Cyclin D_1 initiates cell proliferation by the well-known process CyclinD1 - cdk4 > (E2F - Rb < Rb - P + E2F > / / = Cecy), and *3)* p300 is limited in amount [52, 41, 42, 43, 44] 4) being also required for differentiation (Table III), it provides a molecular vehicle for switching between proliferation and differentiation. A metabolic switch between proliferative and differentiation-promoting pathways based on competition for limiting factors has been proposed to be a central element in the control of cell proliferation [53, 13, 54, 55]. Quantitative *ab initio*-simulations of the cell cycle comprising the rechanneling of metabolic flux into differentiated functions in the quiescent state can be performed based on the notion of such limiting factors [55]. Binding of a few molecules of p300 to those few genes that may be assumed to control the commitment to division at the $G_0 - G_1$ transition would not be sufficient to deplete all the remaining genes of the factor. However, since cyclin D_1 binds to p300; cyclinD1 > (p300 < p300 - cyclinD1) [52], one expects as a consequence the equilibrium

$$(GAP_{Diff}) - p300 \longleftrightarrow p300 - (GAP_{Proliferation}) + p300 - cyclinD1 \tag{30}$$

to be shifted to the right and the proposed gene kinetics would be realized. The differentiated state may then consolidate itself, for example by reprogramming the roles of ambivalent transcription factors such as E2F [56] or by DNA methylation [57], but this represents an additional (younger or older?) level of control.

6 Suppression of Clonal Expansion

A paper on the topic clonal expansion must also include a section on its therapeutic control. This is what the general public expects of the research councils to deliver in this field of research anyway. Not one week passes without a report on some new discovery in fundamental biomedical research that is expected to have some important oncotherapeutic applications. This also remains a challenging intellectual task.

The use of agents suppressing angiogenesis can be taken as an introductory example of the difficulties involved in achieving this goal. Anti-angiogenic agents are known to be oncosuppressive by inhibiting neovascularization. For example, antibodies against VEGF have been used successfully to suppress the growth. Knowing that hypoxia > VEGF / in neoplastic tissue, the rationale for such an approach is to even more deplete the already hypoxic tissue of oxygen and of nutrition. Many other molecular vehicles that accomplish angiogenesis are now being discovered. A low oxygen pressure will tend to induce the expression of the apoptotic protein p53 [58], but will also promote glycolysis at the expense of fatty acid oxidation, which is known to drive transformation [59] and to increase the supply of anabolic substrates as discussed in Ch. 5. An adequate or improved oxygen supply is required for host defense mechanisms like the encapsulation of the affected tissue. Reactive oxygen species, ROS, produced increasingly under high oxygen pressure are required for and promote differentiation from hematologic stem cells [60] and promote apoptosis (the cell's self destruction) but they stimulate invasiveness [61, 62]. A low oxygen pressure on the other hand, promotes autophagy [39] which increases the cells' survival, and leads to a characteristic epigenetic signature/phenotype that may promote transformation [63], possibly also inhibit progression [64]. Hence, the tumor cell benefits from the treatment no matter which approach is taken, whether one 'pushes' against the proliferation-sustaining pathways (VEGF-production, that is) or uses these pathways (adequate oxygen supply enhancing vascularization) to 'pull' the cell into the harness of the organism's self-defense.

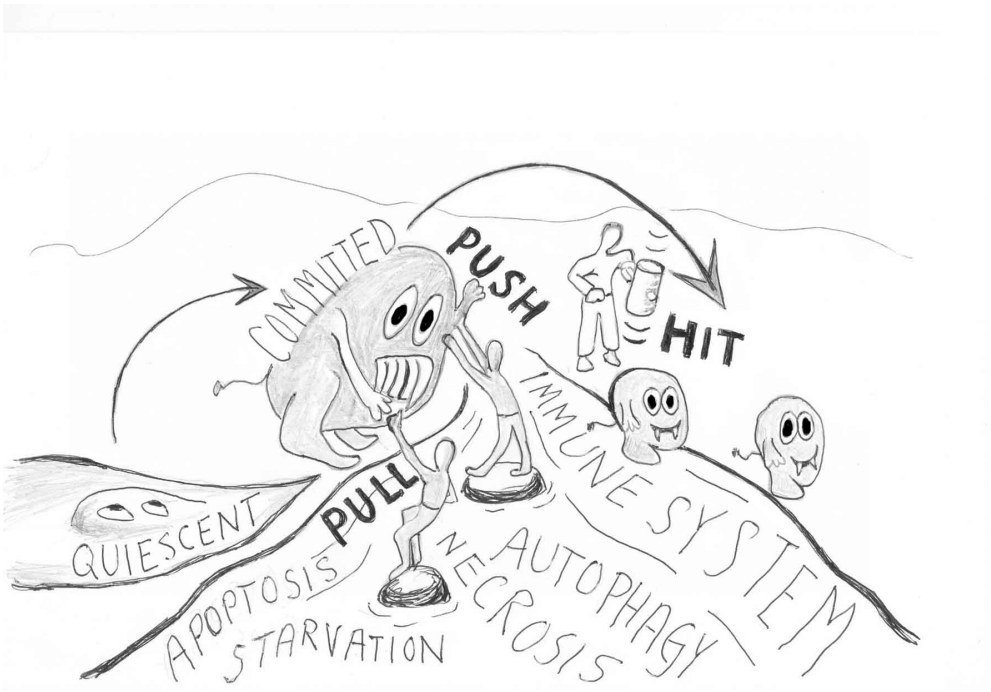

Figure 1: Cartoon illustrating strategies against proliferative disorders, 1) pushing against the cell's proliferation-enhancing pathways, 2) pulling the cell into the harness of the organism's self defenses, and 3) hitting the cell where its metabolic plasticity does not reach.

This scenario is quite general. The oncotherapeutic strategies may be divided into categories as illustrated in Fig. 1. A. *push* ('antiproliferometabolic') strategy (the classical approach) is defined as counteracting a metabolic pathway or faculty that enhances proliferation by inhibiting the action of its molecular vehicles. A *pull* strategy is defined as benefiting from or enhancing the cells' aberrantly expressed pathways and faculties in order to direct it into any of the organism's natural defense mechanisms, like for example apoptosis-autophagy [65] and immune defense [66, 67, 68]. Hyperthermia, which enhances the cell's metabolism to the point of self-destruction may be taken as another example of a 'pull' strategy. There may also be metabolic competition between these pathways, as has been documented for apoptosis *versus* autophagy [39].

Over time, especially the push strategy is usually subdued by the tissue's metabolic plasticity, i.e. the capability of its cells to adapt metabolically. Furthermore, push and pull strategies focussing on the same pathway tend to be antagonistic. For example, if a pull strategy requires the expression of a certain gene, then pushing against that gene or against the faculty (set of genes) with which it is linked (a 'push-pull combo' so to speak) may be counterproductive, like in the VEGF example above.

Besides the strategies that are focussed on existent pathways and faculties of cell and organism a third strategy may be identified. Namely, a *hit* strategy is defined as the use of a method of eliminating the proliferating cells that circumvents the metabolic responses that the cells and the organism have at their disposal. A good example is melittin [69]. In practice, hit strategies are made possible by advances in targeting the active agent to the tissue affected, for example using peptides [70], liposomes [71], nanoparticles [69, 72, 73, 74, 75], viruses [76], or anaerobic bacteria [77, 78, 79, 80, 81, 82]. The latter target to acidic (hypoxic) tissue where they proliferate with some oncolytic potential on their own [83], perhaps by acting as an adjuvant, but they may also be genetically engineered with onco-

suppressive metabolic pathways and genes [78, 79, 80, 81, 82].

Cellular metabolism resembles cognitive processes in that enzymes and pathways are put together dynamically in various constellations depending on the environment just like words and grammar are combined to provide an intellectual response [53, 13]. The eucharyotic cell has perfected its language in the course of a billion years and it is only beginning to be learned. Using the push strategy described above in order to talk to the cell in its own language will always produce a better counter-argument. The cell has all its metabolic plasticity for answering and should that not suffice, the tissue where it resides will provide another better fitted member to stage a better response, which, of course, is the principle of Darwinian evolution again, backed up by billions of years of experience. Is it really necessary to enter into such a lengthy discussion? In the fight against infections, for example, no such cumbersome characterization of the control of proliferation was needed because of the early discovery of the cell wall-targeting inhibitor penicillin. Similarly to bacteria, the eucharyotic cell exposes the plasma membrane as its most vulnerable and energy-consuming organelle. Plasma membrane trafficking, build-up, movement, and nutrient transport constitute a hallmark of neoplastic cells, which have escaped the self-imposed starvation characteristic of the multi-cellular organism. The plasma membrane of failing cells is also the target of the host's immunoresponse. With all the advanced targeting techniques now being developed it is possible to focus directly on 'hit' strategies, to the extent available complemented with immunoresponse-enhancing methods.

In the case of targeted drug delivery using non-live carrier materials the limited capacity of the carrier may be a disadvantage. Once the biocompatibility of the carrier has been ascertained the controlled release of the contained (or bound) drugs may still present a problem. The live carriers, viruses and bacteria, usually employed as vectors for genes, circumvent these problems. On the other hand, they must be genetically engineered with specific genes, one or a few at a time, which is insufficient against the metabolic plasticity of the targeted cells. Because of these disadvantages one may envisage a drug delivery system based on bioluminescent nonpathogenic and anaerobic/acidophilic bacteria whose light emission catalyzes photolysis of photodegradable prodrugs present in plasma and interstitial fluid at the tumor sites. These bacteria can be expected to grow also in small-sized neoplastic tissue. The following three paragraphs elaborate on the design of such a drug delivery system.

The proof of principle that a bacterium engineered to express the luciferase gene can deliver a luciferase that catalyzes emission of light in slightly acidic media and near 37 degrees has already been provided [84, 85]. The luciferase gene employed in these experiments or that from any other suitable species, mutated or not, should then be engineered into the genome of a nonpathogenic acidophilic and/or anaerobic bacterium, for example any of those quoted in refs. [74] - [79]. The luciferases are sensitive to many poisons, intracellular pH, temperature, ATP supply, etc. which means that a successful experimental outcome not is granted. It may, for example be necessary to add a gene coding for some proton pump to the bacterial genome.

The concept of photolytic activation of prodrugs is already established in the literature [86, 87, 88, 89, 90] and is now a very active field of research. In most cases, the photoactivation of the synthesized bioactive substances is done at wavelengths below 300-400 nm whereas the emission from bioluminescence typically does not stretch below green color at about 500 nm (Coelenterazine-based bioluminescence though, may stretch down to about 400 nm, blue). The scarcity in the published literature of prodrugs and sample organic compounds cleavable in the visible light regime is likely due to the impracticality of performing organic synthesis in the dark rather than to any limitation of principal nature. Probably, several compounds that were designed for activation by ultraviolet light can be modified by standard procedures (substitution of chemical groups at the cleavage site or some neighboring atoms) to make them more suitable for activation by visible light.

Many bioactive compounds are inactivated if they are even slightly modified and it may be difficult (but not impossible, cf. [91]) to design a covalent carrier that releases them intact on illumination. Therefore, an alternative may be to enclose them non-covalently into a photo-sensitive molecular wrap. In the case that macromolecular bioactive compounds are used (like siRNAs, peptides, enzymes, etc.) bactericidal effects are not a concern but if low-molecular weight plain poisons are used the engineered bacteria that activate them must be protected, for example by adding a suitable gene coding for some protective metabolic pathway or enzyme into their genome. Alternatively, poisons can be used that are taken up by phagocytosis or pinocytosis into the targeted cells and then released a second time by endogenous enzymatic catalysis (hydrolysis), after having been internalized.

Once a drug delivery system as described above has been established in the host, it would provide flexibility of drug selection combined with targeted delivery, which is an advantage compared with previous techniques.

7 Discussion

In this paper, the old problem of the control of cell proliferation is seen from a new perspective. 1) A convenient notation was introduced that allows the compression of verbal information about gene kinetical events into packages of concise formal language. The general adoption of this formal language would promote the accessibility of data on the genes involved in control of proliferation. 2) An evolutionary approach was taken in order to lift to the foreground cellular faculties that are of primordial importance for cell proliferation. This may explain why some pathways and control mechanisms of proliferation are overridden or redundant in clonal expansions. Similar arguments indicate that failure to deliver glucose may protect the cells against proliferative disorders 3) Specifically, the role of p300/CBP as a molecular vehicle for a metabolic switch between proliferative pathways and cellular differentiation was examined. Such a switch may be at the core of the control of proliferation, something which is supported by quantitative *ab initio* computations of proliferation including differentiated functions. 5) Finally, therapeutic approaches to suppressing cell proliferation were categorized, allowing easy identification of synergistic and antagonistic strategies. Some anticipated improvements of currently available strategies were also discussed.

8 Acknowledgement

Quoted literature is intended to provide evidence of principle, not to constitute an exhaustive review. Publications that were not free public access at the time of reading were avoided. Because of the extensive literature survey undertaken as a platform for this work some statements of fact could not easily be retraced to the source on the local computer and are quoted without specification of source. This applies particularly to Ch. 6. The following Internet engines are gratefully acknowledged: http://www.ncbi.nlm.nih.gov/pmc/journals/ ; http://www.uniprot.org ; http://www.sciencedaily.com/ ; http://www.google.com/advanced_search?hl=en

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