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Evidence for immortality and autonomy as criteria for neoplasms has not been shown for the lesions resulting from most animal models of carcinogenesis

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Abstract

Modern research into carcinogenesis has undergone three phases. Surgeons and pathologists started the first phase and established autopsy and biopsy as routine pathology services, in turn establishing morphological traits for tumors and establishing immortality and autonomy as indispensable criteria for neoplasms. A century ago medical doctors and biologists initiated "experimental cancer research" as the second phase, in which they, with help from chemists, established many chemical-induced animal models of carcinogenesis. In this phase, the two-hit theory and stepwise carcinogenesis of "initiation-promotion" or "initiation-promotion-progression" were established, with an illustrious finding that outgrowths induced in animals depend on the inducers, and thus are not authentically neoplastic, until late stages. For the last 40 years, molecular biologists have gradually dominated the carcinogenesis research fraternity and have established numerous genetically-modified animal models of carcinogenesis. However, evidence has not been provided for immortality and autonomy of the lesions from most of these models. Probably, many peers had already collected the lesions from animals for analyses of "cancer" mechanisms before the lesions became autonomous. We herein review monumental work of many predecessors to reinforce that evidence for immortality and autonomy is essential for confirming a neoplastic nature. We extrapolate that immortality and autonomy are established early during sporadic human carcinogenesis, unlike the late establishment in all animal models. It is imperative to resume many forerunners' work by determining the genetic bases for initiation, promotion and progression, the genetic bases for immortality and autonomy, and which animal models are, in fact, good for identifying such genetic bases.

Keywords:

Transgenic; Cancer; Carcinogenesis; Immortality; Autonomy; cancer stem cells; senescence

Running title: Many tumors induced in animals are inauthentic

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Introduction

Cancer research has been going on for 2,700 years [1], although systematic study of cancer might not be started until 1775 when Pott reported his chirurgical observation of cancers in several body sites [2]. Therefore, its consequent literature is so enormous in size that it becomes a problem for researchers. Actually, even in 1955, Alexander Haddow (1907-1976) had pointed this out, writing that "the mere abundance of the data …presents a growing problem, towards which there are two extreme types of reaction: first, that of the happy researcher who is content to ignore the original literature, and to rely upon others for his information; and secondly, the reaction of those whom the literature totally enslaves" [3]. Few of today's researchers peruse the ancient literature, due to their many tiers of stress, such as dwindling funding and increasing difficulty in obtaining tenured faculty positions, besides the sheer volume of the literature to read. As a repercussion, cancer research manifests a discontinuous growth, just like cancer itself that is a discontinuous growth from its normal parental cell, meaning that few of today's cancer students know and address the questions raised by their predecessors.

We write this perspective article to review some seminal findings by different trailblazers in "carcinogenesis research", which is herein defined as the study 1) on the procedures that convert a normal cell to a malignant one and then to more malignant states, and 2) on the mechanisms underlying these steps. The clinical aspects of cancer will not be discussed to avoid digression. The idiom of "tumorigenesis", which is of broader scope as it also covers the formation of benign tumors, is used sometimes, partly because many animal models produce both benign and malignant lesions. In our opinion, modern research on tumorigenesis, mainly carcinogenesis, has undergone three phases. The first one was the epoch of Rudolf Ludwig Carl Virchow (1821-1902), who proposed the theory that cancer formation is a result of chronic irritation [4-7], mainly chronic inflammation. The cancer research fraternity in this phase was dominated by surgeons and pathologists, who established autopsy and biopsy as the routine pathology practice that leads to the establishment of the morphological traits of tumors [8]. The second phase had its inception roughly at the beginning of the 20th century and was coined by cancer pathologist Harold Leroy Stewart (1908-1998) as an epoch of "experimental cancer research" [8]. In this lengthy second incarnation, medical doctors and biologists, with great help from chemists, established and characterized many animal models of chemical-induced tumorigenesis. With these models, they established the two-hit theory [9], mutation theory [10-12] and clonal evolution theory [13-16], as well as the multi-stage [17-19], i.e. initiation-promotion [20-25] or initiation-promotion-progression [26-29], models of carcinogenesis. Moreover, animal models of irradiation-induced carcinogenesis also emerged during this period [30].

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Starting about 40 years ago when molecular cloning techniques began to disperse swiftly over the whole of biomedical research, molecular biologists, many lacking strict training and clinical experience in surgical pathology or oncology, have gradually replaced medical doctors and traditional biologists to now dominate the fraternity of carcinogenesis research [31, 32], thus moving "experimental cancer research" into a new phase. In this latest incarnation, molecular biologists have established numerous genetically manipulated animal models of carcinogenesis and in vitro systems of neoplastic transformation of normal cells. With these in vivo and in vitro models, we now know deeper mechanisms of how genes regulate behaviors of normal and neoplastic cells. Indeed, thanks to these achievements by molecular biologists, we now enjoy enormous amounts of information and great details on molecular signaling pathways for almost all physiological functions and pathological alterations in the human body. However, few of the genetic animal models established so far address the traditional multiple stages of "initiation-promotion-progression" [28], leaving those mavens who are familiar with their predecessors' work to wonder how to couple the stepwise biological changes observed previously with the molecular alterations seen in these genetic models. Moreover, few of the publications reporting these genetic models provide material evidence for immortality and autonomy of the resulting lesions. To warrant this statement, we encourage readers to search published reports of these genetic models for "immortal", "autonomous" or similar keywords, to see how many of them describe these properties of the resulting lesions. By reviewing work of many forerunners, most being preeminent cancer pathologists, we attempt in this essay to reinforce immortality and autonomy as the cardinal, yet long-neglected, criteria to qualify outgrowths as neoplasms, even the benign ones.

Many chemical-induced tumors in animals depend on the chemical until late stages

To our knowledge, the first experimentally induced tumors in animals were reported, in the German literature, by Fischer in 1906 (B. Fischer, Münch. med Wochnschr. 1906; 42: 2041). According to Davis [33, 34] and Vasiliev [35], Fischer showed that subcutaneous injections of Scarlet Red into the ears of rabbits induced papillomas, which are benign tumors, but the tumors regressed upon discontinuation of the injections, although they could be quickly re-induced with Scarlet Red. According to Davis [33, 34], these phenomena were confirmed by Helmholz in 1907 and by Werner in 1908. Between 1914 and 1924, Katsusaburo Yamagiwa (1863-1930), after he left Virchow and returned to Japan [36, 37], was able to induce papillomas and papillocarcinomas in rabbits' ears by painting the ears with coal tar, with metastases to nearby lymph nodes in some cases. Yamagiwa also found that the tumors, including some carcinomas with metastases, regressed after cessation of the tar-painting, but repainting could quickly

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cause the recurrence of the tumors [38, 39]. Yamagiwa thus concluded that "carcinomas do not develop as carcinomas from the beginning, and do not always continue as carcinomas" [39]. This "do not always continue" is the first statement in the literature, to our knowledge, stating that induced cancer can disappear spontaneously. During the 1930s and 1940s, Peyton Rous (1879-1970), a Nobel laureate, had also performed a series of studies on tar-induced tumors in rabbits' ears, and observed the regression of the lesions upon tar discontinuation and their quick reappearance upon tar repainting [25, 40, 41]. Actually, according to Rous, Des Ligneris had already confirmed in 1930 that,"…a second period of tarring brings out warts sooner than the first" [40]. Realizing that the reversible lesions could not be authentic neoplasms, Rous described them as warts, which is a hyperplastic type of lesion, and wrote in 1940 that, "…it will be seen that the tar warts of rabbits are tumors by all of the standard criteria except two. They have no capacity for independent growth like that exhibited by most (but not all) classical tumors; and the changes in their cells may conceivably be reversible since they often become smaller and vanish [40]. The two unmet criteria in Rous' observations, i.e. "no capacity for independent growth" and being "reversible", are later referred to by many cancer savants as "autonomy" and "immortality". Rous further wrote that, "…in the current definition of a tumor no allowance is made for neoplasms which depend upon favoring factors for existence, and it cannot be used to rule them out" [40]. Here, the lesion inducer is dubbed as "favoring factors".

Chronic treatment of rats with 7,12-diemthylbenz(a)anthracene (DMBA) could induce mammary tumors, but maintenance of the tumors is dependent on the continuous administration of DMBA [42-45]. Continuous feeding of rats with 3-methylcholanthrene could induce palpable mammary tumors as early as the 20th day from the start of the experiment [46]. Painting the skin of C57 brown mice with 3-methylcholanthrene could also induce palpable tumors as early as the 31st day [47]. However, 15 of the 22 induced skin tumors regressed completely upon cessation of the inducer, with hair eventually growing in the epilated areas; of the seven papillomas that remained, three eventually evolved to lesions with malignant morphology and four persisted [47]. Similarly, a large number of papillomas could be induced by painting the skin of albino mice with 3:4-benzyprene, but the tumors actually sloughed off, and only a few continuously progressed to carcinomas [48]. After having studied successive stages of carcinogenesis [49-52], Rusch wrote in the 1950 that carcinogenesis generally consisted of "induction, reversibility and progression" [53], which clearly points out that the lesions can be immortal and autonomous only at a late stage. The typical inducer-dependency until a late stage can be exemplified by the skin carcinogenesis model presented by Berenblum in 1947 [21], which, in Haddow's words, "proceeds from the normal epithelium first to an early non-specific hyperplasia, second to a specific pre-neoplastic hyperplasia, and

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then to the emergence of papillomata, and how later stages can be recognized in the progressive growth of such papillomata, their conversion into carcinoma, and the uncontrolled growth of the latter…This general sequence takes place equally well whether exposure to the carcinogen is continued or not" [54].

Hormone-induced tumors in animals are also inducer-dependent until late stages

Chronic treatment of rats and mice with estrogens can induce cancers in the bladder and mammary glands as well as benign tumors in the pituitary and testes [55-70], and can also induce uterine tumors [71]. The ACI strain (August strain crossed with Copenhagen strain, also called AxC) of rats may be more susceptible than other strains in the induction of the mammary and pituitary tumors [56, 72], but we once found that about one-fourth of the females lacked one side of the uterus and ovary (DJ Liao's unpublished data), suggesting that the ACI strain may bear a recessive mutation in a relevant but not yet identified gene. Treatment of mice with estrogen or with both estrogen and androgen can induce benign and malignant tumors in the cervix and vagina, and the malignant tumors are transplantable to other mice treated with the hormones [73-79]. Administration of androgens to rats can induce prostate [80-84] and uterine [85, 86] cancers. Concomitant treatment of rats with estrogen and androgen can induce mammary and prostate cancers much more quickly than administration of androgen or estrogen alone [80-84, 87-92], and can induce uterine leiomyomas as well [93]. Administration of estrogen to hamsters can induce malignant renal tumors with abdominal metastases [94-99], while administration of both estrogen and androgen to hamsters can induce malignant tumors in the kidneys and can induce benign and malignant tumors in the uterus, in the skin, and in the epididymal tail and adjacent ductus deferens [80, 100, 101]. In addition, gonadal and gonadotrophic hormones have also been shown to possess the ability to induce endocrine cell tumors in ovaries or testes [68, 102-105].

Estrogen-induced mammary cancer as well as pituitary and testicular tumors have been known, ever since the 1930s, to regress partially or completely upon withdrawal of the hormone, and the tumors can sustain themselves without estrogen treatment only at very late stages [55, 65, 78, 106-123] (and DJ Liao's personal experience). Initially, estrogen-induced pituitary tumors can be transplantable only to the animals treated with estrogens, but not to the untreated animals, evincing their dependency on an excessive amount of estrogen [55]. However, they can eventually evolve to estrogen-independency [124, 125]. The Nobel laureate Charles B. Huggins (1901-1997) had shown in both animal studies and human clinics that castration or treatment with estrogens could cause regression of prostate cancer at certain stages, signifying that this cancer is hormone-dependent until a late stage [126-128]. In the words of Jacob Furth (1896-1979), a renowned pathologist [129-131], "this (prostate) tumor is an example of a growth in

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man with a spectrum ranging from conditioned to highly autonomous type. The cases of Huggins that were controlled by castration (that is, removal of sources of androgens) may be regarded as dependent; those which partially or temporarily regressed after castration or estrogen treatment, as partially dependent; those not influenced by such therapy, as autonomous" [132]. Similarly, it is well known, with the earliest reference adduced herein dating back to 1919 [133], that human breast cancer at certain stages is estrogen dependent, and thus is often treated with antiestrogens. Estrogen-induced renal tumors in hamsters, including their abdominal metastases, will regress upon cessation of the estrogen treatment unless the tumors are at very advanced stages [134-137] (and DJ Liao's empirical knowledge). Initially, these renal tumors can be transplantable only to those hamsters that are treated with estrogen, but not to the untreated hamsters, bespeaking that the tumors still depend on an excessive amount of estrogen. However, these nonautonomous tumors can be converted to autonomous ones by manipulating the estrogen treatment of the recipient animals [97, 137, 138].

Treatment of mice with iodine-131 (I-131) can induce pituitary tumors that secrete thyroid stimulating hormone (TSH) [55, 139-142]. This is because I-131 damages the thyroid and thus decreases the levels of thyroid hormones, which in turn stimulates proliferation of TSH secretory cells in the pituitary with formation of TSH-secreting adenomas as the sequel [143-147]. By the same principle, partial thyroidectomy of rats and mice can cause pituitary adenomas as well [148-153]. The tumors can be transplantable [154]; initially only to those mice treated with thiouracil or other goitrogenic compounds that induce TSH, and then gradually to normal mice [155], which again shows the trajectory of "initial dependence and then autonomy", as said by Furth in 1953 [132].

Thyroid neoplasms can be induced in mice by treatment with thiouracil or other goitrogenic compounds that block thyroid hormone secretion and, in turn, induce TSH secretion from the pituitary [148, 151, 152, 156-166]. It is also the high level of TSH that induces the thyroid tumors, similar to the aforesaid induction of pituitary tumors. By the same principle, I-131 can induce thyroid tumors as well [145, 167-170]. Initially, the tumors can be transplantable only to the mice treated with thiouracil, but in a course of subpassages, the tumor cells can eventually be transplantable to normal mice, which, once more, shows the conversion from hormone (TSH) dependent to independent states [132, 148, 151, 152, 157]. These thyroid tumors, though dependent on TSH, often metastasize to lymph nodes [132] and the lungs [132, 148, 159]. In 1953, Furth wrote that "conditions can be created whereby uncontrolled proliferation of one cell type is obtained, resulting in a tumor-like growth. Manipulations attaining this need not involve any intrinsic alteration in cells causing them to behave as cancer cells. Whether or not such tumors and the similar human metastasizing thyroid adenomas are considered neoplastic depends

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on the definition of a neoplasm. In our terminology such thyroid tumors are conditioned neoplasms. In the course of subpassages in thiouracil-treated mice the dependent growths give rise to autonomous growths which possess individual features of their own and can be grafted on normal mice. Thyroid adenomas induced by TSH-secreting pituitary tumors are indistinguishable from those induced by thiouracil" [132]. Here, Furth used "tumor-like growth", "behave as cancer cells" and "conditional neoplasms" to express his reservation in considering the induced pituitary and thyroid tumors, even the spontaneous human thyroid tumors, as authentic; despite their ability to metastasize. In his opinion, "dependent tumors are those in which apparently normal cells proliferate in an altered host; autonomous tumors are those in which permanently altered cells proliferate in normal hosts" [132], although, based on our training in human pathology, we opine that dependent tumor cells are not normal but are hyperplastic.

Immortality and autonomy had already become indispensable criteria for neoplasms over a century ago

To our knowledge, the abovementioned studies on chemical- or hormone-induced outgrowths are among the earliest ones that point out the problem of "inducer-dependency" and set immortality and autonomy as criteria for neoplasms in experimental animals. Actually, before these animal studies became well recognized, some German pathology textbooks published in the 1910s had already outlined these criteria for human neoplasms, according to Rous [40]. Jacob Furth [132] and transcendent pathologist James Ewing (1866-1943) [171] considered that all tumors, including those benign ones that would not progress to malignancy, should be in some form of autonomy. Haddow wrote in the 1947 that, "…we now know that, while constitutional and genetic factors can greatly influence susceptibility to cancer, and many even determine the site of its spontaneous occurrence, the disease is one of the individual cells as a separate organism and with no relation to the needs of the body as a whole. It is this which gives cancer its unique position in pathology, accounts for its intractable nature, and explains its growth, in Paget's words 'irrespective of the maintenance of the rest of the body, discordant from its normal type, and with no seeming purpose' (Paget, 1853)" [54]. The quoted words of Paget had already, in 1853, pointed out tumors' autonomous nature. Indeed, according to Haddow [54] and Knauss et al [172], a cancer has long been regarded as a new race or new strain of organism, which is another way of describing autonomy, dating back to 1897 by David Hansemann, 1903 by G. Hauser (Beitr. Path. Anat., 1903; 33, 1) and 1926 by Menetrier. Many other former pundits also described carcinogenesis as an atavistic procedure, further pointing out that the resulting "new race of organism" is evolutionarily-lower on the life-tree than its host animal [173-176].

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What do "immortality" and "autonomy" really mean pertaining to a neoplasm? Immortality of tumor cells does not mean that each cell can survive forever. On the contrary, "that cancer cells are often sick cells and die young is known to every pathologist", as Rous had pointed out in 1941 [40]. Instead, immortality betokens that a tumor can survive as a "newly developed independent organism" [40] that parasitizes the host patient [173, 174] and forever maintains its life by continuous replication of its cells, just like bacteria that maintain their strains by ongoing cell propagation, as we described before [175, 177, 178]. Harry S.N. Greene (1904-1969), a preeminent surgical pathologist at Yale University, elaborated on the autonomy by writing in 1951 that, "…the definition of a tumor as an autonomous growth has enjoyed persistent popularity in textbooks of pathology. In such definitions the adjective 'autonomous' is employed to express the idea of independence with respect to two different particulars. One of these relates to freedom from the laws restraining and coordinating normal tissue growth, and the other concerns release from the necessity of a continued stimulus" [179]. According to Furth's translation [132], in an article in the German language from 1951, Bungeler considered that a dependence seen in a large variety of outgrowths in humans indicates that the outgrowths are not true tumors. More critically, there is no transition between the dependent and autonomous outgrowths, according to Furth's translation of Bungeler's words [132]. This "no transition" means that whether an outgrowth is autonomous or not is a black-and-white demarcation between neoplasms and non-neoplasms without leaving any room for us to confuse or admix the two. Describing human cancer's properties, Emmanuel Farber (1918-2014), an illustrious cancer pathologist and former chair of the American Association of Cancer Research, also accentuated autonomy as a cornerstone of cancer biology [180]. Notwithstanding, it also needs to be pointed out that autonomy of tumor cells may be achieved in part via non-autonomous mechanisms, i.e. various interactions with other cell types [181-183].

Some tumors from genetically manipulated animals are inducer-dependent as well

The c-myc gene or a mutant of the k-ras gene in transgenic mice can induce malignant tumors in all target tissues or organs studied so far, such as in the liver, pancreas, lungs and mammary glands, as we have shown or reviewed before [116, 184-191]. However, many of these tumors have been shown to regress upon turning off the transgene and can be sustained without the expression of the transgene only at very advanced stages, although, once they have regressed, they can be quickly re-induced through reactivation of the transgene [191-200]. Xmrk, c-myc, mutant k-ras, or SV40 large T oncogene can also induce liver cancer in transgenic Zebrafish, and, again, the tumors will regress after inactivation of the transgene [201-206]. Conversely, inactivation of the tumor suppressor gene p53 via conditional knockout

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can beget tumor formation, but reactivation of the p53 leads to regression of the tumors [205-211]. This phenomenon of "regression upon the inducer withdrawal and quick repopulation upon reintroduction of the inducer" is a full reflection of the same phenomenon seen in the chemical- or hormone-induced carcinogenesis in animals described above, and has become a rationale for targeting therapy in cancer [212-214]. Our contemporaries in the third phase of carcinogenesis research consider "regression upon inducer withdrawal" as "oncogene addiction" and "tumor dormancy" as the reason for "the tumor repopulation upon reintroduction of the inducer" [192, 196, 199, 212-218], but, peculiarly, without mentioning the same phenomenon observed by our predecessors.

Most animal models have not yet been tested for the trajectory of "induction, reversibility and progression"

There are still many animal models of carcinogenesis induced by chemicals or hormones that have not yet been determined for inducer-dependency. Even worse, only a few genetically-modified models, as described above, have been tested for the dependency, whereas the remaining vast majority have not yet been tested, presumably because many molecular biologists have not realized that immortality and autonomy are prerequisite criteria for neoplasms. For visceral tumor induction, like N-nitrosobis(2-hydroxypropyl)amine-instigated lung tumors [219], this situation is especially true, because the determination requires sacrifice of the animals. We surmise that most of the undetermined animal models may also show an inducer dependency until a late stage, with their carcinogenic procedures following the trajectory of "induction, reversibility and progression" described by Rusch in 1950 [53]. Considering that c-myc and mutant k-ras are the most potent oncogenes and the lesions they induced already manifest such dependency, probably other genetic models will show this trajectory as well. Nevertheless, this conjecture needs to be substantiated by studying untested animal models, especially the new ones to be established in the future, using a conditional transgenic or knockout approach.

Spontaneous regression of human neoplasms occurs but is rare

In humans, spontaneous regression of a solid tumor or remission of a liquid neoplasm, such as leukemia or myeloma, is extremely rare, but it is recurrently shown in case reports [220-235]. The frequency of spontaneous regression or remission is actually uncertain, as most relevant studies adduced only old figures that are between 1/60,000-100,000 cases [222, 236, 237]. The frequency may vary greatly among different cancer types. To our knowledge, malignant melanoma may have the highest rate of spontaneous regression [238-244], as it has been reported that 10-50% of cutaneous malignant

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melanoma cases show partial or complete regression without a treatment [245, 246], and that even 0.23% of the metastatic cases show spontaneous regression [245].

Using very strict criteria, there may not be pure spontaneous regression or remission, because in reality probably no patient will do absolutely nothing for his illness. In our cogitation, spontaneous regression or remission may occur in one of four scenarios: 1) Some patients' self-management or self-treatment towards the neoplasm is actually effective, although their doctors may not realize or appreciate it. 2) The patients may have experienced severe infection, especially a febrile one, since a host of studies have shown that infection or fever may be an effective cancer remedy [236, 247-266], as we have reviewed before [175, 267]. 3) The neoplasm is inauthentic, either because of a misdiagnosis or because it has not yet evolved to an authentic one, as to be expanded upon below. 4) The spontaneous regression or remission is genuine via a currently unclear mechanism, but, after the above three scenarios are culled away, its frequency is much lower than what is reported in the literature.

Ewing once summarized spontaneous regression of tumors and suggested some possible mechanisms. He wrote in 1935 that "…moles and neurofibromas become sclerosed, myomas calcify, chondromas ossify. Very few cancer cells may be found in the bodies of subjects dead of scirrhous carcinomas; gastric cancer may end in linitis plastica; lymphosarcoma is sometimes fatal without leaving demonstrable tumor tissue in the body. Partial removal may prove such an insult to the momentum of growth that ovarian cancer, chorio-adenoma, lymphosarcoma, and some adenomatous tumors may regress after such an experience" [268]. Since malignant tumors keep randomly mutating, theoretically some mutations may be good ones that direct the cells towards differentiation. Conversely, since the most common genetic changes found in tumors are large chromosomal deletions [269, 270], genomic damage that is too severe may lead to the loss of those genes required for cell survival. Moreover, some pernicious mutants may undergo mutation again, back to the wild type or to a better version, which presumably has reverse evolution as its essence [271] and may cause differentiation of the cells. This so-called "back mutation" or "reverse mutation" is occasionally discerned in drosophila [272] as well as in some human genetic diseases [273-276] and in some cancers treated with chemotherapeutic agents [277-279].

Inauthenticity of the tumors described above as the third scenario may also be a reason for spontaneous regression. For instance, hepatomas, and even hepatocellular carcinomas, in women chronically using estrogen-rich oral contraceptives, with regression upon termination of the contraceptive use, were frequently reported in the 1970s-1980s [280-286], which substantiates the human relevance of estrogen-induced hepatomas in rodents reported in the 1950s-1960s [287-289]. The regression upon hormone cessation intimates that estrogen serves as an inducer to incessantly coerce hepatocytes into

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neoplastic appearance without really bearing the genetic mutation(s) responsible for immortality and autonomy. As another example, low-grade lymphomas can result from infection by Helicobacter pylori (HP). These tumors are basically curable by eradication of the bacteria with antibiotic treatment [290-295], but, if left untreated, some of them will progress and become incurable, as reviewed by Park and Koo [296]. Similarly, Chronic HTLV-I (human T cell lymphotropic virus type I) infection may spawn adult T cell leukemia or lymphoma, but the neoplasm can be well controlled or even cured by antiviral treatment against HTLV-1 [297-299]. To us, these properties of these bacterium- or virus-caused outgrowths resemble those induced in many animal models described above, and thus are not authentically neoplastic at their early time point, although their diagnoses meet pathological criteria for lymphoma or leukemia and they, if left untreated, will eventually evolve to genuine neoplasms. Or, we can take a non-pathological definition of cancer proposed by Robert Axelrod, who majored in political science but became a prominent cancer ecologist [300], that incipient cancer cells might just have been partly transformed, and not yet fully malignant, thus requiring collaboration with each other for survival and for collective presentation of a cancer phenotype [301].

Loss of allegiance to the animal's body is the essence of a cell's immortality and autonomy

Sporadic tumors can only be derived from those cell types that are renewable, i.e. have a lifelong ability to replicate, but not from nonrenewable cell types, i.e. those that have lost their replicative ability in adulthood, such as neurons and cardiac myocytes [132, 302]. We tag those highly renewable cell types as "anabolic" for their great susceptibility to cancers, and those nonrenewable cell types as "catabolic" for their role in the development of type 2 diabetes [303]. The reason for why only renewable cell types can develop to neoplasms is that DNA mutation needs to be perpetuated by at least one round of DNA replication, and to be passed to filial cells via cellular divisions. The permanence becomes possible is because fitness testing of cells is usually conducted after the mutation is made permanently heritable [304]. Even for those renewable cells, it will take about one-fourth to one-third of the lifespan to complete the procedure of sporadic carcinogenesis, which is about 20-30 years for human beings [180], although it could take 50 years by others' estimation [9]. Therefore, the aforesaid tumors induced by 3-methylcholanthrene in just 3-4 weeks [46, 47] cannot be authentically neoplastic, since the lifespan of experimental mice and rats is three years or longer, although their counterparts in the wild live much shorter [12]. Indeed, we have not been aware of any rodent model in which a genuine cancer can be induced in a space less than a few months, except those genetic models in which the genetic manipulation

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has already been effective during an embryonic stage, thus mimicking a pediatric (but not a sporadic) carcinogenesis, as to be enlarged upon late.

All cell types in a given human individual have a physiological total number. For instance, the white blood cell count in a normal adult should be somewhere between 4,000-8,000 cells per cubic microliter of blood. For renewable cell types, if the cell number is decreased for some reason, the body will trigger cell proliferation to restore the physiological number, which is usually coined as "regeneration". On the other hand, if the number is higher than normal, as seen in over-regeneration that often happens following a regeneration procedure, the body will goad some of the cells into apoptosis to avoid cell redundancy. This denotes that all cells in an animal are allegiant to the animal's body, as we described before [175, 178, 305-308], or "conform with the law of organisms", as put by Rous in 1941 [40]. This allegiance of all normal cells to the animal's body as the "law of organisms" allows the animal's body to require some renewable cells to sacrifice their lives for the body's ultimate interest. A good example is that white blood cells are put on the frontier by the body to fight against infectious micropathogens and die in the battle, so that the body as a whole can survive [307, 308]. However, sometimes some renewable cells, such as select bone marrow cells, epidermal keratinocytes and mucal cells in the gastric-intestinal tract, have lost their altruism, usually due to acquisition of tumor-driving mutations that make the cells egocentric. These selfish cells want to survive such stress as micropathogen infection, over-regeneration-trigged apoptosis, etc., and become independent of the body, i.e. become autonomous. Reiterated, this disloyalty or loss of loyalty to the animal's body is the essence of, or the reason for, autonomy of some cells. "Fail to conform with the law of organisms" as said by Rous [40], or "become autonomous" as outlined by Ewing to be the pathological concept of tumor [268], was set as "the signature of a genuine neoplasm" by Borst in 1903 [309] and has, until today, been an salient feature of tumors, including the benign ones.

Leslie Foulds (1902-1974) split growth rate into "the responsive" component and "the intrinsic" component, and the total growth of cells is the sum of the two [132]. He wrote in 1953 that "all cells which can give rise to cancer possess the ability to multiply at a given rate, provided the environmental conditions are constant. They also have the capacity to respond to nutritional and hormonal growth factors, temperature, pH, etc. The intrinsic growth rate of normal cells is in general low; their responsive growth rate is high. The cancerous change goes with acquisition of a greater intrinsic growth rate and diminished responsiveness; the more malignant a cell, the greater the intrinsic and the less the responsive growth" [132]. Translated into today's language of cancer research, "the responsive growth" is the regenerative type of cell proliferation that is controlled by the animal's body and dwindles away during

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carcinogenesis, whereas “the intrinsic growth” is the autonomous proliferation that is controlled by the cells themselves and is strengthened during carcinogenesis.

Animal models can generally be dichotomized

Animal models established since the 1910s have evolved from using, as the inducer, a single agent, such as Scarlet red or tar, to using a complex regimen or a combination of several manipulations. Nevertheless, we try to split all animal models into two groups, based on whether or not the inducer is a potent genotoxic agent, although there are many intermediate models in which the inducer is a combination of both genotoxic and non-genotoxic agents. In one group, the inducers are potent mutagenic agents or regimens, including genotoxic chemicals, irradiations, and those genetic manipulations that impair DNA repair. In this group of animal models, mutation(s) responsible for the initiation, the first step of carcinogenesis, occur very early. An exemplary one is the Solt-Farber’s “resistant hepatocyte” model of hepatocarcinogenesis in the rat (Fig 1) [310, 311], or our modified version of it in which the promoting agent 2-acetylaminofluorene is routed via gavage instead of by feeding *ad libitum* [312-315]. Carcinogenesis in this group follows a trajectory of “initiation-promotion” or “initiation-promotion-progression”, as detailed by Farber [26-28, 316, 317]. It is clear that the gene(s) and their mutation(s) responsible for initiation are not those responsible for immortality and autonomy. This can be discerned in the Solt-Farber model wherein spontaneous proliferation, which reflects immortality and autonomy, occurs only in the lesions coined by Farber as “phenotype 4” that appear months after the establishment of initiated cells and after the completion of the carcinogenic regimen (Fig 1) [317].

The other group of animal models uses nonmutagenic agents as the inducers, which in the literature are often dubbed as “epigenetic carcinogens or agents” [318-323], “non-genotoxic carcinogens” [322, 323], or “cocarcinogens” [21, 324-326]. In our opinion, carcinogenesis in this group often incepts with promotion, but not with initiation, unlike that in the aforesaid group. This is because in this group, the nongenotoxic inducer cannot cause mutation(s) to establish initiated cells at an early time point, and thus the early proliferative lesions are not of initiated cells. Alternatively, there is some evidence suggesting that initiation might not involve mutations [181, 327-334], which might be the case in this group of animal models. Initiated cells are established much later by stochastically occurring mutation(s) during the relentless proliferation of normal cells induced by the inducer. In our opinion, which still awaits experimental corroboration, lesions turn to be authentically neoplastic, especially malignant, much later in these animal models than in those involving potent mutagens as the inducers.

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Why do lesions depend on the inducer and what does it mean?

Although the two groups of animal models described above differ in the potency of initiation, they all provide potent promotion, the essence of which is to drive cell replication, and they all can induce malignant tumors at a high incidence eventually. During promotion, the inducers function as aggressive coercers of the target cells, forcing them to replicate robustly and to manifest neoplastic, often malignant, cellular and histological morphologies with or without aggressive behaviors. In other words, an ominous morphology with or without diabolical behaviors occurs neither because mutation(s) responsible for immortality and autonomy have occurred nor as a repercussion of mutation(s) responsible for neoplastic morphologies and behaviors. It occurs simply under the inducer's duress, and thus the lesion disappears once the duress ends, because the lesion's cells still have allegiance to the animal's body. This remaining allegiance bespeaks the untransformed or non-autonomous nature, categorizes the lesion to hyperplasia, and is the reason for why the lesion's cells regress via apoptosis after the coercion is removed, so that the host organ or tissue does not have cell redundancy, as we have expounded before for what apoptosis really is [175, 178, 189, 305-308, 335]. Actually, we infer that the lesions may manifest a higher rate of apoptosis than their normal surrounding tissues because of their hyperplastic nature, although probably most, if not all, of the inducers may have a function to suppress apoptosis of the hyperplastic cells as part of their coercive mechanism. In an analogy, nice people may commit outrageous crimes if they are coerced, but they may still have good hearts and may become model citizens again once they are unfettered from the duress. However, if a person is incessantly coerced to commit crimes one after another, he may eventually evolve into a real criminal. The inducers prod cells into relentless proliferation while restraining them from committing apoptosis, which together will sooner or later lead to the genetic mutations responsible for immortality and autonomy as well as for neoplastic morphology and behaviors, making the lesions truly neoplastic. In conclusion, judging lawbreakers simply by the crimes they have committed is not completely right, and the reasons behind their criminal activities need to be taken into consideration. Similarly, diagnosis of outgrowths induced in animals should not solely rest on the pathological morphology. This "coercion hypothesis", proposed by us a few years ago [336] and recently [32] on the essence of animal models of carcinogenesis, deserves experimental testing.

Immortality and autonomy may occur early in most human lesions but occur late in animal models

When are immortality and autonomy established during a lengthy tumorigenesis in humans? It is an enthralling brainteaser, so far without a clear answer. For several reasons we infer that they occur at an early time point (Fig 2). First, spontaneous regression of tumors, many of which are diagnosed at early

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stages, is rare and thus nearly all tumors are considered immortal. Second, in our pathology service and in the literature, we occasionally encounter very tiny malignant tumors in patients, in most cases found fortuitously. Albeit the tiny tumor had already been surgically removed, some patients still died of its metastases years later, which substantiates the malignant authenticity of the primary tumor. Third, autopsies of humans that died of various causes found about 3-27% of the bodies had an occult pituitary adenoma [337-341] (and DJ Liao's empirical knowledge), and magnetic resonance imaging of normal human volunteers found this tumor in about 10% of normal persons [342]. Similarly, it has also been known since 1934 that a large number of men over 40 years of ages have occult prostate adenomas or adenocarcinomas, although many of the lesions do not develop to clinical cancer before the men die from other reasons [343-345]. Nevertheless, more tangible proofs for the speculative early-establishment of immortality and autonomy are still wanting. In humans, tumor-promoting momentum is much weaker, including the thrust provided by those relatively potent promoters such as chronic viral hepatitis, compared with that provided in various animal models. Therefore, human lesions grow and progress much more slowly, allowing immortality and autonomy to occur much earlier with respect to the size of the lesions, and allowing the neoplastic transformation to occur as the result of some relevant mutation(s), long before the patients feel something wrong and go to see their doctors. This is partly because a lengthier course allows accumulation of more haphazardly-occurring mutations, including the one(s) required for immortality and autonomy, if we accepted the notion that tumors, especially cancers, occur as repercussions of mutations that have cell-autonomous modes of action [17-19, 328, 329, 346-353]. Reiterated, because in a sporadic human carcinogenesis the promoting impetus provided by the ascribed factors such as cigarette smoking or chronic hepatitis is weak, the course is lengthy, which allows mutations to occur in a random and spontaneous manner as additional factors to contribute to the carcinogenesis.

The undeniable fact that immortality and autonomy occur only in an advanced stage in many, if not most or all, animal models annunciates that few, if at all, animal models established so far can reflect most cases of sporadic carcinogenesis in humans, germane to the time point for the establishment of immortality and autonomy (Fig 2), no matter how much the lesions' morphologies resemble their human counterparts. Fortunately, many animal models still nicely reflect some rare human situations in which immortality and autonomy are established in a late stage. For example, familial colorectal polyps that will sooner or later progress to cancer are developed due to inherited mutations in some genes, like the APC (adenomatous polyposis coli) gene [354-358]. The constant presence of the mutation serves as a durative coercion on colorectal mucal cells, keeping them in an unremitting state of proliferation to form polyps.

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These polyps are considered in pathology as premalignant lesions, based on their morphology and on the fact that cancer likely ensues. Notwithstanding, we are curious about whether the polyps would regress if we have a way to correct the mutation. Probably, from the point of immortality and autonomy, the polyps are just "preneoplastic", an elegant jargon used by Haddow [54] and Rubin [359], or are "precursor lesions", another good appellation used by Farber [360]. Other good examples of late establishment of immortality and autonomy in human outgrowths include the abovementioned hepatomas and hepatocellular carcinomas caused by long-term consumption of oral contraceptives [280-285], as well as lymphomas or leukemia caused by HP [290-296] or HTLV-1 [297-299], as these lesions at a relative early time point can be cured with removal of the inducer. It has also been suspected that some thyroid tumors may not be immortal and will probably not evolve to authentic neoplasm until the patients die of other causes [361], which dovetails with Forth's opinion in 1953 [132].

Tissue culture and transplantation once were major methods to determine immortality and autonomy

As aforementioned, even over a century ago whether or not a patient's tumor was immortal and autonomous had been a concern of, and thus often tested by, surgical pathologists, because they had realized that morphological traits should not be the only criteria, and the tumor's behavior should also be considered, for an infallible diagnosis of cancer. The tests had been conducted, ever since 1901 [362, 363], mainly by culturing surgically removed tumor tissues in dishes or by transplanting the tissues to animals, the two modern techniques aforetime. Actually, a technique involving both transplantation and culture is to inoculate tumor cells into a fertile egg and then hatch it [364-374], which is the parentage of some modern chick embryo assays for cancer studies [375-387], such as the chick heart invasion assay [388-393]. The rationale for using tissue culture is that all neoplastic cells in a tumor are immortal and can self-renew to forever maintain the tumor as a "new organism" by incessant cell division. Even after the patient has died, the "organism" can be maintained as cell lines. Indeed, the Hela cell line was established in 1951 from the cervical cancer of the late patient Henrietta Lacks [394], and is still widely used today in biomedical research. Therefore, the essence of this approach is to test tumor cells' immortality or self-renewal ability. With this regard, in many cases those "transformed" cells that can be reversed back to the normal in cell culture, as mentioned by Harry Rubin and Andrew Rubin [395], may not have reached a truly neoplastic state.

Tumor tissue transplantation to animals, since it was started by Hanau in 1889 [396], has been heavily used in cancer research, as extensively reviewed many decades ago [396-411]. The rationale for using this approach to determine a tumor's authenticity is to observe the tumor cells' behaviors, mainly autonomy.

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Greene was one of the pioneers in this line of work during the 1940-1960s [179, 412-419]. He elegantly selected the brain or the anterior chamber of an eye of animals as the recipient site. As shown in table 1, transplantations of animal tumors can generally be divided into five different types [179, 413], i.e. 1) autologous transplantation, or transfer back elsewhere in the same animal; 2) homologous I transplantation, or transfer to a tumor-bearing animal of the same species; 3) homologous II transplantation, or transfer to a normal animal of the same species; 4) heterologous I transplantation, or transfer to a tumor-bearing animal of a different species; and 5) heterologous II transplantation, or transfer to a normal animal of a different species. Obviously, transplantation of human tumors can only be done heterologously.

A seminal finding by Greene et al. in the 1940s is that some cancers are not transplantable to normal animals but are transplantable to animals that bear a spontaneous tumor, especially one of the same tissue origin [179, 413]. For instance, the Brown-Pearce rabbit tumor typically does not grow in normal C3H mice but it grows rapidly in those C3H mice bearing spontaneous tumors [414], and Rous chicken sarcoma grows upon subcutaneous transfer to tumor-bearing C3H mice but growths have not been observed in normal C3H mice [179]. These results led Greene to a conclusion that the factors affecting the take of transplanted tumors "are constitutional in distribution and are not localized at the site of the primary growth" [412]. As exceptions, lymphoblastic leukemia and lymphosarcomas are graftable to every normal genetically compatible host but do not produce tumors in the anterior chamber of an eye of an alien host, showing difference from other solid tumors [179]. The difference between normal and tumor-bearing hosts in response to a tumor graft suggests that tumor-bearing animals possess some factors affecting the graft's survival. A possible interpretation is that the spontaneous tumor preexisting in the host has already suppressed the host's immune function that is supposed to reject the graft. Studies of these inhibitory effects of the tumor-bearing host on the grafted tumors have later been extended to the studies on the interaction between normal cells and tumor cells, not only in vivo but also in vitro [420-429], as has been reviewed by us [302], by Rubin [430-433], by Aktipis [434, 435] and by Thomas et al [436-438] from different slants. For instance, it has been shown that normal cells suppress the growth of adjacent tumor cells in culture [439] and in skin grafts on mice [440]. Unfortunately, identifying these tumor or host factors has largely been neglected, although it is important because manipulation of these factors may be helpful in curing cancer.

Another trailblazing finding by Greene et al. in the 1940s is that the malignant tumors that have acquired an ability to metastasize are heterologously transplantable, i.e. can be transplanted to host animals of a different species, whereas cancers that are still incapable of metastasizing cannot [416, 418],

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as has been tested for many human tumors [413, 416, 418, 419]. Based on these observations, Greene concluded that "cancer is not a sudden transformation of normal cells but, on the contrary, represents the final step in a development process". He also concluded that only those lesions which can metastasize are fully autonomous and can be regarded as cancers, whereas those which have not yet possessed this ability are still conditionally autonomous and thus should not be regarded as malignancy [416, 418]. Although in pathology textbooks metastasis is not a required criterion for diagnosis of a malignancy, it is the only reliable yardstick to distinguish malignant neoplasms from benign ones [441]. Unfortunately, as a practical matter, clinicians cannot put a hold onto their diagnosis for months or years until they have seen whether or not their patients develop metastatic lesions. Considering that even today, compared with Green's epoch, in the surgical pathology service we still do not have a simpler or more reliable approach to determine whether a primary tumor removed from a patient has encompassed the ability to metastasize, it is a pity that Greene's simple but reliable test has not been routinely used in clinical service until now. Probably, a graft into an animal's eye may be an ethical concern, and thus a constraint, today.

What do the two genetic hits do in carcinogenesis, and is a third hit needed?

The traditional "two-hit" theory [9] may hold true in many animal models, if we accept the century-old concepts that carcinogenesis results from genetic alterations and that cancer cells owe their properties to mutations [430, 442, 443], although the concepts may not necessarily be true and, actually, continue receiving arguments [181, 334, 395]. The first genetic hit is responsible for the establishment of initiated cells that differ from their surrounding uninitiated cells in the response to the promoting environment. In Rubin's opinion, the cells in the skin papillomas produced in the aforementioned animal models that regress upon withdrawal of the inducer are initiated ones [431]. According to Farber [26-29, 316, 317, 360, 444-449], in most cases promoting agents or regimens cause "mitoinhibition", i.e. inhibition of mitosis or proliferation, of normal cells, whereas initiated cells are resistant to this inhibitory effect (Fig 1) [186]. Actually, in cell culture, a condition disfavoring cell growth, such as a lower serum concentration or a cell confluence, is more potent in driving neoplastic transformation as well [269]. Therefore, in a promoting environment, probably also in the human [269], only initiated cells can robustly proliferate to form lesions, especially when many of their adjacent normal cells die and the organ or tissue has a strong demand for a compensatory regeneration [186, 187, 450]. This "mitoinhibiton" theory is accordant with the hypothesis of Rozhok and DeGregori that cancer occurs more often in old age because normal cells in the young have a stronger capacity of proliferation, thus being less "mitoinhibited" and providing a weaker promoting impetus, whereas the situation in the old is the opposite [12], making the spontaneously-

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occurring initiated cells need to undergo a more robust compensatory proliferation. The molecular mechanisms of promotion via mitoinhibition still remain enshrouded. We extrapolate, sans evidence though, that mitoinhibited normal cells promote proliferation of initiated cells in part via a mechanism identical to that used by senescent cells to promote carcinogenesis of their adjacent cells, since senescence is a state of permanent growth arrest [451-455], i.e. “mitoinhibition”. This mechanism is coined as SASP (senescence-associated secretory phenotype) [456-458], and its effect on carcinogenesis has been extensively reviewed in the literature [452, 459-467].

In our meditation, the second hit is responsible for creating immortality and autonomy, thus establishing neoplastic cells, benign or malignant. This second hit occurs in a later promotion stage of the “initiation-promotion” models or in the progression stage of the “initiation-promotion-progression” models. In sporadic carcinogenesis in humans, initiated cells may also exist, although they are technically difficult to identify. Nevertheless, “preneoplastic” cells in humans may have already experienced the first hit, while “pre-cancerous cells”, which may or may not be benign, may have also experienced the second hit, in our opinion. Genotoxic agents or regimens can quickly establish initiation but have no way to establish immortality and autonomy until a late stage. Indeed, so far there has not been any animal model of sporadic carcinogenesis suggesting an early establishment of immortality and autonomy. While this fact remains to be an enchanting mystery, it insinuates that animal cells put more guards on the genes relevant to immortality and autonomy, compared to the guards on the genes relevant to initiation, meaning that the first hit is easy, but the second is difficult. Actually, so far no exogenous agent, whether a chemical, an irradiation or whatsoever, has been identified that can break through the second defensive line of cells to directly cause mutations to establish immortality and autonomy. A lengthy promotive period in all animal models of sporadic carcinogenesis established so far evinces that this breaking-through can only be made by currently-unknown intrinsic factor(s), although our manipulations can instigate the factor(s). Probably, one day we may identify the relevant genes and in turn find a way to break through this defensive line.

In some carcinogenic procedures wherein a malignancy does not require a benign lesion as a precursor and thus a second hit is sufficient, the mutation(s) responsible for immortality and autonomy may also be responsible for malignant morphologies and behaviors (Fig 3). However, in other animal models and human situations, the mutations for establishing immortality and autonomy may not be the ones for establishing malignant morphologies and behaviors, since benign neoplasms have also experienced the second hit. Therefore, in these situations a third genetic hit may be required to establish malignant morphologies and behaviors (Fig 3). Of course, malignant neoplasms continue evolving via the

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fourth, fifth and subsequent hits to be more and more heinous, but this is irrelevant to the issue described here.

An old question: how many mutations are needed to complete a carcinogenesis?

The target or targets of the three genetic hits remain unknown to us. Initiation created by the first hit likely involves only one or several genes, since initiated cells are morphologically indistinguishable from uninitiated ones [27, 29, 317, 468]. Immortality and autonomy created by the second hit may involve only one or several genes as well. This conjecture is based on the observation in our pathology service that many benign tumor cells, such as uterine leiomyoma cells, are quite similar to, basically indistinguishable from, their normal counterparts in cellular morphology. These types of benign tumors can only be diagnosed by their macroscopic and histological traits, and not by their cellular morphology. If mutations in many genes are involved, cellular morphology should change. Moreover, immortalization of a mortal cell to establish a cell line has been proven to be easy, especially in vitro [116, 469, 470]. It is even easier when the cell has a small rodent parentage, presumably because these rodent cells have their telomerase constantly "on" and have only a single barrier to immortalization controlled by the RB (retinoblastoma protein) pathway [469, 471, 472]. For instance, targeting both the p16ink4 and c-myc genes can immortalize human mammary epithelial cells [473], and the IgEGF and SV40T bi-transgenes can immortalize mouse cells [474]. Actually, even just knocking out the p53 gene alone can immortalize mouse hepatocytes [474]. The third hit, if it is needed, may occur to more genes compared to the first two, since cellular and histological morphologies and behaviors can vary greatly among different cancer cases. Nevertheless, the sum of the three hits may still be congruent with the estimation by Armitage and Doll in 1954 [19] and by Vogestein in 1993 [475] that carcinogenesis requires only six or seven mutations. Liquid cancers such as leukemia may require even fewer mutations and thus may be relatively easier to cure, generally speaking, as to be explained later. A caveat is that different hits in different cancer cases may involve different genes, making the sum of "initiator genes", "immortalizer genes" or "malignant morphology responsible genes" large, and the sum of all three even larger. This feature, dubbed as cancer heterogeneity, is a major reason why such a large number of genes have been found to be cancer-relevant.

Are immortality and autonomy independent of each other?

In 1983 Land et al showed that a mutant ras gene could transform embryonic fibroblasts without immortalizing the cells, and immortalization of the transformed cells could later be achieved by an ectopic expression of the c-myc or a viral oncogene [476]. Concomitant expression of the CDK4 gene and a mutant

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ras can transform cells, as evidenced by the facts that the cells can form colonies in soft agar and develop into invasive tumors in animals; however, the "transformed" cells remain mortal as evidenced by their limited passages in culture [477]. These and other studies show that transformation and immortality can be extricated from each other. However, this segregation is not discerned in some tests with traditional approaches involving chemical carcinogens, such as the "Syrian hamster embryo cell transformation assay" (the so-called SHE assay) [478, 479], and conflicts with the opinion of Newbold et al that immortality is an early and prerequisite step of transformation [480-484]. This discrepancy among different studies with different approaches makes the reliability of the criteria for in vitro transformation [116, 485] dubious to us, because in human situations immortality and autonomy are inextricable from each other. Moreover, in vitro transformation is often reversible [395], which also collides with the aforementioned fact that human cancers rarely regress spontaneously. Probably, a gene like a mutant ras can coerce cells into behavior like being transformed in vitro without really transforming them by mutating the relevant genes, just like variegated inducers of carcinogenesis in animal models described earlier in this essay, although such mutations and true transformation are corollaries of the incessant coercion. In other words, ectopic expression of an oncogene or some other in vitro genetic manipulation may just function as a coercer to the cells, i.e. resemble the inducers in animal models that coerce the target cells in the animals into manifesting neoplastic morphology. Moreover, although malignant tumor cells dissected from patients or animals may indeed form colonies in soft agar, which is an in vitro test for transformation established by Freedman in 1974 [485] and has been widely-used, the other way around may not always be correct, meaning that forming colonies in soft agar may not necessarily be a token of a transformed, i.e. malignant, state. In our opinion, if an in vitro study shows that immortality and autonomy are extricable, more tenable proofs for the neoplastic state may be needed. This is to say that once the cells are shown to be capable of forming colonies in soft agar, they need to be tested for immortality before we can announce that they have been transformed. Unfortunately, few published studies of in vitro transformation show this additional evidence.

We still lack a good strategy to determine molecular pathways leading normal cells to cancers

Not only the genes mediating in the three genetic hits described above, but also the molecular pathways regulated by these genes, remain unknown. One of the reasons is that we have been encountering a logical plight for decades regarding our research strategies and approaches, as repeatedly pointed out by us before [32, 175, 336]: The results from the approaches we used, such as genetic engineering, can only tell us that certain manipulations or alterations, like concomitant overexpression of

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the c-myc and a k-ras mutant, and the ensuing cascade of molecular changes, can cause cellular immortality and autonomy, i.e. neoplastic transformation. However, we still do not know whether cells in humans or in untreated animals spontaneously develop to neoplasms really via these cascades of changes. In an analogy, we have built the highway Interstate-95 (I-95) and know that Mr. Trump can go from New York City (NYC) to Washington DC by taking it, but we do not actually know whether this is indeed the path, but not others, he took. If we cannot come up with a novel strategy to solve this tribulation, our attempt to learn why and how some cells in humans become neoplastic will continue to be prodigal financially and in effort, because we will continue identifying (more correctly, creating) many more molecular pathways leading normal cells to neoplasms, besides the many pathways already known [486], while remaining unable to hold any particular pathway(s) accountable for sporadic carcinogenesis in humans. Restated, we are creating, but not identifying or discovering, pathways by such as creating otherwise non-existing transgenic mice, and assume that these man-made paths are the carcinogenic procedures occurring in patients' bodies. In another analogy, we already have many paths leading from NYC to DC but will endlessly build many more while remaining unable to know which path(s) Mr. Trump took or will take. Probably, we have been upending things or putting the cart before the horse in our research.

Many animal models are overpraised, due to neglect of the immortality issue

As aforementioned, evidence for immortality and autonomy has not been provided for the lesions resulting from the vast majority of genetically-manipulated animal models of carcinogenesis. This is an uncomfortable but undeniable flaw of the relevant studies, although we should somewhat be content with the enormous amount of information provided by these models on the functions and underlying mechanisms of the genes manipulated. It is possible that many peers have already harvested the "cancers" from their host animals for the mechanistic analyses before the lesions, probably large in size, have evolved to genuine neoplasms. This slip is made probably because the surgical pathologists involved made their diagnoses solely based on their experience in human tumor pathology, unlike their predecessors such as Greene and Furth who were familiar with the inducer-dependency of animals' lesions. Actually, although human tumor pathology is generally reliable in reflecting the neoplasms' prognosis, as proven by over a century of clinical practice, the lesion's behavior should be taken as an additional criterion for diagnosis. Greene had pointed it out in 1947 by saying that "the problem of cancer is primarily a problem of behavior. A pathologist who examines tumor tissue under the microscope may observe significant details of form and structure, but he can never determine its malignancy from its appearance alone; only

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by its behavior in the living body can malignant tissue be unmistakably identified. Of two tumors with cells that look exactly alike, one may remain static or even disappear while the other inexorably spreads and kills the patient. Unfortunately many kinds and conditions of tissue which are not malignant bear a remarkable resemblance to cancer" [413]. This extra criterion is especially needed in diagnosis of outgrowths induced in animals because they differ greatly from their human counterparts in two particular aspects. First, immortality and autonomy may not have been established when the lesions are harvested from the animals. Therefore, these animal "cancers", even large in size, can be cured easily, simply by withdrawal of the inducer or by chirurgic removal, whereas most human cancers are not curable, at least not so easily. Second, "malignant" tumors in many animal models, such as mammary tumors from the MMTV-c-myc transgenic mice we have used [184, 188], do not metastasize within the lifespan of the animals, which starkly contrasts most human cancers that, if untreated, will metastasize. Some tumors from animal models can metastasize, but the metastases may still be inducer-dependent. Although this speculation has not been broadly tested, examples have been described herein, like the abdominal metastases from estrogen-induced renal tumors in hamsters [134-136] (and also DJ Liao's own experience), and the lung metastases from TSH-dependent thyroid tumors [132, 155, 159]. In a nutshell, the mortality and non-autonomy, the inducer-dependency, and the inability to metastasize are telltale evidence that many animal cancers are easily curable and thus are disarming, which starkly contrasts with most human cancers. An overarching theme in the second phase of carcinogenesis research had already been to determine immortality and autonomy of the lesions induced in animals, which, unfortunately, had been abandoned about three decades ago when the research entered into its third incarnation by shifting from chemical-induced to genetically-manipulated carcinogenesis. Therefore, there is an exigency to reincarnate this task.

Neglecting immortality causes confusion on aging-caused cell death in outgrowths

Normal animal cells undergo aging and eventually die of it [303, 307, 487-497], and so do cells in overt lesions from many animal models, which of course occurs more often in morphologically-benign than in morphologically-malignant ones [492, 494-496]. We define cell death via aging as "senescent death" (SD) [177], because normal cells have a lifespan [480-484, 498] and because, ever since it was first observed in 1965, this senescent phenomenon has been immediately linked to aging [499, 500]. Indeed, a host of studies have shown that aging and senescence are highly interrelated [12, 452-455, 500-509], although senescence itself is defined as a permanent growth arrest that does not necessarily lead to death of the cell [451-455]. This type of cell death has established "cancer cell senescence" as a popular new

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research bailiwick [307], although many relevant studies do not involve lesions from animals but, peculiarly, use human cancer cell lines that are immortal. Because its essence is "dies from aging", the "cancer cell senescence" concept is illogical and against the immortality and autonomy criteria of neoplastic cells, as it connotes that immortal cells still undergo aging and eventually die of it. In our logic, neoplastic cells are immortal and thus cannot undergo senescence and eventually die from it, whereas cells that can undergo senescence and die from it cannot be regarded as neoplastic no matter how much their morphology and behavior resemble those of neoplastic cells. In all those lesions that are morphologically malignant and even metastatic but are still inducer-dependent, such as the TSH-dependent thyroid tumors and their metastases [132, 148, 159], cells may undergo aging and die via the SD mechanism because they have not yet been authentically neoplastic.

Neglecting immortality causes confusion on cancer stem cells

In the past 20 years or so, "cancer stem cell" (CSC) has become a new popular research province, although the definition of CSC remains erratic or, in the words of Dumont et al [510, 511], fuzzy and evolving. Indeed, its concept in the literature has never been lucid in distinguishing CSCs on the one hand from normal stem cells and on the other hand from ordinary cancer cells [512]. The initial CSC concept was derived from some observations in the 1990s that many leukemia cells showed different extents of differentiation, leading to the theory that neoplasms might be derived from transformed undifferentiated pluripotent stem cells [513-515]. This concept annotates CSCs as those organ- or tissue-specific stem cells that somehow go awry, presumably due to mutation(s), and evolve to cancers [487, 516-518], although sometimes they are also called "transformed stem cells" [519]. According to a denomination of carcinogenic mechanisms, in a renewable cell type a stem cell that has gone awry may stop differentiation during an embryonic stage or during a tissue regenerative procedure, and continues proliferating to form a neoplasm, as we described before [302, 336]. Actually, this "stop-differentiation" mechanism is presumably a reason why nonrenewable cell types still develop childhood neoplasms: Pediatric tumorigenesis, instigated by such reasons as in utero exposure to a carcinogen [520], had already incepted during an embryonic stage when the cells still had their replicative ability [302], and might occur via a stop-differentiation mechanism. For this reason, we have suggested that molecular biologists should be wary of using those DNA elements that are activated during an embryonic stage [185, 191], such as the Mist-1 promoter [521, 522], as the promoters to drive transgenes, because the resulting transgenic animal models of carcinogenesis mimic only formation of childhood cancer via this "stop-differentiation" mechanism, whereas most cancers in humans are sporadic [185, 191].

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With the number of CSC studies soaring, CSCs seem to have been redefined. In Clarke's words, "…a subset of cancer cells within some tumors, the so-called cancer stem cells, may drive the growth and metastasis of these tumors" [515]. More detailed by Chiodi, "in many types of cancers a subset of cells shows peculiar characteristics, such as the ability to induce tumors when engrafted into host animals, self-renew and being immortal, and give rise to a differentiated progeny. These cells have been defined as cancer stem cells (CSCs) or tumor initiating cells" [523]. Similarly, in the words of Weinberg's group, "the CSC hypothesis posits the existence of subpopulations of neoplastic cells within a tumor that exhibit an elevated ability to seed new tumors upon experimental implantation in appropriate animal hosts" [524]. By this definition, which is widely used in many publications [321, 516, 525-529], CSC is a diminutive group of cancer cells in the cancer mass, but not the single normal stem cell that undergoes a carcinogenic procedure to evolve to a cancer. Indeed, Weinberg's group clearly separates these two types of stem cells by saying "evidence is accumulating that both normal and fully neoplastic cell populations harbor subpopulations of stem cells (SC) that can both self-renew and spawn more differentiated progeny" [530]. This definition hints slyly that except a tiny fraction of cells in a cancer mass, the vast majority of cancer cells are not immortal and are not able to self-renew, which is obviously against the definition of neoplasm in all pathology textbooks published since the 1900s.

Benign and malignant cells relentlessly undergo symmetrical binary fission, just like bacterial cells that unremittingly divide without a clear distinction between somatic line and germ-line, to maintain their strains, although some pundits consider that bacterial cells also undergo asymmetrical division and thus undergo aging as well [531-533], probably in part for maintaining their vitality [534]. Moreover, malignant cells are highly plastic and can differentiate to various cell types [513, 535]. For instance, quite different types of cancer of epithelial origin [536-546], and even pre-cancerous lesions [547], manifest bone histology, or osseous metaplasia in pathological phraseology. Therefore, a strong pluripotent ability should not be used to dichotomize cancer cells to CSC and non-CSC groups. It is true that in cancers many cells die at a much higher rate than their surrounding normal counterparts due to various stressors, such as insufficient oxygen or nutrient nourishment or overly severe genetic damage [349, 350, 548]. The opposite is also true that in a cancer mass some cells' ability of self-renewal via symmetrical division is much more potent than that of the others. Actually, many ancient studies, started by Furth and Kahn in 1937 [549], have already shown that single cancer cells in late progression stages were highly transplantable and could grow rapidly in recipient animals [550-554], as extensively reviewed by George Klein six decades ago [397]. However, these quantitative differences simply reflect the well-known heterogeneity of malignant cells [433, 555, 556], which is largely ascribed to the stemness of some cells

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[557] or great genetic variation among most cells [548, 558], and should not be used to split cells into CSC and non-CSC groups either. More critically, "ability of self-renewal *vs* inability of self-renewal" is actually "immortality *vs* mortality", which is a black-white demarcation between neoplasms and non-neoplasms and thus should not be used again as the demarcation between CSCs and non-CSCs. We proffer that, since the above-defined CSCs differ from other cancer cells only quantitatively in such as the competency of self-renewal, metastasis, therapy-resistance, etc., clear quantitative parameters in these vicious behaviors should be established to separate those highly-competent cells, tagged as CSCs or not, from those less-competent counterparts, just like the establishment of the normal ranges for blood pressure, blood sugar, etc., as criteria for diagnosis. Identification of biomarkers for these cells, as performed by many cancer students now, is part of this line of work. Once these quantitative parameters have been set as criteria, these heinous cells can be more easily identified and studied for their behaviors in the aspects of chemotherapy, metastasis, patients' prognosis, etc., without calling them CSCs. For example, it is unnecessary to call "CD44(+)/CD24(-) and ALDH1(+)" breast cancer cells as CSC [559-561], since "CD44(+)/CD24(-) and ALDH1(+)" defines them more specifically and clearly than the equivocal "CSC".

It needs to be mentioned that many other studies prefer not to provide a pellucid CSC definition, such as the one by Lapinska et al [562], but, instead, to sway between the two different definitions described above. A few others, such as Chaffer and Weinberg [530], consider that CSCs may be derived from tissue-specific normal stem cells in a stepwise manner, which elegantly accommodates the second CSC definition into the first one described above but still indirectly suggests that only CSCs, and not the vast majority of cancer cells in the lump, can self-renew, and thus still denies immortality as an indispensable criterion for a neoplastic state.

Where lies the demarcation between benign and malignant neoplasms?

Not only have immortality and autonomy as a lucid demarcation between non-neoplastic and neoplastic cells been neglected by many of today's cancer students, including some pundits, but also the demarcation between benign and malignant neoplasms has often been blurred. For instance, most of "cancer hallmarks" described by Hanahan and Weinberg [563, 564] are actually marks of tumors, including the benign ones, but not of cancer, as pointed out first by Lazebnik [441] and later by us [336]. Actually, Blagosklonny's comment is more frank and straightforward: "…hallmarks can be observed without cancer" [565]. In our rumination, a genetic criterion to segregate the two is: Benign neoplasms do not bear mutation(s) at mutator gene(s), i.e. the gene(s) some alteration(s) of which allow mutations to occur to other genes. In contrast, malignant neoplasms bear mutation(s) at the mutator gene(s), and thus

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accumulation of more mutations in more genes is a corollary of the unremitting cell replication, which, in turn, is the genetic basis for tumor cells to continue to progress to more malignant states [302]. Of course, benign neoplastic cells are also immortal and thus keep replicating, which increases the chance for new mutations to occur. Once new mutations occur to mutator gene(s), the benign neoplasm gets a chance to progress to malignancy, which is a reason for why benign neoplasms are at risk for progression.

Malignant neoplasms have three behaviors distinguishable from their benign counterparts, i.e. 1) encroachment into their normal surrounding tissue, which can be considered as local metastases, 2) consumption of their normal surrounding tissue, partly via cannibalism at the cellular level [566-570], and 3) metastasis to distant body sites. However, none of these three is unique to malignant cells, as some normal cells have these behaviors as well [302, 571] and progression of solid tumors does not always follow the stepwise procedure of "growth, invasion and then metastasis" [302, 572]: Trophoblasts make inroads into the uterine wall to establish gestation and may even encroach into the blood vessels and home in on the lungs of the mother and many organs of the newborn [572]; the function of osteoclasts is to eat up bone tissue; and many bone-marrow-derived or thymus-derived cells can enter into the blood or lymphatic circulation and home in on almost all body sites. Probably because these normal cells already possess the ability to migrate to and home in on most body sites, i.e. to "metastasize", in all pathology textbooks all the neoplasms of the bone-marrow and lymphatic origins, without exception, are classified as malignancy. Actually, this property seems to have its good aspect, as liquid cancers may bear fewer mutations and be easier to cure than most solid cancers, generally speaking, because they inherit from their parental normal cells the ability to metastasize, i.e. to migrate to and home in on most body sites, and thus do not need additional mutation(s) for acquiring this ability. Bearing these bits of knowledge in mind, many "surprising findings" in animal models are actually not so surprising, such as the observations that epithelial cells can be evasive and disseminating and can enter into the bloodstream before they form primary tumors [573], that cancer cells can enter into the blood circulation before invading adjacent stroma [574], and that mammary epithelial cells can be manipulated to metastasize and colonize in the lungs before they are malignantly transformed [575, 576].

Concluding remarks

Over a century ago, pathologists had set immortality and autonomy as indispensable criteria for neoplasms, including the benign ones. It has been known ever since the 1910s that many, if not all or most, outgrowths resulting from experimental animals are inducer-dependent until very late stages. Unfortunately, indubitable evidence for the immortality and autonomy has not been provided for the

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lesions resulting from most genetically manipulated animal models of carcinogenesis at the time when the lesions are collected from the animals. Although these lesions have indeed provided us with sheer amount of information on the functions and underlying mechanisms of the manipulated genes, especially on the aspects of cell proliferation and cell death, the lack of this needful evidence still makes us apprehensive, as much confusion on the behaviors of these animal lesions may be caused by their neoplastic inauthenticity. Furth's opinion should be taken that autonomy has two categories: "conditional" and "full" [132]. "Conditional" should be confined within the physiological range with a physiological factor as the conditioner. For example, breast and prostate cancers are genuine, although castration, which decreases the corresponding hormone to a level below the physiological one, can cause their regression. However, the mammary and prostate tumors in experimental animals have not yet become authentic if they still require a continuous hormone treatment to maintain a pharmacological or toxicological level of the hormone for their sustenance. "Full autonomy" means that sustainment of the tumors does not require a physiological factor as a conditioner. For instance, breast and prostate cancers can sustain and continue to progress after castration. Since achievements obtained many decades ago in the second incarnation of carcinogenesis research had already set immortality and autonomy as cardinal criteria for neoplasms and had identified initiation, promotion and progression stages of carcinogenesis, we should, with numerous genetic models created, continue on identifying the genomic bases for immortality and autonomy and for initiation, promotion and progression. Future carcinogenesis studies need to embark on the following brainteasers: 1) What is the genomic basis for immortality or autonomy? 2) What is the genomic basis for initiation, promotion or progression, or for the first, second or third genetic hit outlined herein? 3) How are immortality and autonomy related to initiation, promotion and progression? 4) Which are good animal models for us to use in tackling the above three tasks? Concerns raised in this essay are obviously provocative but deserve reconsideration by cancer research gurus, especially those at the pinnacle.

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Abbreviations

APC, adenomatous polyposis coli; ACI, August strain crossed with Copenhagen strain; CSC, cancer stem cell; DC, Washington DC; DMBA, 7,12-diemthylbenz(a)anthracene; HTLV-1, human T cell lymphotropic virus type I; HP, helicobacter pylori; MMTV, mouse mammary tumor virus long terminal repeat; RB, retinoblastoma protein; NYC, New York City, SASP, senescence-associated secretory phenotype; SD, senescent death; SHE, Syrian hamster embryo cell transformation assay; TSH, thyroid stimulating hormone.

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Fig 1: The Solt-Farber's "resistant hepatocyte" model of liver carcinogenesis in the rat. A toxic dose of diethylnitrosamine (DEN) will 1) cause liver necrosis and 2) create initiated hepatocytes. Two weeks later, when the liver has recovered from the necrosis, the rat will be given a low dose of 2-acetylaminofluorene (AAF) for two weeks, function of which is to inhibit proliferation, so-called mitoinhibition, of hepatocytes, but initiated cells are resistant to this inhibition. In the middle of AAF treatment, hepatectomy will be performed to remove two-thirds of the liver, which provides a strong impetus for regeneration. Because normal hepatocytes are mitoinhibited, all regeneration pressure is imposed onto the initiated cells, driving them to proliferate robustly and form nodules. The image at the left shows these nodules immunohistochemically stained for the P form of glutathione S transferase, a marker for the nodular cells, in the three remaining lobes of the liver four weeks post cessation of AAF treatment [187, 577]. These nodules will regress afterwards but some new focal cells, which can proliferate spontaneously and are coined by Farber as "phenotype 4", will later develop at some of the sites of these nodules and progress to overt cancers [310, 311].

Fig 2: Illustration of a speculative difference at the time point for the establishment of immortality and autonomy between tumorigenesis in most animal models and that in most human situations. In humans, immortality (Immort.) and autonomy (Auto.) may occur at a very early time point, thus establishing small lesions as genuinely benign or malignant neoplasms. In contrast, tumorigenesis in most animal models is a stepwise procedure of initiation, promotion and, in some cases, progression as well. Initiated cells are still mortal and thus are not neoplastic. Immortality and autonomy in animal models occur at late promotion or at the progression.

Fig 3: Illustration of our three-hit hypothesis. To couple the traditional two-hit principle with the initiation-promotion theory, we meditate that the first genetic hit establishes initiated cells that are still mortal and non-autonomous, whereas the second hit creates immortality and autonomy that establish neoplastic cells, either benign or malignant. Since formation of benign neoplasms also requires two genetic hits, we extrapolate that, in some animal models and probably also in many human situations, establishment of malignant morphologies and behaviors requires a third hit on the relevant gene(s).

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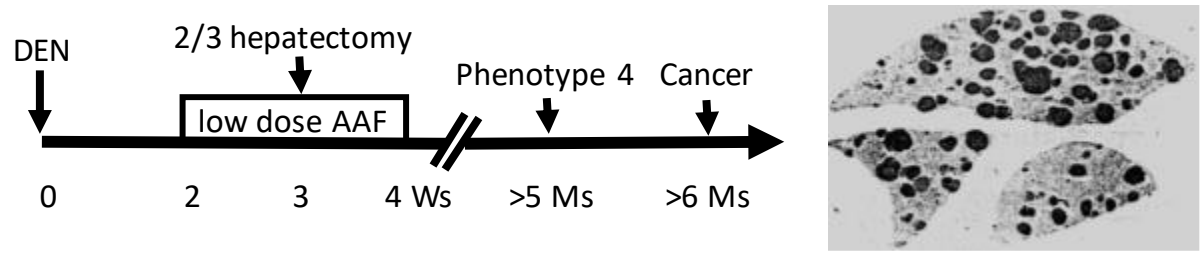

Figure 1

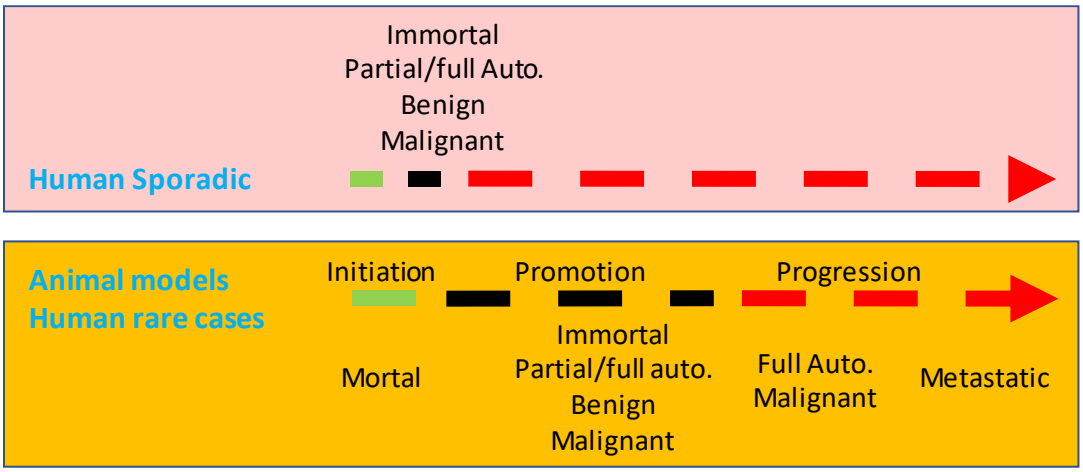

Figure 2

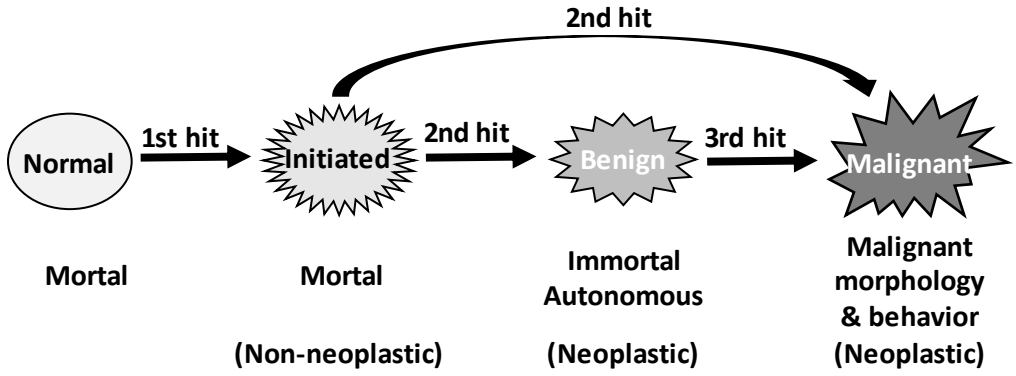

Figure 3