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Review Article

What are the Origins and Characteristics of Cancer Stem Cells?

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ABSTRACT

The two hypotheses of the origin of cancer are the "stem cell" hypothesis and the "Dedifferentiation" or "Re-programming" hypothesis. Cancers are derived from a single cell, with all the cells within the tumor exhibiting heterogeneity of genotypes and phenotypes. Cancer cells, which do not contact inhibit; have growth control; terminally differentiate; are"immortal"; also, do not have function gap junctional intercellular communication (GJIC).The interpretation of animal experiments has suggested that the carcinogenic process consists of three phases, namely, the "initiation" phase; the "promotion" phase and the "progression" phase. With the isolation of embryonic-, induced pluripotent-, somatic nuclear transfer- and adult - stem cells, observations have shown that stem cells express the embryonic gene, Oct4A, but not the gap junction genes. With the isolation of "side population" cells from cancer cell lines, which were shown to sustain the growth of tumors, the terms, "cancer-initiating" and "cancer stem cells", were born. Use of human adult stem cells, expressing Oct4A, were shown to give rise to "initiated" Oct4A- positive cells which, after further modification, could give rise to "cancer stem cells", whereas their differentiated daughters, not expressing Oct4A, never gave rise to "initiated" cells. There are "cancer stem cells" that do not express Oct4A. However, they do express their connexin gene but are unable to have functional GJIC. Therefore, the strategy to target "cancer stem cells" for cancer therapy must take recognize that there are two different "cancer stem cells" and therefore, two different approaches will have to be developed.

Keywords: Cancer stem cells, Adult, Organ-specific stem cells; Connexins, Gap junctions, Oct4A

Quote

"What clearly lies ahead is an era of research on intercellular communication at both local and systemic levels. The balance between proliferation and differentiation must be examined at the molecular level, with emphasis on the interaction between growth factors, growth inhibitors, and their receptors and ultimate targets." V.R. Potter, Biol.Med.2:243 (1981).

Introduction

What clearly lies ahead is an era of research on intercellular communication at both local and systemic levels. The balance between proliferation and differentiation must be examined at the molecular level, with emphasis on the interaction between growth factors, growth inhibitors, and their receptors and ultimate targets." V.R. Potter, Biol.Med.2:243 (1981).

A Complicated Origin of the Concept of the Cancer Stem Cell

While the concept of "cancer stem cells" is relatively recent, there seems to be multiple historic and multi-disciplinary contributions to this term. Not everyone in the field of cancer research will have the same definition of "cancer stem cells", let alone, have a universal understanding of their characteristics, origin or "biomarkers". Some even resist the term, "cancer stem cells" and use the term, "cancer initiating cells". Even today, the use of the term, "cancer cells", helps to confuse the distinction made today between "cancer stem cells" and "cancer non- stem cells" that comprise a cancerous tumor.

There have been some very distinct hypotheses as to the origin of cancers, and by implication, "cancer stem cells", namely, the Stem Cell hypothesis [1-9] versus the De-differentiation or more recently,

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the "Reprogramming" [10], hypothesis. In the stem cell hypothesis, long before the isolation of human embryonic stem cells [11,12], "induced pluripotent stem" cells [13], somatic nuclear transfer stem cells [14] or human organ- specific stem cells [15-23], various disciplines, such as cancer research, embryology, developmental biology, had conceived the concept of stem cells. The late Van R. Potter viewed cancer as "oncology as partially blocked ontogeny" [4], although he had no technical knowledge of any biologically-defined stem cell being the origin of a cancer, let alone the "cancer stem cell".

Ignoring, for the moment, early ideas as to how a normal cell ultimately became a cancer cell or a "cancer stem cells", some early biological characteristics of normal versus cancer cells will be used later to distinguish between two type types of "cancer stem cells" [24,25]. One of those early characteristics of a normal cell and a cancer cell was the notion that a "normal" cell was "mortal" and "contact inhibited" [26], while the cancer cell was "immortal" and non-contact inhibited [27]. Here is where one runs against a definition that has misled cancer researchers, namely, what is a "normal" mortal cell?

Recent attempts to induce, experimentally, neoplastic human cells by exposing primary in vitro cultures of human fibroblasts or epithelial cells to various "carcinogens", have failed [28-30]. Yet, when Land, et al. [31], exposed primary *in vitro* cultures to the myc oncogene, they obtained clones of non-cancerous cells which were "immortalized". When these cells were transfected with another oncogene (Ha-ras), they finally isolated metastatic cancer cells. Therefore, they helped to establish a powerful paradigm that has, to this day, influenced many to believe that the first step in the carcinogenic process was to "immortalize" a somatic differentiated "mortal" cell, which then could live long enough to accrue other needed genetic and epigenetic changes to acquire the "hallmarks of cancer" [32,33].

Before elaborating on the misinterpretation of this solid set of experiments, one must examine another early characterization of the carcinogenic process, namely, the multi-stage, multi-mechanism concept of carcinogenesis [34-36]. In addition, the notion that a tumor, containing billions of cells, albeit being of heterogeneous genetic and phenotypic types, actually were derived from a single "normal" cell [2,3]. Together, these two observations must be viewed together.

The first stage of this carcinogenic process had to be an irreversible event in a single "normal" cell, which is referred to as the initiation stage. While the irreversible event is only "operationally" defined, that single cell is now unable to differentiate or become "mortal". This initiation event either caused a "normal" stem cell to be blocked from terminal differentiation or it "re-programmed" a somatic differentiated cell to become an "embryonic-like stem cell". While at this stage, the underlying mechanism of initiation appeared to be a mutagenic event, it could only be assumed.

These "initiated" cells, next, had to be exposed to non-mutagenic agents, such as phorbol esters, DDT, phenobarbital, polybrominated biphenols, etc. [37-39], or growth conditions, such as growth hormones, growth factors, and cytokines [40-44], compensatory mitogenic conditions, such as wounding or massive cell killing [45], before an appearance of some benign lesions , such as a papilloma in the skin, enzyme altered foci of the liver, polyp of the colon or nodule in the breast could be seen. These benign lesions were clonally expanded "initiated" cells. This process of expanding the single initiated cell is the promotion phase [46], which occurs, operationally, by stimulating, mitogenically, the initiated cells and by preventing the apoptosis of these cells [47].

The final step, the "progression" step, operationally, brings about one of these clonally expanded "initiated" cells to acquire the phenotypes of invasiveness into other tissues and widespread metastatic spread [36]. Therefore, it appears that this "initiation", "promotion" and "progression" process, starts from an irreversible step occurring in a single cell, prevents it from terminally differentiating, and developing into to a malignant metastatic "cancer stem cell". Consequently, one must now examine what is that single normal cell that ultimately becomes the "cancer stem cell" and what are the distinguishing characteristics of the "cancer stem cell" from the "cancer non-stem cell" that might provide targets for prevention and treatment.

What is that Single "Normal" Cell that is the Target Cell that becomes the "Cancer Stem Cell"?

In an important, but rarely cited, paper had shown that, using baby Syrian hamster embryo cells, one could only neoplastically transform primary *in vitro* cultures of these cells if, in the population, there existed a few morphologically distinct cells that seemed to be unable to "contact inhibit" [48]. When Loewenstein and Kanno [49] and Borek and Sacks [27] showed that normal cells could contact inhibit but cancer cells could not, a major clue was discovered. Cancer cells had no growth control, could not terminally differentiate, could not apoptose, but they had extended life spans or were "immortal". These cells also had no gap junctional intercellular communication (GJIC). These three basic cellular functions in "normal" cells were associated with functional gap junctions [50]. Therefore, "normal"cells refer to either (a) normal adult organ-specific stem cells, which do not express connexin genes or have functional GJIC [51], or (b) normal progenitor cells that have expressed gap junctions and functional GJIC. It must be remembered that free standing normal non- stem cells or progenitor cells do not need gap junctions, as these gap junctions are not only communicating channels between conjoined cells, they also contribute to adherence of cells. Also, terminally differentiated cells might or might not have expressed connexins or have functional GJIC. Hepatocytes have functional gap junctions, whereas red blood cells do not. Important, also, to note is that, while normal stem cells do not express connexin genes or have functional gap junctions, they are growth controlled by either or both the extracellular matrix proteins in their niches [52] or by soluble growth factors from other differentiated tissue cells [53-55].

When "normal" stem cells, those cells that were isolated, showed that they were embryonic-like because, in their undifferentiated state, they did not have functional gap junctional intercellular communication [51]. This conclusion was made because they were isolated and perpetuated on mitogenically- suppressed "feeder layer" of fibroblasts [15]. If the isolated or "iPS" (induced pluripotent stem cells) or human adult organ-specific stem cells had functional GJIC, they would communicate with the underlying cells of the monolayer feeder layer cells, that have functional GJIC, and be "contact-inhibited" [15]. Only cancer cells, which lack GJIC, can growth on these feeder layers [15]. In fact, normal human adult kidney, normal human breast [16] and lens [20] adult stem cells have been shown to lack GJIC and the expression of their connexin (gap junction) genes.

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One must now explain what is meant by the word "normal" cell. If the "normal" cell is an adult organ specific-adult stem cell, it is a cell that exist in all organs that can, depending on external conditions, divide either by symmetrical cell division to give two daughters that maintains "self- renewal" properties or by asymmetrical cell division that gives rise to one daughter that maintains self-renewal property and one daughter that can go down the pathway of terminal differentiation [56].

Evidence of Unique Cancer Cells in a Tumor that Sustains Tumorigenicity

There are several observations during the early days of determining if some agent might be able to neoplastically transform normal cells. By exposing a population of primary cells to a suspected "carcinogen" and by suspending the exposed cells in soft agar, one could find a few clones of cells that would grow. "Normal" cells would not grow under these conditions (Later, it was shown that normal stem cells would show limited growth, whereas "normal" non-stem cells die by anoikis when grown in suspension [57]). If those clones, which grew in soft agar, were injected in immune-deficient mice and formed tumors, then the assay was interpreted as indicating the tested agent was a "carcinogen". Yet, what was puzzling at the time was that millions of these soft-agar-positive clones had to be injected into these mice to form a tumor. Titrating that population down to small numbers rarely, if ever, gave rise to tumors. Many explanations were given to try to explain this observation as to why one needed millions of soft agar grown cells to produce a tumor, when it was assumed that each of these cells of these soft agar clones must have been tumorigenic.

It was only after it was discovered that the *in vivo* tumor and cell lines, derived from these tumors, contained a heterogeneous mixture of cells. The introduction of the "cancer stem cell" concept was tied to the discovery that there were only a few cells in this tumor or tumor cell line that could transfer the "tumorigenicity". "Side population cells", detected by staining living cells, derived from a tumor, in the culture medium with Hoechst 33342 dye, were shown to give rise to a tumor [58-67].

Clearly, it was demonstrated that fewer of these "side population cells" than the total tumor cell population were needed to form tumors when injected back into an immune-deficient mouse. These "side-population" cells were then associated with the term, "cancer stem cells" [68] and they were the cells that sustained the growth of the tumors and were able to reproduce the same characteristics of the original tumor from which they were derived [69]. These findings have dramatically altered new research approaches to the understanding the origins of cancer [7,24,70-91] and cancer therapy [92-97].

While these "side-population", "cancer initiating cells" and "cancer stem cells" were similar to "normal stem cells", namely, they express Oct4A and they do not have functional GJIC, they obviously are very distinct. The major difference is when the normal stem cell is suspended in soft agar they do grow and form 3-dimensional organoids, but they eventually stop growing. Upon, examination, one sees that there has been differentiation of these stem cells, as the 3-D organoids create microenvironments that induce the stem cells to differentiate into its normal lineages, plus, it creates a low oxygenated niche to protect the stem cells from differentiation. On the other hand, the "cancer stem cell", treated the identical manner, will continue to grow, provided they have access to nutrients. While there is some "partial" differentiation of these "cancer stem cells" into non-sustaining "cancer non-stem" cells, as one sees in real *in vivo* tumors, the fact that these "cancer stem cells" are "initiated" or inhibited from terminal differentiation, that is, they cannot perform asymmetric cell division very easily.

Aside from this characteristic difference that the normal stem cell, when exposed to oxygen, they tend to differentiate and to seek protection from low oxygen microenvironments in their niche. This, then, leads to other features of normal stem cells, namely, they express Oct4A [7,16-20,51]. This is one reason that the few stem cells in a primary culture, when grown under normal *in vitro* culture conditions of normoxia, eventually senesce [98]. When these primary cells, with the few sustaining adult organ-specific stem cells, are cultured in low oxygen, they can have their life span extended [99]. This is not the case for "cancer cell lines", for they can grow in normoxic conditions *in vitro*. Yet these cancer cell lines are always heterogeneous, as they are in the *in vivo* tumor from which they were derived [69].

Role of Oct4A as a Marker for both Normal Stem Cells and Cancer Stem Cells

One of the critical genes associated with the ES, "iPS", somatic nuclear transfer stem cells, and adult human organ specific- stem cells is the expression of Oct4A and the non-expression of the connexin genes or the non-function of gap junctional intercellular communication [100].Cancer stem cells express the Oct4 gene [70,80,101], as well as drug transporter genes [71].

When Yamanaka showed that a series of embryonic genes, POU domain class 5 transcription factor 1 [Oct-3/4], SRY-box containing box 2 [Sox2], cellular myelocytomatosis oncogene [c-Myc], and Kruppel-like factor 4 [Klf4] when genetically introduced into a population of primary cells, one could recover "induced pluripotent stem cells" (iPS). As an operational test that these were embryonic-like cells, they had to form teratomas when injected by into an adult animal. This was interpreted as a "re-programming" of the somatic differentiated fibroblasts to the embryonic state. However, an alternative interpretation is that, in that primary population of cells, there exist a few adult stem cells [102]. The reasoning was that, while the embryonic genes, when inserted in all the primary cells, including the few adult stem cells, these induced pluripotent stem cells, expressed exogenous Oct4, together with the expressed endogenous Oct4 gene in the adult stem cell, gave them a selective advantage to survive and they were interpreted as a "re-programmed", "induced pluripotent stem cells". Given that these pluripotent stem cells seem to express the differentiated expressed genes of the original cells of the primary culture [103], it seems that this alternative hypothesis supports the origin of the "iPS" cells as being the original normal adult organ-specific stem cell of the primary population. Additional evidence for the lower frequency of "iPS" cells exists when late passages of the primary cultures are used. This is because late passages of primary cultures would have fewer adult stem cells in their population. That is why primary cultures ultimately senesce. In addition to this line of reason, it was shown that, when populations of adult MUSE cells are used to isolate "iPS" cells, they are far more efficient in the production of "iPS" cells than primary cultures [23].

However, probably the strongest direct experimental evidence that adult origin specific stem cells are the "target" cells for the induction of "cancer stem cells" comes from the demonstration that

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normal adult human breast stem cells, expressing Oct4A, estrogen receptor, ABCG2, but not Cx43 gene, and having no functional gap junctional intercellular communication, could be prevented from terminal differentiation by introducing the SV40 large T gene into the stem cells [7]. This produced clones that, while not tumorigenic, still expressed the Oct4A, estrogen receptor, and ABCG2 genes, but still did not express their connexin 43 (Cx43) gene, nor did these cells have functional GJIC. On the other hand, when the normal human breast stem cell was induced to differentiate, the endogenous Oct4A was shut down, transcriptionally, while they expressed Cx43 and had functional GJIC. These normal differentiated breast epithelial cells could not be "re-programmed" with the SV40 large T gene. When the "immortalized" human breast stem cell population was X -ray treated, clones of weakly tumorigenic were isolated that still expressed Oct4A, ABCG2, and the estrogen receptor gene, but did not express their Cx43 gene nor did they have functional GJIC.

Finally, when these weakly tumorigenic cells were transfected with the Neu oncogene, clones were isolated that were highly tumorigenic. These cells still had their Oct4A, ABCG2 and estrogenic receptor genes expressed and had no CX43 expressed nor did they have functional GJIC. This clonal series, starting from the normal breast adult stem cells to the highly tumorigenic breast "cancer stem cells" demonstrates that the SV40 large T did not re-program the original OCT4A gene, but that this endogenous Oct4A gene stayed expressed throughout this process. It also demonstrated that, in this case, the SV40 "immortalizing" viral gene, did not "immortalize" a mortal adult breast stem cell but it kept these adult breast stem cells from "mortalizing" or terminally differentiating. It, also, demonstrated, *in vitro*, the same multi-stage, multi-mechanism- "initiation", "promotion", "progression" steps as seen *in vivo*. The SV40 Large T caused the normal adult breast stem cell, which has unlimited proliferation potential, to have its asymmetrical cell division mechanism blocked, while it could still proliferate symmetrically upon mitogenic stimuli. This is the "initiation" step.

Further mitogenic stimuli by some radiation-induced chromosomal or point mutation, induced in an "initiated SV40 clone", it now is kept in the "immortalized" stage, since it could not divide asymmetrically. Further addition of the neu oncogene to provide additional mitogenic self- stimuli, it now became high tumorigenic.

The consequence of these observations and interpretation suggests that, to prevent "initiation" of a normal organ specific stem cell, one must prevent, as much as possible, stable blockage of the asymmetric cell division mechanism. Prevention of exposure to "immortalizing" viruses [104] or minimizing mutagenesis would be recommended [105]. While this strategy to reduce these stable down regulation events, especially mutagenesis, is possible, one can never reduce mutagenesis to a zero probability. Since there are two means to form point mutations, "errors in DNA repair" [106-109], and by "error in DNA replication" [110,111], to prevent errors in DNA replication would be impossible. Every time a stem cell must replicate during normal growth, wound healing or surgery, there will always be a finite chance that a mutation will be acquired. All human beings have "initiated" organ-specific adult stem cells in each of our organs. The older we get, the more we will accrue. A tumor will occur in those that are exposed, chronically, at threshold levels, and in the absence of "anti-promoters" and to these "epigenetic", mitogenic tumor promoting agents or conditions [112].

One major observation might challenge this hypothesis. Namely, there are some "cancer stem cells" that do not express Oct4A but do express the connexin genes. However, as Loewenstein postulated, cancer cells did not have growth control, do not "contact -inhibit", do not terminally differentiate and are "immortal". How can this be? The answer seems to be quite simple, namely, if a stem cell just started to differentiate (shut off Oct4A and turn on the connexin genes, but at the same time, turn on an oncogene, such as src, ras, or neu), these oncogene proteins can post-translationally modify the connexin proteins to render the gap junctions to be non-functional [113]. Therefore, while Loewenstein was unaware of these facts at the time he proposed that which provides a universal phenotype for all "cancer stem cells", namely the inability to perform GJIC for growth control, terminal differentiation, immortality and apoptosis, whether the connexin gene is transcriptionally suppressed or that the connexin protein is post-translationally modified, the cell is unable to have functional GJIC. Thus, these two "cancer stem cells" are unable to have functional GJIC, but one "cancer stem cell" never expressed the connexin genes or had functional GJIC, while the other "cancer stem cell" shut down the expression of Oct4A but started to transcribed the connexin gene, but their GJIC was inhibited by post-translation of the connexin protein. Therefore, treating both types with the same cancer chemotherapeutic agents will not be effective against both. For the embryonic -like cancer stem cell, one would have to treat them with some agent that will activate transcription of the connexin gene, while repressing the Oct4A gene. For the other partially-differentiated type of cancer stem cells, inhibiting the action of the expressed oncogene to restore the ability of the connexin protein to forge function GJIC would be the strategy. The example of the treatable polyp-type colon tumors versus the non-treatable "flat-type"- colon tumors could illustrate this point [84].

To be fair in testing the opposing hypothesis that cancers and "cancer stem cells" are derived from the "de-differentiation" or "reprogramming" of somatic differentiated cells, one must examine the consequence of this mechanism happening in an adult organism, such as a human being. If a single somatic differentiated cell is "initiated" by the "re-programming" by some stable event to turn on the repressed Oct4A gene and to repress the expressed connexin gene, such as a mutation, then, the resulting "iPS" cell *in vivo* would lead to a teratoma. Since the majority of cancers induced in adult human beings are either sarcomas or carcinomas, not teratomas, it seems that this hypothesis could not explain the real facts of the origin of most human cancers.

Conclusion

Cancer stem cells are derived from "initiated" or benign non-cancer stem cells, which, in turn are derived from normal adult "organ-specific stem cells"

A hypothesis has been offered, based on the fact that normal organ-specific adult stem cells exist in all organs. Moreover, these normal adult organ stem cells express Oct4A and are unable to perform functional GJIC, and can be transformed into "cancer stem cell" by, first, inhibiting asymmetric cell division ("initiation" event) and subsequently clonally amplified by mitogenic processes to accrue all the genetic/epigenetic changes to achieve the "hallmarks of cancer" of invasion and metastasis of other tissues. These cells will express Oct4A and not express connexin genes or have functional GJIC. On the other hand, a second type of "cancer stem cell" will be

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derived from an adult stem cell that just started to differentiate by repressing the transcription of the Oct4A gene and transcribing the connexin gene. However, if these cells are "initiated" by the activation of expression of some oncogene, the connexin proteins are post-translational modified to render the gap junctions unable to function. These cells will be Oct4A negative and connexin positive but GJIC negative.

In other words, both "cancer stem cells" will be negative for GJIC, while the former will be Oct4A positive and the other will be Oct4A negative but also GJIC negative. Strategies for the prevention and treatment of each type will require a completely different approach.

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Conflict of Interest

The author has no conflict of interests concerning the content, attribution or support of this publication.

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