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The STAT3-Ser/Hes3 signaling axis in cancer

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1. ABSTRACT

Disrupting the regenerative capacity of tumorigenic cells is a major focus in medicine. These regenerative properties are carried by a subpopulation of cells within the tumor, termed cancer stem cells. Current therapies don't effectively tackle the disease suggesting these cells employ yet unidentified molecular mechanisms allowing them to evade targeting. Recent observations in neural stem cells reveal an extraordinary plasticity in the signaling pathways they utilize to grow. These findings are being extended to the cancer stem cell field, illuminating conceptually novel treatment strategies. Tumorigenic cells can make use of distinct, even opposing pathways, including JAK/STAT and the non-canonical STAT3-Ser/Hes3 signaling axis. This plasticity may not be confined to the cancer stem cell population, but may be shared by various cell types within the tumor, blurring the line distinguishing cancer stem cells from other tumor cell types. The implications to anti-cancer medicine are highly significant, since these findings demonstrate that inhibiting one cell growth pathway may actually enhance the activity of alternative ones. Drug discovery programs will also benefit from these concepts.

2. SIGNALING STATE OF THE CELLS COMPRISING A TUMOR

A sharp distinction is often made among various subpopulations of cells within a given tumor (1). Initial studies showed that particular cells isolated from tumors based upon differential expression of cell surface biomarkers were significantly more capable of phenocopying the tumor of origin in standard xenotransplantation experiments. Given this stem cell-like regenerative ability, they were termed Cancer Stem Cells ("CSCs"). CSCs are thought to comprise a small percentage of the cells in a tumor, although highly aggressive tumors such as glioblastoma multiforme may contain a higher number of CSCs (2-6). One explanation for the typically low percentage of CSCs is that cells comprising the bulk of the tumor ("Cancer Cells") are able to proliferate more rapidly (7-12). This reflects the proliferation state of non-cancerous stem cells which also exhibit a relatively slow rate of division. An example is the stem cell population of the skin epidermis which is actually identified by its ability to incorporate proliferation labels like BrdU (demonstrating that it divides) and to retain the label for long periods of time, demonstrating that it divides

slowly (13). Similarly, somatic stem cells, such as neural stem cells ("NSCs") also proliferate slowly in the adult brain (14). CSCs, Cancer Cells and NSCs can be placed in culture where the molecular mechanisms of their growth and survival can be studied. In this article we discuss how emerging understanding of the signal transduction state of these cell types can be utilized to appropriately model the signaling state of CSCs in culture, an essential factor in elucidating the cellular machinery that regulate their growth in the context of regeneration and cancer and identifying novel putative therapeutic targets.

Specific biomarkers for CSCs vary depending on the origin of the tumor. The pentaspan transmembrane glycoprotein prominin-1 (also known as CD133) is widely used in the identification of CSCs from glioblastoma multiforme, hematopoietic, pancreatic, and colorectal cancers (15-18). Combinations of biomarkers are often required to define a cell subpopulation as putative CSCs, and even then, additional functional assays are required to ascertain their stem cell properties (19). This lack of sufficient biomarkers that can identify these cells with confidence underscores the need to discover additional biomarkers. Despite the initial studies showing that biomarker positive cells can phenocopy a tumor in experimental settings, subsequent work demonstrated that biomarker negative cells could also recapitulate the tumor provided that adequate experimental conditions were provided (20). Additional evidence comes from multiple reports suggesting that cell lines in culture are at an equilibrium between a Cancer Cell and a CSC state (21-23). A cell might therefore be perceived as having more stem-like properties in part due to environmental conditions and not for purely cell autonomous reasons, blurring the distinction between these cell types that are often considered quite disparate. Therefore, understanding the intracellular signal transduction pathways controlling CSC number and survival is key to identifying and targeting the cells responsible for disease progression.

Numerous signal transduction cascades that regulate the growth of transformed cells have been proposed as potential targets for the treatment of different cancer types. Among these are pathways activated by receptor tyrosine kinases (e.g. the Ras – Raf – Erk MAP Kinase, and PI3 kinase – Akt – mTOR, and JAK/STAT) (1, 24-26). However, clinical translation of the basic findings from cell culture systems has proven notoriously challenging. One potential reason for this difficulty lies in the inability to correctly model the *in vivo* environment under the more accessible *in vitro* culture conditions (27-30). A telling example comes from certain breast cancer models. The efficacy of a gamma secretase inhibitor was compared *in vivo* and *in vitro* utilizing a number of breast cancer cell lines as well following xenotransplantation of these cell lines into immunocompromised mice. Not only did the effects of this drug vary among cultured cell lines, but also its potency *in vitro* (specifically, its anti-proliferative effect) and *in vivo* did not match (31). In other words, cells in culture responded differently and unpredictably to the drug than the same cells did following xenotransplantation. These results highlight the importance of defining culture conditions in which the cells are in a similar signaling state as they are *in vivo*. In fact, depending upon the composition of the growth medium, a given cell can efficiently grow employing mutually opposing signaling pathways. As we begin to appreciate the extraordinary plasticity of tumorigenic cells at the level of signal transduction, the implications to anti-cancer efforts are immediate. Certain culture conditions might be inadvertently "locking" cells into a particular signal transduction state. As a consequence, we are only allowing a cell to utilize a subset of the signal transduction choices it would normally have available *in vivo*, gravely limiting the usefulness of culture techniques as drug discovery tools that attack its self-renewal capabilities. Recently, alternative ("non-canonical") signaling pathways have appeared as major regulators of the growth of cancer. Members of the Hes/Hey family of transcription factors serve as indicators of the signal transduction state of tumorigenic cells as different members of this family are regulated by different pathways in addition to being putative drug targets themselves (Figure 1) (31, 32). Understanding this signal transduction plasticity in tumorigenic cells will lead to vast improvements in drug discovery programs, greatly expanding the number of viable candidate targets (Figure 2).

3. THE STAT3-SER/HES3 SIGNALING AXIS

Notch signaling plays diverse and profound roles in development, cell proliferation, differentiation, and cancer (33-41). Following Notch activation by ligands, a proteolytic cleavage catalyzed by gamma secretase releases the intracellular domain of the receptor into the cytoplasm. This can now interact with other proteins and regulate gene transcription. Of the many targets of Notch signaling, the Hes/Hey gene family of transcription factors is extensively studied and widely used as assays for Notch activation (42-44).

Hes1 is a direct target of Notch and a staple of canonical Notch signaling. Following induction of Hes1 transcription, it can help JAK to phosphorylate STAT3 on the tyrosine residue at position 705 (45). In this way, Notch, via Hes1, contributes to the activation of the JAK/STAT pathway, which is recognized to promote the survival and proliferation of cancer cells (46-50).

On the other hand, increased tyrosine 705 phosphorylation on STAT3 drives the differentiation of neural stem cells towards the astroglial fate (51, 52). This observation suggests that alternative signaling pathways can promote survival and proliferation of neural stem cells and challenge the question of whether tumorigenic cells also utilize these other options (53).

The presence of the STAT3-Ser/Hes3 signaling axis was first shown in rodent neural stem cell cultures (34). Activating the Notch receptor by treatment with soluble ligands (Delta4, Jagged1) increased mRNA levels of Hes3. Although all members of the Hes/Hey gene family were expressed, only Hes3 was regulated. Since Hes3 is not a direct target of Notch, this suggested the involvement of

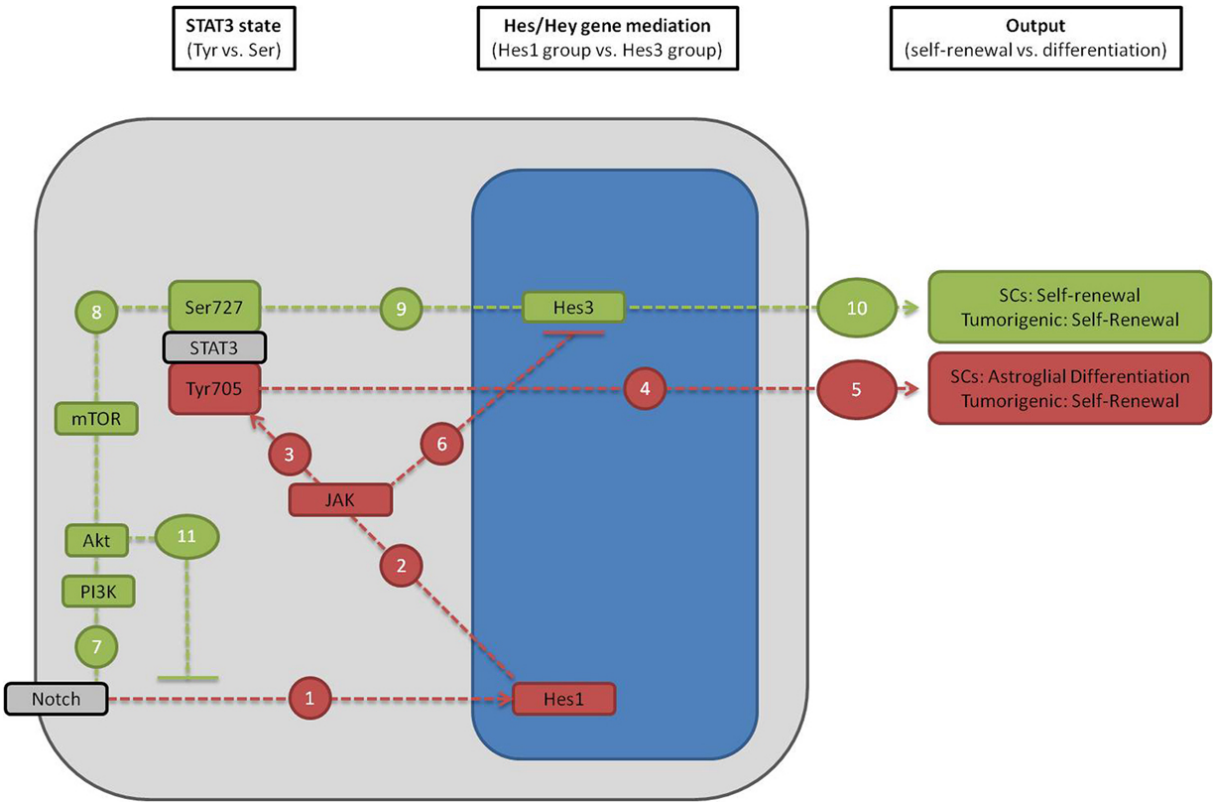

Figure 1. Distinct signaling pathways regulate the cell state of normal and transformed cells. Canonical Notch signaling activates downstream target genes, including Hes1 (1). Hes1 can facilitate the association between JAK and STAT3 (2), promoting the phosphorylation of STAT3 on tyrosine 705 (3). Tyrosine-phosphorylated STAT3 can enter the nucleus and regulate the transcription of several target genes (4). In many transformed cells cultured under standard conditions, this can lead to increased survival and proliferation. In non-cancerous neural stem cells, this can lead to irreversible differentiation towards the astroglial fate (5). JAK kinase also suppresses the expression of Hes3, a target gene downstream of non-canonical Notch signaling (6). A non-canonical branch of Notch signaling leads to the PI3 kinase – dependent activation of Akt (7). This is followed by phosphorylation of mTOR and subsequent phosphorylation of STAT3 on serine 727 (8) in the absence of detectable phosphorylation on the tyrosine residue. These events are followed by elevation in transcription of Hes3 (9). In neural stem cells and glioblastoma – derived CSCs, this is associated with the self-renewal state and increased survival (10). Recent work suggests relevance also in cancer cells. Akt has been suggested to negatively regulate the canonical Notch pathway that leads to Hes1 induction through the cytoplasmic retention of Notch intracellular domain and its partner, RBP-Jk (75). ("SCs" Stem Cells).

non-canonical signal transduction mediators downstream of Notch (44, 54). Eventually, it was found that STAT3 was involved, through phosphorylation on serine 727, in the absence of phosphorylation on tyrosine 705. In fact, JAK activity, which leads to STAT3 tyrosine phosphorylation, inhibits Hes3 induction by Notch ligands and inclusion of a JAK inhibitor improves cell survival and yield in culture. Other signaling components, including PI3 kinase, Akt, and mTOR, were incorporated into the pathway, all of which increase STAT3 serine phosphorylation without activating the JAK-STAT pathway. Modulators of the pathway include insulin, as well as Angiopoietin 2 and cholera toxin, which act through the Tie2 receptor and GM1+ gangliosides respectively, on neural stem cells (35, 36, 37). Recently, Macrophage migration inhibitory factor (MIF), which is expressed by dendritic cells and neural stem / progenitor cells, was demonstrated to provide additional paracrine and autocrine mechanisms supporting the maintenance, survival, and proliferation of neural stem cells (55). Many of these factors are present within their neurovascular microenvironment, with both pro-and anti-angiogenic cytokines being expressed by vascular cells (39, 56-61). Therefore, not surprisingly, treatments using combinations of these compounds not only stimulate NSC growth in culture but also in the living rodent and primate (34-37, 62-64).

Extending these observations to tumorigenic cells, serine phosphorylated STAT3 has significant consequences for regulating CSC growth. Recent reports suggest it is an important mediator of STAT3 nuclear localization and early stage carcinogenesis in hepatocarcinomas (65). Similarly, STAT3-Ser phosphorylation was shown to be important to the growth of prostate cells with CSC properties in culture, independently of STAT3-Tyr phosphorylation (66). In chronic lymphocytic leukemia patients, STAT3 is constitutively phosphorylated on the serine residue and this

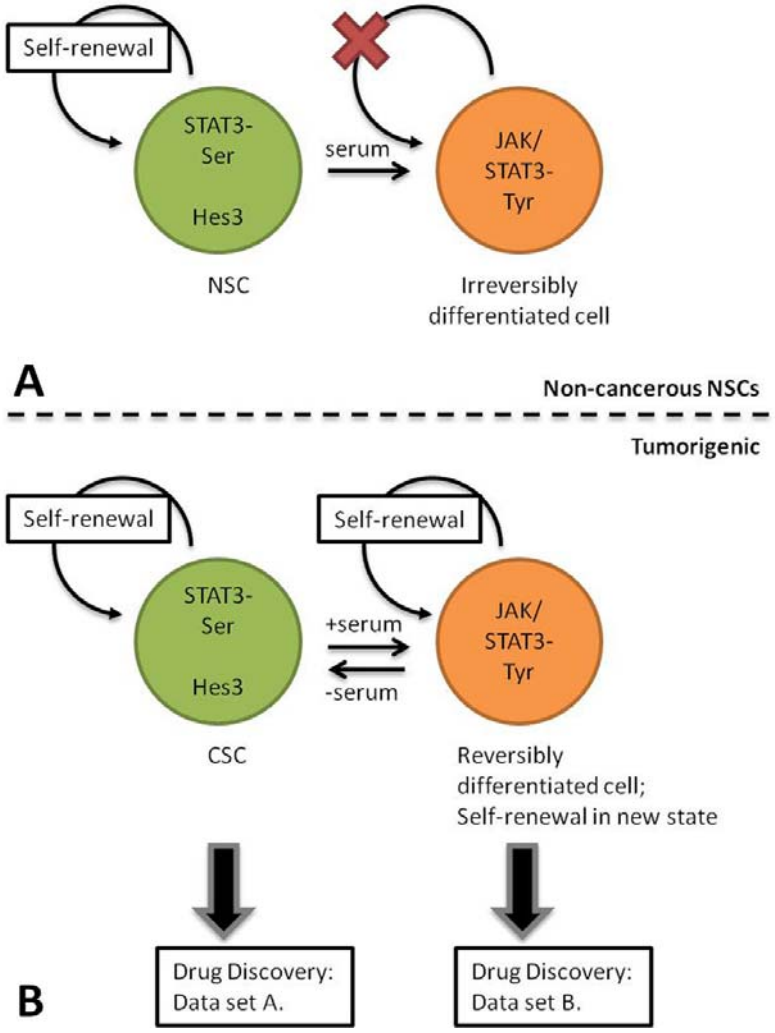

Figure 2. Culture conditions can have significant implications for identifying novel therapeutic hits. (A) Conditions that promote STAT3-Ser phosphorylation in the absence of STAT3-Tyr phosphorylation can be identified by Hes3 expression and support the self-renewal state of neural stem cells. In contrast, conditions that promote the JAK/STAT3-Tyr phosphorylation oppose Hes3 expression and promote cell differentiation. (B) On the other hand, tumorigenic cells can co-opt signaling pathways used by non-cancerous stem cells for differentiation to support their proliferation. Therefore the selection of culture conditions for screening compound libraries is critical. For example, serum tends to promote the JAK/STAT3-Tyr pathway whereas serum-free conditions may promote the STAT3-Ser/Hes3 pathway. It is reasonable to expect that different target "hit sets" will be obtained under different growth conditions. Identifying the ones that best reflect the *in vivo* state of these cells will allow for the discovery of new classes of therapeutics. ("CSCs": Cancer Stem Cells; "NSCs": Neural Stem Cells).

modification promotes the nuclear localization of STAT3 which is able to bind to gene targets (67). Taken together, these reports suggest that different STAT3 phosphorylation states have distinct cellular consequences.

4. A ROLE OF THE STAT3-SER/HES3 SIGNALING AXIS IN CELL CYCLE REGULATION

Hes/Hey genes have important roles in the developing central nervous system where they function as transcriptional repressors to regulate the maintenance of the neural stem cell population (42). In mice simultaneously lacking Hes1, Hes3, and Hes5, neural stem cells undergo premature differentiation into neurons by embryonic day 10 as observed in the developing spinal cord (68). Later in development, however, certain Hes/Hey genes change their function to direct the differentiation of neural stem cells towards a glial fate (69). A recent report identified the avian homolog of Hes5 as a regulator of cell cycle length, cooperating with the proneural protein ATOH7 in the conversion of progenitor cells into retinal ganglion cells (70). These findings suggest this gene family can regulate aspects of the cell cycle and cell cycle exit in neural stem cells.

The ability of Hes/Hey genes to regulate cell proliferation goes beyond their role as transcriptional repressors. Hes1 and Hes5 physically interact with Janus

kinase 2 (JAK2) in the cytoplasm and promote the phosphorylation of STAT3 by JAK2 on tyrosine 705, giving them a direct link to this major regulator of many cellular functions, including G1 to S progression (45, 46, 71). There is much less information about the specific involvement of Hes3 in proliferation versus differentiation decisions. Recent evidence from our laboratory demonstrates that when fetal mouse neural stem cell cultures are treated with soluble ligands of the Notch receptors (Delta 4), Hes3 mRNA levels increase significantly, cell death and cell cycle exit are reduced, but cell cycle duration does not change (34). Hes3 is detected in both the nucleus and the cytoplasm of neural stem cells during expansion in culture (36). When these cells are induced to differentiate by removal of bFGF, Hes3 becomes exclusively cytoplasmic within days as cells exit the cell cycle. However, treatment with cholera toxin promotes the nuclear localization of Hes3 and induces proliferation even in the absence of mitogen (62). In the adult rodent brain, Hes3 expression in quiescent cells is stronger in the cytoplasm of putative neural stem cells than in the nucleus (37). While this data is correlative, it suggests that tight control over both the expression and distribution of Hes3 are important for regulating the cell cycle entry and exit of neural stem cells.

It will be of great value to incorporate Hes3 in studies that aim to elucidate the role of Hes/Hey genes in regulating cell cycle. A key question is whether Hes3, like Hes1, has cytoplasmic functions that are accessible to pharmacological targeting in the context of cancer and regenerative medicine. Sequestration of Hes3 in the cytoplasm may have a more complex role than simply separating it from its DNA binding sites. For example, since Hes1 facilitates the phosphorylation of STAT3 on tyrosine 705, could Hes3 have a similar role on serine 727? It is intriguing to think that two members of the same gene family may induce the phosphorylation of distinct sites on STAT3, each of which has specific consequences to the fate of the cell. In the case of neural stem cells, STAT3-Tyr phosphorylation induces differentiation whereas STAT3-Ser phosphorylation promotes growth (34, 46). Hes3 is also a passive repressor that can form protein-protein interactions with other transcriptional regulators, thereby suppressing their activity (72). Therefore, one could imagine that both cytoplasmic and nuclear Hes3 have broad implications to controlling cell growth. Understanding these roles could provide a strategy to specifically target one set of functions of Hes3 over the other.

5. CONCLUSIONS

Tumors evade treatment by exhibiting extraordinary plasticity at many levels, including switching between proliferation and quiescence as well as among different metabolic states (73, 74). Another recently appreciated ace up their sleeve is their ability to switch between different signal transduction pathways. In this article, written during the early days of these signaling concepts, we discuss a prototypical paradigm: Tumorigenic cells in culture can efficiently grow utilizing the JAK-STAT pathway, but they can also efficiently grow utilizing the STAT3-Ser/Hes3 signaling pathway. The impact of this feat to drug therapy is showcased by the fact that these two signaling pathways are reciprocally opposing. Therefore, inhibiting one pathway in the context of therapy, may inadvertently promote the other. Indeed, JAK inhibition, a deleterious treatment to the growth of most cancer cells, including CSCs cultured under commonly used conditions, is actually beneficial when the same CSCs are cultured in conditions permissive to the STAT3-Ser/Hes3 state. This signaling "trick" that CSCs employ, does introduce a new level of complexity to the field of cancer biology. However, it also provides a new logic for drug discovery programs. The value of cell culture as a drug discovery tool will be greatly boosted by taking into account this extraordinary plasticity of tumorigenic cells at the level of signal transduction. A case in point is Hes3, which is expressed and mediates growth only in conditions that support STAT3-Ser phosphorylation in the absence of STAT3-Tyr phosphorylation, leading to activation of the STAT3-Ser/Hes3 pathway. However, the same cells can grow in a STAT3-Ser/Hes3 – independent way when cultured under common, serum-containing conditions. As a consequence, Hes3 has evaded detection as a putative drug target. Eventually, a set of different culture conditions may need to be defined that maintain cells in as many of their signal transduction states as possible, revealing the full range of their exploitable weaknesses. One of these states is maintained by culture conditions that are currently used in common practice, so the value of such systems is far from gone. This will lead to combined treatment regimes that simultaneously attack multiple signaling states, blocking any attempts towards evasion. Crucially, findings from *in vitro* studies must be compared to *in vivo* findings to assess the predictive ability of culture systems. This task is made simpler by the currently available gamut of biomarkers, such as Hes3 and serine phosphorylated STAT3, which have so far been employed on brain tumors and more recently, prostate and breast cancer cells. It will be of great value to expand these observations to many other cancer types.

The signaling plasticity described here may also help shed some light to the issue of how fundamentally distinct cancer cells and CSCs are. Both appear to be able to perform this switching (although initial observations suggest that CSCs may be significantly more adept at it) blurring the line between these two cell types. This ability to switch may be a hallmark of cancerous cells, as non-cancerous counterparts (e.g., neural stem cells) are unable to accomplish this feat due to their irreversible differentiation in response to JAK/STAT activation. In fact, it was this limitation that led to the original efforts to culture neural stem cells in defined, serum-free media, and the subsequent elucidation of alternative growth mechanisms employed by different stem cell populations. Now it appears that these mechanisms are not limited to CSCs but they may also contribute to cancer cell growth. It is imperative to elucidate these alternative signaling pathways as they may hide a treasure trove of previously not considered drug targets. Hes3 and its partners are likely just the tip of the iceberg.

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