[Frontiers in Bioscience 13, 995-1002, January 1, 2008]

Genes that affect both cell growth and polarity mediate stem cell quiescence

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1. ABSTRACT

Stem cells possess the capacity to expand and self-renew and do so by dividing in either a symmetrical or an asymmetrical manner. Under particular circumstances, some stem cell populations can undergo prolonged cell cycle arrest or quiescence, until they are triggered to divide by a given stimulus. In cancer treatment, these populations represent a significant roadblock to efficient therapies as their non-dividing state renders them refractory to most commonly used cytotoxic interventions. In certain organisms, germline stem cells undergo quiescence if animals experience inappropriate growth conditions, and recent studies have determined that the level of insulin signaling is key in the regulation of their proliferation rate, and that it functions through at least two tumor suppressor genes, PTEN and LKB1. These gene products regulate both growth and polarity in diverse cellular contexts, while it remains unclear how they can modulate cell division and prevent tumorigenesis through each of these functions, and whether indeed these functions are separable. We hope that understanding how these tumor suppressor genes impinge on quiescent stem cell populations could provide us with a means of designing more effective therapies to reduce the frequency of stem cell-derived tumor growth that occurs following treatment.

2. INTRODUCTION

The generation of cellular diversity and its organization into the tissues that make up a functional animal relies largely on the coordination of cell growth and patterned division. Several complementary mechanisms impinge on the cell division machinery to control how and when partition of the cell will occur. Much of the "how" is governed by polarity cues, which determine whether a cell will divide symmetrically into daughter cells of equivalent fates, or asymmetrically such that the resulting daughter cell fates are different, often due to biased partitioning of cellular determinants into the daughters. In the latter case, the establishment of a polarity axis prior to, or during, the division of the mother cell is essential to the unequal segregation of cell fate determinants into each of the daughter cells, and progression through the cell cycle is tightly linked to polarity status. In other circumstances, such as in neural development, the establishment of polarity is associated with terminal differentiation and thus inhibits any further cell divisions (1). Therefore, the manner in which cellular polarity pathways are established and the information they convey may not only affect how a cell divides, but also when and at what frequency these divisions will ensue.

Growth pathways regulate the rate at which cells increase their mass, and when this exceeds a critical cellular threshold in certain cell types, it triggers cell division (2). Therefore, although it seems intuitive, fast growing cells, wherein growth pathways are fully activated are likely to divide more frequently. The word "growth" therefore qualifies the pathways that truly affect both cell mass/volume and quite often cellular proliferation. In contrast, situations of nutrient deprivation negatively affect cell division rates, while at the same time these poor growth conditions also exert measurable effects on certain aspects of cell polarity establishment/maintenance, since they can cause polarity defects in a sensitized genetic background (3).

Stem cells are undifferentiated or partially differentiated cells that self-renew at each division. They may divide symmetrically to increase their numbers, or asymmetrically to maintain their own population while contributing daughter cells to a more differentiated or "committed" population. These unique properties enable stem cells to play key roles throughout embryonic and juvenile development to adulthood and then again during the post-reproductive life of multi-cellular organisms. They participate in several aspects of tissue generation and homeostasis ranging from populating fields within tissues during development or wound healing, to the continual generation of gametes, essential for reproduction during adulthood. The stem cells from which gametes are derived are termed Germline Stem Cells (GSCs), and we review here the implications of polarity and growth pathways in the regulation of their proliferation. We define the GSC proliferation rate as the frequency at which a GSC divides (equally or unequally) into two daughter cells.

3. NICHE SIGNALING AND STEM CELL FATE

To maintain their identity, stem cells require intrinsic factors, but also extrinsic signals that originate from surrounding somatic cells, and which are only available to a defined area. This restricted area where stem cells are maintained is called the niche, and the cells that provide the extrinsic signals are termed niche cells (4). The cell-cell signaling events that occur between these two cell populations sometimes provide a basis to establish an asymmetry within the receiving cell. This event then mediates the downstream signals that ultimately dictate the final localization of the developmental determinants required to distinguish the two resulting daughter cells after the division. The nature and the role of the signals that emanate from the various types of niche cells have been well reviewed and will not be discussed here (5). Many of these signals have general roles in establishing and maintaining stem cell fates in numerous organ contexts, while others are very specific to each individual niche situation and may play critical roles during the downstream differentiation processes characteristic of each emerging cell type/tissue. Among the general signals, Notch signaling has a conserved role in specifying binary cell fate decisions in daughter cells downstream of extrinsic cues received from neighboring ligand producing cells that constitute the niche. In invertebrates this is most clearly observed in the *C. elegans* germ line where the activation of a Notch-like receptor called GLP-1 in the GSCs blocks these cells from executing the meiotic program, while simultaneously promoting mitotic divisions (6). In *Drosophila*, recent evidence has implicated Notch signaling in the asymmetric divisions that occur in the intestinal stem cells. In this unusual system, the observed asymmetry is also established through differential Notch activation in the daughter cells to distinguish the terminal cell types of this lineage thus giving rise to enterocytes or enteroendocrine cells (7). In mammals, stem cell self renewal in the small intestine also requires Notch signaling, while the same pathway is successively reutilized to specify enterocyte versus secretory cell fates (8). Therefore, Notch plays a highly conserved function to transduce asymmetries in order to bias cell fates following division in many different developmental contexts.

4. CYTOSKELETAL CHANGES

When asymmetries are established, the downstream events often impinge on the cytoskeletal network responsible for segregating developmental determinants and/or specify the positioning of the spindle to promote the unequal division that defines this cell population. This signaling, whether it be extrinsic or intrinsic, must therefore converge on regulatory components of the microtubule and actin cytoskeleton to transduce the information required for the non-equivalent separation of cellular components. This basic problem has been addressed in numerous contexts of asymmetric cell division and some of these signals appear to be conserved. In *Saccaromyces cerevisiae*, Kar9, a molecule that shares significant similarity with the Wnt regulator APC, is loaded onto microtubules exclusively at the spindle pole destined to reside within the emerging bud, clearly distinguishing the daughter from the mother spindle pole (9, 10). The function of Kar9/APC may be highly conserved, since an APC homolog APR-1 also specifies asymmetric divisions in *C. elegans*, presumably through its canonical function in Wnt modulation (11). Most strikingly, in the GSCs of the *Drosophila* male germ line, Apc1/Apc2/APC are required for the proper positioning of the mother and daughter centrosomes, and thus participate in the process that eventually distinguishes mother and daughter cell fates (12, 13).

The physical force generation associated with these various events has been most accurately estimated during the asymmetric cell division that occurs in the one cell *C. elegans* zygote (14). During the cell division, antagonistic interactions between anterior and posterior Partitioning defective (PAR) proteins act through various G-protein complexes to regulate force generation on the spindle. This leads to asymmetric spindle positioning, while these PAR proteins also induce the non-equivalent separation of key cellular determinants during the first cell division. To date, the role of these complexes in the polarization of various cell types and developmental contexts has been described and are very well conserved at the structural and functional level (15).

4.1. Asymmetric divisions

The asymmetric division of a cell can be achieved through at least two general mechanisms. The first one relies on the physical asymmetric localization of fate determinants at both ends of the dividing cell, and proceeds with the spindle axis oriented in parallel to the polarity axis, such that the daughters inherit different determinants. However, to our knowledge, there is no known example of a GSC population that utilizes this strategy to divide asymmetrically, although the intestinal stem cells of *Drosophila*, for which the requirement for a niche has not been confirmed, may utilize such a mechanism (7).

Alternatively, a stem cell may divide in an essentially symmetrical manner in terms of fate potential, but achieve asymmetric outcomes due to the different positions of the two daughter cells relative to the niche (16). This is clearly exemplified in the male gonad in *Drosophila*, where the stem cell fate is specified by direct cellular contact with a niche cell (17). In this case, the mitotic spindle is oriented perpendicularly relative to the attachment surface of the GSC on the niche cell, so that one daughter cell remains positioned close to the niche, while the other is displaced away and loses signaling from the niche (12). The stereotyped orientation of the spindle is due to a difference between the mother and daughter centrosomes. Indeed, the mother centrosome is permanently anchored to the niche interface by astral microtubules, while the daughter centrosome remains unattached and is instructed to migrate to the opposite side of the cell before spindle formation (13). A similar mechanism has also been observed in *Drosophila* neuroblasts, suggesting that it may have a conserved role in asymmetric stem cell divisions (18, 19).

4.2. Symmetric divisions

The symmetric division of a stem cell gives rise to two stem cells that are indistinguishable from each other, and from their mother. In the *C. elegans* germ line, a GSC population is contained within a portion of the tubular gonad where they are under the influence of a niche cell called the Distal Tip Cell (DTC), which closes one end of the tube, referred to as the distal tip. Within this area, the GSCs seem to divide symmetrically both physically and in terms of fate potential. The size, morphology and molecular content of the daughter cells appear identical, while unlike the divisions typical of the *Drosophila* GSCs, the orientation of the cleavage plane is essentially random with respect to the proximal/distal axis (16, 20, 21). It is however possible that some potential asymmetries have escaped detection. Indeed, no detailed lineage analysis has been performed, the localization of only a limited number of molecules has been determined, and the mitotic spindle could in principle be aligned non-randomly relative to an axis different from proximal/distal. Until proven otherwise, the GSCs are therefore considered as a more or less (see below) homogeneous population that is maintained constant due to a continual outflow of GSCs at the proximal extremity of the niche, which then progressively enter the path to differentiation as they move away from the niche.

The position of a given GSC within the tubular *C. elegans* niche does however influence the rate at which it divides. Indeed, the GSCs that are physically located at the distal and proximal extremities of the niche divide at a lower frequency than those located in the center. The reason underlying this phenomenon is unknown, and is difficult to explain solely based on differences in the level of signaling from the niche along the axis where the GSCs reside (22). In any case, the differential rate of proliferation along the distal/proximal axis within the GSC population does not seem to cause, or to be associated with any obvious detectable asymmetry; be it physical or in terms of developmental potential.

5. GERMLINE STEM CELL QUIESCENCE

Cellular quiescence is common to many self-renewing cell populations. During normal growth conditions these cells, or a subset of these cells, can undergo a complete arrest of cell growth and division and maintain this G_0-like arrest over a prolonged duration until their proliferation is triggered by some event or signal. This quiescent population of stem or stem-like cells is of particular importance in cancer as they would be refractory to many current cytotoxic regimens that preferentially target actively dividing cells while in this state and can therefore contribute to the resumption of tumor growth following treatment. Understanding how this quiescence is maintained in this self-renewing cell population is therefore of significant interest, not only to better comprehend how such cells contribute to healthy tissues, but also to design ways of specifically targeting this cell population during cancer treatment strategies.

Quiescence of a particular type of stem cell, the GSCs, naturally occurs in diverse organisms when they enter a stage of developmental arrest, such as a diapause: a period during which animals are more likely to encounter harsh conditions, including starvation, that could impair the integrity of GSC divisions and potentially affect the survival of the organism or the next generation. The elucidation of the mechanisms that naturally establish and maintain GSC quiescence in organisms that undergo developmental arrest may help to define the factors that regulate the proliferation of GSCs, and perhaps more generally, other stem cell populations.

5.1. Nutrient/insulin regulation

Under optimal conditions, all GSCs divide continually in adult organisms such as *C. elegans* and *Drosophila*. However, inappropriate growth conditions such as nutrient limitation can negatively influence the rate at which the GSC population generates gametes (23, 24). In *C. elegans* the response of the GSCs to nutrient stress can be as extreme as complete quiescence. This can occur at two different occasions during the *C. elegans* life cycle. Indeed, if *C. elegans* hatchlings do not readily encounter a food source, their primordial germ cells remain quiescent for up to several days, until the animals begin to feed (25). Instead, if the young larvae are able to ingest food/nutrient, they will initiate development and the primordial germ cells will begin to proliferate and generate the GSC population.

However, if the young growing larva senses overcrowding through the perception of a pheromone and/or nutritional stress, it may enter a specialized developmentally-arrested stage called dauer which is non-feeding, although the animals are nonetheless mobile and adapted for dispersal. When the initiation of dauer development is executed, proliferation of the GSC population is slowed down until it completely arrests for the duration of the diapause, which can last for up to several months.

In both of these cases, the establishment and maintenance of GSC quiescence depends primarily on the appropriate down-regulation of the level of insulin signaling (26-28). Hence, from these analyses, it appears that the level of signaling by a pathway that is considered to affect growth plays a predominant role in the regulation of the division status of the GSCs.

5.2. Downstream effectors

The downstream effectors that mediate insulin-dependent GSC quiescence in *C. elegans* include *daf-18*/PTEN, *akt-1*/Akt/PKB, *par-4*/LKB1 and *aak-1*/*aak-2*/AMPK (26, 28). *daf-18*/PTEN is a tumor suppressor in humans which encodes a protein phosphatase known to dephosphorylate phosphatidylinositol 3,4,5-triphosphate (PIP_3), generating phosphatidylinositol 4,5-biphosphate (PIP_2) (29). As such, its main function is to counteract the activity of phosphatidyl inositol 3-kinase (PI3K), a well-characterized component of the insulin signaling cascade which is positively regulated by the activity of the insulin receptor (30). PIP_3 subsequently activates a complex that contains the AKT-1/Akt kinase, which then phosphorylates several targets, including the *daf-16*/FOXO transcription factor, TSC2 and GSK-3β among others, some of which are involved in polarity; some of which are implicated in growth/cell division control (31-34). In *C. elegans*, *daf-16*/FOXO is largely dispensable for GSC quiescence (26, 28) and is thus unlikely to be a key downstream effector in the regulation of the rate at which the GSC population expands.

par-4/LKB1 is a tumor suppressor gene which encodes a protein kinase that phosphorylates several related downstream kinases, including PAR-1 and the AMP-activated protein kinase (AMPK), through which it regulates both cellular polarity and growth/cell division (3, 35-38). During dauer formation in *C. elegans*, both *par-4*/LKB1 and *aak-1*/2/AMPK are required to appropriately establish GSC quiescence, implicating this cascade in insulin-dependent regulation of the rate of GSC divisions (26, 28).

So far, no obvious molecular link has been found between PTEN or Akt and the LKB1 – AMPK cascade, and thus the two pathways are thought to function in parallel. However, in mammalian cell culture, both Akt and AMPK have been shown to affect TOR signaling through the direct phosphorylation of TSC2, another tumor suppressor gene (39). Therefore, even though the two pathways may initially act in parallel, they are believed to converge on TSC2, which in turn regulates the level of TOR signaling (40). TOR signaling links the cellular nutritional status to the rate of protein synthesis and thus affects cellular growth (41). It remains however unclear whether the regulation of TOR signaling plays a role in GSC quiescence in *C. elegans*, for which a TSC2 ortholog has not yet been identified. Hence, although insulin signaling seems to primarily affect growth, it has not been clearly established whether the downstream effectors that mediate the insulin-dependent control of GSC proliferation and quiescence, do so solely through the regulation of growth in *C. elegans*.

5.3. Quiescence in haematopoietic stem cells – a link?

Haematopoietic Stem Cells (HSCs) are adult stem cells that reside in the bone marrow, and which give rise to all blood cell types. At any one time, even during optimal growth conditions, only a limited fraction of the HSC pool enters the cell cycle, while most remain in G_0 phase. Indeed, each HSC divides, on average, every 57 days, and it is thought that this quiescence is important to maintain the HSC pool (42, 43). Despite that the upstream mechanisms that control GSC quiescence during poor growth conditions and those that regulate HSC quiescence under normal conditions may be considerably different, the downstream, cell autonomous factors or processes that they target to establish and maintain quiescence may share some similarity. Indeed, like the GSCs of *C. elegans*, HSCs require PTEN activity to remain quiescent, and its deletion promotes HSC proliferation to the point that it causes the complete depletion of the HSC pool (44). Here, it is believed that the perturbation of TOR signaling is the relevant downstream effector of PTEN, since the artificial downregulation of TOR signaling using rapamycin partially suppresses the defects of PTEN deficient HSCs (44). Nonetheless, this does not positively demonstrate that the overproliferation of HSCs in PTEN mutants indeed results exclusively from a hyperactivation of TOR signaling.

6. DOWNSTREAM EFFECTORS REGULATE BOTH GROWTH AND POLARITY

The downstream effectors that link the level of insulin signaling to the regulation of GSC proliferation in *C. elegans* have been implicated in the control of growth, proliferation, and polarity in diverse cellular populations (Figure 1). In this section, we explore newly discovered roles of the LKB1/AMPK cascade and of the intermediate insulin signaling components (PTEN and Akt) in the regulation of cell polarity. This work reveals that in addition to polarity, growth and proliferation are often also affected in the same cells. A major question that remains unresolved is whether a subset of the growth/cell proliferation defects that are observed when these genes are mutated occur secondary to the loss of polarity, or whether or not growth/cell proliferation and polarity status are indeed interrelated. Perhaps this knowledge will further our understanding of the regulation of GSC proliferation and quiescence.

6.1. The LKB1 network in neuronal and epithelial development

In addition to the aforementioned role for the LKB1 kinase in growth/cell division control through

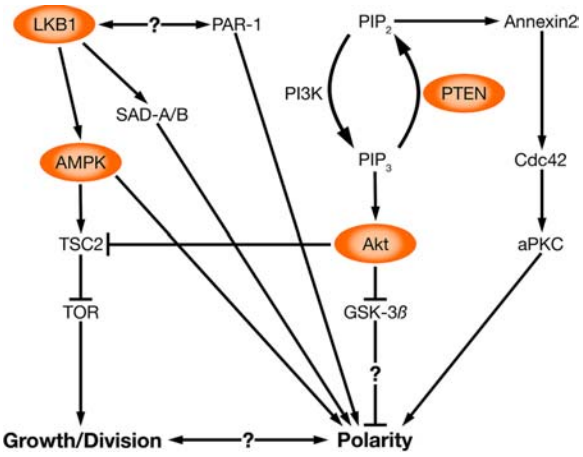

Figure 1. Genes required for GSC quiescence in *C. elegans* (orange ellipses) affect both polarity and growth/division in diverse systems. Arrows and bars indicate direct or indirect activation/induction or inhibition, respectively. See text for details. The placement of a bar or an arrow between GSK-3β and polarity was ambiguous, since its global activation/inactivation does not seem to affect polarity establishment *per se*, but its local inactivation is somehow required downstream of polarity cues for polarized growth.

AMPK and the TOR pathway in mammalian cell culture, LKB1 regulates polarity in several different cell types, and through multiple targets, including AMPK. Indeed, LKB1 is a master kinase that possesses the ability to phosphorylate at least 14 AMPK-related kinases (45). Among these are the SAD-A/B kinases (also known as Brsk2 and Brsk1), the LKB1-dependent activation of which is required for and induces polarization and axonal outgrowth in undifferentiated neurites (46-49). In this case, although this outcome is not discussed, the acquisition of polarity following LKB1 activation participates in terminal neural differentiation and indirectly prevents further cellular division.

LKB1 activity is also required for and actively induces polarization in multiple epithelial tissues. It was first recognized that LKB1 activity is required for the proper establishment of anterior/posterior polarity in the *C. elegans* zygote and in the *Drosophila* oocyte, while also affecting apical/basal polarity of *Drosophila* epithelia (37, 38). Since PAR-1 was found to phosphorylate LKB1 *in vitro* while LKB1 overexpression suppressed some of the defects of PAR-1 mutants in *Drosophila*, it was proposed that PAR-1 activates LKB1, which then regulates polarity (37), although this sequence of events has not found further support. It therefore remains uncertain whether LKB1 functions upstream or downstream of PAR-1 in the establishment of anterior/posterior and epithelial polarity (50). Interestingly, the loss of a PAR-1 homolog (Par-1b) in mice causes diverse metabolic and growth problems in addition to polarity defects (51, 52). Thus, a gene initially isolated as a key regulator of polarity also affects growth signaling, much like LKB1 and PTEN.

The activation of LKB1 in mammalian intestinal epithelial cells in culture induces the polarized formation of an apical brush border while this also blocked their proliferation (35). Surprisingly, recent evidence suggests that the main downstream target of LKB1 in this particular case is AMPK, since its artificial activation induced the intestinal epithelial cells to polarize, much like LKB1 activation (36). Moreover, it was found that the removal of AMPK activity in differentiated *Drosophila* epithelia disrupts polarity, specifically during energetic stress, producing a phenotype that recapitulates the loss of LKB1 activity in this tissue. Furthermore, since the transgenic expression of a phosphomimetic constitutively active version of AMPK rescued most of the LKB1-dependent polarity defects, it was concluded that AMPK is the main target that ensures the LKB1-dependent maintenance of epithelial polarization during energetic stress. Here, the loss of polarity was often accompanied with mitotic misregulation and overproliferative defects (3, 36). Thus, it appears that LKB1 activity is required to establish polarity during normal development in a pathway that somehow encompasses PAR-1 and/or other downstream kinases, but is also necessary to induce and maintain polarity and cell cycle arrest during energetic stress through AMPK regulation.

In the regulation of GSC quiescence in *C. elegans*, it is unclear whether AMPK is the sole relevant LKB1 target (28), and most importantly, whether the loss of LKB1 affects only growth signaling, likely upstream of TOR, or whether it could also affect previously unidentified aspects of polarity, through AMPK or other targets.

6.2. PTEN and Akt in neuronal and epithelial development

Interestingly, the establishment of neuronal and epithelial polarity also depends on PTEN and Akt, two intermediate components of the insulin signaling cascade that have been shown to affect GSC proliferation/quiescence. Indeed, the accumulation of PIP_3 at the tip of an undifferentiated neurite is essential to its specification into an axon (53, 54). Further, overexpression of PTEN in unpolarized neurons prevents axon formation, while its knockdown induces the formation of multiple axons (55). This local PIP_3 accumulation at the tip of one neurite causes the activation of Akt, which is then thought to phosphorylate and inactivate GSK-3β, thereby promoting directional axonal outgrowth, although this is still controversial (1, 56).

The polarity of *Drosophila* epithelia and neuroblasts is established following the asymmetric distribution of the PAR proteins. PTEN directly binds to Bazooka/PAR-3 in these cells and thereby gets recruited to their apical cortex (57). Consistent with this, in an *in vitro* system for the study of tubulogenesis, PTEN is localized to the apical membrane of epithelial cells, where it locally enriches PIP_2 levels. PIP_2 subsequently recruits Annexin 2, which binds Cdc42, thereby finally recruiting aPKC to the apical surface. Disrupting this sequence of events at any step blocks the polarized formation of the apical domain

(58). Unfortunately, it is not clear from this study whether proliferation of the differentiating epithelial cells is somehow affected when PTEN and/or polarization are disrupted.

A very interesting study was performed with a breast cancer cell line that displays growth and polarity defects, both of which can be reverted by an inhibitor of PI3K activity. From their analysis, it appeared that these two processes can be separated in this context; that is, the growth/cell proliferation defects are phenocopied by constitutive Akt activation, whilst the loss of polarity is mimicked by constitutive Rac1 activity, both of which are putative PI3K downstream targets (59). Unfortunately, the data fall short of documenting whether the severity of the growth/cell proliferation defects is similar regardless of polarity status, and thus whether the two processes are truly not interrelated.

7. SUMMARY AND PERSPECTIVES

The downstream effectors that link nutrient sensing through insulin signaling to the regulation of GSC proliferation and quiescence are implicated in the regulation of both polarity and growth/cell proliferation in diverse systems. Most importantly, from the work that we have highlighted, it seems that, at least in some cell types such as in epithelia, there is an intriguing correlation between the polarity and division status of cells, with the loss of polarity often being associated with growth/proliferation defects. Indeed, the loss of polarity in specified tissues appears to promote abnormal excessive growth/proliferation and the disruption of genes that regulate cell polarity often tend to cause a more dramatic impact on final organ size and tissue organization than the effects caused by perturbation of the cell cycle or basal growth control mechanisms (60). Thus, not only are the growth and division rates of these cells closely linked to their polarity status, it appears that these two factors are controlled by an overlapping set of genes. Strikingly, the genes that control GSC proliferation/quiescence seem to reside precisely within this overlap. Although some indirect evidence already suggests that the regulation of TOR signaling (and thus of growth) by these genes may account for at least some of the regulation of the GSC division rate in certain systems (44, 61), it will be of primary interest to confirm whether this is indeed the case, but also to determine whether the loss of PTEN or LKB1 in stem cells could cause any polarity defects. Indeed, even though it may seem counter-intuitive that polarity could be affected in *a priori* unpolarized cells such as the dividing GSCs of *C. elegans*, because of the prevalent roles of PTEN and LKB1 in polarity establishment and maintenance in other systems, it will be important to search for evidence of molecular polarization within the GSCs during diapause versus reproductive development. If the GSCs do exhibit some sign of polarization during diapause, then its establishment could potentially contribute to (or be associated with) the down-regulation of their division rate, and to the maintenance of cell cycle quiescence afterward. Thus, in this yet hypothetical case, the GSC overproliferation that results from the absence of PTEN or LKB1 could partially be secondary to polarity defects. Alternatively, if polarity and growth/proliferation are not related, one would not necessarily expect to find any changes in the GSC polarity status in proliferating versus quiescent GSCs.

It is also striking that two of the downstream effectors that regulate the global GSC division rate, namely PTEN and LKB1, are tumor suppressor genes. Indeed, mutations in either of these two genes in humans lead to dominantly inherited syndromes that share many features, among them a severe predisposition to developing benign and malignant tumors (62). The identification of their relevant downstream targets in the regulation of GSC proliferation may therefore represent new potential targets for therapy while providing further indications of how these genes maintain appropriate cell numbers and tissue architecture during normal development and stress conditions.

8. ACKNOWLEDGEMENTS

We thank Jean-Claude Labbé and Michaël Hebeisen for critically reading the manuscript and for useful suggestions and comments. We also thank two anonymous reviewers for their suggestions which contributed significantly to the quality of this review. P.N. is supported by an NSERC studentship. R.R. is a CIHR New Investigator. Research from this laboratory is supported by an award from the Canadian Cancer Society.

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Key Words: Cell Growth, Polarity, *Caenorhabditis elegans*, Germline Stem Cells, Stem Cell Quiescence, Niche Signaling, Asymmetric Division, Tumor Suppressor Genes, Growth Pathways, Cytoskeletal Network, Haematopoietic Stem Cells, Neuronal and Epithelial Polarity, Downstream Effectors, Review

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