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Environmental Impact on Intestinal Stem Cell Functions in Mucosal Homeostasis and Tumorigenesis

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Abstract

Multiple cell compartments at or near the base of the intestinal crypt have been identified as contributing intestinal stem cells for homeostasis of the rapidly turning over intestinal mucosa and cells that can initiate tumor development upon appropriate genetic changes. There is a strong literature establishing the importance of the frequently dividing Lgr5+ crypt base columnar cells as the fundamental cell in providing these stem cell-associated functions, but there are also clear data that more quiescent cells from other compartments can be mobilized to provide these stem cell functions upon compromise of Lgr5+ cells. We review the data that vitamin D, a pleiotropic hormone, is essential for Lgr5 stem cell functions by signaling through the vitamin D receptor. Moreover, we discuss the implications of this role of vitamin D and its impact on relatively long-lived stem cells in regards to the fact that virtually all the data on normal functioning of mouse Lgr5 stem cells is derived from mice exposed to vitamin D levels well above those that characterize the human population. Thus, there are still many questions regarding how dietary and environmental factors influence the complement of cells providing stem cell functions and the mechanisms by which this is determined, and the importance of this in human colorectal tumor development.

Keywords

INTESTINE; STEM CELLS; HOMEOSTASIS; TUMORIGENESIS; PLASTICITY; ENVIRONMENT

The intestinal mucosa is a rapidly self-renewing tissue in which most epithelial cells turn-over in about 5 days as they move from the proliferative compartment at the bottom of the crypts to the top of the villi where they are sloughed into the lumen (illustrated in Fig. 1). During this period, the cells undergo differentiation along multiple lineages, regulated by both intrinsic and extrinsic—including environmental—signals. It has been suggested that the cells undergo apoptosis before being extruded into the lumen, but this is generally not the case, with apoptotic rates very low along the entire length of the villi, and along the cephalocaudal axis of the small and large intestine, for example, [Augenlicht et al., 1999]. Thus, the final expulsion of cells from the top of the villi into the lumen may only be due to

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pressure from the columns of new cells migrating upward from the crypts, and not triggered by cell death. However, the cells certainly do die once they are released. Whether this is by a “programmed” process, such as anoikis [Gilmore, 2005] arising from lack of structural and/or physiological support from their surrounding environment is not clear.

Thus, the intestinal mucosa can be divided into several zones (Fig. 1B): the zone of proliferation, including the stem cells which give rise to all epithelial cells necessary for function of the organ; the zone of expansion, usually referred to as the transit amplifying region, where the progeny of these stem cells undergo several rounds of proliferation to expand their numbers; the zone of differentiation, where markers of specific functions are expressed, though the physical locations of where steps in reprogramming of the cells are initiated are not known; and finally, the zone of extrusion, where the cells are lost into the lumen, to be replaced by other cells migrating up the villi behind them. In the mouse intestine, the total transit time from the stem cell compartment at the bottom of the crypt is about 5 days. During this 5 day transit, the altered functions and phenotypes of the cells at different stages are reflected in their altered gene expression profiles, with cell position and altered programming highly interactive [Velcich et al., 2004; Mariadason et al., 2005; Wang et al., 2010].

This continuous turnover of cells in the intestinal mucosa, and its proper coordination to maintain homeostasis, are keys to maintaining homeostasis and avoiding neoplastic disease. There is strong evidence for this: expansion of the proliferative compartment was one of the first markers of increased probability of tumor development in response to both genetic and environmental factors [Deschner and Lipkin, 1975; Lipkin et al., 1981a,b, 1983]. Further, discovery that the *Apc* gene product regulates Wnt signaling by targeting β -catenin for degradation, and that inherited or somatic mutations in the *Apc* gene, or genes encoding other proteins involved in β -catenin degradation and/or perturb Wnt signaling, firmly established that normal regulation of proliferation and cell maturation are central in determining the probability of tumor development [Kinzler and Vogelstein, 1996, 1997], and that altered cellular gene programs along the crypt-luminal axis characterize the intestinal mucosa at elevated probability for tumor development [Wang et al., 2010, 2011]. It was also long suspected that the architecture of the tissue suggested there is a stem cell population at the bottom of the crypt with properties of both self-renewal and proliferative capacity to give rise to daughter cells that can populate the villi. However, while there were many speculations regarding the nature of these cells—some of which were prescient regarding what we now understand—the identity and thus physiology and regulation of the intestinal stem cells were elusive.

We will not review the historical literature on putative stem cells in the intestinal mucosa, but begin with the 2007 report that crypt base columnar (CBC) cells located at the very crypt bottom express the marker *Lgr5* on their cell surface (Fig. 2A). As these cells divide, their progeny populate the intestinal villi and provide all the necessary cell lineages [Barker et al., 2007]. This can be visualized in an appropriately marked genetic mouse by a continuous “ribbon” of red cells emigrating from the CBC cells (see Fig. 2B), with replacement of almost all cells in the villi by progeny of *Lgr5*⁺ cells taking approximately 5 days. However, an almost coincident report described a relatively quiescent cell population at the +4 position

(i.e., 4th cell position from the crypt base) that expresses Bmi1, a member of the Polycomb gene family of transcriptional regulators characteristically expressed in pluripotent progenitor cells of immune and neuronal cells, and that these Bmi1+ cells could also populate all lineages of the intestinal mucosa, albeit over a longer period of time [Sangiorgi and Capecchi, 2008]. Data for both the more frequently dividing Lgr5+ cells, and the more rarely dividing Bmi1+ cells showed that each could initiate tumor development upon introduction of a tumor inducing genetic alteration [Sangiorgi and Capecchi, 2008; Barker et al., 2009]. The debate regarding which cell was the “true” intestinal stem cell responsible for routine maintenance of homeostasis of the intestinal mucosa is not yet resolved, and as will be discussed, is a complex issue.

Lgr5+ STEM CELLS

A rapid succession of elegant papers revealed many other key properties of Lgr5+CBC stem cells: the cells express high Lgr5 levels, an important receptor for R-spondin that can amplify Wnt signals characteristic of stem and rapidly proliferating cells, but they express this Lgr5 marker only as long as they remain at the bottom of the intestinal crypt in their functional niche [de Lau et al., 2011; Sato et al., 2011; Clevers and Bevins, 2013; Koo and Clevers, 2014]; that a key component of the niche that secrete Wnts and other growth factors necessary for Lgr5+ stem cell function are Paneth cells, a secretory cell type for which physical contact with Lgr5+ cells is maximized so that signal exchange is optimized [Sato et al., 2011; Clevers and Bevins, 2013]; that Lgr5 cell division is symmetric, with subsequent stochastic allocation of one daughter cell to the crypt bottom as a self-renewing Lgr5^{hi} stem cell, while the other daughter cell migrates out of niche, and ceases to express Lgr5 as it begins its journey of lineage specific differentiation [Lopez-Garcia et al., 2010; Snippert et al., 2010]. Key support for the conclusion that Lgr5+ cells are the fundamental intestinal stem cell is that these cells can be used to grow intestinal organoids in culture that contain all normal intestinal cell lineages [Sato et al., 2009; Jung et al., 2011; Yui et al., 2012]. However, this is also true of Bmi1+ cells, though this may be complicated by the interconversion of these cells to replenish Lgr5 cells [Ootani et al., 2009].

“RESERVE” INTESTINAL STEM CELL POPULATIONS IN GENERAL

There are two lines of evidence that intestinal epithelial cells show great plasticity in their ability to serve as stem cells, complicating the issue of the unique identity and nature of the canonical stem cell in the mucosa. The first comes from genetic manipulation in vivo showing that even differentiated epithelial cells can function as stem and tumor initiating cells under particular circumstances. For example, activating NF-κB signaling in differentiated villus cells in vivo can prompt them to de-differentiate, adopt stem-cell properties and serve as tumor initiating cells [Schwitalla et al., 2013]. Further, long-lived secretory cell precursors [Buczacki et al., 2013], and cells that express Paneth cell markers [Roth et al., 2012] also have similar ability to “de-differentiate” into cells with stem cell properties that can maintain the intestinal mucosa.

The second line of evidence is a series of reports that a number of relatively quiescent cell types, similar to the Bmi1+ cell, but expressing different complements of markers, can

lineage trace and form tumors, especially when the more rapidly dividing Lgr5⁺ cells are damaged by radiation exposure, or chemical or biological insult. Thus far, this has been shown to be the case for cells marked by expression of Bmi1 (as discussed); Hopx [Takeda et al., 2011]; Lrig1, a suppressor of ErbB growth factor signaling that itself is a repressor of intestinal cell proliferation [Powell et al., 2012; Wang et al., 2013]; Tuft cells which are positive for the marker DCLK1 [Westphalen et al., 2014]; and Krt19 cells that express keratin 19, and can regenerate the Lgr5 stem cells when they are ablated [Asfaha et al., 2015]. The relationships among cells from these compartments are not clear: all are located “above” the Lgr5⁺ stem cell compartment, but not in coincident positions and with different complements of marker expression and phenotype. On the one hand, it has been suggested that the Lgr5^{hi} cell expresses many of these markers of the more quiescent cells that can be remobilized, and therefore cells from each compartment are variations of the more rapidly dividing Lgr5 cell [Munoz et al., 2012]. Alternatively, expression profiling has shown not only quantitative but also qualitative differences in gene expression for each population for example [Powell et al., 2012]. The safest conclusion thus far may be that any cell that can be prompted to exhibit stem cell properties must share some features (e.g., pathway activation/repression), hence expression of the markers common to the Lgr5^{hi} cell population. However, there is no agreement—partly semantic, but also functionally important—regarding how different a cell must be on the molecular level to be considered a distinct cell type. In this regard, even different lineages in the intestinal mucosa share expression of many gene sets, albeit at different quantitative levels [Velcich et al., 2005].

What is clear is that for each of the more quiescent cell compartments, the “mobilization” of cells back into the cell cycle and ability to exhibit stem cell properties has been considered part of a “repair” response to the damage/destruction that Lgr5 cells undergo due to physical, chemical, or biological injury. This was shown for each of the cell types discussed above, and particularly for Bmi1⁺ cells [Tian et al., 2011]. Indeed, there are efforts to harness the potential mobilization of these usually quiescent cells to repair the intestinal mucosa damaged by radiation therapy, chemical injury or colitis, or even exposure to nuclear accidents or fallout from nuclear weapons, to which rapidly dividing cells, such as Lgr5 stem and transit amplifying cells in the intestine, are highly sensitive. However, as we shall discuss, environmental factors may play a normal role in determining which stem cell compartments contribute to mucosal maintenance.

ROLE AND POTENTIAL MECHANISM OF VITAMIN D IN REGULATING INTESTINAL STEM CELL FUNCTION

Vitamin D is a highly pleiotropic hormone that affects developmental processes and physiology of dozens of different cell types and tissues [Norman, 2008; Feldman et al., 2014]. In regards to intestinal homeostasis, there is considerable evidence that vitamin D induces pathways of intestinal cell maturation (e.g., cell cycle arrest, differentiation, apoptosis) [Fleet, 2008; Fleet et al., 2012]. In regards to colorectal cancer, there is a very clear inverse relationship between vitamin D levels in the human population and the propensity of individuals to develop colorectal tumors. This association of lower vitamin D levels with higher risk for colorectal cancer is convincingly established by multiple

epidemiological studies involving many thousands of subjects and meta-analyses of up to a million subjects [Garland et al., 1989; Braun et al., 1995; Tangrea et al., 1997; Feskanich et al., 2004; Wactawski-Wende et al., 2006; Gorham et al., 2007; Gonzalez and Riboli, 2010; Jenab et al., 2010; Lee et al., 2011; Ma et al., 2011], and reviewed in [Feldman et al., 2014; Albanes, 2015].

We have therefore studied the role of vitamin D₃ as a nutrient that has profound influence on tumor development in a unique mouse model of sporadic colon tumorigenesis. Sporadic tumorigenesis, rather than tumors initiated by inheritance of a mutation in an oncogene or suppressor gene, characterizes >80% of colon cancer, and the term reflects the unknown etiology of the disease that is most influenced by environmental factors. Moreover, there is significant a priori evidence that vitamin D₃ exposure levels may exert a profound influence on functions of intestinal stem cells, thought to be the cell of origin of intestinal tumors. This comes from determination by the Clevers' lab of an Lgr5^{hi} stem cell signature [Munoz et al., 2012]. This work utilized the mouse model shown in Figure 2A, in which Lgr5⁺ cells fluoresce "green" as long as they remain in their stem cell niche at the crypt bottom, but rapidly reduce green fluorescence in the immediate daughter cell that begins to move up the villus on its journey of lineage commitment and differentiation. Thus, canonical Lgr5 stem cells were isolated by fluorescent activated cell sorting (FACS) as those most highly fluorescent (Lgr5^{hi}) and compared to daughter cells also isolated by FACS that were Lgr5^{lower}. The two cell populations were compared by two RNA expression and one proteomic platform: 510 genes were differentially expressed in the Lgr5^{hi} versus Lgr5^{lower} cells as detected by at least two of the three platforms, but differential expression was found by all three platforms for only 33 of these 510. One of the 33 was the vitamin D receptor (Vdr), characteristically expressed in the Lgr5^{hi} cells but down-regulated in the Lgr5^{lower}—that is, as soon as the stem cell left the crypt bottom. This is strong a priori evidence that stem cell functions of Lgr5^{hi} cells depend on vitamin D signaling through the Vdr. There is precedent for this: it has been established that both hair follicle stem cells [Li et al., 2001; Kong et al., 2002; Alonso and Fuchs, 2003] and oligodendrocyte progenitors [de la Fuente et al., 2015] depend on Vdr signaling for their functions.

We therefore pursued this in the mouse model of sporadic intestinal tumorigenesis. This is a dietary model of sporadic intestinal and colonic tumorigenesis developed by Newmark and Lipkin: the diet they designed was NWD1 (NewWesternDiet1), which adjusts major nutrient risk factors for human CRC to the same relative intake levels commonly consumed in western societies with a high CRC incidence [Newmark, 1987]. Based on AIN76A control diet (American Institute of Nutrition 76A), NWD1 increases lipid from 5% to 20%, and decreases vitamin D₃, calcium, fiber, and methyl-donors (folic acid, choline and methionine) [Newmark et al., 2001]. NWD1 is protumorigenic, amplifying and accelerating intestinal/colon tumors in every mouse genetic model investigated, regardless of etiology or aggressiveness, initiated by single or compound mutations, for example, [Yang et al., 2001, 2003, 2005, 2008b].

More important, we and two other groups showed feeding NWD1 to wild-type *C57Bl/6* mice for 1–2 years causes colon and intestinal tumors, reflecting lag (2/3 of life-span), incidence (25%), frequency (1–2 tumors/mouse) and ratio of carcinoma to adenoma (~10%)

characterizing human *sporadic* CRC [Newmark et al., 2001; Yang et al., 2008a; Aslam et al., 2010]. Gross and microscopic tumor histopathology were published [Newmark et al., 2001; Aslam et al., 2010] and reviewed by us [Newmark et al., 2009]. Vitamin D₃ levels are lower in NWD1 than in chow diets, or control AIN76A on which it is based, but as discussed below, vitamin D₃ levels in such control diets are high, establishing serum 25(OH)D levels in the mouse that are well above the range in the US population [Peregrina et al., 2015]. Feeding mice NWD1 reduces 25(OH)D serum to lower levels that, as predicted by the dietary formulation, characterize individuals at higher risk for CRC in the population, but does not lower serum calcium or PTH, with no significant effect on bone mineral density [Bastie et al., 2012]—that is, mice fed NWD1 are healthy, not obese, and have a “normal” life span of >2 years.

We showed that appearance of sporadic intestinal and colonic tumors in ~20% of the wild-type C57Bl/6 mice after 1–2 years of feeding NWD1 mice is preceded by altered cell maturation, metabolism and stem cell function in the histologically normal flat mucosa >1 year before tumors are detected [Yang et al., 2008a; Wang et al., 2011; Peregrina et al., 2015]. Altered epithelial cell maturation was documented by elevated and expanded Wnt signaling throughout crypts and villi by NWD1 [Yang et al., 2008a; Wang et al., 2011], perturbation of lineage specific markers and high ectopic expression of Paneth cell markers throughout villi and crypts in epithelial cells of the small and large intestine that do not exhibit Paneth cell histology [Wang et al., 2011]. All of these changes are prevented by elevating vitamin D₃ and calcium in NWD2 rescue diet.

Therefore, we determined how the diets affected intestinal stem cell function [Peregrina et al., 2015]. This showed that the essential stem cell function of lineage tracing—giving rise to daughter cells that populate the mucosa with the different cell types needed for maintaining homeostasis and proper mucosal function—was compromised in Lgr5⁺ cells by feeding NWD1 (compare Fig. 2Bi–iii to iv–vi). This is indeed predicted by the stem cell signature of Lgr5⁺ cells that implicated Vdr signaling in Lgr5^{hi} stem cell functioning [Munoz et al., 2012]. This compromised lineage tracing is eliminated by elevating vitamin D in the diet (Fig. 2Bvii–ix). Further, definitive evidence that vitamin D signaling is necessary for Lgr5 stem cell function came from inactivating the gene that encodes the vitamin D receptor specifically in the Lgr5 cells, which showed that within 3 day, this recapitulated the compromise of their stem cell functions by feeding the NWD1 (Fig. 2C compare i–iii to iv–vi).

Another important property of stem cells is that upon accumulation of specific gene mutations, stem cells can initiate tumor development. To test whether this too was sensitive to vitamin D for Lgr5⁺ cells, a conditional *Apc* mutation was introduced into Lgr5⁺ cells in vivo in mice that had been maintained on different diets for several months [Peregrina et al., 2015]. The mice then continued on their respective diets for an additional 6 months to assess intestinal tumor development dependent on loss or inactivation of the second, wild-type allele. Mice fed either AIN76A control diet, or NWD2 diet developed intestinal tumors at about the same frequency as sporadic tumors that develop in humans (i.e., 1–3/mouse). However, in mice fed NWD1, in which lineage tracing from Lgr5⁺ cells was compromised, no tumors were detected (Fig. 3). Thus, there is correspondence between the ability of Lgr5⁺

cells to function as stem cells as assayed by lineage tracing, and their ability to initiate tumor development [Peregrina et al., 2015].

IMPORTANT IMPLICATIONS OF THE PROFOUND EFFECTS OF VITAMIN D SIGNALING ON Lgr5 STEM CELLS

These data raise many questions. First, since at lower dietary vitamin D levels Lgr5+ stem cell function is compromised, how do these mice live out a normal life span with an intestinal mucosa that is only subtly perturbed? The hypothesis is that the other more quiescent cell populations that can serve as stem cells when Lgr5 cells are eliminated or damaged are mobilized to do just that. It has in fact been shown that Bmi1+ cells are completely able to maintain the mucosa upon elimination of Lgr5 stem cell functions [Tian et al., 2011], and our data suggest that these cells also contribute to homeostasis in mice fed NWD1 (unpublished). However, it is as yet unclear whether there are contributions of multiple different potential stem cell populations, and if so, whether relative contribution from different cell compartments is stochastic, which could give rise to heterogeneity in how the mucosa is maintained both within a mouse and between mice. The alternative is that there may be other factors that dictate the relative contributions of cells from different compartments. Regardless of the outcome, the finding demonstrates that environmental factors can play a major role in determining the underlying cell biology of tissue homeostasis and function.

Second, as other cells are mobilized to function as stem cells in place of Lgr5 cells, do these cells become capable of tumor initiation? The fact that NWD1 is protumorigenic, yet impairs the tumorigenesis of Lgr5 cells, suggests that this is the case, and is under investigation.

A third question is what are the mechanisms that drive the mobilization of other cells to function as stem cells? While not yet definitive for the effect of vitamin D, our collaborators have recently addressed this in investigating the impact of Notch signaling on intestinal Lgr5 stem cell function. Notch is a key developmental pathway well-established to be critical for intestinal stem cell function as well as for driving lineage specific differentiation of intestinal stem cell progeny. In this new work, Srinivasan et al. [2016a,b] show that elevating Notch signaling drives asymmetric Lgr5+ cell division that causes a fast cycling Lgr5 stem cell to produce a slower cycling Bmi1+ cell. Although Lgr5 cell division has been reported to be generally symmetric rather than asymmetric [Lopez-Garcia et al., 2010; Snippert et al., 2010], a recent report provides a mechanism by which this may not always be the case, even under conditions in which Lgr5 cells maintain normal mucosal homeostasis. In dissection of short distance Wnt signals necessary for stem cell function, it has been shown that after 1 or 2 divisions of Lgr5+ cells—that is, as the daughter cells are just moving up from the bottom of the crypt—there is a discordance of Wnt signals on opposite sides of the Lgr5+ cell, with the trailing side of the cell still receiving Wnt signals from Paneth cells while the leading edge, no longer in contact with Paneth cells, is exposed to decreased Wnt signals [Farin et al., 2016]. This can lead to asymmetric cell division [Habib et al., 2013]. Thus, Notch signaling may induce asymmetric division of Lgr5 cells either because of a direct interaction

of Wnt signals in regulating Notch [Estrach et al., 2006; Katoh and Katoh, 2006; Rodilla et al., 2009; Guilmeau et al., 2010], or by a similar mechanism in which Notch receptors distributed on the cell membrane have differential contact with neighboring cells that express Notch ligands. An issue, then, is whether asymmetric Wnt or Notch signals are the mechanism through which vitamin D may alter the outcome of an Lgr5 cell division and subsequent functions of the progeny. Preliminary data suggest that it may.

Finally, despite the compelling data that level of vitamin D exposure is a key in determining probability of intestinal tumor development, prospective intervention studies in humans have not yet shown that vitamin D supplements can prevent colon tumor recurrence. This can be attributed to the fact that prospective intervention trials have thus far only modestly elevated vitamin D exposure (e.g., 25(OH)D serum levels) and/or for only relatively short periods of time, whereas interventions to lower cancer risk may require a decade or more to be effective discussed in [Feldman et al., 2014]. There is, however, another explanation which grows from the fact that vitamin D is a key to function of long-lived intestinal stem cells. Since prospective vitamin D supplementation studies for cancer have been carried out only on mid-aged to elderly higher risk individuals, the exposure to lower levels of vitamin D that establish relative risk may “fix” genetic and epigenetic changes in long-lived progenitor cells that cannot be readily reversed at an older age. This is critical because it would shift focus to levels of exposure to vitamin D—and potentially other nutrients and environmental factors—to childhood and early development in order to effectively avoid excessive cancer development much later in life [Dolinoy et al., 2007a,b; Weidman et al., 2007]. Consistent with the fact that epigenetic changes can persist and cause disease over lengthy time frames is that altered probability to develop cancer can be established by maternal or paternal dietary patterns, and that elevated “risk” can persist across generations linked to epigenetic changes [Jirtle and Skinner, 2007; Fontelles et al., 2016a,b].

A NOTE ON MOUSE HUSBANDRY AND IMPLICATIONS FOR THE REAL WORLD

Genetically inbred mouse strains have had unmatched impact in standardization of biologic—especially immunologic—background of these models, and in limiting experimental variability among mice. A parallel to this has been the standardization of mouse care and minimization of their exposure to xenobiotics and pathogens. Together, control of genetic and environmental background has led to relatively long-lived mice that are maintained under rigidly controlled conditions. There is no underestimating this in terms of driving progress across virtually every aspect of cell, molecular, and developmental biology.

It is important, however, to understand the origins and nature of diets that have come into standard use for mouse maintenance. This has involved optimization of macro- and micronutrients to promote rapid growth and reproduction of mouse strains. Rodent diets generally fall into two classes: purified (or semi-purified) that are formulated and comprised of specific nutrients, and, in contrast, what are broadly classified as “chow” diets. Chow diets are less expensive alternatives comprised not of purified components, but of natural plant materials such as ground corn, oats, and wheat, and alfalfa and soybean meal, with

vitamins, minerals, and fat added as required (e.g., see <http://www.researchdiets.com/>). These diets are highly useful: reliable manufacturers formulate and test these natural plant diets to maintain fairly standard composition reflecting what are thought to be optimal nutrient levels. However, failure to carefully consider dietary formulation and composition leads to serious issues. For example, the literature on intestinal stem cells, including publications in even the most high profile and ostensibly rigorous journals, rarely—if ever—mention the diet fed the mice. Thus, it is safe to assume that almost all the literature reviewed above on intestinal stem cells was generated from mice fed chow diets. Because these control diets are formulated to promote the most rapid growth and reproduction of mouse strains, the diets do not necessarily reflect nutrient exposures in the human. For example, standard chow diets provide very high vitamin D₃, generating 25 (OH)D serum levels of ~125 nmol/L [Bastie et al., 2012; Wang et al., 2012; Bolton et al., 2013]. Extensive CDC-NHANES data shows this is well above the range for the US population, and especially those at highest risk for development of colorectal cancer (Fig. 4). Our data, reviewed above (and unpublished), show that when dietary vitamin D is reduced to the lower levels characteristic of a large segment of the US population, or when the vitamin D receptor gene is inactivated in Lgr5⁺ cells, stem cell functions of Lgr5⁺ cells are compromised. Thus, the uncomfortable fact is that one should question whether mouse models fed standard control diets containing very high vitamin D₃ are an accurate model in which the Lgr5 CBC cells are functioning similarly to the corresponding cells in the human population, or the extent to which the human homologues of Lgr5^{hi} cells contribute to intestinal homeostasis or tumor development in the human at much lower vitamin D₃ levels. The other side of this coin is that relatively quiescent cell compartments that contribute stem cells for intestinal maintenance and tumor development in the mouse when Lgr5 stem cells are compromised (e.g., radiation injury) may play a much greater role in the human because of the nutritional environment to which the population is routinely exposed. This is not likely an issue of different required levels of vitamin D for the two species: there is a wealth of data that humans might be healthier in many ways if vitamin D levels could be increased for the general population, and the much higher levels to which mice are routinely exposed are somewhat arbitrarily determined to provide their optimum health, which may not correspond to either requirements or exposure of feral mice in the wild. Thus, the exposure differences between experimental mice and humans reflect what exists, and likely not major differences in physiologically optimum levels. Moreover, vitamin D is probably not the only nutrient or environmental factor that directly or indirectly influence intestinal stem cell functioning, though this is not yet well-investigated. Thus, we have pointed out that failure to reflect human nutritional exposures accurately may be why mouse models so often do not serve as reliable surrogates for human marker and drug development [Augenlicht, 2014], a problem frequently discussed in the literature [Perrin, 2014; Reardon, 2016].

Failure to appreciate the profound differences among rodent diets leads to unreliable data. For example, in a recent report, Lgr5 stem cells from mice fed a high fat experimental purified diet were compared to these cells from mice fed a control chow diet [Beyaz et al., 2016]. The different classes of diets used for the experimental and control groups, from different manufacturers, differed in levels of a very large number of nutrients besides fat. Even more important is that the control chow diet certainly contained very high levels of

phytoestrogens derived from the processed plant material used in its manufacture. These compounds, such as genestein and equol, have well documented effects on key metabolic and regulatory pathways in many tissues, and are elevated in mice fed chow diets to serum levels that are 10^2 to 10^4 fold higher than levels of endogenous estrogen, but are undetectable in mice fed purified diets [Brown and Setchell, 2001; Wang et al., 2005]. Thus comparing mice fed a purified diet to those fed a chow diet generates data that is uninterpretable. Most investigators who use chow diets without much consideration of the implications would be shocked if it were proposed that such high levels of biologically potent chemicals were to be added to the diets fed to their mice. Ironically, however, they ignore that this is exactly the circumstance under which their experiments are carried out. Failure to consider these factors in experimental design can only complicate progress in understanding the impact and mechanisms of environmental modulation of tissue homeostasis and modulation of disease phenotype, and how this is mediated by stem cell functions.

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Fig. 1. Architecture of the intestinal mucosa. (A and B) Hematoxylin/eosin stained sections of the mouse small and large intestine; (C) schematic of the small intestine showing the various functional zones of along the crypt-luminal axis.

Fig. 2.

Stem cells and their functioning in the mouse small intestine. (A) *Lgr5*⁺ crypt base columnar cells from a mouse engineered so that these cells fluoresce green (*Lgr5*^{EGFP-cre:ERT2}, as reported in [Barker et al., 2007]); (B) *Lgr5*^{EGFP-cre:ERT2}, *Rosa26*^{RFP} mice in which Tamoxifen injection causes the *Lgr5*⁺ cells and their daughters to permanently fluoresce red so that their fate can be tracked over time. Mice were fed different diets for 3 months from weaning before tamoxifen injection: AIN76A control diet, i–iii; NWD1 diet, iv–vi; NWD2 diet, vii–ix (reprinted from [Peregrina et al., 2015]); (C) *Lgr5*^{EGFP-cre:ERT2}, *Rosa26*^{RFP} mice that are also either wild-type for the vitamin D receptor, or are homozygous for a conditional knockout (floxed) allele that encodes the vitamin D receptor. Tamoxifen injection in the latter not only marks the *Lgr5* cells and their progeny red, but also simultaneously inactivates expression of a functional vitamin D receptor. All mice were fed AIN76A control diet for 3 months from weaning (reprinted from [Peregrina et al., 2015]).

Fig. 3. Tumorigenesis initiated from *Lgr5* cells in mice fed different diets. (A) Number of tumors and their histopathology in individual *Lgr5^{EGFP-cre:ERT2}, Apc^{flox/+}* mice in which tamoxifen injection inactivates the floxed allele of the tumor suppressor gene *Apc*. Mice were fed AIN76A, NWD1, or NWD2 for 3 months from weaning, injected with tamoxifen, then each mouse continued on its respective diet for an additional 6 months. The difference in tumor frequency between AIN76A and NWD1 mice was significant at $P < 0.03$. (B) Examples of tumor histopathology (reprinted from [Peregrina et al., 2015]).

Fig. 4. Spectrum of vitamin D exposure across the US population. Data are plotted from those made available by the National Health and Nutrition Examination Survey (NHANES) conducted by the Centers for Disease Control (http://www.cdc.gov/nchs/nhanes_products.htm). The arrows show the levels reached in mice by feeding a standard chow diet, control AIN76A purified diet, or NWD1 diet.