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Vitamin D and the nutritional environment in functions of intestinal stem cells: implications for tumorigenesis and prevention

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Abstract

Sporadic colon cancer accounts for ~80% of CRC, with high incidence in western societies strongly linked to dietary patterns. The only mouse model for sporadic CRC results from feeding mice a purified rodent western-style diet (NWD1), establishing mouse intake of several common nutrients that mimic for each its level consumed in western populations at higher risk for colon cancer (higher fat, lower vitamin D_3, calcium, methyl donors and fiber). This causes sporadic colon and small intestinal tumors at an incidence and frequency similar to that of humans. NWD1 perturbs intestinal cell maturation and Wnt signaling throughout villi and colonic crypts before tumors are detected. Surprisingly, feeding NWD1 decreases mouse $Lgr5^{hi}$ intestinal stem cell contribution to homeostasis and tumorigenesis, associated with extensive $Lgr5^{hi}$ cell transcriptional reprogramming, with nutrient levels interactive in these effects. There is a key impact of the lower vitamin D_3 in NWD1 and its signaling through the Vdr. The DNA mismatch repair pathway is elevated in $Lgr5^{hi}$ cells by lower vitamin D_3 and/or calcium in NWD1, reducing accumulation of relevant somatic mutations detected by single cell exome sequencing. There are also alterations in metabolic pathways, including down-regulation of oxidative phosphorylation. In compensation for compromise of $Lgr5^{hi}$ cells, NWD1 also reprograms cells derived from the Bmi1+ population, defined as those cells marked in *Bmi1*^{creERT2}*, Rosa26*^{tom} mice following tamoxifen injection, and at least a portion of these cells then function and persist as stem-like cells in mucosal homeostasis and tumorigenesis.

The data establish a key role of the nutrient environment, and vitamin D signaling, in defining contribution of at least two different stem cell populations to mucosal homeostasis and tumorigenesis. This raises significant questions regarding impact of variable human diets on which and how multiple potential intestinal stem cell populations function in the human and give rise to tumors. Moreover, genetic and epigenetic changes in long-lived stem cells have important

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implications for understanding the effects of vitamin D and other nutrients on intestinal homeostasis and on intervention strategies for altering probability of tumor development.

Keywords

intestinal stem cells; nutritional environment; vitamin D; intestinal homeostasis; intestinal tumors

Introduction:

Continuous turnover of epithelial cells of the intestinal and colonic mucosa requires continuous production and differentiation of these cells to maintain mucosal homeostasis and normal functions. In the mouse, this is accomplished primarily by division of crypt base columnar (CBC) cells located at the bottom of the crypt that express a high level of the marker Lgr5 (Lgr5^{hi} cells) [1–3]. However, there is extensive plasticity in the ability of other cell populations to take over this responsibility when Lgr5^{hi} cells are damaged or ablated experimentally by radiation, chemicals, targeting of toxins, or genetic manipulation [1]. Here we summarize evidence that this plasticity can also be marshalled by changes in the nutritional environment, and in particular by the status of vitamin D signaling of the Lgr5^{hi} cells. The implications of this for understanding the impact of environmental influence on the incidence of colorectal cancer (CRC), and for how this may inform intervention strategies to prevent CRC, are discussed.

1.1 Intestinal stem cells in homeostasis.

It was long appreciated that there must be a stem cell population underlying the constant turnover of the intestinal mucosa (reviewed in [4]). Two reports identifying specific cell populations with properties of stem cells were turning points in understanding how the mucosa constantly renewed itself. First, CBC cells at the crypt bottom of the mouse small intestine were identified as interdigitated with Paneth cells, and shown to be able to give rise to all mucosal epithelial cells [1–3] (Fig 1A–C). Soon thereafter, it was reported that cells at about the +4 position from the bottom of the mouse crypt, expressing the marker Bmi1, could also give rise to all cell lineages of the mucosa [5](Fig 1A). These reports initiated an explosion of research – not to be reviewed here – investigating these and other intestinal cell populations. A summary of the model that dominates the field is that the CBC Lgr5^{hi} cells are normally responsible for maintaining the mucosa. A key feature of these cells is that they express high levels of the surface marker Lgr5, a receptor for the growth factor Rspondin that amplifies Wnt signals [6, 7] (Fig 1B,C). However, it is clear that when these Lgr5^{hi} cells are ablated or damaged by radiation, chemicals or other experimental procedures, other cell types can “seamlessly take over maintenance of the mucosa” [1]. This is accomplished by mobilization of cells from other cell compartments to function as stem or progenitor-like cells. Several of the cell compartments that can contribute such cells are depicted in Fig 1A: Bmi1+ cells at the +4 position [8], although there is heterogeneity in this population in terms of mucosal distribution and co-expression with other stem cell markers [5, 8, 9]; differentiated cells that can revert to a more “stem- or progenitor-like” state [10, 11]; L-rig1+ cells [12]; Tuft cells expressing DCLK1 [13]; Krt19+ cells [14]; and Paneth cells [15–17]. Molecular analysis has also suggested that there may not be dedicated pre-existing cells

that take over when Lgr5^{hi} cells become non-functional as stem cells, but that a multi-potential progenitor cell is recruited as needed [18].

In summary, these and other studies have established that there is tremendous plasticity of intestinal epithelial cells to serve as stem or progenitor cells, and that there may be a hierarchy of such cells that can be mobilized as needed. It is important, however, that although mechanisms have been dissected that can potentially mobilize different populations, there are key unanswered questions: how do the different cell populations "sense" that Lgr5^{hi} cells are no longer functioning as stem cells; are signals transduced uniformly to each potential reserve population or are there conditions which specify which cell population is mobilized?

Stem cell populations and intestinal tumorigenesis.

The definition of what constitutes an adult tissue-specific stem cell population is somewhat fluid, especially with the evidence that multiple populations can acquire the characteristics of stem cells under different conditions. However, among important characteristics of stem cells are the ability to self-renew and to give rise to many or all of the differentiated cell types of a tissue. In the case of the intestine, Lgr5^{hi} cells have been shown to divide, and then by a stochastic process, one of the daughter cells can become a self-replicating stem cell while the other begins the process of differentiation into the many epithelial cell types that comprise the intestinal mucosa [19, 20]. This distinction in the fate of daughter cells likely resides in the environment in which each finds itself. The Lgr5^{hi} stem cell divides symmetrically, with the daughter cell remaining in the crypt – and thus exposed to stem cell niche signals – retaining functions of a stem cell, while the cell that begins to migrate up from the bottom of the crypt loses short-range Wnt signals and/or is exposed to other signals that initiate the process of differentiation [4]. These additional signals likely encompass important pathways that involve cell-cell contacts, such as Notch signaling [21–24], as well as the making and breaking of contacts with cells of the myofibroblast sheath and Paneth cells residing at the crypt bottom (reviewed in [4]).

The shift from a stem to a differentiating cell is rapid and complex: comparison of the Lgr5^{hi} cell gene expression signature compared to that of the immediate daughter cells that are Lgr5^{lower} (i.e. following the first symmetric cell division and movement out of the stem cell niche, as shown in Fig. 1C) identified 512 genes that differed in expression between the two states [9]. This demonstrates a considerable reprogramming that takes place immediately in the cells destined to give rise to all epithelial lineages.

In addition to the ability of stem cells to give rise to a plethora of differentiated cell types, stem cells are likely the cell of origin of tumors by virtue of their ability to continuously divide and self-renew. For example, both human and mouse intestinal tumors are most frequently initiated by inactivating mutations in the *Apc* gene, which removes a brake on Wnt signaling (reviewed in [25]). And indeed when inactivation of the *Apc* gene was targeted to Lgr5^{hi} cells, intestinal tumors were initiated [26–28]. Moreover, in accord with the necessity of cells to have features of "stemness" in order to initiate tumors, targeting an *Apc* mutation to Bmi1+ cells also can give rise to intestinal tumors, as does targeting the mutation to other cell types when they are mobilized to take over for the canonical Lgr5^{hi}

stem cell when it is damaged. This included targeting the mutation to differentiated cells, but importantly, only when such cells had been induced to revert to a more stem cell-like state [10, 11]. Therefore, “stemness” seems to be a requirement for cells to be able to initiate tumor development once they acquire an initiating mutation or complement of mutations.

Dietary and vitamin D_3 impact on intestinal stem cells and tumorigenesis

An aspect of our work on intestinal homeostasis and tumorigenesis has focused on the role of nutritional interactions in stem cell functions. This has made use of a novel dietary model of human *sporadic* colon cancer (sCRC) – the form of the disease that accounts for about 80% of all human CRC. To model conditions that may increase the probability for development of sCRC, Newmark and Lipkin developed the “western-style diet” (NWD1) based on the control purified diet AIN76A [29–33]. The principle of nutrient density was used to adjust the dietary formulation to generate intake for the mouse of several common nutrients each at its level consumed in western populations that are at higher risk for colonic cancer. The changes to NWD1 compared to AIN76A control are higher fat, lower vitamin D_3, calcium, methyl donors (folate/methionine) and fiber, since there are epidemiological data associating each of these with elevated probability of developing sporadic human CRC. As a result of these nutritional changes, feeding NWD1 to mice is highly pro-tumorigenic: in mouse genetic models of intestinal tumor development, feeding NWD1 increased tumor number and caused more rapid tumor development than in mice fed control diet [34–37]. This was independent of the genetic driver(s) in each model that caused the tumors, or the aggressiveness of tumor development in each model when fed control diet. Therefore, there were common pro-tumorigenic effects of the diet on the intestinal mucosa independent of genetic etiology. Most important is that wild-type C57Bl/6 mice rarely - if ever - develop intestinal tumors. However, when fed the NWD1 from weaning, cohorts of these mice developed 1–2 intestinal tumors in the small and/or large intestine in 20% of the mice after 1–2 years, or about one-half to two thirds of their lifespan [33, 38, 39]. This is similar to the incidence, frequency and lag for tumor development found in the general US population undergoing routine screening by colonoscopy. Therefore, this is the only model of sporadic intestinal tumorigenesis, accounting for the majority of the human disease, and the form of the disease for which incidence is most influenced by long term nutritional patterns.

Mice fed NWD1 are generally healthy until tumors develop; they gain more weight, but do not become obese, and consistent with the higher fat content of NWD1, mice fed the diet shift to greater utilization of fat relative to carbohydrate as an energy source [40]. Despite the lower levels of vitamin D_3 and calcium in NWD1, the mice do not suffer from rickets or altered bone mineral density [40]. Moreover, although the mucosa is “primed” to cause higher probability for tumor development, it appears histologically normal. This concept – that there are field effects in a tissue that alter the probability of eventual tumor development and progression – was proposed in 1953 [41], and is considered a milestone in chemoprevention research [42]. In the NWD1 fed mice, such alterations that precede tumor development by 1 or more years include expansion of the proliferative compartment, altered balance of lineage specific cell markers, a shift of cells towards glycolytic metabolism, ectopic expression of Paneth cell markers in the villi and crypts of the small and large

intestine, and elevated Wnt signaling throughout the villi and crypts of the small and large intestine [38, 43].

These distinct alterations in the normal appearing and functioning intestinal mucosa, especially perturbed maturation of epithelial cells and elevated Wnt signaling, prompted investigation of whether NWD1 had altered the function of intestinal stem cells. The first approach investigated lineage tracing of the progeny of the $Lgr5^{hi}$ stem cells at the bottom of the crypt [44]. This used a mouse model in which Lgr^{hi} stem cells expressed a green fluorescent marker as long as they were at the crypt bottom, but that their progeny were induced to permanently express a red fluorescent marker, and thus could be tracked for their subsequent fate. In mice fed control AIN76A diet for 3 months from weaning, the results recapitulated that published for mice fed other control diets: from 1 to 5 days, “red” progeny emerged from the “green” $Lgr5^{hi}$ cells at the crypt base, and progressively made their way up the villi, populating the mucosa with newly generated epithelial cells (Fig 2A-top row). However, when fed NWD1 for 3 months, many of these red cells remained at the bottom of the crypt: $Lgr5^{hi}$ cells which were now green and red appeared yellow at the crypt bottom, with many fewer red cells migrating into, and then up, the villi (Fig 2A-middle row). The same was true for the mice fed NWD1 vs AIN76A for 1 year (Fig 2B). However, when a rescue diet was fed, in which vitamin D_3 and calcium were elevated in the NWD1 (ie, NWD2), normal lineage tracing of the $Lgr5^{hi}$ cell progeny was present (Fig 2A-bottom row; 2B).

Therefore, $Lgr5^{hi}$ cell stem cell function was dependent on higher levels of vitamin D_3 and calcium in the diet fed the mice. In reviewing the literature, it was noted that the $Lgr5^{hi}$ cell stem cell signature had identified 512 genes altered in expression in comparing $Lgr5^{hi}$ cells to their immediate $Lgr5^{lower}$ progeny [9]. Thirty-three of these were altered most robustly, and one of these encoded the vitamin D receptor (Vdr). The Vdr gene was expressed significantly at both the RNA and protein level in $Lgr5^{hi}$ CBC cells but was reduced in the $Lgr5^{lower}$ immediate daughter cells that were exiting the stem cell niche [9]. This strongly suggested that Vdr signaling was necessary for $Lgr5^{hi}$ cells to function as stem cells, but not for cells that were no longer undergoing self-renewal. Therefore, in retrospect, the reduction in lineage tracing from $Lgr5^{hi}$ cells in mice fed NWD1 in which vitamin D_3 was greatly reduced was predicted by this $Lgr5^{hi}$ stem cell signature [44–46].

To pursue this, a mouse was bred in which induction of the red fluorescence in the $Lgr5^{hi}$ cells and their progeny was accompanied by genetic inactivation of the Vdr gene, eliminating vitamin D signaling specifically in these same $Lgr5^{hi}$ cells. In these mice, there was a reproduction of the NWD1 phenotype: the $Lgr5^{hi}$ cell progeny remained at the crypt bottom, even though these mice were fed control AIN76A diet (Fig 2C-bottom row). Thus, the ability of $Lgr5^{hi}$ cells to function as stem cells to populate the mucosa is dependent on vitamin D signaling, which can be compromised either by lower dietary vitamin D_3 or genetic inactivation of its receptor.

Since the mice fed NWD1 were healthy and lived to 2–3 years of age, it was then asked whether other cells had replaced $Lgr5^{hi}$ cells in functioning as stem cells. The first investigation was with a genetic strain in which the progeny of Bmi1+ cells could be

tracked. When these mice were fed control diet, there were occasional bursts of lineage tracing distributed throughout the mucosa, but these were limited in number and extent (Fig 3a,d,g). However, when mice were fed NWD1, the number of such tracks of lineage tracing increased substantially, and by 5 days, many crypt-villi were populated by the progeny of these cells (FIG 3b,e,h). Moreover, this was greatly reduced when the mice were fed the "rescue" diet – NWD1 with elevated vitamin D_3 and calcium (Fig 3c,f,i). Therefore, these progeny of Bmi1+ cells populated the mucosa when the diet suppressed $Lgr5^{hi}$ cell stem cell function. Moreover, the ability of a cell population expressing *Bmi1*^{creERT2} to do this persisted out to at least 60 days following marking of the cells, as long as the mice were fed NWD1, but was reduced by 30 days after switching the mice back to the control diet [46]. The conclusion is that the nutritional environment of the cell can influence relative contribution of cells from different compartments to maintain the mucosa.

The ability of diet to influence stem cell contribution to tumor development was then investigated. When an *Apc* mutation was targeted to $Lgr5^{hi}$ cells, tumors developed in mice fed AIN76A or the rescue diet – that is, when the $Lgr5^{hi}$ cells were functioning efficiently as stem cells – but not when fed NWD1 (Fig 4A). However, when the *Apc* mutation was targeted using $Bmi1^{creERT2}$, most efficient tumor development was with NWD1 – when the Bmi1 marked cells had been mobilized to function as stem cells (Fig 4B). Therefore, there is a direct correspondence between the ability of cells from each of these two compartments to function as stem cells and their efficiency in causing tumors when an initiating mutation is introduced.

Mechanisms impacted by nutritional changes.

RNAseq analysis of both $Lgr5^{hi}$ and Bmi1+ intestinal cells from mice fed different diets provided insight into mechanisms underlying the dietary induced altered contribution of the two stem cell populations to homeostasis. Moreover, to gain insight into potential nutrient interactions, the diets interrogated were: AIN76A control diet, NWD1, NWD1 + elevated vitamin D_3 *and* calcium = NWD2), NWD1+ elevated vitamin D_3 *or* calcium; and AIN76A with only lower vitamin D_3 (Fig 5A). Clustering of the data from each dietary group based on principal components analysis showed that in each cell type, there was a major shift in profile of gene expression when comparing either $Lgr5^{hi}$ or Bmi1 + cells from mice fed NWD1 compared to those from mice fed control AIN76A (Fig 5B,C). Feeding NWD2 – in which both vitamin D_3 and calcium were elevated – caused the NWD1 gene expression pattern for each cell type to shift back towards that of the cells from AIN76A fed mice (Fig 5B,C). However, adding back either vitamin D_3 or calcium to NWD1, or lowering only vitamin D_3 in AIN76A, all produced distinguishable results, suggesting that changes in transcriptional programming of the cells was a function of interaction among nutrient levels [46].

For the $Lgr5^{hi}$ cells, interrogation of the expression data by gene set enrichment analysis (GSEA) showed many pathways altered in these cells by the dietary change to NWD1. High on the list was an elevation in DNA mismatch repair (MMR) in $Lgr5^{hi}$ cells from mice fed NWD1 (Fig 5D), but not in Bmi1+ cells (not shown). However, the MMR pathway was significantly decreased in comparing $Lgr5^{hi}$ cells from mice fed NWD2 to those fed NWD1

(Fig 5E), thus reversing the NWD1 induced increase. Because MMR is a major contributor to the extent of DNA mutation accumulation [47], we then determined how mutations accumulated in the Lgr5^{hi} and Bmil1+ derived cells under different dietary conditions. This required single cell analysis, since the goal was to identify mutation accumulation in the presumably unselected cell populations, along with development of a rigorous bioinformatics pipeline to unambiguously identify these mutations and to distinguish them from potential germ line variants [46]. Coincident with the elevation of the MMR pathway in Lgr5^{hi} cells by feeding NWD1, there was decreased accumulation of mutations, but no change in the Bmi1+ cells, in which the MMR pathway was not altered (Fig 5F). The combination of fewer mutations per Lgr5^{hi} cell and fewer of these cells indicated the mutational burden presented by Lgr5^{hi} cells was decreased by feeding NWD1. In contrast, although there was no change in the mutations per Bmi1+ derived cell, the vastly increased number of these cells in NWD1 fed mice indicates that the mutational burden presented by the cells derived from Bmi1+ cells greatly increased under these dietary conditions. Moreover, analysis of the mutational signature showed the mutations that accumulated were relevant to those that accumulate in intestinal epithelial cells [46].

GSEA also showed that many metabolic pathways were altered in Lgr5^{hi} cells by feeding the NWD1, including a major reduction in mitochondrial oxidative phosphorylation and the TCA cycle (Fig 5G). Extensive experiments are underway to determine the role this plays in potentially reducing the contribution of Lgr5^{hi} cells to maintaining the intestinal mucosa and in reducing their ability to form tumors on targeted introduction of an *Apc* mutation. This is important since it has been reported that mitochondrial oxidative phosphorylation is necessary for Lgr5^{hi} cells to function as stem cells [48, 49].

Implications of these studies for understanding intestinal homeostasis, tumorigenesis and chemoprevention.—There are key unanswered questions regarding the impact of the western diet on intestinal stem cells.

A principle biological question is the nature of the Bmi1+ cells mobilized by feeding the NWD1. Bmi1+ derived intestinal epithelial cells are heterogeneous both in biochemical/molecular identity and in their distribution throughout the crypt-villus architecture. Indeed, Lgr5^{hi} CBC cells are known to also express Bmi1, and the Lgr5^{hi} population can, under some experimental conditions of abrogating Lgr5^{hi} cell stem cell function, be replenished from the Bmi1+ cell population. This may not be a mechanism involved in response to NWD1, since with uninterrupted feeding of this diet, the Lgr5^{hi} cells would be continuously suppressed in giving rise to progeny. However, there could be a subpopulation of Lgr5^{hi} cells at the crypt base that can function as stem cells under the nutrient conditions established by feeding NWD1, including lower vitamin D availability. There is some precedent for this idea, although the data are indirect : random drift is responsible for development of crypt stem cell clonality, but some mutations can impart selective pressure for a given population [50]. It can therefore be envisioned that during long-term feeding of NWD1, genetic or epigenetic changes could select for Lgr5^{hi} cells that robustly function as stem cells under the nutritional conditions conferred by feeding NWD1. However, since the reduction of ability for Lgr5^{hi} progeny to lineage trace extends to at least a year of feeding NWD1 ([44], and Fig 2B), this may not be likely. Therefore, a major effort underway uses single cell RNAseq to

identify the subset of cells that function as stem cells in mice fed the NWD1, and their reprogramming that permits this.

A second biological question is what the signal is that notifies other epithelial cells in the mucosa that Lgr5^{hi} cells are compromised, triggering a compensatory response. These signals can be biochemically transmitted between cells in an exocrine or endocrine manner, but can also include mechanical signals since the generation of cells at the bottom of the crypt likely provides mechanical force to the crypt-villus structure as cells are produced and migrate. Further, since many different cell populations can respond [1], are there multiple signals that integrate to favor the response of one cell population over another? In this context, adaptive radiation – the rapid expansion of species and higher taxa to occupy new niches – is thought to be driven principally by ability of organisms to adapt to and utilize new sources of food [51]. Therefore, an interesting hypothesis is that the plasticity of cells to function as stem cells may have arisen to provide flexibility to organisms to function in new ecosystems.

The data also raise clinically important issues. We have discussed that standard rodent diets used in stem cell research differ considerably in the levels of key nutrients they provide compared to levels that characterize the human population, especially that of populations at higher risk for sporadic colon cancer. A prime example is the level of 25(OH)vitamin D. There is a broad range of exposure to this vitamin in the human population that varies somewhat with sex, age and demographics, but with a maximum of about 100nmol/L serum. In mice fed standard chow diets, the level reaches ~125 nmol/L and with purified control AIN76A about 100nmol/L. Therefore, serum levels of 25(OH)D in the laboratory mouse fed standard control diets are well above levels for almost 100% of the US population (data shown and discussed in [44, 46, 52]. Since the data demonstrate that Lgr5^{hi} cells require Vdr signaling to function as stem cells, these cells may be considerably reduced in this function in the population, and especially for those with lower vitamin D levels that are at elevated risk for CRC [53–55]. Therefore, there may be greater heterogeneity in which and how intestinal cells are functioning as stem cells in the human than is currently appreciated. This may also be in flux for an individual as a function of their daily vitamin D, and/or other, nutrient exposures.

This raises important questions regarding which and how stem cells initiate tumor development, with fundamental implications for early detection, prevention, prognosis and therapy. Are tumors heterogeneous, depending on which stem cell population harbors the initiating mutation(s) giving rise to the tumor, and does this influence efficacy of prevention, treatment and prognosis? Moreover, as regards prevention, we established that the nutritional environment extensively alters expression profile of both Lgr5^{hi} and Bmi1+ intestinal cells [46]. The extent to which these changes are relatively stable, or if they can be reversed by altering vitamin D levels and levels of other nutrients, is under investigation. This is important since the epidemiological evidence is strong that low vitamin D levels are related to higher incidence of colon tumors [53–55], but relatively short-term intervention studies with vitamin D supplements later in life has been ineffective in preventing tumor recurrence or development. However, if there are stable alterations in long-lived stem cells that can initiate tumorigenesis, the study design of intervening later in life for too short a period -

largely dictated by the cost and logistics of such trials - may be “too little, too late”. For example, in familial adenomatous polyposis (FAP), individuals inherit a mutant *APC* allele but only develop tumors when the wild-type allele is also mutated or lost [25]. In carriers of the germ line mutation, inactivation or loss of the wild-type allele and tumor initiation seems to take at least 10–15 years, based on the age at which multiple tumors are detected. This would therefore seem to be a minimum length of time before one might expect an intervention that targets initiation to show beneficial effects, and perhaps longer for patients that do not inherit a mutant allele. However, a recent meta-analysis that determined there was no effect of vitamin D supplementation on cancer incidence included trials with follow-up ranging from about 4 to 7 years [56]. Moreover, in the very large and well-conducted Vital trial, reported to have shown no effect of vitamin D supplementation on incidence of colorectal or other cancer [54], this was at 6 years of follow-up. Thus, it is too early in any of this work to conclude there is no benefit to vitamin D, or of potentially other nutritional interventions that may be epidemiologically linked to lower incidence of tumors.

The caution regarding intervention studies that are conducted in adult subjects at or beyond mid-life is reinforced by recent studies regarding intestinal stem cells, which are long-lived and likely the cell of origin of CRC. Stem cells in a single crypt take 2 to 3 years to reach “clonality” in the human [50]. This is usually through a process of “neutral drift” in which there is no selective pressure for the any of the 5–10 stem cells in each crypt to eventually become clonal [19, 20]. However, it was demonstrated that mutations in key oncogenic genes/pathways can impose selective pressure for a stem cell to outcompete the others in the crypt [50]. Therefore, individuals at mid- to late adulthood may already harbor many crypts that are clonal as regards their stem cells, all of which share mutation(s) that may support tumor development and be refractory to nutritional intervention. Thus again, study design of intervening later in life may have a high probability of failing.

The stakes in this are high: data from migrant populations, and shifts in cancer incidence in countries as their populations alter long-term dietary patterns, suggest that a considerable amount of cancer might be delayed or avoided altogether if we understood long-term impact and mechanisms better, leading to a greater imperative to change dietary habits. This is not simple to achieve, especially when the shorter-term trials not only confuses the public, but leads to premature conclusions in the scientific community that there is not significant value in such interventions. This is perhaps unavoidable. However, understanding fundamental mechanisms by which vitamin D and other nutrients alter stem cell functions and mucosal homeostasis can be important in pursuing these public health goals, and in potentially reducing the cancer burden in a population.

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Highlights:

- In mice fed control diets, $Lgr5^{hi}$ crypt base columnar cells serve as the principle intestinal stem cell in continually regenerating the intestinal mucosa as cells differentiate and are lost
- There is remarkable plasticity among intestinal epithelial cell populations to serve as stem-like cells when $Lgr5^{hi}$ cells are damaged or inhibited in functioning as stem cells
- Nutritional alterations can also marshal this plasticity, especially reduction in level of vitamin D, effects that are recapitulated by genetic inactivation of the gene that encodes the vitamin D receptor. This raises fundamental questions regarding which and how cells in the intestinal mucosa contribute to stem cell functions in homoeostasis and tumorigenesis in human populations in which there are widely varying exposures to different nutrients and to level of vitamin D. This may contribute to the biological and clinical heterogeneity of tumors.
- The potential that epigenetic and genetic changes persist in long-lived stem cells suggests that intervention trials of vitamin D and other nutrients to reduce incidence of colon cancer have been much too short to be conclusive.

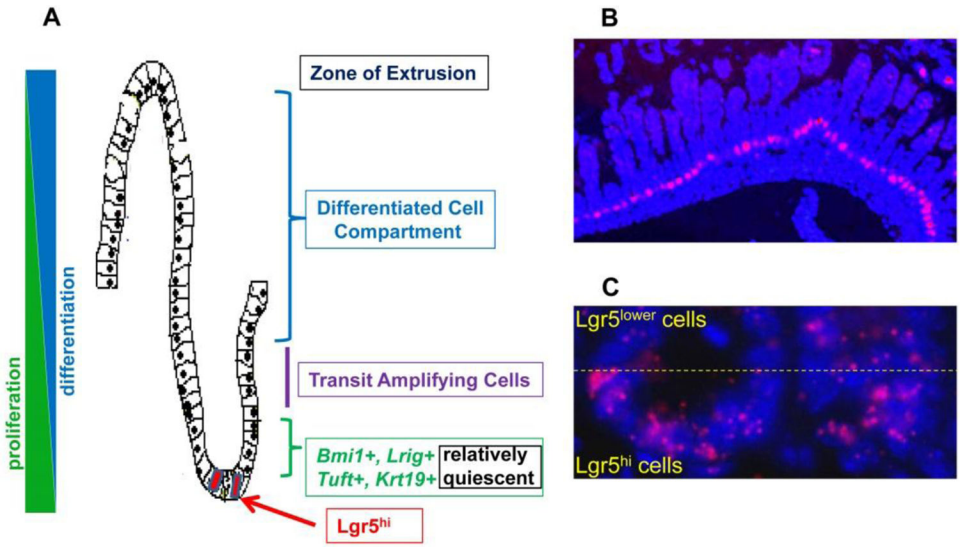

Fig 1.

A) the architectural organization of cell compartments of the intestinal crypt; **B)** low magnification of intestinal tissue showing Lgr5 expression (red) from *in situ* hybridization of RNAscope probes specific for Lgr5 mRNA at the crypt bottom; **C)** higher magnification depicting $Lgr5^{hi}$ cells at the crypt base, and $Lgr5^{lower}$ cells just above these. In B and C, the slides are counterstained with DAPI to reveal the position of nuclei.

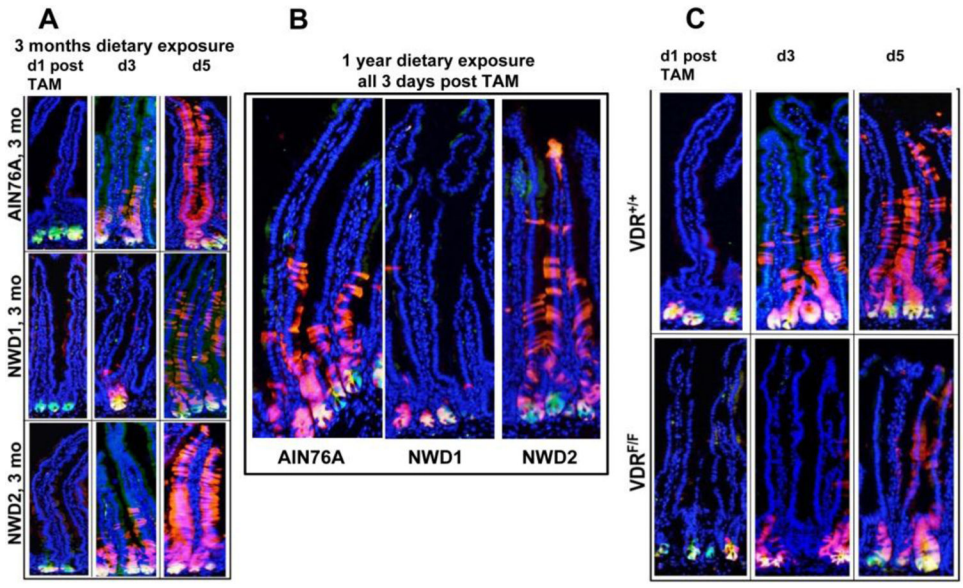

Fig 2: Dietary and genetic effects on lineage tracing from Lgr5 cells.

Lgr5$^{EGFP\text{-}creERT2}$, *Rosa26*^{tom} mice were fed either AIN76A, NWD1 or NWD2 (NWD1+ higher vitamin D and calcium) for 3 months from weaning, injected with tamoxifen, and sacrificed 1–5 days thereafter. Frozen sections of the intestine were observed for fluorescence. **B)** Mice of the same strain were maintained on diets for 1 year from weaning, injected with tamoxifen and sections examined 3 days post Tamoxifen. C) The same strain of mice in A and B, but in the bottom panel also Vdr$^{flox/flox}$, were fed control AIN76A diet for 3 months from weaning, injected with tamoxifen, and the sacrificed 1, 3 or 5 days later. All data are reprinted from [44].

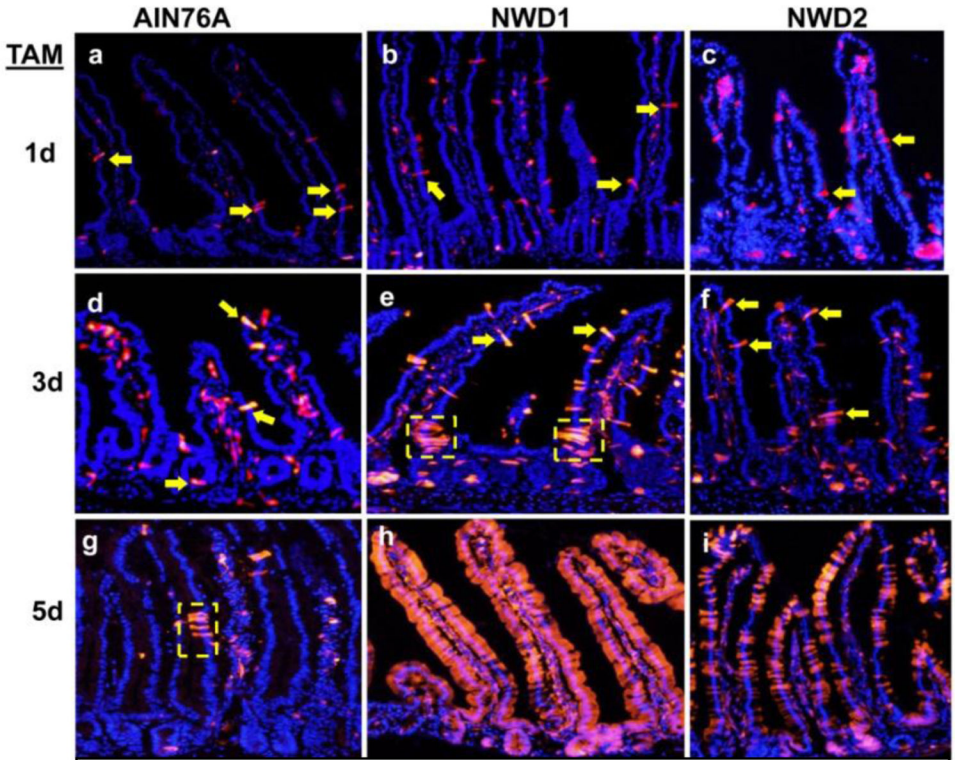

Fig. 3: Mobilization *of Bmi1+ cells* in mice fed different diets.

Bmi1^{creERT2}*, Rosa26*^{tom} mice were fed AIN76A, NWD1 or NWD2 diets for 3 months from weaning, injected with tamoxifen, and then sacrificed 1, 3 or 5 days later. Yellow arrows: examples of red fluorescently marked epithelial cells; Yellow dotted boxes: examples of short bursts of lineage tracing of marked cells. All data are reprinted from [46].

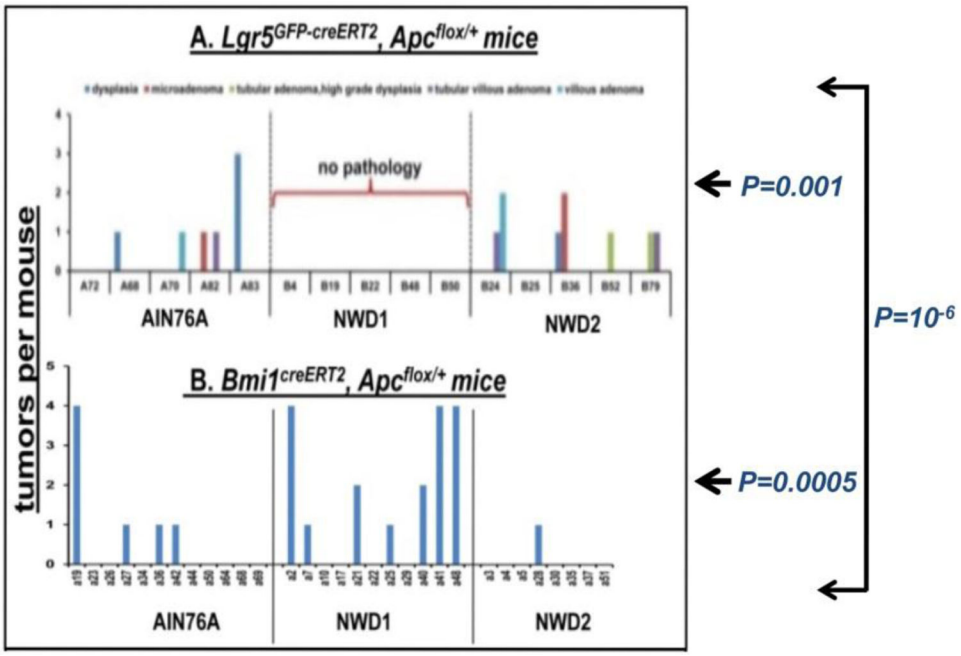

Fig 4: Apc initiated tumor development as a function of diet.

Apc$^{loxp/+}$ mice that were also *Lgr5*^{EGFPcreERT2} **(A-top)** or *Bmi1*^{creERT2} (**B–bottom**) were fed different diets from weaning for 3 months, then received a single injection of tamoxifen, and each mouse then continued on its respective diet until 9 months of age. On sacrifice, swiss rolls were prepared, and formalin fixed, H and E stained sections of these were used to score tumor incidence. Reprinted from [46].

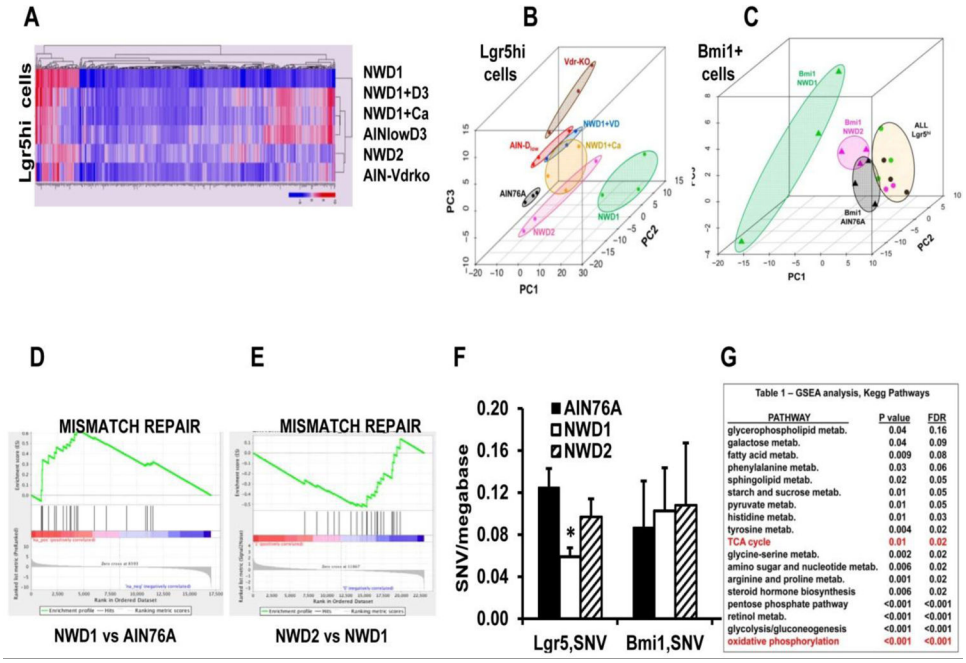

Fig. 5: Transcriptional and mutational profiles of Lgr5^{hi} and Bmi1+ cells are profoundly influenced by diet.

Lgr5$^{EGFP\text{-}creERT2}$ or *Bmi1^{creERT2}, Rosa26^{tom}* mice were randomized to different diets at weaning, the Bmi1 group given a single injection of TAM at 3 months, and all mice sacrificed 2 days later. Crypts were purified from each mouse, and single cell suspensions of each used for isolation of Lgr5^{hi} or Bmi1^{creERT2} marked cells by FACS. RNAs from these cells were then used for RNAseq analysis. An additional group was RNAseq analysis for Lgr5^{hi} cells from *Lgr5^{EGFP,creERT2}, Vdr$^{loxp/loxp}$* mice fed AIN76A control diet from weaning and given a single injection of Tamoxifen to genetically inactivate the *Vdr* gene in Lgr5^{hi} cells 3 days before sacrifice at 3 months of age. **A)** heat map of transcript expression of *Lgr5^{hi}* cells as a function of diet. **B and C)** Principal Component Analysis of *Lgr5^{hi}* or *Bmi1^{creERT2}* marked cells. **D,E)** GSEAof the DNA mismatch repair pathway comparing mice fed NWD1 to AIN76A *(P=0.003, FDR=0.021)*, or NWD2 to NWD1 *(P=0.02, FDR=0.06)*. **F)** Single nucleotide variants (i.e. point mutations) determined for *Lgr5^{hi}* and *Bmi1^{creERT2}* marked cells from mice fed different diets for 3 months from weaning, determined by single cell whole exome sequencing, *(* P=0.01)*. **G)** Metabolic pathways significantly altered by feeding NWD1 compared to AIN76A for 3 months, determined by GSEA. All data are from [46].