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Chapter

Oncometabolite L-2-Hydroxyglutarate Promotes Oncogenesis of Renal Cell Carcinomas by Down-Regulating Differentiation

Mary Taub

Abstract

L-2-Hydroxyglutarate (L2HG) overproducing Renal Cell Carcinomas (RCCs) arise in the kidney due to the genetic loss of L-2HG Dehydrogenase (L2HGDH), the enzyme responsible for the metabolism of L2HG. The overproduced 2-Hydroxyglutarate (2HG) promotes tumorigenesis by inhibiting α-ketoglutarate (αKG)-dependent dioxygenases, including Ten-eleven-Translocation 5-methylcytosine (5mC) dioxygenase (TET) enzymes as well as histone demethylases. The resulting epigenetic changes alter the phenotype of renal proximal tubule (RPT) cells, the cells of origin of RCCs. This report describes the consequences of increased L2HG on the differentiation of RPT cells, one of the initial steps in promoting tumorigenesis. Presumably, similar alterations promote the expansion of renal cancer stem-cells and tumorigenesis.

Keywords: oncometabolite, L-2-Hydroxyglutarate, renal cell carcinoma, renal proximal tubule, epigenetic, tubulogenesis, dedifferentiation, matrigel

1. Introduction

Oncometabolites are components of intermediary metabolism whose abundance increases dramatically during the metabolic rewiring that occurs during tumorigenesis. These oncometabolites act to promote, and/or sustain tumor growth and metastasis. The hypothesis that metabolic rewiring plays a major role in tumorigenesis was initially supported by the work of Warburg in the 1920s. Warburg’s experimental results indicated that aerobic glycolysis becomes the predominant means of generating the metabolic demands of developing tumors, rather than oxidative metabolism [1]. In recent years, the Warburg effect has been substantiated in studies of Clear Cell RCCs (ccRCCs) with von Hippel-Lindau (VHL) mutations, in addition to studies with other tumors [1]. In these tumors, aerobic glycolysis predominates over the tricarboxylic acid cycle (TCA) and oxidative phosphorylation. As a consequence,
these tumor cells use glucose more efficiently than normal cells, producing lactic acid. The lactic acid is used as an alternative source of acetyl-Coenzyme A (acetyl-CoA) for the production of fatty acids and cholesterol [2]. Glutamine, is another essential component for the production of fatty acids and the lipid droplets characteristic of ccRCCs [2]. Following its entry into the mitochondria, glutamine (Gln) is converted to glutamate (Glu) by glutaminase, followed by conversion to αKG, which as we will see, is not only an important intermediate in the TCA cycle, but is also a precursor for the oncometabolite 2HG.

Numerous investigations have shown that the Warburg effect arises due to major changes in the expression of a number of glycolytic and TCA cycle enzymes [3]. In the majority of ccRCCs, both alleles of the von Hippel-Lindau (VHL) gene are mutated, leading to dysregulation of Hypoxia-inducible factor 1 (HIF-1) transcriptional activity. HIF-1, a master regulator of oxygen homeostasis, is hydroxylated on its α subunit during normoxia by a ubiquitin protein ligase, whose activity depends upon VHL. In the absence of normal VHL, HIF-1 reprograms glucose and energy metabolism through its transcriptional activities, such that glycolysis and lactate production become predominant over respiration, even during normoxia [3].

More recent studies indicate that mutations which specifically effect the expression of 3 different types of metabolic enzymes (fumarate hydratase (FH), succinate dehydrogenase (SDH) and isocitrate dehydrogenase (IDH)) similarly promote tumorigenesis because of the accumulation of “oncometabolites” [1]. Oncometabolites are defined as “small-molecule components (or enantiomers) of normal metabolism whose accumulation dysregulates signaling so as to establish a milieu that promotes carcinogenesis” [4]. The fumarate accumulation, which occurs as a consequence of FH mutations, results in the formation of hereditary papillary renal carcinomas, whereas the succinate accumulation, that occurs as a consequence of SDH mutations, results in the formation of hereditary paragangliomas (PGLs). In contrast, cancer-related mutations in the IDHI and IDH2 genes, result in the formation of mutant IDH enzymes which produce D-2-hydroxyglutarate (D2HG) from αKG. This is particularly serious, because IDH1 and IDH2 mutations are associated with 70–80% glioblastomas, and 20% of acute myeloid leukemias (AMLs) [5]. All 3 oncometabolites, D2HG, fumarate and succinate, have been found to act by similar mechanisms, i.e. by inhibiting multiple αKG-dependent dioxygenases, which ultimately dysregulates signaling in a manner that promotes tumorigenesis [1, 5].

![Figure 1](image.png)

Figure 1. The D- and L-enantiomers of L-2Hydroxylutarate (L-2HG).2HG is a chiral molecule, with D- and L-enantiomers as illustrated above.
Notably, oncometabolites such as 2HG have also been used as prognostic markers. In individuals with cancer-related IDH mutations, D2HG is produced to such an extent, that the elevated D2HG can be detected in serum [6]. Remarkably, 87% of the AML patients with high serum 2HG had IDH1/IDH2 mutations, although only 29% of AML patients with moderately high 2HG had IDH1/IDH2 mutations. This latter finding suggests that other genetic events are also responsible for elevated 2HG. Of particular interest in these regards, 2HG is a chiral molecule, with both D- and L- enantiomers (as show in Figure 1). Both IDH1 and IDH2 mutations result in increased synthesis of the D- enantiomer (as shown in Figure 2). However, a loss of copy number of L2HGDH results in increased levels of the L- enantiomer of 2HG, rather than the D- enantiomer. For this reason, Struys [7] has stressed the importance of employing analytical methods that differentiate between L2HG and D2HG (e.g. chiral derivatization followed by Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC–MS/MS)), rather than measuring total 2HG.

During the initial time frame of the studies in which elevated 2HG was detected in serum, an inherited metabolic defect, L-2-Hydroxyglutarate aciduria (L2HGA) was being studied [8]. L2HGA is an autosomal recessive neurometabolic disorder, characterized by abnormalities of the subcortical cerebral white matter dentate nucleus, globus pallidus, putamen and caudate nucleus. The gene associated with this disorder was found to be a mutant L2HGDH, which was defective in metabolizing L2HG to αKG. Consistent with this observation, the biochemical hallmark of L2HGA is elevated levels of L2HG in the urine (such that L2HG levels are 10–300 fold more than normal controls). A consequence of L2HGA is an increased propensity for tumor formation. Kranendijk et al. [8] have reported that L2HG is produced from αKG by L-malate dehydrogenase (MDH), as a promiscuous side reaction (Figure 3). The primary
function of MDH is to convert L-malate to oxaloacetate. Lactate Dehydrogenase (LDH) has similarly been found to produce L2HG as a side reaction during oxygen deprivation under acidic conditions (Figure 3) [9]. In contrast, D2HG is produced from γ-hydroxybutyrate by hydroxyacid-oxoacid transhydrogenase (HOT) [8].

As stated above, patients with L2HGA exhibit neurological disorders, associated with progressive damage to the brain. Biochemical alterations in brain tissues include reduced creatine kinase activity, oxidative stress, and increased Glutamate (Glu) uptake into synaptosomes and synaptic vesicles. Precisely why the brain is primarily affected is not well understood.

Subsequent to the findings that the D2HG enantiomer is elevated in glioblastomas and AMLs, other investigators found that other enantiomer, L2HG, is indeed elevated in another class of tumors, ccRCCs [10]. The elevated L2HG has been attributed to a loss of copies of the L2HGDH gene [11], encoding for the enzyme that metabolizes L2HG to αKG [10]. Elevated L2HG and reduced L2HGDH was observed in a number of human RCC cell lines, including A498, RXF-393 and Caki-1, and in addition was a common attribute of ccRCCs obtained from patients [10]. In order to determine whether the decreased L2HGDH contributes to tumorigenicity, A498 cells were transduced with a WT L2HGDH vector (generating A498-L2HGD). Not only were the L2HG levels reduced in A498-L2HGDH cells, but the volume of A498-L2HGDH tumors was reduced, as compared with controls [10]. L2HG levels are determined by its biosynthetic rate, as well as by its degradation. Shelar et al. [11] reported that L2HG in RCC cells is generated from αKG by MDH1 and MDH2, via promiscuous reactions.

2. Epigenetic effects of L- and D-2HG

Both L2HG and D2HG are competitive inhibitors of αKG-dependent dioxygenases. The specific αKG-dependent dioxygenase(s) which are the targets of elevated L2HG
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In the brain of patients with L2HGA has not yet been identified. However, the most notable known targets of L2HG and D2HG in tumors are epigenetic targets, including αKG-dependent dioxygenases that regulate either DNA or histone demethylation. Included amongst these αKG-dependent dioxygenases that are inhibited by L2HG and D2HG are Jumonji domain-containing histone-lysine demethylases (Jmj-KDMs), that demethylate histones, as well as TETs, which demethylate 5-methyl-cytosine (5mC) residues in genomic DNA. Consequences of the inhibition of these dioxygenases by L2HG and D2HG include the increased methylation of histone marks, as well as an increase in 5mC residues in CpG islands [12]. While both enantiomers of 2HG inhibit Jmj-KDMs as well as TETs, overall L2HG is a more potent inhibitor than D2HG [13].

The Jmj-KDMs demethylate lysine residues on specific classes of methylated histones, their specificity being determined by specific reader domains present within each type of protein [14]. For example, JmjD2A and JmjD3 remove a methyl group from the repressive histones H3K9me3, and H3K27me3, respectively, whereas JARID1A removes a methyl group from the activating histone H3K4me3 (Table 1) [14]. In this manner the Jmj-KDMs reverse the methylation events caused by corresponding Histone Lysine Methyltransferases (abbreviated as either KMTs or HMTs) (Table 1) [15].

The TETs demethylate 5mC residues in genomic DNA in a number of steps. Initially, TETs oxidize 5mC, generating 5-hydroxy-mC (5hmC), followed by further oxidation of 5hmC into 5-formylcytosine (5fC), and finally 5-carboxycytosine (5caC). Subsequently, the modified base is removed, and excision repair occurs [16]. Point mutations and deletion mutations are often observed in human cancers, particularly those affecting TET2 (as observed in AML). This latter observation is consistent with the hypothesis that the inactivation of TETs by D2HG plays a similar role in AML [16].

In their studies, Chowdhury et al. [13] found that 2HG is a weak antagonist of αKG (requiring a 100-fold molar excess of 2HG over αKG). This molar excess of 2HG can nevertheless be achieved in cells with IDH mutations. The concentration of D2HG increases to as high as 35 mM in cells with IDH mutations, exceeding the IC50 of 2HG for Jmj-KDMs [13]. In such IDH mutant cells, αKG is itself consumed, being the substrate of 2HG, further increasing the inhibition by 2HG. Although the evidence is convincing that D2HG and L2HG alter the epigenetic landscape in cells, inhibitory effects of 2HG on other classes of αKG-dependent dioxygenases, may also promote tumorigenesis. For example, L2HG inhibits Prolyl Hydroxylase 2 (PHD2), thereby preventing the hydroxylation of HIF1α, and the degradation of HIF1α by the

<table>
<thead>
<tr>
<th>H3K9me2/3</th>
<th>H3K27me2/3</th>
<th>H3K4me2/3</th>
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<tr>
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<td>G9a/EHMT2</td>
<td>EZH1</td>
<td>SETD1A</td>
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<tr>
<td>KMT</td>
<td>GLP/EHMT1</td>
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<tr>
<td>Jmj-KDM</td>
<td>JmjD2A (KDM4A)</td>
<td>JmjD3 (KDM6B)</td>
<td>JARID1A</td>
</tr>
<tr>
<td>Jmj-KDM</td>
<td>JmjD2C (KDM4C)</td>
<td>UTX (KDM6A)</td>
<td>JARID1B</td>
</tr>
</tbody>
</table>

Methylated Histones are listed in the top row, which have been methylated by the histone lysine methyltransferases (KMTs) in the first 2 rows listed directly below the particular KMT. In the third and fourth rows are listed the Jmj-KDMs (in particular the Jmj-KDMs) which demethylate the histones listed in the same column as the particular Jmj-KDMs.

Table 1.
Representative histone lysine Methyltransferases (KMTs), with associated Jmj-KDMs and histone substrates.
proteosome [9]. Collagen hydroxylase is also inhibited by L2HG and D2HG, which perturbs basement membrane function [17]. Finally, D2HG and L2HG inhibit the repair of DNA alkylating damage (via ALK Homolog, i.e. ALKBH, enzymes), which promotes oncogenesis [17].

3. Effects of D-2HG on adipocyte and myocyte differentiation

Of particular interest to this review are the L2HG mediated effects on renal differentiation, so as to increase the propensity of renal cells to become tumorigenic. However, previous studies concerning the effects of D2HG on the differentiation of other cell types will first be described, to facilitate our understanding of effects of L2HG on renal differentiation, including epigenetic changes. Both D2HG and L2HG inhibit αKG-dependent dioxygenases, although admittedly the 2 enantiomers have different binding affinities for a number of αKG-dependent dioxygenases. Nevertheless, previous studies of the effects of D2HG on differentiation in other tissues, will facilitate our understanding of how L2HG alters kidney development.

The effects of D2HG on differentiation were initially studied because gliomas with IDH mutations had a gene expression profile enriched for genes expressed in neural progenitor cells [18]. Moreover, increased levels of repressive H3K9me3 and H3K27me3 were observed in oligodendrogliomas with IDH1 mutations compared to tumors with wild type IDH1 [18]. Presumably, this was a consequence of the elevated D2HG in these tumors. Consistent with this hypothesis increased histone methylation was observed in 293 cells expressing a mutant IDH1 (or mutant IDH2) as compared to the wild type IDH allele.

In order to determine whether D2HG could block the differentiation of non-transformed cells, studies were conducted with 3T3-L1 cells which could be induced to differentiate into adipocytes using a differentiation cocktail [18]. 3T3-L1 cells transduced with an R172K mutant IDH2, overproduced D2HG, unlike cells transduced with WT IDH2 or empty vector. After induction of differentiation, the mutant IDH2 expressing 3T3-L1 cells had a markedly reduced ability to accumulate lipid droplets and were defective in the expression of transcription factors required for adipocyte differentiation, including CEBPA (CCAAT Enhancer Binding Protein α) and PPARγ ( Peroxisome Proliferator-Activated Receptor γ, encoded by the Pparg gene). The impaired differentiation was associated with increased levels of H3K9me3 and H3K27me3. H3K9me3 in particular was located on the promoters of the Cebpa and Pparg genes. The increased H3K9me3 was attributed to the inhibition of the histone demethylase KDM4C by D2HG. Indeed, KDM4C was induced during the differentiation of 3T3L1 cells into adipocytes, and a knockdown of KDM4C with siRNA inhibited differentiation. Of particular interest in these regards KDM4C (which demethylates H3K9me3), is a member of the JHDM family of histone demethylases (i.e. JmjC domain-containing histone demethylases, or jmj-KDMs). Notably, H3K9me3 is the product of the G9a methyltransferase, which is known to produce repressive histones [14].

Subsequently, Schvartzman et al. [19] presented evidence indicating that the elevated D2HG (produced by oncogenic IDH1/2 mutations) similarly blocked the differentiation of 10T1/2 cell into myocytes by preventing H3K9 demethylation. Indeed, the HMT inhibitor UNC0638 (which blocks the H3K9 methylation by EHMT1/2) restored the ability of 10T1/2 cells expressing IDH2-R172K to form fused myotubes. Furthermore, similar results were obtained by a Clustered Regularly Interspaced...
Short Palindromic Repeat (CRISPR) mediated deletion of EHMT1/2. The differentiation of 10T1/2 cells into myocytes depends upon the MyoD transcription factor. Very importantly, a Chromatin Immunoprecipitation Sequencing (ChIP-Seq) analysis indicated that the IDH2-R172K mutant did not have a global effect on chromatin accessibility in differentiating 10T1/2 cells. Instead, the IDH2-R172K mutant specifically prevented a MyoD-mediated increase in chromatin accessibility at myogenic regions. Thus, the authors conclude that the histone methylations that occur within genetic regions present within “facultative” heterochromatin are responsible for the 2HG-mediated block in differentiation, rather than random methylation events. Further studies of these 2HG-mediated blocks in differentiation, will reveal the precise nature of the 2HG-mediated genomic changes that contribute to tumorigenesis.

4. Effects of L2HG on ccRCCs

The transcriptional basis of nephron-specific gene expression patterns that emerge during nephrogenesis are incompletely understood. However, alterations in histone and DNA methylation caused by oncometabolites such as 2HG may very well upset the network of interactions established by transcription factors (TFs), so as to alter “normal” patterns of differentiated gene expression, and to cause “dedifferentiation.” Nevertheless, Lindgren et al. [20] were able to identify remnants of cell type specific gene expression programs in a number of kidney cancers, and in this manner identify their lineage. Although many fundamental genetic alterations which contribute to the formation of RCCs have been identified, an understanding of the changes which occur in the specific cell subpopulation(s) that are the cells of origin of RCCs is nevertheless of importance. Lindgren et al. [20] were able to identify a gene cluster (cluster B) that was expressed in ccRCCs that corresponds with genes expressed in the renal proximal tubule (including trans-membrane transporters regulated by the Hepatocyte Nuclear Factor (HNF) TF family, most notably HNF4α which plays a role in RPT differentiation [21]). In addition, another gene cluster (cluster C) was over-expressed in ccRCCs (including genes regulated by HIF1α and expressed during hypoxia, angiogenesis as well as the epithelial-mesenchymal transition (EMT)). A number of the changes in gene cluster C are often associated the loss of VHL. In contrast, chromophobe RCCs expressed a Forkhead box protein L1 (FOXl1) gene signature. FOXI1 plays a critical role in the differentiation of intercalated cells in the connecting tubules and collecting ducts during renal development [22].

Although some remnants of normal transcription may remain, transcriptional changes that result in a loss of differentiation is a hallmark of many tumors. When considering ccRCCs in particular, evidence for changes in the activity of histone demethylases (including jmj-KDMs) has been obtained [23]. These changes alter the genetic landscape, resulting in the reduced expression of genes that encode for differentiated functions. Included amongst these genes, are genes encoding for renal transporters, proteins which maintain the polarized epithelial morphology, as well as proteins required for progression through the cell cycle, and apoptosis [24]. Gene silencing due to the hypermethylation of DNA and histones, is a strong candidate mechanism underlying the block in differentiation in ccRCCs. Of particular interest, is the role of elevated L2HG in such renal tumors, because increased L2HG is associated with increases in histone as well as DNA methylation. Indeed, Shelar et al. [11] observed increased H3K27 trimethylation in A498 RCC cells that have elevated levels of L2HG. In addition, there was a decrease in the expression of genes targeted by the
Polycomb Repressor Complex 2 (PRC2), including genes targeted by Suz12, that bear the H3K27me3 mark in human embryonic stem cells [11]. These results suggest there is an interrelationship between the changes in the gene expression profiles observed during embryonic development, and the gene expression changes caused by L2HG in renal carcinomas.

5. Epigenetic and transcriptional changes during renal development

Strikingly, during renal development, there is an interplay between the repressive effects of PRC2 proteins, and stimulatory effects of Trithorax proteins [25]. Repressive histones, including H3K9me2 and H3K27me3, are generated by PRC2 in the metanephric mesenchyme which surrounds the ureteric bud (which includes quiescent cells with low levels of the Six2 and Lhx1 transcription factors). As development proceeds, the mesenchyme condenses. The condensed mesenchyme becomes enriched with the activating histone H3K4me3, indicating it was poised for activation. Somewhat later, nascent nephrons emerge, which have high levels of H3K4me3 and low levels of repressive H3K9me3 and H3K27me3, activating such genes as Lhx1. After nascent nephron cells emerge, Notch2 appears, an important transcription factor in RPT development.

Of particular interest, are the developmental events that occur during segmentation of the nephron, because during this period Renal Proximal Tubules (RPTs) appear, RPTs being the cell of origin of ccRCCs and papillary RCCs [26, 27]. HNF TFs emerge during this developmental stage, including HNF4α, which facilitates the formation of H3K4me2 to maintain active chromatin during this developmental period [28]. HNF1α is similarly active during this developmental period, recruiting KDM6A (i.e. UTX), resulting in the demethylation of H3K27me3, thereby relieving polycomb repression [29]. In contrast, loss of HNF1α function (which has been observed in ccRCCs) [30] results in polycomb repression. A similar loss of HNF1α function has been reported in other cancers, including non-small cell lung cancers [31], oral squamous carcinomas [32], and pancreatic cancers [29]. In the case of pancreatic cancers, re-expression of HNF1α reactivated differentiated acinar cell programs, and in this manner suppressed the emergence of pancreatic cancers [29]. Presumably, there is a potential to similarly override 2HG mediated blocks in differentiation programs in other cancers, including RCCs, by re-establishing required regulation by HNFs. For this reason, an evaluation of the effects of elevated 2HG on the differentiation of renal cells is important, including effects of 2HG on such transcription factors as HNF1α.

6. Effects of L2HG on renal differentiation and development in vitro

L2HG mediated effects on the differentiation of RPT cells have been examined, utilizing a well characterized primary culture system of normal renal cells. Primary cultures of kidney epithelial cells derived from purified rabbit RPTs were grown in serum free medium, supplemented with 5 μg/ml human insulin, 5 μg/ml human transferrin and 5 x 10⁻⁸ M hydrocortisone. Epithelial monolayers formed with a polarized morphology, and membrane transport systems distinctive of the RPT, including an apical Na⁺/glucose cotransport system (SGLT2), a Na⁺/phosphate cotransport system (NPT2a), a Na⁺/H⁺ antiport system (NHE3), and a basolateral p-Amino Hippurate (pAH) transport system (i.e. OAT1) (Figure 4) [33, 34]. The
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primary RPT cells also possess a parathyroid hormone (PTH) sensitive adenylate cyclase, Angiotensin II (Ang II) receptors [35], dopamine receptors, as well as α and β adrenergic receptors [36], which are critical for the regulation of ion transporters (Figure 4). Finally, the cultures retain normal metabolism, including gluconeogenesis, glutathione metabolism and other drug metabolic capabilities distinctive of the RPT (Table 2).

The effects of L2HG on primary RPT cells were examined both in 3-Dimensional (3D) matrigel cultures, as well as monolayer cultures. Such 3D culture systems are the method of choice for examining malignant cells ex vivo [37], including the effects of oncometabolites on differentiation (which relate to tumor progression). Matrigel, a reconstituted basement membrane from the Engelbreth-Holm-Swarm (EHS) tumor, is particularly important to use in 3D studies. Matrigel is a well-characterized 3D system used to study differentiation in both normal and malignant tissues [38]. Matrigel was initially employed to study baby mouse kidney epithelial cells ex vivo. Tubulogenesis was observed, provided that either Epidermal Growth Factor (EGF)
or Transforming Growth Factor α (TGFα) were added to the culture medium [39]. Electron micrographs indicated that the baby mouse kidney tubules resembled collecting ducts [39]. Subsequently, a 3D system of renal proximal tubulogenesis was developed, using primary rabbit RPT cells [40]. The primary RPT cells form tubules in matrigel (Figure 5). The tubules possess transepithelial transport capacity, as indicated by their ability to secrete lucifer yellow (an organic anion) into the luminal space of the tubules [41]. This was indicated by the green fluorescence observed in the lumen of lucifer yellow treated cultures in matrigel. Tubulogenesis by RPTs was stimulated by EGF (similar to baby mouse kidney cells), as well as Hepatocyte Growth Factor (HGF). Because the RPT is the cell of origin of ccRCCs, this primary RPT cell culture system is an appropriate model system to examine effects of oncometabolite L2HG on RPT differentiation.

As described above, the elevated L2HG detected in RCCs, has been attributed to decreased expression of L2HGDH, the enzyme that breaks down L2HG to αKG. The results of a microarray study indicate that the expression of genes targeted by the Polycomb protein Suz-12 was reduced in L2HGDH deficient ccRCC cells, similar to the metanephric mesenchyme [42]. Thus, the hypothesis was examined, that reduced expression of L2HGDH blocks differentiation of renal cells.

In order to examine this hypothesis initially, primary RPT cell cultures were transduced with lentiviral particles containing a vector (pLKO-TRC) encoding either L2HGDH shRNA or control shRNA [43]. The effect on tubulogenesis in matrigel was examined. Figure 6 shows the lack of tubules in cultures treated with L2HGDH shRNA, under conditions where L2HGDH mRNA was reduced by 80%. Tubulogenesis was similarly inhibited in primary RPT cells using L2HGDH siRNA, which similarly reduced L2HGDH mRNA by 80%. The effect of L2HGDH siRNA on intracellular L2HG and D2HG levels was examined by Gas Chromatography–Mass Spectrometry (GC–MS) analysis. The L2HG level increased more than 4-fold in

<table>
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<tr>
<th>Brush Border Enzymes</th>
<th>Apical Membrane Transporters</th>
<th>Basolateral Membrane Transporters</th>
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<tr>
<td>Alkaline Phosphatase</td>
<td>Na+/glucose cotransporter: SGLT2</td>
<td>Organic Anion (OAT1; p-Amino-hippurate transporter)</td>
<td>Hormones: Insulin, Estrogen, Hydrocortisone, Parathyroid Hormone</td>
<td>Gluconeogenesis</td>
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<tr>
<td>γ Glutamyl Transpeptidase</td>
<td>Na+/Pi cotransporter: NPT2a</td>
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<td>Aquaporin 1: AQ1</td>
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Primary rabbit RPT cells are cultured in serum free medium supplemented with insulin, transferrin and hydrocortisone. The method for culturing the primary RPT cells and their extensive characterization has been described by Taub [33].

Table 2.
Characteristics of primary RPT cells in culture.
primary RPTs treated with L2HGDH siRNA, L2HG becoming the predominant enantiomer.

Shelar et al. [11] observed that L2HG is primarily generated from Glutamine (Gln) in human ccRCCs, initially via the breakdown of Gln to Glu by glutaminase (as illustrated in Figure 3). Thus, the inhibition of Gln metabolism to Glu by glutaminase would be expected to reduce the L2HG level, and in this manner overcome the effect of an L2HGDH Knockdown (KD) on intracellular L2HG levels. In order to examine this hypothesis, the ability of primary RPT cells with an L2HGDH KD to form tubules in the presence of glutaminase inhibitor CB-839 was examined. The results (illustrated in Figure 7) indicated that CB-839 relieved the inhibition of tubulogenesis caused by an L2HGDH KD, presumably by reducing L2HG levels (a consequence of inhibiting the metabolism of Gln to Glu, and ultimately to L2HG). The involvement of the elevated L2HG in mediating the inhibition of tubulogenesis in cultures with an L2HGDH KD was further substantiated by the observed inhibitory effect of cell permeable L2HG octyl ester on tubulogenesis [43].

The block in tubulogenesis in primary RPT cells with an L2HGDH KD may very well be associated with a generalized loss of differentiated function. Consistent with this hypothesis, the expression of differentiated transporters was reduced in the primary RPT cells with an L2HGDH KD. This is exemplified by the reduction in the mRNA levels for the apical Na+/Phosphate cotransporter (NPT2a), the Na+/glucose cotransporter (SGLT2), and Aquaporin 1 (AQP1), as well as the basolateral pAH transporter (OAT1) in monolayers with an L2HGDH KD.
In matrigel cultures, the level of expression of NPT2a and SGLT2 increased relative to plastic in control cultures, consistent with the hypothesis that the overall state of differentiation was enhanced in matrigel. Nevertheless, the expression of these 2 transporters continued to be substantially reduced in cultures with an L2HGDH KD. In contrast, AQP1 expression increased in matrigel cultures with an L2HGDH KD. This observation can be explained by differences in transcriptional regulation between NPT2a and SGLT2 as compared with AQP1. Both NPT2a and SGLT2 regulation depends upon HNF1α which is down regulated in primary RPT cells with an
L2HGDH KD, unlike AQP1, whose transcription is controlled by Tonicity Enhancer Binding Protein (TonEBP) and HIF1α. As described above, HNF1α is involved in the appearance of RPTs during kidney development. Thus, it will be of interest to determine whether during tubulogenesis in vitro, HNF1α is involved in the downregulation of L2HGDH levels. The downregulation of the expression of the differentiated transporters, and HNF1α in primary RPT cells with an L2HGDH KD is presumably a consequence of...

Figure 7. Effect of Glutaminase inhibitor CB-839 on tubulogenesis. A. Primary RPT cells were either a. transduced with lentivirus containing either L2HGDH shRNA or control TRC shRNA vectors, or B. transfected with L2HGDH shRNA or negative control siRNA. The cultures were passaged into matrigel, and treated with either with 1 μM CB-839 or untreated. Subsequently, the frequency of tubule formation was determined [41]. C. the effect of 1 μM CB-839 on the level of glutamine and glutamate was determined [41]. D. Model for the effect of CB-839 on L2HGDH levels. Values are averages (+/-) SEMs of triplicate determinations. With permission from Frontiers.

HNF1α has also been reported to play a role in maintaining the polarized morphology of epithelial cells. Indeed, inhibition of HNF1α gene expression with siRNA triggered the Epithelial to Mesenchymal Transition (EMT) in the liver cancer cell lines HEPG2 and HEP1B [44]. The expression of mRNAs encoding for such proteins as E-cadherin (CDH1) and plasminogen activator (PLAU) was similarly reduced in primary RPTs with an L2HGDH KD, as well as cell migration. Further studies are necessary to elucidate whether the downregulation of HNF1α under these conditions is responsible for these changes.

The downregulation of the expression of the differentiated transporters, and HNF1α in primary RPT cells with an L2HGDH KD is presumably a consequence of...
the inhibition of αKG dependent dioxygenases. Included amongst these αKG dependent dioxygenases are Jmj-KDM histone demethylases, as well as TET 5mC demethylases, which are inhibited not only in tumor cells with IDH1 and IDH2 mutations [45], but also in ccRCCs with reduced L2HGDH levels [11]. Consistent with this hypothesis, 5hmC blots of genomic DNA derived from primary RPT cells with an L2HGDH KD indicated that the level of 5hmC was reduced in primary RPT cells with an L2HGDH KD (vs. controls) [43]. In addition, Western blots indicated increases in the levels of a number of different classes of methylated histones in primary RPT cells with an L2HGD KD [43]. Not only was there an increase in the level of the activating histone, H3K4me3, but also an increase in the level of the repressive histone H3K27me3. Shelar et al. [11] similarly observed an increased level of H3K27me3 in A498 ccRCC cells as compared to A498 ccRCC cells which express exogenous L2HGDH. H3K27me3 is generated by EZH2, a component of the repressive PRC2 complex expressed in the primitive mesenchyme.

However, as described above, Schvartzman et al. [19] reported that the inhibitory effect of D2HG on the MyoD-mediated differentiation of myocytes was the specific consequence of increased H3K9 methylation generated by EHMT1/2. Schvartzman et al. [19] found that 5mC DNA hypermethylation could be excluded from the differentiation block in 10 T1/2 cells, because the DNA methyltransferase inhibitor 5-azacytidine was unable to rescue the differentiation block caused by elevated D-2HG. The hypothesis that the inhibition of renal proximal tubulogenesis is similarly the specific consequence of increased H3K9 methylation cannot be excluded, in the absence of results with H3K9me3. However, L2HG does not necessarily act on renal differentiation via the same mechanism as observed with D2HG in myocytes. Indeed, L2HG has a higher affinity than D2HG for a number of αKG-dependent dioxygenases, including TET 5mC hydroxylases [45]. Furthermore, L2HG (unlike D2HG) is an effective inhibitor of such αKG dioxygenases as Prolyl Hydroxylase 2 (PDH2) (which promotes the degradation of HIF1α during normoxia) [17]. The effects of L2HG on other aspects of basic metabolism have not been extensively investigated. Thus, it is unclear whether L2HG, like D2HG causes a reduction in Nicotine Adenine Dinucleotide (NAD+) levels, associated with a reduction in the level of Naprt (Nicotinate Phosphoribosyltransferase), a rate limiting enzyme in the NAD+ salvage pathway that replenishes intracellular NAD+ levels [46]. In any case, the effects of L2HG on differentiation of RPT cells ultimately depend upon the unique sets of epigenetic mechanisms that regulate kidney development, unlike other tissues.

Nevertheless, the L2HG-mediated inhibition of kidney proximal tubulogenesis very likely results at least in part from the inhibition of Jmj-KDMs. Previous studies indicate that histone demethylases, as well as histone methyltransferases play an important role in kidney development. It is well-known, as stated above, that during kidney development there is an interplay between events mediated by repressive PRC2 and stimulatory Trithorax complexes [25]. Different classes of methylated histones are produced by these 2 sets of complexes. While the histone methyltransferase Ash21 (associated with Trithorax complexes) produces activating H3K4me histones, Ezh1, Ezh2 and Suz12 (associated with PRC2) produce repressive H3K9me2/3 and H3K27me3. During the segmentation of the nephron, H3K4me histone levels increase as proximal tubules appear, while H3K9me2/3 and H3K27me3 levels decline. Remarkably, an increase in the level of both H3K9me3 and H3K27me3 is associated with the block in the ability of 10T1/2 cells with a mutant IDH2-R172K to differentiate into myocytes.
A decline in H3K9me2/3 and H3K27me3 during kidney development depends not only upon the activity of the pertinent histone methyltransferases, but also upon the continued activity of pertinent JmjC domain containing histone demethylases. While KDM4A, B, C and D demethylate H3K9me3, the transcriptionally activating KDM7A demethylates H3K9me1/2. Similarly, the demethylation H3K27me3 involves KDM4D, as well as KDM7A. However, given that these KDMs are αKG dependent dioxygenases, they are subject to inhibition by elevated D- and L-2HG. In the case of 10T1/2 cells expressing a mutant IDH enzyme, inhibition of KDM4C (which normally demethylates H3K9me3) was proposed to be responsible for their inability to differentiate into myocytes. Consistent with this hypothesis, these investigators observed that the EHMT1/2 inhibitor UNC0638 restored the ability of 10T1/2 cells expressing IDH2-R172K to form fused myotubes. Similarly, primary RPT cells with an L2HGDH KD can overcome the block in tubulogenesis in the presence of UNC0638 (lowering levels of H3K9me3), although the cultures can also overcome the block in tubulogenesis in the presence of GSK343 (Taub, unpublished).

Shelar et al. [11] presented evidence that increased levels of methylated histones as well as DNA in ccRCCs with elevated L-2HG contribute to tumorigenesis. However, Shelar et al. [11] also provided evidence that the increased levels histone H3K27me3 in ccRCCs with elevated L-2HG are responsible for altering the genetic program of these tumor cells, and, as a consequence their state of differentiation. Indeed, the studies of Taub et al. [43] indicate that increased L2HG establishes a block in RPT differentiation, with associated epigenetic changes. Thus, it is of importance to consider the development of new avenues of epigenetic therapy for ccRCCs.

7. Conclusion

Recent clinical trials of epigenetic-based therapies in RCCs have examined the use of DNA Methyltransferase (DNMT) inhibitors such as decitabine and azacytidine [47]. DNA methylation of tumor suppressor genes in RCCs is thought to contribute tumorigenesis, and the hypermethylation of an EGF response element (recognized by Krueppel-like factor 5 (KLF5)) is associated with poor prognosis of RCC patients [47]. In vitro studies concerning the effects azacytidine with RCC cell lines have also shown promise [48], and combination therapies are in progress [47]. This adds to presently prescribed medications including VEGF inhibitors (e.g. sunitinib) mTOR inhibitors (e.g. everolimus), and HIF2α inhibitors (e.g. belzutifan) [49]. However metastatic ccRCC continues to carry a 5 year survival rate of 13% [49]. Thus, new treatment options are critical.

Epigenetic therapy is pertinent not only for ccRCCs with elevated L2HG, but also for ccRCCs with other genetic alterations (which may occur in addition to reduced L2HGH levels). Recent genome-wide sequencing studies have indicated that a number of epigenetic modifiers and chromatin remodelers are frequently altered in ccRCCs including PBRM1, SETD1, KDM5C, KDM6A, and BAP1. Notably, ccRCCs often have a 50 kb deletion on chromosome 3p where VHL, PBRM1, BAP1 and SETD2 are located, which has opened up the door to individualized epigenetic therapy. SETD1 in particular is an H3K36me3 histone methyltransferase that plays an important role in DNA repair and genomic stability [50]. While mutations in SETD1 result in reduced histone methylation, mutations in KDM5C and KDM6A often result in increased methylation of H3K4me3 and H3K27me3, respectively. The genes encoding for these 2 histone demethylases are located on the X chromosome, and thus, when altered, can a permit an escape from X-inactivation of tumor suppressor genes. Unlike
patients with SETD mutations, RCC patients with KDM5C and KDM6A mutations would be expected to benefit from inhibitors of specific histone methyltransferases. In addition to epigenetic therapies, therapies developed against cancer stem cells may also prove protective against ccRCCs with elevated L-2HG. Several theories have been proposed regarding the origin of cancer stem cells, including that, a) cancer stem cells arise from normal progenitor cells which become tumorigenic due to undefined mutation(s), and b) that cancer stem cells arise from normal somatic cells that acquire stem-like properties through similarly undefined genetic mutations [51]. Thus, the block in renal proximal differentiation caused by elevated L-2HG is a mechanism that potentially results in the development of renal cancer stem cells. Recently, Fendler et al. [52] isolated ccRCC cancer stem cells which depend upon signals sent through the Wingless-related integration site (WNT) and NOTCH networks, which direct the formation of RPTs during renal development. Further studies are needed to assess whether the WNT and NOTCH pathways are active in ccRCCs with elevated L2HG, and whether the targeting of such pathways can alleviate the differentiation block caused by increased L2HG in normal RPT cells.

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