Published in final edited form as:

Curr Pharm Des. 2014 ; 20(11): 1698–1705.

Epigenetic Regulation of EMT: The Snail Story

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Abstract

While the epithelial-mesenchymal transition (EMT) plays a fundamental role during development, its deregulation can adversely promote tumor metastasis. The phenotypic and cellular plasticity of EMT indicates that it is subject to epigenetic regulation. In this review, we try to embrace recent findings on the mechanisms of the transcription factor Snail-mediated E-cadherin silencing, which is a hallmark of EMT. Our studies as well as those of others have clearly demonstrated that Snail can recruit multiple chromatin enzymes including LSD1, HDAC1/2, PRC2, G9a and Suv39H1 to the E-cadherin promoter. These enzymes function in a highly orchestrated fashion to generate heterochromatin and promote DNA methyltransferase (DNMT)-mediated DNA methylation at the promoter region. Disruption of the connection between Snail and these chromatin-modifying enzymes may represent an efficient strategy for the treatment of EMT-related diseases.

Keywords

EMT; epigenetic regulation; Snail

EMT is subject to dynamic epigenetic regulation

As a commanding cellular event during embryogenesis and tumor metastasis, epithelial-mesenchymal transition (EMT) has been a long-time research interest in the field of molecular biology. Upon receipt of microenvironmental stimuli such as tumor growth factor β (TGF- β), Wnt and tumor necrosis factor- α (TNF- α) [1–5], epithelial cells lose their ability to adhere to each other, gain mesenchymal cell characteristics and become more mobile [6–8]. Speaking specifically of tumor cells, EMT confers them not only with the capability to migrate and invade, but also with stem cell-like properties, which largely account for immunosuppression and tumor recurrence [9–12]. Remarkably, EMT is a reversible process, with its counterpart mesenchymal-epithelial transition (MET) functioning to restore cells with epithelial characteristics, again under the guidance of extrinsic signals. The phenotypic and cellular plasticity of EMT is reminiscent of involvement of an epigenetic regulation program [13].

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Conflict of interest statement: The authors have declared that no conflict of interest exists

The epigenetic code, which was introduced to denote the determinants of gene features other than DNA sequence [14–16], is maintained/regulated through a dedicated system highlighted by dynamic histone modifications and DNA methylation [17–19]. While histone modifications generate reversible local chromatin structures, DNA methylation provides reinforcement as well as establishment of gene silencing by keeping chromatin in a relatively stable long-term repression state [18]. Among the diversified histone modifications, acetylation and methylation, as well as their counterparts, i.e. deacetylation and demethylation, play essential roles in conferring genes with specific transcriptional potential [17]. Histone acetylation and deacetylation are mediated by histone acetyltransferases (HATs) and deacetylases (HDACs), respectively. While acetylation introduces an “open chromatin” state and increases the accessibility of transcription complexes, deacetylation does exactly the opposite to impede gene transcription [20]. In terms of histone methylation/demethylation, things become more complicated. For example, methylations on histone H3 lysine 4, 36 and 79 are generally considered as transcription activation marks, whereas methylations on histone H3 lysine 9 and 27 are linked to transcription repression [21]. Histone modifications are dynamically regulated by specific chromatin enzymes and their cofactors, which have been critically reviewed by others [22–26]. DNA methylation, on the other hand, is executed by a family of highly related DNA methyltransferase enzymes (DNMT1, DNMT3a, and DNMT3b), which function by transferring a methyl group to the cytosine in a CpG dinucleotide. Typically, the maintenance of DNA methylation in somatic cells is attributed to DNMT1, whereas *de novo* DNA methylation during embryonic development is credited to DNMT3a and DNMT3b [27, 28]. While DNA methylation is executed by DNMTs, DNA demethylation was initially considered as a passive process, i.e., through DNMT suppression [28]. Not until recently has an active DNA demethylation mechanism been discovered, with the identification of ten-eleven translocation 1 (TET1) to be responsible for removing the 5'-methylcytosine (5 mC) during the DNA mismatch repair process [29–31].

It is established that DNA methylation and diverse histone modifications are interdependent events with specific hierarchies and/or feedback loops [18, 32–34]. On one hand, a specific histone modification pattern would predispose DNA methylation. Basically, as an initial step in gene repression, removal of methylation on histone H3 lysine 4 (H3K4) releases the inhibitory effect on the approaching of DNMT3a and DNMT3b to the nucleosome, therefore presetting a welcome environment for *de novo* DNA methylation to take place [35]. Subsequently, deacetylation of histone H3/H4 opens the door for the landing of histone methyltransferases such as G9a and SUV39H1, which use different mechanisms to further promote the recruitment of *de novo* DNA methyltransferases [18]. On the other hand, once the pattern of DNA methylation has been set, it serves as a template for the reconstruction of epigenetic state during the cell differentiation process. Indeed, there are previous studies demonstrating that methyl-CpG-binding domain proteins (MBDs) residing in the chromatin region with DNA methylation mediate the recruitment of HDACs; in addition, DNA methylation predisposes H3K9 dimethylation (H3K9me2) while excluding methylation on H3K4 [36, 37]. Overall, the epigenetic landmark is maintained in such a sophisticated fashion that disruption of the balance of epigenetic stage, either at the genome scale or even restricted to a certain gene, is usually the cause of many diseases, including cancer [38, 39].

Currently, many mechanistic details of the crosstalk between histone modifications and DNA methylation remain to be fully uncovered. An intriguing fact, for example, lies in that tumor cells harbor genome-wide DNA hypomethylation, which creates genomic instability due to the loss of transcription silencing of many transposons [28], whereas they usually have the tumor suppressor gene promoters hypermethylated compared to their normal siblings. It seems reasonable to argue that the DNA methylation machineries selectively target the promoters of tumor suppressor genes to silence their expression, therefore providing tumor cells with a growth advantage [18, 40]. Identification of the key molecules that mediate this selection process will undoubtedly help us better appreciate the epigenetic regulation program in cancer.

As mentioned, EMT, with its hallmark being loss expression of E-cadherin [7, 41], is intimately associated with an epigenetic regulation program. Indeed, in basal-like breast cancer (generally referred to as triple-negative breast cancer) that has typical EMT characteristics and high metastatic potential [42–46], DNA methylation at the E-cadherin promoter is not uncommon. What is the driving force for DNA methylation to take place at the E-cadherin promoter? Are there additional epigenetic events that happen before the long-term repression by DNA methylation takes effect? How can these events get organized into a hierarchic fashion to eventually boost promoter DNA methylation in specific biological context of EMT? To answer these questions, efforts should be made to decipher the potential built-in connection between histone modifications and DNA methylation during this dynamic cellular event.

Earlier studies indicate that Snail plays crucial roles in assembling repressor complexes on E-cadherin promoter

Several transcription factors, such as Snail, Twist and ZEB1, have been recognized as E-cadherin suppressors and EMT inducers [6, 7]. Not surprisingly, these transcription factors are well known for their roles in early embryogenesis as well as tumor metastasis. Typically, the expression of the transcription factor Snail, which was originally identified in *Drosophila* as a suppressor of *shotgun* (an E-cadherin homologue), is essential for the formation of the mesoderm and neural crest, indicating its fundamental role in morphogenesis [47–49]. Furthermore, Snail expression correlates with tumor grade and nodal metastasis in invasive breast ductal carcinoma, and overexpression of Snail is indicative of poor clinical outcome in patients with breast cancer [50–53]. As an ideal target for the study of EMT, Snail has been under extensive investigation for decades. However, not until recently has the linkage between Snail and epigenetic regulation of EMT been discovered.

According to the chromatin “reader and writer” idea, as a transcription factor, Snail is able to “read” its target gene (E-cadherin in this case) promoter using its DNA-binding zinc fingers; however, Snail does not have chromatin modulation activity and falls short of “writing” either DNA methylation or histone modifications by itself. It is hypothesized that transcription factors (as chromatin readers) cooperate with chromatin enzymes and cofactors (as chromatin writers) to modulate epigenetic information in favor of gene transcription activation or repression. Indeed this reader-writer model ideally fits in the case of Snail.

Rapidly emerging were study results convincingly demonstrating that Snail forms complexes with and then recruits different chromatin modifying enzymes and cofactors to E-cadherin promoter for transcription repression.

Snail associates with the SIN3 repressor complex containing histone deacetylase (HDAC) activity

All Snail family members share a highly conserved zinc finger domain at the C-terminal, which functions to bind DNA on a sequence-specific basis. Typically, the domain is comprised of four to six C₂H₂ type zinc fingers and recognizes the E-box 5'-CACCTG-3' present in the promoter region of E-cadherin, among others [48]. *Drosophila* Snail also contains an N-terminal binding domain for the corepressor CtBP (C-terminal binding protein); upon binding, Snail can recruit CtBP to its target gene promoters for transcription repression [48, 54]. In the case of Vertebrate Snail proteins, namely Snail, Slug and Smuc, they share a conserved SNAG (Snail/Gfi) domain at the N-terminal, which contributes to their repressive function [48].

The first evidence for the interaction of the SNAG domain with repressors came from studies of Peinado and colleagues. They demonstrated that mouse Snail uses its SNAG domain to associate with HDAC1/2 and corepressor mSin3A, and recruits the repressor complex to E-cadherin promoter, where HDAC1/2 deacetylate histone H3 and H4 to create the repressive chromatin environment [55]. Treatment of cells with histone deacetylase inhibitor Trichostatin A (TSA) efficiently abolished the repressive effect of Snail [55]. Based on previously studies demonstrating the connection and mutual communication between histone deacetylation and DNA methylation [56–59], they hypothesized that Snail recruits HDACs to E-cadherin promoter for histone deacetylation, which functions to maintain DNA methylation and gene silencing. Furthermore, Peinado and colleagues found that the level of histone H3 lysine 9 dimethylation (H3K9me₂), which is a transcription repression mark, was increased at the E-cadherin promoter region upon overexpression of Snail in epithelial cells, indicating the involvement of other repressor complexes containing histone methyltransferase activities during the regulation process [55].

Snail cooperates with Polycomb repressive complex 2 (PRC2) for histone H3 lysine 27 trimethylation (H3K27me₃)

Herranz and colleagues have recently demonstrated the recruitment of PRC2 by Snail to E-cadherin promoter, providing a second example for Snail-mediated epigenetic regulation machinery [60]. Polycomb proteins (PcG) play fundamental roles on chromatin remodeling and gene silencing during embryonic development as well as stem cell differentiation [61–63]. As one of the PcG complexes, PRC2 contains methyltransferase activity on histone H3 lysine 27, and functions to initiate the transcription silencing process [64]. Herranz and colleagues found that PRC2 was required for Snail-mediated E-cadherin suppression, as knockdown of essential PRC2 components Suz12 or Ezh2 abolished the suppressive effect of Snail in pancreatic cancer cell line RWP-1 as well as colon adenocarcinoma cell line SW-620 [60]. While the interaction between Snail and PRC2 could be direct or indirect, they demonstrated the necessity of the SNAG domain of Snail for recruiting PRC2 [60]. Other than their studies, researches from two independent groups showed that the SNAG

domain of Snail could interact with both the Ajuba LIM and PRMT5 proteins, the latter of which then associated with Suz12 through a mediator MEP50 [65, 66]. Based on these results, it seems more than likely that the SNAG domain of Snail recruits PRC2 in an Ajuba/PRMT5-dependent fashion. Although not supported by comprehensive data, Herranz and colleagues suggested another possibility that the interaction of Snail with PRC2 is mediated by HDACs, according to the facts that HDACs can associate with both Snail and PRC2, and that histone deacetylation supports PcG-mediated transcription suppression [67, 68].

Together, earlier studies have strongly indicated the crucial role of Snail on epigenetic silencing of E-cadherin, typically through recruiting multiple repressor complexes to the E-cadherin promoter region. How does Snail efficiently organize these epigenetic machineries, allowing the regulation events to continue smoothly to the final step of inhibition, i.e. promoter DNA methylation? Is there any correlation between the expression of Snail and those of chromatin enzymes in tumor cells that are undergoing EMT? And more importantly, how can the Snail story help us rationalize epigenetic drug design to efficiently rectify EMT deregulation? Based on extensive experiments as delineated in the following part, we are trying to draw a conclusive remark on the Snail-mediated epigenetic network.

Snail links transcriptional control of E-cadherin with epigenetic regulation during EMT

Snail uses its SNAG domain as a molecular hook for recruiting histone lysine-specific demethylase 1 (LSD1) complex

We recently applied an unbiased protein affinity purification-mass spectrometry coupled technology to identify Snail-interacting proteins using HEK293 cells stably expressing dual-tagged human Snail [69]. Among the Snail partners, we found several interesting candidates, including LSD1, G9a, and Suv39H1. As the first identified histone demethylase, LSD1 forms a repressor complex together with HDAC1/2, CoREST and BHC80 [70], and functions by specifically removing methylation marks on H3K4 and initiating the transcription repression process [71]. Previous studies have established that LSD1 can cooperate with different transcription factors to selectively repress gene transcription [72–75]. Most importantly, LSD1 plays an essential role during development, and overexpression of LSD1 has been correlated with malignant progression of multiple cancers, including primary neuroblastic tumors, prostate cancer, and ER-negative breast cancer [76–78].

According to our preliminary results, we speculated that Snail and LSD1 form a repressor complex to downregulate E-cadherin and induce EMT. Indeed, we found that the SNAG domain of Snail and the amine oxidase (AO) domain of LSD1 are responsible for protein interaction [69]. Strikingly, we noticed that the sequence of the SNAG domain highly resembles that of histone H3 tail, and both of them are rich in positively charged lysine and arginine residues. Computer modeling analysis further revealed that the SNAG domain can adopt a conformation that is superimposed by the histone tail and bind the enzymatic cleft of LSD1 with high affinity [69]. We dug further to find that Arg3, Arg8 and Lys9 of the SNAG domain participate in critical contacts with LSD1, similar to the case of their counterparts

Arg2, Arg8 and Lys9 of histone H3. Based on our studies, we proposed a model in which Snail uses its histone H3-mimicking SNAG domain as a molecular “hook” (or pseudo-substrate) to recruit LSD1 and potentially other molecules such as HDAC1/2 and the corepressor CoREST to the E-cadherin promoter, where LSD1 demethylates histone H3K4 and HDACs deacetylate both histone H3 and H4 to initiate the transcription repression process [69]. Consistently, Baron and colleagues performed a more detailed structural analysis on Snail-LSD1 binding and found that the SNAG domain and histone H3 tail both harbor positively charged groups and hydroxyl side chains, which enable them to fit into the catalytic cavity of LSD1 in a similar conformation [79]. Furthermore, we performed functional studies to determine the role of Snail and LSD1 during EMT. Knockdown of Snail and LSD1 expression can significantly suppress the migration and invasion activities of colon cancer cell line HCT116, as well as breast cancer cell lines PC3 and MDA-MB231, which further consolidate our findings of the role of the Snail-LSD1 duo in EMT induction [69].

Snail recruits histone H3K9 methyltransferase G9a and Suv39H1 to further promote DNA methylation

Methylation on H3K9 is a well-conserved epigenetic mark for heterochromatin formation and transcriptional silencing [80, 81]. Among various histone methyltransferases, G9a is responsible for H3K9 mono- and dimethylation (H3K9me1 and H3K9me2) at euchromatin and facultative heterochromatin [82, 83]. Our recent study demonstrated that Snail interacted with G9a both *in vitro* and *in vivo* and was required for the enrichment of G9a and corresponding H3K9me2 at the E-cadherin promoter. The domains responsible for their interaction were mapped to the ankyrin-repeat and SET domains of G9a and C-terminal zinc-finger region of Snail, respectively [84]. As mentioned, DNA methylation is executed by DNMTs and commonly occurs in the promoter region of genes [27, 28]. According to our study, Snail can also interact with DNMT1, DNMT3a and DNMT3b, and these interactions are likely to be indirect, as knockdown of G9a expression disrupted the interaction of Snail with DNMTs. Interestingly, H3K9me2 coincided with DNA methylation at the E-cadherin promoter, and knockdown of G9a expression significantly inhibited DNA methylation at the promoter region and reactivated E-cadherin expression in MDA-MB-231 breast cancer cells. This is consistent with previous studies demonstrating that DNA methylation can be remarkably affected in G9a knockout embryonic cells [85]. Also in line with our findings, G9a is enriched at the promoters of aberrantly methylated genes in multiple cancer cells, and co-recruitment of G9a, DNMT1, and HP1 to the promoter of the survivin gene stimulates H3K9me2 and DNA hypermethylation [86]. Together, our study as well as those of others suggests that G9a-mediated H3K9me2 is one of the key events in the maintenance of silencing gene promoters in cancer.

Another Snail-interacting protein, Suv39H1, is a histone methyltransferase responsible for the trimethylation of H3K9, which is typically associated with constitutive heterochromatin. Our results indicated that the SNAG domain is required for the association of Snail with Suv39H1, with amino acid residues Phe5, Lys9, and Ser11 within the domain playing key roles in the protein interaction. Furthermore, we found that the interaction of Suv39H1 with Snail is critical for H3K9 trimethylation (H3K9me3) on the E-cadherin promoter in breast

cancer cells. According to previous studies, H3K9me3 catalyzed by Suv39H1 commonly links to *de novo* gene silencing by promoting promoter DNA methylation. Indeed, DNA methylation at major satellite repeats is apparently decreased in Suv39H1/Suv39H2 double-knockout embryonic stem cells [87]. In addition, it has been demonstrated that Suv39H1 creates an H3K9me3 binding and docking site for the adaptor molecule HP1, which in turn recruits DNMT3b and HDAC to catalyze DNA methylation and histone de-acetylation, respectively [33]. Consistent with these findings, we showed that increased H3K9me3 is accompanied by decreased acetylation of H3K9 and increased DNA methylation on the E-cadherin promoter, and knockdown of Suv39H1 can reverse these events and re-activate E-cadherin expression, indicating the critical role of Suv39H1 during Snail-mediated E-cadherin suppression.

The interaction of Snail with G9a and Suv39H1 gave us a couple of implications. Methylation on H3K9 is a sequential process, with the lysine residue subject to modifications of up to three methyl groups. While G9a catalyzes the mono- and dimethylation processes, Suv39H1 uses the product of G9a, i.e. H3K9me, as a substrate, and further converts it into H3K9me3 [88, 89]. We reasoned that G9a and Suv39H1 cooperate to establish the silencing heterochromatin at the E-cadherin promoter, typically with the help of Snail. Indeed, G9a, Suv39H1 and SETDB1, which is a H3K9 mono-methyltransferase, can assemble to form a giant complex in both euchromatin and heterochromatin areas, and the protein stability of one enzyme may affect the stability of the other two components [90]. Furthermore, there seems to be two distinct routes for H3K9 methyltransferases to promote DNA methylation at the E-cadherin promoter. In the first route, G9a directly interacts with DNMTs and recruits them to the E-cadherin promoter through the association with Snail. In the second route, both G9a and Suv39H1 relies on their catalytic activities to respectively create H3K9me2 and H3K9me3, with both marks being able to recruit HP1 and DNMTs to the promoter area [27]. This dual-mode of regulation may provide a double safety to guarantee the efficient methylation of E-cadherin promoter.

Snail coordinates histone modifications and DNA methylation: conclusive mark and perspective

Based on our results as well as those of others, it becomes clear that Snail manipulates epigenetic outputs and masters EMT in a multi-step fashion. First, Snail uses its molecular hook, i.e. the SNAG domain, to associate with the LSD1/HDAC complex. Once recruited to the E-cadherin promoter region, the complex exerts its histone-modifying activities to create the initial repressive chromatin environment, i.e. H3K4 demethylation and H3/H4 deacetylation. Previous studies indicated that H3K9me2/3-enriched histones devoid of H3K4me2/3 and histones depleted of H3K4me2/3 have elevated H3K9me2/3, due to the fact that H3K9 methyltransferases including G9a and Suv39H1 fail to bind and methylate H3K4me2/3 substrates [91, 92]. We reasoned that demethylation of H3K4 by LSD1 provides a condition that favors Snail-mediated association of G9a and Suv39H1 on the E-cadherin promoter. In addition, HDACs may mediate the interaction of PRC2 with Snail, therefore promoting PRC2 to land in the promoter region. Second, G9a associates with the C-terminal region of Snail and is brought to the promoter region for H3K9me2, while

Suv9H1 is drafted by the SNAG domain to land in the chromatin and uses H3K9me2 as substrate to generate H3K9me3. Since H3K9 methylation and acetylation are considered mutually exclusive, the level of acetylation would be further decreased. Third, the landing of G9a and Suv39H1 on chromatin serves as an intermediate step that bridges the initial H3K4 demethylation to the last modulation event, as it promotes the recruitment of DNMTs to work on DNA methylation, a final touch for gene silencing. Collectively, a picture appears in which Snail coordinates multiple epigenetic events to function in great synergy to suppress E-cadherin and induce EMT (Figure 1).

As our understanding on Snail-mediated EMT regulation continues to get improved, still many mechanistic details remain to be clarified. For example, after binding and bringing enzymes to chromatin, how will the SNAG domain get released to make room for histones? While we hypothesized that it is overabundant amount of histone proteins at the chromatin region that outcompetes the binding of SNAG, further experiments are required to address this issue. In addition, we are wondering whether the histone-mimicking SNAG domain can be modified during the recruitment process. Development of antibodies specifically recognizing SNAG domain with different modifications would definitely help to clarify this question. Once specific modifications on SNAG are confirmed, we can take one step further to find out how these modifications facilitate/obstruct the following epigenetic events during EMT. Chan and colleagues recently applied a pull-down approach in combination with LC-MC/MC analysis to successfully identify nuclear proteins that associate with histone H3 peptides harboring different lysine modifications [93]. Given the resemblance between the histone H3 tail and the SNAG domain, there seems to be a good chance to identify interacting proteins for diversely modified SNAG using a similar strategy. As a third enigma, while PRC2-mediated H3K27me3 is associated with gene repression, its enrichment is unexpectedly anti-correlated with DNA methylation [94, 95]. It is hypothesized that the expression of some developmentally important genes is governed by an “epigenetic switching” reprogramming event. Basically, these genes are repressed by PRC2 in normal cells, whereas they are subject to hypermethylation in cancer [96]. While the specific mechanism remains unclear, this hypothesis is compatible with our model that PRC2 recruitment precedes the approaching of DNMTs during cancerous EMT, and may partially explain the reduced epigenetic plasticity and permanent silencing of E-cadherin in multiple cancers. Future studies are required to clarify the role of PRC2 in this epigenetic modulation cascade.

Last but not least, the refreshing Snail story not only reminds us of the integrity and plasticity of epigenetic network, but also helps us for the development and optimization of epigenetic therapies targeting EMT-related diseases. As early as 2004, Azacitidine (trade name Vidaza), a potent inhibitor of DNA methyltransferases, got approval from FDA and became the first epigenetic drug on the market to treat bone-marrow cancer and blood cancer [97]. Since then, epigenetic drugs emerge as novel treatment strategies. For instance, Vorinostat (formerly known as SAHA) is a histone deacetylase inhibitor used to treat the rare cancer cutaneous T cell lymphoma (CTCL) [98]. Also there are plenty of preclinical studies underway to evaluate the therapeutic potential of LSD1 inhibitors on multiple types of cancers [78, 99, 100]. Most recently, Schenk and colleagues applied LSD1 inhibitor

tranylcypromine (TCP) in combination with all-*trans* retinoic acid (ATRA) therapy to efficiently sensitize acute leukemias of myeloid (AML) cells to undergo differentiation and decrease their leukemia-initiating capability in human xenograft models [101]. Consistently, the studies of Harris and colleagues also indicated the treatment potential of LSD1 inhibitors on AML, albeit the long-term effect of LSD1 inhibitors remains to be evaluated [102]. In addition, inhibitors targeting histone methyltransferases including G9a are under development and will potentially enter clinical trials in the coming years [103]. Finally, considering the hierarchic and interdependent nature of the EMT regulation program, SNAG-mimicking compounds may efficiently interrupt this epigenetic modulation cascade by competing with Snail for binding of histone-modifying enzymes such as LSD1, HDACs and Suv39H1, therefore inhibiting their recruitment by Snail to the E-cadherin promoter. In this regard, these compounds may work in great synergy with other treatment strategies for the cure of patients with EMT-related diseases.

Acknowledgments

We thank Hope D. Johnson for critical reading and editing of this manuscript. This work was supported by grants from NIH (RO1CA125454), Susan G Komen Foundation (KG081310), Mary Kay Ash Foundation (to B.P. Zhou) and pre-doctoral fellowship (BC101068) from DoD Breast Cancer Research Program (to Y. Lin).

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Figure 1.