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DNA Replication Inhibitor Geminin and Retinoic Acid Signaling Participate in Complex Interactions Associated With Pluripotency

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Abstract. *Background/Aim: Several links between DNA replication, pluripotency and development have been recently identified. The involvement of miRNA in the regulation of cell cycle events and pluripotency factors has also gained attention. Materials and Methods: In the present study, we used the g:Profiler platform to analyze transcription factor binding sites, miRNA networks and protein-protein interactions to identify novel links among the aforementioned processes. Results and Conclusion: A complex circuitry between retinoic acid signaling, SWI/SNF components, pluripotency factors including Oct4, Sox2 and Nanog and cell cycle regulators was identified. It is suggested that the DNA replication inhibitor geminin plays a central role in this circuitry.*

The maintenance of genome stability in living cells is associated with the tight regulation of DNA replication and integrity, so that the genome is fully and accurately replicated during each cell cycle. In eukaryotes, the initial steps of replication consist of the sequential assembly of pre-replicative complex (pre-RC) proteins onto the origins of replication. This process is named replication licensing and takes place during a restricted window of time from late mitosis to early G1 (1, 2). The pre-RCs consist of several proteins, including ORC, Cdt1, Cdc6 and MCM 2-7. Restriction of replication licensing from the end of mitosis to early G1 occurs by regulating Cdt1 levels, either by ubiquitin-mediated degradation of Cdt1 or inhibition by geminin (3). Geminin plays a central role in preventing DNA re-replication, a process that can lead to genomic instability and cancer development (3-5).

Geminin is a small nuclear protein (~25 kDa) that plays a critical role in cell cycle regulation by inhibiting DNA replication (6, 7). Geminin binds to and inhibits the DNA replication factor Cdt1. It is expressed in the S and G_2 phases of the cell cycle and is degraded by the anaphase-promoting complex during the metaphase-anaphase transition (8).

Geminin has been found to up-regulate transcription of the *geminin* gene, suggesting that its expression may be regulated by a molecular feedback loop (9). Although *GMNN* is transcriptionally regulated by E2F family members, the mechanism by which geminin modulates E2F-mediated transcriptional regulation of the *GMNN* gene is not fully understood (10). Geminin ablation has been reported to enhance colon and lung carcinogenesis (4) while it has also been found to be overexpressed in several human cancers including colon, rectal, oral and breast cancer (11-13).

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Similarly to other pre-RC components, geminin has been implicated in development and differentiation (14-16). In Xenopus embryos, it has been shown to induce cell differentiation contributing to the formation of the neural tube (17), while it has also been found to regulate the Hox homeobox proteins, controlling differentiation and proliferation (18). In another study with embryonic stem cells, geminin ablation was found to lead to loss of pluripotency and mesendodermal differentiation (19).

In the present article, we explored the interplay that seems to link the areas of DNA replication, pluripotency, development and cancer (14, 15, 20-22). Our main focus was to identify common regulatory nodes among networks of pluripotency and oncogenic factors, development and components of DNA replication. In this direction, we re-examined recent experimental data, in conjunction with *in silico* predictions placing retinoic acid and geminin on the forefront of this network.

Materials and Methods

The web-based g:GOSt tool from the g:Profiler platform was used to identify functional information and enriched pathways and processes from gene lists (23-25). Data for predictions of transcription factor binding sites were derived from the TRANSFAC database (26), protein-protein interactions from the BioGRID database (27) and miRNA target sites from the miRBase database (28). In all cases, multiple testing correction was performed using the g:SCS algorithm that is the default and most stringent algorithm for multiple testing corrections that are not independent of each other (23). A p-value<0.05 was considered to indicate statistically significant differences. The organism parameter was set to 'Homo sapiens (human)'. The generated data of transcription factor predictions and protein-protein interactions are depicted in Figure 1 while miRNA-mRNA UTR binding targets were used to construct an interaction network, and visualized using the open source software Cytoscape (version 3.3.0, USA) (Figure 2).

Results and Discussion

The present bioinformatic analysis is discussed along with significant findings from the literature. Our analysis was divided in several sub-sections in order to examine the involvement of geminin in specific interactions and signaling, shedding light to its pivotal role in certain complex regulatory processes in the mammalian cell machinery.

Geminin, pluripotency factors, and retinoic acid interactions

Geminin has been reported to be essential for maintaining Oct4, Sox2 and Nanog expression (19, 29) by antagonizing Brg1, a chromatin remodeling protein, and indirectly activating the Sox2 SRR2 enhancer (19); thus, keeping cells in a pluripotent state. In the chick embryo, there is strong evidence that it induces expression of the Sox2 SRR1 enhancer as well, through Brm, a subunit of SWI/SNF (30). Geminin has also been reported to act downstream of retinoic acid (RA) signaling; during primary neurogenesis, RA up-regulates the ERF and ETV3L transcriptional repressors which, in turn, have been reported to restrict geminin expression (31).

Bioinformatic analysis results concur with current literature

Evidence for Oct4 and geminin regulation by RAR. In the present study we used the g:Profiler platform (23, 24) in order to identify potential shared transcription factor (TF) binding sites from the TRANSFAC database (26). Interestingly, the *Oct4* and *geminin* genomic loci were predicted to have binding sequences for the retinoic acid receptor (RAR) (p=0.016; g:SCS algorithm) (Figure 1), which is a TF as well as a nuclear receptor (32).

Retinoic acid (RA) has been reported to inhibit *Oct4* expression during embryonic stem (ES) cell differentiation indirectly, by repressing a *cis* enhancer element (33), as well as silencing its promoter (34). However, in these experiments, the role of RAR in mediating the RA effects was not assessed.

RAR has been reported to modulate the expression of c-myc as well as several *Hox* genes (including *HoxB4, HoxB7, HoxA9* and *HoxA10*) (35), while our recent microarray data have shown that geminin ablation in the murine haematopoietic system results in significant RAR up-regulation (36, 37). Interestingly, RA has also been shown to suppress *Nanog, Oct4, geminin* and *Hox* gene expression; however, the exact mechanism and whether it acts directly or indirectly, through RAR and/or other factors, is not known (Figure 1). More importantly, in a recent study, RA was reported to induce chromatin remodeling close to the *Oct4* and *Nanog* genes and suppress their expression. This effect was dependent on a complex of RAR, receptor-interacting protein 140 (RIP140) and Brm. Using chromatin immunoprecipitation, the authors showed that Brm replaces another SWI/SNF subunit, Brg1, in this complex upon RA-induced repression, in the promoters of the aforementioned genes (38). In accordance with these data, Flajollet *et al*. (39) have also shown that RAR physically interacts with Brg1, as well as the SMARCD3/BAF606 complex, a core SWI/SNF subunit, which was eventually identified as a co-activator for RAR-induced transcription (Figure 1).

An interesting point is that during neural development, geminin has also been shown to directly interact with Brg1 and antagonize its activity, in order to maintain the cells in a multipotent state (29, 40, 41). Adding another layer of complexity, geminin is also known to interact with Hox genes, both directly and indirectly, through Polycomb (18, 36) (Figure 1) while BRG1 is known to control Nanog transcription through histone deacetylation (42) and occupy the promoters of Oct4, Sox2 and Nanog (43).

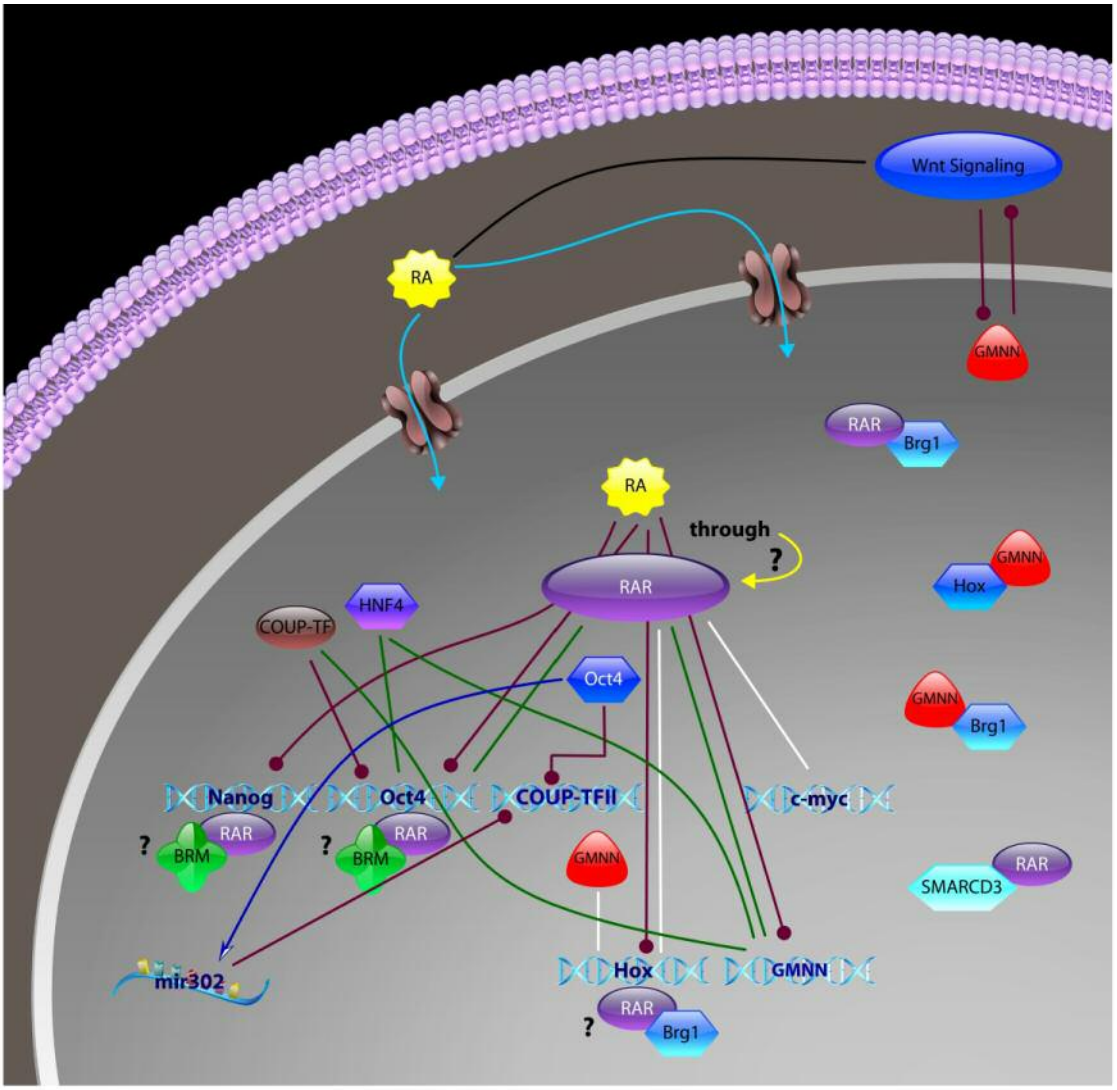

Figure 1. *The signaling events involving geminin, RA signaling and pluripotency factors. RA modulates Wnt signaling (shown in black). Once inside the nucleus, it inhibits Nanog, Oct4, geminin (Gmnn) and Hox gene expression (shown in red), however, whether it mediates these effects through RAR is not known. In turn, RAR modulates Hox and c-myc expression (shown in white) and physically interacts with Brg1 and Smarcd3, while it may bind to the Oct4 and Gmnn genomic loci (shown in green). RAR and Brm are postulated to induce chromatin remodeling and inhibit Nanog and Oct4 expression, upon RA induction, while RAR also modulates Hox gene expression, possibly in co-operation with Brg1. Gmnn regulates Hox expression (shown in white), interacts with Hox and Brg1 proteins and shares bidirectional inhibition with the Wnt signaling pathway (shown in red). HNF4 is predicted to bind to Oct4 and Gmnn sequences (shown in green), while COUP-TF is known to inhibit Oct4 (shown in red) and is predicted to bind to Gmnn (shown in green). Oct4 inhibits COUP-TFII (shown in red) and induces expression of mir-302 (shown in blue), which in turn, inhibits COUPT-TFII (shown in red).*

Moreover, RA has been shown to repress canonical and activate the non-canonical Wnt pathway in ES cells (44) and, in line with this, there is evidence that geminin expression is also regulated by Wnt. More specifically, geminin 5' regulatory sequences and endogenous geminin positively feedbacked to exogenous Wnt signals in *Xenopus laevis* embryos (45) while geminin down-regulation was shown to enhance Wnt signaling (46). This complex signaling cascade is summarized in Figure 1.

Evidence for Oct4 and geminin regulation by HNF4 and COUPTF. The results of the present study predict that, *Oct4* and *geminin*, apart from RAR, have common binding sequences for HNF4 (p=0.016; g:SCS algorithm) and COUPTF (p=0.0266;

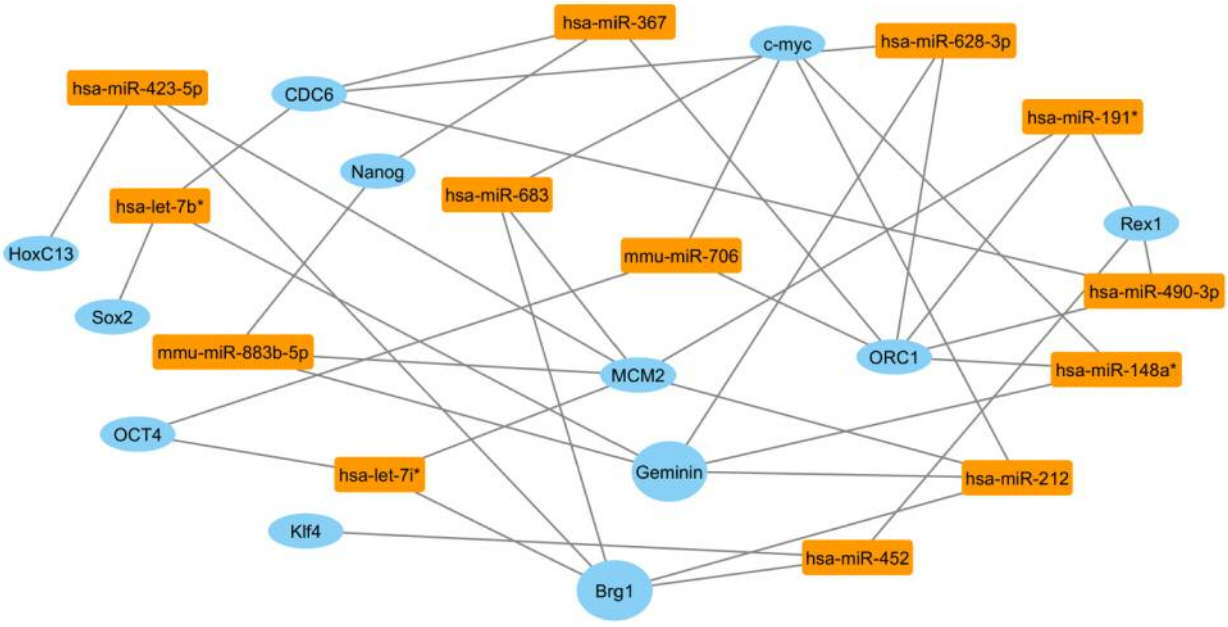

Figure 2. *Network of miRNA UTR binding targets, predicted using miRBase. miRNAs are depicted in yellow and genomic UTRs in blue. Network representation was generated by Cytoscape (version 3.3.0, USA). Node distances in the network are not to scale.*

g:SCS algorithm). Common regulation of *Oct4* and *geminin* by HNF4 seems to be in line with the recent finding that geminin together with the GATA6 TF can induce the generation of induced-pluripotent stem cells (iPSCs), without the need for *Oct4* and *Sox2* expression (47). Interestingly, our previous RNA-seq has shown that upon geminin ablation, HNF4a is highly up-regulated in the fetal liver (36).

Additionally, there is experimental evidence that COUP-TF is a ligand-activated nuclear receptor, with RA as a ligand (48), while other studies had shown that this receptor serves as a RAR accessory protein (49) and is involved in RA signaling (50-52). Interestingly, a regulatory network has also been identified, involving the miRNA miR-302 and the TFs OCT4 and COUPTFII (53) (Figure 1).

Geminin, miRNAs, GABA signaling and retinoic acid

Recent data have revealed an important role for miRNAs in pluripotency as well as regulation of the cell cycle. miRNAs can maintain the pluripotency state (54) or facilitate an exit, by repressing core pluripotency factors (55, 56). There is also increasing evidence about their role in the cell cycle and replicative stress (57, 58). It has been shown, for example, that the miR-34 family targets the MCM proteins of the pre-RC complex (59-61).

Geminin and mir-452. Geminin has only recently been reported to be targeted by miR-571, the only miRNA known to date to prevent aberrant DNA replication (62). Besides MiR-571, no other miRNA has been reported to target geminin or any pre-RC component associated with the previously described circuitry. Nevertheless, geminin appears to share a spatiotemporal expression pattern with mir-452.

Firstly, this miRNA is enriched during mouse neural crest development where it plays a role in the epithelial-mesenchymal signaling; mir-452 down-regulation affects the Sonic hedgehog and Fgf8 signaling in the first branchial arch, through Wnt5a down-regulation, resulting in craniofacial defects (63). Similarly, a study by Emmett and O'Shea has shown that geminin knockdown resulted in E9.5 embryos with smaller and abnormally oriented first branchial arch with reduced *Fgf8* expression (64). In line with this, our previous results have shown that mice lacking geminin expression have a reduced number of neural crest cells at E9.5 and 10.5 (65). Another study has reported similar results by E10.5 (66), whereas, in a reciprocal approach, FGF8 has been reported to induce geminin expression (30). Geminin down-regulation has also been reported to up-regulate Wnt5a in the primitive streak (46) and has been associated to the epithelial-mesenchymal transition (EMT), even though there is conflicting evidence as to whether its down-regulation (46) or overexpression (64, 67) promotes EMT.

Secondly, mir-452 overexpression has been reported to down-regulate the pluripotency regulators Klf4, Sox2, Oct4, Nanog and c-Myc as well as Bmi1, LEF1 and TCF4 in

glioma cells (68). In hepatocellular carcinoma cells (HCC), mir-452 directly targeted Sox7, which has been shown to interact with TCF4. HCC treatment with all-trans retinoic acid (ATRA) promoted cell differentiation and apoptosis and suppressed metastasis in mouse models (69). Regarding geminin, as already mentioned, its expression is required for maintaining Oct4, Sox2 and Nanog expression in ES cells (19, 29), while the geminin promoter contains binding sites for the TCF transcription factor (45). In addition, Caronna *et al*. have reported that geminin directly binds and represses the Lef1 promoter (46).

Thirdly, E2F1 directly activates mir-452 by transactivating its host gene, GABAA receptor ε, in melanoma cell lines. In turn, mir-452 induces EMT and down-regulates TXNIP, a metastasis suppressor (70). Similarly, TXNIP expression induces p27 (71) which promotes EMT *via* Twist1 up-regulation (72). Surprisingly, the geminin promoter has E2F-responsive sequences and E2F1-4 have been shown to up-regulate geminin (10) while geminin dysregulation is associated with increased Twist1 (46, 67).

GABA signaling, geminin and H2AX. As mentioned above, E2F1 can activate GABAA receptor ε, which in turn induces mir-452 expression (70). Interestingly, signaling through GABAA receptors has been reported to be mediated through H2AX and inhibit the proliferation of ES cell and neural crest stem cells, independently of differentiation or DNA damage (73). Similarly, H2AX phosphorylation through GABAA activation negatively regulates proliferation of neural stem cells in the subventricular zone (74). γH2AX is well-known to be induced upon geminin down-regulation, as a result of re-replication and DNA damage (75, 76). However, it is plausible that geminin-induced γH2AX can also affect cell proliferation. So far, geminin is known to affect proliferation-differentiation decisions through different factors (77-82) but not H2AX. Nevertheless, inactivation of geminin at E3.5 has been shown to be lethal due to proliferation defects concurrently with an increase in γH2AX (83).

Retinoic acid, pluripotency and cell cycle miRNA regulation. In order to identify mRNA UTR binding targets of miRNAs, an *in silico* analysis was carried out using g:Profiler (23, 24), employing the miRBase database (28). This analysis identified several miRNAs that were predicted to bind to UTRs of cell cycle and pluripotency factors, pointing to a common regulatory mechanism. Within this miRNA network, cell cycle factors *i.e*. geminin, MCM2, ORC1 and CDC6 are predicted to be coregulated with pluripotency factors Nanog, Oct4, Sox2 and Rex1, as well as Brg1, HoxC13 and Klf4. More specifically, mmu-miR-883b-5p is predicted to bind to Nanog as well as geminin and Mcm2. According to similar predictions, mmu-miR-706 binds to Oct4, Orc1 and c-myc. hsa-miR-367 binds to Nanog as well as Cdc6 and Orc1. hsa-miR-490-3p binds to Rex1 as well as CDC6 and ORC1. hsa-miR-148a* binds to c-myc, geminin and ORC1. hsa-miR-212 binds to Brg1, geminin, Mcm2 and c-myc. hsa-let-7b* binds to Sox2, geminin and CDC6. hsa-miR-423-5p binds to HoxC13, Brg1 and Mcm2. hsa-miR-452 binds to Klf4, Brg1 and Rex1. All the above including some further predictions are graphed as a network in Figure 2.

Several of these miRNAs have been experimentally reported to be modulated by retinoic acid. let-7b, predicted to bind to Sox2, geminin and CDC6 UTRs, has been found to be up-regulated in response to all-trans retinoic acid treatment of the NB4 cells, a human acute promyelocytic leukemia cell line (84). Similarly, miR-883b-5p, predicted to bind to Nanog, MCM2 and geminin UTRs, has been found to be highly up-regulated in J1 mouse ES cells upon RA-induced differentiation (85), while miR-423, predicted to bind to HoxC13, Brg1 and MCM2 was up-regulated in the neuroblast-like SH-SY5Y cells, again, upon RA induction (86). In the latter cell line, RA has also been reported to up-regulate miR-628-3p (predicted to bind to the UTRs of geminin, ORC1 and CDC6) and down-regulate miR-490-3p (predicted to bind to CDC6, ORC1 and Rex1) (87).

Conclusion

Based on the results of the present study, along with extensive evidence from the literature, it is evident that there is a circuitry between RA signaling, SWI/SNF, pluripotency factors and cell-cycle regulators. The role of geminin in this circuitry is shown to be of great significance.

While being essential for the maintenance of genome stability, we have previously shown that geminin acts as a tumor suppressor in the murine colon and lung cancer model (4). In addition, it is frequently overexpressed in several human cancers and a recent study has shown that geminin overexpression promotes breast cancer metastasis through FoxO3 deacetylation (88). Geminin is, therefore, involved in cancer, development and pluripotency. It has also recently been reported to be targeted by miR-571, the first miRNA to prevent aberrant DNA replication (62).

Further transcriptional and miRNA interactions could be examined by molecular dynamic simulations (89-92) and verified *in vitro* by chromatin immunoprecipitation, miRNA/mRNA co-expression and the study of miRNA effects on target proteins (93), along with analysis of possible epigenetic changes. A better understanding of this crosstalk will be invaluable for delineating the cell-cycle links to the loss of pluripotency, subsequent cell differentiation and oncogenesis.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

SCT and ST designed the study and SCT wrote the paper. GJD and MP wrote portions of the paper. SCT and DV performed the bioinformatic analysis. SCT, GJD, VB, GTS and ST analyzed the data relating to transcription factor binding sites. SCT, AP, AKA, MV and GTS analyzed the data relating to miRNA interactions. All authors critically reviewed the final version of the paper.

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