Review article

Transcription factors controlling E-cadherin down-regulation in ovarian cancer

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Transcription factors (TFs), such as Snail, Slug and Twist, control the down-regulation of cell–cell adhesion molecule E-cadherin in ovarian cancer. Low E-cadherin aids tumour cells in undergoing epithelial–mesenchymal transition (EMT) to motile morphology, disseminating to other organs. High TF levels have also correlated with chemoresistance and poor prognosis. This review aims to discern mechanisms of E-cadherin reduction by TFs and identifies hypoxia-inducible factor 1α (HIF1α) as an upstream regulator in hypoxic conditions. Association with chemoresistance is investigated, and whether its reversal is possible. Snail was found to bind more strongly to the E-cadherin promoter than Slug; it was suggested that Snail maintained EMT whilst Slug induced it. The use of differential zinc fingers by Snail and Slug to bind to the E-cadherin promoter supported this. HIF1α was shown to lie upstream of all three TFs and protein degradation or post-transcriptional regulation using miR-548c may regulate of Twist downstream. Further study into this is needed. High Snail expression correlated with cisplatin resistance, with knockdown of Snail reversing it. The same may be true for Twist and Slug, though some studies conflicted this. These findings show promising potential of TFs and HIF1α as therapeutic targets for EMT prevention and even chemoresistance reversal.

Key words: E-cadherin, ovarian cancer, N-cadherin, twist, snail, slug, EMT, OSE, HIF-1α, miR-548c, chemoresistance

Introduction

Ovarian cancer is one of the largest causes of cancer death among women (The American Cancer Society, 2017), with the highest death rate of female reproductive cancers. It was estimated that around 22 440 women in the US would be newly diagnosed with ovarian cancer in 2017, with 14 080 deaths as a result. The disease is particularly lethal due to its vague symptoms and thus, lack of early diagnosis. Though there are many histological types of ovarian tumours, most originate from the ovarian surface epithelium (OSE), and disseminate through the lymphatic system or peritoneal fluid, reaching the omentum and peritoneum (Vergara et al., 2010). This dissemination results in high malignancy ~90% from OSE (Cho and Shih, 2009). For dissemination to occur, cells must detach from the ovary and become motile, transitioning from epithelial to mesenchymal, a fibroblast-like morphology (Vergara et al., 2016). This is epithelial–mesenchymal transition (EMT). It occurs through down-regulation of cell–cell adhesion molecules—in particular E-cadherin. Several transcription factors controlling this expression have been identified—Snail, Slug, Twist1/2, Zeb1/2 and Sip1 (Vergara et al., 2016). Snail and Slug are zinc-finger proteins involved in changing cell shape and morphology in migratory cells

(Dattoli *et al.*, 2015). Twist is a basic helix-loop-helix involved in embryonic development and cellular differentiation (Nuti *et al.*, 2014). All three bind to E-boxes in the E-cadherin promoter and have been associated with its down-regulation and resulting EMT (Vergara *et al.*, 2016). Furthermore, these transcription factors have been associated with chemoresistance (Kurrey *et al.*, 2009; Nuti *et al.*, 2014). A few upstream regulators of Snail, Slug and Twist, for instance hypoxia-inducible factor 1α (HIF1α), have been identified (Thiery *et al.*, 2009). These are potential therapeutic targets for the repression of transcription factors and subsequent reversal of chemoresistance, but the mechanisms through which these transcription factors act must first be understood. This review will focus on the mechanisms of Snail, Slug and Twist in down-regulation of E-cadherin and differences between them. Focus will be placed on HIF1α as an upstream regulator of these factors. Their association with cisplatin resistance will also be investigated. Cisplatin is a standard chemotherapeutic agent, commonly used to treat ovarian carcinoma (The American Cancer Society, 2017). Resistance of the tumour to cisplatin is a major hindrance to the effectiveness of treatment (Miow *et al.*, 2014). Should Snail, Slug or Twist be associated with this resistance, their inhibition might aid in cisplatin resistance reversal.

Differences between Snail and Slug transcription factors

Snail and Slug have been shown to correlate with reduced level of E-cadherin expression (Batlle *et al.*, 2000; Bolós *et al.*, 2003). However, there is evidence that the two act in differential ways. Immunoblotting of SKOV3 OSE carcinoma cell lines (Kurrey *et al.*, 2005) showing ectopic expression of mSnail and mSlug individually resulted in EMT. It was found that Snail overexpression repressed E-cadherin, β-catenin, occludin and Zo1—adherens and tight junction components. Interestingly, Slug overexpression also resulted in the repression of desmosomal junction components. This is indicative of the two's differential roles in EMT induction. Correspondingly, mutation of zinc fingers of Snail and Slug in mice HEK293T and OSE carcinoma cells (Villarejo *et al.*, 2014) showed the use of different zinc fingers in repression of E-cadherin. Mutation of ZF1 and ZF2 simultaneously in Snail mice leads to complete the loss of E-cadherin repression. Contrastingly, repression of ZF3 or ZF4 individually in Slug mice was required for the same effect. This further supports the idea that the two act differently on E-cadherin, using different zinc fingers. It is plausible that Snail is required to maintain EMT, whilst Slug aids in its induction. The study by Kurrey *et al.* (2005) using hypoxic conditions showed initial increased Slug mRNA levels, which then reduced to the basal level after 12 h. Snail, however, was only detected after 60 h. This supports the theory that Snail stabilises rather than induces EMT, being triggered at a later stage. Indeed, Snail was found to have a stronger binding affinity for the E-cadherin promoter than Slug. Band shift studies of MDCK (lung cancer) cells in another study (Bolós *et al.*, 2003)

supported this. This indicates Snail's ability to stably regulate E-cadherin expression compared to Slug.

Though this evidence is fairly conclusive, it should be remarked that reliability of the studies above is questionable. Stable transfection of Snail and Slug into SKOV3 cells in the study by Kurrey *et al.* (2005) was only successful in two and one clones respectively. Without repeat stable samples to check, E-cadherin repression could be concluded to be a result of random mutation. Furthermore, a study by Bolós *et al.* (2003) using *pcDNA3-Slug* and Snail transfection separately into ovarian cancer cells showed the ability of Slug to induce EMT independently of Snail. This contradicts the hypothesis of Snail and Slug work synergistically in this process. Nonetheless, this study uses MDCK cells rather than ovarian cancer cells. Snail and Slug may interact differently in these two tissues. Future studies are required, using ovarian cancer cells and investigating E-cadherin levels over a longer time period. It may have been that Snail expression would have increased in MDCK cells, given time.

Interestingly, the study by Kurrey *et al.* (2005) showed Slug upregulation as a result of hypoxia, suggesting the involvement of HIF1α upstream. Hypoxia induced expression of Snail, HIF1α and Slug. E-cadherin also decreased, most likely as a result. It is possible, therefore, that Slug may induce E-cadherin repression under adverse conditions such as hypoxia. Snail may then act later to maintain EMT. HIF1α may therefore be a target for therapeutic knockout. Studies should be undergone to investigate the effect of this. It may be that in the absence of sufficient oxygen, HIF1α is released to cause dissemination through Slug, Snail and Twist upregulation. CRISPR/cas9 could be used to knockout HIF1α to test this theory. The effect of differing oxygen levels on tumour dissemination might also be a valid area of study.

Twist repression of E-cadherin and HIF1α involvement

More recently, Twist was identified as a transcriptional repressor of E-cadherin. Examination of Twist and E-cadherin expression in ovarian cancer tissues by PCR testing (Wang *et al.*, 2013) showed high Twist expression and low E-cadherin compared to normal ovarian tissue. Use of RNAi to knockout expression resulted in significant increase in E-cadherin, as well as β-catenin. This corroborates results found by Kim *et al.* (2014) in a similar study using immunohistochemistry to examine expression in 123 ovarian cancer cases. Twist expression correlated to reduced E-cadherin expression, as well as overall survival. Both studies indicate the direct repression of E-cadherin by Twist and its further association with poor prognosis.

Interestingly, the study by Kim *et al.* also showed increased HIF1α expression corresponding to Twist. This relationship was noted in other studies, perhaps indicating the role of a HIF1α/TWIST/E-cadherin pathway in EMT. Microarray

evaluation of expression in normal vs carcinoma endometrial samples (Feng *et al.*, 2013) showed a significant (p < 0.01) increase in HIF1α and Twist along with decreased E-cadherin. HIF1α overexpression correlated with increased Twist. This further supports the hypothesis that HIF1α lies directly upstream of Twist. However, though significant (*p* < 0.01), the correlation found using a Spearman's rank test was weak (*r* = 0.249). This suggests either a coincidental relationship between the two, or that other factors may be required to interact with HIF1α to induce Twist. This would explain why upregulation of HIF1α resulted in increased Twist expression in some, but not all cases. Furthermore, a larger sample size of endometrial carcinoma patients than normal endometrial samples was used. This might influence the rise in significance of the results. Further research is necessary to prove this mechanism of E-cadherin repression, perhaps by using CRISPR/Cas9 to knockout HIF1α.

Another explanation for the weak correlation between HIF1α and Twist is the activity of post-transcriptional or translational repression. In one study (Yin *et al.*, 2013), Twist was transfected into epithelial ovarian cancer stem cells using plasmids containing the full-length gene. As expected, this resulted in a significant increase in Twist mRNA expression. However, protein expression did not increase alongside. This implies the involvement of factors repressing the protein after transcription. This could occur through the use of microRNAs, or degradation of the protein. The use of MG132 to block proteasome activity restored expression of Twist protein alongside its mRNA, suggesting regulation to be through protein degradation. In contrast, the use of luciferase reporter assay by

Sun *et al.* (Sun *et al.*, 2016) showed Twist to be a target of *miR-548c*. *miR-548c* is a microRNA involved in translational repression or degradation of RNA. This contests the former study (Yin *et al.*, 2013) and suggests that Twist is regulated through the repression degradation of RNA rather than proteasomal degradation. It is of note, however, that it is possible for both mechanisms of Twist regulation to occur (Fig. 1). Further study of upstream regulatory factors of Twist is required, not only to prove or disprove these mechanisms, but also to identify potential targets for treatment. If *miR-548c* does indeed repress Twist, it has potential for future therapeutic treatment.

Negative regulation of Snail and Slug transcription factors via Twist

Not only is Twist negatively regulated, but burgeoning evidence suggests that it may regulate Snail and Slug expression. In a study by Forghanifard *et al.* (2017) ectopic expression of Twist via transfection into KYSE-30 cells drastically reduced Snail expression, with a 7-fold reduction in *Snail* mRNA. Furthermore, both TWIST and Snail are induced downstream of nuclear factor kappa B (NF-κB)—a signalling pathway heavily involved in inflammation (Šošić *et al.*, 2003). This upregulation appears to act as a negative feedback loop, with Twist inhibiting further NF-κB activation. Whether Snail may also aid in this negative regulation is yet to be uncovered. It may be that, as well as inhibiting NF-κB, Twist mediates Snail expression in EMT. Chip immunoprecipitation by Lander *et al.* (2013) revealed binding of Snail and Slug to E-box sequences via zinc-finger interaction with the Twist C-terminal. Indeed, deletion of the C-terminal WR domain abolished Twist association with Snail, as well its inhibition of Slug chromatin recruitment. Furthermore, co-expression of Snail with Twist stabilised the protein. This stabilisation was hypothesised to occur via Snail interference with other Twist-binding proteins (Lander *et al.*, 2013). Interestingly, inhibition of Twist phosphorylation by GSK-3β led to decreased Twist/Slug interaction. These findings outline a complex, hierarchical method of Snail, Slug and Twist regulation, with each factor mediating the other. While Snail and Slug control early stage EMT, Twist may act later to downregulate Snail and Slug, thus regulating late stage EMT.

The discovery of GSK-3β interaction with Twist via phosphorylation adds further complexity. It is plausible that Twist association with Snail and Slug is regulated by GSK-3β, ensuring its occurrence only in late stage EMT. Despite the conceivability of this idea, it is notable that study into this interaction by Lander *et al.* (2013) was performed in the context of *Xenopus* neural crest development. Thus, extrapolation of these findings to ovarian tumours is a stretch. It is advised, therefore, that further studies of Twist, Snail and Slug interaction be performed specifically in OSE tissue. Nonetheless, these studies present an intriguing model of EMT regulation to be corroborated by study in other tissues.
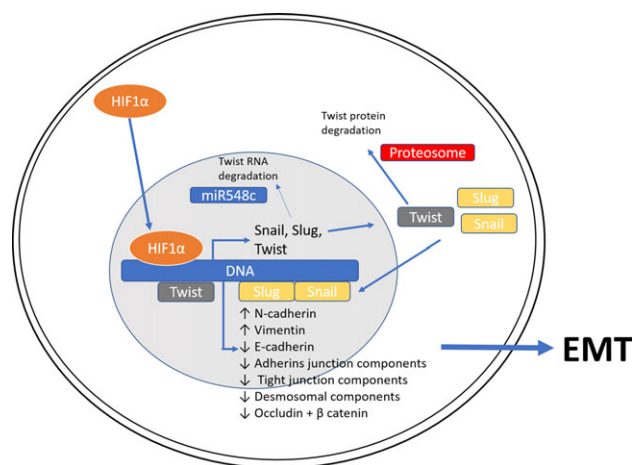

Figure 1. Summary of HIF1α induced activation of Snail, Slug and Twist and subsequent EMT. HIF1α binds DNA, resulting in TF transcription. *miR-548c* may bind and degrade *Twist* RNA. Proteasome activity could also regulate Twist protein levels. Translated TFs bind DNA inducing several expression alterations, including E-cadherin repression. Evidence indicates Slug induces EMT whilst Snail binds later to stabilise and maintain repression of adhesion molecules. Repression of said molecules aids in transition to mesenchymal morphology in EMT.

Transcription factors and cisplatin resistance

Evidence that EMT may be related to cisplatin resistance was noted in recent studies, with Snail's contribution largely evidenced. A study on A459 lung carcinoma cell lines (Wang et al., 2014) showed increased Snail levels when treated with cisplatin to develop resistance. Knockout of Snail using siRNA led to a decrease in cell viability under cisplatin treatment. In another study (Kaufhold and Bonavida, 2014), transcriptome microarrays between ovarian tumour A2780 cells and their cisplatin-resistant daughter A2780cis cells also showed Snail overexpression. Again, use of SiRNA to knockout Snail transcripts resulted in EMT reversion and increased cisplatin sensitivity. This reveals promising potential for future methods of treatment of cisplatin-resistant tumours, though further study, perhaps on live animals may be required. Furthermore, siRNA is prone to mistakes (Latifi et al., 2011), so studies using more accurate methods of genome editing such as CRISPR/cas9 could be performed.

Interestingly, the study by Kaufhold and Bonavida (2014) also revealed overexpression of Slug, Twist2 and Zeb2 in cisplatin-resistant cells, whereas analysis of A459 lung carcinoma cell lines by Wang et al. (2014) did not demonstrate such observations. A point of note in this study, however, is that actual resistance of the cell after exposure to cisplatin seems to have been assumed, not tested. Conclusions as to Snail, Slug and Twist's effect on these cells cannot therefore be fully associated with its effect on cisplatin resistance. Furthermore, this study used lung carcinoma cell lines, whereas Kaufhold and Bonavida (Kaufhold and Bonavida, 2014) investigated ovarian carcinoma lines. Thus, results from this are deemed more reliable. Nonetheless, both studies indicate a crucial role of Snail in cisplatin resistance homologous in both ovarian and lung cancers. One study (Latifi et al., 2011) on ovarian cancer cells showed increased mRNA levels of Snail, Slug and Twist in response to cisplatin. This may have been the tumour acquiring resistance through transcription factor upregulation. This further suggests the involvement of Slug and Twist in cisplatin resistance. Further studies into Twist and Slug's involvement should be undergone by using CRISPR/cas9 to reduce their expression and observe the effects. More physiological methods of transcription factor repression could also be used, perhaps by utilising an inhibitor such as oligonucleotide-Co(III) Schiff base conjugate, which inhibits Snail, Slug and Sip1 (Harney et al., 2009) by preventing binding to DNA. Other transcription factors are not inhibited. This may therefore have potential for development as a less error prone cancer therapeutic.

E-cadherin in normal ovarian epithelium

Interestingly, E-cadherin presence in normal OSE is limited compared to other epithelial tissues. Sundfeldt et al. (1997) found E-cadherin expression was confined to OSE in inclusion cysts and deep clefts. Immunochemical staining revealed no expression on the ovary surface. This is corroborated by multiple studies investigating E-cadherin in OSE (Maines-Bandiera and Auersperg, 1997). Maines-Bandiera and Auersperg suggested that the mesenchymal-like morphology of OSE cells allows rapid progression to EMT in monolayer culture. However, investigation of metaplastic OSE cells by Maines-Bandeira and Auersperg (1997) found an abundance of E-cadherin expression. Furthermore, prominence of E-cadherin increased in columnar and cuboidal OSE compared to flat. This suggests that, though absent in flat OSE, E-cadherin plays a role in specific intercellular adhesion in cuboidal and columnar OSE, for instance, in epithelial inclusion cysts. Furthermore, its expression in OSE may indicate progression to metaplasia (Maines-Bandiera and Auersperg, 1997). Interestingly, morphology and E-cadherin expression of metaplastic OSE resembles oviductal, endometrial and endocervical epithelium (Auersperg et al., 1999). This may be a result of OSE differentiation towards these related tissue types during metaplastic progression. Indeed, transfection of E-cadherin cDNA into mouse OSE resulted in initiation of a metaplastic, epithelialized phenotype resembling neoplastic OSE (Auersperg et al., 1999). This suggests that though E-cadherin may act as a tumour suppressor in late stage carcinomas, its upregulation in OSE aids progression to metaplasia and early neoplasia.

This fluctuation in E-cadherin levels indicates a complex and combinatorial regulation which is yet to be fully understood. Although Snail Slug and Twist have been shown to decrease E-cadherin expression in the transference from normal to malignant tissue, their presence in benign tumours may not have the same effect. Indeed, though levels of these TFs were significantly higher in benign tumours (Yi et al., 2014), E-cadherin expression was present in 40.4% more benign tumour samples than normal or malignant tissue. This prompts the consideration that yet another transcription factor or miRNA may inhibit Snail Slug and Twist in benign tissue, thus negating subsequent E-cadherin down-regulation. Identifying this factor might give an insight into the mechanisms of malignancy formation and targets for its reversal. Furthermore, the increase of E-cadherin in benign tumours suggests a direct upregulation via not only inhibition of Snail, Slug and Twist, but another transcription factor. Should this factor or mechanism of E-cadherin upregulation be identified, its use *in vivo* to prevent EMT (and malignancy) might be considered. Interestingly, in the same study, borderline ovarian tumours showed a 50% presence of E-cadherin after western blotting (Yi et al., 2014). Though high, this level of expression is 7% lower than in benign samples. This lower expression may be a result of conversion from benign to malignant phenotype. Thus, examination of transcription factor expression between benign and borderline ovarian tumours would be a prudent area of study. Inspection of differentially expressed genes in these tissues might identify factors responsible for benign to malignant transition. Such

factors would be promising targets for gene therapy. Though E-cadherin plays a role in cancer progression, the specifics remain unclear. Davidson (2001) suggested E-cadherin expression occurs intermittently during expression. This idea is supported by the differential E-cadherin expression depending on the stage of tumour (Yi *et al.*, 2014). However, the reasoning for this discontinuous expression requires further investigation. Until this fluctuation can be fully explained, manipulation of E-cadherin expression in cancer therapy may not be a viable treatment. Understanding this complex expression is vital to determining both E-cadherin's role in EMT and its potential as a therapeutic target.

Conclusion

In conclusion, all three transcription factors reduce E-cadherin expression and the mechanisms of this process are beginning to be understood in more detail. Understanding this could allow methods of knockdown of Snail, Slug and Twist *in vivo* to be developed. Evidence that Snail and Slug work cooperatively must be taken into account, as one might compensate for the other's loss. Twist, too, may play a role in this hierarchical regulation. HIF1α has been found to be an upstream regulator of Twist and perhaps Snail and Slug. It may be that post-translational or transcriptional regulation is involved, and if correct, this regulatory method could be augmented *in vivo* to regulate Snail, Slug or Twist expression. Though interesting, this requires far more research, as well as confirmation of HIF1α's involvement. Its involvement would indicate hypoxic conditions to be stimulator of metastasis. In addition, Snail has been shown to correlate with cisplatin resistance. The involvement of other transcription factors remains largely unknown and requires more detailed research. Nonetheless, the discovery of Snail's connection could hold therapeutic values. If drugs can be developed to remove or reduce Snail in patient tumours, reversal of cisplatin resistance might be possible. It is also plausible that reduction of other transcription factors as well as Snail might aid to prevent EMT, which may prevent metastasis of the primary tumour. Despite these promising discoveries, E-cadherin regulation in ovarian carcinoma remains a complex process. E-cadherin fluctuation between normal, benign and malignant tissue has yet to be explained. Though the role of Twist, Snail and Slug in this intermittent expression is becoming more defined, much work is yet required before therapies can be developed. Nonetheless, frequent investigation into this process is beginning to fill in the blanks. Once this picture is complete, therapeutic treatment of ovarian cancer may become far more plausible.

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