Details of the research work duly signed by the applicant, for which the Sun Pharma Science Foundation Research Award is claimed, including references and illustrations (not to exceed 6000 words)

We were the first in India to initiate research on high-grade serous ovarian cancer (HGSC), which is recognized to be a very aggressive disease and challenging for basic and clinical research. We work towards understanding HGSC at the molecular, cellular and disease levels with a long-term aim of identifying novel targets for effective cancer therapy.

Though our research in the area of cancer stem cell biology is considered as pioneering, we were one of the first research groups in India to recognize the contribution of epithelial to mesenchymal transition (EMT) to cancer. We have achieved the following in the field of EMT and Cellular Plasticity -

Contribution 1: Identification of a definitive role of transcriptional factors (TFs) Snail and Slug in ovarian cancer

The cues to investigate EMT came from the initial work in the lab when we were attempting to establish primary ovarian surface epithelial (OSE) cultures and compare with ovarian cancer (unpublished). EMT was a feature known to be associated with normal wound healing and hence these cultures rapidly progressed to a mesenchymal phenotype that were associated with a high expression of Snail and Slug (reported in literature). This was rather annoying and we had to transform these primary cells with SV40 Ag or hTERT, which changed the relevance of the cells for our studies. Later, we also identified that the HGSC epithelial cancer stem cell-like cell lines established by us also had an intrinsically high expression of these 2 TFs.

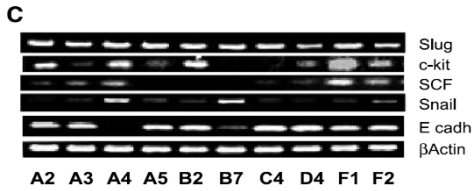
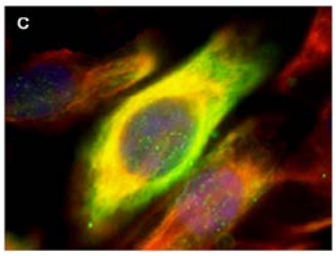


Fig.1C.RT-PCR analyses of expression of E-cadherin, Snail, SCF, c-kit, and Slug mRNA in the 10 clones that indicate heterogeneity of expression of epithelial-mesenchymal transition and survival-associated molecules; Bapat et al. Cancer Research. 2005



We thus initiated detailed experimentation to understand the contribution of the two TFs to HGSC, which was not reported at the time. We observed that ectopic expression of Snail or Slug in ovarian cancer cells lacking intrinsic expression resulted in epithelial–mesenchymal transition (EMT). This was confirmed through the downregulation of the cytoskeletal component Cytokeratin 18 and upregulation of Vimentin [Fig. 3c from the publication Kurrey et al. 2005_Immunofluorescence staining confirmed the co-expression of both CK18 (red) and Vimentin (green)]. At a functional level, this correlates with enhanced in vitro clonogenecity, motility and wound healing, and *in vivo* tumorigenecity, invasion and metastases.

In addressing the mechanism by which Snail and Slug lead to loss of intercellular adhesion, specific repression of adherens junction components (E-cadherin and b-catenin), tight junction components (Occludin and ZO-1) and desmosomal junction components (desmoglein, Dsg2) were observed. Snail suppresses expression of adherens and tight junction components, while Slug suppresses expression of all the three junction components; concertedly, bringing down the intercellular adhesion between cells. Further activation of these transcriptional factors in hypoxic conditions revealed a rapid upregulation of Slug expression as an immediate reaction that probably triggers off a signaling cascade leading to Snail expression.

This effectively identified the crucial functioning of these two transcription factors in association with the aggressiveness of ovarian cancer and work through transcriptional repression of components of several cell-cell junctions besides E-cadherin (which was earlier reported) to mediate an altered phenotype and mediating invasion and migration. Moreover, Slug and Snail may have common as well as distinct roles in ensuring tumor cell survival by signaling the onset of adverse conditions and mediating EMT.

The study published in **Gynecologic Oncology** in 2005, was one of the top 10 cited papers of the journal, and has received over 200 citations.

Contribution 2: Establishment of a mechanistic association of EMT with post-therapy CSC enrichment

A curious observation that we soon noted after publication of our earlier report was that, the two EMT factors continued to be expressed even when the ovarian cancer cells exhibited a distinctly epithelial phenotype. This suggested that both Snail and Slug possibly had alternative / additional roles in the transformed cell besides mediating EMT. EMT-TFs recognize a hexameric (CANNTG) consensus sequence in the promoter regions of their target genes. To explore this hypothesis, we performed a genome-wide profiling of the transcription targets of these TFs through chromatin immunoprecipiration using promoter microarrays (ChIP-on-chip), in 2 cellular contexts – (i) At a steady, proliferative state of cell growth, and (ii) During cell recovery following exposure to g-irradiation / lethal doses of paclitaxel.

These studies revealed non-canonical modulation of EMT at the transcription level through a shift in the transcriptional targets of these TFs under conditions of stress including irradiation or chemotherapy. Differential binding affinities of Snail and Slug to certain gene promoters and their dynamic modulation were observed in steady-state and g-Irradiated ovarian cancer cells. Upregulation of Snail and Slug was associated with a differential modulation of several pathways through altered transcriptional targets of Snail and Slug. Most significant of these were –

1. Enhanced Cell Survival and Acquisition of Radio-/ Chemo-resistance – This involved de-repression of several genes including NANOG, HDAC1, CLDN3, OCLDN, TCF4, HDAC3, KLF4, GPC3, LAMA3, TANK, MUC1, and PLAU, concurrently with upregulation of stem cell markers including OCT4, NESTIN, and c-KIT (CD117). A fourfold to fivefold increase in the putative stem cell populations was evident in both the resistant cell types.
2. Acquisition of resistance to p53 mediated apoptosis through derepression of ATM, PTEN, and BBC3/PUMA, were components of the p53 feedback loop 2 as well as additional components of p53 signaling including KLF4, CDKN1A, BCL2, BID, EPHA2, and CASP9.

Effectively, during stress Snail and Slug elegantly orchestrate their target genes leading to acquisition of pro-survival features of migration, resistance to p53-mediated apoptosis and self-renewal, that culminates in enrichment of CSCs in tumors after therapy. Such a mechanistic elucidation led these molecules to be considered as deterministic markers of aggressive disease. Such derepression of specific target genes and their contribution to acquisition of resistance, thus appears to be one of the key contributory mechanisms in ovarian cancer resistance.

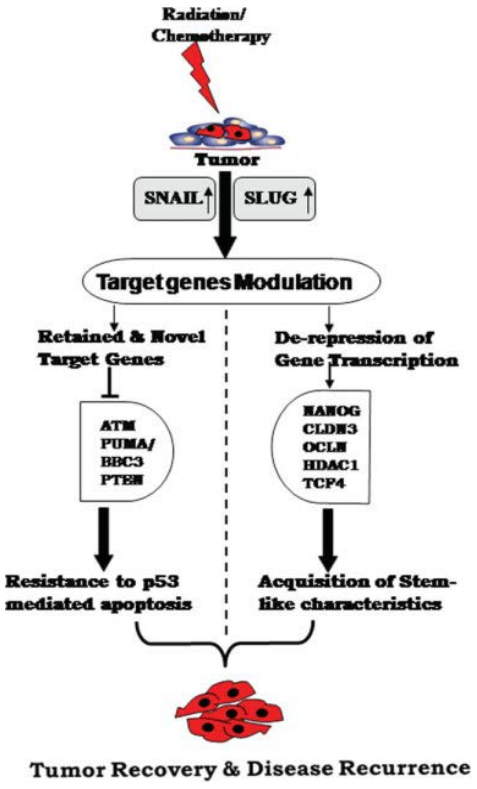


Figure 5. Model summarizing the mechanism of action of Snail and Slug in ovarian cancer cells exposed to stress. Snail and Slug expression is trigged in response to radiation and chemotherapy. These molecules perform two diverse effects, namely (a) repression of genes involved in p53-mediated apoptosis, which leads to enhanced cell survival, and (b) derepression of self-renewal genes, which leads to the acquisition of a stem cell-like phenotype. The outcome of these effects is failure of therapy, tumor cell recovery, and disease recurrence.

Contribution 3: Resolution of discrete molecular classes and specific biological functions in high-grade serous ovarian cancer that assign relevance to clinical disease

Since metastasis is a major reason for the high mortality of HGSC patients, we further explored this theme toward tumor classification through two approaches of gene expression pattern clustering: (i) derivation of a core set of metastases-associated genes and (ii) resolution of independent weighted correlation networks. Further identification of appropriate cell and xenograft models was carried out for resolution of class-specific biologic functions.

Both clustering approaches achieved resolution of three distinct tumor classes, two of which validated in other datasets. Networks of enriched gene modules defined biologic functions of quiescence, cell division-differentiation-lineage commitment, immune evasion, and cross-talk with niche factors. Although deviant from normal homeostatic mechanisms, these class-specific profiles are not totally random. Preliminary validation of these suggests that Class 1 tumors survive, metastasize in an epithelial– mesenchymal transition (EMT)-independent manner, and are associated with a p53 signature, aberrant differentiation,DNAdamage, and genetic instability. These features supported by association of cell-specific markers, including PAX8, PEG3, and TCF21, led to the speculation of their origin being the fimbrial fallopian tube epithelium. On the other hand, Class 2 tumors activate extracellular matrix–EMT–driven invasion programs (Slug, SPARC, FN1, THBS2 expression), IFN signaling, and immune evasion, which are prospectively suggestive of ovarian surface epithelium associated wound healing mechanisms.

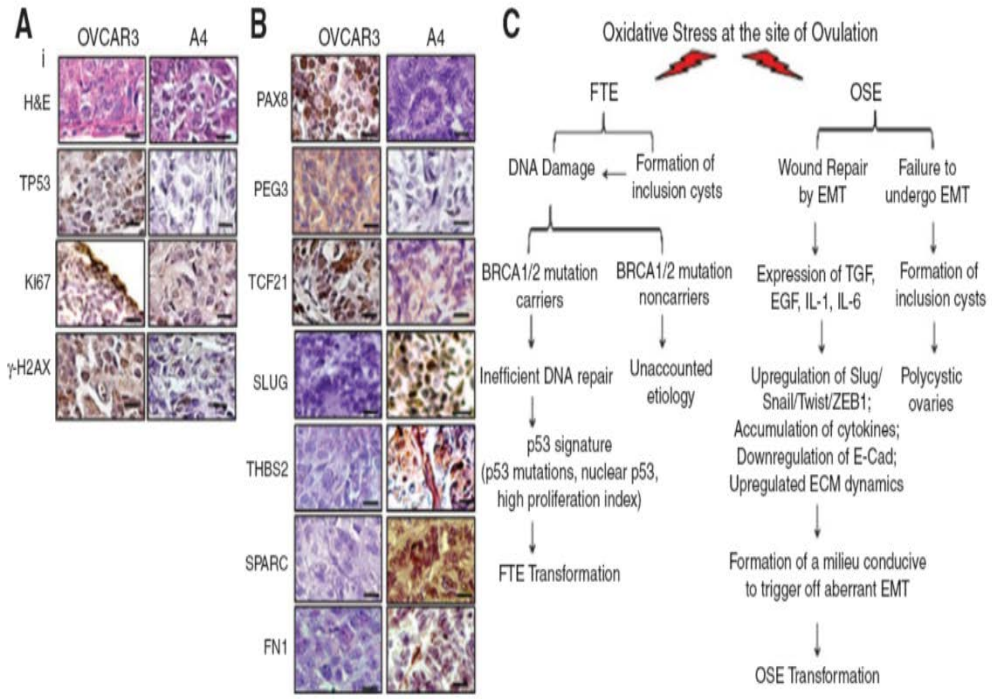


Figure. Class-specific features in OVCAR3 and A4 cells. A, p53 signature comprising of TP53, proliferation marker Ki67 and DNA-Damage marker H2AX staining in OVCAR3 (left) and A4 (right) xenografts. H&E (hematoxyline–eosin staining). Scale bar 25 mm; B, representative immune-histochemistry images associated with FTE (Pax8), Stroma (PEG3),MET (TCF21), EMT (Slug), cell migration (THBS2), extracellular matrix proteins (SPARC, FN1); Scale bar-25 um; C,schematic representation of possible events in transformation of the FTE andOSE triggered by

ovulation stress, FTE transformation is brought by activation of a p53 signature, DNA damage, defective DNA repair due to germline BRCA1/2 mutations (in patients) as also modeled inOVCAR3 cells. OSE transformation is a consequence of ECM dynamics and cross-talk initiated by imbalanced release of cytokines and hormones during ovulation that triggers aberrant EMT.

In conclusion, our systems-based analyses of gene expression data stratified HGSC tumors into 3 discrete groups and associated Class1 tumors with Cooperative Cell Migration (CCM)**,** Class2 tumors with EMT, while Class3 tumors were heterogeneous. Validation of predicted class-specific networks and biological features of quiescence, copy number alterations and epigenetic modifications in cell and xenograft models.

Interesting, a molecular signature of EMT was observed across different cancer gene expression data. Hence we performed a detailed Weighted gene correlation network Analysis of gene expression datasets in eleven cancer types, which identified modules of highly correlated genes and interactive networks conserved across glioblastoma, breast, ovary, colon, rectal and lung cancers. This enabled the extraction of a universal classifier for tumor stratification. Specific conserved gene modules were validated across different microarray platforms and datasets. Strikingly, preserved genes within these modules defined regulatory networks associated with immune regulation, cell differentiation, metastases, cell migration, metastases, oncogenic transformation, and resistance to apoptosis and senescence, with AIF1 and PRRX1 being suggested to be master regulators governing these biological processes. PRRX1 was thus suggested to be a master regulator of EMT across different cancers. Correlation analysis further identified a panel of 15 risk genes with potential prognostic value, termed as the GBOCRL-IIPr panel [(GBM-Breast-Ovary-Colon-Rectal-Lung)–Immune–Invasion–Prognosis]. This panel may now be integrated in predicting patient outcomes in the six cancers.

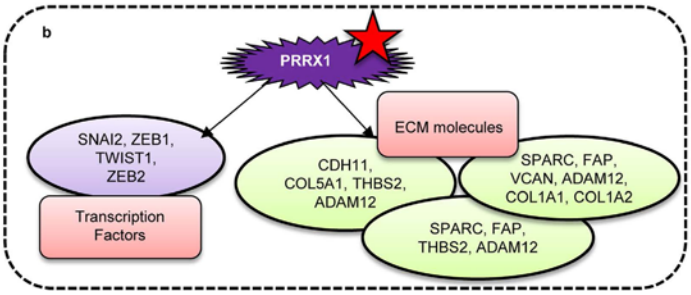


Figure 2. PRRX1 as a master regulator, interacts with several genes to mediate specific functionalities.

Contribution 4: Regulation of the EMT-TF Slug at the transcriptional level

Slug is a five C2H2 zinc finger (ZF) motif transcription factor. At the molecular level, its functioning involves recognition and interactions with a E-box (CACC/GGTG) consensus elements within target gene promoters to achieve transcriptional repression. However, precise elucidation of events involved in this DNA recognition and binding of specific promoters to regulate target genes have not been achieved. In this study, we demonstrated that besides transcriptional repression in mediating EMT and stemness under conditions of stress, Slug can also directly activate its own expression by preferential binding to specific E-box elements in the distal binding region of its promoter. Our findings suggest that while the first ZF does not contribute to the transcription-associated functions of Slug, all the remaining four ZFs are involved in regulating the expression of target geneswith ZF3 and ZF4 being more crucial than ZF2 or ZF5. We also report that recognition and binding preferences of ZFs are defined through intrinsic differences in the E-box core base pairs and/or flanking sequences, with the S2 E-box element being most critical during autoregulation. However, specific target E-box recognition and binding are also defined by the cellular context, which implies that in silico and/or biochemical DNA binding preferences may not necessarily be able to accurately predict in situ events. Thereby, this study constitutes a novel understanding of transcriptional regulation.

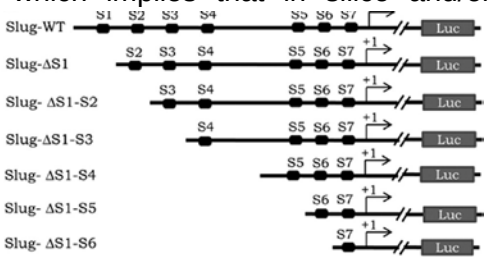
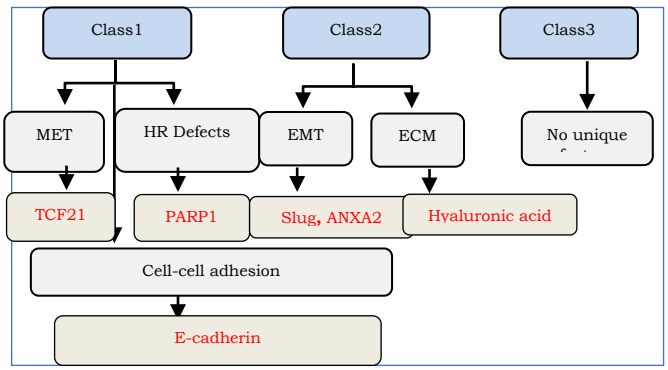


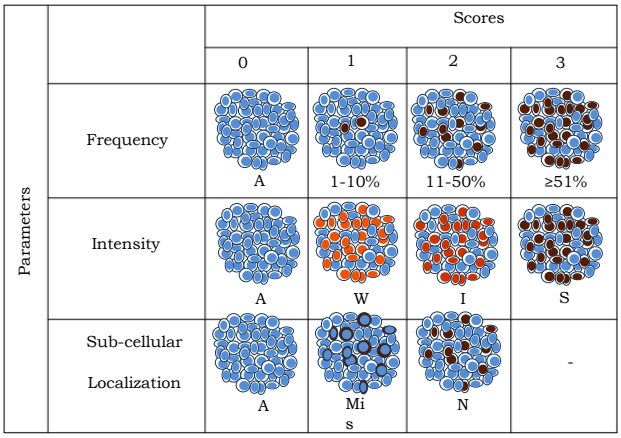
Fig.4.D. Schematic representation of luciferase reporter constructs of wild type Slug promoter (Slug-WT) containing seven E-box elements and its deletion mutants Slug-ΔS1, Slug-ΔS1–S2, Slug-ΔS1–S3, Slug-ΔS1–S4, Slug-ΔS1–S5 and Slug-ΔS1–S6 that were used in the study. +1 indicates the transcription start site.

Another mechanism of *SNAI2* (Slug) regulation by the epithelial transcription factor TCF21 was suggested as a part of the elegant TF regulatory circuitry largely dominated by TCF21 (in Class1) and Slug (Class2) that determines tumor behavior, with TCF21 possibly being a transcriptional repressor of SNAI2. These correlations emerged through the earlier systems networks analyses in which an inverse association was observed between the 2 molecular classes vis-à-vis several expression profiles and corresponding functions. We observed that luciferase activity (reporter assay for *SNAI2* promoter harboring Tcf21-binding consensus E-box sequences) was severely reduced in HGSC cells of the EMT class in which *Tcf21* was exogenously overexpressed. *In vitro* binding assays also affirmed physical interactions between recombinant Tcf21 and S1, S3, S5 *SNAI2* E-boxes. Further probing of Tcf21-bound chromatin complexes in OVCAR3 cells through immunoprecipitation indicated affinity for S2, S3, S6 *SNAI2* E-boxes. A reverse regulation of Tcf21 expression by Slug was not observed. Varied Tcf21 binding affinities in cell-free and cell-based systems emphasize the significance of cellular context in target recognition and imply possible involvement of other coregulatory factors in the process. These findings suggest transcriptional repression of Slug by Tcf21 toward maintenance of epithelial properties in HGSC and are a part of the study reported in Carcinogenesis in 2020.

Contribution 5: Clinical identification of the predicted molecular classes in human HGSC tumors leading to validation of the EMT sub-type

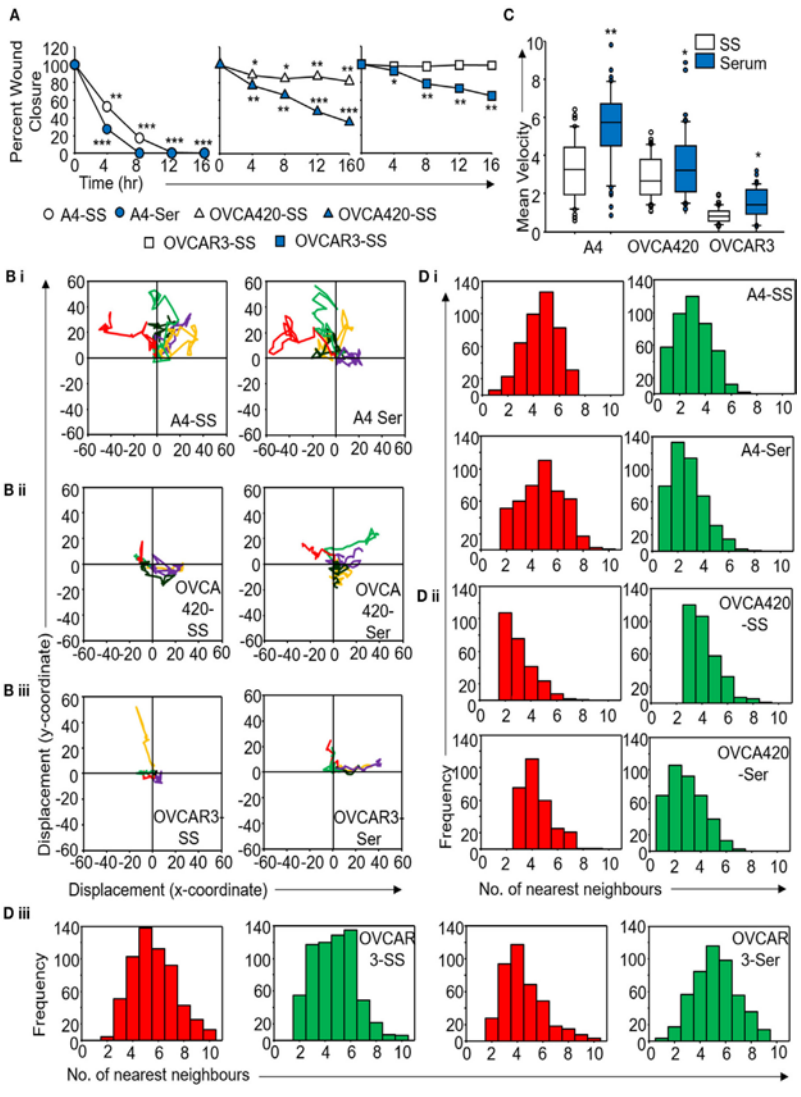
The clinical relevance of the molecular stratification was explored through collaboration with well-established pathologists from AFMC, KEM and Inlaks-Budhrani Hospital (Pune) and TMC (Kolkata). Development of standard operating protocols (SOPs) for immunohistochemistry - based detection of a panel of 6 biomarkers identified from the systems networks along with a robust scoring system for quantifying their expression in FFPE sections of patient derived tumors was undertaken. Biomarker expression was observed to vary significantly between primary and metastatic tumors suggesting class switching during disease progression. Another interesting feature in the study was of enhanced CCM-marker expression in tumors following disease progression and chemotherapy. This led to a successful validation of the predicted HGSC sub-types at the clinical level, and further revealed transcriptional heterogeneity mediating cellular plasticity and class-switching following chemotherapy. These stratification principles and the new information thus generated is the very exciting since they not only validated predicted HGSC stratification at a clinical level, but is a first step towards class-specific personalized therapies in HGSC.





Contribution 6: Identification of different modes of cell migration through Live cell imaging / real time assays

Experimental approaches to detect metastatic dissemination of tumor cells employ several in vitro and in vivo assays toward quantification of these functionalities. A large majority of *in vitro* assessments often employ endpoint assays that rely on the efficacy of wound closure and thwart quantification of migratory phenotypes observed during metastatic dissemination. Recent studies highlight the distinct signatures associated with individual vs. collective cell migration and necessitate the incorporation of these modalities into routine analyses. Advances in live cell imaging that permit real-time visualization of pathophysiological processes can be employed toward elucidating phenotypic plasticity associated with cell migration to overcome caveats inherent to end-point assays. We corroborated live cell imaging with the in vitro scratch assay toward quantification of migratory modalities in transformed cells. This was achieved through development of a protocol of live cell imaging of the classical wound healing assay, and detailed analyses toward definition of three quantitative metrics viz., displacement, velocity and number of nearest neighbors, which provided global/single-cell resolution of migratory phenotypes as opposed to the classical endpoint assays. EMT vs.cooperative cell migration (CCM). These findings were strongly substantiated during an exploration of CCM and EMT as derivatives of three quantitative metrics viz., cell displacement - velocity and number of nearest neighbors during live imaging of cell migration and invasion. Routine application of this protocol in cancer biology can aid the design of therapeutic regimes targeting specific migratory modalities and significantly contribute to the dissection of associated molecular networks.



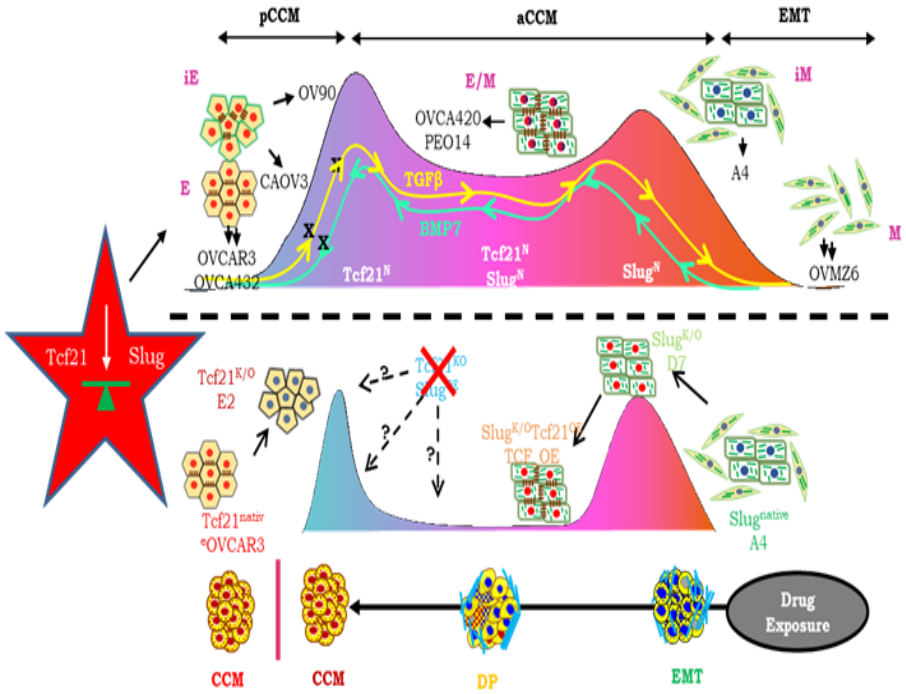
Derivation of quantitative metrics for migration in ovarian cancer cell lines. (A) Percent wound closure derived from in vitro scratch assays for A4, OVCA420, and OVCAR3 cells in the absence (SS) and presence of serum (Ser); (B) Trajectories depicting direction of migration for (i) A4, (ii) OVCA420, and (iii) OVCAR3 cells derived from : "x" and "y" positional co-ordinates over a 16 h duration of live cell imaging.; (C) Representative boxplots depicting mean migratory velocity for A4, OVCA420, and OVCAR3 cells; (D) Frequency of nearest neighbors for (i) A4, (ii) OVCA420, and (iii) OVCAR3 cells at 0 h (red) and 16 h (green) time points. Experiments were performed in the absence and presence of serum and altered migratory metrics were duly noted

We further wrote a review because we felt we need to emphasize and change the current dogma of performing end-point based invasion and migration assays.

Contribution 7: Mapping of associations between phenotypic plasticity and migration-metastases in ovarian cancer –

Over the years, the understanding of EMT has been transitioning from being a two-step phenomenon to a gradient involving intermediate transitionary phases. Recently, we have achieved a correlation between the epithelial, mesenchymal and intermediate states in HGSC and show that cells may have an increased propensity to exist in one particular state. We identified a Tcf21–Slug cross-talk in maintenance of intrinsic cellular states, wherein Tcf21-mediated Slug repression emerged as a feature of the epithelial phenotype. While previous studies exclusively implicate Slug as an EMT–TF, Tcf21 exhibits a tissue-specific activity in facilitating EM transitions. A balance of these TFs was associated with cellular plasticity as gauged by molecular and functional responses to extrinsic stimuli. Cells with native Tcf21 were conferred a highly rigid epithelial phenotype whereas dual nuclear positivity (Tcf21 and Slug) was associated with enhanced plasticity. Rigidity of epithelial and mesenchymal states was challenged by deriving intermediates (iE, EM, iM) and correlating phenotypes with discrete modes of migration despite comparable invasion.

We also explored the physiologically relevant BMP7–TGFβ to identify the imposition of reduced migration with induction of nuclear Tcf21 and epithelial features through BMP7 exposure, while TGF-β enhanced EMT/aCCM mediated migration and mesenchymal features triggered by Slug expression. While TGFβ has been extensively associated with HGSC pathology, previous reports on BMP7 have indirectly implied its role in therapy resistance, tumor progression and inhibition of TGFβ signaling in ovarian cancer. However, our findings conclusively identify a reciprocal role for these cytokines in governing cellular plasticity of ovarian cancer cells, which is reminiscent of their physiological activity during ovulation. These findings may be extrapolated to derive similarities between EMT–aCCM cooperation and OSE wound healing, while maintenance of the rigid, disparate epithelial state during pCCM could be akin to the FT-p53 signature-associated precursor lesions. Serum withdrawal as well as paclitaxel exposure promoted a Tcf21 driven epithelial state and induced aCCM that could represent a pro-survival mode of cooperative drug resistance. Moreover, Tcf21 expression associated with reduced sensitivity, and in return higher IC50, of HGSC cell to the drug when tested across the HGSC panel. Paclitaxel-induced shifts were further associated with Slug repression *via* direct promoter binding by the Tcf21. Interestingly, while the S2 E-box was most crucial for Slug inhibition, occupancy of multiple E-box sequences by Tcf21 may achieve a greater degree of inhibition.



Our study identifies novel regulatory cross-talks between Tcf21 and Slug in mediating phenotypic and migration plasticity in HGSC. Differential

expression and subcellular localization associate Tcf21, Slug with epithelial, mesenchymal phenotypes, respectively; however, gene manipulation approaches identify their association with additional intermediate phenotypic states, implying the existence of a multistep epithelial-mesenchymal transition program. Tcf21–Slug balance identified across a phenotypic spectrum in HGSC cell lines, associated with microenvironment-induced transitions and the emergence of an epithelial phenotype following drug exposure. Phenotypic transitions and associated functionalities following drug exposure were affirmed to ensue from occupancy of Slug promoter E-box sequences by Tcf21. Our study effectively provides a framework for understanding the relevance of plasticity as a function of two transcription factors and presents Tcf21–Slug as a crucial axis in cellular plasticity with genuine clinical implications.

Contribution 8: Development of a monoclonal antibody (mAb150) that is indicated to be useful in treatment of the EMT subtype of HGSC

We have developed a monoclonal antibody that targets a unique antigenic epitope in the Anxa2 protein. The efficacy of mAb150-mediated targeting of AnxA2 has been evaluated across a a panel of cancer cell lines and xenografts. This has revealed a high specificity for targeting cells that express the specific epitope. The association of Anxa2 with EMT is pre-established; additionally, we established it as a biomarker for the EMT subtype of HGSC tumors in our clincal stratification program. Tumor cells expressing AnxA2 were targeted by mAb150, and the treatment led to delayed migration through the aCCM and EMT modes, besides invasion. This assigns a cell phenotype-based context to the efficacy of Anxa2 inhibition, which is a novel feature of the present study. We additionally report that that epigenetic potentiation of AnXA2 by 5-Aza-dC or HMATi in combination with mAb150 improved the efficacy of mAb150 and may deliver further prognostic benefits as is reported in immunotherapy. Therapeutic relevance of mAb150 was finally affirmed through findings in PDX models in which formation of ascites/intraperitoneal spheroids (marked by high AnxA2 levels) were significantly delayed along with extended survival in treated mice.

This suggests that the monoclonal antibody can be used in a class-specific manner for patients who present with tumors that stratify into the EMT class. This is an exciting development. An Indian patent # 374150 has been recently sealed for mAb150 and our manuscript for the same is currently under review with the journal Translational Oncology.

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30/9/21

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