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)

The nominee's group was 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. Her research over the last 22 years has focused on understanding HGSC at the molecular, cellular and disease levels with a long-term aim of identifying novel targets for effective cancer therapy.

Although her research in the area of cancer stem cell biology is considered as pioneering and has been widely appreciated, concurrently, she had been studying the contribution(s) of epithelial to mesenchymal transition (EMT) to cancer and had resolved its implications in acquisition of a 'stem-like' state and phenotypic plasticity of tumor cells as is outlined below-

Contribution 1: Mechanistic elucidation of the association of EMT transcriptional factors (TFs) Snail and Slug in ovarian cancer

Cues to investigate EMT came from initial work in the lab when we were attempting to establish primary cultures from normal ovarian surface epithelial (OSE; unpublished). EMT was a feature known to be associated with normal wound healing following ovulation cycles; hence these epithelial cultures rapidly progressed to a mesenchymal phenotype associated with high expression of Snail and Slug as was reported in literature. This was a challenge, and we would immortalize primary OSE with SV40 Ag or hTERT, which however changed their relevance for our studies in cancer. Later, we identified that some of the HGSC epithelial cancer cell lines established by us intrinsically had high expression of these 2 TFs that suggested an involvement with the disease. This was the first global association of EMT in ovarian cancer.

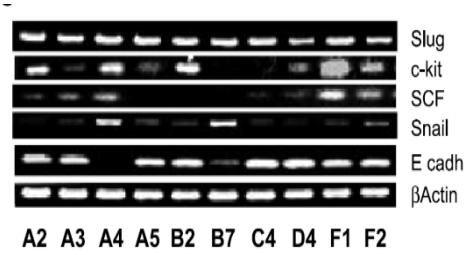


Fig.1.RT-PCR analyses of expression of E-cadherin, Snail, SCF, c-kit, and Slug mRNA in 10 clones that indicate heterogeneity of expression of EMT and stemness molecules; Bapat et al. Cancer Research. 2005

Contribution 2: Assignment of the role(s) of Snail and Slug in ovarian cancer metastases

We thus initiated detailed experimentation to understand the specific contribution of the two TFs to HGSC. 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 (Kurrey et al. 2005). At a functional level, this correlates with enhanced in vitro clonogenecity, motility and wound healing, and *in vivo* tumorigenecity, invasion and metastases.

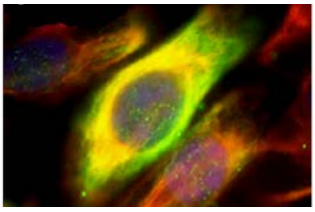


Fig.2. Co-expression of epithelial CK-18 (red) and mesenchymal Vimentin (green) in ovarian cancer cells

In addressing the mechanism by which Snail and Slug mediate 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 additionally represses components of gap junctions; concertedly, weakening the intercellular adhesion. 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 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** being one of the first to resolve the molecular mechanisms of EMT in cancer is highly cited (>200 citations).

Contribution 3: Elucidation of the orchestration of global transcription mediated by Snail and Slug leading to enrichment of cancer stem cells (CSCs) following therapy

A curious observation that we soon noted was that, the two EMT factors were expressed even when ovarian cancer cells exhibited a distinctly epithelial phenotype. This suggested alternative / additional roles in the transformed cell of both these TFs besides mediating EMT. Towards a deeper understanding of the mechanistic implications of the 2 EMT-TFs in cancer, we compared their genome-wide transcirptional targets in in 2 cellular contexts using promoter microarrays (ChIP-on-chip) – (i) At a steady, proliferative state of cell growth, and (ii) During cell recovery following exposure to γ-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 γ -Irradiated ovarian cancer cells that reflected on altered functional pathways –

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.

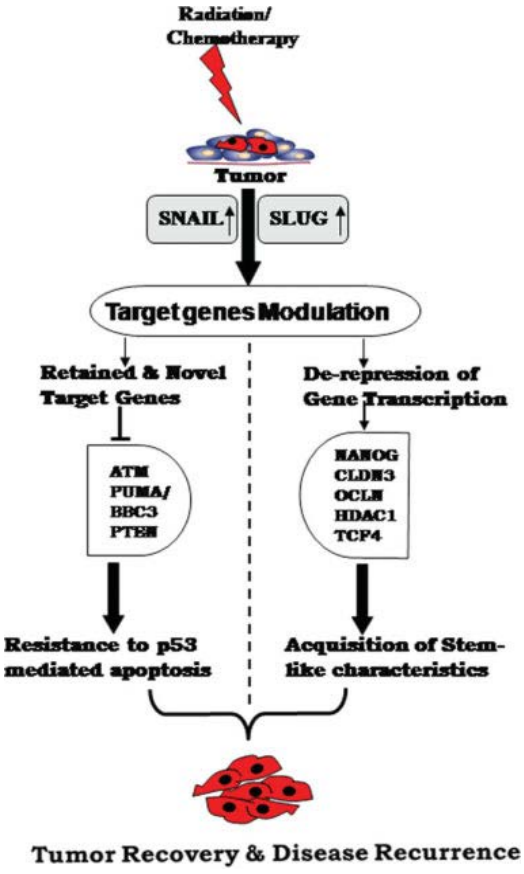


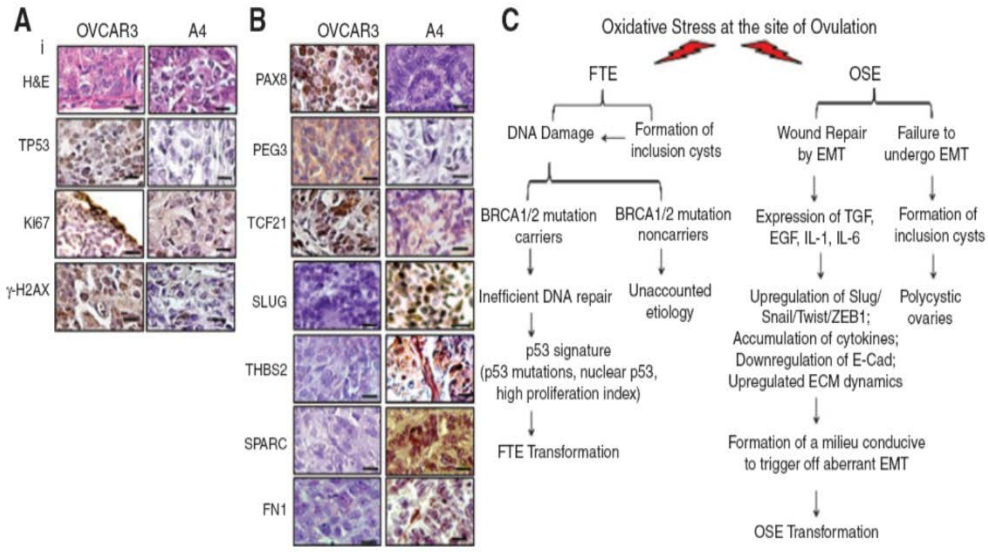
Fig.3. Schematic representing the altered mechanism of action of Snail and Slug in ovarian cancer cells exposed to stress (γ–irradiation and chemotherapy) to perform two diverse effects, namely (a) repression of genes involved in p53-mediated apoptosis, which leads to enhanced cell survival, and (b) de-repression of self-renewal genes, which leads to acquisition of a stem cell-like phenotype.

Effectively, 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, and may be considered as deterministic markers in acquisition of disease resistance in ovarian cancer.

Contribution 4: Resolution of discrete molecular sub-classes and specific biological functions in high-grade serous ovarian cancer that assign relevance to clinical disease

Since aggressive metastasis is associated with the high mortality of HGSC patients, we further explored this theme towards tumor classification, also drawing from our confidence in analyses of expression datasets developed in the earlier study. Thus, two approaches were explored, (i) derivation of a core set of metastases-associated genes, and (ii) resolution of independent weighted correlation gene networks (WGCNA). Both 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. Further validation of class-specific biological functions performed in appropriate cell and xenograft models suggested that Class 1 tumors survive, metastasize in an EMT-independent manner and are associated with a p53 signature, aberrant differentiation, DNA damage 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.

Fig.4. 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 of markers 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.



Importantly, besides stratification of HGSC tumors into 3 discrete groups, our systems-based analyses and validation associated Class1 tumors with Cooperative Cell Migration (CCM), Class2 tumors with EMT, while Class3 tumors were heterogeneous. These findings provided several leads at the basic and translational level that were explored further

Contribution 5: Identification of an 'EMT-module' in pan-cancer expression datasets

In helping other research groups in India understand their expression data, the nominee recognized exclusive "Epithelial" and "EMT" molecular patterns in expression datasets across several other tumor types. To formalize these observations, we performed a detailed WGCNA 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 PRRX1, AIF1 and Slug being suggested to be master regulators governing these biological processes. PRRX1 was thus suggested to be a master regulator of EMT across different cancers.

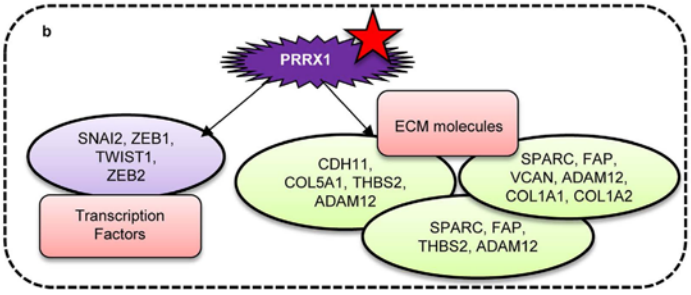


Fig.5. PRRX1 as a master regulator, interacts with several genes to mediate specific functionalities

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] that potentially predicts patient outcomes in these six cancers.

Contribution 6: Mechanistic understanding of regulation of SNAI2 expression (auto-regulation vs. transcriptional repression by TCF21)

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 were not understood. In this study, we undertook a deeper biochemical examination, which revealed that Slug directly activates its own expression by preferential binding to specific E-box elements in the distal binding region of its promoter under conditions of stress. 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 realized 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.

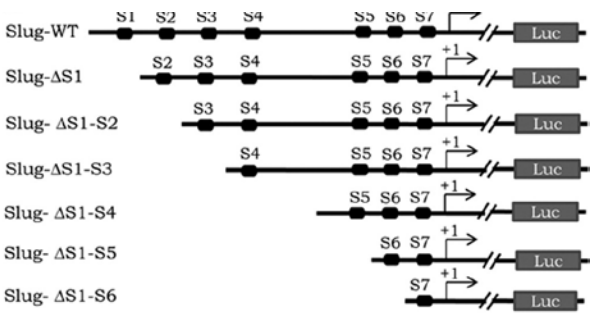
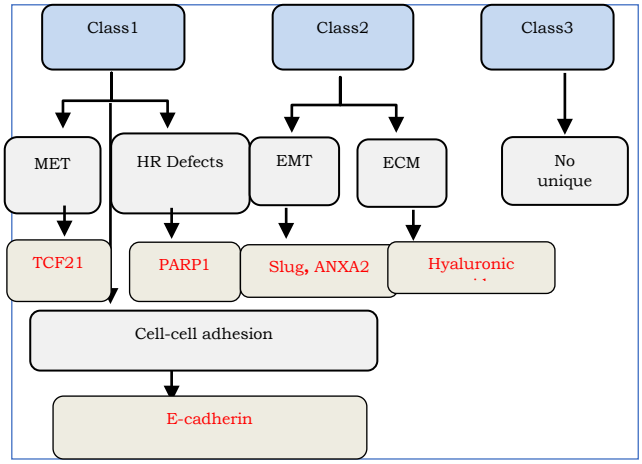


Fig.6. Schematic representation of luciferase reporter constructs of wild type Slug promoter (Slug-WT) containing seven E-box elements and its deletion mutants Slug-ΔS1, Slug-ΔS1–S2, Slug-ΔS1–S3, Slug-ΔS1–S4, Slug-ΔS1–S5 and Slug-ΔS1–S6 that were used in the study. +1 indicates the transcription start site.

Another mechanism of *SNAI2* 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.

Contribution 7: 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.



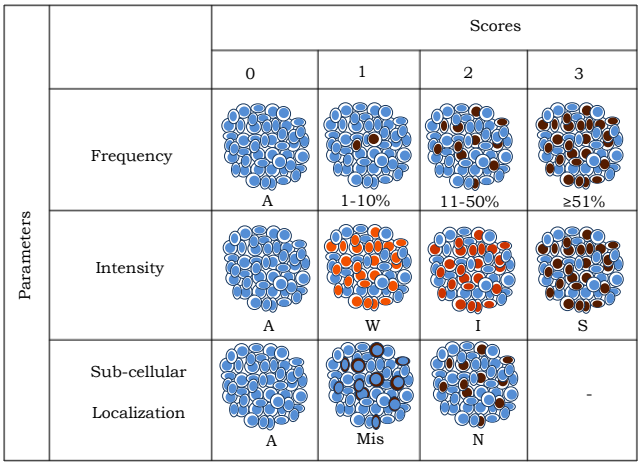


Fig.7. Schematic representation of the panel of biomarkers (left panel) and scoring system applied for the clinical stratification of HGSC based on our systems networks analyses (right panel)

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 8: 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. Our identification of 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 further explored the physiologically relevant BMP7–TGFβ defined microenvironment to identify the imposition of reduced migration. BMP7 induced nuclear localization of Tcf21 and epithelial features, while TGF-β triggered Slug expression to induce EMT and mesenchymal features. These findings conclusively assign a reciprocal role for these cytokines in governing cellular plasticity of ovarian cancer cells, which is reminiscent of their physiological activity (EMT and wound healing of OSE, maintenance of the rigid, disparate epithelial state being akin to the FT-p53 signature-associated precursor lesions). Serum withdrawal as well as paclitaxel exposure promoted a Tcf21-driven epithelial state that could represent a pro-survival mode of cooperative drug resistance, since Tcf21 expression associated with reduced sensitivity, and in return IC50 of HGSC cells to Paclitaxel; such shifts were also associated with Slug repression *via* direct promoter binding by the Tcf21.

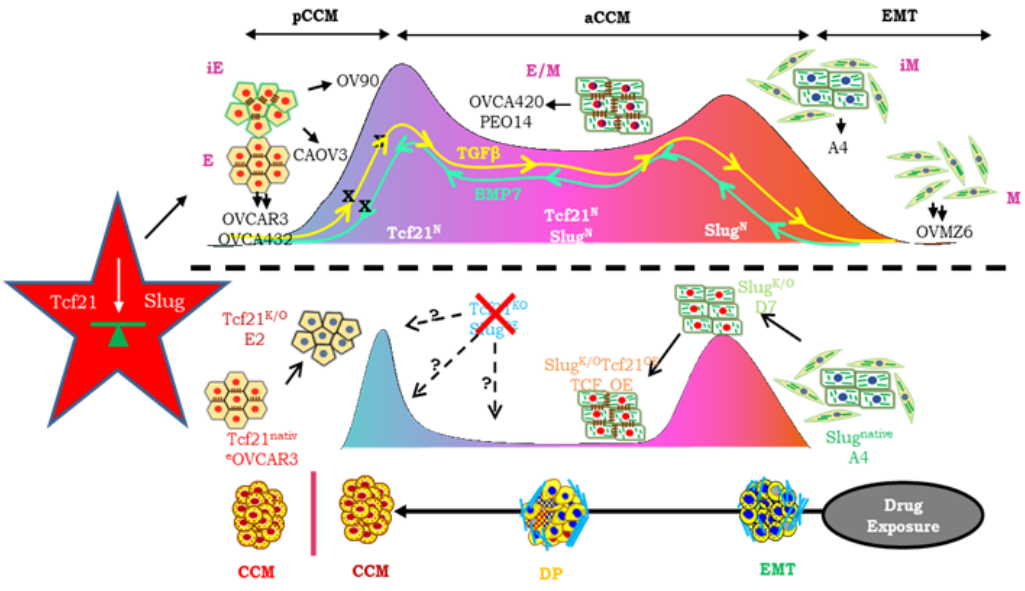


Fig.8.Schematic illustration depicting governance of cellular states as a function of the balance between Tcf21 & Slug expression

These novel regulatory cross-talks between Tcf21 and Slug in mediating phenotypic and migration plasticity in HGSC along with differential expression and subcellular localization not only associate these with epithelial and mesenchymal phenotypes respectively, but also specify their association with additional intermediate phenotypic states, implying the existence of a multistep epithelial-mesenchymal transition program. Our study thus 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 specific physiological and clinical implications.

Contribution 9: Identification of different modes of cell migration through live cell imaging / real-time assays

Experimental approaches to detect metastatic dissemination of tumor cells employ *in vitro* and *in vivo* assays toward identification of these functionalities. Most current *in vitro* assessments 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. 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 neighbours, which provided global/single-cell resolution of migratory phenotypes *viz.* cooperative cell migration (CCM) *vs.* EMT as opposed to the classical endpoint assays. These findings were further strongly substantiated during an exploration of CCM and EMT as derivatives of three quantitative metrics viz., cell displacement - velocity and number of nearest neighbours during live imaging of cell migration and invasion.

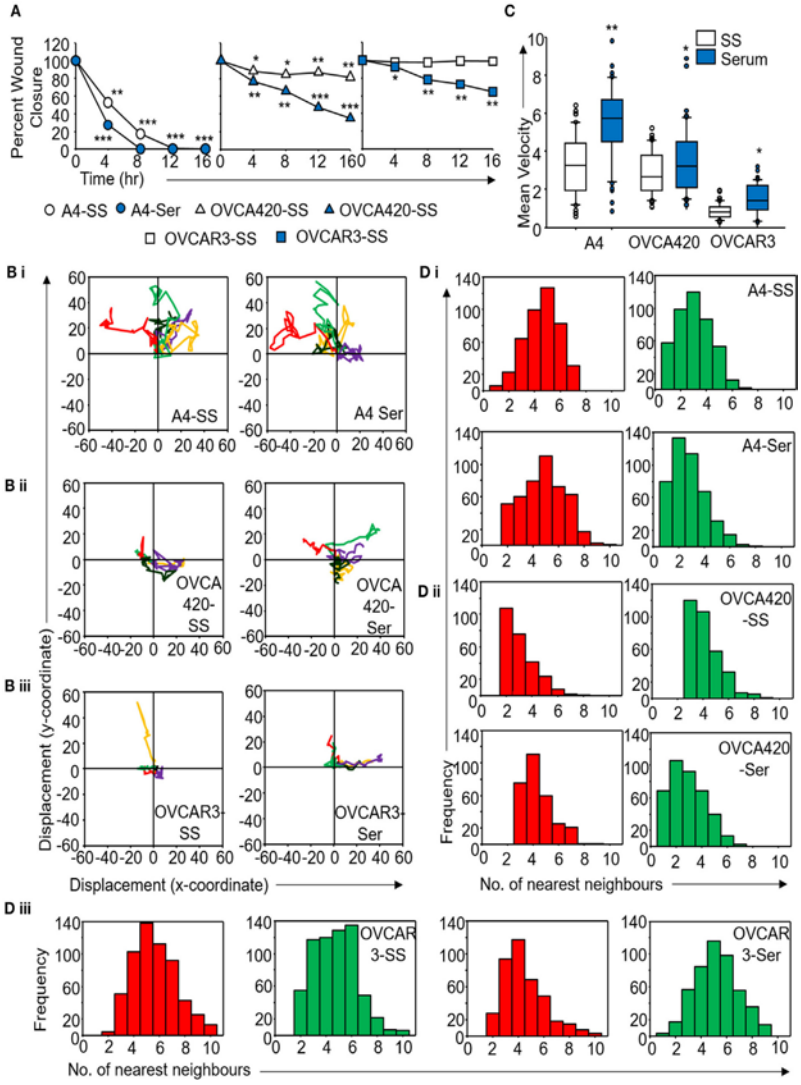


Fig.8. Derivation of quantitative metrics for migration in ovarian cancer cell lines. A. Percent wound closure derived from in vitro scratch assays for A4, OVCA420, and OVCAR3 cells in the absence (SS) and presence of serum (Ser); B. Trajectories depicting direction of migration for (i) A4, (ii) OVCA420, 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 noted

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. In our opinion, these features are needed to be emphasized to change the current dogma of performing end-point based invasion and migration assays that have limited physiological and clinical relevance.

Contribution 10: Development of a monoclonal antibody (mAb150) that is indicated to be useful in treatment of the EMT subtype of HGSC

Resolution of the HGSC molecular subclasses with a defined understanding of the driving pathways and functionalities provided an opportunity for the development of targeted therapies. Towards this aim, mice were immunized with tumor cell membrane proteins of the *in vitro* Class 1 and Class 2 HGSC models (cell lines), and their spleens harvested for development of monoclonal antibodies. On screening these, one (mAb150) was identified as being cytotoxic. Further characterization of this revealed that mAb150 targets a unique antigenic epitope at the N-terminal of the Anxa2 protein, which is essential for membrane localization and interactions with binding partners such as S100A10 that are involved in EMT-mediated metastases. The efficacy of mAb150 treatment across a panel of cancer cell lines and xenografts directly correlated with AnxA2 expression, which was reflected by lower IC50 values in AnxA2-high cells along with a high specificity in blocking its target. Tumor cells expressing AnxA2 were targeted by mAb150, and the treatment led to delayed migration through the aCCM and EMT. AnxA2 expression was specifically higher in the CSC and progenitor populations in xenografts, hence treatment with mAb150 could significantly reduce tumor burden by targeting these populations. This assigns a cell phenotype-based context to the efficacy of Anxa2 inhibition, which is a novel feature of the present study. Further epigenetic potentiation of AnXA2 with 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.

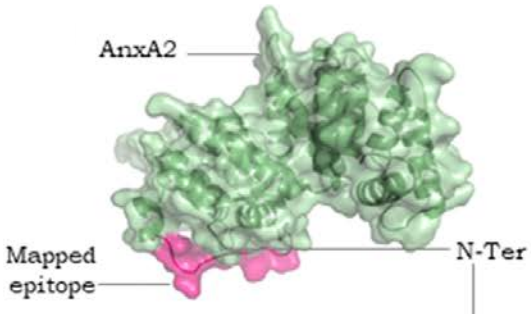


Fig.10. Schematic representation of mAb150 epitope mapped on AnxA2 protein (green) indicating its localization in proximity of the N-terminal region (marked in pink)

This suggests that mAb150 can be used in a class-specific manner for patients who present with tumors that stratify into the EMT class. An Indian patent # 374150 has been recently granted for mAb150.

Sharmila A. Bapat, Ph.D
NCCS