Gould et al, “Population Heterogeneity in…”

Response to reviewers

Dear Dr. Mac Gabhann,

We thank both the editors and reviewers for their thorough evaluation of this manuscript. We are pleased to read of the enthusiasm for which they viewed the depth and complexity of our computational modeling strategy for identifying principal signaling components within EMT and predicting downstream phenotypes. We have thoroughly reviewed the comments, and our responses and resulting manuscript edits are detailed below in blue font.

Editor comment: In particular it was difficult to evaluate this manuscript without clearer definition of the architecture and implementation of the model(s). The comments of reviewer 1 can be a starting point here, and in general we note that the manuscript should be capable of allowing a suitably skilled researcher to recreate the methods herein. As an additional question, the authors should give some analysis on the meaning of robustness coefficients in the region of 10^10 and 10^-12. Values of this magnitude, and with this range, make the model appear unbounded, or at least beyond plausible biological variability.

We thank the editor for this insight. The model architecture was detailed in the SBML file that was included with the manuscript submission. It contains the lists of all the species and connections modeled in the network. We further uploaded the entire model to a freely accessible repository for anyone to replicate our results at GitHub. The SBML format is a convention for presenting extremely large species and network lists. In terms of Reviewer 1’s comments, we address them below. Additionally, we have made all code and parameter sets publicly available via GitHub. We have added the following line of text to the beginning of the Materials and Methods section:

“The simulation code and parameter ensemble used in this study can be downloaded from GitHub (<https://github.com/jeffreyvarner/TGFb-VEGFA-Model_v1.git>).”

The specifics of the robustness coefficients have been detailed in several previous publications (see references below, and in the manuscript). The robustness coefficients within these regions reflect a numerical ratio of the integrand of the model response with respect to an EMT agent being present over the integrand of the response with the source being absent. In this manuscript, the training data for the response included Western Blot data, which is inherently difficult to quantify for numerical analyses that require specific values for “heavily expressed” and “absent”. The data we used from over 40 published papers and predicted responses include situations where a very large effect was identified with the addition of a ligand, and a very small/absent effect was reported at baseline (For example in Figure 2). When a highly expressed result is divided by a highly non-expressed background, a result near 10^10 is very reasonable to be expected. The converse is also true for generating robustness coefficients approaching 10^-12. In machine space, a very small number is represented by an epsilon and is commonly employed in a similar fashion. This large robustness coefficient space therefore was critical to accommodate these large expression differences, as evidenced by faithful replication of the training data in both time and dose (Figure 2). Furthermore, the robustness coefficients for the key signaling mediator identified lied within a much smaller range of 10^-2 to 10^4 (Figure S2). As further evidence, the actual concentration space from which these robustness coefficients were derived (nmol/L) was very plausible biologically (10^-4 to 10^5). We have now included this data for E-cadherin and Vimentin expression following TGFβ exposure in the supplement Figure S9.

To better explain robustness coefficients, we added the following text (and associated references) to the Results section:

“… We have previously used robustness coefficients to systematically quantify response of a system to structural or operational perturbations, for example gene deletions or the addition of a growth factor or hormone (references).“

Song S§, Chakrabarti A#, and J. Varner (2010) Identifying ensembles of signal transduction models using Pareto Optimal Ensemble Techniques (POETs). Biotechnol. J. 5:757 – 769

Tasseff R #, Nayak N #, *Salim S*, Kaushik P, *Rizvi N* and J. Varner (2010) Analysis of the Molecular Networks in Androgen Dependent and Independent Prostate Cancer Revealed Fragile and Robust Subsystems. PLoS ONE 5:e8864

Lequieu J# ,Chakrabarti A# , Nayak S# and J.Varner (2011) Computational Modeling and Analysis of Insulin Induced Eukaryotic Translation Initiation. PLoS Comp. Biol., 7: e1002263

Tasseff R#, Nayak S#, Song S§, Yen A, and J. Varner (2011) Modeling and Analysis of Retinoic Acid Induced Differentiation of Uncommitted Precursor Cells. Integr Biol. 3:578-591

Reviewer #1: In this manuscript Gould et al. used a very comprehensive model studies in combination with some experimental confirmations to study the EMT process induced by TGFb and VEGF. The authors built a model with 995 genes, 1700 interactions, and 1756 unknown parameters. The parameters are trained with 40 data sets. This problem is of no doubt very important and requires some combined systems biology studies. However, after reading the manuscript I found in a position being unable to evaluate the validity of this work. Here are some serious concerns,

1. What is the model? The authors talked about 995 genes, and Figure 1 indicates 80 genes forming the EMT gene expression program. What are they? How are they connected (and how is the connectivity information obtained)? For example, in the supplemental materials and methods part, ZEB1/2 and Twist are also the master transcription regulators in EMT in addition to Snail1/2 the authors mentioned. How are they regulated in the network? How do they contribute to hybrid state? They are barely mentioned in the main text. Therefore it is impossible to even understand this work without the information.

The most common way to identify every component of a complex signaling model is with a connectivity diagram that positions each species within the network (DNA, RNA, Protein) in concentric rings with connecting lines representing each interaction. It is impractical to present data tables of all of these interactions as commonly done in models where few genes were considered. The details of all of these species and their interactions are contained in the SBML file that was uploaded with the document. This file also allows anyone to investigate the network with considerable detail and also replicate our results. Additionally, all model code, and the parameter ensemble have been archived on GitHub (linked via [varnerlab.org](http://varnerlab.org)).

This method, while accurate computationally is very challenging to parse biologically. We chose instead to present the model in a biologically friendly format in the paper, and focused in on the major agonist ligands (TGFβ and VEGFA) and their most common downstream signaling networks. Our model contains both DNA, RNA, and protein interactions that regulate EMT inducing and inhibiting genes, resulting in epithelial and or mesenchymal protein expression. It is impractical to diagram all of these interactions in the figure, so we therefore focused on major signaling feedback.

Regarding the authors specific comments regarding the twist pathway, this was included in the model as detailed in the SBML file. Our objective in this paper was to analyze EMT response to TGFβ and/or VEGF ligand signaling. As noted in the text, there are many different ligands that could also initiate an EMT response, including BMP, Notch/Jag/Dll, and Wnt. The fact that direct testing of all these possible ligands is experimentally intractable elevates the importance of the computational strategy presented herein. In addition, our model includes both smad-dependent and independent signaling paths downstream of TGFβ. While many investigators isolate a very small subset of this modeling network to identify the possibility of the contribution of its components, the broader quantitative understanding of the impact of this subset on the entire network is lost. This is especially relevant when considering dose dependent effects rather than complete blockage of network connections (as is commonly done in experimentation). Our model is completely unbiased and dosage sensitive such that any relevant interaction between other signaling partners in response to either TGFβ or VEGFA stimulation would be identified.

In our subsequent analysis of the literature in response to the reviewers comment, we found that Zeb1/2 signaling is most commonly induced by TNFα and not TGFβ (e.g. Oncogene. 2007 Feb 1;26(5):711-24). Though a potential upregulation and secretion of TNFα by TGFβ cannot be ruled out, the direct effects of TGFβ is likely to dominate the model in the short timespan (which it does as evidenced by the training data). Importantly, there is an alternative signaling mode recently identified by which TGFβ acts in concert with miRNAs to regulate EMT (e.g. Zhang et al Science Signaling 2014). Our current network does not include miRNAs within it because very little is known about the connectivity and regulation of miRNAs at this time. The current papers that evaluate miRNA signaling employ broad screening/sequencing approaches to identify a small subset, and then test those experimentally in isolation. Undoubtedly miRNA signaling is important in EMT, but its integration into large scale signaling models is premature at this time. We will of course update the model as this information is established.

Regarding twist, there are indeed several studies that identify changes in Twist expression in response to TGFβ signaling. Indeed direct relationship between twist and snail regulation of EMT is not established, and experimental investigation using knockdown/deletion plasmids do not address dosage effects of interacting components that are necessary to ascertain principal components in signaling. Recent data supports that twist expression is dispensable for early EMT, but critical for stabilizing EMT long term (Mol Cancer Res. 2011 Dec;9(12):1644-57).

Detailed investigation of individual regulatory components in the context of the broader signaling network is possible with the computational model presented in this study, including those of interest to the reviewer. We can’t possibly satisfy every readers specific interest, which is why we give the model to the field. We hope all of these types of questions are asked, but ultimately answering each one would likely be a manuscript in itself. We believe this model will greatly assist these investigators in generating experimentally tractable predictions. However, there is much to do in this field; we have therefore identified a number of potential future directions, including addressing the miRNA limitation in the last section of the discussion.

Toward this concern, we added a sentence highlighted the need to consider miRNA in a next generation model:

“ … Lastly, while we have modeled classical protein signaling, we have not considered the role of regulatory RNAs on EMT. There is growing evidence that microRNAs (miRNAs) play a strong role in EMT, where several miRNAs, for example miR-21 and miR-31 are strongly associated with TGF-$\beta$ exposure \cite{Bullock:2012aa}. …”

Bullock MD, Sayan AE, Packham GK, Mirnezami AH (2012) Micrornas: critical regulators of epithelial to mesenchymal (emt) and mesenchymal to epithelial transition (met) in cancer progression. Biol Cell 104: 3-12.

2. Even with the parameter space search and ensemble model approach, the number of molecular species and the number of parameters are still unbelievably large. With all these efforts, what can one learn from it that are not possible with even just looking at the network topology? I am a fan of Sethna’s statistical mechanics analysis of systems biology models, but need to see in this work that one can learn something new, and there is no problem of overfitting of 1756 parameters out of 40 data sets.

This is in fact the value of our computational strategy, and systems biology in general. Genetic regulation in vitro and in vivo for the simplest responses requires integration of hundreds of genes and proteins, which are simply intractable to experiment on simultaneously. Lacking a computational strategy to model and predict interactions, science will not easily be able to identify global signaling mediators that act across multiple sub-networks. The strategy and model presented herein was painstakingly assembled from the literature and cross-validated by over 40 experimental datasets. These datasets include effect changes with both ligand dose and time (Figure S1). While we appreciate the insight that the reviewer may have to analyze signaling networks, simultaneously analyzing the dose and time interactions of nearly 1000 species in our view can only be reasonably achieved by computational modeling. These simulations took approximately 2 weeks to run via supercomputer. Furthermore, the POETs analysis in combination with cross validation specifically addresses the concern of the reviewer. We optimally balance competing training objectives, while simultaneously performing cross validation gauge the level of overfitting. Lastly, we performed random parameter controls and found that we could predict system response to TGFβ exposure. A description of these studies, and their results is in the first paragraph of the results section.

Through this analysis we established ranges of values for each parameter that result in notable expression differences, as well as the parameter ranges for which no expression change occurs. This enables us to simulate across huge parameter spaces for all network species simultaneously to identify those species whose expressions are highly effected by parameter choice AND exhibit outsized effect on the overall network response. Without this strategy we agree that overfitting would be a significant problem. This approach is also unbiased and captures as broad a signaling space as available. More common agent based modeling interrogates much smaller network subsets, with more obvious conclusions. As previously mentioned, we believe it would be impossible fro anyone to “look” at the complexity of our network and readily identify a dose and time specific dominating species.

3. The authors used 40 data sets from different cell lines. Can they justify that they can extract parameters from data out of different resources? EMT dynamics is highly cell type dependent. MCF7 cells can finish EMT in 2 days while MCF10A needs at least 5 days.

Extracting data from multiple cell types and conditions is commonly employed for computational modeling. In this case, we acquire data from multiple cell types, which exhibit underlying kinetic differences in response as pointed out by the reviewer. These differences could be directly attributable to differences in parameter sets for all species, which articulate cell type specific sensitivities to ligand dose. The fact that our cross-validation strategy maps not only different time and dose dependent results, but also faithfully recapitulates different sensitivities in our view further supports the robustness of the model.

4. The authors talk about ensemble of models, for example, on page 3 of the ms “POETs identified more than 15,000 likely TGF-beta induced EMT models”. What are these models? Do they have the same connectivity? How are these models generated?

The POET strategy has been previously published and used on several other publications (see example POETs references below). Additionally, we’ve published several other studies that have explored ensemble modeling (see references below). We’ve already referenced several of our previous studies (and ensemble studies from other labs) in the manuscript. Ensembles estimated by POETs (or other parameter estimation techniques used in our lab such as particle swarm optimization) simulate the same possible network topology over and over (thousands of times), varying the parameter space for each species within the network, and identifies parameter ranges for each species that 1) are sensitive to the ligand perturbation, and 2) influence the outcome of the simulation (epithelial vs. mesenchymal phenotype). Essentially, we utilize this response space as a virtual “population” of potential cellular responses (e.g. Figures 3,4) that can be interrogated to identify unique use of the signaling network for the represented outcome. For example, in Figure 3 we identify 4 response subtypes, each utilizing different levels of NFAT and p-Sp1 to achieve. It is precisely this approach that enabled unbiased prediction of the importance of these two proteins in regulating EMT response from TGFβ and VEGFA.

Sagar A# and J. Varner (2015) Dynamic Modeling of the Human Coagulation Cascade using Reduced Order Effective Kinetic Models. Processes, 3:178 - 203(invited; *Special Issue Modeling and Analysis of Signal Transduction Networks*)

Wayman J#, Sagar A# and J. Varner (2015) Dynamic Modeling of Cell Free Biochemical Networks using Effective Kinetic Models. Processes, 3:138-160(invited; *Special Issue Dynamic Approaches to Metabolic Modeling and Metabolic Engineering*)

Luan D#, Szlam F, Tanaka K.A, Barie P.S, and J. Varner (2010) Ensembles of uncertain mathematical models can identify network response to therapeutic interventions. Mol. BioSys. 6:2272 - 2286

Lequieu J# ,Chakrabarti A# , Nayak S# and J.Varner (2011) Computational Modeling and Analysis of Insulin Induced Eukaryotic Translation Initiation. PLoS Comp. Biol., 7: e1002263

Song S§, Chakrabarti A#, and J. Varner (2010) Identifying ensembles of signal transduction models using Pareto Optimal Ensemble Techniques (POETs). Biotechnol. J. 5:757 – 769

To clarify this point, we’ve added text the Results section (first paragraph) better explaining the model ensemble (second paragraph results section):

“ … The selected models all had the same possible molecular connectivity, but different values for model parameters and extrinsic factors such as RNA polymerase or ribosome abundance. …”

5. The network in Fig 1 shows that TGFβ1/2 only activates a SMAD-independent pathway. This is in contradiction to what generally documented in the literature (see, e.g., Feng and derynck, Annu. Rev. Cell Dev. Biol, 2005, 21:659-93).

We are confused by the reviewers interpretation of Figure 1, which identifies *both* Smad-dependent and Smad-independent signaling pathways. Also, as indicated in the methods, the signaling machinery of many other EMT signaling pathways are also included, including VEGF, Notch, BMP, and Wnt. Therefore, our simulations would identify a principal role of one of these other contributors in response to TGFβ if it existed. As previously stated, diagramming all ~1000 species, while preserving an overall understanding of the signaling architecture, would be impossible for the printed space allowed. Therefore, we submitted the complete SBML file for all scientists to evaluate and use.

6. The authors seem to be unaware of a number of studies on the hybrid state. For example, Yu et al (Science, 2013, 339:580) shows that circulating tumor cells are likely in the hybrid state. More relevant to the present work is that of Zhang et al. (Science Signaling, 2014, 7:ra91). These authors used integrated computational and experimental studies to show how the hybrid state and other cell phenotypes are generated using TGF-b1 induced EMT in MCF10A as a model system. In the present work the authors conclude that the treatment with TGF-beta or VEGF-A alone, MCF10A and DLD1 cell were either mesenchymal or epithelial, while only treatment with both can trigger cells to the hybrid state. What is the mechanism behind this? Is there any possibility that change the dose or the duration of the treatment with TGF-beta can also induce cell to the hybrid state?

As previously mentioned and detailed in the text, there are definitely dose and time dependent effects of EMT that these cells, and our model replicates these responses (Figure S1). This figure is presented for X cell type, and would be different for the Y cell type as hypothesized by the author. With respect to TGFβ alone inducing a hybrid state as referenced by the studies the authors indicate, without simultaneously analyzing VEGFA expression in those cells, it is impossible to determine conclusively whether hybrid EMT was caused by TGFβ only. Furthermore, we recognize with the reviewer that completely blocking signaling nodes is likely to cause dramatic changes in EMT response, including hybrid EMT, but these don’t reflect the more common and nuanced situation of dosage. We are very interested in evaluating other cell types and applications, including circulating tumor cells and the contribution of miRNA species, and will do so once this information becomes available. In the mean time, we reiterate the significant need for computational technology to assess net responses to multiple signaling pathways acting in concert, which is experimentally intractable otherwise.

Reviewer #3: The authors generated the novel dynamic mathematical models to understand the operational hierarchy of the molecular programs governing the epithelial-mesenchymal transformation (EMT). Simulations based on the author’s model predict that the transcription factors phosphorylated SP1 and NFAT are the master regulators of EMT and simultaneous treatment with TGF and VEGFA can lead to heterogeneity of epithelial and mesenchymal phenotypes. The authors further tested whether their mathematical prediction of phenotypic heterogeneity is consistent with their experimental observations and found that the experimental data support their mathematical model.

This paper provides the newer understanding of EMT regulation. Specifically, finding of the phenotypic heterogeneity is critical to understand molecular regulation of EMT and therefore this article would significantly contribute to the EMT research field.

We very much appreciate the reviewers enthusiastic support for the impact of our work, both in the simulations and experimentally validated predictions.

1. 6/S-15, line 150.------Fig. 2G does not show TGFß3 expression. Fig. 2G. indicates that TGFß3 induces LEF-1.

We apologize for the error. We’ve updated the results section to correctly reference the appropriate panel of Fig 2.

2. 6/S-15, line 153.------Description about Fig. 2I could be for Fig2G.

We apologize for the error. We’ve updated the results section to correctly reference the appropriate panel of Fig 2.

3. 6/S-15, line 156.------Fig. 2M does not exist in the Fig.2 plate.

We apologize for the error. We have removed the spurious reference to panel M of Fig 2.

4. 10/S-15, line 302.-----An increase in NFATc1 expression is evident but it is not clearly visible in Fig. 5BC and Fig. S3C, E if NFATc1 nuclear localization is significantly increased in MCF10 and DLD1 cells.

As the reviewer indicates, the NFATc1 expression is nuclear associated, indicating transcriptionally active. The confusion may be that all nuclei in the figure are positive (green) rather than negative (blue, compare against the control condition). This treatment was highly effective in stimulating NFATc1. For a heterogeneous example to compare, the T+V example in Figure 5B shows both blue and green cells.

5. 10/S-15, line 318-343.------- There is noticeable difference between MCF10A and DLD1 cells in E-cadherin expression responsive to VEGF +TGFbeta2. DLD1 cells do not appear to show heterogeneity. Combined TGFbeta1/2 and VEGF-A do not appear to increase both Vimentin and E-cadherin in DLD1 cells. Is there any cell type specific response? If so, it should be mentioned here.

Figure S4C demonstrates that DLD1 cells also acquire a significant heterogeneous state (43.7%) as determined by flow cytometry that is not present in either DLD1 control or TGFb stimulation alone. We do agree that there are different underlying sensitivities to heterogeneous EMT in each cell type, including the robustness of the end response, but they are both competent. We clarify this in a revised sentence added on line 373 p11/S-15:

“ … supporting that MCF10A and DLD1 cells have some cell-type specific EMT sensitivity despite their underlying competency for acquiring a heterogeneous phenotype. This suggests that initial EMT sensitivity of a cell influences downstream functional response from TGF-b and VEGFA stimulation. …”

It is also not clear why the authors believe that their results described here demonstrate that NFATc and phosphorylated Sp1 are critical for regulating E-cadherin and Vimentin expression during phenotype heterogeneity in MCF10A and DLD1.

We believe our data supports this conclusion because of 1) our rigorous computational simulations that predict the existence of the NFAT/p-SP1 bottleneck for EMT, 2) the correlation between EMT fate and NFAT/p-SP1 expression that maps directly to our predictions, and 3) the validation of the essential nature of these signaling partners through the computational and experimental gain and loss of function studies presented. The fact that these predictions were validated in two different cell types (though to somewhat different ultimate degrees) further supports the robustness of our conclusions.

6. 11/S-15, line 362------ It is not clear what cell circularity means and how the cell circularity is assessed.

We thank the reviewer for highlighting this important shortcoming. Circularity refers to the morphology of the cells. In general, a quiescent epithelial cells assumes a circular morphology in culture, while an active mesenchymal cell is highly elongated. Circularity index is a common mode for quantifying cell morphology, which relates cell area to perimeter. A perfect circle = 1.0, while a straight line = 0.0. This index has been a standard feature in image analysis packages such as ImageJ for some time.

Toward this concern, we’ve added the following text to the Results section discussing Ductal branching:

“ … Circularity refers to the morphology of the cells. In general, a quiescent epithelial cells assumes a circular morphology in culture, while an active mesenchymal cell is highly elongated. The circularity index, a common means of quantifying cell morphology, relates cell area to perimeter. A perfect circle has a circularity index equal to 1.0, while a straight line has a circularity index equal to 0.0, see Butcher et al. \cite{Butcher:2004aa}. …”

Butcher JT, Penrod AM, Garc ́ıa AJ, Nerem RM (2004) Unique morphology and focal adhesion development of valvular endothelial cells in static and fluid flow environments. Arterioscler Thromb Vasc Biol 24:1429-34.

7. 11/S-15, line 369-----It is necessary to indicate how the tubulogenesis and tubular branches relative to total acini were assessed.

We thank the reviewer for highlighting this important shortcoming. Toward this concern, we added the following text and references to the material and methods (three dimensional cell culture section):

Tubulogenesis was defined as a typical nonmalignant acini structure.  This includes a polarized epithelial cell, hollow lumen, and the basal sides of the cell are surrounded by ECM proteins (Figure 6-A, Controls or VEGF treated). Previous work has shown that change in the morphological characteristics of nontumorigenic MCF10A epithelial acini occur over time and exploiting them to growth in 3D culture can be quantified. For example, using image segmentation, Chang et al. (2007) examined the elongation of the MCF10A acini at 6, 12, and 96 hours after a particular treatment.  Polizzotti et al. (2012) also suggested a computational method to quantify acini structure based on morphological characteristics in nonmalignant, noninvasive, and invasive conditions.  Adapted from these approaches, we first fluorescently labeled our cultures and captured the acini structures by 3D confocal microscopy.  Next individual acini structures in the images were segmented by imageJ and labeled.  We then extracted the number of ductal branches.  Ductal branching was defined as any elongated cell cluster extending away from the total acini structure, which was manually segmented and counted in imageJ.  A total of 5 images for each condition were used, and approximately 12 acini were analyzed in each image.  Total branching was normalized to the amount of acini present, and provides an overall general assessment to the extent of acini remodeling.

Chang H, Park C, Parvin B (2007) Quantitative representation of three-dimensional cell culture models. In: Proceedings of the 2007 IEEE International Symposium on Biomedical Imaging: From Nano to Macro, Washington, DC, USA, April 12-16, 2007. pp. 89–92. doi:10.1109/ISBI.2007.356795. URL http://dx.doi.org/10.1109/ISBI.2007.356795.

Polizzotti L, Basak O, Bjornsson C, Shubert K, Yener B, et al. (2012) Novel image analysis approach quantifies morphological characteristics of 3d breast culture acini with varying metastatic potentials. J Biomed Biotech 2012: 1-16.

8. 11/S-15, line 370--------Fig. 6A for tubulogenesis for T+V does not supports the description given here. Arrows indicate tubulogenesis? This information should be described in the figure legends.

The bottommost horizontal panel in Figure 6A represents a representative confocal imaging of the region indicated by the arrow, or a representative imaging of the gel in other cases. The box in the control condition gives an example, which wasn’t repeated in the other panels for clarity. We added the following sentence to the legend to clarify:

“ … Boxes in the left-most panel identify regions identified by arrows that were then imaged in greater zoom in the panel immediately below. The box diagram was not repeated for arrows in the other panels for clarity, but the same method was applied. ..”

9. 11/S-15, line 375-----This statement does not appear to be fully supported by the data presented here.

We respectfully affirm that the total of our data supports the overall conclusion statement, and submit that the edits and responses to the preceding comments clarify this for the reviewer. We are not aware of an additional alternative interpretation of our data as clarified.