

The Selective Control of Glycolysis, Gluconeogenesis and Glycogenesis by Temporal Insulin Patterns

Rei Noguchi, Hiroyuki Kubota, Katsuyuki Yugi, Yu Toyoshima, Yasunori Komori, Tomoyoshi Soga, Shinya Kuroda

Corresponding author: Shinya Kuroda, University of Tokyo

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

21 December 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, several concerns on your work, which should be convincingly addressed in a revision of this work.

Of particular concern are the following issue:

- the validity of the conclusion beyond the single cell line used
- the lack of measurements of a marker of glycolysis after pulse stimulation

Reviewer #2 raises additional very important concerns that should be addressed with the required additional analyses.

We would also kindly ask you to submit 'source data' files corresponding to the quantitative figure panels shown in this study (see guidelines at <http://www.nature.com/msb/authors/index.html#a3.4.3>).

Please include also in supplementary information a machine-readable version (SBML format) of your model. We would also ask you to deposit the model to Biocompare and include the accession number in the Materials & Methods section.

REFeree REPORTS:

Reviewer #1 (Remarks to the Author):

In this study the authors model the impact of insulin-induced Akt phosphorylation on glycolysis, glycogenesis and gluconeogenesis. They also compare the effect of a single step gradient, of a 10-minute insulin pulse, and or a ramp increase in insulin concentrations. This modeling is based on the actual measurement of several glucose metabolites, the expression of some enzymes, and also data from a previous study on the modeling of the insulin-Akt phosphorylation pathway.

Whereas the model allows some interesting predictions of the response to insulin pulses or to absolute insulin concentrations, these predictions may be valid for the cell line studied, FAO cells, which is a commonly used model for hepatocytes, but may not really represent the behavior of a normal hepatocyte in vivo. This limitation should be discussed.

One apparent limitation of the model is that it fails to consider glucokinase expression and activity. Glucokinase is highly regulated by insulin at the transcriptional level and its activity is also strongly regulated by interaction with its regulatory protein. These regulations are key determinants of hepatic glucose utilization in hepatocytes. In addition, glucose and G6P are allosteric regulators of glycogen phosphorylase and of glycogen synthase. Thus, to correctly model glucose utilization by insulin the precise, and changing, extracellular glucose concentrations of the culture medium should be considered. These points are not discussed and should be addressed, at least in the Discussion. As the Discussion makes global conclusion on the mode of action of insulin in vivo, these reservations should be mentioned.

Reviewer #2 (Remarks to the Author):

In their manuscript, Noguchi and co-authors measure and model the response of metabolic signaling pathways to various patterns of insulin stimulation. Using their data from step-stimulation of FAO hepatoma cells, they re-parameterize their published mass action model of insulin-dependent glucose signaling, and connect it to a new, simplified model of insulin signaling-dependent glucose metabolism. The new model recapitulates experimental results for step stimulation (used in the parametrization), as well as additional data from cell stimulated with a 10 minute pulses of insulin. By identifying network motifs in the model that fits the experiments, the authors can explain the selective control of glucose metabolism. They find that glycolysis and glycogenesis respond to temporal changes of insulin via ***, whereas gluconeogenesis responds to absolute concentrations of insulin via ***.

This manuscript presents an interesting follow-on to work that the same group recently published (Kubota et al. Mol Cell 2012), by connecting metabolic pathways, instead of signaling events, to the pAKT signals induced by insulin stimulation. This allows them to make inferences about the regulations of the metabolic pathways that were hinted at by their signaling study (Kubota et al., Mol Cell 2012 and accompanying commentary by Purvis and Lahav, Mol. Cell 2012).

Nevertheless, in the current manuscript, the presentation of the results lacks in clarity and this must be addressed to allow readers to better assess the strength of the evidence shown by the authors.

1. Matching 'abstract variables' to measured metabolites. The authors aggregated highly correlated neighboring metabolites into a single 'abstract variable' in the newly constructed model (p. 7-8), yet measure (and represent these variables) as actual measured metabolites (i.e. 'G1P' and 'F16P' in Figs. 2, S2 & S3). More details about how these abstract variables constructed would be helpful (is there a real aggregation process or is the metabolism reduced to a single "proxy" metabolite?). For F16P, experimental and simulated results are compared in Fig. 4B - why is the F16P metabolite compared with an 'abstract variable' in the model? In this case would it make sense to create a simplified model that uses F16P as a representative surrogate for the cluster of correlated metabolites, and compare F16P directly between model and experiment?

2. Model assumptions. In the section entitled 'Computational model of insulin-dependent glucose metabolism', connections between pAKT and various processes (via G1P, F16P and PEPCCK), in addition to the connection between G6P and pGP, are described as 'assumed'. The credibility of the

model and its biological significance would be much improved if these assumptions were better rationalized and supported by literature citations. What is the impact of these assumptions on the results?

3. On page 10, the authors state that F16P is used as a measure of glycolysis, however it was not measured in response to pulse stimulation. Because neither pyruvate nor lactate is measured in response to pulse stimulation, this leaves no data quantifying glycolysis in response to pulsed insulin. Consequently, it seems that important conclusions such as "it can be deduced that a transient pAKT signal is sufficient to induce glycolysis..." and "Although gluconeogenesis and glycogenesis show similar responses to the step and pulse stimulations with insulin, the different temporal patterns between gluconeogenesis and glycogenesis suggest that insulin regulates glycolysis and glycogenesis through distinct mechanisms." (p. 11) are not substantiated by data, only predicted by the model. The authors' conclusion would be significantly strengthened by measurements a marker of glycolysis after step stimulation.

4. On p. 12-3, there is some confusion in the description of the results presented in Figure 4. In addition, these experiments are not well enough sampled in the dose-response to be truly conclusive (only 2 or 3 doses of insulin were used). Specifically, the authors state 1) "GLCex exhibited a sustained response, and the amplitude of GLCex at the sustained phase increased in a dose-dependent manner" when the results in Fig. 4 seem to indicate a dose-dependent decrease; 2) "PEPCK exhibited a rapid increase in response to a low concentration... which indicates the high sensitivity of PEPCK to the concentration of insulin" when again Fig. 4 shows the opposite, PEPCK exhibits a rapid decrease in response to low concentrations of insulin; 3) "GLCex at the final time point increased rapidly...", again, the opposite of what figure 4 shows - GLCex at the final time point decreases in response to insulin. A more condensed description of the results, highlighting similarities and dissimilarities across metabolites may be more helpful.

5. The effects of ramp stimulations were only studied with the model, and no data is presented supporting these model predictions. Either this should be emphasized as predictions still untested or new data should be added to validate the predictions.

6. Overall, Figure 6 summarizes important results, these should be better emphasized in the write-up of the results which too often reads like a list of observations.

Other issues:

1. Figure 1: the graph for G6P appears disjointed, is data missing?
2. Figure 1. Glycogen detected in response to 100 nM (red) is missing
3. Results where concentrations of metabolite are unchanged can also be important and should not necessarily be relegated to the supplements (Supp. Fig 1).
4. Overall Fig. 1 and 2 are nice representations of the modeling and experimental results; it would be helpful if Fig 2 was a closer mimic of Fig. 1 in its structure (even if it would then occupy a bit more space).
5. P.7 "This contradictory finding may be due to the different experimental conditions used", here the authors should briefly describe what those differences are.
6. P. 8 "slightly modified the parameters to fit the experimental data" - a more detailed description of how the authors made decisions about which parameters to change and how sensitive the outputs are to changing these parameters is important - is there a unique fit, or would many different parameter sets do equally well at reproducing the data. This could be included in the main text or in at least in the more detailed methods description.
7. Figure 2 and supplementary figure 3. It is not clear why inhibitory interactions of the block diagram are depicted as an arrowhead with a '-' symbol at the end. The more standard 'bar-headed line' is used to denote inhibition (A--|B) elsewhere in the paper (e.g., Figure 6). Standardized notation (e.g. from CADlive or Kitano et al. Nature biotechnology 2005 for standard notations) should be used throughout.
8. Does pAKT suppress PEPCK or induce its expression? Compare model description in the materials and methods with page 8. Figure S2E, reaction v11 suggests that increased pAKT levels reduce mRNA production of PEPCK.

9. Figure 3 refers to 'Fold change' of Glycogen. Figures 5 & 6 refer to 'Difference of fold change': What is 'difference of fold change'?
10. Fig. 4 - should also include the trends for the early time point GLCex and PEPCK dose-response data (even if it is not influenced by dose)- contrast between dose-dependence and dose-independence is important.
11. Fig. 4a & 5: Units of insulin are missing in axis title
12. p13.: EC50s are provided for GLCex, PEPCK, FP16 and glycogen. Citations or methods are required.
13. Arguably the 3-node network of pAKT, PEPCK and F16P (pAKT--|PEPCK, pAKT F16P and PEPCK F16P) form an Incoherent Type 4 motif with substrate depletion (Mangan and Alon, PNAS 2002) which might more accurately describe the glycolysis time profile.
14. The glycogenesis pathway is governed by an Incoherent *Type 1* motif.
15. P. 14 "the feedforward loop that consists of feedforward activation via the ... and feedforward inhibition via..." - it is clearer to state that the feedforward loop consists of "activation" and "inhibition", rather than "feedforward activation" and "feedforward inhibition". The "feedforward" requires 2 pathways.
16. Figure 6 might be improved by including separate diagrams of the 'simplified' network motifs governing glycolysis, glycogenesis and gluconeogenesis.
17. P. 18 , please define "integral effects".
18. P. 18, The statement "the different EC50 and time constants of the protein and mRNA regulation are responsible for the different responses observed between PEPCK..." would be better supported by simulated response curves to changes in these values in the model (or a sensitivity analysis).
19. P.23 - the generation of the G6Pase and pGP(Ser14) antibodies should be described in greater details.
20. P.18, please reference the Kubota et al Mol. Cell paper for the upstream pAkt signaling pathway model.
21. There are some typos and awkward phrasings to be corrected (e.g. p2 "Insulin selectively governs... However how insulin-signalling pathway selectively..." removing the first "selectively" makes the logic of argument clearer; p7: "We developed thus..." should read "We developed this...", and " p.10 "PEPCK pathways prevented the decrease" perhaps could be replaced by "reduced the decrease") and several redundancies in the text. The manuscript would benefit from careful editing.
22. Overall, figure referencing should be enhanced to help point the readers to important data, (e.g. Supplementary Figure 3c is discussed in the main text but not cited.)

1st Revision - authors' response

09 January 2013

The reviewers' remarks are underlined and numbered, followed by our responses.

The editor comments.

Of particular concern are the following issue:

- the validity of the conclusion beyond the single cell line used
- the lack of measurements of a marker of glycolysis after pulse stimulation

Reviewer #2 raises additional very important concerns that should be addressed with the required additional analyses.

Our response: Thank you for giving us an opportunity for the revision. According to the reviewers' suggestions, we have thoroughly revised the manuscript except the lack of measurements of a marker of glycolysis, F16P, after pulse stimulation.

Since no quantitative convenient enzymatic assay for the detection of F16P is available, we should measure F16P by capillary electrophoresis coupled to time-of-flight mass spectrometry (CE-TOF-MS). We estimate that it will take more than 3 months to construct the measurement system and perform the validation experiments of F16P. Instead of the measurements of F16P, we additionally performed a ramp stimulation experiment and validated the computational prediction (Figure 5). Importantly, this validation experiment strengthened our conclusion of selective control of glucose metabolism by temporal patterns of insulin (see our response to the comment 2-5 by the reviewer #2 in detail). We think that this is the key finding in our study and more important than

measuring F16P. We toned down the finding of F16P as a prediction that has to be validated by experiment in the future.

We believe that the current results are sufficient to support our conclusion. However, if needed, we will be willing to measure F16P after pulse stimulation. We would appreciate it if the editor would ask reviewer #2 if necessary.

Reviewer #1 (Remarks to the Author):

In this study the authors model the impact of insulin-induced Akt phosphorylation on glycolysis, glycogenesis and gluconeogenesis. They also compare the effect of a single step gradient, of a 10-minute insulin pulse, and or a ramp increase in insulin concentrations. This modeling is based on the actual measurement of several glucose metabolites, the expression of some enzymes, and also data from a previous study on the modeling of the insulin-Akt phosphorylation pathway.

Our response: We thank the reviewer for reading and understanding of our study and for the encouraging remarks.

1-1) Whereas the model allows some interesting predictions of the response to insulin pulses or to absolute insulin concentrations, these predictions may be valid for the cell line studied, FAO cells, which is a commonly used model for hepatocytes, but may not really represent the behavior of a normal hepatocyte in vivo. This limitation should be discussed.

Our response: We thank the reviewer for pointing out the limitation of our study. As the reviewer pointed out, some cellular responses may be different between FAO hepatoma cells and primary hepatocytes, and others may be similar between them. For example, we found that the expression of glucokinase in FAO cells is much lower than that in primary hepatocytes, and that signalling activities of pAKT, pS6K and GSK3 β and expression of G6pase are similar responses between FAO cells and primary hepatocytes (Kubota et al., *Mol. Cell*, 2012). According to the suggestion, we discussed the limitation of our study based on the above facts in Discussion in detail (p.22, lines 5 to 17). Moreover, we plan to launch the similar project in primary hepatocytes as a separate study, and examine the similarity and dissimilarity in metabolic responses between FAO cells and primary hepatocytes.

1-2) One apparent limitation of the model is that it fails to consider glucokinase expression and activity. Glucokinase is highly regulated by insulin at the transcriptional level and its activity is also strongly regulated by interaction with its regulatory protein. These regulations are key determinants of hepatic glucose utilization in hepatocytes. In addition, glucose and G6P are allosteric regulators of glycogen phosphorylase and of glycogen synthase. Thus, to correctly model glucose utilization by insulin the precise, and changing, extracellular glucose concentrations of the culture medium should be considered. These points are not discussed and should be addressed, at least in the Discussion. As the Discussion makes global conclusion on the mode of action of insulin in vivo, these reservations should be mentioned.

Our response: As the reviewer pointed out, glucokinase expression and activity is critical for regulation of glucose metabolism by insulin. We found that the protein abundance of glucokinase was not changed by insulin in FAO cells (see the below figure). In addition, glucokinase activity in FAO cells has been reported to be much lower than that in primary hepatocytes (Argaud et al, 1997), and, therefore, glucokinase was not incorporated into the current model. However, as the reviewer pointed out, given that glucokinase plays a key role in regulating glucose metabolism *in vivo*, we will incorporate the glucokinase in the hepatocyte model as a separate study. We described the dissimilarity of glucokinase expression between FAO cells and primary hepatocytes and the limitation of our study in Discussion in detail (p.22, lines 5 to 17).

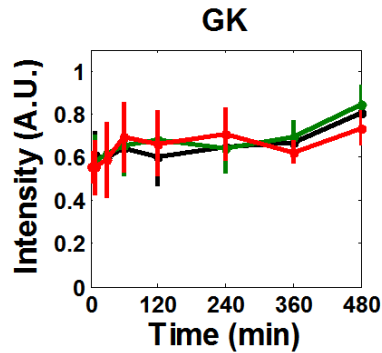


Figure The responses of glucokinase to insulin step stimulation. Protein abundance of glucokinase in response to insulin was measured by western blotting. The mean values and SEMs of four independent experiments are shown. The black, green and red lines indicate the various insulin concentrations used (0, 1 and 100 nM, respectively).

(response continued) As the reviewer pointed out, glycogen phosphorylase (GP), and glycogen synthase (GS) is allosterically regulated by glucose and G6P. For GP, we implicitly incorporated the allosteric effect of glucose and G6P on GP by lumping the allosteric regulations by glucose and G6P together as “allosteric regulation of pGP by G6P” in the model (Supplementary Figure 2). For GS, the allosteric activation of GS by G6P has been reported to be mediated by the facilitation of dephosphorylation (Villar-Palasi & Guinovart, 1997); however, we did not find the dephosphorylation of GS at Ser641, which is an important phosphorylation site for the regulation of glycogenesis (Skurat & Roach, 1995). This suggests that the allosteric regulation of GS does not play a major role in FAO cells. However, given that the allosteric regulation of GS plays a key role in regulating glycogen *in vivo*, we will incorporate the allosteric regulation of GS in the hepatocyte model as a separate study.

Reviewer #2 (Remarks to the Author):

In their manuscript, Noguchi and co-authors measure and model the response of metabolic signaling pathways to various patterns of insulin stimulation. Using their data from step-stimulation of FAO hepatoma cells, they re-parameterize their published mass action model of insulin-dependent glucose signaling, and connect it to a new, simplified model of insulin signaling-dependent glucose metabolism. The new model recapitulates experimental results for step stimulation (used in the parametrization), as well as additional data from cell stimulated with a 10 minute pulses of insulin. By identifying network motifs in the model that fits the experiments, the authors can explain the selective control of glucose metabolism. They find that glycolysis and glycogenesis respond to temporal changes of insulin via ***, whereas gluconeogenesis responds to absolute concentrations of insulin via ***.

This manuscript presents an interesting follow-on to work that the same group recently published (Kubota et al. Mol Cell 2012), by connecting metabolic pathways, instead of signaling events, to the pAKT signals induced by insulin stimulation. This allows them to make inferences about the regulations of the metabolic pathways that were hinted at by their signaling study (Kubota et al., Mol Cell 2012 and accompanying commentary by Purvis and Lahav, Mol. Cell 2012). Nevertheless, in the current manuscript, the presentation of the results lacks in clarity and this must be addressed to allow readers to better assess the strength of the evidence shown by the authors.

Our response: We greatly appreciate the reviewer for careful reading and deep understanding of our manuscript, for appropriate comments that significantly strengthen our conclusions and for information of network motif. We perfectly agree with the reviewer’s suggestions, and have changed the entire manuscript accordingly. We also cited the reference by Purvis and Lahav, Mol. Cell 2012, which elegantly highlights the physiological roles and mechanical insight of a series of our insulin study.

2-1) Matching 'abstract variables' to measured metabolites. The authors aggregated highly correlated neighboring metabolites into a single 'abstract variable' in the newly constructed model (p. 7-8), yet

measure (and represent these variables) as actual measured metabolites (i.e. 'G1P' and 'F16P' in Figs. 2, S2 & S3). More details about how these abstract variables constructed would be helpful (is there a real aggregation process or is the metabolism reduced to a single "proxy" metabolite?). For F16P, experimental and simulated results are compared in Fig. 4B - why is the F16P metabolite compared with an 'abstract variable' in the model? In this case would it make sense to create a simplified model that uses F16P as a representative surrogate for the cluster of correlated metabolites, and compare F16P directly between model and experiment?

Our response: The reviewer pointed out the critical issue of the comparison of the abstracted variable in the model to F16P in experiment. We used F16P as a representative surrogate for the cluster of correlated metabolites to reproduce the temporal patterns of F16P and correlated metabolites together, because, among the correlated metabolites, F16P has been reported to be directly regulated by insulin through PFKFB1 and is thought to be a key factor in the glycolysis regulation by insulin. We explained how these abstract variables are constructed in detail (p.9, lines 3 to7).

Therefore, the pathways in the model such as connection to the AKT pathway do not directly correspond to the real biochemical pathway. Because our aim is to explore how specific temporal patterns of insulin are selectively decoded by temporal patterns of the metabolites, we compared the experimental data of actual F16P with the simulated data of F16P in abstracted model in terms of temporal patterns. Therefore, the absolute concentration of F16P can not be directly compared between the experimental and simulated data. We described this issue in the Materials and Method.

2-2) Model assumptions. In the section entitled 'Computational model of insulin-dependent glucose metabolism', connections between pAKT and various processes (via G1P, F16P and PEPCK), in addition to the connection between G6P and pGP, are described as 'assumed'. The credibility of the model and its biological significance would be much improved if these assumptions were better rationalized and supported by literature citations. What is the impact of these assumptions on the results?

Our response: The connection of pAKT to PEPCK and that of G6P to pGP have previously been reported. We cited these references (Aiston et al, 2003; Nakae et al, 1999; Sutherland et al, 1996). Note that the equations in the abstracted model implicitly involve these multiple steps of biochemical equations. Although the regulation of F16P via PFKFB1 by insulin has also been reported (Probst & Unthan-Fechner, 1985), it remains unclear what kind of signalling molecule regulates PFKFB1. Therefore, we assumed that insulin regulates PFKFB1 via pAKT, which has been shown to play a major role in insulin signalling in liver (Whiteman et al, 2002). We assumed that G1P is regulated by pAKT, because the transient response of G1P could not be reproduced without the regulation of G1P via pAKT. This is the prediction of a missing pathway. We also examined the impact of these pathways in Supplementary Figure 2.

2-3) On page 10, the authors state that F16P is used as a measure of glycolysis, however it was not measured in response to pulse stimulation. Because neither pyruvate nor lactate is measured in response to pulse stimulation, this leaves no data quantifying glycolysis in response to pulsed insulin. Consequently, it seems that important conclusions such as "it can be deduced that a transient pAKT signal is sufficient to induce glycolysis..." and "Although gluconeogenesis and glycogenesis show similar responses to the step and pulse stimulations with insulin, the different temporal patterns between gluconeogenesis and glycogenesis suggest that insulin regulates glycolysis and glycogenesis through distinct mechanisms." (p. 11) are not substantiated by data, only predicted by the model. The authors' conclusion would be significantly strengthened by measurements a marker of glycolysis after step stimulation.

Our response: As mentioned in the original version, the availability of CE-TOF-MS was limited and no quantitative enzymatic assay is available for the detection of F16P, we did not experimentally measure F16P in response to pulse insulin stimulation. Since it will take more than 3 months to construct the measurement system and perform the validation experiments of F16P to the pulse stimulation by use of CE-TOF-MS, we do not have enough time to meet the deadline for the revision. Therefore, we toned down the conclusion about F16P and glycolysis by describing the result as "prediction" in Figure 6 and Table I. Moreover, we are presently trying to measure the response of glycolysis to insulin in primary hepatocytes, and are planning to report the results as a separate study, which will support the prediction of the current study.

Instead of the measurements of F16P, we additionally performed a ramp stimulation experiment and validated the computational prediction. Importantly, this validation experiment strengthened our conclusion of selective control of glucose metabolism by temporal insulin patterns. We think that this is the key finding in our study and more important than measuring F16P.

We believe that the current results are sufficient to support our conclusion. However, if the reviewer thinks it necessary to measure F16P after pulse stimulation, we will be willing to do so.

2-4) On p. 12-3, there is some confusion in the description of the results presented in Figure 4. In addition, these experiments are not well enough sampled in the dose-response to be truly conclusive (only 2 or 3 doses of insulin were used). Specifically, the authors state 1) "GLC_{ex} exhibited a sustained response, and the amplitude of GLC_{ex} at the sustained phase increased in a dose-dependent manner" when the results in Fig. 4 seem to indicate a dose-dependent decrease; 2) "PEPCK exhibited a rapid increase in response to a low concentration... which indicates the high sensitivity of PEPCK to the concentration of insulin" when again Fig. 4 shows the opposite, PEPCK exhibits a rapid decrease in response to low concentrations of insulin; 3) "GLC_{ex} at the final time point increased rapidly...", again, the opposite of what figure 4 shows - GLC_{ex} at the final time point decreases in response to insulin. A more condensed description of the results, highlighting similarities and dissimilarities across metabolites may be more helpful.

Our response: As the reviewer pointed out, the description of increase of both GLC_{ex} and PEPCK were wrong. In addition, as the reviewer suggested, we used more doses of insulin, and incorporated Foxo1, an upstream regulator of PEPCK, to reproduce the dose response. We made a correction of the description and added the new experimental result, and highlighted similarities and dissimilarities across metabolites. We appreciate the reviewer for careful reading.

2-5) The effects of ramp stimulations were only studied with the model, and no data is presented supporting these model predictions. Either this should be emphasized as predictions still untested or new data should be added to validate the predictions.

Our response: According to the suggestion, we performed the ramp stimulation in FAO cells and validated the prediction (p.13, line 8 to p.14, line 13, Figure 5). As predicted, PEPCK, a marker of gluconeogenesis, responded to ramp stimulation, whereas glycogen, a marker of glycogenesis, did not respond to ramp stimulation. GLC_{ex} also responded to ramp stimulation. Importantly, this experimental result highlights the selective control of gluconeogenesis, glycogenesis and GLC_{ex} by ramp stimulation. Thus, these experimental results further verified our key conclusion of selective control of glucose metabolism by temporal patterns of insulin. We think that this result supports our main conclusion of temporal coding of metabolic regulation by insulin.

2-6) Overall, Figure 6 summarizes important results, these should be better emphasized in the write-up of the results which too often reads like a list of observations.

Our response: According to the suggestion, we emphasized the result in Figure 6 and Table I by adding more detailed description (p.17, line 14 to p.18, line 2).

Other issues:

1. Figure 1: the graph for G6P appears disjointed, is data missing?

Our response: The data are not missing. We did not detect the metabolites in some points. We described this in legend.

2. Figure 1. Glycogen detected in response to 100 nM (red) is missing

Our response: We did not show the result because of the large variation and low reliability of the data (p.24, lines 17 to 19 and p.39, lines 12 to 14)

3. Results where concentrations of metabolite are unchanged can also be important and should not necessarily be relegated to the supplements (Supp. Fig 1).

Our response: According to the suggestion, we inserted Supplementary Figure 1 into Figure 1.

4. Overall Fig. 1 and 2 are nice representations of the modeling and experimental results; it would be helpful if Fig 2 was a closer mimic of Fig. 1 in its structure (even if it would then occupy a bit more space).

Our response: According to the suggestion, we inserted the experimental results in Figure 2 so that readers can directly compare the experimental data and simulation results.

5. P.7 "This contradictory finding may be due to the different experimental conditions used", here the authors should briefly describe what those differences are.

Our response: There are many differences in experimental conditions between the previous study and our study such as different cell lines (primary hepatocytes and FAO cells), different extracellular glucose (high glucose and low glucose) and other nutrients in the media, and so on. Because of too many possible reasons, describing these differences is not informative. Therefore, we deleted the sentences.

6. P. 8 "slightly modified the parameters to fit the experimental data" - a more detailed description of how the authors made decisions about which parameters to change and how sensitive the outputs are to changing these parameters is important - is there a unique fit, or would many different parameter sets do equally well at reproducing the data. This could be included in the main text or in at least in the more detailed methods description.

Our response: We needed to modify the parameters because we obtained the data at higher insulin doses (0 to 100 nM of insulin) in the current study than those (0 to 1 nM of insulin) in the previous study. Therefore, we re-estimated the parameters of the insulin-dependent AKT pathway model to reproduce the time course data of pAKT in Figure 1 and Figure 3. After 200 independent estimations for the model were made, we selected the model that had the minimum residual error between the experimental data and simulated data. Among the best five models, we also confirmed that the model had similar characteristics to the insulin-dependent AKT pathway in terms of the time courses and dose responses. According to the reviewer's suggestion, we included these detailed description in the Materials and Methods.

7. Figure 2 and supplementary figure 3. It is not clear why inhibitory interactions of the block diagram are depicted as an arrowhead with a '-' symbol at the end. The more standard 'bar-headed line' is used to denote inhibition (A--|B) elsewhere in the paper (e.g., Figure 6). Standardized notation (e.g. from CADlive or Kitano et al. Nature biotechnology 2005 for standard notations) should be used throughout.

Our response: According to the suggestion, we used standard notation throughout the text.

8. Does pAKT suppress PEPCK or induce its expression? Compare model description in the materials and methods with page 8. Figure S2E, reaction v11 suggests that increased pAKT levels reduce mRNA production of PEPCK.

Our response: pAKT suppress PEPCK expression, which indicated by (-) in Figure 2.

9. Figure 3 refers to 'Fold change' of Glycogen. Figures 5 & 6 refer to 'Difference of fold change': What is 'difference of fold change'?

Our response: "Fold change" in Figure 3 and "Difference of fold change" in Figure 5 & 6 have the same meaning. We used "Fold change" throughout the text.

10. Fig. 4 - should also include the trends for the early time point GLC_{ex} and PEPCK dose-response data (even if it is not influenced by dose)- contrast between dose-dependence and dose-independence is important.

Our response: We included dose-response of GLC_{ex} and PEPCK at early time point in Figure 4B.

11. Fig. 4a & 5: Units of insulin are missing in axis title

Our response: The units (nM) were included in Figure 4A and Figure 5.

12. p13.: EC50s are provided for GLCex, PEPCK, FP16 and glycogen. Citations or methods are required.

Our response: The EC₅₀s were determined by the simulated results in Figure 4. We described how we determined the EC₅₀s in Figure 4.

13. Arguably the 3-node network of pAKT, PEPCK and F16P (pAKT--|PEPCK, pAKT∠F16P and PEPCK∠F16P) form an Incoherent Type 4 motif with substrate depletion (Mangan and Alon, PNAS 2002) which might more accurately describe the glycolysis time profile.

14. The glycogenesis pathway is governed by an Incoherent *Type 1* motif.

15. P. 14 "the feedforward loop that consists of feedforward activation via the ... and feedforward inhibition via..." - it is clearer to state that the feedforward loop consists of "activation" and "inhibition", rather than "feedforward activation" and "feedforward inhibition". The "feedforward" requires 2 pathways.

Our response: We thank the reviewer's instruction for networks motif. We changed the description of the network motif accordingly.

16. Figure 6 might be improved by including separate diagrams of the 'simplified' network motifs governing glycolysis, glycogenesis and gluconeogenesis.

Our response: According to the suggestion, we included the simplified network motifs for glycolysis, glycogenesis and gluconeogenesis.

17. P. 18 , please define "integral effects".

Our response: We changed the integral effects to easier expression; "irreversible effect by the large time constant" (p.21, line 7 and line 10).

18. P. 18, The statement "the different EC50 and time constants of the protein and mRNA regulation are responsible for the different responses observed between PEPCK..." would be better supported by simulated response curves to changes in these values in the model (or a sensitivity analysis).

Our response: We described the EC₅₀s and time constants of PEPCK and *G6Pase*, and compared the results of PEPCK with that of *G6Pase* (Supplementary Figure 5).

19. P.23 - the generation of the G6Pase and pGP(Ser14) antibodies should be described in greater details.

Our response: We described the generation of the antibodies in Materials and Methods in detail.

20. P.18, please reference the Kubota et al Mol. Cell paper for the upstream pAkt signaling pathway model.

Our response: We cited the reference.

21. There are some typos and awkward phrasings to be corrected (e.g. p2 "Insulin selectively governs... However how insulin-signalling pathway selectively..." removing the first "selectively" makes the logic of argument clearer; p7: "We developed thus..." should read "We developed this..." and " p.10 "PEPCK pathways prevented the decrease" perhaps could be replaced by "reduced the decrease") and several redundancies in the text. The manuscript would benefit from careful editing.

Our response: We thank the reviewer for careful reading. We corrected typos accordingly.

22. Overall, figure referencing should be enhanced to help point the readers to important data, (e.g. Supplementary Figure 3c is discussed in the main text but not cited.)

Our response: We referred Supplementary Figure 3C (currently Supplementary Figure 2C) in the main text (p.11, line 3). We also referred figures in the text.

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2nd Editorial Decision

18 March 2013

Thank you again for submitting your revised work to Molecular Systems Biology. I apologize again for the delay in getting back to you. We have now finally heard back from the two referees who accepted to evaluate your revision. As you will see, the referees are supportive but reviewer #2 still raises a number of concerns that we would kindly ask you to address in a minor revision. The points refer to the need of further clarifications in the text and the recommendation provided by reviewer #2 are clear in this regard.

Thank you for submitting this paper to Molecular Systems Biology.

REFeree REPORTS:

Reviewer #1 (Remarks to the Author):

This revised version of the manuscript addresses my few remarks by adding in the Discussion the importance of comparing FAO cells with primary hepatocytes that have different regulatory mechanisms.

Reviewer #2 (Remarks to the Author):

The manuscript by Noguchi et al. has improved in the first revision, in particular, the added analysis and data with ramp stimulation goes far in bolstering model predictions and the manuscript's impact.

However, there are still several issues, mostly regarding the write-up of the study, that, when addressed, will greatly improve clarity and value of the manuscript to its readers.

At a general level, the flow and clarity of the manuscript still suffers from a 'reporting lists of facts' writing style. The authors should make a concerted effort to avoid lengthy lists of results, and instead discuss similar results grouped together and highlight differences (ex. p. 11, top. Most of what is described there seems superfluous or its importance to the main arguments of the paper were lost in the lengthy description; p. 12, bottom, again a list; also, p. 15-16, section: "Sensitivity of glycolysis, gluconeogenesis and glycogenesis to the concentration of insulin"; Here an additional thought: Generally speaking, the response of glycogenesis and glycolysis is the same even though they are regulated by different motifs. Does this suggest that an I4-FFL with 'substrate depletion' is similar to an I1-FFL?).

Overall, discussing how the implications of these data fit within the framework of the network and insulin responses, with particular attention to flow and clarity (throughout), will raise the readability of the manuscript to the high standards expected from articles published in MSB.

Of particular importance is the discussion section, the discussion is poorly written and doesn't really 'close the circle' on what the abstract promises. The authors should avoid just restating results (the first paragraph of the discussion is rather redundant with the last paragraph of the results), should better highlight what are the **new** insights from their study and how they fit in the greater context of what was known before. In particular:

In stating, "To extract the essential mechanism by which insulin selectively controls glucose metabolism". The authors don't emphasize that different network motifs detect different signals. In this regard, regulatory sub-network motifs have properties that explain why each pathway responds to particular features of insulin stimulation. Of potential interest to readers is how these network motifs converge with switch/pulse/ramp signaling regimes. The discussion could perhaps be improved by elaborating how these motifs function in the response to insulin: are these motifs known to detect temporal change vs absolute levels? Are these motifs known to produce adaptive responses? Do these motifs suggest any other details about how information propagates in this system?

The discussion paragraph that starts with: 'We previously found that G6Pase, which...' was difficult to make sense of. The 'irreversible effect by the large time constant' should be defined or explained. The discussion paragraph that starts with: "In a previous study, we found that..." is also difficult to read and needs to be re-written to clarify its logic.

The data for F16P that had been requested in prior review (measurement of F16P after pulse stimulation) may not be required (as stated above, the measurements in the context of ramp stimulation already strengthen the manuscript). However, for the statement -- "Taken together, these results demonstrate that glycolysis and glycogenesis respond to temporal changes of insulin concentration, whereas gluconeogenesis and extracellular glucose concentrations respond to the absolute insulin concentration but not to its temporal changes" -- as F16P is a surrogate for glycolysis, and the response of F16P to pulse or ramp stimulation is not quantified, the wording 'demonstrate that glycolysis...' is too strong; where is it demonstrated that F16P responds to temporal changes in insulin concentration? Perhaps wording it as a suggestion i.e. "These results suggest..." would be more appropriate.

p. 13. In discussing transient vs. sustained pAKT signal, shouldn't some thought also be given to the **amplitude** of this signal which is also very different across conditions?

Overall, better care should be taken in highlighting what are **simulation** vs. **experimental** results.

Other issues and suggestions:

Figures 1 and 2. Instead of repeating all the experimental results in a figure structured like the results of the simulations, why not structure Figure 2A just like Figure 1 and replace graphs or nodes with simulation results, (and replace nodes that were not simulated with just the node names).

p. 3. The statement that starts with "Hepatic glucose metabolism is..." seems entirely redundant and unnecessary.

- p. 7. When first introducing the FAO cells, please add a brief description of what they are.
- p. 7. "... were also enzymatically measured..." should be rephrased as "... were also measured, using an enzymatic assay..." (as the other measurements they are compared to were not done enzymatically!
- p.12. Instead of stating "we did not experimentally measure F16P further in this study.", why not instead write "we were only able to characterize glycolysis in simulations."
- p. 12. "We found that pAKT exhibited only a transient..." please refer to figure.

Fig 4. Assuming $n = 1$ and PEPCK data and model are not in agreement... Still good that the experiment was done and the results generally make sense. Of note: why did they only ramp up to 0.1 nM whereas the other experiments used 1 or 10 nM...

Figure 5B: X-axis legend is missing.

Caption 5B "EC50s of glycogen at the early time point, F16P at the early time point, PEPCK at the final time point and GLCex at the final time point are shown." The caption does not distinguish that the EC50s were determined from simulated data as opposed to experimental data.

Table 1: The 'network motifs' column of Table 1 contain grey boxes with network motifs with arrow diagrams drawn in. These diagrams are incorrect and unnecessary where they are drawn. Perhaps instead draw attention to supplemental fig 4.

Figure S4: This figure should only highlight the I1-FFL and the I4-FFL that participate in the insulin-dependent glucose metabolism network. There is no need for the other 2 motifs as they are not part of the system under study. Also, it would make sense to actually name the nodes of the motif (instead of x, y and z) i.e.

I4-FFL: pAKT--> Glycolysis; pAKT --| PEPCK -->Glycolysis
 I1-FFL: pAKT--> pGP --| Glycogen; pAKT --> Glycogen

Finally, for the I1-FFL the pAKT --> pGP connection is more of a 'black box' that signals through multiple components.

2nd Revision - authors' response

26 March 2013

The reviewers' remarks are underlined and numbered, followed by our responses.
The editor comments.

Thank you again for submitting your revised work to Molecular Systems Biology. I apologize again for the delay in getting back to you. We have now finally heard back from the two referees who accepted to evaluate your revision. As you will see, the referees are supportive but reviewer #2 still raises a number of concerns that we would kindly ask you to address in a minor revision. The points refer to the need of further clarifications in the text and the recommendation provided by reviewer #2 are clear in this regard.

We would also be grateful if you could send us high resolution production-quality files for the figures.

Our response: Thank you for giving us an opportunity for the revision again. According to the reviewers #2's suggestions, we carefully removed redundant description of the results and summarized the related results and interpretation. We believe that the readability of our manuscript is now greatly improved. Moreover, we have sent you higher resolution production-quality files for the figures.

Reviewer #1 (Remarks to the Author):

This revised version of the manuscript addresses my few remarks by adding in the Discussion the importance of comparing FAO cells with primary hepatocytes that have different regulatory mechanisms.

Our response: We thank the reviewer for reading again and for the acceptance of the revision.

Reviewer #2 (Remarks to the Author):

The manuscript by Noguchi et al. has improved in the first revision, in particular, the added analysis and data with ramp stimulation goes far in bolstering model predictions and the manuscript's impact. However, there are still several issues, mostly regarding the write-up of the study, that, when addressed, will greatly improve clarity and value of the manuscript to its readers.

Our response: We thank the reviewer for careful reading and understanding of the impact of the ramp stimulation experiment.

2-1) At a general level, the flow and clarity of the manuscript still suffers from a 'reporting lists of facts' writing style. The authors should make a concerted effort to avoid lengthy lists of results, and instead discuss similar results grouped together and highlight differences (ex. p. 11, top. Most of what is described there seems superfluous or its importance to the main arguments of the paper were lost in the lengthy description; p. 12, bottom, again a list; also, p. 15-16, section: "Sensitivity of glycolysis, gluconeogenesis and glycogenesis to the concentration of insulin"; Here an additional thought: Generally speaking, the response of glycogenesis and glycolysis is the same even though they are regulated by different motifs. Does this suggest that an I4-FFL with 'substrate depletion' is similar to an I1-FFL?).

Our response: According to the reviewer's suggestion, we removed the redundant description of the results (p.11, p.12, and p.15-p.16 in the old version), and grouped them together as the new section in Discussion in the revised version (p.16 and p.17, lines 1 to 13).

As the reviewer pointed out, glycolysis and glycogenesis showed the similar responsiveness to step, pulse and ramp stimulation of insulin despite their different network motifs. We described this in the main text (p.17, lines 7 to 9). The network motif of F16P is a feedforward with substrate depletion rather than an I4-FFL with substrate depletion as the reviewer mentioned. This is because the transient response of F16P is mainly generated by an increase via feedforward by pAKT and a decrease due to substrate depletion, rather than the negative regulation via PEPCK (Supplementary Figure 2C).

2-2) Overall, discussing how the implications of these data fit within the framework of the network and insulin responses, with particular attention to flow and clarity (throughout), will raise the readability of the manuscript to the high standards expected from articles published in MSB.

Of particular importance is the discussion section, the discussion is poorly written and doesn't really 'close the circle' on what the abstract promises. The authors should avoid just restating results (the first paragraph of the discussion is rather redundant with the last paragraph of the results), should better highlight what are the *new* insights from their study and how they fit in the greater context of what was known before. In particular:

In stating, "To extract the essential mechanism by which insulin selectively controls glucose metabolism". The authors don't emphasize that different network motifs detect different signals. In this regard, regulatory sub-network motifs have properties that explain why each pathway responds to particular features of insulin stimulation. Of potential interest to readers is how these network motifs converge with switch/pulse/ramp signaling regimes. The discussion could perhaps be improved by elaborating how these motifs function in the response to insulin: are these motifs known to detect temporal change vs absolute levels? Are these motifs known to produce adaptive responses? Do these motifs suggest any other details about how information propagates in this system?

Our response: We greatly appreciate the reviewer for the instruction. According to the reviewer's suggestion, we summarised new insights from our study in the discussion section (p.19, lines 2 to 13). Moreover, we deleted the first paragraph of the discussion section to remove the redundancy,

and instead, we moved the description about Figure 6 and Table I into Discussion to summarise the motif function for temporal change and absolute level, and the related other detailed information (p.16 and p.17, lines 1 to 13).

2-3) The discussion paragraph that starts with: 'We previously found that G6Pase, which...' was difficult to make sense of. The 'irreversible effect by the large time constant' should be defined or explained.

Our response: We deleted "the irreversible effect by the large time constant" and instead used a simpler explanation (p.16, line 7).

2-4) The discussion paragraph that starts with: "In a previous study, we found that..." is also difficult to read and needs to be re-written to clarify its logic.

Our response: According to the reviewer's suggestion, we rewrote the description and clarified the logic (p.19, lines 8 to 13).

2-5) The data for F16P that had been requested in prior review (measurement of F16P after pulse stimulation) may not be required (as stated above, the measurements in the context of ramp stimulation already strengthen the manuscript). However, for the statement -- "Taken together, these results demonstrate that glycolysis and glycogenesis respond to temporal changes of insulin concentration, whereas gluconeogenesis and extracellular glucose concentrations respond to the absolute insulin concentration but not to its temporal changes" -- as F16P is a surrogate for glycolysis, and the response of F16P to pulse or ramp stimulation is not quantified, the wording 'demonstrate that glycolysis...' is too strong; where is it demonstrated that F16P responds to temporal changes in insulin concentration? Perhaps wording it as a suggestion i.e. "These results suggest..." would be more appropriate.

Our response: We changed the description to "these results suggest..." as the reviewer suggested (p.14, line 1).

2-6) p. 13. In discussing transient vs. sustained pAKT signal, shouldn't some thought also be given to the *amplitude* of this signal which is also very different across conditions?

Our response: We previously found that temporal change and absolute concentration of insulin are encoded into the transient response and sustained response of pAKT, respectively (Kubota et al., 2012). Therefore, in terms of responsiveness to the temporal patterns of insulin, we focused on the temporal patterns of pAKT rather than the amplitude of pAKT in itself.

2-7) Overall, better care should be taken in highlighting what are *simulation* vs. *experimental* results.

Our response: We clearly described which data are simulated results or experimental results throughout the text.

Other issues and suggestions:

2-8) Figures 1 and 2. Instead of repeating all the experimental results in a figure structured like the results of the simulations, why not structure Figure 2A just like Figure 1 and replace graphs or nodes with simulation results, (and replace nodes that were not simulated with just the node names).

Our response: Figure 1 indicates the detailed biochemical network of metabolic map, and Figure 2A indicates abstracted metabolic pathways in the model. Figures 1 and 2A can not be directly compared. Therefore, we do not merge Figures 1 and 2 to avoid confusion. However, if the reviewer would think that Figures 1 and 2 are redundant, we will remove Figure 2B.

2-9) p. 3. The statement that starts with "Hepatic glucose metabolism is..." seems entirely redundant and unnecessary.

Our response: According to the suggestion, we deleted the statement.

2-10) p. 7. When first introducing the FAO cells, please add a brief description of what they are.

Our response: We introduced FAO cells in the text (p.5, lines 15 to 17).

2-11) p. 7. "... were also enzymatically measured..." should be rephrased as "... were also measured, using an enzymatic assay..." (as the other measurements they are compared to were not done enzymatically!

Our response: We thank the reviewer's instruction for the description. We changed the sentence accordingly (p.7, line 8).

2-12) p.12. Instead of stating "we did not experimentally measure F16P further in this study.", why not instead write "we were only able to characterize glycolysis in simulations."

Our response: We thank the reviewer's instruction for the description. We changed the sentence accordingly (p.11, line 13).

2-13) p. 12. "We found that pAKT exhibited only a transient..." please refer to figure.

Our response: According to the suggestion, we added the reference to Figure 3A in the text (p.11, line 15).

2-14) Fig 4. Assuming n = 1 and PEPCK data and model are not in agreement... Still good that the experiment was done and the results generally make sense. Of note: why did they only ramp up to 0.1 nM whereas the other experiments used 1 or 10 nM...

Our response: According to the suggestion, we added additional experimental data of the ramp experiment, and showed the mean values and SEMs of three independent experiments in Figure 4. As we described in the legend of Figure 4, the reason why we used 0.1 nM of insulin for the ramp experiment is because it directly reflects the basal secretion of insulin *in vivo*, which is secreted at a lower concentration than the additional secretion of insulin.

2-15) Figure 5B: X-axis legend is missing.

Our response: We thank the reviewer for careful reading. We added X-axis legend in Figure 5B.

2-16) Caption 5B "EC50s of glycogen at the early time point, F16P at the early time point, PEPCK at the final time point and GLCex at the final time point are shown." The caption does not distinguish that the EC50s were determined from simulated data as opposed to experimental data.

Our response: The EC₅₀s were determined from the simulated data. We described it in the legend of Figure 5B.

2-17) Table 1: The 'network motifs' column of Table 1 contain grey boxes with network motifs with arrow diagrams drawn in. These diagrams are incorrect and unnecessary where they are drawn. Perhaps instead draw attention to supplemental fig 4.

Our response: According to the suggestion, we deleted the grey boxes with network motifs from Table I, and instead, added a reference to Supplementary Figure 4.

2-18) Figure S4: This figure should only highlight the I1-FFL and the I4-FFL that participate in the insulin-dependent glucose metabolism network. There is no need for the other 2 motifs as they are not part of the system under study. Also, it would make sense to actually name the nodes of the motif (instead of x, y and z) i.e.

I4-FFL: pAKT--> Glycolysis; pAKT --| PEPCK -->Glycolysis

I1-FFL: pAKT--> pGP --| Glycogen; pAKT --> Glycogen

Our response: According to the suggestion, we deleted the other two motifs from Supplementary Figure 4, and changed the node names to the actual molecule names.

2-19) Finally, for the I1-FFL the pAKT --> pGP connection is more of a 'black box' that signals through multiple components.

Our response: We added a statement that the pAKT --> pGP connection actually consists of multiple components in the legend of Figure 6.