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## Impaired tissue regeneration corresponds with altered expression of developmental genes that persists in the metabolic memory state of diabetic zebrafish

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### Abstract

As previously reported by our lab streptozocin-induced Diabetes mellitus (DM) in adult zebrafish results in an impairment of tissue regeneration as monitored by caudal fin regeneration. Following streptozocin withdrawal, a recovery phase occurs to re-establish euglycemia, via pancreatic beta-cell regeneration. However, DM-associated impaired fin regeneration continues indefinitely in the metabolic memory state (MM) allowing for subsequent molecular analysis of the underlying mechanisms of MM. This study focuses on elucidating the molecular basis that explains the DM-associated impaired fin regeneration and why it persists into the MM state with the aim of better understanding MM. Using a combination of microarray analysis and bioinformatics approaches our study found that of the 14,900 transcripts analyzed, aberrant expression of 71 genes relating to tissue developmental and regeneration processes were identified in DM fish and the altered expression of these 71 genes persisted in MM fish. Key regulatory genes of major development, and signal transduction pathways were identified among this group of 71. The aberrant expression of key regulatory genes in the DM state that persist into the MM state provides a plausible explanation for how hyperglycemia induced impaired fin regeneration in the adult zebrafish DM/MM model.

### Keywords

diabetes; metabolic memory; regeneration; zebrafish; epigenetics

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### Conflict of Interest Statement, and Contributions to study:

No potential conflicts of interest relevant to this article were reported. A.O. researched data. M.P.S. Jr. contributed to discussion of the data, writing and editing the manuscript. A.A.L. conducted the bioinformatics data analysis and editing the manuscript, and R.V.I. researched data, discussed the data, contributed to writing and editing the manuscript. As indicated on the Title page, M.P.S. Jr. and A.A.L. contributed equally to this study and are considered Co-First Authors.

## INTRODUCTION

Increased blood glucose levels in patients with diabetes mellitus lead to complications including cardiovascular disease, retinopathy, nephropathy, neuropathy, and decreased wound healing. Our laboratory has previously reported that an adult zebrafish model of type I diabetes mellitus can be induced by administration of the diabetogenic drug, streptozocin (STZ) and in this diabetic state (DM) there is an impairment of caudal fin regeneration and skin wound healing (1,2). Additionally, in these studies, we showed that withdrawal of STZ results in regeneration of pancreatic beta cells and return of these previously diabetic fish to a euglycemic state within 2 weeks; however, in contrast, fin regeneration and skin wound healing impairment is permanently observed even in this normal glycemic state (2). In regard to the latter point, several large scale diabetes mellitus clinical trials suggest that once initiated, diabetic complications persist and continue to progress unimpeded even when glycemic control is achieved through pharmaceutical intervention (3–6). This persistence has also been supported by multiple lines of experimental laboratory evidence (2,7–12) and collectively indicate that the initial hyperglycemic period results in permanent abnormalities in the target organs. This harmful phenomenon has been termed metabolic memory (MM) (13,14) and its underlying molecular mechanism(s) remain unknown.

The zebrafish offers advantages over other diabetes mellitus model organisms in that it is an epimorphic species which allows for the study of processes in a background of diabetic and metabolic memory states (1,2). Using this model, it is possible to analyze gene expression patterns within a wide variety of tissues as the fish transverse through the normal, DM, and MM states; thus allowing us to further study important regulatory systems underlying both diabetes and metabolic memory.

In the current study, we focused on the molecular mechanisms that could explain why tissue regeneration (as monitored by caudal fin regeneration) was impaired in acute DM fish and why this impairment continued into the MM state after euglycemia had returned the fish to a normal physiological homeostasis in regards to blood glucose. To accomplish this aim, we have analyzed gene expression changes at four time points of fin regeneration using microarray technology. There are a number of possibilities to explain why fin regeneration is impaired in the DM state and why this impairment continues in the MM state. In this study we studied the hypothesis that “Fin regeneration impairment in the DM and MM state occurs because genes underlying this impairment are similarly affected in both states”. We then applied bioinformatics analysis to our microarray data to test this hypothesis. This approach has allowed us to provide insight into alterations of regulatory gene expression patterns that might explain the persistence of impaired tissue regeneration (i.e. fin regeneration) that occurs in the DM state and continues into the MM state of adult zebrafish.

## METHODS

### **Zebrafish Husbandry, STZ injection, Fasting Blood Glucose Determination, and Fin Regeneration Methods**

The maintenance of zebrafish stocks (*Danio rerio*), the induction of hyperglycemia, blood glucose determinations, and fin regeneration methodology were performed as previously described (1). The fish used in these studies were approximately 4–7 months of age. All procedures were performed following the guidelines described in “Principles of Laboratory Animal Care” (NIH publication no. 85-23, revised 1985) and the approved Rosalind Franklin University IACUC animal protocols 08–19 and B11–16.

## RNA Extraction

Caudal fin samples were obtained from intact and regenerate fin tissue for acute diabetic fish (DM), 60 day metabolic memory fish (MM), and their appropriate controls (CDM) and (CMM). For clarification purposes, 1) CDM fish represents normal adult zebrafish vehicle injected for the diabetic group; 2) DM represents zebrafish that have been induced into an acute diabetic state (for a three week period) using STZ; 3) CMM represents normal adult zebrafish vehicle injected and maintained/manipulated in parallel with the MM group; and 4) MM represents zebrafish that were initiated and were maintained in a diabetic state for the three week period, but returned to a euglycemic state following withdrawal of STZ (referred to as MM fish throughout the article). At 30 days post drug removal, the caudal fins were amputated to eliminate any residual effects within the fin tissue from the hyperglycemic period as previously described (2). After an additional 30 day growth phase, the fish were considered to be the MM group. In all cases, RNA was extracted at multiple time points from the fin regenerate tissue and utilized for microarray analysis. Time 0 ( $T_0$ ) refers to intact fins prior to fin amputation initiating the regeneration process. Regenerate caudal fin tissue was obtained from time 12, 24, and 48 hrs ( $T_{12}$ ,  $T_{24}$ , and  $T_{48}$  hpa) post amputation fish for all four groups. For each time point, triplicate samples of 15 caudal fins, from DM, MM fish, and their appropriate controls were collected and immediately placed into Trizol (Life Technologies Corporation, Carlsbad, CA). This method of triplicate samples from 15 fins statistically strengthens the subsequent microarray data to a high confidence level. The samples were homogenized with 20 strokes of both A and B pestles using a 2 ml dounce homogenizer (Fisher Scientific, Pittsburgh, PA). The debris was pelleted by centrifugation in a microcentrifuge at maximum speed. The supernatant was then collected and the RNA was extracted via the Purelink RNA mini-kit (Life Technologies Corporation, Carlsbad, CA) without exception. The final RNA concentration was determined by an absorbance reading at 260 nm.

## Microarray Analysis

Each of the triplicate samples generated above for each condition were independently used to probe the previously established Affymetrix GeneChip® Zebrafish Genome Array which contains 15,509 probe sets designed to interrogate expression of 14,900 *Danio rerio* transcripts. As such, for each time point 12 arrays were utilized, 3 for DM control fish, 3 for DM fish, 3 for MM control fish and 3 for MM fish. This was done for each time point and therefore a total for 48 array experiments were performed. Microarray analysis was conducted according to manufacturer's instructions for the Affymetrix 3' IVT Express kit. Briefly, RNA quality was assessed by the Agilent Bioanalyzer. Reverse transcription and second strand cDNA synthesis was performed from 100 ng of good quality total RNA. Subsequently, the products were column-purified (Affymetrix) and then *in vitro* transcribed to generate biotin-labeled cRNA. The IVT products were next column-purified, fragmented, and hybridized onto Affymetrix GeneChip® Zebrafish Genome Arrays at 45° C for 16 h. Subsequent to hybridization the arrays were washed and stained with streptavidin-phycoerythrin, then scanned in an Affymetrix GeneChip® Scanner 3000. All control parameters were confirmed to be within normal ranges before normalization and data reduction was initiated. Partek® GS 6.5 software was used for microarray data analysis. The raw data (.CEL files) was processed with the GCRMA algorithm and differentially expressed genes were identified using ANOVA test. A gene was considered as differentially expressed if expression fold change between two groups was equal or greater than 1.5. The data from this microarray experiment, was deposited to NCBI GEO data base under accession number GSE37165.

## Quantitative reverse transcription PCR

RNA samples were used to generate cDNA using the superscript III first strand synthesis kit (Life Technologies Corporation, Carlsbad, CA). Gene expression was analyzed using the cDNA as template for real-time RT-PCR analysis employing the SYBR green system based on real-time detection of fluorescence accumulation under the manufacturers' recommended cycle conditions. (Life Technologies Corporation, Carlsbad, CA). During preliminary foundation studies it was determined that the 3 reference genes *EF1alpha*, *Rpl13alpha* and *18S*rRNA were suitable for our samples. Additionally, the oligonucleotides pairs (supplemental table 3) used in these reactions were designed and tested to ensure that the amplification efficiency was approximately 2 (ie a doubling of product in each cycle) (data not shown). The  $\Delta\Delta C_t$  method (15) was employed to determine the relative expression difference in the experimental samples as compared to controls. Each experiment was performed on three different samples with three replicates of each for a total of nine reactions per sample. The statistical significance of the values obtained was examined by p value determination as described previously for the  $\Delta\Delta C_t$  method (16).

## Gene Enrichment Analysis

Gene enrichment analysis of Gene Ontology was performed using BinGo (The Biological Network Gene Ontology Tool) (17) plug-in for the Cytoscape 2.8 (18) software program. Cytoscape was used to visualize the results of this analysis, namely represent relations between functional groups, significance of their enrichment by the genes which were differentially expressed in our experiments and compare with enrichment in Schebesta et. al (19). Pairwise comparison of experimental and control groups was performed using t-test. The Gene Ontology annotation (Revision 1.385, Submission Date 3/19/2012) of the *Danio rerio* genome build ZV9 (Jul 2010) was used for this analysis.

## RESULTS

### Comparison of microarray data to previous reports

The microarray data presented in this study is an extension of studies initially described in our previous manuscript on metabolic memory in diabetic zebrafish (2). The previous study only analyzed diabetic (DM) and metabolic memory (MM) fish at the  $T_0$  time frame (non-regenerating) of caudal fin or skin tissue. The current study focuses on the regenerating caudal fin and examined four time points to include:  $T_0$ ,  $T_{12}$ ,  $T_{24}$ , and  $T_{48}$  hours post caudal fin regeneration in normal, diabetic, and metabolic memory zebrafish. This approach was employed to reveal why fin regeneration is impaired in the DM state and why this impairment continues in the MM state when the fish return to normal serum glucose levels following pancreatic beta-cell regeneration. It should be noted that for each time point, triplicate samples from 15 caudal fins were obtained which statistically strengthens the microarray data to a high confidence level. To confirm the validity of our methodology, we have compared our data from the control groups (CDM and CMM) at each of the time points ( $T_0$  –  $T_{48}$  hours post fin amputation [hpa]) to previously published studies on normal fin regeneration by Schebesta et. al (19). Our experimental design paralleled that of Schebesta et al and specifically utilized the same Affymetrix GeneChip® Zebrafish Genome Array so that our microarray data and biological results could be viewed in the context of their previously published studies. Exact expression fold changes are not expected to be reproduced due to variations in technical details of experimental practices from different laboratories as well as biological variations between experimental groups of fish in different labs. Therefore, instead of using correlation measures such as correlation coefficient or Pearson correlation coefficient, we measured the proportion of genes which changed concordantly in the same direction (up or down) in our experiment and those reported by Schebesta et al. (19). This analysis demonstrated that our data correlates well with

concordance percentages of 85%, 92%, and 98%, respectively for T<sub>12</sub>, T<sub>24</sub>, and T<sub>48</sub> hpa. This confirms the validity of our procedures and microarray data based on Schebesta et al. (19).

### **Gene enrichment analysis of genes altered in the DM and MM states relative to their corresponding controls**

We approached our bioinformatics analysis with the premise that to account for the occurrence of impaired fin regeneration in the DM state and the persistence of this impairment into the MM state, then genes underlying this impairment had to be similarly affected in both states. In other words, if a gene was up-regulated in the DM state relative to controls, then it should also be up-regulated in the MM state and the converse would hold for down-regulated genes. We applied a 1.5 fold cut-off (only) as the threshold required for altered expression of a gene to be considered significant relative to controls, and first determined all genes from the microarray data that were altered relative to controls in the DM and MM states (Table 1 DM and MM columns. From this analysis we then determined those genes that were common to both states. As shown in the last column of Table 1, we identified 173, 416, 165, and 94 transcripts that were common between the DM and MM states for time points T<sub>0</sub>, T<sub>12</sub>, T<sub>24</sub>, and T<sub>48</sub> hpa, respectively.). In order to validate both the microarray technique and the biological process the changes documented through the microarray analysis were confirmed via a quantitative reverse transcriptase pcr approach for a subset of genes at each time point (supplemental table 4). Since some of the genes were identified at more than one time point, this analysis identified 753 genes in total. We then performed a Gene Ontology analysis of these genes to elucidate a network of functional processes (genes falling into particular canonical biological processes) that were important to fin regeneration common to the DM and MM states. Applying CYTOSCAPE software we created a network based on the gene enrichment analysis of these genes (Figure 1) Central to this study was the gene enrichment analysis of the Gene Ontology domain “Biological process” (BP, Figure 1) which identified three subtrees of overrepresented functional categories that include: 1) Developmental and Regeneration Process 2) Metabolic Process and 3) the Regulation of Metabolic Process. The smallest p-value represented on the map of enriched biological processes is 0.1. This p-value is derived from the hypergeometric test performed during BiNGO analysis. This rather relaxed cut-off was accepted in order to keep coherence of the map where significantly enriched functions may be connected via less significantly (p-value=0.1) enriched functions. The shade of the node represents significance of enrichment (darkness of shade is in proportion to the group enrichment p-value; a scale bar is shown in each figure as a reference standard)

Because developmental and regeneration processes are central to impaired fin regeneration, the current study focused exclusively on this subtree and our analysis indicated that 71 genes were aberrantly expressed (relative to controls) in the DM and MM states (see supplemental Table 1 for a complete list). In order to establish potential functional interrelationships, we performed a gene enrichment analysis and generated the hierarchical tree seen as Figure 2. This diagrammatic representation not only confirms the functional importance of the 71 genes we identified but also illustrates the complexity of gene pathways to be dissected in future studies.

### **Cluster analysis of those Development/Regeneration genes common to the DM and MM states for time points, T<sub>0</sub>, T<sub>12</sub>, T<sub>24</sub>, and T<sub>48</sub> hpa of fin regeneration**

Hierarchical cluster analysis was performed using Partek Genomic Suite software. The aim of this analysis was only to cluster genes according to similarity of their expression in experimental groups. Therefore, supervised cluster analysis was applied. Clustering of the above identified 71 genes, revealed groups of genes that were consistently up-regulated or



down-regulated for each of the four time points (Figure 3). In most cases, the trend indicated that expression in the DM state was greater (either positive or negative relative to controls) than that observed for the MM state; likely reflecting the constant state of insult from the hyperglycemia existing in the DM state. For example, *Bambi* (a BMP blocking regulator) was up-regulated 8.6 fold in the DM state and up-regulated 1.7 fold in the MM state (Figure 2, 48h time point group); while *sox3* (a transcription factor related to cell fate determination during normal fin regeneration in zebrafish) was down-regulated -4.0 fold in the DM state and was down-regulated -1.6 fold in the MM state (Figure 3, 0hr time point group). In this analysis we observed a gene expression range of from +49.4 to -4.2 in the fold change differentials.

## DISCUSSION

We have taken advantage of the adult zebrafish Type I diabetic model in which we can monitor gene expression patterns in zebrafish during the transition from the normal, diabetic, and metabolic states; in order to gain insight as to why the impaired fin regeneration initiated in the DM state persists in the MM state. Through microarray analysis we analyzed the specific gene products that could be correlated to the impairment observed in caudal fin regeneration. Using this approach we have determined which general functional processes and specific genes are altered in the diabetic and metabolic states using bioinformatics programs and public zebrafish genome annotations ([www.geneontology.org](http://www.geneontology.org)). We hypothesized that “the impairment of fin regeneration in the DM and MM state results as a consequence of genes underlying this impairment being similarly affected in both states genes and we focused on this group in this study”.

We first verified our technical approach and microarray data by comparing our data to that previously reported by Schebesta et al. (19) who developed the first microarray data set for normal caudal fin regeneration in adult zebrafish. Our data correlated well with that of Schebesta et al (19); thereby establishing the validity of our experimental and microarray techniques.

Applying additional bioinformatics analysis we then determined 1) those genes whose aberrant expression relative to controls were common for the DM and MM states (either consistently up-regulated or consistently down-regulated in both states), 2) the global network of functional categories for genes common to DM and MM, 3) the hierarchy of functional processes within the Developmental and Regeneration subtree of the global network (composed of three subtrees), and 4) the expression patterns for the 71 genes of the Developmental and Regeneration subtree that were found to be common for the DM and MM states. The following discussion will expand on these four points.

We selected transcripts whose expression was altered (up- or down) at least 1.5 fold relative to control samples and changed in the same direction (up- or down) both in DM and MM states. Of the 14,900 zebrafish transcripts we screened by microarray analysis, 753 transcripts (5.05%) satisfied these criteria and the transcript number varied between the time points as shown in Table 1. Central to this study was the gene enrichment analysis of the Gene Ontology domain “Biological process” which identified “Development and Regeneration Process” as one of the three significantly enriched subtrees comprising the entire network of the selected domain as shown in Figure 1. Obviously, genes related to development and regeneration are critical to understanding why fin regeneration is impaired in the DM and MM states. With this in mind, we determined the 1) relevance of genes comprising the Development and Regeneration subtree and 2) the potential functional inter-relationship of these genes by constructing a hierarchical tree of functional processes (Figure 2).

We expected to shed light on the underlying mechanisms that account for the impaired fin regeneration by determining the time points at which the 71 genes from the Developmental and Regeneration Process subtree were altered in their expression pattern. This analysis revealed that the timing of altered expression was not clustered to a particular time point but rather was distributed throughout the time course examined (18 genes at T<sub>0</sub>, 30 genes at T<sub>12</sub> hrs, 27 genes at the T<sub>24</sub> hrs, and 20 genes at T<sub>48</sub> hrs). As is shown in Figure 3 (T<sub>0</sub> column) the classes of genes aberrantly expressed at T<sub>0</sub> include: 1) developmental transcription factors (e.g. *sox3*, *emx2*, *dlx4a*, etc), 2) DNA modification-related genes (*dnmt1*, *apex1*, *mcm2*, *uhrf1*, etc.), and 3) stress response/wound healing genes (*hsp70*, *hspd1*, *mmp13*, etc). Our previous observation that hyperglycemia induces DNA-hypomethylation which persists in the MM state (2) correlates well with the expression of DNA modification-related proteins reported here. The persistent expression of stress response genes is also consistent with that which has been reported within the in vitro studies of others (9,12–14). The expression of the developmental transcription factors is not as obvious and may relate to repair signal transduction pathways induced by the continued state of hyperglycemic insult. Further studies are under way to better understand how intact fin tissue is responding to the DM/MM states at Time 0.

If we analyze each of the three time points of fin regeneration studied, we find mechanisms that may explain why regeneration is impaired in the DM and MM states. At the earliest phase of fin regeneration that we assayed (T<sub>12</sub>), we see thirty genes either up or down regulated (Figure 2, 12 hrs column). Some of these genes overlap with those aberrantly expressed at the T<sub>0</sub> time point (*ets1a*, *mmp13*, and *inhbaa*), but most are unique to T<sub>12</sub>. These genes are predominately 1) developmental-related transcription factors such as homeobox genes, 2) signal transduction molecules such as inhibin (*inhbaa*), EGF-like protein (*hbegf*), and *jag1b* of the Notch pathway, and 3) ECM related genes such as *mmp13*, *vcanb*, and *chst11* which can also function in signal transduction pathways (20). As reported by Jazwinska et al (21) the inductive influence of growth factors such as activin are essential for blastema formation and therefore high expression levels (34 fold in DM and 1.7 fold in MM) of an inhibitor such as inhibin might be expected to have a deleterious effect on fin regeneration (22). There are numerous other aberrantly expressed genes that could contribute to the impairment in regeneration observed (e.g. *hbegf*, *jag1b*, and *mmp13*, etc.).

At T<sub>24</sub> hpa we see 27 genes either up or down regulated and these genes fall into similar functional groups as observed with the T<sub>12</sub> time point (Figure 2, 24 hrs column). Here we observe both a wnt ligand (*wnt2*) and a canonical wnt receptor (*fzd9*) altered, which together, may amplify their aberrant expression effects on the regenerative process. Additionally, at this time point a connective tissue growth factor (*ctgf*) that has not previously been implicated in fin regeneration is down-regulated (−1.8 in DM and −1.7 in MM). The *ctgf* gene product is reported to promote endothelial growth, migration, adhesion, and survival; thus implicated in endothelial cell function and angiogenesis (23), both critical to fin regeneration. This is occurring in parallel with the up-regulation of *FGF10a* that is known to be a stimulatory growth factor (24) but has not been previously reported to play a role in fin regeneration.

At the final time point (T<sub>48</sub> hrs) the same classes of genes are affected that were observed in the T<sub>12</sub> and T<sub>24</sub> hrs time points with 20 genes being either up or down regulated (Figure 2, 48 hrs column). Again, in regard to impaired fin regeneration; we find that the *BMP* inhibitor *bambi* is up-regulated at this time point (8.6 fold in DM and 1.7 fold in MM) (25). Because of the importance of *bmp* as a positive factor in fin regeneration at the 48 hrs time point (25) the inhibition of *bmp* activity by *bambi* could significantly contribute to the impaired fin regeneration that is observed in the DM and MM states. The same is true for the expression of the *notch* inhibitor lunatic fringe (*lfng*) (26), also seen to be up-regulated at

this time point. Again, there are numerous other genes whose aberrant expression could contribute to the impairment observed (e.g. *ctfg*, *igfbp3*, *fgf10a*, etc.).

It should be noted that the magnitude of altered gene expression is almost always significantly greater in the DM state than in the MM state (average fold change: 7.0 (DM) versus 2.0 (MM) for up-regulated genes and 2.4 (DM) versus 1.9 (MM) for down-regulated genes) but the altered expression in the MM state is still sufficient to vary normal signaling pathways. We attribute this reduction in magnitude to the fact that fish are in a constant state of hyperglycemia in the DM state while this insult is completely absent in the MM state because fish have regenerated their beta-cells and have returned to a euglycemic state with glucose levels maintained at normal physiological levels (2). Therefore our findings indicate that, for each time point there are a wide variety of altered signaling events that can be proposed to explain impaired fin regeneration. Given the complexity of these events it is unlikely that correcting one gene's or even multiple gene's expression dysfunction at any one or multiple time points would restore normal fin regeneration. We have considered these experiments, but as the laboratory that developed the technique for morpholino injection into fins for analysis of this regenerative process we feel that such an approach is futile at this time (27)

In our previous manuscript, we were able to correlate the induction and persistence of aberrant gene expression with hyperglycemia induced DNA hypomethylation for a subset of genes. Here we were able to identify CpG islands for 61 of the 71 genes described above and found that seven of these had at least a two-fold decrease in methylation status within their promoter regions (data not shown). We are now investigating this correlation further.

Finally, it is reported that the pathways and genes responsible for regeneration in zebrafish are common across a wide spectrum of tissue types including the fin (a connective tissue structure), retina (a CNS structure) (28) and heart (a muscle structure) (29). This becomes important from a regenerative medicine perspective as it allows us to potentially extrapolate the findings in our study to the more basic process of zebrafish tissue regeneration in general. In fact, 16 of the genes identified here (Fig 3) are common to the regenerative processes in the retina and/or heart. Additionally our model allows us to analyze molecular aspects of the DM and MM state in ways that cannot be experimentally approached with traditional diabetic models. Most relevant here is that our MM zebrafish represent a population akin to other organisms that have received pancreas transplant or beta-islet cell therapy. In support of this, our results parallel that which was reported when patients with type I diabetes mellitus resulting in end stage renal disease were compared to patients that were previously a part of this patient population but received a double pancreas and kidney transplant (30). In that study, although gene expression remained aberrant in transplant patients (similar to our metabolic memory group) the magnitude of the change was reduced when compared to the patients with end-stage renal disease similar to what we observed. Therefore the combination of our study and above clinical study suggests that restoration of physiologically normal glycemic control may not rescue altered target tissues from the changes induced by diabetes mellitus and thus may have implications for the post-transplant environment.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



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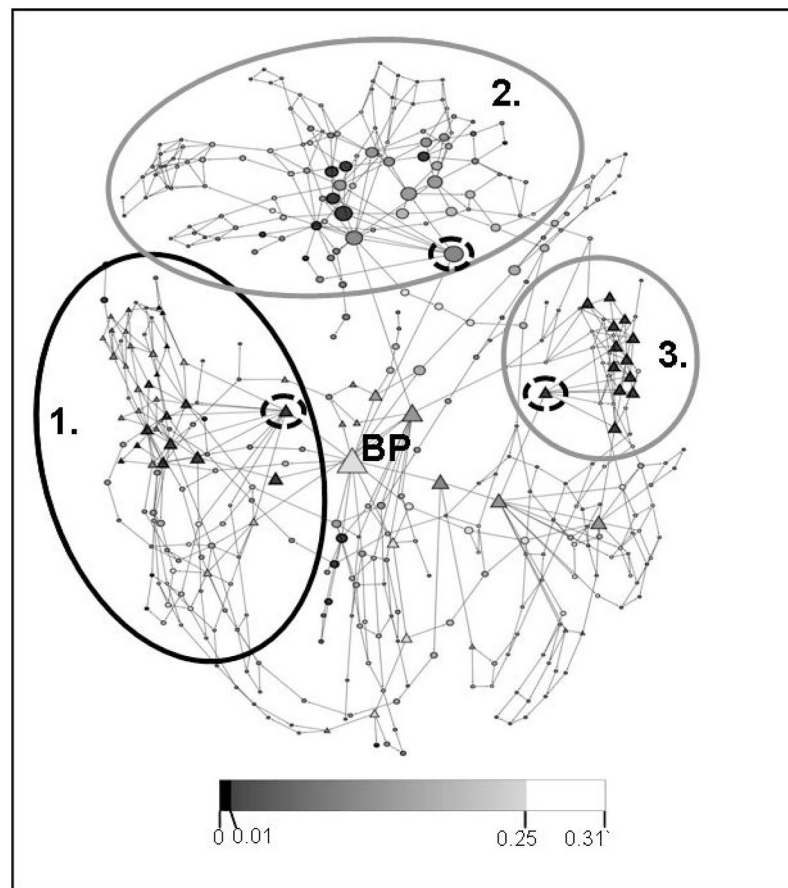
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**Figure 1.**

A network of the Gene Ontology categories overrepresented in a set of 753 genes similarly over-or under expressed at 0h, 12h, 24h and 48 h post amputation in DM and MM states. Overrepresented groups were identified using BiNGO software with Bengamini- Hochberg corrected p- value cut-off of 0.2. The analysis was done on GO domain “Biological process” (BP). The map was constructed with Cytoscape software using the result of BiNGO analysis. The nodes are shaded in proportion to the group enrichment p-value (indicated by scale bar at the bottom) and sized in proportion to the number of genes in the group. Triangles represent functional categories enriched by the genes identified in the Schebesta et al.(15) as well as in this study. Circled are the three major subtrees (1. Developmental and Regenerative Processes, 2. Metabolic Processes, and 3. Control of Metabolic Processes) comprising the whole network and the central node of each sub tree is circled with a dashed line.

Figure 2A

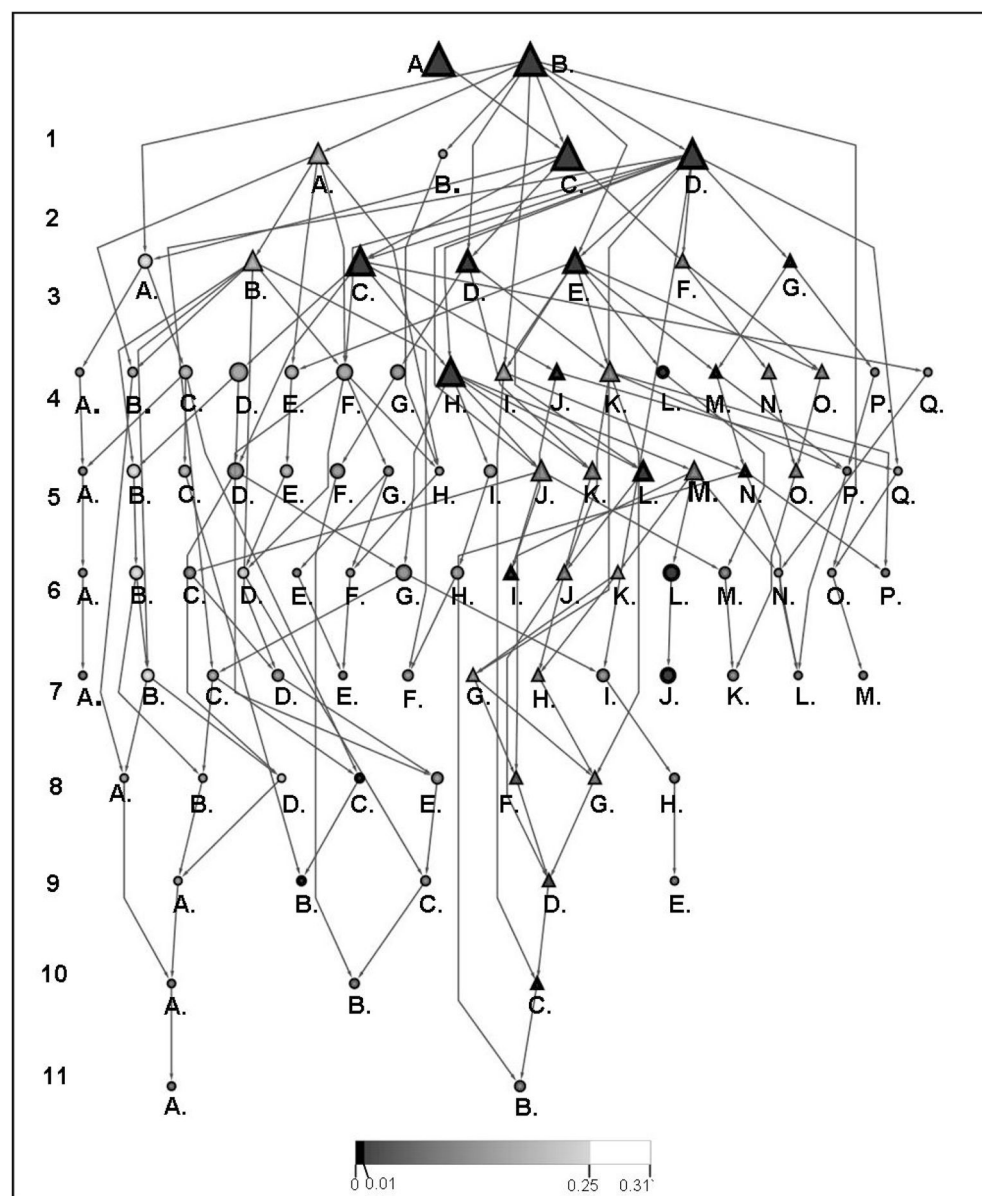
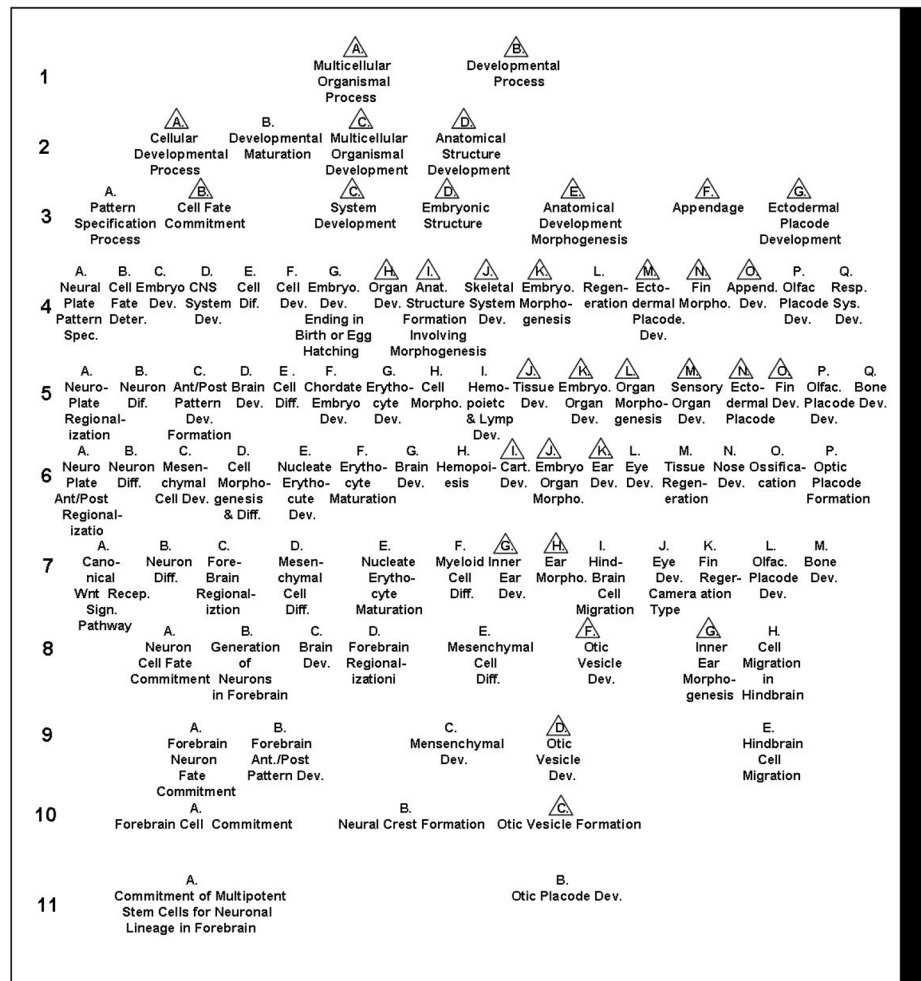
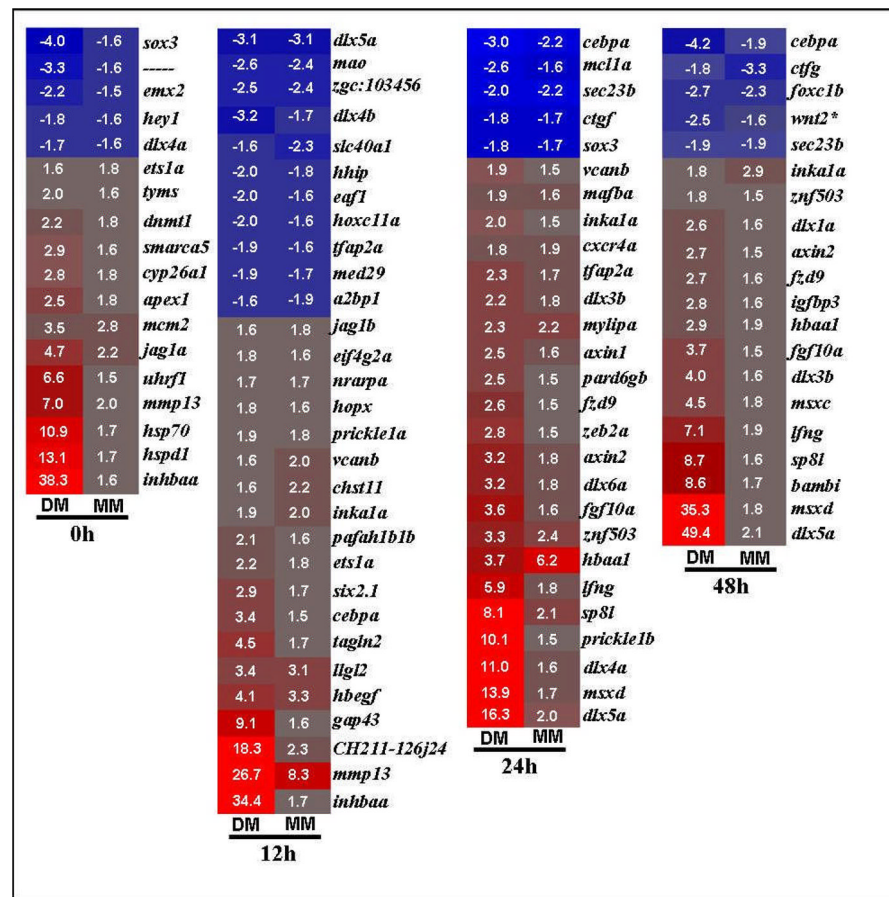


Figure 2B

**Figure 2.**

Hierarchy of functional categories overrepresented by gene whose expression was affected at 0h, 12h, 24h or 48 h post amputation in DM and MM conditions. A) Hierarchical map of the Gene Ontology categories overrepresented in a set of 753 genes similarly over or under expressed at 0h, 12h, 24h and 48 h post amputation in DM and MM states. The nodes are colored in proportion to the group enrichment p-value (indicated by scale bar at the bottom) and sized in proportion to the number of genes in the group. Triangles represent functional categories enriched by the genes identified in the Schebesta et al.(15) as well as in this study. B) Gene Ontology terms corresponding to the groups shown in the map, A.



**Figure 3.**

Cluster analysis was performed for each time point of the 71 genes identified in this study. The time points of 0h, 12h, 24h and 48 h post amputation are indicated at the bottom of each heat map along with the sample designation (DM or MM). The blue color indicates a relative decrease in expression and the red indicates a relative increase. Additionally, printed on each heat map is the value of the fold change. The symbol for each gene is indicated to the right of each heat map.

**Table 1**

The number of altered transcripts at each time point for DM & MM conditions

Time	DM	MM	Common to Both
0	4,856	428	173
12	5,216	2,163	416
24	3,076	532	165
48	3,048	690	94

The number of transcripts altered by at least 1.5 fold between DM and control or MM and control were determined prior to amputation (0), 12 hours post amputation (h.p.a.) (12) 24 h.p.a., and 48 h.p.a. The two identified genes were compared and the number of transcripts common to both groups were determined and are shown in the last column.