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Use of Zebrafish as a model to investigate the role of epigenetics in propagating the secondary complications observed in Diabetes mellitus★

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Abstract

Diabetes mellitus (DM) is classified as a disease of metabolic dysregulation predicted to affect over 400 million individuals world-wide by 2030. The debilitating aspects of this disease are the long term complications involving microvascular and macrovascular pathologies. These long term complications are related to the clinical phenomenon of metabolic memory (MM) that is defined as the persistence of diabetic complications even after glycemic control has been pharmacologically achieved. The persistent nature of MM has invoked involvement of epigenetic processes. Current research with the DM/MM zebrafish model as described in this review as well as human and mammalian studies has established that changes in DNA methylation patterns appear to contribute to tissue dysfunctions associated with DM. This review will describe studies on an adult zebrafish model of type I diabetes mellitus that allows analysis of both the hyperglycemic (HG or DM) phase and MM phase of the disease. The review will discuss the model in regards to: 1) its hyperglycemic phase, 2) its MM phase, 3) biochemical pathways underlying changes in DNA methylation patterns observed in the model, 4) loci specific changes

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in DNA methylation patterns, and 5) strengths of the adult zebrafish model as compared to other MM animal models.

Keywords

diabetes; metabolic memory; epigenetics; zebrafish

Introduction

This review will focus on how DNA methylation changes contribute to the long term secondary complications of diabetes mellitus (DM) using an adult zebrafish model of type I DM that is uniquely qualified for this type of epigenetic analysis. In this regard, diabetes mellitus is classified as a disease of metabolic dysregulation that results in reduced life expectancy due to disease specific microvascular (retinopathy, nephropathy, neuropathy, impaired wound healing) and macrovascular (heart disease and stroke) complications (Brownlee, M., 2005). A unifying mechanism for the induction of complications due to hyperglycemia has been proposed by Brownlee and central to this mechanism is the increased production of reactive oxygen species (ROS) which in turn promotes flux through the polyol, hexosamine, protein kinase C and AGE formation pathways leading to altered gene expression profiles of affected cells (Baynes, J. W. 1991; Brownlee, M., 2005). The results of several large scale clinical trials indicate that once initiated, these complications persist and continue to progress unimpeded even when glycemic control is achieved through pharmaceutical intervention (Gaede, P. H. et al. 2003; Holman, R. R. et al. 2008; Turner, R. C. et al. 1999). This persistence was first documented in a canine model of DM and has been supported by multiple lines of experimental evidence using a variety of animal models and *in vitro* culture systems (El-Osta, A. et al. 2008; Ihnat, M. A. et al. 2007b). Collectively, these studies clearly show that the initial hyperglycemic period results in permanent abnormalities (including aberrant gene expression) in the target organs/cells and this harmful phenomenon has been termed, Metabolic Memory (MM) (Ceriello, A. et al. 2009; Ihnat, M. A. et al. 2007a). The ability to sustain these complications in the absence of hyperglycemia invokes a role for the epigenome in perpetuating diabetic complications and MM.

A. Description of the adult zebrafish type I model of diabetes mellitus and metabolic memory

As many cellular processes are highly conserved throughout vertebrate evolution, zebrafish models spanning a wide range of human pathologies including genetic disorders and acquired disease have been developed (Lieschke, G. J. et al. 2007). In the context of diabetes mellitus, recent data indicate that zebrafish regulate glucose metabolism via the same enzymes and pathways as mice and humans. For example, high glucose in zebrafish stimulates insulin expression (Elo, B. et al. 2007), negatively regulates gluconeogenesis (Elo, B. et al. 2007), increases cortisol levels (Powers, J. W. et al. 2010) and induces expression of VEGF (Alvarez, Y. et al. 2010). In addition, characterization of the GLUT transporter family has revealed remarkable conservation of structure, function and glucose affinity from zebrafish to human (Castillo, J. et al. 2009). From a pharmacological perspective, zebrafish have been documented to respond to anti-diabetic drugs reducing

blood glucose levels which further illustrates the physiological conservation of glucose regulation (Elo, B. et al. 2007). As in mammals, the zebrafish pancreas is comprised of two types of both exocrine and endocrine tissue with the later responsible for regulation of glucose metabolism through secretion of insulin, somatostatin, and glucagon directly into the bloodstream (Gnugge, L. et al. 2004). Due to the above factors it was hypothesized that the zebrafish would make a suitable model for examination of type I DM and its complications.

Experimental models of type I diabetes can be induced through pancreatic beta-cell destruction utilizing the diabetogenic drug streptozotocin (STZ) and this approach was used to induce hyperglycemia in the zebrafish X. Characterization of this model demonstrated that zebrafish exhibit the similar characteristics as patients with diabetes mellitus including: 1) hyperglycemia (HG), FBGLs increasing from 59mg/dl to 307mg/dl, 2) loss of pancreatic beta-cells 3) significantly reduced serum insulin 4) increased serum nonenzymatic glycated proteins and 5) the known secondary complications of diabetes mellitus including: retinal thinning, renal glomerular basement membrane thickening, and impaired epithelial wound healing (Olsen, Ansgar S. et al. 2010). Other endocrine cells of zebrafish islets are not affected by STZ treatment as previously reported (Olsen, A. S. et al. 2012; Olsen, Ansgar S. et al. 2010). It should be noted that STZ-treated fish also exhibit the additional complication of impaired tissue regeneration which uniquely provides a quantifiable bioassay of hyperglycemia induced tissue dysfunction (Olsen, Ansgar S. et al. 2010). Recently published studies have extended this work to the process of angiogenesis in the zebrafish (Sarras, M. P., Jr. et al. 2014).

In the human, the histopathology of type 1 diabetes is defined by a decreased β -cell mass in association with insulinitis, a characteristic lymphocytic infiltration limited to the islets of Langerhans and prominent in early stage disease in children (In't, Veld P. 2011). Animal models designed to mimic type 1 diabetes use various approaches, both genetic and pharmacological. In the later case, as indicated above, streptozotocin is often used as a diabetogenic drug (see Table 2). Depending on the dose of STZ used, beta-cell death may or may not be accompanied by insulinitis in mammalian animal models (Rossini, A. A. et al. 1977). Recent studies indicate however, that onset of hyperglycemia is not related to insulinitis, but rather is solely the result of STZ-induced beta-cell death (Deeds, M. C. et al. 2011; Lu, W. T. et al. 1998; O'Brien, B. A. et al. 1996). Our studies do not report the occurrence of insulinitis associated with STZ treatment of zebrafish (Olsen, Ansgar S. et al. 2010).

One of the main reasons zebrafish was chosen is because as a regeneration competent organism it was hypothesized that if allowed, the fish would restore glucose control via beta-cell replenishment. When STZ was removed, the fish did indeed regain glucose control and as expected this was accompanied by new beta cell production (Olsen, A. S. et al. 2012). In contrast, tissue regeneration, wound healing and angiogenesis remained impaired to the same extent in the newly euglycemic fish as their acutely DM counter parts (Olsen, A. S. et al. 2012). Moreover it was shown that the impairment was transmissible from mother to daughter cell indicating a (epi)genetic component to the persistence of these complications (Olsen, A. S. et al. 2012). As such, this model provides a unique opportunity to examine

hyperglycemia-induced changes within a wide variety of tissues as the fish transverse through the normal, DM, and MM states. This allows for the study of important regulatory systems underlying both DM and MM linking the relationship between the two. Furthermore, the contribution of epigenetic mechanisms to the MM phenomenon can be studied independent of the potentially complicating factors of AGE, ROS, etc, of the previous diabetic state (Olsen, A. S. et al. 2012).

B. Correlation of DNA epigenetic changes to DM and MM zebrafish

Studies with the DM/MM zebrafish model (Olsen, A. S. et al. 2012; Olsen, Ansgar S. et al. 2010) were begun to address the epigenetic aspect of MM by first examining hyperglycemia induced DNA methylation changes as, it is well documented that the methylation status of DNA is heritable through mitosis. Through methylated DNA immuno-precipitation followed by sequencing experiments, it was documented that hyperglycemia-induced specific CpG island demethylation (assayed by genome wide CpG island methylation status identification at a fifty base pair resolution) which was followed by re-methylation in the MM state, but this re-methylation was not restored to “normal levels” for a subset of loci (Olsen, A. S. et al. 2012). When this data was viewed within the context of global gene expression (via microarray analysis), a correlation of CpG island DNA demethylation changes and altered expression was observed (Olsen, A. S. et al. 2012; Sarras, Michael P. et al. 2013). Therefore, the persistence of hyperglycemia-induced retardation of fin regeneration correlated directly with aberrant DNA methylation and continued gene expression alterations in the MM state. This led us to conclude that the epigenetic DNA methylation mechanism may be responsible, in part, for the metabolic memory phenomenon. Additional DNA methylation studies (data not published) indicate that this epigenetic event is not limited to fin tissue but is also observed in others zebrafish tissues with clinical relevance to the human diabetic condition, to include: 1) renal, 2) retinal, and 3) skin tissue. The clinical relevance of these findings are strengthened by the recent report that DNA demethylation is also observed in the case of patients with diabetics mellitus as related to epigenetic changes in the Connective Tissue Growth Factor Gene (Zhang, H. et al. 2014). No other diabetic zebrafish models have focused on epigenetic changes associated with the DM and MM states. It should be emphasized that a number of controls were conducted to determine that the changes associated with DM and MM were not due to spurious affects of the STZ. For example, direct injection of STZ into fins does not affect fin regeneration or induce DNA methylation changes. Other controls are described in detail in the original article (Olsen, Ansgar S. et al. 2010). In summary, these controls indicate that tissue deficits, epigenetic changes, and gene expression pattern changes are solely due to hyperglycemia and not due to a non-specific effect of STZ.

Preliminary studies not yet published related to the DNA methylation state of the caudal fin in control, DM, and MM conditions indicate that DNA de-methylation has very specific characteristics. These studies focus on 1) what functional gene groups are prominent during DM, 2) which genes of these groups persist into MM, and 3) what is the positional genomic relationship of DNA methylation to these genes. Our data indicates that the DM/MM states are associated with alterations in gene expression within the *DNA replication and DNA metabolism* groups. Methylated DNA regions (MRs) were found 6-13 kb upstream of the

transcription start site for a subset of functionally important genes (*dnmt1*, *mcm2*, and *orc3*) within these groups and MRs were associated with *in silico* identified transcription factor (TF) binding sites whose methylation is known to perturb TF binding. Translational application to the human genome is currently being investigated as it applies to the human *DNMT1*, *MCM2* and *ORC3* genes and these studies suggest that zebrafish MRs correspond to high identity regions in the counterpart human genes. Initial results indicate that these high identity regions do track to DNA methylation changes in diabetic patients thereby representing the first translation of an epigenetic change pertaining to DNA methylation in a DM/MM animal model directly to a matching epigenetic change in the gene locus of human diabetic patients.

One of the main advantages of the zebrafish model is that it is experimentally very approachable and amenable to genetic manipulation thereby allowing for the dissection of molecular pathways and mechanisms. The above mentioned demethylation events occurring during hyperglycemia raise the question as to the pathways involved in this process. Studies from human cells, rats, and zebrafish have documented that hyperglycemia induces the demethylation of specific cytosines throughout the genome. The authors have published studies that at least partially establish the components of the DNA de-methylation pathway in the zebrafish Type I model of DM./MM (Dhliwayo, N et al. 2014). These studies found that RNA expression and enzymatic activity assays indicate that the ten-eleven translocation (Tet) family of enzymes are activated by hyperglycemia. Furthermore, through the detection of intermediates generated via conversion of Q:3 5-methyl-cytosine back to the unmethylated form, the data were consistent with the use of the Tet-dependent iterative oxidation pathway. In addition, evidence was provided that the activity of the poly-ADP ribose polymerase (Parp) enzyme is required for activation of Tet activity because the use of a Parp inhibitor prevented demethylation of specific loci and the accumulation of Tet-induced intermediates. This inhibition was accompanied by a complete restoration of the tissue regeneration and angiogenesis deficit that is also induced by hyperglycemia, thereby establishing a link between the de-methylation process and the tissue dysfunction observed in this zebrafish model during hyperglycemia.

C. Strengths of the adult zebrafish model of Type I diabetes as compared to other available animal models for analysis of metabolic memory

Based on the extensive clinical evidence that confirms metabolic memory as a critical process related to the long-term (secondary) complications found in those with type I or type II diabetes, additional research is required to understand the molecular basis for MM. To this aim, both *in vitro* systems and animal models of MM have been developed over the past 27 years. These models are summarized in Table 1 [*In vitro* MM models (El-Osta, A. et al. 2008; Ihnat, M. A. et al. 2007b) and Table 2 (Animal MM models (Capiotti, K. M. et al. 2014; Chan, P. S. et al. 2010; Engerman, R. L. et al. 1987; Hammes, H. P. et al. 1993; Kowluru, R. A. 2003; Kowluru, R. A. et al. 2004; Kowluru, R. A. et al. 2007; Olsen, A. S. et al. 2012; Roy, S. et al. 1990)]. It is important to point out the strengths and weaknesses of these MM models as compared to the zebrafish DM/MM model described in this review. There are many more models that just focus on the hyperglycemic state, but for the purposes

of this review, studies are discussed that focus on both the diabetic and metabolic memory states.

In regard to the *In vitro* MM models (Table 1), criticisms relate to the artificial versus systemic basis of the analysis. For example, these cell culture based systems make assumptions about the duration of hyperglycemia that do not necessarily comport to that existing in the *in vivo* condition. Secondly, their experimental design is apart from normal systemic body systems and therefore creates an artificial environment that brings into question the relevance of the data obtained. While *in vitro* experiments are important to understanding molecular mechanisms, they normally require extension into the *in vivo* setting for confirmation.

As shown in Table 2, animal MM models have been developed to compliment what has been discovered through *in vitro* experimentation. Since 1987, animal MM models have been developed in dogs (Engerman, R. L. et al. 1987) rats (Chan, P. S. et al. 2010; Hammes, H. P. et al. 1993; Kowluru, R. A. 2003; Kowluru, R. A. et al. 2004; Kowluru, R. A. et al. 2007; Roy, S. et al. 1990), and zebrafish (Capiotti, K. M. et al. 2014; Olsen, A. S. et al. 2012). In the case of rats, a number of variations of these rodent MM models exist. All but one utilizes streptozotocin for induction of DM with glycemic control modulated by insulin injections to establish “poor glycemic control” followed by “good glycemic control” in order to compare hyperglycemic conditions to MM conditions (Chan, P. S. et al. 2010; Kowluru, R. A. 2003; Kowluru, R. A. et al. 2004; Kowluru, R. A. et al. 2007; Roy, S. et al. 1990). One exception to this approach is the use of the Cohen rat system (Hammes, H. P. et al. 1993) for induction of DM followed by IP injection of syngeneic pancreatic islets for return of the animal to euglycemic conditions. If one is studying the role of epigenetic-related mechanisms in diabetes, these animal models have weaknesses. These weaknesses relate to the contributions of cellular and extra-cellular pathologies that are on-going in the disease. The major weakness relates to the episodic fluctuation of insulin levels because of the investigator's inability to perfectly control this hormone's systemic levels. This leads to continuous abnormal production of ROS and AGEs and therefore does not allow for a pure metabolic state to exist. While this is also true for the diabetic patient and may argue for a model that exactly mimics the diabetic conditions, if one focuses on underlying epigenetic mechanism, a MM model that reduces experimental variables is desirable, especially in the case of ROS molecules whose presence can produce epigenetic changes. In the DM/MM zebrafish animal model, ROS and AGEs are no longer abnormally produced in the MM state because a pure state of euglycemia is re-established due to regeneration of normal pancreatic beta-cell (Olsen, A. S. et al. 2012). The best rodent model to match the DM/MM zebrafish model is the Cohen rat MM model because it does re-create an euglycemic state following islet transplantation so that the episodic fluctuation of insulin levels do not exist in its MM state (Olsen, A. S. et al. 2012). However, as described in our 2012 article (Olsen, A. S. et al. 2012); DNA methylation analysis and gene expression analysis was performed on regenerated fin tissue that was shown to completely lack ROS and AGE molecules in the MM fin tissue using a number of techniques. Despite the fact that euglycemia has been established and ROS and AGE molecules are not present, impairment of fin regeneration persists in this MM tissue. This is not the case for the Cohen rat MM model that has residual

ROS and AGE molecules throughout the rodent body in its MM state. Additionally, compared to the DM/MM zebrafish model; the Cohen rat model is technically more challenging to create because of the expertise required for the isolation and injection of pancreatic beta cells. In the case of the DM/MM zebrafish model, one simply has to withdrawal STZ injections for creation of a subsequent pure MM state.

This then takes us to the zebrafish DM/MM model. Because of the epimorphic capacity of the zebrafish, one can use its regenerative capacity as an advantage so that streptozotocin-induced ablation of pancreatic beta cells can be followed by conditional *de novo* regeneration of these insulin producing cells. This result allows for a completely normal and permanent euglycemic state to be re-established following the initial hyperglycemic episode. Coupling this fact with the ability of the investigator to induce repeated fin regenerations and capillary blood vessel regenerations creates new daughter tissue that has none of the residual cellular and extra-cellular pathologies of DM such as the presence of AGEs and ROS (Olsen, A. S. et al. 2012). This important ability allows the investigator to test cellular characteristics of the control, DM, and MM cells without the noise associated with normal DM tissues that complicate interpretation of the results. Data obtained from the zebrafish DM/MM model is solely related to alterations that have occurred as a consequence of initial hyperglycemic event and are not due to other DM-associated factors such as AGEs or ROS that also contribute to the disease's secondary complications. This model therefore allows one to drill down to the pure contribution of such mechanisms as epigenetics. This enables a clear and unambiguous interpretation of those results pertaining to the role of DNA and DNA-associated molecules (e.g. histones) in diabetes. It should be noted that another zebrafish DM/MM model has recently been published (Capiotti, K. M. et al. 2014) that uses a different approach. This model induces fish into a hyperglycemic state by immersing them into a solution of 111 mM glucose for 14 days and then induces a metabolic memory state by returning the fish to normal aquatic conditions or by using anti-diabetic drugs such as metformin while the fish are hyperglycemic. As with our studies, they found that fish exhibited tissue deficits in the MM state following the initial 14 day hyperglycemic state. In contrast to our model which is reflective of type I diabetes, they categorize their model as reflective of type II diabetes and did not perform either epigenetic or gene expression analyses.

Conclusion

In aggregate, these studies support the use of the DM/MM Zebrafish model for analysis of Type 1 diabetes (and likely, Type II diabetes given that the initiation of the epigenetic changes are induced by hyperglycemia, a pathology common to both Type 1 and Type II diabetes) and indicate that the model faithfully represents the conditions clinically observed in the human diabetic state. The model's unique characteristics allow for both a cellular and molecular analysis of the mechanisms underlying the long term complications of diabetes and indicate that epigenetic processes such as DNA methylation are a contributing factor in the persistence of tissue dysfunctions observed in the disease. Preliminary data also suggest that the model is a true translational system that will allow one to take the data obtained from DM/MM zebrafish model to directly test parallel pathologies in human cells of diabetic patients. This will allow one to identify mechanisms in the zebrafish and then test them in

human tissues and cells; mechanisms previously unknown to exist in the human diabetic state.

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Abbreviations

HG	Hyperglycemia
DM	Diabetes mellitus
MM	Metabolic memory
DM/MM	Diabetes mellitus/Metabolic Memory
GLUT	Glucose transporter
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor
STZ	Streptozotocin
AGE	Advanced glycation end product
Tet	Ten-Eleven Translocase
CpG	Cytosine–Phosphate–Guanine
MR	Methylated DNA region
Parp	Poly-ADP ribose polymerase
TF	Transcription factor
dnmt1	DNA methyl transferase (gene 1)
mcm2	Mini-chromosome maintenance protein (gene 2)
orc3	Origin of replication (gene 3)

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Table 1

In vitro MM models utilizing various cell types and experimental conditions.

In vitro Models to Study Metabolic Memory (MM)		
In vitro Models of MM (Ref. #)	Description of Model	Summary of Study's Findings
Primary Bovine aortic endothelial cells *1 or Primary Human aortic endothelial cells *2 [2008, El-Osta et al.] (10)	16 hrs 30mM to 6 days 5mM Glucose	Transient hyperglycemia-induced long lasting activating epigenetic changes via histone 3 lysine 4 mono-methylation (H3K4me1) in the promoter of the nuclear factor kappaB (NF-kappaB) subunit p65 in endothelial cells
HUVEC *3 or ARPE19 Retinal cells *4 [2007, Ihnat et al.] (18)	3 weeks 30mM Glucose or 2 weeks 30mM Glucose to 1 week 5mM Glucose	ROS-mediated cellular persistence of vascular stress after glucose normalization.

*1. BAECs

*2. HAECs

*3 Human Umbilical Vein Endothelial Cells.

*4 Spontaneously arising human retinal pigment epithelia (RPE) cell line derived in 1986

Table 2

In vivo Animal MM models using various species and experimental conditions.

Animal Models to Study Metabolic Memory		
Animal Models of MM (ref. #)	Description of Model	Summary of Results
Dog [1987, Engerman and Kern] (12)	Alloxan-induced Diabetes (DM) 1) 5 Yrs poor glycemic control (PGC) using insulin & 5 Yrs good glycemic control (GGC) using insulin or 2) 2.5 Yrs GGC & 2.5 Yrs PGC.	Following hyperglycemic, dogs developed retinopathy under both PGC and GGC conditions.
Rat [1990, Roy, et al] (30)	Streptozotocin (STZ)-induced DM with 2 wk PGC & 2 wk GGC.	Over-expression of ECM fibronectin Under both PGC and GGC conditions.
Rat [1993, Hammes, et al] (15)	Cohen Rat strain used for dietary (Cu++ deficiency/sucrose supplement) induction of DM (PGC). Syngeneic islet transplantation for GGC was performed at 6 or 12 wk following DM.	Irreversible changes induced by antecedent hyperglycemia play a central role in the progressive development of diabetic retinopathy
Rat [2003, Kowluru, et al] (20)	STZ-induced DM for 2-6 months (PGC) followed by 7 mo GGC.	Retinopathy develops following following hyperglycemia under both PGC and GGC.
Rat [2004, Kowluru, et al] (21)	STZ-induced DM for 6 months (PGC) followed by 13 months GGC.	Activation of apoptosis and NF-KB in The retina persists under both PGC and GGC.
Rat [2007, Kowluru, et al] (22)	STZ-induced DM for 12 or 6 months (PGC) followed by 6 months of GGC.	Peroxyntirite accumulation in the retinal microvasculature fails to normalize under both PGC and GGC conditions.
Rat [2010, Chan, et al] (7)	STZ-induced DM for 12 months (PGC) followed by 12 months GGC.	No significant changes in pro-inflammatory mediators was observed between PGC and GGC groups.
Zebrafish [2012, Olsen, et al] (26)	STZ-induced DM for 4 wks followed by conditional de-novo regeneration of normal islet cells for permanent euglycemia and MM.	Tissue deficits observed in DM (hyperglycemia), persisted after pancreatic B-cells regenerated and fish return to a permanent euglycemic state. Global (but loci-specific) loss of DNA methylation accompanied DM as well as gene expression patterns and this persisted into the MM state.
Zebrafish [2014, Capiotti, et al] (4)	Immersion in a 111 mM glucose solution for 14 days to induce hyperglycemia (DM). A MM state was induced by 1) treatment of hyperglycemic fish with anti-diabetic drugs (glimepiride and metformin) or 2) returning fish to normal aquatic conditions for at least 7 days.	Metabolic changes in the DM state that persist into the MM state when glucose levels are returned to normal.