Use of Zebrafish as a Disease Model Provides a Unique Window for Understanding the Molecular Basis of Diabetic Metabolic Memory

Michael P. Sarras Jr.
Department of Cell Biology and Anatomy
Chicago Medical School at Rosalind Franklin University, USA

Robert V. Intine
Department of Biomedical Sciences, Dr. William M. School
College of Podiatric Medicine at Rosalind Franklin University, USA
1 Introduction

As reported by the World Diabetes Foundation and the International Diabetes Federation, Diabetes mellitus (DM *A “List of Abbreviations” is placed at the end of the article) now affects seven percent of the world’s adult population or approximately 346 million people worldwide and these numbers are projected to increase DM above 400 million by 2030 (Shaw, Sicree, & Zimmet, 2010, pp. 4-14). The regions with the highest comparative prevalence rates are North America, where 10.2 % of the adult population have diabetes, followed by the Middle East, and the North Africa Region with 9.3%. The regions with the highest number of people living with diabetes are Western Pacific, where some 77 million people have diabetes and South East Asia with 59 million. India has the greatest number of people with diabetes, with a current figure of 50.8 million, followed by China with 43.2 million. This is followed by the United States (26.8 million); the Russian Federation (9.6 million); Brazil (7.6 million); Germany (7.5 million); Pakistan (7.1 million); Japan (7.1 million); Indonesia (7 million), and Mexico (6.8 million). Besides individual suffering, the costs to world governments are staggering.

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, 2005, pp. 1615-1625). 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, 1991, pp. 405-412; Brownlee, 2005, pp. 1615-1625). 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 (1993, pp. 977-986; Turner, Cull, Frighi, & Holman, 1999, pp. 2005-2012; Gaede et al., 2003, pp. 2658-2661; Holman, Paul, Bethel, Matthews, & Neil, 2008, pp. 1577-1589). 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 (Engerman & Kern, 1987, pp. 808-812; Hammes et al., 1993, pp. 2092-2096; Kowluru, 2003, pp. 818-823; Kowluru, Chakrabarti, & Chen, 2004, pp. 194-199; Roy, Sala, Cagliero, & Lorenzi, 1990, pp. 404-408; Li et al., 2006, pp. 2611-2619; Olsen, Sarras, Jr., Leontovich, & Intine, 2012). 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) (Ihnat et al., 2007, pp. 1523-1531; Ceriello, Ihnat, & Thorpe, 2009, pp. 410-415). The ability to sustain these complications in the absence of hyperglycemia invokes a role for the epigenome in perpetuating diabetic complications and MM, as will be discussed in more detail later in the chapter.

2 In vitro and In vivo Systems for Analysis of MM

Experimental approaches to understanding the underlying mechanisms of MM are limited by the physiological complications associated with diabetes. This is because the primary dysfunctions associated with glucose control are never completely resolved even with the most stringent pharmacological interven-
Throughout the course of a diabetic’s life, there will always be, to some extent, abnormal variations (hyperglycemia to hypoglycemia) in glucose serum levels. As stated, animal and clinical studies clearly indicate that an immediate consequence of these glycemic variations is an 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, 1991, pp. 405-412; Brownlee, 2005, pp. 1615-1625). Further analysis of diabetic complications indicates that additional molecular abnormalities are occurring related to perpetuation of continued tissue defects. Previous clinical studies and the heritable nature of these defects (Olsen et al., 2012 and others) points to epigenetic changes in the diabetic’s tissues as an additional significant factor in contributing to the ongoing secondary complications observed in MM. To separate the primary effects of DM (lack of glycemic control), from the immediate secondary pathologies (ROSs, AGEs, etc.), from other molecular pathologies (epigenetic changes); new experimental design paradigms have been developed for analysis of MM. These experimental paradigms include both in vitro and in vivo approaches as will be discussed below.

The advantage of in vitro approaches lies in the ability of the investigator to simplify the variables involved in the experimental design. In the case of MM, these approaches typically involve using either 1) established or 2) primary cell lines for analysis. In such experiments, cells would typically be exposed to hyperglycemic conditions for a defined amount of time (DM conditions) or cells would be derived from hyperglycemic tissue, and then the media would be returned to normal glucose levels (MM conditions) and some aspect of cell function compared between the DM and MM states. Much data has accumulated from such studies. For example, these studies have documented a continued increase in extracellular matrix production in human umbilical endothelial cells and vascular smooth muscle cells (Roy et al., 1990, pp. 404-408). Additionally, cells derived from obese, insulin-resistant, and diabetic db/db mice significantly increased oxidant stress, activation of key signaling kinases, transcription factors, cAMP response element-binding protein, and NFkB65 in response to in vitro hyperglycemic conditions (Li et al., 2006, pp. 2611-2619). Collectively these studies show some of the uses of in vitro models in dissecting the molecular basis of diabetic complications and metabolic memory. However, the problem with such studies is twofold: 1) they are by design inherently artificial and 2) they lack physiological relevance when viewed through the prism of multisystem organisms. In regard to analysis of molecular mechanism underlying MM, these limitations are painfully illustrated by recent reviews by Rando (Rando, 2012) and Henikoff and Shilatifard (Henikoff & Shilatifard, 2011, pp. 389-396).

In vivo analyses include both animal and human studies. These in vivo studies can also be integrated with in vitro experiments to strengthen the conclusions that can be drawn from their data, as highlighted in this section. Such studies stem from the premise, that once initiated, diabetic complications are propagated by metabolic memory even when glycemic control is achieved (Ihnat et al., 2007, pp. 1523-1531; Ceriello et al., 2009, pp. 410-415). The presence of these MM phenomena was recognized clinically around 2002 and has been supported by multiple clinical trials. However, it was first documented in the mid 1980s in a pioneering study that reported the maintenance and progression of retinopathy after restoration of normal glycemia in a canine model of diabetes mellitus (Engerman & Kern, 1987, pp. 808-812). A similar uninterrupted progression of retinopathy was documented using a diabetes mellitus sucrose fed rat model in which diabetic rats received pancreatic islet transplants at various times post disease onset (Hammes et al., 1993, pp. 2092-2096). Since these initial studies, several reports utilized a STZ-induced diabetic rat model where the animals were either maintained at normal glucose levels (NG)
and were hyperglycemic for the duration of the study (HG) or were initially kept in hyperglycemic conditions but were then switched to normal glycemia part way through (HG/NG). Collectively these studies showed that the HG/NG group had indistinguishable increases of oxidative stress when compared to the HG group as assayed by the presence of increased lipid peroxides, peroxynitrates, nitrosotyrosines and capase 3 activity (Kowluru, 2003, pp. 818-823; Kowluru et al., 2004, pp. 194-199). Additionally, it was reported that nuclear factor kappa B p65 subunit (NFKBp65) expression was increased in both the HG and HG/NG groups (Kowluru et al., 2004, pp. 194-199). As indicated previously, more recent studies point to epigenetic changes as a significant contributing factor in the MM state and this molecular process may in fact, be the basis of MM in the long term. Epigenomes consist of all the chromatin modifications for a given cell type and are responsible for a cell’s unique gene expression pattern, are dynamic during development supporting cell differentiation, are responsive to external stimuli, and can be altered in disease (Ho & Crabtree, 2010, pp. 474-484; Jaenisch & Bird, 2003, pp. 245-254; Jirtle, Sander, & Barrett, 2000, pp. 271-278). Chromatin modifications resulting from epigenetic processes are stable in the absence of the signal that initiated them and are heritable through cell division (Dolinoy & Jirtle, 2008, pp. 4-8; Morgan & Whitelaw, 2008, pp. 394-397), and thus undoubtedly play a role in propagating metabolic memory. Epigenetic mechanisms include: histone modifications, non-canonical inclusion of histone variants in octomers, DNA methylation, chromatin remodeling complexes, and non-coding RNAs (Blomen & Boonstra, 2010; Bogdanovic & Veenstra, 2009, pp. 549-565; Mosammaparast & Shi, 2010, pp. 155-179; Kouzarides, 2007, pp. 693-705). Several studies regarding histone modifications and hyperglycemia have come from the Natarajan lab. Initially, they reported increased acetylation (transcription activator) of histones in the NFKBp65 promoter and two of its downstream target promoters; 1) tumor necrosis factor alpha and 2) cyclooxygenase when monocytes were grown in hyperglycemic conditions (Miao, Gonzalo, Lanting, & Natarajan, 2004, pp. 18091-18097). In experiments probing micro-arrays with chromatin immunoprecipitated DNA prepared with either anti histone 3 lysine 4 (H3K4, activating methylation modification) or anti histone 3 lysine 9 (H3K9) (inactivating modification) from monocytes in culture showed a correlation of the epigenetic mark with the mRNA expression data (Miao et al., 2007, pp. 13854-13863). They then undertook a similar study, but in this case utilizing lymphocytes from diabetic patients, with much the same results (Miao et al., 2008, pp. 3189-3198). Another study from the same lab documented an association of the set7/9 methyltransferase (methylates H3K4) and the NFKBp65 promoter indicating a potential mechanism of increased NFKBp65 expression in hyperglycemic conditions (Li et al., 2008, pp. 26771-26781). In total, these data show that histone modification changes occur in response to hyperglycemic conditions.

Several studies have also documented histone modifications in experiments that mimic the metabolic memory conditions. Aortic endothelial cells grown in transient hyperglycemia conditions for 16 hours maintained: 1) increased transcription of the NFKBp65, 2) increased set7/9 association, and 3) H3K4 monomethylation at the NFKBp65 promoter 6 days post hyperglycemia (El-Osta et al., 2008, pp. 2409-2417). Similar findings by Brasacchio et al. detected an increase set 7/9 methyltransferase and lysine specific demethylase-1 at the NFKBp65 promoter in bovine aortic arch and human microvascular endothelial cells following growth in transient hyperglycemic conditions (Brasacchio et al., 2009, pp. 1229-1236). Approximately 20% of Apo E mice (a diabetic model) spontaneously revert back to normal glycemia for unknown reasons. When these mice were examined, NFKBp65 and its downstream inflammatory targets were still increased to the same levels as their diabetic counterparts and atherosclerotic plaque size was maintained (Brasacchio et al., 2009, pp. 1229-1236). Most recently, histone acetyl-
tion was examined in retinal endothelial cells of STZ-induced diabetic rats that were placed into the three NG, HG or HG/NG groups described above. Hyperglycemia activated histone deacetylases-1,2 and 8 decreased histone acetyl-transferase activity, and H3 acetylation was globally reduced in the HG and HG/NG groups (Zhong & Kowluru, 2010, pp. 1306-1313). Folli et al examined skin samples from patients that had Type I diabetes, Type I diabetes with end stage renal disease, or patients that had received a kidney and pancreas transplant. Using proteomic and ultrastructural approaches, multiple alterations in the expression of proteins involved in oxidative stress, aerobic and anaerobic glycolysis, intracellular signaling, as well as endothelial vascular abnormalities were identified with some reversion after transplant (Folli et al., 2010, p. e9923). Collectively, the above studies indicate an involvement of the histone modification arm of the epigenome in metabolic memory. Additionally, the agouti mouse coat color and susceptibility to developing obesity and diabetes mellitus is governed by DNA methylation at that locus (Morgan, Sutherland, Martin, & Whitelaw, 1999, pp. 314-318). Both histone modifications and DNA methylation were implicated in an investigation where intrauterine growth was retarded in rats and this lead to an increased incidence of Type II diabetes mellitus. In this study, development of Type II diabetes was accompanied by epigenetic silencing of the promoter, Pdx1, a key transcription factor that regulates insulin gene expression and beta cell differentiation (Park, Stoffers, Nicholls, & Simmons, 2008, pp. 2316-2324). In another study, it was shown that, there was increased DNA methylation of the promoter of the peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1γ gene (PPARGC1A), a factor that plays a key role in regulating mitochondrial genes and in the modulation of diabetes in diabetic islets (Ling et al., 2008, pp. 615-622). Although these studies indicate a role for DNA methylation, no studies have been reported regarding DNA methylation alterations induced by hyperglycemia or the potential role of DNA methylation in metabolic memory.

This gives the reader an overview of the in vitro and in vivo approaches and provides highlights of what information can be gleaned from these approaches regarding our understanding of the underlying processes and mechanisms of MM. It is clear that there is much uncertainty regarding what mechanisms explain why tissues are permanently affected by the initial insult of hyperglycemia and what can be used to approach the problem from a clinical treatment standpoint. Much is needed to take us to a position where effective treatment approaches can be investigated to eradicate the secondary complications seen in diabetic MM.

3 STZ-induced Type I DM in Adult Zebrafish

In an attempt to develop a Diabetic/Metabolic Memory in vivo model that would allow one to investigate the molecular basis of MM without the confounding variables associated with traditional DM models, the laboratories of Intine and Sarras (Olsen, Sarras, Jr., & Intine, 2010, pp. 532-542; Olsen et al., 2012) set out to create a new system using the highly regenerative zebrafish animal model. Zebrafish was selected for these studies for a number of reasons to include: 1) as a chordate, it has as part of its physiological make-up, a mammalian-like insulin-based homeostatic regulatory system to control serum glucose levels, 2) the histology of this insulin-based regulatory system is similar to that of mammals with minor modifications, 3) dysfunction of the insulin-based regulatory system of zebrafish had been shown to result in hyperglycemia as is observed in mammalian species, and 4) as an epimorphormic organism, it had the re-
generative capacity to possibly re-grow its insulin-producing beta-cell population; thereby, allowing the organism to regain glycemic control after a prolonged period of DM-induced hyperglycemia.

Various approaches to creating a diabetic state in zebrafish are possible. Attempts by others (communication by R. Thummel to induce a hyperglycemic state by placing fish in water containing elevated glucose levels has proven to be unsuccessful in the long term because the fish compensate for the high glucose over time (presumably by making more beta cells). In addition, no obvious changes in the secondary organs such as the retina could be observed in that model. Another approach involves induction of a Type I diabetic state by ablation of pancreatic β-cells resulting from the administration of the diabetogenic drug STZ (Ganda, Rossini, & Like, 1976, pp. 595-603). As a first phase in development of a diabetic zebrafish model using this approach, the normal FBGLs of adult zebrafish were determined by fasting fish for 24 hours, followed by euthanasia and cardiac puncture. The FBGLs were immediately determined and yielded a value of 58.85 +/-4.79 mg/dL (n=15) (Figure 1A, UN). Zebrafish were administered three intraperitoneal injections (350 mg/kg) of STZ or buffer (as control) on alternating days in Week 1 (W1), and subsequent booster injection/s were administered on a weekly basis to yield the Week 2 (W2) and Week 3 (W3) groups. The FBGLs were determined one week after the final injection. The dose of STZ administered was based on a previous report where hyperglycemia was induced in the teleost, Oreochromis niloticus (Wright, Jr., Abraham, Dickson, Yang, & Morrison, 1999, pp. 431-440). The average FBGL of control-injected fish was 61.94 +/-5.60 (n=287) which is within the error of untreated fish indicating that the injection procedure alone did not alter FBGLs (Figure 1A, GC). In contrast, injection of the zebrafish with STZ induced a distribution of FBGL levels ranging from approximately 60–650 mg/dL with a mean value of 306.3 +/-34 mg/dL, (n=517) one week following administration of the initial dose/s (Figure 1A, W1). Furthermore, the hyperglycemic state was maintained throughout the duration of this study as the glucose concentration for the 2-week group (307.9 +/-37.3 mg/dL, n=513) or 3-week group (311.6 +/-42.1 mg/dL, n=5213) (Figure 1A, W2 and W3) was not statistically different from the week one group. These data indicate that hyperglycemia can be induced and sustained in the zebrafish by intraperitoneal injection of STZ.

One of the methods used to determine the magnitude of glycemic control in human patients during their previous 2–4-week period involves the detection of the level of nonenzymatically glycated serum proteins, fructosamines (Armbruster, 1987, pp. 2153-2163). The enzyme-based fructosamine quantification assay (Diazyme) was utilized to document the presence of nonenzymatic glycation products in the serum of hyperglycemic zebrafish (Olsen et al., 2010, pp. 532-542). As this test required more serum than could be obtained from a single fish, serum from four STZ or control-injected fish (W3) was pooled to perform the assay. These experiments indicated that there was a 320% (p < 0.01, n=53 pools, 12 fish total) increase in the amount of glycated protein in the serum from STZ-treated zebrafish (Figure 1B). This not only documented that nonenzymatic glycation occurred but also supported data demonstrating that the STZ-treated zebrafish were in a persistent hyperglycemic state for 2–4 weeks.

STZ administration in regeneration-incompetent organisms, such as rodents, causes insulinopenia due to ablation of the pancreatic β-cells (Rees & Alcolado, 2005, pp. 359-370). In order to verify a reduction in blood insulin levels, undiluted or a 1 : 5 dilution of pooled serum from four hyperglycemic and control fish (W3) was spotted onto a nitrocellulose membrane and probed for the presence of insulin. The undiluted signal from STZ-treated fish (Figure 2A, bottom row) is comparable with the 1 : 5 dilution of the serum from control fish (Figure 2A, top row) indicating a reduction of approximately 80% in the insulin levels of STZ-treated zebrafish. The zebrafish pancreas is similar in basic structure (containing
Figure 1: Streptozocin (STZ) injection results in sustained hyperglycemia. (A) Graphic representation of fasting blood glucose concentration. UN: uninjected zebrafish, 58.85 +/- 4.79 mg/dL (n=515); C: 61.94 +/- 5.60 (n=5287); W1: 306.3 +/- 32 mg/dL, n=517, zebrafish that only received injections for 1 week; W2: 307.9 +/- 37.3 mg/dL, n=513, zebrafish that received first week injections plus one booster; W3: 311.6 +/- 42.1 mg/dL, n=5224, zebrafish that received first week injections plus two boosters. Student’s t test of statistical significance p < 0.00001 was obtained when W1, W2, and W3 were compared with C and UN. (B) Graphic comparison of serum fructosamine levels in control and STZ-treated zebrafish. The serum from four fish were pooled and the experiment was repeated three times (total n=512). The optical density measurements (output of the assay) indicate a 320% increase (p < 0.05) of fructosamines in STZ-treated zebrafish.

both endocrine and exocrine components), and cellular makeup to the mammalian pancreas (Li, Wen, Peng, Korzh, & Gong, 2009, pp. 128-134). It consists of four distinct regions that lie along the three lobes of the intestine in which four kinds of islets (principal islets, Brockmann bodies, diffusely existing islets, and single β-cells) can be distinguished (Chen, Li, Yuan, & Xie, 2007, pp. 120-125). The largest islet (approximately 300µm wide and 500 µm long) is always observed at the dorsal–anterior position along the first intestinal lobe and may develop from the principle islet observed in embryonic stages (Alvarez et al., 2010, pp. 236-245). In order to further substantiate a decrease in insulin production, immunofluorescence microscopy on pancreatic tissue from control and diabetic fish was performed. Nine control and hyperglycemic zebrafish (W3) were sacrificed, their FBGLs were determined, and serial 10 µm transverse cryo-sections were made. The sections were observed initially by phase contrast microscopy to
identify pancreas tissue (Figure 2B i and ii, P) using the intestine (Figure 2B i and ii, IN) as a stationary landmark. These samples were then processed by double indirect immuno-fluorescence to visualize insulin (red), glucagon (green), and were counterstained with DAPI in order to visualize nuclei (blue). Epifluorescence microscopy indicated that the insulin signal from control fish was consistently much greater than that of hyperglycemic fish (compare the red signal from Figure 2B iii and iv). In contrast, the glucagon signal (green) did not vary significantly between the two groups (compare the green signal from Figure 2B iii and iv). The data was obtained from the largest islet; however, similar differences were seen across all pancreatic tissue and in combination with the immuno-blot data, collectively show reduced insulin production in STZ-treated zebrafish.

**Figure 2**: Streptozocin (STZ) injection results in insulinopenia. (A) Analysis of insulin levels in the blood shows a decrease in the amount of insulin in STZ (W3) treated fish. An undiluted (U) or a 1 : 5 dilution (1 : 5) of pooled serum samples from control (C) and hyperglycemic fish (H), probed with anti-insulin antibody. (B) Phase-contrast images of W3 control (i) and STZ injected (ii) fish are shown where P indicates the pancreas tissue, IN marks the intestine, and a 100 µm scale bar is included. Representative immunofluorescence microscopy images of the same pancreas tissue indicating the presence of glucagon (green), insulin (red), and DAPI (blue) for W3 control (iii), and STZ injected (iv) fish are shown. The scale bar indicates 50 µm.

The coincidence of 1) the loss of pancreatic beta-cells and decreased blood insulin with 2) the simultaneous high elevation of blood glucose (FGBL) provides compelling evidence that STZ is having a physiologically specific effect in regard to its induction of a diabetic state in these fish.
Thickening of the GBM is the earliest detectable feature of diabetic kidney disease (Jefferson, Shankland, & Pichler, 2008, pp. 22-36). In order to assay for the presence of this complication in hyperglycemic zebrafish, glomeruli from eight control and hyperglycemic zebrafish (W3) were imaged via TEM (Figure 3A and B). The glomeruli were easily identifiable due to the presence of podocytes (P), the GBM (G), and endothelial cells (EC). The mean area for each glomerulus was determined by drawing 15 non-overlapping rectangles (250nm length) of the GBM area. This analysis revealed that there was a 61% increase (Control, 1056 +/-29.5 U, STZ: 1695 +/-93.5 U, p < 0.00001) in the thickness of the GBM in the hyperglycemic fish.

![Hyperglycemic fish show thickening of the glomerular basement membrane (GBM) at 3 weeks. (A) A X40,000 image of a glomerulus from a control-injected zebrafish. The podocytes (P), endothelial cells (EC), and GBM (G) are indicated on the figure and the inset scale bar indicates 500 nm. (B) A X40,000 image of a glomerulus from a streptozocin (STZ)-injected fish showing an increased thickness of the GBM. The letter designations are as in (A). (C) Graphic comparison of the mean values of GBM thickness (15 measurements for each). The bars represent the mean thickness measurement of all GBMs in each group with the error bar representing the standard error. Control: 1056 +/-29.5 U, STZ: 1695 +/-93.5 (p < .000005).](image)

Figure 3: Hyperglycemic fish show thickening of the glomerular basement membrane (GBM) at 3 weeks. (A) A X40,000 image of a glomerulus from a control-injected zebrafish. The podocytes (P), endothelial cells (EC), and GBM (G) are indicated on the figure and the inset scale bar indicates 500 nm. (B) A X40,000 image of a glomerulus from a streptozocin (STZ)-injected fish showing an increased thickness of the GBM. The letter designations are as in (A). (C) Graphic comparison of the mean values of GBM thickness (15 measurements for each). The bars represent the mean thickness measurement of all GBMs in each group with the error bar representing the standard error. Control: 1056 +/-29.5 U, STZ: 1695 +/-93.5 (p < .000005).

Diabetes mellitus is the leading cause of adult onset blindness and it is reported that early changes in humans and animal models include retinal thinning (Aizu, Oyanagi, Hu, & Nakagawa, 2002, pp. 161-170). The retina consists of multiple identifiable layers of specialized cells. Previous studies of STZ-induced diabetes mellitus in rats showed that within three weeks of hyperglycemia, the thickness of the
inner plexiform layer (IPL), and photoreceptor layer (PSL) [also commonly referred to as the outer nuclear layer, ONL] were reduced (Aizu et al., 2002, pp. 161-170). Additionally, cone receptor dysfunction and neuronal damage independent of vascular defects were documented in zebrafish exposed to high glucose concentrations in their water (Aizu et al., 2002, pp. 161-170).

For analysis of retina, eight hyperglycemic and control zebrafish (W3) were euthanized, FBGLs determined, and eyes were removed. One-micrometer sections that included all of the retina layers were obtained and images from the thin sections were printed for morphometric analysis (Figure 4A and B). This analysis revealed an 18.7% (Control: 43.67 +/- 1.52, STZ 535.5 +/-2.02, p < 0.005) and 26.5% (IPL control 539.2 +/-1.28 and IPL STZ 528.8 +/-1.15, p < 0.000001) decrease in the retinal PSL and IPL layers; respectively, in hyperglycemic zebrafish when compared with the controls (Figure 4C). From these data, it was concluded that early retinal changes occur in hyperglycemic zebrafish and this provides further evidence that a true diabetic state had been achieved.

**Figure 4**: Hyperglycemic fish show retina layer thinning. Representative images of retina from (W3) control and streptozocin (STZ)-injected fish. A. Control fish retina; the thickness of the photoreceptor layer (PSL; also commonly referred to as the ONL or outer nuclear layer) is indicated by the yellow bars on the left of the images, the thickness of the inner plexiform layer (IPL) is indicated by the red bars on the right of the images and the white scale bar indicates 200 µm. B. Hyperglycemic fish retina with PSL (ONL) and IPL designations as in (A). (C) Graphic representation comparing the thickness (arbitrary units) of the IPL and PSL (ONL) layers of hyperglycemic (black bars) and control (white bars) fish. The bars represent the mean thickness measurement of all retinas in each group (n=58 for each) with the error bar representing the standard error. PSL (ONL) control = 43.67 +/-1.52, PSL (ONL) STZ = 35.5 +/-2.02, IPL control = 39.2 +/-1.28 and IPL STZ = 28.8 +/-1.15. p < 0.005, PSL (ONL) and p < 0.0000001, IPL.
While we have not studied affects of DM on the vascular system in zebrafish, this system has a fundamental role in the progression of this disease. As just one example, in vitro studies on human umbilical vein cells point to the role of micro RNAs (miRNA) in modulation of the diabetic state. Feng et al exposed human umbilical vein endothelial cells to high glucose and reported an increase in fibronectin (FN) expression (observed with several diabetic complications) with an accompanying decrease in miR146a expression (Feng et al., 2011, pp. 2975-2984). The 3'UTR of the FN mRNA houses a target site for miR146a and both the binding of miR146a and control of FN expression by this miRNA was established through transfection experiments. The decrease in miR146a expression was then confirmed to occur in vivo using STZ induced diabetic mice. Additionally, the authors provided evidence that the glucose-induced miR-146a down-regulation is mediated through the HDAC p300 and that the FN/p300/miR146a triad is seen in heart and kidney tissue as well. Therefore, this study not only establishes the control of FN expression by miRNA, but also provides a functional link for the control of gene expression for miRNA and histone modification that has not previously been documented in the context of DM.

4 Diabetic Zebrafish Display Impaired Tissue Regeneration

Adult zebrafish can regenerate many structures such as the heart, retina, spinal cord, pancreas, and fins (Akimenko, Mari-Befa, Becerra, & Geraudie, 2003, pp. 190-201; Poss, Keating, & Nechiporuk, 2003, pp. 202-210). The caudal fin has become a popular model for studying the molecular mechanisms regulating regeneration due to its accessibility for amputation and its relatively simple structure (Thummel et al., 2006, pp. 336-346). In order to assess the effect of hyperglycemia on limb regeneration, caudal fins of control and hyperglycemic zebrafish (W1, W2, and W3) were amputated immediately proximal to the most anterior branch point in the lepidotrichia of the fin. Images were collected at 24, 48, and 72 hours postamputation using NIS Elements Imaging software and images were printed for morphometric analysis (Figure 5A and B). The newly regenerated tissue was traced five independent times using a digitizing pad and Image J software, yielding the area of regeneration. The average area was recorded as the amount of regeneration in each animal. In order to normalize for the differences in initial fin size, this area was divided by the dorsal/ventral length of the fin at the amputation site. The amount of fin regeneration in hyperglycemic zebrafish was expressed as a percent of control fin regeneration to illustrate the differences that were observed (Figure 5C). After 1 week of hyperglycemia, there was no statistical difference in fin regeneration between control and hyperglycemic zebrafish at 24, 48, or 72 hours following amputation (101.0, 106.6, and 100%, respectively). However, after 2 weeks of hyperglycemia, zebrafish exhibited a statistically significant reduction at all three time points (79.1, 61.0, and 57.6%), which was further reduced by a longer duration of hyperglycemia (3 weeks) (43.6, 52.8, and 48.6%). A range in FBGLs of treated fish was determined and therefore a linear regression and a Pearson Product Moment Coefficient analysis was performed to determine the relationship between FBGL of each STZ-injected fish, and the amount of fin regeneration. This analysis resulted in a Correlation Coefficient of -0.92 and an R– value of 0.86, which documents an inverse relationship between the increase in FBGLs and fin regeneration ability. Collectively, this indicates that the extent of sustained hyperglycemia has a direct impact on the regenerative capacity of the zebrafish and the amount of this reduction can be correlated to the duration of hyperglycemia.
Figure 5: Hyperglycemic fish have a reduced rate of caudal fin regeneration. (A) Caudal fin image from a control-injected fish showing a normal amount of regenerative growth 72 hours postamputation. The area of regeneration was determined and the original cut line (black line in image) was used to normalize fin size differences. (B) A 72-hour postamputation caudal fin from a W3 hyperglycemic fish showing a smaller area of regenerated tissue. (C) Graphic presentation of the relative regeneration rate of hyperglycemic zebrafish as compared with control fish at week 1 (white bars), week 2 (gray bars), and week 3 (black bars). Week 1: 101.0, 106.6, and 100% for 24, 48, and 72 hours, respectively, n=510 for control and 14 for hyperglycemic fish. Week 2: 79.1, 61.0, and 57.6% for 24, 48, and 72 hours, respectively, n=511 for control and 9 for hyperglycemic fish. Week 3: 43.6, 52.8, and 48.6% for 24, 48, and 72 hours, respectively, n=532 for control and 19 for hyperglycemic fish. When values within an experiment where statistically compared using Student’s t test, a value of p < 0.05 or less was obtained.

Tissue regeneration requires the coordination of cell proliferation and apoptosis (Bai et al., 2005, pp. 247-260) and it was hypothesized that the reduction documented above could be due to alterations in either of these two processes. In order to address this, indirect immuno-fluorescence and TUNEL assays on 10 µm cryo-sections of regenerating fins 72 hours following amputation was performed. Through initial visual analysis, no difference could be detected in the number or distribution of apoptotic cells (Figure 6 v and vi); however, there appeared to be a marked decrease in the number of proliferating cells in the hyperglycemic fish (Figure 6A i and iv). In order to quantify this difference, proliferative cells were counted (n=58 fins for each group) and expressed as a percentage of the total number of cells (Figure 6B). When all of the cells of the proliferative unit were examined, there was a 48% decrease in the number of PCNA-positive proliferating cells (Control: 56.8% +/-3.4 vs. STZ 29.7% +/-2.9, p < 0.01), and a
39.3% decrease (Control: 34.9% +/-3.0 vs. STZ 21.2% +/-2.5, p < 0.01) when examined by phospho-histone H3 (Ser 10). When only the blastema was considered, this reduction was 39.1% (Control: 64.1% +/-5.3 vs. STZ 39.6 +/-2.5, p < 0.01) and 28.5% (Control: 29.9% +/-1.9 vs. STZ 21.4 +/-1.4, p < 0.01) for each antigen, respectively. Therefore, it appears that hyperglycemia does not cause increased cell death but does decrease the proliferative competency of the regenerating limb.

**Figure 6:** Reduced proliferation is observed in the regenerating caudal fins of hyperglycemic fish. (A) Representative fluorescence images of the regenerative area for cell proliferation and apoptosis assays are presented as indicated below. Adjacent 10 µm sections of W3 control (i, iii, and v, n58) or streptozocin (STZ)-injected fish (ii, iv, and vi n58) were processed with either anti-proliferating cell nuclear antigen antibody (PCNA) antibody (i and ii), phosphohistone H3 (Ser10) (H3P) (iii and iv), or processed via the TUNEL assay to detect apoptosis (v and vi). A white 50 µm scale bar is included. B. Quantification of proliferation in regenerating fins. The number of PCNA or H3P positive cells (n=512 for each group) was determined and is expressed as a percentage of the total number of cells (determined by DAPI staining). PCNA: Epithelium+Blastema: control 56.8 +/-3.4%, STZ 29.7 +/-2.9%. Blastema only: control 64.1 +/-5.3%, STZ 39.6 +/-2.5. p < .01 for both. H3P: Epithelium+Blastema: control 34.9 +/-3.0%, STZ 21.2 +/-2.5%. Blastema only: control 29.9 +/-1.9%, STZ 21.4 +/-1.4, p < .01, p < .01 for both.

In order to ensure that this impaired regeneration effect was not something unique to caudal fins, the rate of skin wound healing for the same three groups was investigated. Following wounding, the percentage of wound closure was determined by comparing the remaining open-wound area (24 h post-wounding) with the original wound area (see Table 1). These results indicated that wounds of DM fish
heal slower than those of their control counterparts and these data clearly demonstrate that tissue regeneration (to include wound healing) is a systemic problem in STZ-induced diabetic zebrafish and not something unique to caudal fin regeneration.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Percent Wound Closure</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 C</td>
<td>87.5 +/- 1.41</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Day 0 DM</td>
<td>62.2 +/- 5.45</td>
<td>17</td>
<td>0.0028</td>
</tr>
</tbody>
</table>

**Table 1:** Wound healing is impaired in DM Zebrafish. Data are means +/- SEM unless otherwise indicated. The P value was calculated for the day-0 time point.

Although STZ is known to be selectively toxic to pancreatic β-cells, it was important to ensure that nonspecific harmful effects of the drug were not confounding the results. During the course of these studies, drug-treated fish were observed that appeared to be resistant to the hyperglycemia-inducing effects of STZ treatment as has been reported previously in rodents (Lerco, Macedo, Silva, Pinheiro, & Spadella, 2006, pp. 87-91). When these fish (W3) were assayed for their regenerative capacity, a range of 96.8–105.9% (n=518) was determined. This showed that the reduction of regeneration is directly correlated with the hyperglycemic state of the zebrafish and not simply correlated with the presence of STZ, and suggests that the reduction in regeneration is not due to nonspecific toxic effects of the drug. This issue was further addressed by directly injecting the caudal fin with the same concentration and injection series of STZ used to induce and maintain the hyperglycemic state. The dorsal side of the fin was injected between each ray at the proximal lepidotrichia branch point with 350 mg/kg of STZ or buffer only (as control). The fins were then amputated and the fish were allowed to regenerate their fins. When regenerative growth was examined, no differences were detected between the dorsal and ventral sides of STZ or control injection (Olsen et al., 2010, pp. 532-542). These data clearly indicate that STZ exposure to caudal fin tissue does not reduce fin regeneration rate. It has been reported that intravenous STZ injection (350 mg/kg) of Tilapia resulted in several indices of general toxicity including severe hepatic failure characterized by hepatic necrosis, jaundice, and ascites accumulation (Wright, Jr. et al., 1999, pp. 431-440). To address this, the commonly used human liver function tests, serum albumin and serum alkaline phosphatase activity assays were used (Tajiri & Shimizu, 2008, pp. 6774-6785). Serum from 16 control and STZ-treated fish were analyzed, for each assay and no statistically significant differences were detected indicating that liver damage was not present (Olsen et al., 2010, pp. 532-542). Additionally, there was no indication of gross tissue or cell damage during histological examination. To rule out possible damage leading to cell death, the presence of apoptotic cells in kidney, intestine, liver, and pancreas was examined. The results indicated that the only tissue where an increase in apoptosis was observed was the pancreas which is expected due to the known β-cell toxicity of STZ (Olsen et al., 2010, pp. 532-542). Weight gain or loss, significant behavioral changes, or noticeable distress of the STZ-treated zebrafish were not observed. Collectively, the data indicate that no nonspecific effects from STZ were observed using this injection regimen.
5 Regeneration of Pancreatic-cells in Adult Diabetic Zebrafish

As described in this chapter, Olsen et al (Olsen et al., 2010, pp. 532-542) reported that an adult zebrafish model of Type 1 DM can be induced by administration of the diabetogenic drug streptozocin. Based on previous studies (Moss et al., 2009), it was hypothesized that removal of streptozocin exposure would allow for the endogenous regeneration of pancreatic β-cells, resulting in a return to normal glycemia without the need for insulin injection or pharmaceutical intervention. In order to test this, hyperglycemia (315 mg/dL) was induced in a group of zebrafish by the standard 3-week induction protocol (the end of induction considered day 0) and fasting blood glucose levels (FBGLs) of these fish were assessed at various time points post–drug removal (Figure 7A). Within the first 7 days of drug removal, the diabetic fish were still hyperglycemic but to a lesser extent (194 mg/dL); however, by 14 days the fish had returned to a euglycemic state (60 mg/dL). Zebrafish that were previously hyperglycemic and recovered to normal FBGLs following streptozocin drug removal are considered to be in a metabolic memory state. In this section, control fish = C, acute diabetic fish = DM, and metabolic memory fish = MM.

The mechanism by which streptozocin induces diabetes is the selective ablation of pancreatic β-cells resulting in reduced serum insulin levels and, ultimately, poor glycemic control. As such, it was determined whether the glycemic recovery observed in day-14 MM fish was accompanied by a repopulation of β-cells and increased serum insulin levels. Pooled samples of serum insulin (six fish per pool) and pancreas tissue were collected and analyzed from DM fish, day-14 MM zebrafish, and accompanying control fish. This analysis revealed that the serum insulin levels of DM fish were approximately 11% those of control fish and that by 14 days after drug removal, these levels had been restored to normal (Figure 7B). Pancreas tissue was fixed, and serial 10 μm transverse cryosections were made. The sections were then processed using double indirect immunofluorescence to visualize insulin (red) and glucagon (green), and the slides were counterstained with DAPI to visualize nuclei. Epifluorescence microscopy indicated that the insulin signal was greatly reduced in DM islets when compared with controls, as was previously documented (Figure 7B and C). Although full recovery was not seen by day 14 (Figure 7D and E), there was a significant repopulation of insulin-producing β-cells in pancreas tissue taken from MM zebrafish versus zebrafish in the acute DM state at this time point (compare Figure 7C and E). Nonetheless, serum insulin levels returned to normal within 14 days (Figure 7B).

6 β-cell Regrowth in the DM Zebrafish Model

As previously shown, Type 1 DM zebrafish not only display the known secondary complications of retinopathy and nephropathy but also exhibit an additional complication; impaired caudal fin regeneration (Olsen et al., 2010, pp. 532-542). Initially, studies were designed to determine whether this complication was susceptible to the MM phenomenon by observing the regenerative capacity of controls, acute DM (fish that were maintained in the acute diabetic state via continued streptozocin injection beyond time 0), and MM fish. Regeneration in these groups was determined by measuring caudal fin outgrowth 48 h post-amputation at 30, 60, and 90 days post–drug removal of the MM group (Figure 8). Fin regeneration of DM fish was impaired compared with control fish, as previously described (Olsen et al., 2010, pp. 532-542). Unexpectedly, the MM group’s ability to regenerate was reduced to levels nearly identical to
Figure 7: Fasting blood glucose normalizes and insulin production increases following cessation of streptozocin (STZ) administration. A: Graphic representation of fasting blood glucose concentration. Day 0: DM 315 +/- 40.96 mg/dL, C 55.9 +/- 6.491 mg/dL; P < 0.001. Day 7: DM 194.0 +/- 43.7 mg/dL, C 58.89 +/- 8.83 mg/dL; P < 0.05. Day 14: DM 62.5 +/- 13.6 mg/dL, C 58.88 +/- 13.36 mg/dL. Day 30: DM 59.5 +/- 8.88 mg/dL, C 54.9 +/- 5.19 mg/dL. Day 60: DM 53.1 +/- 8.83 mg/dL, C 58.6 +/- 6.70 mg/dL. Day 90: DM 58.66 +/- 4.59 mg/dL, C 59.9 +/- 6.65 mg/dL. n = 12 for all groups. B: Graphic comparison of insulin levels determined by enzyme-linked immunosorbent assay. Day 0: DM 1.13 +/- 0.14 mIU, C 9.92 +/- 0.40 mIU. Day 14: DM 9.10 +/- 0.41 mIU, C 9.32 +/- 0.42 mIU. Day 60: DM 9.51 +/- 0.08 mIU, C 10.33 +/- 1.1 mIU; n = 3 for all samples. Representative immunofluorescence microscopy images of pancreas tissue indicating presence of insulin (red), glucagon (green), and DAPI (blue). C: Control injected day 0. D: Streptozocin injected day 0. E: Control injected day 14. F: Streptozocin injected day 14. The scale bar indicates 50 µm. (For all immunohistochemistry, n = 10.)
those in the DM fish after their return to a euglycemic state. Therefore, impaired fin regeneration persists into the MM state even after physiological control of serum glucose levels had been achieved.

It was previously shown that DM-induced impaired tissue regeneration was not restricted to fin regeneration, but was also observed in the process of skin wound healing; indicating that the phenomenon was systemic in nature. To determine if impaired wound healing was also impaired in the MM state, the experiments were repeated in MM zebrafish. Following wounding, the percentage of wound closure was determined by comparing the remaining open-wound area (24 h post-wounding) with the original wound area (see Table 2). These results indicated, as previously found, wounds of DM fish heal slower than those of their control counterparts and that this reduction is maintained in 60-day MM fish. These data clearly demonstrate that fin regeneration and skin wound healing both remain impaired after glycemic homeostasis is achieved and therefore, both are susceptible to the MM phenomenon.

**Figure 8**: Impaired fin regeneration persists after recovery from the diabetic state. Graphic representation of the relative fin regeneration (48 h postamputation) of DM and MM fish compared with C fish. Day 0: DM and MM 68.0 +/- 2.18%. Day 30: DM 66.2 +/- 4.24%, MM 66.5 +/- 2.53%. Day 60: DM 56.4 +/- 4.25%, MM 62.1 +/- 3.95%. Day 90: DM 61.4 +/- 4.71, MM 63.6 +/- 3.95%. The number of replicates is as follows. Day 0: C, n = 39; DM, n = 69. Day 30: C, n = 30; DM, n = 32; MM, n = 37. Day 60: C, n = 25; DM, n = 12; MM, n = 16. Day 90: C, n = 20; DM, n = 10; MM, n = 10. (In all cases, P < 0.001 compared with C.) The rate of control (C) regeneration was set to be 100% and is indicated by the dotted line. STZ, streptozocin.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Percent Wound Closure</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 C</td>
<td>87.5 +/- 1.41</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Day 0 DM</td>
<td>62.2 +/- 5.45</td>
<td>17</td>
<td>0.0028</td>
</tr>
<tr>
<td>Day 60 C</td>
<td>86.0 +/- 1.73</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Day 60 MM</td>
<td>63.8 +/- 3.02</td>
<td>17</td>
<td>0.00004</td>
</tr>
</tbody>
</table>

**Table 2**: Wound healing is impaired in DM Zebrafish. Data are means +/- SEM unless otherwise indicated. The P value was calculated for the day-0 and day 60 time points.
7 Impaired Tissue Regeneration in the MM State

Taking advantage of the zebrafish’s regenerative ability, studies were conducted to examine the heritable nature of MM \textit{in vivo}. These experiments required multiple rounds of caudal fin amputation, and the same three groups (C, DM, and MM) were used. At all time points presented below, a subset of fish was used for FBGL determination and yielded results as documented in figure 1 (Olsen \textit{et al.}, 2010, pp. 532-542). At day 0, all groups had their caudal fins amputated, their 48-h regenerative growth was documented, and they were allowed a 30-day period for re-growth. The caudal fins of these fish were then reamputated at 30 days, immediately proximal to the original transection line, and the fish were once again allowed a period of regenerative growth for an additional 30 days. The tissue grown from 30 to 60 days was termed, MM tissue (Figure 9, [hatched bars]). At 60 days, the caudal fins were once again amputated, but at this time the fish were split into two groups (for schematic, see Figure 9). For one-half, the caudal fin was cut immediately proximal to the original amputation site (Figure 9 [dotted line P, proximal cut]), and amputation for the other group was performed within the MM tissue (Figure 9 [dotted line D, distal cut, tissue never exposed to hyperglycemia]).

![Figure 9](image_url)

\textbf{Figure 9}: Schematic of amputation scheme employed to generate metabolic memory tissue. At time 0 caudal fins were amputated and allowed 30 days for regrowth (left panel). At 30 days the fins were again amputated and allowed an additional 30 period of regenerative growth (middle panel). This tissue is called metabolic memory tissue as it was generated outside of the hyperglycemic state. At 60 days the groups were split into 2 and either cut along the proximal (green line, P) or distal line (red line, D) (right panel).

The regeneration ability was determined at 48 h post-amputation and was expressed as the percent regeneration of controls (Figure 10). First, these data document that no differences could be detected in
the amount of regenerative growth, whether the amputation was performed proximally or distally (Compare proximal versus distal for each group [Figure 10]). Second, as expected, the regenerative ability of DM fish was impaired (Figure 10). Third, these experiments revealed that the MM tissue’s ability to regenerate was impaired to the same extent as both the tissue from which it was derived and DM tissue (Figure 10). These results indicate that the effect of MM was transmitted to the daughter cells and by definition, is therefore a heritable process.

Figure 10: Daughter tissue inherits the regeneration deficit of the previous hyperglycemic state. Graphic representation of caudal fin regeneration 48 h postamputation. (C proximal [CP] was set as 100%). C proximal: 100 +/- 3.96%. C distal (CD): 105 +/- 5.45%. DM proximal (DMP): 64.9 +/- 4.58%. DM distal (DMD): 55.8 +/- 4.54%. MM proximal (MMP): 58.7 +/- 4.27%. MM distal (MMD): 55.8 +/- 6.45%. In all cases, P < 0.01 compared with C. n = 12 for all groups except MM proximal and distal (n = 16).

Metabolic memory tissue was examined for the presence of AGEs and oxidative stress, as it has been reported that these molecules may underlie diabetes complications and MM (Brownlee, 2005, pp. 1615-1625; Pirola, Balcerczyk, Okabe, & El-Osta, 2010, pp. 665-675; Reddy & Natarajan, 2011). Immunofluorescence microscopy was performed to detect the presence of AGEs present in control and DM tissue and 60-day MM regenerating caudal fins (48 h postamputation). Serial longitudinal 10 µm cryosections were processed for immunofluorescence to visualize AGEs (green) and were counterstained with DAPI to visualize nuclei (blue) (Figure 11A–D). Tissue in control caudal fins (Figure 11A and C) and regenerating tissue of DM fins (tissue above top amputation line [Figure 11B and D]) were not found to contain AGEs. In contrast, all of the tissue exposed to hyperglycemia (below the distal amputation line of DM and below the proximal line in MM fish) produced a significant AGE-specific signal (Figure 11B and D). Importantly, the MM tissue (tissue between the two amputation lines) in the MM fins (Figure 11D) did not produce any fluorescence and did not appear to accumulate AGEs. The expression of nuclear factor-kB subunit p65 (rela) has been used as a marker of oxidative stress as well as stress signaling in DM and MM research (Kowluru et al., 2004, pp. 194-199; Li et al., 2006, pp. 2611-2619; Li et al., 2008, pp. 26771-26781). Therefore, experiments were conducted to analyze the expression of this stress marker.
via RT–quantitative PCR with RNA that was extracted from caudal fin tissue of C, DM, and MM zebrafish. RNA from DM zebrafish was found to have a 12.8-fold increase in the expression of *rela*. However, RNA extracted from MM tissue of MM fish did not yield a significant increase in the amount of *rela* expressed over that of control fish (Figure 11E). Hence, similar to the above AGE results, there does not appear to be increased reactive oxygen species–induced stress signaling in caudal fin tissue that has not been exposed to the hyperglycemic environment.

**Figure 11:** MM tissue does not accumulate AGEs or markers of oxidative stress. Fluorescence microscopy images indicating the presence of AGEs (green) and DAPI (blue) in regenerating caudal fin tissue are presented as indicated. A: Day 0, control. B: Day 0, streptozocin. C: Day 60, C. D: Day 60, MM. The amputation sites are indicated on the images, and for the 60 day fish both the distal cut (D) and proximal cut (P) are specified. Scale bar represents 50 μm (n = 10). E: Graphic representation of quantitative PCR analysis of *rela* expression. DM: 12.87 fold +/- 1.86 fold increase compared with C fish. MM: 1.3-fold +/- 0.42 fold increase compared with control (C) fish.

### 8 Correlation of DNA Epigenetic Changes to DM and MM Zebrafish

As MM is transmissible in fin tissue, and because it could not be documented that AGEs or increased oxidative stress occurs in metabolic memory tissue; the study turned to potential epigenetic changes. More specifically, the presence of persistent DNA methylation changes induced by the hyperglycemic environment was examined because DNA methylation is a known heritable process. Caudal fins were amputated and DNA was extracted from DM fish and control fish. Sixty day MM fish were then generated from this group, and DNA was extracted from the MM caudal fin tissue. The methylation status of 13,361 CpG islands within these DNAs was identified via the MeDIP-sequencing technique. This investigation revealed that hyperglycemia induced genome-wide demethylation, as the number of fully methylated CpG islands was reduced from 3,489 in control fish to 130 in the acute hyperglycemic state and the number of hypomethylated CpG islands concomitantly increased to 12,705 from 4,895 (Figure 12). CpG island analysis of DNA from the MM fins revealed that a hypomethylated state persisted, as the
The number of fully methylated genes remained very low (C 3,489 vs. MM 526 [Figure 12]), hypomethylated genes remained increased (C 4,895 vs. MM 5,924), and partially methylated genes increased (C 4,977 vs. MM 6,917) compared with control (C) fish. These results were verified for a subset of loci via a MeDIP quantitative PCR approach (Olsen et al., 2012). Lastly, the induced demethylation appeared to be global, given that the results were similar irrespective of island location (intergenic, intragenic, and promoter located CpG islands (See Olsen et al., 2012) for data).

![Figure 12: Hyperglycemia induces heritable persistent global DNA hypomethylation. Graphic representation of the methylation status of the 13,361 CpG islands (CGIs) examined. Fully methylated (FM): C, 3,489; DM, 130; MM, 520. Partially methylated (PM): C, 4,977; DM, 526; MM, 6,917. Unmethylated (U): C, 4,895; DM, 12,705; MM, 5,924.](image)

Not unexpectedly, due to the critical role in gene expression, altered DNA methylation is associated with several human diseases including multiple sclerosis, Alzheimer's disease, and many cancers (Laird, 2005, p. R65-R76; Goll & Bestor, 2005, pp. 481-514; Watanabe & Maekawa, 2010, pp. 145-167; Casaccia-Bonnefil, Pandozy, & Mastronardi, 2008, pp. 368-378; Mastroeni et al., 2011, pp. 1161-1180). Variations in “normal” DNA methylation have been correlated with many aspects of DM including, susceptibility (Morgan et al., 1999, pp. 314-318; Park et al., 2008, pp. 2316-2324; Ling et al., 2008, pp. 615-622; Caramori et al., 2012, pp. 739-744), insulin resistance (Zhao, Goldberg, Bremner, & Vaccarino, 2012, pp. 542-546), complication development (Sapienza et al., 2011, pp. 20-28), and early detection (Akirav et al., 2011, pp. 19018-19023; Toperoff et al., 2012, pp. 371-383; Rakyan et al., 2011, p. e1002300). Very recently, a comprehensive genomic DNA methylation profiling of type 2 diabetic islets revealed that 276 CpG loci displayed a significant hypomethylation phenotype and may provide insight on the dysregulation of diabetic islets and disease pathogenesis (Volkmar et al., 2012, pp. 1405-1426). The first report demonstrating a cause and effect relationship between hyperglycemia and altered DNA methylation documented that genomic hypomethylation was induced within the liver of type 1 diabetic rats as early as 2 weeks post hyperglycemia onset (Williams, Garrow, & Schalinske, 2008, pp. 2064-2069) [104]. In contrast, the same group reported that hepatic DNA hypermethylation was evident at 12
weeks of age in the Zucker diabetic fatty rat (a type II model) (Williams & Schalinske, 2012, pp. 123-131). Pirola et al examined primary aortic endothelial cells exposed to high glucose in vitro and performed a more comprehensive analysis of both histone acetylation and DNA methylation (Pirola et al., 2011, pp. 1601-1615). They used a methyl-capture followed by sequencing technique to assay DNA methylation changes. In this study they observed significant alterations in DNA methylation patterns and showed that induced hypermethylation localized to regions within 5 kilobases of transcriptional start sites. They also observed broad changes to H3K9/K14 acetylation and reported that regionalized hyperacetylation correlated very well with DNA hypomethylation and hyperglycemia induced gene induction. Unfortunately, none of the above studies have looked at the prolonged hyperglycemic or the metabolic memory state.

9 Future Research Directions

This chapter has highlighted the relationship of MM to DM and has provided an overview of both in vitro and in vivo approaches to understanding the underlying processes and mechanisms associated with MM. It has described the relationship of the primary deficit of DM (resulting in dysregulation in the control of glucose serum levels) and the immediate resulting pathologies of this dysregulation (such as abnormal ROS and AGE formation throughout the tissues of the body). It has also touched on current clinical and animal studies that point to epigenetic changes associated with long term tissue dysfunctions that appear to be occurring in the backdrop of the metabolic alterations associated AGE and ROS increases throughout the DM body. Each of these pathologies is equally important, and the current challenge is our ability to dissect these pathologies from one another so that multifaceted treatment approaches can be developed to address the full spectrum of problems resulting from the DM and secondary MM conditions.

The information provided in this chapter indicates that the STZ-induced Type I diabetic zebrafish animal model offers for the first time, an opportunity to dissect out the underlying molecular mechanisms associated with MM using a synchronized in vivo system. To this aim, studies are currently underway to address a multitude of issues related to MM using the diabetic zebrafish model. These studies relate to such questions as: 1) what MM alterations are occurring in tissues classically known to be affected in DM (e.g. kidney, retina, peripheral nervous system, and the skin), 2) what epigenetic changes are associated with tissues of the zebrafish model other than the fin (e.g. kidney, retina, peripheral nervous system, and the skin), 3) are there common inter-relationships of regulatory genes among the various tissues of the DM zebrafish that relate to the MM condition, and 4) can the DM zebrafish model be used to test pharmacological and genetic approaches to intervene in the tissue dysfunctions associated with the MM state. These are just a few of many important questions that can now be approached using the zebrafish DM/MM model.

List of Abbreviations

DM, Diabetes mellitus
ROS, Reactive oxygen species
MM, Metabolic memory
NG, Normal glucose levels
Acknowledgments

This work was supported by a research grant from the Iacocca Family Foundation, Rosalind Franklin University start-up funds, and National Institutes of Health Grant DK092721 (to R.V.I.). No potential conflicts of interest relevant to this article were reported.

References


