Diabetic complications within the context of aging: Nicotinamide adenine dinucleotide redox, insulin C-peptide, sirtuin 1–liver kinase B1–adenosine monophosphate-activated protein kinase positive feedback and forkhead box O3

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ABSTRACT
Recent research in nutritional control of aging suggests that cytosolic increases in the reduced form of nicotinamide adenine dinucleotide and decreasing nicotinamide adenine dinucleotide metabolism plays a central role in controlling the longevity gene products sirtuin 1 (SIRT1), adenosine monophosphate-activated protein kinase (AMPK) and forkhead box O3 (FOXO3). High nutrition conditions, such as the diabetic milieu, increase the ratio of reduced to oxidized forms of cytosolic nicotinamide adenine dinucleotide through cascades including the polyol pathway. This redox change is associated with insulin resistance and the development of diabetic complications, and might be counteracted by insulin C-peptide. My research and others’ suggest that the SIRT1–liver kinase B1–AMPK cascade creates positive feedback through nicotinamide adenine dinucleotide synthesis to help cells cope with metabolic stress. SIRT1 and AMPK can upregulate liver kinase B1 and FOXO3, key factors that help residential stem cells cope with oxidative stress. FOXO3 directly changes epigenetics around transcription start sites, maintaining the health of stem cells. ‘Diabetic memory’ is likely a result of epigenetic changes caused by high nutritional conditions, which disturb the quiescent state of residential stem cells and impair tissue repair. This could be prevented by restoring SIRT1–AMPK positive feedback through activating FOXO3.

INTRODUCTION
The present review is based on my research experiences and written as a historical perspective review, rather than as a comprehensive review. I used this approach for several reasons, the first of which is that the biochemical consensus view of this topic has not changed in the past 10 years. Second, this format allows me to include more negative data than would otherwise be appropriate. Third, a historical perspective might be more interesting for new readers. For a comprehensive review focusing on the general biochemistry of diabetic complications, see Culcutt et al.1 Diabetic neuropathy was reviewed by Yagi-hashi et al.2, and a review of diabetic nephropathy from the perspective of cell biology was recently written by Maezawa et al.3

POLYOL PATHWAY AND THE PSEUDO-HYPOXIA HYPOTHESIS
When I started PhD courses at Hirosaki University, Hirosaki, Japan, under Professor Yagihashi in pathology, the hottest topic in the field of diabetic complications was the polyol pathway4,5.
This might be the first theory that mechanistically explains how high-glucose-induced changes in metabolism lead to diabetic complications. The polyol (sorbitol) pathway consists of the two enzymatic reactions: (i) glucose + reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) \(\Rightarrow\) sorbitol + oxidized form of nicotinamide adenine dinucleotide phosphate (NADP\(^+\)); and (ii) sorbitol + oxidized form of nicotinamide adenine dinucleotide (NAD\(^+\)) \(\Rightarrow\) fructose + nicotinamide adenine dinucleotide (NADH). The first reaction is catalyzed by aldose reductase, and the second is catalyzed by sorbitol dehydrogenase. When intracellular free glucose increases, flux through the cascade increases. The development of aldose reductase inhibitors (ARI), a class of drugs that specifically targets diabetic complications, accelerated interest in this field and provided the first hope of stopping the development of complications in humans\(^{8,7}\). Using animal models of diabetes, mostly rodents, more than one of these inhibitors has helped prevent retinopathy, nephropathy, neuropathy and vasculopathy\(^{7-9}\).

After receiving my PhD, I continued to study diabetic complications with Professor Joseph Williamson at Washington University in St. Louis, Missouri, USA. At that time, his laboratory had just developed a method to assess albumin vascular permeability and regional blood flow using radio-isotopes\(^{10,11}\). There, I developed the mathematical basis for the measurement of vascular permeability and glomerular filtration rate\(^{12}\), allowing us to more clearly monitor the nature of vascular dysfunction associated with diabetes or high glucose. In addition to using streptozotocin-injected rats (hereafter referred to as STZ diabetic rats), his laboratory also developed a granulation tissue chamber model. Granulation tissue (connective tissue that is generated during wound healing) is grown inside small chambers (typically 2) mounted on the back of a rat. The initial study was carried out in diabetic rats, and found that newly-formed vessels in granulation tissues were leakier than the ones in non-diabetic rats. This leak could be prevented by aldose reductase inhibitors. Subsequently, they found that even in non-diabetic rats, application of 25 mmol/L glucose to granulation tissue directly twice daily for 10 days caused a similar three- to fourfold increase in vascular permeability and blood flow\(^{13}\). This model was quite useful to screen compounds that could potentially ameliorate high-glucose-induced vascular dysfunction. As we published, inhibitors of aldose reductase\(^{13}\) and protein kinase C\(^{14,15}\), aminoguanidine\(^{16}\), acetyl-carnitine\(^{17}\) and insulin C-peptide\(^{18}\) were found to suppress these changes caused by high glucose. In addition to glucose, lactate and non-esterified fatty acids (palmitate) induced similar dysfunction.

Regarding ARI, we hypothesized that ARI not only inhibit the first step of the polyol pathway (glucose to sorbitol), but also decrease flux to the second step (sorbitol to fructose). The second step, which produces NADH from NAD\(^+\), is catalyzed by sorbitol dehydrogenase. It has been known since the 1960s that various tissues from diabetic rats\(^{19}\), including lens\(^4\) and liver\(^{20,21}\), show an increase in the ratio of NADH to NAD\(^+\) compared with non-diabetic rats. In non-diabetic conditions, this is a typical sign of hypoxia (low blood flow), which is caused by impaired utilization of NADH in mitochondria and accumulation of lactate. In diabetes, tissue hypoxia has been implicated in the pathogenesis of complications, retinopathy and neuropathy in particular. However, as glucose increased blood flow in the non-diabetic rats of our tissue chamber model, the vascular dysfunction was not caused by hypoxia. To test whether vascular dysfunction was causally linked to increased cytosolic NADH/NAD\(^+\) ratios, 1 mmol/L of pyruvate was added with high glucose. Pyruvate converts NADH to NAD\(^+\), and produces lactate, thus decreasing the NADH/NAD\(^+\) ratio. Pyruvate treatment reduced vascular dysfunction. In addition, pyruvate also prevented vascular dysfunction caused by 10 mmol/L lactate with 5 mmol/L glucose\(^{19}\). During this experiment, we found that lactate-induced dysfunction could be seen in blood flow just 5 h after incubation without affecting vascular permeability in tissue chambers\(^{22}\), and by bolus injection to the rats. This suggests that blood flow controlled by redox state is an acute process that could be more proximal than a change in permeability. Indeed, we showed that increased regional blood flow by physiological stimuli (muscle blood flow by electrical muscle contraction\(^{23}\), and increased retinal and visual cortex blood flow by visual stimulation\(^{24}\) are the result of similar NADH/NAD\(^+\) redox changes. These changes in blood flow could also be augmented by lactate infusion and suppressed by pyruvate infusion. This phenomenon was also observed in the human brain\(^{25}\). Experiments with longer duration of diabetes in STZ diabetic rats showed different results in blood flow. Increased regional blood flow could be observed up to 8 weeks. However, from 10 to 28 weeks of STZ diabetes, blood flow was no longer different from non-diabetic controls, whereas albumin permeation and NADH/NAD\(^+\) redox change were still present from 6 to 28 weeks of diabetes. This showed that in longer durations of diabetes, the mechanisms to increase blood flow (likely affecting nitric oxide bioactivity) become inoperative.

This series of studies led us to postulate the ‘pseudohypoxia hypothesis’ (increased cytosolic NADH levels without real hypoxia) as a pathogenic mechanism of diabetic complications\(^{19,22,26-28}\). The original theory focused on high-glucose-induced mechanisms (polyol pathway). Later studies showed that pseudohypoxia could be caused by hyperlactatemia (obesity) and high non-esterified fatty acids conditions (type 2 diabetes), which cause similar vascular dysfunctions\(^{29}\). Although the theory is old, the essence of this hypothesis is still valid, and its implications for current cell biology are beginning to unfold. The increased NADH/NAD\(^+\) ratio affects sirtuin 1 (SIRT1) and adenosine monophosphate-activated protein kinase (AMPK) activity, as discussed later. Increasing NADH should also mediate epigenetic regulation through C-terminal binding protein\(^{29}\), whose genome-wide profiles were recently characterized in breast cancer\(^{30}\). Activation of C-terminal binding protein by NADH causes the epithelial-to-mesenchymal transition.
and genome instability, whereas C-terminal binding protein depletion or caloric restriction (decreasing NADH) reverses the gene repression and increases DNA repair. These factors are likely related to cancer risk or progression in diabetes.\textsuperscript{31}

**BRIEF NOTE ON THE CONTROVERSIAL CHANGES IN BLOOD FLOW IN EARLY STZ DIABETIC RATS**

In humans, angiopathy is a late diabetic complication that impairs blood perfusion. However, there were conflicting reports in rats of how retinal and sciatic nerve blood flow were affected by early STZ diabetes. Professor Williamson’s lab used the microsphere method, which is still the ‘gold standard’\textsuperscript{32}, and found increased blood flow in the retina, which contradicted results from Dr King’s group reporting a decreased blood flow as assessed by video fluorescein angiography\textsuperscript{33}. We could not resolve this discrepancy. However, in human diabetes, dilatation of the retinal arterioles was found, leading to speculation that loss of myogenic response and subsequent increased arterial diameter would permit physiologically high blood flow to reach the capillaries in the retina.\textsuperscript{34}

Sciatic nerve blood flow in STZ diabetes is decreased in other researchers’ reports, as shown by the hydrogen clearance and laser-Doppler methods, which also contradicts our observation. However, we showed that exposing the sciatic nerve alone, as occurs during the hydrogen clearance/laser-Doppler method, increased blood flow in control rats, but not diabetic rats. Thus, blood flow appears lower in diabetic rats, and this was corrected by treatment with an ARI\textsuperscript{35}. In human diabetes, it was suggested that a change in vascular reactivity apparently predates the development of neuropathy\textsuperscript{36}. Therefore, a method that measures absolute blood flow might produce a different result than a method that measures changes in blood flow in these tissues.

**INSULIN C-PEPTIDE**

In addition to studying redox effects on the vasculature, I also studied the insulin-connecting peptide (C-peptide). Initially, C-peptide was considered a mere by-product of insulin maturation, a cleavage product without biological activity. However, there were some reports as early as 1975 showing biological actions\textsuperscript{37,38}, including a report that synthetic C-peptide increased exogenous insulin effects in alloxan diabetic rats\textsuperscript{39}. In the 1990s, Professor Wahren and other researchers showed more clearly that C-peptide had beneficial effects. C-peptide increased Na-K-adenosine triphosphatase (Na-K-ATPase) activity in kidney tubulus\textsuperscript{40}, glucose uptake in rat skeletal muscle\textsuperscript{41} and decreased high glomerular filtration rate in diabetes patients\textsuperscript{42}. We investigated C-peptide actions in our systems:\textsuperscript{19} the tissue chamber model and STZ diabetes. In the tissue chamber model, full-length human and rat C-peptide ameliorated high-glucose-induced vascular dysfunction in a dose response fashion. Both human and rat C-peptide was fully effective at 100 nmol/L. A total of 10 amino acids in the middle of the sequence were critical for its function. In the STZ diabetic rat model, 400 μg/kg C-peptide injected subcutaneously twice a day prevented vascular dysfunction in the retina, kidney, sciatic nerve, and aorta at 6 weeks and 3 months’ duration of diabetes. In addition, it prevented diabetes-induced decreases in nerve conduction velocity and nerve Na-K-ATPase activity in diabetes. Other authors\textsuperscript{43,44} also observed the beneficial effects of C-peptide on peripheral neuropathy in STZ-rats.

C-peptide distribution and kinetics in vivo was investigated in both non-diabetic and STZ diabetic rats using \textsuperscript{125}I-labeled C-peptide (unpublished data). Time-dependent distribution showed no clear preferential location for C-peptide accumulation, and the majority of radioactive C-peptide was cleared from the kidney. Non-compartmental analysis showed that compared with non-diabetic rats, diabetic rats had twofold C-peptide whole-body distribution volume and twofold delayed clearance, suggesting that C-peptide distribution and clearance are affected by diabetes.

Although rat C-peptide was slightly more effective at lower concentrations than human C-peptide in the tissue chamber model, the concentration required for correction of vascular dysfunction (10–100 nmol/L) appeared to be more than the physiological concentration of C-peptide (several hundred pmol/L). Peak human C-peptide levels were approximately 9 nmol/L at 10 and 30 min after single subcutaneous injection at the dose of 400 μg/kg in the rats. Professor Wahren’s group suggested that C-peptide treatment is only beneficial in type 1 diabetes, in which endogenous C-peptide levels drop below the physiological levels necessary to maintain normal functions.\textsuperscript{45} As our tissue chamber model is made on non-diabetic rats, the microvessels of granulation tissue are exposed to normal levels of endogenous C-peptide. Therefore, the present results showed that an additional dose (pharmacological rather than physiological dose) of C-peptide was required to prevent high-glucose-mediated insults.

We further tested this hypothesis in an acute glucose infusion model. Intravenous glucose infusion achieving 350–450 mg/dL plasma glucose, into non-diabetic rats for 5 h causes an increase in regional blood flow similar to that observed in diabetic rats. Glucose infusion induces secretion of insulin and C-peptide from pancreatic β-cells, therefore this model is not C-peptide deficient, but has excess C-peptide during glucose infusion. We found that co-infusion of C-peptide did not prevent glucose-induced changes in blood flow in eye tissues (retina, anterior uvea, posterior uvea), the kidney and sciatic nerve (Figure 1). Similarly, C-peptide subcutaneous injection or infusion 1 day before glucose infusion had no effect (data not shown). However, C-peptide fully prevented glucose-induced increases in blood flow if 400 μg/kg C-peptide was injected twice daily for 2 days before glucose infusion (Figure 1). These results suggest that C-peptide action in preventing vascular dysfunction is not acute, and the high concentrations of C-peptide might require eliciting this effect. C-peptide itself does not reverse the redox change caused by high glucose\textsuperscript{18}, yet twice daily injections for 2 days completely prevented blood...
flow increases by lactate infusion (unpublished data). This suggests that C-peptide counteracts the downstream events caused by redox changes. As C-peptide also worked in vascular permeability and peripheral nerve dysfunctions, the point(s) at which C-peptide targets are very close to the redox change, perhaps mediated by transcription factors and changes in gene expression.

Clinical studies of C-peptide have been mainly carried out by Professor Wahren, but they have not yet shown a beneficial effect. The receptor(s) for C-peptide has not been identified, despite high-affinity binding shown by surface plasmon resonance. Discrepancies in reports of C-peptide-mediated signal transduction also make it difficult to understand its beneficial actions in the context of diabetes. However, it has been reported that low nmol/L levels of C-peptide activate AMPK. I also independently found this phenomenon using 100 nmol/L C-peptide when I began to study AMPK in 2001 in cultured human umbilical vein endothelial cells (although the activation was modest compared with the AMPK activator metformin). C-peptide also attenuated 25 mmol/L glucose-induced apoptosis; however, the effect was not impressive compared with AICAR (an AMPK activator). Interestingly, although D-amino C-peptide could also prevent vascular dysfunction in vivo, it was completely ineffective in preventing apoptosis (unpublished data). Therefore, activation of AMPK might not mediate the effect of C-peptide on vascular function in rats. Activation of AMPK by native C-peptide was acute (within 15 min), suggesting that it was likely mediated by calcium/calmodulin-dependent protein kinase kinase beta activation. This phenomenon echoes a recent report that C-peptide binds to a class of G-protein-coupled receptors, analogous to the activation of AMPK by glucagon-like peptide-1, as we reported in endothelial cells. However, in contrast to glucagon-like peptide-1, an early report suggests that C-peptide might suppress insulin secretion, as shown by Toyota et al. Activation of AMPK could explain another early report that C-peptide increased glucose uptake that appeared to be insulin-independent, and activated nitric oxide synthase. However, the report that C-peptide increases Na-K-ATPase activity in kidney tubules is not compatible with observations that AMPK activation decreases Na-K-ATPase activity by inducing its internalization. Our studies using STZ diabetic rats, C-peptide did not change blood glucose levels or body weight with/without a low dose of insulin, which also does not support AMPK activation as a major action.

During our analysis of C-peptide sequence and function, we noticed that effective C-peptide sequences were very flexible. Reverse sequence and D-amino acid sequence were also functional. In addition to the middle part of the peptide, the C-terminus penta-peptide (which can be cleaved-off in vivo) was also functional (unpublished data). However, its randomized sequence was not functional. This indicates that C-peptide action appears to be mediated by at least two different parts of the peptide. Three-dimensional structures of the reverse sequence and D-amino acid sequence should be quite different, and therefore they must be recognized as random structures. The requirement for high concentrations suggests that these parts bind to low-affinity receptors, which might be the reason why it could not be identified by radiolabeling. In addition, this scenario explains the diversity of C-peptide actions, which might strongly depend on the receptors expressed. Alternatively, as suggested by Professor Kahn who commented on our paper, the effect we saw might not be mediated by its receptor(s).

In summary, beneficial effects of C-peptide supplementation have not been confirmed in patients with type 1 diabetes. This could be because C-peptide elicits its beneficial effect at concentrations much higher than physiological concentrations. If so, it might also work in type 2 diabetes. Mechanistically, C-peptide counteracts downstream events caused by increased NADH/NAD⁺, possibly through changing gene expression.
TARGETING INSULIN RESISTANCE AS A GENERAL MECHANISM FOR CELL VULNERABILITY IN DIABETES

On the retirement of Professor Williamson, I began working with Dr Neil Ruderman at Boston University. I continued to study diabetic vascular complications, but decided to shift my focus. At that time, my hypothesis was that insulin resistance could be a fundamental cell biological problem in diabetes and its complications. In vivo, insulin resistance can be a result (type 1 diabetes) and cause (type 2 diabetes) of hyperglycemia. The insulin receptor is found in almost every cell type. A major signaling event of insulin is activation of Akt that can mediate anti-apoptotic signaling. Thus, my hypothesis was that insulin resistance could increase the vulnerability of cells to high-glucose-induced stress. Based on observations that lactate infusion alone causes insulin resistance, I proposed that high-glucose-induced redox changes could be related to insulin resistance.

AMP-ACTIVATED PROTEIN KINASE AND OXIDATIVE STRESS

When I started studying AMPK, it was already known that AMPK activation ameliorates insulin resistance; however, its presence and function were not known in endothelial cells. I first established the AMPK signaling cascade in endothelial cells in culture. I then established a cell culture model to induce apoptosis by high glucose, and examined whether AMPK activation prevented apoptosis by increasing Akt activity. The result was positive, and served as the earliest indication that AMPK activation could attenuate NF-κB signaling. I found that AMPK activation prevented palmitate and tumor necrosis factor-β-mediated NF-κB transactivation. Palmitate is a substrate of ceramide de novo synthesis that was already known to cause insulin resistance in skeletal muscle. In endothelial cells, both high glucose and high palmitate suppressed AMPK activation, and caused insulin resistance. We further investigated this phenomenon in cultured retinal pericytes. Similar to glucose, but at a much faster rate, palmitate induced apoptosis. This was mediated by increases in ceramide, nicotineamide adenine dinucleotide phosphate oxidase-mediated oxidative stress and activation of NF-κB. Apoptosis, as well as palmitate-induced endoplasmic reticulum stress, could be prevented by AMPK activation. In the latter study, activation of AMPK did not prevent oxidative stress, as measured by 2’,7’-dichlorofluorescein fluorescence or electron spin resonance spectroscopy. In addition, 0.1 mmol/L palmitate produced peak levels of 8-iso-prostanones without causing apoptosis, whereas oleate produced more 8-iso prostanes than palmitate, but did not induce apoptosis. These results suggest that oxidative stress is necessary, but not sufficient to produce cell damage in this model.

In 2000, Brownlee et al. showed that incubation of bovine aortic endothelial cells with high glucose induced oxidative stress from mitochondria. We repeatedly tried to reproduce these results. However, we were not able to see any increase in oxidative stress by high glucose alone in aortic endothelial cells from human or bovine, human microvascular, or umbilical vein endothelial cells. In humans, anti-oxidant treatment has not been shown to be effective in any disease conditions, suggesting that although oxidant levels might be increased, the levels themselves might not be enough to cause pathology. On the contrary, it was reported that anti-oxidants prevent health-promoting effects of physical exercise in humans, suggesting that physiological levels of oxidative stress have an important role in inducing adaptive responses to stress as described later through forkhead box O3 (FOXO3).

Brownlee’s study created a lot of interest in the role of mitochondria in diabetes and its associated conditions. There is no question of its importance. However, compared with the time when they published, research focus has shifted from metabolic and biochemical-orientated studies (which examine metabolites and proteins) to cell biology-oriented researches (which examine organelles and cell dynamics). From a cell biology standpoint, sustained mitochondrial dysfunctions are likely to be as a result of problems of mitochondrial quality control mechanisms (mitochondriogenesis, autophagy [mitophagy] and fusion–fusion cycle). In this respect, AMPK was shown to increase mitochondrial genesis by activating PGC-1 and autophagy by phosphorylating Ulk-1 and inhibiting mechanistic target of rapamycin signaling. In recent review articles about diabetic complications, AMPK has been postulated as a potential target in nephropathy, retinopathy, and neuropathy for its putative actions on mitochondria.

SIRT1, SENESENCE AND LONGEVITY GENE PRODUCTS

Around 2004, my study interest became to include understanding diabetes, and its complications within the context of aging, senescence and epigenetics. In their early articles on sirtuins in Caenorhabditis elegans and other lower organisms, Drs Gurante and Sinclair listed longevity gene products including Amp-1 (Ampk), Sir2, Tor, Foxo, p66Shc, Clock, Klotho and Catalase. For myself and other researchers studying AMPK, it was clear some of them (AMPK, SIRT1 [Sir2 ortholog], mechanistic target of rapamycin, FOXO, p66HSC and CATALASE) were interrelated even at that time and more clearly nowadays (Figure 2). However, the connection between AMPK and SIRT1 was not clear, so I decided to study this relationship. Together with Dr Fan Lan, we showed for the first time that sirtuin activators, such as resveratrol and other polyphenols, activate AMPK through liver kinase B1 (LKB1). After a number of experiments, we concluded that SIRT1 deacetylates LKB1 at K48, which induces its translocation to the cytosol where it becomes activated. This cascade was confirmed by
Sinclair’s group\textsuperscript{73}, and is cited as one of the main connections between these two signaling pathways. We also showed that this activation cascade operates in aortic endothelial cells during exercise\textsuperscript{84}, which might explain its beneficial effects.

Around the same time when we published the relationship between AMPK and SIRT1, another group suggested that AMPK might activate sirtuins by increasing NAD\textsuperscript{+} through activation of the key enzyme, nicotinamide phosphoribosyltransferase\textsuperscript{25}, to synthesize NAD\textsuperscript{+} from nicotinamide\textsuperscript{26}. The reaction would increase NAD\textsuperscript{+} and decrease nicotinamide, an endogenous SIRT1 inhibitor. We also found that there seemed to be concurrent regulation of AMPK and SIRT1 by NADH/NAD\textsuperscript{+} redox change induced by the addition of pyruvate\textsuperscript{77}. Similar Sir2 regulation by redox was reported earlier\textsuperscript{78}. These observations suggested an interrelationship between AMPK and SIRT1\textsuperscript{79}. This cascade together with our cascade would create a positive feedback loop (Figure 3). However, as the synthesis of NAD\textsuperscript{+} requires two ATP, this cascade might be operative only when cellular ATP levels are relatively normal. Indeed, the final reaction nicotinamide mononucleotide + ATP ⇔ NAD\textsuperscript{+} + pyrophosphate by nicotinamide mononucleotide adenylytransferase is reversible\textsuperscript{80}, thus it might work for both nicotinamide and NAD\textsuperscript{+} synthesis. Consistent with this, it was reported that cytosolic NAD\textsuperscript{+} content is determined by ATP concentration\textsuperscript{81}. Therefore, if ATP production is compromised by mitochondria dysfunction, the cascade from AMPK–NAD\textsuperscript{+}–SIRT1 might not be operative. Currently, I believe that this positive feedback loop (Figure 2) is the key to maintaining homeostasis. Interestingly, a recent publication by Sinclair\textsuperscript{82} explained that a declining NAD\textsuperscript{+} induces a ‘pseudohypoxic state’, as it accumulates hypoxia-inducible factor-1α.

The SIRT1 activator, resveratrol, has been studied in human subjects and showed some beneficial effects, although there are also reports indicating that it has no effect. At this point (2015), I’m not aware of pharmaceutical companies pursuing the development of a SIRT1 activator. However, the structures of SIRT1 and of its activators became available this year\textsuperscript{83}, which might raise industrial interest again.

**RECENT DEVELOPMENT IN AGEING RESEARCH AND IMPLICATIONS FOR DIABETIC COMPLICATIONS**

Research on aging shown by heterochronic parabiosis experiments suggests that: (i) keeping a healthy population of quiescent residential (or adult) stem cells (which have both the ability to self-renew and differentiate) are key for tissue regeneration\textsuperscript{84–86}; and (ii) there are factors in blood that define age-related tissue regeneration capacity\textsuperscript{87}. As differentiated cells have a limited lifespan, stem cells, rather than differentiated cells already affected by disease, should be the targets for treatment. Nerve Schwann cells, retinal pericytes, glomerular parietal epithelial cells for podocytes and residential endothelial cells are examples of such potential targets for treating diabetic complications.
Longevity gene products (AMPK, SIRT1, etc.) act on these residential stem cells rather than on already differentiated cells, as I observed in human skin keratinocytes and mouse 3T3-L1 pre-adipocytes while investigating the causes of cellular senescence. Contrary to its name, senescent cells are metabolically active, and produce a number of cytokines and chemokines that induce inflammatory signals to surrounding cells. Thus, elimination of senescent cells in vivo delays age-associated disorders. Human skin keratinocytes are adult stem cells that renew indefinitely in vivo. Knockout of insulin receptor in keratinocytes in mice resulted in thinner skin, and interestingly, glucose intolerance. I reported that resveratrol prevented oxidative stress-induced cellular senescence, and restored insulin action in cell proliferation. In these capacities, SIRT1–LKB1–AMPK–FOXO3 cascade played a significant role. Pre-adipocytes are also believed to have stem cell characteristics. They supply additional adipocytes during progression of obesity, and a recent study suggested that proliferated pre-adipocytes become senescent. We found that Sirt1 knockdown in mouse 3T3-L1 cells become senescent after differentiation, and express IL-6 and transforming growth factor-β. These phenotypes were prevented by expression of LKB1K48R, suggesting that LKB1 activity could play a significant role in senescence. Consistent with our observations, LKB1 and FOXO were found to be the main factors that enable stem cells to cope with stress.

**ROLES OF FOXO3 ON EPIGENETICS**

The recent advancement of epigenetics and deep sequencing techniques is simply remarkable. The human genome project is a reference map of human genes and structures, and subsequent efforts to compile polymorphisms will reveal genetic influences on disease progression. Epigenetic differences among individuals can be the result of both genetic variability (polymorphisms) and environmental history to which the cells are exposed. In the case of diabetes, this would include high glucose and fat, oxidants, hormones, cytokines, and so on. It is known that diabetes is a condition that impairs tissue repair. Thus, it is likely that the epigenetics of these stem cells are not normal, which could cause ‘diabetic memory,’ and this might result in sustained tissue damage. If diabetic complications are the result of cell-specific changes in epigenetics, what are they and which transcription factors are involved? This question has not been answered yet. The proteins that recognize DNA sequences are primarily transcription factors. FOXO3 is the only transcription factor so far identified as a longevity gene product. Genotypes of FOXO3 polymorphisms (rs2802292 and rs10457180) and possibly FOXO1 are associated with longevity. FOXO3 directly modifies epigenetic patterns around transcription start sites to enhance gene expression. Perhaps FOXO3 is an aging master regulator. The fresh water creature, Hydra, is famous for its immortality. FOXO mediates immortality by maintaining self-renewal capacity of its three stem cell lineages. FOXO3 activity can be upregulated by SIRT1 and AMPK, and acutely by oxidative stress. In keratinocytes, resveratrol enhances insulin action for proliferation only in the presence of low levels of oxidative stress. Thus, it might work as a center for adaptive responses to stress along or together with nuclear factor (erythroid-derived)-like 2. FOXO3 plays a central role in controlling oxidative stress and insulin signaling, and FOXO3 polymorphisms are associated with insulin sensitivity. Thus, failure to respond properly to oxidative stress might be the feature of diabetes.

**SUMMARY AND THE FUTURE DIRECTIONS FOR THE STUDY OF DIABETIC COMPLICATIONS**

Diabetes research, in particular research in one of its complications, enters the stage in cell biology and molecular medicine from just physiology and biochemistry. To address the ‘diabetic memory’ problem, we should focus on a treatment strategy targeting the epigenetics of progenitor or adult stem cells responsible for tissue regeneration. Ideally, this should be carried out in cultured human cells under the ‘diabetic milieu,’ and apply those results to diabetic animal models, followed by correlation studies in human samples. The ‘diabetic milieu’ is typically described as high glucose; however, serum factors of diabetes...
other than nutrition need to be explored further. From the heterochronic parabiosis experiments, it is clear that aging factors and possibly juvenile factors (GDF11 might not be the one) are present in serum. Although aging research helps to understand some aspect of diabetes, diabetic complications are unique in their pathology, thus different factor(s) or cascade(s) are likely involved. Once a significant genome-wide association of polymorphisms for diabetic complications is established, and cascade-wise polymorphisms (such as the AKR1B1 microsatellite allele [Z-2] and C-106T99) are more readily available, we might be able to use the cells derived from those individuals to differentiate target cell types by induced pluripotent stem cell or transdifferentiation techniques and test the effects of the diabetic milieu on epigenetics. It might also be feasible to carry out drug studies targeting individuals with these polymorphisms prone to diabetic complications (tailored-made medicine).

LKB1 is required for adult stem cells to cope with stress conditions. The Schwann cell-specific LKB1 knockout mouse was created recently100, and shows peripheral neuropathy preferential to sensory nerves and small fibers, resembling diabetic peripheral neuropathy. It is characterized by progressive non-inflammatory axonopathy, and also by increased NADH/NAD+ ratio and an increased AMP/ATP ratio. Similar to the way in which C-peptide antagonizes the effects of changes in redox through a seemingly AMPK-independent mechanism, the effects of LKB1 on redox are AMPK independent. My current research focuses on how LKB1 activation could help diabetes-related pathology, and explores novel ways to measure its activity in situ and on treatment with activators. With advanced molecular technologies, we now have new ways to address problems of diabetic complications.

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