parenchymal and stromal cells in response to tissue stress or malfunction, thereby leading to functional maladaptation and tissue remodeling [12]. Recent studies have demonstrated that obese adipose tissue is characterized by adipocyte hypertrophy, followed by increased angiogenesis, immune cell infiltration, extracellular matrix overproduction, and thus, increased production of proinflammatory adipocytokines during the progression of chronic inflammation (**Fig. 1**) [1, 2, 13, 14]. This is reminiscent of the chronic inflammatory responses in atherosclerotic vascular walls, termed vascular remodeling, which is mediated through complex interactions among vascular endothelial cells, vascular smooth muscle cells, lymphocytes, and monocyte-derived macrophages (Fig. 1) [4]. Thus, the dynamic change seen in obese adipose tissue can be referred to as adipose tissue remodeling, in which stromal cells change dramatically in number and cell type during the course of obesity (Fig. 1). Given the multifunctional roles in a variety of biological contexts, among stromal cells, macrophages should play a central role in adipose tissue remodeling. In this regard, adipose tissue remodeling may be viewed as chronic inflammation that involves adipocyte hypertrophy, macrophage infiltration, and adipocyte-macrophage interaction (**Fig. 2**).

ADIPOSE TISSUE MACROPHAGE INFILTRATION

A previous study with bone marrow transplantation demonstrated that most macrophages in the adipose tissue are derived from the bone marrow [7]. In this regard, increased expression of chemokines in obese adipose tissue has been implicated in the control of monocyte recruitment to the adipose tissue. There is considerable evidence for the pathophysiologic role of the MCP-1/CCR2 pathway in macrophage infiltration into obese adipose tissue {Fig. 2, (ii) [15–18]}. Weisberg et al. [15] reported the attenuation of macrophage accumulation and chronic inflammation in the adipose tissue from mice lacking CCR2 ($CCR2^{-/-}$ mice) during a high-fat diet. Moreover, two previous studies with transgenic mice overexpressing MCP-1 in the adipose tissue and MCP-1-deficient mice ($MCP\text{-}1^{-/-}$ mice) showed that MCP-1 plays a role in the recruitment of macrophages into obese adipose tissue [16, 17]. Through a combination of a real-time horizontal chemotaxis assay in vitro and bone marrow transplantation techniques in vivo, we have also demonstrated that CCR2 expressed in bone marrow cells is involved in macrophage infiltration into obese adipose tissue [18]. In addition to the MCP-1/CCR2 pathway, there are several reports suggesting the potential involvement of other chemotactic factors in obesity-induced macrophage infiltration [19, 20]. For instance, recent evidence suggested the role of osteopontin, angiopoietin-like protein 2, and CXCL14 [19–21]. Importantly, inhibition of macrophage infiltration into obese adipose tissue through genetic and/or pharmacologic strategies improved the dysregulation of adipocytokine production, thereby leading to the amelioration of obesity-induced adipose tissue inflammation and insulin resistance. Understanding the molecular mechanisms underlying increased macrophage infiltration into obese adipose tissue may lead to the identification of novel, adipocyte-derived chemokine(s) and even therapeutic strategies to prevent or treat obesity-induced adipose tissue inflammation.

ADIPOCYTE HYPERTROPHY AND INFLAMMATORY CHANGES

To understand how macrophages are recruited into obese adipose tissue, it is important to know the molecular mechanism underlying increased production of chemokines in the early stages of obesity. Recent studies have demonstrated that multiple intracellular signaling pathways are activated in adipocytes during the course of adipocyte hypertrophy in vitro and in obese adipose tissue in vivo {Fig. 2, (i) [1–3]}. For instance, MAPKs, such as ERK, p38 MAPK, and JNK, are activated in a variety of cellular processes including adipocyte differentiation and hypertrophy [22–24]. Once activated by the upstream ki-

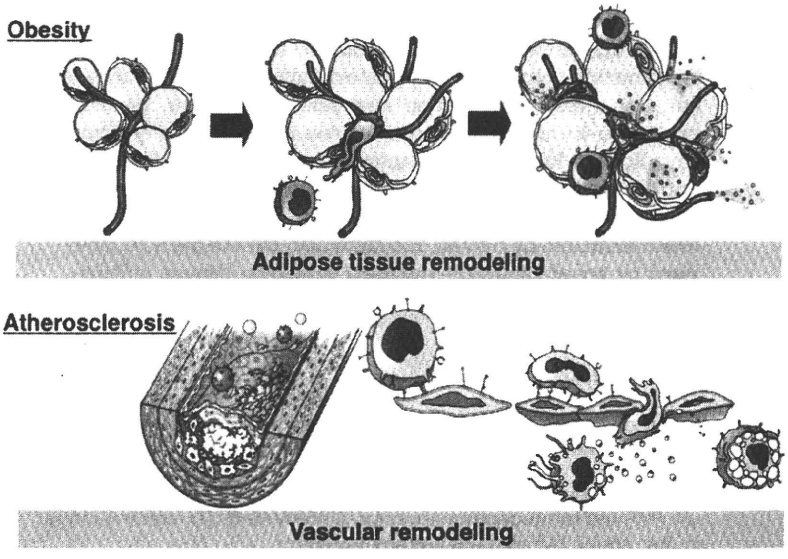

Figure 1. Adipose tissue remodeling. Obesity-induced adipose tissue inflammation is characterized by adipocyte hypertrophy, followed by increases in angiogenesis, immune cell infiltration, extracellular matrix overproduction, and thus, increased production of proinflammatory adipocytokines, which can be referred to as "adipose tissue remodeling." This is similar to chronic inflammatory changes and tissue remodeling in atherosclerotic vascular walls termed "vascular remodeling," which is mediated through complex interactions among vascular endothelial cells, vascular smooth muscle cells, lymphocytes, and monocyte-derived macrophages.

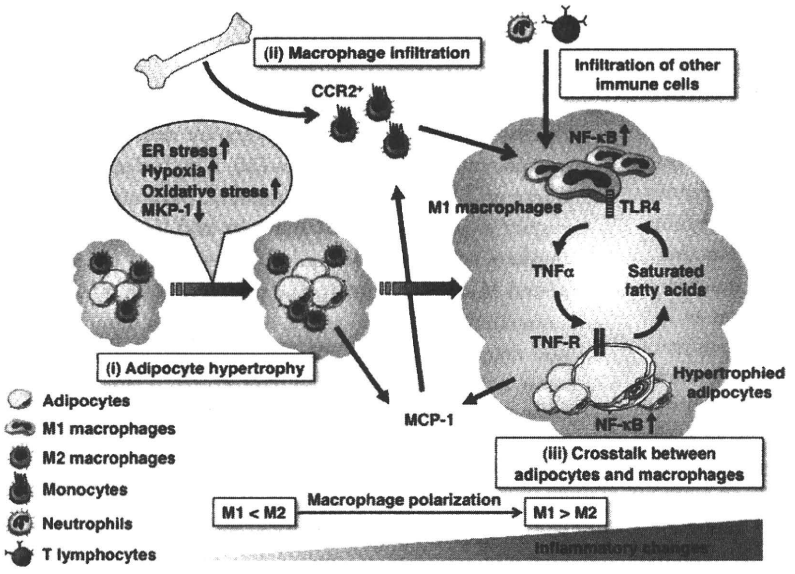

Figure 2. Molecular mechanism underlying adipose tissue inflammation. In the early stages of obesity, adipocytes should be hypertrophied in response to overnutrition (i). Recent evidence suggests that increased metabolic stresses such as ER stress, hypoxia, and oxidative stress and down-regulation of MKP-1 are involved in the induction of inflammatory changes in adipocytes during the course of adipocyte hypertrophy. In the advanced stages of obesity, there are various kinds of stromal immune cells such as neutrophils, T lymphocytes, and macrophages, which infiltrate into obese adipose tissue (ii) and thus, enhance the inflammatory changes through the crosstalk with parenchymal adipocytes (iii). For example, the macrophage-derived TNF-α induces the release of saturated fatty acids from adipocytes via lipolysis, which in turn, induces inflammatory changes in macrophages via TLR4. Such a paracrine loop between adipocytes and macrophages constitutes a vicious cycle, thereby accelerating further adipose tissue inflammation. Recent evidence has also pointed to the heterogeneity of adipose tissue macrophages; i.e., M1 or "classically activated" (proinflammatory) macrophages and M2 or "alternatively activated" (anti-inflammatory) macrophages. Infiltrated macrophages exhibit a phenotypic change from M2 to M1 polarization in obese adipose tissue, thereby accelerating adipose tissue inflammation. TNF-R, TNF-α receptor.

nases, e.g., MEK, MAPKs are inactivated rapidly by a family of protein phosphatases such as MKP-1, an inducible dual-specificity phosphatase [25, 26]. We have demonstrated that down-regulation of MKP-1 is critical for increased production of MCP-1 during the course of adipocyte hypertrophy [27]. On the other hand, Ozcan et al. [28] reported that obesity is associated with the induction of ER stress, predominantly in the adipose tissue and liver, and suggested that ER stress plays a critical role in obesity-induced adipose tissue inflammation. In this regard, Hosogai et al. [29] reported hypoxia-induced ER stress in obese adipose tissue, which is involved in the dysregulation of adipocytokine production. Moreover, Furukawa et al. [30] also showed that reactive oxygen species production is increased in parallel with adipocyte hypertrophy and that oxidative stress induces the dysregulation of adipocytokine production. It is interesting to investigate how such multiple intracellular signaling pathways are integrated during the course of adipocyte hypertrophy and/or in the early stages of obesity.

PARACRINE LOOP BETWEEN ADIPOCTYES AND MACROPHAGES

Once infiltrated into the adipose tissue in the advanced stages of obesity, macrophages participate in the inflammatory pathways that are activated in obese adipose tissue [1, 2]. Using an in vitro coculture system composed of adipocytes and macrophages, we have demonstrated that a paracrine loop involving saturated fatty acids and TNF-α derived from adipocytes and macrophages, respectively, establishes a vicious cycle that augments the inflammatory changes; i.e., marked up-regulation of proinflammatory adipocytokines, such as MCP-1 and TNF-α, and significant down-regulation of anti-inflammatory adiponectin {Fig. 2, (iii) [31]}. As the coculture-induced dysregulation of adipocytokine production is roughly parallel to that in obese adipose tissue in vivo, there may be an intimate crosstalk between adipocytes and macrophages as a potential mechanism that aggravates chronic inflammation in obese adipose tissue. Indeed, TNF-α, which is derived mostly from infiltrated macrophages in obese adipose tissue, acts on TNF-α receptor in hypertrophied adipocytes, thereby inducing proinflammatory cytokine production and adipocyte lipolysis via NF-κB-dependent and -independent (possibly MAPK-dependent) mechanisms, respectively [31]. On the other hand, saturated fatty acids thus released serve as a naturally occurring ligand for the TLR4 complex, which is essential for the recognition of LPS to induce NF-κB activation in macrophages [32, 33].

Evidence has accumulated, suggesting that TLR4 plays an important role in obesity-induced adipose tissue inflammation and systemic glucose and lipid metabolism in vivo [34–37]. As TLR4 is expressed in macrophages more abundantly than in adipocytes, it is likely that chronic inflammatory responses induced by the interaction between adipocytes and macrophages are largely mediated via TLR4 in macrophages. This discussion is supported by a recent report by Saberi et al. [38] showing that hematopoietic cell-specific deletion of TLR4 ameliorates high-fat, diet-induced hepatic and adipose tissue insulin resistance. It is, therefore, likely that inhibition of macrophages activated by adipocyte-derived saturated fatty acids may offer a unique, therapeutic strategy to prevent obesity-induced adipose tissue inflammation. Given the antagonistic relationship between saturated and *n-3* polyunsaturated fatty acids such as EPA [39], we have provided evidence that highly purified EPA increases the otherwise reduced secretion of anti-inflammatory adiponectin in obese adipose tissue, at least partly by interrupting the vicious cycle created by adipocytes and macrophages [40].

The dysregulation of adipocytokine production, which is induced by adipose tissue inflammation, may play a critical

role in the pathophysiology of the metabolic syndrome and atherosclerosis [1–4]. For instance, TNF-α, which is derived mostly from macrophages, is increased in obese adipose tissue [7, 8], and TNF-α-deficient mice are protected from obesity-induced insulin resistance [41]. By contrast, adiponectin, which is expressed exclusively in adipocytes, is markedly down-regulated in obese adipose tissue [42, 43], and supplementation of adiponectin in obese mice effectively reverses insulin resistance in the skeletal muscle and liver [42, 43]. On the other hand, MCP-1 is derived from adipocytes and macrophages in obese adipose tissue [7, 8]. Overproduction of MCP-1 in obese adipose tissue induces macrophage infiltration into the adipose tissue, thereby aggravating adipose tissue inflammation [16, 17]. It also induces insulin resistance directly in the skeletal muscle and liver, suggesting a role as an endocrine hormone [17, 44]. Finally, adipocyte-derived leptin acts directly on the hypothalamus, where it regulates food intake and energy expenditure [45, 46]. Several previous reports demonstrated that vascular remodeling and tissue fibrosis are markedly attenuated in leptin-deficient *ob/ob* mice or leptin signaling-deficient *db/db* mice [47–49]. However, the role of leptin in adipose tissue inflammation still remains to be elucidated.

PHENOTYPIC CHANGE OF ADIPOSE TISSUE MACROPHAGES

Recent studies have pointed to the heterogeneity of macrophages infiltrated into obese adipose tissue; i.e., they follow at least two different polarization states: M1 or classically activated (proinflammatory) macrophages, which are induced by proinflammatory mediators such as LPS and Th1 cytokine IFN-γ, and M2 or alternatively activated (anti-inflammatory) macrophages, which are generated in vitro by exposure to Th2 cytokines such as IL-4 and IL-13 [50, 51]. Evidence has accumulated indicating that macrophages exhibit the phenotypic change from M2 to M1 polarization in obese adipose tissue, thereby accelerating adipose tissue inflammation (Fig. 2) [50–54]. Like LPS, saturated fatty acids, as an endogenous ligand for the TLR4 complex, may contribute to the polarization of infiltrated macrophages toward M1 during the interaction between adipocytes and macrophages.

Through a combination of cDNA microarray analysis of saturated fatty acid-stimulated macrophages in vitro and obese adipose tissue in vivo, we have identified recently ATF3, a member of the ATF/CREB family of basic leucine zipper-type transcription factors, as a target gene of saturated fatty acids/TLR4 signaling in macrophages in obese adipose tissue [55]. Transgenic overexpression of ATF3 in macrophages does not affect adipocyte hypertrophy and macrophage infiltration in obese adipose tissue in vivo [55]. Interestingly, mRNA expression of M1 macrophage markers such as CD11c and TNF-α in macrophage-specific ATF3 transgenic mice is reduced significantly relative to wild-type mice, although there is no significant difference in mRNA expression of M2 macrophage markers (mannose receptor and arginase 1) between the genotypes [55]. These findings, taken together, suggest that ATF3 acts as a transcriptional repressor of saturated fatty acids/TLR4 signaling in macrophages, thereby representing a negative-feedback mechanism that attenuates obesity-induced macrophage activation in obese adipose tissue.

Prior to macrophage infiltration at the site of chronic inflammation, M1 and M2 markers are detected in circulating peripheral blood monocytes [56, 57]. Indeed, monocytes in obese and/or obese type 2 diabetic patients show significantly higher expression of M1 markers and lower expression of M2 markers relative to normal-weight controls [56]. The unbalanced M1/M2 phenotype of peripheral blood monocytes is associated with impairment of several metabolic parameters and arterial stiffness [56]. Interestingly, activation of the nuclear receptor, PPARγ by pioglitazone, a thiazolidinedione class of insulin sensitizer, improves the unbalanced M1/M2 phenotype of monocytes [56, 57], which may contribute to its antidiabetic and antiatherogenic effect. The above discussion is consistent with recent observations that PPARγ and PPARδ can stimulate M2 polarization of adipose tissue macrophages and thus, systemic insulin sensitivity [52–54, 58]. On the other hand, pioglitazone treatment improves the unbalanced M1/M2 phenotype of adipose tissue macrophages in diet-induced obese mice [59]. Moreover, a recent study suggests that macrophage PPARγ is required for full antidiabetic effects of thiazolidinediones [60]. Collectively, phenotypic modulation of adipose tissue macrophages may offer a novel, therapeutic strategy to treat or prevent the progression of obesity-induced complications such as diabetes and atherosclerosis.

OTHER IMMUNE CELLS

In addition to macrophages, other immune cells, such as neutrophils and NK cells, are increased in the adipose tissue during the course of obesity (Fig. 2) [61, 62]. Similar to the sequence of events that comprises acute inflammation, a transient increase in neutrophil infiltration precedes macrophage infiltration in a mouse model of diet-induced obesity [62], suggesting the role of neutrophils in the initiation of the inflammatory cascade. Recent evidence has also revealed a large number of T lymphocytes in the adipose tissue from lean and obese mice [63–66]. For instance, the population of $CD8^+$ T cells in the SVF is increased significantly early in the onset of obesity and continues to increase thereafter [63]. Of note, the increase in $CD8^+$ T cells precedes the accumulation of adipose tissue macrophages [63], suggesting the role of $CD8^+$ T cells in the initiation of adipose tissue inflammation. By contrast, the population of $CD4^+$ T cells and regulatory T cells is decreased in the advanced stages of obesity [63–65]. Such imbalance of the T cell subpopulation may play a role in the progression of obesity-induced adipose tissue inflammation. On the other hand, Moro et al. [67] have reported recently a new type of lymphocytes, "natural helper cells" in a novel lymphoid structure associated with adipose tissues in the peritoneal cavity. They also showed that the novel, innate lymphocytes are capable of producing large amounts of Th2 cytokines [67]. It would be interesting to elucidate the physiologic and pathophysiologic role of natural helper cells in visceral fat obesity.

ADIPOSE TISSUE INFLAMMATION AS "HOMEOSTATIC INFLAMMATION"

In addition to exogenous pathogens such as bacteria and viruses, the immune system is capable of sensing endogenous ligands released from damaged and stressed cells and tissues, thereby inducing sterile inflammation (**Fig. 3**) [12, 68, 69]. The endogenous stress signals, which are called DAMPs or "danger signals," include HMGB1, S100A8, and S100A9, modified low-density lipoproteins, and degradation products of extracellular matrices [12, 68, 69]. The danger signals, which are derived from parenchymal cells, are recognized by immune cells such as macrophages through pathogen sensors or PRRs such as TLRs, nucleotide-binding oligomerization domain-like receptors, retinoid-inducible gene-like receptors, scavenger receptors, and C-type lectin receptors [12, 68, 69].

A previous study showed that macrophages in obese adipose tissue are localized to dead adipocytes, where they fuse to scavenge the residual lipid droplet and ultimately, form multinucleate giant cells, a hallmark of chronic inflammation [70]. Indeed, macrophages aggregate to constitute a CLS surrounding dead adipocytes in advanced obesity [13, 14, 70]. Electron microscopic analysis also revealed lipid-laden phagolysosomes in macrophages within CLS [70]. Given that TNF-α induces proapoptotic and/or death signals in a variety of cell types, it is therefore interesting to speculate that hypertrophied adipocytes, which are stimulated and thus, dying by macrophage-derived TNF-α, can release saturated fatty acids as an endogenous danger signal that report their diseased state to macrophages in obese adipose tissue. Indeed, several lines of evidence indicate that adipocyte death and/or the death receptor Fas signaling contribute to obesity-induced adipose tissue inflammation and systemic insulin resistance [71, 72]. On the other hand, free fatty acids are an important energy source mobilized from triglycerides stored in the adipose tissue, particularly during periods of starvation, but recent evidence has suggested the pathophysiologic roles other than the supply of nutrients in times of fasting or increased energy demand. In this regard, free fatty acids, when released physiologically during fasting or starvation via adipocyte lipolysis, may not act as a danger signal. Similar to the relationship between commensal bacteria and pathogen sensors in epithelial cell homeostasis within the intestinal mucosa, activation of the TLR4 complex by saturated fatty acids may be involved in the regulation of metabolic homeostasis within the adipose tissue (Fig. 3). Sustained interaction between endogenous ligands, which are derived from parenchymal cells and pathogen sensors, expressed in stromal immune cells, should lead to chronic/homeostatic inflammatory responses ranging from the basal homeostatic state to diseased tissue remodeling, which may be referred to as homeostatic inflammation (Fig. 3). Dysregulation of this process can result in a variety of chronic inflammatory diseases, such as obesity, diabetes mellitus, atherosclerosis, malignant cancers, autoimmune diseases, and even neurodegenerative diseases. Collectively, adipose tissue inflammation may represent a prototypic example of homeostatic inflammation.

CONCLUDING REMARKS

The adipose tissue communicates with multiple organs or tissues by virtue of a large number of adipocytokines and thus, influences a variety of physiologic and pathophysiologic processes. Obesity may be viewed as a chronic, low-grade inflammatory as well as a metabolic disease; chronic inflammation within the adipose tissue or adipose tissue remodeling results in the dysregulation of adipocytokine production, thereby contributing to the pathophysiology of the metabolic syndrome. Among stromal cells, macrophages should play a critical role in obesity-related adipose tissue inflammation. During the paracrine interaction between adipocytes and macrophages, saturated fatty acids, which are released from hypertrophied

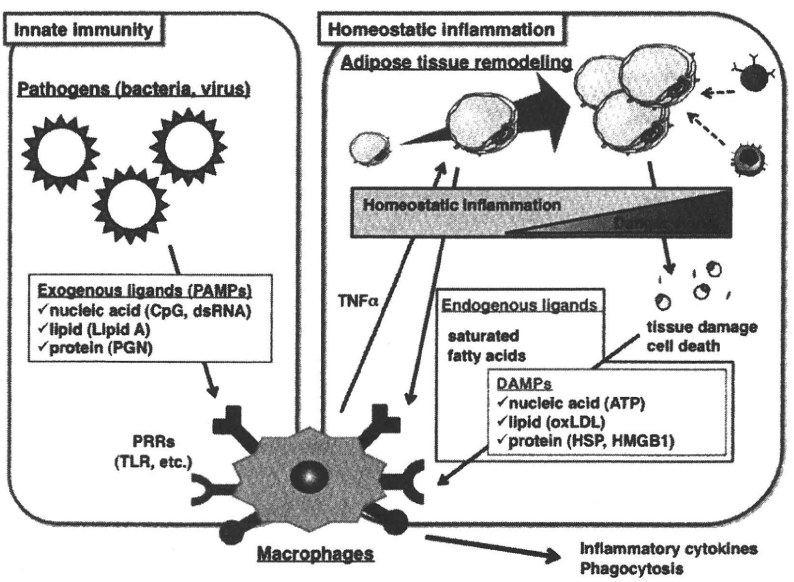

Figure 3. Adipose tissue inflammation as homeostatic inflammation. In "innate immunity," exogenous ligands (PAMPs) are sensed by PRRs, thereby inducing inflammatory changes. On the other hand, DAMPs, released from damaged or stressed cells and tissues, can activate PRRs, thereby inducing homeostatic inflammation ranging from the basal homeostatic state to diseased tissue remodeling. The role of endogenous ligands as a danger signal has been emphasized during the progression of homeostatic inflammation. For instance, free fatty acids, when released as an energy source during fasting or starvation, may not act as a danger signal. However, in adipose tissue inflammation, saturated fatty acids, which are released from hypertrophied adipocytes, can report, as a danger signal, their diseased state to macrophages via the TLR4 complex during the course of obesity. dsRNA, Double-stranded RNA; PGN, peptidoglycan; ATP, adenosine triphosphate; oxLDL, oxidized low-density lipoprotein; HSP, heat shock protein.

adipocytes via the macrophage-induced lipolysis, serve as an endogenous ligand for the TLR4 complex, a major pathogen sensor, to activate macrophages for the regulation of metabolic homeostasis, which is a hallmark of homeostatic inflammation. Understanding the molecular mechanism underlying homeostatic inflammation of obese adipose tissue may lead to novel, therapeutic strategies to prevent or treat obesity-induced adipose tissue inflammation.

ACKNOWLEDGMENTS

Work in the authors' laboratory was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labor and Welfare of Japan. We thank Dr. Kensuke Miyake (The Institute of Medical Science, University of Tokyo) for discussions.

REFERENCES

1. Schenk, S., Saberi, M., Olefsky, J. M. (2008) Insulin sensitivity: modulation by nutrients and inflammation. *J. Clin. Invest.* **118,** 2992–3002.
2. Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. *Nature* **444,** 860–867.
3. Berg, A. H., Scherer, P. E. (2005) Adipose tissue, inflammation, and cardiovascular disease. *Circ. Res.* **96,** 939–949.
4. Rocha, V. Z., Libby, P. (2009) Obesity, inflammation, and atherosclerosis. *Nat. Rev. Cardiol.* **6,** 399–409.
5. Matsuzawa, Y., Funahashi, T., Nakamura, T. (1999) Molecular mechanism of metabolic syndrome X: contribution of adipocytokines adipocyte-derived bioactive substances. *Ann. N. Y. Acad. Sci.* **892,** 146–154.
6. Kadowaki, T., Yamauchi, T., Kubota, N., Hara, K., Ueki, K., Tobe, K. (2006) Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J. Clin. Invest.* **116,** 1784–1792.
7. Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., Ferrante Jr., A. W. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* **112,** 1796–1808.
8. Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., Sole, J., Nichols, A., Ross, J. S., Tartaglia, L. A., Chen, H. (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* **112,** 1821–1830.
9. Clement, K., Viguerie, N., Poitou, C., Carette, C., Pelloux, V., Curat, C. A., Sicard, A., Rome, S., Benis, A., Zucker, J. D., Vidal, H., Laville, M., Barsh, G. S., Basdevant, A., Stich, V., Cancello, R., Langin, D. (2004) Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *FASEB J.* **18,** 1657–1669.
10. Wasserman, F. (1965) *Handbook of Physiology.* Washington, DC, USA, American Physiology Society.
11. Serhan, C. N., Savill, J. (2005) Resolution of inflammation: the beginning programs the end. *Nat. Immunol.* **6,** 1191–1197.
12. Medzhitov, R. (2008) Origin and physiological roles of inflammation. *Nature* **454,** 428–435.
13. Nishimura, S., Manabe, I., Nagasaki, M., Hosoya, Y., Yamashita, H., Fujita, H., Ohsugi, M., Tobe, K., Kadowaki, T., Nagai, R., Sigiura, S. (2007) Adipogenesis in obesity requires close interplay between differentiating adipocytes, stromal cells, and blood vessels. *Diabetes* **56,** 1517–1526.
14. Nishimura, S., Manabe, I., Nagasaki, M., Seo, K., Yamashita, H., Hosoya, Y., Ohsugi, M., Tobe, K., Kadowaki, T., Nagai, R., Sigiura, S. (2008) In vivo imaging in mice reveals local cell dynamics and inflammation in obese adipose tissue. *J. Clin. Invest.* **118,** 710–721.
15. Weisberg, S. P., Hunter, D., Huber, R., Lemieux, J., Slaymaker, S., Vaddi, K., Charo, I., Leibel, R. L., Ferrante Jr., A. W. (2006) CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J. Clin. Invest.* **116,** 115–124.
16. Kanda, H., Tateya, S., Tamori, Y., Kotani, K., Hiasa, K., Kitazawa, R., Kitazawa, S., Miyachi, H., Maeda, S., Egashira, K., Kasuga, M. (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J. Clin. Invest.* **116,** 1494–1505.
17. Kamei, N., Tobe, K., Suzuki, R., Ohsugi, M., Watanabe, T., Kubota, N., Ohtsuka-Kowatari, N., Kumagai, K., Sakamoto, K., Kobayashi, M., Yamauchi, T., Ueki, K., Oishi, Y., Nishimura, S., Manabe, I., Hashimoto, H., Ohnishi, Y., Ogata, H., Tokuyama, K., Tsunoda, M., Ide, T., Murakami, K., Nagai, R., Kadowaki, T. (2006) Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J. Biol. Chem.* **281,** 26602–26614.
18. Ito, A., Suganami, T., Yamauchi, A., Degawa-Yamauchi, M., Tanaka, M., Kouyama, R., Kobayashi, Y., Nitta, N., Yasuda, K., Hirata, Y., Kuziel, W. A., Takeya, M., Kanegasaki, S., Kamei, Y., Ogawa, Y. (2008) Role of CC chemokine receptor 2 in bone marrow cells in the recruitment of macrophages into obese adipose tissue. *J. Biol. Chem.* **283,** 35715–35723.
19. Nomiyama, T., Perez-Tilve, D., Ogawa, D., Gizard, F., Zhao, Y., Heywood, E. B., Jones, K. L., Kawamori, R., Cassis, L. A., Tschop, M. H., Bruemmer, D. (2007) Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *J. Clin. Invest.* **117,** 2877–2888.
20. Nara, N., Nakayama, Y., Okamoto, S., Tamura, H., Kiyono, M., Muraoka, M., Tanaka, K., Taya, C., Shitara, H., Ishii, R., Yonekawa, H., Minokoshi, Y., Hara, T. (2007) Disruption of CXC motif chemokine ligand-14 in mice ameliorates obesity-induced insulin resistance. *J. Biol. Chem.* **282,** 30794–30803.
21. Tabata, M., Kadomatsu, T., Fukuhara, S., Miyata, K., Ito, Y., Endo, M., Urano, T., Zhu, H. J., Tsukano, H., Tazume, H., Kaikita, K., Miyashita, K., Iwawaki, T., Shimabukuro, M., Sakaguchi, K., Ito, T., Nakagata, N., Yamada, T., Katagiri, H., Kasuga, M., Ando, Y., Ogawa, H., Mochizuki, N., Itoh, H., Suda, T., Oike, Y. (2009) Angiopoietin-like protein 2 promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance. *Cell Metab.* **10,** 178–188.
22. Johnson, G. L., Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298,** 1911–1912.
23. Bost, F., Aouadi, M., Caron, L., Binetruy, B. (2005) The role of MAPKs in adipocyte differentiation and obesity. *Biochimie* **87,** 51–56.
24. Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Karin, M., Hotamisligil, G. S. (2002) A central role for JNK in obesity and insulin resistance. *Nature* **420,** 333–336.
25. Farooq, A., Zhou, M. M. (2004) Structure and regulation of MAPK phosphatases. *Cell. Signal.* **16,** 769–779.
26. Keyse, S. M. (2000) Protein phosphatases and the regulation of mitogen-activated protein kinase signaling. *Curr. Opin. Cell Biol.* **12,** 186–192.
27. Ito, A., Suganami, T., Miyamoto, Y., Yoshimasa, Y., Takeya, M., Kamei, Y., Ogawa, Y. (2007) Role of MAPK phosphatase-1 in the induction of monocyte chemoattractant protein-1 during the course of adipocyte hypertrophy. *J. Biol. Chem.* **282,** 25445–25452.
28. Ozcan, U., Cao, Q., Yilmaz, E., Lee, A. H., Iwakoshi, N. N., Ozdelen, E., Tuncman, G., Gorgun, C., Glimcher, L. H., Hotamisligil, G. S. (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* **306,** 457–461.
29. Hosogai, N., Fukuhara, A., Oshima, K., Miyata, Y., Tanaka, S., Segawa, K., Furukawa, S., Tochino, Y., Komuro, R., Matsuda, M., Shimomura, I. (2007) Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* **56,** 901–911.
30. Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., Shimomura, I. (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. *J. Clin. Invest.* **114,** 1752–1761.
31. Suganami, T., Nishida, J., Ogawa, Y. (2005) A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor α. *Arterioscler. Thromb. Vasc. Biol.* **25,** 2062–2068.
32. Suganami, T., Tanimoto-Koyama, K., Nishida, J., Itoh, M., Yuan, X., Mizuarai, S., Kotani, H., Yamaoka, S., Miyake, K., Aoe, S., Kamei, Y., Ogawa, Y. (2007) Role of the Toll-like receptor 4/NF-κB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. *Arterioscler. Thromb. Vasc. Biol.* **27,** 84–91.
33. Lee, J. Y., Sohn, K. H., Rhee, S. H., Hwang, D. (2001) Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J. Biol. Chem.* **276,** 16683–16689.
34. Suganami, T., Mieda, T., Itoh, M., Shimoda, Y., Kamei, Y., Ogawa, Y. (2007) Attenuation of obesity-induced adipose tissue inflammation in C3H/HeJ mice carrying a Toll-like receptor 4 mutation. *Biochem. Biophys. Res. Commun.* **354,** 45–49.
35. Shi, L., Kishore, R., McMullen, M. R., Nagy, L. E. (2002) Lipopolysaccharide stimulation of ERK1/2 increases TNF-α production via Egr-1. *Am. J. Physiol. Cell Physiol.* **282,** C1205–C1211.
36. Poggi, M., Bastelica, D., Gual, P., Iglesias, M. A., Gremeaux, T., Knauf, C., Peiretti, F., Verdier, M., Juhan-Vague, I., Tanti, J. F., Burcelin, R., Alessi, M. C. (2007) C3H/HeJ mice carrying a Toll-like receptor 4 mutation are protected against the development of insulin resistance in white adipose tissue in response to a high-fat diet. *Diabetologia* **50,** 1267–1276.
37. Tsukumo, D. M., Carvalho-Filho, M. A., Carvalheira, J. B., Prada, P. O., Hirabara, S. M., Schenka, A. A., Araujo, E. P., Vassallo, J., Curi, R., Velloso, L. A., Saad, M. J. (2007) Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes* **56,** 1986–1998.
38. Saberi, M., Woods, N. B., de Luca, C., Schenk, S., Lu, J. C., Bandyopadhyay, G., Verma, I. M., Olefsky, J. M. (2009) Hematopoietic cell-specific deletion of Toll-like receptor 4 ameliorates hepatic and adipose tissue insulin resistance in high-fat-fed mice. *Cell Metab.* **10,** 419–429.

39. Lee, J. Y., Plakidas, A., Lee, W. H., Heikkinen, A., Chanmugam, P., Bray, G., Hwang, D. H. (2003) Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. *J. Lipid Res.* **44,** 479–486.
40. Itoh, M., Suganami, T., Satoh, N., Tanimoto-Koyama, K., Yuan, X., Tanaka, M., Kawano, H., Yano, T., Aoe, S., Takeya, M., Shimatsu, A., Kuzuya, H., Kamei, Y., Ogawa, Y. (2007) Increased adiponectin secretion by highly purified eicosapentaenoic acid in rodent models of obesity and human obese subjects. *Arterioscler. Thromb. Vasc. Biol.* **27,** 1918–1925.
41. Uysal, K. T., Wiesbrock, S. M., Marino, M. W., Hotamisligil, G. S. (1997) Protection from obesity-induced insulin resistance in mice lacking TNF-α function. *Nature* **389,** 610–614.
42. Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tobe, K., Nagai, R., Kimura, S., Tomita, M., Froguel, P., Kadowaki, T. (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat. Med.* **7,** 941–946.
43. Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyama, N., Kondo, H., Takahashi, M., Arita, Y., Komuro, R., Ouchi, N., Kihara, S., Tochino, Y., Okutomi, K., Horie, M., Takeda, S., Aoyama, T., Funahashi, T., Matsuzawa, Y. (2002) Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat. Med.* **8,** 731–737.
44. Tateya, S., Tamori, Y., Kawaguchi, T., Kanda, H., Kasuga, M. (2010) An increase in the circulating concentration of monocyte chemoattractant protein-1 elicits systemic insulin resistance irrespective of adipose tissue inflammation in mice. *Endocrinology* **151,** 971–979.
45. Ogawa, Y., Masuzaki, H., Hosoda, K., Aizawa-Abe, M., Suga, J., Suda, M., Ebihara, K., Iwai, H., Matsuoka, N., Satoh, N., Odaka, H., Kasuga, H., Fujisawa, Y., Inoue, G., Nishimura, H., Yoshimasa, Y., Nakao, K. (1999) Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. *Diabetes* **48,** 1822–1829.
46. Friedman, J. M., Halaas, J. L. (1998) Leptin and the regulation of body weight in mammals. *Nature* **395,** 763–770.
47. Tanaka, M., Suganami, T., Sugita, S., Shimoda, Y., Kasahara, M., Aoe, S., Takeya, M., Takeda, S., Kamei, Y., Ogawa, Y. (2010) Role of central leptin signaling in renal macrophage infiltration. *Endocr. J.* **57,** 61–72.
48. Schafer, K., Halle, M., Goeschen, C., Dellas, C., Pynn, M., Loskutoff, D. J., Konstantinides, S. (2004) Leptin promotes vascular remodeling and neointimal growth in mice. *Arterioscler. Thromb. Vasc. Biol.* **24,** 112–117.
49. Stephenson, K., Tunstead, J., Tsai, A., Gordon, R., Henderson, S., Dansky, H. M. (2003) Neointimal formation after endovascular arterial injury is markedly attenuated in *db/db* mice. *Arterioscler. Thromb. Vasc. Biol.* **23,** 2027–2033.
50. Lumeng, C. N., Bodzin, J. L., Saltiel, A. R. (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* **117,** 175–184.
51. Lumeng, C. N., DelProposto, J. B., Westcott, D. J., Saltiel, A. R. (2008) Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes* **57,** 3239–3246.
52. Odegaard, J. I., Ricardo-Gonzalez, R. R., Red Eagle, A., Vats, D., Morel, C. R., Goforth, M. H., Subramanian, V., Mukundan, L., Ferrante, A. W., Chawla, A. (2008) Alternative M2 activation of Kupffer cells by PPARδ ameliorates obesity-induced insulin resistance. *Cell Metab.* **7,** 496–507.
53. Odegaard, J. I., Ricardo-Gonzalez, R. R., Goforth, M. H., Morel, C. R., Subramanian, V., Mukundan, L., Red Eagle, A., Vats, D., Brombacher, F., Ferrante, A. W., Chawla, A. (2007) Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. *Nature* **447,** 1116–1120.
54. Kang, K., Reilly, S. M., Karabacak, V., Gangl, M. R., Fitzgerald, K., Hatano, B., Lee, C. H. (2008) Adipocyte-derived Th2 cytokines and myeloid PPARδ regulate macrophage polarization and insulin sensitivity. *Cell Metab.* **7,** 485–495.
55. Suganami, T., Yuan, X., Shimoda, Y., Uchio-Yamada, K., Nakagawa, N., Shirakawa, I., Usami, T., Tsukahara, T., Nakayama, K., Miyamoto, Y., Yasuda, K., Matsuda, J., Kamei, Y., Kitajima, S., Ogawa, Y. (2009) Activating transcription factor 3 constitutes a negative feedback mechanism that attenuates saturated fatty acid/Toll-like receptor 4 signaling and macrophage activation in obese adipose tissue. *Circ. Res.* **105,** 25–32.
56. Satoh, N., Shimatsu, A., Himeno, A., Sasaki, Y., Yamakage, H., Yamada, K., Suganami, T., Ogawa, Y. (2010) Unbalanced M1/M2 phenotype of peripheral blood monocytes in obese diabetic patients: effect of pioglitazone. *Diabetes Care* **33,** e7.
57. Bouhlel, M. A., Derudas, B., Rigamonti, E., Dievart, R., Brozek, J., Haulon, S., Zawadzki, C., Jude, B., Torpier, G., Marx, N., Staels, B., Chinetti-Gbaguidi, G. (2007) PPARγ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab.* **6,** 137–143.
58. Vats, D., Mukundan, L., Odegaard, J. I., Zhang, L., Smith, K. L., Morel, C. R., Wagner, R. A., Greaves, D. R., Murray, P. J., Chawla, A. (2006) Oxidative metabolism and PGC-1β attenuate macrophage-mediated inflammation. *Cell Metab.* **4,** 13–24.
59. Fujisaka, S., Usui, I., Bukhari, A., Ikutani, M., Oya, T., Kanatani, Y., Tsuneyama, K., Nagai, Y., Takatsu, K., Urakaze, M., Kobayashi, M., Tobe, K. (2009) Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. *Diabetes* **58,** 2574–2582.
60. Hevener, A. L., Olefsky, J. M., Reichart, D., Nguyen, M. T., Bandyopadyhay, G., Leung, H. Y., Watt, M. J., Benner, C., Febbraio, M. A., Nguyen, A. K., Folian, B., Subramaniam, S., Gonzalez, F. J., Glass, C. K., Ricote, M. (2007) Macrophage PPARγ is required for normal skeletal muscle and hepatic insulin sensitivity and full antidiabetic effects of thiazolidinediones. *J. Clin. Invest.* **117,** 1658–1669.
61. Ohmura, K., Ishimori, N., Ohmura, Y., Tokuhara, S., Nozawa, A., Horii, S., Andoh, Y., Fujii, S., Iwabuchi, K., Onoe, K., Tsutsui, H. (2010) Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice. *Arterioscler. Thromb. Vasc. Biol.* **30,** 193–199.
62. Elgazar-Carmon, V., Rudich, A., Hadad, N., Levy, R. (2008) Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding. *J. Lipid Res.* **49,** 1894–1903.
63. Nishimura, S., Manabe, I., Nagasaki, M., Eto, K., Yamashita, H., Ohsugi, M., Otsu, M., Hara, K., Ueki, K., Sugiura, S., Yoshimura, K., Kadowaki, T., Nagai, R. (2009) $CD8^+$ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat. Med.* **15,** 914–920.
64. Winer, S., Chan, Y., Paltser, G., Truong, D., Tsui, H., Bahrami, J., Dorfman, R., Wang, Y., Zielenski, J., Mastronardi, F., Maezawa, Y., Drucker, D. J., Engleman, E., Winer, D., Dosch, H. M. (2009) Normalization of obesity-associated insulin resistance through immunotherapy. *Nat. Med.* **15,** 921–929.
65. Feuerer, M., Herrero, L., Cipolletta, D., Naaz, A., Wong, J., Nayer, A., Lee, J., Goldfine, A. B., Benoist, C., Shoelson, S., Mathis, D. (2009) Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat. Med.* **15,** 930–939.
66. Kintscher, U., Hartge, M., Hess, K., Foryst-Ludwig, A., Clemenz, M., Wabitsch, M., Fischer-Posovszky, P., Barth, T. F., Dragun, D., Skurk, T., Hauner, H., Bluher, M., Unger, T., Wolf, A. M., Knippschild, U., Hombach, V., Marx, N. (2008) T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. *Arterioscler. Thromb. Vasc. Biol.* **28,** 1304–1310.
67. Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J., Ohtani, M., Fujii, H., Koyasu, S. (2010) Innate production of Th2 cytokines by adipose tissue-associated c-$Kit^+Sca\text{-}1^+$ lymphoid cells. *Nature* **463,** 540–544.
68. Medzhitov, R., Janeway Jr., C. A. (2002) Decoding the patterns of self and nonself by the innate immune system. *Science* **296,** 298–300.
69. Zhang, X., Mosser, D. M. (2008) Macrophage activation by endogenous danger signals. *J. Pathol.* **214,** 161–178.
70. Cinti, S., Mitchell, G., Barbatelli, G., Murano, I., Ceresi, E., Faloia, E., Wang, S., Fortier, M., Greenberg, A. S., Obin, M. S. (2005) Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* **46,** 2347–2355.
71. Alkhouri, N., Gornicka, A., Berk, M. P., Thapaliya, S., Dixon, L. J., Kashyap, S., Schauer, P. R., Feldstein, A. E. (2010) Adipocyte apoptosis, a link between obesity, insulin resistance, and hepatic steatosis. *J. Biol. Chem.* **285,** 3428–3438.
72. Wueest, S., Rapold, R. A., Schumann, D. M., Rytka, J. M., Schildknecht, A., Nov, O., Chervonsky, A. V., Rudich, A., Schoenle, E. J., Donath, M. Y., Konrad, D. (2010) Deletion of Fas in adipocytes relieves adipose tissue inflammation and hepatic manifestations of obesity in mice. *J. Clin. Invest.* **120,** 191–202.

KEY WORDS:
adipocytes · homeostatic inflammation · obesity · Toll-like receptor · saturated fatty acids

OBSERVATIONS

Unbalanced M1/M2 Phenotype of Peripheral Blood Monocytes in Obese Diabetic Patients

Effect of pioglitazone

The monocyte-macrophage system exists in at least two distinct phenotypes of differentiation: proinflammatory (M1) and anti-inflammatory (M2) (1,2). Macrophages, when infiltrated into obese adipose tissue, exhibit a phenotypic switch from M2 to M1 polarization, thereby contributing to obesity-induced adipose tissue inflammation and insulin resistance (1). Expression of both M1 and M2 markers is detected in circulating peripheral blood mononuclear cells as well as in atherosclerotic plaques (3). However, there have been no detailed studies on the M1/M2 phenotype of monocytes and their association with cardiovascular risks in obese subjects with type 2 diabetes. On the other hand, we demonstrated that pioglitazone, a thiazolidinedione class of insulin sensitizer, exerts an antiatherogenic effect independent of its antidiabetic effect (4). Here, we investigated the M1/M2 phenotype of peripheral blood monocytes and pulse wave velocity (PWV), an established index of arterial stiffness, and also the effect of pioglitazone in obese diabetic patients.

A total of 161 subjects (95 men and 66 women, mean age 50.4 years), including 45 normal-weight control subjects, 62 obese nondiabetic patients, and 54 obese diabetic patients with or without pioglitazone treatment for 3 months (30 mg daily), were recruited in our clinic. Peripheral blood monocytes were prepared using magnetic-assisted cell sorting and flow cytometry with anti-CD14. Expression of M1/M2 markers was analyzed by real-time quantitative PCR method and flow cytometry. The number and percentage of $CD14^+$ cells among peripheral blood monocytes from obese diabetic patients were significantly increased relative to those of control subjects ($P < 0.05$). The $CD14^+$ cells from obese nondiabetic patients showed significantly higher expression of M1 markers, tumor necrosis factor-α, and interleukin (IL)-6 and lower expression of an M2 marker, IL-10, relative to control subjects ($P < 0.01$). This is consistent with a report that peripheral blood mononuclear cells in obesity are in an inflammatory state (5). In addition, expression of IL-10 and CD163 in $CD14^+$ cells from obese diabetic patients was significantly decreased relative to that of obese nondiabetic patients ($P < 0.01$). Multivariate regression analysis showed that expression of tumor necrosis factor-α is independently associated with age and BMI and that expression of IL-6 is independently associated with BMI and LDL cholesterol ($P < 0.01$); expression of IL-10 was negatively and independently associated with diastolic blood pressure, A1C, and triglycerides, and expression of CD163 was negatively and independently associated with insulin concentration, A1C, and PWV ($P < 0.05$). Moreover, only age and CD163 were independently correlated with PWV ($P < 0.05$). Interestingly, 3-month treatment with pioglitazone significantly increased IL-10 and CD163 and decreased IL-6 ($P < 0.05$) in parallel with the improvement of fasting plasma glucose, A1C, insulin concentration, homeostasis model assessment-insulin resistance index, and PWV in obese diabetic patients. Further studies are required to elucidate more detailed characterization of monocyte subsets in obese diabetic patients and the resulting pathophysiological implication in cardiovascular diseases.

This study provides evidence that an unbalanced M1/M2 phenotype of peripheral blood monocytes is associated with metabolic disorder and arterial stiffness in obese type 2 diabetic patients. We also demonstrate that peroxisome proliferator–activated receptor-γ activation improves the unbalanced M1/M2 phenotype of monocytes in obese diabetic patients, which may contribute to its antiatherogenic effect.

NORIKO SATOH, MD, PHD[1]
AKIRA SHIMATSU, MD, PHD[1]
AKIHIRO HIMENO, MD[1,2]
YOUSUKE SASAKI, BSC[1]
HAJIME YAMAKAGE, BSC[1]
KAZUNORI YAMADA, MD, PHD[2]
TAKAYOSHI SUGANAMI, MD, PHD[3]
YOSHIHIRO OGAWA, MD, PHD[3,4]

From the [1]Clinical Research Institute for Endocrine Metabolic Diseases, National Hospital Organization, Kyoto Medical Center, Kyoto, Japan; the [2]Diabetes Center, National Hospital Organization, Kyoto Medical Center, Kyoto, Japan; the [3]Department of Molecular Medicine and Metabolism, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan; and the [4]Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan.

Corresponding author: Noriko Satoh, nsato@kyotolan.hosp.go.jp.

DOI: 10.2337/dc09-1315

Acknowledgments— This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of the Japan Ministry of Health, Labor, and Welfare of Japan (to N.S. and Y.O.), the Smoking Research Foundation (to N.S.), and Takeda Science Foundation (to Y.O.).

No other potential conflicts of interest relevant to this article were reported.

References

1. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007;117:175–184
2. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005;5:953–964
3. Bouhlel MA, Derudas B, Rigamonti E, Dièvart R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, Marx N, Staels B, Chinetti-Gbaguidi G. PPARγ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab 2007;6:137–143
4. Satoh N, Ogawa Y, Usui T, Tagami T, Kono S, Uesugi H, Sugiyama H, Sugawara A, Yamada K, Shimatsu A, Kuzuya H, Nakao K. Antiatherogenic effect of pioglitazone in type 2 diabetic patients irrespective of the responsiveness to its antidiabetic effect. Diabetes Care 2003;26:2493–2499
5. Ghanim H, Aljada A, Hofmeyer D, Syed T, Mohanty P, Dandona P. Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation 2004;110:1564–1571

●第62回日本自律神経学会総会/シンポジウム1/生体リズム研究の新展開―時計遺伝子のジェネティクス，エピジェネティクス，そして臨床応用まで―
司会：内匠　透・向阪　彰

概日リズムによる生理機能の制御機構

平山　順　　内田好海　　宮村憲央　　沢登健治　　仁科博史

自律神経　第47巻　第4号　別刷
(2010年8月15日)

●第62回日本自律神経学会総会/シンポジウム1/生体リズム研究の新展開―時計遺伝子のジェネティクス，エピジェネティクス，そして臨床応用まで―
司会：内匠　透・向阪　彰

概日リズムによる生理機能の制御機構

平山　順[a]　内田好海[b]　宮村憲央[b]　沢登健治[b,c]　仁科博史[b]

キーワード：概日リズム，分子時計，転写
circadian rhythm, molecular clock, transcription

抄録：概日リズムは生理現象の周期を外環境に適応させ維持する機構であり，分子時計と呼ばれる全身の個々の細胞に存在する転写/翻訳に依存したフィードバックループにより制御されている[1]．精神的ストレスや不規則な生活は概日リズムの異常を引き起こすが，このリズムの異常は精神（時差症候群，不眠，うつ），循環器（心筋梗塞，高血圧），呼吸器（喘息）疾患等の自律神経の異常に起因する疾患の病態に関与する[7]．さらに，近代化がもたらす飽食に伴うメタボリック症候群，老齢化と関連する骨粗鬆症や発癌といった現代生活を脅かす疾患が概日リズムと密接に関係していることが明らかになっている[3]．本総説は，分子時計の制御機構及び分子時計と疾患の関連についての最近の知見を要約する．
（自律神経，47：297～300，2010）

分子時計の転写制御機構

概日リズムは全身の個々の細胞に存在する転写/翻訳に依存したフィードバックループ（分子時計）により制御されている[2]．特に脊椎動物の分子時計はCLOCK，NPAS2，BMAL1，PER（哺乳動物の場合は，PER1，PER2，及びPER3が存在する．）及びCRY（哺乳動物の場合は，CRY1及びCRY2が存在する．）と呼ばれる転写因子（時計蛋白質）により構成される約24時間の周期性をもつ転写/翻訳に依存したフィードバックループである（図1）．CLOCK（NPAS2）とBMAL1は二量体を形成し*Per*及び*Cry*の転写を活性化する．PERおよびCRYは翻訳後二量体を形成し，CLOCK（NPAS2）：BMAL1複合体に直接結合しその転写を抑制する．重要なこととして，CLOCK（NPAS2）：BMAL1二量体は，脂肪細胞の分化に関わる*Peroxisome proliferator-activated receptor* α，肝臓における新陳代謝を制御する*Albumin D element-binding protein*，細胞周期制御因子*Wee1*といった様々な遺伝子の転写調節を担う．特に，細胞内の約10%の遺伝子がCLOCK（NPAS2）：BMAL1二量体により転写調節されていることが報告されている[12]ことから，分子時計は様々な遺伝子の転写調節を介して多くの細胞機能を制御していると考えられている．

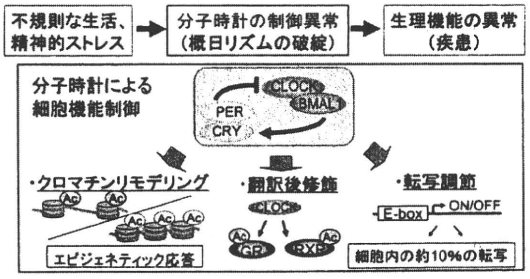

図1　分子時計による細胞機能調節のモデル

[a]東京医科歯科大学難治疾患研究所 Medical Top Track Program
〒113-8510　東京都文京区湯島1―5―45
[b]同　発生再生生物学分野
[c]東京大学大学院薬学系研究科生理化学教室
〒113-8510　東京都文京区本郷7―3―1

表1　時計遺伝子変異マウスの表現型

遺伝子	概日リズムに関する表現型	概日リズム以外の表現型
CLOCK -del.19 mutant	恒暗条件下において行動リズムの周期の約4時間の延長が観察された後，行動リズムが消失する． 分子時計により制御される遺伝子発現の日周性の消失． 視交叉上核における時計遺伝子発現の光誘導の減少． 視交叉上核のニューロンの神経発火は正常であることから，行動リズムの消失は全身の個々の細胞の分子時計の脱同調に起因すると考えられる．	代謝異常及び糖尿病の発症． 加齢に伴う唾液腺増殖の発症． 躁病の発症．
CLOCK KO	行動リズムは正常． 行動リズムの光同調の異常 分子時計により制御される遺伝子発現の日周性の異常．	報告なし．
BMAL1（MOP3）KO	恒暗条件下における行動リズムの消失． 活動量の減少． 分子時計により制御される遺伝子発現の日周性の消失．	老化の促進による寿命の短縮． 末梢血，脾臓，骨髄におけるB細胞の減少． BMAL1ノックアウトマウス胚由来線維芽細胞は脂肪細胞への分化能を消失．
NPAS2（MOP4）KO	恒暗条件下及び明暗条件下で正常な行動リズムを示す．	長期記憶の異常． ノンレム睡眠の異常．
CLOCK：NPAS2 double KO	恒暗条件下の行動リズムの消失．	報告なし．
PER1 KO	恒暗条件下の行動リズムの周期の短縮． 行動の光同調の異常（光による行動周期の前進が観察されない．） PER1：PER3 double KOマウスは，PER1 KOマウスと同じ表現型を示す．	報告なし．
PER2 KO	恒暗条件下において行動リズムの周期性の短縮が観察された後，行動リズムが消失する． 行動の光同調の異常（光による行動周期の後進が観察されない．） PER2：PER3 double KOマウスは，PER2 KOマウスと同じ表現型を示す．	リンパ腫の自然発症率の増加． ガンマ線照射後の癌発症率の増加．
PER3 KO	恒暗条件下及び明暗条件下で正常な行動リズムを示す．	報告なし．
PER1：PER2 double KO	恒暗条件下の行動リズムの消失． PER1：PER2：PER3 triple KOマウスは，PER1：PER2 double KOマウスと同じ表現型を示す．	報告なし．
CRY1 KO	恒暗条件下の行動リズムの周期の短縮．	報告なし．
CRY2 KO	恒暗条件下の行動リズムの周期の延長．	報告なし．
CRY1：CRY2 double KO	恒暗条件下の行動リズムの消失．	野生型マウスと同様のDNA損傷応答を示す．
CRY1：CRY2：p53 triple KO	報告なし．	p53変異マウスの腫瘍形成及び寿命の短縮の表現型が緩和する．
CRY1：PER2 double KO	恒暗条件下の行動リズムの消失．	報告なし．
CRY2：PER2 double KO	恒暗条件下及び明暗条件下で正常な行動リズムを示す．	報告なし．

分子時計制御における時計蛋白質の翻訳後修飾の役割

本来，転写及び翻訳は24時間よりもはるかに短い周期で行われる．従って，分子時計の周期を約24時間に維持するための細胞内機構の存在が考えられる．近年この機構に時計蛋白質の酵素活性及び翻訳後修飾の制御が重要な役割を担うことが報告されている[5]．例えば，時計蛋白質CRYは翻訳後リン酸化修飾され分解されるため，翻訳と細胞内蓄積には時間ラグが生じる．このラグは分子時計に約24時間の周期性を与えるために重要な過程である．

リン酸化以外の分子時計制御に関わる翻訳後修飾として，Sumo化及びアセチル化修飾が報告されている．BMAL1は時間依存的にSumo化修飾され，このSumo化修飾はBMAL1の安定性を制御する．CLOCKは長い間BMAL1と共同的に働く転写因子と考えられていた．近年，CLOCKがヒストンアセチルトランスフェラーゼ（HAT）活性を有することが報告され，CLOCKが酵素であることが明らかにされている[12]．ヒストンのアセチル化は遺伝子の転写活性に関与することが広く知られているが，CLOCKは自身のHAT活性により時間依存的にヒストンをアセチル化し，CLOCK：BMAL1二量体により転写活性化される遺伝子の時間依存的な転写制御を行う．また，CLOCKがそのHAT活性によりBMAL1を時間依存的にアセチル化すること及びこのCLOCKによるBMAL1のアセチル化は転写抑制因子CRYのCLOCK：BMAL1二量体へのリクルートメントを促進することが報告されている[6]．従って，CLOCKのHAT活性は分子時計のターゲット遺伝子の転写活性と転写抑制の両方の過程で重要な役割を担っている．興味深いことに，NAD^+依存的な脱アセチル化酵素SIRT1が時間依存的にBMAL1を脱アセチル化しBMAL1のアセチル化に日周性を与えることが報告されている[10]．SIRT1はDNA損傷応答や脂肪代謝制御等の多くの生理機能を制御することが知られている．従って，CLOCK及びSIRT1による時計蛋白質の翻訳後修飾という知見は分子時計が概日リズム以外の細胞機能をその調節因子の翻訳後修飾を介して制御することを強く示唆している．実際に，最近時計蛋白質CLOCKがアセチル化を介して糖代謝制御に関わるグルココルチコイドレセプター（GR）を機能調節することが報告されている[9]．

分子時計と疾患

躁鬱病の患者には睡眠/覚醒，体温，ホルモンの放出等の生理現象の概日性周期の異常が頻繁に観察されることが知られているように，自律神経疾患を含む多くの疾患の病態に時間因子が関与することが古くから報告されている．近年，時計蛋白質の変異マウスが概日リズムの異常に加え多くの疾患を発症することが報告され（表1），一部その病態メカニズムに分子時計が関与していることが強く示唆されている．例えば，*Clock*変異マウスが通常より高い頻度で躁病になることが報告されている[13]．また，*Per2*ノックアウトマウスは高頻度でリンパ腫を発症し[4]，*Bmal1*ノックアウトマウスは老化の促進という表現型を示す[8]．さらに，*Clock*変異マウスは代謝異常及び糖尿病を発症することが報告されている[14]．

分子時計は睡眠/覚醒，代謝，細胞周期等の様々な生理機能を制御する遺伝子の転写調節を介して他の細胞機能に影響を与える．また，時計蛋白質CLOCKは自身のHAT活性により，概日リズム以外の細胞機能を制御する蛋白質を翻訳後修飾し機能調節する．さらに，分子時計はCLOCKのHAT活性によりターゲット遺伝子の発現調節領域のクロマチンリモデリングを行うが，これは分子時計が細胞のエピジェネティック応答を担う可能性を示唆する（図1）．分子時計は多くの細胞機能の制御を担っていることから，分子時計の制御異常が直接疾患に関連することが考えられる．従って，分子時計の制御機構の詳細な理解は自律神経疾患をはじめとする病態の解明に新たな視点を与え，さらに分子時計の転写制御を指標とした診断法の開発や時計蛋白質を標的とした創薬に還元されることが期待される．

文　献

1) Doi M, Hirayama J, Sassone-Corsi P. Circadian regulator CLOCK is a histone acetyltransferase. Cell 2006; 125: 497—508.
2) Dunlap JC. Molecular bases for circadian clocks. Cell 1999; 96: 271—290.
3) Fu L, Lee CC. The circadian clock: pacemaker and tumour suppressor. Nat Rev Cancer 2003; 3: 350—361.
4) Fu L, Pelicano H, Liu J, et al. The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell 2002; 111: 41—50.

5) Gallego M, Virshup DM. Post-translational modifications regulate the ticking of the circadian clock. Nat Rev Mol Cell Biol 2007; 8: 139—148.
6) Hirayama J, Sahar S, Grimaldi B, et al. CLOCK-mediated acetylation of BMAL1 controls circadian function. Nature 2007; 450: 1086—1090.
7) King DP, Takahashi JS. Molecular genetics of circadian rhythms in mammals. Annu Rev Neurosci 2000; 23: 713—742.
8) Kondratov RV, Kondratova AA, Gorbacheva VY, et al. Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. Genes Dev 2006; 20: 1868—1873.
9) Nader N, Chrousos GP, Kino T. Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications. Faseb J 2009; 23: 1572—1583.
10) Nakahata Y, Kaluzova M, Grimaldi B, et al. The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. Cell 2008; 134: 329—340.
11) Okamura H. Clock genes in cell clocks: roles, actions, and mysteries. J Biol Rhythms 2004; 19: 388—399.
12) Panda S, Antoch MP, Miller BH, et al. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 2002; 109: 307—320.
13) Roybal K, Theobold D, Graham A, et al. Mania-like behavior induced by disruption of CLOCK. Proc Natl Acad Sci USA 2007; 104: 6406—6411.
14) Turek FW, Joshu C, Kohsaka A, et al. Obesity and metabolic syndrome in circadian Clock mutant mice. Science 2005; 308: 1043—1045.

Review

A Common Origin: Signaling Similarities in the Regulation of the Circadian Clock and DNA Damage Responses

Yoshimi UCHIDA,[a] Jun HIRAYAMA,*[,b] and Hiroshi NISHINA[a]

[a] *Department of Developmental and Regenerative Biology, Tokyo Medical and Dental University; and* [b] *Medical Top Track Program, Medical Research Institute, Tokyo Medical and Dental University; 1–5–45 Yushima, Bunkyo-ku, Tokyo 113–8510, Japan.*

Received November 30, 2009

Circadian clocks are intrinsic, time-tracking systems that endow organisms with a survival advantage. Studies of animal models and human tumor samples have revealed that the disruption of circadian rhythms is an important endogenous factor that can contribute to mammalian cancer development. The core of the circadian clock mechanism is a cell-autonomous and self-sustained oscillator system mediated by a transcription/translation-based negative feedback loop that relies on positive and negative elements. Recent studies have implicated these core circadian components in the regulation of both the cell cycle and DNA damage responses (DDR). Indeed, the circadian feedback loop controls the timing of cell proliferation by regulating the expression of key cell cycle genes. Conversely, several intracellular signaling cascades and post-translational modifications that play important roles in the cell cycle and DDR are also essential for circadian clock regulation. Importantly, alteration of a cell's reduction–oxidation (redox) state triggers the transduction of photic signals that regulate circadian clock gene transcription, suggesting that cellular responses to photo-oxidative stress may have been the evolutionary origin of the circadian clock. This review describes selected regulatory aspects of circadian machinery that are evidence of a molecular link between the circadian clock and DDR, focusing particularly on the signaling cascades involved in the light entrainment of the zebrafish circadian clock.

Key words circadian clock; DNA damage response; reduction–oxidation; zebrafish

INTRODUCTION

From bacteria to humans, almost all organisms can adapt the timing of their physiology to the cyclic changes of their environment, thanks to a naturally-selected intrinsic time-keeping system called the circadian clock.[1] The circadian clock enhances the physiological efficiency and survival of an organism by organizing its behavior and body functions.[2,3] During the circadian day, the organism's physiology is given over to catabolic processes, whereas the anabolic functions of growth, repair and consolidation occur at night. To achieve this schedule in mammals, the circadian clock regulates a number of physiological functions, including sleep and wakefulness, food intake, body temperature, cardiovascular and renal activity, hormone production, hepatic metabolism and immune responses.[4,5] Accordingly, disruption of the circadian clock in humans has been linked to profound effects on health, including insomnia, stomach ailments, depression and cancer.[4,5]

In most organisms, the molecular mechanisms underlying the establishment and maintenance of biological rhythms comprise interconnected transcription–translation feedback loops in which some clock factors repress their own transcription once they have attained critical levels.[2,3] These oscillators have the property of being endogenous and cell-autonomous systems that maintain their rhythm in the absence of external time cues.[6] Both vertebrates and invertebrates have circadian oscillators scattered throughout their bodies.[7,8] In mammals, the circadian system is composed of both central and peripheral oscillators.[7] The mammalian central clock is located in the suprachiasmatic nucleus (SCN) within the anterior hypothalamus of the brain.[9] This central clock acts as a coordinator and provides time signals via both neural and humoral routes that entrain independent peripheral clocks. Dysfunction of the central clock does not inactivate the peripheral clocks but instead causes individual peripheral oscillators to become temporally uncoupled.[10—12]

To guarantee that an organism's behavior remains tied to the rhythms of its environment, the circadian clock must be able to reset itself in response to environmental cues.[9,13,14] The main environmental stimulus for organisms is light, which is provided in day-night cycles. Mammals have no photoreceptors in peripheral tissues,[15] so that the effect of light on peripheral clocks is indirect.[13] For the mammalian clock, the SCN integrates photic cues from the retina and uses neural and humoral signals to transmit this information to peripheral clocks, synchronizing them.[16—18] This communication between the central and peripheral clocks results in the seamless regulation of fundamental physiological functions.[4,8] Interestingly, peripheral clocks can also respond directly to SCN-independent signals such as feeding and temperature change.[19,20] However, the physiological role of SCN-independent responses of peripheral clocks is not yet fully understood. A recent study has reported that DNA damage can also act as a resetting cue for the circadian clock in mammalian peripheral cells,[21] and other findings support a major role for the circadian machinery in cellular stress responses.[22] These data point to an intriguing link between the circadian clock and cellular stress responses, and it is the purpose of this review to summarize the evidence and explore the implications of such a link.

THE PHYSIOLOGICAL FUNCTIONS OF CORE CIRCADIAN REGULATORS

The core of the clock mechanism in *Drosophila*, *Neurospora* and mammals is a transcription/translation-based negative feedback loop that relies on positive and negative

* To whom correspondence should be addressed. e-mail: hirayama.mtt@mri.tmd.ac.jp

oscillator elements. In vertebrates, three basic helix–loop–helix Perid-Aryl hydrocardon receptor nuclear translocator-Single-minded (PAS) (PER-ARNT-SIM) domain-containing transcription factors, called CLOCK, NPAS2 and BMAL, constitute the positive elements.[3,23] CLOCK or NPAS2 heterodimerizes with BMAL to form an transcriptionally active complex that binds to E-box elements (CACGTG) present in the promoters of members of the *Period* (*Per*) and *Cryptochrome* (*Cry*) gene families (Fig. 1). Once the PER and CRY proteins have been translated, they form heterodimers that can then translocate to the nucleus to repress CLOCK (NPAS2):BMAL-mediated transcription through direct protein–protein interaction. Importantly, when active, the CLOCK (NPAS2):BMAL complex stimulates the transcription of many other clock-controlled genes. These genes in turn influence functions external to the oscillatory mechanism itself and mediate the "output" function of the clock.[24] This accounts in part for the presence of circadian rhythms in a variety of physiological processes.

A group of eight proteins comprises the basic sprockets of the molecular wheel that controls the mammalian circadian clock: PER1, PER2, PER3, CRY1, CRY2, CLOCK, NPAS2 and BMAL1.[3,23] The phenotypes of mice with targeted disruptions of these genes are summarized in Table 1. Studies of these mutant mice have revealed the distinct roles of clock proteins in regulating circadian rhythms as well as direct links between the circadian clock and non-circadian aspects of animal physiology. For example, mice with mutations in the *Per2* and *Bmal1* genes show increased sensitivity to ionizing radiation and a premature aging phenotype, respectively.[25,26] These findings implicate the core circadian machinery in the regulation of DNA damage response (DDR) and the cell cycle.

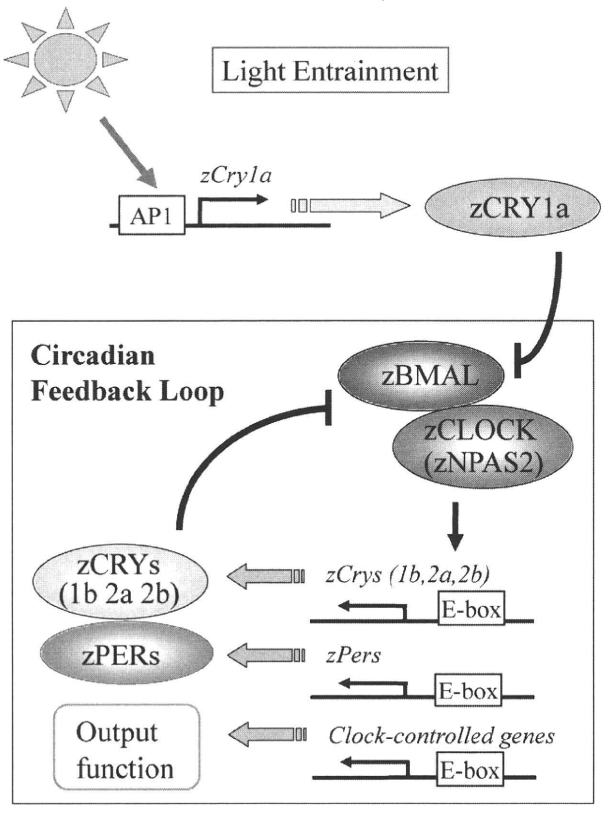

Fig. 1. Key Transcription Factors in the Zebrafish Circadian Feedback Loop

Zebrafish possess an intrinsic circadian oscillator that consists of components similar to those of mammals. CLOCK (NPAS2) and BMAL act as positive elements, whereas CRYs and PERs act as negative regulators. Zebrafish CRY1a, CRY1b, CRY2a, and CRY2b are transcriptional repressors. Expression of *zCry1a* depends on the transcription factor AP-1 and is stimulated by light, whereas *zCry1b*, *zCry2a* and *zCry2b* expression are under the control of the heterodimeric zCLOCK (zNPAS2):zBMAL transcription factor that binds to E-box elements in target gene promoters. Expression of the zPER transcriptional repressors is also stimulated by the zCLOCK (zNPAS2):zBMAL complex. In zebrafish, light-induced zCRY1a inhibits the transcription dependent on zCLOCK (zNPAS2):zBMAL, thereby regulating the light entrainment of the circadian clock.

At the molecular level, the circadian clock controls the timing of cell proliferation by regulating the expression of key cell cycle genes such as *Wee1* and *c-Myc*.[25,27] In addition, PER1 interacts with crucial components of cellular stress response pathways including the ataxia telangiectasia mutated (ATM) and checkpoint kinase 2 (Chk2) proteins.[28] Accordingly, ectopic expression of PER1 or PER2 results in cell growth inhibition, cell cycle arrest, apoptosis, or loss of clonogenic capacity.[28–30] PER1 and PER2 also interact with the androgen receptor (AR) or estrogen receptor (ER), respectively, in that PER1 inhibits AR-dependent transcription and PER2 induces ER degradation.[31,32] These findings are consistent with the idea that clock proteins act as key players in the cell cycle and DDR by interacting directly with and regulating the functions of the proteins mediating these processes.

POSSIBLE CROSSTALK BETWEEN THE CIRCADIAN CLOCK AND CELLULAR PROCESSES THROUGH SHARED POST-TRANSLATIONAL MODIFICATIONS

Post-translational modifications, such as phosphorylation, sumoylation and acetylation, are important modulators of circadian transcription factors and regulate their transcriptional activity, subcellular localization and stability.[6,33] The effects of selected post-translational modifications of various circadian regulators are summarized in Table 2. Below we discuss the role of clock protein acetylation in circadian regulation. The detailed functions of other post-translational modifications of clock proteins have been reviewed elsewhere.[6,33,34]

In mammals, the core circadian regulator CLOCK has intrinsic histone acetyltransferase (HAT) activity[35] that it uses to acetylate its heterodimeric partner BMAL1.[36] This CLOCK-mediated acetylation increases the interaction of the CLOCK:BMAL1 complex with CRY1, providing another level of control in the circadian negative feedback loop.[36] BMAL1 is deacetylated by SIRT1, a nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase (HDAC).[37] Accordingly, BMAL1 acetylation is significantly increased and only mildly rhythmic in livers of liver-specific *Sirt1*-deficient mice.[37] SIRT1 also deacetylates PER2, giving SIRT1 an additional function in circadian transcription regulation.[38] The finding that CLOCK can acetylate non-histone substrates such as BMAL1 has sparked a search for other cellular targets. CLOCK interacts directly with the nuclear receptors retinoic acid receptor (RAR)α and retinoid X receptor (RXR)α through CLOCK's putative nuclear receptor interaction domain (NRID),[39] suggesting that CLOCK may acetylate nuclear receptors. Similarly, CLOCK interacts with and acetylates the glucocorticoid receptor, negatively regulating its transactivation capacity.[40] Thus, in addition to its ef-

Table 1. Phenotypes of Mutant Mice Disrupted in Circadian Clock Genes

Name	Mutation	Circadian phenotype	Non-circadian phenotype
CLOCK -del.19 mutant	Deletion of exon 19 in murine CLOCK gene.[89] Produces a mutant CLOCK protein that functions as a dominant negative regulator.[35]	Circadian period extended by 4h, followed by a complete loss of circadian rhythmicity in DD.[a),89] Displays reduced levels and non-cycling of expression of clock-controlled genes.[89] Displays reduced light induction of immediate early genes in SCN.[90] Shows normal spontaneous firing rhythms of SCN neurons, suggesting that loss of circadian rhythmicity may be due to uncoupling of oscillators.[91]	Diurnal feeding rhythm attenuated.[92] Develops obesity and metabolic syndrome.[92] Develops salivary gland hyperplasia with age.[93] Displays mania-like behavior, including hyperactivity, decreased sleep, lowered depression-like behavior, and lower anxiety.[94]
CLOCK KO	Knockout mutation.[95]	Exhibits almost normal circadian patterns of behavior.[95] Shows altered responses to light, with reduced phase delays and exaggerated phase advances.[95] Displays altered clock-controlled gene expression.[95]	None reported
BMAL1 (MOP3) KO	Knockout mutation.[96]	Displays immediate and complete loss of circadian rhythmicity in DD.[96] Displays altered distribution of activity during LD[a)] and reduced total activity.[96] Shows reduced levels and non-cycling of expression of clock-controlled genes.[96]	Reduced life span with symptoms of premature aging.[26] Displays reduced levels of B cells in the peripheral blood, spleen and bone marrow.[97] BMAL1-deficient embryonic fibroblasts cannot differentiate into adipocytes.[98]
NPAS2 (MOP4) KO	Knockout mutation.[23]	Exhibits almost normal circadian patterns of behavior.[23] Has a slightly shortened circadian period and an altered response to perturbations in the LD cycle.[23]	Displays deficits in the long-term memory arm of the cued and contextual fear task.[99] Displays defect in the homeostatic regulation of non-rapid eye movement sleep time.[100]
CLOCK; NPAS2 double KO	Double knockout.[23]	Displays immediate and complete loss of circadian rhythmicity in DD.[23]	None reported
PER1 KO	Knockout mutation.[101]	Displays a shorter circadian period in DD.[101] Exhibits a defect in circadian clock resetting (unable to advance the clock).[102] Per1; Per3 KO has the same phenotype.[103]	None reported
PER2 KO	Knockout mutation.[103]	Displays a shorter circadian period followed by a loss of circadian rhythmicity in DD.[103] Exhibits a defect in circadian clock resetting (unable to delay the clock).[102] Per2 mutant; Per3 KO has the same phenotype.[103]	Is cancer-prone and shows increased sensitivity to γ-irradiation.[25]
PER3 KO	Knockout mutation.[103]	Has normal circadian pattern of behavior.[103]	None reported
PER1; PER2 double KO	Double knockout.[103]	Displays a complete loss of circadian rhythmicity in DD.[103] PER1; PER2; PER3 triple KO has the same phenotype.[103]	None reported
CRY1 KO	Knockout mutant.[104]	Exhibits accelerated free-running periodicity of locomotor activity.[104]	None reported
CRY2 KO	Knockout mutant.[104]	Exhibits delayed free-running periodicity of locomotor activity.[104]	None reported
CRY1; CRY2 double KO	Double knockout.[104]	CRY1; CRY2 double KO displays immediate and complete loss of circadian rhythmicity in DD.[104]	Normal genotoxic stress-induced morbidity and mortality.[105]
CRY1; CRY2; p53 triple KO	Triple knockout.[106]	None reported	Shows delayed onset of cancer and extended median lifespan upon genotoxic stress compared to mice lacking only p53.[106]
CRY1; PER2 double KO	Double knockout.[107]	Displays an immediate loss of circadian rhythmicity in DD.[107] Normal circadian behavior in LD.[107]	None reported
CRY2; PER2 double KO	Double knockout.[107]	Displays normal circadian behavior in LD and DD.[107]	None reported

a) DD, dark–dark cycle, *i.e.* constant darkness. LD, light–dark cycle.

Table 2. Post-Translational Modifications of Murine Circadian Clock Proteins

Protein	Modification (site)	Regulator	Effect/function
CLOCK	Phosphorylation (unknown)	BMAL1 induces phosphorylation, whereas CRYs induce unphosphorylated form.	Phosphorylated CLOCK is transcriptionally active[108,109] and localized in the nucleus.[110] Phosphorylation may regulate CLOCK's subcellular localization.
	Phosphorylation (Ser-Pro-rich region)	H1 kinase	Unknown.[111]
	Phosphorylation (Ser38 and Ser42)	Unknown	Prevents CLOCK:BMAL1 complex from binding to E-box, thus inhibiting CLOCK:BMAL1-dependent transcription.[34]
	Phosphorylation (Ser427)	GSK-3β	Induces CLOCK degradation.[112] CIPC induces phosphorylation.[34]
	Phosphorylation (unknown)	PKG	Unknown.[113]
	Phosphorylation (unknown)	PKC	PKC-mediated phosphorylation stimulates CLOCK: BMAL1-dependent transcription.[114]
BMAL1	Phosphorylation (unknown)	CLOCK induces phosphorylation, whereas CRYs induce unphosphorylated form.	Phosphorylated BMAL1 is transcriptionally active.[108,109]
	Phosphorylation (unknown)	Casein kinase 1 (CK1)	CKI-mediated phosphorylation stimulates CLOCK: BMAL1-dependent transcription.[115]
	Phosphorylation (Ser-527, Thr-534, Ser-599)	MAPK/ERK	Thr-534 phosphorylation inhibits CLOCK:BMAL1 dependent transcription. Functions of Ser-527 and Ser-599 phosphorylations are unknown.[116]
	Phosphorylation (Ser-90)	Casein kinase 2 (CK2)	Required for BMAL1 nuclear translocation.[43]
	Sumoylation (Lys-259)	CLOCK induces Sumoylation.	Regulates BMAL1 protein half-life.[117]
	Acetylation (Lys-537)	CLOCK acetylates BMAL1, whereas SIRT1 deacetylates it.	CLOCK-mediated BMAL1 acetylation facilitates CRY recruitment to the CLOCK:BMAL1 complex.[36,37]
PER1	Phosphorylation (amino acids 902—916)	CK1	Masks PER1 nuclear localization signal, inducing PER1 cytoplasmic localization.[118]
	Phosphorylation (amino acids 653—663)	CK1	Required for nuclear translocation of PER1.[119]
PER2	Phosphorylation (β-TrCP recognition motif)	CK1	Required for TrCP binding and subsequent PER2 degradation.[120]
	Phosphorylation (unknown)	GSK-3β	Induces nuclear localization of PER2.[48]
	Phosphorylation (Ser-10, Thr-12, Ser-13, and Thr-15)	CK2	Stabilizes PER2.[42]
	Phosphorylation (Ser-53)	CK2	Induces PER2 degradation.[44]
	Acetylation (unknown)	Acetylase is unknown. SIRT1 deacetylates PER2.	Deacetylation of PER2 by SIRT1 induces PER2 degradation.[38]
PER3	Phosphorylation (amino acids 613—626)	CK1	Induces PER3 degradation and nuclear translocation.[121]
CRY1	Phosphorylation (unknown)	CK1	Function unknown. PER acts as a scaffold that brings CK1 and CRY into close proximity.[115]
	Phosphorylation (Ser247)	MAPK/ERK	Reduces transcriptional inhibition activity.[122]
CRY2	Phosphorylation (Ser265)	MAPK/ERK	Reduces transcriptional inhibition activity.[122]
	Phosphorylation (Ser557)	MAPK/ERK (MAPK/ERK phosphorylates the Ser-557 of CRY2 *in vitro*, but it is unlikely to contribute to this phosphorylation *in vivo*.)	Function unknown but phospho-Ser-557-CRY2 is localized specifically in the nucleus and displays robust circadian variation.[51,122]
	Phosphorylation (Ser553)	GSK-3β	Induces degradation.[51] GSK-3β induces this phosphorylation only if Ser-557 is also phosphorylated.[51]

fects on circadian clock elements, CLOCK targets key components of the cell cycle machinery.

It is well established that post-translational modifications are vital for the regulation of the cell cycle and DDR.[41] SIRT1[37,38] and casein kinase 2 (CK2),[42—44] already identified as responsible for post-translational modifications of clock proteins, have also been implicated in post-translational modifications of proteins such as p53, FoxO, and E-cadherin that are involved in cellular metabolism, the cell cycle, and DDR.[45,46] These findings support the hypothesis that the circadian clock may be linked to other cellular processes through shared post-translational modifications.

POSSIBLE CROSSTALK BETWEEN THE CIRCADIAN CLOCK AND CELLULAR PROCESSES THROUGH SHARED INTRACELLULAR SIGNALING CASCADES

Vertebrate circadian feedback loops are affected by an enormous variety of stimuli known to induce intracellular signaling pathways, including pathways involving protein kinase C (PKC), glucocorticoid, Wnt, tumor growth factor (TGF)-β/activin, and mitogen-activated protein kinases (MAPKs).[47—50] Conversely, several studies have demonstrated at a molecular level how these intracellular signaling mediators contribute to circadian regulation. For example, in mammals, glycogen synthase kinase-3β (GSK-3β), the key kinase regulating the Wnt signaling pathway, phosphorylates CRY2 and PER2 and thereby controls CRY2 protein stability and PER2 subcellular localization.[48,51] Another example is the stimulation of *Dec1* gene expression that is induced by the TGF-β/activin signaling pathway during alkaline shock-induced entrainment of mammalian peripheral clocks.[49] The signaling pathways involving these mediators play essential roles in development, proliferation, DDR, and cell death processes, suggesting that these processes and the circadian clock are separate nodes of a common regulatory network. In support of this idea, a recent study in zebrafish has revealed that the same MAPK signaling cascades are involved in both DDR and the light-dependent pathways responsible for entrainment of the circadian clock[52] (see below).

ZEBRAFISH AS A MODEL SYSTEM FOR CIRCADIAN CLOCK STUDY

Zebrafish are a good model organism in which to study the vertebrate circadian clock because the molecular components of the mammalian and zebrafish circadian oscillators are same. Moreover, cell-autonomous circadian oscillators are present throughout the peripheral tissues and organs of zebrafish.[53,54] Importantly, zebrafish peripheral clocks are directly light-responsive.[55] Thus, in zebrafish organs and tissues, an acute light pulse can be used to transactivate clock genes and thus entrain oscillations of clock gene expression to a new light-dark cycle. In addition, cultured lines of embryonal zebrafish cells, which recapitulate most features of the zebrafish clock system, have been established as an attractive vertebrate cell-based model suitable for the examination of the light signaling pathway and its impact on the circadian clock.[56] Studies using these cell lines have revealed critical roles for redox control and MAPK signaling pathways in light-dependent circadian entrainment[57] (Fig. 2).

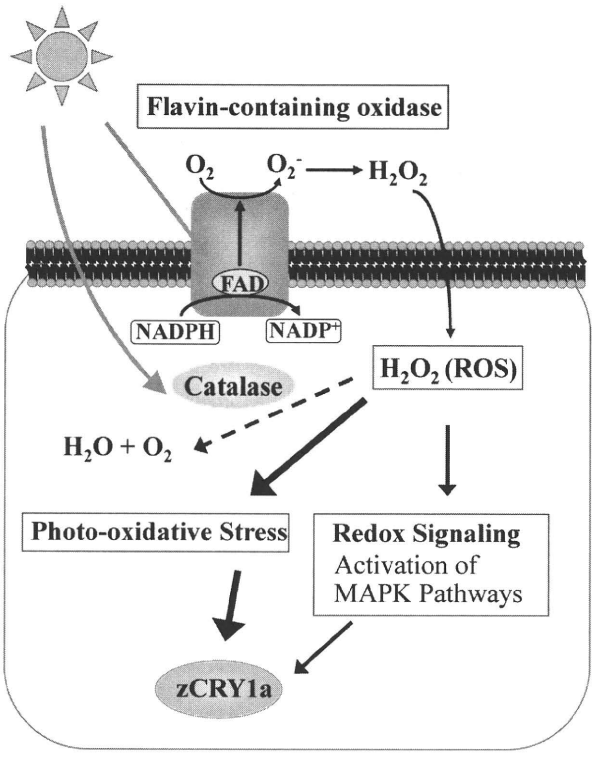

Fig. 2. A Model of a Potential Molecular Mechanism Underlying Light-Dependent Redox Signaling in Zebrafish

In the presence of flavin-containing oxidases, light drives the production of intracellular ROS such as H_2O_2. Excess ROS production has deleterious effects because ROS can react with various cellular targets to cause photo-oxidative stress. However, light-induced ROS can also take on a signaling role by stimulating MAPK pathways that lead to transcriptional activation, including transactivation of the *zCry1a* gene. Importantly, light also increases *catalase* transcription and thus intracellular catalase activity, resulting in degradation of H_2O_2 and decreased photo-oxidative stress. This reduction in ROS also leads to decreased *zCry1a* expression, thus creating a negative feedback loop that directly impinges on the circadian clock.

Other cellular functions, including the cell cycle and DDR, are also directly regulated by light in zebrafish.[52,58,59] The dissection of the light signaling pathways used for circadian entrainment in zebrafish peripheral cells has revealed common mediators also shared by pathways used to regulate the cell cycle and DDR.[52,60]

REDOX SIGNALING IS INVOLVED IN THE LIGHT ENTRAINMENT OF THE ZEBRAFISH PERIPHERAL CLOCK

As a result of whole genome duplication during the evolution of the teleost lineage, the circadian oscillator of zebrafish contains duplications of most clock genes.[53] Seven zebrafish *Cry* genes (*zCry1a*, *1b*, *2a*, *2b*, *3*, *4* and *Dash*) have been cloned.[53,61] Investigation of the *in vitro* functions of these genes has shown that they fall into two groups: one group inhibits CLOCK:BMAL-mediated transcription (repressor type CRYs: zCRY1a, 1b, 2a and 2b), whereas the other group does not inhibit transcription (non-repressor type CRYs: zCRY3, 4 and Dash).[61,62] Despite its structural and functional similarities to the repressor type CRYs, *zCry1a* transcription is quite different from that of the other repressor type CRYs and is strictly light-dependent (Fig. 1, top). *zCry1a* transcription exhibits circadian oscillation in zebrafish cells exposed to a light-dark cycle but this oscillation

dampens quickly after transfer of the cells to constant darkness, indicating that zCRY1a functions only in light-dependent circadian clock regulation.[57,63,64] Indeed, there is evidence that light-induced zCRY1a is a transcriptional repressor essential for the light-dependent entrainment of the circadian clock.[63]

The light-dependent transcription of *zCry1a* is controlled through the production and removal of cellular reactive oxygen species (ROS).[57] ROS were originally thought to act solely as toxic metabolites because they react with components of DNA, proteins and lipids and exert oxidative stress.[65] However, ROS are also ideally suited to be signaling molecules because they are small in size and can easily diffuse short distances within a cell. In addition, mechanisms for ROS production (such as *via* flavin-containing oxidases) and its rapid removal (such as *via* catalase) are present in almost all cell types[65,66] (Fig. 2). In a variety of organisms, light induces ROS production that leads to altered redox status.[65,67] In zebrafish cells, this light-induced redox change stimulates intracellular MAPK signaling that transduces photic signals to *zCry1a* transactivation.[57] Importantly, light also increases intracellular catalase activity by stimulating catalase transcription, an event that occurs after the maximum expression of the *zCry1a* gene has been reached.[57] This increased catalase activity diminishes light-induced cellular ROS levels, resulting in decreased *zCry1a* transcription and creating a negative feedback loop.

The regulation of the circadian clock by redox signaling raises an important issue concerning the identity of the circadian photoreceptor. Because ROS can transduce a photic signal to the circadian machinery, the phototransducing molecules responsible for light-dependent ROS production should function as the circadian photoreceptor in zebrafish cells. We hypothesize that flavin-containing oxidases, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and acyl-CoA oxidase, may be good candidates for the circadian photoreceptors. These enzymes, which produce intracellular ROS, are activated by a plethora of extracellular stimuli, including visible light, wounding, and low and high temperature.[65,67] In addition, we have found that diphenyleneiodonium, an inhibitor of NADPH oxidase, efficiently suppresses light-induced activation of *zCry1a* transcription in cultured zebrafish cells (Uchida *et al.*, unpublished data). Redox signaling also appears to play a significant role in circadian regulation in other organisms. In *Drosophila*, a genome-wide screen identified several redox molecules as essential for the light entrainment of the circadian clock.[68] Similarly, a study in mammals showed that an increase in reduced NADPH and NADH levels enhanced the affinity of the NPAS2 (CLOCK):BMAL1 complex for its target DNA *in vitro*.[69] Thus, redox state may be an important determinant of circadian oscillations in mammalian peripheral tissues.

The circadian clock is thought to have first arisen with the evolution of a eukaryotic lifestyle. This lifestyle requires that the fragile DNA exposed during mitosis be protected from photo-oxidative stress.[70,71] The development of a circadian rhythm would be one way to separate diurnal and nocturnal metabolic processes, with light-dark cycles acting as the selective force. In this scenario, photo-oxidative stress could have been a decisive factor in relegating the anabolic processes of mitosis, growth and consolidation to the dark hours. Thus, it is reasonable to propose that redox signaling is utilized in the control of the circadian clock, and that common regulatory pathways may mediate both cellular responses to photo-oxidative stress and the light entrainment of the circadian clock.

LIGHT-DEPENDENT CIRCADIAN ENTRAINMENT AND DDR SHARE A COMMON SIGNALING PATHWAY

In many organisms, external stimuli are connected to a cell's nucleus *via* MAPK signaling pathways, and roles for MAPKs in circadian clock regulation are well-established.[6,50] There are three major MAPKs: c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK).[72] Light-induced ROS production in zebrafish cells leads to downstream activation of MAPK cascades, which then contribute to the regulation of *zCry1a* transcription[52,57] (Fig. 2). Interestingly, light-induced ERK activation triggers *zCry1a* transcription, whereas light-induced p38 activation suppresses it,[52] highlighting a MAPK-mediated cross-regulatory mechanism of circadian regulation (Fig. 3). We have also found that light directly activates the JNK signaling cascade in zebrafish cells (Uchida *et al.*, unpublished data), an event whose physiological function is under investigation.

Our work has recently shown that light-induced activation of MAPK cascades can contribute to a non-circadian biological process, namely photoreactivation.[52] Photoreactivation is a mechanism by which visible light reverses some of the lethal and mutagenic effects of UV irradiation.[73] Photoreactivation is mediated by DNA photolyases (PHRs), which are DNA repair enzymes.[74] When cells are irradiated with UV light, photoproducts called cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts [(6-4) photoproducts] are produced in the DNA. The PHRs bind to and repair these types of DNA damage using visible light

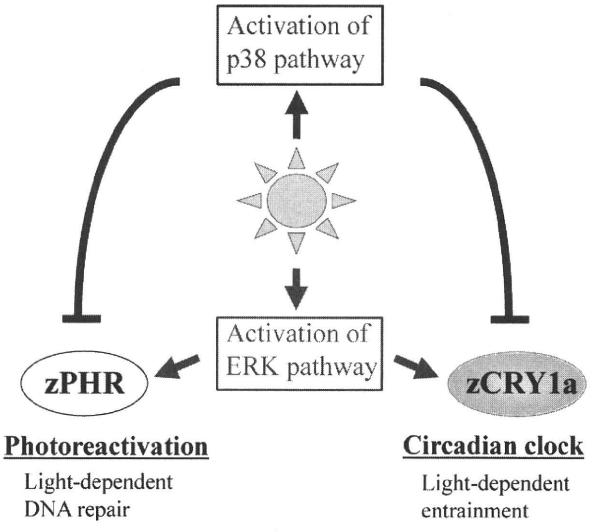

Fig. 3. A Model Depicting the Molecular Mechanisms Underlying Light-Dependent Transcriptional Events Regulating DNA Repair and Circadian Entrainment

Light activates both ERK and p38 MAPK signaling cascades. The former positively regulates expression of both *zPhr* and *zCry1a* genes whereas the latter inhibits it. The light-induced zPHR repairs DNA lesions by utilizing visible light energy. On the other hand, the light-induced zCRY1a entrains the circadian clock by acting as a transcriptional repressor.

as an energy source. Two classes of PHRs have been identified, one specific for CPDs (CPD PHRs) and the other specific for (6-4) photoproducts (64PHRs).[74,75] Both CPD PHRs and 64PHRs are induced by visible light in cultured fish cells.[52,59,76] We have obtained evidence indicating that photoreactivation in zebrafish is a cell-autonomous phenomenon, and that both the induction of 64PHRs in response to light and the subsequent light-dependent repair of DNA by 64PHRs are essential for successful photoreactivation.[52] Notably, the expression levels of the *z64Phr* gene associated with photoreactivation, as well as the *zCry1a* gene associated with the circadian clock, are regulated by the same light-induced MAPK cascades (Fig. 3). Light-induced ERK activation triggers the expression of *z64Phr*, whereas light-induced p38 activation inhibits it.[52] Thus, light-dependent DNA repair and the entrainment of the circadian clock are governed by shared regulatory pathways.

Both CRYs and PHRs belong to the DNA photolyase/cryptochrome protein family and are highly similar in amino acid sequence.[75,77,78] Evolutionary studies have shown that the animal CRY protein first functionally diverged from the CPD photolyase, and then diverged further to generate 64PHR.[79] These facts, together with the observation that *zCry1a* and *z64Phr* share regulatory pathways,[52] strongly indicate an evolutionary link between the circadian clock and DDR. Although solar light has many beneficial uses, including photosynthesis and the entrainment of circadian clock, the UV component of solar energy is harmful to living cells because it produces cytotoxic, mutagenic and carcinogenic lesions in DNA. It is speculated that natural selective pressure must then have forced the development of a self-defense system such as the DNA repair mechanism mediated by DNA PHRs.[75,78] Thus, it is not surprising that two ostensibly very different biological events, repair of UV-damaged DNA and light entrainment of the circadian clock, are governed by the same signaling pathways.

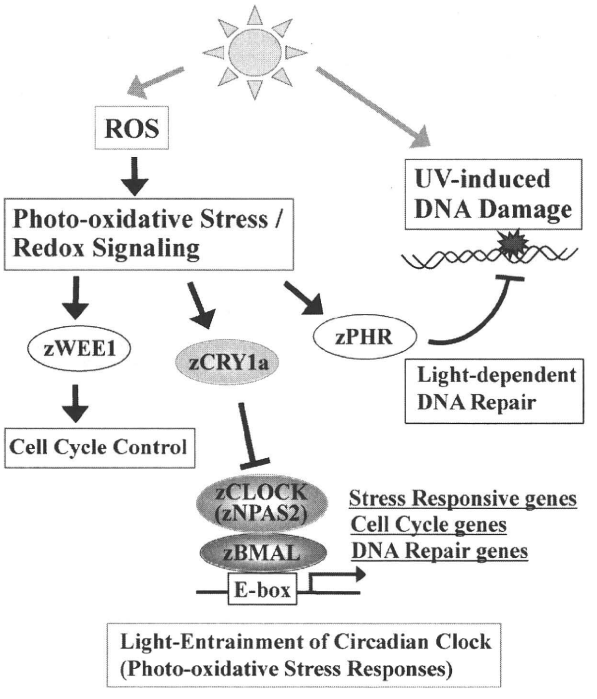

Fig. 4. A Model of Light-Induced Signaling Cascades Potentially Involved in Shared Control of the Circadian Clock, DNA Repair and the Cell Cycle

Sunlight has two major toxic effects: generation of ROS that mediate photo-oxidative stress and induction of UV-mediated DNA damage. In zebrafish, photo-oxidative stress can act as a redox signal that induces expression of a DNA repair gene (*Phr*), a circadian clock gene (*Cry1a*), and a cell cycle regulator gene *(Wee1*). In the presence of solar light, PHR repairs UV-induced DNA damage and WEE1 halts the cell cycle, protecting the genome of the organism. At the same time, ROS induced by the sun trigger expression of zCRY1a. This transcriptional repressor interacts directly with the zCLOCK (zNPAS2):zBMAL complex and inhibits its transcriptional capacity, thereby entraining the circadian clock. Notably, the zCLOCK (zNPAS2):zBMAL complex also regulates the transcription of a variety of genes involved in cellular stress responses. Thus, it is conceivable that light-dependent circadian entrainment may have originated as a cellular stress response against photo-oxidative stress and/or UV-induced DNA damage.

A COMMON LIGHT-INDUCED SIGNALING PATHWAY REGULATES THE CIRCADIAN CLOCK AND THE CELL CYCLE

Both the cell cycle and the circadian clock are endogenous pacemakers. These mechanisms coexist in most eukaryotic cells and share a number of conceptual features. In particular, both rely on interconnected autoregulatory loops that consist of sequential phases of transcription–translation, protein modification, and degradation.[80] Increasing evidence points to functional links between the cell cycle and circadian rhythms in a variety of organisms.[5,80] In zebrafish, the cell cycle is directly regulated by light. Light determines the timing of S phase entry, establishing a circadian rhythm for cell cycle progression.[58] At the molecular level, light induces the expression of zebrafish *Wee1* (*zWee1*), a cell cycle gene.[60] The Wee1 kinase controls the timing of the G_2-M transition by directly phosphorylating and thus inhibiting Cdc2/cyclin B, leading to suppression of mitotic cell division.[81] This mechanism is consistent with the observation that the growth of cultured zebrafish cells is suppressed by light.[60] Because solar light increases intracellular ROS levels that can exert oxidative stress,[57,65] light-induced *zWee1* expression may act as a cellular stress response, suppressing cell growth under conditions where DNA damage is likely (Fig. 4).

The transcription factor AP-1 modulates a wide range of cellular processes, including cell proliferation, apoptosis and the circadian clock.[82] In mammals, various stimuli activate AP-1, which binds directly to the consensus AP-1 motif within the *Wee1* promoter and drives *Wee1* expression.[83] In zebrafish, light can induce AP-1 activation, which then triggers *zWee1* transcription.[60] Components of AP-1 that are acutely light-inducible include *c-fos*, *fos-B* and *jun-B*.[84,85] In mammals, light-dependent activation of AP-1 in the SCN has been implicated in light-induced phase-shifting of the circadian clock.[84,85] Another factor that induces AP-1 activation is alteration of a cell's redox state. ROS-triggered induction of AP-1 is mediated by MAPK signaling pathways.[86] It is therefore conceivable that a light-induced alteration of cellular redox status could stimulate AP-1 to initiate light-dependent *zCry1a* expression. Indeed, light-stimulated AP-1 activity contributes to *zCry1a* transactivation involved in light entrainment of the circadian clock.[60] Taken together, these findings provide strong evidence that the cell cycle and circadian clock are regulated by a common signaling pathway controlled by AP-1 transcription factor.

PERSPECTIVE

Much evidence has accumulated indicating that evolutionary links exist that have resulted in the functional coupling of the circadian clock and DDR. In *Neurospora*, PRD-4, an orthologue of mammalian Chk2, transduces DNA damage signals into the core circadian machinery, resetting the clock.[87] In the diatom *Phaeodactylum tricornutum*, PtCPF1 (Phaeodactylum tricornutum cryptochrome/photolyase family 1) is a novel cryptochrome/photolyase family member that not only repairs UV-induced DNA damage but also acts as a transcriptional repressor of the circadian clock.[88] In addition, the critical role of redox signaling in the light-dependent entrainment of the circadian clock[57,68] strongly implicates cellular responses to the toxic effects of sunlight as the evolutionary origin of circadian rhythms.

The UV component of sunlight and the photo-oxidative stress derived from it are two major sources of harm to cells (Fig. 4). In lower vertebrates such as zebrafish, the light-induced PHRs repair UV-damaged DNA using light as an energy source.[52] Importantly, this light-induced activation of *DNA Phr* expression appears to be stimulated by photo-oxidative stress.[76] These observations are consistent with the idea that photo-oxidative stress may be utilized as a signal to activate DNA repair enzymes that can protect the organism's DNA from UV-induced damage. The fact that light-dependent expression of *zCry1a* is induced by alteration of a cell's redox state,[57] together with the finding that *zCry1a* and *DNA Phr* are governed by shared light-induced signaling pathways,[52] strongly suggests that oxidative stress may act as a signal triggering the light-induced expression of *zCry1a*. Indeed, H_2O_2, a well-known inducer of oxidative stress, can activate *zCry1a* transcription in zebrafish peripheral cells.[57] Once translated, the zCRY1a protein interacts directly with the CLOCK (NPAS2):BMAL complex and regulates its transcriptional capacity, entraining the circadian clock.[63] The circadian machinery regulates a variety of key genes involved in cellular stress responses, DNA repair, and cell cycle regulation.[24] Thus, zCRY1a may be the key integrator of external signals (such as oxidative stress) that controls the core circadian machinery and regulates the transcription of genes responsible for stress responses, DDR and cell cycle adjustments. It is tempting to speculate that, at least in zebrafish, the light entrainment of the circadian clock reflects a long-standing cellular response to photo-oxidative stress.

Acknowledgements We would like to thank all members of the Nishina and Katada (University of Tokyo) laboratories for critical reading and helpful discussions. This work was supported by Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (a Grant-in-Aid for Scientific Research on a Priority Area and Special Coordination Fund for Promoting Science) and the Ministry of Health, Labor and Welfare of Japan. This work was also supported by grant from the Takeda Science Foundation.

REFERENCES AND NOTES

1) Reppert S. M., Weaver D. R., *Nature* (London), **418**, 935—941 (2002).
2) Dunlap J. C., *Cell*, **96**, 271—290 (1999).
3) Okamura H., *J. Biol. Rhythms*, **19**, 388—399 (2004).
4) King D. P., Takahashi J. S., *Annu. Rev. Neurosci.*, **23**, 713—742 (2000).
5) Fu L., Lee C. C., *Nat. Rev. Cancer*, **3**, 350—361 (2003).
6) Hirayama J., Sassone-Corsi P., *Curr. Opin. Genet. Dev.*, **15**, 548—556 (2005).
7) Cermakian N., Sassone-Corsi P., *Nat. Rev. Mol. Cell Biol.*, **1**, 59—67 (2000).
8) Schibler U., Sassone-Corsi P., *Cell*, **111**, 919—922 (2002).
9) Pando M. P., Sassone-Corsi P., *Sci. STKE*, **2001**, RE16 (2001).
10) Pando M. P., Morse D., Cermakian N., Sassone-Corsi P., *Cell*, **110**, 107—117 (2002).
11) Nagoshi E., Saini C., Bauer C., Laroche T., Naef F., Schibler U., *Cell*, **119**, 693—705 (2004).
12) Yoo S. H., Yamazaki S., Lowrey P. L., Shimomura K., Ko C. H., Buhr E. D., Siepka S. M., Hong H. K., Oh W. J., Yoo O. J., Menaker M., Takahashi J. S., *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 5339—5346 (2004).
13) Cermakian N., Sassone-Corsi P., *Curr. Opin. Neurobiol.*, **12**, 359—365 (2002).
14) Tamai T. K., Carr A. J., Whitmore D., *Biochem. Soc. Trans.*, **33**, 962—966 (2005).
15) Underwood H., Groos G., *Experientia*, **38**, 1013—1021 (1982).
16) Balsalobre A., Brown S. A., Marcacci L., Tronche F., Kellendonk C., Reichardt H. M., Schutz G., Schibler U., *Science*, **289**, 2344—2347 (2000).
17) Schibler U., Ripperger J., Brown S. A., *J. Biol. Rhythms*, **18**, 250—260 (2003).
18) Ishida A., Mutoh T., Ueyama T., Bando H., Masubuchi S., Nakahara D., Tsujimoto G., Okamura H., *Cell Metab.*, **2**, 297—307 (2005).
19) Brown S. A., Zumbrunn G., Fleury-Olela F., Preitner N., Schibler U., *Curr. Biol.*, **12**, 1574—1583 (2002).
20) Damiola F., Le Minh N., Preitner N., Kornmann B., Fleury-Olela F., Schibler U., *Genes Dev.*, **14**, 2950—2961 (2000).
21) Oklejewicz M., Destici E., Tamanini F., Hut R. A., Janssens R., van der Horst G. T., *Curr. Biol.*, **18**, 286—291 (2008).
22) Kondratov R. V., Gorbacheva V. Y., Antoch M. P., *Curr. Top. Dev. Biol.*, **78**, 173—216 (2007).
23) DeBruyne J. P., Weaver D. R., Reppert S. M., *Nat. Neurosci.*, **10**, 543—545 (2007).
24) Panda S., Antoch M. P., Miller B. H., Su A. I., Schook A. B., Straume M., Schultz P. G., Kay S. A., Takahashi J. S., Hogenesch J. B., *Cell*, **109**, 307—320 (2002).
25) Fu L., Pelicano H., Liu J., Huang P., Lee C., *Cell*, **111**, 41—50 (2002).
26) Kondratov R. V., Kondratova A. A., Gorbacheva V. Y., Vykhovanets O. V., Antoch M. P., *Genes Dev.*, **20**, 1868—1873 (2006).
27) Matsuo T., Yamaguchi S., Mitsui S., Emi A., Shimoda F., Okamura H., *Science*, **302**, 255—259 (2003).
28) Gery S., Komatsu N., Baldjyan L., Yu A., Koo D., Koeffler H. P., *Mol. Cell*, **22**, 375—382 (2006).
29) Hua H., Wang Y., Wan C., Liu Y., Zhu B., Yang C., Wang X., Wang Z., Cornelissen-Guillaume G., Halberg F., *Cancer Sci.*, **97**, 589—596 (2006).
30) Oda A., Katayose Y., Yabuuchi S., Yamamoto K., Mizuma M., Shirasou S., Onogawa T., Ohtsuka H., Yoshida H., Hayashi H., Rikiyama T., Kim H., Choe Y., Kim K., Son H., Motoi F., Egawa S., Unno M., *Anticancer Res.*, **29**, 1201—1209 (2009).
31) Cao Q., Gery S., Dashti A., Yin D., Zhou Y., Gu J., Koeffler H. P., *Cancer Res.*, **69**, 7619—7625 (2009).
32) Gery S., Virk R. K., Chumakov K., Yu A., Koeffler H. P., *Oncogene*, **26**, 7916—7920 (2007).
33) Gallego M., Virshup D. M., *Nat. Rev. Mol. Cell Biol.*, **8**, 139—148 (2007).
34) Yoshitane H., Takao T., Satomi Y., Du N. H., Okano T., Fukada Y., *Mol. Cell. Biol.*, **29**, 3675—3686 (2009).
35) Doi M., Hirayama J., Sassone-Corsi P., *Cell*, **125**, 497—508 (2006).
36) Hirayama J., Sahar S., Grimaldi B., Tamaru T., Takamatsu K., Nakahata Y., Sassone-Corsi P., *Nature* (London), **450**, 1086—1090 (2007).
37) Nakahata Y., Kaluzova M., Grimaldi B., Sahar S., Hirayama J., Chen D., Guarente L. P., Sassone-Corsi P., *Cell*, **134**, 329—340 (2008).
38) Asher G., Gatfield D., Stratmann M., Reinke H., Dibner C., Kreppel F., Mostoslavsky R., Alt F. W., Schibler U., *Cell*, **134**, 317—328 (2008).
39) McNamara P., Seo S. P., Rudic R. D., Sehgal A., Chakravarti D.,