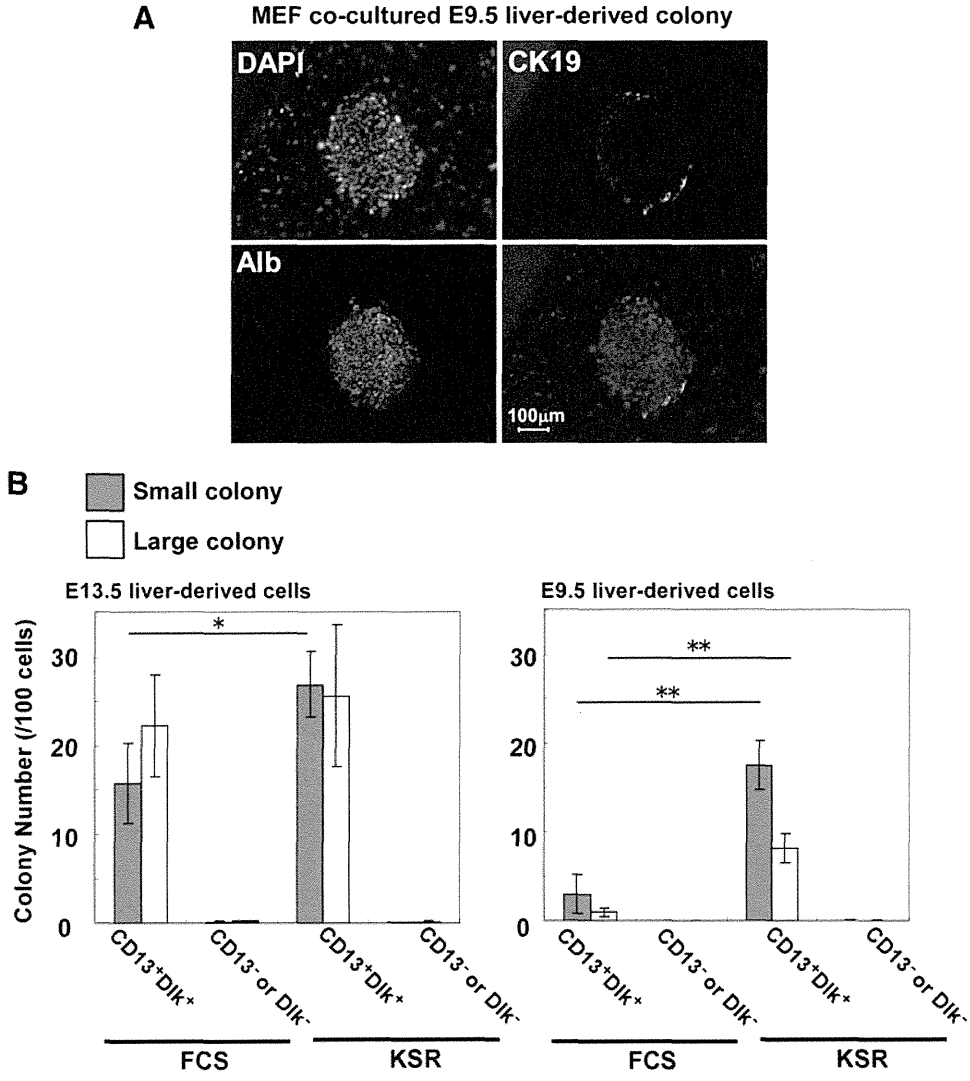

FIG. 3. Early fetal liver $CD45^-Ter119^-CD13^+Dlk^+$ cells could form large colonies in the coculture with MEF. **(A)** Representative view of a colony formed from a single E9.5 $CD45^-Ter119^-CD13^+Dlk^+$ cell cocultured with MEF. **(B)** KSR induced colony formation by E9.5 $CD45^-Ter119^-CD13^+Dlk^+$ cells. E13.5 and E9.5 fetal liver cells were sorted and cocultured with MEF for 6 days. Cells were cultured in H-CFU-C medium supplemented with either fetal bovine serum or KSR. Small colonies (*gray bars*) consisting of 50–100 cells and large colonies (*white bars*) consisting of >100 cells were counted. Results are represented as mean colony count ± SD (triplicate samples; * and ** denote $P<0.05$ and $P<0.01$). MEF, mouse embryonic fibroblast; KSR, KnockOut Serum Replacement.

cells, Y-27632 (a Rock inhibitor), PD0325901 (a MEK inhibitor), CHIR99021 (a GSK3β inhibitor), and A-83-01 (a transforming growth factor β type I receptor inhibitor) were added to cell cultures. The morphology of colonies was not changed by the addition of these inhibitors (Fig. 4A). Although PD0325901, CHIR99021, and A-83-01 did not change the number of large colonies formed by E9.5 $CD13^+Dlk^+$ cells, Y-27632 significantly increased the number of large colonies formed by these cells, indicating that inhibition of Rock is important for proliferation of E9.5 $CD13^+Dlk^+$ cells (Fig. 4B). The addition of Y-27632 induced colony formation of E10.5 $CD13^+Dlk^+$ cells (Fig. 4C). In contrast, the addition of Y-27632 could not induce proliferation of E13.5 $CD13^+Dlk^+$ cells, as previously shown (Fig. 4B) [9].

We used conditioned medium derived from E14.5 liver cells in conventional H-CFU-C culture system for mid-fetal liver hepatoblasts [10,13]. When E9.5 $CD13^+Dlk^+$ cells were cocultured with MEF, however, the addition of fetal liver cell-conditioned medium decreased the number of large and small colonies (data not shown). The number of proliferative cells in individual colonies was not significantly changed (Fig. 5A), suggesting that cell apoptosis might be involved in the inhibition of colony formation by the addition of fetal liver cell-conditioned medium. In consequence, we cultured early fetal liver cells in the following experiments without using fetal liver cell-conditioned medium. We also found that these isolation and culture methods could induce proliferation of early fetal HSPCs derived from C3H mice, in addition to C57BL6 mice (Supplementary Fig. S4A, B).

In addition to $CD13^+Dlk^+$ cells, the $CD13^{mid}Dlk^{mid}$ cells (the intermediate fraction) existed in E10.5 and E11.5 livers. We asked whether the intermediate fractions also contain HSPCs. However, significant colony formation by these fractions was not detected compared with the $CD13^+Dlk^+$ fractions (Supplementary Fig. S4C, D), indicating that most progenitor cells exist in the $CD13^+Dlk^+$ fraction during early- to mid-fetal liver development. These results suggested that the addition of ROCK inhibitor was required for clonal expansion of early fetal $CD13^+Dlk^+$ progenitor cells but not of mid-fetal $CD13^+Dlk^+$ hepatoblasts.

Inhibition of the Rock-myosin II pathway induced colony formation of early fetal liver $CD13^+Dlk^+$ cells

We varied length of exposure to Y-27632 in E9.5 fetal liver cell culture. Short-time exposure to Y-27632 (culture days 0–3

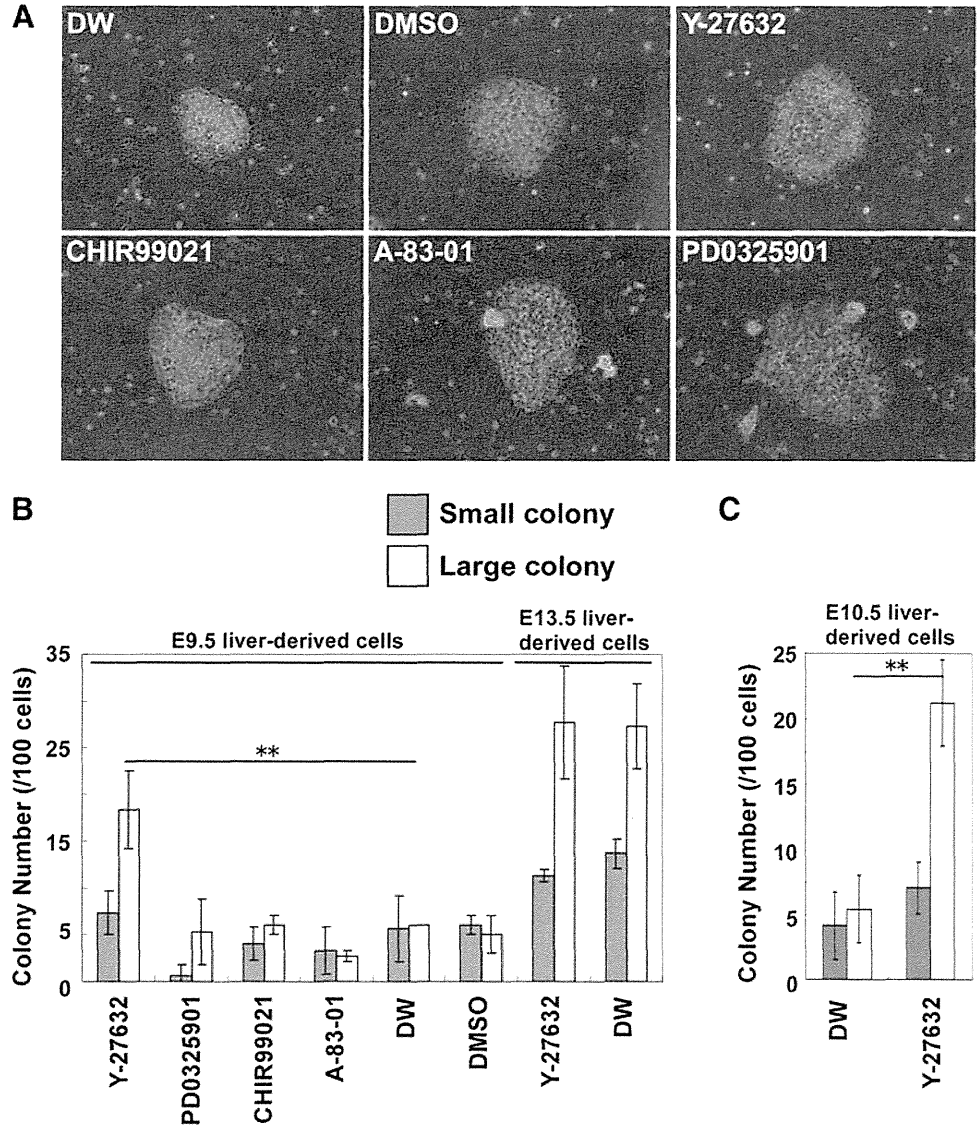

FIG. 4. Addition of Rock inhibitor is important for colony formation of early fetal liver $CD45^-Ter119^-CD13^+Dlk^+$ cells. **(A)** Representative view of colonies formed from a single E9.5 $CD45^-Ter119^-CD13^+Dlk^+$ cell in the presence of signaling inhibitors. E9.5 $CD45^-Ter119^-CD13^+Dlk^+$ cells were cocultured with MEF for 6 days in the presence of either Y-27632 (a Rock inhibitor), PD0325901 (a MEK inhibitor), CHIR99021 (a GSK3β inhibitor), or A-83-01 (a transforming growth factor β type I receptor inhibitor). DW: distilled water (DW) added (control for Y-27632); DMSO: 0.01% DMSO added (control for PD0325901, CHIR99021, and A-83-01). **(B)** E9.5 and E13.5 $CD45^-Ter119^-CD13^+Dlk^+$ cells were cocultured with MEF for 6 days in the presence of inhibitors shown in **A**. The number of colonies derived from E9.5 $CD45^-Ter119^-CD13^+Dlk^+$ cells was significantly increased by culture in medium containing Y-27632, whereas the number of colonies derived from E13.5 $CD45^-Ter119^-CD13^+Dlk^+$ cells did not increase with Y-27632 exposure. **(C)** E10.5 $CD45^-Ter119^-CD13^+Dlk^+$ cells were cocultured for 6 days with MEF in the presence of Y-27632. DW: DW added (control). **(B, C)** Small colonies (*gray bars*) consisting of 50–100 cells and large colonies (*white bars*) consisting of >100 cells were counted. Results are represented as mean colony count ± SD (triplicate samples; * and ** denote $P<0.05$ and $P<0.01$). DMSO, dimethyl sulfoxide.

or 3–6) partially induced progression of colony formation. Interestingly, early-stage addition of Y-27632 (days 0–3) significantly induced formation of large colonies compared with late-stage addition of Y-27632 (days 3–6). Thus, inhibition of Rock is particularly important for the early stage of colony formation by E9.5 cells (Fig. 5B). Rock induces phosphorylation of several substrates, leading to various cellular responses [18]. The inactivation of myosin phosphatase target subunit, which is induced by Rock, protects the phosphorylated form of myosin regulatory light chain. This phosphorylation keeps myosin II in its active form. Blebbistatin, which specifically inhibits myosin II, has an effect similar to that of Y-27632. It inhibits apoptosis of single-suspended human embryonic stem cells [19,20]. Blebbistatin, like Y-27632, significantly induced colony formation by E9.5 cells (Fig. 5B). We analyzed proliferation of colonies in the presence of several inhibitors and found that a number of colonies expressed high levels of Ki67 proliferation marker in the culture stimulated with Y-27632 (Fig. 5A). These results suggest that inhibition of the Rock-myosin II pathway is important in expansion of early fetal HSPCs.

Soluble factors derived from MEF partly induced proliferation of early fetal $CD13^+Dlk^+$ cells

We then assessed whether soluble factors derived from MEF are involved in expansion of E9.5 $CD13^+Dlk^+$ cells. Confluent MEFs were cultured for 2 days in H-CFU-C medium. This medium (now "MEF conditioned") was used as medium for various colony formation assays (Fig. 6A). When cells were cocultured with MEF, use of MEF-conditioned medium made no difference in the efficiency of colony formation by E9.5 $CD13^+Dlk^+$ cells. However, when E9.5 $CD13^+Dlk^+$ cells were cultured on collagen type I, small and

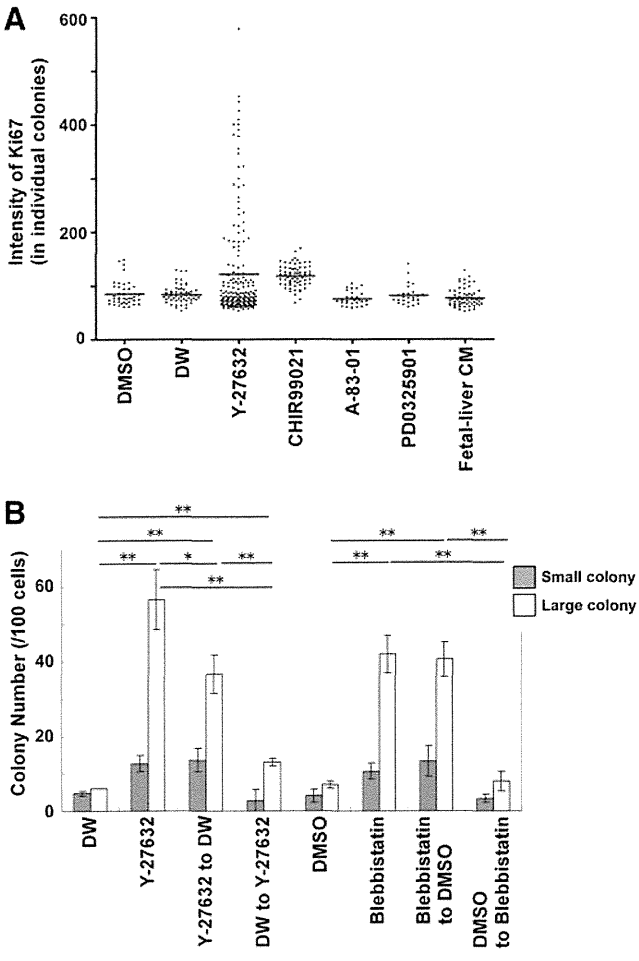

FIG. 5. A Rock inhibitor, Y-27632, and a myosin II synthetic inhibitor, blebbistatin, induced colony formation by early fetal liver $CD45^-Ter119^-CD13^+Dlk^+$ cells. **(A)** Proliferation of early fetal HSPCs regulated by ROCK inhibitor. Intensities of Ki67 in individual albumin-positive colonies were analyzed. DW: distilled water (DW) added (control for Y-27632); DMSO: 0.01% DMSO added (control for PD0325901, CHIR99021, and A-83-01); Fetal-liver CM: fetal-liver conditioned medium added. **(B)** $CD45^-Ter119^-Dlk^+$ cells were cocultured for 6 days with MEF in the presence of either Y-27632 or blebbistatin. DW: DW added (control for Y-27632); DMSO: 0.1% DMSO added (control for blebbistatin); Y-27632 to DW: first 3 days of culture with Y-27632 followed by 3 days of culture without Y-27632; DW to Y-27632: first 3 days of culture without Y-27632 followed by 3 days of culture with Y-27632; Blebbistatin to DMSO: first 3 days of culture with blebbistatin followed by 3 days of culture with DMSO; DMSO to blebbistatin: first 3 days of culture with DMSO followed by 3 days of culture with blebbistatin. Small colonies (*gray bars*) consisting of 50–100 cells and large colonies (*white bars*) consisting of >100 cells were counted. Results are represented as mean colony count ± SD (triplicate samples; * and ** denote $P<0.05$ and $P<0.01$).

large colonies were detected only when MEF-conditioned media were used. Fresh medium not conditioned with MEF did not support colony formation on collagen-coated dishes. These data suggest that expansion of early fetal progenitor cells was partly supported by soluble factors derived from MEF.

Discussion

In this report, we showed that early fetal (E9.5 and E10.5) liver-derived $CD13^+Dlk^+$ cells have characteristics of hepatic progenitor cells: They have a high proliferative capacity and the ability to differentiate into both albumin-positive hepatocytic cells and CK19-positive cholangiocytic cells. In contrast to mid-fetal hepatoblasts, early fetal HSPCs require interaction with MEF to expand clonally. Hlx is a transcription factor expressed in septum transversum mesenchyme and fetal liver expansion is severely deficient in Hlx-knockout mice [17]. Therefore, at an early fetal liver developmental stage, interaction with Hlx-positive mesenchymal cells is important for proliferation of hepatoblasts in vivo. Under our culture conditions, MEF, which express Hlx (data not shown), supported proliferation of early fetal progenitor cells. MEF-conditioned medium partially supported clonal expansion of E9.5 $CD13^+Dlk^+$ cells, suggesting that soluble factors derived from MEF are at least partly necessary for the survival or growth of E9.5 $CD13^+Dlk^+$ cells. Other cell–cell and cell–matrix interactions also appear important for proliferation of early fetal progenitor cells, because large colonies derived from E9.5 $CD13^+Dlk^+$ cells cocultured with MEF were significantly more numerous than when the same population of cells was cultured on collagen-coated dishes in the presence of MEF-conditioned medium.

Not only coculture with MEF but also inhibition of Rock or myosin II activity remarkably improved clonal expansion of E9.5 $CD13^+Dlk^+$ cells. Rock inhibitor Y-27632 promotes the survival and growth of various other types of cells, including human embryonic stem cells and adult liver-derived progenitor cells [9,21]. The molecular mechanisms by which Rock and myosin II inhibitors promoted the colony forming efficiency of early fetal liver cells in our culture system are unknown and await further investigation. As hepatic progenitor cells differentiated from foregut endoderm in E9.5 embryos into the septum transversum mesenchyme, they start to lose epithelial properties and to acquire mesenchymal properties [22]. An epithelium-specific property is that of polarity, established by the segregation of apical and basolateral domains. Epithelial shape is regulated by apical constriction, a process dependent on activated myosin II [23,24]. Rock also participates in apical constriction [25]. Dissociated early fetal progenitor cells, deprived of cell–cell contact and of traction from adjacent cells, may not be able to tolerate the force generated by apical constriction and thus undergo apoptosis. Inhibition of Rock or myosin II activity might thus rescue these cells from apoptosis through inhibition of excessive apical constriction.

We found that E9.5 to E13.5 $CD13^+Dlk^+$ cells in fetal livers can serve as bipotent progenitor cells. However, expression of several hepatic and endodermal genes differed remarkably between early- and mid-fetal $CD13^+Dlk^+$ cells. Levels of albumin mRNA, but not CK19 mRNA, were high in E13.5 $CD13^+Dlk^+$ cells. In contrast, E9.5 $CD13^+Dlk^+$ cells scarcely expressed mRNA of hepatic genes (AFP, albumin, and c-met) but exhibited high Sox17 and CK19 mRNA levels (Fig. 2A, B). Sox17 is expressed in definitive endodermal progenitor cells and CK19, a cholangiocytic marker gene in mid-fetal livers, is also expressed in primitive gut endoderm [16]. Therefore, E9.5 $CD13^+Dlk^+$ cells, which differentiate into mid-fetal hepatoblasts during liver development, seem

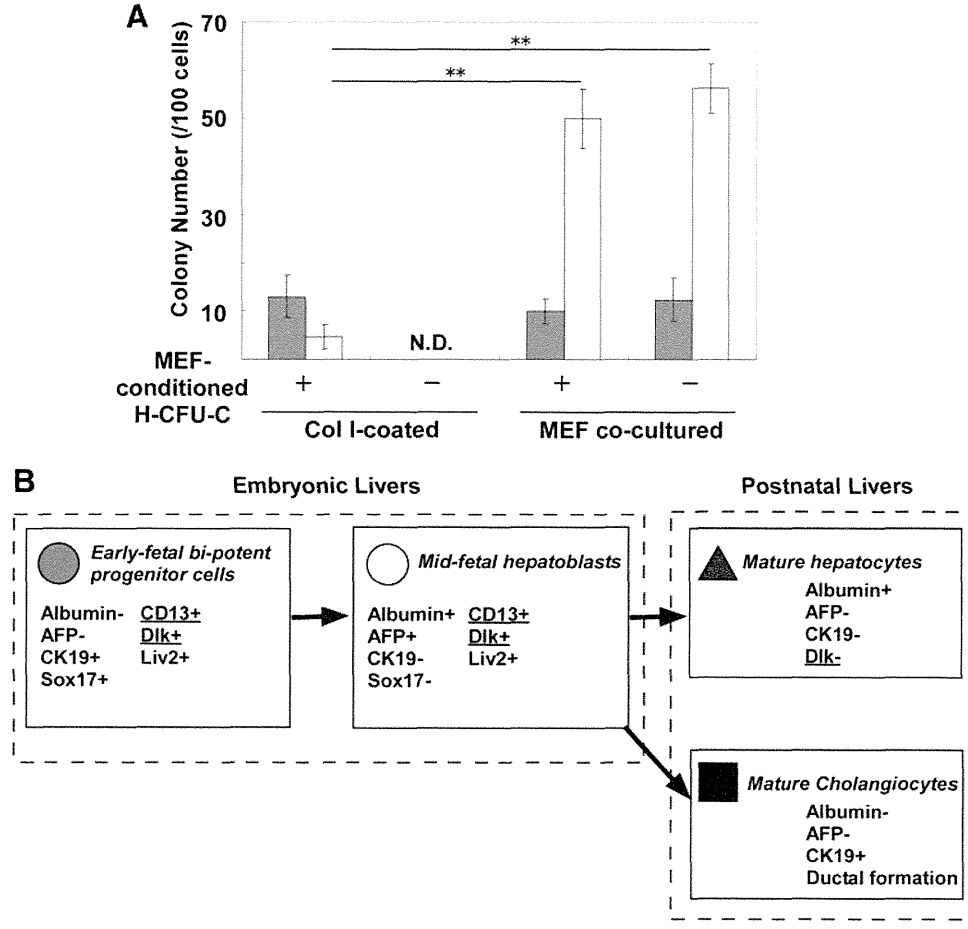

FIG. 6. Soluble factors and cell–cell/extracellular matrix interactions are important for MEF-induced expansion of early fetal cells. **(A)** E9.5 $CD45^-Ter119^-CD13^+Dlk^+$ cells were cultured for 6 days with MEF-conditioned medium or fresh medium in the presence of Y-27632. Cells were sorted onto collagen-coated dishes or dishes containing MEF. Small colonies (*gray bars*) consisting of 50–100 cells and large colonies (*white bars*) consisting of >100 cells were counted. Results are represented as mean colony count±SD (triplicate samples; $^{**}P<0.01$). **(B)** Schema of phenotypes of progenitor cells during fetal liver development. CD13 and Dlk are surface markers common to early fetal progenitor cells and mid-fetal hepatoblasts. However, expression of several genes (albumin, AFP, CK19, and Sox17) differed significantly between early fetal progenitor cells and mid-fetal hepatoblasts. N.D., not detected.

to possess the properties of endodermal progenitor cells (Fig. 6B).

In the present study, we showed that $CD13^+Dlk^+$ cells derived from early fetal livers have high proliferative capacity and can differentiate into both albumin-positive cells and CK19-positive cells, suggesting that at the single-cell level CD13 and Dlk are markers for bipotent progenitor cells in the early fetal liver developmental stage. These cells show gene expression patterns distinct from those of hepatoblasts in the mid-fetal liver. Signaling pathways regulating the proliferative capacity of $CD13^+Dlk^+$ hepatic progenitor cells in vitro also differ between cells derived from early fetal livers and those derived from mid-fetal livers. These findings highlight a biologically important and potentially therapeutic role for mesenchymal cells and for the Rock-myosin II signaling pathway in the differentiation and expansion of hepatic progenitor cells derived from pluripotent stem cells.

Acknowledgments

This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan and Core Research for Evolutional Science and Technology from Japan Science and Technology Agency.

Author Disclosure Statement

There is no conflict of interest to disclose.

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Received for publication May 6, 2011
Accepted after revision August 18, 2011
Prepublished on Liebert Instant Online August 23, 2011

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 42, p. 35443, October 12, 2012
Published in the U.S.A.

Reply to Sun *et al.*: Targeting YAP Acetylation in Cancer

This is a response to a letter by Sun et al. (1).

We appreciate the thoughtful comments made by Sun *et al.* (1) regarding our recent study on Yes-associated protein (YAP) acetylation. As they point out, the Hippo pathway induces the phosphorylation and cytoplasmic retention of TAZ (transcriptional coactivator with PDZ-binding motif) as well as YAP (2). Therefore, it is possible that dephosphorylation of TAZ leading to its nuclear translocation also occurs in response to the S_n2 alkylating agent methyl methanesulfonate (MMS). In addition, because p300 acetyltransferase physically interacts with TAZ in a fashion similar to its interaction with YAP (3), it would be intriguing to study whether TAZ is also acetylated upon MMS treatment.

The growth-promoting and oncogenic activities of YAP have been demonstrated *in vivo* and *in vitro* (2). However, many other studies have reported that YAP functions as a pro-apoptotic regulator in certain tumor-derived cell lines exposed to chemotherapeutic agents (4). Thus, the overall *in vivo* function of YAP in response to chemotherapeutic agents is unclear. Interestingly, YAP protects non-transformed keratinocytes from UV irradiation but promotes the UV-induced apoptosis of squamous cell carcinoma cells (5). All these investigations indicate that YAP functions in a cell context-dependent manner. Our study has indicated that YAP acetylation affects its pro-apoptotic function in HeLa carcinoma cells. Taking our results and previous studies on YAP functions into account, we agree with Sun *et al.* (1) that YAP acetylation may well have differential effects on the response of cancer *versus* non-transformed cells to S_n2 alkylating agents. We also concur that further studies using animal models and broad panels of normal and cancer cell lines are needed.

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DOI 10.1074/jbc.L112.413328

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Hindawi Publishing Corporation
Journal of Signal Transduction
Volume 2012, Article ID 459265, 9 pages
doi:10.1155/2012/459265

Review Article

Diverse Roles of JNK and MKK Pathways in the Brain

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Received 11 August 2011; Accepted 1 November 2011

Academic Editor: M. Gaestel

The c-Jun NH_2-terminal protein kinase (JNK) plays important roles in a broad range of physiological processes. JNK is controlled by two upstream regulators, mitogen-activated protein kinase kinase (MKK) 4 and MKK7, which are activated by various MAPKKKs. Studies employing knockout mice have demonstrated that the JNK signaling pathway is involved in diverse phenomena in the brain, regulating brain development and maintenance as well as animal metabolism and behavior. Furthermore, examination of single or combined knockout mice of *Jnk1*, *Jnk2*, and *Jnk3* has revealed both functional differences and redundancy among JNK1, JNK2, and JNK3. Phenotypic differences between knockouts of MKK4 and MKK7 have also been observed, suggesting that the JNK signaling pathway in the brain has a complex nature and is intricately regulated. This paper summarizes the functional properties of the major JNK signaling components in the developing and adult brain.

1. Introduction

The c-Jun NH_2-terminal protein kinases (JNKs, also called stress-activated protein kinases (SAPKs)) are members of the evolutionarily conserved mitogen-activated protein kinase (MAPK) family [1, 2]. The JNK subfamily consists of three related genes: *Jnk1*, *Jnk2*, and *Jnk3*. In mammals, the JNK1 and JNK2 proteins are ubiquitously expressed, whereas JNK3 is found almost exclusively in the brain and testis. JNKs are activated by many types of external stress, including heat shock, UV irradiation, and inflammatory cytokines. JNKs phosphorylate numerous important substrates, including the transcription factors AP-1 and c-Jun, various apoptotic proteins, and microtubule-associated proteins (MAPs) [3–7]. Through phosphorylation of these substrates, JNKs regulate gene expression governing stress responses as well as the normal physiological processes of cell proliferation, apoptosis, differentiation, and cell migration.

Activation of JNK is catalyzed by two kinases, mitogen-activated protein kinase kinase (MKK) 4 and MKK7 [8–10]. Although MKK4 and MKK7 are both dual-specificity Thr and Tyr kinases, previous studies of JNK activation have shown that MKK4 preferentially phosphorylates the Tyr residue of the TPY motif in the activation loop of JNKs, whereas MKK7 preferentially phosphorylates the Thr residue [11, 12]. The activation of MKK4 and MKK7 is mediated by various MAPKKKs, including mixed lineage protein kinases (MLKs), apoptosis signal-regulating kinases (ASKs) and dual leucine zipper kinase (DLK) [13]. In addition to regulation by these upstream kinases, the JNK signaling pathway is modulated by various scaffold proteins, including JNK-interacting protein (JIP) 1, JIP2, and JIP3 (also known as JNK/SAPK associated protein-1 (JSAP1)) [14–17]. These scaffold proteins assemble multienzyme complexes that involve a specific triad of a MAPKKK, a MAPKK, and a MAPK and provide an insulated physical conduit for signal transduction. The resulting linkage of these kinases forms a functional signaling module that performs transduction duties for a particular purpose.

In most mammalian cell types, JNK activation is tightly controlled and employed at moderate levels in specific circumstances. In contrast, JNK signaling in the brain is

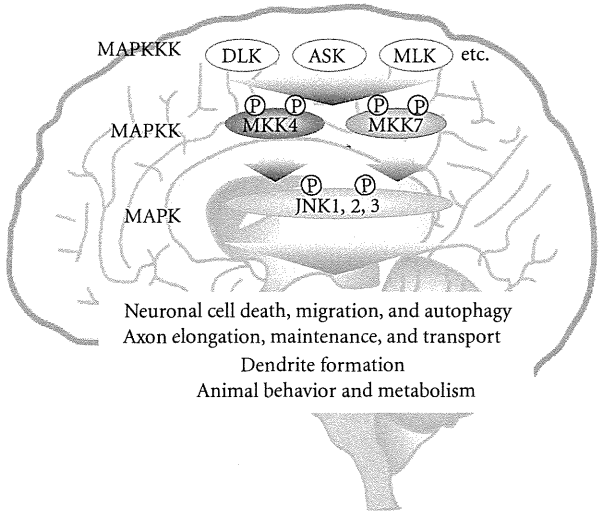

FIGURE 1: The JNK signaling pathway in the mammalian brain. A MAPKKK, such as DLK, ASK, or MLK, phosphorylates (P) and activates a MAPKK such as MKK4 or MKK7. An activated MAPKK in turn phosphorylates and activates a MAPK such as JNK1, JNK2, or JNK3. Activated JNKs then regulate various phenomena in the brain.

highly and constitutively activated. Studies of gene knockout (KO) mice lacking JNKs or their upstream kinases have shown that this sustained activation is necessary to fulfil the diverse and essential roles that JNK signaling plays in the brain (Figure 1). This paper summarizes the phenotypes of these mutant animals and discusses what they reveal about the regulation and various functions of JNK and MKK signaling in the brain.

2. Brain Phenotypes of JNK Knockout Mice

2.1. Jnk1/2 DKO Mice: JNK1/2 Induces Programmed Cell Death during Early Brain Development. Knockout mice lacking one, two, or all JNK isoforms have been generated over the past decade, and their diverse phenotypes have been reported (Table 1). Importantly, embryonic lethality is not caused by the single KO of any *Jnk* gene. Two strains of double KO mice, $Jnk1^{-/-}Jnk3^{-/-}$ (*Jnk1/3 DKO*) and $Jnk2^{-/-}Jnk3^{-/-}$ (*Jnk2/3 DKO*), are viable, but $Jnk1^{-/-}Jnk2^{-/-}$ (*Jnk1/2 DKO*) animals exhibit early embryonic lethality.

During early brain development in mice, the neural folds emerge at embryonic day (E) 7.5 in the cephalic region, close over at E8.5–E9 (neural tube closure), and form the neural tube. The *Jnk1/2* DKO mouse is lethal at E11.5 due to dysregulation of apoptosis in the neural tube [18, 19]. Specifically, there is a marked reduction in cell death in the lateral edges of the hindbrain prior to neural tube closure. In contrast, increased apoptosis and caspase activation are found in the mutant forebrain, leading to precocious degeneration. Interestingly, about 25% of $Jnk1^{-/-}Jnk2^{+/-}$ fetuses display excencephaly that is likely the result of failed neural tube closure, whereas $Jnk1^{+/-}Jnk2^{-/-}$ mice are normal [19]. These results suggest that JNK1 and JNK2 are redundant regulators of programmed cell death during early brain development and that JNK1 is dominant in this particular function of the JNK signaling pathway.

TABLE 1: Phenotypes of JNK knockout mice.

Mouse model	Phenotypes	References
$Jnk1^{-/-}$	Accelerated radial migration Shorter dendrites with increased branching Degeneration of anterior commissure Enhanced glucocorticoid- or insulin-induced food intake	[20–22, 31]
$Jnk2^{-/-}$	Resistance to MPTP-induced neuronal cell death	[24]
$Jnk3^{-/-}$	Resistance to MPTP-induced neuronal cell death Resistance to kainic acid-induced neuronal cell death Resistance to ischemia-induced neuronal apoptosis Resistance to 6-hydrozydopamine-induced neuronal apoptosis	[24–27, 29]
$Jnk1^{-/-}$ $Jnk2^{-/-}$	Embryonic lethal at E11.5 Defective neural tube closure Dysregulated apoptosis in brain (increased in forebrain, reduced in hindbrain)	[18, 19]
$Jnk2^{-/-}$ $Jnk3^{-/-}$	Resistance to MPTP-induced neuronal cell death	[24]
$Jnk1^{flox/flox}$ *Nestin-Cre*	Animal protected from diet-induced glucose intolerance and insulin resistance Reduced serum IGF-1 and GH Increased serum thyroid hormones	[30]
$Jnk1^{flox/flox}Jnk2^{-/-}$ $Jnk3^{-/-}$ *Nestin-Cre*	Early embryonic lethal	[53]
$Jnk1^{flox/flox}Jnk2^{-/-}$ $Jnk3^{-/-}$ *Pcp2-Cre*	Loss of dendritic arborization Axon hypertrophy Increased autophagic vacuoles	[53]

2.2. $Jnk1^{-/-}$ Mice: JNK1 Is Required for Neuronal Migration, Dendrite Formation, and Axon Maintenance during Later Brain Development. After neural tube formation at E8.5–E9, three of its vesicles give rise to various specialized regions of the brain. Neuronal progenitors proliferate and generate immature neurons. These cells subsequently migrate from the proliferative zones to their final positions, where they extend neurites. For example, in the developing cortex, neural progenitors proliferate from E11 to E17 then generate immature neurons that migrate and form the cortical layers, the cortical plate, subplate, intermediate zone, and ventricular zone. The extension and maturation of neurites by these cells then continues during the remaining embryonic and postnatal stages. $Jnk1^{-/-}$ embryos display thicker cortical

plates and thinner ventricular zones than wild-type animals [20]. *Jnk1*$^{-/-}$ embryos also show abnormally accelerated migration of cortical neurons (radial migration), a process whose precise regulation is required for the correct formation of cortical layers. These findings indicate that JNK1 regulates the rate of neuronal migration during cortical development.

Other studies have shown that JNK1 also regulates neurite formation and maintenance. Neurons extend two types of neurites: dendrites and axons. Compared to wild-type mice, dendrites in the cortex and cerebellum of *Jnk1*$^{-/-}$ mice are shorter and have more processes [21], indicating that JNK1 plays an important role in defining dendritic architecture during brain development. In contrast, axon formation is not affected by JNK1 disruption. Axon tracts such as the corpus callosum and anterior commissure appear normal in *Jnk1*$^{-/-}$ mice until postnatal day (P) 6. From P6-P12, however, the anterior commissure degenerates, demonstrating that JNK1 is required for axon maintenance [22].

One of the molecular mechanisms mediating JNK pathway functions during brain development and maintenance is the phosphorylation of MAPs such as MAP1B, MAP2, and superior cervical ganglion 10 (SCG10) [20–22]. MAPs bind to microtubules (MTs) and modulate their stability and structure. Importantly, the phosphorylation of MAPs by one of several protein kinases (including JNK) regulates the binding of these enzymes to MTs and thus the regulation of MT modification. In *Jnk1*$^{-/-}$ brain, the phosphorylation of MAP1B, MAP2, and SCG10 is reduced. Moreover, the introduction of a mutated SCG10 protein that mimics the JNK1-phosphorylated form restores normal neuronal migration in the brains of *Jnk1*$^{-/-}$ embryos [20]. These results demonstrate that, in addition to regulating radial migration, dendrite formation, and axon maintenance in the developing brain, JNK1 controls MT structure through MAP phosphorylation.

2.3. Jnk2$^{-/-}$ *and Jnk3*$^{-/-}$ *Mice: JNK2 and/or JNK3 Are Required for Neuronal Cell Death Induced by Neuronal Stresses in Adult Brain.* The redundancy of many JNK functions is highlighted by the fact that *Jnk2*$^{-/-}$ and *Jnk3*$^{-/-}$ single KO mice show none of the defects observed in *Jnk1*$^{-/-}$ mice. However, *Jnk2*$^{-/-}$ and *Jnk3*$^{-/-}$ mice do show alterations in neuronal stress-induced neuronal cell death that are rarely observed in *Jnk1*$^{-/-}$ mice, implying that each JNK enzyme has a unique function. For example, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin whose administration induces dopaminergic cell demise and so results in symptoms that replicate most of the neuropathological hallmarks of Parkinson's disease [23]. *Jnk2*$^{-/-}$ or *Jnk3*$^{-/-}$ mice (but not *Jnk1*$^{-/-}$ mice) display resistance to MPTP-induced neuronal cell death *in vivo* [24]. Moreover, this resistance to MPTP is enhanced by double mutation of *Jnk2* and *Jnk3*, indicating that JNK2 and JNK3 play partially overlapping roles in this process. *Jnk3*$^{-/-}$ mice also display resistance to kainic acid-induced cell death (excitotoxicity-induced apoptosis) and ischemia-induced cell death *in vivo* [25–27], as well as reduced susceptibility to apoptosis induced by the Alzheimer's disease-related protein beta-amyloid *in vitro* [28]. Finally, *Jnk3*$^{-/-}$ mice intrastriatally injected with 6-hydroxydopamine, which provokes the death of dopaminergic neurons, show a transient prolongation of dopaminergic neuron survival in the substantia nigra compacta compared to injected control mice [29]. These results suggest that the physiological function of both JNK2 and JNK3 in the brain is to induce apoptosis in response to neuronal stress.

2.4. Conditional Jnk1$^{-/-}$ *Mice: JNK1 Activity in Adult Brain Regulates Animal Metabolism.* Intact JNK signaling in the adult brain is required for normal animal metabolism. A high-fat diet induces JNK activation in the hypothalamus and pituitary, which regulate body weight control, glucose homeostasis, and secretion of hormones. Conditional KO (cKO) mice have been generated in which *Jnk1* expression is controlled by Nestin-Cre, which induces Cre recombinase expression in neural stem cells [30]. Upon high-fat feeding, *Jnk1*$^{flox/flox}$ *Nestin-Cre* mice exhibit increased insulin sensitivity (compared to wild-type controls) both in the CNS and in peripheral tissues, improved glucose metabolism and protection from hepatic steatosis and adipose tissue dysfunction. *Jnk1*$^{flox/flox}$ *Nestin-Cre* mice also display reduced somatic growth and altered secretion of growth hormone (GH), insulin-like growth factor (IGF), and thyroid hormones. JNK activity in the brain is thus required for normal metabolism.

JNK signaling in the brain is also involved in the control of feeding. The hypothalamus governs food intake in response to nutrient status, cytokines, and hormones. *Jnk1*$^{-/-}$ mice exhibit enhanced food intake and weight gain upon hypothalamic administration of glucocorticoid [31]. Moreover, JNK1 disruption increases mouse sensitivity to the anorexigenic effects of hypothalamic insulin administration. These data show that JNK signaling in the brain regulates not only hormone secretion and peripheral metabolism but also feeding and thus contributes to the maintenance of energy homeostasis. It remains unclear whether JNK2 and/or JNK3 are also involved in these functions, but improved insulin resistance has been reported for both *Jnk1*$^{+/-}$*Jnk2*$^{-/-}$ mice and *Jnk1*$^{-/-}$ mice fed a high-fat diet [32, 33]. These results suggest that JNK2 may be involved in the central and/or peripheral regulation of glucose homeostasis. Analyses of cKO mice lacking *Jnk1* and/or *Jnk2* will resolve this issue.

3. Brain Phenotypes of MKK Knockout Mice

Because JNK isoforms are required for brain development, and MKK4 and MKK7 activate all JNK isoforms, it is not surprising that disruption of *Mkk4* and/or *Mkk7* results in severe defects in mouse development. *Mkk4*$^{-/-}$*Mkk7*$^{-/-}$ (*Mkk4/7 DKO*) mice die at E8.5 before neural tube formation, and both *Mkk4*$^{-/-}$ and *Mkk7*$^{-/-}$ single KO mice are embryonic lethal at around E11.5 due to impaired liver formation [9, 34–36]. To circumvent this limitation, the functions of MKK4 and MKK7 during brain development have been investigated using Nestin-Cre cKO mice lacking MKK4 and/or MKK7. Analyses of these mutants have proven very helpful in overcoming the conundrum posed by the redundancy of JNK isoforms.

3.1. Mkk4$^{flox/flox}$ Nestin-Cre Mice: MKK4 Is Required for Neuronal Migration and Axon Maintenance in the Developing Brain. In *Mkk4$^{flox/flox}$ Nestin-Cre* mice, total JNK activation in the brain is reduced to 20% of normal but these animals are not embryonic lethal and dysregulated apoptosis is not observed [37]. At birth, *Mkk4$^{flox/flox}$ Nestin-Cre* mice are indistinguishable from their control littermates, but the mutants stop growing a few days later and die at around 3 weeks of age. The brains of *Mkk4$^{flox/flox}$ Nestin-Cre* mice display misaligned Purkinje cells in the cerebellum and delayed radial migration in the cerebral cortex. In their commissural axon tracts, axonal degeneration is observed not only in the anterior commissure, the site of a similar defect in *Jnk1$^{-/-}$* brain [22], but also in the corpus callosum. At the molecular level, hypophosphorylation of MAP1B is observed in *Mkk4$^{flox/flox}$ Nestin-Cre* mice, suggesting that dysregulation of MT dynamics is involved in these phenotypes. The altered neuronal cell migration and axon maintenance observed in *Mkk4$^{flox/flox}$ Nestin-Cre* mice are also seen in *Jnk1$^{-/-}$* mice, but these phenotypes are less severe in the latter. Thus, MKK4 is a regulator of radial migration and axon maintenance in the brain, and MKK4's effects are mediated not only by JNK1 but also by JNK2 and/or JNK3.

3.2. Mkk7$^{flox/flox}$ Nestin-Cre Mice: MKK7 Is Required for Neuronal Migration and Axon Elongation in Developing Brain. Our group has generated *Mkk7$^{flox/flox}$ Nestin-Cre* mice [38]. Unlike *Mkk4$^{flox/flox}$ Nestin-Cre* mice, which survive until age 3 weeks, *Mkk7$^{flox/flox}$ Nestin-Cre* mice die at birth without breathing. Like *Mkk4$^{flox/flox}$ Nestin-Cre* mice, JNK activation is reduced to 20% of normal in the developing brain of *Mkk7$^{flox/flox}$ Nestin-Cre* mutants, and a delay in neuronal migration in the cerebrum is observed. However, other phenotypes do not overlap between *Mkk7$^{flox/flox}$ Nestin-Cre* and *Mkk4$^{flox/flox}$ Nestin-Cre* mice (Table 2). At E18.5, *Mkk7$^{flox/flox}$ Nestin-Cre* mice display enlarged brain ventricles, diminished striatum, decreased forebrain axon tracts, and reduced corticofugal axons; none of these defects has been found in *Mkk4$^{flox/flox}$ Nestin-Cre* mice. In addition, ultrastructural alterations such as abnormal accumulations of filamentous structures and autophagic vacuoles are observed in *Mkk7$^{flox/flox}$ Nestin-Cre* brain but not in *Mkk4$^{flox/flox}$ Nestin-Cre* brain. Thus, *Mkk7* has unique functions in the developing brain that differ from those of MKK4.

Differences between MKK7 and MKK4 functions also appear at the molecular level. In *Mkk4$^{flox/flox}$ Nestin-Cre* brain, phosphorylation levels of MAP1B are reduced but DCX phosphorylation is not altered. In contrast, phosphorylation levels of both MAP1B and DCX are decreased in *Mkk7$^{flox/flox}$ Nestin-Cre* brain, suggesting that the MKK7-JNK and MKK4-JNK signaling modules in this organ are not identical. In line with this hypothesis, the scaffold protein JIP1 binds to JNK, MKK7, and DCX but not to MKK4. We therefore propose that differences in scaffold proteins and/or substrates involved in the MKK7-JNK versus MKK4-JNK pathways could cause the phenotypic divergence observed between *Mkk7$^{flox/flox}$ Nestin-Cre* and *MKK4$^{flox/flox}$ Nestin-Cre* mice (Figure 2).

Table 2: Comparison of phenotypes of *Mkk4 $^{flox/flox}$Nestin-Cre* and *Mkk7 $^{flox/flox}$Nestin-Cre mice.*

Phenotype	*Mkk4$^{flox/flox}$ Nestin-Cre* [37]	*Mkk7$^{flox/flox}$ Nestin-Cre* [38]
Neuronal JNK activity	Suppressed	Suppressed
Age of lethality	Around 3 weeks old	At birth
Brain ventricle size at E18.5	Not reported	Enlarged
Striatum	Not reported	Reduced
Axon tracts (E18.5)	Unaffected	Greatly reduced
Axon tracts (postnatal)	Less fasciculated	Not determined
Intermediate filaments	Not reported	Accumulate in axons
Autophagic vacuoles	Not reported	Accumulate
JNK phosphorylation in axons	Not reported	Suppressed
L1-positive axons (E18.5)	Unaffected	Reduced
TAG-1-positive axons (E18.5)	Not reported	Reduced
Apoptosis	Unaffected	Unaffected
Cortical layer markers	Not reported	Expressed
Radial migration	Delayed	Delayed
Phosphorylation of c-Jun	Suppressed	Suppressed
Phosphorylation of NF-H	Suppressed	Suppressed
Phosphorylation of MAP1B	Suppressed	Suppressed
Phosphorylation of DCX	Unaffected	Suppressed

4. Brain Phenotypes of MAPKKK Knockout Mice

Compared with MAPKKs, MAPKKKs comprise a much larger family of related proteins. Indeed, at least 12 MAPKKKs have been identified as regulating various steps of the JNK signaling pathway [13]. This multiplicity of related functions suggests that each MAPKKK has a specific spatiotemporal role in controlling JNK signaling. However, the precise mechanisms by which most MAPKKKs regulate JNKs and their biological roles in the brain have yet to be fully elucidated. We summarize below evidence supporting the importance of two MAPKKKs, DLK, and ASK1, in the JNK signaling pathway in the brain.

4.1. Dlk$^{-/-}$ Mice: DLK Is Required for Axon Elongation and Neuronal Migration in Developing Brain. DLK is known to be critical in the developing mammalian brain. *Dlk$^{-/-}$* mice die perinatally, with no homozygous mutant surviving until weaning. *Dlk$^{-/-}$* mice display retarded radial migration and impaired fiber tract development by neocortical pyramidal neurons in the cerebrum [40]. Direct and quantitative analysis of pyramidal neuron radial migration using slice culture and a time-lapse imaging system has revealed that *Dlk*

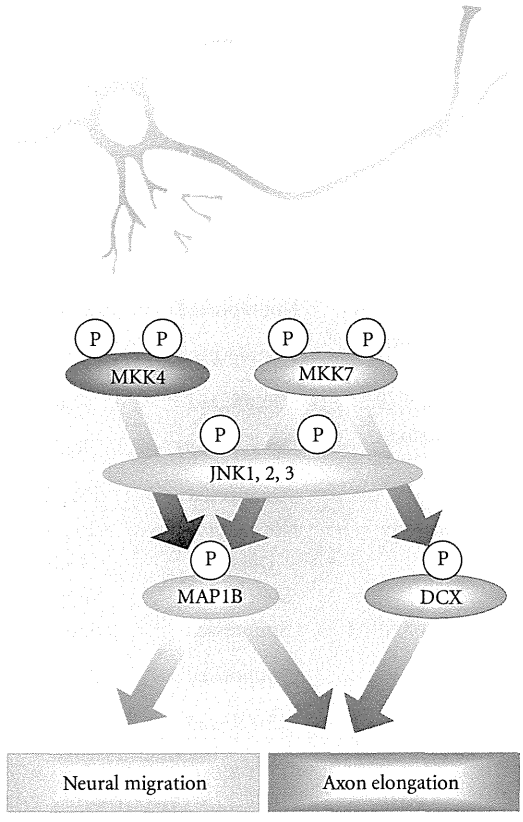

FIGURE 2: MKK4 and MKK7 have different functions in the developing brain. MKK4 and MKK7 both activate JNKs, which phosphorylate MT-associated proteins such as MAP1B and DCX. Activated MAP1B and DCX regulate neuronal migration and axon elongation in the developing brain. However, JNK activated by MKK4 regulates radial migration but not axon elongation, whereas JNK activated by MKK7 controls both radial migration and axon elongation. These differences between MKK7 and MKK4 functions also appear at the molecular level. The phosphorylation of MAP1B requires that JNK be activated by both MKK4 and MKK7. However, the phosphorylation of DCX requires JNK activation only by MKK7 and not by MKK4.

disruption affects acceleration around the cortical subplate. Furthermore, *in vitro* culture of $Dlk^{-/-}$ neurons has shown that DLK is involved in the establishment of neuronal polarity and regulates the MT dynamics driving the transition of stage 1 (nonpolar) neurons to stage 2 (multipolar) neurons and then the transition of stage 2 to stage 3 (axon-forming) neurons [41]. These results demonstrate that DLK regulates radial migration and axon formation during brain development. However, a reduction of only 30% in total phosphorylated JNK is observed in $Dlk^{-/-}$ brain, indicating that other MAPKKKs must be involved in JNK activation in this organ. The identities of these enzymes are under active investigation.

4.2. $Ask1^{-/-}$ Mice: ASK1 Drives Neuronal Cell Death in Adult Brain Following Ischemia or Neurodegeneration and Regulates Animal Behavior. ASK1 is well known as a proapoptotic MAPKKK that is involved in responses to diverse stresses and activates both the JNK and p38 signaling pathways [42]. $Ask1^{-/-}$ mice are born at the expected Mendelian frequency and show no developmental abnormalities as determined by histological analysis [43]. Retinal ganglion cells of $Ask1^{-/-}$ mice do not readily undergo ischemia-induced apoptosis *in vivo* [44], and $Ask1^{-/-}$ primary neurons display resistance to neuronal cell death triggered by polyglutamine *in vitro* [45]. In mice transgenic for a mutation of Cu/Zn-superoxide dismutase, which serve as a model of human amyotrophic lateral sclerosis, deletion of ASK1 mitigates motor neuron loss and extends mouse lifespan [46]. These data indicate that ASK1's proapoptotic functions extend to neurons *in vivo*. ASK1 also has nonapoptotic functions in the brain, since $Ask1^{-/-}$ mice exhibit temporary hyperactivity in an open-field test [47]. Interestingly, this hyperactivity is specific to the novel environment, with $Ask1^{-/-}$ mice displaying normal activities in the familiar field. $Ask1^{-/-}$ mice also show impaired novelty preference at 24 hours after training but superior performance on the rotarod test. These results demonstrate that ASK1 is involved in locomotor activity, novelty preference, and motor coordination requiring dopaminergic transmission.

5. Other Evidence Supporting Roles for JNK Signaling in the Nervous System

5.1. Regulation of Axonal Transport. In *Drosophila*, an absence of the function of either Bsk (*Drosophila* JNK) or Hep (*Drosophila* MKK7) causes a failure of lateral epithelial cells to stretch such that the embryo develops a hole in the dorsal cuticle [48–50]. These data indicate that the MKK7-JNK signaling pathway mediates cell migration in *Drosophila* and regulates dorsal closure during early morphogenesis.

Axonal transport in *Drosophila* is driven along MT by the kinesin motor system, and APLIP (*Drosophila* JIP1) is known as a "cargo linker" that joins kinesin-1 to various vesicle proteins such as the *Drosophila* equivalent of the Alzheimer's APP protein [51]. *Drosophila* axonal transport is believed to be regulated by the JNK signaling pathway because mutation of Wnd (*Drosophila* DLK), Hep, or Bsk results in the abnormal accumulation of synaptic vesicles in the nerves of third instar larvae [39]. Mutation of Wnd or Hep also disrupts the binding of kinesin-1 to APLIP1. Thus, the JNK signaling pathway regulates the attachment of APLIP1 to kinesin-1 in *Drosophila*, ensuring that the dissociation of kinesin-1 from its vesicle protein cargo occurs at the appropriate position (Figure 3(a)).

The involvement of the JNK signaling pathway in axonal transport has also been reported in mammals. JNK can be activated by the pathogenic polyglutamine-containing Huntingtin protein associated with human Huntington's disease. Fast axonal transport is then inhibited because JNK3

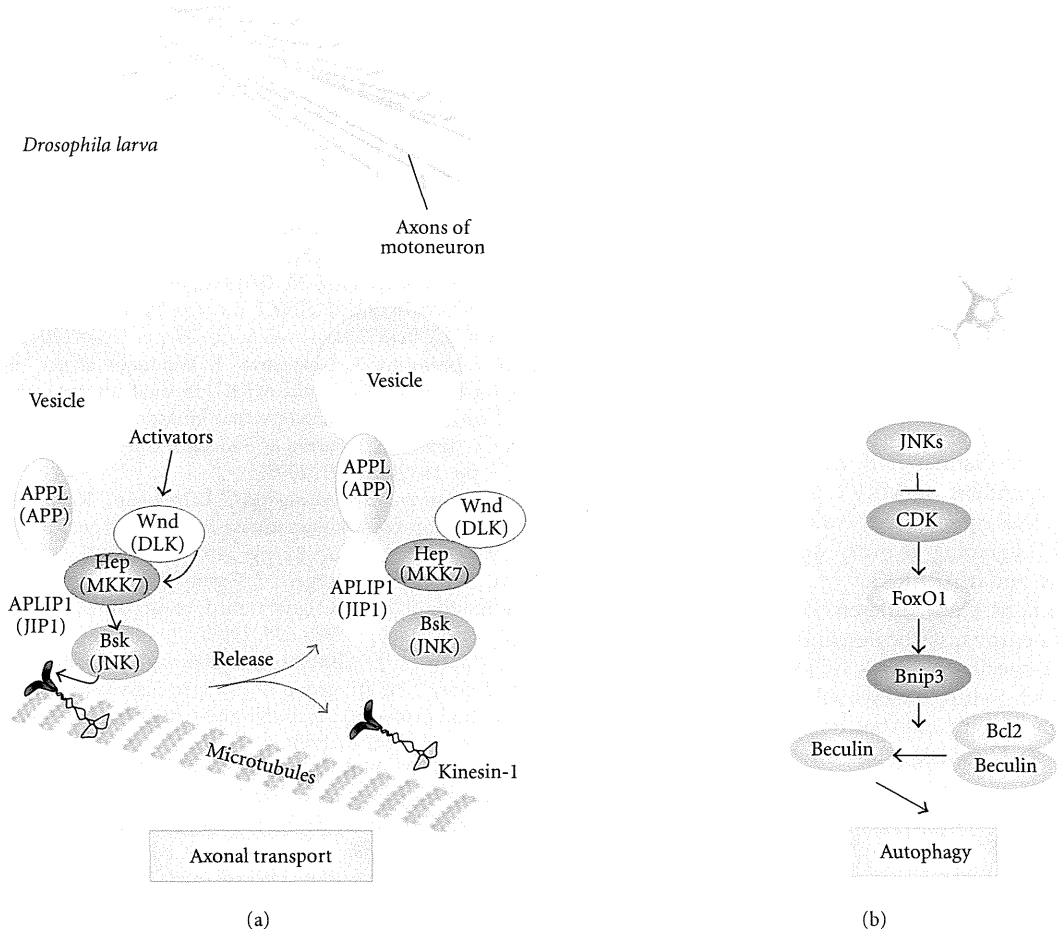

FIGURE 3: The JNK signaling pathway regulates axonal transport and autophagy. (a) Model of how the JNK signaling pathway controls axonal transport in *Drosophila*. Axonal transport is driven along MT by kinesin-1, which binds to the vesicles that make up its cargo through APLIP1 (*Drosophila* JIP1) and APPL (APP). This process is driven by Wnd (DLK), which is activated by unknown upstream signals, and phosphorylates Hep (MKK7). Activated Hep then phosphorylates and activates Bsk (JNK), which then directly or indirectly modifies the linkage complex and causes APLIP1 and the cargo to dissociate from kinesin-1. (This figure is excerpted from [39] with some modifications.) (b) Model of the regulation of neuronal autophagy in mice. In normal neurons, constitutively activated JNKs suppress CDK-induced FoxO1 activation, preventing autophagy. When all three of JNK1, JNK2, and JNK3 are disrupted, CDK-mediated FoxO1 activation increases Bnip3 expression. High levels of Bnip inhibit the binding of Beclin1 to Bcl2, and this freshly released Beclin1 induces autophagy.

directly phosphorylates the kinesin-1 motor domain [52]. It remains to be determined how extensively this molecular mechanism is conserved between *Drosophila* and mammals.

5.2. Regulation of Neuronal Autophagy. Conditional *Jnk1, Jnk2, Jnk3* triple knockout mice (*Jnk1/2/3 cTKO* mice) have been generated to eliminate the problem of functional redundancy among JNK1, 2, and 3 [53]. These animals were created by crossing *Jnk1 flox* mice with $Jnk2^{-/-}$, $Jnk3^{-/-}$, and Cre-expressing strains. *Jnk1/2/3 cTKO Nestin-Cre* mice are embryonic lethal at an early stage, but *Jnk1/2/3 cTKO Pcp2-Cre* mice, which express Cre in Purkinje cells, are viable. While numerous abnormal phenotypes have been reported for *Jnk1/2/3 cTKO Pcp2-Cre* mice, including axon hypertrophy, abnormal mitochondrial transport, and prolonged cellular lifespan in culture, the most striking anomaly observed has been increased neuronal autophagy [53]. Results to date indicate that, in neurons, the JNK signaling pathway suppresses autophagy, whereas, in nonneuronal cells, JNK signaling either induces autophagy or serves as an effector of autophagy-associated cell death [54, 55]. Although the JNK substrate leading to autophagy has yet to be identified, it has been shown that increased autophagy in *Jnk1/2/3 cTKO* neurons is mediated by FoxO1 and not by an mTORC1-dependent mechanism [53]. The combined disruption of

JNK1, JNK2, and JNK3 increases Bnip3 expression through FoxO1 activation that is mediated by CDK (Figure 3(b)). Bnip3 inhibits Beclin1-Bcl2 binding and induces Beclin1 release, which in turn triggers autophagy.

6. Conclusion and Future Perspectives

Studies of KO mice lacking JNKs, MAPKKs or MAPKKKs, have revealed that the JNK signaling pathway is involved in diverse roles in the brain, including induction of neuronal cell death, radial migration, neurite formation, metabolism regulation, and behavioral control. Twelve MAPKKKs, two MAPKs, and three JNKs can be combined in triads with specific scaffold proteins to form a large array of JNK signaling modules. These various modules are believed to facilitate JNK participation in specific biological functions. Indeed, the difference in phenotypes displayed by *Mkk4*$^{flox/flox}$ *Nestin-Cre* and *Mkk7*$^{flox/flox}$ *Nestin-Cre* mice reinforces the notion that there are distinct JNK signaling modules that may be MKK4 dependent or MKK7 dependent. Thus, to truly elucidate the JNK signaling network, it will be necessary to investigate JNK signaling at every step of its hierarchy: MAPKs, MAPKKs, and MAPKKKs.

The brain contains many different cell types, including various classes of neurons, astrocytes, and oligodendrocytes. To date, cell type-specific analyses of JNK signaling have not been performed. Future reviews will no doubt focus on the use of cell type-specific cKO mice to further dissect the striking reach of JNK signaling in normal and abnormal physiology.

Acknowledgments

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health, Labour and Welfare of Japan, and the Japan Society for the Promotion of Science.

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J. Biochem. 2012;152(2):121–123 doi:10.1093/jb/mvs069

JB THE JOURNAL OF BIOCHEMISTRY

JB Commentary

hDlk-1: a cell surface marker common to normal hepatic stem/progenitor cells and carcinomas

Received May 8, 2012; accepted June 7, 2012; published online June 12, 2012

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Advances in stem cell biology have clarified that a tumour is a collection of heterogeneous cell populations, and that only a small fraction of tumour cells possesses the potential to self-renew. Delta-like 1 protein (Dlk-1) is a surface antigen present on foetal hepatic stem/progenitor cells but absent from mature hepatocytes in neonatal and adult rodent liver. Using a monoclonal antibody (mAb) against hDlk-1, Yanai *et al.* (Dlk-1, a cell surface antigen on foetal hepatic stem/progenitor cells, is expressed in hepatocellular, colon, pancreas and breast carcinomas at a high frequency. *J. Biochem.* 2010;148:85–92) have shown that human (h) Dlk-1 is expressed in human foetal, but not adult, liver and that 20% of all hepatocellular carcinomas (HCCs) are hDlk-1$^+$. Importantly, an even higher percentage of HCCs in younger patients are hDLK-1$^+$. These authors also found that hDlk-1 is present at high frequency in colon adenocarcinomas, pancreatic islet carcinomas and small cell lung carcinomas. Here, I discuss the implications of the expression of foetal hepatic stem/progenitor cell antigens on carcinoma cells.

Keywords: carcinoma/cell surface antigen/liver/monoclonal antibody/stem cell.

Abbreviations: AFP, alpha-feto protein; Dlk-1, delta-like 1 protein; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; mAbs, monoclonal antibodies; NASH, non-alcoholic steatohepatitis.

Stem cells have the ability to both maintain proliferation (self-renewal) and differentiate into a variety of cell lineages (pluripotency), even after cell division. In addition to embryonic stem cells, there are tissue-specific populations of tissue (or somatic) stem cells in adult organs. Tissue stem cells supply new cells for individual organs and tissues to sustain their growth and maintain their activities. For example, hematopoietic stem cells in the bone marrow generate all lineages of blood cells, while neural stem cells differentiate into neurons and glial cells. When tissue stem cell functions are lost such that new cells are no longer supplied, various disorders of the vital organs can arise.

It has recently become clear that tumour cells are a heterogeneous population and that only a small fraction of them has the potential to self-renew (*1*, Fig. 1A). In this context, tumourigenesis can be considered a disease of unregulated self-renewal. The notion that a small number of cancer cells with the properties of stem cells (cancer stem/progenitor cells) are the origin of a tumour was originally proposed in the 1970s. However, the existence of these cancer stem/progenitor cells was difficult to prove experimentally. In the 1990s, developments in flow cytometry technology and a surge in our knowledge of cell surface markers made it possible to isolate and identify a single cell from a specific tissue. Studies of non-obese diabetic/severe combined immunodeficiency mice in 1997 revealed the existence of blood-specific cancer stem/progenitor cells in acute myeloid leukemia, followed in the 2000s by their isolation in a range of different cancers (*2*). However, although the expression of specific cell surface markers such as CD133 can be detected on cancer stem/progenitor cells in malignancies of the breast, brain and colon, the precise biological roles of these markers in these tumours are unclear (*3*). Thus, much work remains to be done on the identification and characterization of useful surface markers for cancer stem/progenitor cells.

In mammals, the foetal liver functions as a hematopoietic organ. In adults, the liver is less important for blood cell production but is essential for metabolism, detoxification, and the production of bile and serum proteins (*4*). The adult liver is a huge organ containing blood cells, fibroblasts and liver cells such as hepatocytes, biliary epithelial cells (cholangiocytes), liver sinusoidal endothelial cells and hepatic stellate cells. With respect to blood cells, more than 300 cell surface markers have been identified, and monoclonal antibodies (mAbs) have been raised against most of them. The use of these mAbs in conjunction with cell sorting has proved to be a powerful means of isolating and characterizing various blood cell subtypes. In contrast, very few liver-specific cell surface markers have been identified so that the properties of various liver cell subtypes have yet to be fully elucidated.

Foetal hepatic stem cells (hepatoblasts) are thought to be proliferative cells with the ability to differentiate into both hepatocytes and cholangiocytes (Fig. 1B). Hepatoblasts are derived from intestinal endoderm cells and form a hepatic bud that eventually develops into the foetal liver. In mice and rats, Delta-like 1 protein (Dlk-1), also known as preadipocyte factor 1 (Pref-1), is a transmembrane and secreted protein with epidermal growth factor (EGF)-like repeats (*5*). Dlk-1 is absent from neonatal and adult rodent liver but expressed on hepatoblasts. Dlk-1$^+$ cells isolated from foetal mouse livers form colonies containing cells of the hepatocyte or cholangiocyte lineages when cultured in the presence of hepatocyte growth

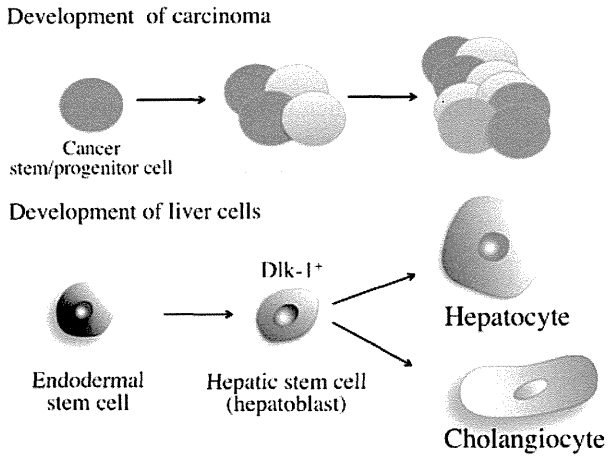

Fig. 1 Carcinogenesis and hepatic stem/progenitor cells.
(A) Proposed model of general carcinoma development. A cancer stem/progenitor cell proliferates and generates a heterogeneous cell population that contains additional cancer stem/progenitor cells (brown) as well as a variety of more differentiated tumour cells (shades of blue) that are not able to initiate tumourigenesis. (**B**) An endodermal stem cell gives rise to a hepatoblast (liver stem cell) that can differentiate into a hepatocyte or cholangiocyte. Hepatoblasts express Dlk-1.

factor and EGF. However, the precise physiological role of Dlk-1 in these situations is not clear. Using a mAb recognizing human (h) Dlk-1, Yanai *et al.* (*6*) showed that hDlk-1 is expressed in human foetal liver but not in adult liver. Significantly, 20% of all human hepatocellular carcinomas (HCCs) are positive for hDlk-1, with an even higher percentage of HCCs in younger patients being hDLK-1^{+}. These authors also demonstrated that hDlk-1 can be detected at high frequency in human colon adenocarcinomas, pancreatic islet carcinomas and small cell lung carcinomas.

Many HCCs (80–90%) occur in association with chronic hepatitis or cirrhosis of the liver. HCC develops in males and females at a ratio of 3:1 and occurs more often in Japan and east Asian countries than in Europe or North America. Recently, the relatively benign disease non-alcoholic steatohepatitis (NASH) has been found to be a precursor to liver cirrhosis and HCC in patients without detrimental drinking habits. Although alpha-feto protein (AFP) has been used as a tumour marker specific for HCC, it is often not expressed in an HCC when the tumour is still small and treatable. Thus, AFP is not useful for timely beneficial HCC diagnosis. If a marker for NASH could be identified, treatment could be instituted before progression to cirrhosis commenced. A similar difficulty exists for small cell lung cancers, which account for about 20% of lung tumours that are closely linked to smoking. No molecular marker specific for the early stages of this malignancy has yet been identified so that these high-grade carcinomas that easily make the transition to other organs are often discovered only when they are already advanced and impossible to remove. Molecular markers are also missing for islet cell tumours, including insulinomas, pancreatic islet carcinomas, glucagonomas and gastrinomas. Therefore, Yanai *et al.*'s discovery that hDlk-1 may be a molecular marker specific for the early stages of many of these tumours may lead to the development of valuable diagnostic tools and therapeutic agents.

Traditionally, pharmaceutical products are relatively low molecular weight compounds that are prepared by organic synthesis and administered orally. Although these low molecular weight drugs are clinically beneficial for many diseases, there are still a significant number of maladies for which they do not have a satisfactory therapeutic effect. Large molecular weight protein drugs were developed to address this gap, such as insulin for the treatment of diabetes, erythropoietin for anaemia and interferon for viral hepatitis. In the 1990s, the pharmaceutical industry was revolutionized by the development of therapeutic humanized mAbs as anti-cancer drugs. For example, rituximab, bevacizumab and trastuzumab are mAbs recognizing the human B cell surface antigen CD20, vascular endothelial growth factor and the growth factor receptor human EGF receptor-related 2, respectively (*7–9*). Yanai *et al.*'s work positions hDlk-1, a cell surface molecule of hepatic stem/progenitor cells, as a molecular marker of cancer stem/progenitor cells in several hard-to-detect malignancies. It may be possible to generate a diagnostic or therapeutic mAb targeting hDlk-1 that will identify early stage carcinomas and bring concrete clinical benefits to patients. Yanai *et al.*'s findings also imply that other cell surface molecules present on normal tissue stem/progenitor cells may emerge as useful markers of cancer stem/progenitor cells in a variety of tumours.

Funding
This work was supported by grants from the Japan Society for the Promotion of Science, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labor and Welfare of Japan.

Conflict of interest
None declared.

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Ⅰ．活性酸素種

3. 活性酸素シグナルと概日リズム

平山　順，仁科博史

ヒトを含む地球上のほぼすべての生物は，睡眠/覚醒やホルモン分泌といった生理機能の日周期を，外環境の明暗周期に同調させることで恒常性を維持している．この生理機能の日周的な変動は概日リズムとよばれ，生物に内在する分子時計により形成される．最近の研究により，活性酸素シグナルが分子時計の制御の過程に主要な役割を担うこと，一方で分子時計が生体内の酸化還元（レドックス）ホメオスタシスを制御することが明らかになってきた．本稿では，活性酸素シグナルと概日リズムの相互作用について最近の知見を解説する．

はじめに

概日リズムとは，遺伝子発現，細胞のエネルギー代謝，動物の行動といった生命現象に観察される約24時間周期の変動であり，地球上のほぼすべての生物に存在する[1)2)]．概日リズムは，生物に内因する機構により制御されるため，一定の温度で外環境の明暗サイクルの情報のない恒常的条件においても形成される（リズム形成の自律性）．また，概日リズムの周期性は外環境の温度変化に対して安定に維持される（温度補償性）．さらに，概日リズムの“約24時間の周期”は，光や温度変化といったシグナルを利用して，外環境の正確な24時間の周期に同調する（外環境周期への同調能）．

概日リズムは，入力系，ペースメーカー，および出力系の3つのコンポーネントにより構成される[1)2)]（**図1**）．ペースメーカーは生命現象の約24時間の周期性をつくり出す機構であるが，その実体は細胞自律的に制御され温度補償性を有する分子時計である．この分子時計に光や温度変化といった外環境からのシグナルが伝達され，その周期を外環境周期に同調させる過程が入力系である．さらに，分子時計が生命現象に日周性を与える過程が出力系である．

最近の研究により，分子時計制御における活性酸素シグナルの重要性が明らかになってきた[3)〜9)]．本稿では，活性酸素シグナルと分子時計の関連について，①ゼブラフィシュ分子時計の光同調，ならび②アカパンカビ分子時計の日周性形成における活性酸素シグナルの機能，また③哺乳動物分子時計の細胞内酸化還元（レドックス）ホメオタシスにおける役割を紹介する．最後に，④細胞内酸化還元ホメオスタシス制御機構と分子時計の共進化により形成された可能性が提唱されている，ペルオキシレドキシン（PRX）リズムについて解説する．

[キーワード&略語]
活性酸素シグナル，概日リズム，分子時計，ペルオキシレドキシン

PRX：peroxiredoxin（ペルオキシレドキシン）

Reduction/oxidation signal and circadian clock

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図1　概日リズムを構成する3つのコンポーネント

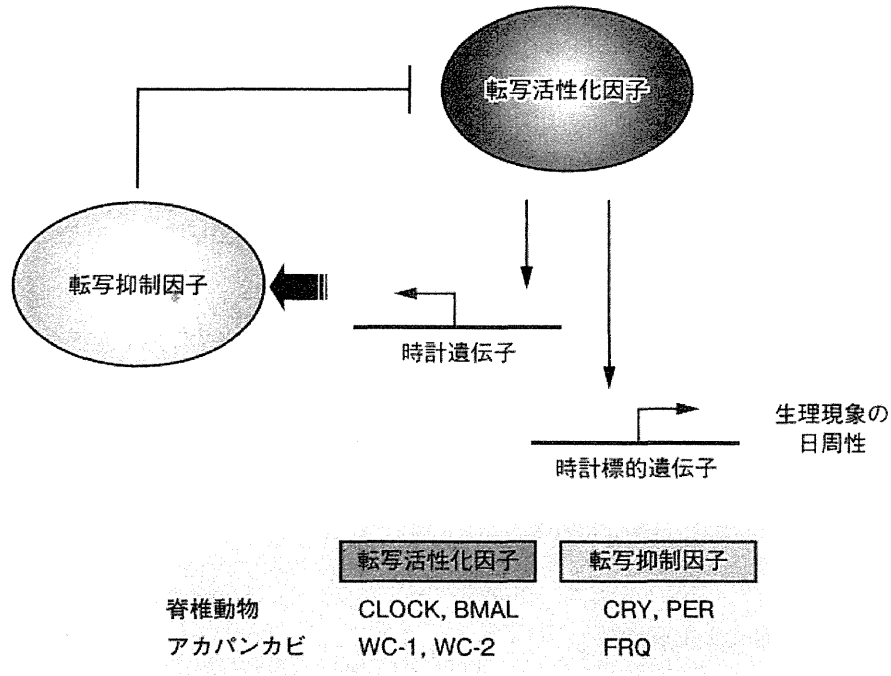

図2　概日リズムを形成する分子時計
分子時計において，転写活性化因子は転写抑制因子の発現を誘導し，転写抑制因子は転写活性化因子の活性を抑制することで自身の転写を負に制御する

1 概日リズムをつくり出す分子時計

現在までに報告されている生物の分子時計は，転写・翻訳に依存したネガティブフィードバックループであることは共通であるが，その構成因子（時計タンパク質）は生物種間で異なっている（**図2**）．また，シアノバクテリアにおいては，転写・翻訳に依存しない時計タンパク質のリン酸化の日周的な変動が報告されている[10]．ここでは，脊椎動物を例にして，分子時計の制御機構について解説する．

脊椎動物の分子時計は転写活性化因子CLOCK，BMALおよび転写抑制因子CRY，PERの時計タンパク質により構成される（**図2**）．CLOCKはBMALと二量体を形成し*Cry*および*Per*遺伝子の転写を活性化する．一方，CRYとPERはCLOCK：BMAL二量体に直接結合し，その転写活性を抑制する．分子時計の転写の活性化と抑制の周期は約24時間になるように調節されており，したがって分子時計の標的遺伝子の発現および標的遺伝子の制御する生理機能には日周性が与えられる．時計タンパク質をコードする遺伝子の変異マウスは，遺伝子の発現や行動リズムの日周性の異常に加え，癲癇，躁鬱病，発がん，代謝異常といった表現型を示