Eight micrometer of frozen sections were stained immunohistochemically using a method described previously (61).

Cell culture and RNA detection by RT–PCR

Mouse embryonic carcinoma P19 cells were maintained in MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells at a density of 1×10^5 cells per 35 mm Petri dish were maintained in MEM supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For each dish, 1, 10 or 100 ng/ml of either FGF2 or FGF8 recombinant protein (Genzyme) was added, and the medium was changed each other day. After 3 days, total RNA was isolated from cells in each dish using an RNA easy Mini Kit (Qiagen) and converted to cDNA by a standard procedure using SuperScript II RNase H- reverse transcriptase and adaptor primers (GibcoBRL) (62). DNA segments for mouse *Pax6* and β-*actin* were amplified in 30 and 19 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min with the following primers: mouse *Pax6*-forward primer 5′-CACAGCGGAGTGAATCAGCTTG-3′ and reverse primer 5′-CCAGAATTTTACTCACACAACCGT-3′ [respective product size:160 bp for *Pax6*(−5*a*) and 202 bp for *Pax6*(+5*a*)]; β-*actin*-forward primer 5′-GTGGGCCGCCC TAGGCACCA and reverse primer 5′-CTCTTTGATGTC ACGCACGATTTC (product size:540 bp).

Reporter plasmid

To obtain clones carrying the promoter region of the *Pax6* gene, we first screened the human BAC Library (Research Genetics) and detected one clone (32H10). A *Hin*dIII–*Psh*AI fragment carrying ∼2 kb *Pax6* promoter region (1285–3381 nucleotides in GenBank accession no. U63833) was excised and inserted into the *Hin*dIII–*Sal*I sites of pCAT Basic (Promega). The insert was verified by sequencing as having the reported sequence. CAT reporter constructs carrying six copies of P6CON or two copies of 5aCON were reported previously (23–25).

Transient transfection and CAT assay

P19 cells at a density of 5×10^5 cells per 60 mm petri dish were transfected with 0.5 μg of reporter plasmid (*Pax6* promoter, P6CON or 5aCON) and 0.05 μg of pSVβgal (Promega) as an internal control coated with polycationic liposome (Lipofectoamine Plus, Life Technology) according to the manufacturer's instruction. For each dish, 10, 30 or 100 ng/ml of FGF2 or FGF8 recombinant protein (Genzyme) was added, and the medium was changed each other day. Cell extracts were prepared after 72 h and assayed for CAT activities using FAST CAT Green Reagent (Molecular Probes) according to the standard procedure (62). The CAT activity was quantified by measurement with a phosphofluor-imager (Molecular Dynamics) and illustrated in a fold-activation compared with the condition without application of FGF.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Surgical Treatment of Recurrent Juvenile Xanthogranuloma of the Eyelid

Juvenile xanthogranuloma, a benign histiocytic disorder, generally arises in the skin of infants and young children and spontaneously regresses. However, surgeons should be aware that multiple recurrences are possible after surgical excision of ocular lesions.[1,2] We report a case of a recurrent eyelid lesion successfully treated by surgical excision and grafting.

Case Report

A 7-year-old girl presented with a mass on the left upper eyelid that had recurred after three excisions. The initial lesion, a 6 × 7-mm solitary subcutaneous nodule, appeared when the patient was 1 year old. The anterior segment and fundus were unremarkable. Computed tomography revealed no orbital involvement; no systemic anomalies were found. Simple excision was initially performed, and histology showed nodular lesions consisting of foamy histiocytes and Touton giant cells (Fig. 1A). The immunoreactivities were $CD68^+$, $CD1a^-$, and $S100^-$, confirming the diagnosis of juvenile xanthogranuloma (Fig. 1B). When the child was 5 years old, the eyelid mass recurred. Simple excision was repeated; however, the mass recurred after 10 months and grew (Fig. 2A). The third operation consisted of a wide excision including the surrounding tissue, but the mass recurred subcutaneously after 3 months.

The fourth operation consisted of complete excision of the mass with surrounding cutaneous and subcutaneous tissue, including the orbital septum. A skin incision was made with esthetic considerations in mind.

Histologic examination confirmed that the surgical margin was lesion-free. Orbital fat tissue was placed on the levator muscle to form a recipient bed (Fig. 2B). To obtain better color and texture matches, a full-thickness skin graft was harvested from the preauricular area. The skin graft was secured with tie-over sutures for 6 days (Fig. 2C). A frost suture was placed for 8 days. The graft adapted well and there has been no recurrence for 2 years (Fig. 2D). In addition to the characteristic morphologic and immunohistochemical features of juvenile xanthogranuloma, histologic study revealed multiple nodular lesions in the dermis (Fig. 1C), in which prominent spindle-shaped cells were arranged in a storiform pattern (Fig. 1D). The remarkable arrangement of spindle cells was only partly seen in the initial lesion but increased in the recurrent lesions.

Comments

In a previous report of a recurrent eyelid lesion, six excisions were performed when the patient was between 5 and 12 years old, but the mass continued to recur. However, histopathologic differences between the original and recurrent lesions were not investigated in that case.[1] Recently, juvenile xanthogranuloma was reported to represent a disorder of the dermal dendrocytes.[3] Within this disease spectrum, solitary spindle-cell xanthogranuloma and progressive nodular histiocytosis, which seldom regress, are characterized by predominantly (>90%) spindle cells arranged in a storiform pattern.[4,5] Although the recurrent lesion in our case had the same immunophenotypic characteristics as the original, the increased number of spindle cells may suggest an evolutionary stage of xanthogranulomatous reaction and the tendency for local recurrence. Our case indicates that juvenile xanthogranuloma of the eyelid may include the refractory variant, and grafting, following complete excision confirmed by biopsy of each edge, may be effective to treat recurrent lesions.

Key Words: eyelid, juvenile xanthogranuloma, surgical treatment

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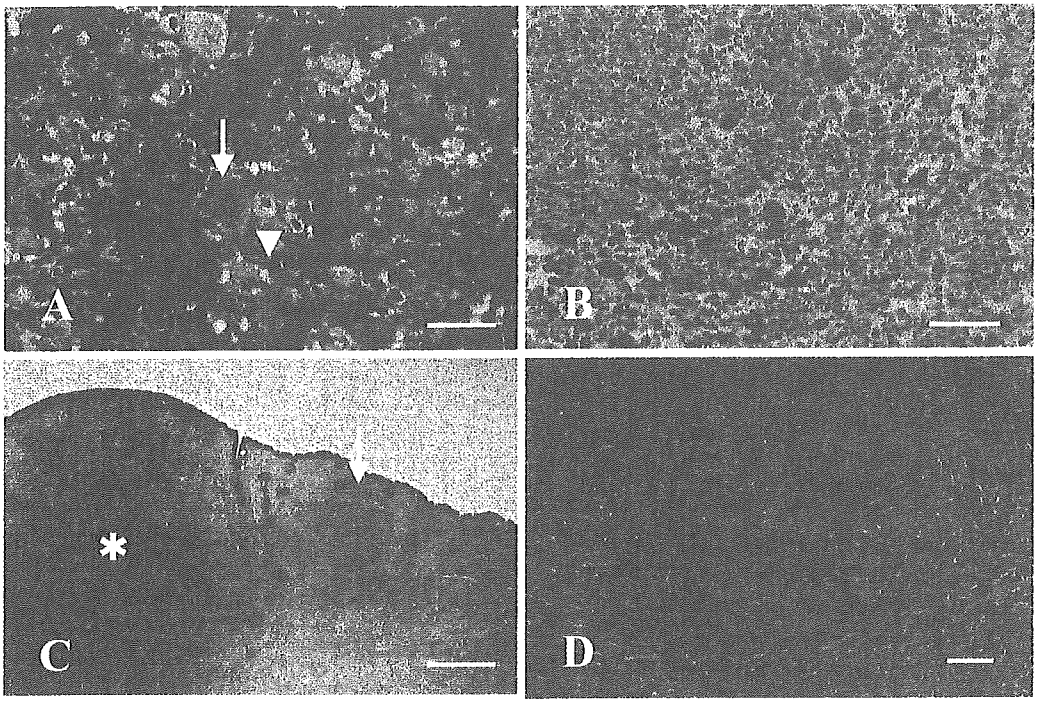

Figure 1. **A** Photomicrograph of the first eyelid excision shows lesion with foamy histiocytes (*arrowhead*) and characteristic Touton giant cells (*arrow*). H&E, bar = 50μm. **B** Immunolabeling for CD68 is positive in all sections. Bar = 100μm. **C** Low-power photomicrograph of the fourth eyelid excision shows the major dermal nodular lesion (*asterisk*) accompanied by satellite lesions (*arrow*). H&E, bar = 1000μm. **D** Prominent spindle-shaped cells are arranged in a storiform pattern. Bar = 100μm.

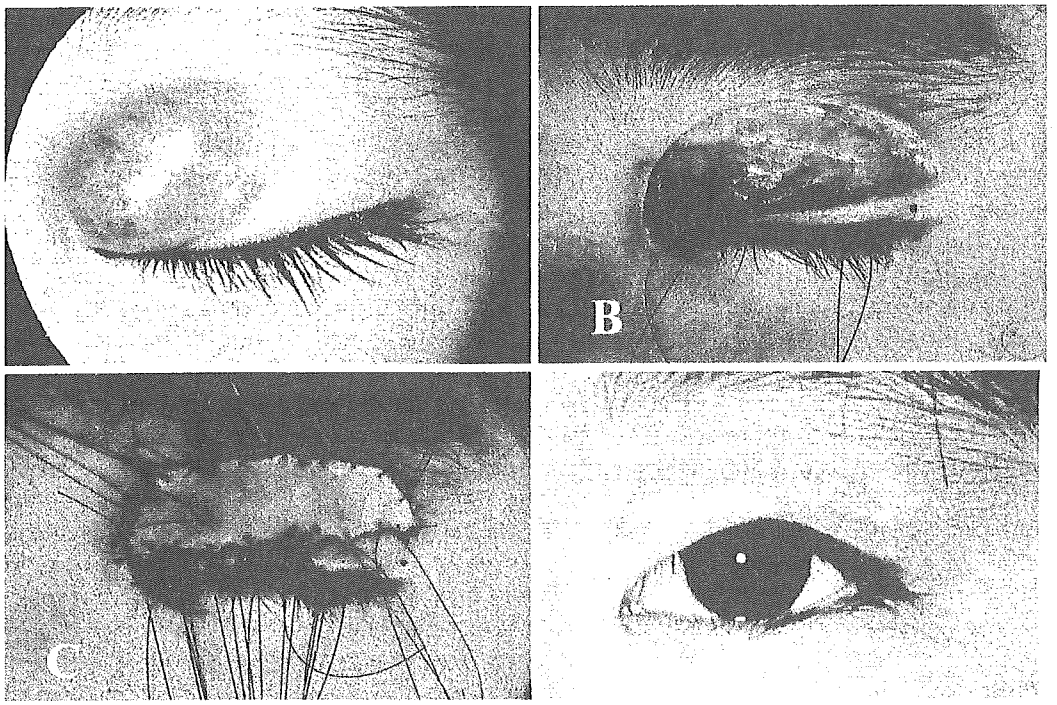

Figure 2. **A** The recurrent lesion has grown to a reddish, 14 × 19-mm mass. **B** The fourth operation: after complete excision of the mass with surrounding cutaneous tissue, orbital fat tissue is placed on the levator muscle to form a recipient bed. **C** The skin graft from the preauricular area is secured. **D** Postoperatively, the graft was well adapted, and there was no recurrence of the eyelid lesion.

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Activation mechanism and physiological roles of stress-activated protein kinase/c-Jun NH_2-terminal kinase in mammalian cells

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ABSTRACT: Stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK), which belongs to the family of mitogen-activated protein kinase (MAPK), is activated by many types of cellular stress or extracellular signals. Recent studies, including the analysis with knockout cells and mice, have led towards understanding the molecular mechanism of stress-induced SAPK/JNK activation and the physiological roles of SAPK/JNK in embryonic development and immune responses. Two SAPK/JNK activators, SEK1 and MKK7, are required for full activation of SAPK/JNK, which responds to various stimuli in an all-or-none manner in mouse embryonic stem (ES) cells. SAPK/JNK activation plays essential roles in organogenesis during mouse development by regulating cell proliferation, survival or apoptosis and in immune responses by regulating cytokine gene expression. Furthermore, SAPK/JNK is involved in regulation of mRNA stabilization, cell migration, and cytoskeletal integrity. Thus, SAPK/JNK has a wide range of functions in mammalian cells. (J Biol Regul Homeost Agents 2003; 17: 295-302)

KEY WORDS: *Stress, MAPK, SAPK/JNK, Apoptosis, Knock out, ES cells*

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MAP kinases (MAPKs) are evolutionary conserved signal-transducing enzymes involved in the regulation of many cellular events. Several MAPK groups have been identified in mammalian cells, including extracellular signal-regulated kinase (ERK), p38, ERK5, and SAPK/JNK. These MAPKs are activated by their specific MAPK kinases (MAPKKs): ERK by MEK1 and MEK2, p38 by MKK3 and MKK6, ERK5 by MEK5, and SAPK/JNK by SEK1 (also known as MKK4) and MKK7 (SEK2). These MAPKKs are also activated by various MAPKK kinases (MAPKKKs) such as Raf, MLK, MEKK1, TAK1, and ASK1. In this review, we focus on recent progress in the SAPK/JNK group of MAPK-signaling pathways in mouse embryonic stem (ES) cells, embryos, and adult mice (Fig. 1). It is becoming clear that SAPK/JNK activation regulates many cellular processes such as gene expression, cell survival and apoptosis (1-4).

Molecular mechanism of SAPK/JNK activation in ES cells

SAPK/JNK is activated not only by many types of cellular stress including changes in osmolarity, UV irradiation, heat shock, cisplatinum, etoposide, thapsigargin, and tunicamycin, but also by serum, lysophosphatidic acid (LPA), and inflammatory cytokines, interleukin-1ß (IL-1β) and tumor necrosis factor α (TNF-α). The activated SAPK/JNK phosphorylates a number of substrates including transcription factors, c-Jun, Jun D, and ATF-2, to regulate gene expression for stress responses. Activation of SAPK/JNK requires the dual phosphorylation of Tyr and Thr residues located in a Thr-Pro-Tyr motif in the activation loop between VII and VIII of the kinase domain (5). The phosphorylation is catalyzed by the dual specificity kinases, SEK1 and MKK7, which are capable of catalyzing the phosphorylation of both Thr and Tyr residues.

Recent studies have shown that SEK1 has a preference for the phosphorylation of Tyr residue, while MKK7 for Thr residue of SAPK/JNK *in vitro* and that both phosphorylation results in the synergistic activation of SAPK/JNK (Fig. 2A) (6-8). Strong support for this activation mechanism has been obtained from studies of SEK1- and MKK7-gene disruption in ES cells. The severe impairment of SAPK/JNK activation observed in $mkk7^{-/-}$ ES cells was accompanied with a loss of the Thr-phosphorylation of SAPK/JNK, without marked reduction in its Tyr-phosphorylated level. On the other hand, Thr-phosphorylation of SAPK/JNK in $sek1^{-/-}$ ES cells was also attenuated, in addition to a decreased level of its Tyr-phosphorylation. These results indicate that the Tyr and Thr residues of SAPK/JNK are sequentially phosphorylated by SEK1 and MKK7, respectively, in stress-stimulated ES cells (Fig. 2B) (9, 10).

Involvement of "scaffold proteins" has also been reported for the correct proceeding of SAPK/JNK-

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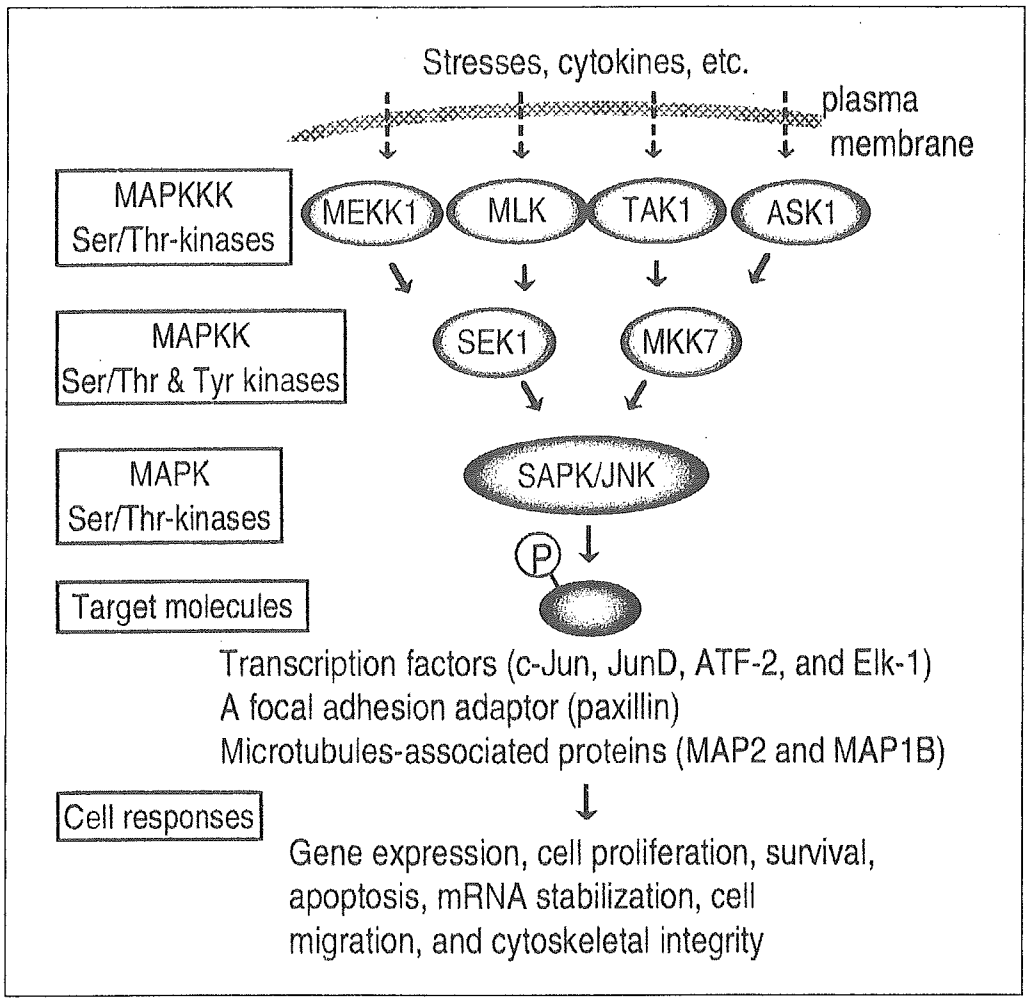

Fig. 1 - SAPK/JNK-signaling pathways involved in a variety of cell responses. SAPK/JNK is activated by extracellular stimuli including stresses and cytokines through kinase cascades. Activated MAPKKKs such as MEKK1, MLK, TAK1, and ASK1 transmit the signal to two MAPKK, SEK1 and MKK7. SAPK/JNK activated by SEK1 and/or MKK7 phosphorylates target molecules such as c-Jun, paxillin, and MAP2 to regulate a wide range of cell functions.

signaling pathway. The scaffold proteins, JIP-1, JIP-2, JIP-3, JSAP1, and JLP, organize the components of SAPK/JNK cascade to secure SAPK/JNK-signaling specificity in mammalian cells (11-15). JIP-1, JIP-2, and JIP-3 bind to SAPK/JNK, MKK7, and mixed-lineage protein kinases (MLKs). JSAP1 is an alternatively spliced variant of JIP3, however, associates with SAPK/JNK, SEK1, and MEKK1. JLP acts as a scaffold protein to bring together Max and c-Myc along with SAPK/JNK and p38, as well as their upstream SEK1 and MEKK3. MEKK1 itself plays a role as a scaffold protein that regulates SEK1 (Fig. 2C) (16). A theoretical consideration indicates that a single set of the scaffold-supported complex, which contains either SEK1 or MKK7, can not induce the sequential phosphorylation, since the synergistic activation of SAPK/JNK requires both SEK1 and MKK7 as observed in ES cells. Therefore, two sets of scaffold complexes, one containing SEK1 and the other MKK7, must exist closely in ES cells. Indeed, we have screened and found a predicted "binder"-like protein, which connects the two MAPKK (MKK7 and SEK1) cascades (Fig. 2D). Characterization of the protein in SAPK/JNK-signaling pathway is currently under investigation in our laboratory.

SAPK/JNK activation as a molecular switch in all-or-none manner

Recently, Ferrell et al have proposed an interesting concept that SAPK/JNK-signaling cascade could, in principle, function as a sensitivity amplifier, which converts graded inputs into more switch-like outputs, allowing the cascade to filter out noise and yet still respond decisively to supra-threshold stimuli (17, 18). They have shown in *Xenopus* oocytes, HeLa cells, HEK293 cells, and Jurkat T cells that SAPK/JNK responds to physiological and pathological stimuli, such as progesterone and sorbitol, in an all-or-none manner. The activation of SAPK/JNK by the stimuli was graded at the level of a population of oocytes, however, at the level of an individual oocyte, the stimulatory response appeared to be switch-like. Indeed, we have also observed a very steep concentration-dependent response in the activation of SAPK/JNK by hyper-osmolar stress, sorbitol, in murine ES cells (Fig. 3) (10). This suggests that the all-or-none type MAPK activation also occurs in mammalian cells at an individual cell level only when the two MAPKKs are simultaneously activated. Therefore, this MAPK signaling should

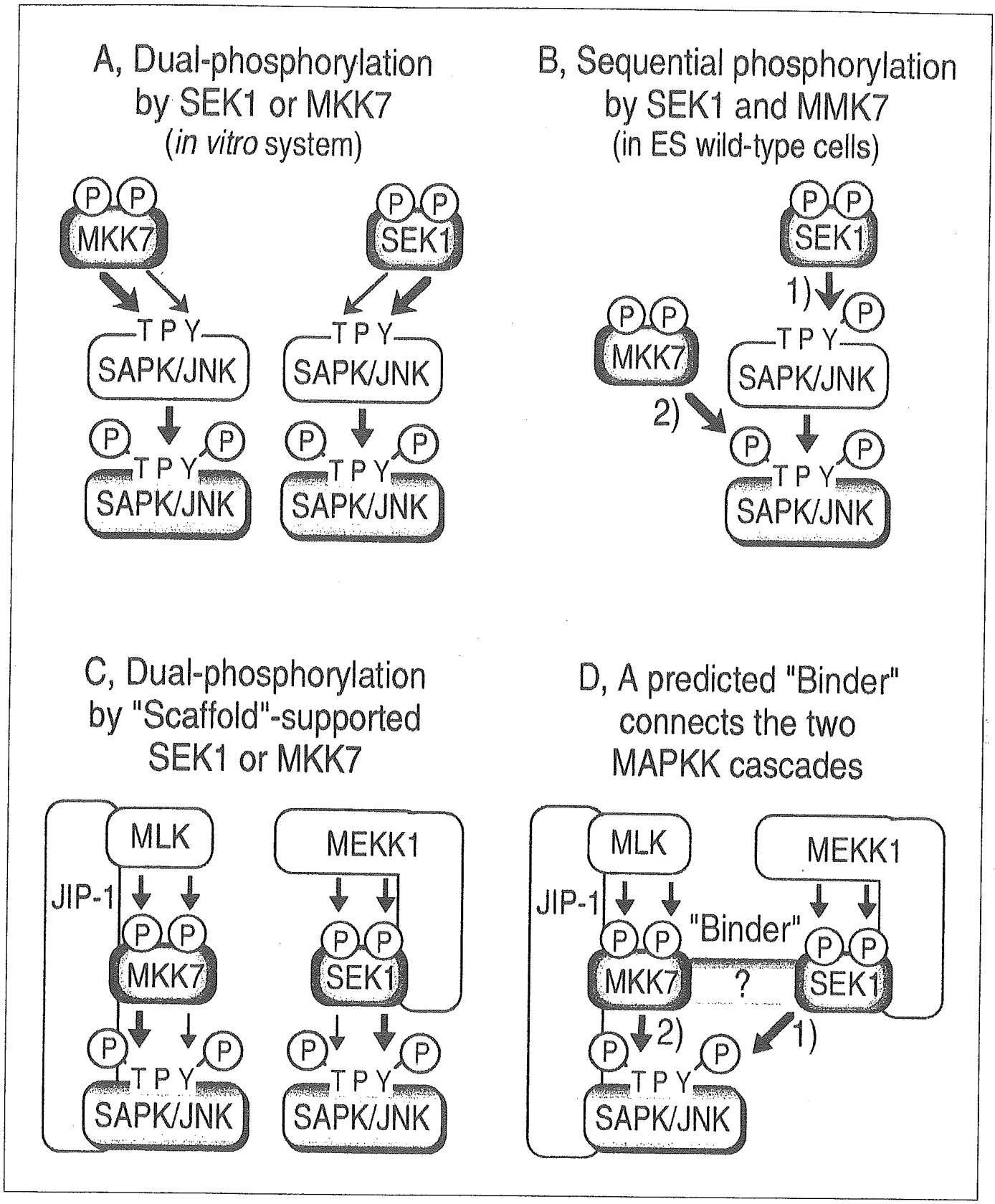

Fig. 2 - Schematic description of SAPK/JNK phosphorylation by SEK1 and MKK7 under various conditions. A) synergistic activation of SAPK/JNK by the dual-specificity kinase, SEK1 or MKK7, which has been reported in *in vitro* conditions (6-8). B) synergistic activation of SAPK/JNK through sequential phosphorylation by SEK1 and MKK7 in murine ES cells (9, 10). C) activation of SAPK/JNK by SEK1 or MKK7 associated with their scaffold proteins, JIP-1 and MEKK1 (11, 16). D) a predicted "binder" connects the two scaffold-supported MAPKK cascades (SEK1 and MKK7) for the sequential phosphorylation of SAPK/JNK as shown in panel B) TPY, Thr-Pro-Tyr motif.

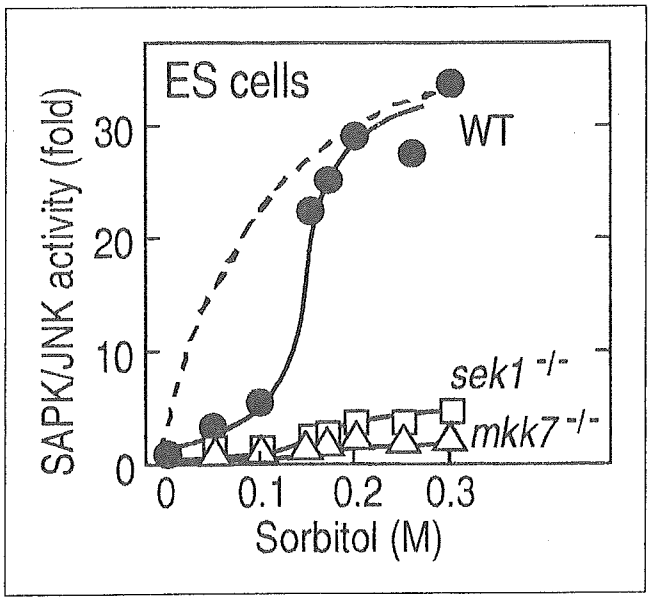

Fig. 3 - SAPK/JNK activation in response to hyper-osmolar stress (sorbitol) requires both SEK1 and MKK7 in ES cells. Wild-type, sek1$^{-/-}$, and mkk7$^{-/-}$ ES cells were stimulated with the indicated concentrations of sorbitol for 30 min.

strictly proceed without errors basically through the two separated signals, one activating SEK1 and another activating MKK7. Although the molecular mechanism whereby the two MAPKKs are simultaneously stimulated by various stress signals remains to be resolved, it is tempting to speculate that the two separated pathways leading to SAPK/JNK activation may exist and physiologically function as a fail-safe mechanism as proposed previously (9).

Role of SAPK/JNK in mouse development

Sek1, mkk7, and all three Jnk (Jnk1, 2, and 3) loci have been knocked out at present. Sek1$^{-/-}$ embryos die between embryonic day 10.5 (E10.5) and E12.5 with impaired liver formation and massive apoptosis (Fig. 4) (19-21). We have recently shown that SEK1-mediated SAPK/JNK pathway downstream TNF-α receptor 1 (TNFR1) participates in embryonic hepatoblast proliferation and survival via a pathway different from NF-κB-induced anti-apoptosis. On the other hand, mkk7$^{-/-}$ embryos die between E11.5–12.5 with similar defects in liver formation. These results indicate that SAPK/JNK activation mediated through SEK1 plus MKK7 plays indispensable roles in hepatoblast proliferation and survival during mouse embryogenesis (Fig. 5) (22).

JNK1 and JNK2 are widely expressed in many tissues, but JNK3 is expressed predominantly in nervous system. Mice deficient in the single gene of Jnk1, Jnk2, or Jnk3, and Jnk1/Jnk3- or Jnk2/Jnk3-double mutant mice all survived normally. Mice lacking both JNK1 and JNK2 die around E11 with severe dysregulation of apoptosis in brain. Specifically, there was a reduction of cell death in the lateral edges of hindbrain prior to neural tube closure. In contrast, increased apoptosis and caspase activation were found in the mutant forebrain. These results assign both pro-and anti-apoptotic functions to JNK1 and JNK2 in the development of the fetal brain (23, 24).

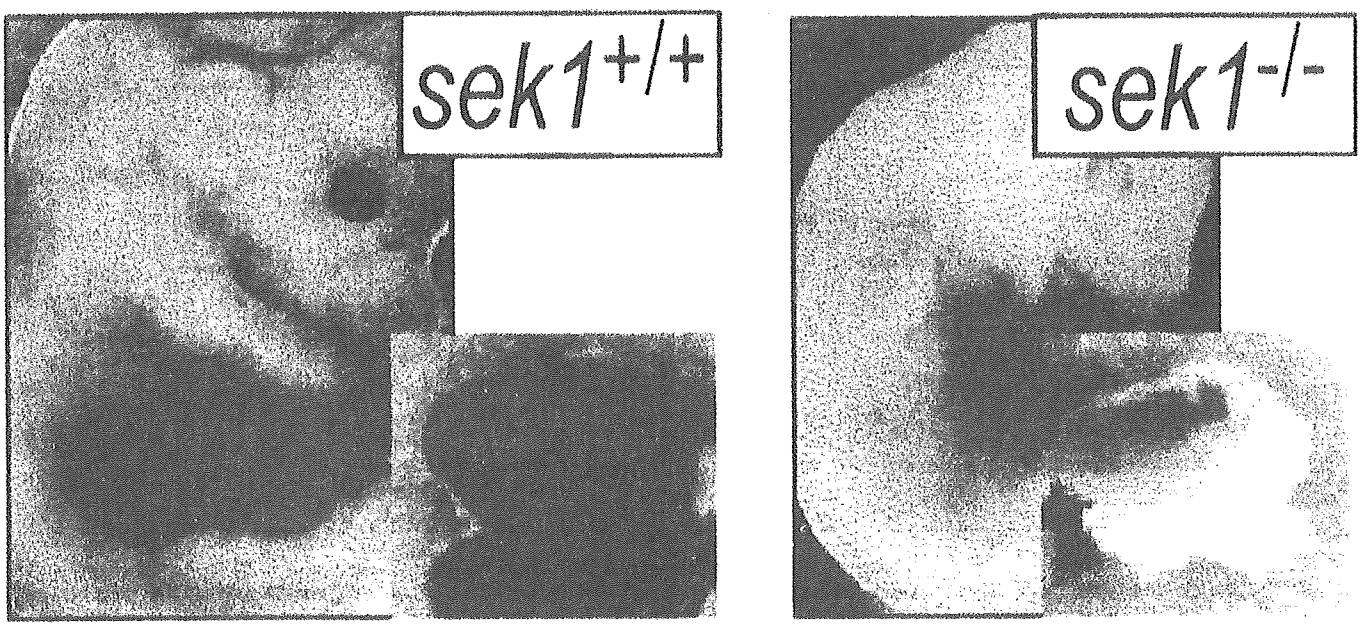

Fig. 4 - Defective liver formation in sek1$^{-/-}$ embryos. Appearance of wild-type and sek1$^{-/-}$ embryos and livers at embryonic day 12.5. Severe anemia is observed in sek1$^{-/-}$ embryos.

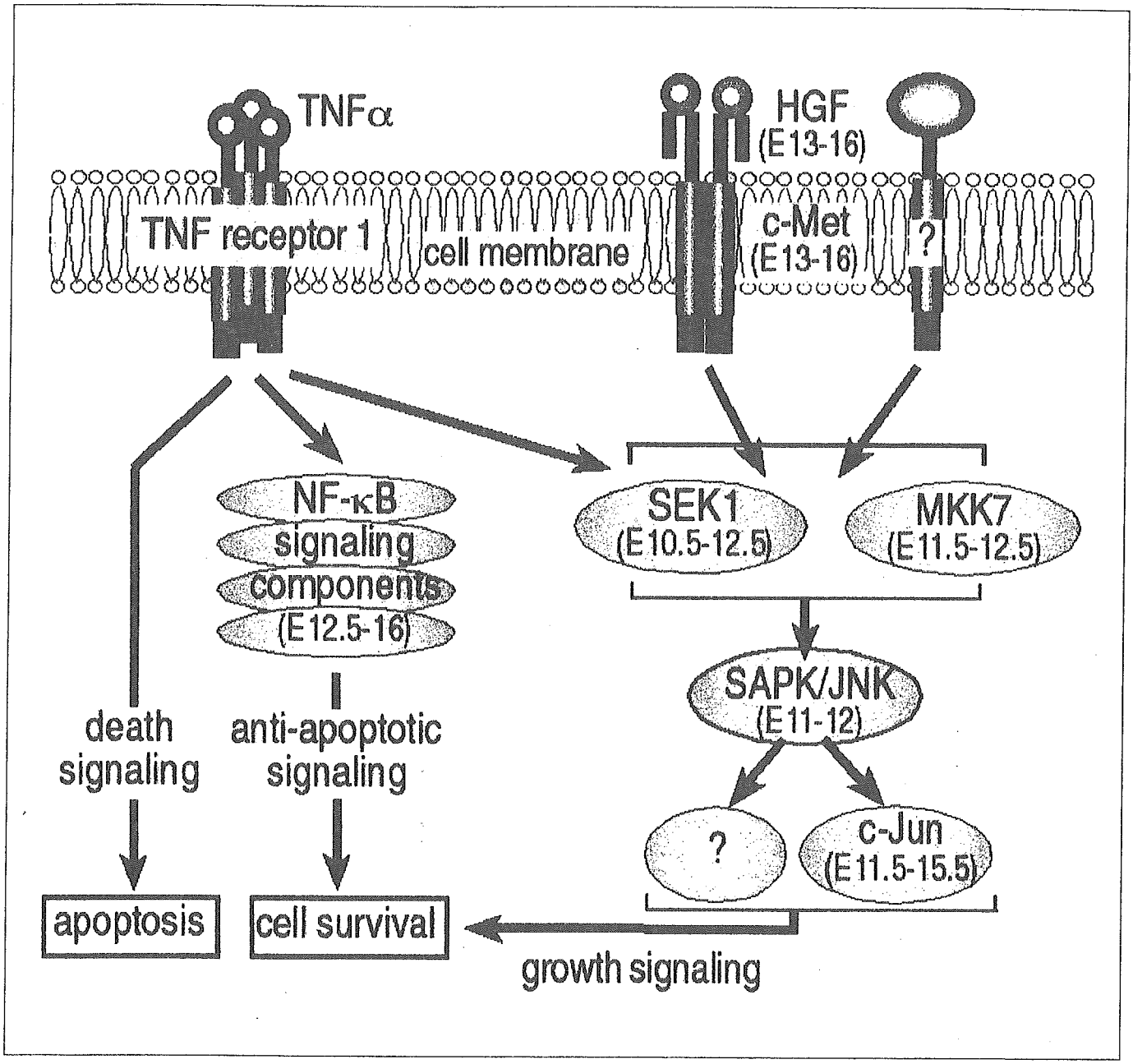

Fig. 5 - A proposed model for SAPK/JNK signaling pathway in hepatoblasts. The numbers in parentheses are dates of embryonic lethality reported in previous papers. TNFα elicits a wide range of biological responses, such as inflammation, tumor necrosis, differentiation, cell proliferation, and apoptosis, through the stimulation of its receptor, TNFR1. The induction of apoptosis, NF-κB activation, and SAPK/JNK activation are simultaneously mediated through TNFR1. SAPK/JNK activation is involved in cell growth, while activation of NF-κB protects against the apoptosis in hepatoblasts (22).

Role of SAPK/JNK in cell survival and apoptosis

In mammalian cells, apoptotic signaling cascades can be divided into two broad categories: the intrinsic (mitochondria-dependent) and the extrinsic (death receptor-mediated) pathways. The initiation of mitochondria-dependent pathway requires a change in the organella membrane permeability that is prevented by anti-apoptotic molecules such as Bcl-2 and Bcl-X_L and promoted by pro-apoptotic molecules including Bax and Bak. The permeability change results in the release of mitochondrial proteins. One of the released proteins, cytochrome c, associates with Apaf1 and caspase 9 to activate the effector caspase 3 (25, 26). Cellular stresses such as UV irradiation and heat shock mediate apoptosis through the mitochondria-dependent pathway (27). However, upstream signaling that regulates the pro-apoptotic molecules remains to be elucidated. Recently, the involvement of SAPK/JNK activation in pro-apoptotic function has been suggested in the study with $Jnk1^{-/-}$ $Jnk2^{-/-}$ and $mkk4^{-/-}$ $mkk7^{-/-}$ mouse

embryonic fibroblasts (MEFs). Both Jnk1$^{-/-}$ Jnk2$^{-/-}$ and mkk4$^{-/-}$mkk7$^{-/-}$ MEFs exhibited profound defects in stress-induced apoptosis (28, 29). Furthermore, it has been reported that JNK activation causes the release of apoptogenic factors such as cytochrome c and Smac from isolated mitochondria in a cell-free system (30, 31). These results strongly suggest that the SAPK/JNK activation directly regulates mitochondria-dependent apoptosis in pro-apoptotic direction.

Recently, we generated ES cells lacking both MKK4 and MKK7 to reevaluate the role of SAPK/JNK activation in the stress-induced and mitochondria-dependent apoptosis. We utilize mouse ES cells in terms of the following advantages: 1) ES cells are a prototype of all cell lineages and can be differentiated into MEF-like cells with retinoic acid, 2) ES cells do not express death receptors including Fas and TNFR1, but have stress-induced, mitochondria-dependent apoptotic pathway, and 3) the molecular mechanism of SAPK/JNK activation is well characterized in ES cells. Interestingly, SAPK/JNK activation is not required for stress-induced and mitochondria-dependent apoptosis in ES and MEF-like cells (submitted). Thus, the physiological role of SAPK/JNK activation in cell survival and apoptosis is still controversial, suggesting an anti-apoptotic, a pro-apoptotic, or no function in these processes dependent on the types of cells and stimuli (32).

Role of SAPK/JNK in mouse immune responses

CD4$^+$ and C8$^+$ T cells are two subsets of peripheral T cells that play important roles during an immune response. After antigen stimulation, CD4$^+$ T cells differentiate into effector Th1 or Th2 cells that secret cytokines to help modulate the type of immune response that is generated. Th1 cell promote cell-mediated immunity against intracellular microbial pathogens by expressing interferon-γ, interleukin (IL)-2, and lymphotoxin, whereas Th2 cells promote humoral immunity against parasites and extracellular pathgens by expressing IL-4, IL-5, IL-9, IL-10, and IL-13. CD8$^+$ T cells differentiate into cytotoxic T cells to help defend the host during the cell-mediated immune response. Thus, defective T cell development to Th1 and Th2 cells results in impaired immune responses. Furthermore, inappropriate activation of T cells initiates and perpetuates many autoimmune diseases including rheumatoid, asthma, inflammatory bowel disease, and multiple sclerosis. It has been reported that T cells from mice deficient in the Jnk1 or Jnk2 gene have a defect in functional differentiation into Th1 or Th2 subsets (33, 34). Sabapathy et al have reported that JNK1 positively regulates mature T cell activation and that JNK1 and JNK2 have similar and overlapping roles in T cell function (35). Conze et al have also reported that JNK1 is required for CD8$^+$ T cell activation, however, that JNK1 and JNK2 have distinct functions in CD4$^+$ T cell differentiation and CD8$^+$ T cell activation (36). Furthermore, we found defective T cell activation, whereas Swat et al. found normal activation of T cells lacking SEK1 (37, 38). These results indicate that the magnitude of the stimuli, the expression of JNK isoforms, and the activation level of SAPK/JNK are important factors determining the direction and efficiency of T cell activation (35). Thus, SAPK/JNK appears to be involved in the differentiation and activation of T cells, though its precise molecular mechanisms are still controversial.

Other physiological roles and target molecules of SAPK/JNK

As described above, SAPK/JNK regulates embryonic development including cell proliferation, survival, and apoptosis, and immune responses including T cell differentiation and activation. Furthermore, it has been reported that SAPK/JNK regulates mRNA stabilization, cell migration, and cytoskeletal integrity (Fig. 1). Turnover of mRNA is an important mechanism for the regulation of gene expression in organisms from bacteria to mammals. Regulation of mRNA half-life can influence normal cell proliferation, differentiation and oncogenesis. PB-3c mast cells produce IL-3 upon stimulation with extracellular signals, and its short-lived (half-life is about 30 min) mRNA is stabilized by Ca^{2+} ionophores. Using an active MEKK1 and a dominant-negative mutant of JNK, it has been indicated that SAPK/JNK is involved in the regulation of IL-3 mRNA turnover in mast cells (39). SAPK/JNK is required for *Drosophila* dorsal closure and is also essential for cell migration in mammalian cells. Rat bladder tumor epithelial cells (NBT-II) exhibit rapid keratinocyte-like movement. Interestingly, SP600125, a specific inhibitor of JNK, suppresses the movement. Several experiments indicate that JNK1 phosphorylates serine 178 on paxillin, a focal adhesion adaptor, in NBT-II cells. Expression of a paxillin mutant (Ser178 to Ala) inhibited the migration of the cells. Thus, phosphorylation of paxillin by SAPK/JNK seems to be essential for maintaining the labile adhesions required for rapid cell migration (40). Dynamic assembly and disassembly of microtubules is essential for a variety of cellular functions, such as maintenance of cell morphology and polarity, cell division, cell locomotion, and intracellular trafficking. JNK1-deficient mice exhibit progressive degeneration of long nerve fibers and loss of microtubule integrity in dendrites. Dendritic degeneration of neuronal microtubules is associated with hypo-phosphorylation of microtubule assembly-promoting protein (MAP) 2 and its reduced ability to promote tubulin polymerization. Thus, JNK1 is required for maintaining the cytoskeletal integrity of neuronal cells and is a critical regulator of MAP activity and microtubule assembly (41).

Conclusions

Data continues to emerge implicating the SAPK/JNK-signaling pathway in a number of physiological functions that may be involved in human disease including autoimmune, anti-inflammatory, neurogenerative diseases, and cancers. In fact, mutations in Jnk3 gene were identified in human brain tumors (42). Similarly, mutations in sek1 gene have been identified in human cancers of pancreas, lung, breast, colon, and prostate as tumor suppressor genes (43-45). Under these circumstances, several pharmaceutical companies have been working on the discovery of SAPK/JNK-related drugs such as an anthrapyrazolone and SP600125. Efforts by many researchers in this field may help to find effective drugs in the near future.

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水晶体の形成遺伝子とその変異

Genetics on the Lens Morphogenesis and Its Malformation

東　範行*

要　約

発生は転写因子遺伝子群がカスケードをなして働く一連のシステムである．眼においては*Pax6*を頂点とする遺伝子カスケードが存在する．*Pax6*はまずヒトの先天無虹彩の原因遺伝子として見つかり，下等動物の初期胚に導入すると眼が異所性にできることから，眼形成のmaster control遺伝子であると考えられるようになった．ヒト発生眼でも広範に発現し，無虹彩のほかに，前眼部形成不全，先天白内障，黄斑低形成，視神経形成異常で変異が見つかったことから，発生における働きはヒトでも眼のほぼ全体を網羅していることが判明した．さらに*Pax6*の下流にあって，前眼部を形成する遺伝子*Pitx*，*Eya1*が見つかり，先天白内障と前眼部形成異常でその変異が見出された．また，鶏やアフリカツメガエルでは*Pax6*の下流で水晶体を形成するmaster control遺伝子*L-Maf*が発見された．水晶体の発生において，これらの形態形成遺伝子はクリスタリンをはじめとする水晶体特異蛋白の発現を制御していることも明らかになった．そして，これらの構造蛋白の変異も先天白内障で次々と見つかっている．これら水晶体形成遺伝子に変異が起これば同様な表現型が生ずるので，疾患概念も変化しつつある．これら発生に関する遺伝子システムの解明は，疾患の成因への理解や，遺伝相談に有用であるが，さらには将来の遺伝子治療や再生医学に結びつくことが期待される．

はじめに

眼は複雑な構造をもつ器官であり，遺伝性疾患も多いことから，これまで眼科領域では多くの疾患で原因遺伝子が発見されてきた．代表的なものとしては，Leber視神経症（ミトコンドリア遺伝子の変異による），網膜色素変性症（rhodopsin，peripherin，cGMPなど），小口病（arreston，rhodopsin kinase），色覚異常（red-green opsin），角膜ジストロフィ（kerato-epithelin）などがある．Leber視神経症におけるミトコンドリア遺伝子は特殊であるが，その他多くの遺伝性眼疾患は各組織に特異的に発現する構造遺伝子や酵素をコードする遺伝子の変異が原因である．一方，形態形成（発生）においては組織構築に関わる蛋白をコードする遺伝子も重要であるが，転写因子やシグナル伝達物質がきわめて重要な役割を果たしている．Leber黒内障（guanylate cyclaseの変異による）やNorrie病（Norrie disease，*ND*遺伝子，ムチンや成長因子と類似），網膜分離症（*XLRS1*遺伝子，網膜間の接着に関与）などは前者であり，先天無虹彩（*PAX6*遺伝子）などは後者である．

水晶体は表面外胚葉から形成される特異な組織であり，最近，その発生に関わる多くの遺伝子が発見された．一方，先天白内障は発生期に起こる水晶体の形成異常であるが，これらの発生遺伝子や水晶体に特異的に発現する蛋白の変異によって起こることが明らかになってきた．これら基礎研究と遺伝学研究から，水晶体の発生や疾患に関する概念は大きく変化してきている．ここでは，眼球およびその中の水晶体の形成に関わる遺伝子と，先天白内障の遺伝子変異について最近の知見を述べる．

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I 器官・組織の発生に関わる遺伝子

細胞の核の中に存在する遺伝子は，その翻訳する蛋白の性格によって，構造蛋白，酵素，シグナル伝達物質，転写因子などに大別されるが，発生においては転写因子が重要な役割を果たしている．転写因子は遺伝子調節蛋白ともよばれ，他のDNAのおもにpromoter領域に結合して，その遺伝子の発現スイッチをon offする司令塔の役割をする蛋白である．働きの場は細胞核内で，微量にしか存在しないが，細胞が特異的機能を果たすために適切な遺伝子を適切量発現させるための司令塔の役割をもち，構造蛋白，酵素，シグナル伝達物質などはいずれもその支配下にある．ことに発生においては，複雑な組織・器官が分化するために各時期に適切な遺伝子が発現するネットワークが必要であり，そのプログラムに従って整然と形成過程は進まなければならない．たとえば，手が形成される場合には，ある時期に中胚葉細胞が増殖して手根骨，指骨，筋肉になり，外胚葉細胞から皮膚ができ，さらにある時期に指間の細胞が一斉にアポトーシスを起こすことによって各指が離れる．動物によっては，アポトーシスが完全に起こらなければ指間にヒレが残るわけである．したがって，発生（形態形成）においては転写因子遺伝子ネットワークを解明することは，一連の工程の設計図を明らかにすることを意味する．

発生に関わる遺伝子は，その中にhomeoboxという構造をもつことが特徴である．Homeoboxは生物種を越えて発生に関与する遺伝子のきわめて多くに共通して含まれる180の塩基対であり，これは翻訳されて60アミノ酸homeodomainとなる．Homeodomainは転写因子における標的DNA結合部位として働くが，その普遍性から，homeoboxをもつ遺伝子は発生における転写因子遺伝子，位置や空間を決定する遺伝子の指標であると考えられるようになった．

さらに，遺伝子が意外な場所に転用されるのも特徴である．眼を作る遺伝子は，同時に中枢の発生にも関与していることが多い．眼と中枢は神経系として共通点も多いが，まったく関係のない腎臓や膵臓などで同じ遺伝子が働いていることがある．これは，眼と腎臓が同起源ということではなく，遺伝子（あるいは翻訳される蛋白）が1つの固定した機能に縛られず，融通性に富んでいて，少し条件を換えれば他の働きもできることを意味している．そして，生物は進化して複雑化する際に，しばしばこの転用を利用してきた．新しい遺伝子を作ることもあったが，これは大変なことであり，使えるものはそのままあるいは少し違えて使うほうが，はるかに効率的である．激烈な進化の競争のなかで，生物はゆっくり新しい遺伝子を創作している余裕はなかったのである．そして，遺伝子がこのように多用されているため，同じ遺伝子障害によって多彩な複数臓器障害の症候群が起こることになる．

眼は最も複雑な構造をした器官であり，角膜，水晶体，網膜などその一部の組織をとっても，発生には膨大な転写因子ネットワークがあるはずである．そして，現在までにさまざまな転写因子遺伝子が見つかり，複数臓器に発現しているものも多かった．そして，これらに変異が起こればヒト疾患の原因となることも明らかになってきた．このなかで，後に述べる*PAX6*遺伝子の発見が最も重要である．これが眼全体を作る遺伝子ネットワークの頂点に存在するmaster control遺伝子であるからである．

II 眼形成のmaster control遺伝子*PAX6*の発見と動物間の共通性

*PAX*遺伝子群はpaired boxとhomeoboxを共通モチーフとしてもつ遺伝子familyで，PAX蛋白ではpaired boxとhomeoboxから翻訳される部位paired domainとhomeodomainの2つが標的遺伝子への結合部位となる（図1）．この遺伝子群は最初にショウジョウバエで発見されたが，脊椎動物では9種見つかっており，*PAX6*は6番目に発見された．ヒトでは最初に先天無虹彩の候補遺伝子として染色体11p13領域の欠失部位からpositional cloningによって発見され[1]，多くの変異が発見されて無虹彩の原因遺伝子であることが確定した（図2，3）．その後，マウスやラットで変異があると小眼球になる*small eye (Sey)*遺伝子や，ショウジョウバエで複眼が形成されない*eyeless*遺伝子と同じものであることが判明し，*Pax6*に統一された（遺伝子の表記はすべてが大文字ならヒト，頭だけが大文字ならばマウスなど他のほ乳類，全部小文字ならばショウジョウバエなどの下等動物

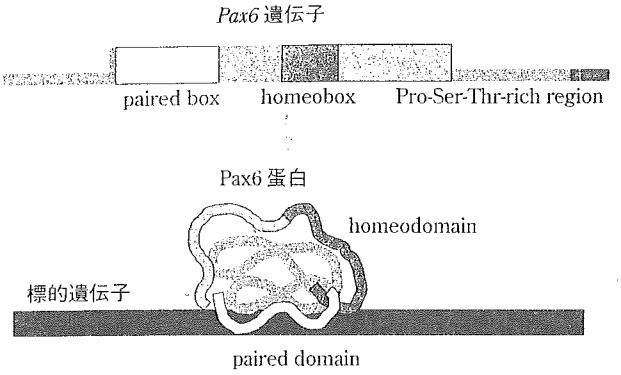

図 1　*PAX6* 遺伝子と，これから翻訳される PAX6 蛋白
PAX6 蛋白はおもに paired domain が標的 DNA に接着して，これを制御する．

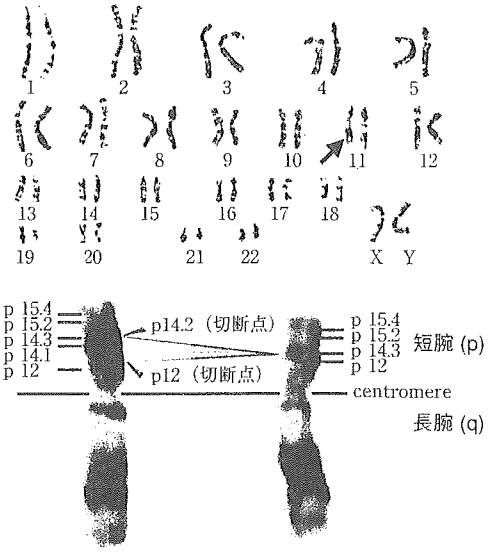

図 2　先天無虹彩の染色体異常
（11 番短腕欠損）

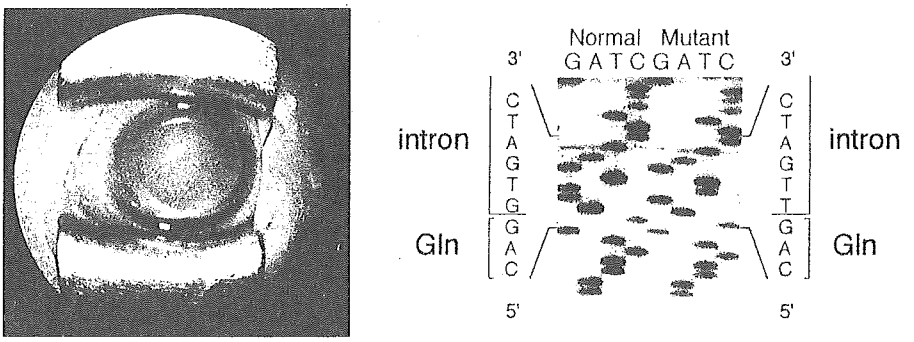

図 3　黄斑と視神経の低形成を伴う先天無虹彩の *PAX6* 遺伝子変異（スプライシング変異）

のものを表す．また遺伝子は斜体文字，翻訳された蛋白は標準体文字で書かれる）．

さらに，ショウジョウバエで初期胚のさまざまな部位にこの遺伝子を発現させたところ（target expression），触覚や翅，肢などで異所性に複眼が発生し，しかもこれらが光を感じたため，眼という器官全体を 1 つ作ってしまうような強力な遺伝子であることが明らかになった[2]．器官が形成される場合，その過程が整然と行われるためには，全体を支配する遺伝子（master control 遺伝子）があるだろうと予測されていたが，昆虫のような下等動物とはいえ，眼という最も複雑な器官でその遺伝子が見つかったのである（図 4）．

その後，さまざまな動物で *Pax6* 遺伝子が見つかり，

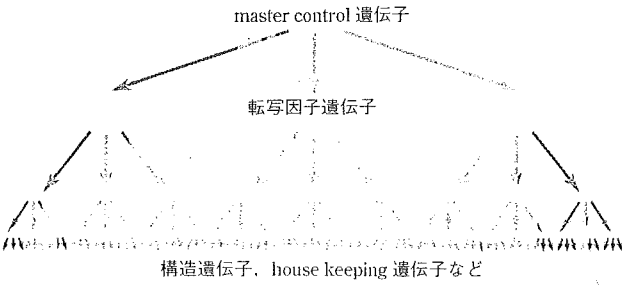

図 4　形態形成遺伝子カスケード

脊椎動物，軟体動物の眼や昆虫の複眼だけではなく，プラナリアの原始的な眼や線虫の光感受性細胞にも存在していることが明らかになった．ほとんどすべての動物の

眼発生に*Pax6*が存在し，しかもその塩基配列が高度に保存されていたことは，眼の起源に関する考えに大きな転換をもたらした．動物には種によって複眼，鏡眼，カメラ眼などさまざまな構造の眼があり，かつては40～60系統の眼が別個に発生したものと考えられていた．しかし，master control遺伝子*Pax6*が共通に存在することは，眼が原始の動物で光を感じる細胞としてただ1度だけ発生し，進化とともに多彩な眼を作るようになったことを意味している[3]．

III 高等動物における*Pax6*の発現とヒト疾患における変異

マウスやヒトにおける*in situ*ハイブリダイゼーションや免疫染色で示されたPax6の発現様式は，発生初期にまず中枢神経や眼原基に現れ，中枢神経では前脳，後脳，神経管脳室腹側，下垂体，嗅脳に発現し，眼ではまず視溝，ついで眼胞，表面外胚葉と水晶体板，網膜，角膜の順に眼球ほぼ全体を網羅している（図5）[4]．以上から，Pax6は高等生物においても眼発生のさまざまな場面に関わっていることが判明した．言い換えれば，太古に光を感ずる細胞から出発した遺伝子が，眼形態形成の中心に居続けて角膜，虹彩，水晶体，網膜など複雑な眼の形成に関わるようになったと考えられる．また，この遺伝子に変異が起こればきわめて多くの先天形成異常を起こすことが推察された．

事実，先に述べたように，先天無虹彩では多くの変異が見出された．さらにPeters奇形のような前眼部形成不全[5]，瞳孔形成異常[6]，家族性角膜ジストロフィ[7]，先天白内障[8]，黄斑低形成[9]，視神経形成異常[10]で変異が見つかっている（図6）．したがって，*PAX6*はヒトでも前眼部から眼底における広い範囲の形成を行っていることが，分子遺伝学からも証明された．

これまでに見つかった*PAX6*の変異型と表現型の間には遺伝子の変異が重篤なほど表現型も重症であるという法則がある．これは*PAX6*に，(1) 一対の対立遺伝子の両方とも揃っていないと正常に機能しない（haploinsufficiency），(2) 遺伝子障害の程度と表現型が相関する（dose dependent），という特徴があるためである．両側の対立遺伝子に変異（homozygous）があれば眼球が

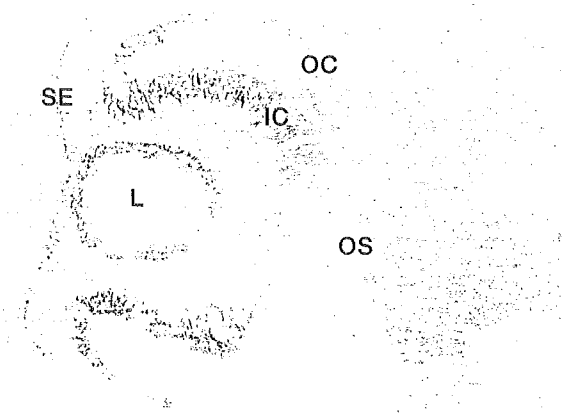

図5 Pax6のモノクローナル抗体による発生ヒト眼（胎齢5週）の免疫染色
表層外胚葉（SE），水晶体胞（L），眼杯（内板IC，外板OC），視茎（OS）と，外胚葉を主体に眼球全体が染まる．

形成されず，重篤な中枢形成異常がある．片側の対立遺伝子の変異（heterozygous）でもstop codonを起こすnonsense変異であれば，その蛋白は機能しない．先天無虹彩で見つかる変異の大部分はnonsense変異であり，その表現型は無虹彩のみならず角膜混濁，白内障，黄斑・視神経低形成など全眼球に及ぶ．一方，1つのアミノ酸だけが置換されるmissense変異では，前眼部形成不全，白内障，黄斑低形成などが，眼球の一部で単独に起こることが多い．

しかし，遺伝子型と表現型の間には相関は一般にみられない．しかも，家族例あるいは孤発例同士で同一の変異をもっていても，臨床像は多彩であった．染色体異常があり，片側alleleがすべて欠損していても，軽度のmissense変異であっても同様に無虹彩症を起こしていた．これは，PAX6が角膜，虹彩，水晶体，網膜の発生において運命づけには重要であっても，個々の組織の細かい形成過程は下流の遺伝子が担っているからである．Pax6/PAX6が下流に従える遺伝子は，後に述べるいくつかの転写因子，クリスタリン（CRYAA，CRYAB，CRYD，CRYZの発現を亢進，CRYBB1を抑制）[11～16]，ロドプシン[17]くらいしかまだ知られていないが，その数は膨大と推測される（図4）．しかも時間や場所を違えても，発生期を通じ多くの組織で発現するので，これら下流遺伝子のわずかな発現様式の差や，細胞内環境や

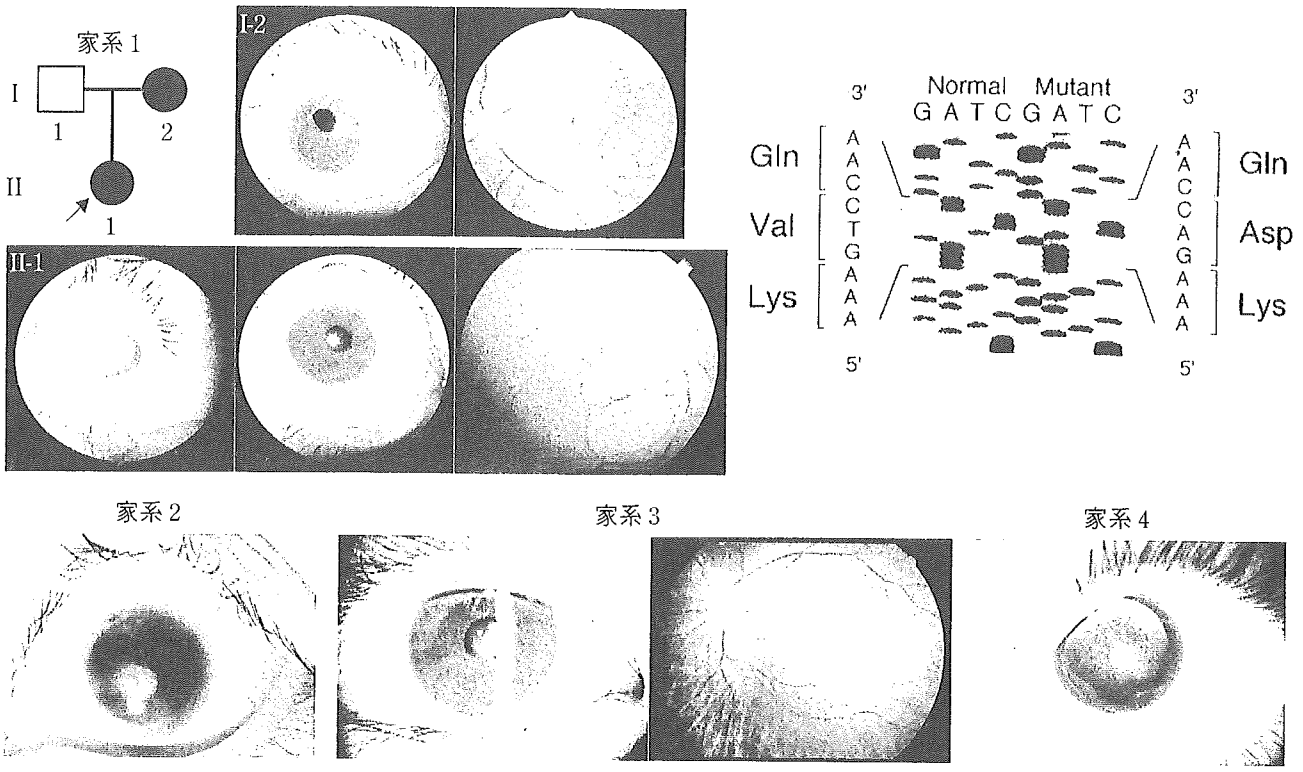

図 6　*PAX6*遺伝子の同一変異（exon 5a 内）による多彩な表現型

家系1の発端者は右眼前眼部形成不全，左眼角膜輪部形成不全と黄斑低形成，その母親は先天白内障術後の無水晶体と黄斑低形成，家系2は前眼部形成不全と白内障，家系3はAxenfelt異常と黄斑低形成，家系4は先天白内障が両眼にみられた．

co-factorの影響によって表現型に違いが起こると考えられる．同一変異をもつ家族間で表現型が異なる一方で，一個人では左右眼の所見に差が少ないこともこれで説明できる．

IV　前眼部を形成する*EYA1*遺伝子

最近，ショウジョウバエにおいて*eya*（*eyes absent*）に，*so*（*sine oculis*），*dac*（*dachshund*）などの眼形成遺伝子が見つかった．これらは*eyeless*（*Pax6*）の支配下（下流）にあり，いずれもtarget expressionすると*Pax6*ほどではないが小さい異所性の複眼ができる．眼形成の準マスターコントロール遺伝子と言えるものである．このうち，*eya*蛋白の機能はほとんどわかっていないが，apoptosisに関わると考えられている．その後，*eya*の相同遺伝子が哺乳類で見つかり，*Eya1*，*Eya2*，*Eya3*の3種に分かれてfamilyを形成し，*Eya3*はごく早期の眼に，

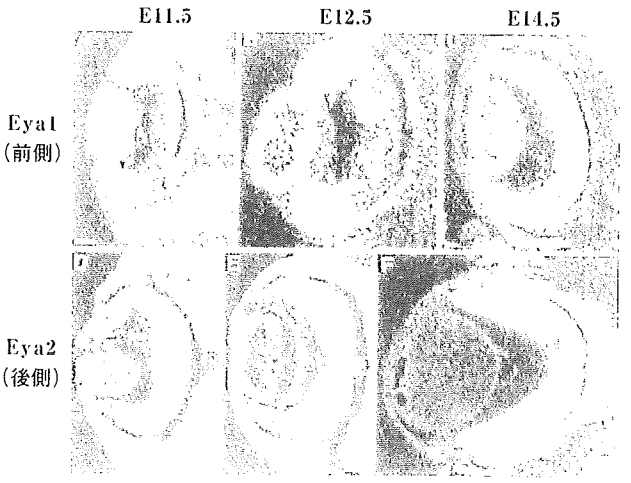

図 7　マウス胚におけるEya1, Eya2の発現（*in situ* hybridization）

眼球前方ではEya1が，後方ではEya2が発現し，眼球の前後軸を決定すると考えられる．（文献18より許可を得て転載）

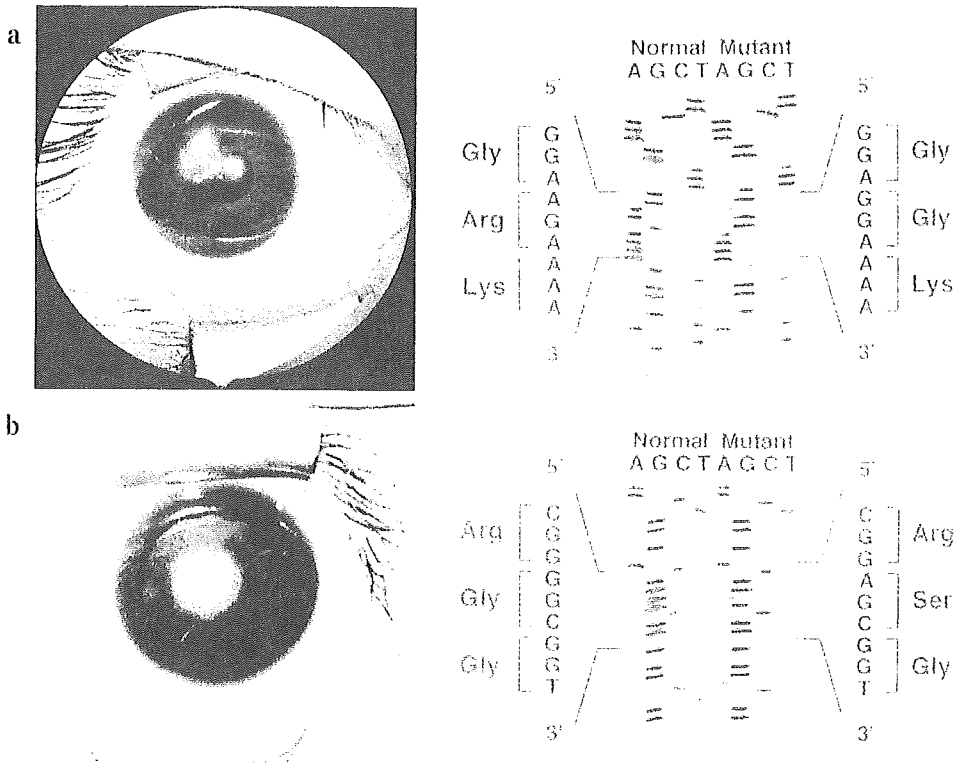

図 8　Peters 奇形 (a) と先天白内障 (b) にみられた *EYA1* 遺伝子変異 (missense)
b の症例は Branchio-oto-renal 症候群であり、先天白内障とともに左の先天嚢胞性腎形成不全，伝音性難聴，頸部瘻管を伴っていた．

Eya1 は眼球の前半に，*Eya2* は眼球の後半に発現することが判明した (図 7)[18]．*Eya1* は眼だけでなく腎や鰓弓の形成でも発現している．*EYA1* は，ヒトでまず Branchio-oto-renal 症候群 (第一鰓弓由来の頸部瘻孔と耳小骨形成不全による伝音性難聴，腎形成不全) の原因遺伝子として発見された．その後筆者らは，*EYA1* の変異によって先天白内障や前眼部形成異常で *EYA1* の変異を発見し，ヒトでも前眼部形成に関与していることを明らかにした (図 8)[19]．

V　*PITX/RIEG* 遺伝子

ショウジョウバエの卵の最も初期に体軸の前後を決めるために極性決定遺伝子が発現するが，その前部を決定する遺伝子に *bicoid* がある．マウスではこれと類似する遺伝子として *Pitx* が見出され，3 つの family (*Pitx1*, *Pitx2*, *Pitx3*) に分かれていることが判明し，ヒトでも同様 *PITX1*，*PITX2*，*PITX3* が見つかった．そして，Rieger 症候群の遺伝子が 4 番染色体長腕から positional cloning されたが[20]，これは *PITX2* であり，*PITX2/RIEG* と記載されるようになった．ついで虹彩低形成でも *PITX2/RIEG* の変異が発見され，さらに *PITX3* の変異によって先天白内障と前眼部異形成が起こることが報告された[21]．この遺伝子群は前眼部形成を行っているらしい．

VI　水晶体の形成遺伝子 *L-Maf*

Maf family はレンズ細胞特異的転写因子であり，水晶体の形成において重要な役割を果たしている．近年，ニワトリおよびアフリカツメガエルにおいて Large Maf (*L-Maf*) が水晶体形成の master control 遺伝子であることが判明した[22]．*L-Maf* は *Pax6* の下流に存在しており，これを培養細胞 (*in vitro*) や鶏初期胚の表層外胚葉 (*in vivo*) に導入すると，クリスタリンなどの水晶体特

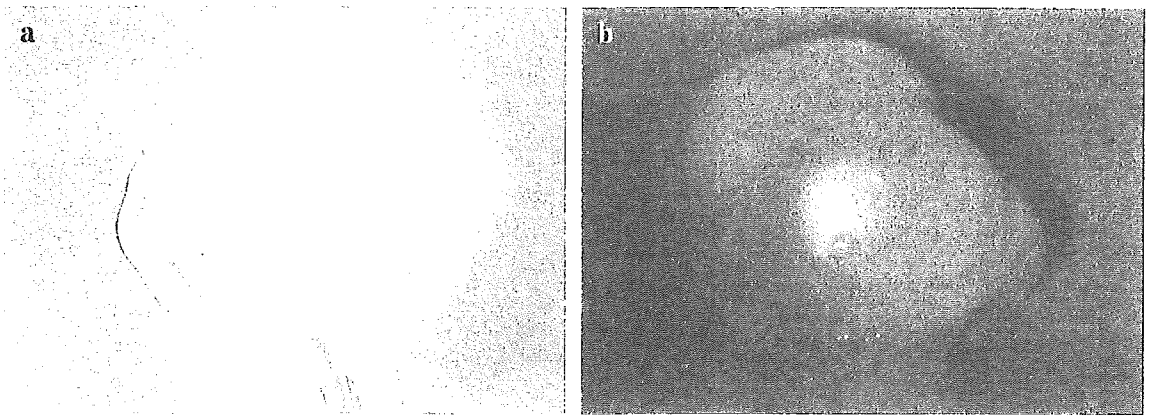

図 9　*L-Maf* 導入による異所性水晶体形成（stage14 に導入，stage28 の所見）
a：頭部の透明な異所性水晶体組織，b：同時に導入した GFP の発色により，*L-Maf* の発現部位がわかる．

異蛋白を発現し，*in vivo* で *L-Maf* の発現を抑制するとこれら水晶体特異蛋白の発現，ひいては水晶体の形成が抑制される．*L-Maf* は表面外胚葉が水晶体板から水晶体胞となって眼杯に向かって陥入する時期に発現して水晶体形成の場を決定し，他の Maf family 発現を誘導する．さらにその後は水晶体線維の形成にも関わり，種々のクリスタリン（CRYD，CRYAA，CRYBB1）や水晶体線維特異蛋白の発現を誘導する[23]．

L-Maf の相同遺伝子はマウス，ヒトにおいて *MafA/MAFA* として同定された．MAF family の変異が先天白内障の原因になることは指摘されているが[24]，*L-Maf/MafA* のヒト水晶体発生および疾患に変異はまだ明らかにされていない．

VII　水晶体特異蛋白の遺伝子

細胞の構造遺伝子としては，水晶体特異蛋白である種々のクリスタリン（CRYAA[25]，CRYBA3/A1[26]，CRYAB[27]，CRYBB2[28]，CRYG[29]，CRYGD[30]），細胞間 gap junction channel 蛋白（Connexin 46[31]，Connexin 50[32]），細胞構成蛋白（beaded filament protein gene BFSP2[33]，MIP[34]），水晶体上皮アクアポリン[35] の変異によって先天白内障が起こることが次々に報告されている．これら構造蛋白は水晶体発生においては最終産物と言えるものであり，水晶体に特異的に存在するので，表現型は白内障のみであることが多い．興味深いことに，これらクリスタリン遺伝子を中心とした構造蛋白遺伝子の変異では，白内障混濁の形態に特徴があるとの報告が多い［posterior polar cataract（CRYAB）；zonular cataract（CRYβA3/A1）；cerulean cataracts（CRYGD）sutural cataracts（CRYG）；pulverulent cataract（Connexin 50）］．これらはまだ症例数が少ないので正確な相関はわからないが，クリスタリンは発生期に応じて発現するタイプが異なるので，水晶体線維の成長や層形成に伴って特異な部位に混濁が起こることが考えられる．かつて J. Francois や S. Duke-Elder の眼科教科書には先天白内障の形態分類が詳細に記載されていた．これらは一部を除いて顧みられることが少なくなっていたが，ふたたび脚光を浴びることになるかもしれない．

これらの遺伝子変異は最初にヒトで発見された例もあるが，マウスなどの動物で眼と遺伝子の異常が見つかり，ついでヒトでも疾患を起こすことが明らかになったものも多い[36]．

最近，マウス DNA 分解酵素の 1 種がマウスの水晶体細胞で特異的に発現し，この欠損マウスでは核白内障が生じることが報告された[37]．したがって，この DNA 分解酵素の変異がヒトでも先天白内障を起こす可能性がある．

VIII　原因遺伝子の発見による疾患概念の変化

これまでに述べてきたように，眼形成には Pax6 を頂点とする遺伝子カスケードが存在し，角膜を作る遺伝子群，水晶体を作る遺伝子群，網膜を作る遺伝子群などに

分かれていると考えられる．眼の形成に関わる遺伝子は膨大な数にのぼり，ショウジョウバエのような下等動物でも1,000を越えると試算されている[37]．水晶体形成カスケードは，*Pax6*が最上流に位置し，その下で*Eya*，*Pitx*や*L-Maf*が働き，最下流にクリスタリンなどの構造蛋白をコードする遺伝子が位置すると大まかながら推測されるが，この上下にも数多くの遺伝子が関与していると考えられる（図4）．このカスケード内の遺伝子のどれに変異があっても先天白内障が起こる可能性がある．言い換えれば，非常に多くの遺伝子が先天白内障の原因遺伝子として記載されるようになるであろう．今の技術では遺伝子変異のスクリーニングには膨大な労力を要し，また疾患概念の混乱にも通じかねない．網膜色素変性症では，原因遺伝子（おもに網膜視細胞の酵素や構造蛋白）が多数見つかり，一方，同一遺伝子で白点状網膜炎のような異なる疾患が起こるなど，疾患概念の混乱が言われている．ネットワークの複雑さから考えて，発生に由来する疾患の概念の混乱ははるかに深刻である．

ただ，水晶体形成の遺伝子カスケードの位置によってある程度の違いはある．*Pax6*の変異では，その支配領域から考えて，眼のあらゆる部位で形成異常が起こり，表現型が白内障単独であることは稀である．*Eya1*，*Pitx*は前眼部の形成に関わるので，その変異では白内障とともに角膜，虹彩の異常を伴う．さらに，これまで見てきたように，転写因子など上流の遺伝子はまったくタイプの異なる臓器でも働くので（*Pax6*は中枢，Eya1は鰓弓，腎），その変異は多臓器障害をもつ症候群を起こすことがある．しかし，クリスタリンなどの水晶体構造蛋白遺伝子の変異では，ほとんどが白内障のみを起こす．

遺伝子のほうから疾患の再分類を行おうとするのは，混乱を招くだけで頴けない．また，先天異常の多くは遺伝病でなく，感染や炎症，中毒，環境の変化などによって起こることも注意すべきである．これまでに確立された疾患単位が臨床経過，合併症，治療方針の面で明確であることを考えれば，これを遵守し遺伝子の異常はその原因として検索し併記すべきものと考える．

IX　遺伝子治療，再生医学への応用

現在臨床に応用できるのはまず遺伝相談である．原因遺伝子の候補が多数あるので，検索には労を要し，情報の告知や利用には注意が必要であるが，患者や家族が希望する場合は疾患の原因に関する有用な情報を得ることができる．

先天異常の遺伝子治療はきわめて困難である．ジストロフィで試みられているような正常（野生型）遺伝子の補充は，根本的治療にならない．白内障で補充療法ができたとしても，混濁が軽いか強いかの違いが起こるくらいで，手術が必要になるのであれば何にもならない．正常遺伝子を導入すると，本来存在する変異遺伝子を相対的に押さえることもあり，かえって変異遺伝子が強調されたり，他の遺伝子に影響を与えるような予想外の動きをすることも考えられる．変異遺伝子をそっくり正常遺伝子に置換する方法として，相同組み換え法があるが，組み換え効率が低いので今のところ非現実的である．一方，点突然変異では，変異部位を正常構造に置換する方法（DNA-RNAキメラオリヌクレオチド法）が近年報告された[38]．この方法は，修復させるべき塩基配列と，その前後にDNAとRNAを混在させたDNA-RNAキメラオリヌクレオチドを作製し，これを変異DNAに結合させると，DNAミスマッチ修復酵素によって，点変異が正常塩基に置換されるというものである．これによって，*in vitro*ではあるが，鎌状貧血の遺伝子校正に成功している．この遺伝子修復効率はきわめて高く，哺乳類細胞で30％にも達する．この方法の開発によって，先天異常の遺伝子治療が，概念的ではあっても可能となった．

むしろ，水晶体の形成に関わる遺伝子は再生医学へ応用されるほうが早いかもしれない．未分化幹細胞に遺伝子を適切に発現させれば，臓器ないしは組織の再生が可能となる．オートメーション工場のスイッチを押すようなもので，工場（細胞）の環境が良ければ，かなりの行程を進めることができる，自己細胞から組織を作れば拒絶反応も起こらない．*Pax6*遺伝子の異所導入によってアフリカツメガエルのオタマジャクシでは不完全な眼を作ることができ，その中には構造が乱れているが水晶体も存在している[39]．しかし，*Pax6*で水晶体を単独に作成した報告はまだない．*L-Maf*を用いれば，鶏胚組織に異所水晶体組織を作成することができる[15]．筆者らは*L-Maf*を鶏胚頭部に導入し，異所性の透明水晶体組織