Midkine (MK), the product of a retinoic acid responsive gene, and pleiotrophin constitute a new protein family regulating growth and differentiation

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ABSTRACT Using mouse teratocarcinoma system, we found a novel retinoic acid responsive gene. Midkine (MK), the product of the gene is a secreted, heparin-binding protein of molecular weight 14,000. MK gene is intensely expressed in the midgestation period, and in the adult mouse, the kidney is the principal site of its expression. MK and pleiotrophin have 50% sequence identity and constitute a new protein family regulating growth and differentiation. They share neurite outgrowth activity; other activities, either specific for one or common to both, have been reported. Furthermore, MK is of significant interest in cancer biology.

KEY WORDS: *embryonal carcinoma cells, growth differentiation factor, heparin-binding protein, midkine (MK), neurite outgrowth, pleiotrophin (PTN)*

Introduction

Retinoic acid, a metabolite of Vitamin A, exerts profound effects on differentiation and development. As an example, retinoic acid mimics the action of morphogen in limb development, and local administration of retinoic acid produces duplication in the finger pattern (Tickle *et al.*, 1982). Furthermore, retinoic acid produces abnormalities in the development of the central nervous system along the anterior-posterior axis (Durston *et al.*, 1989). Retinoic acid is also known to induce differentiation of certain cancer cells (Strickland and Mahdavi, 1978). Retinoic acid receptors belong to the nuclear receptor superfamily (Petkovich *et al.*, 1987), and retinoic acid complexed with the receptor binds to the control region of genes, some of which are thought to be important in the regulation of differentiation and development. In order to discover one such «control gene», we used the retinoic acid-induced differentiation system of embryonal carcinoma (EC) cells, which are stem cells of teratocarcinoma resembling the multipotential cells of early embryos (Matin, 1980). Midkine (MK) thus discovered is a low molecular weight heparin-binding protein with neurotrophic activity (Kadomatsu *et al.*, 1988; Tomomura *et al.*, 1990a,b; Muramatsu and Muramatsu, 1991). Although fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) are heparin-binding growth factors, MK is not structurally related to any of them. MK is the first member of a new family of proteins thought to be important in the control of development.

Isolation of MK cDNA

MK cDNA was isolated from HM-1 EC cell line, which was established from a spontaneous testicular teratocarcinoma of 129 mouse (10). When aggregates of HM-1 cells were treated with 10^{-6} M retinoic acid for 2 days, cells started to differentiate, and 8 days later, fully differentiated cells such as nerve cells, myoblasts and extraembryonic endoderm cells were produced. When HM-1 cells were sparsely cultured and continuously treated with 10^{-6} M retinoic acid, cells differentiated only into myoblasts; the full differentiation along this line takes about 14 days. We used the latter differentiation conditions to clone a cDNA whose expression increased after induction of differentiation. A cDNA library was constructed from cells treated with retinoic acid for 1 day, and a clone named MK-1 was isolated by differential hybridization (Kadomatsu *et al.*, 1988). The clone was so named because the message was strongly expressed in the midgestation period of mouse embryogenesis, and in the adult mouse, the kidney was the principal site of its expression.

Abbreviations used in this paper: EC, embryonal carcinoma cells; MK, midkine; PTN, pleio-trophin; RIHB, retinoic acid-induced heparin binding protein.

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0214-6282/93/$03.00

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  1  Met Gln His Arg Gly Phe Phe Leu Leu Ala Leu Leu Ala Leu Leu Val Val Thr Ser Ala
 21  Val Ala Lys Lys Lys Glu Lys Val Lys Lys Gly Ser Glu Cys Ser Glu Trp Thr Trp Gly
 41  Pro Cys Thr Pro Ser Ser Lys Asp Cys Gly Met Gly Phe Arg Glu Gly Thr Cys Gly Ala
 61  Gln Thr Gln Arg Val His Cys Lys Val Pro Cys Asn Trp Lys Lys Glu Phe Gly Ala Asp
 81  Cys Lys Tyr Lys Phe Glu Ser Trp Gly Ala Cys Asp Gly Ser Thr Gly Thr Lys Ala Arg
101  Gln Gly Thr Leu Lys Lys Ala Arg Tyr Asn Ala Gln Cys Gln Glu Thr Ile Arg Val Thr
121  Lys Pro Cys Thr Ser Lys Thr Lys Ser Lys Thr Lys Ala Lys Lys Gly Lys Gly Lys Asp
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Fig. 1. Amino acid sequence of mouse MK. *Signal sequence was shown by a dashed line. Cysteine, lysine residues and other basic amino acids were encircled, doubly underlined and singly underlined, respectively (based on Tomomura et al., 1990a).*

Structure of MK cDNA and its protein product

Upon continued survey of the cDNA library, we found heterogeneity in MK cDNAs (Tomomura *et al.*, 1990a). So far, we have identified three types of MK cDNAs, MK1, MK2 and MK3. They share a common coding sequence, and have distinct 5' untranslated sequences. Primer extension and ribonuclease protection revealed that MK2 was the major retinoic acid-inducible MK mRNA. There was another heterogeneity in an oligo-A stretch in the 5' region. This heterogeneity affected the length of the open reading frame. In the majority of cases, where the number of A was 9, the deduced protein product had a molecular weight of 17,000 with a typical signal sequence. When the number of A was 11 or 10, the open reading frame was truncated to yield a protein of MW 10,000. The fundamental structural features are conserved in the two forms of putative MK polypeptide. The reason for this heterogeneity is not clear. Although a likely explanation is an erroneous action of the reverse transcriptase used to construct the cDNA library, the possibility should not be excluded that by a novel mechanism of RNA editing, minor MK RNA species are produced *in vivo* leading to production of truncated cytoplasmic forms of MK. Since the number of A residues was 9 in the genomic sequence (Matsubara *et al.*, 1990), we further studied MK2 cDNA with 9 A residues.

The structure of MK protein was not related to any other proteins at the time of publication (Kadomatsu *et al.*, 1988; Tomomura *et al.*, 1990a), and has distinctive characteristics; they are unusually rich both in cysteine and basic amino acids (Fig. 1). From the rule of signal peptide cleavage, the mature MK polypeptide was predicted to have a molecular weight of 13,000. When MK2 mRNA was translated by rabbit reticulocyte lysate in the presence of dog pancreatic microsomes, MK protein was processed and a product of MW 13,000 entered the microsomal lumen (Tomomura *et al.*, 1990a). The experiment verified that the putative signal sequence indeed worked.

The *in vitro* transcribed MK polypeptide tightly bound to heparin (Tomomura *et al.*, 1990b). This property of MK protein was used to identify it in the culture medium of differentiating HM-1 cells. A band with a molecular weight of 13,000 was detected in the culture medium of these cells by heparin affinity column chromatography. That the band was MK protein was confirmed by immunoprecipitation using anti-MK antibody raised to a synthetic MK peptide (Tomomura *et al.*, 1990b). As above, MK protein was a low molecular weight, secreted protein with heparin-binding activity. This property of MK protein is reminiscent of a growth factor. Thus, we proceeded on the hypothesis that MK protein is a novel growth factor.

MK activity

To test biological activity, recombinant MK protein was necessary. MK2 cDNA was placed under the ß-actin promoter and RSV enhancer, then transfected into L cells. The cell line thus established continuously secreted MK protein (Tomomura *et al.*, 1990b). Addition of heparin to the culture medium was required for efficient MK recovery. The conditioned medium containing MK promoted growth of PC12 pheochromocytoma cells, when examined using the conditioned medium of parental L cells as a control (Tomomura *et al.*, 1990b). MK protein was purified to homogeneity by SP-Sephadex column chromatography, and heparin agarose column chromatography (Muramatsu and Muramatsu, 1991). When the purified MK protein was sequenced, most of it yielded the MK sequence starting from lysine 23 in Fig. 1 (Muramatsu, H., unpublished results). These results confirmed the deduced signal peptide cleavage site. Purified MK protein promoted the growth of 3T3 cells, although the effect was much weaker as compared with FGF (Muramatsu and Muramatsu, 1991). When the dissociated embryonic brain cells were cultured on MK coated plastic dishes, cells vigorously extended neurites (9), (Fig. 2). One question is whether MK simply increased the cell-substratum adhesion of embryonic brain cells. The dissociated single cells extended neurites and survived better on MK-coated dishes than on synthetic poly-L-lysine coated dishes (Muramatsu, H., unpublished results). Thus, we have concluded that MK has neurotrophic activity. Recently, Dr. H. Maruta produced recombinant MK in *E. coli*. This MK suppressed growth of HL-60 cells at 50 ng/ml level (Maruta *et al.*, submitted for publication).

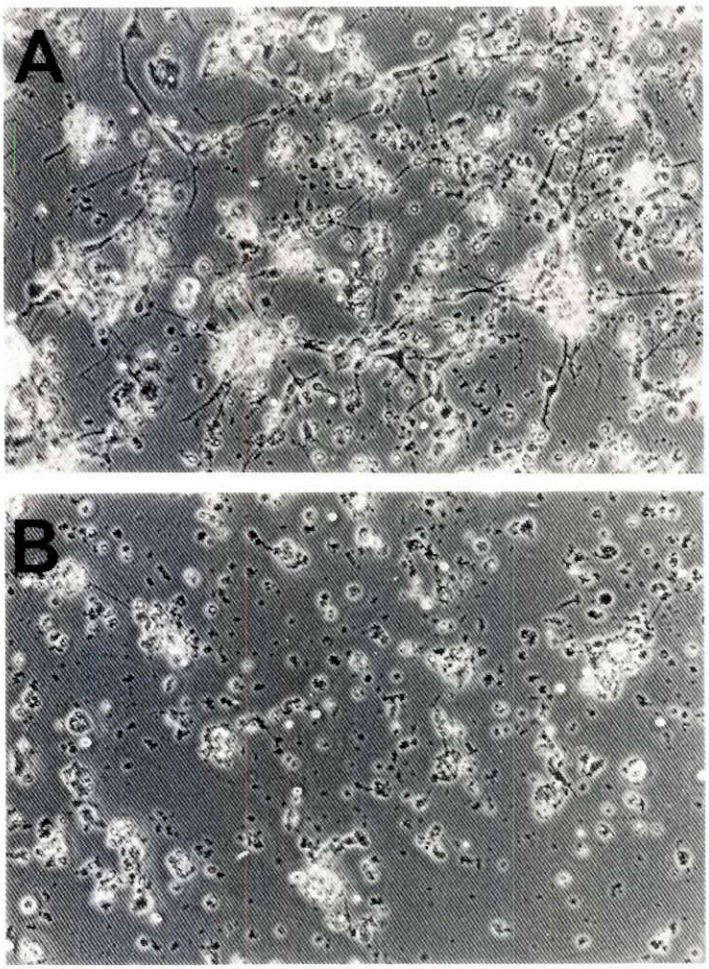

Fig. 2. Neurite extension of embryonic brain cells by MK. **(A)** *Cells were cultured on an MK-coated dish for 48 h.* **(B)** *Cells were cultured on an uncoated dish for 48 h (based on Muramatsu and Muramatsu, 1991).*

From these results, it is proper to conclude that MK can be classified as a growth differentiation factor with various functions. On the basis of these findings, MK protein was named "midkine" (it is expressed in the *mid*gestation period and *ki*dney, and is probably a cyto*kine* in the *mid*dle of the differentiation pathway triggered by retinoic acid), and the abbreviation MK (midkine) was retained.

Developmentally controlled expression of MK

The mode of developmentally controlled expression of MK has been extensively studied in mouse embryos using Northern blots, slot blots and *in situ* hybridization (Kadomatsu *et al.*, 1988, 1990). While on day 5 of gestation MK was not detected, strong expression of MK was noted in all germ layers of the embryo proper on day 7. On day 9, the strong expression continued all over the embryo. On day 11, and thereafter, MK expression gradually decreased and became restricted to specific regions. The MK message was strong in the nervous tissues, epithelial tissues where epithelial mesenchymal interactions occur (i.e. those in the small intestine, stomach, pancreas and lung), mesodermal tissues undergoing remodeling (the jaw, vertebrae, and limbs) and the kidney (Kadomatsu *et al.*, 1990). In addition, the anterior lobe of the hypophysis and the retina were intensely positive. These modes of MK expression are interesting for two aspects. 1) MK is often expressed in one of two interacting layers (i.e. epithelial tissues interacting with mesenchymal tissues, anterior lobe of hypophysis interacting with the posterior lobe, and retina interacting with lens layer). 2) MK is highly expressed where teratogenic effect of retinoic acid is observed; i.e. the brain, limbs and jaw. On the 15th day of gestation, the kidney was the only site where an intense MK message was detected.

While the message was quite strong in the brain on 11-13th day of gestation, a weak signal was detected in the brain of later stage embryos (Nakamoto *et al.*, submitted for publication). In the neonatal period, this message increased slightly, then again decreased. In the adult mice, the kidney continued to express MK significantly. In addition, considerable expression was observed in the ovary (Huang *et al.*, 1990).

The mode of MK gene expression was also extensively studied in the retinoic acid-induced differentiation system of HM-1 embryonal carcinoma cells (Huang *et al.*, 1990). The results can be summarized as follows. 1) MK expression became maximum at 48 h after initial exposure to retinoic acid, then rapidly decreased, both in monolayers and in cell aggregates. 2) The continued presence of retinoic acid was required for maximum induction by retinoic acid. 3) The intensity of MK message correlated with retinoic acid concentration, especially between 10^{-8} - 10^{-7} M. 4) Cycloheximide, an inhibitor of protein synthesis, partly inhibited MK induction (Tomomura *et al.*, 1990a).

MK gene

Southern blot analysis of Hind III digest of mouse genomic DNA revealed two bands of 3 kb and 4 kb, which reacted with MK cDNA probe (Matsubara *et al.*, 1990). Both of the genomic DNA fragments were cloned, and the sequence was analyzed. The 4 kb fragment contained all the sequences found in MK cDNA, while the 3 kb one had sequences closely related to but distinct from MK. Thus, we concluded that the 4 kb Hind III fragment contained MK gene. The structure of MK gene is schematically shown in Fig. 3. The coding sequence was divided into 4 exons. The 5' non-coding sequences specific for each MK cDNA were arranged in the order of MK3, MK2 and MK1 (Matsubara *et al.*, 1990).

Chromosomal localization of MK gene was determined using interspecific hybrid mice, using restriction fragment polymorphism of MK gene between the two mouse species (Simon-Chazottes *et al.*, 1992). MK gene (officially named as *Mdk*) was located in chromosome 2, near *Surf* and *Hox 4-2*. The order of arrangement was Surf-17.24±4.96 cM-*Mdk*-1.82±1.80 cM-*Hox*-4.2. It is interesting to note that expression of Hox-4 series is affected in case of retinoic acid-induced alteration of limb morphogenesis in the chicken (Nohno *et al.*, 1991).

Since MK2 is the major, retinoic acid inducible MK cDNA, we studied 5' upstream region of the MK2-specific region in order to understand the mechanism of retinoic acid-induced expression of MK2. There was no TATA box in the vicinity of transcription initiation site, but several GC boxes were present. When the 2 kb 5' upstream region of MK2-specific sequence was fused with CAT gene and transfected into F9 EC cells, retinoic acid-induced activation of CAT activity was observed (Matsubara *et al.*, unpublished results). We are currently trying to locate the retinoic acid responsive sequence in the 2 kb fragment.

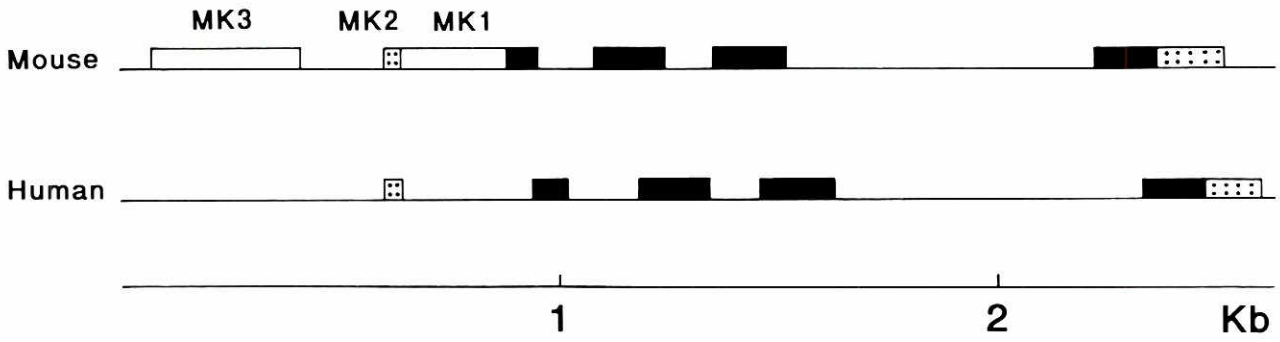

Fig. 3. Organization of MK genetic region in the mouse and human. ■ *Coding exons;* ⊡ *non-coding exons in MK2 mRNA;* ❒ *non-coding exons in MK1 and MK3 mRNA (based on Matsubara et al., 1990; Uehara et al., 1992).*

When the same 2 kb fragment was fused with bacterial β-galactosidase and introduced into fertilized mouse eggs, the mode of β-galactosidase expression was generally similar to that of MK: the transgene was not expressed in the early egg cylinder stage, but was intensely expressed in the somite stage (Kaname *et al.*, unpublished results). The expression then became restricted, and in the adult mice, only the kidney continued significant expression. Thus, the 2 kb fragment also contains most of the elements that dictate the developmentally controlled expression of MK gene. Upon closer examination, there were some differences between β-galactosidase expression and MK gene expression, and the details are under investigation.

Human MK

We studied human MK for 2 reasons. First, we wished to know how MK cDNAs and the gene are conserved between 2 mammalian species, the human and mouse. Second, we intended to use a human MK cDNA probe to examine its possible abnormal expression in certain diseases.

Human MK cDNA was isolated from human fetal kidney cDNA library using mouse MK cDNA as a probe (Tsutsui *et al.*, 1991). At the amino acid level, human and mouse MK were 83% identical, and most amino acid changes were conservative. All the characteristic cysteine residues were conserved. This high degree of evolutional conservation reinforces the important role of MK during embryogenesis.

Human MK gene was isolated using a human MK cDNA as a probe (Uehara *et al.*, 1992). The organization of human MK gene was quite similar to that of mouse MK gene (Fig. 3). Furthermore, all exon-intron boundaries were conserved between the mouse and human MK. When the 5' upstream sequence of MK2 was compared, we found 4 blocks composed of 100-200 nucleotides, which have a high degree of sequence homology between the human and the mouse. These homologous blocks should play important roles in developmentally regulated expression of MK gene.

We then examined MK gene expression in human carcinomas and found that in all 7 cases of Wilms tumor, a kind of kidney carcinoma, MK gene was intensely expressed (Tsutsui *et al.*, unpublished results). The level of expression was much higher than that in the normal kidney and in other kidney carcinomas. High levels of MK expression were also experienced frequently in lung, colon and pancreatic carcinomas (Tsutsui *et al.*, unpublished results). It should be noted that during the midgestation period, MK gene is expressed in epithelial tissues of these organs. Thus, MK is of interest from the view point of cancer etiology and diagnosis.

The MK family

At the end of 1990, the sequence of a protein with strong homology to MK was reported from 5 different laboratories (Kovesdi *et al.*, 1990; Kuo *et al.*, 1990; Li *et al.*, 1990; Merenmies and Rauvala, 1990; Tesuka *et al.*, 1990). This protein has several names, i.e. heparin-binding growth associated molecule (HB-GAM) (Merenmies and Rauvala, 1990), pleiotrophin (PTN) (Li *et al.*, 1990), OSF-1 (Tezuka *et al.*, 1990), heparin-binding neurotrophic factor (HBNF) (Kovesdi *et al.*, 1990) or P18 (Kuo *et al.*, 1990). Here, the term PTN is adopted, because of simplicity. PTN was initially identified as a heparin-binding protein with neurite extension activity (Rauvala, 1989), or mitogenic activity (Milner *et al.*, 1989), or as a protein expressed in osteoblasts (Tezuka *et al.*, 1990). PTN structure is highly conserved between species. Human and mouse PTN are more than 99% identical as regards amino acid level. MK and PTN have about 50% sequence identity at the amino acid level, and all cysteine residues are conserved (Fig. 4). Thus, MK is the first member of a new family of proteins which we shall call the MK family. The functions of MK and PTN so far disclosed are similar. Recently, a protein that may be identical to PTN has been reported to have angiogenic activity (Courty *et al.*, 1991). It is not known whether MK has angiogenic activity.

The mode of developmental control of PTN expression is entirely different from that of MK. In the head/brain region, PTN was only weakly expressed between 12-15 days of gestation in the mouse (Nakamoto *et al.*, submitted for publication). The level of expression was lower in later embryos, and after birth, PTN expression was strongly induced in the brain. Thus, the mode of expression of MK and PTN can be described as redundant but reciprocal during brain development. Why two closely related but distinct proteins with similar functions are expressed in an entirely different manner during development is a challenging question.

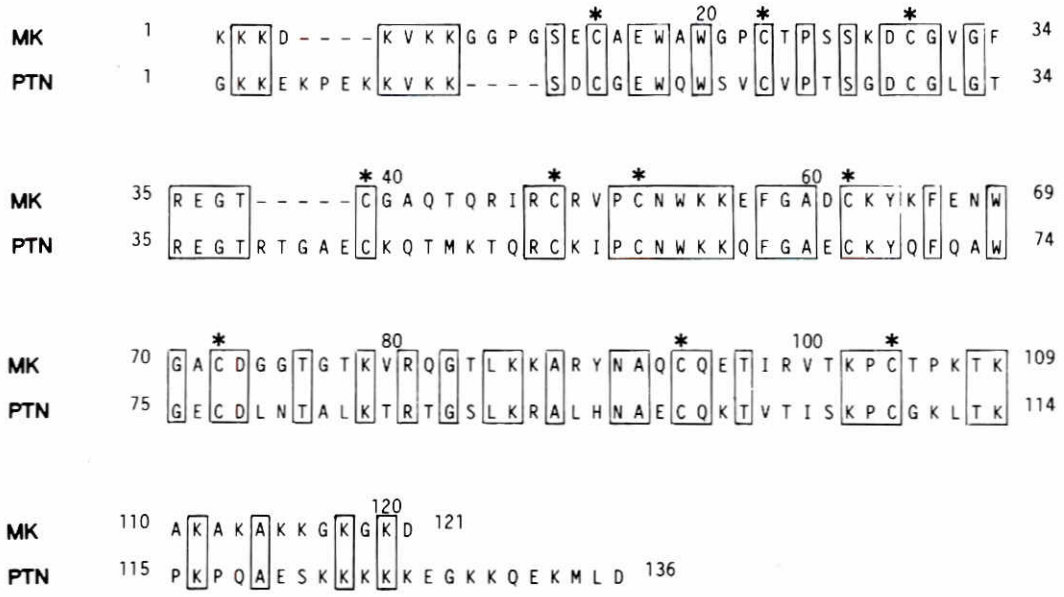

Fig. 4. Comparison of human MK and human PTN amino acid sequences *(based on Li et al., 1990; Tsutsui et al., 1991).*

At the beginning of 1991, another protein called RIHB (retinoic acid-induced heparin-binding protein) isolated from the chicken embryo was shown to have 65% sequence identity with MK (Urios *et al.*, 1991). RIHB was initially found as a heparin-binding protein in the chicken embryo (Vigny *et al.*, 1989). Because of retinoic acid-induced expression, RIHB may be the chicken homolog of MK. If so several embryological experiments, such as limb development and neural crest migration, could be more easily performed using the chicken system.

That MK and PTN form a family has accelerated research in this field, since a finding for one member of the family can be tested in the other member. It is also of interest to determine whether the MK family consists of only two members. The 3 kb MK-related gene found in mouse genome (Matsubara *et al.*, 1990) coded for a protein sequence different from MK and PTN (Obama *et al.*, unpublished results). However, it remains to be elucidated whether the 3 kb gene is expressed.

Comments

MK gene found in the mouse teratocarcinoma system is becoming an important molecule with which to resolve questions both in development and cancer. This is in line with the idea of Dr. G. Barry Pierce to use mouse teratocarcinoma as a system to resolve these questions. I am happy to be part of this tribute to Dr. G. Barry Pierce, who influenced my research both through his publications—especially the key one (Kleinsmith and Pierce, 1964)—and personally at international conferences. Many experiments need to be performed to clarify the role of MK during embryogenesis. We expect that homologous recombination, which is also developed from the teratocarcinoma system, will be a decisive experiment.

Acknowledgments

I thank Miss Kumiko Sato for her expert secretarial assistance. This work was supported by grants from the Ministry of Education, Science and Culture.

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