Expression profile of mouse *Mterfd2*, a novel component of the mitochondrial transcription termination factor (MTERF) family

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Mterfd2 is a component of mitochondria transcription termination factor (MTERF) family which belongs to the MTERF4 subfamily. In this report, we characterized the expression profile of mouse *Mterfd2* during embryogenesis by *in situ* hybridization (ISH), quantitative real-time PCR (qRT-PCR) and northern blot. The whole mount ISH at E9.5, E10.5 and E11.5 showed that *Mterfd2* was dynamically expressed in the brain. Besides, at E9.5 and E10.5 stages, *Mterfd2* was persistently expressed in the lateral plate mesoderm and heart; at E10.5 and E11.5 stages, it showed an abundant expression in the limb buds. The tissue ISH of E13.5 and E15.5 suggested that *Mterfd2* was ubiquitously expressed, and has the higher expression in the forebrain, diencephalon, midbrain, spinal cord, dorsal root ganglion, tongue, lung, liver and kidney. This ubiquitous expression profile in the late embryogenesis was further confirmed by qRT-PCR and northern blot at E12.5, E15.5 and E18.5 stages. Besides, the results of co-location of EGFP-*Mterfd2* fusion protein indicated that *Mterfd2* was targeted to the mitochondria. Collectively, these data suggested that *Mterfd2* showed a dynamic expression pattern during embryogenesis. It might play an important role in the organ differentiation which was probably resulted from its role in the mitochondrial transcription regulation.

Key words: *Mterfd2*, expression profile, *in situ* hybridization, northern blot, immunocytochemistry

INTRODUCTION

Mitochondrion is the metabolic control organelle in which oxidative phosphorylation takes place. Besides, it plays pleiotropic roles in numerous regulatory and developmental processes, such as apoptosis, senescence and so on (Chan, 2006; Kuo et al., 2009). Mitochondrial dysfunctions contribute to many neurological disorders and metabolic diseases, including Parkinson's disease, MELAS syndrome and diabetes (Mandemakers et al., 2007; Tan et al., 2009; Yoon et al., 2010). Thus, insights into the mitochondrial regulation would give light on the regulation in developmental process and probably on clinical therapy.

The mitochondrion is a semi-autonomic organelle which is widely accepted as developing from an α -proteobacterium

(Gray et al., 1999). The regulation of mitochondrial transcription level is dual which results from its genetic origin and is controlled at both initiation and termination level (Asin-Cayuela and Gustafsson, 2007; Scarpulla, 2008). Three proteins, the mitochondrial RNA polymerase (POLRMT), the mitochondrial transcription factor A (TFAM), and the mitochondrial transcription factor B1 or B2 (TFB1M or TFB2M) were reported to be sufficient to initiate mtDNA transcription *in vitro* (Falkenberg et al., 2002). However, the detailed mechanisms are not well understood despite that most of the components in mitochondrial initiation are well defined. Recent years, a consistent amount of studies have been focused on a nucleus encoded novel protein family-the mitochondrial transcription termination factors (MTERFs).

The MTERFs family is a wide spread protein family which is identified in metazoan and plants, which consists of 4 subfamilies named *MTERF1* through *MTERF4* (Linder et al., 2005). *MTERF1* (*mTERF*) is the founder

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and also the most well characterized as one of the MTERFs. *MTERF1* was firstly purified from human mitochondrial lysate and identified to be a mitochondrial transcription terminator. It promotes transcription termination by binding to a 28-base pair region within the tRNA^{Leu (UUR)} gene at the position immediately adjacent and downstream of the 16S rRNA gene (Kruse et al., 1989). Through bioinformatics tools, its paralogues like sea urchin *mtDBP*, *Drosophila DmTTF*, *Chlamydomonas reinhardtii Moc1*, *Mus musculus mTERF*, *Homo sapiens MTERF2* (*mterfd3*, *mTERFL*) and *MTERF3* (*mterfd1*) (Chen et al., 2005; Fernandez-Silva et al., 2001; Li et al., 2005; Roberti et al., 2003, 2006; Schonfeld et al., 2004) with similar structure were found, divided into four subfamilies, and then further characterized. The salient feature of proteins in MTERF family is the containing of variable number of “mterf” motifs of about 30 amino acids in length. This feature is already verified in several MTERF family proteins, for example, Hs-mTERF, Pl-mtDBP and Dm-DmTTF (Daga et al., 1993; Fernandez-Silva et al., 2001; Roberti et al., 2003). MTERF2 is found to be a nucleoid component in mammalian and binds to the heavy strand promoter (HSP) region of the mtDNA (Wenz et al., 2009). MTERF3 function as a negative regulator in mammalian mtDNA transcription (Park et al., 2007).

Retrospective studies of three other subfamilies mainly focused on their role in mitochondria transcription regulation. To date, little information of the gene expression profile was reported, especially on the embryonic stage. Moreover, barely any information was elucidated about MTERF4 subfamily despite the fact that a consistent amount of studies has been poured on other subfamilies. With the aim to shed light on the MTERF4 subfamily in mouse, we characterized the expression profile of mouse *Mterfd2* during development and its protein subcellular localization.

MATERIALS AND METHODS

Bioinformatics Protein architecture analysis of the “mTERF motifs” was carried out by taking advantage of the Web tool SMART in Normal mode (<http://smart.embl-heidelberg.de/>). Multiple alignments of mterf domains were performed with DNAMAN 6.0.40 Edition and subsequently processed with Adobe Illustrator CS2. Subcellular localization of Mm-Mterfd2 was predicted using MitoProt II- v1.101 (Claros and Vincens, 1996), TargetP (Emanuelsson et al., 2000), and PSORT (Nakai and Horton, 1999).

Animals and preparation of embryos BALB/cByJ male and female mice were obtained from Beijing Laboratory Animal Research Center (Silaike, Beijing, China). Four animals per cage were fed at the maximum

in a 12 h light/dark cycle controlled chamber (FengShi, Suzhou, China) at room temperature and supplied with commercial feed and tap water with a drop of HCl per litter. All the experiments conducted with accordance to the “Rules for experiments’ animals” published by Chinese Government (Beijing, China). The presence of a vaginal plug on the next morning was counted as 0.5 day post conception (E0.5).

RNA and first strand cDNA synthesis Total RNA was isolated from the embryos and organs according to the standard protocols of Trizol Reagent (Invitrogen, San Diego, USA). The cDNAs were synthesized using a SuperscriptTM III RNase H⁻Reverse Transcriptase kit (Invitrogen); all the experiment procedures were performed according to the manufacturer’s protocol.

In situ hybridization (ISH) For the whole mount ISH analysis, embryos were fixed in 4% paraformaldehyde with phosphate buffered saline (PBS) and incubated at 4°C overnight. For tissue ISH, the fixed embryos were dehydrated and subsequently drenched completely in xylene and eventually embedded in paraffin. Sections (10 µm) of the embryos were sliced with a microtome (YAMATO, RV-240, Japan) and then adhered on the slides filmed with polylysine. ISH was performed according to the standard protocol described previously (Breitschopf et al., 1992). Digoxigenin (DIG)-UTP labeled RNA probe was synthesized using a DIG RNA labeling kit (Roche, Mannheim, Germany). Forward primer (5'-gttggtcttcgcttggtc-3') and reverse primer (5'-cacccctcatttggttg-3'), plasmid pBluescript II KS (+) (Stratagene, California, USA) were used to generate template for RNA synthesis. All the experiment procedures were performed according to the manufacturer’s protocol.

Quantitative Real Time PCR (qRT-PCR) analysis

The qRT-PCR was performed by ABI PRISM 7500 Real-Time PCR System (ABI, California, USA) with a Prefect Real Time SYBR Premix Ex TaqTM Kit (TaKaRa, Tokyo, Japan). Primers (forward 5'-tgaagaggcgttcaggtac-3' and reverse 5'-gaacaagcactctccctga-3') were used. The β -actin gene was used as control. The primers of β -actin were forward: 5'-taccacaggcattgtgatggact-3', and reverse: 5'-ttgatgtcacgcagattccct-3'.

Northern blot analysis The concentration and purity of the total RNA was evaluated by NanoDrop 2000c (Thermo, Massachusetts, USA). 2 µg mRNA was separated through 1% formaldehyde gels electrophoresis and subsequently blotted onto nylon membrane (Amersham, USA) and subsequently crosslink by exposing to ultraviolet ray (120 mJ). All the procedures were performed according to the manufacturer’s instructions (Roche). *GAPDH* was used as a loading control.

Sub-cellular localization of Mterfd2 The mouse *Mterfd2* fragment lacking its natural stop codon was amplified from E15.5 mouse brain cDNA using primers forward (5'-tactca**ctcgag**atggtcttcgctggga aggcaggttc-3') and reverse (5'-tactca**ctgcag**cagtagctctctctcttct-3'). "ctcgag" and "ctgcag" refer to the restriction sites recognized by *Xho*I and *Pst*I respectively. "atg" highlights the transcription start site. This PCR product was digested

by *Xho*I and *Pst*I, and subsequently cloned into the expression vector pEGFP-N1 (Clontech, USA). The resultant construct, designed as pEGFP-N1-Mterfd2, was sequencing certified and then transiently transfected into NIH3T3 cells using GeneJuice® Transfection Reagent (Merck, Darmstadt, Germany) according to manufacturer's protocol. The pEGFP-N1 vector was transfected in parallel as a control. Transfected cells were grown in

Fig. 1. Bioinformation of Mterfd2. (A) Genomic organization of *Mterfd2*. The rectangles represent the exons while the broken lines show the introns. ATG and TGA indicate the translation start codon and the termination codon. PF1 and PR1 is the forward and reverse primer used for the generation of the RNA probe, respectively. PF2 and PR2 are for qRT-PCR. (B) Architecture of Mterfd2 and three other well characterized proteins of the mterf family. Black lines show the amino acid chains and grey ellipses indicate mterf motifs of the proteins, respectively. The grey line of the Mterfd2 (Mm) is a coiled-coil region on the C terminal. Specific abbreviations used are Mm (*M. musculus*), Hs (*H. sapiens*), Pl (*Paracentrotus lividus*), Dm (*Drosophila melanogaster*) respectively. (C) Multiple alignments of the mterf motifs in (B). The motif numbers of the species increase from the N terminal to the C terminal. (D) Multiple alignments of the *Mterfd2*. D1 to D4 highlights the four mterf motifs. New specific abbreviations used are Bt (*Bos taurus*), Cf (*Canis familiaris*), Ec (*Equus caballus*), Rn (*Rattus norvegicus*), others are identical to (b).

DMEM supplemented with 5% FBS for 48 h. The chamber slides were then incubated in regulator medium with 100 mmol/L MitoTracker Red CMXRos (Invitrogene) for 40 min. The chamber slides were then viewed under a Zeiss confocal fluorescence microscope (LSM510 META, Zeiss).

RESULTS

Bioinformation of the *Mterfd2* The mouse *Mterfd2* gene spans 6.7 Kb on chromosome 1 and consists of four

exons which encode a protein of 346 amino acids (Fig. 1A) with a molecular mass of 40.2 kDa and an estimated isoelectric point (pI) value of 6.2. The mature protein is about 37.3 kDa and has a pI of 5.9. Similarly to the structure of other well characterized proteins in the family, *Mterfd2* exhibits four typical “mterf” motifs (Fig. 1B). Multiple alignment of the amino acid sequence within the motif revealed that the amino acid sequence were highly conserved in the presumptive functional positions, namely a proline residue at position 8, a leucine at position 11, 18, 25 that can be substituted for another

Fig. 2. *Mterfd2* expression analysis during mouse development by ISH. ISH were carried out at embryonic stage E9.5 (A), E10.5 (B) and E11.5 (C) with whole mount mouse embryos, E13.5 (D) and E15.5 (E) with sagittal sections of mouse embryos. CSPall: central subpallium, Di: diencephalon, Dpall: dorsal pallium, DRG: dorsal root ganglia, EP: ear pinna, Fb: forebrain, flB: fore limb bud, Hb: hindbrain, hLB: hind limb bud, Ht: heart, IC: inferior colliculus, In: intestine, Ki: kidney, Li: liver, Lpm: lateral plate mesoderm, Lu: lung, Mb: midbrain, NT: neural tube, OE: olfactory epithelium, OV: otic vesicle, RhB: roof plate of the hindbrain, Sc: spinal cord, Str: striatum, Su: subpallium, TA: trigeminal area, To: tongue.

hydrophobic amino acid, a glutamic acid in preference at position 29 (Fig. 1C). To verify the conservation state of *meterfd2*, we performed multiple alignments of the amino acid residues among species including mouse, cattle, dogs, horse, rats and human beings (Fig. 1D). The results showed that *Mterfd2* was highly conserved among mammals, especially in the “*mterf*” motifs and the C-terminal tail.

In situ hybridization of *Mterfd2* To characterize the mouse *Mterfd2* expression in embryonic development, whole mount ISH (WISH) was carried out on E9.5 through E11.5 embryos and section ISH (SISH) in E13.5 and E15.5 embryos. The expression of *Mterfd2* emerged from the brain, lateral plate mesoderm and heart at E9.5 (Fig. 2A). At E10.5, *Mterfd2* expression was found in forebrain, ear pinna, trigeminal area, limb buds, lateral plate mesoderm and heart (Fig. 2B). At E11.5, *Mterfd2* expression was abundant in forebrain, midbrain and limb buds; very weak signals could be detected in the otic vesicle and neural tube (Fig. 2C). At E13.5, *Mterfd2* was highly expressed in the brain, spinal cord, dorsal root ganglia, olfactory epithelium and tongue (Fig. 2D). At E15.5, *Mterfd2* was highly expressed in the forebrain, midbrain, spinal cord, tongue, lung, liver and kidney (Fig. 2E). These data showed that *Mterfd2* was comprehensively expressed. As a whole, *Mterfd2* shows a persistent and dynamic expression pattern in the early embryonic development stage E9.5 through E15.5.

qRT-PCR and Northern blot analysis of *Mterfd2*

To confirm the ubiquitous expression of *Mterfd2* in E13.5 and E15.5 tissue ISH and whether this phenomenon was common during late embryogenesis, we carried out qRT-PCR in multiple tissues obtained from E12.5, E15.5 and

Fig. 3. *Mterfd2* expression analysis during embryogenesis. (A) qRT-PCR analysis of *Mterfd2*. qRT-PCR analysis was performed in six tissues at embryonic stage E12.5, E15.5 and E18.5. Data were tripled at least and presented as the mean with standard deviation as the bar. (B) Northern blot analysis of *Mterfd2* by using mRNA isolated from E12.5, E15.5 and E18.5 embryos.

Fig. 4. Sub-cellular localization of mouse *Mterfd2* in NIH3T3 cells. Reconstructed plasmid pEGFP-N1-*Mterfd2* (upper panel) or pEGFP-N1 vector (down panel) were transiently transfected into NIH3T3 cells. The mitochondria were stained in red with CMXRos. The green signals indicate the GFP or *Mterfd2*-GFP fusion protein.

E18.5 embryos. The results showed that *Mterfd2* was expressed in all tested tissues (Fig. 3A). To further investigate the expression characteristic of *Mterfd2*, we performed northern blot on mRNAs extracted from E12.5, E15.5 and E18.5 embryos. The results showed that *Mterfd2* was persistently expressed in tested stages, with an up regulation in E18.5 (Fig. 3B). This data further certificated the ubiquitous expression of *Mterfd2* during late embryogenesis.

Sub-cellular localization of Mterfd2 Firstly, we used three online software programs to predict sub-cellular localization of the mouse *Mterfd2* protein, MitoProt II - v1.101 (67%), TargetP (56.3%), and PSORT (82%), with a cleavage side between amino acid residue 25 and 26, suggesting a high probability of mitochondrial localization.

To get direct evidence for this prediction, we transiently transfected NIH3T3 cell with reconstructed plasmids pEGFP-N1-*Mterfd2* which encodes a fusion protein consisting of *Mterfd2* fused at its carboxyl terminus to enhanced green fluorescent protein (*Mterfd2*-EGFP). At the same time, the pEGFP-N1 vector was transfected as a control. The intracellular localization of *Mterfd2*-EGFP overlaps with mitochondria labeled with the fluorophore mitotracker while the green fluorescence in the control cells distributed throughout the cell (Fig. 4).

DISCUSSION

In this report, we characterized a new component of the MTERF4 subfamily, Mm-*Mterfd2*, which contains 4 mterf motifs unique to MTERF family. The conservation analysis of *Mterfd2* reveals that this protein is highly conserved among mammals, which might be indicative of an evolutionarily conserved function. Differently from other components in the family, Mm-*Mterfd2* had a 31 amino acids leucine rich coiled-coil region in the C-terminal tail which is highly conserved among mammals. It is reported that coiled-coil structure plays an important role in protein-protein interaction. For example, in a study concerns Sir4p, it is reported that the C-terminal coiled-coil mediated dimerization played an essential role in silent chromatin at telomeric and silent mating type loci (Murphy et al., 2003). Another research on the yeast transcriptional activator GCN4 showed that coiled-coil dimerization can interact with DNA (Ellenberger et al., 1992). Thus, *Mterfd2* might interact with other proteins through the C-terminal domain.

As part of the research, we focus on the expression profile of *Mterfd2* by ISH, qRT-PCR and Northern blot. Our data demonstrated that *Mterfd2* is dynamically expressed during embryogenesis. It emerged from the brain, lateral plate mesoderm, and the heart and gradually extended to the whole tissue. These data are consistent with the result that *Mterfd2* encoded a mitochondria

related protein, and it probably results from the participation in the mitochondrial transcription termination process.

Previous studies have verified the pronounced role of mitochondria dysfunction in neurological disorders and metabolic diseases, including Parkinson's disease, MELAS syndrome and diabetes (Mandemakers et al., 2007; Tan et al., 2009; Yoon et al., 2010). Thus, we paid special attention to the *Mterfd2* expression in the nervous system, especially the brain. Our results demonstrated that *Mterfd2* expression region became gradually restricted, from the whole brain expression at E9.5 to the highly restricted expression in the pallium of the fore-brain and inferior colliculus of the midbrain (E15.5). These results might reflect the dynamic regulation of the *Mterfd2* in the brain. This data is supported by the down regulation of *Mterfd2* in the brain by qRT-PCR. Besides, *Mterfd2* expression in the spinal cord emerged from E11.5 mouse neural tube. It was quite abundant in the spinal cord at E13.5 and E15.5 stages. It might be reasonable that *Mterfd2* has a role in cognition since the expression was persistently high in the pallium and spinal cord.

Moreover, WISH results showed that *Mterfd2* was expressed in the lateral plate mesoderm. At the later embryogenesis stage (E13.5 and E15.5), *Mterfd2* was ubiquitously expressed in the main organ and other supportive tissues. These data suggested that *Mterfd2* might have multiple roles in organogenesis.

Since the founder of the MTERF family, Hs-mTERF is purified from mitochondrial lysate (Kruse et al., 1989), Hs-MTERF2 (*mTERFL*) and MTERF3 and Mm-mTERF are all found to be localized in the same organelle (Chen et al., 2005; Li et al., 2005; Park et al., 2007). It is highly possible that their paralogue *Mterfd2* is also targeted to mitochondria. Our result clearly demonstrated this possibility.

Recent reports suggest that mitochondrial regulation is rather complicated and it might be carried out by a complex. Proteins like Hs-mTERF, Pl-mtDBP and Dm-DmTTF have been shown to participate in the regulation of transcription termination (Daga et al., 1993; Fernandez-Silva et al., 2001; Kruse et al., 1989; Roberti et al., 2003). The significant protein sequence similarity between mTERF and *Mterfd2*, as well as the same sub-cellular localization of these proteins, predicts the important role of *Mterfd2* in the modulation of mitochondria transcription. As our study showed that *Mterfd2* is targeted to mitochondria, it is predictable that *Mterfd2* might execute its transcription regulation function by binding to the regulation elements of the mtDNA, probably the HSP and transcription initiating region. Previous studies have showed that MTERF1 and MTERF2 and MTERF3 co-immunoprecipitate with each other in the HSP region (Wenz et al., 2009). Based on the unique

structure and the same sub-cellular localization, Mterfd2 may potentially bind to the HSP region and play a central role in coordinating the complex orientation. It is intriguing to us whether and how these factors coordinate with each other in their performance.

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