

# Expression Patterns of Ubiquitin Conjugating Enzyme UbcM2 During Mouse Embryonic Development

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Ubiquitin conjugating enzyme UbcM2 (Ubiquitin-conjugating enzymes from Mice, the number reveals the identification order) has been implicated in many critical processes, such like growth-inhibiting, mediating cell proliferation and regulation of some transcription factor, but the expression profile during mouse embryo development remains unclear. Hereby, during mid-later embryonic stage, the expression patterns of *UbcM2* were examined using in situ hybridization and quantitative real-time PCR (qRT-PCR). The signals were significantly intense in central nervous system and skeletal system, weak in tongue, heart, lung, liver, and kidney. In the central nervous system, *UbcM2* was principally expressed in thalamus, external germinal layer of cerebellum (EGL), mitral cell layer of olfactory bulb, hippocampus, marginal zone and ventricular zone of cerebral cortex, and spinal cord. In the skeletal system, *UbcM2* was primarily expressed in proliferating cartilage. Furthermore, qRT-PCR analysis displayed that the expression of *UbcM2* was ubiquitous at E15.5, most prominent in brain, weaker in lung liver and kidney, accompanied by the lowest level in tongue and heart. During brain development, the expression level of *UbcM2* first ascended and then decreased from E12.5 to E18.5, the peak of which sustained starting at E14.5 until E16.5. Together, these results suggest that *UbcM2* may play potential roles in the development of mouse diverse tissues and organs, particularly in the development of brain and skeleton.

Key words: *UbcM2*; In situ hybridization; Mouse embryo development

## INTRODUCTION

The ubiquitin (Ub) system has a greater-than-average involvement in regulating numerous crucial cellular processes, such as protein quality control (26) and transcription (11,22) and so on. Most proteins are targeted for proteasome-mediated degradation by covalent attachment of multiple ubiquitin molecules (24). Ubiquitination of a protein requires an enzyme cascade composed of a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub protein ligase (E3) (10). Ub is first activated in an ATP-dependent manner by the E1, the cysteine active site of which forms a thiol ester intermediate with the C-terminal

glycine residue of ubiquitin. After that, Ub is transferred to the active site of the E2 cysteine residue. An E3 ligase facilitates the transfer of ubiquitin from the E2 to the substrate (6,24).

UbcM2, one of class II E2s that have amino-terminal but lack carboxyl-terminal extensions, is located in mouse chromosome 2. UbcM2 was first identified as the closest homologs of yeast UBC4/UBC5, sharing an exceptionally highly conserved domain called UBC with its counterparts UBE2E3 from humans. The UBC domain is all E2s' characteristic that encompasses ~150 residues to form a globular domain, containing an active site of catalytic cysteine (13,23). This domain is necessary to mediate the

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interaction with Nedd4-2, consequently involved in the regulation of ENaC cell surface expression (3). So far, the field of UbcM2's characteristics and functions has been widely digged up. UbcM2 localizes to the nucleus and can shuttle in and out of the nucleus in the Ub-charging state by importin-11 (20,21). It is identified as a growth-inhibitory gene that enzymatic activity of UbcM2 may lead to inhibition of cell cycle progression, affecting the activity of some critical proteins that are involved in cell cycle (8,16). Moreover, UbcM2's counterpart UBE2E3 is required for retinal pigment epithelial cell proliferation (18), and it is necessary to protect photoreceptors from acute photo-oxidative toxicity (14). *UbcM2* is overexpressed in cancer compared with normal prostate tissue, becoming a specific marker of prostate cancer (1). Although Western blotting demonstrates that UbcM2 is most prominently expressed in the retina and ubiquitously expressed in most tissues at lower levels in 7-month-old mice (14), the expression patterns of *UbcM2* during mouse mid-late embryonic stage have not been reported thus far.

In this study, we examined the expression of *UbcM2* in diverse tissues and developmental stages by in situ hybridization and qRT-PCR. The result showed that *UbcM2* was ubiquitously expressed during mouse embryonic stage, with significantly heavy signals in the central nervous system (CNS) and skeletal system. In addition, the data of qRT-PCR displayed that the expression of *UbcM2* was ubiquitous at E15.5, most abundant in the brain. During the mid-late embryonic stage, the expression of *UbcM2* in the brain formed a crest between E14.5 and E16.5. These data suggested that *UbcM2* may be involved in the development of diverse mouse tissues or organs, particularly brain and skeleton.

## MATERIALS AND METHODS

### *Animal*

Mice (C57BL/6 J) were time-mated and pregnant females were sacrificed to collect the embryos and tissues at distinct embryonic stages. The age of the embryos was determined by the presence of a vaginal plug representing embryonic day 0.5 (E0.5). The day of birth was designated as postnatal day (P). All procedures were carried out following the "Rules for experiments' animals" promulgated by Chinese Government (Beijing, China).

### *In Situ Hybridization (ISH)*

The whole embryos (E10.5, E12.5, E14.5, and E16.5) were fixed with 4% paraformaldehyde in

phosphate-buffered saline (PBS) at 4°C overnight. Embryos were dehydrated, paraffin embedded, and subsequently sectioned at 9 µm. For preparing *UbcM2* probe, a mouse cDNA fragment (sense: 5'-aagatgccagtgacagcgaag-3', antisense: 5'-tctgctcctcaccactgcatac-3') was subcloned into pBluescript II KS<sup>(+)</sup> (Stratagene, CA, USA) T-vector by using DNA Ligation Kit (TARAKA, Dalian, China) following the manufacturer's instructions. Antisense and sense RNA probes specific to *UbcM2* gene were synthesized by in vitro transcription in the presence of digoxigenin-UTP (Roche, Mannheim, Germany). ISH (section and whole-mount) was performed as previously described (2,27).

### *RNA Preparation and Quantitative*

#### *Real-Time-PCR (qRT-PCR)*

Total RNAs were extracted from diverse tissues of E15.5 and brain tissues from E12.5 to E18.5 by Trizol reagent (Invitrogen, Eugene, OR, USA). The cDNAs were synthesized using a SuperScript<sup>TM</sup>III RNase H<sup>-</sup> Reverse Transcriptase kit (Invitrogen). Subsequently, the cDNAs were used for *UbcM2* expression analysis by qRT-PCR. Sequences of *UbcM2* primers used in qRT-PCR were: 5'-caggttctgtatatgaagtg-3' (sense), 5'-gtcaataactgagtgctatg-3' (antisense). qRT-PCR analysis was performed by the ABI Prism 7500 Real-Time PCR System, and reactions were prepared using the SYBR Green PCR master mix kit (Applied Biosystems, CA, USA) according to manufacturer's instructions. The expression level of *UbcM2* gene was normalized by that of the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene. All samples were analyzed in triplicate at least.

## RESULTS

### *UbcM2 Gene Structure and Amino Acid Sequence Analysis of UbcM2 With Its Orthologues*

The *Mus musculus UbcM2* gene (Gene ID: 22193) is located in mouse chromosome 2 2D region, includes 6 exons, and encodes a 207-amino acid protein. A predicted CpG island is situated from -507 bp upstream to +969 bp downstream harboring 140 CpG sites in a stretch of the *UbcM2* promoter region, the exon 1 and intron 1 (Fig. 1A).

The UBC core domain is located at amino acids 59 to 207. It contains a conserved catalytic cysteine (145) that is required for enzyme-ubiquitin thiol ester formation. The noncatalytic cysteine (136) was identified as a putative redox sensor (19). The alignment of mUBE2E3 sequences from other species indicated that this domain is highly conserved, only a serine

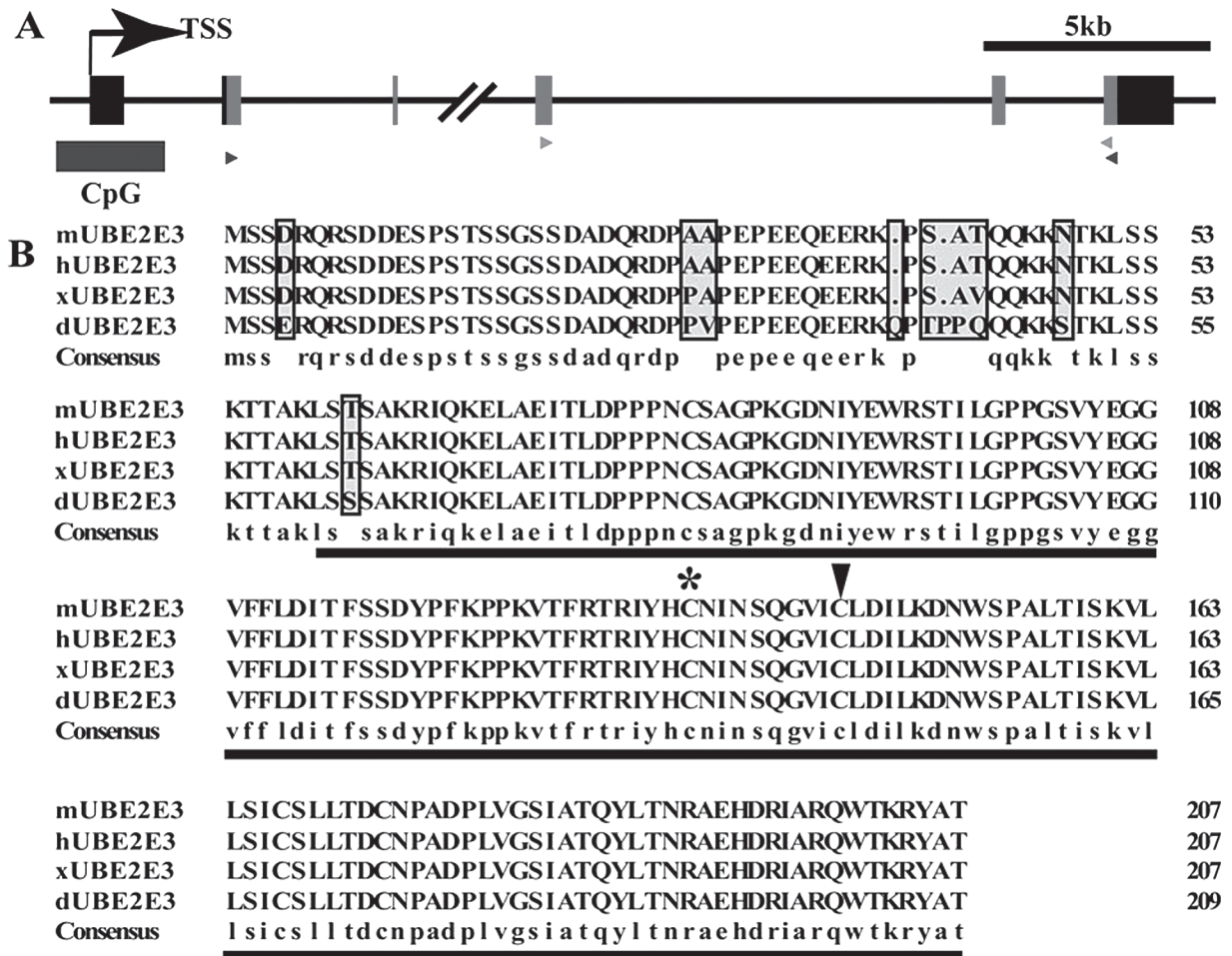


Figure 1. *UbcM2* was highly conserved in vertebrates. (A) Schematic representation of the *UbcM2* gene structure (*Mus musculus*, chromosome 2 D). Gray boxes represent the coding region of *UbcM2*. Dark rectangle demonstrates a predicted CpG island harboring a stretch of 140 CpG sites (<http://genome.ucsc.edu>). The black and gray triangles label the primers for specific sense and antisense probe preparation and qRT-PCR, respectively. (B) Amino acid sequence alignment of the primary sequences of mouse *UbcM2* (mUBE2E3) and its orthologues in human (hUBE2E3), *Xenopus laevis* (xUBE2E3), *D. rerio* (dUBE2E3). The highly conserved UBC core domain is underlined. The conserved catalytic cysteine (145) is indicated by an arrowhead. The noncatalytic cysteine (136), which is identified as a putative redox sensor, is indicated by an asterisk.

(S) representing a threonine (T) in *Danio rerio* (dUBE2E3) (Fig. 1B). Furthermore, it was more notable that the amino acid sequences of mouse (mUBE2E3) and human (hUBE2E3) exhibited 100% identity, demonstrating the conservative property of *UbcM2* enzyme in the phylogenetic evolution. Comparison of the conservation among these orthologues was generated by multiple amino acid sequence alignment using DNAMAN (Fig. 1B).

*Expression of UbcM2 During Mouse Embryonic Development*

To analyze the expression of *UbcM2* in mouse embryos during mid-later gestation period, whole-mount and section in situ hybridizations were carried

out, respectively. Specific RNA probe using the primers, shown as blue triangles in Figure 1A, was prepared.

By E10.5, the strong expression signals were detected in the cortical anlage and mesenchyma of the telencephalon (Te), mesencephalon (Me), and limb buds (LB) (Fig. 2A). The signals can be observed at the rhombencephalon (Rh) and neural tube (NT) from dorsal of the identical embryo as well. In addition, *UbcM2* was also detected in the mandibular arch (MA), hyoid arch (HA), and lateral nasal process (LNP), consistent with the signals viewed at the upper lip (UL) and tongue (To) of subsequent section in situ hybridization. As a negative control, no signals were detected using the sense probe (data not shown).

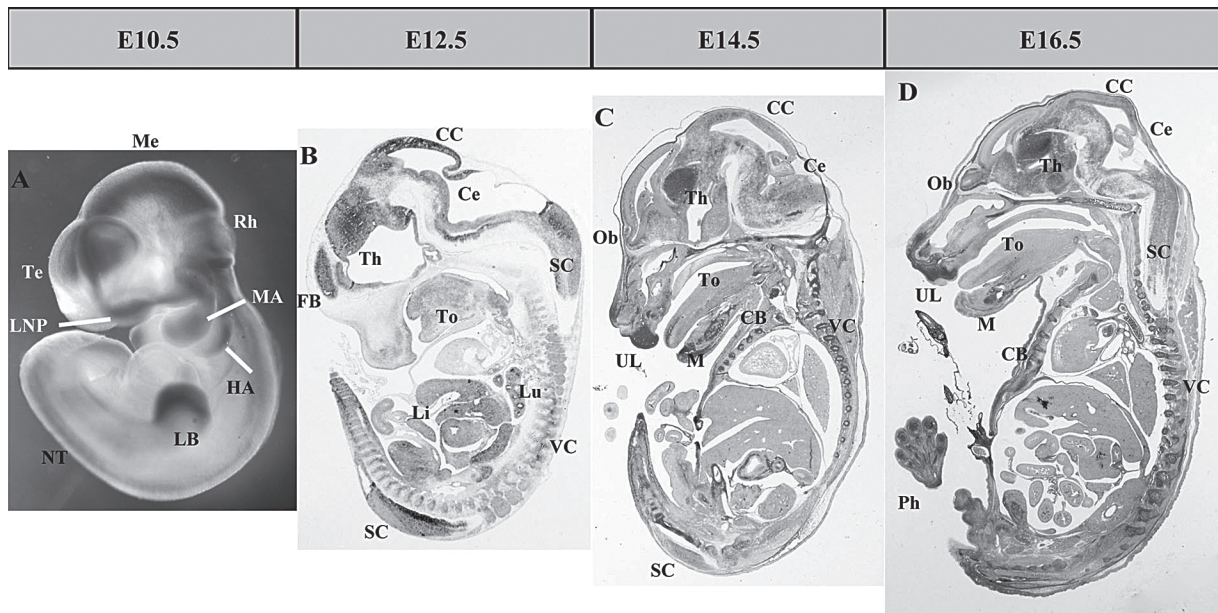


Figure 2. *UbcM2* overall expression pattern during mid-later gestation period. (A) The expression of *UbcM2* in E10.5 embryo by whole-mount ISH. (B–D) The expression of *UbcM2* in E12.5, E14.5, and E16.5 embryo by section ISH. FB, forebrain; Th, thalamus; CC, cerebral cortex; Ce, cerebellum; SC, spinal cord; Th, thalamus; To, tongue; Li, liver; Lu, lung; VC, vertebral column; Ob, olfactory bulb; UL, upper lip; M, Meckel's cartilage; CB, costal bone; Ph, phalange.

From E12.5 to E16.5, the expression signals of *UbcM2* mainly distributed in the CNS and skeletal system. By E12.5, the signals were primarily detected in brain (Fig. 3A, a) liver (Li, Fig. 3A, e) lung (Lu, Fig. 3A, d), and spinal cord (SC), weaker in the tongue (To, Fig. 3A, b), kidney (Fig. 3A, f), and vertebral column (VC), while no signals were detected in heart (Fig. 3A, c). *UbcM2* in the brain was mainly located at the fore brain (FB), thalamus (Th), cerebral cortex (CC), and cerebellum (Ce) (Fig. 2B), most of which was in accordance with those derived from the signal location of E10.5.

By E14.5 and E16.5, the expression of *UbcM2* became more intensive by *in situ* hybridization (Fig. 2C, D), principally in the CNS and skeletal system, with low expression level of tongue (Fig. 3A, h, n), heart (Fig. 3A, i, o), liver (Fig. 3A, j, p), lung (Fig. 3A, k, q), and kidney (Fig. 3A, l, r). In the developing CNS, the strong signal was detected in the regions of cerebral cortex (CC), thalamus (Th), cerebellum (Ce), olfactory bulb (OB), hippocampus (Hi), and spinal cord (SC). In cerebral cortex, the signals of *UbcM2* were detected mainly in the marginal zone (MZ) and ventricular zone (VZ), accompanied by low expression in intermedial zone (IZ) (Fig. 3B, d, i). The strongest expression signals were observed in thalamus (Fig. 3B, b, g). By 14.5, the signals appeared in the whole regions of thalamus, including the dorsal (DTh), anterior (ATH), ventral (VTh), and posterior (PTh) areas, which was different from the appearance

of individual signals in ventral thalamus by E16.5. In cerebellum, only the external granular layer (EGL) exhibited *UbcM2*'s expression signal (Fig. 3B, e, j). In olfactory bulb (Fig. 3B, a, f), *UbcM2* mainly distributed in the mitral cell layer (MC), with weaker signals detected in the granule cell layer (GC). *UbcM2* also showed a high expression level in immature hippocampus (Fig. 3B, c, h) and in the central nucleus of the dorsal horn, gelatinous substance, motoneurons of spinal cord (SC). In addition to the expression in CNS, the evident signals also presented in Meckel's cartilage (Fig. 3C, a, f), sphenoid bone (Fig. 3C, b, g), costicartilage (Fig. 3C, c, h), cartilages of vertebral column (Fig. 3C, d, i), and skull (Fig. 3C, e, j), primarily visible in proliferating chondrocytes, rather than in the hypertrophic chondrocytes.

#### Expression Analysis of *UbcM2* in Different Tissues

To further investigate *UbcM2* expression pattern, we performed qRT-PCR to detect its expression among diverse tissues. At E15.5, *UbcM2* was most abundant in brain, moderate in lung, liver, and kidney, accompanied by the lowest level in tongue and heart. This result was in accordance with the trends we discovered by ISH in mouse embryos. The prominent signals of *UbcM2* in developmental brain showed high specificity, prompting us to examine its expression in brain in more detail. We then analyzed the expression of *UbcM2* by qRT-PCR during mouse

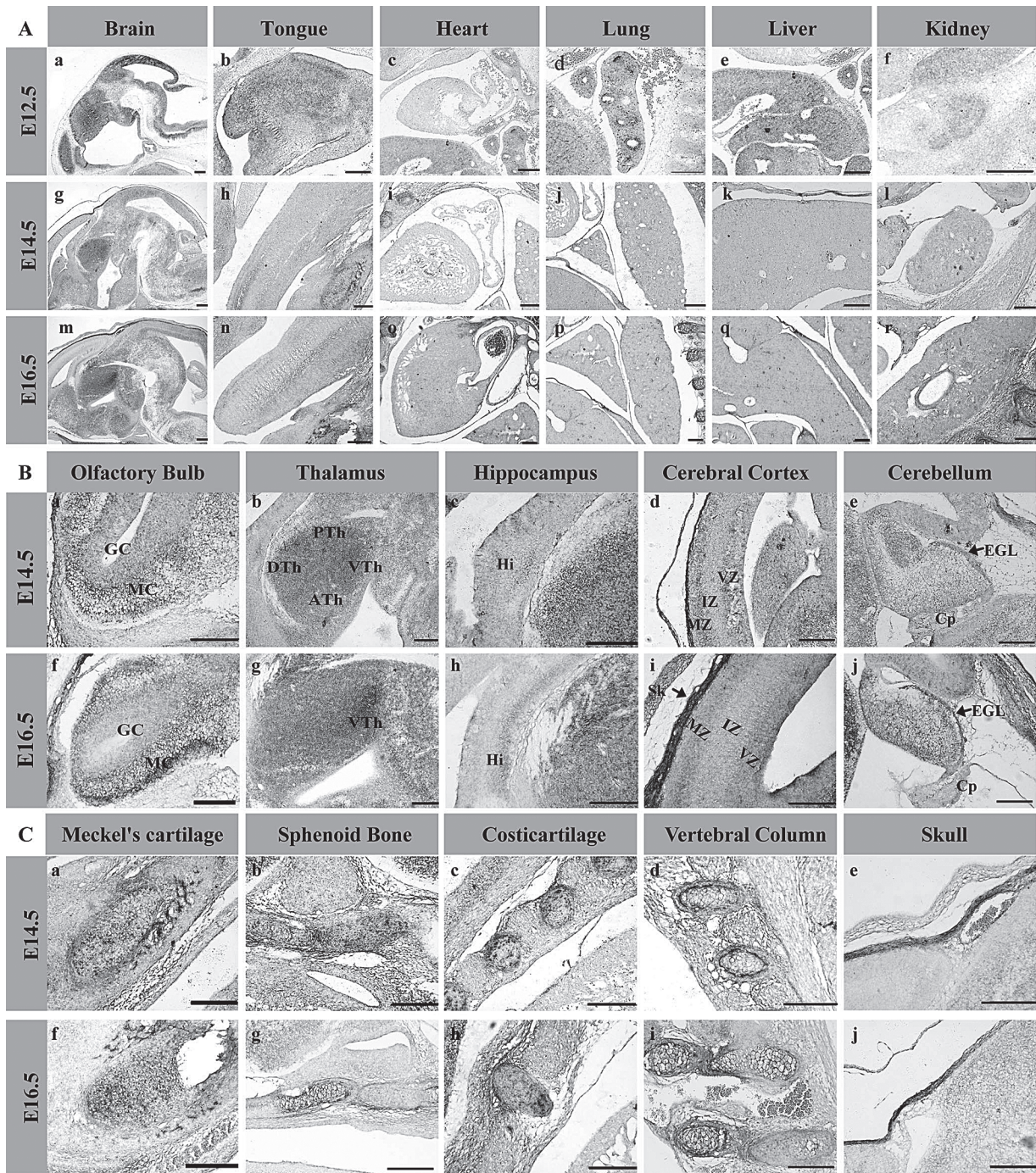


Figure 3. The expression of *UbcM2* in diverse tissues of different development stages. (A) The expression pattern of *UbcM2* in main tissues of E12.5, E14.5, and E16.5. (B) The expression pattern of *UbcM2* in the brain of E14.5 and E16.5. GC, granule cell layer; MC, mitral cell layer (a, f); DTh, the dorsal area of thalamus; ATH, the anterior area of thalamus; VTh, the ventral area of thalamus; PTh, the posterior area of thalamus (b, g); Hi, immature hippocampus (c, h); MZ, marginal zone; VZ, ventricular zone; IZ, intermedial zone (d, i); EGL, external germinal layer; Cp, choroid plexus (e, j). (C) The expression pattern of *UbcM2* in the skeletal system of E14.5 and E16.5. Scale bars: 200  $\mu$ m.

brain development. The result showed that the *UbcM2* dynamic expression ascended from E12.5, reached a plateau at E14.5, maintained the status until E16.5, and then decreased during the mid-later embryonic stage (Fig. 4B).

## DISCUSSION

Despite the discovery of numerous events *UbcM2* can be involved in (3,10,20,21), to date the expression profile of this enzyme still needs to be identified. In this report, we performed in situ hybridization to analyze the expression patterns of *UbcM2* during mouse embryogenesis. It exhibited intense signals in developing CNS and skeletal system, compared to those signal-nonsignificant tissues or organs. This result is in accordance with the Western blot data of adult mouse tissues, which show a strong expression

in retina and brain, but weak in heart, lung, and liver (14).

The CNS of vertebrates (such as mouse) consists of the brain and spinal cord. The first sign of the nervous system is the appearance of the neural plate. The inner portion of the neural plate is destined to turn into the CNS, which is organized around a rostral–caudal axis of bilateral symmetry. With the development of the neural plate, it gets surrounded by neural folds, which finally become the neural tube. In our study, the embryo of E10.5 revealed a strong expression in the CNS, including telencephalon, mesencephalon, rhombencephalon, and neural tube, which indicated that *UbcM2* may involve in the morphogenesis or development of the CNS in mid-gestation embryos. As the CNS develops, telencephalon, diencephalon, mesencephalon, and rhombencephalon differentiate into the hippocampus and neocortex, the

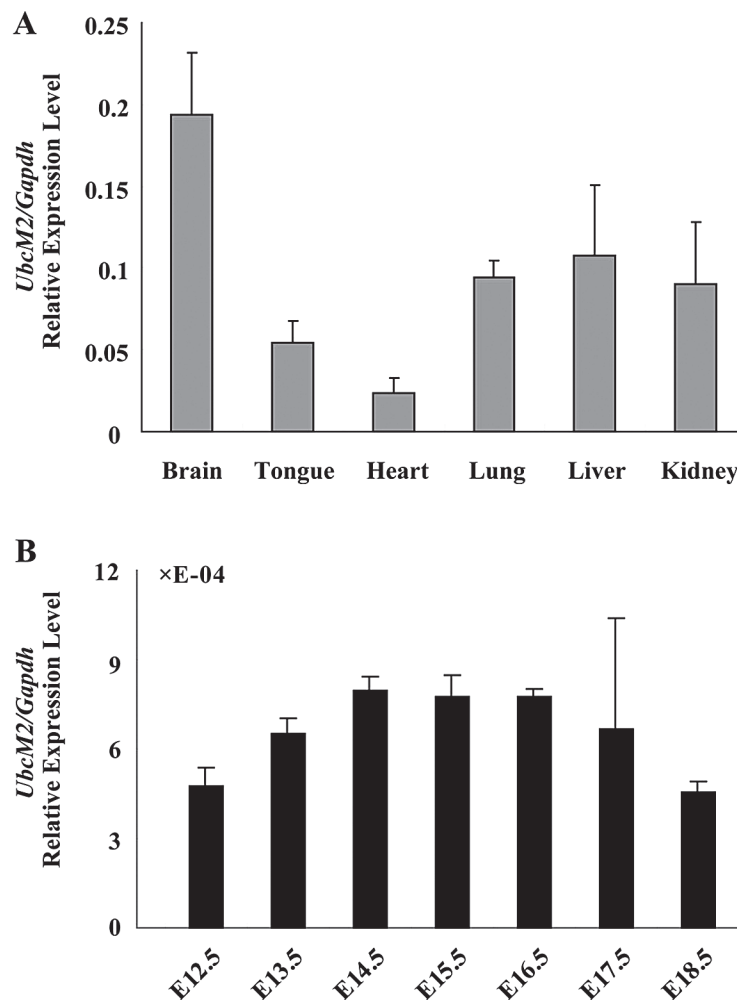


Figure 4. The qRT-PCR analysis of *UbcM2* in E15.5 and in developing brain. (A) The expression of *UbcM2* in different tissues of E15.5. (B) The expression profiles of *UbcM2* in developing brain from E12.5 to E18.5. Data were expressed relative to the corresponding values for *Gapdh*. Error bars represented the standard deviation in three independent experiments.

thalamus, the tectum, the cerebellum, and the medulla oblongata, respectively, in accordance with the signals detected in those parts of brain by ISH from E14.5 to adulthood. At the E14.5 stage, organogenesis is largely complete, applicable to study organ architecture, with cell differentiation still occurring. In addition, early corticogenesis is taking place at this stage, whereas middle telencephalic development is occurring at E16.5.

These results indicated that *UbcM2* could act a role in the developing brain as well as the spinal cord. Because of the character E2 enzyme UbcM2 played, the expression patterns may relate to the distribution of E3 ligases and relevant substrates. Nedd4-1, as a HECT E3 ubiquitin ligase, is abundant in mammalian neurons (12). It is a positive regulator of dendrite development; moreover, HECT-type ubiquitin-protein ligases (Nedd4 and Nedd4-2) can have an interaction with UBE2E3, a homolog of UbcM2 (5). It interprets the expression of *UbcM2* in brain to some extent. It also seemed possible that these expression patterns in brain appeared due to the relevance between *UbcM2* and *Mdm2*, a negative regulator of p53 activity (7,9). *Mdm2* is involved in the DDD-E2 complex, which consists of UbcM2 as a critical member (17). *Mdm2* is expressed in the early CNS (4); in addition, loss of *Mdm2* lead to the neonatal lethality (28). *UbcM2* null mice as disruption of both alleles is embryonic lethal (14), implicating the important role *UbcM2* played during mouse embryogenesis. We also used qRT-PCR to examine *UbcM2*'s expression in brain, the tendency may result from the differentiation

and proliferation of neurocytes. *UbcM2* is required for RPE cell proliferation, and *UbcM2* is downregulated during RPE maturation (18), which is applicable in the brain neurocytes. These inferences still need to be investigated in our further study.

In the skeletal system, the signals of *UbcM2* are located in the proliferating chondrocytes, which may infer that *UbcM2* is involved in skeletogenesis. Nedd4 is expressed abundantly in proliferating chondrocytes, but is undetectable in hypertrophic chondrocytes (25), which is in accord with our results. Moreover, knocking down an E2 enzyme Ubc9 in zebrafish will lead to defects in cranial cartilage, and it is required during vertebrate organogenesis (15), which makes our data evident as well.

In conclusion, the present research reveals that the *UbcM2* is ubiquitously expressed during mouse embryonic stage and it is developmentally regulated in the CNS and skeletal system. Our data also indicate a spatial and temporal expression pattern of *UbcM2* within various structures of the developing brain. This may be helpful for dissection of the regulation mechanism of the proteasome proteolytic system in mouse brain in the future.

#### ACKNOWLEDGMENTS

This work was supported by grants from the Fundamental Research Funds for the Central Universities (Grant No. HIT.NSRIF.2010027) and the National Natural Science Foundation of China (No. 30971645 and 31171383).

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