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Predominant expression of Sir2α, an NAD-dependent histone deacetylase, in the embryonic mouse heart and brain[1]

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Abstract **Sir2 is an NAD-dependent histone deacetylase that functions in longevity, gene silencing, heterochromatin formation, DNA repair, and suppression of DNA recombination in yeast. The mammalian homolog Sir2α (SIRT1) has been shown to inhibit p53-dependent apoptosis, but its physiological roles are still not known. We found that the level of Sir2α expression during embryogenesis was high. The highest Sir2α mRNA expression was detected as early as embryonic day (E) 4.5. Although the level was down-regulated during embryogenesis, a high level of expression was still found in the late embryonic stage (E18.5). In embryos, Sir2α was expressed at high levels in the heart, brain, spinal cord, and dorsal root ganglia. The expression levels in these organs were high on E10.5–E13.5 and low on E16.5. Quantitative reverse transcription polymerase chain reaction showed a 60% reduction in Sir2α mRNA content in the heart between E12.5 and E14.5. After E14.5, the expression level in the heart remained constant up to 27 months of age. The expression pattern of Sir2α protein in embryonic hearts was consistent with that of mRNA. These results suggest new roles of Sir2α not only in early embryogenesis but also in cardiogenesis and neurogenesis with a stage-specific manner.**

Key words: Silent information regulator; Histone deacetylase; Cardiogenesis; Neurogenesis; Gene expression

1. Introduction

The silent information regulator 2 (Sir2) has been shown to be an essential component for silencing of yeast silent mating type loci [1]. In yeast, Sir2 functions in gene silencing, heterochromatin formation, DNA repair, and suppression of DNA recombination [2–4]. Sir2 is a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase and has been shown to deacetylate histone H3 and H4 in the presence of NAD [5]. Overexpression of Sir2 induces longevity of yeasts [6] and *Caenorhabditis elegans* [7]. Calorie restriction extends the lifespans of a broad range of organisms, from yeasts to mammals [8,9]. Sir2 is necessary for extension of yeast lifespan by calorie restriction [10]. Sir2 is evolutionarily conserved from bacteria to humans. Seven human Sir2 homologs, named SIRT1–7, have so far been identified [11,12]. SIRT1, 2, and 3 have NAD-dependent protein deacetylase activities. SIRT2 colocalizes with microtubules and deacetylates tubulin [13]. SIRT3 is a mitochondrial NAD-dependent deacetylase that is located in the mitochondrial matrix [14]. Sir2α (SIRT1) is a nuclear protein that has been shown to deacetylate not only histone but also the tumor suppressor p53 [15,16]. Sir2α directly binds to p53 and suppresses p53-dependent apoptosis in response to DNA damage. It has been reported that the fly Sir2 ortholog affects segmentation and sex determination [17] and that developmental defects in the brain [18], heart [18,19], and retina [19] were observed in Sir2α-null mice. These studies suggest that Sir2α is important in embryonal development. Although it has been shown that two-cell embryos express Sir2α [18], little is known about the expression of Sir2α in the embryo.

In this study, we investigated the expression of Sir2α in mouse embryos to try to obtain an insight into the function of Sir2α in the developmental process. We unexpectedly found that Sir2α is expressed at high levels in mouse embryos. Sir2α was found to be widely expressed in embryonic organs, and its expression levels in the heart and brain were high in specific embryonic stages, suggesting that Sir2α has an as yet undetermined role in embryonic development.

2. Materials and methods

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and to the Guideline for Animal Experiments in Sapporo Medical University.

2.1. cDNA cloning of Sir2α

A lambda zap mouse brain cDNA library (Stratagene) was screened using a mouse Sir2α (SIRT1) cDNA fragment corresponding to nucleotides 968–1261 in the coding region of mouse Sir2α cDNA (AF214646). The cDNA fragment was obtained by reverse transcription polymerase chain reaction (RT-PCR) of mouse brain total RNA using a primer set of 5′-AATATATCCCGGACAGTTC-3′ and 5′-AAGACAATCTCTGGCTTCA-3′. For the expression of Sir2α cDNA, the coding region of mouse Sir2α cDNA was subcloned in pIRES-hrGFP-1a (Stratagene).

2.2. Tissue distribution of the Sir2α mRNA and quantitative RT-PCR

For tissue distribution analysis, total RNA was purified from various normal mouse tissues using an RNeasy Mini kit (Qiagen). RT-PCR was performed using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech). The primer sets used were 5′-CCCCATGAAGTGCCTCAAAT-3′ and 5′-GATTACCCTCAAGCCGCTTA-3′, cor-

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[1] The nucleotide sequence reported in this paper has been submitted to the GenBank data bank with accession number AY377984.

Abbreviations: Sir, silent information regulator; NAD, nicotinamide adenine dinucleotide; bHLH, basic helix-loop-helix

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responding to nucleotide positions 1342–1361 and 1872–1891, respectively, in the Sir2α cDNA and the expected size of the PCR product is 550 bp. The cDNA clones were used as a positive control and a negative control was done in which the first step of reverse transcription was omitted in the RT-PCR. The conditions for PCR amplifications were: denaturation at 95°C for 30 s; annealing at 55°C for 30 s, and then elongation at 72°C for 1 min. The amplified products were analyzed on a 2% agarose gel and visualized by staining with ethidium bromide.

Real-time quantitative RT-PCR was performed using an ABI Prism 7700 sequence detection system and TaqMan One-Step RT-PCR Master Mix Reagents (ABI). Primers for the Sir2α mRNA were designed using Primer Express software (ABI), and the Sir2α primer sequences used were 5′-TCCTCACTAATGGCTTTCATTCCT-3′ and 5′-CGCGGAGTCCAGTCACTAGAG-3′. The probe sequence was FAM-ATGACGATGACAGAACGTCACACGCC-TAMRA. The conditions of one-step RT-PCR were as follows: 30 min at 48°C, 10 min at 95°C, and then 45 cycles of amplification for 15 s at 95°C, 60 s at 55°C and 30 s at 72°C. Commercially available glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and a probe (TaqMan Rodent GAPDH Control Reagent, ABI) were used to quantify GAPDH for standardization of the reaction. The expression levels of Sir2α mRNA were normalized by the contents of GAPDH mRNAs.

2.3. Preparation of anti-Sir2α polyclonal antibody

The polyclonal antibody for mouse Sir2α was raised in a rabbit against a synthetic peptide corresponding to amino acid residues 722–737 (ATRQELTDVNYPSDKS) in the C-terminal region of mouse Sir2α. The antiserum was purified with antigenic peptide-coupled Sulfolink resin (Pierce).

2.4. Western blot analysis

COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum with 5% CO_2 at 37°C. Cells plated in six-well culture dishes were transfected with a pIRES-hrGFP-1a vector containing Sir2α cDNA or an empty vector using LipofectAMINE2000 (Life Technologies) following the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed in a lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.1% (w/v) sodium dodecyl sulfate (SDS)) and vortexed vigorously. The debris in cell lysates was removed by centrifugation. Lysates were analyzed in 7.5% SDS–polyacrylamide gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were probed with an anti-Sir2α antibody and incubated with an horseradish peroxidase-conjugated antibody against rabbit IgG (Santa Cruz). For a control experiment, an anti-Sir2α antibody preabsorbed with excess antigenic peptide was used. To investigate the developmental changes in the amount of Sir2α in the heart, embryos of ddY mice were soaked in phosphate-buffered saline, and embryonic hearts at embryonic day (E) 12.5, E14.5 and E16.5 were dissected. Hearts were also obtained from 1-day-old, 10-week-old and 27-month-old ddY mice. Hearts were homogenized in a lysis buffer and lysates were transferred onto PVDF membranes as described above, and the membranes were incubated with an anti-Sir2α antibody. As a protein loading control, membranes were also incubated with anti-GAPDH antibody (Chemicon). Immunoreactive bands were visualized by SuperSignal enhanced chemiluminescence (Pierce) using the luminescent image analysis system LAS-1000 (Fujifilm).

2.5. Fluorescent immunostaining

COS7 cells transfected with Sir2α cDNA or the control vector were fixed in 4% paraformaldehyde and 0.1 M sodium phosphate, pH 7.4, 48 h after transfection and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. The cells were incubated with anti-Sir2α antibody (1:500 dilution) for 30 min at room temperature and then with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (Molecular Probes). For a control experiment, Sir2α antibody preincubated with an excess of peptide was used. The cell nucleus was stained with 1 μg/ml Hoechst 33342 dye (Sigma). Stained cells were examined using a laser scanning confocal fluorescent microscope (Radiance 2100, Bio-Rad).

2.6. Immunohistochemistry of mouse embryos

Timed pregnant ddY mice were obtained from Sankyo Labo Service. Mice were deeply anesthetized and killed at various gestation stages. Embryos were dissected from extra-embryonic membranes, washed in phosphate-buffered saline, embedded in the Tissue-Tek OCT compound (Sakura Finetechnical), and immediately frozen. Samples were kept at −80°C until use. Sagittal cryostat sections were cut (5 μm) and collected on MAS-coated slides (Matsunami). Sections were fixed for 7 min in methanol:acetone (1:1) at −20°C, incubated with 3% H_2O_2 for 30 min to block endogenous peroxidase activity, treated with 5% normal goat serum, and then incubated overnight with Sir2α antibody (1:500 dilution) at 4°C. Immunodetection was carried out using a biotinylated goat anti-rabbit antiserum and a Vectastain ABC kit (Vector Laboratories). Sections were photographed on an Olympus SZX9 dissection microscope or a Nikon Eclipse E600 microscope.

2.7. Quantitation of Sir2α mRNA in the mouse heart at different stages

Mouse hearts at various ages were collected for measurements of Sir2α mRNA levels. Isolated hearts were flash-frozen in liquid nitrogen and stored at −80°C until RNA purification. Total RNA was isolated using an RNeasy Mini kit (Qiagen). Quantitative RT-PCR was carried out as described above to determine Sir2α mRNA levels in the heart. The expression levels of Sir2α mRNA were normalized by the contents of GAPDH mRNAs and mean Sir2α mRNA levels were compared between groups by analysis of variance followed by a post-hoc multiple comparison test. A P value <0.05 was considered statistically significant.

3. Results

3.1. Sir2α cDNA cloning

We cloned mouse Sir2α cDNA from a mouse brain cDNA library. One full-size Sir2α cDNA clone (3.9 kb) was isolated from 6×10^5 phage clones. The nucleotide sequence and deduced amino acid sequence were identical to those previously reported [5].

3.2. Expression of Sir2α mRNA in adult mouse tissues

The tissue distribution of Sir2α mRNA was examined by RT-PCR. As illustrated in Fig. 1A, we detected the expression of Sir2α transcript in all tissues examined. The expression levels of Sir2α mRNA in various tissues were determined by quantitative RT-PCR (Fig. 1B). Sir2α mRNA levels were normalized to the contents of GAPDH mRNA. The quantity varied considerable among the tissues and the highest expression was detected in the lung followed by the testis. Moderate levels of expression were observed in the ovary, spleen, thymus, heart, and E14.5 embryos.

3.3. Developmental changes in Sir2α mRNA expression

It has been demonstrated that the fly Sir2 ortholog affects segmentation and sex determination [17] and that Sir2α-null mice exhibit developmental defects [18,19]. It has also been reported that two-cell-stage embryos expressed high levels of Sir2α [18]. These results prompted us to examine the expression profile of Sir2α mRNA during embryogenesis. Total RNAs of mouse whole embryos from E4.5 to E18.5 were analyzed by Northern blotting. As shown in Fig. 1C, embryos expressed abundant amounts of Sir2α mRNA. The highest expression levels were found in early-stage embryos. The expression levels of Sir2α mRNA continuously decreased with progression of embryonic development, but, unexpectedly, a high expression level of Sir2α mRNA persisted until late developmental stages. Densitometric analysis of blots normalized by S28 ribosomal RNA intensity showed that the expression level of Sir2α mRNA on E18.5 had decreased by 60% compared with that on E4.5 (Fig. 1D). Thus, we found that

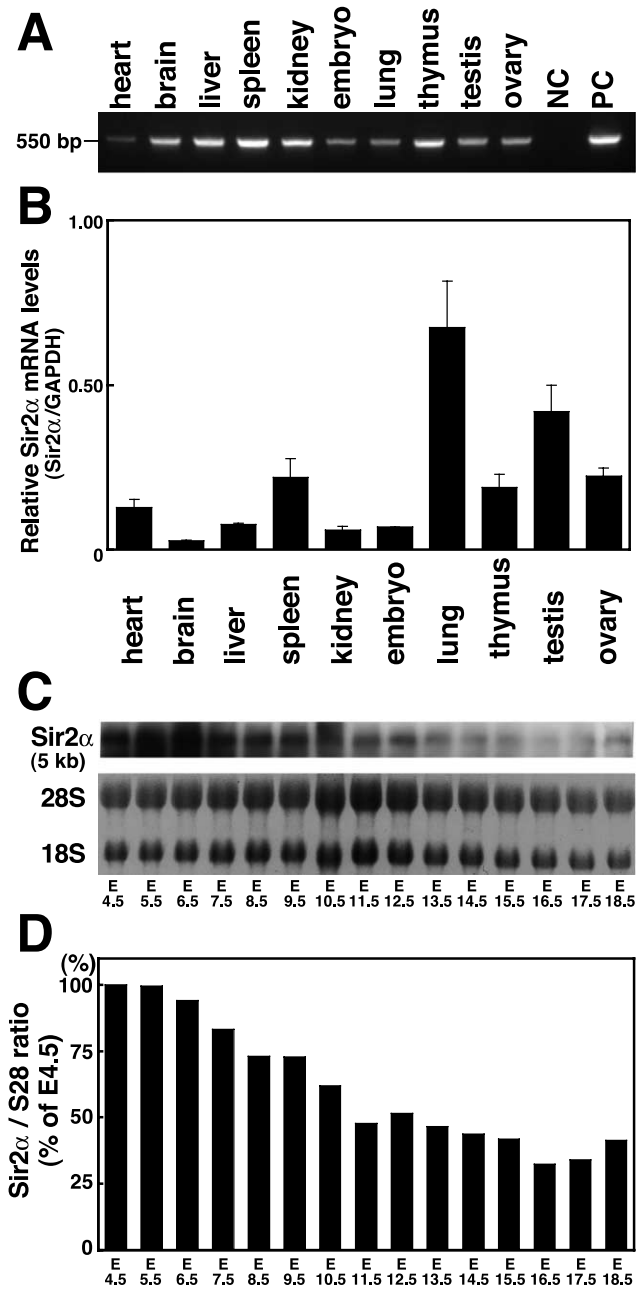

Fig. 1. Expression levels of Sir2α mRNA in various mouse tissues and embryos. A: Expression of Sir2α mRNA in various mouse tissues. RNAs from mouse tissues were subjected to RT-PCR using specific primers for Sir2α. NC, negative control; PC, positive control. B: Quantitative analysis of Sir2α mRNA in mouse tissues. Expression levels of Sir2α mRNA were assessed by quantitative RT-PCR using a Prism 7700 sequence detector. Sir2α mRNA levels were normalized to GAPDH mRNA levels. Data are presented as the mean ± S.E.M. ($n = 3$). C: Northern blot analysis of Sir2α during embryogenesis. Expression levels of Sir2α mRNA in the mouse embryo at the indicated different stages. A mouse embryo blot (20 μg of total RNA/lane) was probed with a ^{32}P-labeled cRNA fragment of Sir2α (upper panel, numbers indicating embryonic days). Levels of 28S and 18S ribosomal RNA were used as internal standards (lower panel). D: The content of Sir2α mRNA of each embryonic stage was normalized using each content of 28S ribosomal RNA and represented as a percentage of the value on E4.5.

Sir2α mRNA is expressed in a highly stage-specific manner in the embryo.

3.4. Characterization of a polyclonal antibody and expression of Sir2α protein in mammalian cells

To investigate the tissue distribution of Sir2α protein in embryos, we developed a polyclonal antibody against the carboxy-terminal region of mouse Sir2α. The raised anti-Sir2α antibody could be used in Western blotting (Fig. 2A,B) and also in immunostaining (Fig. 2C). In accordance with previously reported results [15,16], a band at approximately 120 kDa was detected in COS7 cell lysates transfected with Sir2α cDNA (Fig. 2A, left lane). The band was specifically abolished in the presence of an antigenic peptide (Fig. 2B, left lane). Immunofluorescent microscopic examination of COS7 cells transfected with Sir2α cDNA showed specific immuno-

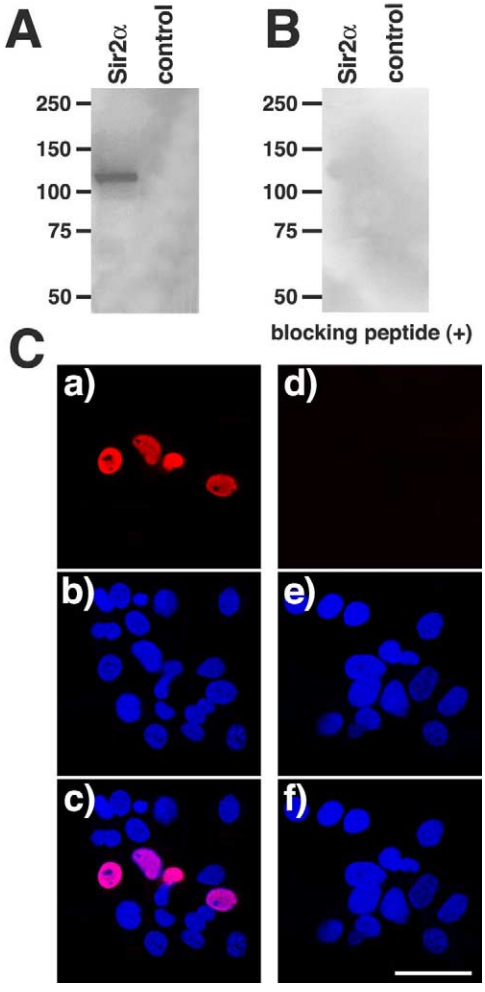

Fig. 2. Western blot and immunohistochemical detection of Sir2α in COS7 cells. A: Anti-Sir2α antibody recognizes 120-kDa Sir2α protein in COS7 cells transfected with Sir2α cDNA (left lane). Positions of molecular mass markers are indicated on the left side of the panel. B: The addition of an excess of antigenic peptide abolished the signal. C: Immunostaining of COS7 cells transfected with Sir2α cDNA. Transiently transfected cells were stained with anti-Sir2α antibody (a–c). Anti-Sir2α antibody preabsorbed with antigenic peptide showed specificity of the antibody (d–f). Hoechst staining of the same field is shown in panels b and e to indicate nuclei of the cells. A merged image shows the nuclear localization of Sir2α protein (c). The scale bar represents 50 μm.

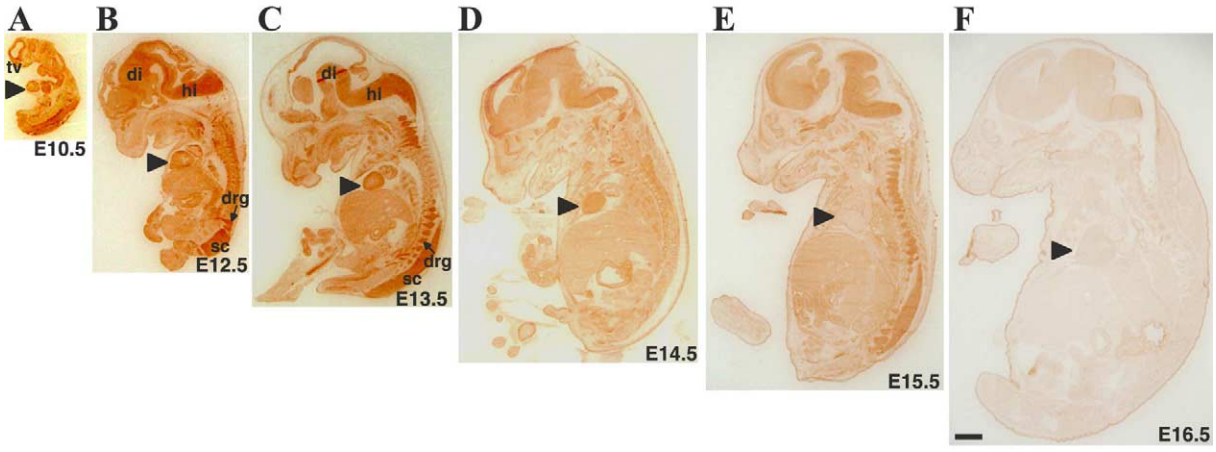

Fig. 3. Immunohistochemical detection of Sir2α protein in mouse embryos from E10.5 to E16.5. Sections of fixed whole embryos (E10.5–16.5) of ddY mice were stained with anti-Sir2α antibody. Sir2α protein was prominently expressed in the embryonic heart and brain, and the expression levels gradually decreased. Arrowheads indicate embryonic hearts. tv, telencephalic vesicle; di, diencephalon; hi, hindbrain; sc, spinal cord; drg, dorsal root ganglion. The scale bar represents 2 mm.

staining of Sir2α (Fig. 2C). The specificity of the staining was also validated by the results of an absorption experiment using an excess of an antigenic peptide (Fig. 2C, panel d).

Sir2α localized in the nuclei of transfected cells, and the nuclear localization of Sir2α is consistent with results of previous studies [15,16].

3.5. Immunolocalization of Sir2α protein in the mouse embryo

Abundant expression of Sir2α mRNA in middle and late developmental stages suggests the involvement of Sir2α in organogenesis. Therefore, we investigated the tissue distribution and developmental change of Sir2α protein in mouse fetuses from E10.5 to E16.5. To compare each section with others we carefully standardized our experimental conditions. Although Sir2α protein was expressed ubiquitously in embryos at all stages examined, we found specific and prominent immunoreactivity in the heart, brain, spinal neural tube, and dorsal root ganglion at E10.5–E13.5. Histological abnormalities in the pancreas and lung in Sir2α-null mice had been found in a previous study [18], but these organs did not show predominant expression of Sir2α protein. In the embryonic heart, the most intense immunostaining was seen from E10.5 to E12.5 (Fig. 3A,B). The expression of Sir2α in the heart of E13.5 embryos had slightly decreased (Fig. 3C), and the immunoreactivity had clearly decreased on E14.5 and E16.5 (Fig. 3C–E). In the developing brain, strong staining for Sir2α was detected at the wall of the telencephalic vesicle on E10.5 (Fig. 3A). As brain formation proceeded, strong immunoreactivity was evident in the diencephalon, hindbrain, spinal cord, and dorsal root ganglia of E12.5 and E13.5 embryos (Fig. 3B,C). Immunoreactivity of Sir2α in the central nervous system was slightly decreased on E14.5 (Fig. 3D). We found that the neural expression level of Sir2α on E16.5 was reduced compared with those on E10.5–E15.5 (Fig. 3F). Hey2, a Hairy-related basic helix-loop-helix (bHLH) transcription factor, has recently been reported to bind to Sir2α [20]. Hey2 is thought to contribute to ventricular development of the embryo. Sir2α may interact with Hey2 and regulate ventricular formation. We compared the expression pattern of Sir2α in the heart ventricles with that in the atrium (Fig. 4). In the E12.5 heart, Sir2α was expressed strongly in the atrium (Fig. 4D) and the ventricle (Fig. 4G) at almost the same levels. Strong immunostaining of nuclei was observed in cells in the atrium and ventricle. In the E14.5 embryo, the expression levels of Sir2α in both chambers (Fig. 4E,H) were reduced to comparable extents, and intensity of nuclear staining was also reduced. The E16.5 heart showed weak expression of Sir2α similar to the levels of expression in other organs (Fig. 4F,I). Myocytes in the atrium and ventricle showed identical time courses of Sir2α expression.

3.6. Expression levels of Sir2α in the developing heart

To examine the expression levels of Sir2α mRNA and protein in developing hearts, total RNA and tissue lysates were

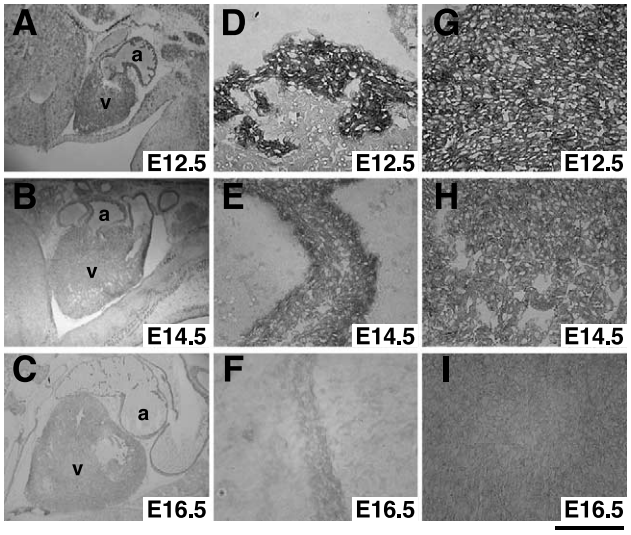

Fig. 4. Expression of Sir2α protein in developing hearts on E12.5 (A), E14.5 (B) and E16.5 (C). Whole embryonic sections (5 μm) were immunostained with anti-Sir2α antibody. Middle panels (D–F) and right panels (G–I) show a higher magnification of the atrium and ventricle of the heart, respectively. Prominent expression of Sir2α was found in the atrium and ventricle in E12.5 embryos, and the expression levels gradually decreased. a, atrium; v, ventricle. The scale bar represents 1 mm in panels A–C and 100 μm in panels D–I.

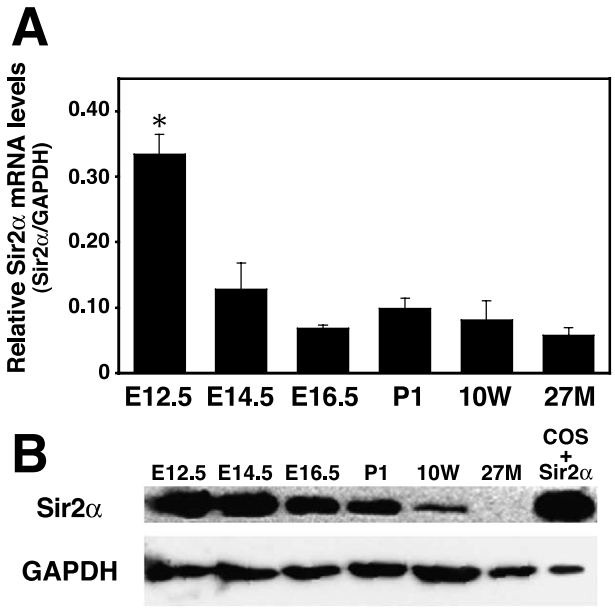

Fig. 5. Sir2α mRNA and protein expression levels in mouse hearts at different developmental stages. A: Sir2α mRNA expression levels in the embryonic heart (E12.5, E14.5, E16.5), neonatal heart from day 1 (P1), and adult heart from 10 weeks and 27 months were examined. Real-time quantitative RT-PCR was performed, and Sir2α mRNA levels were normalized to GAPDH mRNA levels. Values are averages ± S.E.M. ($n = 4$). *$P < 0.05$, significantly different from 10 W. B: Developmental changes in the amount of Sir2α protein in the mouse heart were determined by Western blot analysis. Heart lysates were prepared at the indicated time points. Each lane contained 100 μg of protein. The amount of protein loaded on the gels was determined by reprobing the same membrane with an anti-GAPDH antibody. Cell lysates from COS7 cells that had been transfected with Sir2α cDNA were used as controls.

prepared from embryonic hearts on E12.5, E14.5 and E16.5, from hearts on postnatal day 1 (P1), and from hearts of 10-week-old (10W) and 27-month-old (27M) mice. Real-time quantitative RT-PCR analysis was used for precise measurement of the Sir2α mRNA expression levels (Fig. 5A). Sir2α mRNA was detected not only in the embryonic and newborn hearts but also in the young adult and senile hearts. The E12.5 heart expressed the highest level of Sir2α mRNA, which is consistent with our immunohistochemical observation. The content of Sir2α mRNA on E14.5 was about 40% of that on E12.5, indicating that a rapid decrease in Sir2α mRNA had occurred between E12.5 and E14.5. The expression level of Sir2α mRNA on E16.5 was 21% of that on E12.5 and almost the same as that on P1. After birth, the level remained constant up to 27 months of age. Expression of Sir2α protein in hearts of developmental stages are shown in Fig. 5B. In accordance with Sir2α mRNA levels, the amounts of 120-kDa bands decreased gradually with development of the heart. The protein content was further reduced after birth. These data are consistent with results of Northern blot analysis (Fig. 2) and immunohistochemical analysis of embryos (Figs. 4 and 5).

4. Discussion

Northern blot analysis of embryos from E4.5 to E18.5 showed that there was abundant expression of Sir2α mRNA in these embryonic stages. The highest level of expression was detected in embryos as early as E4.5. Since two-cell-stage embryos have been shown to express Sir2α [18], Sir2α may be continuously expressed throughout embryogenesis and may play a role in early embryonic development. We also found that Sir2α mRNA was still expressed in the middle and late embryonic stages. In E10.5–E14.5 embryos, prominent expression of Sir2α was detected in the heart and nervous system. Immunohistochemical analysis also showed down-regulation of Sir2α expression after E13.5 in the embryonic heart and after E15.5 in the nervous system. The results of a quantitative RT-PCR experiment also showed a rapid decline in Sir2α mRNA content in the heart between E12.5 and E14.5. Expression profiles of Sir2α in both the heart and brain suggest that Sir2α plays a critical role in the formation of these organs. The results of our study are consistent with those of previous studies demonstrating developmental defects in the brain [18,19] and heart [19] in Sir2α-null embryos.

The heart is the first organ to differentiate and function for delivery of nutrients in the embryo. Development of the heart starts around E7.5, and beating of the heart, which is just a simple tube, is observed at the end of E8. A four-chambered heart appears by about E12 [21]. Sir2α is strongly expressed on E10.5–12.5, the period of development in which the chambers of the heart are formed. These findings led us to speculate that strong expression of Sir2α may function as one of the regulators of cardiac morphogenesis. Since cardiomyocytes continue to proliferate and enlarge until the postnatal period, the significance of a high level of expression of Sir2α may be secondary to the proliferation of undifferentiated cells. The brain also begins to differentiate at early stages of embryogenesis. The earliest appearance of what can be considered brain tissues is distinguishable on E7.5. By E12, the diencephalon differentiates into the thalamus, epithalamus and hypothalamus, and the hindbrain differentiates into the cerebellum and medulla oblongata [21]. Differentiation of the neural tissues continues after birth. The diencephalon and hindbrain exhibited intense expression of Sir2α on E12.5 (Fig. 3B). The first notable reduction in the level of expression of neural Sir2α occurred between E13.5 and E14.5, which is similar to cardiac Sir2α (Fig. 3C,D). There may be a second decrease in Sir2α expression level in the brain between E15.5 and E16.5 (Fig. 3E,F). The slower decline in Sir2α expression level in the brain compared to that in the heart may reflect developmental differences between the two organs. Detailed examination of temporal and spatial expression patterns of Sir2α in the brain may be necessary to understand the function of Sir2α in brain formation.

Many transcription factors have been delineated as regulators of cardiogenesis [22,23] and neurogenesis [24]. Hes1 and Hey2, which are classified as bHLH transcription factors, have been reported to bind to Sir2α [20]. Sir2α enhanced transcriptional repression activities of Hes1 and Hey2. Hes1 is expressed in neural precursor cells, and its expression level decreases as neurogenesis proceeds [25]. It prevents premature neural differentiation, and down-regulation of Hes1 is required for precursor cells to enter the differentiation process. Comparable expression patterns of Hes1 and Sir2α suggest that the two proteins are coordinated in the process of neural development. Sir2α may function in the inhibition of differentiation and/or proliferation of undifferentiated cells. Hey2, which is detectable on E7.5 in the primitive heart tube, exhibits ventricle-specific expression and is presumed to regulate morphogenesis of the ventricle [26,27]. Since we detected al-

most the same levels of immunoreactivity of Sir2α in the ventricle and atrium (Fig. 4D,G), transcriptional factors other than Hey2 may be involved in the development of the atrium. Although Hey2 and Hes1 are attractive candidates for transcription factors that orchestrate the heart and brain formation with Sir2α, further studies are required to identify target proteins of Sir2α. Recently, Sir2α was reported to interact with MyoD, a bHLH transcription factor, and to inhibit differentiation of muscle precursor cells to mature muscle cells [28]. Thus, Sir2α may function as an inhibitor of differentiation.

The anti-apoptotic property of Sir2α [15,16] may be another regulatory mechanism involved in organ formation. In addition to cell proliferation, apoptosis is also a component of cardiogenesis [29,30] and neurogenesis [31,32]. Interestingly, TUNEL-positive cells have been detected in the mouse cardiac ventricle from E13 [33], when a decrease in Sir2α expression level was observed. Thus, there is a possibility that Sir2α inhibits apoptosis in the developing heart, though an anti-apoptotic function of Sir2α has been demonstrated only for apoptosis induced by p53 and the role of p53 in cardiogenesis is still unknown.

A quantitative RT-PCR experiment and Western blot analysis detected Sir2α expression in adult hearts. Although Sir2α is thought to contribute to embryogenesis, it may play another role in adulthood.

The results of this study suggest that Sir2α is involved in embryogenesis, particularly in the development of the heart and brain. Functions of Sir2α appear worth investigating further for a better understanding of mammalian organogenesis.

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