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CK2 α is Essential for Embryonic Morphogenesis

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Abstract

CK2 is a highly conserved serine-threonine kinase involved in biological processes such as embryonic development, circadian rhythms, inflammation and cancer. Biochemical experiments have implicated CK2 in the control of several cellular processes and in the regulation of signal transduction pathways. Our laboratory is interested in characterizing the cellular, signaling and molecular mechanisms regulated by CK2 during early embryonic development. For this purpose, animal models, including mice deficient in *CK2* genes, are indispensable tools. Using *CK2 α* gene deficient mice, we have recently shown that CK2 α is a critical regulator of mid-gestational morphogenetic processes, as *CK2 α* deficiency results in defects in heart, brain, pharyngeal arch, tail bud, limb bud and somite formation. Morphogenetic processes depend upon the precise coordination of essential cellular processes in which CK2 has been implicated, such as proliferation and survival. Here we summarize the overall phenotype found in *CK2 α* ^{−/−} mice and describe our initial analysis aimed to identify the cellular processes affected in *CK2 α* mutants.

Keywords

CK2; morphogenesis; embryo development; proliferation; apoptosis

Introduction

CK2 is a highly conserved serine-threonine kinase with orthologs in all eukaryotes from plants to yeast to mammals. CK2 is involved in important biological processes such as embryonic development [1–4], circadian rhythms [5], inflammation [6] and cancer [7, 8]. CK2 regulates essential cellular processes such as viability [9], cell proliferation and growth [10], cell survival [11], cell fate specification [12–14], differentiation [15, 16], metabolism [17], cell cytoarchitecture [18] and migration [19].

In mammals, two different genes encode for CK2 kinases, *CK2 α* and *CK2 α '*. *CK2 α* and *CK2 α '* can be found as monomeric kinases and as a tetrameric holoenzyme composed of two catalytic subunits, *CK2 α* and/or *CK2 α '*, and two regulatory subunits, *CK2 β* [20–22]. *CK2 β* changes *CK2 α* / α ' substrate specificity and confers CK2 holoenzyme membrane binding ability [23, 24]. *CK2 β* also has *CK2 α* / α '-independent roles [25, 26]. The importance of CK2 in morphogenetic processes during mammalian embryonic development is reflected in the phenotypes obtained upon genetic depletion of the *CK2* genes in mice [1–3, 27]. *CK2 α '* gene deficiency results in viable offspring, but leads to male sterility with oligospermia and sperm displaying defective head morphogenesis [1]. *CK2 β* gene

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deficiency leads to early post-implantation lethality at embryonic day (E) 6.5 with embryos showing no preamniotic canal and an inner cell mass with a small, probably blastocoelic, cavity [2]. Conditional deletion of *CK2 β* in embryonic neural stem cells leads to telencephalic defects including absence of oligodendroglial cells at the corpus callosum level and of the emerging hippocampal dentate gyrus [27]. *CK2 α* gene deficient mice die by embryonic day (E)11 and display abnormal heart tube, hypoplastic limb buds and pharyngeal aches, open neural tube and tailbud defects [3, 7]. In this report we revise the phenotypic features found in *CK2 α* ^{-/-} mice and describe initial analysis identifying cellular processes affected in *CK2 α* mutant embryos.

Materials and methods

Freshly dissected embryos were collected at embryonic day E9.5 and E10.5, photographed in an Olympus SZX16 stereomicroscope, washed in PBS and fixed overnight at 4°C with 4% paraformaldehyde in 1× PBS. The following day, embryos were dehydrated and stored at 4°C, or embedded and sectioned [12]. To examine differences in somite size, photographs from four somite-matched pairs of *CK2 α* ^{+/+} and *CK2 α* ^{-/-} embryos were used to measure the area and perimeter of selected somites using CANVAS software. Measured area and perimeter of somites 5 and 16 was analyzed with a t-test (Excel).

For whole-mount TUNEL/ anti-phospho-histone H3/Ser10 (anti-phH3) staining, three pairs of somite-paired *CK2 α* ^{-/-} and *CK2 α* ^{+/+} embryos were hydrated, treated with 10 µg/ml of proteinase K/ 10mM Tris HCl pH 7.5 for 10–15 min at room temperature (RT) and stopped in 2 mg/ml glycine in 1× PBS/0.1% Tween (PBSt) for 10 min at RT. Embryos were then washed 3× in PBSt and stained for TUNEL with an *in situ* cell death detection kit, fluorescein (ROCHE) following manufacturer's instructions. After TUNEL staining, embryos were washed in PBSt and blocked in 10% goat serum/PBSt for 1 h at RT. Embryos were then washed, incubated with anti-phH3 (Upstate) at 1:500 in PBSt for 1.5 h at RT, washed and incubated with anti-rabbit AlexaFluor 594 (Invitrogen) at 1:1000 in the same buffer for 1 hour at RT. Then, embryos were washed, counterstained with DAPI (Invitrogen) at 1:10,000 for 5 min at RT, washed and stored in PBSt at 4°C. Embryos were rocking in all the steps except for the TUNEL incubation. In each experiment, as a positive control for TUNEL, an additional embryo was treated with RQ1-DNase (Promega), and, as a negative control, another embryo was treated with a solution without TUNEL enzyme and incubated without primary antibody. Stained embryos were photographed in an Olympus SZX16 stereomicroscope. Pictures were pseudocolored using ImageJ (NIH).

For TUNEL/ pH3 staining in sections, slides with comparable sections of two pairs of somite-paired *CK2 α* ^{-/-} and *CK2 α* ^{+/+} embryos were warmed, deparaffinized and hydrated. Sections were circled with a Pap Pen (Ted Pella, Inc.) and slides were treated with 10 µg/ml of proteinase K/ 10mM Tris HCl pH 7.5 for 15 min at RT. Slides were rinsed twice in 1× PBS and stained for TUNEL as described above. After TUNEL staining, slides were washed in 1× PBSt and blocked in 10% goat serum/PBSt for 1 h at RT. Slides were then washed, incubated with antiphH3 at 1:200 in 1% goat serum/PBSt for 1 h at RT, washed and incubated with anti-rabbit AlexaFluor 594 at 1:400 in the same buffer for 1 h at RT. One section of each slide was treated with RQ1-DNase as a positive control for TUNEL, and another section, incubated with a solution without TUNEL enzyme and no pH3 antibody, served as a negative control. Slides were counterstained with DAPI as described above, mounted with ProLong Gold (Molecular Probes, Inc.) and photographed in a Nikon Eclipse TE-2000E/Photometrics CoolSnap *HQ²* camera with NIS-elements software. Pictures were pseudocolored and overlaid using ImageJ. Total nuclei and antibody-stained nuclei were counted, percentage of positive cells/total number of cells calculated (mitotic and apoptotic indexes), and significance analyzed with a Wilcoxon rank sum test (R software). p values

<0.05 were considered significant. Represented mitotic index values are mean $\pm$ standard deviation.

RT-qPCR analysis was performed as described in [28] and radioactive RT-PCR as described in [12] in two pairs of somite-paired *CK2 α* ^{-/-} and *CK2 α* ^{+/+} embryos. The number of amplification cycles was optimized to ensure that the PCR reaction was within its linear range. Primers: 5'-TCTTCAAAGACCCAGGGATG-3' and 5'-GGACTGAAAGCCAGACGAAG-3' for *delta-like 1 (DIII)*; 5'-TGAGGGAGAGCGCAGGCTCAAG-3' and 5'-TGCTGTCCACGATG GACGTAAGG-3' for *myogenin*; 5'-GCCCTCAACTGTCTCTCTG-3' and 5'-GGGAGCTGTTTCTCGACTG-3' for *Sal-like 4 (Sall4)*.

Results and discussion

Heterozygous *CK2 α* mutant mice have a normal life span and, so far, seem phenotypically normal. In contrast, *CK2 α* ^{-/-} embryos die *in utero* around E11. Embryonic defects in *CK2 α* ^{-/-} embryos were apparent at E9.5. At E9.5, 95% of *CK2 α* ^{-/-} embryos displayed open neural tubes at the midbrain level (Table 1) in contrast to 18% of *wild-type (WT)* embryos. Defects in the pharyngeal arches (precursors of cranial nerves, skeletal and muscle elements in the face and pharynx) are also observed at E9.5. Thus, 84% of *WT* embryos had developed first and second pharyngeal arches while 85% of *CK2 α* ^{-/-} embryos had only a hypoplastic first pharyngeal arch and 10% of embryos had no pharyngeal arches (Table 1). Defects in pharyngeal arch formation persist as embryos age, and at E10.5 the total number of pharyngeal arches is diminished in *CK2 α* ^{-/-} embryos compared to *WT* embryos [3]. E9.5 *CK2 α* ^{-/-} mutants also show hypoplastic fore and hindlimb buds compared to *WT* embryos (Table 1) and defective tail bud shape (Table 1, Fig. 1). In addition to these defects, we also observed hypoplastic somites (masses of mesoderm on the sides of the neural tube that will form vertebrae, muscle, and dermis) (Fig. 2A). In order to quantify the effect of *CK2 α* depletion on somite size, we measured the area and perimeter of somites number 5 and 16 in four E9.5 somite-matched embryo pairs. There was a statistically significant decrease in the somite area of both somites (approximately 50%, $p=0.006$) and a significant decrease in somite perimeter of both somites (approximately 30%, $p<0.02$) (Fig 2B). In addition, *CK2 α* ^{-/-} embryos display cardiac malformations described and analyzed elsewhere (Degano *et al.*, submitted). *CK2 α* ^{-/-} embryos probably die of cardiac malfunction, as none of the other defects would be embryonic lethal at this stage of development, although this needs to be confirmed in conditionally deleted *CK2 α* mice.

Previously, we found that *engrailed-1*, a neural marker gene, and *Tbrachyury*, a notochord and primitive streak marker gene, were expressed at similar levels in *CK2 α* ^{-/-} embryos compared to *WT* embryos [3, 7]. In contrast, *myogenin*, a transcription factor involved in skeletal muscle development, was downregulated in *CK2 α* ^{-/-} embryos compared to *CK2 α* ^{+/+} (Fig. 2C) by radioactive RT-PCR. In addition, preliminary RT-qPCR analysis showed that, compared to *CK2 α* ^{+/+}, *CK2 α* ^{-/-} embryos had a decrease of 15 % on *Delta-like 1 (DIII)*, a Notch ligand involved in somite development, and of 28 % on *Sal-like 4 (Sall4)*, a transcription factor involved in pattern formation and cell specification important for heart, limb and brain development [29]. These data are in accordance with the defects in somites, limbs and heart observed in *CK2 α* ^{-/-} embryos.

Since several embryonic regions were hypoplastic in *CK2 α* ^{-/-} embryos, we tested whether proliferation or apoptosis were affected in *CK2 α* ^{-/-} embryos. To test this, we used embryos at E9.5 and E10.5 (before *CK2 α* ^{-/-} embryonic death) to analyze proliferation by phospho-histone H3/Ser10 (pH3) staining and apoptosis by TUNEL staining by whole-mount immunohistochemistry (WIHC). In *WT* embryos, pH3+ cells were readily detected while

TUNEL+ cells were rarely detected (Fig. 3). In *CK2α*^{-/-} embryos there was a decrease in pH3+ cells particularly noticeable in pharyngeal arches, limb buds, somites, tailbud and the heart (Fig. 3, 4). However, we did not observe an effect on TUNEL staining in tissues affected in *CK2α*^{-/-} embryos including limb buds, somites, heart and pharyngeal arches (Fig. 3). Negative controls showed no staining and positive control for TUNEL showed staining in all nuclei (not shown). To quantify the effects on proliferation and apoptosis, we performed immunohistochemistry (IHC) in sections of forelimb buds and somites, from two pairs of E10.5 *WT* and *CK2α* mutants (32–35 somites)(Fig. 5A). Similar to the WIHC staining, a number of pH3+ cells were detected by IHC in forelimb buds and somites while little TUNEL staining was observed. The mitotic index (number of pH3+ cells/ number of DAPI+ cells) in *CK2α* mutants was decreased by 40% in limb buds ($p<0.0001$) and by 50% in somites ($p<0.0001$) compared to *WT* embryos (Fig. 5B). In contrast, the apoptotic index (number of TUNEL+ cells/ number of DAPI+ cells) did not change in the forelimb buds (apoptotic index=0.12, $p=0.6$) and somites (apoptotic index=0.75, $p=0.28$) among genotypes. Negative controls showed no staining and positive controls for TUNEL showed staining in all nuclei (not shown). These results show that CK2α is required for proliferation during early embryogenesis. These data suggest that diminished proliferation, but not increased apoptosis, may explain the defects observed in *CK2α*^{-/-} embryos. We are currently determining the mechanism(s) underlying the proliferation defects in *CK2α*^{-/-} mice.

This predominant role for CK2α in proliferation during early mouse development was somehow surprising, as *in vitro* experiments showed a role for CK2 in both cell proliferation and survival. For example, depletion of CK2 activity with antisense oligonucleotides (AS ODN) and siRNA technology in cells in culture leads typically to a 40–50% decrease in CK2 activity correlating with 50% decreased cell viability and/or 50–100% decrease in proliferation [30–34]. In contrast, genetic *CK2α* depletion leads to an 80% decrease in CK2 activity [7] with no apparent effects in apoptosis (Fig. 3). Similar to *CK2α* mutants, *CK2β* gene deficiency leads to lethality at E6.5 probably due to defects in proliferation and not apoptosis [2]. The mechanism underlying the decreased proliferation is not yet known; analysis of *CK2β*^{+/-} embryonic stem (ES) cells showed no effects on growth rate and G1- and G2-checkpoints [35]. Similarly, conditional deletion of *CK2β* in embryonic neural stem cells leads to telencephalic defects caused by decreased proliferation and defective oligodendrogenesis but not to defects in apoptosis [27]. These data suggest that, during embryonic development, *CK2α* and *CK2β* are mainly controlling cell proliferation. *CK2α* mutants display reduced levels of CK2β at E10.5, therefore, part or all the effects of *CK2α* depletion on proliferation could be due to the downregulation of CK2β. Rescue experiments with stabilized forms of CK2β should address the role that CK2β plays in the *CK2α*^{-/-} proliferative defects.

In contrast to *CK2α* and *CK2β* mutants, *CK2α'* gene deficiency results in viable offspring but leads to male sterility [1]. Sperm display defective head morphogenesis and spermatids had increased apoptosis, however, the underlying cause for the morphogenetic and apoptotic defects are not known [36]. These data suggest that CK2α' may be involved in cell survival during embryonic development. *CK2α*^{-/-} embryos seem to have normal levels of CK2α' by immunoblot and that may explain the lack of effect in apoptosis in this mutant mice. One question that arises is whether the combined depletion of *CK2α/CK2α'* or *CK2α/CK2α'* will result in defects in both proliferation and apoptosis.

Determining the cellular processes controlled by the different CK2 subunits during embryonic development requires a detailed analysis of their endogenous expression pattern. CK2 is expressed in all tissues by northern and immunoblot analysis [1, 37, 38], however, detailed analysis of CK2α and CK2β transcripts and proteins in sections in late-gestation

mouse embryos revealed that, even though CK2 α and CK2 β are expressed in all mouse embryo tissues, there is a spatial and temporal regulation in their expression [39]. Determining the effect that deficiency on one of the three genes has on the levels and expression pattern of the others will help us to address the potential compensation between subunits. For example, could the restriction of the CK2 α ' phenotype to the testis due to lineage restricted expression pattern and/or it could be due to compensation by CK2 α ?; and are the levels of CK2 α ' elevated in specific tissues in CK2 α mutant mice? This could be inferred from data showing that in lung cell lines, CK2 α ' protein levels can rise after CK2 α depletion by siRNA [40], however, this is not true for other cell lines [41]. Subsequent studies of the mechanism of CK2 differential expression in embryos may help understand how CK2 expression is upregulated in cancers.

In summary, CK2 loss of function and gain of function experiments in animal models show the key role that the different CK2 subunits have during animal embryonic development, in particular in morphogenesis. These animal models are also helping decipher which of the cellular functions assigned to CK2 are affected at different times of development and also in adulthood (see articles by David Seldin and Heike Rebholz in this issue); and they allow us to test and confirm predictions derived from biochemical experiments, such as the dependence of CK2 β levels on the presence of CK2 α [41, 30, 40, 7]. Molecular studies in these animal models will help address the biological role of CK2 in signaling pathways, such as Wnt, EGF, TGF β , FGF, Activin, Notch and adiponectin [42–44, 8, 45–52, 13]; and future proteomic analysis in these animal models will be helpful to determine which of the *in vitro* identified substrates [53] plays a role in controlling the development or function of particular tissues during embryogenesis and in adulthood.

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Fig. 1. Photographs of tail buds of E9.5 WT and *CK2α*^{-/-} embryos

Dorsal and right views of *CK2α*^{+/+} and *CK2α*^{-/-} embryos at somite pairs (22–23). Arrows show the increased tail bud width in *CK2α*^{-/-} embryos. Representative photographs of over 70 embryo litters.

Fig. 2. Phenotype of E9.5 *CK2 α ^{-/-}* embryos

(A) Right: right view of 22 somite pair *CK2 α ^{+/+}* and *CK2 α ^{-/-}* embryos (Scale bar: 0.5 mm); left: magnified images of the squares on the right (Scale bar: 0.25 mm). Arrow indicates somite number 16. Somites 5 (s5) and 16 (s16) were analyzed for area and perimeter. Histogram showing average somite area (B) and perimeter (C) $\pm$ standard deviation. (*) denotes $p < 0.05$; (**) denotes $p < 0.005$. (D) Decrease in *Myogenin* transcript expression in *CK2 α ^{-/-}* mutant compared to WT embryos by radioactive RT-PCR. Increasing PCR cycles for both *CK2 α ^{-/-}* and *CK2 α ^{+/+}* are shown. (-RT) Control minus reverse transcriptase.

Fig. 3. Proliferation and apoptosis in E9.5 and E10.5 $CK2\alpha^{-/-}$ embryos
 (A, B) E9.5 embryos (22–24 somite pairs) were stained by WIHC for pH3 (red, A) and TUNEL (green, B). (C) E10.5 embryos (32–34 somite pairs) were stained by WIHC for pH3 (red) and TUNEL (green). Photographs show bright field (BF), and pH3 and TUNEL staining. Fluorescent images were pseudo-colored. Scale bar: 0.5 mm. Arrowheads point to different embryonic tissues. Abbreviations: fl: forelimb bud; h: heart; hl: hindlimb bud; p1: first pharyngeal arch; s: somite.

Fig. 4. Head, limb buds and tailbud of E10.5 *CK2α*^{-/-} embryos

Magnified images of E10.5 embryos (32–34 somite pairs) stained with pH3. Abbreviations: AER: apical ectodermal ridge; di: diencephalon; fl: forelimb bud; hl: hindlimb bud; mes: mesencephalon; met: metencephalon; mye: myelencephalon; p1: first pharyngeal arch; p2: second pharyngeal arch; s: somite; tb: tail bud; tel: telencephalon.

Fig. 5. Proliferation in E10.5 forelimb buds and somites

(A) Composite pH3 (red)/ DAPI (blue) photographs (a to d) and TUNEL (yellow)/ DAPI (blue) photographs (e to h) of limbs and somites. Bracket indicates one somite. Arrows point to TUNEL+ cells. Fluorescent images were pseudo-colored. Scale bar: 50 μ m (B) Histogram represents the average mitotic index (total number of pH3+ cells/ number of DAPI+ cells) $\pm$ standard deviation. A total of 15 sections per tissue were counted from two pairs of *CK2α^{+/+}* and *CK2α^{-/-}* embryos. (***) denotes $p < 0.0005$.

Table 1Phenotype of *CK2a*^{-/-} embryos at E9.5

Tissue	Percent embryos	<i>CK2a</i> ^{+/+}	<i>CK2a</i> ^{+/-}	<i>CK2a</i> ^{-/-}
Pharyngeal arches	Two	84	88	15
	One	16	12	85
	None	0	0	10
	# embryos	19	34	20
Neural tube	Closed	82	88	5
	Open	18	12	95
	# embryos	22	34	21
Tailbud	Normal	62	63	5
	Broad	38	37	95
	# embryos	21	30	20
Forelimb	Present	75	88	43
	Not present	25	12	57
	# embryos	20	33	21