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ORIGINAL ARTICLE

Expression of *Six1* homeobox gene during development of the mouse submandibular salivary gland

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BACKGROUND: Members of the Six family of homeoproteins are expressed in numerous tissues during vertebrate embryogenesis, and are critical regulators of both cell proliferation and survival. Here we report the temporal and spatial expression of *Six1* during maturation of the mouse submandibular salivary gland (SSG) from embryonic day 18.5 (E18.5) to postnatal day 28. Additionally, we examine the role of *Six1* during SSG development using *Six1*-deficient mice.

METHODS: *Six1* expression was assessed by reverse transcription-polymerase chain reaction, Western blot, and immunofluorescence. Proliferation was measured by bromodeoxyuridine (BrdU) incorporation index, and apoptosis was evaluated by TUNEL assay.

RESULTS: Six1 mRNA and protein levels are high in the epithelial SSG cells at E18.5 and decrease progressively in the postnatal maturing SSG. Although SSGs from *Six1*$^{-/-}$ embryos are significantly smaller than wild type SSGs, the histological structures of the SSG acini and ducts are similar. *Six1*$^{-/-}$ salivary epithelial cells exhibit an intrinsic defect in cell proliferation accompanied by a significant reduction in the *Six1* target gene cyclin A1, previously shown to be a critical mediator of *Six1*-induced proliferation.

CONCLUSION: Our results suggest that the reduction in size of *Six1*$^{-/-}$ SSGs is result of a decrease in cell proliferation during development/maturation.

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Introduction

Homeobox genes encode a large and diverse group of DNA binding proteins that act as 'master regulators' of development, performing critical functions in specifying cell proliferation, differentiation, survival, and migration (Pearson *et al*, 2005). The Six family of homeoproteins (Six1-6) are characterized structurally by a divergent homeodomain that is involved in DNA binding, and an N-terminally localized Six domain, which confers cooperative interactions with co-factors (Kawakami *et al*, 2000; Christensen *et al*, 2008). Animal studies demonstrate that the *Six* family members play critical roles in organogenesis via regulating cell growth and survival as well as tissue specification (Christensen *et al*, 2008). Importantly, mutations in *Six* family members are found in numerous human genetic disorders, underscoring their importance in human embryonic development (Christensen *et al*, 2008).

Recently, *Six1*$^{-/-}$ mice were generated, which exhibit perinatal death due to severe muscle hypoplasia that results in inadequate diaphragm muscle development and suffocation at birth. In addition, these animals exhibit hypoplastic or completely lacking kidneys and thymus, craniofacial structure malformations, and defects in neurogenesis (Laclef *et al*, 2003; Li *et al*, 2003; Xu *et al*, 2003; Zheng *et al*, 2003; Ozaki *et al*, 2004; Zou *et al*, 2004; Ikeda *et al*, 2007). Interestingly, cells in the *Six1*$^{-/-}$ affected organs exhibit an increase in apoptosis and a decrease in proliferation (Li *et al*, 2003; Xu *et al*, 2003; Ozaki *et al*, 2004), suggesting that *Six1* is important for the expansion of tissue-specific progenitor cell populations in early development. This expansion is believed to occur because Six1 homeoprotein directly activates numerous cell cycle regulators, including gdnf, c-myc, and cyclin D1 during development (Li *et al*, 2003; Yu *et al*, 2006). Recently, our group identified the tissue-restricted cyclin A1 as a transcriptional target of Six1, uncovering yet another mechanism by which Six1 promotes cell cycle progression (Ford *et al*, 1998; Coletta *et al*, 2004).

Salivary gland development is a dynamic process in which cellular proliferation and survival are carefully controlled during branching morphogenesis, directed, in

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part, by transcriptional regulation (Melnick and Jaskoll, 2000). Although a reduction in salivary gland size has been observed in Six1-deficient mice (Laclef *et al*, 2003), the mechanism by which Six1 influences submandibular salivary gland (SSG) development/maturation is unknown. To address this question, we have analyzed the expression profile of the *Six1* gene during normal SSG development/maturation, and have characterized the effect of *Six1* deficiency in this gland.

Material and methods

Animals

Generation and characterization of Six1 null mice have previously been described (Ozaki *et al*, 2004). The mice were housed at the Center for Laboratory Animal Care at the University of Colorado Denver (UCD) and treated in accordance with the NIH Guide to Humane Use of Animals in Research. All animal protocols were approved by the UCD-Institutional Animal Care and Use Committee (IACUC). To determine the normal temporal expression pattern of Six1, wild type C57Bl6/J mice were used. For Six1, cyclin A1 and cyclin A2 expression analysis, at each time point, SSG from three animals were sampled.

Reverse transcription-polymerase chain reaction

RNA was isolated from SSGs according to the manufacturer's protocol for Trizol reagent (Invitrogen, Carlsbad, CA, USA). Before the RT reactions, all RNA samples were treated with DNaseI for 10 min at room temperature to eliminate genomic DNA contamination. Two micrograms of total RNA per sample was used to generate cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen). The resulting cDNAs were subsequently amplified in a 50 μl reaction mixture containing 1 μM of each primer, 2 mM $MgCl_2$, 0.8 mM dNTPs, and 0.025 U/μl Taq DNA polymerase. Actin was used as a housekeeping control. Primer pairs used to amplify mouse *Six1* (WT allele) were 5′ GAA TCA ACT CTC TCC TCT GG 3′ and 5′TTA GGA ACC CAA GTC CAC CA 3′; EGFP (mutated allele) primers were 5′ CTG GTG ACC ACC CTG ACC TAC 3′ and 5′ TGA TCC CGG CGG CGG TCA CGA A 3′; and actin primers were 5′ TAT CCT GAC CCT GAA GTA CC 3′ and 5′ GGT CAG GAT CTT CAT GAG GT 3′. After denaturation for 2 min at 94°C, 30 cycles of amplification were performed using a thermocycler, followed by a final extension of 10 min at 72°C. The amplification cycling parameters were: denaturation for 30 s at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. After amplification, 20 μl of PCR products was electrophoresed on a 1% agarose gel containing 0.5 μg/ml of ethidium bromide.

Quantitative real-time PCR

Quantitative real-time PCR was performed using a model 7000 instrument (Applied Biosystems, Foster City, CA, USA). Amplicons were detected using Taqman fluorescence probes as described elsewhere (Lie and Petropoulos, 1998). The primers and probes used for this study were as follows: for Six1 5′ AAC TGC AGC AGC TGT GGC T 3′, 5′ GTC GGC CGC GAA GTT TC 3′, and 5′ AAA GCG CAC TAC GTG GAG GCC G 3′ (probe), and for cyclin A1 5′ TTT CCC CAA TGC TGG TTG A 3′, 5′ AAC CAA AAT CCG TTG CTT CCT 3′, and 5′ CCC ACC ACC CAT GCC CAG TCA 3′ (probe). The cyclin A2 and 18S rRNA primers and probes were purchased as Assays-on-Demand gene expression from Applied Biosystems. Target genes were analyzed using standard curves to determine relative levels of gene expression, and individual cDNA samples were normalized according to the levels of 18S rRNA.

Western blot analysis

Submandibular salivary glands were washed with cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% deoxycholic acid, 0.5% SDS, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4 and 1 μg/ml leupeptin). After centrifugation, protein concentrations were measured using a protein assay (Bio Rad, Hercules, CA, USA) according to the manufacturer's instructions. Western blot analysis was performed using anti-Six1 and anti-actin antibodies as described (Ford *et al*, 2000).

Immunofluorescence

Submandibular salivary glands from $Six1^{-/-}$ animals at embryonic day 18.5 (E18.5) were fixed in 4% paraformaldehyde in PBS for 16 h at 4°C. Following fixation, the tissues were washed in PBS, paraffin-embedded, and sectioned at 3 μm. After dewaxing and hydrating in graded alcohol solutions, the sections were treated with 10 mM citric acid pH6.0 in a microwave for 20 min. To prevent non-specific binding, the tissues were blocked with the M.O.M. mouse Ig blocking reagent (Vector Labs, Burlingame, CA, USA) in PBS for 16 h at 4°C. The tissues were then incubated with monoclonal mouse anti-GFP antibodies (Chemicon Int., Temecula, CA), washed with PBS, and incubated with goat anti-mouse IgG conjugated with fluorescein (Calbiochem, San Diego, CA, USA). Tissues were examined under an Olympus BX51 fluorescence microscope equipped with a Penguin 600CL camera. Tissues untreated with primary antibodies were used as negative controls.

SSG measurement, histology, cell proliferation and cell death assays

Pregnant female mice at day 18.5 of gestation were intraperitoneally injected with 100 mg of bromodeoxyuridine (BrdU) per kg body weight. Embryos were collected 2 h later and the SSG volumes were calculated using the formula: volume = $0.5 \times \text{Length} \times \text{Width}^2$. After measurement, the SSGs from wild $Six1^{+/+}$ and $Six1^{-/-}$ embryos were dissected with the aid of a stereoscopic microscope, fixed in 70% ethanol, embedded in paraffin, and sectioned at 3 μm. The sections were either hematoxylin&eosin (H&E) stained or analyzed for cell proliferation or cell death. Cell proliferation was measured via BrdU incorporation using an immunohistochemical analysis kit (GE Healthcare,

Piscataway, NJ, USA), whereas cell death assays were performed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis using an apoptosis detection kit (Intergen, Norcross, GA, USA).

Statistical analysis

All assays were performed at least twice. Student's *t*-test was used for statistical analysis, and $P \leq 0.05$ was considered to indicate statistical significance.

Results

Six1 is dynamically expressed in the maturing SSG

To elucidate the potential role of *Six1* in SSG maturation, we first evaluated the temporal and spatial expression of Six1 in wild-type mouse SSGs from E18.5 to postnatal day 28. Six1 mRNA levels were high in SSGs from E18.5 to postnatal day 4, after which they declined progressively (Figure 1a,b). In accordance with Six1 mRNA expression levels, the amount of Six1 protein

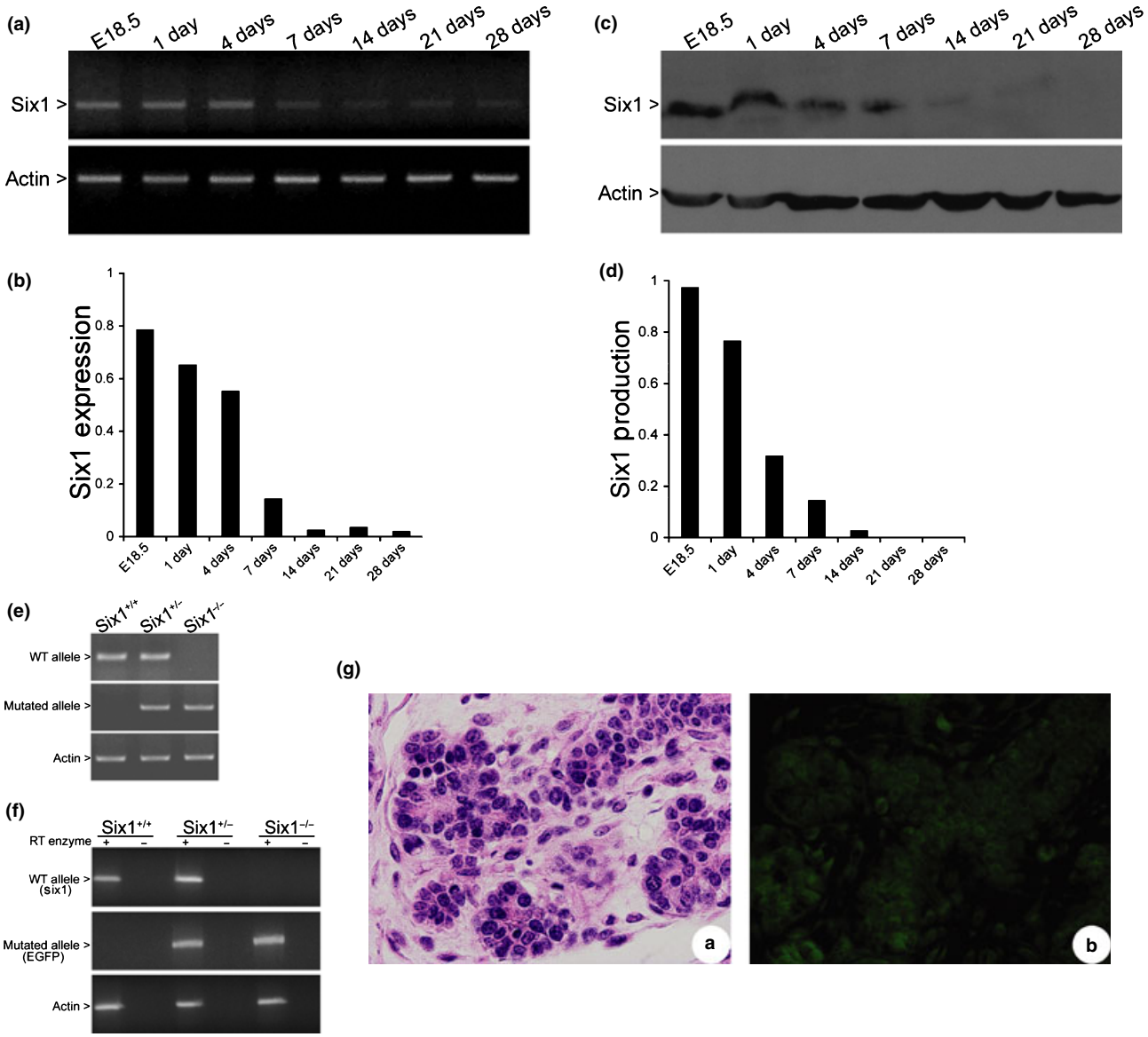

Figure 1 Temporal and spatial expression of *Six1* during submandibular salivary gland (SSG) late embryogenesis is dynamic. **(a)** RNA isolated from wild-type SSGs was subjected to reverse transcription-polymerase chain reaction (RT-PCR) assays using specific primers for *Six1* (actin was used as a control). **(b)** Comparison of *Six1* expression throughout SSG development by densitometric analysis indicates that Six1 mRNA levels progressively decrease from E18.5 to postnatal day 28. Values are expressed in arbitrary units as the ratio of the optical density of Six1/actin. **(c, d)** Western blot and densitometric analyses demonstrate that the Six1 protein is present at high levels in SSG development, and levels decrease throughout the course of development. **(e)** PCR analyses used to genotype $Six1^{+/+}$, $Six1^{+/-}$, and $Six1^{-/-}$ neonates. Tail DNA was isolated and PCR was performed using specific primer pairs as described. **(f)** RT-PCR analyses of SSG from $Six1^{+/+}$, $Six1^{+/-}$, and $Six1^{-/-}$ at day E18.5 confirms the absence of Six1 mRNA and the expression of EGFP in the $Six1^{-/-}$ SSG. **(g)** H&E staining and immunostaining against EGFP, which was knocked into the Six1 locus, demonstrate the expression of Six1 in the ductal and acinar cells of the SSG at E18.5. (Magnification, ×40)

decreased steadily in the developing postnatal SSG as shown by Western blot analysis (Figure 1c,d). To determine which cell types within the salivary gland express Six1, immunofluorescence was performed on E18.5 SSGs isolated from Six1 heterozygote embryos, in which enhanced green fluorescent protein (EGFP) had been knocked into the Six1 locus (Figure 1e,f). EGFP expression was strongly observed in the developing ductal and acinar cells, with some mesenchymal cells showing very low levels of expression (Figure 1g).

Six1$^{-/-}$ *SSGs are significantly smaller than wild-type littermates as a consequence of decreased proliferation*

Our results confirm those from the previous findings (Laclef *et al*, 2003) revealing that *Six1*$^{-/-}$ SSGs are significantly smaller than Six1$^{+/+}$ SSGs (Figure 2a). The SSG volume in *Six1*$^{+/+}$ animals ($n = 28$) ranged from 0.67 to 2.45×10^3 mm^3, with a mean of $1.41 \pm 0.38 \times 10^3$ mm^3, whereas the SSG volume in Six1$^{-/-}$ animals ($n = 21$) ranged from 0.24 to 0.86×10^3 mm^3, with a mean of $0.51 \pm 0.14 \times 10^3$ mm^3 (Figure 2b). Although the Six1$^{-/-}$ SSGs were significantly smaller than wild-type SSGs, the histological structures of the *Six1*$^{-/-}$ SSG acini and ducts showed normal morphogenesis (Figure 2c).

Six1 stimulates proliferation and inhibits apoptosis in both cell culture and in mouse model systems (Li *et al*, 2003; Xu *et al*, 2003; Coletta *et al*, 2004; Ozaki *et al*, 2004; Ikeda *et al*, 2007), suggesting that the loss of *Six1* leads to an exit from the cell cycle and to the premature death of epithelial cells. Thus, BrdU incorporation and TUNEL assays were performed to determine whether the decreased size of *Six1*$^{-/-}$ SSGs was the result of attenuated cell proliferation and/or increased cell elimination by apoptosis. The percentage of total BrdU-positive cells in *Six1*$^{+/+}$ and *Six1*$^{-/-}$ SSGs was 33.92 ± 8.05 and 7.62 ± 4.54% at E18.5, respectively, demonstrating a marked decrease in the number of proliferating epithelial cells in the *Six1*$^{-/-}$ SSGs (Figure 3). However, the number of TUNEL-positive cells was very low in both *Six1*$^{+/+}$ and *Six1*$^{-/-}$ SSG epithelial cells, and no significant difference was observed (data not shown).

Six1 transcriptional target cyclin A1 is decreased in Six1$^{-/-}$ *SSGs*

To investigate the molecular mechanism underlying the proliferation defect in *Six1*$^{-/-}$ SSG epithelial cells, expression levels of cyclin A1, a known transcriptional target of *Six1* that mediates its effects on proliferation, was analyzed. First, the temporal expression of cyclin A1 and its functionally related gene, cyclin A2, was determined throughout SSG development. The temporal expression of cyclin A1 was very similar to that of *Six1*, with high levels of expression in the embryonic SSG. By contrast, cyclin A2 was most highly expressed in the postnatal SSG, although its levels were significantly higher than cyclin A1 throughout the course of SSG development (Figure 4a). Expression of cyclin A1 was significantly lower in *Six1*$^{-/-}$ SSGs as compared

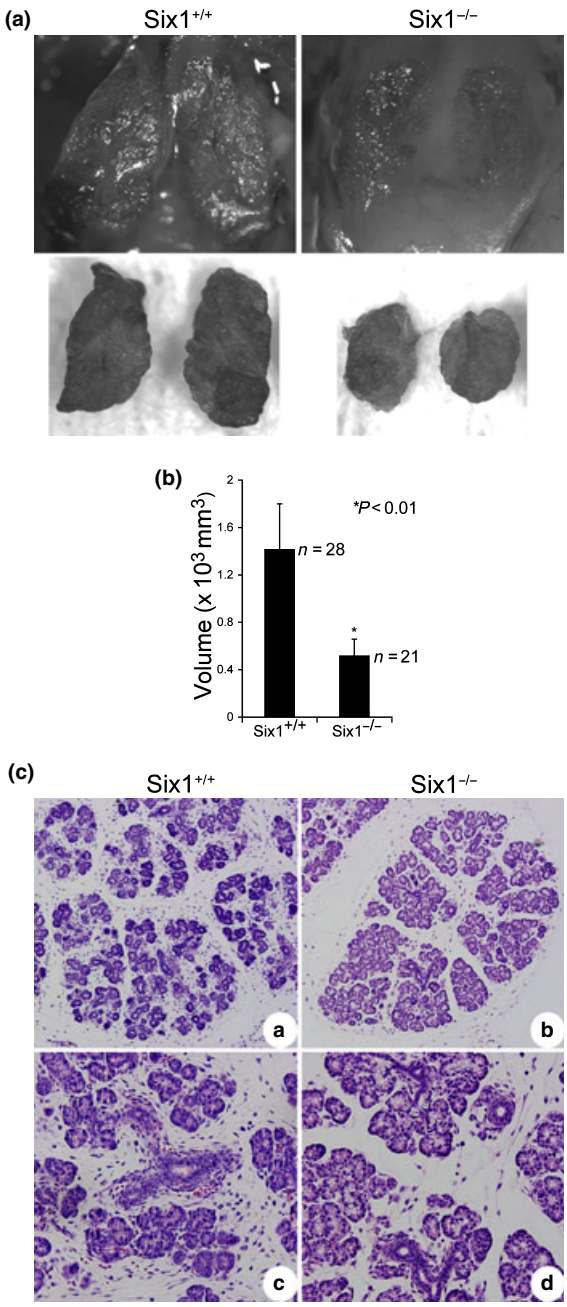

Figure 2 Submandibular salivary gland (SSGs) are reduced in size in *Six1*-deficient embryos. **(a)** Photographs of SSGs from a Six1$^{+/+}$ embryo (left) and from a Six1$^{-/-}$ littermate (right) at E18.5. Top panels represent SSG within the mouse, whereas bottom panels represent glands after dissection from the mouse. **(b)** Measurements of SSG size demonstrate that Six1$^{-/-}$ SSGs are significantly smaller than Six1$^{+/+}$ SSGs $*P < 0.01$. **(c)** E18.5 SSGs from Six1$^{-/-}$ mice are completely normal in structure when compared with litter-matched wild type mice. (Magnification: a, b ×25; c, d ×40)

with *Six1*$^{+/+}$ SSGs ($P < 0.01$), whereas cyclin A2 levels were not changed in a statistically significant manner (Figure 4b). Thus, the proliferation defect observed in *Six1*$^{-/-}$ SSG cells was accompanied by a downregulation of the Six1 transcriptional target cyclin A1, which is highly expressed in the SSG during late embryogenesis.

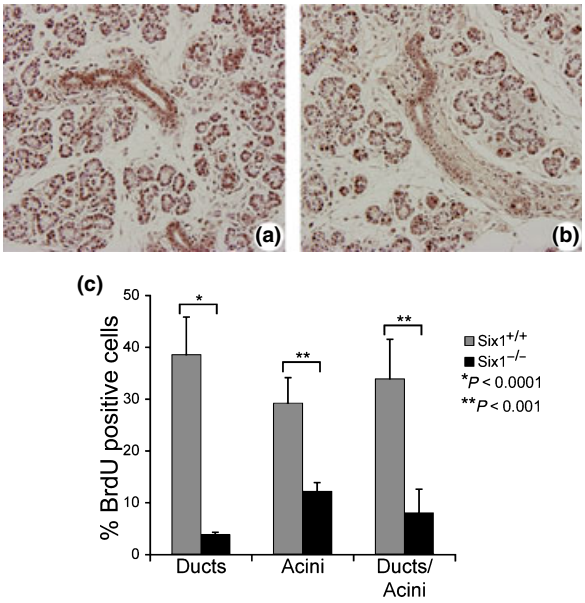

Figure 3 Proliferation is dramatically decreased in *Six1*$^{-/-}$ submandibular salivary gland (SSG) epithelial cells. BrdU-staining in SSGs from Six1$^{+/+}$ **(a)** and Six1$^{-/-}$ **(b)** embryos at E18.5. **(c)** The BrdU indix, expressed as the percentage of positive cells, was determined by counting 1500 cells in five independent samples for each genotype
$^{*}P < 0.0001$ and $^{**}P < 0.001$

Discussion

The SSG develops through a process of branching morphogenesis, undergoing complex stages of cell proliferation and differentiation, which are tightly coordinated by transcriptional regulatory pathways (Melnick and Jaskoll, 2000). However, the molecular mechanisms underlying these events within the SSG remain largely unknown. The homeobox gene family encodes proteins with DNA-binding and transcriptional activities that are critical in normal development (Pearson *et al*, 2005). To date, from a family with more than 200 members, only few homeobox genes are reported to be expressed in the mouse SSG (Raju *et al*, 1993; Jaskoll *et al*, 1998; Tanaka *et al*, 2000; Biben *et al*, 2002). In this study, we have identified the expression of another homeoprotein in the mouse SSG, and we have examined the dependence of SSG maturation on this important developmental molecule. Expression of *Six1* is high during late stages of embryonic development (E18.5), and its levels progressively decline during postnatal SSG development. These findings are consistent with the general role of Six family members, which function in regulating cell proliferation and in specifying cell fate in the developing embryo (Christensen *et al*, 2008).

Studies examining *Six1* deficiency in mice suggest that it is a key participant in development by controlling the expansion of progenitor cell populations in specific organs (Li *et al*, 2003; Xu *et al*, 2003; Ozaki *et al*, 2004). Inner ear development in *Six1*$^{-/-}$ embryos arrests at the otic vesicle stage and all components of the inner ear fail to form because of an increase in apoptosis and a decrease in proliferation of the cells of the otic epithelium (Zheng *et al*, 2003; Ozaki *et al*, 2004). In the kidney, loss of Six1 leads to a failure of ureteric bud proliferation and invasion, with subsequent apoptosis of mesenchymal cells (Xu *et al*, 2003). Recently, it has been shown that Six1 stimulates proliferation and tumor growth through direct activation of cyclin A1 (Coletta *et al*, 2004). In contrast to the studies which demonstrated that Six1 alters both cell proliferation and apoptosis during inner ear and kidney development, no alterations in cell death were observed in *Six1*$^{-/-}$

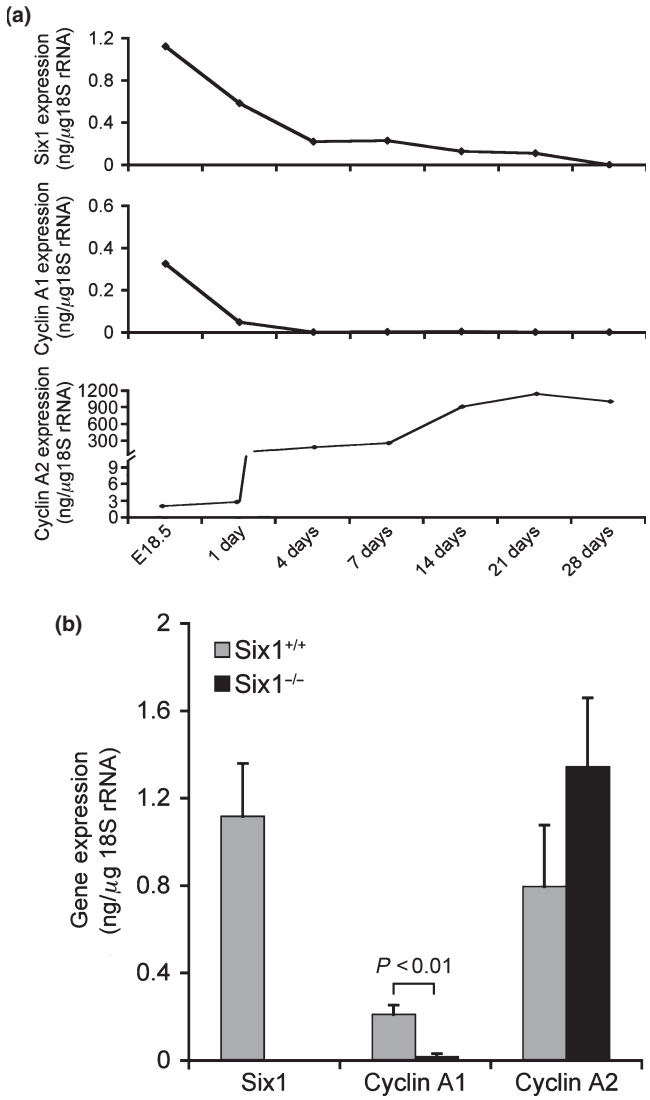

Figure 4 Six1 and cyclin A1 expression are coordinately regulated during embryogenesis, and expression of cyclin A1 is significantly reduced in *Six1*$^{-/-}$ submandibular salivary gland (SSG). SSGs were dissected, RNA isolated and converted into cDNA, and Six1, cyclin A1, and cyclin A2 expression levels determined by quantitative real-time PCR. **(a)** Representative expression of Six1, cyclin A1, and cyclin A2 during different developmental stages of the mouse SSG. Note that Six1 and cyclin A1 have a similar temporal expression during late development of SSG. **(b)** Expression levels of Six1, cyclin A1, and cyclin A2 in the SSG from Six1$^{+/+}$ and Six1$^{-/-}$ embryos at day 18.5. Results represent the average ± s.d. of three independent samples, each sample containing the pooled SSGs of five mice for each genotype. SSGs from Six1$^{-/-}$ mice demonstrated a significant reduction in cyclin A1 expression as compared with Six1$^{+/+}$

SSGs. However, Six1 deficiency in the mouse SSG resulted in a profound decrease in proliferation. Therefore, we conclude from our studies that the significant reduction in size of $Six1^{-/-}$ SSGs occurs as a result of decreased proliferation. Importantly, the lack of an apoptotic effect may be due to the developmental stages analyzed in this study. During SSG development, proliferation seems to be a constant event, whereas apoptosis is rare, except for a few apoptotic cells observed in a large duct at E15 in mouse (Melnick and Jaskoll, 2000), a stage which we did not include in our analysis. Interestingly, apoptosis is associated with rat intercalated duct development (Hayashi *et al*, 2000; Hecht *et al*, 2000).

Although our data clearly demonstrate that Six1 deficiency decreases cell proliferation, the SSG is still properly formed and no difference in the degree of maturity of the gland was observed. The absence of morphological alterations in $Six1^{-/-}$ SSGs confirms that the primary role of Six1 in the SSG is to expand the SSG cell population and is not to influence differentiation or morphogenesis *per se*. It is likely that the SSG is not completely absent in $Six1^{-/-}$ mice because additional factors are involved in the proliferation of SSG cells during development, and these can, in part, compensate for the loss of *Six1*. One possibility is that other *Six* family members compensate for the loss of *Six1*. At this time, the expression pattern of the other four *Six* family members throughout SSG development has not been investigated. Given the documented redundancy between *Six* family members during development (Grifone *et al*, 2005; Konishi *et al*, 2006; Kobayashi *et al*, 2007), another *Six* family member might compensate for the loss of *Six1* and promote proliferation to establish the SSG. Redundancy between *Six1* targets might also compensate for the loss of *Six1*. For example, the Six1 transcriptional target cyclin A1 is, at least in part, functionally redundant with cyclin A2 during embryogenesis (Winston, 2001). Both A-type cyclins bind to and activate the cyclin-dependent kinases 1 and 2 and are required at critical points in the cell cycle, including the progression though S phase and the G2/M transition (Yang *et al*, 1999; Liu *et al*, 2000; Romanowski *et al*, 2000). Here we demonstrate that cyclin A1 expression is dramatically reduced in the $Six1^{-/-}$ SSG, whereas cyclin A2 levels remain unchanged. Thus, it is possible that cyclin A2 partially compensates for the loss of cyclin A1 in the *Six1*-deficient SSG. Furthermore, as no SSG size alterations have been reported in cyclin A1 null mice (Liu *et al*, 1998), it is likely that other cyclin A1-independent proliferative pathways important for SSG development are affected by *Six1*.

In summary, we demonstrate that *Six1* is important for SSG development by controlling the proliferation that is necessary for the expansion of SSG epithelial cells. Recent evidence suggests that *Six1* may play important roles in multiple tumor types, as its overexpression has been observed in breast (Ford *et al*, 1998; Coletta *et al*, 2004, Reichenberger *et al*, 2005), ovarian (Behbakht *et al*, 2007), hepatocellular carcinoma (Ng *et al*, 2006), cervical carcinoma (Wan *et al*, 2008), Wilms' tumor (Li *et al*, 2002), and alveolar rhabdomyosarcoma (RMS) (Khan *et al*, 1999; Yu *et al*, 2004). Interestingly, in some cases, *Six1* expression is high in cancers derived from tissues where it is normally expressed and where it plays a functional role during development, including kidney and muscle (Khan *et al*, 1999; Li *et al*, 2002). As the molecular pathways involved in carcinogenesis often represent aberrations of normal processes that control embryogenesis (Abate-Shen, 2002), it is of interest to determine whether *Six1* can contribute to salivary gland tumorigenesis.

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Conflict of interest

There is no conflict of interest.

Author contributions

Dr McCoy and Dr Coletta were responsible for the experiments and manuscript preparation. Dr Ford and Kawakami were responsible for manuscript revisions.

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