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RESEARCH REPORT

Cholesterol metabolism plays a crucial role in the regulation of autophagy for cell differentiation of granular convoluted tubules in male mouse submandibular glands

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ABSTRACT

It has been long appreciated that sex hormone receptors are expressed in various non-gonadal organs. However, it remains unclear how sex hormones regulate the morphogenesis of these non-gonadal organs. To address this issue, we used a male mouse model of androgen-dependent salivary gland morphogenesis. Mice with excessive cholesterol synthesis in the salivary glands exhibited defects in the maturation of granular convoluted tubules (GCTs), which is regulated through sex hormone-dependent cascades. We found that excessive cholesterol synthesis resulted in autophagy failure specifically in the duct cells of salivary glands, followed by the accumulation of NRF2, a transcription factor known as one of the specific substrates for autophagy. The accumulated NRF2 suppressed the expression of *Foxa1*, which forms a transcriptional complex with the androgen receptor to regulate target genes. Taken together, our results indicate that cholesterol metabolism plays a crucial role in GCT differentiation through autophagy.

KEY WORDS: Cholesterol metabolism, Autophagy, Salivary glands, Differentiation, Duct formation

INTRODUCTION

Rodent submandibular glands (SMGs) are composed of acini and duct systems that contain intercalated ducts (IDs), striated ducts (SDs), granular convoluted tubules (GCTs) and excretory ducts (Pinkstaff, 1980). The GCTs are located between the IDs and SDs, and develop from SDs with the onset of puberty, in an androgen-dependent manner, in male rodents (Dunn and Wilson, 1975). Therefore, rodent SMGs display morphological and functional sexual dimorphism (Caramia, 1966a). Previous studies showed a prominent role for testosterone in the maintenance of the GCTs (Berkman and Kronman, 1970). For example, castrated male mice have regressed GCTs that resemble those of female mice (Bhoola et al., 1973; Caramia, 1966a,b; Chrétien, 1977; Kaiho et al., 1975; Rogers and Brown-Grant, 1971). Moreover, mice with testicular feminization (*Tfm*), a spontaneous single-base deletion in the androgen receptor (*Ar*) gene, have GCTs with fewer secretory granules and increased cytoplasmic vacuoles (Matsuura et al., 1984). A recent study shows that mice deficient for *Ar* ($Ar^{F/F}$;*CAG-Cre* mice) exhibit GCT maturation defects (Adthapanyawanich et al., 2015). Thus, GCT maturation in the SMGs in male mice is one of the established models for studying androgen-dependent non-gonadal tissue differentiation.

Autophagy – Greek for 'the eating of oneself' – is a major catabolic process that degrades cytoplasmic components and proteins via the ubiquitin-proteasome system (Shintani and Klionsky, 2004). A growing number of studies indicate that autophagy and autophagy-related molecules play a crucial role in cell differentiation (Mizushima and Levine, 2010). In addition, a relationship between cholesterol metabolism and autophagy regulation has been suggested in several pathological conditions (Barbero-Camps et al., 2018; Cheng et al., 2006; Seo et al., 2011); however, the nature of this relationship has not yet been clarified in development and organogenesis.

In this study, we show that excessive cholesterol synthesis in the salivary glands results in differentiation defects in the duct system, through the inactivation of autophagy, in mice with an epithelial tissue-specific deletion of either *Insig1/2*, an endogenous inhibitor for an HMG-CoA reductase that enhances cholesterol synthesis, or *Atg7*, a key enzyme for autophagy.

RESULTS

Male mice with excessive cellular cholesterol synthesis display maturation defects in the GCTs of the SMGs

To investigate the contribution of cellular cholesterol metabolism to salivary gland development, we analyzed mice with an epithelial tissue-specific deletion of the *Insig1* and *Insig2* genes (*K14-Cre;* $Insig1^{F/F}$;$Insig2^{-/-}$ mice, hereafter referred to as *Insig1/2* cKO mice). To investigate the early developmental stage of salivary duct formation, we conducted histological analysis of the SMGs in male and female wild-type and *Insig1/2* cKO mice at E14.5, and from P0 to P21, and detected no developmental defects in both sexes until P21 (Fig. S1). However, male *Insig1/2* cKO mice exhibited impaired GCT formation. GCTs differentiated from SDs after postnatal day (P) 28 when the male mice reached sexual maturity (Fig. 1A). The ductal area in the submandibular glands was significantly reduced from 48% and 53% to 15% and 30% at P28 and P56, respectively, in male *Insig1/2* cKO mice without altered cell proliferation and death (Fig. 1B; Fig. S2). Consistent with the GCT maturation defects in *Insig1/2* cKO mice, there were a few eosinophilic granules in the apical cytoplasm of GCT cells, while GCTs in wild-type control mice contained abundant granules (Fig. 1C). We confirmed that loss of *Insig1/2* had less impact on other cell types (Fig. S3).

Compromised androgen receptor signaling in the GCTs in *Insig1/2* cKO mice

To examine the androgen receptor (AR) signaling pathway, we performed quantitative RT-PCR analyses for the cysteine-rich

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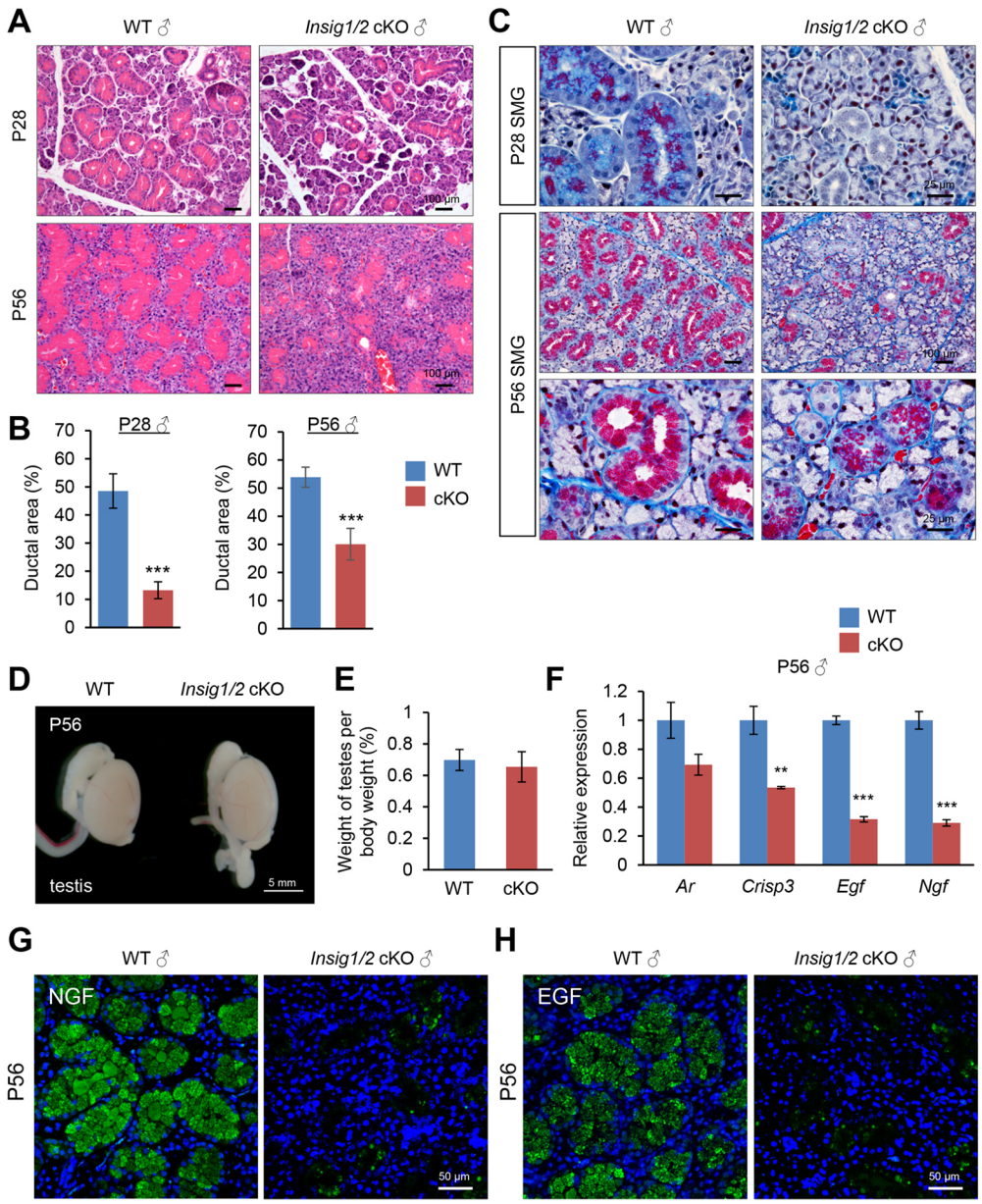

Fig. 1. Phenotypic analyses of the salivary glands of *Insig1*$^{Fl/F}$;*Insig2*$^{-/-}$; *K14-Cre* cKO mice. (A) Hematoxylin and Eosin staining of the submandibular glands (SMGs) from P28 and P56 male wild-type (WT) control and *Insig1/2* cKO mice. Scale bars: 100 µm. (B) Quantification of ductal area per total gland area in P28 and P56 male wild-type (blue bars) and *Insig1/2* cKO (red bars) SMGs. n=6 per group. ***P<0.001. (C) CAB staining of the SMGs from P28 and P56 male wild-type and *Insig1/2* cKO mice. Scale bars: 25 µm in top and bottom panels; 100 µm in middle panels. (D) Gross pictures of the testes from P56 wild-type control and *Insig1/2* cKO mice. Scale bar: 5 mm. (E) Testis weight as a percentage of body weight at P56. Wild type, blue bar; cKO, red bar. n=6 per group. (F) Quantitative RT-PCR analyses for the indicated genes in the SMGs of P56 male wild-type control (blue bars) and *Insig1/2* cKO (red bars) mice. n=6 per group. **P<0.01; ***P<0.001. (G) Immunohistochemical analysis of NGF expression (green) in the SMGs of P56 male wild-type control and *Insig1/2* cKO mice. Nuclei were stained with DAPI (blue). Scale bars: 50 µm. (H) Immunohistochemical analysis of EGF expression (green) in the SMGs of P56 male wild-type control and *Insig1/2* cKO mice. Nuclei were stained with DAPI (blue). Scale bars: 50 µm.

secretory protein 3 (*Crisp3*), epidermal growth factor (*Egf*) and nerve growth factor (*Ngf*), which are known as downstream targets of the AR pathway (Schwidetzky et al., 1995), in the SMGs of wild-type control and *Insig1/2* cKO mice. We confirmed that development of the testes, the main source of androgens, was not affected in *Insig1/2* cKO mice (Fig. 1D,E; Fig. S3E). Although *Ar* expression was not altered in the SMGs of *Insig1/2* cKO mice, compared with that of wild-type control mice, expression of *Crisp3*, *Egf* and *Ngf* was significantly downregulated in P28 and P56 SMGs of *Insig1/2* cKO mice (Fig. 1F; Fig. S4). The GCTs contain numerous secretory granules that contain EGF and NGF in the cytosol on the apical side of the nucleus (Barka, 1980; Gresik et al., 1981; Mudd and White, 1975). As expected, we detected EGF- and NGF-positive GCT cells in the SMGs of wild-type mice. By contrast, these cells were dramatically decreased in number in *Insig1/2* cKO mice (Fig. 1G,H; Figs S4 and S5). Taken together, our results indicate that excessive cholesterol synthesis results in impaired GCT maturation in *Insig1/2* cKO mice.

Increased cholesterol synthesis suppresses *Atg7* expression

Several studies suggest that cholesterol metabolism may be linked to the regulation of autophagy and/or of the autophagic machinery (Cheng et al., 2006; Seo et al., 2011). In addition, increasing numbers of studies indicate that molecules involved in autophagy play crucial roles in a variety of non-autophagy pathways, including differentiation (Subramani and Malhotra, 2013). To investigate the link between aberrant cholesterol metabolism and autophagy activity, we performed immunohistochemical analyses for LC3, ubiquitin (Ub) and p62/SQSTM1, an adaptor/receptor protein for ubiquitylated proteins (Komatsu et al., 2007), in the SMGs of *Insig1/2* cKO and wild-type mice. Previous studies have shown that Ub and p62/SQSTM1 increase when autophagy is suppressed (Ichimura et al., 2008; Komatsu et al., 2005). We found that Ub and p62/SQSTM1 accumulated only in SMG duct cells of *Insig1/2* cKO mice (Fig. 2A). We further confirmed these findings by immunoblotting (Fig. 2B). In addition, we treated SMG explants

Development (2019) 146, dev178335. doi:10.1242/dev.178335

from P28 male wild-type and *Insig1/2* cKO mice with rapamycin, an autophagy inducer, or bafilomycin, an autophagy inhibitor that blocks fusion of autophagosomes with the lysosomes, in order to investigate the flux of autophagy (Klionsky et al., 2016) (Fig. 2C). We found that the steady-state level of autophagy was decreased due to decreased autophagic flux in the SMGs of *Insig1/2* cKO mice compared with wild-type controls (Fig. 2D). Taken together, our results strongly suggest that autophagy is compromised in *Insig1/2* cKO mice, resulting in a failure of GCTs to differentiate in the SMGs. To investigate whether and how the autophagic machinery was involved in the altered GCT differentiation in the SMGs of *Insig1/2* cKO mice, we performed PCR array analyses for molecules related to the autophagic machinery [*Atg3*, *Atg5*, *Atg7*, *Atg10*, *Atg12* and beclin 1 (*Becn1*)]. Among them, expression of *Atg7*, a key enzyme for autophagy and autophagic machinery, was specifically and significantly decreased (Fig. 2E). Previous studies indicate that *Insig1/2* deficiency results in increased translocation of the sterol regulatory element-binding protein (SREBP) to the nucleus (Engelking et al., 2005). As expected, SREBP1 and SREBP2 protein levels were increased in the nucleus in *Insig1/2* cKO SMGs compared with controls, suggesting that these increased SREBPs suppressed *Atg7* expression (Fig. 2F). Bioinformatics analyses revealed that there was a putative sterol regulatory element (SRE; 5′-CTGGGGTGT-3′), where SREBP can bind, in the *Atg7* promotor region (−384 bp to −376 bp). To test the binding of SREBP proteins (SREBP1 and SREBP2) to the *Agt7* promoter region, we performed chromatin immunoprecipitation (ChIP) assays and found that both SREBP1 and SREBP2 could bind to the *Atg7* promoter region in wild-type SMGs, but not in *Insig1/2* cKO SMGs (Fig. 2G). Thus, while SREBP levels increased in the nucleus of the cells in SMGs of *Insig1/2* cKO mice, these SREBPs failed to bind the *Atg7* promoter region in the *Insig1/2* cKO background. The epigenetic mechanism involved in the regulation of *Atg7* promoter accessibility is currently unknown and likely complex.

Male mice deficient for *Atg7* display similar maturation defects in the GCTs of the SMGs

To confirm that autophagy deficiency results in GCT maturation defects, we analyzed mice deficient for *Atg7*, a key enzyme for autophagy, in the salivary glands (*Atg7*$^{F/F}$*;K14-Cre* mice; hereafter *Atg7* cKO mice). As expected, male *Atg7* cKO mice exhibited GCT maturation defects, as seen in *Insig1/2* cKO mice. GCT differentiation was remarkably suppressed in the SMGs of *Atg7* cKO mice at P28 and at a later age, although there was no duct developmental defect before P21 (Fig. 3A,B; Figs S6-S9). The ductal area was significantly decreased in the SMGs of P56 *Atg7* cKO mice, compared with those of wild-type control mice (Fig. 3C). There was less impact on duct formation in female P56 *Atg7* cKO mice (Figs S10 and S11). Without defects in testis development (Fig. 3D,E; Fig. S8F), the expression of molecules regulated through the AR signaling pathway was significantly downregulated in the SMGs of male *Atg7* cKO mice, as seen in *Insig1/2* cKO mice (Fig. 3F-H; Fig. S9). The autophagy deficiency in *Atg7* cKO mice was confirmed with immunohistochemical staining and immunoblotting for Ub and p62 (Fig. S12).

Autophagy regulates GCT differentiation through NRF2-mediated *Foxa1* expression

Previous studies indicate that the cytosolic accumulation of p62/SQSTM1 consequently results in the activation of the NRF2 cascade (Ichimura and Komatsu, 2018). To evaluate NRF2 activation/translocation to the nuclei, we carried out immunohistochemical staining of NRF2 in the SMGs of *Insig1/2* cKO and *Atg7* cKO mice. We found that NRF2 was accumulated in the nuclei of duct cells in *Insig1/2* cKO and *Atg7* cKO mice, but not in wild-type control mice (Fig. 4A). *Ar* expression was not altered in either *Insig1/2* cKO or *Atg7* cKO mice, compared with wild-type control mice (Figs 1F and 3F; Figs S4D and S9A). The gene expression of AR downstream targets is regulated through a complex of forkhead box protein A1 (FOXA1) and AR (Augello et al., 2011). *Foxa1* expression was decreased in the SMGs of *Insig1/2* cKO and *Atg7* cKO mice, compared with wild-type controls, at P56 (Fig. 4B). Moreover, gene expression related to endoplasmic reticulum (ER) stress was not altered in the SMGs of *Insig1/2* cKO and *Atg7* cKO mice, compared with wild-type controls (Fig. S13). We therefore hypothesized that the accumulated NRF2 suppressed *Foxa1* expression, but not directly *Ar* expression. To examine whether and how NRF2 regulates *Foxa1* expression, we performed a bioinformatics analysis for the *Foxa1* promoter region and found that it contains four putative NRF2-binding sites [also known as the antioxidant response element (ARE)] (Fig. 4C; Fig. S14). To validate the binding of NRF2 to the *Foxa1* promoter region, we conducted ChIP assays and found that NRF2 bound to NRF2-binding sites (BSs) 3 and 4, but not to the NRF2 BSs 1 and 2, in the *Foxa1* promotor region (Fig. 4D). Furthermore, we confirmed that FOXA1 expression was decreased in the SMGs of *Insig1/2* cKO and *Atg7* cKO mice (Fig. 4E). Taken together, our results indicate that impaired autophagy results in GCT maturation defects through the NRF2-FOXA1 mechanism (Fig. S15).

DISCUSSION

NRF2 was originally proposed to be a cytoprotective factor against reactive oxygen species, as well as against chemical, electrophile and oxidative stresses. Under normal conditions, NRF2 binds kelch-like ECH-associated protein1 (KEAP1), the sole ubiquitin ligase adaptor protein for NRF2, and is degraded by the ubiquitin-proteasome pathway. In presence of a cellular stress, KEAP1 is inactivated so that NRF2 is stabilized and translocates into the nucleus, where it induces the expression of genes involved in cytoprotective roles (also known as the canonical KEAP1-NRF2 pathway). On the other hand, p62 interacts with KEAP1 at the NRF2-binding site, which inhibits binding of KEAP1 to NRF2 in the noncanonical KEAP1-NRF2 pathway. This interaction leads to the release of NRF2 from a degradation complex; the stabilized NRF2 then translocates into the nucleus to regulate target gene expression (Jain et al., 2010). The noncanonical KEAP1-NRF2 pathway has been suggested to have a role in development, disease and, specifically, cancer. For example, a deficiency of *Atg7* in paired box 7 (Pax7)-positive muscle satellite cells (*Atg7*$^{F/F}$*;Pax7-Cre* mice) results in dwarfism and decreased skeletal muscle mass in male mice only after P14, both of which are due to cell proliferation defects through decreased NRF2-target gene expression in the satellite cells (Zecchini et al., 2018). In this study, we found that an activated noncanonical KEAP1-NRF2 pathway resulted in a maturation defect of the GCTs in the SMGs of *Insig1/2* cKO and *Atg7* cKO mice through suppressed *Foxa1* expression. GCT differentiation is distinct after the onset of puberty in male rodents. Therefore, the GCT in the SMGs is a suitable model tissue for studying how sex hormones contribute to non-gonadal organ development. The mechanism revealed in this study may be conserved in the development of various non-gonadal organs with a variety of degrees, as sex hormone receptors are differently expressed in each tissue and cell type.

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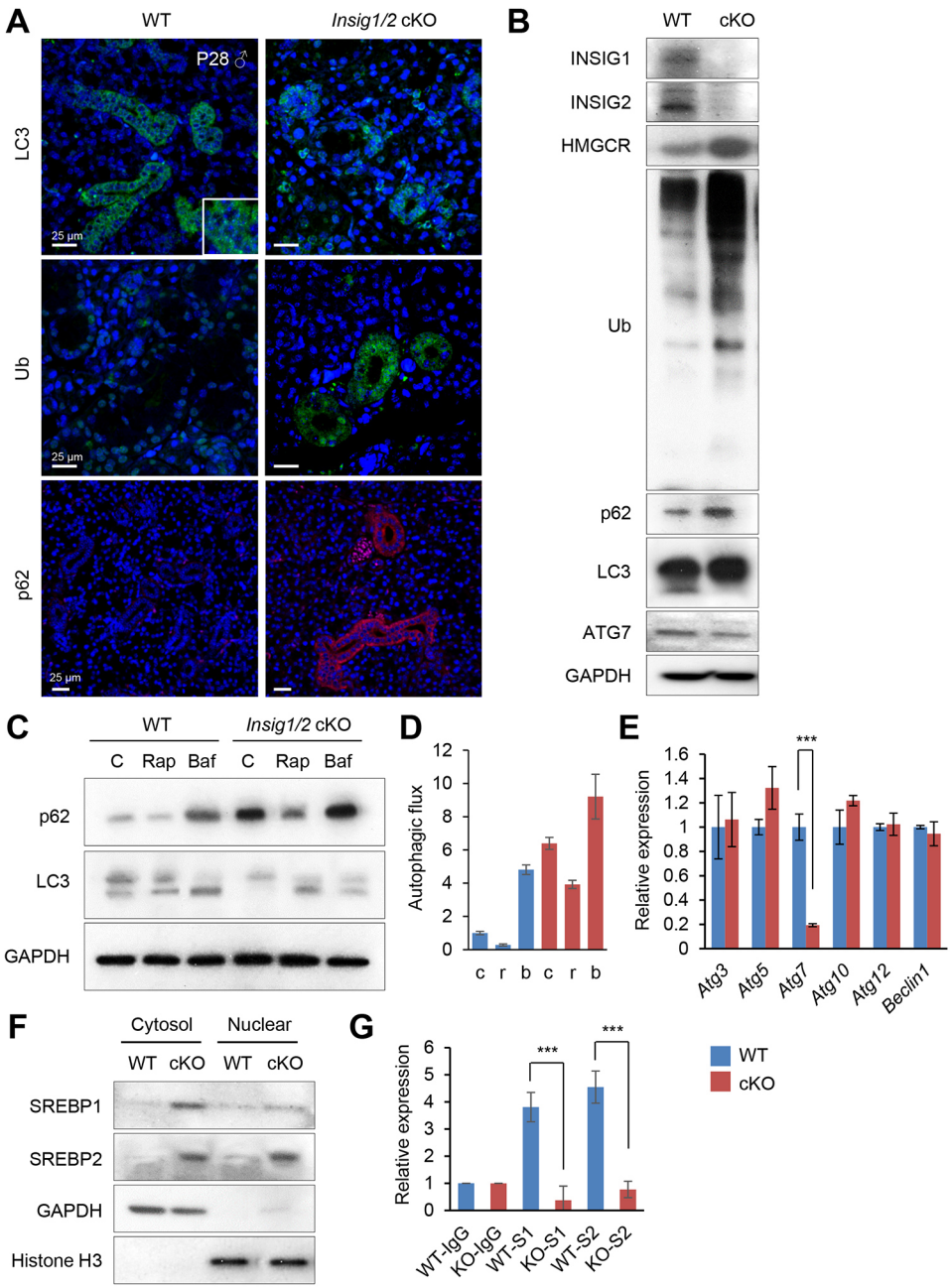

Fig. 2. Impaired autophagy during development of the submandibular glands in *Insig1/2* cKO mice.
(A) Immunohistochemical analysis of LC3 (green), ubiquitin (Ub, green) and p62 (red) in the SMGs of P28 male wild-type and *Insig1/2* cKO mice. Nuclei were stained with DAPI (blue). Scale bars: 25 µm. (B) Immunoblotting for the indicated molecules in the SMGs of P28 male wild-type and *Insig1/2* cKO mice. (C) Immunoblotting for the indicated molecules, after treatment with vehicle control (C), 100 nM rapamycin (Rap) and 200 nM bafilomycin (Baf) for 24 h in SMG explants from wild-type and *Insig1/2* cKO mice. (D) Autophagic flux was measured through quantification of three independent blots for p62 after treatment with control vehicle (c), rapamycin (r), and bafilomycin (b) in wild-type (blue bars) and *Insig1/2* cKO (red bars) mice. (E) Quantitative RT-PCR analyses for genes related to the autophagic machinery in the submandibular glands of P56 wild-type control (blue bars) and *Insig1/2* cKO (red bars) mice. n=6 per group. ***P<0.001. (F) Cell fractionation and subsequent immunoblotting for the indicated molecules in the SMGs of P28 male wild-type and *Insig1/2* cKO mice. (G) ChIP assays of IgG control and SREBP1 (S1) or SREBP2 (S2) for a SREBP-binding site in the *Atg7* promoter region in wild-type (blue bars) control and *Insig1/2* conditional knockout (KO; red bars) SMGs. n=6 per group. ***P<0.001.

Salivary gland cancers occur with a prevalence of 6% among head and neck cancers in the USA. Although there is no salivary duct carcinoma in *Insig1/2* cKO and *Atg7* cKO mice, these mice may have a higher probability of developing cancer when exposed to some carcinogens. Expression of AR in the salivary glands of individuals with salivary duct carcinoma varies by tumor type and accounts for 0 to 86% of cases (Dalin et al., 2017). Among them, salivary duct carcinoma is the most frequent AR-expressing cancer, ranging from 64 to 98% of the cases. Excessive activation of AR signaling is strongly associated with the prognosis and malignancy of cancers in the salivary glands, prostate and breast (Dai et al., 2017; Giovannelli et al., 2018; Robinson et al., 2015). Interestingly, several studies indicate that expression of *NRF2* and *NRF1* is associated with prostate cancers via AR transactivation in humans and mice (Frohlich et al., 2008; Schultz et al., 2014). Indeed, NRF2 activation with either bardoxolone, sulforaphane or curcumin suppresses AR signaling in prostate cancer cell lines (Khurana and Sikka, 2018). Thus, impaired autophagy accelerates NRF2 accumulation, which suppresses AR signaling not only during GCT maturation but also in some cancers.

In addition to FOXA1 binding directly to the consensus sequence (the FKHD motif) to regulate target gene expression, FOXA1 opens chromatin and recruits other transcription factors, such as AR and estrogen receptor α (ERα), to their binding sites in the target genes (Jin et al., 2014). *Foxa1* null (*Foxa1*$^{-/-}$) mice die within 2 weeks of birth and display impaired differentiation of secretory luminal epithelial cells and dysregulated ductal pattern in the prostate (Gao et al., 2005). FOXA1 also plays important roles in bile duct differentiation (Li et al., 2009). Mice with a tissue-specific deficiency for *Foxa1* in the mammary glands or epithelial cells (*Foxa1*$^{F/F}$*;MMTC-Cre* and *Foxa1*$^{F/F}$*;K14-Cre* mice, respectively) exhibit impaired differentiation of the mammary gland ducts

Development (2019) 146, dev178335. doi:10.1242/dev.178335

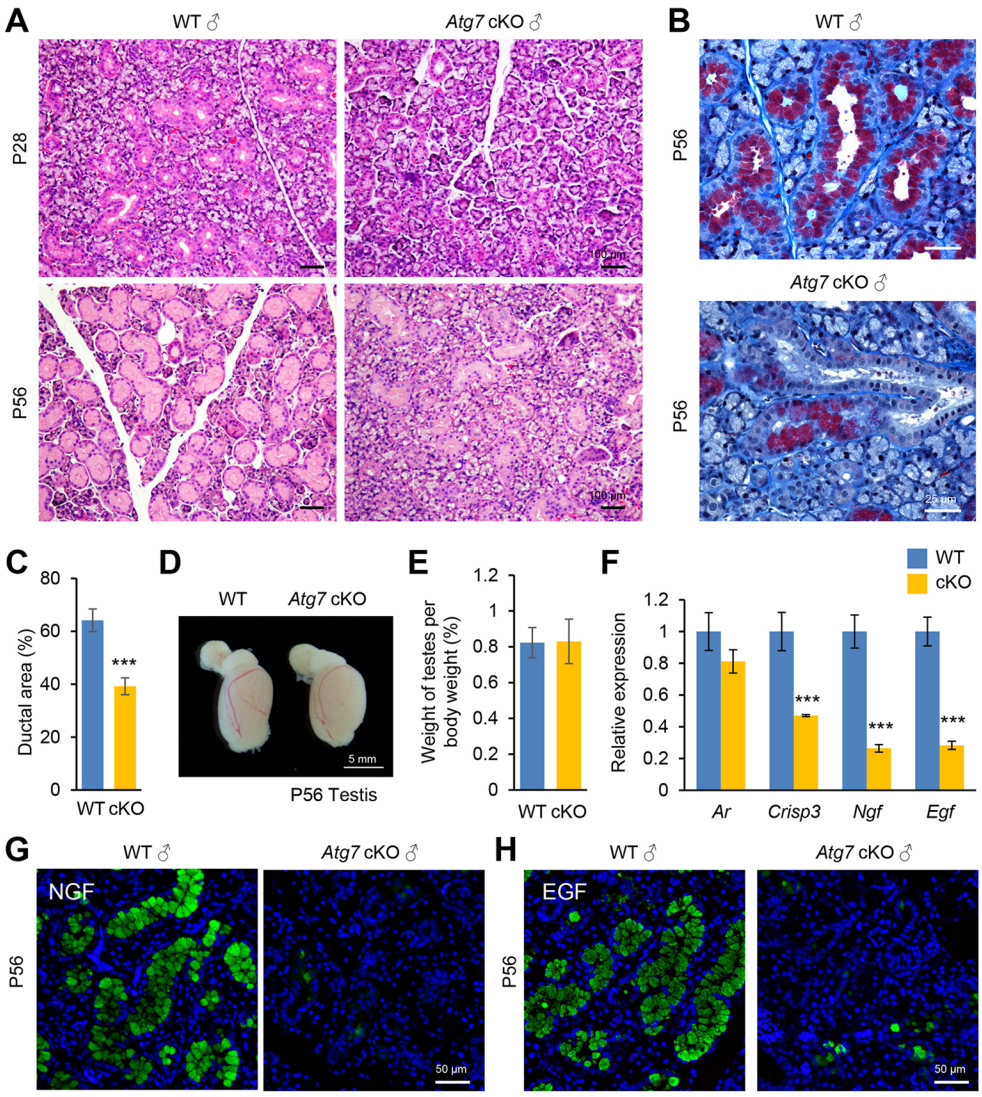

Fig. 3. Phenotypic analyses of salivary glands of *Atg7*$^{F/F}$;*K14-Cre* cKO male mice. (A) Hematoxylin and Eosin staining of the SMGs from P28 and P56 male wild-type control and *Atg7* cKO mice. Scale bars: 100 µm. (B) CAB staining of the SMGs from P56 male wild-type and *Atg7* cKO mice. Scale bars: 25 µm. (C) Quantification of ductal area per total gland area in the SMGs of P56 male wild-type (blue bar) and *Atg7* cKO (yellow bar) mice. n=6 per group. ***P<0.001. (D,E) Gross pictures of the testes (D) and testis weight as a percentage of body weight (E, n=6 per group) for P56 wild-type control (blue bar) and *Atg7* cKO (yellow bar) mice. Scale bar: 5 mm. (F) Quantitative RT-PCR analyses for the indicated genes in the SMGs of P56 male wild-type control (blue bars) and *Atg7* cKO (yellow bars) mice. n=6 per group. ***P<0.001. (G) Immunohistochemical analysis for NGF expression (green) in the SMGs of P56 male wild-type control and *Atg7* cKO mice. Nuclei were stained with DAPI (blue). Scale bar: 50 µm. (H) Immunohistochemical analysis for EGF expression (green) in the SMGs of P56 male wild-type control and *Atg7* cKO mice. Nuclei were stained using DAPI (blue). Scale bar: 50 µm.

through decreased ERα expression, while the alveolar-lineage cells develop and differentiate normally (Bernardo et al., 2010). Thus, *Foxa1* is a promising target gene of AR signaling in duct development. Mutations in the *FOXA1* gene are also associated with the prognosis of human salivary duct carcinoma (Urano et al., 2018), breast cancer (Rangel et al., 2018; Robinson and Carroll, 2012) and prostate cancer (Robinson and Carroll, 2012).

In summary, we have found that, in *Insig1/2* and *Atg7* cKO mice, *Foxa1* expression was compromised through the ATG7-mediated NRF2 pathway, which in turn resulted in the suppression of AR signaling during duct formation, without developmental defects in acinar cells. This mechanism may be applicable to the development of other ductal and non-gonadal organs.

MATERIALS AND METHODS

Animals

Insig1$^{F/F}$;*Insig2*$^{-/-}$ (Engelking et al., 2005) and *K14-Cre* (Dassule et al., 2000) mice were obtained from The Jackson Laboratory. *Atg7*$^{F/F}$ mice (Komatsu et al., 2005) were a gift from Dr Masaaki Komatsu (Juntendo University, Tokyo, Japan). *Insig1*$^{F/F}$;*Insig2*$^{-/-}$ and *Atg7*$^{F/F}$ mice were crossed with *K14-Cre* mice to generate *Insig1/2* cKO and *Atg7* cKO mice, respectively. Genotyping was performed using PCR primers, as previously described (Dassule et al., 2000; Engelking et al., 2005; Komatsu et al., 2005).

Histology

Hematoxylin and Eosin staining, Chromotrope-Aniline blue (CAB) staining, and immunohistochemistry were performed as previously described (Suzuki et al., 2018). The antibodies used for immunohistochemistry were rabbit polyclonal antibodies against EGF (Abcam), LC3 (Cell Signaling Technology), NGF (Abcam), ubiquitin (DAKO), α-SMA (Abcam), AQP5 (Alomone labs) and LAMP2 (Abcam); rabbit monoclonal antibodies against AR (Abcam), NRF2 (Abcam), KIT (Cell Signaling Technology), Ki67 (Abcam), KRT17/19 (Cell Signaling Technology) and MIST1 (Cell Signaling Technology); and a mouse monoclonal antibody against p62/SQSTM1 (Abcam) (catalog codes and dilutions are provided in Table S1). Fluorescence images were obtained using a confocal microscope (Ti-C2, Nikon). Color images were obtained using a light microscope (BX43, Olympus) (n=6 per group). TUNEL staining was performed using a Click-iT Plus TUNEL Assay kit with Alexa 594 (C10618, Molecular Probes), according to the manufacturer's instructions. Fat deposits were investigated using Oil Red O staining. Briefly, frozen sections were fixed in 4% paraformaldehyde (PFA), washed with distilled water, rinsed with 60% isopropanol, stained with Oil Red O (0.18% w/v in isopropanol) for 15 min, and then rinsed with 60% isopropanol and counterstained with 0.04% methylene blue for the nuclei. For Nile Red staining, a stock solution of Nile Red (500 µg/ ml) in acetone was prepared and stored chilled and protected from light. A fresh staining solution of Nile Red was made by adding 2-10 µl of the stock solution to

Development (2019) 146, dev178335. doi:10.1242/dev.178335

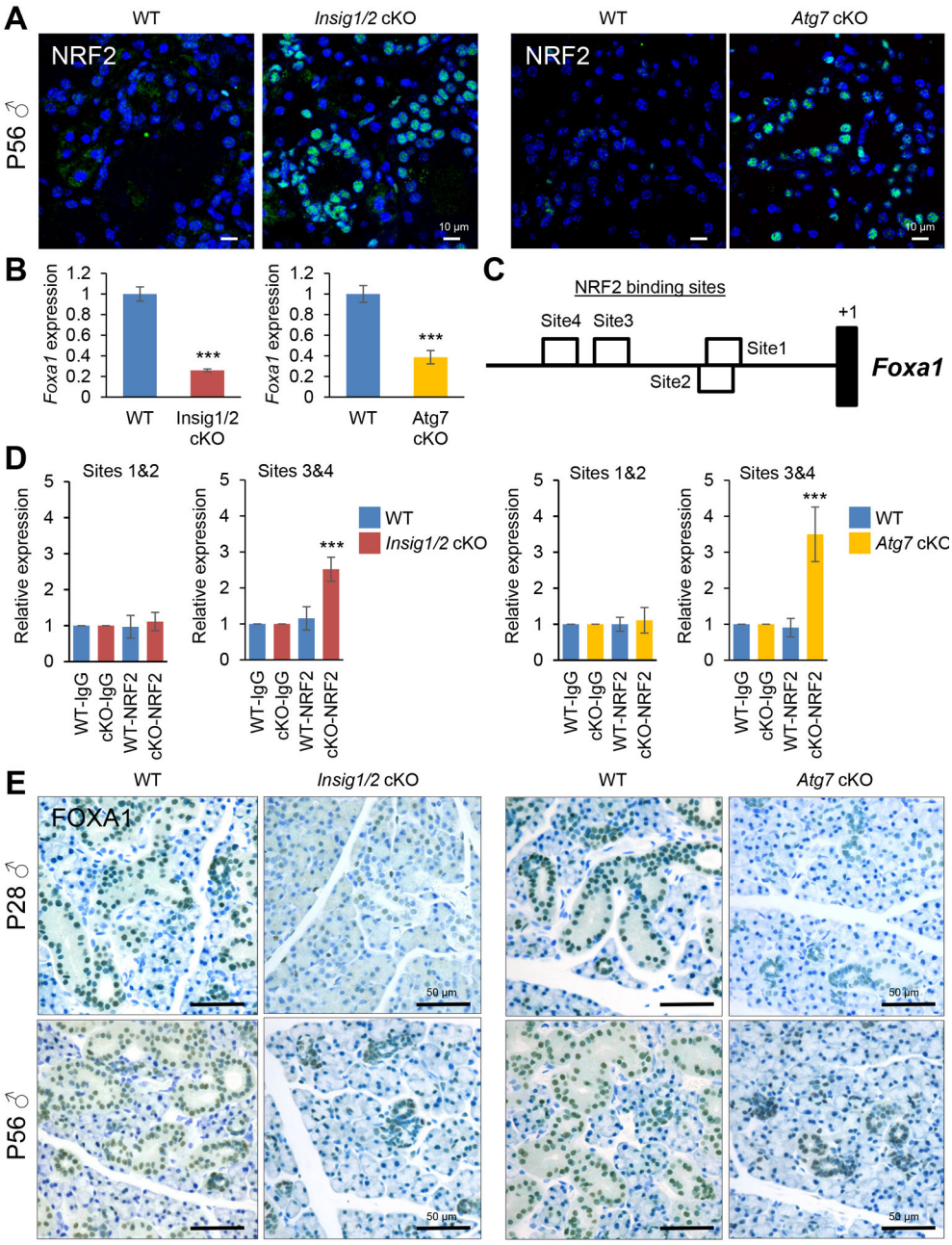

Fig. 4. Compromised *Foxa1* expression during GCT maturation in *Insig1/2* cKO and *Atg7* cKO mice.
(A) NRF2 expression in wild-type, *Insig1/2* cKO (left) and *Atg7* cKO (right) GCTs of male mice at P56. Nuclei were stained with DAPI (blue). Scale bars: 10 µm. (B) Quantitative RT-PCR analyses of *Foxa1* expression in the SMGs of male wild-type (blue bars), *Insig1/2* cKO (red bar) and *Atg7* cKO (yellow bar) mice at P28 and P56. n=6 per group. ***P<0.001. (C) Schematic drawing of the *Foxa1* promoter. (D) ChIP assays for NRF2 binding to the NRF2-binding sites in the *Foxa1* promotor region, using SMG tissues from P56 male wild-type control (blue bars), *Insig1/2* cKO (red bars) and *Atg7* cKO (yellow bars) mice. n=6 per group. ***P<0.001. (E) Immunohistochemical analysis for FOXA1 (brown) in the SMGs of male wild-type, *Insig1/2* cKO and *Atg7* cKO mice at P28 and P56. Nuclei were stained using 0.04% Methylene Blue. Scale bars: 50 µm.

1 ml of 75% glycerol, followed by brisk vortexing. To stain frozen tissue sections, a drop of the glycerol staining solution was added to each section. After 5 min, the sections were examined under a fluorescence microscope (Nikon, ECLIPSE Ti). For Periodic acid-Schiff (PAS) staining, the slides were rehydrated and quenched in 0.5% Periodic acid solution for 5 min, followed by 15 min in Schiff's reagent.

Organ cultures

SMG explants were dissected out from P28 mice and sliced at 1 mm then incubated in BGjB medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 0.1 mg/ ml ascorbic acid and penicillin/streptomycin in a rotor incubator at 37°C for 24 h. The SMG explants were treated with or without rapamycin (LC Laboratories; 200 nM) and bafilomycin (Sigma; 100 nM) for 24 h.

Immunoblotting

SMG tissues were lysed in T-PER Tissue Protein Extraction Reagent (Thermo Scientific). Cell fractionation was performed using a NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific). Equal amounts of protein were analyzed by SDS-PAGE, followed by electrophoretic transfer to PVDF membranes (Millipore). The membrane was blocked for 1 h with blocking buffer (5% skim milk in TBS) and incubated overnight at 4°C with a primary antibody. The antibodies used for immunoblotting were as follows: anti-INSIG1 rabbit polyclonal (Abcam), anti-INSIG2 rabbit polyclonal (Abcam), anti-HMGCR rabbit polyclonal (Santa Cruz), anti-ubiquitin rabbit polyclonal (Dako), anti-p62 mouse monoclonal (Abcam), anti-LC3 rabbit polyclonal (Cell Signaling Technology), anti-ATG7 rabbit polyclonal (Cell Signaling Technology), anti-SREBP1 mouse monoclonal (Novus Biologicals), anti-SREBP2 rabbit polyclonal (Novus Biologicals), anti-histone H3 rabbit polyclonal (Cell Signaling Technology) and anti-GAPDH mouse monoclonal (Millipore) (catalog codes and dilutions are provided in Table S1).

Quantitative RT-PCR

Total RNAs isolated from the submandibular salivary glands (n=6 per group) were dissected with the QIAshredder and RNeasy mini extraction kit (QIAGEN), as previously described (Suzuki et al., 2015). *Gapdh* was used as an internal housekeeping control. The ΔΔ-CT method was applied for the

Development (2019) 146, dev178335. doi:10.1242/dev.178335

analyses. The following PCR primers were used for further specific analysis: *Atg3*, 5′-CATGCAGGCATGCTGAAGTGATG-3′ and 5′-TCGTCTGACAGGGGAAACCC-3′; *Atg5*, 5′-ACCTCGGTTTGGCTTTGGTT-3′ and 5′-TCAGGGGTGTGCCTTCATATTC-3′; *Atg7*, 5′-ATGCCAGGACACCCTGTGAACTTC-3′ and 5′-ACATCATTGCAGAAGTAGCAGCCA-3′; *Atg10*, 5′-CGAGCGAGCGGGTTCTCATT-3′ and 5′-CCAGAGCTAACGGTCTCCCA-3′; *Atg12*, 5′-TGCTGAAGGCTGTAGGAGAC-3′ and 5′-TTACCATCACTGCCAAAACACTCA-3′; *Beclin1*, 5′-ACTCACAGCTCCATTACTTACCAC-3′ and 5′-CTGTGCATTCCTCACACAGC-3′; *Ar*, 5′-TCTGCCTCCGAAGTGTGGTA-3′ and 5′-ACTTCTGTTTCCCTTCAGCGG-3′; *Crisp3*, 5′-ACAGTGGCCATTATCCAAGCA-3′ and 5′-GCATGTAGCTAGGCAACGTTTT-3′; *Egf*, 5′-TAAGGATCCTGACCCCGAACTT-3′ and 5′-CAAATCCTGTGGGGCATGTG-3′; *Ngf*, 5′-GGAGCGCATCGAGTGACTT-3′ and 5′-CCTCACTGCGGCCAGTATAG-3′; *Foxa1*, 5′-GACGCCAAGACATTCAAGCG-3′ and 5′-ATCGTGCCACCTTGACGAAA-3′; *Fas*, 5′-GACCTCAGGCTGCAGTGAAT-3′ and 5′-GTCCCACTTGATGTGAGGGG-3′; *Scd1*, 5′-GAGTAGCTGAGCTTTGGGCT-3′ and 5′-ACTTCATCAGCGGGGACTTG-3′; *Foxo1*, 5′-TGTACAGCGCATAGCACCAA-3′ and 5′-CCGATGGACGGAATGAGAGG-3′; *Foxo3*, 5′-GAGCTGGAGCTCGAACCTT-3′ and 5′-TGTGCCGGATGGAGTTCTTC-3′; *Chop*, 5′-CCTGGTATGAGGATCTGCAG-3′ and 5′-GTTTCCTAGTTCTTCCTTGCTCTTC-3′; *Ire1a*, 5′-CACTGCCTGAGACCTTGTTG-3′ and 5′-TGTTGGGACCTGCAGGAC-3′; *β-actin*, 5′-ACAATGAGCTGCGTGTGG-3′ and 5′-GACAGCACAGCCTGGATG-3′; and *Gapdh*, 5′-AACTTTGGCATTTGGAAGG-3′ and 5′-ACACATTGGGGGTAGGAACA-3′.

Comparative analysis of transcription factor binding sites

The UCSC genome browser was used to obtain the genomic sequences of the murine *Ar* gene (NC_000086.7), *Atg7* gene (NC_000072.6) and *Foxa1* gene (NC_000078.6), including the 5 kb sequence upstream of the respective transcription start site. The sequence was then mapped to seven additional mammalian genomes [human (Build 38), chimpanzee (Build 2.1.4), orangutan (Build 2.0.2), rhesus macaque (Build 1.0), rat (Build 5), dog (Build 3.1) and horse (Build equCab2)] with the BLAST tool, as previously described (Suzuki et al., 2015). The multiple alignments were obtained using the Clustal Omega tool with default parameters and settings (Sievers et al., 2011). The antioxidant response element (ARE), which resembles the NRF2 binding motif (5′-TGAG/CNNNGC-3′), and the sterol response element (SRE), which resembles the sterol regulatory element-binding protein (SREBP) binding sites (5′-TCACNCCAC-3′), were searched in the aligned DNA sequences.

ChIP assay

Tissue extracts from the salivary glands were incubated with either rabbit polyclonal NRF2 antibody (Abcam, ab62352, 5 µg), normal rabbit IgG (Santa Cruz Biotechnology, sc-2027, 1:100), mouse monoclonal SREBP1 antibody (Santa Cruz Biotechnology, sc-13551 X, 5 µg), mouse monoclonal SREBP2 antibody (Santa Cruz Biotechnology, sc13552 X, 5 µg) or normal mouse IgG (Santa Cruz Biotechnology, sc-2025, 1:100) overnight at 4°C, followed by precipitation with magnetic beads. Washing and elution of the immune complexes, as well as DNA precipitation, were performed according to standard procedures, as previously described (Suzuki et al., 2015). The putative ARE on the *Foxa1* promoter in the immune complexes was detected by PCR using the following primers: for sites 1 and 2, 5′-CCAGGTCGGCCTAACGTC-3′ (−1002 bp to −985 bp) and 5′-ACTACCCCTACTTCCCGGC-3′ (−904 bp to −886 bp); for sites 3 and 4, 5′-ATCCGCCTGCCTTATCACAC-3′ (−1201 bp to −1182 bp) and 5′-GAAACTCGTACCTGCGGCT-3′ (−1053 bp to −935 bp). The putative SRE on the *Atg7* gene in the immune complexes was detected by PCR using the following primers: 5′-TCCCAGGCCAAGGATACATG-3′ (−641 bp to −622 bp) and 5′-AGTCTCTTAGCTATCCAGGCA-3′ (−356 bp to −336 bp). The positions of the PCR fragments correspond to NCBI mouse genome Build 38 (mm 10).

Statistics

The two-tailed Student's *t*-test was used for statistical analysis. $P<0.05$ was considered statistically significant. For all graphs, data are mean±s.d.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.S., J.I.; Methodology: A.S., J.I.; Validation: A.S., J.I.; Formal analysis: A.S., J.S., K.O., H.Y., J.I.; Investigation: A.S., J.S., K.O., H.Y., J.I.; Data curation: A.S., K.O., J.I.; Writing - original draft: J.I.; Writing - review & editing: A.S., J.I.; Supervision: J.I.; Funding acquisition: J.I.

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Supplementary information

Supplementary information available online at
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