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The Slc26a4$^{-/-}$ mice thyroid dysfunction

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ABSTRACT

Pendred syndrome is caused by mutations in the anion exchanger pendrin (SLC26A4) and is characterized by deafness, post-pubertal goiter and iodide organification defects. Studies were performed on thyroids from Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at various ages: postnatal days 10 (P10), P15 (corresponding to the time point of maximal thyroid gland activity), P30 (post- weaning) and P80 (young adults). Global gene expression levels in the thyroid were evaluated by gene arrays performed at P15. Thyroxine (T4) levels in the serum were quantified by immunoassay. Gene expression was quantified by qRT-PCR. Western blotting was used to evaluate the expression of two proteins involved in thyroid hormone synthesis, Tpo and Duox1, as well as the chloride channel, ClC-5, oxidized proteins, eNOS and proteins involved in iron metabolism. Nitrated and oxidized proteins were quantified by ELISA. Total iron was measured by ferrozine spectrophotometry. Slc26a4 mRNA expression was modest in the thyroid of Slc26a4$^{+/-}$ mice at all ages. Decreased levels of nitrated proteins were observed in the thyroid of Slc26a4$^{-/-}$ mice at P35. Between the Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice: a) no differences in the serum T4 levels were observed, b) no differences in the expression of Tpo and Duox1 were observed and c) no differences in the levels of oxidized proteins were observed. The absence of elevated levels of oxidized proteins suggests that increased oxidative stress is not present in the thyroid of Slc26a4$^{-/-}$ mice. The low expression of Slc26a4 in mouse thyroid and the normal thyroid gland function in the Pendred syndrome mouse model suggests a distinction between the roles of pendrin in murine and human thyroids.

1. Introduction

Pendred syndrome is an inherited autosomal recessive condition that primarily affects the thyroid and the inner ear. It is characterized by a positive perchlorate discharge test, goiter, and prelingual sensorineural deafness (Cremers et al., 1998; Morgans et al., 1958; Pendred et al., 1896). Pendred syndrome is caused by loss-of-function mutations in the gene SLC26A4, which encodes pendrin. Pendrin is primarily expressed in the inner ear, the thyroid and the kidney (Everett et al., 1999; Royaux et al., 2000; Soleimani et al., 2001). In the thyroid, pendrin localizes to the apical membrane of thyrocytes and mediates iodide transport (Everett et al., 1997; Royaux et al., 2000; Yoshida et al., 2002). Pendrin has been hypothesized to play a role in iodide organification by transporting iodide to the follicular lumen (Bidart et al., 2000). Therefore, lack of pendrin function has been suggested to limit the iodination of thyroglobulin, thereby affecting the synthesis of thyroid hormone (Everett et al., 1997; Morgans et al., 1958; Sheffield et al., 1996).

Clinical studies suggest a role for pendrin in thyroid hormone metabolism. Patients with non-functional pendrin can present with goiter, which can be either hypothyroid or euthyroid. Moreover, mutations in Slc26a4 have been found in congenital hypothyroidism (Banghova et al., 2007). Decreased expression of proteins that are involved in thyroid iodide metabolism, including pendrin, has been shown in thyroid cancer in humans (Elisei et al., 1994; Sheils et al., 1999; Xing et al., 2003). Thyroid carcinomas have also been reported in patients with Pendred syndrome (Abs et al., 1991; Bashir et al., 2004; Ozluk et al., 1998). In the mouse, the thyroid gland activity peaks around postnatal day 15 (P15)

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(Campos-Barros et al., 2000). However, a study that investigated the thyroid histology and serum T3, T4 and TSH levels in adult mice identified no differences between Slc26a4$^{+/+}$ and Slc26a4$^{-/-}$ mice (Everett et al., 2001). Because the previous study was carried out on adult mice, we were prompted to re-evaluate the thyroid pathology in thyroid of Slc26a4$^{-/-}$ mice in a developmental study (Everett et al., 1997; Royaux et al., 2001). Thus, the first aim of the present study was to determine the time course of Slc26a4 expression in the mouse thyroid and to evaluate the effect of absence of pendrin on the expression of genes/proteins involved in the synthesis of thyroid hormone and on serum T4 levels. Pendrin-mediated iodide/Cl- exchange in the apical membrane of the thyrocyte requires the presence of Cl- in the follicular lumen. It has been suggested that the Cl-channel, ClC-5, may assist in Cl- cycling by transporting Cl- into the follicular lumen. If Cl-transport by ClC-5 and iodide transport by pendrin are a coupled mechanism, it is conceivable that lack of pendrin will have an effect on the expression of ClC-5. In one study, Clcn5$^{-/-}$ (Clcn5 encodes ClC-5) mice have been shown to develop euthyroid goiter that correlates with delayed iodide organification and reduced pendrin expression (van et al., 2006). The second aim of this study was to determine whether absence of pendrin affects ClC-5 protein expression in the mouse model of Pendred syndrome. The third aim of this study was to address whether free radical stress and iron metabolism are altered in the thyroid of Slc26a4$^{-/-}$ mice. Absence of pendrin is associated with increased free radical stress and altered iron metabolism in the stria vascularis of Slc26a4$^{-/-}$ mice (Singh et al., 2007), raising the possibility that it also may lead to increased oxidative stress in the thyroid gland. This is particularly feasible, given that hydrogen peroxide (H2O2), a well-known oxidant, is involved in the process of thyroid hormone synthesis (Nakamura, 1994; Ramasarma, 1990). Indeed, cumulative oxidative stress in the thyroid could underly the goiter formation and thyroid carcinomas observed in human patients with Pendred syndrome (Abs et al., 1991; Karbownik et al., 2003; Reardon et al., 1997).

2. Methods

2.1. Animal use

The Slc26a4$^{-/-}$ and Slc26a4$^{+/-}$ mice used in the experiments were raised in a colony at Kashan/Tehran (CRFK), established with breeders kindly provided by Institute of Veterinary Researches, Kashan, Iran.

Deeply anesthetized mice(tribromoethanol, 560 mg/kg, i.p.) were sacrificed either by decapitation or by transcardial perfusion with Cl--free solution (150 mM Na- gluconate, 4 mM Ca2+-gluconate, 5 mM glucose 1.6 mM K2HPO4, 1 mM MgSO4, 0.4 mM KH2PO4, pH 7.4) and dissection was carried out in the same solution at -10 °C. All procedures performed on animals were approved by the Iran national Institute of Veterinary Researches, Tehran, Iran.

2.2. Gene array

Total RNA was isolated from the thyroid of three P15 Slc26a4$^{-/-}$ and Slc26a4$^{+/-}$ mice (RNeasy micro, Qiagen, Valencia, CA, USA) and stored at -80 °C until further processing. RNA was processed at the Gene Expression Facility at Kashan/Tehran (CRFK). A total of six arrays were run from sex-matched littermates. Three chips, each, hybridized with cDNA from thyroids of Slc26a4$^{+/-}$ and Sl c26a4$^{-/-}$ mice, were compared. RNA was amplified to produce cDNA, which was fragmented, biotinylated and hybridized to high-density oligonucleotide gene chips (Ovation™ RNA Amplification System V2, Cat # 3100-12, NuGen, San Carlos, CA and FL- Ovation™ cDNA Biotin Module V2, Cat # 4200-12, NuGen; mouse 430 2.0 gene chip, Cat # 900496, Affymetrix, Santa Clara, CA, USA). Gene array data were analyzed using commercial software (GCOS, Affymetrix) and with custom-written macros (Excel, Microsoft, Redmond, WA, USA), as described previously (Jabba et al., 2006). Present/absent calls were used to determine the expression, and averaged signal intensities (average of data obtained from three chips) were used to determine changes in expression levels. 'Intensity' for gene array data from Slc26a4$^{+/-}$ and for Slc26a4$^{-/-}$ samples represents averages of data from one or more probes. For example, the gene Tg is represented on the chip by two probes. Present calls (P) were summarized for all three chips. For example, 6/6 indicates that this gene was called present by all 6 probes (2 × 3 = 6); 4/6 indicates that the gene is represented by 2 probes on the 3 chips (2 × 3 = 6) and that the gene was called present by 4 of the 6 probes. Ratios of intensity values (Slc26a4$^{-/-}$ to Slc26a4+/-) were calculated for each probe and averaged.

2.3. Quantitative RT-PCR

Total RNA was isolated from the thyroid (RNeasy micro, Qiagen) and kidney (RNeasy min i, Qiagen) of sex-matched Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ littermates and stored at -80 °C until further processing. Real time RT-PCR in the presence of SYBR green (Molecular Probes, Eugene, OR) was carried out in 96-well plates (QuantiTect SYBR Green RT-PCR Kit Cat# 204243, Qiagen, Valencia, CA; iCycler, BioRad, Hercules,CA) using gene specific primers (Table 1).

The RT-PCR plate was set up by programming an automatic pipetting station (Biomek NXp, Beckman Coulter, and Fullerton, CA). RT-PCR was performed as described previously (Singh et al., 2007). Single product amplification was verified by gel electrophoresis and the identity of the product was confirmed by sequencing. The number of template molecules (T) was calculated according to the following formula: T = 10^ log (number of molecules at Ct) / (PCR efficiency ^ Ct), where Ct represents the

cycle at which the fluorescence of the product molecules reached a set threshold. Samples that failed to reach the set threshold within the 40 cycles of PCR were assigned a default Ct value of 40. The number of molecules at Ct was calibrated by amplifying known numbers of 18S rRNA molecules. The content of 18S in total RNA was estimated under the assumption that total RNA consists of 90% 18S and 28S rRNA. PCR efficiency was obtained from the slope of the log-linear phase of the growth curve (Ramakers et al., 2003).

2.4. Serum T4 measurements

Blood samples were collected by cardiac puncture, transferred into microcentrifuge tubes and allowed to clot for 30 min at room temperature. The clotted samples were centrifuged at 1500 rpm for 10 min at room temperature. The serum was collected and centrifuged again at 1500 rpm for 10 min at room temperature. The collected supernatant was stored at -20 °C until further processing. The total serum T4 was measured by a chemiluminescent enzyme immunoassay (Immulite® total T4, Siemens Healthcare Diagnostics, and Deerfield, IL) according to the manufacturer's instructions.

Table 1: Gene specific primers

Gene	Primers	Product
size 18S	gag gtt cga aga cga tca ga (sense) gtt ctt agt tgg tgg agc ga (antisense)	316 bp
Slc26a4	tcg gaa cat caa gac aca tc (sense) acc tca cta tga atc caa tct g (antisense)	252 bp
Ferritin heavy chain (Fth)	ttt gag cct gag ccc ttt (sense) tca aag aga tat tct gcc atg c (antisense)	706 bp
Actb	gga cct gac aga cta cct c (sense) tcg ttg cca ata gtg atg ac (antisense)	210 bp

2.5. Isolation of protein

Freshly dissected thyroids were transferred into microcentrifuge tubes and excess Cl--free solution was removed. Freshly dissected spleens were flash-frozen in liquid nitrogen, then pulverized. Proteins from both tissues were extracted by adding 30 µl of Tris-Triton buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X) and bath sonicated for 30 min at 0 °C (Fisher Scientific, Pittsburgh, PA). The samples were centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant fractions were collected and either used immediately or stored at -80°C.

2.6. Quantification of nitrated proteins

The level of nitrotyrosine in isolated proteins was quantitated by two different immunoabsorbent assays (Cat # HK501, Cell sciences, Canton, MA; Cat #17-376, Millipore, Billerica, MA) according to the manufacturers' instructions. (The total protein content of the sample was measured (NanoOrange protein quantitation kit, Cat # N666, Invitrogen, Carlsbad, CA). Differences in the amount of nitrotyrosine were evaluated by comparison of nitrated protein content to the total protein content.

2.7. Quantification of oxidized proteins

Two different techniques were used to quantitate the oxidized proteins in tissue samples. Oxyblots: Carbonyl groups of oxidized proteins were derivatized with dinitrophenyl hydrazine (Oxyblot Kit, Cat # S7150, Millipore,) to form denitrophenyl hydrazone (DNP). DNP-labeled proteins were separated by SDS gel electrophoresis (5 µl/lane) and detected in western blots. Procedures were carried out according to the manufacturer's recommendations. Differences in the presence of oxidized proteins were evaluated by comparison to the expression of actin or GAPDH. ELISA: Protein was isolated and the level of oxidized proteins was qua ntified by an immunoabsorbent assay (Cat # STA-310, Cell Biolabs, San Diego, CA ;) according to manufacturer's recommendation.

2.8. Quantitative western blotting

An equal volume of Laemmli buffer containing 5% β-mercaptoethanol was added to the protein samples. Protein samples were either incubated at 75 °C for 10 min or at 37 °C for 1 hour. Protein samples (15 µl) were separated by SDS-PAGE (4-15% Tris-SDS Polyacrylamide Precast Gels, Cat # 161-1104, BioRad Laboratories, Hercules, CA). After se paration, proteins were transferred to PVDF membranes (0.2 µm pore size, Cat # 162-0174, BioRad), blocked with 5% dry milk in TBS-Tween (137 mM NaCl, 20 mM Tris-Cl, 0.1% Tween-20, pH 7.6) and probe d w ith primary antibodies (rabbit anti-ClC-5, 1:1000, a gift from Professor Thomas Jentsch, FMP/ Max-Delbrück-Centrum für Molekulare Medizin, Berlin, Germany; goat anti-Tpo, 1:200, Cat # sc-48951, Santa Cruz Biotechnology, Santa Cruz, CA; goat anti-Duox1, 1:200, Cat # sc-49939, Santa Cruz Biotechnology; rabbit anti-Trf, 1:750, Cat #A76, Biomeda, Foster City, CA; mouse anti-Tfrc 1:2,000, Cat #13-6800, Zymed, San Francisco, CA; rabbit anti-eNOS, 1:500, Cat # 610296, BD Biosciences, San Jose, CA; rabbit anti-actin, 1:1000, Cat # A2066, Sigma, St. Louis, MO; mouse anti-GAPDH, 1:300, Cat # MAB374, Chemicon International, Billerica, MA; rabbit anti-tubulin, 1:500, Cat # ab6046, Abcam Inc., Cambridge, MA). Membranes were washed 4x for 15 min each in TBS-Tween and incubated with the appropriate HRP-conjugated secondary antibodies (anti-rabbit,

1:5,000, Cat # 1858415; anti-mouse, 1:5,000, Cat # 1858416, Pierce, Rockford, IL; anti-rabbit, 1:5,000, Cat # anti-goat, 1:5,000, Cat # sc-2033, Santa Cruz Biotechnology). After washing 4x for 15 min each in TBS-Tween, HRP was detected by chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Cat # 34095, Pierce, and Rockford, IL) using a camera-based imaging workstation (4000MM, Kodak). Membranes were stripped and reprobed with an antibody to a reference protein. Differences in protein expression were evaluated by comparison to the expression of actin, GAPDH or tubulin.

2.9. Quantification of total tissue Fe content

A ferrozine-based assay was modified and used for total iron measurement in the tissue (Fish, 1988). Iron measurement in thyroid: Each freshly dissected thyroid was transferred into a microcentrifuge tube and excess Cl--free solution was removed. Guanidine hydrochloride (2 µl) was added to the tissue and mixed by a pulse spin. After incubation for 45 min at 60°C, 2 µl of FAT solution (0.5 M ferrozine, 0.5 M ascorbic acid and 1 M Tris-Cl, pH 7) was added. After mixing by pulse-spinning, the tissue was incubated for 45 min at 60°C. Absorbance at 562 nm of the supernatant was measured (ND1000, Nanodrop Technologies) and standards containing FeCl2 were processed in parallel. Measurements of the total iron (Tissue-Fe) are reported per mg of wet tissue weight. Iron measurement on spleen: Freshly dissected spleens were flash- frozen in liquid nitrogen, then pulverized. Guanidine hydrochloride (100 µl) was added to the tissue and mixed by a pulse spin. After incubation for 45 min at 60°C, 100 µl of FAT solution was added. Mixing was facilitated by a pulse spin and the tissue was incubated for 10 min at room temperature. Absorbance at 562 nm was measured in the supernatant of the 200 µl sample (ND1000, Nanodrop Technologies) and standards containing FeCl2 were processed in parallel. Measu rements of the Tissue-Fe are reported per mg of wet tissue weight.

2.10. Statistic alanalysis

Data for the quality metrics on the gene array experiment are expressed as mean ± S.D. For all other experiments, data are reported as mean ± sem. Data throughout the manuscript were compared by unpaired t-test. Significance was assumed at $P < 0.05$.

3. Results

3.1. Slc26a4 transcript expression does not correlate with the thyroid gland activity

To determine whether pendrin expression correlates with the thyroid gland activity, the mRNA expression of Slc26a4 in the mouse thyroid was evaluated at various ages (Fig 1). RNA was isolated from two pendrin-expressing tissues, thyroid and kidney, of Slc26a4+/– mice at the following ages: P10, P15, post-weaning mice (P30) and young adults (P80), and quantitative RT- PCR was performed. As a quality control to ensure that the RT-PCR reaction worked and that sufficient level of RNA was present, transcript levels coding for ferritin heavy chain (Fth) and ActB were evaluated in parallel with Slc26a4 from the RNA samples. In comparison to the kidney where Slc26a4 was highly expressed at all ages, Slc26a4 expression in the thyroid of Slc26a4$^{+/-}$ mice was modest. No age dependence was observed in the expression pattern of Slc26a4 in either the thyroid or the kidney. Moreover, in a few experiments, Slc26a4 expression was below the level of detection in the thyroid samples since the fluorescence failed to reach the set threshold. Consistent with these results, Slc26a4 was called absent in the gene arrays performed on the thyroid of Slc26a4$^{+/-}$ mice. The relatively low and constant expression of Slc26a4 at all ages in the mouse thyroid indicates that Slc26a4 expression does not increase during the peak of thyroid gland activity at P15 in the mouse.

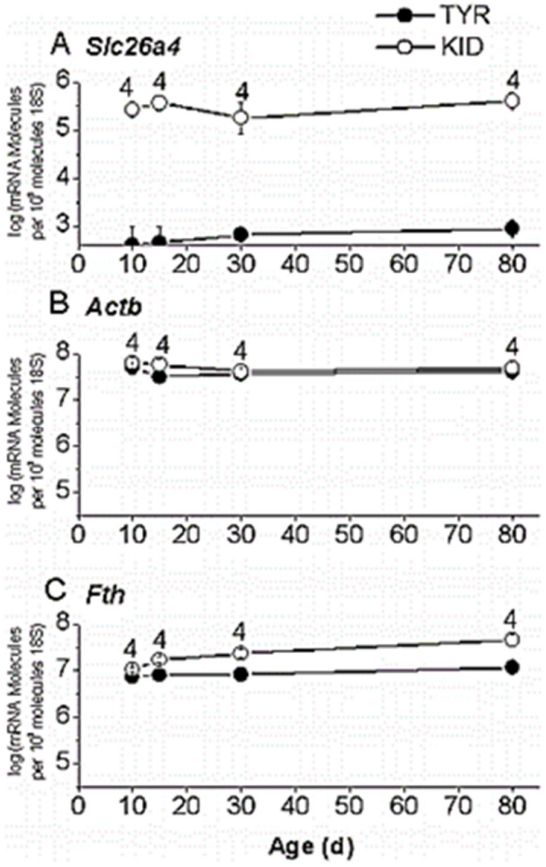

Fig. 1: Slc26a4 mRNA expression in the thyroid is constant over various ages Transcripts coding for A) Slc26a4, B) Actb and C) ferritin heavy chain (Fth) were quantified relative to 18S RNA isolated from thyroid (TYR) and kidney (KID) from Slc26a4$^{+/-}$ mice at P10, P15, P30 and P80 (n=4). Slc26a4 was highly expressed in the kidney and

modestly expressed in the thyroid at all ages. Fth and Actb were highly expressed in the thyroid and kidney of S lc26a4$^{+/-}$ mice at all ages.

3.2. Lack of pendrin does not affect the expression of genes involved in thyroid hormone Synthesis

Gene array data from P15 Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice were analyzed to evaluate the expression of genes involved in thyroid hormone synthesis (Table 2). Gene array data analysis revealed no differences in the expression of Tg, Tpo, Duox2, Dio1 and Dio2 between the Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice.

Table 2: Genes involved in thyroid hormone synthesis

#	Gene	Description	*Slc26a4*$^{+/-}$ Intensity	P	*Slc26a4*$^{-/-}$ Intensity	P	Fold	Significance
1	*Tg*	Thyroglobulin	6,619	6/6	6,507	6/6	1.25	n.s
2	*Tpo*	Thyroid peroxidase	2,737	3/3	2,967	3/3	1.08	n.s
3	*Duox2*	Dual oxidase 2	43	2/3	34	2/3	1.28	n.s
4	*Dio1*	Deiodinase 1	4,028	3/3	3,830	3/3	1.05	n.s
5	*Dio2*	Deiodinase 2	735	9/9	1114	9/9	1.56	n.s
6	*Slc26a4*	Pendrin	7	0/3	8	0/3	-	-
7	*NIS*	Sodium iodide symporter	1,157	6/6	935	6/6	1.12	n.s

For parameters 'P', and 'Fold' see section: Methods.Genearray. Fold differences are given when a gene was called Present (P) in Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ samples in at least half of the available probes.

Western blots were performed to evaluate the protein levels of Tpo and Duox1 in the Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at various ages (Fig 2). Although densitometry was not performed, visual inspection did not reveal obvious differences in the protein expression of Tpo and Duox1 between Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice. These observations suggest that the absence or presence of pendrin does not affect the expression of genes/ proteins that are involved in the synthesis of thyroid hormone.

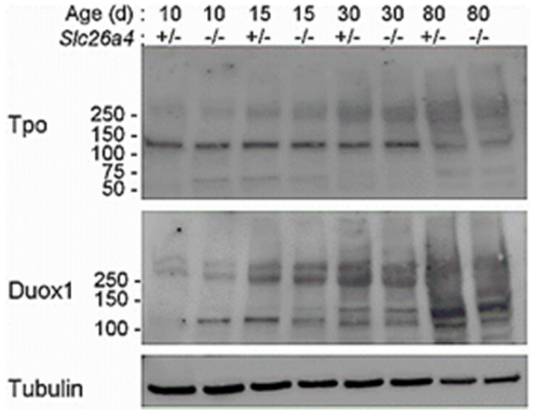

Fig. 2: No visible difference between the protein expression of Tpo and Duox1 in the thyroid of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice. Representative western blots for Thyroid peroxidase (Tpo), dual oxidase 2 (Duox1) and tubulin in thyroid of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at P10, P15, P30 and P80 (n=3). Consistent with the gene expression data, no differences in the protein expression of Tpo and Duox1 were detected in the thyroid of Slc26a4$^{-/-}$ mice as compared to Slc26a4$^{+/-}$ mice. Data were collected by Christa Linsenmeyer and Ruchira Singh

3.3. Lack of pendrin does not affect serum T4 levels

Fig. 3 shows a time course of serum T4 level as measured by immunoassay of the serum samples from Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice. No differences in the T4 levels in the serum of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice were observed at any time point included in this study. These data are consistent with results from previous studies suggesting the lack of overt hypothyroidism in Slc26a4$^{-/-}$ mice (Everett et al., 2001).

3.4. Lack of pendrin does not affect ClC-5 protein expression in the thyroid

Clcn5$^{-/-}$ mice show selective loss of pendrin expression in the thyroid (van et al., 2006). This finding led us to investigate whether the knockout of pendrin affects ClC-5 expression of ClC-5 was compared between the Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ protein expression. The mice using western blots (Fig 4). No difference in the expression of ClC-5 in the thyroids from Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice was found at any time point included in this study. This finding suggests that lack of pendrin does not affect the expression of ClC-5 in the thyroid.

3.5. Lack of pendrin leads to reduced nitrative stress in the thyroid

The finding that free radical stress is increased in the stria vascularis of Slc26a4$^{-/-}$ mice led us to investigate oxidative and nitrative stress in other tissues (Singh et al., 2007). Oxidative stress was

evaluated in: a) a pendrin-expressing tissue, thyroid, and b) a non-pendrin-expressing tissue, spleen. Oxidized proteins, using DNP-labeling of carbonyl groups by derivatization with DNPH, were detected by: a) western blot at P35 (Fig5A, 5B) and b) ELISA at P15 (Fig 5C). Note that no labeling was observed in the absence of derivatization in western blot (Fig 5A). No difference in the levels of oxidized proteins was found between the thyroids of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice. It is important to note that two different techniques were used to quantitate oxidized proteins at P15 and P35. Although data from two different techniques cannot be compared, both data sets are presented. (The reason for using two different techniques at P15 and P35 is due to the use of P35 mice for our initial experiments. In later experiments we decided to focus on P15, an age that corresponds to the maximal activity of thyroid gland.)

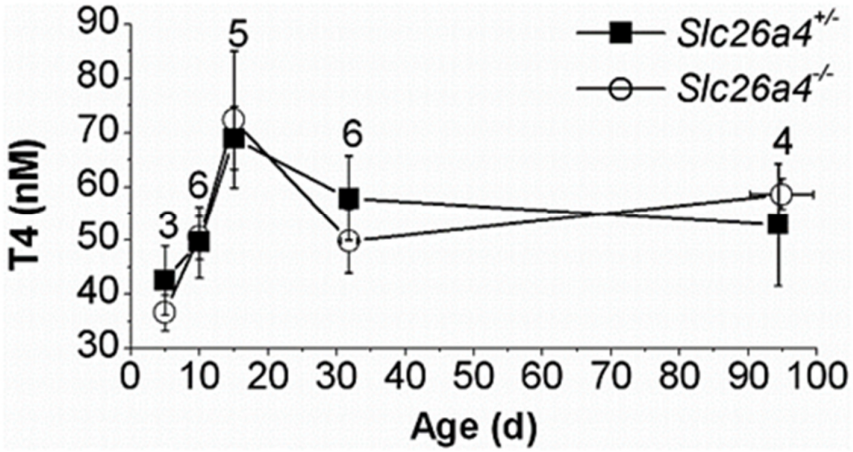

Fig. 3: No difference in serum T4 levels is observed between Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice. Measurement of serum T4 levels in Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at ages P5, P10 P15, P30 and ~ P90. Numbers next to the bars represent the number of observations. No differences in the T4 levels in the serum of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice were found at any age, indicating that Slc26a4$^{-/-}$ mice do not develop systemic hypothyroidism. Data were collected by Christa Linsenmeyer.

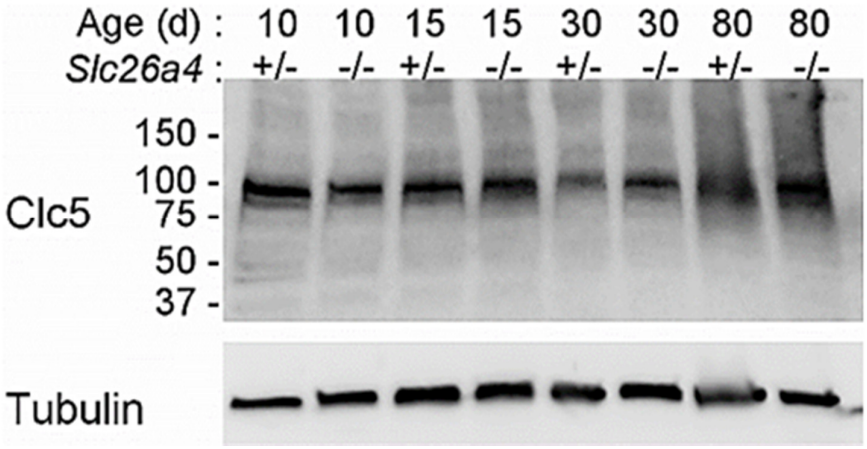

Fig. 4: No visible difference is observed in the ClC-5 protein expression level in the thyroid of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice. Representative western blots for ClC-5 protein and tubulin in thyroid of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at P10, P15, P30 and P80 (n=3). Based on visual inspection, no difference in thyroid ClC-5 protein expression was seen at any age in Slc26a4$^{-/-}$ mice as compared to Slc26a4$^{+/-}$ mice. Data were collected by Dr. Peying Fong, Christa Linsenmeyer and Ruchira Singh.

The protein expression of eNOS was evaluated by western blots in a developmental study (Fig 6A, 6B). The amount of nitrated proteins in relationship to the total protein was quantified at P15 and P35 in the thyroid of Slc26a4$^{-/-}$ and Slc26a4$^{+/-}$ mice (Fig 6C). No difference in the levels of eNOS was found between the thyroid of Slc26a4$^{-/-}$ and Slc26a4$^{+/-}$ mice. Although Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice show similar levels of nitration at P15, nitration is reduced at P35 in Slc26a4$^{-/-}$ mice, suggesting that thyroid of Slc26a4$^{-/-}$ mice experience reduced nitrative stress. It is conceivable that reduction of nitrative stress occurs between P15 and P35.

3.6. Lack of pendrin does not affect the expression of Trf and Tfrc in thyroid and spleen.

The observation that the expression of genes involved in iron metabolism is altered in the stria vascularis of Slc26a4$^{-/-}$ mice led us to investigate the expression of Trf and Tfrc, two proteins involvedin iron metabolism, in a pendrin-expressing tissue, thyroid, and in a non- pendrin expressing tissue,

spleen (28). Protein levels of Trf and Tfrc were quantified in the thyroid and spleen of Slc26a4$^{+/-}$and Slc26a4$^{-/-}$ mice at P35 (Fig 7A). Further, the tissue iron levels (Tissue-Fe) were measured at P15 corresponding to the time point of maximal activity of thyroid gland (Fig 7B). No differences in protein expression of Trf and Tfrc were found in the thyro ids and spleens of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at P35. No differences in tissue-Fe were found in the thyroids and spleens of Slc26a4$^{-/-}$ compared to Slc26a4$^{+/-}$ mice at P15.

4. Discussion

The most salient finding of the present study is that no difference between the thyroid gland function was observed between littermates of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice. The inference that Slc26a4 expression is not essential for thyroid gland function is derived from the following observations: 1) Slc26a4 mRNA expression is very low in the mouse thyroid and its levels do not increase at the time point corresponding to maximal thyroid gland activity, 2) no observable effect of lack of pendrin is seen on the expression of genes involved in thyroid hormone synthesis and 3) serum T4 levels are unaffected in Slc26a4$^{-/-}$ mice.

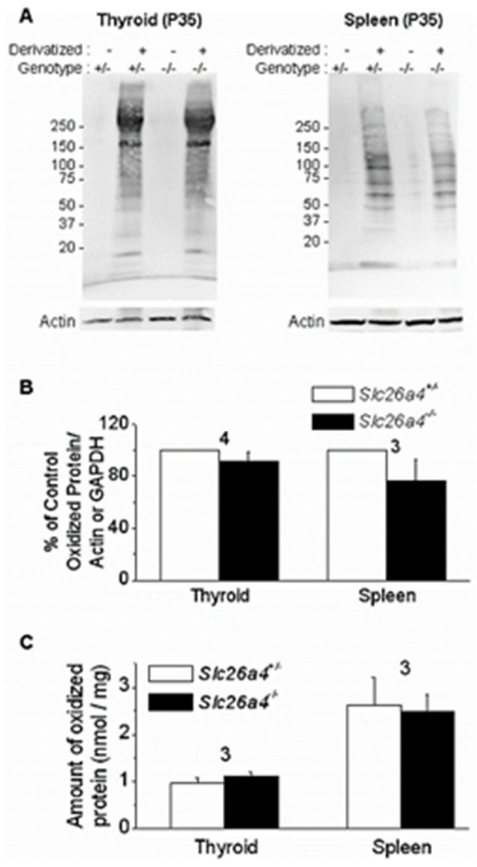

Fig. 5: The levels of oxidized proteins were not different in the thyroid of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice. (A-B) Oxidized proteins were quantified against actin or GAPDH in thyroid and spleen from Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at P35. Representative western blots and data summaries are shown. (C) Relative amount of oxidized protein was determined by ELISA in thyroids from Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at P15. No difference in the levels of oxidized proteins was found between thyroid and spleen of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice.

It is conceivable that the variability in thyroid phenotype between human patients and Slc26a4$^{-/-}$ mice is due to differences in Slc26a4 expression between humans and rodents. Slc26a4 transcript is highly expressed in the human thyroid at levels much higher than in the kidney (Everett et al., 1997). In contrast, our results show very little Slc26a4 mRNA expression in the mouse thyroid at all-time points, including those of maximal gland activity. Consistent with our finding, another group has reported that Slc26a4 mRNA expression in thyroids from adult mice is only 2% of the kidney expression (van et al., 2006). Pendrin in the thyroid has been implicated in iodide transport across the apical membrane of the thyrocytes (Royaux et al., 2000; Yoshida et al., 2002). Iodide organification defects are seen in human Pendred syndrome patients, and reduced expression of pendrin has been implicated as the cause of euthyroid goiter due to delayed iodide organification in Clcn5$^{-/-}$ mice (van et al., 2006).

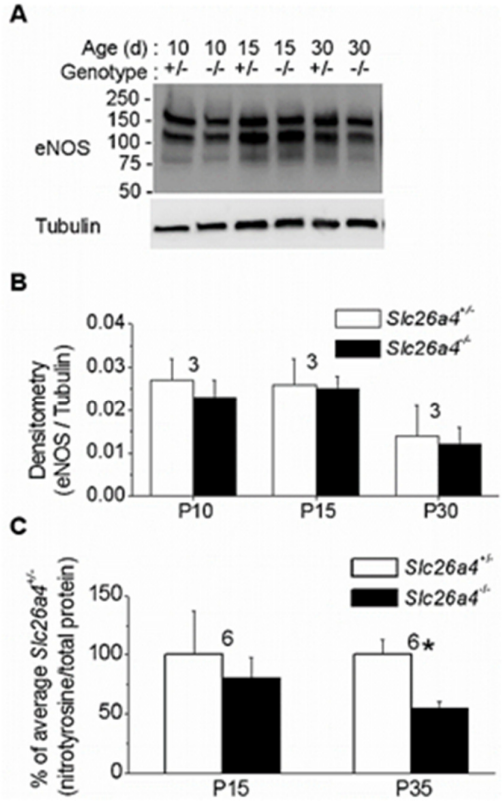

Fig. 6: The amounts of nitrated proteins in the thyroid of Slc26a4$^{-/-}$ mice were decreased in comparison to the thyroid of Slc26a4$^{+/-}$ mice. (A-B) Protein expression of eNOS was quantified relative to tubulin in thyroid of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at P10, P15 and P30. Representative western blots and data summaries are shown. No difference in eNOS expression was found between Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at any age. (C) The level of nitrotyrosine by ELISA was quantified against total

protein in thyroids from Slc26a4$^{+/-}$ and S lc26a4$^{-/-}$ mice at P15 and P35. Significant changes are marked (*) and numbers next to the bars represent the number of observations. Reduced levels of nitrated protein were seen at P35 in the thyroid of Slc26a4$^{-/-}$ mice.

However, our current observations suggest that pendrin is not necessary for thyroid gland function, at least not in the 129Sv/Ev strain of mice. Two possible reasons for the presence of euthyroid goiter in Clcn5$^{-/-}$ mice could be that 1) although a lack of ClC-5 is associated with reduced pendrin expression, the euthyroid goit er observed in Clcn5$^{-/-}$ mice is caused by a different mechanism and 2) the two mouse strains, 129Sv/Ev and C57BL/6J, respond differently to absence of pendrin in the thyroid gland.

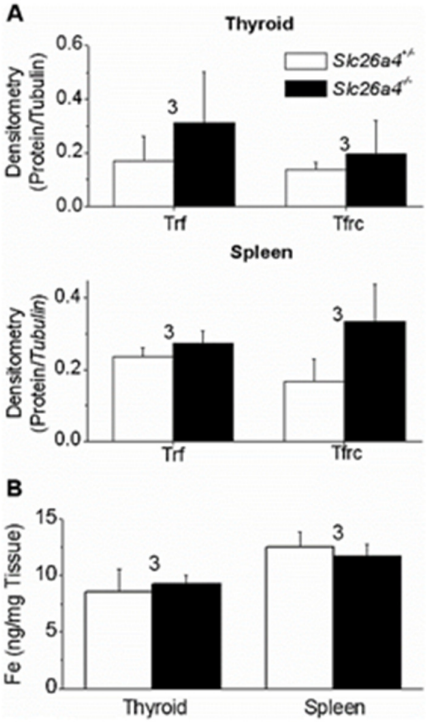

Fig. 7: Tissue iron levels and protein expression of Trf, Tfrc was unchanged in the thyroid and spleen of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice. (A) Transferrin (Trf) and transferrin receptor (Tfrc) were quantified relative to tubulin in thyroid and spleen of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice at age P35. (B) Tissue-iron was quantified in thyroid and spleen of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ at age P15. Numbers next to the bars represent the number of observations. No difference was found in tissue-Fe or the levels of Trf and Tfrc between thyroid and spleen of Slc26a4$^{+/-}$ and Slc26a4$^{-/-}$ mice.

Pendrin has not been shown to transport iodide in vivo, which could imply that 1) the apical iodide transporter in the thyroid remains to be identified, 2) the apical iodide transporter differs between human (pendrin) and mouse thyroid (unidentified) or 3) an unidentified iodide transporter compensates for the absence of pendrin in the mouse thyroid. The absence of Slc26a4 leads to increased free radical stress in the stria vascularis of Slc26a$^{-/-}$ mice (Singh et al., 2007). The absence of increased levels of free radical stress markers in the thyroid of Slc26a4$^{-/-}$ mice suggests that complete absence of Slc26a4 in itself may not be sufficient to cause free radical stress. Alternatively, it is possible that increased free radical stress is present in the thyroid but is restricted to a few cells and hence was not detected in analysis of whole tissue lysate. One of the consistent features of Pendred syndrome in human patients is deafness (Fraser, 1965). Hypothyroidism at birth is known to lead to deafness. Since most Pendred syndrome patients are deaf at birth and mutations in Slc26a4 are found in congenital hypothyroidism, it difficult to completely rule out systemic hypothyroidism as the cause of deafness in Pendred syndrome (Banghova et al., 2007).

Our present finding that the overt hypothyroidism is not present in Slc26a4$^{-/-}$ mice together with the fact that deafness is a consistent feature of Pendred syndrome mouse model establishes that absence of Slc26a4 in the cochlea is sufficient to affect hearing. However, it is still possible that lack of Slc26a4 leads to conditions of local hypothyroidism in the cochlea that could contribute to deafness in Pendred syndrome. Tissue-specific hypothyroidism limited to the cochlea has been demonstrated as the cause of deafness in Dio2$^{-/-}$ mice (Ng et al., 2004). The normal function of the thyroid gland in the Pendred syndrome mouse model and the difference in Slc26a4 expression between mouse and human thyroid suggest that pendrin has a different functional role in mouse and human thyroids.

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