Schnurri-2 regulates Th2-dependent airway inflammation and airway hyperresponsiveness

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Abstract

Schnurri (Shn)-2 is a large zinc finger-containing protein, which plays a critical role in cell growth, signal transduction and lymphocyte development. In Shn-2-deficient (Shn-2−/−) CD4 T cells, the activation of nuclear factor-κB is up-regulated and their ability to differentiate into Th2 is enhanced. Here, we extend our investigation and demonstrate that Shn-2 regulates Th2 responses in vivo using an ovalbumin-induced allergic asthma model. Eosinophilic inflammation, mucus hyperproduction and airway hyperresponsiveness (AHR) were all enhanced in Shn-2−/− mice. Moreover, eosinophilic infiltration and AHR were enhanced in mice given a transfer of Shn-2−/− effector Th2. Shn-2 in Th2 is thus considered to play an important role as a negative regulator in allergic airway inflammation.

Introduction

Drosophila Schnurri (Shn) is a large zinc-finger containing protein with a molecular weight of ~270 kDa. Drosophila Mad–Medea and Shn interact with each other and act as nuclear targets in the Drosophila decapentaplegic-signaling pathway (1–3). In vertebrates, this signaling pathway may equate to the bone morphogenetic protein/transforming growth factor-β/activin-signaling pathways which play diverse roles in the developmental processes. Vertebrates have at least three orthologs of Shn, namely Shn-1, Shn-2 and Shn-3 (4). mRNA expression of Shn-2 was detected primarily in the brain, heart and spleen. The vertebrate homologs of Shn were originally identified as proteins that bind to the nuclear factor-κB (NF-κB) site of various genes (5). Recently, the role of Shn-2 in the positive selection of thymocytes has been reported (6), and Shn-3-deficient CD4+CD8+ thymocytes were shown to exhibit a defect in cell survival (7). We recently demonstrated that Shn-2 binds to the NF-κB motif directly, thus resulting in the repression of the transcriptional activity of NF-κB through the competition of NF-κB binding in T cells (8). Shn-2-deficient (Shn-2−/−) CD4 T cells showed an increased capability to differentiate into Th2, due to the constitutive activation of NF-κB and the subsequent up-regulation of GATA3 expression (8). However, the precise physiological roles of these Shn family member proteins in in vivo immune responses still remain largely unknown.

Th2 play an important role in allergic asthma by inducing allergen-specific IgE production, airway inflammation, airway hyperresponsiveness (AHR) and mucus hyperproduction (9–13). The administration of allergens adsorbed with alum induces reproducible allergen-specific acquired immune responses that are dependent on Th2 producing IL-4, IL-5 and IL-13. A subsequent allergen challenge via the airway causes the rapid activation of Th2, mast cells and B cells. This activation results in increased vascular permeability, cellular infiltration into the lung tissue, smooth muscle contraction and mucus secretion.

In this study, we investigated the role of Shn-2 in allergic inflammation using Shn-2−/− mice. Our results suggest that Shn-2 plays a crucial role in the regulation of allergic inflammation and AHR.

Methods

Mice

Shn-2−/− mice have been described previously (6, 8). The animals used in this study were backcrossed to BALB/c >12 times and were 7–9 weeks old. Anti-ovalbumin (OVA)-specific TCRαβ (DO11.10) transgenic (Tg) mice were provided by Dennis Loh (Washington University School of Medicine, St Louis, MO, USA) (14). BALB/c mice were purchased from Clea Inc., Tokyo, Japan. Mice used in this study were at 7–9 weeks of age. Three independent experiments were performed for each experiment. All mice used in this study were maintained under specific pathogen-free conditions. All animal care was conducted in accordance with the guidelines of Chiba University.
Schnurri-2 controls Th2 responses in vivo

Sensitization and airway challenge with OVA

The mice were sensitized by an intraperitoneal injection of 100 μg OVA (Sigma–Aldrich, St Louis, MO, USA) adsorbed to 1 mg alum (LSL, Tokyo, Japan) on day 0. OVA solution in PBS (100 μg per 30 μl) was administered intranasally to each mouse on days 7 and 9.

Measurement of AHR

The degree of AHR was assessed by methacholine-induced airflow obstruction 24 h after the last antigen challenge. The respiratory parameters were obtained by exposure of mice to 0.9% saline mist, followed by incremental doses of aerosolized methacholine (0, 3, 6, 12, 24 and 48 mg ml⁻¹ in saline). Airflow obstruction was monitored and analyzed by whole-body plethysmograph (Buxco Electronics, Wilmington, NC, USA) as described previously (15). The results are expressed as the average in percentages of baseline enhanced paused values. The degree of AHR was also expressed as the average in percentages of baseline resistance (RL) was recorded during tidal breathing every 10 s. The maximum values of RL were determined and expressed as the percent changes from baseline after saline exposure.

Collection of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was performed 48 h after the last OVA challenge as described previously (17). All BAL fluid was collected and the cells were counted in 100-μl aliquots. One hundred thousand viable BAL cells were cytocentrifuged onto slides by a Cytospin 4 (Thermo Electron, Waltham, MA, USA) and stained with May-Grunwald–Giemza solution (MERCK, Darmstadt, Germany). Two hundred leukocytes were counted on each slide. Cell types were identified using morphological criteria. The percentages of each cell type were calculated. Cytokine levels in the BAL fluid were measured 6 h after the last OVA challenges. IL-5, IL-13 and eotaxin-2 levels in BAL fluid were measured by ELISA as previously described (18).

Lung histology

The mice were sacrificed by asphyxiation at 48 h after the last OVA challenge, and the lungs were infused with 10% (v/v) formalin in PBS for fixation. The lung samples were sectioned, stained with hematoxylin and eosin (H&E) reagents or periodic acid–Schiff (PAS) reagent and examined for pathological changes under a light microscope at ×200. The number of infiltrated mononuclear cells in the peribronchioral regions was calculated by direct counting in four different fields per slide.

Lung mononuclear cell preparation and a flow cytometry analysis

The lungs were sliced into small cubes and then incubated for 30 min in 5 ml RPMI 1640 solution containing collage-nase (20 U ml⁻¹) (Worthington, Lakewood, NJ, USA) and trypsin inhibitor (0.3 mg ml⁻¹) (Sigma–Aldrich). Lung mononuclear cells were separated by centrifugation on Percoll (GE Healthcare, Buckinghamshire, UK). For staining, one million cells were incubated on ice for 30 min with the appropriate staining reagents, according to a standard method (19). The reagents used in this study were anti-CD8α–PE (63.6.7) and anti-CD4–APC[K2] (RM4-5) purchased from PharMingen (San Diego, CA, USA). A flow cytometry analysis was performed on FACScalibur™ (Becton Dickinson, Franklin lakes, NJ, USA) and the results were analyzed using the CELLQUEST™ software program (Becton Dickinson).

Adoptive cell transfer of Tₘ₂ for the development of airway inflammation and AHR

Effector Tₘ₂ were generated as previously described (20). In brief, splenic CD4 T cells purified from DO11.10 OVA-specific TCR Tg or Shn-2⁻/⁻ DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide (Loh15, 3 μM) plus antigen presenting cells under Th2 culture conditions for 6 days in vitro. These effector Tₘ₂ (5 × 10⁶) were transferred intravenously into BALB/c recipient mice on day 0. These recipient mice were not irradiated. On day 1 and 3, OVA solution (100 μg per 30 μl) was administrated intranasally to each mouse. The degree of AHR was measured on day 4. BAL fluid was collected on day 5.

Quantitative PCR analysis

Total RNA was isolated from the lung (three mice in each group) using the TRIzol reagent (Sigma–Aldrich). Reverse transcription (RT) was carried out with Superscript II RT (Invitrogen, Carlsbad, CA, USA). Samples were then subjected to real-time PCR analysis on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) under standard conditions. The primers and TaqMan probes for the detection of Muc5ac, thymus activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC) and hypoxanthine-guanine phosphoribosyltransferase (hprt) were purchased from Applied Biosystems. The expression of mRNA was normalized using the hprt signal.

Data analysis

The statistical analysis was performed using the two-tailed Student’s t-test. Mann–Whitney U-tests were used to determine the level of difference in the degree of AHR. The values are the mean ± SD.

Results

Enhanced eosinophilic infiltration in BAL fluid and AHR in Shn-2⁻/⁻ mice

We recently reported that the ability to differentiate into Tₘ₂ in vitro was enhanced in Shn-2⁻/⁻ naive CD4 T cells (8). The aim of this study was to clarify the role of Shn-2 in Tₘ₂-dependent in vivo immune responses, such as OVA-induced allergic airway inflammation. Wild-type and Shn-2⁻/⁻ mice were immunized with OVA–alum on day 0 and challenged with OVA intra-nasally on day 7 and 9. On day 11, BAL fluid
was harvested and examined. The absolute numbers of eosinophils, lymphocytes, neutrophils and macrophages were determined by cell counts based on morphological criteria. As shown in Fig. 1(A), total cell numbers of infiltrating leukocyte significantly increased in the Shn-2\(^{-/-}\) allergy-induced mice. A significant increase in the absolute number of eosinophils was also observed. In Shn-2\(^{-/-}\) mice, both OVA immunization and OVA challenge were required for the induction of allergic inflammation. The levels of IL-5, IL-13 and eotaxin-2 were increased in allergy-induced Shn-2\(^{-/-}\) mice in comparison to the levels in wild-type mice (Fig. 1B). No IL-4 was detected in the BAL fluid (data not shown).

We examined the degree of AHR in the allergy-induced Shn-2\(^{-/-}\) mice by measuring methacholine-induced airflow obstruction with a whole-body plethysmograph (Fig. 1C) and a mechanical ventilator (Fig. 1D). The degree of AHR in Shn-2\(^{-/-}\) mice was enhanced in comparison to that of wild-type mice. These data indicate that OVA-induced airway inflammation and AHR are therefore enhanced in allergy-induced Shn-2\(^{-/-}\) mice.

Enhanced lung inflammation and mucus production in the lung of Shn-2\(^{-/-}\) mice

We examined the histological changes in the lungs of allergy-induced Shn-2\(^{-/-}\) mice by H&E staining (Fig. 2A, left panels). No massive inflammatory cell infiltration was noted in the lungs of wild-type and Shn-2\(^{-/-}\) mice that did not receive the OVA challenge (Fig. 2A, panels a and c). Substantial numbers of mononuclear cells were infiltrated in the peribronchiolar regions in wild-type mice after the OVA challenge (Fig. 2A, panel b), and the infiltration extended to the surrounding area in Shn-2\(^{-/-}\) mice (Fig. 2A, panel d). The number of infiltrated cells also increased in Shn-2\(^{-/-}\) mice (Fig. 2A, right panels).

We then examined the levels of mucus hyperproduction by PAS staining. Representative staining profiles of the bronchiolar regions in allergy-induced Shn-2\(^{-/-}\) mice are shown (Fig. 2B). No specific staining was detected in wild-type and Shn-2\(^{-/-}\) mice without the OVA challenge (Fig. 2B, panels a and c). Moderate staining was noted in wild-type bronchioles, whereas the staining levels increased in the

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**Fig. 1.** Enhanced airway inflammation and AHR in Shn-2\(^{-/-}\) mice. Airway inflammation and AHR were induced with OVA sensitization and challenges. (A) The absolute numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym) and macrophages (Mø) in the BAL fluid are shown. The results were obtained using the values from cell counting, the percentages of the cells, total cell number per milliliter and the volume of BAL fluid recovered. Samples were collected 48 h after the last OVA challenge. Mean values with SDs (\(n = 5\)) are shown. Four independent experiments were done with similar results. \(-/-\): without OVA priming or OVA challenge, OVA/\(-\): with OVA priming but not OVA challenge and OVA/OVA: with OVA priming and OVA challenge. (B) The levels of IL-5, IL-13 and eotaxin-2 in BAL fluid were determined by ELISA. Samples were collected 6 h after the last OVA challenge. Mean values with SDs (\(n = 5\)) are shown. Four independent experiments were done with similar results. (C and D) One day after the last OVA challenge, AHR in response to increasing doses of methacholine was assessed by measuring enhanced pause (C) and RL (D). Five animals from each group were individually examined, and the mean values and SDs are indicated. Four independent experiments were done with similar results. The differences were statistically significant between wild-type and Shn-2\(^{-/-}\) mice with OVA sensitization and OVA challenge (\(*P < 0.05\) and \(**P < 0.01\)).
Shn-2⁻/- bronchioles (Fig. 2B, panels b and d). Consequently, we examined the expression of Muc5ac in the lungs of Shn-2⁻/- mice, and a slight, but significant increase in the expression was noted in the Shn-2⁻/- mouse lungs (Fig. 2C). These results indicate that the levels of mucus hyperproduction were moderately enhanced in the lungs of the allergy-induced Shn-2⁻/- mice in comparison to those of the wild-type mice.

The increased number of lung CD4 T cells in Shn-2⁻/- mice is accompanied with an enhanced production of TARC and MDC.

Previous studies have reported a reduced number of CD4 T cells in the spleen of Shn-2⁻/- mice (8). We therefore examined whether the number of CD4 T cells were reduced in OVA-sensitized Shn-2⁻/- mice. Lung leukocytes were stained with anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry. The percentages of CD4⁺ and CD8⁺ cells in Shn-2⁻/- mice significantly decreased in comparison to those in wild-type mice (Fig. 3A). After the OVA challenge, the percentage of CD4 T cells increased substantially in the Shn-2⁻/- mice (12.1 versus 23.4%). The absolute numbers of total leukocytes and CD4 T cells in the lung decreased significantly in Shn-2⁻/- mice (Fig. 3B, upper panels). However, a dramatic increase in the total numbers of lung leukocytes and CD4 T cells was observed in the Shn-2⁻/- mice after the OVA challenge (Fig. 3B, lower panels).

In our previous study, the proliferative ability of CD4 T cells in the Shn-2⁻/- mice was comparable to that in the wild-type mice (8). To investigate the reason why the CD4 T cell levels increased in the allergy-induced Shn-2⁻/- mice, we examined the mRNA expression of TARC and MDC. These chemokines are known to be selective attractants for Th2 migration (21). As shown in Fig. 3(C), the mRNA expression levels of TARC and MDC in the lung from the Shn-2⁻/- mice were significantly higher than those in the wild-type mice. The increased expression of TARC and MDC may thus explain the dramatic increase observed in the number of CD4 T cells in the lungs of Shn-2⁻/- mice.

Shn-2⁻/- effector T(h)2 enhanced AHR and eosinophilic infiltration into the lungs in recipient mice

We performed adoptive transfer experiments to determine whether the enhancement of airway inflammation and AHR observed in Shn-2⁻/- mice is mediated via Shn-2⁻/- T(h)2. Effector T(h)2 from Shn-2⁻/- DO11.10 OVA-specific TCR Tg mice were prepared as described in the Methods. On days

Fig. 2. Enhanced leukocyte infiltration into the lung and mucus production in allergy-induced Shn-2⁻/- mice. The levels of OVA-induced airway inflammation and mucus production in Shn-2⁻/- mice were examined by a histological analysis and a quantitative real-time RT-PCR. (A) Antigen-induced leukocyte infiltration into the lung was evaluated using H&E staining (left panels). The numbers of infiltrated mononuclear cells in the perivascular and peribronchiolar regions were calculated by direct counting from four different fields per slide (right panels). The mean values with SDs (n = 5) are shown (*P < 0.05). (B) Antigen-induced goblet cell hyperplasia was evaluated by PAS staining. Representative photomicrographic views of wild-type and Shn-2⁻/- mice are shown. (C) Total mRNA was prepared from the lung of allergy-induced wild-type or Shn-2⁻/- mice, and mRNA levels of Muc5ac were examined. The data represent the mean values of Muc5ac mRNA expression normalized with hprt expression. Three independent experiments were done with similar results (*P < 0.05).
1 and 3 after effector T\(_{h2}\) transfer into syngeneic BALB/c mice, airway inflammation was induced by the OVA intranasal administration. A significant increase in the absolute number of eosinophilic infiltration in BAL fluid was observed in the mice that received Shn-2\(^{-/-}\) T\(_{h2}\) (Fig. 4A). The levels of IL-5 and eotaxin-2 in the BAL fluid from the mice receiving Shn-2\(^{-/-}\) T\(_{h2}\) increased markedly more than that from the mice receiving wild-type T\(_{h2}\) (Fig. 4B). The levels of IL-4 were not increased but moderately decreased. The levels of IL-13 were comparable. The degree of AHR in the mice receiving Shn-2\(^{-/-}\) T\(_{h2}\) also increased more than that in the mice receiving wild-type T\(_{h2}\) (Fig. 4C and D). These results suggest that the hyperactivation of effector T\(_{h2}\) in Shn-2\(^{-/-}\) mice exacerbates the development of allergic airway inflammation.

We then examined the levels of mucus hyperproduction by PAS staining. Representative staining profiles of the bronchiolar regions in the mice that received wild-type or Shn-2\(^{-/-}\) T\(_{h2}\) are shown (Fig. 4E). Moderate staining was noted in both bronchioles (Fig. 4E, panels a and b). The mRNA expression of Muc5ac in the lung of mice that received Shn-2\(^{-/-}\) T\(_{h2}\) was examined, and it was found to be comparable to that in the mice receiving wild-type T\(_{h2}\) (Fig. 4F). These results indicate that the levels of mucus hyperproduction in the lungs of the mice that received Shn-2\(^{-/-}\) T\(_{h2}\) are therefore comparable to those of the mice that received wild-type T\(_{h2}\).

**Discussion**

We previously reported that the activation of NF-kB is up-regulated in Shn-2\(^{-/-}\) CD4 T cells, and their ability to differentiate into T\(_{h2}\) was enhanced (8). In this study, we demonstrated that OVA-induced allergic inflammation and AHR are enhanced in the Shn-2\(^{-/-}\) mice as well as in the wild-type mice transferred with Shn-2\(^{-/-}\) effector T\(_{h2}\). These results indicate that Shn-2 regulates OVA-induced airway inflammation and AHR through the control of CD4 T cell activation.

We observed an increased IL-5, IL-13 and eotaxin-2 level in the BAL fluid in OVA-sensitized and OVA-challenged Shn-2\(^{-/-}\) mice (Fig. 1B). IL-13 is known to induce AHR in the absence of inflammatory cells (13). IL-5 and eotaxin-2 are known to attract eosinophils (22). Since eosinophils release granule proteins that are cytotoxic to the airway epithelium such as major basic proteins, eosinophilia may exacerbate the airway obstruction and AHR (23). Therefore, it is likely that the overproduction of these factors (IL-5, IL-13 and eotaxin-2) resulted in the enhanced eosinophilic infiltration and AHR in the airways of the Shn-2\(^{-/-}\) mice. The hyperproduction of mucus also plays an important role in the pathogenesis of various asthmatic features and is linked with the Shn-2\(^{-/-}\) mice without OVA challenge (**P < 0.01). The differences in the number of CD4 T cells were statistically significant between the wild-type mice and the Shn-2\(^{-/-}\) mice with OVA sensitization and OVA challenge (**P < 0.01). (C) Total mRNA was prepared from the lung of allergy-induced wild-type or Shn-2\(^{-/-}\) mice. A real-time RT-PCR analysis for TARC and MDC as well as hprt (as a control) was performed. Representative data of three individual animals from three independent experiments are shown (**P < 0.05).
IL-13 has been shown to induce mucus hypersecretion in vivo and in vitro (26). Therefore, an overproduction of IL-13 may induce severe mucus secretion in the Shn-2-/- lung. IL-13 was also shown to induce TARC and MDC production from keratinocytes and bronchial epithelial cells (27, 28). The increased number of CD4 T cells in the lungs of the Shn-2-/- mice may be due to the increased amount of IL-13, which thus resulted in the overproduction of TARC and MDC.

To investigate whether the exacerbation of airway inflammation and AHR was due to the deficiency of Shn-2 in Tn,2, we performed a set of experiments with adoptive transfer of Tn,2 (Fig. 4). As a result, an enhanced degree of eosinophilic infiltration and increased levels of IL-5 and eotaxin-2 in BAL fluid were observed in wild-type recipient mice transferred with Shn-2-/- Tn,2. The degree of AHR was also amplified. Therefore, the exacerbation of airway inflammation and AHR appears to be at least in part due to the enhanced Th2 activities of Shn-2-/- Tn,2.

However, the levels of IL-13 in the BAL fluid from the mice that received Shn-2-/- Tn,2 were comparable to those from the mice that received wild-type Tn,2. The levels of mucus hypersecretion did not increase in the mice that received Shn-2-/- Tn,2 (Fig. 4E and F). These results may indicate that the overproduction of IL-13 in the BAL fluid of Shn-2-/- mice was not only from CD4 T cells but also from other IL-13-producing cells such as mast cells, basophils and eosinophils.

We observed the Shn-2 expression in naive CD4 T cells as well as bone marrow-derived mast cells (BMMCs) (Supplementary Figure 1A, available at International Immunology Fig. 4. Enhanced airway inflammation and AHR in mice receiving Shn-2-/- effector Tn,2. Naive CD4 T cells from DO11.10 OVA-specific TCR Tg or Shn-2-/- DO11.10 OVA-specific TCR Tg mice were cultured under Th2 conditions for 6 days. The effector Tn,2 (5 x 10^6) were transferred into BALB/c mice 1 day before the first OVA challenge. (A) The absolute number of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym) and macrophages (Mø) in the BAL fluid are shown. The mean values with SDs (n = 5) are shown. Three independent experiments were done with similar results (***P < 0.01). (B) The levels of IL-4, IL-5, IL-13 and eotaxin-2 in the BAL fluid were determined by ELISA. Samples were collected 6 h after the last OVA challenge. The mean values with SDs (n = 5) are shown. Three independent experiments were done with similar results (*P < 0.05 and **P < 0.01), n.d. not detectable. (C and D) AHR was monitored by measuring enhanced pause (left panel) and RL (right panel) as described in the Methods. The mean values with SDs (n = 5) are shown. (E) Antigen-induced goblet cell hyperplasia was evaluated by PAS staining. Representative photomicrographic views of the lung in the mice receiving wild-type or Shn-2-/- Tn,2 are shown. (F) The data represent the mean values of Muc5ac mRNA expression in the lung of mice receiving wild-type or Shn-2-/- Tn,2. It was normalized with hprt expression. Three independent experiments were done with similar results. Differences in AHR were statistically significant between wild-type and Shn-2-/- T cell transfer groups (*P < 0.05).
Shn-2 exacerbates airway inflammation and AHR in the development of asthma (9, 22, 33). Although we need to await the exacerbation of airway inflammation and AHR independently from conventional Th2 over-expressing NF-κB (p65) (C. Iwamura and T. Nakayama, unpublished observation). Furthermore, some Shn-2−/− mice after 16 weeks of age died from severe whole-body inflammation and AHR. Therefore the down-regulation of regulatory T cells may exacerbate the airway inflammation in Shn-2−/− mice. Not only T cells but also for non-T cell populations, such as airway smooth muscle cells, eosinophils and epithelial cells, which may thus enhance both airway inflammation and AHR.

In summary, OVA-induced eosinophilic airway inflammation, AHR and mucus hyperproduction were all found to be enhanced in Shn-2−/− mice. Therefore, Shn-2 appears to play a key role as an in vivo negative regulator of the Tj2-dependent allergic airway responses.

Supplementary data
Supplementary Figure 1 is available at International Immunology Online.

Acknowledgements
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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>AHR</td>
<td>airway hyperresponsiveness</td>
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<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<td>BMPC</td>
<td>bone marrow-derived mast cell</td>
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<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<td>hprt</td>
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References

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