A New Mouse Gene, SRG3, Related to the SWI3 of Saccharomyces cerevisiae, Is Required for Apoptosis Induced by Glucocorticoids in a Thymoma Cell Line

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Summary
We isolated a new mouse gene that is highly expressed in thymocytes, testis, and brain. This gene, SRG3, showed a significant sequence homology to SWI3, a yeast transcriptional activator, and its human homolog BAF155. SRG3 encodes 1,100 amino acids and has 33–47% identity with SWI3 protein over three regions. The SRG3 protein contains an acidic NH2 terminus, a myb-like DNA binding domain, a leucine-zipper motif, and a proline- and glutamine-rich region at its COOH terminus. Rabbit antiserum raised against a COOH-terminal polypeptide of the SRG3 recognized a protein with an apparent molecular mass of 155 kD. The serum also detected a 170-kD protein that seems to be a mouse homologue of human BAF170. Immunoprecipitation of cell extract with the antiserum against the mouse SRG3 also brought down a 195-kD protein that could be recognized by an antiserum raised against human SWI2 protein. The results suggest that the SRG3 protein associates with a mouse SWI2. The SRG3 protein is expressed about three times higher in thymocytes than in peripheral lymphocytes. The expression of anti-sense RNA to SRG3 mRNA in a thymoma cell line, S49.1, reduced the expression level of the SRG3 protein, and decreased the apoptotic cell death induced by glucocorticoids. These results suggest that the SRG3 protein is involved in the glucocorticoid-induced apoptosis in the thymoma cell line. This implicates that the SRG3 may play an important regulatory role during T cell development in thymus.

Progenitor T cells arise in the bone marrow and migrate to thymus, where they continue to develop. During the T cell development, more than 95% of developing immature thymocytes die by apoptosis as a consequence of negative selection or lack of positive selection (1). This apoptotic death targets mainly the cortical double-positive (CD4+CD8+) thymocytes. In thymocytes, apoptosis can be triggered by several exogenous stimuli such as glucocorticoids (2–4), removal of growth factors (5, 6), exposure to γ-irradiation (7), and antigen binding involving the CD3/TCR (8–10). The effect of glucocorticoids (GCs) is selective; the immature CD4+CD8+ thymocyte fraction is rapidly killed by GC treatment, whereas both the precursor population (TCR-CD4-CD8-) and mature thymocytes (CD4+ or CD8+) are relatively resistant (11). It was reported that GC is produced within the thymus (12), and that transgenic expression of anti-sense RNA to glucocorticoid receptor (GR) significantly affects the thymocyte development (13). These results suggest that endogenous GC produced in thymus may participate as an important regulatory molecule of normal thymic development (14, 15).

GCs, when complexed with an activated receptor, can induce or inhibit the expression of specific genes, which may be related to the induction of apoptosis. The transcriptional regulation of downstream genes by GCs requires not only GR itself but several additional transcription factors such as the SWI–SNF protein complex (16–20). For example, the rat GR, when expressed in yeast, requires SWI–SNF proteins for transcriptional activation of GR-responsive genes and the GR–SWI3 complexes were coimmunoprecipitated in yeast extract (19, 20). In addition, antibodies against SWI3 interfere with the ability of rat GR to activate transcription in Drosophila melanogaster nuclear extracts (19).

SWI3 is a subunit of the SWI–SNF complex that seems

1 Abbreviations used in this paper: GC, glucocorticoids; GR, glucocorticoid receptor; GST, glutathione-S-transferase; IP, immunoprecipitation; MACS, magnetic activated cell sorter; PI, propidium iodide.
to facilitate transcriptional activation by antagonizing the repressive actions of chromatin (21–23). The other subunits of the SWI–SNF complex so far identified include the SWI1 (ADR6), SWI2 (SNF2), SNF5, SNF6, SNF11, and SWP73. The complex was initially identified in Saccharomyces cerevisiae (S. cerevisiae) as a positive regulator of HO, a gene involved in mating type switching (24, 25), and SU12, a glucose-repressible gene that encodes the enzyme invertase (26, 27). These SWI gene products were subsequently found to be required for the transcriptional activation of many other genes (28–31). Such activities of the SWI–SNF proteins are closely interconnected and they seem to function as components of a complex that associates with genespecific activators (31–35). The functional significance of the SWI–SNF complex is reflected by the evolutionary conservation of these genes in higher eukaryotes. Several higher eukaryotic homologues of SWI–SNF genes such as Drosophila homeotic gene activator brm, hbrm (also known as hSNF F2/α) and BRG1 (also known as hSNF F2/β) have been identified (36–39). In addition, a human protein homologue of SNF5 (40, 41) and mouse BAF60 (42), which is homologous to SWP73, have been identified. Recently, distinct complexes containing the BRG1 or hbrm that have an in vitro activity similar to yeast SWI–SNF have been purified from human cell lines (42, 43). From these complexes, the human BAF155 and BAF170 proteins that are homologous to SWI3 protein were identified (42).

In this paper, we describe a newly isolated mouse gene, the SWI3-related gene (SRG3), expressed in thymus and encoding a protein that shows significant amino acid sequence homology to both yeast SWI3 and human BAF155 proteins. The SRG3 protein coimmunoprecipitates with a mouse SWI2-like protein, suggesting their forming a protein complex in vivo. In addition, our data show that the SRG3 is expressed at much higher level in thymus than in peripheral lymphocytes. Because the GC is proposed to be a regulatory molecule in thymocyte development in thymus, and the SWI-related proteins have an important role in GC-mediated gene regulation, the high level expression of SWI3-related gene (SRG3) in thymocytes may imply that SRG3 has a crucial role in thymocyte development as a mediator of GC-induced transcriptional activation and apoptotic cell death of thymocytes. As a first step of testing this hypothesis, we analyzed the effect of downregulation of SRG3 expression in a GC-sensitive thymoma cell line on GC-induced apoptosis.

Materials and Methods

Mice and Cells. C57BL/6 J mice were maintained in the Institute for Molecular Biology and Genetics (Seoul National University, Seoul, Korea). The yeast strain C Y165 (MATα, swi3Δ::tpi1Δ1, H0-ΔlacZ, ura3-52, leu2Δ, his3Δ200, ade2-101, lys-801) cells, and yeast P50 plasmid containing the SWI3 gene were gifts from C. Peterson (University of Massachusetts, Worcester, MA). Yeast cells were grown in synthetic minimal medium (0.67% Bacto-yeast nitrogen base without amino acids; Difco BRL, Gaithersburg, MD) supplemented with leucine, histidine, adenine, and glycine to a mid-log phase. The mouse thymoma cell line, S49.1, was purchased from American Type Culture Collection (ATCC, Rockville, MD) and grown in DMEM supplemented with 10% fetal bovine serum.

Isolation and Purification of Poly(A)⁺ RNA. Total RNA was isolated by CsCl banding, as described by Chomczynski and Sacchi (44). Intact thymic and spleen cells were collected from 3-5-wk-old mice and used as sources for RNA. Poly(A)⁺ RNA was isolated by oligo(dT)–cellulose chromatography (45).

Preparation of Subtractive Probe. ~10 μg of poly(A)⁺ RNA was heated at 65°C for 2 min and annealed with 1 μg of magnetic beads containing oligo (dT25) (Dynabeads oligo (dT25), Dynal, Inc., Great Neck, N.Y.) for 30 min at room temperature (46). The annealed poly(A)⁺ RNA was separated in magnetic field, and used as templates for the synthesis of the first-strand cDNA. The second-stranded cDNA was synthesized by random priming using hexanucleotides and 200 μCi of [α-32P]dCTP (3,000 Ci/mmole). To prepare a subtractive probe, 200 μg of the first-stranded spleen cDNA conjugated with magnetic beads were mixed with the labeled probe. After incubating at 55°C for 1 h, the labeled probe DNAs hybridized to the first-stranded spleen cDNA were removed using magnetic field, and the remaining subtractive probe was used for the screening of thymic cDNA library. The cDNA library was obtained from M. M. Davis at Stanford University (Stanford, CA).

DNA Sequencing and Computer Analysis. To determine the nucleotide sequences, the restriction fragments of the cloned gene were subcloned into pBluescript (SK⁻) vector (Stratagene Inc., La Jolla, CA). Nested deletions were generated by the Erase-A-Base system (Promega Corp., Madison, WI). The nucleotide sequence was determined by dideoxy chain termination method (47) using Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH). Homology searches of the nucleotide and deduced amino acid sequences with sequences were performed at the National Center for Biotechnology Information, using the BLAST network service (48).

Separation of T and B Cells. Single cell suspensions were prepared from intact spleens and lymph nodes of C57BL/6J mice. After the red blood cells were removed, cells were resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3). To isolate T cells, single cell suspensions were sequentially reacted with biotinylated H57-597 antibody, which is specific to αβ TCR, and streptavidin-conjugated microbeads. The B cells were reacted with microbead-conjugated goat anti-mouse IgM. The cell-magnetic bead conjugates were separated by a magnetic cell sorter (MACS; Milteny Biotec, GmbH, Bergisch Gladbach, FR G) (49). The purity of isolated populations were confirmed by FACS® (Becton Dickinson, Mountain View, CA) analysis and Northern blot assay using TCF-1, a T cell–specific gene, as a probe.

Overexpression and Purification of GST–fusion Protein. For the construction of GST–fusion protein, COOH-terminal region of the SRG3 gene was inserted into pGEX 4T-2 vector in frame. DH5α cells harboring recombinant plasmids with GST–3C fusion were grown overnight and diluted to 1:200 in 200 ml of LB medium. After incubation at 37°C for 2 h with vigorous shaking, the culture was treated with 1 mM IPTG and then incubated for 3 h to induce expression of the fusion protein. Cells were harvested and resuspended in a sample loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 4% SDS, 0.2% BPP, 20% glycerol), and boiled for 2 min. These lysates were analyzed by electrophoresis on polyacrylamide gel. The overexpressed protein was purified by glutathione–sepharose 4B affinity chromatography as described.
by Smith and Johnson (50). The polyclonal antiserum was prepared by immunizing New Zealand white rabbit with the purified fusion protein.

Immunoprecipitation and Immunoblot Analysis. The immunoprecipitation of the SR G3 and SW12-like protein was performed by a method described by M.uchardt et al. (40) with some modifications. The single cell suspension of the mouse thymus was harvested in immunoprecipitation (IP) buffer (20 mM Heps, pH 7.6, 10% glycerol, 25 mM MgCl2, 0.1 mM EDTA, 0.2% NPR-40) containing 0.1 M potassium acetate and 2.25 μg/ml leupeptin, 1 μg/ml soybean inhibitor, 2 mM PM SF, and 0.1 mM DTT. The cells were sonicated and debris were pelleted by centrifugation. The extracts were preclariied with protein A–sepharose suspension and anti-SRG G3 or anti-HSW12 rabbit antiserum were added. After overnight incubation at 4°C, the extracts were incubated with protein A–sepharose suspension. The beads were washed three times in IP buffer containing 0.6 M potassium acetate, and once with IP buffer without Heps. The precipitate was eluted by boiling in SDS-PAGE loading buffer. For immunoblot analysis, the proteins separated on SDS-PAGE were electrotransferred to nitrocellulose paper, and incubated in blocking solution (100 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1% non-fat dry milk). After incubation with the SRG3 antiserum or hSWI2 antiserum, the specific precipitate was eluted by boiling in SDS-PAGE loading buffer. For immunoprecipitation (IP), the SRG3 and SWI2-like protein was performed by Muchardt et al. (40) with some modifications. The isolated gene has an open reading frame of 3,300 bp encoding 1,100 amino acids (Fig. 1). The homology search in the GenBank sequence database of NCBI using the BLASTP program showed amino acid sequence similarity to the SWI3 protein of S. cerevisiae. The isolated gene has an open reading frame of 3,300 bp encoding 1,100 amino acids (Fig. 1). The homology search in the GenBank sequence database of NCBI using the BLASTP program showed amino acid sequence similarity to the SWI3 protein of S. cerevisiae (Fig. 2). The new gene was named as SRG3 to emphasize its relatedness to the SWI3 gene.

Results

Cloning and Characterization of the Mouse SWI3-related Gene (SRG3). We made an attempt to isolate genes that are specifically expressed in thymus but not in spleen by subtractive hybridization. One of the clones isolated was found to be expressed preferentially in thymus and was found to have similar amino acid sequences to a part of the SWI3 protein of S. cerevisiae. The isolated gene has an open reading frame of 3,300 bp encoding 1,100 amino acids (Fig. 1). The homology search in the GenBank sequence database of NCBI using the BLASTP program showed amino acid sequence similarity to the SWI3 protein of S. cerevisiae (Fig. 2). The new gene was named as SRG3 to emphasize its relatedness to the SWI3 gene.

Figure 1. Amino acid sequence of the SRG3 gene predicted from cDNA sequence. The predicted leucine zipper motif is indicated by asterisks, and the myb-like tryptophan repeat is indicated by the closed triangles. The regions showing highest homology to the yeast SWI3 are unnumbered. The myb-like tryptophan repeat is indicated by the closed triangles. The regions showing highest homology to the yeast SWI3 are unnumbered.
The NH$_2$-terminal part of the SRG3 is highly acidic; 23% of 231 amino acids (211–441) are either aspartate or glutamate. The COOH-terminal part has the leucine-zipper motif (53), and the proline- and glutamine-rich region. The proline and glutamine residues make up 44% over 150 amino acids (952–1,100). In addition, the myb-like tryptophan repeat domain or SANT (SWI3, ADA2, N-CoR, and TFIIIB) domain (54), which may be involved in interaction with DNA, was also found in the middle part of this protein (Fig. 2A). Therefore, this gene product seems to act as a transcriptional activator probably by interacting with other proteins or by interacting with DNA.

Recently, the human homologues of yeast SWI3, BAF155, and BAF170, were identified and designated as BRG1-associated factors (42). We found that the SRG3 protein has very high amino acid sequence homology to BAF155 protein (Fig. 2). They show 91% identity in their complete amino acid sequences, with the major deviations occurring at the NH$_2$ termini. Therefore, we conclude that the SRG3 protein is the murine counterpart of the human BAF155 protein.

Identification of the SRG3 Gene Product. To identify the protein product of the SRG3 gene, polyclonal antibody was produced against the SRG3–GST fusion protein. The COOH-terminal part of the SRG3 gene was inserted in-frame into the pGEX4T-2 plasmid containing the glutathione-S-transferase (GST) gene. The overexpressed GST–3C fusion protein with 85 kD of molecular mass was used to immunize rabbits through subcutaneous injection. After primary and booster injections, polyclonal antiserum was confirmed to recognize specifically the fusion protein (data not shown). To identify the SRG3 gene product, immunoblot analysis was performed with crude extracts prepared from thymus and lymph node. As shown in Fig. 3A, two bands of 155 and 170 kD were observed; the 155-kD protein is likely to be SRG3. This is supported by the observations that the protein matches well to the size of

![Figure 2.](image-url)
possibly with other SWI–SNF proteins.

The SRG3 associates with a SWI2-like protein and is immunoprecipitated with the SRG3 antiserum and blotted with the hSWI2 antiserum. When thymocyte extract was immunoprecipitated with the hSWI2 antiserum, a specific band corresponding to the mouse SWI2-like protein as well as SRG3. Interestingly, SRG3 is expressed highly in thymocytes, brain, and testis. Northern blot analysis of SRG3 gene expression showed that the transcripts of this gene were about 5 and 3.5 kb in size (Fig. 4A). It seems that the 5.0-kb mRNA encodes the SRG3 protein because the 3.5-kb mRNA is not long enough to include the 3,300-base open reading frame, 5′- and 3′-untranslated regions, and poly(A) tail. At this point, it is not clear what the 3.5-kb mRNA species encodes for. However, when a 1.5-kb PstI fragment from the 3′-end of the SRG3 gene was used as a probe, only the 5-kb transcript was detected (data not shown), suggest-

Figure 3. Immunoblotting and immunoprecipitation of the SRG3 protein. The overexpressed COOH-terminal part of SRG3 gene in Escherichia coli system was used to immunize rabbits to produce the polyclonal antiserum. When thymus and lymph node extract were blotted with the SRG3 antiserum, bands at 155 and 170 kD were detected (A). When the extract was blotted with the hSWI2 antiserum, a band at 195 kD (B, top) was detected. After immunoprecipitating the extract with the SRG3 antiserum, the precipitates were blotted with the SRG3 antiserum (B, bottom), displaying the 155- and 195-kD bands, respectively. Immunoprecipitation with preimmune serum and blotting with the SRG3 antiserum do not show any band (B). TCL, total cell lysate; IP:Pre, immunoprecipitation with the pre immune serum; IP: anti-SRG3, immunoprecipitation with the SRG3 antiserum.

Figure 4. Northern blot analysis of SRG3 gene expression in different organs (A) and cell types (B). The same amount (15 μg) of total RNAs isolated from various tissues were analyzed by probing with a 1.8-kb HindIII fragment (bases 653–2361). (A) The lanes represent thymus (1), spleen (2), brain (3), lymph nodes (4), testis (5), and lung (6). Two transcripts of about 5 and 3.5 kb in size were expressed highly in thymus (lane 1), brain (lane 3), and testis (lane 5). (B) Both T and B cell expressed the SRG3. Lane 1, thymus; lane 2, T cells; lane 3, B cells. Normal T and B cells were separated from spleen and lymph nodes by magnetic activated cell sorter (miniMACS). The purity of the separated population was tested by probing the RNA blot with TCF-1 (dotted arrowhead). Both B and T cells expressed the same levels of SRG3, as judged by the control β-actin probe (open arrowhead).
SRG3 protein (and the 170 kD protein) exists independently of the SWI–SNF complex and that they may play a special role in developing thymocytes. This was similarly reported for BAF155 and BAF170 proteins; some BAF155 and BAF170 are not associated with human SWI–SNF complexes (42).

One of the roles played by the SRG3 or the SWI–SNF complex in thymus may be mediating GR-induced transcriptional activation. One consequence of it is the induction of GC-mediated apoptosis in immature thymocytes. Thus, we have tested whether the SRG3 is required for the GC-mediated apoptosis in GC-sensitive thymocyte cell line. A plasmid expressing the SRG3 gene in anti-sense orientation under the control of CMV promoter (pRcASRG3) was constructed. The pRcASRG3 construct was introduced into a GC-sensitive thymoma cell line, S49.1, through DNA transfection, and two clones, clone A and B, displaying reduced expression of SRG3 were selected. As shown in Fig. 6, clone A and B express ~50% and 30% SRG3 protein of the vector transfectants, respectively. The DNA fragmentation induced by GC treatment was greatly reduced in these clones and this effect was more dramatic in clone B, which expressed lower level of SRG3 protein than clone A (Fig. 6 B). The reduction in apoptotic cell death of the pRcASRG3 transfectants was also confirmed by FACS analysis of the DNA contents of the cells. After GC treatment, about 46% of the vector transfectants were subdiploid and apoptotic (Fig. 6 C, d); however, only about 4% of the treated clone B transfectants were apoptotic (Fig. 6 C, d). These results suggest that the SRG3 protein is involved in the GC-induced apoptosis of the thymoma cell line.

Discussion

The subunit proteins of the SWI–SNF complex were initially identified in S. cerevisiae as transcriptional activators of a set of genes. The SWI–SNF proteins seem to facilitate transcription by antagonizing the repressive actions of chromatin (21–23). The subunit proteins seem to function as components of a complex that associate with gene-specific activators. Mutations in any of the subunit genes resulted in very similar phenotypes and the phenotype of multiple defective swi-or snf- mutants was identical to that of a single swi- or snf- mutant (33). Besides, SWI–SNF proteins functioned interdependently in transcriptional activation (32). In addition, the SWI–SNF proteins were copurified and coimmunoprecipitated (31, 34) and were shown to be components of a large multisubunit complex (34, 35). Recently, human SWI–SNF complexes were shown to be present in multiple forms made up of 9–12 proteins and several of them were purified using the BRG1 antiserum (42, 43). There seems to exist several different forms of SWI–SNF complexes in a single cell and in different differentiated cell types (42). Based on this, it has been suggested that these complexes are involved in tissue-specific and developmental process-specific chromatin remodeling activity needed for the differentiation of a cell (42). The human homologues of yeast SWI3, the BAF155 and BAF170, were
also identified and the two proteins seem to exist as core components in the same protein complex (42). SRG3 identified in the present study is homologous to human BAF155. The overall sequences are 94% similar to each other and the protein products are quite similar in size. The antiserum produced against SRG3 recognized an additional 170-kD protein, which seems to correspond to human BAF170, implying that there also exists two SWI3 homologues in mouse. In spite of this similarity, the expression patterns of BAF155 and SRG3 seem to be somewhat different. BAF155 is selectively expressed in muscle and heart but expressed at relatively low levels in liver and brain (42); however, SRG3 is expressed at much higher level in brain than in liver (Fig. 4 A). The selective expression patterns of BAF155 and BAF170 in different tissues were quite similar to each other. However, mouse SRG3 is expressed at higher level in thymus than in peripheral lymphoid tissues (Fig. 3); on the other hand, the 170-kD protein is expressed at similar levels in these tissues. Interestingly, some SRG3 proteins and the 170-kD proteins seem to exist independently of the SWI–SNF complex (Fig. 5). It is not yet conclusive whether some SRG3 and 170-kD proteins exist as heterodimers or homodimers. These results suggest that even though the human and mouse homologues of yeast SWI3 are structurally and functionally similar, they may play distinct roles in each species and in different tissues.

In yeast, the native GR can activate transcription from a promoter bearing the GR-responsive element in the presence of glucocorticoid (GC) (57, 58). It has been reported that transcriptional activation by GR, which regulates the expression of a network of genes in a tissue-specific manner, is dependent on SWI1, SWI2, SWI3, SWP73 gene functions in yeast (19, 59). Furthermore, SWI3 was coimmunoprecipitated with GR in yeast extracts (19), suggesting that the two proteins interact directly upon activation of GR. Our study showed that SRG3 was highly expressed in developing thymocytes compared with mature peripheral T and B lymphocytes (Fig. 3). When the level of SRG3 protein was reduced to ~50–30% of normal level of thymoma cells by expressing anti-sense RNA to the gene, apoptosis induced by GC on these cells was significantly reduced (Fig. 6). These results indicate that SRG3 is required for GC-induced apoptosis in the thymoma cells and suggests that SRG3 is an important factor for GC-mediated regulation of thymocyte development.

It has been hypothesized that GC might affect thymocyte development in a number of ways. Thymocytes respond to GC by apoptosis both in vitro and in vivo (2, 60). In vivo, the immature CD4−CD8+ thymocyte population is rapidly killed in the presence of GC, whereas both the CD4−CD8− precursor population expressing no TCR and mature thymocytes (CD4+ or CD8+) cells expressing high levels of TCR are relatively resistant. Using anti-CD3 monoclonal antibodies as a model for negative selection, it has been found that pretreatment of mice with a GR antagonist, RU486, protects immature CD4−CD8+ thymocytes from apoptosis (61). Furthermore, it has been reported that radiosensitive thymic epithelial cells constitutively produce GC (12). Thus, GC and GR may function as important regulators in normal thymic differentiation (14, 15). At this point, it is not clear whether GC-induced apoptosis of thy-
moma cells requires SR G3 as a component of the SWI–SNF complex or as a separate entity. In yeast, transcriptional activation by GR is blocked by disrupting any one of SWI1, SWI2, and SWI3 genes, suggesting the possibility that the SWI–SNF complex is required for the GC sensitivity of thymocytes. However, it is noteworthy that SR G3 protein is expressed at a much higher level in thymocytes than in peripheral T lymphocytes and that the major part of SR G3 protein may exist independently of the SWI–SNF complex in thymus. Furthermore, even though the antisense RNA expression reduced the level of SR G3 protein in the transfected cells, there still remained ~50% (clone A) or 30% (clone B) of normal level of SR G3 protein (Fig. 6).

Even in the case of the clone B transfectant, this is at least the similar level of protein found in peripheral T lymphocytes (Fig. 3). Therefore, it is likely that there still may be enough SR G3 protein left to form SWI–SNF complexes in the transfectants. These results suggest a possibility that SR G3 protein may function independently of the SWI–SNF complex in GC-mediated apoptosis. No matter how SR G3 functions in GC-mediated apoptosis in thymoma cells, either as a component of SWI–SNF complex or as an independent factor of the complex, our present results show that SR G3 protein is required for the process and possibly plays an important regulatory role during thymocyte development.

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