SOX13, A γδ T CELL-SPECIFIC GENE, IS A WNT-SIGNALING ANTAGONIST REGULATING T CELL DEVELOPMENT

A Dissertation Presented

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DEDICATION

This thesis is dedicated to two intelligent and beautiful women,
Dr. Cynthia Chambers and my mother, Carole Melichar.

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ABSTRACT

Mature αβand γδ T cells arise from a common precursor population in the thymus. Much debate has focused on the mechanism of T cell lineage choice made by these multi-potential precursor cells. It is widely believed that the decision of these precursor cells to commit to the γδ or αβ T cell lineages is regulated primarily by a specific instructive signal relayed through the appropriate T cell receptor. Contrary to this model, we present evidence for a TCR-independent lineage commitment process. Comparison of global gene expression profiles from immature αβ and γδ lineage thymocytes identified *Sox13*, an HMG-box transcription factor, as a γδ T cell-specific gene. Unlike other HMG-box transcription factors such as TCF1, LEF1 and SOX4, that are critical for proper αβ T cell development, *Sox13* expression is restricted to early precursor subsets and γδ lineage cells. Importantly, SOX13 appears to influence the developmental fate of T cell precursors prior to T cell receptor expression on the cell surface. Transgenic over-expression of *Sox13* in early T cell precursors strongly inhibits αβ lineage development, in part, by inhibiting precursor cell proliferation and concomitantly, leading to increased cell death among αβ lineage subsets. Steady-state γδ T cell numbers, however, appear unaffected. Strikingly, the DP αβ lineage cells that do develop in *Sox13* transgenic mice are imprinted with a γδ- or precursor-like molecular profile, suggesting that SOX13 plays an active role in the lineage fate decision process or maintenance. *Sox13*-deficient mice, on the other hand, have selectively reduced numbers

of γδ thymocytes, indicating that SOX13 is essential for proper development of γδ T cells. We present additional data demonstrating that SOX13 is a canonical WNT signaling antagonist modulating TCF1 activity, raising a strong possibility that WNT signals, and their modulators, are at the nexus of γδ versus αβ T cell lineage commitment.

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CHAPTER I

INTRODUCTION

At some point in its lifetime, a stem cell is directed along a likely irreversible path that ends with its differentiation into a mature, functionally-specific cell type. As there are over 200 different cell types in mice (and humans), the mechanisms that regulate lineage choice are of paramount importance. The immune system, itself, contains an extraordinarily diverse repertoire of cell types that contribute unique protective functions. While the processes that account for lineage fate decisions in the lymphoid system continue to be intensely studied, many aspects remain elusive. Therefore, identifying the pertinent genes, how their inherent functions are responsible for differentiation of lymphoid precursor cells, and how they participate in a cascade of signals that imprints specific lineage fates upon daughter cells is central to our understanding of the immune system. In addition, elucidating the determinants of lineage choice goes much farther than revealing the fundamental nature of immune system formation, as these factors are undoubtedly linked to regulation of cell proliferation, and aberrant expression of lineage determining genes has been linked to tumorigenesis (1).

Murine T cells arise from pluripotent hematopoietic stem cells (HSC) that reside in the fetal liver and adult bone marrow (2). HSCs subsequently differentiate through discrete intermediates that have increasingly restricted lineage potentials. Along the differentiation pathway, the precursor cells are directed through numerous lineage choices via intrinsic and extrinsic cues. Eventually, most precursor cells destined for the T cell lineage migrate to the thymus where additional cell lineage choices are made. In

particular, this thesis addresses how bi-potent thymic T cell precursors choose between two distinct cell fates to become αβ or γδ lineage T cells. These two T cell subsets arise at distinct stages of ontogeny, preferentially localize to distinct tissues, and perform unique, as well as overlapping, immune functions (3, 4).

T cell development

Lymphoid precursor cells that seed the thymus remain multi-potent, capable of generating not only αβ and γδ T cells, but also NK cells, NK T cells, some B cells, and a subset of thymic dendritic cells (5, 6). Cell surface expression of lineage markers is low or absent on these precursor cells (Lin^-). Routinely, these precursor cells are termed triple negative (TN), since they lack high expression of CD4, CD8, and CD3 on their cell surface, markers that are indicative of more mature cells of the αβ and γδ T cell lineages (7, 8). To become mature αβ and γδ T cells, the lymphoid precursor cells that have immigrated to the thymus transit through several, partially overlapping, developmental intermediates. These intermediates are identified by differential cell surface expression of specific markers, most notably CD44, CD25, and CD117 (c-kit) (9-11). Additional cell surface antigens are being characterized to further differentiate even more specific thymocyte precursor subsets with increasingly restricted developmental potentials. In addition, it should be noted that although the delineation of developmental intermediates in the lymphoid, and specifically T cell, lineage is exquisite compared to that of other cell types, the categorization of the progenitor populations is not absolute, and markers of the

precursor subsets are constantly updated. Hence, a basic, but widely accepted model of T cell development is described (Figure I-1).

It is generally accepted that the earliest T cell progenitor population in the thymus is characterized by cell surface expression of CD44 and absence of CD25 on TN cells (TN1). This population is regarded as the most multi-potent of the thymic precursors. TN1 precursor cells expressing CD117, however, are thought to be more restricted to the T cell lineage, and are therefore called early T lineage progenitors (ETPs) (11, 12). Upregulation of CD25 expression on these cells indicates further, but not absolute, commitment to the T cell lineage (TN2). This TN2 precursor population can give rise to both αβ and γδ T cells. However, the exact stage at which T cells are diverted to either the αβ or γδ lineage remains unknown. While it has been shown that the TN2 subset is heterogeneous in lineage potential based on IL-7Rα expression (13), others contend that lineage diversion of these cell types does not occur until the TN3 stage when both the αβ and putative γδ selection processes occur. The TN3 stage is marked by the subsequent loss of both CD44 and CD117 expression and is also the point at which precursors are fully committed to the T cell lineage. Rearrangement and expression of the γ, δ, and β T cell receptor (TCR) chains occurs as cells mature from the TN2 to the TN3 stage (14, 15). A positive selection process for cells expressing the γδ TCR has recently been suggested (16, 17), but the timing and specifics of this process are still unclear. It is known, however, that successful rearrangement and expression of the TCRβ chain complexed with a surrogate α chain, termed the preTCR, is required for the survival, proliferation, and further differentiation of αβ lineage T cells (18, 19). In addition, this selection

Figure I-1

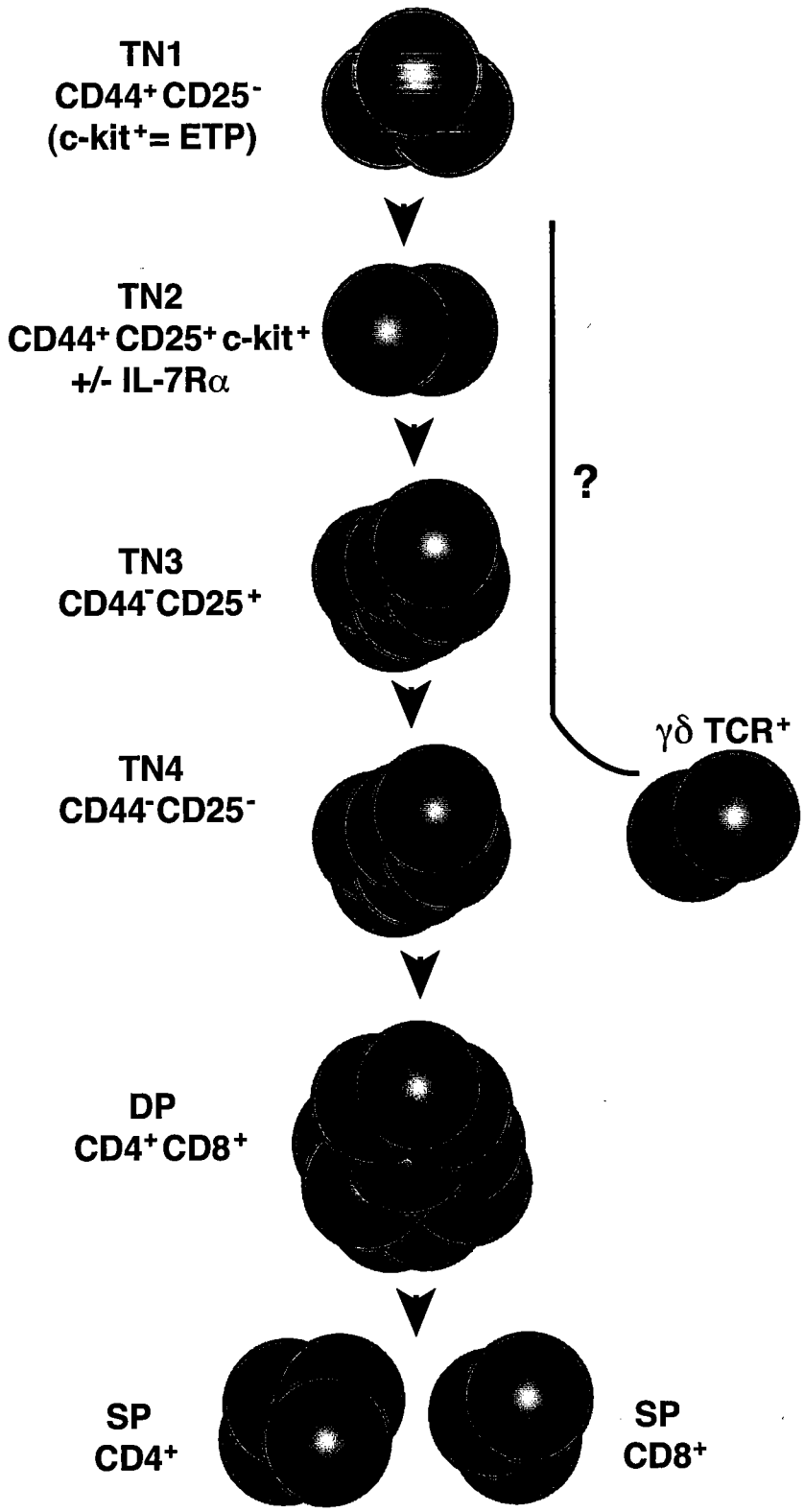

TN1
CD44+ CD25-
(c-kit+= ETP)
TN2
CD44+ CD25+ c-kit+
+/- IL-7Rα
?
TN3
CD44- CD25+
γδ TCR+
TN4
CD44- CD25-
DP
CD4+ CD8+
SP
CD4+
SP
CD8+

Figure I-1. Schematic of thymocyte development. Thymocyte precursor cells that are $CD4^-CD8^-CD3^-$ (TN) transit through several intermediaries identified by differential expression of cell surface antigens. TN1: $CD44^+CD25^-$ (and c-kit^+, identifies ETPs), TN2: $CD44^+CD25^+$c-kit^+, TN3: $CD44^-CD25^+$c-kit^-, and TN4: $CD44^-CD25^-$c-kit^-. TCR selection processes occur at TN3. When γδ T lineage cells diverge from this pathway is not known. αβ lineage T cells continue through a $CD4^+CD8^+$ DP stage before down-regulating expression of one co-receptor to become mature SP αβ lineage T cells.

process, termed β-selection, is required to initiate the rearrangement and expression of the TCR α chain. αβ T cells quickly transit through the TN4 stage, characterized by the loss of CD25 expression, as well as an immature $CD8^+$ single positive (ISP) stage, and on to become immature αβ lineage cells that express both TCR co-receptors, CD4 and CD8, and are thus called double positive (DP) cells. According to one report, cells categorized within the TN4 subset still have a limited ability to still divert toward the γδ T cell lineage (20). However, by the DP stage, this cell population is absolutely committed to generating αβ lineage T cells. After further TCR selection processes and cell fate choices, DP cells down-regulate expression of one of the TCR co-receptors, either CD4 or CD8, to become more mature single positive (SP) cells that exit the thymus to perform T helper ($CD4^+$) or cytotoxic ($CD8^+$) functions. In contrast to these known developmental check-points and intermediates of the αβ lineage, additional γδ T cell-specific intermediates have yet to be identified, and the only known cell surface marker for this lineage is the γδ TCR itself.

Distinct properties of αβ and γδ T cells

Although αβ and γδ T cells arise from a common progenitor population in the thymus, one critical distinguishing property of these two cell lineages is their rate of cell division: intrathymic αβ T cell development is accompanied by extensive cell expansion—up to 10 cell divisions post-β-selection (21), whereas γδ lineage cells undergo only one to three cell divisions prior to exiting the thymus (22). Thus, the proliferative burst that occurs after β-selection, appears to be reserved for αβ lineage T

cell precursors. This asymmetrical cell expansion property, at least in part, leads to the predominance of αβ T cells over γδ T cells in the thymus and peripheral lymphoid organs. However, γδ T cells are abundant in mucosal epithelia, equaling or surpassing the size of the αβ T cell pool in these tissues in many vertebrates (4).

Another important difference in αβ and γδ T cell generation is when the majority of development occurs during ontogeny. In general, γδ T cells develop at earlier stages than αβ T cells. It is estimated that ETPs migrate to the thymus between embryonic days 10 to 11 (E10-11) (23, 24), and initially develop exclusively into γδ lineage T cells. Significant αβ T cell development does not occur until several days later (25). This skewing of the thymocyte repertoire suggests the environment in the fetal and adult thymus may also be dramatically different, or that the precursors that seed the thymus have significantly different developmental potential during the early waves of thymic development to direct differentiation of subtypes of γδ T cells. For example, in adult mice, it has been shown that normal γδ T cell development requires the presence of wild-type αβ lineage DP cells for proper development (26). This cross-talk between the two lineages occurs via lymphotoxin mediated signals (27). However, in the early stages of thymic γδ development, DP cells are not yet detected, and yet these cells develop normally. One explanation proposed for this observation suggests that as αβ lineage cells eventually expand and dominate the thymus, γδ interactions with stromal cells that may also convey this essential lymphotoxin-mediated signal are overwhelmed and disrupted, making developing γδ T cells reliant upon lymphotoxin-producing DP cells. However, in *TCRβ*$^{-/-}$ mice in which the γδ/stromal cell ratio is increased relative to a

wild-type adult thymus, γδ development remains abnormal due to the lack of appropriate lymphotoxin-mediated signals. Hence, γδ T cell subsets that arise at different stages of ontogeny may differ in their thymic requirements. Correspondingly, it has been shown that distinct types of γδ T cells develop in the fetal vs. adult thymus. For instance, early fetal T cell development is dominated by the production of dendritic epidermal γδ T cells (DETCs) (28). DETC progenitors require unique properties within the fetal thymus as the development of these cells cannot be supported by an adult thymus (29).

Upon reaching maturity, αβ and γδ T cells emigrate from the thymus to reside in secondary locations. The majority of αβ lineage T cells home to secondary lymphoid organs such as the lymph nodes and spleen, as well as circulate through the blood. Although some γδ T cells also inhabit these secondary lymphoid niches, they generally make up only five to ten percent of the total T cells in these organs. Rather, many γδ T cells localize to mucosal epithelia and the skin to perform specialized functions (4). αβ T cells participate in the adaptive immune response to pathogen by recognizing peptide ligand in the context of major histocompatibility complex (MHC) presented by antigen presenting cells. In contrast, γδ T cells are early responders, and generally considered part of innate immunity, and although few γδ T cell ligands have been identified, they are thought to sense physiological disturbances and respond to "stress-induced" self-ligands in the context of non-classical MHC molecules (30-32). Thus, a role for γδ T cells in wound healing and tumor surveillance has been suggested, but γδ T cells also appear to play a role in protective immunity against certain infections (33-37).

Regulators of T cell lineage choice

Three major cell fate decisions are made before mature T cells exit the thymus: the initial decision to become a T cell, whether or not to enter the γδ or αβ lineage, and once determinedly an αβ T cell, whether to adopt the T helper or cytotoxic T cell fate. Within the T cell lineage, two "master regulators" of these cell fate processes have been described. Notch1, a transmembrane signaling receptor that plays important roles in lineage specification in a plethora of invertebrate and vertebrate cell types, also plays a decisive role in mouse B vs. T cell lineage commitment. Enforced expression of constitutively active *Notch1* in early hematopoietic precursors leads to extra-thymic development of immature T cells, specifically in the bone marrow, at the expense of B cell development (38). Furthermore, in the absence of Notch1 signals, HSCs fail to produce mature T cells, and immature B cells inappropriately develop in the thymus (39). These data persuasively imply that Notch1 signaling is both necessary and sufficient for T cell specification from a precursor population capable of giving rise to both the T and B cell lineages.

A second transcription factor, Th-POK, has recently been described as the ultimate regulator of CD4 vs. CD8 SP T cell specification within the αβ lineage. Mirroring the observations reported for *Notch1* signaling deficiency and over-expression, two separate groups nearly simultaneously identified Th-POK (also known as, cKrox) as a master regulator of the $CD4^+$ T helper cell lineage. Over-expression of this gene results in the re-direction of MHCI restricted cells to the $CD4^+$ T cell lineage (40, 41). Conversely, a spontaneous mouse mutant with a point mutation in Th-POK showed

aberrant conversion of MHCII restricted αβ T cells to the $CD8^+$ lineage (40, 42). The factor(s) that influences Th-POK expression, however, is unknown, although it has been suggested that expression is not stochastic, but rather directed by TCR signals during positive selection.

Our knowledge of αβ vs. γδ T cell lineage choice, however, is ambiguous at best. No single gene has yet been identified that is capable of converting precursors exclusively to the γδ or αβ lineage. Thus, several hypotheses have been bandied about as to how these two cell types diverge from a common precursor population even before identification of the TCRδ genes, and though these models have incrementally gathered circumstantial support, definitive resolution of this phenomenon remains unsettled. From the beginning, two basic schools of thought on this subject collided, one that favored TCR-centric models and others whom put forth TCR-independent models of lineage commitment. These two basic models of lineage commitment have evolved over time as knowledge of about these two T cell subsets has increased. One early model suggested that lineage determination occurred prior to TCR gene rearrangement and that lineage determination would direct which TCR genes would be rearranged. However, early studies identified some rearranged γ transcripts that were found in functional αβ T cell lines and T cell leukemias, though non-functional in their rearrangements, arguing against this early stochastic model of lineage commitment. Several years later, it was noted that β and γ TCR genes rearranged their loci significantly earlier than the TCRα chain. This and several other pieces of evidence led to the postulation of a step-wise rearrangement model. More specifically, since evidence suggested that γδ T cells did not

usually express full-length α or β mRNA (though this is not absolute), αβ T cells invariably have rearranged γ genes (including some in-frame rearrangments), and that β and γ chains rearrange simultaneously, Allison and Lanier suggested that the γ TCR chain would complete rearrangement before the β TCR chain as it required only V-J recombination in contrast to the V-DJ recombination necessary for full β chain rearrangment (43). At about the same time, evidence supporting an ordered gene rearrangement model or stochastic model lineage choice (depending on one's interpretation) surfaced. The identification of the TCRδ chain was solidified and found embedded within the TCRα locus (44). Excision products of Vα-Jα recombination therefore contained δ genes. Although it was initially thought that these circular DNA excision products contained only unrearranged δ genes (45), it was later shown that some of these excision circles contained functionally rearranged δ chains (46). Though this did not confirm co-presence of functionally rearranged γ in these cells, this data added fuel to the on-going debate. Concurrently, an additional instructive or competitive model suggested that positive selection through either the γδ or αβ-specific preTCR dictated lineage choice (47). This model could explain why functional rearrangements of TCR genes of the opposite lineage are sometimes found within a T cells as this model requires expression of the appropriate TCR on a pre-committed cell, but does not rule out random rearrangement of other TCR genes.

It was apparent that rearrangement and expression of TCR genes is required for the development and survival of T cells, but the role of the TCR in lineage determination was still controversial. Though evidence suggested that rearrangement and expression of

αβ or γδ TCR may inhibit other lineage development, further analysis of TCR transgenic and knock-out models suggested that, perhaps, TCR type is not the ultimate regulator of lineage fate. For example, transgenic expression of rearranged αβ or γδ TCRs does not appear to inhibit development of the opposite lineage (48). Further, additional data suggested that a γδ TCR transgene could actually support αβ T cell development in the absence of functional TCRβ (49, 50).

Though much of this data is contradictory or multiple interpretations are plausible, relatively recent reports that have gained significant attention propose a variation on the instructive mechanism suggesting that TCR isotype is not the ultimate lineage director. Rather, the signal strength model suggests that it is the quantity of signal rather than the TCR isotype that regulates cell specification (51, 52). This model was proposed based on observations from γδ TCR transgenic mouse models in which the TCR signal strength is manipulated by regulating the amount of cell surface γδ TCR, the availability of cognate ligand, or the signaling response (51, 52). In this TCR-limited model system, preferential development of αβ lineage cells at the expense of γδ lineage T cells was observed when γδ TCR signal strength was lower, and *vice versa*. However, the qualitative or quantitative differences in TCR signaling that the instructive and signal strength models predict would need to imprint the receiving cells with lineage-specific molecular programs. While intriguing, supporting evidence that the TCR isotype or strength of signal promotes expression of αβ or γδ-specific genes is currently lacking.

The experiments described above and additional observations suggesting that the TCR is the central regulator of αβ and γδ lineage choice do not preclude the possibility

that precursor cells are pre-committed to a T cell lineage and that appropriate TCR signals function as a necessary check-point for differentiation to a programmed lineage fate. Thus, a contrary model, the stochastic model of lineage commitment, proposes that T cell lineage fate is determined prior to, and independent of, TCR signaling (48, 53). Although the importance of TCR signaling in determining lineage choice is de-emphasized in this model, it is not ignored, as it is well known, at least for the αβ T cell lineage, that TCR signaling is necessary for the survival, proliferation, and further differentiation of precursor cells to maturity (18, 19). Rather, this model proposes that the precursor population capable of giving rise to αβ and γδ lineage T cells is heterogeneous in its lineage potential, and that lineage decisions are made before TCR signaling occurs.

The three major predictions of the stochastic model of lineage commitment are supported by several observations. First, this model predicts a heterogeneous precursor population. It has previously been shown that the TN2 precursor subset, permissible to both αβ and γδ T cell lineage development, is heterogeneous in lineage potential based on IL-7Rα expression, such that the IL-7Rα^{hi} TN2 cells preferentially give rise to γδ lineage T cells, and IL-7R$\alpha^{neg\text{-}lo}$ cells are developmentally biased toward the αβ T cell lineage (13). Second, the stochastic model predicts that lineage commitment occurs independently of TCR isotype. While several pieces of evidence support this prediction, one of the most striking examples is that αβ lineage DP cells develop in *Tcrβ*$^{-/-}$ mice and are dependent upon γδ TCR signals (22). In addition, αβ TCR transgenic mice are known to develop αβ TCR expressing CD4$^-$CD8$^-$ cells with some phenotypic and

functional characteristics of γδ T cells (54). Although this evidence is less concrete, only because the only absolute identifier of the γδ T cell lineage is the γδ TCR itself, cumulatively, these data suggest that at least the type of TCR does not restrict lineage specification. Lastly, this TCR-independent model would necessitate lineage-restricted gene expression patterns. Until recently, very little was known about the molecular differences between these two T cell lineages. Recently, comparison of gene expression profiles from αβ and γδ lineage T cells fulfilled this final prediction of the stochastic model, a γδ-lineage restricted gene expression pattern (26). These data led to the identification of several γδ-biased genes and at least one new signaling pathway necessary for normal γδ T cell development in adult mice, but unfortunately, it lent no insight as to how γδ T cell lineage choice occurred, particularly because no known lineage-specific markers have been identified prior to cell surface TCR expression. One of the γδ-biased genes identified down-stream of lymphotoxin signaling, ICER, is a transcription factor that is also expressed in early thymocyte precursor populations, but not in αβ-lineage T cells. Interestingly, ICER appears to be expressed in most progenitor cells suggesting that it is not a marker of a subset of pre-committed γδ T cells (27). Although these data support a stochastic model of αβ and γδ T cell lineage specification, the evidence is circumstantial rather than absolute. Hence, identification and purification of a lineage-determined precursor subset is required to definitively settle this on-going debate.

Morphogen and other signaling pathways in T cell development

In lower organisms, such as in the *Drosophila* model system, a number of unique signaling proteins, dubbed morphogens, relay concentration-dependent specific cellular signals and regulate almost every aspect of cell fate specification from the head to the tip of the wing. In addition to the morphogen concentration gradient itself, the effects on the cell from these signaling proteins depends on the expression level of their receptor and on additional endogenous proteins expressed by the cell that regulate the effect of incoming signals. It is apparent that these signals are exquisitely interrelated and interdependent, as well as enormously important for proper development (55). A number of these morphogens have mammalian signaling protein counterparts, many of which are expressed in the murine thymus and are implicated in lineage-specific developmental regulation and perhaps lineage determination. Following is a brief discussion about the role of select morphogen pathways and other signaling proteins in thymocyte development or lineage commitment.

One morphogen pathway implicated in maintaining appropriate αβ T cell development is the Hedgehog signaling pathway. There are three mammalian secreted Hedgehog (Hh) proteins that bind to a receptor composed of transmembrane proteins, Patched (Ptc) and Smoothened (Smo). In the absence of Hh proteins, Ptc inhibits Smo activity. In the presence of Hh signal, this inhibition is relieved such that Smo may activate members of the Gli family of zinc finger transcription factors that subsequently regulate, positively or negatively, the expression of target genes (56). In thymocyte development, the three Hh proteins are expressed by different components within the

human thymus (57), while only two have been identified in the murine fetal and adult thymus (58). The receptor component Smo, is most highly expressed on TN2 cells and then incrementally down-regulated during further stages of differentiation (57-59). Several different model systems have been employed to study Hh signaling in thymocytes. At the surface, the results appeared incongruent in many aspects as one model suggested that Hedgehog signaling inhibited differentiation, whereas another suggested that Hedgehog was necessary for αβ lineage development (58-60). These disparate findings may be reconciled with the observation that the dose of Hh signaling is of paramount importance at different stages of development. Strong Hh signaling may be required in the precursor population, where, correlatively, the Hh transducer, Smo, is at its highest expression, to maintain precursor cells in an undifferentiated state and promote their proliferation, while lower doses of Hh signals appear to be necessary for the differentiation of αβ lineage T cells (60). Unfortunately, differential expression of Hh signaling pathway proteins between αβ and γδ T cells has not yet been studied, but it is possible that Hh signals may differentially affect development of these two lineages.

In addition, another evolutionarily conserved signaling pathway through Notch has been implicated in a number of lineage fate choices within the lymphoid system including T/B specification, αβ vs. γδ T cell determination, and $CD4^+$ or $CD8^+$ αβ-lineage fate choice (61, 62). The influence of Notch signaling, or lack thereof, on directing thymocyte precursors to the αβ or γδ lineages is a veritable conundrum. In part, some of the confusion in the literature can be attributed to partial redundancy in the pathway due to the multiplicity of Notch ligands coupled with the fact that several Notch

receptors are expressed in the thymus (63). Therefore, examples presented herein will focus on experimental mouse model systems that limit potential redundancy issues.

Activation of the Notch signaling pathway upon ligand binding leads to cleavage of the intracellular fragment of Notch (icNotch) and its translocation into the nucleus where it binds to RBP-J to mediate transcription. RBP-J can interact with all four known mammalian Notch proteins and can activate transcription of target genes. In theory, conditional inactivation of RBP-J should inhibit signaling through each of the different Notch receptors (64). However, a RBP-J independent signaling pathway has been described (65). Nevertheless, directed deletion of floxed *Rbp-J* by *Lck* promoter driven CRE recombinase in early thymocyte precursor development leads to early developmental arrest of the αβ T cell lineage with a significant block at the TN3 stage of development (66). In contrast, in the absence of Notch signals through RBP-J, an increase in the absolute numbers of γδ T cells was observed, suggesting that Notch signals are required early in αβ T cell development but not in γδ T cell commitment or development (66). Complementary studies using a mouse model with enforced expression of a constitutively active form of Notch1 support this finding. In one study, constitutively active Notch signaling appears to promote abnormal development of γδ T cells expressing the CD8αβ heterodimer which is generally associated only with αβ lineage T cells (67). These data suggest a clear influence of differential Notch signals on αβ and γδ lineage development. However, these experiments are not without caveats, as dissenting data has been presented. As mentioned briefly, different Notch signaling family members may impart specific signals to recipient cells. For example, the Notch

ligand Delta, but not Jagged, can regulate T/B differentiation (19). However, *Jagged* deficiency results in a reduction of fetal γδ T cells while αβ lineage development appears unaffected (68). This observation suggests more complexity in the signaling pathway, but may reflect specific differences among Notch signaling family members to influence αβ and γδ T cell development and/or differences in fetal and adult thymic development.

Another signaling pathway, via IL-7/IL-7R interactions, has been implicated, not necessarily in lineage choice, but in lineage-specific processes. IL-7 is secreted by the thymic stroma, and the IL-7R is dynamically expressed during T cell development (69). This receptor is composed of two chains, the unique IL-7Rα chain and a common γ chain (γc) that is promiscuously shared among other cytokine receptors including those of IL-2, IL-4, IL-9, IL-15, and IL-21 (70, 71). In the thymus though, IL-7/IL-7R interactions are non-redundant with other signaling pathways that share γc, as it is indispensable for normal T cell development. Correlating with the highest levels of IL-7Rα expression (69), loss of the IL-7/IL-7R signal primarily impacts early thymocyte precursors and γδ T cell development. In IL-7Rα-deficient mice, the absence of signaling leads to a significant decrease in thymic cellularity due, in part, to increased cell death among precursor cells. This, in turn, leads to an overall decrease in the αβ T cell compartment (72-75). γδ T cells, however, are nearly, if not completely absent without IL-7/IL-7R signaling (76, 77). It is proposed that this signaling pathway is absolutely required for the expression of the TCR γ locus, a prototypical marker of the γδ lineage (78, 79). Of note, it has been implied that the effects of this signaling may be dose-dependent: low doses of IL-7 may benefit αβ T cell development, whereas high doses that impede the αβ

lineage may actually enhance γδ T cell differentiation (80). This effect may be regulated by the amount of cell surface expression of the IL-7R, as different levels of receptor expression correlate with lineage potential (13).

WNT, a mammalian homologue of the *Drosophila* morphogen, Wingless, also impacts T cell development. The canonical WNT signaling pathway acts through β-catenin. In the absence of WNT signaling, β–catenin is sequestered in the cytoplasm by an inhibitory complex that includes GSK3β. This complex directs the ubiquitination and subsequent proteolytic degradation of β-catenin. In the presence of WNT signals, β-catenin is released from this inhibitory complex and translocates to the nucleus where it can bind to transcriptional co-activators of the TCF/LEF family that are otherwise bound to the transcriptional repressor, Groucho (Figure I-2) (56).

Suggestions that WNT signaling may be involved in thymocyte development came from the identification of T cell factor 1 (TCF1) as a necessary factor in αβ T cell development. In *Tcf1*$^{-/-}$ mice, there is a significant, but incomplete block at the ISP stage, as TCF1 plays an important role in the differentiation, proliferation, and survival of αβ lineage cells (81, 82). TCF1, and fellow HMG-box transcription factor family member, Lymphoid enhancer-binding factor 1 (LEF1), play partially redundant roles in αβ T cell development. In mice doubly deficient for these two transcription factors, αβ lineage T cell development is blocked prior to the DP stage (83). There are as many as eight TCF1 isoforms in the thymus (84). One of the dominant isoforms, however, contains a β-catenin interaction domain (82). Over-expression of this isoform is capable of rescuing, for the most part, the αβ T cell deficiency in *Tcf1*$^{-/-}$ mice, suggesting that TCF1 is indeed

Figure I-2

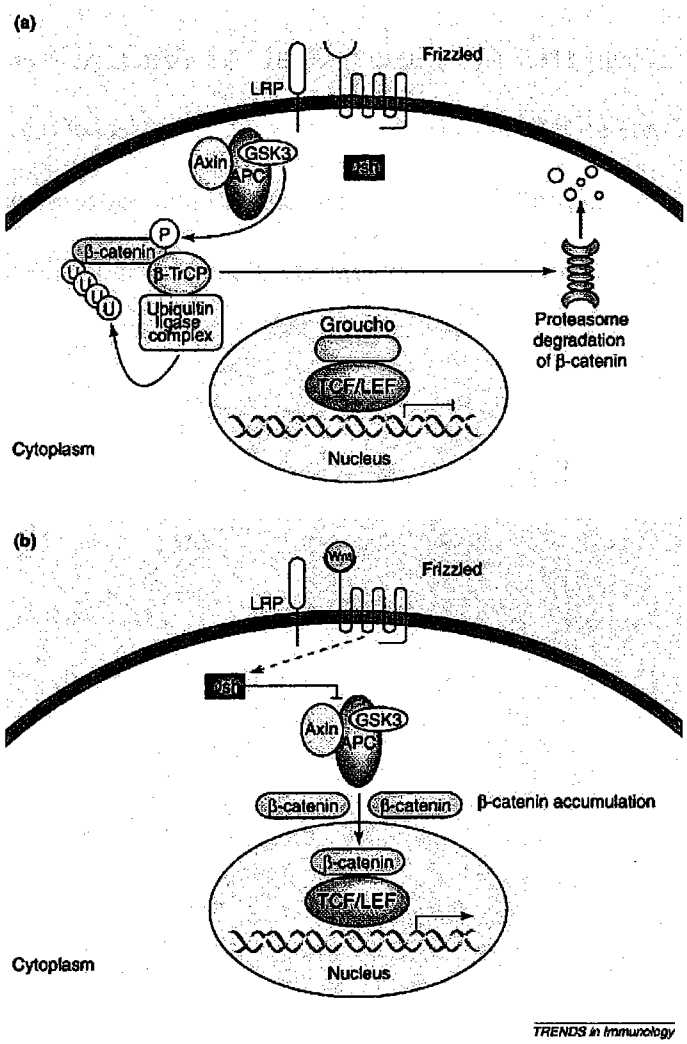

(a)
Frizzled
LRP
Axin
GSK3
APC
Dsh
P
β-catenin
β-TrCP
U
Ubiquitin ligase complex
Proteasome degradation of β-catenin
Groucho
TCF/LEF
Cytoplasm
Nucleus
(b)
Wnt
Frizzled
LRP
Dsh
Axin
GSK3
APC
β-catenin
β-catenin
β-catenin accumulation
β-catenin
TCF/LEF
Cytoplasm
Nucleus
TRENDS in Immunology

Figure I-2. Over-view of canonical WNT signaling pathway via β-catenin. In the absence of WNT, β-catenin is sequestered in the cytoplasm by an inhibitory complex that contains, among others, GSK3β and Axin. This complex directs the phosphorylation, ubiquitination, and subsequent proteolytic degradation of β-catenin. In the absence of nuclear β-catenin, TCF, and other transactivators, are bound to a repressor, Groucho, and target gene expression is inhibited. In the presence of a WNT glycoprotein binding to its receptor, frizzled, Disheveled (Dsh) mediates the release of β-catenin from the inhibitory complex and translocation into the nucleus. Binding of β-catenin to transcription factors, such as TCF, activates target gene transcription. Reproduced from Varas, et al., **Trends Immunol.,** 2003 (56).

acting down-stream of β-catenin in aspects of T cell development (82). Correspondingly, mice deficient is some of the predominant WNT glycoproteins expressed in the thymus, WNT1 and WNT4 for example, also exhibit reduced thymic cellularity, though other WNT family members may partially compensate for a necessary role in αβ T cell development (85). Interestingly, although TCF1 is also expressed in γδ lineage T cells, there appears to be no affect on thymic γδ T cell development in the absence of TCF1 (86). Expression of a constitutively active form of β-catenin appears to promote αβ T cell differentiation even in the absence of preTCR signals (87). This differentiation, however, is not accompanied by the extensive proliferation associated with preTCR signaling, and the DP αβ lineage T cells that do develop are more susceptible to apoptosis (87). This suggests that the WNT and preTCR signaling pathways play distinct roles in αβ T cell development. In addition, two similar, but independent, β-catenin conditional knock-out mouse models have been described. Deletion in early thymocyte precursors under the direction of *Lck* promoter driven CRE resulted in reduced αβ lineage thymocytes, and normal number of γδ T cells as predicted by earlier observations (88). It should be noted, however, that in a more recent study in which β-catenin was conditionally deleted in bone marrow stem cells, no disturbance in hematopoiesis or T cell development was observed (89). Based on a significant amount of data, the importance of β-catenin in T cell development is generally accepted. At present, the range of significant to absent phenotypes in these mouse models is not definitively explicable, but may reflect the timing of the β-catenin deficiency.

Many of the WNT molecules, of which there are almost twenty identified in mice, are differentially expressed in the fetal and adult thymus, mainly by the thymic epithelium (85, 90). In addition, thymocytes (and the thymic epithelium) appear to express different frizzled receptors in a developmentally regulated pattern (90). Further, three β-catenin-independent WNT signaling pathways have been described (91). Together, these observations imply that WNT signaling may play very specific roles at discrete points in the development of thymocytes and that expression and usage of different ligands and receptors may impart subtly different signals to the receiving cells. Determining the specific roles played by each of these WNT signaling components at particular times in T cell development is an important task at hand.

In lower organisms, as well as in mice, many of these pathways are have been shown to interact to impart specific cell fates upon differentiating cells. Specifically, during hematopoiesis, both WNT and Notch act to maintain the self-renewal capabilities of the stem cell population. As in other cell types, it has recently been observed that these two pathways intersect to maintain a population of undifferentiated stem cells in the bone marrow. The study found that WNT activation led to the modulation of Notch target gene expression but that additional WNT-independent Notch pathways were necessary to maintain the self-renewal capacity of HSCs and inhibit differentiation (92). It is possible that some overlap between these two signaling pathways involves the β-catenin destabilizer protein, GSK3β, as it has previously been presented that GSK3β may also initiate the degradation of icNotch (93). Hence, in the presence of WNT signaling, both β-catenin and icNotch may be stabilized to promote transcriptional activation of

target genes. As both WNT and Notch are invaluable for proper thymocyte development, it is likely that this or a similar interaction will be identified during thymocyte development. Further, down-modulation of TCF1 and LEF1 expression, transcriptional transactivators down-stream of WNT/β-catenin activation, by IL-7/IL-7R signals has been suggested (94). The IL-7/IL-7R signaling pathway is essential for early precursor maintenance and γδ T cell development. However, β-catenin dependent, TCF1 transcriptional promotion is not necessary for normal γδ lineage differentiation. Further, Sonic Hedgehog (Shh), at least in human thymocytes, may regulate the levels of IL-7R in precursor populations (95). Effects on the amount of IL-7/IL-7R signaling may, in turn, differentially promote γδ and αβ lineage development. Determining when and how these signals precisely interact may illuminate the mechanism of lineage specification in thymocyte precursor cells. These signaling pathways are impressively regulated in their own right, but in context with additional "morphogen" signals, including those of the fibroblast growth factors (FGF), bone morphogenetic proteins (BMP), and others that also appear to play key roles in the thymus, an exquisite balance must be maintained to define T cell development. Further studies to clarify the interactions between these pathways during thymocyte development, and perhaps identification of lineage-restricted factors that may influence the outcome of "morphogen" signals may be complicated, but are of the utmost importance.

It is, indeed, of great importance to delineate how these crucial signaling pathways interact, but these pathways rely on cell-intrinsic genes to orchestrate the transcriptional outcome from a plethora of extrinsic signals. Hence, the transcription

factor pool within a cell will dictate how specific signals are interpreted. Therefore, by identifying lineage-specific genes that regulate signaling cascades known to influence T cell development and lineage choice, we may be able to clarify how αβ and γδ T cell specification occurs. This thesis identifies and describes the function of a γδ T cell-specific gene that acts, in part, by modulating morphogen signaling, to influence T cell development.

CHAPTER II

IDENTIFICATION AND EXPRESSION OF SOX13

Introduction

Although a number of transcription factors have been identified as important for the development of αβ T cells (96, 97), a comparable set of genes paramount for the differentiation and maintenance of the γδ T cell lineage is virtually nonexistent. In fact, apart from the different types of antigen-specific TCRs expressed on the cell surface, little is known regarding the molecular distinction between these two cell types. One of the central questions in understanding how these two T cell lineages arise is whether the lineage choice is directed by distinct instructional signals from the γδ TCR or the αβ lineage-specific preTCR complex, or if the lineage fate process occurs independently of, and prior to, TCR expression and signaling. While the question of lineage choice has raged for well over a decade, it has been difficult to unequivocally resolve this issue or to provide irrefutable evidence for the existence of γδ or αβ T cell precursors before TCR expression, as there are no definitive markers that can distinguish these lineages prior to cell surface TCR expression and no known markers that can impose a lineage-specific fate.

To identify potential markers of the γδ T cell lineage or genes involved in the development of this T cell subset, thorough gene expression profiling was performed to compare immature DP αβ lineage T cells with thymic γδ T cells using Affymetrix Microarray gene chip technology. While these two populations may not be at the same

level of maturity, due to the lack of knowledge of γδ developmental intermediates, these are the earliest identifiable subsets unquestionably committed to the αβ and γδ T cell fates and were so chosen. Of multiple genes that were differentially expressed between these two populations, this thesis will focus on the identified γδ T cell-specific transcription factor, *Sox13*.

Sry-related HMG box 13 (SOX13) is a member of the High Mobility Group (HMG) box family of transcription factors (98). HMG transcription factors function by their unique ability to bind to the minor groove of DNA, with or without DNA sequence specificity, and induce a significant structural bend (99). Altered DNA architecture may facilitate access to the DNA by diverse groups of transcription regulators and DNA repair/recombination machinery. The HMG-box transcription factors are broadly classified into two subfamilies, UBF/HMG and TCF/SOX, which have been evolutionarily conserved through yeast, plants, insects, and vertebrates (100). The HMG-box transcription factor superfamily contains a number of members that are thought to play indispensable roles in the development and differentiation of numerous cell types and tissues (101). Specifically, in T cell development, several TCF/SOX family members have been shown to play important roles in the development and differentiation of αβ lineage T cells. TCF1, LEF1, and SOX4 are essential for proper proliferation, survival, and differentiation of αβ T cells (81-83, 102). More recently, TOX has also been implicated in the positive selection of more mature αβ lineage thymocytes (103). Very little is known about the function of SOX13, but due to the importance of several

family members in T cell development, SOX13 was identified as a candidate transcription factor that may be important for γδ T cell development.

Results and Discussion

Sox13 is a γδ T cell-specific gene.

Comparison of the global gene expression profiles of immature αβ and γδ lineage thymocytes using Affymetrix DNA microarrays identified *Sox13* as a γδ T cell-specific gene. To confirm the gene chip results, *Sox13* expression was determined in sorted DP αβ lineage and γδ TCR^+ thymocytes by RT-PCR (Figure II-1A). Interestingly, while *Sox13* expression is restricted to the γδ T cell lineage, other HMG-box transcription factors that are necessary for normal development of αβ lineage T cells, are expressed in both αβ and γδ lineage thymocytes (Figure II-1A.) In addition, the HMG-box transcription factor expression profiles in the thymus hold true in peripheral αβ and γδ T cells (J. Kang, unpublished.) Further, *Sox13* expression was determined, by RT-PCR, in a human γδ T cell clone, $V\gamma2V\delta2^+$ Isoamyl 5.C7 cells (104), as well as in human PBMCs. Importantly, we could detect *Sox13* in these samples containing human γδ T cells (Figure II-1B). Previous characterization of *Sox13* expression had suggested a restricted pattern of expression in mice, with detection of *Sox13* in embryonic arterial walls, the inner ear, hair follicles, and the thymus (98). In adults, *Sox13* transcripts were reported in the kidney, pancreas, and ovary, but not the thymus (105). The previous failure to detect *Sox13* expression in the adult thymus in earlier studies may be attributed to the fact that

Figure II-1

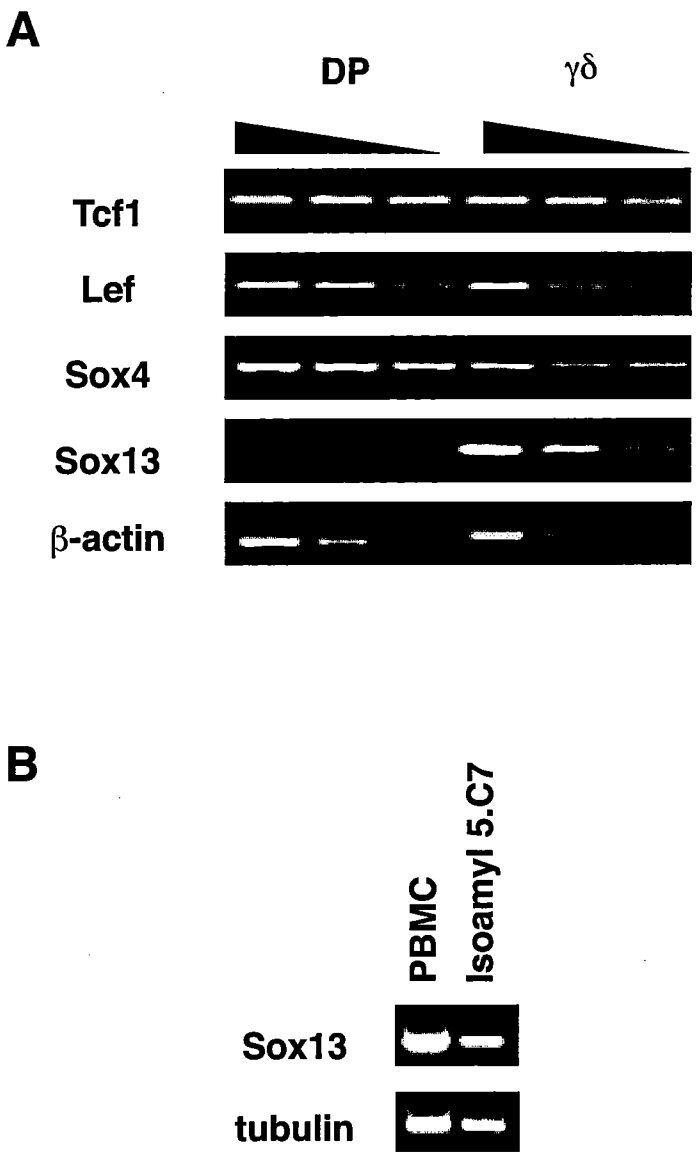

A
DP
γδ
Tcf1
Lef
Sox4
Sox13
β-actin
B
PBMC
Isoamyl 5.C7
Sox13
tubulin

Figure II-1. *Sox13* is a γδ T cell lineage HMG-box transcription factor. (A) The relative expression levels of *Sox13*, *Tcf1*, *Lef1*, and *Sox4* in sorted αβ lineage DP cells (pooled thymocytes from > 2 mice) and γδ lineage (pooled thymocytes from > 15 mice wild-type (WT) C57BL/6 thymocytes was determined by semi-quantitative RT-PCR. cDNA was serially diluted four-fold (n=2). **(B)** RT-PCR assay to determine *Sox13* expression in human PBMC sample and a human γδ T cell clone, Isoamyl 5C.7 (n=1).

more than 95% of adult thymocytes belong to the αβ T cell lineage and do not express *Sox13*.

Sox13 is expressed in thymocyte precursor cells.

Analysis of T cell developmental intermediates by RT-PCR determined that, in addition to being expressed in γδ TCR^+ thymocytes, *Sox13* is also expressed in the TN progenitor population from which both αβ and γδ lineage T cells develop (Figure II-2A). When the TN precursor subsets were analyzed for Sox13 expression, it was found that expression was highest in TN1 ($CD44^+CD25^-$) cells (Figure II-2B). The TN1 subset is a multi-potential population, capable of giving rise to T, NK, and dendritic cells. In addition, the TN1 subset also contains the earliest T cell precursors in the thymus. *Sox13* expression is sustained as cells transit to the TN2 stage of development (Figure II-2B). Here, it is generally agreed that the majority of cells in this subset are bi-potent cells restricted to the T cell lineage and are capable of giving rise to both αβ and γδ lineage T cells. Expression of *Sox13* at these early time points in T cell development correlates with a potential role in early γδ T cell development or perhaps maintenance of an undifferentiated cell state. It is important to note that TN1 and TN2 cells do not express significant levels of rearranged αβ or γδ TCR genes, suggesting that γδ TCR signaling is not required to promote transcription of *Sox13*. β, γ, and δ TCR gene rearrangement occurs as cells transit from the TN2 to the TN3. Intriguingly, the expression of *Sox13*, although not absent, is significantly decreased in cells at the TN3 stage of development (Figure II-2B), coincident with αβ-specific β-selection. By the DP stage, *Sox13*

Figure II-2

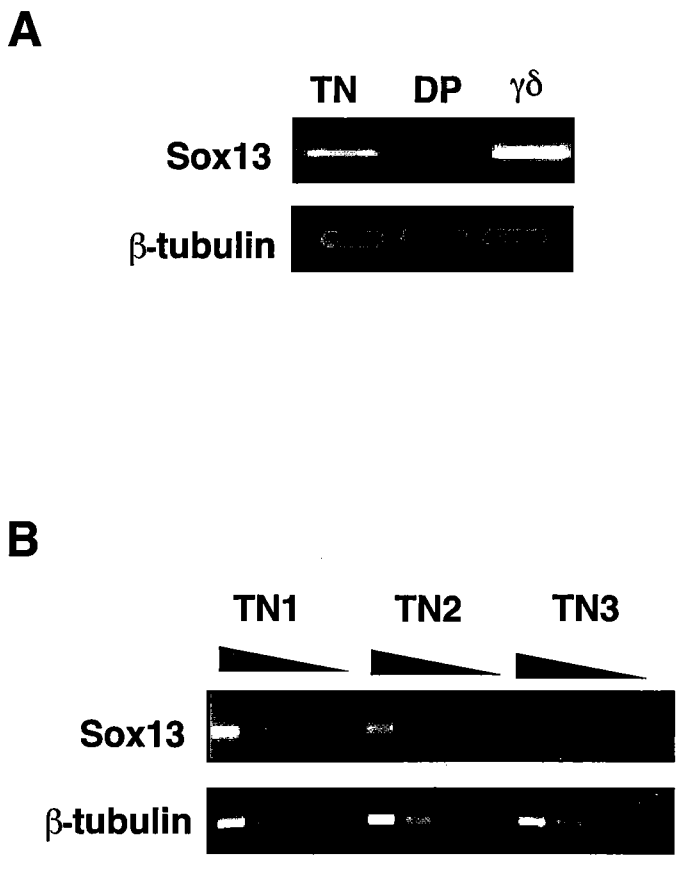


Figure II-2. *Sox13* is differentially expressed in thymocyte precursor populations. **(A)** RT-PCR assay to determine *Sox13* expression in sorted TN ($CD4^-CD8^-CD3^-$), DP ($CD4^+CD8^+$), and γδ ($CD4^-CD8^-\gamma\delta TCR^+$) thymocytes from WT mice (thymocytes pooled from > 10, 2, or 10 mice respectively.) (n=2) **(B)** Semi-quantitative RT-PCR assay (four-fold serial dilutions) to determine relative amounts of *Sox13* mRNA in TN precursor populations from WT mice. TN1: $CD44^+CD25^-$TN; TN2: $CD44^+CD25^+$TN; TN3: $CD44^-CD25^+$TN (thymocytes pooled from > 20 mice) (n=2).

transcripts are no longer detected (Figure II-1 and Figure II-2A.) The restricted pattern of *Sox13* expression has also been identified in other tissues. For example, SOX13 was identified in a subset of neural progenitors that correlated with the end of mitosis and the commencement of differentiation (106). Also, the expression pattern of *Sox13* in the pancreas suggests a role in quiescence or differentiation, as it appears to be limited, in humans and adult mice, to the islet progenitor population (107, 108).

Heterogeneous expression of Sox13 in thymocyte precursor cells.

It has previously been shown that TN2 precursor cells are heterogeneous in T cell lineage potential: when intrathymically injected into *Rag1*$^{-/-}$ recipients, it was demonstrated that IL-7Rα^{+} TN2 cells are developmentally biased toward the γδ T cell lineage at the expense of αβ lineage T cells. Conversely, IL-7Rα$^{neg\text{-}lo}$ TN2 cells are developmentally skewed toward the αβ lineage (13). Hence, we sought to determine if *Sox13* expression correlated with the γδ-biased lineage potential of TN2 cells as determined by IL-7Rα expression. Real-time quantitative PCR (qPCR) was performed on several sets of sorted TN2 IL-7Rα$^{neg\text{-}low}$ and IL-7Rα^{+} cells to determine if *Sox13* was differentially expressed between these two populations. Indeed, *Sox13* expression is, on average, ~2-fold higher in the γδ-biased IL-7Rα^{+} TN2 cells (Figure II-3A), suggesting that *Sox13* is heterogeneously expressed in T cell precursors. In contrast, *Tcf1* expression is not significantly different in these two subsets (Figure II-3B). It is important to point out, however, that IL-7Rα cell surface expression is not an absolute marker of γδ T cell lineage commitment, as some αβ-lineage DP cells do develop when IL-7Rα^{+} TN2 cells

Figure II-3

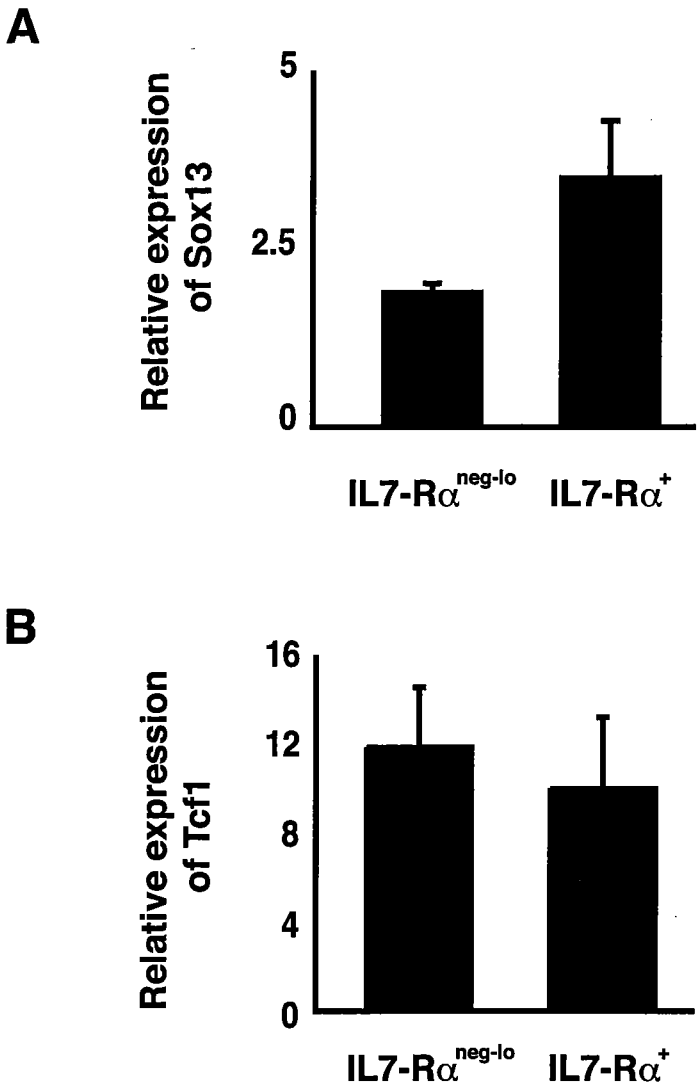

A
Relative expression of Sox13
5
2.5
0
IL7-Rα^neg-lo
IL7-Rα^+
B
Relative expression of Tcf1
16
12
8
4
0
IL7-Rα^neg-lo
IL7-Rα^+

Figure II-3. *Sox13* is preferentially expressed in γδ lineage-biased precursor cells. Quantitative real-time RT-PCR assay for **(A)** *Sox13* or **(B)** *Tcf1* expression (presented as *Sox13*/β-*actin* or *Tcf1*/*β-actin* ratio, ratios are presented as arbitrary units) based on seven and nine independently sorted sample sets (run in duplicate or triplicate) of IL-7R$^{lo\text{-}neg}$ and IL-7R^{+} TN2 cells, respectively, from WT mice. Error bars represent standard error of the mean. (for *Sox13* expression, $p<0.05$ based on one-tailed student's *t*-test, unequal variance.)

are intrathymically transferred into *Rag1*$^{-/-}$ mice (13). Therefore, the difference in *Sox13* expression between these two "lineage-biased" subsets may be an underestimate of the true difference between *bona fide* "lineage-restricted" precursor cells, though it remains possible that *Sox13* expression is not "lineage-biased" within the precursor subset. Ultimate interpretation of these data requires purification of *Sox13* expressing precursor cells and subsequent analysis of their developmental potential. In addition, qPCR does not allow the nature of the difference in *Sox13* expression to be identified. For example, it cannot be distinguished whether the differential expression of *Sox13* between these subsets is due to different levels of transcript on a per cell basis or if *Sox13* is being expressed in twice as many cells.

To discriminate between these two possibilities, and determine if *Sox13* expression marks a distinct population of T cell precursors, single cell RT-PCR was performed. Precursor cells from the TN2 subset were sorted as single cells into individual wells of 96-well plates and a reverse transcription reaction was performed. For each well, two PCRs were performed, the first to detect expression of a *β-actin* control, and a second PCR to determine the relative frequency of *Tcf1* and *Sox13* expressing precursor cells. Among the individual wells in which *β-actin* was detected, 100% expressed *Tcf1* (Figure II-4). In contrast, *Sox13* expression was found in only 46% of cells with sufficient amounts of RNA for testing (Figure II-4). These results suggest that *Sox13* expression is restricted to a specific population of TN2 precursor cells. Collectively, these data indicate that TCR^{neg} TN2 precursor cells are molecularly and developmentally heterogeneous. Furthermore, *Sox13* expression segregates, more often,

Figure II-4

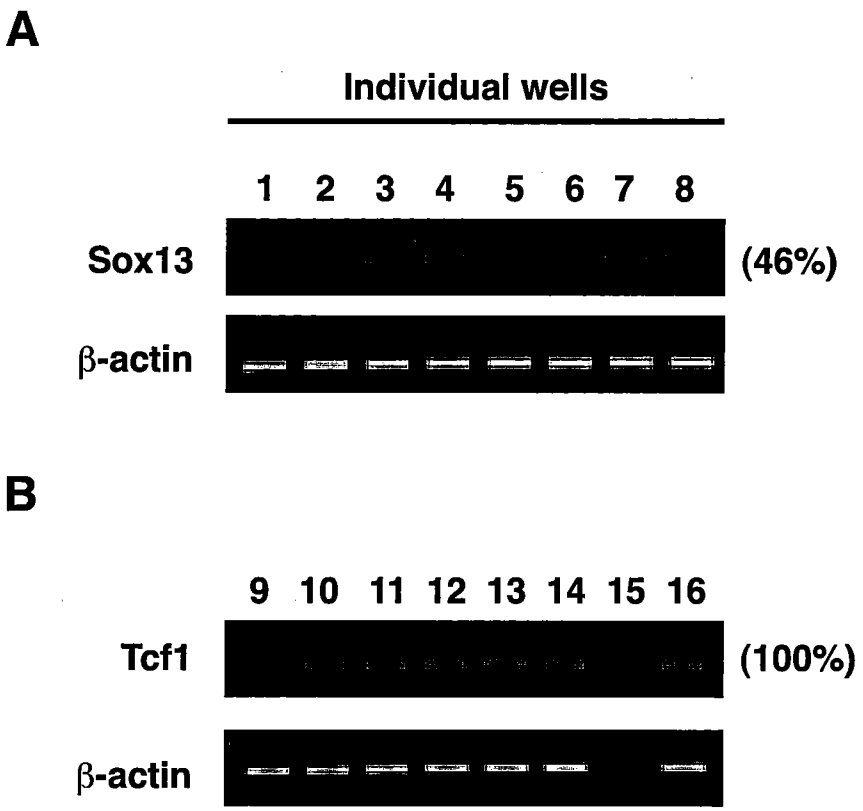

A
Individual wells
1 2 3 4 5 6 7 8
Sox13
(46%)
β-actin
B
9 10 11 12 13 14 15 16
Tcf1
(100%)
β-actin

Figure II-4. *Sox13* expression marks a distinct population of T cell precursors. A representative RT-PCR assay for **(A)** *Sox13* and **(B)** *Tcf1* expression in sorted single $CD4^-CD8^-CD3^-$c-$kit^+CD44^+CD25^+$ TN2 thymocytes from WT mice (n=3). Numbers above indicate individual clones. Of 13 single cells that were positive for β-*actin*, all 13 also gave signals for *Tcf1* (frequency of positive cells in the parentheses) while only 13 out of 28 β-*actin* positive cells expressed *Sox13*. In a separate control experiment, using the γδ T cell line, DN2.3, *Sox13* mRNA was detected in every single cell tested.

with γδ lineage-biased IL-7Rα$^+$ TN2 cells, thereby potentially marking cells more likely to become γδ T cells.

Regulation of Sox13 expression.

Due to the lineage-biased expression of *Sox13*, it was important to determine if *Sox13* expression was regulated or influenced by TCR signals. *Sox13* expression in TN1 and TN2 precursor subsets in which expression of rearranged γδ and αβ TCRs has not yet occurred (Figure II-2B), suggested at least, that γδ TCR signals were not required to induce expression of *Sox13* in γδ T cells. To confirm this observation, RT-PCR analysis of *Sox13* expression in *Rag1*$^{-/-}$ TN cells was undertaken. In the absence of *Rag1* expression, T (and B) cells cannot rearrange their receptors. As predicted, *Sox13* expression was indeed detected in *Rag1*$^{-/-}$ precursor subsets showing that the onset of *Sox13* expression occurs independent of, and prior to, TCR signals (data not shown) (K. Narayan and J. Kang, unpublished).

Interestingly, *Sox13* expression was significantly reduced at the TN3 stage in which αβ lineage-specific β-selection occurs (successful pairing of rearranged TCRβ with a surrogate preTα chain is required for progression to the DP stage.) This observation could imply that αβ T cell specific receptor signaling was necessary to eliminate *Sox13* expression in αβ lineage cells. To determine if this was true, we determined if *Sox13* was expressed in *Tcrβ*$^{-/-}$ DP cells. "αβ lineage" DP thymocytes in *Tcrβ*$^{-/-}$ arise via γδ TCR-dependent signaling. Therefore, if signals through the αβ-

specific preTCR were required to suppress *Sox13* expression, *Sox13* expression would be expected in *Tcrβ*$^{-/-}$ DP cells. If *Sox13* expression is regulated independently of the preTCR, we would not expect to detect *Sox13* transcript in DP cells from *Tcrβ*$^{-/-}$ mice. Significantly, no *Sox13* expression was detected by RT-PCR in *Tcrβ*$^{-/-}$ DP cells (Figure II-5A). Together, these results suggest that the onset of *Sox13* expression is not linked to TCR expression or signaling and that γδ TCR signaling is insufficient to maintain *Sox13* expression in αβ lineage thymocytes. Therefore, the expression pattern of *Sox13* correlates with T cell lineage but not TCR type. It should be noted, however, that recent reports suggest that TCR signal strength rather than TCR isotype govern lineage choice. It is therefore possible that the quantity of signal that γδ TCR-selected "αβ lineage" thymocytes in TCRβ-deficient mice receive may result in *Sox13* gene silencing.

We also sought to determine if IL-7R signals were necessary for *Sox13* expression. *Sox13* expression is increased in precursor populations that express IL-7Rα, and IL-7R signals are necessary for γδ T cell development (76, 77). In addition, it has recently been suggested that IL-7R signaling regulates expression of other HMG-box transcription factors, *Tcf1* and *Lef1* (94). However, when we analyzed expression of *Sox13* in thymocytes from IL-7R$^{-/-}$ mice by RT-PCR, we could detect *Sox13* expression suggesting that IL-7R signaling is not necessary for *Sox13* expression (Figure II-5B). The observation that *Sox13* expression is biased towards IL-7Rα^{hi} subsets is also suggestive that SOX13 may regulate *Il-7R* expression. Although we do have evidence suggesting that SOX13 is not sufficient to drive *Il-7R* expression (data not shown), we

Figure II-5

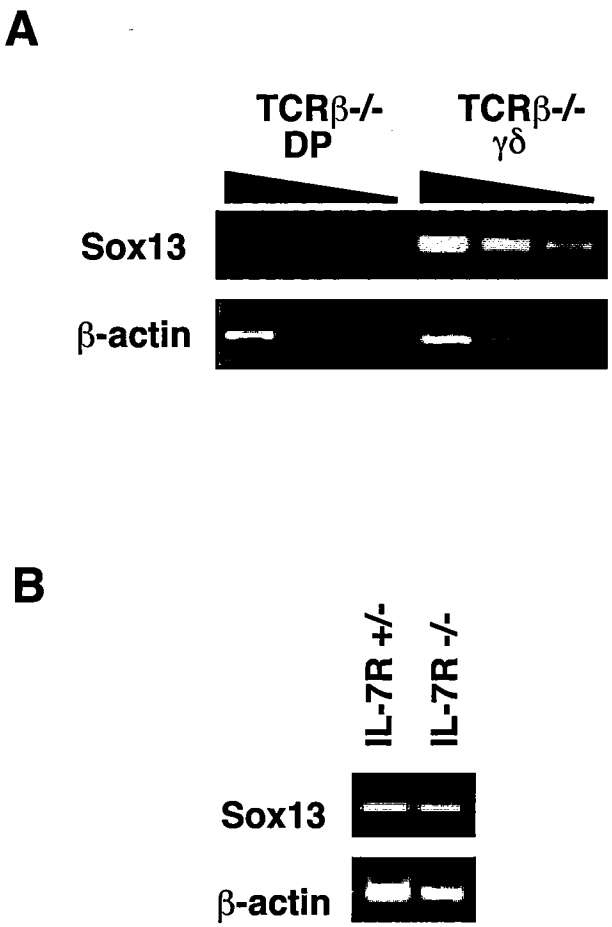


Figure II-5. *Sox13* expression is regulated independently of TCR and IL-7R signals. **(A)** Semi-quantitative RT-PCR assay to determine *Sox13* expression in sorted DP and γδ thymocytes from TCRβ$^{-/-}$ mice (four-fold serial dilutions) (thymocytes pooled from > 5 mice) (n=1). **(B)** Analysis of *Sox13* expression in total thymocytes from *IL-7R*$^{+/-}$ and *IL-7R*$^{-/-}$ mice (thymocytes from 1 or 3 mice pooled, respectively) (n=1).

have not ruled out that it may play a role in its expression in conjunction with other cellular factors.

Summary and Future Directions

We have identified *Sox13* as a γδ T cell-specific gene that is also expressed among thymocyte precursor populations. The onset of *Sox13* expression appears to be regulated independently of TCR gene expression and signaling. The heterogeneous expression of *Sox13* within the precursor subset segregates with γδ lineage-biased precursor subsets suggesting that it may be a marker of γδ precursor cells. To determine if *Sox13* expression is indeed a marker of the γδ T cell lineage in precursor cells, we are generating reporter transgenic mice in which GFP expression is regulated by the *Sox13* promoter. This model may allow us to purify *Sox13* expressing precursor cells based on GFP expression, and then determine their developmental potential via fetal thymic organ culture and intrathymic transfer of $Sox13^{+}$ and $Sox13^{-}$ precursor populations.

Experiments presented in this chapter identified a restricted pattern of *Sox13* expression during thymocyte development. The pattern of expression is suggestive of SOX13 playing a role in T cell development, lineage choice, or perhaps precursor cell maintenance. The following chapters will examine the role of SOX13 in T cell development.

Materials and Methods

Mice

All mice used in these experiments were housed in a pathogen-free rodent barrier facility. The University of Massachusetts Medical School Institutional Care and Use Committee approved all animal experiments. All mouse strains used for expression analysis including *IL-7R*$^{-/-}$, *Rag1*$^{-/-}$, and *TCRβ*$^{-/-}$ mice were maintained on the C57BL/6 background.

Cell sorting

Thymocytes were stained with the following antibodies purchased from eBiosciences or BD Pharmingen: antibodies specific for mouse CD4 (PE, PE-Cy5, and biotin), CD8 (FITC, PE-Cy5, and biotin), CD3 (PE-Cy5 and biotin), γδTCR (biotin), IL-7Rα (PE), CD25 (FITC), CD44 (PE), and CD117/c-kit (APC). Streptavidin-PE and streptavidin-Pacific Blue were purchased from eBiosciences and Molecular Probes, respectively. Samples were sorted using the DakoCytomation MoFlo or FACSVantage SE/Diva.

RT-PCR, Real-time qPCR, and single cell RT-PCR

For RT-PCR and real-time qPCR, total RNA was isolated from cells using Trizol reagent (Invitrogen) and cDNA was prepared using AMV RT (Roche). The following primer pairs were used for PCR: *β-tubulin*-for 5'-CAG GCT GGT CAA TGT GGC AAC CAG ATC GGT-3' and *β-tubulin*-rev 5'-GGC GCC CTC TGT GTA GTG GCC TTT GGC CCA-3', *β-actin*-for 5'-CTA GGC ACC AGG GTG TGA TGG-3' and *β-*

actin-rev 5'-TCT CTT TGA TGT CAC GCA CGA-3', *Sox13*-for 5'-CGG AAC AGC AGC CAC ATC AAG AGA-3' and *Sox13*-rev 5'-ATG GTG TAG CTT TGG CGA GCA C-3', *Tcf1*-for 5'-GCC AGC CTC CAC ATG GCG TC-3' and *Tcf1*-rev 5'-GCT GCC TGA GGT CAG AGA ATA A-3', *Lef*-for 5'-GTC CTC TCA GGA GCC CTA CC-3' and *Lef*-rev 5'-CAT CTG ACG GGA TGT GTG AC-3', *Sox4*-for 5'-ATG GTA CAA CAG ACC AAC AAC GCG G-3' and *Sox4*-rev 5'-GGC GAG CAT CCC GGG CCT CCA T-3'.

qRT-PCR was performed using SYBR green (Applied Biosystems) fluorescence detection using the BioRad iCycler system. The following primers were used for qRT-PCR: q*Sox13*(3047)-for 5'-CCC TAT TTC TCT CCA GAC TGT TTC TT-3' and q*Sox13*(3142)-rev 5'-GCT GGT TAA GTT ATT CAT CAT TAT CTT CTT-3', q*β-actin*-for 5'-CGA GGC CCA GAG CAA GAG AG-3', q*β-actin*-rev 5'-CGG TTG GCC TTA GGG TTC AG-3' (109), *Tcf1*-for, and *Tcf1*-rev.

For single cell RT-PCR, thymocytes were sorted into 96-well plates containing 5μl of lysis buffer (0.4% NP40, 25μM DTT, 0.5U RNasin, and 65μM dNTP) at one cell per well. To each well, 5μl of 2x Sensiscript RT (Qiagen) reaction mix was added and incubated at 37°C for one hour. For first round PCR reactions, 1μl (β-actin) or 4μl (Tcf1 or Sox13) of the RT reaction was amplified in a 25μl PCR reaction mix for 35 cycles. For nested PCR, 1μl of the first round PCR reaction was amplified with an additional 35 cycles using internal primers. The sensitivity of Sox13 PCR reactions was tested on single γδ T cell clones (DN2.3), where all single cells tested gave Sox13-specific products. Repeated samplings (n >50) of 4-fold diluted RNA samples from single

precursor cells also gave differential expression pattern for *Tcf1* and *Sox13* when expression of these genes was tested simultaneously from the same clone. The following primers were used for single cell RT-PCR: *β-actin*-for and *β-actin*-rev, q*β-actin*-for and q*β-actin*-rev, *Sox13*-for and *Sox13*-rev, 1303-for and 1384-rev, *Tcf1*-for and *Tcf1*-rev, *Tcf1nest*-for 5'-CCC CAG CTT TCT CCA CTC TA-3' and *Tcf1nest*-R 5'- AAT CCA GAG AGA TCG GGG GT-3'.

CHAPTER III

SOX13 ANTAGONIZES THE WNT/TCF1 SIGNALING PATHWAY IN T CELL DEVELOPMENT

Introduction

Sox13, determined by gene expression profiling and later confirmed by RT-PCR, to be a γδ T cell-specific gene, was identified as a potential marker of precursors destined for the γδ T cell lineage and/or an important gene in γδ T cell development. Since a number of other HMG-box transcription factor family members have been implicated in αβ T cell development, *Sox13* was the focus of intensive research to determine if it, too, played an important role in differentiation and lineage choice. In addition, contrasting with *Tcf1*, *Lef1*, and *Sox4*, the restricted pattern of *Sox13* expression suggested a unique role for SOX13 in γδ T cell development.

Preliminary over-expression studies of *Sox13* in an αβ lineage DP cell line indicated that SOX13 may act to inhibit cell proliferation and oppose αβ lineage differentiation (K. Narayan and J. Kang, unpublished.) Therefore, to test the possibility that SOX13 is a physiological antagonist of αβ lineage development, we generated several *Sox13* transgenic founder lines that over-express *Sox13* under the control of the *Lck* proximal promoter, which is active during early thymocyte development (110). There are two obvious outcomes that one could predict with respect to lineage commitment: 1. *Sox13* over-expression has no affect on lineage commitment or thymocyte development as seen by normal proportions and numbers of thymocyte

subsets in the transgenic model, or 2. *Sox13* is a master regulator of γδ T cell lineage, causing all *Sox13* over-expressing thymocyte precursors to develop into γδ T cells at the expense of αβ lineage development. This chapter reports thorough analysis of the role of *Sox13* function in T cell development.

Results and Discussion

SOX13 inhibits αβ lineage development.

To determine the effect of *Sox13* over-expression in thymic precursors on T cell development, we analyzed fetal and adult thymocytes from *Sox13* transgenic and littermate control (LMC) mice by flow cytometry. Flow cytometric analysis of *Sox13* transgenic embryonic day 17 (E17) thymuses revealed only a modest reduction (less than 2-fold) in thymic cellularity as compared to LMCs. However, αβ lineage T cell development was nearly ablated in *Sox13* transgenic E17 thymuses as evidenced by the near absence of DP cells (Figure III-1A). In contrast, the percentage and calculated cellularity of the γδ T cell compartment appeared largely unaffected (Figure III-1B).

Analysis of adult (4-6 week old) transgenic mice demonstrated that αβ lineage development was severely impaired as demonstrated by the significant reduction in the proportion of αβ lineage DP thymocytes (Figure III-2A). Strikingly, the absolute numbers of thymocytes in the *Sox13* transgenic mice were reduced by 10-50 fold depending on age and founder line (Figure III-2D). This significant decrease was due, for the most part, to the significant reduction in αβ lineage DP cells (Figure III-2D).

Figure III-1

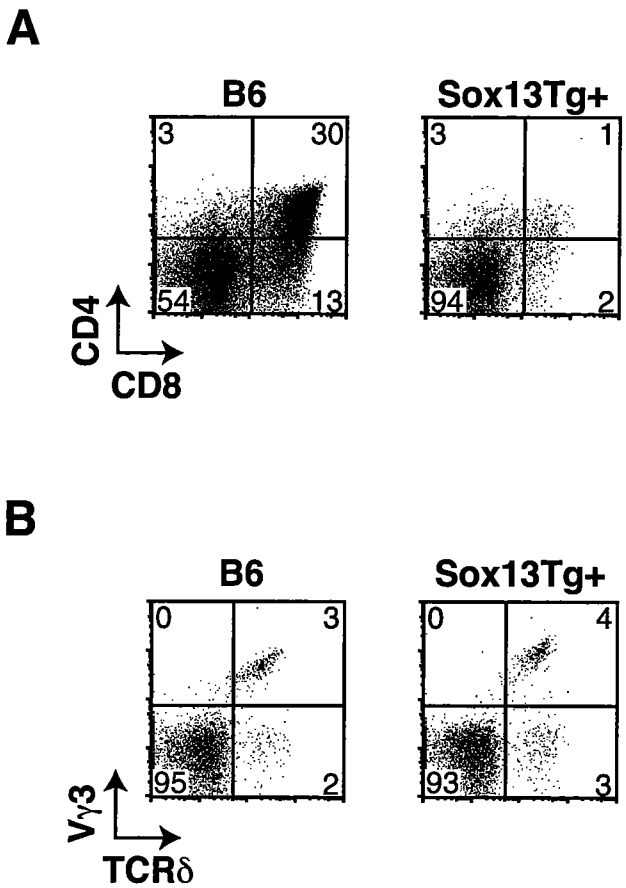

A
B6
Sox13Tg+
3
30
54
13
3
1
94
2
CD4
CD8
B
B6
Sox13Tg+
0
3
95
2
0
4
93
3
Vγ3
TCRδ

Figure III-1. SOX13 inhibits αβ lineage development in the fetal thymus. Thymocytes from WT and *Sox13* transgenic E17 fetuses were analyzed by flow cytometry for **(A)** αβ (CD4 and CD8) and **(B)** γδ (TCRδ and Vγ3, gated on $CD4^-CD8^-$ cells) lineage markers. Representative profiles from analyses of three litters of two Tg founder lines are shown.

Figure III-2

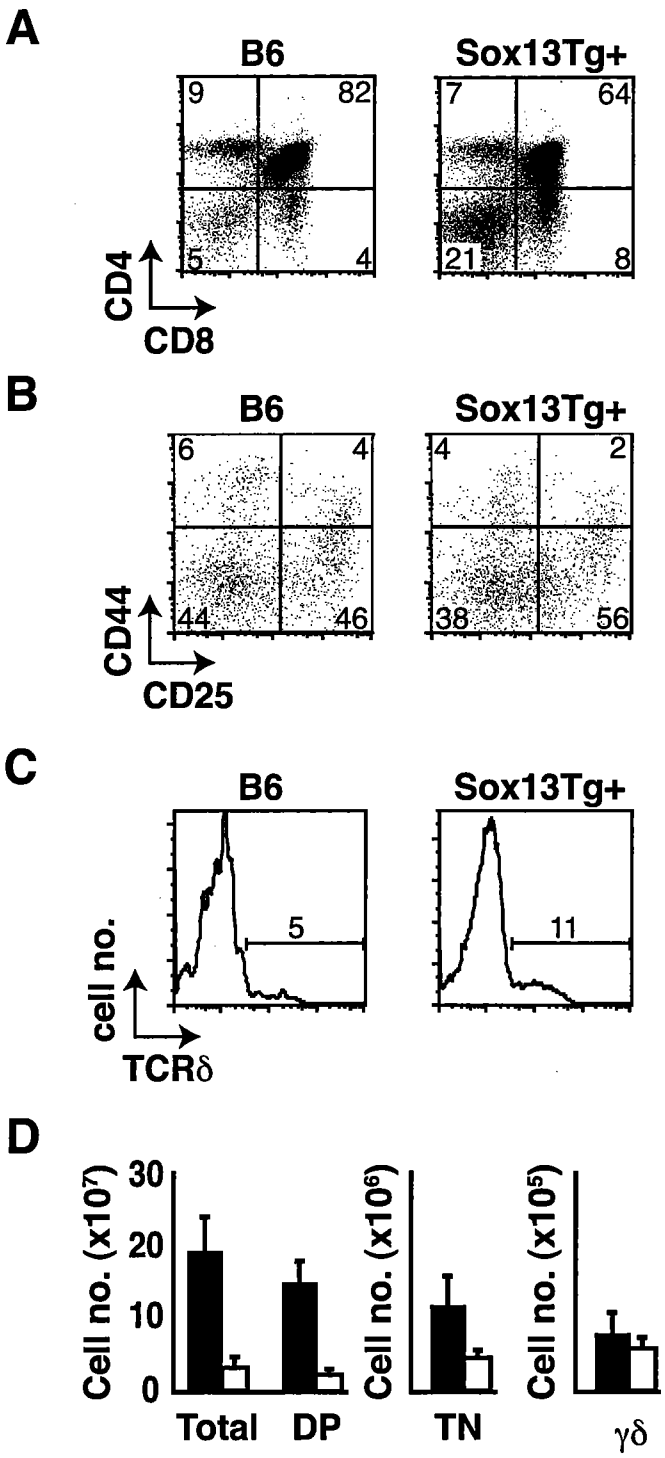

A
B6
Sox13Tg+
9
82
5
4
7
64
21
8
CD4
CD8
B
B6
Sox13Tg+
6
4
44
46
4
2
38
56
CD44
CD25
C
B6
Sox13Tg+
5
11
cell no.
TCRδ
D
Cell no. (x10^7)
30
20
10
0
Total
DP
Cell no. (x10^6)
TN
Cell no. (x10^5)
γδ

Figure III-2. αβ lineage T cell development is inhibited in adult *Sox13* transgenic mice. Thymocytes from 6-8 week old adult WT and *Sox13* transgenic mice were analyzed by flow cytometry for **(A)** αβ (CD4 and CD8) lineage markers, **(B)** precursor subsets (CD44 and CD25, gated on $CD4^-CD8^-CD3^-$ cells), and **(C)** γδ (TCRδ, gated on $CD4^-CD8^-$ cells) lineage markers. Representative profiles from analyses of multiple mice (2 to >7 per transgenic founder line) are shown. Seven out of nine total transgenic founder lines showed defects in T cell development. **(D)** Absolute numbers of thymocyte subsets from WT (filled bars, n=7) and *Sox13* transgenic (open bars, n=8) mice. Error bars represent standard deviation.

Although the number of TN precursor cells was also modestly decreased in the *Sox13* transgenic mice, the TN subset distribution, based on CD25 and CD44 expression, was largely normal (Figure III-2B and D). Similar to the *Sox13* transgenic fetal thymocytes, and despite an increase in the proportion of γδ TCR^+ thymocytes (Figure III-2C), the absolute numbers of γδ lineage thymocytes were similar to that of the LMCs (Figure III-2D). Importantly, the αβ lineage thymocytes that do develop in *Sox13* transgenic mice express the αβ TCR with no detectable γδ TCR on their cell surface (Figure III-3A and B). Collectively, these data suggest that over-expression of *Sox13* in thymocyte precursors inhibits one or more aspects of αβ T cell lineage development, while γδ T cell steady-state numbers appear normal.

SOX13 acts independently of the type of TCR expressed.

To determine whether the effects of SOX13 on αβ lineage development was associated with the lineage differentiation program *per se* or the type of TCR signals, *Sox13* transgenic mice were crossed to $TCR\beta^{-/-}$ mice. $TCR\beta^{-/-}$ mice generate "αβ lineage" DP thymocytes via selection through γδ TCR signals (22). *Sox13* transgenic expression in $TCR\beta^{-/-}$ mice suppresses the proportion and absolute numbers of αβ lineage γδ TCR-dependent DP cells (Tg^-: $9.3 \times 10^5 \pm 8.9 \times 10^5$ and Tg^+: $1.2 \times 10^5 \pm 4.8 \times 10^4$, p-value < 0.05) while not affecting the cellularity of the γδ lineage compartment (Tg^-: $8.6 \times 10^5 \pm 1.1 \times 10^5$ and Tg^+: $1.0 \times 10^6 \pm 5.0 \times 10^5$, p-value = 0.7) (Figure III-4A and B). Therefore, these data suggest that SOX13 acts independently of the type of TCR expressed to impact T cell development, and its expression is specifically detrimental to the αβ T cell lineage.

Figure III-3

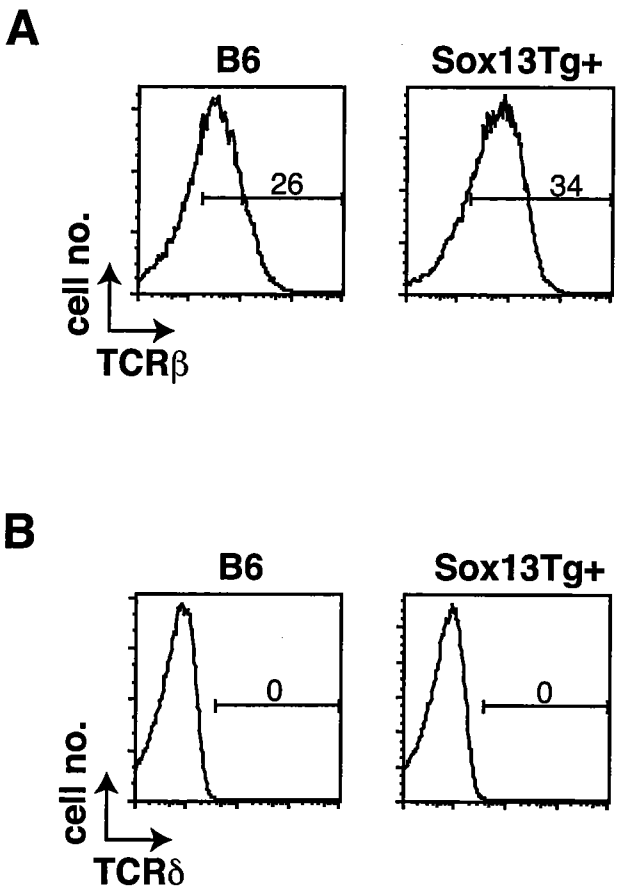


Figure III-3. DP cells from *Sox13* trasngenic mice do not express the γδ TCR. DP thymocytes from *Sox13* transgenic mice were analyzed by flow cytometry for expression of **(A)** TCRβ and **(B)** TCRδ expression. Representative flow cytometric profiles are shown. (WT: n=7, Tg: n=8).

Figure III-4

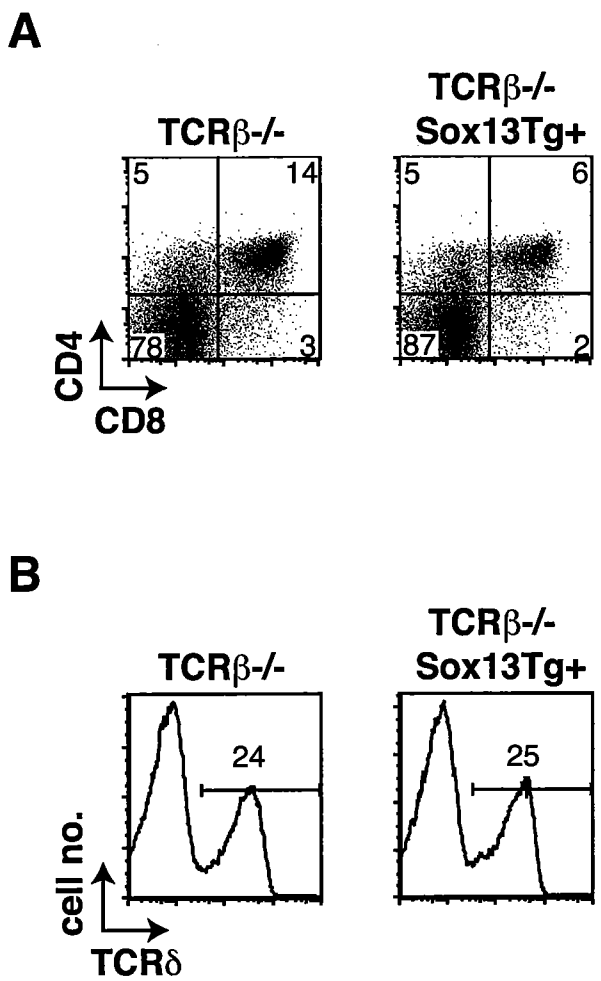

A
TCRβ-/-
TCRβ-/-
Sox13Tg+
5
14
78
3
5
6
87
2
CD4
CD8
B
TCRβ-/-
TCRβ-/-
Sox13Tg+
24
25
cell no.
TCRδ

Figure III-4. SOX13 inhibits αβ lineage development independent of the type of TCR expressed. Representative flow cytometric profiles of thymocytes from *Tcrβ*$^{-/-}$ (n=4) and *Tcrβ*$^{-/-}$*Sox13* transgenic (n=8) mice analyzed by flow cytometry for **(A)** αβ (CD4 and CD8) and **(B)** γδ (TCRδ, gated on CD4^{-}CD8^{-} cells) lineage markers.

SOX13 inhibits cell-turnover and survival.

Many HMG-box transcription factors have been implicated in modulating both cell cycle and cell survival. For example, thymocyte subsets in *Tcf1*$^{-/-}$ mice have reduced proliferative capacity and inhibited survival, not unexpectedly as it has been suggested that cyclinD1, c-Myc, and Bcl-xL are proposed targets of TCF transcription factors (82, 111-113). Other HMG-box transcription factors, such as HBP1, however, appear to inhibit cell cycle progression (114). Therefore, we sought to determine if the significant decrease in development of αβ lineage T cells in *Sox13* transgenic mice was caused by a decrease in cell proliferation and/or increased cell death. Adult *Sox13* transgenic mice and LMCs were injected with Bromodeoxyuridine (BrdU) and sacrificed one-hour post-injection to determine the percentage of cells in S phase of the cell cycle. There was approximately a 2.5-fold decrease in BrdU incorporation in the TN precursor population in *Sox13* transgenic mice as compared to LMCs (Figure III-5A). This inhibition of proliferation was limited to the precursor population as no significant differences in BrdU incorporation were detected in the DP, CD4 or CD8 single positive, or γδ lineage thymocytes from *Sox13* transgenic mice as compared to LMCs (Figure III-5A and data not shown). Since the vast majority of proliferating TN precursor cells in a normal thymus are αβ lineage cells undergoing β-selection and destined to become DP thymocytes, SOX13 likely inhibits the proliferative burst associated with the αβ T cell lineage-specific TN to DP developmental transition.

To determine if *Sox13* over-expression was also affecting cell survival, we examined cell surface AnnexinV expression, an early marker of apoptosis, on *Sox13*

Figure III-5

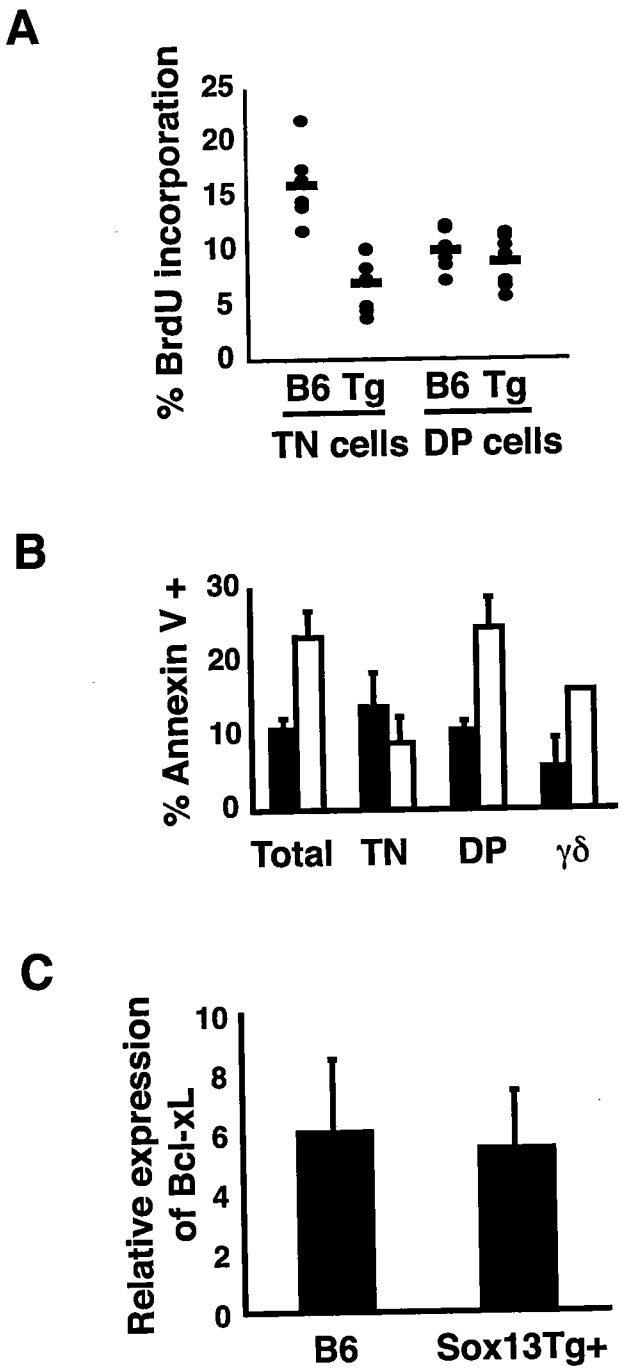

A
% BrdU incorporation
25
20
15
10
5
0
B6 Tg
B6 Tg
TN cells
DP cells
B
% Annexin V +
30
20
10
0
Total
TN
DP
γδ
C
Relative expression of Bcl-xL
10
8
6
4
2
0
B6
Sox13Tg+

Figure III-5. SOX13 inhibits cell-turnover and survival. **(A)** Analysis of BrdU incorporation in thymocytes from WT and *Sox13* transgenic adult mice that were treated with BrdU for 1 hour. Thymocytes were stained with mAbs specific for lineage markers and BrdU, and analyzed by flow cytometry. Filled circles represent the percentage of BrdU incorporation in individual mice; the bar represents the average BrdU incorporation in each subset. **(B)** Thymocytes from WT (filled bars, n=4) and *Sox13* transgenic (open bars, n=4) mice were stained with mAbs specific for lineage markers and AnnexinV, and analyzed by flow cytometry. Error bars represent standard deviation. **(C)** Quantitative real-time RT-PCR assay (presented as *Bcl-xL*/β-*actin* ratio, ratios are presented using arbitrary units) to determine relative *Bcl-xL* expression in thymocytes from WT control and *Sox13* transgenic mice. Error bars represent standard deviation (thymocytes pooled from >2 mice of each type, samples run in duplicate, n=1).

transgenic and LMC thymocytes. Although no difference in AnnexinV staining was observed in the TN precursor population, increased rates of apoptosis were identified in DP and γδ TCR^+ thymocytes in *Sox13* transgenic mice as compared to LMCs (Figure III-5B). In control mice, approximately 10% of αβ lineage DP cells express AnnexinV on their cell surface (Figure III-5B). In contrast, DP thymocytes from *Sox13* transgenic mice showed a greater than two-fold increase in the proportion of cells expressing cell-surface AnnexinV (Figure III-5B). Similarly, γδ lineage thymocytes exhibited a similar increase in AnnexinV staining (Figure III-5B). The increase in cell death among γδ TCR^+ thymocytes from *Sox13* transgenic mice suggests that *Sox13* over-expression is not a neutral event for γδ lineage thymocytes. Normal steady-state numbers of γδ thymocytes in *Sox13* transgenic mice despite an increased rate of cell-death, and coupled with normal cell proliferation, suggests that there may be an increase in the frequency of precursor cell differentiation in the γδ T cell lineage. Collectively, these results indicate that the decrease in αβ lineage thymic cellularity in *Sox13* transgenic mice is the result of reduced proliferation and altered differentiation of the precursor compartment, further compounded by increased cell death in differentiated DP thymocytes.

The mechanism of increased cell death in αβ lineage DP cells in *Sox13* transgenic mice is unknown. In thymocyte development, another HMG-box transcription factor family member, TCF1, is thought to regulate levels of genes involved in cell survival, specifically, *Bcl-xL* (82). Hence, we sought to determine if over-expression of *Sox13* was leading to disregulated expression of anti-apoptotic genes. Although not comprehensive in scope, initial studies suggested that their were no significant differences in the relative

levels of certain anti-apoptotic genes, such as *Bcl-xL*, in *Sox13* transgenic DP cells as compared to DP cells from LMCs (Figure III-5C). From this we may only infer that SOX13 is regulating pro- or anti-apoptotic genes that we have not tested and/or that some of the cell death seen in the DP compartment in the *Sox13* transgenic mice is due to SOX13 imposing an aberrant developmental program on αβ lineage precursors or inhibiting their differentiation such that these cells have difficulty passing through selection processes.

SOX13 imposes a γδ-biased molecular profile in DP cells from Sox13 transgenic mice.

The effects of ectopic *Sox13* expression *in vivo* are most consistent with SOX13 acting as an inhibitor of αβ T cell lineage proliferation and/or survival. We suggest an additional hypothesis that SOX13 is acting as an inhibitor of the αβ lineage differentiation program as well as cell division and survival. This would implicate SOX13 as a molecular switch that prevents the initiation of molecular programs associated with αβ T cell differentiation in γδ T cell biased precursor cells, and/or that its shutdown permits the initiation of αβ T cell lineage-specific molecular program in precursors destined to become αβ lineage cells. In addition, it is equally possible that SOX13 is required to impose a γδ lineage molecular differentiation program on developing T cells. To test this possibility, we took advantage of the fact that some "αβ lineage" DP cells did develop in adult *Sox13* transgenic mice, and sought to determine if they were molecularly "normal." We reasoned that analyses of molecular changes in this phenotypically classified αβ lineage population should reveal the extent to which SOX13 enforces γδ lineage-specific gene expression. To define the γδ lineage-specific gene

Figure III-6

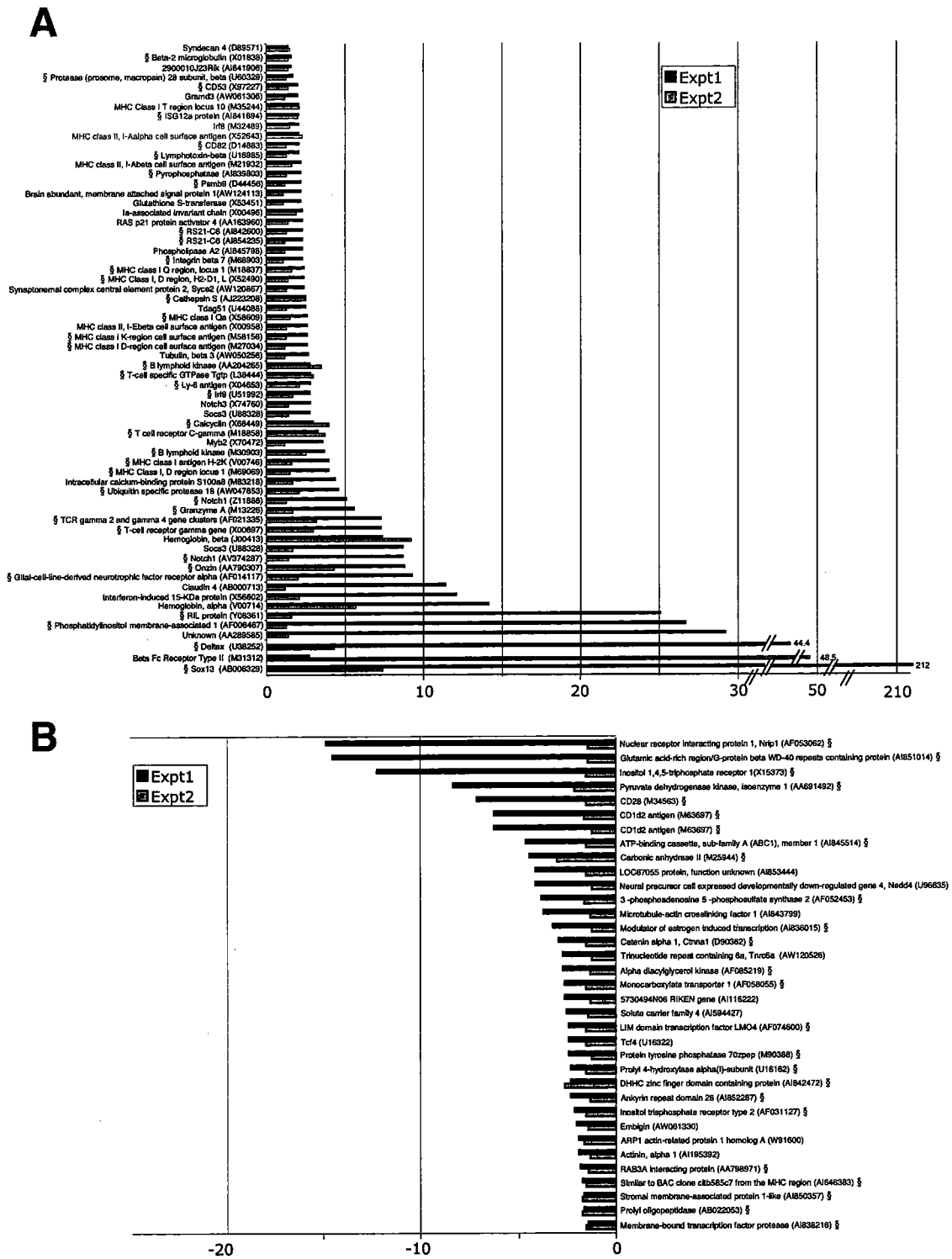

A
Expt1
Expt2
Syndecan 4 (D89571)
§ Beta-2 microglobulin (X01838)
2900010J23Rik (AI841906)
§ Protease (prosome, macropain) 28 subunit, beta (U60329)
§ CD53 (X97227)
Gramd3 (AW061306)
MHC Class I T region locus 10 (M35244)
§ ISG12a protein (AI841894)
Irf8 (M32489)
MHC class II, I-Aalpha cell surface antigen (X52643)
§ CD82 (D14883)
§ Lymphotoxin-beta (U16985)
MHC class II, I-Abeta cell surface antigen (M21932)
§ Pyrophosphatase (AI839803)
§ Pemt2 (D44556)
Brain abundant, membrane attached signal protein 1(AW124113)
Glutathione S-transferase (X53451)
Ia-associated invariant chain (X00496)
RAS p21 protein activator 4 (AA163960)
§ RS21-C6 (AI842600)
§ RS21-C6 (AI854235)
Phospholipase A2 (AI845798)
§ Integrin beta 7 (M68903)
§ MHC class I Q region, locus 1 (M18837)
§ MHC Class I, D region, H2-D1, L (X52490)
Synaptonemal complex central element protein 2, Syce2 (AW120857)
§ Cathepsin S (AJ223208)
Tdag51 (U44088)
§ MHC class I Qa (X58609)
MHC class II, I-Ebeta cell surface antigen (X00958)
§ MHC class I K-region cell surface antigen (M58156)
§ MHC class I D-region cell surface antigen (M27034)
Tubulin, beta 3 (AW050256)
§ B lymphoid kinase (AA204265)
§ T-cell specific GTPase Tgtp (L38444)
§ Ly-6 antigen (X04653)
§ Irf9 (U51992)
Notch3 (X74760)
Socs3 (U88328)
§ Calcyclin (X68449)
§ T cell receptor C-gamma (M18858)
Myb2 (X70472)
§ B lymphoid kinase (M30903)
§ MHC class I antigen H-2K (V00746)
§ MHC Class I, D region locus 1 (M69069)
Intracellular calcium-binding protein S100a8 (M83218)
§ Ubiquitin specific protease 18 (AW047853)
§ Notch1 (Z11886)
§ Granzyme A (M13226)
§ TCR gamma 2 and gamma 4 gene clusters (AF021335)
§ T-cell receptor gamma gene (X00697)
Hemoglobin, beta (J00413)
Socs3 (U88328)
§ Notch1 (AV374287)
§ Onzin (AA790307)
§ Glial-cell-line-derived neurotrophic factor receptor alpha (AF014117)
Claudin 4 (AB000713)
Interferon-induced 15-KDa protein (X56602)
Hemoglobin, alpha (V00714)
§ Rit protein (Y08361)
§ Phosphatidylinositol membrane-associated 1 (AF006467)
Unknown (AA289585)
§ Deltex (U38252)
Beta Fc Receptor Type II (M31312)
§ Sox13 (AB006329)
44.4
48.5
212
0
10
20
30
50
210
B
Expt1
Expt2
Nuclear receptor interacting protein 1, Nrip1 (AF053062) §
Glutamic acid-rich region/G-protein beta WD-40 repeats containing protein (AI851014) §
Inositol 1,4,5-triphosphate receptor 1(X15373) §
Pyruvate dehydrogenase kinase, isoenzyme 1 (AA691492) §
CD28 (M34563) §
CD1d2 antigen (M63697) §
CD1d2 antigen (M63697) §
ATP-binding cassette, sub-family A (ABC1), member 1 (AI845514) §
Carbonic anhydrase II (M25944) §
LOC67055 protein, function unknown (AI853444)
Neural precursor cell expressed developmentally down-regulated gene 4, Nedd4 (U96635)
3 -phosphoadenosine 5 -phosphosulfate synthase 2 (AF052453) §
Microtubule-actin crosslinking factor 1 (AI843799)
Modulator of estrogen induced transcription (AI836015) §
Catenin alpha 1, Ctnna1 (D90362) §
Trinucleotide repeat containing 6a, Tnrc6a (AW120526)
Alpha diacylglycerol kinase (AF085219) §
Monocarboxylate transporter 1 (AF058055) §
5730494N06 RIKEN gene (AI116222)
Solute carrier family 4 (AI594427)
LIM domain transcription factor LMO4 (AF074600) §
Tcf4 (U16322)
Protein tyrosine phosphatase 70zpep (M90388) §
Prolyl 4-hydroxylase alpha(I)-subunit (U16182) §
DHHC zinc finger domain containing protein (AI842472) §
Ankyrin repeat domain 28 (AI852287) §
Inositol trisphosphate receptor type 2 (AF031127) §
Embigin (AW061330)
ARP1 actin-related protein 1 homolog A (W91600)
Actinin, alpha 1 (AI195392)
RAB3A interacting protein (AA798971) §
Similar to BAC clone clb585c7 from the MHC region (AI846383) §
Stromal membrane-associated protein 1-like (AI850357) §
Prolyl oligopeptidase (AB022053) §
Membrane-bound transcription factor protease (AI838216) §
-20
-10
0

Figure III-6. SOX13 imposes a γδ-biased molecular profile on DP cells from *Sox13* transgenic mice. Genes showing differential expression between *Sox13* transgenic DP and WT DP thymocytes as determined by analyses using Affymetrix Mu11K (expt.1) and MuU74Av2 (expt.2) DNA microarrays. Total RNA (>5ug) was made from DP cells sorted from pooled thymocytes of minimum of two 4-6 week old mice (expt.1: *Sox13* transgenic, n=4 and WT, n=3; expt.2: *Sox13* transgenic, n=2 and WT, n=2) Biotinylated cRNA used for hybridization was obtained after one round of *in vitro* transcription. Fold-change values (*y-axis*) were calculated with Affymetrix GeneChip software with expression values from LMC DP thymocytes as the baseline. Only those genes whose expression are increased, and designated as Present **(A),** or decreased, from positive expression values of the baseline designated as Present, **(B)** in *Sox13* transgenic DP thymocytes in both experiments are shown. § denotes genes whose expression are also differentially expressed in γδ thymocytes as compared to DP thymocytes (baseline, >2-fold change, increased (A) and decreased (B)) based on two independent experiments using Mu11K or MuU74Av2 DNA microarrays (S. Der and J. Kang, unpublished).

expression pattern, we compared the global gene expression profiles of sorted DP thymocytes from *Sox13* transgenic and LMCs using Affymetrix gene chip technology. Importantly, the majority of differentially expressed genes between these two DP populations (70% and 57% of genes whose expression was decreased or increased, respectively, in *Sox13* transgenic DPs relative to baseline LMC DP cells) were also the genes that are normally differentially expressed between wild-type γδ and αβ lineage DP thymocytes. A full list of the "γδ lineage molecular signature" in *Sox13* transgenic DP thymocytes that was repeatable over two different gene chip experiments is presented in Figure III-6. Confirmation of select genes differentially expressed between *Sox13* transgenic and LMC DP populations was achieved by semi-quantitative RT-PCR (Figure III-7). Notably, among the differentially expressed genes in DP cells from *Sox13* transgenic and wild-type DP cells, TCRγ genes, which are generally silenced in αβ lineage cells from the DP stage onward, were aberrantly expressed in *Sox13* transgenic DP cells (Figure III-6A and 7). TCRγ is one of the prototypic γδ T cell lineage markers. Collectively, these results suggest that SOX13 is regulating a central molecular feature of the γδ T cell lineage. Importantly, many, but not all, genes differentially expressed in the γδ T cell lineage as compared to the αβ lineage are also shared by the precursor population. Therefore, some of the genes that are altered in *Sox13* transgenic DP cells are also "precursor-like". In addition, it has previously been suggested that γδ-biased gene expression that is also evident in precursor cells is actively down-regulated as cells mature into the αβ T cell lineage (27). Hence, interpretation of these gene expression profiling results from *Sox13* transgenic DP cells suggests that SOX13 may act, in part, by

Figure III-7

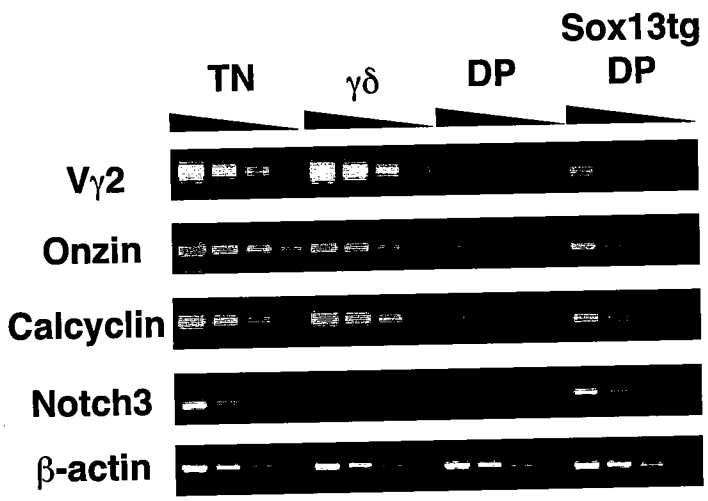


Figure III-7. Confirmation of precursor and γδ-like molecular profile of DP cells from *Sox13* transgenic mice. TN and γδ thymocytes from WT mice, and DP thymocytes from WT and *Sox13* transgenic mice were sorted and analyzed by semi-quantitative RT-PCR assay to confirm select genes identified by Affymetrix DNA microarrays as differentially expressed between *Sox13* transgenic and WT DP thymocytes (thymocytes were pooled from > 3 mice per subset using a different *Sox13* transgenic founder line than those used for gene-chip analysis, n=1).

Figure III-8

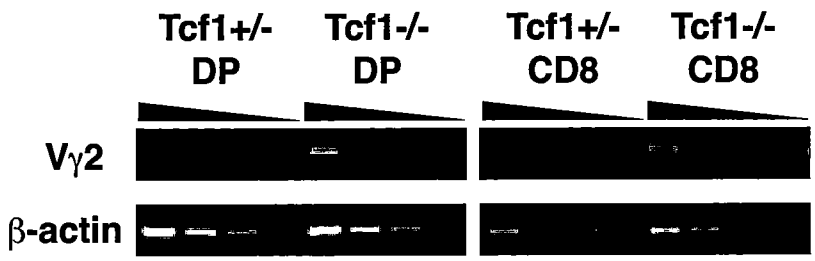


Figure III-8. TCRγ gene expression in *Tcf1*$^{-/-}$ mice. Semi-quantitative RT-PCR analysis of the rearranged Vγ2-Jγ1 gene expression in sorted DP thymocytes and CD8^{+} T cells from *Tcf*$^{+/-}$ and *Tcf1*$^{-/-}$ mice (four-fold serial dilutions) (n=2).

imposing a γδ-like molecular signature on the developing αβ lineage thymocytes and/or by inhibiting appropriate differentiation of αβ T cells.

SOX13 inhibits TCF1-dependent gene expression.

To date, the only other *in vivo* model in which TCRγ is mis-expressed in αβ lineage T cells is in *Tcf1*$^{-/-}$ mice (W. Held and J. Kang, unpublished.) Expression of the dominant Vγ2-Jγ1.1 gene rearrangement is evident in both DP thymocytes and peripheral CD8^{+} αβ T cells from *Tcf1*$^{-/-}$ mice (Figure III-8). Intriguingly, aside from this molecular similarity, the overall phenotype of *Sox13* transgenic and *Tcf1*$^{-/-}$ mice are strikingly alike (Table III-1). Both mouse models have significant decreases in total thymic cellularity due, in part, to inhibition of αβ T cell lineage development. Thymocytes in *Sox13* transgenic and *Tcf1*$^{-/-}$ mice proliferate less, and an increase in apoptosis has been noted. Also, thymic γδ T cell development appears unimpaired in both models. In concert with the phenotypic and molecular similarities between *Sox13* transgenic and *Tcf1*$^{-/-}$ mice, we hypothesized that SOX13 may act, at least in part, by antagonizing TCF1 in T cell development. TCF1 acts as a transcriptional transactivator, but importantly, although SOX13 is known to bind to similar concensus sequences in DNA, it does not appear that SOX13 can promote transcription of reporter genes containing multiple SOX binding sites, at least in the cell line tested (98). The activity of certain TCF1 isoforms, along with the closely related LEF1, is regulated by morphogenetic WNT signaling via the canonical β-catenin pathway (82, 115). In lower organisms, as well as in mice, some SOX family members have been shown to negatively influence WNT signaling (116-118). To test if SOX13 also inhibits the WNT/β-catenin signaling pathway, specifically

Table III-1

	Lck-Sox13Tg	TCF1$^{-/-}$
αβ lineage development:	Inhibited	Inhibited
γδ lineage development:	Normal	Normal
Thymic cellularity:	Decreased	Decreased
Thymocyte proliferation:	Inhibited	Inhibited
Thymocyte survival:	Decreased	Decreased

Table III-1. Comparison of observed phenotypes in *Sox13* transgenic and *Tcf1*$^{-/-}$ mice.

through TCF1, we took advantage of previously published luciferase reporter assay systems.

First, in 293T (human embryonic kidney) cells expressing endogenous TCF1 and optimal levels of co-transfected constitutively active *β-catenin*, we sought to determine if SOX13 could antagonize transcription of a *Luciferase* reporter gene controlled by an upstream element containing multiple TCF1 binding sites (TOPFLASH) (119). SOX13 did, indeed, inhibit the transcriptional activation of the TOPGAL reporter construct in a dose-dependent manner (Figure III-9A). To extend this finding for a physiological target gene of TCF1 in lymphocytes, we tested whether SOX13 can interfere with the expression of the TCF1-regulated *Ly49A* gene. It has previously been shown that a regulatory upstream region of the *Ly49A* gene contains functionally relevant TCF1 binding sites (120). An *Ly49A* promoter-*Luciferase* reporter construct was transiently co-transfected into EL4 (T cell lymphoma) cells in the presence or absence of an expression vector containing *Sox13* cDNA. A significant, dose-dependent decrease in the relative Luciferase activity was observed with increasing amounts of transfected *Sox13* expression plasmid (Figure III-9B), demonstrating that SOX13 can inhibit expression of TCF1 target genes in T cells *in vitro*.

Importantly, TCF1 transactivation of the *Ly49A* gene promoter appears to be independent of WNT/β-catenin signaling, as the *Ly49A* promoter reporter is active in the absence of co-transfected constitutively active *β-catenin* expression plasmid. In EL4 cells, the generic TCF1 reporter TOPFLASH requires constitutively active *β-catenin* for expression (Figure III-9C). These data suggest that SOX13 can, indeed, antagonize β-

Figure III-9

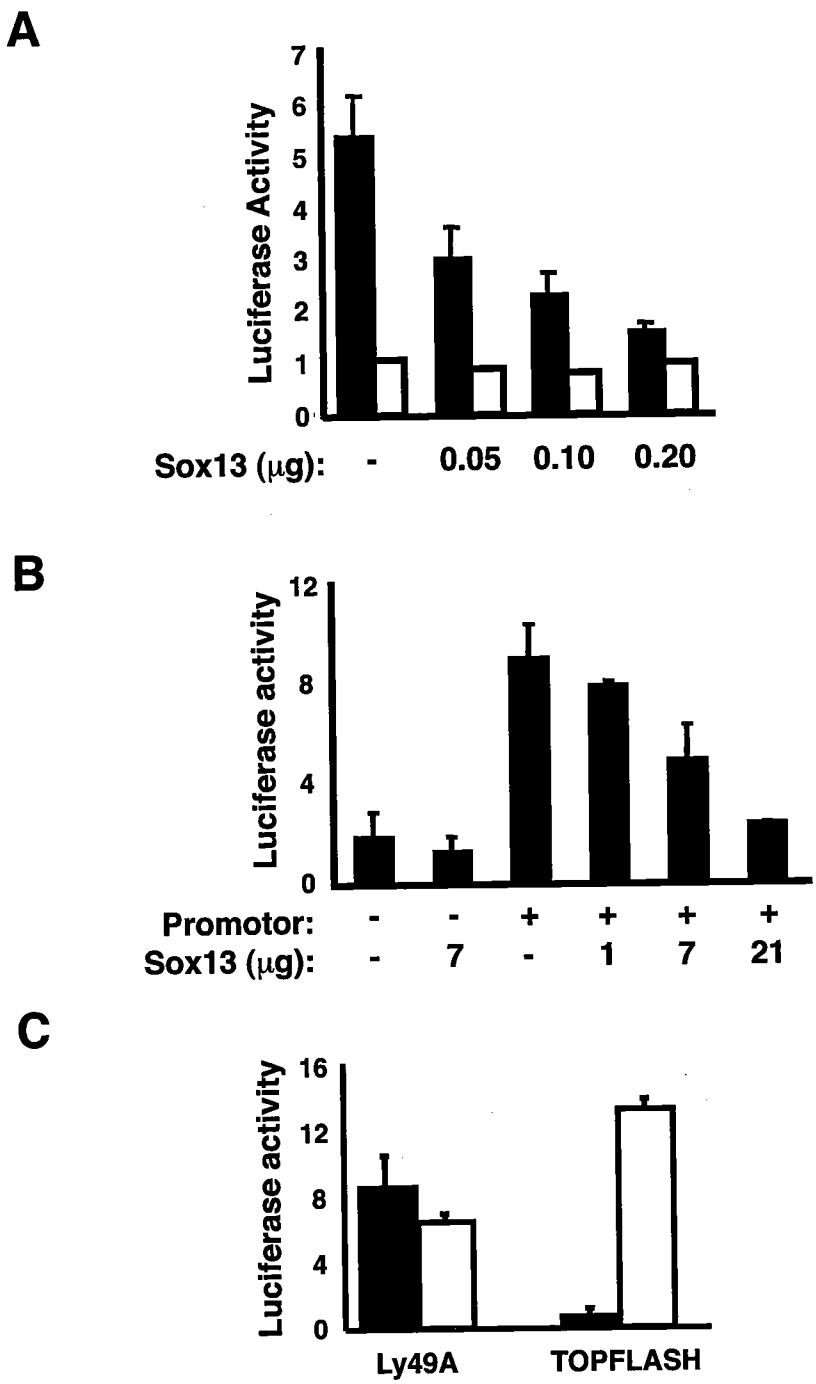

A
Luciferase Activity
0
1
2
3
4
5
6
7
Sox13 (μg): - 0.05 0.10 0.20
B
Luciferase activity
0
4
8
12
Promotor: - - + + + +
Sox13 (μg): - 7 - 1 7 21
C
Luciferase activity
0
4
8
12
16
Ly49A
TOPFLASH

Figure III-9. SOX13 antagonizes TCF1-dependent target gene transcription. (A) SOX13 inhibits β-catenin dependent TCF1 transcription activation. 293T cells were transiently transfected with a pGL3 firefly luciferase plasmid containing multiple TCF1 consensus binding sites, TOPFLASH (filled bars), or the luciferase reporter plasmid in which the TCF1 consensus DNA binding sites have been mutated, FOPFLASH (open bars). The cells were co-transfected with an expression plasmid pEF-Bos containing constitutively active β-catenin and increasing concentrations of pEF-Bos containing *Sox13* cDNA. A Renilla luciferase plasmid was included as a transfection control. After 32-36 hours, cells were lysed to measure luciferase activity. Results are presented as firefly/Renilla luciferase ratio. Error bars represent standard deviation. (Transfections performed in duplicate or triplicate within each experiment, and performed ≥ 2 times.) **(B)** SOX13 inhibits TCF1-controlled *Ly49A* gene transcription. EL4 cells were transiently co-transfected with a promotorless pGL3 firefly luciferase plasmid or the pGL3 firefly luciferase plasmid containing the 5' promotor region of the *Ly49A* gene, along with increasing concentrations of an expression plasmid pEF-Bos containing *Sox13* cDNA, and the assay was performed as in (A). (Transfections performed in duplicate or triplicate within each experiment, and performed ≥ 2 times.) **(C)** β-catenin is necessary for TOPGAL reporter transcription. EL4 cells were transiently co-transfected with either the TOPGAL or *Ly49A* promotor reporter construct in the absence (filled bars) or presence (open bars) of constutively active β-catenin, and the luciferase assay performed as in (A). (Transfections performed in duplicate, n=1).

catenin dependent and independent TCF1 transactivation of targets. In the thymus, there are several TCF1 isoforms, only some of which include the β-catenin transactivation domain that is required for mediating the survival of DP αβ lineage thymocytes (82). The role of other isoforms has not been solidified at present, but may be responsible for the activation of some TCF1 targets such as the TCRα enhancer and the LY49A promoter.

Summary and Future Directions

The significant, but incomplete, inhibition of αβ lineage development when *Sox13* is over-expressed in early thymocyte precursor cells, in combination with the γδ- and precursor-biased molecular profile that over-expression of *Sox13* imposes on DP αβ lineage thymocytes, suggests that SOX13 plays an important role in T cell development. The precise function of SOX13, however, has not been fully resolved. It is apparent that SOX13 acts to inhibit cell proliferation and survival, but it is also possible that SOX13 affects the molecular program of developing thymocytes, either by imposing a γδ T cell expression profile or by inhibiting genes necessary for αβ T cell differentiation. Additionally, since conversion of all precursors to the γδ T cell lineage is incomplete, these results would suggest that SOX13 is not a "master regulator" of lineage fate, redirecting all cells in which it is expressed into the γδ T cell lineage. However, it is possible that this mode of over-expression is limiting. First, it is possible that the *Lck* proximal promoter driving *Sox13* expression, active from TN2 onward, may not be early

enough to convert precursors to the γδ T cell lineage. Also, it is not known if *Lck* is expressed in every precursor cell, and at the same level among cells. Therefore, to determine if SOX13 is a "master regulator" of γδ T cell lineage fate, we are attempting to retrovirally over-express *Sox13* in bone marrow hematopoietic stem cells that we can transfer into *Rag1*$^{-/-}$ mice to determine the developmental potential of cells when *Sox13* is expressed in the earliest precursors at high levels.

We have also presented data that SOX13 may regulate thymocyte development, at least in part, by inhibiting TCF1 target gene transcription *in vitro*. In addition, mis-expression of TCRγ genes in DP cells in both the *Sox13* transgenic and *Tcf1*-deficient mouse models is intriguing, but perhaps not definitive evidence of this antagonism *in vivo*, as we cannot decisively rule out the possibility that SOX13 inhibits the αβ differentiation program necessary to silence the TCRγ locus. This question is the focus of on-going research in the lab as we are attempting to determine the effect of SOX13 on expression of known TCF target genes *in vivo*, as well as develop additional model systems to more definitively address this issue. In addition, we are attempting to determine the mechanism of TCF1 antagonism by SOX13. One mode of SOX inhibition of the WNT signaling pathway is competition with TCF for β-catenin binding, though alternate modes of action may exist. It is possible that SOX13 is blocking TCF1 target gene transcription by binding to and sequestering β-catenin, TCF1 itself, and/or perhaps by competing with TCF1 for DNA concensus sequence binding sites. Due to the lack of anti-mouse SOX13 antibody, the answer to this question has been delayed. We have,

however, generated a V5-tagged SOX13 construct that we may be able to use to determine possible protein interactions to resolve this issue.

Materials and Methods

Mice

All mice used in these experiments were housed in a pathogen-free rodent barrier facility. The University of Massachusetts Medical School Institutional Care and Use Committee approved all animal experiments. To generate *Sox13* transgenic mice, a full-length mouse *Sox13* cDNA (105) was cloned downstream of the *Lck* proximal promoter in the p1017mod vector. The transgenic construct was injected directly into fertilized day 0.5 C57BL/6 embryos and maintained on the C57BL/6 background. Transgenic mice were identified by PCR of tail DNA (*Sox13*-for 5'-CGG AAC AGC AGC CAC ATC AAG AGA-3' and *Sox13*-rev 5'-ATG GTG TAG CTT TGG CGA GCA C-3'.) All other mouse strains including $Tcr\beta^{-/-}$ and $Tcf1^{-/-}$ mice were maintained on the C57BL/6 background.

FACS and cell sorting

The following antibodies were purchased from eBiosciences or BD Pharmingen: antibodies specific for mouse CD4 (PE and PE-Cy5), CD8 (FITC and PE-Cy5), CD3 (PE-Cy5), γδTCR (biotin), Vγ2 (FITC), Vγ3 (FITC), CD25 (FITC), CD44 (PE), and AnnexinV (PE). Streptavidin-PE was purchased from eBiosciences. For BrdU experiments, mice were injected intraperitoneally with 0.1mg of BrdU twice at one hour intervals and thymocytes analyzed one hour after the second injection. Cells were stained

with the appropriate lineage antibodies and with anti-BrdU FITC. Samples were analyzed on the EPICS XL cytometer (Coulter), and data analyzed using FloJo software (Tree Star, San Carlos, CA.) Samples were sorted using the DakoCytomation MoFlo system.

RT-PCR and Real-time qPCR

For RT-PCR and real-time qPCR, total RNA was isolated from cells (Trizol Reagent, Invitrogen) and cDNA prepared using AMV RT (Roche). The following primer pairs were used for PCR: *β-tubulin*-for 5'-CAG GCT GGT CAA TGT GGC AAC CAG ATC GGT-3' and *β-tubulin*-rev 5'-GGC GCC CTC TGT GTA GTG GCC TTT GGC CCA-3', *β-actin*-for 5'-CTA GGC ACC AGG GTG TGA TGG-3' and *β-actin*-rev 5'-TCT CTT TGA TGT CAC GCA CGA-3', *Vγ2*-for 5'-CTG GGA ATT CAA CCT GGC AGA TGA-3' and *Jγ1.1*-rev 5'-CTT ACC AGA GGG AAT TAC TAT GAG-3' (22), *Onzin*-for 5'-TGC TCC CCA AAA TTC CAA CTG-3' and *Onzin*-rev 5'-AAA ATA AAT CAA AAA GCC CAA CTA-3' (121), *Calcyclin*-for 5'-CAG TGA TCA GTC ATG GCA TGC C-3' and *Calcyclin*-rev 5'-ACG GTC CCA TTT TAT TTC AGA GCT-3' (122), *Notch3*-for 5'-GAT GTC ACA TAT GAC TGT GCT TGC-3' and *Notch3*-rev 5'-GAT ATT CTG ACT GCA GCT CTC ACC-3'.

qRT-PCR was performed using SYBR green (Applied Biosystems) fluorescence detection using the BioRad iCycler system. The following primers were used for qRT-PCR: q*β-actin*-for 5'-CGA GGC CCA GAG CAA GAG AG-3' and q*β-actin*-rev 5'-CGG TTG GCC TTA GGG TTC AG-3' (109), q*Bcl-xL*-for 5'-ATT GGT GAG TCG GAT TGC-3', and q*Bcl-xL*-rev 5'-CAC AGT CAT GCC CGT CAG-3'.

Luciferase Assays

Full-length *Sox13* cDNA was cloned into the pEF-Bos expression vector. Other vectors used in luciferase assays have previously been described (120). Briefly, 2.5 x 10^5 293T cells were transiently transfected, using Lipofectamine and Plus Reagent (Invitrogen), with 0.5μg of a constitutively active form of β-*catenin* in the expression plasmid pEF-Bos, increasing amounts of pEF-Bos-*Sox13*, TOPFLASH or FOPFLASH firefly luciferase reporter, and pRL-TK (Renilla luciferase) as a transfection control. Cells were washed and lysed in Passive Lysis Buffer (Promega) by freeze-thaw, 32-36 hours post-transfection, and analyzed using the Dual-Luciferase Reporter Assay System (Promega). For EL4 cell transfection, 10^7 cells were electroporated with a pGL3 firefly luciferase reporter vector containing the WT *Ly49A* promoter or a promoterless pGL3 vector along with increasing amounts of pEF-Bos-*Sox13* plasmid, and pRL-TK as the transfection control. Luciferase activity was measured as above.

DNA microarrays

Cell subsets were sorted by FACS using total pooled thymocytes from minimum of two mice and immediately lysed in Trizol. Gene expression profiling was performed according to the manufacturer's protocol (Affymetrix). Labeled cRNA (from total RNA) was generated and applied to Affymetrix Mu11K(A and B) or muU74Av2 microarrays. All experiments were performed twice using RNA samples from two independently sorted cell subsets. Results were analyzed using Microarray Analysis Software v4 and v5 (Affymetrix).

CHAPTER IV

SOX13 IS NECESSARY FOR NORMAL γδ T CELL DEVELOPMENT

Introduction

We have demonstrated that SOX13 is sufficient to impose significant molecular γδ and/or precursor T cell like qualities in developing αβ lineage precursor cells. Observations of the impact of *Sox13* over-expression in αβ lineage cells demonstrate that SOX13 can inhibit proliferation, increase apoptosis, and impose an aberrant molecular profile on non-γδ T cells. One of the few known critical differences in αβ and γδ T cell lineage development is the extent of cell division during development before thymic egress. αβ lineage T cells undergo as many as ten cell divisions before becoming mature αβ T cells (21). In contrast, γδ T cells, before maturation, only divide two or three times (22). Hence, it is possible that SOX13 is responsible, in part, for determining differences in this intrinsic cell cycling property that may be intimately associated with the differentiation process. Therefore, it is important to determine if SOX13 is playing these same roles in the γδ T cell lineage where it is endogenously expressed. Given that other HMG-box transcription factors are necessary for various aspects of αβ lineage T cell development, and that over-expression of *Sox13* significantly alters the development of cells in which it is not normally expressed, we sought to determine if SOX13 was necessary for γδ T cell development. To this end, we have generated *Sox13*-deficient mice to determine if SOX13 is necessary for proper development of γδ lineage T cells.

Results and Discussion

Generation of Sox13-deficient mice.

Within the *Sox13* gene, three protein domains are predicted. The leucine zipper motif is encoded in exon 4, a glutamine rich region in exons 4 and 5, and the HMG-box is encoded in exons 11 and 12 (Figure IV-1A) (123). To create *Sox13* null alleles, exons 4 through 11, approximately 5 kilobases of genomic sequence, were excised via homologous recombination in ES cells of a targeting construct containing loxP sites and subsequent *in vitro* CRE expression, deleting the regions encoding the leucine zipper, the glutamine rich region, and the majority of the DNA-binding HMG-box (Figure IV-1A). Deletion of the appropriate genomic region was confirmed by Southern blot (Figure IV-1B). As expected, we could not detect any *Sox13* transcript in homozygous *Sox13* null mice generated from these targeted ES cells by RT-PCR (Figure IV-1C).

Sox13-deficient mice have significant developmental abnormalities.

In mice, there are over 20 SOX transcription factor family members (Figure IV-2) (101). Several knock-out mouse models of these genes have been developed, and due to the important role of many SOX transcription factors in various aspects of development, many of these *Sox* knock-out models result in embryonic or peri-natal lethality (124-127). Specifically, a deficiency in *Sox5* or *Sox6*, the most closely related *Sox* gene family members to *Sox13*, leads to early post-natal lethality. $Sox5^{-/-}$ mice die shortly after birth due to respiratory distress, while *Sox6*-deficient mice show severe dwarfism about one week after birth, stop feeding days later, and die by three weeks of age (127). Similarly,

Figure IV-1

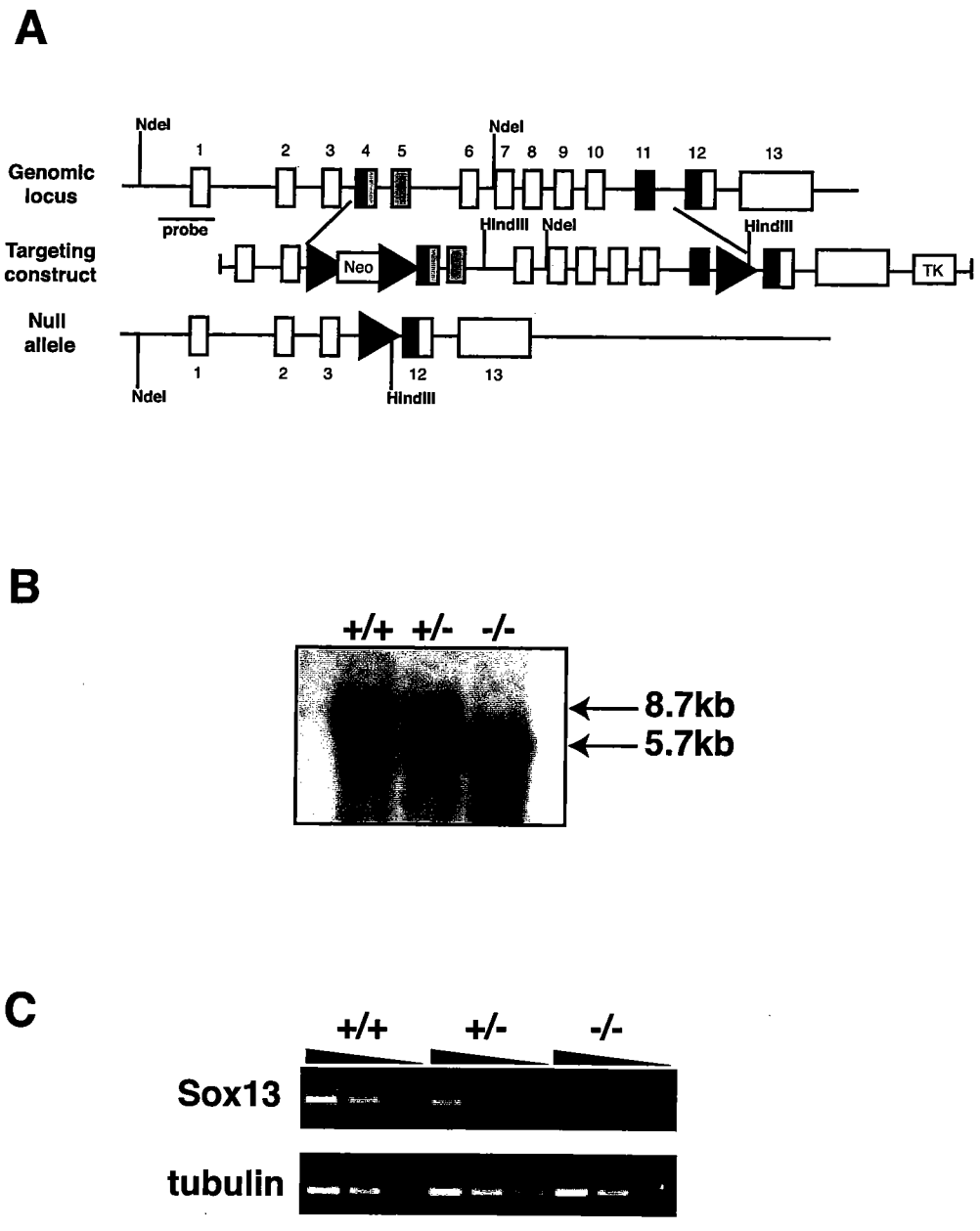

A
NdeI
NdeI
1
2
3
4
5
6
7
8
9
10
11
12
13
Genomic locus
probe
HindIII
NdeI
HindIII
Targeting construct
Neo
TK
Null allele
1
2
3
12
13
NdeI
HindIII
B
+/+
+/-
-/-
8.7kb
5.7kb
C
+/+
+/-
-/-
Sox13
tubulin

Figure IV-1. Generation and genotyping of *Sox13*$^{-/-}$ mice. **(A)** *Sox13* genomic locus and targeting construct. Genomic region (not to scale) encoding leucine zipper shaded in black (exon 4), exons encoding glutamine rich region shaded in light grey (exons 4 and 5), and exons encoding the HMG box shaded in dark grey (exons 11 and 12.) Correctly targeted ES cell clones were subjected to transient CRE expression to recombine the most 5' and 3' loxP sites to generate a null allele. Triangles indicate loxP sites. **(B)** Representative Southern blot to identify *Sox13*$^{+/+}$, *Sox13*$^{+/-}$, and *Sox13*$^{-/-}$ mice. Tail DNA was digested with HindIII and NdeI. Digested DNA was subject to Southern blot. (WT allele is ~8.7kb, and null allele is ~5.7kb.) **(C)** Semi-quantitative RT-PCR for *Sox13* expression in total thymus of *Sox13*$^{+/+}$, *Sox13*$^{+/-}$, and *Sox13*$^{-/-}$ mice.

Figure IV-2

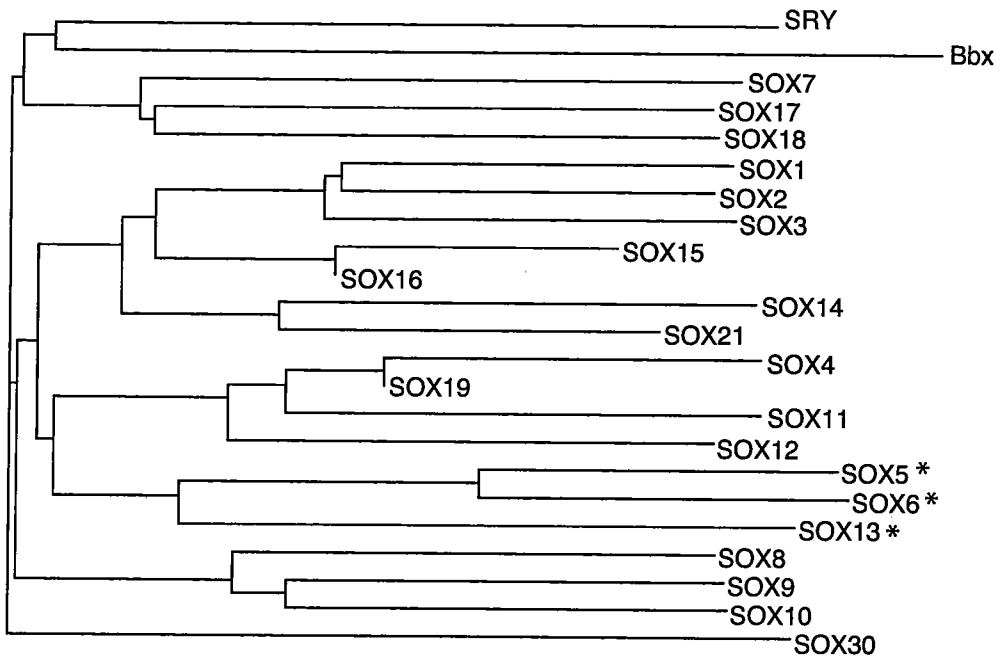

SRY
Bbx
SOX7
SOX17
SOX18
SOX1
SOX2
SOX3
SOX15
SOX16
SOX14
SOX21
SOX4
SOX19
SOX11
SOX12
SOX5 *
SOX6 *
SOX13 *
SOX8
SOX9
SOX10
SOX30

Figure IV-2. Phylogenentic analysis of SOX family members. Phylogenic tree of the SOX family of transcription factors was compiled using ClustalW. Similar trees and family annotation has been previously been published, but exclude one or more of the more recently described family members. Of note, SOX13 is most closely related to family members, SOX5 and SOX6 as indicated by *.

some *Sox13*-deficient mice died shortly after birth, while in others, we observed significant dwarfism and death by three weeks of age (Figure IV-3A). These severe, but as yet uncharacterized, abnormalities are observable in the majority of $Sox13^{-/-}$ mice. However, some *Sox13*-deficient mice do survive to adulthood and appear, at least superficially, normal, leading to skewed Mendelian ratios from $Sox13^{+/-}$ intercrosses. Less than half of the predicted number of $Sox13^{-/-}$ mice are actually viable at 3 weeks of age.

γδ T cell development is impaired in the absence of Sox13.

Due to the significant generalized defects between post-natal LMC and $Sox13^{-/-}$ mice, we sought to determine the effect of *Sox13*-deficiency in embryonic development where there are no overt signs of dwarfism. In the thymus, however, there is a gene-dose dependent decrease in total thymic cellularity in $Sox13^{+/-}$ and $Sox13^{-/-}$ mice as compared to LMC E18.5 fetuses (WT: $5.3 \times 10^6 \pm 4.1 \times 10^5$, het: $3.9 \times 10^6 \pm 1.0 \times 10^6$, KO: $2.7 \times 10^6 \pm 1.0 \times 10^6$, p-values between each genotype ≤ 0.05). Despite this decrease in total thymic cellularity, no significant differences in the proportions of αβ lineage T cells were observed at E18.5 (Figure IV-4A). There was however, a significant decrease in $CD4^+$ and $CD8^+$ SP cell numbers in $Sox13^{-/-}$ mice as compared with LMC (WT: $3.0 \times 10^5 \pm 5.1 \times 10^4$, KO: $1.8 \times 10^5 \pm 7.2 \times 10^4$, p-value = 0.02). In contrast, there was a gene-dose dependent decrease in the proportion of γδ lineage thymocytes in $Sox13^{+/-}$ and $Sox13^{-/-}$ mice as compared to LMC (Figure IV-4A). The absolute number of total γδ TCR^+ thymocytes were also decreased in $Sox13^{+/-}$ and $Sox13^{-/-}$ mice (Figure IV-4B), resulting in a significantly increased ratio of the absolute numbers of SP αβ lineage thymocytes

Figure IV-3

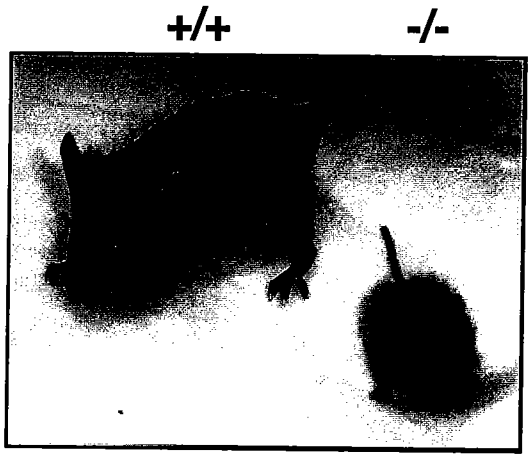


Figure IV-3. *Sox13*$^{-/-}$ mice have severe growth abnormalities. Representative *Sox13*$^{-/-}$ and WT mice. 2.5 week-old littermates from a *Sox13*$^{+/-}$ F2 intercross. Significant dwarfism is apparent approximately one week after birth, and the time to death is variable.

Figure IV-4

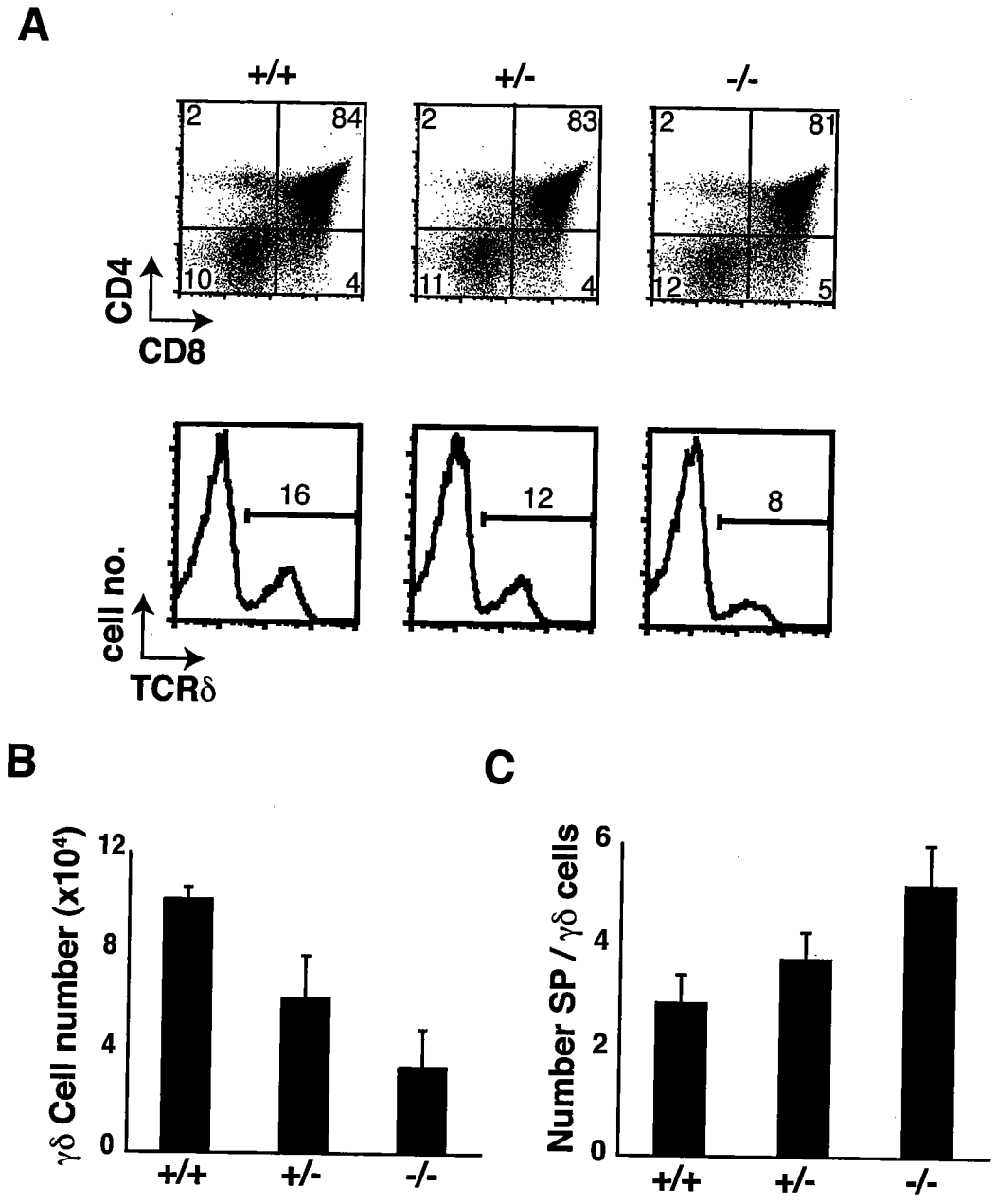

A
+/+
+/-
-/-
2
84
10
4
2
83
11
4
2
81
12
5
CD4
CD8
16
12
8
cell no.
TCRδ
B
γδ Cell number (x10⁴)
12
8
4
0
+/+
+/-
-/-
C
Number SP / γδ cells
6
4
2
0
+/+
+/-
-/-

Figure IV-4. *Sox13*-deficient mice have impaired γδ T cell development. **(A)** Representative flow cytometric profiles of thymocytes from E18.5 *Sox13*$^{+/+}$, *Sox13*$^{+/-}$, and *Sox13*$^{-/-}$ fetuses for αβ (CD4 and CD8, top panels) and γδ (TCRδ) lineage markers on total and CD4$^-$CD8$^-$ thymocytes, respectively. **(B)** Absolute numbers of CD4$^-$CD8$^-$ γδ cells from *Sox13*$^{+/+}$ (n=3), *Sox13*$^{+/-}$ (n=11), and *Sox13*$^{-/-}$ (n=13) fetuses and **(C)** ratio of the absolute number of mature αβ lineage cells (CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ thymocytes) to the absolute number of γδ cells. Error bars represent standard deviation.

relative to γδ TCR^+ thymocytes (Figure IV-4C). These results suggest that deficiency in SOX13 preferentially impacts γδ T cell development.

Sox13 may differentially influence γδ T cell subsets.

The different subsets of γδ T cells develop at different stages of ontogeny, rearranging the five different Vγ genes at specific times during development. At E18.5, the predominant γδ T cell subsets developing in the fetal thymus are those expressing Vγ3 and Vγ2. As it is possible that the requirement for SOX13 varies in different γδ T cell subsets, we co-stained γδ TCR^+ thymocytes from E18.5 fetuses with anti-Vγ3 or anti-Vγ2 antibody. Data from these experiments indicate that there is no significant decrease in the proportion of $V\gamma3^+$ γδ TCR^+ thymocytes in *Sox13*$^{-/-}$ at E18.5 (Figure IV-5A). In contrast, preliminary results suggest that there may be a gene-dose dependent decrease in the proportion of $V\gamma2^+$ γδ TCR^+ thymocytes in *Sox13*$^{+/-}$ and *Sox13*$^{-/-}$ mice (Figure IV-5B). Although preliminary, these data suggest that SOX13 may play different roles in the development or maintenance of subsets of γδ T cells. Additional experiments will be required to confirm these observations, and it will also be interesting to determine the role of SOX13 in other γδ T cells subsets.

Sox5 may partially compensate for the absence of Sox13.

As mentioned earlier, *Sox13* is most closely related to SOX family members, *Sox5* and *Sox6 (101)*. These three members of SOX transcription factors make up the Group D *Sox* genes. All contain a leucine zipper and glutamine rich region, as well as an HMG box that is highly conserved among group members (Figure IV-6A). It has previously

Figure IV-5

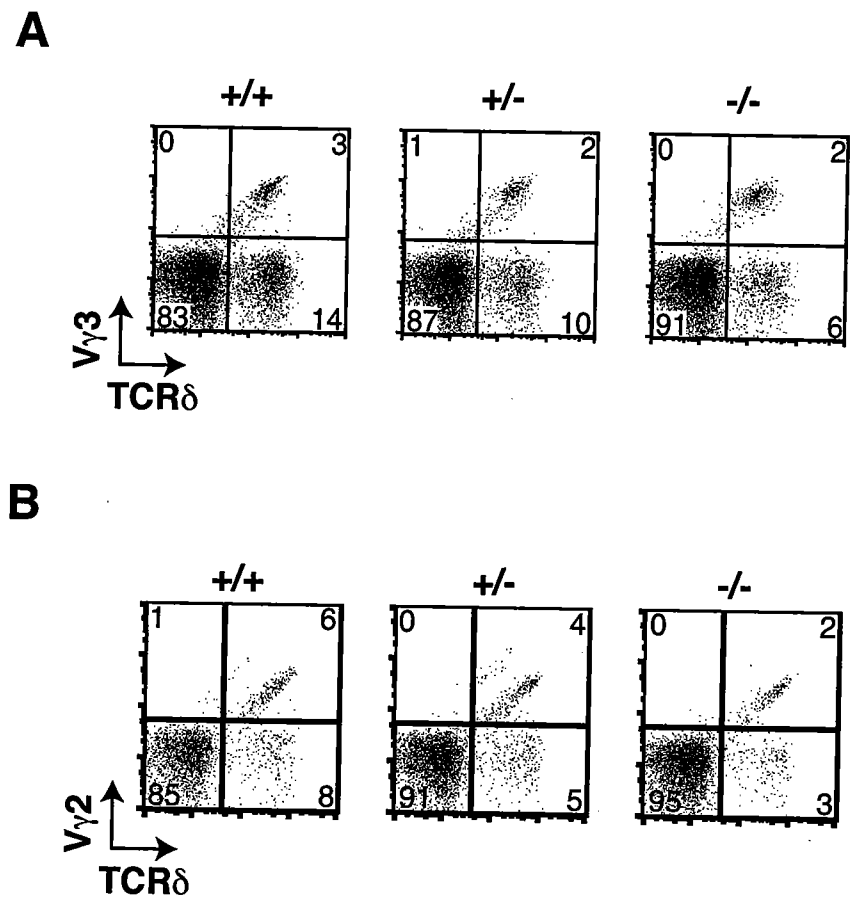


Figure IV-5. *Sox13*-deficiency may differentially affect γδ T cell subsets. Representative flow cytometry profiles of $CD4^-CD8^-$ thymocytes from E18.5 $Sox13^{+/+}$, $Sox13^{+/-}$, and $Sox13^{-/-}$ fetuses stained with mAbs to identify **(A)** Vγ3 (n=3, 11, and 13, respectively) and **(B)** Vγ2 (n=1, 6, and 5, respectively) expressing γδ T cells.

Figure IV-6

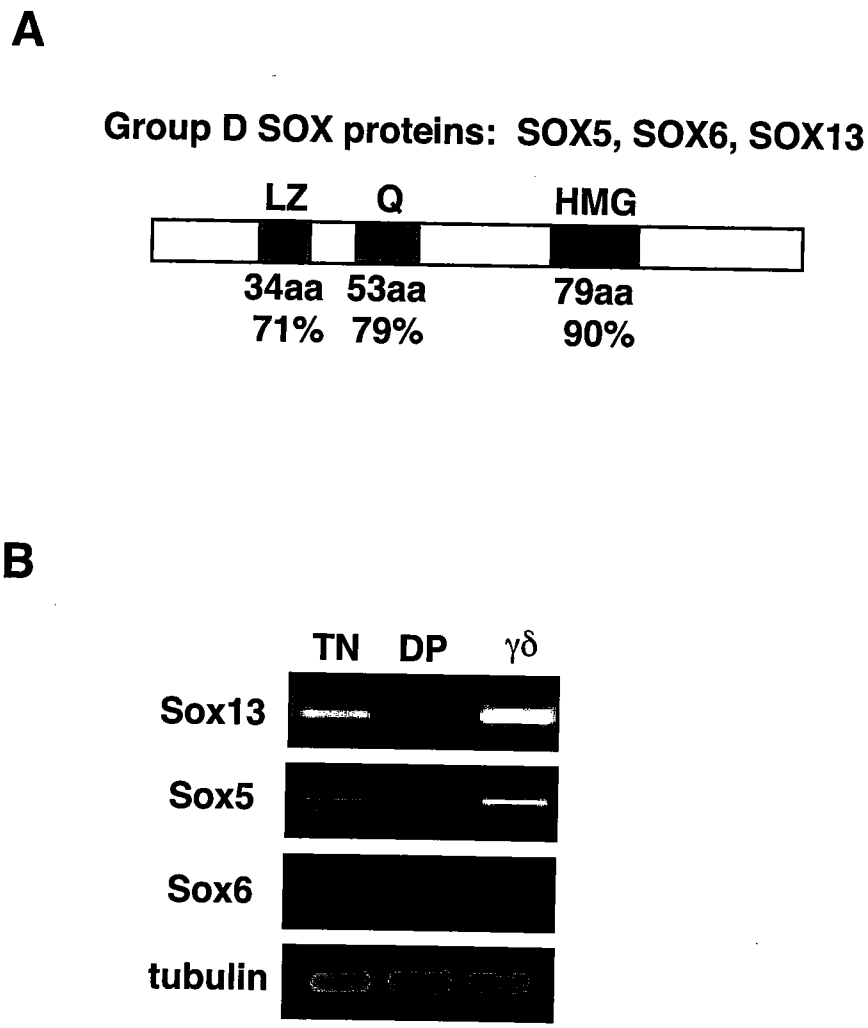

A
Group D SOX proteins: SOX5, SOX6, SOX13
LZ
Q
HMG
34aa
71%
53aa
79%
79aa
90%
B
TN
DP
γδ
Sox13
Sox5
Sox6
tubulin

Figure IV-6. *Sox5* is also expressed in γδ-lineage thymocytes. **(A)** Amino acid sequence similarity in known protein motifs among Group D SOX family members, SOX5, SOX6, and SOX13. Percent amino acid homology is reported for regions encoding the Leucine zipper, glutamine-rich region, and HMG-box. Regions of the protein outside of these domains is not significantly conserved. Adapted from Wegner, **Nuceic Acids Res**, 1999 (101). **(B)** RT-PCR analysis of expression of closely related *Sox* gene family members, *Sox5* and *Sox6*, in sorted TN, DP, and γδ thymocytes from WT mice. (Thymocytes pooled from > 3 mice per subset, n=2).

been shown that some closely related SOX family members with over-lapping expression patterns have redundant or partially redundant functions (127). Therefore, it was important to determine the thymic expression patterns of closely related *Sox5* and *Sox6*. *Sox6* is not detected by RT-PCR in sorted TN precursors, αβ lineage DP cells, or γδ TCR^+ thymocytes (Figure IV-6B). *Sox5*, however, shares an over-lapping expression pattern with *Sox13*, with expression restricted to thymocyte precursors and γδ lineage cells, but absent in DP thymocytes inviting the possibility that *Sox5* may partially compensate in the absence of *Sox13* (Figure IV-6B). Thus, the adverse effects of *Sox13*-deficiency in γδ T cell development that were evident despite the fact that *Sox13*$^{-/-}$ mice still express *Sox5*, suggest that developing γδ T cells may be exquisitely sensitive to levels of SOX13, and likely SOX5, and that SOX13 is necessary for normal γδ T cell development. To this end, we are currently generating *Sox13*$^{-/-}$*Sox5*$^{-/-}$ compound knock-out mice. It is also plausible, albeit less likely, that *Sox13* and *Sox5* play redundant roles, but in different subsets of γδ T cells. These hypotheses remain to be tested.

Summary and Future Directions

Sox13$^{-/-}$ mice have significant generalized developmental abnormalities that limit the study of the role of *Sox13* in thymocyte development. Analyses to date, have been limited to studying the effect of *Sox13*-deficiency in the fetal thymus. From these experiments, it is apparent that *Sox13* is necessary for normal γδ T cell development, as there is a gene-dose dependent decrease in the proportion and absolute numbers of γδ

TCR^+ thymocytes in *Sox13*$^{+/-}$ and *Sox13*$^{-/-}$ mice. Preliminary experiments suggest that the effect of the *Sox13*-deficiency may be γδ T cell subset specific. This observation requires additional experimentation. Not only will it be necessary to determine the function of SOX13 in the other γδ T cell subsets, it will be interesting to determine whether *Sox13* is also expressed differentially among these various γδ T cell subsets. Finally, due to the overlapping expression of *Sox5*, the mostly closely related family member to *Sox13*, we suggest that *Sox5* and *Sox13* may have partially redundant functions in γδ T cell development.

The initial analysis of the *Sox13*$^{-/-}$ mice suggests that SOX13 is necessary for normal γδ T cell development. However, some of these observations appear incongruent with expectations based on analyses of the *Sox13* transgenic mouse model presented in Chapter III. The previous data suggested that SOX13 impacts T cell development, at least in part, by inhibiting the proliferation and survival of thymocyte subsets. One obvious prediction of the *Sox13*$^{-/-}$ phenotype would be, then, an increase in proliferation and cell survival in the absence of *Sox13* leading to an increase in cell populations, such as thymocyte precursor subsets and γδ lineage T cells that endogenously express this gene. In contrast, we observe a decrease in these populations. In addition, if SOX13 acts to inhibit differentiation of αβ lineage T cells, it is possible that an increase in DP or SP αβ lineage thymocytes would be observed. Again, this appears not to be the result of *Sox13*-deficiency. Although the exact mechanism of γδ T cell impairment in the absence of *Sox13* is not known, it is possible that SOX13 is necessary for appropriate differentiation of this subset through direction of a γδ-specific molecular program. As

analysis of this mouse model system has commenced only recently, significant effort to elucidate the function of SOX13 is underway.

Sox13 conditional knock-out mice were generated in parallel with the *Sox13*$^{-/-}$ mice. Breeding of the *Sox13* conditional knock-out mice with transgenic lines expressing *Cre* recombinase under the control of a number of different promoters expressed at various stages of lymphocyte development will allow more thorough understanding of the role of *Sox13* at different stages of thymocyte cell development. In addition, since current analysis of *Sox13*$^{-/-}$ is limited to the fetal thymus, these conditional knock-out lines will allow us to study the effect of *Sox13*-deficiency in adult mice, and therefore, in peripheral γδ T cells.

Additionally, it is apparent that SOX13 alone is not absolutely necessary for γδ T cell development. Due to the over-lapping expression pattern of *Sox5* and its similarity to *Sox13*, we have hypothesized that SOX5 may partially compensate for the loss of *Sox13*, though we have not ruled out a plethora of other possibilities. At present, this hypothesis is solely theoretical so to clarify any redundant roles between *Sox13* and *Sox5* during γδ T cell development, we are currently generating compound *Sox13*$^{-/-}$*Sox5*$^{-/-}$ mice. It is hypothesized that these compound knock-out mice will be embryonic lethal similar to the *Sox5*$^{-/-}$*Sox6*$^{-/-}$ double knock-out mice which die around E16.5 due to generalized heart failure. However, we hope to determine if compound knock-out mice are able to generate γδ lineage thymocytes and if SOX13 and SOX5 affect different subsets of γδ lineage T cells.

Materials and Methods

Mice

All mice used in these experiments were housed in a pathogen-free rodent barrier facility. The University of Massachusetts Medical School Institutional Care and Use Committee approved all animal experiments. The *Sox13* targeting vector was cloned from a BAC clone identified by a screen of a 129/Sv genomic library. The targeting construct included a *neo* resistance gene flanked by *loxP* sites between exons 3 and 4, and an additional *loxP* site in the intron between exons 11 and 12. A ~500bp deletion in the intron between exons 5 and 6 was generated for ES cell screening purposes. A *thymidine kinase* (TK) gene was inserted at the end of the 3' homology arm. ES cells were electroporated and selected for Neomycin (G418) resistance and TK sensitivity. Correctly targeted clones were exposed to transient CRE recombinase expression, and subsequently analyzed for recombination of the most 5' and 3' loxP sites. *Sox13* gene-deleted (exons 4 to 11) clones were injected into C57Bl/6 blastocysts to generate $Sox13^{-/-}$ mice. $Sox13^{-/-}$ mice were backcrossed one or two generations to C57Bl/6 mice. $Sox13^{-/-}$ mice were identified by Southern analysis (genomic DNA digested with HindIII and NdeI, and probed with radiolabeled ~750bp probe corresponding to exon 1 and flanking sequence) or PCR (WT allele: 285-for 5'-CAA CCG CAA CTT ACA GGA GGT T-3' and 1384-rev 5'-GCT GTT TCT CCT GGT TGG TCA T-3', KO allele: 1303-for 5'-GCC TTC CCA GAC ATG CAT AAC-3' and 1384-rev 5'-GCT GTT TCT CCT GGT TGG TCA T-3'.)

FACS and cell sorting

The following antibodies were purchased from eBiosciences or BD Pharmingen: antibodies specific for mouse CD4 (PE and PE-Cy5), CD8 (FITC and PE-Cy5), CD3 (PE-Cy5), γδTCR (biotin), Vγ2 (FITC), and Vγ3 (FITC). Streptavidin-PE was purchased from eBiosciences. Samples were analyzed on the EPICS XL cytometer (Coulter), and data analyzed using FloJo software (Tree Star, San Carlos, CA.) Samples were sorted using DakoCytomation MoFlo system.

RT-PCR

For RT-PCR, total RNA was isolated from cells (Trizol Reagent, Invitrogen) and cDNA prepared using AMV RT (Roche). The following primer pairs were used for PCR: *β-tubulin*-for 5'-CAG GCT GGT CAA TGT GGC AAC CAG ATC GGT-3' and *β-tubulin*-rev 5'-GGC GCC CTC TGT GTA GTG GCC TTT GGC CCA-3', *Sox13*-for 5'-CGG AAC AGC AGC CAC ATC AAG AGA-3' and *Sox13*-rev 5'-ATG GTG TAG CTT TGG CGA GCA C-3', *Sox5*-for 5'-TGG AGA TTC TGA CGG AAG CG-3' and *Sox5*-rev 5'-CTT GTC CCG CAA TGT GGT T-3' (107), *Sox6*-for 5'-CAT ATG CCT GAC GAA GGG AGT CGG GA-3' and *Sox6*-rev 5'-GGA TCC CAT GTG CCC CTG AAC CTG GA-3'.

CHAPTER V

GENERAL DISCUSSION

The work presented herein describes a γδ T cell-specific gene, *Sox13*, that regulates T cell development from a multi-potent precursor population of cells in the thymus. Cumulatively, we have demonstrated that, unlike other HMG-box transcription factor family members, *Sox13* expression is restricted during thymocyte development, being present in both the precursor subset and γδ T cells, but not in αβ lineage thymocytes. As *Sox13* expression is highest in the earliest thymocyte precursor populations, before any TCR rearrangement occurs, γδ TCR signals cannot be responsible for directing *Sox13* expression in the γδ T cell lineage. In fact, within the precursor population, *Sox13* is heterogeneously expressed in a lineage-biased manner suggesting that *Sox13* expression may identify a subset of precursor cells restricted to the γδ T cell lineage prior to rearrangement and expression of TCR chains. This would be in conflict with several models of lineage specification that suggest that divergence of these two T cell lineages is dependent upon TCR signals. We have ruled out the possibility that αβ-specific signals are responsible for suppressing *Sox13* expression as β-selected cells further differentiate to the DP stage and have shown that γδ TCR signals cannot maintain expression in αβ lineage cells. As mentioned briefly, however, we cannot, at present, rule out that the strength of TCR signal impacts *Sox13* expression. Therefore, *Sox13* expression is likely, but not absolutely, regulated independently of TCR signals.

Complementary over-expression and deletion studies further imply an important role for SOX13 in T cell development. Ectopic expression of *Sox13* inhibits normal αβ T cell development, in part by inhibiting proliferation of TN cells and leading to increased apoptosis in immature αβ lineage DP cells. The most convincing evidence that *Sox13* is also altering the differentiation program of αβ lineage T cells is the abnormal expression profile expressed by DP cells in *Sox13* transgenic mice. Significant changes in gene expression occurr that resemble that of γδ and/or precursor cell types implying, that SOX13 may impart a γδ lineage-specific program on expressing cells or by inhibiting an αβ-specific differentiation program. One potential mechanism of action of SOX13 appears to be antagonism of the WNT/β-catenin signaling pathway via inhibition of TCF1 target genes. Together with the fact that *Sox13*-deficient thymocytes have impaired generation of γδ lineage T cells, these data support an important role for SOX13 in γδ T cell development.

Based on these data, we suggest a model for the role of SOX13 in a stochastic T cell specification process (Figure V-1). We have identified a heterogeneous precursor population in the thymus based on *Sox13* expression. We propose that precursor cells expressing *Sox13* are precursors fated to the γδ T cell lineage. SOX13, acting, in part, by antagonizing WNT/β-catenin signals, would direct cells toward a γδ-specific differentiation pathway which would include expression of lineage-specific genes as well as limited proliferation. γδ lineage precursor cells receiving the appropriate TCR signals would then progress to maturation. Conversely, in the absence of SOX13, αβ lineage programmed precursor cells with requisite WNT/β-catenin signaling through TCF1,

Figure V-1

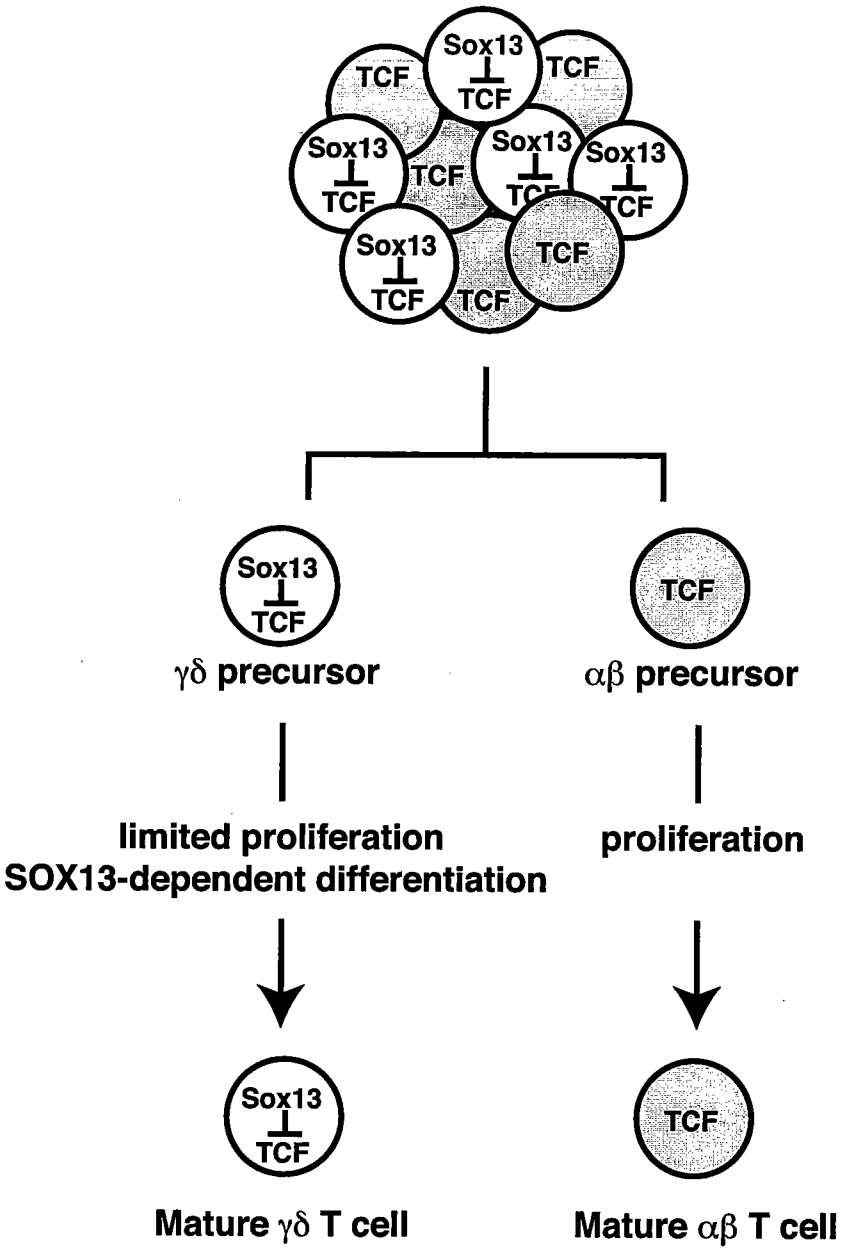

Sox13
TCF
γδ precursor
αβ precursor
limited proliferation
SOX13-dependent differentiation
proliferation
Mature γδ T cell
Mature αβ T cell

Figure V-1. Model of SOX13 function in thymocyte development. We have identified heterogeneity within the thymocyte precursor population based on *Sox13* expression. We propose that *Sox13* expression identifies γδ-lineage precursor cells prior to TCR expression and signaling. *Sox13* expressing cells with a successfully rearranged γδ TCR will go on to differentiate into mature γδ T cells. We propose that SOX13 acts to limit the proliferative capacity of these cells and to impart a γδ-specific differentiation program, in part, by antagonizing TCF1 during development.

would undergo multiple rounds of proliferation and differentiation into mature αβ lineage cells given that they pass necessary TCR check-points. Only successful purification of *Sox13* expressing progenitors will allow definitive support of this model. We are currently generating *Sox13* reporter mice that will potentially allow such purification, and subsequently, determination of the lineage potential of *Sox13* expressing and non-expressing precursor cells.

In addition to *Sox13* and several other HMG-box transcription factors, such as *Tcf1*, *Lef1*, and *Sox4*, that are implicated in thymocyte development and differentiation, it stands to reason that there are likely other SOX transcription factor family members involved at discrete stages of T cell development. We have presented evidence of differential SOX gene expression in thymocyte populations. In addition, gene expression profiles of peripheral αβ and γδ T cells, using gene chip technology, also suggests differential expression of SOX family members (J. Kang, unpublished). Although a more thorough expression analysis through dissection of more discrete subsets of lymphocytes is needed, these data suggest that *Sox* genes may play intricate roles at various aspects of development as the interplay between these molecules appears to be important in regulating specification within other tissues.

How the expression of *Sox13* and other family members is regulated during thymocyte development remains to be seen. Although an obvious candidate, our data suggests that TCR nor IL-7/IL-7R signals are necessary for the commencement or maintenance of *Sox13* expression within the thymus. However, a number of *Sox* genes are known to interact with other morphogen signaling pathways. As this interaction

appears to be dependent on tissue, cell type, and SOX family member, there is no absolute mechanism of action or expression regulation. SOX family members have been found to interact positively or negatively, upstream or downstream, of a number of morphogen and other signaling pathways, including WNT, BMPs, FGF, and Notch in various tissues and cell types (117, 128-130). We have presented evidence that SOX13 is capable of antagonizing WNT signaling to inhibit αβ T cell differentiation, but the mechanism of action is currently unknown. In addition, the interplay of SOX13 with other signaling pathways remains to be seen. Identification of SOX13 binding partners, and perhaps analysis of its transcriptional regulation, will clarify some of these issues.

We have proposed a role for SOX13 in the stochastic lineage commitment of γδ T cells from a common progenitor population in the thymus. If this model holds true through additional experimentation as suggested earlier, it becomes logical to question when this lineage specification occurs. We have identified heterogeneous expression within the TN2 precursor population, but also detect strong expression in TN1 progenitor cells, suggesting that these cells may be lineage committed at the earliest stages of thymic development.

In conclusion, we have presented convincing evidence that SOX13 is likely an important regulator of T cell development, yet the mechanism and extent of the impact of SOX13 on this process and lineage commitment is not fully realized. Our experimental endeavors have raised a plethora of additional questions concerning the mechanism of SOX13 action as well as how SOX13 may be interacting globally with a number of other regulatory signaling processes. The task of identifying how these networks interact to

determine αβ vs. γδ lineage commitment will, undoubtedly, be a complex and exciting task for future experimentation.

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