Transcription factors controlling B-cell development

Two structurally dissimilar transcriptional regulators, E2A and Pax-5, have been shown to play essential roles in B-cell development; the p50 subunit of NFκB, by contrast, is required for normal immune responses.

B-lymphocyte development has long been studied because of its unique biological objectives, which include the generation of immunoglobulin diversity and the shaping of the antibody repertoire. B lymphopoiesis, like all other developmental processes, entails the ordered, sequential expression of specific sets of structural and regulatory genes. A sizable body of work — mostly correlative — has implicated a diverse group of regulatory proteins as important for the regulation of gene expression during B-cell development. Nonetheless, direct assessment of the roles of individual transcription factors has only recently been made possible by the targeted disruption of specific genes in mice. In the past several months, this approach has been used to test the dependence of B-cell development on three structurally dissimilar transcription factors — E2A [1,2], Pax-5 [3] and the p50 subunit of NFκB [4] — whose involvement in B-cell development had been indicated by previous work.

The E2A gene encodes a pair of closely related proteins, E12 and E47, which are generated by alternative splicing of RNA transcripts [5]. The E2A proteins, and the product of a homologous gene called E2-2, were first identified by their ability to bind a class of specific consensus DNA sites, the E2 motifs, which are found in the intronic immunoglobulin μ and κ enhancers. E2 and related motifs (collectively termed E-boxes) were defined by in vivo footprinting of the proteins bound at sites in the intronic immunoglobulin heavy chain enhancer that are occupied specifically in lymphoid cells. Similar sequence elements were subsequently found in all immunoglobulin enhancers and in several regulatory regions that are active in non-lymphoid settings, including the muscle creatine kinase enhancer and the insulin promoter. Mutational analysis has shown that the E-boxes, and the E2 motifs in particular, are critical for immunoglobulin enhancer function.

E12, E47 and E2-2 are members of the basic helix–loop–helix (bHLH) family of transcription factors. These proteins share two characteristic structural features: a pair of amphipathic helices, separated by a loop of variable length (the helix–loop–helix or HLH region); and an adjacent, conserved basic region. The bHLH proteins bind to DNA as homo- or heterodimers; in addition to dimerization, which is mediated by the HLH region, DNA binding requires the basic region. Some bHLH proteins, including E12, E47 and E2-2, are expressed ubiquitously. Others are expressed in a tissue-specific fashion; these include the MyoD family, which is required for muscle development, and the Achaete–Scute family, which functions in neurogenesis.

The ability of bHLH proteins to form heterodimers provides a potentially rich source of combinatorial diversity — distinct bHLH dimers show dramatic differences in DNA binding. For example, the non-tissue-specific bHLH proteins can bind the E2 motif as homodimers, as heterodimers with each other, or as heterodimers with tissue-specific bHLH proteins. In contrast, tissue-specific bHLH proteins bind the E2 motif only as heterodimers with non-tissue-specific proteins. In the B-cell lineage, the predominant E2-binding activities are contributed by the E2A proteins and E2-2.

Several observations have suggested that B-cell development might be critically dependent on E2A (and perhaps E2-2). A specific E2 site, μE5, which is situated within the immunoglobulin μ intronic enhancer, acts as a positive transcriptional element in lymphoid cells and a suppressor of immunoglobulin transcription in non-lymphoid cells. E12 and E47 can activate the intronic μ enhancer when cotransfected with a reporter construct into non-B cells [6]. Furthermore, overexpression of E47 can induce transcription and rearrangement of endogenous immunoglobulin heavy-chain genes in pre-T cells [7]. Curiously, despite the presence of E2A proteins in other cell types, only extracts of B-lymphoid cells have yielded E2A homodimers capable of binding to the E2 motif [8,9].

Nonetheless, until the recent 'gene knockout' experiments, an essential role for E2A in B-cell development was far from certain. Indeed, targeted disruption of the E2A gene, despite its broad expression pattern, had no effect on the ability of cultured embryonic stem cells to differentiate into muscle, cartilage, nerve or erythroid lineages [10]. In this context, the effect of E2A inactivation on murine B-cell development, described recently by two independent teams, is especially striking. The approaches of the two groups differed somewhat but the results are similar overall. Zhuang and coworkers [2] effected a complete knockout of both E2A products. In contrast, Bain and coworkers [1] selectively disrupted the E12-coding sequence; for reasons that are not entirely clear, this also interfered with expression of E47. In either case, homozygous E2A-deficient mice exhibited a profound block in B-cell development.

On the basis of immunoglobulin expression, developing B cells can be divided into three broad groups: pro-B

cells, in which no immunoglobulin chains are expressed; pre-B cells, in which immunoglobulin μ heavy chains, but not light chains, are expressed; and B cells, in which immunoglobulin heavy and light chains are expressed at the cell surface. These stages reflect the underlying sequence of immunoglobulin gene rearrangements — light chains are generally (but not necessarily) assembled after heavy chains. Immunoglobulin heavy chain gene rearrangement occurs in a $CD45R^+$ c-kit$^+$ $CD25^+$ $CD43^+$ population (termed proB/pre-B-I by Melchers and co-workers [11]); upon productive heavy chain gene rearrangement these cells give rise to a $CD45R^+$ c-kit$^-$ $CD25^+$ population (large pre-B-II), which is heterogeneous with respect to CD43 expression. Large preB-II cells subsequently give rise to a resting, $CD43^-$ population (small pre-B-II), in which most immunoglobulin light-chain gene rearrangement occurs (Fig. 1).

B cells are absent from the spleens of homozygous E2A-deficient ($E2A^{-/-}$) mice, and in bone marrow the percentage of $CD45R^+$ $CD43^-$ cells is reduced at least ten-fold. This is consistent with a block in development at or before the transition from pro-B/pre-B-I cells to large pre-B-II cells (Fig. 1). Immunoglobulin gene rearrangements are undetectable in the fetal liver or bone marrow of $E2A^{-/-}$ mice, indicating a failure of B-cell development to progress to the pre-B cell stage. Interestingly, B-cell development is blocked at a similar stage in immunoglobulin heavy chain deficient mice, and in mice carrying homozygous disruptions of the recombination activating genes, RAG-1 or RAG-2 [12,13]. In contrast, the effect of E2A disruption on T-cell development seems to be minimal. Modest alterations in the ratios of intrathymic T-lymphoid subsets are observed in $E2A^{-/-}$ mice, beginning several weeks after birth. These changes may, however, be a secondary effect of the B-cell deficiency.

What is the nature of the E2A requirement at this developmental stage? The available evidence is consistent with possible effects on transcription and immunoglobulin gene rearrangement. Several B-lineage-specific transcripts, including those for RAG-1 and the transcriptional activator pax-5, are substantially reduced in $E2A^{-/-}$ mice [1]. Assuming that the absence of these transcripts does not merely reflect reduced cell number (which remains a possibility), diminished expression of these genes — and perhaps others — could contribute to the observed developmental defect. As an example of the possible ramifications of E2A inactivation, consider Pax-5, itself a transcription factor whose production in adult mice is restricted to the B lineage and testes [14]. Pax-5 has been implicated as a positive regulator of several B-cell-specific genes. Remarkably, homozygous disruption of the pax-5 gene results in a B-lymphoid developmental arrest similar to that seen in E2A knockout mice: B cells are absent or greatly diminished, and large $CD45R^+$ $CD43^+$ B-cell progenitors are detectable in the bone marrow but fail to progress to the small $CD43^-$ stage [3]. In contrast to the $E2A^{-/-}$ mice, the pax-5 knockout mice exhibit a low but detectable level of immunoglobulin μ gene rearrangements, suggesting that Pax-5 normally acts downstream of E2A. Nonetheless, it remains possible that diminished expression of pax-5 plays a role in determining the E2A-deficient phenotype.

A link between the inactivation of E2A and failure to rearrange immunoglobulin genes could stem from effects on the production of RAG proteins, which are essential for immunoglobulin gene assembly, or on the accessibility of the immunoglobulin heavy chain locus to the recombinase machinery. As the transcription and assembly of immunoglobulin genes share common elements of control, E2A might normally potentiate rearrangement through its effects on the heavy chain intronic enhancer. In normal mice, unrearranged heavy chain loci give rise to two transcripts, μ^0 and I_μ, which are initiated upstream of the J_H cluster and within the intronic immunoglobulin μ enhancer, respectively. In the fetal livers of $E2A^{-/-}$ mice, μ^0 and I_μ transcripts are both
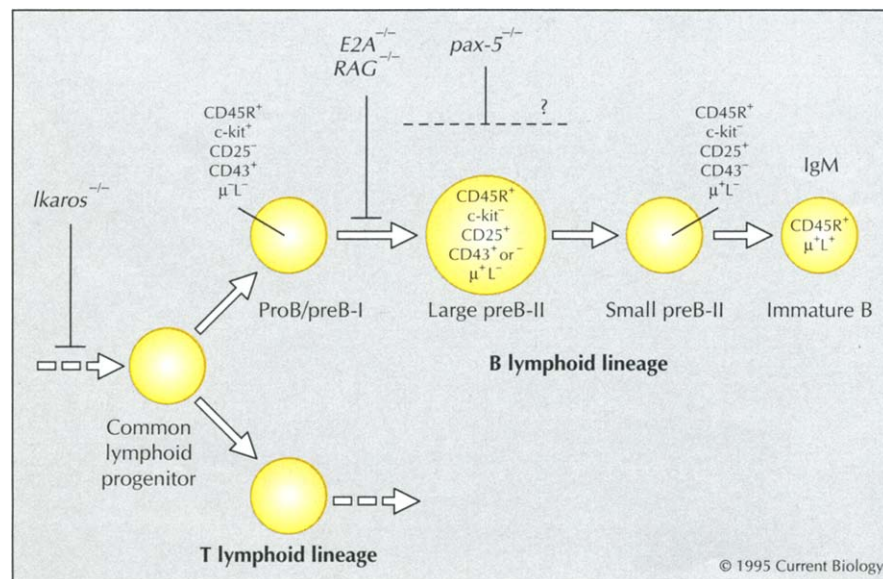
Fig. 1. Blockade of B-cell development by inactivation of the E2A or pax-5 genes. B-cell developmental stages are depicted according to the nomenclature of Melchers and coworkers [11]. Developmental blocks associated with inactivation of Ikaros ($Ikaros^{-/-}$), E2A ($E2A^{-/-}$), RAG-1 or RAG-2 ($RAG^{-/-}$) and pax-5 ($pax-5^{-/-}$) are indicated. The position of the $E2A^{-/-}$ developmental block is based on the absence of immunoglobulin heavy chain gene rearrangements and $CD45R^+$ $CD43^-$ bone marrow cells in homozygous E2A-deficient mice. The position of the $pax-5^{-/-}$ effect is less clear; the published data are consistent with a developmental block at the transition from pro-B/pre-B-I cells to large pre-B-II cells, or within the large pre-B-II stage.

reduced but the reduction in I_μ RNA is far greater, suggesting that E2A proteins are specifically required for I_μ transcription [1]. As the transcription of immunoglobulin loci is correlated with their ability to undergo rearrangement, the lack of I_μ transcription may also reflect inaccessability of the immunoglobulin μ locus to the recombinase machinery; in this scheme, the resulting failure to express immunoglobulin μ would be an immediate cause of the developmental block.

Remarkably, heterozygous $E2A^{+/-}$ embryos have about half as many B-lymphoid cells in their fetal livers as wild-type littermates [2]. In principle, random inactivation of one E2A allele could produce such a phenotype, but this mechanism is considered unlikely because in transgenic animals the E2A promoter remains active in the majority of B cells [2]. Positive autoregulation of E2A expression could also provide an explanation, but this also seems unlikely because E2A RNA levels seem to correspond to gene dosage. Zhuang and coworkers [2], rather, favor a threshold effect, effected by the inhibitory proteins Id1 or Id2, which are normally expressed in pro-B cells but which are down-regulated later in B-cell development. The Id proteins form inactive heterodimers with bHLH proteins [15]; thus, if Id1 or Id2 were to mop up any E2A protein below a certain threshold level, this could account for the observations in $E2A^{+/-}$ heterozygotes. The ability of Id1 to interfere with E2A function is underscored by an experiment in which constitutive Id1 gene expression was targeted to the B lineage in transgenic mice [16]. Under these conditions, B-cell development fails to progress to the $CD45R^+ CD43^-$ pre-B-cell stage — a defect reminiscent of the E2A-deficient phenotype and consistent with inhibition of E2A by Id1.

Although the most striking effect of E2A inactivation is the B-cell developmental block, an additional defect is observed in $E2A^{-/-}$ mice — an apparent failure to thrive [2]. Among progeny of crosses between $E2A^{+/-}$ mice, Zhuang and colleagues [2] observed that homozygous $E2A^{-/-}$ progeny were represented normally (about 1/4) in embryos at embryonic day 18.5, but were under-represented (about 1/10) by one week of age. Postnatal death in $E2A^{-/-}$ mice is accompanied by cessation of growth, weight loss and dehydration. The differential rate of growth between homozygous and heterozygous littermates is apparently not a result of a failure to feed, nor does the B-cell deficiency alone seem to be responsible, as sterile care did not overcome this defect. Nonetheless, E2A is clearly inessential for determination of most cell types and for embryonic development. The results are consistent with compensatory effects in those processes for which E2A is not essential (perhaps through the action of homologous genes such as E2-2), and imply that a lack of E2A cannot be compensated for in the B lineage.

In contrast, inactivation of the p50 component of the NFκB transcription factor illustrates that the phenotypes of knockout mice cannot be predicted from in vitro studies [4]. NFκB is a ubiquitous, heterodimeric factor comprising p50 and p65 subunits. Despite its broad expression pattern, NFκB DNA-binding activity is undetectable in most cell types. A low level of constitutive activity is detectable in B-lymphoid cells, however, and this activity is substantially increased in response to a variety of stimuli, including exposure to bacterial lipopolysaccharide and engagement of surface immunoglobulin. Induction of NFκB activity involves dissociation of the p50–p65 heterodimer from an inhibitor, IκB, and translocation of the heterodimer to the nucleus. Target genes that are regulated by NFκB include those for immunoglobulin κ, the type I and II major histocompatibility complex proteins and a number of cytokines (including IL–1β, IL–2, IL–6, IFN–β and TNF–α).

NFκB is one of several related transcription factors that can be assembled from products of the NFκB/rel gene family. This family includes NFκB-1 (encoding the p105 precursor of p50), NFκB-2 (encoding the p100 precursor of a 52 kD homologue of p50), relA (encoding p65), c-rel and relB. All of these polypeptides can bind as homodimers or heterodimers to consensus κB sequence motifs, and all have been shown to affect transcription of κB-containing reporter constructs positively or negatively in transfection experiments. Previous work had indicated an ordered appearance of NFκB/Rel complexes during B-cell development: p50 and p65 dominate in pre-B and non-B cells; p50 and Rel in mature B cells; and p52 and RelB in plasma cells ([8] and references therein).

Despite a large body of correlative evidence suggesting a critical role for p50 in early B-cell development, homozygous disruption of the p50 gene failed to perturb the development of B or T cells [4]. The relB gene has also been disrupted recently, without discernable effects on lymphocyte development; relB-deficient mice do, however, show a striking reduction in the numbers of dendritic cells in the thymic medulla [17,18]. On the other hand, p50 is clearly essential for normal immune responses: $p50^{-/-}$ mice exhibit a failure of B-cell proliferation in response to lipopolysaccharide, diminished production of non-M immunoglobulin isotypes, diminished T-cell-dependent production of immunoglobulins, and failure of T-cell proliferation in response to crosslinking of the T-cell antigen receptor to the co-stimulatory molecule CD28 [4]. Overall, the phenotype of p50-deficient mice would have been difficult to predict from previous studies.

The role of E2A in hematopoiesis can be contrasted with those of two other transcription factors, PU.1 and Ikaros. Inactivation of PU.1 results in ablation of the granulocytic, monocytic and lymphoid lineages, but leaves megakaryoid and erythroid development intact [19]. PU.1 may act, therefore, at the level of a multipotent granulocytic, lymphoid and myeloid progenitor cell. Inactivation of Ikaros ablates the B- and T-lymphoid compartments, but spares the development of all other haematopoietic lineages [20]. This suggests that Ikaros acts downstream of PU.1 to promote the differentiation

of a multipotent progenitor into a component of the lymphocyte pathways (Fig. 1). Both PU.1 and Ikaros seem to act earlier in hematopoiesis than E2A, whose disruption affects only the B lineage. As B- and T-cell development are similar in many details, it will be interesting to see whether there is a protein that, like E2A, acts downstream of Ikaros but which is required selectively for T-cell development.

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