Novel Developmental, Cellular and Biochemical Functions of Fucosylated Glycans In Mammals

by

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Dedication

To My Parents and Xiangfei

Acknowledgments

"If it wasn't hard, everyone would do it. It's the hard that makes it great."

I would like to thank my mentor, Dr. John Lowe first. In the past four years, and the best years in my life, I have had this opportunity to work in his lab and learned how to think about science from him. Although it was saying that ideas are cheap, I've found that it would have been impossible for me to finish my thesis work without his tremendous amount of advice and support on a daily basis. In Dr. Lowe's lab, I've exposed to the independence and freedom of scientific research from the first day together with the support from the senior post-doctoral scholars and technicians. I am also grateful for the options John ever gave me on the way to finish this exciting yet painful work in my curiosity towards this developing story. Personally, I really appreciate that John and his wife Kim have taken me as one of their daughters for years, and they have been with me in the best time and worst. I would not have been enjoyed my life as much without their love.

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List of abbreviations

ADAM	A disintegrin and metalloprotease
ATCC	American Type Culture Collection
BM	Bone Marrow
CL17	Clone 17
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
Dll	Delta Like
DN	Double Negative
DNMAML	Dominant Negative form of M
DP	Double Positive
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescence Protein
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-Linked Immuno Sorbent Assay
ER	Endoplasmic reticulum
ETP	Early T cell progenitor
FCS	Fetal Calf Serum
Flt3	FMS-like tyrosine kinase 3
FUT	Fucosyltransferase Gene
FX	3,5 epimerase/4-reductase
GalNAc	N-acetylgalactosamine
GATA-3	GATA binding protein
GDP	Guanosine diphosphate
GMD	GDP-mannose 4,6-dehydratase
GMP	Granulocyte/macrophage lineage-restricted progenitors
HES	Hairy and enhancer of split
hIgG	Human Immunoglobulin G
HSC	Hematopoietic Stem Cell
ICN	Intracellular domain of Notch
IL-7	Interleukin 7
IMDM	Iscove's Modified Dulbecco's Medium
IRES	Internal ribosome entry site
LAD	Leukocyte adhesion deficiency
LSK	Lineage- Sca-1+ c-Kit+
MAP-kinase	Mitogen-activated protein kinase
MEF	Mouse Embryonic Fibroblast
MEP	Megakaryocyte/erythrocyte lineage-restricted progenitors
NADP	Nicotinamide adenine dinucleotide phosphate
NCBI	The National Center for Biotechnology Information
NIH	National Health Institute
NK	Natural killer
NOD	Non-Obese Diabetic Mice

Notch EC	Notch extracellular domain
Notch IC	Notch intracellular domain
PCR	Polymerase chain reaction
POFUT1	Protein O-fucosyltransferase 1
PSA	Pisum sativum agglutinin
PSGL-1	P-selectin glycoprotein ligand 1
RAG	Recombination Activating Gene
SP	Single positive

Abstract

Cell surface glycans are branched structures composed of linear and branched chains of monosaccharides that include fucose. Fucose has important biological functions during ontogeny and cellular differentiation, suggested by pathological phenotypes observed in different strains of mice, including mice with a null mutation in the 3', 5'-epimerase/4'-reductase locus (FX-/- mice). FX-/- mice are conditionally deficient in all fucosylated glycans, and exhibit server thymic atrophy. This phenotype is cell autonomous and characterized by a fucose-dependent complete deficiency of mature T cell with loss of early thymic progenitors. Interestingly, Notch1 signaling deficient mouse models have been reported with the similar phenotypes, and there are reports that O-fucosylation is required in some in vitro experiments for Notch1 signaling. These results strongly suggested that lack of Notch1 signaling in FX-/- progenitors accounts for the thymic atrophy phenotype. By introducing the Notch1 intracellular domain into fucose-deficient FX-/- bone marrow-derived lymphoid progenitors, the T cell developmental defect characteristic of FX-/- lymphoid progenitors is rescued and thus implicated a fucose-dependent requirement for Notch1 signaling in this process. *In vitro*, OP9 cells that are bearing Notch ligand of Delta-like1 (Dll-1), Delta-like 4 (Dll-4) or Jagged2, instruct cells to assume a T lymphoid differentiation identity, whereas while OP9 cells bearing Jagged1 or Dll3 do not. However, in fucose-deficient experimental situation, FX-/- bone marrow cells fail to assume a T lineage identity when co-cultured with OP9-Dll1, OP9-Dll4 or OP9-Jagged2, and fail to initiate Notch1 signaling events. These results indicate that fucosylation is required for Notch1 signaling-dependent T cell differentiation. In effort to define the molecular mechanisms that account for fucosylation-controlled Notch1 signaling activation, I conclude

from a series of experiments that (1) fucosylation controls the strength of binding between Notch1 and its ligand Dll4, and (2) fucosylation controls Notch1 receptor density at the surface of an E2a/Pbx1 immortalized bone marrow progenitor cell line. These results indicate that fucosylation controls Notch1 signaling strength and thus regulates the development of T lymphocytes. My studies have revealed novel biochemical, cellular and developmental functions of fucosylation in the development and signaling pathways characteristic of the mammalian lymphoid lineage.

Chapter I

Introduction

Although complex carbohydrate research began in the nineteenth centuries, it was not until the last fifty years that the field has received substantial experimental attention from biologists and biochemists. Together with nucleic acids, proteins, and lipids as the main components of biological system, complex carbohydrates are linear and branched chain sugar molecules found in most eukaryotic cells and organisms, including mammals, and are expressed through ontogeny to adulthood. Glycan structures vary in complex tissue-specific and developmentally regulated expression patterns. At the molecular level, complex carbohydrates are primarily found covalently linked to proteins or to lipids, which has strongly suggested that sugar structures are involved in the regulation of biological systems. Recently, the term glycobiology has been used to refer to structure, biosynthesis and biology of saccharide chains that are widely distributed in nature (1).

The structures of glycans have been extensively studied with the emergence of modern analytic methods such as mass spectrometry. The basic unit of carbohydrates is called a monosaccharide that cannot be hydrolyzed into a simpler unit. These units include widely known sugar molecules like glucose, fucose and mannose. Monosaccharides in ring forms utilize potential carbonyl groups at the end of the carbon chain or at an inner carbon to be linked together. This results in the formation of oligosaccharides, comprised of linear and branched chains of monosaccharides. Polysaccharides usually refer to longer and more complex forms composed by multiple oligosaccharides. Unlike nucleic acids and proteins

that are composed of linear structures, glycans display enormous structural complexity and combinatorial diversity with combination of different branches (1).

When polysaccharides are linked to a noncarbohydrate moiety (usually proteins), the resulting structures are termed as glycoconjugates. Glycoproteins are proteins with sugar molecules attached either in a N-linked or in an O-linked fashion. The most common form of polysaccharides on proteins are N-linked glycans, in which an N-acetylglucosamine (GlcNAc) is linked to some asparagine residues of membrane and secreted proteins (2, 3). O-glycans are linked to serine or threonine residues of proteins via typically N-acetylgalactosamine (GalNAc) residues (4-6). They are also synthesized in the secretory pathway with diversification steps leading to linear and branched O-glycan structures (7). In addition to GalNAc, fucose, glucose and mannose may also be linked directly to come serine/threonine residues to form the base of other oligosaccharide during modifications (8-10).

Biosynthesis of glycoconjugates is typically associated with components of the protein synthesis pathway as occurs in different compartments of the cell (11, 12). The synthetic pathway contains not only elongations of the glycans but also modifications and even shortening. As one example, Figure 1-1a shows the synthesis of a branched N-linked glycan structure. This synthetic pathway of oligosaccharide assembly is highly ordered, so that each step shown is fully dependent on the previous one. Initially N-linked glycans arise in the endoplsmic reticulum (ER) of the early secretory pathway (13). A previously synthesized core structure containing of 14 monosaccharides including two GlcNAc, nine mannose residues and three glucose residues are added in a co-translational fashion to newly synthesized proteins. During maturation, three different enzymes, (glucosidase I, glucosidase II and ER mannosidase), modify the structure with cleavage of all three glucose residues and one mannose residue, resulting in a 10-unit structure. Up to this stage of protein synthesis, the variations of oligosaccharide structures are relatively limited. When synthesis moves into the Golgi lumen, more extensive modifications apply to the structure

resulting in very different structures depending on the set of enzymes expressed in a specific cell type (13, 14).

One of the most well known O-linked glycan structure is shown in Figure 1-1b, as an example of linear oligosaccharides. The Epidermal Growth Factor (EGF) repeat is a component of many proteins and contains serine and threonine residues that are enzymes that may be covalently modified by a fucose molecule (15) by protein-O-fucosyltransferase 1 (16, 17). The Fringe molecules are enzymes that catalyze the attachment of GlcNAc to O-fucose (18). This glycan structure is further elongated by catalytic activities of β-4-Gal Transferase 1 (19) and either α-2,3-sialic acid transferase (shown in Figure 1-1b) or α-2,6-sialic acid transferase (15, 18). This synthetic pathway is, like the biosynthesis of proteins characterized by a series of highly ordered linear single molecule additions.

The structural characteristics of polysaccharides potentially allow them to carry more biological information than linear forms of nucleic acids and proteins (1). This is because that 1) polysaccharides are both linear and branched molecules, and 2) there are many distinct monosaccharides that contribute as components of polysaccharides. Nonetheless, the functional roles of glycans have remained enigmatic relative to the more extensively investigated nucleic acids and proteins. In the past decade, glycans have been discovered to serve as intermediates in protein folding (13) and cellular interactions (20), and as structural components of the cells and cell matrix (21). For example, alteration of glycosylation has been observed in cancer (22), ulcerative colitis (23), and cystic fibrosis (24). Recently, important functional roles have been ascribed to glycans in different signaling pathways. For example, O-fucosylated glycans have been shown to be involved in Notch signaling (25), which relates to many developmental process in mammals (26-29). However, it has not yet been shown if fucosylation is required for mammalian development, including the development of the immune system where Notch function is required (30, 31).

My thesis work emphasizes the novel physiological, cellular, and biochemical roles of fucosylated glycans in mammalian development, specifically in the development of T lymphocytes in the immune system. In this introduction a few key concepts, including the fucosylated glycans, Notch signaling and immune development, are briefly explained.

Fucosylated glycans

L-fucose, also known as 6-deoxy-L-galactose, is a monosaccharide commonly seen in many glycan structures from Drosophila to humans (32-35). The L-configuration and lack of a hydroxyl group on the carbon 6-position distinguish it from other small sugars. Fucose exits primarily as a terminal modification of N-glycans. Recent O-glycan structures have been found in which fucose is direct linked to serine/threonine residues (15). Numerous fucosylated glycan structures exist, and vary depending on the position of fucose added to proteins and/or oligosaccharides (Figure 1-2). N-linked fucose can exit as a terminal or subterminal modification of glycans. O-linked fucose is directly added to proteins via serine or threonine residues. Like other monosaccharides, fucose is added to glycans or proteins by fucosyltransferases, enzymes that use GDP-fucose as the nucleotide sugar donor, linking to specific acceptors (Top of Figure 1-2). The catalytic domain of fucosyltransferases typically reside in the lumen of the Golgi lumen or endoplastic reticulum (ER) (36). Many fucosyltransferases display a pattern of tissue-specific regulation, suggesting important roles for fucosylated glycans in specific tissues or cell types.

The biological significance of fucosylated glycans include the following:

(a) ABO blood group (37).

The fucosyltransferase genes FUT1 and FUT2 encode the H transferase and the Secretor (Se) transferase, respectively. The H transferase is expressed in erythroid precursors while the Se transferase is expressed in epithelial tissues and salivary glands. They are both responsible for synthesis of a specific fucosylated glycan structure (H antigen) with fucose added in a $\alpha(1,2)$ fashion to the terminal galactose residue (Figure 1-2). Humans whose red blood cells express only H antigen found are blood type O. H antigen can be modified further by the A transferase (an N-acteylgalactosaminyltransferase) or the B transferase (an galactosyltransferase) to form A or B antigen. Humans whose red blood cells express only A antigen are blood type A, while individuals with red cells bearing only the B antigen are blood type B. Blood type AB individuals have both A and B antigens on their red blood cells.

Mixing blood from two individuals can cause blood clumping or agglutination. Big clumps of red cells can clog blood vessels and stop the circulation of the blood to various parts of the body. The clumped red cells may burst and its contents leak out in the body. Hemoglobin outside the cells becomes toxic to the body and resulted effects may be fatal. Thus, identification of an individual's blood type is essential when blood transfusion is required. It's necessary to ensure that blood transfusion recipients receive ABO compatible donor red cells. This compatibility study initiated the modern transfusion medicine.

Lots of studies have shown the susceptibility of individuals with different blood types is different in many diseases. These studies include associations of blood type A with gastric cancer (38), blood type A and B with gastric atrophy (39), non-O group with increased severity of fibrosis in chronic hepatitis C infection (40), blood type O with low Factor VIII and von Willebrand factor expressed in blood that can lead to excess bleeding (41), non-O group with increased Factor VIII and von Willebrand factor expressed in blood that increase the risk of ischaemic heart disease and venous thromboembolic disease (41). For example, a bacteria strain of Helicobacter pylori is associated with the development of

gastritis, gastric ulcers and adenocarcinomas in humans. Bacterium Helicobacter pylori binds preferentially to Lewisb antigens found on the surface of gastric epithelial cells especially in stomach (42). Lewisb antigens are part of the blood group antigens that determine blood group O. However, the bacteria do not attach to the structure of blood type A antigen (42). Moreover, this result confirmed the epidemiological study decades ago that showed association of blood type O with gastric ulceration (43).

(b) Host-microbe interaction.

The microbial pathogen Helicobacter pylori can attach to host epithelial cells in the stomach by using a Lewisb glycan structure (determined by FUT2 and FUT3 product) (44). A and B blood group individuals have shown decreased peptic ulcer disease incidence that correlates with A antigen expression, which masks the fucosylated Lewisb structure (45). In mammalian experimental systems, overexpression of the FUT3 gene leads to Lewisb expression in non-Lewisb expressing cells, resulting in an increased severity of gastritis by Helicobacter pylori exposure (46). Furthermore, the bacteria itself express the Lewisb structure and related glycans (47), and the expression of these structures increase at acid pH similar to the gastric environment (48). Taken together, these observations indicate that fucosylated glycans are important for host-microbe interactions.

(c) Selectin-dependent leukocyte adhesion.

Selectins are among the best-studied glycoproteins that are expressed on the cell surface. Three types of selectins have been found: P-selectin expressed on platelets and endothelial cells, E-selectin expressed by endothelial cells and L-selectin found on most of leukocytes (49). During the process whereby leukocytes are recruited from the vascular compartment to an inflammatory site, or in trafficking to lymph nodes, an initial step involves the deceleration of the rapidly moving leukocytes by a process called rolling, mediated by selectin and their counter-receptors, so that they may then firmly attach to the

endothelial wall of the vessels followed by migration to the extravascular compartment (49). Selectin ligands are modified with terminal fucose by the product of the FUT4 and FUT7 fucosyltransferase loci, two α(1,3) fucosyltransferases (50, 51). Study of genetically engineered mice deficient in FUT4 and/or FUT7 demonstrate that α(1,3)-fucosylated glycans are essential for selectin-ligand interaction and related cell trafficking (52, 53).

(d) Signal transduction in development.

Fucosylated glycans are found on many proteins involved in cell signaling events during development. These include well-studied Notch receptors in Notch signaling (15), the cripto-1 receptor in Tumor Growth Factor (TGF)-beta signaling (54) and urokinase-type plasminogen activator as a growth factor (55) in mammalian development. Fucose is added to these proteins in a O-linked fashion by protein O-fucosyltransferase 1 (16). This enzyme adds fucose directly to serine/threonine residues in epidermal factor like (EGF) domains (56). Abolishing fucosylated glycans of urokinase-type plasminogen activator inactivates its mitogenic activity without disturbing the cell surface binding ability (55). Inactivation of the protein O-fucosyltransferase 1 gene results in the absence of O-fucosylated glycans on EGF domains, a failure of embryonic development (57), dysfunction of Notch signaling (58). Interestingly, absence of POFUT1 leaves cripto-related TGF-beta signaling intact (54). These observations indicate that fucosylated glycans play specific and essential roles in particular signaling pathways during development.

Notch1 signaling pathway

The Notch signaling pathway was first studied in Drosophila melanogaster and this pathway plays a role in lineage decision of neural precursor cells, directing them to become either neuroblasts or neuroepidermal cells (59-62). When Notch is overexpressed in

precursor cells, an increase of neuroepidermal cells has been observed at the expense of neuroblasts. By contrast, lack of functional Notch protein yields increased numbers of neuroblasts at the expense of neuroepidermal cells. These observations indicate that the Notch signaling pathway plays an important role in lineage decisions during development. Similar functions of the Notch signaling pathway have been demonstrated in other developmental systems.

The Notch signaling pathway includes a family of highly conserved cell surface receptors (Notch proteins) on signaling receiving cells, and a set of structurally conserved cell surface ligands (Notch ligands) on signaling sending cells. In Drosophila, there is one Notch receptor, and two Notch ligands (Serrate and Delta). However, mammals possess four different Notch receptors (Notch 1, 2, 3, 4) and five different Notch ligands (Jagged1, 2, Delta-like 1, 3, 4), suggesting much more complex regulations in mammalian species.

Notch-dependent signal transduction is quite similar in flies and in mammals. As shown in Figure 1-5, mouse Notch1 is synthesized as a large polypeptide precursor, in ways similar to most cell surface transmembrane proteins that originate on the ER membrane. During this process, Notch1 receptors are also fucosylated on some serine and threonine residues. Fucosylated Notch1 proteins then move to the Golgi for further modifications. Within the Golgi, Notch1 proteins are cleaved by a Furin-like protease, and thus form heterodimer, and eventually are presented on the cell surface.

Interactions between Notch and its ligand(s) are characterized by a series of subsequent proteolytic cleavages that are required for downstream signal transduction events. In Drosophila, the force applied by a Notch ligand to Notch extracellular domain (EC) has been shown to be essential to change the protein structure of Notch so that the ADAM (a distintegrin and metalloprotease) protease is recruited to cleave, and release the extracellular portion of Notch. Subsequently, γ-secretase cleaves the Notch intracellular domain (Notch IC) at a site near or within the cell membrane. Notch IC, a 120 KD

polypeptide, then travels to the nucleus, where it displaces a transcriptional suppressor and then forms a multi-protein complex to initiate downstream gene expression.

Unlike the MAP-Kinase signaling pathway, Notch signaling is without multiple cascades of protein phosphorylation and interactions before the downstream targets are transcribed in nucleus. Once Notch IC is cleaved from the anchored receptor, it directly travels into nucleus and the downstream genes are activated. There are few intermediates involved in the Notch signaling transduction pathway once the ICN is cleaved. However, many of the direct target genes activated by Notch are transcription factor genes, such as Hes-1 and Hes-6, which themselves are able to activate other target genes. These considerations imply that Notch signal transduction can yield very complicated gene activation cascades after direct targets were transcribed, and may affect as many as several hundred genes.

Notch signaling has been implicated in many developmental processes, including in neurogenesis (26), somite formation (63), eye development (64), inner ear and hair cell development (29), blood vessel formation (65), cardiogenesis (66), and very importantly, immune cell differentiation (67). For example, in fetal lung development, Notch signaling seems to be essential for lung size control, because losing Notch downstream target Hes1 expression in mouse resulted in 20% decrease of lung size compared to wild type littermates (68). Jagged-mediated Notch signaling maintains proliferating neural progenitors and regulates cell diversity in the ventral spinal cord (69).

Aberrant Notch signaling is associated with, or causes many diseases, including breast cancer (70), lung cancer (71), and leukemia (72). Transgenic mice that ectopically express the intracellular domain of Notch4 (designated as INT-3) develop mammary gland tumors (73). A truncated transcript derived from the human Notch4 gene (h-Int-3sh) expressed in human breast cancer cell line of MCF-10A (74). These observations indicated the association of Notch4 to breast cancer.

A truncated Notch1, named as TAN1 (translocation-associated Notch homologue 1), is highly associated with T-cell acute lymphoblastic leukemia (T-ALL) patients, and formed by a chromosome translocation (75). TAN1 transduced mouse hematopoietic stem cells developed T-ALL after being transplanted in vivo (76), indicating its tumorgenic effects. Interestingly, immature TAN1 expressing T cells were found in the bone marrow, where they should not reside. These results indicate that the activation of Notch1 signaling promote T cell development. Conditional Notch1 knockout mice loss mature T cells in thymus (77), with B cell populations are enriched (78). These results suggested that the lymphoid progenitors adopt B cell fate in the absence of Notch1 signaling. Above observations indicated that Notch1 signaling plays an essential role in T lineage decision.

Immune system and hematopoiesis

The components of our immune system include cells within the bone marrow, thymus, spleen, lymph nodes, blood and the mucosal-associated lymphoid tissues (79). The bone marrow is the site of origination of the cellular components of immune system including B and T lymphocytes, and natural killer (NK) cells (80). The thymus is an organ located in the upper part of the mediastinum, behind the sternum, extending upwards into the root of the neck. The function of thymus is specialized for the development of T lymphocytes and other types of lymphocytes, which contribute to the adaptive immune system (81). The spleen is located in the upper-left part of the abdomen. It functions primarily to surveil for and collect antigens from the blood and to dispose of senescent red blood cells (82). Lymph nodes are located where the vessels of the lymphatic system converge. These secondary lymphoid organs contain both B and T lymphocytes that, together with dendritic cells, allow mammals to respond to foreign antigens (83). Blood enables the circulation of red cells and leukocytes, and delivers these cells, and protein

components of the immune system, including antibodies to lymphoid organs, and to sites of inflammation and antigen challenge (84).

The developmental biology of the immune system includes hematopoiesis, the development of the cells of the blood (hematopoietic) cells (Figure 1-3). Different blood cell types are categorized by their shapes, cell surface markers, properties on staining, and by their functions. Hematopoietic cells develop from hematopoietic stem cells (HSCs) in the bone marrow (85). HSCs are capable of self-renewal, can differentiate into more mature cells, and can reconstitute the hamatopoietic system in of irradiated recipients (86). HSCs can be identified with cell surface markers by antibody staining, are negative for all mature lineage (Lin-) markers, and are positive for Sca-1 and c-Kit proteins (Sca-1+, c-Kit+) (87). Typically they are within a population of cells termed LSKs, standing for Lin-, Sca-1+, c-Kit+. Long-term HSCs (Flt-3- LSKs) are capable of hematopoietic reconstitution for months after the bone marrow transplantation (88), while short-term HSCs (Flt-3+ LSK) are capable of conferring transient (a few weeks) bone marrow reconstitution (89). Both HSC populations are capable of generating all mature lineages including myeloid and the lymphoid cells (86, 90).

Cells of the myeloid lineage are thought to derive from a common myeloid progenitor (CMP) derived from hematopoietic stem cells (91). These cells are lineage negative, IL-7 Receptor-alpha negative, Sca-1 negative, c-Kit positive, Fc-gamma receptor negative and CD34 positive (91). CMPs give rise to granulocyte/macrophage lineage-restricted progenitors (GMPs) and megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs) (91). GMPs and MEPs then differentiate into various specific cell types within the myeloid lineage, including monocytes and neutrophils (GMPs) and red cells and platelets (MEPs).

Lymphocytes are a class of leukocytes that mediate the adaptive immune response involving antigen-specific reactions (79). In humans and mice, there are two main populations, B lymphocytes and T lymphocytes that share common lymphoid progenitors

(CLPs) in the bone marrow (92) (Figure 1-3). During lineage decisions in the lymphoid compartment, CLPs may be directed to assume a B lymphocyte phenotype in the bone marrow. Alternatively, CLPs and/or their later progeny may migrate out of bone marrow and develop into mature T thymocytes in thymus.

Our knowledge of T lymphocyte development is extensive, yet still, incomplete. It is widely accepted that once T cell progenitors arrive in the thymus, they are instructed to progress through different developmental stages, beginning with a double negative (DN) phenotype (CD4- CD8a-), followed acquisition of CD4 and CD8a markers as the more mature double positive (DP) phenotype (Figure 1-4). Later, upon different selection signals provided by the thymic stromal cells, DP thymocytes will differentiate into one of two types of single positive (SPs, CD4+CD8a- or CD4-CD8a+) T cells, which are then exported to the blood stream, where the assure their immune functions. Our understanding of developmental processes in the thymus, blood, and marrow, proximal to the DN stage, is incomplete.

Fucosylation, Notch signaling and the immune system

Notch receptors are large proteins with 36 epidermal growth factor-like (EGF) repeats containing serine and threonine residues, some of which are modified by O-linked fucose, and extended O-linked fucosylated glycans (15, 18). In Drosophila, O-fucosylated glycans on Notch receptor are thought to be essential for Notch-dependent signal transduction (25). In mammalian cells, lack of O-linked fucosylation on Notch1 has the effect of reducing downstream signaling in the Notch pathways (58). These observations imply that fucosylation is important in Notch signal transduction.

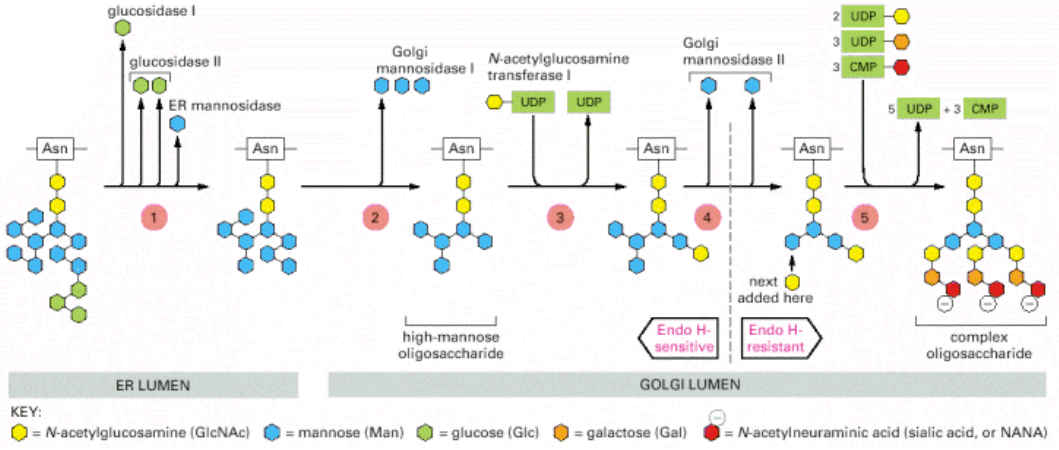
Notch receptors and Notch ligands are widely distributed in different cell types during hematopoietic development and differentiation (Figure 1-6). As Notch signaling is

known to be critical for T cell development (77), it is very likely that O-linked fucosylation controls T cell development through modulating Notch1 signaling strength. Thus the important roles of fucosylated glycans in T cell development were hypothesized in this thesis. In principle, the hypothesis that O-fucosylation and T cell development are linked, could be examined by inactivating O-fucosyltransferase 1 in mice and ask if T cell developed in the animals. Unfortunately, as reported in 2005, POFUT1(-/-) mice are not able to grow to the age when T cells fully develop (57).

In Dr. Lowe's lab, a global-defucosylated mouse modal has been made in the effort to discover novel functions for fucosylated glycans, by deleting the locus encoding an enzyme termed FX (93). As I will described in subsequent chapters of this thesis, the FX(-/-) mouse can be maintained as an essentially wild type animal when reared on water or chow supplemented with fucose, yet sustains a global fucosylation deficiency when the mice are reared without fucose supplementation. We observed a thymic atrophy phenotype in FX(-/-) mice in fucose-deficient environment, suggesting an important physiological role for fucosylated glycans in T cell development in these mice (discussed in Chapter 2). This hypoplastic thymic atrophy phenotype is very similar to the phenotype observed in mice subjected to inactivation of Notch1 signaling (77). These observations suggested that the T cell developmental deficiency characteristic of the fucose-dependent thymic atrophy phenotype in FX(-/-) mice is due to a fucosylation-dependent loss of Notch1 signaling in their T cell progenitors. Experiments in Chapter III indicate that Notch1 downstream signaling is indeed fucose-dependent, and that generation of mature T cells is fucose-dependent. Moreover, biochemical and cell biology experiments reported in Chapter IV disclose that absence of O-linked fucosylation of Notch1 diminishes the ability of Notch ligands to interact with Notch1, and down-regulates cell surface expression of Notch1.

Figure 1-1

a



b

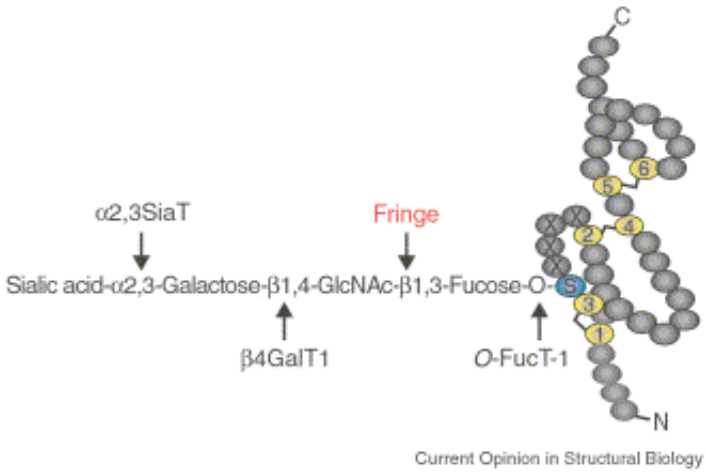


Figure 1-1 Examples of glycan biosynthesis and glycan structures. a. N-linked oligosaccharide processing in the ER and the Golgi apparatus. (Molecular Biology of the Cell, 4th Edition, Alberts B and et al, New York and London: Garland Science; c2002) b. Structure of O-linked glycans on an Epidermal Growth Factor repeat. (Haltiwanger RS, Current Opinions of Structural biology, 2002 Oct; 12(5): 593-8)

Figure 1-2

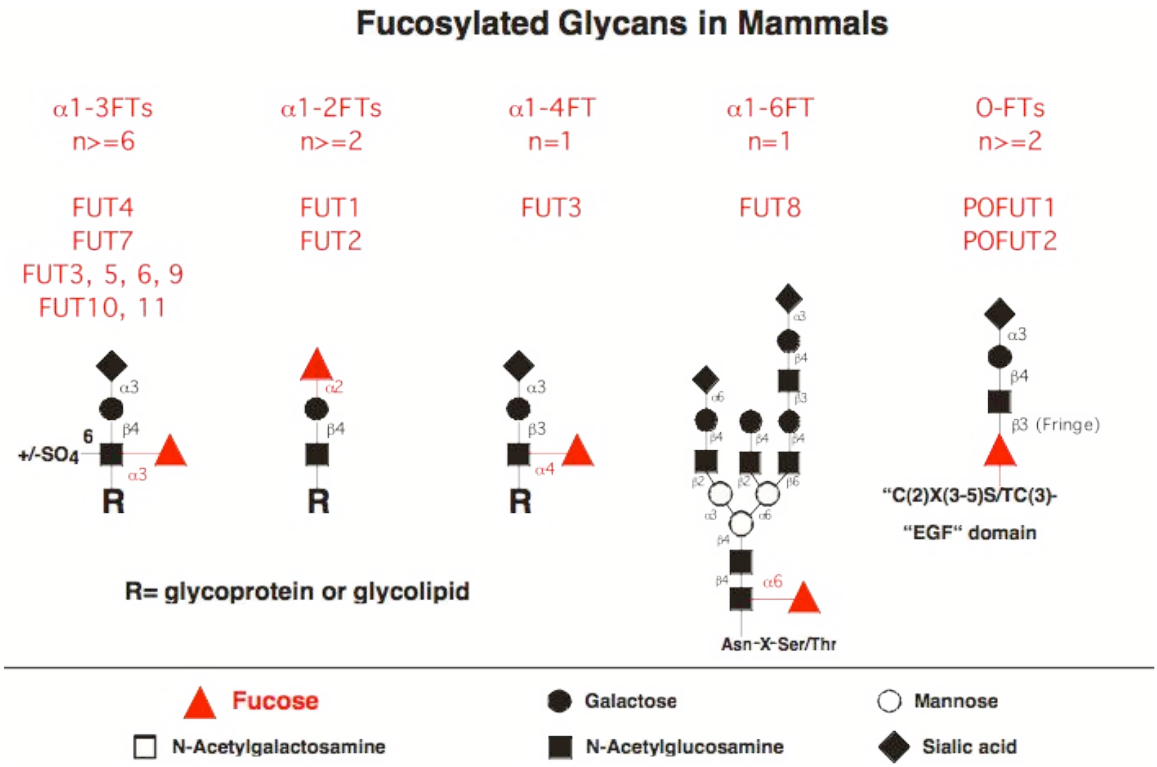


Figure 1-2: Fucosylated glycans in Mammals. Structures of selected fucosylated glycans are shown here. N=number of genes. (From Dr. John Lowe)

Figure 1-3

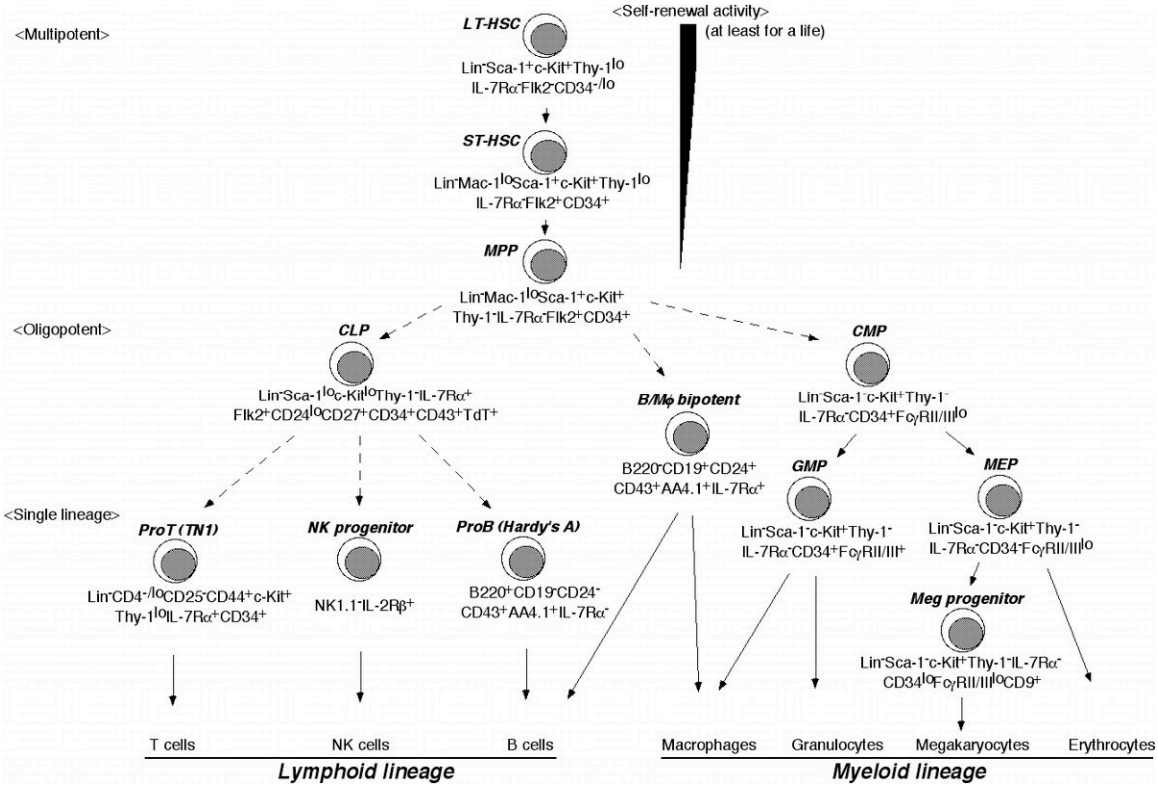


Figure 1-3: Conceptual hematopoietic trees in adult mice. Indicated cell populations can be purified based on the cell surface phenotype. Not all of the linear relationships in this figure have been proven. Multipotent progenitors (MPPs), at least at the population level, can differentiate into all types of hematopoietic cells, but have no detectable self-renewal potential in vivo. (Kondo M, et al. Annual Review of Immunology Vol. 21: 759-806, 2003)

Figure 1-4: Anatomical microenvironments in the adult thymus. The thymus is a lobed organ divided by mesenchymal septae. Lobes are organized into discrete cortical and medullary areas, each of which is characterized by the presence of particular stromal cell types, as well as thymocyte precursors at defined maturational stages. Thymocyte differentiation can be followed phenotypically by the expression of cell-surface markers, including CD4, CD8, CD44, CD25, CD69 and CD62L (Mel-14), as well as the status of the T-cell receptor (TCR). Interactions between thymocytes and thymic stromal cells are known to be important in driving a complex program of T-cell maturation in the thymus, which ultimately results in the generation of self-tolerant CD4+ helper and CD8+ cytotoxic T cells, which emigrate from the thymus to establish the peripheral T-cell pool. (4, CD4; 8, CD8; 44, CD44; 25, CD25; 69, CD69; TCRlow, expressing the TCR at low levels; TCRhi, expressing the TCR at high levels.) (Modified from Anderson G & Jenkinson EJ, Nature Reviews Immunology 1, 31-40, 2001)

Figure 1-4

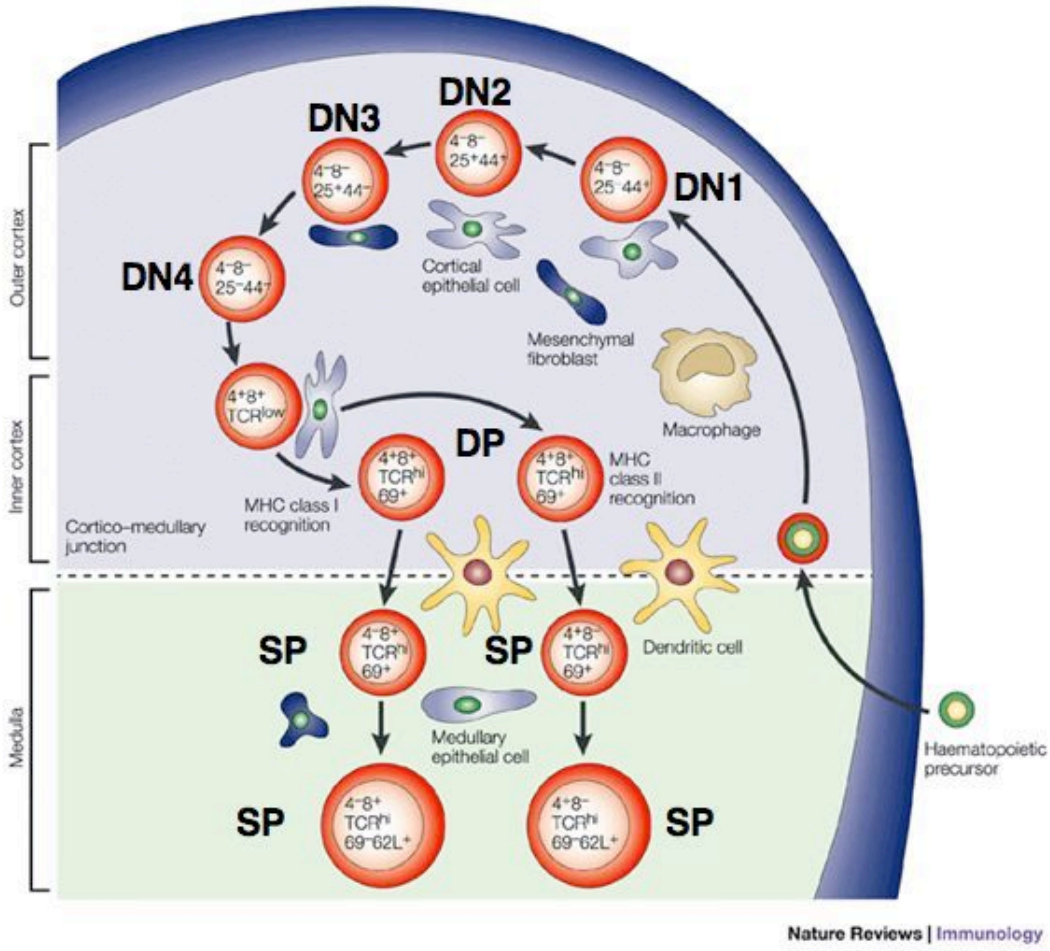

DN3
DN2
DN1
DN4
Outer cortex
Inner cortex
Medulla
Cortical epithelial cell
Mesenchymal fibroblast
Macrophage
DP
MHC class I recognition
MHC class II recognition
Cortico–medullary junction
SP
SP
SP
SP
Dendritic cell
Medullary epithelial cell
Haematopoietic precursor
Nature Reviews | Immunology

Figure 1-5: Notch expression and activation. Notch proteins are synthesized as a single peptide of ~300KDa. a. In the endoplasmic reticulum (ER), the Notch1 polypeptide is fucosylated by protein O-fucosyltransferase 1. b. Notch1 protein is shuttled to the trans-Golgi, where it is cleaved by a furin-like protease (S1-cleavage) to generate the non-covalently bounded heterodimerized surface receptor. c. The Notch1 heterodimer is on the plasma membrane where it is available to bind with its ligands on signal sending cells. d. The interaction with Notch ligand is induced by ADAM metalloproteinase TACE (S2 cleavage) close to the cell membrane. Notch1 extracellular domain will get into signal sending cells by endocytosis. e. S2 cleavage is followed by mono-ubiquitylation of the intracellular portion of the transmembrane Notch fragment. f. This results in endocytosis of the transmembrane fragment of the Notch protein, presumably facilitating cleavage by γ-secretase (S3 cleavage) on transmembrane portion. g. Intracellular Notch is released and translocated to nucleus, where it competes out binding of CSL to suppressor protein complex. After recruiting transcriptional co-activators, intracellular Notch1 induces downstream gene expression. Abbreviations: CoR (Co-repressor), CoA (co-activator). (Osborn BA and Minter LM, Nat Rev Immunol. 2007 Jan; 7(1): 64-75. Epub Dec 15, 2006.)

Figure 1-5

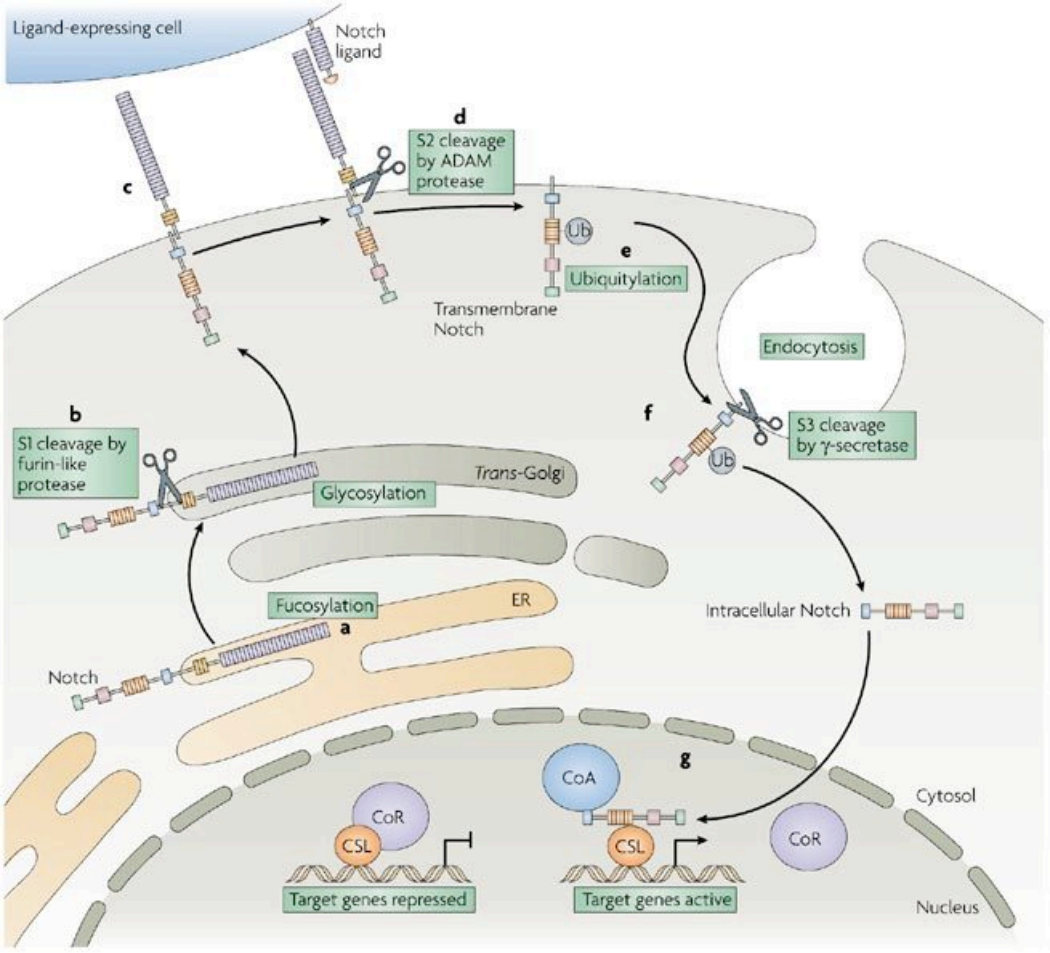


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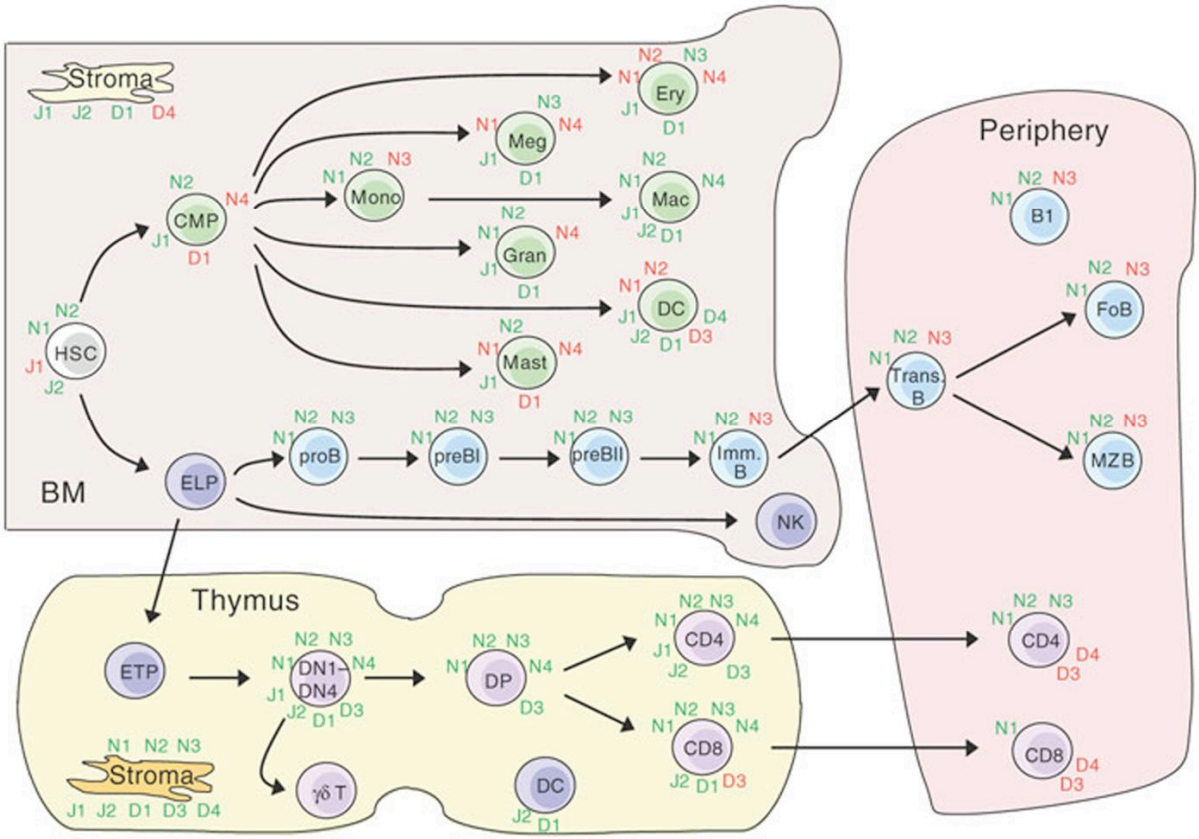
Figure 1-6: Notch receptor-ligand expression during hematopoiesis in the mouse. Expression pattern of Notch receptors (above cells) and ligands (below cells) in different hematopoietic lineages in the organ of developmental origin: green, expressed genes; red, absence of detectable expression. Controversial results are not indicated unless a consensus has been reached. Abbreviations and references: N1–4, Notch1–Notch4; J1–2, Jagged1–Jagged2; D1,3,4, Delta1, Delta3, Delta4; BM, bone marrow; BM stromal; HSC; CMP, common myeloid progenitor; ELP, early lymphoid progenitor; Ery, erythroblast; Meg, megakaryocyte; Mono, monocyte; Mac, macrophage; Gran, granulocyte; DC, dendritic cell; myeloid DC; thymic DC; Mast, mast cell; proB, pro-B cell; preBI, pre-BI cell; preBII, pre-BII cell; Imm.B, immature B cell; Trans.B, transitory B cell; FoB; MZB; B1, B1 cell; NK, natural killer cell; ETP, early thymic progenitor; DN1–4, double-negative (CD4-CD8-) thymocyte subsets; T, T cell; DP, double-positive (CD4+CD8+) thymocyte; thymic CD4; CD4, CD4+ T cell; thymic CD8; CD8, CD8+ T cell; peripheral T; thymic stroma. (Radtke F, et al. Nature Immunology 5, 247-253, 2004)

Figure 1-6


Stroma
J1 J2 D1 D4
Ery
Meg
Mono
Mac
CMP
Gran
DC
Mast
HSC
BM
ELP
proB
preBI
preBII
Imm. B
NK
Periphery
B1
FoB
Trans. B
MZB
Thymus
ETP
DN1–DN4
DP
CD4
CD8
γδ T
DC
Stroma
N1 N2 N3
J1 J2 D1 D3 D4

Chapter II

Characterization of thymic atrophy phenotype of FX(-/-) mice

Abstract

As has been established in previous studies, glycoprotein fucosylation deficiency results in leukocyte adhesion deficiency (LAD) type II. This disease is a rare human congenital disorder resulting in developmental abnormalities, a deficiency of selectin-dependent leukocyte trafficking and adhesion, and disabled O-linked glycan structure extensions in Notch receptors and thrombospondin repeat containing proteins. Mice genetically engineered with a mutation in FX locus, which encodes an enzyme controlling the last step in the de novo pathway for GDP-fucose synthesis, has global fucosylation deficiency, an extreme neutrophilia, myeloproliferation, and an absence of leukocyte selectin ligand expression. Furthermore, a severe thymic atrophy phenotype is observed in FX(-/-) mice, in temporal association with the loss of fucosylated glycan structures on soluble and cell surface proteins. However, restoration of fucosylation with a fucose-supplemented diet restores thymic development in the FX(-/-) mice within 2 weeks. In the FX(-/-) hypoplastic thymus, there is a loss of more than 95% of total thymocyte and mature T cells subsets ($CD4^+CD8a^+$, $CD4^-CD8a^+$, and $CD4^+CD8a^-$). Further, T cell progenitor populations, including the lineage negative DN2, DN3, and the recently proposed DN1a-b cells, are

absent in the FX(-/-) hypoplastic thymus after rearing the mice off fucose for 4 weeks. These observations strongly indicate that T cell development is disrupted in FX(-/-) mice in the absence of fucosylation.

Introduction

Leukocyte adhesion deficiency type II (LADII) is a rare human syndrome characterized by psychomotor defects, persistent leukocytosis, facial and skeletal abnormalities and developmental retardation (94). Cells from LADII patients display a global deficiency of fucosylated carbohydrate structures on the cell surface (95, 96). The molecular basis of LADII corresponds to a disruption in the de novo pathway of GDP-fucose biosynthesis (Figure 2-1) (97).

In this GDP-fucose synthetic pathway, GDP-fucose is the high-energy form of fucose and is used as a substrate in the synthesis of fucosylated glycans (44). In the *de novo* synthetic pathway, GDP fucose is converted first from GDP-mannose (98-100) to an intermediate product, GDP-4-keto-6-deoxymannose, by the GDP-mannose 4,6-dehydratase enzyme (GMD) (Figure 2-1) (101). The sequence of GMD is substantially conserved among *E.coli* (102-104), mice (105) and humans (106, 107), suggesting that this synthetic pathway is essential for living organisms. The GMD mRNA transcript is expressed in almost all human tissues, although at considerably different levels (106), and has been detected in several human cell lines (107). Crystallography studies have shown that *E.coli* GMD protein belongs to the short-chain dehydrogenase/reductase family of proteins with threonine133-tyrosine-lysine catalytic triad and glucose135 as an active-site base (103). Crystallization and biochemical studies indicate that the GMD enzyme forms a domain-swapped homodimer for intersubunit communication, with each monomer consisting of an $NADP^+$ binding N-terminal domain and a GDP-mannose binding C-terminal domain (103,

107, 108). GDP-fucose, as a final product, inhibits the activity of GMD, which has been shown to function as a classic negative feedback in enzyme regulation (103, 104, 106-110).

GDP-4-keto-6-deoxymannose is converted to GDP-fucose by a dual functional enzyme, 3,5 epimerase/4-reductase called FX (Figure 2-1) (111, 112). As an epimerase, FX speeds up the slow, spontaneous epimerization of the GDP-4-keto-6-deoxymannose stereo conversion to yield GDP-4-keto-6-deoxygalactose (113). The reductase activity of FX protein then catalyzes an NADPH-dependent transfer of a hydride to the keto group of GDP-4-keto-6-deoxygalactose to generate GDP-fucose (113). As first seen during the purification of glucose-6-phosphate dehydrogenase from human erythrocytes in 1975, FX was annotated as an NADP(H)-binding protein with unknown functions (114). It was not until twenty years later that FX was shown, by sequence comparisons to enzymes in the pathway for GDP-fucose synthesis in *E.coli,* to be involved in mammalian GDP-fucose synthesis (115). The FX locus has been highly conserved during evolution, with a 50% sequence identity between the human and *E.coli* enzymes, and with approximately 90% sequence identity among mammalian FX homologues (112). The structural study of the crystallized *E.coli* FX homologues assigns the FX protein to the reductase-epimerase super family (116). Somers and colleagues report a single substrate-binding pocket for GDP-4-keto-6-deoxymannose in FX, indicating that the epimerization and reduction reactions take place at the same site (117). At 1.45-1.6 Angstrom resolution, X-ray crystallographic analysis of mutant *E.coli* FX molecules with site-specific changes in amino acid sequence identify Cys109 and His179 as components of the N-terminal NADPH-binding domain and a C-terminal domain that forms the substrate-binding pocket (118).

After being synthesized in the cytosol, GDP-fucose is transported by a multi-pass transmembrane transporter to the lumen of the Golgi (119), where it is used for fucosylated glycan synthesis. O-fucosylation occurs in the ER (36) indicating the existence of a GDP-fucose transporter in ER that transports GDP-fucose into the lumen of the ER (120). The catalytic domains of fucosyltransferases, as shown here residing in the Golgi lumen, direct

the GDP-fucose-dependent transglycosylation reaction that ultimately links fucose to specific acceptors (Figure 2-1) (44). Eventually, fucose-containing glycoconjugates/glycolipids are transported out of Golgi, to be presented on the extracellular side of the cell membrane, or to be released as a soluble molecule to execute their biological functions.

Components of the de novo GDP-fucose synthetic pathway have been shown to be involved in many physiological and pathological pathways. The expression level of FX protein is elevated in human hepatocellular carcinoma/hepatocyte cell lines (121), in highly metastatic colorectal cancer variants (122), and in activated T and B cells (123). Further, synchronized upregulation of GMD, FX, GDP-fucose transporter and fucosyltransferase VII in inflammation and in tumorigenesis has been reported (124).

Tonetti and colleagues first demonstrated that cells from LADII patients display a significant deficit in GMD activity (125). Thus those patients lack fucose-containing glycan structures and fucose-specific biological functions as reviewed in (126). The fucosyltransferases in LADII patients were expressed at normal levels (127), suggesting that the deficiency of fucosylation was due to the unavailability of GDP-fucose. Later it was shown that the GDP-fucose transport activity of Golgi preparations was reduced in cells from LADII patients (128, 129). Further, missense mutations (130) or deletion (131) found in locus of SLC35C1 gene, a GDP-fucose transporter protein on Golgi apparatus (132), corresponded to the LADII phenotype. Mice deficient in SLC35C1 displayed severe growth retardation, elevated postnatal mortality rate, dilatation of lung alveoli, hypocellular lymph nodes, and a deficiency of selectin-ligand function resembling the symptoms of LADII patients (133). Interestingly, adding fucose to cells from these SLC35C1 deficient mice partially rescued the phenotype, implying the existence of alternative mechanisms for transporting GDP-fucose into the Golgi lumen (133).

These observations assign important roles to fucose and fucosylated glycans in physiology and pathogenesis. In order to uncover novel and specific functional roles for

fucose and fucosylated glycans, a genetically engineered mouse model was made in which the FX locus was disrupted to induce global fucosylation deficiency (112). Interestingly, FX(-/-) mice displayed a partially penetrate embryonic lethal phenotype, with the loss of the majority of embryos by 12.5 dpc (93). Intercrosses between FX(+/-) and FX(+/-) were carried out in an attempt to generate FX(-/-) mice. These intercross yielded only 6 live-born FX(-/-) mice, from a total of 360 progeny genotyped at weaning, which suggested that most FX(-/-) mice die in the uterus. These live-born FX(-/-) mice exhibit a postnatal failure to grow and have a life span of 1 to 2 months. These observations made it almost impossible to study the adult phenotypes of FX(-/-) mice on the original strain background.

Fortunately, as will be elaborated below, a salvage pathway has evolved that allows the supply of GDP-fucose to be restored when the FX-dependent de novo synthetic pathway from GDP-mannose is disrupted (Figure 2-1), and was used in combination with breeding experiments to generate sufficient numbers of live-born FX(-/-) mice to study. The salvage pathway starts from free L-fucose molecules that are transported into cytosol from the extracellular milieu (121,134). Yorek and colleagues reported that the uptake of L-fucose in various mammalian cell lines is mediated by a fucose-specific process that is not receptor-mediated endocytosis but is characteristic of a facilitated diffusion system (135). They further purified the L-fucose transporter, a 57kD cell-membrane protein, from mouse brains (136). This transporter can be found in almost all major mouse tissues, such as the kidney, lung, spleen, heart, and thymus (136), suggesting that the GDP-fucose synthetic salvage pathway is not tissue-specific, but is instead a rather generally expressed pathway.

In a two-step reaction, the salvage pathway in the cytosol converts exogenous L-fucose. First L-fucose is phosphorylated by fucokinase, a 110kD protein that is widely distributed in mammalian tissues, to form fucose-1-phosphate (137-139). The only other sugar that can be phosphorylated by fucokinase is D-arabinose with the generation of α-D-arabinose-1-P, at about 10% the rate of L-fucose, indicating the high specificity of this enzyme (138). Fucose-1-phosphate is subsequently converted to GDP-fucose by GDP-L-

fucose pyrophosphorylase (128,129). This enzyme is a 61kD protein with specificity for beta-L-fucose-1-P, although it can use α-D-arabinose-1-P to produce GDP-α-D-arabinose (140). Similar to fucokinase, L-fucose pyrophosphorylase was widely distributed in all tissues tested (141), indicating that the salvage pathway is a universal mechanism for mammalian cell types. The activity of fucokinase and L-fucose pyrophosphorylase may change in various conditions, such as after the acquisition of a brightness discrimination reaction in the rat hippocampus (142), a day after passive avoidance training in chicks right forebrains (143), after incubation of dopamine with rat hippocampal slices (144), or by administrate rats with dopaminergic drugs in water (145). Although it's not fully understood yet how fucokinase is regulated, L-fucose and its analogs inhibit fucokinase activity *in vitro*, indicating a negative feedback system similar to GMD (146).

The degree to which the salvage pathway contributes to GDP-fucose synthesis under normal physiological conditions is not understood. In a quantitative study using *in vitro* HeLa cell culture, it has been observed that greater than 90% of GDP-fucose is generated from the de novo pathway in the presence of L-fucose (147), indicating a supporting role for the salvage pathway in GDP-fucose synthesis. However, this pathway may fully reconstitute cellular GDP-fucose levels in Chinese Hamster Ovary Lec13 cells, which lack GMD and are thus deficient for the de novo synthetic pathway (105), if L-fucose is added to the Lec13 cells in culture (Becker DJ & John Lowe, unpublished data).

Taking advantage of the salvage pathway, fucose was added to the water or chow of two of the original FX(-/-) male mice. These FX(-/-) male founders, when reared on fucose, were found to be fertile, and when crossed with FX(+/-) females, gave rise to a nearly Mendelian ratio of progeny (50% -/-, 50% +/-expected, ~30%-/-, ~70% +/-) when the females were kept in cages with fucose supplementation (93). Breeding experiments further improved the yield of null progeny, which is essentially fully Mendelian when FX(+/-) males are crossed with FX(+/-) females, when the FX null allele is on a C57Bl/6J background (93).

Under these circumstances, FX(-/-) mice are able to grow to adulthood on fucose supplementation. Experimental evidence has shown that FX(-/-) mice on a fucose-supplemented diet display an essentially wild-type phenotype in growth (by weight), viability (by survival analysis), and cellular functions (by histology and expression of fucosylated-glycans) (93).

In addition, fucosylation is easily manipulated by simply supplying FX(-/-) mice with a fucose-containing diet, or maintaining the mice on water or chow not supplemented with fucose. Most experiments in my thesis were done using FX(-/-) mice fed with fucose from weaning until the age of 8 weeks. In some instances, these mice were then placed on a non-fucose supplemented diet, yielding fucosylation deficiency and the fucose-dependent phenotypes described previously (148). This approach to the conditional control of fucosylation makes it possible to observe phenotypes controlled by fucosylation in FX(-/-) mice.

This chapter will focus on the thymic atrophy observed in adult FX(-/-) mice upon the withdrawal of fucose from their diet. Mature T cells were almost completely lost among total thymocytes isolated from these FX(-/-) mice. By phenotypic analysis of cell surface markers, T lymphocytes within the FX(-/-) hypoplastic thymus accumulated in an early stage of development. As discussed in the following chapters of this thesis, these observations initiated further investigations that focus on the novel functional roles of fucosylated glycans.

Methods

Materials- All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals and within the approved animal protocols of the University of Michigan and Case Western Reserve University

Animal Care and Use Committees. C57B/6 mice were obtained from Jackson laboratory. FX knockout mice, as FX(-/-), were made as previously described (93). FUT4/7 double knockout mice (DKO) were made, again, as previously described (53). Thymus was isolated from the mice and mechanically disrupted to make a single cell suspension. Thymocytes were first resuspended in red blood cell lysis buffer (Sigma) at room temperature for 5 minutes, and washed twice in Hank's Balanced Salt Solution (HBSS, Gibco) with 0.1% Bovine Serum Albumin (BSA, Sigma) (working medium). Cells were pelleted by centrifugation and resuspended for cell counts by Trypan blue or phenotypic analysis by flow.

Thymocyte enrichment- After red blood cell lysis, thymocytes were first incubated on ice for 10 minutes in HBSS+0.1% BSA with anti-mouse Fc block (1:400, BD Pharmingen). After being washed, the cells were then incubated on ice for 30 minutes with biotinylated antibodies from BD Pharmingen: anti-mouse CD3ε (553059), anti-mouse CD4 (553045) (1mg/ml stock as 20ul/100x10^6 cells). The cells were then washed twice in HBSS+0.1% BSA and were incubated on ice for 15 minutes with rat-anti-mouse IgG magnetic beads (Miltyni Biotech) with a concentration of 120x10^6 cells/830ul beads. Then the bead-labeled cells were passed through magnetic columns for negative selection so that lineage positive cells were left bound on the columns. Cells that flowed through were collected for further surface antibody staining. In FX(-/-) mice rearing off fucose for 4 weeks, this thymocyte enrichment procedure was typically skipped, since very few mature T cells were found.

Flow cytometry- Thymocytes were stained with anti-mouse antibodies from eBiosciences and BD pharmingen and were incubated on ice for 30 minutes. Stained cells were then washed twice before being loaded into the cytometor for analysis. The following antibodies were used for CD4 and CD8a analysis and PSA staining: FITC-anti mouse CD4, PE-anti mouse CD8a, FITC-PSA (*Pisum sativum* agglutinin). As a negative control for lectin binding specificity, 100mM mannose was added in a staining medium of PSA

tubes. In lineage depleted DN1-4, ETP and DN1a-b analysis, biotinylated anti-mouse CD3, CD4, CD8a, CD11b, CD19, Gr-1, Ter-119, NK1.1 were first incubated with thymocytes on ice for 30 minutes. After being washed twice, cells were further stained with SA-PE-TxRd. Other antibodies used included APC-anti mouse CD44, APC-Cy7-anti mouse CD25, FITC- anti mouse CD24, FITC-anti mouse CD127 (IL7-Rα), PE-anti mouse CD117 (c-Kit). FACSAria (BD biosciences) FACScan (BD biosciences) and ELITE (Coulter) were used for 2-6 colored analysis.

Bone marrow cells isolation- Bone marrow cells from femur and tibia of the mice were flushed out in HBSS with 0.1% Bovine Serum Albumin (tissue culture tested, Sigma). Red blood cells were lysed after isolation with red cell lysis buffer (Sigma). Lineage depletion was carried out by incubating the cells with biotinylated anti-mouse CD3, CD4, CD8a, δγ-TCR, B220, Gr-1, CD11b, Ter-119, NK1.1 (BD, Pharmingen) antibodies, followed by magnetic bead depletion (Miltyni Biotec).

Intravenous injection- Lineage depleted bone marrow progenitors were intravenously injected into FX(-/-) recipient mice off fucose for 4 weeks. $11x10^6$ lineage depleted cells on average were isolated from one wild-type mouse and injected intravenously into a FX(-/-) recipient mouse off fucose for 4 weeks (12wks old). Recipient mice were analyzed four weeks after injection.

Results

Fucose depletion and abnormal FX(-/-) thymic development in an off fucose environment.

Encouraged by previous evidence of the important roles of fucosylated glycan structures in the innate and adaptive immune systems (49), FX(-/-) mice were genetically constructed to uncover novel functional roles of fucosylated glycans (93). The *de novo*

GDP-fucose synthesis pathway was disrupted in the FX(-/-) mice by abolishing the function of the FX enzyme that controls the last step of the GDP-fucose synthesis (Figure 2-1). As expected, FX(-/-) mice displayed a global fucosylation deficiency (93). In most cases, FX (-/-) mice mostly died around 12.5 days in the uterus, displaying the embryonic lethal phenotype. However, this lethality of FX(-/-) mice embryos was rescued by a fucose-supplemented diet to mothers and the newborn babies. These FX (-/-) mice on a diet including fucose displayed wild-type phenotypes. As a result, there was no defect observed in these FX(-/-) "on fucose" mice and they were able to grow to adulthood (93). The size of the thymus of these FX(-/-) on-fucose mice was similar to their age-matched wild-type littermates, as shown in Figure 2-2. These observations indicate that by restoring fucosylation with a fucose-supplemented diet normal thymic development is sustained.

After being fed with a fucose-supplemented diet until 8 weeks old, these FX(-/-) mice were fed on a non-fucose-supplemented diet to induce phenotypes that related to the loss of fucosylation (Figure 2-2 time bar). At a two-day interval, the thymus of FX(-/-) was isolated for analysis. The size of the FX(-/-) thymus decreased at the time of fucosylation loss and reached a steady state after 4 weeks off fucose (Figure 2-2). The size of the FX(-/-) thymus did not change when mice were maintained on a non-fucose supplemented diet for up to 13 weeks (data not shown).

When FX(-/-) mice off fucose for 4 weeks were again fed a fucose-supplemented diet at two-day intervals, within two weeks the size of the thymus of these mice increased to a level comparable to that of their age-matched wild-type littermates, indicating that restoring fucosylation may fully rescue the atrophy phenotype in FX(-/-) mice.

The number of total thymocytes of FX(-/-) mice dramatically decreased at the time of fucosylation loss and increased when fucosylation was restored, as shown in the same time frame of fucose supplementation to mice during analysis (Figure 2-2). Notably, as counted by trypan blue exclusion, when losing fucosylation for 4 weeks, the number of live total thymocytes of FX(-/-) mice dropped on average by a factor of 1000, from 1.38×10^8

(on fucose since birth, 12 weeks old) to $2.15x10^5$ (4 week off fucose, 12 weeks old) (Figure 2-2). These results indicate that one or more thymocyte populations were lost in FX(-/-) thymus off fucose for 4 weeks. In the following analysis, if not specifically indicated, "FX(-/-) off fucose" mice refer to FX(-/-) mice having been off fucose for 4 weeks.

However, FX(-/-) mice that had always been fed the fucose-supplemented diet had comparably sized thymuses (Figure 2-2) and comparable numbers of thymocytes (Figure 2-3) relative to their age-matched wild-type littermates, indicating that the atrophy phenotype of FX(-/-) mice off fucose was not age-dependent. Rather, the atrophy phenotype of FX(-/-) thymus is only fucosylation-dependent.

In our analysis, the fucosylation status of FX(-/-) thymocytes was monitored by the flow cytometry staining of P-lectin, also known as PSA (*Pisum sativum* agglutinin), which is a reagent that recognizes α(1,6)-fucosylated N-glycans (149) on the cell surface (93, 150). PSA may bind to cells specifically and non-specifically. Free D-mannose can compete with fucosylated glycans on the cells and abolish the specific recognition of PSA to its fucosylated glycan target. Thus if PSA stains specifically on the cells, this binding can be abolished by adding D-mannose (data not shown) (93, 151). By 4 weeks off fucose, FX(-/-) thymocytes are mostly PSA negative, indicating the absence of fucosylated glycans on the cell surface and the fucosylation-deficient status of the thymocytes. When FX(-/-) mice off fucose for 4 weeks were again fed with a fucose-supplemented diet, the PSA positive thymocytes of these mice increased to 95% of their original total in two days, indicating the presence of fucosylated glycans on the cell surface and the fucosylation-sufficient status of the thymocytes.

It is interesting to notice that FX(-/-) thymocytes needed about 8 days to completely lose the α(1,6)-fucosylated N-glycans on their cell surface. However, in only 2 days, the α(1,6)-fucosylated N-glycans were detected again on FX(-/-) thymocytes. This time difference indicates that the fucosylated glycans may be turned over and fucose may be re-used by the cells or the tissues of FX(-/-) mice; thus the fucosylated structures were still

being made in FX(-/-) thymocytes after a few days off fucose. These results also imply that the time frame from the synthesis of fucosylated structures to their presentation on the cell surface is about 2 days.

These observations suggest that the thymic atrophy phenotype of FX(-/-) mice is fucosylation-dependent and reversible. We were particularly interested in understanding the mechanisms of this fucosylation-dependent thymocyte development, because in this way we might learn why and how fucosylation contributes to thymocyte development, a very important process in the formation of our adaptive immune system.

The Complete Block of Mature T cell Development in FX(-/-) Mice off Fucose.

The thymus is the organ in which T cell development occurs during the development of adaptive immune system. The total number of thymocytes include all T cells at various developmental stages within the thymus, thus they are very heterogeneous. $CD4^+CD8^+$ double positive (DP) cells compose 90% of the total thymocytes. We first tested whether this DP population was present in FX(-/-) off-fucose thymus by using two-parameter flow cytometry. Around 90% of cells in the thymus of a wild-type mouse were CD4 and CD8a double positive (Figure 2-4 a). However, FX(-/-) mice off fucose 4 weeks typically have less than 1% of DP cells (Figure 2-4 a). Instead, around 60% of FX(-/-) off-fucose thymocytes were in the CD4-CD8a- (DN) stage. CD4+CD8a- and CD4-CD8a+ (SP) cells in FX(-/-) off-fucose thymus were enriched up to 30% of total number of thymocytes. These results indicate that the missing DP population is probably the major defect in FX(-/-) off-fucose thymus and accounts for the thymic atrophy phenotype.

Since there was a dramatic change in the total thymocyte number of FX(-/-) off-fucose mice, the percentage of each population could not represent the change in number within the thymus. The numbers of DP, DN and SP populations were further enumerated by multiplying the percentage of each population with the total thymocyte number (Figure

2-4 b). The numbers of $CD4^+CD8a^+$ DP, $CD4^+CD8a^-$, and $CD4^-CD8a^+$ cells were graphed according to the time frame shown in Figure 2-2. Similar to the changes of total thymocytes, the numbers of all of these mature thymocyte populations in FX(-/-) mice first decreased upon the time of fucose depletion. FX(-/-) mice off fucose 4 weeks had only $3.03x10^3$ DP cells in average, while FX(-/-) on-fucose littermates had $1.21x10^8$ DP cells on average, and age matched wild-type mice had $1.47x10^8$ DP cells on average. Despite the enrichment of SP populations, as shown by percentage in Figure 2-4 a, the numbers of $CD4^+CD8a^-$ and $CD4^-CD8a^+$ cells in FX(-/-) off-fucose thymus actually decreased during fucose depletion, indicating that the maturation of T cells is abolished upon the time of fucosylation loss in FX(-/-) mice.

When fed with a fucose-supplemented diet, FX(-/-) mice had an increase in all these mature thymocytes and reached levels comparable to their 14-week-old wild-type littermates (Figure 2-4 b). These results indicated that the generation of mature thymocytes could be restored by fucosylation.

Lin- DN1 T Cell Progenitors Missing in FX(-/-) Mice off Fucose.

Loss of mature thymocytes in FX(-/-) mice on a non-fucose supplemented diet strongly indicated a "block" during thymocyte development. Most of the thymocytes in the FX(-/-) off-fucose thymus were in the $CD4^-CD8a^-$ (DN) stage, which is earlier than the DP and SP stages. To further refine the developmental stages within DN cells, CD44 and CD25 were used to divide the $CD4^-CD8a^-$ cells into four different stages (152-154). $CD44^+CD25^+$ (DN1) cells within DN population progress through $CD44^-CD25^+$ (DN2), $CD44^-CD25^+$ (DN3) to $CD44^+CD25^-$ (DN4), and then up-regulate CD4 and CD8a expression to be DP cells as a traditional model for T cell development. FX(-/-) mice off fucose 4 weeks had in average 76.51(±0.53)% of $CD4^-CD8a^-$ cells in $CD44^+CD25^-$ (DN1) stage of the T cell developmental pathway (data not shown), which indicated a possible accumulation of T cell progenitors. However, within the DN1 population, other lineage

positive cells, including B cells as $B220^{+}CD19^{+}$ and NK cells as $NK1.1^{+}$, were found (155, 156) (data not shown). These observations indicated that lineage positive cells should be excluded in the analysis of T cell progenitors that usually don't express any of the lineage markers. A previous study indicated that B220 is also expressed on T progenitors in addition to B cell progenitors (157). To avoid the depletion of this potential T progenitor population, CD19 was used as a marker for mature B cells in our analysis, while in other studies B220 might be used during lineage depletion (157-159).

The DN1 population is thus extremely heterogeneous, and we analyzed the T cell progenitor populations after excluding mature hematopoietic cells. Wild-type thymocytes and FX(-/-) on-fucose thymocytes usually occur in the range of 10^{8} per thymus, and lineage negative thymocytes were enriched by passing them through the columns that bind to magnetic beads conjugated on lineage positive cells. Due to the very limited cell numbers of FX(-/-) off-fucose thymus, lineage depletion was carried out simply by electronic gating during analysis without lineage depletion by magnetic columns. In fact, around 8 to 10 thymuses of FX(-/-) off fucose for 4 weeks were combined to generate one flow profile in each analysis.

Propidium iodide (PI) was used at a final concentration of 1ug/ml in every staining tube in order to separate the live cells from the dead, because it can stain nuclear chromatin when cell membrane is disrupted (160). Live FX(-/-) thymocytes were electronically gated on PI negative staining first, and then lineage negative (Lin-) cells were analyzed by CD44 and CD25 surface markers to be grouped as Lin-DN1 to Lin-DN4 cells (161). Compared with age-matched FX(-/-) on fucose and wild-type thymocytes, Lin-DN2 and Lin-DN3 cells greatly decreased. 60% of lineage negative FX(-/-) mice off-fucose thymocytes were in the Lin-DN1 stage, while 40% of them were in the Lin-DN4 stage (Figure 2-5 a). Although there seems to be an accumulation of Lin-DN1 cells in FX(-/-) off-fucose thymus percentage-wise, the numbers of the FX(-/-) off-fucose Lin-DN1 cells actually decreased (Figure 2-5 b). The complete absence of Lin-DN2 and Lin-DN3 cells in FX(-/-) off-fucose

thymus suggests that there is a "block" during the transition of the DN1 stage to the DN2 stage in early thymocyte development. The presence of Lin-DN4 cells in the FX(-/-) off-fucose thymus and the absence of DP cells that are a direct downstream population of Lin-DN4, indicate that there is another "block" during the transition from the DN4 stage to the DP stage. These multiple "blocks" suggest that fucosylation is required in all stages of early T cell development.

Early T Cell Progenitors Missing in the FX(-/-) Off-fucose Thymus

Interestingly, Lin-DN1 cells are also very heterogeneous. These cells do not equally express cell surface markers such as CD117 (c-Kit), CD127 and CD24, according to the studies that attempted to define the earliest thymic immigrants (158, 159, 162).

Bhandoola and colleagues proposed ETPs (early T cell progenitors) defined as Lin^{-} $CD117^{+}CD127^{-}$ DN1, which represented a very small population of thymocytes, giving rise only to T cells or B cells *in vivo* (158). In their studies, ETPs were the major population, 87% (158) or 69% (162) within lin-DN1 cells, however in our study we typically found that 3% of Lin-DN1 cells were ETPs (Figure 2-6). The difference might depend on the different definitions of lineage negative/low populations during analysis with the obscure standards in history. Another possibility is that the CD19 marker is used in our analysis to include B220 positive T cell progenitors, which is different from others (157-159), since previous studies have shown that B220 may be expressed by T cell progenitors (157). Compared to wild type and FX(-/-) on-fucose littermates, the ETP population was missing in FX(-/-) mice off fucose for 4 weeks (Figure 2-6).

In another study, Petrie and colleagues proposed five different populations within lineage negative DN1 cells defined by the surface CD24 and CD117 expression. Among these five different populations, DN1a-b were the T cell progenitors (159). However, the DN1a and DN1b populations could not be fully separated in our analysis (Figure 2-7) nor in Bhandoola's study (162). DN1a-b cells may generate only T cells *in vitro* (159), and T

and B cells *in vivo* (162). Petrie's group found around 12% of Lin-DN1 cells were in the DN1a-b stage (159), while Bhandoola's group found 69% Lin-DN1 cells are DN1a-b cells (162) by using the same analytical method. Thus, there is an inconsistency in the literature regarding the percentage of DN1a-b cells per thymus, which may possibly be due to the lineage negative gating. In our study, we consistently found that 2% of the lineage negative DN1 cells were in DN1a-b stages in wild-type thymocytes.

In their study, Bhandoola and colleagues further proposed that ETPs are the same as DN1a-b cells (162). Our results, that similar percentages of ETPs and DN1a-b cells were found in lineage DN1 thymocytes, seem to be consistent with this proposal. Similar to the result of ETP analysis, the DN1a-b population was missing to a large extent in FX(-/-) off-fucose mice (Figure 2-7). To some extent DN1c and DN1d were also missing in FX(-/-) off-fucose thymus, and to some extent DN1e was enriched.

These results suggest that early T cell progenitors could not develop or survive in the FX(-/-) off-fucose thymus. These observations also suggest that early T cell progenitors may not be able to get into the FX(-/-) off-fucose thymus. To answer these results raised, we further analyzed several possible reasons for the FX(-/-) thymic atrophy.

Proposed Causes of FX(-/-) Thymic Atrophy:

1. Notch1 signaling deficiency

Notch1 conditional knockout mice were reported with a thymic atrophy phenotype similar to FX(-/-) off-fucose mice (77). Most of the cells lost in Notch1 knockout mice were mature DP thymocytes, which is very similar to the observations in FX(-/-) mice. Further, the DN1a-e populations observed in the FX(-/-) hypoplastic thymus are very similar to the DNMAML1 (Dominant negative mastermind like molecule-1) transduced bone marrow cells in the bone marrow transplantation experiments (162). In this study, a dominant negative form of MAML1 was introduced into the bone marrow progenitors, in order to compete out functional MAML1, a transcription co-activator required in Notch1

signaling (163). Progenitors with inactivation of Notch1 signaling populated the thymus *in vivo* with an enriched DN1e population and, most importantly, with an absence of DN1a-b populations (162). These observations suggest that the thymic atrophy of FX(-/-) off-fucose thymus is due to the disruption of Notch signaling.

2. Thymic Environmental Defects.

T cell development occurs only within the thymus of mammals, indicating the thymic environment is unique for T lineage commitment. Another possibility is that the FX(-/-) off-fucose thymic environment may not be capable of supporting T progenitor development. Defects in the thymus epithelial cells could result in the reduced production of T cells (164). To identify whether the thymic environment of FX(-/-) off-fucose mice can support T cell development, the developmental potential of healthy bone marrow stem cells from wild-type mice were tested. Lineage depleted donor bone marrow cells isolated from one wild-type mouse ($11x10^6$ cells in average, with CD45.1 expression) were injected intravenously into an FX(-/-) (CD45.2) recipient mouse that had been off fucose for 4 weeks (Figure 2-8a). The chimera mice were analyzed 4 weeks after the bone marrow transplantation (165).

The total number of thymocytes in the recipient mice ($1.20\ x10^8$ /thymus in average of 6 mice, 3 independent experiments) was found comparable with age-matched wild-type mice (14 weeks old, $1.39x10^8$/thymus in average of 6 mice). The wild-type donor-derived $CD45.1^+$ cells were the major population (96%) in the recipient thymus and were PSA positive (Figure 2-8b). Around 96% of CD45.1+ donor derived thymocytes were DPs, which was comparable to the wild-type mice (Figure 2-8).

CD45.2+ endogenous cells were PSA negative (Figure 2-8b). The absence of fucosylated glycan structures on recipient cell surfaces indicated that there was no fucose transfusion from donor cells to recipient cells. Phenotypically, CD45.2+ endogenous cells remained in the DN stage, which was similar to the mock-injected recipients (data not

shown) or age-matched FX(-/-) mice off fucose (Figure 2-8). The absence of the DP population in the recipient endogenous thymocytes indicates that donor cells did not alter the development of recipient cells.

These results indicate that wild-type healthy hematopoietic cells develop to mature T cells when fucosylation is absent in the thymic environment; thus the fucosylated thymic environment is dispensable for T cell development. These results suggest that the thymic atrophy in FX(-/-) mice off fucose is cell-autonomous.

3. Thymic Progenitor Homing Defects.

Due to the absence of ETPs in FX(-/-) off-fucose thymus, one possibility is that the fucose-deficient thymocyte progenitors may not home to thymus. It has been reported that bone marrow cells losing P-selectin glycoprotein ligand 1 (PSGL-1) showed defective thymocyte homing ability in competition with wild-type thymocytes (166). PSGL-1 is a heavily glycosylated protein with alpha-1,3 fucosylated moiety required for its normal functions (167) that binds selectively to P-selectin (168, 169) and E-selectin (170, 171). It contributes greatly in the leukocyte and neutrophils rolling process (172, 173). Fucosyltransferase 4 and 7 are in charge of adding terminal fucose to functionalize PSGL-1 (174) and play important regulatory roles in leukocyte rolling (52, 53). Thus FUT4/7 double knockout mice lack functional PSGL-1 and mimic the major phenotypes of PSGL-1 knockout mice. However, neither FUT4/7 double knockout mice nor PGSL-1 knockout mice have thymic developmental abnormalities, indicating that PSGL-1 and alpha-1,3 fucosylated glycans are dispensable for thymocyte homing and development (52, 53, 166). Thus the defective homing is apparently not the major reason for FX(-/-) thymic atrophy.

4. Increased Apoptosis.

Another reason for the FX(-/-) thymic atrophy may lie in the increased apoptotic events in thymocytes. Apoptotic DP thymocytes were found throughout the cortex region of

the normal thymus and were thought to correspond to the consequences of positive and negative selection (175). If apoptosis is the main reason for the loss of DP cells in FX(-/-) thymus, the apoptotic events have to happen before the DP stage. To address this possibility, lineage negative DN1 cells were stained by Annexin V, a reagent detecting early apoptotic cells (176). Thymic early progenitors of 4-week off-fucose FX(-/-) mice showed a similar percentage of apoptotic cells (Annexin V positive) within Lin-DN1 and other populations, as the age-matched littermate FX(-/-) on-fucose mice and the age-matched wild-type mice (data not shown). These results indicate that the decrease in the total thymocytes is not likely due to the apoptosis of FX(-/-) thymic progenitors off fucose. It could be possible that early T cell progenitors undergo apoptosis at such a high rate that we cannot detect their presence within the thymus.

To summarize the above analysis, the deficiency of multiple populations of T cells and their progenitors in FX(-/-) off-fucose thymus indicates that the normal thymocyte development pathway is disrupted. Of the above possibilities, it is most likely that this thymic atrophy is cell autonomous and related to the disruption of the Notch1 signaling pathway.

Discussion

The results presented here described a novel phenotype of FX(-/-) mice in the function of fucosylation. It was very interesting for us to find out how fucosylation and/or fucosylated glycans control thymocyte development, which is an important process during immune system development. The enumeration of total thymocyte numbers of FX(-/-) off-fucose mice indicates that there were populations missing within the atrophic thymus. In order to find out which populations were missing in FX(-/-) off-fucose thymus, almost all known populations within the thymocyte developmental pathway, such as DP, SP, DN, Lin-

DN1-4 and early thymic progenitors, were analyzed. This analysis was our first step in determining the mechanism of FX(-/-) off fucose thymic atrophy.

No Thymic Atrophy Reported in Known Fucosyltransferase Deficient Mice.

FX enzyme controls the total de novo production of GDP-fucose, the substrate of all the fucosyltransferases. Disruption of GDP-fucose synthesis *in vivo* ablates the supply of GDP-fucose as the substrate for all of the fucosyltransferases (FUT) and disrupts all the functions of the fucosyltransferases. In theory, FX(-/-) mice display all the phenotypes that could be found in fucosyltransferase deficient mice.

Glycans synthesized by fucosyltransferases contribute to many different developmental, physiological, and pathophysiological processes, such as ABO blood type (by FUT1 and FUT2 gene products) (37), host-microbe interactions (e.g. by FUT2 and FUT3 gene products) (45), selectin-dependent leukocyte adhesion (by FUT4 and FUT7 gene products) (52, 53) and neonatal development (e.g. by FUT9 gene product) . So far, 13 fucosyltransferase genes have been identified in the human genome, FUT1, FUT2, FUT3 (177), FUT4 (178, 179), FUT5 (180), FUT6 (181, 182), FUT7 (183, 184), FUT8 (185), FUT9 (186), FUT10 and FUT11 (187-189), OFUT1 (17), and OFUT2 (188).

Among these fucosyltransferases, FUT10 and FUT11 are putative fucosyltransferases based on sequence comparisons only and as yet have unknown functions and no known enzymatic activities. The remaining genes are grouped by the glycan structures they make: α-1,2 FUT (FUT1 and FUT2), α-1,3 FUT (FUT3,4,5,6,7 and 9), and α-1,6 FUT (FUT8) and O-FUT (OFUT1 and OFUT2). Mice deficient in FUT1, FUT2, FUT4, FUT7, FUT8, or FUT9 have no reported thymic developmental defects (53, 190-192). FUT3, FUT5, and FUT6 are clustered in the human genome (193) but do not have functional homologues in mice (194, 195). FUT9 product CD15 has no expression in adult thymocyte subsets and LSKs in mice (Yunfang Man & John Lowe, unpublished data). In summary, the absence of genes accounting for constructing N-fucosylated glycans

does not result in thymic atrophy. It seems that N-fucosylated glycans may not play a role in thymic development.

In recent studies, OFUT1 and OFUT2 were found encoding enzymes with O-fucosylation abilities that add fucose directly to EGF repeats or thrombospondin type I repeats (TSRs) respectively (17, 196, 197). Recently, Protein O-fucosyltransferase (POFUT) 1 has been extensively studied for the important role it plays in Notch signaling in Drosophila (25) and mammals (58). OFUT1 knockout mice display the embryonic lethal phenotype and most of the mice die on day 8 of postgastrulation, strongly indicating the important role of O-linked glycans in early developmental stages (57). POFUT2 adds fucose specifically to the TSRs of many important proteins (196), and mice lacking POFUT2 also die early in embryo (Lowe, Haltiwanger, unpublished data). Thus, it is not known yet whether POFUT1 or POFUT2 regulates adult thymic development.

O-fucosylation (Notch signaling) May Contribute to FX(-/-) Thymic Atrophy

GDP-fucose is added to different glycans as a terminal modification in Golgi or directly to the Serine/Threonine of proteins in ER. Most fucosyltransferases seem to reside in Golgi except POFUT1 found in ER. It's not known yet if POFUT2 resides in ER. Thus we could ask whether the FX(-/-) thymic atrophy relates to ER fucosylation or Golgi fucosylation.

Mice lacking the Golgi GDP-fucose transporter SLC35C1 do not have N-fucosylated glycans, since GDP-fucose cannot get into Golgi. Since transportation of GDP-fucose to ER is not disrupted, SLC35C1(-/-) mice have ER O-fucosylated glycans expressed with their normal functions. In other words, SLC35C1 deficient mice are considered to resemble a putative mouse model with all fucosyltransferase genes knocked out but with OFUT1 left intact. FX(-/-) mice are considered to have all fucosyltransferases genes knockout out due to the global fucosylation deficiency. Thus in theory the phenotype

differences of FX(-/-) mice and SLC35C1 mice should represent the functions of O-fucosylation.

However, thymic atrophy was not observed in SLC35C1 mice but was a major phenotype for FX(-/-) mice. This strongly indicates that thymic atrophy is due to a lack of O-fucosylation in FX(-/-) mice. As it has been reported, O-fucosylation is critical for Notch signaling, which regulates thymic development. Finally then, we reach the hypothesis that the FX(-/-) thymic atrophy phenotype is due to a lack of O-fucosylation and results in Notch signaling deficiency.

This hypothesis initiates the study to confirm the contribution of Notch signaling in FX(-/-) thymic atrophy *in vivo* and *in vitro*, as described on the cellular level in Chapter III. Chapter IV further pursues, using the methods of biochemistry, the mechanism of how fucose contributes to Notch signaling.

Notes to Chapter II

[1]Acknowledgments: We thank Peter Smith for advice on phenotype analysis.

[2]Attribution of data: Jay Myers performed the thymus dissection depicted in Figure 2-2 and contributed to Figure 2-5, Figure 2-6 and Figure 2-7. Clare Rodgers performed most of the FACS analysis in Figure 2-2,Figure 2-3 and Figure 2-4.

Figure 2-1

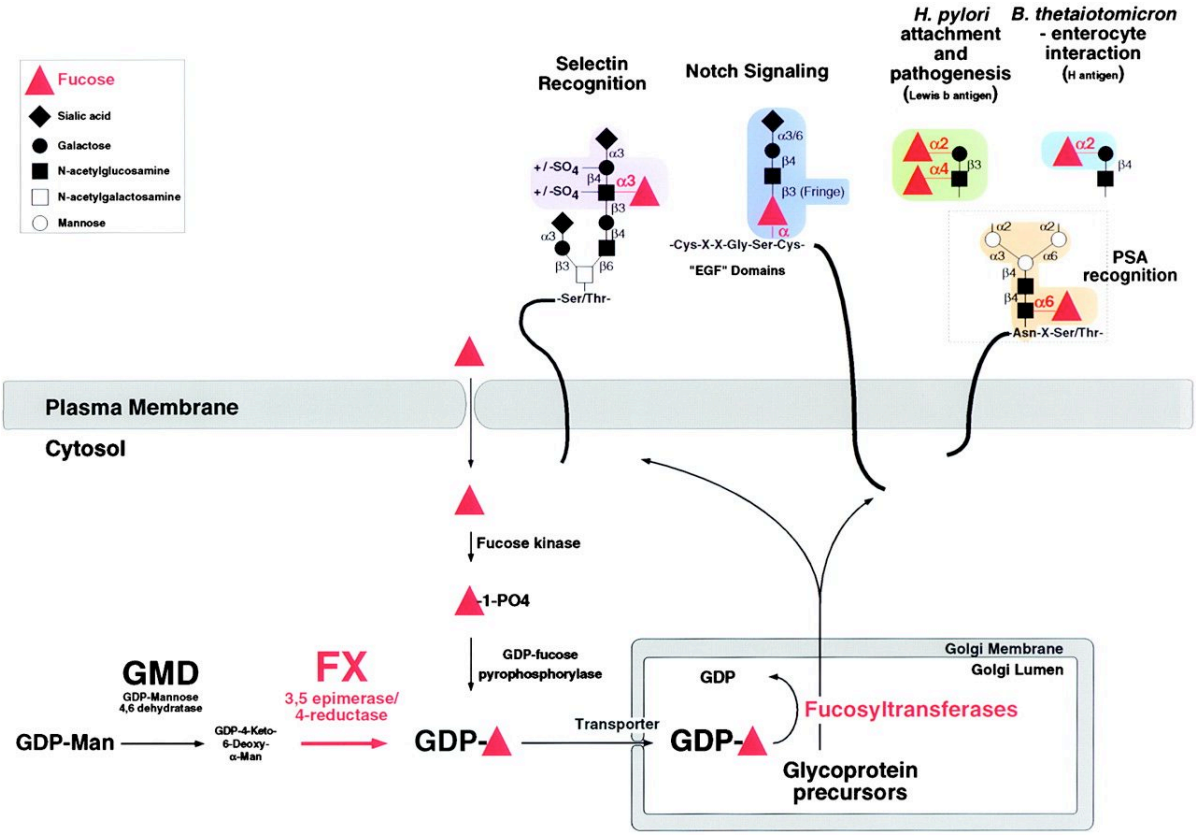


Figure 2-1 Fucosylation pathways in mammals. Two different cytosolic pathways lead to formation of GDP–fucose. The constitutively active de novo pathway converts GDP–mannose into GDP–fucose via oxidation, epimerization, and reduction catalyzed by two enzymes (GMD and FX). The salvage pathway initiates with free fucose, delivered to the cytosol from extracellular sources (shown) or from intracellular (lysosomal) sources (not depicted). (Smith PL, et al, The Journal of Cell Biology, Volume 158, Number 4, August 19, 2002 801-815)

Figure 2-2 Thymic atrophy and total thymocyte decreased in FX-/- mice upon the time off fucose. Each bar shows the average of 3 and more animals while N=37 on FX-/-off fucose 4 weeks. Error bar represents standard deviation of the mean (SEM).

Figure 2-2

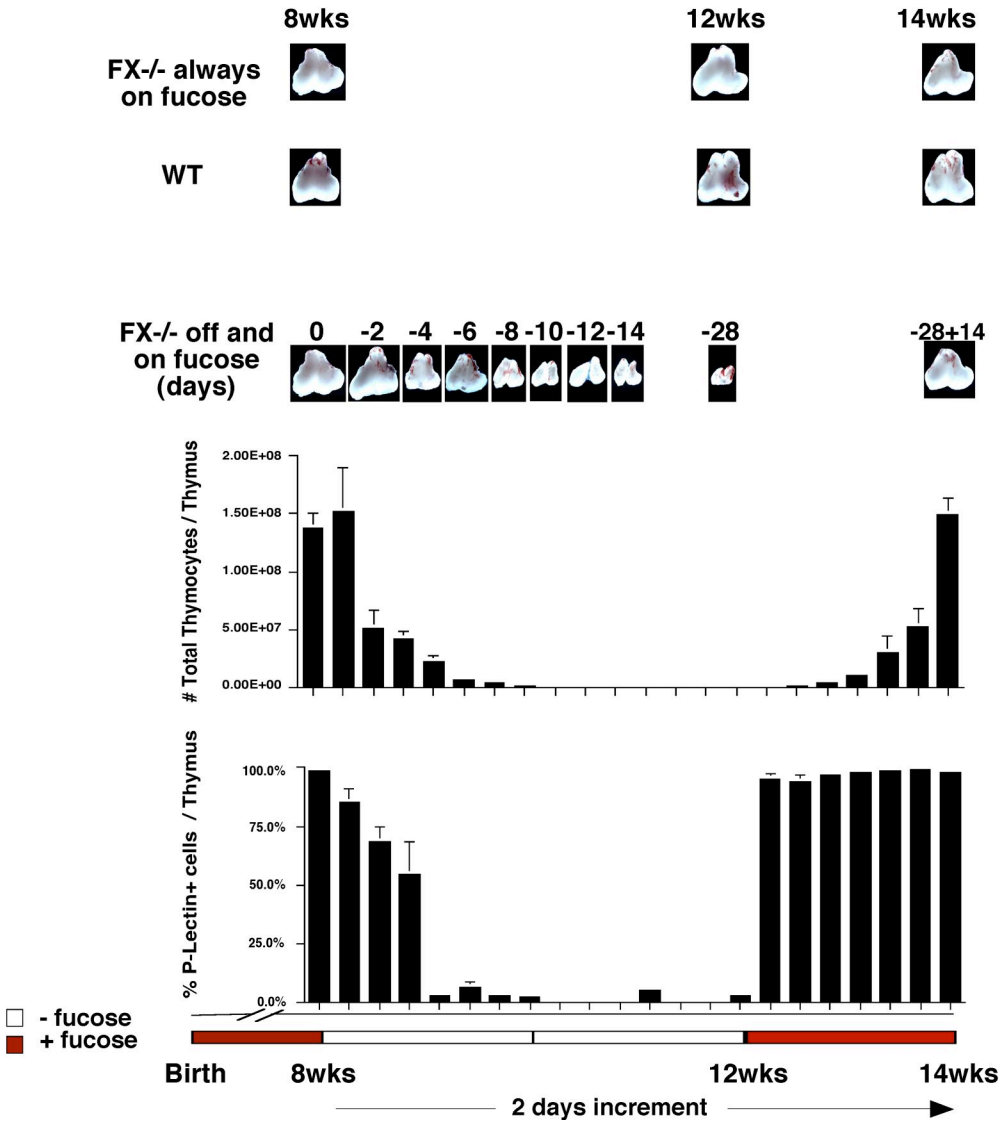

8wks
12wks
14wks
FX-/- always on fucose
WT
FX-/- off and on fucose (days)
0
-2
-4
-6
-8
-10
-12
-14
-28
-28+14
Total Thymocytes / Thymus
2.00E+08
1.50E+08
1.00E+08
5.00E+07
0.00E+00
% P-Lectin+ cells / Thymus
100.0%
75.0%
50.0%
25.0%
0.0%
- fucose
+ fucose
Birth
8wks
12wks
14wks
2 days increment

Figure 2-3

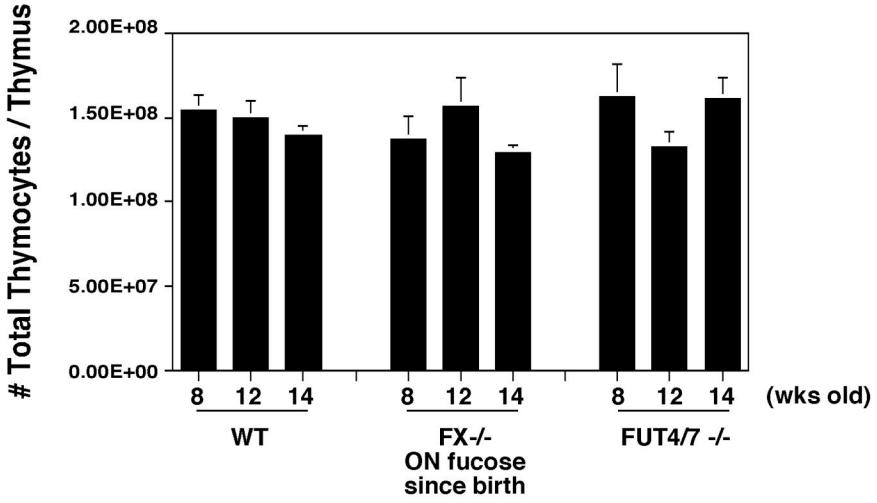
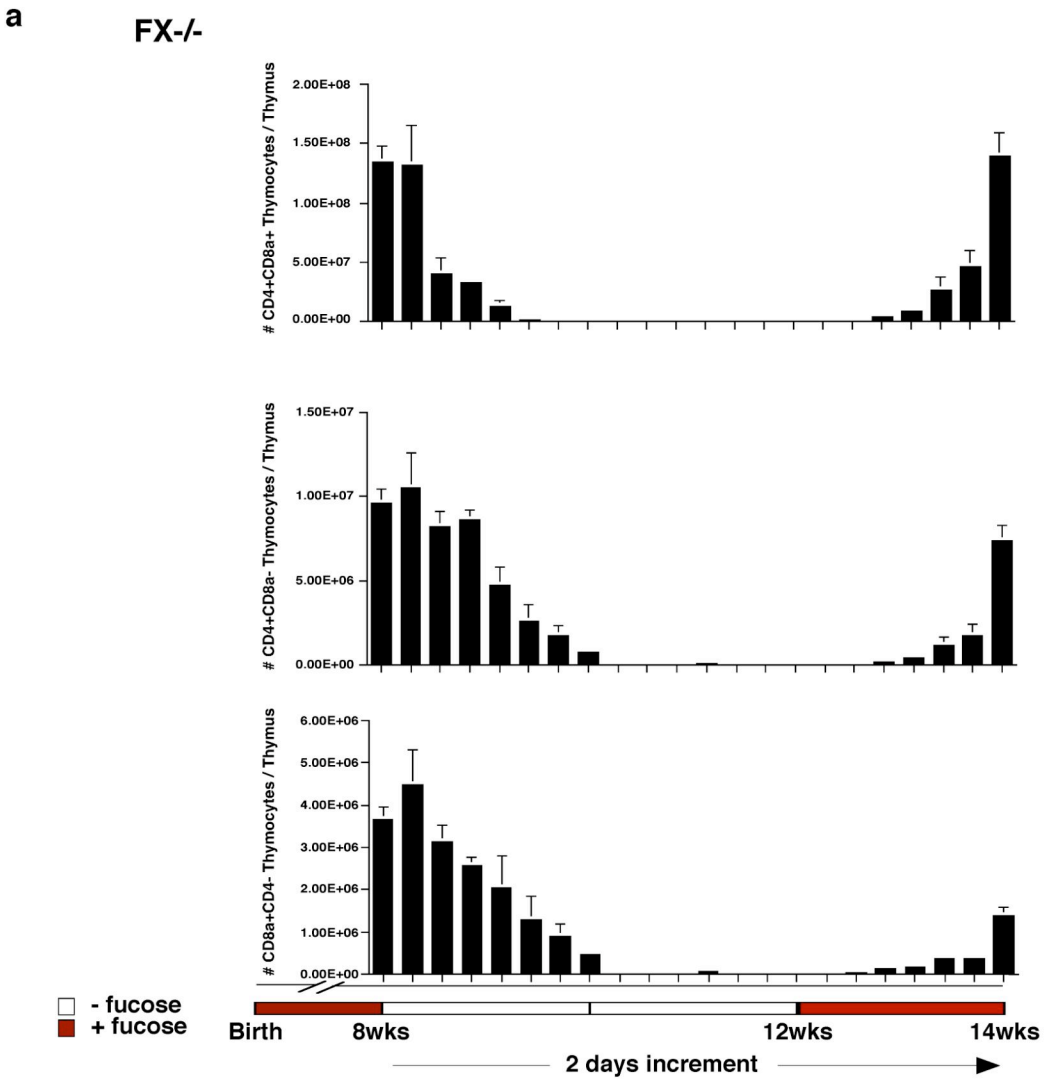


Figure 2-3 FX-/- on fucose mice have normal thymic development. N> or =3 for each data point. Error bars represent SEM.

Figure 2-4 Mature T cells greatly decreased in FX(-/-) thymus. a. CD4+CD8a+. CD4+CD8a-, and CD4-CD8a+ subsets of cells in FX(-/-) total thymocytes decrease upon the time off fucose in FX(-/-) mice. N>=3 for each data points. N=37 for off fucose 4 weeks. Error bars show SEM. b. Representative flow cytometry data of 3 or more experiments on indicated genotypes on PI negative total thymocytes.

Figure 2-4


a
FX-/-
CD4+CD8a+ Thymocytes / Thymus
2.00E+08
1.50E+08
1.00E+08
5.00E+07
0.00E+00
CD4+CD8a- Thymocytes / Thymus
1.50E+07
1.00E+07
5.00E+06
0.00E+00
CD8a+CD4- Thymocytes / Thymus
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
- fucose
+ fucose
Birth
8wks
12wks
14wks
2 days increment

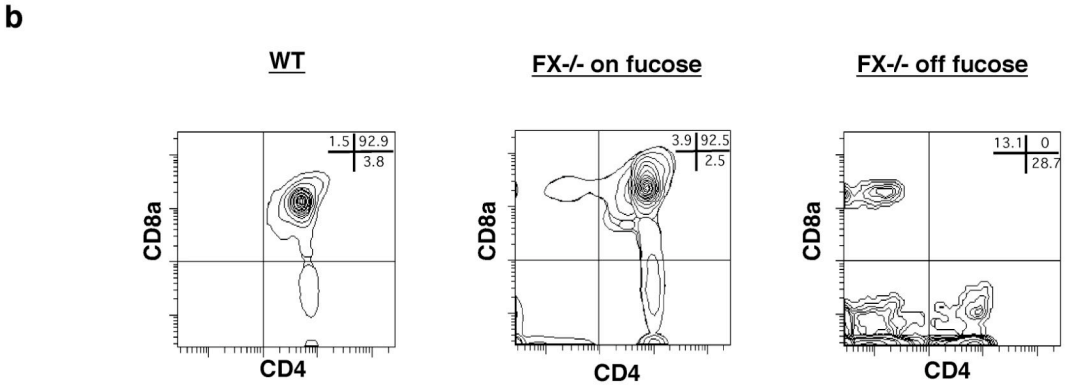

b
WT
FX-/- on fucose
FX-/- off fucose
CD8a
CD4
1.5
92.9
3.8
3.9
92.5
2.5
13.1
0
28.7

Figure 2-5

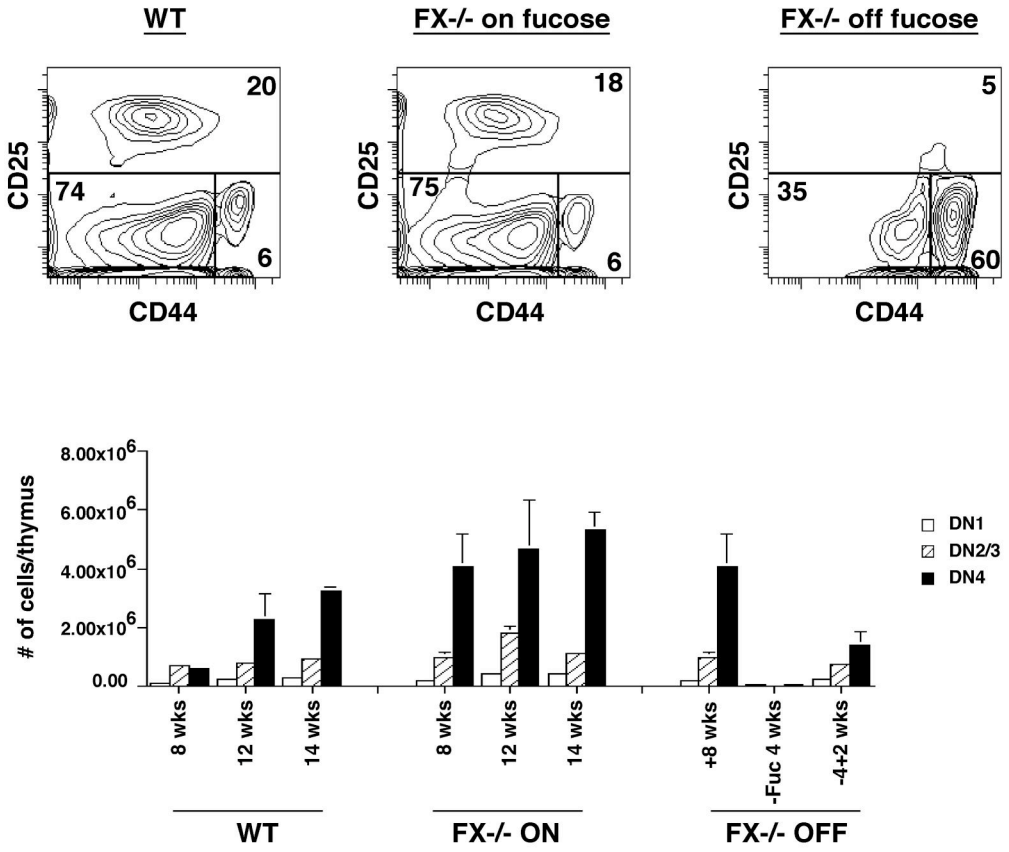


Figure 2-5 Lineage negative DN1-4 cells. Top panel shows the representative flow profiles of 4 individual mice in 3 different experiments. CD44 and CD25 on lineage negative, PI negative thymocytes. Lin-DN1: Lin-CD44+CD25- (lower right population), Lin-DN2&3: Lin-CD25+ (upper population) Lin-DN4: Lin-CD44-CD25- (lower left population) Bottom panel shows the numbers per thymus of each DN subsets of different ages and genotypes. Each bar shows the average of 4 mice, with error bar showing SEM.

Figure 2-6

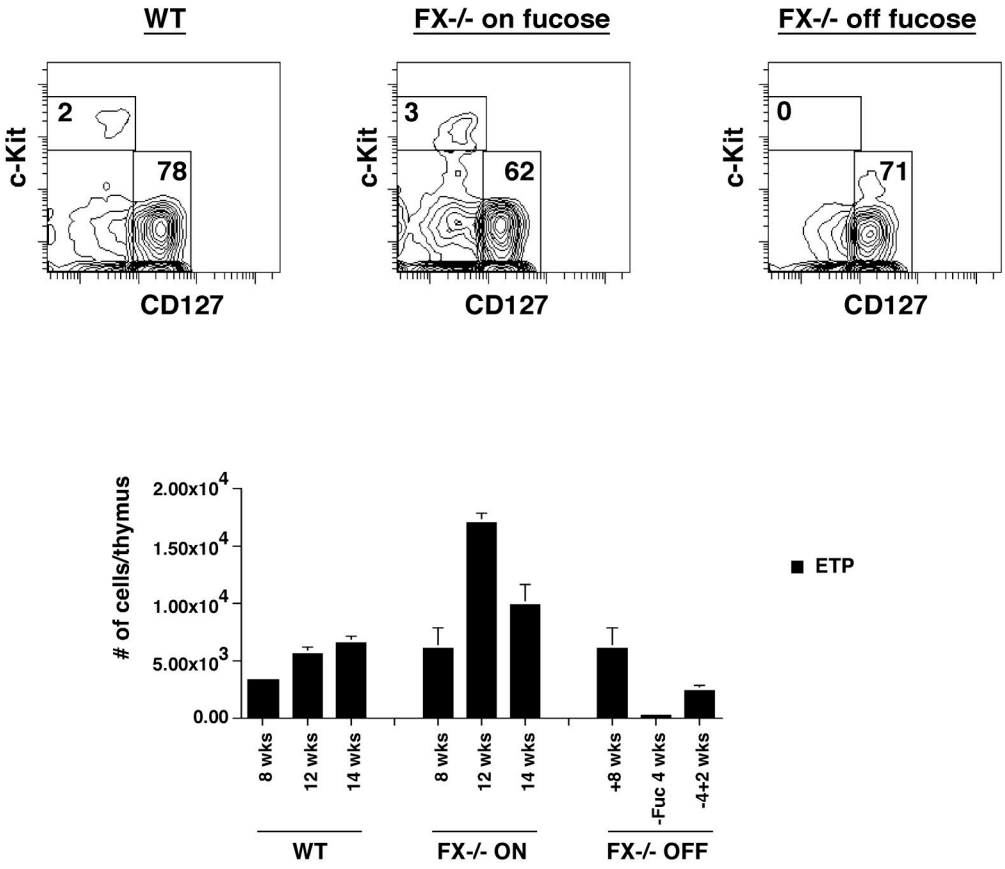


Figure 2-6 Early T cell progenitors. Top panel shows the representative flow profiles of 4 individual mice in 3 different experiments. CD127 and CD117 (c-Kit) on lineage negative, PI negative DN1 thymocytes. ETP: $Lin^{-}DN1CD127^{-}c\text{-}Kit^{+}$ Bottom panel shows the numbers of ETPs per thymus of different ages and genotypes. Error bars=SEM.

Figure 2-7

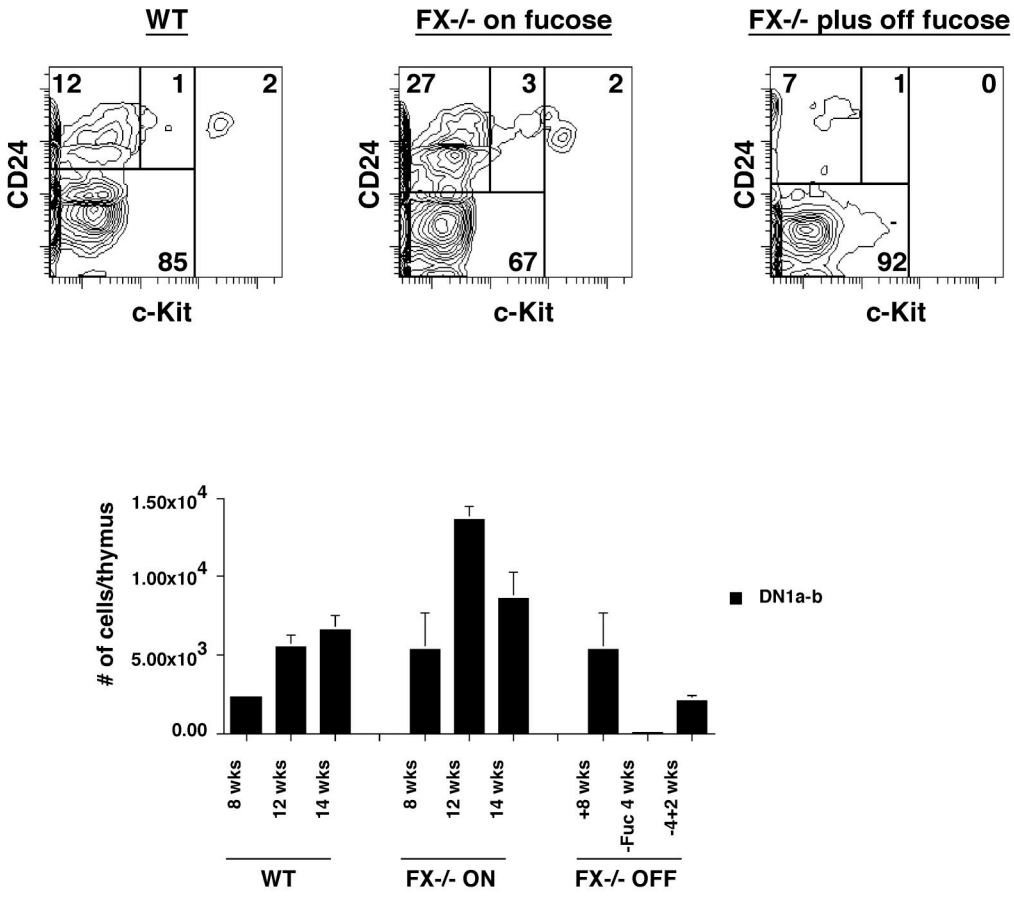
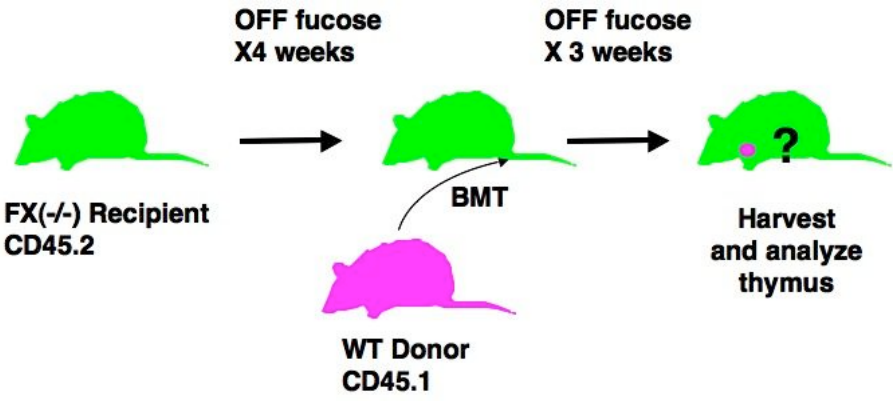
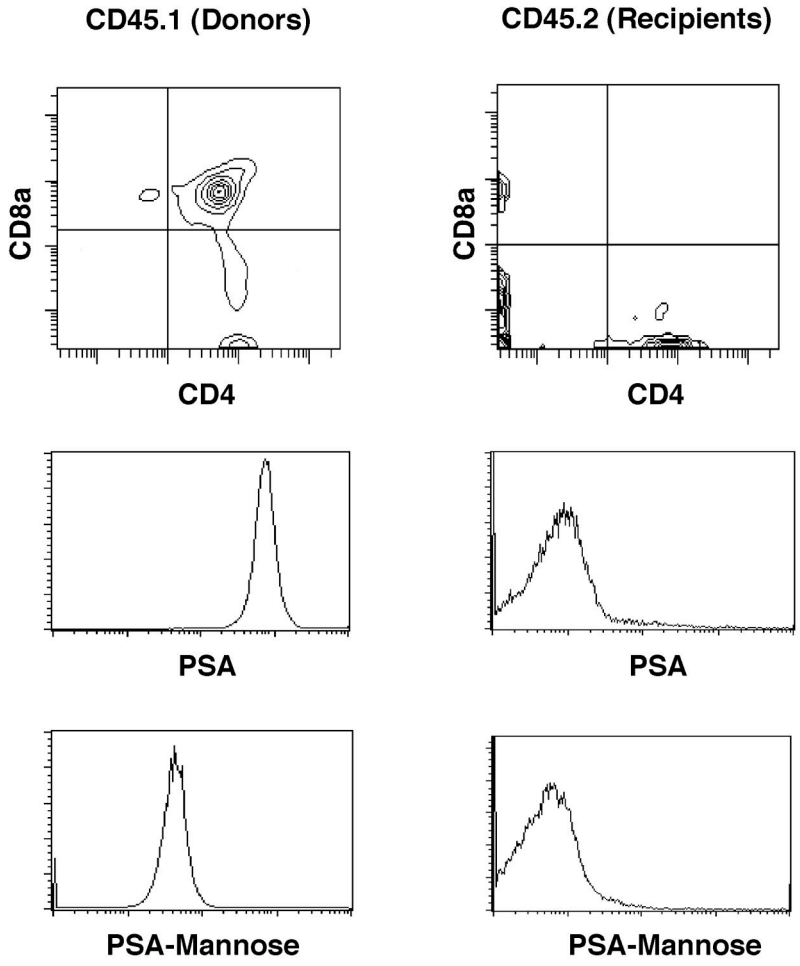


Figure 2-7: DN1a-b T cell progenitors. Top panel shows the representative flow profiles of 4 individual mice in 3 different experiments. CD24 and CD117 (c-Kit) on lineage negative, PI negative DN1 thymocytes. DN1a-b: $CD24^{+}CD117^{+}$ Bottom panel shows the numbers of DN1a-bs per thymus of different ages and genotypes. Error bars=SEM.

Figure 2-8: Cell autonomous defect in FX(-/-) hypoplastic thymus. FX(-/-) off fucose thymic epithelial cells and soluble cytokine are taken together as "thymic environment in the absence of fucosylation" tested by intravenous injecting wild type bone marrow cells (Top panel). Four weeks after bone marrow transplantation, donor (CD45.1) and recipient (CD45.2) thymocytes were analyzed.

Figure 2-8


OFF fucose
X4 weeks
OFF fucose
X 3 weeks
FX(-/-) Recipient
CD45.2
BMT
?
Harvest
and analyze
thymus
WT Donor
CD45.1


CD45.1 (Donors)
CD45.2 (Recipients)
CD8a
CD4
CD8a
CD4
PSA
PSA
PSA-Mannose
PSA-Mannose

Chapter III

Dysfunction of Notch1 signaling in pathogenesis of FX(-/-) thymic atrophy

Abstract

FX(-/-) mice lacking the *de novo* fucose synthesis pathway displayed a severe cell autonomous thymic atrophy, with a loss of multiple mature thymocyte populations. This phenotype is very similar to that reported in Notch1 conditional knockout mice, and thus it is very likely that the Notch1 signaling pathway is disrupted in FX(-/-) mice. To test this hypothesis, the Notch1 intracellular domain was introduced into fucose-deficient FX(-/-) bone marrow-derived lymphoid progenitors, and the cells were assayed for T lymphoid developmental potential. This experimental maneuver rescued the T cell developmental defect characteristic of FX(-/-) lymphoid progenitors and thus implicated a fucose-dependent requirement for Notch1 signaling in this process. *In vitro*, when genetically modified to express some Notch ligands, a bone marrow-derived stromal cell line termed OP9 can support the generation of the T-lymphoid lineage from bone marrow stem cells, providing another platform to test our hypothesis. OP9 cells bearing the Notch ligands Delta-like1 (Dll-1), Delta-like 4 (Dll-4) or Jagged2, when co-cultured with WT bone marrow stem cells, instruct such cells to assume a T lymphoid differentiation identity, whereas while OP9 cells bearing Jagged1 or Dll3 do not. However, in a fucose-deficient experimental situation, FX(-/-) bone marrow cells fail to assume a T lineage identity when

co-cultured with OP9-Dll1, OP9-Dll4 or OP9-Jagged2, and fail to initiate Notch1 signaling events. These results indicate that fucosylation is required for Notch1 signaling-dependent T cell differentiation.

Introduction

Precisely correlating a phenotype with a specific cellular event in knockout mice is sometimes difficult due to factors such as the multiple functions of a protein, systematic defects, the crosstalk of signaling pathways, and environmental effects. In our study of FX(-/-) mice, it is even more difficult, since fucose is integrated into all different kinds of glycan structures. Fucosylated glycans can be found on a tremendous number of cell surfaces and intracellular proteins. Thus it is almost impossible to determine which exact fucosylated protein contributes to the thymic atrophy of FX(-/-) mice off fucose. However, similar thymic atrophy phenotypes can be found in other mouse models, which could hint at ways to narrow down the possibilities. In Chapter II, all of the proposed T cell progenitors in FX(-/-) hypoplastic thymus were enumerated, and the cell distribution patterns were compared.

Disruption of the Notch Signaling Pathway Results in Mature Thymocyte Loss

The FX(-/-) off-fucose thymus contains very few CD4+CD8a+ DP cells, a decrease of about 1000 fold compared with the wild-type thymus. DP cell loss has also been reported in a few strains of genetically engineered mice. Notch1 conditional knockout mice have partially suppressed Notch1 gene expression in the thymus, and 90% of thymocytes were lost in the thymus of these mice (77). DP and SP populations were 3 to 5 fold decreased in cell numbers in Notch1 conditional knockout mice. Since Notch1 conditional

knockout mice still had 75% of the Notch1 gene expression left in the thymus, the greater cell loss was expected if the Notch1 gene was completely suppressed. The second mouse model was the conditional knockout mouse of RBP-J, which encodes a Notch1 downstream signaling partner. This mouse displayed a 90% total cell loss 3 months after being induced and an approximately 10 fold of reduction of DP and SP T cells (198). RBP-J conditional knockout mice had a 25% RBP-J gene expression left in the thymus and were also expected to have a more severe phenotype in a complete knockout condition. The above results strongly indicate that disruption of Notch1 signaling immediately results in a loss of mature thymocytes.

Disruption of Notch Signaling Accounts for the Decrease of ETPs

Secondly, early thymic progenitors were missing in the FX(-/-) thymus off fucose for 4 weeks. In Chapter II, the DN1a-b population of the FX(-/-) hypoplastic thymus was greatly reduced, while some enrichment of DN1e population was observed. This pattern is very similar that in another study, in which the thymic progenitors lacked Notch1 signaling (162). Transduction of DNMAML1, a dominant negative regulator that inhibits Notch1 downstream signaling into bone marrow progenitors, resulted in the reduction of DN1a-b population within early T cell progenitors, with a slight accumulation of DN1e population. This DN1a-e distribution pattern is very similar to that of the FX(-/-) off-fucose thymocytes and strongly suggests a probable interruption of Notch1 downstream signaling in FX(-/-) mice.

In summary, comparing with the mice or the cells that lack of Notch 1 signaling, the FX(-/-) thymic atrophy has the similar defect during T lineage commitment. The hypoplastic thymus of FX(-/-) mice is very likely due to a similar disruption of Notch1 signaling.

O-fucosylation is essential for Notch1 Signaling

Within the components of Notch1 signaling, Notch receptors and Notch ligands are subjected to fucosylation on the EGF domains (15, 199). Fucose was known found as the terminal differentiation for glycan structures, but recently found also in an O-linked fashion. The O-fucose glycans are found at Serine/Threonine residues of consensus sequence C^2XXGGS/TC^3 within the second and the third cysteine of the EGF repeats (56). The enzyme responsible for adding fucose to the EGF repeats was first cloned in hamsters (16), then in humans and mice (17), and later in Drosophila (25). This enzyme was designated with the name of protein O-fucosyltransferase 1 (POFUT1).

Biochemical experiments have proved the important roles of fucosylation to Notch downstream signaling in Drosophila and mammalian systems. By using RNA interference, knock down of OFUT1 in Drosophila results in a notched wing phenotype resembling that of Notch null mutants (25). The Notch downstream target gene expression was regulated by POFUT1. In another study, the disruption of O-fucosyltransferase 1 gene in Drosophila, also known as Neurotic, resulted in a wing-notched phenotype similar to Notch mutants (200). These two independent studies indicated the requirement of POFUT1 in Notch signaling. Furthermore, the GMD mutant Drosophila has a global fucosylation deficiency in theory and displays a wing development defect similar to Notch mutant flies (201). In the mammalian system, O-fucosyltransferase 1 knockout mice display a embryonic lethal phenotype (58) that resembles Notch1 knockout mice (77), indicating defects in signaling pathways related to development. These observations suggest an essential role of O-fucosylation in the Notch1 signaling pathway.

In this chapter, we hypothesized that the FX(-/-) thymic atrophy phenotype was due to a lack of O-fucosylation and resulted from a Notch1 signaling deficiency. We first reconstituted Notch1 signaling *in vivo* and *in vitro* and tested whether Notch1 signaling was sufficient to rescue T lymphocyte development. Further, Notch1 downstream signal targets and the T cell specific transcription factor gene were studied with regard to the function of fucosylation. Our results indicate that Notch1 signaling is regulated by the function of

fucosylation in FX(-/-) hematopoietic progenitors and that fucosylation is required for T lymphocyte development.

Methods

Materials- All experiments were conducted in accordance with National Institutes of Health guidelines for the care and use of animals and with an approved animal protocol form the University of Michigan and Case Western Reserve University Animal Care and Use Committees. C57B/6 mice were obtained from Jackson laboratory. FX knockout mice were made by as previously described(93). FUT4/7 double knockout mice (DKO) were made, again as previously described (53).

*Total thymocytes isolation-*The thymus was isolated from the mice and mechanically disrupted to make a single cell suspension. Thymocytes were first resuspended in red blood cell lysis buffer (Sigma) at room temperature for 5 minutes, and washed twice in Hank's Balanced Salt Solution (HBSS, Gibco) with 0.1% Bovine Serum Albumin (BSA, Sigma). Cells were collected by centrifugation and resuspended for cell counts by Trypan blue

Bone marrow isolation- Bone marrow cells were flushed out in HBSS with 0.1% Bovine Serum Albumin (Tissue culture tested, Sigma) from the femur and tibia of the mice. Red blood cells were lysed after isolation with red cell lysis buffer (Sigma). Lineage depletion was carried out by incubating the cells with biotinylated anti-mouse CD3, CD4, CD8a, δγTCR, B220, Gr-1, CD11b, Ter-119, NK1.1 (BD, Pharmingen) antibodies followed by magnetic bead depletion (Miltenyi Biotec). Lineage depleted cells were used for retrovirus transduction. For LSK isolation, the lineage-depleted cells were further stained with FITC-anti-mouse Sca-1 and APC anti-mouse c-Kit antibodies.

As one alternative method, 5'-Fluorouracil treatment was used to deplete mature bone marrow cells, as previously described (202). In brief, 5'-Fluorouracil was

intraperitoneally injected with 150mg/kg to each mouse. Five days after injection, treated bone marrow cells were isolated and used directly as donors.

Retrovirus- Notch 1 intracellular domain (ICN1) (1735-2531) was inserted into pMIG vector to form pMIG-Noch1IC-EGFP, which was subsequently transfected into Phoenix E packaging cells. Viral supernatant was collected 2 days after the transfection and kept frozen at -80°C. The titer of retrovirus was determined by transduction to NIH3T3 cells in a serial dilution assay (data not shown). The transduction efficiency to NIH3T3 was above 90% on average (data not shown).

Retroviral transduction- ICN1 retrovirus was transduced as previously described (202). In detail, the isolated bone marrow progenitors were plated in 6 well plates in IMDM (Gibco) with 10%FCS (Hyclone), 1xL-glutamine (Gibco), 1xPrecillin/straptmycin (Gibco), 6ng/ml IL-3, 10ng/ml IL-6, and 100ng/ML Stem Cell Factor (R&D system). On the second day, the cells were centrifuged down in the plates at room temperature for 5 minutes at 1300rpm. Undiluted retrovirus supernatant was added to each well for maximum transduction efficiency. Polybrene was added to each well at the final concentration of 4ug/ml. The virus cell mixture was sealed in the dish and centrifuged at room temperature for 2 hours at 900g (2500rmp of table top centrifuge). Right after transduction the IMDM medium was changed and fresh IMDM medium was added to the cells. On the third day, the cells were again transduced with the same procedure and again cultured overnight.

Intrathymic injection- Donor cells were analyzed by flow cytometry prior to injection. For each recipient, $5x10^5$ donor cells were injected intrathymically after being reared off fucose for 6 days. On day 17, the thymus of recipients was isolated and the thymocytes were analyzed.

OP9 co-culture- OP9 stromal cells (ATCC) were transduced with bicistronic Ret10-EGFP vector alone or with a different full length Notch ligand inserted. Different OP9 cell lines were sorted with the same EGFP expression level to ensure the same expression level

of transduced genes. The function of different Notch ligands on OP9 has been tested by co-culture OP9 cell lines with C2C12 myoblasts as previously described (203).

1000 LSK cells were cultured on different OP9 cell lines with 1ng/ml of IL-7 and 5ng/ml of Flt3 ligand (carrier free, R&D system), as previously described (204). The culture was disrupted by vigorously pipetting on day 8 and day 12. One fifth of each well was transferred to the newly plated OP9 cells in each passage with new cytokines added in each passage. Analysis of the culture was performed on day 20.

$5x10^4$ to $1x10^5$ total thymocytes (equivalent to one FX/- off-fucose thymus) were cultured on different OP9 cell lines with 1ng/ml of IL-7 and 5ng/ml of Flt3 ligand (carrier free, R&D system), as previously described (204). On day 8, the co-culture was disrupted by vigorously pipetting, and all of the cells were passed to new layers of OP9 cells, with fresh cytokines added. On day 13, half of the disrupted culture was kept for analysis, while the other half of the culture was transferred onto new OP9 layers. The remaining culture was analyzed on day 20.

Real-time quantitative PCR- LSK cells cultured on OP9 cells were sorted via flow cytometry after 8 days in different OP9 co-cultures. The LSK progeny was gated by the right lymphoid-like size in the side and forward scatter gate, together with the GFP negative gate. Post-sorting efficiency was examined to ensure that the purity of LSK progeny was over 98%. RNA was isolated by using RNeasy plus (Qiagen) and then reversely transcribed by iScript kit (Bio-Rad). cDNA amplification was carried out using SYBR green Supermix reagents (Bio-Rad) on iCycler (Bio-Rad).

Primers--Primers were designed by MacVector software and the products of amplification were ensured within 100-150bp. Primer efficiencies were tested prior to the experiments by the serial dilution of the total thymocyte cDNA samples (data not shown). The primer pairs used had a typical efficiency of around 2.0 by experiment and in theory. GAPDH (forward: TCAAGAAGGTGGTGAAGCAGGC, backward: AAGGTGGAAGAGTGGGAGTTGC), Hes-1 (Forward:

AAATGACTGTGAAGCACCTCCG, Backward: TCACACGTGGACAGGAAGC), Deltex-1 (Forward: AAAGCGACGTGAAGCCTGTGC, Backward: TCACCTTCTGCATGTACCTCCG), GATA-3 (Forward: TCTGGAGGAGGAACGCTAATGG, Backward: TTTCGGGTCTGGATGCCTTC).

Results

Notch1 Signaling Rescues the Defect of T cell Development in FX(-/-) Mice off Fucose *In Vivo*.

To determine the mechanism for the thymic atrophy in FX(-/-) mice reared in a fucosylation-deficient state, we first asked if similar phenotypes had been reported in other genetically engineered mouse models. The majority of the thymocytes lost in the absence of fucosylation are due to the sizable decease of CD4+CD8a+ mature T cells as well as downstream single positive populations. The loss of mature T cell populations has been observed in several mouse models, including NOD/LtSz-scid mice (205), IL-7 Receptor -/- mice (206), Rag1-/- mice (207), and notably, inducible Notch1 knockout mice (77). Notch1 knockout mice had a dramatic decrease of DP cells and SP cells. More excitingly, Notch1 proteins are fucosylated, while IL-7 receptor and Rag1 proteins have not been reported as fucosylated glyco-conjugates. Thus, losing fucosylation does not likely disturb the functions of the IL-7 receptor and Rag1 recombinase. Rather, these observations indicate that the loss of T differentiation potential in FX(-/-) progenitors was very likely due to abolishing the functions of Notch1 receptors on the cell surface.

In the absence of fucosylation, FX(-/-) mice lost multiple T cell progenitor populations, including the early T cell progenitors (ETPs) defined by $CD117^{+}CD24^{-}IL7R\alpha^{-}$ within DN1 cells. The ETPs are generated in a Notch-dependent manner (162). Loss of the ETPs has also been observed in the thymic progenitors where Notch1 signaling was

suppressed by DNMAML1 (162). These observations pointed to a Notch1 signaling deficiency in non-fucosylated T cell progenitors as a mechanism that accounts for thymic atrophy in the FX(-/-) mice.

To determine whether Notch signaling can rescue T cell differentiation in FX(-/-) mice off fucose for 4 weeks, Notch1 signaling was introduced by retroviral transduction of the Notch1 intracellular domain (ICN1) in bone marrow progenitors, as previously described (202). ICN1 was inserted into the bicistronic retroviral vector pMIG that has an internal ribosome entry site (IRES). IRES on pMIG allows the inserted gene (ICN1) to be independently expressed with an Enhanced Green Fluorescence Protein (EGFP) cassette. Thus the ICN1 gene expression may be monitored by the EGFP expression in the same cell. The pMIG-ICN construct was transfected into packaging cells, Phoenix E, and the viral containing medium was then collected for the transduction (208). Virus viability has been tested by transduction to NIH3T3 fibroblast cells with a typical >95% efficiency.

Donor bone marrow progenitors (CD45.1) from FX(-/-) mice off fucose for 4 weeks were enriched via two different methods. First, 5-fluorouracil was used for injection to the animal *in vivo* in order to deplete mature bone marrow cells. In the second method, lineage depletion via magnetic beads was carried out on untreated total bone marrow cells *ex vivo*. Since not every single donor cell was transduced, cells expressing ICN1 were traced by EGFP expression and CD45.1 expression. Lineage analysis was carried out before injection. No CD4 and CD8a expression were detected after transduction in the progenitor cells (data not shown).

To bypass the possible homing deficiency of FX null cells, $5x10^5$ transduced bone marrow progenitors in 10μl of PBS with 1% BSA were injected intrathymically into the FX(-/-) ($CD45.2^+$) recipient mice off fucose for 6 days, when the thymus was big enough for the injection. As shown in Figure 3-1, three populations were injected separately in each experiment. The viral vector transduced bone marrow donors from wild-type mice were used as positive controls. The viral-vector-transduced donor FX(-/-) off-fucose cells were

used as negative controls. The experimental group was the ICN1 transduced FX(-/-) off-fucose progenitor cells. The recipient thymus was analyzed 17 days post-transplantation by flow cytometry, in accordance with the previous studies (165).

Figure 3-2 show one representative flow data out of 10 experiments on day 17 post-injection. Transduced donor cells were gated by CD45.1 positive expression and GFP positive expression. The left-panel shows that the wild-type bone marrow progenitors transduced with the pMIG vector were capable of differentiating into $CD4^{+}CD8a^{+}$ mature T cells in the FX(-/-) recipient thymus. The FX(-/-) bone marrow progenitors transduced with the pMIG vector remained in the $CD4^{-}CD8a^{-}$ stage (middle panel) and were not able to differentiate into mature T cells. However, when ICN1 was expressed in the FX(-/-) bone marrow progenitors, these progenitor cells were able to differentiate into CD4+CD8a+ T cells (right panel). These observations indicate that the *in vivo* restoration of Notch1 signaling rescued the ability of FX(-/-) bone marrow progenitors along the T lineage developmental pathway in the fucose-deficient environment. These results suggest that the hematopoietic progenitors of FX(-/-) mice were deficient regarding Notch1 signaling.

Fucose-dependent Notch1 Signaling Rescues FX(-/-) off Fucose in T Cell Development *In Vitro*

To further confirm that restoring Notch1 signaling in non-fucosylated FX(-/-) T cell progenitors can fully recover mature T cell generation, an *in vitro* assay for T cell differentiation was used, including the co-culture system with OP9 stromal cells (209). As has been shown in recent studies, the generation of CD4+CD8+ T lymphocytes from the different multi-potent progenitors can be achieved by using Dll1 transduced OP9 cells (159, 204, 210, 211). In our study, each of the five mammalian Notch ligands was tested to define their specific functions in the T cell development pathway.

The OP9 cells from ATCC were transduced with different constructs in Ret10 bicistronic EGFP-containing retroviral vector (as shown in Figure 3-1). Each construct

contained one of the five full-length Notch ligand mouse cDNAs (Dll1, Dll3, Dll4, Jagged-1, or Jagged-2). The control OP9 cell line was transduced with an "empty" Ret10 vector. Transduction efficiency was examined by the EGFP protein expression. All six transduced OP9 cell lines were sorted for the same medium/low EGFP expression level (data not shown). Further, these OP9 cell lines were tested in the C2C12 myotube differentiation assay to test the functionality of each ligand (Stephanie Chervin and John Lowe, unpublished data). Each of the OP9-ligand cell lines, except the OP9-Dll3, have inhibitory effects on the myotube differentiation (data not shown), as previously reported (212, 213).

The LSK (Lineage$^-$ c-Kit$^+$ Sca-1$^+$) cells sorted from 12-week-old wild-type mice were first used to characterize this co-culture assay. Post-sort analysis was carried out to ensure purity (90% in average). On day 0 of the culture, 1000 LSK cells from wild-type mice were seeded on OP9 cell lines (204, 210, 214) in the presence or absence of 1mM fucose. A IL-7 concentration lower than 5ng/ml of the original reported system was used to optimize T cell generation (210). Since our purpose was to provide the *in vitro* assay that favors T cell development, 1ng/nl IL-7 was consistently used, as in the previous report (210).

On OP9 cells transduced with Ret10 or with Ret10-Dll3 or with Ret10-Jagged-1, wild-type LSK differentiated along the B lineage and myeloid lineages in the presence and absence of fucose, which confirmed previous reports that B and myeloid lineage differentiation proceeds in the absence of Notch1 signals in some circumstances (204, 214). Dll3 and Jagged-1 were designated as "non-functional ligands" for T cell development. However, B cell generation in OP9-Dll3, OP9-Jagged1 or control OP9 culture in WT and FX(-/-) LSK has occurred with differing incidence among experiments, and even among the wells in the same experiment, which indicates that the culture condition was not optimized for B cell development, as previously seen (162). In contrast to the B cells, myeloid cells were consistently generated, indicating that myeloid lineage is the default pathway in the absence of Notch1 signaling.

Among all the culture conditions, OP9 cells expressing Dll1, Dll4 or Jagged2 supported the generation of CD4+CD8a+ T cells from the wild-type bone marrow LSKs (Figure 3-3) in the absence and presence of fucose. We designated Dll1, Dll4 and Jagged2 as "functional ligands" for T cell development. Among those ligands, Dll4 and Jagged2 containing cultures generated 6-8 times more T cells percentage-wise, indicating that Dll4 and Jagged2 are more efficient than Dll1 in conferring T cell differentiation in this assay. The cell proliferation abilities, shown as number per input cells, were consistent with this observation (Figure 3-5). More interestingly, our results corresponded to the observations that Dll4 and Jagged2, but not Dll1, were greatly expressed in the thymus (215). These observations suggest that multiple Notch ligands play redundant roles in T cell generation while displaying different abilities. These results also imply a more complicated and finely regulated system in mammalian hematopoietic developmental pathways.

As shown in Figure 3-4, FX(-/-) off-fucose LSK did not progress to mature CD4+CD8a+ T cells in the absence of Notch ligands, or when non-functional Notch ligands were present (OP9, OP9-Dll3, OP9-Jagged1) in culture. These FX(-/-) cells derived to B cell or myeloid lineage cells regardless of the fucose present in culture, indicating that fucosylation is dispensable in B and myeloid lineage decisions. In the absence of fucose, even in the functional-ligand containing cultures (OP9-Dll1, OP9-Dll4, or OP9-Jagged2) myeloid and B cell were generated with full suppression of T cell development. Only when 1mM fucose was added to the culture was the T cell development pathway restored in FX(-/-) off-fucose bone marrow progenitors. These results indicate that T cell development is fucosylation-dependent only in the presence of "functional Notch ligand". These observations also suggest that the function of Notch1 signaling in T lineage commitment is fucosylation-dependent.

Fucosylation Controlled Notch1 Signaling Is Required for Intrathymic Development.

T progenitors developed from stem cells in bone marrow and travel via the blood to the thymus, where they further mature. The precise steps along this differentiation pathway are not yet fully understood (216-219). Although T cell development may be rescued *in vivo* and *in vitro* by restoring Notch1 signaling to FX(-/-) off-fucose bone marrow hematopoietic progenitors as shown in the previous experiments, it was not known if there were "blocks" inside the thymus along T differentiation.

Phenotypic analysis in Chapter II indicated that the multiple-potent T cell progenitors were lost from the thymus of FX(-/-) mice off fucose for 4 weeks (Figure 2-5, 2-6, 2-7). The paucity of the remaining cells within each T progenitor population makes it extremely difficult to complete biochemical or functional assays with these cells. To determine whether fucose is required for intrathymic progenitor development, total thymocytes from FX(-/-) mice off fucose 4 weeks were isolated and co-cultured with the OP9 and OP9-Notch ligand expressing cells in the presence or absence of 1mM fucose. Although multiple potential T progenitors were used in the same culture system, the proliferation potential of cells in OP9 culture would easily help us to find out if progenitors were blocked from maturation along the differentiation pathway.

For each experiment, the thymuses from 39 FX(-/-) mice reared off fucose for 4 weeks was isolated and the thymocytes were isolated by mechanic disruption. The thymocytes were combined in 3 different groups. In each group, 1/13 of the cells were phenotypically examined by flow cytometry to ensure that no DP cells were seeded in the culture (Figure 3-6 day 0). Then each group of cells was equally divided and seeded in 12 different wells (6 different OP9 cell lines with 2 conditions with or without fucose). Thus in each culture condition, 3 different wells were seeded in parallel. For each group of the cells, a fair comparison could be made within all 6 different conditions.

On average, $5x10^4$ to $1x10^5$ cells (equivalent to one FX(-/-) thymus off fucose 4 weeks) were seeded in the wells of a 24-well plate. With a supplement of fucose in the medium, thymocytes proliferated over time in OP9-Dll1, OP9-Dll4 or OP9-Jagged2

cultures (Figure 3-7a). Lineage commitment to $CD4^+CD8a^+$ cells was observed by flow cytometry on day 13 (Figure 3-6) and day 20 (data not shown) in OP9-Dll1, OP9-Dll4 and OP9-Jagged2 cultures. These observations are consistent with OP9 cultured with FX(-/-) off-fucose LSK cells (Figure 3-4). However, in the absence of fucose, the thymocytes did not proliferate and in fact disappeared over time, in OP9-Dll1, OP9-Dll4 and OP9-Jagged2 cultures (Figure 3-6a). Proliferation and differentiation along myeloid ($CD11b^+Gr1^+$) and B ($B220^+CD19^+$) lineages was not observed in these culture conditions (data not shown).

The FX(-/-) thymocytes also disappeared over time on OP9-Ret10, OP9-Dll3 and OP9-Jagged1 cultures regardless of fucosylation, indicating that Notch1 signaling is required for intrathymic development. Proliferation and differentiation along myeloid ($CD11b^+Gr1^+$) and B ($B220^+CD19^+$) lineages was not observed in these culture conditions.

Several potential populations in the FX(-/-) off-fucose total thymocytes have the potential to progress through DP cells, including Lin-DN1, Lin-DN4 and DN1e (159, 214). Although we could not pinpoint the exact populations giving rise to DP cells, these observations indicated that, in one or more developmental transition, within the thymus fucose is required for Notch1 signaling related to T cell generation.

Fucosylation Dependent Notch1 Downstream Target Gene Expression and Lineage Related Gene Expression

Fucosylation is required for Notch1 downstream signaling in many different *in vitro* and *in vivo* systems, including the wing development of Drosophila (25, 58). However, the role of fucosylation in the Notch1 downstream signaling along T cell differentiation of mammals has not yet been examined. To directly confirm our previous observations, we further examined the activation of Notch downstream targets together with lymphocyte lineage related genes in the presence and absence of fucose in the LSK and OP9 cells co-culture system.

After 8 days in culture, 10,000 LSK progeny were sorted directly into lysis buffer by lymphoid-like light scatters and GFP negative, which eliminated contamination by OP9 cell lines that are GFP positive and have higher light scatters. Real-time PCR with SYBRGreen dye was used to monitor the expression levels of different genes. To further normalize the cell number, GAPDH was used as the internal reference.

Two Notch1 downstream targets were tested, Hes-1 (220, 221) representing RBP-J dependent signaling target, and Deltex-1(222, 223) as a RBP-J independent signaling target. In OP9-Dll3, OP9-Jagged1 and OP9 culture, Hes-1 and Deltex-1 were not activated in the cultures, regardless of the presence or absence of 1mM fucose. In OP9-Dll1, OP9-Dll4, and OP9-Jagged2 cultures, Hes-1 mRNA expression were upregulated 25 times or more in the presence of fucose (Figure 3-8), while Deltex-1 mRNA was increased in its expression more than 300-fold (Figure 3-8).

Pre T-alpha is a Notch1 downstream target (224) and is expressed on DN3 populations (224-226), specifically along T cell development. However, pT-alpha mRNA transcripts were so few to be detected in those LSK progenies (data not shown). GATA-3 is so far the specific transcription factor for T lineage (227) and was used in the same experiments to monitor the T lineage potential of cells. Similar to Hes-1 and Deltex-1, GATA-3 was expressed fucosylation-dependently in the FX(-/-) progenitor cells in Dll1, Dll4, and Jagged2 containing OP9 co-cultures (Figure 3-8). Although other fucose-dependent signaling pathways may contribute to T cell development, our observations strongly indicate that fucose is required for the activation of Notch1 downstream signaling as well as the T cell differentiation. The correlation of fucose-dependent Notch1 downstream signaling activation and fucose-dependent T cell differentiation has shown that in the absence of fucose, the LSK progeny displayed the silence of Notch1 downstream activation and they were unable to differentiate to T cells. These results also imply that the pathogenesis of the FX(-/-) thymic atrophy is the disruption of Notch1 downstream signaling and T lineage commitment by the abolition of fucosylation.

Discussion

FX(-/-) mice have a fucosylation-dependent hypoplastic thymus. Analysis of thymocyte populations in these mice pointed out a developmental defect of T cells that correlated with a loss of fucosylation. Early T cell progenitors are missing in the atrophic thymus, due to the loss of fucosylation. These conclusions from Chapter II raised the question of how fucosylation controls T lymphocyte differentiation. In comparison with the phenotypes of other experimental models, it was proposed that fucosylation ceases T cell development by disrupting Notch1 signaling. The experiments outlined in Chapter III were designed to test this hypothesis. Our observations in Chapter III reveal an important role for fucose in Notch1 signaling in the T cell differentiation pathway.

First, we took the approach of reconstituting Notch1 signaling *in vivo* by transducing the intracellular domain of Notch1 into hematopoietic progenitors. Retroviral transduction has been widely used to introduce genes to cells that are difficult to transfect. Bone marrow stem cells are difficult to transfect, and the efficiency of retroviral transduction is relatively low. Several studies indicate that only 0.1% to 1% of the human pluripotent hematopoietic stem cells are potentially targeted during retroviral transduction (228, 229). In murine models, retrovirus has been widely used and nearly every experimental detail of designing retroviral vector to increase transduction efficiency has been greatly improved over time. The Phoenix E packaging cell line (230) was made based on the genetically engineered BOSC 23 cell line capable of producing high-titer helper-free retrovirus (231). Compared with the BOSC 23 cell line, the Phoenix E (Phoenix Ecotropic) cell line is highly transfectable with either calcium phosphate mediated transfection or lipid-based transfection protocols. High-titer retroviruses were obtained in 3 days by transient transfection in

Phoenix E cells. Thus highly active retroviruses were used in transduction in order to increase the transduction efficiency.

Notch1 intracellular domain inserted retrovirus has been used previously to identify the functions of Notch signaling in various experimental systems. Warren Pear and colleagues have defined the transduction protocol in their studies of Notch1 signaling in T cell leukemia (76, 202, 232-234). Spinoculation during transduction was applied in our procedure so that the maximum amount of virus could hit bone marrow stem cells (235). In addition, retroviral transduction on the same stem cell population twice in consecutive days potentially increased the ratio of transduced cells (232).

The intrathymic injection technique was developed to study the process of bone marrow cell homing to the thymus. This is a complicated surgical procedure made even more challenging by the small size of the FX(-/-) recipients with a hypoplastic thymus. Transduced cells were analyzed carefully right before being injected into the recipient mice. EGFP expression has been seen in transduced bone marrow cells to validate the transduction. Cell surface markers of CD4 and CD8a were used in the analysis of transduced bone marrow progenitors in order to ensure the early developmental stage of injected cells. No mature T cell populations were identified in the transduced bone marrow cells that were injected. This observation has been consistent across our 10 or more experiments.

The functions of Notch1 and its ligands in hematopoietic cell differentiation have long been sought in different *in vitro* experimental setups. Soluble human Dll-1 was shown to delay the differentiation of mouse hematopoietic progenitors in a culture dish (236). Meanwhile, hDll-1 promoted the expansion of primitive precursors *in vitro*. Plated-coated Notch ligand of Dll1 was shown to be required for Notch signaling and downstream gene activation (203). However, bone marrow cells cultured on plate-bound Dll1 could only progress to the DN2 (CD25+) stage along T cell development. To identify the role of Notch ligands in lymphoid differentiation pathways, L. Parreira and colleagues expanded

the idea from a co-culture system (237), in which bone marrow stem cells were cultured with a bone marrow stromal cell line. In their studies, bone marrow stromal S17 cell line was transduced with different Notch ligands and was coated as the bottom layer in co-culture with human bone marrow progenitors (238). Our initial adaptation of this co-culture system by Lan Zhou and Stephanie Chervin for the study of T cell differentiation was not able to identify the generation of mature T cells (unpublished data). However, it seems that greater T cell differentiation efficiency is achieved with another bone marrow stromal cell line of OP9 (204). It is not yet known how S17 cells differ from OP9 cells in terms of conferring T cell differentiation *in vitro*.

Originated from OP9-Dll1 co-culture system by Juan Carlos Zúñiga-Pflücker and colleagues (239), OP9 cells have been widely used in co-culture experiments in order to identify the roles of different Notch ligands. OP9-Dll1 cells have been shown to support T lymphopoiesis from mouse fetal liver cells (204) or adult bone marrow stem cells and embryonic stem cells (211). Progenitors from fetal liver were consistently able to progress to mature T cells on OP9-Dll1 (204, 240). However, the generation of CD4+CD8a+ T cells from bone marrow hematopoietic stem cells was not as reproducible on OP9-Dll1 cells (210). In our study, Dll1 had the weakest ability to induce T cell differentiation compared to Dll4 and Jagged2, which has not been reported in previous literature. Dll1 was recently not extensively expressed in the thymus, while Dll4 and Jagged2 were identified with a much higher expression level (215), indicating that Dll4 and Jagged2, but not Dll1, may actually play roles in thymocyte development. This result confirmed our observations of different functional abilities within Notch ligands that are able to induce T cell differentiation. Further, by comparing culturing systems of OP9-Dll1 and OP9-Dll4 (241), a recently published observation indicated that Dll4 has stronger abilities during T cell differentiation *in vitro*, which in part confirms our results.

Moreover, previously only Jagged1 and Dll1 among all five Notch ligands have been extensively compared, in terms of functional roles (214, 238). Our work, again for the

first time, compared all Notch ligands in their functions of T cell differentiation in parallel. It is very interesting that Jagged1 and Jagged2 showed distinct functions during T cell development though in protein sequences they very closely resemble each other. It is not yet known how Jagged1 and Jagged2 transduce different Notch downstream signals. It is also intriguing that Jagged2 and Dll4 functionally resemble each other and yet possess very distinct protein sequences.

Mammalian cells possess five different Notch ligands, while flies have only two homologues. It is important to understand the differences among those ligands in order to understand the regulatory system of mammals in Notch signaling. By providing the functional information, our results potentially initiate the future study of Notch ligands with regard to their structures and signaling mechanisms.

Notch downstream signaling is very complicated in the way that many downstream targets are transcription factors themselves. These transcription factors such as Hes-1, Deltex-1 may activate many other genes, which forms complex signaling cascades. However, in order to induce Notch signaling, a simple process is required, namely the release of the Notch intracellular domain. In a biological context, Notch ligands interact with Notch receptors and induce a conformational change of the receptors, thus Notch receptors are processed by ADAM protease and gamma-secretase sequentially. There are many mechanisms potentially regulating the Notch signaling pathway, and our results have shown one possibility of fucosylation-dependent regulation. In the absence of fucosylation, Notch downstream genes are silenced and the T cell specific transcription factor (GATA-3) was not expressed. When Notch receptors are fucosylated, Notch signaling was transduced and downstream genes were expressed. The level of the fucosyltransferases expressed tightly regulates the presence of fucosylated structures. Recently, missing certain fucosylated glycans on Notch1 receptors was shown to interfere Notch1 downstream signaling (242). Our results and others suggest a mechanism by which regulating the expression of O-fucosyltransferase 1 may control the downstream gene expression of Notch1 signaling in T

cell progenitors in order to sustain the normal amount of mature T cells produced within mammals. This result suggests a novel function of fucosylation in the cellular regulatory system of mammalian development.

The results in Chapter III raise another question: how fucosylation regulates Notch1 signaling. Based on the conclusions introduced in the previous chapters, Chapter IV focuses on our approach to this question.

Notes to Chapter III

[1]Acknowledgments: We thank Peter Smith for technical support on intrathymic injection, Lan Zhou for initialization of OP9 culture, Stephanie Chervin and Melissa Ruffs for transducing OP9 Notch ligand bearing cell lines, Kary Oetjen for effort on setup real time PCR, Ying Plews and Rocio Guardia-Wolff for genotyping mice, and R. Michael Sramkoski at Case Western Reserve University Cancer Center Cytometry Core Facility for technical advice on cell sorting.

[2]Attribution of data: Melissa Ruffs and Bronia Petryniak cloned all of the DNA constructs used. Bronia Petryniak performed the transduction and contributed to intrathymic injection in Figure 3-1. Jay Myers performed the thymus dissection depicted in Figure 3-1. Clare Rodgers performed most of the FACS analysis in Figure 3-1.

Figure 3-1: Experimental designs of intrathymic injection. a. Illustration of DNA constructs used in producing retrovirus. b. Experimental scheme. Bone marrow progenitors were differently transduced and intrathymically injected into FX(-/-) off fucose recipient. 17 days after the injection, thymus was isolated and analyzed by flow cytometry.

Figure 3-1

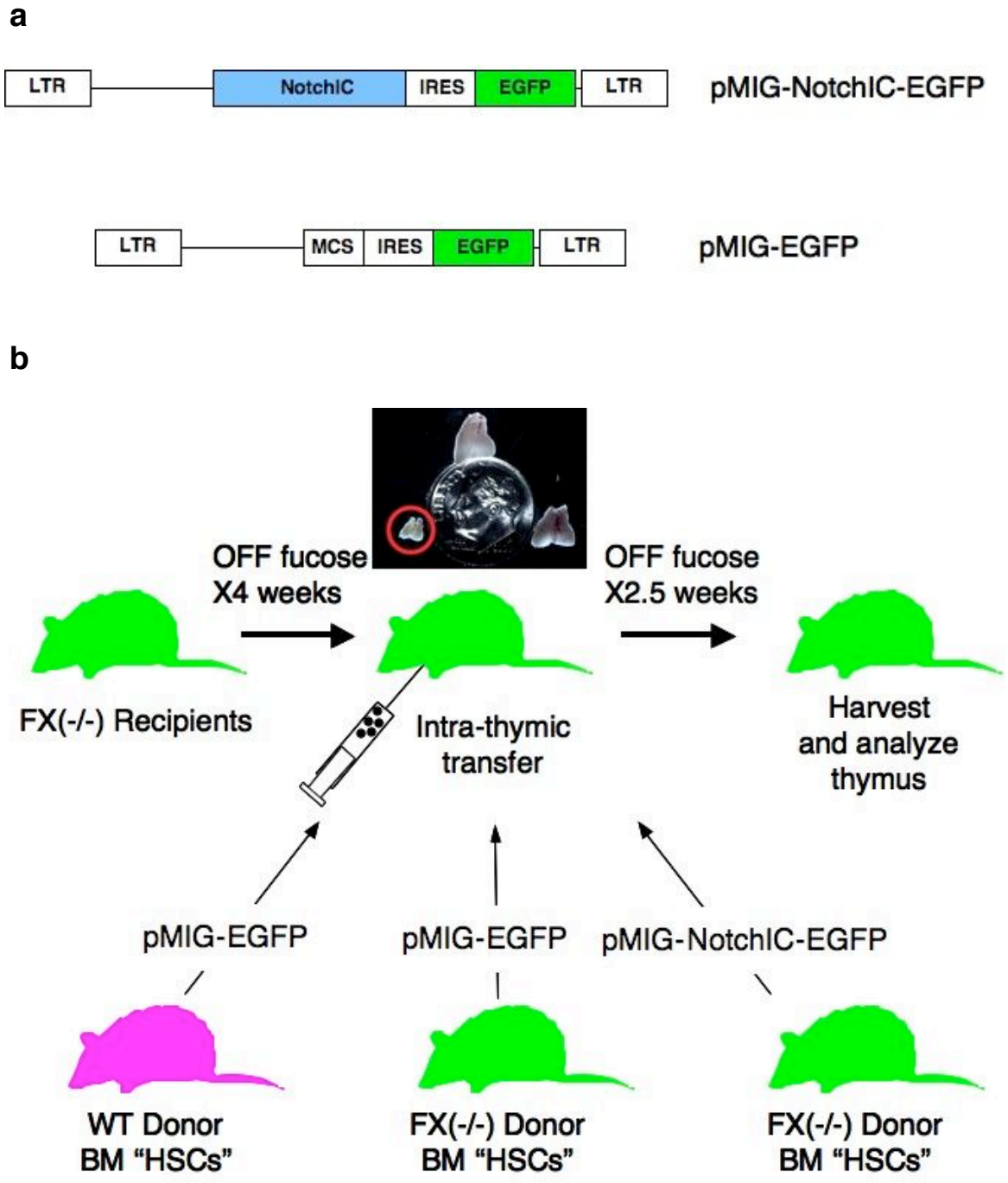

a
LTR
NotchIC
IRES
EGFP
LTR
pMIG-NotchIC-EGFP
LTR
MCS
IRES
EGFP
LTR
pMIG-EGFP
b
OFF fucose
X4 weeks
OFF fucose
X2.5 weeks
FX(-/-) Recipients
Intra-thymic
transfer
Harvest
and analyze
thymus
pMIG-EGFP
pMIG-EGFP
pMIG-NotchIC-EGFP
WT Donor
BM "HSCs"
FX(-/-) Donor
BM "HSCs"
FX(-/-) Donor
BM "HSCs"

Figure 3-2

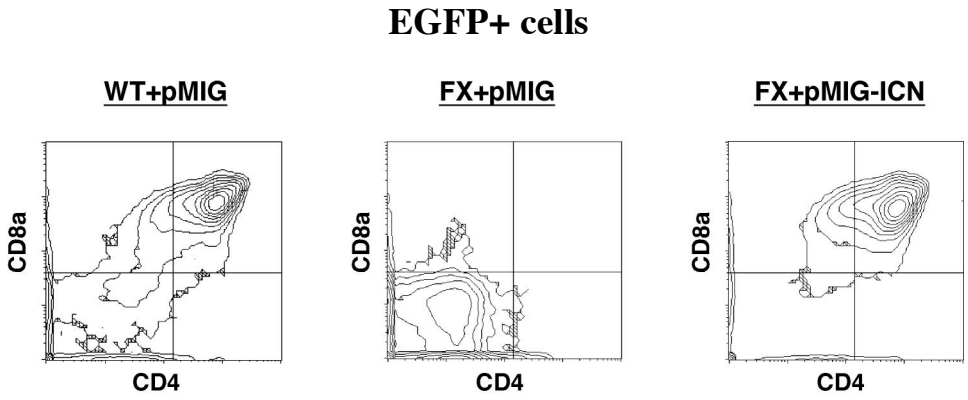


Figure 3-2: Notch1IC rescues FX(-/-) thymic atrophy in vivo. Transduced cells were identified by EGFP expression during analysis 17 days post injection. b. Representative of 10 recipients in 5 different experiments.

Figure 3-3: WT LSK co-cultured with OP9 cell lines. Representative of 6 and more experiments, in which WT bone marrow LSKs were cultured on different OP9 cell lines as indicated for 20 days, in the presence or absence of 1mM fucose.

Figure 3-3

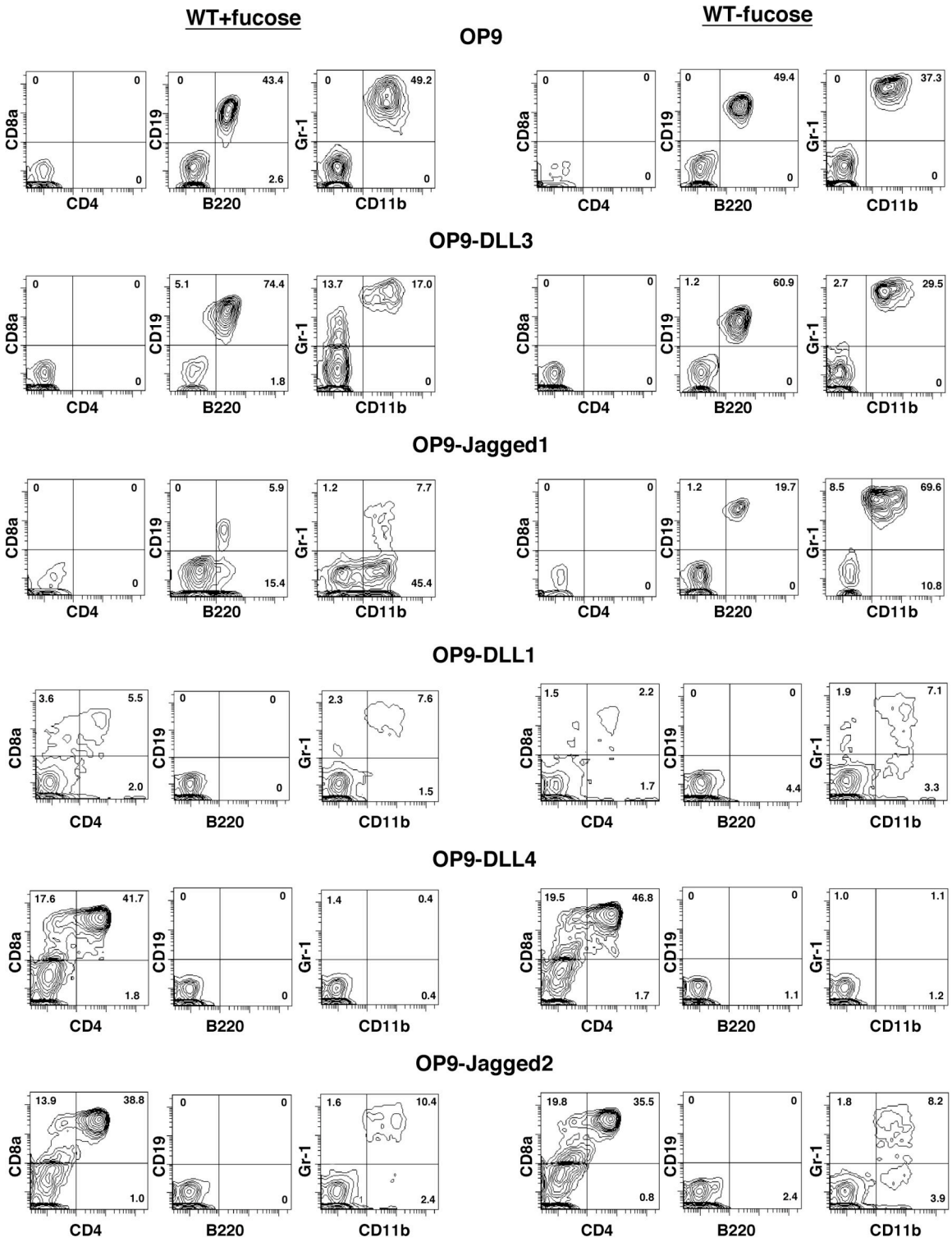

WT+fucose
WT-fucose
OP9
OP9-DLL3
OP9-Jagged1
OP9-DLL1
OP9-DLL4
OP9-Jagged2
CD8a
CD4
CD19
B220
Gr-1
CD11b
0
0
0
43.4
2.6
0
49.2
0
0
0
49.4
0
0
37.3
0
0
0
0
5.1
74.4
1.8
13.7
17.0
0
0
0
1.2
60.9
0
2.7
29.5
0
0
0
0
5.9
15.4
1.2
7.7
45.4
0
0
0
1.2
19.7
0
8.5
69.6
10.8
3.6
5.5
2.0
0
0
2.3
7.6
1.5
1.5
2.2
1.7
0
0
4.4
1.9
7.1
3.3
17.6
41.7
1.8
0
0
1.4
0.4
0.4
19.5
46.8
1.7
0
0
1.1
1.0
1.1
1.2
13.9
38.8
1.0
0
0
1.6
10.4
2.4
19.8
35.5
0.8
0
0
2.4
1.8
8.2
3.9

Figure 3-4: FX(-/-) off fucose LSK co-cultured with OP9 cell lines. Representative of 6 and more experiments, in which FX(-/-) off fucose 4 weeks bone marrow LSKs were cultured on different OP9 cell lines as indicated for 20 days, in the presence or absence of 1mM fucose.

Figure 3-4

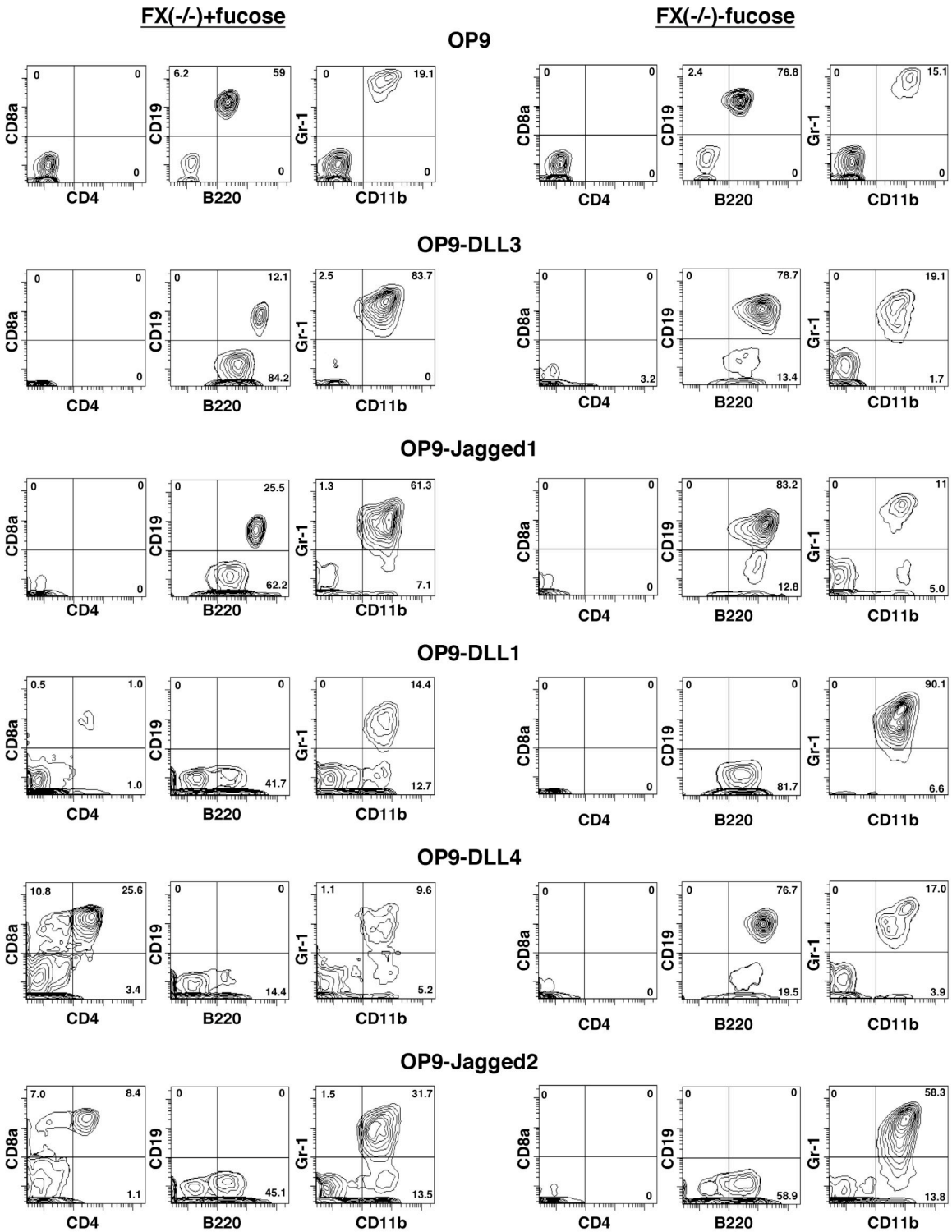

FX(-/-)+fucose
FX(-/-)-fucose
OP9
OP9-DLL3
OP9-Jagged1
OP9-DLL1
OP9-DLL4
OP9-Jagged2
CD8a
CD4
CD19
B220
Gr-1
CD11b

Figure 3-5

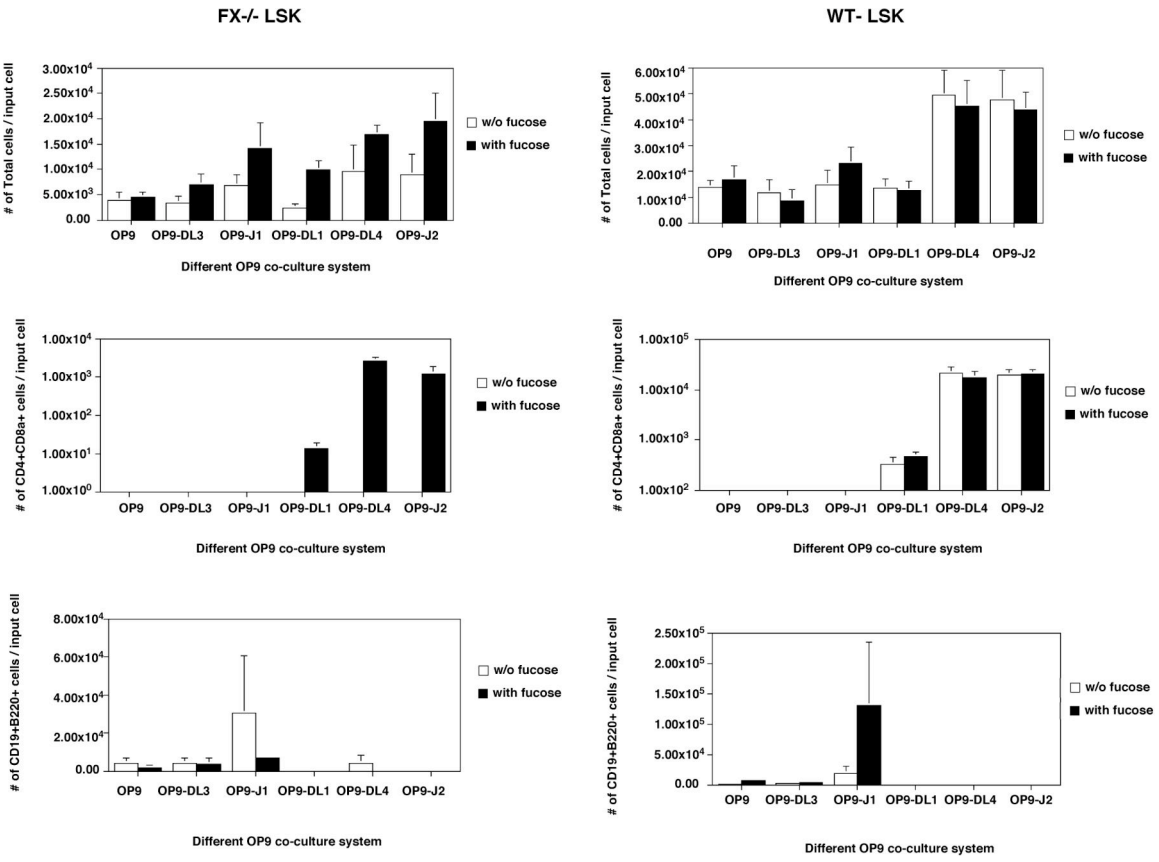


Figure 3-5: Cell proliferation in OP9 cultures. Left panels show the number of different cells per input as day 0 from FX(-/-) OP9 coculture (Figure 3-4) comparing with the ones in right panels from WT OP9 culture (Figure 3-3). Bar graphs represent the average of 10 or more data in 6 experiments, with error bar showing SEM.

Figure 3-6

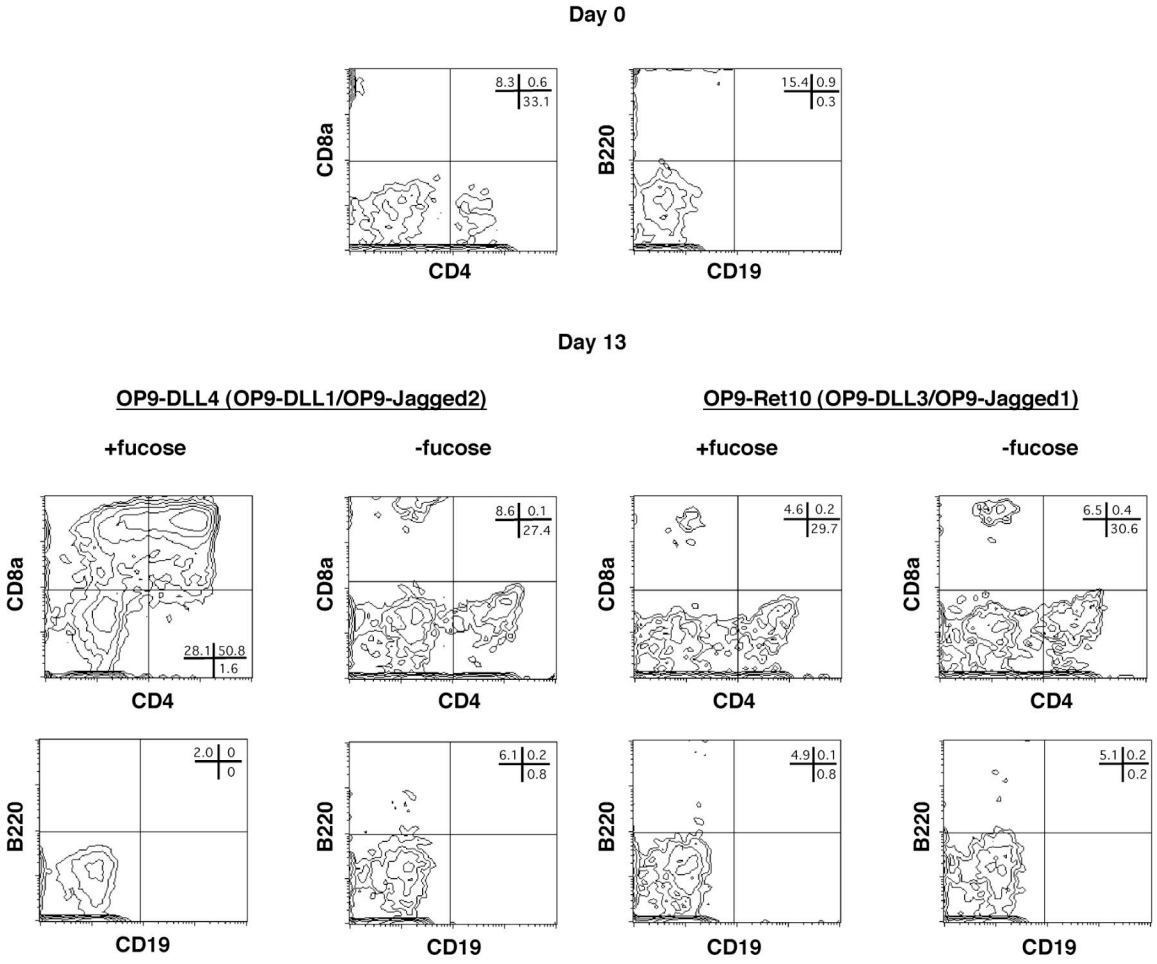


Figure 3-6. Fucosylation controlled Notch1 signaling is required for intrathymic development. Total thymocytes from FX(-/-) off fucose for 4 weeks were flowed at day 0 and seeded on different OP9 Notch ligand-bearing cells. After 13 days, cells were collected and analyzed by flow cytometry. Here shows the representative flow profiles of day0 and day 13 cultures.

Figure 3-7

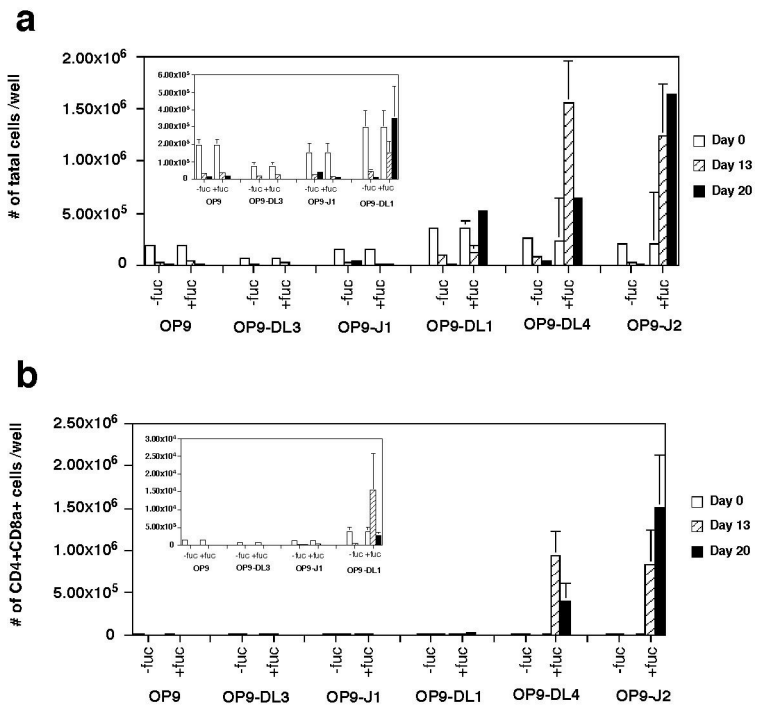


Figure 3-7 Cell proliferation of total thymocyte in OP9 cultures. a. Total cell proliferation upon the time has been shown in different OP9 cultures. b. CD4+CD8a+ DP cells generation upon the time in different OP9 cultures.

Figure 3-8

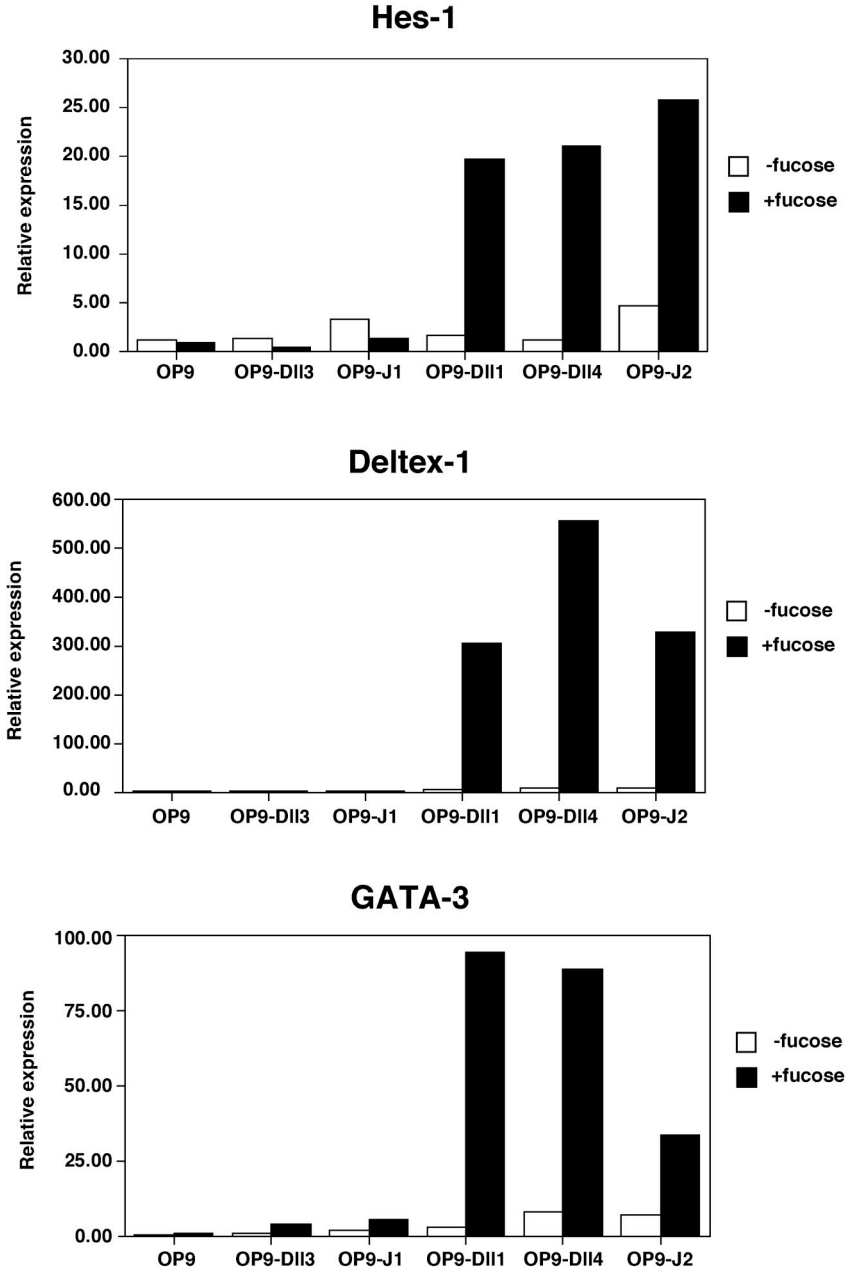


Figure 3-8: Notch1 downstream gene expressions of LSK progenies on day 8 in OP9 co-cultures. One of 3 independent experiments shown here with the mRNA expression monitored by real-time PCR. Hes-1 (top), Deltex-1 (middle) and GATA-3 (bottom) expression detected in 10, 000 LSK progenies sorted after cultured with OP9 cells for 8 days.

Chapter IV

Molecular regulations of Notch1 signaling by fucosylation

Abstract

Fucosylated glycans contribute to important cellular events, including the modulation of the Notch1 intracellular signaling pathway by modifying the epidermal growth factor (EGF) like repeats. Previous evidence suggests that a loss of fucosylation on Notch1 abolishes its signaling ability and its functions in T cell differentiation. To define the molecular mechanisms that account for fucosylation-controlled Notch1 signaling activation, I completed experiments to ask 1) whether fucosylation controls the amount of cell surface Notch1 expressed by cells, and 2) whether fucosylation modulates the strength of the interaction between Notch1 and its ligands. These experiments allowed me to conclude that (1) fucosylation controls the strength of binding between Notch1 and its ligand Dll4, using OP9-Dll4 cells and a recombinant Notch1-IgG fusion protein, and (2) fucosylation controls Notch1 receptor density on the surface of an E2a/Pbx1 immortalized bone marrow progenitor cell line. These results indicate that fucosylation controls Notch1 signaling strength and thus regulates the development of T lymphocytes.

Introduction

In the effort to fully understand the role of fucosylation in mammals, a mouse model with global fucosylation deficiency was generated by disrupting the gene FX that is responsible for making GDP-fucose, the only fucose donor in the de novo pathway. A thymic atrophy phenotype was observed and analyzed in detail. This phenotype resembled the phenotype reported in Notch1 conditional knockout mice, as it exhibited a virtually complete loss of mature thymocyte populations. Further, fucosylation-dependent disruption of Notch1 signaling has been shown to be the key reason for thymic atrophy in FX(-/-) mice deprived off fucose for 4 weeks at the cellular level. In the absence of fucosylation, bone marrow progenitors were not able to differentiate into mature T cells, either *in vitro* or *in vivo*. Moreover, non-fucosylated bone marrow stem cells were not able to express Notch1 downstream genes in conditions where Notch1 downstream signaling was induced. These observations assign an essential role to fucosylation in T cell development and related Notch signaling. However, the mechanisms that account for defective Notch signaling coincident with the loss of fucosylation remain unknown.

To pinpoint the roles of fucosylation and fucosylated glycans in Notch signaling, we took into consideration the details of Notch signaling and the ways in which fucosylation may modulate Notch signaling.

Which Components are Fucosylated in Notch Signaling Pathway?

Notch signaling is composed of Notch ligands on the signaling sending cells and Notch receptors on signaling receiving cells, together with multiple regulators and co-activators. All of the mammalian Notch ligands and receptors are large transmembrane proteins with multiple EGF repeats in extracellular domains (243).

EGF repeats have several structural characteristics. First, each EGF repeat has approximately 40 amino acids with six evolutionarily conserved cysteine residues. These

cysteines form three disulfide bonds between C1-C3, C2-C4 and C5 and C6 (244). Second, EGF repeats contain Ca^{2+} binding sites. However, a comparison between the structures of Ca^{2+}-containing EGF (245) and Ca^{2+}-free EGF (246) suggest that Ca^{2+} performs a minor role in conformation change. Third, two different types of glycosylation have been found on the EGF repeats, O-glucosylation and notably O-fucosylation (15). The O-glucose modification occurs at the serine residues of the first and second conserved cysteins of EGF repeat with the consensus sequence of C1XSXPC2 (10). More importantly, O-fucose is found linking to serine or threonine residue within the putative consensus sequence C2XXGG(S/T)C3 between the second and the third cysteins of the EGF repeat (242).

Potentially, EGF containing proteins are subjected to fucosylation. Because all of the Notch receptors and ligands contain multiple EGF repeats, they are all very likely to be fucosylated. Haltiwanger and colleagues have proved that Notch receptors (15) and Notch ligands (199) are all fucosylated. Other partners of Notch signaling have not yet been known to be fucosylated. Thus we focused on three different possibilities: fucosylated ligands, fucosylated Notch receptors, or both are required for Notch signaling.

Two of the three possibilities are easily ruled out, because fucosylation seems not to be required for Notch ligand function. As discussed in Chapter II, when wild type bone marrow cells were injected into a FX(-/-) recipient off fucose mouse, wild type T cell progenitors developed normally in the non-fucosylated environment of FX(-/-) thymus when deprived of fucose 4 weeks. As is widely accepted, Notch ligands are expressed on the surface of thymic stromal cells and instruct the development of the thymocyte progenitors. The fact that non-fucosylated Notch ligands are apparently able to direct thymocyte development suggests that fucosylation of Notch ligands may not be required for them to induce signal transduction.

Thus fucosylation on Notch receptors must be critical for proper signaling. Non-fucosylated Notch receptors may not signal. This is indicated by the experiments discussed in Chapter III, in which bone marrow stem cells carrying Notch receptors can only progress

through mature T cells in the presence of fucosylation. This conclusion helped us focus our efforts on the fucosylation of Notch receptors and to look for the roles of fucosylation in Notch signaling regulation.

Where and When does Fucosylation Occur on Notch Receptors?

As described above, Notch receptors contain EGF repeats where fucosylated glycans are added. Fucosylation is one of the post-translational modifications to proteins by the enzymes of fucosyltransferases, indicating that the location of fucosylation may be at ER, Golgi, cytosol and/or a cellular compartment other than nucleus. O-fucosylation of Notch EGF repeats is specifically carried out by protein O-fucosyltransferase 1 (197). The location of POFUT1 determines the location of fucosylation.

POFUT1 was first found located in ER in Drosophila (201), which is very different from Golgi-localized fucosyltransferases mediating the addition of N-linked fucosylated glycans. The ER-localized site for POFUT1 has been confirmed in mammalian systems (36). The ER is one of the most important places for protein synthesis, modification, and secretion. For a type 1 transmembrane protein such as a Notch receptor, mRNA is transcribed to protein and folded in the ER, while the signaling peptide immediately directs the protein localizing across the ER membrane. Notch proteins are very likely to be folded during or after synthesis via chaperon, a set of proteins helping polypeptide folding.

Temporally, O-fucosylation happens in ER in the early stage of protein synthesis, before the Notch receptor reaches the Golgi and is subsequently presented on the plasma membrane where it interacts with its ligands. There is evidence that POFUT1 also serves as a chaperon for the Drosophila Notch, suggesting that fucosylation plays a role in protein folding, at least in Drosophila (201).

How does Fucosylation Regulate Notch1 Signaling in Mammals?

From the above analysis, multiple roles of fucosylation in Notch1 signaling may exist; in Chapter IV two major roles of fucosylation in the regulation of Notch signaling have been proposed and tested.

1) The proposed role of fucosylation in ligand binding:

Glycans serve many roles, including their role within glycoprotein interactions. One of the widely known examples is P-selection glycoprotein ligand 1 (PSGL-1). PSGL-1 has a fucosylated moiety that serves as a component recognized by P-selection. Deletion of fucose on PSGL-1 abolishes the binding ability of PGSG-1 to its counter receptors. This could also be the case for O-linked fucosylated glycans, as they serve as a Notch ligand recognition motif on Notch. In flies, mutations of O-fucosylated sites on different EGF repeats have shown that EGF11 and 12 are required for the binding of Notch to its ligand. These two EGF repeats are required and sufficient for the ligand delta (247). A mutation of EGF12 with a substitution of Ala to Serine in order to abolish the fucosylation resulted in defects in wing development that are similar to a Notch null phenotype (248). *In vitro* evidence indicates that missing fucosylated glycans of Drosophila Notch protein at EGF12 enhanced Notch binding to its ligands (248). This observation indicates that, in flies, O-linked fucosylated glycans regulate the binding affinity of Notch receptors to their ligands. However, it remains to be determined whether fucosylation controls the binding of mammalian Notch receptors to its ligands.

Thus in the first part of this chapter, we pursued quantitative methods to characterize the binding activity of mammalian Notch1 and its receptors in two individual experiments as a function of fucosylation *in vitro*.

2) The proposed role of fucosylation in Notch receptor folding and secretion:

Notch receptor secretion was suggested by studies published in 2005 (201). These studies introduced Drosophila protein O-fucosyltransferase 1 and showed that this enzyme acts significantly as a fucosyltransferase, and also functions as a chaperon. Mutational

inactivation of the fucosyltransferase activity of POFUT1 does not seem necessary for Notch secretion, indicating that fucosylation is not required for Notch receptor presentation on the cell surface in flies. The method of confocal microscopy used in assessing the amount of surface Notch receptors in the studies was not satisfying, due to the limited number of subcellular regions focused on each time and the non-quantitative nature of this technique. However, this piece of information sheds light on, and may potentially contribute to new studies in the field of fucosylation.

Mammalian Notch signaling is more complex than that of Drosophila, given that there are more Notch receptors, more ligands, and a more complex mechanism involved. Mutation on EGF 27 of mouse Notch1 that abolished O-fucosylated glycans on that site reduced the amount of cell-surface Notch (242). These data strongly suggested that fucosylated glycans are used in the mammalian system during Notch1 protein folding and secretion. However, no similar data have been published yet on the consequences of the loss of fucosylated glycans on Notch. Given that O-fucosyltransferase would fucosylate all potential sites or none, it is impossible for cells to lose fucosylated glycans only on one particular EGF. Thus testing whether Notch secretion is regulated by global fucosylation seems essential to understanding the details of mammalian regulatory systems. In the second part of Chapter IV, a quantitative biochemical method was used to find out whether fucosylation is relevant to the surface presentation of Notch1 receptors. Considering this together with the binding studies, we hope that our effort will advance glycobiological studies of fucose as functionally important glycans.

Methods

DNA constructs-Full length mouse Dll4 or Notch1(1-15EGF) was cloned into vector pCDNA3.1 containing human IgG1 Fc portion, resulting in a fusion chimera of Dll1-hIgG1 or Notch1(1-15EGF) separately.

Transient transfection- 8x106 HEK 293T cells were plated in a 10-cm dish the day before transfection in DMEM medium with 10% FBS. 24ug of DNA and 60ul Lipofectamin 2000 (Invitrogen) was diluted separately in 1.5ml of OPTI-MEM medium and sat at room temperature for 5 minutes. DNA solution was added to Lipofectamin and sat at room temperature for 20 minutes for integration, before being added to the cells. 24 hours after transfection, attached HEK 293T cells were trypsinized and washed with serum free medium (Hyclone) 3 times. Then the cells were plated into 3 150-cm plates for protein production. The supernatant was collected 3 days after plating and cells were eliminated by centrifugation at room temperature for 15 minutes. To make fucosylated and non-fucosylated Notch1(1-15EGF), similar procedures were followed for Lec13 cells. Fucose was added on the day of transfection and in serum-free medium for CHO cells (Gibco).

ELISA--The ELISA quantification method for human IgG1 Fc chimeras was set up. Each well was coated with 100ul of 2ug/ml anti-hIgG1 (Sigma) at 4°C overnight. After being washed 5 times with PBS-0.5%Tween 20, 100ul human IgG (Sigma) in different concentrations was first added to develop a standard curve with a 2-hour incubation at room temperature. After being washed 5 times, 100ul anti-human IgG Alkaline phosphatase conjugated antibody (Sigma) was incubated for 1 hour at room temperature. 50ul of p-nitrophenyl phosphate (PNPP, Pierce) as the substrate was added for colorimetrical detection. After 15 minutes, the reactions were detected by the ELISA plate reader (Bio-Rad).

Notch1 fusion protein staining and confocal microscopy- OP9-Dll4 and OP9 cells were plated in $25x10^3$ cells/ml in 150mm dishes or on collagen-coated microscope slides

and grew for 2 days. OP9 cells in dishes were dissociated by 3mM EDTA at 37°C for 15min for flow cytometry staining. 70um cell strainers were used to eliminate cell clumps. Each 100,000 cells were aliquoted into each staining tube. 100ul of each Notch1(1-15EGF)-hIgG1 chimera dilution was added to each staining tube in OP9 medium at room temperature for 30min. PE-conjugated anti-human IgG was used as the secondary antibody for flow cytometry detection. The OP9 and OP9-Dll4 cells on the slides were stained by Notch fusion proteins in the same condition as above for 1 hour at room temperature. Texas-Red conjugated anti-hIgG was used as the secondary antibody for Notch1, since the emission wavelength of Texas-Red does not overlap with that of EGFP expressed by OP9 cells. Thus when the Argon and HeNe lasers are simultaneously working, the signaling leakage from green channel by EGFP would be minimized in the Red channel, so that red signals by Notch fusion protein could be sensitively detected. After staining, cells were fixed by 4% formaldehyde and mounted for examination (Leica Microsystems Inc). Typically a series of 30 pictures were taken from several different regions on the slide from the bottom of the cells to the top at about 6um intervals along the z-axis. To minimize the biased comparison among different regions of the cells, a picture of overlapped z-stack pictures was generated afterwards to show the total signal.

Ligand fusion protein staining- Dll4-hIgG1 chimera in serum-free culture medium was added to cells in a 1:1 dilution with culture medium at room temperature for 30 minutes. After being washed with the medium, the cells were incubated with a PE-conjugated anti-human IgG polyclonal antibody. Human IgG was used as a negative control.

Extracellular Notch1 dissociation by EGTA treatment- After being collected, the cells were washed twice in Hank's Balanced Salt Solution with 2.5mM $CaCl_2$ buffer. The Notch1 extracellular domain was dissociated by EGTA in TBS buffer in a 37°C water bath for 15 minutes. After the cells were centrifuged at 1300 rpm for 5 minutes, the dissociated proteins were collected in the supernatant. Cell pellets were treated with 1xRIPA buffer and

DNAs were sheared by being passed through an insulin syringe in a cold room. DNA and membrane debris were removed by centrifugation at top speed for 30 minutes at 4°C.

Western blot- Cell lysate or EDTA dissociated proteins with SDS loading buffer (Invitrogen) were boiled at 70°C for 10 minutes. Samples were loaded in 15-well 3-8% acrylamide gel and transferred to a PMSV membrane overnight. Anti-mouse Notch1 (8G10 clone, 1:200, Santa Cruz) was used to probe the membrane. After washes, anti-hamster HRP (1:5000) conjugated secondary antibody was added. To develop the films, after washing the membrane, ECL substrates (Pierce, Supersignal) were added for 1 minute. The membrane was stripped by Restore Western Blot Stripping Buffer (Pierce) and blotted by anti-mouse beta-actin (mouse monoclonal, 1:10,000, Santa Cruz) as loading controls.

Results

Our results in Chapter II suggested that the loss of fucosylation diminishes Notch1 downstream signaling. In this chapter, we further pursued the molecular mechanisms of fucosylation-dependent Notch1 signaling regulation. Losing fucosylation may disrupt Notch1 signaling in many different ways. It is likely that non-fucosylated Notch1 proteins are not presented on the cell surface; thus there are no Notch1 receptors to bind to its ligands for downstream signal transduction. We first tried to ask whether losing fucosylation perturbed the surface presentation of Notch1 proteins.

The Reduced Surface Presentation of Notch1 in the Absence of Fucosylation

The role of fucosylation in Notch1 protein secretion was tested by quantitative western blot experiments. In these experiments, Homogenous FX(-/-) myeloid progenitors were used. These cells were made by transducing the E2a/Pbx1 gene to FX(-/-) adult bone marrow progenitors. More specifically, the beta-estradiol response unit controlled E2a/Pbx1

gene was inserted into a viral vector pMIG to produce the oncogenic fusion proteins (249). Virus was produced by Phoenix E packaging cell lines and collected in supernatant. The FX(-/-) bone marrow stem cells were enriched *in vivo* by 5-fluorouracil treatment and were isolated by the depletion of lineage positive cells. These FX(-/-) stem cells were cultured for 2 days before transduction. After transduction, the transduced cells were selected by long-term culture and myeloid lineage was driven by the cytokine of GMCSF made by B16 cells (249). The expression of the E2a/Pbx1 gene was enabled by the addition of 1uM beta-estradiol. Only the cells expressing E2a/Pbx1 would exist in the culture and be able to avoid dying out. When the culture was 4 weeks old, individual clones were seeded by serial dilution methods. These clones were expanded and selected for their uniform GFP expression. I discovered 2 independently selected cell lines. These cells were cultured as single-cell suspensions at $1x10^6$/ml and fed on a daily basis. The progenitor cells are large round suspended cells with large nuclei (Figure 4-1a) and do not express mature granulocyte markers, such as Gr-1 (Figure 4-1b). When they were cultured in the absence of beta-estradiol, they further differentiated into polymorphonuclear neutrophils (PMNs) showing the segmented shape of nucleus (Figure 4-1a) and expressing Gr-1 on the cell surface (Figure 4-1b).

An FX(-/-) myeloid progenitor cell line was first characterized in its ability to bind to Notch ligands as a function of fucosylation (Figure 4-1c). Expanded cloned cells were originally made in the absence of fucose. They were divided in half and cultured in the absence or presence of 1mM fucose in the culture medium. Two days later, the cells were collected for staining. PSA staining showed the fucosylation status of the cells right before being used for the quantitative western blot experiments. Gr-1 staining demonstrated the homogenous progenitor-like developmental stage of these myeloid progenitors with all the cells lacking Gr-1 expression. Dll4-IgG chimera binds to fucosylated myeloid progenitors much better than the non-fucosylated ones, indicating the presence of non-fucosylated Notch1 receptors on the cell surface.

EGTA treatment was used to dissociate cell membrane Notch1 proteins, and the experimental setup is shown in Figure 4-2. The 8G10 antibody used on the western blot was made as targeted against the N terminal of mouse Notch1 (Figure 4-3a). At first, the EGTA dilution curve was generated to optimize the dissociation of non-fucosylated Notch1 proteins (Figure 4-3b). It seemed that 5mM EGTA was enough to dissociate all the Notch1 proteins on the cell surface, while 10mM EGTA was used to ensure maximum dissociation results. Because the FX(-/-) myeloid progenitor cell line was made in the absence of fucose, these expanded cloned myeloid cells were cultured in the absence of fucose or in 1mM fucose in parallel for 2 days before shedding experiments.

Dissociated proteins in the supernatants from $1x10^6$ cells were loaded per lane for comparison in the presence or absence of fucosylation (Figure 4-3c). Only one form of Notch was found in the supernatants, by size as known of extracelluar domain of Notch1 (ECN). More ECNs from the fucosylated cells were detected than the ECNs from the non-fucosylated cells. No other forms of Notch1 were detected. The absence of full-length Notch1 that reside inside of the cells indicated that the cells were not lysed by EDTA treatment.

However, in the cell lysate after EGTA treatment, two forms of Notch proteins were found. The smaller form of Notch1 by size is most likely to be the ECN sequestered inside the cells, perhaps within the protein secretion pathway. More ECN from fucosylated cells were found compared to the ECN found in non-fucosylated cells. The larger form of Notch1 is the non-processed Notch protein, the so-called full-length Notch1. In the cell lysate after EGTA treatment, fewer full-length Notch1 proteins from the fucosylated cells were found compared to the full-length Notch1 from the non-fucosylated cells. The presence of more naïve and unprocessed Notch1 proteins in non-fucosylated cells indicated that there was a defect in the Notch1 protein secretion pathway of the non-fucosylated cells.

Notably, a size difference was observed between fucosylated-Notch1 forms and non-fucosylated Notch1 forms, including ECNs and full-length Notch1. It seemed that

fucosylated-glycans on Notch1 EGF domains added a few kilo-Dolton to the protein weight, thus they migrated slower in electrophoresis. These results indicate that fucosylation is required for Notch1 secretion in mammalian cells. It is very different from the reported results in the Drosophila system (201), indicating the more complicated regulatory role of fucosylation of Notch signaling in mammals.

Although in a reduced quantity, there were indeed non-fucosylated Notch1 proteins on the cell surface. Since these non-fucosylated proteins presented on the cell surface, did they bind to the ligand as well as the fucosylated proteins? Non-fucosylated FX(-/-) E2a/Pbx1-immortalized progenitor cells had less binding affinity to the Dll4-hIgG1 chimera (Figure 4-1c). However, the reduced binding ability may result from the reduced amount of the surface Notch1 proteins that were available to bind to the Dll4 ligand. Thus we took a different approach to assess the binding abilities of the fucosylated and the non-fucosylated Notch1 proteins.

Non-fucosylated Notch1 Reduced Ability to Bind to its Ligand of Dll4 *In Vitro*.

A very precisely controlled experimental system has been designed to investigate the influence of fucosylation on Notch1 binding to its ligands (for details see the Discussion). OP9-Dll4 cells were used for Notch ligand Dll4 expression. The cells were dissociated from the plate by EGTA treatment. Because the Ca^{2+} ion does not contribute to forming the structure of EGF repeats, depleting Ca^{2+} by EGTA may not able to change the conformation of Dll4 proteins that contain only 8 EGF repeats. Moreover, Notch ligands are transmembrane proteins with no S1 cleavage (i.e. not heterodimer), which suggested that Notch ligands could not be dissociated by EDTA treatment. Thus the low concentration of the EGTA treatment is a "safe" method for generating individual OP9 cells bearing Notch ligand without losing Notch ligand proteins on surface.

A Notch1(1-15EGF)-hIgG1 chimera was made *in vitro*, in FX-deficient CHO cells (CL17), cells via transient transfection by Lipofectamin 2000. Cells were trypsinized 24

hours after being transfected and expanded in serum-free medium for protein secretion. The fucosylated Notch1(1-15EGF)-hIgG1 chimera was secreted by the transfected CL17 cells in the presence of 1mM fucose. The non-fucosylated Notch1(1-15EGF)-hIgG1 chimera was secreted by the transfected CL17 cells in the absence of fucose.

A human IgG specific ELISA was developed and optimized to measure the concentration of each chimera. IgG Fc portion of the chimera naturally dimerized, thus one hIgG1 chimera protein has two Fc sites available for antibody binding. The coating antibody for capture recognized one Fc site. The other Fc site was recognized by Alkaline Phosphate conjugated anti-human IgG antibody for detection (Figure 4-4a). A standard curve was developed by using the serial dilutions of purified human IgG Fc proteins. The O.D.405 readings were plotted versus different concentrations of chimera, and the resulted curve was linear-regressed (Figure 4-4b). The diluted Notch1 chimeras in this assay had readings in this linear range and the original concentrations were calculated according to the dilution factors. Finally, the Fucosylated and non-fucosylated Notch1 (1-15EGF) chimeras were normalized to 1800ng/ml, in order that the same molar of the proteins were made in serial dilution and were used in later experiments.

OP9-Dll4 cells and OP9 cells were expanded in culture for two different experimental setups (illustrated in Figure 4-5). The same batch of cells was used for staining on slides and in tubes to ensure the same number of Dll4 ligands on the cell surface. The same amount of the fucosylated or non-fucosylated Notch1 chimeras were used to test the binding abilities to the same number of Dll4 ligands.

In flow staining, OP9-Dll4 cells were stained by fucosylated-Notch1(1-15EGF)-hIgG chimera or non-fucosylated Notch1-hIgG chimera in different concentrations (Figure 4-6). Each pair of data showed that the same concentration of the fucosylated chimera and the non-fucosylated chimera were used and compared in parallel. OP9-Dll4 cells bound to the fucosylated-Notch1(1-15EGF)-hIgG more than the non-fucosylated Notch1(1-15EGF)-hIgG in all concentrations tested. These results indicate that the non-fucosylated

Notch1(1-15EGF)-hIgG1 has weaker binding affinity than the fucosylated chimera. As controls, OP9 cells were stained in the same experimental setup but showed binding abilities to neither the fucosylated nor the non-fucosylated Notch1(1-15EGF)-hIgG chimera (data not shown).

The mean fluorescence intensity of stained OP9-Dll4 cells in Figure 4-6 was plotted versus the different concentrations of Notch1 chimera used in staining (Figure 4-7). Low concentration points were plotted by linear regression, with the regression equations shown by the lines (Figure 4-7a). These data fit the linear curve with a coefficient of determination (R^2) very close to 1.00 (perfect fit), suggesting a precisely controlled experimental platform. The fucosylated Notch1 chimera staining curve had a greater slope (1.379) in the linear range, indicating a greater binding affinity than non-fucosylated Notch1 chimera (slope=0.49).

Figure 4-7b shows the full range of the different concentrations of chimeras used. Strikingly, the non-fucosylated chimera reached a binding plateau (MFI=319.59) of about half that of the fucosylated one (MFI=592.99), indicating different binding avidities of Dll4 to different forms of Notch1. These observations have been confirmed by an independent experiment of the surface plasmon resonance (Jeongsup Shim and John Lowe, unpublished data).

Further, confocal microscopy was used to confirm this result in an independent experimental setup. Figure 4-8 showed the representative area of each staining slide with a series of Notch1 chimera concentrations on OP9 and OP9-Dll4 cells. Notch1 chimera bound to Dll4 on the cell surface was represented by the red signals along the edge of the cells. Green signals indicated the intracellular region, since EGFP proteins are soluble and restricted within the plasma membrane. The co-localization of red and green signals was never observed, thus Notch1 proteins bound outside of the cells were not co-localized with EGFP sequestered inside the cells, indicating that there was no leak on the cell surface. In low concentrations, the Notch1(1-15EGF) chimera did not bind well regardless of whether

it was fucosylated. This may be due to the sensitivity of the instrument, since flow experiments showed the binding in low concentrations.

When cells were stained by 180ng/ml of chimeras, the intensity difference was shown that OP9-Dll4 cells bound more fucosylated Notch1 chimeras than the non-fucosylated chimeras. OP9 cells are not stained by any concentration of the fucosylated or non-fucosylated Notch1(1-15EGF)-hIgG1 chimeras as shown in the right panels, indicating that binding to the chimeras to OP9-Dll4 cells was specific to the Dll4 ligand. These observations have been confirmed by three individual experiments.

The above results from two independent experiments indicate that the loss of fucosylation on Notch1 reduces its binding ability to the ligand Dll4. This observation corresponds to the loss of Notch1 downstream signaling in FX(-/-) hematopoietic progenitors and the loss of Notch1-dependent T cell development in the FX(-/-) thymus. This observation also indicates that the monosaccharide fucose plays a very important role in regulating signaling strengths in critical developmental processes in the lymphopoietic system.

Discussion

A Cell Line for Cell Surface Protein Comparison

To compare cell surface protein expression in a function of fucosylation, a specific cell line was pursued. First, these cells have to be a homogenous population that has global fucosylation deficiency. This will ensure the precise comparison in the quantitative methods in the function of fucosylation. We currently have three homogenous populations available to use: 1) Lec13 (mutation on GMD) and CL17 (mutation on FX) are Chinese hamster ovary cells with global fucosylation deficiency and have been used for decades in various research groups; 2) FX(-/-) MEF (Mouse Embryonic Fibroblast) was made from FX(-/-)

embryos in our lab and used in our previous experiments; 3) Four years ago, I made an immortalized myeloid progenitor cell line from FX(-/-) adult bone marrow stem cells, which is also a potent choice for the quantitative experiments.

Second, this cell line will be a progenitor population in hematopoietic development. Lec13 and CL17 cells were made from the hamster ovary, which is not relevant to the blood system. MEF cells are fibroblasts involved in hematopoiesis by their ability to support embryonic stem cell differentiation. So we could exclude Lec13, CL17 and FX(-/-) MEF cells from our pool of selections.

Third, there have to be a constant level of Notch proteins expressed by the cells in order to ensure that only one variable of fucosylation is tested. The binding ability of FX(-/-) myeloid progenitors to Notch ligand Dll4 was tested first (Figure 4-1). In the presence of fucose, FX(-/-) myeloid progenitors were bound to the Dll4-hIgG1 chimera, indicating the existence of Notch1 receptors. This observation encouraged us to test FX(-/-) myeloid progenitors in the surface Notch1 study. Thus only the immortalized FX(-/-) myeloid progenitors were chosen for our experiments.

A Feasible Experimental System for Binding Assay

In order to assess the binding activities of non-fucosylated Notch1 receptors to their fucosylated ligands, it is very important to find out the experimental platform suitable for this defined question. The cell-based systems have long been used in binding assays and may closely represent what is happening *in vivo*. So a cell-based system with Notch1 expressed is desired, while the Notch ligand could be used to quantify the binding ability of Notch1 proteins in the function of fucosylation.

At first, to closely replicate the progress of T cell development, T cell progenitors were proposed with great interest in having Notch1 receptors on the surface. As discussed in the introduction chapter, the earliest T cell progenitor in bone marrow has not yet been defined, which made it impossible to isolate such populations for our study. Stem cell

derivatives from certain *in vitro* OP9 co-culture systems were able to differentiate to T cells, as described in Chapter III, indicating their potential for the binding assay. However, the complexity of multiple Notch ligands on multi-potential progenitors cells made it very hard to interpret our results while accessing their binding ability to Notch ligand chimeras, especially in a quantitative way.

Thus a homogenous population was preferred for our purpose. The FX(-/-) E2a/Pbx1 progenitor cells are homogenous and express Notch1 on the cell surface. We preferred for our experiments, however, for the amounts of fucosylated and non-fucosylated Notch1 proteins on the cell surface to be different. Thus a fair comparison cannot be made due to various amounts of receptors on the cell surfaces.

In light of the difficulties we met in the above attempts, a reversible system was designed. A cellular system bearing Notch ligands was proposed with the availability of fucosylated and non-fucosylated Notch1 chimeras made *in vitro*. Notch ligand transduced OP9 cells were ideal for us to use in the binding assay because of the purity of cells and the equivalent amount of Notch ligand presented on the cell surface. Fucosylated and non-fucosylated Notch1 chimeras were quantified *in vitro* by the ELISA system, which is highly sensitive. The same molars of fucosylated and non-fucosylated chimera were used; thus the only variable in the assay system was the presence or absence of fucosylated glycans on Notch1 proteins.

The Dual Functions of Fucosylation in Notch1 Signaling

Haltiwanger and colleagues have reported their study of fucosylated sites on Notch1 receptors in mammalian cell lines *in vitro* (15, 242, 250). Three EGF repeats on Notch1 receptors were extremely evolutionarily conserved across different species (251). Several other EGF repeats on Notch1 were highly evolutionarily conserved. They made 8 different mutants, in which one EGF repeat was mutated by using Ala substituting Serine or Threonine to delete the site O-fucose may link to. Among the 8 mutants studied, only 3 of

them resulted in aberrant Notch1 downstream signaling. Mutation on either EGF 12 or EGF 27 led to decreased downstream signaling triggered by either Dll1 or Jagged1. On the contrary, mutation on EGF 26 elevated downstream signaling induced by either Dll1 or Jagged1. These studies suggest the important roles of fucosylated glycans on Notch receptors in the regulation of its signaling abilities.

However, it is not known yet whether losing fucosylated glycans on any of the EGF repeats of Notch1 receptors leads to altered ligand-binding abilities. Moreover, these studies were focused on individual EGF domains and/or the fucosylated glycans on these particular domains. There was no evidence yet that showed what would happen if all the fucosylated glycans were lost at one time. Since POFUT1 is the enzyme fucosylating all possible sites on EGF repeats, it is not very likely that only one EGF repeat is not fucosylated while the others are. It would be more likely that all of the possible fucosylated sites are fucosylated or not fucosylated. It is important to find out how losing fucosylation on all of the EGF repeats affects the signaling and functions of Notch1.

N-glycans have been studied for a long time and they are known as essential components of the binding pockets of lots of proteins, including PSGL-1. It is not clear yet if O-fucosylated glycans contribute to the structure of binding pockets in the mammalian system. Our results from Figure 4-3 strongly indicate that a non-fucosylated Notch1 form exists in nature. The presence of non-fucosylated Notch1 on the cell surface indicates that losing fucosylation alone cannot fully stop Notch1 secretion. This result seems to be different from that in Drosophila, in which losing fucosylation did not perturb the secretory pathway of Notch1 proteins (201).

The amount of Notch1 presented on the cell surfaces was evident independent of the fucosylation status of the cells (Figure 4-3). Dll1 density was shown regulating Notch1 signaling strength *in vitro* (252). But the density of Notch ligands within the thymus is more likely to be constant. Conversely, it is very likely that the density of Notch1 on signal receiving cells actually controls the signaling strength. Thus regulating Notch1 receptor

density on T cell progenitors could be a very efficient and direct way to regulate the T lineage commitment. These results suggest the regulatory role of fucosylation in controlling Notch1 signaling strength by attenuating the secretory pathway of Notch1.

The binding ability of the non-fucosylated form of Notch1 was greatly reduced compared to the fucosylated forms of Notch1 (Figure 4-7 and Figure 4-8). Even when non-fucosylated Notch1 was provided at high concentration, the binding of non-fucosylated Notch1 to Dll4 could not be restored to the level of fucosylated Notch1. In the kinetic aspect, the result showed in Figure 4-7 is the statistical summary of an equilibrated status that included dynamic binding and dissociating. Some of the ligands were dissociating from the receptor at the moment others were binding to the receptor. The bound-ligand detected from our assays actually represents the chance of Notch1 binding to OP9-Dll4 cells.

When the Notch1 chimera concentration was increased, the chance of Notch1 chimera binding to ligands was increased. However, the ratio of Notch1 chimera dissociating from OP9-Dll4 cells was also simultaneously increased. When providing enough Notch1 chimeras, dissociating Notch1 and binding Notch1 reached a constant level. In this situation, no more Notch1 was able to bind to the OP9-Dll4 cells, since all the binding pockets were occupied. The plateau in the binding curve shown in Figure 4-7 represents the nature of binding pockets themselves. When the binding pockets were fucosylated, the ligands were not likely to be dissociated as fast as the ones that bound to the non-fucosylated binding pockets. It is also possible that dissociation was the same for non-fucosylated and fucosylated ligands, while fucosylation greatly increased the speed at which ligands were able to bind.

In conclusion, fucosylation in mammalian system plays important and complex roles in the regulation of Notch1 signaling. Mammalian cells can quickly reduce Notch1 downstream strength by lowering the fucosylation level, thus non-fucosylated ligands would be stuck during transportation from ER to Golgi. The cell surface fucosylated receptors were reduced during turnover. Newly synthesized non-fucosylated Notch1 proteins were

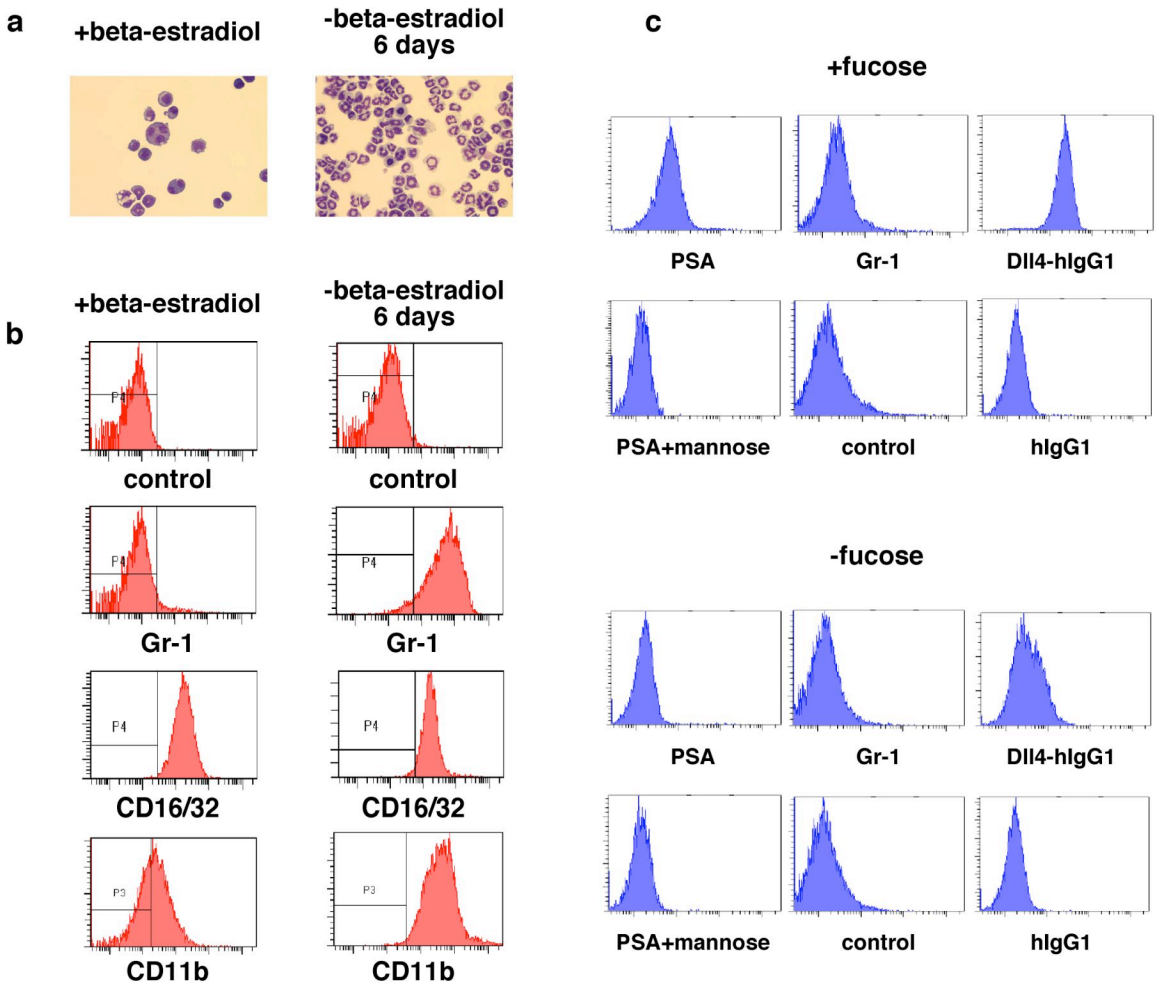
presented on the cell surface with difficulty. Even non-fucosylated Notch1 proteins were able to bind and be presented on the cell surface; their reduced ability to bind to their ligands substantially decreased the Notch1 downstream signaling strength. As a result, the T lineage commitment of hematopoietic progenitors was disrupted.

Notes to Chapter IV

Acknowledgments: We thank Bronia Petryniak for technical supports of immunofluorescence, western blot and immortalizing myeloid progenitors, and Bronia Petryniak together with Jeongsup Shim and Melissa Ruff cloned all of the DNA constructs used.

Figure 4-1: Characterization of E1a/Pbx1 transduced FX(-/-) bone marrow myeloid progenitor cell lines. a. Pictures of the cells cultured in the presence of beta-estradiol (Left) and in the absence of beta-estradiol (Right) for 6 days. Blue staining shows the nucleus. b. Cell surface markers of myeloid progenitor cells in presence or absence of beta-estradiol. (No-fucose added in the culture) c. Staining and binding experiments on myeloid progenitors cultured with beta-estradiol. Fucosylation status of the cells is shown by PSA staining. Arrested cell differentiation statuses are indicated by Gr-1staining. Notch-ligand Dll4-hIgG1 chimera binds to FX(-/-) myeloid progenitor cells in the presence of fucose much better than the cells cultured in the absence of fucose.

Figure 4-1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-beta-estradiol
6 days
b
+beta-estradiol
-beta-estradiol
6 days
control
control
Gr-1
Gr-1
CD16/32
CD16/32
CD11b
CD11b
c
+fucose
PSA
Gr-1
Dll4-hIgG1
PSA+mannose
control
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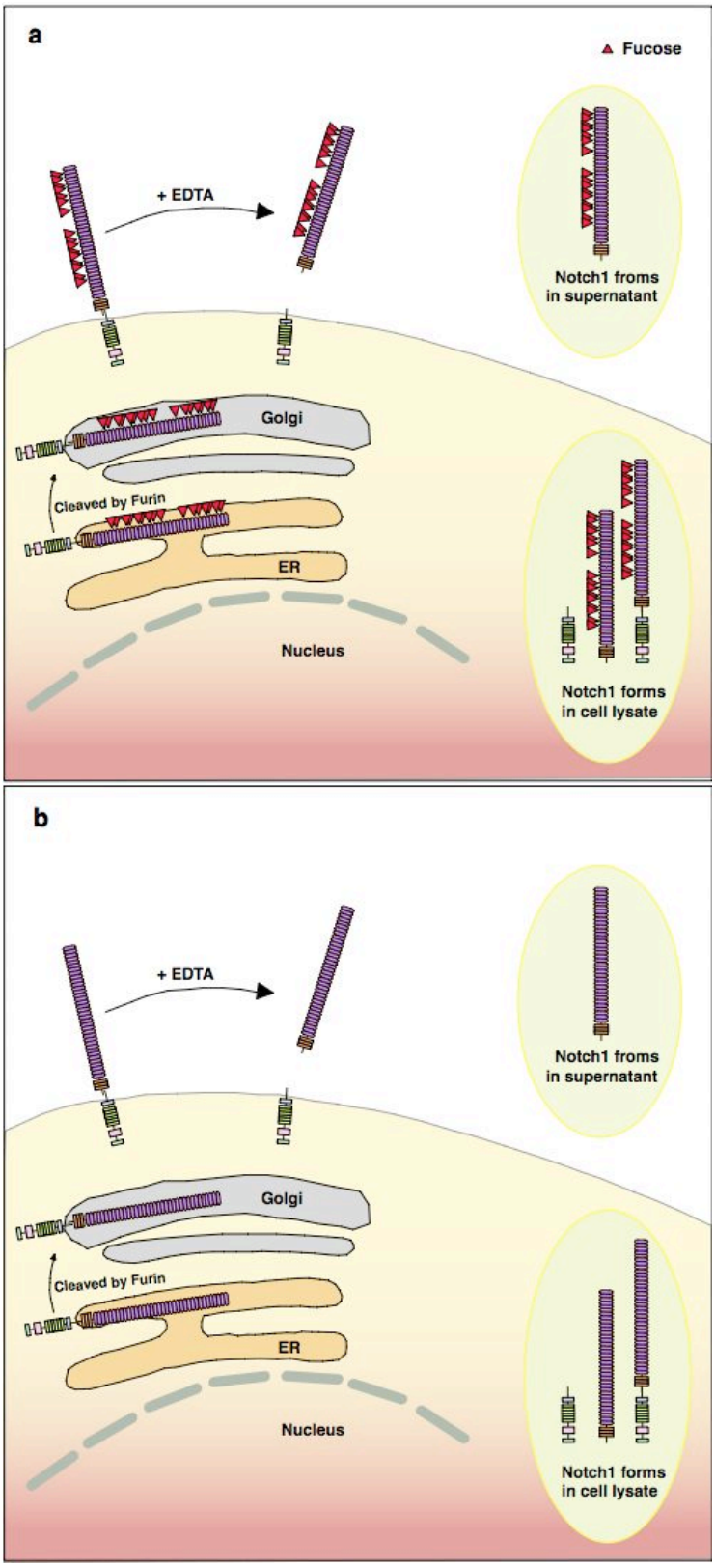
-fucose
PSA
Gr-1
Dll4-hIgG1
PSA+mannose
control
hIgG1

Figure 4-2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Fucose
+ EDTA
Notch1 froms
in supernatant
Golgi
Cleaved by Furin
ER
Nucleus
Notch1 forms
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+ EDTA
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in supernatant
Golgi
Cleaved by Furin
ER
Nucleus
Notch1 forms
in cell lysate

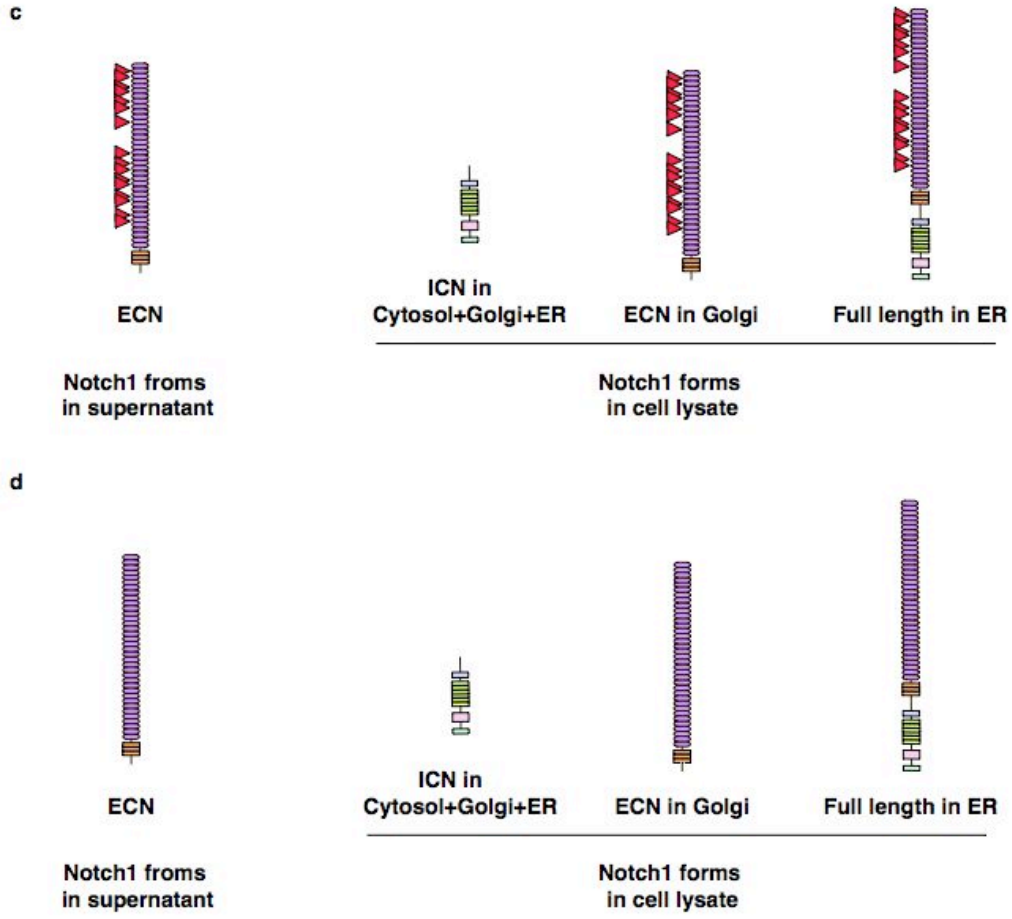
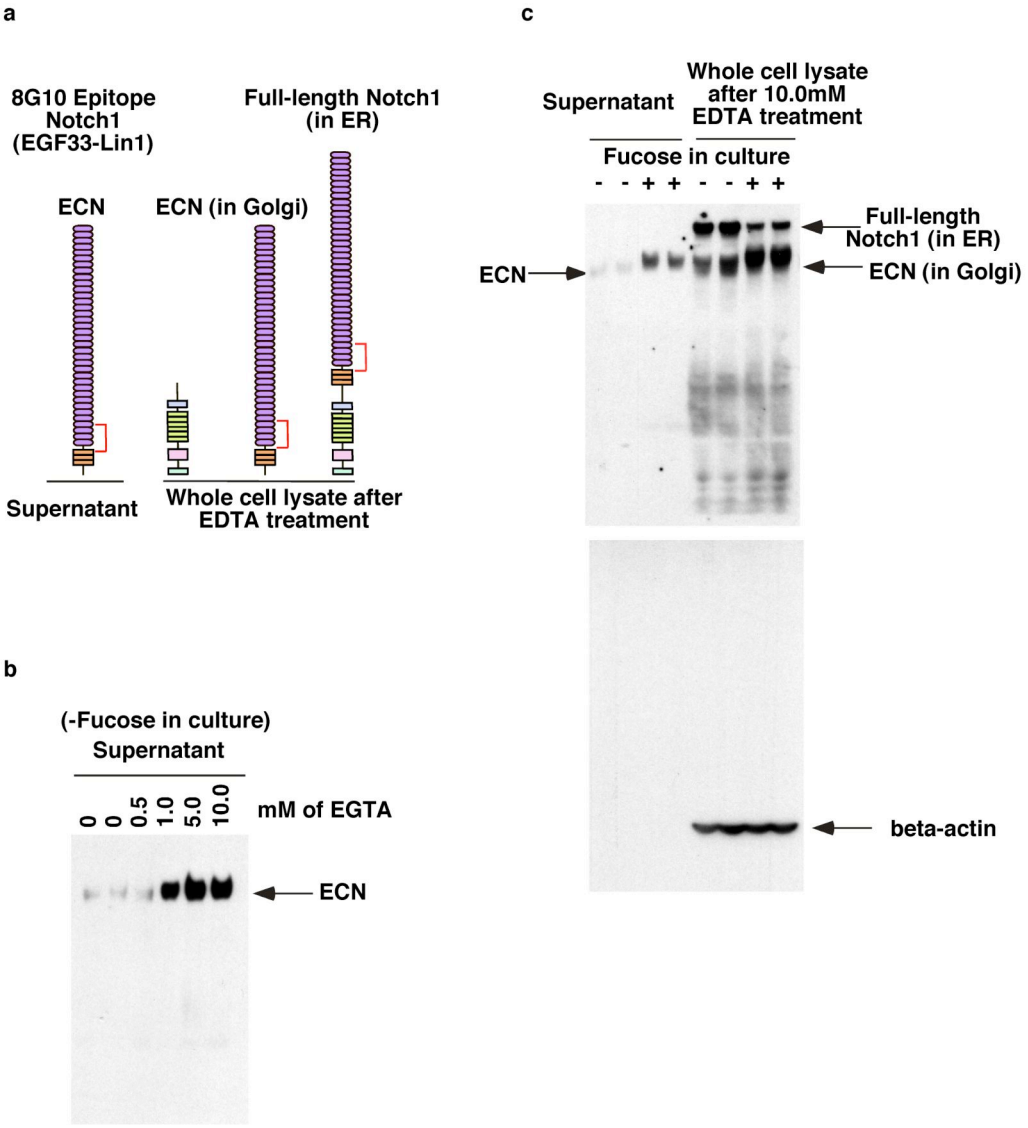


Figure 4-2: Illustration of quantification of Notch1 different forms. Notch1 protein is made in ER and modified by POFUT1. Then during secretion pathway, Notch1 proteins moved through Golgi, where it is cleaved by Furin enzyme, known also as S1 cleavage to form the heterodimer. EDTA dissociate Notch extracellular domain from cell surface. Panel a shows the fucosylated cells while panel b shows the non-fucosylated cells. Panel c and d shows the Notch1 proteins in supernatant and cell lysate after EDTA treatment in fucosylated or non-fucosylated scenario separately.

Figure 4-3: Quantification of Notch1 in different cellular locations. a. Anti-Notch1 antibody 8G10 specifically binds to mouse Notch1 EGF33 to LIN repeat 1 (shown in panel a as in non-fucosylated Notch1 forms). FX(-/-) E2a/Pbx1 transduced myeloid progenitor cell line was made in the absence of fucose. b. EDTA in Notch dissociation experiment has been titrated on those cells in the absence of fucose while maximum amount of Notch1 in supernatant detected in 10mM EDTA treated samples. c. The fucosylated and non-fucosylated FX(-/-) E2a/Pbx1 transduced myeloid progenitors were treated with 10mM EDTA. Supernatant collected after treatment was loaded in left lanes. Cells collected after EDTA treatment were lysed and denatured before loaded in right lanes. Anti-Notch1(8G10) shows the Notch forms as indicated in upper western blot. The same blot was stripped and blotted with anti-beta-actin to show the equivalent loading among the samples as quantitative controls.

Figure 4-3


a
8G10 Epitope
Notch1
(EGF33-Lin1)
Full-length Notch1
(in ER)
ECN
ECN (in Golgi)
Supernatant
Whole cell lysate after
EDTA treatment
b
(-Fucose in culture)
Supernatant
0
0
0.5
1.0
5.0
10.0
mM of EGTA
ECN
c
Supernatant
Whole cell lysate
after 10.0mM
EDTA treatment
Fucose in culture
- - + + - - + +
Full-length
Notch1 (in ER)
ECN
ECN (in Golgi)
beta-actin

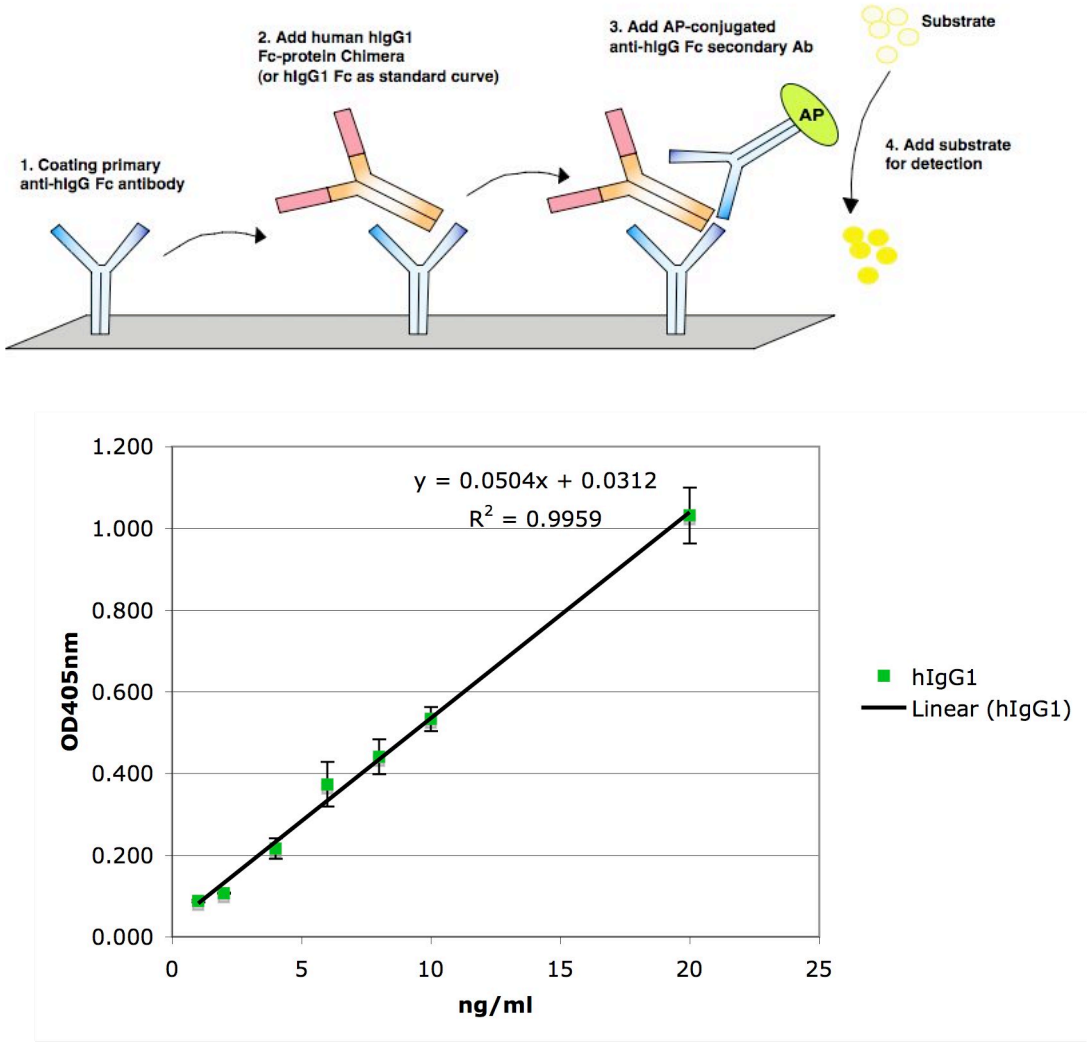


Figure 4-4: Standard curve of hIgG1 Fc chimera ELISA. Top panel shows the experimental setup. Lower Standard panel shows one of the representative standard curves using human IgG1 Fc. In details, Fc dimerized hIgG1 was bought from Sigma and diluted to different concentrations for a standard curve in quantification of soluble Notch1(1-15EGF)-hIgG1. Linear regression was used to develop a correlation of concentration to O.D. 405. R^2 represents the approximation of data points to the linear regression curve, and in this case, 0.9959 of R^2 has shown a very accurate linear relationship within 1 to 20ng/ml range.

Figure 4-5

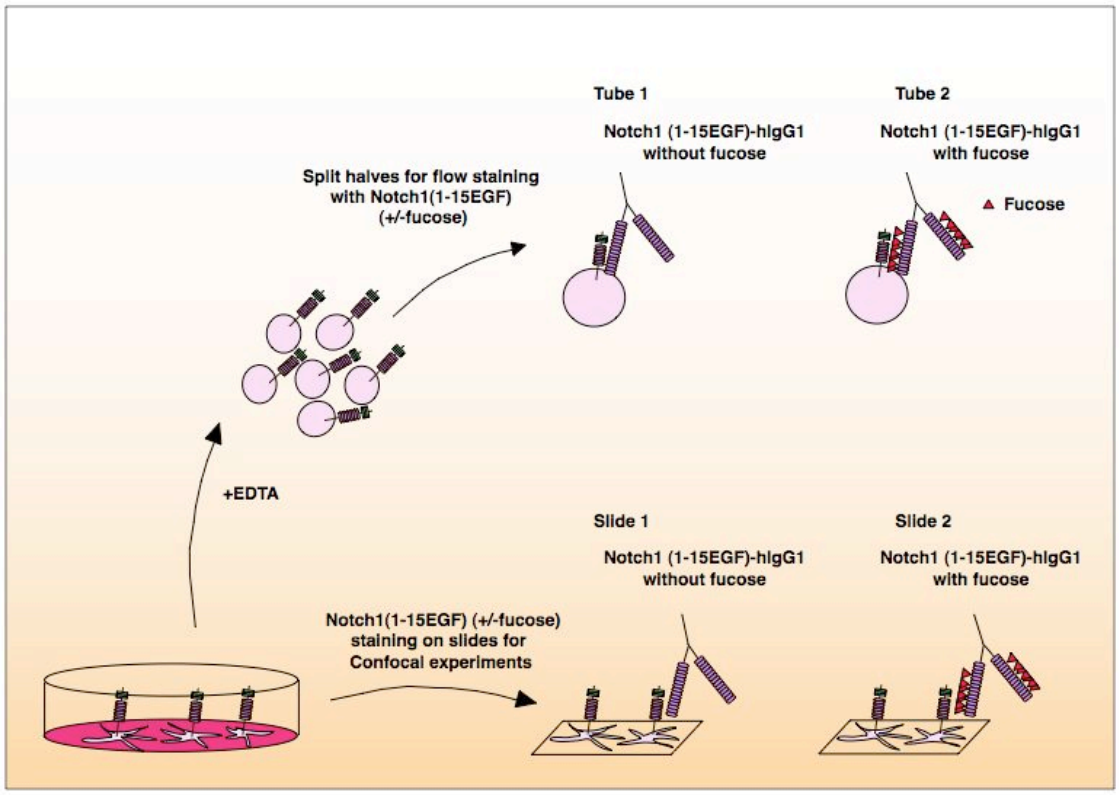


Figure 4-5: Designs of quantitative binding experiments. OP9-Dll4 cells were expanded in culture first. Some of the OP9 cells were directly plated on microscope cover slides for staining with fucosylated or non-fucosylated Notch1 (1-15EGF)-hIgG1 chimera (Bottom). Some of the cells were seeded in very low density to be detached by EDTA treatment. Single cell suspension was used for fucosylated or non-fucosylated Notch1 (1-15EGF)-hIgG1 chimera staining examined by flow cytometry.

Figure 4-6

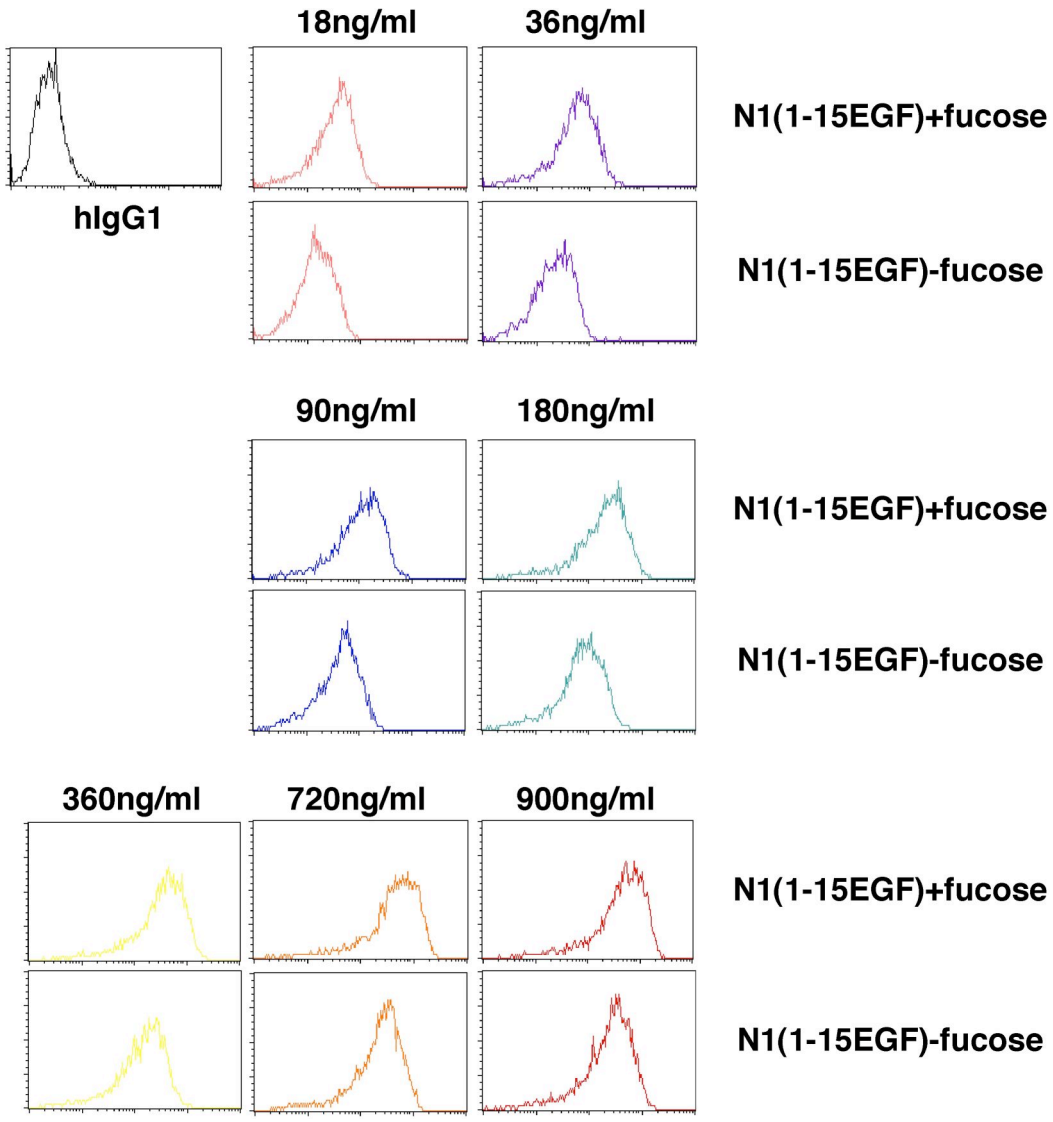


Figure 4-6: Fucosylated Notch1 has a higher binding affinity to Delta-like 4 than does non-fucosylated Notch1. Top left panel shows control staining of hIgG1 to OP9-Dll4 cells. Different concentration of fucosylated Notch1(1-15EGF)-hIgG1 (shown as +fucose panels) and non-fucosylated Notch1(1-15EGF)-hIgG1 (shown as –fucose panels) were used as indicated in flow cytometry staining. In each defined concentration, OP9-Dll4 cells are stained better by the fucosylated Notch1 chimera.

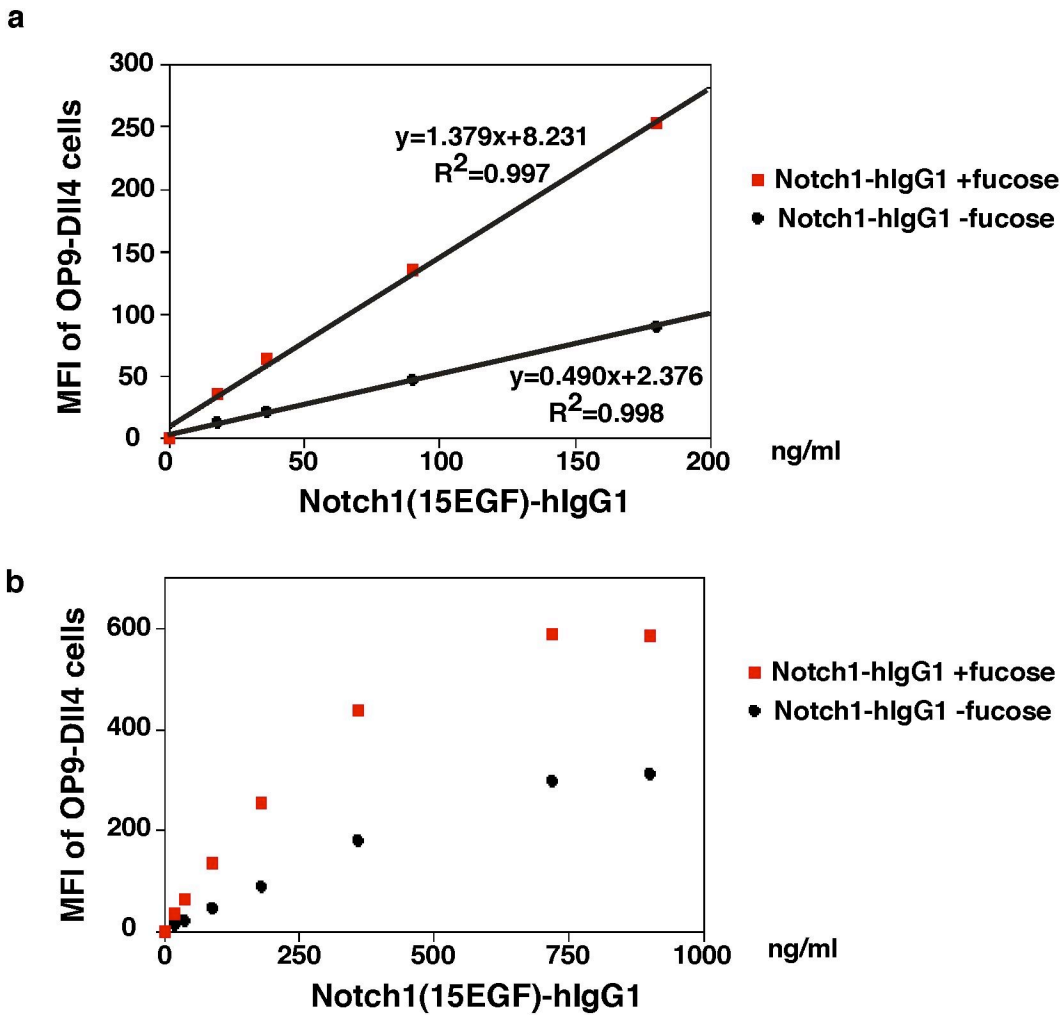


Figure 4-7: OP9-Dll4 cells have different affinity and avidity to fucosylated and non-fucosylated Notch1(1-15EGF)-hIgG1 chimera. Mean fluorescence of intensity (MFI) of each flow sample in previous figure was plot with the increasing concentrations. a. Lower concentration rage (1-180ng/ml) MFI points showed linear fashion. Slopes represent the affinity of OP9-Dll4 to different binding partners. b. Binding of non-fucosylated Notch1(1-15EGF)-hIgG1 to OP9-Dll4 cells reached a lower plateau than fucosylated chimera, indicating avidity of OP9-Dll4 cells to each chimera is different.

Figure 4-8

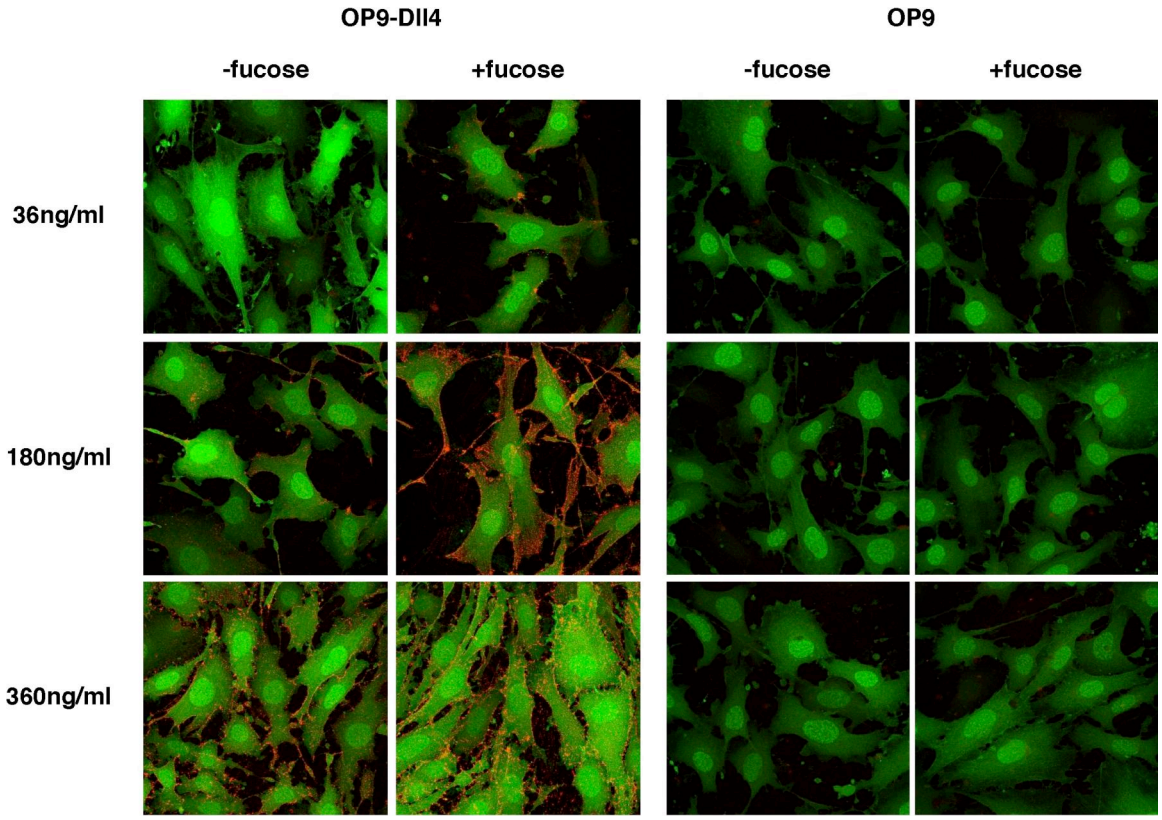


Figure 4-8: Dll4 binds to fucosylated Notch1 with better affinity confirmed by confocal microscopy. OP9-Dll4 cells were plated on microscope cover slide two days before staining. Left panels show OP9-Dll4 cells with right panels showing control OP9 cells. OP9 cells are not stained with any of Notch1 chimera in any concentration. OP9-Dll4 cells show better staining in low concentration to fucosylated Notch1(1-15EGF)-hIgG1 chimera than non-fucosylated ones. Green: GFP. Red: Notch1 chimera.

Chapter V

Conclusions

Glycobiology is still a young field compared with protein biology and nucleic biology. Although it was estimated that 80% of proteins are glycosylated, our knowledge of these sugar structures has remained limited. Searching on the NCBI pubmed database, the entries returned by "glycan" amounted to about one third of the publications returned by "DNA", and one tenth of the publications returned by "protein." From these numbers, and given the fact that fewer labs focus on glycobiological research, it is very possible that the scientific community has underappreciated carbohydrates and the carbohydrate research for a long time (253). The study of glycobiology is complicated by its emphasis it places on three different aspects: structure, biosynthesis, and biology. Scientists new to the field may first experience consternation at the complex linear and branching structures, and the elaborate biosynthetic procedures. Exploring the biological functions of polysaccharides must take into account the required knowledge base of the structures and of biosynthesis. In the past decades, our understanding of the biological functions of carbohydrates has been greatly enhanced by many scientists such as ourselves, enchanted by the concealed wonders that may lie ahead.

Fucose biology was studied as early as the 1960s. The earlier efforts focused on the biosynthesis of GDP-fucose, the fucose donor for all fucosyltransferases. With the identification of various fucosylated structures, the functions of these glycans were brought to Dr. John Lowe's attention. Taking the approach of genetically engineering mouse models, Dr. Lowe and his colleagues successfully disclosed the roles of many fucosylated structures in the immune system. One of his prominent works has focused on the glycans

modified by fucose attached in the alpha-1,3 anomeric linkage and on the fucosyltransferases responsible for their synthesis. In the initial required step during the process used by leukocytes when migrating from the blood to extravascular site of inflammation, fucosylated structures on selectin ligands are required in leukocyte-selectin interactions. Encouraged by the functions discovered, a global fucosylation-deficient mouse model was made in order to uncover more functional roles of fucose-containing structures.

Novel Developmental Functions of Fucosylated Glycans

A genetically engineered mouse model, FX(-/-) mice, was made by the disruption of the FX locus to silence the de novo GDP-fucose synthetic pathway (Figure 2-1). The salvage pathway of GDP-fucose synthesis enables the manipulation of fucosylation status in these mice. By the time of fucosylation loss, each organ of FX(-/-) was extensively examined. The thymus from FX(-/-) mice off fucose was shown to be much smaller than those of their littermates kept on a fucose-supplemented diet during the loss of fucosylation (Figure 2-2). This finding revealed a novel phenotype of FX(-/-) mice: thymic atrophy.

In order to find out the reason for this thymic atrophy, it is important to examine the cellular components within the thymus. The functional role of the thymus in physiology is to support the differentiation of T cells. The great majority of the cells in thymus are T cells. Multiple cell types, including T cells, epithelial cells, and myeloid cells could be found. Thus, cellular components other than T cell in FX(-/-) thymus were considered as the supporting environment for thymocyte development (218). To simplify our study: cells in thymus are considered to be two groups, the thymocytes (T cells) and other cell types (thymic environment). The thymic atrophy defect may be caused by the defects exhibited by the thymocytes, or on the thymic environment, or on both.

Since thymic size in FX(-/-) mice off fucose decreased greatly, it is most likely that the majority of T cells are lost in the thymus. The very first approach was to count the total thymocyte numbers from the thymus of FX(-/-) mice upon the time of fucosylation loss. Surprisingly, thymocytes were lost when fucosylation was shut off for 4 weeks in FX(-/-) mice (Figure 2-2). Although many cell types could be found within thymus, thymus is the organ specialized for T cell development. In a wild-type thymus, more than 90% of total thymocytes are T cells in different stages of development. Further, T cell populations were enumerated in total thymocyte population and refined by their stages during the developmental pathway. Mature CD4+CD8a+ T cells were almost completely lost when fucosylation was disabled in FX(-/-) mice (Figure 2-3). Early T cell progenitors were also missing in FX(-/-) mice off fucose for 4 weeks (Figure 2-4, 2-5, 2-6). These results strongly suggested that T cell development was blocked in the absence of fucose.

It is also likely that defects may occur on any of those cells that eventually harmed T cell development within the FX(-/-) thymus in the absence of fucosylation. For normal ontology to occur, thymocytes must physically interact with thymic epithelial cells in the cortex and the medulla (164). Disruption of thymic epithelial cell expansion in Nude mice resulted in the cystic structure made up of epithelial sheets instead of the meshwork three-dimensional structure in normal mice (254-257). Thymocyte development was greatly reduced in nude mice (258). Moreover, the soluble growth factors and cytokines within the thymus are also required for thymic development. As one example, mice lacking both receptor tyrosine kinase (c-Kit) and the common cytokine receptor gamma chain have severe thymic atrophy with completely abrogated thymocyte development (259).

Thus it is very important to examine the thymic environment of FX(-/-) mice. Wild-type bone marrow stem cells were introduced into FX(-/-) mice after their being off fucose for 4 weeks. Firstly, the emergence of donor wild-type cells in the FX(-/-) off-fucose thymus proved that the thymic epithelial cells have no defect upon receiving bone marrow progenitors in the absence of fucosylation. Secondly, mature donor T cells identified in the

recipient mice proved that the thymic environment is capable of supporting thymocyte development.

These observations strongly suggest that possibilities other than defects on thymocytes are excluded in the absence of fucosylation. The non-fucosylated T cell progenitors are not capable of differentiating to mature T cells. Cell autonomous defects are responsible for thymic atrophy in FX(-/-) when fucosylation is disabled. This physiological function role of fucosylation has not been reported yet in previous literature. In conclusion, a novel functional role of fucosylation in thymocyte development has been identified in FX(-/-) mice.

The Required Presence of Fucosylation in Signaling Events During Thymocyte Development

The observations and conclusions in Chapter II raised a very interesting question: how does loss of fucosylation result in the disruption of thymocyte development? Thymocyte development is a highly controlled process in which multipotent blood stem cells become specialized into T lymphocytes. The differentiation procedures are intricately regulated involving changes in many aspects of cell shape, size, polarity, metabolic activity, and gene expression profiles. T cell development is controlled by sequential signaling events that eventually turn on many gene expressions, including CD4 and CD8. Genes specifically expressed in multipotent progenitors are shut down during this process. However, fucosylation is a biochemical event concerning the macromolecules inside the inner compartments of the cells. How does a biochemical modification to substrates influence a strictly controlled developmental process? Fucosylation has to change the characteristics and functions of some proteins or lipids involved in T cell differentiation. Only through cellular signaling events could a developmental procedure be stopped. To

answer this question, a bridge of cell signaling has to be found to bridge the gap from a chemical modification procedure of fucosylation to the fundamental process of the cells.

Biological developmental procedures are tightly regulated by cellular events both temporally and spatially in order to formulate organisms. During the differentiation of the cells, the programmed events take place in response to the surrounding environment. Cellular response is triggered by the signals transduced from outside the cell to inside. Similar to all other developmental procedures, T cell differentiation is precisely regulated by numerous transcription factors and signaling pathways, including E2a, hematopoietic transcription factor PU.1, growth factor independence (Gfi)-1, T cell factor (TCF)-1, Runx factors, and the Notch1 signaling pathway (260). Many of these transcription factors are also used in many other lineage specifications (261). Most of them promote T cell development through Notch signaling. It was thought that Notch signaling has to be "on" all the time during T cell development in order to restrain the latent diversionary effect of the other factors involved (260). Very luckily, among all these transcription factors and signaling participants for T cell differentiation, only Notch receptors and ligands are fucosylated. This indicates that Notch signaling is the "bridge" we sought to link fucosylation to T cell development. Fucosylation controls T cell development through its influences on Notch signaling. Our results in Figure 3-6 have proven that the presence of fucosylation turns on Notch1 downstream genes and T cell specific transcription factors.

On the other hand, fucosylated glycans related to thymocyte development were searched for in all known fucosylated proteins. Hundred of proteins are potentially fucosylated in a N-linked and/or O-linked fashion mediated by different fucosyltransferases. The fucosylated proteins are categorized in two groups roughly, N-fucosylated proteins and O-fucosylated proteins. N-linked fucosylation is mediated by several different fucosyltransferase, encoded by FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7, FUT8, FUT9, FUT10 and FUT11. However, no reported thymic abnormalities were found in known knockout mice from the genes above. Then O-link fucosylated proteins

were examined extensively. Some of EGF domains are fucosylated by protein O-fucosyltransferase 1 (POFUT1). Many other proteins containing TSR domains are O-fucosylated by POFUT2. Among all of these proteins, Notch1 stood out as the most promising candidate because it is involved in the regulation of T cell development.

This conclusion has been further confirmed by comparing thymic abnormalities in detail in the following experimental models: 1) Notch1 conditional knockout mice and FX(-/-) mice off fucose both had a sizable loss of mature T cell subsets. Notch1 conditional knockout mice had a residue Notch1 gene expression that cannot be ignored. Thus the complete loss of CD4+CD8a+ T cells was not seen in Notch1 conditional knockout mice. Although the thymic phenotypes resulting from fucosylation loss are not exactly the same as the ones resulting from the loss of Notch1 receptors, it has been strongly suggested that T cell progenitors without fucosylation behave to a large extent like the ones without functional Notch1 receptors. 2) RBPJ conditional knockout mice also showed a sizable loss of mature T cell subsets. These mice lack the Notch1 signaling pathway and display phenotypes similar to the Notch1 conditional knockout mice. 3) DNMAML1 transduced bone marrow cells lack Notch1 downstream signaling in early differentiation stages. These cells cannot progress through DN1a-b stages. 4) DN2 and DN3 cells require Notch1 signaling to maintain T lineage specification and progression (214, 262). In the absence of fucosylation, DN2 and DN3 cells were greatly reduced in the FX(-/-) thymus, indicating the loss of Notch1 signaling.

Although other possibilities may exist, from the above evidence it is most likely that fucosylation controls T cell development through modulating Notch1 signaling. Our experimental results have supported this hypothesis. Moreover, Notch1 signaling is not transduced in T cell progenitors in the absence of fucosylation. This result provided direct evidence that fucosylation controls the Notch1 signaling pathway. It is a new function for fucosylation in glycobiology and developmental studies and emphasizes the important regulatory roles of fucose in nature.

Multiple Molecular Functions of Fucosylated Glycans

The studies presented in Chapter II and Chapter III reached the conclusion that fucosylation controls Notch1 signaling transduction and thus influences T lymphocyte development. The molecular mechanisms of fucosylation controlled Notch1 signaling were focused on in Chapter IV. This part of study shifts from the cellular level to the level defined by molecular biology. Our effort included the applications of recombinant DNA technology, protein quantification, functional studies of proteins, and cell imaging.

Kenneth D. Irvine and colleagues have contributed to Notch signaling in Drosophila since early 1990. In their earlier studies, they focused extensively on the formation of Drosophila wings (263). During this developmental process, the interactions between dorsal and ventral cells are required. A soluble protein that modulates dorsal and ventral cells was identified and named Fringe (264). Other researchers have found that Serrate (265), Delta (266) and Notch (267) also modulate the wing disk formation of Drosophila. Serrate and Delta were shown signaling through Notch during wing development (266, 268). The Notch signaling then has been set up with Notch proteins as the receptors and Serrate/Delta as the ligands. Fringe modulates the Notch signaling pathway through a cell-autonomous mechanism (269). The basic scheme of Notch signaling has been set up.

O-fucose was first identified on the EGF domain of factor XII (270) and was soon found on the EGF domains of mammalian Notch (18). A few years later, Fringe was found modulating the Notch-ligand interaction (269) through a glycotransferase activity (271). It turned out to be a beta1,3 N-acetylglucosaminyltransferase activity that initiates the elongation of O-linked fucose residues attached to epidermal growth factor-like sequence repeats of Notch (18). Further, studies from the Irvine lab have shown that O-fucose may regulate Notch signaling in wing development (25). O-fucosyltransferase was cloned (25)

and shown to have chaperon activity in Notch receptor folding (201). Losing fucosylation does not disturb the secretion of the Notch receptor in Drosophila (201).

In the mammalian system, the chaperon activity of POFUT1 has not yet been reported. If mammalian Notch1 were folded in the same way as that of Drosophila, losing fucosylation would not alter the secretion of Notch1. In FX(-/-) mice off fucose, Notch1 receptors accumulated in ER in the full-length format (Figure 4-3), indicating an important role of O-fucose in mammalian Notch1 folding. No quantitative evidence has shown that all of the Drosophila Notch receptor stuck in Golgi. Our results showed that there were little non-fucosylated Notch1 receptors on the cell surface that potentially interacted with their ligand (Figure 4-3). Non-fucosylated Notch1 was also observed in multipotent progenitor surfaces in the absence of fucose (Yunfang Man, Bronia Petryniak, John Lowe, unpublished data), which excludes the artificial abnormalities of E2a/Pbx1 cell line. However, the numbers of non-fucosylated proteins on cell surfaces were scarce compared to those of fucosylated Notch1 (Figure 4-3). Thus, different from Drosophila, mammalian cells require fucosylation to present Notch1 on the cell surface.

The Notch1 receptor without fucosylated glycans has lowered binding abilities to the Dll4 ligand. One possibility is that non-fucosylated Notch1 receptor is properly folded, but that the binding pocket on Notch1 is not in the right shape, due to having lost fucosylated glycans. Dll4 binds to the binding pocket with less affinity. Another possibility is that the non-fucosylated Notch1 receptor is not properly folded. Dll4 binds to other parts of Notch1 (within 1-15EGF). Our results could not differentiate the conformation changes of the Notch1 receptor. Future studies are required to answer this question. By NMR and X-Ray crystallography, the structures of Notch1 could be obtained to compare the conformation of fucosylated Notch1 with non-fucosylated Notch1 in the near future.

A Regulatory Mechanism for Maintaining T cell Generation

Our results in the above chapters strongly suggested that T cell generation depends on the cellular fucosylation level and on the proper transduction of Notch1 signaling. Attenuating the cellular fucosylation level seems able to adjust the surface Notch1 receptors presented. By presenting non-fucosylated Notch1 on the cell surface, the ligand-binding abilities of signaling receiving cells are reduced. Further multipotent progenitors adapt lineage specifications other than T cells.

We proposed a possible regulatory pathway to balance T cell development in the thymus. Since the expression of Notch1 ligands on thymic stromal cells occurs at a constant level, multipotent progenitors are differentiated in the thymus. When Notch1 signaling is constitutively active, these progenitors are enforced to differentiate along the T cell pathway. However, cells other than T lymphocyte subsets were identified within the thymus. Since Notch1 signaling is required for multiple transitions within the thymus, multipotent progenitors have several chances to quit from the T cell differentiation pathway and are quickly differentiated into other cell types, including B cells. Immature and mature B cell subsets were identified within the normal thymus (155). Losing Notch1 signaling led multipotent progenitors to adopt the B cell fate within the thymus (78). These observations indicate that when T cells were surfeit, B cells would be generated within the thymus. Natural killer cells were found within thymus and for a long time it was thought they were being recruited from the peripheral repertoire (272). More evidence showed that thymic natural killer cells developed in situ (273). DN2 cells are able to adopt the NK cell lineage that is normally suppressed by Notch1/ligand interaction (262). It is very likely that these cell types other than T lymphocyte are generated as the result of decreasing T cell generation.

Thus there might be a feedback loop to prevent the excessive generation of T cells, also preventing T cell lymphoma. Excess or an absence of T cells in the periphery may

signal multipotent progenitors before their entry to the thymus. A possible feedback target is the protein O-fucosyltransferase 1 residing in the ER in these progenitor cells. By attenuating the fucosyltransferase activities of POFUT1, the Notch1 signaling strength is adjusted accordingly (Figure 5-1). Whenever more T cells than sufficient are generated around the periphery, multipotent progenitors in the blood may receive a signal to decrease the O-fucosylation level. POFUT1 in multipotent progenitors may be deactivated by various methods, including protein degradation and gene silencing. Once these multipotent progenitors arrive in the thymus, they will no longer receive Notch ligand signals from thymic stromal cells. When T cells are in shortage within the mammal, the cellular activities of POFUT1 may be increased for enhanced Notch1 downstream signaling. Thus these cells will have more Notch1 receptors that would increase the chances of binding to their ligands. Once they find the Notch1 activating ligand, they will bind well and quickly induce intracellular cleavages. Then the chance of this progenitor cell differentiating along the T lineage is greatly enhanced.

Other modulators than POFUT1 can regulate this process as well. For example, Fringe enzymes are famous in their regulatory roles related to Notch1 signaling (274). Mammals have three different Fringe enzymes, Lunatic Fringe, Manic Fringe and Radical Fringe (275). Among them, neither Radical nor Manic Fringe were thought essential for development in any mouse tissue (274). Multipotent progenitors over-expressing the Lunatic Fringe gene were inclined to adopt the B cell fate within the thymus other than T cells (276). Increasing the expression of the Lunatic Fringe in multipotent progenitors may reduce its ability to be a T lymphocyte during competition (277). Lunatic Fringe deficient mice have been made independently by Jackson Lab (278) and Randy L. Johnson's lab (279). However, there is a discrepancy between the two mouse models. Female Lfn(-/-) mice from Johnson's lab were all not fertile, due to a disorganized ovarian morphology (280). But some of the female mice made by Jackson Lab were fertile as reported (281). It is not yet clear why there was such a difference between two mice similarly targeted during

genetic engineering. Moreover, there has not yet been any report of thymic abnormalities of the Lfn(-/-) mice from either lab yet. It is not known whether Lunatic Fringe is dispensable for thymocyte development.

In summary, our studies have concluded the important roles of fucosylation in the regulation of signaling pathways and programmed cellular development. These functions of fucosylation had not previously been discovered. Our FX(-/-) mouse model greatly enhanced our knowledge in fucose biology and has explored a new field in thymocyte development. There are still a few key things left to investigate. The T cell progenitors are still thought to have multiple possibilities (216). It is not clear yet how Notch1 gene is regulated. Upstream of Notch signaling was not extensively focused yet. Further, Notch signaling talks with other signaling pathways forming a very complicated network (282, 283). It will be very interesting to explore the functions of fucose in regulation of this network in lymphocyte development and other developmental processes in mammalian systems.

Figure 5-1

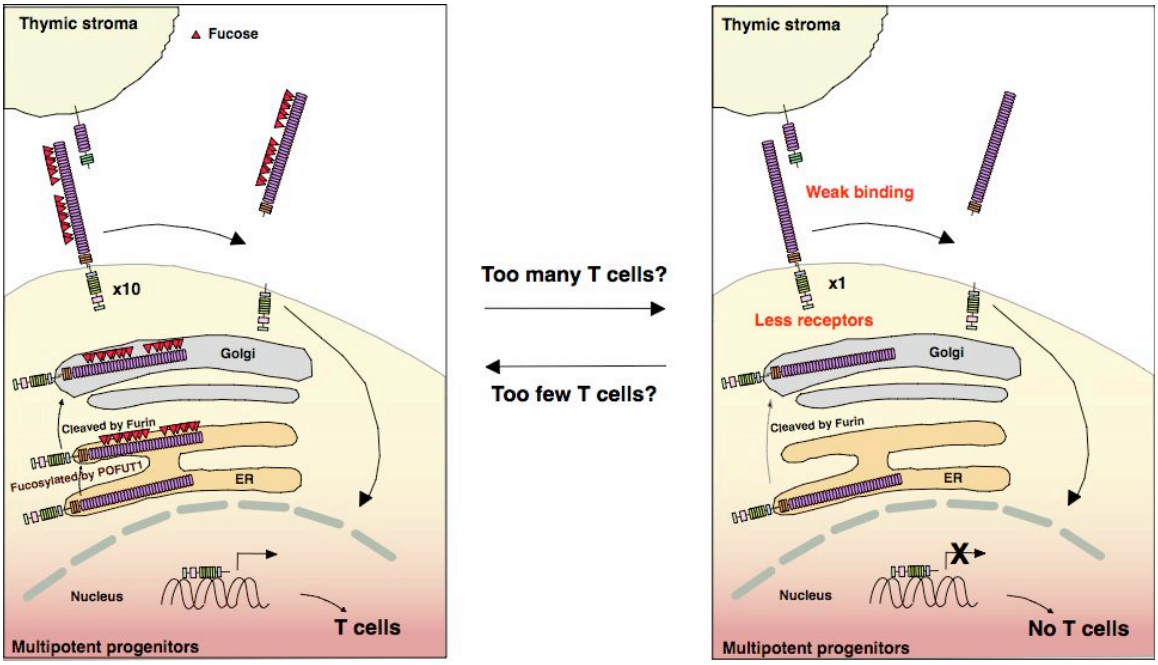


Figure 5-1: Proposed regulatory feed back in T cell development via POFUT1. Left panel shows the situation of T cell generation. Right panel shows when T cells in peripheral are in excess.

Bibliography

1. Varki, A. C., Richard; Esko, Jeffrey; Freeze, Hudson; Hart, Gerald; Marth, Jamey, editors. 1999. *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press.

2. Hubbard, S. C., and R. J. Ivatt. 1981. Synthesis and processing of asparagine-linked oligosaccharides. *Annu Rev Biochem 50:555*.

3. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem 54:631*.

4. Abeijon, C., and C. B. Hirschberg. 1987. Subcellular site of synthesis of the N-acetylgalactosamine (alpha 1-0) serine (or threonine) linkage in rat liver. *J Biol Chem 262:4153*.

5. Van den Steen, P., P. M. Rudd, R. A. Dwek, and G. Opdenakker. 1998. Concepts and principles of O-linked glycosylation. *Crit Rev Biochem Mol Biol 33:151*.

6. Roth, J. 1984. Cytochemical localization of terminal N-acetyl-D-galactosamine residues in cellular compartments of intestinal goblet cells: implications for the topology of O-glycosylation. *J Cell Biol 98:399*.

7. Schweizer, A., H. Clausen, G. van Meer, and H. P. Hauri. 1994. Localization of O-glycan initiation, sphingomyelin synthesis, and glucosylceramide synthesis in Vero cells with respect to the endoplasmic reticulum-Golgi intermediate compartment. *J Biol Chem 269:4035*.

8. Inamori, K., T. Endo, J. Gu, I. Matsuo, Y. Ito, S. Fujii, H. Iwasaki, H. Narimatsu, E. Miyoshi, K. Honke, and N. Taniguchi. 2004. N-Acetylglucosaminyltransferase IX acts on the GlcNAc beta 1,2-Man alpha 1-Ser/Thr moiety, forming a 2,6-branched structure in brain O-mannosyl glycan. *J Biol Chem 279:2337*.

9. Moloney, D. J., and R. S. Haltiwanger. 1999. The O-linked fucose glycosylation pathway: identification and characterization of a uridine diphosphoglucose: fucose-beta1,3-glucosyltransferase activity from Chinese hamster ovary cells. *Glycobiology 9:679*.

10. Shao, L., Y. Luo, D. J. Moloney, and R. Haltiwanger. 2002. O-glycosylation of EGF repeats: identification and initial characterization of a UDP-glucose: protein O-glucosyltransferase. *Glycobiology 12:763*.

11. Hirschberg, C. B., and M. D. Snider. 1987. Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. *Annu Rev Biochem 56:63*.

12. Brockhausen, I., G. Moller, A. Pollex-Kruger, V. Rutz, H. Paulsen, and K. L. Matta. 1992. Control of O-glycan synthesis: specificity and inhibition of O-glycan core 1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 3-galactosyltransferase from rat liver. *Biochem Cell Biol 70:99*.

13. Helenius, A., and M. Aebi. 2001. Intracellular functions of N-linked glycans. *Science 291:2364*.

14. Robbins, P. W. 1991. Genetic regulation of asparagine-linked oligosaccharide synthesis. *Biochem Soc Trans 19:642*.

15. Moloney, D. J., L. H. Shair, F. M. Lu, J. Xia, R. Locke, K. L. Matta, and R. S. Haltiwanger. 2000. Mammalian Notch1 is modified with two unusual forms of O-linked glycosylation found on epidermal growth factor-like modules. *J Biol Chem 275:9604*.

16. Wang, Y., and M. W. Spellman. 1998. Purification and characterization of a GDP-fucose:polypeptide fucosyltransferase from Chinese hamster ovary cells. *J Biol Chem 273:8112*.

17. Wang, Y., L. Shao, S. Shi, R. J. Harris, M. W. Spellman, P. Stanley, and R. S. Haltiwanger. 2001. Modification of epidermal growth factor-like repeats with O-fucose. Molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. *J Biol Chem 276:40338*.

18. Moloney, D. J., V. M. Panin, S. H. Johnston, J. Chen, L. Shao, R. Wilson, Y. Wang, P. Stanley, K. D. Irvine, R. S. Haltiwanger, and T. F. Vogt. 2000. Fringe is a glycosyltransferase that modifies Notch. *Nature 406:369*.

19. Chen, J., D. J. Moloney, and P. Stanley. 2001. Fringe modulation of Jagged1-induced Notch signaling requires the action of beta 4galactosyltransferase-1. *Proc Natl Acad Sci U S A 98:13716*.

20. Roseman, S. 1970. The synthesis of complex carbohydrates by multiglycosyltransferase systems and their potential function in intercellular adhesion. *Chem Phys Lipids 5:270*.

21. Aplin, J. D., and R. C. Hughes. 1982. Complex carbohydrates of the extracellular matrix structures, interactions and biological roles. *Biochim Biophys Acta 694:375*.

22. Kim, Y. J., and A. Varki. 1997. Perspectives on the significance of altered glycosylation of glycoproteins in cancer. *Glycoconj J 14:569*.

23. An, G., B. Wei, B. Xia, J. M. McDaniel, T. Ju, R. D. Cummings, J. Braun, and L. Xia. 2007. Increased susceptibility to colitis and colorectal tumors in mice lacking core 3-derived O-glycans. *J Exp Med*.

24. Lamblin, G., S. Degroote, J. M. Perini, P. Delmotte, A. Scharfman, M. Davril, J. M. Lo-Guidice, N. Houdret, V. Dumur, A. Klein, and P. Rousse. 2001. Human airway mucin glycosylation: a combinatory of carbohydrate determinants which vary in cystic fibrosis. *Glycoconj J 18:661*.

25. Okajima, T., and K. D. Irvine. 2002. Regulation of notch signaling by o-linked fucose. *Cell 111:893*.

26. Louvi, A., and S. Artavanis-Tsakonas. 2006. Notch signalling in vertebrate neural development. *Nat Rev Neurosci 7:93*.

27. Cornell, R. A., and J. S. Eisen. 2005. Notch in the pathway: the roles of Notch signaling in neural crest development. *Semin Cell Dev Biol 16:663*.

28. Callahan, R., and S. E. Egan. 2004. Notch signaling in mammary development and oncogenesis. *J Mammary Gland Biol Neoplasia 9:145*.

29. Kelley, M. W. 2003. Cell adhesion molecules during inner ear and hair cell development, including notch and its ligands. *Curr Top Dev Biol 57:321*.

30. Robey, E. A., and J. A. Bluestone. 2004. Notch signaling in lymphocyte development and function. *Curr Opin Immunol 16:360*.

31. Radtke, F., A. Wilson, and H. R. MacDonald. 2004. Notch signaling in T- and B-cell development. *Curr Opin Immunol 16:174*.

32. Bouyain, S., N. J. Silk, G. Fabini, and K. Drickamer. 2002. An endogenous Drosophila receptor for glycans bearing alpha 1,3-linked core fucose residues. *J Biol Chem 277:22566*.

33. De Luca, L., M. Schumacher, and D. P. Nelson. 1971. Localization of the retinol-dependent fucose-glycopeptide in the goblet cell of the rat small intestine. *J Biol Chem 246:5762*.

34. Sato, M., S. Yonezawa, H. Uehara, Y. Arita, E. Sato, and T. Muramatsu. 1986. Differential distribution of receptors for two fucose-binding lectins in embryos and adult tissues of the mouse. *Differentiation 30:211*.

35. Presper, K. A., M. Basu, and S. Basu. 1978. Biosynthesis in vitro of fucose-containing glycosphingolipids in human neuroblastoma IMR-32 cells. *Proc Natl Acad Sci U S A 75:289*.

36. Luo, Y., and R. S. Haltiwanger. 2005. O-fucosylation of notch occurs in the endoplasmic reticulum. *J Biol Chem 280:11289*.

37. Lowe, J. B. 1993. The blood group-specific human glycosyltransferases. *Baillieres Clin Haematol 6:465*.

38. Lee, H. H., H. Y. Wu, Y. C. Chuang, A. S. Chang, H. H. Chao, K. Y. Chen, H. K. Chen, G. M. Lai, H. H. Huang, and C. J. Chen. 1990. Epidemiologic characteristics and multiple risk factors of stomach cancer in Taiwan. *Anticancer Res 10:875*.

39. Shibata, A., N. Hamajima, Y. Ikehara, T. Saito, K. Matsuo, N. Katsuda, K. Tajima, M. Tatematsu, and S. Tominaga. 2003. ABO blood type, Lewis and Secretor genotypes, and chronic atrophic gastritis: a cross-sectional study in Japan. *Gastric Cancer 6:8*.

40. Poujol-Robert, A., P. Y. Boelle, D. Wendum, R. Poupon, and A. Robert. 2006. Association between ABO blood group and fibrosis severity in chronic hepatitis C infection. *Dig Dis Sci 51:1633*.

41. O'Donnell, J., and M. A. Laffan. 2001. The relationship between ABO histo-blood group, factor VIII and von Willebrand factor. *Transfus Med 11:343*.

42. Boren, T., P. Falk, K. A. Roth, G. Larson, and S. Normark. 1993. Attachment of Helicobacter pylori to human gastric epithelium mediated by blood group antigens. *Science 262:1892*.

43. Clarke, C. A., W. K. Cowan, J. W. Edwards, A. W. Howel-Evans, C. R. Mc, J. C. Woodrow, and P. M. Sheppard. 1955. The relationship of the ABO blood groups to duodenal and gastric ulceration. *Br Med J 2:643*.

44. Becker, D. J., and J. B. Lowe. 2003. Fucose: biosynthesis and biological function in mammals. *Glycobiology 13:41R*.

45. Hooper, L. V., and J. I. Gordon. 2001. Glycans as legislators of host-microbial interactions: spanning the spectrum from symbiosis to pathogenicity. *Glycobiology 11:1R*.

46. Guruge, J. L., P. G. Falk, R. G. Lorenz, M. Dans, H. P. Wirth, M. J. Blaser, D. E. Berg, and J. I. Gordon. 1998. Epithelial attachment alters the outcome of Helicobacter pylori infection. *Proc Natl Acad Sci U S A 95:3925*.

47. Appelmelk, B. J., R. Negrini, A. P. Moran, and E. J. Kuipers. 1997. Molecular mimicry between Helicobacter pylori and the host. *Trends Microbiol 5:70*.

48. Moran, A. P., Y. A. Knirel, S. N. Senchenkova, G. Widmalm, S. O. Hynes, and P. E. Jansson. 2002. Phenotypic variation in molecular mimicry between Helicobacter pylori lipopolysaccharides and human gastric epithelial cell surface glycoforms. Acid-induced phase variation in Lewis(x) and Lewis(y) expression by H. Pylori lipopolysaccharides. *J Biol Chem 277:5785*.

49. Lowe, J. B. 2003. Glycan-dependent leukocyte adhesion and recruitment in inflammation. *Curr Opin Cell Biol 15:531*.

50. Lowe, J. B. 1997. Selectin ligands, leukocyte trafficking, and fucosyltransferase genes. *Kidney Int 51:1418*.

51. Smith, P. L., K. M. Gersten, B. Petryniak, R. J. Kelly, C. Rogers, Y. Natsuka, J. A. Alford, 3rd, E. P. Scheidegger, S. Natsuka, and J. B. Lowe. 1996. Expression of the alpha(1,3)fucosyltransferase Fuc-TVII in lymphoid aggregate high endothelial venules correlates with expression of L-selectin ligands. *J Biol Chem 271:8250*.

52. Maly, P., A. Thall, B. Petryniak, C. E. Rogers, P. L. Smith, R. M. Marks, R. J. Kelly, K. M. Gersten, G. Cheng, T. L. Saunders, S. A. Camper, R. T. Camphausen, F. X. Sullivan, Y. Isogai, O. Hindsgaul, U. H. von Andrian, and J. B. Lowe. 1996. The alpha(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. *Cell 86:643*.

53. Homeister, J. W., A. D. Thall, B. Petryniak, P. Maly, C. E. Rogers, P. L. Smith, R. J. Kelly, K. M. Gersten, S. W. Askari, G. Cheng, G. Smithson, R. M. Marks, A. K. Misra, O. Hindsgaul, U. H. von Andrian, and J. B. Lowe. 2001. The alpha(1,3)fucosyltransferases FucT-IV and FucT-VII exert collaborative control over selectin-dependent leukocyte recruitment and lymphocyte homing. *Immunity 15:115*.

54. Shi, S., C. Ge, Y. Luo, X. Hou, R. S. Haltiwanger, and P. Stanley. 2007. The threonine that carries fucose, but not fucose, is required for cripto to facilitate nodal signaling. *J Biol Chem*.

55. Rabbani, S. A., A. P. Mazar, S. M. Bernier, M. Haq, I. Bolivar, J. Henkin, and D. Goltzman. 1992. Structural requirements for the growth factor activity of the amino-terminal domain of urokinase. *J Biol Chem 267:14151*.

56. Harris, R. J., and M. W. Spellman. 1993. O-linked fucose and other post-translational modifications unique to EGF modules. *Glycobiology 3:219*.

57. Shi, S., M. Stahl, L. Lu, and P. Stanley. 2005. Canonical Notch signaling is dispensable for early cell fate specifications in mammals. *Mol Cell Biol 25:9503*.

58. Shi, S., and P. Stanley. 2003. Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc Natl Acad Sci U S A 100:5234*.

59. Greenwald, I., and G. M. Rubin. 1992. Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. *Cell 68:271*.

60. Ghysen, A., and C. Dambly-Chaudiere. 1993. The specification of sensory neuron identity in Drosophila. *Bioessays 15:293*.

61. Artavanis-Tsakonas, S., K. Matsuno, and M. E. Fortini. 1995. Notch signaling. *Science 268:225*.

62. Simpson, P. 1995. Developmental genetics. The Notch connection. *Nature 375:736*.

63. Gridley, T. 1997. Notch signaling in vertebrate development and disease. *Mol Cell Neurosci 9:103*.

64. Blair, S. S. 1999. Eye development: Notch lends a handedness. *Curr Biol 9:R356*.

65. Hofmann, J. J., and M. L. Iruela-Arispe. 2007. Notch signaling in blood vessels: who is talking to whom about what? *Circ Res 100:1556*.

66. Pedrazzini, T. 2007. Control of cardiogenesis by the notch pathway. *Trends Cardiovasc Med 17:83*.

67. Tanigaki, K., and T. Honjo. 2007. Regulation of lymphocyte development by Notch signaling. *Nat Immunol 8:451*.

68. Ito, T., N. Udaka, T. Yazawa, K. Okudela, H. Hayashi, T. Sudo, F. Guillemot, R. Kageyama, and H. Kitamura. 2000. Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. *Development 127:3913*.

69. Yeo, S. Y., and A. B. Chitnis. 2007. Jagged-mediated Notch signaling maintains proliferating neural progenitors and regulates cell diversity in the ventral spinal cord. *Proc Natl Acad Sci U S A 104:5913*.

70. Politi, K., N. Feirt, and J. Kitajewski. 2004. Notch in mammary gland development and breast cancer. *Semin Cancer Biol 14:341*.

71. Collins, B. J., W. Kleeberger, and D. W. Ball. 2004. Notch in lung development and lung cancer. *Semin Cancer Biol 14:357*.

72. Pear, W. S., and J. C. Aster. 2004. T cell acute lymphoblastic leukemia/lymphoma: a human cancer commonly associated with aberrant NOTCH1 signaling. *Curr Opin Hematol 11:426*.

73. Jhappan, C., D. Gallahan, C. Stahle, E. Chu, G. H. Smith, G. Merlino, and R. Callahan. 1992. Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Dev 6:345*.

74. Imatani, A., and R. Callahan. 2000. Identification of a novel NOTCH-4/INT-3 RNA species encoding an activated gene product in certain human tumor cell lines. *Oncogene 19:223*.

75. Ellisen, L. W., J. Bird, D. C. West, A. L. Soreng, T. C. Reynolds, S. D. Smith, and J. Sklar. 1991. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell 66:649*.

76. Pear, W. S., J. C. Aster, M. L. Scott, R. P. Hasserjian, B. Soffer, J. Sklar, and D. Baltimore. 1996. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med 183:2283*.

77. Radtke, F., A. Wilson, G. Stark, M. Bauer, J. van Meerwijk, H. R. MacDonald, and M. Aguet. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity 10:547*.

78. Wilson, A., H. R. MacDonald, and F. Radtke. 2001. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med 194:1003*.

79. Charles A Janeway, P. T., Mark Walport, Mark Shlomchik. 2001. *Immunobiology*. Garland Publishing.

80. Travlos, G. S. 2006. Normal structure, function, and histology of the bone marrow. *Toxicol Pathol 34:548*.

81. Goldstein, A. L., and A. White. 1971. The thymus gland: experimental and clinical studies of its role in the development and expression of immune functions. *Adv Metab Disord 5:149*.

82. Lockwood, C. M. 1983. Immunological functions of the spleen. *Clin Haematol 12:449*.

83. Castenholz, A. 1990. Architecture of the lymph node with regard to its function. *Curr Top Pathol 84 (Pt 1):1*.

84. Szulman, A. E. 1966. Chemistry, distribution, and function of blood group substances. *Annu Rev Med 17:307*.

85. Spangrude, G. J., L. Smith, N. Uchida, K. Ikuta, S. Heimfeld, J. Friedman, and I. L. Weissman. 1991. Mouse hematopoietic stem cells. *Blood 78:1395*.

86. Siminovitch, L., E. A. McCulloch, and J. E. Till. 1963. The Distribution Of Colony-Forming Cells Among Spleen Colonies. *J Cell Physiol 62:327*.

87. Spangrude, G. J., S. Heimfeld, and I. L. Weissman. 1988. Purification and characterization of mouse hematopoietic stem cells. *Science 241:58*.

88. Morrison, S. J., and I. L. Weissman. 1994. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity 1:661*.

89. Yang, L., D. Bryder, J. Adolfsson, J. Nygren, R. Mansson, M. Sigvardsson, and S. E. Jacobsen. 2005. Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term

hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood 105:2717*.

90. Wu, A. M., J. E. Till, L. Siminovitch, and E. A. McCulloch. 1967. A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. *J Cell Physiol 69:177*.

91. Akashi, K., D. Traver, T. Miyamoto, and I. L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature 404:193*.

92. Kondo, M., I. L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell 91:661*.

93. Smith, P. L., J. T. Myers, C. E. Rogers, L. Zhou, B. Petryniak, D. J. Becker, J. W. Homeister, and J. B. Lowe. 2002. Conditional control of selectin ligand expression and global fucosylation events in mice with a targeted mutation at the FX locus. *J Cell Biol 158:801*.

94. Anderson, D. C., and T. A. Springer. 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu Rev Med 38:175*.

95. Etzioni, A., M. Frydman, S. Pollack, I. Avidor, M. L. Phillips, J. C. Paulson, and R. Gershoni-Baruch. 1992. Brief report: recurrent severe infections caused by a novel leukocyte adhesion deficiency. *N Engl J Med 327:1789*.

96. Etzioni, A., R. Gershoni-Baruch, S. Pollack, and N. Shehadeh. 1998. Leukocyte adhesion deficiency type II: long-term follow-up. *J Allergy Clin Immunol 102:323*.

97. Karsan, A., C. J. Cornejo, R. K. Winn, B. R. Schwartz, W. Way, N. Lannir, R. Gershoni-Baruch, A. Etzioni, H. D. Ochs, and J. M. Harlan. 1998. Leukocyte Adhesion Deficiency Type II is a generalized defect of de novo GDP-fucose biosynthesis. Endothelial cell fucosylation is not required for neutrophil rolling on human nonlymphoid endothelium. *J Clin Invest 101:2438*.

98. Ginsburg, V. 1960. Formation of guanosine diphosphate L-fucose from guanosine diphosphate D-mannose. *J Biol Chem 235:2196*.

99. Foster, D. W., and V. Ginsburg. 1961. Biosynthesis of L-fucose by mammalian tissue. *Biochim Biophys Acta 54:376*.

100. Ginsburg, V. 1961. Studies on the biosynthesis of guanosine diphosphate L-fucose. *J Biol Chem 236:2389*.

101. Oths, P. J., R. M. Mayer, and H. G. Floss. 1990. Stereochemistry and mechanism of the GDP-mannose dehydratase reaction. *Carbohydr Res 198:91*.

102. Stevenson, G., K. Andrianopoulos, M. Hobbs, and P. R. Reeves. 1996. Organization of the Escherichia coli K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. *J Bacteriol 178:4885*.

103. Somoza, J. R., S. Menon, H. Schmidt, D. Joseph-McCarthy, A. Dessen, M. L. Stahl, W. S. Somers, and F. X. Sullivan. 2000. Structural and kinetic analysis of

Escherichia coli GDP-mannose 4,6 dehydratase provides insights into the enzyme's catalytic mechanism and regulation by GDP-fucose. *Structure 8:123*.

104. Sturla, L., A. Bisso, D. Zanardi, U. Benatti, A. De Flora, and M. Tonetti. 1997. Expression, purification and characterization of GDP-D-mannose 4,6-dehydratase from Escherichia coli. *FEBS Lett 412:126*.

105. Ohyama, C., P. L. Smith, K. Angata, M. N. Fukuda, J. B. Lowe, and M. Fukuda. 1998. Molecular cloning and expression of GDP-D-mannose-4,6-dehydratase, a key enzyme for fucose metabolism defective in Lec13 cells. *J Biol Chem 273:14582*.

106. Sullivan, F. X., R. Kumar, R. Kriz, M. Stahl, G. Y. Xu, J. Rouse, X. J. Chang, A. Boodhoo, B. Potvin, and D. A. Cumming. 1998. Molecular cloning of human GDP-mannose 4,6-dehydratase and reconstitution of GDP-fucose biosynthesis in vitro. *J Biol Chem 273:8193*.

107. Bisso, A., L. Sturla, D. Zanardi, A. De Flora, and M. Tonetti. 1999. Structural and enzymatic characterization of human recombinant GDP-D-mannose-4,6-dehydratase. *FEBS Lett 456:370*.

108. Snook, C. F., P. A. Tipton, and L. J. Beamer. 2003. Crystal structure of GDP-mannose dehydrogenase: a key enzyme of alginate biosynthesis in P. aeruginosa. *Biochemistry 42:4658*.

109. Kornfeld, R. H., and V. Ginsburg. 1966. Control of synthesis of guanosine 5'-diphosphate D-mannose and guanosine 5'-diphosphate L-fucose in bacteria. *Biochim Biophys Acta 117:79*.

110. Broschat, K. O., S. Chang, and G. Serif. 1985. Purification and characterization of GDP-D-mannose 4,6-dehydratase from porcine thyroid. *Eur J Biochem 153:397*.

111. Chang, S., B. Duerr, and G. Serif. 1988. An epimerase-reductase in L-fucose synthesis. *J Biol Chem 263:1693*.

112. Tonetti, M., L. Sturla, A. Bisso, U. Benatti, and A. De Flora. 1996. Synthesis of GDP-L-fucose by the human FX protein. *J Biol Chem 271:27274*.

113. Menon, S., M. Stahl, R. Kumar, G. Y. Xu, and F. Sullivan. 1999. Stereochemical course and steady state mechanism of the reaction catalyzed by the GDP-fucose synthetase from Escherichia coli. *J Biol Chem 274:26743*.

114. DeFlora, A., A. Morelli, U. Benatti, and F. Giuliano. 1975. An improved procedure for rapid isolation of glucose 6-phosphate dehydrogenase from human erythrocytes. *Arch Biochem Biophys 169:362*.

115. Tonetti, M., M. Rizzi, P. Vigevani, L. Sturla, A. Bisso, A. De Flora, and M. Bolognesi. 1998. Preliminary crystallographic investigations of recombinant GDP-4-keto-6-deoxy-D-mannose epimerase/reductase from E. coli. *Acta Crystallogr D Biol Crystallogr 54:684*.

116. Rizzi, M., M. Tonetti, P. Vigevani, L. Sturla, A. Bisso, A. D. Flora, D. Bordo, and M. Bolognesi. 1998. GDP-4-keto-6-deoxy-D-mannose epimerase/reductase from Escherichia coli, a key enzyme in the biosynthesis of GDP-L-fucose, displays the

structural characteristics of the RED protein homology superfamily. *Structure 6:1453*.

117. Somers, W. S., M. L. Stahl, and F. X. Sullivan. 1998. GDP-fucose synthetase from Escherichia coli: structure of a unique member of the short-chain dehydrogenase/reductase family that catalyzes two distinct reactions at the same active site. *Structure 6:1601*.

118. Rosano, C., A. Bisso, G. Izzo, M. Tonetti, L. Sturla, A. De Flora, and M. Bolognesi. 2000. Probing the catalytic mechanism of GDP-4-keto-6-deoxy-d-mannose Epimerase/Reductase by kinetic and crystallographic characterization of site-specific mutants. *J Mol Biol 303:77*.

119. Capasso, J. M., and C. B. Hirschberg. 1984. Effect of nucleotides on translocation of sugar nucleotides and adenosine 3'-phosphate 5'-phosphosulfate into Golgi apparatus vesicles. *Biochim Biophys Acta 777:133*.

120. Ishida, N., and M. Kawakita. 2004. Molecular physiology and pathology of the nucleotide sugar transporter family (SLC35). *Pflugers Arch 447:768*.

121. Noda, K., E. Miyoshi, J. Gu, C. X. Gao, S. Nakahara, T. Kitada, K. Honke, K. Suzuki, H. Yoshihara, K. Yoshikawa, K. Kawano, M. Tonetti, A. Kasahara, M. Hori, N. Hayashi, and N. Taniguchi. 2003. Relationship between elevated FX expression and increased production of GDP-L-fucose, a common donor substrate for fucosylation in human hepatocellular carcinoma and hepatoma cell lines. *Cancer Res 63:6282*.

122. Zipin, A., M. Israeli-Amit, T. Meshel, O. Sagi-Assif, I. Yron, V. Lifshitz, E. Bacharach, N. I. Smorodinsky, A. Many, P. A. Czernilofsky, D. L. Morton, and I. P. Witz. 2004. Tumor-microenvironment interactions: the fucose-generating FX enzyme controls adhesive properties of colorectal cancer cells. *Cancer Res 64:6571*.

123. Eshel, R., M. Besser, A. Zanin, O. Sagi-Assif, and I. P. Witz. 2001. The FX enzyme is a functional component of lymphocyte activation. *Cell Immunol 213:141*.

124. Niittymaki, J., P. Mattila, and R. Renkonen. 2006. Differential gene expression of GDP-L-fucose-synthesizing enzymes, GDP-fucose transporter and fucosyltransferase VII. *Apmis 114:539*.

125. Sturla, L., A. Etzioni, A. Bisso, D. Zanardi, G. De Flora, L. Silengo, A. De Flora, and M. Tonetti. 1998. Defective intracellular activity of GDP-D-mannose-4,6-dehydratase in leukocyte adhesion deficiency type II syndrome. *FEBS Lett 429:274*.

126. Becker, D. J., and J. B. Lowe. 1999. Leukocyte adhesion deficiency type II. *Biochim Biophys Acta 1455:193*.

127. Shechter, Y., A. Etzioni, C. Levene, and P. Greenwell. 1995. A Bombay individual lacking H and Le antigens but expressing normal levels of alpha-2- and alpha-4-fucosyltransferases. *Transfusion 35:773*.

128. Lubke, T., T. Marquardt, K. von Figura, and C. Korner. 1999. A new type of carbohydrate-deficient glycoprotein syndrome due to a decreased import of GDP-fucose into the golgi. *J Biol Chem 274:25986*.

129. Sturla, L., L. Puglielli, M. Tonetti, P. Berninsone, C. B. Hirschberg, A. De Flora, and A. Etzioni. 2001. Impairment of the Golgi GDP-L-fucose transport and unresponsiveness to fucose replacement therapy in LAD II patients. *Pediatr Res 49:537*.

130. Lubke, T., T. Marquardt, A. Etzioni, E. Hartmann, K. von Figura, and C. Korner. 2001. Complementation cloning identifies CDG-IIc, a new type of congenital disorders of glycosylation, as a GDP-fucose transporter deficiency. *Nat Genet 28:73*.

131. Hidalgo, A., S. Ma, A. J. Peired, L. A. Weiss, C. Cunningham-Rundles, and P. S. Frenette. 2003. Insights into leukocyte adhesion deficiency type 2 from a novel mutation in the GDP-fucose transporter gene. *Blood 101:1705*.

132. Luhn, K., M. K. Wild, M. Eckhardt, R. Gerardy-Schahn, and D. Vestweber. 2001. The gene defective in leukocyte adhesion deficiency II encodes a putative GDP-fucose transporter. *Nat Genet 28:69*.

133. Hellbusch, C. C., M. Sperandio, D. Frommhold, S. Yakubenia, M. K. Wild, D. Popovici, D. Vestweber, H. J. Grone, K. von Figura, T. Lubke, and C. Korner. 2007. Golgi GDP-fucose transporter-deficient mice mimic congenital disorder of glycosylation IIc/leukocyte adhesion deficiency II. *J Biol Chem 282:10762*.

134. Kaufman, R. L., and V. Ginsburg. 1968. The metabolism of L-fucose by HeLa cells. *Exp Cell Res 50:127*.

135. Wiese, T. J., J. A. Dunlap, and M. A. Yorek. 1994. L-fucose is accumulated via a specific transport system in eukaryotic cells. *J Biol Chem 269:22705*.

136. Leck, J. R., and T. J. Wiese. 2004. Purification and characterization of the L-fucose transporter. *Protein Expr Purif 37:288*.

137. Miller, E. N., A. L. Rupp, M. K. Lindberg, and T. J. Wiese. 2005. Tissue distribution of L-fucokinase in rodents. *Comp Biochem Physiol B Biochem Mol Biol 140:513*.

138. Park, S. H., I. Pastuszak, R. Drake, and A. D. Elbein. 1998. Purification to apparent homogeneity and properties of pig kidney L-fucose kinase. *J Biol Chem 273:5685*.

139. Butler, W., and G. S. Serif. 1985. Fucokinase, its anomeric specificity and mechanism of phosphate group transfer. *Biochim Biophys Acta 829:238*.

140. Pastuszak, I., C. Ketchum, G. Hermanson, E. J. Sjoberg, R. Drake, and A. D. Elbein. 1998. GDP-L-fucose pyrophosphorylase. Purification, cDNA cloning, and properties of the enzyme. *J Biol Chem 273:30165*.

141. Niittymaki, J., P. Mattila, C. Roos, L. Huopaniemi, S. Sjoblom, and R. Renkonen. 2004. Cloning and expression of murine enzymes involved in the salvage pathway of GDP-L-fucose. *Eur J Biochem 271:78*.

142. Popov, N., S. Schmidt, S. Schulzeck, R. Jork, B. Lossner, and H. Matthies. 1983. Changes in activities of fucokinase and fucosyltransferase in rat hippocampus after acquisition of a brightness discrimination reaction. *Pharmacol Biochem Behav 19:43*.

143. Lossner, B., and S. P. Rose. 1983. Passive avoidance training increases fucokinase activity in right forebrain base of day-old chicks. *J Neurochem 41:1357*.

144. Jork, R., M. Schmitt, B. Lossner, and H. Matthies. 1984. Dopamine stimulated L-fucose incorporation into brain proteins is related to an increase in fucokinase activity. *Biomed Biochim Acta 43:261*.

145. Hocher, B., F. Abou-Rebyeh, and C. Bauer. 1993. Influence of dopaminergic agonists/antagonists on fucose metabolism in the rat brain. *Eur J Clin Chem Clin Biochem 31:347*.

146. Zeitler, R., S. Danneschewski, T. Lindhorst, J. Thiem, and W. Reutter. 1997. Inhibition of L-fucokinase from rat liver by L-fucose analogues in vitro. *J Enzyme Inhib 11:265*.

147. Yurchenco, P. D., and P. H. Atkinson. 1975. Fucosyl-glycoprotein and precursor polls in HeLa cells. *Biochemistry 14:3107*.

148. Luhn, K., T. Marquardt, E. Harms, and D. Vestweber. 2001. Discontinuation of fucose therapy in LADII causes rapid loss of selectin ligands and rise of leukocyte counts. *Blood 97:330*.

149. Kornfeld, K., M. L. Reitman, and R. Kornfeld. 1981. The carbohydrate-binding specificity of pea and lentil lectins. Fucose is an important determinant. *J Biol Chem 256:6633*.

150. Reitman, M. L., I. S. Trowbridge, and S. Kornfeld. 1980. Mouse lymphoma cell lines resistant to pea lectin are defective in fucose metabolism. *J Biol Chem 255:9900*.

151. Chapman, J. A., O. W. Wiebkin, and W. G. Breed. 2000. Interspecific variation of zona pellucida glycoconjugates in several species of marsupial. *J Reprod Fertil 119:111*.

152. Pearse, M., L. Wu, M. Egerton, A. Wilson, K. Shortman, and R. Scollay. 1989. A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. *Proc Natl Acad Sci U S A 86:1614*.

153. Godfrey, D. I., J. Kennedy, T. Suda, and A. Zlotnik. 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol 150:4244*.

154. Godfrey, D. I., J. Kennedy, P. Mombaerts, S. Tonegawa, and A. Zlotnik. 1994. Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3-CD4-CD8- thymocyte differentiation. *J Immunol 152:4783*.

155. Akashi, K., L. I. Richie, T. Miyamoto, W. H. Carr, and I. L. Weissman. 2000. B lymphopoiesis in the thymus. *J Immunol 164:5221*.

156. Matsuzaki, Y., J. Gyotoku, M. Ogawa, S. Nishikawa, Y. Katsura, G. Gachelin, and H. Nakauchi. 1993. Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. *J Exp Med 178:1283*.

157. Martin, C. H., I. Aifantis, M. L. Scimone, U. H. von Andrian, B. Reizis, H. von Boehmer, and F. Gounari. 2003. Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. *Nat Immunol 4:866*.

158. Allman, D., A. Sambandam, S. Kim, J. P. Miller, A. Pagan, D. Well, A. Meraz, and A. Bhandoola. 2003. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol 4:168*.

159. Porritt, H. E., L. L. Rumfelt, S. Tabrizifard, T. M. Schmitt, J. C. Zuniga-Pflucker, and H. T. Petrie. 2004. Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity 20:735*.

160. Krishan, A. 1975. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J Cell Biol 66:188*.

161. Ceredig, R., and T. Rolink. 2002. A positive look at double-negative thymocytes. *Nat Rev Immunol 2:888*.

162. Sambandam, A., I. Maillard, V. P. Zediak, L. Xu, R. M. Gerstein, J. C. Aster, W. S. Pear, and A. Bhandoola. 2005. Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nat Immunol 6:663*.

163. Wu, L., T. Sun, K. Kobayashi, P. Gao, and J. D. Griffin. 2002. Identification of a family of mastermind-like transcriptional coactivators for mammalian notch receptors. *Mol Cell Biol 22:7688*.

164. Gray, D. H., T. Ueno, A. P. Chidgey, M. Malin, G. L. Goldberg, Y. Takahama, and R. L. Boyd. 2005. Controlling the thymic microenvironment. *Curr Opin Immunol 17:137*.

165. Goldschneider, I., K. L. Komschlies, and D. L. Greiner. 1986. Studies of thymocytopoiesis in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. *J Exp Med 163:1*.

166. Rossi, F. M., S. Y. Corbel, J. S. Merzaban, D. A. Carlow, K. Gossens, J. Duenas, L. So, L. Yi, and H. J. Ziltener. 2005. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat Immunol 6:626*.

167. Moore, K. L., S. F. Eaton, D. E. Lyons, H. S. Lichenstein, R. D. Cummings, and R. P. McEver. 1994. The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, O-linked poly-N-acetyllactosamine. *J Biol Chem 269:23318*.

168. Sako, D., X. J. Chang, K. M. Barone, G. Vachino, H. M. White, G. Shaw, G. M. Veldman, K. M. Bean, T. J. Ahern, B. Furie, and et al. 1993. Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell 75:1179*.

169. Yang, J., J. Galipeau, C. A. Kozak, B. C. Furie, and B. Furie. 1996. Mouse P-selectin glycoprotein ligand-1: molecular cloning, chromosomal localization, and expression of a functional P-selectin receptor. *Blood 87:4176*.

170. Lenter, M., A. Levinovitz, S. Isenmann, and D. Vestweber. 1994. Monospecific and common glycoprotein ligands for E- and P-selectin on myeloid cells. *J Cell Biol 125:471*.

171. Asa, D., L. Raycroft, L. Ma, P. A. Aeed, P. S. Kaytes, A. P. Elhammer, and J. G. Geng. 1995. The P-selectin glycoprotein ligand functions as a common human leukocyte ligand for P- and E-selectins. *J Biol Chem 270:11662*.

172. Norman, K. E., K. L. Moore, R. P. McEver, and K. Ley. 1995. Leukocyte rolling in vivo is mediated by P-selectin glycoprotein ligand-1. *Blood 86:4417*.

173. Moore, K. L., K. D. Patel, R. E. Bruehl, F. Li, D. A. Johnson, H. S. Lichenstein, R. D. Cummings, D. F. Bainton, and R. P. McEver. 1995. P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J Cell Biol 128:661*.

174. Niemela, R., J. Natunen, M. L. Majuri, H. Maaheimo, J. Helin, J. B. Lowe, O. Renkonen, and R. Renkonen. 1998. Complementary acceptor and site specificities of Fuc-TIV and Fuc-TVII allow effective biosynthesis of sialyl-TriLex and related polylactosamines present on glycoprotein counterreceptors of selectins. *J Biol Chem 273:4021*.

175. Surh, C. D., and J. Sprent. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature 372:100*.

176. Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods 184:39*.

177. Kukowska-Latallo, J. F., R. D. Larsen, R. P. Nair, and J. B. Lowe. 1990. A cloned human cDNA determines expression of a mouse stage-specific embryonic antigen and the Lewis blood group alpha(1,3/1,4)fucosyltransferase. *Genes Dev 4:1288*.

178. Lowe, J. B., L. M. Stoolman, R. P. Nair, R. D. Larsen, T. L. Berhend, and R. M. Marks. 1990. ELAM-1--dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA. *Cell 63:475*.

179. Kumar, R., B. Potvin, W. A. Muller, and P. Stanley. 1991. Cloning of a human alpha(1,3)-fucosyltransferase gene that encodes ELFT but does not confer ELAM-1 recognition on Chinese hamster ovary cell transfectants. *J Biol Chem 266:21777*.

180. Weston, B. W., P. L. Smith, R. J. Kelly, and J. B. Lowe. 1992. Molecular cloning of a fourth member of a human alpha (1,3)fucosyltransferase gene family. Multiple homologous sequences that determine expression of the Lewis x, sialyl Lewis x, and difucosyl sialyl Lewis x epitopes. *J Biol Chem 267:24575*.

181. Koszdin, K. L., and B. R. Bowen. 1992. The cloning and expression of a human alpha-1,3 fucosyltransferase capable of forming the E-selectin ligand. *Biochem Biophys Res Commun 187:152*.

182. Weston, B. W., R. P. Nair, R. D. Larsen, and J. B. Lowe. 1992. Isolation of a novel human alpha (1,3)fucosyltransferase gene and molecular comparison to the human Lewis blood group alpha (1,3/1,4)fucosyltransferase gene. Syntenic, homologous,

nonallelic genes encoding enzymes with distinct acceptor substrate specificities. *J Biol Chem 267:4152*.

183. Natsuka, S., K. M. Gersten, K. Zenita, R. Kannagi, and J. B. Lowe. 1994. Molecular cloning of a cDNA encoding a novel human leukocyte alpha-1,3-fucosyltransferase capable of synthesizing the sialyl Lewis x determinant. *J Biol Chem 269:16789*.

184. Sasaki, K., K. Kurata, K. Funayama, M. Nagata, E. Watanabe, S. Ohta, N. Hanai, and T. Nishi. 1994. Expression cloning of a novel alpha 1,3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis x carbohydrate determinants in leukocytes. *J Biol Chem 269:14730*.

185. Yamaguchi, Y., J. Fujii, S. Inoue, N. Uozumi, S. Yanagidani, Y. Ikeda, M. Egashira, O. Miyoshi, N. Niikawa, and N. Taniguchi. 1999. Mapping of the alpha-1,6-fucosyltransferase gene, FUT8, to human chromosome 14q24.3. *Cytogenet Cell Genet 84:58*.

186. Kaneko, M., T. Kudo, H. Iwasaki, Y. Ikehara, S. Nishihara, S. Nakagawa, K. Sasaki, T. Shiina, H. Inoko, N. Saitou, and H. Narimatsu. 1999. Alpha1,3-fucosyltransferase IX (Fuc-TIX) is very highly conserved between human and mouse; molecular cloning, characterization and tissue distribution of human Fuc-TIX. *FEBS Lett 452:237*.

187. Oriol, R., R. Mollicone, A. Cailleau, L. Balanzino, and C. Breton. 1999. Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. *Glycobiology 9:323*.

188. Roos, C., M. Kolmer, P. Mattila, and R. Renkonen. 2002. Composition of Drosophila melanogaster proteome involved in fucosylated glycan metabolism. *J Biol Chem 277:3168*.

189. Baboval, T., and F. I. Smith. 2002. Comparison of human and mouse Fuc-TX and Fuc-TXI genes, and expression studies in the mouse. *Mamm Genome 13:538*.

190. Domino, S. E., L. Zhang, P. J. Gillespie, T. L. Saunders, and J. B. Lowe. 2001. Deficiency of reproductive tract alpha(1,2)fucosylated glycans and normal fertility in mice with targeted deletions of the FUT1 or FUT2 alpha(1,2)fucosyltransferase locus. *Mol Cell Biol 21:8336*.

191. Wang, X., S. Inoue, J. Gu, E. Miyoshi, K. Noda, W. Li, Y. Mizuno-Horikawa, M. Nakano, M. Asahi, M. Takahashi, N. Uozumi, S. Ihara, S. H. Lee, Y. Ikeda, Y. Yamaguchi, Y. Aze, Y. Tomiyama, J. Fujii, K. Suzuki, A. Kondo, S. D. Shapiro, C. Lopez-Otin, T. Kuwaki, M. Okabe, K. Honke, and N. Taniguchi. 2005. Dysregulation of TGF-beta1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. *Proc Natl Acad Sci U S A 102:15791*.

192. Kudo, T., M. Kaneko, H. Iwasaki, A. Togayachi, S. Nishihara, K. Abe, and H. Narimatsu. 2004. Normal embryonic and germ cell development in mice lacking alpha 1,3-fucosyltransferase IX (Fut9) which show disappearance of stage-specific embryonic antigen 1. *Mol Cell Biol 24:4221*.

193. Reguigne-Arnould, I., P. Couillin, R. Mollicone, S. Faure, A. Fletcher, R. J. Kelly, J. B. Lowe, and R. Oriol. 1995. Relative positions of two clusters of human alpha-L-

fucosyltransferases in 19q (FUT1-FUT2) and 19p (FUT6-FUT3-FUT5) within the microsatellite genetic map of chromosome 19. *Cytogenet Cell Genet 71:158*.

194. Gersten, K. M., S. Natsuka, M. Trinchera, B. Petryniak, R. J. Kelly, N. Hiraiwa, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, and J. B. Lowe. 1995. Molecular cloning, expression, chromosomal assignment, and tissue-specific expression of a murine alpha-(1,3)-fucosyltransferase locus corresponding to the human ELAM-1 ligand fucosyl transferase. *J Biol Chem 270:25047*.

195. Costache, M., P. A. Apoil, A. Cailleau, A. Elmgren, G. Larson, S. Henry, A. Blancher, D. Iordachescu, R. Oriol, and R. Mollicone. 1997. Evolution of fucosyltransferase genes in vertebrates. *J Biol Chem 272:29721*.

196. Luo, Y., K. Koles, W. Vorndam, R. S. Haltiwanger, and V. M. Panin. 2006. Protein O-fucosyltransferase 2 adds O-fucose to thrombospondin type 1 repeats. *J Biol Chem 281:9393*.

197. Luo, Y., A. Nita-Lazar, and R. S. Haltiwanger. 2006. Two distinct pathways for O-fucosylation of epidermal growth factor-like or thrombospondin type 1 repeats. *J Biol Chem 281:9385*.

198. Han, H., K. Tanigaki, N. Yamamoto, K. Kuroda, M. Yoshimoto, T. Nakahata, K. Ikuta, and T. Honjo. 2002. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol 14:637*.

199. Panin, V. M., L. Shao, L. Lei, D. J. Moloney, K. D. Irvine, and R. S. Haltiwanger. 2002. Notch ligands are substrates for protein O-fucosyltransferase-1 and Fringe. *J Biol Chem 277:29945*.

200. Sasamura, T., N. Sasaki, F. Miyashita, S. Nakao, H. O. Ishikawa, M. Ito, M. Kitagawa, K. Harigaya, E. Spana, D. Bilder, N. Perrimon, and K. Matsuno. 2003. neurotic, a novel maternal neurogenic gene, encodes an O-fucosyltransferase that is essential for Notch-Delta interactions. *Development 130:4785*.

201. Okajima, T., A. Xu, L. Lei, and K. D. Irvine. 2005. Chaperone activity of protein O-fucosyltransferase 1 promotes notch receptor folding. *Science 307:1599*.

202. Pui, J. C., D. Allman, L. Xu, S. DeRocco, F. G. Karnell, S. Bakkour, J. Y. Lee, T. Kadesch, R. R. Hardy, J. C. Aster, and W. S. Pear. 1999. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity 11:299*.

203. Varnum-Finney, B., L. Wu, M. Yu, C. Brashem-Stein, S. Staats, D. Flowers, J. D. Griffin, and I. D. Bernstein. 2000. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *J Cell Sci 113 Pt 23:4313*.

204. Schmitt, T. M., and J. C. Zuniga-Pflucker. 2002. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity 17:749*.

205. Shultz, L. D., P. A. Schweitzer, S. W. Christianson, B. Gott, I. B. Schweitzer, B. Tennent, S. McKenna, L. Mobraaten, T. V. Rajan, D. L. Greiner, and et al. 1995. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol 154:180*.

206. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, J. D. Meyer, and B. L. Davison. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med 180:1955*.

207. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell 68:869*.

208. Nolan, G. P., and A. R. Shatzman. 1998. Expression vectors and delivery systems. *Curr Opin Biotechnol 9:447*.

209. Zuniga-Pflucker, J. C. 2004. T-cell development made simple. *Nat Rev Immunol 4:67*.

210. Huang, J., K. P. Garrett, R. Pelayo, J. C. Zuniga-Pflucker, H. T. Petrie, and P. W. Kincade. 2005. Propensity of adult lymphoid progenitors to progress to DN2/3 stage thymocytes with Notch receptor ligation. *J Immunol 175:4858*.

211. Schmitt, T. M., R. F. de Pooter, M. A. Gronski, S. K. Cho, P. S. Ohashi, and J. C. Zuniga-Pflucker. 2004. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat Immunol 5:410*.

212. Nofziger, D., A. Miyamoto, K. M. Lyons, and G. Weinmaster. 1999. Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. *Development 126:1689*.

213. Ladi, E., J. T. Nichols, W. Ge, A. Miyamoto, C. Yao, L. T. Yang, J. Boulter, Y. E. Sun, C. Kintner, and G. Weinmaster. 2005. The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. *J Cell Biol 170:983*.

214. Lehar, S. M., J. Dooley, A. G. Farr, and M. J. Bevan. 2005. Notch ligands Delta 1 and Jagged1 transmit distinct signals to T-cell precursors. *Blood 105:1440*.

215. Heinzel, K., C. Benz, V. C. Martins, I. D. Haidl, and C. C. Bleul. 2007. Bone marrow-derived hemopoietic precursors commit to the T cell lineage only after arrival in the thymic microenvironment. *J Immunol 178:858*.

216. Bhandoola, A., and A. Sambandam. 2006. From stem cell to T cell: one route or many? *Nat Rev Immunol 6:117*.

217. Wu, L. 2006. T lineage progenitors: the earliest steps en route to T lymphocytes. *Curr Opin Immunol 18:121*.

218. Takahama, Y. 2006. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol 6:127*.

219. Kondo, M., D. C. Scherer, A. G. King, M. G. Manz, and I. L. Weissman. 2001. Lymphocyte development from hematopoietic stem cells. *Curr Opin Genet Dev 11:520*.

220. Tomita, K., M. Hattori, E. Nakamura, S. Nakanishi, N. Minato, and R. Kageyama. 1999. The bHLH gene Hes1 is essential for expansion of early T cell precursors. *Genes Dev 13:1203*.

221. Jarriault, S., O. Le Bail, E. Hirsinger, O. Pourquie, F. Logeat, C. F. Strong, C. Brou, N. G. Seidah, and A. Isra l. 1998. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol Cell Biol 18:7423*.

222. Storck, S., F. Delbos, N. Stadler, C. Thirion-Delalande, F. Bernex, C. Verthuy, P. Ferrier, J. C. Weill, and C. A. Reynaud. 2005. Normal immune system development in mice lacking the Deltex-1 RING finger domain. *Mol Cell Biol 25:1437*.

223. Ordentlich, P., A. Lin, C. P. Shen, C. Blaumueller, K. Matsuno, S. Artavanis-Tsakonas, and T. Kadesch. 1998. Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol Cell Biol 18:2230*.

224. Reizis, B., and P. Leder. 2002. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. *Genes Dev 16:295*.

225. von Boehmer, H. 2005. Unique features of the pre-T-cell receptor alpha-chain: not just a surrogate. *Nat Rev Immunol 5:571*.

226. Reizis, B., and P. Leder. 2001. The upstream enhancer is necessary and sufficient for the expression of the pre-T cell receptor alpha gene in immature T lymphocytes. *J Exp Med 194:979*.

227. Rothenberg, E. V., and T. Taghon. 2005. Molecular genetics of T cell development. *Annu Rev Immunol 23:601*.

228. Van Beusechem, V. W., J. A. Bart-Baumeister, T. A. Bakx, L. C. Kaptein, R. J. Levinsky, and D. Valerio. 1994. Gene transfer into nonhuman primate CD34+CD11b- bone marrow progenitor cells capable of repopulating lymphoid and myeloid lineages. *Hum Gene Ther 5:295*.

229. Dunbar, C. E., M. Cottler-Fox, J. A. O'Shaughnessy, S. Doren, C. Carter, R. Berenson, S. Brown, R. C. Moen, J. Greenblatt, F. M. Stewart, and et al. 1995. Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood 85:3048*.

230. http://www.stanford.edu/group/nolan/retroviral_systems/phx.html.

231. Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci U S A 90:8392*.

232. Pear, W. S., J. P. Miller, L. Xu, J. C. Pui, B. Soffer, R. C. Quackenbush, A. M. Pendergast, R. Bronson, J. C. Aster, M. L. Scott, and D. Baltimore. 1998. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood 92:3780*.

233. Aster, J. C., L. Xu, F. G. Karnell, V. Patriub, J. C. Pui, and W. S. Pear. 2000. Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1. *Mol Cell Biol 20:7505*.

234. Allman, D., F. G. Karnell, J. A. Punt, S. Bakkour, L. Xu, P. Myung, G. A. Koretzky, J. C. Pui, J. C. Aster, and W. S. Pear. 2001. Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. *J Exp Med 194:99*.

235. Kotani, H., P. B. Newton, 3rd, S. Zhang, Y. L. Chiang, E. Otto, L. Weaver, R. M. Blaese, W. F. Anderson, and G. J. McGarrity. 1994. Improved methods of retroviral vector transduction and production for gene therapy. *Hum Gene Ther 5:19*.

236. Han, W., Q. Ye, and M. A. Moore. 2000. A soluble form of human Delta-like-1 inhibits differentiation of hematopoietic progenitor cells. *Blood 95:1616*.

237. Rawlings, D. J., S. G. Quan, R. M. Kato, and O. N. Witte. 1995. Long-term culture system for selective growth of human B-cell progenitors. *Proc Natl Acad Sci U S A 92:1570*.

238. Jaleco, A. C., H. Neves, E. Hooijberg, P. Gameiro, N. Clode, M. Haury, D. Henrique, and L. Parreira. 2001. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J Exp Med 194:991*.

239. de Pooter, R., and J. C. Zuniga-Pflucker. 2007. T-cell potential and development in vitro: the OP9-DL1 approach. *Curr Opin Immunol 19:163*.

240. Ciofani, M., G. C. Knowles, D. L. Wiest, H. von Boehmer, and J. C. Zuniga-Pflucker. 2006. Stage-specific and differential notch dependency at the alphabeta and gammadelta T lineage bifurcation. *Immunity 25:105*.

241. Besseyrias, V., E. Fiorini, L. J. Strobl, U. Zimber-Strobl, A. Dumortier, U. Koch, M. L. Arcangeli, S. Ezine, H. R. Macdonald, and F. Radtke. 2007. Hierarchy of Notch-Delta interactions promoting T cell lineage commitment and maturation. *J Exp Med 204:331*.

242. Rampal, R., J. F. Arboleda-Velasquez, A. Nita-Lazar, K. S. Kosik, and R. S. Haltiwanger. 2005. Highly conserved O-fucose sites have distinct effects on Notch1 function. *J Biol Chem 280:32133*.

243. Osborne, B. A., and L. M. Minter. 2007. Notch signalling during peripheral T-cell activation and differentiation. *Nat Rev Immunol 7:64*.

244. Bork, P., A. K. Downing, B. Kieffer, and I. D. Campbell. 1996. Structure and distribution of modules in extracellular proteins. *Q Rev Biophys 29:119*.

245. Banner, D. W., A. D'Arcy, C. Chene, F. K. Winkler, A. Guha, W. H. Konigsberg, Y. Nemerson, and D. Kirchhofer. 1996. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature 380:41*.

246. Muranyi, A., B. E. Finn, G. P. Gippert, S. Forsen, J. Stenflo, and T. Drakenberg. 1998. Solution structure of the N-terminal EGF-like domain from human factor VII. *Biochemistry 37:10605*.

247. Rebay, I., R. J. Fleming, R. G. Fehon, L. Cherbas, P. Cherbas, and S. Artavanis-Tsakonas. 1991. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell 67:687*.

248. Lei, L., A. Xu, V. M. Panin, and K. D. Irvine. 2003. An O-fucose site in the ligand binding domain inhibits Notch activation. *Development 130:6411*.

249. Sykes, D. B., and M. P. Kamps. 2001. Estrogen-dependent E2a/Pbx1 myeloid cell lines exhibit conditional differentiation that can be arrested by other leukemic oncoproteins. *Blood 98:2308*.

250. Shao, L., D. J. Moloney, and R. Haltiwanger. 2003. Fringe modifies O-fucose on mouse Notch1 at epidermal growth factor-like repeats within the ligand-binding site and the Abruptex region. *J Biol Chem 278:7775*.

251. Haines, N., and K. D. Irvine. 2003. Glycosylation regulates Notch signalling. *Nat Rev Mol Cell Biol 4:786*.

252. Delaney, C., B. Varnum-Finney, K. Aoyama, C. Brashem-Stein, and I. D. Bernstein. 2005. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood 106:2693*.

253. Finkelstein, J. 2007. Glycochemistry & Glycobiology. *Nature* 446:999.

254. Pantelouris, E. M., and J. Hair. 1970. Thymus dysgenesis in nude (nu nu) mice. *J Embryol Exp Morphol 24:615*.

255. Wortis, H. H., S. Nehlsen, and J. J. Owen. 1971. Abnormal development of the thymus in "nude" mice. *J Exp Med 134:681*.

256. Kingston, R., E. J. Jenkinson, and J. J. Owen. 1984. Characterization of stromal cell populations in the developing thymus of normal and nude mice. *Eur J Immunol 14:1052*.

257. Van Vliet, E., E. J. Jenkinson, R. Kingston, J. J. Owen, and W. Van Ewijk. 1985. Stromal cell types in the developing thymus of the normal and nude mouse embryo. *Eur J Immunol 15:675*.

258. Cordier, A. C., and S. M. Haumont. 1980. Development of thymus, parathyroids, and ultimo-branchial bodies in NMRI and nude mice. *Am J Anat 157:227*.

259. Rodewald, H. R., M. Ogawa, C. Haller, C. Waskow, and J. P. DiSanto. 1997. Pro-thymocyte expansion by c-kit and the common cytokine receptor gamma chain is essential for repertoire formation. *Immunity 6:265*.

260. Rothenberg, E. V. 2007. Regulatory factors for initial T lymphocyte lineage specification. *Curr Opin Hematol 14:322*.

261. Rothenberg, E. V., and M. K. Anderson. 2002. Elements of transcription factor network design for T-lineage specification. *Dev Biol 246:29*.

262. Schmitt, T. M., M. Ciofani, H. T. Petrie, and J. C. Zuniga-Pflucker. 2004. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med 200:469*.

263. Irvine, K. D., S. L. Helfand, and D. S. Hogness. 1991. The large upstream control region of the Drosophila homeotic gene Ultrabithorax. *Development 111:407*.

264. Irvine, K. D., and E. Wieschaus. 1994. fringe, a Boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during Drosophila wing development. *Cell 79:595*.

265. Speicher, S. A., U. Thomas, U. Hinz, and E. Knust. 1994. The Serrate locus of Drosophila and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. *Development 120:535*.

266. Doherty, D., G. Feger, S. Younger-Shepherd, L. Y. Jan, and Y. N. Jan. 1996. Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in Drosophila wing formation. *Genes Dev 10:421*.

267. de Celis, J. F., A. Garcia-Bellido, and S. J. Bray. 1996. Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. *Development 122:359*.

268. Diaz-Benjumea, F. J., and S. M. Cohen. 1995. Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. *Development 121:4215*.

269. Panin, V. M., V. Papayannopoulos, R. Wilson, and K. D. Irvine. 1997. Fringe modulates Notch-ligand interactions. *Nature 387:908*.

270. Harris, R. J., V. T. Ling, and M. W. Spellman. 1992. O-linked fucose is present in the first epidermal growth factor domain of factor XII but not protein C. *J Biol Chem 267:5102*.

271. Bruckner, K., L. Perez, H. Clausen, and S. Cohen. 2000. Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature 406:411*.

272. Di Santo, J. P., and C. A. Vosshenrich. 2006. Bone marrow versus thymic pathways of natural killer cell development. *Immunol Rev 214:35*.

273. Vosshenrich, C. A., M. E. Garcia-Ojeda, S. I. Samson-Villeger, V. Pasqualetto, L. Enault, O. Richard-Le Goff, E. Corcuff, D. Guy-Grand, B. Rocha, A. Cumano, L. Rogge, S. Ezine, and J. P. Di Santo. 2006. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol 7:1217*.

274. Visan, I., J. S. Yuan, J. B. Tan, K. Cretegny, and C. J. Guidos. 2006. Regulation of intrathymic T-cell development by Lunatic Fringe- Notch1 interactions. *Immunol Rev 209:76*.

275. Johnston, S. H., C. Rauskolb, R. Wilson, B. Prabhakaran, K. D. Irvine, and T. F. Vogt. 1997. A family of mammalian Fringe genes implicated in boundary determination and the Notch pathway. *Development 124:2245*.

276. Koch, U., T. A. Lacombe, D. Holland, J. L. Bowman, B. L. Cohen, S. E. Egan, and C. J. Guidos. 2001. Subversion of the T/B lineage decision in the thymus by lunatic fringe-mediated inhibition of Notch-1. *Immunity 15:225*.

277. Visan, I., J. B. Tan, J. S. Yuan, J. A. Harper, U. Koch, and C. J. Guidos. 2006. Regulation of T lymphopoiesis by Notch1 and Lunatic fringe-mediated competition for intrathymic niches. *Nat Immunol 7:634*.

278. Zhang, N., and T. Gridley. 1998. Defects in somite formation in lunatic fringe-deficient mice. *Nature 394:374*.

279. Evrard, Y. A., Y. Lun, A. Aulehla, L. Gan, and R. L. Johnson. 1998. lunatic fringe is an essential mediator of somite segmentation and patterning. *Nature 394:377*.

280. Hahn, K. L., J. Johnson, B. J. Beres, S. Howard, and J. Wilson-Rawls. 2005. Lunatic fringe null female mice are infertile due to defects in meiotic maturation. *Development 132:817*.

281. Xu, J., C. R. Norton, and T. Gridley. 2006. Not all lunatic fringe null female mice are infertile. *Development 133:579; author reply 579*.

282. Collu, G. M., and K. Brennan. 2007. Cooperation between Wnt and Notch signalling in human breast cancer. *Breast Cancer Res 9:105*.

283. Blokzijl, A., C. Dahlqvist, E. Reissmann, A. Falk, A. Moliner, U. Lendahl, and C. F. Ibanez. 2003. Cross-talk between the Notch and TGF-beta signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. *J Cell Biol 163:723*.