α1,3-Fucoslytransferase IX (Fuc-TIX) is very highly conserved between human and mouse; molecular cloning, characterization and tissue distribution of human Fuc-TIX

Mika Kaneko<sup>a</sup>,<sup>b</sup>, Takashi Kudo<sup>a</sup>, Hiroko Iwasaki<sup>a</sup>, Yuzuru Ikehara<sup>a</sup>, Shoko Nishihara<sup>a</sup>, Satoshi Nakagawa<sup>a</sup>, Katsutoshi Sasaki<sup>c</sup>, Takashi Shiina<sup>d</sup>, Hidetoshi Inoko<sup>d</sup>, Naruya Saitou<sup>b</sup>, Hisashi Narimatsu<sup>a</sup>,<sup>*</sup>

<sup>a</sup>Division of Cell Biology, Institute of Life Science, Soka University, I-226 Tango-cho, Hachioji-shi, Tokyo 192-8577, Japan
<sup>b</sup>Department of Genetics, School of Life Science, Graduate University for Advanced Studies, National Institute of Genetics, 1111 Yata, Mishima-shi, Shizuoka 411-8540, Japan
<sup>c</sup>Tokyo Research Laboratories, Kyowa Hakko Kogyo Company, Limited, 3-6-6 Asahi-machi, Machida-shi, Tokyo 194-8533, Japan
<sup>d</sup>Second Department of Molecular Life Science, School of Medicine, Tokai University, Isehara-shi, Kanagawa 259-1193, Japan

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Abstract The amino acid sequence of Fuc-TIX is very highly conserved between mouse and human. The number of non-synonymous nucleotide substitutions of the Fuc-TIX gene between human and mouse was strikingly low, and almost equivalent to that of the α-actin gene. This indicates that Fuc-TIX is under a strong selective pressure of preservation during evolution. The human Fuc-TIX (hFuc-TIX) showed a unique characteristic, i.e. hFuc-TIX was not activated by Mn<sup>2+</sup> and Co<sup>2+</sup>, whereas hFuc-TIV and hFuc-TVI were activated by the cations. The hFuc-TIX transcripts were abundantly expressed in brain and stomach, and interestingly were detected in spleen and peripheral blood leukocytes.

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Key words: Glycosyltransferase; Fucosyltransferase; α1,3-Fucosyltransferase; Lewis x; CD15; SSEA-1

1. Introduction

The Lewis x (Le<sup>x</sup>) carbohydrate epitope, which is defined as Galβ1,4(Fucα1,3)GlcNAc, is synthesized by transferring a fucose (Fuc) to the N-acetylgalcosamine (GlcNAc) residue of the type 2 chain, Galβ1,4GlcNAc, with an α1,3-linkage. This fucose transfer is catalyzed by the α1,3Fuc-T. So far, the human genes encoding five α1,3Fuc-Ts (Fuc-TIII, IV, V, VI and VII or FUT3, 4, 5, 6 and 7) have been cloned [1^6]. In a previous paper, we reported the molecular cloning of a new member of the α1,3Fuc-T family in the mouse and named the enzyme mouse Fuc-TIX (mFuc-TIX or mFUT9) [7].

CD15 is one of the differentiation markers of the cells, and the CD15 epitope has been determined as the Le<sup>x</sup> carbohydrate structure. Several immunohistochemical studies have detected CD15 antigens in certain neuronal cells and glial cells in the central nervous system (CNS) of humans and rodents [8—10]. The expression of CD15 antigens in the CNS is developmentally regulated, and considered to play a role in neuronal development. In our previous study, mFuc-TIX was identified as the most likely candidate for the enzyme synthesizing the Le<sup>x</sup> structure (CD15 epitope) in the mouse CNS [7]. Some α1,3Fuc-Ts are known to be polymorphic in human. Three kinds of null alleles of the Fuc-TII (Le<sup>1</sup>) gene, named le1, le2 and le3 alleles, are widely distributed in the Japanese population [11—13]. Homozygotes of null Fuc-TVI alleles were also found in some populations [14]. In bovine, there is only one gene, named <i>futb</i>, corresponding to the hFuc-TV-hFuc-TIII-hFuc-TVI gene cluster [15]. In addition, the mouse gene orthologous to the <i>futb</i> gene seems to be a pseudogene [16]. In this sense, the hFuc-TII, hFuc-TIV and hFuc-TVI genes are regarded as non-essential for individual ontogeny, and therefore their sequences are not conserved among species.

Mice and humans possess functional Fuc-TIV and Fuc-TVII genes [3—6,16,17]. The distribution of Fuc-TIV is limited to specific tissues and cell populations such as leukocytes and high endothelial cells of the venule [18]. Fuc-TVII apparently participates in the synthesis of sialyl Lewis x (sLex) epitopes in leukocytes: this epitope is required for leukocyte trafficking [18]. However, Fuc-TVII cannot synthesize the CD15 (Le<sup>x</sup>) epitope [4,5,19]. Fuc-TIV is ubiquitously expressed in various tissues, and probably participates in CD15 epitope synthesis in some tissues, but its biological function remains to be elucidated [16].

In this study, we cloned a human gene, named hFuc-TIX, orthologous to the mFuc-TIX gene, and analyzed its characteristics and tissue distribution. Interestingly, the nucleotide and amino acid sequences of hFuc-TIX were very highly conserved in comparison with those of mFuc-TIX [7]. This indicates that Fuc-TIX has been under a strong selective pressure during its evolution, suggesting that it is essential for ontogeny.

2. Materials and methods

2.1. cDNA cloning and sequencing of the hFuc-TIX gene

A cDNA library derived from a human gastric mucosa was constructed in our laboratory (Ikehara et al., unpublished). We screened the human stomach cDNA library with a mFuc-TIX cDNA probe

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encompassing the full-length open reading frame (ORF), and obtained several clones encoding the hFuc-TIX gene. The inserts were subjected to nucleotide sequencing.

2.2. Calculation of the numbers of synonymous and non-synonymous nucleotide substitutions

To calculate the numbers of synonymous and non-synonymous nucleotide substitutions in Fuc-T genes between mouse (or other species) and human, we employed the ODEN program [20] of SuperMac at the National Institute of Genetics, Japan.

2.3. Flow cytometric analysis of Namalwa cells stably expressing the hFuc-TIX gene and assaying of α1,3Fuc-T activity

Namalwa cells stably expressing the hFuc-TIX gene were established, and subjected to flow cytometry analysis, using SSEA-1 (anti-Lea; Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA, USA) [21], AH6 (anti-Lewis y (Ley)) [22] and CSLEX-1 (anti-Lea) [23] and assaying of α1,3Fuc-T activity using pyridylaminated (PA) oligosaccharides, as described previously [7].

2.4. Quantitative analysis of α1,3Fuc-T transcripts in various human tissues using the competitive reverse transcription-polymerase chain reaction (competitive RT-PCR) method

The competitive RT-PCR methods for the measurement of the transcripts for the hFuc-T III, IV, V, VI, VII genes have been described in detail in our previous paper [24]. To quantify the hFuc-TIX transcripts by competitive RT-PCR, a competitor DNA plasmid (pBS-hFT9d) was prepared by deleting the 113-bp NcoI-NolI fragment from the standard DNA (pBS-hFT9s) which encodes the full-length ORF of the hFuc-TIX gene. A pair of PCR primers (forward primer, 5'-GGCAAGCATTTGGAGAATATGTCAAT-3' and reverse primer, 5'-ACCAACAGACTTATATTCTTGATGCC-3') amplified 398-bp and 285-bp fragments from pBS-hFT9s and pBS-hFT9d, respectively. cDNA samples were prepared from various human tissues by the method described previously [7,24]. The PCR conditions for the hFuc-TIX gene were as follows: a pre-PCR heating step at 95°C for 11 min, followed by 45 cycles of 1 min at 95°C, 1.5 min at 65°C, and 2 min at 72°C. To ascertain the efficiency of cDNA preparation from total RNAs, measurement of the β-actin transcripts in each sample was performed.

2.5. In situ hybridization and immunohistochemical analyses

Surgically resected stomach specimens for in situ hybridization were prepared as described previously [7]. The antisense and sense RNA probes, at the nucleotide positions 655–835 of hFuc-TIX cDNA, labeled with digoxigenin were synthesized using a Dig RNA Labeling Kit (Boehringer Mannheim) according to the supplier’s manual. The hybridization and signal detection procedures were described previously [7].

Immunohistochemistry was performed with monoclonal antibodies, PM-81 (anti-Leα) [25] and AH6 (anti-Leβ), by the method described previously [26].

2.6. Construction of a phylogenetic tree of the α1,3Fuc-T family

We used amino acid sequences for six human [1-6], three mouse [7,16,17], one rat [27], one bovine [15], one chicken [28], two zebrafish [29] and four Caenorhabditis elegans [30] genes. These 18 sequences have not been cloned, therefore the deduced amino acid sequences, Clustal W [31] was used for multiple alignment and the neighbor-joining method [32] was used to construct a tree. We also performed a bootstrap analysis to gain a statistical measure of confidence in the phylogenetic tree. By bootstrap analysis, a total of 1000 trees were generated from the initial data set, and the percentage of trees containing a particular clade was measured.

3. Results

3.1. A putative amino acid sequence of hFuc-TIX deduced from the cDNA sequence which is highly conserved between mouse and human

The 3019-bp nucleotide sequence of the hFuc-TIX cDNA was registered by us in DDBJ/EMBL/GenBank with the accession number AB023021. The putative amino acid sequence predicted from the nucleotide sequence encodes a typical type II membrane protein, and is very similar to that of mFuc-TIX, i.e. only three amino acid residues, Val-37 to Ile, Thr-237 to Ala and Phe-292 to Tyr, differ among 359 amino acids (Fig. 1). The consensus amino acid sequence for the members of the α1,3Fuc-T family [33], FxLVxFENs/TxxxYxTEK, is conserved in the hFuc-TIX sequence.

3.2. Synonymous and non-synonymous substitutions of the members of the Fuc-T gene family

We calculated the numbers of synonymous (ds) and non-synonymous (dn) nucleotide substitutions between mFuc-TIX and hFuc-TIX genes to establish if there is some significant evolutionary pressure for maintaining the primary structure of the enzyme (ds; number of substitutions per site which cause no amino acid replacement; dn; number of substitutions per site which cause amino acid replacement) (Table 1). The other Fuc-T genes, H (FUT1), Fuc-TIII (FUT3), Fuc-TIV (FUT4), Fuc-TVII (FUT7) and FUT8 genes, were also analyzed to determine their ds and dn values (Table 1). The mouse Fuc-TIII and FUT8 genes have not been cloned, therefore the bovine orthologue (futh) for hFuc-TIII gene and the porcine FUT8 gene were compared with the respective human genes. We considered that it was essentially not a problem to use different species to calculate ds and dn, because they started to diverge more or less at the same time. The Fuc-TIX gene showed the lowest ds value (0.005), followed by the FUT8 gene (0.021), among the six members of the Fuc-T gene family. The difference in ds values between the Fuc-TIX gene and the others was almost 40-fold. On the other hand, the dn

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>La</th>
<th>ds</th>
<th>dn</th>
<th>Species pair</th>
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<tbody>
<tr>
<td>H (FUT1)</td>
<td>368</td>
<td>0.938</td>
<td>0.148</td>
<td>mouse vs. human</td>
</tr>
<tr>
<td>Fuc-TIII (FUT3)</td>
<td>366</td>
<td>0.516</td>
<td>0.218</td>
<td>bovine vs. human</td>
</tr>
<tr>
<td>Fuc-TIV (FUT4)</td>
<td>408</td>
<td>0.734</td>
<td>0.159</td>
<td>mouse vs. human</td>
</tr>
<tr>
<td>Fuc-TVII (FUT7)</td>
<td>343</td>
<td>0.950</td>
<td>0.111</td>
<td>mouse vs. human</td>
</tr>
<tr>
<td>FUT8</td>
<td>576</td>
<td>0.331</td>
<td>0.021</td>
<td>porcine vs. human</td>
</tr>
<tr>
<td>Fuc-TIX (FUT9)</td>
<td>360</td>
<td>0.527</td>
<td>0.005</td>
<td>mouse vs. human</td>
</tr>
<tr>
<td>histone H4°</td>
<td>101</td>
<td>0.228</td>
<td>0.001</td>
<td>mouse vs. human</td>
</tr>
<tr>
<td>α-actin°</td>
<td>376</td>
<td>0.587</td>
<td>0.002</td>
<td>mouse vs. human</td>
</tr>
</tbody>
</table>

aNumber of codons compared.
bLi et al. [40].
3.3. Flow cytometry analysis of Namalwa cells transfected with the hFuc-TIX gene and measurement of \( K_{1,3}Fuc-T \) activity of hFuc-TIX towards oligosaccharides

Namalwa-hFT9 cells, stably expressing the hFuc-TIX cDNA, were analyzed by flow cytometry. Namalwa-hFT9 cells showed a positive peak with SSEA-1 (anti-Le\(^a\)) and AH6 (anti-Le\(^v\)), but not with CSLEX-1 (anti-sLe\(^x\)) (data not shown). In contrast, Namalwa-hFT4 cells (stable transformant with the hFuc-TIV gene) showed positive peaks with all three antibodies, SSEA-1, AH6 and CSLEX-1, although the positive peak with CSLEX-1 was very weak (data not shown).

The \( \alpha_{1,3}Fuc-T \) activities of hFuc-TIX were measured against PA oligosaccharides, i.e. lacto-N-neotetraose-PA (LNnT-PA), lacto-N-tetraose-PA (LNT-PA) and \( \alpha_{2,3} \)-sialyl lacto-N-tetraose-PA (sialyl-LNnT-PA). hFuc-TIX exhibited activity against LNnT-PA, resulting in the synthesis of Le\(^a\) epitope, but no activity against LNT-PA or sialyl-LNnT-PA, resulting in no synthesis of the Lewis a (Le\(^a\)) or sLe\(^x\) epitope, respectively (data not shown).

Thus, hFuc-TIX showed almost the same results of flow cytometry analysis and \( \alpha_{1,3}Fuc-T \) activities as mFuc-TIX [7].

3.4. Activation of \( \alpha_{1,3}Fuc-Ts \) in the presence of Mn\(^{2+}\) or Co\(^{2+}\)

Three human \( \alpha_{1,3}Fuc-Ts \), i.e. hFuc-TIX, hFuc-TIV and hFuc-TVI, were examined for activity against LNnT-PA in the presence of Mn\(^{2+}\) or Co\(^{2+}\) (Fig. 2). Interestingly, hFuc-TIX was not activated at any concentration of Mn\(^{2+}\) or Co\(^{2+}\), moreover it was markedly suppressed by Mn\(^{2+}\) and Co\(^{2+}\) (Fig. 2A). In contrast, hFuc-TIV was markedly activated in the presence of Mn\(^{2+}\), but less so in that of Co\(^{2+}\) (Fig. 2B). hFuc-TVI was also markedly activated by both Mn\(^{2+}\) and Co\(^{2+}\) (Fig. 2C).

3.5. Tissue distribution and quantitative measurement of six human \( \alpha_{1,3}Fuc-T \) transcripts

The amounts of six human \( \alpha_{1,3}Fuc-T \) transcripts expressed in various tissues were measured by competitive RT-PCR (Table 2). hFuc-TIII was abundantly expressed in gastrointestinal tissues, i.e. stomach, jejunum and colon, and faintly expressed in spleen, lung, kidney and cervix uteri. hFuc-TIII was not detected in brain, liver, adrenal cortex or peripheral blood leukocytes (PBL). hFuc-TIV was found to be ubiquitously expressed in all tissues examined and it was abundantly expressed in cervix uteri. hFuc-TIV was not expressed in any tissues examined, except for spleen in which it was faintly expressed. The tissue distribution of hFuc-TVI was similar to that of hFuc-TIII, except that hFuc-TVI was expressed positively in liver whereas hFuc-TIII was not. hFuc-TVI was much more abundant in kidney than hFuc-TIII. hFuc-

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<th>Table 2</th>
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<tr>
<th>Expression levels of ( \alpha_{1,3}Fuc-Ts ) relative to the level of ( \beta )-actin (%)</th>
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<tbody>
<tr>
<td>Forebrain</td>
</tr>
<tr>
<td>Stomach (antrum)</td>
</tr>
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<td>Stomach (corpus)</td>
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<tr>
<td>Jejunum</td>
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<tr>
<td>Colon</td>
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<td>Liver</td>
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<td>Spleen</td>
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<td>Lung</td>
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<td>Kidney</td>
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<td>Adrenal cortex</td>
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<td>Cervix uteri</td>
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<td>PBL</td>
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</table>

Fig. 2. Activation of hFuc-TIX, hFuc-TIV and hFuc-TVI by Mn\(^{2+}\) or Co\(^{2+}\). The activities toward LNnT-PA were measured at various concentrations of Mn\(^{2+}\) (E) or Co\(^{2+}\) (F). The activity in the absence of the dications is presented as 100% activity.
TVII was detected in spleen and PBL. hFuc-TIX was markedly expressed in forebrain and stomach, i.e. in both the antrum and corpus of the stomach, and, interestingly, was detected in spleen and PBL. hFuc-TIX was not expressed in kidney at all. In this regard, hFuc-TIX differs from mFuc-TIX which is abundantly expressed in mouse kidney [7].

3.6. Immunohistochemical analysis of Le$^{x}$ and Le$^{y}$ expression and in situ hybridization analysis of hFuc-TIX transcripts in the antrum portion of human gastric mucosa

A stomach specimen of a secretor individual, whose secretor (Se) genotype is Se$^{1}$/Se$^{1}$ [26], was subjected to immunohistochemical analysis for Le$^{x}$ and Le$^{y}$ expression and in situ hybridization with the hFuc-TIX probe. As can be seen in Fig. 3A,B, the Le$^{x}$ antigens were mainly positive in the glandular compartments of the pyloric mucosa. In contrast, the Le$^{y}$ antigens were detected both in the glandular compartments and in the pit of the gastric mucosa. The hFuc-TIX transcripts were localized in the glandular compartments alone, being well co-localized with the Le$^{x}$ antigens (Fig. 3C).

3.7. A phylogenetic tree of the α1,3Fuc-T gene family

We performed a FASTA search [34], and BLAST search [35] of the DDBJ/EMBL/GenBank International Nucleotide Sequence Database to identify the genes homologous to the hFuc-TIX gene. We used two specific search programs, tfastx and tblastn, which compare amino acid sequences with a DNA database. Mammalian, chicken, zebrafish, C. elegans, Helicobacter pylori, Drosophila melanogaster, and Vibrio cholerae genes were retrieved, while no members of the K$^{1,2}$ or K$^{1,6}$Fuc-T gene families were found to show homology with the hFuc-TIX gene. The root was determined by assuming that C. elegans genes are located as outgroups (Fig. 4). The phylogenetic tree indicates that the Fuc-T genes evolved by independent gene duplication between vertebrates and C. elegans. There are four clusters in the vertebrate Fuc-T gene family, the Fuc-T gene subfamily being the first to diverge from the ancestral gene. Two zebrafish α1,3Fuc-Ts, zFT1 and zFT2, which are developmentally regulated in embryogenesis, form a cluster with mFuc-TIX and hFuc-TIX.
4. Discussion

hFuc-TIX can synthesize the Leα and Leβ epitopes, but not the Leα or sLeα epitopes, in contrast to hFuc-TIV's low, but apparently positive activity of sLeα synthesis. These characteristics of hFuc-TIX were almost the same as those of mFuc-TIX. In the present study, we found that Mn²⁺ and Co²⁺ ions did not activate the hFuc-TIX activity at all.

Mollicone et al. reported that human brain homogenates exhibited activity for Leα synthesis, but not for sLeα synthesis [36,37]. The enzymes expressed in brain and leukocytes had similar levels of activity, i.e. both showed similar Km values for GDP-fucose and H-type 2 compounds, the same optimum pH, and were resistant to N-ethylmaleimide. However, they differed in the enzyme activity when activated by dications. The enzyme(s) in leukocytes exhibited only 10–15% of the activity in the presence of Co²⁺ as compared to Mn²⁺, whereas the activity of brain enzyme(s) did not differ in the presence of Mn²⁺ or Co²⁺ [36,37]. Foster et al. indicated that the enzyme purified from human neuroblastoma cells exhibited a level of activity in the presence of 10 mM Co²⁺ that was 75% of that in the presence of 10 mM Mn²⁺ [38]. The characteristics of hFuc-TIX determined in the present study were most similar to those of the enzyme(s) in adult brain [36]. However, there was some discrepancy between previous studies [36–38] and our study as to the characteristics of α1,3Fuc-T activities which can be explained as follows. As demonstrated in Table 2 in the present study, many tissues contain multiple α1,3Fuc-Ts. The previous studies measured the activities directed by mixed enzymes in tissue homogenates, while we measured the activities of the recombinant enzymes. In tissue homogenates, there may still be unknown α1,3Fuc-Ts, the genes of which have not been cloned.

Cameron et al. [39] reported the tissue distribution of three hFuc-Ts, i.e. hFuc-TIII, V and VI, on Northern analysis and semi-quantitative RT-PCR. We employed the competitive RT-PCR method in the present study because of the following advantages. Competitive RT-PCR enabled us for the first time to compare all six α1,3Fuc-Ts in terms of the level of relative amounts of transcripts in various human tissues. The small amounts of transcripts, which could not be detected by Northern analysis, were quantitatively detected by this sensitive method. Fuc-TIV is believed to be a single enzyme capable of Leα (CD15) synthesis in myeloid tissues. It is worth noting that hFuc-TIX was also expressed in spleen and PBL almost at the same level as hFuc-TIV. We will examine whether hFuc-TIX and hFuc-TIV are expressed in cells of different subpopulations in PBL or together in the same cells. hFuc-TIX was not expressed in human kidney at all, whereas mFuc-TIX was most abundantly expressed in mouse kidney as demonstrated in our previous study [7]. There may be species-specific and tissue-specific regulation of the Fuc-TIX gene. In this study, we detected both hFuc-TIX and hFuc-TIV expression almost at the same level in forebrain, while we did not detect mFuc-TIV in a previous study [7]. This discrepancy may be attributed to the different areas of brain examined, or to a species-specific regulation as in kidney.

The co-localization of hFuc-TIX transcripts with the Leα antigens in the glandular compartments of the stomach strongly suggested that hFuc-TIX is involved in the synthesis of Leα antigens in stomach. However, the absence of hFuc-TIX in the foveolar cells in the gastric pits indicated that some other α1,3Fuc-T(s) are involved in Leα synthesis, because gastric mucosa expressed four kinds of α1,3Fuc-Ts, i.e. hFuc-TIII, IV, VI and IX, as shown in Table 2.

The position of Fuc-TIX in the phylogenetic tree indicates that the Fuc-TIX subfamily was the first to diverge in vertebrates. CFT1, a chicken homologue (probably orthologue) of Fuc-TIV, is clustered with the mammalian Fuc-TIV subfamily. This suggests that a chicken orthologue of Fuc-TIX may be found in the Fuc-TIX subfamily. It is of interest to know to what extent the Fuc-TIX sequence is evolutionarily conserved in non-mammal vertebrates. The numbers of non-synonymous substitutions (ds) of protein coding gene regions are known to vary considerably. Genes for conservative proteins such as histones and actins show very low ds values (0.001 and 0.002, respectively, between mouse and human [40]), because selective constraints are strong. It should be emphasized that the ds value of the Fuc-TIX gene is as low as those of histone and α-actin genes. The very low ds value of the Fuc-TIX gene clearly indicates a strong selective pressure for the preservation of the Fuc-TIX amino acid sequence during the mammalian evolution. Recently, two zebrafish α1,3Fuc-T genes, zFT1 and zFT2, were found to be expressed in embryogenesis [29]. hFuc-TIX and mFuc-TIX form a cluster with zFT1 and zFT2 in the phylogenetic tree. Fuc-TIX may possibly be involved in mammalian embryogenesis, and play an essential role for ontogeny.

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