Epithelial Rat Embryonic Development Exhibits Differential Glycan Expression According to Organ Localization

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Abstract

The development of the different systems involved glycoproteins including mucins present in most epithelia. Mucins are glycoproteins which 50 - 60% of their molecular masses consisted on carbohydrates. It has been largely described changes on their glycosylation mainly related to tumors with the appearance of short carbohydrate as well as Lewis group associated antigens. At present, the expression of these carbohydrate antigens during embryonic development has not been studied. Here we have performed a detailed immunohistochemical investigation of mucin associated glycans such as Tn (GalNAcα1-O-Ser/Thr, CD175), T (Thomsen-Friedenreich, Galβ1-3GalNAcα1-O-Ser/Thr, CD176), and sTn (CD175s, sialyl-Tn antigen), Lewis x and sialyl Lewis x (sLewis x) during rat epithelia embryonic development as well as in neonates and adults. A high expression of Tn, T, and Lewis x was found in most intestinal organs and kidney; the skin expressed T and Lewis x antigens, salivary glands showed Tn, T and these glands were the only tissues that expressed sTn. Respiratory organs, liver, and pancreas were negative for all analyzed antigens while sLewis x was not expressed at any sample. In most organs, Tn, T, sTn and Le x antigens showed an increased percentage of stained cells along with developmental progression. Our findings would suggest a role of carbohydrate epitopes probably modifying cell-cell and cell-matrix interactions and cytodifferentiation during embryonic development.

Keywords: Rat; Epithelial Embryonic Development; Mucin Associated Glycans

Introduction

Cells of the body are decorated with a variety of carbohydrates that serve many diverse functions. These sugars not only act as a protective barrier on the outside of the cell, but are also involved in communication and signaling events [1].

Glycosylation is a very common modification of proteins and lipids; although the transfer of initial sugar(s) to glycoproteins or glycolipids occurs in the ER or on the ER membrane, the subsequent addition of the many different sugars that make up a mature glycan is accomplished in the Golgi. Golgi membranes are studded with glycosyltransferases, glycosidases, and nucleotide sugar transporters arrayed in a generally ordered manner from the *cis*-Golgi to the *trans*-Golgi network, such that each activity is able to act on specific substrate(s) generated earlier in the pathway [2].

The two major types of sugar linkages present on membrane and secreted proteins are *N*-linked and *O*-linked GalNAc; this last type, known as mucin-type *O*-linked glycosylation, is a conserved protein modification which influences basic biological processes in development and disease such as cell adhesion, communication, morphology, and proliferation [3-5].

Mucins are glycoproteins which 50 - 60% of their molecular masses consisted on carbohydrates; mucin protein core has a variable number of tandem repeats (VNTR) rich in serines and threonines, which constitute potential sites for *O*-glycosylation. It has been demon-

strated that this glycosylation is initiated in the Golgi complex by N-Acetyl-galactosaminyl-transferases which add N-acetyl-galactosamine (GalNac) to a serine or threonine in the VNTR region. Each *O*-glycan is then elongated by the addition of hexoses like galactose (Gal), N-acetyl-glucosamine (GlcNAc), and fucose, or sialic acid by specific glycosyl-transferases (Figure 1), which are also expressed in a specific spatial and temporal pattern during embryogenesis [5-8]. However, the roles for *O*-linked glycosylation have been difficult to decipher given the functional redundancy present among the 20 enzymes (ppGalNAcTs) responsible for initiation of this type of glycosylation in mammals [9,10].
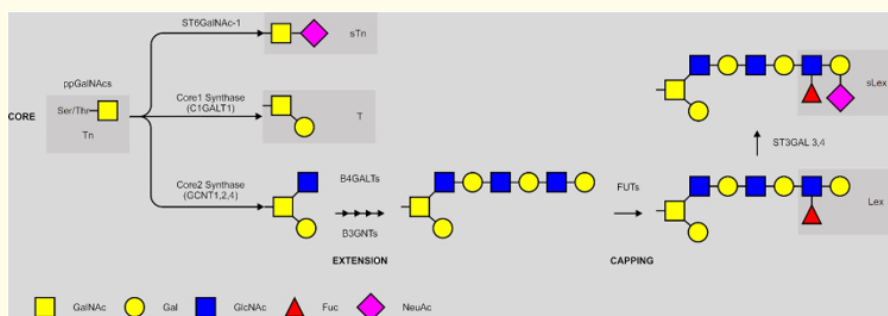
Figure 1: Mucin type O-glycosylation.

O-glycosylation is initiated by the activity of ppGalNAcTs, which attaches an N-acetylgalactosamine to Ser/Thr (highlighted in yellow) amino acid residues in the tandem repeat region, resulting in the formation of a Tn antigen. This Tn antigen formed can be extended by activity of C1GALT1 to form the T antigen. Alternatively, Tn can be modified by a sialyltransferase to form sialyl Tn (sTn). T antigen can be further elongated and extended to several structures, including Lewis x and sLewis x. Tn can also be extended by activity of other glycosyltransferases to form several other structures.

Since early reports, short *O*-glycan antigens like Tn (GalNAcα1-O-Ser/Thr, CD175), T or TF (Thomsen-Friedenreich, Galβ1-3GalNAcα1-O-Ser/Thr, CD176), and sTn (CD175s, sialyl-Tn antigen) have been extensively associated to cancer [11-16]. Adult normal cells may express these antigens but they have been mainly detected in embryonic tissues constituting oncofetal antigens [17,18]. Mucin associated glycans also include Lewis-type antigens (Figure 1) which are a group of terminal fucosylated epitopes altered during cancer development and are present in the glycocalyx in normal as well as transformed cells [19-26]. Lewis x oligosaccharide has been identified in blastomeres of early murine embryo in eight cells stage, which correlates with the beginning of the compaction process of the morula [27-29]. Furthermore, sialyl Lewis x (sLewis x) would be important for recognition and cell adhesion at the time of embryo implantation [30].

Our laboratory has extensively revealed differential mucin expression during rat epithelia development [31-33]. *O*-glycosylation would play an essential role during the process but the specific *O*-glycans expressed remain unknown. The present research was undertaken to provide information regarding cell-specific expression mucin associated carbohydrates Tn, TF, sTn, Lewis x and sLewis x in several organs during rat embryonic development and their relationship with patterns of epithelial cytodifferentiation.

Materials and Methods

Animals and samples

A total of 80 animals were included in this study: 64 rat embryos and fetuses were collected at 13 - 20 days of gestation (stages D13-D20) from pregnant females (WKAH/Hok), neonates of 15 days post birth and adults of 12 weeks; 8 individuals from each stage of development were included. Eight independent samples belonging to each embryonic day were examined and scored. Copulation was determined by the presence of a vaginal plug; the middle of the *artificial night* was designed as day 0 of pregnancy [34].

The following organs were studied: esophagus, stomach, small intestine, large intestine, liver, pancreas, salivary glands, trachea, lung, kidney and skin.

All animal experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the Institute for Laboratory Animal Research (Washington DC, USA, 2011). The animals were sacrificed in chambers containing CO_2. Immediately, were dissected and the embryos and fetuses were removed. Sterile surgical material was used and worked on ice.

Samples of embryos and fetuses processing

Embryos and fetuses were obtained by laparotomy and hysterectomy of the pregnant females. The number of breeding in each gestation ranged from 4 to 12 which were processed and studied.

After the extirpation of the embryonic sac, whole embryos were washed with 0.01M phosphate buffer saline pH 7.4 (PBS) and fixed in 10% formaldehyde solution for 3h. After this period of time, the embryos were washed in water, dehydrated and clarified with xylol, embedded in paraffin, and finally blocked with paraffin. Sections were made with a microtome with a thickness of 6 μm (fetuses) and 4 μm (neonates and adults), placed in slides treated with silane (silicon tetrahydride) followed by hematoxylin and eosin staining and immunohistochemical analysis.

Samples of neonates and adults processing

The organs obtained from neonates and adults were fixed in 10% formaldehyde in PBS for 3h dehydrated in ethanol, clarified, embedded and blocked with paraffin for immunohistochemical analysis as described above.

Antibodies

The monoclonal and polyclonal antibodies employed are summarized in table 1.

Antigen	Epitope structure	Antibody	Isotype and source	Producer or Reference
Tn	GalNAcα-R	HB-Tn1	IgM, mouse MAb	DAKO
TF	Galβ1-3GalNAcα1-R	HB-T1	IgM, mouse MAb	DAKO
sTn	NeuAc2-3GalNAcα-R	HB-STn1	IgG, mouse MAb	DAKO
Lewis x	Galβ1-4GlcNAcβ1-3Gal-R	KM380	IgM, mouse MAb	Hanai., *et al.* 1986
sLewis x	NeuAc2-3Galβ1-4GlcNAcβ1-3Gal-R	KM93	IgM, mouse MAb	Hanai., *et al.* 1986

Table 1: *Antibodies assayed.*

TF: Thomsen-Friedenreich Antigen (T antigen); MAb: Monoclonal Antibody.

Immunohistochemical analysis (IHC)

Immunohistochemistry was performed according to standard procedures as reported in a previous study [31]. Briefly, dewaxed sections were treated with 10 mM sodium citrate buffer at 100°C for 5 minutes for antigen retrieval and were placed in methanol with 0.3% H_2O_2 to block endogenous peroxidase activity; after three washes in PBS, sections were blocked for non-specific binding with normal horse serum diluted 1:10 in 1% bovine serum albumin in PBS. Samples were then incubated overnight at 4°C with the primary antibodies, whereas negative controls were incubated with PBS under the same conditions. Immunodetection was performed with the Dako Cytomation LSAB+System-HRP (Dako, Denmark). Finally, sections were counterstained with hematoxylin (SIGMA), dehydrated and coverslipped with mounting media. Samples were evaluated under light microscope and the percentage of cells positively stained in one sample was quantified: 0 - 5%= 0; 5 - 30%= I; 31 -6 0%= II and 61 - 100%= III. The patterns of reaction were: L=linear membrane, C=cytoplasmic, and M=mixed: linear and cytoplasmic [35]; apical and non-apical staining was also recorded as well as nuclear reactivity (N). Staining intensity was scored in a semiquantitative manner and was graded as negative (-), low (+), moderate (++) and strong (+++).

Statistical analysis

Multivariate analysis was performed employing Principal Component Analysis (PCA).

Three main factors were extracted and the scores for each case were recorded (81% of the variances results were explained); PCA was carried out with varimax rotation. The Chi-Square test was employed to analyze differences in frequencies. Correlations between carbohydrate antigens were assessed employing the Kendall's tau-b coefficient. The basic significance level was fixed at $p < 0.05$ and all data were analyzed with SPSS® statistical software (SPSS Inc., Chicago, IL, USA).

Results

As a first step on the study of O-glycan implications on rat epithelial tissue development, the expression of carbohydrate antigens was analyzed at organs of the digestive and respiratory systems as well as renal and skin specimens; in this sense, both simple as well as stratified epithelial development were included. Immunohistochemical results are summarized in tables 2-5.

Considering Tn expression, at D17 onwards, a positive reaction was found at epithelial cells that cover renal tubules (Figure 2a) and intestinal lining epithelial cells (Figure 2b). At D18 expression was detected in developing acini of salivary glands (Figure 2c) which increased at D19. From D19 onwards, an increased reaction was observed at intestinal epithelial cells (Figure 3a and 3b) while goblet cells were negative. In the case of salivary glands, from D20, mucous and serous acinar cells were possible to be detected; Tn reaction was restricted to the mucous acinar cells although epithelial cells of excretory ducts were also reactive (Figure 3c, 3d and Figure 4a, 4b). From D20, gastric secretory epithelial cells showed a moderate reaction at its secretory surface as well as at glands (Figure 3e, 3f). In neonates and adults, expression was present in all positive tissues above described (Figure 4). The pattern of reaction changed according to embryologic development; at early stages and in the majority of the organs described, the pattern was linear while from D19 onwards a predominant mixed pattern was found (Figures 2 and 3). In the case of salivary glands, although at early stages the linear pattern was maintained in the ducts, the mucous acinar cells showed a mixed one, which was present in all epithelial reactive cells at post-birth stages (Figure 3c, 3d and Figure 4a, 4b).

antigen Tn

| Organs | Embryos and fetuses gestational days | | | | | Neonates postnatally days | Adults |
	16	17	18	19	20	2 - 15	10 - 12 weeks
Esophagus	-	-	-	-	-	-	-
Stomach	-	-	-	-	++ (4/8) II	++ (7/8) III	++ (7/8) III
Small Intestine	-	++ (4/8) II	++ (4/8) II	+++ (5/8) III	+++ (5/8) III	+++ (6/8) III	+++ (6/8) III
Large Intestine	-	++ (5/8) III	++ (5/8) III	+++ (6/8) III	+++ (6/8) III	+++ (6/8) III	+++ (6/8) III
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	++ (4/8) III	+++ (5/8) III	+++ (5/8) III	+++ (6/8) III	+++ (6/8) III
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	++ (7/8) I	+++ (8/8) I	+++ (8/8) II	+++ (7/8) II	+++ (7/8) III	+++ (7/8) III
Skin	-	-	-	-	-	-	-

antigen T (TF)

| Organs | Embryos and fetuses gestational days | | | | | Neonates postnatally days | Adults |
	16	17	18	19	20	2 - 15	10 - 12 weeks
Esophagus	++ (4/8) III	+++ (5/8) III	+++ (6/8) III	+++ (7/8) III	+++ (7/8) III	-	-
Stomach	++ (4/8) II	++ (5/8) II	++ (5/8) II	+++ (6/8) II	+++ (7/8) III	+++ (5/8) III	+++ (6/8) III
Small Intestine	-	-	-	++ (3/8) II	++ (3/8) II	++ (4/8) II	++ (3/8) II
Large Intestine	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	++ (5/8) II	+++ (5/8) II	+++ (5/8) II	+++ (4/8) II	+++ (5/8) II
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	-	++ (4/8) I	+++ (5/8) I	+++ (5/8) II	+++ (4/8) III	+++ (5/8) III
Skin	-	-	++ (5/8) III	+++ (5/8) III	+++ (6/8) III	+++ (6/8) III	+++ (6/8) III

antigen sTn

| Organs | Embryos and fetuses gestational days | | | | | Neonates postnatally days | Adults |
	16	17	18	19	20	2 - 15	10 - 12 weeks
Esophagus	-	-	-	-	-	-	-
Stomach	-	-	-	-	-	-	-
Small Intestine	-	-	-	-	-	-	-
Large Intestine	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	++ (5/8) I	+++ (5/8) II	+++ (6/8) II	+++ (6/8) II	+++ (7/8) II
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-
Skin	-	-	-	-	-	-	-

Table 2: Intensity of the immunohistochemical staining for carbohydrate Tn in different organs from rat embryos, fetuses, neonates and adults. The intensity of reaction was scored as absent (-), low (+), moderate (++) and strong (+++). N° positive cases/Total samples was indicated between brackets. The percentage of cells positively stained in each sample was quantified: 0 - 5%= 0; 5 - 30%= I; 31 - 60%= II and 61 - 100%= III.
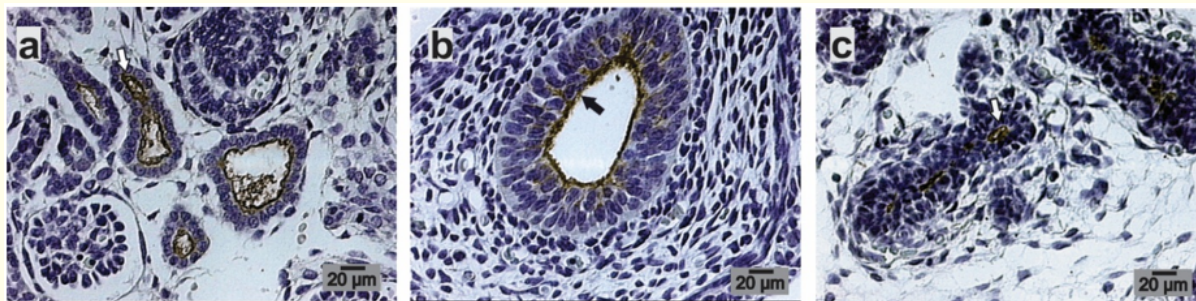
Figure 2: Immunoperoxidase staining with anti-Tn MAb are shown. A linear apical reaction at: (a) D17 renal tubules, (b) D17 intestinal epithelium, and (c) D18 salivary gland ducts. Black and white arrows show a positive reaction (x40).
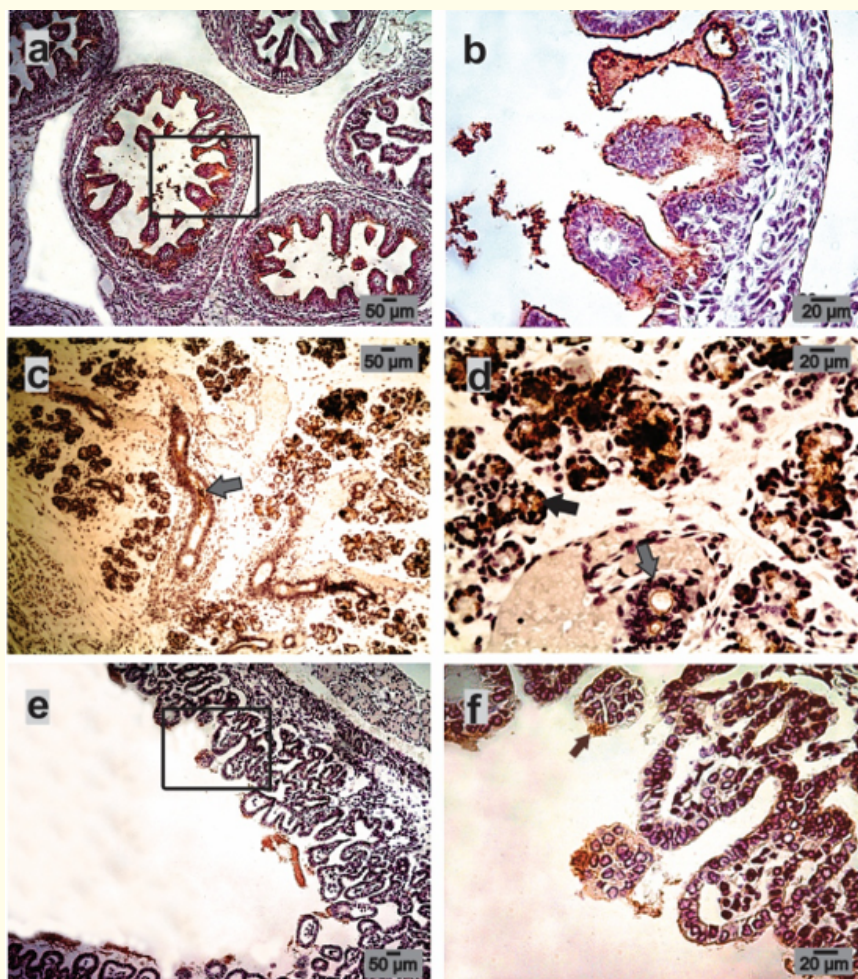
Figure 3: Immunohistochemical findings of Tn antigenic expression; (a) and (b) D19 small intestine (a) x10 (b) x40; (c) and (d) D20 salivary gland, (c) x10 (d) x40 while (e), and (f) D20 stomach (e) x10 (f) x40. In (a)-(d) microphotographs a strong reactivity with a mixed pattern is depicted; in (c) and (d) grey arrows show a positive reaction at excretory ducts of salivary glands. In (e) and (f) gastric secretory surface and glands show a moderate reaction. Black arrows show a positive expression in salivary gland acini and at gastric mucosae. Boxes in (a) and (e) are augmented in (b) and (f) respectively.
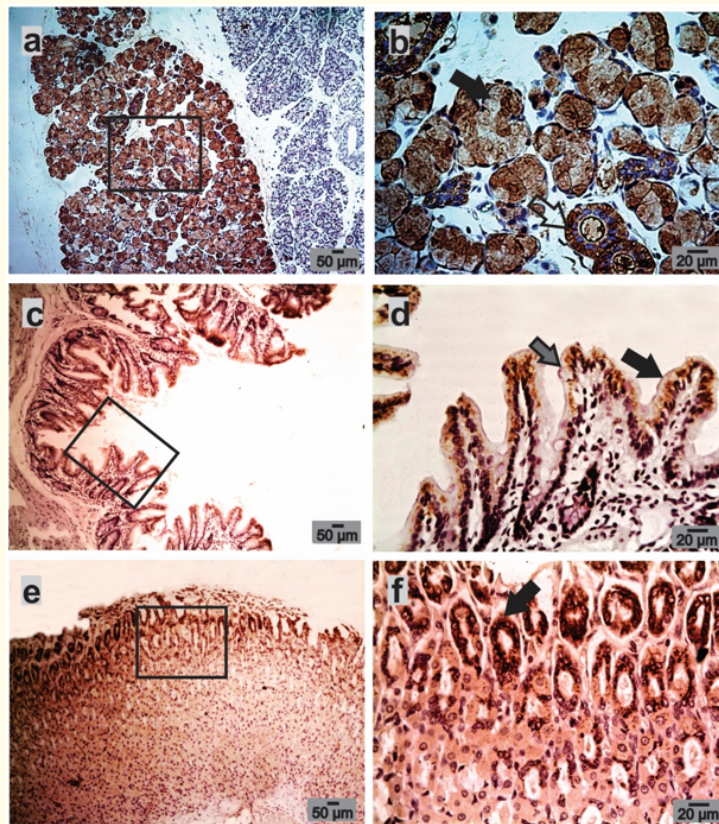
Figure 4: *Immunohistochemical Tn expression at adult organs. (a) and (b) show a strong and mixed reaction at salivary gland: (a) x10 and (b) x40; in (c) and (d) a mild and mainly cytoplasmic reactivity at the small intestine is shown; grey arrows show negative goblet cells; (c) x10 and (d) x40. In (e) and (f) stomach mucosae shows a strong mixed expression; (e) x10 and (f) x40. Boxes in (a), (c) and (e) are augmented in (b), (d), and (f) respectively. Black and white arrows denote a positive reaction.*

On respect to TF reactivity, esophageal and gastric epithelia showed an early (D16), moderate, and linear expression (Figure 5a). At this point, the esophagus and stomach presented a stratified epithelia but only the upper layer revealed reaction. Between D17 and D18 early gastric glands were found and at D18 two gastric regions may be distinguished; a glandular one with simple epithelia and a non-glandular (adult forestomach) with stratified epithelia; both regions revealed TF expression. At D18, same reactivity was also found at excretory ducts of salivary glands (Figure 5b) and renal tubules (Figure 5c) while keratinocytes of the upper layers of the epidermis exhibited a mixed expression (Figure 5d). At D19, intestinal epithelial cells also exhibited a moderate and mixed reaction (Figure 5e and 5f); goblet cells were negative. From D20 onwards, TF renal expression was restricted to the Bowman corpuscles (Figure 6a and 6b) with a mixed pattern while the renal tubules became negative. Neonates and adults showed a positive expression in the same tissues, except for the esophageal (Figure 6f) as well as the forestomach epithelium which were negative. In adults, salivary glands exhibited a strong TF reaction in the excretory ducts but also at mixed acini (Figure 6c-6e). In all positive tissues, the pattern of reaction was predominantly mixed.

antigen Tn

Organs	Embryos and fetuses gestational days					Neonates postnatally days	Adults
	16	17	18	19	20	2 – 15	10 – 12 weeks
Esophagus	-	-	-	-	-	-	-
Stomach	-	-	-	-	++ (4/8) II	++ (7/8) III	++ (7/8) III
Small Intestine	-	++ (4/8) II	++ (4/8) II	+++ (5/8) III	+++ (5/8) III	+++ (6/8) III	+++ (6/8) III
Large Intestine	-	++ (5/8) III	++ (5/8) III	+++ (6/8) III	+++ (6/8) III	+++ (6/8) III	+++ (6/8) III
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	++ (4/8) III	+++ (5/8) III	+++ (5/8) III	+++ (6/8) III	+++ (6/8) III
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	++ (7/8) I	+++ (8/8) I	+++ (8/8) II	+++ (7/8) II	+++ (7/8) III	+++ (7/8) III
Skin	-	-	-	-	-	-	-

antigen T (TF)

Organs	Embryos and fetuses gestational days					Neonates postnatally days	Adults
	16	17	18	19	20	2 – 15	10 – 12 weeks
Esophagus	++ (4/8) III	+++ (5/8) III	+++ (6/8) III	+++ (7/8) III	+++ (7/8) III	-	-
Stomach	++ (4/8) II	++ (5/8) II	++ (5/8) II	+++ (6/8) II	+++ (7/8) II	+++ (5/8) III	+++ (6/8) III
Small Intestine	-	-	-	++ (3/8) II	++ (3/8) II	++ (4/8) II	++ (3/8) II
Large Intestine	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	++ (5/8) II	+++ (5/8) II	+++ (5/8) II	+++ (4/8) II	+++ (5/8) II
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	-	++ (4/8) I	+++ (5/8) I	+++ (5/8) II	+++ (4/8) III	+++ (5/8) III
Skin	-	-	++ (5/8) III	+++ (5/8) III	+++ (6/8) III	+++ (6/8) III	+++ (6/8) III

antigen sTn

Organs	Embryos and fetuses gestational days					Neonates postnatally days	Adults
	16	17	18	19	20	2 – 15	10 – 12 weeks
Esophagus	-	-	-	-	-	-	-
Stomach	-	-	-	-	-	-	-
Small Intestine	-	-	-	-	-	-	-
Large Intestine	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	++ (5/8) I	+++ (5/8) II	+++ (6/8) II	+++ (6/8) II	+++ (7/8) II
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-
Skin	-	-	-	-	-	-	-

Table 3: Intensity of the immunohistochemical staining for carbohydrate T (TF) in different organs from rat embryos, fetuses, neonates and adults. The intensity of reaction was scored as absent (-), low (+), moderate (++) and strong (+++). N° positive cases/Total samples was indicated between brackets. The percentage of cells positively stained in each sample was quantified: 0-5%= 0; 5-30%= I; 31-60%= II and 61-100%= III.
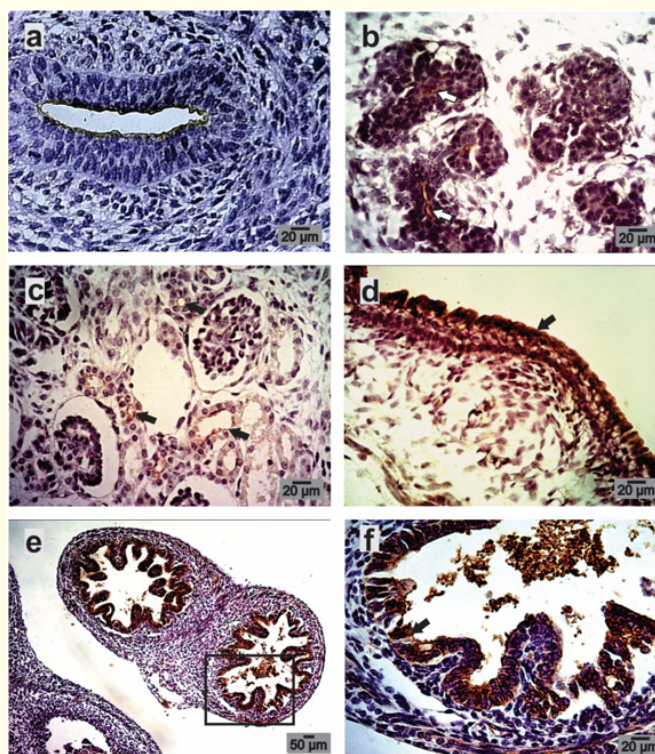
Figure 5: Immunoperoxidase T expression: (a) a moderate, linear reactivity at D16 esophageal epithelia, x40; (b) an apical mixed D18 salivary gland, x40; c) D18 distal renal tubules show an apical linear reactivity at distal renal tubules, x 40; d) D18 shows an apical, mixed expression at upper layers of epidermis, x40; In e) and f) a strong and mixed small intestine (e) x10 and (f) x40. Box in (e) is augmented in (f). Black and White arrows point to the positive reaction.
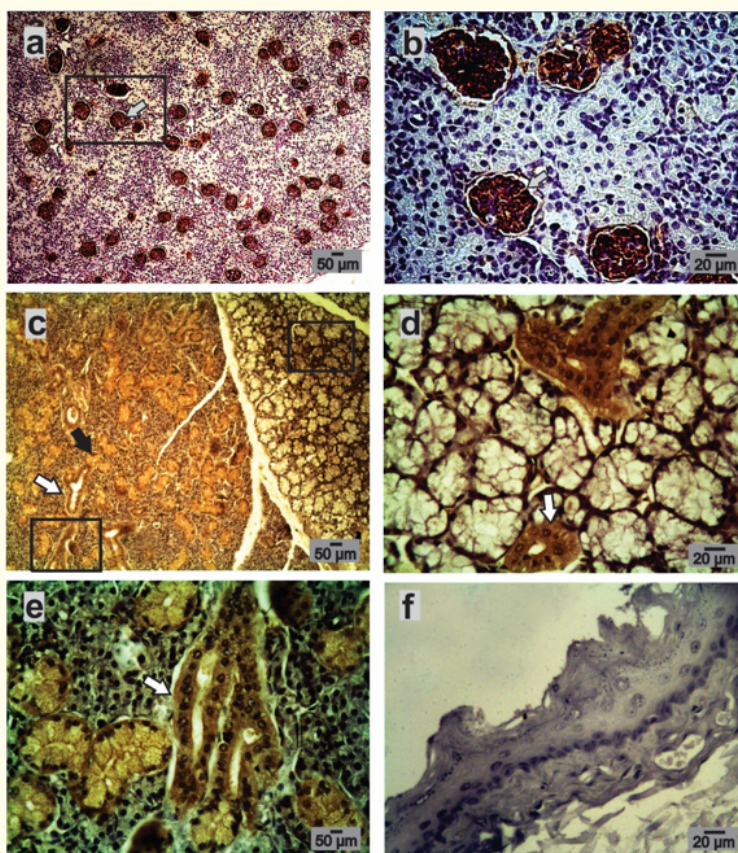
Figure 6: Immunostaining with anti-T MAb showing expression in adult samples. In (a) and (b) a mixed reaction in renal corpuscles is depicted (grey arrow) while renal tubules are negative; (a) x10 and (b) x40; (c), (d), and (e) show the cytoplasmic expression at salivary gland acini (black arrow) as well as ducts (white arrow); (c) x10, (d) and (e) x40. In (f) a negative reaction at the esophagus is shown. Boxes in (a) and (c) are augmented in (b), (d), and (e) respectively.

On the other hand, sTn expression was restricted to salivary glands. At D18, a few cells at the primordial ducts showed a moderate reaction with a linear pattern (Figure 7a) while at D19 an increased percentage of positive cells with a high intensity was observed (Figure 7b). At this stage, acini were not possible to be distinguished while from D20 onwards, mixed acini and excretory ducts were differentiated; all structures were reactive with a strong intensity and a mixed pattern (Figure 7c). In neonates and adults, acini still showed a strong reaction while, at excretory ducts, a weak expression was observed (Figure 7c and 7d), with a mixed pattern in both cases.

antigen Tn

| Organs | Embryos and fetuses gestational days | | | | | Neonates postnatally days | Adults |
	16	17	18	19	20	2 – 15	10 – 12 weeks
Esophagus	-	-	-	-	-	-	-
Stomach	-	-	-	-	++ (4/8) II	++ (7/8) III	++ (7/8) III
Small intestine	-	++ (4/8) II	++ (4/8) II	+++ (5/8) III	+++ (5/8) III	+++ (6/8) III	+++ (6/8) III
Large intestine	-	++ (5/8) III	++ (5/8) III	+++ (6/8) III	+++ (6/8) III	+++ (6/8) III	+++ (6/8) III
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	++ (4/8) III	+++ (5/8) III	+++ (5/8) III	+++ (6/8) III	+++ (6/8) III
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	++ (7/8) I	+++ (8/8) I	+++ (8/8) II	+++ (7/8) II	+++ (7/8) III	+++ (7/8) III
Skin	-	-	-	-	-	-	-

antigen T (TF)

| Organs | Embryos and fetuses gestational days | | | | | Neonates postnatally days | Adults |
	16	17	18	19	20	2 – 15	10 – 12 weeks
Esophagus	++ (4/8) III	+++ (5/8) III	+++ (6/8) III	+++ (7/8) III	+++ (7/8) III	-	-
Stomach	++ (4/8) II	++ (5/8) II	++ (5/8) II	+++ (6/8) II	+++ (7/8) III	+++ (5/8) III	+++ (6/8) III
Small intestine	-	-	-	++ (3/8) II	++ (3/8) II	++ (4/8) II	++ (3/8) II
Large intestine	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	++ (5/8) II	+++ (5/8) II	+++ (5/8) II	+++ (4/8) II	+++ (5/8) II
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	-	++ (4/8) I	+++ (5/8) I	+++ (5/8) II	++ (4/8) III	+++ (5/8) III
Skin	-	-	++ (5/8) III	+++ (5/8) III	+++ (6/8) III	+++ (6/8) III	+++ (6/8) III

antigen sTn

| Organs | Embryos and fetuses gestational days | | | | | Neonates postnatally days | Adults |
	16	17	18	19	20	2 – 15	10 – 12 weeks
Esophagus	-	-	-	-	-	-	-
Stomach	-	-	-	-	-	-	-
Small intestine	-	-	-	-	-	-	-
Large intestine	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	++ (5/8) I	+++ (5/8) II	+++ (6/8) II	+++ (6/8) II	+++ (7/8) II
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-
Skin	-	-	-	-	-	-	-

Table 4: Intensity of the immunohistochemical staining for carbohydrate sTn in different organs from rat embryos, fetuses, neonates and adults. The intensity of reaction was scored as absent (-), low (+), moderate (++) and strong (+++). N° positive cases/Total samples was indicated between brackets. The percentage of cells positively stained in each sample was quantified: 0-5%= 0; 5-30%= I; 31-60%= II and 61-100%= III.
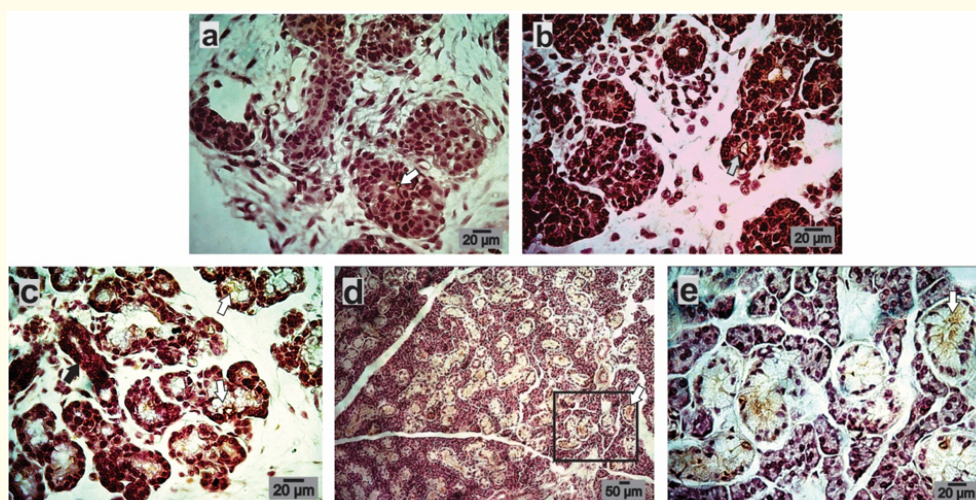
Figure 7: *Immunoperoxidase sTn expression at salivary gland. At (a) a D18 embryonic sample shows a linear reactivity restricted to a few cells; (b) at D19, a high intensity reaction is detected in most cells with a mixed pattern; at (c) D20 a strong reaction with a mixed pattern is depicted; (d) and (e) show adult samples with a strong reaction at acini and a moderate at excretory ducts. (a), (b), (c), and (e) x40 and (d) x10. Box in (d) is augmented in (e). White and black arrows denote the positive reaction.*

Lewis x expression, from D17 onwards a moderate and mixed apical reaction was found at the gastric mucosa (Figure 8a), renal distal tubules, and at all layers of the skin stratified epithelium (Figure 8b, d); also, large intestinal lining epithelial cells showed a moderate and linear expression (Figure 8c) while goblet cells, which were first detected at D19, did not show any reaction (Figure 8e and 8f). At this stage, renal expression did not change since it was restricted to distal tubules (Figure 9a and 9b). Adult samples showed reaction at the same tissues with the same pattern of expression (Figure 9c and 9d) except for the epidermis (data not shown).

<div style="text-align:center">**Le x**</div>

| Organs | Embryos and fetuses gestational days | | | | | Neonates postnatally days | Adults |
	16	17	18	19	20	2 - 15	10 - 12 weeks
Esophagus	-	-	-	-	-	-	-
Stomach	-	++ (4/8) **II**	+++ (4/8) **II**	+++ (5/8) **II**	+++ (5/8) **II**	+++ (6/8) **III**	+++ (6/8) **III**
Small Intestine	-	-	-	-	-	-	-
Large Intestine	-	++ (4/8) **II**	+++ (3/8) **II**	+++ (5/8) **II**	+++ (6/8) **II**	+++ (6/8) **II**	+++ (7/8) **II**
Pancreas	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Salivary glands	-	-	-	-	-	-	-
Trachea	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-
Kidney	-	++ (3/8) **I**	+++ (5/8) **I**	+++ (6/8) **II**	+++ (6/8) **III**	+++ (7/8) **III**	+++ (8/8) **III**
Skin	-	++ (3/8) **II**	++ (4/8) **II**	++ (2/8) **II**	++ (2/8) **II**	-	-

Table 5: *Intensity of the immunohistochemical staining for carbohydrate Lex in different organs from rat embryos, fetuses, neonates and adults. The intensity of reaction was scored as absent (-), low (+), moderate (++) and strong (+++). N° positive cases/Total samples was indicated between brackets. The percentage of cells positively stained in each sample was quantified: 0-5%= 0; 5-30%= I; 31-60%= II and 61-100%= III.*
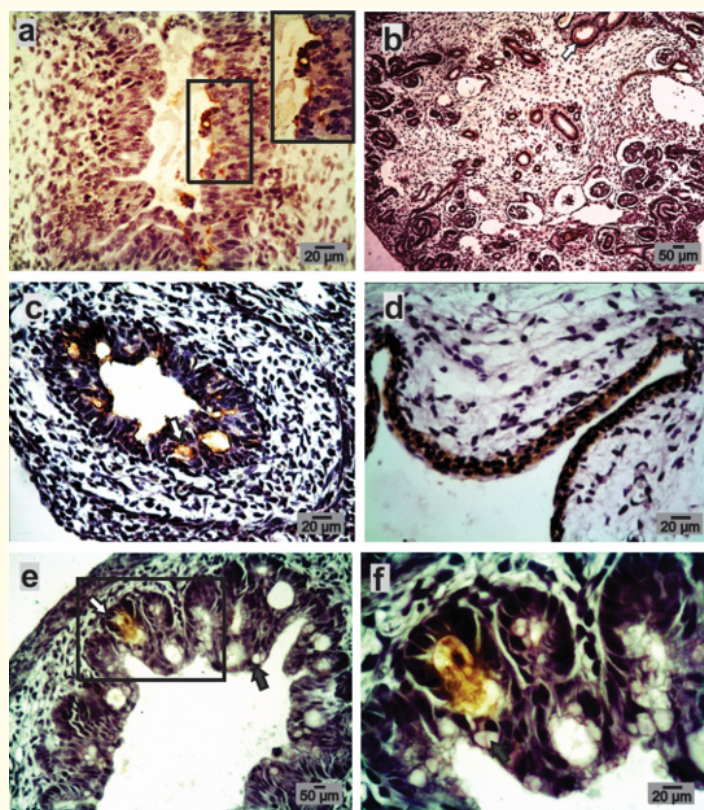
Figure 8: *Immunostaining with anti-Lewis x MAb show a positive mixed apical reaction at (a) gastric mucosae, (b) distal renal tubules, (c) large intestine, (d) epidermis, and (e) and (f) a mild restricted expression at D19 large intestine sample; (a), (c), (d) and (f) x40 and (b) and (e) x10. Box in (a) is augmented in the same picture. Box in (e) is augmented in (f). Black and white arrows show the positive reaction while the grey arrow denotes the negative goblet cells.*
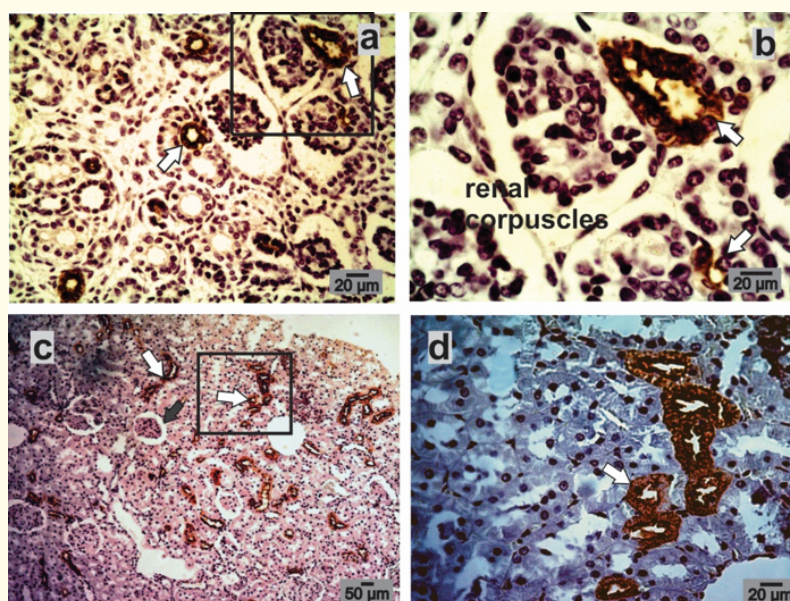
Figure 9: *Immunperoxidase reactivity of Lewis x in kidney at D19 ((a), x40 and (b), x100) and adults; (c) x10 and (d) x40). A positive reaction is restricted to renal tubules. Box in (a) is increased in (b) while box in (c) is augmented in (d). White arrows denote distal renal tubules. Grey arrows show negative renal corpuscles.*

In most organs included, Tn, TF, sTn and Le x antigens showed an increased percentage of stained cells along with developmental progression.

Finally, sLewis x reaction was not observed at any of the analyzed tissues. Furthermore, the respiratory organs and the annexed glands of the digestive system, liver and pancreas, did not show reaction to any of the analyzed antigens.

Statistical analysis of carbohydrate expression during rat development (Figure 10)

Statistical analysis employing PCA showed a strong correlation between Tn and Lewis x antigen ($r = 0.571$; $p < 0.001$), whereas Tn and TF also showed a significant correlation ($r = 0.361$; $p < 0.001$). Likewise a positive correlation between TF and Lewis x epitopes was found ($r = 0.447$; $p < 0.001$) while sTn did not show any significant association with the other carbohydrate antigens.

When embryonic stage was considered, a positive association with T antigenic expression ($r = 0.382$; $p < 0.001$), Lewis x expression ($r = 0.310$; $p < 0.001$) and with Tn antigenic expression ($r = 0.278$; $p < 0.001$) was found. Lewis x and T expression explained factor 1 data variability while T antigenic expression as well as stage explained factor 2 data variability. Although significant, sTn showed a lower correlation with stage ($r = 0.187$; $p < 0.001$) and explained factor 3 data variability.
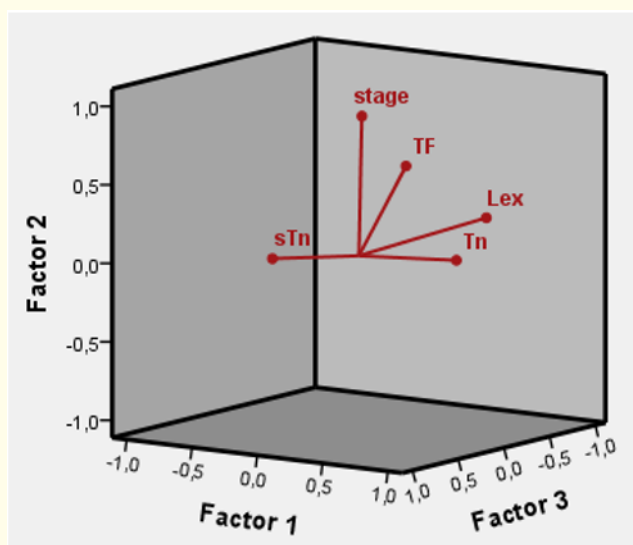
Figure 10: *Three-dimensional representation of component plot in rotated space showing factors positively correlated.*

Discussion

The development of the different systems is regulated by numerous glycoproteins including mucins, which are present in most tissues and cells and modify many membrane and secreted proteins [18,37,38]. Here we have performed a detailed immunohistochemical investigation of mucin associated glycans and we have identified their expression during rat epithelia embryonic development as well as in neonates and adults. Among the carbohydrate antigens, we studied the Tn antigen which was first described in 1969 [39]; then, Springer et al. reported that it was highly expressed in 90% of breast carcinomas, which indicated the relationship between Tn antigen and cancer [12]; further research confirmed that it is widely expressed in tumors [40]. Studies on mouse and humans found the Tn antigen mainly associated to the development of the nervous system and gut [40-42] while Tian and Ten Hagen [43] studying *Drosophila melanogaster* detected Tn on a number of ectodermally derived tissues such as the salivary glands, developing gut, and the tracheal system. Here, Tn was found early at D17 stage in intestinal epithelia and renal tubules. Afterwards, developing acini of salivary and gastric glands showed Tn expression at D18 and D20, respectively. Interestingly, other studies performed in mouse demonstrated that the absence of several glycosyltransferases involved in Tn synthesis produced alterations in the morphogenesis of salivary glands [44].

In this report, we have also detected the Thomsen Friedenreich (TF, T) antigen which has been attracted scientific interest for long time [12,40] as a carcinoma associated antigen. In human embryonic development, it has been probed that TF antigen is expressed at the syncitotrophoblast layer of the placenta directed to the maternal blood side as well as in cells of the decidua during all the gestation period with a decrease at the third trimester [45]. It has been suggested that this epitope expression may be part of the maternal system which avoid immune recognition and lytic reactions against the fetus [45]. Also, TF antigen has been detected in embryonic organs development; in this sense, we have found it at epithelia belonging to esophagus, both parts of the stomach (glandular and forestomach), intestines, salivary glands, skin, and kidney. Toma., *et al.* [46,47] found this epitope in human embryonic lung and kidney; at this organ, these authors detected TF in a differentiation and developmental stage-related manner, with the most immature and highly proliferating epithelial being reactive. In agreement, we found TF expression at rat embryonic corpuscles and renal tubules although we never detected it at lung samples. Alexander., *et al.* [48] studied mice deficient for *C1galt1*, the gene encoding the enzyme implicated on TF synthesis. It was found that mutations on *C1galt1* did not cause embryonic lethality but the kidneys had distorted glomeruli and proximal tubules, resulting in eventual renal failure and death by 200 days of age. At D19 we found TF expression in intestinal epithelial cells. Fu., *et al.* [49] demonstrated that TF glycan also plays a role preserving integrity and maintenance of the colonic epithelium. *C1galt1*-deficient mice produced less intestinal mucin (Muc2) and had impaired mucosal barrier formation and integrity. In agreement with these findings, in a previous report, we found Muc2 expression in intestinal epithelial cells at D19 stage of rat embryogenesis [33].

The sTn antigen (Neu5Acα2-6GalNAcα-*O*-Ser/Thr) is the truncated Tn containing a sialic acid α-2,6 linked to GalNAc α-*O*-Serine/Threonine (Ser/Thr) (Figure 1). It has been reported that more than 80% of human carcinomas expressed sTn [40,50]; early studies identified sTn as a marker for diagnosis and prognosis in cancer [51,52]. Our present results showed that sTn expression was restricted to salivary glands development. The primordial excretory ducts showed a moderate reaction with a linear pattern at D18 and besides, at D20, the mucous acini were also positive. Furthermore, in neonates and adults, a moderate and strong reaction remained in the excretory ducts and mucous acini, respectively. Kirkeby., *et al.* [53] studied sTn expression in the different human adult salivary glands and their findings showed a similar location predominantly at mucous acini and to a lesser extent in excretory ducts. At present, there is no information about its expression during embryonic development, neither in humans nor in other species. According to Zhang., *et al.* [54] and Julien., *et al.* [55] sTn expression is low or absent in normal adult epithelial cells although Yonezawa., *et al.* [50] reported its expression limited to areas of many normal human tissues. In a previous report in human normal samples, we detected very low expression of this antigen at oral cavity mucosae while breast and colorectal samples did not show any expression [23].

In the present study, expression and localization of the fucosylated antigens Lewis x and sLewis x were analysed. Lewis x is a carbohydrate structure that functions as an adhesion molecule in glycolipids and glycoproteins. It has been found in normal tissues [23,56] while it has been recognized as a marker of bladder cancer, Hodgkin's lymphoma Reed-Sternberg cells as well as a significant prognostic marker in triple negative breast cancer [57], although an association of Lewis x expression with a better outcome in patients with head and neck squamous cell carcinoma has been found [58]. Here, we identified Lewis x in gastric mucosa, large intestine, renal tubules and epidermis from stage D17 to D20 onwards; in neonates and adults, it was found at the same tissues, except for the epidermis which became negative. It has been suggested that this epitope would be a calcium dependent mediator for homeotropic adhesion in mouse embryo cells and it would have an important role in control cellular recognition during aggregation of cells in embryonic development [59].

Finally, sLewis x was also included in this research; this carbohydrate antigen has been found over-expressed in carcinomas [23,60]. It has been largely associated to metastatic behavior in lung, colorectal and breast carcinoma [61-63] and related to poor survival in gastrointestinal tumors [64-67]. In our research, sLewis x reaction was not found in any of the tissues included which could suggest that this antigen does not exert any important function on their development. This observation is not surprising since this antigen and its isomer sLewis a have been related to tumor cell adhesion to vascular endothelia [64]. It has been demonstrated that expression of sLewis x on leucocytes contributes to their function in the inflammatory response via interaction with E- and P-selectin expressed on endothelial cells. By exposing sLewis x at the cell surface, disseminated circulating cancer cells may use a similar mechanism for extravasation from blood to peripheral tissues where selectins are highly expressed on blood vessels [68].

PCA analysis showed principally a strong correlation between Lewis x and Tn which would suggest that glycoproteins such as mucins could carry both epitopes, which has been largely proven [69-72].

Considering the pattern of expression of the positive carbohydrate antigens, in most cases, a linear pattern of reaction was observed at early stages while a predominant change to a mixed pattern was detected at later stage, neonates, and adults. This fact would be related to an increase in *O*-glycosylation through Golgi complex and also, according with Reis., *et al.* [73], may be due to the variability of type/rate of glycosylation of different cells. The development of the different systems is regulated by numerous glycoproteins [38,74] and the pattern of glycans expressed in a cell depends on the glycosyltransferases expressed, their substrate specificity, and location [75].

This study has some limitations, firstly, it is a descriptive analysis and, on the other hand, we included organs that can be easily identified during the developmental process; the mammary gland is difficult to identify and, in consequence, it was excluded [31,76]. Nevertheless, to our knowledge, this is the first detailed investigation of carbohydrate antigens during rat epithelia development. Following Toma., *et al.* [47], the basis for a satisfying model to explain our results may lie in the tissue-specific expression and regulation of the enzymes of the glycosylation machinery, which expression is beginning to be unraveled. Many glycosyltransferases are regulated in an oncofetal manner but very little is known about the molecular mechanisms involved.

Conclusion

Our findings would suggest a role of Tn, TF, sTn, and Lewis x epitopes probably modifying cell-cell and cell-matrix interactions and cytodifferentiation; these antigens may be also involved on the integrity maintenance of epithelia. Mucin carbohydrate expression during embryonic development may be explained, at least in part, on the exposure of epithelial cells/tissues to growing factors. It has been proved that this exposure may result in remodeling of mucin carbohydrate structure, potentially altering the biological properties of the cells [77]. It is interesting that β-galactose-containing glycoconjugates like TF containing glycoproteins would be ligands of galectins, the most widely expressed class of lectins in all organisms. It has been shown that these lectins bind selectively to only some cell-surface and extracellular matrix ligands at low lectin concentrations but at higher concentrations they more often show broad binding to many different glycoproteins. However, the precise physiological ligands for each galectin and the physiological concentrations of galectins at sites of interactions are not well understood [78]. We suggest a potential role for the studied carbohydrates in these interaction with galectins in the context of development.

Conflicts of Interest

The authors declare no conflict of interest.

Disclosure

Prof. María Virginia Croce and Dr. Marina Isla Larrain are members of the research career of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC/PBA).

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