



THE ROLE OF CDX2 AND P63 IN THE DEVELOPMENT OF THE ESOPHAGUS, STOMACH AND INTESTINE

Pathology

Rekun Tetyana

Department of Histology, Vinnytsia National Pirogov Memorial, Medical University, Vinnytsia, Ukraine

Vernygorodskiy Sergii*

Pathological Anatomy, Forensic Medicine and Law Department, Vinnytsia National Pirogov Memorial, Medical University, Vinnytsia, Ukraine* Corresponding Author

ABSTRACT

To better understand the mechanisms underlying transformation of esophageal and gastrointestinal epithelium and its relationship with developmental processes, we studied and compared the expression of the intestine-specific homeodomain protein CDX2 and protein p63 in digestive system of human embryos and fetuses. CDX2 staining was only seen in the nuclei of small and large intestinal epithelium. To obtain more insight into the role of p63 in the morphogenesis of these epithelia, we examined the p63 expression that was detected in the esophagus in early embryonic period and late fetal one. We hypothesized, that p63 may exert essential roles in regulating the switch between different types of epithelia. Based on our results, we suggest that the lack of p63 expression in the esophageal cells results in the transdifferentiation of one epithelial type into another one.

KEYWORDS

CDX2, p63, Immunohistochemistry, Esophagus, Gastrointestinal Tract Development

INTRODUCTION

During early embryogenesis, the gastrointestinal tract (GIT) develops from two endoderm invaginations at the anterior (anterior intestinal portal, AIP) and posterior (caudal intestinal portal, CIP) ends of the embryo. Several transcription factors are expressed in the early AIP and CIP endoderm and their mutant phenotypes suggest roles in endoderm specification and early patterning. For instance, Gata4 (a member of the GATA family of transcription factors) is expressed very early in the definitive AIP endoderm, Foxa2 (a forebrain domain/winged helix transcription factor, previously called Hnf3 β) is also expressed in the definitive endoderm. Some members of the homeobox (HOX) family gene also are expressed in the visceral endoderm^{1,2}.

Different molecular pathways and transcription factors have been described and studies in the processes of esophagus, stomach and intestine development. Key molecular pathways that involved in gastrointestinal tract (GIT) differentiation include the hedgehog (Hh), bone morphogenetic protein (BMP), and Notch signaling pathways, the HOX and SOX transcription factors, transformation-related protein (p63), the Eph receptors/ephrin ligands (Eph-ephrin) signaling system, the Wnt/ β -catenin and TCP signaling pathways. Many of these systems are best known as critical control factors in general body plan developmental processes as well as role in organ pattern formation including having key roles in gastrointestinal development³.

In order to understand better the role of these factors in the upper and lower parts of GIT development, we have decided to choose and investigate CDX2 and p63 expression in epithelia of the lower part of the esophagus (gastroesophageal junction), gastric cardia, small and large intestine.

CDX1 and CDX2 are members of the caudal-related homeobox gene family and are intestine-specific transcriptional factors⁴. In adult mice and humans, expression of CDX1 and CDX2 is strictly confined to the gut, from the duodenum to the rectum⁵. While normal gastric mucosa does not express the transcriptional factors CDX1 and CDX2, aberrant expression of CDX1 and CDX2 is observed in animal and human gastric intestinal metaplasia (IM)^{6,7}.

The human p63 gene is located on chromosome 3q27-29 and comprises 15 exons. Like the other members of the p53 family, p63 is expressed from two different promoters, P1 and P2, that generate two classes of proteins, TAp63, which contains the N-terminal transactivation (TA) domain, and the N-terminal truncated (Np63) isoform, which lacks this transactivation domain. p63 is a key regulator of the expansion of the basal keratinocyte population and is essential for the development of several different epithelia cellular proliferation, differentiation and survival in both physiological and pathological contexts. Despite accumulating evidence about the p63

network, the molecular mechanisms by which p63 participates in epithelial self-renewal and homeostasis are complex and still far from being fully understood⁸.

Danieli et al⁹ indicated that p63 plays a critical role in the development of normal esophageal and tracheobronchial epithelia and appears to control the commitment of early stem cells into basal cell progeny and the maintenance of basal cells⁹.

Substantial debate exists about the cells of origin of Barrett's esophagus (BE) and the underlying molecular mechanisms¹⁰. Several theories have been proposed by Quinlan et al¹¹ including the transdifferentiation of one committed cell type to another or the reprogramming of a progenitor or stem cell population toward a simple epithelial lineage rather than a stratified one¹¹. Molecularly, two key players would be p63 and CDX2, the latter being a transcription factor of the caudal-related homeobox family with an important role in intestinal epithelial development and differentiation. Consistent with this view, immunohistopathological and functional experimental studies have implicated the inappropriate expression of CDX2 in BE^{11,12}. Conversely, conditional CDX2 deletion in the intestine results in squamous metaplasia. Expression of p63 is lost in BE. Thus, one attractive hypothesis is that p63 normally functions as a negative regulator of CDX2 expression. However, now Wang et al¹³ report that CDX2 is not upregulated in esophageal cells lacking p63, in spite of the columnar phenotype of these cells. The authors also note that increased expression of CDX2 is not constantly observed in all forms of BE. This may be a feature of the disease's later steps and/or a reflection of different clinical classifications and heterogeneity of the condition^{11,12}.

Despite intense research efforts, the molecular mechanisms underlying this metaplastic change in epithelial phenotype have not been completely elucidated.

The identity of the cell that gives rise to BE has not been definitively identified¹³. The esophagus originates from the foregut endoderm during early embryonic development. Its epithelium undergoes a series of changes involving the differentiation of stem cells into unique cell types and ultimately forming the mature epithelia⁹. In the esophageal epithelium, stem cells differentiate first into ciliated cells, followed by basal cells and after that into stratified squamous non-keratinized epithelium but mechanisms of these transformations are still unclear up to now. That's why the purpose of our research was to study the role of p63 in differentiation of the esophageal and GI epithelia, to investigate the expression and significance of caudal-related homeobox transcription factor (CDX2) and to determine whether CDX2 plays an essential role in the differentiation of upper GIT.

MATERIALS AND METHODS

The present study included 169 autopsied formalin-fixed human embryos and fetuses with gestational age (GA) 4–38 weeks of

Caucasian ethnic origin. All autopsies were performed between 4 and 24 h after death as pre-arranged protocol followed by the department, after a written consent, which included name of the mother, mode of delivery, sex of the fetus, anthropometry, external and internal examination. The presence of congenital malformations involving the GIT was considered as an exclusion criterion. Crown-rump length (CRL) was the main criterion for estimating fetal age. The fetal ages are indicated in Table 1.

Routine histology included hematoxylin and eosin staining as well as alcian blue and PAS staining. Immunohistochemical studies were conducted in paraffin sections using the streptavidin_biotin visualization method (Dako, Denmark). Antigen demasking was carried out in citrate buffer at pH 6.0. As initial antibodies we used mouse and rabbit monoclonal antibodies. Cell nuclei were counterstained with Mayer hematoxylin during 15–60 s. Gene expression of transcription factor of intestinal differentiation CDX2 was estimated with the use of mouse monoclonal antigens to nucleus antigen CDX2 (clone DAK-CDX2, Dako, Denmark) and p63 Clone DAK-p63. In preparations at 400_fold microscope enlargement, we determined the intestinal differentiation index (nucleus mark CDX2 and p63) in five accidentally selected fields of vision (≤ 500 cells) as a portion in percentage of positively stained nuclei of epithelial cells of the esophagus, stomach and intestine.

CDX2 was evaluated immunohistochemically using anti-CDX2 antibody.

Table 1: Crown-rump length (CRL) of the fetus in relation to gestational age

№	Age (weeks)	Number	CRL, MM
1	4-5	6	6,1±0,3
2	6-7	6	15,1±0,6
3	8-9	7	19,4±0,5
4	10-11	10	39,02±0,5
5	12-13	11	58,72±2,27
6	14-15	12	93,11±5,1
7	16-17	15	122,17±2,7
8	18-20	19	152,9±3,5
9	21-24	20	192,14±1,8
10	25-28	13	230,96±3,1
11	29-32	14	264,83±1,7
12	33-36	20	302,92±1,4
13	37-38	16	341,5±5,7
Total		169	-

All patients were thoroughly informed about the study that was approved by the local ethics committee.

Statistical analysis. Results of immunohistochemical alterations were compared to the clinicopathologic features using chi-square test with two tailed p value, $p < 0.05$ was considered as significant.

RESULTS AND DISCUSSION

In embryos with crown-rump length (CRL) 6,1±0,3 mm (4-5 gestation weeks) and 15,1±0,6 mm (6-7 gestation weeks) esophageal epithelium consists of a layer of two to three cells and the deepest layer is formed by columnar cells with strongly positive reaction for p63 in nuclei that were located on different levels (Figure 1).

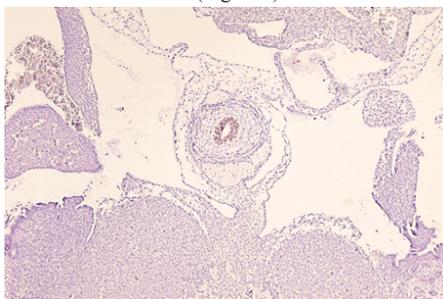


Fig. 1: An intense nuclear staining of p63 in nuclei of the esophagus columnar epithelium. Embryo esophagus, 5 weeks. Immunohistochemistry of anti-p63, x 200.

In positive cases, the immunoreactivity was predominantly nuclear with occasional faint cytoplasmic staining. By the eighth week (CRL 19,4±0,5 mm), the epithelium remains pseudostratified with the appearance of single vacuoles in the cells cytoplasm. However, already in the ninth week, the vacuoles reach their largest volumes. After the tenth week (CRL 37-38 mm), a pseudostratified epithelium starts to get considerably thinner, and starting with CRL 40 mm becomes ciliated multilayered columnar. Starting from this period the nuclei become negative for p63 labeling up to the sixteenth week, when squamous epithelium arises in the lower part of the esophagus and columnar epithelium of the stomach cardia part becomes more differentiated but we are not revealed expression of p63 in the latter. It was interesting that positive expression of CDX2 was observed only in small and large epithelium from the sixteenth week. The positive weak expression of p63 was detected in the esophageal cells by the 25 week. From 25 to 38 gestation weeks, the progressing increase of p63 expressing is observed in nuclei of esophageal epitheliocytes. In contrast with esophageal epithelium p63 expression was found in the gastric and intestinal mucosa during all the time of investigation.

In small and large intestine CDX2 expression was detected in nuclei of columnar epithelium beginning from 16 gestational weeks till 38 weeks and did not express in early embryonic and fetal period (from 4 to 15 weeks). After 16 gestational weeks the nuclei of small intestinal epithelia are CDX2 positive in the majority of cases, usually showing a strong, uniform staining (Figure 2).

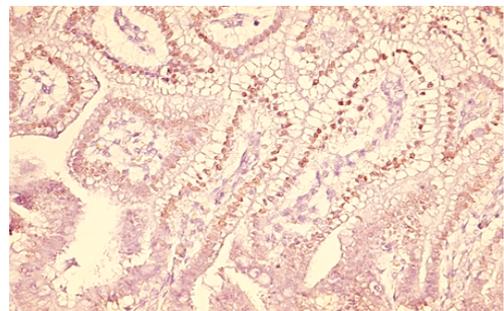


Fig. 2: A strong expression of CDX2 in nuclei of the small intestine columnar cells mucosa. Fetal small intestine, 16 weeks. Immunohistochemistry of anti-CDX2, x 200.

Moderate nuclear staining was observed extensively in large intestinal epithelium (Figure 3).

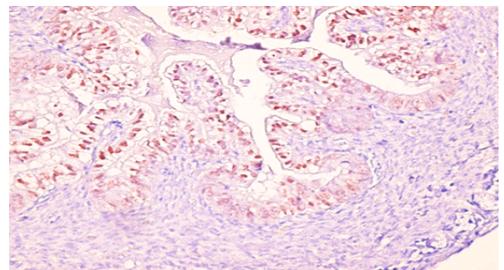


Fig. 3: A moderate expression of CDX2 in nuclei of the large intestine. Fetal large intestine, 16 weeks. Immunohistochemistry of anti-CDX2, x 200.

It is necessary to note that after 16 weeks small and large intestinal epithelia are CDX2 positive in the large majority of cases, usually showing a moderate staining of nuclei with focal staining are included, about 95% of cases. The fine granular cytoplasmic staining was also observed in the perinuclear regions that were PAS and alcian blue positive. When in early embryonic and fetal period (from 4 to 16 weeks) the expression is more often negative in 98%. In compare with intestine CDX2 has not been expressed in normal esophageal or gastric mucosa during the study period.

GIT development may be divided in multiple stages, where the number and type of epithelial cells are sequentially determined. There are different molecular signalling that regulate GIT development and it is possible to observe that the molecular signals are expressed in different stages of embryogenesis. Starting from the 8th week of gestation, the esophageal epithelium is gradually replaced by a ciliated and then a

squamous epithelium until a non-keratinized stratified squamous epithelium is fully developed.

Given that the normal epithelium found in the human esophagus after birth is predominantly squamous (the exception being the epithelium lining submucosal gland ducts and comprising the submucosal glands), two distinct hypotheses developed on how a squamous epithelial cell could give rise to a columnar epithelial cell. First, a fully differentiated squamous epithelial cell could undergo irreversible direct phenotypic conversion through molecular reprogramming into an intestinalized columnar cell without undergoing mitosis, a process termed transdifferentiation. Alternatively, a squamous epithelial precursor or stem cell could undergo molecular reprogramming leading to a change in the cell fate of progeny cells, a process termed transcommitment¹⁵. The other potential source for the BE cell of origin besides a proximally migrating columnar epithelial cell, a native squamous epithelial cell, or a native epithelial cell from an esophageal submucosal gland or duct, is an external circulating stem cell (i.e., from the bone marrow). Potential sources for the cell or tissue of origin for metaplastic Barrett's epithelium are reviewed including native esophageal differentiated squamous cells, progenitor cells native to the esophagus located within the squamous epithelium or in the submucosal glands or ducts, circulating bone marrow-derived stem cells, and columnar progenitor cells from the squamocolumnar junction or the gastric cardia that proximally shift into the esophagus to fill voids left by damaged squamous epithelium¹⁴. The conversion of stratified squamous esophageal epithelium may require an intermediate columnar step and may arise via reprogramming of the differentiation program of basal cells, from the glandular ducts, or from cells at the gastroesophageal junction in order for CDX2 to induce IM. Perhaps, these cell lines do not contain the requisite squamous progenitor or stem cell with the plasticity to become an intestinalized columnar cell. The acquisition of a non-intestinal columnar phenotype could be compared to the cardia-type mucosa that has been proposed as an intermediate in BM. The phenotype switching from squamous to intestinalized columnar may require multiple genetic alterations in a specific combination and sequence. To date, the majority of studies perhaps is to stably express a columnar transcription factor followed by an intestinal transcription factor followed by a mucus-related transcription factor¹⁶.

The various lines of evidence suggest the presence of multiple stem cell pools in the stomach epithelium, but the relationships between these populations and their respective properties and developmental origins remain obscure¹⁷.

In our research CDX-2 is not demonstrable immunohistochemically in the esophageal or gastric cells of the GIT at any stage of maturation. CDX2 expression was highly restricted to the small and large intestinal epithelia in mid-and late gestation. Our investigations coincide with Daniely et al⁹, which demonstrated that lack of p63 expression results in the development of a highly ordered, columnar ciliated epithelium deficient in basal cells, but our data do not concur with Wang et al¹³ which proposed that the metaplasia derives from a reservoir of progenitor cells, which pre-exist in embryonic tissue and then repopulate or "take over" a damaged area when other cell populations have left.

Morphogenesis occurs by numerous genetic and epigenetic factors not just by a single gene and also most of the developmental defects in GIT usually occur as a result of mutations in genes encoding signaling molecules and transcriptional factors. Clearly we still have very limited insight in pathways and genes involved in normal homeostasis of the esophageal epithelium.

CONCLUSIONS

The present results suggest that CDX2 is a key regulator for the development and differentiation of columnar epithelium in small and large intestine in mid and late stages of embryogenesis. We didn't find an ectopic expression of CDX2 in the gastric or esophageal epithelium. Our investigation has shown that p63 protein is essential for the morphogenesis and differentiation of esophageal epithelium. The esophageal squamous epithelium contains only one type of cell which differentiates along one lineage and expresses transcription factors that are absent from the stomach, such as p63.

In this study, we have used immunohistochemistry to characterize the patterns of CDX2 and p63 expression in normal esophageal, gastric

and intestinal mucosa. Our hypothesis is that p63 may exert essential roles in regulating the switch between different types of intercellular contacts that are necessary for the formation, maintenance, differentiation and renewal of esophageal epithelium.

The results show that, in normal development, the squamous esophageal epithelium arises from the columnar epithelium by a direct conversion process.

Many questions still remain about the mechanisms lined in transdifferentiation of esophageal and GI epithelia. Future studies using additional molecular and biological markers are needed in order to shed more light on these problems.

References

1. Roberts DJ, Johnson RL, Burke AC, Nelson CE, Morgan BA, Tabin C. Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development*. 1995; 121(10):3163-3174.
2. Le Guen L, Marchal S, Faure S, de Santa Barbara P. Mesenchymal-epithelial interactions during digestive tract development and epithelial stem cell regeneration. *Cell Mol Life Sci*. 2015; 72(20):3883-3896.
3. Pascal de Santa Barbara, van den Brink GR, Roberts DJ. Development and differentiation of the intestinal epithelium. *Cell Mol Life Sci*. 2003; 60(7): 1322-32.
4. Silberg DG, Swain GP, Suh ER, Traber PG. CDX1 and CDX2 expression during intestinal development. *Gastroenterology*. 2000; 119: 961-71.
5. Jung Mook Kang, Byoung Hwan Lee, Nayoung Kim. CDX1 and CDX2 Expression in Intestinal Metaplasia, Dysplasia and Gastric Cancer. *J Korean Med Sci*. 2011; 26(5): 647-653.
6. Satoh K, Mutoh H, Eda A, Yanaka I, Osawa H, Honda S, Kawata H, Kihira K, Sugano K. Aberrant expression of CDX2 in the gastric mucosa with and without intestinal metaplasia: effect of eradication of *Helicobacter pylori*. *Helicobacter*. 2002; 7: 192-8.
7. Vernygorodskiy SV, Degtiarova LV, Iatsyna OI, Blume YB, Yemets AI. Role of transcription factors in transdifferentiation of the gastric mucosa. *Cytology and Genetics*. 2015; 49(2): 113-117.
8. Candi E, Cipollone R, Pia Rivetti di Val Cervo, Gonfioni S, et al. p63 in epithelial development. *Cell Mol Life Sciences*. 2008; 65(20): 3126-3133.
9. Daniely Y, Liao G, Dixon D, Linnoila RI, Lori A, et al. Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium. *Am J Physiol Cell Physiol*. 2004; 287(1): 171-81ar.
10. Karine Lefort, G. Paolo Dotto. p63 and Epithelial Metaplasia: A Gutsy Choice. *Cell*. 2011; 145(7); 1003-1005.
11. Quinlan, JM, Colleypriest BJ, Farrant M, Tosh D Epithelial metaplasia and the development of cancer. *Biochim Biophys Acta*. 2007; 1776, 10-21.
12. Herfs M., Hubert P., Delvenne P Epithelial metaplasia: adult stem cell reprogramming and (pre)neoplastic transformation mediated by inflammation? *Trends Mol Med*. 2009; 15: 245-253.
13. Wang X, Ouyang H, Yamamoto Y, Kumar PA, Wei T.S, Dagher R, et al. Residual embryonic cells as precursors of a Barrett's-like metaplasia. *Cell*. 2011; 145: 1023-1035.
14. Wang DH, Souza RF. Transcommitment: Paving the Way to Barrett's Metaplasia. *Adv Exp Med Biol*. 2016; 908: 183-212.
15. Wang DH, The Esophageal Squamous Epithelial Cell—Still a Reasonable Candidate for the Barrett's Esophagus Cell of Origin? *Cellular and Molecular Gastroenterology and Hepatology*. 2017; 4(1): 157-160.
16. Mills JC, Sansom OJ. Reserve stem cells. Differentiated cells reprogram to fuel repair, metaplasia, and neoplasia in the adult gastrointestinal tract. *Sci Signal*. 2015; 8(385):re8.
17. Kim TH, Shivdasani RA. Stomach development, stem cells and disease. *Development*. 2016; 143(4): 554-565.