Abstract. Background: Basic research on HPV has focused on identifying the genetic changes that lead to cervical carcinoma. However, while focusing on the molecular biology of the cancer, understanding of its cellular biology has lagged: the target cell of the HPV infection is unknown. Materials and Methods: In this study we identified the stem cell population of the cervical epithelium by monoclonal antibodies against p63, a homologue of the tumor suppressor gene p53 and cytokeratin 17 (CK17). Results: We noted p63 expression consistently in the nuclei of reserve cells, hyperplasia of the reserve cells and the basal layer of the ectocervical epithelium, while CK17 only stained endocervical reserve cells and reserve cell hyperplasia. Conclusion: We conclude that both p63 and CK 17 are suitable markers for cervical stem cell identification. Both markers, therefore, qualify for the identification of the HPV target cell.

The relationship between the development of cervical cancer and infection with certain types of Human Papilloma Viruses (High risk HPV) is well established (1). Cell cycle is influenced by molecular interactions of human papillomavirus gene products, particularly from the E6 and E7 open reading frames. These gene products have the ability to bind host regulatory proteins and lead to degradation of the p53 tumor suppressor gene product E6 and functional inactivation of the (tumor suppressor) retinoblastoma gene protein (pRb) E7 (2,3). However, the target cell of these transforming mutations, caused by high risk HPV infection in the uterine cervical epithelium still remains unknown.

Pierce and Potten suggested that the target cell in the carcinogenic cascade is the stem cell of the epithelium (4-6). Stem cells are defined as cells that have the ability to perpetuate themselves by means of self renewal and to generate mature cells of a particular tissue through differentiation. Signalling pathways that normally regulate stem cell self renewal can lead to carcinogenesis when dysregulated, so stem cells may be the target of transformation. Arguments supporting this hypothesis are the following: firstly, stem cells have the machinery for self renewal already activated; secondly, stem cells often persist for long periods of time, in contrast to dying after short periods of time like mature cells in highly proliferative tissues. This means that there is a much greater opportunity for mutations to accumulate in individual stem cells than in mature cell types (7). Therefore, it may be presumed that the target cell for high risk HPV infection is the stem cell of the uterine cervical epithelium. However, the exact nature of this stem cell is still under debate.

The uterine cervical epithelium consists of ectocervical squamous epithelium, endocervical columnar epithelium and subcolumnar reserve cells, so there are several potential candidates to qualify as stem cells. From morphological studies we know that reserve cells are undifferentiated, omnipotent cells which possess the capacity to undergo squamous differentiation (metaplasia) (8). Basal cells of the ectocervical squamous epithelium are more differentiated, dedicated to the formation of squamous cells, and therefore less suitable as stem cells of the epithelium (9,10). Another problem in the identification of the stem cell is that, so far, reliable markers are not available to identify stem cells of certain epithelia. Earlier studies revealed two potential markers (10,11).

Recently, p63, a homologue of the tumor suppressor gene p53, has been described as a transcription factor operating mainly in the embryonal stage of development (12). In several tissues such as bronchial, prostate and cervical reserve cells, p63 has been immunohistochemically demonstrated and hence is suggested to play a role in the regulation and maturation of epithelium in the adult phase (11,13-15). In pulmonary epithelium, p63 expression was seen in the...
bronchial reserve cells, which is consistent with the role of p63 in maintaining a stem cell population (16). In prostate tissue, p63 specifically labelled basal cells (17). p63 therefore could be a suitable marker for cervical stem cells (13).

Keratin polypeptide patterns can be used for identification or at least sub-classification of epithelial tissues. The keratin expression pattern in cervical tissue has been well defined (18). Previously, we observed that subcolumnar reserve cells, a potential stem cell population of the uterine cervix, showed a typical keratin expression pattern of which cytokeratin 17 was prominent and specific (19). To identify the stem cell of the uterine cervical epithelium, we performed an immunohistochemical study using monoclonal antibodies against p63 and cytokeratin 17 (CK 17) in a well defined subset of normal epithelium of the uterine cervix and in preneoplastic samples (CIN I, II and III).

**Materials and Methods**

*Tissue specimens.* All of the formalin-fixed and paraffin-embedded uterine cervix specimens were retrieved from the files of the Department of Clinical Pathology, Deventer Hospital, The Netherlands. Biopsies and diathermy loop excision specimens were taken from women with cytologically verified dysplasia. Histopathological analyses were performed on H&E-stained sections. The samples comprised CIN I (6 cases), CIN II (7 cases) and CIN III (7 cases). In these samples normal ectocervical squamous epithelium was diagnosed in 14 cases: endocervical columnar cells (20 cases), reserve cells (17 cases) and reserve cell hyperplasia (5 cases) were also identified.

*Immunostaining protocol.* The p63 mouse monoclonal antibody (Ab 4, clones 4A4+Y4A3, titre 1:100) was used to study tissue samples. It was obtained from Neomarkers, Klinipath, NL. To perform the immunological staining procedures we used the Ventana Medical Systems iView™ DAB Detection Kit, an indirect biotin streptavidin system of the Ventana Benchmark™ (Ventana Medical Systems, Inc., Tucson, Arizona, USA). The specificity of the staining with p63 antigen was verified by an internal control system. In each case, stromal cells, that did not show p63 expression in animal models, were used as an internal negative control (11). Mouse anti-cytokeratin 17 (clone E3) titre 1:200 was used to study the paraffin-embedded tissue samples in the same way, also using the Ventana Benchmark™ and the Ventana iView™ DAB detection kit. This antibody labels basal and myoepithelial cells of complex human epithelia (20). The mouse anti-CK17 antibody was obtained from Dakocytomation (Glostrup, Denmark).

*Immunoenzyme double-staining method.* We used an immunoenzyme double-staining method for the simultaneous detection of CK17 and p63 antigens. The first indirect method involved an unlabelled monoclonal antibody CK17 followed by the Ventana Medical Systems iView™ DAB Detection Kit. After a washing step with Tris-HCl buffer pH 7,6, the second indirect method was applied using an unlabelled antibody p63 followed by the visualisation of this antibody by the alkaline phosphatase conjugated Envision™ reagents, based on a unique enzyme conjugated polymer backbone (Dakoymation). The two antigens can be distinguished clearly and selectively by the reaction products of the enzyme activities of horseradish peroxidase (brown) and alkaline phosphatase (blue).

*Evaluation of the staining reactions.* The staining results of p63 and CK17 were independently evaluated by three of the authors (JM, BB and JWA). In cases of discrepancy, slides were reviewed together and consensus was reached in all cases. The number of cells positively stained for p63 and CK 17 was semi-quantitatively evaluated. Four groups could be distinguished, i.e. cases with 1 to 25%, 26-50%, 51-75% and 76-100% of positive cells, respectively. For both p63 (nuclear staining reaction) and CK 17 (cytoplasmatic staining reaction), the expression within the different epithelial layers was studied. CIN lesions were subdivided into three compartments, i.e. basal, intermediate and superficial, each consisting of approximately one-third of the epithelial thickness. Staining in ectocervical squamous epithelium, endocervical columnar epithelium and reserve cells as well as reserve cell hyperplasia was separately identified.

**Results**

The immunohistochemical staining pattern of monoclonal antibody against p63 is schematically represented in Figure 1 and illustrated in Figure 3 A-F. The expression of the monoclonal antibody against CK 17 is represented in Figure 2 and further illustrated in Figure 3G-H. The results of the combined immunohistochemical staining of p63 (nuclear) and CK 17 (cytoplasmic) staining are illustrated in Figure 3 I.
Figure 3. p63 expression in normal ectocervical epithelium (A), endocervical epithelium and reserve cells (B), reserve cell hyperplasia (C), in CIN I (D), CIN II (E), CIN III (F). Cytokeratin 17 expression in endocervical epithelium and reserve cells (G), reserve cell hyperplasia (H) and double-staining of p63 and CK 17 in (I).
Normal cervical epithelia. p63 expression was restricted to the nucleus and was consistently expressed in all slides. Staining intensity varied very little between individual cases. In ectocervical squamous epithelium, nuclear p63 staining was restricted to the basal layer of the epithelium in 76% to 100% of the cells in all cases (Figure 3A). In sharp contrast, endocervical columnar epithelium did not show any expression of p63. The subcolumnar reserve cells in the transitional zone and endocervix, however, showed p63 expression in 76%-100% of cells in all cases. The superficial compartment did not show staining (Figure 3F). In sharp contrast, the other retains its stem cell identity and thus regenerative potential (26). Failure of such an asymmetric cell division process can lead to differentiation of all daughter cells, thereby causing total depletion of stem cells and regenerative capacity (24). Changes that block the normal maturation of cells toward a non dividing, terminally differentiated state or that prevent normal programmed cell death play an essential role in the development of cancer. Such a basal, stem cell population should be p63-positive (23). The combination of CK 17 expression and p63-expression suggests that the reserve cell population is the stem cell population of the uterine cervical epithelium.

In this report we described the identification of the stem cell population of the uterine cervical epithelium using monoclonal antibodies against p63 and CK 17 in normal ectocervical, endocervical and preneoplastic lesions. Reserve cells and reserve cell hyperplasia showed strong staining expression of p63 (nuclear) and CK 17 (cytoplasmatic) in all cases.

In all cases p63 immunostaining was strongly expressed in the basal layer of ectocervical epithelia and in the basal layers of CIN (cervical intra epithelial neoplasia) lesions irrespective of grade. Our findings are consistent with recent studies describing p63 as a marker for basal squamous cells and subcolumnar reserve cells in cervical epithelium (13,21).

The cytoplasmatic staining reaction with the monoclonal antibody against CK 17 was shown expression in the basal compartment of ectocervical epithelium and of CIN lesions irrespective of their grade. Reserve cells and reserve cell hyperplasia showed strong expression in all cases.

p63 is involved in the maintenance of basal, progenitor cell populations and guarantees the capacity of tissues to develop and regenerate (22). The p63 protein appears to play a critical role by regulating stem cell commitment and promotion of squamous differentiation in skin, lung, cervix and other sites (16,23,24). Immunohistochemical studies detected p63 expression in proliferating mouse and human tissues. Inactivation of murine p63 resulted in complex deformities in the late mouse embryo. The abnormalities were accompanied by a non-regenerative differentiation of the epidermis and squamous mucosa including that of the genital tract, indicating that the p63 gene is critical to normal epithelial development and function (11). In cervical carcinogenesis high-risk human papilloma viruses are able to influence cell cycle control mechanisms leading to (pre) malignant transformation (1,25). However, the exact target cell for the HPV infection is unknown. It is plausible that it is an undifferentiated, multiplying cell, that makes it possible for a virus to incorporate into its DNA during the cell cycle. A stem cell can be the origin of tumor growth by disturbance of the "asymmetric" division process and can therefore be a candidate for the target cell of high risk HPV infection (7,22,26).

The main feature of a stem cell population is the ability to undergo "asymmetric" divisions, in such a way that one daughter cell proceeds onto differentiation pathways, while the other retains its stem cell identity and thus regenerative potential (26). Failure of such an asymmetric cell division can lead to differentiation of all daughter cells, thereby causing total depletion of stem cells and regenerative capacity (24). Changes that block the normal maturation of cells toward a non dividing, terminally differentiated state or that prevent normal programmed cell death play an essential role in the development of cancer. Such a basal, stem cell population should be p63-positive (23). The combination of CK 17 expression and p63-expression suggests that the reserve cell population is the stem cell population of the uterine cervical epithelium.

Evidence has already been found for the stem cell role of the reserve cell (9,19). Furthermore, a study on bcl-2, a marker for protection against apoptosis, showed the ubiquitous presence of this marker in reserve cells (27). This protection of bcl-2-positive reserve cells against apoptotic cell death is required to ensure survival of the epithelium and in this way points out their stem cell function.
The well-known process of metaplasia in the transformation zone of the cervix also suggests an important role for the reserve cell as the basal cell for columnar as well as squamous epithelial regeneration (8). In our study we detected two important p63- and CK 17-positive cell populations, i.e. the basal cells of the normal ectocervical epithelium and the subcolumnar reserve cells. In conclusion, our study identified the reserve cell as the most favorable candidate for stem cell of the epithelium of the uterine cervix and showed the value of both CK 17 and p63 as markers for these stem cells.

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References


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