



Modeling and validating three dimensional human normal cervix and cervical cancer tissues *in vitro*

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Abstract

The use of three dimensional *in vitro* systems in cancer research is a promising path for developing effective anticancer therapies. The aim of this study was to engineer a functional 3-D *in vitro* model of normal and cancerous cervical tissue. Normal epithelial and immortalized cervical epithelial carcinoma cell lines were used to construct 3-D artificial normal cervical and cervical cancerous tissues. De-epidermised dermis (DED) was used as a scaffold for both models. Morphological analyses were conducted by using hematoxylin and eosin staining and characteristics of the models were studied by analyzing the expression of different structural cytokeratins and differential protein marker MAX dimerisation protein 1 (Mad1) using immunohistochemical technique. Haematoxylin and eosin staining results showed that normal cervical tissue had multi epithelial layers while cancerous cervical tissue showed dysplastic changes. Immunohistochemistry staining revealed that for normal cervix model cytokeratin 10 was expressed in the upper stratified layer of the epithelium while cytokeratin 5 was expressed mainly in the middle and basal layer. Cytokeratin 19 was weakly expressed in a few basal cells. Cervical cancer model showed cytokeratin 19 expression in different epithelial layers and weak or no expression for cytokeratin 5 and cytokeratin 10. Mad1 expression was detected in some suprabasal cells. The 3-D *in vitro* models showed stratified epithelial layers and expressed the same types and patterns of differentiation marker proteins as seen in corresponding *in vivo* tissue in either normal cervical or cervical cancerous tissue. These findings imply that they can serve as functional normal and cervical cancer models.

Keywords: cervical cancer, MAX dimerisation protein 1, cytokeratins, three dimensional *in vitro* models

Introduction

In vitro studies are commonly used to mimic the physiologic environment of tumors^[1-2] at early stages of drug development. When cells are grown as monolayers in conventional two-dimensional (2-D) models, they lack the natural three-dimensional (3-D) tissue *in vivo*

characteristics^[3]. 2-D cell cultures can only provide some approximate information of normal and cancer tissues due to the highly unnatural geometric and mechanical limitations imposed on cells^[4]. This means that cells grown in conventional 2-D culture conditions commonly fail to mimic tissue structure and functions, and consequently do not provide information about the

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way cancer cells interact with the extracellular matrix (ECM) and its complex environment interactions that exist in human cancers^[5]. Under conventional culture conditions, keratinocytes grow as monolayers and are not able to grow and differentiate in stratified squamous epithelium as observed in the normal human cervix, but only reach an incomplete terminal differentiation^[6-7]. Animal models established in immunocompromised mice reconstitute conditions observed *in vivo* mimicking the physiologic microenvironment of cervical carcinoma, but these may also show false effects on tumor progression and molecular mechanisms of the disease due to the differences between mice and humans^[5]. To overcome the above difficulties, *in vitro* 3-D tumor models with different human cells have been progressively explored to enable accurate human tissue reproduction^[8]. 3-D *in vitro* models have an important role in tumor biology and provide important insights into cancer research. They enhance our understanding of tissue organization, cellular differentiation and provide us a better understanding of tumor behavior. The 3-D ECM and its receptors can promote normal epithelial polarity and differentiation^[9]. Various techniques have been developed for the construction of 3-D *in vitro* tumor models, such as cell-seeding 3-D scaffolds^[10], hydrogel embedding^[11], microfluidic chips^[12] or cell patterning^[13]. Tissue engineering developments have further improved the diversity and quality of 3-D *in vitro* models which take them one step closer to the *in vivo* situation. However, each of the represented models has its advantages and limitations. One model is organotypic epithelial “raft” culture system that allows proliferation and full differentiation of keratinocyte monolayers by culturing cells on collagen gels at the air-liquid interface^[14-16]. Normal keratinocytes grown in this model stratify and fully differentiate in a similar way to normal squamous epithelium^[16]. Another model that mimics the native state in the skin is the system where keratinocytes are grown on a de-epidermized or devitalized dermis, on which cells are able to grow at the air-liquid interface^[17-20]. The latter model is considered more physiologically relevant as the cell's growing conditions are similar to the *in vivo* situation, and the diffusion of nutrients from the underlying dermis into the epidermis can be observed^[16]. In addition, the de-epidermized dermal (DED) scaffold uses a human acellular dermis to construct a new multilayered epidermis to preserve the basement membrane which is critical for keratinocytes attachment *in vitro*^[21].

3-D *in vitro* systems closely recapitulate the 3-D organization of cells and ECM as seen in *in vivo* tissues. To mimic the human cervix *in vitro*, i.e., its multilayered

tissue organization and its interactions with ECM, a 3-D *in vitro* models of normal and cancer cervix were developed and the method used in this current research was adopted and modified from DED model of human skin^[21-22]. The de-epidermized dermis was used as a scaffold for both *in vitro* models. This system is an inexpensive alternative to other commercially available 3-D *in vitro* models and a viable alternative to animal testing. It can be widely used to assess the efficacy of drugs, to estimate dosage of new therapies and to develop tumor vaccines. In this study, a list of biomarkers was used to confirm and validate the reliability of proposed *in vitro* systems. Cytokeratin biomarkers were used to assess the reliability of the 3-D *in vitro* model of the cervix. As in cervical cancer tissues, the pattern of cytokeratins is often disorganized^[23], a differentiation biomarker MAX dimerisation protein 1 (Mad 1) was used to assist in evaluating the 3-D cervical cancer model.

Materials and methods

The immortalized human keratinocytes cell line NTERT and the cervical cancer cell line C33A were purchased from ATCC (ATCC, UK). C33A cell lines were grown in RPMI (Gibco Invitrogen, UK) media supplemented with 10% fetal bovine serum (FBS, BioSera, UK) and 1% penicillin/streptomycin antibiotic (Sigma, UK). NTERT cells were grown in cytokeratinocyte-SFM medium with L-glutamine, without calcium chloride (Gibco Invitrogen, UK) supplemented with cytokeratinocyte-SFM supplement human recombinant epidermal growth factor (2.5 µg/L) and bovine pituitary extract (BPE) (25 mg/L) (Gibco Invitrogen). Both cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂ with the media being changed every 2-3 days as required. To create the desired microenvironment for cells to enable them to grow and differentiate to the required phenotype, estradiol (Sigma, UK) at physiologic concentration level of 1 µmol/L^[24] was added to the 3-D *in vitro* models of normal cervix and cervical cancer. Estradiol can promote differentiation in cervical epithelial cells^[25-26] and can also increase proliferation and inhibit apoptosis in cervical cancers^[27-31].

Human skin (Euro Skin Bank, Netherlands) was first incubated in sterile PBS for 48 hours and then the epidermis was removed using a scalpel. The remaining dermis was cut in small 1.5 × 1.5 cm² pieces and washed three times in sterile PBS. Dermal scaffolds were incubated for 24 hours with different media according to the cell line used. Human keratinocyte NTERT cells and cervical cancer C33A cells were

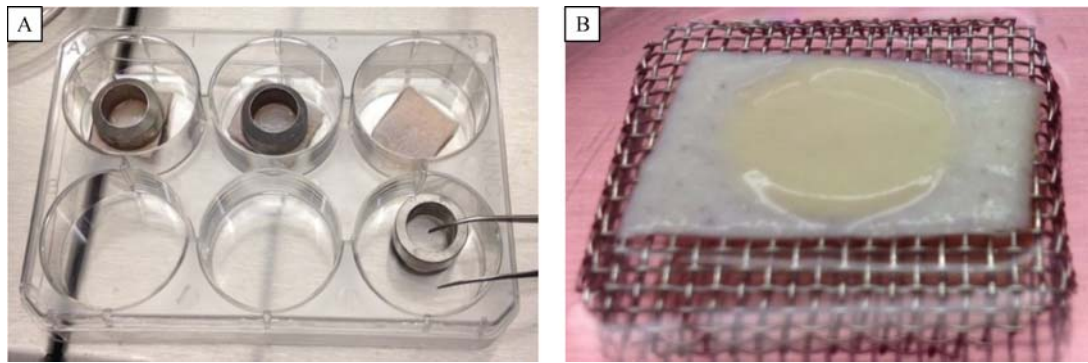


Fig. 1 The 3-D *in vitro* model of the cervix. A: Cells were seeded on dermal scaffold. B: The scaffold is placed on metal mesh to enable air-liquid growing conditions.

respectively seeded inside a metal ring on the top of dermal scaffolds, and left for 2 days to attach (**Fig. 1A**). Rings were then removed and dermal scaffolds were raised and placed on metal meshes for another four to six weeks at the air-liquid interface to allow cell stratification and differentiation (**Fig. 1B**). At least three dermal scaffolds were prepared for each cell line used. After three weeks samples were washed in PBS and fixed in 4% paraformaldehyde for 48 hours at 4°C. After fixation, samples were processed, wax embedded and cut into 6 µm sections for staining.

Slides were stained using standard immunohistochemistry (IHC) methods published elsewhere^[32]. In brief, paraffin embedded tissue blocks were cut into 6µm sections, dewaxed and rehydrated through graded alcohol to water. Sections were then either stained with hematoxylin and eosin, or incubated with primary antibodies (**Table 1**) followed by avidin biotin complex

(ABC) staining methods dehydration, clearing and mounting in DePeX (BHD, UK). Images were captured using the Image-Pro Express 6.3 program. Results were graded by two researchers independently and then recorded according to the number of positive cells in the tissues studied. “+” represents 1 to 5 positive cells; “++” represents 5 to 20 positive cells and “+++” represents 20 or more positive cells. Sections where no staining was detected were classified as negative (-ve).

Results

NTERT cells were grown in our 3-D *in vitro* model and served as a control model of cervical tissue. After 4-6 weeks of incubation, human keratinocytes managed to grow on the de-epidermised dermis and formed multi-layered structures. IHC analysis revealed that the

Table 1 Antibodies used in immunochemical staining

1° Ab	Ig type	Concentration	Antigen retrieval	Serum	2 ° Ab
Anti-cytokeratin [CK5] (Rabbit monoclonal) (Abcam)	IgG	1 µg/mL	Citrated buffer x1, heated for 20 minutes	horse	Biotinylated anti-rabbit IgG
Anti-cytokeratin 10 [CK10] (Mouse monoclonal) (Abcam)	IgG1	1 µg/mL	Citrated buffer x1, heated for 20 minutes	horse	Biotinylated anti-mouse IgG
Anti-cytokeratin 19 [CK19] (Mouse monoclonal) (Abcam)	IgG2a	1 µg/mL	Citrated buffer x1, heated for 20 minutes	horse	Biotinylated anti-mouse IgG
Mad1 (Rabbit polyclonal) (Santa Cruz Biotechnology)	IgG	2 µg/mL	0.1% Triton-100, 10 minutes	horse	Biotinylated anti-rabbit IgG
Isotypic control (Goat anti-mouse) (Abcam)	IgG1	5 µg/mL 10 µg/mL	Citrated buffer x1, heated for 20 minutes 0.1% Triton-100, 10 minutes	horse	Biotinylated anti-goat IgG
Isotypic control (Goat anti-mouse) (Abcam)	IgG2a	1.1 µg/mL 38 µg/mL	0.1% Triton-100, 20 minutes	horse	Biotinylated anti-goat IgG
Isotypic control (Goat anti-mouse) (Abcam)	IgG	2 µg/mL 1.53 µg/mL	0.1% Triton-100, 20 minutes	horse	Biotinylated anti-goat IgG

NTERT model was positively stained with cytokeratin 10 with the staining seen in the upper most stratified layer of the epithelium as seen in a normal cervix. Cytokeratin 5 expression was seen mostly in either the entire cell layer or in the basal part of tissue depending on the 3-D *in vitro* model thickness achieved and this is consistent with the staining pattern seen in a normal cervix (**Fig. 2**).

Cytokeratin 19 is normally seen in the basal cell layer of the normal cervix. The NTERT 3-D *in vitro* model sections were mostly negative for cytokeratin 19, with only weak staining for single cells found in the basal layer (**Fig. 2**). Two negative controls were included, one of which was an internal negative control where primary antibody was omitted and the other a negative isotypic control, where primary antibody was replaced with normal IgG from the same species using the same isotype at the same concentration.

C33A cells were then grown in our 3-D *in vitro* model to serve as a model of cervical cancer. Staining results showed that the cytokeratin distribution pattern resembled a typical cervix cancerous tissue, where

weak or no staining for cytokeratin 10 and cytokeratin 5 was detected whereas cytokeratin 19 was expressed in different layers of artificial tissue model (**Fig. 3**).

Expression of Mad 1 differentiation marker

MAX dimerization protein 1 (Mad1) is a transcriptional repressor that is produced in differentiating cells and was used as a differentiation marker to characterize the cell maturation and differentiation processes observed when cells were grown at a air-liquid interface in the 3-D *in vitro* model of cervical cancer. In the C33A 3-D *in vitro* model, cells in the upper, most differentiated layer were stained with Mad1 (**Fig. 4**).

Discussion

To gain a better insight into mechanisms of human cancer pathology and to find new anticancer therapies, reliable, physiologically relevant and cost effective *in vitro* models are needed. In this study, we have developed a 3-D *in vitro* model to allow *in vitro* reconstruction of the normal cervix and cervical cancer.

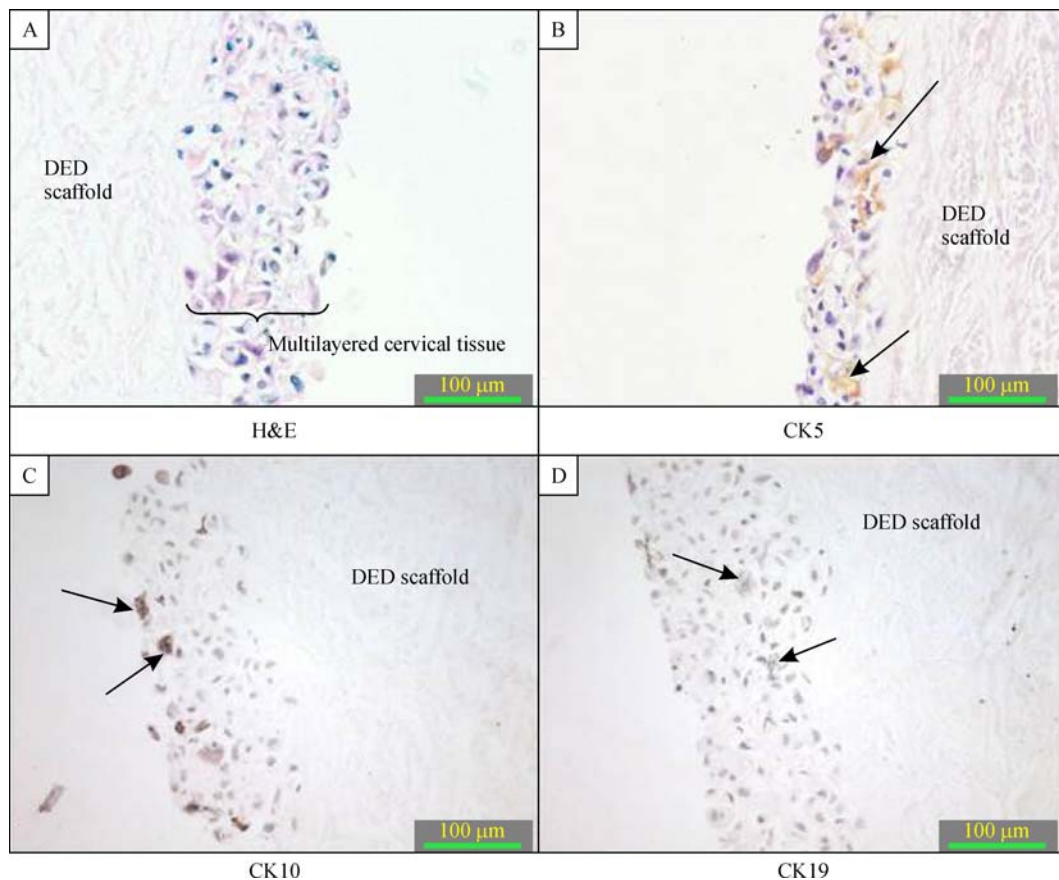


Fig. 2 The 3-D *in vitro* models of normal cervix (magnification 400 \times). A: H&E staining; B-D: immunohistochemistry of the cervix model; B: cytokeratin 5 positively stained cells are found in the basal cell layer. C: Cytokeratin 10 positively stained cells are seen in the upper layer. D: Cells were negative for cytokeratin 19 with few positively stained cells in the basal layer. Arrows point to positively stained cells.

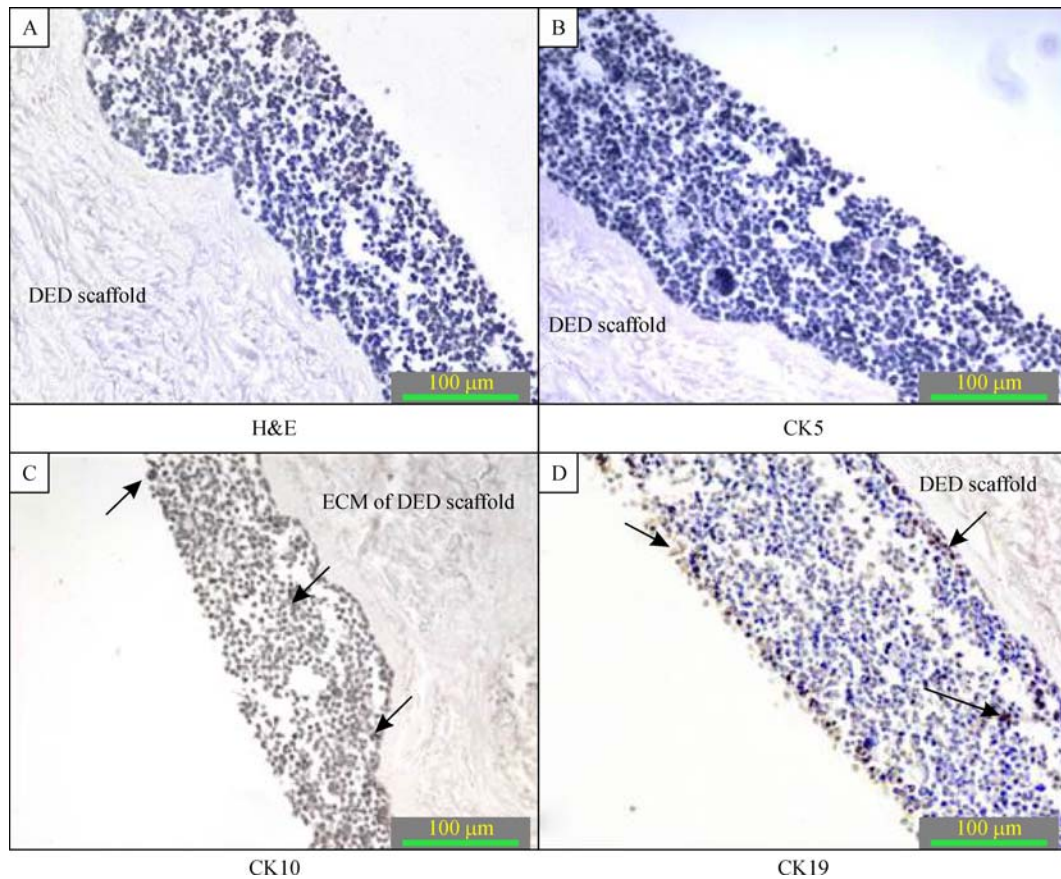


Fig. 3 The C33A 3-D *in vitro* model of cervical cancer at magnification of 200 \times . A: H&E staining; B-D: immunohistochemical staining of the cervical cancer model; B: cytokeratin 5 negatively stained; C: cytokeratin 10 weakly stained; D: Cytokeratin 19 is detected in different layers of the 3-D *in vitro* model. Arrows point to positively stained cells.

Previous studies showed that under conventional culture conditions where keratinocytes were grown in plastic petri dishes or flasks as monolayers, they could only reach an incomplete terminal differentiation and

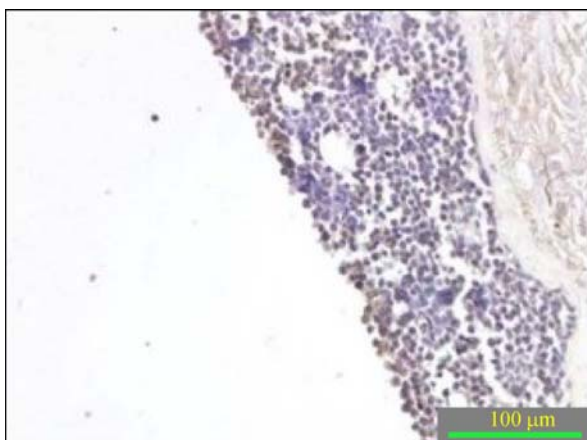


Fig. 4 Immunohistochemical staining of Mad 1 from C33A 3-D *in vitro* model of cervical cancer. Positive stained cells are detected in the upper most differentiated layer of the model. Arrows point to positively stained cells. The image is taken at magnification of 200 \times .

were not able to form multiple cell layers as observed in the human cervix^[6-7,19]. Terminal differentiation of NTERT cells was observed in our multilayered 3-D model of normal cervix. Cultured C33A cancer cells in the proposed model resulted in significant tumor formation in a 3-D fashion, with some differentiated cells found in the most superficial layer. One of the possible reasons for the cells to be able to form tissue like structures in this *in vitro* model proposed in our study is that they are surrounded by a natural environment of human dermis, where nutrients are naturally transported from the underlying media and at the same time most superficial cells are exposed to the liquid air interface.

To evaluate the accuracy of our models, we compared the expression of cytokeratins 5, 10 and 19 in the created 3-D *in vitro* system of normal cervix with the expression of the same cytokeratins seen in normal human cervix *in vivo*. Cytokeratins (CK), also known as keratins (K) constitute the largest intermediate filament protein subgroup and represent a multigene family with more than 20 different types of polypeptides. They are the major structural proteins of epithelial cells and are

Table 2 Different patterns of cytokeratins expression in non-malignant and malignant cervical tissue, where “-” represents negative staining, “+” represents weak positive, “-/+” represents negative or weak positive, “++” represents positive and “+++” represents strongly positive staining.

		Non- malignant tissue					Malignant tissue				
		Neutral-basal		Acidic			Neutral-basal		Acidic		
Types of Keratin	Expression levels of different keratins in stratified squamous epithelia of cervix	Cytokeratin -1	Cytokeratin -5	Cytokeratin -10	Cytokeratin -19	Cytokeratin -14	Cytokeratin -1	Cytokeratin -5	Cytokeratin -10	Cytokeratin -19	Cytokeratin -14
		Non-keratinizing intermediate and superficial cells		+	+	+	-	++	-/+	+	-/+
Basal cells		-	+++	-	++	+++	-/+	+	-/+	+++	+

divided into acidic type I (CK9-CK20) and basic type II (CK1-CK8) cytokeratins^[33-34]. The main function of cytokeratins is to maintain the epithelial cell integrity and to form the cytoskeleton of epithelial cells^[34-35]. Cytokeratins have a specific distribution pattern in epithelial tissues^[36-37]. Changes in the pattern of cytokeratin expression during neoplastic transformation in the cervix have been shown in several studies^[38-40]. **Table 2** represents different patterns of cytokeratin expression in non-malignant and cancerous cervical tissue^[23,34,40-47]. Of particular interest are the changes of cytokeratins 19, 10 and 5.

Cytokeratin 10 is a member of the type I keratin family and it is a suprabasal differentiation marker in ectocervical epithelium restricted to skin and cervix^[43]. Whenever cytokeratin 10 is expressed in invasive carcinomas, it is associated with the grade of differentiation and is expressed in well-differentiated areas and keratin pearls of squamous carcinomas^[23,40,42]. Cytokeratin 19 is an intermediate type I keratin, the smallest known acidic keratin of 40 kDa, that is not paired with a basic keratin in epithelial cells. This keratin is specifically found in the periderm, the transiently superficial layer that envelops the developing epidermis^[34,44]. Cytokeratin 19 has not been detected in the epidermis of adult human skin and its presence is restricted to the outer root sheath of the hair follicle^[45-46]. This cytokeratin is found only in the basal layer of normal epithelium of the cervix and in the full thickness of metaplastic cervical epithelium^[43]. Cytokeratin 5 is a neutral-basic cytokeratin expressed during differentiation of simple and stratified epithelial tissues. Cytokeratin 5 is type II keratin that is expressed mostly in the basal layer of the epidermis with its family member acidic keratin 14^[47]. In this study, cytokeratin 5 and 10 expression in NTERT 3-D *in vitro* model corresponded with staining seen in the normal cervix. Cytokeratin 19 was weakly expressed and found only in

a few NTERT cells, but the localization of these positively stained cells corresponded to its *in vivo* expression in the normal cervix. Moreover, we found that C33A cells when grown in our 3-D system showed cytokeratin distribution characteristic of typical cancerous tissue, where no positivity for cytokeratin 5, and weak or no positivity for cytokeratin 10 was observed, whereas cytokeratin 19 was expressed in different layers. In addition to cytokeratins, another differentiation marker, Mad 1, was used to indicate whether any differentiation process could be observed in our cervical cancer model. The Mad1 differentiation marker is a basic helix-loop-helix-leucine zipper protein that is a transcriptional repressor produced in differentiating cells^[48-49]. Mad1 is detected in differentiating epithelial cells of the suprabasal layers of normal epidermis^[50] and its expression extends from the spinous to superficial layer^[51]. This transcriptional repressor is expressed at low levels in proliferating cells and its expression increases during differentiation of epithelial cells^[50,52]. Mad 1 expression is associated with growth arrest whereas loss of its expression is related with the progression to invasive, poorly-differentiated cancers^[50,52]. With increasing severity of dysplasia, the expression of Mad 1 is progressively shifted to more superficial layers and the immunostaining intensity is reduced^[51]. In this study, the expression of Mad1 confirmed that the partial differentiation process occurred in cancer cells after culturing them in the 3-D conditions.

Advances of the presented 3-D model have enabled direct assembly of normal human keratinocytes, cervical cancer cells and ECM of human de-epidermised dermis to form *in vitro* like cellular models. This promising technique has offered an inexpensive solution to culture a variety of cell types under 3-D culture conditions, also enabled us to manipulate a particular cell of interest and to study specific interactions in a

well-defined environment. The functionality of the proposed model may provide us better insights in human cervical cancers and create effective therapeutic designs for patients with cervical carcinoma.

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