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A comparison of the epithelium to mesenchyme transformation process in cultured embryonic chick neural crest cells and human metastatic cervical cancer cells

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A Comparison of the Epithelium to Mesenchyme Transformation Process in Cultured Embryonic Chick Neural Crest Cells and Human Metastatic Cervical Cancer Cells

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Abstract

Embryonic neural crest cells (NCCs) and metastasizing cancer cells both undergo an epithelium to mesenchyme transformation (EMT) during which they disengage from neighboring cells and basement membrane and actively migrate to new locations. NCCs, small groups of cells that emerge from the neural tube, move directionally through adjacent tissues to reach specific destinations where they differentiate into a diverse array of specific cells and tissues. Caski cells are an established line of metastatic cervical cancer cells that contain HPV. We compared NCCs to Caski cells during EMT with respect to the expression of key proteins related to cell migration—integrin, paxillin, and N-cadherin, and cell shape, size, and motility behavior. Cultured NCCs and Caski cells were immunostained with anti-integrin, -paxillin, and -N-cadherin. Staining patterns were similar for both types of cells. Cells emerging from epithelium and newly motile cells showed higher levels of paxillin and integrin but lower levels of N-cadherin compared to the cells that had not yet emerged. Image analysis showed that the Caski cells became more circular, while the neural crest cells moved with greater velocity and showed greater area and perimeter dynamic change. There was little difference in mean elongation index, breadth, length, or direction changing. Caski cells showed more edge-ruffling behavior and migrated as small continuous patches, pulling away from the monolayer. NCCs more frequently crawled as individuals and elongate clusters. At higher magnification, focal contacts immunostained with anti-integrin were longer and more prominent in Caski cells. The borders of both showed numerous filapodia. Overall the cells have some interesting contrasts and similarities during EMT that give valuable information about the EMT, a change that can influence both the possibility of neural tube defects and the spreading of cancerous cells.

A COMPARISON OF THE EPITHELIAL TO MIGRATORY TRANSFORMATION PROCESS IN CULTURED EMBRYONIC CHICK NEURAL CREST CELLS AND HUMAN METASTATIC CERVICAL CANCER CELLS

Analyzing patterns in nature can give incredible insight about how and why living cells have a specific structure and how they function as they do. It can also give clues about how to overcome life threatening problems by finding solutions for one aspect that might be able to be used on a similar aspect in nature. One unique question of pattern investigates possible similarities and differences in neural crest cells and cervical cancer cells during the epithelial to migratory transformation (EMT). EMT is vitally important for the development of both embryos and metastasizing tumors. It contributes to the progression of a tumor toward malignancy (Fischer and Quinlan, 1998).

During EMT, cells leave an epithelial layer to become mesenchyme cells which can migrate individually. EMT occurs when an epithelialization gene expression program is inactivated, as a mesenchymal gene expression program is activated (Fischer & Quinlan, 1998). It is defined by the loss of intercellular junctions and attainment of cell motility and is associated with cell scattering (Boyer, Valles, & Edme, 2000).

During embryonic development, neural crest cells emerge, originating from the neural folds. They go through the epithelial-mesenchymal transformation and migrate, generating specialized cell types that include skeletal and tissue components of the head, pigment-containing epidermal cells, neurons and glial cells, and adrenal gland cells. The frontal, parietal, exoccipital, and squamosal bones, are all formed by neural crest cells that specialize (Holleville, Quilhac, Bontoux, & Monsoroburg, 2003).

One type of metastasizing tumor cell is Caski cells. These cells are cervical cancer keratinocytes that contain the human papilloma virus (HPV). More than 99% of invasive cervical carcinomas harbor high risk HPV, and high risk HPV is associated with decreased apoptosis and increased cell proliferation (Bernard, 2003).

In embryos and metastasizing tumors, cells must lose their cadherin based-junctions, erode their basement membrane, activate their cytoskeletons, forming and breaking focal contacts which require integrin and paxillin, and change shape and become motile, extending and contracting as they crawl for EMT to occur. Newgreen and Minichiello (1996) found that a loss of cell to cell adhesion through a loss of N-cadherin and adherens junctions induces neural crest cell migration in culture. Cadherin is a primary adhesion molecule of epithelia. It stabilizes epithelial organization because it mediates the junctions that hold the cells together. Therefore when the cells migrate the cadherin deactivates or the levels of cadherin decrease. Irregularity of cadherin expression or function is a common characteristic of epithelial cancers; moreover, cadherin is actually thought to be a tumor suppressor (Casanova, 2002). According to Takeichi (1995), cadherins are expressed in the neural fold of chick embryos and continue to be expressed during the fusion of the neural folds. This suggests that cadherin plays a role in neural tube closure.

Activation of the cytoskeleton during EMT is another connection between neural crest cells and metastasizing cells. The cytoskeleton has three major types of protein polymers – actin filaments, microtubules, and intermediate filaments. Actin filaments account for force generation and contractions within cells that result in a change in shape (Gilbert, 2003). Regulated changes in the organization of the actin cytoskeleton influence motility for both neural crest cells and metastasizing cells (Casanova, 2002).

Both neural crest cells and Caski cells exhibit standard features of motility – extension, adhesion, contraction. For extension cells form shelf-shaped processes known as lamellapodia and fine, spike-like processes referred to as filopodia that extend out to test the immediate environment (Small et al, 2002). Filopodia are probably created by bundling of lamellipodium filaments called fascin and fimbrin, both of which bundle actin filaments in vitro (Bartles, 2000). Adhesion occurs when cells form focal contacts at their membrane interface with the surface. These tiny "cellular feet" depend on integrin, paxillin, and other proteins to link the extra-cellular adhesion sites with the cytoskeleton thus enabling contraction. Focal adhesions have central roles in cell migration and morphogenesis, and they transmit information across the cell membrane that regulates extra-cellular matrix assembly, cell proliferation, differentiation, and death (Geiger et al, 2001). Actin-based cables known as stress fibers that stretch between focal contacts can shorten, pulling up the posterior regions of the cells to produce a contraction.

In addition to cadherins, other key proteins are known to be related to cell migration. For example, integrins function as cell adhesion receptors. Integrins comprise a family of glycoprotein receptors that mediate the adhesion of numerous cell types such as neural crest cells to ECM, and are required for normal development (Kil et al, 1998.) They mediate interactions with the extracellular environment through which the cells migrate. When integrin-mediated cell adhesion occurs, paxillin is phosphorilated which leads to the phosphorilation of their proteins that can alter localized areas of the cytoskeleton to allow for adhesion and movement. Thus paxillin is known as a focal adhesion adapter. The interaction between α_4 integrin and paxillin is thought to lead to enhanced rates of cell migration and

reduced rates of cell spreading, focal adhesion, and stress fiber formation (Ambroise et al, 2002).

The present study compares neural crest cells to Caski cells during epithelium to mesenchyme transformation with respect to the expression of these three key proteins known to be related to cell migration – integrin, paxillin, and N-cadherin. It also evaluates the cell shape, size, and motility behavior for neural crest cells and Caski cells.

Procedure

Obtaining Neural Crest Cells

Fertilized Red Rock hen eggs (Welp Inc., Bancroft, IA) were incubated at 38°C for approximately 48 hours to acquire chick embryos at Hamburger-Hamiliton stages 10 to 11. Using a filter paper ring the embryo was removed and placed in Earl's saline solution. Trunk neural tube (see Figure 1) was microdissected from the embryo and sliced into segments using glass needles.

Figure 1.

Using a plastic pipette segments were removed from the Earl's solution and cultured in 35 mm Falcon *Primaria* dishes (Falcon, Becton-Dickenson, Lincoln Park, NJ) at 38°C in Medium 199 with HEPES modification, pH 7.35 (Sigma, St. Louis, MO), 10% fetal bovine

serum (Sigma, St. Louis, MO), and 5% donor horse serum (Atlanta Biologicals, Norcross, GA) for 24 hours. This allowed for the segments to adhere to the surface of the dish and for the epithelial-mesenchymal transformation to begin with the outward migration of neural crest cells.

Obtaining migratory Caski Cells

Caski (human squamous carcinoma of the cervix uteri) cells were derived from the small bowel mesentery of a forty-year-old Caucasian woman with the human papilloma virus. The cells were grown to nearly confluent monolayers on a 35 mm Falcon *Primaria* dish, and a cell-clear lane was created by scratching the surface with a 0.5 mm pipette tip. Migration of cells into the lane was then observed.

Image Capture and Analysis

To collect motility and morphometry data, images were captured and analyzed using a method similar to the method described by Fuller et al (2002). Each dish was placed on a preheated aluminum block stage warmer on an Olympus CK2 inverted phase contrast microscope equipped with a Hitach HVC20 3 CCD video camera. The microscope stage was maintained at 38°C so that sequences of phase contrast images could be captured of migrating cells for both neural crest cells and Caski cells using a 10X objective. Images were captured every 3 minutes for one hour and saved.

The images were analyzed using Optimas and Excel programs. Five border Caski cells or five border neural crest cells depending on the dish being analyzed were selected. The cells chosen had to be visible in all 20 frames and recognizable as a separate cell. Each cell was assigned its own label, and the cell borders were traced by hand using the computer mouse for all 20 frames. Image analysis using Optimas and statistical analysis performed in

Microsoft Excel were used to acquire quantitative values for area, perimeter, maximum length, maximum width, circularity, elongation, velocity, dynamic perimeter change, dynamic area change, and direction change. According to Optimas, circularity is the ratio of the perimeter length squared, divided by the area. The longest axis of the outlined area is the maximum length, and the greatest distance measured perpendicular to the longest axis is the maximum width. The mean and median values of each parameter for each frame were calculated in Excel. The elongation index was also calculated using the spread sheet data by dividing the maximum width of a cell by the maximum length of a cell. To find the dynamic perimeter change and dynamic area change, the absolute value of the difference in perimeter or area for each cell from frame to frame was divided by the time period.

Significance was tested for using a t-test (two-sample assuming unequal variances) between the results for the Caski cells and the results for the neural crest cells with a significance level of .05.

Immunostaining

Neural crest cells migrating out from the neural tube epithelium and Caski cells migrating out from the epithelial monolayer into the cell-clear lane were fixed and processed for imuunolocalization. To do this, the cultured explants of the trunk neural tube were washed in phosphate buffered saline (PBS) and fixed in 20% DMSO in methanol for 30 minutes. The cells were washed again in PBS and 0.1% Triton X-100 in PBS was added to be used as a permeabilizing buffer. The cells were then blocked and immunostained. The cells were washed with PBS, and monoclonal antibodies to integrin, paxillin, and N-cadherin were used to reveal red staining in the cells, which were counterstained with hematoxcylin. The control dishes were processed identically except that the primary antibody was omitted. They

were mounted in GVA Mount (Zymed, Inc., S. San Francisco, CA) and images of the explants were obtained using an Olympus BX40 microscope equipped with an Optronics CEI 750 video camera (Optronics Engineering, Goleta, CA). Images were taken with a 40X objective and bright field optics.

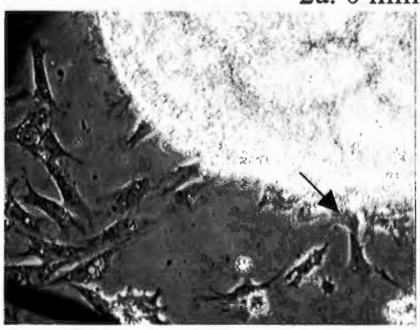
Results

Image Capture

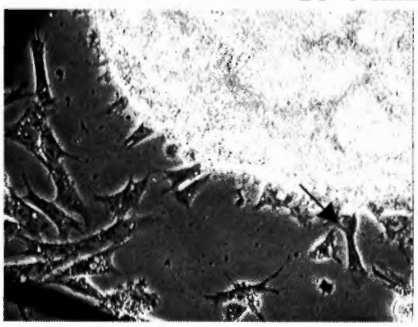
Sequential time-lapse images of neural crests cells and Caski were taken during the epithelial to mesenchymal transformation, and samples of the images are illustrated in Figures 1 and 2.

Neural Crest Cells

2a. 0 min.



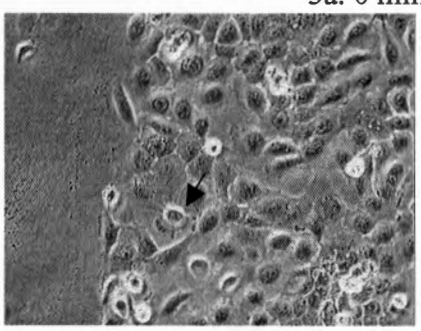
2b. 3 min.



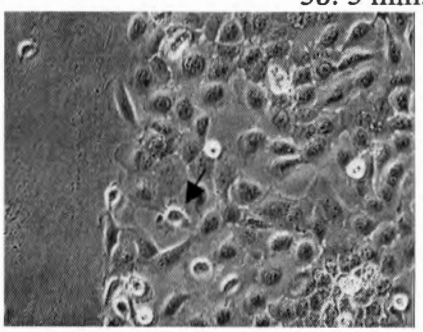
2c. 9 min.

Ca Ski Cells

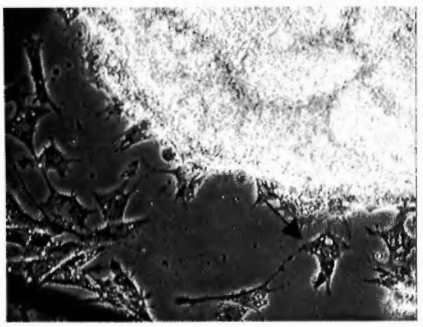
3a. 0 min.

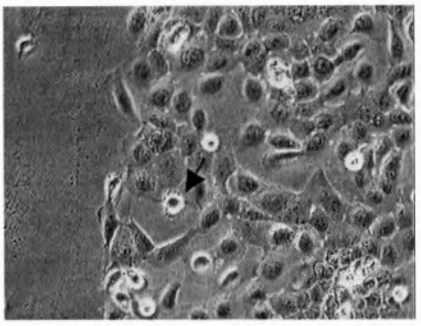


3b. 3 min.

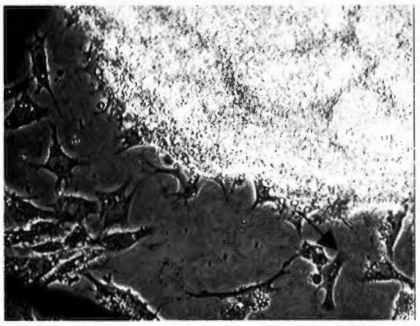


3c. 9 min.

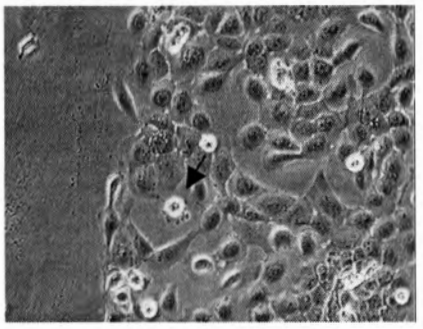




2d. 15 min.



3d. 15 min.



Figures 2 & 3. Emergence of cells from the epithelial source.
Images were taken with in phase contrast optics with the 10X objective.

In Figures 2a through 2d, the neural crest cells can be observed emerging from the neural tube epithelium mainly as individual cells that have irregular and dynamically changing shapes. An example of a cell leaving the neural tube and changing shape as it migrates was indicated in all four of these figures. The Caski cells are seen in Figures 3a through 3d as a nearly confluent monolayer, with the cell-free lane on the left side of the images. With time many of the cells slowly spread outward as small patches of contiguous, flat cells into the lane. Some, however, move as individuals, usually with a prominent ruffling edge leading the cell such as one particular cell pointed out in each image.

Image Analysis

Image analysis measurements of cells emerging from epithelium in culture are given in Tables 1 and 2.

	Neural Crest Cells	Ca Ski Cells	Significance (<P)
Mean Cell Area (μm^2)	523	348	10^{-4}
Mean Cell Perimeter (μm^2)	169	95	10^{-9}
Mean Circularity	60	27	10^{-10}
Mean Maximum Length (μm)	43	31	10^{-8}
Mean Cell Breadth (μm)	29	19	10^{-7}
Elongation Index (Breadth/Length)	0.67	0.61	10^{-1}

Table 1.
$n \geq 10$ cells through 20 frames of images

	Neural Crest Cells	Ca Ski Cells	Significance (<P)
Area Change per Minute (μm2)	126	44	10^{-6}
Perimeter Change per Minute (μm)	46	14	10^{-7}
Direction Changing	41	39	10^{-1}
Velocity (μm/minute)	0.035	0.013	10^{-2}

Table 2. Motility Parameters

The measurements are derived from image frames captured every three minutes during 60 minutes on a 37°C microscope stage using phase contrast optics. Values shown are the means of 5 cells analyzed through 20 frames for each parameter. The six morphometry parameters - area, perimeter, circularity, maximum length, cell breadth, and elongation index - are addressed in Table 1. For the elongation index the more elongated cells (i.e., less rounded), show a lower the value, and are typically more migratory. A significant difference [$p < .05$] was found between neural crest cells and Caski cells for mean cell area, mean perimeter, mean circularity, mean maximum length, and mean cell breadth with neural crest

cells having a higher value in for each comparison. The neural crest cells also had a greater elongation, but not enough to be considered significant.

Three motility parameters – area change per minute, perimeter change per minute, and direction changing - were also compared between neural crest cells and Caski cells (see Table 2). Area and perimeter change were the amount of positive change (i.e., cell expansion) or negative change (i.e., cell shrinkage) in these measurements observed per minute, and may be viewed as a measure of the amount of dynamic activity of the cells. The area and perimeter change per minute was significantly higher for neural crest cells compared to Caski cells [$p < .05$]. There was no significant difference for direction changing, but the neural crest cells had a significantly higher velocity than the Caski cells.

Immunostaining

Immunolocalization of the focal adhesion protein paxillin in emerging cells was captured using an Optronics video camera for both neural crest cells and Caski cells. Figures 4a and 4b are examples of these images.

Figure 4a. Paxillin--Neural Crest Cells

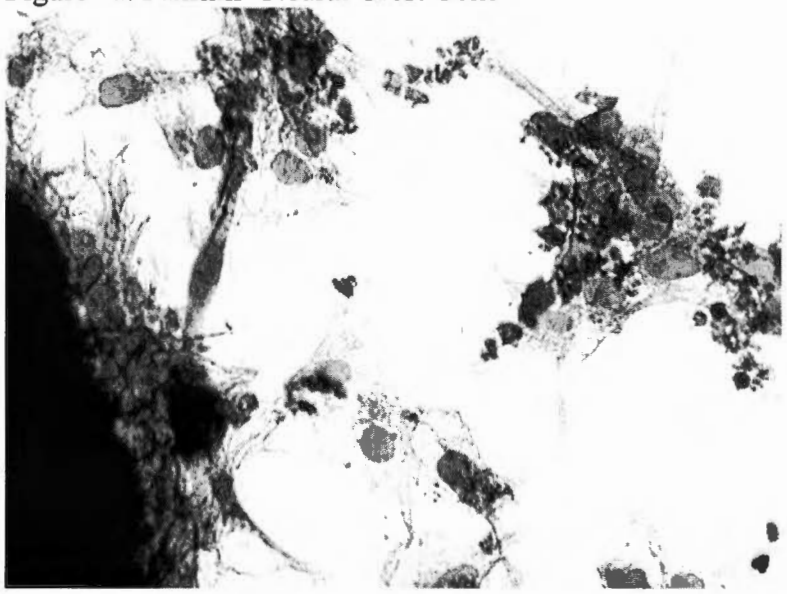
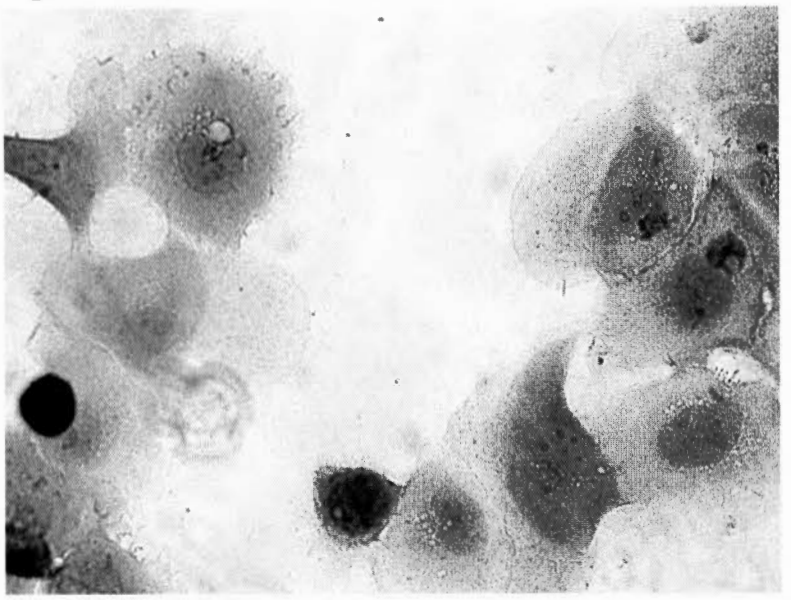


Figure 4b. Paxillin--Caski Cells



Figures 4a & 4b. Immunolocalization of focal adhesion protein paxillin in emerging cells of each type.
Images taken with 40X objective, bright field optics.

Paxillin was observed in flat and spread neural crest cells that had migrated away from the neural tube fragment that was located in the lower left corner of the image (Figure 4a). In these cells paxillin was visible in focal contacts as tiny spots and near cell peripheries more diffusely. In Caski cells the pattern was similar but the stained focal contacts were longer and there was more diffuse staining around the nucleus (Figure 4b). In both cell types the amount of paxillin varied considerably from cell to cell. Similar results were found for integrin. Cells emerging from the epithelial showed higher amounts of integrin than those cells that were still considered to be epithelium for both the neural crest cells and the Caski cells, but the focal contacts were longer and more prominent for the Caski cells. In contrast lower levels of N-cadhedrin were observed in emerging cells compared to those cells in epithelium.

Discussion

Integrin and paxillin tended to be similarly distributed in both types of cells, but focal contacts immunostained with anti-integrin and anti-paxillin were longer and more prominent in Caski cells. Cells emerging from epithelium and newly independent, motile cells showed higher levels of paxillin and integrin but lower levels of N-cadherin compared to the cells that had not yet emerged . Such findings are supported by past research. Down regulation of cadherin may directly result in mesoderm migration and can also account for certain types of cancers (Boyer, Valles, & Edme, 2002). A large amount of research supports the idea that integrin would show higher levels in motile cells. Shoenwaelder and Burridge (1999) suggested that integrins link the extra-cellular matrix and actin cytoskeleton, acting as signal transducers from the extra-cellular matrix. Integrins are often clustered into specialized adhesive structures such as focal adhesions and focal complexes (1999). Initial contact of the lamellipodia with the extra-cellular matrix results in the formation of integrins localized at the tip of the protrusion (Bailly, 2003). Immunolocalization of integrin was observed in these adhesive structures for both neural crest cells and Caski cells stained with integrin.

Image analysis showed that the Caski cells became more circular, while the neural crest cells moved with greater velocity and showed greater area and perimeter dynamic change. There was little difference in mean elongation index, breadth, length, or direction changing. Caski cells showed more edge-ruffling behavior and migrated as small continuous patches, pulling away from the monolayer, while neural crest cells more frequently crawled as individuals and elongated clusters. The borders of both showed numerous filapodia.

Possible limitations for this study would be a small sample size currently analyzed. Some of the images captured have not yet been analyzed and such an analysis might give a

more thorough picture. In addition more images of the immunostained cells need to be taken., particularly of those stained with N-cadherin. It would also be beneficial to look at other molecules involved in the epithelium to mesenchyme transition such as fibronectin to see if similarities are found there also. Another interesting aspect to look at would be how integrated the molecules are with each other or if they work independently.

Overall the cells have some interesting contrasts and similarities during EMT that give valuable information about the EMT, a change that can influence both the possibility of neural tube defects and the spreading of cancerous cells. Continued comparison of neural crest cells and Caski cells would be beneficial because both can have debilitating effects. If the neural fold does not fuse properly facial disfigurations, skin discolorations, and nervous system impairments are possible effects of such a malfunction. Metastasizing cancer cells can be deadly and very detrimental to a person's health. It is evident that the epithelial to mesenchyme transformation is vitally important in both cases, and if a preventive measure and a treatment could be found to help the EMT run smoothly in one, a similar measure could be used for the other if motility and other factors are similar enough. Examining patterns such as this could lead to medical breakthroughs.

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