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Ectopic expression of HNF4α in Het1A cells induces an invasive phenotype

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ABSTRACT

Barrett's oesophagus (BO) is a pathological condition in which the squamous epithelium of the distal oesophagus is replaced by an intestinal-like columnar epithelium originating from the gastric cardia. Several somatic mutations contribute to the intestinal-like metaplasia. Once these have occurred in a single cell, it will be unable to expand further unless the altered cell can colonise the surrounding squamous epithelium of the oesophagus. The mechanisms by which this happens are still unknown. Here we have established an *in vitro* system for examining the competitive behaviour of two epithelia. We find that when an oesophageal epithelium model (Het1A cells) is confronted by an intestinal epithelium model (Caco-2 cells), the intestinal cells expand into the oesophageal domain. In this case the boundary involves overgrowth by the Caco-2 cells and the formation of isolated colonies. Two key transcription factors, normally involved in intestinal development, HNF4α and CDX2, are both expressed in BO. We examined the competitive ability of Het1A cells stably expressing HNF4α or CDX2 and placed in confrontation with unmodified Het1A cells. The key result is that stable expression of HNF4α, but not CDX2, increased the ability of the cells to migrate and push into the unmodified Het1A domain. In this situation the boundary between the cell types is a sharp one, as is normally seen in BO. The experiments were conducted using a variety of extracellular substrates, which all tended to increase the cell migration compared to uncoated plastic. These data provide evidence that HNF4α expression could have a potential role in the competitive spread of BO into the oesophagus as HNF4α increases the ability of cells to invade into the adjacent stratified squamous epithelium, thus enabling a single mutant cell eventually to generate a macroscopic patch of metaplasia.

1. Introduction

Barrett's oesophagus (BO) is a pathological condition in which the squamous epithelium of the distal oesophagus is replaced by intestinal-like columnar epithelium and occurs in response to acid-biliary reflux from the stomach. The study of BO is important because it is the only known precursor to oesophageal adenocarcinoma (Dunbar et al., 2016; Peters et al., 2019; Zhang et al., 2021), a disease with a poor prognosis and five-year survival of only 10-22% (Kauppila et al., 2018).

The cellular origin of BO has been controversial but recent studies involving single-cell transcriptomics and chromatin analysis show that it comes from the gastric cardia, from which tongues of columnar cells extend into the distal oesophagus. This is accompanied by metaplasia to a mixed gastric-intestinal phenotype (Nowicki-Osuch et al., 2021; Singh et al., 2021). The formation of BO involves many somatic mutations (Leedham et al., 2008; Wright, 2018), among which are the expression of two key transcription factors: Hepatocyte Nuclear Factor 4α (HNF4α), a nuclear receptor type factor, and CDX2, a homeodomain protein, both of which are involved in the regulation of intestinal gene expression (Chen et al., 2011). Introduction of the genes for these transcription factors into gastric organoids can cause a transformation into mixed phenotype cells similar to BO (Singh et al., 2022). Moreover, the developmental pathways of intestinal cell development in gastric intestinal metaplasia and in BO appear very similar (Nowicki-Osuch et al., 2023).

Although BO may originate from individual cells with appropriate somatic mutations, a macroscopic patch of metaplasia cannot develop unless the Barrett's epithelium can expand into the oesophagus. The

Abbreviations: BO, Barrett's oesophagus; ECM, extracellular matrix.

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mechanisms by which this happens are still unknown. However, there must be an element of competition between columnar and squamous cell populations resulting in preferential expansion of the columnar epithelium. BO progression has been described as an evolutionary process by which heterogeneous populations of clones compete with each other for space and resources (Merlo et al., 2011).

In vitro co-culture assays have previously been used to study the mechanisms underlying competitive cell-cell interactions (Moitrier et al., 2019; Porazinski et al., 2016; Rodriguez-Franco et al., 2017). Here, we introduce a new type of *in vitro* system which enables the confrontation and continued culture of two epithelia. As a model for oesophageal epithelium, we used the human cell line Het1A. This was originally isolated from an oesophageal biopsy in 1986 and is transformed with SV40 large T to facilitate continued replication (Stoner et al., 1991). It has an epithelial morphology with tonofilaments, desmosomes and cytokeratin expression. However, there are some limitations in using this cell line as it has lost some squamous characteristics such as E-cadherin, cytokeratins 5/6, and DNp63 (Underwood et al., 2010). For an intestinal model we used the human Caco-2 cell line. This was originally isolated from a colon adenocarcinoma (Hidalgo et al., 1989). In confluent monolayers Caco-2 cells become polarised, acquiring a distinctive apical brush border with microvilli, then form tight junctions and express enzymes characteristic of enterocytes, such as lactase, SI and aminopeptidase (Lea, 2015). Due to these properties, Caco-2 cells have been used as a surrogate *in vitro* enterocyte model for studying BO (Marsman et al., 2007). However, as for Het1A cells, Caco-2 cells also exhibit limitations. In the context of the cell type of origin for BO, using enterocyte-like cells may reflect a latter stage of the metaplastic process (Biswas et al., 2018), rather than using a progenitor from the gastric cardia. Caco-2 cells also require some time to differentiate and do not exhibit the full repertoire of differentiated enterocyte properties (Sun et al., 2008).

In this study we first investigated whether Caco-2 cells have a natural tendency to overgrow Het1A cells. As Caco-2 cells express high levels of HNF4α and CDX2 we examined whether expression of either HNF4α or CDX2 would confer the ability to overgrow another epithelium. Each gene was separately overexpressed in Het1A cells, and the effect examined in competition with unmodified Het1A cells. Given the role of the extracellular matrix (ECM) in growth and development (Rozario and DeSimone, 2010; Walma and Yamada, 2020) and the changes in components of the ECM during development of Barrett's and progression to dysplasia and adenocarcinoma (Leppänen et al., 2017; Rickelt et al., 2022), the experiments were carried out both on plastic and on different ECM substrata with Matrigel as a more classical basement membrane; and collagen and fibronectin, which are highly associated with oesophageal tumorigenesis and patients' prognosis (Li et al., 2019; Sudo et al., 2013).

In summary, our data show four conclusions. First, intestinal (Caco-2) epithelium will displace oesophageal-like (Het1A) epithelium. Second, the boundary between these cell types is not sharp but involves overgrowth and colony formation. Third, that HNF4α, but not CDX2, expressed in Het1A cells confers a competitive advantage enabling a displacement of unmodified Het1A cells. In this case the boundary between the epithelia is sharp, as observed in patients with BO. Finally, the effects occur on all substrates but are most marked on fibronectin or Matrigel.

2. Materials and methods

2.1. Cell lines

The immortalised human oesophageal squamous cell line, Het1A, was acquired from the American Type Culture Collection (ATCC, Middlesex, UK). The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, Porton Down, UK). The stable Het1A Cell Line expressing HNF4α clone 1 (Het1A-HNF4α) was previously generated as described by Colleypriest et al. (2017). The lentiviral vector (pL-S-HNF4α-I-EGFP) used to generate the cell line encodes two sequences: the transcription factor, HNF4α, and an enhanced green fluorescence protein (EGFP).

The Het1A-CDX and Het1A-RFP control cells were generated by lentiviral transduction of Het1A cells. To this end, the Precision LentiORF Viral Particle Starter Kit (Horizon Discovery, OHS5836-EG1045) was used according to the manufacturer's instructions. The Lentiviral ORF constructs include the CDX2 gene together with a GFP reporter as a surrogate to track protein expression or RFP for control cells. At 48 h post-transduction, Het1A cells were microscopically examined for the presence of GFP/RFP expression. To create a stable cell line, Blasticidin S (4 μg/ml, Gibco, A1113902) selection was used. After 10 days of selection, we enriched GFP-expressing cells by FACS (BD FACSAria III).

2.2. Culture of cell lines

All cell lines were maintained in low-glucose Dulbecco's Modified Eagle's (DMEM, Sigma, D6046) supplemented with 10% FBS (Gibco, F7524), Penicillin/Streptomycin (100x, Gibco, 15140122), L-Glutamine (100x, Sigma, G7513), Non-Essential Amino Acids (100x, Gibco, 12084947) and Sodium Pyruvate (100x, Gibco, 12539059), which we call "DMEM complete". Cell cultures were maintained in a humidified atmosphere at 37 °C with 5% CO_2 and the medium was replenished every 2-3 days.

2.3. Extracellular matrix protein coatings

Cell culture plates were used uncoated or coated with ECM proteins. Bovine plasma fibronectin (Gibco 33010018) coated surfaces were prepared through overnight evaporation at a final concentration of 4 μg/cm^2 in sterile PBS. Collagen I rat tail (4 μg/cm^2, Gibco, A1048301) was diluted in 20 mM acetic acid and cell culture plates were coated at room temperature for 1 h. Prior to cell seeding, the solution was aspirated from the wells and rinsed twice with sterile PBS. Matrigel (Corning, 354277) was prepared in advance by dissolving one frozen (-80 °C) Matrigel aliquot in 10 ml of ice-cold DMEM complete. 100 μl/24 well were added and incubated at room temperature for 1 h before adding the cells. For a 24-well cell culture plate, 100 μl of each ECM coating solution was added per well.

2.4. In vitro migration/competition assay

Cell migration and competition were studied using 2 well culture inserts (Ibidi, 81176), which consist of two wells separated by a wall of 0.5 mm (Fig. 1A). Prior to the beginning of each experiment, inserts were placed in a 4-well cell culture plate. With a black marker, several lines were drawn across the bottom of the wells. This was used as a point of reference to visualize cells under the microscope. 3.5-4 x 10^4 cells were seeded into each chamber in 70 μl of culture medium and allowed to attach until they reached confluency. The inserts were removed carefully with sterile tweezers. To reduce the effect of cell proliferation during cell migration, cells were serum starved in medium for 12 h before commencing the experiment. The medium was aspirated and then replaced with DMEM complete. Images were taken at 0, 24 and 48 h on an EVOS FL microscope (Invitrogen) and the gap between the migrating cell areas measured using the open-source software ImageJ/Fiji in combination with a published wound healing plugin (Suarez-Arnedo et al., 2020). For non-competitive migration assays in the first 48 h the gap at 0 h represents 100% of the area. After contact, the movement of the boundary between the cell populations was tracked for up to 20 days by analysing both brightfield and fluorescent images, which allows tracking of the boundary. In these assays -25% and +25% represent the starting position of the migration fronts.

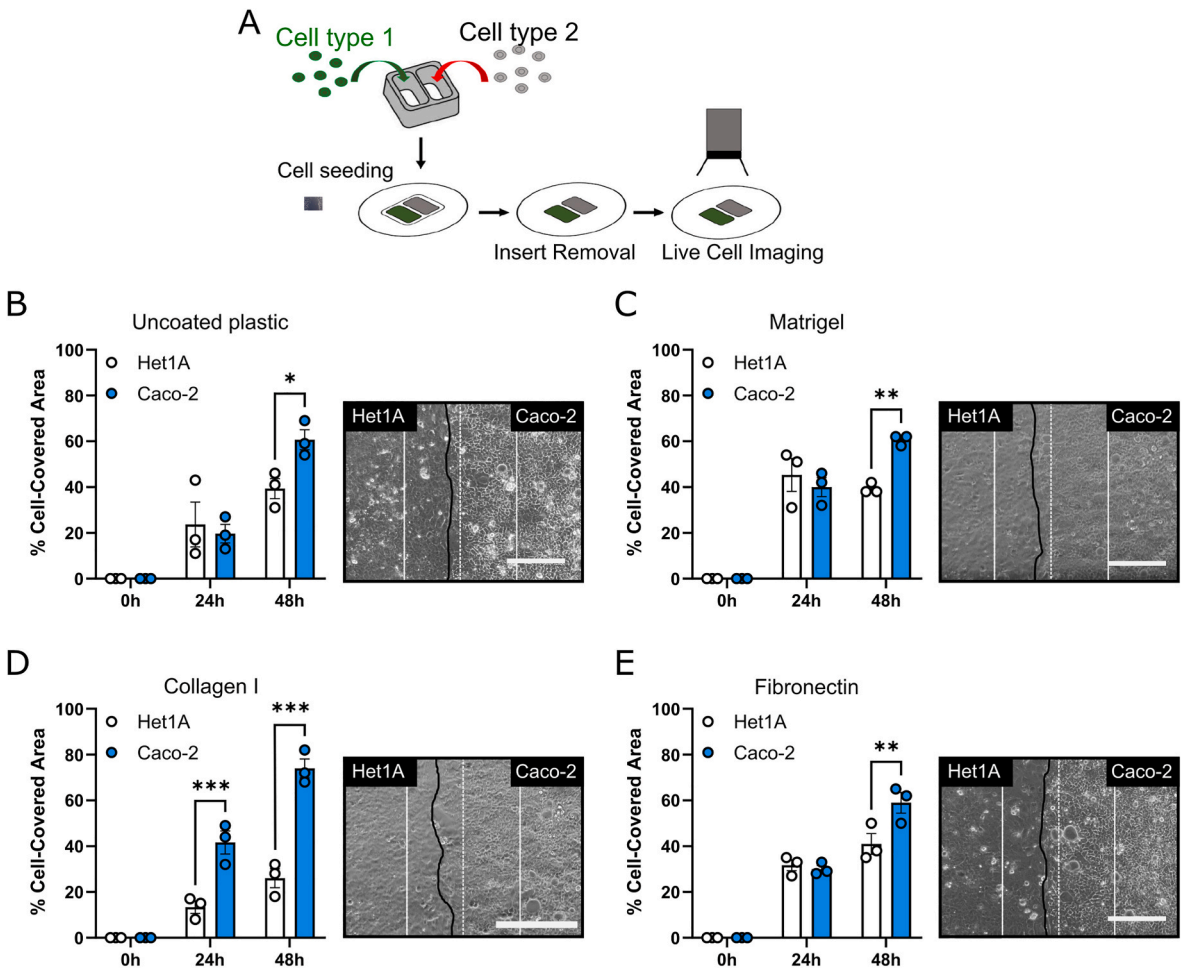

Fig. 1. Caco-2 cells cover area faster in heterotypic cell migration assay. (A) Experimental setup: The two cell populations are seeded into separate wells of a silicone chamber, which is removed when they reached confluency. The movement of the epithelial cell fronts is recorded both before and after contact with the other cell population. Cell populations are distinguished either by morphology or GFP expression. (B-E) Caco-2 cells cover the area faster than Het1A cells. Quantitative analysis of migration of Het1A and Caco-2 cells on (B) uncoated plastic, (C) Matrigel, (D) collagen I and (D) Fibronectin. Graphs show percentage area covered by each cell type over 48 h. Data are presented as percentages of cell-covered area at various time points (0, 24 and 48 h). Bars represent the mean ± SEM of three independent experiments (*P <0.05, **P <0.01 and ***P <0.001, two-way ANOVA with Šidák's post-hoc test). Representative phase contrast images of migration (marked by black lines, original wound marked by white lines and dashed line shows 50%, scale bar, 200 μm).

2.5. Immunofluorescence analysis

For immunofluorescence experiments 2-well inserts were placed on heat sterilised Ø 11-13 mm glass coverslips and cells cultured as described above. Cells were fixed with cold (4 °C) 4% paraformaldehyde (PFA) (Gibco, 15670799) diluted in PBS for 15-20 min at room temperature. PFA-fixed cultures were permeabilised by adding 0.1% (v/v) Triton-X100 (Sigma-Aldrich, 93443) in PBS for 20 min at room temperature. Nonspecific binding sites were blocked by incubating all cultures in a blocking buffer (Roche 11096176001, 2% in PBS) for 1-3 h at room temperature. After the incubation, the blocking buffer was removed, and primary antibodies (see Supplementary Table 1) at desired dilutions were added and incubated overnight at 4 °C. The next day, cultures were washed three times (5 min each) with PBS. After the final wash, cultures were incubated with secondary antibodies in the dark for 1-2 h at room temperature followed by washing twice in PBS (5 min each). To stain the cell nuclei, fixed cultures were incubated with 0.5 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI, Sigma, D9564) in PBS for 5-10 min at room temperature. Lastly, the DAPI solution was removed, and coverslips were directly mounted onto microscopy slides using fluorescence mounting medium (DAKO, Agilent S3023). Images were taken on a Zeiss LSM880 Inverted Confocal Microscope and process by the Zen Black software (v2.3).

2.6. Western blotting

Cells were lysed in 1x RIPA buffer, sonicated and boiled, prior to western blotting. Cell lysates were subject to immunoblotting with the Bio-Rad Western blot system according to the manufacturer's protocol. Blots were blocked with 5% BSA before incubation with primary and secondary antibodies at the dilutions indicated (Supplementary Table 1).

2.7. Statistical analysis

Graphs were drawn and statistical analyses were performed with GraphPad Prism software version 9 (GraphPad Software, San Diego, CA, USA). The two-way ANOVA, (or two-factor ANOVA), was used to determine the statistical significance observed between different groups that have been split on two factors (or independent variables). Post hoc tests were carried out by using the GraphPad Prism Software. Differences between groups were considered to be significant at a P value of <0.05.

3. Results

3.1. Competition between Het1A and Caco-2

Initially we were interested to determine what happens when an oesophageal-like epithelium directly confronts an intestinal one. To model the oesophageal epithelium, we used the Het1A cell line and to represent intestinal epithelium we used the Caco-2 cell line. Investigation of competition between these epithelia was performed by creating confluent sheets of Het1A cells and of Caco-2 cells and then allowing them to come into contact (Fig. 1A). Due to the morphological differences between the two cell types, it was easy to distinguish them under the microscope, with Caco-2 cells forming a more distinct cylindrical cobble stone layer. Experiments were performed on uncoated plastic, and on dishes coated with Matrigel, representing a basement membrane in healthy tissue, in comparison to collagen I or fibronectin, which are often upregulated in oesophageal cancer (Li et al., 2019; Sudo et al., 2013).

Growing cells in 2 well culture inserts allowed the creation of two epithelial patches formed out of either Het1A or Caco-2 cells, separated by a 0.5 mm gap. Initially both cell populations start to expand across the gap, the rate of movement of the free edge depends on the cell type and on the substrate applied to the dish. Once the epithelia are in contact, which usually takes about 48 h, the phase of competition starts. The position of the epithelia was recorded daily until contact and then every 5 days up to a maximum of 20 days.

Results from the early phase during which the cell sheets move towards one another are shown in Fig. 1B and C. The Caco-2 cells move somewhat faster such that they occupy about 60% of the former gap by the time of contact, with the Het1A occupying about 40%. This is consistent with the movement of the monotypic cultures of the two cell models alone (Supplementary Figs. 1A and B). In the heterotypic culture the Caco-2 advantage is more pronounced on the collagen substrate with on average a 74% coverage after 48h. Next, we investigated the change of the migration front after the cells met. During the longer phase (20 days) of competition between the cell sheets in contact, there is a continued displacement of the boundary in favour of Caco-2, on plastic (Fig. 2A and B), Matrigel (Fig. 2C) and fibronectin (Fig. 2E), but not on collagen where the boundary stagnated (Fig. 2D). A combination of the short-term and long-term data is also shown in Supplementary Fig. 2. Not surprisingly the rate of movement of the boundary in the competition situation is much slower than when it was expanding over the substrate with a free edge. The rate of cell division in the standard medium is higher for Caco-2 cells than for Het1A in a monotypic culture. As the competition takes place under conditions of confluence of both cell sheets the percentage Ki67 and total cell numbers were further investigated. While there was no difference in the percentage of Ki67 cells at the point of confluency, Caco-2 cells had reached over time a higher cell density (Supplementary Figs. 1C–E), and it might be considered that this proliferative advantage provides a sufficient explanation for its overall advantage.

Initially we supposed that the two cell sheets meet head on and that one pushes the other sideways. However, when the junction region was examined from the side as a confocal microscope Z-stack, it became clear that this was not the case. In fact, the Caco-2 cells migrate over the top of the Het1A cells. Also, the Caco-2 sheet can break up leading to formation of small proliferating colonies on top of the Het1A sheet (Fig. 3).

3.2. Effect of HNF4α and CDX2 on migratory and competitive behaviour

Two important transcription factors in BO are HNF4α and CDX2. *HNF4α* is not expressed in the normal human oesophagus but is upregulated in BO (Green et al., 2014; Piessen et al., 2007; Wang et al., 2009). Similarly, *CDX2* has been described as marker of epithelial intestinal differentiation in BO (Colleypriest et al., 2010; Groisman et al., 2004). While both factors are highly expressed in Caco-2 cells they are, as expected, absent in Het1A cells (Supplementary Figs. 3A and B). To assess whether expression of *HNF4α* or *CDX2* could confer a competitive

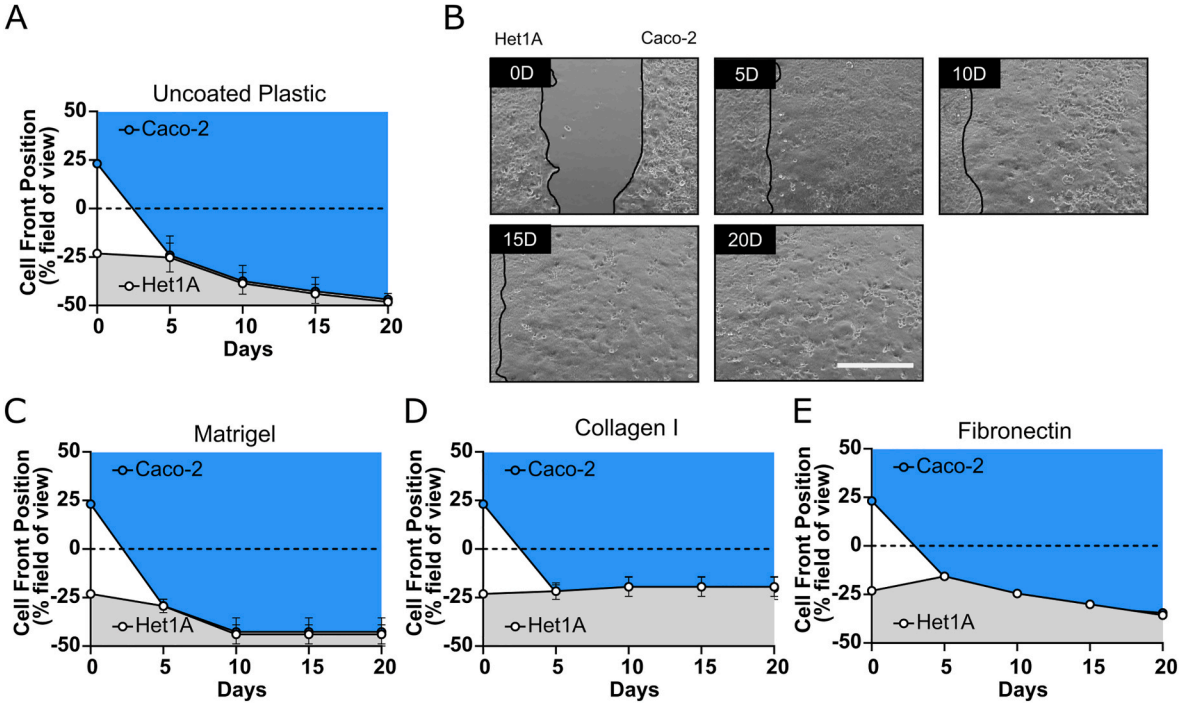

Fig. 2. Caco-2 cells push into the Het1A domain. (A) Het1A and Caco-2 cell front positions at 0, 5, 10, 15 and 20 days cultured on uncoated cell culture plastic. Bars represent the mean ± SEM of three independent experiments. (B) Representative phase contrast images of movement of confluent Caco-2 cells into the Het1A domain on uncoated cell culture plastic. The vertical black wavy lines mark the front position of Caco-2 cells (scale bar, 400 μm). (C) Het1A and Caco-2 cell front positions at 0, 5, 10, 15 and 20 days cultured on Matrigel, collagen I or fibronectin. Bars represent the mean ± SEM of three independent experiments. -25 and + 25% represent the starting position of the migration fronts.

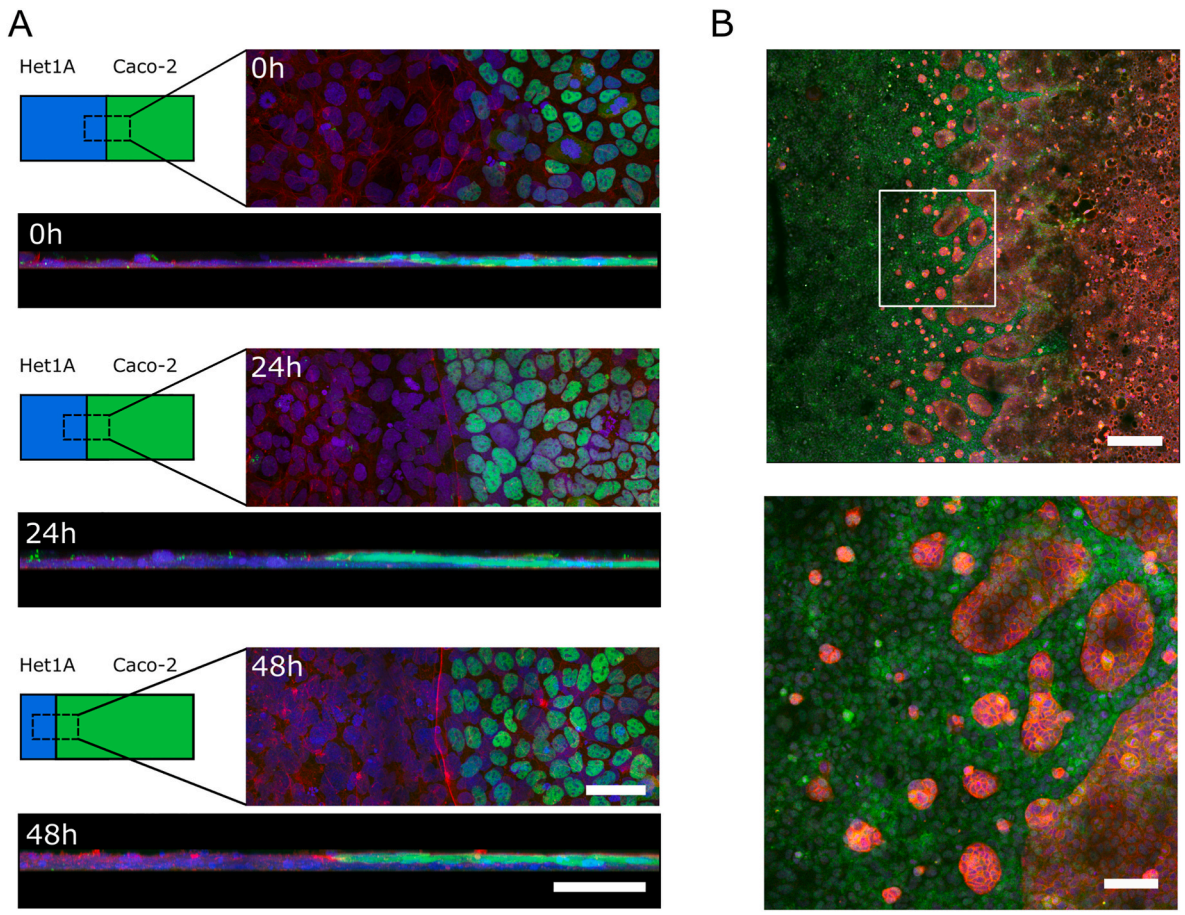

Fig. 3. Caco-2 cells migrate above the monolayer of Het1A cells after contact. (A) Boundary between Het1A cells and Caco-2 cells stained for HNF4α (green), Phalloidin (red) and DAPI (blue). Co-cultures were fixed at 0, 24 and 48 h after contact. Representative images of maximum intensity projection of Z-stack section and orthogonal view showing Caco-2 cells growing on top of the Het1A monolayer (scale bars, 50 μm). (B) Confocal image of Caco-2 colonies in the Het1A domain stained for N-cadherin (green, Het1A cells), E-cadherin (red, Caco-2 cells), and DAPI (blue). Bottom image represents a 3.5x magnification of the highlighted field of view (scale bars: top image 350 μm, bottom image 100 μm).

advantage, the study was expanded to Het1A cells overexpressing either transcription factor.

To achieve this, two sublines of the Het1A cells expressing either *HNF4α* or *CDX2* were used. The stable Het1A cell line expressing *HNF4α* was previously generated by Colleypriest et al. (2017). A new stable cell line expressing *CDX2*, together with a control cell model expressing RFP, was made by lentiviral transduction of Het1A as described in the Methods. Expression of HNF4α and CDX2 respectively was confirmed by Western blotting (Supplementary Figs. 3A and B). Het1A- HNF4α and Het1A-CDX2 cell lines also express EGFP or GFP which enables the cell type boundary to be tracked in real time by fluorescence microscopy.

To obtain baseline information on migration we compared the behaviour of Het1A with Het1A-HNF4α and Het1A-CDX2, in each case putting the same cells in the two wells to create a symmetrical situation. Similar to the study of Caco2 cells, migration assays were performed on plastic that was either uncoated or coated with Matrigel, collagen I or fibronectin, and migration of the cell sheets was measured and expressed as the percentage of the area covered after 24 and 48 h (Supplementary Fig.1B and 3C,D). We found that migration of all three cell types was faster on all the substrates than on uncoated plastic. The two modified cell lines Het1A-HNF4α and Het1A-CDX2 both migrated faster than the unmodified Het1A, in all four culture conditions. Despite this faster migration of the modified cell lines and in contrast to Caco-2 cells, no difference in proliferation of the confluent sheets was observed, measured by cell counting over time, and DAPI counts and percentage Ki67 of confluent layers (Supplementary Figs. 3E–J).

Next, we investigated the competitive behaviour of Het1A-HNF4α and Het1A cells in co-culture. As before, experiments were conducted on uncoated plastic, Matrigel, collagen I and fibronectin. To make the experiment more relevant to *in vivo* conditions, the length of the co-culture assays was increased to 20 days and data collected every 5 days. During the initial period of migration as a free edge, the modified cell line moved faster than the unmodified Het1A (Fig. 4), just as it had in the experiments on single cell types (Supplementary Fig. 3C). Once the sheets had made contact, the Het1A-HNF4α cell population was able to push into the wild-type Het1A cell domain on uncoated plastic (Fig. 4A) as well as on all three substrates (Fig. 4B-D).

We were interested to know the nature of the junction between the two cell populations. Did it represent a migration of one cell type over the other, as was the case with the Caco-2 cells, or a more robust boundary? To answer this, Z-stack sections obtained by confocal microscopy of the boundary on uncoated plastic were taken. This revealed that the two cell populations remain coherent after contact, and that the junction is an abrupt boundary between them (Fig. 5). These observations also revealed that both cell populations become multilayered by 48 h.

To examine whether CDX2 has a similar impact on competitive behaviour we compared Het1A and Het1A-CDX2 cells under the same conditions as above. In this case we found that there was no significant difference in the position of the boundary with the unmodified cells. This was true on uncoated plastic and on the three substrates. Both cell lines filled the gap equally between 0 and 24 h. Following cell contact,

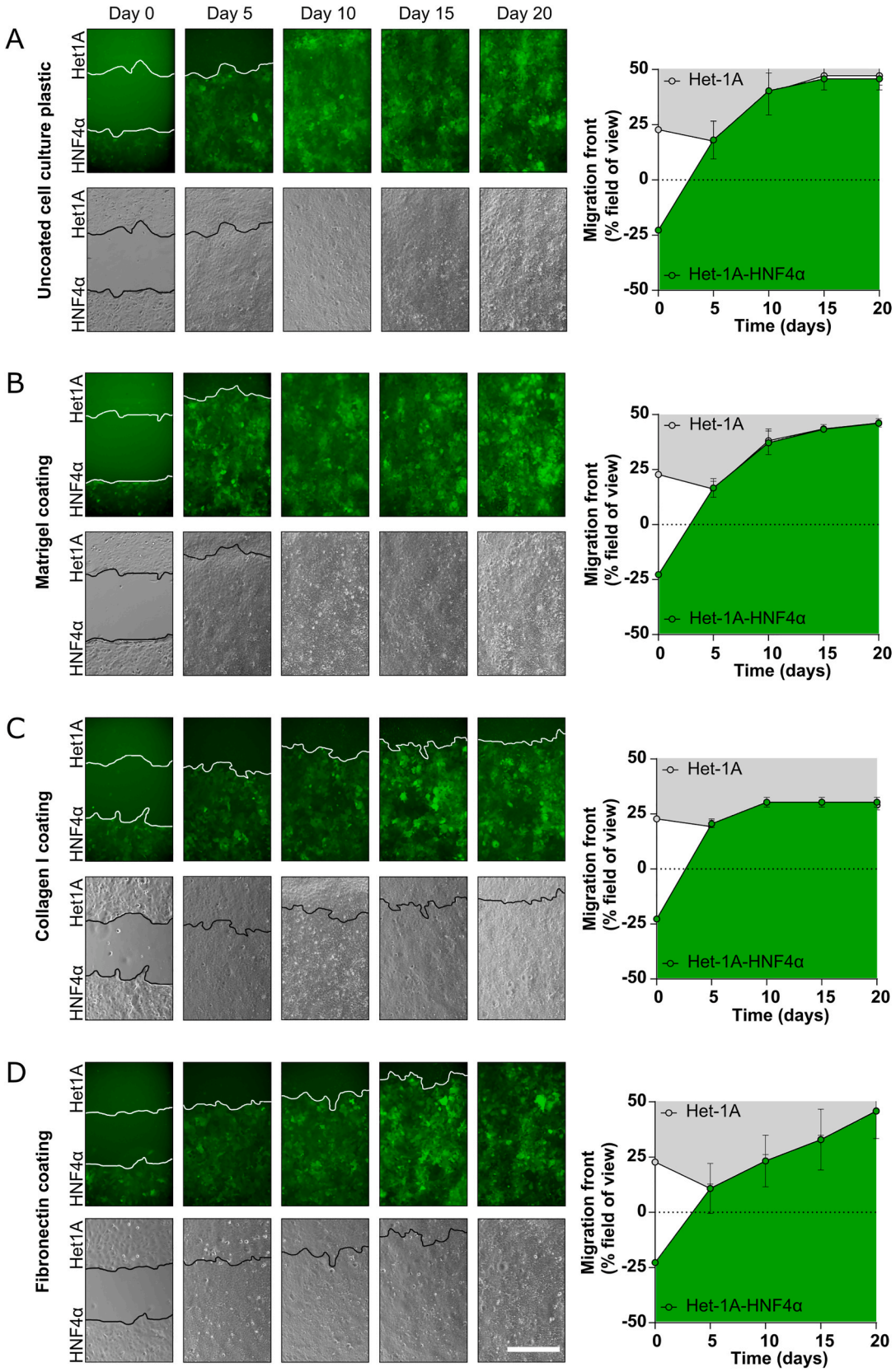

Fig. 4. Het1A-HNF4α epithelium pushes into the unmodified Het1A cell domain. Heterotypic cultures of wildtype Het1A and Het1A-HNF4α (GFP-positive) on (A) uncoated plastic, (B) Matrigel, (C) collagen I or (D) fibronectin were monitored up to 20 days. Graphs show unmodified Het1A and Het1A-HNF4α cell front positions at 0, 5, 10, 15 and 20 days. -25 and + 25% represent the starting position of the migration fronts (mean ± SEM, n = 3 independent experiments). Representative phase contrast and immunofluorescence are next to the graphs. Black lines indicate the cell front between unmodified Het1A and GFP-positive Het1A-HNF4α cells (scale bar, 400 μm).

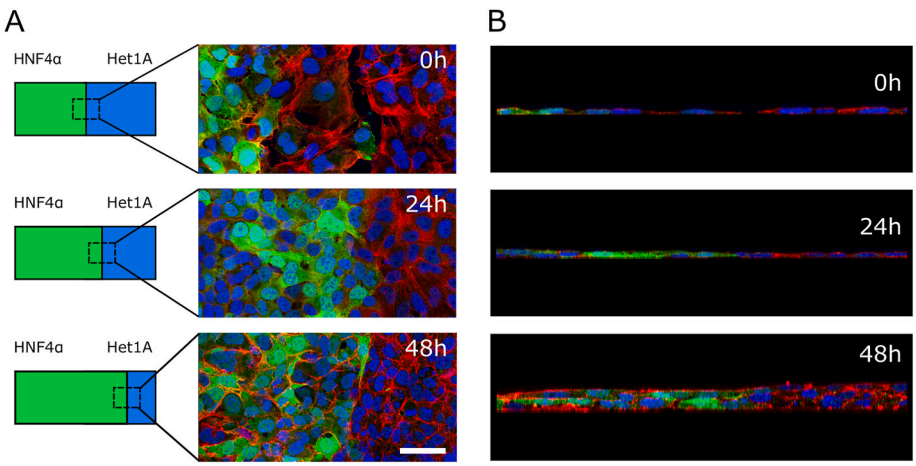

Fig. 5. Het1A-HNF4α and Het1A epithelia show a sharp junction. (A) Orthogonal view of the boundary between GFP-labelled Het1A-HNF4α cells and Het1A cells stained with EGFP (green), Phalloidin (red) and DAPI (blue). (B) Z-stack sections. Co-cultures on uncoated plastic were fixed at 0-, 24- and 48-h after contact (scale bar, 50 μm).

neither cell line showed a significant competitive advantage. While there is an undulating front with both Het1A and Het1A-CDX2 cells pushing forward in different areas, overall, the position of the boundary between the cell types does not significantly change between 5 and 20 days. At day 20 it can be observed that the cell types start to mix, however, without a clear winner or loser. This shows that, in contrast to HNF4α, the transcription factor CDX2 does not confer a competitive advantage on the Het1A cells (Fig. 6). Similar results were obtained when Het1A-HNF4α and Het1A-CDX2 cells were compared to Het1A-RFP control cells (Supplementary Fig. 4). While no difference was observed between Het1A-RFP cells and unmodified Het1A and Het1A-CDX2 cells. A greater field of view in these experiments shows that the Het1A-HNF4α cells continued to push against Het1A cells.

4. Discussion

An important question concerning the nature of Barrett's oesophagus (BO) is how the patches of columnar-like cells can expand into the oesophagus and what is the molecular and cellular basis of this competition. To address this question, we have developed an *in vitro* model of cell competition between two epithelial sheets. The experiments on competition between Caco-2 and Het1A cells show that the intestine-like Caco-2 cells do displace the oesophagus-like Het1A cells. However the boundary between the epithelia does not resemble the sharp boundary seen in BO *in vivo* (Biddlestone et al., 1998) and the higher proliferation of Caco-2 cells might explain its advantage. A limitation of the direct comparison of Het1A and Caco2 cells is that the two cell models are grown in different media. Consequently, a mixed medium composition was used, which was kept consistent throughout the study. While it was ensured that the original growth behaviour had not changed, there is still the possibility that the medium used in this study provided an advantage to Caco-2 cells.

Previous studies have shown that the transcription factors HNF4α and CDX2 are expressed in BO, and that HNF4α drives at least some aspects of columnar intestinal differentiation (Colleypriest et al., 2017). We were interested to know whether these specific transcription factors could impart the competitive advantage to the cells. To investigate this possibility, we used the Het1A cell line in combination with a Het1A cell lines stably overexpressing the HNF4α or CDX2.

CDX2 is an important transcription factor in the regional patterning of the gut during embryonic development, and in the differentiation of the intestinal epithelium (Gao et al., 2009; Silberg et al., 2000). Ectopic expression of CDX2 in the stomach of transgenic mice can cause the formation of heterotopic intestinal epithelium (Mutoh et al., 2004; Silberg et al., 2002). Conversely, selective deletion of gut endodermal CDX2 during development results in the expression of squamous differentiation markers in the intestine (Gao et al., 2009).

Hnf4α is expressed in the tissues of the developing gut including the intestine, stomach, liver and pancreas (Taraviras et al., 1994; Zhong et al., 1993). Importantly, HNF4α is not expressed in normal human oesophagus, but is expressed in BO (Colleypriest et al., 2017; Piessen et al., 2007a,b). In addition to its role in promoting the columnar phenotype of BO, HNF4α has been shown to drive clonal expansion in gastrointestinal tumours by promoting cell proliferation (Chang et al., 2016). CDX2 has been suggested to act as tumour suppressor in colorectal cancer (Bonhomme et al., 2003), where expression correlates inversely with colorectal cancer grade (Bakaris et al., 2008). In contrast, expression analysis of a range of different adenocarcinomas showed increased CDX2 expression in human colorectal carcinoma samples (Werling et al., 2003).

In the early, migratory, phase of the co-culture assays, the Caco-2 epithelium covers the area faster than Het1A. Also, the Het1A-HNF4α cells migrated faster compared to unmodified Het1A (despite these cells lacking E-cadherin expression) while the Het1A-CDX2 did not. Consistent with the HNF4α findings, a pro-migratory role for this transcription factor has been shown in SGC7901 gastric cancer cells (Hu et al., 2018). By contrast, an anti-migratory role for CDX2 has been reported in the human colon adenocarcinoma cell line, Caco-2/TC-7 (Gross et al., 2008). Fibronectin, collagen I and Matrigel all promoted the migration of the tested cell models in monotypic cultures in comparison with uncoated plastic. These findings are consistent with the role previously observed by others of fibronectin (Ou et al., 2019), collagen I (Chen and Nalbantoglu, 2014) and Matrigel (Benton et al., 2011) in promoting cancer cell migration. Interestingly the impact of the matrix coating was less pronounced in heterotypic cultures and long-term competition assays.

Following cell contact (of Het1A with Het1A-HNF4α cells and Het1A with Het1A-CDX2 cells), HNF4α but not CDX2 demonstrated a role in conferring a competitive advantage. Here, the Het1A-HNF4α cells were capable of pushing into the unmodified Het1A domain and colonising the observed area over 20 days. Z-stack sections of the boundary revealed that Het1A-HNF4α push and compact the Het1A cells, generating a sharp boundary, such as is seen in clinical BO (Biddlestone et al., 1998). This suggests the possibility of mechanical stress-induced cell competition in oesophageal-like Het1A cells as observed by Wagstaff et al. (2016). Based on these findings, we proposed that activation of HNF4α in BO is not only associated with the induction of intestinal markers, such as VILLIN (Colleypriest et al., 2017; Nowicki-Osuch et al.,

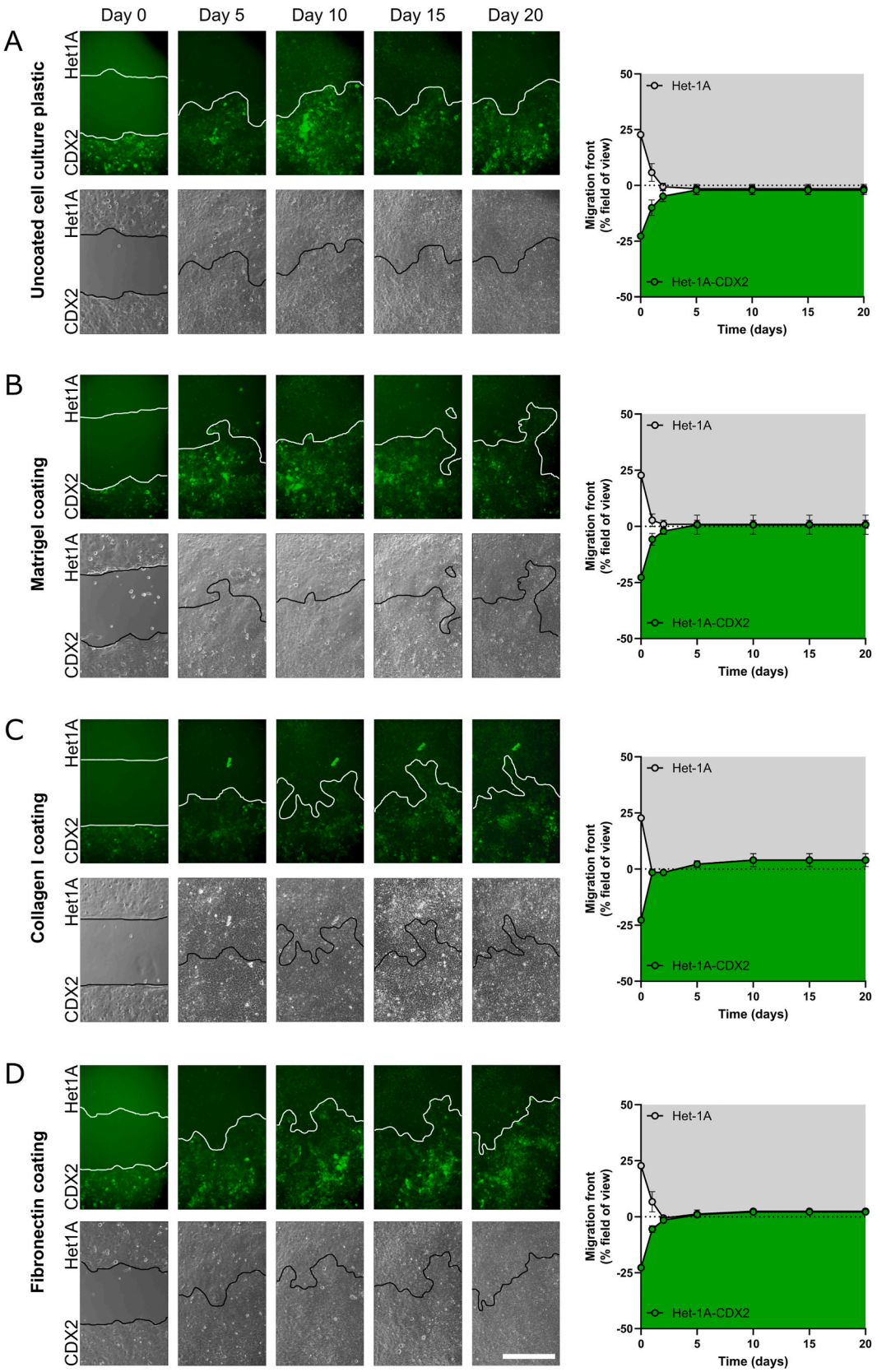

Fig. 6. Het1A-CDX2 cells are not able to push into the unmodified Het1A domain. Heterotypic cultures of wildtype Het1A and Het1A-CDX2 (GFP-positive) on (A) uncoated plastic, (B) Matrigel, (C) collagen I or (D) fibronectin and were monitored for up to 20 days. Graphs show unmodified Het1A and Het1A-CDX2 cell front positions at 0, 5, 10, 15 and 20 days. -25 and + 25% represent the starting position of the migration fronts (mean ± SEM, n = 3). Representative phase contrast and immunofluorescence are next to graphs. Black lines indicate the cell front between unmodified Het1A and GFP-positive Het1A-HNF4α cells (scale bar, 400 μm).

2021), but also with promoting cell competition. By contrast, Het1A-CDX2 cells did not show any ability to push into the Het1A domain. However, it has to be noted that while the Het1A-CDX2 cells ectopically express CDX2 (the wildtype Het1A cells do not express the transcription factor), levels are still lower in comparison to Caco-2 cells. CDX2 levels might not be high enough to trigger any downstream effect on migration.

HNF4α exists in 12 different isoforms that are generated by transcription from two different promoters (termed P1 and P2) and by two alternative 3' splicing events. The P1 isoforms are predominantly expressed in the differentiated upper half of human colonic crypts, whereas the P2 isoforms are expressed in the proliferative lower half. Activation of the WNT/β-catenin pathway inhibits the HNF4α P1 isoforms in colorectal cancer cells and the P2 isoform gene signature is associated with pro-oncogenic functions (Babeu et al., 2018). Therefore, it will be of interest in future studies to determine the differential expression of HNF4α P1 and P2 isoforms in BO, dysplasia and progression to adenocarcinoma. Based on the previously discussed studies on HNF4α, we hypothesize that expression of P1 HNF4α isoforms may be associated with epithelial differentiation of BO cells, while P2 HNF4α isoforms could be linked to the proliferation of BO cells and malignant progression.

We observed differences in the degree of cell competition depending on the ECM component. Before cell contact, Het1A-HNF4α cells migrated more rapidly than Het1A cells on all ECM coatings, but the largest differences were observed on collagen I. Despite this increased migratory behaviour, after contact, Het1A-HNF4α cells showed poor ability to infiltrate into the Het1A domain on collagen I. This emphasizes the fact that the capability to infiltrate into the opposite domain does not depend on how rapidly cells migrate before the boundaries touch. Further studies are needed to determine the molecular and cellular mechanisms by which Het1A-HNF4α cells maintained on collagen I exhibit a lower degree of competition than on fibronectin and Matrigel. The question arises whether interactions with ECM integrin receptors play a role in the colonisation of the Het1A area. Previously it has been reported that Het1A cells exposed to bile acids impacts the expression of integrin av, which binds to RGD motives in ECM proteins, but not collagen (Prichard et al., 2017) and increases ICAM-1 (Gergen et al., 2022). Furthermore, it has been suggested that changes in integrin pattern and expression could play a role in the disease progression of oesophageal cancer (Vay et al., 2014). Changes in extracellular matrix composition and integrin signalling via focal adhesion (FA) kinases can have a multiple effects, including MAPK and phosphatidylinositol 3-kinase (PI3K) signalling impacting survival and proliferation, Rho signalling and FA turnover and epithelial plasticity (Cooper and Giancotti, 2019). However, the present work did not demonstrate proliferative differences between Het1A-HNF4α and Het1A cells in a confluent situation (Supplementary Fig. 3).

In conclusion, the data showed that stable expression of HNF4α, but not CDX2, conferred a competitive ability to Het1A cells regarding cell migration and infiltration into the wild-type domain. These findings highlight further the importance of HNF4α as a causal factor in BO and a potential therapeutic target. In future the work could usefully be extended by examining the effects of the two genes in additional cell models, including gastric-derived cells and BO-derived cells (Mytar et al., 2018; Palanca-Wessels et al., 2003), as well as investigating further the cellular and molecular mechanisms involved.

Author contributions

C Grimaldos Rodriguez: investigation, data analysis, writing-original draft. *E Rimmer*: investigation, data analysis. *B Colleypriest*: conceptualization, writing-original draft, funding acquisition. *D Tosh*: conceptualization, supervision, writing-original draft, funding acquisition. *JMW Slack*: conceptualization, supervision, writing-original draft. *U Jungwirth*: conceptualization, supervision, data analysis, writing-original draft, funding acquisition.

Declarations of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diff.2023.08.003.

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