Echinococcus granulosus **cyst fluid enhances epithelial - mesenchymal transition**

Ahmed A. Mohammed*, J. Allen & M.T. Rogan†. Biomedical Research Centre, School of Environment & Life Sciences, University of Salford, M54WT, UK.

*Current address: Branch of Clinical Laboratory Sciences, College of Pharmacy, University of Al-Mustansiriyah, Iraq.

† Corresponding author

Running title: *E. granulosus* cyst fibrosis

ACKNOWLEDGEMENTS

This work was funded through a PhD studentship from the Branch of Clinical Laboratory Sciences, College of Pharmacy, University of Al-Mustansiriyah, Iraq.

Abstract

Aims

Cystic Echinococcosis is characterised by fluid filled hydatid cysts in the liver and lungs. The cysts are surrounded by a host fibrous layer (the pericyst) which acts to isolate the parasite from surrounding tissues. Previous studies in liver cysts have indicated that the parasite may be stimulating fibrosis. The aim of this study was to investigate whether Hydatid Cyst Fluid (HCF) could influence the potential for fibrosis to occur in lung tissue by stimulating epithelial to mesenchymal transition (EMT) in a human lung epithelial cell line.

Methods and Results

An adenocarcinoma-derived alveolar basal epithelial cell line (A549) was used as a model for human alveolar epithelial cells (AEC II). These were cultured *in vitro* with HCF (UK sheep origin). Assays to investigate cell proliferation, cell migration and expression of cytoskeletal markers showed that HCF could stimulate changes indicative of EMT, including enhanced cell proliferation and migration; increased expression of mesenchymal cytoskeletal markers (fibronectin and vimentin) accompanied by a down regulation of an epithelial marker (E-cadherin).

Conclusions

Molecules within hydatid cyst fluid are capable of inducing phenotypic changes in A549 cells indicating that the parasite has the potential to modify lung epithelial cells which could contribute to fibrotic reactions.

Key words: Echinococcosis; hydatid cyst; fibrosis; Epithelial – Mesenchymal transition; A549 cell; pulmonary

Introduction

Cystic Echinococcosis (CE) is a zoonotic infection caused by the metacestode of the tapeworm *Echinococcus granulosus* and is characterised by the development of slow growing hydatid cysts, mainly in the liver and lungs of domestic livestock or humans. The typical hydatid cyst is a unilocular fluid filled chamber which, when fertile, contains the protoscoleces. The hydatid cyst fluid (HCF) is a complex biological mixture of various soluble components including glycolipids proteins, carbohydrates and salts and includes host molecules as well as parasite macromolecules (including major diagnostic molecules, Antigen 5 (Ag5) and Antigen B (AgB) (1-3). The fluid is surrounded by a cyst wall of three layers, the inner germinal layer which in turn is enclosed by an acellular laminated layer and surrounded by a fibrous outer layer (referred to as either the adventitial layer or pericyst) which is of host origin (4-7).

The host-parasite relationship in Cystic Echinococcosis is extremely complex and cyst survival, growth and development is dependent on a balance between host immunological activity from one side, and the expression of parasite immunoevasive strategies from the other (5,8-10). The fibrotic host response around the cyst is part of this relationship and the relative thickness of this layer can vary depending on the anatomical location of the cyst. In general, within animal hosts, it is most developed in hepatic cysts and to a lesser extent in lung cysts (11-13). It has also been suggested that this fibrotic activity may help in restricting growth of the cysts with lung cysts being perceived as having a higher growth rate than hepatic cysts (13). This suggestion is also supported in rare cases of brain cysts where there is little fibrosis and where very high growth rates have been estimated (14). Whilst the fibrous pericyst is clearly a mechanism by which the host is attempting to isolate the parasite and restrict its development, it could also conceivably be a mechanism which is protective to the parasite, acting as a further barrier to potentially damaging immune effector mechanisms. (15-16)

In hepatic Echinococcosis (CE and AE) the parasites are known to both upregulate and down regulate several hundred host genes associated with metabolism, the immune system (complement cascade and antigen processing) and cell signaling and transport. (17-20) and it has recently been shown that HCF from *E. granulosus* may promote fibrosis in hepatic stellate cells by down regulation of miR-19B (21). Similar studies

have not been carried out in relation to lung tissue where it might be expected that fibrogenic activity would be reduced. Indeed, cellular pathology and immunoregulation of pulmonary CE has been somewhat neglected.

Fibrogenesis and deposition of collagen can be initiated and regulated in different tissues by different mechanisms. In the lungs, one such mechanism involves alveolar epithelial cells (AECII) which, under certain conditions, can undergo an epithelial to mesenchymal transition (EMT), i.e. the transformation of the epithelial cells to mesenchymal cells with a myofibroblast-like phenotype with subsequent hyperplasia that is pathognomonic for human lung fibrosis with subsequent collagen deposition (22, 23). Epithelial cells which undergo EMT reorganize their cytoskeleton (23, 24) and acquire increased migratory characteristics (25, 26), accompanied by down-regulation of epithelial differentiation markers including E-cadherin and cytokeratin, and transcriptional induction of mesenchymal markers such as vimentin, fibronectin and N-cadherin (24, 26-30).

Parasite induced initiation of fibrogenesis in liver tissue is likely to involve a range of cells such as macrophages and hepatic stellate cells but there is currently no information on how the parasite can modify lung tissue. In order to establish whether metacestodes of *E. granulosus* are actively involved in altering the environment within the lungs leading to possible fibrosis, the A549 lung epithelial cell line was used to investigate EMT-like changes resulting from exposure to parasite-derived molecules present in HCF. A549 are often used in initial studies as a model for investigating effects on human alveolar epithelial cells type II (AECII) because they demonstrate many of the properties of normal lung AEC (31,32), particularly their metabolic, structural and transport characteristics (25). Previous investigations into lung EMT have made use of this cell line and have shown it to exhibit EMT-like responses which are similar to those obtained with normal alveolar epithelial cells.

Materials and methods

Hydatid Cyst Fluid (HCF) samples

Samples of HCF were obtained from fertile cysts in individual naturally infected sheep from UK abattoirs. All samples were filter sterilized and stored frozen at -20^{o}C until use. The condition of each batch of cyst fluid was assessed by checking the relative antigenicity of samples from different sheep by ELISA against sera from human CE cases and the six highest reacting samples were pooled together for use in the cell culture experiments (data not shown). The protein concentration of the pooled HCF sample was estimated using a colourimetric assay (Biorad) to be 0.18mg/ml. All samples were checked to be free from the presence of bacterial endotoxins using the ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (GenScript).

Cell proliferation assay.

A549 cells (Sigma Aldrich) were seeded at a density of 5×10^4 cells per well in 6 well plates (Falcon) and maintained in RPMI-1640 medium (Labtech International) supplemented with 10% foetal bovine serum FBS (Fisher Scientific), 1% L-Glutamine (Lonza) and 1% Penicillin-Streptomycin-Amphotericin B (Lonza), termed complete medium (CM). For experiments, cells were changed to serum limited medium (LSM) containing 0.5% FBS. Cell number and FBS concentration were optimised prior to finalising culture conditions (see Supplementary Data, Figs i and ii). Cells were incubated in 37°C incubator (Sanyo-Japan) in a humidified 5% CO_2 atmosphere. Pooled HCF (initial concentration of 0.18mg/mL protein) was diluted 1 in 10 (18 μg/mL), 1 in 20 (9 μg/mL), 1 in 50 (3.6 μg/mL) and 1 in 100 (1.8 μg/mL) in LSM and added to the plates. Cells were incubated for 120 hours with one medium change after 72 hours. Cell growth from three replicate wells was estimated on each day using direct microscopic examination and a cell proliferation assessment where cell numbers were determined by crystal violet staining according to Chiba *et al*. (33). Briefly, wells were washed 2 times with PBS and stained with 1ml 0.1% Crystal Violet (CV) for 15 minutes at room temperature. The stain solution was aspirated and wells washed 3 times with dH_2O before being allowed to air dry. 1 mL of methanol was then added to each well to extract the dye taken up by living cells. After 15 minutes, 100μL was transferred to a 96 well microtitre plate and absorbance was measured at 620nm in an Ascent Multiscan reader (Thermo Scientific). A calibration curve was used to relate optical density to estimated cell number.

Cell migration assay.

Cell migration was assessed using a wound healing assay according to Liang *et al*. (34) with minor modifications. Cells were cultured in CM as described above until 70% confluent, then washed in LSM before a longitudinal scratch (wound) was made in the middle of each well using a 100µL pipette tip. HCF (18 µg/mL) in LSM, or LSM alone was applied as a treatment. Cells were examined after 24 hours and 48 hours on an inverted microscope and the distance between the two sides of wound was measured at five equidistant points along the wound, (0.6mm between each point).

EMT

To investigate expression of EMT markers, sub-confluent A549 cells were treated with either 10% HCF (final protein concentration = 0.18mg/mL) in LSM, or with 5ng/mL TGF-β1 (PeproTech-USA) in LSM, as a known inducer of EMT (35), or LSM alone. Cells were cultured for a further 3 days before immunocytochemistry and microscopic examination. Cells were washed 2 times in PBS, fixed for 5 mins with 4% formaldehyde (Sigma-Aldrich), washed 2 times in PBS, then permeabilised by 1% Triton X-100 (Sigma-Aldrich) for 5 minutes (for E-cadherin) and 0.5% Triton X-100 for 10 mins (Cytokeratin, Fibronectin and Vimentin), followed by 3 washes for 5 minutes with PBS. Non-specific binding was blocked with 3% BSA (Sigma-Aldrich) for 1 hour at room temperature and then cells were exposed to 1:1000 dilution (in PBS plus 1% BSA) of either: mouse anti-human E-cadherin (Clone HECD-1, Takara, Japan); mouse anti-human pan cytokeratin (clone C-11, Abcam, UK); mouse anti-human fibronectin (clone IST-9 Abcam, UK); or mouse anti-human vimentin (Sigma-Aldrich) and incubated overnight at 4°C in a wet tray. The next day, cells were washed 3 times in PBS, and incubated for 2 hours in the dark at room temperature with 1:2000 Alexa Fluor 546 goat anti-mouse IgG (Life Technologies-USA) in PBS plus 1% BSA. Cells were finally washed 3 times in PBS and mounted in 10µL mounting medium containing DAPI (Thermo Fisher Scientific) to stain cell nuclei for 1 hour. Wells were examined using an Eclipse TE2000-S microscope system (Nikon UK Ltd, Surrey) and Image-Pro Plus (Media Cybernetics UK, Berkshire).

Semi-quantification of EMT marker expression was carried out by analysis of images at six fields in each well under the same image exposure conditions. The position of fields was chosen from a pre-defined grid pattern determined for all slides using the x-

y co-ordinates on the microscope stage. Cells were counted within each field, and the percentage of positively labelled cells (cells expressing a certain marker) calculated for each group. Two patterns of distribution within individual cells were sometimes evident and were defined as either extensive (E), throughout the whole cytoplasm or condensed (C) in small areas in the perinuclear region. Where these patterns were evident, the proportion of cells showing each pattern was also determined.

Statistical analyses

For the cell proliferation and cell migration studies results are presented as mean values for three replicate wells in each treatment. Data was analysed using a paired t-test comparing treated and controls at each time point and for the cell proliferation study a one way ANOVA was also performed (IBM SPSS Statistics 20). Each assay was repeated three times and similar trends were seen in all three occasions. For the cell marker studies counts were taken at six points in each well for three replicate wells. A one way ANOVA was carried out for all treatments and all markers and also to compare all markers between HCF treated and control slides, and between HCF treated and TGF-β1 treated cells. In all cases values of $p < 0.05$ were considered statistically significant.

Results

Cell proliferation

There were no significant differences in proliferation between HCF treated cells and controls over the first 72 hours. However, from 3 to 5 days of culture there was a dose dependent increase in proliferation observed with HCF (Fig. 1) compared to controls ($p < 0.001$) with the highest concentration of HCF resulting in a 250% increase in cell number.

Cell migration

Semi-quantitative analysis of scratch assay plates showed an enhanced cell migration in the presence of HCF compared to control. At 24 hours, HCF-treated cells (Fig. 2A)

had migrated further into the "scratch" than control cells in some areas but the mean gap between each side of the "scratch" was not significantly different from controls (Fig 2B, $p > 0.05$). However, at 48 hours the gap between the sides of the wound was closed in many areas of the HCF treated cells and the mean gap was significantly smaller compared to controls (Fig. 2B, $p < 0.05$).

EMT

Epithelial markers (Fig. 3): cells treated with HCF showed downregulation of E-cadherin (E-Cad) expression with 40.8% positive for E-Cad in HCF treated cells, compared to 76.1% in controls ($p < 0.01$). By comparison, TGF-β1 as a known EMT inducer completely inhibited expression of E-Cad (Fig. 3A). Cytokeratin was ubiquitously expressed and there were no significant differences between treated and control cultures (Fig. 3B). With respect to the intracellular distribution of expression, the 'extensive' pattern is more typical of an epithelial phenotype and although HCF treated cultures appeared to demonstrate fewer cells with this distribution pattern, The differences in numbers did not reach significance, compared to those found in the control cultures ($p > 0.05$). It was also noted that the staining intensity of the HCF treated cells was less than controls but this was not quantified. TGF-β1 also did not reduce cytokeratin expression and had a similar pattern of staining to controls.

Mesenchymal markers (Fig 4): cells treated with HCF showed upregulation of fibronectin (Fn) expression with 39.6% positive for Fn in HCF treated cells, compared to just 13% in controls ($p < 0.01$) which only weakly expressed this marker. In comparison, cells treated with TGF-β1 also upregulated Fn expression with 51.7% positive cells. Importantly, Fn expression in TGF-β1-treated cells was not statistically different to Fn expression in HCF treated cells, ($p > 0.05$). The number of cells positive for Vimentin (Vm) was not significantly different between HCF treated and control cultures (Fig. 4B), although the distribution of the marker varied. In control cells virtually all vimentin expression was limited to 'condensed' areas in the perinuclear region. However, in HCF and TGF-β1 treated cultures significantly more cells showed more 'extensive' marker distribution throughout the cytoplasm ($p < 0.01$). However expression in TGF-β1 treated cells was significantly greater than in HCF treated cells ($p < 0.05$). In addition to mesenchymal marker expression, it was noted that some cells

in HCF treated cultures also displayed morphological features consistent with a mesenchymal phenotype, in producing extended cytoplasmic processes (Fig 4B). Statistical analysis by ANOVA indicated when all treatments and all markers were compared, significant differences were evident ($p < 0.01$). The ANOVA comparing HCF treated cells to controls for all markers showed significant differences ($p < 0.05$) but the differences between HCF treated cells and TGF-β1 treated cells was also significant ($p < 0.05$).

Discussion

The results of this study indicate that HCF can affect A549 cells by increasing their proliferation and migratory activity. In addition, exposure to HCF also alters the expression of some phenotypic markers i.e down regulation of epithelial markers such as E-cadherin and upregulation of mesenchymal markers such as fibronectin and vimentin. Although HCF treatment modified expression of some markers its effects were different from TGF-β1, a known initiator of EMT, mainly in relation to the level of regulation. It is also interesting to note that both treatments did not affect cytokeratin expression. Recent investigations note that EMT is not a binary process and cells can display a range of hybrid states that include but are not limited to, fully epithelial and fully mesenchymal, and in fact a hybrid partial EMT phenotype with cells co-expressing markers of both epithelial and mesenchymal states has been suggested to be a stable phenotype that cells can adopt, depending on external factors (36).

Taken together, these alterations are supportive of HCF-mediated induction of EMT. Although there was no significant effect on cytokeratin expression, vimentin expression was considered to be more extensive throughout cells, a feature which is associated with mesenchymal cytoskeletal reorganisation (37). These effects on A549 cells in themselves are not indicative of a full fibrotic reaction which would involve monitoring collagen deposition and other elements such as α- smooth muscle actin (αSMA), but do indicate that parasite derived molecules can lead to transformations in host cells which make fibrogenesis in lung tissue more likely. EMT is a frequent feature in patients with lung fibrosis caused by other factors. Hydatid cyst fluid from *E. granulosus* is known to have significant effects on a variety of host immune cells, from acting as a mitogen, non-specifically stimulating lymphocyte proliferation (38), to

altering accessory cell function and antigen processing (39). It has also been shown to modify the phenotype of cells such as monocytes (40). In most cases these effects are generally believed to influence the nature of the immune response in favour of parasite survival and often linked to a more permissive Th2 type response (8,9, 4, 42). However there have been no studies which have indicated that the phenotype of epithelial cells can be modified by HCF. The findings from this study in a lung cell line are supportive of the findings in LX-2 hepatic stellate cells (21) where HCF from *E. granulosus* has been shown to increase expression of α-SMA, COL1A1 and COL3A1 along with increased TβRII expression and inhibition of miR-19b. These authors interpretation is that *E. granulosus* metacestodes may actively promote fibrosis through the increase in TβRII, the activation of hepatic stellate cells and extracellular matrix production.

It may be argued that the concentration of HCF used in these studies (18μg/mL) is in excess of the true *in vivo* situation. HCF does leak out in large amounts, of cysts which have been damaged and may lead to anaphylactic reactions (43). However there are no definitive studies which have looked at release of cyst fluid from intact cysts. But it is known that antibodies to major cyst fluid antigens are present in most CE patients and that antibody levels may fluctuate within individuals indicating periodic release of antigen. Circulating cyst fluid antigens are also detectable in some CE patients (44). It should also be noted that HCF is a complex mixture of both host and parasite molecules and that the total protein concentration used in these studies will reflect all molecules present. The active molecules responsible for the observed effects are therefore likely to be present at much smaller concentrations.

In the current study it is evident that there is a mechanism by which cyst fluid components derived from lung hydatid cysts could also promote fibrosis via EMT of AECII cells and subsequent differentiation into myofibroblasts. Such events would suggest that fibrosis is potentially beneficial for the parasite by actively stimulating the building of fibrous layers. The parasite recruits host cells to migrate toward the parasite lesion and stimulates their proliferation and transition to the fibroblast-like phenotype which subsequently deposits ECM components.

The exact molecules involved in transitional events are not known. It is valid to question whether or not host components may be responsible for the observed cellular changes in A549 cells but it is important to remember that the cell line is of human origin whilst the HCF will contain host molecules which are of sheep origin. In addition, proteomic analysis of HCF has not shown the presence of known cytokines (2, 45). Additional studies also indicate that HCF is not simply acting as a source of nutritional protein as the same proliferative effects were not evident when HCF was substituted with bovine serum albumen (BSA) at the same concentration. (See Supplementary Data, Fig iii).

The mechanism by which HCF induces EMT is also not known. Direct stimulation of the cells via a ligand/receptor interaction may be possible but initiation of autocrine secretion of TGF-β may also be possible. With *E. multilocularis* infections in mice chronic hepatic fibrosis is a classical feature of the infection and acts to both isolate the parasite and contributes to pathology. TGF-β / Smad interactions are thought to be the driving mechanism of this process in hepatic cells, with TGF-β being produced as a result of either parasite induced inflammation or potentially by parasite derived molecules which can interact with the TβRI or TβRII receptor (19). It is known that HCF contains many parasite molecules, some of which have cytokine-like properties, but it is interesting to note that the proliferative and migratory responses observed in this study were retained after heating the cyst fluid to 95^{o}C for 5 minutes, a process that would affect the structural integrity of typical mammalian cytokines. Similar activity was also evident in a semi-purified extract of Antigen B indicating that this known, heat-stable immunomodulatory molecule may have a role. (see Supplementary Data, Figs iv and v).

Extensive fibrosis and pericyst formation in CE is often seen as a mechanism by which the host restricts the growth and proliferation of the metacestode. Conversley recent studies in experimental mice have shown that IL-17A can reduce fibrosis which leads to a reduced parasite biomass (46) indicating that a fibrotic reaction is favourable to the parasite. The thick pericyst, in addition to acting as a barrier, also provides considerable mechanical strength to the fluid filled cyst which may be under considerable hydrostatic pressure. From a transmission perspective it is important that cyst integrity is maintained in the natural intermediate host (domestic/wild livestock) to be available to

carnivorous canids. The fibrotic pericyst may therefore be essential to prevent cyst degeneration within the internal organs of dead livestock. This could be particularly important in lung cysts where the surrounding pulmonary tissue is less rigid.

References

1. Larrieu EJ, Frider B. Human cystic echinococcosis: contributions to the natural history of the disease. Ann Trop Med & Parasitol. 2001; 95: 679-687.

2. Aziz A, Zhang W, Li J, Loukas A, McManus DP, Mulvenna J. Proteomic characterisation of *Echinococcus granulosus* hydatid cyst fluid from sheep, cattle and humans. J Proteomics 2011; 74: 1560-1572.

3. Li ZJ, Wei Z. Analysis of Protoscoleces-specific Antigens from *Echinococcus granulosus* with Proteomics Combined with Western Blot. Biomed Environ Sci 2012; 25:718-723

4. Rogan MT, Hai WY, Richardson R, Zeyhle E, Craig PS. Hydatid cysts: does every picture tell a story? Trends Parasitol. 2006; 431-8.

5. Vuitton DA. The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? Acta Tropica 2003; 85: 119-132.

6. Díaz A, Casaravilla C, Irigoín F, Lin G, Previato JO, Ferreira F. Understanding the laminated layer of larval *Echinococcus* I: structure. Trends Parasitol; 27: 204-213.

7. Golzari SEJ, Sokouti M. Pericyst: The outermost layer of hydatid cyst. World J Gastroenterol : 2014; 20: 1377-1378.

8. Siracusano A, Delunardo F, Teggi, A., *et al*. Host-parasite relationship in cystic echinococcosis: an evolving story. Clin Dev Immunol. 2012; 639362

9. Wang J, Gottstein B. Immunoregulation in larval *Echinococcus multilocularis* infection. Parasite Immunol 2016; 38: 182-192

10. Brehm K, Koziol U. *Echinococcus*-Host Interactions at Cellular and Molecular Levels. Adv Parasitol 2017. 95; 147-212

11. Njoroge EM, Mbithi PM, Gathuma JM, et al. Study of cystic echinococcosis in slaughter animals in three selected areas of northern Turkana, Kenya. Vet Parasitol 2002. 104: 85–91

12. Beigh AB, Darzi, MM, Bashir, S et al. Gross and histopathological alterations associated with cystic echinococcosis in small ruminants J Parasit Dis. 2017 41: 1028.

13. Barnes TS, Hinds LA, Jenkins DJ, Bielefeldt-Ohmann H, Lightowlers MW, Coleman GT Comparative Pathology of Pulmonary Hydatid Cysts in Macropods and Sheep. J Comp Pathol 2011. 144: 113-122

14. Valour F, Khenifer S, Della-Schiava N *et al*. Unusual growth rate during cystic echinococcosis. Parasitol Int. 2014; 63: 275-277.

15. Rogan MT, Craig, PS. Immunology of *Echinococcus granulosus* infections, Acta Tropica 1997; 67:7-17

16. Peng XY, Wu XW, Zhang SJ, Niu JH. The new aspects in recognition of pathogenesis of the pericyst of human hepatic *Echinococcus granulosus*. World Chin J Digestol. 2005. 13: 276–9

17. Gottstein B, Wittwer M, Schild M *et al*. Hepatic gene expression profile in mice perorally infected with Echinococcus multilocularis eggs. PLoS One 2010. 5; e9779

18. Lin R, Lü G, Wang J *et al*. Time Course of Gene Expression Profiling in the Liver of Experimental Mice Infected with *Echinococcus multilocularis*. PLoS One 2011; 6: e14557.

19. Wang J, Zhang C, Wei, X, et al. TGF-beta and TGF-beta/Smad Signaling in the interactions between *Echinococcus multilocularis* and its hosts. PLoS One 2013. 8: e55379

20. Hui W, Jiang S, Liu X, Ban Q, Chen S and Jia B. Gene Expression Profile in the Liver of Sheep Infected with Cystic Echinococcosis. PLoS One 2016; 11: e0160000.

21. Zhang C, Wang L, Ali T *et al*. Hydatid cyst fluid promotes peri-cystic fibrosis in cystic echinococcosis by suppressing miR-19 expression. Parasit Vectors 2016; 9: 278.

22. Pirozzi G, Tirino V, Camerlingo R *et al*. Epithelial to Mesenchymal Transition by TGFβ-1 Induction Increases Stemness Characteristics in Primary Non Small Cell Lung Cancer Cell Line. PLoS One 2011; 6: e21548.

23. Ramos C, Becerril C, Montano M *et al*. FGF-1 reverts epithelial-mesenchymal transition induced by TGF-b1 through MAPK/ERK kinase pathway. Am J Physiol Lung Cell Mol Physiol 2010; 299: L222-231.

24. Shintani Y, Okimura A, Sato K *et al*. Epithelial to Mesenchymal Transition Is a Determinant of Sensitivity to Chemoradiotherapy in Non-Small Cell Lung Cancer. Ann Thorac Surg 2011; 92: 1794-1804.

25. Mikami Y, Yamauchi Y, Horie M *et al*. Tumor necrosis factor superfamily member LIGHT induces epithelial–mesenchymal transition in A549 human alveolar epithelial cells. Biochem Biophys Res Commun 2012. 428: 451-457.

26. Suh SS, Yoo JY, Cui R *et al*. FHIT Suppresses Epithelial-Mesenchymal Transition (EMT) and Metastasis in Lung Cancer through Modulation of MicroRNAs. PLoS Genetics 2014; 10: e1004652.

27. Kalluri R, Neilson E G. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 2003, 112: 1776-1784.

28. Pozharskaya V, Torres-Gonzalez E, Rojas M *et al*. Twist: a regulator of epithelial-mesenchymal transition in lung fibrosis. PLoS One 2009; 4: e7559.

29. Corbiere V, Dirix V, Norrenberg S, Cappello M, Remmelink M and Mascart F. Phenotypic characteristics of human type II alveolar epithelial cells suitable for antigen presentation to T lymphocytes. Respir Res 2011; 12: 15.

30. Sueki A, Matsuda K, Iwashita C et al. Epithelial–mesenchymal transition of A549 cells is enhanced by co-cultured with THP-1 macrophages under hypoxic conditions. Biochem Biophys Res Commun 2014; 453: 804-809.

31. Barkauskas CE, Noble PW. Cellular Mechanisms of Tissue Fibrosis. 7. New insights into the cellular mechanisms of pulmonary fibrosis Am J Physiol Cell Physiol. 2014. 306: C987-C996

32. Kawami M; Deguchi J; Yumoto R, et al. Effect of COA-Cl on transforming growth factor-beta 1-induced epithelialemesenchymal transition in RLE/Abca3 cells Drug Metab Pharmacokinet 2017. 32: 224-227

33. Chiba K, Kawakami K, Tohyama K. Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. Toxicol In Vitro 1998; 12: 251-258.

34. Liang CC, Park AY and Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protocols 2007; 2: 329-333.

35. Kasai H, Allen JT, Mason RM, Kamimura T and Zhang Z. TGF-β1 induces human alveolar epithelial to mesenchymal cell transition (EMT). Respir Res 2005; 6: 56.

36. Jolly MK, Ward C, Eapen MS; *et al*. Epithelial-mesenchymal transition, a spectrum of states: Role in lung development, homeostasis, and disease. Dev Dynam 2018 247: Pages: 346-358

37. Mendez MG, Kojima S-I and Goldman RD. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. The FASEB Journal 2010; 24: 1838-1851.

38. Dixon JB, Jenkins P, Allan D. Immune recognition of *Echinococcus granulosus*. 1. Parasite-activated, primary transformation by normal murine lymph node cells. Parasit Immunol 1982; 4: 33-45.

39. Kanan JHC Chain BM. Modulation of dendritic cell differentiation and cytokine secretion by the hydatid cyst fluid of *Echinococcus granulosus*. Immunology 2006; 118: 271-278.

40. Silva-Alvarez, V, Folle, AM, Ramos, AL, *et al*. Echinococcus granulosus Antigen B binds to monocytes and macrophages modulating cell response to inflammation. Parasit Vectors 2016. 9 Article Number: 69

41. Tamarozzi F, Mariconti M, Neumayr A and Brunetti E. The intermediate host immune response in cystic echinococcosis. Parasit Immunol 2016; 38: 170-181.

42. Siracusano A, Delunardo F, Teggi A and Ortona E. Host-Parasite Relationship in Cystic Echinococcosis: An Evolving Story. Clin Dev Immunol 2012: 12.

43. Vuitton, DA. Echinococcosis and allergy. Clin Rev Allerg Immunol 2004: 26: 93-104

43. Tappeh KH, Uner A. Circulating Antigen Detection in Patient Serum for Diagnosis of Cystic Echinococcosis. J. Pure App Microbiol 2012: 6: 551-556

45. Zeghir-Bouteldja R, Polome A, Bousbata S *et al*. Comparative proteome profiling of hydatid fluid from Algerian patients reveals cyst location-related variation in *Echinococcus granulosus* Acta Tropica 2017. 171 : 199-206

46. Labsi M, Soufli I, Khelifi L, Amir Z, Touil-Boukoffa C. *In vivo* treatment with IL-17A attenuates hydatid cyst growth and liver fibrogenesis in an experimental model of echinococcosis. Acta Tropica 2018. 181: 6-10

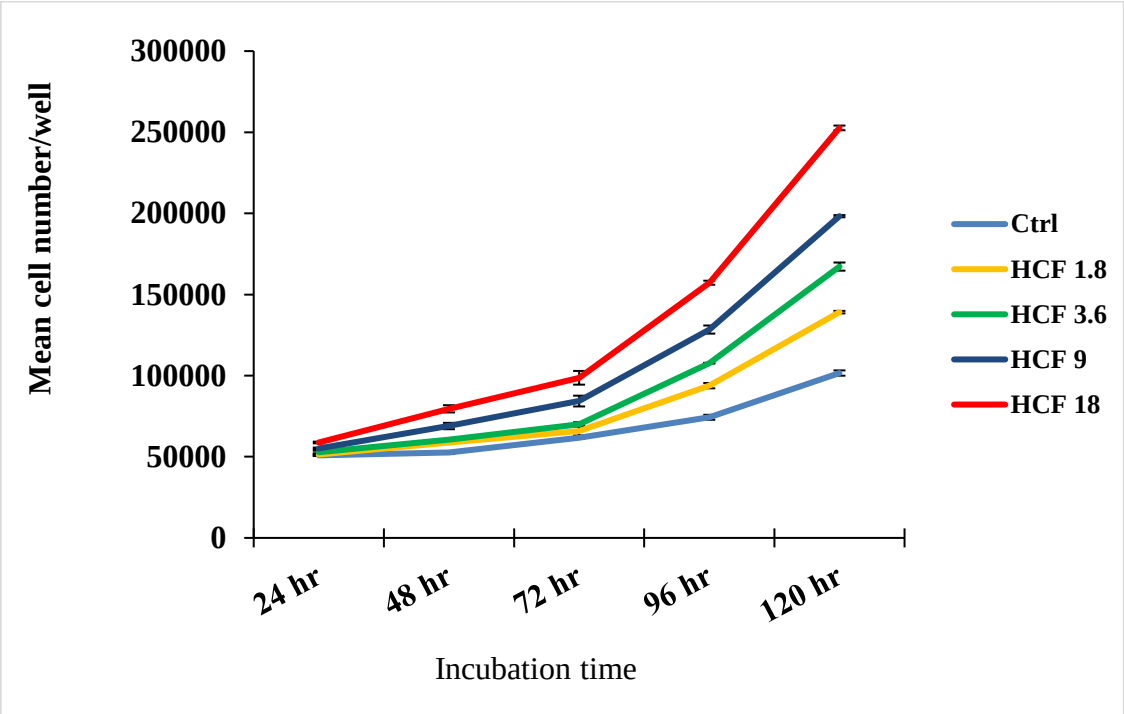


Figure 1

Fig. 1: Proliferation of A549 cells exposed to different concentrations of HCF (1.8-18 µg/mL) over 120 hours in culture. The control is medium plus 0.5% PBS. Standard errors (SE) are shown for the mean of three replicate wells for each treatment. All concentrations of HCF showed significantly more cell proliferation compared to controls at 96 hours ($p<0.05$) and 120 hours ($p<0.01$) but not at sample times before that.

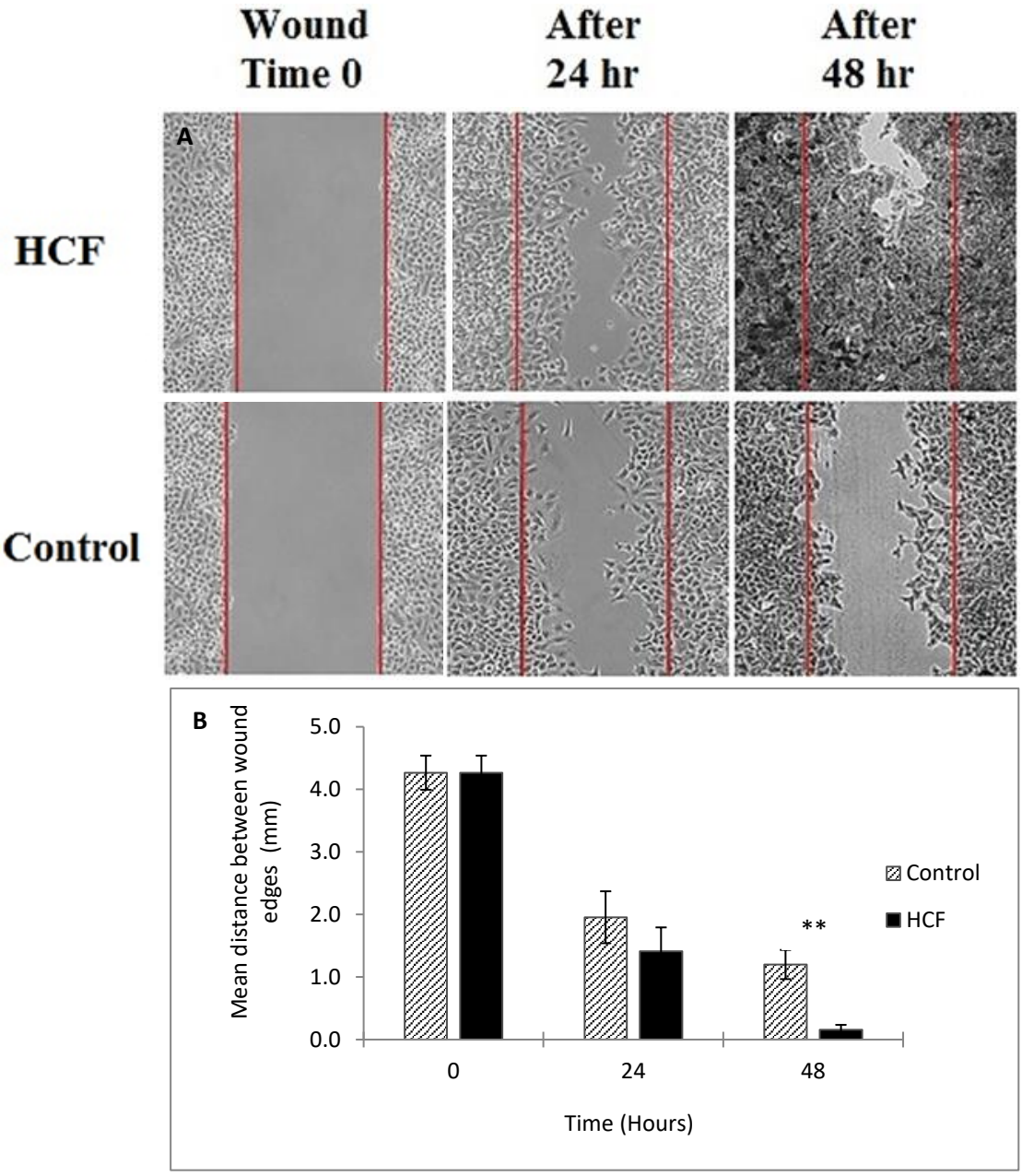
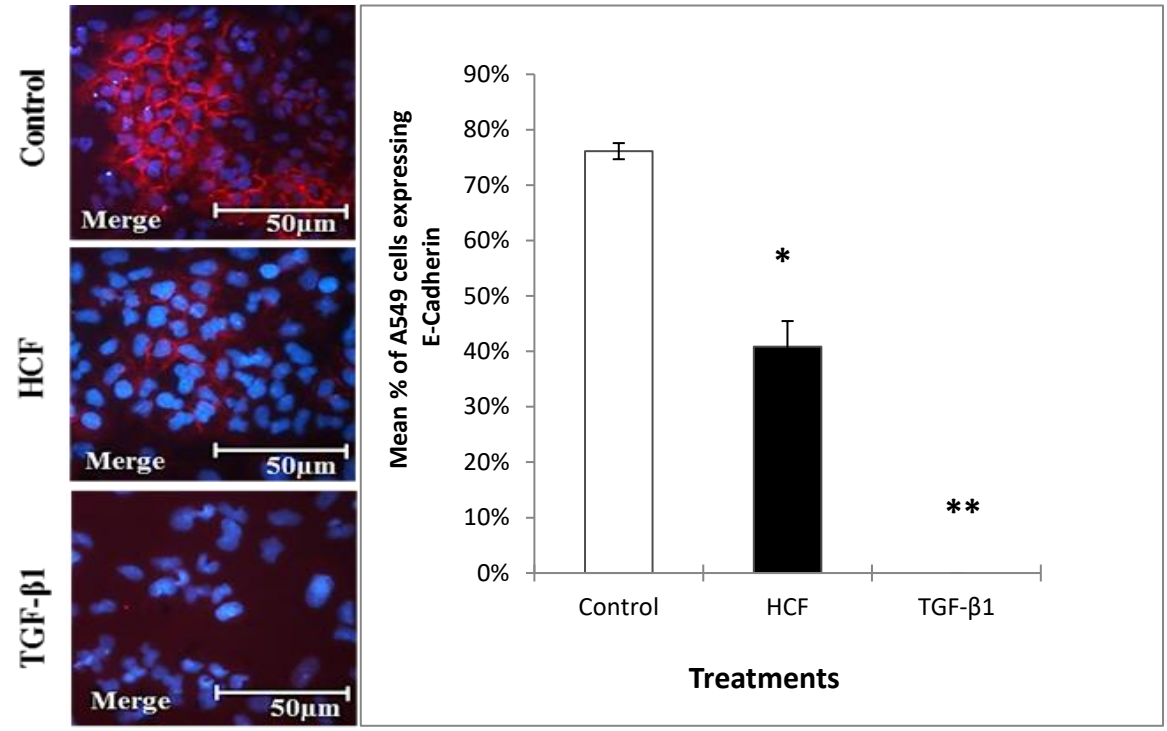
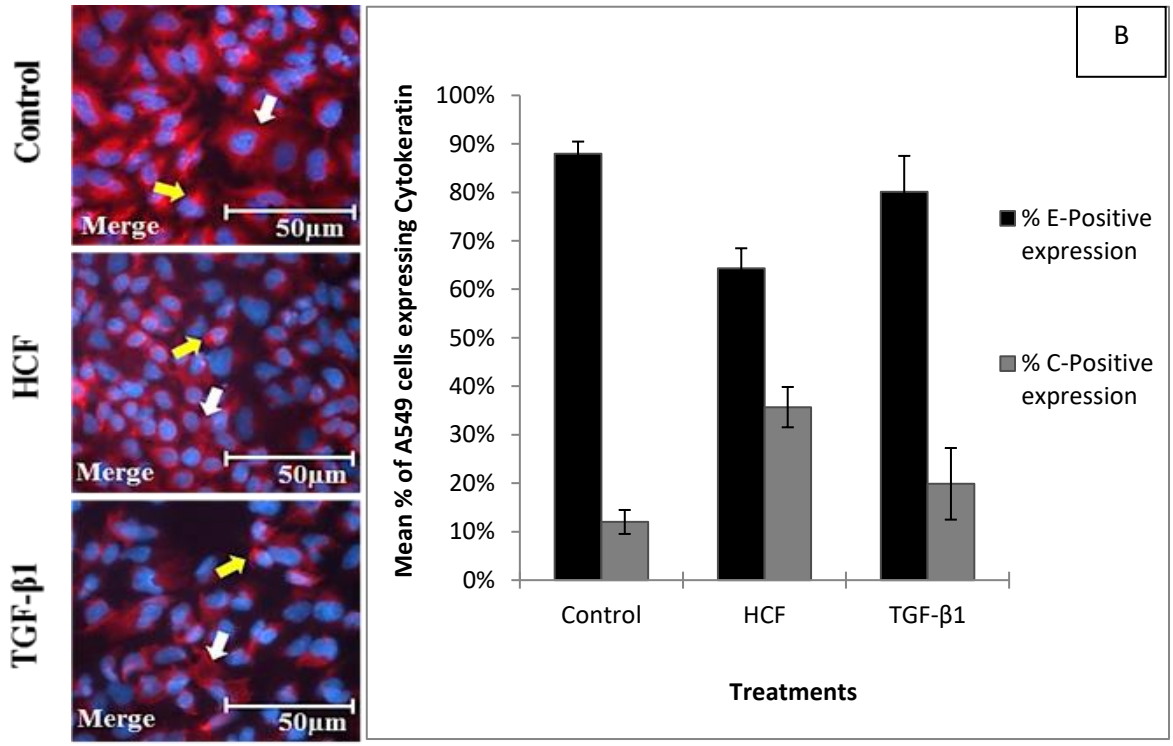


Figure 2

Fig. 2A: Representative views of cell migration in the "wound healing" scratch assay after 0, 24 and 48 hours in culture in the presence of HCF (18 μg/mL) and in control cultures (no HCF). Red lines represent the edge of the initial wound scratch (X100).
Fig. 2B: Mean distance between either side of the scratch after 0 hour, 24 hour and 48 hours of cell migration in HCF treated (18 μg/mL) and control cells (no HCF). The results represent the mean ± SEM of 3 replicates. By 48 hours the gap in HCF treated cells had almost closed. The differences between treated and control cells after *24 hours was not significant ($p > 0.05$) but was after 48 hours** ($p < 0.01$).


Control
HCF
TGF-β1
Merge
50µm
Mean % of A549 cells expressing E-Cadherin
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
**
Control
HCF
TGF-β1
Treatments


B
Control
HCF
TGF-β1
Merge
50µm
Mean % of A549 cells expressing Cytokeratin
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% E-Positive expression
% C-Positive expression
Control
HCF
TGF-β1
Treatments

Figs. 3-4. Merged DAPI/immunofluorescence images of biomarker expression in A549 cells treated with HCF (18 μg/mL), TGF-β1 (5ng/mL) and controls (LSM alone). Original magnification X400. Cell counts represent the mean ± SEM of 3 replicate wells.

Fig. 3 Panel A: E-cadherin expression. Cell counts indicate significantly less cells express E-cadherin in HCF treated cultures* compared to LSM controls ($p < 0.01$) but that there was significantly more expression in HCF treated cultures compared to TGF- β1** ($p < 0.01$) where no cells expressed the marker.
Fig. 3 Panel B. cytokeratin expression. Cell counts indicate no difference in the number of cells expressing cytokeratin in HCF or TGF- β1 treated cultures ($p>0.05$) compared to LSM controls ($p > 0.05$). Two different patterns of staining were evident; extensive across the whole cell (E - white arrow) and condensed around the perinuclear region (C – yellow arrow), but there were no significant differences in the proportions of cells showing either pattern although the intensity of staining in HCF treated cells was less than that of controls ($p>0.05$).

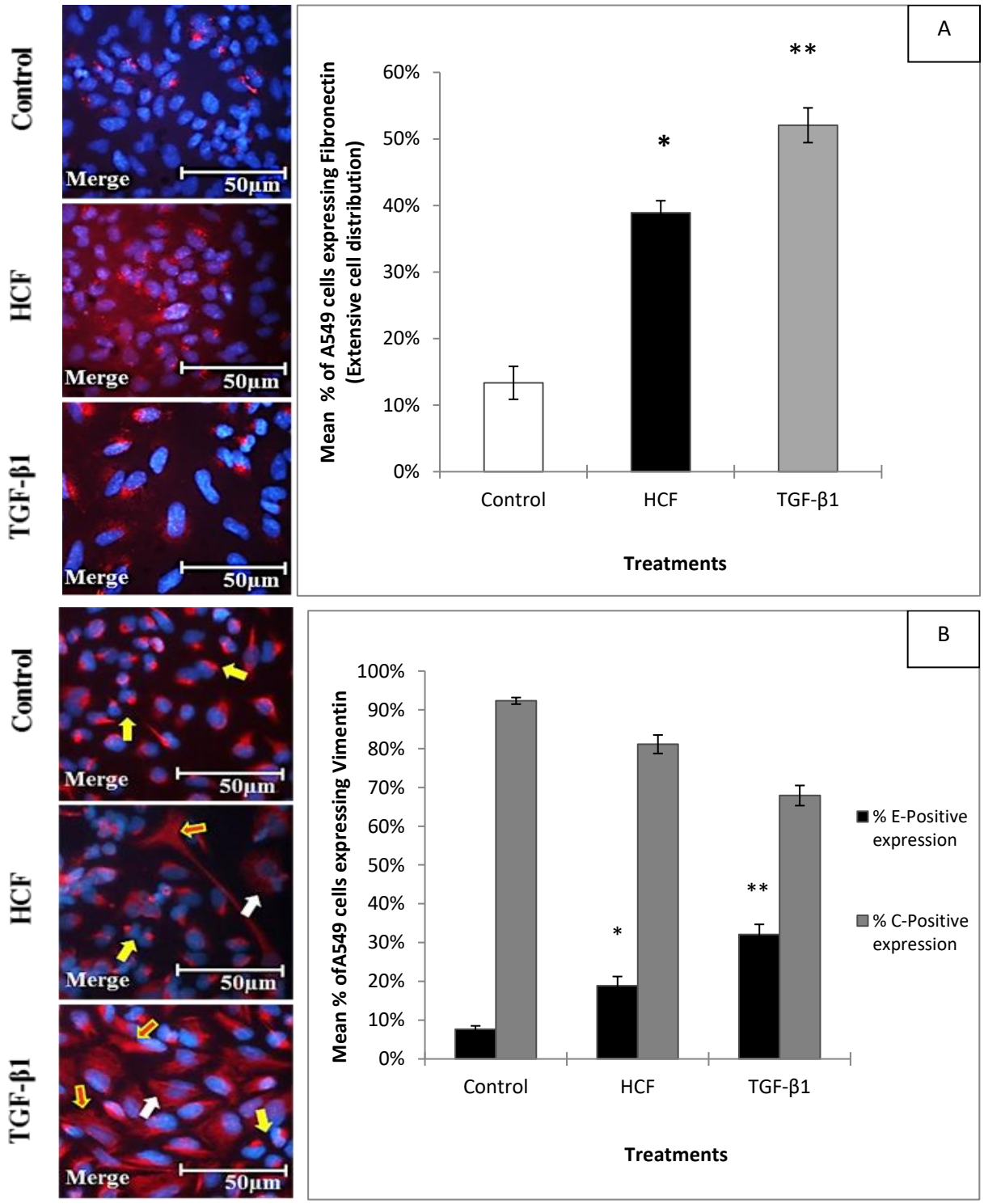


Fig. 4 Panel A: Fibronectin expression. Fibronectin labelling was evident in virtually all cells however two different patterns of staining were evident; extensive across the

whole cell (E - white arrow) and condensed around the perinuclear region (C – yellow arrow), Cell counts indicated that the extensive distribution pattern of the marker was significantly greater in HCF* and TGF-β1** treated cells than LSM controls ($p < 0.01$), but that there was significantly less expression in HCF treated cells than in TGF-β1 treated cells ($p < 0.05$)

Fig. 4 Panel B: Vimentin expression. Most cells in all treatments showed expression of vimentin, however the distribution of the marker differed. In HCF* and TGF-β1** treated cells the extensive staining pattern was present in significantly more cells than controls ($p < 0.05$ and $p < 0.01$ respectively). White arrows show the extensive type of vimentin expression, while yellow arrows show the condensed type of marker expression. Some cells also showed morphological changes involving production of extensive cytoplasmic processes (yellow/orange arrow).