Reviewer’s report

Title: Fibroblast Phenotypes in Different Lung Diseases

Version: 3 Date: 19 June 2014

Reviewer: Omar Franco

Reviewer’s report:

Response to Authors

Major Compulsory Revisions

1) The manuscript requires “substantial” English language editing.
Authors response: According to the reviews suggestion, we invited the company named “Bioedit” (Company no. 04150179 (UK)) to help us edit our manuscript. The reviewers of this company were native English speaker. We believed that our manuscript being edited may make you satisfied.

Reviewer: The revised version only shows a minimum (if none) improvement. Authors should request a revised version to the company or use a different more reliable one. I will leave this matter to the Editor’s discretion.

Examples of wrong grammar use highlighted:

a) We analyzed fibroblasts in a set of samples which including chronic inflammation…

b) CAFs can be formed through several ways and the one of which is named EMT (epithelial-mesenchymal transition)

c) Fibroblasts in lung tissue were investigated recent years. This sentence does not make sense

d) It was not that easy for us to counted fibroblasts or CAFs just like cancer cells

And many more sentences that need editing……

2) Page 1, Line 3: “The role of CAF in lung cancer has been previously investigated”, This statement is not supported in the main text, there is no reference. Also if the role has been previously studied, please provide the main findings.

Authors response: We searched the Medline used “lung”, “cancer-associated fibroblasts” and “stromal fibroblasts”. Several recent articles related were found. We added the main findings about the CAFs in lung tissue in our main text. (see Page 2)

Fibroblasts in lung tissue were investigated recent years. CAFs in lung cancer promoted lung cancer cells proliferation. Vicent S et. al made a cross-species functional characterization of mouse and human lung CAFs and finally found that CAFs supported the growth of lung cancer cells in vivo by secretion of soluble
factors that directly stimulate the growth of tumor cells. They also found that IL-6 (interleukin-6) secreted by CAFs might play roles in promoting cancer cells growth. Besides, CXCL-12/CXCR4 axis existed between the cross-talk of CAFs and non-small lung cancer cells also contributed to the proliferation of cancer cells. Some proteins expressed by CAFs such as matrix metalloproteinase (MMP)-2, α-SMA, podoplanin and carbonic anhydrase (CA) IX may also have a role in predicting the prognosis of lung cancer.

Reviewer: Authors presented relevant literature. However Reference #13 explaining the work by Vincent S et al is missing! Reference #13 and #14 are the same!

The sentence “Besides, CXCL-12/CXCR4 axis…” should be “Besides, CXCL-12/CXCR4 axis…” The chemokine CXCL12 (also known as SFD1-#) binds to two distinct receptors CXCR4 or CXCR7.

3) Page 1, Line 4: “CAFs may also play a role in inflammatory disease”, here there is a fundamental misunderstanding of the term CAF by the authors. CAF as they stated stands for “CARCINOMA associated fibroblasts” emphasizing the presence “only” in the microenvironment of malignant disease. Reactive stroma, activated fibroblasts or myofibroblasts can be seen in inflammatory diseases and in wounds during the process of repair. Please provide a better explanation of the term or use an alternative.

Authors response: We are sorry that we use a wrong word. We reviewed the manuscript and used the word “fibroblasts” or “activated fibroblasts” instead of “CAFs” in inflammatory diseases and in healthy tissue into fibroblasts.

Reviewer: I’m delighted that the authors acknowledged the importance of this fundamental difference to refer CAF as tumor-associated cells vs. those present in non-malignant disease. However there are similarities that need further study and may require a new nomenclature in the future.

In page 2 “In previous studies, the role of CAF was investigated by assessing their tissue-specific expression in one type of human cancer” please provide a reference and specify which type of “human cancer” the authors refer to.

4) Page 1 second line in the Background section: What does “bad” cancer cells mean? Cancer cells are malignant in nature, perhaps the authors meant cancer cells that will show progression vs. those that will remain localized? Please explain.

Authors response: The word “bad” we used in the background section meant that cancer cells showed several features such as progression, invasion and distance metastasis. All these features were not friendly to patients and indicated a poor prognosis. We deleted the word “bad” in order to make it easy for readers to understand. (see Page 1).

Reviewer: Readers understand the difference however the word “bad” should be presented using the proper scientific term(s) to make it clear. Authors changed the expression “bad cancer cells” for “malignant cancer cells”, again cancer cells
are MALIGNANT! However the degree of aggressiveness and whether we can identify cancer cells that have the potential to leave the primary site and metastasize is the main focus of current research. The sentence should read as follows: “The high degree of malignancy and heterogeneity of cancer cells have led researchers to focus on the study of malignant cells present in the epithelial compartment”.

Authors response: Background The “seed and soil” hypothesis explains the importance of interactions between tumor cells and their microenvironment.

Reviewer: Actually the “seed and soil” hypothesis does not “explains” but rather “emphasizes” the importance of interactions….

Authors response: CAFs (Cancer associated fibroblasts) are important components of the tumor microenvironment. The role of CAFs in lung cancer has been investigated previously. Fibroblasts may also play a role in inflammatory disease. We hypothesized that fibroblasts phenotypes may vary among different types of lung disease.

5) Reference 3 by Xing et al is a review in which the authors describe the characteristics of CAF (expression of #-SMA, vimentin, FAP and other markers) and lack of CD31 and epithelial markers without mentioning CK19 loss. Please provide a reference of the lack of CK19 expression by CAF.

Authors response: We reviewed the reference 3 by Xing et al and found that they mentioned the epithelial marker cytokeratin, so we revised this sentence. Besides, we add a reference 4 by who mentioned the epithelial marker E-cadherin. (see Page 2)

CAFs express high levels of certain proteins, including #-SMA (smooth muscle actin) and vimentin (3,10,11), whereas they show negative expression of the epithelial markers cytokeratin and E-cadherin (3)(4).

Here is the text in the reference 3 by Xing et al:

In most cases, CAFs are negative for epithelial or endothelial markers such as cytokeratin and CD31. It was reported that in breast invasive ductal carcinoma (IDC), alpha SMA + myofibroblasts are increased in the cancer regions and CD34+ fibrocytes gradually disappear (19). Reference 4. Gravdal K, Halvorsen OJ, Haukaas SA et al: A Switch from E-Cadherin to N-Cadherin Expression Indicates Epithelial to Mesenchymal Transition and Is of Strong and Independent Importance for the Progress of Prostate Cancer. Clin Cancer Res 2007, 13:7003-7011.

Reviewer: CAF lack expression of CK, however CK19 increases with lung cancer progression. If EMT is considered an important contributor to CAF formation, as the authors suggest, do cancer cells in their transition to EMT express more or less CK19?

6) Results, 3.2.1 Detection of CAFs and epithelial markers: In the first sentence the authors state that a-SMA is expressed in blood vessels and airway ducts in normal and in H (atypical adenomatous hyperplasia) tissues, however 6 lines
below the state” N and H tissues were negative for #-SMA expression (?), Please explain. In figure 1 #,-SMA can be seen expressed in all groups with the highest percentage (optical) and intensity in I>AM>CIS>A>H>N

Authors response: We are sorry that we could not make it easy to understand for authors. In fact, we meant that in N and H, #,-SMA was only expressed in blood vessels and airway ducts. The pulmonary parenchyma cells did not express #,-SMA. We have revised this paragraph as follows: (see Page 4-5)

#,-SMA was detected in benign and malignant tissues and its expression was localized to blood vessels and airway ducts in N, I and H. Stromal cells stained positive for #,-SMA in the three pathological stages of adenocarcinoma, although with different staining intensity. #,-SMA expression was lower in CIS (SI ranged from 1 to 4 and the median was 2) than in A (SI : 1 to 6, median was 4) (P = 0.026), whereas AM (SI: 4 to 9, median was 6) showed higher expression than A (P = 0.009).

Reviewer: The updated is clear, however based on the pictures it can be appreciated that strong ##SMA expression is present in the I group (inflammatory pseudotumor), a feature characteristic of wounds. Perhaps a more accurate description should be “#,-SMA was detected in benign and malignant tissues and its expression was localized to blood vessels and airway ducts in N and H. Stromal cells stained positive for #,-SMA in inflammatory pseudotumors as well as in all three pathological stages…..”

Authors response: Vimentin expression was negative in N and H but positive in I. In the cancer samples, Vimentin expression was higher in A (SI: 2 to 6, median was 3.5) than in CIS (SI: 0 to 4, median was 2) (P = 0.017) and it was higher in AM (SI: 3 to 9, median was 6) than in A (P = 0.022). In general, #,-SMA and vimentin expression increased gradually from N to AM (Fig. 1).

Reviewer: Authors claimed negative expression of Vimentin in H group, however the picture shows clear expression of fibroblasts in closed proximity with the basal cells.

7) The differences between the groups should be presented with a graphical representation using bars or dot plot along with IHC images to see a better stratification of patients within each group.

Authors response: In order to present the differences between the groups, box plot was used. (see figure 4)

Reviewer: The authors should present the box plot along with statistical analysis. Labels (X and Y axis) are too small to read when printed.

In graphs showing correlation of Twist and aSMA or E-cadherin, instead of “Twist” is Twist.

There is no consistency in the use of fonts in graphs (different types), nor size of fonts.

8) Quantitation of the intensity is a somewhat subjective assessment. There are
several digital
methods published in the scientific literature that can be used to validate the
results. If the authors have access to frozen tissues a quantitative measure by
qRT-PCR or protein analysis can be more informative.

Authors response: We have tried the software named “Imagepro_plus” when we
validated our results. We found that this software was very practical. We used
the IOD (integrated optical density) recommended by some scientific literature to
measure the sections. But the IOD values measured by this software were
unsatisfactory for the software mechanically assessed the results. Many factors
may had an effect on the IOD. Compared with other kinds of tissues which were
very “clean”, lung tissues were always contaminated by the dust in the air. The
dust in samples had a great influence on the IOD value measured by software
though we tried out best to avoid it. Finally, we had to give up using software to
assess the results. We searched many literatures which also used IHC in their
studies and finally we decided to use SI (staining index, just as mentioned in our
study) to validate the results. We had to acknowledge that quantitation of the
intensity was a somewhat subjective assessment. Our sections were from
pathology department of our hospital and all the samples were fixed using
formalin and embedded by paraffin, so we could not obtained any samples which
could be used for frozen tissues and qRT-PCR. We used IHC in order to made a
preliminary analysis of the phenotypes conversion of fibroblasts in different lung
disease and we planned to collect fresh samples and used qRT-PCR in our next
studies.

Reviewer: The method used by the authors to quantitate SI is rather confusing.
SI was calculated by multiplying intensity (graded 0-3) and percentage of staining
(0-3)? Is this a standard method used? Please reference. Perhaps a more
widespread method is the addition of both values.

To my knowledge there is no scientific literature addressing the problematic of
dust in IHC sections.

However, authors could isolate RNA from FFPE tissues and perform qPCR
analysis to show increased expression of TGFβ.

9) Figure 1, vimentin expression, same concerns as #-SMA

Authors response: We are sorry that we could not make it easy to understand for
authors. In fact, we meant that in

N and H, #-SMA was only expressed in blood vessels and airway ducts. The
pulmonary parenchyma cells did not express #-SMA. We have revised this
paragraph as follows: (see Page 4-5)

 #-SMA was detected in benign and malignant tissues and its expression was
localized to blood vessels and airway ducts in N, I and H. Stromal cells stained
positive for #-SMA in the three pathological stages of adenocarcinoma, although
with different staining intensity. #-SMA expression was lower in CIS (SI ranged
from 1 to 4 and the median was 2) than in A (SI : 1 to 6, median was 4) (P =
0.026), whereas AM (SI: 4 to 9, median was 6) showed higher expression than A
Vimentin expression was negative in N and H but positive in I. In the cancer samples, Vimentin expression was higher in A (SI: 2 to 6, median was 3.5) than in CIS (SI: 0 to 4, median was 2) \( (P = 0.017) \) and it was higher in AM (SI: 3 to 9, median was 6) than in A \( (P = 0.022) \). In general, \#-SMA and vimentin expression increased gradually from N to AM (Fig. 1).

Reviewer: See comments above.

10) Cytokeratin 19 expression in Figure 2; poor tissue in CIS sample, please use consecutive slices of tissues.

Authors response: As we all know that lung cancer is very easy to progression and metastasis. Unlike other pathological stages of lung cancer, samples of CIS (carcinoma in situ) of lung cancer is relatively hard to found and the size of samples was very small. We could not obtained enough sections from a single patient. As our samples came from pathology department of our hospital, we were very sorry that we could not found any other samples for our study.

Reviewer: Authors should note in the text that collection of CIS samples were limited.

11) Figure 3. TGF-#: assessment of TGF-# expression by IHC is very difficult. This secreted protein can be present in the epithelial and stromal compartment, perhaps staining with phosphor-Smad2/3 can show activation of the TGF-# pathway in tumor or stromal cells. Again if frozen tissues are available it can be coupled to the total expression of TG-# by the tissue, but it cannot identify the cell secreting the protein. Sorting cells by flow cytometry (epithelial vs stromal cells) will answer this question.

Authors response: In the present study, the most important thing we wanted to prove was that the fibroblasts had a phenotype conversion in different lung diseases. The purpose we tested the expression of TGF-# was to prove that the EMT (epithelial-mesenchymal transition) might play an critical role in the phenotype conversion of fibroblasts. Yoko Katsuno et al. had proved that TGF-# played a role in EMT and expressing of \#-SMA. (Yoko Katsuno, Samy Lamouille, Rik Derynck: TGF-# signaling and epithelial–mesenchymal transition in cancer progression. Curr Opin Oncol 2013, 25:76–84. András Masszi, Caterina Di Ciano, Gábor Sirokmány et al: Central role for Rho in TGF-#-induced \#-smooth muscle actin expression during epithelial-mesenchymal transition.) Am J Physiol Renal Physiol 2003, 284: F911–F924.) Besides, all the samples were from the pathological department of our hospital, and all tissues were embedded with paraffin. We could not obtain any fresh tissues for frozen. So, we planned to research the mechanism and the TGF-# pathway using frozen tissues and flow cytometry in our next step.

Reviewer: The role of TGFβ in EMT or the activation of fibroblasts is well established. The literature presented by the authors corroborates this observation. However in this manuscript authors are attempting to attribute increased expression of TGFβ using IHC (again this is not the standard method
to assess TGFβ expression/secretion) and the expression of some proteins present during EMT or during the conversion of fibroblasts to myofibroblasts. Since this is the main purpose of the authors, it is imperative to show some “causative” effect by activation of TGFβ signaling in these tissues. A classical, reliable and simple approach is the staining against the phosphorylated Smad2/3 complex and show nuclear translocation. This method can be easily quantitated in an automated or semi-automated manner and can add more relevance to the role of TGFβ, a message that the authors are eagerly pursuing.

12) It will be useful to have the N, I and H groups in Fig3 to compare the level of TGF-#, Twist and FAP with the other groups

Authors response:As mentioned in our results (see Page 5 results 3.2.2), TGF-#, FAP, and Twist expression was detected in the cytoplasm of cancer cells. The three factors were expressed in I, CIS, A, and AM, whereas N, H, and tissues 2 cm away from the tumor focus were negatively stained. As Fig 1 and 2 did not exhibited tissues 2 cm away from the tumor focus and the reason that TGF-#, FAP, and Twist were mainly expressed in lung cancer samples, we showed these samples in Fig 3. N and H did not expressed these factors.

Reviewer: Inclusion of control samples are always informative. Unfortunately authors decided not to shown them.

It is to note that the use of arrows to indicate positive staining should be described in the main text or Fig. Legends. Authors never mention whether readers should be looking at the nuclear or cytoplasmic staining. When staining for nuclear proteins is performed the use of hematoxylin is preferable to differentiate to fast red.

13) Twist expression seems to be present in the nucleus. Authors claim to be in the cytoplasm. Explain

Authors response:Study by Guanghui Wang et al. demonstrated that twist was located in both the cytoplasm and nucleus. (Guanghui Wang, Wei Dong, Hongchang Shen et al: A comparison of Twist and E-cadherin protein expression in primary non-small-cell lung carcinoma and corresponding metastases. European Journal of Cardio-thoracic Surgery 2011, 39:1028—1032.)

We found that our results were in accordance with Wang’s and we had revised our results as follows.(see Page 5)

TGF-# and FAP expression was detected in the cytoplasm of cancer cells while twist was located in both cytoplasm and nucleus. The three factors were expressed in I, CIS, A, and AM, whereas N, H, and tissues 2 cm away from the tumor focus were negatively stained (the results of N, I, and H were not included in Fig. 3). TGF-# expression followed a similar pattern than that of #-SMA and vimentin, with positive expression in I, CIS, A, and AM. TGF-# was expressed at higher levels in AM (SI: 3 to 9, median was 6) than in A (SI: 2-9 median was 3.5) (P<0.001). No statistical significance was seen between CIS and A (P = 0.392). On the other hand, Twist expression was seen in A and AM.

Reviewer: Twist is a transcription factor and its function correlates with its nuclear
localization. Do the authors quantitated nuclear and/or cytoplasmic stainings?

14) FAP is considered a stromal marker (PubMed; PMID: 23835897), however the authors found localization in cancer cells with increased expression in CIS compared to the other groups based on the IHC images. Was the quantitation made considering only stromal FAP?

Authors response: In our results we found that FAP was located in the matrix of cancer cells and the CAFs in the stromal of cancer tissues did not expressed FAP. Though we also have known that FAP was considered to be a stromal marker. We had discussed this novel results in our manuscript as follows. (see Page 8)

However, our results showed that FAP was expressed in the cellular matrix of cancer cells rather than in the cellular matrix of CAFs, and no statistically significant differences in its expression were detected among CIS, A, and AM tissues. TGF-# expression was also localized to the matrix of cancer cells, which was not consistent with the results reported by Xing et al. who showed that the secretion of TGF-# by CAFs promotes their proliferation. These two novel phenomena revealed by our findings indicated that cancer cells might have a similar function than CAFs and CAFs may not originate exclusively from normal epithelial cells but may also be derived from mutant epithelial cells, including cancer cells.

Reviewer: Authors used the expression “cellular matrix” throughout the manuscript referring to the cytoplasmic compartment of cells. Please use the correct scientific term.

15) The A group show “nuclear” FAP? Authors used a rabbit antibody purchased from Santa Cruz Biotechnology. Santa Cruz sells five antibodies against FAP (three goat, one mouse and one rabbit), none of them are recommended for IHC. The rabbit antibody FAP# (clone H-56) is a protease that can be found in the cytoplasm or ECM. Can the authors provide validation of this antibody for IHC?

Authors response: The A group showed FAP was located in the matrix of cancer cells. The antibody of FAP was from Assay Biotech, USA. (FAP antibody #C0188)

Reviewer: The authors “changed” the FAP antibody used for IHC from Santa Cruz to Assay Biotech, however the images were the same (?).

16) Several papers have addressed the complex nature of the tumor stroma. Due to the high heterogeneity and the lack of a “single” marker of CAF, attempts have been made to show co-expression at the cellular level of these potential markers. Thus double/triple co-expression of the most abundant primers and categorizing with each group should be presented.

Authors response: In each group, we detected three CAFs markers which were #-SMA, FAP and vimentin, though we found that in our study FAP was located in cancer cells. Fig 1 showed the results.

Reviewer: Have the authors attempted to show co-expression by
immunofluorescence of #SMA and vimentin? Myofibroblasts (or activated fibroblasts), the major component of CAF express both markers during the transition from their basal fibroblastic nature.

17) Figure 4. This graphs are rather confusing. Were the comparisons made for each group or all the groups combined? What do the individual circles represent (patient/s)? Why are there different number of circles in each graph? Are the X and Y axis representing SI index? If so why the scale is different in some, instead of 10 scale (first graph) up to 4 or 6 in the others? Please show consistency and better labeling. Perhaps a better representation would be to show a table and correlations.

Authors response: The comparisons were made for all the 3 groups of lung cancer samples (including 3 groups: CIS, A and AM, totally 66 samples). And the individual circles represent patients in this group. But some cycles were overlapped because these samples had a same score. All the four pictures in Fig 4 contained 66 samples, but some patients had the same score, so these cycles were overlapped. The X and Y axis representing SI index. The SI values of the factors we detected had different ranges. The SI of both #SMA and TGF-# ranged from 0 to 9, the SI of E-cadherin ranged from 0 to 4, the SI of twist ranged from 0 to 6. So the scale is different in some, instead of 10 scale (first graph) up to 4 or 6 in the others.

We have revised in our manuscript and made a detailed explanation (see Page 5):
All the lung cancer samples (including CIS, A and AM, totally 66 samples) expressed different levels of #SMA, vimentin, E-cadherin, Twist and TGF-#. We performed a correlation analysis between TGF-# and #SMA, TGF-# and vimentin, TGF-# and E-cadherin, Twist and #SMA, Twist and E-cadherin and Twist and #SMA in all the cancer samples.

The figure legends of Fig. 4 was revised according to the reviewer’s reports and number of patients, type of tissues and the meaning of each cycle was supplemented. (see figure legends of Fig. 4 in Page 12): Correlation analysis between TGF-# and #SMA, TGF-# and E-cadherin, Twist and #SMA, Twist and E-cadherin. Correlation analysis between TGF-# and #SMA, TGF-# and E-cadherin, Twist and #SMA and Twist and E-cadherin were performed in all the 66 lung cancer samples (including CIS, A, AM). Each cycle in the figure represented one lung cancer sample. But some cycles were overlapped because these samples had a same score.

Reviewer: Box plots are show a better stratification of each groups and show an increased expression of aSMA, vimentin and TGFß in tumor samples and a decreased expression of E-Cadherin.

Changes in formatting and statistical analysis were discussed above (see point#7)

18) The major question(s) of the paper has not been answered. Do the CAF phenotype vary in lung disease, if so, can this variation explain tumor
Authors response: Our study demonstrated that CAFs phenotype varied in lung disease. But we only used IHC and we did not detect gene expression. We planned to use CAFs phenotype conversion to explain tumor progression using western blot, qRT-PCR, PCR and animal disease models in our next steps.

Reviewer: These results suggest that there is an increased expression of aSMA and Vimentin as tumor progresses. However, similar changes can be observed in non-malignant tumors (inflammatory disease). So instead of a variation, fibroblasts are prone to show the same changes highlighting the importance to understand fibroblasts activation in benign and malignant disease.

In page 2 authors state: “We hypothesized that TGF-ß and Twist play key roles in lung carcinogenesis”, however no direct experimental evidence of the “key role” of TGFß or Twist is presented but rather weak associations using IHC.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Not suitable for publication unless extensively edited

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I declare that I have no competing interests