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Lineage tracing of mesothelial cells during parietal adhesion formation

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Abstract

Peritoneal adhesions (PAs) are fibrotic tissues that form after abnormal healing between organs, bowel and parietal peritoneum and limit mobility. They usually form after surgeries and induce further health problems that do not have an effective treatment. Mesothelial cells (MCs) are known to undergo mesothelial to mesenchymal differentiation (MMT) post surgeries and to contribute to the pool of fibroblasts that aid the PAs development. In this study, we performed a lineage tracing experiment to analyse the function of Wt1 positive mesothelial cells in PAs formation in a mice model. The tamoxifen inducible Cre recombination system labels the Wt1 positive MCs cells in green, allowing tracing after the PA formation. Immunofluorescence was used to compare the mesothelial cells with specific markers for mesenchymal phenotype and Wt1 expression. Our results show that Wt1 positive MCs do have a role in PA formation, however we also hypothesize that other key factors might have important roles in maintaining fibroblasts and fibrin production high.

Key words: Parietal adhesion, Wilms' tumor 1, CreERT/loxP system, mesothelial cells, mesothelial to mesenchymal differentiation
Abbreviations: Wt1- Wilms' tumour 1; MC- Mesothelial cells; PA- Peritoneal adhesions; MMT- mesothelial to mesenchymal differentiation

Introduction

The peritoneum is a complex organ constituted of submesothelial connective tissue, basal lamina and mesothelium cell layer. Its main function is maintaining friction free movement between the peritoneal wall and abdominal viscera [1]. Surgeries that breach and injure the peritoneum onset inflammatory signals that lead to an immune response, a wound healing response and a subsequent fibrosis [2]. The tipping point in PA formation is the local equilibrium between fibrinolysis and fibrin production [3]. If the cell mass resulting after injury is not inhibited by fibrinolysis and persists after peritoneal repair (normally 3 to 5 days), fibroblasts migrate in and deposit extracellular matrix, such as fibronectin and collagen. This leads to the scaffold formation on top of the mesothelial cells, followed by re-epithelialization and adhesion formation [4]; hence peritoneal adhesion formation is defined as an abnormal fibrous connection joining caused by an atypical tissue healing process [5].

Frequency of PAs is very high in surgery situations; occurring in 55% of women who undertake laparoscopies. This occurring rate is similar to most surgical procedures involving intra-abdominal injury in both men and woman, and the rate of PA reformations [5,6].

Consequently, the clinical and life quality costs are significant: most patients suffer from bowel obstruction, chronic abdominal and pelvic pain, extensive adhesiolysis and even infertility. Additionally, there are economical drawbacks; a study in the U.S. calculated 1.3 billion dollars annually spent only on the hospitalization costs, without taking into account the possible social shortcomings [7].

The pathology of PAs is largely not known, with most used prevention methods being optimization of surgical techniques, including the handling of protective barriers during surgeries [8]. Clinical research focuses on developing treatments to arrest adhesion formation pathways and tip the balance for adhesion resorption. One way to achieve this is regulating the inflammatory response; a number of steroidal and anti-inflammatory drugs have been considered, such as aspirin and dexamethasone, however none of which showed exciting potential. Macrophage recruitment is a crucial factor in triggering postoperative inflammation during PAs formation [9]. Studies to counteract this have demonstrated that inhibiting OPN levels, a chemokine like protein that plays key roles in several processes connected to tissue repair, leads to significant decrease in scar formation and tissue granulation [10]. Other studies have been focussed on stopping the process of angiogenesis in its early stages to limit tissue remodelling [11].

Recently literature has placed high emphasis on fibrin formation and degradation, how to discontinue the influx of fibroblasts and fibrin production during wound repair. Fibroblasts endorse fibrin production by producing inflammatory cytokines, chemokines and growth factors, promote angiogenesis, secrete metalloproteinases and extracellular matrix proteins and act as a contractile force for wound closure via smooth muscle actin (SMA) expression [12,13]. Different hypothesises have been proposed on the source of fibroblasts in tissue scarring; one of the most common hypothesis supports epithelial-mesenchymal transition (EMT). The process of EMT is an auto-controlled mechanism, however in certain conditions the regulatory processes fail and there is formation of fibrous tissue instead of normal healing [14]; consequently, EMT has been associated in several fibrogenic disorders. Different studies have shown EMT happening in wound healing situations by trans-differentiation of epithelial cells into active fibroblasts [15–17].

In PA formations, similar processes take place where mesothelial cells differentiate (MMT), and after the reprogramming, cells lose specific traits and/or markers and acquire a phenotype distinctive to mesenchymal and fibroblasts [18]. Mesothelial cells transformed into fibroblasts attain a strong capacity to produce extracellular matrix components, such as collagen and fibronectin, but also angiogenic factors; this being a key factor in adhesion formation [19]. Different studies have shown that MMT is involved in the PA formations and is upkept by regulating expression of different factors such as TGF-β, IL-6 and PDGF receptor [20,21]. Also, Suzuki *et al* demonstrated the significance of mesothelial cells as a barrier against adhesion formation, showing that maintaining the mesothelial cell layer intact could lead to PA prophylaxis [22].

Wt1 it is a crucial factor in the regulation of transition between the mesenchymal and epithelial states, it can drive either transition depending on its temporo-spatial expression. For example, while Wt1 expression in the heart generates cardiovascular progenitors from epicardium (EMT transition), in renal development it is a key regulator in the reverse process (EMT transition) and produces nephron cells. Its power to change the balance between the epithelial and mesenchymal states has also been demonstrated in the intestines; Wt1 positive mesothelial cells undergo ETM reprogramming and contribute to the angiogenesis process [23,24]. Moreover, it was also proved that knockdown of WT1 in human adult epicardiac tissue induces the EMT transition [25].

In this study, we used a lineage tracing approach using tamoxifen inducible Cre-Lox system, that allowed us to analyse the fate of Wt1 positive MCs in a parietal adhesion mice type model (Figure 1). We hypothesize that Wt1 expression in mesothelial cells might have a front function in not only formation of adhesion fibrosis but also in influencing other key pathways that add to this phenotype.

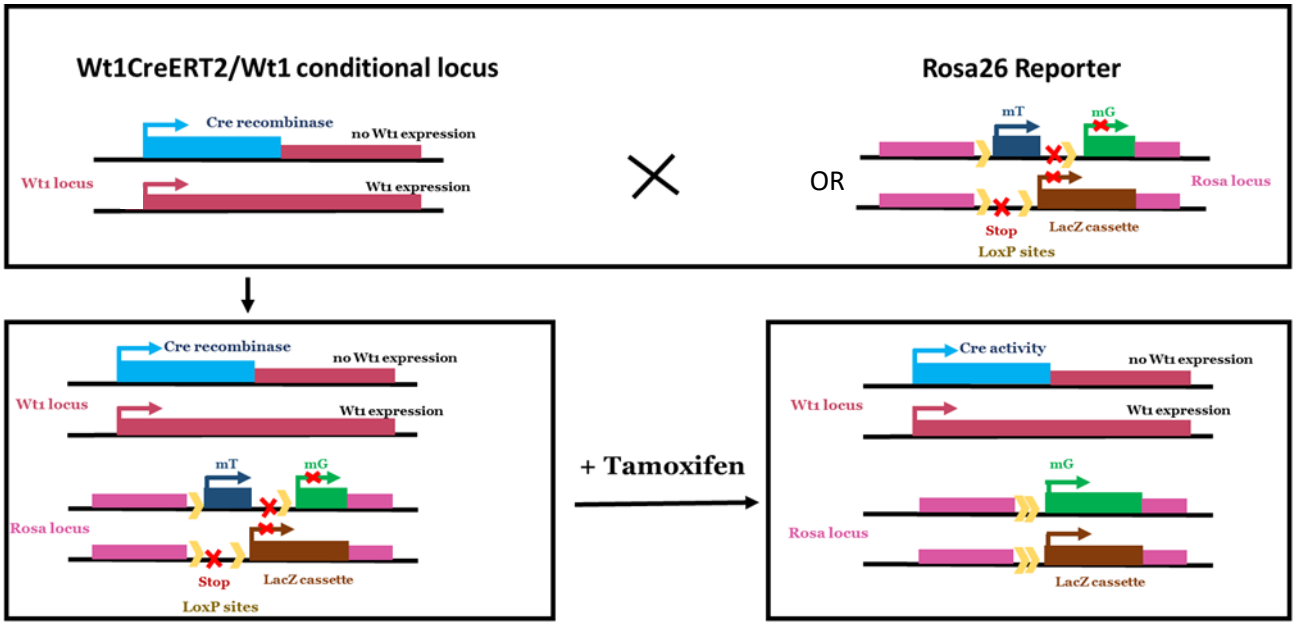

Figure 1| Construction of the tamoxifen inducible Cre-lox system used for the lineage tracing of Wt1 positive mesothelial cells. Mice with the Wt1CreERT2/Wt1 genotype were crossed with mice positive for the Rosa26 Reporter. Two different Rosa26 Reporter homozygous genotypes were used in this experiment: coERT2mTmG and coERT2LacZ. The resulting progenitors were genotyped and the positive ones for both alleles were used in this experiment. After tamoxifen was administrated, the Cre recombination would become activated and the membrane bound GFP protein (mG) or LacZ cassette would be expressed.

Materials and Methods

Animal handling and experimental procedures

All animals used for these experiments were housed and bred in the Animal handling facility (BSU building) of the University of Liverpool and were kept in compliance with the Home Office regulations. Two mice genotypes were used (**Figure 1**): coERT2mTmG from which the frozen sections were produced, and coERT2LacZ that generated the paraffin sections. In day 1 to day 5 tamoxifen was administrated at 4mg/40g body weight (40mg/ml in con oil), this being followed by a wash out phase during day 6 to day 14. In day 15 the surgery was performed to create the adhesion model; the cecum was attached to the parietal wall forming an adhesion like structure. In day 14 post surgery the mice were culled and the adhesions structures together with the kidneys were dissected and further processed. Body weights were measured daily throughout the experiment. All animal experiments were performed by Dr. Thomas Wilm under permission of the license and with conformity of Home Office regulations.

Tissue fixation and handling

The adhesions and kidney fragments were kept in PBS on ice until pictures of endogenous GFP expression were taken. The tissue fixation was performed overnight in 4% PFA on a rocker. All tissues were then washed for 3x 1 hour in 1% PBS.

For the frozen sections, the fixed tissue was kept overnight in 30% sucrose on a rocker, then the samples were embedded on dry ice in OCT (Richard-Allan Scientific™ Neg-50™ Frozen Section Medium). Both adhesion and kidney tissue were cut at 7 µm thickness sections with a Thermo Scientific HM525NX machine; the resulting slides were maintained at 80˚C until usage.

For the paraffin sections, the tissues were dehydrated through a series of ethanol concentrations, following washes with isopropanol for 2x1 hour, then 1hr incubation at 60˚C in 50:50 paraffin to isopropanol mixture. The tissues were incubated at 60˚C in paraffin overnight, embedded plastic moulds and then kept at 4˚C until sectioning. The sections were acquired with a Thermo Scientific Shandon Finesse 325 microtome with a thickness of 7µm. Before collection, the tissues sections were kept for 2 minutes at 40˚C in a water bath. The resulting slides were kept at 37˚C overnight to dry.

HIAR protocol optimization

The slides were dewaxed for 2x5 min in HistoClear II (National Diagnostics) and then rehydrated in a series of ethanol concentrations (100%, 96%, 75% and 50%). Three different protocols were tried prior to the immunofluorescence:

- Protocol 1: All sections were microwaved at 800V for 15 min in 10mM citrate buffer (pH 6). The slides were then left for another 15 min in the hot buffer.
- Protocol 2: All sections were incubated at 60˚C overnight in with 0.2M boric acid (pH 7).
- Protocol 3: All sections were incubated at 60˚C for 7 hrs in with 0.2M boric acid (pH 7).

Immunofluorescence protocol

All frozen sections were kept for 45 min at RT to dry before the start of the protocol. Both frozen and paraffin sections were then washed in 1% PBS for 3 x 5 minutes, then 10 minutes in 1% PBS with 0.25% Triton, following 3 washes in 1% PBS. The slides were then blocked for 1hr in 1% PBS with 2% BSA. The primary antibodies were incubated at 4˚C overnight and then washed off with 1% PBS for 3x15 min. To avoid additional background, we used primary labelling kits for the primary anti-mouse antibodies (The Zenon® Alexa Fluor®). The secondary antibodies and DAPI staining were added for 1hr at RT in the dark. Following 3 washes with 1% PBS and one wash with ddH_2O, the slides were mounted with Fluoro-Gel (Electron Microscopy Sciences) and left to dry for 45 min at 4˚C in the dark. The primary and secondary antibodies used together with best conditions for each are presented in **Table 1**.

The resulting images were captured with a Leica DM2500 microscope attached to Leica DFC350FX camera and then processed in Adobe Photoshop programme.

Table 1| List with primary antibody used and protocols for each one. *indicates the protocol that generated best results for that specific primary antibody; mono-monoclonal; poly-polyclonal

Antibody	*Product nr.*	*Company*	*Isotype*	*Tissue used*	*Optimised Protocol**
Wt1	Ab89901	Abcam	Rabbit mono	Frozen sections	2nd Abs
	Sc-192	Santa Cruz	Rabbit poly	Frozen + Paraffin	2nd Abs
	GTX15249	GeneTex	Rabbit poly	Frozen + Paraffin	2nd Abs
	05-753	Millipore	Mouse IgG1	Frozen + Paraffin	Direct labelling
Ki67	NCL-Ki67p	Novocastra	Rabbit poly	Frozen sections	2nd Abs
α-SMA	A2547	Sigma Aldrich	Mouse IgG2a	Frozen + Paraffin	Protocol 2 + 2nd Abs
Desmin	A0611	DakoCytomation	Rabbit poly	Frozen sections	2nd Abs
CD31	Ab2864	Abcam	Rabbit poly	Frozen sections	2nd Abs
	550274	BD Pharmingen	Rat poly	Frozen sections	2nd Abs
Vimentin	Ab8978	Abcam	Mouse mono	Frozen sections	-
P4/80	ab170192	Abcam	Mouse IgG2b	Frozen sections	-
GFP	Ab6556	Abcam	Rabbit poly	Frozen sections	2nd Abs

Results

Lineage tracing with the Cre-lox system was successful

After dissection of the adhesion, we looked under the microscope to confirm that the Cre recombination was successful. As we can see in **Figure 2A-D**, the GFP positive cells near the adhesion have a more mesenchymal phenotype in comparison to the ones further away, which look more similar to mesothelial cells. Moreover, we can see that the cells present a movement towards the formation of the adhesion; more GFP expressing cells are present in the vicinity of the formation. This specific pattern of GFP expression is the first confirmation that the Cre-Lox system has worked.

Moreover, Dapi staining on frozen sections with the endogenous expression mTmG allele also showed us that the Cre recombination was successful. As we can see in **Figure 3A**, the endogenous GFP was activated after the administration of tamoxifen and the Wt1 cells are visible in green in the glomeruli of the kidney. When we look at the endogenous expression in the adhesion, we can see how the Wt1 expressing cells are on the mesothelial layer next to the adhesion, moving slowly toward the centre of it. In the same time the tomato is fading in a very specific pattern in both organs, this illustrating that only certain cells expressed Wt1 at the time of tamoxifen administration. Consequently, the precise expression of the tomato positive cells is another marker of Cre recombination's success.

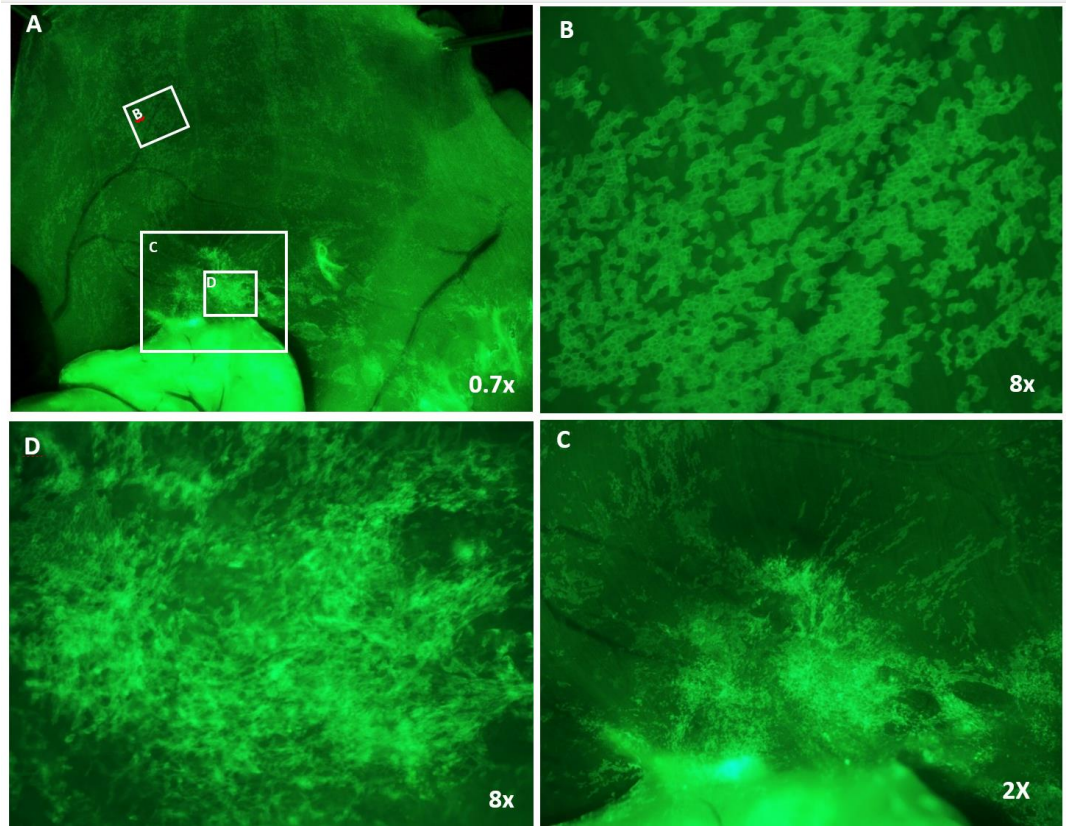

Figure 2| Tracing Wt1 positive mesothelial cells near the adhesion formation. A) Overall picture of the adhesion and parietal wall that shows the GFP positive cells; as we get nearer to the adhesion we can see a more pronounced GFP positive cells with different phenotype. **B)** Further away from the adhesion the GFP expressing cells have mesothelial type morphology, however as we move along the parietal wall nearer the adhesion formation **(C+D)** the cells morphology changes and becomes more like a mesenchymal phenotype.

Wt1 expressing mesothelial cells tend to differentiate towards mesenchymal lineage

After verifying that the Cre recombination succeeded, we looked at what lineage did the Wt1 expressing cells migrated towards. We achieved this by trying different markers on frozen and paraffin sections for the mesenchymal lineage and markers for the Wt1 expression.

To analyse the expression of Wt1 in the adhesion we have tried four different primary antibodies in different conditions. Wt1 antibody from Abcam (**Table 1**) on frozen sections gave us the most conclusive results with the least background and unspecific staining. Due to the endogenous expression of GFP being very low, we have added an anti-GFP antibody primary. As we can see in **Figure 3B-C**, Wt1 is expressed around and in the adhesion in certain cells. It is interesting to notice that some cells express Wt1 in both colours (before and after the adhesion formation) whereas other cells express Wt1 only after the adhesion formation or only before the injury that lead to the adhesion. While we expected to see the cells that maintain a constant expression of Wt1 or switch it off after they proliferate and differentiate into mesenchymal cells, we did not expect that specific cells will turn on the expression only after the injury event. This occurrence can be also seen in the caecum close to the adhesion formation (**Figure 3D**); Wt1 expressing cells from the mesothelial layer should all be GFP positive cells, however only selective few are expressing GFP and less so still express Wt1 after the injury.

To better understand this phenomenon, we have firstly tried Ki67 marker, a marker used to stain proliferating cells. Results from both caecum and parietal peritoneum near the adhesion showed us that cells do proliferate. In the parietal peritoneum, there is a gradient layer that starts next to the adhesion and as we go further away, we see less proliferating cells (**Figure 4A-E**). This also sustains our hypothesis that cells from further away come in, differentiate and participate in the formation of the adhesion at different stages.

Mesothelial cells usually express both epithelial and mesenchymal markers, however as they differentiate into mesenchymal cells they start expressing specific proteins. To attest that the GFP positive cells are not only proliferating but also differentiating into mesenchymal lineage, we have tested for specific markers. Firstly, we have looked at α-SMA expression pattern (**Figure 5A-C**) in the peritoneum close to the adhesion. Certain GFP positive cells also express α-SMA and start moving in the peritoneum in the direction of the adhesion formation. This confirms that mesothelial cells expressing Wt1 before injury proliferate into mesenchymal cells and participate in the formation of the adhesion.

Desmin expression in the adhesion provides a similar pattern with the α-SMA marker. As we can see in **Figure 6A**, the GFP positive cells are moving towards the centre of the formation and in a fraction of them we can see that they also express Desmin. It is important to notice that on the other side of the adhesion formation there is a higher expression of Desmin, which could imply that there are other crucial factors that are involves in this injury response.

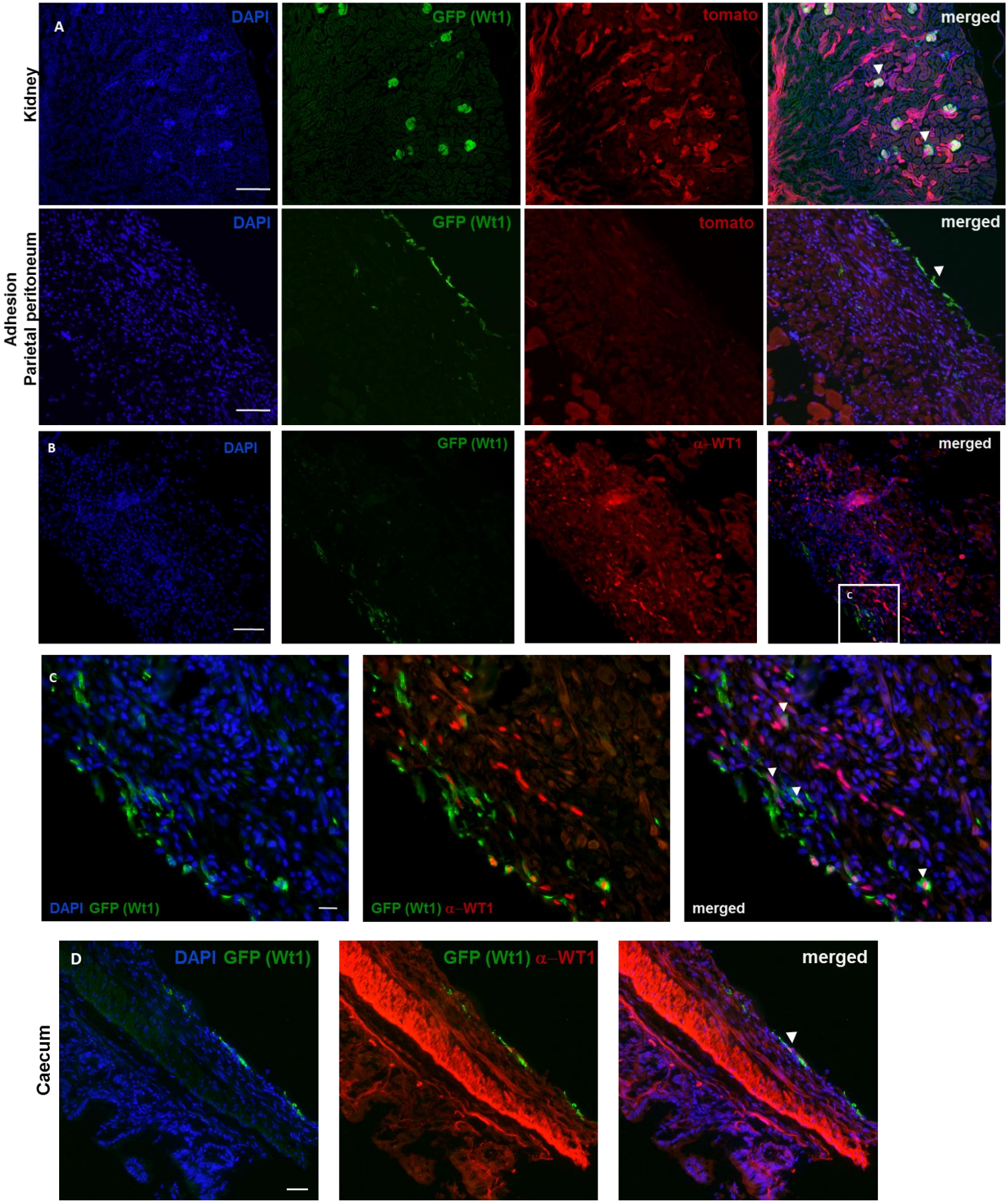
A
DAPI
GFP (Wt1)
tomato
merged
Kidney
DAPI
GFP (Wt1)
tomato
merged
Adhesion
Parietal peritoneum
B
DAPI
GFP (Wt1)
α–WT1
merged
C
C
DAPI GFP (Wt1)
GFP (Wt1) α–WT1
merged
D
DAPI GFP (Wt1)
GFP (Wt1) α–WT1
merged
Caecum

Figure 3| Successful recombination of Cre protein presents specific endogenous expression of GFP and tomato and expression of Wt1. A) In the kidney and parietal peritoneum the pattern of GFP positive cells matches with the expected location of the Wt1 expressing mesothelial cells; in the kidney, they are mostly expressed in the podocytes (white arrows) and in the peritoneum, they form a thin cell layer that enfolds the entire tissue (white arrows). As the tomato is only knocked out when the Wt1 gene is active in that specific cell, it is expected to see different patterns of tomato brightness in the tissue, as certain cells still maintain its expression. **B)** There is a high expression of Wt1 in the adhesion (red cells) while the endogenous GFP positive cells are few cells that seem to be coming towards the middle of it. **C)** When we look more closely, we can conclude that certain cells maintain the expression of Wt1 after the injury and adhesion formation (white arrows) while others change. **D)** In the caecum, we can observe the GFP positive cells are as expected in the mesothelial layer however most of them have lost expression of Wt1 at this moment (white arrow). Scales- A+B-100µm, C+D-50µm

To further sustain this hypothesis, we have tried to analyse the expression of vimentin, however due to high tomato background we could not say with certainty that what we see is the expression of the marker or the endogenous tomato expression. Similarly, we have tried macrophage markers such as P4/80 to see the immune response participation in relation to mesothelial cells and adhesion formation, however we did not attain an accurate result. These markers would require further protocol improvements before we could make a certified assumption on their expression.

Pecam is found on the surface of certain immune cells and is known to be a factor of angiogenesis. As it is known that angiogenesis is vital player in the process of PAs formation, we attempted to see how GFP positive cells localize in correlation to blood vessels. We have tried different antibodies to achieve this, however due to a very high tomato background we were required to change the colours of the secondary antibody (**Figure 6B**). Although the background was drastically reduced, we cannot effectively distinguish between the endogenous GFP cells and the immunofluorescence stained ones.

Discussion

In this report, we have successfully induced a mice model for parietal type adhesion formations and performed a lineage tracing experiment on the Wt1 expressing mesothelial cells. We have shown that Wt1 cells tend to move towards the adhesion, proliferate and differentiate into mesenchymal cells; however due to the endogenous expression of tomato, immunofluorescence exhibited a high background on frozen sections. This problem could be avoided in the future if we use other colour ranges for the secondary antibodies, such as orange-yellow or even blue.

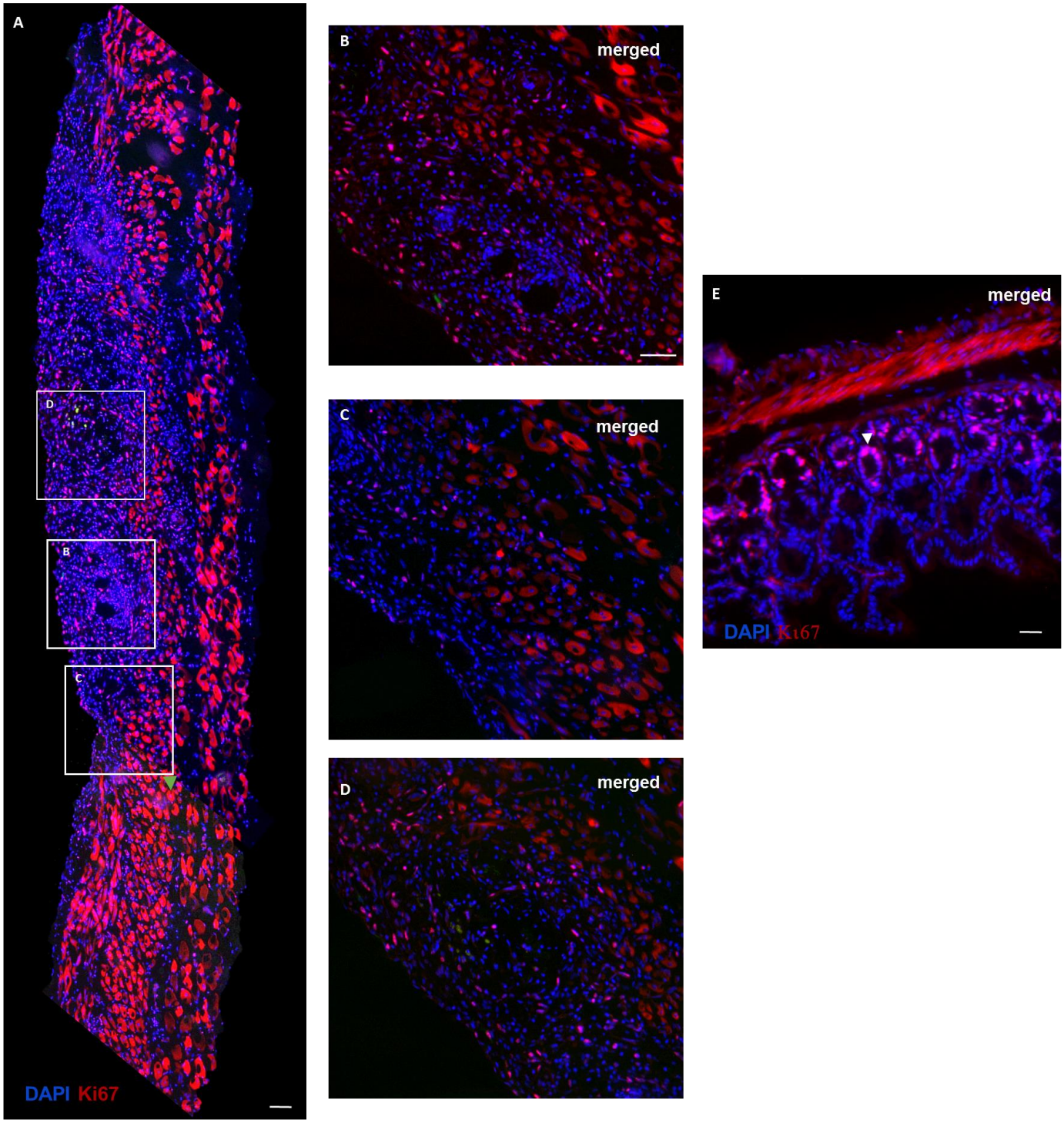

Figure 4| Ki67 expression in the parietal peritoneum and adhesion. A) Overall picture of Ki67 expression in the peritoneum presents a gradient; more cells are proliferating near the adhesion formation. Although there is a high background, the Ki67 marker is a nucleus marker while the background noise left from the endogenous tomato is membrane bound (green arrow). Looking more closely (**B, C, D**), we notice that Ki67 positive cells move towards the centre of the adhesion. **E)** High expression of Ki67 in the crypts of the intestines validates that the staining was successful (white arrow). Scales: A+B+C+D- 100µm, E-50µm

Having more than two simultaneous markers on top of the endogenous GFP positive cells would allow us to more deeply analyse the peculiar Wt1 expression pattern. Currently, we can see that most mesothelial cells expressing Wt1 seem to switch expression, few cells seem to maintain Wt1 expression constantly on. It would be interesting to use Ki67 and Wt1 markers together and see if the switching in expression could be due to the proliferating and reprogramming of cells.

Performing immunofluorescence and qPCR on markers specific for the epithelial phenotype, such as E-catherin and cytokeratin, we could ensure that all Wt1+ mesothelial cells have differentiated. We could see if there are any significant changes in expression between epithelial and mesenchymal markers in mesothelial cells between the firsts days after the injury compared with 14 days later. This might also give us some indication of how and/or when the Wt1 expression switches on and off and if it has any link with the differentiation of mesothelial cells.

Another possibility would be that due to Wt1 gene having 36 isoforms [26], antibodies stain only specific proteins and leaving others non-stained. Considering that not all MCs seem express Wt1 at the same time (data not shown), another option could be that WT1 expression is switched on/off by the Arcadian rhythms. If this was to be true, we would still expect to have all cells traced considering the tamoxifen administration lasted for 5 days consecutively, but the antibody staining would only target a part of the population. In both cases, we did not analyse the entire MCs pool that is participating in the adhesion formation. It would be interesting to re-do the lineage tracing experiment with another gene specific to the mesothelial lineage and compare the results.

In this report, we have showed that Wt1 positive mesothelial cells are not only moving towards the PA formation, but they also express markers specific for the mesenchymal lineage. We hypothesize that mesothelial cells differentiate by MMT into fibroblasts and take an active role into the adhesion formation. However, Desmin staining indicated that other key players may take part in this process. Also, the number of Wt1 stained cells inside the adhesion is lower than expected if they would be the main influence of fibroblasts, which confirms that there are probably other decisive factors involved in this process.

LeBleu *et al* showed that in kidney fibrosis, a significant number of myofibroblasts attracted to the site are mostly from differentiation from bone marrow rather than from EMT or MET transitions [27]. Fibrocytes are matrix-producing cells of the peripheral blood [28], however they are originally from bone marrow. They have been shown to be involved in the tissue healing process – fibrocytes start expressing α-smooth muscle actin (α-SMA) in the healing environment together with the influence of TGF-β and differentiate into myofibroblasts [29]. Consequently, fibrocytes could be another major player in the formation of parietal adhesion. One way to analyse what role both mesothelial cells and fibrocytes have, would be to evaluate the qPCR levels of specific genes that mark each phenotype, and compare it with the immunostaining location of the cells in the very earlies stages of adhesion formation (1-5 days).

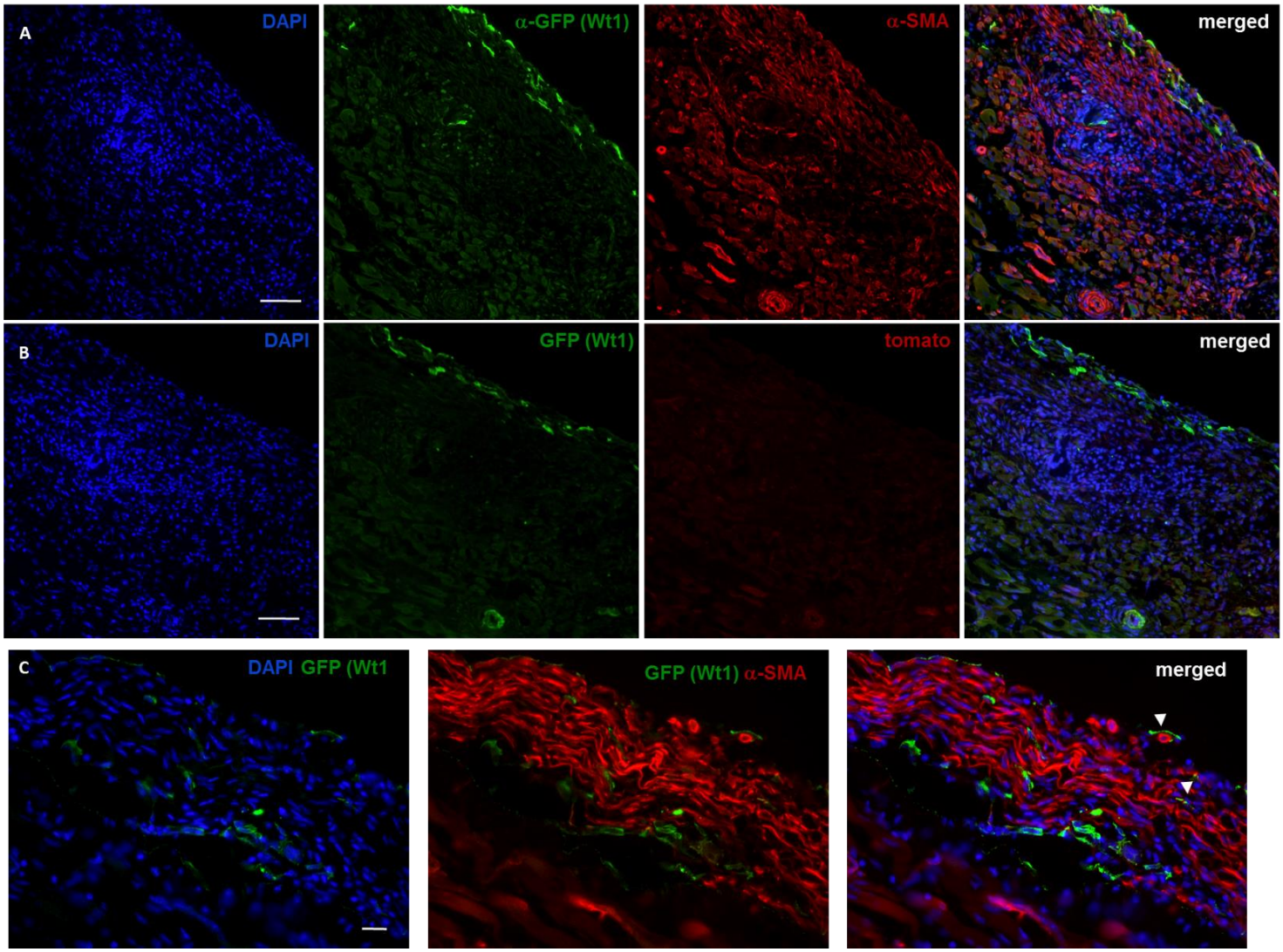

Figure 5| SMA expression in the parietal peritoneum in relation to the GFP positive cells. A) The expression of SMA in the proximity of the adhesion appear hectic and non-specific, however when we compare it to the control **(B)** we can see that the tomato background is significantly lower. **C)** As we look more in depth we see that specific GFP positive cells not only start moving inside the peritoneum but also express SMA (white arrows). Scales: A+B- 100µm; C- 50µm

We know that macrophages have an important role in regulation of inflammation, however the presence of T lymphocytes is what gives away the function of adaptive immune response in PA formation and leads to the conclusion that the immune response is not limited to the early postoperative phase [30,31]. Therefore, performing a co-localization of mesothelial cells with T cells and/or macrophages in both early and late formation phases would tell us if one could influence the other. Considering that T cells influence the differentiation of fibrocytes from CD14+ monocytes [32], one could believe that they might also affect mesothelial transformation indirectly.

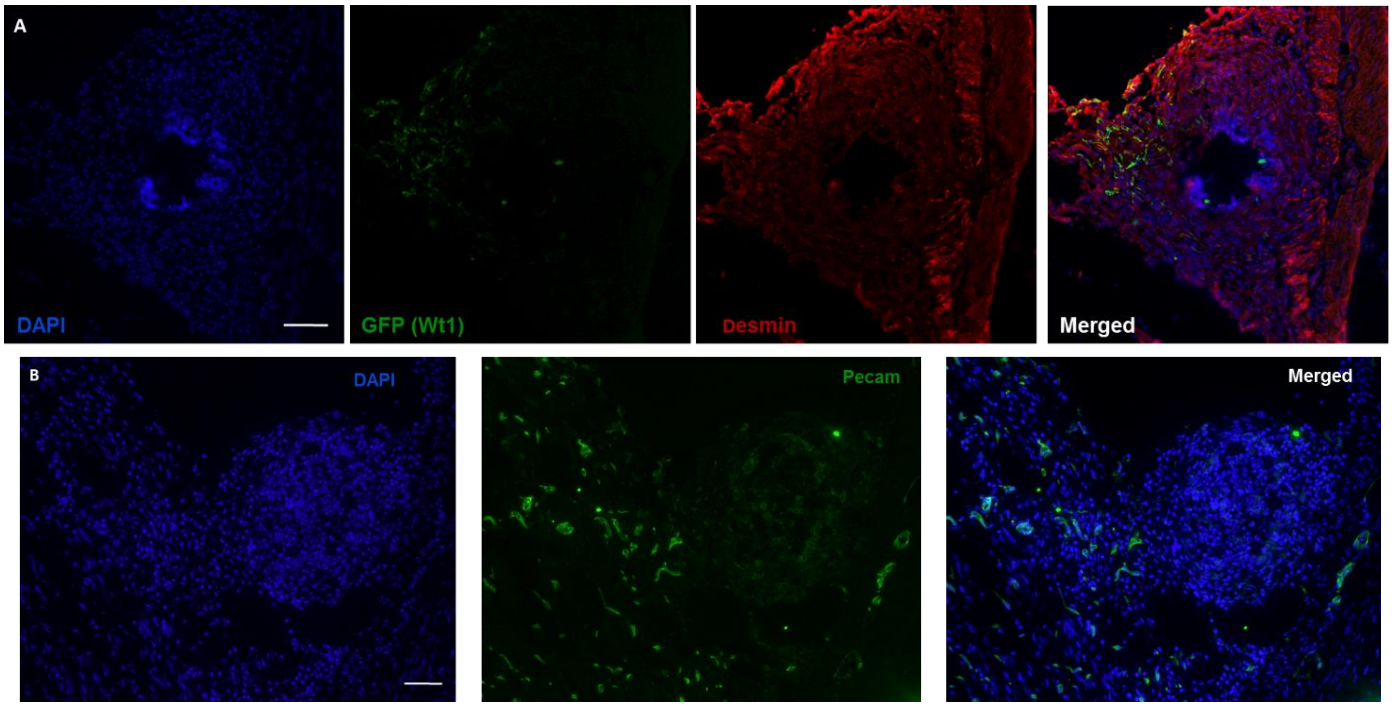

Figure 6| Desmin and CD31 (Pecam) expression inside the adhesion. A) There is a high overlap between the expression of GFP positive cells and Desmin, as shown by the white arrows, however there is still a noise from the endogenous tomato expression. **B)** CD61 staining of blood vassals demonstrates that angiogenesis process is actively happening on the vicinity of the adhesion, however not inside. Scales: A+B-100µm

Conclusion

Parietal adhesion is continuing to be an important pathology that has a high impact on life quality long term. Our experiments have shown that Wt1 positive mesothelial cells is not only moving in the adhesion during its formation, but also that they actively help with the fibrin deposits by differentiating into fibroblasts. This could be very important for any future therapy development based on tackling the balance between fibrinolysis and fibrin production during healing.

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References

1 Asahina, K., Zhou, B., Pu, W. T. and Tsukamoto, H. (2011) Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. Hepatology **53**, 983–995.

2 Grzybowski, A. and Breborowicz, A. (1997) [Peritoneum as a dialysis membrane. II. Pathology]. Przegl Lek **54**, 52–61.

3 Maciver, A. H., McCall, M. and James Shapiro, A. M. (2011) Intra-abdominal adhesions: Cellular mechanisms and strategies for prevention. Int. J. Surg., Elsevier Ltd **9**, 589–594.

4 Saed, G. M. and Diamond, M. P. (2003) Modulation of the expression of tissue plasminogen activator and its inhibitor by hypoxia in human peritoneal and adhesion fibroblasts. Fertil. Steril. **79**, 164–168.

5 Awonuga, A. O., Saed, G. M. and Diamond, M. P. (2009) Laparoscopy in gynecologic surgery: adhesion development, prevention, and use of adjunctive therapies. Clin. Obstet. Gynecol. **52**, 412–22.

6 Keckstein, J., Ulrich, U., Sasse, V., Roth, a, Tuttlies, F. and Karageorgieva, E. (1996) Reduction of postoperative adhesion formation after laparoscopic ovarian cystectomy. Hum. Reprod. **11**, 579–82.

7 Ray, N. F., Denton, W. G., Thamer, M., Scott, C., Henderson, C. and Perry, S. (1998) Abdominal adhesiolysis: Inpatient care and expenditures in the United States in 1994. J. Am. Coll. Surg. **186**, 1–9.

8 Risberg, B. (1997) Adhesions: preventive strategies. Eur. J. Surg. Suppl. 32–9.

9 Hoshino, A., Kawamura, Y. I., Yasuhara, M., Toyama-Sorimachi, N., Yamamoto, K., Matsukawa, A., Lira, S. a and Dohi, T. (2007) Inhibition of CCL1-CCR8 interaction prevents aggregation of macrophages and development of peritoneal adhesions. J. Immunol. **178**, 5296–304.

10 Andrews, S., Ford, D. and Martin, P. (2012) Knockdown of osteopontin reduces the inflammatory response and subsequent size of postsurgical adhesions in a murine model. Am. J. Pathol., Elsevier Inc. **181**, 1165–1172.

11 Herrick, S. E., Mutsaers, S. E., Ozua, P., Sulaiman, H., Omer, A., Boulos, P., Foster, M. L. and Laurent, G. J. (2000) Human peritoneal adhesions are highly cellular, innervated, and vascularized. J. Pathol. **192**, 67–72.

12 Quan, T. E., Cowper, S., Wu, S. P., Bockenstedt, L. K. and Bucala, R. (2004) Circulating fibrocytes: Collagen-secreting cells of the peripheral blood. Int. J. Biochem. Cell Biol.

13 Metz, C. N. (2003) Fibrocytes: A unique cell population implicated in wound healing. Cell. Mol. Life Sci.

14 Sandoval, P., Jiménez-heffernan, J. A., Guerra-azcona, G., Pérez-, M. L., Rynne-vidal, Á., Albar-vizcaíno, P., Gil-vera, F., Coronado, M. J.,

Barcena, C., Dotor, J., et al. No Title.

15 Iwano, M., Plieth, D., Danoff, T. M., Xue, C., Okada, H. and Neilson, E. G. (2002) Evidence that fibroblasts derive from epithelium during tissue fibrosis. J. Clin. Invest. **110**, 341–350.

16 Zeisberg, E. M., Tarnavski, O., Zeisberg, M., Dorfman, A. L., McMullen, J. R., Gustafsson, E., Chandraker, A., Yuan, X., Pu, W. T., Roberts, A. B., et al. (2007) Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat. Med. **13**, 952–961.

17 Marmai, C., Sutherland, R. E., Kim, K. K., Dolganov, G. M., Fang, X., Kim, S. S., Jiang, S., Golden, J. a, Hoopes, C. W., Matthay, M. a, et al. (2011) Alveolar epithelial cells express mesenchymal proteins in patients with idiopathic pulmonary fibrosis. Am. J. Physiol. Lung Cell. Mol. Physiol. **301**, L71-8.

18 Thiery, J. P., Acloque, H., Huang, R. Y. J. and Nieto, M. A. (2009) Epithelial-Mesenchymal Transitions in Development and Disease. Cell.

19 Lopez-Cabrera, M. (2014) Mesenchymal Conversion of Mesothelial Cells Is a Key Event in the Pathophysiology of the Peritoneum during Peritoneal Dialysis. Adv. Med. **2014**, 473134.

20 Jin, X., Ren, S., Macarak, E. and Rosenbloom, J. (2016) Pathobiological mechanisms of peritoneal adhesions: The mesenchymal transition of rat peritoneal mesothelial cells induced by TGF-??1 and IL-6 requires activation of Erk1/2 and Smad2 linker region phosphorylation. Matrix Biol., International Society of Matrix Biology **51**, 55–64.

21 Chen, Y.-T., Chang, Y.-T., Pan, S.-Y., Chou, Y.-H., Chang, F.-C., Yeh, P.-Y., Liu, Y.-H., Chiang, W.-C., Chen, Y.-M., Wu, K.-D., et al. (2014) Lineage tracing reveals distinctive fates for mesothelial cells and submesothelial fibroblasts during peritoneal injury. J. Am. Soc. Nephrol. **25**, 2847–58.

22 Suzuki, T., Kono, T., Bochimoto, H., Hira, Y., Watanabe, T. and Furukawa, H. (2015) An injured tissue affects the opposite intact peritoneum during postoperative adhesion formation. Sci. Rep. **5**, 7668.

23 Martínez-Estrada, O. M., Lettice, L. A., Essafi, A., Guadix, J. A., Slight, J., Velecela, V., Hall, E., Reichmann, J., Devenney, P. S., Hohenstein, P., et al. (2010) Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin. Nat. Genet. **42**, 89–93.

24 Wilm, B., Ipenberg, A., Hastie, N. D., Burch, J. B. E. and Bader, D. M. (2005) The serosal mesothelium is a major source of smooth muscle cells of the gut vasculature.

25 Bax, N. A. M., Van Oorschot, A. A. M., Maas, S., Braun, J., Van Tuyn, J., De Vries, A. A. F., Gittenberger-De Groot, A. C. and Goumans, M. J. (2012) In vitro epithelial-to-mesenchymal transformation in human adult epicardial cells is regulated by TGFbeta-signaling and WT1. Cardiovasc Res **93**, S56–S57.

26 Hohenstein, P. and Hastie, N. D. (2006) The many facets of the Wilms ’ tumour gene , WT1 **15**, 196–201.

27 LeBleu, V. S., Taduri, G., O’Connell, J., Teng, Y., Cooke, V. G., Woda, C.,

Sugimoto, H. and Kalluri, R. (2013) Origin and function of myofibroblasts in kidney fibrosis. Nat. Med. **19**, 1047–53.

28 Bucala, R., Spiegel, L. a, Chesney, J., Hogan, M. and Cerami, a. (1994) Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol. Med. **1**, 71–81.

29 Mori, L., Bellini, A., Stacey, M. A., Schmidt, M. and Mattoli, S. (2005) Fibrocytes contribute to the myofibroblast population in wounded skin and originate from the bone marrow. Exp. Cell Res. **304**, 81–90.

30 Binnebösel, M., Rosch, R., Junge, K., Lynen-Jansen, P., Schumpelick, V. and Klinge, U. (2008) Macrophage and T-lymphocyte infiltrates in human peritoneal adhesions indicate a chronic inflammatory disease. World J. Surg. **32**, 296–304.

31 Binnebösel, M., Klink, C. D., Serno, J., Jansen, P. L., von Trotha, K. T., Neumann, U. P. and Junge, K. (2011) Chronological evaluation of inflammatory mediators during peritoneal adhesion formation using a rat model. Langenbecks. Arch. Surg. **396**, 371–8.

32 Abe, R., Donnelly, S. C., Peng, T., Bucala, R. and Metz, C. N. (2001) Peripheral Blood Fibrocytes: Differentiation Pathway and Migration to Wound Sites. J. Immunol. **166**, 7556–7562.