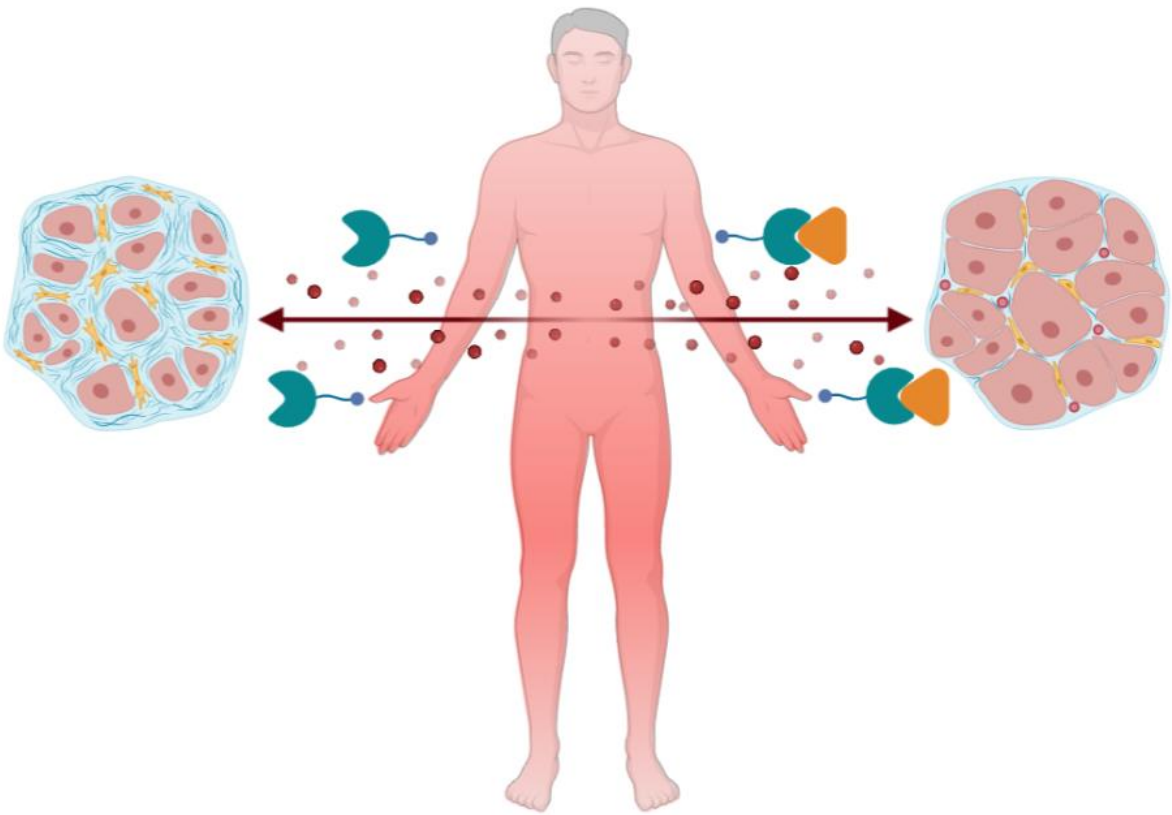
UNIVERSITY OF GRONINGEN

FACULTY OF SCIENCE AND ENGINEERING

Galectin-3 induced fibrotic response, generic or cell specific?



Master of Science thesis

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Abstract

The leading causes of death and morbidity worldwide are cancer and cardiovascular disease. While these two diseases share hallmarks and risk factors which connects them, recent studies now show a causal relationship between cancer and heart failure. Hallmarks which are shared in both cancer and cardiovascular disease include inflammation and fibrosis, which both play an important role in the pathophysiology of the diseases. One fibrosis biomarker which is both related to cancer and heart failure is galectin-3. Galectin-3 is a key player in the formation of fibrosis, specifically myocardial fibrosis and neoplastic stromal deposition. Fibrosis has been hypothesized to initiate cancer onset through the disruption of the tumour microenvironment, and galectin-3 has also been indicated to downregulate T-cell activation which could facilitate tumour growth. Galectin-3 is therefore an interesting target in both cancer and heart failure. The main cellular effectors of fibrosis are the myofibroblasts, which are modulated fibroblasts. Both genetic and pharmacological inhibition of galectin-3 has been shown to prevent cardiac remodelling in mouse and rat models, but inhibition of galectin-3 has yet to be researched *in vitro.* Therefore, this research project focused on the role of galectin-3 mediated fibrosis in different types of fibroblasts, and postulate whether this is a generic reaction or cell specific. Furthermore, this research will include a pilot murine study with the intention of creating a functional 6-week-old transaortic constriction mouse model to use for galectin-3 inhibition research.

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Introduction

Cardiovascular disease (CVD) and cancer are amongst the main mortalities and morbidities worldwide (1). With the world population ageing, the incidence of both diseases is expected to increase. The main complication of CVD is heart failure (HF), and incidence of HF has surged over the last few decades (2,3). It is well known that cancer patients can develop HF after cancer treatment due to cardiotoxic effects (4). HF and cancer share common risk factors and comorbidities, which could explain the correlation of the illnesses (3). However, more recent studies are suggesting a causal relationship between HF and cancer (Figure 1) (5).

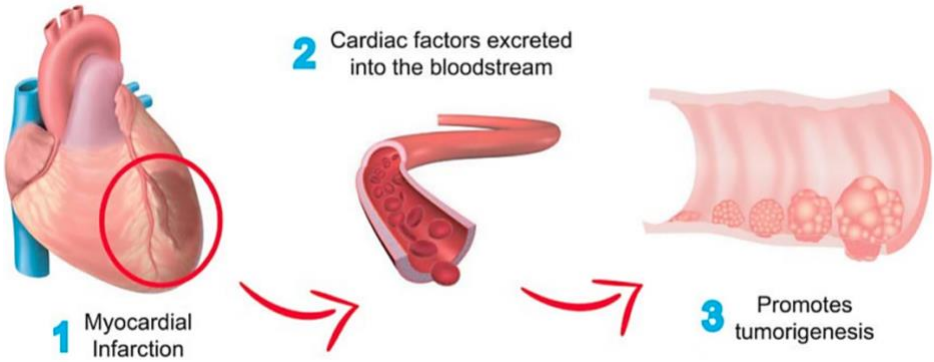

Figure 1: The hypothesis of the failing heart enhancing tumorigenesis (5).

The epidemiology of cancer in the HF population has gotten more attention, and recent studies have been focused on exploring the incidence of cancer in patients prior to HF diagnosis. Hasin et al. explored the risk of cancer incidence in HF patients in a case control study (N = 596 patients with HF and N = 596 controls), showing that even when adjusted for common risk factors, patients with HF were 60% more likely to develop malignancies than the control group (6). The same study group then proceeded with a prospective cohort study, where the risk of cancer was explored in a homogenous group of myocardial infarction (MI) survivors. Patients with myocardial infarction who developed HF had indeed an increased risk of developing cancer (7). More important epidemiolocal data is from a study by Banke et al., where the incidence of cancer in a HF patient group (N = 9307) demonstrated that the HF population had an increased incidence rate of malignancies as compared to the background population. They found that all types of cancer except for prostate cancer were more frequent in the HF population (4).

Recent preclinical studies have shown that HF can also directly affect tumour growth. A murine study provided evidence that HF was indeed directly associated with tumorigenesis. The group from Meijers et al. subjected C57BL/6-Apc^{Min} mice, which are susceptible to spontaneous intestinal adenoma formation, to MI (model for ischaemic myocardial injury) or a sham procedure. Six weeks post operation, the intestinal polyps observed in the MI mice were more frequent and larger in size than in the sham mice (5). A different study showed that MI also accelerated breast cancer outgrowth and cancer-specific mortality in both mice and humans (8). Here researchers saw an increased risk of cancer reappearance

and death in patients who suffered from early-stage breast cancer and experienced cardiovascular events after cancer diagnosis. Lastly, in a study with a trans aortic constriction (TAC) HF model (pressure overload-induced cardiac hypertrophy) to investigate the effect of early cardiac remodelling on tumour growth and metastasis, it was observed that TAC mice developed tumours with higher proliferation rate and larger in size than the control group (9). *In vitro* tests with serum from the TAC mice resulted in potentiated cancer cell proliferation, indicating TAC may result in the secretion of secreted tumour-promoting factors. These studies suggest that HF can indeed cause tumorigenesis.

Important to note are the pathophysiologic mechanisms that connect cancer and HF, which, as previously mentioned, could be an explanation as to why HF patients have a higher incidence of cancer. Ageing, BMI and smoking are common risk factors shared between cancer and HF (10). Cancer and HF share multiple hallmarks, including inflammation (11,12), fibrosis ,and metabolic remodelling (13). Fibrosis and inflammation also play major roles in the pathophysiology of the diseases. Inflammation in carcinogenesis has been of interest since the 19th century, and the CVD risk factor of chronic inflammation is well known (14). Fibrosis accumulates in the tumour microenvironment (TME), which consists of tumour cells as well as stromal cells such as fibroblasts, and macrophages. Increased deposition of matrix proteins interferes with cell-cell interaction and amplification of growth factor signalling, resulting in the promotion of tumorigenesis. The accumulation in HF results in the contribution of progression of diastolic dysfunction (15,16).

Galectin-3 (gal-3) is one of the prognostic biomarkers associated with both HF and cancer. It has been demonstrated to be associated with an increased risk of death or HF rehospitalisation, independent of established risk factors in several HF cohorts (17–19). Cancer studies also show that elevated gal-3 levels and disease progression had a close relationship (20,21). Surprisingly, recent evidence suggests that gal-3 is not just a marker for fibrosis, but a key player in its formation (22). Furthermore, gal-3 is associated with both myocardial fibrosis (19,23) and neoplastic stromal deposition (20,21). In cancer onset, fibrosis has been hypothesized to initiate cancer onset through the disruption of the tumour microenvironment (21). Interestingly, gal-3 has also been shown to suppress T-cell activation, which could facilitate tumour growth (24). These findings suggest that gal-3 is an interesting target to develop a treatment strategy for both cancer and HF.

Fibrosis is a dynamic process, and there have been clear implications for therapeutic interventions that are designed to profit from this inherent plasticity. Despite the recent progress made in the understanding of fibrosis, there remains to be a translation gap between the identification of antifibrotic targets and using these targets in the clinic for human treatment (25). To dissect what is necessary for gal-3-induced fibrosis to become a therapeutic target, the formation of fibrosis will be discussed below.

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The main cellular effectors of fibrosis are the myofibroblasts, which are modulated fibroblasts that express contractile proteins such as a-smooth muscle actin (α-SMA). Myofibroblasts differ from fibroblasts in the amount of matrix proteins they can produce, but also phenotypically. Transforming growth factor (TGF)-β is considered a main activator of the fibrotic pathway (26), and gal-3 levels were markedly upregulated in several cardiac fibrotic phenotype animal models (22,23). Gal-3 presence induces fibroblasts to proliferate and deposit collagen, which contributes to cardiac fibrosis and adverse remodelling. Both genetic and pharmacological inhibition of gal-3 has been shown to prevent cardiac remodelling in mouse and rat models, but inhibition of gal-3 has yet to be researched mechanistically *in vitro* (27). In the tumour microenvironment, cancer associated fibroblasts (CAFs) are the most common cells. Resident fibroblasts transition under TGF-β1 mediation to cancer-associated fibroblasts. Studies with trans-well migration assays determined that TGF-β1 mediated transition of gastric fibroblasts to CAFs enhanced tumour cell migration (28). This study suggests that there is a difference in response to TGF-β1 and possibly other activators of the fibrotic pathway such as gal-3 between different fibroblasts.

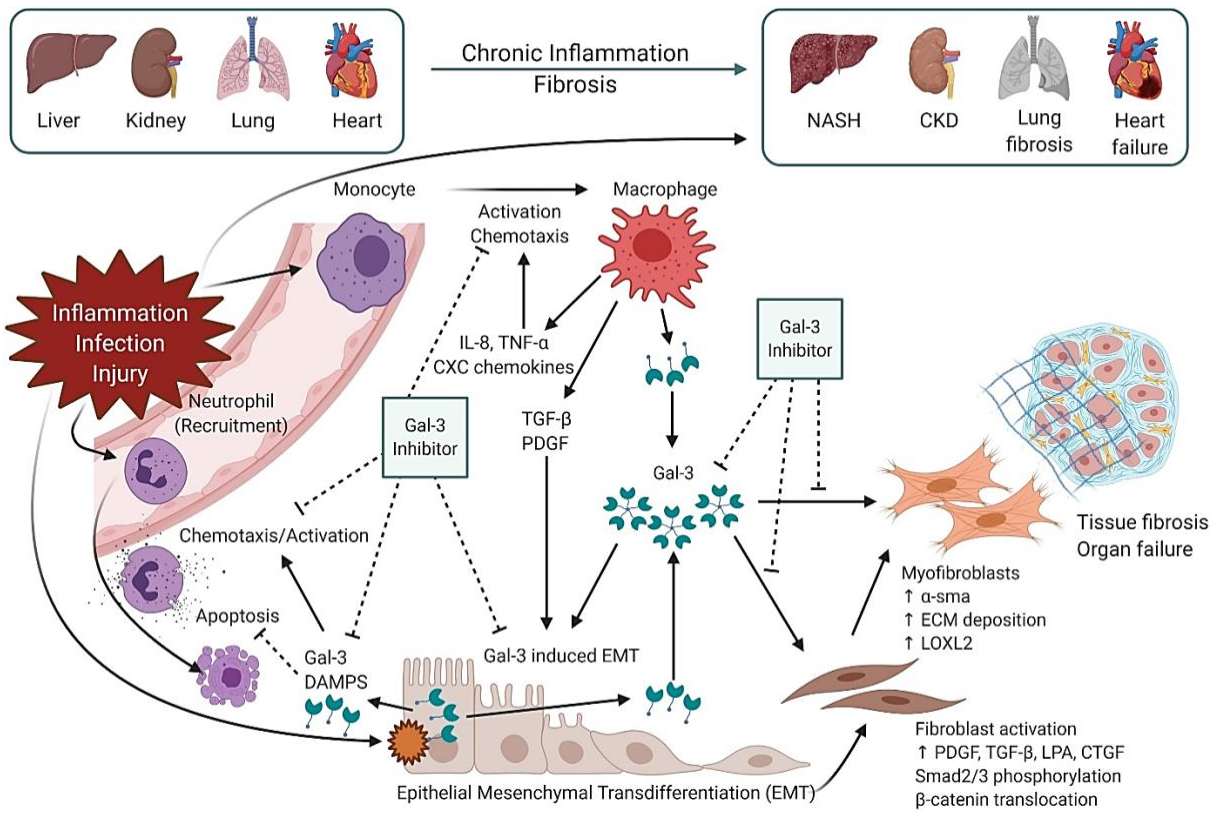

Figure 2: The inflammatory, infection and injury pathways driven by gal-3 which result in fibrosis in different organs (20).

Therefore, this research project will focus on the role of gal-3 mediated fibrosis *in vitro* and aid in a pilot study to create a functional 6-week-old TAC mouse model to use for gal-3 inhibition research. The effect of gal-3 will be studied on dermal, cardiac, and cancer associated fibroblasts. A pectin-based inhibitor from pumpkin puree, G3P-01, will be tested to see if it attenuates the fibrotic progress from gal-3 in the cells (Figure 2, (20)). We hypothesize that gal-3 treatment in fibroblasts will lead to an upregulation of fibrotic gene expression, and that gal-3 treatment will also increase a-SMA stained cells, signifying an increase of myofibroblasts present. We also hypothesise that there will not be a generic fibrotic response induced by gal-3, but that this is fibroblast type specific. Furthermore, the pilot will be done with 6-week-old mice to determine the ring size necessary to create an *in vivo* cardio-oncology model suitable for future research on, for instance, gal-3. In this future research, this model will be used to study the effect of inhibiting gal-3 with G3P-01 on reducing fibrosis formation in the heart and in tumour.

Methods

Cell cultures

Three types of fibroblasts were used: human dermal fibroblasts (HDFs, medium DMEM high glucose with 1% pen/strep and 10% foetal calf serum (FCS), Merck), cancer associated fibroblasts (CAFs, medium Vitroplus III low serum with 1% pen/strep, Neuromics) and cardiac fibroblasts (CF, medium FGM-2 Fibroblast growth medium-2 Bulletkit, Lonza). Cells were seeded in a 6 well plate with a density of 100.000 cells per well (was dit voor RNA?), or a 12 well glass coated well plate seeded with a density of 30.000 cells per well for 24 hours (dit voor IF?), to reach a confluency of 80-90%, and then starved overnight. Cells were treated with 50 to 200 ng/mL gal-3 for 24 hours.

RNA isolation and real-time q-PCR

Cells from the 6 wells were put in TRI-reagent in the freezer for RNA isolation and qPCR analysis for fibrotic gene expression. RNA was extracted from the cells which were kept at -80 °C before isolation. Subsequent steps of adding chloroform for phase separation, isopropanol for precipitation and 70% EtOH for washing were performed. RNA was dissolved in 20 uL miliQ and the concentration was measured with Nanodrop. RNA was then converted into cDNA with the use of the Quantitect reverse transcriptase kit (Qiagen). qPCR was performed using a Bio-Rad CFX384 Real-Time PCR system (Bio-rad, CA, USA) with SYBR green PCR mix. qPCR was performed for the following genes in Table 1. The qPCR program was set to 35 cycles of 3 minutes 95°C, 15 seconds 95°C, 30 minutes 60°C and then a dissociation step. mRNA levels were calculated relative to a reference gene, GAPDH.

Tabel 1: Primers used for qPCR

Gene	FW primer	RV primer
GAPDH	CATCAAGAAGGTGGTGAAGC	ACCACCCTGTTGCTGTAG
col1a1	GCCTCAAGGTATTGCTGGAC	ACCTTGTTTGCCAGGTTCAC
col3a1	CTGGACCCCAGGGTCTTC	CATCTGATCCAGGGTTTCCA
fibronectin	TTCCACACCCCAATCTTCAT	GGGGTCTTTTGAACTGTGGA
TGFβ1	CCCGAGACTGACACACTGAA	ACCCTGACTTTGGCGAGTAA

Immunofluorescence staining and analyses

For immunofluorescence (IF) imaging, cells in 12 wells were fixed with 50/50 methanol/acetone and stained with primary antibodies vimentin (1:50, Sigma Aldrich, secondary antibody goat anti-rabbit IgG, Southern biotech, 1:1000, green), DAPI nuclear staining (blue) and α-SMA (1:50, Sigma Aldrich, secondary antibody donkey anti-mouse IgG, Thermo Fisher, 1:1000, red) staining. Vimentin is a marker for fibroblasts and α-SMA for myofibroblasts. Subsequently, a dose response curve with gal-3 treatment was performed on both HDF and CF, with treatment of gal-3 ranging from 0 to 200 ng/mL. Positive controls were treated with 5 ng/mL TGF-β1. To determine the number of cells which showed α-SMA

staining, 4 pictures were taken of each condition based on the DAPI staining. Microscope settings were set on Exposure: 400 (except DAPI on 80), Gain: 4, Intensity: 4 for all pictures. Pictures were then exported as Tiff files to ImageJ, where they were converted to 16-bit. ImageJ was used to count the nuclei and the original photos were used to determine the number of cells showing α-SMA (red cells). Cells were counted only if they were morphologically myofibroblasts. Cell were only counted if the entire nucleus of the cell was in the photograph, for each condition a minimum of 200 cells were counted. Photos were all brightened in Power Point to increase visibility for presentations.

Mouse model

All animal experiments were conducted on male mice from the C57BL/6J (BL6) mouse strain from Charles River. Only male mice were used for the pilot study to rule out variations in HF severity due to possible size differences in the aorta. For future studies the C57BL/6J Apc^{Min} model will be used to create a cardio-oncology model (currently new IvD waiting for approval). Experimental procedures were performed in accordance with the European Union guidelines (DEC 6944, the Netherlands). All animals were housed individually to monitor their food intake (monitored twice a week). Mice had an *ad libitum* supply to food and water, under 12:12 light:dark cycles. Mice were checked twice a week for signs of discomfort and their weight was measured weekly.

Cardiac surgeries

TAC operations were carried out on BL6 mice (N=15) when they were 6 weeks old. For both sham and TAC surgeries, mice were anesthetised with isoflurane and mechanically ventilated and underwent thoracotomy. TAC was inflicted by constricting the ascending aorta with a Nitrile O-ring (or in the case of the sham mice, a sham procedure). The small pilot study was performed with mice receiving TAC surgery with either an O-ring size of 0.50mm (n = 6) or an O-ring size of 0.46 (n = 6). The sham surgery group consisted of 3 mice. Analgesics in the form of Carprofen (5mg/kg, subcutaneously) were administered during surgery, and if necessary, 24 hours after the procedure. The mice were monitored daily during the first week after their procedure for body weight changes and signs of discomfort.

Echocardiography

Two and six weeks after surgery, *in vivo* cardiac dimensions were assessed with transthoracic echocardiography (Vivid 7 equipped with 12 MHz linear array transducer; GE Healthcare, Chalfont St. Gilles, UK). During echocardiography, mice were anesthetised with a 2% isoflurane/oxygen mixture, and their body temperature was maintained utilising a heating pad on which they were placed during the procedure. With echocardiography at two points in time, cardiac function can be followed-up closely.

Euthanising

The sacrifice of the animals took place at 12 weeks of age. The mice were anesthetized with a 2% isoflurane/oxygen mixture and were euthanized by cardiac puncture and heart removal.

Results

Myofibroblast presence higher in gal-3 treated CFs for 24 hours

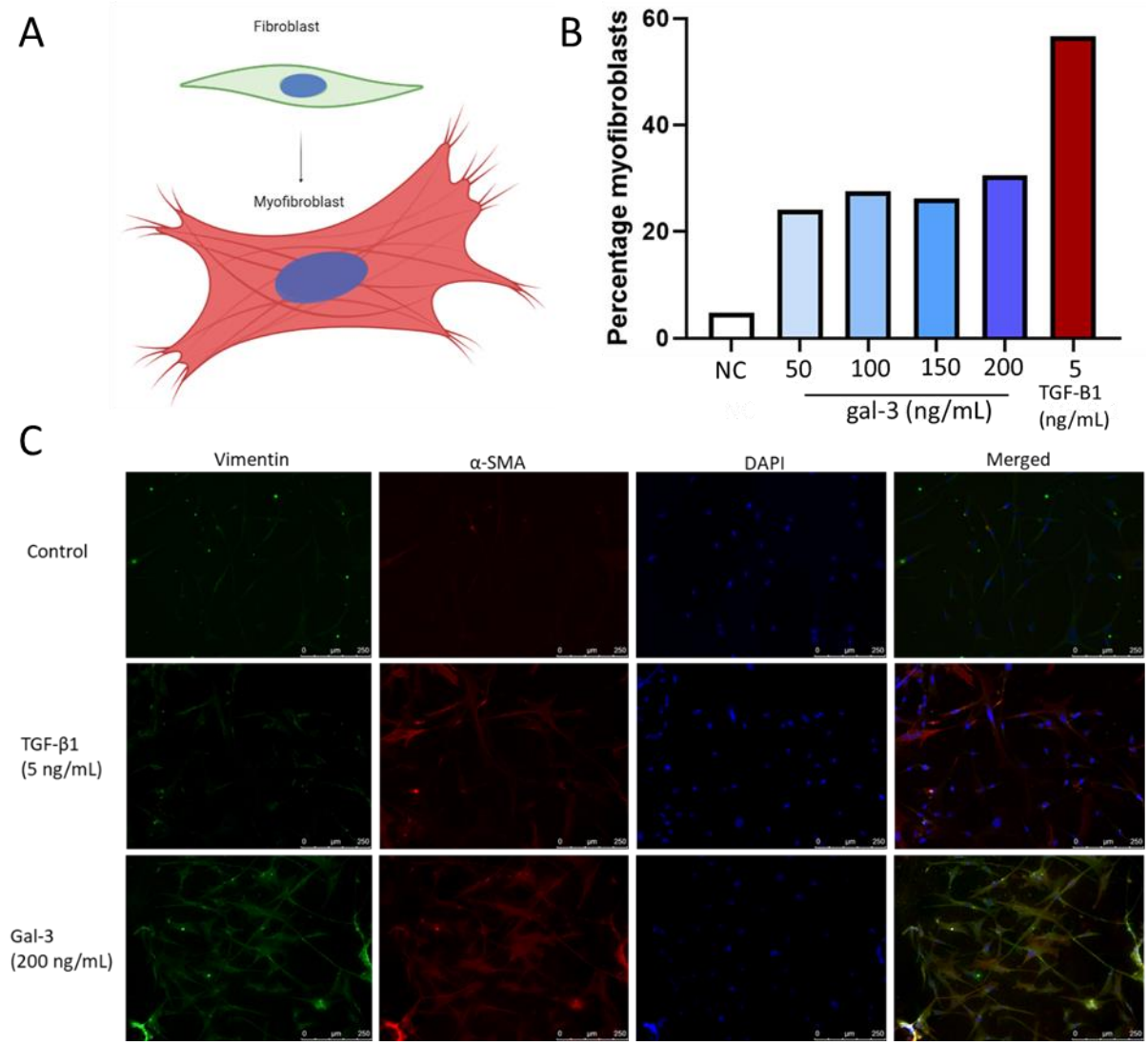

Figure 4: A) Morphological and colour differences between a fibroblast and a myofibroblast. Fibroblasts will be green due to vimentin, while myofibroblasts will be red due to a-SMA. Both will have a blue nucleus because of DAPI, Picture created in Biorender. B) The percentage of myofibroblasts present per treatment after 24 hours, N=1. C) The IF pictures per channel and combined of the negative control, TGFβ-1 and gal-3 200 ng/mL, treatment lasted 24 hours. Scale is 0-100 µm.

IF staining was performed on CFs to determine the number of fibroblasts which had differentiated to myofibroblasts (see Figure 4A). In untreated (Control) CFs 4.6% myofibroblasts were present in the sample (see Figure 4B and 4C), and TGF-β1 treated CF cultures consisted of 56.3% myofibroblasts. The gal-3 dose response curve shows an increase in myofibroblasts between 24 and 32% (IF stainings of all treatments of the CFs can be found in Appendix II).

Dermal fibroblasts fibrotic gene expression after gal-3 treatment

To determine whether there was a change in fibrotic gene expression in the HDFs after gal-3 treatment, a qPCR was performed on several fibrotic genes (Figure 5). These genes included *Col1A1*, *Col1A3*, *TGF-β1* and fibronectin and housekeeping gene *GAPDH*. Because these results are only based on technical replicates from one experiment, they may not be trustworthy enough to make strong statements.

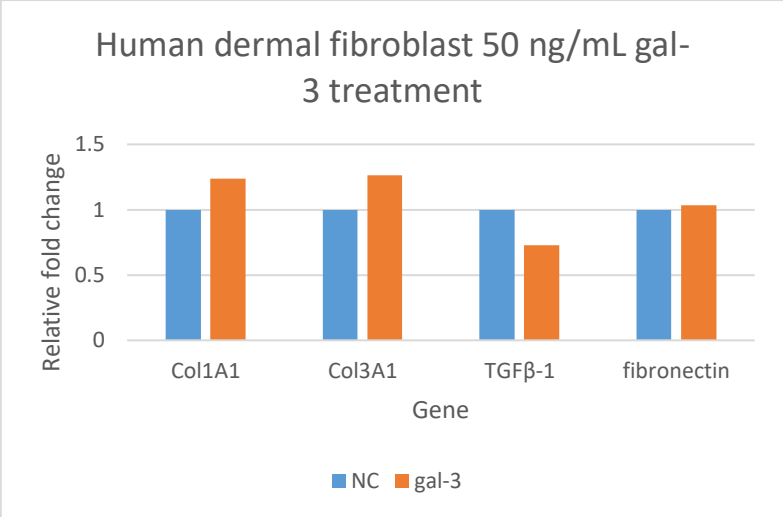

Figure 5: The fibrotic expression of the human dermal fibroblasts after 24-hour treatment of 50 ng/mL gal-3 (N=1).

Myofibroblast presence increased in gal-3 treated HDFs

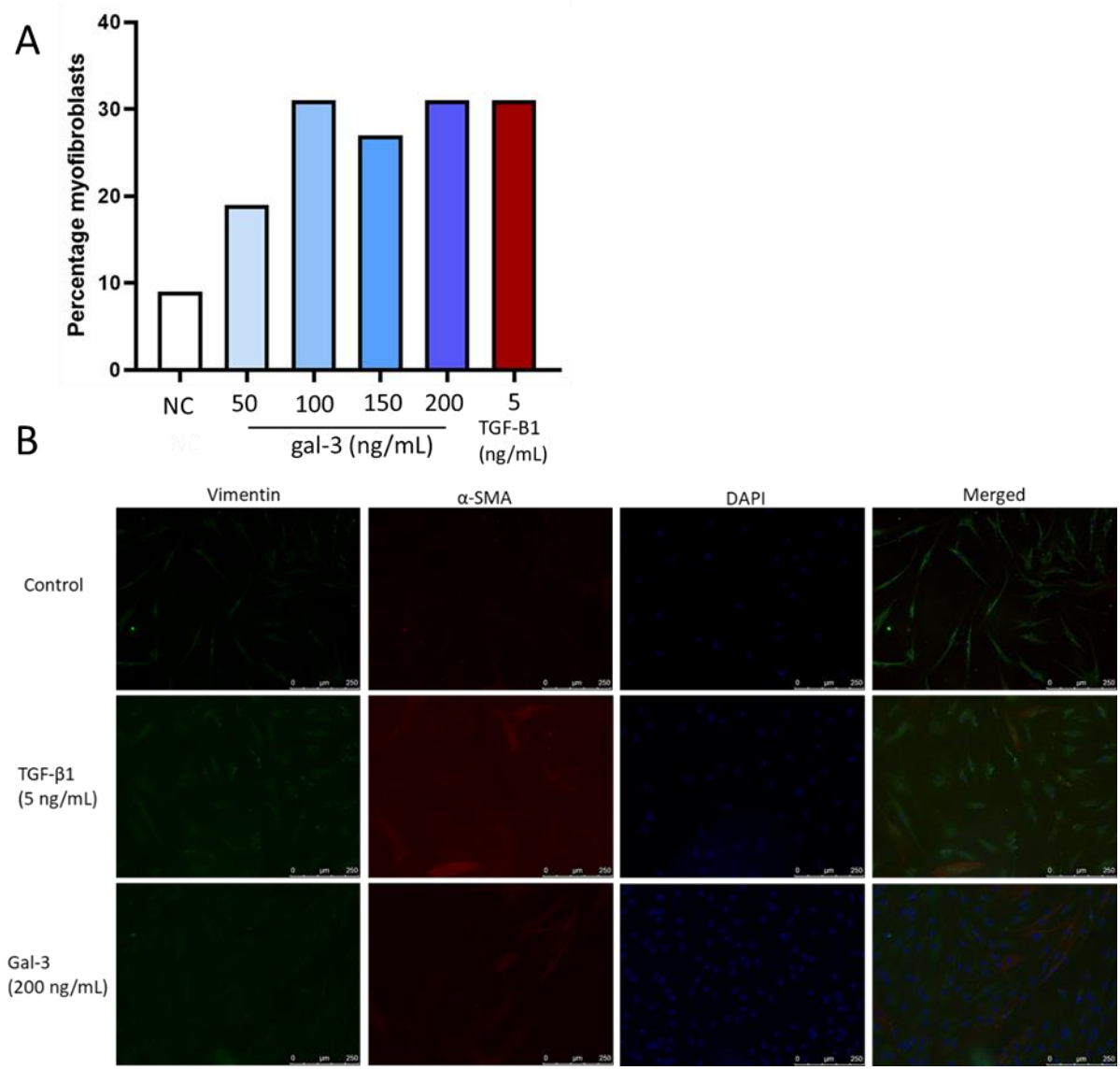

Figure 6: A) The percentage of myofibroblasts present per treatment after 24 hours, N=1. B) The IF pictures per channel and combined of the negative control, TGFβ -1 and gal-3 200 ng/mL, treatment lasted 24 hours. Scale is 0-100 µm.

The IF staining was performed on HDFs to determine the number of fibroblasts which had differentiated to myofibroblasts (see Figure 6A). Untreated/control HDFs cultures consisted of 9.4% myofibroblasts (see Figure 6A and 6B), and in TGF-β1 treated HDFs 31% of the cells were myofibroblasts. The gal-3 dose response curve shows an increase in myofibroblasts ranging from 28% to 31% (IF stainings of all gal-3 treatments of the HDFs can be found in Appendix III).

BL6 TAC ring surgery with 0.46- and 0.50-mm nitrile O-ring

Fifteen BL6 mice were used for the pilot experiment. The original plan was to have 3 sham mice and 6 mice for each TAC ring size. However, mice receiving an 0.46 mm nitrile O-ring (N=3) did not manage to awaken from their intubation, and all three of them died almost immediately. After this severity and the loss of the mice became evident, no more mice received a 0.46 mm nitrile O-ring. The plan was then to give the remaining mice either a sham operation or a 0.50 mm nitrile O-ring. The sham group consisted of 3 mice, of which 2 received an operation, one did not due to failed intubation, and subsequent death. The other two sham mice survived, and one more mouse was used to increase the sham group to three mice again. One mouse died during intubation, and from the remaining mice who underwent TAC surgery (N=8) with a 0.50 mm nitrile O-ring, 4 survived. These mice are currently 8 weeks old and survived the first week after operation. All surviving mice showed no signs of discomfort after 1 week after operation, they move and behave similarly and their wounds all healed without problems. The wellness diary of the mice can be found in Appendix I.

Discussion

This study focused on the gal-3 induced response in fibroblasts and whether this was cell specific or a generic response. Our results demonstrate that gal-3 does induce a fibrotic expression from both HDFs and CFs, which is proven with IF and fibrotic gene expression. Based on the data from the IF, gal-3 seems to induce a generic profibrotic response in these two types of fibroblasts. Moreover, this study contained an *in vivo* murine pilot study to determine the TAC nitrile-O ring size necessary for 6-week-old BL6 mice. Both the ring sizes which were used for this study proved too severe for the mice, so it can only be concluded that the nitrile-O ring size for future TAC surgeries in 6-week-old mice should be larger than 0.50mm.

Our data demonstrates that gal-3 does indeed have an impact on the number of myofibroblasts present in HDF and CF cultures. Results from the CAFs are still pending. In the HDFs, there was an increase of collagen1A1 and collagen3A1 expression after gal-3 treatment, but TGF-β1 expression was decreased and fibronectin expression stayed similar to that of the negative control. The increased expression of collagen genes is in line with literature, where even in *in vivo* models showed an increase after gal-3 treatment (29). However, gal-3 increases the expression of collagens through the activation of the TGF-β signalling pathway. This would indicate that the TGF-β1 expression we observed does not match with the expression in literature and that gal-3 activates the TGF-β signalling pathway through increased expression of another TGF-β, such as TGF-β2 or TGF-β3 (30). Fibronectin expression was also expected to increase, as myofibroblasts exhibit enhanced fibronectin assembly when compared to fibroblasts (31). Repeating the experiment and increasing technical replicates for the qPCR should validate our results.

Regarding the IF stainings, both the gal-3 and TGF-β1 treated cells clearly showed larger numbers of myofibroblasts present, suggesting gal-3 does indeed induce a fibrotic response. This was evident from the morphological changes between gal-3 or TGF-β1 treated and untreated cells, both gal-3 and TGF-β1 treated cells showed an increase in cells with morphological similarity to myofibroblasts (15). Should a similar result be obtained when the N has been increased, then it can be assumed that gal-3 does indeed generate a profibrotic response in both HDFs and CFs. Furthermore, there was a visible increase of α-SMA IF staining in the treated cells, which for the TGF-β1 treated cells was substantiated in literature (32,33). Based on the IF results available now, it suggests that gal-3 induces a similar fibrotic response effect in concentrations ranging from 50 to 200 ng/mL in both HDFs and CFs. Moreover, the fibrotic response induced by gal-3 is around 30% in both CFs and HDS regardless of concentration, suggesting a general fibrotic response on gal-3 treatment. However, these are all resident fibroblasts and the currently pending results from the CAFs will enlighten whether CAFs will behave similar to the

resident fibroblasts when treated with gal-3. Interestingly, the effect of the positive control TGF-β1 was different in CFs and HDFs, with TGF-β1 treatment in CFs resulting in a 55% differentiation to myofibroblasts as compared to 30% in HDFs.

A similar study, where fibroblasts were also treated with 5 ng/mL TGF-β1 for 48 hours, saw a higher percentage of myofibroblasts than our study (33). This may indicate that the fibroblasts could be treated a bit longer to see if there is an additional effect.

One important factor that could impact the effect of the gal-3 and TGF-β1 treatment is that the cell lines require different media in this study, which could play a role in the differentiation. However, perhaps a medium can be found which can sustain both cardiac and cancer associated fibroblasts so they can be co-cultured.

Should the fibrotic response to gal-3 be validated with an increased number of replicates, then it could be an indication that the gal-3 induced fibrotic process may also be attenuated by a possible gal-3 inhibitor in future studies. Other murine studies have indeed seen a decrease in fibrosis when using a gal-3 knock out model or a gal-3 inhibitor (30,35,36). These studies also indicate that gal-3 expression in chronically inflamed tissue could result in myofibroblast activation and subsequent scar formation.

Regarding the BL6 mice from the pilot study, previous experiments done at the department of Experimental Cardiology have shown that mice of 8 weeks old who received an O-ring of 0.56 mm with TAC gives a HF phenotype with fibrosis. However, this O-ring size does not give a sufficient effect in mice of 6 weeks of age. Therefore, two smaller ring sizes were studied to determine whether the 0.46 or the 0.50 mm ring would prove effective to give a HF phenotype in a 6-week-old mouse model. During the pilot of the mouse study (N=15), it became evident that the mice could not be weaned from the ventilator when they were operated with the 0.46 mm nitrile O-ring. Three mice in a row were lost due to the effects induced by the 0.46 ring, and it was decided to cease the TAC surgery with this size due to the severity and loss of the mice. In case of the 0.50 ring, 50% of the mice survived the first week after surgery. While this is an improvement, the losses are still too great to continue operating the 6-week-old mouse model with the 0.50 ring, regardless of outcome. Should the 0.50 ring prove sufficient in HF severity and fibrosis formation at the end of the pilot, the optimal ring size between 0.50 and 0.56 should be studied to determine the optimal balance. This balance should be determined by HF validation, fibrosis formation and also the number of mice surviving the procedure. Alternatively, a different type of oncology mouse model must be chosen which can be fitted with a 0.56 mm nitrile-O ring at 8 weeks of age.

Future perspectives

The first future perspective which is already in progress is monitoring the fibrotic gene response of both the CFs and the CAFs, including an IF staining of the CAFs similar to the IF staining on CFs and HDFs. Moreover, the similar response to the gal-3 treatment of the dose-response curve could also be an indication that the dose response curve could be decreased to lower than 50 ng/mL and there would still be a pro-fibrotic effect. Lower dosages still affecting pro-fibrotic gene expression and an increase in myofibroblast differentiation would support this research translation to the clinic, as high gal-3 of 21,4 ng/mL predicts CV death (34). Furthermore, while the effect of the gal-3 induced fibrotic response has been measured in different types of fibroblasts, the interplay between them remains unknown. We hypothesise that due to excreted factors in either the treated cardiac fibroblasts or the treated cancer associated fibroblasts would result in increased proliferation of the cancer associated fibroblasts or an increase of myofibroblasts present in the cardiac fibroblasts. This due to the fact that cardiac excreted factors in the *in vivo* study by Meijers et al. showed an increase of tumour count from transplanted hearts with heart failure, suggesting this effect through circulating factors (5). More genes and perhaps proteins related to deposited extracellular matrix could be checked with qPCR to determine an increase of both fibrotic gene expression and an increase in myofibroblasts. A possible future prospect could be a co-culture of two types of fibroblasts to see whether previously treated cells would secrete fibrosis inducing agents which would call for an increased fibrotic response in their co-cultured cells. The same effect can possibly also be achieved through interchanging the (non-) activated cell medium. In both co-culture and conditioned medium swap, cells would require the medium to be the same. Furthermore, the possible fibrosis attenuating effect of the gal-3 inhibitor G3P-01 has not been determined during this study, and has to be assessed at a later time. A similar study with a gal-3 inhibitor based on modified citrus pectin was performed which showed no decrease in either gal-3 blood plasma levels or fibrotic gene expression (37). However, other pectin-based inhibitors have been shown to be effective *in vivo* against fibrosis formation. Therefore, the G3P-01 can both be effective but not result in a decreased fibrotic gene expression in humans. Currently, an omics-based literature study is being performed to determine how gal-3 inhibition results in a decreased fibrotic phenotype.

Regarding the murine pilot study, future steps should be based on the outcome of the 0.50 mm nitrile-O TAC operated mice. If these mice show a HF and fibrotic phenotype, a model must be chosen with a ring between 0.50 and 0.56 mm if available. If this has been determined, the next step would include attenuating the fibrotic process with G3P-01 (see figure 7). If gal-3 does cause an increase of fibrosis in the HF model, then a cardio-oncology model could be tested. This would possibly be a mouse model for spontaneous intestinal adenoma formation (BL6 ApcMin).

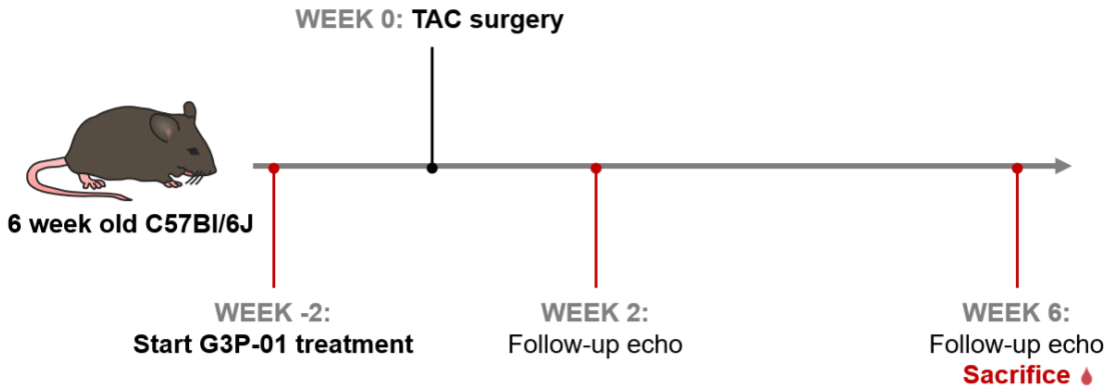

Figure 7: The mouse study design for the 6-week-old HF model which will receive the G3P-01 inhibitor. Treatment lasts the entire study.

Eventually, if the cardio-oncology model works and the G3P-01 inhibitor does attenuate fibrosis formation, this could be tested in humans in a similar intervention type study. People from the LifeLines study would be included with an overall good health (including no pre-existing CVD or cancer) and receive the G3P-01 inhibitor or a placebo in pill form for 30 days. Individuals would then be tested if biomarkers related to fibrosis changed after their treatment. People could be stratified based on their gal-3 levels, which would result in four groups to see whether there is an impact or not.

Should all of these future perspectives be met, then gal-3 inhibition would prove useful for patients who suffer from heart disease or cancer, and perhaps even other diseases which share the fibrosis hallmark, should the fibrosis be induced by gal-3. This also depends on the timing where G3P-01 can attenuate gal-3 induced fibrosis. Clinical implications could be that gal-3 levels should be measured after cardiac events such as myocardial infarction and treatment can be given with G3P-01 to perhaps prevent further fibrosis formation in the heart and to prevent cancer as a result from heart failure.

Limitations

Regarding the fibroblasts, both qPCR and IF are only performed as technical replicates of one experiment, so of the number of experiments need to be increased to determine more accurately the effect of gal-3 on the cells. Moreover, in HDFs TGF-β1 expression was lower and fibronectin expression maintained stable in gal-3 treated cells when compared to the negative control. No firm conclusions can be drawn about the difference in myofibroblast percentage between TGF-β1 treated cardiac and dermal

fibroblasts, as different media were used for each fibroblast type. Cell counting for α-SMA expression was done by one researcher, meaning that there will always be a bias. This bias was limited by making a picture from the DAPI channel for counting and that cells were only considered myofibroblasts if they showed both α-SMA expression as well as presented a myofibroblast morphology. However, due to the large differences, bias may not have a large impact on the result of this study. To remove bias, a recount can be done by someone else who is not familiar with the study.

The pilot mouse study of course consists of fewer mice than an ordinary study to determine a new possible HF model, but in this case has perhaps resulted in a very severe outcome in the mice with the 0.50 mm TAC ring. Several of these mice were hard to intubate and also perhaps died due to their inability to recover from their weaning from intubation, which may result in a low survival rate not due to the TAC alone. This may explain why these mice died quite soon after an otherwise perfectly performed operation.

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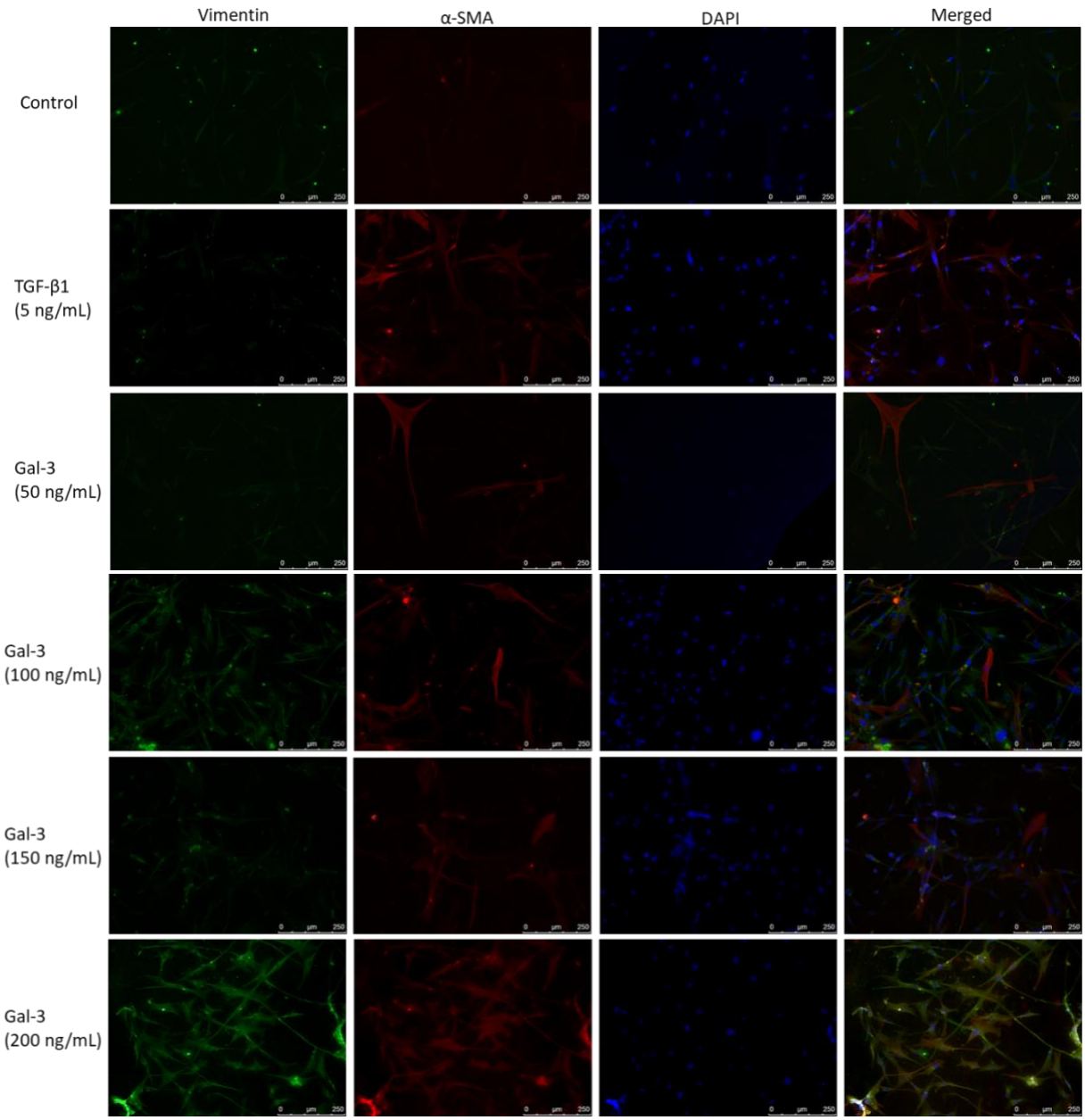
Appendix I: Wellness diary BL6 mice pilot study O-rings

MOUSE NO.	SURGERY	ALIVE	WEIGHT BEFORE SURGERY (G)	WEIGHT 1 WEEK AFTER OP (G)	COMMENT
0608523	TAC 0.50	No	18,9	NA	Died 1 night post op
0608523	TAC 0.50	Yes	18,5	18,7	
0608524	TAC 0.50	Yes	19,1	19,6	
0608525	TAC 0.50	No	21,7	NA	Died 2 hours post op
0608526	TAC 0.46	No	18,7	NA	Died after weaning intub.
0608527	TAC 0.46	No	19,4	NA	Died after weaning intub.
0608528	TAC 0.46	No	19,9	NA	Died after weaning intub.
0608529	Sham	No	19,5	NA	Died during intubation
0608530	Sham	Yes	21,2	21,6	
0608531	Sham	Yes	19,4	20,3	
0608532	TAC 0.50	No	17,7	NA	Died 2 hours post op
0608533	TAC 0.50	Yes	19	19,6	
0608534	TAC 0.50	Yes	19,7	19,5	
0608535	TAC 0.50	No	20,3	NA	Died 2 hours post op
0608536	Sham	Yes	19,9	20,7	

Appendix II: All channels and treatments IF staining CF



Appendix III: All channels and treatments IF staining HDF