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„The Influence of Desmin and SPARC on Cardiomyogenesis of Cardiovascular Progenitor Cells"

verfasst von / submitted by

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Abstract

Ever since the view of the heart as a post-mitotic organ was challenged and stem cells were found in the adult heart, they have become subject of intense research for stem cell therapies. This work deals with a specific population of cardiovascular progenitor cells and ultimately contributes to the understanding of the signalling networks within. There are two proteins of special interest, Desmin and SPARC, and the Group Weitzer aims to understand their role in cardiomyogenesis. For this, one part of this work deals with creating homozygous *sparc* and *desmin* knock-out cell lines by selecting for spontaneous inter-chromosomal recombination of a knock-out allele in our established heterozygous cell lines. Unfortunately, no *sparc*$^{-/-}$ or *desmin*$^{-/-}$ clone could be identified, however we could establish a reliable method of genotyping our cells for sparc.

The second part of this work aims to answer the question, whether the genes *sparc* and *desmin*, as well as other genes of interest (GOIs), are directly influenced by TGFβ signalling and inhibition thereof by Specific Inhibitor of Smad3 (SIS3). The most consistent influence of exogenous TGFβ1 and SIS3 were seen on the gene *brachyury*, as well as an upregulation of *desmin* by SIS3. Untangling the signalling pathways proved to be a complicated feat, as secondary, regulatory effects between our GOIs might play into our results.

Future experiments could profit from trying different Smad inhibitors or TGFβ2 in a combination with SPARC, as well as a culture method that allows basolateral uptake of our additives.

Acknowledgements

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I am also grateful to my parents and family, who supported me in every possible way during the long journey of my studies and I am proud to now present them with this degree.

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1. Introduction

For the longest time, the existence of regenerating stem cells in the adult heart was undiscovered. In the year 2001, Beltrami et al. published their findings of a non-terminally differentiated sub-population of cells in the myocardium. These “cardiovascular progenitor cells” (CVPCs) are a rare occurrence in the adult organ and are physiologically not able to regenerate lager tissue damage. They are, however, very relevant for developing potential therapies.

Cardiovascular diseases are the leading cause of death globally. They made up an estimated 32% of all gobal deaths in 2019 (World Health Organisation, accessed 23.4.2023) and damage to the heart, like after myocardic infarction, is as of now not fully recoverable. Due to the low self-renewal abilities of the organ and the high number of congenital or acquired heart diseases, there is a big interest in stem cell therapies. One such approach could be to use the CVPCs in the adult heart and stimulate them to proliferate and differentiate and ultimately regenerate diseased tissue.

The CVPCs are defined by an expression pattern involving stemness-factors as well as transcription factors (TFs) of the early myocardium. As such they represent a population in an in-between phase of mesodermal cells differentiating to various cell-types of the heart. Under the influence of leukemia inhibitor factor (LIF) the cells used in this study have the ability of self-renewal, while without LIF they differentiate to endothelial cells, cardiomyocytes and smooth muscle cells (Hoebaus et al. 2013).

1.1 CVPC Characterisation

CVPCs are a very heterogeneous group of cells that are found to be residing in different sites of the cardiac tissue, such as in the epicardium, the ventricles or atria, or the endomyocardium. They are traditionally defined by their self-renewability and multipotency, being able to differentiate into the three main cell types of cardiac tissue (cardiomyocytes, smooth muscle cells and endothelial cells) and can be sorted into several groups based on specific markers they test positive for. A rough categorisation would differentiate between Islet-1^+ cells, Sca-1^+ cells, CSPCs (cardiac side population cells), PDGFRα^+ cells, CDCs (cardiosphere-derived cells), c-Kit^+ cells and EPDCs (epicardium-

derived cells). Those different groups of CVPCs are specific for certain areas of the heart and their occurrence in the human or murine embryonic or adult heart (Ge et al. 2015).

Probably the most intensively studied and discussed group is the c-Kit$^+$ CPCs (cardiac progenitor cells). Early embryonic c-Kit positive cells without any cardiac markers yet were already identified very early in embryogenesis. During later stages of development, as well as in the adult heart, there are also several subpopulations of c-Kit$^+$ cells co-expressing various other factors. For example, c-Kit$^+$/VEGFR-2$^+$ cells are classified as vascular progenitor cells and c-Kit$^+$/VEGFR-2$^-$ but myocyte TFs positive cells are classified as myocardial progenitor cells. Generally, c-Kit$^+$ CPCs are said to be declining in frequency post-natal and become quiescent (Ge et al. 2015).

The population of c-Kit$^+$ CPCs was first discovered by Beltrami et al. 2001 in the murine heart. They were also described as self-renewing, clonogenic, multipotent and most importantly able to regenerate viable myocardium after transplantation into infarcted regions of the heart (Beltrami et al. 2003). These claims were later contested, especially after a controversy regarding falsified data by the connected laboratory surrounding Dr. Anversa, which led to a call to retraction of 31 papers by this group in 2018, all concerning c-Kit$^+$ cell characterisation and therapeutic use (Bolli et al. 2021). Specifically the paper regarding the clinical trial results of the SCIPIO project was retracted, with concerns regarding the precise character of the cells used in this trial (Bolli et al. 2011, The Lancet Editors 2019). Still, an extensive review of papers unrelated to this specific group, published in 2021, states that there is evidence for benefits of c-Kit$^+$ CPC administration after infarct (Bolli et al. 2021). However, across all 50 assessed papers, there was no evidence that implanted cells led to formation of significant amounts of new cardiomyocytes, neither by the implanted cells themselves, nor by resident CVPCs in the heart. The number of remaining c-Kit$^+$ cells after implantation was negligible and they did not engraft or exhibit the morphology and structure of mature cardiomyocytes. Instead, a paracrine function of these cells is proposed, which could for example improve the contractile abilities of the host heart (Tang et al. 2010, Bolli et al. 2021). In the newest phase II trial with c-Kit$^+$ CPCs and MSCs (mesenchymal stromal cells), CONCERT-HF, an improvement in clinical outcome was noted, albeit without reduction in scar size or left ventricle function improvement. Although the mechanism by which this improvement in heart function is achieved is still

unclear, the results do align with other studies (TAC-HFT and ixCELL-DCM) and allow a positive outlook for further stem-cell facilitated therapies (Bolli und Tang 2022).

The cells used in our lab and in this experiment are primarily extracted from the whole heart of mice and are tested positive for Sca-1 and Mdr. They are indeed self-renewing in vitro under the influence of LIF, can differentiate into endothelial cells, cardiomyocytes and smooth muscle cells and are able to form beating cardiac bodies (Weitzer et al. 1995, Hoebaus et al. 2013)

1.2 Transcription Factor Network During Cardiogenesis

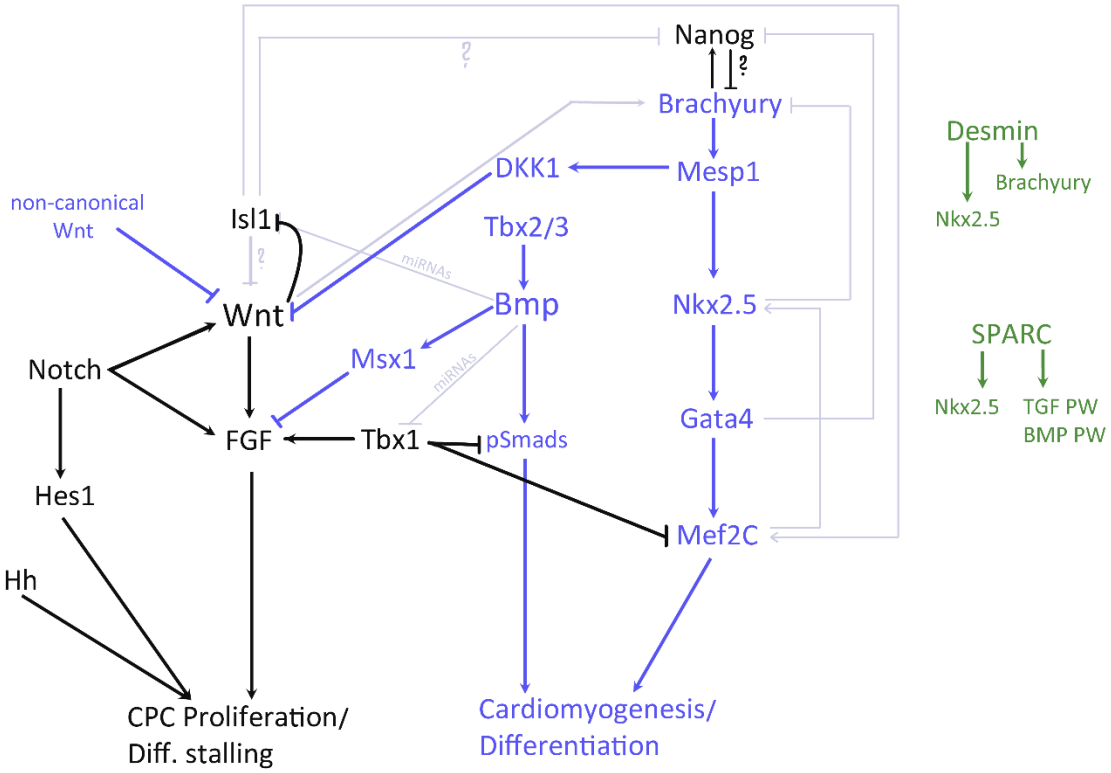

Figure 1 Schematic of the signalling network *during cardiomyogenesis based on the sources of this chapter. It is important to note that not all connections and effects are fully understood yet and also might not be effective simultaneously and in the same regions during development. The suggested main effect of a factor is categorised as either "CPC proliferation" (black) or "Differentiation" (blue), but there are also suggested auto-regulatory feedback loops in place. The proteins of our main focus and their known influences are shown separately in green (PW = pathway).*

During embryogenesis, cardiac progenitor cells are one of the earliest to immigrate through the primitive streak and differentiate into mesoderm. During this early stage, the transcription factor (TF) Mesp1 is expressed and can be used as a marker for the early mesodermal cells. Due to new single-cell RNA sequencing approaches, it was revealed that

the first signs of branching into either cardiomyocytes (CMs) or endocardial cells (ECs) might exist as early as embryonic day 6.75 in mice. This would mean that CM and EC lineage fate could already be determined at primitive streak formation. The exact relationship between these two lineages is still under discussion, with some experiments supporting the idea that they both arise from a common progenitor, and others showing that ECs migrate into the heart tube later (in zebrafish) (Sendra et al. 2022).

Later signalling cues for differentiation and primitive heart tube formation are provided from the adjacent endoderm. For example Nodal, or its mimic Activin, activates anterior visceral endoderm formation and thus contributes to cardiogenesis more indirectly (Liu und Foley 2011). Nodal/Activin/TGFβ signalling are commonly attributed similar function, one of the most important ones being epithelial-mesenchymal transition (EMT) (Tan et al. 2015). Generally, the involved signalling pathways can be attributed to one of two seemingly opposing purposes: Progenitor cell proliferation, or differentiation into e.g. cardiomyocytes. The balance between these two is meticulously orchestrated by a complicated network of factors, not all of which are fully known and understood yet. The following overview only covers selected factors and it is important to note, that gene expression of said factors may differ depending on developmental stage, but also might vary between different areas of the primitive heart at the same timepoint (figure 1).

Especially in the early stages of primitive streak formation and progenitor cell proliferation, the Wnt/β-Catenin (canonical Wnt) signalling pathway is a major player. Its activity follows a specific timing to prevent premature CM differentiation and is sometimes transiently activated in different stages, to ensure maintenance and proliferation. Without β-Catenin, there is no axis formation in the embryo and no mesoderm development (Liu und Foley 2011). Another important factor for cardiac progenitor cell (CPC) proliferation in specific regions of the growing heart tube is FGF. Especially Fgf8 seems to play a major role in heart tube extension at both poles. These relevant FGF TFs are also regulated by Wnt/β-Cat and Notch signalling. In this network, the Wnt/β-Cat signal itself was also shown to act downstream of Notch for CPC proliferation (Francou et al. 2013). Notch1 seems to interfere with general CM maturation and even marks cells differentiating into EC (Sendra et al. 2022). Hedgehog (Hh), which is one of the factors expressed in the adjacent ventral endoderm, also has a positive effect on CPC proliferation in the second heart field and acts

at least partially parallel to or upstream of Tbx5 (Xie et al. 2012). Similarly, Tbx1 may link signals from the endoderm to Fgf8 (Vitelli et al. 2002).

After appropriate elongation of the heart tube and sufficient proliferation of CPCs, the genetic program in certain areas shifts towards myocardial formation. The major factors to drive CM differentiation are the BMP family. The differentiation is to some extent promoted indirectly by antagonising canonical Wnt/FGF signals. This can be achieved either via factors like Msx1, or via microRNA expression which block for example *tbx1* transcripts (Tirosh-Finkel et al. 2010). The exact role of BMP signalling is still controversial, with different implications on cardiogenesis and cardiomyocyte development detected in different model species. Generally, it was proposed that BMP signalling is not required for initial CM specification, but rather for maintenance of CM gene expression. Also, specifically the role of regulating the number of atrial CMs is attributed to BMP. It was found to play a role in cardiogenesis via both the classical Smad-dependent pathways, but also Smad-independently via TAB1/Tak1 of the MAPK pathway (Liu und Foley 2011).
Other key cardiac transcription factors are Nkx2.5, Tbx20, Mef2c, Gata4 and Hand2. Mesp1 is known to activate Nkx2.5, which in turn can activate Bmp4, Gata4 and Mef2C, all of which promote cardiomyogenesis (Tirosh-Finkel et al. 2010, Francou et al. 2013). Mef2C can also be activated by Gata4 itself and Isl1, but is blocked by Tbx1. Nkx2.5 and Mesp1 also indirectly support differentiation by blocking Wnt/β-Cat signalling. It was also shown that Desmin upregulates *brachyury* (T) and *nkx2.5* and thus also stimulates cardiomyocyte differentiation (Hofner et al. 2007). SPARC seems to increase the effectivity of Bmp2 and TGFβ signalling and also Nkx2.5. Thus, it also stimulates CM differentiation and the general downstream activities of these two pathways (Francki et al. 2004, Stary et al. 2005). This highlights the importance of Desmin and SPARC as potential regulators of cardiomyogenesis and hence these two proteins and their roles in this complex network are the special focus of this group.

1.3 About Sparc

“Secreted protein acidic and rich in cysteine” is a matricellular protein, also called BM-40 and osteonectine. It is a protein that associates with the ECM and is collagen-binding, however it does not have a structural function itself. More so, it is described to have a

regulatory function, for example on cell proliferation and adhesion, as well as on matrix assembly and cytoskeletal rearrangements. In accordance with this, SPARC-null mice primarily show a deficit in fibrillar collagen and anomalous ECM or fibril morphology (Bradshaw 2016).

The exact function of SPARC in angiogenesis is not well understood yet. As of 2011, it was stated that its activity can, depending on the circumstances, promote angiogenesis or act anti-angiogenic (Harris et al. 2011). Similarly, the effect on cardiomyogenesis during embryonic development or in adult CVPCs is still under investigation. An example of promotion of cardiomyogenesis by SPARC is its positive influence on Nkx2.5. Some transcription factors known to initiate cardiac development by starting the myocardial gene expression program are Gata4, Mef2C and the aforementioned Nkx2.5. It was shown that in embryoid bodies and fetal cardiomyocytes, SPARC enhanced *bmp2* and *nkx2.5* expression. In addition, inhibition of either SPARC or Bmp2 in both cases led to lower *nkx2.5* expression and with it a negative effect on cardiomyogenesis. Hence it can be deduced, that SPARC and Bmp2 follow a similar pathway and have a similar effect in regards to Nkx2.5 and cardiomyogenesis. Also, it appears that this upregulatory effect of both proteins is co-dependent on each other (Stary et al. 2005). A similar effect of SPARC is observed in combination with TGFβ1, so SPARC might exert its influence on CM differentiation by co-acting with TGF superfamily ligands (Francki et al. 2004). A closer inspection of their relation is provided in the sub-chapters 1.6 and 1.7.

It is important to note that the available research is mainly based on fetal cardiomyocytes or embryonic stem cells. Although some aspects of the role of SPARC in cardiac development are known in these cell types, several of these functions still need verification in adult CVPCs. Our goal in this study is, however, to uncover a way in which *sparc* expression itself is regulated in CVPCs and if this regulation is manipulable by specific reagents. A positive interference on *sparc* expression could in turn lead to the described promotion of cardiomyogenesis in these adult progenitor cells.

1.4 About TGFβ Pathway

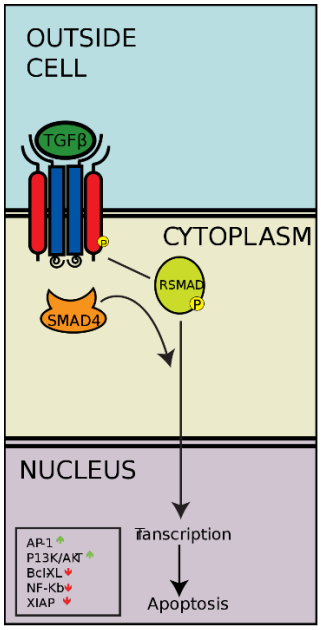

Figure 2 Simplified TGFβ pathway *and the roles of R-SMAD and SMAD4. (Picture by* Jerome Walker 2006 *under CC license)*

Well-known effects of the TGFβ-superfamily pathways are regulation of cell growth and differentiation as well as apoptosis. In this work, we used the protein Transforming Growth Factor beta1 (TGFβ1) in order to study its influence especially on *sparc* and *desmin* expression, and its interplay with innate SPARC and Desmin levels. TGFβ1 ligands bind to TGF-beta receptor type II dimer on the cell surface and induce a hetero-tetramer formation of the type II dimer with a receptor type I dimer. Through auto- and trans-phosphorylation processes of the receptors, they get activated and induce a signal cascade via various adapters and Smads, which ultimately results in transcription factor (TF) activity in the nucleus (figure 2).

A bit more specifically, Type I receptors can phosphorylate so called receptor-activated Smads (R-Smads) like Smad2 and Smad3. This leads to dissociation of the Smads from the receptors and in the cytoplasm, they form hetero-oligomeres with Co-Smad (Smad4). Phosphorylated Smad3 complex can translocate into the nucleus with the help of Importin-β1, whereas phosphorylated Smad2 complex enters the nucleus without any Importin assistance. In the nucleus, either of the R-Smad/Co-Smad oligomeres has gene-regulating activity. Once again, Smad3 and Smad2 act via slightly different mechanisms. The Smad3/4 complex is able to bind so-called Smad-binding elements (SBEs) on the DNA, while the

Smad2/4 complex can only interact with DNA via Smad4 interaction. Additionally, both the R-Smads and Co-Smad are able to interact with a variety of TFs or co-activators due to their MH1 and MH2 domains. A specific way of inducing transcription is via a R-Smad-Smurf (Smad-specific E3 ligase family) complex, which can attack and degrade transcriptional repressors in the nucleus. But it is also worth mentioning that Smads can in contrast to co-activators also recruit co-repressors, which can for example lead to histone deacetylase (HDAC) activity, a repressive chromatin modification (Moustakas 2002).

The effect of the TGFβ signalling can be controlled via regulating the available Smad pool in the cell. One of such ways is by ubiquitination of free Smad3 in the nucleus and subsequent export to the nucleoplasm and proteosomal degradation. The aforementioned Smurfs can also ubiquitinate phosphorylated R-Smads in the nucleus, which leads to degradation. Another pathway-inhibitory mechanism is via the inhibitory Smads (I-Smads) like Smad7 in association with Smurfs. These complexes act on the cytoplasmic site of the TGFβ receptors and basically block activation of R-Smads (Moustakas 2002).

As quite apparent, there are already many regulatory possibilities in even just the core-players of the TGFβ pathway. We suspect our proteins of interest (Desmin and SPARC) to be influenced and influence gene expression via some sort of TGFβ pathway interaction, but, as this introduction aims to highlight, finding the exact regulatory mechanism is a complicated feat, influenced by many small factors.

1.5 About SIS3

Specific inhibitor of Smad3 (SIS3) can act as an inhibitor of the TGFβ signalling in several ways (figure 3). Certain doses of SIS3 can reduce ALK-5 activity and specifically reduce the phosphorylation of Smad3, as well as the interaction of Smad3 with Co-Smad (Smad4). No effect of SIS3 on the phosphorylation of Smad2 was found. SIS3 was indeed reported to be able to attenuate transcription, for example of TGFβ1-induced type I procollagen, and inhibit differentiation of fibroblasts to myofibroblasts, as would also be induced by TGFβ1 (Jinnin et al. 2006).

Similar experiments to ours were able to report increased HIF-1α gene and protein expression in human periodontal ligament cells due to TGFβ1 treatment, as well as increased Smad3 phosphorylation and decreased PHD2 expression. Treatment with SIS3 on

the other hand reduced Smad3 phosphorylation, TGFβ1 -induced HIF-1α expression and protein stability and increased PHD2, which seems to be the negative controlling factor of HIF-1α protein stability (Watanabe et al. 2013).

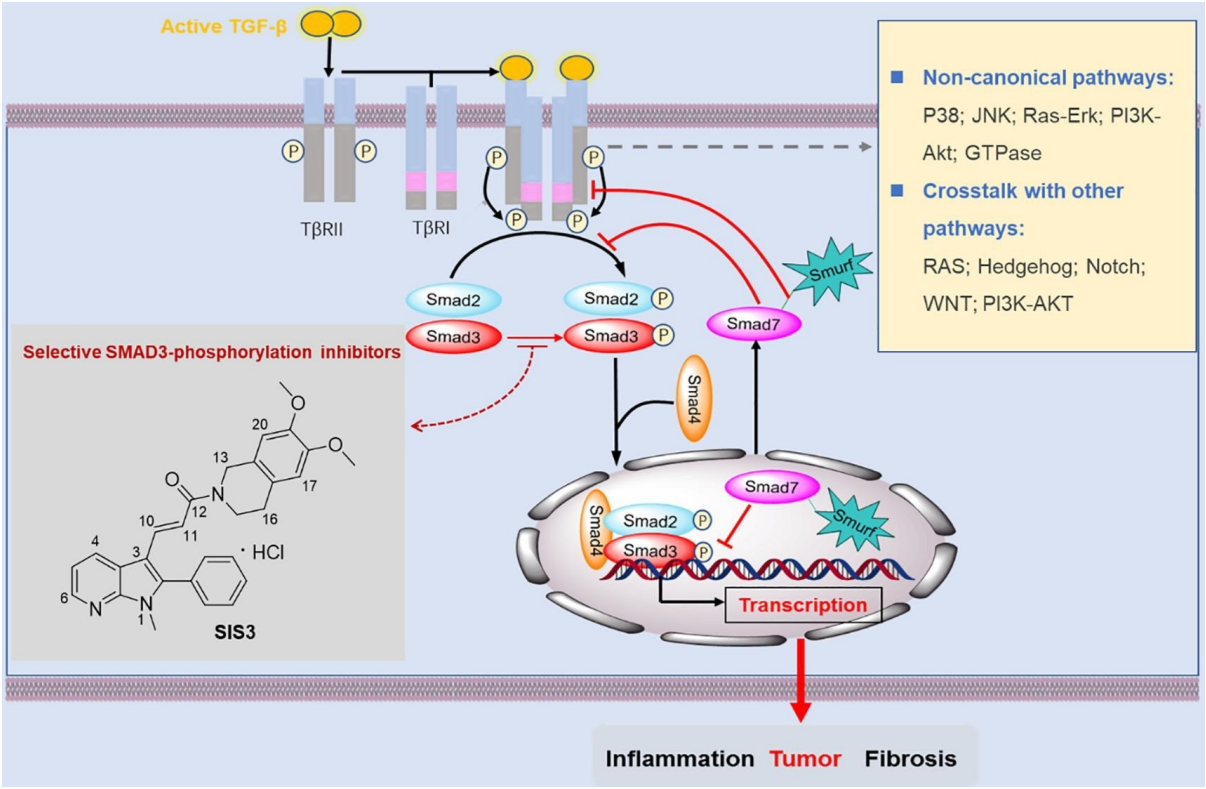

Figure 3 *The role of SIS3 in the TGFβ signalling pathway. As seen in here, TGFβ can bind a TGFβRI dimer, which then tetramerises with a TGFβRII dimer and it auto-phosphorylates/phosphorylates Smad2 and/or Smad3. Shown in the bottom left corner is SIS3, which blocks this Smad3 phosphorylation and thus the rest of the downstream pathway (picture by* Wu et al. 2020*).*

1.6 TGFβ and SPARC Interactions

Experiments to determine the relationship between SPARC and TGFβ were already done for example by Wrana et al. 1991, however in human fibroblast cells and not in CVPCs. The results of that study showed that an addition of 1,0 ng/ml TGFβ leads to a maximum 3,5-fold increase of SPARC protein after 24h. SPARC mRNA levels were similarly increased, with a maximum of 3,9-fold increase after 24h. This increase is not due to a higher TGFβ mediated stability of the SPARC mRNA. The stimulation of mRNA synthesis was successfully blocked by cycloheximide, a translation inhibitor. Their conclusion was that a nuclear post-transcriptional mechanism is responsible for the regulation of SPARC by TGFβ.

It was further shown that SPARC can regulate TGFβ1 expression via a TGFβ1-dependent pathway and thus proposed a reciprocal, autocrine regulatory feedback loop between the two (Francki et al. 2004). Data suggested that SPARC regulates the TGFβ1-dependent phosphorylation of Smad-2 in murine primary mesangial cells. In SPARC-null cells, the amount of phosphorylated Smad-2 was significantly lower than in WT cells, while the total amount of Smad-2 stayed the same. Other Smad family proteins like Smad-3, -4, -6 and -7 were not affected in this way. Also, mRNA expression of the mentioned Smads, as analysed by RT-PCR, did not change in SPARC-null cells. So, a lack of SPARC effects the TGFβ pathway in such a way that Smad-2 phosphorylation is reduced.
In summary, they showed that SPARC interacts with the TGFβ-receptor complex in a ligand-dependent manner, hence under the condition that TGFβ1 is bound to receptor type II. This interaction can lead to structure alterations of the receptors and changes in the type I receptor kinase activity or downstream signalling. They observed TGFβ-pathway "enhancing" changes, such as phosphorylation (=activation) of Smad-2 or JNK. This either happened by exogenous SPARC in synergy with exogenous TGFβ1 in WT cells, or independently of TGFβ1 in cells lacking endogenous SPARC. This suggests not only a different effect of endo- and exogenous SPARC, but also a TGFβ1-dependent and -independent influence of SPARC on the TGFβ1 pathway. A general lack of intrinsic SPARC expression in any case showed reduced Smad-2 phosphorylation and a reduced positive effect of recombinant TGFβ1 and SPARC. It was also proposed, that only in presence of SPARC the type I receptor kinase might alter its binding efficiency to different downstream signalling molecules and thus alter the downstream effect from TGFβ1-only to TGFβ1+SPARC (Francki et al. 2004).

A study in a different model cell line, human trabecular meshwork endothelial cells, showed a clear upregulation of SPARC via TGFβ2 on protein and mRNA level. Increased phosphorylation of Smad3 was detected about 1 hour after TGFβ2 treatment and selective inhibition of Smad3 was able to suppress the induced *sparc* expression again. Inhibitors against other players in the TGFβ2 pathways also proved to effectively suppress the *sparc* up-regulation, leading to the conclusion that TGFβ2 works via a Smad-dependent and -independent signalling pathway. It is proposed that JNK signalling provides the baseline of

upregulation, while Smad2/3 and p38 signalling pathways lead to an additional increase in *sparc* expression (Kang et al. 2013).

1.7 TGFβ and BMP Interactions

BMPs are a group of signalling molecules of the TGFβ superfamily, which bind to their own receptors and, in contrast to TGFβ, use Smad1/5/8 as downstream effectors. However, TGFβs and BMPs share the component Smad4 in their signalling cascade, which lead to studies of the potential cross-talks of these pathways. Contrary to earlier proposals, it seems like BMP and TGFβ (specifically BMP4 and TGFβ3) do not inhibit each other's downstream signalling or compete for Smad4, as nuclear localisation of their respective pathways' transcription factors was not decreased. Even with low abundance of Smad4 present in the cells, there was no evidence found that competition over Smad4 lead to negative regulation between the pathways. However, it was discovered that TGFβ3 could also cross-activate pSmad1/5/8, but BMP4 did not cross-activate Smad2/3. So in summary this means TGFβ3 and BMP4 do not inhibit each other's signal transduction and TGFβ3 can have an activating effect on BMP signals but not vice versa (Coster et al. 2016). Taking this into account, TGFβ1 treatment of our cells might also lead to increased expression of BMP2 or BMP4 target genes.

1.8 About Desmin

Desmin is an intermediate filament protein which plays a key role in the integrity and coordination of the physical structure of cardiomyocytes. It is also an important element in early heart development, as it is the earliest myogenic marker described in mice and is necessary for healthy heart development (Weitzer et al. 1995, Milner et al. 1999).

During the mesoderm formation, *desmin* expression and synthesis is increased. This leads to the up-regulation of the genes *brachyury* and *nkx2.5* and also to an accelerated cardiomyogenesis. It was further reported that cells with active *desmin* genes developed into large and proliferating cardiomyocyte clusters, which were highly interconnected and were able to contract synchronously. In contrast to this, *desmin*$^{-/-}$ cells showed the opposite phenotype (Hofner et al. 2007).

This is already a strong indicator that Desmin plays a key role in cardiomyocyte differentiation and hence our group also has special focus on this gene and its effects.

Furthermore, Desmin most likely up-regulates *nkx2.5* directly in cardiac progenitor cells, since Desmin was able to activate transcription of *nkx2.5* reporter genes and even rescue *nkx2.5* haploinsufficiency. This idea of a direct regulatory mechanism is further supported by the fact that Desmin was detected in the nucleus, if temporarily, and is able to physically interact with TFs on *nkx2.5* promoters and enhancers (Fuchs et al. 2016).
Another strong indication for Desmin being effective via a direct interaction with TFs in the nucleus is a study showing that a functioning amino-terminus is necessary for Desmin's described function and also for nuclear localisation (Hofner et al. 2007).

1.9 Hypothesis and aim of the Study

The works of our group and the study on hand deal with certain signalling pathways in CVPCs and how they could be intervened to stimulate differentiation into cardiomyocytes. Important players in early myogenic development are the proteins SPARC and Desmin, which this work will specifically focus on. Of special interest is the regulation of their expression, potentially via the TGFβ pathway and the signalling molecules Smad2/3.
Specifically, we aim to answer the question whether TGFβ signalling and inhibition of Smad3 phosphorylation by SIS3 alter gene expression of *sparc*, *desmin* and other GOIs in self-renewing and differentiating cardiac stem cells, and if so, whether these effects are influenced by the intra-cellular expression level of SPARC and Desmin.
We approached this question by conducting several differentiation experiments with either exogenous TGFβ1 or SIS3 added for 0-49 hours in cell culture and subsequent reverse-transcriptase quantitative-PCR (qPCR) to determine expression levels of GOIs. Investigation of whether innate levels of Desmin or SPARC influence the signalling in question was attempted by using *sparc* and *desmin* wild-type cells, as well as a *sparc*$^{+/-}$ and *desmin*$^{+/-}$ cell line.

Additionally, there was an attempt at creating sub-clones of our heterozygous *sparc* and *desmin* cell lines, which should exhibit a full knock-out of either gene. The chosen method to achieve this was by selecting for spontaneous inter-chromosomal recombination of the KO-alleles in single clones. In the past, it was not possible to successfully create homozygous KO lines for either gene, as especially *sparc* KOs seemed to be not viable.

2. Materials and Methods

2.1 Media and Stocks

Culture Medium M10Gi: 10%Vol Gibco Fetal Bovine Serum, 89%Vol DMEM, 1%Vol GPS. Medium for feeding SNLs and FCs.
Culture Medium M15Hy: 15%Vol Hyclone Fetal Bovine Serum, 83%Vol DMEM, 1%Vol GPS, 1%Vol β-Mercaptoethanol. Medium for feeding CVPCs
Freezing Medium: 60%Vol DMEM; 20%Vol FBS Hyclone; 20%Vol DMSO
DMEM (Dulbeco's Modified Eagle Medium): with 4,5g/L glucose, by Thermo-Fisher Scientific
GPS (Glutamin-Penicillin-Streptomycin): 4,25g NaCl; 1,5g Penicillin; 2.5g Streptomycin; 14,6g L-(t)-Glutamin; filled to 500ml with H2O
DMSO (Dimethylsulfoxid): by ACROS Organics
PBS (Phosphate Buffered Saline): 10x Stock Solution contains 80g NaCl; 2g KCl; 10,72g Na2HPO4●H20; 2g Kh2PO4; filled up to 1l with H2O; pH 7,2
Trypsin: 3,5g NaCl; 0,5g D-Glucose; 0,11g Na2HPO4●H20; 0,185g KCl; 0,12g KH2PO4; 0,2g EDTA; 1,25g Trypsin (Thermo-Fisher Scientific); 1,5g Tris; filled up to 500ml with MilliQ H2O; pH 7,6
Mitomycin C: 0,5mg/ml in 1x PBS, for mitotic inactivation of SNL76/7 cells. By ABCAM
G418: Gibco™ Geneticin™ Selective Antibiotic (G 418 disulfate salt), by Sigma-Aldrich. For selection. Used in a final concentration of 0.96mg/ml in medium for selecting for homozygous KO cells. Considering the product possessed only 45% potency, 40mg/ml were used for the stock solution, instead of 18mg/ml.
SIS3 stock: 0,5mM, 2,45 µg/ml, dissolved in DMSO, final conc. on cells 12,25ng/ml, by Sigma
TGFb1 stock: dissolved in 5mM HCL + 0,1mM BSA, 10x stock conc. 50µg/ml, 1x stock conc. 5µg/ml diluted right before use in M15Hy Medium. Final conc. on cells 50ng/ml. By R&D.
Lysisbuffer for RNA purification: 350yl FARB Puffer from the Kit (Favorgen: FavorPrep Total RNA MiniKit) + 3,5yl β-Mercaptoethanol per well or sample
Lysisbuffer for gDNA purification: 10mM Tris pH7,5 + 10mM EDTA pH8,0 + 10mM NaCl + 0,5% Sarcosyl.
1x TAE Buffer: 40mM Tris, 20mM Acetate, 1mM EDTA

2.2 Cell lines

Cell type	Cell Line Name	Abbreviation
Fibroblasts	SNL76/7	FCs (after mito. inactiv.)
CVPC WT	A5B8/1	A5 or WT
CVPC Sparc +/-	A5-SparcKO-K11	K11
CVPC Desmin +/-	A5-DesKO-2H	2H

Table 1 Overview of the cell lines used in this study. *The fibroblasts served as LIF producing feeder cells, while the various CVPC lines were the actual model system.*

The cardiovascular progenitor cells (CVPCs) used in this experiment stem from the cell line "A5 wildtype" (WT). This original WT cell line was extracted from heart tissue of 2 days old mice and carried the neomycin resistance vector ("neo-cassette") on one *hdac1* allele (histone-deacetlyase) (Hoebaus et al. 2013). As a wildtype control in our experiments, we used the sub-subclone A5B8/1. Further we used the cell line A5-SparcKO-K11 (K11), which is an A5 derivative with another neo-cassette in one *sparc* allele, hence a *sparc* +/- line (Gilmour et al. 1998). Also the line A5-DesKO-2H (2H) was used, which are also A5 derivative cells with a neo-cassette in one *desmin* allele, so a *desmin* +/- cell line (Weitzer et al. 1995).

FCs are SNL76/7 fibroblasts which were mitotically inactivated and serve to produce LIF (Leukemia Inhibitory Factor), which slows down CVPCs differentiation. This FC cell line was produced by McMahon and Bradley by also introducing a neo-cassette and additionally another LIF gene into fibroblasts (McMahon und Bradley 1990).

2.2.1 Culture of SNL76/7 fibroblasts

SNL76/7 cells were cultivated on 10cm plates at 37°C with 10ml M10Gi medium and were split about once a week. To do so, the medium was removed and the cells washed with 5ml PBS, then 1ml trypsin was added and incubated with the cells for about 6 minutes, before stopping the trypsin reaction with 3ml M10Gi medium and splitting the cells suspension at the desired ratio onto fresh 10cm plates. These cells do not require a gelatine coating on the wells.

2.2.2 Making of Feeder Cells

To turn SNL76/7 into the FCs used in CVPC culture, they have to be mitotically inactivated. This is achieved by first reducing the amount of medium per plate to 4ml, then adding 80µl of Mitomycin C and incubating the plates for 4h at 37°C. Meanwhile, 24-well plates were coated with 500µl 0.1% gelatine per well, the supernatant of which was carefully removed again after approximately two hours. After the incubation with Mitomycin C, the cells were washed twice with 5ml PBS and then trypsinated with 1ml for 6min. After stopping the reaction by suspending the cells in 3ml M10Gi medium, the cell density was counted on a cell counter (DeNovix® CellDrop FL) and diluted to achieve $30*10^4$ cells/ml. 500µl of this FC suspension were transferred onto each of the previously prepared 24-well plate wells. Feeder cells kept in culture alone as stock were fed every 7 days with 500µl M10Gi medium.

2.2.3 Culture of CVPCs

All CVPCs are cultivated on cell culture plates with a layer of 0.1% gelatine and a layer of "Feeder Cells" (FCs). Feeding of CVPCs in culture was carried out approximately every 24 hours with M15Hy medium, if not stated otherwise. In case of the culture plates being confluent (fully covered by cells), the contents were split, usually 1:4, onto fresh FC wells. To transfer cells to fresh wells, the culture medium was first removed, then the cells were washed with 500ml PBS and then incubated at 37°C with 200µl trypsin for about 20min. The trypsin reaction was stopped by adding 600µl M15Hy and thoroughly suspending the cells in the medium. Then, a respective part of the suspension was transferred onto a new well with FCs and about 1ml H15Hy medium.

2.2.4 Thawing of cells

The desired cryovials of any cell line were gathered from the -150°C storage freezer and thawed as quickly as possible in an approximately 37°C warm water bath. The entire content (ca. 1ml) was transferred to a 15ml Falcon tube and 1ml M10Gi medium were slowly added in single droplets. Subsequently, another 7ml of M10Gi were added and mixed gently. The tubes were centrifuged for 7min at 1000rpm, the supernatant was carefully removed and the pellet resuspended in 1ml M15Hy. The entirety of the cell suspension was plated onto one 24-well plate well, containing FCs and 1ml M15Hy medium beforehand.

2.2.5 Freezing of cells

To freeze living cells, they were fed with 1,5ml fresh M15Hy 2hrs prior to freezing. Then the cells were washed with 500µl PBS and detached from the wells with 200µl trypsin for 20min, which was countered then by adding 800µl M15Hy. At first, 200µl of freezing medium were added, then an additional 800µl were added and mixed well with the cells. After filling cryovials with 1ml of cell suspension each, they were frozen in a tightly sealed Styrofoam box at -70°C and after about a week transferred to either a -150°C freezer or liquid nitrogen.

2.3 EXPERIMENT 1: Homozygous Desmin and Sparc KO lines

2.3.1 Setup of selection and genotyping

This experiment aimed at creating homozygous *desmin* and *sparc* KO cell lines by selecting for spontaneous interchromosomal recombination and thus duplication of the already introduced *sparc* or *desmin* KO allele, respectively.

To select for potentially homozygous KO cells, the 2H and K11 cell lines were cultivated on 10cm culture dishes under the influence of 0,96mg/ml G418 (426µl of the actively 18mg/ml stock in 8ml M15Hy medium). However, already after 3 days of cultivation, the plates were too confluent to guarantee a successful selection, so all plates were split 1:10000 onto new 10cm plates. Following another week of G418 selection, fresh FCs were added, to secure continuous LIF production under the damaging influence of the antibiotic. Therefor, a 10cm plate confluent with FCs was trypsinated, the reaction stopped with 4ml M15Hy and 2ml of the cell suspension were added to one 10cm CVPC plate, replacing 2ml of fresh medium. Another four days later, so effectively after 12 days of selection, the surviving clones were picked. This was realised by gathering one single clone on the plate at the time with a 200µl pipet tip and transferring the cells onto a 96-well containing 30µl of trypsin, to dissolve the colony. The trypsin reaction was halted with 70µl M15Hy and the suspension transferred onto a fresh 96-well with FCs and a final amount of 210µl medium. With this method, 34 potential homozygous clones per +/- cell line were extracted.

These 96-well wells were continuously fed with 200µl M15Hy medium and the same G418 concentration as before. Upon reaching confluency, they were transferred onto 24-well

wells with FCs. Finally, after freezing some backups, the cells were cultivated on just gelatine wells for two passages, to remove FCs which could influence future gDNA analysis.

Originally, cell density on the culture plates was not determined prior to harvesting them for gDNA purification. Hence, living backups of 10 potential *sparc* -/- clones were thawed and cultivated again at a later date, without additional G418. The clones in question were A5-SparcKOK11- K3, K10, K11, K16, K19, K20, K21, K24, K26, K35. This time, they were "pre-adsorbed" to remove FCs by trypsinating the wells and transferring the content onto gelatine plates for 45min. This leads to FCs accumulating on the gelatine coating, while CVPCs will largely still stay in suspension and can be removed again by gathering the supernatant carefully. Subsequently the cells were also kept on gelatine wells for two passages, to remove as many FCs as possible.
These 10 clones were, in contrast to the usual treatment noted in "gDNA purification", first washed with 500µl PBS, trypsinated with 200µl for 20min, then suspended in 800µl medium and the cell density was counted at a cellcounter (DeNovix® CellDrop FL).

2.3.2 gDNA purification

The cells of the confluent wells were, unless they were counted as stated above, scraped off in 500µl PBS and the suspension transferred into 2ml tubes. The tubes were centrifuged for 7min at 1000rpm and the pellet with supernatant frozen in liquid nitrogen and stored at -70°C.
Upon further use, all tubes (counted or not counted) were centrifuged again with the same settings, then the supernatant was discarded carefully and the pellet was washed in 500µl PBS, centrifuged again, the supernatant again discarded and the pellet dissolved in 500µl lysis buffer. The tubes were sealed with Parafilm and incubated overnight on a shaker with 60°C.
The following day, 1ml cold precipitation ethanol were added (EtOH absolute with 0,075M NaCl) and the tubes were inverted until the DNA precipitated. Afterwards, the samples need to be stored at -20°C for at least 5 hours or overnight.
Subsequently all samples were centrifuged for 30min at 14.000rpm, preferably in cooled conditions. The supernatant was discarded, pellets resuspended in 500µl cold EtOH 70% and the tubes again centrifuged for 15min at 14.000rpm, the supernatant discarded and the pellets again resuspended in 500µl cold EtOH 70%. After another centrifugation step of

15min at 14.000rpm and discarding the supernatant, the pellets are dried in the open tubes for about 45min. Then the DNA is solved in 50µl diethylpyrocarbonate (DEPC) treated H2O, which is free of endo-, exodeoxyribonucleases, ribonucleases and phosphatases.
DNA concentration was measured on a spectrophotometer (NanoDrop 2000c). DNA in solution was stored at -70°C.

2.3.3 Standard PCR (gDNA)

Gene Name	Primer Name	Sequence [5'-3']		Length (bp)
		fwd	rev	
Sparc	I5-gen-fw	AGCCAGAGCCAACTTATGCT		1717/ 6013
	3_I6-r		GCCTAGTGAGTCATCTCCTGAGC	
Desmin	Des-dKO-1	GGGCTACAAATAGTGCAGACAG	CCGGGGAATGACCTTTTCCA	806/ 2034
Desmin	Des-dKO-2	GGGCTACAAATAGTGCAGACAG	GGAGCCACTCCCTTGAAGTC	1650/ 2878
Hdac	Hdac-I7-NEO	TCGCCTTCTTGACGAGTTCTTCT	GTGGCTCACTAAGCTGGGTT	300 (KO)
Hdac	Hdac-E5	GCCTTGTGTCTTGGAAGAGC	GCTGAAGGAAGGTGGAAGAGTGG	317 (WT)
Hdac	Hdac-E5-I7	GCCTTGTGTCTTGGAAGAGC	GTGGCTCACTAAGCTGGGTT	1918/ ~7000

Table 2 *Primers used on genomic DNA of all three cell lines. Most primers were designed to span a different number of base pairs, depending on whether they bind a WT allele or KO allele. Hdac-I7-NEO was designed to span from the hdac Intron 7 into the NEO cassette and thus only detect KO. Hdac-E5 on the other hand binds in the Exon 5, which is only present in WT.*

To confirm the existence of the KO allele and to determine the ratio of KO allele to WT allele in the selected, potential homozygous KO cell lines, standard PCRs were performed.

Here, a primer pair in *sparc* Intron 5 and Intron 6 was used, which would produce amplicons with different lengths in the WT allele and the KO allele. On the following gel electrophoresis, bands at different heights would be visible.
The same principle was utilised for the design of the *desmin* primers and to confirm the

expected *hdac*$^{+/-}$ genotype also for *hdac*. Since the concept didn't prove to be very fruitful for these two genes, a few different primer pairs had to be tested and are listed above (table 2).

The purified gDNA samples of the potential *desmin*$^{-/-}$ and *sparc*$^{-/-}$ clones were normalised to 2µg/20µl and measured once more on the Nanodrop. Then they were diluted 1:10. The optimal concentration of DNA is 40ng per 20µl reaction. Hence, different amounts of DNA and water were used for each sample, based on the stock concentration measured prior.

The following master mix was prepared per sample/PCR tube:

10µl Mastermix Invitrogen Platinum Superfy Green II
3µl H2O DEPC
5µl gDNA (40ng=x µl gDNA + x µl H2O to reach 5µl)
2µl Primer Mix (10µM)

Cycle Protocol Sparc:

1) 98°C – 30 sec
2) 98°C – 10 sec
3) 60°C – 15 sec
4) 72°C – 3 min 30 sec
repeat 2)-4) 25x
5) 72°C – 5 min
4°C hold

The cycle protocol for the PCR with *desmin* and *hdac* primers differed in step 4, where the extension time was 1 min 30 sec instead. Standard PCRs were conducted on a Biometra TRIO (Analytik Jena) thermocycler. The PCR products were run on a 1% agarose gel in 1x TAE buffer for about 1h at 90V. Per lane, 15µl PCR product or 4µl Quick-Load® Purple 1kb Plus DNA Ladder (New England Biolabs) were used. No extra dye was added, since the PCR Mastermix already contains dye for gel electrophoresis.

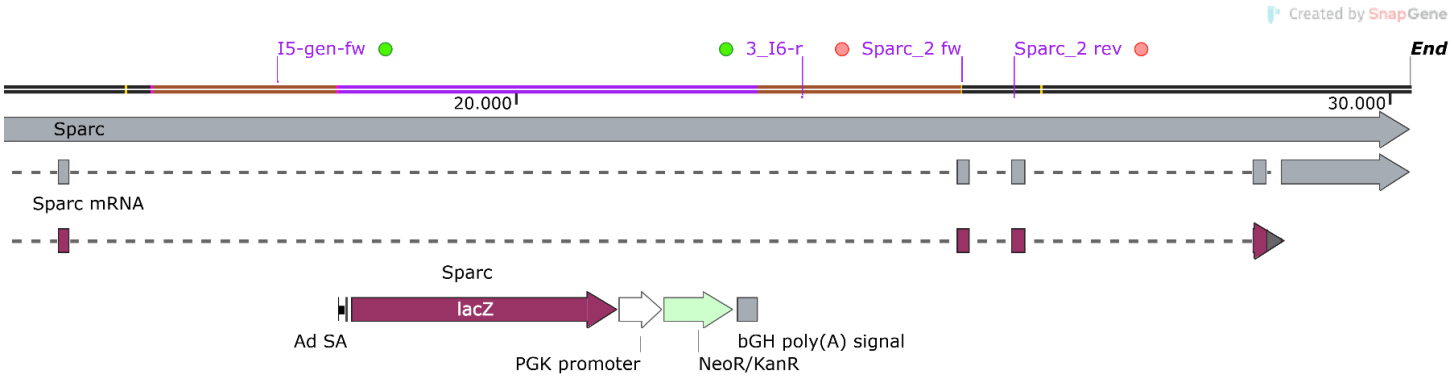

Figure 4 Sparc gene map from exon 5 until the end*. Exons are depicted as boxes, introns as a dashed line. The KO insert is shown as the coloured stretch, replacing exon 6, whereby the brown area is still WT sequence and the purple part is the lacZ/NEO insert depicted below. The primer pair used in this experiment is shown marked in green, in this case spanning the longer KO insert. Also shown marked in red is the Sparc_2 primer used for WT RNA/cDNA quantification, which didn't lead to suitable results and is therefore not included in this report. Sparc_2 rev is exon-spanning, meaning it is partially spanning exon 7 and 8.*

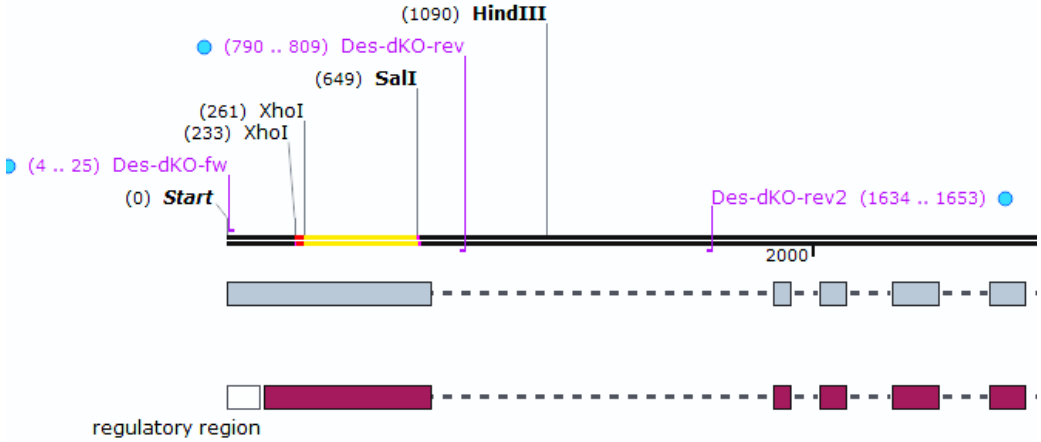

Figure 5 Desmin WT gene map from transcription start until exon 5. *Exons are depicted as boxes, introns as a dashed line. The KO insert partially replaces exon 1 (vector site yellow) on the KO allele. Des-dKO-fw/rev are the forward and reverse primers of the Des-dKO1 pair, while Des-dKO-fw/Des-dKO-rev2 are the primers of the Des-dKO2 pair. With the KO vector inserted, longer amplicons than depicted in this WT allele are expected.*

2.4 EXPERIMENT 2: Gene expression experiment

2.4.1 Experimental setup

In this experiment, the usual three cell lines (A5-WT, K11 and 2H) were subjected to exogenous SIS3 or TGFβ1 for 49 hours. RNA samples were taken at 0hrs (no stimulant added), 3hrs, 6hrs, 20hrs, 28hrs and 49hrs. All time points consisted of three replicate wells and three control wells respectively (see figure 6).

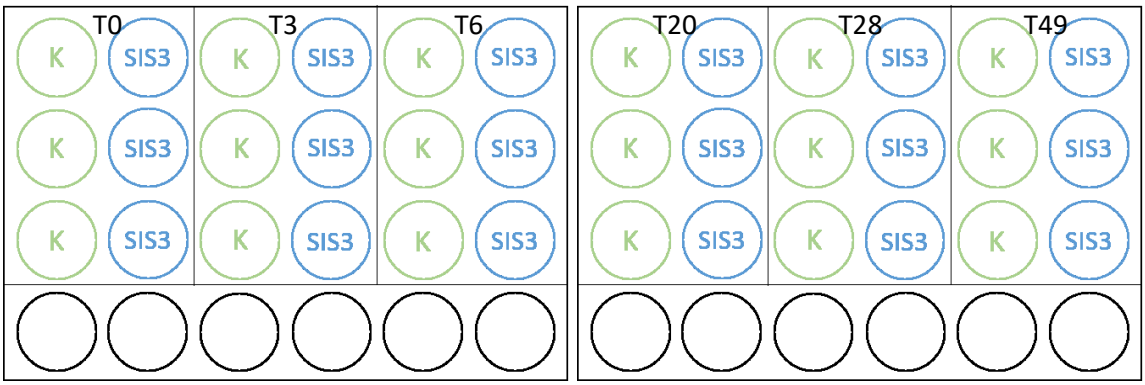

Figure 4 *gene expression experiment, cell culture setup on 24-well plates. Each time point had three replicates of control and test group each.*

On the first day for each round of this experiment, the cells of the desired cell line were trypsinated from confluent 24-well plates and transferred to 10cm gelatine plates for 45min for pre-adsorption and removal of FCs. Afterwards, the supernatant of the 10cm plates was carefully collected and pooled into a tube and the cell number per ml was determined on a cellcounter (DeNovix® CellDrop FL). 500.000 cells were seeded onto each of the 36 wells (figure 6).

Exactly 22hrs later, all wells were fed with 1990µl or 1980µl fresh M15Hy medium for SIS3 or TGFβ1 experiments respectively. Two hours later, so exactly 24hrs after seeding the cells, the six T0 wells were already washed and trypsinated for 20min. This constitutes as T0. Meanwhile, to all other test wells 10µl SIS3 stock (end conc. 12,25ng/ml) or 20µl of 1x TGFβ1 stock (end conc. 50ng/ml) were added. For SIS3 experiments, the control groups were subjected to 10µl DMSO and for TGFβ1 experiments, the controls had 20µl of TGFβ1 solvent (5mM HCL + 0,1mM BSA, 1:10 diluted in medium).

At each time point, after trypsination with 200µl, 800µl of medium were added and the concentration of cells was measured on the cellcounter. An amount equal to 500.000 cells was transferred to new tubes and they were centrifuged for 7min at 1000rpm. The supernatant was discarded, 350µl RNA Lysisbuffer (prepared as 2100µl FARB-buffer + 21µl

β-Mercaptoethanol) were added and the pellets were shock-frozen in liquid nitrogen, then kept on -70°C until further use.

This experimental setup was carried out for all three cell lines with both TGFβ1 and SIS3. The A5 culture experiment was performed twice, however only one set was used for further qPCR analysis. Hence, this cell culture setup was carried out a total of 8 times.

2.4.2 RNA isolation and cDNA

The further RNA isolation from our lysed cell samples was performed as according to the "Isolation of Total RNA from Human Whole Blood" protocol of the "Favorgen: FavorPrep Total RNA MiniKit", without the optional steps. In the final step, the RNA was eluted in 40µl DEPC H2O and the concentration was measured at the Nanodrop. Samples were stored at -70°C until further use.

For cDNA preparation, RNA samples were diluted to 1µg per 8µl DEPC H2O. The "RevertAid First Strand cDNA Synthesis Kit" (Thermo Scientific) was used. To the 8µl of normalised sample, 1µl 10x Reaction Buffer and 1µl DNAseI were added. Then, the samples were incubated at 37°C for 30min. After adding 1µl 50mM EDTA, samples were incubated for another 10min on 65°C before adding 1µl Oligo dT primer, 4µl 5x Reaction Buffer, 1µl RiboLock Rnase Inhibitor, 2µl 10mM dNTP mix and 1µl RevertAid Reverse Transcriptase (RT) in that order. Simultaneously, NTCs without RNA (instead 8µl pure water) and NRTs without reverse transcriptase (instead 1µl water) were produced. The last incubation step consisted of 60min on 42°C and another 5min on 70°C. A 1:50 dilution of all samples was prepared.

Alternatively, for cDNA preparation, the "LunaScript® RT SuperMix Kit" by New England Biolabs was used (for 2H+SIS3 and 2H+TGFβ1 experiments). This kit uses 4 µl LunaScript® RT SuperMix (5X) a varying amount of RNA to reach 1µg RNA total and a varying amount of nuclease-free water (from the kit) to 20µl total reaction volume. For NRTs, the No-RT Control Mix (5X) is provided in the kit. Then, samples are incubated at 25°C (left on room temperature) for 2min, then moved to 55°C for 10min and finally 95°C for 1min. Preparation of cDNA proved to be much simpler and faster using this kit.

A 1:50 dilution of all samples was prepared before using them for qPCR, meaning usually 10µl cDNA plus 490µl H2O.

2.4.3 qPCR (for cDNA)

Luna® Universal qPCR Master Mix Protocol (New England Biolabs, per 1x reaction):

10µl Luna Mastermix

4µl H2O

1µl Primer Mix (10µM)

5µl cDNA

5µl of the 1:50 diluted cDNA was used. Luna MM + H2O mix was prepared first for all reactions, then split onto different tubes to add the different primers (see table 3). 15µl of this mix was pipetted onto the 96-well plates and then 5µl cDNA were pipetted into each well individually. Plates were centrifuged for about 1min at 2500rpm to remove bubbles and assure all liquids were pooled at the bottom of the wells.

Cycle Protocol for Luna qPCR:

1) 95°C, 1min

2) 95°C, 15sek

3) 60°C, 30sek

4) repeat steps 2)-3) 39 more times

5) 65°C to 95°C in increments of 0,5°C per 5sek.

For the measurements of the GOIs *desmin*, *sparc*, *tgf*β in A5+SIS3, a different Master Mix and protocol were used, due to availability issues. Only these qPCRs were repeated, leading to twice as many data points as the other measurements. All qPCRs were carried out on a CFX Opus 96 Real-Time PCR System (Bio-Rad).

SYBR Green qPCR Master Mix Protocol (Bimake, per 1x reaction):

10µl SYBR Green Ready Mastermix

7,2µl H2O

1µl DNA sample

1,8µl Primer mix (10µM)

qPCR Cycle Protocol:

1) 95°C, 10min
2) 95°C, 15sek
3) 60°C, 30sek
4) 72°C, 60sek
5) repeat steps 2)-4) 39 more times
6) 65°C to 95°C in increments of 0,5°C per 5sek.

2.4.4 Statistical evaluation of cDNA PCR results

Outlier analysis and dCq normalisation were performed in Microsoft Excel 2013. In an initial step, all technical and biological replicates of one cell line and one GOI per time point and treatment were averaged and always one representative (technical replicate) of each biological replicate was divided by said average. This resulted in the normalization factor for each biological replicate, by which then each single technical replicate was multiplied correspondingly. The same was done for the reference gene *atp5f1*. Then the delta-Cq method was performed for the whole ref.gene-GOI matrix. So each single GOI data point was normalised to each ref.gene data point using the following formula:

$$2\text{^} - (GOI - ref.gene)$$

To remove outliers, an excel function was used which removes any values above or below 1,5xStd.Dev of the matrix average from the matrix. In the final step, averages and standard deviations of the outlier-corrected matrix were calculated (example matrix see App. 4-6). Plots were created in R (version 4.1.1) using the package ggplot2 (version 3.35) and ggpubr (version 0.4.0) including p-values based on a Student's T-test (R Script see App. 8).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 ATP5f1 T0-K-1	Unk-1 ATP5f1 T0-K-1	Unk-2 ATP5f1 T0-T-1	Unk-2 ATP5f1 T0-T-1	Unk-3 ATP5f1 T3-K-1	Unk-3 ATP5f1 T3-K-1	Unk-4 ATP5f1 T3-T-1	Unk-4 ATP5f1 T3-T-1	Unk-5 ATP5f1 T6-K-1	Unk-5 ATP5f1 T6-K-1	Unk-6 ATP5f1 T6-T-1	Unk-6 ATP5f1 T6-T-1
B	Unk-7 Nanog T0-K-1	Unk-7 Nanog T0-K-1	Unk-8 Nanog T0-T-1	Unk-8 Nanog T0-T-1	Unk-9 Nanog T3-K-1	Unk-9 Nanog T3-K-1	Unk-10 Nanog T3-T-1	Unk-10 Nanog T3-T-1	Unk-11 Nanog T6-K-1	Unk-11 Nanog T6-K-1	Unk-12 Nanog T6-T-1	Unk-12 Nanog T6-T-1
C	Unk-13 Nodal T0-K-1	Unk-13 Nodal T0-K-1	Unk-14 Nodal T0-T-1	Unk-14 Nodal T0-T-1	Unk-15 Nodal T3-K-1	Unk-15 Nodal T3-K-1	Unk-16 Nodal T3-T-1	Unk-16 Nodal T3-T-1	Unk-17 Nodal T6-K-1	Unk-17 Nodal T6-K-1	Unk-18 Nodal T6-T-1	Unk-18 Nodal T6-T-1
D	Unk-19 FH_Nkx2.5 T0-K-1	Unk-19 FH_Nkx2.5 T0-K-1	Unk-20 FH_Nkx2.5 T0-T-1	Unk-20 FH_Nkx2.5 T0-T-1	Unk-21 FH_Nkx2.5 T3-K-1	Unk-21 FH_Nkx2.5 T3-K-1	Unk-22 FH_Nkx2.5 T3-T-1	Unk-22 FH_Nkx2.5 T3-T-1	Unk-23 FH_Nkx2.5 T6-K-1	Unk-23 FH_Nkx2.5 T6-K-1	Unk-24 FH_Nkx2.5 T6-T-1	Unk-24 FH_Nkx2.5 T6-T-1
E	Unk-25 ATP5f1 T20-K-1	Unk-25 ATP5f1 T20-K-1	Unk-26 ATP5f1 T20-T-1	Unk-26 ATP5f1 T20-T-1	Unk-27 ATP5f1 T28-K-1	Unk-27 ATP5f1 T28-K-1	Unk-28 ATP5f1 T28-T-1	Unk-28 ATP5f1 T28-T-1	Unk-29 ATP5f1 T49-K-1	Unk-29 ATP5f1 T49-K-1	Unk-45 ATP5f1 T49-T-1	Unk-45 ATP5f1 T49-T-1
F	Unk-30 Nanog T20-K-1	Unk-30 Nanog T20-K-1	Unk-31 Nanog T20-T-1	Unk-31 Nanog T20-T-1	Unk-32 Nanog T28-K-1	Unk-32 Nanog T28-K-1	Unk-33 Nanog T28-T-1	Unk-33 Nanog T28-T-1	Unk-34 Nanog T49-K-1	Unk-34 Nanog T49-K-1	Unk-46 Nanog T49-T-1	Unk-46 Nanog T49-T-1
G	Unk-35 Nodal T20-K-1	Unk-35 Nodal T20-K-1	Unk-36 Nodal T20-T-1	Unk-36 Nodal T20-T-1	Unk-37 Nodal T28-K-1	Unk-37 Nodal T28-K-1	Unk-38 Nodal T28-T-1	Unk-38 Nodal T28-T-1	Unk-39 Nodal T49-K-1	Unk-39 Nodal T49-K-1	Unk-47 Nodal T49-T-1	Unk-47 Nodal T49-T-1
H	Unk-40 FH_Nkx2.5 T20-K-1	Unk-40 FH_Nkx2.5 T20-K-1	Unk-41 FH_Nkx2.5 T20-T-1	Unk-41 FH_Nkx2.5 T20-T-1	Unk-42 FH_Nkx2.5 T28-K-1	Unk-42 FH_Nkx2.5 T28-K-1	Unk-43 FH_Nkx2.5 T28-T-1	Unk-43 FH_Nkx2.5 T28-T-1	Unk-44 FH_Nkx2.5 T49-K-1	Unk-44 FH_Nkx2.5 T49-K-1	Unk-48 FH_Nkx2.5 T49-T-1	Unk-48 FH_Nkx2.5 T49-T-1

Figure 5 Example for qPCR plate setup. *Each biological replicate was measured in duplicates with three biological replicates per cell line, time point and treatment. To make the measurements as comparable as possible, it was decided to favour having all time points and both treatments of one biological replicate each on one 96-well plate.*

Gene Name	Primer Name	Sequence [5'-3']		Length (bp)
		fwd	rev	
ATP synthase peripheral stalk-membrane subunit b	Atp5f1	GTCCAGGGGTATTACAGGCAA	TCAGGAATCAGCCCAAGACG	112
Desmin	Des_2	ACACCTAAAGGATGAGATGG	GAGAAGGTCTGGATAGGAAG	147
Secreted Protein Acidic And Cysteine Rich	Sparc_2	CTCAAAAATGTCCTGGTCAC	CTCATGGATCTTCTTCACAC	93
Transforming Growth Factor beta1	Tgfb1	GATACGCCTGAGTGGCTGTC	TTTGGGGCTGATCCCGTTG	156
Mesoderm Posterior BHLH Transcription Factor 1	Mesp1	GTACACGCTCTAAAGATGAAG	TTTTGACACTAGCACAATCG	88
GATA binding protein 4	FH_Gata4	AGCAGGACTCTTGGAACAGC	GCCCCAGCCTTTTACTTTGC	138
Brachyury	T	AGAATGAGGAGATTACAGCC	GGTTCCTCCATTACATCTTTG	103
Nanog	Nanog	GTTTCAGAAGCAGAAGTACC	TCAGACCATTGCTAGTCTTC	158
Nodal	Nodal	TTCTTCTCAGGTCACGTTTGC	GGTGGGGTTGGTATCGTTTCA	519
NK2 Homeobox 5	FH_Nkx2.5	CCACCTTTAGGAGAAGGGCG	TGGATCGGAGAAAGGTCCCA	111

Table 3 *List of primers used for measuring expression of respective genes.*

3. Results

3.1 RESULTS EXPERIMENT 1

3.1.1 *Sparc* homozygous KOs

As seen in figure 8, the first results of PCRs with the Sparc I5-gen-fw + 3_I6-r primer pair show, that the A5 WT control group exhibits a large band slightly below 2kb (expected PCR fragment size is 1717kb). Also following our expectations, the *sparc*$^{+/-}$ control shows two bands on the gel. One of these bands is at the same height as the WT allele and is in respect to the A5 WT control weaker in saturation. The other band is exhibited between 6kb and 8kb, which corresponds to the KO allele (expected size is 6013kb).
The potential *sparc*$^{-/-}$ clones exhibit variable amounts of both bands, in most cases the WT band appearing stronger. The band saturation patterns closest to the *sparc*$^{+/-}$ control are created by the samples K3-2 and K10-1. Since all selected, potential *sparc*$^{-/-}$ sub-clones are descendants of the *sparc*$^{+/-}$ cell line "K11", it is expected to at least show a heterozygous KO genotype, which is met by all samples. However, although the tested clones vary in WT allele amounts according to the provided gel electrophoresis pictures, none of the samples shows a clear homozygous KO genotype.

After testing all potential *sparc*$^{-/-}$ clones created in this experiment, this data leads to the preliminary result that the methodology described in this report did not successfully select for homozygous sparc knockout cells.
However, the protocol of genotyping and the primers used to differentiate between *sparc* WT and KO allele worked and at least confirmed the expected genotypes of our A5 WT and SparcKO-K11 lines. This is also important for future experiments, as possible switch ups of cell lines can be ruled out as an explanation for controversial gene expression results.

3.1.2 *Desmin* homozygous KOs and *hdac* tests

Following the same principle of primer design as used for the sparc genotyping, there were also attempts to create *desmin* and *hdac* primers. As we've already had no positive homozygous KO result for *sparc*, we thought one potential reason for the cells surviving the selection pressure without having two *sparc* KO alleles was to instead have two *hdac* KO alleles. Hence the decision to also genotype our clones for said gene.

Unfortunately, neither of the two tested *desmin* primer pairs worked as intended. The gel picture (figure 9) shows tests conducted with three reference cell lines: A5 WT, SparcKO-K11 and DesKO-2H, the latter one being a heterozygous *desmin* knockout line. Two primer pairs for *desmin* genotyping were tested (Des-dKO-1 and Des-dKO-2). In WT and K11, only one band representing the WT allele were expected. In 2H, the WT band and an additional KO band were expected. However, each of the cell lines only exhibits a band at the WT length. Due to these results, no further PCRs of the potential *desmin*$^{-/-}$ clones were conducted.

The same figure 9 shows the tests for the *hdac* gene. In this case, three different primer pairs were used on the same three cell lines, while the last primer pair (HDAC-E5-I7) was also tested on one of the potentially homozygous *sparc* KO clones, K11-K1.

According to these results, none of the primers was able to detect the *hdac* KO allele, or successfully differentiate between the WT and KO allele. The primer pair Hdac-I7-NEO should only detect the KO, since one of the primers binds in the NEO cassette. This pair showed no bands and hence no KO detection. The Hdac-E5 has the forward and backward primer binding in the Exon 5, which should only be available in the WT allele. There is a band in all tested cell lines. Hdac-E5-I7 follows the same principle as the *sparc* and *desmin* primers, it spans the vector insertion site and should produce a shorter band for WT and a longer band for KO alleles. Therefore, in case of a *hdac*$^{+/-}$, two bands are expected. Neither of the four tested cell lines show two bands, which supports the results of the previous two tested primers that the cell lines are all *hdac*$^{+/+}$. This disproves the theory that our clones spontaneously doubled their *hdac* KO vector, but also raises the new question of why they don't present the expected *hdac*$^{+/-}$ genotype.

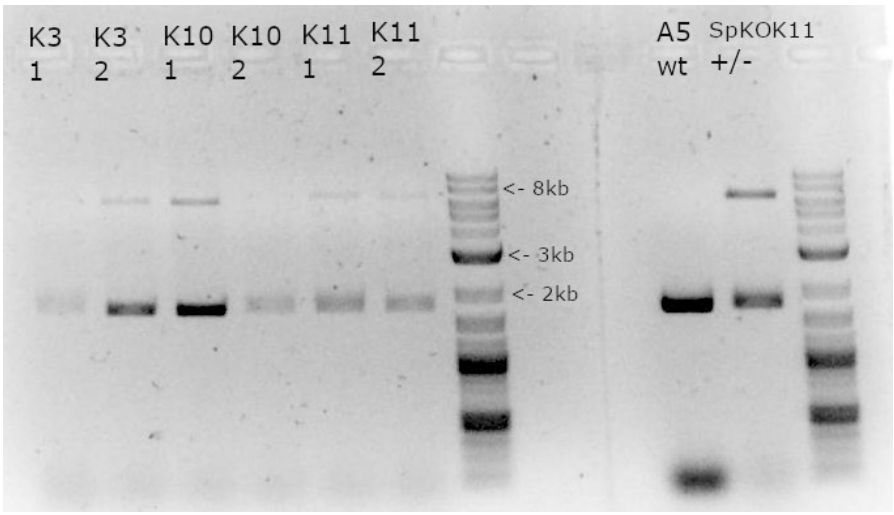

Figure 6 *Exemplary picture of gelelectrophoresis results of sparc genotyping. In a first test round, three potentially sparc$^{-/-}$ sub-clones of K11 were measured in duplicates. They show both the WT and the KO bands to varying degrees, a pattern which is seen throughout the rest of all sub-clones, although usually with an intensity similar to K10-1*

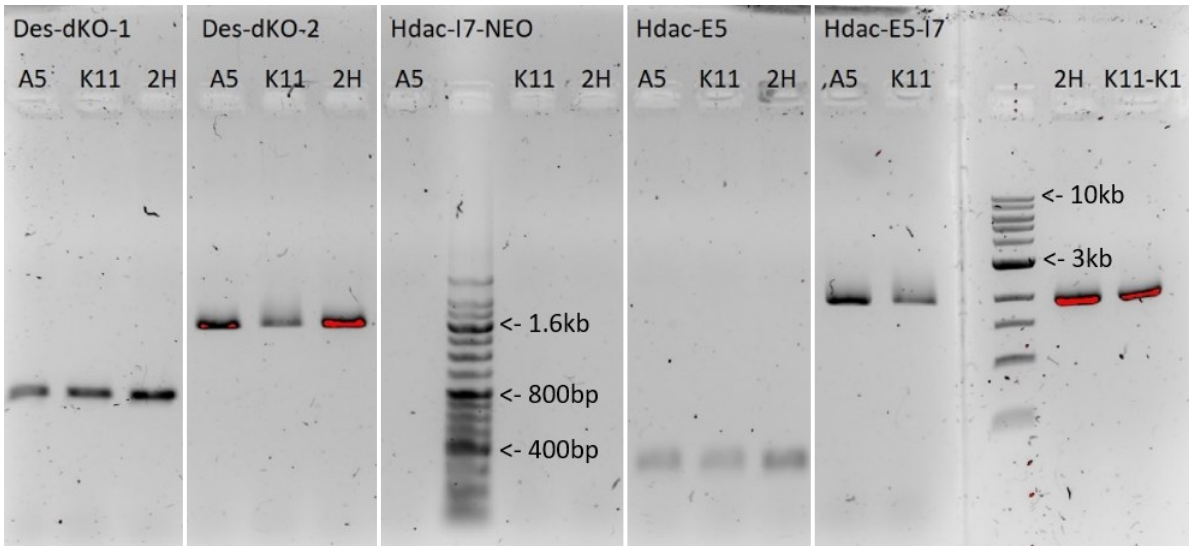

Figure 7 *Primer tests for desmin and hdac loci. The primers were tested on three reference cell lines (A5 WT, SparcKO-K11, DesKO-2H) and Hdac-E5-I7 was also tested on one of the potentially sparc$^{-/-}$ clones (K11-K1). According to these results, neither of the five primer pairs was able to detect a KO allele of their respective gene.*

3.2 RESULTS EXPERIMENT 2

This experiment aimed at showing the influence of exogenous TGFβ1 or SIS3 on several GOIs over a time period of 49h on differentiating CVPCs.

TGFβ1 and SIS3 are expected to have an opposite effect, if any, because TGFβ1 acts as a ligand for TGFβRI which phosphorylates Smad2/3 downstream of TGFβ1 binding and SIS3 is, as the name suggests, a specific inhibitor for Smad3 which blocks Smad3 phosphorylation. In very basic terms, TGFβ1 is expected to activate the canonical TGFβ pathway and SIS3 is expected to block that activation. Furthermore, whether different levels of endogenous SPARC or Desmin have an influence on the function of TGFβ1 and SIS3 or not is addressed by using a *sparc*$^{+/-}$ and a *desmin*$^{+/-}$ cell line in addition to a WT line.

3.2.1 Brachyury

A good example of this regulatory opposition is seen for the gene *brachyury* at T6 hours. As seen in figure 10, in all three cell lines, addition of TGFβ1 to the culture medium lead to increased *T* expression at T6, while addition of SIS3 lead to downregulated *T*. Residual effects of the additives can still be seen after 49 hours, with some differences between the cell lines. At T49, SIS3 did no longer downregulate *brachyury* in A5, however it did still do so in K11 and 2H. TGFβ1 on the other hand still upregulated *brachyury* at T49 in A5 and 2H, but now seemingly downregulated it in K11. Hence 2H is the only line where the same effect of both additives is observed at T6 and T49. Still, there is no consistent evidence that less Desmin or SPARC in CVPCs has an influence on the effects of TGFβ1 or SIS3 on *T* expression.

Similar to other tested GOIs, no clear effect was detectable before T6. However, of the affected genes, *T* is one with an effect showing earlier than most others.

3.2.2 Desmin

One protein we were highly interested in was the early myogenic marker Desmin. Generally, *desmin* was consistently upregulated in SIS3 test groups of all three cell lines, starting at T20 (figure 11). The effect on *desmin* is visible later than on *T*, which might be in line with *T* being significant for CM differentiation at an earlier time than *desmin*.

The influence of TGFβ1 is slightly less consistent, the expected downregulation by TGFβ1 to oppose the SIS3 effect is only clearly visible in A5 starting at T28 and in the other cell

lines possibly even later at T49. At T20, A5 shows an upregulation by TGFβ1, which is neither in line with T28 nor the fact that it should contrast the SIS3 effect.

Neither in 2H nor in K11, a consistent regulation of *desmin* by TGFβ1 can be determined, as most time points show no significant difference to controls or only very little but contradicting effects over time.

Viewing the progression over all time points (see Appendix1), in K11 and 2H the TGFβ1 T and K groups show very similar expressions throughout all time points. Only at T49, the control is rising a bit above the treated group. A5 shows a similar behaviour, although the T group spikes upwards a little at T20 and declines again at T28, staying below the control at T28 and T49. SIS3, however, shows a consistent increase in *desmin* expression throughout all cell lines and starting earliest at T6.

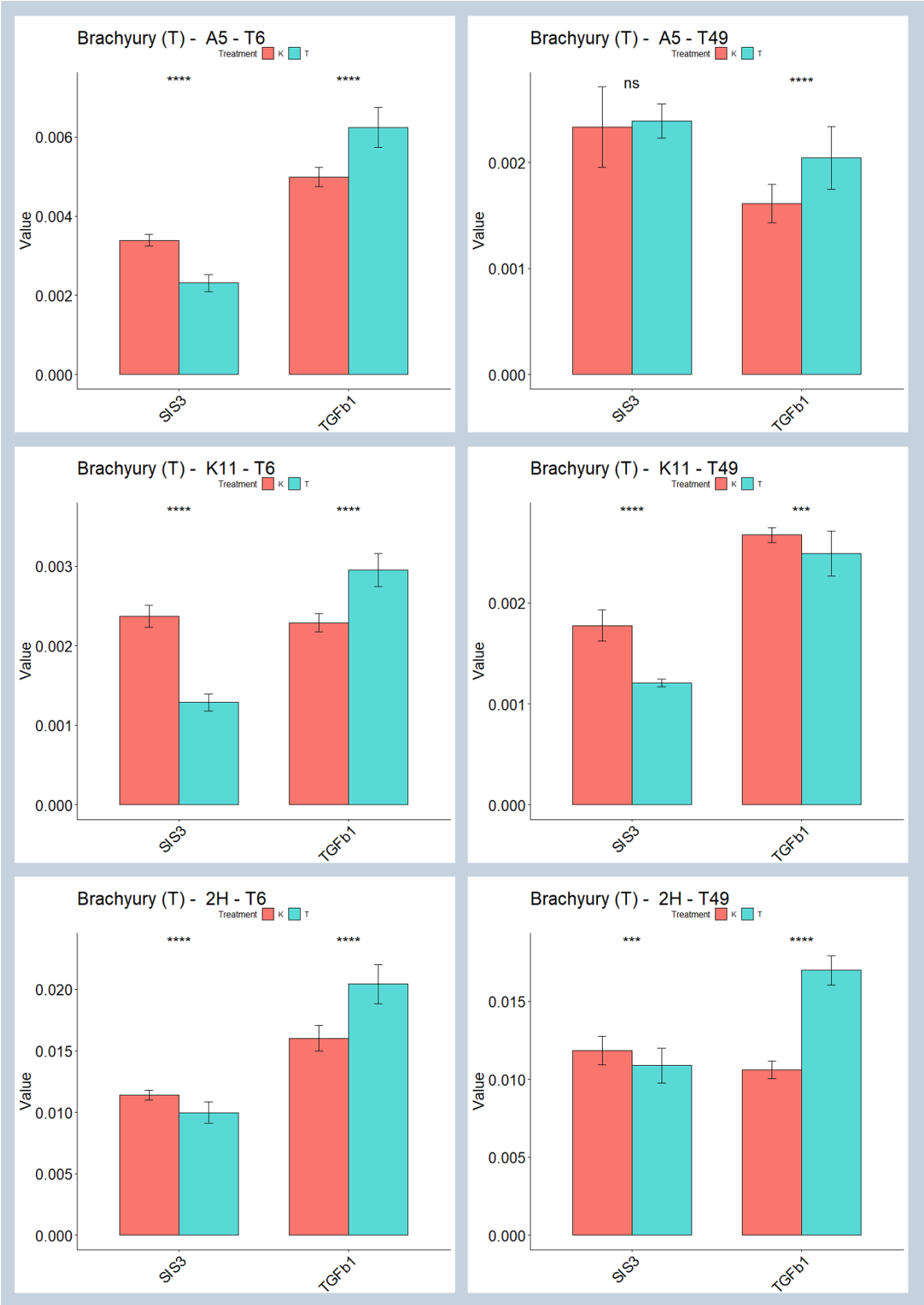

Figure 8 Brachyury expression after 6 and 49 hours. *This figure shows brachyury (T) expression at two representative time points in all three cell lines (A5, K11, 2H). One plot each shows one cell line at either T6 or T49 and both control group (K, red) and treated group (T, turquoise) for both treatments (SIS3 or TGFβ1). As demonstrated here, in all three cell lines SIS3 lowered T expression and TGFβ1 increased T expression at T6. This trend does not extend to T49, where SIS3 only lowered T expression in K11 and 2H and TGFb1 only increased T expression in A5 and 2H.*

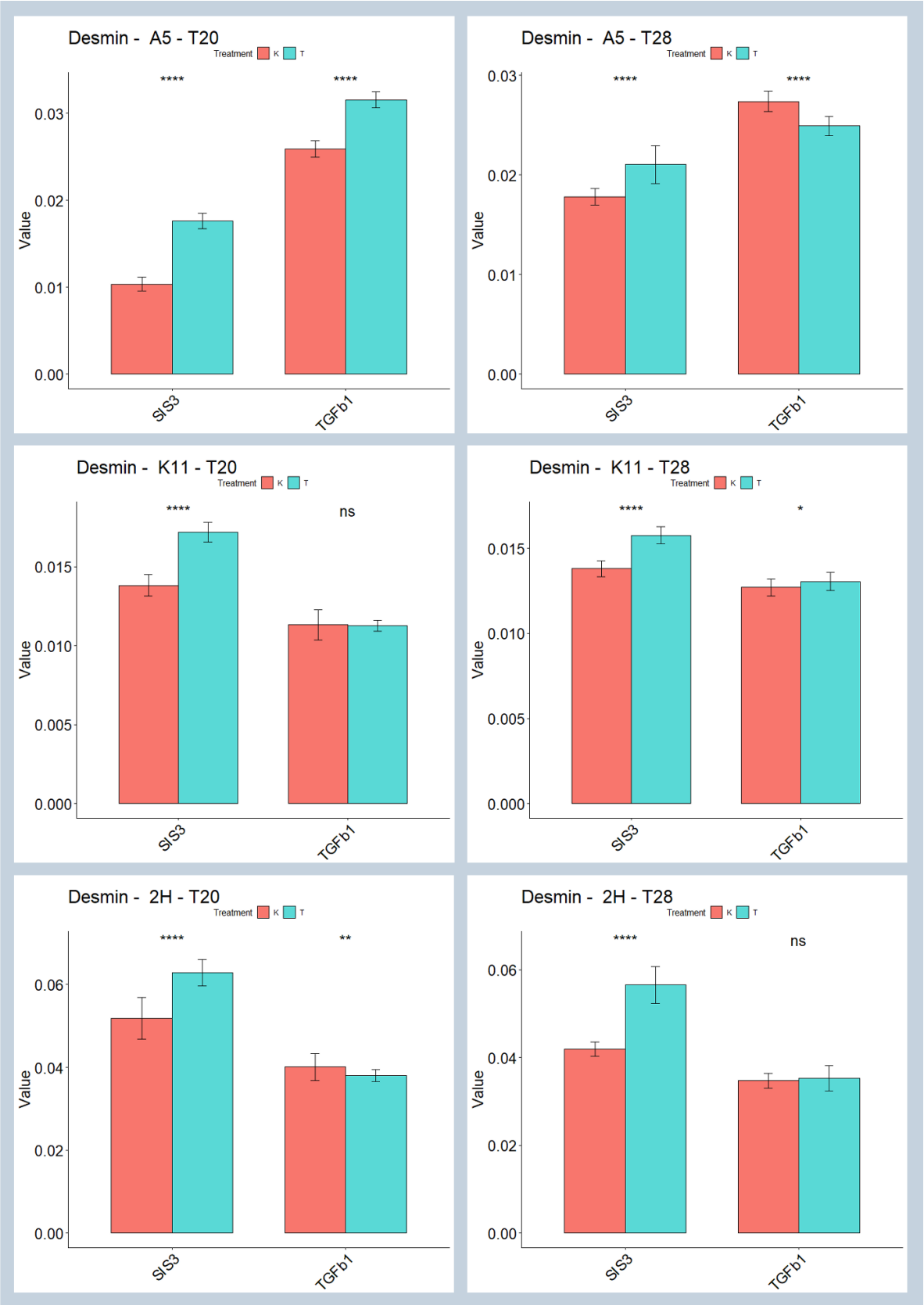

Figure 9 Desmin expression after 20 and 28 hours. *Each cell line shows clear upregulation of desmin by SIS3 at T20 and T28. Influence of TGFβ1 is not as straightforward. While TGFβ1 shows almost no to no significant influence on desmin expression in K11 and 2H, in A5 it first shows and upregulation at T20 and then a downregulation at T28.*

3.2.3 Sparc

The next gene of interest was *sparc*, already known to itself influence the TGFβ-Smad2/3 pathway.

While SIS3 seems to consistently downregulate *sparc* in A5 starting at T6, TGFβ1 upregulation is only clearly visible at T20. No specific pattern of expression following the addition of TGFβ1 or SIS3 could be found in 2H and K11 (figure 12).
It is currently undeterminable whether seemingly opposing effects in the other cell lines or over the different time points are an artefact of this experiment or a true reaction that depends on either the cell type or the state of differentiation.

Even though A5 T20 shows an increase in expression in figure 12, upon viewing the whole trend over time, this increase seems to be a slight spike only at T20.

3.2.4 Tgfβ

No consistent patterns or relations between the influence of the different additives, cell lines or time points can be determined. For example, at T20, both reagents seem to upregulate *tgfβ* in A5 and K11 and have no effect in 2H (figure 13).
At T28 SIS3 still upregulates *tgfβ* in A5 and K11, but downregulates it in 2H. Meanwhile, TGFβ1 only shows the expected opposite effect to SIS3 in A5 by barely downregulating *tgfβ* and in 2H by upregulating it.

Generally, in K11 both factors seem to upregulate *tgfβ* expression, SIS3 starting at T20 and TGFβ1 already starting at T6.

In A5, both factors seem to upregulate *tgfβ* expression at T20, however at T28 expression seems to be slightly downregulated by TGFβ1 and at T6 it seems strongly downregulated by SIS3 (additional time-points see Appendix2).

In 2H, there is no effect visible at T20, but at T6 also both factors seem to upregulate *tgfβ* expression. At T28 SIS3 slightly downregulates *tgfβ* expression and TGFβ1 upregulates it.

Due to the inconsistencies and many opposing statements these results give, no definitive statement on the influence of either SIS3 or TGFβ1 on the gene *tgfβ* will be made. However, it seems that a higher *tgfβ* expression under the influence of SIS3 might be dependent on innate Desmin levels, as 2H is the only cell line not showing this effect.

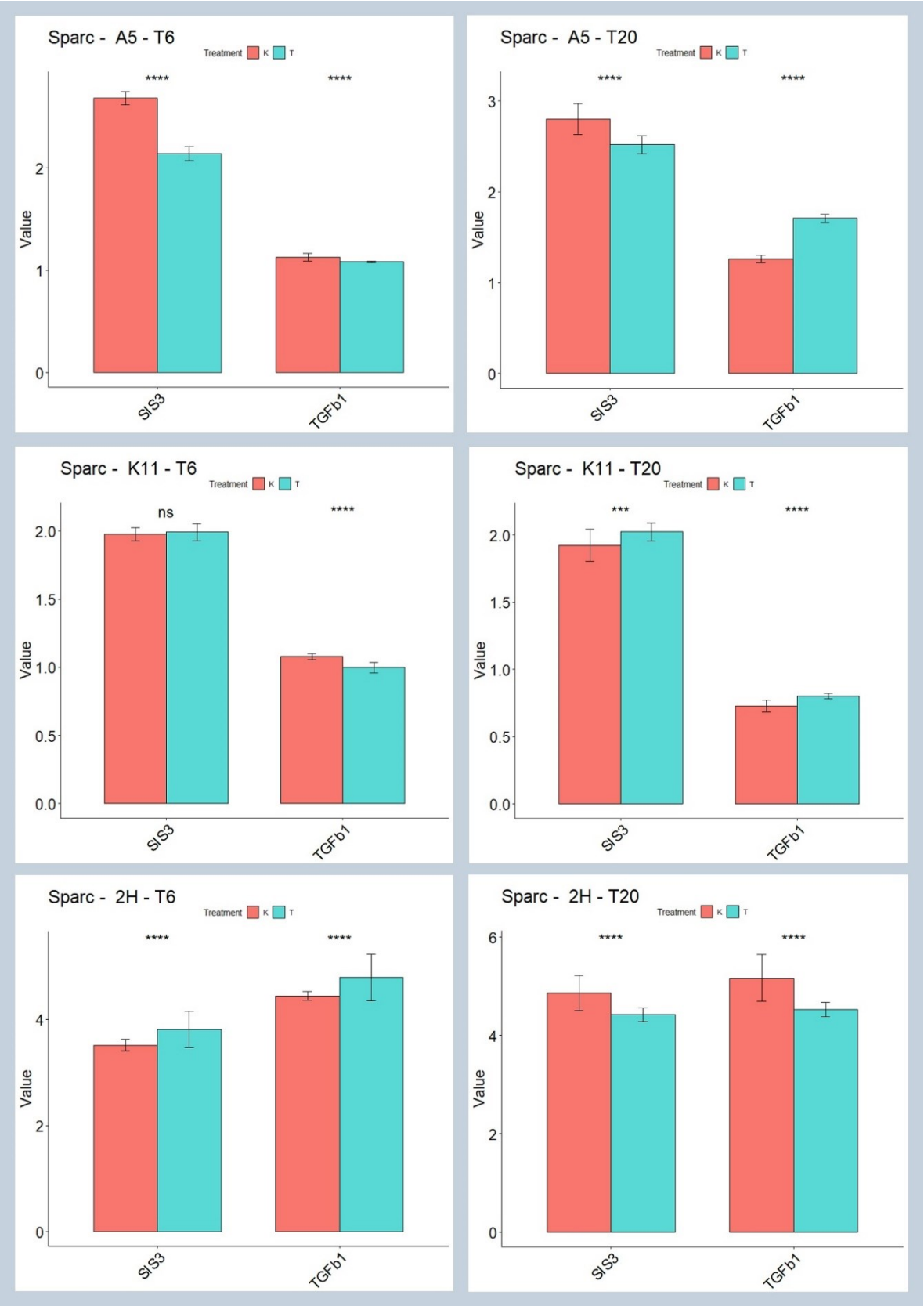

Figure 10 Sparc expression after 6 hours and 20 hours. *SIS3 seems to downregulate sparc starting at T6, however at that time also TGFβ1 seems to have the same effect. At T20, the expected opposite effects of the two factors is visible in A5. The other cell lines show no consistent and interpretable results.*

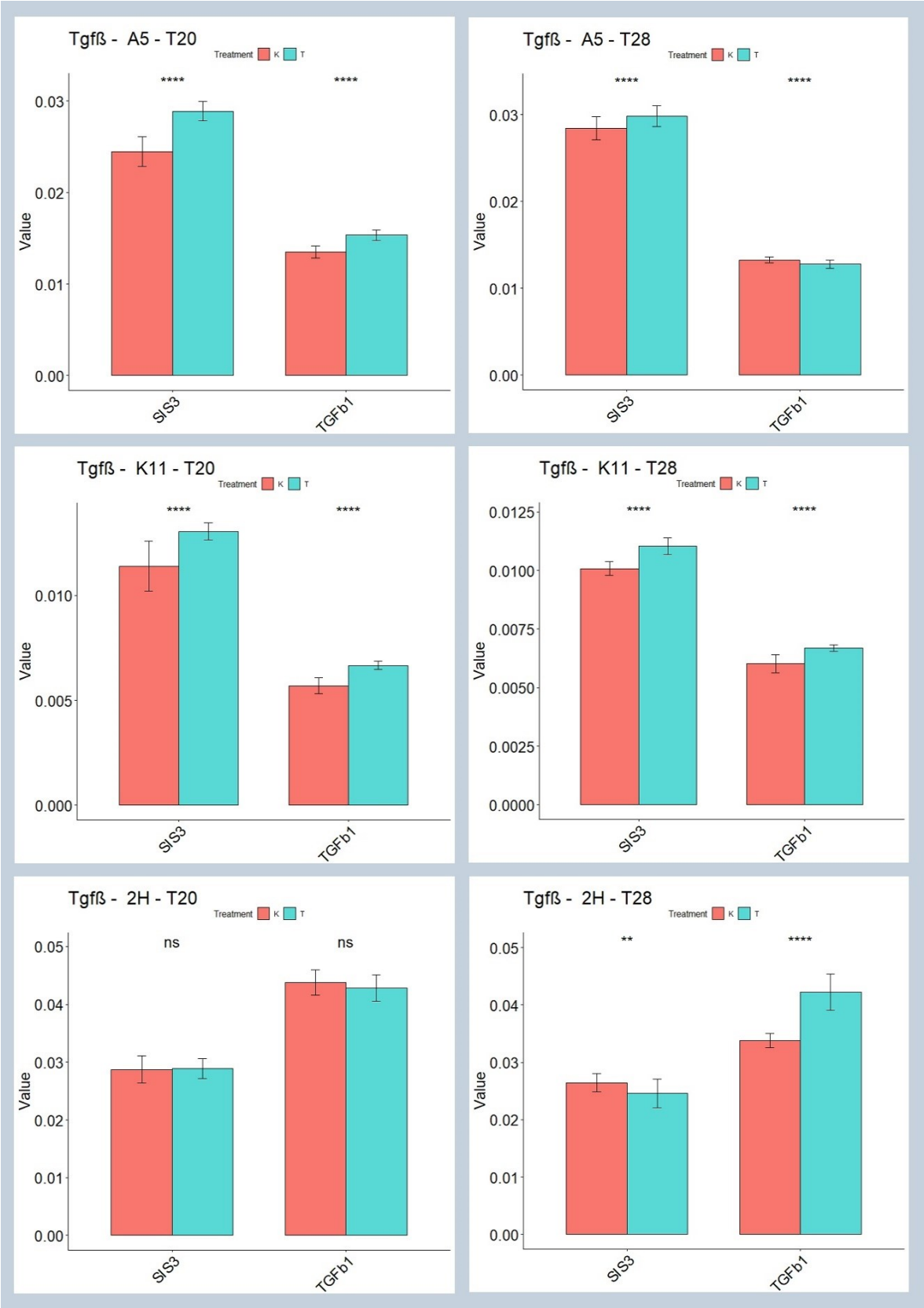

Figure 11 Tgfβ expression at 20 hours and 28 hours. *In A5, SIS3 consistently increases tgfβ expression starting at T20, while the effect of TGFβ1 is less consistent over the same time period. In K11, both factors increased expression starting at T20. In 2H, no effect is seen at T20, however at T28 SIS3 lead to a slight decrease and TGFβ1 to an increase of tgfβ expression.*

3.2.5 Nkx2.5

An influence on *nkx2.5* is barely detectable (figure 14). For example, at T20, neither SIS3 nor TGFβ1 had a significant influence in A5. The two factors seem to have the opposite effect in K11 than in 2H at T20. K11 T20 and A5 T28 seem to be the only situations in which SIS3 and TGFβ1 have opposing effects as expected. At 2H T28 and K11 T28, TGFβ1 seems to have miniscule to no effect. The two cell lines once again behave in opposite ways, in the sense that SIS3 raised *nkx2.5* expression in K11 and lowered it in 2H. It is important to note that the values are generally very low, which means that error margins might appear as an even bigger effect and might also be the reason for the several not significant results. 2H results are in a severely higher range, a pattern which is also seen for some other genes. Currently there is no explanation for this phenomenon.

In the overall trend over time (see Appendix3 figure) the SIS3 T and K groups and the TGFβ1 T and K groups seem to completely switch trend around T20. While at first the TGFβ1 T and K groups showed increasing expression, it declined starting after T6 and ended at T49 slightly lower than T0. The SIS3 T and K groups first showed barely an increase compared to T0 and had lower expression than the TGFβ1 groups, but then had a steep incline after T20 and ended with a much higher expression at T49 than T0.

3.2.6 Nanog

In all three cell lines, SIS3 raised *nanog* expression, but so did TGFβ1. This effect seems to be reduced by T28, with SIS3 in A5 having no significant influence anymore, in K11 still raising expression and in 2H marginally lowering it. TGFβ1 at T28 reduced expression in A5, has no influence in K11 and still raises it in 2H.

Viewing the overall trend of all time points (see Appendix3), the treated groups and their respective controls seem to follow the same trends though, especially in K11 where basically all groups have the same progression over time. In A5, the TGFβ1 treated group always shows higher expression than the control, except for T28. Hence the reduced expression in A5 T28 seen in figure 15 might be an outlier.

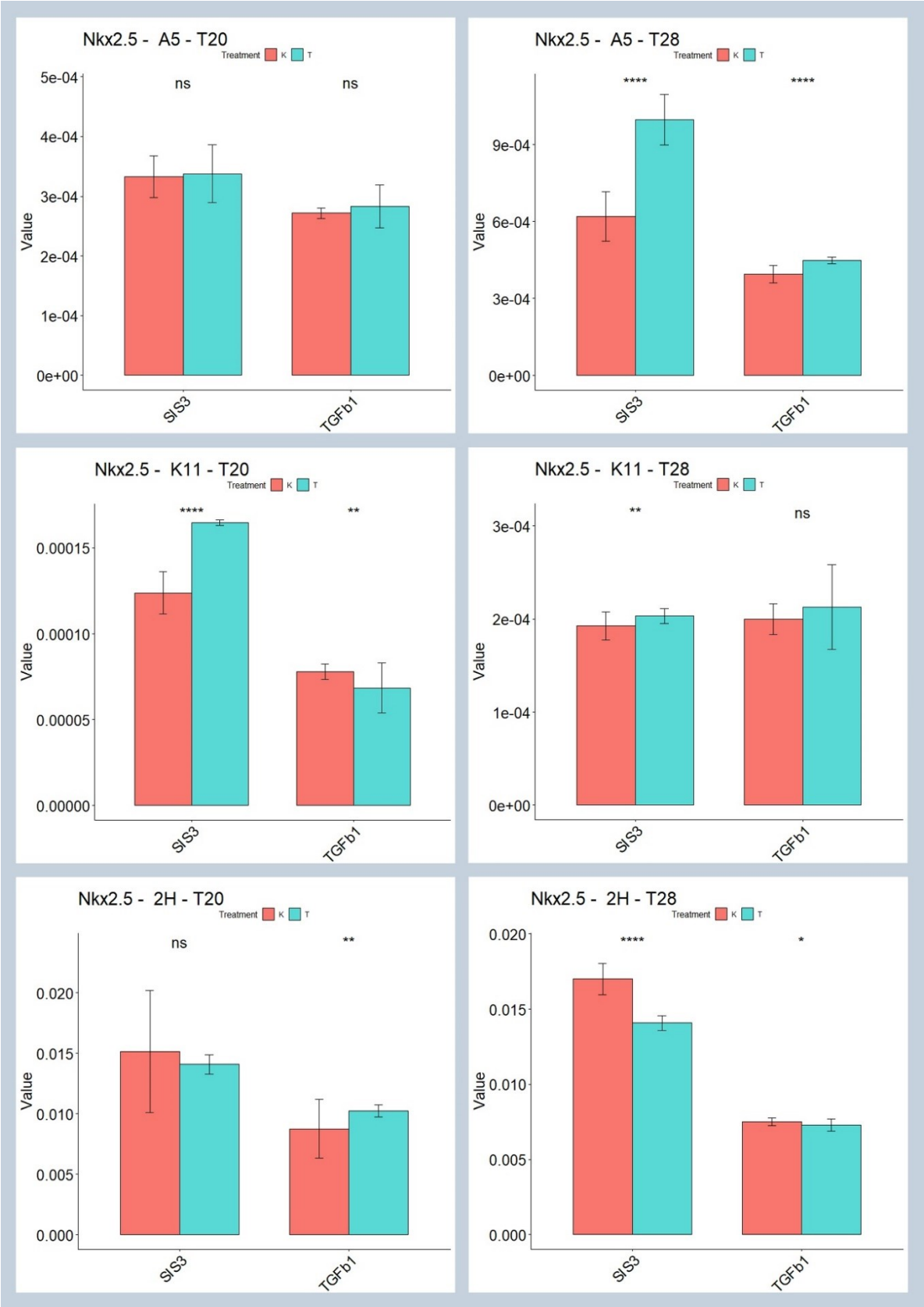

Figure 12 Nkx2.5 expression at 20 hours and 28 hours. *The effects on nkx2.5 seem comparatively low. 2H tends to show opposite effects to K11 and A5 and it is noteworthy that the expression range in 2H is higher by at least two orders of magnitude.*

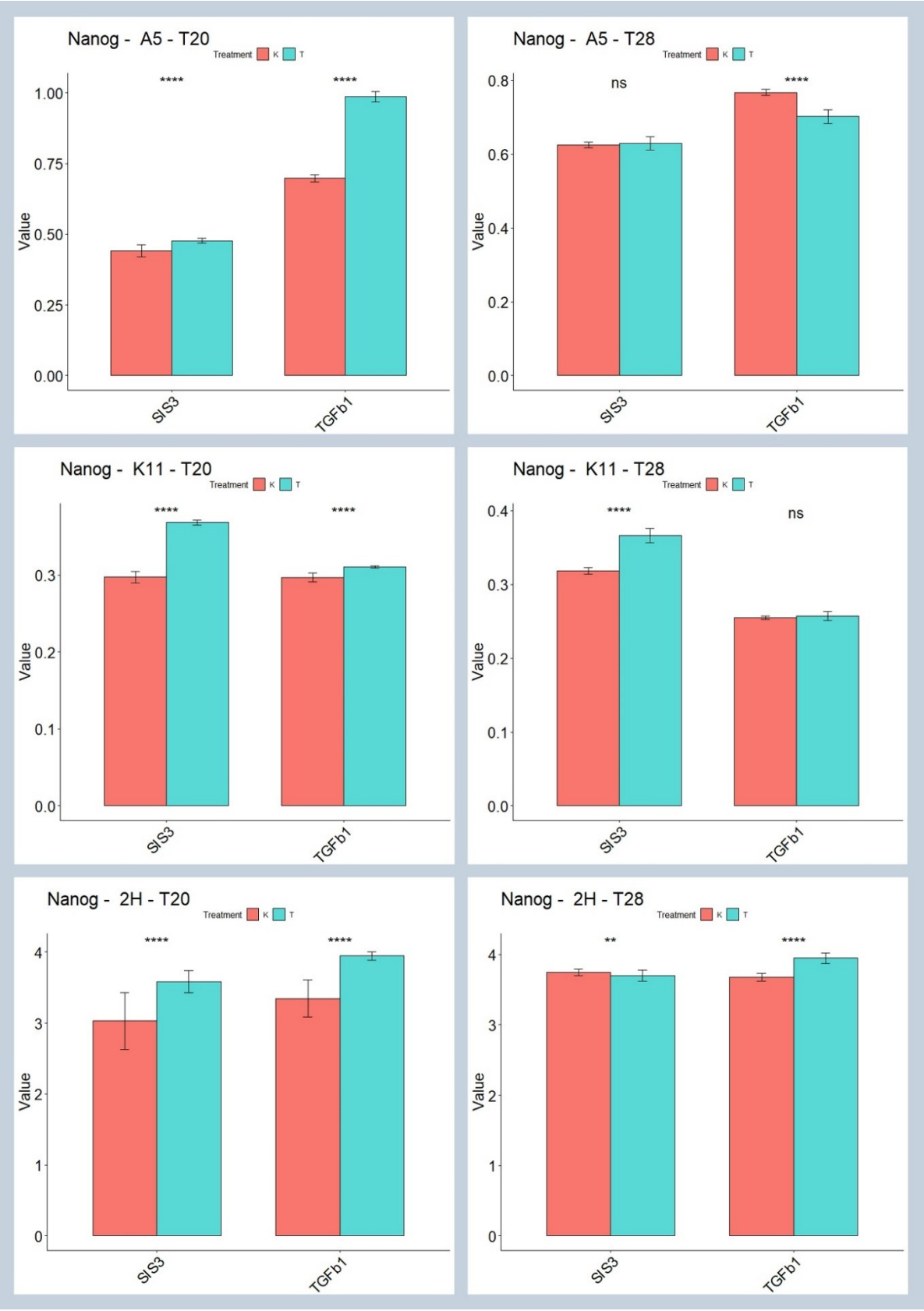

Figure 13 Nanog expression at 20 hours and 28 hours. *SIS3 and TGFβ1 both significantly raise nanog expression at T20 in all three cell lines. By T28, these effects seem to be slightly reduced. The significantly reduced nanog expression by TGFβ1 in A5 T28 might be an outlier, if viewed in context of the whole development over time (see Appendix3).*

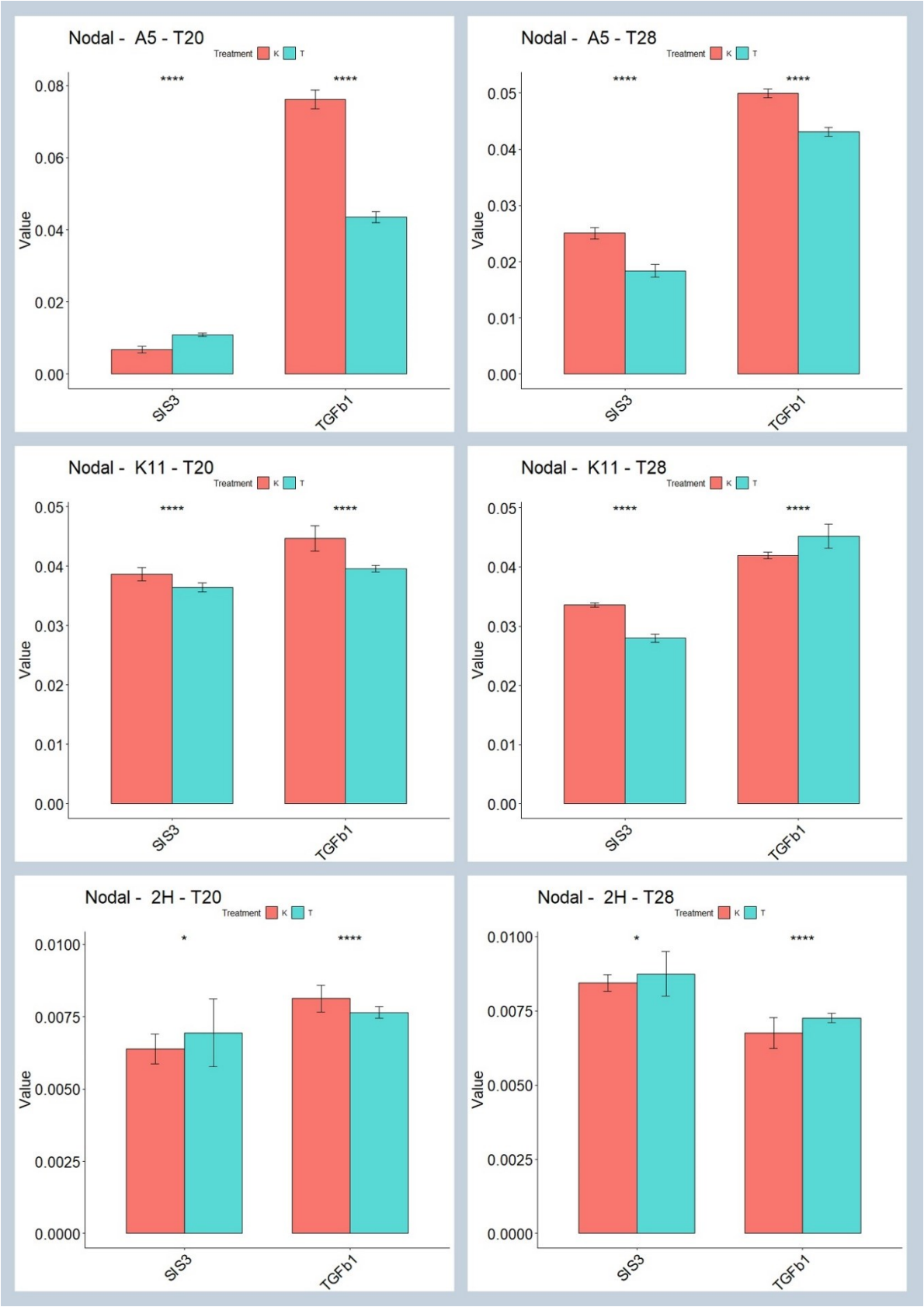

Figure 14 Nodal expression at 20 hours and 28 hours. *No consistent pattern can be observed, except for TGFβ1 lowering nodal expression at T20 in all three cell lines.*

3.2.7 Nodal

The only consistency observed is TGFβ1 lowering *nodal* expression in all cell lines at T20. Otherwise, there is no discernible pattern based on additive, cell line or time point (figure 16).

Based on the plots of all time points (see Appendix3), especially in A5 and K11 the treated groups follow a similar progression as their respective controls. In A5, at T0 and T3 the SIS3 treated group is lower than the control (in concordance with T28) and at T6 and T49 there is no significant difference. Hence, T20 is the only time point where the SIS3 treated group shows a higher expression. The TGFβ1 control group shows a large peak at T20 in A5, which is not observed anywhere else and hence might be an artefact.

3.2.8 Gata4

At T20, there is no clear influence of any of the two factors (figure 17). Four out of six cases have no significant result. The only result deemed as highly significant at T20 is TGFβ1 raising *gata4* in A5. At T28, the only consistent result is TGFβ1 lowering *gata4* in all three cell lines. SIS3 seems to lower *gata4* in A5 and 2H at T28, but raises it in K11.
Similar to *nkx2.5* and *mesp1* results, 2H shows much higher *gata4* expression than K11 and A5 (about one order of magnitude).

3.2.9 Mesp1

As seen before in *nkx2.5* and *gata4*, the total expression values are very low, with 2H again showing a higher expression by about two orders of magnitude. SIS3 has the opposite effect in K11 than in 2H or A5, as it raises *mesp1* expression only in K11 in both time points. TGFβ1 could lead to a slight decrease in expression in all circumstances except A5 T20. This means the K11 cell line and A5 T20 are the only instances in which SIS3 and TGFβ1 create opposing effects, although the directions of the effects are the opposite in the two cell lines (figure 18).

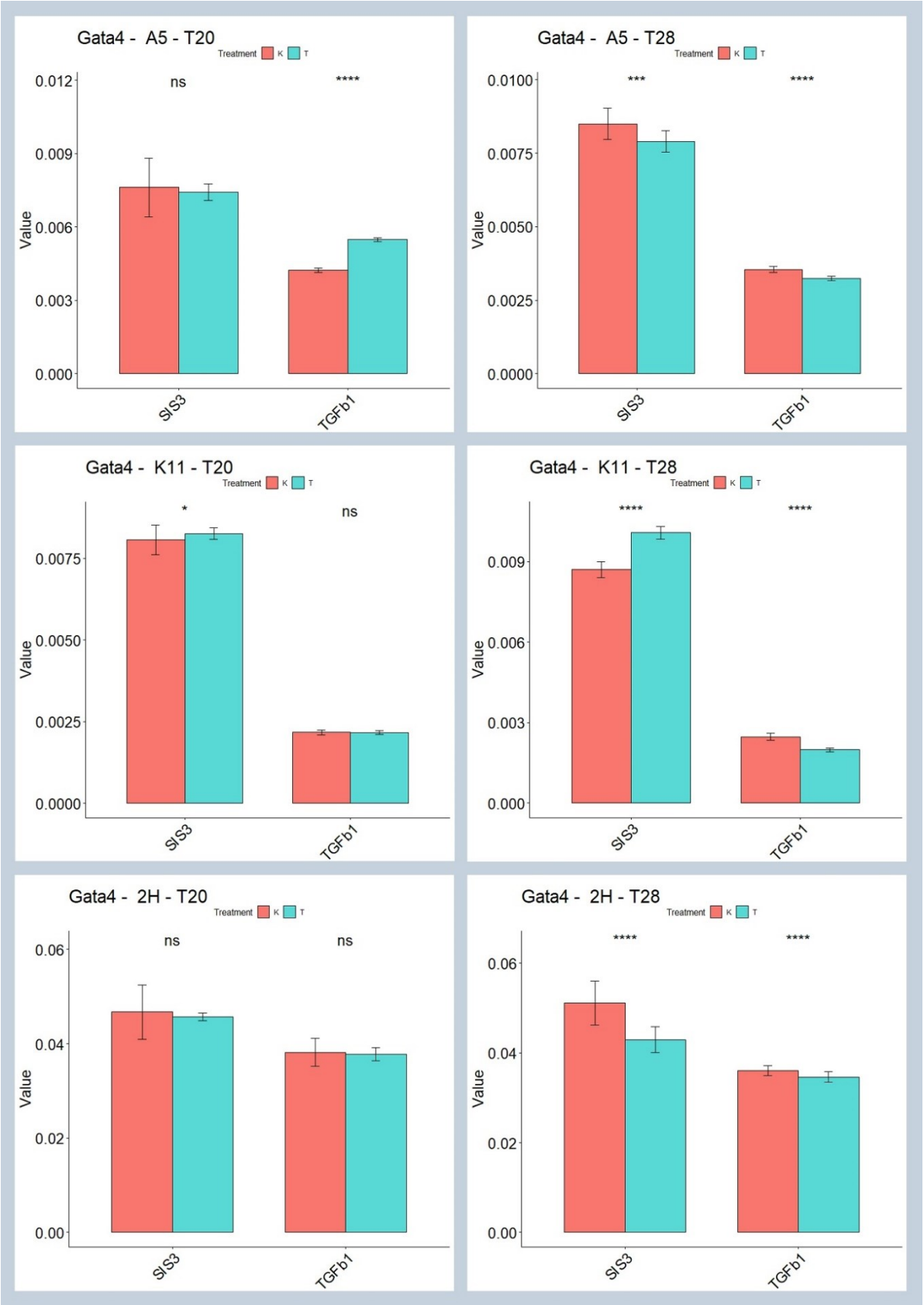

Figure 15 Gata4 expression at 20 hours and 28 hours. *At T20 barely any effect of TGFβ1 or SIS3 is observed. At T28, in five out of six cases, gata4 expression is reduced. Only SIS3 seemingly increased the expression in K11 at T28.*

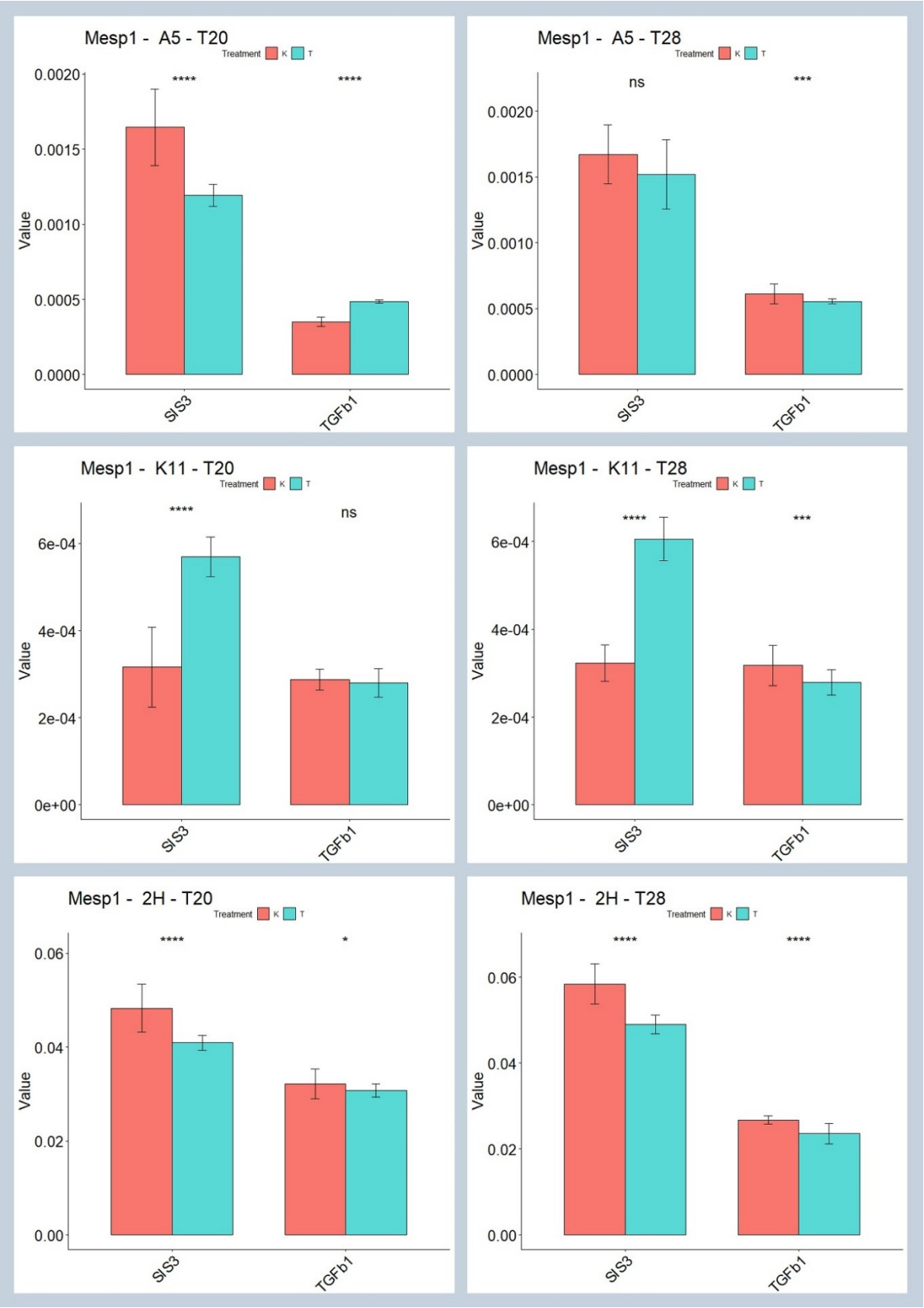

Figure 16 Mesp1 expression at 20 hours and 28 hours. *TGFβ1 only raises mesp1 expression in one out of six cases (A5 T20). SIS3 reduces or has no significant effect in A5 and 2H at any time point, but raises mesp1 expression in K11 at both time points.*

4. Discussion

4.1 Discussion Experiment 1

As mentioned in the results of the selection experiment, no *sparc*$^{-/-}$ clone was detected, but as proven by the control groups, the primers and method of genotyping the cells worked for the *sparc* gene. No clear statements can be made about the Desmin 2H sub-clones, as a similar setup of primers did not work on control groups.

Considering the SPARC K11 sub-clones clearly did not show a homozygous *sparc* KO genotype, this bodes the question as to why they were able to survive the selection pressure of G418 in the amount provided. A concentration of 18mg/ml active G418 should select for three NEO-cassettes, in our case one in one of the *hdac* alleles and two *sparc* alleles. As this was not a directed mutation performed, but rather selection of a spontaneous doubling of one allele, it would be possible that the *hdac* locus instead of the *sparc* locus got recombinated. However, it seems statistically improbable, that this would happen in 34 out of 34 cases with no case of *sparc* recombination. Still, to rule out this possibility, or even the possibility of the progenitor cell lines A5 or K11 already carrying two *hdac* KO alleles, we attempted to genotype them for *hdac* as well. As seen in figure 9 of the results, none of the three tested primers was able to detect a *hdac* KO allele. This would disprove the theory of the cells being homozygous *hdac* KOs, but it is still an unexpected result, as all lines should carry at least one NEO cassette on the *hdac* locus. At the current time it is unclear whether these results represent the actual genotype or are an artefact of incorrect primer design. However, since more than one primer pair lead to the same result in both the *desmin* and *hdac* cases, the problem seems to at least go beyond one faulty primer sequence.

Unfortunately, even further attempts at diverse NEO primer plus *desmin* or *hdac* primer combinations did not lead to any positive results for *desmin* or *hdac* KO alleles in the selected K11, 2H and A5 clones (results not provided). The NEO primers on their own have shown positive results before (results not provided).

Apart from the possibilities of human errors in applying the selective pressure or simply no recombination having happened in our cells, another possibility supported by previous,

unpublished experiments of our group, is that *sparc*$^{-/-}$ cells of this specific origin are not viable. However, this does not explain why single clones of our originally heterozygous population survived the given G418 concentration. Unfortunately, due to a bacterial infection, the planned A5 control group had to be discarded, so we have no comparison on how the selection pressure affected a population with only one NEO-cassette.

The first attempt to determine *sparc*$^{-/-}$ levels in our selected clones was actually done via quantitative real-time PCR. However, this detection method proved to be insufficient to differentiate between the three different genotypes (*sparc WT, sparc*$^{+/-}$, *sparc*$^{-/-}$). Since no definitive protocol could be established, the results of our attempts are not provided in this work. To give a short overview, the same gDNA as for the standard PCR of three potential *sparc*$^{-/-}$ clones (K3, K10 and K11) was measured. A variety of primers for *sparc* exon 6 (only present in WT) and different NEO-intron combinations were tested, with *sdha* as the reference gene. All Ct values of the qPCRs were above 30, even the reference gene, which might imply too diluted DNA samples. The qPCR curves were all exponential and also the primer melt curves were homogenous, but some primers had NTC values in the same range as sample values. Some qPCR products were run in a gel-electrophoresis and no sample showed bands for KO detecting primers, but all of them showed a band for the WT allele, also leading to the same conclusion as standard PCR of definitely no full *sparc*$^{-/-}$ clone being detected. Delta-Delta-Cq evaluation of the qPCRs should in theory lead to SparcKO-K11 (*sparc*$^{+/-}$) control samples showing 50% of the *sparc* WT allele signal of A5 WT controls and a full *sparc*$^{-/-}$ showing no WT signal. Primers measuring the KO allele would be expected to show 100% signal in *sparc*$^{-/-}$, 50% in *sparc*$^{+/-}$ and no signal in A5 WT. Since these ranges were not met with our control groups, the method of qPCR was suspended for the time being, and replaced by the efforts of standard PCR, as described in this work.

For future trials with the qPCR approach, a big change to make would be to switch out the reference gene to *atp5f1*, like in Experiment 2. Since these first tests, we have also made upgrades to our statistical methods, which could also be of use in the evaluations here. However, all together, standard PCR appears to be the more reliable and sturdy method and at least for *sparc* worked as intended on control groups. It appears more promising to continue with this method in the future and refine it for *desmin* characterisation as well.

4.2 Discussion Experiment 2

The question this experiment aimed to answer was whether or not TGFβ1 and the Smad3 inhibitor SIS3 alter the gene expression of several genes of interest for cardiac development, primarily *desmin* and *sparc*. Also, the dependency of the effect on intra-cellular expression levels of SPARC and Desmin was tested by using not only a WT model, but also a *sparc*$^{+/-}$ and *desmin*$^{+/-}$ model cell line. The changes in expression pattern between self-renewing and differentiating cardiac stem cells and their possible influence on TGFβ1 and SIS3 effects were addressed by subjecting the cells to the two factors over time periods between 0 and 49 hours.

Since all the GOIs are connected in a network, TGFβ1 or SIS3 might only indirectly influence them, so by up- or downregulating a factor upstream of the GOI. At the same time, this might provide an explanation for seemingly no influence of TGFβ1 or SIS3 on certain GOIs: Two upstream factors of a gene might be influenced in opposite ways by our additives, resulting in cancelling each other's effects out. This can possibly be observed in *nkx2.5* with SIS3, where *desmin* and *sparc* expressions change in opposite ways and *nkx2.5* expression stays the same in the majority of cases (see chapter 4.2.4). Hence it is important to also look beyond the direct influence of added TGFβ1 or SIS3 on one gene alone and keep the already known pathways in mind, which is why this discussion also deals with interplays possibly affecting our results.

4.2.1 *Brachyury* regulation via a TGFβ1/SIS3 pathway

The clearest results are the effects of SIS3 and TGFβ1 on the expression of *brachyury* (*T*) at T6. As a specific inhibitor of Smad3, SIS3 is expected to interfere with TGFβ1 signalling by inhibiting Smad3 phosphorylation. This means a gene which depends on the TGFβ pathway would most likely be influenced by TGFβ1, a ligand to the TGFβRI, and SIS3 in opposite ways. This is given in rare single cases of our results, most consistently across all cell lines for the gene *brachyury*.

According to our results, *brachyury* expression is increased by TGFβ1 and decreased by SIS3, meaning that this gene is in some way activated or enhanced through the TGFβ pathway and this effect can be sufficiently hindered by addition of SIS3. Although it was found that Desmin increases *brachyury* expression (Hofner et al. 2007), the *desmin*$^{+/-}$

genotype of 2H had no influence on the *T* regulation via SIS3 or TGFβ1. As discussed under 4.2.3, SIS3 did lead to increased *desmin* expression, but our results still show a decrease of *brachyury* via SIS3. Their regulation and influence of each other in our specific experiment is hence unclear at the current time.

4.2.2 Nanog and Brachyury interplay

Another connection is possibly between *nanog* and *T*. TGFβ signalling directly leads to an increase in *nanog* promoter activity via Smads, BMP signalling leads to a decrease, at least in ESCs (Xu et al. 2008). In human acute myleoid leukemia cells, it was reported that elevated levels of BMP4 ultimately lead to increased Nanog as well (Voeltzel et al. 2018). Further, it was reported that *nanog* expression is upregulated by Brachyury and STAT3 binding to its promoter in murine ES cells and *brachyury* itself is upregulated by canonical Wnt/β-Catenin in ESCs and mesenchymal-like colorectal cancer cells. In turn Nanog blocks BMP-induced mesoderm differentiation by hindering Smad1 activity (figure 19) (Suzuki et al. 2006, Tarafdar et al. 2013, Sarkar et al. 2012). Again, it is important to note that most studies were made in embryonic stem cells or human cancer cells and that these factors are not active at all times in all tissues.

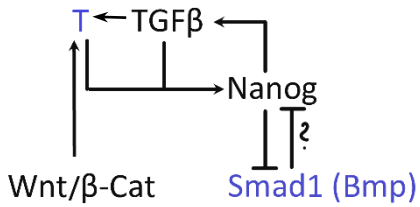

Figure 17 *Possible interactions between T and Nanog. The interaction between BMP signals and Nanog seem not entirely clear yet, or might be subject to spacial and temporal differences. The upregulation of T by TGFβ1 was shown in our experiment.*

Still, this would first lead us to believe TGFβ1 treatment in and of itself would upregulate *nanog*, and SIS3 treatment would downregulate *nanog*. Additionally, the observed increase in *T* by TGFβ1 treatment would, probably starting at a later time point than *T*, further lead to an upregulation of *nanog*. On the other hand, the decreased *T* by SIS3 might contribute to downregulation of *nanog* or have at least no enhancing effect. Only the expected upregulatory effect of TGFβ1 treatment on *nanog* could be verified by our experiment in four out of six presented cases. At T20, all cell lines had increased *nanog* by TGFβ1, but also

by SIS3. One possible explanation could be different roles of Smad2 and Smad3 in *nanog* regulation. While TGFβ1 could activate *nanog* via Smad2, Smad3 might have a more repressive role, hence a block of Smad3 via SIS3 also leads to an upregulation. However, there is a lot more evidence needed to confirm this theory. How the TGFβ receptors could control varying downstream effects is not well understood yet.

4.2.3 *Desmin* is upregulated under the influence of SIS3

Desmin results show repeatedly that blockage of TGFβ signalling by SIS3 increases *desmin* expression. That seems to suggest, the TGFβ pathway has a repressive role in *desmin* regulation. However, TGFβ1 itself shows varying effects. The influence of TGFβ1 seems to be generally lower, with two cases even showing no significant difference to control groups. Only A5 shows the expected decrease of *desmin* at T28, but contrarily also an increase at T20.

A possible theory for the reduced influence of TGFβ1 is provided by Francki et al. 2004, suggesting that added SPARC in combination with TGFβ1 has a bigger influence on downstream signalling than TGFβ1 alone. However, they mainly observed an influence on Smad2 phosphorylation and not Smad3 phosphorylation, which was targeted in this study by SIS3. Their observation that low levels of intrinsic SPARC lead to an even smaller effect of added TGFβ1 could not be clearly verified in our *sparc*$^{+/-}$ cell line, however, SPARC protein levels were not measured for this particular experimental setup. Earlier works with the A5-SparcKO-K11 cell line provided at times conflicting results regarding the mRNA levels, with some repeated results showing higher Sparc mRNA levels than in WT cells. However, protein levels of SPARC were lower than in WT, as expected (L. Leitner and C. Rudolf, results not provided). Although there is no explanation yet as to why the mRNA levels are that high, since the protein levels were nonetheless reduced, one would still assume a lower innate SPARC level in K11 cells of our experiment. Direct comparison of mRNA levels between cell lines might only be insightful to a small extent, as the cell culture experiments as well as the qPCRs were conducted independently of each other. That being said, while K11 generally showed slightly lower Sparc mRNA levels than A5 and 2H, the *desmin*$^{+/-}$ line 2H had higher mRNA levels than the other two *desmin*$^{+/+}$ lines in our results.

Generally, the influence our different genotypes had on the results could not clearly be determined yet.

4.2.4 Correlations between changes in *sparc/desmin* expression and *nkx2.5* expression

Previous studies reported an upregulation of *bmp2* by SPARC, as well as a joint positive influence of SPARC and Bmp2 on *nxk2.5* expression (Stary et al. 2005). With either SPARC or Bmp2 attenuated by added antibodies in the medium, *nkx2.5* expression was reduced. When comparing this to the TGFβ1 and SPARC joint effect, there seems to be a similar pattern of SPARC supporting ligand binding or even changing the downstream effect of the ligand into the opposite (Francki et al. 2004).

Is there a correlation between changes in *sparc* expression and *nkx2.5* expression?

At the representative time point T20 in the two *sparc* WT lines (A5, 2H), SIS3 downregulates *sparc*. At the same time in the same cell lines, *nkx2.5* has no significant change in expression due to SIS3 and only a minor increase of expression in 2H due to TGFb1.

In K11, which should express less Sparc mRNA in total, the *sparc* levels are slightly increased by SIS3. *Nkx2.5* in K11 also shows an increased *sparc* levels due to SIS3, which would be the only common change of their expression.

Does *desmin* upregulation in *desmin* WT cells correlate with nkx2.5 upregulation?

Desmin is also reported to upregulate *T* and *nkx2.5* (Hofner et al. 2007). However, in the *desmin* WT lines (A5 and K11), the upregulated *desmin* by SIS3 did not explicitly correlate with upregulated *T*.

Desmin upregulation via SIS3 correlated with *nkx2.5* upregulation in A5 and K11, at both T20 and T28, in 3 out of 4 cases. In *desmin*$^{+/-}$ cells, SIS3 still raises *desmin* expression, but *nkx2.5* shows no significant difference in T20 and a downregulation at T28.

What does this mean for *sparc* and *desmin* together?

At T20, SIS3 has the opposite effects on *desmin* and *sparc* expression in A5. The *nkx2.5* expression remains unaffected, which can be seen as equilibrium between an increased positive effect of more Desmin and a reduced expression by less SPARC (possibly via the Bmp2 route).

T20 2H with SIS3 has again an upregulation of *desmin* and a downregulation of *sparc*, resulting in no significant difference in *nkx2.5* expression. Whether the lower innate *desmin*

levels lead to lower total *nkx2.5* expression in comparison to the other cell lines cannot be clearly stated, as the experiments with each line were conducted separately and comparability of total expression levels might not be given.

At the same time in K11, *desmin* and *sparc* are both increased by SIS3 and *nkx2.5* is also strongly increased. Although unclear why SIS3 raises *sparc* expression in *sparc* deficient cells but not in *sparc* WT cells, this at least correlates with the idea that more Desmin upregulates *nkx2.5* and there is no *sparc* downregulation which would possibly inhibit *nkx2.5*.

On the other hand, the influence of added TGFβ1 does not fit the pattern observed with SIS3 at all. In fact, results of the TGFβ1 group seem to always be the opposite of what's expected. In A5, both *desmin* and *sparc* are increased by TGFβ1 but *nkx2.5* shows no significant difference. In K11, changes in *desmin* expression are not significant and *sparc* expression is increased, but *nkx2.5* is decreased. In 2H, *desmin* and *sparc* expression are both decreased and this time *nkx2.5* is increased. It is still unclear which role exogenous TGFβ1 on its own plays for any of the involved genes.

4.2.5 Further correlations to known pathways

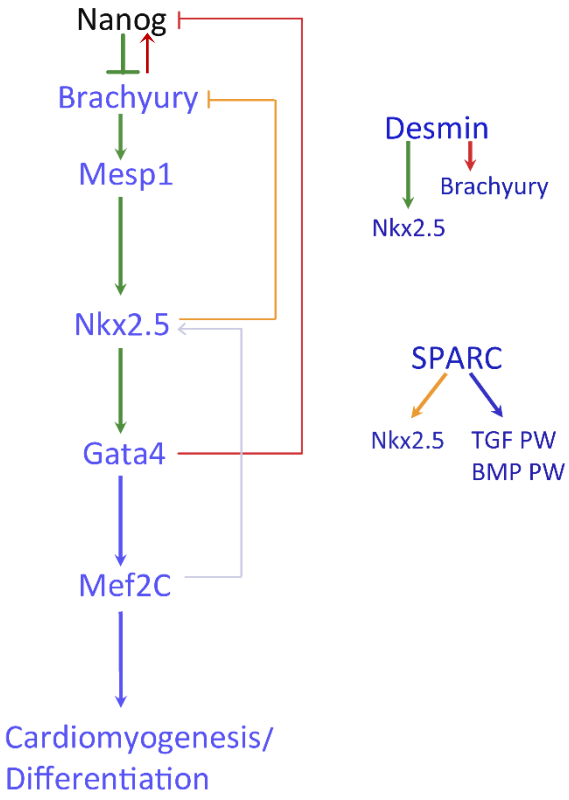

Figure 18 *Previously described pathways and whether these connections could be reflected in our results or not. As results of the TGFβ1 test groups are not consistent enough to allow conclusions about general up- or downregulation by this protein, this diagram is only based on the SIS3 results. A green symbol means the expected correlation was observed in the majority of cases, over the timepoints and cell lines depicted in the results. A red symbol means the connection was not seen in our results. This might have varying reasons, such as autoregulatory feedback loops possibly not being active at the same time. Blue/grey symbols mean this relationship was not part of the experiment.*
Nkx2.5 inhibiting T is a special case, due to many ns results in nkx2.5. The expected negative effect of more nkx2.5 on T was only observed in K11, which is why the symbol is orange.

In most cases of our results, the direct influence of TGFβ1 or SIS3 on our GOIs is not clearly interpretable. This is why we attempted to see the expression changes in context of the known signalling network. Figure 20 depicts a comparison between the known connections between our GOIs and correlating expression changes in our experiment after SIS3 treatment. For example, *T* expression is down regulated by SIS3 in six out of six cases and *mesp1* is downregulated in four cases, the only exception being in the K11 cell line. This fits the fact that *mesp1* expression was described to be directly related to *T,* but could be interpreted as the regulation of *mesp1* via *T* being SPARC dependent. However, it could still also mean that *mesp1* is directly, negatively regulated via a Smad3 pathway, and that innate SPARC levels are important for this downregulation, due to K11 showing an increase in *mesp1* expression. The only consistent influence of TGFβ1 or Smad3 inhibition on any of our main genes of interest, *desmin* and *sparc*, was an upregulation of desmin due to SIS3. This could mean that *desmin* is indeed subject to downregulation via a Smad3 pathway, but maybe initiated another ligand than TGFβ1 or TGFβ1 in combination with a co-ligand like SPARC. The missing correlating expression increase in *desmin* and *T* might be explained by the influence of *nanog*, which is upregulated in all but one cases in which *desmin* is upregulated. As of now it is difficult to differentiate between direct and secondary effects of our additives, as also described at the *nkx2.5* interpretation (see 4.2.4).

4.2.5 General Troubleshooting and Outlook

The influence of the genotype on our studied signalling pathway is still unclear. Both when it comes to the change in expression due to TGFβ1 and SIS3 and the total amount of RNA, which is not entirely comparable between cell lines due to being handled separately.

Also, shedding light on the role of TGFβ1 turns out to be more complex than this experiment was able to visualise. As stated before, TGFβ1 and also Bmp2 are dependent on SPARC, exogenous and or intrinsic, to act out their full potential. Future experiments would benefit from another group which has Sparc and TGFβ1 added simultaneously. Since TGFβ signalling is often described as enhancing self-renewing activities and pluripotency factors, and BMP signalling on the other hand as driving differentiation, SPARC interacting with the receptors of both pathways together with their canonical ligand might explain some of the already previously observed dichotomy of SPARC.

A recent study showed that in hiPSCs, the epithelial integrity is of great importance to cell-fate signals like BMP4, ACTIVIN A and also TGFβ. In fact, local disruption of the epithelial integrity prolonged the activation of TGFβ signalling pathway. It is suggested that epithelial organisation as well as the polarity of ESCs is relevant for accessibility of TGFβ superfamily ligands and that their receptors are located basolaterally instead of apically. Also, when cultured over a prolonged period of time, BMP4 activation (Smad1,5 phosphorylation) was found to be decreasing with increasing cell density (Legier et al. 2023). These mechanisms might be another explanation as to why TGFβ1 influence was not as consistent as SIS3 in our experiments, since SIS3 already acts inside the cell and might thus not be influenced by cellular organisation to the same extent.

This already led to implementation of a culture method with cell culture inserts for multi-well plates for different experiments in our group. These cell culture inserts allow growth of cells on a membrane, with medium and thus also exogenous factors reaching the cells basolaterally. A further study could involve this method of cultivation in combination with an added TGFβ1+SPARC test group. Other possible changes could be the use of TGFβ2 as an opposition to SIS3, or to try different Smad inhibitors. As summarised in chapter 4.2.3, TGFβ1 in combination with SPARC was only found to change Smad2 phosphorylation, but not Smad3. However, SIS3 did yield better results for this study than TGFβ1, so a possible change to TGFβ2 might prove more fruitful.

As evaluation over several time points shows, the effects are not always consistent over the 49 hours period and usually the earliest time point to observe significant results is T6.

Altogether, finding the exact involved elements by repeating this experiment with a variety of proteins and factors, together with possibly a new culture method, would provide new insights into the regulation of *desmin*, *sparc* and the other GOIs of this study.

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Picture citations

Figure 2: *Simplified TGFβ pathway and the roles of R-SMAD and SMAD4.* Page 10. Picture by Jerome Walker, 23.7.2006, under CC license.
https://commons.wikimedia.org/wiki/File:SMAD_apoptosis.svg#filelinks

Figure 3: *The role of SIS3 in the TGFβ signalling pathway.* Page 12. Picture by Wu N, Lian G, Sheng J, Wu D, Yu X, Lan H, Hu W, Yang Z. 2020. Discovery of a novel selective water-soluble SMAD3 inhibitor as an antitumor agent. Bioorganic & Medicinal Chemistry Letters, 30(17):127396.

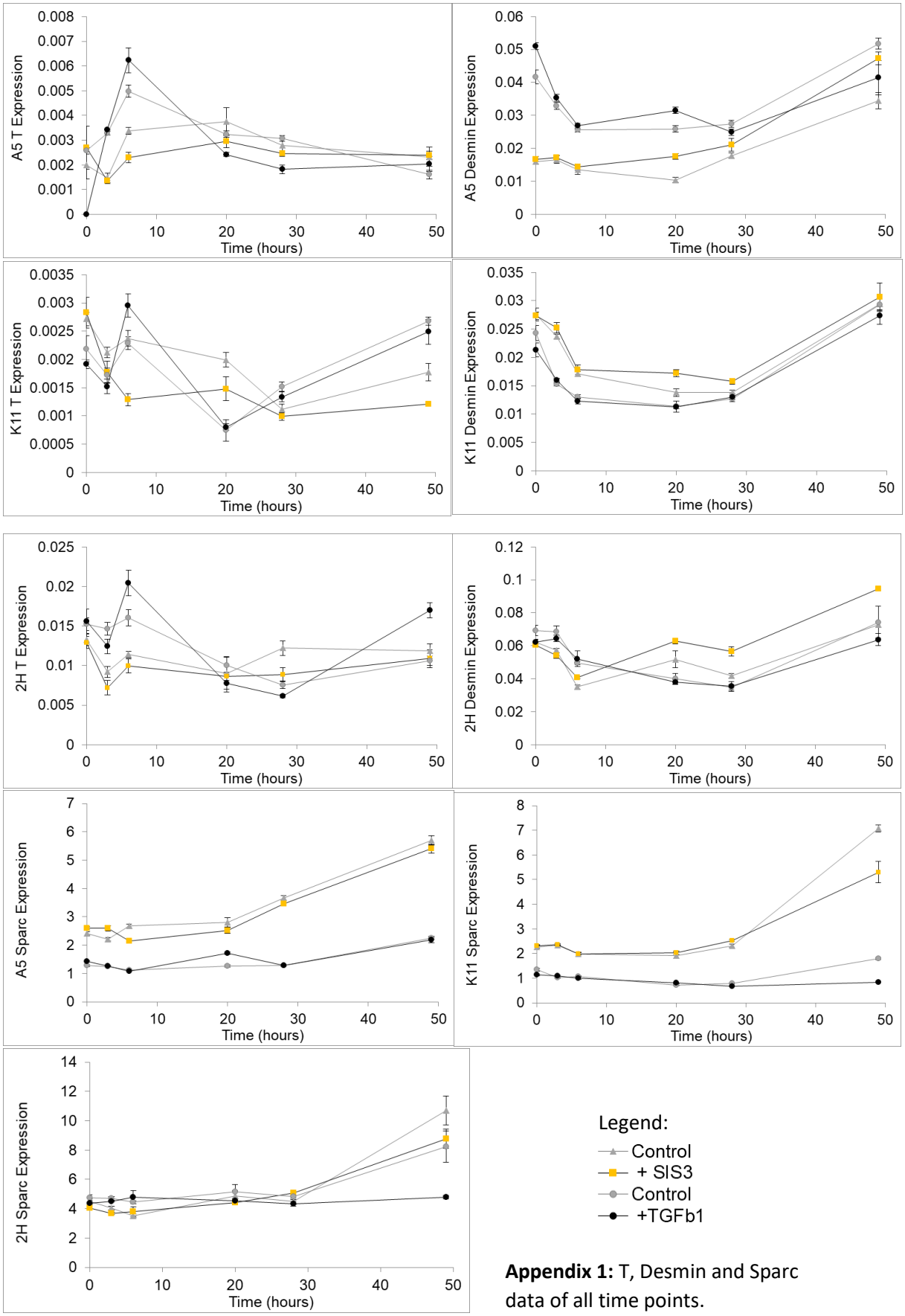
Figures 4 and 5: Gene maps. Page 25. SnapGene® software (from Dotmatics; available at snapgene.com)

All other pictures and graphics are of the author's own creation.

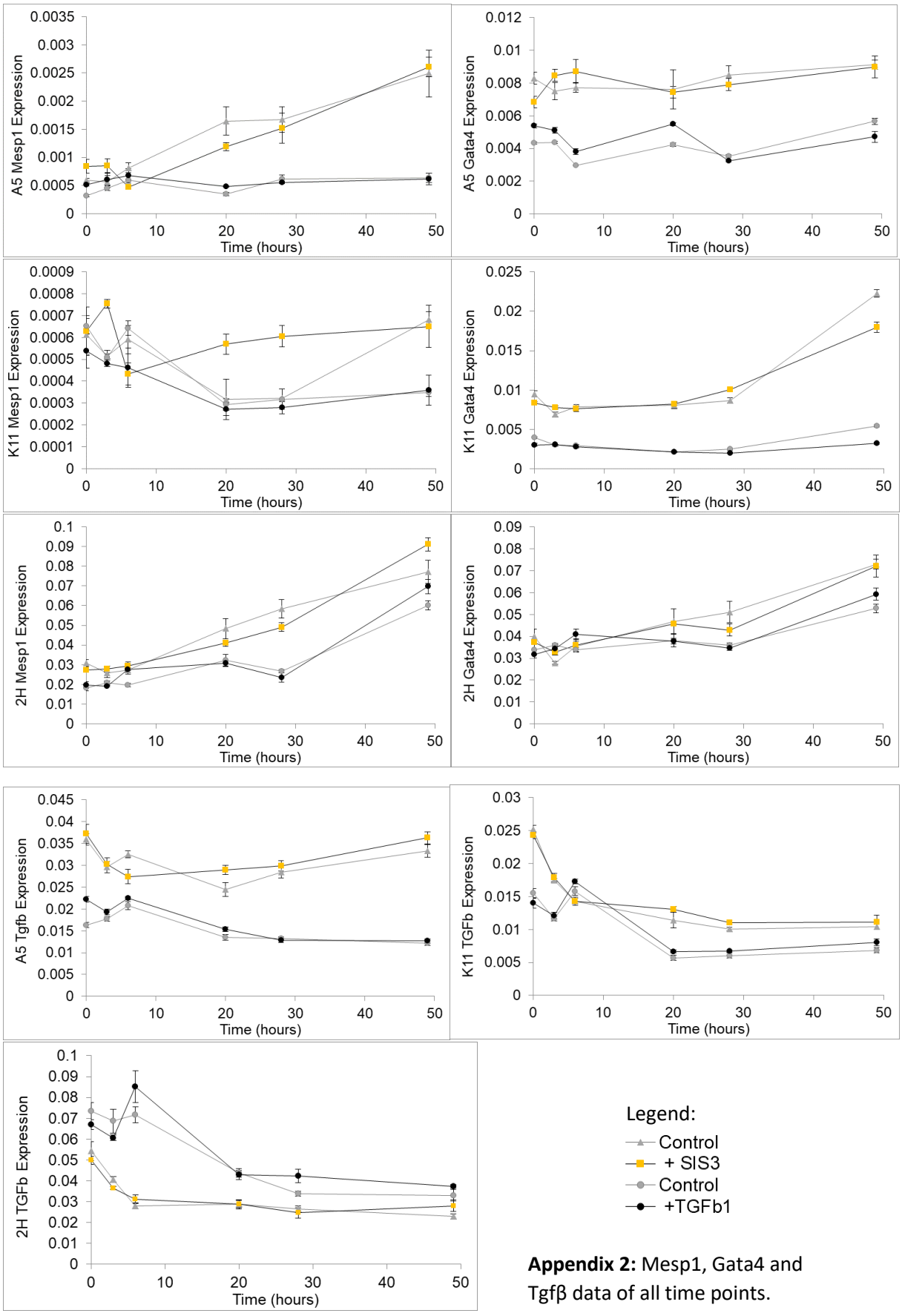
Zusammenfassung

Seit dem Nachweis von Stammzellen im adulten Herzen wurden diese ein wichtiger Aspekt der Forschung zur Prävention und Heilung kardiovaskulärer Erkrankungen. Diese Arbeit befasst sich mit einer spezifischen Population an kardiovaskulären Progenitorzellen und soll zum Verständnis der Signalwege in diesen beitragen. Die zwei Proteine Desmin und SPARC und deren Rolle während der Kardiomyogenese sind dabei von besonderem Interesse. Zur Untersuchung dieser Rolle beschäftigt sich der erste Teil der Arbeit mit der Etablierung von homozygoten *desmin* und *sparc* knock-out Zelllinien, indem auf spontane, inter-chromosomale Rekombination des KO Allels in etablierten heterozygoten Zelllinien selektioniert wird. Leider konnte kein $sparc^{-/-}$ oder $desmin^{-/-}$ Klon identifiziert werden, allerdings konnte eine verlässliche Methode zur Genotypisierung von *sparc* etabliert werden. Der zweite Teil der Arbeit befasst sich mit der Frage, ob die Expression der Gene *desmin* und *sparc*, sowie andere "genes of interest", direkt durch TGFβ Signale bzw. deren Blockade via Specific inhibitor of Smad3 (SIS3) beeinflusst werden. Der größte Einfluss beider Komponenten zeigte sich am Gen *brachyury*. Ebenso schien *desmin* durch SIS3 hochreguliert zu werden. Die Aufklärung des vollständigen Signalnetzwerkes zeigt sich allerdings weiterhin als schwierig, da sekundäre Effekte zwischen den "genes of interest" ebenfalls eine Rolle spielen und unsere Resultate beeinflussen können. Zukünftige Experimente könnten davon profitieren, verschiedene Smad Inhibitoren sowie TGFβ2 in einem ähnlichen experimentellen Setup zu testen, sowie auf eine Kulturmethode mit basolateraler Aufnahmemöglichkeit für die Zellen umzusteigen.

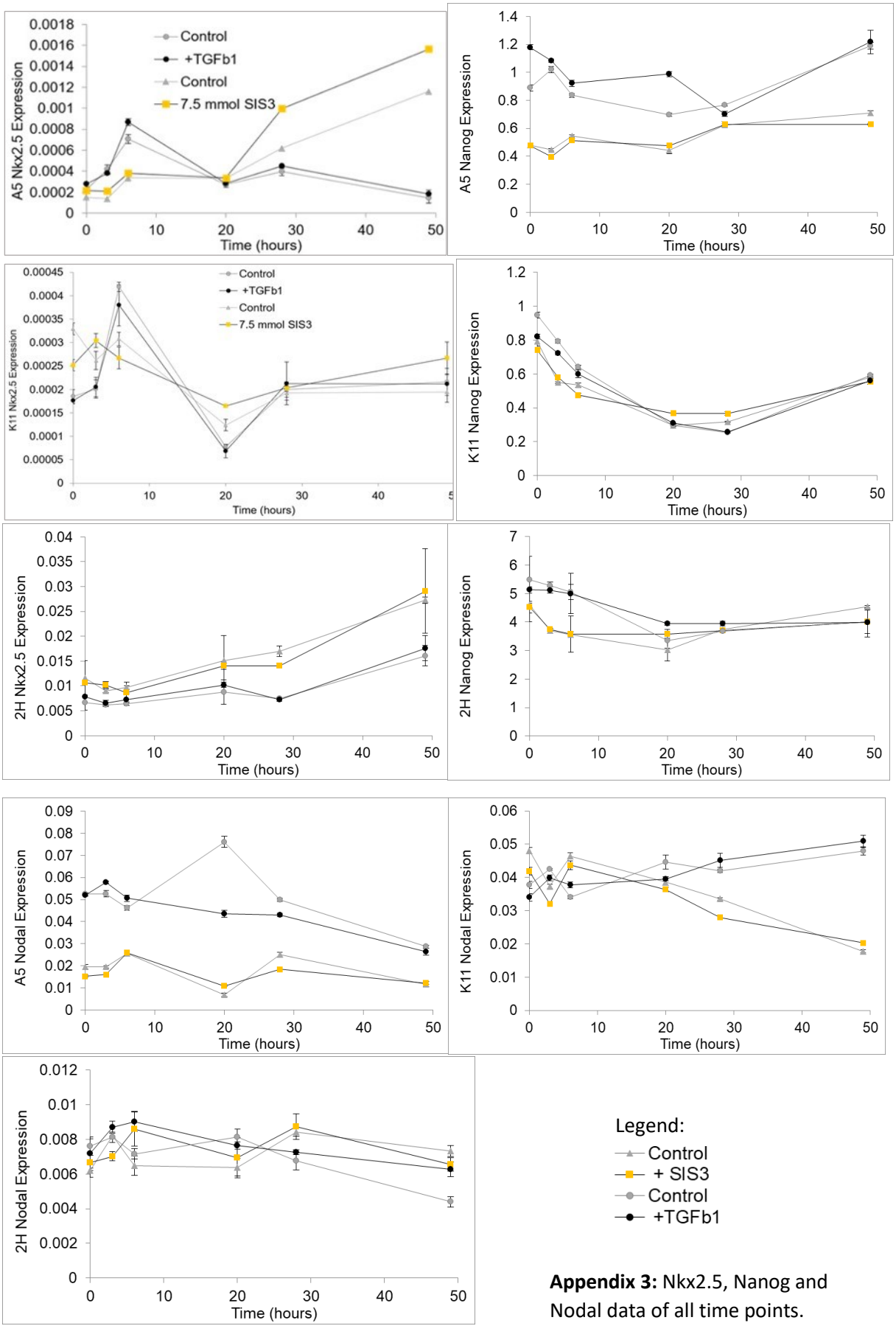
Appendix



Appendix 1: T, Desmin and Sparc data of all time points.



Appendix 2: Mesp1, Gata4 and Tgfβ data of all time points.



Appendix 3: Nkx2.5, Nanog and Nodal data of all time points.

Kontrolle zur Zeit t=0, ...									
Rohdaten									
	GOI	TB1-1	TB1-2	TB2-1	TB2-2	TB3-1	TB3-2	MW	SD
Ref. Gen		25.72	25.58	25.96	25.82	26.07	25.88	25.8383333	0.17394444
TB1-1	21.11	0.04094979	0.04512279	0.03467405	0.03820751	0.03212856	0.03665109		
TB1-2	21.09	0.04038603	0.04450157	0.03419668	0.03768149	0.03168623	0.03614651		
TB2-1	21.20	0.04358574	0.04802735	0.03690602	0.04066693	0.03419668	0.03901033		
TB2-2	21.27	0.04575268	0.05041511	0.03874087	0.04268876	0.03589682	0.04094979		
TB3-1	21.23	0.04450157	0.04903651	0.03768149	0.04152143	0.03491522	0.03983002		
TB3-2	21.16	0.04239389	0.04671404	0.03589682	0.03955489	0.03326157	0.03794359	MW	0.03978946
MW	21.1766667							SD	0.00483087
SD	0.0697615								

Appendix 4: Example matrix of the control group at T0 with blue being the Cq values of the reference gene *atp5f1* and red being the Cq values of desmin. The matrix is filled with the delta-delta-Cq calculations (=2^-(GOI-ref.gene)) before outlier removal, which here only serve illustrative purpose.

	GOI	TB1-1	TB1-2	TB2-1	TB2-2	TB3-1	TB3-2		
Ref. Gen		25.8383333	25.6976892	25.8383333	25.6989895	25.8383333	25.6500217		
		1.00460083	1.00460083	0.9953133	0.9953133	0.99111367	0.99111367	Normierungsfaktoren	
TB1-1	21.1766667	1.00315806							
TB1-2	21.1566035	1.00315806							
TB2-1	21.1766667	0.99889937							
TB2-2	21.2465896	0.99889937							
TB3-1	21.1766667	0.99748783							
TB3-2	21.1068425	0.99748783							
		Normierungsfaktoren							

Appendix 5: Start of outlier analysis of appendix 4. The values in the white boxes are calculated first by always dividing the first of two technical replicates (e.g. TB1-1 or TB2-1) by the mean (MW) of all "biological" replicates. This constitutes as the "normalisation factor" with which now each raw value (GOI or ref.gene Cq value) of one biological replicate is multiplied (grey boxes). So, the two technical replicates of one biological replicate (e.g. TB1-1 and TB1-2) are normalised by the same factor.

	GOI	TRBR1-1	TRBR1-2	TRBR2-1	TRBR2-2	TRBR3-1	TRBR3-2	MW	SD
Ref. Gen		25.8383333	25.6976892	25.8383333	25.6989895	25.8383333	25.6500217	25.7602834	0.08730176
TRBR1-1	21.1766667	0.03950922	0.04355486	0.03950922	0.04351562	0.03950922	0.04501797		
TRBR1-2	21.1566035	0.03896358	0.04295334	0.03896358	0.04291465	0.03896358	0.04439625		
TRBR2-1	21.1766667	0.03950922	0.04355486	0.03950922	0.04351562	0.03950922	0.04501797		
TRBR2-2	21.2465896	0.04147128		0.04147128		0.04147128			
TRBR3-1	21.1766667	0.03950922	0.04355486	0.03950922	0.04351562	0.03950922	0.04501797		
TRBR3-2	21.1068425		0.04149707		0.04145969		0.04289106	MW (K)	0.04164217
MW	21.1733393							SD	0.00212365
SD	0.04494604							N	30
								MW-1,5SD	0.03845669
								MW+1,5SD	0.04482765

Appendix 6: The next step is filling the matrix again with delta-delta-Cq calculations based off of the normalised values of appendix 5. Outliers which are ±1,5 times the standard deviation off from the mean are removed from this matrix and the remainder is used for significance tests and plots.

	Cellline	Gene	Time	Additive	Treatment	Value
1	A5	Sparc	T6	SIS3	K	2.719485
2	A5	Sparc	T6	SIS3	K	2.588693
3	A5	Sparc	T6	SIS3	K	2.719485
4	A5	Sparc	T6	SIS3	K	2.719485
5	A5	Sparc	T6	SIS3	K	2.719485
6	A5	Sparc	T6	SIS3	K	2.623216
7	A5	Sparc	T6	SIS3	K	2.719485

Appendix 7: Example data frame containing all outlier corrected delta-delta-Cq normalised values of one gene and one time point but for all cell lines. Alternatively, the T20 and T28 data of all genes and cell lines are in one data frame. These data frames were used as input for the following R script.

```r
library(ggplot2)
library(ggpubr)

input=read.table("C:/Users/ random path /Sparc-T6.csv",sep=";",h=TRUE,dec=".")
input$Value <- as.numeric(as.numeric(input$Value))

celllines <- c("A5", "K11", "2H")
additives <- c("SIS3", "TGFb1")
times <- c("T6")

# ____________________________________________
# export in 800x800

for (line in celllines) {
  for (time in times) {
    subtime <- subset(input, input$Cellline == line & input$Time == time)
    plottitle <- paste("Sparc - ", line, "-", time)
    newbarplot <- ggbarplot(subtime, x="Additive", y="Value", fill="Treatment", position=position_dodge(0.7),
                            title = plottitle, add="mean_sd", fun="mean_sd")+
      scale_fill_manual(values = c("#f8766d", "#59d9d6")) +
      stat_compare_means(method = "t.test", label = "p.signif", aes(group=Treatment), size=7) +
      font("title", size = 25, vjust=-1.5) +
      font("xlab", size=20) +
      font("ylab", size=20) +
      font("x.text", size=20, angle=45, hjust=1) +
      font("y.text", size=20)
    plot(newbarplot)
  }
}
# ____________________________________________________________________________
# ____________________ Endgültige Version FÜR EITHER T20 OR T28 ______________________
# export in 800x800

input=read.table("C:/Users/ random path /AlleT28-WerteTabelle.csv",sep=";",h=TRUE,dec=".")
input$Value <- as.numeric(as.numeric(input$Value))

celllines <- c("A5", "K11", "2H")
additives <- c("SIS3", "TGFb1")

subgene <- subset(input, input$Gene == "Gata4")
for (line in celllines) {
  subtime <- subset(subgene, subgene$Cellline == line)
  plottitle <- paste("Gata4 - ", line, "- T28")
  newbarplot <- ggbarplot(subtime, x="Additive", y="Value", fill="Treatment", position=position_dodge(0.7),
                          title = plottitle, add="mean_sd", fun="mean_sd")+
    scale_fill_manual(values = c("#f8766d", "#59d9d6")) +
    stat_compare_means(method = "t.test", label = "p.signif", aes(group=Treatment), size=7) +
    font("title", size = 25, vjust=-1.5) +
    font("xlab", size=20) +
    font("ylab", size=20) +
    font("x.text", size=20, angle=45, hjust=1) +
    font("y.text", size=20)
  plot(newbarplot)
}

#------------------------------------------------------------------------
```

Appendix 8: R Script for creating plots with significance tests.