



Article

Serum-Induced Proliferation of Human Cardiac Stem Cells Is Modulated via TGF β RI/II and SMAD2/3

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Abstract: The ageing phenotype is strongly driven by the exhaustion of adult stem cells (ASCs) and the accumulation of senescent cells. Cardiovascular diseases (CVDs) and heart failure (HF) are strongly linked to the ageing phenotype and are the leading cause of death. As the human heart is considered as an organ with low regenerative capacity, treatments targeting the rejuvenation of human cardiac stem cells (hCSCs) are of great interest. In this study, the beneficial effects of human blood serum on proliferation and senescence of hCSCs have been investigated at the molecular level. We show the induction of a proliferation-related gene expression response by human blood serum at the mRNA level. The concurrent differential expression of the TGF β target and inhibitor genes indicates the participation of TGF β signalling in this context. Surprisingly, the application of TGF β 1 as well as the inhibition of TGF β type I and type II receptor (TGF β RI/II) signalling strongly increased the proliferation of hCSCs. Likewise, both human blood serum and TGF β 1 reduced the senescence in hCSCs. The protective effect of serum on senescence in hCSCs was enhanced by simultaneous TGF β RI/II inhibition. These results strongly indicate a dual role of TGF β signalling in terms of the serum-mediated effects on hCSCs. Further analysis via RNA sequencing (RNA-Seq) revealed the participation of Ras-inactivating genes wherefore a prevention of hyperproliferation upon serum-treatment in hCSCs via TGF β signalling and Ras-induced senescence is suggested. These insights may improve treatments of heart failure in the future.

Keywords: human cardiac stem cells; human blood serum; proliferation; senescence; ageing; RNA-Seq; TGF β 1



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1. Introduction

During life the adult body requires mechanisms to renew organs and tissues as well as for damage repair after injury [1]. These regenerative processes are driven by ASCs which remain quiescent in their niche until the need for proliferation or differentiation due to injury, disease, or normal turnover of the cells [2–4]. These regenerative and homeostatic capacities of the body decline during the ageing process, which is defined as a progressive loss of physiological integrity leading to an increased vulnerability to age-related diseases [1,5]. In 2013, López-Otín and colleagues defined eight hallmarks of ageing including the exhaustion of stem cells and the increase of senescent cells, which both strongly contribute to the decline of the regenerative potential of the body [1]. The exhaustion of stem cells is mainly characterized by the accumulation of DNA-damage, expression of cell cycle inhibitory proteins and an overall decrease of cell cycle activity

leading to reduced proliferation [1]. Accordingly, the investigation of stem cell proliferation at the molecular level is of great interest to counteract age-related diseases and increase the relative health span during life. Hence, the development of new cell-based methods and stem cell application is a fast-growing field in regenerative medicine. To this day, the treatments of bone marrow failure, muscular dystrophy or neurodegenerative diseases as well as the support of myocardial regeneration have been reported in this context so far [6–8].

Notably, CVDs are the leading cause of death worldwide and are strongly linked to the ageing phenotype. CVDs occur along with decreased proliferation of resident cardiomyocytes and decreased regeneration potential of cardiac stem cells (CSCs) [9,10] which makes the development of rejuvenating stem cell-based strategies a promising approach to enhance the clinical outcome of CVD treatments. In terms of regeneration, particularly the adult human heart was long considered as an organ with low regenerative capacity until clonogenic and multipotent cardiac stem cells with the ability to self-renew were identified [6,11–13]. In previous studies we successfully isolated a Nestin⁺/S100⁺/c-kit⁻ cell population from human left atrial appendages (LAA) which expresses the cardiac progenitor markers CD105, CD31 and Sca-1. This stem cell population, namely human cardiac stem cells (hCSCs), was able to form cardiospheres after clonal growth and was shown to successfully differentiate into cardiomyocytes in vitro. In general, the transplantation of CSCs has already been shown to enhance the functional outcome for cardiac regeneration after heart failure in animals [14], which underlines the high medical applicability of stem-cell-based approaches for treatment of CVDs. However, studies targeting the activation of endogenous cardiac stem cells in order to overcome the need for surgical intervention were only carried out at a very basal level so far. Interestingly, young blood and plasma have been reported to have rejuvenating effects by several studies. For instance, the exposure of old mice to the blood of young mice by heterochronic parabiosis led to elevated regeneration of the muscle, the liver, the brain, the spinal cord, the olfactory system, and the heart [15–17]. Additionally, beneficial effects of young blood, plasma or serum in old tissues and organs as well as in various stem cell populations were described [16,18]. We could successfully underline these effects by showing serum-mediated proliferation and senescence reduction in hCSCs after application of human blood serum in vitro [11,19]. However, for the evaluation of a potential clinical application of young blood or its components, a deeper understanding of the molecular signalling networks is crucial. At a molecular level, the p38-MAPK/HSP27 axis was identified as a mediator of the reported serum effects in terms of hCSC migration, proliferation and senescence [19]. To fully elucidate the molecular mechanisms of these effects, further analysis of upstream or downstream pathways is necessary. In this association, the investigation of the TGFβ signalling pathway as a potential upstream mediator of the serum-induced effects is of great interest as it is known to be involved in proliferation and senescence [20–25].

TGFβ family members include several cytokines such as bone morphogenic molecules (BMPs), growth differentiation factors (GDFs), activins, Nodal and TGFβs occurring in three isoforms (TGFβ 1-3) [26]. All isoforms are translated with a large amino-terminal prodomain, which is necessary for correct folding and dimerization [27]. The latent form of TGFβ with furin cleaved but associated with the prodomain is also found in serum [28]. Activation of latent TGFβ1 can occur through interaction with extracellular matrix and α(v)β(6) integrin [29]. These ligands bind to the serine and threonine kinase receptors type II (TβRII) and type I (TβRI) on the outer cell membrane by building a receptor heterocomplex. Binding of a ligand to the homodimeric TβRII initiates conformational adaptation of the ligand and TβRII creates a new binding site with high affinity for TβRI. After recruitment of two TβRI units, the receptor heterocomplex is formed and the TβRII phos-

phorylates the serine residues of T β RI. During canonical SMAD signalling, the receptor-activated (R-) SMADs are subsequently phosphorylated by T β RI leading to downstream signal transmission [26]. Besides, induction of the TGF β pathway also happens to activate non-SMAD signalling which comprises context-dependent induction of several pathways such as ERK-MAPK, p39/JNK, NF- κ B, PI3K/AKT/mTOR, Rho-GTPase, JAK/STAT and p38-MAPK signalling [26]. In the heart in particular, the TGF β signalling pathway plays an important role, as it is known to contribute significantly to cardiac development and vascularization [30–36]. Moreover, TGF β 1 is secreted by cardiac fibroblasts and cardiomyocytes thereby inducing myofibroblast differentiation [37,38]. Overall, these studies show that the investigation of this pathway is important in the context of cardiac regeneration.

In the present study, a proliferation-related response of hCSCs to serum treatment at the gene expression level was revealed by Gene Ontology. Additionally, the involvement of the TGF β signalling pathway was indicated by differential gene expression analysis. Within this study, we demonstrate a dual role of the TGF β signalling pathway in terms of proliferation and senescence in hCSCs. Differential gene expression analysis showed significant upregulation of proliferation-related and Ras-inactivating genes in hCSCs treated with serum and the TGF β RI/II inhibitor, compared to cells treated with serum alone. Our data suggest a prevention of hyperproliferation upon serum treatment in hCSCs via TGF β signalling and Ras-induced senescence.

2. Results

2.1. RNA-Seq Analysis Reveals the Enrichment of Proliferation-Related Processes after Serum Treatment in hCSCs

To clarify the molecular mechanism underlying human blood serum-mediated proliferation in hCSCs, RNA sequencing (RNA-Seq) was performed. We previously published a proliferative and senescence reducing effect of human blood serum and compared differential gene expression after induction with human blood serum from old and young donors, revealing the p38-MAPK signalling pathway to be crucial for serum-mediated proliferation in hCSCs [11]. Here, the application of human blood serum resulted in the significant upregulation of 213 differential expressed genes and downregulation of 31 genes which underline the inductive effect of human blood serum on gene expression in hCSCs (Figure 1). Interestingly, the TGF β signalling target gene ATF3 [39] and the endogenous TGF β inhibitor NR4A1 [40] were differentially expressed after serum-treatment. The here observed upregulation of both targets and inhibitors of the TGF β pathway indicate a crucial role of TGF β in regulating the serum-mediated effects on hCSCs at the molecular level. GO term analysis between untreated and serum-treated hCSCs likewise showed a significant enrichment of several biological processes (BP) which are strongly related to proliferation. These comprise terms like ‘M phase of mitotic cell cycle’, ‘nuclear division’, ‘mitosis’, ‘cell division’, ‘microtubule-based process’, ‘microtubule cytoskeleton organization’, ‘regulation of microtubule-based process’, ‘mitotic prometaphase’ and ‘regulation of microtubule cytoskeleton organization’ (Figure 2A). With respect to cellular components (CC), GO terms including ‘microtubule organizing center’, ‘organelle inner membrane’, ‘spindle’, ‘microtubule’, ‘chromosome centromeric region’, ‘intermediate filament cytoskeleton’ and ‘centrosome’ were significantly enriched (Figure 2B). Accordingly, the molecular functions (MF) ‘microtubule binding’ and ‘tubulin binding’ were enriched (Figure 2C). The enriched GO terms revealed here with respect to BP, CC and MF emphasize the inductive effect of blood serum and strongly correlate with the reported proliferative effect of human blood serum on hCSCs.

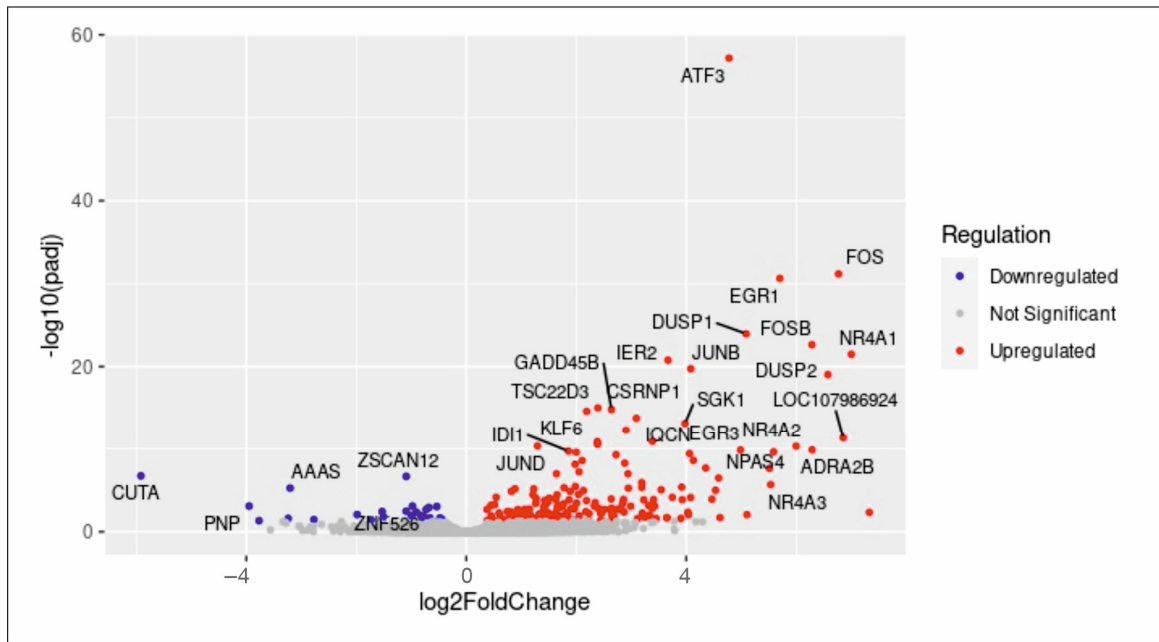


Figure 1. Human blood serum induces differential gene expression. Volcano plot shows upregulation of 213 differentially expressed genes after serum treatment and downregulation of 31 genes.

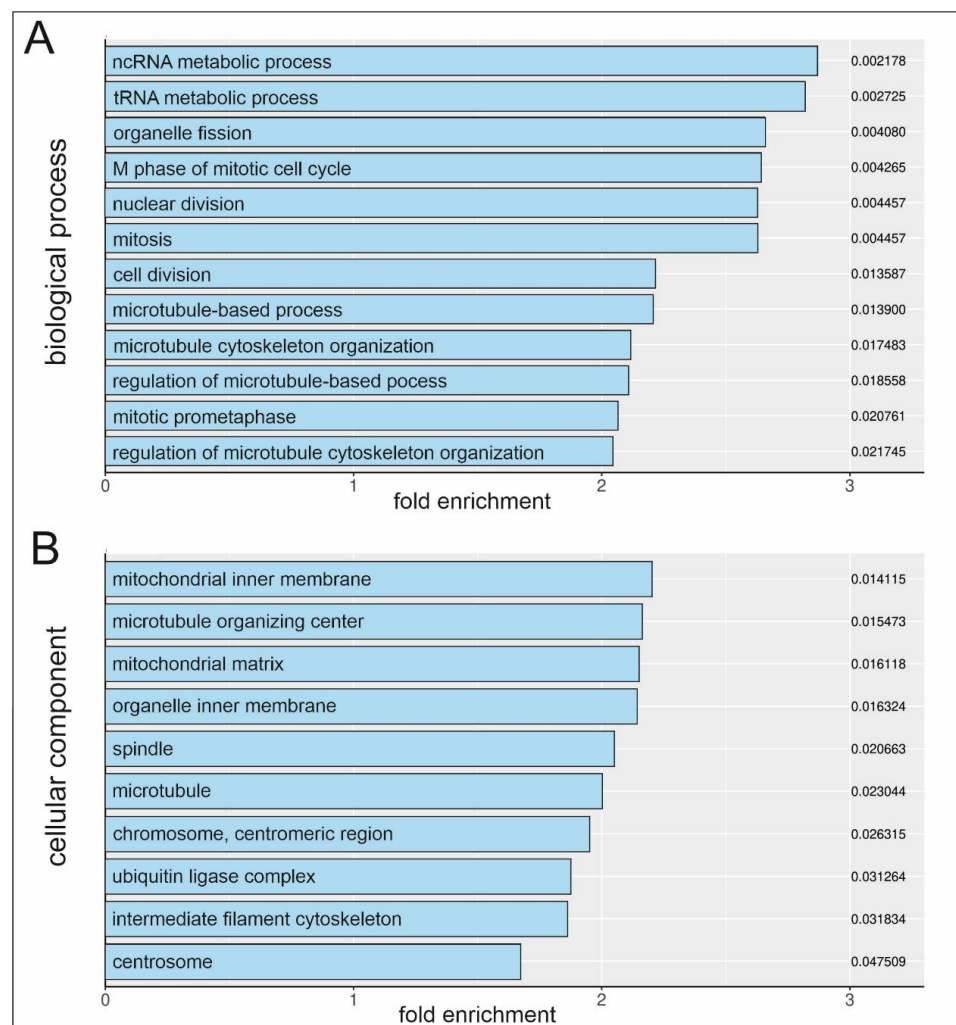


Figure 2. Cont.

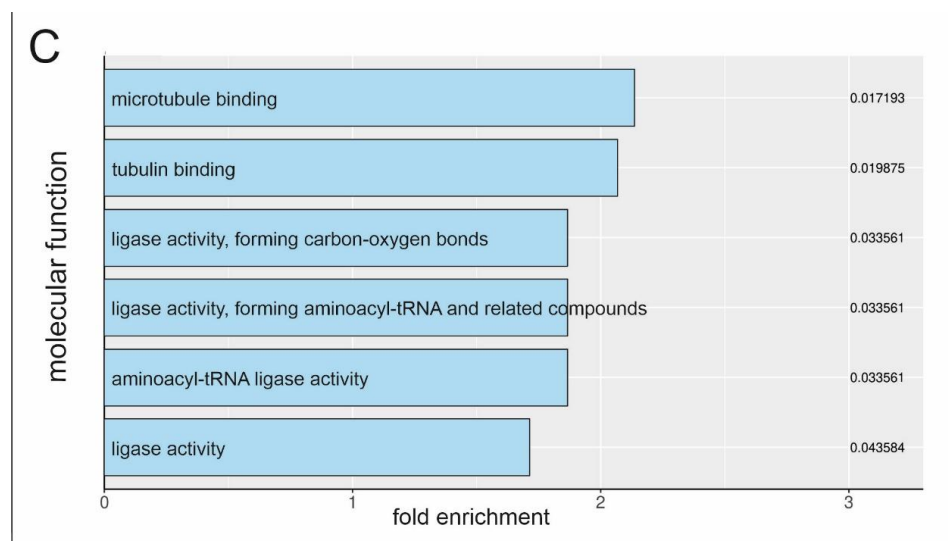


Figure 2. Human blood serum induces proliferation-related GO term enrichment. Upregulated GO terms regarding to (A) biological process, (B) cellular component and (C) molecular function.

2.2. Inhibition of TGF β RI/II Enhances Proliferation While TGF β Possesses a Dual Role in Regard to Proliferation and Senescence in hCSCs

RNA-Seq revealed the differential gene expression of targets and inhibitors of the TGF β 1 pathway and the enrichment of proliferation-related GO terms after serum treatment in hCSCs. In addition, the downstream p38-MAPK signalling pathway was shown in previous studies to be significant for serum-mediated proliferation and migration of hCSCs [11,19], further underlining the potential role of TGF β 1 signalling in this regard. Consequently, the TGF β 1 signalling pathway was investigated to further clarify the molecular mechanisms of the beneficial effects of serum. Supplementation with increasing concentrations of TGF β 1 led to reduced proliferation of hCSCs compared to untreated cells, which indicates a suppressive role of TGF β 1 in this context. To gain deeper insights of the involvement of TGF β in the observed suppressive effect on proliferation, the selective TGF β RI/II inhibitor LY2109761 [41] was applied upon serum treatment, leading to enhanced proliferation, further highlighting the central role of TGF β signalling in this context. The proliferation assay showed increased proliferation of hCSCs correlating with increased concentration of inhibitor, with a maximum effect of a 6.2-fold induction of proliferation at 50 μ M LY2109761 and an IC₅₀ of 6.252 μ M (Figure 3A–C).

In order to determine the role of TGF β -signalling in serum-treated hCSCs, we performed proliferation and senescence assays using 50 μ M of the TGF β RI/II inhibitor LY2109761. A strong and significant increase in hCSC proliferation was observed upon inhibition of TGF β RI/II and serum treatment. Notably, the application of the TGF β RI/II inhibitor LY2109761 also increased the proliferation of cells which were cultured in serum-deprived medium to a higher extent than in serum-treated cells (Figure 4D).

Besides the induction of proliferation, application of LY2109761 resulted in morphological changes. Untreated hCSCs appeared as long spindle shaped cells with thin long processes and single granular cells. In contrast, serum-treated hCSCs were formed as single granular and spindle shaped cells with y-shaped cytoplasm. Supplementation with serum and LY2109761 also induced a spindle shaped morphology while here no single granular cells were visible. Particularly, a strong increase in cell–cell interactions were visible after TGF β RI/II inhibition as here each cell showed connections to other cells (Figure 4A–C). We investigated the amount of total TGF β 1 in several serum samples (Table 1) and found TGF β 1 levels ranging from 0.39 ng/mL to 1.10 ng/mL. Taken together, pooled serum induced proliferation of hCSCs in the same manner as application of 1 ng/mL TGF β 1 (see below).

We tested after inhibition with LY2109761, the effects of TGF β 1 on proliferation. They were investigated via supplementation of increasing TGF β 1 concentrations of 1 ng/mL, 5 ng/mL or 10 ng/mL, revealing a highly inductive effect. The application of low concentrations of TGF β 1 (1 ng/mL) could significantly increase proliferation whereas application of 10 ng/mL TGF β 1 had the same inductive effect as serum (Figure 4E). Strikingly, simultaneous application of the TGF β RI/II inhibitor and TGF β 1 highly increased the cell proliferation of hCSCs regardless of the applied concentration of TGF β 1 (Figure 4F–H), indicating a potential dual role of TGF β signalling in hCSCs. Regarding senescence, serum significantly reduced the senescence-associated β -galactosidase (SA- β -Gal) activity in hCSCs, and this effect was further enhanced after addition of the inhibitor LY2109761. Application of TGF β 1 reduced senescence in hCSCs compared to untreated cells while the protective effect of TGF β 1 was smaller compared to serum-treated cells (Figure 4I). Here, a dual role of TGF β 1-signalling in regard to senescence in serum-treated hCSCs is likewise suggested.

Table 1. TGF β 1 levels in the human blood serum samples. Quantification of the TGF β 1 levels in the serum range from 0.39 ng/mL to 1.10 ng/mL.

Sample	TGF β 1 [ng/mL]
Serum 1	0.39
Serum 2	0.84
Serum 3	1.10
Serum 4	1.17
Serum 5	0.93
Serum 6	1.10

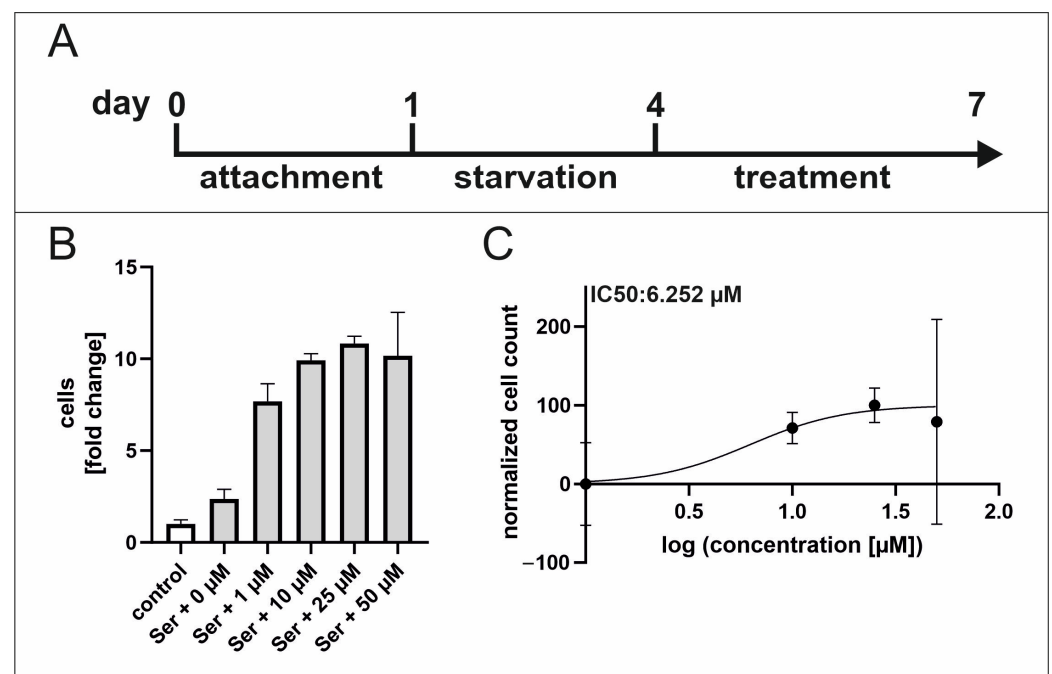


Figure 3. 50 μ M LY2109761 is effective for induction of proliferation in hCSCs. (A) After starvation, hCSCs were treated with human blood serum and TGF β RI/II inhibitor. (B) Increasing concentrations of the selective TGF β RI/II inhibitor LY2109761 upon serum treatment indicate a proliferation enhancing effect in hCSCs with an effective concentration at 50 μ M. (C) The IC₅₀ value of the TGF β RI/II inhibitor LY2109761 is 6.252 μ M.

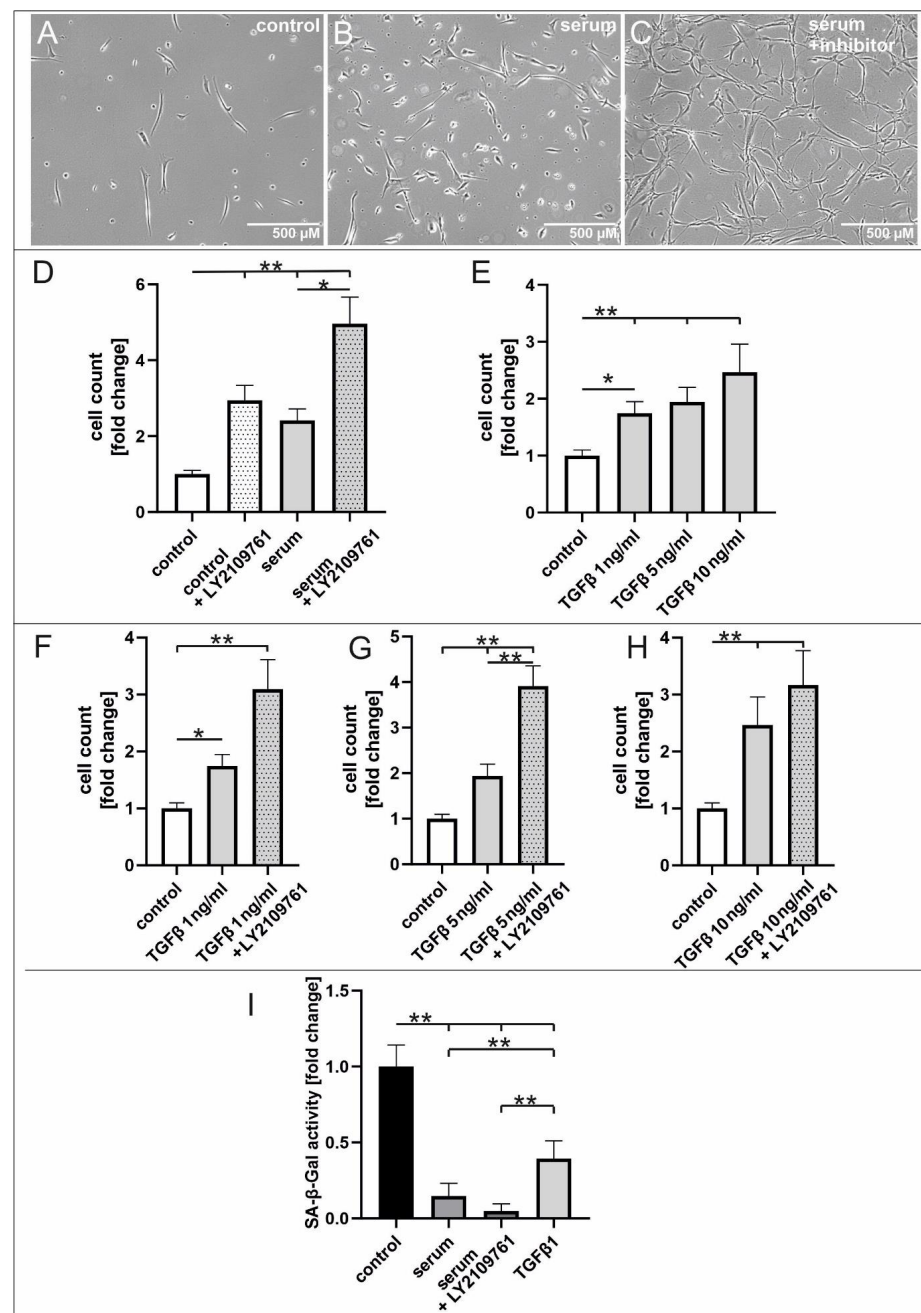


Figure 4. The dual role of TGF β signalling in serum-mediated effects in hCSCs. (A–C) Upon serum treatment, the supplementation with LY2109761 leads to morphological changes of hCSCs. (D) The application of LY2109761 alone increases the proliferation higher than treatment with serum alone, while the proliferative effect of the serum is enhanced by LY210976. (E) TGF β 1 mediates proliferation in concentrations from 1 ng/mL to 10 ng/mL. (F–H) Simultaneous application of LY2109761 and TGF β 1 highly increases the cell proliferation of hCSCs regardless of the applied concentration of TGF β 1. (I) Serum reduces the SA- β -Gal activity in hCSCs, and this effect is further enhanced after addition of LY2109761. Application of TGF β 1 reduces senescence in hCSCs compared to untreated cells while it is significantly increased compared to serum-treated cells. (Mann–Whitney U two-tailed, ** $p < 0.01$; * $p < 0.05$).

2.3. Human Blood Serum Strongly Induces the Phosphorylation of SMAD2/3 in the Nucleus Which Is Significantly Reduced by the Application of the Selective Inhibitor LY2109761

To investigate the effect of human blood serum on TGF β 1 signalling and the function of LY2109761, phosphorylated SMAD2/3 (pSMAD2/3) was immunocytochemically

determined in the nucleus of hCSCs. Serum treatment induced a three-fold increase of nuclear pSMAD2/3 compared to untreated hCSCs. Addition of the TGF β RI/II inhibitor LY2109761r significantly decreased this effect while the amount of nuclear phosphorylated SMAD2/3 was higher than in untreated cells. Supplementation with TGF β 1 resulted in an eight-fold increase of phosphorylated SMAD2/3 while this effect was reduced after the addition of LY2109761 (Figure 5A,B). These data underline the inductive effect of human blood serum on TGF β 1 signalling via SMAD2/3 phosphorylation and the successful reduction of this effect via the selective inhibitor LY2109761.

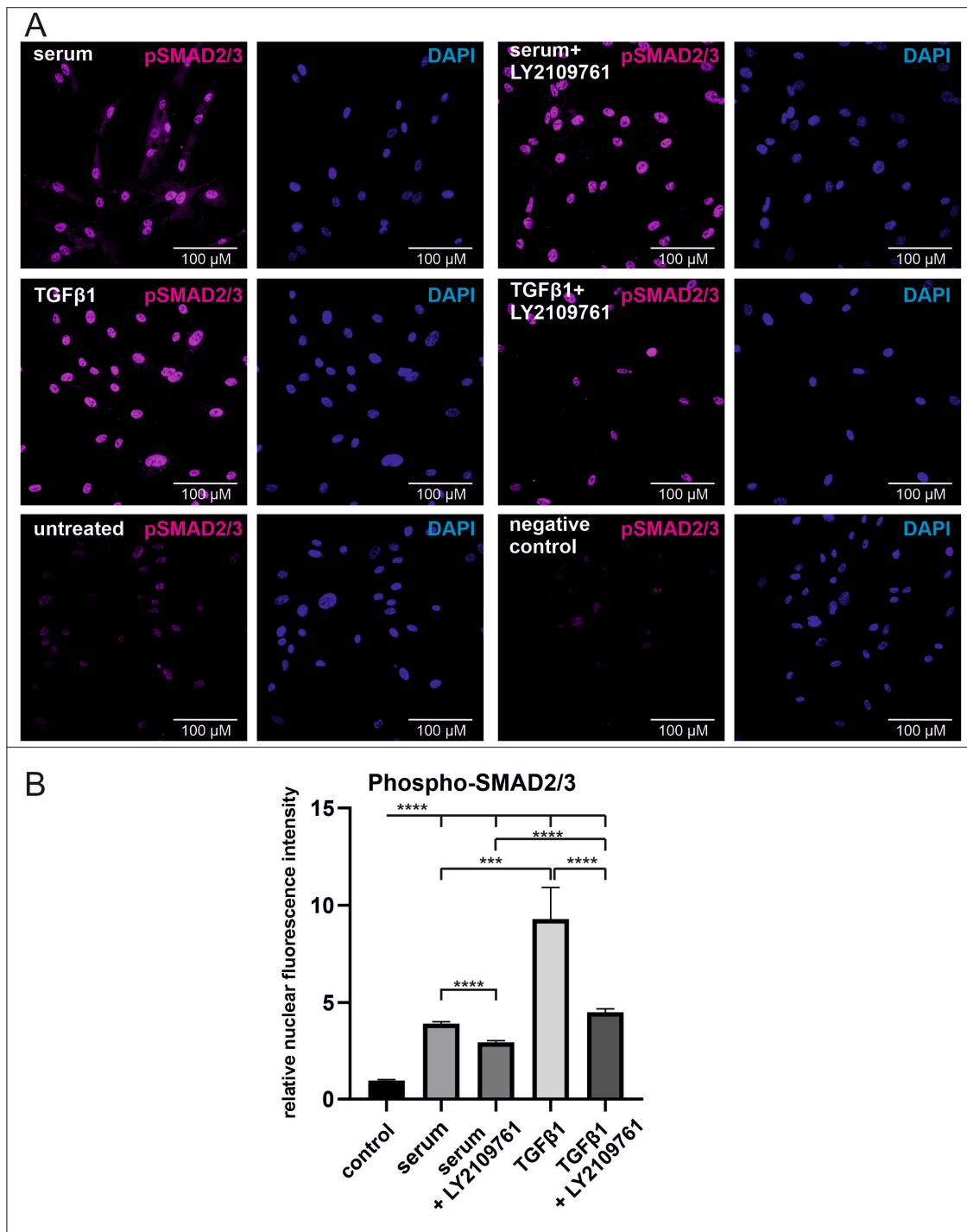


Figure 5. Phosphorylation of SMAD2/3 after serum treatment and inhibition of TGF β RI/II in hCSCs. (A) Immunocytochemical staining of hCSCs shows enriched amounts of pSMAD2/3 in the nucleus

after treatment with serum or TGF β 1 which further increases after inhibition of TGF β RI/II, magnification $\times 63$. (B) Quantification of the fluorescence signal reveals a significant decrease of nuclear pSMAD2/3 after application of TGF β RI/II inhibitor. (Kruskal–Wallis, *** $p < 0.001$; **** $p < 0.0001$).

2.4. TGF β RI Inhibition Reduces Serum-Mediated hCSC Proliferation

Following the striking observations of TGF β 1 and TGF β RI/II inhibition which both increased hCSC proliferation, a more detailed analysis was performed. The selective TGF β type I receptor inhibitor, SB431542, was applied to serum-treated hCSCs. Contrary to the proliferative effect of TGF β RI/II inhibition, application of the TGF β RI inhibitor SB431542 resulted in a strong and significant reduction in the serum-mediated hCSC proliferation with an IC₅₀ of 0.553 μ M (Figure 6A,B).

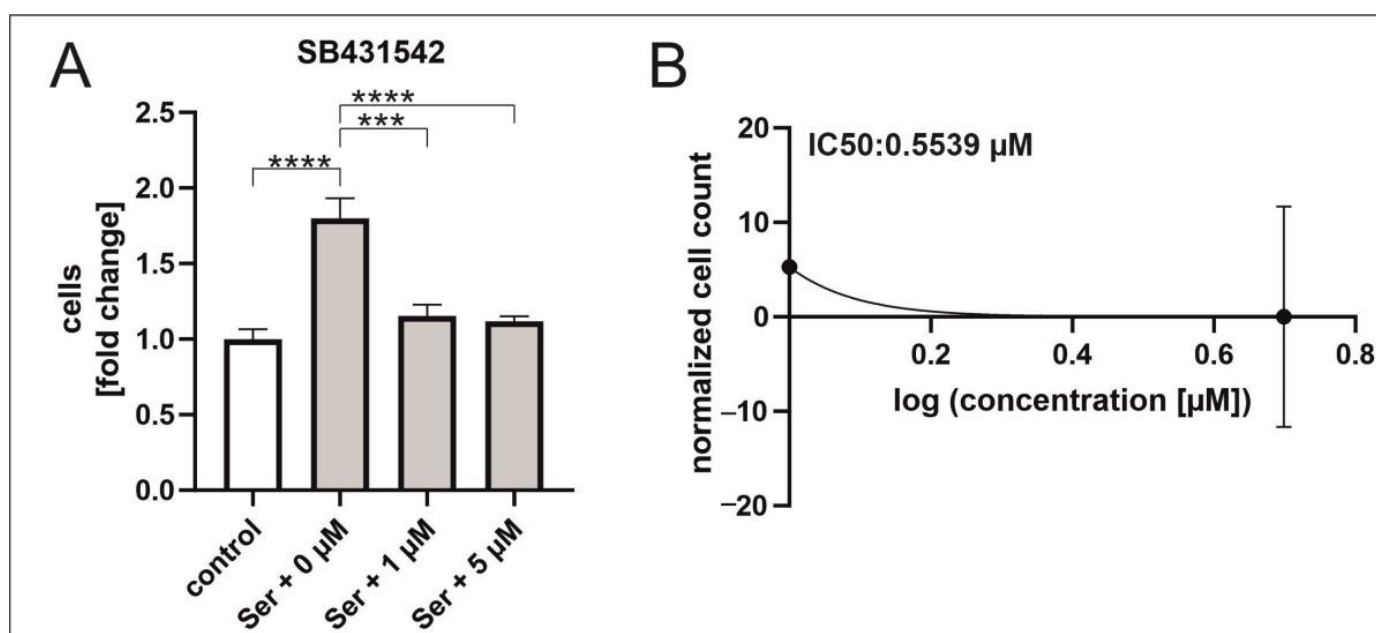


Figure 6. TGF β RI inhibition reduces serum-induced hCSC proliferation. (A) Application of TGF β RI inhibitor SB431542 reduces serum-mediated hCSC proliferation with an (B) IC₅₀ of 0.5339 μ M. (Ordinary one-way ANOVA, **** $p < 0.0001$; *** $p < 0.001$).

2.5. Differential Global Gene Expression Indicates Upregulation of Proliferation-Related and Ras-Inactivating Genes after TGF β RI/II Inhibition and Serum Treatment in hCSCs

To further clarify the underlying molecular basis of the observed effects, RNA-Seq was performed with serum- and LY2109761-treated hCSCs. The differential gene expression analysis revealed significant upregulation of proliferation-related genes, such as *WEE1* (*WEE1* Checkpoint Kinase), *CETN2* (*Centrin2*) and *MLF1* (*Myeloid Leukemia Factor 1*) in cells treated with serum and LY2109761 compared to serum treatment (Figure 7A). Additionally, Ras-inactivating genes such as *RIN1* (*Ras and Rab interactor 1, Ras inhibitor 1*) and *SYNGAP1* (*Synaptic Ras GTPase Activating Protein 1*) were differentially expressed (Figure 6A). Interestingly, the previously identified genes *FOS*, *FOSB*, *NR4A* and *EGR* were similarly upregulated in hCSCs treated with serum or serum with LY2109761 compared to untreated hCSCs (Figure 7B).

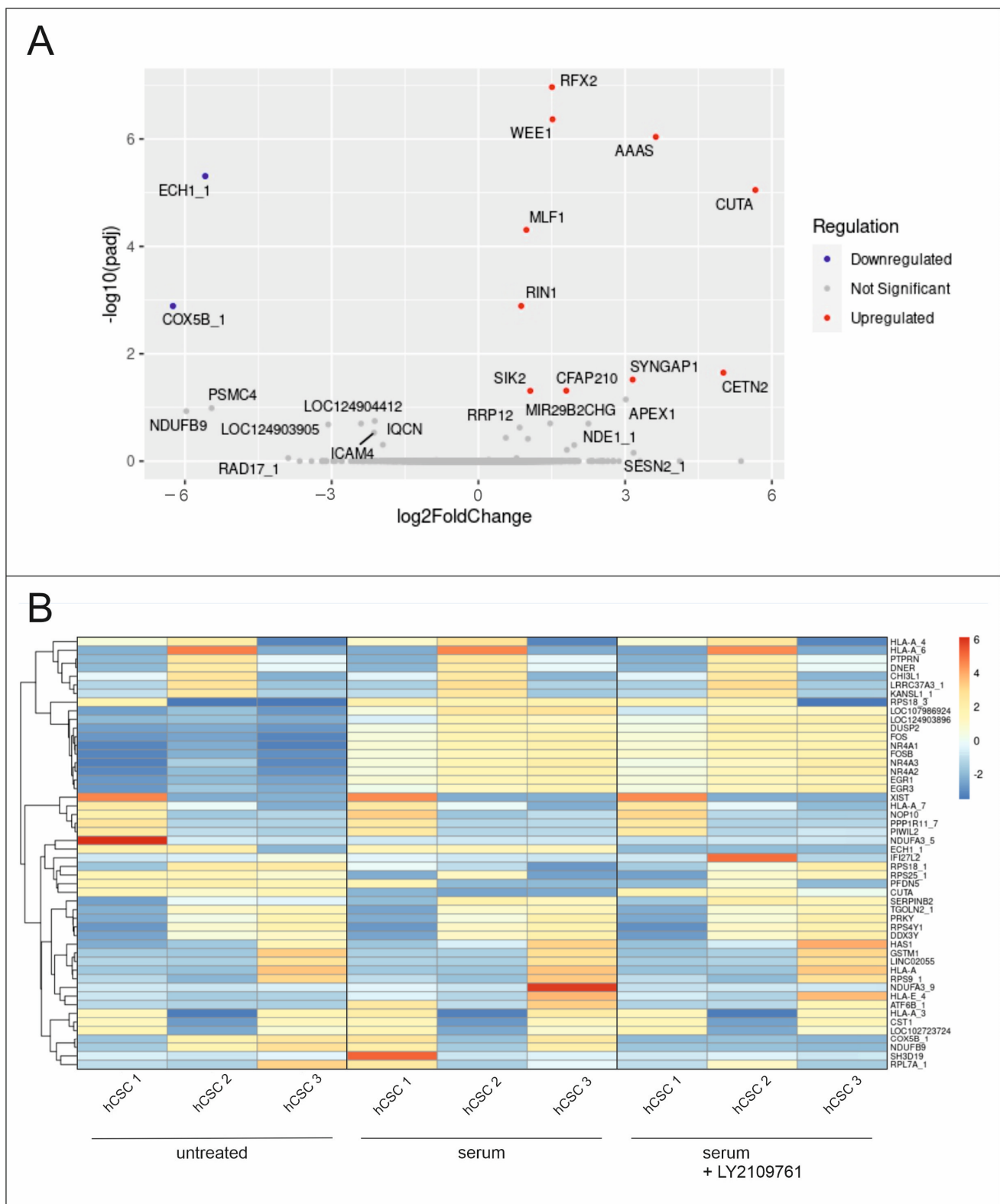


Figure 7. Gene expression analysis of serum- and LY2109761-treated hCSCs. (A) Volcano plot of differentially expressed genes in serum-treated hCSCs after TGF β RI/II inhibition shows upregulation of ten genes and downregulation of two genes. Proliferation-related and Ras-inactivating genes are significantly upregulated after serum and inhibitor treatment in hCSCs. (B) The genes FOS, FOSB, NR4A and EGR are similarly upregulated in hCSCs treated with serum or serum with LY2109761 compared to untreated hCSCs.

3. Discussion

The characteristics of adult human stem cells, such as the ability to self-renew and the high differentiation potential, make their presence crucial for the regeneration and homeostasis of the human organism [2]. Hence, the application of adult stem cells is a fast-growing field in regenerative medicine. This includes treatment of bone marrow failure, muscular dystrophy or neurodegenerative diseases as well as the support of myocardial regeneration [6–8,42]. The exhaustion of stem cells and the increase of senescent cells both are hallmarks of ageing and contribute to the decline of the regenerative potential of the body [1,5]. Accordingly, the investigation of stem cell proliferation as a marker for stem cell fitness is necessary for developing new cell-based applications in regenerative medicine to counteract age-related diseases. Especially the human heart which was long considered as an organ with low regenerative capacity until self-renewing, clonogenic and multipotent cardiac stem cells were identified [6,11,12]. We previously isolated a Nestin⁺/S100⁺ cell population from human left atrial appendages which expresses the cardiac progenitor markers CD105, CD31 and Sca-1. This human cardiac stem cell population was able to form cardiospheres after clonal growth and differentiated into cardiomyocytes in vitro [11]. In terms of rejuvenation, several studies showed a beneficial effect of young blood, plasma or serum on old tissues and organs as well as on various stem cell populations [16,18]. We could successfully underline these effects by showing serum-mediated proliferation and senescence-reduction in hCSCs after application of human blood serum in previous studies [11,19].

Within this study, we showed that human blood serum induces enrichment of proliferation-related GO terms in hCSCs which is consistent with our previously observed serum-mediated proliferative effects [11]. Here, the upregulation of the biological processes associated with mitosis strongly indicate an immediate response to serum at a global gene expression level coherent to the observed proliferative phenotype. We previously identified the p38-MAPK/HSP27 axis as a partial mediator of serum-induced proliferation in hCSCs [19]. Consequently, making examinations of upstream signalling pathways is of great interest to further unravel the underlying molecular mechanisms of the serum-mediated proliferative effects in hCSCs. In this study we show the upregulation of 213 differentially expressed genes induced by serum. These comprise the TGF β signalling target activating transcription factor 3 (ATF3) [39] and the endogenous TGF β inhibitor nuclear receptor subfamily 4 group A member 1 (NR4A1) [40], which strongly implies the involvement of TGF β -signalling in the serum-mediated response in hCSCs. ATF3, a known stress response gene belonging to the ATF/CREB family, has been reported to be induced by TGF β -activated SMAD3 signalling in human epithelial cells [39]. Additionally, the transcription factor has not only been shown to be a target of TGF β 1 but also as a mediator of TGF β signalling. In detail, it has previously been found that ATF3 induces proliferation and invasion and inhibits apoptosis via TGF β 1/SMAD2/3 signalling in keloid fibroblasts [43]. In contrast, the NR4A1 receptor has been shown to be an endogenous inhibitor of TGF β signalling which mediates the repression of TGF β target genes. The receptor therefore has been focused on in other studies to counter TGF β -mediated signalling in several diseases [44,45]. The here observed involvement of both targets and inhibitors of the TGF β pathway, makes further investigation to unravel the molecular mechanisms in hCSCs crucial to gain deeper insights on the serum-mediated effects in relation to proliferation. Especially focusing on cardiac development, the TGF β signalling pathway has already been reported to be involved by several studies. Interestingly, the cardiac progenitor marker endoglin (CD105) is also known to be an ancillary TGF β receptor and has been shown to be required for extraembryonic angiogenesis and heart development [46]. In several species, bone morphogenic proteins (BMPs), members of the TGF β superfamily, have also been found to mediate cardiac development. This comprises the participation of BMPs and SMADs during heart development in terms of endocardial cushion formation, septation and specification of the left/right axis [30–35]. Further, the TGF β signalling pathway has been reported to be crucial during vascular development [36]. Additionally, previous studies

showed the secretion of TGF β 1 by cardiac fibroblasts and cardiomyocytes resulting in the induction of myofibroblast transdifferentiation [37,38,47]. Based on the here-demonstrated induction of TGF β -related genes after serum treatment in hCSCs and the reported involvement of TGF β signalling in several cardiac processes, we analysed proliferation. We analysed six different samples of human serum with a TGF β 1-specific ELISA and detected amounts of about 1 ng/mL TGF β 1, which is in accordance with the current literature [48]. In line with these concentrations, we observed a similar induction of proliferation (2-fold) with either pooled sera or recombinant TGF β 1 (1 ng/mL). A selective TGF β RI/II inhibitor LY2109761 which negatively affects the phosphorylation of SMAD2/3 was utilized. The serum-mediated proliferative effects of human blood serum on hCSCs were significantly increased by LY2109761. A strong proliferation inducing effect of TGF β 1 on hCSCs was measured. Strikingly, within this study, the application of TGF β RI/II inhibitor significantly increased the proliferation of serum-deprived cultivated hCSCs whereby the effect was higher than in serum-treated cells. Coherently, a strong and significant increase in hCSC proliferation was also observed after serum treatment and inhibition of the TGF β RI/II. As the application of different concentrations of TGF β 1 and simultaneous application of the TGF β RI/II inhibitor LY2109761 increased proliferation, while application of TGF β 1 alone also induced proliferation, a dual role of TGF β -signalling in hCSCs is strongly suggested. Furthermore, human blood serum significantly reduced senescence as measured by SA- β -Gal activity, while supplementation with LY2109761 further increased this beneficial serum effect. Application of TGF β likewise reduced senescence in hCSCs; however, this effect was to a lesser extent than in serum or serum- and LY2109761-treated hCSCs. Our results again indicate a dual role of TGF β 1 signalling regarding senescence in hCSCs and a prevention of serum-induced hyperproliferation in hCSCs via TGF β 1 signalling. In this context it has been previously reported that the loss of TGF β 1 expression resulted in hyperproliferation of skin and skin tumours of mice [49]. Further, a senescence-inducing effect of TGF β has been shown in human diploid fibroblasts, bronchial epithelial cells and cancer cells [25,50–52]. Additionally, an inductive effect of human blood serum on TGF β 1 signalling via SMAD2/3 phosphorylation and the successful reduction of this effect via LY2109761 was demonstrated by analysing phosphorylation of nuclear SMAD2/3 in hCSCs. The observed higher inductive effect of TGF β 1 compared to the effect of the serum may be due to lower amounts of TGF β 1 in the serum or TGF β inhibitors present in the serum.

To extend our understanding of the proliferative effects of TGF β RI/II inhibition, the selective TGF β RI inhibitor SB431542 was applied, and it significantly reduced proliferation in serum-treated hCSCs. This proliferation-reducing effect after selective inhibition of TGF β RI has also been seen in human tenon's fibroblast [53]. Moreover, prevention of SMAD2/3 phosphorylation via TGF β RI inhibition potentially induced the upregulation of the TGF β -non-SMAD pathway, which comprises the activation of Ras and Rho signalling, which have been strongly linked to cell proliferation [11,54–58]. While LY2109761 inhibits the kinase domains of both, TGF β receptor type I and II [41], SB431542 selectively inhibits ALK5 TGF β receptor type I [59]. We therefore conclude that the proliferative effect of LY2109761 could be a result of TGF β receptor type II inhibition. This hypothesis could be strengthened by the fact that TGF β receptor type II is constitutively active by autophosphorylation and this autophosphorylation is blocked by LY2109761 [41]. In turn, we suggest that TGF β RII potentially prevents hyperproliferation by activating pathways or signal cascades negatively regulating proliferation. In this regard, an increased TGF β RII expression has already been shown to directly correlate with a higher CDKN1A/p21 expression which is associated with senescence induction [60,61]. Further, the mutational inactivation of TGF β RII has been discussed to increase G1/S transition kinase activity of CDK4 leading to colon cancer cell proliferation [62]. Additionally, the type II receptor has been shown to act as a G-protein-coupled receptor kinase (GRK), thereby showing that activity of the receptor alone can independently induce other signalling pathways [63]. Besides, previous studies have been demonstrating a direct regulatory function of TGF β RII on non-canonical Rho-associated protein kinase (Rho-ROCK) pathway [64]. Interestingly, likewise to our

observations after LY2109761 treatment of hCSCs, the inhibition of ROCK via Y-27632 has been shown to promote cell viability and proliferation of ESCs, ESC-derived endothelial cells and primary keratinocytes while it also has been shown to reduce senescence [65]. Notably, ROCK inhibition by utilizing Y-27632 has been previously reported to reduce Ras-induced senescence in skin keratinocytes [66]. Within our study, RNA-Seq revealed the upregulation of Ras-inactivating genes after serum and TGF β RI/II inhibition via LY2109761 which strongly indicates the involvement of the ROCK-signalling pathway in this context. Based on these results, we conclude that TGF β RII potentially regulates Ras-mediated prevention of hyperproliferation in hCSCs.

We used RNA-Seq to investigate the effects of TGF β RI/II inhibition in contrast to the proliferation reducing effects of TGF β RI inhibition in hCSCs. Interestingly, in accordance with our observed proliferative effects of TGF β RI/II inhibition upon serum treatment, proliferation related genes were significantly upregulated. These comprise *WEE1* (*WEE1 Checkpoint Kinase*), *CETN2* (*Centrin2*) and *MLF1* (*Myeloid Leukemia Factor 1*). In previous studies we showed that human blood serum induces proliferation and Ras signalling in hCSCs via Gene Ontology (GO) Term Analysis [11]. The Ras signalling pathway is known to be involved in proliferation, differentiation, morphology and apoptosis [67,68]. A direct target of Ras signalling is the induction of the cell cycle via expression of D cyclins which subsequently activate Cdk4 and Cdk6 through phosphorylation allowing them to inactivate the pocket proteins resulting in cell cycle progression from G1 to S phase [57,69–71]. The activation of Ras signalling, therefore, has been shown to lead to proliferation and migration in several cell types, such as mouse embryonic fibroblasts [72]. Ras has also been reported to act as a proto-oncogene with mutations of the Ras genes highly occurring in pancreatic, lung and colorectal carcinomas [67,73,74]. On the other hand, Ras has been shown to play a role in senescence in primary human or rodent cells, resulting in a permanent G1 arrest [75]. Here, differential gene expression analysis revealed a significant upregulation of Ras-inactivating genes such as *RIN1*, and *SYNGAP1* after serum treatment and TGF β RI/II inhibition. *RIN1* (Ras and Rab interactor 1, Ras inhibitor 1) is a known Ras inhibitor while *SYNGAP1* (Synaptic Ras GTPase Activating Protein 1) is known to negatively regulate Ras [76,77]. Best to our knowledge the presence of *SYNGAP1* has not been reported in the context of cardiac regeneration so far. Considering the here observed selective effects of TGF β RI/II inhibition in the context of proliferation and senescence in hCSCs, it is suggested that TGF β RII-mediated regulation of non-SMAD signalling prevents serum-mediated hyperproliferation of hCSCs via TGF β RII-mediated Ras-induced senescence. Further comparison of the differential gene expression of hCSCs treated with either serum or serum with LY2109761 and untreated hCSCs, revealed the upregulation of the previously identified genes *FOS*, *FOSB*, *NR4A* and *EGR* as mediators of hCSC proliferation. In accordance, the *FOS* protein family has been reported to modulate proliferation in several cell types, such as osteosarcoma cells, osteoblasts and human lung cancer cell lines [78–81]. Further, the *NR4A* nuclear receptor family is known to induce endothelial cell proliferation, smooth muscle cells and cancer cells [82–86]. Likewise, the transcription factor *EGR1* has been demonstrated to modulate the proliferation of astrocytes, smooth muscle cells and gastric cancer cells [87–90]. Overall, our results underline the upregulation of these genes in the context of serum-induced proliferation of hCSCs.

In conclusion, we demonstrate proliferation-related responses of hCSCs to serum treatment at a gene expression level which were revealed by Gene Ontology while involvement of the TGF β signalling pathway was indicated. Subsequent inhibition of the TGF β RI/II and simultaneous serum treatment significantly increased proliferation and reduced senescence. Both, human blood serum and TGF β 1, strongly induced the phosphorylation of nuclear SMAD2/3. Altogether, a dual role of TGF β 1 signalling in hCSCs is suggested. Contrary to inhibition of TGF β RI and II, inhibition of TGF β RI resulted in reduced proliferation after serum treatment. RNA-Seq revealed the significant upregulation of proliferation-related and Ras-inactivating genes of hCSCs treated with serum and TGF β RI/II. As observed here, the induction of proliferation is potentially based on the TGF β RII inhibition; we

conclude that TGF β RII regulates (non-canonical) pathways which mediate the prevention of hyperproliferation in hCSCs. In detail, we suggest that the observed proliferation is mediated by repression of Ras signalling. This dual role of TGF β may help to explain the characteristics of molecular regulation in tissues with low turnover such as the heart and lead to new therapeutic approaches in the future.

4. Materials and Methods

4.1. Isolation and Cultivation of Human Cardiac Stem Cells

Adult human cardiac cells (hCSCs) have been isolated and cultivated via explant cultures from human atrial left appendages which have been routinely removed during surgery in accordance with local and international guidelines (declaration of Helsinki). Ethical approval for isolation and experimental procedures was conducted by the ethics commission of the Ruhr-University Bochum (Faculty of Medicine, located in Bad Oeynhausen, with the approval number eP-2016-148). Isolation and cultivation were performed as described previously [11].

4.2. Proliferation Assay

For the proliferation assays, hCSCs were pre-cultivated in TC25 flasks (Sarstedt AG and Co., Nürmbrecht, Germany). After detachment using Trypsin-EDTA (Sigma Aldrich, St Louis, MO, USA), 1×10^3 cells per well were seeded in a 0.1% gelatin-type-B (Sigma Aldrich, Taufkirchen, Germany)-coated 96-TC plate (Sarstedt AG and Co., Nürmbrecht, Germany) in hCSC medium which consisted of DMEM-F12 (Life Technologies, Darmstadt, Germany), 10% foetal calf serum, L-glutamine (2 mmol/L), bFGF (5 ng/mL) (Miltenyi Biotech, Bergisch Gladbach, Germany), EGF (10 ng/mL) (Preprotech, Hamburg, Germany) and Penicillin/Streptomycin (10 mg/mL) (Sigma Aldrich, Taufkirchen, Germany). Triplicates were performed for each condition. After 24 h, the medium was changed to the starvation medium which contained DMEM-F12 (Life Technologies, Darmstadt, Germany), L-glutamine (2 mmol/L), bFGF (5 ng/mL) (Miltenyi Biotech, Bergisch Gladbach, Germany), EGF (10 ng/mL) (Preprotech, Hamburg, Germany) and Penicillin/Streptomycin (10 mg/mL) (Sigma Aldrich, Taufkirchen, Germany) for 72 h. To calculate the IC50 value, 10% pooled blood serum and concentrations from 0 to 50 μ M of selective TGF β RI/II inhibitor LY2109761 (Selleckchem) or 0 to 10 μ M TGF β RI inhibitor SB431542 (abcam, Cambridge, UK) dissolved in DMSO (Sigma Aldrich, Taufkirchen, Germany) was applied. The amounts of DMSO in each well were equal to the maximum concentration of DMSO, respectively. For determination of the role of TGF β RI/II in the serum-mediated proliferative effects on hCSCs human blood serum, 50 μ M LY2109761 was applied. After 72 h at 37 °C under hypoxic conditions with 5% CO₂ and 5% O₂ at 37 °C, 10 μ L Orangu Cell Counting Solution (Cell Guidance Systems, Cambridge, UK) was added to each well for 2 h at 37 °C. Absorbance was measured at 450 nm and the cell count was calculated by the formula derived from a previously determined calibration straight line. For calibration, hCSCs from the same suspension were seeded in a 0.1% gelatin-type-B-coated TC-96 plate with 0, 250, 500, 750, 1000, 1500, 2000, 2500 and 3000 cells per well. After allowing the cells to attach to the surface for 1.5 h, 10 μ L Orangu Cell Counting Solution (Cell Guidance Systems, Cambridge, UK) was added for 2 h and the absorbance was measured at 450 nm. Clinically accredited therapeutic human blood plasma samples were collected from six healthy young (age < 20 years) male donors via a routine blood donation service (Institute for Laboratory- and Transfusionmedicine, Bad Oenhausen, Germany). Serum was isolated as described previously [11]. Levels of TGF β 1 were quantified using an ELISA Kit (88-8350-22, Invitrogen, Thermo Fisher, Waltham, MA, USA) according to the manufacturer's guidelines. Values within the linear range of the serum dilution series were used for calculation of the TGF β 1 amount.

4.3. Senescence-Associated Quantitative β -Galactosidase Assay

A total of 1×10^5 human cardiac stem cells were seeded in 0.1% gelatin-type-B-coated TC100 dishes. Cultivation and treatment were equal to the conditions described for the proliferation assay. Triplicates were performed. After washing six times with PBS, 300 μ L $1 \times$ lysis buffer containing 5 mM CHAPS, 40 mM citric acid, 40 mM sodium phosphate, 0.5 mM benzamidine and 0.25 mM PMSF was added. The cells were detached by scraping and transferred to a 1.5 mL reaction tube and froze at -80°C . Subsequent to thawing, the samples were centrifuged at $12,000 \times g$ for 5 min and 100 μ L of the lysate was mixed with 100 μ L $2 \times$ reaction buffer containing 40 mM citric acid, 40 mM sodium phosphate, 300 mM NaCl, 300 mM β -mercaptoethanol, 4 mM MgCl_2 and 1.7 mM MUG. After incubation at 37°C for one hour, 50 μ L of the reaction mix was added to 150 μ L 400 mM sodium carbonate stop solution. Then, 150 μ L was transferred to a black 96-well plate and quantification was performed via a TECAN reader with excitation 360 nm, emission 465 nm and integration 40 μ s. The senescence-associated β -galactosidase activity was normalized to the amount of the total isolated protein.

4.4. RNA Sequencing and Analysis

In total, 5×10^5 hCSCs from three male donors were seeded in 0.1% gelatin-type-B (Sigma Aldrich, Taufkirchen, Germany)-coated TC100 dishes (Sarstedt AG and Co., Nürmbrecht, Germany) in hCSC medium (described previously) and cultivated for 24 h. Subsequently, the medium was changed to the starvation medium (described previously) for a further 24 h. An amount of 50 μ M of LY2109761 or the equivalent amount of DMSO was then applied for one hour for pre-treatment, followed by one-hour treatment with either 10% pooled human blood serum from six young males or 10% pooled human blood serum from six young males and 50 μ M LY2109761. RNA was isolated using the NucleoSpin RNA Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's guidelines. For determination of the amount of isolated RNA, a nanodrop (Peqlab, Erlangen, Germany) was used. The samples were stored at -80°C . mRNA library preparation and sequencing were conducted by Novogene (Cambridge, UK) with NovaSeq 6000 30 M reads PE150. Using HISAT2 (version 2.1.0-4), the reads were aligned to the reference genome GRCh38.p14. Feature Counts (version 2.0.0) was used to quantify the read number after mapping. For differential gene expression analysis, the DESeq2 R package was used. Gene Ontology (GO) term enrichment and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed by using the gage package in R (version 1.3.1093). For KEGG analysis, the Pathview R package and for GO-Term analysis, the org.Hs.eg.db R package provided databases.

4.5. Immunocytochemical Staining

For immunocytochemical staining, cells were cultivated in 8-well slides (ibidi, cells in focus, Gräfelfing, Germany) as described for the RNA sequencing analysis. The medium was discarded, and the cells were washed one time with PBS followed by fixation at room temperature with 4% PFA for 15 min. After washing three times with PBS, the cells were permeabilized and blocked with 0.02% PBT containing 5% goat serum for 30 min. Subsequently, cells were washed one time with PBS. The primary antibody phosphoSMAD2/3 (Carlsbad, CA, USA) was applied overnight at 4°C , diluted 1:300, in blocking solution. The second antibody goat anti rabbit alexa 555 (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) was diluted 1:300 in blocking solution and applied for one hour in the dark at room temperature. After washing three times with PBS, nuclei were stained via 49,6-diamino-2-phenylindole (DAPI, 1 μ g/mL) (Sigma-Aldrich, Taufkirchen, Germany) for 15 min, followed by two washing steps with PBS and one time with H_2O . Mowiol-4-88 was mounted to cover the cells. Fluorescence imaging was performed by confocal laser scanning microscopy (LSM780; Carl Zeiss; Jena, Germany). For analysis, ZEN software (version 2011 SP7) and ImageJ (<https://imagej.net/ij/>, accessed on 8 January 2024) were used.

5. Patents

Parts of this research article were filed for a patent application entitled “Method and medium for cultivating animal cells”.

Author Contributions: Conceptualization, K.E.S., C.K. (Christian Kaltschmidt) and C.K. (Cornelius Knabbe); methodology, K.E.S. and K.T.; software, S.K.Z.; validation, K.E.S., A.L.H., B.K., C.K. (Christian Kaltschmidt) and C.K. (Cornelius Knabbe); formal analysis, K.E.S., K.T. and S.K.Z.; investigation, K.E.S., K.T. and S.K.Z.; resources, C.K. (Christian Kaltschmidt) and C.K. (Cornelius Knabbe); data curation, K.E.S., A.L.H., B.K., C.K. (Christian Kaltschmidt) and C.K. (Cornelius Knabbe); writing—original draft preparation, K.E.S.; writing—review and editing, K.E.S., A.L.H., B.K., C.K. (Christian Kaltschmidt) and C.K. (Cornelius Knabbe); visualization, K.E.S., K.T. and S.K.Z.; supervision, A.L.H., B.K., C.K. (Christian Kaltschmidt) and C.K. (Cornelius Knabbe); project administration, C.K. (Christian Kaltschmidt) and C.K. (Cornelius Knabbe); funding acquisition, C.K. (Christian Kaltschmidt) and C.K. (Cornelius Knabbe). All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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