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Original

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(Article begins on next page)

Strain-sensitive fibrotic programming of human cardiac stromal cells can be reverted by interfering with YAP-dependent transcriptional activation

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28 **Abstract**

29 **Background** - Conversion of cardiac stromal cells into myofibroblasts is typically associated with hypoxia
30 conditions, metabolic insults, and/or inflammation, all of which are predisposing factors to cardiac fibrosis and
31 heart failure. We hypothesized that this conversion could be also mediated by response of these cells to
32 mechanical cues through activation of the Hippo transcriptional pathway. The objective of the present study
33 was to assess the role of cellular/nuclear straining forces acting in myofibroblast differentiation of cardiac
34 stromal cells under the control of YAP transcription factor and to validate this finding using a pharmacological
35 agent that interferes with the interactions of the YAP/TAZ complex with their cognate transcription factors
36 TEADs, under high-strain and pro-fibrotic stimulation.

37 **Methods** - We employed high content imaging, 2D/3D culture, atomic force microscopy mapping and
38 molecular methods to prove the role of cell/nuclear straining in YAP-dependent fibrotic programming in a
39 mouse model of ischemia-dependent cardiac fibrosis and in human-derived primitive cardiac stromal cells. We
40 also tested treatment of cells with Verteporfin, a drug known to prevent the association of the YAP/TAZ
41 complex with their cognate transcription factors TEADs.

42 **Results** – our experiments suggested that pharmacologically targeting the YAP-dependent pathway overrides
43 the pro-fibrotic activation of cardiac stromal cells by mechanical cues *in vitro*, and that this occurs even in the
44 presence of pro-fibrotic signaling mediated by TGF- β 1. *In vivo* administration of Verteporfin in mice with
45 permanent cardiac ischemia reduced significantly fibrosis and morphometric remodeling but did not improve
46 cardiac performance.

47 **Conclusions** - Our study indicates that preventing molecular translation of mechanical cues in cardiac stromal
48 cells reduces the impact of cardiac maladaptive remodeling with a positive effect on fibrosis.

49 ***Non-standard abbreviations and acronyms***

50 YAP: Yes-associated protein
51 TAZ: Transcriptional co-activator with PDZ-binding motif
52 TEAD: TEA domain transcription factor
53 TGF- β 1: Transforming growth factor beta-1
54 ECM: Extracellular matrix
55 MI: Myocardial infarction
56 LV: Left ventricle
57 VTP: Verteporfin
58 CF: cardiac fibroblast
59 CM: Cardiomyocyte

Introduction

Maladaptive remodeling of the myocardium is one of the earliest hallmarks of heart failure (HF). This is characterized by inflammation and a progressive fibrosis that leads to replacement of the parenchyma with a stiff, fibrotic, tissue¹. In the adult healthy myocardium, the turnover of the extracellular matrix (ECM) is controlled by stromal interstitial cells, which can be mapped into several different phenotypes based on transcriptional and functional features²⁻⁴. Under conditions causing ventricular mechanical overload (e.g. hypertension), in the presence of metabolic alterations such as hyperglycemia, injury such as hypoxia, or just depending on the aging process, resident un-activated stromal cells evolve into pro-fibrotic cells, the so called myofibroblasts (Myo-Fbs) that contribute to inflammation, altered ECM accumulation, and myocardial stiffening⁵⁻¹⁰.

The term mechanosensation refers to the ability of the cells to sense the physical characteristics of the surrounding environment through the activation of intracellular signaling cascades elicited by mechanical cues¹¹. The Hippo signaling pathway, an essential component of the machinery translating cell mechanical responses into discrete transcriptional activation is well contextualized in cancer biology as a *primus movens* in cancer stem cells determination, metastatic activity, drug resistance and cell plasticity¹². A direct connection between static/dynamic mechanical cues and transcriptional activation of downstream targets has been established with the finding that cytoskeletal tensioning resulting from cellular adhesion to extracellular components with specific geometric arrangement and viscoelastic properties¹³ translates into reversible nuclear shuttling of the main Hippo transcriptional component, the YAP/TAZ complex¹⁴⁻¹⁶. In addition to promoting shuttling from the cytoplasm to the nucleus of the YAP/TAZ complex by inhibiting the Hippo kinase pathway, the acto-myosin cytoskeleton also directly forces the YAP/TAZ into the nucleus by physically deforming the nuclear lamina and opening the nuclear pores¹⁷. The relevance of the Hippo signaling for cardiac biology has emerged from studies highlighting the role of YAP in neonatal myocytes proliferation and cardiac regeneration¹⁸⁻²². Moreover, specific deletion of upstream components of the Hippo pathway Lats1/2 in cardiac fibroblasts determines a permanent activation into Myo-Fbs^{23, 24}, strongly suggesting implication of the YAP/TAZ complex in homeostatic control of cardiac matrix, and a specific function in myocardial remodeling after injury. In keeping with these evidences, elevated levels of nuclear YAP have been found in infarcted hearts in mice²⁵, and blockade of YAP/TAZ complex activity with Verteporfin (VTP), a drug that interferes with the binding of the complex to TEADs DNA binding proteins, attenuates injury-dependent cardiac fibrosis^{26, 27}. The link between the abnormal distribution of the straining forces in the infarcted heart and activation of the YAP-dependent transcriptional activation is, however, not clear. It is also not clear whether human myocardial stromal cells respond directly to strain forces with activation of YAP-dependent pro-fibrotic signaling. In the present study, we provide evidences that the straining forces acting upon remodeling of the myocardial matrix after infarction activate the Hippo transcriptional pathways through a mechanism determining nuclear straining and preferential cellular polarization in the infarct scar. We also show that cardiac stromal cells (cSt-Cs) activate a pro-fibrotic pathway in response to sensing of tissue compliance and geometry, thus linking YAP nuclear translocation to the force-dependent matrix remodeling process occurring

97 during the cardiac healing after infarction. This process can be reverted by treatment with VTP even under
98 high-strain conditions and in the presence of TGF- β 1, a key paracrine stimulator of cardiac fibrosis²⁸. These
99 findings were obtained in mice carrying permanent ligation of the left anterior descending coronary, in which
100 treatment with VTP reduced fibrosis at short- and long-term after ischemia.

101 **Methods**

102 **Ethics**

103 Experimental investigations involving human-derived cells and tissues were approved by the local ethical
104 committee at Centro Cardiologico Monzino, IRCCS (approval date: 19 May 2012 and subsequent renewal on
105 16 May 2016) and Policlinico San Donato, IRCCS (protocol 2438, 27/01/2009 and CE 85/int/2016 9/6/2016).
106 Collection of material and experiments were performed in compliance with the Declaration of Helsinki.
107 Patients were required to sign an informed consent to donate small fragments of cardiac tissue (right atrial
108 appendage), during coronary bypass grafting or aortic valve substitution interventions. Experiments in mice
109 were conducted in keeping with the guidelines from Directive 2010/63/EU of the European Parliament on the
110 protection of animals used for scientific purposes, and in accordance with experimental protocols approved by
111 the University Committee on Animal Resources at the University of Milan (668-2015). The protocol of VTP
112 *in vivo* administration in mice with permanent MI was approved by the Italian Ministry of Health
113 (Authorization number 861-2021) and were executed in an authorized facility.

114 **Mouse model of myocardial infarction (MI)**

115 A mouse model of permanent ligation of the left anterior descending coronary artery (LAD) was employed to
116 create a myocardial infarction. Mice were sacrificed at short (3 days), intermediate (7 days) and long (4weeks)
117 follow-up times, after which hearts were prepared for histological analysis. The protocol of *in vivo* treatment
118 of mice with VTP was performed by administering VTP dissolved in corn oil at a concentration of 50 mg/kg
119 every third day. Monitoring of the treatment effect was performed by echocardiography at specific steps during
120 the observation period. At the end of the treatment, the hearts were harvested and processed for histology. For
121 further information on animal procedures see the extended methods available in the supplementary material.

122 **Microfabrication methods**

123 For the manufacturing of the controlled stiffness gels with polyacrylamide (PAA), we used a protocol that was
124 previously employed by us²⁹. For investigating the traction forces generated by single cells, the micropillar
125 method was adopted^{30, 31}. The manufacturing of the micropatterned substrate of poly(dimethylsiloxane)
126 (PDMS, Sylgard 184, Dow Corning, United States), a dedicated silicon mold with a negative pattern of the
127 micropillar arrays was designed using the software Solidworks (Dassault Systemes, Vélizy-Villacoublay,
128 France). Further details about design and fabrication of the microfabricated structures are provided in the
129 extended methods available in the supplementary material.

130 **Primary cell culture, human tissue harvesting and main immunofluorescence methods.**

Primary human cardiac stromal cells were derived from “cardiospheres” as previously described^{32, 33}. Briefly, cellular outgrowths of small myocardial explants were sub-cultured to obtain cardiospheres and cardiospheres-derived primitive cSt-Cs. Cardiospheres and cSt-Cs were used for immunofluorescence staining and high content image analysis using CARE, an algorithm originally developed by us to analyze microscopic images³⁴. Controls of immunofluorescence/histochemistry included samples stained with pre-immune antibodies/sera and also secondary antibody-only stained samples to determine the background level. For exhaustive information on cell culture methods and cell analyses see the extended methods. For the observations on human myocardium, left ventricle cardiac biopsies were harvested from patients affected by dilated hypokinetic ischemic cardiomyopathy undergoing surgical ventricular reconstruction procedure. Formalin-fixed paraffin-embedded consecutive serial sections (1 μ m thickness) of the border zone samples (peri-infarct area) were dewaxed and hydrated through graded decrease alcohol series and stained for histological analysis. Hematoxylin/Eosin staining (Mayer’s Hematoxylin/Eosin; Bio Optica, Milan, Italy) and Masson Trichrome staining (Masson trichrome with aniline blue Bio-Optica) were carried out using standard protocols. The microscopic images contained in the figures are those that best represent the observed phenotypes.

Atomic force microscopy

Force spectroscopy measurements were performed with a commercial AFM mounted on an inverted microscope using a borosilicate glass microsphere of about 18 μ m in diameter manually glued at the end of the cantilever. Cell rigidity was evaluated in 30 randomly chosen cells for each condition, acquiring three force spectroscopy curves in the center of each cell nuclei. Further details are provided in the online supplementary material.

Molecular methods

For Western blotting analyses, cells were lysed in standard sample buffer and run (30 μ g) into 4-12% gradient SDS polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes followed by incubation with the indicated primary and secondary antibodies (see Major Resource Table). For RNA-sequencing analysis, total RNA was extracted from 6 independent cSt-Cs cultured \pm TGF- β \pm VTP using TRIzol. After quality checking and quantification, the Poly(A)⁺ RNA was enriched and then processed for RNAseq. Differential gene expression analysis was performed using the R software. Validation was performed in independent RNA samples of cSt-Cs by RT-qPCR. Indications about the primers sequences and reagents are provided in the Major Resource Table. The effects of TGF- β 1 \pm VTP on cSt-Cs contraction were determined using a cell contraction assay kit (Cell Biolabs), as per the Manufacturer's instructions. Collagen plug areas were measured using ImageJ software. Soluble collagen release was quantified using Sircol colorimetric assay (Biocolor) on conditioned medium of cSt-Cs. A full description of the methods employed in molecular analyses is provided in the online supplementary material.

Statistical analyses

Data were graphed and analyzed using the Prism Graph Pad statistical software. Normality tests on normal/log-normal distributions were run using the Shapiro-Wilk methods fixing $\alpha = 0.01$. The number of replicates, the

167 *P* values, and the type of tests that were used for statistical comparisons are specified by the dots/numbers over
168 the histograms in the figures and the figure legends, respectively.

169 Results

170 Orientation- and cell strain-dependent activation of YAP in the infarct scar

171 To assess the activation of the YAP-dependent transcriptional pathway in the context of the late myocardial
172 remodeling occurring after ischemia, we performed immunohistochemistry on hearts of mice with permanent
173 ligation of the left coronary artery at early (3 days) and late (4 weeks) follow up³⁵. **Figure 1A** shows the results
174 of YAP immunolocalization in transversal sections of the infarcted heart at three days post-MI. Cells with the
175 morphology of monocytes/macrophages invading the infarct area exhibited, in line with the literature³⁶, high
176 levels of the protein in the nucleus. Cells showing nuclear-localized YAP included also putative myofibroblasts
177 and cardiomyocytes (CMs). In the remote zone, a reduced number of cells were positive for YAP
178 (independently of nuclear/cytoplasmic localization), suggesting an ischemia- and or inflammatory-dependent
179 activation of the pathway in the infarcted region. At 4 weeks after infarction, infarct scars with abundant
180 collagen deposition were clearly visible (**Figures 1B**). **In this fibrotic tissue, collagen bundles and cells with**
181 **Myo-Fbs morphological characteristics were clearly recognizable with a preferential**
182 **equatorial/circumferential orientation (see insets in the figure). Analysis of YAP expression in the fibrotic**
183 **tissue by immunohistochemistry revealed cells with Fibroblasts (Fbs) morphology bearing high levels of YAP**
184 **nuclear localization (YAP⁺) or negative for expression of the transcription factor (YAP⁻) (Figure 1C).**
185 Examining the IHC images at high magnification, it came to our attention that YAP⁺ cells were not evenly
186 distributed in the collagen-rich matrix, but exhibited a preferential nuclear orientation along the equatorial
187 cutting plane of the left ventricle (LV), suggesting a relationship between nuclear translocation of the
188 transcription factor and strain forces transmitted within the circumferentially arranged collagen fibers (**Figure**
189 **1B**)³⁷. **To quantify this finding we determined the nuclei orientation and deformation in these cells using**
190 **CARE, an algorithm that we previously tailored to perform automatic segmentation of microscope images³⁴**
191 **(Figures 1C, D, S2).** We then correlated these values to the presence of nuclear YAP. **Figure 1E** contains the
192 results of this analysis, showing a significantly higher nuclear deformation and a more frequent equatorial
193 alignment of YAP⁺ compared to YAP⁻ cells. This suggests that strain forces, known to direct anisotropic
194 collagen deposition in the forming scar³⁷, are also involved in the activity of the YAP/TAZ transactivation
195 pathway connected to nuclear deformation and equatorial alignment of **cells with CFs morphological**
196 **characteristics in the fibrotic areas¹⁷.** These results were validated in myocardial tissue of patients with severe
197 heart failure undergoing surgical ventriculoplasty. As shown in **Figure 1F**, the interstitial fibrosis, well evident
198 in the tissue, was associated with presence of cells with YAP staining in the nucleus, suggesting activity of the
199 pathway in cells of the fibrotic scar.

200 YAP nuclear translocation is mechanically regulated by cytoskeleton tensioning in human cardiac 201 stromal cells and is correlated to **expression** of fibrotic markers

202 To prove that YAP nuclear translocation is mechanically connected to cytoskeleton tensioning in cells with
203 the ability to differentiate into Myo-Fbs, we derived cardiac stromal cells from human primary explant cultures
204 from human right atrial fragments³², selected through spontaneous spheroid growth (the so-called

205 ‘cardiosphere’)³⁸ for a primitive mesenchymal phenotype. In line with the existing literature³⁹, cardiosphere-
 206 derived cells exhibited a variety of mesenchymal markers including CD29, CD44, CD90 and CD105, but not
 207 endothelial markers CD31 and CD144 (**Figure S2**). We first employed 2D poly-acrylamide gels (PAGs) with
 208 a discrete stiffness in the range of 17 - 58 kPa (Young’s elastic modulus, detected by atomic force microscope
 209 - AFM)²⁹ and, as a reference, glass surfaces to promote cell attachment onto Fibronectin. Results of
 210 cytoskeleton staining by phalloidin-TRITC, revealed a decrease in stress fibers polymerization and consequent
 211 changes in cellular forms factors (area and circularity) onto softer gels (**Figure 2A**). To unravel the role of
 212 environmental mechanics on YAP-dependent nuclear signaling^{15, 16}, we performed YAP staining of the cells
 213 plated onto PAGs followed by quantitative evaluation of the cells with nuclear-localized YAP (nYAP⁺ cells)
 214 and of the cytoplasmic/nuclear YAP signal ratio (**Figure 2B, S3**). As expected^{16, 40}, results showed a stiffness-
 215 dependent trend of YAP to be confined in the nucleus, and this was also directly connected to cell proliferation,
 216 as detected by labeling cells with Ki-67 antibody (**Figure 2C**).
 217 Since the changes in proliferation and YAP nuclear/cytoplasm ratio observed in cells plated onto PAGs with
 218 controlled stiffness could be due to variations in cytoskeleton polymerization/tensioning¹⁷ we explored the
 219 possibility that cSt-Cs in a 3D structure could sense geometric or positional information translating into
 220 discrete YAP nuclear translocation and activation of pro-fibrotic signaling. To validate this hypothesis, we
 221 analyzed the distribution of YAP localization inside the cardiospheres. These structures have been in fact
 222 described as 3D models of cardiac ‘niches’ with undifferentiated cells located in the ‘core’ of the sphere and
 223 more differentiated cells with mesenchymal characteristics located in the external shell^{32, 41, 42}. **Figures 3A-B**
 224 show, respectively, the structure of the cardiospheres and the localization of YAP protein and Ki67
 225 proliferation marker in the 3D structures. By employing CARE³⁴ to analyze these structures in high resolution
 226 images serially acquired along the vertical axis of the spheroid cell clusters (z-stack), we were able to clearly
 227 distinguish in the 3D volume an external ‘shell’ of the cardiospheres, defined as the outer volume
 228 encompassing one-third of the radius of the spheroids, where cells with intra-nuclear YAP and Ki67
 229 localization were preferentially localized, and an inner ‘core’ where YAP was mainly cytoplasmic and Ki67
 230 signal was absent (**Figures 3A, S4**). As a first control in this experiment, we used immunostaining with
 231 antibodies specific for GATA-4, a cardiac-specific transcription factor that is not known to be mechanically
 232 regulated and, indeed, showed no preferential nuclear localization neither in the shell nor in the core of the
 233 cardiospheres (**Figure 3C, S4**). Second, given that the asymmetric distribution may simply reflect from
 234 possible metabolic stress of the cells in the core, due to limited distribution of nutrients and oxygen, we cultured
 235 cSt-Cs in hypoxia, low glucose and low serum conditions, and various combinations thereof. As shown in
 236 **Figure S5**, none of these conditions affected the YAP nuclear distribution in the cells, suggesting that
 237 asymmetric YAP nuclear localization results from a differential response of the cells to compression forces
 238 resulting from topological cues. Finally, since YAP nuclear translocation is directly connected to cell
 239 mechanics-controlled proliferation and pro-fibrotic activation⁴³, we assessed the distribution of Ki67 and
 240 markers typically expressed in pro-fibrotic cells in the heart (Collagen I and α SMA) in the cardiospheres.
 241 Results showed an overlapping between the regions of the cardiospheres where nuclear YAP, Ki67 and fibrotic

242 markers were more frequently detected (**Figures 3B, D, E**), suggesting a cause relationship between
 243 topological activation of YAP signaling and differentiation of cSt-Cs in pro-fibrotic cells.

244 To establish a direct relationship between YAP localization and transmission of forces from the cytoskeleton
 245 to the nucleus, we investigated the nuclear ellipticity of cSt-Cs plated on 2D gels with differential stiffness
 246 and, in parallel, in the 3D cardiospheres volume. As shown in **Figures 4A, B**, there were significantly more
 247 round nuclear shapes in cells plated onto gels with low stiffness and in the core of the cardiospheres compared
 248 to gels with higher stiffness and the outer cardiospheres layer. We then directly interfered with the
 249 polymerization of stress fibers by treating cSt-Cs plated onto glass (stiffness in the MPa range) with
 250 Blebbistatin (BB) and Y27632^{44, 45}. Although these two compounds inhibit cytoskeleton tensioning with
 251 different mechanisms, both determined a completely reversible effects on stress fibers generation and nuclear
 252 YAP localization (**Figure 4 C, D**). To assess whether nuclear straining by cytoskeleton tensioning is directly
 253 associated with modifications in nuclear geometry and mechanics we used confocal imaging and AFM. Results
 254 of these analyses showed that both inhibitors reversibly increased the dimension along the *z*-axis of the nuclei
 255 of cSt-Cs, and determined a relaxation of their chromatin as detected by a decrease in nuclear stiffness (**Figure**
 256 **4E**). Finally, to contextualize the results in the framework of the YAP-dependent transactivation, we analyzed
 257 the expression of three YAP canonical targets (*CTGF*, *CYR61*, *ANKRD1*) in cells treated with BB or Y27632
 258 using RT-qPCR (**Figure 4F**). This experiment showed a completely reversible reduction in the expression
 259 level of the three genes *vs.* controls. This establishes a connection between the expression of *pro*-fibrotic genes
 260 and strain-dependent YAP transcriptional signaling in human cSt-Cs.

261 **Override of the pro-fibrotic mechanical programming of human cSt-Cs by Verteporfin**

262 Results showing the reversible changes in nuclear shape and relaxation in cells treated with Actin cytoskeleton
 263 inhibitors indicated a possible way to interfere with YAP signaling and, thus, reverse the cSt-Cs pro-fibrotic
 264 activation controlled by cell and nuclear mechanics. On the other hand, these inhibitors are quite unspecific
 265 and display an elevated cytotoxicity *in vivo*, thereby calling for more specific pharmacological inhibitors of
 266 the YAP nuclear functions. A more specific inhibitor was found in the Verteporfin (VTP) molecule, a drug
 267 with anti-fibrotic properties^{46, 47}, recently found to inhibit cardiac remodeling in mice^{26, 48}.

268 In the first series of experiments, cSt-Cs were treated with VTP under the maximal strain condition (culture
 269 onto glass) and then assayed for the expression of YAP target genes and genes involved in cardiac fibrosis²⁶.
 270 To exclude that VTP has toxic effects on cells, we performed live/dead staining of cells treated with increasing
 271 amounts (1 – 10 μ M) of the drug for 5 hours, followed by a 48 hours observation period (**Figure S6**). Having
 272 excluded toxic effect of VTP, we then analyzed its biological effects. **Figure 5A** shows that a 5hrs treatment
 273 with the drug did not modify the cellular shape, and did not affect YAP nuclear localization. By contrast, RT-
 274 qPCR experiments clearly showed a reduction in the expression of *CTGF*, *CYR61* and *ANKRD1*, as well as of
 275 *Coll1A1*, *CollA3* and *Thy-1*, which are typical markers of cells with a fibrotic phenotype⁴⁹.

276 We then analyzed the effects of a more chronical treatment with VTP (3 days) in the context of the signaling
 277 induced by TGF- β 1, one of the most potent inducers of cardiac fibrosis⁵⁰. In these experiments, we co-treated
 278 cSt-Cs with TGF- β 1 (\pm VTP) and assessed the expression of fibrotic genes after 72 hours using an RNA

279 sequencing approach. Following data processing and raw count filtering criteria, we identified 17781
 280 expressed genes, which included 13112 protein coding genes (74%), 2823 pseudogenes (16%), 1770 long non-
 281 coding genes (10%), and a small fraction of small non-coding genes (<<1%) (Gene ID details in
 282 **supplementary bioinformatics data – a**). Paired-sample analysis adjusted for confounding “latent” variables
 283 allowed reducing the effects of heterogeneity among subjects, thus unveiling specific changes between each
 284 treatment *vs.* CTRL. We report the overall results and statistics for each statistical model in the supplementary
 285 material, and summarize the main findings in **Figure S7A, B** with a representation of the differentially
 286 expressed genes in the comparisons of the treated cells *vs.* control cells, or in the VTP+TGF- β 1 *vs.* TGF- β 1
 287 condition. In brief, we found a substantial number of differentially expressed (DE) genes by comparing the
 288 mRNAs of VTP-treated cells (\pm TGF- β 1) *vs.* CTRL and to a lesser extend also for the treatment with TGF β
 289 alone. On the other hand, 103 out of the 196 genes up-regulated by TGF β were down-regulated by co-treatment
 290 with VTP, indicating that VTP has a down-regulatory effect on genes induced by the pro-fibrotic cytokine
 291 (**Figure S8A**). This evidence was supported by the results of unsupervised hierarchical clustering, which
 292 allowed us to distinguish between the diverse treatment conditions and, in particular, indicated a clear
 293 distinction in the transcriptional signature of cells treated with VTP (\pm TGF- β 1) and those that were cultured
 294 without the drug (**Figure 5B**). We validated the transcriptional signature by performing single RT-qPCR
 295 assays with specific primers for *CTGF*, a pro-fibrotic factor expressed under the direct control of
 296 YAP/TAZ/TEAD complex⁵¹, for the two collagen-encoding genes *ColA1*, *ColA3*, and for *Thy-1* and *ACTA2*,
 297 the genes encoding for CD90 and α SMA, two markers of cardiac Myo-Fbs^{49, 52}. In all cases, treatment of the
 298 cells with VTP reverted the upregulation of these genes even below the level exhibited in control cells (**Figure**
 299 **5B**), again confirming a potent transcriptional inhibitory effect of VTP in human cSt-Cs. Since interference
 300 with YAP signaling may also result in an imbalance in the expression of the Hippo pathway, we investigated
 301 the relative expression of genes with a functional annotation in the pathway. As shown in **Figure 5C**, a number
 302 of DE genes were up- or downregulated in VTP-treated (\pm TGF- β 1) *vs.* untreated cells (\pm TGF- β 1). Among the
 303 upregulated genes, we noticed the expression of *DVL2*, that in analogy to DVL, is involved in balancing the
 304 YAP cytosol/nuclear ratio^{53, 54} and that of *AMOTL1*, a member of the Angiomotin proteins family, which has
 305 a specific inhibitory function of YAP activity by promoting phosphorylation via LATS proteins⁵⁵. It was also
 306 remarkable that VTP treatment determined downregulation of the transcripts of *LATS1/2*, the transcripts
 307 encoding for the kinases of the Hippo pathway directly promoting YAP phosphorylation and functional
 308 activity⁵⁶, of *TJP1*, encoding for a tight junction protein positively regulating the TAZ/TEAD function⁵⁷ and
 309 of *YAP* mRNAs itself. Together, these results suggest that treatment with VTP determines an imbalance in
 310 numerous checkpoints of the finely regulated mechanism tuning the activity of the YAP/TAZ transcriptional
 311 function. In order to substantiate the action of VTP on cSt-Cs with the attribution of specific biological
 312 functions, we performed a Gene Ontology (GO) enrichment analysis of the DE genes in the various treatments.
 313 As expected, this showed a majority of pathways that were downregulated with high significance in VTP-
 314 treated cells (\pm TGF- β 1) connected with extracellular matrix organization, cell migration, inflammatory
 315 responses and cytokines production (**Figure S9** and complete description of the pathways in **supplementary**

316 **bioinformatics data – b**), consistent with a reduction of the inflammatory and matrix remodeling activity of
317 cells treated with VTP. Finally, in order to contextualize the inhibitory effect of VTP in the framework of the
318 TGF- β -dependent transcriptional signaling, we performed *cis*-regulatory sequence analysis on down-regulated
319 genes by VTP, and identified binding motifs and tracks associated with TEAD 1/3/4 and SMAD4 transcription
320 factors among the most significant. As shown in **Figure S9** (see also **supplementary bioinformatics data –**
321 **c**), the analysis identified groups of genes that are likely co-downregulated by VTP, due to connection of the
322 YAP/TAZ complex with transcriptional mediators downstream of TGF- β 1 (SMADs), through cooperation of
323 TEADs.

324 **VTP inhibits proliferation and reduces matrix remodeling activity of human cSt-Cs.**

325 Since conversion of cardiac stromal cells into Myo-Fb is accompanied by transitioning into a rapid
326 proliferation phase under the control of TGF- β 1 signaling⁵⁰, or even mechanical cues regulated by YAP^{15, 43},
327 we determined the percentage of cells exhibiting PCNA staining in cSt-Cs cultured with or without TGF-
328 β 1±VTP onto glass slides. Results evidenced a sharp increase in the percentage of proliferating cells in the
329 presence of TGF- β 1 (**Figure 6A**), blunted by VTP treatment. In a previous study, we established a method to
330 assess cell strain-dependent Myo-Fb differentiation onto substrates with discrete stiffness, by determining the
331 loading of α SMA onto the F-actin cytoskeleton as a measure of the ability of the cells to evolve toward a
332 contractile phenotype²⁹. Adopting this system, we found a clear effect of TGF- β 1 treatment in increasing the
333 number of the fluorescence peaks showing co-localization of F-Actin and α SMA onto stress fibers, using
334 confocal imaging (**Figure 6B**), thus confirming promotion of a Myo-Fb phenotype. On the other hand, cells
335 treated with VTP reduced, at least in part, the loading of α SMA onto the actin cytoskeleton, suggesting
336 reversion of the Myo-Fb phenotype due to TGF- β 1 treatment. To further validate the effect of VTP in reversing
337 the Myo-Fb activation of cSt-Cs, we assessed the expression of α SMA using fluorescence quantification on
338 confocal images and Western blotting, and by measuring the collagen secretion in the culture medium (**Figure**
339 **6C**). In line with the previous results, VTP reduced the expression of the Myo-Fb marker and the secretion of
340 collagen even below the level of control cells. Finally, since the ability to remodel the extracellular matrix in
341 the context of myocardial infarction is a specific activity of Myo-Fbs, we measured the matrix compacting
342 ability of the cSt-Cs treated with TGF- β 1, and the reversal of this activity by VTP, using a collagen compaction
343 *in vitro* assay. Results (**Figure 6D**) showed a clear contraction of the 3D gel containing cSt-Cs treated with
344 TGF- β 1, as opposed to control cells, and cells treated with TGF- β 1+VTP. Interestingly, cell force analysis by
345 measuring the bending of PDMS micropillars did not show differences between the average forces developed
346 by the cells treated under the different conditions (**Figure 6E**). Together, these results show that interfering
347 with YAP-dependent transcriptional activity blocks differentiation of cSt-Cs into myofibroblasts induced by
348 TGF- β 1 and limits their matrix remodeling and compacting ability, without affecting the physiological
349 intracellular transmission of mechanical forces.

350 **Anti-fibrotic activity of VTP in a mouse model of ischemic cardiac fibrosis**

351 To verify the possible anti-fibrotic activity of VTP *in vivo*, we set a mouse model of ischemic cardiac
352 remodeling in mice. The treatment protocol of mice was in line with existing literature on the anti-fibrotic

353 effects of VTP in other organs, such as kidney⁵⁸. Two time-points were chosen to assess the effects of the
354 continuous drug administration in relationships with the known biphasic myocardial remodeling process
355 characterized by a precocious inflammatory phase (7 days), followed by an anti-inflammatory phase
356 characterized by scar formation and collagen deposition (day 28). The experiment was controlled by evaluating
357 the cardiac function by echocardiography and, finally by histological examination of the explanted heart at 7
358 and 28 days. The results of this experiment are summarized in **Figure 7**, where it is clearly shown that
359 administration of VTP reduced the extent of the fibrotic areas at either 7 or 28 days post MI (Figure 7A-D). It
360 was interesting to note that the fibrotic areas in VTP-treated mice were less compact (compare insets in panels
361 **7C** vs. those in **7D**) and, in some cases, exhibited a higher number of CMs resembling the interstitial fibrosis
362 detected in patients with ischemic cardiomyopathy (**Figure 1**). The anti-fibrotic effect of VTP also determined
363 a significant increase in infarct thickness and a reduction of the infarct size at 7 and to a lesser extent at day 28
364 (**Figure 7D**). The positive effect on fibrosis of VTP was not, however, counterbalanced by an improvement in
365 cardiac function, as shown by the failure of the treatment to increase ejection fraction and left ventricle
366 fractional shortening as well as to decrease the end-diastolic/systolic volumes.

Discussion

Relevance of extracellular matrix remodeling for activation of YAP-pathway *in vivo*

Previous investigations highlighted the importance of the YAP signaling in cardiac fibrosis. For example, it was found that cells in the infarct zone in mice exhibit YAP nuclear staining²⁵ and that in mice lacking Lats1/2 kinases, two components of the Hippo kinase pathway, cardiac fibroblasts transition into Myo-Fbs spontaneously, giving rise to a hyper-secretory phenotype worsening the outcome of myocardial infarction²⁴. Since Lats proteins phosphorylate directly YAP preventing its nuclear translocation⁵⁶, the Authors investigated the promoter occupancy of the chromatin in wt and Lats^{-/-} CFs and found significant enrichment of YAP-bound enhancer sites in *pro*-fibrotic genes in knockout cells. This evidence led them to conclude that YAP/TAZ complex promotes cardiac fibroblasts transitioning into myo-Fbs by stable modifications of chromatin architecture and activity. In keeping with these conclusions, selective genetic ablation of YAP in cardiac fibroblasts, reduced cardiac fibrosis and improved cardiac function after MI^{26, 59}.

To assess whether the active matrix remodeling creates permissive conditions for YAP nuclear translocation in cells with morphological characteristics of Myo-Fbs, we employed a mouse model of myocardial infarction and characterized the features of the cells exhibiting nuclear YAP localization as a criterion to determine its transcriptional activity *in vivo*^{25, 56}. In this analysis, we took as a reference evidences emerging from computational models describing the regional deposition of collagen fibers following myocardial injury, where non-random patterns have been observed, likely depending on the alignment of the cells depositing the matrix caused by the anisotropic distribution of the strain forces³⁷. Strikingly, as revealed by the nuclear ellipticity and orientation analyses performed **in cells with the morphology of CFs in the scars at 4 weeks post-MI (Figure 1)**, we noticed a more frequent occurrence of nuclei with a more elongated shape and a more consistent alignment along the equatorial cutting plane of the left ventricle in cells with nuclear-localized YAP. Since the orientation of the nuclei in the infarct and, more in general, inside 3D matrices subjected to strain with a principally uniaxial component aligns the cells and the ECM deposition along the principal force vector⁶⁰, the prevalent nuclear translocation of YAP in cells with a higher nuclear ellipticity and a more frequent cellular circumferential orientation might be part of a strain-dependent anisotropic activation of the *pro*-healing response to infarct reinforcing the cardiac wall and preventing its immediate rupture after infarction, but promoting chronic ventricular remodeling (hypothetical model described in **Figure 8**)⁶¹. This hypothesis is corroborated by results showing that matrix remodeling in the infarcted heart involves a complex series of structural modifications, changes in mechanical properties and hyper-activation of *pro*-fibrotic signaling⁶², as well as by evidences in other tissues, e.g. the cardiac valves, where the deformation of the nuclei in interstitial cells has been connected to cyclic deformations due to straightening and compaction of the fibrous ECM components (i.e. collagen)⁶³. This validates YAP as a transcriptional ‘sensor’ of the dynamic remodeling of the cardiac ECM consequent to ischemic damage. Its expression in nuclei of CFs in the fibrotic areas in myocardial samples of patients with severe heart failure (**Figure 1**) suggests the possible activity of the pathway in the human pathology.

Cell/nuclear strain/compression-dependence of a pro-fibrotic pathway in human cardiac stromal cells

Human primitive cardiac stromal cells have been shown to display pro-fibrotic features when exposed to the native extracellular matrix from remodeled hearts, or even to the altered substrates synthesized *ex vivo* by pathological cardiac fibroblasts from failing hearts^{62, 64, 65}. To get insights in the mechanically-activated pro-fibrotic evolution of the primitive human stromal cells, we employed a 2D cell culture systems to test the effects of discrete cytoskeleton tensioning on the activation of YAP target genes, and to assess reversion of the *pro*-fibrotic phenotype by treating cells with inhibitors of the F-Actin cytoskeleton. In line with evidences provided in other studies, performed either on cardiac or valve stromal cells^{29, 43, 52, 66}, human cSt-Cs exhibited a stiffness-dependent YAP nuclear translocation resulting into cell proliferation (**Figure 2**). When treated with inhibitors of the cytoskeleton tensioning, cSt-Cs reversibly extruded YAP from the nucleus and downregulated canonical transcriptional targets and *pro*-fibrotic genes (**Figure 4**). It was finally interesting to note that nucleus geometry and compliance were also reversibly affected by the release of cytoskeleton tensioning by the two inhibitors (**Figure 3**). The change in nuclear geometry due to relaxation along the *z*-axis of the microscopic view likely reflects the release of the nuclear tensioning - acting mainly in the 2D (*x*, *y*) dimensions - due to adherence of the cells to a rigid flat surface¹⁷, similarly to the effect of plating the cells onto soft substrates (**Figure 2**). The decrease in nuclear compliance suggests a possible effect of releasing cytoskeleton tensioning not only on the reduction of the strain at the level of the nuclear lamina, but also generalized changes in the chromatin compaction possibly leading to modifications in the epigenetic setup. In line with these evidences, it was demonstrated that biophysical cues can alter the methylation and acetylation status of histones, favoring epithelial to mesenchymal transition⁶⁷, and that cells embedded into matrices simulating stiff pathological tissues exhibit a mechanical ‘memory’ establishing permanent activation of pathological programs⁶⁸. Whether, and to what extent, the nuclear straining imposed by cytoskeleton tensioning in human cSt-Cs has an epigenetic readout is the subject of ongoing investigations.

The connection between YAP nuclear translocation, nuclear straining and activation of *pro*-fibrotic signaling was optimally validated in cardiospheres. Apart from the description of this model as an *in vitro* ‘niche’ of cardiac mesenchymal progenitors³⁹, the cardiosphere appeared to fulfill the characteristics of a suitable 3D system to assess the impact of cell/nuclear straining on YAP-dependent pro-fibrotic activation in cSt-Cs, in analogy to what we observed in the fibrotic scar in mice (**Figure 1**). We adopted this model by extending concepts validated in the earliest stages of mammalian embryogenesis, where mechanical-dependent inhibition of the Hippo pathway and a robust YAP nuclear translocation have been described as a topological determinant of the primitive trophectoderm cells differentiation in the cellular shell of the forming blastocyst⁶⁹. To validate this model, we computer-analyzed the deformation of the nuclei in the cellular spheroids in relationship with the position of the cells in the core *vs.* the shell of the cardiospheres, the presence of nuclear-localized YAP, the expression of cell proliferation and *pro*-fibrotic cellular markers, and the deposition of ECM components (**Figure 3**). The suitability of this method, based on the CARE algorithm³⁴, allowed to establish rapidly, and with a high level of significance, a cause-effect relationship between the extent of nuclear deformation, the YAP nuclear translocation and the presence of Myo-Fb features (such as proliferation, expression of α SMA

440 and collagen) in the cells depending on their 3D positioning, with cells in the shell characterized by higher
441 strain vs. those positioned in the core subjected to lower compression forces and strain. These evidences
442 support, finally, the *in vivo* findings showing the effects of topological cues on the activity of the YAP signaling
443 in a 3D multicellular cardiac fibrotic context (**Figure 1**).

444 **Targeting downstream effectors of mechanical cues reverts TGF- β -dependent and independent CF** 445 **fibrotic programming**

446 The data of our RNAseq analysis support the notion that treating cells with VTP disassembles the bridging of
447 YAP to TEADs regardless of cell strain and has unbalancing effects for the activity of the Hippo pathway with
448 possible readouts on the level of YAP phosphorylation, transcriptional cooperation with the Wnt pathway,
449 *YAP1* gene silencing (**Figures 5B, C**) and downregulation of several pathways connected with extracellular
450 matrix remodeling, inflammation and cell polarity/migration (**Figure S9A**). Interestingly, cells treated with
451 VTP also downregulated more than a half of the genes that were induced by TGF- β 1, one of the most potent
452 cardiac fibrosis-inducing factors^{50, 62} (**Figure S8**). These genes included canonical YAP targets such as *CTGF*
453 (*CCN2*) and *ACTA2*, genes connected to the CFs pro-fibrotic phenotype (*Thy-1*), and genes encoding for major
454 scar components such as *COL1A1* and *COLA3*. The centrality of the YAP/TAZ transcriptional signaling in the
455 context of fibrotic activation of human cSt-Cs emerged, finally, from the bioinformatics search of the *cis*-
456 regulatory elements potentially involved in the generalized gene downregulation observed in VTP-treated cells
457 (\pm TGF- β 1) vs. controls (**Figure S9B**). This search identified TEAD 1/3/4 and the common transcriptional
458 transducer of TGF- β signaling SMAD4 as candidates for the co-regulation of genes involving YAP/TAZ
459 complex as a common factor. While this is in line with the current literature identifying cooperation of these
460 transcriptional modulators⁷⁰, it also highlights the centrality of the YAP/TAZ complex in the context of the
461 TGF- β 1 transcriptional signaling⁷¹, suggesting effectiveness of ‘mechano-therapeutic’ approaches to reduce
462 the extent of fibrosis in the cardiovascular system and other organs controlled by the pro-fibrotic factor.

463 In agreement with the variation in gene expression signatures, the blockade of YAP/TAZ/TEAD interaction
464 by VTP also appeared to completely override the TGF- β 1-dependent *pro*-fibrotic activation of cSt-Cs on a
465 phenotypic and functional points of view. This was substantiated by the reduction in cell proliferation (**Figure**
466 **6C**), the unloading of SMA from the F-Actin cytoskeleton (**Figure 6B**), and the inhibition of collagen
467 secretion/matrix compaction ability of the cells treated with TGF- β 1 (**Figure 6C, D**). Opposite to our
468 hypothesis that cells treated with TGF- β 1 might increase pulling forces, the experiment on micropillar arrays
469 did not show any variation in cell contractility (**Figure 6E**). This finding is only in apparent contradiction with
470 the reduction in matrix compaction due to VTP treatment, considering that other factors such as secretion of
471 matrix remodeling enzymes may concur to the increased collagen compaction of the cells treated with TGF-
472 β 1.

473 **Anti-fibrotic effect of VTP treatment *in vivo* does not coincide with improvement of cardiac function**

474 The interest for manipulating the Hippo pathway as a strategy to repair/regenerate the heart has produced a
475 growing course of studies with remarkable, but sometimes antagonistic results (reviewed in⁷²). In fact the
476 existing literature clearly distinguishes between the function of the YAP/TAZ complex in the heart, depending

477 on the localization of the loss/gain of function effects. For example, while upregulation/stabilization of YAP
 478 in CMs primes the contractile cells to divide, with potential cardiac regenerating effects^{19, 20, 22}, the
 479 hyperactivation of the YAP/TAZ complex in cardiac fibroblasts primes these cells toward a chronic scarring
 480 phenotype that result in accelerated and injury-independent myocardial remodeling²⁴ (also discussed in ²³). On
 481 the other hand, more recent studies showed that selective ablation of YAP in CFs reduces ischemia or pressure
 482 overload-dependent fibrosis with an improvement of myocardial function^{26,59}, thus opening the way to possible
 483 mechano-therapeutic strategies to limit cardiac fibrosis.

484 Prompted by the positive effects of VTP administration in models of ischemia/reperfusion-dependent kidney
 485 fibrosis⁵⁸, in the present study we used a classical VTP continuous administration protocol in mice with a
 486 chronic cardiac ischemia setting. Although this clearly reduced the accumulation of collagen and, at least in
 487 part, preserved the infarct wall from the extreme thinning observed in control mice, the administration of the
 488 drug did not relief the detrimental effects of ischemia on cardiac function (**Figure 7**). Different possibilities
 489 may account for this effect, which makes the results of our findings clearly different from those achieved in
 490 genetic models of selective YAP inhibition in CFs. A first element that may play a role in this difference is the
 491 possibility that VTP alters/retards the timing of the pro/anti-inflammatory phases after cardiac damage. For
 492 example Freeman and colleagues showed that selective ablation of YAP in early stage macrophages after MI
 493 promoted cardiac repair by shifting the phenotype of toward an anti-inflammatory phenotype³⁶. In line with
 494 this, our treatment with VTP could have an initially positive effect on selective ablation of pro-inflammatory
 495 macrophages but, thereafter, a pleiotropic inhibiting function of anti-inflammatory macrophages with
 496 detrimental effects for cardiac functional recovery. A second possibility may derive from the ambivalent
 497 function of the YAP/TAZ complex in cardiac myocytes vs. fibroblasts. For example, YAP loss of function in
 498 CMs leads to impaired survival after cardiac injury with worsening effects on maladaptive ventricular
 499 remodeling after myocardial injury⁷³. In such a case, the beneficial effect of VTP administration on reduction
 500 of fibrosis might be counteracted by a reduced survival of myocytes in the infarcted heart, thus limiting the
 501 therapeutic efficacy of bulk administration of the drug. In order to substantiate our results in translational and
 502 functional perspectives, we are currently evaluating, *i*) other treatment modalities (e.g. to start or interrupt
 503 administration of the drug at defined times after the initial pro-inflammatory period) to better distinguish
 504 between possible antagonistic effects of the continuous VTP treatment on myocardial functional recovery, *ii*)
 505 the adoption of imaging-derived parameters of global cardiac function such as the myocardial strain⁷⁴, *iii*) the
 506 use of 3D imaging tools such as the second harmonic generation microscopy⁶², and *iv*) of force-based mapping
 507 by nanoindentation²⁹ of the cardiac tissue.

508 **Conclusions**

509 The emerging role of YAP in fibrotic progression in several diseases⁷⁵, including myocardial remodeling after
510 infarction²⁵, provides a strong rationale for a potential anti-fibrotic therapy of the failing heart, based on local
511 mechanical desensitization of the *pro*-fibrotic cells. Translation of genetic approaches into protocols of
512 selective pharmacological inhibition of the complex in CFs but not in CMs is, **in fact, not currently amenable**
513 **using systemic administration protocols.**

514 In the present study, we provide evidences that the pro-fibrotic programming of human cSt-Cs *in vitro* is
515 subjected to the cooperation of mechanical, topological and paracrine cues likely originating from the known
516 effects of strain forces resulting from the anisotropic arrangement of the collagen matrix occurring during scar
517 formation^{37, 76} (**Figure 8**), and the results of the nuclear strain/orientation analyses performed on YAP⁺ and
518 YAP⁻ nuclei in the infarct fibrotic scar. We extend this conclusion also to an *in vivo* cardiac remodeling
519 situation, where the administration of VTP caused a significant inhibition of the fibrotic progression in the
520 ischemic hearts, even though this improvement was not accompanied by a net increase in cardiac performance.
521 A final and more general conclusion of our investigation is that, in analogy to what already described in other
522 cardiovascular pathological settings such as the aortic valve disease^{29, 77} or vascular pathological conditions^{78,}
523 ⁷⁹, understanding the cooperation between mechanical cues and paracrine factors in cardiovascular diseases
524 will be a possible key to achieve innovative and targeted anti-fibrotic therapies. Indeed, if administered with
525 systems specifically designed to perform drug delivery in the fibrotic tissues (e.g. nanotechnology), these
526 therapies will be optimal candidates to mechanically desensitize the pro-fibrotic cells, with hopes for robust
527 reverse remodeling effects.

528 **Novelty and significance**

529 Emerging evidences suggest that mechanical signaling is crucial for acquisition of pathological phenotypes in
530 cardiovascular tissues remodeling. While the identity and the function of mechanically regulated
531 transcriptional activators in cardiac pathology has been clarified mainly with genetic studies, a direct
532 connection between cell mechanics and progression of ischemia-dependent fibrosis was missing. In the present
533 contribution, we show that nuclear translocation of the Yes-Associated Protein (YAP) transcription factor
534 occurs by exposing cardiac fibroblasts to incremental strain/compression forces either *in vivo* or *in vivo*. We
535 also provide evidences that pharmacological interfering with nuclear function of YAP is sufficient to override
536 the TGF- β 1-dependent pro-fibrotic programming *in vitro*, and to reduce the extent of cardiac fibrosis *in vivo*.
537 Our results open the way to ‘mechano-therapeutics’ of the fibrotic heart.

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545 **Disclosures**

546 The Authors declare no conflicts of interest.

547 **Supplemental materials**

548 Expanded Materials & Methods

549 Major resource table

550 Online figures S1 – S9

551 Supplementary references: 80 - 99

552 Figure Legends

553 **Figure 1. Activity of YAP-dependent signaling is subjected to oriented nuclear straining and cellular**
554 **alignment in the infarct scar.** (A) Equatorial section of the infarcted heart showing the left and right ventricles
555 (LV and RV, respectively) at a short follow-up time (3 days post-MI) stained with anti-YAP antibodies. The
556 higher magnification on the left side of the panel shows the presence of cardiomyocytes (cm) and fibroblasts
557 (Fbs) characterized by low levels of nuclear YAP. A preferential localization of YAP⁺ cells was observed in
558 the infarct zone (IZ) magnified in the right side of the panel, which at this time point is not yet subjected to
559 extensive remodeling, but is affected by a high degree of inflammation. At this stage, YAP nuclear signal was
560 detected in inflammatory infiltrate cells (infl), in some myofibroblasts (Myo-Fbs) and cardiomyocytes (cm).
561 (B) Masson trichrome staining of a terminal stage (four weeks post-MI) cardiac remodeling event,
562 characterized by the presence of a collagen-rich scar extending from the wall of the right ventricle (RV) to a
563 wide portion of the left ventricle (LV). On the left side of the panel it is represented the whole equatorial
564 section of the heart to show the totality of the remodeling process. In the center and the right of the panel are
565 represented two consecutive magnifications of the areas of the infarct zone (IZ) encircled by the blue dotted
566 squares, to show the orientation of the collagen (Coll) bundles along a preferential equatorial plane (red
567 arrows). Note the the presence of numerous fibroblasts exhibiting a similar orientation. (C) YAP
568 immunohistochemical labeling of the same heart cut with the same equatorial orientation shown in panel B.
569 The magnification of the two areas encircled by the red dotted squares show the presence of collagen bundles
570 (Coll; blue arrows) and of fibroblast-resembling cells characterized by presence of YAP in the nucleus (YAP⁺)
571 or absence of the transcription factor (YAP⁻), indicated by red arrows. Note in both panels that cells with YAP⁺
572 nuclei appeared in both areas to show a higher nuclear aspect ratio (ellipticity). (D) Virtual reconstruction of
573 the nuclei orientation in the infarct scar as detected by CARE. On the left side of the panel it is represented the
574 actual image of an equatorial section of the left and right ventricle (LV and RV, respectively) of a heart with
575 an end-stage remodeled infarct zone (red dotted square), with an indication of the profiles of the external
576 cardiac wall and the internal LV wall (both indicated by blue lines) and the midline of the wall (green line).
577 The panel in the center shows the margins of the scar (green lines) defined by the two concentric lines
578 contouring the collagen-rich region in the scar, and the scar centerline (red). The orientation of each cell
579 nucleus was measured by determining the tilting of the major nuclear axis with respect to the line intersecting
580 perpendicularly the scar midline in manually selected zones with a **clearly visible fibrotic appearance in the**
581 **scars** (green boundaries in the right panel; see also **Figure S1**). (E) Comparison of nuclear ellipticity and
582 orientation as computed by CARE for YAP⁺ and YAP⁻ nuclei in the terminally remodeled infarct of 3 mice
583 with a 4 weeks post-MI follow-up. The box plot on the left represents the min-max distribution, the median
584 and the mean (+) of the nuclear aspect ratio in YAP⁻ (blue) and YAP⁺ (brown) nuclei. **Data were analyzed by**
585 **Mann-Whitney t-test** (n = 3329 YAP⁻ and = 4559 YAP⁺ nuclei, respectively). The two distributions on the
586 right side of the panel show the percentage of the cells with nuclei with an orientation (θ) $\pm 10^\circ$ in a $0^\circ < \theta <$
587 180° range with respect to the perpendicular direction to the scar center line (See **panel E** and **Figure S1**).
588 Data in the two distributions are indicated as percentage (\pm SE) of YAP⁻ (blue) or YAP⁺ (brown) nuclei of the

total amount of computable nuclei in the scar of 3 mice within the 18 resulting $\pm 10^\circ$ orientation categories. As shown, the percentage of YAP⁺ nuclei with an orientation $\pm 10^\circ$ within the $70^\circ < \theta < 110^\circ$ range were significantly higher than that of YAP⁻ nuclei. **Data were compared by unpaired multiple t-test analysis.** (F) Low and high magnification of representative sections of human myocardium from patients with dilated hypokinetic ischemic cardiomyopathy stained with Masson's trichrome and YAP IHC. In the lower insets it is evident the presence of numerous fibroblasts with YAP⁺ nuclei populating the collagen scar.

Figure 2. Strain-dependent activation of YAP transcriptional pathway in human cSt-Cs. (A) Geometric features (cell spreading area; circularity) of human cSt-Cs are subjected to stiffness-dependent control. Cells were plated onto glass and onto a series of polyacrylamide gels with controlled stiffness ranging from 58kPa to 17kPa (Young's modulus)²⁹ and were photographed to derive geometrical information. The pictures show the staining of the nuclei (blue) and the F-Actin cytoskeleton by Phalloidin-TRITC (white color). The two graphs include areas [μm^2] and circularity factor from more than cells from each of the indicated number of experimental replicates performed with cSt-Cs lines from different donors. (B) The expression of YAP (green fluorescence) was visualized by IF along with the nuclear and F-Actin staining by DAPI (blue) and phalloidin-TRITC (red), respectively. The percentage of cells with nuclear localized YAP (arrows) and the YAP_{NUCL/CYTO} expression ratio were determined using ImageJ and CARE³⁴, respectively (see also **Figure S3**). Graphs were derived with data obtained in at least 10 cells from each of the indicated number of experimental replicates performed with cell lines from different donors. (C) Cells plated onto glass and PAGs with differential stiffness were labelled with Ki-67 proliferation marker (green, arrows) along with F-Actin (red) and nuclear (blue) staining. The graph shows the quantification of the results and indicates the statistical significance in the comparison between experimental groups. **In all graphs in the panels, the P values of the statistical comparisons between the cells plated onto glass vs. PAGs with the different stiffness, or between cells plated on the differential stiffness PAGs are calculated by one-way pairwise Anova with Dunnet and Tukey post-hoc tests, respectively.**

Figure 3. Topological cues support an asymmetric distribution of cells with high YAP_{NUCL/CYTO} expression ratio and proliferation/fibrotic markers in a 3D context. (A) Phase contrast view of primary cardiospheres derived from human myocardial tissue (left) and confocal image of a cardiosphere whole mount-stained with YAP-specific antibody (green, right), F-actin (red), and nuclei. The image on the right shows, on the top, the 3D projection of the cardiosphere and the x, y, z dimensions (μm) as detected by high-resolution confocal imaging. The three images on the bottom show three discrete x, y equatorial images of the internal view of the same cardiosphere at the indicated distances (-35 μm , -20 μm and -10 μm) from the top of the sphere (set at 0 μm) along the z axis. In these images it is evident the asymmetric distribution of cells with YAP nuclear signal in proximity of the surface of the cardiosphere compared to its core. The arrows in different colors show examples of nuclei exhibiting a high content of nuclear YAP in the three equatorial images, also indicated with the same color code in the 3D projection on the top. (B) On the right side of the panel it is represented a confocal imaging 3D projection (top) and three x, y equatorial views (bottom) of a cardiosphere stained with Ki-67 antibody (white fluorescence) and nuclei (blue fluorescence) as described for **panel A**. The two graphs

on the left represent, respectively, the $\text{YAP}_{\text{NUCL/CYTO}}$ expression ratio (top) and the percentage of the cells expressing Ki67 (bottom) as computed by CARE algorithm. (C) The whole mount IF of GATA-4, a cardiac specific transcription factor, which is not known to be mechanically regulated, did not show differences in the nuclear/cytoplasm expression ratio between the shell and the core of the cardiospheres at any of the equatorial x, y projections. Data computation by CARE confirmed no statistically significant differences in the distribution of the ratios in the two compartments. (D - E) Whole mount IF staining of cardiospheres with Collagen-I and αSMA antibodies, respectively, visualized as in **panels B** and **C**. As shown by the images and the x, y projections, these markers tended to be more expressed at the periphery and not in the center of the cardiospheres. Data computation with CARE performed on the fluorescence distribution in the shell vs. the core of the spheres showed a clear asymmetry, similar to that found for YAP. **In all graphs, the P -value of statistical comparison by paired student's t-test is shown.** The number of cardiospheres (obtained from 4 independent tissue donors) introduced in the analyses are represented by the orange circles overlapped to the bars.

Figure 4. Nuclear geometry and tensioning regulates activity of YAP transcriptional pathway in human cSt-Cs. (A) Comparison between the shape of the cSt-Cs when in contact with hard (58kPa) and soft (17kPa) substrates. The low magnifications on the left show the cellular shape along with the YAP labeling (green); the magnifications of the areas encircled with the dotted squares on the right show the major (a) and the minor (b) axes, used to calculate the aspect ratio (Ellipticity) of the nuclei. Note that in cells in contact with the soft substrate, YAP fluorescence was almost localized almost entirely in the cytoplasm, while in cells adhering onto the stiff substrate it was almost entirely into the nucleus (see also **Figure 2** for quantifications). The graph on the right shows the quantification of nuclear ellipticity in cSt-Cs plated onto the whole series of PAGs (plus glass as a control). **The P values of the statistical comparison of the nuclear aspect ratio in cells the cells plated onto glass vs. PAGs with the different stiffness, or between cells plated on the differential stiffness PAGs are indicated. These values were calculated by one-way pairwise Anova with Dunnet and Tukey post-hoc tests, respectively.** (B) Calculation of the nuclear aspect ratio in the cardiospheres with CARE. The two images show, respectively, the equatorial section of a cardiosphere labeled as in **Figure 3**, with YAP antibody (green), for F-Actin (red) and nuclei. Arrows indicate filamentous Actin labeling, showing putative stress fibers. The image on the right is the virtualization of the nuclei image in the left with an indication of the shell and the core of the sphere (see also **Figure S4**). This image contains a color-coded nuclear structural information according to a nuclear ellipticity scale comprised in this panel between 0.40 and 0.50. It is evident that the majority of the nuclei with higher aspect ratio were present in the shell of the sphere and that more round nuclei were abundant in the core. The graph on the right is a statistical comparison of the average nuclear ellipticity in the shell and the core in the indicated number of cardiospheres. **The P value of data statistical comparison by paired student's t-test is indicated above the graph.** (C) Cellular effects of treating cSt-Cs with ROCK inhibitor Y27632 and Myosin II inhibitor Blebbistatin on substrate with maximal stiffness (glass). Cells are labeled with YAP antibody (green), F-Actin label (Phalloidin-TRITC, red) and nuclei (DAPI, blue). Before treatment (CTRL, left), cells exhibited a normal fibroblastoid/mesenchymal phenotype and nuclei with a high

level of nuclear YAP (arrows). Treating them with both inhibitors (center panels in **C**) reduced the number of stress fibers and induced a change in cell shape, with a consistent reallocation of the YAP fluorescence in the cytoplasm. A re-tensioning of the stress fibers was observed with washout of both drugs (right panels), along with a return to normal level of YAP nuclear confinement (arrows). **(D) Quantification of YAP nuclear confinement.** *P* values of data statistical comparison by one-way pairwise ANOVA with Tukey post-hoc test are indicated above each graph. **(E)** Nuclear geometry and compliance are affected by release of cytoskeleton tensioning. The pictures on the left show the normal (*x*, *y*) microscopic view of glass-adhering cSt-Cs labeled with YAP antibody (green), F-Actin probe (red) and nuclear label (blue). The pictures on the right of each panel show the projection of the cells circled with a dotted square, along the *x*, *z* and the *y*, *z* axes, as detected by super-resolution confocal imaging. It is evident that treatment with inhibitors determined a relaxation of the nuclei along the *z* axis and that after washout of the cells, the re-tensioning of the stress fibers caused a return to a nuclear flattening condition (in keeping with YAP nuclear segregation). The effect of nuclear geometry on nuclear mechanical characteristics is shown in the panels on the right, where a quantification of the nuclear geometrical changes along the *z*-axis by both treatment (and the treatments washouts) is shown, together with the physical ‘softening’ of the nucleus, as detected by AFM force imaging. **In both graphs, the *P* values of data statistical comparison by one-way pairwise ANOVA with Tukey post-hoc test are indicated above each graph.** **(F)** Transcriptional readout of cytoskeleton inhibition consisted of downregulation of canonical YAP targets, as shown by RT-qPCR amplification of *CTGF*, *CYR61* and *ANKRD1* gene transcripts. Note that the expression level of these genes returned to baseline after drugs washout. Data are represented as fold changes (FC) in the expression of each gene in the indicated condition with respect to the expression level in untreated cells (indicated with a C corresponding to a level=1) calculated by the $2^{-\Delta\Delta C_t}$ method. **Statistics was calculated on the corresponding ΔC_t values. Above all graphs, the *P* values of RT-qPCR Delta-CT data statistical comparison by one-way pairwise ANOVA with Tukey post-hoc test indicate the significance of differences in the expression of each tested gene in VTP-treated vs. control cells.**

Figure 5. Global transcriptional effects of VTP. **(A)** cSt-Cs pulse-chased with verteporfin (VTP) for 5 hours exhibited a partially reversible downregulation of YAP target genes (*CTGF*, *CYR61*, *ANKRD1*) and pro-fibrotic markers (*Col1A1*, *Col3A*, *Thy-1*). Transcriptional inhibition was not accompanied by cell morphological changes and YAP cytoplasm reallocation, as in experiments performed with cytoskeleton inhibitors Y27632 and blebbistatin (**Figure 4**). **Data are represented as fold changes (FC) in the expression of each gene in the indicated conditions with respect to the expression level in untreated cells (indicated with a C corresponding to a level=1) calculated by the $2^{-\Delta\Delta C_t}$ method. Above all graphs, the *P* values of RT-qPCR Delta-CT data statistical comparison by one-way pairwise ANOVA with Tukey post-hoc test indicate the significance of differences in the expression of each tested gene in VTP-treated vs. control cells.** **(B)** Results of an RNAseq analysis of RNA samples extracted from control cSt-Cs, and cSt-Cs cultured \pm TGF- β 1 (\pm VTP) for three days. Hierarchical clustering was performed by Euclidean (sample) and 1-pearson correlation (genes) metric and average linkage method; gene expression levels are displayed as gradient colors from higher (dark red) to lower (dark blue). The heat map on the left shows the results of DE gene unsupervised clustering,

700 exhibiting a nearly perfect clusterization of genes significantly regulated by the treatment with the drug. The
 701 table on the bottom indicates the number of the DE genes (adj. P -Value<0.05 and $|\log_2FC|>0.58$) for each
 702 comparison between treatment vs. CTRL. DE genes are also distinguished between those that are up- or down-
 703 regulated in each treatment vs. CTRL. On the top right side of the panel, we report a representation of RT-
 704 qPCR analysis of five genes regulated by YAP whose modulation was consistent with the observed changes
 705 in the RNAseq profiling, in independent cellular replicates. **In all panels, graphs were generated using the fold**
 706 **changes (FC) in the expression of each gene in the indicated conditions with respect to the expression level in**
 707 **untreated cells (indicated with a C corresponding to a level=1) calculated by the $2^{-\Delta\Delta Ct}$ method. Above all**
 708 **graphs, the P values of RT-qPCR Delta-CT data statistical comparison by one-way pairwise ANOVA with**
 709 **Tukey post-hoc test indicate the significance of differences in the expression of each tested gene in VTP-**
 710 **treated vs. control cells or between treatments.** The heat map on the bottom right indicates the variation in the
 711 expression of the same genes as represented in the general dataset of the DE mRNAs (**bioinformatics data**
 712 **supplement – a**). (C) Differential expression of genes with functional annotation in the Hippo pathway. With
 713 * are indicated differentially expressed genes (adj. P -Value < 0.05 and $|\log_2FC|>0.58$) found in at least one of
 714 the treatments vs. CTRL comparison.

715 **Figure 6. Biological effects of VTP treatment in vitro.** (A) Treatment with VTP reduces proliferation of
 716 human cSt-Cs, as assessed by IF staining with antibodies specific for PCNA (green fluorescence) and F-Actin
 717 (red fluorescence)/nuclear staining (blue staining). Note that the drug reduced the percentage of PCNA⁺ cells
 718 (arrows) below the control level even in the presence of TGF- β 1, supporting a strong reduction of cellular
 719 proliferation, a recurrent feature in conversion of primitive stromal cells into myo-FBs. **Above the graph, the**
 720 **P values indicate the results of data statistical comparison by one-way pairwise ANOVA with Tukey post-hoc**
 721 **test.** (B) cSt-Cs treated with VTP loose myo-FB characteristics promoted by TGF- β 1, as detected by the
 722 unloading of α SMA from F-Actin cytoskeleton. The three images on the top show three cells labelled with F-
 723 Actin (red fluorescence) and nuclear staining (blue fluorescence) along with α SMA antibody (green
 724 fluorescence). As shown by the fluorescence intensity profile along the indicated dotted lines, treatment with
 725 VTP reduced the co-localization of α SMA and F-Actin signals (indicated by * in each of the plots
 726 corresponding) that was elevated by treatment with TGF- β 1. (C) Reduction of α SMA protein expression and
 727 of collagen-1 secretion by VTP treatment. The top left panel indicates the integration of the α SMA IF signal
 728 calculated as the integrated fluorescence density using ImageJ software. The panel on the top right represent
 729 an example of a Western blotting analysis performed with whole protein extracts from cSt-Cs treated as
 730 indicated. Note the decrease of the α SMA band intensity in VTP-treated cells compared to controls and TGF-
 731 β 1 treatment, also indicated in the bar graph in the low right, showing quantification of the normalized
 732 α SMA/GAPDH in all the conditions. The panel on the low left indicates the reduction in collagen secretion by
 733 the cells treated with VTP vs. CTRL and TGF- β 1 treatment. Also in this case, this reduction occurred also in
 734 the combined VTP+ TGF- β 1 treatment. **Above all graphs, the P values indicate the results of data statistical**
 735 **comparison by one-way pairwise ANOVA with Tukey post-hoc test.** (D) The ability of the cells to remodel
 736 the matrix was assessed by the collagen plug contraction assay. The panels on the top show the top-view

images of the plugs containing cells treated as indicated, while the bar graph on the bottom shows the quantification of the area occupied by the plug at 24hrs after matrix release. Lower areas are indicative of a higher contraction activity, in response to Myo-Fb differentiation of human cSt-Cs determined by TGF- β 1. VTP treatment completely reverted the contractile phenotype of the cSt-Cs. Above the graphs, the *P* values indicate the results of data statistical comparison by one-way pairwise ANOVA with Tukey post-hoc test. (F) Untreated and TGF- β 1-treated cSt-Cs \pm VTP were seeded onto PDMS micropillars to calculate the average traction force. The picture on the top illustrates the phase contrast image of a cell deposited onto the pillar array (left) and its corresponding F-Actin (red)/nuclear (blue) staining. The picture on the bottom is a virtual representation of the traction forces exerted by the cell onto each individual pillar, its direction (arrows) and intensity (color code). The graph on the bottom represents the average force exerted by a total of 12 cells (3 cells per donor) in the four treatment conditions.

Figure 7. Effects of VTP treatment on cardiac fibrosis and function in a mouse model of permanent cardiac ischemia. (A) Side by side comparison of equatorial sections of infarcted hearts harvested at 7 days post-MI from control (CTRL) and VTP-treated mice after staining with Masson's trichrome. It is evident the higher extension of the infarct, the higher collagen deposition, and the thinning of the scar in CTRL vs. VTP mice. (B – C) Side by side comparison of the scars at 28 days after MI in CTRL and VTP-treated mice. Also at this time point VTP reduced fibrosis and increased thickness of the infarct. Less evident was the effect of the drug on reduction of the infarct size. (D) Quantification of the infarct morphometry as detected in the tissue sections stained with Masson's trichrome at 7 and 28 days after MI. VTP reduced significantly the fibrosis and the infarct size and increased the wall thickness indicative of a lower LV remodeling. This effect was more pronounced at 7 days given that the reduction of the infarct size at 28 days post MI did not reach statistical significance. Above all graphs, the *P* values indicate the results of data statistical comparison by pairwise student's t-test. (E) None of the echocardiographic parameters (Ejection Fraction, LV end diastolic/systolic volumes and fractional shortening) were affected by the treatment at neither time.

Figure 8. Proposed model of strain-dependent YAP transcriptional signaling activation in the infarct scar. Myocardial remodeling after a transmural infarction involves matrix deposition and this occurs, according to literature, with a preferential equatorial orientation. This deposition pattern is due to a strain-dependent alignment of the collagen depositing cells along the principal strain vector. The findings in this work show that the variation in the nuclear aspect ratio in the collagen-depositing cells is connected to nuclear translocation of YAP. We propose that this creates a permanent activation condition of these cells toward a pro-fibrotic phenotype. Given that existing models of anisotropic collagen deposition in injured hearts describe this phenomenon only for regions of the cardiac wall at a distance from the LV apex³⁷, we cautiously extend our hypothetical model only to regions of the cardiac wall, where the distribution of strain forces occurs principally with an equatorial direction, and not in the apex. Further studies involving *in vivo* cardiac kinematics and serial sectioning of the scars at different sectioning planes would be necessary to further validate this hypothesis for the whole ventricular wall.

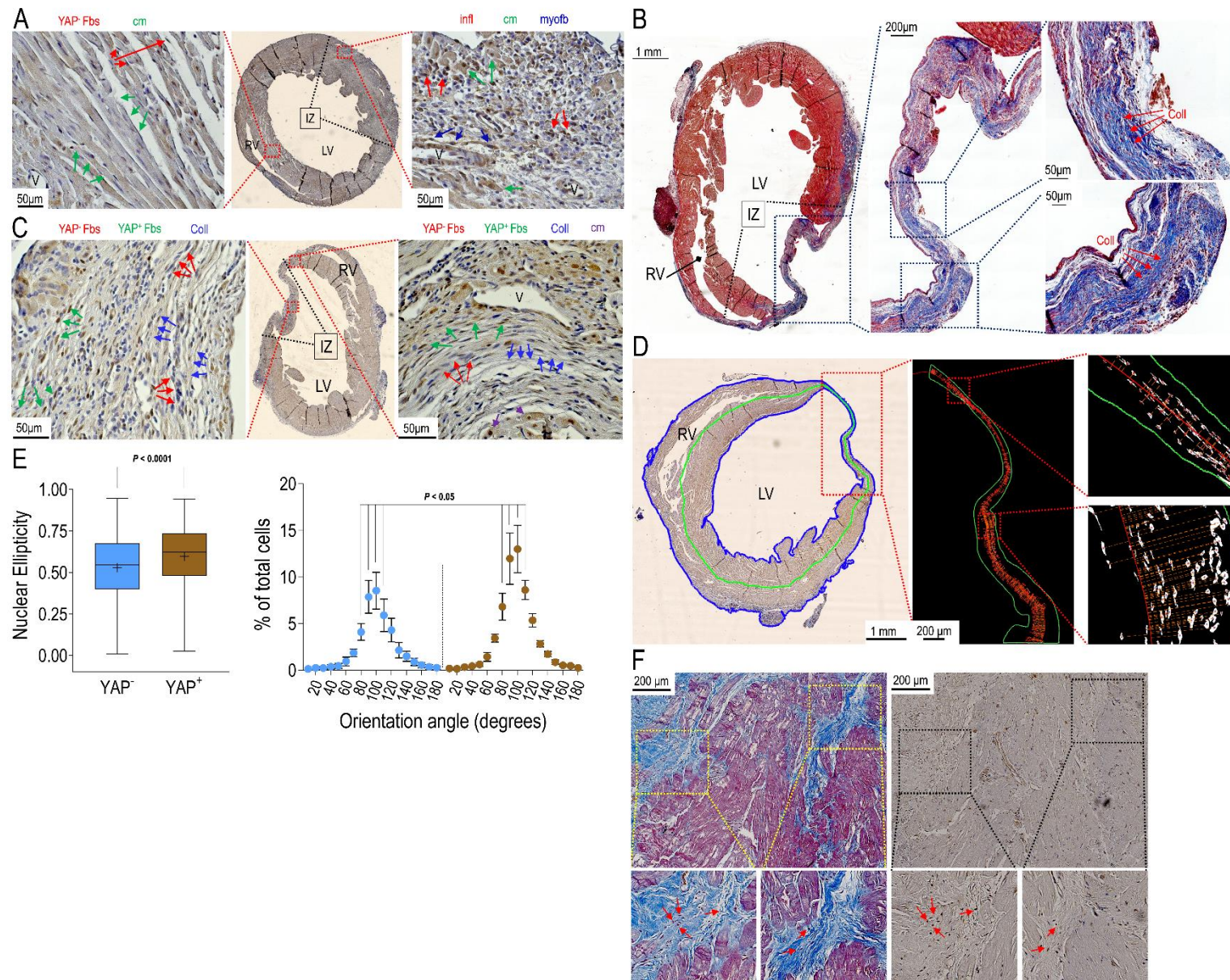


Figure 1

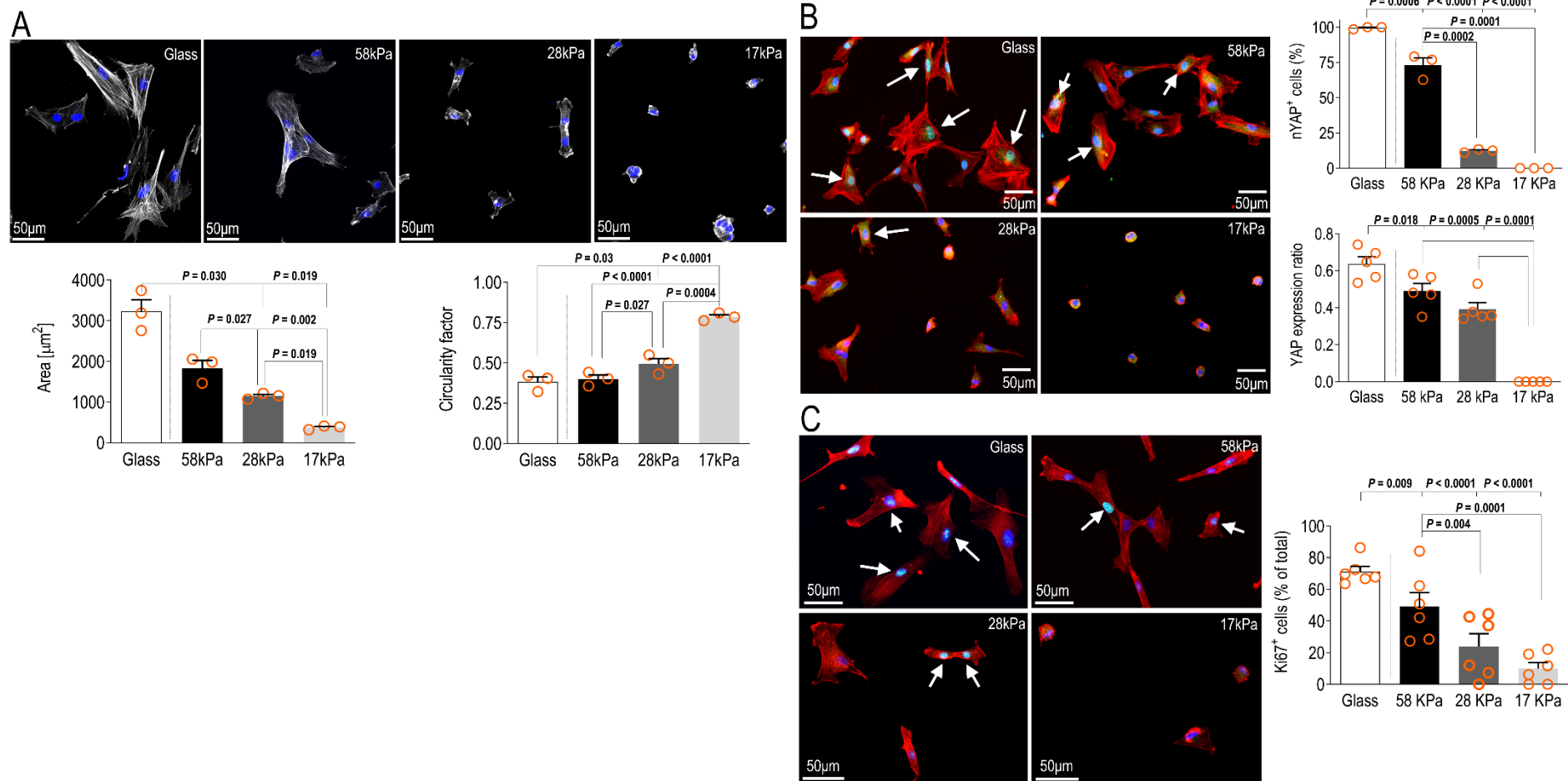


Figure 2

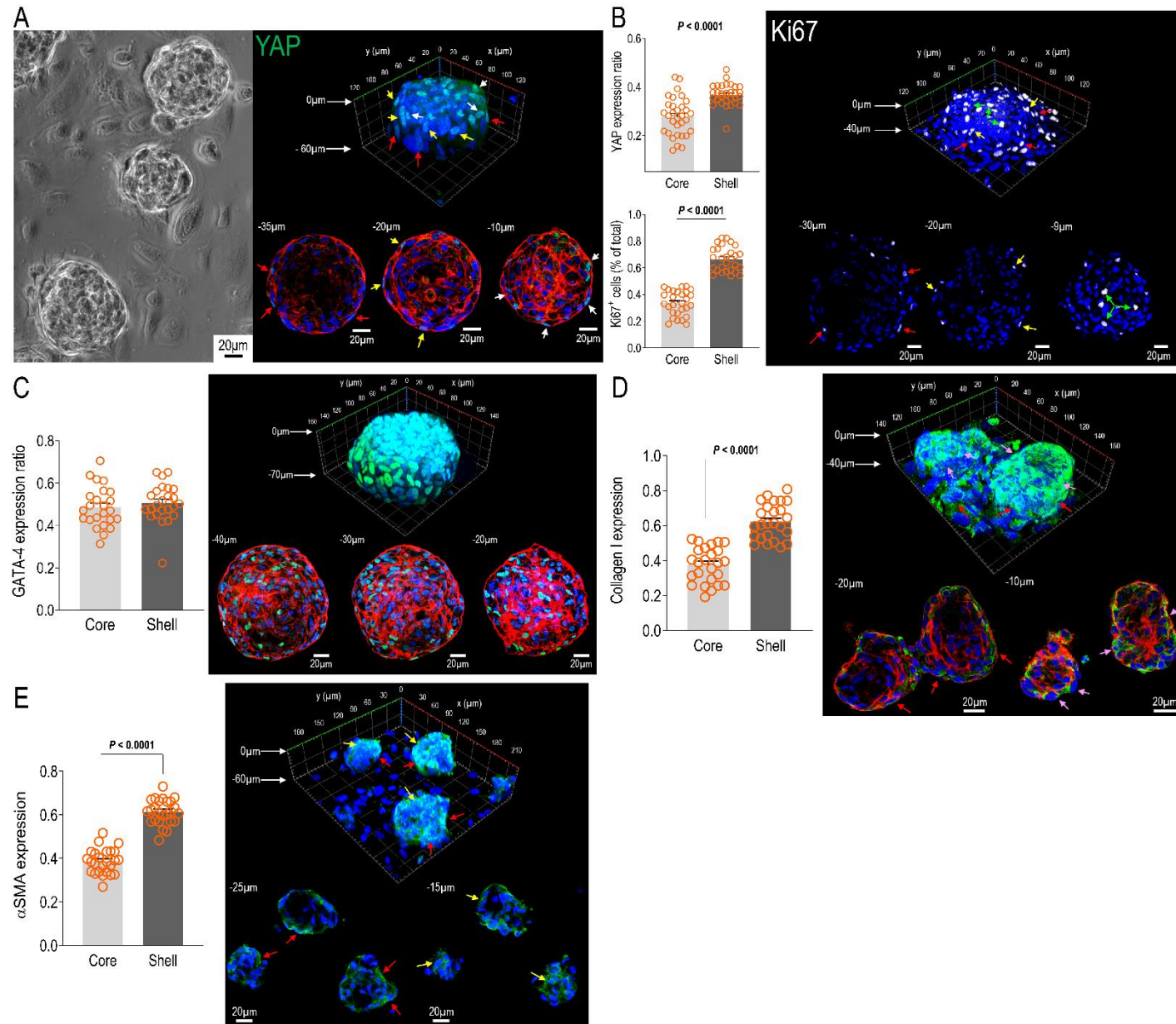


Figure 3

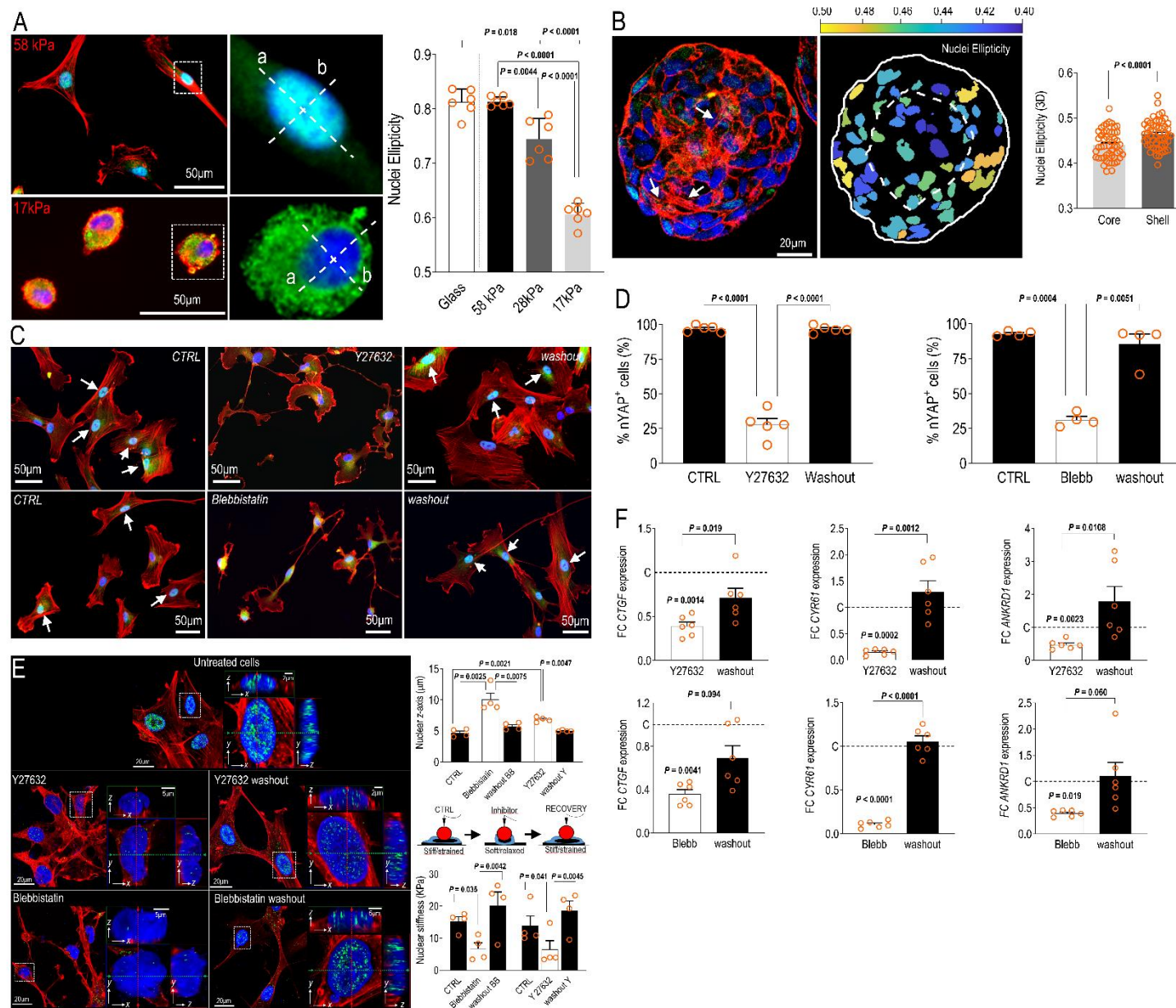


Figure 4

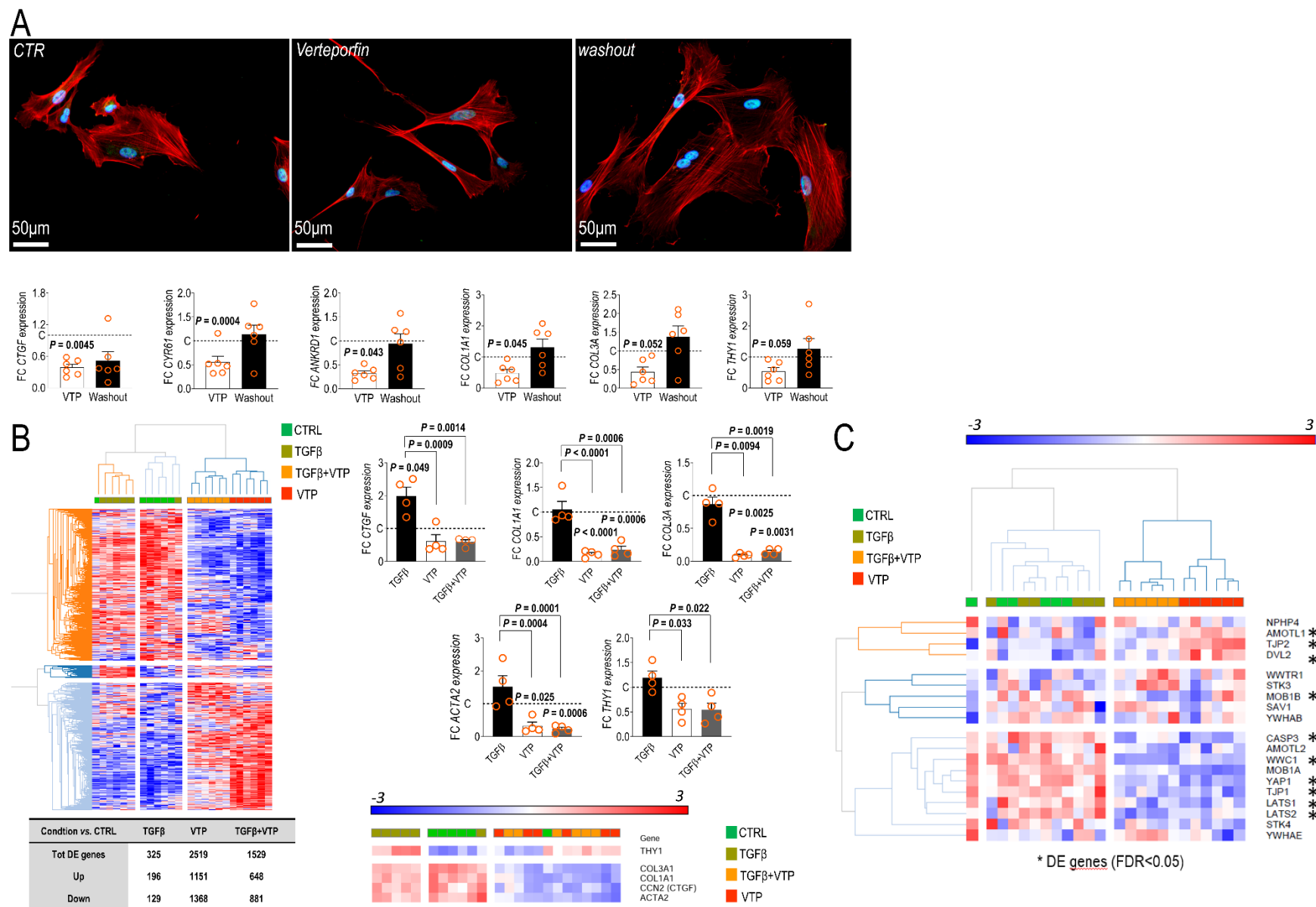


Figure 5

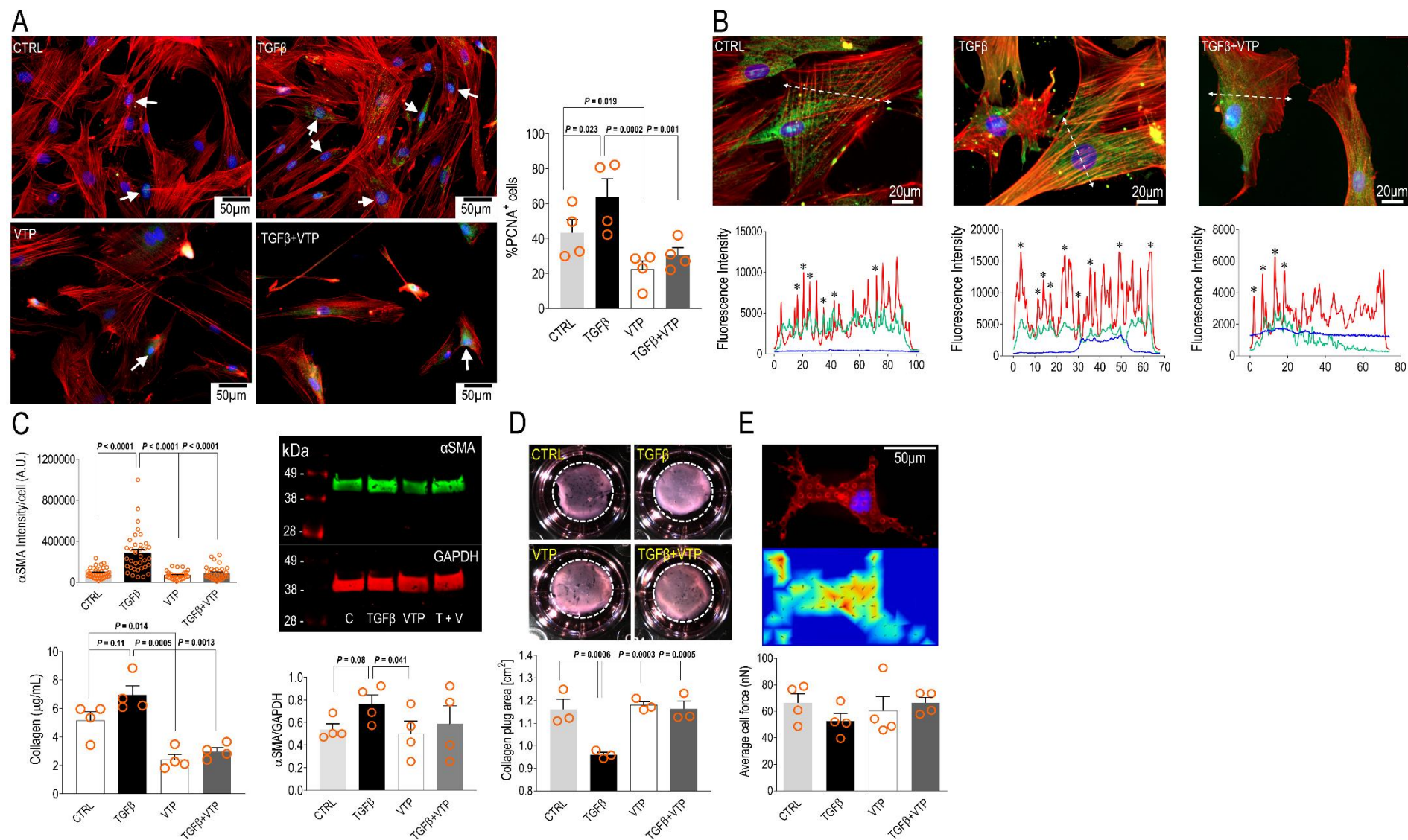


Figure 6

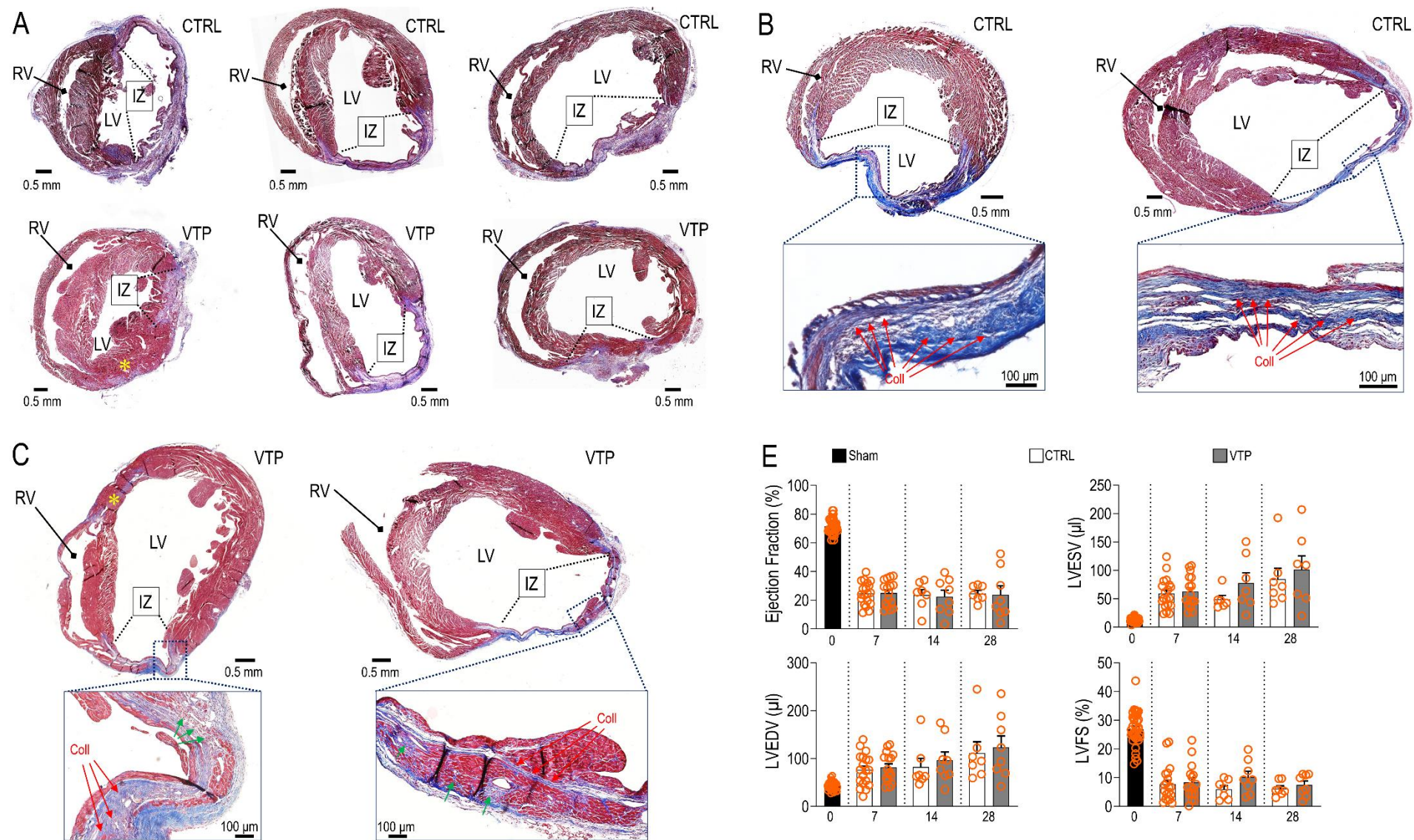


Figure 7

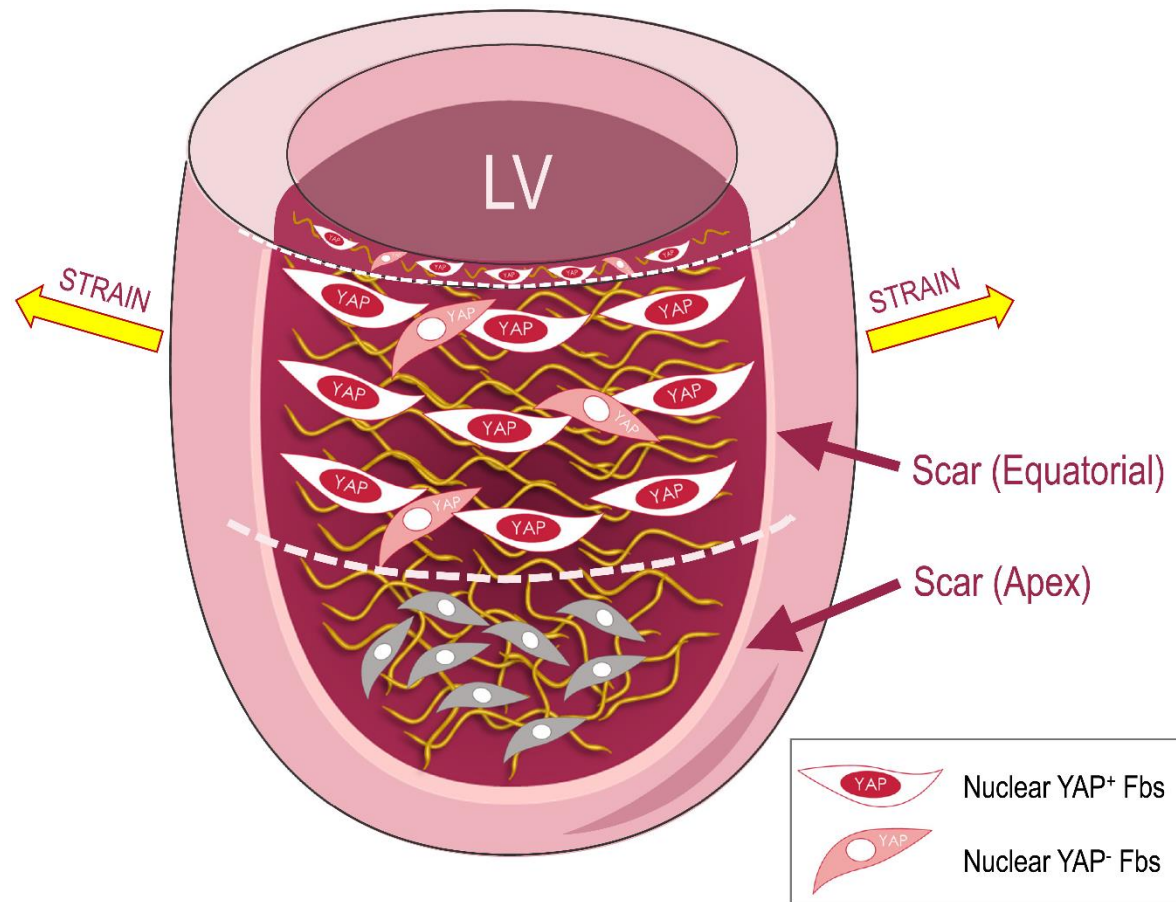


Figure 8

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