Mechanotransduction in cardiac stem cells: role of YAP/TAZ in the cellular response to the microenvironment

Diogo Miguel Mosqueira Alves Moreira da Silva

Mestrado Integrado em Bioengenharia – ramo Biotecnologia Molecular

**Supervision:** Dr. Giancarlo Forte and Dr. Perpétua do Ó

Porto, July 2012
MECHANOTRANSDUCTION IN CARDIAC STEM CELLS:
ROLE OF YAP/TAZ IN THE CELLULAR RESPONSE TO THE
MICROENVIRONMENT

Dissertation for Master Degree in Bioengineering – Molecular Biotechnology branch

**Workplace:**
Smart Biomaterials Group, Biomaterials Unit, International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS) – Tsukuba, Japan

**Supervision:**
Giancarlo Forte¹, PhD
Perpétua Pinto do Ó²,³, PhD

**Affiliation:**
1 - Smart Biomaterials Group, Biomaterials Unit, International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS) – Tsukuba, Japan
2- INEB – Instituto de Engenharia Biomédica, Porto, Portugal
3- ICBAS – Instituto de Ciências Biomédicas de Abel Salazar, Porto, Portugal

**Category:**
1- MANA scientist and NIMS senior researcher
2- Assistant investigator
3- Affiliate professor

The present work was funded by the Japan Society for the Promotion of Science (JSPS) through the “Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program), “MANA Grand Challenge Program 2012”, the project PTDC/SAU-ORG/118297/2010 and in the framework of the project PEst-C/SAU/LA0002/2011 from the Portuguese Foundation for Science and Technology (FCT), Quadro de Referência Estratégico Nacional (QREN) and FEDER funds through the Programa Operacional Factores de Competitividade – COMPETE. DMS was the recipient of NIMS Internship Program Fellowship and PPÓ was granted by Ciência2007.

**Aprovado em provas públicas pelo júri**

**Presidente:** Prof. Dr. Alexandre Quintanilha (IBMC/UP)
**Arguente:** Dr. João Bettencourt Relvas (IBMC/UP)
**Orientador:** Dra. Perpétua Pinto-do-Ó (INEB/ICBAS/UP)
Acknowledgments

This section is to thank all those who helped me throughout my academic path, not only for this thesis, but also for the whole Master programme.

First of all, I would like to express my deepest gratitude to Prof. Perpétua Pinto do Ó, for all the dedication she showed since we first met. I have learned a lot from our collaboration in these last three years, in all the different projects I could work with you as my supervisor. Your never-ending efforts in getting me the opportunity to work with you at INEB, and further enrich my research experience by sending me to Japan will never be forgotten. You have greatly inspired me and even influenced the hard choices I had to make and also shaped my determination towards the future.

Dr. Giancarlo Forte has also greatly contributed to my background as a bioengineer by receiving me in Japan, acting not only as a great supervisor but mostly as a friend I hope to keep in the future. Your enthusiasm is incredibly contagious, and I thank you for all you had to endure to receive me in your lab (the famous Japanese bureaucracy really is demanding but it works) and for everything we have been through together, both professionally and personally.

Next, I would like to acknowledge all the other supervisors I worked with during these 5 years (I realize now they are quite a few). As such, I would like to thank Prof. Diamantino Freitas (FEUP), Dr. Paulo Pereira (IBMC), Prof. Ülo Langel (SU), Prof. Nuno Azevedo (LEPAE/FEUP) and Dr. Takao Aoyagi (NIMS) and as already mentioned Prof. Perpétua do Ó (INEB) and Dr. Giancarlo Forte (NIMS). All the respective groups led by these supervisors also provided a great work environment, so my thanks to all them, especially Dr. Diana Nascimento, Ana Freire and Mariana Valente at INEB; mina svenska vänner: Henrik Helmfors, Andrés Alarcón, Staffan Lindberg, Dr. Kariem Ezzat, Dr. Oana Tudoran and Daisy Helmqvist på Stockholms Universitet; miei amici italiani Dr. Stefania Pagliari and Sara Romanazzo and 日本人の友達 (Nihon-jin no tomodachi): Takaharu Okada, Dr. Koichiro Uto and Dr. Janice Tam at NIMS.

Moreover, all my professors and colleagues in the Master program in Bioengineering, especially the ones in Molecular Biotechnology branch, had an important contribution in making me the bioengineer I aspire to be. You have not only contributed to a great learning (and sometimes rather funny!) environment but also provided some friendly competition. So my thanks to all of you, especially my previous workmates and friends: Andreia Silva, Ana Catarina Fonseca, José Pedro Quintanilha and Diogo Rodrigues.
Last but not least, I would like to thank my whole family who has been behind me all the way, ever since I started to study. The love and support you have given me has been unequaled and words cannot express how thankful I am to all of you. Por isso agradeço à minha mãe, ao meu pai, à Eva e ao Zé, aos meus primos: João, Marta, Ana, Rui, Leonor e tios/padrinhos, bem como à minha avó. Para finalizar um agradecimento muito especial à Sara que sempre foi o meu pilar emocional, tão importante para que eu chegasse onde estou hoje tendo a certeza que tomei as decisões correctas, sempre com o teu apoio incondicional. A tua dedicação, lealdade e amor nunca serão esquecidos e serão certamente retribuídos.

To finish, I would like to generalize these acknowledgements in a nerdy/funny way (which has been my hallmark for quite some time), by relating them with my thesis: thank all of you for presenting all the extracellular cues needed for me to differentiate into a Bioengineer!
Abstract

Cardiac diseases represent the first cause of death worldwide. Stem cell-based therapies constitute a promising therapeutic approach, yet the first attempts of clinical translation have met modest success. This is partly due to the limited understanding of biological mechanisms mediating interaction of cells with the surrounding microenvironment, be it their native milieu, or the one presented in therapeutic strategies. This dissertation addresses this issue by studying the effect of mechano-structural cues on cardiac stem/progenitor cell fate determination, via two transcriptional modulators, YAP and YAZ, implicated in mechanotransduction pathways. YAP/TAZ regulation and activity were studied in a murine Sca-1+ cardiac stem/progenitor cell line (cMPC<sup>Sca-1</sup>), by the identification of intracellular localization of these proteins, along with processes in which they are involved. It was concluded that YAP/TAZ are influenced by a plethora of different extracellular cues, being negatively regulated by confluence and requiring actin cytoskeleton for nuclear localization, which was enhanced by suppressing ROCK signaling and cell tension. Furthermore, YAP/TAZ nuclear localization gradually increases with stiffness of the interacting surface. Cell shape also regulates YAP/TAZ localization in cMPC<sup>Sca-1</sup>: round cells display cytoplasmic expression whereas spread morphologies correlate with nuclear expression. Dynamic changes in stiffness and nanotopography of the surfaces trigger YAP/TAZ nuclear localization. Moreover, YAP/TAZ were shown to be involved in several cellular processes such as proliferation, cardiac commitment and differentiation, adhesion and gene expression. Altogether, YAP/TAZ are mechanical sensors involved in cardiac stem/progenitor cell sensing of the microenvironment, responding to several mechano-structural cues and controlling important cellular responses related with stem cell fate determination.

**Key words:** mechanotransduction; YAP/TAZ; cardiac stem/progenitor cells; extracellular microenvironment; mechano-structural cues
Resumo

Doenças cardíacas representam a principal causa de morte a nível mundial. Terapias baseadas em células estaminais constituem uma abordagem terapêutica promissora, embora o seu sucesso na clínica tenha sido modesto. Tal deve-se a um conhecimento limitado dos mecanismos biológicos que medeiam a interacção de células com o microambiente extracelular, quer o meio nativo onde residem, quer o apresentado numa estratégia terapêutica. Nesta tese aborda-se esta questão pelo estudo do efeito de factores mecanoestruturais na determinação do destino celular de células estaminais/progenitoras cardíacas, através de dois moduladores transcricionais, YAP e TAZ, implicados em vias de mecanotransdução. A regulação e actividade de YAP/TAZ foram estudadas numa linha celular representativa de células estaminais/ progenitoras cardíacas Sca-1⁺ (cMPC Sca-1⁺), através da identificação da localização intracelular destas proteínas, bem como dos processos em que estão envolvidas. Concluiu-se que YAP/TAZ são influenciados por uma série de diferentes factores extracelulares, sendo negativamente regulados pela confluência celular e requerendo citoesqueleto de actina para localização nuclear, que é favorecida ao inibir vias mediadas por ROCK e tensão celular. Além disso, a localização nuclear de YAP/TAZ aumenta gradualmente com a dureza da superfície com a qual as células interagem. A morfologia celular também regula a localização de YAP/TAZ em cMPC Sca-1⁺: células arredondadas apresentam expressão citoplasmática ao passo que as estendidas exibem nuclear. Alterações dinâmicas da rigidez e nanotopografia das superícies activam a localização nuclear de YAP/TAZ. Além disso, mostrou-se que YAP/TAZ estavam envolvidos em vários processos celulares como proliferação, comprometimento cardíaco e diferenciação, adesão e expressão gênica. Concluindo, YAP/TAZ são sensores mecânicos envolvidos na perceção do microambiente das células estaminais /progenitoras cardíacas, respondendo a vários factores mecanoestruturais e controlando respostas celulares importantes, relacionadas com a determinação do destino celular.

Palavras-chave: mecanotransdução; YAP/TAZ; células estaminais/progenitoras cardíacas; microambiente extracelular; factores mecanoestruturais
# Table of Contents

Acknowledgments........................................................................................................ ii

Abstract .................................................................................................................... v

Resumo..................................................................................................................... vi

List of Figures and Tables........................................................................................... ix

List of Abbreviations................................................................................................. xi

1) Introduction .......................................................................................................... 1

1.1) Cardiac diseases and tissue engineering strategies ............................................ 1

1.1.1) Scaffolds and bioreactors used in cardiac tissue engineering ......................... 1

1.1.2) Stem cell sources for cardiac tissue engineering ............................................. 3

1.2) Stem cell fate determination ............................................................................. 6

1.2.1) Overview ........................................................................................................ 6

1.2.2) Influence of mechano-structural factors ....................................................... 8

1.3) Mechanotransduction signaling pathways ....................................................... 10

1.3.1) Integrins as mechanoreceptors ..................................................................... 10

1.3.2) Focal adhesions as mechanochemical signaling complexes ......................... 11

1.3.3) Actin cytoskeleton as a global signal integrator .......................................... 11

1.3.4) Nuclear shuttling proteins ........................................................................... 12

1.4) YAP and TAZ transcriptional modulators ....................................................... 14

1.4.1) Structural characterization .......................................................................... 14

1.4.2) Function in different cell types .................................................................... 15

1.4.3) Regulation by cellular pathways .................................................................. 15

1.4.4) Role in mechanotransduction ................................................................. 16

1.5) Objective of the experimental work ............................................................... 18

2) Materials and Methods .................................................................................. 19

2.1) Cell culture, transfection and treatment with pharmacological inhibitors .......... 19

2.2) *In vitro* wound healing assay ........................................................................ 20

2.3) Immunofluorescence staining and confocal microscopy .................................. 20

2.4) Protein extraction, cell fractioning and Western Blot ....................................... 20

2.5) Matrigel™ preparation and experimental setup .............................................. 21

2.6) Polyacrylamide gels preparation .................................................................... 22

2.7) Poly-ε-caprolactone film preparation and stiffness control ......................... 23

2.8) Alamar Blue® assay ..................................................................................... 24

2.9) RNA extraction and real time PCR array ................................................... 25
2.10) Biostatistical analysis ................................................................. 25

3) Results .............................................................................................. 26
   3.1) YAP/TAZ characterization and regulation by confluence and migration .............. 26
   3.2) Signaling pathways involved in YAP/TAZ localization and activity ..................... 28
   3.3) Role of YAP/TAZ in CPC proliferation ......................................................... 33
   3.4) Role of cell tension and YAP/TAZ in CPC cardiac commitment .......................... 35
   3.5) YAP/TAZ activity in substrates displaying different stiffness .............................. 37
       3.5.1) Activity on soft substrates: Matrigel™ ..................................................... 37
       3.5.2) Activity of YAP/TAZ on physiologically relevant surfaces: coated polyacrylamide gels with controlled stiffness ......................................................... 39
       3.5.3) Activity of YAP/TAZ on stiff surfaces (PCL films) ....................................... 44
   3.6) Activity of YAP/TAZ in response to dynamic changes in surface stiffness ........... 46
   3.7) Influence of cell shape in YAP/TAZ activity .................................................... 48
   3.8) Differential gene expression with surface stiffness and YAP/TAZ supression ........ 52

4) Conclusions and discussion .................................................................. 54
   4.1) Overview of YAP/TAZ regulation and activity .................................................. 54
   4.2) Future perspectives ..................................................................................... 56

5) References ........................................................................................... 62
List of Figures and Tables

Figure 1 – Current strategies for cardiac tissue engineering. .......................................................... 5
Figure 2- Stem cell fate determination .......................................................................................... 7
Figure 3 – Mechano-structural factors influence stem cell fate. .................................................... 9
Figure 4 – General mechanotransduction pathways. .................................................................. 13
Figure 5 – YAP and YAZ structure. ............................................................................................... 14
Figure 6 - Regulation and functions of YAP/TAZ transcriptional modulators ............................ 17
Figure 7– Polyacrylamide gels preparation .................................................................................. 23
Figure 8 - Preparation of cross-linked PCLs.................................................................................. 24
Figure 9 - YAP and TAZ display an overlapping signal both in cytoplasmic and nuclear expression in cMPC<sup>Sca-1</sup> ................................................................................................................ 26
Figure 10 – YAP colocalizes with GATA-4 and phosphorylated paxillin in cMPC<sup>Sca-1</sup> ....... 27
Figure 11 - YAP/TAZ activity is regulated by confluence but not by migration in cMPC<sup>Sca-1</sup> ...... 27
Figure 12 - Percentage of cMPC<sup>Sca-1</sup> expressing nuclear YAP in different conditions .......... 28
Figure 13 – Effect of pharmacological inhibitors on cMPC<sup>Sca-1</sup> morphology and focal adhesion organization. ............................................................................................................................... 29
Figure 14– Effect of pharmacological inhibitors of defined signaling pathways involved in cell mechano-transduction on cMPC<sup>Sca-1</sup> cells. ................................................................................ 30
Figure 15 – Effect of pharmacological inhibitors on YAP/TAZ nuclear activity on cMPC<sup>Sca-1</sup> .... 30
Figure 16- Percentage of cMPC<sup>Sca-1</sup> expressing nuclear YAP when treated with pharmacological inhibitors. .................................................................................................................................... 31
Figure 17 - Analysis of YAP expression in cMPC<sup>Sca-1</sup> by Western Blot. ........................................ 32
Figure 18- Influence of pharmacological inhibitors’ treatment on cMPC<sup>Sca-1</sup> migration and zyxin nuclear shuttling. ....................................................................................................................... 33
Figure 19- Western Blot for YAP and TAZ expression in control cMPC<sup>Sca-1</sup> and silenced cells. . 34
Figure 20- Role of YAP/TAZ in CPC proliferation ....................................................................... 34
Figure 21 – Role of tension and YAP/TAZ on CPC cardiac commitment ...................................... 36
Figure 22– YAP/TAZ localization in cMPC<sup>Sca-1</sup> grown on soft substrates ............................. 37
Figure 23 – Regulation of YAP/TAZ in cMPC<sup>Sca-1</sup> vasculogenesis, on soft substrates .......... 39
Figure 24 – YAP/TAZ activity on cMPC<sup>Sca-1</sup> seeded onto polyacrylamide gels................. 41
Figure 25 – Percentage of cMPC<sup>Sca-1</sup> expressing nuclear YAP or TAZ when seeded onto polyacrylamide gels coated with either collagen or fibronectin................................. 42
Figure 26 – Role of YAP/TAZ in cell adhesion onto PA gels........................................................... 43
Figure 27 – Influence of YAP/TAZ in cellular adhesion to substrates displaying different stiffnesses................................. 44
Figure 28 – Activity of YAP/TAZ on stiff PCL films ................................................................. 45
Figure 29 – Percentage of cells expressing nuclear YAP when seeded onto PCL films exhibiting different stiffnesses ........................................................................................................ 46
Figure 30 – YAP/TAZ activity in dynamic surface changes .......................................................... 47
Figure 31 – Role of YAP/TAZ during dynamic changes of the surface where cMPC^Sca-1 is seeded. ................................................................. 48
Figure 32 – cMPC^Sca-1 shape when seeded onto micropatterned surfaces displaying different adhesive areas .................................................................................................................. 49
Figure 33 - Regulation of YAP/TAZ activity by cell shape .......................................................... 50
Figure 34 - Regulation of cMPC^Sca-1 cardiac commitment and focal adhesion maturation by cell shape ............................................................................................................................... 50
Figure 35 – Role of YAP/TAZ on TBX5 activation and vinculin maturation in micropatterned surfaces ........................................................................................................................... 51
Figure 36 - Role of YAP/TAZ in mechanotransduction of cardiac stem/progenitor cells .......... 56

Table 1 – Differences in gene expression between YAP/TAZ^-/- and YAP/TAZ^+/+ cMPC^Sca-1, both seeded onto TCPS .................................................................................................................. 52
Table 2 – Differences in gene expression between cells seeded onto Pa gels displaying 0.5 KPa and 40 KPa stiffness, respectively ........................................................................................................ 53
Table 3 – Proposed future studies - purpose and strategy ........................................................................ 60
List of Abbreviations

CVD- cardiovascular disease
MI- myocardial infarction
CTE- cardiac tissue engineering
ECM- Extracellular matrix
PEG - poly (ethylene glycol)
MSC - mesenchymal stem cells
ES - embryonic stem
CPC- cardiac stem/progenitor cells
Lin - lineage markers
Sca-1 - Stem Cell Antigen-1
DMSO - dimethyl sulfoxide
PGS - poly glycerol sebacate
PGA - poly glycolic acid
PLA - poly lactic acid
FA- focal adhesions
FAK - focal adhesion kinase
PY- phosphotyrosine
MLC- myosin light chain
MLCK - myosin light chain kinase
ROCK - Rho kinase
YAP - Yes-associated protein
TAZ - transcriptional co-activator with PDZ-binding motif
EMT - epithelial to mesenchymal transition
PBS - Phosphate buffer saline
PA - Polyacrylamide
PCL - Poly-ε-caprolactone
SEM - standard error of mean
TCPS - tissue culture polystyrene
1) Introduction

1.1) Cardiac diseases and tissue engineering strategies

Cardiac diseases represent a dominant cause of death worldwide, including low and middle income countries\(^1\). In fact, in 2005, the total number of cardiovascular disease (CVD) fatalities (mainly coronary heart disease, stroke, and rheumatic heart disease) had increased globally to 17.5 million from 14.4 million in 1990. The World Health Organization estimates there will be about 20 million CVD casualties in 2015, accounting for 30 percent of all deaths worldwide\(^2\). Thus, CVD is today the largest single contributor to global mortality and is predicted to dominate mortality trends in the future\(^3\). One of the most common cardiac pathologies is ischaemic heart disease, which is characterized by reduced blood supply to the heart muscle. Obstruction of coronary arteries leads to myocardial infarction (MI) with the associated loss of cardiomyocytes\(^4\). MI typically results in fibrotic scar formation and permanently impaired cardiac function because the myocardial tissue exhibits a very limited regenerative capacity\(^5\).

Currently, the only suitable treatment for heart failure is cardiac transplant, which is restricted by the shortage of organ donors for transplantation and the process of graft rejection over time, with the subsequent need for immunosuppressants\(^6\). Therefore, new therapeutic strategies addressing this problem are eagerly sought. As such, cardiac tissue engineering (CTE) may provide a novel approach to treat heart disease, by replacing, repairing or regenerating the damaged myocardium using tissue- and/or cell-based strategies\(^6\).

In the classical tissue engineering approach, different cell types are combined with scaffolds or hydrogels (that may present cells growth factors along with mechanical support) and cultivated in bioreactors\(^6\). As such, in order to achieve a successful therapeutic outcome, it is necessary to optimize all these parameters so that cells are efficiently integrated in the scaffold prior to transplantation, and their phenotype is adequate for the desired therapy. Alternatively, when scaffold-free approaches are used, a right number of cells have to be delivered to the injured site, and cell viability needs to be maintained in order to accomplish tissue repair/regeneration.

1.1.1) Scaffolds and bioreactors used in cardiac tissue engineering

The scaffolds so far proposed for CTE are very diverse, reflecting the lack of success achieved so far, which led to the investigation of alternatives. Both naturally-derived and
synthetic scaffolds have been suggested as suitable cell delivery systems for CTE applications\textsuperscript{5,6}. Natural scaffolds include polymers such as alginate\textsuperscript{7}, chitosan\textsuperscript{8}, collagen-based\textsuperscript{9}, fibrin\textsuperscript{10}, gelatin\textsuperscript{11} and glycosaminoglycan\textsuperscript{12}, and fibrous or porous scaffolds (that aim at mimicking the nanoscale structure of the native extracellular matrix (ECM) where cells are embedded) composed of collagen, elastin, fibronectin and laminin fibers or sponges\textsuperscript{6}. Synthetic scaffolds typically include poly (glycerol sebacate)(PGS), poly glycolic acid (PGA) and poly lactic acid (PLA)\textsuperscript{6}. Moreover, engineered cardiac tissue has also been generated using scaffolds derived from decellularized native tissues, resulting in acellular structures that preserve the architecture of native ECM of the heart\textsuperscript{13}. Alternatively, functional engineered cardiac tissue can be generated by a scaffold-free approach, for instance by a cell sheet strategy wherein individual cell monolayers or cell sheets were stacked to create thick cardiac tissue\textsuperscript{14}. Furthermore, cells can be injected intravenously into coronary arteries or directly into the myocardium. However, this approach revealed several adverse effects\textsuperscript{15}, e.g. extensive cell loss through the vasculature, very low efficiency of engraftment\textsuperscript{16} and survival in the inflammatory environment of infarcted myocardium. Moreover, it has been reported that a major fraction (approx. 90\%) of the delivered cells vanish within a week\textsuperscript{17}.

However, the native myocardium displays a very complex macro- to nano-scale structural organization, which is essential to proper cardiac function\textsuperscript{6}. Some researchers have tackled this problem by focusing on the functional improvement of engineered cardiac tissue by mimicking the aligned structure of the native myocardium\textsuperscript{18}. Topographical cues can influence properties of cardiomyocytes, including cell attachment, cell hypertrophy, binucleation, remodelling of ion channels, release of atrial natriuretic peptide, biomechanical stresses, and structural remodeling\textsuperscript{19}. In order to provide such anisotropic cues, different strategies have been explored such as nanopatterning of poly (ethylene glycol) (PEG) hydrogels\textsuperscript{20}, rotary spinning of polymer nanofibers\textsuperscript{21}, and stamping of ECM proteins in lanes on thin films and hydrogels\textsuperscript{19}. Other scaffolds have been designed with controllable stiffness and anisotropy, suitable for cardiac tissue engineering\textsuperscript{22}. Micro- and nanoscale techniques were even used to create stem cell niches to regulate their differentiation towards the myocardial lineage\textsuperscript{23,24}.

Cells seeded in scaffolds have also been cultured in dynamic conditions using bioreactors able to provide mechanical stimuli favorable to the desired phenotype and enhance oxygen flow and the removal of catabolic products from scaffold inner layers. For this, different setups were used such as perfusion bioreactors that provide adequate oxygen supply to the scaffold, otherwise impaired in static conditions\textsuperscript{25}. Furthermore, since previous studies demonstrated the relevance of physical stimuli for the morphology, mechanical properties and
function of engineered cardiac tissues\textsuperscript{6}, cyclic mechanical stimulation was used to create thick cardiac tissues that were implanted in a rat MI model\textsuperscript{26}. In addition, it is essential that cells within engineered cardiac tissues are capable of synchronously responding to electrical pacing in order to develop proper excitation-contraction coupling\textsuperscript{27}. As such, bioreactors that provide electrical field stimulation (e.g. with byphasical pulses\textsuperscript{28}) were used to induce synchronous beating of cardiomyocytes in engineered cardiac tissues\textsuperscript{27}.

1.1.2) Stem cell sources for cardiac tissue engineering

If the number of possibilities concerning scaffolds and bioreactors is already huge, then the variety of cell types considered to achieve cardiac regeneration is absolutely stunning\textsuperscript{4,5,29}. In fact, different cell populations have been explored that include: (i) skeletal myoblasts, whose ability to give rise to cardiac-like contractile cells\textsuperscript{30} is acknowledged together with a strong resistance to ischemia\textsuperscript{31}, although they do not make the proper electric coupling\textsuperscript{32}; (ii) fetal/neonatal cardiomyocytes which can engraft the host heart with relative success and engage in appropriate electrical coupling\textsuperscript{33}; (iii) endothelial progenitor cells, a subset of hematopoietic cells found in the bone marrow and in blood that have the potential to differentiate into endothelial cells, thus promoting angiogenesis\textsuperscript{34}; (iv) mesenchymal stem cells (MSC), that play an important role in paracrine growth factor signaling and allow MHC-mismatched allogeneic transplantation due to their low immunogenicity\textsuperscript{35}; (v) embryonic stem (ES) cells, whose wide differentiation potential can be driven towards the cardiomyocyte lineage\textsuperscript{36} provided that the correct signals are given; and even other populations less extensively studied such as monocytes\textsuperscript{37}, aortic valve interstitial cells\textsuperscript{38} and smooth muscle cells\textsuperscript{39}. However, each of these sources introduce a particular grade of disadvantage, such as incomplete characterization, requirement of labor-intensive procedures with low efficiency of cell extraction and expansion, controversial ethical/moral issues (mainly in the case of ES cells) and risk of immunogenicity in case of allogenic transplant (lower for MSC)\textsuperscript{29,40}.

Endogenous cardiac stem/progenitor cells

Besides the aforementioned cell sources, the heart harbors several populations of putative cardiac stem/progenitor cells (CPC)\textsuperscript{41}, either autochthonous, e.g. set aside in the developing heart and thus entrapped in the differentiated adult tissue, or allochthonous, e.g. recruited from the bone marrow (or other organs) through bloodstream\textsuperscript{42}. These cardiac stem/progenitor cell populations can be identified by the presence of cell surface proteins, along with other markers such as lineage-associated transcription factors, and were shown to follow the hallmarks of stemness: self-renewal capability, multipotential and clonogenicity\textsuperscript{43}.
Moreover, contrarily to what was previously thought, the heart is not a terminally differentiated organ, i.e. moderate renewal of cardiomyocytes throughout life was already reported\(^{44-46}\). Nevertheless, this process is very slow\(^{46}\), and in pathological conditions such as MI, limited numbers of cardiac stem cells, scar formation, an unfavorable milieu associated with the injured tissue (inflammatory environment and lack of vascular supply), along with continuous contractile activity of the injured heart may all limit myocardial repair and regeneration\(^{47}\).

Several CPC populations have been identified within the mammalian heart, and will be briefly described in this section. (i) Cells expressing the LIM-homeodomain transcription factor Islet-1 (Isl-1\(^+\)), that can be identified and isolated from embryonic and post-natal mouse, rat and human myocardium and were shown to expand \textit{in vitro} on a cardiac mesenchymal feeder layer and differentiate into functional cardiomyocytes\(^{48}\). (ii) Cells expressing the Stem Cell Factor (SCF) receptor c-kit and lacking mature lineage markers (Lin) comprise another CPC population. Lin\(^-\) c-Kit\(^+\) CPC are self-renewing, clonogenic, and multipotent, giving rise to myocytes, smooth muscle cells, and endothelial cells. When injected into an ischemic heart, these cells or their clonal progeny reconstitute contractile myocardium\(^{43,49}\). (iii) CPC expressing Stem Cell Antigen-1 (Sca-1\(^+\)) constitute another important cell subset\(^{50}\). The latter can be isolated from adult mouse hearts (where cardiomyocytes comprise 20–30% of the total cellular fraction, the remainder including fibroblasts, vascular smooth muscle, and endothelial cells) by immunomagnetic sorting techniques\(^{51}\). Sca-1\(^+\) CPC express early cardiac transcriptional regulators (e.g. GATA-4, MEF-2c and TEF-1) but do not display cardiac structural proteins, e.g. α and β myosin heavy chain and cardiac actin\(^{51}\). \textit{In vitro} differentiation into cardiomyocytes is modest and, as with the c-Kit\(^+\) CPCs, appears to require prior treatment with demethylating agents such as cytosine analog 5-azacytidine (5-Aza) and dimethyl sulfoxide (DMSO). Overall, only a small subpopulation of cells express cardiac transcription factors, display sarcomeric structures, and form spontaneously beating cardiomyocytes with calcium transients\(^{51}\). Nevertheless, when freshly isolated Sca-1\(^+\) cells were injected intravenously into mice after ischemia-reperfusion, they were shown to target the border zone of the injured myocardium and differentiate into cardiomyocytes, expressing important cardiac markers such as sarcomeric α-actin, cardiac Troponin I, and connexin-43, with and without fusing with host cells\(^{52}\). The presence of a resident cell population having similar characteristics has been recently described in the human heart\(^{53}\). (iv) An additional multipotent and clonogenic cell population has been identified within the myocardium as well as in other tissues by their ability to efflux toxic compounds like Hoechst and rhodamine dyes. This so-called side population (SP), represent 2% of total cardiac cells and express Sca-1, ATP-binding cassettes
(Abcg2), and multi drug resistance-1 (MDR-1), but not c-Kit, CD34, and CD45 (hematopoietic markers). When injected into an injured heart, SP cells could differentiate into cardiomyocytes, endothelial cells, or smooth muscle cells\textsuperscript{54}. Finally, CPCs can be clonally expanded from human myocardial biopsies, as these cells are spontaneously shed from human surgical specimens and murine heart samples in primary culture and self-organize into spontaneously beating clusters (cardiospheres). Cells from cardiospheres differentiate in co-culture with neonatal rat ventricular cardiomyocytes and, when implanted into a model of mouse infarction, they establish functional cell–cell connections and differentiate into the three main cardiac lineages (cardiomyocytes, endothelial cells, smooth muscle cells)\textsuperscript{55}.

Current strategies for cardiac tissue engineering are summarized in Figure 1.

**Figure 1 – Current strategies for cardiac tissue engineering.** Cells from different sources are harvested, purified and expanded in culture, and seeded into appropriate scaffolds inside bioreactors that provide several types of stimulation to enhance cell proliferation and integration with the scaffold. Alternatively, scaffold-free systems can be used such as direct injection of cells (intrapericardial, intravenously, among other routes of delivery) and cell sheets. Finally, cells are implanted into the heart.
1.2) Stem cell fate determination

1.2.1) Overview

Even though the different CTE strategies explored above seem promising, there are still serious challenges that need to be solved before translation into clinical practice\textsuperscript{56,57}. The most obvious is the choice of the appropriate cell source for cell-based therapy. Thus far, autologous bone marrow derived stem cells, skeletal myoblast, MSCs, and circulating blood-derived progenitor cells have been used in human clinical trials\textsuperscript{57} and others using c-Kit\textsuperscript{+} cardiac resident stem cells\textsuperscript{58}, or CPC derived from cardiospheres\textsuperscript{59} are currently in progress\textsuperscript{60}. As stated above, all the sources currently explored have disadvantages that need to be overcome for a successful and sustainable cell-therapy, e.g. need to obtain large cell numbers, immunological rejection in case of allogeneic settings; autologous sources might also be compromised by low “off-the-shelf” availability\textsuperscript{57}. Furthermore, some bioengineering roadblocks need to be addressed in order to improve existing CTE strategies, such as (i) insufficient scaffold seeding with non-proliferative cells (i.e. cardiomyocytes), to the point of tissue-like cell densities\textsuperscript{17}; (ii) insufficient nutrient delivery to the inner layers of the engineered bio-constructs, for which perfusion bioreactors are being developed\textsuperscript{61} and (iii) cell delivery with sufficient engraftment rates, low death rates of transplanted cells\textsuperscript{62} and suitable mechanical properties of the scaffold and/or delivery system\textsuperscript{56}. Finally, the limited understanding of the mechanisms that modulate the repair of the myocardium, likely related to the control/ modulation of the stem cell fate, might explain the modest success of the existing strategies\textsuperscript{57}. This problem comprises the central topic of this thesis and will be explored in the sections below.

It has been proposed that tissue-resident stem cell fate determination (e.g. cell response - proliferation, self-renewal, differentiation, migration) to different stimuli is influenced by the concerted integration of multiple parameters. These include overall extracellular matrix (ECM) composition, along with biochemical (cytokines, hormones, growth factors), physico-chemical (oxygen tension, pH, temperature) and mechano-structural (stiffness, rugosity, porosity) signals coming from the microenvironment in which stem cells reside\textsuperscript{42} (Figure 2A). Therefore, a complete understanding of the composition of the cardiac natural microenvironment in physiological conditions as well as insight on how it is affected by pathological events is required. Most studies agree that progenitor cells (single or clustered) are settled in specific anatomic and functional locations (niches)\textsuperscript{62}, that mediate signals maintaining stem cell self-renewal and multipotency or inducing their commitment in response to tissue specific needs\textsuperscript{63}. It is believed that the heart stem/progenitor cell niches have only functional and temporal dimensions and are characterized by a dynamically
symmetric array of signals delivered by neighboring non-progenitor cells (including fibroblasts and cardiomyocytes) and ECM soluble and insoluble components (mostly collagen) (Figure 2B). It has been proposed that stem cells are inherently unstable (i.e., prone to chaotically adopt multiple phenotypes) and only a complex array of opposite symmetric signals maintains progenitor cells in a metastable quiescent state, even for long periods of time, preventing aging processes and preserving their multipotency. A very small modification in local or long-range extracellular signaling can attract progenitor cells towards one of many possible states. Once the symmetry of signals maintaining the metastable equilibrium has been broken by internal and/or external perturbations, progenitor cells engage in differentiating pathways and tissue assembly processes in an environment characterized by the aforementioned parameters, changing with time to generate a dynamic multicomponent template of microenvironmental symmetry (Figure 2C).

**Figure 2- Stem cell fate determination.** A- CPC fate determination results from the concerted integration of different parameters: biochemical, physico-chemical and mechano-structural factors. B- The native microenvironment of the heart comprehends different cell types such as cardiac stem/progenitor cells, fibroblasts, smooth muscle cells, endothelial cells, and cardiomyocytes, embedded in ECM rich in collagen. It presents different cues to CPC. C- The microenvironment of the heart dynamically changes with time either maintaining CPC in a metastable quiescent state or inducing different cellular responses such as migration proliferation and differentiation. Adapted from 42.
This concept has been demonstrated in vitro, as the differentiation of different CPC populations to cardiomyocytes was shown to result from a fine-tuned combination of specific biological and physical factors, including scaffold geometry and stiffness\textsuperscript{64,65}, rather than solely biological factors. Since the work developed for this thesis is focused on the influence of mechano-structural parameters in the determination of CPC fate, these factors will receive special attention.

1.2.2) Influence of mechano-structural factors

Several mechano-structural factors, including physical signals such as tensile, compressive, shear, osmotic, and fluid stresses often arising from interactions with the ECM, control stem cell fate\textsuperscript{66}. Cell shape has been pointed out as a regulator of stem cell fate as, for instance, growth and differentiation of capillary endothelial cells are in part regulated by ECM-induced changes in cell shape\textsuperscript{67}. Cell shape can be modulated by several approaches such as artificial matrices, type of culture system (flattened in 2D vs. round in 3D) and micropatterned surfaces where the area of cell attachment is controlled (by selective coating with extracellular matrix proteins in restricted areas)\textsuperscript{66,68} (Figure 3A). Another example illustrating the relevance of this parameter is the control of MSC lineage commitment between adipogenic and osteogenic phenotype in this micropatterning approach\textsuperscript{69} (different shapes favor alternative phenotypes).

Another relevant parameter is surface/ECM nanotopography as cells have the ability to sense micro-and even nanoscale geometric cues from their environment, such as differences in molecular conformation, surface topography or roughness and fiber diameter\textsuperscript{66}. In fact, the nanoscale geometry and size of the features of the ECM may have significant effects on a number of cell properties, such as attachment/adhesion, migration, and proliferation, although the molecular mechanisms associated with these processes remain to be elucidated\textsuperscript{66}. As illustrated in Figure 3B, changes in the nanotopography of the substrate may influence adhesion by altering the degree of cell spreading, along with clustering of cell surface receptors (integrins) and other cell-adhesion molecules\textsuperscript{70}.

Importantly, the stiffness of the ECM/surface is also a relevant mechanical parameter sensed by the cells and modulating stem cell fate. Cells that attach to a substrate have been shown to exert contractile forces, resulting in tensile stresses in the cytoskeleton\textsuperscript{71}. The relationship between these forces and the mechanical stiffness, or elasticity, of the ECM may have a major influence on cell behavior such as migration, apoptosis, and proliferation\textsuperscript{66}. In the case of stem cell fate, the most illustrative example is the modulation of lineage commitment and differentiation of MSC by changes in stiffness of the surface where cells were seeded.
Human MSC were shown to specify lineage and commit to phenotypes with extreme sensitivity to tissue-level elasticity, as soft matrices that mimic brain are neurogenic, stiffer matrices that mimic muscle favor myogenic commitment, and rigid matrices that mimic collagenous bone proved to be osteogenic\cite{72,73}.

Finally, other mechanical factors play a role in cell fate determination, as cells are constantly exposed to a variety of mechanical stimuli through the actions of muscle forces, gravity, blood flow, and other physical processes\cite{74} (Figure 3C).

**Figure 3 – Mechano-structural factors influence stem cell fate.** A- Cell shape, modulates cellular morphology and actin cytoskeleton tension with downstream responses controlling cell fate. B- Surface/ ECM nanotopography is also a relevant factor as it leads to clustering of integrins and subsequent alterations in cell signaling. C- Cells are exposed to a variety of physical signals including tensile, compressive, shear, osmotic and fluid stresses. These, along with ECM/ surface stiffness induce different degrees of ECM stretching and tension of the cytoskeleton and nucleus through focal adhesions, generating different cell responses such as modulation the activity of osmotically sensitive ion channels. Adapted from \cite{66} and \cite{125}.
Several examples of mechanical modulation of biological responses can be presented: dynamic mechanical compression can significantly increase the expression of chondrocytic markers (e.g., Sox-9, type II collagen, and aggrecan) in bone-marrow-derived MSCs encapsulated in a hydrogel. However, changes in mechanical parameters of the environment affect other factors as well, resulting in complex physical environments that consist of time-varying stress, strain, fluid flow, and pressure and, potentially, other biophysical changes such as osmotic pressure which may alter the structure of ECM proteins and the activity of soluble growth factors and cytokines. As such, it is difficult to isolate the in vivo effects of mechanical force from indirect effects associated with mechanically driven changes in adhesive cues and/or paracrine signaling and, as described earlier, subsequent changes in cell shape.

1.3) Mechanotransduction signaling pathways

Taking into account all the aforementioned mechano-structural factors, a central question is to understand how cells can perceive such mechanical stimuli in order to generate an appropriate response. This process is termed mechanotransduction and encompasses the molecular mechanisms by which cells sense and respond to mechanical changes. Central pathways and players will be explored in this section.

1.3.1) Integrins as mechanoreceptors

Travelling from the outside to the inside of the cells, first-line players in mechanotransduction are transmembrane adhesion receptors that mediate mechanical coupling between ECM and the cytoskeleton. In this field, integrins are considered mechanoreceptors of excellence as, unlike other receptors, when applied with direct mechanical stress using magnetic twisting cytometry techniques, cell stiffness (elastic modulus) increases in direct proportion as the level of stress that was raised. Integrins are heterodimeric transmembrane proteins formed by an α and a β chain that require Mg$^{2+}$ or Ca$^{2+}$ to bind to the ECM proteins. Integrin regulation and function is very complex as it comprises a multitude of different families and because they are involved in different cellular processes. Yet for the purpose of the herein thesis, relevance is mainly given to the role of integrins in mechanotransduction. As such, integrins act as bidirectional signal transducers: interactions with cytoskeletal adaptor proteins that associate with the cytoplasmic tails of integrins control the ligand-binding activity of the receptors and, conversely, the extracellular ligand-binding, clustering or pulling on integrins triggers the recruitment of cytoskeletal adaptor proteins.
1.3.2) **Focal adhesions as mechanochemical signaling complexes**

Cells exert tractional forces on the substratum to which they are attached through adhesive structures such as focal adhesions (FA), composed of multiprotein complexes, which function as molecular scaffolds and mediate signaling events. The formation of these structures requires tension generated by actomyosin fibers within the cell. FAs are initiated by the activation of integrin extracellular heads’ affinity for ECM through association of their cytoplasmic tails with the vinculin and actin-binding protein talin. Early after integrin activation, the adapter protein paxillin is recruited by an unknown mechanism, and more integrins cluster into FA. Further FA growth is accompanied by the recruitment of the actin-bundling protein α-actinin, with which talin may establish a link between integrins and the actin cytoskeleton. Myosin II confers cell tension as it crosslinks actin fibers. It is also part of the α-actinin–actin network, connected to the integrin–ECM linkage. This tension promotes elongation of an adhesion-associated actin bundle where cytoskeletal adaptor proteins vinculin and zyxin accumulate. Recruitment of phosphorylating proteins (kinases) such as Focal Adhesion Kinase (FAK) induces tyrosine phosphorylation of early FA proteins, including FAK (by auto-phosphorylation), paxillin, and p130cas, that then act as scaffolds for phosphotyrosine (PY)-binding SH2 domain–containing proteins. As such, FAs comprise dynamic, multi-protein structures sense the ECM both chemically and physically, and respond to external and internal forces by changing their size and signaling activity. The complex nature of FAs and the myriads of proteins it includes, along with their interaction with the mechanoreceptors (integrins) have led to a view of the focal adhesion as a mechanochemical signaling machine. These sites represent points of convergence for signals from soluble chemicals, insoluble adhesive molecules, and mechanical stresses.

1.3.3) **Actin cytoskeleton as a global signal integrator**

Even if the mechanical stresses are sensed locally at the FAs, the changes in cell response/behavior are exerted globally. For example, studies carried out with cells labeled with GFP-mitochondria showed that stress application to integrins results in mitochondrial displacements over twenty micrometers from the site of force application. As such, there is a long-range force transfer within the cells mediated by the cytoskeleton, which is therefore considered the global signal integrator. In short, although cells may sense and respond locally to forces applied to integrins, somehow the cell is able to integrate these cues with information conveyed by the overall state of the global cytoskeleton in order to decide along a particular response.
FAs are generally linked by actin stress fibers that are in a state of balanced isometric contraction, as some proteins such as non-muscle myosin II crosslink actin fibers conferring them additional stability and tension. The force transmitted to sites of adhesion derives from the interaction of myosin II with actin filaments that attach to these sites. Myosin II activity is regulated by myosin light-chain (MLC) phosphorylation, which is either directly positively regulated by MLC kinase (MLCK) or Rho kinase (ROCK) or negatively regulated by MLC phosphatase, which is itself phosphorylated and inhibited by ROCK. MLC phosphorylation activates myosin, resulting in increased contractility and transmission of tension to sites of adhesion. Interplay of all these pathways and signaling proteins (along with other structures not reviewed in this thesis such as microtubules) makes mechanotransduction a very complex field so that more in depth studies are required in order to fully understand cell mechanobiology. As an example, clustering of integrins into focal adhesions and focal complexes is regulated by the actin cytoskeleton. In turn, actin dynamics are governed by Rho family GTPases. Integrin-mediated adhesion activates these GTPases, triggering assembly of stress fibers.

1.3.4) Nuclear shuttling proteins

Importantly, other mechanisms for conveying long-range and long-term cell responses after mechanical stresses are based on protein shuttling to the nucleus. Integrin-mediated signaling has shown to induce profound changes in gene expression that can affect cell fate, progress through the cell cycle, and state of cellular differentiation. Since integrins do not exhibit any catalytic activity, it is thought that signaling occurs via the ability of the receptors to regulate the activities of non-covalently associated signaling partners. As such, some proteins that constitute the focal adhesions have shown to possess the ability to shuttle between the cytoplasm and the nucleus. These include the zyxin and paxillin protein families, which have two major domains, an N-terminal half that contains proline-rich sequences (some of which are SH3 domain binding sites) and a C-terminal LIM domain region, comprised of double zinc finger structures that dock specific protein partners. Furthermore, zyxin and paxillin protein families exhibit a leucine-rich nuclear export signal (NES) in the N-terminal domain, and treatment of cells with leptomycin B, an inhibitor of Crm1-dependent nuclear export, causes zyxin/paxillin family proteins to accumulate in the nucleus. Several functions were attributed to these shuttling processes, the most relevant being alteration in gene expression, either by direct binding to DNA, or (most likely) by sequestering nuclear-active proteins (such as transcriptional co-activators/ repressors) from the cytoplasm to the nucleus. An illustration of such pathways is displayed in Figure 4.
Figure 4 – General mechanotransduction pathways. 1) Cells sense the external microenvironment or ECM by the binding of proteins of the ECM to integrins (mechanoreceptors). 2) Recruitment of several proteins to sites of ECM-integrin linkage leads to the formation of focal adhesions. Phosphorylation of proteins by different kinases provides binding sites in scaffolding proteins such as vinculin and talin. 3) After focal adhesion maturation, actin cytoskeleton is assembled, acting as a global signal integrator in mechanotransduction. Activity of signaling pathways such as RhoA/ROCK/MLCK confers F-actin tension and contractility due to the activation of proteins crosslinking the cytoskeleton. 4) Some of the proteins involved in focal adhesion dissociate from the multiprotein complexes and shuttle to the nucleus either alone or carrying partners with nuclear activity with them. 5) Once in the nucleus, nuclear active proteins such as transcription factors and modulators trigger gene expression. 6) mRNA exits the nucleus leading to the production of important proteins in response to the mechanosensing. 7) Different cellular responses are triggered after external stimuli are perceived, namely regulation of stem cell fate.
In addition, other mechanotransduction pathways exist based on conformational changes of cell surface receptors mediated by stretching induced by external forces. These include direct stretching of integrins, deformation of gap junctions containing calcium-sensitive stretch receptors, alteration of ion channel permeability on the cell membrane and activation of growth factor receptor signaling cascades in the absence of the ligands. They converge on the activation of phosphorelay systems (Mitogen-activated protein (MAP) kinases), with subsequent downstream changes in gene expression. However, because the latter do not constitute the aim of this thesis so they will not be further reviewed.

1.4) YAP and TAZ transcriptional modulators

1.4.1) Structural characterization

Identifying key molecules involved in stem cell determination is crucial for achieving a higher understanding of stem cell biology to be harnessed in tissue engineering therapies. In this context, Yes-associated protein (YAP) and highly related (structurally and functionally) transcriptional co-activator with PDZ-binding motif (TAZ) appear to be promising candidates. Structurally, both TAZ and YAP contain (i) a 14-3-3 binding motif; (ii) a single or duplicated WW domains (1 in TAZ, 2 in YAP); (iii) an extended coiled-coiled region within a larger transcriptional regulatory domain; (iv) multiple sites of phosphorylation, and (v) a C-terminal motif that can interact with PDZ domain-containing proteins, as represented in Figure 5. The WW domains of TAZ and YAP bind strongly to the sequence motif Pro-Pro-X-Tyr, which can be found within the regulatory regions of a large number of transcription factors, suggesting that TAZ and YAP may function as general transcriptional modulators during the execution of many developmental programs. The C-terminus of YAP/TAZ likely interacts directly with core transcriptional machinery to stimulate gene expression. Moreover, the PDZ motifs allow interaction of YAP/TAZ with several transcription factors such as: Runx2 (runt-related transcription factor 2), PPAR (peroxisome proliferator-activated receptor), TBX5 (T-box transcription factor 5), TEADs (TEA domain family members), with subsequent transcriptional modulating activity.

[Figure 5 – YAP and YAZ structure. Both proteins have WW domains, an extended coiled-coil region, multiple sites of phosphorylation, a 14-3-3 binding motif and a C-terminal motif that can interact with PDZ domain-containing proteins. Adapted from]
1.4.2) Function in different cell types

The discovery of the YAP/TAZ interactome led to the finding of the involvement of these molecules in different processes, either acting as transducers of Hippo pathway or responding to other stimuli. These include: (i) stem cell fate determination: favoring MSC osteogenic differentiation rather than adipogenic and enhancing MyoD-induced myogenic differentiation in myoblasts; (ii) stem cell proliferation: inducing expansion of intestinal stem cells, restraining cardiomyocyte proliferation and heart size and maintaining human embryonic stem (hES) cell self-renewal; (iii) in cancer: overexpression of YAP/TAZ in normal mammary cells causes morphologic changes characteristic of tumorigenesis, promoting cell migration and invasion, with enhanced proliferation and epithelial to mesenchymal transition (EMT). Moreover, the physiological function of TAZ could be investigated in vivo by the generation of knockout mice, which, surprisingly, just suffer a minor skeletal defect though TAZ plays a crucial role in MSC differentiation. Nonetheless, TAZ knockout (KO) mice develop two severe abnormalities: polycystic kidney disease and emphysema, implicating TAZ role in renal and lung development.

1.4.3) Regulation by cellular pathways

Regulation of YAP/TAZ activity is extremely complex due to the involvement of several signaling pathways and external stimuli, and is represented in Figure 6. YAP/TAZ nuclear activity is negatively regulated by the Hippo tumor supressor pathway, composed by a kinase cascade wherein MST1/2 (macrophage stimulating 1/2), complexed with its regulatory subunit SAV1, phosphorylates and activates LATS1/2 in complex with its regulatory subunit MOB1, resulting in phosphorylation of YAP/TAZ. When phosphorylated, YAP/TAZ bind to 14-3-3 proteins and remain sequestered in the cytoplasm, thus cannot go to the nucleus where transcriptional regulation occurs. On the other hand, dephosphorylation of YAP/TAZ by PP1 (phosphoprotein phosphatase 1) coupled with ASPP2 (ankyrin repeat-containing, SH3 domain-containing, and proline-rich region-containing protein 2) prevents 14-3-3 sequestration and enhance YAP/TAZ nuclear levels, activating these proteins. Highlighting the complexity of this regulation, α-catenin prevents YAP/TAZ dephosphorylation by PP1/ASPP2, negatively regulating YAP/TAZ activity, e.g. impairing epidermal stem cell proliferation. Furthermore, YAP/TAZ protein stability is controlled by a phosphodegron recognized by the F-box protein β-TrCP (beta-Transducin repeat containing protein) and ubiquitylated by the SCF/CRL1β-TrCP E3 ligase. Phosphorylation of a phosphodegron YAP/TAZ by LATS primes it for further phosphorylation by CK1ε and subsequent binding by β-TrCP, leading to YAP/TAZ proteasomal
Cell confluence is a known negative regulator of YAP/TAZ activity in vitro, both by activating Hippo pathway, and through formation of protein complexes in tight or adherens junctions (structures connecting adjacent cells), where interacting partners of YAP/TAZ such as Angiomotin and zonula occludens proteins can retain the proteins in their vicinity impairing their activity in the nucleus. Nevertheless, a cytoplasmic function of YAP/TAZ has also been documented, due to their interaction (via PDZ domain) with proteins belonging to other pathways, such as in Wnt signaling.

1.4.4) Role in mechanotransduction

Importantly, YAP and TAZ activity has also been shown to be modulated by external mechanical contexts. In fact, Dupont et al. have studied extensively the role of YAP/TAZ in mechanotransduction, and concluded that in hMSC, YAP/TAZ are predominantly nuclear when cells are seeded onto stiffer substrates, while cells on softer substrates display cytoplasmic YAP/TAZ. Importantly, inhibition of Rho or disruption of actin cytoskeleton by pharmacological treatment inhibited YAP/TAZ activity on stiff substrates, demonstrating their requirement for YAP/TAZ to act as mediators of mechanical signals. In this stem cell type, both active ROCK and non-muscle myosin II-mediated cell tension were required for YAP/TAZ to be nuclear, as shown by treatment with the respective inhibitors. Furthermore, the response of YAP/TAZ to the matrix rigidity seems to be independent from the Hippo pathway, pointing out the existence of an independent mechanism. Cell shape has shown to modulate YAP/TAZ intracellular localization and activity, as concluded through the use of micropatterned substrates with either large islands that cause individual cells to adopt a flat, spread morphology or small islands that induce a round, compact shape. These experiments revealed that spread-out cells have higher levels of nuclear YAP than round cells. In addition, cell attachment also interferes with YAP/TAZ activity, as detached cells have lower YAP/TAZ activity. Moreover, actin cytoskeleton has shown to regulate YAP/TAZ activity, as expected by its involvement in mechanotransduction, where it is considered the global signal integrator. Experiments in mammalian cells showed that the amount of F-actin was proportionally correlated with YAP/TAZ activity: (i) treatment of cells with Cytochalasin D inhibits F-actin polymerization and prevents YAP/TAZ translocation to the nucleus; (ii) F-actin stress fibers are abundant in cells at low cell density and rare in high-density cultures and flat cells have more stress fibers than round cells, consistent with the observed YAP/TAZ activity in confluence vs. non-confluence and in different cell shapes. Altogether, YAP/TAZ respond to different conditions of the extracellular environment and its regulation comprises several mechanisms, as shown in Figure 6.
Figure 6 - Regulation and functions of YAP/TAZ transcriptional modulators. Different stimuli are involved in YAP/TAZ regulation, including: 1) Cell confluence, that inhibits YAP/TAZ nuclear shuttling due to the activation of 2) Hippo signaling pathway, wherein a kinase cascade ultimately phosphorylates YAP/TAZ which either drives 3) YAP/TAZ ubiquitinilation due to the recruitment SCF/CRL1β-TrCP E3 ligase of and subsequent proteasomal degragation or 4) binding to 14-3-3 proteins and consequent cytoplasmic retention. On the other hand, 5) dephosphorylation of YAP/TAZ is mediated by PP1 and enhances its nuclear localization and activity, though some proteins such as 6) α-catenin inhibit this reaction. In addition, some proteins can bind YAP/TAZ retaining them in the cytoplasm, preventing their nuclear function, as is the case of 7) amot and 8) zonula occludens proteins. 9) In case YAP/TAZ can shuttle to the nucleus, they can bring transcription factors therefore modulating gene expression. 10) Transcriptional modulation mediated by YAP/TAZ has been associated with different cellular processes such as stem cell proliferation and differentiation along with induction of tumorogenic phenotypes. YAP/TAZ activity are also regulated by mechanical factors as explored in the text (not shown in this Figure).
1.5) Objective of the experimental work

Taking into account the reviewed topics in this introductory section, the aim of the experimental work developed for this dissertation is the dissection of molecular pathways associated with YAP/TAZ nuclear shuttling in mechanotransduction, in the cardiac stem/progenitor cell context. Factors like elasticity, nanorugosity and three-dimensional pore geometry are known to affect cell cytoskeleton organization, which is responsible of a shift in intracellular signal transduction and, eventually, in changes in gene activation. Therefore, it is conceivable that the expression of genes involved in stem/progenitor cell maintenance, survival or differentiation could be switched off or on in response to slight changes in the mechano-physical signals coming from the microenvironment. As such, the knowledge obtained with this work is expected to shed some light into cardiac stem cell fate determination in relation with YAP/TAZ activity as a transducer of these mecanostructural effects. Bearing this in mind, one central question to be addressed is “how can cells perceive their microenvironment?”

In brief, this work was performed in the cardiac context by the use of a cardiac progenitor cell line characterized by the expression of Sca-1 cell surface protein hereafter referred to as cMPC\textsuperscript{Sca-1}\textsuperscript{(Freire AG, Nascimento DS, Forte G et al., manuscript)}. The experimental plan was conceived to address how is YAP/TAZ activity regulated in mechanotransduction, in terms of substrate stiffness, nanotopography, cell shape and interacting proteins. As such, nuclear vs. cytoplasmic expression of YAP/TAZ was evaluated, with subsequent downstream responses related with cardiac stem/progenitor cell biology such as proliferation and differentiation/commitment to mature cardiac cell types, resorting to immunostaining relevant markers, Western blot and real time PCR for assessing protein and gene expression. Influence of migration in YAP/TAZ activity in cMPC\textsuperscript{Sca-1} was also evaluated using the in vitro wound healing assay, whereas involvement in cell adhesion was assessed using Alamar blue\textsuperscript{®} assay. Involvement of signaling pathways into YAP/TAZ activity was also sought, and was analyzed by the use of specific chemical inhibitors.
2) Materials and Methods

2.1) Cell culture, transfection and treatment with pharmacological inhibitors

Murine Sca-1\(^+\) cardiac stem/progenitor cell line (cMPC\(^{Sca-1}\)) were grown in DMEM:F12 1:1 mixture with 15mM HEPES and L-Glutamine (Lonza, Switzerland) supplemented with 10% Fetal Bovine Serum (FBS) (Equitech-BioInc., Kerrville, TX), 200 U/ml Penicillin and 200 µg/ml streptomycin (Invitrogen, USA), ascorbic acid (50µg/ml) (WAKO chemicals, Japan), 0,2% EGM-2 basal medium (Lonza, Switzerland) and 0,1 mM MEM Non-essential aminoacids (Invitrogen, USA), hereafter referred to as CT medium. Human mesenchymal stem cells (hMSC) were grown in Mesenchymal stem cell growth medium (MSCGM) composed of MSC Basal medium (Lonza, Switzerland), supplemented with Single Quots Kit (Lonza, Switzerland).

For the experiments done in tissue culture polystyrene (TCPS), cMPC\(^{Sca-1}\) were detached using 0.25% Trypsin 1mM EDTA (Nacalai Tesque, Japan) and subcultured in CT medium onto plates of different sizes: 8 chamber-slides (BD Biosciences, USA), 24 well plates (IWAKI, Japan), 6-well plates (Nuclon Delta,USA) or micropatterned adhesive islands slides (Cytoo, USA). This was done either in non-confluent (7,5 x10\(^3\) cells/cm\(^2\)) or confluent conditions (25x10\(^3\) cells/cm\(^2\)). For cMPC\(^{Sca-1}\) seeding onto polyacrylamide gels or poly ε-caprolactone films, low cell adhesion 24 well plates (IWAKI, Japan) were used after pre-coating of gels or films with CT medium for 2h. The cells were analyzed at 3h, 5h, 8h and 24h.

For the short-interfering RNA (siRNA) transfections, 7,5x10\(^3\) cells/cm\(^2\) were seeded in antibiotic-free CT medium onto 24 well plates, 18h prior to transfection. Cells were washed with PBS 1h before the transfection, YAP siRNA duplexes (1 µg/ well) and transfection reagent were mixed in a 1:1 (v/v) ratio and added at 10% in transfection medium. All these reagents were purchased from Santa Cruz Biotechnology, USA. Thereafter, growth medium was replaced with transfection medium containing siRNA complexes comprising 20% of the final volume. After 7h, CT medium supplemented with 2x normal FBS and antibiotics concentration was added to the cells. Thereafter, cMPC\(^{Sca-1}\) were incubated for 24h, followed by replacement of transfection medium by regular growth medium. YAP and TAZ expression was subsequently assayed by Western Blot 24h after last medium replacement, and/or cMPC\(^{Sca-1}\) YAP/TAZ \(^{-/-}\) were subcultured afterwards.

Cytochalasin D (1mg/ml) (Sigma, USA), Y27632 (50µM) (WAKO chemicals, Japan) and Blebbistatin (50µM) pharmacological inhibitors (Merck Biosciences, USA) were dissolved in PBS and added to growth medium (10% of the final treatment volume), for 4h. Leptomycin B (10ng/ml) (Sigma, USA) was dissolved in 70% methanol (Wako chemicals, Japan) whereas okadaic acid (0,7 nM) (Sigma, USA) was dissolved in DMSO (Wako chemicals, Japan). Both were
added 100x concentrated to the medium (1% of the final treatment volume), for 2h. Appropriate controls were performed using methanol and dimethyl sulfoxide alone.

2.2) In vitro wound healing assay

In order to analyze the effect of migration in regulating YAP/TAZ localization and activity, an established in vitro migration assay was performed. Herein, cells are grown until confluence, followed by a scratch (wound) made with a pipette tip on the cell monolayer, allowing cells to migrate and close the wound. Different time points (2h, 4h and 8h) were analyzed.

2.3) Immunofluorescence staining and confocal microscopy

Cells seeded on every setup (TCPS well plates, polyacrylamide gels, PCL films, micropatterned surfaces) were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) in PBS for 15 minutes at room temperature, and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, USA) in PBS for 2 minutes at room temperature. Depending on the marker analyzed, cells were incubated for 1,5h with primary antibodies against: YAP (1:200), TAZ (1:200), Vinculin (1:100); 405-conjugated Alexa Fluor® Zyxin (1:100), phosphoPaxillin (1:150) (Invitrogen, USA), GATA-4 (1:200), TBX5 (1:300), Ki67 (1:100) (Abcam, UK). Unless stated otherwise in the text above, all antibodies were purchased from Santa Cruz Biotechnology, USA. The appropriate fluorophore-conjugated secondary antibodies (1:300) were as follows: Alexa fluor 488 goat anti mouse; Alexa fluor 488 goat anti rabbit; Alexa fluor 546 goat anti mouse; Alexa fluor 546 goat anti rabbit; Alexa fluor 488 donkey anti goat; Alexa fluor 546 donkey anti goat. All antibodies were dissolved in bovine serum albumin (Wako chemicals, Japan) 1% in PBS, to block unspecific binding. Nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA). Moreover, filamentous actin was stained using rhodamine phalloidin (1:500, Invitrogen, USA). The images were taken using a Leica DMRB microscope equipped with a digital camera or using a confocal laser scanner microscope (LEICA SFS), after excitation at 405 nm, 488 nm, and 543 nm wavelengths for blue, green, and red channels acquisition, respectively. All the pictures obtained were processed both with Image J and Adobe Photoshop CS5 (USA).

2.4) Protein extraction, cell fractioning and Western Blot

Protein extraction of total cell lysates was performed using a Ready prep extraction kit (Bio-RAD, USA), according to the manufacturer’s instructions. In short, ready prep protein extraction buffer supplemented with protease inhibitor cocktail (Thermo Scientific, USA) was
added to cell pellets and the samples were sonicated using 30 sec bursts 4 times, being placed on ice between each burst. Subsequently, samples were centrifuged at 16,000 x g for 20–30 min at 18–20°C and supernatant was recovered. In order to distinguish between nuclear and cytoplasmic fractions an alternative extraction kit was used (Thermo Scientific, USA), according to the manufacturer’s instructions. Briefly, cell pellets were resuspended with cytoplasmic extraction reagent supplemented with protease inhibitor cocktail and centrifuged at 16000 x g, followed by the recovery of the supernatant (cytoplasmic fraction). Thereafter, pellets were resuspended with nuclear extraction reagent, centrifuged in the same setting and the supernatant was recovered (nuclear fraction). Concentration of proteins extracted was determined by Bradford assay, using a kit (Bio-RAD, USA).

Western blot was performed as hereby described. Approximately 25 µg protein samples were loaded on a loading buffer (Tris-HCL pH 6.8/ Glycerol/ SDS/β-mercaptoethanol/ bromophenol blue) per well onto a 10% Mini-PROTEAN TGX Precast Gel, placed in running buffer (Tris/Glicine/SDS), and SDS-PAGE was run at 100V for 2 h. Afterwards, proteins in the gel were transferred to a polyvinyl difluoride (PVDF) membrane, using the standard blotting procedure (placing the gel tight between the PVDF membrane, filter paper and cotton pads and applying voltage - 65V for 2h) in a Transfer Blot core. Thereafter, PVDF membrane was washed in PBS-Tween (0,15%) and non-specific immunodetection was blocked by washing the membrane in Tris Buffered Saline (TBS) with 1% Casein for 1h with shaking. Subsequently, PVDF membrane was incubated with primary antibodies (in TBS 1% casein) against YAP (1:200), TAZ (1:200) and α-smooth muscle actin (1:500) for 2h with shaking, followed by membrane washing and incubation with secondary antibodies: Goat Anti-Rabbit IgG (H+L)-HRP Conjugate and Goat Anti-Mouse IgG (H+L)-HRP Conjugate, at 1:8000 in TBS 1% casein for 2h with shaking. Finally, chemiluminescence reaction was performed by adding 1:1 luminol substrate/enhancer and peroxide buffer, followed by incubation for 5 minutes in the dark and short exposition (less than 1 minute) to photographic paper. All the reagents/ materials were purchased from Bio-RAD, USA.

2.5) Matrigel™ preparation and experimental setup

BD Matrigel™ Basement Membrane Matrix Growth Factor Reduced, Phenol Red Free (BD Biosciences, USA) was prepared for the thick gel method according to the manufacturer’s instructions. Firstly, Matrigel was thawed overnight at 4°C on ice and the vial was swirled to assure homogeneity. Afterwards, 50 µL of Matrigel solution were added per well of cooled 96 well plate (IWAKI, Japan), using cooled pipettes. Thereafter, plates were placed at 37°C for 30 minutes, followed by the culture of cMPC^{Sca-1} cells (20x10^3 per well) in CT medium (100 µl), on
top of the thick gel. At every relevant time point (3h, 6h, 8h) brightfield pictures were taken using an Olympus IX71 microscope equipped with a digital camera. After 8h, cells were fixed and immunostained for YAP (1:200), GATA-4 (1:200) and nuclei were counterstained with DAPI, according to the procedure described in 2.2.

2.6) Polyacrylamide gels preparation

Polyacrylamide (PA) gels were prepared as described by Tse and Engler\textsuperscript{128}, with minor modifications. Firstly, glass coverslips (Matsunami glass, Japan) were amino-silanated as follows: (1) 13 mm diameter glass coverslips were covered with 0.1 M NaOH and heated at 80°C until the solution evaporated, (2) coverslips were placed in a nitrogen-filled tent and 3-Aminopropyltriethoxysilane (APES) was added to their surfaces for 5 minutes; (3) coverslips were washed repeatedly in destilled H\textsubscript{2}O and subsequentially immersed in 0.5% glutaraldehyde in PBS for 30 min, followed by air drying. Squared thick glass slides were fluoro-silanated as follows: (1) glass slides were dipped in 0.1 M potassium hydroxide overnight, followed by washing with ethanol and acetone and drying by blowing nitrogen into them; (2) glass slides were placed in vacuum box and 200 µl of Fluorosilane (Trichloro (1H, 2H,2H-perfluoroctyl)silane) (Aldrich co., USA) inside a pipette tip were placed inside the box followed by vacuum induction. The box was placed at 37°C overnight.

Afterwards, PA gels were prepared by the addition of the adequate amounts of 40% (w/v) acrylamide stock solution (Sigma, USA), 2% (w/v) bis-acrylamide (N,N'-Methylenebisacrylamide) (Tokyo Chemical Industry, Tokyo, Japan) stock solution and PBS, towards achieving the desired stiffness (Young modulus, E) according to the published protocol\textsuperscript{128}. E of a material represents the intrinsic resistance of organs and tissues to stress, and in can be expressed as the tensile stress, σ, or force applied per unit area, divided by the resultant strain, ε, or relative change in length\textsuperscript{129}.

The mixture was placed under strong vacuum for 30 minutes, and polymerizing catalysts were added (10% (wv) ammonium persulfate (APS) at 1% of final volume and Tetramethylethylenediamine (TEMED) at 0.1% final volume). Polymerizing solution was added to the fluorosilanated glass slide and aminosilanated coverslip was placed on top, followed by polymerization for 30 min and subsequent washing with PBS to remove unpolimerized PA, resulting in glass coverslip with PA gel attached (Figure 7A-E).

Finally, PA gels were coated with a protein of the ECM to allow cell attachment as follows: (1) 0.2 mg/ml sulfosuccinimidyl-6-(4’-azido-2’-nitrophenylamino)-hexanoate (sulfo-SANPAH; Pierce Biotechnology) was added to the gel surface, followed by photoactivation by exposition to 365-nm UV light source at a distance of 8 cm for 10 min; (2) gels were rinsed with
50 mM HEPES buffer pH 8.5 (Sigma, USA) and incubated overnight with either fibronectin (10 µg/ml) or collagen (100 µg/ml) in HEPES buffer. (Figure 7F-H) (3) Finally, gels were washed with PBS and sterilized by UV exposure for 30 min (each side).

**Figure 7– Polyacrylamide gels preparation.** The gel-glass composite includes: A- the aminosilanated coverslip; B- polymerizing solution and C- fluoro-silanated glass slide. D- The polymerizing solution is placed between the coverslip and the glass slide until polymerization is finished. E- After removal of unpolimerized polyacrylamide (PA) the gel is attached to the coverslip and functionalization can proceed. F- the surface of the gel becomes activated upon addition of sulfo-SANPAH, a reaction catalyzed with 365-nm UV light. G- Overnight attachment of ECM protein in a 50 mM HEPES solution, pH 8.5. H- Completed functionalization of ECM protein to the PA hydrogel. Adapted from 128

2.7) **Poly-ε-caprolactone film preparation and stiffness control**

Poly-ε-caprolactone (PCL) materials were prepared by cross-linking tetra-branched PCL with acrylate end-groups in the presence of linear PCL telechelic diacrylates, according to a previously reported protocol. Briefly, two-branched and four-branched PCL were synthesized by ε-caprolactone (CL) ring-opening polymerization that was initiated with tetramethylene glycol and pentaerythritol as initiators, respectively. Then, acryloyl chloride was reacted to the end of the branched chains. The structures and the molecular weights were estimated by $^1$H NMR spectroscopy (JEOL, Tokyo, Japan) and gel permeation chromatography (JASCO International, Tokyo, Japan). The average degrees of polymerization of each branch on two-branched and four-branched PCL were 18 and 10, respectively. The obtained PCL macromonomers were then dissolved in xylene containing benzoyl peroxide (BPO), and the solution was injected between a glass slide with a 0.2 mm thick Teflon spacer to prepare the
substrate layers. The PCL macromonomers were cured for 180 min at 80°C. The synthesis of PCL films is briefly represented in **Figure 8**.

![Figure 8 - Preparation of cross-linked PCLs. Layers with various stiffness were obtained by simply mixing and curing two- and four-branched PCL with acrylate end-groups. PCL macromonomers were dissolved in xylene containing benzoyl peroxide (BPO), cured for 180 min at 80°C and the solution injected between a glass slide with a 0.2 mm thick Teflon spacer to obtain planar layers. Adapted from 150.](image)

The mechanical properties of the crosslinked materials were characterized by a tensile test (EZ-S 500N, Shimadzu, Kyoto, Japan). The contact angles on the PCL layers were also determined by a sessile drop method. They were measured 30 seconds after a water drop was placed on the surface at 37°C.

### 2.8) Alamar Blue® assay

The alamar blue® Assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresce and change color in response to chemical reduction of growth medium resulting from cell growth131. In some of the experimental setups used, the time point selected (8h after seeding) is very early after subculturing, so that the reduction of the indicator is correlated with cell attachment instead of proliferation. In the selected time points for the assay, medium was removed and cells were washed with PBS. Thereafter, medium + 10% alamar blue solution were added to the cells, and incubated for 30 min (in the short time point-8h) or 1h (longer time points- 24h). Afterwards, 100 µl of medium containing alamar blue solution were transferred (in triplicates) to a 96 well plate with black
bottom (Iwaki, Japan) and fluorescence was read at 570 nm. Values were normalized to those obtained by YAP/TAZ<sup>+/+</sup> cells (control) in TCPS.

### 2.9) RNA extraction and real time PCR array

Real-time PCR was used to determine the expression profile of 84 key genes involved in cell-cell and cell-matrix interactions in cMPC<sup>Sca-1</sup>. Cells were harvested and collected after 24 hours of seeding into different substrates. Total RNA was extracted by RNeasy Mini Kit (Qiagen Valencia, CA) and reverse-transcribed by RT<sup>2</sup> First Strand Kit (Qiagen). After synthesis of first-strand cDNAs, RT-PCR was performed by the 7500 Real Time PCR System (Applied Biosystems, USA) using the RT<sup>2</sup> Profiler PCR Array Mouse Extracellular Matrix and Adhesion Molecules PCR Array (PAMM-013A; Qiagen) with the RT<sup>2</sup> SYBR Green/ROX PCR Master mix (Qiagen) according to the manufacturer’s protocol. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence reaches 10-fold standard deviation of the baseline (from cycle 3 to 11). The specificity of the SYBR PCR signal was confirmed by melt curve analysis. Data were analyzed by the RT<sup>2</sup> Profiler PCR Array Data Analysis version 3.5 (Qiagen).

### 2.10) Biostatistical analysis

The quantitative analysis represents counting of 5 picture fields per condition with approximately 150 cells / field and the results are presented as mean ± standard error of mean (SEM) of three independent experiments. The cell counts and band intensity estimations were assessed by Image J software v1.45 (USA). The significance of differences (in all the measured variables) in multigroup comparison was evaluated by unpaired Student’s t-test, run by GraphPad Prism v5.0 software (USA). A final value of p<0.05 was considered statistically significant.
3) Results

3.1) YAP/TAZ characterization and regulation by confluence and migration

Since YAP/TAZ has never been studied in cardiac stem/progenitor cells, a preliminary characterization of its activity under conditions already explored in other cell types has been carried out. To rule out different activities/mechanisms between YAP and TAZ both proteins were co-immunostained, as shown in Figure 9. YAP and TAZ were shown to display an overlapping signal, both when present in the cytoplasm and in the nucleus. Thus, due to this overlapping expression pattern, activity of both proteins was inferred from the readout of one of them, in the remainder of this thesis. Nevertheless, that was further verified for each experimental setting. Additionally, relevance of YAP/TAZ in the CPC context was firstly analyzed by co-staining YAP with the transcription factor GATA-4 (Figure 10A), revealing co-localization of both proteins, thus rendering GATA-4 a possible interacting partner (given that YAP/TAZ is a transcriptional co-activator and GATA-4 a transcriptional factor). Moreover, co-localization of YAP with a nuclear shuttling protein paxillin (phosphorylated) was also found, hinting into the mechanism of YAP nuclear translocation and/or interaction with nuclear shuttling proteins (Figure 10B).

![Figure 9 - YAP and TAZ display an overlapping signal both in cytoplasmic and nuclear expression in cMPC<sup>Ca<sup>-1</sup></sup>. As such, YAP and TAZ activity was considered redundant throughout this thesis. Nuclei were counterstained with DAPI.](image-url)
Furthermore, known regulatory mechanisms of YAP/TAZ were addressed in the CPC context, such as confluence and migration. As such, cMPC<sup>Sca</sup>-1 were seeded either in confluent or non-confluent conditions and immunostained for YAP, as depicted in Figure 11A-B. A preferential cytoplasmic expression of YAP in confluent cells is detected (holes devoid of signal in the picture represent the nuclei), whereas YAP expression is preferentially nuclear when cells are non-confluent/sparse. Moreover, since YAP/TAZ have been implicated in tumorigenesis and enhanced migration<sup>132</sup>, nuclear vs. cytoplasmic localization was addressed in an in vitro model of migration, the wound healing assay. In this setup there were no differences relative to confluent conditions, indicating that migration did not significantly enhance YAP/TAZ nuclear localization (Figure 11C).

**Figure 10 – YAP colocalizes with GATA-4 (A) and phosphorylated paxillin (B) in cMPC<sup>Sca</sup>-1, rendering these two proteins as possible interacting partners, and suggesting involvement of YAP with CPC cardiac commitment.**

**Figure 11 – YAP/TAZ activity is regulated by confluence but not by migration in cMPC<sup>Sca</sup>-1.** YAP localization was predominantly cytoplasmic in confluent cells (A) whereas it was mostly nuclear in non-confluent conditions (B). In an in vitro migration assay (C), YAP localization was not different from that seen on dense conditions.
In order to understand the relevance of these effects in a large cell population, the percentage of cells expressing nuclear YAP was determined in each condition and the results are plotted in Figure 12. For the migrating assay, only cells in the border of the wound (actively migrating) were counted. The percentage of cells expressing nuclear YAP in confluent cells (10.5% ± 1.4) was statistically different (p<0.05) as compared to that of non-confluent cells (54.2% ± 5.2), and similar to that of migrating cells (13.7% ± 1.8).

3.2) Signaling pathways involved in YAP/TAZ localization and activity

Regulation of YAP/TAZ localization and activity by mechano-structural factors in cMPC<sup>Sca-1</sup> was evaluated by subjecting the cells to pharmacological inhibitors, namely Cytochalasin D (inhibits actin polymerization and disrupts formed filamentous actin), Y27632 (inhibits ROCK activity thereby preventing its involvement in multiple signaling pathways such as the phosphorylation of myosin light kinase and consequent activation of myosin II) and blebbistatin (inhibits non-muscle myosin II directly, impairing actomyosin contractility and cell tension).

Firstly, the inhibitors’ effect in cell morphology and focal adhesions was investigated by vinculin immunostaining. As shown in Figure 13, the typical expression pattern of vinculin in adherent cMPC<sup>Sca-1</sup> is disrupted by cytochalasin D treatment, leading to impaired focal adhesion assembly. Moreover, the treatment of cMPC<sup>Sca-1</sup> with Y27632 and blebbistatin caused minor changes in focal adhesions, vinculin expression being slightly disarranged in treated
cells, and phosphorylated paxillin no longer being accumulated in the perinuclear region. Importantly, cells treated with either Y27632 or Blebbistatin exhibit elongated protrusions, most likely due to the lack of cell tension as a result of the treatment.

Figure 13 – Effect of pharmacological inhibitors on cMPC<sup>Sca-1</sup> morphology and focal adhesion organization. CytoD treatment causes disruption of focal adhesions as vinculin spiked signal is absent. Treatment with Blebbistatin or Y72632 causes slight modifications in vinculin in terms of organization/alignment, impairs phosphopaxillin accumulation in the perinuclear region, and leads to the appearance of elongated protrusions in the cells.

The pharmacological inhibitors’ effects on actin cytoskeleton were analyzed by immunostaining F-actin and the α-actinin crosslinker (confers contractility and cell tension). As expected, Cytochalasin D treatment disrupts actin cytoskeleton, and α-actinin expression appears heavily affected (Figure 14). Treatment with Y72632 and blebbistatin induces changes in F-actin cytoskeleton as the cells are bent (consistent with the extended protrusions seen in Figure 13, as a result of disruption of cell tension), and α-actinin expression pattern appears scattered in cell cytoplasm and less intense than in untreated cells.

Following characterization of the influence of the inhibitors on cell cytoskeleton and focal adhesion structure, the drugs’ influence on YAP/TAZ localization/activity was analyzed by immunofluorescence (Figure 15). In the cMPC<sup>Sca-1</sup> line, cytochalasin D treatment impairs YAP nuclear localization, implying that F-actin is required for YAP/TAZ localization in the nucleus.
Figure 14—Effect of pharmacological inhibitors of defined signaling pathways involved in cell mechanotransduction on cMPC $^{Sca-1}$ cells. Treatment with Cytochalasin D strikingly disrupts the F-actin cytoskeleton organization and alters α-actinin expression. cMPC$^{Sca-1}$ cells treated with Y27632 exhibit a bent phenotype consistent with the lack of tension resulting from the treatment, as evaluated by F-actin and α-actinin expression.

Figure 15—Effect of pharmacological inhibitors on YAP/TAZ nuclear activity on cMPC$^{Sca-1}$. Treatment with Cytochalasin D impaired YAP nuclear localization, indicating that cytoskeleton integrity is required for its nuclear localization and activity. Treatment with Y27632 and Blebbistatin enhanced YAP nuclear localization suggesting that functions exerted by ROCK and cell tension negatively regulate YAP nuclear localization.
Surprisingly, treatment with either Y27632 or Blebbistatin significantly enhanced YAP nuclear levels, suggesting that ROCK –mediated signaling pathways and cell tension – as transduced through Myosin II pathway - impair YAP/TAZ nuclear translocation. This observation is interesting as treatment with these two inhibitors caused opposite effects in hMSC^{125}, suggesting different regulation mechanisms of YAP/TAZ localization and activity in different stem cell types. Percentages of cells displaying YAP in the nucleus were estimated by cell counting, and the results are plotted in Figure 16. The percentage of cells expressing nuclear YAP was statistically different between the untreated cells (non-confluent: 54.2% ± 5.2) and cells treated with each of the inhibitors (Cytochalasin D: 11.7% ± 1.5; Y27632: 94.1% ± 1.6; Blebbistatin: 98.1% ± 0.8).

In order to further validate these data, YAP expression was quantified by Western Blot, discriminating between nuclear and cytoplasmic fractions, only for control and blebbistatin treatment (Figure 17A). Band intensity was quantified using Image J software and the percentage of nuclear expression (normalized to α-SMA) relative to the sum of both fractions is shown in Figure 17B.
The Influence of the abovementioned inhibitors of known mechano-structural signaling pathways in zyxin cellular sub-localization was also assessed. Zyxin is a focal adhesion protein which has been previously shown to shuttle to the nucleus in response to cell stretching and modifications in substrate mechanical properties\(^\text{133}\). Moreover, the impact of pharmacological inhibition on migration was also analyzed taking advantage of an in vitro wound healing assay. The experimental setup was designed as to treat cells with the selected inhibitors immediately after inflicting the wound in the cell monolayer. The results represented in Figure 18 show that the treatment with Cytochalasin D impaired migration, whereas treatment with either Y27632 or Blebbistatin hastened it relatively to control conditions, as both sides of the wound can be seen in the same picture, indicating faster migration. Moreover, zyxin localization accounts for its shuttling activity in migrating cells. Zyxin expression in migrating cells (control) is mostly nuclear (right-hand panel for higher magnification), while it translocates to the cytoplasm when Cytochalasin D and Blebbistatin are used. Interestingly, Y27632 exposure did not trigger any change in zyxin sub-localization.

Although these observations do not clearly explain zyxin shuttling regulation in the cardiac cell progenitor line used in the experiments, they indicate that zyxin expression in migrating cells does not follow the same trend as YAP (deemed clear by blebbistatin treatment that triggers YAP to the nucleus and zyxin to the cytoplasm), ruling out the possibility of interaction and involvement of these two proteins, in the contexts analyzed. More
interestingly, these results suggest that the localization of YAP/TAZ and zyxin is differentially regulated in migrating cardiac stem/progenitor cells.

Figure 18- Influence of pharmacological inhibitors’ treatment on cMPC<sup>Sca-1</sup> migration and zyxin nuclear shuttling. Treatment with Cytochalasin D impaired migration whereas cells treated with either Y27632 or blebbistatin migrated faster relatively to untreated cells. Zyxin localization is influenced by the treatment as well, as shown in detailed pictures in the right hand side: zyxin is predominantly nuclear in untreated cells and cMPC<sup>Sca-1</sup> treated with Y27632 and cytoplasmic in cells treated with Cytochalasin D or blebbistatin.

3.3) Role of YAP/TAZ in CPC proliferation

In order to investigate the relevance of YAP/TAZ in the context of Sca-1<sup>+</sup> cardiac stem/progenitor cells, its suppression was achieved using siRNA-mediated mRNA silencing. As shown in Figure 19, both YAP and TAZ were silenced by the siRNA directed to YAP, accounting for the high similarity in mRNA sequence between both these players.

Subsequently, by exploiting cells’ knock-down for YAP/TAZ expression, the relevance of such proteins in different cellular processes was assessed. Firstly, the role of YAP/TAZ in CPC proliferation was evaluated by immunostaining of Ki67, a known marker of cell proliferation<sup>134</sup>. As shown in Figure 20A, the expression of Ki67 is significantly higher in YAP/TAZ<sup>-/-</sup> cells, the
percentages of nuclear ki67\(^*\) cells in control and YAP/TAZ \(-/-\) cells being 26.6 ±2.1% and 54.6±5.5%, respectively (Figure 20B).

![Figure 19- Western Blot for YAP and TAZ expression in control cMPC\(^{Sca-1}\) and silenced cells. Successful knock-down of both YAP and TAZ expression is achieved by siRNA directed against YAP mRNA.]

![Figure 20- Role of YAP/TAZ in CPC proliferation. Immunostaining of ki67 (A) resulted in a higher expression in YAP/TAZ -/- cells, as quantified and plotted (B). **** p<0.0001]
3.4) Role of cell tension and YAP/TAZ in CPC cardiac commitment

Since YAP/TAZ were found to co-localize with the early cardiac transcription factor GATA-4 in cMPC^{Sca-1} (Figure 10), and an effect of cell tension release in triggering nuclear shuttling of such factors was demonstrated, the involvement of myosin II pathway in CPC cardiac commitment was investigated. GATA-4 was immunostained in control and YAP/TAZ^{−/−} cells treated with blebbistatin to inhibit Myosin II activity (thus cell tension) (Figure 21A). Nuclear GATA-4^{+} cells were quantified by immunofluorescence, yielding the following results: control: 23.4%± 1.2; YAP/TAZ^{−/−}: 5.7% ± 1.1; control + blebbistatin: 46.6% ± 3.7; YAP/TAZ^{−/−} + blebbistatin 4.3% ± 0.3) (Figure 21B).

The results suggest that YAP/TAZ is needed for GATA-4 nuclear localization in the cMPC^{Sca-1} cardiac progenitor cell-line model. Interestingly, nuclear GATA-4 expression seems to be enhanced when cell tension is released. These results indicate YAP/TAZ as a relevant factor in the transcriptional modulation of CPC commitment in response to a mechano-structural cues arising from the extracellular environment. Nonetheless, controlled differentiation experiments will be necessary to confirm the data.
Figure 21 – Role of tension and YAP/TAZ on CPC cardiac commitment. A- Immunofluorescence of cMPC<sup>Sca-1</sup> stained with GATA-4 in different conditions (Control vs YAP/TAZ<sup>-/-</sup>, either untreated or treated with blebbistatin). Nuclei were counterstained with DAPI. B- Percentage of cells expressing nuclear GATA-4 in the different conditions assessed. (****p< 0.0001)
3.5) YAP/TAZ activity in substrates displaying different stiffness

3.5.1) Activity on soft substrates: Matrigel™

To investigate YAP/TAZ regulation by the stiffness of the extracellular environment, cMPC<sup>Sca-1</sup> were seeded onto substrates exhibiting different stiffness. Matrigel™ is a commercially available basement membrane (BM)-like complex whose stiffness was reported to be approximately 450 Pa, as determined by atomic force microscopy<sup>135</sup>. Given its structure, Matrigel™ is also used for tumor invasion and endothelial differentiation assays (vasculogenesis), as metastatic and/or endothelial cells can migrate within the gel, forming capillary-like structures.

cMPC<sup>Sca-1</sup> were seeded on top of Matrigel™ and migration occurred with formation of capillary-like structures (Figure 22), accounting for the endothelial differentiation potential of these cardiac progenitor cells. Among the cells seeded onto Matrigel™, different morphologies could be recognized, with some cells displaying rounded, sprouting and migrating morphologies, the last being predominant over time. Immunostaining of cells after 8h migration against YAP revealed that it was exclusively located in the cytoplasm, thus not exerting its transcriptional modulation function. YAP/TAZ cytoplasmic localization in cMPC<sup>Sca-1</sup> grown on Matrigel™ was detected in all cell types, independently of their morphology. Moreover, cells did not exhibit active (nuclear) GATA-4, further validating previous data of co-localization, and thus possible interaction, between YAP/TAZ and GATA-4.

![Figure 22 – YAP/TAZ localization in cMPC<sup>Sca-1</sup> grown on soft substrates. cMPC<sup>Sca-1</sup> were seeded onto Matrigel™ and actively migrated forming capillary-like structures. Within the gel, cells acquired alternative shapes. Immunostaining of YAP and and GATA-4 revealed that both were exclusively localized in the cytoplasm independently of cell shape, indicating that soft substrates do not induce their activation.](image-url)
In order to further understand the involvement and regulation of YAP/TAZ in soft substrates and also endothelial differentiation, different pharmacological inhibitors were employed. Since a role for the pathways involved in cell mechanosensing was previously demonstrated in migrating cells (see please 3.2), cMPC\textsuperscript{Sca-1} were exposed to Y27632 and blebbistatin, to understand whether ROCK-mediated signaling and cell tension, respectively, could interfere with vasculogenesis. Moreover, YAP nuclear localization was manipulated using leptomycin B, an inhibitor of nuclear export, thus keeping YAP/TAZ inside the nucleus. Additionally, okadaic acid, an inhibitor of protein serine/threonine phosphatase 1, 2A, and 2B\textsuperscript{136}, was used, as it is known to prevent YAP/TAZ dephosphorylation, consequently enhancing its cytoplasmic retention by binding to 14-3-3 proteins\textsuperscript{137}. Remarkably, these last two inhibitors cannot be considered to affect specifically YAP/TAZ expression and/or localization, since they also interfere with other cellular processes (for instance okadaic acid is highly cytotoxic and had to be used in subnanomolar concentrations). Finally, YAP/TAZ \textsuperscript{-/-} cMPC\textsuperscript{Sca-1} were used to address the role of these factors in cardiac progenitor cell migration and endothelial differentiation in Matrigel\textsuperscript{™}.

Control cells, treated or YAP/TAZ \textsuperscript{-/-} cells were seeded onto Matrigel\textsuperscript{™} and the occurrence of vasculogenesis was assessed in a time-course experiment by brightfield microscopy. As seen in Figure 23, the inhibition of either ROCK or cell-tension enhances the migration of cMPC\textsuperscript{Sca-1} in Matrigel\textsuperscript{™}, as after 6h and 8h of vasculogenesis, capillary like-structures are in higher quantity as compared to the control. Cells where YAP is retained in the cytoplasm (okadaic acid treated) were neither able to migrate nor to form capillary-like structures. This result, suggesting a role for cytoplasmic YAP/TAZ in vasculogenesis is surprising, since migrating cells exhibit YAP in their cytoplasm, as shown in Figure 22. On the other hand, cMPC\textsuperscript{Sca-1} treated with leptomycin B do not exhibit drastic changes in vasculogenesis relative to control, probably not only due to the low efficiency of this inhibitor in retaining YAP/TAZ in the nucleus (data not shown) but mainly to a wash-out effect (leptomycin B-treated cells were seeded onto Matrigel\textsuperscript{™} and assayed after 8h). Importantly, siRNA specific suppression of YAP/TAZ resulted in impairment of vasculogenesis clearly indicating a role of these two proteins in CPC vasculogenesis/ endothelial differentiation, and also further validating the involvement of a YAP/TAZ cytoplasmic function in this cellular process.
Since the origin of cMPC<sup>Sca-1</sup> cells is related to the cardiac muscle, it is important to evaluate the cellular response when these cells are seeded onto scaffolds displaying stiffness values similar to that of the muscle. At the physiologically appropriate strains, the degree of stiffness (Young modulus, E) varies dramatically between tissues: brain (E<sub>brain</sub> ~ 0.1-1 kPa) is clearly softer than striated skeletal muscle (E<sub>muscle</sub> ~ 8-17 kPa), which is more compliant than precalcified bone (E<sub>precalcified bone</sub> = 25-40 kPa)<sup>72</sup>. As such, YAP/TAZ activity was explored in cMPC<sup>Sca-1</sup> cells grown onto surfaces displaying physiological stiffness values, by the use of polyacrylamide gels with tunable stiffness. Moreover, the biological effect of extracellular matrix proteins on YAP/TAZ expression was studied by coating these gels with fibronectin or collagen I. Therefore, cMPC<sup>Sca-1</sup> cells were seeded onto PA gels and immunostained against YAP and TAZ by using two different antibodies after 24h, as shown in Figure 24A (collagen coating) and Figure 24B (fibronectin coating). The cells positive for YAP or TAZ were counted and expressed as a percentage of total cells (Figure 25). As shown in Figure 24, cMPC<sup>Sca-1</sup> cells are sensitive to substrate stiffness, responding by activating YAP/TAZ on stiffer substrates (higher than 10kPa), for collagen coated gels (Figure 25A). However, in gels coated with fibronectin, YAP/TAZ expression seems less
sensitive to stiffness changes, as the percentage of cells expressing these proteins in the nucleus does not increase gradually with stiffness (Figure 25B). This indicates that, apart from the mechanical properties of the ECM, its composition is also a parameter to consider in cardiac progenitor cell mechanosensing. Hence, the concept that the regulation of cell fate depends on several factors of different nature (biological, mechano-structural and physico-chemical) is further validated. Moreover, because hMSC were shown to respond to stiffness under different thresholds (TAZ is cytoplasmic at 0.7kPa and nuclear at 40kPa), differences in mechanosensing between different stem cell populations might be inferred by these results.
Figure 24 – YAP/TAZ activity on cMPC<sup>Sca-1</sup> seeded onto polyacrylamide gels. In (A) collagen-coated PA gels, nuclear expression of YAP and TAZ increase with stiffness whereas in (B) fibronectin-coated gels there is not a clear correlation between stiffness and YAP/TAZ expression.
Since YAP/TAZ is involved in cell response to substrate mechanical cues, it is likely that its role is exerted early at the cell adhesion, as the formation of the adhesion processes and focal adhesion is directly related to the nature of the surface on which cells grow. In order to address this topic, cells seeded onto PA gels were analyzed 3h and 5h post-seeding, to get a glimpse of the initial steps of cell adhesion. As shown in Figure 26A, a switch in YAP/TAZ localization occurs early at stiffness 0.7 KPa, these proteins migrating towards the cytoplasm after 5 hours and shuttling back to the nucleus after 24h (even though only about 25% of the cells display nuclear YAP when cells are seeded under this stiffness condition). This movement suggests that YAP/TAZ is required in the initial steps of cell adhesion to soft substrates, and

Figure 25 – Percentage of cMPC<sup>Sca-1</sup> expressing nuclear YAP or TAZ when seeded onto polyacrylamide gels coated with either collagen (A) or fibronectin (B). (A) cMPC<sup>Sca-1</sup> can respond to stiffness on collagen-coated PA gels by activating YAP/TAZ at stiffer gels whereas in fibronectin-coated gels (B) that response is not so clear.

Since YAP/TAZ is involved in cell response to substrate mechanical cues, it is likely that its role is exerted early at the cell adhesion, as the formation of the adhesion processes and focal adhesion is directly related to the nature of the surface on which cells grow. In order to address this topic, cells seeded onto PA gels were analyzed 3h and 5h post-seeding, to get a glimpse of the initial steps of cell adhesion. As shown in Figure 26A, a switch in YAP/TAZ localization occurs early at stiffness 0.7 KPa, these proteins migrating towards the cytoplasm after 5 hours and shuttling back to the nucleus after 24h (even though only about 25% of the cells display nuclear YAP when cells are seeded under this stiffness condition). This movement suggests that YAP/TAZ is required in the initial steps of cell adhesion to soft substrates, and
also for long-term cell mechanosensing (24h). The experiment was repeated using hMSC, which revealed that a similar shift in YAP/TAZ localization occurs earlier in time (3h post-seeding, Figure 26B) in mesenchymal stem cells. This result once again points out the existence of different mechanisms regulating YAP/TAZ activity between different types of stem cells.

Given that these results indicate a relationship between YAP/TAZ activity and cell adhesion onto surfaces with different stiffness values, an additional experiment was devised to further validate this theory. AlamarBlue® assay was used, wherein metabolism of a fluorometric dye by adherent cells is correlated with successful adhesion processes, 8h post-seeding. In order to determine the relevance of YAP/TAZ in this process, siRNA treated cells (YAP/TAZ 

Figure 26 – Role of YAP/TAZ in cell adhesion onto PA gels. A- cMPC<sup>SCa-1</sup> seeded onto fibronectin-coated PA gels were fixed at different time points and YAP immunostaining revealed a shift between the nucleus and the cytoplasm at 5h, at the softest gels. B- hMSC in the same setup exhibited the shift at an earlier time point (3h). This implies that YAP/TAZ are involved in cell adhesion, yet the mechanisms regulating their activity may differ between these two stem cell types.

Figure 27, expressed as the
fluorescence ratio (in percentage) between control cells and YAP/TAZ 
/- cells, for each stiffness-condition tested (collagen-coated gels).

At softer stiffness, the ratio is about 100%, implying that YAP/TAZ -/- cells attach as successfully as control cMPC Sca-1. However, at stiffness higher than 10 kPa the ratio is higher than 100%, indicating that YAP/TAZ -/- cells are less adherent as compared to control cells. This result further validates our data suggesting that YAP/TAZ is required for cell adhesion mostly on stiff substrates.

Interestingly, these data correlate with evidence showing that YAP/TAZ nuclear expression is significantly higher in cells grown onto stiff surfaces (Figures 24 and 25).

3.5.3) Activity of YAP/TAZ on stiff surfaces (PCL films)

To evaluate whether regulation of YAP/TAZ activity still applies when cells are seeded onto substrates having supra-physiological stiffness values, cMPC Sca-1 were seeded onto PCL films displaying stiffness values in the MPa range, and immunostained for YAP (Figure 28). Moreover, GATA-4 staining was also carried out to assess if the co-localization of the two proteins is also occurring at this stiffness range. When supra-physiological stiffness values are
used to challenge cardiac progenitor cells, YAP expression shows no direct correlation with substrate stiffness. Moreover, YAP is not always co-localizing with GATA-4 (e.g. 130 MPa), indicating that their activity is not connected in such experimental conditions.

Cells displaying nuclear YAP were counted and percentages relative to total cells were calculated and plotted in Figure 29. From this graph it is clear that YAP nuclear localization is

![Figure 28 – Activity of YAP/TAZ on stiff PCL films. cMPC^{Sca-1} cells were seeded onto PCL films exhibiting stiffness values over MPa range. No clear correlation between nuclear YAP expression and stiffness in the MPa range seems to exist. Moreover, GATA-4 expression is not always correlated with YAP (for example at 130 MPa), implying that their function is no longer connected when cMPC^{Sca-1} cells are seeded onto stiff surfaces.](image-url)
not correlated with stiffness at the MPa range, as the differences in percentages are not statistically significant.

These results are somewhat expected because cells do not encounter such stiff surfaces in vivo. The stiffest surfaces correspond to the bone, whose stiffness is lower than 100KPa, whereas PCL films tested display stiffness over 900 KPa. This suggests that the regulation of YAP/TAZ activity by surface stiffness only applies when the stiffness of these surfaces span those encountered in vivo. This might have an effect on the downstream processes regulated by YAP/TAZ such as cardiac commitment, as GATA-4 expression no longer co-localizes with that of YAP/TAZ in these stiffness range.

3.6) Activity of YAP/TAZ in response to dynamic changes in surface stiffness

So far, it has become clear that YAP and TAZ are involved in several processes in cMPC<sup>C<sub>sca</sub>-1</sup> and also respond to extracellular cues. However, this was only done in static conditions. So as to increase the relevance of these results, the involvement of YAP/TAZ in dynamic conditions was explored. For this, a thermoresponsive PCL polymer was used. The PCL polymer derives from the structural arrangement of two monomers, and is constituted by 50% of four-branched PCL monomers and 50% of two-branched PCL monomers. This composition results in the formation of a semi-crystalline arrangement at 32 °C. However, when the
temperature is shifted to 37°C, structural rearrangements occur turning the polymer into a complete amorphous structure, thereby decreasing its stiffness. Using this system, both the nanotopography and the stiffness are changed with temperature, providing an interesting model to test cell responses to dynamic changes.

Thus, cMPC<sup>Sca-1</sup> were seeded onto this material at 32°C overnight and then moved to 37°C to induce the shift in substrate elasticity. Thereafter, YAP was immunostained and localization compared to non-shifting control (TCPS). Pictures are shown in Figure 30A, and percentage of cells expressing nuclear YAP were calculated by manual cell counting and plotted in Figure 30B.

---

**Figure 30– YAP/TAZ activity in dynamic surface changes.**

**A** - cMPC<sup>Sca-1</sup> were seeded onto a temperature responsive PCL polymer and YAP nuclear localization was assessed by immunofluorescence. **B** - Percentage of cells expressing nuclear YAP is higher when the material where they were seeded change its nanotopography and stiffness (PCL) in response to temperature, relative to non-changing TCPS control.
In static conditions, the percentage of cells expressing nuclear YAP when seeded onto PCL films ranges from around 50 to 75% (Figure 29B). This number is enhanced to over 95% when cells suffer a dynamic change, indicating that cells can sense the change in stiffness and nanotopography imposed by the temperature responsive polymer. To prove that YAP/TAZ is involved in this process, Alamar Blue assay was used to assess adhesion of cMPC<sup>Sca-1</sup> exposed to the dynamic change, in comparison to YAP/TAZ<sup>-/-</sup> cells. Data obtained were represented as a ratio between fluorescence measured after the dynamic change and that measured before it (Figure 31). Since this ratio is less than 100% for YAP/TAZ<sup>-/-</sup> cells it can be inferred that some cells have detached from the polymer, contrarily to what happened in control cells. This indicates that YAP/TAZ is needed during dynamic changes (in this case stiffness and nanotopography) of the surface where cMPC<sup>Sca-1</sup> are seeded, participating in the maintenance of proper cell attachment.

**3.7) Influence of cell shape in YAP/TAZ activity**

Study of the regulation of YAP/TAZ by cell shape can be performed by the use of micropatterned surfaces portraying different adhesive areas (islands coated with fibronectin). As shown in Figure 32, these areas induce the cells to acquire different cell shapes, either round (due to area constraint) in the smaller areas or flattened in the larger areas. Moreover, this system allows single cell level studies avoiding the impact of cell-to-cell contact.
Thus, regulation of YAP/TAZ activity by cell shape can be studied using this tool. As shown in Figure 33, cMPC<sup>Sca-1</sup> seeded onto the micropatterned surfaces can only express nuclear YAP on the two largest surfaces (2025 and 10000 µm<sup>2</sup>). This implies that cell shape is another regulator of YAP/TAZ activity as cMPC<sup>Sca-1</sup> have to be flattened for YAP/TAZ to translocate to the nucleus. In the same Figure, zyxin staining is predominantly nuclear in all the shapes displayed by the cells, further validating the observation of a different mechanism regulating zyxin intracellular localization relative to YAP/TAZ. Moreover, actin staining demonstrates that actin fibers can only be formed at the two largest shapes, concomitant with nuclear YAP activation, which reinforces the theory that actin cytoskeleton is required for YAP/TAZ to shuttle to the nucleus.

cMPC<sup>Sca-1</sup> commitment to cardiac lineage and mature focal adhesion formation were also studied using this system as it allows a better single cell resolution. Given that GATA-4 is not expressed even at the larger islands (data not shown), the nuclear expression of another transcription factor involved in cardiac commitment, TBX5 was analyzed, along with mature focal adhesion formation. Thus, as shown in Figure 34, TBX5 is only nuclear in the two largest islands, concomitant with YAP nuclear localization. Moreover, mature vinculin signal (spikes) was also detected in these islands, indicating that cell spreading is needed for focal adhesions to fully mature. This last observation is expected as the amount of focal adhesions containing vinculin were shown to increase in direct proportion to cell spreading<sup>138</sup>. 

![Figure 32 – cMPC<sup>Sca-1</sup> shape when seeded onto micropatterned surfaces displaying different adhesive areas. On the smaller areas (300 and 1024 µm<sup>2</sup>), the adhesion is restricted so cells are constrained and acquire a round shape. On the larger areas (2025 and 10000 µm<sup>2</sup>), cells can acquire flattened shapes, similar to what they display in normal culture conditions.](image)
Figure 33 - Regulation of YAP/TAZ activity by cell shape. cMPC<sup>Sca-1</sup> seeded onto smaller areas (300 and 1024 µm<sup>2</sup>) do not express nuclear YAP, and the actin fibers are not formed. On the other hand, when seeded onto larger areas (2025 and 10000 µm<sup>2</sup>) YAP is nuclear, concomitant with actin fibers formation.

Figure 34 - Regulation of cMPC<sup>Sca-1</sup> cardiac commitment and focal adhesion maturarion by cell shape. TBX5 expression was only expressed in cMPC<sup>Sca-1</sup> seeded onto larger islands, were YAP was shown to be nuclear, suggesting a possible link between cardiac commitment and cell shape. Vinculin was only fully formed in the two largest islands as well, indicating that a flat shape is required for mature focal adhesion formation.
In order to determine if YAP/TAZ is controlling the two processes analyzed in Figure 34 (TBX5 activation and focal adhesion formation), YAP/TAZ−/− cMPC^{Sca-1} were challenged with the micropatterned surfaces. Interestingly, suppression of YAP/TAZ prevents TBX5 nuclear localization in the larger islands, as well as mature vinculin formation (Figure 35). This provides a direct link between YAP/TAZ and TBX5 activation (even though they do not colocalize in standard TCPS culture-system), and focal adhesion formation, shedding light on the intracellular processes where YAP/TAZ is involved.

Nevertheless, the lack of co-localization of GATA-4 and YAP even in the largest islands indicate that cell shape also interferes in GATA-4-mediated cardiac commitment although the putative interacting partner YAP is nuclear in this context, suggesting the participation of different mechanisms regulating YAP activity and cardiac commitment.
3.8) Differential gene expression with surface stiffness and YAP/TAZ supression

Since YAP/TAZ are transcriptional modulators, analysis of gene expression in different conditions was carried out in order to understand if differential modulation applies when extracellular cues are provided. In this setting, 4 experimental conditions were chosen: cells were seeded in TCPS either untreated (control) or YAP/TAZ silenced (YAP/TAZ\(^{-/-}\)), and in fibronectin-coated PA gels displaying 0.5KPa or 40KPa stiffness. The expression of genes involved in ECM formation and cell adhesion was analyzed by Real Time PCR-array. The results were expressed as the fold differences in gene expression between each condition and the control. Only changes in gene expression higher than 4-fold were considered. The results obtained must be interpreted carefully, i.e. as a screening-strategy for future studies so as to clearly demonstrate the role of the gene-candidates in the different conditions tested. Nevertheless, possible explanations can be speculated for the results observed, and will be provided in this section.

Firstly, the relevance of YAP/TAZ in modulating gene expression was evaluated by seeding YAP/TAZ\(^{-/-}\) cells onto TCPS, and comparing gene expression relative to control cells (in TCPS), as shown in Table 1.

Table 1 – Differences in gene expression between YAP/TAZ\(^{-/-}\) and YAP/TAZ\(^{+/+}\) cMPC\(^{5ca-1}\), both seeded onto TCPS.

<table>
<thead>
<tr>
<th>Genes overexpressed</th>
<th>Gene name</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen, type III, alpha 1</td>
<td>7.22</td>
<td>A disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 8</td>
</tr>
<tr>
<td>Elastin microfibril interfacer 1</td>
<td>5.824</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>8.8275</td>
<td>Selectin, platelet</td>
</tr>
<tr>
<td>Integrin α2</td>
<td>7.6848</td>
<td>Thrombospondin 1</td>
</tr>
<tr>
<td>Integrin α4</td>
<td>7.6848</td>
<td></td>
</tr>
<tr>
<td>Integrin β2</td>
<td>18.2775</td>
<td></td>
</tr>
<tr>
<td>Laminin, alpha 1</td>
<td>6.242</td>
<td></td>
</tr>
<tr>
<td>Matrix metalloproteinase 10</td>
<td>695.545</td>
<td></td>
</tr>
<tr>
<td>Matrix metalloproteinase 13</td>
<td>72.6052</td>
<td></td>
</tr>
<tr>
<td>Matrix metalloproteinase</td>
<td>22.1925</td>
<td></td>
</tr>
</tbody>
</table>
The high number of genes being differentially expressed between these conditions further validates the role of YAP/TAZ as a transcriptional modulator on cMPC\textsuperscript{Sca-1}. These differences span integrin composition, matrix metallopeptidase and also some proteins of the ECM, being upregulated in YAP/TAZ \textsuperscript{-/-} cells. Regarding that, suppression of YAP/TAZ has been implicated in impaired vinculin maturation, so it is likely that, since cells cannot strongly attach to a surface, they tend to migrate, thereby enhancing expression of matrix metallopeptidases. Nonetheless, this putative explanation needs to be confirmed in order to obtain greater understanding of YAP/TAZ relevance in adhesion and migration in terms of gene expression.

In order to understand if cMPC\textsuperscript{Sca-1} can perceive subtler changes in stiffness, differences in gene expression between cells seeded onto 0.5 and 40KPa were analyzed, as shown in Table 2.

### Table 2 – Differences in gene expression between cells seeded onto Pa gels displaying 0.5 KPa and 40 KPa stiffness, respectively.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold difference</th>
<th>Gene name</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contactin 1</td>
<td>5.0771</td>
<td>Laminin, alpha 3</td>
<td>-4.7437</td>
</tr>
<tr>
<td>Ectonucleoside triphosphate diphosphohydrolase 1</td>
<td>7.9118</td>
<td>Matrix metallopeptidase 10</td>
<td>-14.2808</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>23.3282</td>
<td>Matrix metallopeptidase 3</td>
<td>-4.3954</td>
</tr>
<tr>
<td>Matrix metallopeptidase 14 (membrane-inserted)</td>
<td>4.3289</td>
<td>Matrix metallopeptidase 1a (interstitial collagenase)</td>
<td>-8.9755</td>
</tr>
<tr>
<td>Matrix metallopeptidase 15</td>
<td>13.306</td>
<td>Matrix metallopeptidase 3</td>
<td>-11.5195</td>
</tr>
</tbody>
</table>

These data demonstrates that cMPC\textsuperscript{Sca-1} can sense different stiffness of the extracellular environment where it is embedded, further validating results obtained for regulation of YAP/TAZ activation by small differences stiffness. The main differences concerned matrix metallopeptidase expression which was different between cells seeded onto surfaces exhibiting different stiffness.

Albeit preliminary, these results confirm that YAP/TAZ have a role in cell adhesion and matrix sensing, modulating gene expression for generating appropriate cell responses.
4) Conclusions and discussion

4.1) Overview of YAP/TAZ regulation and activity

Stem cell homeostasis *in vivo* has been demonstrated to be tightly regulated by the surrounding microenvironment, biological as well mechano-structural cues playing a key role in stem cell determination\(^\text{72}\). *In vitro*, the role of substrate stiffness and nano-pattern has been shown to exert a direct effect on mesenchymal stem cell differentiation through the YAP/TAZ axis, a complex able to shuttle from the cytoplasm to the nucleus in response to changes in matrix composition\(^\text{125}\). In the present study, we demonstrated that YAP/TAZ expression and sub-localization can be regulated by a number of ECM mechano-structural parameters. Firstly, cell confluence was shown to negatively regulate YAP/TAZ nuclear activity in the cMPC\(^\text{Sca-1}\) line, as previously demonstrated in other cell types such as hMSC and endothelial cells\(^\text{125}\). On the other hand, *in vitro* migration did not seem to regulate YAP/TAZ activity, as demonstrated in the wound healing and Matrigel™ assay. Actin cytoskeleton integrity is required for YAP/TAZ nuclear shuttling, as previously observed in hMSC\(^\text{125}\). In contrast, ROCK activity, actomyosin contractility and tension were shown to inhibit YAP/TAZ nuclear activity, its suppression resulting in higher percentage of cells expressing nuclear YAP/TAZ. In addition, stiffness is a strong modulator of YAP/TAZ nuclear localization/ activity, as cells seeded onto soft surfaces (displaying stiffness values up to 0,7 KPa) exhibit very low levels of nuclear YAP/TAZ, in contrast to cells seeded onto stiffer surfaces (higher than 10 KPa). However, this modulation is effective when cells are exposed to substrates having a Young modulus in the KPa range, as there is no correlation between nuclear YAP/TAZ localization and increasing stiffness of PCL polymers in the MPa range. Still, biological factors such as the dominant protein of the ECM with which the surfaces were coated also interfere with the mechanosensing process. Importantly, dynamic changes of surface properties such as stiffness and nanotopography were sensed by cardiac progenitor cells, resulting in the activation of nuclear YAP/TAZ. Additionally, cell shape also modulates YAP/TAZ activity, as round cells cannot express nuclear YAP/TAZ, as compared to spread cells.

YAP/TAZ activity was exerted in several cellular processes, demonstrating its relevance in determining stem cell fate of cardiac stem/progenitor cells. YAP/TAZ were implicated in CPC proliferation, cardiac commitment, endothelial differentiation, cell-surface adhesion and focal adhesion maturation. These functions probably resulted from the transcriptional modulating activity of YAP/TAZ, as differences in gene expression were evident between YAP/TAZ\(^{-/-}\) cells as compared to its YAP/TAZ\(^{+/+}\) counterparts, namely concerning genes coding integrins and
different matrix metallopeptidases. Differences in gene expression between cardiac progenitor cells seeded onto surfaces displaying diverse stiffness were also evident, a total of 20 genes being identified as candidates for differential mechanosensing activities, in terms of cell adhesion molecules and proteins of the ECM. A proposed model depicting regulatory mechanisms and activities involved with YAP/TAZ function in cardiac stem/progenitor cells is represented in Figure 36.

![Figure 36 - Role of YAP/TAZ in mechanotransduction of cardiac stem/progenitor cells. Regulation of YAP/TAZ is made at different levels such as confluence, actin cytoskeleton and cell tension, stiffness of interfacing surface and its dominant extracellular protein, cell shape and dynamic changes of substrate where cells are seeded. In CPC, YAP/TAZ is involved in cell proliferation, cardiac commitment/differentiation, and adhesion, modulating gene expression concerning at least matrix metallopeptidases and integrins.

Altogether, YAP/TAZ appear to constitute mechanical sensors involved in cardiac stem/progenitor cell sensing of the microenvironment, responding to mechano-structural cues and controlling important cellular responses related with stem cell fate determination.
Results in the herein study are rather interesting when compared with findings reported by others. For instance, cardiac stem/progenitor cells and mesenchymal stem cells both seem to rely on YAP/TAZ as a mechanosensor, yet with different regulation mechanisms. For instance, ROCK-mediated signaling and cell tension have opposite effects on these two stem cell systems as, contrarily to its action in the cardiac system, these elements are required to activate YAP/TAZ nuclear localization/activity in hMSC\textsuperscript{125}. Moreover, YAP/TAZ nuclear shuttling occurs at different time points (in early adhesion steps) in the two cell types. Furthermore, YAP/TAZ response to stiffness has slightly different thresholds, as in hMSC YAP/TAZ displays a nuclear expression at 40 KPa whereas in cardiac progenitor cells this event takes place at 10KPa. Cell shape and spreading appears to exert similar responses in both stem/progenitor cell types.

YAP/TAZ are known to act as transcriptional co-activators in the nucleus, thus requiring additional factors to exert their function on gene expression. The interaction of YAP/TAZ with nuclear shuttling proteins was not fully determined, yet (phosphorylated) paxillin is a putative partner as concluded by co-localization experiments. On the contrary zyxin does not seem to be a suitable partner for YAP/TAZ, since different intracellular localizations were detected in response to pharmacological inhibitors in the in vitro wound healing migration assay.

A novelty in this study was the demonstration that suppression of cell tension can induce CPC cardiac commitment in a mechanism dependent on YAP/TAZ activity. This result states that a mechano-structural cue can effectively control stem cell fate determination, and points at YAP/TAZ as one of the mechanotransducer involved in this mechanism, highlighting its importance in the stem cell field. In the hMSC context, however, cytoskeletal tension and actomyosin contractility were shown to promote osteogenesis in tri-dimensional microcarrier system\textsuperscript{139}, further demonstrating the importance of mechano-structural factors as regulators of stem cell fate determination.

Importantly, since YAP/TAZ have shown to be involved in CPC proliferation, cardiac commitment and endothelial differentiation, it is plausible that these two proteins are key molecular switches, redirecting CPC fate either to self-renewal or differentiation into different lineages, in response to mechano-structural factors characteristic of the microenvironment in which CPC reside. This knowledge could be harnessed to improve existing CTE strategies.

4.2) Future perspectives

Despite the fact that this study already contemplates a large scope of events involved in YAP/TAZ regulation and activity, taking the work developed for this MSc. Degree dissertation...
to the verge of publication, several experiments could still be performed in order to further validate/ optimize some of the observations reported.

First of all, it would be interesting to perform immunohistochemistry studies on murine heart tissue, so as to understand if YAP and/or TAZ are expressed in different resident putative cardiac stem/progenitor cell subsets, i.e. Sca-1$^+$, c-Kit$^+$ or Isl-1$^+$ CPCs.

Secondly, confirmation of cell counting experiments with Western Blot should be finished, providing a more sensitive and accurate method to assess YAP/TAZ nuclear activity.

Thirdly, the use of additional pharmacological inhibitors could further validate the data acquired so far concerning YAP/TAZ regulation by mechanical forces. For instance, thrombin has shown to increase cell tension concomitant with myosin II phosphorylation. Treatment with thrombin could be anticipated to produce the opposite effects induced by blebbistatin, and therefore confirm tension as a powerful modulator of YAP/TAZ activity.$^{140}$

Fourthly, additional migration experiments could be performed in order to further investigate the role of YAP/TAZ activation during cell migration. This assertion is due to the fact that migration is involved in many processes including tissue repair and regeneration$^{92}$, wherein involvement of actin cytoskeleton dynamics and myosin II is well studied$^{91}$, and could thus provide interesting models for studying mechanotransduction pathways in CPC. As such, further dissection of molecular mechanisms activated during CPC migration could provide useful data for the purpose of scaffold implantation/seeding, possibly ameliorating therapeutic outcomes for cell-based therapies.

Moreover, the reported co-localization and regulation of YAP and GATA-4 is an interesting process, deserving future studies. Protein interaction should be confirmed, considering that our co-immunoprecipitation attempts were so far less succeeded. Thus, this experiment should be completed in order to confirm the interaction, as it further validates the data acquired in this thesis.

RNAi experiments provided very important results. It would be interesting to discriminate between the effects mediated by YAP and TAZ alone, by the use of specific suppression of each protein, either by the use of more specific siRNA sequences or lentiviral-transfection of shRNA for either YAP or TAZ. This strategy would allow understanding the relative contribution of each protein for the cellular responses reported in this study.

Next, even though a broad range of stiffnesses was analyzed in this work, the chemical composition of the materials used differed between the Pa range (Matrigel), kPA range (Polyacrylamide) and MPa range (Poly-ε-caprolactone). In order to solve this issue, the same surface composition should be used throughout all the stiffness range, yet, to the best of our knowledge, such material does not exist. Nevertheless, attempts were made towards reducing
the contribution of biological factors, such as the use of growth factor-reduced Matrigel, and determination of the impact of biological factors in mechanosensing of PA gels. Still, additional experiments could be performed in order to address this problem, such as the use of other hydrogels comprising different chemical compositions such as agarose-based in order to further validate stiffness-specific sensing by cardiac progenitor cells, regardless of the chemical composition of the surface.

Dynamic changes were evaluated in this thesis, but more stimuli could be used in order to challenge the mechanosensing of cells by YAP/TAZ. In the experimental setup used, nanotopography but mostly stiffness are modulated by temperature. As such, only one of the mechnostructural parameters should be changed at a time. For this purpose materials (PCL polymers) that only change their nanotopography have already been developed, so these should be used as well, in order to provide a more detailed understanding of the external cues modulating YAP/TAZ activity.

The influence of cell shape on YAP/TAZ nuclear activity was also studied, yet some questions remain to be elucidated. This is so because measurements of tension of the different adhesive areas of the used micropatterned plates were previously performed, revealing that tension increases proportionally with adhesion area. As such, the used system does not solely contemplate varying shapes, but also tension, a factor known to be relevant in modulating YAP/TAZ activity. However, since cells seeded in the larger islands (acquiring flattened shape) displayed positive nuclear YAP whereas tension negatively regulates nuclear YAP in cTERT, this possibly implies that cell shape is a stronger cue than tension in modulating YAP/TAZ nuclear activity.

Real time PCR gene array experiments provided candidate genes being differentially expressed due to regulation by stiffness changes and as a result of YAP/TAZ suppression. These results are of difficult interpretation due to the complexity of the analyzed genes. For instance, matrix metallopeptidases are known to be regulated by several mechanical forces, including tensile strain, dynamic compression and hydrostatic pressure. Thus, as mentioned in the results section, more thorough experiments should be performed, at the single gene level (such as siRNA-mediated suppression), in order to clearly demonstrate their relevance in mechanotransduction of stiffness changes and absence of YAP/TAZ activity. Moreover, since some results pointed out involvement of YAP/TAZ in several responses such as cardiac commitment by activation of transcription factor GATA-4, additional genes should be studied, such as the transcription factors involved in stem cell commitment, along with other markers correlated with differentiation. As such, high-throughput experiments aiming at the detection
of protein interactions between YAP and transcription factors could also be performed, such as protein microarrays, yeast two hybrid or phage display systems.

In order to reach a higher understanding of the molecular pathways involved in YAP/TAZ mechanotransduction in CPC, participation of additional proteins in YAP/TAZ activity should be investigated. In this sense, several candidates arise, namely other cytoskeletal protein kinases (a group in which studied myosin light chain kinase is included), such as titin, which is known to be involved in mechanosensing, as mechanical modulation of its catalytic activity might allow cytoskeletal signaling proteins to create feedback mechanisms between cytoskeletal tension generation or cellular remodeling\textsuperscript{143}. Moreover, lessons from the cytoskeleton and focal adhesion rearrangements during migration, as well as interplay of microtubules and Rho GTPase signalling in cell polarization and motility can be useful for understanding how YAP/TAZ involvement in focal adhesion maturation is regulated. Thus, the regulation of YAP/TAZ activity by microtubules and Rho GTPases could constitute a topic for further studies, for which pharmacological inhibitors could be used (such as nocodazole for disrupting microtubules\textsuperscript{144}). Moreover, focal adhesion kinase (FAK) is a central player in focal adhesion maturation, and has been implicated in cardiac development, playing a critical role at the cellular level in the responses of cardiomyocytes and cardiac fibroblasts to biomechanical stress and to hypertrophic agonists such as angiotensin II and endothelin\textsuperscript{145}. FAK has also been shown to be essential for angiogenesis in the embryo and cardiomyocyte response to pressure overload in adult mice\textsuperscript{146}. As such, modulation of YAP/TAZ activity by FAK is also a possible relevant study. In addition, integrins are the first line of mechanosensing so should also be studied, for example by the generation of chimeric structures allowing the identification of the subtype of monomers involved in particular mechanotransduction signaling pathways.

Furthermore, since YAP is the transducer of Hippo signaling, an important pathway regulating organ size control\textsuperscript{147} in multiple species, it would be interesting to understand whether Hippo signaling is behind most of the regulatory processes of YAP/TAZ. Independence of Hippo pathway in YAP/TAZ regulation in hMSC has been demonstrated\textsuperscript{125}, so the same experimental framework could be applied to the cardiac field. This could be explored by making CPC insensitive to Hippo pathway (for example by siRNA-mediated suppression of its kinase LATS1/2 which acts directly on YAP/TAZ) and then check if cardiac progenitor cells respond to the presented stimuli (confluence, tension inhibition, etc.) in the same manner comparing with normal cells. Finally, interaction of YAP/TAZ with established signaling pathways modulating cardiac stem cell fate determination (such as Notch\textsuperscript{148} and Wnt\textsuperscript{149}) would be an interesting topic to address in future studies.
All these proposed future studies and strategies to perform them are summarized in **Table 3.**

**Table 3 – Proposed future studies - purpose and strategy.**

<table>
<thead>
<tr>
<th>Purpose of study</th>
<th>Possible strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investigate YAP/TAZ expression in resident cardiac stem/progenitor cells in murine heart tissue</td>
<td>Immunohistochemistry of YAP/TAZ in conjunction of markers identifying resident CPC (Sca-1, c-Kit, Isl-1)</td>
</tr>
<tr>
<td>Confirm cell counting results</td>
<td>Finish Western Blot experiments</td>
</tr>
<tr>
<td>Further validate YAP/TAZ regulation by mechanical forces</td>
<td>Additional pharmacological inhibitors such as thrombin for enhancing cell tension</td>
</tr>
<tr>
<td>Confirm role of migration in YAP/TAZ activity</td>
<td>Additional migration studies</td>
</tr>
<tr>
<td>Confirm YAP/TAZ – GATA-4 protein interaction</td>
<td>Finish co-immunoprecipitation experiments</td>
</tr>
<tr>
<td>Relative contribution of YAP/TAZ towards the observed effects</td>
<td>Use more specific RNAi technology for suppressing only YAP or TAZ</td>
</tr>
<tr>
<td>Confirm stiffness-sensing specificity of cMPC&lt;sub&gt;Sca-1&lt;/sub&gt;</td>
<td>Use materials with same stiffness but different chemical composition</td>
</tr>
<tr>
<td>Distinguish between dynamic changes in stiffness and nanotopography</td>
<td>Use nanopatterened PCL polymers that lose the pattern (without affecting stiffness) by temperature responsiveness</td>
</tr>
<tr>
<td>Confirm relevance of differentially expressed candidate genes in response to different stiffness and suppression of YAP/TAZ</td>
<td>Single gene experiments (such as siRNA-mediated suppression)</td>
</tr>
<tr>
<td>Determine involvement of YAP/TAZ in modulating expression of other genes – transcription factors and focal adhesion proteins</td>
<td>Other PCR arrays; screening by high throughput experiments for determining protein-protein interactions (protein arrays, yeast two hybrid, phage display)</td>
</tr>
<tr>
<td>Investigate involvement of other relevant players in mechanotransduction in YAP/TAZ activity – titin, microtubules, Rho GTPases and integrins</td>
<td>Pharmacological inhibitors, RNAi technology</td>
</tr>
<tr>
<td>Determine involvement of Hippo pathway in YAP/TAZ regulation and activity in CPC</td>
<td>Pharmacological inhibitors, siRNA of effector kinase (LATS 1/2)</td>
</tr>
</tbody>
</table>
An additional degree of relevance would also be given to this work if the results would be extended to human cardiac stem/progenitor cells of the same population (Sca-1⁺).

Despite the fact that the work developed for this thesis and the knowledge generated from it comprise a fundamental study in biology, the information acquired might be important to design applications useful for clinical purposes. As such, future stem cell-based therapies targeting the heart and relying on the use of bioengineered scaffolds should contemplate YAP/TAZ as modulators of stem/progenitor cell fate, possibly by triggering appropriate cellular responses towards achieving the desired outcome.

<table>
<thead>
<tr>
<th>Investigate interaction of YAP/TAZ with established pathways controlling CPC fate – Notch and Wnt</th>
<th>Pharmacological inhibitors, RNAi technology</th>
</tr>
</thead>
</table>

Notch and Wnt
5) References


41. Messina, E., Giacomello, A. & Marbán, E. Chapter 7 - Endogenous Stem Cells. 

42. Di Nardo, P., Forte, G., Ahluwalia, A. & Minieri, M. Cardiac progenitor cells: 


44. Anversa, P., Kajstura, J., Leri, A. & Bolli, R. Life and death of cardiac stem cells: a 

45. Quaini, F. *et al.* Chimerism of the transplanted heart. *New England Journal of 


47. Leri, A., Kajstura, J. A. N. & Anversa, P. Cardiac Stem Cells and Mechanisms of 

48. Laugwitz, K., Moretti, A. & Lam, J. Postnatal isl1 1 cardioblasts enter fully 

49. Beltrami, A. P. *et al.* Adult Cardiac Stem Cells Are Multipotent and Support 

50. Holmes, C. & Stanford, W. L. Concise review: stem cell antigen-1: expression, 

51. Oh, H. *et al.* Cardiac progenitor cells from adult myocardium : Homing, 

52. Wang, X. *et al.* The role of the sca-1+/CD31- cardiac progenitor cell population in 
   postinfarction left ventricular remodeling. *Stem cells (Dayton, Ohio)* **24**, 1779–88 

53. Smits, A. M. *et al.* Human cardiomyocyte progenitor cells differentiate into 
   functional mature cardiomyocytes: an in vitro model for studying human cardiac 

54. Oyama, T. *et al.* Cardiac side population cells have a potential to migrate and 
   differentiate into cardiomyocytes in vitro and in vivo. *Journal of Cell Biology* **176**, 

55. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human 


